MECHANISM OF DIATOMIC LIGAND BIOSYNTHESIS BY RADICAL S-ADENOSYLMETHIONINE [FEFE]-HYDROGENASE MATURASE HYDG

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

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DEDICATION

This work is dedicated to Scott and Kathryn, for their everlasting impact on me as my first mentors in science and helping form my inquisitive scientific mind.
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# LIST OF ABBREVIATIONS

- 5’-dAdo: 5’-deoxyadenosine
- 5’-dAdo•: 5’-deoxyadenosine-5’-yl radical
- AdoCbl: Adenosylcobalamin
- AdoHcy: S-Adenosyl-L-homocysteine
- AdoMet: S-Adenosylmethionine
- anAdo•: 5’-deoxy-3’, 4’-anhydroadenosine-5’-yl radical
- anSAM: S-3’,4’-anhydroadenosyl-L-methionine
- Arg: Arginine
- His: Histidine
- BioB: Biotin Synthase
- BtrN: Butirosin dehydrogenase
- CD: Circular dichroism
- CN−: Cyanide
- CO: Carbon monoxide
- Cys: Cysteine
- deoxyHb: Deoxyhemoglobin
- deoxyMb: Deoxymyoglobin
- DHG: dehydroglycine
- DOPA: 3,4-dihydroxy-L-phenylalanine
- DTT: Dithiothreitol
- ENDOR: Electron nuclear double resonance
- EPR: Electron paramagnetic resonance
- ESI-MS: Electrospray ionization mass spectrometry
- FAS: Ferrous ammonium sulfate
- Fe–S: Iron–sulfur
- FTIR: Fourier transform infrared
- GDP: Guanosine-5’-diphosphate
- GTP: Guanosine-5’-triphosphate
- HbCO: Carboxyhemoglobin
- HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
- HPLC: High performance liquid chromatography
- HYSCORE: Hyperfine sublevel correlation spectroscopy
- LC–MS: Liquid chromatography–mass spectrometry
- MbCO: Carboxymyoglobin
- Met: Methionine
- metHb: Methemoglobin
- NH2–Y: 3-amino-L-tyrosine
- NTM: HydG variant with N-terminal tricysteine CX3CX2C motif substituted for alanines
- PFL–AE: Pyruvate-formate lyase activating enzyme
- PFL: Pyruvate-formate lyase
- Phe: Phenylalanine
RNR: Ribonucleotide reductase
SAM: S-Adenosylmethionine
SF-FTIR: Stopped-flow fourier transform infrared
SPL: Spore photoprodut lyase
TIM: Triose phosphate isomerase
Tris: 2-Amino-2-hydroxymethyl-propane-1,3-diol
Tyr: Tyrosine
WT: wild-type
XAS: X-ray absorption spectroscopy
ΔCTD: HydG variant with truncated C-terminal domain
Iron–sulfur (Fe–S) clusters are ubiquitous in biology, and serve as catalysts in a vast array of chemical transformations that comprise central metabolic reactions and small molecule interconversions. Complex Fe–S clusters such as the [FeFe]-hydrogenase “H-cluster” cofactor are part of a distinct subgroup of metalloenzymes that have evolved from reduced Fe–S mineral phases, as the H-cluster catalyzes H–H bond formation through reduction of protons with electrons. Biosynthesis of this cofactor is unique in its involvement of two radical S-adenosylmethionine (SAM) enzymes HydG and HydE, and a scaffold GTPase HydF. Together, these proteins synthesize a unique Fe–S cluster that coordinates a bridging dithiolate ligand as well as two CN− and three CO ligands. However, many mechanistic details relating to the biosynthesis are not well known. In this work, the radical SAM enzyme HydG has been shown to synthesize CO, CN−, and p-cresol through a radical-initiated fragmentation of the substrate tyrosine. The catalytic mechanism is complex, because an accessory C-terminal Fe–S cluster is required for catalysis. The exact role of this cluster in the biosynthetic mechanism is unresolved, but is proposed to serve a modular role as a potential scaffold for diatomic ligand synthesis. To understand the catalytic mechanism, a combined biochemical and spectroscopic approach was applied. In this work, it is shown that the C-terminal Fe–S cluster is essential for the formation of both CO and CN− products. Spectral characterization of the enzyme has shown the formation of diatomic ligand products that are bound to the coordinated Fe–S clusters. Also, an H atom abstraction profile of HydG has been recently characterized to provide insight to the involvement of the 5′-deoxyadenosyl radical in catalysis. Further mechanistic insight into catalysis has also been investigated through site-directed mutagenesis and through using substrate analogues. The work presented as a whole, by establishing parallels to the radical SAM enzyme superfamily in character to biosynthesis, reveals unifying themes in complex metal cluster assembly related to radical-initiated modification of ordinary Fe–S clusters via product organometallic complex formation.
CHAPTER 1

INTRODUCTION

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

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Co-Author: Trinity L. Hamilton

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Contributions: Wrote section in one review article, provided new results in review article, and edited manuscript drafts.

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Contributions: Wrote sections in one review article, prepared figures, and edited manuscript drafts.

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Contribution of Authors and Co-Authors - Continued

Co-Author:  John W. Peters

Contributions:  Provided revisions to manuscript draft for one review.
Iron–Sulfur Clusters in Biology

Iron–sulfur (Fe–S) clusters are ubiquitous in nature and serve as catalysts in a vast array of chemical transformations that comprise central metabolic reactions and small molecule interconversions. The most prevalent Fe–S clusters observed in biology include [2Fe-2S], [3Fe-4S], and [4Fe-4S] rhombs and cubes (Figure 1.1). These clusters are typically covalently bound to the protein through cysteine thiolate ligation arising from cysteine residues typically organized in conserved motifs. Fe–S proteins can be described as finely tuned transition metal sulfide catalysts that have evolved from an inorganic, abiotic environment, as remnants of an ancient geochemistry that was part of the early Earth. Modern acetogens and methanogens that live off the H₂–CO₂ redox couple thrive from chemical reactivity of complex Fe–S enzyme catalysts, that likely

![Figure 1.1. Common Fe-S Clusters Found in Biology.](image)
catalyzed the earliest bioenergetic events in primitive living cells before the great oxidation event on primordial Earth.\textsuperscript{10,11}

An essential component in early bioenergetic evolution concerns “ligand accelerated catalysis” or the modification of Fe–S clusters with ligand sets that convey distinct reactivity.\textsuperscript{12,13} Ligand accelerated catalysis was likely a common process on the early Earth and thus could have played a central role in the generation of the chemical building blocks of life from simple precursors such as the gaseous substrates CO\textsubscript{2}, N\textsubscript{2}, and H\textsubscript{2}.\textsuperscript{12-14} By association, the Fe–S active site catalysts observed in contemporary biology constitute finely tuned remnants of Fe–S mineral phase catalysis, and have employed ligand accelerated catalysis to tailor ancient small molecule biotransformations catalyzed by the mineral phase via organic “nesting” into a protein architecture.\textsuperscript{15} The composition of reduced Fe–S mineral phases on early Earth as characterized from alkaline hydrothermal vents from the deep ocean are similar in structure and reactivity to transition metal sulfide catalysts found in modern anaerobic autotrophs toward small molecule biotransformations, in terms of their chemical and thermodynamic commonalities.\textsuperscript{16} For example, pyrite (FeS\textsubscript{2}) catalyzes the reduction of dinitrogen (N\textsubscript{2}) to ammonia (NH\textsubscript{3}) under conditions under conditions that mimick those those in hydrothermal vent environments.\textsuperscript{17,18} Furthermore, sulfur vacancies present on Fe pyrite act to increase the retention time of adsorbed amino acids at the FeS\textsubscript{2}–H\textsubscript{2}O interface and enhance the reactivity of the Fe and sulfur atoms at the defect site.\textsuperscript{19-21} In a manner analogous to FeS mineral surfaces modified with ligands, the chemistry catalyzed by these metalloenzymes is a direct consequence of the composition, structure, and ligand environment of their active site metallocofactors.\textsuperscript{22}
The parallels between life and properties of the early Earth are strong where it appears primordial life learned how to make Fe–S compounds by mimicking catalysis present in rock formations in nonliving Earth. Molecular “fossils” such as Fe–S clusters in metalloenzymes serve as ancient artifacts of catalysis from the early earth, where insight toward the evolved mimicry noted can be reflected in the carrier, scaffold, and enzymes associated with assimilation and assembly. The most common types of clusters (Figure 1.1) are synthesized in vivo by complex biological machinery that include iron chaperones, cysteine desulfurase enzymes, electron transfer proteins on which the nascent cluster is built prior to insertion into a target apo-protein. The primary systems involved in simple iron–sulfur cluster assembly are the isc and suf pathways, which function to build Fe–S clusters under normal and oxidative stress conditions, respectively.

By contrast, the more complex Fe–S centers such as the FeMo cofactor found in Mo-nitrogenase and the H-cluster found in [FeFe]-hydrogenase (Figure 1.2) are more advanced than the simpler Fe–S cluster assemblies noted in Figure 1.1. Both active sites can be described as modular, inorganic nanocrystals having minimal protein ligation that are “nested” within the protein environment at the active site and tuned by nonprotein ligation. While Mo-nitrogenase and [FeFe]-hydrogenase are evolutionarily unrelated, they have developed intriguingly similar pathways for the synthesis and insertion of their respective complex metallocofactors. For example, standard Fe–S cluster assembly machinery is employed to synthesize the basic building blocks ([4Fe-4S] and [2Fe-2S] clusters) for both the H-cluster and FeMo-co. Modifications are introduced to these standard FeS clusters through the utilization of scaffold (e.g. assembly site) and carrier
Figure 1.2. Examples of Complex Fe-S Clusters in Biology. Left, [FeFe]-hydrogenase H-cluster cofactor (PDB 3C8Y). Right, FeMo cofactor from Mo-nitrogenase (PDB 1M1N). Structures are depicted as sticks and spheres, with irons colored chocolate, sulfurs colored yellow-orange, carbons colored gray, oxygens colored red, nitrogens colored blue, and molybdenum colored cyan.

(e.g. transport) proteins in combination with the activities of radical S-adenosylmethionine (AdoMet) enzymes, culminating in metalloclusters that have enhanced catalytic reactivity and substrate specificity.  

To understand the transition between prebiotic and biotic life on Earth, the structure and reactivities of Fe–S enzymes within extant biology can be informative regarding primordial catalysis. Partnerships between elements rooted in the prebiotic, particularly with respect to the molecules, pathways, structures, and assemblies of ancient life, are integrally tied to Fe–S enzymes such as the radical SAM enzymes.  

As will be described in more detail in the following sections, radical SAM enzymes fuse the information-based nucleotide and metabolism-based Fe–S chemistries to catalyze radical-initiated biotransformations through generation of organic radicals. The partnership encompassed between the nucleotide-amino acid conjugate SAM and Fe–S clusters likely catalyzed many of the early biochemical processes of the early Earth.
Radical SAM Enzymes

Radical $\text{S}-\text{adenosylmethionine}$ (SAM) enzymes constitute a large group of enzymes that catalyze radical-initiated reactions, generating organic radicals as part of their catalytic operation.\textsuperscript{30-32} Similar to other metalloenzymes that catalyze the formation of organic radicals (e.g. cytochrome P450, methane monooxygenase, ribonucleotide reductase (RNR), and adenosylcobalamin ($\text{B}_{12}$) enzymes), radical SAM enzymes generate an organic radical species at the interface between a Fe–S cluster and the organic molecule SAM.\textsuperscript{33} Classified as a superfamily using bioinformatic techniques in 2001,\textsuperscript{34} approximately 48,000 members currently have been identified,\textsuperscript{35} although research on radical SAM enzymes has been going on for several decades, well preceding the more recent classification.\textsuperscript{36} Radical SAM enzymes are part of a larger group of enzymes that challenged the early paradigm in biochemistry in which nearly all reactions in biology were thought to be catalyzed by mechanisms involving paired electron species.\textsuperscript{37}

Fundamental studies on radical SAM enzymes by its founding members have provided a template for understanding the richness of Fe–S catalyzed radical chemistry. The radical SAM enzyme lysine-2,3-aminomutase (LAM) was initially characterized in 1970, where a strict dependence on iron and SAM was determined;\textsuperscript{36} early work on the enzyme pyruvate-formate lyase (PFL) showed that it generated a stable protein radical,\textsuperscript{38} and that it was stimulated by addition of iron, SAM, and an activating component in the cell extract,\textsuperscript{39} now known to be the radical SAM enzyme pyruvate-formate lyase activating enzyme (PFL–AE). Both PFL–AE and LAM were found to contain a
catalytically essential [4Fe-4S] cluster,\textsuperscript{40,41} and while PFL–AE was initially shown to use SAM as an essential component for PFL activation,\textsuperscript{40,42} the adenosyl moiety of SAM for LAM mediated hydrogen transfer in a manner similar to adenosylcobalamin-dependent rearrangements, which implicated radical intermediates.\textsuperscript{43}

Radical SAM enzymes generate the organic 5’-deoxyadenosyl radical via electron transfer from a reduced [4Fe-4S]\textsuperscript{+} that is coordinated at a CX\textsubscript{3}CX\textsubscript{2}C motif (Figure 1.3). SAM itself is coordinated to the unique iron of the [4Fe-4S] cluster by the amino and carboxylate moieties of the methionine amino acid backbone, forming a classical 5-member chelate ring (Figure 1.3).\textsuperscript{44,45} While it is well known that amino acids can chelate metal ions, the [4Fe-4S]-SAM complex was a novel structure in biology when it was first determined.\textsuperscript{44,45} The CX\textsubscript{3}CX\textsubscript{2}C motif provides three cysteine ligands that are oriented to coordinate three iron ions, where with incubation of sulfide, generates a [3Fe–4S] cluster. The remaining iron ion to make a [4Fe-4S] cluster is not coordinated by a cysteine ligand; in the absence of SAM, spectroscopic evidence is ambiguous, but a
small-molecule thiol from the buffer likely coordinates in selected cases. 46

It was determined from early studies that radical SAM enzymes cleaved SAM generate methionine and 5’-deoxyadenosine (5’-dAdo) (Figure 1.4). Further, in some cases use of isotopically labeled substrate provided evidence for H atom transfer from substrate to 5’-dAdo during catalysis. These observations, together with the recognition that both LAM and the anaerobic RNR catalyzed reactions that were directly analogous

Figure 1.4. Formation of the 5’-dAdo• Radical through Reductive Cleavage of SAM at a Site-differentiated [4Fe-4S] Cluster (PDB ID 3CB8).

Figure 1.5. Structures of (A) Adenosylcobalamin and (B) SAM. Figure reprinted with permission from Reference 32. Copyright 2014 American Chemical Society.
to adenosylcobalamin (AdoCbl)-dependent reactions, led to the hypothesis that the radical SAM enzymes generated the same intermediate, the 5’-deoxyadenosyl radical (dAdo•), that AdoCbl enzymes generated (Figure 1.4, Figure 1.5). While the 5’-dAdo• radical has not been detected directly for either AdoCbl or radical SAM enzymes, radical analogues have been employed to understand the radical (Figure 1.6). 3’,4’-Anhydroadenosylcobalamin was synthesized by Magnusson and Frey and was shown to give rise to the relatively stable allylic radical species, 5’-deoxy-3’, 4’-anhydroadenosine-5’y-yl (anAdo•), upon reaction with the enzyme diol dehydrase. AnS-3’,4’-anhydro adenosyl-L-methionine (anSAM), which upon reaction with reduced LAM, substrate and S-3’,4’-anhydroadenosyl-L-methionine (anSAM) gave rise to the same allylic radical species anAdo• (Figure 1.6).

The reductive cleavage of SAM to generate 5’-dAdo• and methionine requires the input of one electron, now known to come from the reduced site differentiated [4Fe-4S] cluster (Figure 1.3, Figure 1.4). The [4Fe-4S]⁺ state is the catalytically active oxidation state for the Fe–S cluster in the radical SAM enzymes. This was unequivocally

![Figure 1.6. Formation of the Stable Allylic Radical Species 5’-deoxy-3’,4’-anhydroadenosine-5’y-yl Radical (anAdo•). Reductive cleavage of S-3’,4’-anhydroadenosyl-L-methionine (anSAM) yields the anAdo•. Figure reprinted with permission from Reference 32. Copyright 2014 American Chemical Society.](image-url)
demonstrated for PFL-AE by controlled generation of defined quantities of the $[4\text{Fe}-4\text{S}]^+$ state, followed by the addition of the substrate PFL and the observation using EPR that the quantity of glycyl radical generated on PFL was equivalent to the quantity of $[4\text{Fe}-4\text{S}]^+$ originally on the PFL-AE. Further, it was shown that upon generation of the glycyl radical, the $[4\text{Fe}-4\text{S}]^+$ state was oxidized to the EPR-silent $[4\text{Fe}-4\text{S}]^{2+}$ state, indicating that the $[4\text{Fe}-4\text{S}]^+$ cluster provides the electron required for the reductive cleavage of SAM. Concurrently, it was shown for LAM that the $[4\text{Fe}-4\text{S}]^+$ state was the active state, by correlating the quantity of $[4\text{Fe}-4\text{S}]^+$ signal with the activity, although direct stoichiometric conversion of reduced cluster to product could not be shown because LAM utilizes SAM as a cofactor and therefore the Fe–S cluster is re-reduced after each catalytic cycle. These observations for PFL-AE and LAM, together with the requirement of a strong reducing agent in all radical SAM enzymes activity assays, has led to the general acceptance of the $[4\text{Fe}-4\text{S}]^+$ state being the catalytically active oxidation state for these enzymes.

**Framework Mechanism**

A mechanism for the involvement of SAM in radical SAM enzymes is summarized in Figure 1.32 This unifying preliminary mechanism involves a site-differentiated $[4\text{Fe}-4\text{S}]^{2+}$ cluster with SAM chelating the unique iron. The cluster is reduced by one electron to the $[4\text{Fe}-4\text{S}]^+$ state; *in vivo* the reducing system requires flavodoxin or other single electron donors, while *in vitro* strong reductants such as dithionite or photoreduced 5-deazariboflavin are used. The reduced $[4\text{Fe}-4\text{S}]^+$ cluster can
transfer one electron to SAM to homolytically cleave the S-C(5') bond, generating methionine (still bound to the unique iron) and a 5'-dAdo•. This reductive cleavage of SAM occurs in most radical SAM enzymes in vitro even in the absence of substrate, producing as products methionine and 5'-dAdo, with the 5'-dAdo presumably resulting from quenching of the 5'-dAdo• with solvent or a protein moiety. In the presence of substrate, however, the rate of the reductive cleavage reaction is generally considerably enhanced. The 5'-dAdo• produced by reductive cleavage in the presence of substrate abstracts an H atom from substrate in a regio- and stereo-specific manner to generate a substrate radical. In some cases, this substrate radical is the end product of the reaction, for example in the case of PFL-AE where the glycyl radical on PFL is generated. In most cases, however, the substrate radical is an intermediate, and undergoes simple or complex
transformations and may react with additional substrates prior to product formation. In most radical SAM enzymes characterized to date, methionine and 5’-dAdo are produced in a 1:1:1 stoichiometry with product, indicating that SAM is being used as a cosubstrate and is consumed during catalysis. Several characterized radical SAM enzymes, however, use SAM catalytically; in these cases, rearrangement of a substrate radical intermediate produces a product radical intermediate, and this latter species abstracts a H-atom from 5’-dAdo to regenerate 5’-dAdo•, which recombines with methionine to regenerate SAM (Figure 1.7).

Energetics

The [4Fe-4S]⁺ electron transfer to SAM is energetically very unfavorable, yet it is one that occurs in the radical SAM enzyme superfamily. Trialkylsulfonium compounds such as SAM are known to have extremely negative reduction potentials that approximate −1.8 V,⁵⁶,⁵⁷ while the 2+/+ redox couple for biological [4Fe-4S] clusters is rarely more negative than −500 to −600 mV. Measured reduction potentials measured for the radical SAM enzymes LAM, BioB, and MiaB range from −479 to −505 mV.⁴⁶,⁵⁸,⁵⁹ Given the differences in reduction potential noted above, the redox potentials of the Fe–S cluster and of SAM alone do not provide an adequate measure of the redox environment, since SAM and the [4Fe-4S] cluster are in proximity. The radical SAM enzyme LAM has been used as a model for understanding this relationship, where interactions in the active site decrease the barrier associated with SAM cleavage.⁴⁶ While the respective potentials are mediated by coordination of substrate and SAM to the [4Fe-4S] cluster to make a Michaelis complex, a significant difference in reduction potential as unfavorable is observed, by −390 mV.⁴⁶,⁶⁰ Additional factors that may contribute to make for favorable
electron transfer include the difference in coordination environment at the [4Fe-4S] cluster upon cleavage, the tightness in binding of methionine to the cluster, as well as the polarity surrounding the active site.

Regioselective Cleavage and Inner-Sphere Electron Transfer

Radical SAM enzymes principally catalyze homolytic bond cleavage of the S-C(5') bond to generate methionine and 5'-dAdo• (Figure 1.8). Some exceptions have been observed, where the B12-independent glycerol dehydratase activating enzyme (GD-AE) cleaves an alternate S-C bond (S-C(γ)), while the diphthamide biosynthetic enzymeDph2, an enzyme not in the radical SAM superfamily but uses SAM in radical chemistry, also cleaves the S-C(γ) bond (Figure 1.8). This discrimination in S-C cleavage is largely dictated by the details in the cluster-SAM interaction, where

![Figure 1.8. Regioselective Cleavage of the S-C Bonds of SAM. Bonds that may undergo enzymatic-based homolytic cleavage are indicated in varying colors. S–C(5’), blue; S–C(γ), red; S–C(methyl), magenta. Reprinted with permission from Reference 32. Copyright 2014 American Chemical Society.](image)
orientation of the 2p antibonding orbital of the sulfonium sulfur with the Fe–S enzyme controls the regioselectivity of the homolytic bond cleavage.\textsuperscript{67,68} These effects are controlled by both the coordination chemistry and the protein environment, by which electronic perturbations to the [4Fe-4S] cluster and the antibonding S-C(5') orbital affect the activation barrier for bond cleavage. For example, SAM binding to the radical SAM enzyme spore photoproduct lyase (SPL) [4Fe-4S] cluster induces elongation of the Fe…Fe distances within the ferromagnetically coupled $M_s = +9/2 [2\text{Fe-2S}]$ rhomb of which the site differentiated iron ion resides, as well as between the two antiferromagnetically coupled $[2\text{Fe-2S}]$ rhomb pairs.\textsuperscript{69}

Computational studies based on the radical SAM enzyme HydE have predicted an energy barrier associated with SAM cleavage and have provided a picture of the transition state structure that may be general to all radical SAM enzymes.\textsuperscript{61} Major contributors to the transition state HOMO include the carbon based radical of 5’-dAdo•, the methionine based Sδ, and the site differentiated iron. Inner-sphere electron transfer to the C5’ group of SAM from the [4Fe-4S] cluster involves a direct path between the unique iron ion of the cluster and the p orbitals of the sulfonium group.\textsuperscript{61} The bidentate coordination of SAM coupled with the close proximity of the sulfonium moiety to the iron sulfur cluster causes a perturbation in the electronic distribution of the cluster away from the standard sulfur-centered\textsuperscript{70} to more iron-centered redox chemistry.\textsuperscript{61} Similar results have been obtained following XAS studies with PFL-AE,\textsuperscript{62} although recent computational studies with the radical SAM enzyme biotin synthase (BioB) have suggested that the electron transfer step from the [4Fe-4S]$^+$ cluster into the antibonding S-C orbital likely occurs via a sulfide-sulfonium interaction.\textsuperscript{71}
Structural Basis for Radical SAM Catalysis

Several radical SAM enzymes have been structurally characterized by X-ray crystallography. A unifying component for all of these enzymes is a full or partial triose phosphate isomerase (TIM) barrel. A full TIM barrel consists of eight alpha helices alternating with eight beta strands, which form a barrel-like structure with the beta strands on the interior and the alpha helices surrounding them on the protein surface (Figure 1.9A). The radical SAM enzymes BioB, thiamine pyrimidine biosynthetic enzyme ThiC, the hydrogenase biosynthetic enzyme HydE, and the lysine mutase PylB have crystal structures solved with complete \((\alpha\beta)_8\) TIM barrels. The remaining structurally characterized radical SAM enzymes contain partial TIM barrels, including

Figure 1.9. Examples of Structurally Characterized Radical SAM Enzymes. (A) PylB (PDB 3TV7) with a full \((\alpha\beta)_8\) TIM barrel. (B) PFL–AE (PDB 3C8B) with a \((\alpha\beta)_6\) TIM barrel. In both structures, Fe–S clusters depicted in chocolate and yellow orange spheres, SAM in stick structure with carbons colored black, nitrogens colored blue, and oxygens colored red. Methylornithine in PylB structure is colored similar to SAM. 7-mer peptide colored in magenta, with the glycine position colored in green.
PFL–AE (Figure 1.9B), LAM, molybdopterin cofactor biosynthetic enzyme MoaA, oxygen-independent coproporphyrinogen oxidase HemN, the rRNA methyltransferase RImN, QueE, the tRNA ring cyclase TYW1, the anaerobic sulfatase maturase enzyme SME, the butirosin dehydrogenase BtrN, the ribosomal protein methylthiotransferase RimO, and SPL. The smallest known radical SAM enzyme, the activating component of the anaerobic RNR (RNR–AE) is predicted to have an \((\alpha\beta)_4\) partial TIM barrel, although it has yet to be structurally characterized. The most primitive members of the radical SAM family are PFL-AE and aRNR-AE, comprised of \((\alpha\beta)_6\) and \((\alpha\beta)_4\) folds, respectively, possibly indicating the evolution of a subunit fold from \((\alpha\beta)_2\) precursor units. Recently, the crystal structure for the radical SAM enzyme QueE has been solved to contain a modified \((\beta_6/\alpha_3)\) TIM barrel structure.

Radical SAM enzyme TIM barrels vary from having either closed or open, splayed barrel structures. The conserved cluster binding CX\_3\_CX\_2\_C motif is found on the loop that follows the first \(\beta\) strand and the \([4Fe-4S]\) cluster itself is located 7 to 10 Å from the closest protein surface. The positioning of the cluster is buried by loop regions at the top of the barrel and additional protein elements and SAM act to sufficiently shield the cluster and active site environment from bulk solvent. SAM coordination to the cluster positions the molecule across the top of the barrel, forming contacts with residues originating from each of the core \(\beta\) strands. A conserved “GGE” motif forms H-bonds to the amino portion of the methionyl group of SAM, acting to further position this group for coordination to the unique iron of the cluster. Amino acids that interact with the carboxylate functionality are more assorted, with H-bonding interactions among the different structures originating from either arginine, lysine, histidine, or serine and
H-bonding interactions with the ribose hydroxyl groups are accomplished by charged or polar residues that originate mainly from strands $\beta 4$ and $\beta 5$, while the adenine moiety forms a multitude of interactions that are hydrophobic, H-bonding, and $\pi$-stacking in nature. For example, mutational studies on the two $\beta 4$ strand residues in BioB have implicated their roles in enzyme binding and cleavage of SAM and proper 5’-dAdo’ positioning for abstraction on the substrate. Several of the radical SAM enzymes exhibit additional C-terminal associated structural features outside the core TIM barrel that may confer substrate specificity, however substrates are consistently observed to bind within the TIM barrel, if anything to help minimize the deleterious effects on radical chemistry of incidental exposure to other cellular components.

**Radical SAM Enzymes with Auxiliary Fe–S Clusters**

Radical SAM enzymes initiate radical-based catalysis at a site differentiated [4Fe-4S] cluster, but additional Fe–S clusters present participate in various capacities with respect to catalysis (Table 1.1). One subclass of radical SAM enzymes with additional clusters are ones that use the Fe–S cluster as a reactant in the biotransformation, formally a cannibalization reaction. One representative example includes BioB, which coordinates an auxiliary [2Fe-2S] cluster in addition to the radical SAM [4Fe-4S] cluster. BioB catalyzes a sulfur insertion reaction, where it has been shown that the sulfur from the [2Fe-2S] cluster is incorporated into the product. Similar catalysis has been proposed with the radical SAM enzymes lipolyl synthase (LipA), where two sulfur atoms from the auxiliary [4Fe-4S] cluster have been proposed.
Table 1.1. Radical SAM Enzymes that Coordinate Auxiliary Fe-S Clusters

<table>
<thead>
<tr>
<th>Radical SAM Enzyme</th>
<th>Auxiliary Cluster(s)</th>
<th>Role of Auxiliary Cluster</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoaA</td>
<td>[4Fe-4S]</td>
<td>Lewis Acid; Scaffold</td>
<td>80,100</td>
</tr>
<tr>
<td>HydE</td>
<td>[2Fe-2S]; [4Fe-4S]</td>
<td>Unknown</td>
<td>75</td>
</tr>
<tr>
<td>HydG</td>
<td>[4Fe-4S]</td>
<td>Scaffold; Fe source</td>
<td>101,102</td>
</tr>
<tr>
<td>NifEN-B</td>
<td>[4Fe-4S]</td>
<td>FeMo-co protocluster</td>
<td>103</td>
</tr>
<tr>
<td>Hpd–AE</td>
<td>[4Fe-4S]</td>
<td>Unknown</td>
<td>104</td>
</tr>
<tr>
<td>anSME</td>
<td>[4Fe-4S]</td>
<td>Electron Transfer Conduit</td>
<td>85</td>
</tr>
<tr>
<td>BtrN</td>
<td>[4Fe-4S]</td>
<td>Substrate-radical oxidation</td>
<td>86</td>
</tr>
<tr>
<td>AtsB</td>
<td>[4Fe-4S]</td>
<td>Electron Transfer Conduit</td>
<td>105</td>
</tr>
<tr>
<td>PqqE</td>
<td>[4Fe-4S]</td>
<td>Unknown</td>
<td>108</td>
</tr>
<tr>
<td>TYW1</td>
<td>[4Fe-4S]</td>
<td>Substrate Scaffold</td>
<td>110</td>
</tr>
<tr>
<td>AlbA</td>
<td>[4Fe-4S]</td>
<td>Substrate-radical Oxidation</td>
<td>111</td>
</tr>
<tr>
<td>FbiC</td>
<td>[4Fe-4S]</td>
<td>Second 5’-dAdo• Source</td>
<td>112</td>
</tr>
<tr>
<td>SkfB</td>
<td>[4Fe-4S]</td>
<td>Substrate Scaffold</td>
<td>113</td>
</tr>
</tbody>
</table>

to be incorporated into the octanoyl-acyl carrier protein. Analogous chemistry is posited to occur with the methylthiotransferase enzymes MiaB, YqeV, and RimO, although the sulfur source for the RimO enzyme has been assigned to a polysulfide group attached to the auxiliary cluster, not strictly the [4Fe-4S] cluster sulfides.

A second subclass of radical SAM enzymes that coordinate auxiliary clusters include those that use the cluster to orient or interact with substrates or intermediates for proper catalysis. Radical SAM enzymes believed to have this function include MoaA, the sporulation killing factor biosynthetic enzyme SkfB, TYW1, as well as HydG. As a foundational example, the radical SAM enzyme MoaA catalyzes the first step in the synthesis of molybdopterin from GTP, a cyclization reaction,
forming (8S)-3′,8-cyclo-7,8-dihydroguanosine 5′-triphosphate (3′,8-cH₂GTP)\textsuperscript{117,118} with two site-differentiated [4Fe-4S] clusters.\textsuperscript{119} ENDOR studies of the enzyme that coordinated only the auxiliary cluster has shown that at least one nitrogen from the [\textsuperscript{14}N or \textsuperscript{15}N]-5′-GTP complexed with the MoaA was coordinated to the unique iron of the C-terminal cluster; using natural abundance [\textsuperscript{14}N]-ITP, which lacks the amino group of GTP, it was demonstrated that the purine ring nitrogen N1 was coordinated as an enol tautomer.\textsuperscript{100}

A third subclass of radical SAM enzymes with auxiliary Fe–S clusters include those that mediate electron transfer with the substrate and product, as well as with generated intermediate radicals. Examples in this group include the radical SAM enzymes anSME,\textsuperscript{85,120} BtrN,\textsuperscript{86} TYW1,\textsuperscript{110} as well as the subtilosin A biosynthetic enzyme AlbA.\textsuperscript{111} As a foundational example, the radical SAM enzyme BtrN catalyzes a two electron oxidation of 2-deoxy-scyllo-inosamine to make amino-2-deoxy-scyllo-inosose.\textsuperscript{121,122} Recent structural characterization of the BtrN enzyme has shown that the auxiliary cluster is fully coordinated by cysteine residues, but is only 9.6 Å away from the C-3 position of the substrate abstraction position.\textsuperscript{86} This cluster is poised to serve as an electron acceptor, perhaps to help drive the oxidation of the intermediate radical species toward the dehydrogenation reaction, and to avoid an elimination reaction catalyzed by the radical SAM enzyme DesII.\textsuperscript{86}
Radical SAM Enzymes in the Biosynthesis of Complex Fe–S Clusters

[FeFe]-Hydrogenase: Complex Metallocofactor Structure

Hydrogenases are metalloenzymes that are integral components of metabolic pathways in a variety of microorganisms, either accepting electrons from reduced Fe–S carrier proteins like ferredoxin that accumulate during fermentation, or coupling the oxidation of H₂ to energy yielding processes according to the reaction \( H_2 \rightarrow 2 H^+ + 2 e^- \). \(^{123,124}\) Two classes of hydrogenase enzymes found in nature are the [NiFe]- and [FeFe]-hydrogenases; these enzymes are phylogenetically unrelated, with the [NiFe] enzymes routinely biased towards H₂ oxidation and the [FeFe] enzymes biased towards H₂ evolution. \(^{125,126}\) [FeFe]-hydrogenases are found in Bacteria and Eukarya, and contain a complex metal center, referred to as the H-cluster, that is responsible for proton reduction. The composition of the H-cluster was determined by X-ray crystallographic

Figure 1.10. Structural Crystallographic Model of the [FeFe]-Hydrogenase H-cluster Active-site Environment. The \( \text{CpI HydA} \) structure (PDB 3C8Y) is depicted from Reference 133. For clarity, Phe417 and Gly418 have been omitted.
analysis of the [FeFe]-hydrogenase (HydA) enzymes from Clostridium pasteurianum (CpI)\textsuperscript{127} and Desulfovibrio desulfuricans,\textsuperscript{128} complemented by FTIR spectroscopic studies.\textsuperscript{129,130} The unique active site metal cluster (Figure 1.10) consists of a [4Fe-4S] cubane that is bridged to a 2Fe subcluster through a cysteiny1 thiolate linkage; the 2Fe subcluster contains three carbon monoxides (CO), two cyanides (CN\textsuperscript{−}), and a bridging dithiolate moiety as ligands.\textsuperscript{127,128} These π-acid ligands participate in back bonding with the metal ions, thereby stabilizing lower oxidation states of the Fe and facilitating the rapid and reversible oxidation and reduction reactions associated with hydrogen catalysis.\textsuperscript{131} The 2Fe subcluster in as purified HydA is described as a low spin S = 1/2 state with an Fe(II)/Fe(I) pair,\textsuperscript{132-134} and the distal Fe atom is likely the site for proton binding and reduction.\textsuperscript{135}

H-Cluster Maturation Machinery

Early efforts to heterologously express HydA in Escherichia coli (which lacks an endogenous [FeFe]-hydrogenase) yielded inactive enzyme,\textsuperscript{136,137} demonstrating that E. coli is unable to properly assemble the active site H-cluster. In 2004, Posewitz and King\textsuperscript{138,139} identified three hydrogenase accessory genes hydE, hydF, and hydG (hydE and hydF existing either as separate gene products or as a fused gene depending on the organism) that were absolutely conserved in all organisms containing [FeFe]-hydrogenases. They also demonstrated that active HydA is obtained when the hydrogenase gene was co-expressed with these accessory genes. Moreover, it was soon reported that inactive HydA overexpressed in E. coli alone (HydA\textsuperscript{AEFG}) could be activated through the \textit{in vitro} addition of an E. coli lysate containing overexpressed hydE,
hydF, and hydG together, indicating that HydE, HydF, and HydG were the only unique components required to properly assemble the H-cluster. 140

Analysis of the amino acid sequences of HydE and HydG revealed the presence of the canonical CX₃CX₂C motif characteristic of the radical SAM superfamily, while HydF contained several putative C-terminal Fe–S cluster binding ligands and was anticipated to be a GTPase given the presence of N-terminal Walker A P-loop and Walker B Mg⁺² binding motifs. 138,139,141,142 Importantly, site directed mutagenesis studies have defined the [4Fe-4S] cluster binding motifs for the maturation proteins, as well as the GTPase binding region of HydF as required to achieve proper H-cluster assembly. 139 These observations have led to the development of a hypothetical pathway for H-cluster assembly that served as a platform for biochemical studies (Scheme 1.1). 143

In this proposal, HydE and HydG were involved in synthesizing the 2Fe subcluster on the scaffold HydF through the modification of a [2Fe-2S] cluster moiety; alkylation of the sulfide groups was thought to be a first step followed by the decomposition of a

Scheme 1.1. Preliminary Working Proposal for [FeFe]-Hydrogenase H-cluster 2Fe Subcluster Biosynthesis, based on Reference 143.
glycyl radical into CO and CN\textsuperscript{−}. It was proposed that HydE, HydF, and HydG were solely directed at 2Fe subcluster synthesis as standard Fe-S cluster assembly machinery could be expected to synthesize the [4Fe-4S] cubane of the H-cluster.\textsuperscript{143}

HydA Expressed Without HydE, HydF, and HydG Contains a [4Fe-4S] Cluster

All [FeFe]-hydrogenases contain a common active site domain but have a variety of distinct arrangements of accessory cluster domains.\textsuperscript{144} The simplest [FeFe]-hydrogenases from chlorophycean algae, such as \textit{Chlamydomonas reinhardtii}, contain only the active site domain,\textsuperscript{145-147} and accordingly have become attractive targets for H-cluster directed studies.\textsuperscript{148-152} Characterization of the \textit{C. reinhardtii} HydA enzyme expressed in \textit{E. coli} in the absence of hydE, hydF, and hydG demonstrated that the purified protein contained UV-visible, EPR, and Mössbauer spectroscopic features characteristic of [4Fe-4S]\textsuperscript{2+/+} clusters.\textsuperscript{149} Moreover, neither the as purified nor metal free forms of HydA\textsuperscript{AEFG} were active towards H\textsubscript{2} production, but activity could readily be restored either through addition of \textit{E. coli} cell-free lysates containing \textit{C. acetobutylicum} HydE, HydF, and HydG, or addition following chemical reconstitution of the [4Fe-4S] cluster. The results provided evidence for the requirement of the pre-assembled [4Fe-4S] cubane of the H-cluster (presumably synthesized by standard iron sulfur cluster assembly machinery) prior to activation by the hydrogenase maturation enzymes.\textsuperscript{149}

The nature of the immature [FeFe]-hydrogenase, as well as insights into its activation, were clarified by the X-ray crystal structure of \textit{C. reinhardtii} HydA\textsuperscript{AEFG} (PDB ID 3LX4).\textsuperscript{152} This structure showed the presence of the [4Fe-4S] cubane of the H-cluster and an absence of electron density associated with the 2Fe subcluster. Together with the
spectroscopic data, the structure presented direct experimental evidence that HydE, HydF, and HydG are solely directed at 2Fe subcluster maturation. The HydA\textsuperscript{AEFG} structure showed the presence of an electropositive channel filled with H\textsubscript{2}O molecules leading to the active site; comparison with the CpI WT HydA structure suggests that the channel is formed by two conserved loop regions that are disordered.\textsuperscript{144,152} Collectively, the data advocated for the stepwise synthesis of the H-cluster, with 2Fe subcluster insertion into HydA\textsuperscript{AEFG} and structural rearrangement of the disordered loop regions over the channel enclosing the active site.\textsuperscript{144} Additional support for this mechanism of assembly was provided by recent NRVS and EPR data, where \textsuperscript{56}Fe containing CpI HydA\textsuperscript{AEFG} was activated \textit{in vitro} using \textsuperscript{57}Fe labeled HydE, HydF, and HydG lysate mixtures enriched with exogenous \textsuperscript{57}Fe.\textsuperscript{134} The \textsuperscript{57}Fe isotope becomes associated with the 2Fe subcluster of the activated hydrogenase and not the [4Fe-4S] cubane of the H-cluster, revealing that the cubane is not synthesized by the hydrogenase maturation machinery.

**HydF: Assembly Scaffold or Carrier Protein for Radical SAM Chemistry**

\textit{In vitro} activation experiments of HydA\textsuperscript{AEFG} demonstrated that H\textsubscript{2} evolution was only observed when the immature hydrogenase was mixed with a strain of \textit{E. coli} expressing HydE, HydF, and HydG in concert; activation of HydA\textsuperscript{AEFG} could not be attained when the maturation enzymes were either expressed individually or in varying combinations.\textsuperscript{140,149} Analysis of \textit{C. acetobutylicum} HydE, HydF and HydG proteins purified from \textit{E. coli} based co-expressions in which all three proteins were present (HydE\textsuperscript{FG}, HydF\textsuperscript{EG} and HydG\textsuperscript{EF}) revealed that as-purified HydF from this genetic background activated HydA\textsuperscript{AEFG}, whereas HydF expressed in the absence of HydE and
HydG (HydF<sup>ΔEG</sup>) could not. These observations established that HydF acts as a scaffold or carrier protein transferring an H-cluster like species to HydA in the final step of hydrogenase maturation.

Given the presence of putative Fe–S cluster binding residues in HydF sequences and the requirement of these ligands in achieving hydrogenase maturation, it was postulated that this enzyme bound an Fe-S cluster that was somehow directly involved in the H-cluster assembly process. Several studies have now probed Fe-S cluster binding in both wild type and variant forms of heterologously and homologously expressed HydF from <i>T. maritima</i>, <i>C. acetobutylicum</i>, <i>Thermotoga neapolitana</i>, and <i>Shewanella oneidensis</i>. Low temperature EPR studies on <i>C. acetobutylicum</i> HydF<sup>EG</sup> and HydF<sup>ΔEG</sup> proteins heterologously expressed in <i>E. coli</i> revealed two overlapping cluster signals in samples photoreduced using 5-deazariboflavin. Based on temperature dependence studies, the more prominent signal in samples of both HydF<sup>EG</sup> and HydF<sup>ΔEG</sup> was assigned to a fast relaxing [4Fe-4S]<sup>+</sup> cluster (g = 1.89, 2.05), while the overlapping feature was a slower relaxing signal attributed to a [2Fe-2S]<sup>+</sup> cluster (g = 2.00, 1.96). FTIR studies on the HydF<sup>EG</sup> and HydF<sup>ΔEG</sup> proteins revealed Fe-CO (1940 and 1881 cm<sup>-1</sup>) and Fe-CN (2046 and 2027 cm<sup>-1</sup>) vibrations in as purified HydF<sup>EG</sup>, bands which were clearly absent in HydF<sup>ΔEG</sup>. Similarly, Czech and coworkers observed a high field component in the EPR spectrum of homologously expressed <i>C. acetobutylicum</i> HydF<sup>EG</sup> that exhibited slower relaxation and was attributed to a Fe-S species belonging “to a cluster which contains three or less irons”; additionally, Fe–CN, Fe–CO, and Fe–CO–Fe species were observed in FTIR spectra, fully consistent with a binuclear nature of the H-cluster intermediate bound to this enzyme. Interestingly, purified <i>S. oneidensis</i>
HydF\textsuperscript{EG} displays very low hydrogen reduction/oxidation activity, behavior consistent with the presence of a 2Fe subcluster like moiety.\textsuperscript{160} XAS studies of \textit{C. acetobutylicum} HydF provided evidence for [4Fe-4S] and [2Fe-2S] clusters bound to HydF\textsuperscript{AEG}, while the iron species on HydF\textsuperscript{EG} were highly similar to those in HydA, leading the authors to suggest that the 2Fe subcluster bound by HydF was directly bridged to the [4Fe-4S] cluster in a manner analogous to the H-cluster itself.\textsuperscript{156} Taken collectively, these results help to clarify the nature of the H-cluster like moiety bound to HydF and point to a role for HydE and HydG in modifying a [2Fe-2S] cluster precursor into the 2Fe subcluster.\textsuperscript{27,161}

The nature of 2Fe subcluster binding to HydF is currently unresolved, although biomimetic studies using synthetic analogs of the 2Fe moiety have shown that these entities can be loaded into \textit{T. maritima} HydF.\textsuperscript{159} In addition, spectroscopic characterization provides evidence for the unique coordination of a CN\textsuperscript{-} ligand where the carbon atom binds to an Fe ion of the [4Fe-4S] cubane while the nitrogen atom complexes the 2Fe unit;\textsuperscript{159} further studies are warranted to determine if this unique bridging coordination exists in the natural enzyme. It is notable that EPR data collected on the \textit{S. oneidensis} HydF\textsuperscript{AEG} protein show no evidence of [2Fe-2S]\textsuperscript{+} cluster coordination.\textsuperscript{115} This result coupled with the observation that low levels of hydrogenase activation could be attained using \textit{in vitro} cell lysate mixtures that lacked either HydE or HydF (but contained HydG) led to the proposal that HydG itself synthesizes the 2Fe subcluster (termed HydG-co) which is subsequently transferred to HydF and then HydA\textsuperscript{AEG} to accomplish hydrogenase maturation.\textsuperscript{115} Additional experimental evidence is
needed to demonstrate whether the 2Fe subcluster is first built on HydG or is directly synthesized on HydF.

**HydE: Dithiolate Ligand Biosynthesis**

Early hypotheses regarding the function of the radical SAM enzyme HydE was tied to HydG in playing a similar role in the biosynthesis (Scheme 1.1). As is described in the section below, because the substrate and function of HydG have been determined, HydE’s role may be in the synthesis of the non-protein, bridging dithiolate ligand. While some biochemical studies have been reported on HydE, little is resolved about its role in maturation and the substrate for this enzyme still remains to be identified. It is very likely that the substrate is a common metabolite, as [FeFe]-hydrogenase activation is readily accomplished in *E. coli* cell lysate mixtures coexpressing the three maturation genes. A report exploring the effect of exogenous additives on *in vitro* HydA<sup>ΔEFG</sup> activation levels discovered that both tyrosine and cysteine individually and cooperatively enhanced H<sub>2</sub> consumption levels. While the effects of tyrosine can be attributed to the activity of HydG, the stimulation obtained from cysteine addition may either be a consequence of HydE’s activity or is an artifact of more efficient cluster assembly due to the generalized Fe–S machinery present in the cell lysate.

One of the outstanding issues in H-cluster structure over the last decade has been the identity of the bridgehead atom in the dithiolate ligand, an especially important question when defining the precursor to this molecule. Initially modeled as 1,3-propanedithiolate, the assignment was quickly revised to dithiomethylamine given the ability of the amine functionality to act as a proton donor/acceptor. While
computational studies suggested that the dithiolate ligand could be dithiomethylether. Spectroscopic, computational, and functional biomimetic studies have demonstrated unequivocally a dithiomethylamine ligand. Along these lines, it is plausible that HydE generates a carbon based radical species upon H atom abstraction by the 5′-dAdo• radical that then reacts with the sulfide groups of a coordinated [4Fe-4S] or nearby [2Fe-2S] cluster that may be similar to LipA and BioB; it is also possible that the sulfur atoms of the bridging dithiolate are derived from the substrate molecule itself.

HydE structural characterization has shown that it accommodates a full \((\alpha\beta)_8\) TIM barrel fold (Figure 1.11). Elucidation of the SAM-bound and methionine/5′-dAdo-bound structures have provided insight toward the active site in two states, where SAM might be utilized as a cofactor. HydE shares significant sequence similarity with PylB (with a root mean square deviation value of only 1.3 Å) and BioB, and contains an accessory cluster binding site near the protein surface located 20 Å away from the

![Figure 1.11. HydE Crystal Structure (PDB 3IIZ). Fe–S clusters are colored in chocolate and yellow-orange. Cysteines and arginine involved in ligating clusters are depicted in sticks. SAM coordinated to the site-differentiated [4Fe-4S] cluster is depicted in sticks with carbons colored black, oxygens colored red, and nitrogens colored blue.](image-url)
radical SAM cluster (Figure 1.11). Three cysteine residues and a water molecule act to coordinate an auxiliary Fe–S cluster, although occupancy of this cluster is highly variable. Notably, the cysteines used to coordinate this cluster (Cys311, Cys319, and Cys322) are only conserved in ~ 48% of available HydE sequences; substitution of these residues to alanine showed no adverse effects towards hydrogenase activation in whole cell extracts, suggesting that this auxiliary cluster plays no role in the H-cluster maturation process. Intriguingly, the HydE structure reveals a large internal electropositive cavity that spans the full length of the (αβ)$_8$ barrel and is the site of three distinct anion binding sites. Thiocyanate was discovered to bind with high affinity in the third anion-binding site at the bottom of the barrel. It still remains unclear as to why HydE would have such high affinity for thiocyanate, although this observation may define a pathway wherein substrate reacts at the top of barrel near the radical SAM cluster and the product molecule then migrates to the bottom of the barrel for transfer to either HydF or HydG.

The hydrogenase maturation enzymes are part of an interactive machinery, where each interacts with one another in an intimate fashion as part of the biosynthesis. An early reaction sequence was proposed where the alkylation of the sulfide ions of a [2Fe-2S] cluster was suggested to precede CO and CN$^-$ ligand addition, as modification of the sulfides would protect these groups against further alteration and shift chemical reactivity towards the Fe ions (Figure 1.11). This in turn suggested that the enzyme responsible for dithiolate ligand biosynthesis (HydE) likely acts first. Recent surface plasmon resonance studies support this hypothesis, where HydE was found to bind to HydF with a $K_D$ value of $9.19 \times 10^{-8}$ M, an order of magnitude lower value than the $K_D$
for HydG binding to HydF (1.31 x 10^{-6} \text{ M}). Interestingly, HydG is unable to displace HydE when it is bound to HydF and that HydG and HydE do not appear to interact with one another. Additional experiments aimed at probing the GTPase functionality of HydF show that GTP addition to either the HydF-HydE or HydF-HydG complexes during dissociative phases results in an increase in the rates of dissociation, suggesting that the GTPase activity of HydF promotes displacement of the interacting partners. In our own work, HydE and HydG both independently stimulate HydF GTP hydrolysis rates, suggesting that GTP binding and/or hydrolysis may act to gate the interactions between the other maturation proteins during 2Fe subcluster assembly. These data certainly speak towards the tightly controlled stepwise reaction between HydE and HydG with HydF, and the observation of the high binding affinity between HydE and HydF is certainly not surprising given the existence of fused \textit{hydEF} genes in certain organisms.

**HydG: Similar to ThiH**

Relative to HydE, significant progress in understanding HydG catalysis has been made. Early analysis of \textit{T. maritima} HydG demonstrated that the reconstituted enzyme bound up to 4 irons and 5 sulfides per protein, contained a $S = 1/2 \ [4\text{Fe-4S}]^{+}$ cluster upon dithionite reduction, and cleaved SAM nonproductively. However, HydG exhibited considerable (27%) amino acid sequence homology with ThiH, a radical SAM enzyme that forms $p$-cresol and dehydroglycine (DHG) from tyrosine during thiamine biosynthesis. Driven by this sequence similarity, Pilet and coworkers confirmed that in the presence of tyrosine, HydG exhibited enhanced rates of SAM cleavage, a hallmark attribute of SAM enzymes when assayed in the presence of their substrates.
Further, by using LC-MS techniques the authors verified that $p$-cresol was formed during catalysis.\textsuperscript{175} Independent support for the role of tyrosine in H-cluster biosynthesis came from \textit{in vitro} $\text{HydA}^{\Delta\text{EFG}}$ activation experiments that monitored $\text{H}_2$ consumption levels following treatment with cell extracts containing $\text{HydE}$, $\text{HydF}$, and $\text{HydG}$; while either exogenously added tyrosine or 3,4-dihydroxy-L-phenylalanine resulted in stimulated levels of $\text{H}_2$ depletion, other tyrosine analogs lacking a $p$-hydroxyl group failed to provide any stimulation.\textsuperscript{162} This observation led to the hypothesis that the initial H atom abstraction event by the $5'$-dAdo• radical occurred at the \textit{para}-position on the phenyl ring, similar to the proposed mechanism for ThiH.\textsuperscript{176,177}

Biochemical characterization of the ThiH enzyme, by commonality of substrate, has served as a foundation for understanding HydG’s catalytic activity. ThiH catalyzes DHG formation as part of a multistep process involving ThiG, ThiF, ThiI, IscS, and 1-deoxyxyulose 5-phosphate (Dxp) (Figure 1.12).\textsuperscript{178} Early characterization of ThiH showed that it purified with nearly 1:1 stoichiometry with ThiG.\textsuperscript{179} Importantly, thiazole synthase activity in \textit{E. coli} lysate mixtures was stimulated upon addition of purified ThiGH, SAM, and a reducing agent and depended upon addition of tyrosine, leading to the proposal that the deoxyadenosyl radical was responsible for initiating tyrosyl radical formation.\textsuperscript{176} Turnover experiments performed with L-[U-$^{14}$C]-tyrosine and S-adenosyl-L-[methyl-$^{14}$C]-methionine showed that concurrent consumption resulted in the formation of two radiolabeled products, $p$-cresol and glyoxylate (Figure 1.12).\textsuperscript{174} Kinetics experiments further demonstrated the reaction stoichiometry proceeded with formation of 1.3 equivalents of $5'$-dAdo to 1 equivalent of $p$-cresol and glyoxylate, respectively.
Figure 1.12. Proposed Mechanism of Radical SAM Enzyme ThiH Substrate Radical Initiation and Propagation. The heterolytic cleavage mechanism is depicted, but it is also possible that a homolytic cleavage mechanism forming $p$-quinone methide and a glycyl radical that oxidizes to make dehydroglycine (DHG) can occur. Adapted from reference 174. Aspects of the mechanism left of the dotted line are performed in proximity to the radical SAM [4Fe-4S] cluster.

Interestingly, kinetics for both ThiGH and ThiH were observed to be biphasic in nature, with a burst preceding a slower steady-state phases of $53 \pm 6 \times 10^{-4}$ s$^{-1}$ and $1.6 \pm 0.2 \times 10^{-4}$ s$^{-1}$, respectively, for ThiGH formation of $p$-cresol, suggesting product release was rate limiting.\(^\text{177}\) During the burst phase, efficient coupling of 5'-dAdo production to tyrosine cleavage occurs, but during the steady-state phase the uncoupled cleavage of SAM increases dramatically as the tyrosine kinetics become more strongly influenced by the accumulation of products.

Glyoxylate formation occurs following the hydrolysis of dehydroglycine (DHG) (Figure 1.12).\(^\text{174}\) Interestingly, addition of exogenous glyoxylate and ammonia to ThiH assay mixtures inhibited tyrosine turnover, possibly as a consequence of glyoxylate (or DHG) binding in the active site. Challand et al. have suggested that the hydrolysis event
might modulate uncoupled cleavage of SAM as a mechanism ensuring DHG production and incorporation into the thiazole carboxylate by ThiH and ThiG.\textsuperscript{177} DHG formation has been proposed to occur through two mechanisms, both initiated by an H-atom abstraction from the hydroxyl group on the phenol moiety of tyrosine by the 5’-deoxyadenosyl radical.\textsuperscript{174} Assays performed with several tyrosine analogs have shown a strict dependence on the phenol group for SAM cleavage.\textsuperscript{177} The resulting tyrosyl radical then undergoes C\textsubscript{\(\alpha\)}-C\textsubscript{\(\beta\)} bond cleavage through a heterolytic process forming dehydroglycine directly or a homolytic process forming a glycyl radical which may oxidize to dehydroglycine.\textsuperscript{174}

While HydG and ThiH use a common substrate and a common radical-initiated chemistry to facilitate their biochemical transformation, they principally differ in their products formed. HydG contains an additional C-terminal 88 amino acid extension lacking in ThiH, which contains an additional CX\textsubscript{2}CX\textsubscript{22}C motif.\textsuperscript{175,180} Some variation in the ligation of the C-terminal cluster is predicted to exist, but the first and last cysteine in the motif are conserved in homologs identified.\textsuperscript{169} In vitro work that followed

![Figure 1.13. HydG-catalyzed Cyanide Formation Detected by Derivatization. Trapping of synthesized cyanide to the fluorescent 1-cyanobenz[f]isoindole compound was performed, and was reported in Reference 181. Detection constituted the first report that HydG synthesizes diatomic ligands from tyrosine.](image-url)
identification of tyrosine as a substrate of HydG, however, demonstrated that CN\(^-\) was produced in a 1:1 stoichiometric ratio with \(p\)-cresol in the HydG-catalyzed reaction, indicating that HydG synthesizes the CN\(^-\) ligands of the H-cluster.\(^{181}\) Enzymatic assays of HydG reconstituted with Fe and sulfide showed turnover-dependent formation of the fluorescent CN\(^-\) adduct 1-cyanobenz[f]isoindole over time (Figure 1.13).\(^{181}\) Importantly, the amount of CN\(^-\) correlated well to \(p\)-cresol production; quantified 5’-deoxyadenosine was found in slight excess relative to both \(p\)-cresol and CN\(^-\), representing partial uncoupled AdoMet cleavage. Studies with uniformly-labeled \(^{13}C,^{15}N\)-tyrosine resulted in a CN\(^-\) adduct with an increase in mass of two m/z units, reflecting incorporation of the \(^{15}N\) amino and \(^{13}C\)\(-\alpha\)-carbon of tyrosine.\(^{181}\)

**Research Directions**

The work presented in this dissertation was directed by four primary goals. The first objective of these studies was to elucidate the functional role of the radical SAM enzyme HydG in the [FeFe]-hydrogenase H-cluster biosynthetic scheme. Early hypotheses regarding the biosynthetic roles of the radical SAM enzymes HydE and HydG were limited by identifying the specific chemistry catalyzed by the enzyme, in particular the substrate consumed by either enzyme (Scheme 1.1). Subsequent identity of HydG’s functional role was skewed by a lack of thorough characterization of all products.\(^{175}\) Pursuing our own investigation, we showed that the previously undetected CO product was formed in addition to CN\(^-\).\(^{114,181}\) This foundational work has defined the roles played by the radical SAM enzymes in the H-cluster biosynthetic scheme.
The second objective of these studies was to provide mechanistic insight into the biosynthesis of the diatomic ligands CO and CN\(^-\) at the level of the role the coordinated Fe–S clusters serve in catalysis. HydG catalysis is facilitated at two site-differentiated [4Fe-4S] clusters; one serves as the radical SAM [4Fe-4S] cluster involved with SAM cleavage to generate the 5’-dAdo•, while a second site-differentiated [4Fe-4S] cluster participates by undefined role. Preliminary mutational studies suggested that the second cluster was not strictly involved in diatomic ligand formation.\(^{180,182}\) Our work has extended the preliminary study to understand the role of the Fe–S clusters and the role of substrate binding to respective clusters.\(^{101}\)

In addition, HydG catalyzes diatomic ligand formation under conditions where the products themselves can form an organometallic product from HydG. Part of a larger interest to understand how the diatomic ligands are transferred to the hydrogenase active site, we have employed a novel approach to understand organometallic complex formation by HydG, by characterizing exogenous diatomic ligand binding to HydG Fe–S clusters. A different approach has been undertaken by others to understand the mechanism of HydG product formation,\(^{102}\) but collectively the approaches complement an understanding of product binding to HydG Fe–S clusters.

The third objective of these studies was to obtain a mechanistic basis for the stoichiometry of diatomic ligand formation. CN\(^-\) product formation was found to be stoichiometric to other byproducts of catalysis, however CO product formation was not.\(^{114,181}\) Methods to improve catalysis along the confines of which the cyanide assay occurred resulted in negligible CO formation, which implicated an effect of small molecules on the interaction of HydG Fe–S clusters and respective catalysis. This work
has mechanistic significance to the catalytic mechanism of diatomic ligand formation, because formation of CO and CN\(^{-}\) from the DHG intermediate involves an oxidative decarbonylation mechanism to form CN\(^{-}\) and CO that may be affected by small molecules that interact with the C-terminal cluster, including products or substrates that are part of the overall biosynthesis.

Lastly, the fourth objective of these studies was to extract mechanistic detail regarding the involvement of organic radical in the diatomic ligand biosynthetic mechanism. CO and CN\(^{-}\) formation from tyrosine involves the cleavage of two C–C bonds. Tyrosine substrate radical generation by H atom abstraction from the 5’-dAdo\(^{•}\) is sufficient to cleave the C\(_{\alpha}\)–C\(_{\beta}\) bond, but the events that result in cleavage of the second bond are largely unclear, due to the requirement of a second Fe–S cluster. The work depicted herein documents our efforts to understand the mechanism of abstraction; the tyrosine 4-phenol group is the abstraction site, but also undergoes solvent exchange. Our work has shown that SAM is regenerated as part of abstraction mechanism, but also is consumed as a substrate. Additional mechanistic approaches have been employed to understand radical propagation by using tyrosine substrate analogues as well as site-directed mutants that putatively interact with the generated 5’-dAdo\(^{•}\). Collectively, the results in this dissertation provide a deeper understanding of the mechanism of diatomic ligand formation by the HydG in the biosynthesis of the H-cluster 2Fe subcluster.
References Cited


Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

Author: Eric M. Shepard

Contributions: Performed preliminary UV-Vis and EPR spectroscopies on as-isolated and Fe–S reconstituted HydG. Collected EPR data and performed EPR spectral simulations. Performed preliminary activity experiments with non-CO productive HydG in the presence and absence of hemoglobin and performed product analysis by HPLC. Worked with B.R.D. to carry out experiments with CO-productive HydG. Prepared FTIR samples. Generated figures and co-wrote the manuscript.

Co-Author: Benjamin R. Duffus

Contributions: Prepared HydG enzyme by which CO product evolution could be detected. Performed initial activity experiments with CO-producing HydG enzyme, and performed preliminary kinetic characterization of the active enzyme. Assisted in product characterization with CO-productive HydG by HPLC. Generated figures and co-wrote the manuscript.

Co-Author: Simon J. George

Contributions: Performed stopped-flow FTIR spectroscopy with HydG samples incubated with $^{13}$C,$^{15}$N Tyr isotopologues and hemoglobin. Generated a figure for the manuscript.

Co-Author: Shawn E. McGlynn

Contributions: Contributed to the design of the study, discussed results, and edited the manuscript.

Co-Author: Martin R. Challand

Contributions: Contributed to the design of the study, discussed results, and edited the manuscript.
Co-Author: Kevin D. Swanson

Contributions: Assisted in developing methodology for detecting enzymatically generated CO with hemoglobin.

Co-Author: Peter L. Roach

Contributions: Discussed results and implications and provided comments to the manuscript.

Co-Author: Stephen P. Cramer

Contributions: Discussed the results and the implications of the FTIR data, and edited the manuscript.

Co-Author: John W. Peters

Contributions: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

Co-Author: Joan B. Broderick

Contributions: Devised the idea for using Hb as a probe of CO production. Directed experimental design and interpretation. Obtained funding and resources, and co-wrote the manuscript.
CHAPTER 2

[FEFE]-HYDROGENASE MATURATION: HYDG-CATALYZED SYNTHESIS OF CARBON MONOXIDE

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Abstract

Biosynthesis of the unusual organometallic H-cluster at the active site of the [FeFe]-hydrogenase requires three accessory proteins, two of which are radical AdoMet enzymes (HydE, HydG) and one of which is a GTPase (HydF). We demonstrate here that HydG catalyzes the synthesis of CO using tyrosine as a substrate. CO production was detected by using deoxyhemoglobin as a reporter and monitoring the appearance of the characteristic visible spectroscopic features of carboxyhemoglobin. Assays utilizing $^{13}$C-
tyrosine were analyzed by FTIR to confirm the production of HbCO and to demonstrate that the CO product was synthesized from tyrosine. CO ligation is a common feature at the active sites of the [FeFe], [NiFe], and [Fe]-only hydrogenases; however, this is the first report of the enzymatic synthesis of CO in hydrogenase maturation.

The organometallic metal centers at the active sites of all known hydrogenases are unique in biology. In addition to containing unusual ligands such as carbon monoxide (CO) and cyanide (CN⁻), these organometallic centers serve as the catalytic sites for the reversible reduction of protons, a reaction central to both early and present-day microbial life and future bioenergy applications. The unique H-cluster of the [FeFe]-hydrogenase is comprised of a [4Fe-4S] cluster bridged by a cysteinyl residue to a 2Fe cluster coordinated by three CO, two CN⁻, and a bridging dithiolate (Chart 2.1). Three accessory proteins, two radical S-adenosylmethionine (AdoMet) enzymes (HydE and HydG) and a GTPase (HydF), are necessary for the synthesis of the 2Fe subcluster of the

![Chart 2.1 Overall Structure and H-Cluster of the [FeFe]-Hydrogenase from Clostridium pasteurianum](image)
HydF serves as the carrier for an H-cluster precursor that is transferred to the structural protein to yield an active hydrogenase. HydG has been shown to catalyze the cleavage of tyrosine to produce \( p \)-cresol, and we have recently demonstrated that this tyrosine cleavage leads to formation of \( \text{CN}^- \). We report here that HydG also catalyze formation of CO, monitored via binding of CO to deoxyhemoglobin. These results provide the first example of enzymatic production of CO in the maturation of any hydrogenase.

HydG was heterologously produced in \textit{Escherichia coli} BL21 using the plasmid pCDF::HydG-his. The protein was purified in an anaerobic chamber (Coy). Anaerobically purified HydG contains up to 2.8 Fe/protein and exhibits visible and paramagnetic resonance (EPR) spectroscopic properties consistent with the presence of iron–sulfur clusters, with the EPR spectra suggestive of the presence of both [4Fe-4S] and [2Fe-2S] clusters (Appendix Figure A.1, A.2). Reconstitution of this as-isolated enzyme with iron and sulfide produced protein containing up to \( 8.7 \pm 0.7 \) Fe/protein and was absolutely necessary in order to observe the catalytic activity observed here. These iron numbers, together with the conservation of two cysteine motifs in HydG, suggest that holo-HydG contains two iron–sulfur clusters, a possibility that has also been suggested by others. Our reduced reconstituted HydG shows a single fast-relaxing signal (\( g = 2.03, 1.92, 1.90 \)) characteristic of \( [4\text{Fe-4S}]^+ \) clusters (Figure 2.1) and similar to a recently reported EPR spectrum of HydG. Addition of AdoMet to the reduced reconstituted enzyme splits this signal into two distinct rhombic signals (\( g_{\text{cluster1}} = 2.02, 1.93, 1.91 \) and \( g_{\text{cluster2}} = 2.00, 1.87, 1.83 \)), presumably arising from two different iron–sulfur clusters on HydG. Both signals have a temperature dependence characteristic of
Figure 2.1 X-band EPR Spectra of Reduced Reconstituted HydG. (A) Reduced HydG in the absence (65 µM enzyme, 8.7 ± 0.7 Fe/protein, black line) and in the presence (65 µM enzyme, 8.7 ± 0.7 Fe/protein, red line) of 1 mM AdoMet. EPR parameters: 12 K, 9.37 GHz, 1.84 mW. (B) Temperature dependence of reduced HydG in the presence of AdoMet. EPR parameters as in (A) with temperatures indicated.

[4Fe-4S]$^+$ rather than [2Fe-2S]$^+$ clusters (Figure 2.1 and Appendix Figure A.3).

Assays for HydG-catalyzed CO formation were carried out in an anaerobic chamber (Mbraun, O$_2$ < 1 ppm) and sealed in 1.4 mL anaerobic cuvettes. Reconstituted HydG was assayed for CO production using deoxyhemoglobin (deoxyHb) as a reporter. Assays contained HydG (20–65 µM) in buffer (50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol) containing 1–4.8 mM dithionite. DeoxyHb was added to a final concentration of ~10 µM heme. UV–visible difference spectra before and after addition of deoxyHb were used to quantify deoxyHb, which shows characteristic $\lambda_{max}$ values at 430 (Soret band) and 555 nm (visible band). Tyrosine was then added, and the assay was initiated by addition of AdoMet. The results (Figure 2.2) reveal formation of HbCO, dependent on the presence of HydG, tyrosine, and AdoMet. The formation of HbCO is evidenced by the shift in the Soret band (430 to 419 nm) and by the shift and splitting of the 555 nm
Figure 2.2 HydG-dependent CO Formation. (A) Time-dependent conversion of deoxyHb to HbCO as monitored by changes in the Soret and visible bands (top inset). Reaction contained 26 \( \mu \text{M} \) HydG (6.4 ± 0.2 Fe/protein), 4 mM dithionite, 1 mM Tyr, 1.1 mM SAM, and 9.2 \( \mu \text{M} \) heme (as Hb). Bottom inset shows FTIR spectra of HbCO formed from \(^{13}\text{C-tyrosine (a)}\) and a control spectrum (b) in which \(^{13}\text{CO}\) was added to Hb (see Appendix A for details). (B) Single-wavelength (419 nm) kinetics illustrating the biphasic nature of HbCO formation at 30 °C. Arrows in panel B represent where single wavelength kinetics scan was paused and aliquots were removed for HPLC analysis (C and Appendix A). Reaction contained 45 \( \mu \text{M} \) HydG (7.2 ± 0.2 Fe/protein), 10 \( \mu \text{M} \) heme, 1 mM AdoMet, and 500 \( \mu \text{M} \) tyrosine.

band, which together give rise to a visible spectrum characteristic of HbCO. \(^{13}\) Tyrosine was confirmed as the source of CO by observing the appropriate isotopic shifts of the
HbCO FTIR spectra for reactions using U-\textsuperscript{13}C-tyrosine as the substrate (Figure 2.2 and Appendix Figure A.6).

Reconstituted HydG with 8.7 ± 0.7 Fe/protein provided the greatest levels of HbCO formation. Single-wavelength kinetic experiments at 419 nm showed biphasic HbCO formation, with a linear burst phase corresponding to $k_{\text{cat}} = 11.4 (±0.09) \times 10^{-4}$ s\textsuperscript{-1} and a slow phase with $k_{\text{cat}} = 1.71 (±0.01) \times 10^{-4}$ s\textsuperscript{-1} (Figure 2.2, Appendix Figure A.4). Although it was not possible to quantify both CN\textsuperscript{-} and CO under identical conditions in a single assay due to the particular requirements of each assay, the rate of the fast phase of HbCO formation is consistent with the rates we have previously reported for CN\textsuperscript{-} formation ($k_{\text{cat}} = 20 \times 10^{-4}$ s\textsuperscript{-1} at 37 °C), given the lower temperatures utilized in the current assays.\textsuperscript{10} The total amount of CO detected is limited by the quantity of deoxyHb present in the assay (~10 µM heme). Although attempts were made to utilize higher concentrations of deoxyHb (in shorter path length cuvettes due to the high extinction coefficient of the Soret band), we were unable to significantly improve the quantity of CO detected. Further, HPLC analysis reveals the formation of 5\textsuperscript{-}deoxyadenosine and p-cresol concomitant with CO (Figure 2.2). The quantities of these organic products are consistent with our previously reported CN\textsuperscript{-} assay but always exceed the detected quantities of CO, suggesting perhaps that, under our current assay conditions, CO synthesized by HydG is partially sequestered within the protein. Our CN\textsuperscript{-} assays utilized protein denaturation prior to CN\textsuperscript{-} analysis, which may account for the differences in total amounts of product detected.\textsuperscript{10} Efforts are underway to improve the yields of CO in these assays.
Although the three known types of hydrogenases ([FeFe]-, [NiFe]-, and [Fe]-) all contain CO ligands at their active sites, the work reported herein is the first demonstration of enzymatically catalyzed CO synthesis in the maturation of a hydrogenase. The results presented here, when coupled with our recent report that HydG catalyzes the formation of CN\(^-\) from tyrosine, point to a novel radical AdoMet reaction in which generation of an amino acid radical on tyrosine leads to decomposition into diatomic ligands; an analogous reaction was proposed in our original hypothesis for H-cluster assembly\(^{14}\). The radical-mediated interconversion between small molecules (CO and CN\(^-\)) and an amino acid is potentially an interesting link between biochemistry and prebiotic chemistry. The stoichiometric formation of \(p\)-cresol in the HydG-catalyzed reaction\(^{10}\) suggests that dehydroglycine may be an intermediate that undergoes chemically precedented decarbonylation\(^{15}\) to produce CO and CN\(^-\).

The diatomic ligands synthesized by HydG are presumably transferred to HydF, which has been shown to contain CO and CN\(^-\) ligands when expressed in the presence of HydE and HydG (Hyd\(^{EG}\)\(^{12,16}\)). Further, we have shown that HydA\(^{AEFG}\), which can be activated by HydF\(^{EG}\), contains a [4Fe-4S] cluster but lacks the 2Fe subcluster of the H-cluster.\(^{7,17,18}\) The cumulative data thus point to a mechanism for H-cluster assembly in which a 2Fe precursor to the H-cluster is assembled on HydF by the activities of HydE and HydG and is subsequently transferred to HydA.

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Supporting Information Available: Experimental procedures; spectroscopic and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org/, and can also be found in Appendix A.
References Cited


CHAPTER 3

BIOCHEMICAL AND KINETIC CHARACTERIZATION OF RADICAL S-ADENOSYL-l-METHIONINE ENZYME HYDG

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

Author: Rebecca C. Driesener

Contributions: Purified and characterized the HydG\textsuperscript{WT}, HydG\textsuperscript{C386S}, and HydG\textsuperscript{ΔCTD} enzymes in the study. Performed UV-visible absorption and EPR spectroscopies on the above enzymes. Obtained the Michaelis–Menten constants and apparent product turnover numbers, and quantified time-dependent product formation by HPLC. Optimized the assays detecting for cyanide and glyoxylate. Prepared figures and co-wrote the paper.

Co-Author: Benjamin R. Duffus

Contributions: Purified and characterized the HydG\textsuperscript{WT} and HydG\textsuperscript{C96/100/103A} enzymes employed in the study. Performed UV-visible absorption and EPR spectroscopic studies on the above enzymes. Performed CO formation assays on all variants included in the study. Performed and directed simulation of EPR spectra obtained from Southampton and Montana samples. Provided complementary product time course data to the published figures via HPLC analysis. Prepared figures and edited the manuscript.

Co-Author: Eric M. Shepard

Contributions: Purified and provided preliminary characterization of the HydG\textsuperscript{C386S} and HydG\textsuperscript{ΔCTD} enzymes employed in the study. Performed UV-visible absorption and EPR spectroscopies on the enzymes listed above, and performed preliminary CO formation assays on the HydG\textsuperscript{C386S}. Discussed simulation results, and edited the manuscript.

Co-Author: Ian R. Bruzas

Contributions: Performed site-directed mutagenesis to generate the HydG\textsuperscript{C96/100/103A} variant and assisted in preliminary purification of the enzyme. Assisted in the simulation of the EPR data.
Contribution of Authors and Co-Authors - Continued

Co-Author: Kaitlin S. Duschene
Contributions: Assisted in purifying HydG$^{C386S}$ and HydG$^{ΔCTD}$ enzymes for initial characterization in Montana.

Co-Author: Natalie J.-R. Coleman
Contributions: Assisted in the purification of the HydG variants prepared in Southampton.

Co-Author: Alexander P.G. Marrison
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Co-Author: Enrico Salvadori
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Co-Author: Christopher W.M. Kay
Contributions: Provided oversight in EPR spectral acquisition of Southampton-prepared HydG samples.

Co-Author: John W. Peters
Contributions: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

Co-Author: Joan B. Broderick
Contributions: Directed experimental design and interpretation. Obtained funding and resources, and edited the manuscript.

Co-Author: Peter L. Roach
Contributions: Directed experimental design and interpretation. Obtained funding and resources, and co-wrote the manuscript.
CHAPTER 3

BIOCHEMICAL AND KINETIC CHARACTERIZATION OF RADICAL S-
ADENOSYL-L-METHIONINE ENZYME HYDG

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Keywords: [FeFe]-Hydrogenase, S-adenosylmethionine, Carbon Monoxide, Cyanide

Abstract

The radical S-adenosyl-l-methionine (AdoMet) enzyme HydG is one of three maturase enzymes involved in [FeFe]-hydrogenase H-cluster assembly. It catalyzes L-tyrosine cleavage to yield the H-cluster cyanide and carbon monoxide ligands as well as p-cresol. Clostridium acetobutylicum HydG contains the conserved C\textsubscript{X3}C\textsubscript{X2}C motif coordinating the AdoMet binding [4Fe-4S] cluster and a C-terminal C\textsubscript{X2}C\textsubscript{X22}C motif
proposed to coordinate a second [4Fe-4S] cluster. To improve our understanding of the roles of each of these iron–sulfur clusters in catalysis, we have generated HydG variants lacking either the N- or C-terminal cluster and examined these using spectroscopic and kinetic methods. We have used iron analyses, UV-visible spectroscopy, and electron paramagnetic resonance (EPR) spectroscopy of an N-terminal C96/100/103A triple HydG mutant that cannot coordinate the radical AdoMET cluster to unambiguously show that the C-terminal cysteine motif coordinates an auxiliary [4Fe-4S] cluster. Spectroscopic comparison with a C-terminally truncated HydG (ΔCTD) harboring only the N-terminal cluster demonstrates that both clusters have similar UV-visible and EPR spectral properties, but that AdoMET binding and cleavage occur only at the N-terminal radical AdoMET cluster. To elucidate which steps in the catalytic cycle of HydG require the auxiliary [4Fe-4S] cluster, we compared the Michaelis–Menten constants for AdoMET and L-tyrosine for reconstituted wild-type, C386S, and ΔCTD HydG and demonstrate that these C-terminal modifications do not affect the affinity for AdoMET but that the affinity for L-tyrosine is drastically reduced compared to that of wild-type HydG. Further detailed kinetic characterization of these HydG mutants demonstrates that the C-terminal cluster and residues are not essential for L-tyrosine cleavage to p-cresol but are necessary for conversion of a tyrosine-derived intermediate to cyanide and CO.

Introduction

Hydrogenases catalyze the efficient formation of molecular hydrogen through reduction of protons. Three phylogenetically unrelated hydrogenase classes can be distinguished on the basis of their active site metal content: [FeFe]-, [NiFe]-, and [Fe]-
The [FeFe]-hydrogenase cofactor consists of a [4Fe-4S] cluster bridged to a 2Fe subcluster containing CO and cyanide ligands as well as a bis(thiomethy)amine bridge. Recent evidence indicates that the cofactor is assembled in a stepwise process, where the loaded 2Fe subcluster is transferred from HydF to the [FeFe]-hydrogenase protein already containing the [4Fe-4S] cluster. The demonstration that purified *Clostridium acetobutylicum* (Ca) HydF heterologously expressed in the background of Ca HydE and Ca HydG (HydFEG) is sufficient to activate the from *Clostridium pasteurianum* (CpI) heterologously expressed in the absence of the maturases led to the proposal of HydF as a scaffold protein. This was supported by the observation of cluster-bound cyanide and CO ligands in the Fourier transform infrared (FTIR) spectrum of HydFEG. The demonstration that *Desulfovibrio vulgaris* HydF does not interact with its putative [FeFe]-hydrogenase implies that the proposed action of HydF as a scaffold for H-cluster biosynthesis might not be strictly conserved. Similarly, *Shewanella oneidensis* HydG was the only maturase necessary for CpI [FeFe]-hydrogenase activation in the presence of a desalted *Escherichia coli* lysate and additional small molecule substrates like L-tyrosine (tyrosine hereafter) and AdoMet, suggesting that HydG might be acting like a scaffold protein. HydG has been shown to produce p-cresol, cyanide, and CO. Evidence that the diatomic molecules are incorporated into the H-cluster came from characteristic shifts in the vibrational cyanide and CO energies of the CpI [FeFe]-hydrogenase produced *in vitro* with natural abundance or isotopically labeled tyrosine. HydE has been proposed to have a role in the biosynthesis of the dithiolate bridge or translocation of the 2Fe subcluster, but despite
Scheme 3.1. Role of HydG in [FeFe]-Hydrogenase Cofactor Assembly

the availability of the crystal structure of HydE, the substrate still needs to be identified. Even with more information about [FeFe]-hydrogenase cofactor assembly and transfer becoming available, detailed mechanistic insights into HydG-based cyanide and CO ligand synthesis are lacking.

HydG and HydE both belong to the radical AdoMet superfamily of enzymes, which was initially characterized by a conserved N-terminal CX₃CX₂C motif and the requirement for a reduced [4Fe-4S]⁺ cluster and AdoMet for activity. Three iron atoms of this [4Fe-4S] cluster (cluster I) are coordinated by the conserved cysteines, whereas the fourth, unique iron is coordinated by the α-amino and α-carboxy groups of AdoMet. The transfer of a single electron from the cluster to AdoMet initiates homolytic C–S bond cleavage, generating a highly reactive 5’-deoxyadenosyl radical. This radical in turn abstracts a hydrogen atom from the substrate to generate
a substrate radical, which undergoes further rearrangement or cleavage to the final product(s).

Sequence analyses suggest that HydG belongs to a subclass of radical AdoMet enzymes that act on amino acids during cofactor biosynthesis\textsuperscript{16,30} and include tyrosine lyase (ThiH) for anaerobic thiamine biosynthesis,\textsuperscript{31-33} NocL and NosL for nosiheptide biosynthesis,\textsuperscript{34,35} and FbiC (CofG/CofH in cyanobacteria) for the biosynthesis of the F\textsubscript{420} cofactor.\textsuperscript{30} The sequence of HydG is 27\% identical with that of ThiH,\textsuperscript{15} and under anaerobic conditions, both cleave the C\textsubscript{\alpha}–C\textsubscript{\beta} bond of tyrosine in an AdoMet-dependent manner to yield \textit{p}-cresol.\textsuperscript{15,32} An energetically more favored homolytic cleavage reaction yields a C-centered glycine radical as the nonaromatic intermediate, while heterolytic cleavage results in the formation of dehydroglycine (Scheme 3.1).\textsuperscript{32} During turnover of ThiH, glyoxylate accumulates and is presumed to be the product of dehydroglycine hydrolysis,\textsuperscript{32} whereas little glyoxylate is formed during HydG catalysis.\textsuperscript{16} Instead, stoichiometric quantities of cyanide\textsuperscript{16} with respect to \textit{p}-cresol were detected as well as substoichiometric amounts of CO.\textsuperscript{17}

Initial spectroscopic characterization of chemically reconstituted \textit{Thermotoga maritima} HydG indicated the presence of one and possibly two [4Fe-4S] clusters.\textsuperscript{20} The primary sequence of \textit{Ca} HydG contains two cysteine motifs that both have been shown to be essential for activation of the [FeFe]-hydrogenase.\textsuperscript{36} While the radical AdoMet CX\textsubscript{3}CX\textsubscript{2}C triad at the N-terminus is well-conserved, the additional CX\textsubscript{2}CX\textsubscript{2}C motif at the C-terminus, proposed to coordinate the second [4Fe-4S] cluster (cluster II),\textsuperscript{36} displays some variation.\textsuperscript{37} For 301 HydG homologues investigated, only the first and last cysteine residue (C386 and C412, respectively, in the \textit{Ca} HydG sequence) of the C-
terminal motif were found to be conserved. Clear evidence of the presence of an accessory [4Fe-4S] cluster II came from iron content analyses and EPR spectra of reconstituted Ca HydG in the absence and presence of AdoMet.

Other radical AdoMet enzymes containing an auxiliary (mostly [4Fe-4S]) cluster have been identified and some studied in great detail. The functional importance of the HydG auxiliary cluster II in cyanide synthesis was inferred in a mutagenesis study: approximately 50 and 100% decreases in the amount of cyanide formation were observed for a Ca HydG SX2CX22C double mutant and a mutant lacking the entire C-terminal domain, including the cysteine motif (ΔCTD), respectively. In the mechanistic proposal for HydG (Scheme 3.1) the reaction step being affected by these mutations could have been either tyrosine cleavage (step A) or the synthesis of cyanide and CO (step B). This question was addressed by further studies in which Thermaanaerobacter tengcongensis (Tte) ΔCTD HydG was shown to have a tyrosine cleavage activity more than 98% reduced compared to that of wild-type (WT) enzyme, forming only 0.04 molar equiv (1 µM) of p-cresol. These very low levels of p-cresol may indicate poor turnover of tyrosine, and this may explain the apparent absence of detected cyanide. The lack of CO formation and the decreased iron content of the SX2SX22C and ΔCTD HydG variants compared to the values of the WT enzyme were rationalized by the proposal that cluster II is not required for cyanide but essential for CO synthesis. The proposal that the second cluster is required for the formation of a stable iron–carbonyl complex is consistent with the substoichiometric detection of CO formed by WT HydG.

In this study, we prepared an N-terminal C96/100/103A mutant that cannot coordinate the radical AdoMet [4Fe-4S] cluster, a single C386S mutant, and a C-terminal
deletion mutant. Characterization of WT HydG and these mutants by iron, UV-visible, and EPR spectral analyses supports a hypothesis in which the C-terminal CX₂CX₂₂C motif coordinates an auxiliary [4Fe-4S] cluster II, which is unable to coordinate AdoMet. To establish the role of the auxiliary cluster II and C-terminal residues during tyrosine cleavage, the formation of p-cresol, cyanide, CO, and glyoxylate by WT HydG and C-terminal mutants has been compared. A prerequisite for these studies was the determination of the respective Michaelis-Menten constants for the AdoMet and tyrosine substrates. The Michaelis-Menten constants permitted the use of near-saturating substrate concentrations upon comparison of WT and mutant HydG enzymes, which in turn allowed us to assess the effect of mutations on the rate of reaction and on apparent substrate binding. Our results indicate that mutations affecting cluster II coordination are, at worst, mildly deleterious to the rate of reductive AdoMet cleavage and cleavage of tyrosine to p-cresol. Proteins lacking cluster II formed no detectable CO, highlighting the importance of the C-terminal cluster for CO formation.

**Materials and Methods**

Except where otherwise stated, chemicals were reagent grade or better, purchased from commercial sources, and used without further purification. All solutions were prepared inside an anaerobic glovebox using deoxygenated buffers unless stated otherwise. Expression of *E. coli* AdoMet synthetase was conducted using overproducing strain DM22 (pK8) as described previously, with slight modifications. Enzymatically synthesized AdoMet was estimated by ¹H nuclear magnetic resonance to be 95% biologically active S,S-AdoMet and by HPLC analysis to contain <0.05%
adenine and <1.5% 5’-methylthioadenosine and was used throughout these studies unless otherwise state to prevent impurities in commercial AdoMet samples from inhibiting HydG. The concentration was determined using its extinction coefficient ($\varepsilon_{260}$) of 15400 M$^{-1}$cm$^{-1}$. Protein concentrations were determined using the Bradford assay and bovine serum albumin as a standard. Tyrosine solutions were prepared by addition of 25.7 mM stock in 200 mM HCl (350 µL) to 1 M NaOH (80 µL) and buffer D [(50 mM HEPES and 0.5 M KCl (pH 7.4, 20 µL)], giving a 20 mM stock solution that was further diluted as needed.

Site-Directed Mutagenesis

Mutant genes *hydG_C96/100/103A*, *hydG_C386S*, and *hydG_ΔCTD*, were prepared using a Quikchange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene or Agilent Technologies, La Jolla, CA) using the *Ca hydG_WT* gene as a template. Incorporation of the mutations and the absence of secondary mutations were confirmed by sequencing (Eurofins UK or Idaho State University Molecular Research Core Facility, Pocatello, ID).

Protein Expression, Purification, and Reconstitution

The pCDFDuet-1 plasmids encoding HydG variants from *C. acetobutylicum* were transformed into *E. coli* BL21(DE3) (Stratagene) and overexpressed as described previously, with slight modifications. Briefly, single colonies obtained from transformations were grown overnight in LB medium and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose, and 50
mM potassium phosphate buffer (pH 7.2). The cultures were grown at 37 °C while being shaken at 225 rpm until the OD_{600} reached 0.5, at which point 0.06g/L ferrous ammonium sulfate (FAS) and isopropyl β-D-thiogalactopyranoside (final concentration of 1 mM) were added. The cultures were grown for an additional 2.5 h at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N\textsubscript{2} overnight. Cells were harvested by centrifugation, and the resulting cell pellets were stored at -80 °C until further use. Protein expressions in Southampton were conducted as described previously,\textsuperscript{16} but without the addition of FAS. Protein purifications were conducted as previously published\textsuperscript{16} with the following modifications. Cell lysis was performed in the absence of PMSF, while sonication was achieved over five or six 10 min cycles of 1 s bursts (20 W). Buffer-exchanged protein fractions were pooled and concentrated to 30-40 mg/mL and flash-frozen in liquid N\textsubscript{2}. Protein reconstitution was achieved by dropwise addition (over 20 min) of FeCl\textsubscript{3} followed by Na\textsubscript{2}S•9H\textsubscript{2}O (10 equivalents for WT and C386S HydG and 5 equivalents for ΔCTD HydG) as 20 mM stock solutions in buffer C [20 mM HEPES, 0.5 M NaCl, %\%(w/v) glycerol, and 5 mM DTT (pH 7.4)] to protein solutions previously supplemented with DTT (5 mM). After the mixture had been gently stirred for 2 hours, precipitated protein and excess iron sulfide were removed by centrifugation (SS-34, 13000 rpm, 4 °C, 20 min), and the protein was flash-frozen in liquid N\textsubscript{2} and stored as 0.5 mL aliquots at -80 °C until further use. Prior to experiments, proteins were freshly desalted into the required buffer via a pre-equilibrated PD-10 column packed with Sephadex G-25 (GE Healthcare). The efficiency of chemical reconstitution was then assessed by UV-visible spectroscopy and iron content analyses using the method of Fish.\textsuperscript{46} Protein purification,
reconstitutions, and assays performed in Montana followed previously published methods with slight modifications.  

**EPR Spectroscopy**

Reconstituted WT, C386S, and ΔCTD HydG proteins (~500 µM) in buffer C were thawed inside an anaerobic glovebox before being desalted into buffer D. Proteins were mixed with substrate(s) [1 mM commercial AdoMet, 1 mM S-adenosyl-l-homocysteine (AdoHcy), or 1 mM AdoHcy and 1 mM tyrosine] prepared in buffer D before the addition of sodium dithionite (1 mM). Following a 20 minute incubation period, the mixture (160 µL) was transferred into an EPR tube (Wilmad Quartz, CFQ, 4 mm outer diameter) and sealed with a rubber septum before being flash-frozen in liquid N₂ outside the glovebox. EPR measurements at University College London (UCL) were performed on a Bruker EMXplus spectrometer operating at 9.4 GHz (X-band) equipped with a 4122SHQE resonator, with an Oxford Instruments ESR900 cryostat for measurements in the temperature range of 10-40 K. Measurements were performed with a magnetic field sweep from 0 to 600 mT, a microwave power of 2 mW, a modulation amplitude of 0.5 mT, and a modulation frequency of 100 kHz. EPR samples analyzed at Montana State University (MSU) were prepared as described previously. Briefly, the HydG enzyme was supplemented with 50 mM Tris (pH 7.4), 100 µM 5-deazariboflavin, and 5 mM DTT in buffer E [50 mM HEPES, 0.5 M KCl, and 5% (w/v) glycerol (pH 7.4)] and placed in an ice-water bath in the MBraun anaerobic glove box. Following illumination with a 300 W Xe lamp for 1 h, enzymatically synthesized AdoMet (1 mM) was added in the absence of light. Within 3 min, the EPR tube was sealed with a rubber
stopper and the sample was flash-frozen in liquid N\textsubscript{2}. Low-temperature EPR spectra were recorded using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller from Oxford Instruments. Typical EPR parameters were as follows: sample temperature 12 K; microwave frequency, 9.37 GHz; microwave power, 1.59 mW; time constant, 20.48 ms. Experimental spectra were baseline corrected and plotted using OriginLab (version 8.6.0, OriginLab Corp., Northampton, MA). All simulations of EPR data were performed using EasySpin\textsuperscript{47} and yielded the $g$ values reported throughout the text and summarized in Appendix B.

**Determination of Michaelis–Menten Constants**

Tyrosine cleavage was assessed by measuring initial rates of $p$-cresol formation as a function of AdoMet or tyrosine concentration. The AdoMet dependence was measured in assays (200 $\mu$L) containing reconstituted HydG (5 $\mu$M), tyrosine (400 $\mu$M for WT and 3 mM for C386S and $\Delta$CTD HydG) sodium dithionite (1 mM), and varying concentrations of AdoMet (5–500 $\mu$M), while the dependence of the rate on tyrosine was determined in similar assays containing AdoMet (100 $\mu$M for WT and 200 $\mu$M for C386S and $\Delta$CTD HydG) and varying concentrations of tyrosine (13 $\mu$M to 4 mM) instead. Control assays were devoid of sodium dithionite to accurately measure the nanomolar $p$-cresol impurities observed in tyrosine preparations. Assays were equilibrated at 37 °C for 5 min before initiation with sodium dithionite. Reactions were stopped at four time points between 1 and 14 minutes by addition of 20% perchloric acid (15 $\mu$L) and then mixtures stored at -80 °C before HPLC analysis. All assays were cleared by centrifugation (13000 rpm and 25 °C for 20 min) before the supernatant was analyzed for
p-cresol using a modified HPLC procedure described by Challand et al. In short, assay supernatants (40 µL) were applied to a Gemini C18 reverse phase HPLC column (4.6 mm × 250 mm, 5 µM, 110 Å, Phenomenex) equilibrated with 70% solvent A [0.1% (v/v) acetic acid in water, 0.8 mL/min]. After injection, the column was eluted for 5 min with 70% solvent A, followed by a gradient to 40% solvent B [0.1% (v/v) acetic acid in acetonitrile] over 3 min. An additional gradient over 7 min to 42% solvent B was applied before a step to 100% solvent B over 1 min where it was held isocratically for 8 min and then returned to 70% solvent A over 1 min. Before the next injection, the column was re-equilibrated with 70% solvent A for 5 min (total sampling time of 30 min). The fluorescence detector (Shimadzu RF-10Axl) measured the excitation and emission at 274 and 312 nm, respectively. The amount of p-cresol (tR = 15.9 min) was quantified using a calibration curve of synthetic standards (7.8–125 nM) analyzed in parallel. The quantification limit was 15 nM.

In vitro Activity Assays

Time course assays with all HydG variants contained reconstituted HydG (40 µM), AdoMet (0.5 mM), and tyrosine (4 mM) and were initiated with sodium dithionite (1 mM) as described above. Negative control assays were devoid of tyrosine. Duplicate reactions were stopped (1–60 min) by addition of 20% perchloric acid (15 µL), and then mixtures were immediately buffered by addition of 0.5 M HEPES (pH 7.5, 20 µL) and 1 M NaOH (30 µL) before being stored at -80 °C. HPLC analysis for 5'-deoxyadenosine (5'-dAdo) and p-cresol was conducted as previously described. Formed cyanide was quantified as the fluorescent 1-cyanobenz[f]isoindole derivative with slight
briefly, a solution to be analyzed (10 µL) was added to a freshly prepared working solution of MeOH, 20 % NH₃, and 83 mM taurine in water (9:1:3, 65 µL) before addition of naphthalene-2,3-dicarboxaldehyde (10 mM in MeOH, 25 µL). After being incubated for 30 min at 20 °C, the solution was diluted 1:1 with water and immediately injected (40 µL) onto a pre-equilibrated HyperClone BDS C₁₈ reverse phase HPLC column (4.6 mm × 150 mm, 5 µM, 130 Å, Phenomenex, 0.8 mL/min) for the most accurate results. Following sample injection, the column was eluted isocratically for 5 min using 60% solvent A (2 mM ammonium formate buffered to pH 3 with formic acid), before a gradient to 100% solvent B (MeOH) over 10 min was applied and then held isocratically for 10 min. The gradient was reversed to 60% solvent A over 1 min before the column was re-equilibrated for 9 min (total sampling time of 35 min). Under these conditions, the fluorescent 1-cyanobenz[f]isoindole eluted with at tᵣ of 11.9 min (λₑₓ = 418 nm; λₑₘ = 454 nm). Cyanide recovery in the presence of protein is reduced, while sodium dithionite affects the fluorescence of the cyanide derivatization product. To accurately quantify cyanide, a calibration curve under assay conditions was employed where duplicate KCN standards (final concentrations of 1.25–160 µM) in 2.5 mM NaOH were added (80 µL) to activity assays (200 µL) lacking AdoMet and tyrosine. These assays were incubated for 12 and 38 minutes before being subjected to the precipitation–derivatization conditions described above. Glyoxylate was quantified as the fluorescent 2-quinoxalinol derivative using a previously published method, with slight modifications. Briefly, cleared assay supernatants (10 µL) were diluted with 50 mM HEPES (pH 7.5) to 50 µL before being acidified with 0.5 M HCl (100 µL) and addition of freshly prepared o-phenylene diamine in 0.5 M HCl (10 mg/mL, 50 µL). Using a
polymerase chain reaction machine, all samples were incubated at 25 °C for 1 min, before
being heated at 95 °C for 10 min and then cooled to 25 °C for 10 min before addition of
1.25 M NaOH (120 µL). After a 5 min period at 4 °C, the samples were stored at -80 °C
and thawed only shortly before HPLC analysis. The derivatization mixture (40 µL) was
injected onto an equilibrated HyperClone BDS C18 reverse phase HPLC column (4.6 mm
× 150 mm, 5 µM, 130 Å, Phenomenex) connected to a fluorimeter (λ_ex = 350 nm; λ_em =
420 nm), and the column was washed with 85% solvent A (100 mM ammonium
bicarbonate, 0.8 mL/min) for 5 min, followed by a gradient to 50% solvent B
(acetonitrile) over 15 min. The gradient was increased to 100% solvent B over 1 min,
held isocratically for 4 min, and return to 85% solvent A over 0.5 min. Before the next
injection, the column was re-equilibrated with 85% solvent A for 9.5 min (total sampling
time of 35 min). Under these conditions, 2-quinoxalinol eluted between 6.2 and 6.6 min.
Sodium dithionite present in activity assays affects the fluorescence of the 2-quinoxalinol
derivative. Quantitative estimates for glyoxylate were obtained from a calibration curve
of derivatized synthetic glyoxylate standards (3–100 µM) prepared and incubated as
described for the activity assays lacking AdoMet, HydG, and tyrosine.

Data Analysis

Data were analyzed and graphs prepared using GraphPad Prism (version 6.00 for
Windows, GraphPad Software, La Jolla, CA). At saturating concentrations, the velocity
data of all HydG variants were fit (R^2 > 0.98, except for WT K_{M AdoMet}, where R^2 = 0.93)
to classical Michaelis–Menten kinetics^{49,50} (Equation 3.1).
\[
\frac{v}{[\text{HydG}]} = \frac{k_{\text{cat}} [S]}{K_{M} + [S]}
\]  

(3.1)

where \( v \) is the initial velocity, \( k_{\text{cat}} \) is the turnover number, \([S]\) is the substrate concentration, and \( K_{M} \) is the Michaelis–Menten constant. Time courses were fit \( (R^2 > 0.97) \) to a first-order kinetic process (Equation 3.2).

\[
[P] = [P]_{\text{max}} (1 - e^{-kt})
\]  

(3.2)

where \([P]\) is the observed product concentration, \([P]_{\text{max}}\) is the maximal amount of product formed, and \( k \) is the observed first-order rate constant, or fit to linear product formation. The slope of this curve at time zero gives the initial rate of product formation \( (v = [P]_{\text{max}}k) \). Dividing the initial rate by the concentration of HydG gives the apparent turnover number, \( k_{\text{cat}}^{\text{app}} \), as shown in Equation 3.3.

\[
k_{\text{cat}}^{\text{app}} = \frac{k[P]_{\text{max}}}{[\text{HydG}]}
\]  

(3.3)

Results

HydG Contains Two [4Fe-4S] Clusters

\( C. \ acetobutylicum \) HydG was anaerobically purified by Ni-affinity chromatography and contained substoichiometric amounts of iron. After chemical reconstitution with 10 molar equivalents of iron and sulfide in the presence of DTT, WT HydG contained on average \( 7.1 \pm 1.1 \) irons per protein and showed a broad UV-visible
absorption band around 410 nm (Figure 3.1), characteristic of [4Fe-4S]$^{2+}$ clusters. On the basis of the protein concentration determined using the Bradford assay, the extinction coefficient at 400 nm ($\varepsilon_{400}$) was calculated to be 34 mM$^{-1}$cm$^{-1}$, in agreement with the presence of two [4Fe-4S]$^{2+}$ clusters assuming an $\varepsilon_{400}$ of 16 mM$^{-1}$cm$^{-1}$. To characterize the individual clusters bound to the N- and C-terminal cysteine motifs of HydG, we prepared an N-terminal C96/100/103A triple mutant that cannot coordinate the radical AdoMet cluster and a ΔCTD mutant that is lacking the CX$_2$CX$_2$C motif proposed to coordinate cluster II and amino acids thereafter. Both mutants were expressed as soluble and stable proteins, suggesting that neither cluster is required for the folding of the protein in a soluble form. Iron analyses after in vitro reconstitution with 5–6 molar equivalents of iron and sulfide indicated the presence of 3.4 ± 0.1 irons per C96/100/103A HydG and 3.1 ± 0.4 irons per ΔCTD HydG (Appendix Table B.1). The corresponding UV-visible spectra are characteristic of a [4Fe-4S]$^{2+}$ cluster Figure 3.1,

![UV-visible Characterization of HydG Variants](image)

Figure 3.1. UV-visible Characterization of HydG Variants. Representative UV-visible spectra of WT [(–) 8.0 ± 0.9 Fe atoms/protein], C96/100/103A [(---) 3.4 ± 0.1 Fe atoms/protein], and ΔCTD HydG (···) 3.6 ± 0.2 Fe atoms/protein].
and the calculated $\varepsilon_{400}$ of approximately 16 mM$^{-1}$ cm$^{-1}$ suggest the presence of a single [4Fe-4S]$^{2+}$ cluster in C96/100/103A and ΔCTD HydG, in agreement with the iron analyses.

EPR spectroscopy was used to confirm the presence of [4Fe-4S] clusters and to allow a more detailed characterization. Photoreduced, reconstituted WT HydG has previously been characterized and displayed $g$ values of 2.03, 1.92, and 1.90 in the absence of AdoMet, while two distinct rhombic signals were observed ($g_1$ values of 2.02, 1.93, and 1.91; $g_2$ values of 2.00, 1.87, and 1.83) in the presence of AdoMet. For ease of comparison, these spectra are reproduced in Figure 3.2A. The observation of 0.48 spin/protein in photoreduced WT HydG samples in the absence and 0.88 spin/protein in the presence of AdoMet suggests that binding of AdoMet to cluster I increases its redox potential. This is in accord with spectroelectrochemical results that showed that the redox potential of the [4Fe-4S]$^{2+/+}$ couple in the radical AdoMet enzyme lysine-2,3-aminomutase is increased from -480 to -430 mV in the presence of AdoMet. It should be noted that some batch to batch variability was observed in the extent of additional cluster I reduction for the WT enzyme when AdoMet was present (data not shown). Addition of the AdoMet analogue S-adenosyl-L-homocysteine (AdoHcy, 1 mM) to WT HydG had no observable effect on the reduced WT HydG spectrum (data not shown).

While there is little difference between photoreduced and dithionite-reduced HydG spectra with regard to overall line shape and $g$ values, the fraction of reduced AdoMet-bound cluster was found to be slightly higher in dithionite-reduced than in photoreduced HydG samples as exemplified for WT HydG in Figure 3.2A,B and Appendix Figure
Figure 3.2. X-band EPR Spectra of Reduced, Reconstituted HydG Variants in the Presence of 1 mM AdoMet (12 K). (A) Photoreduced WT HydG (65 µM, 8.7 ± 0.7 Fe atoms/protein). (B) Dithionite-reduced WT HydG (101 µM, 6.5 ± 0.1 Fe atoms/protein). (C) Photoreduced ΔCTD HydG (424 µM, 2.4 ± 0.2 Fe atoms/protein). Experimental spectra are colored black, while composite simulations are colored red. Spectral components are depicted in the top part of each panel. (D) The bottom part of the panel shows the spectrum for photoreduced C96/100/103A HydG (90 µM, 3.4 ± 0.1 Fe atoms/protein) in the absence (black) and the presence of 1 mM AdoMet (red), and the top part of the panel shows the experimental spectrum of the photoreduced enzyme (black) and the corresponding simulation spectrum (red). Samples were prepared in 50 mM HEPES, 0.5 M KCl, and 5% (w/v) glycerol (pH 7.4). See Materials and Methods for EPR spectrometer parameters.

B.1A,B (see also Appendix Table B.2).

The \( S = 1/2 \) signal in the EPR spectrum of photoreduced, reconstituted ΔCTD HydG closely resembles the WT resonance, with \( g \) values of 2.03, 1.92, and 1.90 (Appendix Figure B.1C). As seen for WT HydG, addition of AdoMet generates a
rhombic signal (\(g\) values of 2.00, 1.88, and 1.84) and a mostly axial signal (\(g\) values of 2.04, 1.92, and 1.90) representing AdoMet-bound and unbound cluster I, respectively (Figure 3.2C). Simulation of the data suggests that 73% of the overall signal arises from AdoMet-bound cluster I. Spin quantification in the absence (0.31 spin/protein) and presence of AdoMet (0.39 spin/protein) indicates that the radical AdoMet \([4\text{Fe-4S}]\) cluster is only partially reduced in either case.

Photoreduced, reconstituted C96/100/103A HydG displayed a broad signal with \(g\) values of 2.03, 1.92, and 1.88 (Figure 3.2D, bottom panel, black trace). This spectrum is similar to that of reduced WT HydG and unambiguously demonstrates that the C-terminal cysteine residues can also coordinate a \([4\text{Fe-4S}]\) cluster. The observed values of 0.41 spin/protein in the absence and 0.43 spin/protein in the presence of AdoMet are very close to those of the \([4\text{Fe-4S}]^+\) clusters quantified for \(\Delta\text{CTD HydG}\) and suggest similar redox potentials for clusters I and II. Although the similarity of these EPR resonances point toward comparable electronic environments of each cluster, addition of AdoMet to the C96/100/103A mutant in > 10-fold excess did not cause a significant signal perturbation (Figure 3.2D, bottom panel, red trace), suggesting that cluster II cannot substitute for cluster I with regard to AdoMet binding.

EPR analyses of the HydG variants lacking the N- or C-terminal cluster allowed extraction of the signals deriving from the individual clusters. Spectral summation of these N- and C-terminal clusters in a near 1:1 ratio accurately simulates the reduced WT HydG spectrum (Appendix Figure B.1A, B and Appendix Table B.2), suggesting that both clusters behave independently, with no observable coupling in the absence of AdoMet. The observation that AdoMet does not interact with cluster II allowed for
detailed simulation of the reduced WT spectrum in the presence of AdoMet by additionally including the AdoMet-bound cluster I signal.

The C-terminal cysteine motif contains only three conserved cysteine residues, including that cluster II is site differentiated, with one iron available for potential tyrosine coordination in a mode similar to that of binding of AdoMet to cluster I. Coordination of the substrate to the auxiliary [4Fe-4S] cluster was experimentally observed for MoaA and was implicated for TYW1. The presence of 1 mM tyrosine or AdoHcy and tyrosine (1 mM each) caused subtle perturbations of the EPR spectrum of the dithionite-reduced WT enzyme (Appendix Figure B.2), which may be indicative of coordination of tyrosine to cluster II. In the absence of additional experimental evidence, however, it is difficult to verify this. The absence of signal perturbation in the dithionite-reduced WT HydG spectrum upon addition of AdoHcy suggests that it does not substitute for AdoMet. In a separate experiment, we explored the possibility that the binding of tyrosine to cluster II occurs only in the presence of AdoMet by using the C96/100/103A variant protein (data not shown). These EPR spectra revealed similar slight perturbations to the paramagnetic signatures associated with the reduced cluster II signals as shown in Appendix Figure B.2, indicating that either tyrosine does not coordinate cluster II or its coordination does not substantially perturb cluster g values.

These results collectively support a role for the C-terminal cluster and/or amino acid residues in the C-terminus in contributing to the proper orientation of substrates in the active site, as well as ensuring efficient N-terminal cluster reduction.
Characterization of the C386S HydG Single Mutant

A C386S HydG single mutant was reconstituted with 10 molar equivalents rather than 5 molar equivalents of iron and sulfide to investigate whether the single amino acid change and the similarity of the ability of serine and cysteine to act as cluster ligands\textsuperscript{56-58} are sufficient for partial or full assembly of cluster II. Reconstituted C386S HydG contained on average 4.9 ± 1.1 irons per protein and showed a slightly decreased absorbance at 410 nm compared to that of reconstituted WT HydG (Appendix Figure B.3). This suggests that cluster II might be present in only a fraction of C386S HydG or, alternatively, may only be partially assembled, for example, as a [3Fe-4S] cluster.\textsuperscript{59} The UV-visible spectrum supports the absence of [2Fe-2S] clusters that have strong characteristic absorption maxima at 330, 420, and 460 nm.\textsuperscript{60} Parallel EPR studies of dithionite-reduced C386S HydG (Appendix Figure B.4A) also excluded [2Fe-2S] clusters as the $S = 1/2$ signal can be observed only below 30 K (data not shown). This EPR resonance is more rhombic than that of WT HydG and was best simulated as 86% reduced cluster I (\textit{g} values 2.03, 1.93, 1.89) and 14% reduced cluster II (\textit{g} values of 2.02, 1.93, 1.86). Addition of AdoMet gave rise to a clear rhombic signal (\textit{g} values of 2.00, 1.89, and 1.84) for AdoMet-bound cluster I, with an approximately 10% contribution from the reduced C-terminal cluster (\textit{g} values of 2.04, 1.93, and 1.87) (Appendix Figure B.4B). The small amount of reduced cluster II required to simulate the spectrum is in accord with the decreased amount of iron quantified relative to that for WT HydG.
Determination of Substrate Michaelis–Menten Constants for WT and Mutant HydG

We set out to determine the Michaelis–Menten constants for AdoMet and tyrosine ($K_{M_{\text{AdoMet}}}$ and $K_{M_{\text{Tyr}}}$) (i) to investigate whether any observed reduction in the level of formation of cyanide by HydG mutants$^{39,40}$ was due to a decrease in the rate of tyrosine cleavage or a decreased affinity of the AdoMet and/or tyrosine substrates for these enzymes and (ii) to use this information to optimize activity assays for more accurate detection of AdoMet- and tyrosine-derived cleavage products. Unsurprisingly, the N-terminal C96/100/103A triple mutant ($100 \mu M$) was unable to cleave AdoMet to 5’-dAdo upon being incubated with 1 mM AdoMet, 1 mM tyrosine, and 5 mM sodium dithionite at 37 °C for 60 minutes because the lack of AdoMet binding cluster I. Accordingly, this mutant was not included in the following study.

To assess tyrosine cleavage, initial rates of $p$-cresol formation were determined. The slow reaction catalysis by HydG ($k_{\text{cat}} < 2 \times 10^{-3} \text{ s}^{-1}$)$^{16}$ limited the number of turnovers that could be measured. As a result, the initial rates may have not been determined under ideal steady state conditions. Another consequence of slow turnover was the requirement, at low substrate concentrations, to add relatively high concentrations of HydG ($5 \mu M$) to achieve measurable turnover. This gave equimolar concentrations of the enzyme and substrate for the lowest AdoMet and tyrosine concentrations, conditions at which the free ligand assumption ([S]$_{\text{total}}$ = [S]$_{\text{free}}$ for Michaelis–Menten kinetics) is not valid. For these two reasons, the associated Michaelis–Menten constants should be treated as approximate values.
Figure 3.3. Substrate-dependent Initial Rates of p-cresol Formation by HydG Variants. Initial rates (v₀/[E]) of formation of p-cresol by HydG (5 µM) in 50 mM HEPES, 0.5 M KCl (pH 7.4) at 37 °C. Assays additionally contained 1 mM sodium dithionite and (A) tyrosine (400 µM for WT and 3 mM for mutants) and varying AdoMet concentrations or (B) AdoMet (100 µM for WT and 200 µM for mutants) and varying tyrosine concentrations. The data were fit to Michaelis–Menten kinetics (Equation 3.1). Calculated kinetic parameters for WT (▲), C386S (●), and ΔCTD HydG (■) are summarized in Table 3.1.

Table 3.1. AdoMet- and Tyrosine-Dependent Kinetic Parameters for WT and Mutant HydG (Figure 3.3)

<table>
<thead>
<tr>
<th>HydG</th>
<th>K_M AdoMet (µM)</th>
<th>k_cat AdoMet (×10⁻³ s⁻¹)</th>
<th>K_M Tyr (mM)</th>
<th>k_cat Tyr (×10⁻³ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.6 ± 1.1</td>
<td>2.6 ± 0.1</td>
<td>0.3 ± 0.0 (3)</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>C386S</td>
<td>17.3 ± 2.6</td>
<td>2.9 ± 0.1</td>
<td>1.6 ± 0.2ᵇ</td>
<td>4.3 ± 0.3ᵇ</td>
</tr>
<tr>
<td>ΔCTD</td>
<td>3.6 ± 0.7</td>
<td>1.1 ± 0.0 (3)</td>
<td>10.6 ± 0.6ᵇ</td>
<td>3.0 ± 0.1ᵇ</td>
</tr>
</tbody>
</table>

ᵃInitial rates of AdoMet- and tyrosine-dependent p-cresol formation were fit to Michaelis–Menten kinetics (Equation 3.1). ᵇThese values should be regarded as approximate. For the ΔCTD HydG, the apparent K_M is above the highest concentration investigated, and for the C386S mutant, only two data points were obtained above K_M. The solubility of tyrosine limited the measurements that were possible at higher tyrosine concentrations.

At saturating tyrosine concentrations (400 µM for WT and 3 mM for mutant HydG), the velocity data of all HydG variants were best fit to classical Michaelis–Menten kinetics⁴⁹,⁵⁰ (Equation 3.1 and Figure 3.3A). The K_M AdoMet values for WT (2.6 ± 1.1 µM) and ΔCTD (3.6 ± 0.7 µM) are identical within error, whereas the K_M AdoMet for
C386SHydG was calculated to be 17.4 ± 2.6 µM (Table 3.1). At higher AdoMet concentrations (>300 µM) an activity decrease for WT but not for mutant HydG was observed, which may be due to a decreased assay pH caused by the presence of residual trifluoroacetic acid in the employed AdoMet samples.

At saturating AdoMet concentrations (100 µM for WT and 200 µM for mutants), the p-cresol velocity data were also fit to Equation 3.1 (Figure 3.3B), and the $K_{M \text{Tyr}}$ for WT HydG was calculated to be 278.9 µM ± 34.0 µM (Table 3.1). This $K_M$ value is 1 order of magnitude higher than most reported substrate $K_M$ values of other radical AdoMet enzymes, but it lies below the intracellular tyrosine concentration of C. acetobutylicum ATCC824 during acidogenesis (640 µM) and solventogenesis (660 µM), ensuring that HydG would be able to respond to fluctuations in tyrosine concentration during both stages of cell growth. Bearing in mind the assumption required with treating a $K_M$ as an apparent binding constant, we found the increased $K_{M\text{Tyr}}$ constants for C386S and ΔCTD HydG of 1.6 ± 0.2 and 10.6 ± 0.6 mM, respectively, clearly show that modifications to the C-terminal cluster decrease the apparent affinity for tyrosine. The similarity of the turnover number ($k_{cat}$) values for p-cresol formation between ΔCTD HydG (3.0 × 10⁻³ s⁻¹) and WT and C386S values (4.3 × 10⁻³ s⁻¹) (Table 3.1) is consistent with a model which the rate of radical formation and tyrosine cleavage is unaffected in the variant proteins. This suggests that catalytic steps can be rescued at higher tyrosine concentrations. Experimentally, this is observed for C386S HydG, but it is experimentally difficult to fully saturate ΔCTD HydG because of the limited solubility of tyrosine in assay buffer at physiological pH.
Kinetic Characterization of WT and Mutant HydG

We previously observed catalytic formation of \( p \)-cresol and cyanide in a 1:1 ratio by WT HydG in the presence of AdoMet, tyrosine, and sodium dithionite (1 mM each).\textsuperscript{16} Taking advantage of the determined Michaelis–Menten constants for AdoMet and tyrosine (Table 3.1), we repeated these time course experiments under optimized assay conditions (40 \( \mu \)M HydG, 0.5 mM AdoMet, 4 mM tyrosine, and 1 mM sodium dithionite) to compare formation of the tyrosine cleavage products cyanide and glyoxylate as well as the AdoMet cleavage products 5’-dAdo relative to the formation of \( p \)-cresol. Under these conditions, all HydG proteins use AdoMet as a substrate, as reflected in the formation of 5’-dAdo, and cleave tyrosine catalytically (Figure 3.4). The employed concentration of AdoMet represents a compromise between the observed inhibition of WT HydG (significant above 300 \( \mu \)M AdoMet) and the problem of AdoMet becoming limiting upon multiple turnovers (increasingly a problem at low AdoMet

![Figure 3.4](image.png)
Table 3.2 Apparent Turnover Numbers for HydG-Catalyzed AdoMet and Tyrosine Cleavage (Figure 3.4)\textsuperscript{a}

<table>
<thead>
<tr>
<th>product</th>
<th>WT</th>
<th>C386S</th>
<th>ΔCTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textsuperscript{'}-dAdo</td>
<td>2.3 ± 0.3</td>
<td>6.9 ± 0.3</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>p-cresol</td>
<td>1.8 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>cyanide</td>
<td>0.6 ± 0.0 (2)\textsuperscript{b}</td>
<td>1.6 ± 0.4</td>
<td>NA\textsuperscript{c}</td>
</tr>
<tr>
<td>glyoxylate</td>
<td>0.5 ± 0.1</td>
<td>4.2 ± 0.9</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data were fit to first-order kinetics (Equation 3.2), and the apparent turnover number was extracted using Equation 3.3. \textsuperscript{b}A fit to Equation 3.2 was not successful, and cyanide formation was modeled with linear kinetics. \textsuperscript{c}Not applicable.

we observed a 50\% decrease in the level of p-cresol formation by WT HydG \([k_{\text{cat}}^{\text{app}} = (1.8 \pm 0.2) \times 10^{-3} \text{ s}^{-1}]\) compared to that of C386S HydG \([k_{\text{cat}}^{\text{app}} = (4.6 \pm 0.2) \times 10^{-3} \text{ s}^{-1}]\) (Figure 3.4A,B; Table 3.2). Over a 60 min time period, ΔCTD HydG formed quantities of p-cresol similar to that formed by the (inhibited) WT enzyme with a \(k_{\text{cat}}^{\text{app}}\) of \((1.1 \pm 0.1) \times 10^{-3} \text{ s}^{-1}\) (Figure 3.4C and Table 3.2). This observed apparent \(k_{\text{cat}}^{\text{app}}\) compares very well with the rate for the previously observed tyrosine-dependent p-cresol formation (Figure 3.3B), and bearing in mind that ΔCTD HydG cannot be experimentally saturated with tyrosine, we suggest that neither the auxiliary cluster II nor the C-terminal residues (after C386) are essential for the cleavage of tyrosine to form p-cresol.

The nonaromatic product of ThiH-catalyzed tyrosine C\textsubscript{α}–C\textsubscript{β} bond cleavage is hypothesized to be dehydroglycine. Dehydroglycine is hydrolytically unstable and yields glyoxylate \textit{in vitro}. While stoichiometric quantities of glyoxylate with respect to p-cresol were identified in ThiH activity assays, little glyoxylate accumulated during HydG turnover. Instead, a 1:1 ratio of cyanide to p-cresol was observed\textsuperscript{16} suggesting that dehydroglycine (or the closely related glycine C\textsubscript{α}-centered radical) is an intermediate during cyanide synthesis (Scheme 3.1, step B). In this study, we observed some variation
in the cyanide stoichiometry compared to that with \( p \)-cresol for different WT HydG samples. At the 60 min time point for batch A, a \( p \)-cresol:cyanide ratio of 2.5:1 was observed, whereas for batch B, the ratio was 1.3:1 (Figure 3.4A). The reduced level of cyanide formation compared to that of \( p \)-cresol was completely ascribed to detection of increased amounts of glyoxylate. We hypothesize that glyoxylate accumulates \textit{in vitro} if the reaction step from dehydroglycine (or a closely related intermediate) to cyanide and CO formation (Scheme 3.1, step B) is very slow or indeed aborted. The varying amount of cyanide formed indicate that some batch to batch variation can be observed in reconstituted HydG samples, although no significant differences in the iron quantification and UV-visible spectra were observed. This variation makes it difficult to accurately assess the role of the C-terminal cysteine motif and coordinated cluster II with respect to cyanid formation for the C386S HydG mutant. The turnover number for cyanide formation by C386S HydG was on average 65% lower than that of \( p \)-cresol (Table 3.2), which is equivalent to a \( p \)-cresol:cyanide ratio of 2.2:1 after 60 min (Figure 3.4B). Again, the remaining tyrosine cleavage product is accounted for by glyoxylate. It is interesting to note that while the WT enzyme formed 1.5–2.3 molar equivalents of cyanide with respect to protein concentration, the C386S mutant catalyzed 2.8 molar equivalents of cyanide. Although \( \Delta \)CTD HydG catalyzed formation of 100 \( \mu \)M \( p \)-cresol over a 60 min period (Figure 3.4C), representing a 60-fold improvement in activity compared to observations by Tron\textsuperscript{40} for \textit{Ca} or \textit{Tie} \( \Delta \)CTD HydG, the amounts of cyanide reported herein were very small (4.6 ± 0.6 \( \mu \)M, 0.12 mol/mol of \( \Delta \)CTD). Instead, almost 100% glyoxylate with respect to \( p \)-cresol was formed. Importantly, none of the C-terminally modified HydG variants formed detectable amounts of CO.
Discussion

In addition to the canonical radical AdoMet CX$_3$CX$_2$C motif found close to the N-terminus, Ca HydG contains a C-terminal CX$_2$CX$_{22}$C cysteine triad, proposed to coordinate a second [4Fe-4S] cluster. While the C-terminal motif is not completely conserved among different organisms, its requirement for HydG activity during [FeFe]-hydrogenase activation has been demonstrated by mutagenesis. Using an N-terminal C96/100/103A mutant and a truncated ΔCTD HydG variant lacking the 87 C-terminal residues, we have used UV-visible and EPR spectroscopy together with iron analyses to independently characterize the [4Fe-4S] cluster in these variants, and the results unambiguously demonstrate that the C-terminal cysteine triad in WT HydG coordinates an auxiliary [4Fe-4S] cluster. The EPR spectra of reduced C96/100/103A and ΔCTD HydG both displayed remarkably similar $g$ values compared to that of WT HydG, which suggests the N- and C-terminal clusters occupy similar electronic environments. Analysis of the EPR spectra does not, however, unequivocally identify the nature of the fourth ligand to the C-terminal cluster. While addition of AdoMet to a reduced ΔCTD HydG samples gave a rhombic EPR signal, characteristic of an AdoMet-bound [4Fe-4S] cluster, a similar change in line shape was not observed for the triple N-terminal mutant. These results suggest that while the N- and C-terminal clusters are both site-differentiated, AdoMet binding occurs only at the N-terminal radical AdoMet cluster. This explains the observed inability of the C96/100/103A HydG mutant to cleave AdoMet.
Independent characterization of each [4Fe-4S] cluster by EPR using the N- and C-terminal HydG variants allowed for the successful reconstruction of the WT reduced signal in the absence (Appendix Figure B.1A, B) and presence of AdoMet (Figure 3.2A, B). These results clearly show that the reduced WT signal is best simulated as a near 1:1 mixture of N- and C-terminal [4Fe-4S]$^+$ clusters, while the observed signal change following AdoMet addition can be simulated by additionally including the AdoMet-bound cluster I signal. These simulations define the $g$ values associated with the individual [4Fe-4S] cluster states in WT HydG more clearly (Appendix Table B.2) than simulations we previously employed.\textsuperscript{17} As a result, we identified that the nearly doubled spin quantification in the WT HydG•AdoMet sample compared to that of WT HydG mostly reflects an enhanced reduction of the radical AdoMet and not the C-terminal auxiliary [4Fe-4S] cluster (Appendix Table B.2). For WT, C386S, and ΔCTD HydG, the extent of cluster reduction in the presence of AdoMet varied between protein preparations, likely depending on efficiency of cluster reconstitution, and this variability restricts a detailed interpretation of the data.

We used enzymatically synthesized AdoMet to determine the $K_M$ AdoMet in the presence of saturating tyrosine concentrations for WT, C386S, and ΔCTD HydG to be between 3 and 17 $\mu$M. Given that AdoMet coordinates to cluster I and is additionally involved in well-characterized hydrogen bonding interactions to residues not expected to be affected by C386S mutation and C-terminal deletion,\textsuperscript{23-25,67,68} it is not surprising that the affinity for AdoMet is very similar for these HydG variants. It further suggests that the introduced mutations do not strongly affect the positioning of AdoMet binding.
residues, implying correct folding of the characteristic core triosephosphate isomerase (TIM) barrel observed among radical AdoMet enzymes.\textsuperscript{69}

The C386S HydG mutant showed a 5-fold decrease in apparent tyrosine affinity compared to that of WT HydG, suggesting a significant role for the auxiliary [4Fe-4S] cluster in the recognition and/or binding of tyrosine. On the basis of these results, it is intriguing that addition of tyrosine with AdoHcy introduced slight changes into the EPR spectrum of dithionite-reduced WT HydG, but additional experiments are required to verify the binding of tyrosine to cluster II. Deleting the HydG C-terminal domain decreases the apparent affinity for tyrosine an additional 7-fold compared to that of C386S HydG and could point toward additional binding interactions between tyrosine and amino acids in the C-terminal domain.

Using the determined Michaelis–Menten constants, we conducted comparative \textit{in vitro} activity assays. The WT, C386S, and $\Delta$CTD HydG enzymes all cleaved tyrosine catalytically with apparent $p$-cresol $k_{cat}$ values ranging between 1.1 and $4.6 \times 10^{-3}$ s$^{-1}$. This agrees well with our previous WT HydG characterization\textsuperscript{16} and product formation rates observed for other radical AdoMet enzymes.\textsuperscript{70} Bearing in mind that the $\Delta$CTD HydG cannot be saturated with tyrosine because of experimental limitations (the solubility of tyrosine), we find the observed similarity in the amounts of $p$-cresol formed compared to that for (partially inhibited) WT HydG indicates that tyrosine cleavage can continue in a manner predominantly independent of the C-terminal domain. With the observed affinity decrease for tyrosine after removal of the C-terminus (suggesting some interactions with these amino acid residues), it appears that spatially distinct and additive binding contacts to tyrosine can be established but that initiation of tyrosine cleavage
occurs inside the TIM barrel. This is in accord with HydG and ThiH sharing similar patches of conserved amino acid residues positioned at the internal face of the β-sheets of the TIM barrel.\textsuperscript{15}

Spectral EPR simulations furthermore allowed the deconvolution of the cluster components present in reduced C386S HydG to be approximated as 13% cluster II compared to 46% simulated for WT HydG. The observation that WT and C386S HydG catalyzed cyanide formation with the same relative rates compared to that of \textit{p}-cresol implies that cluster II is not required for cyanide synthesis, as previously suggested by Nicolet and co-workers.\textsuperscript{39} While substoichiometric detection of CO in WT HydG assays\textsuperscript{17,39} indicated the formation of a strong iron–carbonyl complex, the formation of 2.8 molar equivalents of cyanide by C386S HydG (after 60 min) argues against the coordination of cyanide to cluster II.

The levels of cyanide apparently formed by ΔCTD HydG are very low (4 – 5 μM); in fact, they are so close to our quantification limit (5 μM) that they may represent a “false positive”. In any case, the formation of 100 μM \textit{p}-cresol and glyoxylate but negligible cyanide by ΔCTD HydG supports the notion that C-terminal amino acid residues are required for cyanide synthesis.\textsuperscript{39} In conclusion, our collective data support a model in which deletion of the \textit{Ca} HydG C-terminal domain is at worst only mildly deleterious to the AdoMet and tyrosine cleavage steps but strongly affects the steps leading to cyanide and CO formation. In this regard, C-terminally truncated \textit{Ca} HydG strongly resembles ThiH.\textsuperscript{32}

The auxiliary [4Fe-4S] clusters of the radical AdoMet-dependent Cys- and Ser-type anaerobic sulfatase-modifying enzymes\textsuperscript{71,72} and the 2-deoxy-\textit{scyllo}-inosamine
dehydrogenase BtrN\textsuperscript{73,74} have been proposed to act as electron acceptors. This was evidenced in BtrN by an EPR silent mutant not containing the radical AdoMet cluster and the appearance of a new EPR signal during WT BtrN turnover, suggesting a very low redox potential for auxiliary cluster II in WT BtrN, which is however reduced during catalysis of the reaction. A similar electron transfer role could be envisaged for the Ca HydG C-terminal cluster to permit the interconversion between the tyrosine-derived dehydroglycine and the glycine radical (Scheme 3.1). EPR analyses of the C96/100/103A HydG mutant showed a broad signal, confirming that (in contrast to BtrN) cluster II in HydG can be readily reduced. In principle, tyrosine can be cleaved homolytically or heterolytically to form a C-centered glycine radical or dehydroglycine, respectively (Scheme 3.1, step A). While formation of the glycine radical is thermodynamically favored,\textsuperscript{32} our \textit{in vitro} observations that significant amounts of the dehydroglycine hydrolysis product glyoxylate are formed by the C386S and ΔCTD HydG mutants (which contain little or no C-terminal cluster) point towards heterolytic cleavage of tyrosine to form dehydroglycine. Because of the reducing assay conditions and the absence of cluster II, it is not evident how the glycine radical could be oxidized to dehydroglycine. When considering the stoichiometry of reaction products, decarbonylation of the dehydroglycine intermediate can potentially yield cyanide and CO products.\textsuperscript{16} We have confirmed the observation\textsuperscript{39} that none of the C-terminal \textit{Ca} HydG mutants formed CO. The apparent requirement for cluster II supports an alternative hypothesis that holds that the reduced cluster II may provide one electron for reversible reduction of dehydroglycine to the C-centered glycine radical (Scheme 3.1). In this hypothesis, deletion of the HydG C-terminal prevents formation of the glycine radical.
from dehydroglycine. The mechanism by which one of these intermediates is converted to cyanide and CO will require further elucidation.\textsuperscript{75}

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CHAPTER 4

REVERSIBLE H ATOM ABSTRACTION AT THE TYROSINE PHENOL POSITION CATALYZED BY THE RADICAL SAM ENZYME HYDG

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

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Contributions: Implemented the study. Prepared all samples, carried out all LC–MS and HPLC assays, analyzed the data, and wrote the manuscript.

Co-Author: Shourjo Ghose
Contributions: Provided preliminary expertise in LC–MS instrumentation, developed the LC method used, and discussed results.

Co-Author: John W. Peters
Contributions: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

Co-Author: Joan B. Broderick
Contributions: Conceived the study, and directed experimental design and interpretation. Obtained funding and resources, and edited manuscript drafts.
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CHAPTER 4

REVERSIBLE H ATOM ABSTRACTION AT THE TYROSINE PHENOL POSITION
CATALYZED BY THE RADICAL SAM ENZYME HYDG

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Abstract

The organometallic H-cluster at the active site of [FeFe]-hydrogenases is synthesized by three accessory proteins, two of which are radical S-adenosylmethionine (SAM) enzymes (HydE, HydG) and one of which is a GTPase (HydF). In this work we probed the specific role of H atom abstraction in HydG-catalyzed carbon monoxide and cyanide production from tyrosine (Tyr). The isotope distribution of 5’-deoxyadenosine (5’-dAdo) was evaluated using deuterium-labeled tyrosine substrates in H2O and D2O. The deuterium atoms observed in the 5’-dAdo product provide unambiguous evidence for dAdo radical-mediated abstraction of a hydrogen atom from the solvent-exchangeable Tyr 4-phenol as the principal radical initiation event.
Hydrogen metabolism in microorganisms is largely facilitated by the activation of dihydrogen through complex iron-sulfur (Fe–S) active site catalysts. The [FeFe]-hydrogenase H-cluster contains an unusual organometallic 2Fe subcluster in which the iron ions are coordinated by three carbon monoxides (CO), two cyanides (CN⁻), a dithiomethylamine, and a cysteine thiolate that bridges this entity to a [4Fe-4S] cluster (Figure 4.1). The 2Fe subcluster is synthesized by dedicated maturation machinery consisting of HydF, a GTPase that serves as a scaffold or carrier for synthesis of the 2Fe subcluster, and HydE and HydG, both radical SAM enzymes. While HydE has been proposed to synthesize the dithiolate ligand, HydG synthesizes the diatomic ligands of the H-cluster from Tyr in a reaction that produces p-cresol, CO from the C-1 carboxylate, and CN⁻ from the C-2 carbon in a reaction that requires two discrete HydG catalyzes the reductive cleavage of SAM at an N-terminal site-differentiated [4Fe-4S] cluster coordinated by a CX₃CX₂C motif (Scheme 1). Radical SAM enzymes generally catalyze the reductive cleavage of SAM to produce methionine and a 5’-deoxyadenosyl radical (5’-dAdo•); the latter species abstracts a hydrogen atom from substrate to produce 5’-deoxyadenosine. In the case of HydG, 5’-dAdo has been shown to be produced in a

Figure 4.1. [FeFe]-Hydrogenase H-cluster Active Site from Clostridium pasteurianum (3CBY).
1:1 stoichiometric ratio to two products of tyrosine cleavage: p-cresol and cyanide.\textsuperscript{7} HydG also coordinates a second [4Fe-4S] cluster near the C-terminus at a CX\textsubscript{2}CX\textsubscript{2}C motif\textsuperscript{8} that is essential for catalysis.\textsuperscript{9} While the N-terminal cluster of HydG is required for reductive cleavage of SAM and tyrosine cleavage, the C-terminal cluster and/or residues in the C-terminal domain are essential for the production of the diatomic products.\textsuperscript{10-12} Beyond these clues, however, few mechanistic details for the HydG-catalyzed reaction have been elucidated.

To investigate the mechanism by which HydG initiates tyrosine cleavage, specifically deuterated Tyr substrates and/or deuterated solvent were used in enzymatic assays and deuterium incorporation into 5'-dAdo was monitored. \textit{Clostridium acetobutylicum} HydG was expressed in \textit{E. coli}, purified, and reconstituted with iron and sulfide; this protein was found to exhibit visible and electron paramagnetic resonance
Figure 4.2. ESI-MS HydG (100 μM; 7.1 ± 0.2 Fe/protein) Product 5’-dAdo Isotope Distribution Performed in 92% tris-D₂O (50 mM tris, 300 mM KCl, 5% glycerol, pH 8.1) buffer. (A) Full Reaction. (B) Reaction lacking Tyr. (C) Reaction Containing 1 mM Phe. (D) 5’-dAdo Reference in H₂O. Spectra are depicted as normalized, extracted ion chromatograms.

(EPR) spectroscopic properties similar to those previously reported. Assays for HydG-catalyzed 5’-dAdo formation were carried out in an anaerobic chamber (Mbraun, O₂ < 1 ppm) in sealed 1.5 mL eppendorf tubes, containing HydG (100 μM with 7.1 ± 0.2 mol Fe per mol enzyme) in buffer (50 mM tris, pH 8.1, 300 mM KCl, 5% (w/v) glycerol), 5 mM dithionite, 1 mM Tyr, and 1 mM SAM at 37 °C, similar to assays reported previously. Reaction products were analyzed by ESI-MS following enzyme precipitation with 1 M HCl (13% v/v).

HydG reactions carried out using Tyr deuterium isotopologs ([β-D₂]-Tyr, [ring-D₄]-Tyr, and [α,ring-D₃]-Tyr) in H₂O yielded 5’-dAdo isotope distributions comparable to natural abundance 5’-dAdo (Figure 4.2D and Appendix Figure C.1). Since these isotopologs span all nonexchangeable positions on Tyr, the results indicate that the 5’-
dAdo• generated upon reductive cleavage of SAM abstracts only at a solvent-exchangeable position. This hypothesis was confirmed when the HydG reaction was carried out with natural abundance Tyr in D$_2$O buffer (50 mM tris, 300 mM KCl, 5% glycerol, pD 8.1, 92% D), where significant changes in the isotope distribution of 5’-dAdo were observed (Figure 4.2A). The 5’-dAdo product isolated from the reaction in D$_2$O buffer consisted of a mixture of singly, doubly, and triply deuterated 5’-dAdo, with 69% of 5’-dAdo containing at least one deuterium (Appendix Table C.1). When Tyr was omitted from the assay, HydG catalyzed uncoupled SAM cleavage, however only 16% of the 5’-dAdo product contained a single deuterium label (Figure 4.2B and Appendix Table C.1); these results indicate that the substantial multiple deuterium incorporation into 5’-dAdo observed in Figure 4.2A requires the presence of tyrosine. Similarly, when

![Figure 4.3. Quantitative $p$-cresol Product Detection in tris-H$_2$O (filled squares) and 95% tris-D$_2$O (filled triangles). Assays contained 40 µM HydG (9.5 ± 0.2 Fe/protein), 1 mM AdoMet, 1 mM Tyr, 5 mM dithionite, performed at 37 °C in 50 mM tris, 300 mM KCl, pH/pD 8.1.](image-url)
Tyr was replaced with phenylalanine (Phe) (which is not a substrate of HydG), a comparably small amount of deuterium incorporation was observed (Figure 4.2C; Appendix Table C.1). Only samples that contained enzyme, reductant, SAM, and Tyr in D$_2$O buffer resulted in significant deuterium label transfer to dAdo.

Abstraction of a hydrogen atom from substrate by 5’-dAdo• is generally accepted as a rate-limiting step in radical SAM enzymes, and thus substitution with deuterium at the abstracted position should give rise to an isotope effect. In order to examine the solvent isotope effect on HydG catalysis, p-cresol production was compared in identical assays performed in tris-H$_2$O and tris-D$_2$O buffer (Figure 4.3). The rate of p-cresol production in H$_2$O and D$_2$O was fitted to a first-order exponential, revealing an apparent deuterium isotope effect of 1.71 ($k_{\text{cat-H}_2\text{O}}/k_{\text{cat-D}_2\text{O}} = 1.71$). The magnitude of this $k_{\text{H}}/k_{\text{D}}$ is consistent with a primary isotope effect and provides unambiguous evidence that tyrosine cleavage to produce p-cresol is initiated by H atom abstraction from the solvent-exchangeable phenolic O-H of Tyr. Similar solvent isotope effects have been measured in the radical SAM enzyme DesII, where the observed KIE was diminished by other kinetic steps.

The observation of multiple deuterium atoms being incorporated into 5’-dAdo (Figure 4.2A, Appendix Table C.1) was surprising, since the simple mechanistic model would involve 5’-dAdo• abstracting a D from substrate to produce singly deuterated 5’-dAdo. Multiple deuterium incorporations into 5’-dAdo product requires a more complex mechanism in which, after formation of 5’-dAdo, the 5’-dAdo• is regenerated and can abstract an additional H/D. Because the percent unlabeled 5’-dAdo product in Figure 4.2A exceeded the estimated percent H$_2$O expected, the experiment was repeated under
conditions that minimized contaminating protons from solvent, substrate, and protein (Figure 4.4A; details see the Appendix C text). This optimization resulted in a modest increase in D atom incorporation into 5’-dAdo (from 69 % to 82 % of total 5’-dAdo containing at least one D, Figures 4.2A, 4.4A; Appendix Table C.1), and a 40% increase in the percentage of 5’-dAdo containing two or three deuterium atoms. It is clear from these results that HydG catalyzes the cleavage of Tyr by a mechanism involving regeneration of 5’-dAdo• from 5’-dAdo, followed by additional H atom abstractions.

Because the incorporation of multiple deuterium atoms into 5’-dAdo requires the reversible generation of 5’-dAdo•, which in turn could be coupled to reversible cleavage of SAM to form 5’-dAdo•, we investigated the possibility of deuterium incorporation into
Figure 4.5. ESI-MS Optimized HydG (100 μM; 8.5 ± 0.1 Fe/protein) Product SAM Isotope Distribution Performed in 95% D₂O buffer (50 mM tris, pD 8.1). (A) Full Reaction (B) SAM Reference in H₂O. Spectra are represented as normalized, extracted ion chromatograms.

SAM during the HydG catalyzed reaction. SAM was isolated from the optimized assay described above, and ESI-MS revealed that 56% of the SAM contained at least one deuterium (Figure 4.5A; Appendix Table C.2). In the absence of HydG, 9% of the SAM incorporated deuterium during the assay, consistent with previous reports of slow exchange of the C-5’ hydrogens of SAM with solvent; 16-18 assays run in the absence of Tyr showed similar levels of SAM deuteration (Appendix Figure C.2), showing that the significant incorporation of deuterium into SAM shown in Figure 4.5A requires the presence of both enzyme and substrate (Appendix Figure C.2). Considering that SAM is present in a 10-fold excess over HydG, and that HydG produces 4 equivalents of 5’-dAdo and 3 equivalents of p-cresol under the assay conditions utilized herein, 12 the extent of deuteration of both SAM and 5’-dAdo exceeds the amount of turnover products
produced. In addition, the levels of deuterium incorporation into SAM and 5’-dAdo are comparable. Together, these observations point to a SAM $\rightleftharpoons$ 5’-dAdo• + Met equilibrium in which the 5’-dAdo• can be deuterated by interaction with Tyr or Tyr cleavage products as outlined below.

The results presented herein provide evidence for a mechanism of HydG-catalyzed Tyr cleavage in which a SAM-derived 5’-dAdo• abstracts a hydrogen atom from the phenolic O-H of Tyr to generate a tyrosyl radical as a reversible event. A single deuterium incorporation into 5’-dAdo when the assay is carried out in D2O would be indicative of an irreversible substrate radical initiation event in which the substrate radical proceeds on in the reaction without further interaction with the 5’-dAdo; the observation of multiple deuterium incorporations into 5’-dAdo, and deuterium incorporation into SAM, must invoke additional steps. For example, the resulting tyrosyl radical can either proceed forward with Cα–Cβ bond cleavage, or can re-abstract a hydrogen atom from 5’-dAdo, quenching the substrate radical to regenerate 5’-dAdo• and ultimately SAM (Figure 4.6A). Iterative abstraction–reabstraction events carried out several times prior to productive Tyr Cα–Cβ bond cleavage would result in multiple deuterium atom incorporations into 5’-dAdo and SAM. An energetic problem with this scenario is that the abstraction of a hydrogen atom from the 5’-methyl of 5’-dAdo (BDE(C–H) = 100 kcal/mol)$^{19}$ by a tyrosyl radical to form Tyr (BDE(O–H) = 86 kcal/mol)$^{20}$ would be thermodynamically uphill, although the uphill process could be coupled to the downhill process of re-forming SAM. Alternatively, Tyr Cα–Cβ bond cleavage may produce alternate radical species that could conceivably abstract a hydrogen from 5’-dAdo to generate 5’-dAdo•, which could then abstract an additional
Figure 4.6. Mechanistic Proposals for Observed HydG H Atom Abstraction–Reabstraction Events and 5′-dAdo• Regeneration.

phenolic O-H from Tyr or recombine with Met to form SAM (Figure 4.6B). Evidence supporting this latter scenario includes recent spectroscopic detection of the intermediate-based $p$-cresolate phenoxy radical. In either mechanism however, a tyrosine-derived radical participates in hydrogen atom abstraction from 5′-dAdo.

The reversible abstraction events catalyzed by HydG bear resemblance to B$_{12}$ chemistry as a reversibly functioning “radical source”, similar to radical SAM enzymes that employ SAM catalytically, however HydG consumes SAM as a cosubstrate. Evidence for a reversible H atom abstraction by 5′-dAdo• has also been reported for enzyme DesII, where multiple deuterium labels from labeled substrate have been detected in the SAM cosubstrate, although the abstraction occurs from a non-exchangeable position. Elucidating the specific role of the reversible radical chemistry in the HydG-
catalyzed synthesis of CO and CN$^{-}$ from Tyr, and the interplay of the radical intermediates with the C-terminal cluster, await further investigations of this fascinating enzyme.

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DEFINING A BASIS FOR DIATOMIC LIGAND PRODUCT BINDING TO THE RADICAL SAM ENZYME HYDG

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

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Contributions: Conceived of and directed the study. Developed exogenous ligand method of comparison and performed all spectral simulations reported. Performed stopped-flow IR and CO photolysis experiments, prepared \textit{C. acetobutylicum} HydG\textsc{WT} and HydG\textsc{NTM} samples used herein. Wrote the manuscript.

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Contributions: Helped perform stopped-flow FTIR experiments, and provided technical expertise related to stopped-flow FTIR experimental design and interpretation.

Co-Author: Aubrey D. Scott

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Contributions: Prepared the HydG\textsuperscript{\textsc{ΔCTD}} EPR sample and collected EPR data. Provided feedback and helpful comments related to EPR spectral simulation.

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Contributions: Prepared the HydG\textsuperscript{\textsc{ΔCTD}} enzyme and collected EPR data.

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Co-Author: John W. Peters

Contributions: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

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Contributions: Directed experimental design and interpretation. Obtained funding and resources, and co-wrote the manuscript.
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DEFINING A BASIS FOR DIATOMIC LIGAND PRODUCT BINDING TO THE RADICAL SAM ENZYME HYDG

Abstract

The [FeFe]–hydrogenase H-cluster is a unique organometallic cofactor in biology that catalyzes the reduction of protons to produce dihydrogen in photosynthetic algae. The radical SAM enzyme HydG catalyzes an important step in the biosynthesis of the H–cluster, namely the synthesis of the coordinated carbon monoxide (CO) and cyanide (CN⁻) diatomic ligands through a radical-initiated tyrosine lyase reaction mechanism.¹² Biochemical characterization of HydG from Clostridium acetobutylicum (Ca) has implicated free release of the diatomic ligands upon formation, while studies of HydG from Shewanella oneidensis (So) point to coordination of the diatomic ligands to HydG iron-sulfur (Fe–S) clusters. Here we use electron paramagnetic resonance (EPR) and

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fourier transform infrared spectroscopy (FTIR) to characterize and compare the interactions of diatomic ligands with the two enzymes. Spectral characterization of the Ca enzyme during turnover yields similar characteristics to enzyme doped with exogenous diatomic ligands. These observations, which are described herein for the Ca enzyme, are distinct from those for the So enzyme.

Introduction

Hydrogen metabolism in microorganisms is facilitated through use of complex Fe–S active site catalysts. The [FeFe]-hydrogenase H-cluster is an unusual organometallic complex that coordinates three CO and two CN⁻ ligands as part of a 2Fe subcluster. These diatomic ligands are synthesized by the actions of radical SAM biosynthetic maturase enzyme HydG on the amino acid substrate tyrosine. These diatomic products of catalysis are incorporated as part of the H-cluster in a maturation scheme that involves a GTPase protein HydF that has been proposed to serve as an assembly scaffold, as well as a second radical SAM enzyme HydE that by elimination is proposed to synthesize the dithiolate bridge in a manner similar to radical SAM enzyme BioB. HydG-catalyzed formation of the diatomic ligands requires two site–differentiated [4Fe-4S] clusters, an N-terminal radical SAM [4Fe-4S] cluster, and a C–terminal [4Fe-4S] cluster that acts upon the proposed intermediate dehydroglycine (DHG) by an unknown mechanism.

The form in which the diatomic ligands are transferred from HydG to the hydrogenase active site is an intriguing question in the 2Fe subcluster biosynthetic scheme. The HydG enzyme singularly produces all diatomic ligands of the 2Fe
subcluster, but its catalysis alone is not sufficient for maturation of the H-cluster; HydE and HydF are also required for biosynthesis. Initial characterization of diatomic ligand formation by *Clostridium acetobutylicum (Ca)* HydG performed in the absence of HydE and HydF resulted in detection of CO and CN⁻ as “free” ligands, captured either by deoxyhemoglobin (deoxyHb) or by derivatization. Additional support for release of diatomic ligand products from HydG came from the observation of multiple equivalents of *p*-cresol and CN⁻ products formed in a 1:1 stoichiometry, which would presumably require cyanide to be released from the active site for multiple catalytic events to occur. However, an *in vitro* maturase assembly study following transfer of Fe from *S. oneidensis* (So) HydG to *C. reinhardtii* HydAΔEFG has recently provided evidence that the 2Fe subcluster iron content (along with the diatomic ligands)

![Figure 5.1](image_url)

**Figure 5.1.** *S. oneidensis* HydG-catalyzed Fe Delivery to HydA and Fe–CO/CN Complex Formation as published by Kuchenreuther et al. (A) ⁵⁷Fe ENDOR of *C. reinhardtii* HydA1 with full enrichment (line A) and of ⁵⁷Fe enrichment with only HydG (line B). (B) Fe–CO/CN species identified by stopped-flow FTIR spectroscopy. Complex A forms immediately upon catalysis, while Complex B forms after 100 seconds of incubation. Reprinted with permission from reference 17. Copyright 2014 American Association for the Advancement of Science
in the holo-HydA enzyme originates from HydG (Figure 5.1A). Comparison of Q-band Davies ENDOR spectra of $^{57}$Fe-enriched HydA$_{EFG}$ to natural Fe abundance HydA$_{EFG}$ incubated with cell-free lysates HydE, HydF (both natural Fe abundance), and $^{57}$Fe-enriched HydG pointed to the transfer of $^{57}$Fe from HydG to the 2Fe subcluster. Further, time-resolved Fe–CO/CN FTIR vibrational bands on HydG were detected, suggesting that HydG-associated catalytic events yield enzyme-bound diatomic ligands (Figure 5.1B). Evidence for the formation of CO and CN$^-$ coordination to Fe on the HydG enzyme, together with the evidence for iron transfer from HydG to HydA, suggests that the diatomic ligands are not released freely, but are rather transferred together in an iron-bound state.

HydG is unusual within the radical SAM enzyme superfamily in that its products serve as potential ligands to the active site metal cofactors. For example, the CN$^-$ anion is a strong field ligand that can bind to biological metal sites in a way that is deleterious to function. In excess, this can lead to formation of hexacyanometallate complexes, dependent on the thermodynamics of CN$^-$ versus protein ligand binding. However, the unique Fe of site-differentiated [4Fe-4S] clusters is coordinated by non-protein ligands that can undergo facile displacement by molecules of interest, such as SAM for members of the radical SAM superfamily, citrate in the case of aconitase, and diatomics such as CN$^-$ in the case of Pyrococcus furiosus (Pf) ferredoxin.

In this work, we characterize the Ca HydG enzyme under turnover conditions to resolve differences observed between it and So HydG. These enzymes share considerable sequence similarity and have several identical amino acid residues within the TIM barrel that likely participate in catalysis, suggesting indifference in the
catalytic mechanism. Because differences at the catalytic level seem untenable by the common substrate and sequence identity, insight regarding the mechanism of diatomic ligand biosynthesis remains limited in understanding a basis for organometallic complex stability at the C-terminal cluster. Also, we employ an unusual strategy to understand the nature of diatomic ligand binding to the Ca HydG enzyme through exogenous ligand addition, in order to differentiate endogenous and exogenous ligand binding between the enzymes. The results presented herein provide a basis for understanding HydG diatomic ligand complex formation at two participatory Fe–S clusters.

**Experimental Section**

**Materials**

All chemicals and other materials used herein were from commercial sources and of the highest purity where available. KCN, CO, K₄[Fe(CN)₆], K₃[Fe(CN)₆], exoskeleton equine myoglobin, and natural abundance tyrosine were obtained from Sigma-Aldrich (St. Louis, MO), while ¹³CO was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Tris, HEPES, IPTG and DTT were obtained from RPI (Mt. Prospect, IL), while KCl and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite and sodium sulfide were obtained from Acros Organics (Fair Lawn, NJ). Iron(III) chloride and ferrous ammonium sulfate were obtained from Fisher Scientific (Fair Lawn, NJ). H64L myoglobin and hemoglobin were received as kind gifts from Professor John S. Olson (Rice University) and Professor David J. Singel (Montana State University), respectively. SAM²² and 5-deazaflavin²³-²⁶ were synthesized and purified as reported elsewhere, with slight modifications.
Heterologous overexpression of *Clostridium acetobutylicum* HydG in *Escherichia coli*, and purification and chemical reconstitution with iron and sulfide were prepared as described previously \(^2,11\) with slight modifications (Appendix A and Chapter 3). Briefly, single colonies obtained from transformations were grown overnight in LB media and used to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50 mM potassium phosphate buffer pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD\(_{600}\) = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N\(_2\) overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber (Grass Lake, MI), as described \(^2,11\) with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4, 20 mM MgCl\(_2\), 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, ~ 0.6 mg lysozyme per gram cell. The lysis mixture was stirred for one hour, after which time the lysate was centrifuged in gastight bottles (Nalgene; Rochester, NY) at 18,000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrapTM Ni\(^{2+}\)-affinity column (GE Healthcare,
Uppsala). The column was pre-equilibrated with 50 mM HEPES 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Pure fractions (gauged by SDS-PAGE) were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 and concentrated using either an Amicon Ultra centrifugal unit (Millipore; Billerica, MA) fitted with a YM-10 membrane, or using a Minicon B15 static protein concentrator (Millipore). Protein was flash frozen in liquid N2 and stored at – 80 °C or in liquid N2 until further use.

Reconstitution of as-purified HydG was carried out following the general procedures described previously. Enzyme (50 – 150 µM) was incubated with 6 – 7 fold excess of FeCl3 and Na2S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 for approximately 3 hours with gentle agitation in a Coy anaerobic chamber at 22 °C. Following a 5 minute incubation with DTT, FeCl3 was added slowly and stirred for 20 minutes prior to the stepwise addition of Na2S. Following the reconstitution period, the mixture was centrifuged to remove exogenous Fe–S clusters in the mixture. Following initial concentration using an Amicon centrifugal unit, the supernatant was treated over a Sephadex G-25 column (GE Healthcare; Piscataway, NJ) to remove excess ions. Following this, the darkest brown fractions were pooled and concentrated using an Amicon Ultra centrifugal unit. UV-visible spectroscopy was used to confirm the Fe–S cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Enzyme concentration was
determined by performing a Bradford assay,\textsuperscript{27} while the iron content was determined through the colorimetric ferrozine method.\textsuperscript{28}

**Stopped-Flow FTIR Spectroscopy**

FTIR spectra were measured using a Bruker IFS/66S FTIR spectrometer (Billerica, MA) interfaced to a home-built stopped-flow drive system with the sample cuvette and drive system maintained inside an anaerobic chamber (Belle Technology Weymouth, UK, O$_2$ < 1.1 ppm) as described elsewhere.\textsuperscript{29} The IR cuvette was thermostatted at 25 °C. For time-resolved experiments characterizing HydG turnover, a sample containing HydG, dithionite, tyrosine, and buffer was loaded on one side of the drive system, with the other side loaded with AdoMet, dithionite, tyrosine, and buffer. Time-resolved experiments typically ran for approximately 40 minutes. For experiments where exogenous CO and CN$^-$ were added, the diatomic ligands were loaded in the non-protein containing syringe. A saturated CO buffer solution was prepared by adding two mL of CO atmosphere into a syringe containing 1 mL of buffer, and was thoroughly mixed for two minutes. Spectra were measured at 4 cm$^{-1}$ resolution. The IR cuvette path length was calibrated at 47.6 $\mu$m. Arbitrary background corrections were applied to yield flat baselines for measured spectra.

**HydG CO Photolysis FTIR Experiments**

The sample photolysis was conducted in a Oxford liquid-He flow cryostat using a Sutter Instruments (Novato, CA) 300 W Lambda LS xenon arc lamp, in a manner similarly described elsewhere.\textsuperscript{30,31} Spectra were recorded at 4 cm$^{-1}$ resolution with a Bruker V-70v FTIR spectrometer and a MCT detector. For photolysis, the lamp was
illuminated through the side of the cryostat oriented 90° to the IR-light path. The sample was held at 45° to both beams. This allowed the use of quartz windows for the visible light.

Samples subjected to turnover were prepared in Montana in an anaerobic glovebox (MBraun; Stratham, NH) at O₂ levels ≤ 1 ppm. Samples were prepared identically to HydG CO formation experiments described below with deoxyHb or H64L deoxymyoglobin (deoxyMb), with slight modifications. 62 μM HydGWT (6.4 ± 0.4 Fe/protein) was incubated for 1 hour at 37 °C with 1 mM C₁⁻¹³C-tyrosine, 1 mM SAM, 5 mM dithionite in 250 mM tris, 300 mM KCl, 5% glycerol, and 90 μM deoxyHb at pH 8.5. An experiment was performed using the Cary 6000i spectrophotometer (Agilent; Santa Clara, CA) to confirm that CO formation occurred (at 37 °C), and then was subsequently frozen for transfer to Davis for CO photolysis experiments. In Davis, the sample was loaded into a sample holder in a Vacuum Atmospheres anaerobic chamber (Hawthorne, CA) that contained < 3 ppm O₂ before flash freezing in liquid N₂.

**EPR Spectroscopy**

EPR samples were prepared in an MBraun box at O₂ levels < 1 ppm. HydG samples undergoing turnover with dithionite reductant were prepared as a mixture of tyrosine, buffer, and HydGWT. After the enzyme mixture was loaded into an X-band EPR tube, a mixture of SAM and dithionite was added by glass pipet and the mixture was briefly mixed before incubating for the indicated time at ambient temperature prior to quenching in liquid N₂. Sample handling required about 20 seconds before flash-freezing was possible. Turnover samples contained final concentrations of 3 mM Tyr, 3 mM
SAM, and 3 mM dithionite in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. Non-
turnover samples that were reduced by dithionite were typically incubated in the presence
of dithionite for 15 minutes at ambient temperature before ligands were added. Samples
were frozen within 60 seconds of ligand addition. Photoreduced samples of reconstituted
HydG^{WT}, HydG^{ΔCTD}, and HydG^{NTM} were made by supplementing the protein with 50
mM Tris, pH 7.4, 100 µM deazariboflavin, and 5 mM DTT using enzyme concentrations
reported in the results sections. Samples were then placed in an ice water bath in the
MBrannon glovebox and illuminated with a 300 Watt Xe lamp for 1 hour. Following
photoreduction, 10 mM CN⁻ was added to the enzyme sample (where the added CN⁻
volume comprised 2% of the final volume) to make a total volume of 260 µL in the
absence of light and the sample was immediately flash-frozen in liquid N₂. For single
turnover experiments, SAM and tyrosine were added after the sample was briefly
centrifuged. Approximately five minutes elapsed between sample removal from the light
and sample freezing, while three minutes elapsed between reagent addition and sample
freezing. For the CN⁻ titration performed, incremental volumes of CN⁻ were added, but
the total EPR sample volume was 260 µL. Samples were prepared in 250 mM tris, 300
mM KCl, 5% glycerol, pH 8.5. It should be noted that ionic strength was not held
constant, however samples prepared with identical CN⁻ concentrations in higher salt
buffer but lower pH (50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4) gave a similar
perturbation in signal (data not shown). Low temperature EPR spectra were collected
using a Bruker EMX X-band spectrometer (Billerica, MA) equipped with a liquid helium
cryostat and temperature controller (Oxford instruments; Abingdon, U.K.). Typical EPR
parameters included sample temperature, 12 K; microwave frequency, 9.37 GHz;
microwave power, 1.0-1.8 mW; time constant, 81.92 ms; sweep time, 167.77 s. Simulation of EPR data was performed with the EasySpin software program\textsuperscript{32} and yielded the g-values in the chapter.

HydG CN\textsuperscript{−} UV-visible Absorbance Experiments

Experiments to assess the effect of CN\textsuperscript{−} on the UV-visible absorbance were performed on reconstituted HydG\textsuperscript{WT} in buffer (50 mM tris, 300 mM KCl, 5\% glycerol, pH 8.5). Experiments were performed on a Cary 6000i spectrophotometer at room temperature, where scans were made in the 300-800 nm range. The assay buffer was degassed on a schlenk line vacuum manifold in a schlenk flask, where it was subjected to iterative vacuum (3 x 10 min) each followed by N\textsubscript{2} backfills. The degassed volume was diluted with degassed dd H\textsubscript{2}O to make the original volume. Samples were prepared in an anaerobic MBraun glovebox, where degassed buffer and HydG (40 \(\mu\)M; 3.9 \(\pm\) 0.1 Fe/protein) were combined, mixed, and centrifuged at 14,000 rpm for five minutes. Potassium cyanide (KCN) was prepared by weighing out the solid, then by dissolving it in the assay buffer. CN\textsuperscript{−} was loaded into a pre-purged, 25 \(\mu\)L gastight syringe (Hamilton; Reno, NV) while the enzyme was loaded into a 1 cm pathlength anaerobic cuvette with a screwtop cap (Starna Cells; Atascadero, CA) and a teflon membrane insert (Thermo–Fisher; Bellefonte, PA). After performing an initial 300-800 nm scan, CN\textsuperscript{−} was injected, in a manner similar to CO formation experiments. The penetrated teflon membrane was sealed with vacuum grease, and the cuvette was inverted a few times before the initial scan was made. Scans were performed periodically until no spectral change was observed.
HydG CO Formation Experiments

HydG CO formation was performed similarly to methods reported elsewhere (Chapter 2), with slight modifications. Briefly, HydG CO formation experiments were performed on a dual pathlength Cary 6000i spectrophotometer with a Peltier temperature module (Strumenti Scientific; Padova, Italy), using a 1 mm pathlength far UV (170-2200 nm) quartz cuvette with a screwtop cap (Spectrocell; Oreland, PA) held in place with a cell mount insert. Baseline absorbance data was obtained in dual pathlength mode, using reaction buffer as a blank across the sampled 300-800 nm range with the scan program, and at 423 nm (the Soret band $\lambda_{\text{max}}$ for H64L MbCO) for the kinetics program in the Cary software package. Preparation of samples monitoring HydG CO formation was performed under strict anoxic conditions under $\leq 1$ ppm O$_2$ in an anaerobic MBraun glovebox. Tyr solid was dissolved in degassed 1.2 M HCl in the anaerobic chamber, and then was diluted with 100 mM tris, pH 7.4 buffer to make a concentrated 67.7 mM stock. For experiments, a working 2 mM Tyr stock was prepared by diluting 29.6 $\mu$L of the concentrated stock into the buffer of interest to 1000 $\mu$L total. The pH of the working stock was found to be slightly acidic (as gauged by pH range 6-8 pH paper), but the final assay solution approximated to the original pH of the assay buffer. The H64L myoglobin was prepared for use by treating it with K$_3$[Fe(CN)$_6$] (15 mM final) in the anaerobic chamber. The sample was irradiated with light in an ice bath for 10 min before being transferred to a Coy chamber, where it was desalted using a PD-10 desalting column. To ensure complete removal of the ferricyanide, the myoglobin was dialyzed into a working buffer (50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4) before it was stored in a 2.0 mL screwcap cryovial, flash-frozen in liquid N$_2$, and stored in a -80 °C freezer.
Reductant sodium dithionite was weighed under aerobic conditions in a 1.5 mL eppendorf tube and was immediately brought into the anaerobic chamber. To minimize solid sample loss upon transfer into the anaerobic chamber, the tubes were wrapped with parafilm and penetrated with a needle to subject the solid to vacuum-N₂ backfill cycles.

HydG CO assays were all performed similarly to methods reported elsewhere,² with a few slight modifications. Reconstituted HydG₇⁰ was combined with tyrosine, 5 mM dithionite, H₆₄L deoxyMb and assay buffer of interest to appropriate dilution. HydG was added last to minimize enzyme precipitation from the addition of the acidic tyrosine stock. After centrifuging the sample for 5 minutes at 13,000 rpm, the sample was loaded by gastight syringe into an anaerobic 1 mm pathlength UV-visible cuvette with a pre-purged 500 µL gastight syringe, while the required SAM and CN⁻ volumes were combined and loaded into a pre-purged, sharp-ended 25 µL gastight syringe that was transferred to the instrument by inserting the sharp end into a rubber stopper. After equilibrating the sample at 37 °C for 5 minutes, an initial 300-800 nm scan was recorded. In the kinetics program, the initial A₄₂₃nm was also recorded. SAM was injected into the sample by penetrating a 12 mm Tuf-bond teflon insert (Thermo-Fisher; Bellefonte, PA) in the screwcap of the cuvette, and the absorbance at 423 nm was monitored every second over the course of 15 or 30 minutes. The hole generated by the penetration was sealed with vacuum grease to minimize sample exposure to O₂. The cuvette immediately was inverted four times before replacement back in the instrument, where A₄₂₃nm was monitored every second for 30 minutes at 37 °C. The time delay between the SAM injection and initial absorbance readings ranged from 10 to 15 seconds. At the end of the program, a final absorbance scan was made, and an 80 µL aliquot was removed, and 15%
(v/v) of 1 M HCl was added and the sample was flash frozen.

Results

Spectral Characterization of *Ca* HydG under Turnover Conditions

During *Ca* HydG catalysis, the detected levels of CO are consistently lower than those of *p*-cresol and CN\(^-\).\(^2\) We originally proposed that binding or sequestration of CO by the HydG Fe–S clusters might account for this low stoichiometry.\(^2\) To investigate this hypothesis, stopped-flow FTIR spectroscopy was performed on *Ca* HydG\(^{WT}\) at Lawrence Berkeley National Laboratory (Berkeley, CA) to examine whether distinct metal–CO/CN vibrational bands could be observed. Experiments where HydG, dithionite, and tyrosine (natural abundance) were shot against SAM, dithionite, and tyrosine at 25 °C yielded no

![Figure 5.2](image_url)

**Figure 5.2.** Stopped-flow FTIR Experiment of *Ca* HydG\(^{WT}\) Undergoing Turnover with Myoglobin (equine exoskeleton) performed at 25 °C. Samples contained HydG\(^{WT}\) (100 µM, 4.7 ± 0.2 Fe/protein), 2 mM SAM, 1 mM natural abundance tyrosine, 194 µM myoglobin in 50 mM HEPES, 500 mM KCl, pH 7.4. (A) Time-averaged, baseline-subtracted spectrum of HydG-catalyzed CO formation of the final four minutes of the reaction. The band at 1943 cm\(^{-1}\) represents \(^{12}\)CO-bound myoglobin. (B) Time course of the vibrational band at 1943 cm\(^{-1}\), representing MbCO formation.
Figure 5.3. CO Photolysis of \( Ca \) HydG\textsuperscript{WT} Undergoing Turnover with \( C_1\text{-}^{13}C \)-tyrosine Following 2 minutes of Photolysis at 8 K. HydG\textsuperscript{WT} (62 \( \mu \)M; 6.4 \( \pm \) 0.4 Fe/protein) was incubated with 1 mM tyrosine, 1 mM SAM, 5 mM tyrosine and 90 \( \mu \)M deoxyHb for 60 minutes at 37 \( ^\circ \)C.

observable vibrational bands (data not shown). In addition to SF-FTIR, we utilized flash photolysis of the HydG after turnover to look for evidence of bound photolabile CO. These flash photolysis experiments also did not yield observable FTIR bands following sample irradiation (data not shown). However, inclusion of equine exoskeleton deoxyMb to each syringe in the stopped-flow experiment noted above resulted in time-dependent formation of CO as indicated by a vibrational band at 1943 cm\(^{-1}\) (Figure 5.2) assigned to Mb–CO; CO is thus being formed by HydG, although it is not observed bound to HydG. The band observed in the 2070–2110 cm\(^{-1}\) region (Figure 5.2) can be assigned as an artifact of baseline subtraction. CO photolysis experiments in the presence of human deoxyHb provided similar results, with \( ^{13}CO\text{-}Hb \) detected from a sample where HydG and deoxyHb were incubated with \( ^{13}C\text{-}C_1 \)-tyrosine with a principal vibrational band at 1906 cm\(^{-1}\) (Figure 5.3). These observations are qualitatively different from the results presented in Figure 5.1B, which pointed to CO binding directly to \( So \) HydG.
FTIR spectroscopy is one of several complementary spectroscopic approaches that can be utilized to probe the binding of diatomic ligands that might serve as products. While FTIR is particularly useful for looking at metal–CO/CN vibrational modes, other methods can also provide insight into the binding of ligands that perturb Fe–S electronic character. For example, EPR spectroscopy has shown that the addition of SAM perturbs the N-terminal cluster g-values.\(^2,\text{16}\) If the CO and CN\(^-\) were sequestered by the HydG enzyme at an Fe–S cluster, a perturbation of the EPR spectroscopic properties would be expected.

To investigate EPR spectral changes associated with turnover, photoreduced Ca HydG was prepared with SAM alone and with SAM and tyrosine (Figure 5.4). Photoreduction with 5-deazariboflavin allows for single turnover conditions due to the ability to remove excess reductant by removing the light. Relative to the sample that only

![Figure 5.4. X-band EPR spectra (9.37 GHz, 12K) of Photoreduced Ca HydG\(^WT\) Undergoing a Single Turnover Event. To HydG\(^WT\) (30 µM; 9.7 ± 0.1 Fe/protein) containing 50 mM tris, 5 mM DTT, and 100 µM 5-deazariboflavin prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 was added 1 mM SAM (black trace) or 1 mM SAM and 1 mM Tyr (red trace) following 60 minute photoreduction. EPR spectral parameters can be found in the experimental section.](image)
contained SAM (Figure 5.4, black trace), the turnover sample containing SAM and tyrosine (Figure 5.4, red trace) yielded comparable spectral features but also a drop in integrated spin, from 0.55 to 0.29 spins/protein. An acceptable, three-component spectral simulation was performed for the turnover sample using a combination of N-terminal SAM-bound, and N- and C-terminal reduced, unbound clusters simulated from the sample lacking tyrosine (Appendix Figure D.1), and is similar to our recent simulation model. The decreased signal intensity observed when substrate is present is consistent with [4Fe-4S] cluster oxidation, which would yield a diamagnetic, EPR-silent species. Interestingly, a feature at 3330G present in the turnover sample (Figure 5.4, red trace) was absent in the sample lacking tyrosine, suggesting an organic radical; however, the low signal-to-noise suggested that the signal likely was artifactual.

While photoreduction limits the extent of catalysis by removal of reductant in the absence of light, dithionite reduction provides a continuous source of reductant during multiple turnovers. Because dithionite generally maintains the Fe–S clusters of HydG in an EPR-active state, we used dithionite reduction to assess Fe–S cluster character under turnover conditions. In the absence of tyrosine, dithionite-reduced HydG<sub>WT</sub> in the presence of SAM was similar to our previous report under 10-fold excess of SAM (Appendix Figure D.2). The addition of SAM yielded an increase in integrated spin from 0.39 to 0.78 spins per protein, and corresponded to 71% of the simulated signal (Appendix Figure D.2).

Interestingly, the addition of tyrosine to the above mixture with SAM resulted in observable perturbation in the [4Fe-4S] cluster signal, in addition to the appearance of a new organic radical signal (Figure 5.5). The latter organic radical has been identified
Figure 5.5. X-band (9.37 GHz, 12 K) EPR Spectroscopy of HydG\textsuperscript{WT} Undergoing Turnover with Dithionite, Tyrosine, and SAM. HydG\textsuperscript{WT} (100 \mu M; 5.2 ± 0.1 Fe/protein) was prepared in the presence of 3 mM tyrosine, 3 mM SAM, and 3 mM dithionite, incubated at 23 °C. Black trace, 30 second incubation; red trace, 90 second incubation; blue trace, 5 minute incubation; magenta trace, 15 minute incubation. Incubation times correspond to the time between SAM and dithionite addition and sample flash-freezing. EPR spectral parameters used can be found in the experimental section, while spectral simulations can be found in Figure 5.6.

Table 5.1. Spectral Comparison of Integrated Spin for \textit{Ca} HydG\textsuperscript{WT} Turnover Samples of Figure 5.5

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<th>Incubation Time (min)</th>
<th>Integrated Spin (spins/protein)</th>
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<tr>
<td>0.5</td>
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<td>1.5</td>
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<td>5</td>
<td>0.35</td>
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Figure 5.6. X-band EPR Spectral Simulation of Ca HydG<sup>WT</sup> Turnover Samples from Figure 5.5. Left panels depict composite simulation (red traces) compared to experimental spectra (black traces). Right panels correspond to simulated components. Black traces denote the composite simulation. Red trace, reduced N- and C-terminal clusters; blue trace, SAM–bound N-terminal cluster; magenta trace, unique axial signal #1; green trace, unique axial signal #2; violet trace, organic radical. Normalized percent simulation is reported. A percent simulation of zero represents a detected signal that rounded less than 1% of the total signal.
Table 5.2. EPR Spectral Simulation Parameters to Data Presented in Figure 5.6.

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<th>$g_3$</th>
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for the So HydG system to be the $p$-cresolate phenoxy radical.\(^{33}\) The Fe–S spectral features appeared to not change remarkably with increasing incubation time, as a comparable quantity of integrated spin was observed for each time point, from 0.31 to 0.35 spins per protein (Table 5.1). Discrete spectral changes were observed with incubation time that did not change the amount of integrated signal; the organic radical
signal decayed and the feature at 3560 G decreased with time, the sharpening and shift of features at 3288 and 3482 G increased with time (Figure 5.5; Table 5.1).

Spectral simulation was performed to deconvolute the different paramagnetic species present during catalysis. Each spectrum was simulated as a five-component simulation, corresponding to (i) reduced (unbound) N- and C-terminal clusters, (ii) SAM-bound N-terminal cluster, (iii) and (iv) two discrete, axial signals, and (v) the organic radical signal (Figure 5.6, Table 5.2). The simulation model was set up based on previous photoreduced spectral simulations, assigning firm simulation parameters for reduced and SAM-bound signals identified previously. The signals (iii), (iv), and (v) were unusual, as it appeared that these signals originated from catalytic turnover by the enzyme. The unique axial signal #2 (iv) increased with time as the as the organic radical (v) decayed. Also, unique axial signal #1 (iii) was present in the initial turnover sample and appeared to decrease with added incubation time, suggesting the presence of new or perturbed cluster species that originated from catalysis (Table 5.2; Figure 5.6).

HydG Fe–S Clusters and Activity Following Exogenous CN⁻ Addition

_Ca_ HydG yielded detectable CO vibrational bands when myoglobin or hemoglobin was included in the incubation mixture, and spectral changes observed by EPR spectroscopy under turnover conditions suggested that complex formation might be occurring (Figure 5.5). While these results were distinct from the _So_ HydG FTIR results, clearly cluster changes occurred for the _Ca_ enzyme. To investigate these distinctions, an alternative exogenous ligand addition approach was investigated by using stopped-flow FTIR spectroscopy (Figure 5.7). The addition of $^{13}$CO to the HydG reaction mix
Figure 5.7. Stopped-flow FTIR Spectra of Ca HydG in the Presence of Exogenous CO and CN⁻. Samples contained HydG^{WT} (100 µM; 6.0 ± 0.3 Fe/protein), 2 mM SAM, 1 mM tyrosine, 5 mM sodium dithionite. The opposite syringe contained either saturated $^{13}$CO only (red and blue traces, lower set) or $^{13}$CO and natural abundance CN⁻ (red and blue traces, upper line) making a final concentration of 5 mM. Samples lacking HydG (green trace) were performed, as well as control 50 µM potassium ferrocyanide (orange trace) and 5 mM KCN (black trace) prepared in 50 mM HEPES, 500 mM KCl, pH 7.4.

containing SAM, tyrosine, and dithionite did not result in detectable vibrational bands outside of the selected baseline (Figure 5.7; red and blue traces, lower set). However, addition of both $^{13}$CO and natural abundance CN⁻ resulted in formation of bands at 2093 and 2038 cm⁻¹ within the first second, along with an observable shoulder at 2020 cm⁻¹ that did not appear to change with added incubation time (Figure 5.7; red and blue traces, upper spectra). When compared to experimental controls for CN⁻ and ferrocyanide, however, these bands appeared to be identical to the controls (Figure 5.7, black and orange traces, respectively), thus providing no evidence for CO and CN⁻ binding to
Figure 5.8. Effect of CN\(^{-}\) Addition to Non-reduced HydG\(^{WT}\) by UV-visible Absorbance Spectroscopy. Samples contained 40 \(\mu\)M HydG\(^{WT}\) (3.5 ± 0.1 Fe/protein) in 50 mM tris, 300 mM KCl, 5% glycerol, pH 8.5. Left panel, 40 \(\mu\)M CN\(^{-}\) added. Right panel, 1 mM CN\(^{-}\) added. Blue trace represents the scan before CN\(^{-}\) was added, while the red trace represents the final spectrum following 45 minutes (A) and 83 minutes (B) of incubation.

HydG.

The ability to form CN\(^{-}\) as a product of catalysis by an Fe–S containing metalloenzyme is remarkable, because CN\(^{-}\) itself is a strong-field ligand that can efficiently coordinate to metal ions in biological systems.\(^1\)\(^8\) To investigate the potential affect that CN\(^{-}\) has on the [4Fe-4S] clusters of HydG, UV-visible spectroscopy was performed (Figure 5.8). Two experiments were performed with the CN\(^{-}\) concentration varied, both at the concentration formed after a single turnover event (40 \(\mu\)M), as well as in 25-fold excess (1 mM) (Figure 5.8). For both experiments, CN\(^{-}\) caused changes in absorbance spectral features. 40 \(\mu\)M CN\(^{-}\) addition yielded slight decreases and increases near 500 nm and below 400 nm, respectively (Figure 5.8A). By comparison, 1 mM CN\(^{-}\) addition immediately decreased the absorbance below 420 nm and increased the absorbance at 595 nm, forming an isobestic point at 530 nm (Figure 5.8). Interestingly, addition of 1 mM CN\(^{-}\) to HydG coordinating approximately 140 \(\mu\)M Fe after 83 minutes
of incubation yielded detectable Fe–S absorbance features. The CN⁻ concentration was in excess 7-fold relative to the enzyme Fe concentration, providing ample CN⁻ to form ferrocyanide as in Figure 5.7. However, for each of the CN⁻ concentrations investigated, the enzyme appeared to be binding the ligand (Figure 5.8).

To assess native HydG activity in the presence of CN⁻, CO formation experiments were performed where CN⁻ was injected along with SAM at 25 °C; these assays were carried out under modified conditions from what were reported previously, but with SAM

![Figure 5.9. HydG^{WT} CO Formation in the Presence of CN⁻.](image)

Table 5.3. Kinetic Parameters for CO Formation for the Figure 5.6 Spectral Data

<table>
<thead>
<tr>
<th>Sample Mix</th>
<th>Burst phase $k_{cat}^0$ ($\times 10^{-4}$ s⁻¹)</th>
<th>Slow phase $k_{cat}^0$ ($\times 10^{-4}$ s⁻¹)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CN⁻</td>
<td>58.2 ± 0.85</td>
<td>3.62 ± 0.04</td>
<td>0.99</td>
</tr>
<tr>
<td>40 μM CN⁻</td>
<td>60.8 ± 0.86</td>
<td>3.34 ± 0.04</td>
<td>0.99</td>
</tr>
<tr>
<td>200 μM CN⁻</td>
<td>59.0 ± 0.68</td>
<td>2.13 ± 0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>1 mM CN⁻</td>
<td>69.6 ± 2.34</td>
<td>N.D.</td>
<td>0.99</td>
</tr>
<tr>
<td>N.D.</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and tyrosine concentrations above their respective $K_M$ values\textsuperscript{16}, using H64L deoxyMb (Figure 5.9). This CO-binding variant has nanomolar affinity,\textsuperscript{34} and was used as part of ongoing studies to improve \textit{in situ} CO detection, as is described in more detail elsewhere (Chapter 6, Appendix Figure D.3). A combined SAM and CN$^-$ mixture was added to initiate the HydG reaction. With one equivalent of CN$^-$ relative to enzyme, no immediate affect on catalysis was observed (Figure 5.9, black and red traces). However, when the CN$^-$ concentration was increased to 200 $\mu$M, differences were observed in the rate and the relative amount of CO formed. Interestingly, addition of 1 mM CN$^-$ resulted in a small amount of CO formed, although after fifteen seconds no additional CO was formed (Figure 5.9, Appendix Figure D.4). Aside from the 1 mM CN$^-$ experiment (which was fitted to a first-order exponential function), CO formation was fitted to a biphasic exponential function (Table 5.3). For all experiments, the burst phase rate was comparable; a slight increase was observed for the 1 mM CN$^-$ sample because it was fitted to a smaller set of data points. The second, slower phase rate, however, decreased with increasing CN$^-$ concentration (Table 5.3).

The collective UV-visible absorbance and CO formation data above pointed to a likely interaction between the exogenous CN$^-$ added and the HydG Fe–S clusters. To investigate this hypothesis, EPR samples of photoreduced HydG$^{WT}$ were prepared in the presence of 10 mM CN$^-$ following photoreduction with 5-deazariboflavin, and the samples were frozen within three minutes of CN$^-$ addition. Addition of CN$^-$ (Figure 5.10A, red trace) caused a spectral shift in the axial reduced spectrum, generating three discrete features in the 3200-3300 G region, as well as a feature near 3470 G (Figure 5.10A, black trace). The spectrum appeared consistent with the coordination of CN$^-$ at
both [4Fe-4S] clusters, but spectral subtraction of the reduced spectrum gave a complex difference spectrum that was intractable to differentiate binding to one or both Fe–S clusters (data not shown). However, preparation of photoreduced HydG$^{\Delta CTD}$ (a C-terminal truncated version of HydG coordinating only the N-terminal cluster) and HydG$^{NTM}$ (a tricysteine site-directed mutant of HydG with only the C-terminal cluster) in the presence of 10 mM CN$^-$ resulted in differences relative to the signal for reduced enzyme in the absence of CN$^-$ (Figures 5.10B, C). The HydG$^{\Delta CTD}$ and HydG$^{NTM}$ samples prepared in the presence of CN$^-$ could be adequately simulated as a combination of reduced (unbound) (see Chapter 4) and cyanide-bound cluster signals, and simulation parameters for the latter could be determined for each cluster (Appendix Table D.3). A rational simulation model could be constructed for HydG$^{WT}$ as a four-component spectrum, comprising mixtures of bound and unbound N- and C-terminal clusters (Figure 5.11A, Appendix Table D.3). It should be noted that a slight shift in assigned unbound cluster g-values for HydG$^{WT}$ relative to the variants was required to achieve an acceptable simulation (Figure 5.11A, C blue traces; Appendix Table D.2); while the HydG$^{\Delta CTD}$
coordinates an N-terminal [4Fe-4S] cluster, slight changes in cluster g-values is not unexpected as a consequence of truncation of the C-terminal 88 amino acid residues.\textsuperscript{16}

With assignment of spectral features possible through simulation, the effect of CN\textsuperscript{−} addition to HydG\textsuperscript{WT} could be determined (Appendix Table D.2). For each of the HydG variants, addition of CN\textsuperscript{−} was associated with a loss in integrated spin. Addition of CN\textsuperscript{−} decreased the integrated spin for reduced HydG\textsuperscript{ΔCTD} and HydG\textsuperscript{NTM} from 0.31 to 0.19 and from 0.42 to 0.33 spins per protein, respectively (Figure 5.10B, C). However, for most sample preparations of HydG\textsuperscript{WT} a doubling in integrated spin was observed with the addition of CN\textsuperscript{−} that appeared to depend on the reductant. While generally an increase was observed for HydG\textsuperscript{WT} in the course of photoreduction, dithionite reduction was more amenable for observing a signal increase. For example, the dithionite-reduced HydG\textsuperscript{WT} spectrum increased from 0.39 spins to 1.01 spins per protein when CN\textsuperscript{−} was added (Appendix Figure D.2). Simulation values for dithionite–reduced HydG\textsuperscript{WT} in the
presence of 10 mM CN\textsuperscript{−} were found to be comparable to the photoreduced sample depicted in Figure 5.10A (Appendix Table D.2, D.3).

To investigate the cyanide-binding ability of HydG more fully, a titration was performed on photoreduced HydG\textsuperscript{WT} (Figure 5.12). A spectral shift was observed that was similar to the spectral data simulated in Figure 5.10A. An increase in integrated spin was observed up to 10 mM CN\textsuperscript{−}, increasing from 0.21 to 0.72 spins per protein (Table 5.4). At concentrations greater than this, however, a drop in integrated spin was observed (Figure 5.12; Table 5.4). Interestingly, an additional, low-field feature was present at 3200 G; this feature was present in photoreduced samples (Figure 5.12) but was absent for dithionite-reduced samples (Appendix Figure D.2). Because this feature was only observed for photoreduced samples, this observation appeared to suggest that the signal arose from 5-deazaflavin, not from CN\textsuperscript{−}. Also, reflected in the higher concentration CN\textsuperscript{−} spectra was the growth of a spectral band at 3330 G that appeared similar to a [3Fe–4S] cluster signal (Figure 5.12; violet trace). Finally, unidentified spectral features in the 100 mM CN\textsuperscript{−} spectrum were present, including features at 3400 and 3450 G. However, the integrated spin was very low, at 0.03 spins per protein (Table 5.4).

An EPR signal increase associated with exogenous addition of CN\textsuperscript{−} up to 10 mM concentration (Figure 5.12) was a bit strange, because the UV-visible absorbance trend from Figure 5.5 showed time-dependent Fe extrusion by CN\textsuperscript{−}, while the EPR sample was a time point. Thus, the time dependence of CN\textsuperscript{−} addition was compared by EPR spectroscopy, with dithionite-reduced HydG that was treated with excess CN\textsuperscript{−} and was incubated to assess the [4Fe-4S]\textsuperscript{+} signal (Figure 5.13). Similar to the photoreduced enzyme, dithionite-reduced enzyme yielded a substantial increase in integrated spins per
Figure 5.12. X-band EPR (9.37 GHz, 12K) CN⁻ Titration of HydG<sup>WT</sup>. Photoreduced HydG<sup>WT</sup> (113 µM; 5.8 ± 0.4 Fe/protein) in the presence of CN⁻ in 250 mM tris, 300 mM KCl, 5% glycerol, pH 8.5. Samples also contained 5 mM DTT and 100 µM 5-deazariboflavin. Typical EPR spectral acquisition parameters are indicated in the experimental section.
Table 5.4. Spectral Comparison of Integrated Spin for CN\(^-\) Titration Experiment from Figure 5.12

<table>
<thead>
<tr>
<th>[CN(^-)] (mM)</th>
<th>Integrated Spin (spins/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.21</td>
</tr>
<tr>
<td>1</td>
<td>0.54</td>
</tr>
<tr>
<td>5</td>
<td>0.40</td>
</tr>
<tr>
<td>10</td>
<td>0.72</td>
</tr>
<tr>
<td>25</td>
<td>0.35</td>
</tr>
<tr>
<td>50</td>
<td>0.13</td>
</tr>
<tr>
<td>100</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 5.13. X-band EPR Spectra (9.37 GHz, 12K) of Dithionite-reduced HydG\(^\text{WT}\) in the Absence and Presence of CN\(^-\). HydG\(^\text{WT}\) (101 \(\mu\)M, 6.5 ± 0.1 Fe/protein) was in the absence (black trace) and presence (red and blue traces) of 10 mM CN\(^-\) prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. (A) high-field spectra; (B) Low-field spectra. All samples were reduced with 1 mM dithionite; the blue trace represents a sample that incubated for an additional 15 minutes in the presence of dithionite and CN\(^-\), relative to the black and red traces. The spin percentage of the Panel B spectral features constituted less than 1 % of the total integrated signal.
protein upon addition of CN⁻ (Appendix Figure D.2, black and blue traces; Figure 5.10A). However, added incubation time of HydG with CN⁻ and dithionite for 15 minutes resulted not in a decrease, but rather a slight increase in integrated signal (Figure 5.13A). Interestingly, the signal in the g = 4.3 region, corresponding to adventitiously bound, high-spin Fe(III), increased upon the initial addition of CN⁻, but decayed in intensity with added incubation time (Figure 5.13B).

The observation that CN⁻ perturbs HydG Fe–S clusters suggested that spiking HydG with CO, like CN⁻, might also cause perturbations observable by EPR spectroscopy. Despite our inability to observe CO–associated vibrational bands with HydG using stopped-flow FTIR spectroscopy, we have shown that unique paramagnetic

Figure 5.14. Effect of CO Diatomic Ligand Binding to HydG^{WT}. Black trace, photoreduced HydG^{WT}; red trace, photoreduced HydG^{WT} plus 12% (v/v) saturated CO solution; blue trace, photoreduced HydG^{WT} plus 5 mM CN⁻; magenta trace, photoreduced HydG^{WT} plus 5 mM CN⁻ and plus 12% (v/v) saturated CO. Photoreduced HydG^{WT} (102 μM; 5.9 ± 0.1 Fe/protein) was prepared in 250 mM tris, 300 mM KCl, 5% glycerol, pH 8.5 and contained 5 mM DTT and 100 μM 5-deazariboflavin. EPR spectral parameters are indicated in the experimental section.
signals have been observed under catalysis that might reflect coordination of CO and CN\textsuperscript{−} (Figure 5.5), while parallel work on the HydF scaffold protein has shown that exogenous addition of CO and CN\textsuperscript{−} to HydF\textsuperscript{EAG} can result in the formation of discrete vibrational bands at 1987, 2073, and 2124 cm\textsuperscript{−1} (Appendix Figures D.5, D.6 and D.7). Addition of a saturated CO solution (12% v/v) to photoreduced HydG\textsuperscript{WT} resulted in slight sharpening and broadening at 3460 G and 3527 G, respectively (Figure 5.14, red trace). Its addition to photoreduced HydG\textsuperscript{WT} in the presence of CN\textsuperscript{−} however produced a similar spectrum to that of CN\textsuperscript{−} added alone, suggesting that CO did not contribute to the observed signal (Figure 5.14, blue and magenta traces). It is important to note here that CO was added after CN\textsuperscript{−} was added, and it has been shown by stopped-flow FTIR that ferrocyanide forms almost instantaneously, and CO might not be able to compete with CN\textsuperscript{−} for binding after CN\textsuperscript{−} is added. Nevertheless, the addition of CO and CN\textsuperscript{−} yielded a spectrum similar to added CN\textsuperscript{−} EPR samples described above (Figure 5.14).

EPR Spectral Simulation of Turnover-Associated Signal with So HydG

Recent FTIR spectroscopic studies on So HydG have suggested evidence for enzyme-coordinated Fe–CO/CN species on HydG,\textsuperscript{17} and are thus distinct from results obtained with Ca HydG; the reasons for these different observations is currently under investigation. Our observation of unique EPR spectral signals (Figure 5.5) during turnover of the Ca HydG are, however, similar to the results recently reported for So HydG.\textsuperscript{33} For both enzymes, an organic radical is observed at 3330 G, and discrete cluster-associated signals different from the reduced or SAM-bound Fe–S cluster signals are observed (Figure 5.5, Figure 5.15). Britt and coworkers reported spectral simulation
of the $So$ HydG Fe–S signals during turnover as a three-component simulation, representing a single reduced N- and C-terminal cluster signal, a single N-terminal SAM-bound signal, and a single C-terminal “bound” cluster signal. $^{33}$ While the Britt model assigned the reduced N-, C-terminal cluster signal as rhombic under turnover conditions, $^{33}$ we have shown here and elsewhere with $Ca$ HydG simulation models that the reduced N- and C-terminal cluster signals are and remain axial upon addition of SAM and upon turnover (Figure 5.6). Our basis for this assignment comes from our simulation model of $Ca$ HydG, in which a mixture of unbound and ligand–bound signals are observed, comprising the original axial signal and a new signal associated with binding of the ligand. $^{16}$ Thus the simulations reported by Britt for $So$ HydG do not appear to be consistent with the behavior for a reduced cluster in the absence of SAM or diatomic ligand products.
Figure 5.16. Alternative Spectral Simulation Model of HydG Fe–S EPR Spectrum (9.37 GHz, 20 K, 0.1 mW) Undergoing Turnover (containing tyrosine, SAM, and sodium dithionite) for 30 seconds. This spectrum is reported in Figure 2D in Kuchenreuther et. al in Reference 33. Composite simulation (left panel) was performed using EasySpin as a superimposition of four discrete signals (black trace), omitting the organic radical signal. Signal components included N-,C-terminal cluster reduced signal (red trace), N-terminal cluster SAM-bound signal (blue trace), C-terminal cluster bound signal #1 (magenta trace), and C-terminal cluster bound signal #2 (olive trace). For spectral parameters, please see Table 5.4. Normalized signal percentage is provided relative to the composite spectrum.

We conducted our own simulation of the published So HydG EPR turnover spectra using the simulation model constructed for Ca HydG reported above to compare the discrete cluster–associated signals formed for both enzymes (Figure 5.16). Similar to the Ca HydG simulation model shown in Figure 5.6, the So HydG Fe–S spectral data was modeled as four overlapping \( S = 1/2 \) signals, corresponding to (i) reduced (unbound) N- and C-terminal clusters, (ii) SAM-bound N-terminal cluster, and (iii) (iv) two additional, unique signals (Figure 5.16, Table 5.5). Spectral parameters (\( g \)-values, \( g \)-strain) determined for the reduced N- and C-terminal cluster signal, and for N-terminal, SAM-bound cluster signals for So HydG (Figure 5.16, right panel, red and blue traces) were similar to our model (Table 5.2) and reported values for Ca HydG (Chapter 3, Appendix B). The residual signal was fitted to a two component spectrum, constituting a mixture
Table 5.5. EPR Spectral Simulation Parameters from Figures 5.16 and 5.17. Organic radical is omitted for clarity of Fe–S cluster simulation.

<table>
<thead>
<tr>
<th></th>
<th>g-value</th>
<th>g-strain</th>
<th>g-value</th>
<th>g-strain</th>
<th>g-value</th>
<th>g-strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal &amp; C-terminal Reduced (unbound)</td>
<td>2.0400</td>
<td>0.0160</td>
<td>1.9350</td>
<td>0.0110</td>
<td>1.9080</td>
<td>0.0400</td>
</tr>
<tr>
<td>N-terminal Reduced (SAM bound)</td>
<td>2.0060</td>
<td>0.0110</td>
<td>1.8770</td>
<td>0.0190</td>
<td>1.8460</td>
<td>0.0300</td>
</tr>
<tr>
<td>Unique Signal #1</td>
<td>2.0580</td>
<td>0.0170</td>
<td>1.9180</td>
<td>0.0130</td>
<td>1.8810</td>
<td>0.0180</td>
</tr>
<tr>
<td>Unique Signal #2</td>
<td>2.0580</td>
<td>0.0190</td>
<td>1.9100</td>
<td>0.0140</td>
<td>1.9010</td>
<td>0.0110</td>
</tr>
</tbody>
</table>

of axial and rhombic signals that principally involved perturbation of the 3250 G and 3450-3550 G regions (Figure 5.16, right panel, green and magenta traces; Table 5.5).

Following development of our simulation model for the So HydG data, the So HydG time course data (Figure 5.15) was simulated to assess the progression of the unique Fe–S cluster signals associated with catalysis (Figure 5.17). In all cases, adequate spectral simulation was made possible by simply changing the simulation weight for each component signal determined from Figure 5.16 and Table 5.5. Qualitatively, the EPR spectra changed with increasing incubation time for the So HydG. First, an intensity increase (that is, an increase in the simulated spectral weight) for all the assigned signals was observed with increasing incubation time, with the largest increase observed for the reduced N-,C-terminal cluster signal (Figure 5.17, right panels, red traces). However,
Figure 5.17. Simulation of Time-dependent So HydG Fe–S EPR Spectra (9.37 GHz, 20 K, 0.1 mW) From Figure 5.15. Composite simulations (left panels) constituted a superimposition of four discrete signals (right panels), and are colored relative to time course data in Figure 5.15. For clarity, the non-Fe–S organic radical signal was omitted from the simulation. Black trace denotes composite simulation. Signal components included N-, C-terminal cluster reduced signal (red trace), N-terminal cluster SAM-bound signal (blue trace), C-terminal cluster bound signal #1 (magenta trace), and C-terminal cluster bound signal #2 (green trace). For spectral parameters, please see Table 5.4. Normalized signal percent is provided relative to the composite spectrum.
dithionite, tyrosine, and SAM were added together with enzyme to initiate the reaction; the observed increase in signal intensity therefore may simply reflect an increase in [4Fe-4S] cluster reduction with time. Second, percent contributions of the axial and rhombic C-terminal “bound” signals appear to change over time. With longer incubation periods, the percent contribution for the bound C-terminal axial signal (Figure 5.17, right panels, green traces) increased while the corresponding rhombic signal (Figure 5.17, right panels, magenta traces) decreased in percent contribution, despite an observed increase in spectral simulation weight. Interestingly, the simulation weight for the rhombic signal (Figure 5.17, right panels, magenta traces) increased between the thirty- and ninety-second incubation period, and did not increase in contribution beyond this time point, which accounted for its decreased percent simulation contribution with respect to time.

**Discussion**

Herein, *Ca* HydG has been characterized by EPR and FTIR spectroscopies under turnover conditions to identify catalytic reaction intermediates associated with HydG catalysis. The C-terminal [4Fe-4S] cluster coordinated at a CX₂CX₂₂C motif has been proposed to facilitate the conversion of DHG to make the diatomic ligands of the H-cluster.²¹⁶,³³³ We originally rationalized the apparent substoichiometry of detected CO, relative to CN⁻ and *p*-cresol, on the grounds that the diatomic ligand was sequestered in some way by the enzyme.² The work described in this chapter has probed the possibility of CO sequestration by binding to Fe–S clusters on HydG.

*Ca* HydG was subjected to FTIR spectroscopy to identify CO and cyanide-bound species potentially generated by the enzyme. Complementary stopped-flow and CO
photolysis methods did not yield detectable vibrational bands that could be assigned to
diatomic ligands on HydG, but inclusion of hemoglobin or myoglobin in the experiments
yielded detectable CO that increased with time (Figure 5.2, 5.3). CO photolysis
performed HydG undergoing turnover with $^{13}$C-C$_{1}$-tyrosine in the presence of
hemoglobin yielded a vibrational band at 1906 cm$^{-1}$, consistent with Hb--$^{13}$CO as reported
in our initial publication (Figures 5.2, 5.3),$^2$ and corresponded to formation of 30 $\mu$M CO
produced after incubation for 60 minutes at 37 °C. Therefore while CO was produced by
HydG in these experiments, no evidence for its binding to iron in HydG could be found
under catalytic conditions, suggesting that a CO-bound species is not formed on the
enzyme itself (Figure 5.2).

EPR spectroscopy provides information on the electronic environment of a
paramagnetic species, and can complement FTIR spectroscopy of [4Fe-4S] clusters that
coordinate ligands with vibrational bands, provided that the Fe–S cluster remains
paramagnetic. EPR spectra of photoreduced Ca HydG undergoing turnover revealed a
decrease in EPR-active signal with time, with the signal similar to that of photoreduced
HydG plus SAM with no unusual features (Figure 5.4). In these experiments the EPR
spectra represent a single turnover event, as once light is removed the enzyme is in
absence of reductant. While no distinct spectral shifts were observed for the enzyme
aside from SAM coordination to the N-terminal cluster, the possibility of a coordinated
diatomic species making the resultant [4Fe-4S] cluster diamagnetic cannot be ruled out,
since diamagnetic clusters cannot be characterized by this technique.

Exogenous diatomic ligand addition was explored as an alternative strategy to
explore the perturbation of HydG Fe–S clusters that might occur due to binding of HydG
products. FTIR spectroscopy of *Ca* HydG with added CN\(^-\) revealed coordination and extrusion of Fe to make a hexacyanoferrate complex (Figure 5.7), based on the vibrational band at 2038 cm\(^{-1}\), which is comparable to the band for the [Fe(CN)\(_6\)]\(^4-\) standard. Consistent with this observation was UV-visible spectral data, in which a drop in absorbance was consistent with the destruction of Fe–S clusters for ferrocyanide formation (Figure 5.8). Correspondingly, EPR spectroscopy of photoreduced HydG\(_{WT}\) with addition of 250-fold excess or greater of CN\(^-\) resulted in a drop in integrated signal, indicating loss of paramagnetic species (Figure 5.12).

As CN\(^-\) is a product of the HydG reaction, it can either coordinate or extrude iron from the Fe–S clusters. As was noted above, UV-visible spectral data for HydG\(_{WT}\) subjected to 25-fold excess of CN\(^-\) resulted a drop in absorbance (representing extrusion by CN\(^-\)), but Fe–S were intact, suggesting a toleration towards CN\(^-\) that might be reflected in coordination ability (Figure 5.8). HydG has been shown to produce over three equivalents of CN\(^-\) following incubation for 60 minutes at 37 °C, and CN\(^-\) inhibition has been observed for the HydG\(_{WT}\) under optimized conditions.\(^1\) CO formation ability is affected by the addition of CN\(^-\), in which the slow phase rate of CO formation was impacted by the exogenous CN\(^-\) concentration (Figure 5.9). Interestingly, all samples produced comparable burst phase CO formation rates, which suggested that CN\(^-\) binding did not affect initial catalytic events, but primarily affected the enzyme undergoing multiple catalytic events (Table 5.3). It should be noted that all samples formed CO, even a small amount was formed initially with the 1 mM CN\(^-\) added (Appendix Figure D.4). The slow phase rate scaled very well to the amount of competent
cluster expected to remain after the iron is extruded from Fe–S clusters of HydG to make ferrocyanide (Table 5.3).

Spectral perturbation of the [4Fe-4S] cluster paramagnetic signal was observed when exogenous CN⁻ was added (Figure 5.10). Spectral simulation of cyanide-bound C-terminal clusters was fit with g-values of 2.09, 1.94, and 1.91; the cyanide-bound N-terminal cluster was simulated with the g-values of 2.06, 1.93, and 1.90 (Table 5.11; Appendix Table D.2). These values resemble the CN-bound [4Fe-4S] cluster g-values from Pf ferredoxin, in which the reduced S = 1/2 species (with g-values of 2.10, 1.87, and 1.80) shifted to g-values 2.09, 1.95, and 1.92 upon 250-fold addition of CN⁻. Detailed ¹³C,¹⁵N ENDOR spectroscopic studies of this Fe–S cluster have shown that a single CN⁻ ion binds directly with the cluster in a fashion similar to a Cys residue, but resulting in a notably small contact interaction. CN⁻ titration to HydG<sup>WT</sup> showed that additions up to 10 mM resulted in a net increase in integrated spin, and this behavior is consistent with CN⁻ serving as a coordinating ligand (Figure 5.12). Interestingly, this trend was only observed for reduced HydG<sup>WT</sup> enzyme, as addition of 10 mM CN⁻ to either reduced HydG<sup>ΔCTD</sup> or HydG<sup>NTM</sup> resulted in net loss of integrated spin (Figure 5.10). For Pf ferredoxin, addition of CN⁻ converted the observed S = 3/2 [4Fe-4S] cluster to S = 1/2 cluster that accounted for the spin increase; a similar trend might be expected here, but no S = 3/2 signal was observed for prepared samples.

The CN⁻ titration performed on HydG<sup>WT</sup> exhibited peculiar behavior, both in the concentration of CN⁻ required to cause a decrease in integrated signal and in the concentration required to form a bound complex (Figure 5.12). To understand this apparent increase in signal, an alternative approach that subjected dithionite-reduced
HydG to CN– for different incubation periods was employed. If CN– served to extrude Fe from the complex, a decrease in signal intensity would be observed with added incubation time, as similar to UV-visible absorbance data reported above (Figure 5.8). Instead, addition of CN– to dithionite-reduced HydGWT that incubated for additional time did not result in a loss of the $S = 1/2$ [4Fe-4S] cluster, but rather a loss in the $g = 4.3$ feature corresponding to adventitiously bound, low-spin, Fe(III) (Figure 5.13). These observations suggest that the cyanide-bound Fe–S cluster is not affected by additional incubation events that would otherwise extrude the Fe from the enzyme and decrease the paramagnetic signal, presuming a constant CN– concentration (Figure 5.13).

An organic radical is observed for Ca HydG under turnover conditions in the presence of dithionite (Figure 5.5). While this radical is very similar to the radical to that observed previously for the So HydG under turnover,17 some notable differences in the EPR of the Fe–S cluster signals in the two enzymes can be made. Under otherwise identical conditions, Ca HydG generates an organic radical that decays, representing less than 1% of the total integrated $S = 1/2$ envelope. The contribution of the reduced Fe–S cluster integrated spin does not change under conditions of excess dithionite, suggesting that an increase in reduced signal is not observed with added incubation time (Table 5.1). Interestingly, the two simulated axial signals (unique signal #1 and #2 in Table 5.2) are similar in character to the exogenous cyanide–treated samples reported above (Figure 5.11, Appendix Table D.2). Unique signal #1 (which is similar to the C-terminal cyanide-bound signal in Figure 5.11A) is present in the initial thirty second sample (Figure 5.5 black trace). With added incubation time, an additional feature at $g = 2.05$ grows in the spectrum, coupled with a spectral shift near 3500 G that is similar in
character to the exogenous, cyanide–treated HydG\textsuperscript{ACTD} signal (Figure 5.11B, Appendix Table D.2). Finally, it should be noted that HydG in the presence of exogenous CO and CN\textsuperscript{−} has been characterized by EPR spectroscopy, as no difference relative to CN\textsuperscript{−} alone addition was observed (Figure 5.14).

Similarly, addition of SAM and tyrosine to dithionite-reduced \textit{So} HydG\textsuperscript{WT} similarly generates an organic radical, as well as discrete Fe–S cluster species associated with turnover (Figure 5.15). The published simulation model for \textit{So} HydG\textsuperscript{WT} turnover was proposed as a minimal superposition of N-terminal SAM bound, N-terminal reduced, C-terminal reduced, and an unique rhombic signal, in addition to an organic radical.\textsuperscript{33} To achieve an acceptable fit of simulation, however, assignment of the reduced, unbound N-terminal (radical SAM) cluster signal under turnover conditions was assigned to be rhombic, and an axial signal corresponding to reduced, unbound signal was ignored.\textsuperscript{33} Considering that the N-terminal cluster generates the 5’-deoxyadenosyl radical, that the C-terminal cluster generates CO and CN\textsuperscript{−}, and that tyrosine cleavage does not require the C-terminal cluster, perturbation of the N-terminal cluster from axial to rhombic would implicate an additional role of the N-terminal cluster aside from SAM cleavage. Further, in our characterization of the \textit{Ca} HydG enzyme in the presence of SAM, perturbation of the N-terminal, reduced signal was not necessary to sufficiently simulate the spectra.\textsuperscript{16}

We here propose an alternative simulation for modeling the EPR-active \textit{So} HydG [4Fe–4S] cluster signals, in which the unique Fe–S cluster perturbation originates from the C-terminal cluster (Figures 5.16, 5.17). This model more accurately assigns the effect of catalysis on the [4Fe–4S] cluster that is expected to interact with the diatomic ligand products, since bound CO and CN\textsuperscript{−} ligands have been observed by FTIR spectroscopy
Figure 5.1). Our simulation of the So HydG thirty second turnover sample Fe–S signal was modeled as a four-spin model representing (i) reduced N- and C-terminal clusters, (ii) SAM-bound N-terminal cluster, and (iii) and (iv) product-bound C-terminal clusters. This model simulated the published data accurately, and identified an axial and rhombic signal associated with catalysis at the C-terminal cluster (Figure 5.16, 5.17). In this model, 34% of the total intensity (excluding the organic radical signal) corresponded to reduced, unbound N- and C-terminal cluster signal, 17% to N-terminal, SAM-bound signal, and 49% to catalysis-associated paramagnetic signal.

It is intriguing to speculate on what the simulated C-terminal “bound” components represent as coordinated [4Fe-4S] clusters for either the Ca or So HydG enzymes from the simulation model proposed herein. Consistent with parallel ENDOR, HYSCORE, and stopped-flow FTIR studies, the signals from Ca and So HydG likely represent diatomic ligand-bound [4Fe-4S] clusters by which the C-terminal Fe–S unique Fe is coordinated by CO and CN– ligands similar to discrete species identified by FTIR spectroscopy. Our simulation model for the time-dependent EPR spectral data for So HydG is congruent as a complex mixture of [4Fe-4S] cluster bound and unbound species similar to our model for Ca HydG (Figure 5.6, Figure 5.17). Measured by EPR simulation of So HydG, formation of “Complex B” (assigned to be a Fe(CO)2(CN) complex) is concomitant with “Complex A” (assigned as a Fe(CO)(CN) complex) within 30 seconds, while a greater percentage of Complex B is formed after 2 min of incubation (Figure 5.17). While spectral summation of all simulated signals of So HydG appears to increase with incubation time overall, comparably large normalized percent increases in signal contribution were observed for reduced, unbound [4Fe-4S] cluster and for the
Complex B signal. Interestingly, the So HydG normalized percent contribution dropped by one half for the Complex A signal despite an apparent increase in simulation weight, perhaps consistent with its role as an intermediate complex at the C-terminal cluster. Consistent with the parallel FTIR study, reduced simulation intensity after 90 seconds of incubation might reflect processing or reaction of complex A.

The detection of EPR-active species that are distinct from reduced [4Fe-4S] cluster and SAM-bound N-terminal cluster under turnover conditions provides additional, corroborative insight to the Fe–S cluster species for both the Ca and So HydG enzymes (Figure 5.6, Figure 5.17). Simulation of the EPR time course spectra support the presence of at least two distinct C-terminal bound species, which is consistent with the time-resolved FTIR data observed for So HydG. The supporting ENDOR/HYSCORE methods have assigned interaction of $^{13}\text{C},^{15}\text{N}$ nuclei with the C-terminal cluster with comparable hyperfine interactions with $^{13}\text{CN}$–bound Pf [4Fe-4S] ferredoxin and $^{13}\text{CO}$ coordinated to the hi-$^{13}\text{CO}$ form of the nitrogenase MoFe protein. Because the incubation period for the So HydG ENDOR/HYSCORE sample is comparable to the time scale that the Fe–CO/CN vibrational bands were observed, the identified ENDOR/HYSCORE spectral features are more consistent as coordinated diatomic ligands than as a “tyrosine-derived fragment” (Figure 5.1, 5.17). Interestingly, reported So HydG data showed that after the ninety second time point, CO was released (detected via myoglobin incubation), which would represent loss of CO from a HydG-associated Fe–S cluster (Chapter 7, Figure 7.14); as is discussed more in Chapter 7, similar behavior with CO formation in the presence of cysteine is observed for Ca HydG, and this compound may be present in the supplemented E. coli cell lysates added to So HydG.
FTIR samples. Our simulation model of *So* HydG time course samples show a significant increase in unbound cluster and C-terminal bound #2 signals, and a negligible increase in C-terminal bound #1 signal after the ninety second sample incubation, respectively (Figure 5.17). Because an increase in observable signal also likely represents an increase in Fe–S clusters that are reduced, the loss of CO may in principle originate from lack of an increase in signal in C-terminal signal #1 by *So* HydG, as reflected by EPR.

The identification of Complex B from *So* HydG as a Fe–(CO)$_2$CN species is intriguing, since in principle it may represent an Fe species that has the correct composition of ligands observed in the CO–inhibited H$_{ox}$ 2Fe subcluster. Mechanistic details of its synthesis, however, are not well understood because the stoichiometry requires generation of an additional equivalent of CN$^-$ that does not coordinate to the complex. Because complex A [Fe–(CO)(CN)] can also be detected, control of the diatomic ligand products via coordination constraints at the unique Fe could serve to differentiate DHG processing as part of the second turnover event. As we have shown here with the *Ca* HydG enzyme, CN$^-$ coordination yields distinct EPR spectral features, either formed as a product or added externally (Figure 5.5, Figure 5.10). DHG is a bidentate ligand that is proposed to coordinate the unique Fe through chelation of the carboxylate oxygen and the imine nitrogen atoms, though monodentate coordination of DHG might also be expected, to differentiate final processing of the DHG intermediate by H-atom abstraction (see Chapter 7). While coordination of the carboxylate (producing CO) would leave the unique Fe coordinatively saturated, the remaining equivalent of CN$^-$ would be unable to coordinate to the unique Fe, leaving it to possibly coordinate to the N-
terminal [4Fe-4S] cluster in a fashion perhaps similar to exogenous addition of cyanide to HydG Fe–S clusters (Figure 5.5, Figure 5.10A).

In conclusion, the Ca and So HydG enzymes have been characterized using similar spectroscopic methods in order to compare their catalytic activity and the organometallic intermediates formed by each. Spectral characterization for each has highlighted notable similarities that have been determined by EPR spectroscopy that implicate comparable mechanisms in the formation and delivery of the diatomic ligand products. Our alternative approach toward understanding diatomic ligand product formation through comparison of exogenous ligand addition provides a foundational basis for diatomic ligand stability at a site-differentiated [4Fe-4S] cluster. The observation that CO does not appear to bind to the Ca HydG enzyme is unique to the biosynthetic mechanism, but is consistent with the exogenous diatomic ligand addition experiments presented herein and the initial burst phase production of CO by Ca HydG observed experimentally. These differences in catalysis could be an artifact of loading of the C-terminal [4Fe-4S] cluster, as perhaps under catalytic conditions the unique Fe becomes labile and C-terminal [3Fe–4S] cluster generation occurs. This catalysis is reminiscent to the HydG enzyme characterized elsewhere (Chapter 3), where CN⁻ and CO formation is affected by substitution of a C-terminal cysteine motif residue (Chapter 3). Nevertheless, EPR spectral simulation of Ca HydG unique signal #1 is similar to ligand binding at the C-terminal cluster, which suggest products are bound to a reduced [4Fe-4S] cluster. Identification of a bound Fe–CO/CN species and ⁵⁷Fe species transfer by the So HydG enzyme suggests an efficient mode of diatomic ligand transfer to HydA that may be common to all HydG enzymes. Future work to identify the
mechanism of formation of this fascinating organometallic species is ongoing in our laboratory, and should provide added insight to the catalytic mechanism.

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CHAPTER 6

HYDG CARBON MONOXIDE FORMATION STOICHIOMETRY: THE ROLE OF PHOSPHATE IN DIATOMIC LIGAND BIOSYNTHESIS

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

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Contributions: Conceived of the study. Prepared enzyme stocks used in study. Performed and optimized all CO experiments, prepared EPR samples, prepared and performed LC–MS on D2O H atom abstraction experiments, and wrote the manuscript.

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Contributions: Performed complementary cyanide experiments in phosphate and provided helpful feedback regarding product formation in phosphate buffer.

Co-Author: Eric M. Shepard

Contributions: Prepared EPR samples, contributed to the design of the study, collected EPR data, and discussed results.

Co-Author: Peter L. Roach

Contributions: Discussed results and implications and provided comments to the manuscript.

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Contributions: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

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Contributions: Directed experimental design and interpretation. Obtained funding and resources, and co-wrote the manuscript.
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CHAPTER 6

HYDG CARBON MONOXIDE FORMATION STOICHIOMETRY: THE ROLE OF PHOSPHATE IN DIATOMIC LIGAND BIOSYNTHESIS

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Abstract

The [FeFe]-hydrogenase H-cluster active site is a novel organometallic cofactor in biology that catalyzes the reversible reduction of protons to generate dihydrogen. The five coordinated diatomic ligands of the H-cluster are synthesized by the radical S-adenosylmethionine (SAM) enzyme HydG, which catalyzes a unique, radical-initiated reaction that produces carbon monoxide (CO) and cyanide (CN–) from the substrate tyrosine. While the 1:1 stoichiometry of CN– formation relative to substrate has been demonstrated, CO has only been detected with less than 1:1 stoichiometry. Herein, investigation of the substoichiometry of CO formation is presented, and method optimization and comparison to methods for CN– detection has shown a general
improvement of enzyme activity and CO detection. Interestingly, the buffering material phosphate has a detrimental impact on CO formation by the HydG enzyme, with \( p \)-cresol, \( 5' \)-deoxyadenosine (\( 5' \)-dAdo), and \( \text{CN}^- \) formed as expected, but not CO. Involvement of the phosphate ion has been investigated spectroscopically and biochemically, and its relevance to the biosynthetic mechanism of the 2Fe subcluster is described herein.

**Introduction**

The radical SAM enzyme HydG catalyzes a remarkable tyrosine lyase reaction that involves the cleavage of two C–C bonds to yield the products CO, \( \text{CN}^- \), and \( p \)-cresol.\(^{1,2} \) HydG catalyzes this complex reaction at two [4Fe-4S] clusters, with reductive cleavage of SAM leading to radical formation on the substrate Tyr, followed by cleavage of the Tyr C\text{\( \alpha \)}–C\text{\( \beta \)} bond to produce \( p \)-cresol and the diatomic products. Dehydroglycine (DHG)\(^2 \) has been proposed to serve as the intermediate that precedes formation of the CO and \( \text{CN}^- \) products (Chapter 2), however details regarding the transformation of DHG to make \( \text{CN}^- \) and CO is not well understood. The N-terminal [4Fe-4S] cluster is coordinated by SAM at its unique iron, and provides the electron required for the reductive cleavage of SAM to produce the \( 5' \)-deoxyadenosyl radical (\( 5' \)-dAdo•) and methionine. The \( 5' \)-dAdo• abstracts a hydrogen atom from substrate to initiate catalysis. In the case of HydG, this N-terminal cluster is the only cluster required to catalyze the cleavage of the Tyr C\text{\( \alpha \)}–C\text{\( \beta \)} bond.\(^3 \) The C-terminal [4Fe-4S] cluster, although not required for tyrosine cleavage, is essential for diatomic ligand synthesis.\(^3-5 \) Formation of CO and \( \text{CN}^- \) from DHG requires a second C–C bond cleavage event that is likely facilitated by the C-terminal cluster,\(^6,7 \) but the mechanism remains to be determined. However, one
equivalent of DHG has been proposed to result in the concerted formation of one CO and CN− molecule (Chapter 2). ²

Given this stoichiometry, generation of the diatomic ligands from Tyr by HydG catalysis would require three turnover events to generate the three CO ligands bound to the 2Fe subcluster of the H cluster. ⁸ However formation of three equivalents of CO would be accompanied by three equivalents of CN− as well, however only two equivalents of the latter present in the H cluster. ⁹,¹⁰ HydG was found to catalyze the formation of CN− at a rate comparable to that for the formation of p-cresol when incubated in the presence of SAM, tyrosine, and the reductant sodium dithionite.¹¹ Concurrent work showed that HydG also catalyzed CO formation with burst-phase kinetics, with the burst rate comparable to the rate of CN− formation, although the quantity of CO was found to be substoichiometric relative to the amount of p-cresol produced due to the slower post-burst rate for CO (Chapter 2). ² The detection methods for the diatomic ligand formation have some notable differences; CN− was detected following enzyme denaturation and cyanide derivatization, while CO was detected by binding to deoxyhemoglobin (deoxyHb) in situ, without enzyme denaturation. Recent characterization of the HydG enzyme from S. oneidensis has shown that these diatomic ligand products may also be detected as part of the HydG enzyme. ⁶,⁷

As part of the biosynthetic mechanism, the diatomic ligands are transferred from HydG to HydA through involvement of the maturase protein HydF. ¹¹,¹² HydF appears to serve as a receptacle of the individual activities of radical SAM enzymes HydE and HydG to synthesize the 2Fe subcluster. While HydF possesses several amino acid residues associated with the coordination of Fe–S clusters, ¹³,¹⁴ it also contains an N-
terminal GTPase domain, including Walker A P-loop and Walker B Mg\(^{2+}\) binding motifs that perform hydrolysis of GTP to produce GDP and inorganic phosphate. Recent studies have shown that GTP addition to either HydF–HydE or HydF–HydG complexes during dissociative phases increases the dissociation rates for each, suggesting that HydF GTPase activity promotes partner dissociation from its scaffold. To what effect these actions are reflected in the catalytic activity of individual radical SAM enzymes, however, is not well understood.

Both CO and CN\(^{-}\) are synthesized from discrete fragments of the substrate tyrosine. This observation is expected to yield one equivalent of CO and one equivalent of CN\(^{-}\) per molecule of substrate, following a concerted oxidative decarbonylation mechanism (Chapter 2). While differences in the method of diatomic ligand detection could explain the deficit in the quantity of CO observed relative to CN\(^{-}\), it is unclear whether this is the case. We originally proposed that the deficit could be due to diatomic ligand sequestration by the enzyme (Chapter 2). As we have shown in Chapter 5, the *Clostridium acetobutylicum* (Ca) HydG Fe–S clusters undergo discrete changes under turnover conditions that may reflect possible product diatomic ligand complex formation, but that are distinct from the *Shewanella oneidensis* HydG enzyme. Alternatively, efficient HydG-catalyzed CO formation might require the presence of the HydF scaffold; however the burst-phase kinetics of CO formation, together with the observation of multiple equivalents of CO bound to the enzyme within seconds of catalysis in the absence of HydF argue against this possibility.
The aim of this study was to optimize CO assays and accurately determine the stoichiometry of CO formed in the HydG reaction in the presence and absence of the scaffold protein HydF. CO assay optimization and comparison of CO formed to CN−:p-cresol showed that HydG can catalyze production of multiple CO equivalents, even though only modest improvement in CO detection was obtained. Surprisingly, we found that the assay conditions utilized for CN− assays resulted in a complete lack of CO, and that HydG CO formation is stimulated by the presence of HydFΔEG. Such observations have mechanistic relevance to the mode of diatomic ligand formation catalyzed by HydG, as discussed herein.

**Experimental Section**

**Materials**

All chemicals and other materials used herein were from commercial sources and of the highest purity where available. Natural abundance tyrosine, guanosine-5′-triphosphate (GTP), guanosine-5′-diphosphate (GDP), ferricyanide were obtained from Sigma-Aldrich (St. Louis, MO). Tris, HEPES, and DTT were obtained from RPI (Mt. Prospect, IL). KCl, acetonitrile (HPLC grade) and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite, D₂O, and sodium sulfide were obtained from Acros Organics (Fair Lawn, NJ). Iron(III) chloride and acetic acid (99%, HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Sperm whale H64L myoglobin was received as a kind gift from Professor John S. Olson (Rice University), while hemoglobin was received as a kind gift from Professor David J. Singel (Montana State University). SAM^{17} and 5-deazaflavin^{18-21} were purified as reported elsewhere, with slight
modifications. Experiments containing HydF$^{\Delta EG}$ was used were prepared by E.M.S. according to as described previously.$^{11,15}$

HydG Overexpression, Purification, and Chemical Reconstitution

Heterologous overexpression of Ca HydG in Escherichia coli, purification and chemical reconstitution with iron and sulfide were prepared as described previously$^{2,11}$ with slight modifications (Appendix A and Chapter 3). Briefly, single colonies obtained from transformations were grown overnight in LB media and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50 mM potassium phosphate buffer, pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD$_{600}$ = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N$_2$ overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber (Grass Lake, MI), as described$^{2,11}$ with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES 500 mM KCl, 5% glycerol, 10 mM imidazole, 20 mM MgCl$_2$, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, and approximately 0.6 mg lysozyme per gram cell. This mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles (Nalgene; Rochester, NY) at 18,000 rpm for 30 minutes. The resulting
supernatant was loaded onto a 5 mL HisTrapTM Ni\(^{2+}\)-affinity column (GE Healthcare, Uppsala). The column was pre-equilibrated with 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Pure fractions (gauged by SDS-PAGE) were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4, and concentrated using an Amicon Ultra centrifugal unit (Millipore; Billerica, MA) fitted with a YM-10 membrane or using a Minicon B15 static protein concentrator (Millipore). Protein was flash frozen in liquid N\(_2\) and stored at -80 °C or in liquid N\(_2\) until further use.

HydG\(^{WT}\) was reconstituted in a fashion similar to that reported elsewhere,\(^2,3\) with slight modifications. Briefly, DTT, FeCl\(_3\), and Na\(_2\)S were added to HydG\(^{WT}\) over the course of an hour, and the final concentration of added FeCl\(_3\) and Na\(_2\)S equaled 6.5 times the final concentration of HydG\(^{WT}\) in a Coy anaerobic chamber. The reconstitution buffer used was 50 mM HEPES, 500 mM KCl, 5% glycerol, pH = 7.4, while the DTT, FeCl\(_3\) and Na\(_2\)S solutions were prepared in dd H\(_2\)O. Samples were incubated for 2.5 hours following additions, stir slowly (using a flea-sized magnetic stir bar) in a scintillation vial. Following centrifugation and pelleting of excess iron-sulfur cluster produced in the reconstitution; the reconstituted sample was desalted over a GE Healthcare PD-10 column (Piscataway, NJ), where all the colored fractions were pooled together. The resultant protein was then concentrated, using an Amicon centrifugal concentration unit, was aliquoted and was flash frozen. Protein concentration was
determined through the Bradford assay, while Fe quantitation was determined using the method reported by Fish. For preparation of enzyme in phosphate, the above protein following reconstitution and a subsequent freeze-thaw was loaded into a dialysis bag and was dialyzed for five hours into 50 mM potassium phosphate, 300 mM KCl, 5% glycerol, pH = 8.0 (including a change of buffer at 2.5 hours) in an anaerobic chamber.

HydG CO Formation Experiments

HydG CO formation experiments were performed on a Cary 6000i spectrophotometer (Agilent; Santa Clara, CA) with dual pathlength functionality and a Peltier module temperature (Strumenti Scientific; Padova, Italy), using a 1 mm pathlength far UV (170-2200 nm) quartz cuvette with a screwtop cap (Spectrocell; Oreland, PA) held in place with a cell mount insert. Baseline absorbance data was obtained in dual pathlength mode, using reaction buffer as a blank across the sampled 300-800 nm range with the scan program in the Cary software package. For kinetics experiments using deoxyHb or deoxymyoglobin (deoxyMb), the 419 or 424 nm absorbance wavelength was also blanked with reaction buffer with the kinetics program in the Cary software package, respectively.

Preparation of samples monitoring HydG CO formation was performed under strict anoxic conditions under <1 ppm O₂ in an anaerobic chamber (Mbraun; Stratham, NH). All buffers used were degassed in schlenk flasks on a schlenk line (3 x 10 minute vacuum cycles each followed by N₂ backfill). Upon bringing buffers into the anaerobic chamber, degassed H₂O was added to buffers to bring them up to their original volume, to account for solvent evaporation upon degassing. Tyr solid was dissolved in 1.0 M HCl (22.2% v/v) and was diluted with 100 mM tris, pH 7.4 buffer to make a concentrated 67.7
mM stock. For experiments, a working 2 mM Tyr stock was prepared by diluting 29.6 
µL of the concentrated stock into the buffer of interest to 1000 µL total. The pH of the 
working stock was found to be slightly acidic (as gauged by pH range 6-8 pH paper), 
however the final assay solution approximated to the assay buffer pH. The obtained 
carboxyhemoglobin (HbCO) or carboxymyoglobin (MbCO) was prepared for use by 
treating with K₃[Fe(CN)₆] (15 mM final) in the MBraun chamber. The sample was 
irradiated with light in an icebath for 10 min before the sample was transferred to a Coy 
Chamber, and it was desalted using a PD-10 desalting column. To ensure complete 
removal of the ferricyanide, the hemoglobin or myoglobin was dialyzed into a working 
buffer (50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4) for 5 hours at 22 °C with a 
buffer change after 2.5 hours before it was stored in a 2.0 mL screwcap cryovial, flash-
frozen in liquid N₂, and stored in a -80 °C freezer. Reductant sodium dithionite was 
weighed under aerobic conditions in a 1.5 mL eppendorf tube and was immediately 
brought into the anaerobic chamber; to minimize solid sample loss upon transfer into the 
anaerobic chamber, eppendorf tubes were wrapped with parafilm and penetrated with a 
noodle to subject the solid to vacuum–N₂ backfill cycles.

HydG CO assays were all performed similarly to methods reported elsewhere 
(Chapter 2),² with a few slight modifications. Reconstituted HydGWT was combined 
with tyrosine, 5 mM dithionite, deoxyHb or H64L deoxyMb and assay buffer of interest 
to appropriate dilution. HydG was added last to minimize enzyme precipitation from 
addition of the acidic tyrosine stock. After centrifuging the sample for 5 minutes at 
13,000 rpm, the sample was loaded by gastight syringe into an anaerobic 1 mm 
thickness UV-visible cuvette with a pre-purged 500 µL gas-tight syringe (Hamilton;
Reno, NV), while the required SAM volume was measured with a pre-purged, sharp-ended 25 μL gas-tight syringe that was transferred to the instrument by inserting the sharp end into a rubber stopper. After equilibrating the sample at 37 °C for 5 minutes, an initial 300-800 nm scan was performed in the scan program. In the kinetics program, the initial A_{419nm} (A_{424nm} for H64L deoxyMb) was also recorded. SAM was injected into the sample by penetrating a 12 mm Tuf-bond teflon insert (Thermo-Fisher; Bellefonte, PA) in the screwcap of the cuvette, and the absorbance at 419 nm (for deoxyHb; 423 nm for H64L deoxyMb) was monitored every second over the course of 15 or 30 minutes. The hole generated by the penetration was sealed with vacuum grease to minimize sample exposure to O₂. The cuvette immediately was inverted four times before replacement back in the instrument, and A_{419nm} was monitored every second for 30 minutes at 37 °C. The time delay between SAM injection and initial absorbance readings ranged from 10 to 15 seconds. At the end of the program, a final absorbance scan was made, and an 80 μL aliquot was removed, and 15% (v/v) of 1 M HCl was added and the sample was flash frozen. Experiments that were prepared in mixtures of tris and phosphate buffer were performed analogously to what was described above, with percent mixtures of two stock buffers (250 mM tris, 300 mM KCl, 5% glycerol, pH 8.5; 250 mM phosphate, 300 mM KCl, 5% glycerol, pH 8.5) that were combined and used to prepare the buffer and dithionite stock used. Spectral data were fitted using OriginPro (version 9.1.0; OriginLab Corp., Northampton, MA), applying a biexponential plot as reported elsewhere for radical SAM enzyme ThiH. In some cases, it was necessary to fit as a single exponential plot, that is indicated as necessary below.
Experiments that were performed in the presence of HydF$^{\Delta E}\text{G}$ were prepared analogously to the above procedure with slight modifications. HydF$^{\Delta E}\text{G}$ was purified and dialyzed by Dr. Eric M. Shepard. Experiments were performed at 37 °C with 62 $\mu$M HydG$^{\text{WT}}$ (4.7 ± 0.3 Fe/protein) with 2 mM MgCl$_2$, 1 mM Tyr, 1 mM SAM, 5 mM sodium dithionite, and 80 $\mu$M deoxyHb (per heme) in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. Where applicable, 62 $\mu$M HydF$^{\Delta E\text{F}}$ (1.3 ± 0.1 Fe/protein), 2 mM GTP, 2 mM GDP was used as well.

CO Formation by Enzyme Denaturation/H64L deoxyMb Detection

An alternative assay to detect CO formed by HydG was developed for use in this study. Experiments described above were performed in the absence of deoxyHb or deoxyMb at 37 °C in a 3 mL crimped vial (40 $\mu$L) that also contained a flea-sized teflon stir bar. A parallel sample containing 80 $\mu$M deoxyHb (per heme) in assay buffer with 5 mM dithionite was prepared, and an initial 300 – 800 nm scan was performed on the Cary 6000i spectrophotometer in an anaerobic 1 mm pathlength cuvette with a screwtop. Following a defined period of incubation, the HydG assay mixture was boiled for 2 minutes to precipitate the enzyme, and was then was cooled in an icebath for 2 minutes. The prepared deoxyHb sample was loaded into a 500 $\mu$L gastight syringe, after which it was injected into the precipitated enzyme mixture, which was stirred at room temperature at 150 rpm for 4 minutes before the sample was brought back into the MBraun glove box. The sample was retrieved from the crimped vial and centrifuged (5 min at 14,000 rpm), and the supernatant was loaded back into the cuvette for a final scan. To compare this method, CO evolved from denaturing MbCO was performed in a similar fashion, that
was incubated, denatured, and then a prepared deoxyMb solution was added. Difference spectra between the deoxyMb prior to addition to precipitated enzyme, followed by after was obtained to detect the amount of CO released, based on the Soret $\lambda_{max}$ ($\Delta A_{424\text{nm}}$ and $\Delta \varepsilon_{424\text{nm}}$) for H64L MbCO. This value was compared to prepared MbCO standards to estimate an MbCO concentration (per heme). Because this method involved using the same enzyme for denaturation and detection and because incomplete denaturation would overestimate detection, an additional control was performed to assess completeness of denaturation. In place of deoxyMb, buffer was added to the denatured MbCO to determine the amount of soluble MbCO remaining. By comparison of a standard MbCO solution that did not undergo denaturation, estimation of the percent enzyme that denatured was made. Denaturation of MbCO by the above method was over 95%.

**Product Quantitation**

The products $p$-cresol and 5’-dAdo were quantified by HPLC, using UV-visible absorbance. Samples were boiled for 50 seconds, and centrifuged for 2 x 10 minutes at 14,000 rpm and 4 °C, and the supernatant was subjected to HPLC analysis. Samples were injected onto a Phenomenex (Torrance, CA) Curosil 5 $\mu$m 4.6 x 150 mm analytical column that had been equilibrated at 98% solution A (H$_2$O plus 0.1% acetic acid) and 2% solution B (CH$_3$CN plus 0.1% acetic acid) at 1 mL/min. An isocratic mobile phase at these percentage values ran for 7 minutes following sample injection at which time a linear gradient to 40% solution A, 60% solution B was run over the next 17 minutes. At this time the gradient was held isocratic for 3 minutes and then the column was re-equilibrated to 98% solution A and 2% solution B for another sample injection. The
column temperature during runs was held at 30 °C and total run time was 35 minutes. Elution of reaction products was monitored via $A_{280\text{nm}}$ and $A_{254\text{nm}}$, since tyrosine phenol rings absorb stronger at 280 nm, while adenine rings absorb more optimally at 254 nm. AdoMet eluted at ~ 2 minutes, tyrosine eluted at ~ 4 minutes, 5-dAdo eluted at ~ 12 minutes, and $p$-cresol eluted at ~ 20 minutes. Integration of peak area using Origin 8.6 (OriginLab Corp; Northampton, MA) relative to standard samples run in parallel allowed for quantification of reaction product concentrations.

**Hemoglobin CO Binding Experiments**

CO-hemoglobin binding experiments followed a similar method of detection for enzymatic CO formation as described above, with a few modifications. Experiments were performed in a quartz, 1 cm (1.4 mL) UV-visible cuvette (Starna Cells; Atascadero, CA) with a single pathlength Agilent Cary Bio 50 spectrophotometer at ambient temperature. Like the CO experiments reported above, experimental baseline scans and blanks with aerobic reaction buffer were collected in the scan and kinetics programs, respectively. A 2.4 mL reaction mixture was prepared in the anaerobic chamber, and contained reaction buffer, dithionite, and 8 µM deoxyHb (per heme); the volume was chosen to completely fill the cuvette, to minimize CO gas escape into the headspace, considering that it has a mole fraction solubility of $1.774 \times 10^{-5}$ in H$_2$O at 25 °C. An anaerobically-sparged 25 µL gastight syringe was kept anaerobic by inserting its needle into a rubber stopper while transferring it to the spectrophotometer. A 25 mL crimped vial (crimped anaerobically) was sparged with natural abundance CO for 3 min at 5 psi with a purge needle in a fume hood. An initial 300-800 nm scan was performed on the
deoxyHb solution. Injection of 1 µL CO gas to the cuvette denoted the start of the experiment, and the penetrated hole was filled in with vacuum grease and the cuvette was inverted a few times. Following this, the cuvette was replaced in the sample mount and the absorbance at 419 nm was monitored. While an increase in absorbance could be observed, it was only after additional sample mixing. Therefore, the kinetics program was paused, and the sample cuvette was inverted manually for 15 to 20 seconds before subsequent A$_{419\text{nm}}$ absorbance was monitored. This cycle was repeated as necessary until negligible absorbance changes could be observed with the hemoglobin sample. Following a 300-800 nm absorbance scan, 25 µL of CO was added, and the cuvette was inverted again a few times until no more A$_{419\text{nm}}$ absorbance changes were observed.

**EPR Spectroscopy**

EPR samples were prepared as described previously with slight modifications.$^{2,3}$ Briefly, the HydG enzyme was supplemented with 50 mM tris (pH 7.4), 100 µM 5-deazariboflavin, and 5 mM DTT in buffer (100 mM potassium phosphate, pH 7.5), and was placed in a ice-water bath in the MBraun box. Following illumination with a 300 W Xe lamp for 1 hour, enzymatically synthesized SAM (1 mM) was added in the absence of light. Within three minutes, the EPR tube was frozen in liquid N$_2$, and low-temperature EPR spectra were recorded using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller (Oxford Instruments; Abingdon, U.K.). Typical EPR parameters included a sample temperature of 12 K, a microwave frequency of 9.37 GHz, a microwave power of 1.59 mW, and a time constant
of 20.48 ms. Experimental spectra were baseline corrected and plotted using OriginPro (version 9.1.0; OriginLab Corp., Northampton, MA).

**H Atom Abstraction Experiments**

Assays were performed in D$_2$O containing buffer, under conditions described in the main text and similar to methods described elsewhere (Chapter 4). Experiments were performed in an anaerobic chamber (Mbraun) under strict anaerobic conditions (<1 ppm O$_2$). D$_2$O buffers were prepared by lyophilization of H$_2$O buffer on a Schlenk line for 16 hours. D$_2$O buffers were prepared by lyophilization of H$_2$O buffer on the schlenk line for 16 hours. The resultant salt was brought into the M Braun box and was dissolved with degassed D$_2$O to original volume. Prior to suspending the salt in D$_2$O, the schlenk flask was briefly subjected to a heat gun to drive off remaining H$_2$O moisture. Upon resuspending the buffer salt in D$_2$O, the pD was measured with pH paper (Micro Essentials; Brooklyn, NY) and was as expected acidic by 0.4 units. Working 2 mM Tyr stocks in D$_2$O were prepared in a similar fashion to buffers described above, via dilution from an aerobic 67.7 mM stock in H$_2$O (prepared by dissolving Tyr in 1 M HCl, then dilution with 100 mM tris, pH 7.4) that was lyophilized overnight.

Experiments were performed at 37 °C in an isotemp heatblock (Fisher), containing 100 $\mu$M HydG (8.5 ± 0.2 Fe/protein), 1 mM AdoMet (enzymatically prepared), 1 mM Tyr, and 5 mM dithionite (80 $\mu$L volume) for 60 minutes, and then the enzyme was precipitated by addition of one volume equivalent of acetonitrile (HPLC grade, EMD) or 1 M HCl (13 % v/v). A concentrated HydG stock at ~ 2 mM was used to minimize the H$_2$O contribution for performed experiments. Use of either quenching
medium did not affect the observed product isotope distribution. Samples were centrifuged for 3 x 10 minutes at 14,000 rpm, and the supernatant was collected. Samples were then used for LC-MS or HPLC, as described below. Reported assay pDs were corrected for changes related to temperature.  

**LC-MS Methods**

Deuterium isotope distribution in AdoMet and 5’-dAdo was assessed using LC-MS with as-obtained samples following centrifugation of the precipitated enzyme. Quantitation of 5’-dAdo, AdoMet, and tyrosine was performed using an Agilent 1290 series UHPLC coupled to an Agilent 6538 Q-TOF mass spectrometer equipped with the dual-ESI source and an autosampler. Samples were injected onto a Microsolv (Eatontown, NJ) normal phase Cogent “Diamond Hydride” HPLC column (150 x 2.1mm) equilibrated with solution B (CH₃CN + 0.1% formic acid) at 0.8 mL/min. An isocratic mobile phase was maintained for 2 minutes following sample injection, and then a linear gradient from solution A (H₂O + 0.1% formic acid) to 50% solution B mixture was run over 4 minutes. This was followed by isocratic elution for 2.5 minutes at 50% solution B after which was immediately re-equilibrated to 100% B for 1.5 minutes. Total run time was 10 minutes and the column temperature was maintained at 50 °C. Under these conditions, 5’-dAdo eluted at ~ 3 minutes, AdoMet eluted at ~ 6 minutes, and tyrosine eluted at ~ 4 minutes. The capillary exit voltage was 120 V and gas temperature was 300 °C. All data was recorded in positive mode between 25 m/z and 750 m/z in profile mode. Hardware summation time was 1 second. Peaks were validated using high-accuracy formula confirmation and elution matching from individual reference
standards. Quantitation was performed using the MassHunter Quantitative Analysis package (Agilent). An extracted ion chromatograph was generated using the accurate mass of each compound with a -0.04 to +0.04 ppm m/z extraction window. Samples were injected in duplicate, and between sample injections, a water blank injection was performed to minimize sample-to-sample carryover.

Sample isotope distributions for 5’-dAdo and SAM were assessed using the natural abundance distribution as a template distribution. Incorporation of a deuterium atom corresponds to a net mass increase of one, but results in an otherwise identical distribution to the template distribution. Respective isotopic mass abundances were subtracted, and quantities for each were normalized to 100% total. Sample 5’-dAdo and SAM were quantitated using the HPLC method described above.

Results

Optimizing CO Formation Assay

The initial report of CO formation by HydG revealed substoichiometric CO relative to the amount of p-cresol detected and amount of CN⁻ expected (Chapter 2).¹² In order to determine whether the differences in the CO and CN⁻ assay conditions played a role in the observed low stoichiometry of CO, we worked to make the CO assay more comparable to the CN⁻ assay. The CO detection assay was initially performed in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4, while the CN⁻ detection assay was performed in 100 mM phosphate, pH 7.4 (Chapter 2).¹² In principle, these differences appeared to be trivial, with the key differences being the ionic strength, the presence or absence of glycerol, and the identity of the buffer. We therefore examined a range of
Figure 6.1. Effect of Salt and Glycerol on HydG CO Formation. Assays contained 40 μM HydG$^{\text{WT}}$ (6.6 ± 0.7 Fe/protein), 1 mM Tyr, 1 mM SAM, 1 mM dithionite, and 80 μM (per heme) H64L deoxyMb, at 37 °C. Magenta trace, buffer 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4; blue trace, buffer 50 mM HEPES, 45 mM KCl, 0.2% glycerol, pH 7.4; black trace, 50 mM tris, 300 mM KCl, 5% glycerol, pH 8.5; red trace, 50 mM tris, 46 mM KCl, 0.2% glycerol, pH 8.5.

Table 6.1. Effect of Ionic Strength on $p$-cresol and 5’-dAdo Assays from Figure 6.1.$^a$

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$[p$-cresol] (µM)</th>
<th>$[5'$-dAdo] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES, 45 mM KCl, 0.2% glycerol, pH 7.4</td>
<td>265 ± 1 µM</td>
<td>283 ± 1 µM</td>
</tr>
<tr>
<td>HEPES, 500 mM KCl, 5% glycerol, pH 7.4</td>
<td>146 ± 1 µM</td>
<td>262 ± 3 µM</td>
</tr>
<tr>
<td>tris, 46 mM KCl, 0.2% glycerol, pH 8.5</td>
<td>305 ± 14 µM</td>
<td>514 ± 4 µM</td>
</tr>
<tr>
<td>tris, 300 mM KCl, 5% glycerol, pH 8.5</td>
<td>252 ± 26 µM</td>
<td>279 ± 28 µM</td>
</tr>
</tbody>
</table>

$^a$HPLC quantitation via UV-visible absorbance (DAD) integration after 30 min incubation at 37 °C. Each sample constitutes two sample injections.

buffer conditions for the CO assay, in order to probe the effects of ionic strength, glycerol, buffer, and pH (Figure 6.1). Activity assays were performed at 37 °C for 30 minutes, under which conditions no protein precipitation was observed. In general, an increase in CO formation was observed for assays which the salt and glycerol was minimized in HEPES buffer at pH 7.4 (Figure 6.1). Assays performed at a more alkaline pH (in tris buffer) likewise exhibited an increase in CO formation with removal of salt, although the increased pH also stimulated the CO product formed. To examine whether
the increase in CO formation reflected an improved stoichiometry relative to \( p \)-cresol, product quantitation was performed on aliquots taken after 30 minutes of incubation. The results revealed that the improved CO formation was accompanied by an increase in \( p \)-cresol and 5'-dAdo formation (Table 6.1). An improvement in CO:\( p \)-cresol stoichiometry was observed in the tris pH 8.5 buffer, while a more modest increase was observed in HEPES pH 7.4 buffer. It should be noted that H64L deoxyMb was used in place of deoxyHb; this CO-binding variant has nanomolar affinity for CO, and was used as part of our studies. Experiments performed with H64L deoxyMb yielded improved CO formation with comparable \( p \)-cresol and 5'-dAdo formation ability (Appendix Figure D.3), consistent with an improvement due to increased affinity for CO.

Figure 6.2. Comparison of Difference UV-visible Absorbance Spectra of H64L MbCO Formation by HydG Catalysis. Red trace, deoxyMb incubation with HydG; blue trace, deoxyMb addition after HydG precipitation. Experiments represent 30 min incubation at 25 °C with 1 mM Tyr, 1 mM SAM, and 5 mM dithionite, and 16 nmol of HydG\(^{WT}\) (7.0 ± 0.1 Fe/protein). Detecting solution contained 80 \( \mu \)M (per heme) H64L deoxyMb with 5 mM dithionite in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. The inset shows a zoomed-in view of the visible absorption bands near 550 nm for respective samples.
Because CN\textsuperscript{−} assays followed enzyme denaturation while CO assays were performed on intact enzyme, we hypothesized that the low CO stoichiometry could be due to CO being trapped in pockets in the native protein. An alternative mode of CO detection was therefore developed to better mimic experiments used to quantify CN\textsuperscript{−} formed by the enzyme.\textsuperscript{1,3} Assay mixtures containing HydG, tyrosine, dithionite, and SAM were prepared in a sealed, crimped vial to trap the CO within the vial. Following the enzyme assay period of 30 minutes, the enzyme was denatured, a solution of reduced deoxyMb was added to the precipitated enzyme mixture, and the mixture was allowed to stir to bind to CO released from HydG (Figure 6.2). While CO could be detected, it was at lower levels than what was observed through the standard assay. By comparison to assays performed with 400 µL reaction volume, the 40 µL sample contained the same number of moles of enzyme, differing only with respect to volume; the sample that underwent denaturation comprised a volume of 40 µL while the sample in which Mb was present during HydG turnover possessed a volume of 400 µL. To provide a positive control where CO detection by denaturation could be assessed, known concentrations of MbCO were denatured by the methods described above, followed by detection with deoxyMb. Relatively optimal recovery yields of CO by this method using MbCO were observed, on upwards of 70\% (Appendix Figure E.1). Because this positive control uses the same biomolecule as a CO and as a detection molecule, an added control where buffer was added to the denatured MbCO was subjected to UV-visible absorbance spectroscopy. In optimal experiments, over 95\% of MbCO was denatured, relative to prepared MbCO standards of known concentration (Appendix Figure E.2).
Figure 6.3. Buffer-dependent HydG CO Formation Kinetics. Single point A<sub>419nm</sub> absorbance kinetics was monitored by incubation with deoxyHb with 40 µM HydG<sup>WT</sup> (13.3 ± 0.2 Fe/protein), 1 mM Tyr, 1 mM SAM, 5 mM dithionite and 80 µM deoxyHb (per heme) at 37 °C. Black trace, 50 mM tris, 10 mM KCl, pH 8.5; red trace, 50 mM HEPES, 10 mM KCl, pH 7.4; blue trace, 50 mM potassium phosphate, 10 mM KCl, pH 7.4; magenta trace, 50 mM potassium phosphate, 10 mM KCl, pH 8.5.

Table 6.2. Product Quantitation and Kinetic Parameters of Assay Samples from Figure 6.3.<sup>a</sup>

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;b&lt;/sup&gt;</th>
<th>[p-cresol] (µM)</th>
<th>[5'-dAdo] (µM)</th>
<th>CO Efficiency&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Burst &lt;em&gt;k&lt;/em&gt;&lt;sub&gt;cat&lt;/sub&gt; (× 10&lt;sup&gt;-4&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM HEPES, pH 7.4</td>
<td>272 ± 24 µM</td>
<td>324 ± 37 µM</td>
<td>11.7</td>
<td>6.22 ± 0.03</td>
</tr>
<tr>
<td>50 mM tris, pH 8.5</td>
<td>376 ± 23 µM</td>
<td>447 ± 24 µM</td>
<td>14.0</td>
<td>14.8 ± 0.2</td>
</tr>
<tr>
<td>50 mM phosphate, pH 7.4</td>
<td>265 ± 32 µM</td>
<td>329 ± 8 µM</td>
<td>0.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>50 mM phosphate, pH 8.5</td>
<td>304 ± 33 µM</td>
<td>366 ± 22 µM</td>
<td>1.8</td>
<td>0.879 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>HPLC quantitation via UV-visible absorbance (DAD) integration. Each sample constitutes three sample injections. <sup>b</sup>Samples also contained 10 mM KCl. <sup>c</sup>CO:p-cresol ratio. N.D. Not detected, negligible CO formed.

In order to examine the effect of phosphate buffer utilized in the CN<sup>-</sup> assays, HydG activity assays were performed in 50 mM phosphate buffer at pH 7.4 and pH 8.5; in each case CO formation was only ~ 10% of that seen with HEPES or tris buffers.
HydG assayed in pH 7.4 phosphate buffer produced only 0.5 µM CO over the course of 30 min at 37 °C. The products p-cresol and 5’-dAdo were also quantified for assays performed in phosphate and the non-phosphate buffer (Table 6.2). Interestingly, the experiments performed in HEPES and phosphate pH 7.4 buffer yielded comparable quantities of p-cresol and 5’-dAdo. By comparison, experiments performed in tris and phosphate pH 8.5 buffers yielded an observable difference in product formation (Table 6.2). No increase in A800nm baseline was observed, suggesting that the small Soret band absorbance increase was not an artifact of precipitation (Table 6.2).

Figure 6.4. The Effect of Phosphate on HydGWT CO Formation. Time course experiments monitoring the A419nm increase, corresponding to HbCO formation were performed with reconstituted enzyme in phosphate or tris buffer, before and after dialysis into phosphate buffer. Red trace, reconstituted enzyme performed in tris buffer; black trace, phosphate-dialyzed enzyme performed in tris buffer; magenta trace, reconstituted enzyme performed in phosphate buffer; blue trace, phosphate-dialyzed enzyme performed in phosphate buffer. Black and magenta traces represent experiments that also contained 3 mM phosphate and 3 mM tris buffer in the experiment, respectively. Assays included 52 µM HydGWT, 1 mM AdoMet, 1 mM tyrosine, 4.73 mM dithionite in the buffers listed in the text. Assays were conducted at 37 °C in a 1 mm pathlength cuvette, and deoxyHb (~ 100 µM per heme) was added.
Table 6.3. Product Quantitation of Phosphate-treated Assay Samples from Figure 6.4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[p-cresol] (µM)</th>
<th>[5’-dAdo] (µM)</th>
<th>CO Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-reconstituted, tris buffer</td>
<td>264 ± 17 µM</td>
<td>483 ± 28 µM</td>
<td>9.5</td>
</tr>
<tr>
<td>Tris-reconstituted, phosphate buffer</td>
<td>116 ± 1 µM</td>
<td>193 ± 1 µM</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphate-dialyzed, tris buffer</td>
<td>93 ± 4 µM</td>
<td>145 ± 2 µM</td>
<td>6.1</td>
</tr>
<tr>
<td>Phosphate-dialyzed, phosphate buffer c</td>
<td>43 ± 1 µM</td>
<td>83 ± 3 µM</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

aHPLC quantitation via UV-visible absorbance integration (DAD) after 30 minutes of incubation at 37 °C. Each sample constitutes three sample injections. bCO:p-cresol ratio. cAliquot taken after 19 minutes. N.D. Not detected. No increase in absorbance observed.

The buffer-dependent differences in CO, 5’-dAdo, and p-cresol produced at pH 7.4 (Table 6.2) suggested the possibility that the phosphate buffer was directly affecting the active sites, possibly by coordinating the Fe–S clusters. To understand this more fully on a catalytic level, assays containing HydGWT reconstituted in tris buffer (5.9 ± 0.2 Fe/protein) were performed in either tris (250 mM tris, 300 mM KCl, 5% glycerol, pH = 8.5) or phosphate (250 mM potassium phosphate, 300 mM KCl, 5% glycerol, pH = 8.5) buffer. The resultant phosphate-dialyzed HydGWT had a depressed iron number (4.1 ± 0.2 Fe/protein). The results of these assays were compared to those for the same enzyme stock that was dialyzed in 50 mM phosphate, 300 mM KCl, 5% glycerol pH 8.5 after reconstitution (Figure 6.4). The effect of dialysis into phosphate buffer resulted in a loss of product formation. Interestingly, recovery of CO formation occurred when phosphate-dialyzed HydG assayed in tris buffer (Figure 6.3; black trace) formed 3 µM CO after 15 minutes (5.6 µM after 30 minutes), yielding a CO efficiency of 6.1% at 30 minutes (Table 6.3). By comparison, non-dialyzed enzyme assayed in phosphate buffer (Figure 6.4; magenta trace) produced 0.5 µM CO, 116 µM p-cresol, and 193 µM 5’-dAdo after
30 minutes. While it is important to note that the black and blue traces in Figure 6.4 represented protein that underwent an additional freeze-thaw cycle relative to the red and magenta traces in Figure 6.4 (which could affect activity), our evidence suggests that the phosphate buffer is the main variable causing loss of activity.

Given the surprising behavior of phosphate in inhibiting CO formation by HydG, the enzyme’s sensitivity to phosphate was assessed by performing a titration experiment. The concentration of phosphate was varied using a mixture of tris and phosphate buffers from stocks (250 mM tris, 300 mM KCl, 5% glycerol, pH = 8.5; 250 mM potassium phosphate, 300 mM KCl, 5% glycerol, pH = 8.5). Kinetics traces for CO formation (A419nm vs. time) are shown in Figure 6.5, while quantities of products assayed are shown

![Figure 6.5. Titration of Phosphate on HydG^{WT} CO Formation. Time course experiments monitoring the A_{419nm} increase, corresponding to HbCO formation with varied phosphate concentrations were performed. Assays included 51 µM HydG^{WT}, 1 mM SAM, 1 mM tyrosine, and 5 mM sodium dithionite prepared in the buffers listed in the text. Assays were conducted at 37 °C in a 1 mm pathlength cuvette, and deoxyHb (~ 100 µM) was added. Total buffer concentration for each experiment was prepared to be similar for each experiment. Black trace, 230 mM tris, no phosphate; red trace, 229 mM tris, 1 mM phosphate; magenta trace, 221 mM tris, 9 mM phosphate; blue trace, 207 mM tris, 23 mM phosphate; green trace, 5 mM tris, 225 mM phosphate.](image-url)
Table 6.4. Product Quantitation and Kinetic Parameters of Phosphate-treated Assay Samples from Figure 6.5.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>[(p)-cresol] (µM)</th>
<th>[5(^{ \prime})-dAdo] (µM)</th>
<th>CO Efficiency (%)\textsuperscript{b}</th>
<th>Burst (k_{\text{cat}}) (\times 10^{-4}) s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>230 mM tris</td>
<td>172 ± 54 µM\textsuperscript{c}</td>
<td>421 ± 51 µM\textsuperscript{c}</td>
<td>13.9</td>
<td>7.46 ± 0.13</td>
</tr>
<tr>
<td>229 mM tris, 1 mM phosphate</td>
<td>105 ± 5 µM</td>
<td>296 ± 8 µM</td>
<td>18.9</td>
<td>10.7 ± 0.18</td>
</tr>
<tr>
<td>221 mM tris, 9 mM phosphate</td>
<td>67 ± 2 µM</td>
<td>186 ± 8 µM</td>
<td>23.1</td>
<td>5.99 ± 0.14</td>
</tr>
<tr>
<td>207 mM tris, 23 mM phosphate</td>
<td>43 ± 1 µM</td>
<td>120 ± 2 µM</td>
<td>17.0</td>
<td>1.74 ± 0.02</td>
</tr>
<tr>
<td>5 mM tris, 225 mM phosphate</td>
<td>55 ± 3 µM</td>
<td>95 ± 8 µM</td>
<td>1.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}HPLC quantitation via UV-visible absorbance integration (DAD) following 30 minutes of incubation at 37 °C. Each sample constitutes three sample injections.

\textsuperscript{b}CO: \(p\)-cresol ratio.

\textsuperscript{c}Sample comprised five sample injections. N.D. Not determined, no CO formed.

in Table 6.4. Addition of even small concentrations of phosphate resulted in a net decrease in detected HbCO, \(p\)-cresol and 5\(^{ \prime}\)-dAdo, with concentrations greater than 25 mM phosphate resulting in a 70% reduction in the amount of CO formed. Interestingly, an increase in percent CO efficiency was observed with concentrations of 1-10 mM phosphate, despite an overall drop in CO and other products formed.

EPR Spectroscopy of Phosphate-Treated Enzyme

Phosphate-dependent differences in catalytic activity suggested that it might be coordinating or interacting with the Fe–S clusters of HydG. To investigate this hypothesis, EPR samples of photoreduced HydG\textsuperscript{WT} following enzyme dialysis into phosphate buffer (50 mM phosphate, 500 mM KCl, 5% glycerol, pH 7.4) from enzyme prepared in reconstitution buffer (50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4) were prepared (Figure 6.6). EPR samples prepared prior to dialysis into phosphate had
Figure 6.6. Comparison of X-band (9.37 GHz, 12K) EPR spectra Prepared in Phosphate Buffer. (A) HydG<sup>WT</sup> reconstituted in HEPES buffer (65 µM, 8.7 ± 0.7 Fe/protein). (B) Reconstituted HydG<sup>WT</sup> following dialysis into phosphate buffer (134 µM, 2.75 ± 0.01 Fe/protein). Black traces correspond to an as-isolated sample, while the red traces correspond to photoreduced protein. EPR spectral parameters are reported in the experimental section.

Small contributions of g = 4.3 signal (Figure 6.6A, black trace) that disappeared upon photoreduction (Figure 6.6A, red trace), indicative of a non-specifically bound, low-spin Fe(III) species. By comparison, the EPR spectrum of a sample dialyzed into phosphate yielded a large signal in the g = 4.3 magnetic field region that was significantly more intense than the g = 2.01 [3Fe-4S] cluster signal (Figure 6.6B, black trace). Photoreduction of the phosphate-dialyzed enzyme resulted in a large, broad feature in the low-field region (Figure 6.6B, red trace). Addition of SAM to the phosphate-treated enzyme did not perturb the paramagnetic signal (data not shown), contrasting our initial report (Chapter 2, Figure 2.1).<sup>2</sup>
CO Binding to Hemoglobin Experiments

The binding of CO to deoxyHb or deoxyMb and its associated UV-visible spectral changes is well-documented.\textsuperscript{32-34} As noted in Chapter 2, a spectral change in the Soret $\lambda_{\text{max}}$ from 430 nm to 419 nm, as well as splitting of the 555 nm visible band to 540 nm and 569 nm is reflective of CO binding to the Fe(II) heme in human hemoglobin.\textsuperscript{25} Because our HydG rates of CO production appeared to be affected by the buffer, we wanted to determine whether any of the effects we were seeing were due to buffer effects on CO binding to Hb, rather than production of CO by HydG. At ambient temperature, injection of 1 $\mu$L of 1 atm CO gas to a 1 cm pathlength cuvette lacking headspace volume containing approximately 8 $\mu$M (per heme) of reduced deoxyHb resulted in observable, buffer dependent differences in CO binding (Figure 6.7, Table 6.5). In all experiments,

![Figure 6.7. Time Course CO Binding to deoxyHb under Assay Buffer Conditions used for Detecting HydG CO Formation. $\Delta A_{419\text{nm}}$ was monitored to calculate bound [CO]. Experiments were performed with minimal headspace volume to maximize hemoglobin CO binding ability. Experiments were performed at 25 °C with 8 $\mu$M deoxyHb (per heme) and 5 mM dithionite. 1 $\mu$L of CO gas was injected into the cuvette. Black trace, 50 mM phosphate, pH 7.4; red trace, 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4; blue trace, 50 mM HEPES, pH 7.4; magenta trace, 50 mM tris, pH 8.5.](image-url)
Figure 6.8. Difference UV-visible Spectra of Figure 6.7 deoxyHb Time Course, Following Excess CO Addition. Excess CO (25 µL) added to assay mixtures reported in Figure 6.7 after the depicted experiment. Black trace, 50 mM phosphate, pH 7.4; red trace, 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4; blue trace, 50 mM HEPES, pH 7.4; magenta trace, 50 mM tris, pH 8.5.

Table 6.5. Kinetic Parameters for Figure 6.7 CO Binding to deoxyHb

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$k_{on}^0 (\times 10^{-4} \text{s}^{-1})^a$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM HEPES, pH 7.4</td>
<td>26.7 ± 0.3</td>
<td>0.99</td>
</tr>
<tr>
<td>50 mM Phosphate, pH 7.4</td>
<td>10.2 ± 0.1</td>
<td>0.99</td>
</tr>
<tr>
<td>50 mM tris, pH 8.5</td>
<td>31.7 ± 0.3</td>
<td>0.99</td>
</tr>
<tr>
<td>50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4</td>
<td>15.4 ± 0.1</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Time course plots were fitted to exponential function, and reported were multiplied by calculated [P]max and divided by [deoxyHb]tot

the time course for CO binding was exceptionally slow, requiring frequent inversion of the cuvette to yield a change in 419 nm absorbance (Table 6.5). Decreasing the buffer ionic strength resulted in an increase in the initial rate of CO binding (Figure 6.7, blue and red traces; Table 6.5). Substitution of HEPES for tris performed at an alkaline pH (8.5) resulted in a similar experimental CO binding rate (Figure 6.7, blue and magenta traces; Table 6.5). Interestingly, phosphate buffer resulted in a depressed rate of binding to hemoglobin (Figure 6.7, black trace; Table 6.5) relative to tris or HEPES buffers (blue
and magenta traces). Following a 300-800 nm absorbance scan, an additional 25 µL of CO was injected to the volume to confirm the assay heme concentration, and to affirm the differences in CO binding (Figure 6.8). Low salt HEPES and tris buffers had the smallest change, consistent with near saturation of CO. Consistent with the CO time course in Figure 6.7, phosphate had a slower CO binding rate; injection of saturated CO resulted in a larger absorbance change, reflecting a lack in CO binding ability relative to HEPES or tris buffers (Figure 6.8).

**H Atom Abstraction Experiments of HydG Performed in Phosphate Buffer**

In order to further explore the phosphate effect on HydG catalysis, we investigated whether differences in the details of H atom abstraction would be observed in the presence of phosphate. Product 5’-dAdo and SAM isotope distributions from experiments performed in D$_2$O/phosphate buffer were compared to those observed in non-phosphate buffers (Figure 6.9). Samples of HydG incubated with reductant, SAM, and tyrosine incubated in D$_2$O/phosphate buffer exhibited stimulated 5’-dAdo formation relative to samples lacking tyrosine, similar to previously published results in phosphate–H$_2$O$^1$ and in non-phosphate D$_2$O buffers$^{28}$ (Table 6.2). Figure 6.9 illustrates the total amount of SAM and 5’-dAdo with at least one deuterium incorporated at the 5’-position. A 40% increase in the amount of 5’-dAdo with label was observed in phosphate buffer at pD 8.5 relative to phosphate pD 7.4 (red, blue, and magenta bars), while a modest 13% increase in labeled SAM was observed (white and green bars). By contrast, assays performed in the absence of phosphate under similar conditions yielded a substantial (~100%) increase in both labeled SAM and 5’-dAdo products at pD 8.1 relative to pD 7.2
Figure 6.9. Quantitation of labeled 5’-dAdo and SAM in Phosphate Buffer. 60 minute incubations of HydG<sup>WT</sup> (100 µM; 9.1 ± 0.1 Fe/protein) with 1 mM Tyr, 1 mM SAM, 5 mM dithionite in 95% D<sub>2</sub>O solution (final) were performed. Distributions of labeled product were estimated from respective EICs from natural abundance 5’-dAdo and SAM isotope distributions. Unlabeled 5’-dAdo is omitted from the graph, however it should be noted that comparable amounts of unlabeled 5’-dAdo were detected in samples where the pD was similar or constant. Error bars represent the scaled error associated from HPLC, UV-visible based product quantitation. Samples were subjected to HPLC were performed in triplicate. Structures of 5’-dAdo and SAM are provided to show label at the 5’-position, corresponding to the deuterated species depicted.

(Figure 6.9). Individual species of labeled 5’-dAdo at the 5’- position (red, blue, and magenta bars) were comparable in non–phosphate and phosphate buffers at pD 7.2 and 7.4, respectively. However, increasing the assay pD to 8.5 in phosphate yielded modest changes in the 5’- dAdo and SAM distribution (Figure 6.9). By contrast, the 5’-dAdo and SAM distribution in tris buffer at pD 8.1 resulted in a substantial increase of deuterium label in each. These differences appeared to indicate that phosphate was interacting with the generated 5’-dAdo•.

The distribution of labeled SAM and 5’-dAdo in D<sub>2</sub>O buffer presumably reflect an equilibrium between [4Fe-4S]<sup>2+</sup>/SAM and [4Fe-4S]<sup>2+</sup>/Met/5’-dAdo• in the active site, with the latter capable of abstracting the solvent-exchangeable H/D from substrate. The
distribution differences between phosphate and non–phosphate buffers suggests that phosphate may affect the equilibrium indicated above. In order to evaluate these numbers in another way, quantities of 5’-dAdo and SAM with at least one deuterium label were normalized (Figure 6.10). Experiments performed in tris or HEPES buffers yielded significantly more SAM with label relative to product 5’-dAdo with label, while in phosphate these proportions were reversed. As is noted elsewhere, the apparent reversibility of H atom abstraction in HydG\textsuperscript{WT} enzyme has been interpreted to occur either before or after cleavage of the Tyr C\textsubscript{α}–C\textsubscript{β} bond at a solvent-exchangeable position (Chapter 4). The results in Figure 6.10 therefore suggest that phosphate affects the partitioning between the forward (producing 5’-dAdo) and reverse reactions (reforming SAM from 5’-dAdo\textsuperscript• that is deuterated by interaction with substrate).
HydG CO Experiments with HydF

HydG is a maturase radical SAM enzyme that acts in conjunction with the maturase HydF in 2Fe subcluster biosynthesis and assembly. The work described in the preceding sections involved HydG characterized in the absence of HydF, however HydG is hypothesized to deliver its products to HydF via direct protein-protein interactions, and thus we were interested in probing the effects of HydF on HydG catalysis. First, HydG assays were performed in the presence of 2 mM GTP or GDP (HydF substrate and product, respectively) to determine effects of the phosphate-containing nucleotides on HydG-catalyzed CO formation (Figure 6.11A). Addition of these nucleotides produced some effects on HydG-catalyzed CO formation, specifically

![Figure 6.11. HydGWT CO Formation Activity in the Presence of Nucleotides and HydFΔEG. (A) HydGWT CO Formation Activity in the Presence of Nucleotides. Black trace, HydG turnover assay with no nucleotide addition; red trace, HydG turnover assay with 2 mM GTP; magenta trace, HydG turnover assay with 2 mM GDP. (B) CO Formation activity of HydGWT in the presence of HydFΔEG and nucleotides. Black trace, HydG turnover assay with 1:1 HydGWT and HydFΔEF without nucleotide addition; red trace, 1:1 HydG:HydF with 2 mM GTP; magenta trace, 1:1 HydG:HydF with 2 mM GDP; green trace, HydG turnover assay with no HydF or nucleotide addition. Experiments were performed at 37 °C with 62 µM HydGWT (4.7 ± 0.3 Fe/protein) with 2 mM MgCl2, 1 mM Tyr, 1 mM SAM, 5 mM sodium dithionite, and 80 µM (heme) deoxyHb in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. Where applicable, 62 µM HydFΔEF (1.3 ± 0.1 Fe/protein), 2 mM GTP, 2 mM GDP was used as well.](image-url)
Table 6.6. Kinetic Parameters for Figure 6.11 HydG<sup>WT</sup> Catalysis in Presence of nucleotides and HydF<sup>ΔEG</sup>

<table>
<thead>
<tr>
<th>Sample Mix</th>
<th>Burst $k_{cat}^0$ ($\times 10^{-4}$ s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Slow $k_{cat}^0$ ($\times 10^{-4}$ s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; Only</td>
<td>3.63 ± 0.09</td>
<td>1.14 ± 0.14</td>
<td>0.99</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; + GTP</td>
<td>1.95 ± 0.02</td>
<td>N.D.</td>
<td>0.99</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; + GDP</td>
<td>4.75 ± 0.19</td>
<td>1.78 ± 0.04</td>
<td>0.99</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; + HydF&lt;sup&gt;ΔEG&lt;/sup&gt;</td>
<td>6.12 ± 0.08</td>
<td>2.32 ± 0.02</td>
<td>0.99</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; + HydF&lt;sup&gt;ΔEG&lt;/sup&gt; + GTP</td>
<td>6.36 ± 0.11</td>
<td>1.55 ± 0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt; + HydF&lt;sup&gt;ΔEG&lt;/sup&gt; + GDP</td>
<td>3.21 ± 0.07</td>
<td>1.37 ± 0.04</td>
<td>0.99</td>
</tr>
</tbody>
</table>

N.D. Not determined. Fitted to first-order exponential function.

On the burst phase $k_{cat}^0$, with GDP causing an increase while GTP caused a decrease (Table 6.6). Due to the effects of phosphate on HydG-catalyzed CO formation described in this chapter, the phosphate produced from HydF-catalyzed GTP hydrolysis was expected to result in a drop in HydG CO, p-cresol, and 5'-dAdo formation. HydG assays in the presence of HydF were performed with a 1:1 HydG<sup>WT</sup> and HydF<sup>ΔEG</sup> with 2 mM MgCl<sub>2</sub> (an essential cofactor for GTP hydrolysis). In the absence of nucleotides, HydG CO formation was stimulated in the presence of HydF (Figure 6.11B, black trace) with the burst phase and slow phase $k_{cat}^0$ values doubled relative to no HydF addition (Figure 6.11B, green trace) (Table 6.6). Interestingly, GTP addition in the presence of HydF did not affect the burst-phase $k_{cat}^0$; the decreased slow-phase rate relative to GTP’s absence resulted in a decreased [CO]<sub>tot</sub>. Experiments containing both HydF and nucleotides were incubated for approximately 15 minutes before SAM was added to initiate the reaction; the reported HydF<sup>ΔEG</sup> GTP hydrolysis rate ($k_{cat}$) is $2.43 \pm 0.36$ min<sup>-1</sup> at 30 °C<sup>15</sup> but because the GTP was in excess and the sample was handled under ambient conditions (22 °C), the ratio of GTP:GDP was assumed to be high (Figure 6.11B, red trace).
Discussion

The results presented herein have been directed towards understanding HydG CO substoichiometry through assay optimization and interaction with HydF. The HydG diatomic ligand product has served as a basis for defining product transfer to HydF and HydA. Several different approaches have been employed to understand this process, including chemical derivatization, as well as UV-visible and FTIR spectroscopy. However, limitations in defining diatomic ligand product stoichiometry might be due to detection methods that sampled different pools (protein-bound vs. free in solution) of diatomic ligands produced.

One qualitative difference between the assays used to define stoichiometry concerned the assay conditions employed to detect the diatomic ligand. The CN⁻ derivatization assay was developed under low salt, low glycerol conditions that initially resulted in enzyme precipitation when the CO assay was attempted under similar conditions (Chapter 2). After obtaining enzyme that was less susceptible to precipitation, however, performing the CO assay in low salt buffer provided clear improvements in CO production (Figure 6.1). While the increase in CO was encouraging, it was also accompanied by increases in products (5'-dAdo and p-cresol) and thus did not improve the CO:p-cresol ratio. Nonetheless, that CO formation could be performed in the absence of salt and glycerol without the enzyme undergoing significant denaturation following assay incubation was a significant advance in making the two assay experiments amenable for comparison (Figure 6.1).
A second qualitative difference between the detection methods for CO and CN\textsuperscript{−} formation was that the former was detected continuously \textit{in situ} while the latter was detected after complete enzyme denaturation.\textsuperscript{1} In order to determine whether CO was being sequestered in HydG during the in situ assay, we developed CO assays involving complete enzyme denaturation. HydG experiments performed in a sealed environment (trapping produced CO) followed by denaturation and addition of reduced deoxyMb did not result in remarkably improved CO formation relative to inclusion of deoxyHb or deoxyMb in the real-time assay (Figure 6.2). An important distinction in denaturation CO detection methods is that CO can be detected; the behavior however is consistent with the \textit{in situ} in terms of the amount of CO detected. To validate the developed method, control experiments with MbCO have shown that high percent CO recovery yields are observed from the denaturation CO detection methods employed here (Appendix Figure E.1). Thus these results suggest that CO does not appear to be sequestered by the enzyme, because a larger increase in CO formed would be detected from the precipitated volume.

The physiologically relevant buffer phosphate is present in the cytosol of photosynthetic algae and thus would be present upon \textit{in vivo} expression of the HydG maturases. \textit{In vitro} experimental assays directed to detect CO have been shown herein to be severely impacted in the presence of phosphate, with significant suppression in CO detected relative to the other products detected (Figure 6.3, Table 6.2). Such a drop in CO formation was not anticipated on the basis of the buffer composition, since stoichiometric formation of CN\textsuperscript{−} was observed elsewhere,\textsuperscript{1} and foundational CO binding kinetics to hemoglobin were reported in phosphate buffer.\textsuperscript{33,34} In optimized assays,
phosphate had a specific effect on CO formation (Figure 6.3); at pH 7.4 HydG catalyzed comparable ouch-cresol and 5’-dAdo products, but differed on the amount of CO formed (Figure 6.3, Table 6.2).

Phosphate buffer appears to have an impact on HydG Fe–S clusters involved in catalysis. Preparation of samples dialyzed into phosphate tended to depress iron numbers overall. By comparison, titration of phosphate buffer as the buffering medium into HydG prepared in Tris or HEPES resulted in less CO, ouch-cresol, and 5’-dAdo products formed (Figure 6.5, Table 6.4). For samples where the reconstituted HydG stock was divided in half and was dialyzed in a phosphate buffer, observable differences in the iron number, activity, and CO efficiency were observed (Figure 6.4, Table 6.3). Interestingly, an improvement in CO efficiency was observed for phosphate-dialyzed enzyme assayed in tris buffer than was observed for tris-reconstituted enzyme assayed in phosphate buffer. The amounts of ouch-cresol formed with the respective samples are comparable, considering that the phosphate-dialyzed sample underwent an additional freeze-thaw event, and considering that the samples differ in the assay phosphate concentration. This suggests a potential interaction of phosphate with the C-terminal cluster, because more CO was formed with the phosphate-dialyzed protein in tris buffer than was formed with tris-reconstituted enzyme performed in phosphate buffer. Thus, phosphate appears to play a specific role in the CO formation mechanism, not only by affecting tyrosine cleavage but also in the steps that follow to form the CO product (Table 6.3, 6.4).

Corroborative data supporting a drop in iron number for samples prepared in phosphate includes spectral characterization of the enzyme paramagnetic signals. EPR spectroscopy of photoreduced HydGWT enzyme dialyzed and prepared in phosphate
buffer exhibited a decrease in as-isolated [3Fe-4S]$^+$ and reduced [4Fe-4S]$^+$ signal, and an increase in non-specifically bound Fe species with growth of an observable $g = 4.3$ feature (Figure 6.6). These results were coupled with a decreased amount of SAM-bound cluster observed by EPR, which is likely indicative of Fe–S cluster degradation. Such an observation is consistent with the loss in $p$-cresol and 5’-dAdo product formation observed for most samples prepared in phosphate.

Hemoglobin–CO binding kinetics were investigated in different buffers to determine whether the buffer effects were in part due to our method of detecting CO. Such experiments were designed to mimic the expected catalytic amounts of CO produced in the first minutes by HydG, and are similar to experiments with human hemoglobin in phosphate buffer published elsewhere. Differences in the apparent binding rate of CO with deoxyHb were observed, as identical volumes of CO gas injected into deoxyHb resulted in different rates of CO formed (Figure 6.7). While HEPES and tris buffers performed at different pH yielded comparable binding rates, the phosphate buffered Hb bound CO at a much slower rate, approximately 1/3 of the rate observed in tris and HEPES buffers (Table 6.5). This in turn yielded a smaller amount of CO detected by hemoglobin, meaning that CO detected in phosphate may be underestimated relative to HEPES or tris buffers, as independent of HydG CO formation. Applying these observations to catalysis, even with a slower rate of CO binding of binding, less CO is detected by phosphate-buffered HydG than is formed in HEPES buffers (for example, see Figure 6.3). Interestingly, it should be noted that we have observed improvement in percent CO efficiency by using H64L deoxyMb in place of deoxyHb (Appendix Figure D.3) in reactions that catalyzed comparable $p$-cresol product. In turn, these results
definitely speak toward the limitations encountered by detecting CO with hemoglobin, where stoichiometric CO formation might be probable, but is not observed.

In addition to the work noted above, a comparative profile of H atom abstraction by HydG has been performed in different buffers. Differences in the quantity and distribution of label in 5′-dAdo and SAM were observed in phosphate buffer at pH 7.4 and 8.5 relative to HEPES buffer at pH 7.2 and tris buffer at pH 8.1 (Figure 6.9). Phosphate buffer yields a similar distribution of labeled 5′-dAdo to non-phosphate buffer that does not appear to change with increasing the pD. The H atom abstraction in phosphate buffer is largely pD-invariant with respect to distribution of label in SAM and 5′-dAdo, and yields modest pD-dependent changes in product formed is different from non phosphate buffers assayed. In contrast, non-phosphate buffers have pD-dependent abstraction distributions in both SAM and 5′-dAdo, with an increase in pD resulting in an increase in SAM and 5′-dAdo with label (Figure 6.9).

The apparent decrease in abstraction reversibility in phosphate buffer has potential implications on the mechanism of CO and CN⁻ formation. Diatomic product formation follows cleavage of a second C–C bond, perhaps from H atom abstraction by the 5′-dAdo• or alternatively by the p-cresolate phenoxyl radical. Differences in the abstraction serve as a proxy to perturbations the catalytic mechanism. Regeneration of the 5′-dAdo• can occur as part of events the precede or follow cleavage of the Tyr C\textsubscript{α}–C\textsubscript{β} bond\textsuperscript{28}. Additional reabstraction (regenerating more 5′-dAdo• and more SAM with label) appears to occur with the non-phosphate buffer at comparable pD. Phosphate has an effect on the distribution of 5′-dAdo and SAM possessing deuterium, as a larger percentage of the labeled species is 5′-dAdo (Figure 6.10) is observed. It should be noted
that a shift in the equilibrium towards production of more 5′-dAdo and less SAM with deuterium signifies an effect of the HydG’s ability to recycle the 5′-dAdo•; production of less 5′-dAdo and more SAM would signify an increase in enzyme ability to reabstract a hydrogen atom from 5′-dAdo, recycling the radical for recombination with methionine. Whether or not this shift in equilibrium is part of the C-terminal cluster catalytic involvement is unclear at this point, but the associated loss of CO formed suggests a potential association. It should be noted that preliminary ENDOR studies examining $^{31}$P–Fe coupling in the presence of phosphate were unsuccessful at observing a coupling between phosphate and HydG Fe–S clusters (M. Horitani, B.M. Hoffman, unpublished results) that would be expected if phosphate were coordinated in proximity to the [4Fe-4S] clusters.

HydG CO formation was employed as an indirect tool to understand the likely protein–protein interactions expected between HydG, HydF, and the potential effect of endogenous phosphate production from GTP hydrolysis on HydG CO formation. Spectrophotometric CO detection is relevant to the biochemical reaction studied, because CO is a product that is modulated by exogenous phosphate and CN$^-$ (Chapter 5). HydF serves as a transfer agent of the diatomic ligands to HydA, and might affect CO produced if it received the diatomic ligands. The stimulated HydG CO formation in the presence of HydF$^{ΔEG}$ is noteworthy (Figure 6.11B), since a stimulation might be expected if HydF and HydG interacted. Surface plasmon resonance experiments have measured an affinity between HydG and HydF$^{ΔEG}$. 16 The radical SAM matrerase HydE has a higher affinity for HydF than does HydG, 16 but a measurable binding affinity between HydG and HydF implicates an interaction. The cluster states of HydF have suggested that a [2Fe-2S]
cluster is modified by HydG and HydF, but the possibility that HydG supplies Fe to the HydF protein for modification cannot be ruled out. Nevertheless, stimulated CO formation in the presence of HydF likely reflects HydF’s ability to function as an allosteric effector in the HydG catalytic reaction, in that its presence stimulates CO formation.

Delineating the involvement of HydF GTP binding and hydrolysis in the biosynthetic mechanism has been difficult. The observation that phosphate inhibits HydG CO diatomic ligand formation was intriguing, since phosphate is a product of HydF GTPase activity. Because HydF likely receives HydG products, HydF-catalyzed GTP hydrolysis (generating phosphate) might have an effect on gating HydG diatomic ligand formation ability. HydF GTP binding and hydrolysis has an observable affect on CO produced by HydG in the presence of HydF, and depression in the slow-phase CO formation rate accounts for the decreased CO formation overall (Figure 6.11). In the absence of HydF, the HydG burst phase $k_{\text{cat}}^0$ value for the sample containing GTP was depressed, signifying inhibition; that a stimulation in the rate is observed with HydF and GTP suggests that GTP is binding to HydF over HydG (Table 6.6). Interestingly, the burst phase $k_{\text{cat}}^0$ values for HydF alone and with HydF and GTP were nearly identical, while HydF and GDP resulted in a drop in rate that was comparable to the $k_{\text{cat}}^0$ value reported for HydG alone without HydF around. The combination of HydF with GTP not affecting the burst phase $k_{\text{cat}}^0$ is interesting, since the sample incubated for 15 minutes in the presence of GTP, facilitating hydrolysis to form phosphate. The comparable burst phase and different slow phase rates of CO formation observed for HydG and HydF in
the presence or absence of GTP suggests that hydrolysis has an impact that is largely dependent on the rate of HydF GTP hydrolysis (Table 6.6).

This preliminary study on the effect of HydF GTP hydrolysis on HydG CO formation provides foundational work toward the interface the maturase enzymes have with respect to the biosynthetic mechanism. GTPase activity is associated with protein dissociation that may be serving here between HydE or HydG with HydF. CO formation rates are impacted by HydF and the molecules involved with with GTP hydrolysis. With respect to the interfacial role that endogenous phosphate serves in affecting HydG CO formation, additional experiments characterizing HydG CO formation with HydF in the presence of non-hydrolyzable analogs (ITP and IDP), as well as with HydF that has completely hydrolyzed GTP (GDP and phosphate) would help differentiate the observed effects as being a consequence of phosphate binding and coordination to HydG. Finally, while a stimulated burst $k_{\text{cat}}^0$ was observed for GDP addition only, addition of phosphate is likely to cause a depression in the rate of CO formation.

As of this writing, the precise involvement of phosphate in HydG catalysis remains largely speculative. While HydG does not appear to possess obvious structural motifs that would support phosphate ion binding, phosphate is a known biological metal ion ligand that in principle could extrude iron from HydG’s [4Fe-4S] clusters. Sequence homology modeling of the HydG sequence from *C. acetobutylicum* has shown that the two [4Fe-4S] clusters are oriented at opposite ends of the TIM barrel, and each have distinct coordination environments around respective [4Fe-4S] clusters (Chapter 8, Figure 8.8) that may be susceptible to phosphate binding and extrusion. In phosphate buffer, while HydG undergoes multiple turnover events to produce CN$^-$, 5'-dAdo, and $p$-cresol
products, preliminary ENDOR measurements have shown that added phosphate does not appear to coordinate the HydG Fe–S clusters (M. Horitani, B.M. Hoffman; unpublished results). While p-cresol and 5’-dAdo are formed in the presence of phosphate at the radical SAM [4Fe-4S] cluster, the interplay of phosphate at the C-terminal [4Fe-4S] cluster in generating CN– but not CO could simply serve as a modulator between oxidative decarbonylation (producing CN– and CO from DHG) and oxidative decarboxylation (producing CN– and CO2). The lack of CO detected, coupled with the stoichiometric quantities of CN– detected would appear to suggest that phosphate might interact with the DHG intermediate. Alternatively, the binding of phosphate (as reflected in the abstraction difference) could afford a conformational change that tunes the catalytic reaction from an oxidative decarbonylation to an oxidative decarboxylation event.

It should be noted that the effects of phosphate on HydGWT is reminiscent of the observations with the HydG C386S variant, where CN– but no CO could be detected (Chapter 3) in non-phosphate buffer. The effect of the point substitution of Cys386 to serine on the C-terminal [4Fe-4S] cluster state is at present unknown, but the similar product trend observed for HydGWT in phosphate buffer suggests a potentially comparable effect on the C-terminal [4Fe-4S] cluster, and perhaps phosphate coordination and extrusion of the C-terminal unique Fe renders the resultant cluster to be [3Fe-4S] in character. We have shown here that phosphate affects the equilibrium between [4Fe-4S]+/SAM and [4Fe-4S]2+/Met/5’-dAdo• in the active site that may be related to a lack of an unique Fe at the C-terminal [4Fe-4S] cluster. By comparison to the initial report of CN– detection, its stoichiometric formation with p-cresol under buffer
conditions associated with iron loss and lack of CO formation may reflect a divergent, off-pathway mechanism of diatomic ligand formation. Whether this effect is due to catalysis from a C-terminal [3Fe-4S] cluster following phosphate Fe extrusion, or due to phosphate active site coordination and modulation of catalysis during processing of the DHG intermediate is currently speculative. However, future spectroscopic studies of the HydG_C386S enzyme to identify the C-terminal cluster state undergoing turnover would provide a controlled model for which the effect of phosphate can be understood.

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CHAPTER 7

EFFECTOR AND INTERMEDIATE MOLECULE INTERACTION WITH RADICAL SAM [FEFE]-HYDROGENASE MATURASE HYDG

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 2, 3, 4, 5, 6, 7, 8

Author: Benjamin R. Duffus
Contribution: Conceived and carried out the comparison study. Prepared enzyme samples, performed CO experiments, performed UV-visible CD and absorbance spectroscopy, prepared EPR samples, and performed LC–MS and HPLC product quantitation. Analyzed the data and wrote the manuscript.

Co-Author: Eric M. Shepard
Contribution: Collected EPR data.

Co-Author: John W. Peters
Contribution: Provided guidance to the experiments. Obtained funding and resources, and edited manuscript drafts.

Co-Author: Joan B. Broderick
Contribution: Directed experimental design and interpretation. Obtained funding and resources, and co-wrote the manuscript.
CHAPTER 7

EFFECTOR AND INTERMEDIATE MOLECULE INTERACTION WITH RADICAL SAM [FEFE]-HYDROGENASE MATURASE HYDG

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Abstract

The diatomic ligands of the H-cluster of the [FeFe]-hydrogenase active site are synthesized by the radical S-adenosylmethionine (SAM) enzyme HydG. HydG catalyzes a complex radical initiation reaction that involves the cleavage of two C–C bonds, and utilizes two site-differentiated [4Fe-4S] clusters to form the products carbon monoxide (CO) and cyanide (CN⁻). Dehydroglycine (DHG) is thought to be an intermediate product of tyrosine Cα–Cβ bond cleavage, however mechanistic insight regarding involvement to form these diatomic products is largely unknown. HydG acts in conjunction with the scaffold GTPase HydF and the radical SAM enzyme HydE to synthesize the 2Fe subcluster of the H cluster. HydG catalysis is stimulated in the presence of cysteine, a proposed substrate for the maturase HydE. Biochemical and spectroscopic characterization of HydG and its interaction with cysteine and DHG is presented herein.
Cysteine appears to interact with the HydG enzyme, although it appears to not affect the D atom incorporation observed when HydG is assayed in D₂O. DHG confers a unique spectral perturbation observed by CD and EPR spectroscopy, yet it alone appears to not catalyze CO formation, requiring additional steps. Mechanistic implications of these results are described.

Introduction

The [FeFe]-hydrogenase H-cluster is an unique organometallic active site in biology that facilitates the reversible reduction of proton to produce dihydrogen gas. The H-cluster is composed of a [4Fe-4S] cluster that links to a 2Fe subcluster by a bridging cysteine; the 2Fe subcluster is also coordinated by a non-protein bridging dithiomethylamine ligand, three CO and two CN⁻ ligands. Synthesis and assembly of this unique 2Fe subcluster requires a coordinated maturation machinery comprising of two radical SAM enzymes and a scaffold GTPase. These maturases act by modifying standard Fe–S clusters synthesized by the housekeeping Fe–S cluster biosynthetic genes from the nif, isc, and suf operons. Only three proteins are required for the synthesis of the 2Fe subcluster in addition to the housekeeping Fe–S biosynthetic machinery described above. While it has been shown recently that abiological structural mimics to the 2Fe subcluster can be incorporated as part of the H-cluster without the need for the maturation proteins, biosynthesis of the 2Fe subcluster requires functional activity of HydE, HydF, and HydG.

While HydG catalyzes the formation of the diatomic ligands, HydE (by elimination) has been proposed to synthesize the dithiolate ligand using an unknown
substrate. Preliminary substrate screening for the HydE enzyme has suggested that it acts upon cysteine.\textsuperscript{14} Consistent with this observation was a preliminary study that identified additives stimulating hydrogenase activity included cysteine and tyrosine.\textsuperscript{15} While exogenous tyrosine might be expected to increase hydrogenase activity by increasing HydG-catalyzed synthesis of CO and CN\textsuperscript{−} ligands required for the H-cluster, the cysteine effect could arise from enhanced Fe–S biosynthesis due to the presence of cysteine desulfurases in the lysate used in the assay. Alternatively, preliminary indications of deuterium transfer from cysteine to 5’-dAdo suggested that cysteine serves as a substrate for HydE.\textsuperscript{16}

As part of our larger objective to understand substoichiometric CO product formation (described in more detail in Chapter 6), it was proposed that control of product stoichiometry might be dependent on the presence of other maturases and their associated molecules. HydG forms the CO and CN\textsuperscript{−} products as part of a coordinated biosynthetic machinery with the HydE and HydF maturases, along with their associated substrates and products. For example, reconstitution of hydrogenase activity using maturases from \textit{S. oneidensis} was not successful without addition of an \textit{E. coli} cell lysate.\textsuperscript{17,18} Also as noted in Chapter 5, product diatomic ligands have been shown to coordinate to HydG and HydG-derived Fe has been shown to be transferred to HydA,\textsuperscript{19} likely via transfer to HydF. However, the involvement of two discrete radical SAM enzymes in the biosynthetic scheme is unique, as the product of one enzyme in principle could serve as the substrate for the other.

The mechanism of HydG diatomic ligand product formation is complex, requiring two site-differentiated [4Fe-4S] clusters. While heterolytic tyrosine C\textsubscript{α}–C\textsubscript{β}
bond cleavage via 5’-dAdo• abstraction of the 4-phenol hydrogen atom forms p-cresol, 5’-dAdo, methionine, and dehydroglycine (DHG) at the N-terminal [4Fe-4S] cluster, the steps that follow to produce CO and CN– are largely unknown. Detection of glyoxylate (the hydrolysis product of DHG) has been used to infer intermediacy of DHG, but additional corroborative evidence is largely lacking. It has been proposed that the HydG C-terminal [4Fe-4S] cluster processes DHG to produce CO and CN–. The C-terminal cluster is redox active and in principle electron transfer between it and DHG would be sufficient to generate diatomic ligand products. Alternatively, additional activation or deprotonation events might be required to yield an oxidative decarbonylation reaction that would yield CO and CN–.

Presented herein is biochemical and spectroscopic characterization of the HydG enzyme in the presence of cysteine and in the presence of DHG, directed towards providing insight into its role in the biosynthesis of the H-cluster. Cysteine appears to serve a unique role in stimulating HydG product formation without conferring a difference in H atom abstraction. By comparison, DHG confers a unique spectral perturbation observed by electron paramagnetic resonance and CD spectroscopy, but does not result in CO formation. These observations have relevance to the HydG biosynthetic mechanism, which are described.

Experimental Section

Materials

All chemicals and other materials used herein were from commercial sources and of the highest purity where available. Tyrosine, glyoxylic acid, and the p-cresol and 5’-
dAdo standards were obtained from Sigma-Aldrich (St. Louis, MO). Triton X-100 and imidazole were obtained from Alfa Aesar (Ward Hill, MA). Tris, HEPES, IPTG, PMSF, tryptone, yeast extract, potassium phosphate, DTT, and streptomycin were obtained from RPI (Mt. Prospect, IL). MgCl₂, KCl, acetonitrile (HPLC grade) and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite, sodium sulfide, and D₂O was obtained from Acros Organics (Fair Lawn, NJ). DNase I, RNase A, and lysozyme (hen egg) were obtained from Roche (Indianapolis, IN). Ferrous ammonium sulfate, Iron(III) chloride, ammonium chloride, acetic acid (HPLC grade) and cysteine was obtained from Fisher Scientific (Fair Lawn, NJ). H64L myoglobin and hemoglobin were received as kind gifts from Professor John S. Olson (Rice University) and Professor David J. Singel (Montana State University), respectively. SAM²⁰ and 5-deazaflavin²¹-²⁴ were synthesized and purified as reported elsewhere, with slight modifications.

HydG Overexpression, Purification, and Chemical Reconstitution

Heterologous overexpression of *Clostridium acetobutylicum* HydG in *Escherichia coli*, purification and chemical reconstitution with iron and sulfide were prepared as described previously²⁵,²⁶ with slight modifications (Appendix A and Chapter 3). Used in these studies was the intact, unmodified enzyme (HydGWT) as well as a tricysteine-substituted variant that contained only the C-terminal cluster, with C96A/C100A/C103A substitutions incorporated (HydGNTM); the HydGNTM was prepared as described previously with slight modifications (Chapter 3).²⁷ Briefly, single colonies obtained from transformations were grown overnight in LB media and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50
mM potassium phosphate buffer, pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD<sub>600</sub> = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N<sub>2</sub> overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber (Grass Lake, MI), as described<sup>25,26</sup> with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES 500 mM KCl, 5% glycerol, 10 mM imidazole, 20 mM MgCl<sub>2</sub>, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, and approximately 0.6 mg lysozyme per gram cell. This mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles (Nalgene; Rochester, NY) at 18,000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrap<sup>TM</sup> Ni<sup>2+</sup>-affinity column (GE Healthcare, Uppsala). The column was pre-equilibrated with 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Pure fractions (gauged by SDS-PAGE) were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4, and concentrated using an Amicon Ultra centrifugal unit (Millipore; Billerica, MA) fitted with a YM-10 membrane or using a Minicon B15 static protein
concentrator (Millipore). Protein was flash frozen in liquid N$_2$ and stored at –80 °C or in liquid N$_2$ until further use.

Reconstitution of as-purified HydG was carried out following the general procedures described previously.$^{26}$ Enzyme (50-150 µM) was incubated with 6 – 7 fold excess of FeCl$_3$ and Na$_2$S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 for ≈ 3 hours with gentle agitation in a Coy chamber at 22 °C. Following a 5 minute incubation with DTT, FeCl$_3$ was added slowly and stirred for 20 minutes prior to the stepwise addition of Na$_2$S. Following the reconstitution period, the mixture was centrifuged to remove exogenous FeS clusters in the mixture. Following initial concentration using an Amicon Ultra centrifugal unit (Millipore), the supernatant was treated over a sephadex G-25 column (GE Healthcare; Piscataway, NJ) to remove excess ions. Following this, the darkest brown fractions were pooled and concentrated using the centrifugal unit noted above. UV-visible spectroscopy was utilized to confirm the Fe–S cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Enzyme concentration was determined by performing a Bradford assay,$^{28}$ while the iron content was determined through the colorimetric ferrozine method.$^{29}$

UV-visible Absorbance/Circular Dichroism Experiments

UV-visible CD experiments were performed in duplicate under anaerobic conditions using a Jasco J-710 spectropolarimeter (Easton, MD) at room temperature. Samples were prepared in an anaerobic Coy chamber in a cold room (4 °C), and a Hellma 1.0 cm pathlength screwcap anaerobic cuvette (Plainview, NY) was used for all CD
experiments. Buffers used for CD experiments were sparged with N₂ gas for at least 1 hour before use. UV-visible absorbance spectra were performed in series, using a Starna 1.5 mL screwcap anaerobic cuvette (Starna cells; Atascadero, CA) on an Agilent Cary 60 single pathlength spectrophotometer (Santa Clara, CA). Typical CD instrument parameters included a sensitivity of 100 millidegrees, a scan speed of 100 nm/min, a data pitch of 0.1 nm, a bandwidth of 1.0 nm, a response time of 4 seconds, and an accumulation of 3 scans across the 300-800 nm spectral window. Samples were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 buffer, which also served as the buffer blank for UV-visible absorbance scans, across the 200-1000 nm spectral range. Samples contained HydG enzyme concentrations 24-46 µM that were confirmed by the Bradford assay, while sample iron concentrations were assessed using the method developed by Fish. Samples also contained freshly prepared 1 mM DTT. Optimal spectral data was obtained when the enzyme had undergone minimal freeze-thaw cycles. While additions of cysteine, glyoxylate, and ammonium were added in a Coy chamber, glyoxylate was handled in subdued light to minimize photodegradation. Samples that were expressed in molar ellipticity were calculated with the Equation 7.1:

\[
[\theta] = \frac{\theta}{10cI}
\]  

(7.1)

**CO Activity Experiments**

HydG CO formation was monitored via incubation with deoxyhemoglobin (deoxyHb) under strictly anaerobic conditions at 37 °C in mixtures that contained HydG (40 µM), sodium dithionite (5 mM), tyrosine (1 mM), deoxyHb (80 µM per heme),
cysteine at various concentrations reported throughout, and SAM (1 mM) in 50 mM tris, pH 8.5. Sample preparation was performed in an anaerobic glove box (MBraun; Stratham, NH). H$_2$O buffers were prepared by degassing on a schlenk line (3 x 10 minute vacuum cycles followed by N$_2$ backfill). Buffer volume lost to evaporation was replaced with anaerobic H$_2$O in the glovebox. DeoxyHb was oxidized with potassium ferricyanide and was desalted over a PD-10 column filled with Sephadex G-25 resin prior to use. Sodium dithionite was prepared fresh in the degassed buffer, while the tyrosine stock was prepared aerobically by dissolving tyrosine solid in 1 M HCl, and it was then diluted with 100 mM tris, pH 8.5 buffer to make a 67.7 mM concentrated stock that was degassed on the schlenk line manifold (3.59:1 buffer:HCl).

Samples containing the above components aside from SAM were centrifuged at 14,000 rpm to pellet unwanted precipitates. Meanwhile, SAM was loaded into a 25 µL gastight syringe, which was capped by insertion in a rubber stopper. The prepared solution was loaded by 500 µL gastight syringe into a 1 mm pathlength UV-visible cuvette (Spectrocell; Oreland, PA) and was capped with a screwcap with a penetratable teflon insert. Experiments were performed on a Cary 6000i dual pathlength spectrophotometer (Agilent) that was pre-blanked with the cuvettes used with aerobic reaction buffer. After an initial scan (300-800 nm) was performed, SAM was injected into the cuvette, which was inverted a few times, and the penetrated hole was filled in with vacuum grease. After about 12 seconds, $A_{419\text{nm}}$ was monitored every second for 30 minutes at 37 °C. The $\Delta A_{419\text{nm}}$ and $\Delta \varepsilon_{419\text{nm}}$ were used to calculate the concentration of Hb-CO at each time point to determine the rate of CO formation. Following the incubation period, a final scan was performed to confirm the extent of CO binding, and
following this a sample aliquot was saved for HPLC product quantitation.

EPR Spectroscopy

EPR samples were prepared as described previously with slight modifications.26,27 Briefly, the HydG enzyme was supplemented with 50 mM tris (pH 7.4), 100 µM 5-deazariboflavin, and 5 mM DTT in buffer (50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4), and was placed in a ice-water bath in the MBraun box. Following illumination with a 300 W Xe lamp for 1 hour, cysteine (1 mM) was added in the absence of light; after three minutes, the X-band EPR tube was frozen in liquid N₂. Samples subjected to CD and UV-visible absorbance spectroscopy that contained glyoxylate and ammonium were loaded into X-band EPR tubes in the presence and absence of dithionite. Low-temperature EPR spectra were recorded using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller (Oxford Instruments; Abingdon, U.K.). Typical EPR parameters included a sample temperature of 12 K, a microwave frequency of 9.37 GHz, a microwave power of 1.59 mW, and a time constant of 20.48 ms. Experimental spectra were baseline corrected and plotted using OriginPro (version 9.1.0; OriginLab Corp., Northampton, MA).

H Atom Abstraction Experiments

Assays were performed in D₂O containing buffer, under conditions described in the main text and similar to methods described elsewhere (Chapter 4). 30 Experiments were performed in an anaerobic chamber (MBraun) under strict anaerobic conditions (<1 ppm O₂). D₂O buffers were prepared by lyophilization of H₂O buffer on the schlenk
line for 16 hours. The resultant salt was brought into the M Braun box and was dissolved with degassed D$_2$O to original volume. Prior to suspending the salt in D$_2$O, the schlenk flask was briefly subjected to a heat gun to drive off remaining H$_2$O moisture. Upon resuspending the buffer salt in D$_2$O, the pD was measured with pH paper (Micro Essentials; Brooklyn, NY) and was as expected acidic by 0.4 units.$^{31,32}$ Working 2 mM Tyr stocks in D$_2$O were prepared in a similar fashion to buffers described above, via dilution from an aerobic 67.7 mM stock in H$_2$O (prepared by dissolving Tyr in 1 M HCl, then dilution with 100 mM tris, pH 7.4) that was lyophilized overnight and was dissolved in D$_2$O.

Experiments were performed at 37 °C in an IsoTemp heatblock (Fisher), containing 100 $\mu$M HydG (8.5 $\pm$ 0.2 Fe/protein), 1 mM AdoMet (enzymatically prepared), 1 mM Tyr, and 5 mM dithionite (80 $\mu$L volume) for 60 minutes, and upon incubation the enzyme was precipitated via 1:1 volume addition of acetonitrile (HPLC grade) or addition of 1 M HCl (13 % v/v). A concentrated HydG stock at ~ 2 mM was used to minimize the H$_2$O contribution for performed experiments. Use of either quenching medium did not affect the observed product isotope distribution. Samples were centrifuged for 3 x 10 minutes at 14,000 rpm, and the supernatant was collected. Samples were then used for LC-MS or HPLC, as described below. Reported assay pDs were corrected for changes related to temperature.$^{33}$

**LC-MS Methods**

Deuterium isotope distribution in AdoMet and dAdo was assessed using LC-MS with as-obtained samples following centrifugation of the precipitated enzyme.
Quantitation of 5’-deoxyadenosine, AdoMet, and tyrosine was performed using an Agilent 1290 series UHPLC coupled to an Agilent 6538 Q-TOF mass spectrometer equipped with the dual-ESI source and an autosampler. Samples were injected onto a normal phase MicroSolv (Eatontown, NJ) Cogent “Diamond Hydride” HPLC column (150 x 2.1mm) equilibrated with solution B (CH$_3$CN + 0.1% formic acid) and 0.8 mL/min. An isocratic mobile phase was maintained for 2 minutes following sample injection, and then a linear gradient with solution A (H$_2$O + 0.1% formic acid) was run for 4 minutes, making a 50% solution B mixture. Isocratic elution was carried out for 2.5 minutes at 50% solution B and then re-equilibration to 100% B for 1.5 minutes. Total run time was 10 minutes and the column temperature was maintained at 50 °C. Under these conditions, dAdo eluted at ~ 3 minutes, AdoMet eluted at ~ 6 minutes, and tyrosine eluted at ~ 4 minutes. The capillary exit voltage was 120 V and gas temperature was 300 °C. All data was recorded in positive mode between 25 m/z and 750 m/z in profile mode. Hardware summation time was 1 second. Peaks were validated using high-accuracy formula confirmation and elution matching from individual reference standards. Quantitation was performed using the MassHunter Quantitative Analysis package (Agilent). An extracted ion chromatograph was generated using the accurate mass of each compound with a -0.04 to +0.04 ppm m/z extraction window. Samples were injected in duplicate, and between sample injections, a water blank injection was performed to minimize sample-to-sample carryover.

Sample isotope distributions for dAdo and SAM were assessed using the natural abundance distribution as a template distribution. Incorporation of a deuterium atom corresponds to a net mass increase of one, but results in an otherwise identical
distribution to the template distribution. Respective isotopic mass abundances were subtracted, and quantities for each were normalized to 100% total.

Glyoxylate/Ammonium CO Turnover Experiment

Experiments designed to detect CO from glyoxylate and ammonium were performed similarly to those described above for CO turnover, with a few modifications. Experiments were performed using a Cary Bio 60 spectrophotometer in a Starna 1.5 mL anaerobic UV-visible cuvette with a screwtop cap. Sample preparations were performed in an anaerobic glove box (MBrAn) containing < 1 ppm O2. A mixture containing active HydGWT (40 µM; 5.2 ± 0.1 Fe/protein) was mixed with deoxyhemoglobin (10 µM heme), as well as 25 mM glyoxylic acid and 25 mM NH4Cl in degassed 50 mM tris, pH 8.5. The glyoxylic acid solid was dissolved with degassed 1 M NaOH and stored in subdued light, while the NH4Cl was dissolved in the above buffer. Sodium dithionite was also dissolved from solid in the above buffer, and was loaded into a 1.5 mL eppendorf tube placed in a 25 mL crimped vial. Prepared sample was loaded into the cuvette and iterative scans were performed, along with mixing via inversion until no spectral change was observed. Using a 25 µL gastight syringe, 5 mM dithionite was injected into the mixture, and iterative scans were performed, with intermittent manual mixing via inversion.
Results

Cysteine Stimulates HydG Activity

HydG turnover experiments in the presence of cysteine were performed to assess the effect, if any, on HydG product formation. As expected, replacement of tyrosine with cysteine in a turnover sample resulted in no CO and a very small amount of 5’-dAdo resulting from non-productive SAM cleavage (data not shown). However, incubation of cysteine and tyrosine together with HydG under turnover conditions yielded stimulated CO formation relative to no cysteine addition (Figure 7.1). This stimulation appeared to be specific to cysteine, as addition of DTT (another thiol reducing agent) did not have a stimulatory effect on CO formation (data not shown). To confirm that the improved CO formation was associated with an improvement in CO efficiency, thirty minute time

Figure 7.1. HydGWT CO Formation in the Presence of Cysteine. 62 µM HydGWT (7.0 ± 0.4 Fe/protein) containing 1 mM tyrosine, 1 mM SAM, 5 mM sodium dithionite, 80 µM (per heme) deoxyHb, and in the absence (black trace) or presence (red trace) of 1 mM Cys in 250 mM tris, 300 mM KCl, 5% glycerol, pH 8.5 at 37 °C. (A) Full reaction. (B) The first ten minutes of reaction.
points for both assays were subjected to HPLC quantitation. The HPLC results revealed that an increase in \( p \)-cresol and 5’-dAdo was associated with the stimulated CO formed. After thirty minutes at 37 °C, the reaction that contained cysteine produced 227 ± 9 \( \mu \)M \( p \)-cresol and 546 ± 35 \( \mu \)M 5’-dAdo, while the reaction that lacked cysteine produced 191 ± 6 \( \mu \)M \( p \)-cresol and 439 ± 13 \( \mu \)M 5’-dAdo (Figure 7.1). The percent CO efficiency (CO:\( p \)-cresol) increased with the addition of cysteine, producing 26% relative to 21% in its absence (Figure 7.1A). Interestingly, addition of cysteine conferred a difference in the kinetic profile for CO formation under turnover conditions. Instead of the burst phase CO formation observed in the absence of cysteine (Figure 7.1B, black trace), a lag was observed for at least thirty seconds before CO product was detected (Figure 7.1B, red trace). Over the course of the experiment the rate of CO formation following the lag phase was faster than the slow phase of CO formation in the absence of cysteine, which accounted for the overall increase in CO formed (Figure 7.1A).

The lag in CO formation was peculiar, and suggested the possibility that cysteine might be interacting with one of the site-differentiated [4Fe-4S] clusters. To investigate this possibility, UV-visible CD spectroscopy was performed on HydG\(^{WT}\) and HydG\(^{NTM}\) to examine the potential interaction between cysteine and the Fe–S clusters (Figure 7.2). HydG\(^{NTM}\) is a tricysteine substitution of the radical SAM CX\(_3\)CX\(_2\)C motif that only coordinates the C-terminal [4Fe-4S] cluster;\(^{27}\) HydG\(^{NTM}\) was compared here to differentiate interaction of cysteine as involving the C-terminal cluster. Addition of 200 \( \mu \)M cysteine to HydG\(^{NTM}\) resulted in negligible changes in ellipticity (Figure 7.2; red trace). UV-visible absorbance of HydG\(^{NTM}\) in the presence of cysteine resulted in an increase at 390 nm, a decrease at 530 nm, and an isobestic point at 464 nm (Appendix
Figure 7.2. Effect of Cysteine on HydG\textsuperscript{WT} UV-visible CD Signal. UV-visible CD spectroscopy of HydG\textsuperscript{WT} (46 \(\mu\)M; 4.9 \(\pm\) 0.1 Fe/protein) (black lines) and HydG\textsuperscript{NTM} (46 \(\mu\)M; 2.5 \(\pm\) 0.1 Fe/protein) (red lines) in the presence (thick full lines) and absence (thin dotted lines) of 200 \(\mu\)M cysteine. Spectra were obtained at 23 °C in an anaerobic, 1 cm pathlength CD cuvette in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 with 1 mM DTT.

Figure F.1). However, addition of cysteine to HydG\textsuperscript{WT} resulted in observable changes in the CD spectrum (Figure 7.2; black trace). A positive change in ellipticity was observed at \(\lambda_{\text{max}}\) of 300, 380, and 460 nm, as well as the \(\lambda_{\text{min}}\) at 340 nm. Sharpening of the 550 nm \(\lambda_{\text{min}}\) feature was observed with positive changes at 525 and 590 nm (Figure 7.2; black trace). It is important to note that HydG\textsuperscript{NTM} contains one site-differentiated cluster (the C-terminal cluster), however discrete spectral changes were observed only with the HydG\textsuperscript{WT} enzyme, which contains both clusters.

To further investigate the interaction of cysteine with the HydG Fe–S clusters, the enzyme was characterized using EPR spectroscopy. Addition of cysteine to photoreduced HydG\textsuperscript{WT} resulted in minor, yet discernible changes in paramagnetic signal (Figure 7.3, red trace). By comparison, a lack in spectral perturbation has been observed following addition of the substrate tyrosine to the enzyme (Appendix Figure F.2).
Interestingly, the addition of cysteine resulted in sharpening of the 3445 G feature, and loss of an inflection at 3475 G (Figure 7.3, red trace). Both features arise from overlapping axial signals; cysteine addition appears to resolve the line broadening associated with cluster anisotropy. An observable drop in signal intensity was observed upon cysteine addition, perhaps due to the presence of cystine that likely re-oxidized some of the photoreduced enzyme.

The changes in ellipticity for HydG\textsuperscript{WT} (Figure 7.2) appeared to implicate an interaction with either Fe–S cluster. To differentiate this interaction as cluster–specific, UV-visible CD experiments containing cysteine were performed in the presence of SAM, since SAM binds to the N-terminal cluster (Figure 7.4). First, to assess changes in ellipticity associated with SAM binding with HydG\textsuperscript{WT}, a titration experiment was performed (Appendix Figure F.3). Spectral changes with isobestic points at 365 and 410
nm and broadening of the $\lambda_{\text{max}}$ values at 300, 385, and 465 nm and $\lambda_{\text{min}}$ values at 340, 475, and 550 nm were observed. A difference spectrum (Appendix Figure F.4) showed a positive change in ellipticity at 300, 350 and 425 nm, and negative changes at 385 nm, however large amounts of SAM were required to elicit an observable change in the CD spectrum (Appendix Figure F.3). Addition of 1.2 mM SAM (Figure 7.4A, red trace) before addition of 400 $\mu$M cysteine resulted in a negative change and broadening of the $\lambda_{\text{max}}$ at 385 nm that increased with cysteine addition (Figure 7.4A, blue trace). By comparison, addition of cysteine (Figure 7.4B, blue trace) before addition of SAM yielded no changes to this feature, even with SAM added afterward (Figure 7.4B, red trace). Also, the broad spectral feature between 450–500 nm appeared to increase only with the addition of cysteine, as addition of SAM alone did not sharpen the feature (Figure 7.4). Comparison of molar ellipticities for HydG$^{\text{WT}}$ and HydG$^{\text{NTM}}$ yielded estimation of spectral features associated with specific clusters (Appendix Figure F.5), that allowed for assignment of cysteine-specific perturbation in signal. Given the
observation that SAM binds only to the N-terminal cluster, the spectral perturbation associated with cysteine appeared to affect both the N-terminal and C-terminal [4Fe-4S] clusters (Figure 7.4).

As noted above, differences in initial time point CO formation involving cysteine appeared to indicate an interaction between cysteine and either Fe–S cluster. Because tyrosine cleavage to form the 5’-dAdo and p-cresol products has been shown to not require the C-terminal cluster, substrate Cα–Cβ bond cleavage and CO formation events are discrete and might be different with the addition of cysteine. To investigate this proposal, early time points were sampled for p-cresol and 5’-dAdo in the presence and absence of cysteine, while parallel CO formation experiments were performed (Figure 7.5). After a 60 second incubation at 37 °C, the sample with added cysteine had produced doubled the quantity of p-cresol and 5’-dAdo relative to no addition (Figure 7.5A). Similar to Figure 7.1, samples containing cysteine exhibited a lag phase in CO
Figure 7.6. Cysteine Invariance on the H Atom Abstraction Deuterium Isotope Distribution. Quantitation of labeled 5’-dAdo and SAM, following a 60 min incubation of HydG^{WT} (100 µM; 9.1 ± 0.1 Fe/protein) with 1 mM Tyr, 1 mM SAM, 5 mM dithionite in 95% tris-D_{2}O solution (final), pD 8.1. Distributions of labeled product were estimated from respective EICs from natural abundance 5’-dAdo and SAM isotope distributions. Unlabeled 5’-dAdo is omitted from the graph, however it should be noted that comparable amounts of unlabeled 5’-dAdo were detected in samples having similar pD. Error bars represent the scaled error associated from HPLC, UV-visible based product quantitation. Samples were subjected to HPLC were performed in duplicate.

formation than the control sample near sixty seconds (Figure 7.5B). These differences here in the amount of CO to p-cresol and 5’-dAdo appeared to suggest that cysteine affected both the initial cleavage of tyrosine substrate, in addition to CO formation (Figure 7.5).

To discern an interaction between cysteine and 5’-dAdo•, its effect on deuterium atom incorporation into 5’-dAdo and SAM during assays in D_{2}O was examined (Figure 7.6). The assays containing cysteine exhibited increased deuterium incorporation into SAM and 5’-dAdo (Figure 7.6). The distribution of deuterated species did not change however; normalization of total labeled species gave identical distributions in the presence and absence of cysteine (data not shown). Similar to the other data depicted (Figure 7.1, Figure 7.5) cysteine appears to stimulate the amount of p-cresol and of 5’-dAdo and SAM D atom incorporation, without affecting the isotope distribution for the
A lag phase in HydG CO formation in the presence of cysteine was intriguing, because it is a component in *E. coli* cell lysates and was therefore likely present in *So* HydG samples assayed in the presence of lysate. 17,19,34 An observed lag phase in free CO formation was observed by *So* HydG that contained a 5% v/v *E. coli* cell lysate, and in principle this could be explained by the presence of cysteine in the assay mixture (Figure 7.1). The time course for CO formation was therefore evaluated on a cysteine basis to assess its impact on the duration of the lag phase across several orders of concentration magnitude (Figure 7.7). Addition of cysteine resulted in a lag phase, even at substoichiometric cysteine concentrations (Figure 7.7A). Excess cysteine to HydG also yielded a detectable lag phase with resultant kinetics slowed relative to lower concentrations of cysteine. Interestingly, the substoichiometric cysteine addition resulted in a lag phase similar to other cysteine concentrations, and afterward proceeded to have
similar CO kinetics observed in the absence of cysteine (Figure 7.7B). This appeared to suggest that cysteine was consumed, because the resultant trend exhibited behavior observed in the absence of cysteine.

As it has been noted elsewhere, substoichiometric CO formation is likely limited by the inefficiency of the detection method (Chapter 6). Concurrent with ongoing studies to understand HydG CO formation, cysteine incubation improved the percent CO formation efficiency over a thirty minute incubation period (Figure 7.1). The linear CO formation rates following the initial lag over the course of the experiment suggested that “improvement” in CO efficiency might be attainable with longer incubation times and using the H64L deoxyMb that exhibited nanomolar CO affinity. This linear rate is faster than the slow rate of biphasic CO formation observed in the absence of cysteine, and appeared constant throughout the time course (Figure 7.1). Small quantities of CO binding to hemoglobin has been shown to be relatively inefficient (Chapter 6), suggesting that a longer incubation might improve the percent CO efficiency (CO:p-cresol).

Experiments were performed over long periods of time under substrate–limited concentrations to determine percent CO formation efficiency in the presence of cysteine (Figure 7.8). A tyrosine concentration greater than 2.5 fold relative to the HydG<sup>WT</sup> enzyme resulted in 38 μM CO formed after two hours when incubated with cysteine (Figure 7.8, red and black traces). By contrast, the enzyme formed 26 μM CO in the absence of cysteine after 45 minutes of incubation (Figure 7.8, blue trace). Over longer sampling periods, CO formation eventually stopped, however initial formation of CO followed a lag phase in the presence of cysteine, indicating its presence in the mixture (Figure 7.8A, black and red traces). Addition of excess cysteine relative to substrate
concentration did not appear to improve the CO quantity (Figure 7.8B, black and red traces); however, removal of cysteine yielded biphasic CO formation, and produced less CO relative to the presence of cysteine (Figure 7.8, blue trace).

Glyoxylate and Ammonium Binding to HydG

DHG is a proposed intermediate of the HydG-catalyzed reaction that was anticipated to interact with HydG Fe–S clusters. DHG can be produced exogenously in the presence of excess glyoxylate and ammonium chloride. Therefore, the HydGWT enzyme was subjected to a combination of UV-visible CD and absorbance spectroscopy to probe DHG binding to the C-terminal [4Fe-4S] site-differentiated Fe site on HydG (Figures 7.9, 7.10). Addition of glyoxylate (25 mM) to HydGWT yielded discrete changes at the 344, 440, and 550 nm $\lambda_{\text{max}}$ and 383 nm $\lambda_{\text{min}}$ features. A positive change in ellipticity was observed at 344 and 383 nm, while a spectral decay at 466 and 550 nm was observed (Figure 7.9A, red trace). Addition of ammonia to HydGWT in the absence
Figure 7.9. UV-visible CD Spectra of HydG<sup>WT</sup> and HydG<sup>NTM</sup> in the Presence of Glyoxylate and Ammonium Chloride. HydG<sup>WT</sup> (A),(B) and HydG<sup>NTM</sup> (C),(D) in the presence of 25 mM glyoxylate and ammonium, under anaerobic conditions. Samples contain 1 mM DTT. Black traces correspond to HydG<sup>WT</sup> (25 µM; 5.2 ± 0.1 Fe/protein) or HydG<sup>NTM</sup> (25 µM; 6.8 ± 0.3 Fe/protein). (A) and (C) Red trace, 25 mM glyoxylate added; blue trace, 25 mM glyoxylate and 25 mM ammonium chloride added. (B) and (D) Red trace, 25 mM ammonium chloride added; blue trace, 25 mM ammonium chloride and 25 mM glyoxylate added. Samples contain 1 mM DTT and were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4.

Addition of glyoxylate yielded minor perturbations in the features noted above for glyoxylate (Figure 7.9B, red trace). The UV-visible absorbance spectrum was altered in the presence of glyoxylate, with a decrease in the 392 nm feature observed (Figure 7.10A, red trace). Ammonium chloride addition had no effect on the UV-visible spectrum (Figure 7.10B, red trace). Addition of glyoxylate and ammonium together gave comparable CD spectra regardless of addition order (Figure 7.9A,B; blue traces). However, addition of both glyoxylate and ammonium resulted in a measurable perturbation of the absorbance spectrum. A broad drop in absorbance was observed.
Figure 7.10. UV-visible Absorbance Spectra of HydGWT and HydGNTM in the Presence of Glyoxylate and Ammonium Chloride. HydGWT (A),(B) and HydGNTM (C),(D) were prepared in the presence of 25 mM glyoxylate and ammonium, under anaerobic conditions. Samples contain 1 mM DTT. Black traces correspond to HydGWT (25 µM; 5.2 ± 0.1 Fe/protein) or HydGNTM (25 µM; 6.8 ± 0.3 Fe/protein). (A) and (C) Red trace, 25 mM glyoxylate added; blue trace, 25 mM glyoxylate and 25 mM ammonium chloride added. (B) and (D) Red trace, 25 mM ammonium chloride added; blue trace, 25 mM ammonium chloride and 25 mM glyoxylate added. Samples contain 1 mM DTT and were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. The inset for each figure shows a zoomed in view of the baseline absorbance from 700-800 nm.

between 300 and 650 nm, with the largest decay centered near the Fe–S $\lambda_{\text{max}}$ (Figure 7.10A,B; blue traces). The absorbance feature shifted from being a discrete peak at 392 nm to a shoulder at 409 nm.

HydGWT contains two site-differentiated [4Fe-4S] clusters that each could in principle support DHG coordination. The HydGNTM variant has only the C-terminal cluster, however, so assignment of spectral features is simpler. HydGNTM yielded similar trends in UV-visible absorbance to HydGWT; while glyoxylate decreased the Fe–S cluster absorbance, simultaneous addition of glyoxylate and ammonium resulted in a more
Figure 7.11. Difference UV-visible CD Spectroscopy of HydG<sup>WT</sup> and HydG<sup>NTM</sup> with DHG Formation. Difference denote changes in HydG<sup>WT</sup> (black line) and HydG<sup>NTM</sup> (red line) associated with glyoxylate and ammonium addition relative to their absence. Samples contained 25 mM glyoxylate and 25 mM ammonium chloride was subtracted by spectra lacking glyoxylate and ammonium chloride. Spectra were obtained at 23 °C in an anaerobic, 1 cm pathlength CD cuvette in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 with 1 mM DTT. Molar ellipticity was calculated using Equation 7.1.

substantial decrease (Figure 7.10C, red trace; Figure 7.10C, D, blue traces). Addition of glyoxylate to HydG<sup>WT</sup> resulted in a positive change in ellipticity at 344 and 383 nm, while the broad feature between 430–500 nm became more negative than the HydG<sup>WT</sup> sample (Figure 7.9C, red trace). Difference molar ellipticity plots were generated to discriminate cluster-specific interactions with glyoxylate or DHG between HydG<sup>WT</sup> and HydG<sup>NTM</sup> (Figure 7.11). Differences between HydG<sup>NTM</sup> and HydG<sup>WT</sup> included changes in spectral features at 375, 425, and 575 nm observed for HydG<sup>NTM</sup> but was absent for HydG<sup>WT</sup> (Figure 7.11). These differences can be assigned to reflect distinctions in DHG or glyoxylate ligation to individual [4Fe-4S] clusters.

The combined addition of glyoxylate and ammonium (forming exogenous DHG) caused an observable decay in absorbance that was suggestive of changes in Fe–S cluster oxidation state or coordination for HydG<sup>WT</sup> and HydG<sup>NTM</sup> samples (Figure 7.10, blue
Figure 7.12. X-band EPR Spectroscopy of Reconstituted (as-isolated) HydG\textsuperscript{WT} and HydG\textsuperscript{NTM} in the Presence of Glyoxylate and Ammonium Chloride. Red traces correspond to samples prepared in Figure 7.10 that each contain 25 mM glyoxylate and ammonium, while black traces lack glyoxylate and ammonium. (A) HydG\textsuperscript{WT}. (B) HydG\textsuperscript{NTM}.

Samples containing glyoxylate and ammonium from Figures 7.9 and 7.10 were subjected to EPR spectroscopy to evaluate the paramagnetic clusters, and to correlate CD spectral changes with the EPR signal (Figure 7.12). Samples subjected to CD spectroscopy were loaded EPR tubes for spectral analysis in the presence and absence of dithionite, however the addition of dithionite to the dilute HydG (23 μM) used in CD experiments yielded a weak signal not discernible above baseline noise (data not shown). However, samples in the absence of dithionite yielded discernible spectral features in the \(g = 2.00\) and \(g = 4.3\) regions consistent with [3Fe–4S] clusters and high-spin Fe(III) species, respectively (Figure 7.12). Interestingly, the lineshape of the \(g = 2.00\) signal shifted for the HydG\textsuperscript{WT} enzyme relative the absence of glyoxylate and...
ammonium, while for the HydG$^{\text{NTM}}$, no spectral shift was observed. Addition of glyoxylate and ammonium shifted the isotropic signal from 2.00 to 2.01, while a shoulder feature at $g = 2.03$ appeared (Figure 7.12A, red trace), and was similar to the HydG$^{\text{NTM}}$ spectra obtained (Figure 7.12B). It should be noted that the samples prepared in the presence of glyoxylate and ammonium were subjected to an additional freeze–thaw event relative to samples that lacked glyoxylate and ammonium, the $g = 4.3$ contribution was large, comprising a majority of the total signal. However, added glyoxylate and ammonium appeared to perturb the [3Fe–4S] cluster signal for the HydG$^{\text{WT}}$ enzyme in a way that was not observed by the HydG$^{\text{NTM}}$ enzyme (Figure 7.12).

The collective CD, absorbance, and EPR spectral data support the intermediacy of DHG, and support its interaction with the iron-sulfur clusters of HydG. To investigate the possibility that CO and CN$^-$ could be formed from DHG by HydG, glyoxylate and ammonium were combined with HydG and deoxyHb in the absence of SAM to test for

![Image](image.png)

**Figure 7.13.** Effect of Glyoxylate and Ammonium Chloride on HydG in the Presence of deoxyHb. Black trace, HydG, deoxyHb, with 10 mM glyoxylate and 10 mM ammonium chloride; red trace, above mixture after mixing for 20 minutes; blue trace, injection of 5 mM sodium dithionite; magenta trace, incubation of above mixture after 30 minutes.
CO formation (Figure 7.13). A mixture of HydG with methemoglobin (metHb) with glyoxylate and ammonium resulted in a shift in the Soret band $\lambda_{\text{max}}$ from 408 to 430 nm without addition of dithionite (Figure 7.13, black and red traces). Changes in the Soret band were consistent with the conversion of metHb to deoxyHb. These results appeared consistent with a component in the reaction causing the heme to reduce; given the small concentration of DHG expected in the assay mixture, glyoxylate was thought to reduce the heme. 5 mM dithionite was added to reduce HydG to generate reduced Fe–S clusters that might participate in the transformation, however dithionite reduction did not affect the Soret $\lambda_{\text{max}}$ band (Figure 7.13, magenta traces), suggesting no catalytic activity by HydG. Dithionite appeared to be consumed with incubation time; loss of the reduced dithionite-associated absorption at 313 nm upon incubation at ambient temperature for 30 minutes was observed, however no shift in the 430 nm $\lambda_{\text{max}}$ was observed to indicate potential CO binding. Thus it appears that conditions favoring DHG formation did not result in detectable CO formation, possibly requiring additional components lacking in the experimental assay.

Discussion

Effect of Cysteine on HydG Catalysis

HydG produces the diatomic ligands of the H-cluster. HydG catalysis is performed at two [4Fe-4S] clusters, and a $p$-cresolate phenoxyl radical has been observed as a transient species. As HydG functions in vivo in the presence of substrates and products that are part of HydE and HydF activity, its catalytic activity in the presence of molecules identified as relevant to the other maturase proteins is relevant. A
preliminary study investigating HydG activity in the presence of nucleotides is presented in Chapter 6. In this chapter, the putative HydE substrate cysteine is shown to affect HydG catalysis, stimulating tyrosine cleavage and CO formation and causing discrete changes in Fe–S spectral properties. Cysteine-stimulated H₂ production in cell–free lysates containing maturase proteins, as well as preliminary work in our lab, has pointed to the possibility of cysteine serving as a substrate for HydE.

Cysteine affects HydG product formation, stimulating CO, as well as p-cresol and 5'-dAdo, respectively (Figure 7.1). While cysteine cannot substitute for tyrosine in terms of diatomic ligand-forming ability, it likely serves a secondary role in activating the enzyme. Similar behavior has been observed with use of cysteine to reductively active lysine-2,3-aminomutase.³⁹,⁴⁰

CD spectroscopy can be a powerful tool to determine interaction of molecules of interest with an enzyme that binds them. Addition of cysteine has an observable effect, as CD spectral features are enhanced for HydGWT enzyme (Figure 7.2). Slight shifts in wavelength yielded sharper features overall, and dramatic spectral shifts corresponding to changes in Fe–S ellipticity were not observed with its addition. It should be noted that tyrosine did not confer a spectral change (data not shown), so cysteine likewise was not anticipated to confer drastic changes to the signal. However, the observed sharpening of signal likely reflected a potential interaction. Similarly, EPR spectroscopy of HydG in the presence of cysteine resulted in differentiation of the axial values, suggesting that cysteine specifically interacted with a Fe–S cluster (Figure 7.3). However, addition of cysteine did not yield a CD spectral change for the HydGNTM variant, which only contains the C-terminal cluster. This difference may reflect an inability for cysteine to reductively
activate the C-terminal cluster; alternatively the presence of both clusters may be required to affect a change (Figure 7.2). It should be noted that UV-visible absorbance changes are observed for HydG<sup>NTM</sup> with cysteine addition (Appendix Figure F.1) that may indicate an interaction without causing change in ellipticity. The absorbance range that yielded CD spectral differences upon cysteine addition was associated with regions that possess assignable features for both the N-terminal and C-terminal clusters (Appendix Figure F.5). Additional support for this includes sequential addition of cysteine and SAM, as cysteine affected sample ellipticity, regardless of addition order (Figure 7.4). These observations collectively suggest that cysteine serves as an allosteric effector in the HydG reaction.

Unique in the incubation of cysteine in the HydG reaction is that a lag phase in CO formation is observed (Figure 7.1, 7.7). An immediate absorbance increase was observed upon SAM addition, but it stayed constant for thirty to forty-five seconds before an increase (corresponding to CO formation) was observed. This difference appeared to suggest that cysteine either repressed CO product formation or repressed binding to hemoglobin. However, we have shown that stimulated p-cresol and 5’-dAdo is formed in the presence of cysteine after sixty seconds of incubation (Figure 7.5). Considering that cysteine did not affect the HydG<sup>NTM</sup> ellipticity but affected UV-visible absorbance (Appendix Figure F.1), an interaction might be possible without conferring CD spectral change. As noted in the previous paragraph, cysteine’s CD spectral change only with HydG<sup>WT</sup> may indicate cluster-dependent changes in ellipticity may require both clusters. Product analysis clearly shows that tyrosine cleavage is not inhibited by cysteine incubation (Figure 7.5).
The basis for a stimulatory effect of cysteine on tyrosine cleavage without a burst-phase increase in CO formation is unusual, since it suggests that cysteine might be binding or interacting with the product intermediate of substrate cleavage. In D$_2$O, cysteine stimulates formation of 5'-dAdo and SAM with deuterium, but the label distribution is unchanged relative to its absence (Figure 7.6). If cysteine interacted with the 5'-dAdo', a shift in distribution would be observed. While cysteine does not appear to change the C-terminal Fe–S ellipticity, it sharpens and decays the C-terminal cluster 390 nm and 540 nm absorbance features, respectively (Appendix Figure F.1). Under catalytic conditions, it might be possible that the cysteine thiol might be coordinating to the unique Fe of the C-terminal cluster similar to non-protein thiols that have been reported to coordinate to the unique Fe site of [4Fe-4S] clusters such as the radical SAM enzyme LAM. Alternatively, it might be interacting with the p-cresolate phenoxyl

Figure 7.14. Time Scale of Generation of the p-cresolate Phenoxy Radical, the Fe–CO/CN Species, and Free CO by S. oneidensis HydG. The reconstituted enzyme used in these studies was chemically reconstituted in the presence of cysteine, which is similar to the study described in this chapter. Reprinted with permission from Reference 19. Copyright 2014 American Association for the Advancement of Science.
radical following tyrosine Cα–Cβ bond cleavage, yielding the observed lag. It should be noted that the lag phase in CO formation observed here is similar to the lag phase reported for *Shewanella oneidensis* (*So*) HydG (Figure 7.14). *So* HydG forms enzyme-associated vibrational bands immediately upon turnover, but free CO (detected via myoglobin incubation) is detected after approximately 90 seconds, measured via FTIR by the Mb–CO vibrational frequency (Figure 7.14). For these experiments, cysteine was added to *So* HydG as part of chemical reconstitution, and was not removed prior to the assay.

Concomitant with work reported in Chapter 6, the addition of cysteine improved enzyme percent CO efficiency relative to its absence (Figure 7.1). To determine whether an overall CO efficiency relative to substrate concentration, HydG CO formation experiments were performed over long periods to improve the CO detected from catalysis (Figure 7.8). Over longer incubation time, samples that included cysteine resulted in an improvement in CO formation. Relative to the substrate concentration, 40% of the CO expected was detected in the presence of cysteine, an improvement relative from samples lacking cysteine (Figure 7.8). Similar to data presented in Chapter 6, CO formation as detected by hemoglobin or myoglobin detection may not be binding all the CO formed in the experiment. Our work has shown here that incubation of cysteine helps to improve the ratio of CO detected relative to the substrate or product concentration.

**Dehydroglycine as a Reaction Intermediate**

DHG is proposed to be an intermediate following tyrosine Cα–Cβ cleavage. The radical SAM enzyme ThiH 41,42 similarly uses tyrosine to generate DHG as a synthon for
producing the thiazole ligand in thiamin phosphate. Exogenous glyoxylate and ammonium addition has been used to infer involvement of DHG in thiamine biosynthesis, as reconstitution of thiazole phosphate formation in the absence of ThiH (or the aerobic equivalent ThiO) was shown to occur in the presence of glyoxylate, ammonia, sulfide, DXP and enzyme ThiG. Because ThiH and ThiO produce DHG from the substrates tyrosine and glycine respectively, DHG formation (as a product of ThiH or ThiO) from a defined, exogenous addition of glyoxylate and ammonia has been used to identify the intermediate.

The objective for preparing HydG samples in the presence of DHG (via glyoxylate and ammonia) was to determine whether evidence for DHG coordination to an Fe–S cluster could be obtained, and whether HydG-catalyzed CO and CN⁻ formation could be detected starting with this intermediate. In principle exogenous DHG might bind to either site-differentiated Fe–S cluster (similar to CN⁻ from Chapter 5), however binding to the C-terminal cluster would be expected to have more mechanistic relevance. Interestingly, the largest shift in the CD spectrum observed for either both clusters (HydGWT) or for the C-terminal cluster (HydGNTM) followed addition of glyoxylate (Figure 7.9). An insignificant change was observed once ammonium was added along with glyoxylate to make exogenously produced DHG; ammonium itself did not perturb the signal at all. These results suggested that glyoxylate (not DHG) caused the spectral ellipticity changes observed. However, UV-visible absorbance changes conferred by DHG (as glyoxylate and ammonia) formation differed from those with glyoxylate only for either HydGWT or HydGNTM (Figure 7.10). These absorbance changes appear specific to conditions that form DHG that glyoxylate alone was not able to reproduce as an
absorbance feature. Interestingly, the change in CD signal here is qualitatively similar to work performed on ThiH in the presence of glyoxylate and ammonium, where it was shown that exogenous DHG and glyoxylate stimulated decoupled SAM cleavage relative to \( p \)-cresol product.\(^{42}\) The observed CD and absorbance changes observed with exogenous DHG formation by HydG might be correlated to an altered ThiH activity reported, perhaps in perturbing the environment around the Fe–S clusters.

The effect of DHG on HydG was also explored by EPR spectroscopy. Reduction of the dilute CD samples with dithionite yielded very little detectable EPR signal, limiting assessment of the reduced clusters (data not shown). However, a detectable signal at \( g = 2.01 \) was observable, corresponding to \( S = 1/2 \) [3Fe-4S] clusters. Interestingly, a spectral shift was observed for the HydG\(^{WT}\) [3Fe–4S] \( g = 2.00 \) feature to \( g = 2.01 \) upon addition of glyoxylate and ammonium that was not observed for the HydG\(^{NTM}\) variant (Figure 7.12). The samples subjected to glyoxylate and ammonium were underwent an additional freeze-thaw event, which may have caused the increase in the \( g = 4.3 \) signal, and may have degraded the HydG\(^{WT}\) signal. While the mechanistic relevance of coordinated [3Fe-4S] clusters in catalysis is not high, it should be noted that if a Fe–CO/CN complex is formed and released by HydG,\(^{19}\) then a [3Fe-4S] cluster would be generated in the active site. HydG DHG formation, as a product of tyrosine \( C_\alpha–C_\beta \) bond cleavage at the N-terminal cluster, does not require the C-terminal cluster for generation; in absence of an intact C-terminal cluster, the DHG intermediate might interact with a coordinated [3Fe-4S] cluster. Our work has shown here that DHG does not appear to affect the C-terminal [3Fe-4S] cluster signal (Figure 7.12).
If DHG is an intermediate in catalysis, it appears that under the assay conditions reported that it alone is not sufficient to catalyze HydG CO formation. Incubation of excess glyoxylate and ammonium to drive formation of DHG\textsuperscript{35} with HydG resulted in no CO formation, even following dithionite addition to reduce the HydG enzyme (Figure 7.13). Considering the complexity of the radical-initiated reaction catalyzed by HydG, DHG processing to form the diatomic products might require involvement of a generated radical. A surprising observation in these experiments was that the hemoglobin underwent reduction to deoxyHb in the presence of glyoxylate and ammonium. It is unclear what would be reducing the hemoglobin, however the Soret $\lambda_{\text{max}}$ becomes shifted upon incubation with glyoxylate and ammonium from 408 to 430 nm, consistent with reduction of methemoglobin (metHb) to form deoxyHb.\textsuperscript{36} Because glyoxylate is in excess relative to both HydG\textsuperscript{WT} and the exogenous DHG concentration, glyoxylate might be serving as a reducing agent; glyoxylate can undergo oxidation of its hydrated aldehyde group to form oxalate as a product under the assay conditions the experiment was performed in.\textsuperscript{37} Even with dithionite added, no change in the deoxyHb Soret band was observed, which suggests that even under conditions that HydG is reduced, exogenous DHG does not undergo turnover to form CO. Additional work towards understanding the basis of HydG and methemoglobin reduction with DHG or glyoxylate is needed, however these results clearly show that CO is not formed (Figure 7.13).

In conclusion, insight toward the catalytic mechanism of HydG has been facilitated by characterization of small molecules and intermediates that have relevance to the biosynthetic H-cluster mechanism. In the case of cysteine, a stimulatory effect on HydG catalysis may simply reflect reductive activation akin to that observed for lysine-
2,3-aminomutase.\textsuperscript{39,40} Given the presumably complex radical initiation and propagation mechanism of HydG catalysis and the unprecedented chemistry catalyzed by radical SAM enzymes, perhaps cysteine radical interaction (such as from the $p$-cresolate phenoxy radical) helps direct nascent product radicals away from the active site, stimulating catalytic turnover. Additional studies extending the role of small molecules may likewise have a beneficial effect in improving CO detection efficiency. By comparison, DHG confers discrete spectral changes to the UV-visible spectrum that may reflect binding, however the excess glyoxylate may be affecting the results interpreted to occur with DHG as reflected in CD spectral changes in ellipticity. Under reducing conditions, DHG alone cannot catalyze CO formation, which may simply reflect an additional requirement for a radical to activate the DHG intermediate. Future spectral characterization with respective clusters and associated radicals in catalysis (see Chapter 5) should provide additional insight toward the mechanistic events following tyrosine $C_\alpha$–$C_\beta$ bond cleavage.

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CHAPTER 8

DELINEATING H ATOM ABSTRACTION IN HYDG CATALYSIS WITH TYROSINE ANALOGUES AND SITE-DIRECTED MUTAGENESIS

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Abstract

Enzymes of the radical S-adenosylmethionine (SAM) superfamily catalyze radical-initiated catalysis through direct H atom abstraction of substrate. The [FeFe]-hydrogenase maturase enzyme HydG is a radical SAM enzyme that catalyzes the synthesis of carbon monoxide (CO) and cyanide (CN⁻) diatomic ligands through H atom abstraction of Tyr at the 4-phenolic position. H atom abstraction by HydG occurs through apparent abstraction-reabstraction events that regenerates SAM, however it is consumed as a cosubstrate in the reaction. Mechanistic details regarding the role of the 5’-deoxyadenosyl radical (5’-dAdo•) in the events following initial H atom abstraction are unclear with respect to the apparent radical regeneration event. Abstraction at a solvent-exchangeable position is dependent on the acidity of the abstraction position, the
substrate oxidation potential and other participating factors that affect abstraction and
catalytic events. To investigate this further, substrate analogs were used to determine an
effect of oxidation potential and \( pK_a \) on abstraction. Also, site-directed mutagenesis was
performed on HydG, and differences in product formation, spectral characterization, and
deuterium isotope distribution were observed relative to the wild-type enzyme. The
collective data supports a radical-initiated model for substrate activation and C–C bond
cleavage events that are dependent on substrate radical stability and that also requires
participation of active site bases near the C-terminal [4Fe-4S] cluster. The implications
of these results with respect to reversible abstraction-reabstraction events on the catalytic
mechanism and involvement of the C-terminal cluster are discussed.

Introduction

Biosynthesis of the five diatomic ligands that become coordinated to the 2Fe
subcluster of the [FeFe]-hydrogenase H-cluster requires the involvement of the radical
SAM enzyme HydG.\(^1,2\) HydG utilizes tyrosine as a substrate, and through a radical-
initiated decomposition mechanism the products CO, CN\(^-\), \( p \)-cresol, 5’-deoxyadenosine,
and methionine are formed.\(^3-6\) HydG catalyzes a complex diatomic ligand formation
from the substrate tyrosine with two [4Fe-4S] clusters, and substrate radical initiation
results in cleavage of two C–C bonds and controlled oxidation of the dehydroglycine
(DHG) intermediate forms the diatomic products.\(^7\)

HydG belongs to the subclass of the radical SAM enzyme superfamily utilizing
SAM as a cosubstrate; the 5’-dAdo\(^*\) is consumed stoichiometrically relative to substrate.\(^8\)
However, we have demonstrated that solvent-derived deuterium label is incorporated into
both 5’-dAdo and SAM, the latter would only occur if the 5’-dAdo• was regenerated following initial H atom abstraction. Two mechanisms rationalizing the apparent abstraction–reabstraction events, either as i) preceding or ii) following tyrosine Cα–Cβ bond cleavage. For the first mechanism, the substrate tyrosine radical would be in equilibrium with the 5’-dAdo•. An energetic problem with this scenario, however, is that the abstraction of a hydrogen atom from the 5’-methyl of dAdoH (BDE(C–H) = 100 kcal/mol) by a tyrosyl radical to form Tyr (BDE(O–H) = 86 kcal/mol) is thermodynamically uphill, although the uphill process could be coupled to the downhill process of re-forming SAM. For the second mechanism, a product radical generated after tyrosine Cα–Cβ bond cleavage would abstract a hydrogen atom from 5’-dAdo, regenerating 5’-dAdo• for subsequent recombination with methionine to reform SAM. The recent observation of the p-cresolate phenoxyl radical following H atom abstraction and tyrosine Cα–Cβ bond cleavage may implicate additional involvement of radicals in the steps that process the DHG intermediate. Like what was shown in Chapter 7, DHG alone does not appear to catalyze CO formation. In turn, a detailed mechanism regarding the processing of DHG at the HydG C-terminal cluster remains largely speculative.

HydG undergoes H atom abstraction from a solvent-exchangeable position that appears to be pH-dependent. A large body of data has shown that turnover is pH-dependent, and that elevating the pH from 7.4 generally increases product formation; pH 8.5 has trended to improve general catalysis (Chapter 6). Considering that the tyrosine 4-phenolic hydrogen and sidechain ammonium groups have pK values of 9.03 and 10.08, the pH-dependent differences were not due to substrate deprotonation; alternative
mechanisms yielding the distribution differences were occurring. While HydG remains to be crystallographically characterized, the generated 5’-dAdo• might be interacting with solvent-exchangeable residues within the TIM barrel. However, tyrosine undergoes electron transfer within proteins,\textsuperscript{11,14} so reversible electron transfer might be expected before a productive \(C_\alpha-C_\beta\) bond cleavage event as a substrate.

Herein, we provide insight toward the apparent reversibility in H atom abstraction mechanism. By comparing the pD-dependent abstraction pattern with tyrosine substrate, by comparing abstraction with substrate analogs, and by using sequence homology modeling of the \textit{C. acetobutylicum} HydG sequence to identify a series of active site residues that likely interact with the 5’-dAdo• with solvent-exchange ability, catalysis within the HydG TIM barrel is more clearly defined in the complex reaction catalyzed by HydG. A mutagenesis study regarding the functional role for three of these residues has been performed on the substitution variants HydG\textsuperscript{H212A}, HydG\textsuperscript{H272A}, and HydG\textsuperscript{R136A}, each have been characterized spectroscopically and have been assayed in their CO forming and H atom abstracting ability. These results collectively provide a model by which initial substrate H atom abstraction is facilitated at the radical SAM [4Fe-4S] cluster, and help define the C-terminal amino acid residues that participate in the processing of the DHG intermediate within the radical SAM TIM barrel.

\textbf{Experimental Section}

\textbf{Materials}

All chemicals and other materials used herein were from commercial sources and of the highest purity available. Tyrosine, \textit{p}-cresol, ferric ammonium citrate, and 5’-dAdo
were obtained from Sigma-Aldrich (St. Louis, MO). Triton-X100, l-DOPA, 4-methylcatechol, and imidazole were obtained from Alfa Aesar (Ward Hill, MA). Tris, HEPES, IPTG, PMSF, tryptone, yeast extract, MOPS, DTT, and streptomycin were obtained from RPI (Mt. Prospect, IL). MgCl₂, KCl, acetonitrile (HPLC grade) and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite, sodium sulfide, sodium fumarate and D₂O was obtained from Acros Organics (Fair Lawn, NJ). DNase I, RNase A, and lysozyme (hen egg) were obtained from Roche (Indianapolis, IN). 3-amino-l-tyrosine was obtained from MP Biomedicals (Solon, OH). Iron(III) chloride, ammonium chloride, acetic acid (HPLC grade) and cysteine was obtained from Fisher Scientific (Fair Lawn, NJ). H64L myoglobin and hemoglobin were received as kind gifts from Professor John S. Olson (Rice University) and Professor David J. Singel (Montana State University), respectively. SAM¹⁵ and 5-deazaflavin¹⁶-¹⁹ was synthesized and purified as reported elsewhere, with slight modifications.

Sequence Homology Modeling

The amino acid sequence encoded from the hydG_WT gene from Clostridium acetobutylicum (NP_347984.1) was submitted to the Protein Homology/analogy Recognition Engine v 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), or to the structure homology-modeling server, via the ExPASy web server (http://swissmodel.expasy.org).

Mutant Enzyme Overexpression, Purification, and Reconstitution

Mutant genes hydG_R136A, hydG_H212A, and hydG_H272A were prepared
using a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) using the *C. acetobutylicum* hydG<sub>WT</sub> gene encoded on the pCDFDuet-1 plasmid as a template. Incorporation of the mutations and the absence of secondary mutations were confirmed by sequencing (Idaho State University Molecular Research Core Facility, Pocatello, ID). Mutant constructs encoding *C. acetobutylicum* HydG were transformed into *E. coli* BL21(DE3) (Stratagene) cells for protein expression, as described previously with slight modifications. Briefly, care was taken to ensure overexpression through small-scale 5 mL pilot growths performed in LB (Miller) broth, and selected colonies were plated onto LB agar and were inoculated into 5 mL of LB media. Pre-induction and post-induction samples were obtained, and overexpression was determined via SDS-PAGE. Colonies that showed overexpression were used for large-scale enzyme preparation. Mutant and wild-type HydG was prepared using the modified procedure described below. Single colonies of interest were grown overnight in LB media and were utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L glucose (dry) and 50 mM MOPS buffer pH 7.40, in addition to 500 mg/L of ferric ammonium citrate (dry) and 1 mL/L of streptomycin (50 mg/mL; sterile filtered). The cultures were grown at 25 °C and 225 rpm shaking until an OD<sub>600</sub> = 0.5 was reached at which point 1.6 g/L of sodium fumarate (dry) and 242 mg/L of l-cysteine (dry) was added. Cultures were sparged with N<sub>2</sub> for 15 minutes at ambient temperature before 500 μL/L of 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each flask. The cultures were then sparged overnight (16 hours) with N<sub>2</sub> at ambient temperature. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.
Protein purification was performed according to previously published procedures (Chapter 2, 3, 4), with slight modifications. Briefly, Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber (Grass Lake, MI). Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 with 20 mM MgCl₂, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, and ~ 0.6 mg lysozyme per gram cell. The lysis mixture was stirred for one hour with a magnetic stir bar, and was made homogeneous by pipetting through a syringe. The mocha colored lysate was centrifuged in gas tight bottles (Nalgene; Rochester, NY) at 18,000 rpm for 30 minutes. The resulting coffee-brown supernatant was loaded onto a 5 mL HisTrap™ Ni²⁺-affinity column (GE Healthcare; Piscataway, NJ) that was pre-equilibrated with 50 mM HEPES 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Fractions of interest were pooled, and were concentrated via ultracentrifugation units at 6000 rpm (Millipore; Billerica, MA) to 50-100 mg/mL using an anaerobic clinical centrifuge. Concentrated fractions were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4; protein purity was gauged by SDS-PAGE prior to dialysis. Protein was flash frozen in liquid N₂ and stored at -80 °C until further use. Typical yields averaged 15 mg HydG per L of growth media.

Chemical reconstitution of as-dialyzed HydG was performed as described elsewhere with slight modifications in an anaerobic Coy chamber at 4 °C. Initial UV-
visible absorbance and CD spectral data was obtained on pre-reconstituted enzyme (109 µM) following dilution into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. Spectroscopic methods for each of these instruments can be found below. The HydG enzyme was then incubated in the presence of 6.5-fold excess of FeCl3 and Na2S in the presence of 5 mM dithiothreitol (DTT) with gentle stirring using a flea bar stir magnet (VWR; Radnor, PA). Following a 5 minute incubation with DTT, FeCl3 was added dropwise over the course of 20 minutes, and was then incubated for 20 minutes before dropwise addition of Na2S was performed over the course of 20 minutes. Samples were incubated with gentle stirring for an additional 2.5 hours under the conditions stated above. Following the chemical reconstitution, the mixture was centrifuged 3 x 10 min using a benchtop minicentrifuge until no precipitated Fe–S pellet could be observed. The supernatant was concentrated to approximately 1 mL via ultracentrifugation units at 6000 rpm with an anaerobic clinical centrifuge, and was loaded over a PD-10 column loaded with Sephadex G-25 resin (GE Healthcare; Piscataway, NJ) that was pre-equilibrated with the above buffer after rinsing with filtered H2O sparged with N2. The darkest fractions were combined, and a final UV-visible absorbance and CD spectrum was obtained on the pooled fraction that was subjected to dilution with the above buffer. The pooled fractions were concentrated a final time, to yield 100 mg/mL enzyme that was subsequently aliquotted to minimize freeze–thaw associated enzyme denaturation. Enzyme concentration was estimated through the spectrophotometric Bradford method, while iron content of the as-purified, reconstituted, and samples subjected to CD were examined by the spectrophotometric method developed by Fish, which uses ferrozine under reductive conditions after protein digestion with 4.5% (w/v) KMnO4 and 1.2 M
CO Activity Experiments

HydG CO formation was monitored via incubation with deoxyhemoglobin (deoxyHb) under strictly anaerobic conditions at 37 °C in mixtures that contained HydG (40 µM), sodium dithionite (5 mM), tyrosine or analogue (1 mM), deoxyhemoglobin (deoxyHb) (80 µM) and SAM (1 mM) in 50 mM tris, pH 8.5. Sample preparation was performed in an anaerobic glove box (MBraun; Stratham, NH); the buffer was degassed on a schlenk line vacuum manifold by subjecting buffer to iterative vacuum (3 x 10 min), followed by N₂ backfill cycles. The volume of evaporated water was replaced with degassed dd H₂O to original volume to ensure a comparable buffer concentration. DeoxyHb was oxidized with potassium ferricyanide and was desalted over a GE Healthcare PD-10 desalting column with sephadex G-25 prior to use. Sodium dithionite was prepared fresh in the degassed buffer, while the tyrosine stock was prepared aerobically by dissolving tyrosine solid in 1 M HCl, and then was diluted with 100 mM tris, pH 8.5 buffer to make a 67.7 mM concentrated stock that was degassed on the schlenk line manifold (3.59:1 buffer:HCl).

Samples containing the above components aside from SAM were centrifuged at 14,000 rpm to pellet unwanted precipitates. Meanwhile, SAM was loaded into a 25 µL gastight syringe (Hamilton; Reno, NV), and was capped by insertion in a rubber stopper. The prepared solution was loaded by 500 µL gastight syringe into a 1 mm pathlength UV-visible cuvette (Spectrocell; Oreland, PA) and was capped with a screwcap with a penetratable teflon insert (Thermo Fisher; Bellefonte, PA). Experiments were performed
on a Cary 6000i dual pathlength spectrophotometer (Agilent; Santa Clara, CA) that was pre-blanked with the cuvettes used with aerobic reaction buffer. After an initial scan (300–800 nm) was performed, SAM was injected into the cuvette, and the sample was then inverted a few times, and the penetrated hole was filled in with vacuum grease. After about 12 seconds, $A_{419\text{nm}}$ was monitored every second for 30 minutes at 37 °C. The $\Delta A_{419\text{nm}}$ and $\Delta \varepsilon_{419\text{nm}}$ were used to calculate the concentration of Hb-CO at each time point to determine the rate of CO formation. Following the incubation period, a final scan was performed to confirm the extent of CO binding, and following this a sample aliquot was saved for HPLC product quantitation.

UV-visible Absorbance/Circular Dichroism Experiments

Visible CD experiments were performed in duplicate under anaerobic conditions using a Jasco J-710 spectropolarimeter (Easton, MD) at room temperature. Samples were prepared in an anaerobic Coy chamber in a cold room (4 °C), and a Hellma 1.0 cm pathlength screwcap anaerobic cuvette (Plainview, NY) was used for all CD experiments. Buffers used for CD experiments were sparged with N₂ gas for at least 1 hour before use. UV-visible absorbance spectra were performed in series, using a Starna 1.0 mL screwcap anaerobic cuvette (Starna Cells; Atascadero, CA) on an Agilent Cary 60 single pathlength spectrophotometer. Typical CD instrument parameters included a sensitivity of 100 millidegrees, a scan speed of 100 nm/min, a data pitch of 0.1 nm, a bandwidth of 1.0 nm, a response time of 4 seconds, and an accumulation of 3 scans across the 300–800 nm spectral window. Samples were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 buffer, which also served as the buffer blank for UV-visible absorbance scans,
across the 200–1000 nm spectral range. Samples contained HydG enzyme concentrations 24-46 µM that were confirmed by the Bradford assay, while sample iron concentrations were assessed using the method developed by Fish. Samples also contained 1 mM dithiothreitol, which was prepared fresh when performing CD experiments. Optimal spectral data was obtained on enzyme had underwent minimal freeze-thaw cycles; immediately following chemical reconstitution of the enzyme with iron and sulfide would CD experiments be typically performed, following enzyme small molecule desalting on a sephadex G-25 (PD-10) column, and the darkest fractions were collected. The sample typically underwent a 1:1 dilution before it was loaded into either cuvette for spectral analysis. Samples that were expressed in molar ellipticity were calculated with the Equation 8.1:

\[ [\theta] = \frac{\theta}{10c l} \]  

(8.1)

H atom Abstraction Experiments and LC-MS Methods

H atom abstraction experiments were performed in D₂O buffer, under conditions described in the main text. Experiments were performed in an anaerobic chamber (Mbraun) under strict anaerobic conditions (<1 ppm O₂). D₂O buffers were prepared by lyophilization of H₂O buffer on the schlenk line for 16 hours. The resultant salt was brought into the M Braun box and was dissolved with degassed D₂O to original volume. Prior to suspending the salt in D₂O, the schlenk flask was briefly subjected to a heat gun to drive off remaining H₂O moisture. Upon resuspending the buffer salt in D₂O, the pD was measured with pH paper (Micro Essentials; Brooklyn, NY) and was as expected
acidic by 0.4 units. Working 2 mM Tyr or analogue stocks in D$_2$O were prepared in a similar fashion to buffers described above, via dilution from an aerobic stock in H$_2$O (prepared by dissolving Tyr in 1 M HCl, then dilution with 100 mM tris, pH 7.4), and was lyophilized overnight. DOPA was prepared similar to Tyr, while NH$_2$–Y was dissolved in 100 mM tris, pH 7.4 without HCl addition.

Experiments were performed at 37 °C in an IsoTemp heatblock (Fisher), containing 100 µM HydG (8.5 ± 0.2 Fe/protein), 1 mM AdoMet (enzymatically synthesized), 1 mM Tyr, and 5 mM dithionite (80 µL volume) for 60 minutes. Following this incubation, the enzyme was precipitated via 1:1 volume addition of acetonitrile (HPLC grade, EMD) or addition of 1 M HCl (13 % v/v). A concentrated HydG stock at ~2 mM was used to minimize the H$_2$O contribution for performed experiments. Reported assay pDs were corrected for changes related to temperature. HPLC and LC–MS methods used for quantitation of 5’-dAdo, p-cresol, and SAM follow methods described in Chapter 4, 6, and 7, with the only difference that LC–MS isotope distributions for some samples were assessed on a Bruker micrOTOF (Billerica, MA) coupled to a Agilent 1100 HPLC with an identical LC protocol as described for the Agilent 6538 Q-TOF instrument. However, isotope distributions between the Agilent 6538 Q-TOF and the Bruker micrOTOF instruments were found to be identical.

EPR Spectroscopy

EPR samples were prepared as described previously with slight modifications. Briefly, the HydG enzyme was supplemented with 50 mM tris (pH 7.4), 100 µM 5-deazariboflavin, and 5 mM DTT in buffer (50 mM HEPES, 500 mM KCl, 5% glycerol,
pH 7.4), was placed in a ice-water bath in the MBraun box in an X-band EPR tube. Following illumination with a 300 W Xe lamp for 1 hour, SAM (5 mM) or cyanide (10 mM) was added in the absence of light, and in three minutes, the X-band EPR tube was frozen in liquid N$_2$. Mutant turnover samples were prepared containing 100 $\mu$M HydG with dithionite (3 mM), SAM (3 mM) and tyrosine (3 mM). Samples were prepared by combining the above buffer with enzyme and tyrosine; after centrifugation for 5 min at 14,000 rpm, sample was loaded into an X-band EPR tube. SAM and dithionite were combined, and combined SAM/dithionite mixture was added by glass pipet and was immediately mixed before freezing. Approximately 20 seconds of handling were required before flash freezing in liquid N$_2$ by hand was possible. Low-temperature EPR spectra were recorded using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller (Oxford Instruments; Abingdon, U.K.). Typical EPR parameters included a sample temperature of 12 K, a microwave frequency of 9.37 GHz, a microwave power of 1.59 mW, and a time constant of 20.48 ms. Experimental spectra were baseline corrected and plotted using OriginPro (version 9.1.0; OriginLab Corp., Northampton, MA).

Results

pD-Dependent H Atom Abstraction Events

HydG tyrosine lyase activity is initiated via reversible, SAM-derived 5’-dAdo• H atom abstraction from the phenolic O–H position. The multiple deuterium labels in SAM and 5’-dAdo appeared to suggest that the phenolic O–H position undergoes an equilibrium exchange between 5’-dAdo• and the Tyr• with 5’-dAdo, respectively. To
understand this mechanism, experiments were performed at acidic and alkaline pHs 6.8 and 7.8 in 50 mM HEPES buffer at 37 °C for 1 hour (Figure 8.1). Quantitation of 5’-dAdo and SAM were shown to contain an observable isotope shift similar to our initial report.9 Significantly more deuterium was detected in SAM and 5’-dAdo at pH 7.8 than at 6.8 (Figure 8.1A,B). The labeled species were normalized to compare the equilibrium and the constitution of labeled species (Figure 8.1). The pH increase not only increased general catalysis, labeled 5’-dAdo comprised a greater percentage of label relative to SAM (Figure 8.1A,C). A shift towards more extensive deuterium label was observed with the increase in pH, and a greater percentage of bis- and tris-labeled 5’-dAdo was observed for experiments performed at pH 7.8 (Figure 8.1).
Tyrosine Analogues and H Atom Abstraction, CO Formation

The amino acid tyrosine is a redox–active substrate that undergoes proton–coupled electron transfer (PCET).\textsuperscript{14} H atom abstraction can be defined as a PCET event, and one-electron oxidation of the 5'-dAdo• coupled to loss of a hydrogen atom occurs, yielding the Tyr•. Reversible abstraction-reabstraction events that regenerate SAM can be described as reversible electron transfer events that are modulated by adjusting the substrate oxidation potential and $pK$ values. Two model tyrosine analogues were chosen to compare H atom abstraction, 3,4-dihydroxy-L-phenylalanine (DOPA) and 3-amino-L-tyrosine (NH$_2$–Y) (Figure 8.2). They have been used elsewhere to understand electron transfer in class Ia ribonucleotide reductases as thermodynamic radical traps to control

![Figure 8.2. Tyrosine and Tyrosine Analogues Used for Experimental Study.](image)

Table 8.1. Oxidation Potentials and $pK$ Values for Tyrosine and Respective Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E^{\circ\prime}$ (V)</th>
<th>Macroconstant</th>
<th>Microconstant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$pK_1$, $pK_2$</td>
<td>$p_{k1}$, $p_{k2}$, $p_{k12}$, $p_{k21}$</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.83$^a$</td>
<td>9.04, 10.08$^d$</td>
<td>9.63, 9.28, 9.69, 10.04</td>
</tr>
<tr>
<td>NH$_2$–Y</td>
<td>0.64$^b$</td>
<td>9.09, 10.91$^e$</td>
<td>9.49, 9.31, 9.79, 9.97</td>
</tr>
<tr>
<td>DOPA</td>
<td>0.57$^c$</td>
<td>8.80, 9.83$^f$</td>
<td>8.93, 9.19, 9.63, 9.43</td>
</tr>
</tbody>
</table>

electron transfer modes between the α2 and β2 subunits.\textsuperscript{25-28} The added meta-position substituent modulates tyrosine’s reduction potential while leaving the associated acid dissociation constants unchanged (Table 8.1, Figure 8.2).\textsuperscript{13,27-33} As applied to HydG catalysis, substitution of tyrosine for DOPA or NH$_2$–Y was proposed to affect the initial substrate H atom abstraction event, since ease of substrate oxidation might stabilize the radical from reverse reabstraction.

To assess productive abstractions with the tyrosine analogues, CO formation was followed under turnover conditions (Figure 8.3). Both analogues were productive in their formation of CO (Figure 8.3, red and magenta traces). The DOPA analogue yielded roughly 70% (37 µM) (Figure 8.3, red trace) of the CO formed by use of tyrosine as a substrate (52 µM) (Chapter 6, Figure 6.3, black trace) under identical conditions. CO formation was fitted to an exponential function with a $k_{cat}$ of (13.2 ± 0.02 x 10$^{-4}$ s$^{-1}$) that
lacked an initial burst phase observed by the native substrate (Chapter 6, Table 6.2). The apparent rate of CO formation with the DOPA analogue was comparable to that observed with the tyrosine substrate performed at comparable pH (Figure 6.3, Table 6.2). By comparison, the NH₂–analogue produced approximately 30% (15 μM) of the CO detected from using tyrosine (Figure 8.3, magenta trace). Interestingly, the tyrosine analogues were more sensitive with CO formation at acidic pH than tyrosine was, as 20% or less of CO detected at pH 8.5 was detected at pH 7.4 for DOPA (Figure 8.3, black and red traces) and NH₂–Y (Figure 8.3, blue and magenta traces) analogues relative to tyrosine (Chapter 6, Figure 6.3, black and red traces). The natural substrate produced substantial CO at pH 7.4, approximately 60% of the CO detected at pH 8.5. These pH-

![Figure 8.4. HydG WT Product 5'-dAdo and SAM Isotope Distributions with Tyrosine Analogues.](image)

Samples contained 100 μM HydG WT (8.5 ± 0.1 Fe/protein), 1 mM tyrosine (analogue), 1 mM SAM, and 5 mM dithionite, incubated at 37 °C for 60 min. Samples containing tyrosine substrate were performed in 50 mM HEPES, pD 7.8 buffer, samples containing DOPA substrate were performed in 50 mM tris, pD 8.1 buffer (95%), while samples containing 3-amino-tyrosine (NH₂–Y) substrate were performed in 50 mM HEPES, pD 7.8 buffer (95%). Spectra are depicted as normalized, extracted ion chromatograms.
Figure 8.5. Product Quantitation of Tyrosine Analogue Formation of (A) 5′-dAdo, (B) SAM, and (C) $p$-cresol. Error bars represent standard deviation following triplicate sample injections on the HPLC, while the isotope distributions in Panels A and B were measured from the sample using LC-MS from Figure 8.4. The $p$-cresol product from the NH$_2$–Y could not be detected by the HPLC method used.

dependent differences in CO formation with the substrate analogues suggested a perturbing role of the substituent, since either can undergo hydrogen bonding at the substituent position that is absent for tyrosine.

H atom abstraction with the HydG$^{WT}$ enzyme was performed with the tyrosine analogues to evaluate their H atom abstraction ability (Figure 8.4). Under comparable conditions to those reported previously (Chapter 4), the substrate analogues conferred a shift in isotope distribution for 5′-dAdo and SAM relative to tyrosine (Figure 8.4). Both analogues shifted the distribution towards a greater percentage of unlabeled 5′-dAdo and SAM following HPLC quantitation (Figure 8.4, Figure 8.5A, B). The DOPA sample was prepared at a more alkaline pD relative to the other samples, but a similar 5′-dAdo and SAM distribution to NH$_2$–Y was observed (Figure 8.5A, B). Less 5′-dAdo and 4-
methylcatechol (the DOPA p-cresol species) products were detected relative to tyrosine substrate (Figure 8.5A, C). Interestingly, all samples produced comparable unlabeled 5’-dAdo, suggesting that baseline, non-relevant SAM cleavage was independent of the substrate (Figure 8.5A). Remarkably, the DOPA and NH₂–Y 5’-dAdo distributions shifted from a mixture of mono-, bis-, and tris-deuterated products to principally monodeuterated; similar trends were also observed for SAM.

The shift in distribution of product with deuterium with the substrate analogues suggested modulated abstraction reversibility. Perturbations to the substrate oxidation potential were anticipated to alter radical stability for reabstraction. In turn, the

![Scheme 8.1](image)

Scheme 8.1. Illustrative Example to Predict a 5’-dAdo Isotope Distribution from an Experimentally Determined SAM Isotope Distribution. Positions on SAM and 5’-dAdo that undergo abstraction–reabstraction are bolded in blue.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Measured 5’-dAdo Distribution</th>
<th>Predicted 5’-dAdo Distribution (SAM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>Measured 5’-dAdo Distribution</td>
<td>Predicted 5’-dAdo Distribution (SAM)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>22.5 34.7 23.7 19.1</td>
<td>32.7 44.9 17.3 5.1</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>37.6 50.3 10.6 1.5</td>
<td>37.0 49.1 13.0 0.9</td>
</tr>
<tr>
<td>NH₂–Y</td>
<td>48.2 46.9 4.9 0.0</td>
<td>45.7 49.8 4.3 0.2</td>
</tr>
</tbody>
</table>
experimentally determined SAM deuterium isotope distributions from Figure 8.4 were used to predict a 5’-dAdo distribution, since 5’-dAdo originates from SAM. The method used for predicting the distribution from the observed SAM distribution is shown in Scheme 8.1. The experimentally detected 5’-dAdo distribution was larger than what was predicted from the SAM distribution for tyrosine substrate (Table 8.2). Interestingly, either analogue yielded a predicted distribution more similar to the experimentally determined 5’-dAdo distribution than for tyrosine, reflecting a more reversible abstraction between product and cosubstrate.

The labeled SAM and 5’-dAdo distributions from Figure 8.5 were normalized to compare the SAM ⇌ 5’-dAdo abstraction equilibrium (Figure 8.6). The natural substrate had the largest percentage of labeled 5’-dAdo (at nearly 50%) relative to NH₂–Y (40%) and DOPA (25%). While tyrosine and NH₂–Y have similar macroscopic and

Figure 8.6. Normalized Sample Distribution of Labeled 5’-dAdo and SAM for Tyrosine Analogues. Excludes unlabeled SAM and 5’-dAdo. Error bars represent standard deviation following triplicate sample injections on the HPLC.
microscopic $pK$ values that favor initial deprotonation of the sidechain ammonium group,\textsuperscript{13,31} DOPA (by the addition of the $m$-hydroxyl group) has altered values that favor initial deprotonation of the $p$-hydroxyl group at the pD assayed,\textsuperscript{29} but a similar distribution of DOPA was observed at acidic pD (Appendix Figure G.1). Thus these distribution shifts (Figure 8.6) appeared to be directly related to the substrate oxidation potential (Table 8.1).

The effect of oxidation potential on apparent product formation and abstraction suggested the possibility that a substrate radical would be stable. EPR spectroscopy was performed with DOPA and NH$_2$–Y under single turnover conditions to assess organic

![Figure 8.7. X-band (9.37 GHz, 12 K) EPR Spectroscopy of Photoreduced HydG$^{WT}$ in the Presence of Tyrosine Analogues and SAM. Black trace, photoreduced HydG$^{WT}$ (100 $\mu$M; 7.0 ± 0.1 Fe/protein) plus 1 mM NH$_2$–Y; red trace, photoreduced HydG$^{WT}$ (100 $\mu$M; 7.0 ± 0.1 Fe/protein) plus 1 mM NH$_2$–Y and 1 mM SAM; blue trace, photoreduced HydG$^{WT}$ (96 $\mu$M; 8.5 ± 0.1 Fe/protein) plus 1 mM DOPA and 1 mM SAM. Samples containing SAM were frozen within 30 seconds of addition. Samples contain 5 mM DTT, 100 $\mu$M 5-deazariboflavin, and 50 mM tris in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 buffer. EPR spectral acquisition parameters can be found in the experimental section.](image)
substrate radical formation ability (Figure 8.7). Photoreduced samples prepared in the presence of DOPA, NH$_2$–Y and SAM (Figure 8.7, red and blue traces) after 30 seconds yielded a perturbed signal similar to the native substrate (Chapter 5, Figure 5.4). An observable increase in integrated spin was observed for the turnover sample containing NH$_2$–Y with the addition SAM relative to its absence, from 0.41 to 0.63 spins per protein (Figure 8.7, black and red traces). The spectral changes observed with the analogues largely reflected SAM binding, not reflecting catalytic turnover (Figure 8.7).

Sequence Homology Results

While HydG lacks crystallographic characterization, its sequence predicts a complete ($\alpha\beta$)$_8$ triose isomerase phosphate (TIM) barrel$^{3,34}$ observed in structurally characterized enzymes such as BioB,$^{35}$ PylB,$^{36}$ and HydE.$^{37,38}$ Alignment of the predicted homology structure of the HydG sequence using the Phyre sequence homology

![Figure 8.8. HydG Phyre2 Sequence Homology Model. C. acetobutylicum HydG (residues 36-394) (NCBI reference NP_347984.1) used as a template, and was aligned to the radical SAM PylB structure (PDB:3T7V).](image-url)
Figure 8.9. Putative Involvement of Active Site Residues of interest near HydG Fe–S Clusters. (A) N-terminal radical SAM [4Fe-4S] cluster; (B) C-terminal [4Fe-4S] cluster. Phyre2 sequence homology model of *C. acetobutylicum* HydG is aligned to the aligned to the radical SAM PylB structure (PDB:3T7V).

environment (Clostridium acetobutylicum) with PylB (Methanosarcina barkeri) and HydE (Thermotoga maritima) showed excellent similarity in secondary structural elements up to HydG amino acid residue 394 that comprised a majority of the catalytic structure for the enzyme (Figure 8.8). HydG possesses structural motifs designed to bind and coordinate SAM reflected in other structurally characterized superfamily members. HydG’s C-terminal [4Fe-4S] cluster tricysteine sequence was difficult to identify as accurate since the C-terminal domain is unique to HydG. A short α-helix capping the end of the TIM barrel is similarly positioned in PylB structure, yet distinct from the ~90 residues of HydG that make up the C-terminal domain. The sequence homology model up to residue 394 included two of the three Cys residues comprising the C-terminal cluster-binding motif for tentative placement (Figure 8.8, 8.9). These residues were modeled on the bottom part of the TIM barrel capping the overall structure (Figure 8.8). Significant uncertainty in the placement of the C-terminal [4Fe-4S] cluster follows a lack of structural characterization of the C-terminal domain, catalytic events facilitated by the
domain would however be protected from interaction with bulk solvent, since the DHG intermediate readily undergoes hydrolysis (Figure 8.9). 41

**Site-Directed Mutagenesis**

Three *C. acetobutylicum* HydG site-directed mutants (HydG$^{H212A}$, HydG$^{H272A}$, and HydG$^{R136A}$) were selected to probe the interaction of active site residues near the N- and C-terminal [4Fe-4S] clusters as relevant to abstraction (Figure 8.9). The residues of interest (His212, His272, and Arg136) are conserved in HydG sequences, 3 though their functional roles in catalysis are unclear, aside from playing a supporting role in stabilizing substrates or intermediates at respective Fe–S clusters. Importantly, each residue has a potential ability to hydrogen bond with the substrate or intermediates at the pDs that abstraction distribution differences have been observed. As is shown in Scheme 8.2, the His212 substitution is in proximity of the N-terminal radical SAM [4Fe-4S] cluster, while the His272 and Arg136 are in proximity of the C-terminal [4Fe-4S] cluster. The HydG variants were overexpressed under conditions modified from our previous

![Scheme 8.2. Active Site Residues of Interest in Proximity to Respective HydG Fe–S Clusters.](image-url)
Table 8.3. HydG Variants used in Study, and Respective Iron Number

<table>
<thead>
<tr>
<th>HydG Variant</th>
<th>Fe # (Fe/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HydG&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;H212A&lt;/sup&gt;</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;R136A&lt;/sup&gt;</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>HydG&lt;sup&gt;H272A&lt;/sup&gt;</td>
<td>5.4 ± 0.1</td>
</tr>
</tbody>
</table>

report<sup>7</sup> and were purified, dialyzed, and chemically reconstituted with iron and sulfide under strictly anaerobic conditions. The as-purified protein coordinated on average ~ 2.0 iron ions per protein, and after chemical reconstitution appeared to coordinate two [4Fe-4S] clusters (Table 8.3).

UV-visible absorbance and CD spectroscopy was performed on the site-directed mutants to compare difference in Fe–S cluster binding. The absorbance spectra for HydG<sup>WT</sup>, HydG<sup>H212A</sup>, and HydG<sup>H272A</sup> had a comparable $\lambda_{\text{max}}$ around 393 nm (Figure 8.10A). The HydG<sup>H212A</sup> sample (Figure 8.10A, red trace) had an additional feature at 615 nm.

![Figure 8.10. UV-visible Absorbance (A) and CD (B) Spectroscopy of HydG Histidine-substituted Variants. The thick black line represents HydG<sup>WT</sup>, the thick red line represents HydG<sup>H212A</sup>, while the thick blue line represents HydG<sup>H272A</sup>. Experiments were performed at 23 °C. HydG concentration was 24 µM with iron numbers reported in Table 8.3. Samples contained 1 mM DTT, and samples were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 at 23 °C. Molar ellipticity was calculated using Equation 8.1.](image_url)
nm, while its baseline was elevated relative to the other samples, consistent with exogenous Fe-S clusters bound to the enzyme. By comparison, the UV-visible CD spectra for HydG\textsuperscript{WT}, HydG\textsuperscript{H212A}, and HydG\textsuperscript{H272A} all were likewise similar, and minor perturbations in signal were observed relative to HydG\textsuperscript{WT} enzyme (Figure 8.10B). Primary spectral differences included sharpening of the 383, 430 and 530 nm $\lambda_{\text{min}}$ for the HydG\textsuperscript{H272A} enzyme, as well as a signal shift at 383 and 550 nm for the HydG\textsuperscript{H212A} enzyme (Figure 8.10B, red and blue traces). The relative positioning of 383 and 430 features were maintained for the HydG\textsuperscript{H272A} sample, while a positive change in molar ellipticity was observed for the HydG\textsuperscript{H212A} sample (Figure 8.10B).

While the HydG\textsuperscript{H212A} and HydG\textsuperscript{H272A} variants conferred relatively minimal spectral perturbation relative to HydG\textsuperscript{WT}, observable spectral differences were observed with the HydG\textsuperscript{R136A} substitution (Figure 8.11). A comparable HydG\textsuperscript{R136A} absorbance

![Figure 8.11](image.png)

**Figure 8.11.** UV-visible Absorbance (A) and CD (B) Spectroscopy of HydG Arg-substituted Variant. The thick black line represents HydG\textsuperscript{WT}, while the thick magenta line represents HydG\textsuperscript{R136A}. HydG concentration was 24 $\mu$M with iron numbers reported in Table 8.3. Experiment was performed at 23 °C. Samples contained 1 mM DTT, and samples were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 at 23 °C. Molar ellipticity was calculated using Equation 8.1.
spectrum was observed relative to HydG<sup>WT</sup> enzyme, indicating comparable [4Fe-4S] cluster coordination ability relative to no substitution (Figure 8.11A). However, the UV-visible CD spectrum of HydG<sup>R136A</sup> was distinct from the HydG<sup>WT</sup> spectrum, yielding a large negative change in ellipticity in the region of 360–460 nm, and a positive change at 315 and 533 nm, respectively (Figure 8.11B). It is important to note that while this substitution did not affect the ability of the enzyme to coordinate [4Fe-4S] clusters, its principal effect caused a substantial change in Fe–S ellipticity.

**EPR Spectral Characterization**

The site–directed mutants were subjected to EPR spectroscopy to correlate the observed CD spectral changes to respective Fe–S clusters. Photoreduction of the HydG<sup>R136A</sup> and the HydG<sup>H272A</sup> enzymes produced typical axial spectra observed for HydG<sup>WT</sup> described in Chapter 2 and 3. The 3400 G feature for the HydG<sup>R136A</sup> was sharp and extended higher than the low-field g<sub>||</sub> feature (Figure 8.12A, black trace); by comparison, for HydG<sup>H272A</sup> the 3400 G feature was more broad, and an additional inflection at 3480 G was observed (Figure 8.12B, black trace). Addition of SAM to HydG<sup>R136A</sup> gave sharp, distinct features indicating a mixture of C-terminal and N-terminal clusters, although addition of SAM did not increase the integrated spin (Figure 8.12A; red trace); all of the reduced HydG<sup>R136A</sup> samples had integrated spins of approximately 0.75 spins per protein. The HydG<sup>H272A</sup> spectrum appeared to coordinate multiple clusters as well, although the reduced cluster signal was a shoulder to the larger perturbing feature associated with SAM binding, with N-terminal SAM-bound feature
Figure 8.12. X-band (9.37 GHz, 12 K) EPR Spectroscopy of HydG Site-directed Mutants near the C-terminal [4Fe-4S] Cluster. (A) Photoreduced HydG<sup>R136A</sup> (82 μM; 7.0 ± 0.6 Fe/protein) (black trace) in the presence of 5 mM SAM (red trace) and 10 mM CN<sup>−</sup> (blue trace). (B) Photoreduced HydG<sup>H272A</sup> (119 μM; 5.4 ± 0.1 Fe/protein) (black trace) in the presence of 5 mM SAM (red trace) and 10 mM CN<sup>−</sup> (blue trace). Samples were prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 and contained 5 mM DTT, 100 μM 5-deazariboflavin, and 50 mM tris. Typical EPR spectral parameters are reported in the experimental section.

more low-field than expected (Figure 8.12B, red trace) for either the HydG<sup>R136A</sup> or the HydG<sup>WT</sup> samples prepared. Addition of SAM resulted in a drop in integrated spin, from 0.58 to 0.22 spins per protein (Figure 8.12B, black and red trace). Addition of CN<sup>−</sup> to HydG<sup>H272A</sup> and HydG<sup>R136A</sup> yielded similar perturbing features to HydG<sup>WT</sup> (Chapter 5). Interestingly, the HydG<sup>R136A</sup> perturbation was unique in yielding two low-field g<sub>||</sub> features that appeared to lack a g = 2.05 feature (Figure 8.12A, blue trace).
By comparison, the effect of the N-terminal residue substitution HydG^{H212A} was also characterized by EPR spectroscopy. The reduced signal was similar to HydG^{WT} samples prepared, with significant broadening of signal, a slight inflection at 3480 G, as well as a low-field feature at 3250 G (Figure 8.13, black trace) similar to photoreduced sample preparations that were absent in dithionite–reduced samples described in Chapter 5. A small inflection was also observed at 3300 G, suggesting the presence of multiple clusters. Interestingly, addition of 5 mM SAM resulted in no perturbation, but rather a drop in signal intensity (Figure 8.13, red trace). Because this substitution was in proximity to where SAM binds, this substitution appeared to SAM’s ability to perturb the paramagnetic signal (Figure 8.13).

Given the observation that HydG^{WT} with Tyr substrate yields detectable organic radical with dithionite reduction (Chapter 5, Figure 5.5), HydG^{H272A} turnover samples
Figure 8.14. X-band (9.37 GHz, 12 K) EPR Spectroscopy of HydG$^\text{H272A}$ under Turnover Conditions. HydG$^\text{H272A}$ (100 $\mu$M; 5.2 ± 0.1 Fe/protein) was prepared in the presence of 3 mM tyrosine, 3 mM SAM, and 3 mM dithionite, incubated at 23 °C. Black trace, 30 second incubation; red trace, 5 minute incubation. Incubation times correspond to the time between when SAM and dithionite was added to the sample and when it was flash frozen. EPR spectral acquisition parameters used can be found in the experimental section.

were prepared to compare evolution of the Fe–S cluster signal in a fashion similar to HydG$^\text{WT}$. In the presence of 3 mM tyrosine, 3 mM SAM, and 3 mM dithionite, spectral changes were observed for the enzyme (Figure 8.14, black trace). An unique signal near 3333 G was present after 30 seconds of incubation that went away with added incubation time (Figure 8.14, red trace). Interestingly, the line shape appeared similar to the HydG$^\text{WT}$ turnover in Chapter 5 (Figure 5.5), with similar features observed, and similar Fe–S features that appeared transient with time (Figure 8.14).

**CO Formation Activity**

CO formation ability was assessed in H$_2$O buffer to correlate spectral behavior to enzyme functional competence (Figure 8.15). Substitution of either His212 or His272 to Ala did not confer significant spectral CD change relative to HydG$^\text{WT}$ enzyme, while
substitution of Arg136 conferred a significant change (Figures 8.10, 8.11). Interestingly, the HydG$^{H212A}$ variant produced significant CO produced, while HydG$^{H272A}$ and HydG$^{R136A}$ yielded negligible CO formed (Figure 8.15). HydG$^{H212A}$ CO formation was fitted to a first order exponential function, and had an observed $k_{cat}$ of $(11.1 \pm 0.02 \times 10^{-4} \text{ s}^{-1})$. By comparison, biphasic carboxyhemoglobin (HbCO) formation of HydG$^{WT}$ with burst-phase $(14.8 \pm 0.2 \times 10^{-4} \text{ s}^{-1})$ and slow-phase $(3.16 \pm 0.12 \times 10^{-4} \text{ s}^{-1})$ $k_{cat}$ values were similar to those reported in Chapter 2. The single HydG$^{H212A}$ $k_{cat}$ was higher than the HydG$^{WT}$ slow-phase $k_{cat}$ value (Chapter 5, Table 5.3). However, either C-terminal substitution resulted in complete loss of CO formation activity (Figure 8.15).

**H Atom Abstraction by HydG Site-Directed Mutants**

H atom abstraction with the HydG variants with the tyrosine substrate was performed to compare their profiles to HydG$^{WT}$. Differences in the isotope distribution
of 5'-dAdo and SAM were observed relative to HydGWT (Figure 8.16). As noted in the above sections, with tyrosine substrate HydGWT produces a mixture of labeled 5'-dAdo and SAM species (Figure 8.16A). By comparison, distribution shifts were observed for each of the variants studied; mono-deuterated 5'-dAdo and SAM were the principal species. The HydGH272A or HydGH212A variants conferred a similar effect on 5'-dAdo, while HydGH212A generated nearly equivalent deuterated SAM relative to HydGWT enzyme (Figure 8.16A,B). HydGR136A had a similar 5'-dAdo and SAM distribution to HydGWT, despite an apparent loss in ability to cleave SAM. All variants demonstrated an ability to cleave the Cα–Cβ bond in the p-cresol product formed (Figure 8.16C). It should be noted that while the HydGR136A sample was prepared at a more alkaline pD relative to the other samples, the variant incorporated less deuterium into SAM and 5'-dAdo relative to HydGWT.

Figure 8.16. Product Quantitation of (A) 5'-dAdo, (B) SAM, and (C) p-cresol Formation by HydG Site-directed Mutants. Error bars represent standard deviation following triplicate sample injections on the HPLC, while the isotope distributions were measured from the sample using LC-MS.
Figure 8.17. Normalized Sample Distribution of Labeled 5’-dAdo and SAM for HydG Site-directed Mutants. Unlabeled SAM and 5’-dAdo have been omitted for clarity. Error bars represent standard deviation following triplicate sample injections on the HPLC.

The substantial differences in the abstraction profile of SAM and 5’-dAdo present between HydG<sub>WT</sub> and the variants were normalized to compare the SAM $\rightleftharpoons$ 5’-dAdo equilibrium associated with abstraction (Figure 8.17). Interestingly, HydG<sub>WT</sub> and HydG<sup>R136A</sup> produced similar SAM to 5’-dAdo ratios, while a larger ratio was observed with the HydG<sup>H212A</sup> (Figure 8.17). For HydG<sup>H212A</sup>, the equilibrium between SAM and 5’-dAdo was similar to HydG<sup>WT</sup> with the substrate analogue DOPA (Figure 8.6). The HydG<sup>H212A</sup> substitution does not abolish CO product formation, but the increased SAM to 5’-dAdo ratio is similar (Figure 8.17). Since this residue likely interacts with the SAM carboxylate,<sup>42</sup> loss of this type of interaction affects coordination of SAM at the radical SAM cluster.
HydG substrate radical initiation via 5’-dAdo• H atom abstraction constitutes the initial step in the biotransformation of tyrosine to make CO and CN\textsuperscript{–}.\textsuperscript{3-5,9} HydG is unique among characterized members of the radical SAM enzyme superfamily in that an abstraction profile from a solvent-exchangeable position has been characterized. HydG catalyzes both stoichiometric consumption of SAM and reversible abstraction–reabstraction events.\textsuperscript{9} Abstraction is not only affected by pH, but also by the oxidation potential associated with H atom abstraction. The substrate tyrosine is a polyprotic acid that has related acid dissociation macroconstants and microconstants, reflective of several protonated and deprotonated species.\textsuperscript{13,43} Concurrent with our interest to optimize percent CO formation ability by HydG, pH elevation between the macroconstant-microconstant pK values and pH 7.4 has both improved general catalytic ability and CO formation, as described elsewhere (Chapter 6 and 7).

Stimulation of product formation without an observed change in distribution was expected to occur with the pD changes studied. As a template for understanding product stimulation, the effector molecule cysteine has been shown to stimulate product formation, but without affecting the distribution of labeled species in D\textsubscript{2}O (Chapter 7). A similar hypothesis was proposed for increasing the pH, since stimulated p-cresol and 5’-dAdo was observed upon making the pH (pD) more alkaline, but well below the associated pK values for the backbone ammonium and phenol hydroxyl groups (Table 8.1). However, the distribution of labeled species shifted, however, with the pD change from 6.8 to 7.8 (Figure 8.1). The assay pDs are below the substrate thermodynamic pK
macroconstants (9.04 and 10.08), yielding deprotonated and protonated species at the ammonium and phenol groups, respectively. A larger shift in distribution of deuterium label was observed for 5’-dAdo than for SAM (Figure 8.1C). The normalized distribution change appeared to suggest that the distribution difference was independent of the substrate, and was instead dependent on the active site environment that 5’-dAdo• would abstract.

H atom abstraction within the radical SAM enzyme superfamily is generally a single, rate-limiting step for members that employ SAM as a cosubstrate. However, we have shown previously (Chapter 4) that abstraction–reabstraction events yielding deuterium label in both SAM and 5’-dAdo require regeneration of 5’-dAdo• to obtain the observed distribution of labeled species in SAM. Thus, the 5’-dAdo and SAM isotope distribution constitutes an equilibrium between abstraction and reabstraction. A predictable 5’-dAdo isotope distribution from the observed SAM isotope distribution would indicate a purely reversible H atom abstraction–reabstraction mechanism controlled by the percent D2O exchanging with the solvent-exchangeable abstraction site. On the other hand, a difference in prediction indicates additional hydrogen atom transfer modes for events preceding or following Cα–Cβ bond cleavage.

Two approaches were employed in this study to understand the observed pD–dependent changes in H atom transfer between SAM and 5’-dAdo: 1) use of tyrosine analogues and 2) site–directed mutagenesis of proposed active site residues that would be anticipated to interact with the generated 5’-dAdo•. First, the objective for using tyrosine analogues was to determine an effect on changing the oxidation potential on the apparent H atom abstraction and product formation. Use of analogues NH2–Y and DOPA have
comparable macroscopic $pK$ values relative to the native substrate tyrosine, but differ in their oxidation potential, making them serve as thermodynamic radical traps following H atom abstraction (Table 8.1). $^{32,33}$ Tyrosine has an oxidation potential of $+830$ mV, while the analogues NH$_2$–Y and DOPA are $190$ mV and $270$ mV easier to oxidize respectively (pH 7.0). In the class Ia ribonucleotide reductases, formation of the NH$_2$–Y• has been characterized to possess an internal hydrogen bond that stabilizes the generated radical from one electron reduction.$^{44}$

Modulation of HydG H atom abstraction–reabstraction was observed by lowering the oxidation potential of the substrate (Figure 8.4, 8.5). Reversible abstraction–reabstraction occurred between SAM and 5’-dAdo regardless of the substrate or analogue, with additional label observed in each with the natural substrate. A shift toward a greater percentage of labeled SAM species with DOPA or NH$_2$–Y is reflective of the increased thermodynamic stability associated with abstraction and radical generation on the analogue substrate (Figure 8.6). Isotope distributions for 5’-dAdo approximated to the expected isotope distribution from the SAM distribution, indicating a more reversible abstraction event (Table 8.2). By comparison, a similar isotope distribution between SAM and 5’-dAdo was observed at acidic pD (Appendix Figure G.1).

Analogue stabilization of the substrate radical was interpreted to yield distinct catalytic behavior at the level of radical generation and propagation. Samples prepared under single turnover conditions to probe the potential formation for a substrate radical were unsuccessful (Figure 8.7), although a product radical has been observed with natural substrate, requiring the continuous reductant dithionite. However, analogue C$_\alpha$–C$_\beta$ bond
cleavage ability was demonstrated via CO formation competency (Figure 8.3). DOPA catalyzed comparable quantities of CO with a fitted exponential function that was slower than the burst-phase rate observed for natural substrate; its rate was faster than the tyrosine slow phase rate, which accounted for the similar quantity of CO formed overall (Figure 8.3). Interestingly, either analogue was more sensitive to pH change than tyrosine was in their CO formation ability. The ability of the substituent to hydrogen bond with the active site residues involved with hydrogen bonding, in principle, could stabilize or inhibit \( C_{\alpha} - C_{\beta} \) bond cleavage from occurring, that would be affected by the pH range the experiments were performed (Figure 8.3). This property is absent from the native substrate (Figure 8.2).

The objective for performing site-directed mutagenesis was to probe interacting active site residues affecting abstraction–reabstraction events. While HydG has yet to be crystallographically characterized, it is predicted to contain a complete \((\alpha\beta)_8\) triose isomerase phosphate (TIM) barrel observed in structurally characterized enzymes such as BioB, PylB, and HydE. The predicted homology structure of HydG (Figure 8.8) shared similar structural motifs reflected in other structurally characterized members of the superfamily. HydG possesses two \([4\text{Fe}-4\text{S}]\) clusters that have predicted to be coordinated at the ends of the cylindrical TIM barrel.\(^{34}\) Several structurally characterized radical SAM enzymes coordinate auxiliary \([4\text{Fe}-4\text{S}]\) clusters, but most are oriented laterally about the TIM barrel diameter from the radical SAM cluster. The auxiliary cluster of radical SAM enzyme BioB has a more posterior orientation about the principal barrel axis, however it is a \([2\text{Fe}-2\text{S}]\) cluster.\(^{35}\) HydG’s predicted C-terminal cluster domain
coordinates a [4Fe-4S] cluster that at present has no structural precedent as a site-differentiated cluster.

The N-terminal radical SAM [4Fe-4S] cluster environment of HydG shares structural motifs with other structurally characterized enzymes of the superfamily. HydG contains the CX₃CXΦC motif (Φ represents an aromatic residue) that coordinates a [4Fe-4S] cluster and along with a “GxIxGxxE”-like motif provides hydrophobic interactions to the adenine ring of SAM. Similar to other radical SAM enzymes characterized, HydG also contains a “GGE” motif that stabilizes the interaction between the SAM methionine amino group with the unique Fe of the [4Fe-4S] cluster (Chapter 1). Additional, secondary interactions are present in the superfamily to help stabilize the coordination of SAM at the [4Fe-4S] cluster, one of which includes an active site arginine residue that is in close proximity to the coordinated carboxylate moiety of SAM. While arginine’s functional role as an active site base subjected to hydrogen bonding would be not entirely intuitive at physiological pH since it would be protonated, however proximity to deprotonated carboxylate residues are known to alter arginine functionality to being a base. In most cases in the radical SAM superfamily this interacting base is arginine, although for HydG (as well as for BtrN), this interacting residue is a histidine (Figure 8.9).

Candidate base residues proposed to rationalize the observed abstraction differences included histidine residues (with an imidazole pKₐ value of 6.04) and arginine residues next to residues with negatively charged sidechains that were in proximity to either of the [4Fe-4S] clusters and conserved among HydG homologues (Figure 8.8). The site directed mutants HydG^{R136A}, HydG^{H212A}, and HydG^{H272A} were principally
selected to probe the ability of the active site residue in H atom abstraction. These substitutions were anticipated to affect the coordination environment of the HydG [4Fe-4S] clusters involved in catalysis. While Arg136 and His272 are oriented near the putative coordination site for the C-terminal [4Fe-4S] cluster, substitution of either residue might be expected to affect tyrosine substrate binding. Only the HydG$^{R136A}$ conferred a substantial perturbation in Fe–S ellipticity associated with the substitution; HydG$^{H212A}$ and HydG$^{H272A}$ produced CD spectra that were similar to HydG$^{WT}$ enzyme (Figure 8.10). Substitution of either residue as a charge–neutral species at the assayed pH did not affect a difference in charge at the Fe–S cluster. In contrast, the arginine substitution removes a positive charge near the C–terminal cluster, which likely rationalizes the changes observed (Figure 8.11).

CO formation by the site-directed mutants was expected to yield different results relative to the tyrosine analogues reported above, since substitution of essential active site residues would affect enzyme ability to produce the diatomic ligands. The only substitution that yielded observable CO formation was the HydG$^{H212A}$ variant, a substitution near the N-terminal radical SAM cluster (Figure 8.15). Substitution for either residue near the C-terminal cluster (variants HydG$^{H272A}$ and HydG$^{R136A}$) resulted in no CO formation, conferring their importance in the catalytic mechanism. It should be noted that similar CO formation kinetics with the HydG$^{H212A}$ variant was observed using the DOPA substrate analogue (Figure 8.3) with HydG$^{WT}$ an exponential without a burst phase, though a mechanistic relation between the two remains unknown. Qualitatively they differ in hydrogen bonding removed and added near the coordinated SAM and as part of the substrate, respectively.
Preliminary EPR spectral characterization of the HydG<sup>R136A</sup>, HydG<sup>H212A</sup>, and HydG<sup>H272A</sup> variants all yielded paramagnetic signals reflective of $[^4\text{Fe}-4\text{S}]^{+} S = 1/2$ spin state upon reduction (Figure 8.12, 8.13, 8.14). However, some notable differences in the electronic environment between these variants and HydG<sup>WT</sup> were observed. First, substitution of the N-terminal His212 resulted in a protein that was not perturbed upon SAM coordination, although SAM was added in considerable (40-fold) excess relative to the enzyme concentration (Figure 8.13). This residue has been implicated in its coordinative interaction with the SAM carboxylate involved with chelation to the site-differentiated $[^4\text{Fe-4S}]$ radical SAM cluster. Substitution of the residue appears to not affect the observed reduced Fe–S cluster signal, but without additional structural information, it is difficult to say whether or not SAM coordination was altered as a consequence of the substitution. Second, addition of SAM to either HydG<sup>R136A</sup> or HydG<sup>H272A</sup> yielded unique signal perturbations relative to HydG<sup>WT</sup>; while addition to HydG<sup>R136A</sup> yielded discrete spectral features not affected by Fe–S anisotropy, addition to HydG<sup>H272A</sup> was more distorted, with a rhombic $g_1$ value more lowfield relative to HydG<sup>WT</sup> (Figure 8.12A, B, red traces). As SAM coordination to the intact, unmodified N-terminal $[^4\text{Fe-4S}]$ cluster provides electronic information relating to the C-terminal cluster, effect of the substitution for HydG<sup>R136A</sup> yielded an intact cluster, while for HydG<sup>H272A</sup> the resultant cluster was either mostly absent or diamagnetic. Consistent with the observed iron number (Table 8.3) addition of CN<sup>−</sup> to either variant resulted in formation of feature at 3250 G, consistent with coordination to a C-terminal cluster (Figure 8.12).
The site-directed mutants selected in this study all facilitated reductive cleavage of SAM and cleavage of the tyrosine $C_\alpha$–$C_\beta$ bond to produce $p$-cresol and 5’-dAdo, but each caused significant shifts in deuterium isotope distribution in SAM and 5’-dAdo (Figure 8.16, 8.17). These distributions are somewhat similar to those observed with the tyrosine analogues described above, but differ in their increase of deuterium label in both SAM and 5’-dAdo. Substitution of either His212 or His272 conferred similar 5’-dAdo isotope, though HydG$^{H212A}$ yielded a greater percentage of SAM with deuterium label and a larger percentage of bis-deuterated 5’-dAdo; this normalized distribution is similar to the DOPA substrate analogue distribution noted above (Figure 8.17). It is noteworthy that similar behavior was observed between HydG$^{H212A}$ with natural substrate and HydG$^{WT}$ with DOPA, since in either case hydrogen bonding ability with either the residue or the analogue is retained. The HydG$^{R136A}$ distribution was most similar to HydG$^{WT}$, likely a reflection of the His212 and His272 residues retained.

The differences in H atom abstraction by the site-directed mutants described above are remarkable, since they represent substitutions at opposite ends of the radical SAM ($\alpha\beta$)$_8$ TIM barrel (Figure 8.8). That a comparable 5’-dAdo isotope pattern is observed for HydG$^{H272A}$ and HydG$^{H212A}$ suggests a common effect on the 5’-dAdo–SAM equilibrium (Figure 8.17). The specific interaction of His272 is unique, since it is positioned well over 20 Å from the N-terminal site-differentiated Fe coordinating SAM. It is possible that His272 helps orient the substrate for correct abstraction, in a manner similar to His212 expected in coordinating the bound SAM carboxylate carbon at the radical SAM cluster. As noted above, EPR spectroscopy supports coordination of a C-terminal cluster by HydG$^{H272A}$ and turnover samples in the presence of SAM and tyrosine
support the generation of unique Fe–S cluster coordination, as reflected in the unique signal generated (Figure 8.14).

To conclude, the collective data presented herein provide insight toward a mechanism of diatomic ligand formation from tyrosine lyase. While natural substrate yields 5’-dAdo and SAM with significant deuterium label incorporation, use of either NH₂–Y or DOPA with HydG\textsuperscript{WT} or HydG\textsuperscript{H212A} with tyrosine results in the forward reaction of tyrosine C\textsubscript{α}–C\textsubscript{β} bond cleavage, catalyzes CO formation, with minimized abstraction–reabstraction events. In the case of DOPA, the distribution of labeled species across SAM and 5’-dAdo are nearly reversible, as predicted from label distributions for the detected products. While this brings into question the mechanistic relevance of multiple labels observed with natural substrate, DOPA appears to minimize reversible abstraction–reabstraction in the events following H atom abstraction since radical generation is more stable on the analogue, as reflected by the decreased substrate oxidation potential. In the case of HydG\textsuperscript{H212A} with natural substrate, a loss of interaction in His212 hydrogen bonding may destabilize the coordinated SAM such that iterative abstraction–reabstraction events are minimized. Future work characterizing the catalytic properties of HydG\textsuperscript{H212A} with native substrate and of HydG\textsuperscript{WT} with DOPA substrate should provide additional insight to this remarkable biosynthetic transformation.

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CHAPTER 9

CONCLUDING REMARKS

Complex metalloenzyme active sites such as the [FeFe]-hydrogenase H-cluster are remarkable in the similarity in structure and catalytic activity observed with Fe–S minerals present during the early Earth. Details toward a chemical mechanism of synthesis and assembly of complex Fe–S clusters in biology at the interface of enzymes, scaffolds, and carrier proteins help better define elements of biosynthesis that originated from a prebiotic Earth. This work clarifies the functional role and chemical mechanism of HydG, a [FeFe]-hydrogenase H-cluster maturase, that applies radical SAM chemistry to transform a biological substrate into the products CO and CN\(^{-}\). The goal of this work was to identify the functional role of HydG, and to develop a molecular-level understanding of product CO and CN\(^{-}\) formation and transfer to the H-cluster active site. These diatomic products were prevalent on the early Earth, and served to drive ligand-accelerated catalysis of Fe–S minerals. Radical SAM enzymes catalyze complex, radical-initiated reactions with Fe–S clusters coordinated within a protein architecture. The radical SAM enzyme HydG represents a modern example of ligand-accelerated catalysis, where the products of catalysis are used to modify an ordinary Fe–S cluster. In turn, the work presented in this dissertation provides a deeper understanding regarding scope of catalysis afforded by radical SAM enzymes in the biosynthesis of ancient, organometallic mineral catalysts found in biology.

One of the outstanding questions regarding H-cluster biosynthesis was the functional involvement of two radical SAM enzymes. The two enzymes HydE and
HydG were proposed to catalyze similar chemistry, but lack of identification of a substrate for each limited initial characterization of each. Owing to the large number of radical SAM enzymes that have been characterized with substrates identified, inspiration helped to differentiate the functionality between the maturases HydG and HydE. The work presented in Chapter 2 on HydG provided the first clear conclusive data to show that HydG serves in the synthesis of not only the CN– product, but also the CO product as well, requiring two [4Fe-4S] clusters. This result, along with detection of CN– showed that HydG functioned to synthesize the diatomic ligands, leaving HydE to synthesize the dithiolate ligand by an unknown mechanism.

Radical SAM enzymes catalyze radical-initiated reactions at a site-differentiated [4Fe-4S] cluster coordinated by a CX₃CX₂C motif. HydG, however, is unique in its requirement of a second site-differentiated [4Fe-4S] cluster for its synthesis of CO and CN–. This property has been understood at the functional level of the maturases involved, but insight at the molecular level was not well defined. Biochemical and spectroscopic characterization of HydG variants probing the role of the C-terminal [4Fe-4S] cluster, presented in Chapter 3, demonstrated that tyrosine Cα–Cβ bond cleavage occurs without the presence of the C-terminal domain or coordinated [4Fe-4S] cluster. This provided compelling data that the enzyme mechanism operates in two parts, defined by cleavage of the substrate Cα–Cβ and amino acid backbone C–C bonds, respectively. Importantly, a simulation model differentiating coordination of SAM to the active site was rationally constructed as a superposition of signals from the two, individual [4Fe-4S] clusters, based off of EPR spectral characterization of the individual clusters through mutagenesis studies, that suggested the two clusters are not electronically coupled.
Given the complex involvement of two [4Fe-4S] clusters in the catalytic reaction, a profile of the H atom abstraction was determined in Chapter 4 to differentiate the involvement of the 5’-dAdo• in the catalytic reaction. Owing to the observation that radical initiation of the substrate constitutes the rate-limiting step of the reaction, and that HydG appeared to consume SAM as a cosubstrate, the abstraction mechanism was proposed to follow a simple label pattern corresponding to label transfer of the 5’-dAdo•. However, multiple labeled species in 5’-dAdo and SAM were observed, which would only occur if the 5’-dAdo• was regenerated from a substrate or intermediate radical. Abstraction yielding deuterium label into 5’-dAdo only occurred at a solvent-exchangeable position, meaning that all other positions, including the substrate Cα Hydrogen, do not undergo abstraction by the 5’-dAdo•. This study identified the basis by which the HydG enzyme and the generated 5’-dAdo• interacts with the substrate, which defined involvement of the SAM-derived radical.

The diatomic ligand products of the HydG reaction are used to synthesize an organometallic complex defined by coordination of iron bound to CO and CN⁻ ligands. In Chapter 5, a comprehensive analysis of product diatomic ligand binding to HydG Fe–S clusters was compared, applying UV-visible, EPR, and FTIR data from HydG isolated from two different organisms. Importantly, under catalytic turnover conditions, both the \textit{C. acetobutylicum} and \textit{S. oneidensis} HydG enzymes undergo electronic changes at respective Fe–S clusters, and both are active in their formation of the diatomic ligand products. Spectral characterization of the \textit{C. acetobutylicum} HydG enzyme in the presence of exogenously added diatomic ligands helped provide a foundational basis for the coordination chemistry supported by the enzyme. Also in Chapter 5, detection of an
organic radical can be observed for each under turnover conditions, which helps support the commonality in the mechanism catalyzed by respective enzymes. Distinctions in the nature of the FTIR data persist between the enzymes, although the two catalyze the same reaction under a similar reaction mechanism.

An outstanding question in the chemical mechanism of HydG has been defining the stoichiometry of the CO product formed. Chapter 6 highlighted a significant body of work dedicated toward CO detection by the enzyme, employing an in situ CO detection through incubation with hemoglobin. Importantly, comparison of binding of CO to hemoglobin and myoglobin variants suggested that detection is relatively inefficient, precluding detection of all CO that is formed. Also in Chapter 6 highlighted an important component of the reaction, where phosphate specifically inhibited CO formation by HydG relative to the other products formed. Phosphate has potential relevance to the biosynthetic mechanism because it is a product of GTP hydrolysis of the scaffold protein HydF; while HydG lacks discernible structural motifs that would suggest phosphate binding to the enzyme, it appeared to modulate a reaction where CN−, but not CO is formed. While chemical details to this distinction remain largely unresolved, phosphate might be modulating the transformation where oxidative decarboxylation of the DHG intermediate (forming CN− and CO2) is favored over oxidative decarbonylation (forming CN− and CO).

In Chapters 6 and 7, selected molecules identified to have an important role in HydG catalysis and in the biosynthetic mechanism were probed to understand their effect on HydG catalysis, including molecules identified or proposed to be substrates for HydF and HydE, respectively. Of these, the molecule cysteine (that has been proposed to be a
substrate for HydE) beneficially improved HydG catalysis, but it affected CO formation during the initial time points of the reaction. The collective experimental data suggested an activating role of cysteine, possibly serving as an allosteric effector in the chemical reaction. Also in Chapter 7, interaction of exogenously produced DHG was characterized to understand its role in catalysis in the absence of generated 5’-dAdo•. These results, along with data presented in Chapter 6 related to phosphate, help to define molecular-based catalytic reactivity modulated by biomolecules involved in the biosynthetic scheme.

One of the difficulties of the reaction catalyzed by HydG is not only that substrate radical initiation occurs from a solvent-exchangeable position, but also that catalysis occurs in two steps, involving two site-differentiated [4Fe–4S] clusters. To address these issues, two complementary approaches were employed to understand product formation and involvement of the 5’-dAdo• that were presented in Chapter 8. First, tyrosine analogues that retained the relevant components for catalysis, but altered the oxidation potential, yielded productive formation of CO, but with differences in the deuterium abstraction pattern in both SAM and 5’-dAdo. In both cases, reducing the oxidation potential for either analogue affected the SAM:5’-dAdo abstraction equilibrium, favoring more SAM formation with less extensive label transfer to 5’-dAdo. These results suggested that the abstraction–reabstraction mechanism occurs as part of the initial part of the reaction. Second, site-directed mutants substituting residues that were proposed to hydrogen bond with the generated 5’-dAdo• or SAM also affected this distribution similarly using tyrosine substrate. Interestingly, of these substitutions, the substitution near the C-terminal [4Fe-4S] cluster (His272) was unable to produce CO, while an N-
terminal substitution (His212) was able to produce CO; both enzymes exhibited differences in EPR spectral behavior in the presence of SAM relative to HydG$^{WT}$ that likely is rooted in differences in SAM binding within the TIM barrel. Also in Chapter 8 was an additional C-terminal residue substitution (Arg136) that principally affected enzyme ability to form CO at an intact C-terminal [4Fe-4S] cluster. These results helped to define a model catalysis performed within the TIM barrel that involves the two participatory Fe–S clusters in the chemical reaction.

Collectively, the results presented in this dissertation provide critical insight regarding the mechanism of diatomic ligand formation and transfer, part of the larger assembly process involving modification of ordinary Fe–S clusters to synthesize more complex variations. The combined biochemical and spectroscopic approaches described herein have enabled a more comprehensive analysis of the complex biochemical transformation occurring by HydG, by defining the products and intermediates formed, the involvement of organic radicals, as well as an understanding of catalysis performed at the auxiliary C-terminal cluster. The cumulative biochemical data provide significant evidence that this reaction occurs in discrete steps, namely that cleavage of the amino acid backbone C–C bond follows substrate C$_\alpha$–C$_\beta$ bond cleavage. Analysis of EPR spectroscopic data strongly suggests the formation of diatomic ligand product-bound Fe–S clusters, with transient generation of the $p$-cresolate phenoxyl radical. As this reaction is sensitive to components and to the coordination environment of the C-terminal Fe–S cluster, insight to the likely catalytic steps yielding the diatomic ligands following substrate C$_\alpha$–C$_\beta$ bond cleavage have been characterized. The work presented herein provides significant insight into the molecular mechanism of formation of diatomic
ligand formation catalyzed by radical-initiated catalysis, and demonstrate the utility of applying a combined biochemical and spectroscopic approach to examine the modification of ordinary Fe–S clusters to synthesize complex metallocofactors found in biology.
APPENDICES
APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2
[FEFE]-HYDROGENASE MATURATION: HYDG-CATALYZED SYNTHESIS OF CARBON MONOXIDE

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Supplemental Figures

Figure A.1. Spectroscopic Characterization of Non-reconstituted HydG. (A) UV-visible spectra of HydG as-isolated (147 µM, 1.3 ± 0.1 Fe/protein, solid line) and after reduction using dithionite (44 µM, 2.2 Fe/protein, dashed line). (B) X-band EPR spectra of photoreduced HydG in the absence (black line, 284 µM, 2.2 ± 0.3 Fe/protein) and presence (red line, 279 µM, 2.2 ± 0.3 Fe/protein) of AdoMet. EPR parameters: 12 K, 9.36 GHz, 1.47 mW.
Figure A.2. Temperature-dependence of the EPR Signals Present in Non-reconstituted, Photoreduced HydG. Sample shown at 11K is the same sample shown in Appendix Figure A.1, panel B, black line. EPR parameters: sample temperature, 12 K; frequency, 9.36 GHz; power, 1.47 mW; time constant, 327.68 ms; conversion time, 81.92 s.

Figure A.3. Spectroscopic Characterization of Reconstituted HydG. (A) UV-visible spectra of non-reconstituted, as-isolated HydG (87 μM, 4.6 ± 0.2 Fe/protein, black line) and reconstituted HydG (63 μM, 6.8 ± 0.1 Fe/protein, red line). The λ\textsubscript{max} shift from 415 nm to 395 nm was consistently observed in all samples following reconstitution. (B) X-band EPR spectra of photoreduced, reconstituted HydG. The sample shown is the same as displayed in Figure 2.1 (65 μM enzyme, 8.7 ± 0.7 Fe/protein). EPR parameters are identical to those in Figure 2.1 and temperatures are indicated.
Figure A.4. Single Wavelength (419 nm) Kinetics Illustrating the Biphasic Nature of HbCO Formation at 30 °C. Reaction contained 33 µM HydG (8.7 ± 0.7 Fe/protein), 4 mM dithionite, 1 mM Tyr, 1 mM SAM, 10.7 µM heme. The fast phase results in 1.4 µM HbCO in ~0.8 min with HbCO formation continuing to 5.5 µM in 30 minutes. The biexponential fit yielding the rates reported in Chapter 2 is shown in red (Origin 7.0).

Figure A.5. HPLC Analysis of HydG Reaction Products shown in Appendix Figure A.4 (see Appendix A text).
Figure A.6. FTIR Demonstrating the Presence of $^{13}$CO–deoxyhemoglobin. (a) HydG reaction mixture containing U-$^{13}$C-tyrosine, deoxyhemoglobin, and SAM. (b) Control reaction mixture which contained all assay components as in (a) with the exception of U-$^{13}$C-tyrosine. This control showed no active IR bands but was subsequently shot against 1 mM $^{13}$CO to yield (b).

Figure A.7. HPLC Analysis Demonstrating Stoichiometric Production of 5‘-deoxyadenosine and $p$-cresol in a HydG CO Assay (see Appendix A text).
Protein Expression and Purification

Constructs encoding HydG from *Clostridium acetobutylicum* were transformed into *E. coli*- BL21(DE3) (Stratagene) cells for protein expression, as described previously with slight modifications. Briefly, single colonies obtained from transformations were grown overnight in LB media and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50 mM potassium phosphate buffer pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD$_{600}$ = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N$_2$ overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber, as described with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol, 10 mM imidazole, 20 mM MgCl$_2$, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, ~ 0.6 mg lysozyme per gram cell in an anaerobic Coy (Grass Lake, MI) chamber. The lysis mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles at 18000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrapTM Ni$^{2+}$-affinity column (GE Healthcare, Uppsala).
The column was pre-equilibrated with 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol, 10 mM imidazole (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol, 500 mM imidazole). Fractions of interest were pooled, dialyzed into 50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol, and concentrated using an Amicon concentrator (Millipore; Bedford, MA) fitted with a YM-10 membrane or using a Prochem BJP 10/40 static protein concentrator. Protein was flash frozen in liquid N$_2$ and stored at -80 °C or in liquid N$_2$ until further use.

**Preparation of Reconstituted HydG**

Reconstitution of as-isolated HydG was carried out following the general procedures utilized for biotin synthase.$^2$ Enzyme (50 – 150 $\mu$M) was incubated with 6 – 7 fold excess of FeCl$_3$ and Na$_2$S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 0.5 M KCl, 5% glycerol for $\approx$ 3 hours with gentle agitation in a Coy anaerobic chamber. Following a 5 minute incubation with DTT, FeCl$_3$ was added slowly and stirred for 20 minutes prior to the stepwise addition of Na$_2$S. Following the reconstitution period, the mixture was centrifuged and the supernatant was run over a Sephadex G-25 column (GE Healthcare; Piscataway, NJ) to remove excess ions. The resulting protein was concentrated using an Amicon concentrator fitted with a YM-10 membrane or a Pro-chem (Littleton, MA) BJP 10/40 static protein concentrator. UV-visible spectroscopy was utilized to confirm the Fe–S cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Iron content of both the as-
purified and reconstituted sample was examined by the spectrophotometric method
developed by Fish, which uses ferrozine under reductive conditions after protein
digestion with 4.5\% (w/v) KMnO$_4$ and 1.2 N HCl.  

UV-visible analysis of reconstituted HydG samples are brown in color and
showed strong ligand-to-metal charge transfer bands at 320 and 415 nm that were more
intense than the as-purified samples. Reconstituted samples used in this study had initial
iron numbers between 2 to 3 per protein. Following reconstitution, iron analysis shows
reconstituted samples to have iron numbers ranging between 4.8 $\pm$ 0.2 to 8.7 $\pm$ 0.7 mol Fe
per protein.

**UV-visible Spectroscopic Analysis**

For UV-visible absorption experiments, samples were transferred to a 1.4 ml
anaerobic cuvette (Spectrocell Inc, Oreland, PA) within an MBraun glove box (MBraun
USA, Stratham, NH). Room temperature UV-visible absorption data were acquired using
a Cary 6000i UV-visible/near-IR spectrophotometer (Varian; Palo Alto, CA). UV-visible
spectra were collected at a data interval of 1.0 nm.

**EPR Sample Preparation and Spectroscopic Analysis**

EPR samples were prepared in an MBraun box at O$_2$ levels <1 ppm. Photoreduced samples of non-reconstituted HydG (284 $\mu$M; 2.2 $\pm$ 0.3 Fe/protein) were
made by supplementing the protein with 50 mM Tris, pH 7.4, 100 $\mu$M deazariboflavin,
and 5 mM DTT. Samples were then placed in an ice water bath in the MBraun box and
illuminated with a 300 Watt Xe lamp for 1 hr. To determine the effects of SAM on
reduced enzyme, an additional aliquot of protein was prepared simultaneously as described above. Following 1 hour photoreduction, 630 μM SAM was added to the enzyme sample (279 μM final; 2.2 ± 0.3 Fe/protein) in the absence of light and the sample was immediately flash-frozen in liquid N₂. Reconstituted samples of HydG (65 μM protein at 8.7 Fe/protein) were prepared in an identical manner as described above, although the final concentration of SAM was 1 mM. Samples were stored in liquid N₂ until low temperature spectral analysis was performed (Figures 2.1, A.1, A.2 and A.3). Low temperature EPR spectra were collected using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller from Oxford instruments. Typical EPR parameters were: sample temperature, 12 K; microwave frequency, 9.37 GHz; microwave power, 1.84 mW; time constant, 81.92 ms; sweep time, 167.77 s. Simulation of EPR data was performed with the EasySpin software program and yielded the g-values reported in Chapter 2.

CO Assays Additional Information

CO formation assays were carried out in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol. Raw data were corrected for the contribution of reduced HydG to the visible absorption. The ΔA₄₁₉nm and Δε₄₁₉nm were used to calculate the concentration of HbCO at each time point in order to determine the rate of CO formation. Data was fit to a biphasic exponential, and kₐₜ values were calculated as previously described. All kinetics assays were performed at 30 °C.

Control experiments lacking AdoMet were carried out using 36 μM HydG at 7.57 ± 0.3 Fe/protein in 3.22 mM DT and 10.5 μM deoxyhemoglobin all in 50 mM HEPES pH
7.4, 0.5 M KCl, 5% glycerol buffer. Tyrosine (1 mM) was added and spectra were monitored for 2 hrs at 30 °C. No spectral changes were observed. At the 2 hour mark, the sample was transferred back to the MBraun box and 1.3 mM SAM was added. Within 15 minutes spectral changes associated with the Soret band consistent with HbCO formation were observed.

Control experiments lacking tyrosine were carried out using 36 µM HydG at 7.57 ± 0.26 Fe/protein in 3.22 mM DT and 10.5 µM deoxyhemoglobin all in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol buffer. AdoMet (1.3 mM) was added and spectra were monitored for 2 hrs at 30 °C. No spectral changes were observed. At the 2 hour mark, the sample was transferred back to the MBraun box and 1 mM tyrosine was added. Within 15 minutes spectral changes associated with the Soret band consistent with HbCO formation were observed.

Several attempts were made to simultaneously detect CO and CN– in a single assay, however these were unsuccessful. One problem was buffer incompatibility, as the CN– derivatization could not be carried out in HEPES buffer (the buffer used for CO assays), and the CO assays did not work in phosphate buffer (the buffer used for cyanide assays). Further, although the cyanide assay required protein denaturation and thus recovered and detected most of the cyanide produced, our method of utilizing hemoglobin to detect CO, together with the problems associated with quantifying a gaseous product, have prevented us from using a parallel denaturation protocol to detect CO. Efforts are currently underway to modify the CO assay in order to improve the quantitative aspects of the assay.
FTIR Sample Preparation and Spectroscopic Analysis

Samples for FTIR analysis were prepared in an MBraun box at O₂ levels <1 ppm in a manner similar as for UV-visible analysis. Briefly, reconstituted HydG (59 µM final concentration, 8.1 ± 1.7 Fe/protein) was mixed with deoxyhemoglobin (115 µM heme final) in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol, 4 mM sodium dithionite containing buffer. L-tyrosine (U-¹³C₀, 97-99%; ¹⁵N, 97-99%) at a chemical purity ≥ 98% was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The mixture was set up in a 1.0 mL screwcap cryovial and was incubated at 30 °C for 5 minutes using an IsoTemp block (Fisher; Waltham, MA). The assay was initiated by addition of AdoMet (1 mM final concentration) and following AdoMet addition the cryovial was immediately sealed. The assay was allowed to incubate for 1 hour at 30 °C at which time the mixture was flash frozen in liquid N₂. Control mixtures were set up alongside the HydG–hemoglobin assay that utilized HydG from the same reconstitution batch but lacked either U-¹³C-labeled tyrosine or deoxyhemoglobin.

FTIR spectra were measured using a Bruker IFS/66S FTIR spectrometer interfaced with a home-built stopped-flow drive system with the sample cuvette and drive system maintained in an anaerobic chamber (Belle Technology (Weymouth, UK), O₂ < 1.1 ppm) as described elsewhere. The IR cuvette was thermostatted at 25 °C. For these measurements, protein sample in 50 mM HEPES pH 7.4 buffer containing 500 mM KCl were introduced on only one side of the drive system, with the other side loaded with buffer. Spectra were measured at 4 cm⁻¹ resolution. The IR cuvette path length was
calibrated at 47.6 μm. Arbitrary background corrections were applied to yield flat baselines for measured spectra.

The presence of the vibrational band at 1907 cm⁻¹ in spectrum spectrum “a” is consistent with the existence of ¹³CO bound to the heme iron of deoxyhemoglobin (Figure 2.2A inset and Appendix Figure A.6). Neither control showed the presence of a band at 1907 cm⁻¹, and differential spectral analysis yield the spectra in Appendix Figure A.6. As an internal control experiment, exogenous ¹³C-labeled CO (1 mM) was shot against the control sample that lacked ¹³C-labeled tyrosine, resulting in spectrum “b” in Appendix Figure A.6. Based on the peak area relative to the control ¹³CO spectrum, we estimate ≈ 4 μM HbCO formation in spectrum “a”.

HPLC Analysis of HydG Reaction Products

At given time points, aliquots of the HydG, deoxyhemoglobin assay mixtures were removed and quenched by addition of 1 M HCl. Samples were then boiled for 40 seconds, then centrifuged two subsequent times at 14,000 rpm at 4 °C and the supernatant was subjected to HPLC analysis. Initial sample aliquots were run through a YM-3 Microcon filter (Millipore) prior to subjecting the flow through to HPLC analysis. All subsequent samples were not subjected YM-3 filter treatment, as it was discovered that p-cresol binds to the YM-3 filter (see below). Samples were injected onto a Phenomenex (Torrance, CA) Curosil 5 μm 4.6 x 150 mm analytical column that had been equilibrated at 98% solution A (H₂O plus 0.1% acetic acid) and 2% solution B (CH₃CN plus 0.1% acetic acid) at 1 mL/min. An isocratic mobile phase at these percentage values ran for 7 minutes following sample injection at which time a linear gradient to 40% solution A,
60% solution B was run over the next 17 minutes. At this time the gradient was held isocratic for 3 minutes and then the column was re-equilibrated to 98% solution A and 2% solution B for another sample injection. The column temperature during runs was held at 30 °C and total run time was 35 minutes. Elution of reaction products was monitored via absorbance at 280 nm and under these conditions AdoMet eluted at ~ 2 minutes, tyrosine eluted at ~ 4 minutes, deoxyadenosine eluted at ~ 12 minutes, and p-cresol eluted at ~ 20 minutes (Appendix Figure A.5). Integration of peak area using Origin 7.0 (Microcal; Northampton, MA) relative to standard samples run in parallel allowed for quantification of reaction product concentrations.

Appendix Figure A.5 shows the reaction components from the experiment shown in Appendix Figure A.4, including unreacted AdoMet and tyrosine and the reaction products 5’-deoxyadenosine and p-cresol. Absorbance changes for the assay mixture in Appendix Figure A.4 were monitored for a total time of 1.5 hours yielding a final HbCO concentration of 6.7 µM (data not shown). HPLC injections were performed in triplicate and quantification of 5’-deoxyadenosine produced during this time was shown to be 103.6 ± 6.6 µM, while p-cresol formed was found to be 55.1 ± 5.5 µM. Sample aliquots for this analysis had been subjected to treatment with a YM-3 filter membrane and following HPLC analysis, it was demonstrated using control solutions that approximately 50% of an applied concentration of p-cresol would bind to the YM-3 filter membrane. All subsequent aliquots for HPLC analysis, such as those shown in Figures 2.2C and Appendix Figure A.7, were not subjected to YM-3 filter treatment. Adjusting the yield of p-cresol for this fact shows that the 33 µM enzyme in Appendix Figure A.5 underwent a
total of ~ 3.08 turnover events during 1.5 hours, which agrees quite well with levels of turnover reported by the Roach laboratory. 

Appendix Figure A.7 shows the end result of a similar experiment. In this case, 64 µM (6.4 ± 0.2 Fe/protein) produced 9.5 µM HbCO in 75 minutes. The amounts of 5’-deoxyadenosine and p-cresol were determined to be 117.5 ± 10.8 µM and 102.1 ± 5.9 µM, respectively. Although lower total turnover was observed in this experiment, which is likely due to the lower iron number of the enzyme sample, it demonstrates nearly stoichiometric 5’-deoxyadenosine:p-cresol formation.

Given the consistent results that showed substoichiometric HbCO formation relative to levels of 5’-deoxyadenosine and p-cresol, we designed an experiment to determine levels of HydG reaction products formed within 5 minutes. Figures 2.2B and 2.2C shows the results of an experiment where 45 µM HydG (7.2 ± 0.2 Fe/protein) was incubated with 10 µM heme deoxyHb, 1 mM AdoMet, and 500 µM tyrosine. The experiment was set up to run in single wavelength absorbance mode (at 419 nm) using a Cary spectrophotometer (similar to data shown in Appendix Figure A.4). At 1 minute time the program was paused and an aliquot of the reaction mixture was removed using a Hamilton (Reno, NV) gastight syringe (denoted by arrow in panel B in Figure 2.2). This aliquot was immediately quenched with 1 M HCl and flash frozen in liquid N₂. The program was reinitiated and at 2 minutes and 5 minutes additional aliquots were taken as described. These aliquots were prepared for HPLC analysis as described above, avoiding the use of YM-3 filter membranes (Figure 2.2C).

The data clearly shows that the amount of HbCO formed is less than the amount of 5’-deoxyadenosine and p-cresol produced. At 1 minute, we observe ~ 1 µM HbCO to
7.3 ± 0.33 μM p-cresol and 8.9 ± 0.04 μM 5’-deoxyadenosine. At 2 minutes, we observe
1.4 μM HbCO, 11.9 ± 1.9 μM p-cresol and 11.2 ± 0.15 μM 5’-deoxyadenosine and at 5
minutes we see 2.2 μM HbCO, 16.9 ± 0.17 μM p-cresol and 19.5 ± 0.38 μM 5’-
deoxyadenosine formation. We hypothesized that the low levels of CO relative to 5’-
deoxyadenosine and p-cresol may represent sequestration of some CO within HydG
itself. The assays developed by the Roach laboratory for cyanide detection used acid
denaturation of HydG and may therefore explain the greater levels of cyanide detection,
especially in light of the similar levels of 5’-deoxyadenosine and p-cresol observed in our
enzyme preparations that yield CO.
Appendix A References Cited


APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3
BIOCHEMICAL AND KINETIC CHARACTERIZATION OF RADICAL S-ADENOSYL-L-METHIONINE ENZYME HYDG

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Further Supporting Materials are available free of charge via the Internet at http://pubs.acs.org/doi/abs/10.1021/bi401143s. This contains 1 additional figure not included here in Appendix B.
Supplemental Figures

Figure B.1. X-band EPR Spectra of Reconstituted HydG Variants (12 K). (A) Photoreduced WT HydG (65 μM, 8.7 ± 0.7 Fe/protein). (B) Dithionite reduced HydG (101 μM, 6.5 ± 0.1 Fe/protein). (C) Photoreduced ΔCTD HydG (424 μM, 2.4 ± 0.2 Fe/protein). Experimental spectra are depicted in black, while composite simulation is shown in red for panels A and B, while panel C shows the simulation in blue. Samples were prepared in 50 mM Hepes, 0.5 M KCl, 5% (w/v) glycerol, pH 7.4. See experimental section in Chapter 3 for EPR spectrometer parameters.
Figure B.2. X-band EPR Spectra of Dithionite Reduced, Reconstituted WT HydG in the Presence of Substrate (Analogues). Reconstituted, dithionite reduced WT HydG (130 µM, 5.3 ± 0.3 Fe/protein) in 50 mM Hepes, 0.5 M KCl, pH 7.4 in the absence (black) or presence of tyrosine (1 mM, red), or AdoHcy and tyrosine (1 mM each, blue). Spectra were recorded at 10 K in the absence and 12 K in the presence of additives. See experimental section in Chapter 3 for EPR spectrometer parameters.

Figure B.3. UV-visible Characterization of C386S HydG. Representative WT (solid, 8.0 ± 0.9 Fe/protein) and C386S (dashed, 6.4 ± 0.8 Fe/protein) spectra.
Figure B.4. X-band EPR Spectra of Dithionite Reduced, Reconstituted C386S HydG at 12 K in the Absence and Presence of 1 mM AdoMet. C386S HydG (141 $\mu$M, 4.3 ± 0.4 Fe/protein) in (A) the absence or (B) the presence of commercial AdoMet (1 mM). Experimental spectra are depicted in black, while the composite simulation is shown in red. Spectral components to the simulated signal are depicted in the upper part of each panel.
Supplemental Tables

Table B.1. Summary of Iron Content in Reconstituted Samples of HydG Variants used in Chapter 3.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>WT</th>
<th>C386S</th>
<th>ΔCTD</th>
<th>C96/100/103A</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPR (Photoreduction)</td>
<td>8.7 ± 0.7</td>
<td>3.7 ± 0.4</td>
<td>2.4 ± 0.2</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>EPR (Dithionite Reduction)</td>
<td>5.3 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>$K_{M\text{AdoMet}}$</td>
<td>5.5 ± 0.4</td>
<td>4.8 ± 0.6</td>
<td>3.0 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td>$K_{M\text{Tyr}}$</td>
<td>7.6 ± 0.6</td>
<td>4.9 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>Time course</td>
<td>8.0 ± 0.4</td>
<td>6.4 ± 0.8</td>
<td>3.6 ± 0.2</td>
<td>NA</td>
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</tbody>
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Table B.2. EPR Characterization of Photo and Dithionite (DT) Reduced HydG Samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>g₁</th>
<th>g₁-strain</th>
<th>g₂</th>
<th>g₂-strain</th>
<th>g₃</th>
<th>g₃-strain</th>
<th>% spin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) N-Terminal Reduced</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>WT</td>
<td>2.0292</td>
<td>0.03019</td>
<td>1.9100</td>
<td>0.02796</td>
<td>1.8933</td>
<td>0.05680</td>
<td>54.3</td>
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<tr>
<td>WT.AdoMet</td>
<td>2.0310</td>
<td>0.03020</td>
<td>1.9199</td>
<td>0.02319</td>
<td>1.9053</td>
<td>0.04987</td>
<td>18.0</td>
</tr>
<tr>
<td>WT - DT</td>
<td>2.0243</td>
<td>0.02999</td>
<td>1.9160</td>
<td>0.02488</td>
<td>1.8971</td>
<td>0.06495</td>
<td>49.0</td>
</tr>
<tr>
<td>WT.AdoMet - DT</td>
<td>2.0352</td>
<td>0.03413</td>
<td>1.9149</td>
<td>0.02784</td>
<td>1.9102</td>
<td>0.05080</td>
<td>13.2</td>
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<tr>
<td>C386S - DT</td>
<td>2.0331</td>
<td>0.03979</td>
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<td>0.05415</td>
<td>1.8868</td>
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<td>ΔCTD</td>
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<td>0.03326</td>
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<td>0.03688</td>
<td>1.9029</td>
<td>0.05968</td>
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<td>ΔCTD.AdoMet</td>
<td>2.0359</td>
<td>0.03028</td>
<td>1.9151</td>
<td>0.03318</td>
<td>1.9047</td>
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<td>WT.AdoMet</td>
<td>1.9984</td>
<td>0.02607</td>
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<td>1.8393</td>
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<tr>
<td>WT.AdoMet - DT</td>
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<td>0.03069</td>
<td>1.8731</td>
<td>0.02484</td>
<td>1.8468</td>
<td>0.05758</td>
<td>63.3</td>
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<tr>
<td>C386S.AdoMet - DT</td>
<td>1.9988</td>
<td>0.01957</td>
<td>1.8919</td>
<td>0.02109</td>
<td>1.8352</td>
<td>0.03543</td>
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<td>ΔCTD.AdoMet</td>
<td>1.9953</td>
<td>0.02835</td>
<td>1.8768</td>
<td>0.02227</td>
<td>1.8402</td>
<td>0.05895</td>
<td>73.4</td>
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<td>(III) C-Terminal Reduced</td>
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<td></td>
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</tr>
<tr>
<td>C386S - DT</td>
<td>2.0167</td>
<td>0.04356</td>
<td>1.9312</td>
<td>0.02607</td>
<td>1.8595</td>
<td>0.06176</td>
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</tr>
<tr>
<td>C386S.AdoMet - DT</td>
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<td>0.06018</td>
<td>1.9325</td>
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<td>0.07669</td>
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<tr>
<td>WT - DT</td>
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<td>1.8747</td>
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<tr>
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<td>1.9198</td>
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<td>1.8803</td>
<td>0.07190</td>
<td>45.7</td>
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<tr>
<td>WT.AdoMet</td>
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<td>0.06251</td>
<td>1.9224</td>
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<td>C96/100/103A</td>
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<td>1.8795</td>
<td>0.07371</td>
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</table>
Supplemental Information

We previously demonstrated CN⁻ formation by WT HydG in 100 mM potassium phosphate pH 7.5 buffer¹ and have since attempted parallel detection of CO using hemoglobin as a reporter. While we are able to measure CO formation in HEPES² and Tris buffers, no CO could be detected in phosphate containing buffers. The current nature of detecting CO using hemoglobin and cyanide after acid denaturation of HydG thus far prevented parallel detection of cyanide and CO. To allow comparison of cyanide and CO quantification, we here used a 50 mM HEPES, 0.5 M KCl, pH 7.4 assay buffer instead.
Appendix B References Cited


APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4
Supplemental Figures

Figure C.1. ESI-MS HydG (100 µM; 4.4 ± 0.3 Fe/protein) Product 5’-dAdo Isotope Distribution Performed in H2O Buffer (50 mM tris, 300 mM KCl, 5% glycerol, pH 8.1). (A) [β-D2]-Tyr (B) [ring-D4]-Tyr (C) [α,ring-D5]-Tyr (D) Unlabeled Tyr. Spectra are scaled, extracted ion chromatograms.
Figure C.2. Control ESI-MS Product SAM Isotope Distributions. Performed in 95% D$_2$O buffer (50 mM tris, pD 8.1) with HydG (100 $\mu$M; 8.5 ± 0.1 Fe/protein). (A) Sample lacking Tyr. (B) Sample lacking HydG. (C) SAM Reference in H$_2$O. Spectra are represented as normalized, extracted ion chromatograms.
**Supplemental Tables**

Table C.1. Percent Sample Isotope Distribution of 5’-deoxyadenosine (5’-dAdo)

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<tr>
<th>Sample</th>
<th>5’-dAdo–CH$_3$</th>
<th>5’-dAdo–CH$_2$D</th>
<th>5’-dAdo–CHD$_2$</th>
<th>5’-dAdo–CD$_3$</th>
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</thead>
<tbody>
<tr>
<td>Figure 4.2A</td>
<td>31</td>
<td>34</td>
<td>19</td>
<td>16</td>
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<tr>
<td>Figure 4.2B</td>
<td>84</td>
<td>15</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Figure 4.2C</td>
<td>83</td>
<td>14</td>
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<td>0</td>
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<tr>
<td>Figure 4.4A</td>
<td>18</td>
<td>33</td>
<td>27</td>
<td>22</td>
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</tbody>
</table>

Table C.2. Percent Sample Isotope Distribution of S-adenosylmethionine (SAM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>SAM–CH$_2$</th>
<th>SAM–CHD</th>
<th>SAM–CD$_2$</th>
</tr>
</thead>
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<tr>
<td>Figure 4.5A</td>
<td>44</td>
<td>37</td>
<td>19</td>
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<tr>
<td>Figure C.2A</td>
<td>90</td>
<td>10</td>
<td>0</td>
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<tr>
<td>Figure C.2B</td>
<td>91</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>
Supplemental Methods

Materials

All chemicals and other materials used herein were from commercial sources and of the highest purity where available. Natural abundance tyrosine was obtained from Sigma-Aldrich (St. Louis, MO). [\(\beta-D_2\)]-tyrosine was obtained from Cambridge Isotope Laboratory (Tewksbury, MA). [ring-\(D_4\)]-tyrosine and [\(\alpha,\text{ring-}D_5\)]-tyrosine was obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Tris, HEPES, and DTT were obtained from RPI (Mt. Prospect, IL). KCl, acetonitrile (HPLC grade) and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite, \(D_2O\), and sodium sulfide was obtained from Acros Organics (Fair Lawn, NJ). Iron(III) chloride and acetic acid (99%, HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ). SAM\(^1\) was purified as reported elsewhere, with slight modifications.

HydG Overexpression, Purification, Reconstitution, and Assessment of Activity

Heterologous overexpression of *Clostridium acetobutylicum* HydG in *Escherichia coli*, purification and chemical reconstitution with iron and sulfide were prepared as described previously\(^2,3\) with slight modifications (Appendix A and Chapter 3). Briefly, single colonies obtained from transformations were grown overnight in LB media and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50 mM potassium phosphate buffer pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD\(_{600}\) = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl \(\beta-D-1\)-thiogalactopyranoside
(IPTG) (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37 °C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10 °C refrigerator and purged with N₂ overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at -80 °C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber (Grass Lake, MI), as described²³ with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4, 20 mM MgCl₂, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNAse and RNAse per gram cell, ~0.6 mg lysozyme per gram cell. The lysis mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles at 18000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrap™ Ni²⁺-affinity column (GE Healthcare, Uppsala). The column was pre-equilibrated with 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Pure fractions (gauged by SDS-PAGE) were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 and concentrated using an Amicon Ultra centrifugal unit (Millipore; Billerica, MA) fitted with a YM-10 membrane or using a Minicon B15 static protein concentrator (Millipore). Protein was flash frozen in liquid N₂ and stored at -80 °C or in liquid N₂ until further use.

Reconstitution of as-purified HydG was carried out following the general
procedures described previously. Enzyme (50 – 150 µM) was incubated with 6 – 7 fold excess of FeCl₃ and Na₂S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 for ≈ 3 hours with gentle agitation in a Coy anaerobic chamber at 22 °C. Following a 5 minute incubation with DTT, FeCl₃ was added slowly and stirred for 20 minutes prior to the stepwise addition of Na₂S. Following the reconstitution period, the mixture was centrifuged to remove exogenous Fe–S clusters in the mixture. Following initial concentration using an Amicon centrifugal unit, the supernatant was treated over a Sephadex G-25 column (GE Healthcare; Piscataway, NJ) to remove excess ions. Following this, the darkest brown fractions were pooled and concentrated using an Amicon centrifugal unit. UV-visible spectroscopy was utilized to confirm the Fe–S cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Enzyme concentration was determined by performing a Bradford assay, while the iron content was determined through the colorimetric ferrozine method.

HydG activity was determined by performing by assessing CO production using methods described previously (Chapter 2 and Appendix A). Briefly, typical samples contained 40 µM HydG with 1 mM Tyr, 1 mM AdoMet, 80 µM deoxyhemoglobin (per heme), and 5 mM dithionite, in 50 mM tris, 300 mM KCl, pH 8.1 (400 µL total volume), and was prepared in an anaerobic chamber (MBraun; Stratham, NH). Tyr solid was dissolved in 1.0 M HCl (22.2% v/v) and was diluted with 100 mM tris, pH 7.4 buffer to make a concentrated 67.7 mM stock. For experiments, a working 2 mM Tyr stock was prepared by diluting 29.6 µL of the concentrated stock into the buffer of interest to 1000 µL total. CO experiments were performed using a Cary 6000i dual pathlength UV-visible
Spectrophotometer (Agilent; Santa Clara, CA) in a 1 mm pathlength anaerobic cuvette (Spectrocell; Oreland, PA) fitted with a teflon membrane. Upon performing an initial 300-800 nm scan (600 nm/min), SAM was added using a stoppered 25 µL gastight syringe (Hamilton; Reno, NV). Upon addition and mixing, 419 nm absorbance (the Soret \( \lambda_{\text{max}} \) for carboxyhemoglobin) was monitored every second for 30 min at 37 °C. Following this, a final 300-800 nm scan was made. Enzyme stocks used for time course and H atom abstraction experiments described herein produced on upwards of 30 µM CO after 30 min at 37 °C, under the conditions reported above.

**H Atom Abstraction Experiments**

Assays were performed in either H2O or D2O containing buffer, under conditions described in Chapter 4. Experiments were performed in an anaerobic chamber (Mbraun) under strict anaerobic conditions (<1 ppm O2). H2O buffers were prepared by degassing on a schlenk line (3 x 10 minute vacuum cycles followed by N2 backfill). Buffer volume lost to evaporation was replaced with anaerobic H2O in the glovebox. D2O buffers were prepared by lyophilization of H2O buffer on the schlenk line for 16 hours. The resultant salt was brought into the MBraun box and was dissolved with degassed D2O to original volume. Prior to suspending the salt in D2O, the schlenk flask was briefly subjected to a heat gun to drive off remaining H2O moisture. Upon resuspending the buffer salt in D2O, the pD was measured with pH paper (Micro Essentials; Brooklyn, NY) and was as expected acidic by 0.4 units. Working 2 mM Tyr stocks in D2O were prepared in a similar fashion to buffers described above, via dilution from an aerobic 67.7 mM stock in H2O (prepared by dissolving Tyr in 1 M HCl, then dilution with 100 mM tris, pH 7.4)
that was lyophilized overnight.

Experiments were performed at 37 °C in an IsoTemp heatblock (Fisher), containing 100 µM HydG (8.5 ± 0.2 Fe/protein), 1 mM AdoMet (enzymatically prepared), 1 mM Tyr, and 5 mM dithionite (80 µL volume) for 60 minutes, where the enzyme was precipitated via 1:1 volume addition of acetonitrile (HPLC grade, EMD) or addition of 1 M HCl (13 % v/v). A concentrated HydG stock at ~ 2 mM was used to minimize the H₂O contribution for performed experiments. Use of either quenching medium did not affect the observed product isotope distribution. Samples were centrifuged for 3 x 10 minutes at 14,000 rpm, where the supernatant was collected. Samples were then used for LC-MS or HPLC, as described below. Reported assay pHs were corrected for changes related to temperature. ⁹

HydG Time Course Assays

Time-dependent production of 5'-dAdo and p-cresol by HydG was performed in a manner similar to H atom abstraction experiments performed above, in an anaerobic chamber. Assays were performed in duplicate at 37 °C in heatblock containing 40 µM HydG (9.5 ± 0.2 Fe/protein), with 1 mM AdoMet, 1 mM Tyr, 5 mM dithionite, in 50 mM tris, 300 mM KCl, pH 8.1 buffer (80 µL total volume). Reported assay pHs were corrected for changes related to temperature. ⁹ Experiments were initiated by the addition of AdoMet, and samples were incubated for 30 sec, 1 min, 2 min, 5 min, 10 min, 15 min, 30 min, or 60 minutes before quenching by the addition of 1 M HCl (13% v/v). Samples were centrifuged for 3 x 10 min to ensure removal of the precipitated enzyme. Samples were injected onto a Phenomenex (Torrance, CA) Kinetex PFP analytical column (150 x
4.60 mm) connected to an Agilent 1100 series HPLC containing an autosampler. The column was equilibrated with 98% solution A (H₂O + 0.1% acetic acid) and 2% solution B (CH₃CN + 0.1% acetic acid) at 1 mL/min. Following sample injection, the mobile phase was held isocratically for 7 minutes, and a linear gradient to 40% solution A, 60% solution B was run over the next 17 minutes. At this point, the gradient was held isocratically for 3 minutes before the column was re-equilibrated to 98% solution A and 2% solution B for subsequent sample injections. The column temperature was held at 30 °C, and the total run time was 35 minutes. Product detection was monitored at 280 and 254 nm. AdoMet eluted at ~2 minutes, tyrosine eluted at ~4 minutes, deoxyadenosine eluted at ~12 minutes, and p-cresol eluted at ~18 minutes. Integration of peak area relative to standard samples ran in parallel using Agilent’s ChemStation data analysis package allowed for quantitation of reaction product concentrations, and data was fitted using OriginPro v. 8.6 (OriginLab Corp; Northampton, MA).

**LC-MS Methods**

Deuterium isotope distribution in AdoMet and 5′-dAdo was assessed using LC-MS. After centrifugation of the precipitated HydG, samples prepared in H₂O were diluted 100-fold in 10% methanol; D₂O samples were treated without further dilution. Quantitation of 5′-deoxyadenosine, AdoMet, and tyrosine was performed using an Agilent 1290 series UHPLC coupled to an Agilent 6538 Q-TOF mass spectrometer equipped with the dual-ESI source and an autosampler. Samples were injected onto a normal phase Microsolv (Eatontown, NJ) Cogent “Diamond Hydride” HPLC column (150 x 2.1 mm) equilibrated with solution B (CH₃CN + 0.1% formic acid) at 0.8 mL/min.
An isocratic mobile phase was maintained for 2 minutes following sample injection, and then a linear gradient from solution A (H₂O + 0.1% formic acid) to 50% solution B was run for 4 minutes. This was followed by isocratic elution for 2.5 minutes at 50% solution B after which it was immediately re-equilibrated to 100% B for 1.5 minutes. Total run time was 10 minutes and the column temperature was maintained at 50 °C. Under these conditions, 5’-dAdo eluted at ~ 3 minutes, AdoMet eluted at ~ 6 minutes, and tyrosine eluted at ~ 4 minutes. The capillary exit voltage was 120 V and gas temperature was 300 °C. All data was recorded in positive mode between 25 m/z and 750 m/z in profile mode. Hardware summation time was 1 second. Peaks were validated using high-accuracy formula confirmation and elution matching from individual reference standards. Quantitation was performed using the MassHunter Quantitative Analysis package (Agilent). An extracted ion chromatograph was generated using the accurate mass of each compound with a -0.04 to +0.04 ppm m/z extraction window. Samples were injected in duplicate, and between sample injections, a water blank injection was performed to minimize sample-to-sample carryover.

Sample isotope distributions for 5’-dAdo and SAM were assessed using the natural abundance distribution as a template distribution. Incorporation of a deuterium atom corresponds to a net mass increase of one, but results in an otherwise identical distribution to the template distribution. Respective isotopic mass abundances were subtracted, and quantities for each were normalized to 100% total.

In addition to the experiments described in the main text, samples were also quenched immediately after AdoMet was added, to assess deuterium label incorporation. Negligible 5’-dAdo product was observed after HCl was added ~ 2 seconds after AdoMet
was added, and the observed AdoMet isotope distribution was identical to a reference standard prepared in H₂O. Finally, for experiments performed in D₂O, no label transfer was observed on tyrosine substrate. The mass spectrum of Tyr was found to be identical to a reference standard prepared in H₂O.
Appendix C References Cited


APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER 5
DEFINING A BASIS FOR DIATOMIC LIGAND PRODUCT BINDING TO THE RADICAL SAM ENZYME HYDG FE–S CLUSTERS

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2Department of Chemistry, University of California–Davis, Davis, CA 95616
3Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Supplemental Figures

Figure D.1. X-band EPR Spectral Simulation Results to Accompany Figure 5.4. Black trace, experimental spectrum; red trace, composite simulation; blue trace, N-terminal cluster (unbound); green trace, N-terminal cluster (SAM-bound); magenta trace, C-terminal cluster (unbound). Spectral parameters are found in Appendix Table D.1.
Figure D.2. X-band (9.37 GHz, 12 K) EPR Spectra of Dithionite-reduced HydG$^{\text{WT}}$. HydG$^{\text{WT}}$ (96 µM; 8.5 ± 0.1 Fe/protein) was reduced with 1 mM sodium dithionite, prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. Black trace, reduced HydG$^{\text{WT}}$; red trace, reduced HydG$^{\text{WT}}$ with 1 mM SAM; blue trace, reduced HydG$^{\text{WT}}$ with 10 mM cyanide. It should be noted that the HydG$^{\text{WT}}$ stock used here, upon photoreduction yields a low-field 3250 G feature similar to that depicted in Figure 5.12 (black and red traces), but is absent upon dithionite reduction. Spectral signals are similar to those reported in Appendix Table B.2 for the SAM-bound cluster, while spectral simulation values for the cyanide-bound sample (blue trace) can be found in Appendix Table D.3.
Figure D.3. Comparison of HydG<sup>WT</sup> CO Formation Ability in the Presence of Reporting Heme Molecules. Black trace, sperm whale H64L deoxyMb; red trace, human deoxyHb. Experiments contained 40 µM HydG<sup>WT</sup> (6.6 ± 0.7 Fe/protein), 1 mM dithionite, 1 mM SAM, and 1 mM tyrosine, and were performed at 37 °C, monitoring A<sub>423nm</sub> and A<sub>419nm</sub> absorbance changes for the carboxyMb and carboxyHb, respectively. HPLC quantitation of the carboxyHb sample produced 305 ± 14 µM p-cresol, while the carboxyMb sample produced 296 ± 21 µM p-cresol following duplicate injections on HPLC, respectively.

Figure D.4. Difference UV-visible Absorbance Spectra of HydG<sup>WT</sup> for Kinetics Data Depicted in Figure 5.9. Experiments were performed with 40 µM HydG<sup>WT</sup> (6.4 ± 0.1 Fe/protein) at 25 °C with 5 mM sodium dithionite, 80 µM H64L deoxyMb, 200 µM SAM and 200 µM Tyr. Difference represents absorbance changes after incubation for 10 minutes.
Figure D.5. Examining the Effect of Exogenous CO and Cyanide on HydF by Stopped-Flow FTIR Spectroscopy. HydF containing dithionite was shot against buffer containing dithionite, $^{12}$CO (500 µM), and cyanide (500 µM). Red traces correspond to time-averaged plot from 0.5 - 5.5 seconds, while blue traces correspond to time-averaged plot from 1515 - 1965 seconds. Magenta trace corresponds to a potassium ferrocyanide reference (50 µM), while the orange trace corresponds to a potassium cyanide reference (5 mM). Experiment was performed with HydF$^{\Delta G}$ (395 µM; 0.9 ± 0.2 Fe/protein) and HydF$^{E\Delta G}$ (305 µM; 0.4 ± 0.2 Fe/protein). Experiments were performed in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. The HydF$^{\Delta G}$ protein was prepared by E.M.S., while the HydF$^{E\Delta G}$ was prepared by B.R.D. Stopped-flow FTIR experiments were performed by B.R.D. and S.J.G.
Figure D.6. Time Course of HydF Vibrational Bands of Interest from Figure D.4. (A) In the HydF$^{\Delta G}$ and added CO, cyanide experiment, a decrease in the vibrational frequency at 2038 cm$^{-1}$ (red trace) is concomitant with an increase at 2074 cm$^{-1}$ (blue trace). (B) In the HydF$^{\Delta G}$ experiment, a vibrational band at 2123 cm$^{-1}$ appears to increase in the first 2 minutes of reaction.

Figure D.7. Zoomed Image of Baseline-subtracted HydF with Added CO and Cyanide Data Depicted from Figure D.4. Experimental scans represent time-averaged plot from 1515-1965 seconds. Frequencies for HydF vibrational bands are assigned and compared to cyanide (blue trace) and ferrocyanide (green trace) controls. Black trace corresponds to experiment performed with HydF$^{E_{\Delta G}}$ (spectrum scaled x 2), while red trace corresponds to experiment performed with HydF$^{\Delta G}$ (spectrum scaled x 0.1). The HydF$^{\Delta G}$ protein was prepared by E.M.S., while the HydF$^{E_{\Delta G}}$ was prepared by B.R.D. Stopped-flow FTIR experiments were performed by B.R.D. and S.J.G.
Table D.1. Spectral Simulation Parameters for EPR Data from Appendix Figure D.1.

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<th>g-value</th>
<th>g-strain</th>
<th>g-value</th>
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Table D.2. Spectral Simulation Parameters for EPR Data from Figure 5.10.

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Table D.3. Spectral Simulation Parameters for EPR Data from Appendix Figure D.2 (blue trace).

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APPENDIX E

SUPPORTING INFORMATION FOR CHAPTER 6
HYDG CARBON MONOXIDE FORMATION STOICHIOMETRY: THE ROLE OF PHOSPHATE IN DIATOMIC LIGAND BIOSYNTHESIS

Benjamin R. Duffus,1 Rebecca C. Driesener,2 Eric M. Shepard,1 Peter L. Roach,2,3 John W. Peters,1 and Joan B. Broderick1

1Department of Chemistry & Biochemistry, Montana State University, Bozeman, MT 59717
2Chemistry, Faculty of Natural and Engineering Sciences, University of Southampton, Highfield SO17 1BJ, U.K.
3Institute for Life Sciences, University of Southampton, Highfield SO17 1BJ, U.K.

Supporting Figures

Figure E.1. Difference UV-visible Absorbance Spectra, Comparing CO Recovery Yield Following Denaturation and Addition of deoxyMb. Black trace, HydG reaction where 100 µM HydGWT (7.0 ± 0.1 Fe/protein) incubated for 30 minutes at 25 °C with 1 mM Tyr, 1 mM SAM, and 5 mM dithionite (40 µL); red trace, denaturation of 46 µM carboxymyoglobin (heme) (40 µL).
Figure E.2. Control UV-visible Absorbance Spectra for CO Detection by Enzyme Denaturation Experiments. Black trace, absorbance spectrum following addition of 340 μL of 50 mM HEPES, 500 mM KCl, 5% glycerol to precipitated 459 μM (heme) carboxyMb (40 μL) mixture following sample boiling for 2 minutes. Red trace, absorbance spectrum of 46 μM (heme) carboxyMb, scaled by 0.05.
APPENDIX F

SUPPORTING INFORMATION FOR CHAPTER 7
EFFECTOR AND INTERMEDIATE MOLECULE INTERACTION WITH RADICAL SAM [FEFE]-HYDROGENASE MATURASE HYD

Benjamin R. Duffus, Eric M. Shepard, John W. Peters, and Joan B. Broderick

From the Department of Chemistry and Biochemistry
Montana State University, Bozeman, MT 59717

Supporting Figures

Figure F.1. UV-Visible Absorbance Spectroscopy of HydGNTM in the Presence of Cysteine. HydGNTM (2.5 + 0.1 Fe/protein) prepared in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 with 5 mM DTT. Absorbance scans represent identical sample data to that depicted in Figure 7.2. Black trace, no cysteine added; red trace, 250 µM cysteine; violet trace, 4 mM cysteine.
Figure F.2. X-band (9.37 GHz, 12 K) EPR Spectroscopy of Photoreduced HydG<sup>NTM</sup> in the Absence and Presence of Tyrosine. Black trace, no tyrosine; red trace, 1 mM tyrosine added. Samples also contain 100 µM 5-deazaflavin, 50 mM tris, and 5 mM DTT in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4. EPR spectral acquisition parameters can be found in the Chapter 7 experimental section.

Figure F.3. HydG<sup>W1</sup> SAM Titration, Monitored by (A) UV-visible CD and (B) Absorbance Spectroscopy. Experiments were performed at 23 °C. Thick black line represents no SAM added, while the thick red line represents [SAM] = 7 mM. Thin, dotted black lines represent titrated amounts of SAM at 1 mM increments.
Figure F.4. Difference CD Spectra of SAM Titration to HydG WT. Experiments were performed at 23 °C. Thick red line represents the difference of 1 mM SAM added relative to no addition, while the thick blue line represents the difference of 7 mM SAM added relative to no addition. Thin black, dotted lines represent the titrated difference spectra between 1 mM and 7 mM SAM at 1 mM increments.

Figure F.5. Molar Ellipticity Plots for HydG \textsuperscript{WT} and HydG \textsuperscript{NTM}. Black trace, HydG \textsuperscript{WT} (N-, C-terminal [4Fe–4S] clusters); red trace, HydG \textsuperscript{NTM} (C-terminal [4Fe–4S] cluster); blue trace, spectral difference between HydG \textsuperscript{WT} and HydG \textsuperscript{NTM} (N-terminal [4Fe–4S] cluster). Molar ellipticity was calculated by determining the Fe concentration for each sample, and calculating the molar ellipticity according to Equation 7.1.
APPENDIX G

SUPPORTING INFORMATION FOR CHAPTER 8
Figure G.1. Product Quantitation of Tyrosine Analogue Formation of (A) 5'-dAdo, (B) SAM, and (C) p-cresol at pH 7.0. Error bars represent standard deviation following triplicate sample injections on the HPLC, while the isotope distributions in Panels A and B were measured from the sample using LC-MS from Figure 8.4. The p-cresol product from the NH₂–Y could not be detected by the HPLC method used.
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