

MECHANISTIC AND SPECTROSCOPIC INVESTIGATIONS OF THE RADICAL
SAM MATURASES HydE AND HydG FOR [FeFe]-HYDROGENASE

by

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DEDICATION

This accomplishment and all others big and small are dedicated to my high school prom date, my goat herder, the mother of my four children, my partner in crime, my fellow sojourner in this crazy beautiful life we have created, my best friend, my loving wife, Megan.

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LIST OF ABBREVIATIONS

aRNR-AE: anaerobic ribonucleotide reductase activating enzyme
ATP: adenosine-5'-triphosphate
BioB: biotin synthase
BLAST: basic local alignment search tool
BtrN: butirosin biosynthetic enzyme
Ca: *Clostridium acetobutylicum*
CBI: 1-cyanobenzy[f]isoindole
Ct: *Clostridium thermocellum*
CoA: coenzyme A
CoM: coenzyme M
Cp: *C. pasteurianum*
Cpl: *C. pasteurianum* I [FeFe]-hydrogenase enzyme
CTC: C-terminal cluster
 Δ CTD: HydG without C-terminal domain
CW: continuous wave
dAdo•: deoxyadenosyl radical
dAdoH: 5'-deoxyadenosine
DALI: distance alignment matrix method
deoxyHb: deoxyhemoglobin
DHG: dehydroglycine
DRF: 5-deazariboflavin
DTMA: 1,3-dithiomethylamine
DTT: dithiothreitol
Ec: *Escherichia coli*
E. coli: *Escherichia coli*
ENDOR: electron-nuclear double resonance
EPR: electron paramagnetic resonance
FeS: iron sulfur
FLD: fluorescence detector
FPLC: fast protein liquid chromatography
FTIR: fourier transform infrared spectroscopy
 γ GC: γ -L-glutamyl-L-cysteine
GSH: glutathione
GTP: guanosine-5'-triphosphate
HbCO: carboxyhemoglobin
HC: homocitrate
Hcy: homocysteine
HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HemN: coproporphyrinogen III oxidase enzyme
HmdB: [Fe-only]-hydrogenase enzyme
HPLC: high pressure liquid chromatography

LIST OF ABBREVIATIONS – CONTINUED

HydA: [FeFe]-hydrogenase
 HydA^{ΔEFG}: HydA expressed without HydE, HydF, and HydG
 HydA^{EFG}: HydA expressed with HydE, HydF, and HydG
 HydF^{ΔEFG}: HydF expressed without HydE and HydG
 HydF^{EFG}: HydF expressed with HydE and HydG
 HydE: [FeFe]-hydrogenase maturase E
 HydF: [FeFe]-hydrogenase maturase F
 HydG: [FeFe]-hydrogenase maturase G
 HYSCORE: hyperfine sublevel correlation spectroscopy
 IPTG: isopropyl β-D-1-thiogalactopyranoside
 ISC: iron sulfur cluster synthesis enzymes
 Δ*iscR*: deletion of the *isc* repressor gene
 LAM: lysine 2,3-aminomutase
 LB: Luria broth
 LC-MS: liquid chromatography mass spectrometry
 LipA: lipoyl synthase enzyme
 βME: β-mercaptoethanol
 MiaB: tRNA modifying enzyme
 MoaA: molybdopterin biosynthetic enzyme A
 MP: mercaptopyruvate
 3-MPA: 3-mercaptopropionic acid
 MTA: methylthioadenosine
 MWCO: molecular weight cut off
 NADPH: nicotinamide adenine dinucleotide phosphate
 NaDT: sodium dithionite
 NCBI: National Center for Biotechnology Information
 NIF: nitrogenase FeS cluster assembly enzymes
 Ni-NTA: nickel nitrilotriacetic acid resin
 NMR: nuclear magnetic resonance
 NosL: tryptophan lyase enzyme
 NRVS: nuclear resonance vibrational spectroscopy
 NTM: HydG N-terminal mutant protein
 PDB ID: Protein Data Bank identification code
 PFL: pyruvate formate lyase
 PFL-AE: pyruvate formate lyase activating enzyme
 PhnJ: phosphonate metabolism enzyme
 PLP: pyridoxal phosphate
 PMSF: phenylmethylsulfonyl fluoride
 PylB: methylornithine synthase enzyme
 RMSD: root-mean-square deviation
 RSC: radical SAM cluster

LIST OF ABBREVIATIONS – CONTINUED

QueE: 7-carboxy-7deazaquanine synthase enzyme
SAH: *S*-adenosylhomocysteine
SAM: *S*-adenosylmethioinine
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFLD: Structure Function Linkage Database
So: *Shewanella oneidensis*
SPL: spore photoproduct lyase enzyme
SUF: FeS cluster assembly proteins
ThiC: thiamin pyrimidine biosynthetic enzyme
ThiH: thiamine biosynthesis enzyme H
TIM: triosephosphate isomerase
Tm: *T. maritima*
Tn: *T. neopolitana*
Tt: *Thermoanaerobacter tengcongensis*
TWB: thesis writing burnout
WT: wild type
XAS: X-ray absorption spectroscopy
YfiD: radical containing enzyme

ABSTRACT

While biochemical, spectroscopic, and analytical investigations helped classify multiple phylogenetically distinct hydrogenases it was not until 2004 that Peters *et al.* gave the world a look at the non-proteinaceous component of the active site of a hydrogenase enzyme. The active site (H-cluster) of [FeFe]-hydrogenase was found to possess a typical [4Fe-4S] cluster bridged by the sulfur of a cysteinyl group to an iron of a uniquely decorated 2Fe subcluster that serves as the site of molecular hydrogen synthesis and oxidation. The subcluster contains two irons bridged by a dithiomethylamine (DTMA) group and a carbon monoxide ligand. In addition each iron is coordinated by a carbon monoxide and cyanide ligand. Posewitz *et al.* in 2004 were the first to shed light on the syntheses of these non-proteinaceous ligands when through an insertional mutagenesis study of a hydrogen producing green alga they found two radical SAM enzymes, HydE and HydG, that were required for the maturation of [FeFe]-hydrogenase. HydG has been extensively studied and been shown to produce the diatomic ligands of the H-cluster from tyrosine. In this work the substrate specificity and active site of HydG was investigated. These investigations led to a refinement of the location and mechanism of H-atom abstraction of the substrate HydG and support the identity of the C-terminal FeS cluster as a [4Fe-4S] cluster that is responsible in the later steps of diatomic production. While several crystal structures of HydE have been published, the work reported herein is the first to propose a substrate and reaction mechanism for HydE. The results point to commonly biologically available low molecular weight thiols such as L-cysteine, L-homocysteine, and mercaptopyruvate as likely substrates. More recent work has implicated mercaptopyruvate as the substrate given glyoxylate was produced under turnover conditions. Our proposed mechanism involves formation of thioformaldehyde from mercaptopyruvate. Two thioformaldehyde units may be condensed with ammonia forming the DTMA precursor. While many details remain unsolved regarding the maturation of [FeFe]-hydrogenase, our findings regarding HydE and HydG are important steps forward in the understanding of biological catalysts of hydrogen production.

CHAPTER 1

INTRODUCTION

Themes in Radical SAM Enzymes

The abstraction or transfer of a hydrogen atom ($H\bullet$) from an unactivated bond *in vivo* is often carried out by transition metal containing enzymes. Due to its multiple biologically accessible oxidation states, iron is more often than not called upon to aid in hydrogen atom abstraction mechanisms. This is illustrated in one major class of heme containing proteins, cytochrome P450 that under anaerobic conditions may utilize an oxyferryl heme group to abstract an H-atom from a C-H bond eventually producing an alcohol.⁽¹⁾ This oxygen insertion reaction is used in solubilizing foreign hydrophobic chemicals and synthesizing biologically important chemicals. The radical *S*-adenosyl-L-methionine (SAM) superfamily,⁽²⁾ on the other hand, is primarily responsible for anaerobic H-atom abstraction events that initiate a wide array of biochemical transformations. Radical SAM enzymes all contain a [4Fe-4S] cluster, in which three of the irons are coordinated by sulfurs from three cysteine residues and one by the carboxyl and amine functional groups of SAM in a bidentate fashion (Figure 1.1). Over 5000 anaerobic and aerobic organisms from all three domains of life possess radical SAM enzymes. Roughly fifty thousand radical SAM enzymes have been putatively identified through bioinformatics analyses of sequences in the NCBI sequence database illustrating their importance in biology.

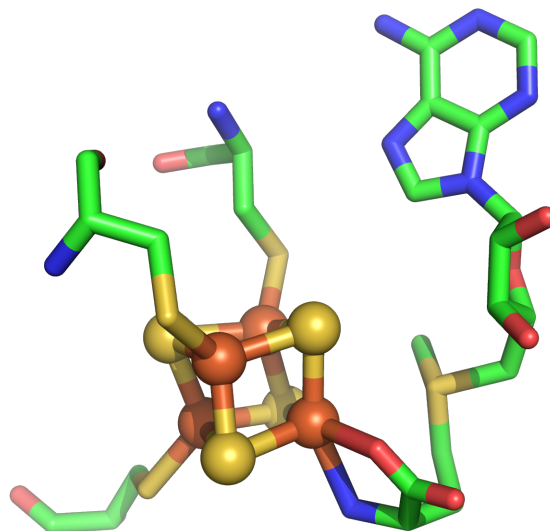


Figure 1.1. The site differentiated [4Fe-4S] cluster coordinated by the Cys residues of the CX₃CX₂C motif and SAM (PDB ID: 3IIZ). Atoms in figures are colored as follows: Green (C), Blue (N), Red (O), Yellow (S), and Orange (Fe) unless otherwise noted.

Early Characterization and Superfamily Classification

As one can only see the forest when the number of trees is large and the vantage point is sufficiently far, the description of the radical SAM superfamily arose only after the discovery and biochemical and spectroscopic characterization of several key enzymes. New bioinformatics tools and low cost sequencing led to the discovery of many representative enzymes further catalyzing the superfamily designation.^(2, 3) The classification scheme is largely built around the highly conserved CX₃CX₂C motif whose thiolate moieties bind three irons of a site differentiated [4Fe-4S] cluster. Early members, which are still currently used as model enzymes for understanding radical SAM mechanisms, include lysine amino mutase (LAM), pyruvate formate lyase activating enzyme (PFL-AE), and biotin synthase (BioB).⁽⁴⁾ While these enzymes

catalyze very different overall chemistries it is held that their early mechanistic steps are conserved, as later described.

Catalytic Reaction Examples

A small sample set of the breadth of reactions that radical SAM enzymes are capable of catalyzing is represented in Figure 1.2: isomerizations (A and E), enzyme activation (B), sulfur insertion (C), amino acid cleavage (D), and complex rearrangements (F). Four examples of some of the best characterized radical SAM enzymes are highlighted in the following sections.

Lysine 2,3-aminomutase (LAM) Radical SAM enzymes are often employed to catalyze reactions involving amino acid metabolism. LAM, for example, stereoselectively converts L-lysine to L- β -lysine in the first step of the lysine fermentation pathway. LAM was shown to require SAM and the cofactor pyridoxal phosphate (PLP) for full mutase activity and activity enhanced with the addition of exogenous iron (Fe^{2+}).⁽⁵⁾ Subsequent investigations showed that four irons were bound per subunit, and these were assigned to a [4Fe-4S] cluster.⁽⁶⁾ Electron-nuclear double resonance (ENDOR) studies demonstrated that SAM coordinated to this [4Fe-4S] cluster via the amino and carboxylate moieties, and suggested that upon reductive cleavage the methionine would be bound as a tridentate chelate with the added coordination of the sulfur.⁽⁷⁾ The X-ray crystal structure of LAM from *Clostridium subterminale* revealed a dimer of dimers where each structural unit contains a splayed $(\beta\alpha)_6$ motif (Figure 1.3A and B) similar to a TIM $(\beta\alpha)_8$ barrel.⁽⁸⁾ LAM is rather unusual amongst radical SAM

enzymes as its quaternary structure is stabilized by both zinc ion binding and domain swapping.⁽⁸⁾

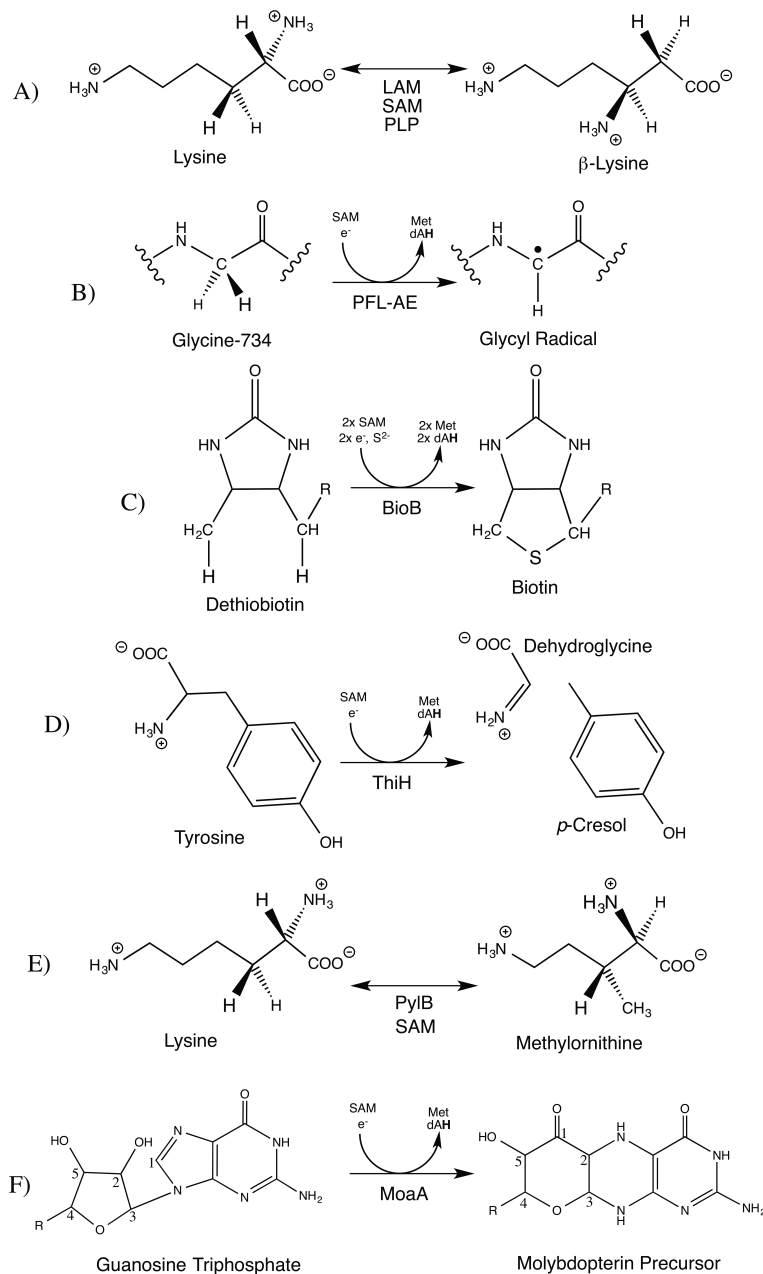


Figure 1.2. Overall reactions of select radical SAM enzymes. A) Lysine 2,3-aminomutase (LAM). B) Pyruvate formate lyase-activating enzyme (PFL-AE). C) Biotin synthase (BioB). D) Thiamine biosynthesis enzyme H (ThiH). E) Pyrrolysine biosynthesis enzyme B (PylB). F) Molybdopterin biosynthetic enzyme A (MoaA). R = triphosphate group.

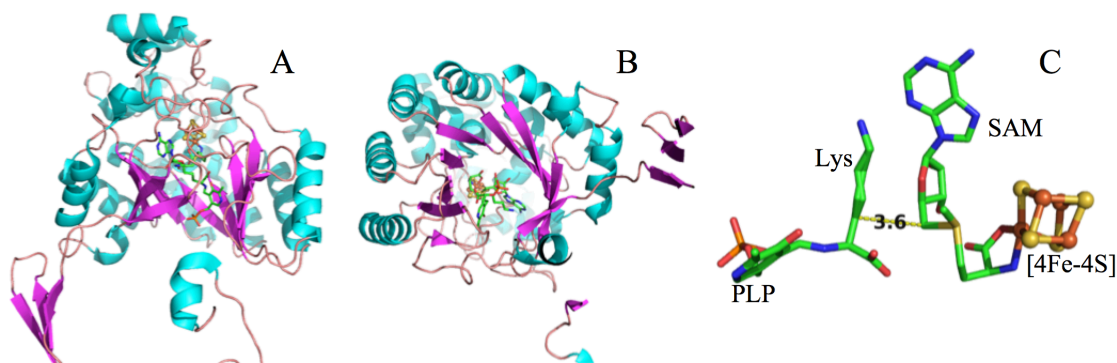


Figure 1.3. Structure of the LAM subunit as viewed from side (A) and bottom (B) of the partial TIM barrel. Alpha helices in light blue and beta strands in magenta. The active site highlighting the [4Fe-4S] cluster, SAM, PLP, and Lys (C). The distance in Å between lysine's carbon with abstractable hydrogen and 5'C of SAM marked with yellow dash (PDB ID: 2A5H).

The LAM isomerization reaction (Figure 1.4) is completely reversible, with SAM used as a cofactor and regenerated after each catalytic cycle.⁽⁹⁾ PLP forms a Schiff base with the α or β amine nitrogen of lysine immobilizing the substrate. Upon reductive cleavage of SAM, the 5'-deoxyadenosyl radical (dAdo•) intermediate is produced, which abstracts a hydrogen atom from lysine to initiate rearrangement. The active site is located within the partial TIM barrel, and the distance between C5' of SAM and the β carbon of lysine was determined to be 3.6 Å (Figure 1.3C);⁽⁸⁾ at this distance the radical can swing over and abstract a hydrogen from either the β carbon of lysine or the α carbon of β -lysine.⁽⁹⁾ The generally accepted isomerization mechanism has a cyclic intermediate that includes the α carbon and nitrogen and the β carbon of lysine (Figure 1.4) in a manner that is likely similar to the analogous enzyme glutamate 2,3-aminomutase.^(10, 11)

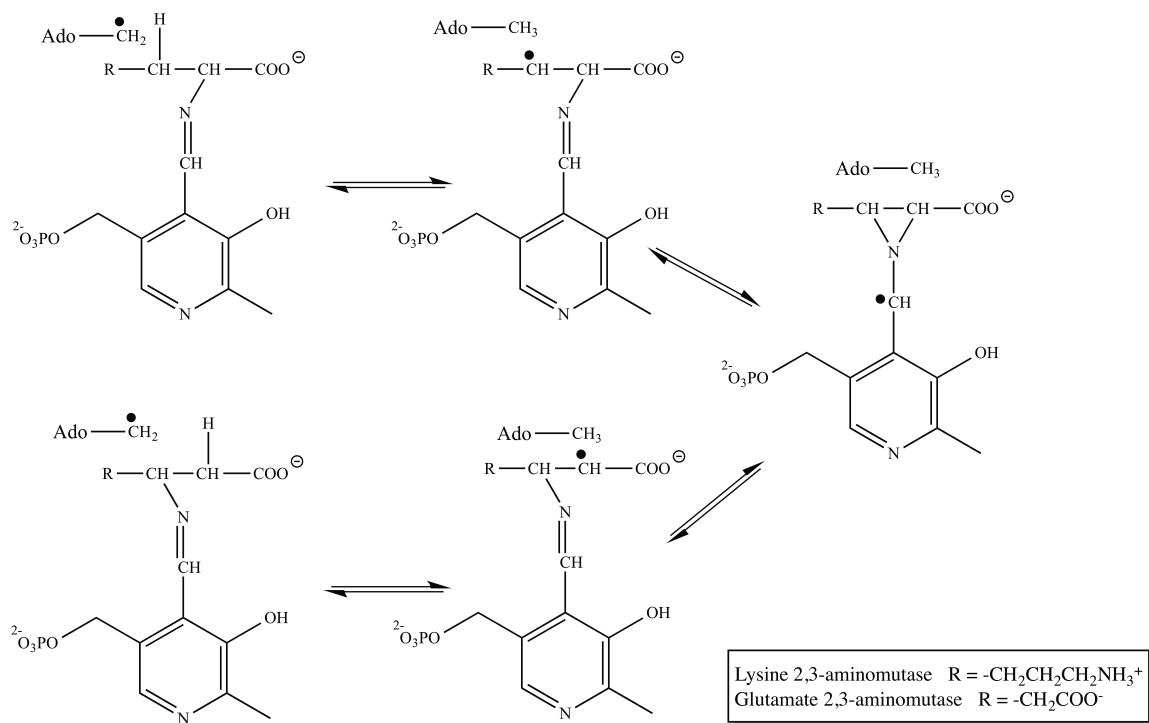


Figure 1.4. Proposed equilibrium driven mechanism for LAM following reductive cleavage of SAM.

Pyruvate Formate Lyase Activating Enzyme (PFL-AE) While LAM has a relatively small substrate, PFL-AE abstracts a hydrogen atom from a conserved glycine residue on the enzyme pyruvate formate lyase (PFL), a 170 kDa dimer. The proposed mechanism begins by coupling the oxidation of a $[4\text{Fe-4S}]^{1+}$ cluster to the reductive cleavage of SAM, which produces methionine and the reactive dAdo• radical.^(12, 13) PFL-AE induces a large conformational shift in PFL causing it to expose Gly-734.⁽¹⁴⁾ The dAdo• radical then directly abstracts the pro-*S* hydrogen from Gly-734 of PFL allowing for the generation of a relatively stable radical on PFL (Figure 1.2B).⁽¹⁴⁻¹⁶⁾ Activated PFL can then reversibly catalyze multiple conversions of coenzyme A and acetate to acetyl-CoA and formate.

Upon exposure to O₂, activated PFL is cleaved into two catalytically inactive peptides with the masses of 3 and 82 kDa.⁽¹⁷⁾ Some organisms produce a “spare part” to repair the inactivated PFL enzyme; in *E. coli* this spare part is the protein YfiD, which can restore activity after binding to the larger PFL degradation product.⁽¹⁸⁾ YfiD contains a catalytic glycine residue located in a 50 amino acid sequence that corresponds to a similar sequence in PFL. While full activity is observed only with these natural substrates, lower level glycy radical formation can be observed when as little as a heptamer peptide fragment of the glycy radical loop is shared by PFL and YfiD.⁽¹⁶⁾

Unlike LAM, PFL-AE employs SAM as a cosubstrate and thus SAM must be continually resupplied for catalytic activity. PFL-AE is the enzyme for which the SAM coordination to the site-differentiated [4Fe-4S] cluster was first demonstrated;^(7, 19) this same coordination mode has now been observed in all radical SAM enzymes for which structural information is available, suggesting that the SAM-cluster interaction is an essential aspect of the radical SAM mechanism.⁽²⁰⁾

Biotin Synthase (BioB) The biologically essential vitamin biotin is synthesized from dethiobiotin *in vivo* by biotin synthase. This enzyme is the first documented and most studied member of the subfamily of radical SAM enzymes that insert sulfur into unactivated C-H bonds. The disputed role or roles of auxiliary [2Fe-2S] and [4Fe-4S] clusters in sulfur inserting enzymes has led to lively discussions between research groups, with the primary point of contention being whether auxiliary clusters are immediately sacrificed to provide the substrate sulfur, or exogenous sulfur supplies are tapped during or even prior to catalysis. In addition to the canonical [4Fe-4S]²⁺ cluster observed in all

other radical SAM enzymes, biotin synthase was found to contain an auxiliary $[2\text{Fe-2S}]^{2+}$ cluster as demonstrated by Mössbauer spectroscopy with differential isotopic iron labeling.⁽²¹⁾ While multiple lines of evidence indicated that SAM was not the sulfur source for biotin biosynthesis,^(22, 23) other experiments demonstrated that when ^{35}S -reconstituted BioB was assayed, ^{35}S was inserted into dethiobiotin to produce labeled biotin.^(24, 25) Further spectroscopic studies indicated that the $[2\text{Fe-2S}]$ cluster was degraded during turnover, suggesting that this cluster served as the sacrificial sulfur donor during biotin synthesis.⁽²⁶⁾ Such a role for the $[2\text{Fe-2S}]$ cluster was also supported by the X-ray crystal structure of BioB, which revealed this cluster optimally positioned to react with the dethiobiotin subsequent to H-atom abstraction.⁽²⁷⁾

The current mechanistic proposal for BioB involves the cannibalistic abstraction of a sulfide from the $[2\text{Fe-2S}]$ cluster by two sequential rounds of radical mediated hydrogen atom abstraction from the methyl and then methylene groups of dethiobiotin to produce an initial 9-mercaptodethiobiotin intermediate and then finally biotin.⁽²⁸⁾ In addition to supplying the sulfur for biotin production, it has been proposed the $[2\text{Fe-2S}]$ cluster may also act as a radical quenching Lewis acid after both hydrogen atom abstraction events.⁽²⁹⁾ The eventual replenishment of the incorporated sulfur *in vivo* is likely supplied by cysteine desulfurase activity. Some have proposed that as biotin synthase is partially degraded during turnover it should be referred to as a substrate and not an enzyme.⁽²⁴⁾ The sources of sulfur in other similar sulfur-insertion enzymes such as lipoyl synthase (LipA) and MiaB are still under investigation.⁽³⁰⁻³²⁾

Molybdenum Cofactor Biosynthetic Enzyme (MoaA) In 1998 investigators shed significant insight into the complex intramolecular rearrangements that yield the precursor to the molybdopterin cofactor (Figure 1.2F).⁽³³⁾ Cells containing a *moaABC* cassette that were supplied with isotopically labeled ribulose 5-phosphate (pentose) or guanine produced labeled molybdopterin precursors as identified by NMR spectroscopy. The pentose and guanine moieties in GTP have since been confirmed to be the sources of the molybdopterin skeleton.^(34, 35) Hänzelmann and Schindelin solved a crystal structure of the MoaA radical SAM enzyme in 2004 and confirmed the presence of an additional [4Fe-4S] cluster located ~17 Å from the canonical N-terminal [4Fe-4S] cluster.⁽³⁵⁾ The same authors solved another MoaA crystal structure with bound substrate, 5'-GTP, and the SAM degradation products methionine and 5'-deoxyadenosine bound which surprisingly revealed that GTP coordinated to the accessory cluster (Figure 1.5).⁽³⁴⁾

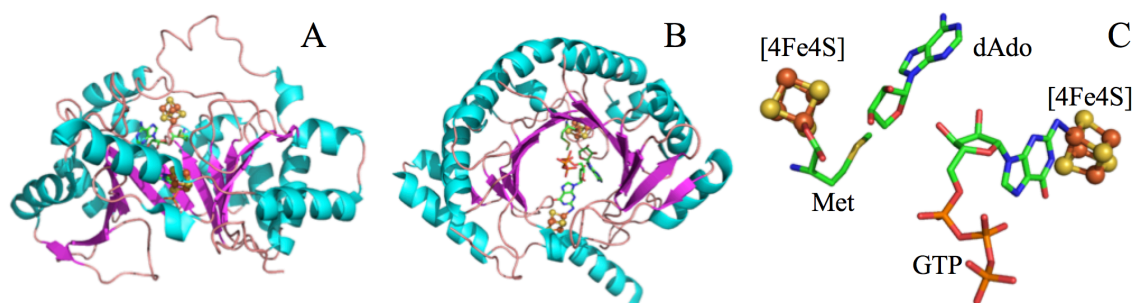


Figure 1.5. Structure of MoaA as viewed from side (A) and bottom (B). The active site highlighting the two [4Fe-4S] clusters, 5'-deoxyadenosine (dAdo), methionine, and GTP (C) (PDB ID: 2FB3).

ENDOR studies carried out in collaboration with Brian Hoffman clarified GTP coordination to the second cluster in an unusual bidentate fashion to the enol tautomer of guanine.⁽³⁶⁾ With structural information from the active site and deuterium labeled GTP,

researchers in 2013 identified the likely hydrogen abstraction site to be the 3'C of the ribose moiety and subsequently proposed a mechanism for GTP rearrangement.⁽³⁷⁾

Architecture and Mechanism of Radical SAM Enzymes

In addition to similarities in the primary structure, radical SAM enzymes share elements of the same tertiary fold.⁽³⁸⁾ Two years after the formal classification of the superfamily the first crystal structure of a radical SAM enzyme (HemN) was solved.^(2, 39) The HemN active site structure was consistent with earlier spectroscopic investigations of PFL-AE that had demonstrated coordination of the amino and carboxy groups of the methionine moiety of SAM to the site differentiated [4Fe-4S] cluster.^(7, 19) HemN was also shown to consist of two domains, one of which was a $(\beta\alpha)_6$ barrel. To date about two dozen other radical SAM crystal structures have been published. Each enzyme is shown to contain a modestly modified TIM barrels, generally either a complete $(\beta\alpha)_8$ or an incomplete $(\beta\alpha)_6$ TIM barrel with the exceptions BtrN and QueE.^(40, 41) Table 1.1 highlights some of the details of these structures. In addition to the core TIM barrel motif, many of the solved structures contain smaller auxiliary domains that are believed to aid in substrate recognition and help seal the active site from bulk solvent. The TIM barrel serves as a convenient container for the unique initiating mechanism common to all radical SAM enzymes.

Table 1.1. Radical SAM enzymes with published crystal structure solutions.

Radical SAM Enzyme (Abbreviation)	Gene	Genome	PDB Deposition	Protein Data Bank ID(s)	Resolution(s) (Å)	Reference
coproporphyrinogen III oxidase (HemN)	<i>hemN</i>	<i>E. coli</i>	13-Aug-2003	1OLT (SAM, [4Fe4S])	2.07	37
biotin synthase (BioB)	<i>bioB</i>	<i>E. coli</i>	30-Sep-2003	1R30 (SAM, [4Fe4S], [2Fe2S], Sub)	3.40	25
molybdenum cofactor biosynthesis protein A (MoaA)	<i>moaA</i>	<i>S. aureus</i>	28-Jun-2004	1TV7 ([4Fe4S]), 1TV8 (SAM, [4Fe4S])	2.80, 2.20	33
L-lysine 2,3-aminomutase (LAM)	<i>kamA</i>	<i>C. subterminale</i>	30-Jun-2005	2ASH (SAM, [4Fe4S], PLP, Sub)	2.10	7
molybdenum cofactor biosynthesis protein A (MoaA)	<i>moaA</i>	<i>S. aureus</i>	8-Dec-2005	2FB2 (Var, SAM, 2x[4Fe4S]), 2FB3 (Var, 2x[4Fe4S], Met, dAdo, PP, Sub)	2.25, 2.35	32
N(1)-methylguanosine cyclizing enzyme (TWY1)	<i>taw1</i>	<i>P. horikoshii</i>	23-Apr-2007	2YX0	2.21	106
N(1)-methylguanosine cyclizing enzyme (TWY1)	<i>taw1</i>	<i>M. jannaschii</i>	28-May-2007	2ZZU	2.40	107
ribosomal protein S12 methylthiotransferase (RimO)	<i>rimO</i>	<i>T. maritima</i>	29-Jun-2007	2QGQ	2.00	108
pyruvate formate-lyase 1-activating enzyme (PFL-AE)	<i>pflA</i>	<i>E. coli</i>	11-Feb-2008	3CBF (disSAM, [4Fe4S]), 3CB8 (SAM, [4Fe4S])	2.25, 2.77	16
FeFe-hydrogenase maturase (HydE)	<i>hydE</i>	<i>T. maritima</i>	12-Mar-2008	3CIW (SAH, [4Fe4S]), 3CIX (SAH, [4Fe4S], [2Fe2S], SCN)	1.35, 1.70	86
HMP-P synthase (ThiC)	<i>thiC</i>	<i>C. crescentus</i>	29-Sep-2008	3EPM, 3EPN (SubA), 3EPO (Prod)	2.79, 2.11, 2.10	109
FeFe-hydrogenase maturase (HydE)	<i>hydE</i>	<i>T. maritima</i>	3-Aug-2009	3IIX ([4Fe4S], Met, dAdo), 3IIZ (SAM, [4Fe4S], [2Fe2S])	1.25, 1.62	48
ribosomal RNA large subunit methyltransferase N (RimN)	<i>rimN</i>	<i>E. coli</i>	5-Apr-2011	3RF9 ([4Fe4S]), 3RFA (SAM, [4Fe4S])	2.20, 2.05	110
methylornithine synthase (PylB)	<i>pylB</i>	<i>M. barkeri</i>	31-Jul-2011	3T7V (SAM, [4Fe4S], Prod)	1.50	87
spore photoproduct lyase (SPL)	GTNG_2348	<i>G. thermodenitrificans</i>	6-Jun-2012	4FHC (SeSAM, [4Fe4S]), 4FHD (SeSAM, [4Fe4S], PP, SubA), 4FHE (Var, SeSAM, [4Fe4S]), 4FHF (Var, SeSAM, [4Fe4S], PP, SubA), 4FHG (Var, SeSAM, [4Fe4S])	2.20, 2.00, 2.30, 2.00, 2.00	111
ribosomal protein S12 methylthiotransferase (RimO)	<i>rimO</i>	<i>T. maritima</i>	20-Feb-2013	4ICO (2x[4Fe4S])	3.30	30
FeFe-hydrogenase maturase (HydE)	<i>hydE</i>	<i>T. maritima</i>	28-Mar-2013	4JXC, 4JY8, 4JY9, 4JYD, 4JYE, 4JYF (see reference)	1.50, 2.90, 1.60, 1.71, 1.65, 1.45	112
anaerobic sulfatase-maturating enzyme (anSMEcpe)	CPF_0616	<i>C. perfringens</i>	10-Apr-2013	4K36 (SAM, 3x[4Fe4S]), 4K37 (SAM, 3x[4Fe4S]), 4K38 (SAM, 3x[4Fe4S], SubA), 4K39 (SAM, 3x[4Fe4S], SubA)	1.62, 1.62, 1.83, 1.78	113
spore photoproduct lyase (SPL)	GTNG_2348	<i>G. thermodenitrificans</i>	20-Apr-2013	4K9R (Var, SeSAM, [4Fe4S])	2.30	114
butirosin biosynthetic enzyme (BtrN)	<i>btrN</i>	<i>B. circulans</i>	12-Aug-2013	4M7S (2x[4Fe4S]), 4M7T (SAM, 2x[4Fe4S], Sub)	2.02, 1.56	38
HMP-P synthase (ThiC)	<i>thiC</i>	<i>A. thaliana</i>	16-Oct-2013	4N7Q	1.60	115
7-carboxy-7-deaza-uridine synthase (QueE)	<i>queE</i>	<i>B. multivorans</i>	10-Nov-2013	4NUG (SAM, [4Fe4S], SubA), 4NUH (SAM, [4Fe4S], Sub), 4NUJ (SAM, [4Fe4S], Sub, Mg2+), 4NUJ (SAM, [4Fe4S], Sub, Mn2+), 4NUK (SAM, [4Fe4S], Prod, Mg2+)	2.60, 1.90, 2.20, 2.70, 1.91	39
ribosomal RNA large subunit methyltransferase N (RimN)	<i>rimN</i>	<i>E. coli</i>	16-May-2014	4PL1 (Var, SAM, [4Fe4S]), 4PL2 (Var, [4Fe4S])	2.58, 2.20	116

Tertiary Structure Similarities The smallest radical SAM enzyme characterized is the anaerobic ribonucleotide reductase activating enzyme (aRNR-AE), which has been predicted to have a $(\beta\alpha)_4$ barrel although its structure has not yet been solved.⁽³⁸⁾ The crystal structures of the radical SAM enzymes, BtrN and QueE, have recently been solved and exhibit significant modifications of the core $(\beta\alpha)_6$ barrel.^(40, 41) BtrN, which catalyzes the anaerobic dehydrogenation of the antibiotic precursor 2-deoxy-*scyllo*-inosamine, was found to have a unique $(\beta_5\alpha_4)$ fold less reminiscent of a typical radical SAM TIM barrel.⁽³⁸⁾ Nevertheless, like other superfamily members, the CX_3CX_2C motif was located in a loop between the first β strand and α helix in the primary sequence (Figure 1.6).

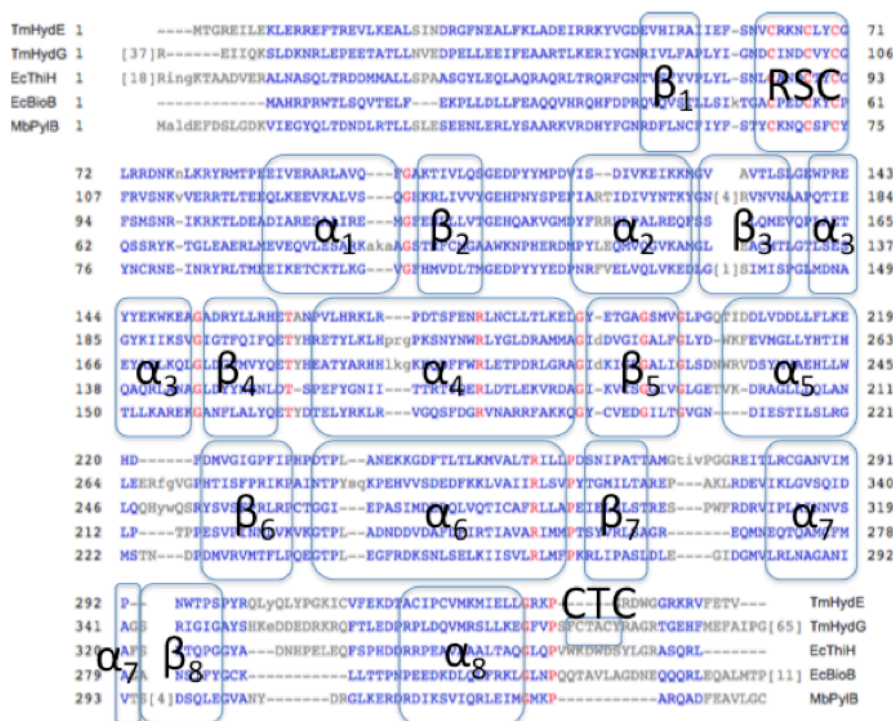


Figure 1.6. Sequence alignment of select radical SAM enzymes with complete TIM barrels. The CX_3CX_2C motif that binds the radical SAM cluster is labeled RSC. HydG's C-terminal domain contains a $CX_2CX_{22}C$ motif of which the first two Cys are in a box labeled CTC.

7-carboxy-7-deazaguanine synthase (QueE) crystalized as a dimer in which each monomer containing a ($\beta_6\alpha_3$) core.⁽⁴¹⁾ The three helices missing from the core ($\beta\alpha$)₆ TIM barrel had been largely replaced largely by loops. Alignments of radical SAM enzymes revealed that sequence similarities between different radical SAM subfamilies end around β_5 and the differences that extend beyond this region might play a role in substrate diversity.^(38, 42) For instance, the completeness of the barrel appears to be inversely correlated with the size of the substrate (Figure 1.7), with the less complete barrels generally accommodating larger substrates (PFL-AE/PFL) and full barrels inherently providing more shielding for radical based chemistry on smaller substrates (BioB/dethiobiotin).⁽⁴³⁾

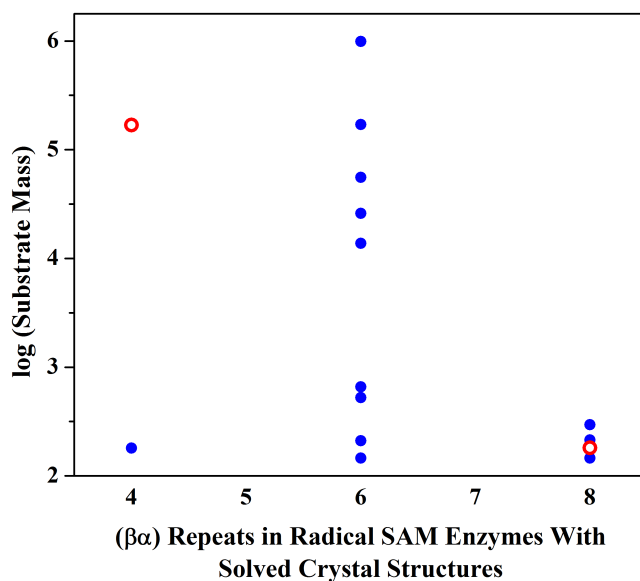


Figure 1.7. Plots of substrate mass (Da) compared to the completeness of the core domain. Blue closed circles: Radical SAM enzymes with crystal structures. Red open circles: aRNR-AE ($\beta\alpha$)₄ and HydG/ThiH ($\beta\alpha$)₈ predicted completeness. SPL is not plotted because the substrate size is variable, however it would follow the general trend of larger substrates with less complete TIM barrels. Due to the highly modified architectures of QueE and BtrN, they have been plotted as having six and four repeats, respectively.

Four exceptions to this overall trend include BtrN, LAM, MoaA, and HemN which all have smaller substrates (< 750 Da) but have only a partial TIM barrel. A stronger correlation occurs with larger substrates, which appear to be completely incompatible with full $(\beta\alpha)_8$ barrels (Figure 1.7).

Conserved Motifs Despite the conserved canonical CX_3CX_2C motif and a common super-secondary structure, radical SAM enzymes have low sequence homology between subfamilies. However, glycine and proline residues are semiconserved, often punctuating the α helices and β strands. These two residues can relieve stress when transitioning between secondary structural elements by adopting unusual dihedral angles. Structurally based sequence alignments have been helpful in uncovering more motifs by aligning primary structures of radical SAM enzymes in different subgroups (Figure 1.6).^(38, 42) These alignments have found two additional semiconserved glycine rich motifs as discussed further below.

In 2001 Sofia *et al.* identified the radical-SAM superfamily, adding over 600 putative members to the five “defining” homologous members.⁽²⁾ This was accomplished through a bioinformatics approach in which researchers mined the available gene databases for DNA sequences coding for the cysteine rich CX_3CX_2C motif where X can be any amino acid (Figure 1.8). This motif along with other similarities, such as cofactors and iron and sulfide requirements, had previously been described in the literature but Sofia *et al.* had not applied these as discriminating factors in the search for other members. Exceptions to the CX_3CX_2C motif have been reported including in the thiamin pyrimidine biosynthetic enzyme ThiC (CX_2CX_4C)⁽⁴⁴⁾ and in an enzyme involved

in phosphonate metabolism, PhnJ (CX₂CX₂₇C).^(45, 46) Other non-canonical motifs have been described but all contain at least three cysteine residues requisite for ligation of an FeS cluster and subsequent catalysis.^(41, 47) For example, while ~20% of QueE sequences contain an additional 11 or 17 residues between the first two cysteines of the three-Cys motif, the insert does not significantly affect the geometry of the cysteines that coordinate the [4Fe-4S] cluster.⁽⁴¹⁾

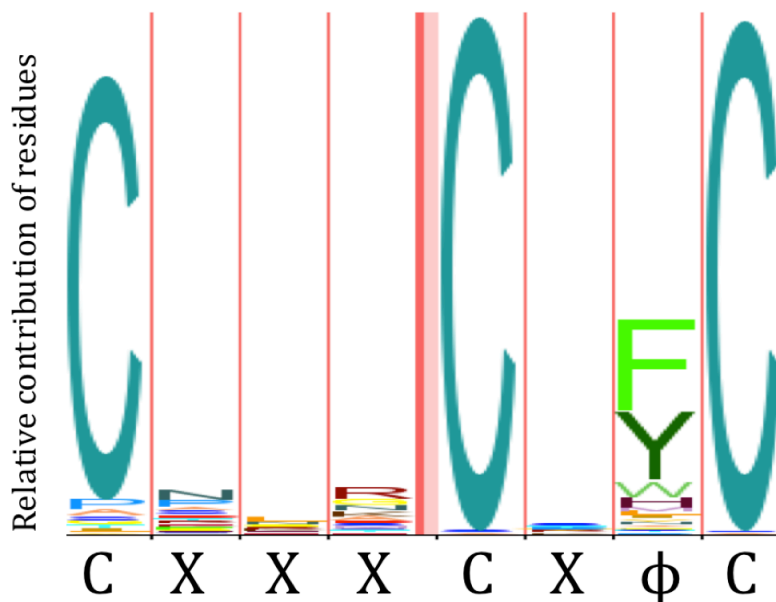


Figure 1.8. A sequence alignment logo generated using the Pfam 27.0 database. Heights of single letter amino acid abbreviations are proportional to their relative abundances in multiple sequences of radical SAM superfamily members.

The three-Cys motif in radical SAM enzymes coordinates a redox active [4Fe-4S] cluster or “radical SAM cluster” whose resting state is 2+; the three-cysteine coordination renders the radical SAM [4Fe-4S] clusters site differentiated, with one of the four irons not coordinated by cysteine. Cuboidal [4Fe-4S] clusters are not unique to radical SAM enzymes and other similarly composed clusters are used in electron shuttling, redox

sensing, substrate binding, and protein structure.⁽⁴⁸⁾ These [4Fe-4S] clusters typically have biologically accessible reduction potentials. Single electron transporting systems such as flavodoxin, flavodoxin reductase, and NADPH can reduce these clusters to the 1+ state, which is the catalytically active state for radical SAM enzymes.⁽¹³⁾

The radical SAM canonical cysteine rich motif is often written CX₃CX ϕ C with ϕ representing an aromatic residue. The graphical representation of the contribution at the penultimate position in Figure 1.8 shows the relative abundance to be F, Y, W, H, and M. Interestingly, the only conserved interaction of this residue is a hydrogen bond (averages distance of 3.0 Å) between the N6 hydrogen of the SAM adenine moiety and the backbone carbonyl on the ϕ residue (Figure 1.9).

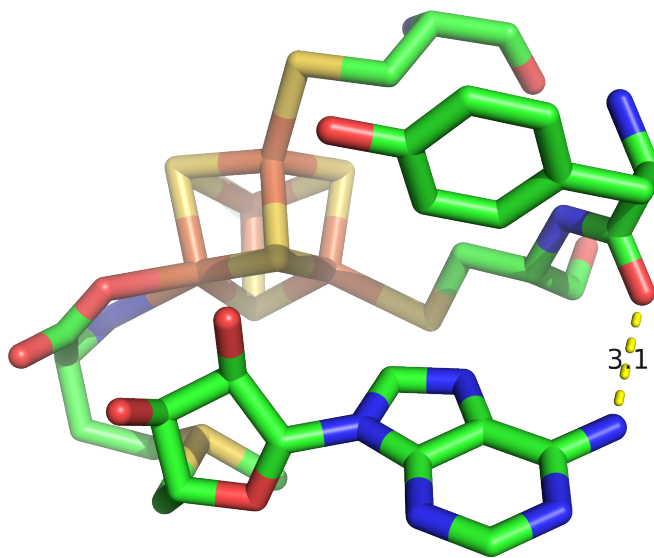


Figure 1.9. The anSMEcpe's hydrogen bond between the N6 hydrogen of SAM's adenine moiety and the backbone carbonyl on the ϕ residue depicted by the yellow dashed line (distance in Å; PDB ID: 4K38).

In a recent spectroscopic and functional characterization of BlsE, a radical SAM enzyme required for blasticidin S biosynthesis, which contains a penultimate methionine

residue, M37F, M37Y, and M37W mutants had near WT activities.⁽⁴⁹⁾ Often radical SAM enzymes without aromatic residues at the ϕ position have such amino acids located within two positions of the C-terminal Cys. The aromatic residue may serve as a conduit for electron transfer to the [4Fe-4S] cluster and/or block solvent access to the active site. A crystal structure of an enzyme with an aliphatic residue would perhaps shed light on the nature of this residue and its role in enzyme function.

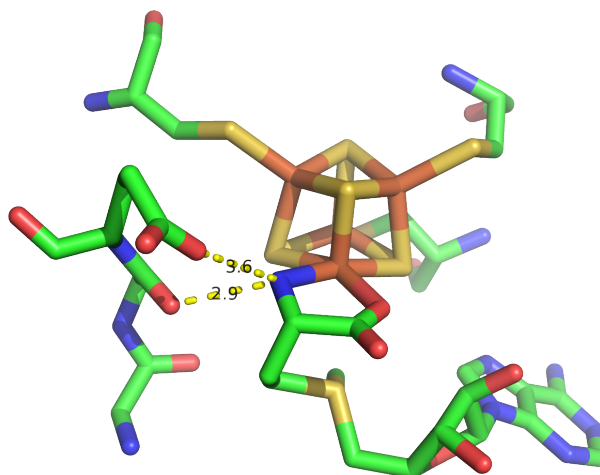


Figure 1.10. The GGE (PFL-AE) motif interacting with methionine moiety on SAM depicted by yellow dashed lines (distance in Å; PDB ID: 3CB8).

The second most prominent radical SAM motif is the conserved GGE sequence starting at the end of the β_4 strand. The first Gly of this motif aids in orienting the second Gly and the following Glu residues so that they may form a hydrogen bond with the amino group on the methionine moiety of SAM (Figure 1.10). Further, this “GXIXGXXE” motif is located along the β_4 strand, running roughly parallel with SAM and appears to provide the adenosine moiety with a hydrophobic binding pocket. While

there are exceptions to these residues in wild type proteins, mutating these residues may lead to inactive proteins.⁽⁴⁹⁾

Common Initial Mechanism While the reactions and substrates vary tremendously, radical SAM enzymes appear to initiate catalysis by a common mechanism.⁽²⁰⁾ Upon *in vitro* or *in vivo* reduction to the 1+ state, the radical SAM cluster can donate an electron through an inner sphere electron transfer to the sulfonium moiety on SAM (Figure 1.11). Computational and experimental evidence suggests this electron transfer is promoted by electron donation from the reduced [4Fe-4S] cluster into the σ^* orbital of the C-S bond in SAM's sulfonium moiety.^(50, 51) Immediately following the electron transfer event, the S-C(5') is cleaved to produce methionine and the dAdo•. It is expected that the hydrogen atom to be abstracted would be poised very near to the site where the dAdo• is generated; several crystal structures are in agreement with this expectation. The crystal structure for LAM bound to SAM and the substrate L- α -lysine, for example, shows how the 5'-deoxyadenosyl moiety of SAM is poised in close proximity to the substrate H-atom (Figure 3C). The dAdo• can readily rotate or bend while anchored by the adenosine group to abstract a hydrogen atom from the nearby substrate. The substrate is held in very close proximity to the radical so that very little movement is needed.⁽⁵²⁾ The substrate-based radicals then embark on a seemingly limitless array of transformations.

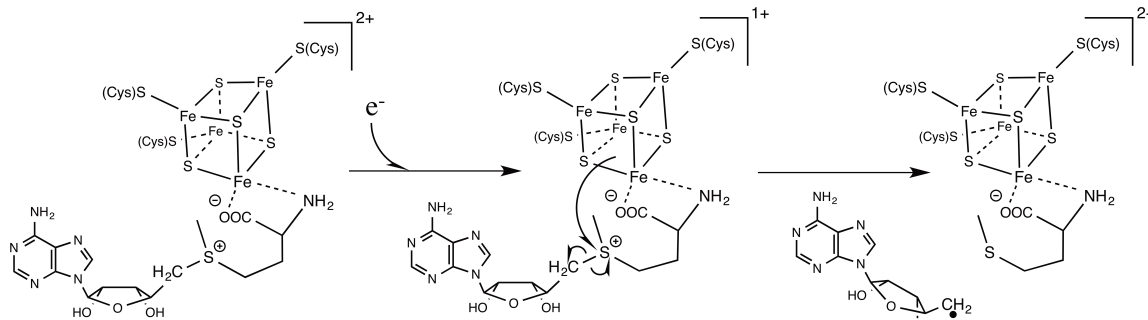


Figure 1.11. The reductive cleavage of SAM and the generation of the 5'-deoxyadenosyl radical (dAdo•).

Iron Sulfur Clusters

The simplicity of iron sulfur cluster structures underrepresents the diversity of biological roles to which they have been called to perform. Of the seven most common metals in earth's crust (aluminum, iron, calcium, sodium, potassium, magnesium, and titanium) iron is unique in its ability to readily adopt multiple oxidation states at or near physiological conditions.⁽⁵³⁾ Sulfur's utility and plasticity, on the other hand, does not necessarily stem from its range of oxidation states (2^- to 6^+) but rather its ability to donate into 3d orbitals forming very covalent bonds with iron. Sulfur, in its acid labile (S^{2-}) and cysteinyl thiolate forms serves as the core for a wide variety of protein cofactors.⁽⁴⁸⁾

Simple Iron Sulfur Clusters

While iron most often adopts a tetrahedral coordination when bound to sulfur, often period two elements increase iron's coordination environment to five or six.^(54, 55) The most common FeS clusters in biology are $[2Fe-2S]$, $[3Fe-4S]$, and $[4Fe-4S]$ as illustrated in Figure 1.12.⁽⁵⁶⁾ All three cluster types can be reduced with biological ferredoxin and flavodoxin reducing systems.^(48, 57) In the radical SAM enzymes involved

in the maturation of [FeFe]-hydrogenase, all three common FeS cluster types are present either in the active enzyme or as degradation products. Formal charges and valence delocalization properties have been assigned to each iron in the three cluster species through analysis of Mössbauer spectra.⁽⁵⁸⁻⁶⁰⁾ Most relevant to radical SAM enzymes are the site differentiated [4Fe-4S] clusters.⁽²⁰⁾ The 2+ oxidation state of the [4Fe-4S] cluster can simply be described as two valence delocalized $\text{Fe}^{2.5+}$ - $\text{Fe}^{2.5+}$ pairs that are antiferromagnetically coupled.⁽⁵⁴⁾ The reduced $[\text{4Fe-4S}]^{1+}$ cluster can be described as a single mixed valence $\text{Fe}^{2.5+}$ - $\text{Fe}^{2.5+}$ pair antiferromagnetically coupled to a Fe^{2+} - Fe^{2+} pair.⁽⁶¹⁾

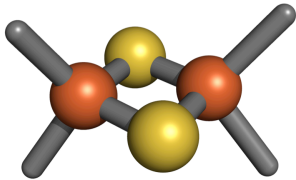
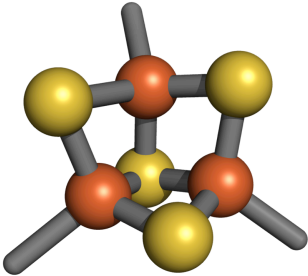
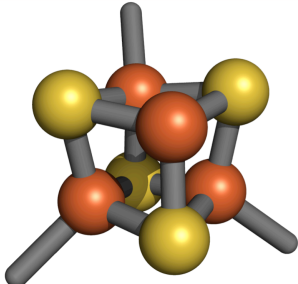
	[2Fe-2S]	[3Fe-4S]	[4Fe-4S]
			
Oxidized	$\text{Fe}^{3+}, \text{Fe}^{3+}$ (net 2+) $S = 0$ EPR Silent	3Fe^{3+} (net 1+) $S = \frac{1}{2}$, Nearly Isotropic g values: 2.03, 2.01, 1.99 EPR $T_{\text{opt}}: \leq 12\text{K}$	$4\text{Fe}^{2.5+}$ (net 2+) $S = 0$, EPR Silent
	λ_{max} (nm) = 325, 420, 465 $E^0 = -240$ to -420 mV	λ_{max} (nm) = 305, 415, 455 $E^0 = -130$ to -260 mV	λ_{max} (nm) = 305, 390 $E^0 = -400$ to -600 mV
Reduced	$\text{Fe}^{3+}, \text{Fe}^{2+}$ (net 1+) $S = \frac{1}{2}$, Nearly Isotropic g values: 2.00, 1.96 EPR $T_{\text{opt}}: 25\text{K}-30\text{K}$	$\text{Fe}^{3+}, 2\text{Fe}^{2.5+}$ (net 0) $S = 0$, EPR Silent	$2\text{Fe}^{2.5+}, 2\text{Fe}^{2+}$ (net 1+) $S = \frac{1}{2}$, Axial to Rhombic $g = 2.03, 1.92, 1.88$ g (+SAM) = 2.01, 1.88, 1.83 EPR $T_{\text{opt}}: \leq 12\text{K}$
	50% bleach when reduced	λ_{max} (nm) = 425	50% bleach when reduced

Figure 1.12. Simple iron sulfur clusters in biology and selected spectroscopic characteristics.

Functions of Iron Sulfur Clusters

In proteins, FeS clusters serve four broad interrelated functions: (1) electron transfer, (2) substrate binding/activation, (3) redox sensing, and (4) tertiary structure stabilization.^(48, 62) The iron sulfur clusters in the radical SAM enzymes HydE and HydG can be assigned all four of these roles as will be described later in Chapters 4 and 7.

Biogenesis of Simple Iron Sulfur Clusters

While *in vitro* reconstitution of iron sulfur clusters can be achieved by reconstitution with free exogenous iron and a source of sulfide in an anaerobic, reducing environment, these conditions are far from physiological and are not achievable inside a cell.^(63, 64) Further, this relatively crude method for loading iron and sulfur clusters into proteins is in stark comparison to the three known systems employed by living organisms.⁽⁶⁵⁾ The study of the *in vivo* methods for cluster assembly has been a multidisciplinary effort and the culmination of bioinformatics and biochemical studies have roughly defined three separate roles for the ISC, SUF, and NIF multicomponent systems.⁽⁶⁵⁾ The ISC system encoded by the *isc* operon is generally described as the primary method to generate clusters *in vivo*. The ISC machinery is largely conserved in prokaryotic organisms and eukaryotic organisms from simple yeasts to human mitochondria.^(65, 66) Interestingly, the functionally overlapping and complementary SUF cluster assembly system encoded by the *suf* operon is usually employed under unfavorable cellular conditions such as limited iron availability, oxidative stress, and the presence of excess heavy metals.⁽⁶⁷⁻⁶⁹⁾ Both these multicomponent systems share two key features. First, they divide the loading of FeS clusters into apo proteins into an assembly

and a transport step. Both possess scaffold proteins upon which a suite of other gene products assemble the clusters. Second, they both have a cysteine desulfurase, which catalyzes the conversion of cysteine to alanine, thereby generating sulfide.⁽⁷⁰⁾ In addition to the ISC and SUF multicomponent systems, the NIF system, originally discovered when looking for the iron sulfur assembly necessary for the maturation of nitrogenase, was found in some organisms without nitrogenase.^(71, 72) Further investigations will likely reveal more similarities between the systems and further breakdown the canonical definitions as many organisms may have fully functional FeS cluster assembly mechanisms with a patchwork of enzymes from the three multicomponent systems.⁽⁷³⁾ While simple [2Fe-2S] and [4Fe-4S] clusters can be assembled by two or three generic FeS cluster assembly systems, more complex FeS cofactor assembly requires specific enzymes whose sole function is the construction delivery of unique clusters.⁽⁷⁴⁻⁷⁷⁾ Figure 1.13 gives four examples of complex iron and sulfur containing cofactors that require their own specific complement of enzymes for construction.

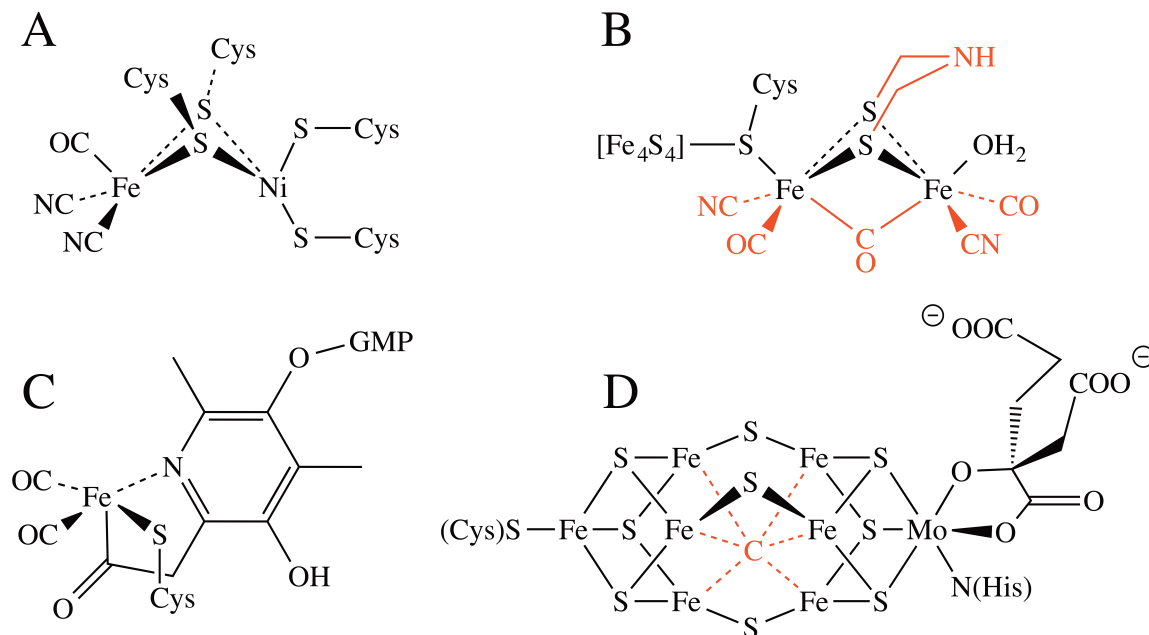


Figure 1.13. Complex metallocofactors. (A) [NiFe]-; (B) [FeFe]-; (C) [Fe-only]-hydrogenases; and (D) nitrogenase active site primary coordination environments. Red atoms and bonds represent those putatively derived radical SAM chemistry.

Nitrogenase Active Site Cluster Maturation The reduction of elemental nitrogen (N_2) to ammonia (NH_3) is a costly reaction both biologically and industrially. It has been estimated that two-thirds of all nitrogen fixation is catalyzed by nitrogenase with a bulk of the remainder by the Haber-Bosch process.⁽⁷⁸⁾ Diazotrophic microbes catalyze the reaction $\text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{MgATP} + 16 \text{H}_2\text{O} \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{MgADP} + 16 \text{P}_i$ at ambient temperatures and pressures whereas industrial processes require much higher temperatures and pressures.⁽⁷⁹⁾

The holoenzyme of nitrogenase is composed of two polypeptides, NifD and NifK, with NifD housing the active site. The crystal structure of the NifH(γ)/NifD(α)/NifK(β) complex with a $(\alpha\beta\gamma)_2$ stoichiometry from *Azotobacter vinelandii* likely represents a snapshot of active nitrogenase.⁽⁸⁰⁾ The most distinctive aspect of nitrogenase is its active

site cluster also known as the M-cluster or FeMo-co (Figure 1.13D) located in NifD. FeMo-co ($\text{Fe}_7\text{MoCS}_7\cdot\text{homocitrate}$) contains a unique heterometallic cluster whose core may be described as a $[\text{4Fe-3S}]$ cubane bridged to a $[\text{Mo3Fe3S}]$ cubane by three sulfides and one hexacoordinated carbide.^(81, 82) The molybdenum at the distal position is coordinated in a bidentate fashion to homocitrate (HC) and anchored by a His ligand from the protein. A Cys ligand also from NifD secures the terminal iron on the other side of the cluster. The “*ex situ*” or external synthesis of the FeMo-co prior to insertion into NifDK is proposed to be accomplished on two discrete proteins.⁽⁸³⁾

Nitrogenase produced in the absence of the *nifB* gene was shown to be inactive as it lacked FeMo-co, implicating that NifB was involved in a critical step in cluster maturation.⁽⁸⁴⁻⁸⁷⁾ The *A. vinelandii nifB* gene product contains a $\text{CX}_3\text{CX}_2\text{C}$ motif and a total of nine Cys and eight His residues highly conserved in NifB proteins, indicating the ligation of a site differentiated $[\text{4Fe-4S}]$ cluster and potentially other FeS clusters.⁽⁸⁴⁾ Anaerobically purified NifB was shown to contain 12 Fe/dimer and could be reconstituted up to 18 Fe/dimer.⁽⁸⁷⁾ UV-vis spectral features of reconstituted NifB decrease upon addition of dithionite in a manner suggestive of the presence of FeS clusters. Activity dropped significantly for NifB synthesis of FeMo-co when Fe^{2+} , S^{2-} , or SAM was omitted in *in vitro* assays. These findings in conjunction with previously noted sequence analysis strongly suggest that NifB is a radical SAM enzyme. NifB likely constructs an all iron FeMo-co precursor that is transferred to the scaffold protein NifEN.^(88, 89) Also, NifB has been assigned to the insertion of the central carbon of the FeMo-co precursor using SAM as the substrate.⁽⁹⁰⁾ On NifEN, either NifH or NifQ and

NifV likely swap the cluster's distal Fe atom for a Mo atom and aid in its coordination by homocitrate to produce the FeMo-co.^(78, 83) This FeMo-co can be transferred to the structural protein complex NifDK where Cys and His residues hold it in place.

Hydrogenase

Over 70 years ago, Gaffron and Rubin demonstrated that under anaerobic conditions algae could produce molecular hydrogen.⁽⁹¹⁾ They also described how previously hypothesized hydrogenase activity could be stimulated by photoillumination. This opened the door for decades of research in harnessing solar energy for the production of clean fuels such as hydrogen gas. In addition to [FeFe]-hydrogenase, two other phylogenetically distinct classes of hydrogen evolving enzymes have been termed [NiFe]- and [Fe-only]-hydrogenases.⁽⁹²⁾

[Fe-only]-Hydrogenase

[Fe-only]-hydrogenases in methanogenic archaea employ an unusual active site where methenyltetrahydromethanopterin (methenyl-H₄MPT⁺) is catalytically (reversibly) reduced with H₂ leading to the production of methane after fixation of CO₂.⁽⁹³⁾ This enzyme harbors a single iron atom which is coordinated by two CO ligands, a cysteinyl residue, and a nitrogen and acyl group from a pyridinol moiety; the moiety has an open ligation site that may interact with H₂ (See Figure 1.13C).⁽⁹⁴⁾ The maturation machinery for [Fe-only]-hydrogenase is comprised of at least seven separate gene products of which one, HmdB, is a member of the radical SAM superfamily.⁽⁹⁵⁾ This maturase enzyme

houses a non-canonical CX_5CX_2C radical-SAM binding motif, however, its exact role in hydrogenase maturation is presently unknown.⁽⁹⁶⁾

[NiFe]-Hydrogenase

[NiFe]-hydrogenase activity reversibly oxidizes molecular hydrogen to electrons and protons but is usually employed *in vivo* to catalyze H_2 oxidation.⁽⁹⁷⁾ The catalytic heart of this subset of hydrogenases contains nickel and iron ions bridged by the thiolate moieties of two Cys residues (see Figure 1.13A). In a similar fashion, two additional Cys residues in a similar fashion further coordinate the redox active Ni atom. The iron ion is also coordinated with two cyanide and one carbon monoxide ligand.⁽⁹⁸⁾ The multistep process of inserting metal ions, generating ligands, assembly, and insertion of the cluster into [NiFe]-hydrogenase are accomplished by a suite of six maturase enzymes encoded by the genes *hypA-F*.⁽⁹⁹⁾ None of these six proteins are radical-SAM enzymes and the production of the CO and CN^- ligands (diatomics) is not analogous to the method employed by HydG, a radical SAM maturase enzyme of [FeFe]-hydrogenase discussed below.

[FeFe]-Hydrogenase

Protein Structure and Active Site H-Cluster [FeFe]-hydrogenase (HydA) is primarily used *in vivo* to catalyze the reduction of protons to hydrogen gas. The first look at [FeFe]-hydrogenase's multiple domain structure and active site was given by Peters *et al.* in 1998.⁽⁵⁵⁾ A more recent structure better depicts the area of H_2 activation composed of a [4Fe-4S] cluster coordinated by four Cys, one of which bridges to a unique 2Fe

subcluster. This smaller cluster (see Figure 1.13B) is ornately decorated with several unusual ligands.

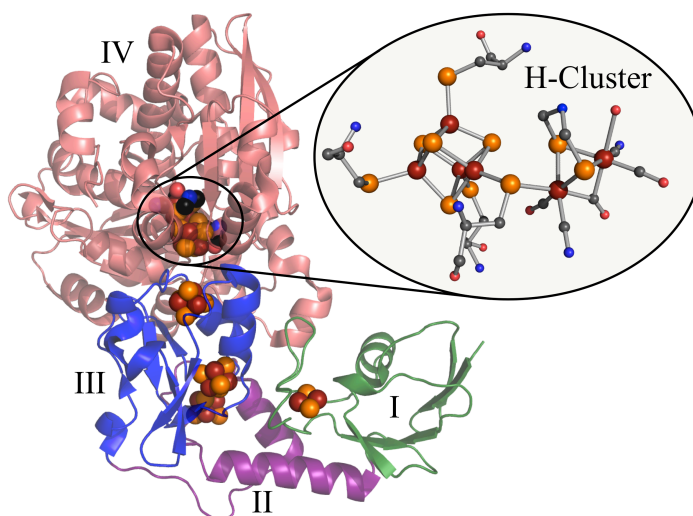


Figure 1.14. Crystal structure for *C. pasteurianum* [FeFe]-hydrogenase CpI at 1.39 Å. Domain IV contains the H-cluster highlighted in the callout. (PDB ID: 3C8Y).

Both iron atoms are singularly coordinated by a carbon monoxide and cyanide ligand and bridged together by a single carbon monoxide as well as two thiolates of 1,3-dithiomethylamine (DTMA).⁽¹⁰⁰⁻¹⁰²⁾ During catalysis the [4Fe-4S] cluster may donate and accept electrons to and from the 2Fe subcluster.⁽¹⁰³⁾ The *in vitro* and *in vivo* activation of this enzyme has been intensely investigated by several research groups whose findings will be highlighted in the following section.

Early Maturation Discoveries Ten years ago, 60 years after Gaffron and Rubin's seminal report on algal hydrogen production, Posewitz *et al.* determined the maturase enzymes required for [FeFe]-hydrogenase activation.⁽¹⁰⁴⁾ Isolates from a *Chlamydomonas reinhardtii* insertional mutagenesis library were screened for H₂

production. A strain unable to produce hydrogen was found to have an insertion in the *hydEF-1* gene destroying the activity of the gene product HydEF. HydEF in *C. reinhardtii* is homologous to two separate proteins (HydE and HydF) found in all prokaryotic organisms that contain [FeFe]-hydrogenase (HydA1 in *C. reinhardtii*). Genomic analysis and comparison of organisms with [FeFe]-hydrogenases revealed that the gene product of *hydG* was also strictly conserved. Heterologous co-expression of [FeFe]-hydrogenase, HydEF, and HydG in *E. coli* was found sufficient and essential to activate HydA1.⁽¹⁰⁴⁾ In the same report the investigators noted that HydE and HydG contain the canonical CX₃CX₂C motif and are putatively radical-SAM enzymes.

Researchers for the past ten years have been uncovering the mechanisms by which [FeFe]-hydrogenase are matured by investigating its maturases individually and collectively. Initial characterization of HydE and HydG demonstrated the requisite spectroscopic and biochemical features that confirmed these two proteins as members of the radical-SAM super family. For example, both were found to contain multiple FeS clusters and reductively cleave SAM.⁽¹⁰⁵⁾ In a hypothesis paper, researchers made several predictions about the roles of the maturation machinery which were later supported experimentally.⁽¹⁰⁶⁾ They speculated that a single enzyme, so as to prevent the build up of one cytotoxic species, would produce both CO and CN⁻; the maturation scheme would utilize one of the enzymes as a scaffold; and the diatomic ligands may be derived from the components of a glycine radical.⁽¹⁰⁶⁾ Also, they reasoned that the dithiolate bridge would help stabilize a [2Fe-2S] cluster prior to the addition of the diatomic ligands. King, Posewitz, and coworkers followed up their work and demonstrated that *C.*

acetobutylicum maturase genes were more stable in prokaryotic expression systems *C. reinhardtii* and could activate [FeFe]-hydrogenases from *C. acetobutylicum*, *Clostridium pasteurianum*, and *C. reinhardtii*.⁽⁷⁶⁾ This further demonstrated that the maturation systems as well as the H-clusters were highly conserved between multiple organisms and left the door wide open for system level investigations.⁽¹⁰⁷⁾

McGlynn *et al.* found that inactive HydA lysate could be activated by a lysate containing heterologously co-expressed HydE, HydF, and HydG.⁽¹⁰⁸⁾ They rationalized that their observation of immediate activation upon mixing resulted from one of the enzymes serving as a scaffold for the synthesis of a H-cluster precursor. The same lab later demonstrated that apo-HydA is not capable of activation by a lysate containing coexpressed HydE, HydF, and HydG (HydEFG).⁽¹⁰⁹⁾ Upon reconstitution of HydA a redox active [4Fe-4S] cluster was spectroscopically observed, and the enzyme could then be activated by HydEFG lysate.⁽¹⁰⁸⁾ These results implied that the [FeFe]-hydrogenase maturation machinery was responsible for building and transferring only the embellished 2Fe subcluster to immature HydA.

A group out of Stanford University found that addition of L-cysteine and L-tyrosine stimulated the activity of purified *C. reinhardtii* HydA1 in the presence of dialyzed heterologously co-expressed HydE, HydF, and HydG of *Shewanella oneidensis*, suggesting possible roles for these amino acids in maturation.⁽¹¹⁰⁾ Collectively, the research from these groups paved the way for determining the individual duties of the maturation enzymes for [FeFe]-hydrogenase.

HydF: The Scaffold Protein While the role of HydA was previously known, the functions of the maturase enzymes were largely speculative. McGlynn *et al.* furthered their HydF-scaffold hypothesis by demonstrating that isolated and purified HydF, co-expressed in a genetic background with HydE and HydG (HydF^{EG}), could activate [FeFe]-hydrogenase expressed devoid of maturation factors (HydA^{ΔEFG}).⁽¹⁰⁸⁾ Following this biochemical result Shepard *et al.* further spectroscopically characterized purified HydF expressed with (HydF^{EG}) and without (HydF^{ΔEG}) HydE and HydG, showing by EPR spectroscopy the presence of both [2Fe-2S]¹⁺ and [4Fe-4S]¹⁺ clusters on HydF^{ΔEG}.⁽¹¹¹⁾ However, when co-expressed with HydE and HydG, the [2Fe-2S]¹⁺ signal was not observed.⁽¹⁰⁸⁾ Spectroscopy revealed however that HydF^{EG} contained carbon monoxide and cyanide ligands suggesting that the [2Fe-2S] cluster had been converted to the 2Fe subcluster.⁽¹⁰⁸⁾ This expanded on a similar finding from Czech *et al.* who also observed FTIR Fe-CN and Fe-CO bands when *C. acetobutylicum* HydF was homologously co-expressed with HydE and HydG.⁽¹¹²⁾ Together these results suggested that the diatomic-ligated 2Fe component of the H-cluster was synthesized on HydF prior to transfer to unactivated HydA.

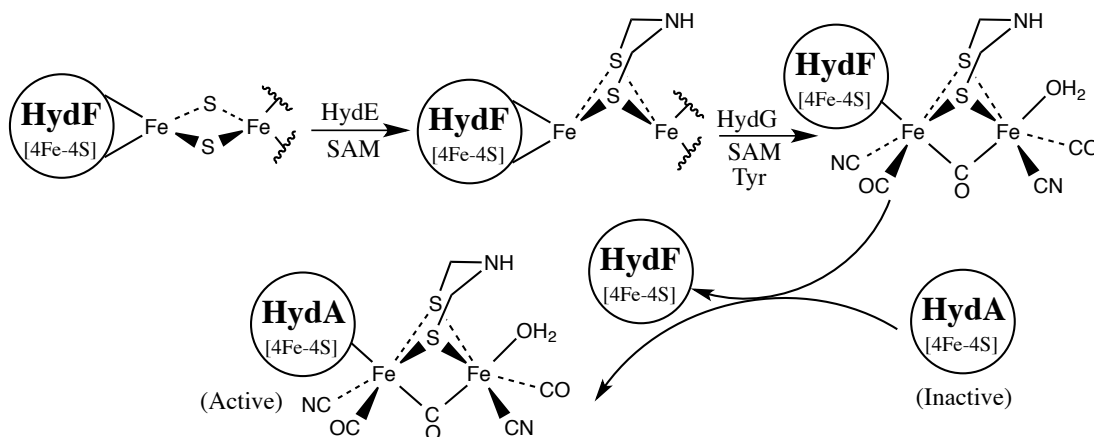


Figure 1.15. Proposed composite [FeFe]-hydrogenase maturase scheme

HydG: Carbon Monoxide and Cyanide Synthase Despite the limited sequence homology of radical SAM enzymes, which generally pushes the limits of alignment algorithms, HydG was found to share a 27% identity with the thiamine biosynthetic enzyme, ThiH.⁽¹¹³⁾ While tyrosine was shown to be a factor in the synthesis of thiamine in the 1970's, this requirement was only recently linked to ThiH.⁽¹¹⁴⁻¹¹⁶⁾ Under the hypothesis that the similarities between HydG and ThiH may extend to a common substrate, Pilet *et al.* found that HydG also demonstrated tyrosine lyase activity, producing *p*-cresol.⁽¹¹³⁾ It was proposed at the time that the dehydroglycine intermediate produced upon cleavage of tyrosine might be a precursor to the DTMA bridge of the H-cluster. Kuchenreuther *et al.* have recently revisited this hypothesis by investigating HydA activation independent of either HydF (HydE and HydG only) or independent of HydE (HydF and HydG only).⁽¹¹⁷⁾ Their results led them to speculate that HydG produces the DTMA bridge (in addition to the diatomic ligands discussed below) to form a pre-H-cluster species termed HydG-co; the involvement of HydG in synthesizing the DTMA bridge has not, however, been conclusively demonstrated. The proposed involvement of tyrosine in HydA maturation was further supported by stimulation of H₂ production by cell lysates containing heterologously overexpressed HydAEFG upon addition of exogenous tyrosine.⁽¹¹⁰⁾

Sequence alignments of *Thermotoga maritima* HydG with *T. maritima* HydE, *Methanosarcina barkeri* PylB, and *E. coli* BioB first indicated that all have (β α)₈ complete TIM barrels. This hypothesis was recently confirmed by the solution of two sets of crystal structures by two separate labs.^(118, 119) The C-terminal domain beginning

immediately after the last alpha helix contains the CX₂CX₂₂C believed to take part in the chemistry that differentiates HydG from ThiH.⁽¹¹³⁾

Following the demonstration that tyrosine served as a substrate for HydG, a race ensued amongst several labs to determine the ultimate product(s) and how these fit into the HydA maturation process. In addition to dAdo and *p*-cresol, HydG was also shown to produce the cyanide ligands likely from a dehydroglycine intermediate.⁽¹²⁰⁾ HydG incubated with SAM, Tyr, and NaDT was shown to produce CN⁻, which was detected via derivatization with naphthalene-2,3-dicarbaldehyde to generate a compound detected by fluorescence and LC-MS. Utilization of U-[¹³C, ¹⁵N]Tyr produced a mass shift in the derivatized product, confirming Tyr as the source of the CN⁻. Shortly thereafter, it was demonstrated that CO was also produced by HydG.⁽¹²¹⁾ The production of CO was detected by monitoring a shift in the λ_{max} values of deoxyhemoglobin included in the HydG assay as it was converted to carboxyhemoglobin. FTIR analysis of the carboxyhemoglobin produced in the assay carried out with U-[¹³C, ¹⁵N]Tyr revealed the expected shifts in the vibrational bands for HbCO, confirming that the CO formed was derived from Tyr. The Swartz lab utilized FTIR analysis to demonstrate that all five diatomic ligands in the H-cluster were derived from tyrosine.⁽¹²²⁾

Conclusive spectroscopic and analytical evidence that two separate [4Fe-4S] clusters are required for HydG activity was provided in a paper by Shepard *et al.*⁽¹²¹⁾ Anaerobically expressed, purified, and reconstituted protein was found to contain 8.7 +/- 0.7 Fe/protein and exhibit EPR spectra similar to those previously reported.⁽¹⁰⁵⁾ Upon addition of SAM to the photoreduced and reconstituted HydG, however, a significant

shift in the g-values for only one of the two clusters was observed. Further, variants of HydG lacking the C-terminal iron-sulfur cluster, made by either deleting the C-terminal domain (Δ CTD) or by changing the first two cysteine residues of the $CX_2CX_{22}C$ motif to serine or alanine, were compromised in their ability to generate the diatomic ligands.^(123, 124) While the Δ CTD variant did not produce measurable CO or CN^- , the C $\rightarrow$ S and C $\rightarrow$ A variants produced half the CN^- compared to wild type HydG, with no CO production.^(123, 124) Insight into the role of the C-terminal [4Fe-4S] cluster was recently inferred from the observation of $Fe(CO)_2CN$ FTIR bands associated with HydG during turnover; this result coupled with ENDOR data tracking ^{57}Fe species suggest that the shuttling of the diatomic species from HydG to HydF may involve cannibalization of HydG's accessory [4Fe-4S] cluster.⁽¹²⁵⁾ A recent crystal structure of HydG was solved with a [5Fe-5S] cluster at the C-terminal terminus; the authors speculate that this may have mechanistic implications for HydG maturation activity.⁽¹¹⁹⁾ Further discussion pertaining to the mechanisms and substrate binding pocket(s) of HydG will be in Chapter 7. Ultimately, the hypotheses relating to the role of the C-terminal cluster in HydG are very exciting and will certainly incite further investigations.

HydE: Putative DTMA Bridge Synthase The same groups that have elucidated the HydF and HydG roles in [FeFe]-hydrogenase maturation have worked on unraveling the function of HydE. An early biochemical characterization of HydE confirmed its place in the radical SAM superfamily by showing that it produced dAdo in the absence of substrate and that it could be reconstituted to contain [4Fe-4S] clusters, but provided little insight into its function or mechanism.⁽¹⁰⁵⁾ The first of several HydE crystal structures

confirmed that HydE contains a complete TIM barrel.⁽¹²⁶⁾ Crystal soaks and *in silico* docking studies indicated that the substrate binding site would accommodate a small compound with a carboxylate group and a partial positive charge.⁽¹²⁶⁾ Also noted was HydE's high affinity for SCN⁻ which led to the speculation that HydE produced CN⁻.⁽¹²⁶⁾

More recently, Costantini *et al.* measured binding constants between HydE, HydF, and HydG by surface plasmon resonance.⁽¹²⁷⁾ They determined that HydE and HydG have K_D values of 9.19×10^{-8} M and 1.31×10^{-6} M, respectively, for binding to HydF. While neither HydG or HydE could displace each other the high affinity, even under aerobic conditions, further supports the proposed maturation scheme (Figure 1.15) in that HydE operates independently of and prior to HydG in the synthesis of the 2Fe H-cluster moiety. Despite the efforts of several groups, however, the substrate, product, and mechanism of HydE have yet to be determined.

HydE has been frequently annotated as biotin synthase (BioB) due to its structural similarities, presence of an auxiliary FeS cluster, and sequence homology to BioB. This has led to proposals that HydE also may have a role in catalyzing a sulfur insertion reaction. However, this proposal is likely inaccurate given that HydE and BioB have significant differences that likely contribute to distinct chemistries. The auxiliary [2Fe-2S] cluster of BioB is located close to the active site (7.6 Å from the 5'-carbon on SAM) and is likely directly involved in providing the sulfur atom for insertion into dethiobiotin through a 9-mercaptodethiobiotin intermediate.⁽²⁸⁾ On the other hand, the auxiliary and radical SAM clusters of HydE are located on opposite sides of the TIM barrel at a distance of over 21 Å (PDB ID: 4JY8). At this distance it is unlikely that the sulfur

atoms of the auxiliary cluster of HydE could be involved in direct insertion into the substrate in a BioB-like reaction. In addition, the auxiliary cluster in HydE is only semi-conserved and not required for HydA activation.⁽¹²⁶⁾

Recently, the crystal structure of methylornithine synthase (PylB) was solved and may provide useful information in the study of HydE.⁽¹²⁸⁾ The gene product of *pylB* is a radical SAM enzyme that catalyzes the isomerization of L-lysine to L-methylornithine in the biosynthetic pathway of pyrrolysine, the 22nd naturally encoded amino acid.⁽¹²⁹⁾ The tertiary structure of PylB is an ($\beta\alpha$)₈ TIM barrel with one conserved [4Fe-4S] cluster coordinated by the canonical CX₃CX₂C motif. The distance alignment matrix method (DALI) was used to compare PylB to all other solved crystal structures with HydE and BioB scoring 29.0% and 20.6% similarity, respectively.^(128, 130) Also, HydE and PylB crystal structures have a root-mean-square deviation (RMSD) of only 1.3 Å compared to BioB and PylB with an RMSD of 1.8 Å. The alignment of PylB and HydE crystal structures show similar SAM and putative substrate binding pockets and together these similarities may point to a parallel mechanism for these two enzymes (Figure 4.7B). Interestingly, HydG and PylB both have proposed mechanisms involving glycine-like intermediates, and perhaps HydE will as well.^(123, 128) Work that I performed investigating HydE is captured in Chapters 3-5.

Research Goals and Highlights

While the maturation of the metallocofactors for [NiFe]-hydrogenase and nitrogenase require several unique maturase proteins, [FeFe]-hydrogenase maturation

requires only three specific gene products. Surprisingly, two of these maturases are radical SAM enzymes. The research that was conducted in close collaboration with the hydrogenase team at MSU focused on the chemistries conducted by these two radical SAM enzymes, HydE and HydG. My work on HydE was a continuance from work conducted largely by two graduate students in the Broderick lab, Nick Boswell and Shourjo Ghose. The aims that I originally described in my research proposal were primarily directed toward testing several new hypotheses about HydE. My last year was spent testing a few new hypotheses and the postulations from other groups regarding the chemistry conducted by HydG. Like all scientific proposals, the science more often than not leads you in exciting and unanticipated directions.

Determining HydE's Substrate, Product, and Reaction Mechanism

The predominant hypothesis when I began this research was that a hydrogen on the β carbon of cysteine, HydE's proposed substrate, was the point of abstraction by the deoxyadenosyl radical. This result was suspect for several reasons, one of which was the instability of the enzyme previously used for biochemical and spectroscopic investigations. My first goal was to develop a HydE enzyme system so as not to be limited by enzyme quantity or quality for assay. Secondly, I wanted to further define the role of cysteine in the HydE reaction. While I hypothesized that cysteine had a non-substrate role and proposed a top down LC-MS screening approach for finding HydE's substrate, I found that the cysteine story was much more interesting than I had previously thought and much of my second and third years were spent elucidating the role of

cysteine, culminating in an alternate hypothesis regarding HydE's substrate and a published manuscript (Chapter 4).

Defining HydG's Substrate Binding Pocket and Tyrosine Lyase Mechanism

Our lab in close collaboration with the Peters Lab has an extensive history with the radical SAM enzyme HydG. My interest in this enzyme was largely stimulated by the solution to the crystal structure of the HydG homolog NosL.⁽¹³¹⁾ This structure challenged previous hypotheses that presumed the sight of H-atom abstraction from tryptophan and tyrosine on NosL and HydG/ThiH, respectively, was at the solvent exchangeable positions of the amino acid side chains. My goals were to test this new theory, further define the roles for the tyrosine substrate binding pocket, and engineer HydG's active sight pocket to accommodate alternate substrates.

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CHAPTER 2

GENERAL METHODOLOGY

Cell Growth and Overexpression of Proteins

Several FeS-cluster containing proteins were expressed and purified employing the following generalized procedure. While the basic principles were unchanged, the hardware utilized varied depending upon instrument availability and the scale of the overexpression/purification. Details regarding the building and mutation of the specific constructs that were used in the discussed biochemical and spectroscopic assays will be described in Chapters 3, 4, and 7.

Following sequence verification, the constructs were transformed into BL21-CodonPlus(DE3)-RIL chemically competent cells which were made using protocols recommended by Agilent Technologies. This cell line contains a plasmid with three tRNA genes that are not commonly found in *E. coli* but necessary for overexpression of the proteins of interest. This plasmid is maintained by chloramphenicol when required. Transformants were used for pilot expressions and subclones that overexpressed were stored in 30% glycerol at -80 °C.

Fernbach Flask 9 L Growths

In short, phosphate buffered low-salt LB supplemented with iron was used as the growth medium for most of the routine overexpressions of the radical SAM enzymes and HydF and the procedure followed similar previously published methods.⁽¹⁾ Table 2.1 lists

the components of the typical recipe for each liter of media. For each 9 L growth the media was divided between six 2.8 L baffled Fernbach flasks, each with 1.475 L, and three 250 mL flasks, each with 50 mL, all of which were then autoclaved.

Table 2.1. Media recipe used for protein overexpression in *E. coli* of radical SAM enzymes and HydF.

<u>Chemical</u>	<u>1 Liter</u>	<u>9 Liter</u>
KH ₂ PO ₄	2.268 g	20.412 g
K ₂ HPO ₄	5.806 g	52.252 g
Tryptone	10 g	90 g
Yeast Extract	5 g	45 g
Dextrose	5 g	45 g
pH adjusted with NaOH	7.2	7.2
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	1 mM	1 mM
Ferrous Ammonium Sulfate	0.12 g	1.08 g
Kanamycin	30 mg/L	30 mg/L
Ampicillin	100 mg/L	100 mg/L
Streptomycin	50 mg/L	50 mg/L

The night before overexpression, the three 50 mL starter flasks with appropriate antibiotic were inoculated with either a single colony from an LB agar plate or with 1 mm³ of a previously made glycerol stock and placed in a shaker incubator at 37 °C and 250 rpm and grown overnight. The next morning the dextrose, appropriate antibiotic, and 7.5 mL of overnight culture were added to each flask and these were incubated at 37 °C and 225 rpm for approximately 3 hrs. At an OD₆₀₀ somewhere between 0.45 to 0.6 the IPTG and half of the ferrous ammonium sulfate were added and the cultures were allowed to incubate at 37 °C and 225 rpm for an additional 2.5 hrs at which time they were removed from the shaker incubator and allowed to cool at room temperature for 15 to 30 minutes. The remaining ferrous ammonium sulfate and two drops of antifoam were added to all six flasks. The flasks were then consolidated down to four flasks and the

cultures were sparged with nitrogen overnight (14 hrs) at 4 °C. The following morning the cells were collected by centrifugation at 6000 rpm for 5 min. The wet cell pellets, which ranged from 16 to 24 grams total, were flash frozen in liquid nitrogen and stored at -80 °C until purification.

Fermenter 10 L Growths

In addition to generating higher cell mass and greater protein yield fermenter growths can also be a simpler choice for protein overexpression with minimal media given the ease associated with adding the trace constituents over the duration of the growth in this method.

The minimal media is composed of a mixture of amino acids supplemented with carbon (glucose), phosphate, sulfate, iron, nitrogen (ammonium), calcium, monovalent salts, and vitamins. Component A (Table 2.2) was autoclaved and added to the fermenter the day prior to overexpression.

Table 2.2. Components used for fermenter growths

<u>Component A</u>	<u>Quantity</u>	<u>Component C</u>	<u>Quantity</u>
18 MΩ Water	9.8 L	Biotin	10 mg
Casamino Acids	100 g	Pantothenic Acid	10 mg
MOPS (Buffer Salt)	84.2 g	Cobalamin	10 mg
Tricine	8.0 g	Thiamine	10 mg
Sodium Chloride	14.7 g	Folic Acid	10 mg
Potassium Hydroxide	16.0 g	Riboflavin	10 mg
Ammonium Chloride	5.1 g	Niacinamide	10 mg
		Thioctic Acid	10 mg
<u>Component B</u>	<u>Quantity</u>	Pyridoxine	10 mg
18 MΩ Water	50 g	Kanamycin (30 mg/L)	10 mL
KH ₂ PO ₄ (1 M)	25 mL	Ampicillin (100 mg/L)	10 mL
K ₂ SO ₄ (276 mM)	12.5 mL	Streptomycin (50 mg/L)	10 mL
CaCl ₂ (0.1 M)	62.5 mL		
Glucose	50 g		
Ferrous Ammonium Sulfate	0.75 g		

Starter cultures were prepared by inoculating 50 mL of LB media and the appropriate antibiotic with a single colony from an LB agar plate or with 1 mm³ of glycerol stock. These overnight cultures were incubated at 37 °C and 250 rpm for less than 18 hours prior to inoculation of the minimal media. The day of the growth the fermenter, with Component A, was warmed to 37 °C using a thermostatically controlled jacket, agitated at 300 rpm, and then bubbled with filtered compressed air at 5 L/min. During equilibration Component B was made, sterile filtered, and added to Component A in the fermenter. Component C was also added as a dry powders. The entire 50 mL overnight starter culture was added and the OD₆₀₀ was monitored. At an OD₆₀₀ between 0.45 and 0.6 IPTG was added for a final concentration of 0.5 mM IPTG and the growth allowed to incubate. After 2 hrs the temperature was actively cooled to 30 °C as the compressed air was removed and nitrogen sparging began. At 20 °C five drops of antifoam and an additional 0.75 g of ferrous ammonium sulfate hexahydrate were added to the fermenter and then it was placed in a 4 °C refrigerator and sparged with nitrogen overnight (14 hrs). The following day the cells were centrifuged at 6000 rpm and the resulting wet cell paste was flash frozen in liquid nitrogen and stored at -80 °C until purification. Pre and post expression along with purification samples were taken throughout this process and eventually run on SDS-PAGE gels that were later stained with Coomassie dye to check overexpression levels and relative protein purity (Figure 2.1).

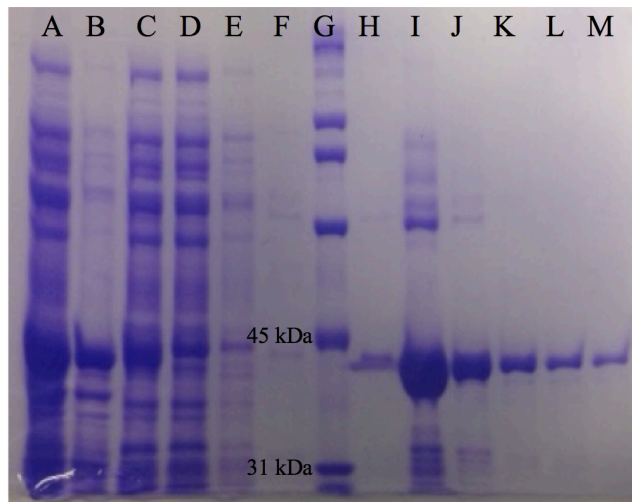


Figure 2.1. SDS-PAGE gel of *C. acetobutylicum* HydE with C-terminal tag. A. Whole cell lysate. B. Precipitated insoluble cell debris. C. Soluble clarified lysate. D. Ni-NTA column flowthrough. E. 10mM imidazole column wash flowthrough. F. 25mM imidazole column wash flowthrough. G. Biorad broad range molecular weight standards. H-M. Single column volume elutions 1-6.

Purification of HydE and HydG

All subsequent preparation of protein for assays was conducted in gas tight containers or in anaerobic Coy chambers with a 5% hydrogen atmosphere balanced with nitrogen. Previous members of the Broderick Lab have purified heterologously overexpressed polyhistidine tagged HydE and HydG from *C. acetobutylicum* in a variety of buffers.^(2, 3) However, due to protein stability issues, activity optimization, and convenience a single common buffer (CaHyd) composed of 25 mM HEPES pH 8.0, 500 mM KCl, and 5% glycerol (w/v) was found to be optimal for most situations (Chapter 3). Cell lysis was accomplished using a method similar to the one previously described⁽¹⁾ where cell pellets containing overexpressed protein were thawed and incubated in a lysis buffer with a 1:2 cells/buffer ratio. The chemical lysis buffer was composed of CaHyd

buffer with 10 mM imidazole and 1% Triton X-100 and supplemented with 9 mg lysozyme, 9 mg PMSF, 1 mg DNase, 1 mg RNase, and 100 mg MgCl_2 per 50 mL. The cell lysis slurry was stirred vigorously for 2-3 hrs in a strictly anaerobic Coy chamber at 4 °C. A clarified lysate was generated by centrifugation at 18K rpm for 30 min at 4 °C in sealed Oakridge tubes. For HydE, the clarified lysate was passed over 9 mL of Qiagen Ni-NTA resin that had been equilibrated in lysis buffer (10 mM imidazole CaHyd buffer). When loaded with HydE, the column turned visibly dark brown (Figures 2.1 and 2.2A). The protein bound column was then washed with 7-10 column volumes of lysis buffer and a 25 mM imidazole wash buffer. Finally, the protein was eluted with 250 mM imidazole buffer (figure 2.2B). Colored fractions that ranged from 35 to 330 μM protein were immediately buffer exchanges or dialyzed at 4°C to remove imidazole. This method routinely generated protein that was >95% pure as judged by SDS-PAGE gel with Coomassie staining (figure 2.1). Following removal of imidazole the protein was aliquoted into screw top vials with a rubber o-ring cap, flash frozen in liquid nitrogen, and stored at -80 °C.

Reconstitution of Radical SAM Enzymes

As both HydE and HydG are purified with substoichiometric (< 8 Fe/protein) iron, reconstitution with exogenous iron and sulfide is necessary to achieve fully loaded and active enzymes. The following method, similar to those previously published by our lab⁽⁴⁾, can be easily scaled to accommodate any quantity of protein and consistently yields

protein with near full loading of iron as judged by EPR and iron quantitation assays (see following section).

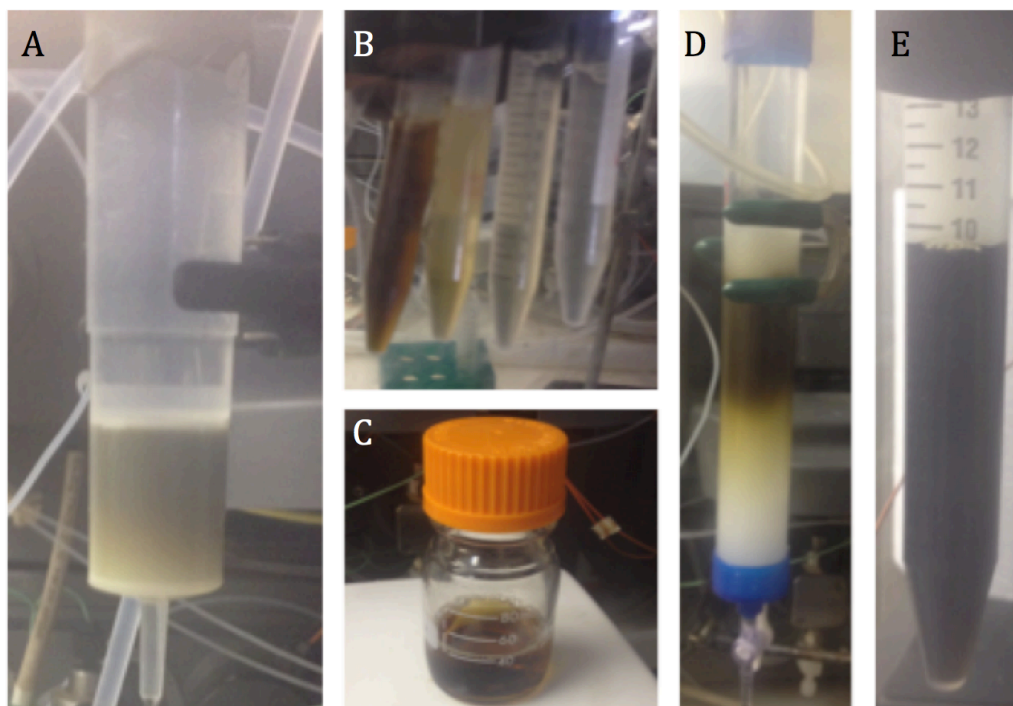


Figure 2.2. Protein purification and reconstitution images. A. Ni-NTA poly-His affinity column loaded with *C. acetobutylicum* HydE. B. HydE eluted with imidazole from column. Fractions 1 through 4. C. Reconstitution of 100 μM HydE. D. Sephadex G25 resin in gravity flow column used to exchange buffer and remove imidazole. E. Reconstituted HydE protein concentrated to 344 μM .

Pooled HydE fractions were adjusted to a 100 μM final protein concentration and DTT was added to a final concentration of 5 mM prior to reconstitution with exogenous Fe^{3+} and S^{2-} at 4°C. Over 30 min, six successive aliquots of a 50 mM S^{2-} in water (final concentration of 600 μM /6x protein) were added to HydE. Following the sulfide, 10 mM FeCl_3 was added in six additions over 30 min to a final concentration of 600 μM . The solution turned noticeably darker upon each addition of the iron solution (Figure 2.2C). After 2 hrs of incubation with stirring the solution was loaded into an Oakridge gas tight

tube and centrifuged for 30 min to remove FeS particulates. The supernatant containing protein was decanted into Amicon 15mL 10 kDa MWCO spin concentrators and spun at 6000 rpm down to ~0.5 mM prior to being buffer exchanged on a 75 mL Sephadex G25 gravity flow column (Figure 2.2D) to remove any remaining DTT, S^{2-} , and Fe^{2+} . The protein (Figure 2.2E) was then aliquoted and flash frozen prior to storage at $-80^{\circ}C$ in screw top tubes.

Protein and Iron Quantitation

Protein was quantified using the Bradford method with a BSA standard solution.⁽⁵⁾ A colorimetric assay using the spectrophotometric reagent ferrozine was used to quantify the iron content of the protein samples in a manner similar to a previously published method.⁽⁶⁾

Synthesis and Purification of S-adenosylmethionine (SAM)

SAM was enzymatically prepared *in vitro* using a crude SAM synthetase lysate similarly to the previously described method.⁽⁷⁾ The components listed in Table 2.3A were added to a scintillation vial and incubated overnight (15-16 hrs) with stirring. The reaction was quenched with 1 mL of 1 M HCl causing a noticeable cloudiness due to protein precipitation. The precipitate was removed by centrifugation at 18K rpm at $4^{\circ}C$ for 30 min and the supernatant loaded on equilibrated Source 15S cation exchange resin in a GE, HR 10/10 column. SAM binds well to the column given its positive charge and after washing with water and 0.1 M HCl, applying a linear gradient (Table 2.3B) up to 1

M HCl was sufficient to elute the SAM. Depending upon the quantity of SAM loaded on the column, the SAM eluted between 0.35 and 0.55 M HCl, which could be easily observed by monitoring the eluent's absorbance at 260 nm. The eluate containing SAM was concentrated using a rotary evaporator and subsequently lyophilized overnight to a powder. The white to light yellow powder was anaerobically resuspended in 50 mM Tris pH 8.0 buffer and the pH of the solution was then adjusted to 7.2 in an MBraun anaerobic chamber. The concentration of SAM was measured using the extinction coefficient of $16000 \text{ M}^{-1} \text{ cm}^{-1}$ and adjusted to 50 mM before aliquoting and storing at -80°C .

Table 2.3. *S*-adenosylmethionine synthesis. A. Components of one pot synthesis of SAM. B. SAM chromatography scheme with cation exchange column.

<u>Component</u>	<u>Quantity</u>	<u>Flow (2 mL/min)</u>	<u>HCl (M)</u>	<u>Time (min)</u>
100 mM Tris-HCl, pH 8.0	8.275 mL	Isocratic	0.0	13
Potassium Chloride	37 mg	Linear Gradient	0.0 to 0.1	6
Magnesium Chloride	53 mg	Isocratic	0.1	8
0.5 M EDTA	18 μL	Linear Gradient	0.1 to 1.0	60
Adenosine Triphosphate	73 mg	Isocratic	1.0	10
L-methionine	18 mg	Linear Gradient	1.0 to 0.0	4
β -mercaptoethanol	800 μL	Isocratic	0.0	20
Inorganic phosphatase	5 μL			
SAM synthetase crude lysate	1 mL			

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CHAPTER 3

HydE CONSTRUCT DEVELOPMENT

Background

The desire for a *hydE* construct that overexpresses well, is stable during purification, and amenable to assay with the other maturase enzyme and HydA has led to testing five different constructs and the development of three. Ultimately, a construct with a C-terminal poly-His tag from *Clostridium acetobutylicum* heterologously expressed in an *E. coli* strain proved to be the best all around construct for pertinent spectroscopic work and biochemical assays. The following will provide a brief description of the process that led to the selection of this expression system and the recent exploits with this protein.

Previous cloning work from the Peters group led to the development of *Thermotoga maritima* and *C. acetobutylicum hydE* constructs.⁽¹⁾ However, N. Boswell's work with *C. acetobutylicum* HydE was met with difficulty as the ensuing overexpressions resulted in a low yield of protein and the protein often precipitated during purification or upon subsequent handling. Further, pilot expressions and 9 L batch growths from his glycerol stocks did not yield noticeable overexpression as observed by SDS-PAGE (not shown).

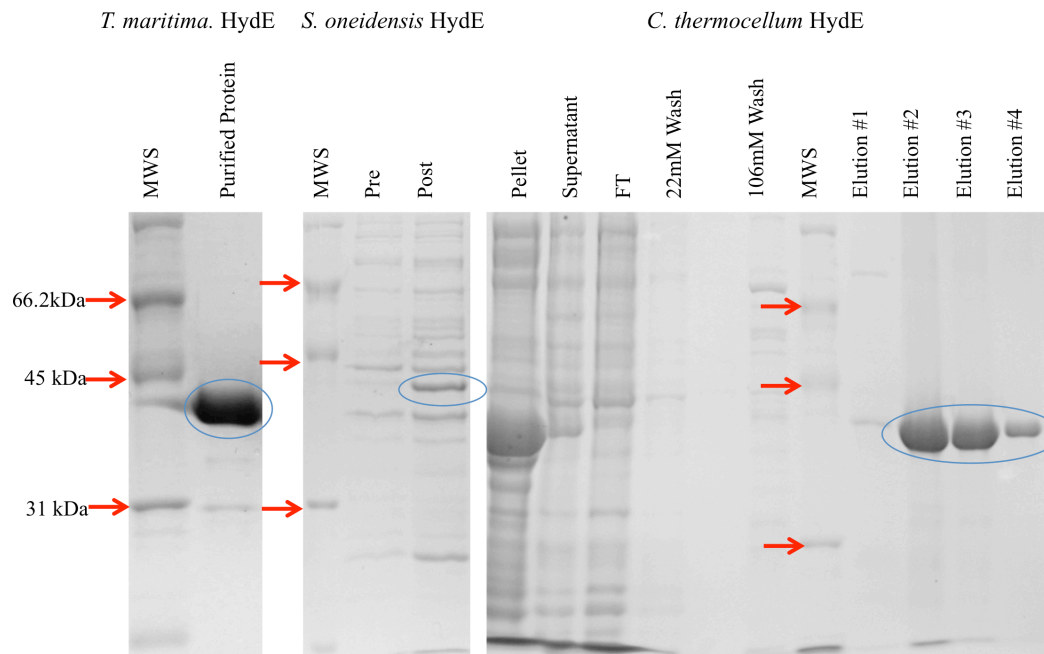


Figure 3.1. HydE overexpression and purification from *Thermotoga maritima*, *Shewanella oneidensis*, and *Clostridium thermocellum*. Samples' contents visualized on 10% SDS-PAGE gel with coomassie staining.

Overexpression of HydE

Thermotoga maritima HydE

Even though the *T. maritima* maturase system has not yet been successfully used to activate HydA, *T. maritima* HydE's strong overexpression and thermal stability made it a good candidate for an individual screening assay (Figure 3.1A). *T. maritima* HydE was grown in 9 L batches in low salt Miller LB media under conditions developed in a collaboration between the Peters and King labs for *C. acetobutylicum* maturase proteins.⁽¹⁾ Cultures were initially grown under aerobic conditions followed by sparging with nitrogen overnight to aid in cluster assembly and folding of anaerobic proteins. The cell lysate was then anaerobically purified by FPLC yielding a modest 2 mg *T. maritima*

HydE per liter of cell culture. Mass spectroscopic analysis calculated the mass of the purified *T. maritima* HydE to be 41966.4 amu, which is in close agreement with the predicted transcript. Upon subsequent dialysis 40% of the protein precipitated as quantified by the Bradford method. During this time *Clostridium thermocellum* and *Shewanella oneidensis* constructs were developed and attention was diverted to characterize these more promising proteins.

Performing a BLAST search using *T. maritima hydE* yields hundreds of homologous proteins. Among these, *S. oneidensis* and *C. thermocellum* were chosen due to accessibility to their genomic DNA for cloning, among other reasons. In fact, the *S. oneidensis* [FeFe]-maturase system had been successfully used by a competitor's lab.⁽²⁾ By using a defined “*in vitro*” maturase system this lab reported that exogenous tyrosine and cysteine stimulate HydA activity. While most of their work involves using exclusively cell lysates, their expression system has been demonstrated to activate [FeFe]-hydrogenase. HydE from *C. thermocellum* was also selected because of its origin from a thermophilic organism and, therefore, its potential to exhibit thermal stability. In addition *C. thermocellum* and the previously studied *C. acetobutylicum hydE* gene products share a 43.7% identity.

Shewanella oneidensis and
Clostridium thermocellum HydE

S. oneidensis and *C. thermocellum hydE* genes were each cloned from genomic DNA into a pET14b vector and sequence verified prior to transformation into *E. coli* expression strains. Pilot growths (See Figure 3.1B) for both constructs were positive for

overexpression as was determined by SDS-PAGE of the total cellular protein. While *S. oneidensis* HydE overexpressed well the protein was likely unstable, folded in a manner that shielded the poly-His affinity tag, or was packaged into inclusion bodies *in vivo*. This was evident upon chemical lysis of the cell pellet and subsequent FPLC purification of the recovered *S. oneidensis* HydE fractions that were nearly devoid of FeS clusters and of very low concentration as determined by absorbance at 426 nm and SDS-PAGE, respectively. Further optimization and characterization of *S. oneidensis* HydE was suspended as concurrent work on the *C. thermocellum* HydE construct proved to be more promising.

As previous HydE constructs often precipitated during the slow loading and washing processes during FPLC, a gravity flow column packed with Ni-NTA resin was used to purify the clarified lysate in order to shorten the purification time. Upon elution with imidazole a recovery of 12.5 mg *C. thermocellum* HydE per liter of cell culture was achieved (See Figure 3.1C). The protein was found to have a mass of 42259.7 amu that corresponded well with the predicted 42259.9 amu of the holoprotein after its cleavage of the N-terminal methionine. A second large peak that could not be attributed to potassium adduct(s) was also observed in the deconvoluted mass spectrum at 42435.1 amu (175.4 amu higher). This peak may have been due to the presence of a persistent [2Fe-2S] cluster which, according to the literature, is consistent with a 175.8 amu mass found in ESI mass spectra of intact [2Fe-2S] clusters.⁽³⁾

During handling *C. thermocellum* HydE, especially during thawing, up to 40% of the protein would often precipitate as was determined by Bradford assays. In a series of

freeze/thaw experiments it was found that rapid thawing resulted in a more stable protein and only 10% of the total protein was subsequently lost. In compliance with this observation, upon removal from the -80 °C freezer the anaerobically sealed screw top tube with protein was consistently thawed in a 42 °C water bath prior to entry into the anaerobic chamber. The protein was then reconstituted with Fe^{2+} and S^{2-} under reducing conditions to attain up to 6.41 iron atoms per protein. The g -values calculated and the temperature relaxation profile from EPR spectroscopy of the purified *C. thermocellum* HydE indicated the presence of a mixture of [4Fe-4S], [3Fe-4S], and [2Fe-2S] clusters. The complicated spectra that contained shoulders on the pertinent peaks may have been caused by substoichiometric occupation of both [4Fe-4S] clusters (See Figure 7A and 7B).

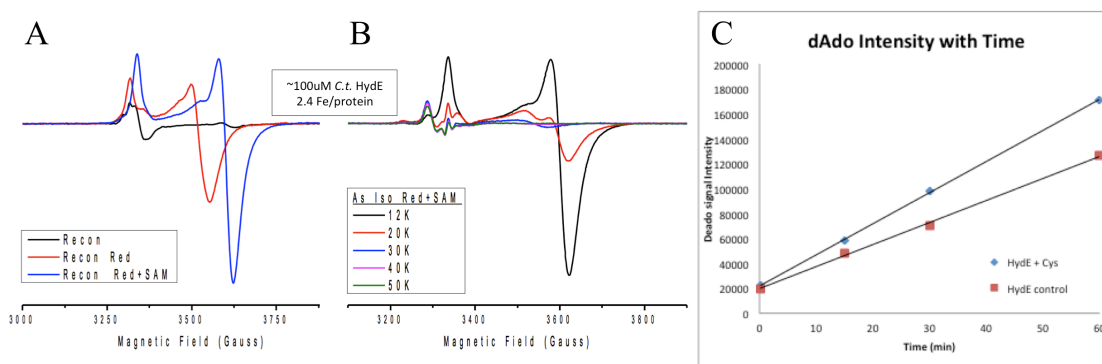


Figure 3.2. A. EPR Spectra of *C. thermocellum* HydE (100 μM) with 2.4 Fe/protein reconstituted, photoreduced, and photoreduced with exogenous SAM (2 mM); B. EPR temperature relaxation profile. The EPR spectra parameters were microwave frequency 9.37 GHz; temperature 12 +/- 2K unless otherwise noted; power, 1 mW; time constant and conversion time, 20.48 ms; receiver gain, 1×10^4 ; modulation frequency, 100 kHz; and modulation amplitude, 10 G; C. Plots of production of 5'-deoxyadenosine with time for *C. thermocellum* HydE (20 μM), SAM (2 mM), and sodium dithionite (1 mM) with and without L-cysteine (200 μM) at 50 °C.

While all kinetic assays with *C. thermocellum* HydE were run at 50 °C (See Figure 2.1C), this was not compatible with *C. acetobutylicum* HydF because it is unstable at this temperature. This presented a problem in the continuation of S. Ghose's research. Based on his data, he suggested that in the presence of *C. acetobutylicum* HydF, *C. acetobutylicum* HydE produced less 5'-deoxyadenosine (dAdo) and abstracted a proton from the C3 position of cysteine. In order to continue S. Ghose's research and confirm his results it was necessary to either develop a *C. thermocellum* *hydF* construct or reclone *C. acetobutylicum* *hydE* in a different vector or coexpress HydE fused to HydF, which has been a successful technique with other similar radical SAM enzymes.⁽⁴⁾

Shewanella oneidensis HydEF Fusion Proteins

To create a fusion of HydE and HydF I chose to take advantage of the *S. oneidensis* *hyd* operon in which *hydE* and *hydF* are syntenous. More specifically, the genes *hydE* and *hydF* are naturally separated by only 40 nucleotides. The two genes and the noncoding linker were amplified using two sets of primers (Table 3.1) in order to make HydEF fusion proteins that were tagged both N- and C-terminally. The vectors pET-14b and pET-23b were used to make the HydEF fusion with N-terminal and C-terminal 6x His tagged peptides, respectively. To make a single fused protein the stop codon from HydE had to be removed and the proteins had to be put in a single frame. By removing a single adenosine from the TAA stop codon with mutagenesis both requirements were fulfilled. All together four constructs were generated with *S. oneidensis* *hydE* and *hydF*. Table 3.1 provides information regarding the details of the primers, restriction sites, and host plasmids used for the cloning work I performed while

conducting my research. The constructs overexpressed well in *E. coli* BL21(DE3) producing brown proteins with the appropriate masses of 86 kDa. The protein, however, was susceptible to precipitation upon concentration and handling and not extensively assayed. While these proteins showed promise and should be revisited in the future, the *C. acetobutylicum* HydE protein described below also began to show encouraging results and so investigations into the HydEF fusion proteins were discontinued.

Clostridium acetobutylicum Revisited

In reviewing radical-SAM and protein purification literature two observations were made. Many radical SAM enzymes such as HydE have the site-differentiated [4Fe-4S] cluster near the N-terminus. Placing a poly-His affinity tag at the C-terminal end of the protein has generally been found to result in more stable protein.⁽⁵⁾ Secondly, purifying and assaying SAM proteins in a buffer with a pH that is more than one pH unit from the protein's pI is ideal.⁽⁶⁾ Perhaps the instability and difficulty with *C. acetobutylicum* HydE experienced by S. Ghose and N. Boswell was caused by a C-terminal poly-His tag and/or the choice of buffer pH.

Indeed, the C-terminal tagged *C. acetobutylicum* HydE protein (pI = 6.87) is stable during purification, dialysis, reconstitution, spin and sponge well concentration, and multiple freeze/thaw events in a pH 8.0 buffer and overexpresses at adequate levels (See Figure 3.3A and B). Off the column ~3 mg of *C. acetobutylicum* HydE was recovered per liter of culture. As isolated protein contained 3.7 Fe/protein and could be reconstituted to consistently contain up to 8 Fe/protein (See Figure 3.3C) as determined by colorimetric ferrozine and Bradford assays and confirmed by spectroscopic analysis.

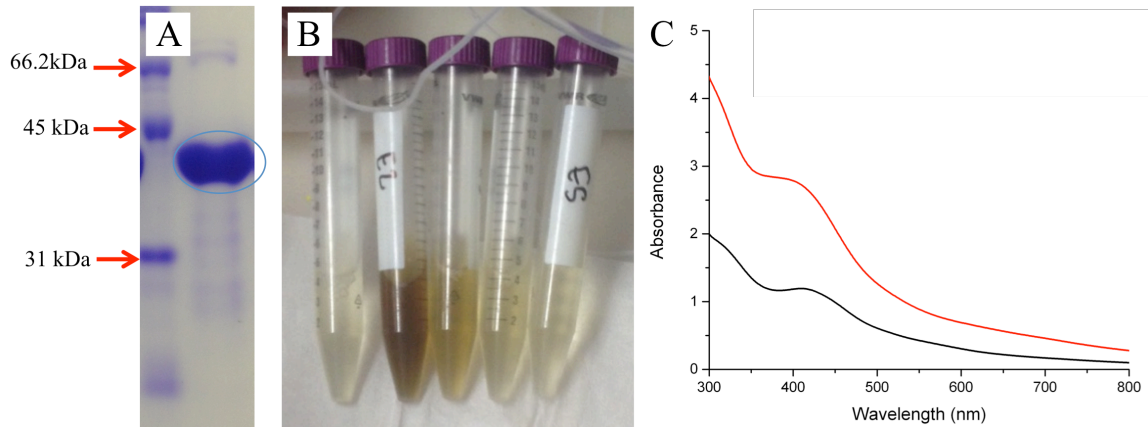


Figure 3.3. *C. acetobutylicum* HydE images. A. Purification HydE elutions; B. SDS-PAGE elution #3; C. UV-vis of pre (black trace) and post-reconstituted *C. acetobutylicum* HydE (red trace) (83 μ M).

Table 3.1. Protein overexpression constructs tested and/or built.

Protein(s)	Organism	Plasmid	Antibiotic	Affinity Tag	PCR Primer Set
HydE	<i>Clostridium acetobutylicum</i>	pET-23b	Amp	C-term 6x His	CCGTCATATGGATAATATAATAAGTTAATTAATAAGCAGAG GGATCTCGAGACCAATAGATTCTTTGTAGCTTTTTTTC
HydE	<i>Clostridium thermocellum</i>	pET-14b	Amp	N-term 6x His	GGACATATGACAAACATGACAAATATGATAAAATTAATTGACA GGAGGATCCTTAATTGGCTTTTTCAGTCGC
HydE	<i>Shewanella oneidensis</i>	pET-14b	Amp	N-term 6x His	AGTCATATGATCACTCGCCCTAGCC GAGGGATCCTTAATGCCTTGACACATATTGAGTCCTTTTG
HydE	<i>Thermatoga maritima</i>	pET-21b	Amp	C-term 6x His	ND ND
HydEF Inline	<i>Shewanella oneidensis</i>	pET-14b	Amp	N-term 6x His (HydE)	CTGCCTCATATGATCACTCGCCCTAGCC GTGCCTGGATCCCTATTGCTGAGGATTGCGGT
HydEF Inline	<i>Shewanella oneidensis</i>	pET-23b	Amp	C-term 6x His (HydF)	CTGCCTCATATGATCACTCGCCCTAGCC GTGCCTAAGCTTTTGTGCTGAGGATTGCGGTTA
HydEF Fusion	<i>Shewanella oneidensis</i>	pET-14b	Amp	N-term 6x His	CTGCCTCATATGATCACTCGCCCTAGCC GTGCCTGGATCCCTATTGCTGAGGATTGCGGT
HydEF Fusion	<i>Shewanella oneidensis</i>	pET-23b	Amp	C-term 6x His	CTGCCTCATATGATCACTCGCCCTAGCC GTGCCTAAGCTTTTGTGCTGAGGATTGCGGTTA
HydF	<i>Clostridium thermocellum</i>	pET-23b	Amp	C-term 6x His	CGATGCTAGCGGATTGAATGAAACACCATCTG CACTCTCGAGAAGAATTTCTGCAAGTATGTCCG
HydG	<i>Shewanella oneidensis</i>	pET-23b	Amp	C-term 6x His	CTGCCACATATGAGCACACGAGCATCA GTTCTCTCGAGCAGATAGATATCCCGTTCACCC
HydG	<i>Shewanella oneidensis</i>	pCDFDuet	Strep	C-term Strep	NA NA

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CHAPTER 4

[FeFe]-HYDROGENASE MATURATION: INSIGHTS INTO THE
ROLE HYDE PLAYS IN DITHIOMETHYLAMINE BIOSYNTHESIS

Contribution of Authors and Co-Authors

Manuscripts in Chapters 4

Author: Jeremiah N. Betz

Contributions: Conducted bulk of experimental work, and preparation of most figures and text of the manuscript.

Co-Author: Nicholas W. Boswell

Contributions: Conducted preliminary assays that contributed to the hypotheses.

Co-Author: Corey J. Fugate

Contributions: Conducted molecular docking experiments, suggested alternate experiments, and helped interpret results.

Co-Author: Gemma L. Holliday

Contributions: Prepared sequence similarity network, prepared selected figures and parts of the text of the manuscript, and interpreted results.

Co-Author: Eyal Akiva

Contributions: Prepared genomic context network, prepared selected figures and parts of the text of the manuscript, and interpreted results.

Co-Author: Anna G. Scott

Contributions: Prepared protein and reagents and conducted assays.

Contribution of Authors and Co-Authors - Continued

Co-Author: Patricia C. Babbitt

Contributions: Provided important insight and overview for sequence similarity and genomic context networks. Aided in interpretation of results and preparation of the manuscript and figures.

Co-Author: John W. Peters

Contributions: Provided important insight and overview for biochemical assays and hydrogenase maturation context. Aided in interpretation of results and preparation of the manuscript and figures.

Co-Author: Eric M. Shepard

Contributions: Conducted preliminary investigations with HydE. Provided preliminary insight and overview for biochemical assays and hydrogenase maturation context. Aided in preliminary interpretation of results and preparation of the preliminary manuscript and figures.

Co-Author: Joan B. Broderick

Contributions: Provided key insight and overview for entire research program. Facilitated interpretation of results and preparation of the manuscript and figures.

Manuscript Information Page

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[FeFe]-Hydrogenase Maturation: Insights into the Role HydE Plays in Dithiomethylamine Biosynthesis

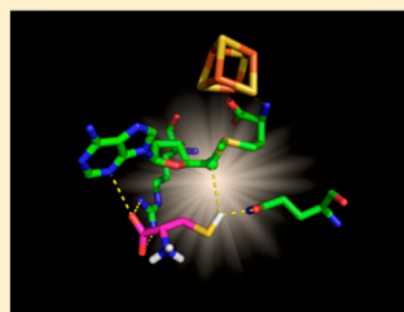
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S Supporting Information

ABSTRACT: HydE and HydG are radical S-adenosyl-L-methionine enzymes required for the maturation of [FeFe]-hydrogenase (HydA) and produce the nonprotein organic ligands characteristic of its unique catalytic cluster. The catalytic cluster of HydA (the H-cluster) is a typical [4Fe-4S] cubane bridged to a 2Fe-subcluster that contains two carbon monoxides, three cyanides, and a bridging dithiomethylamine as ligands. While recent studies have shed light on the nature of diatomic ligand biosynthesis by HydG, little information exists on the function of HydE. Herein, we present biochemical, spectroscopic, bioinformatic, and molecular modeling data that together map the active site and provide significant insight into the role of HydE in H-cluster biosynthesis. Electron paramagnetic resonance and UV–visible spectroscopic studies demonstrate that reconstituted HydE binds two [4Fe-4S] clusters and copurifies with S-adenosyl-L-methionine. Incorporation of deuterium from D₂O into 5'-deoxyadenosine, the cleavage product of S-adenosyl-L-methionine, coupled with molecular docking experiments suggests that the HydE substrate contains a thiol functional group. This information, along with HydE sequence similarity and genome context networks, has allowed us to redefine the presumed mechanism for HydE away from BioB-like sulfur insertion chemistry; these data collectively suggest that the source of the sulfur atoms in the dithiomethylamine bridge of the H-cluster is likely derived from HydE's thiol containing substrate.



The interconversion between H₂ and 2H⁺ and 2e[−] within microorganisms is catalyzed by the [FeFe]- and [NiFe]-hydrogenases.¹ Despite a lower tolerance for O₂, [FeFe]-hydrogenase is the more promising catalyst for economical hydrogen production due to its higher catalytic rates that are on the order of 10⁴ turnovers per second.^{2–4} [FeFe]-hydrogenases (HydA) contain a catalytic 6Fe-cluster, termed the H-cluster, which through biochemical, analytical, spectroscopic, and crystallographic investigations was found to contain a [4Fe-4S] cluster bridged by a cysteine thiolate to a uniquely decorated 2Fe-subcluster (Figure 1).^{5–7} The two irons of the subcluster are bridged by one carbon monoxide and a dithiomethylamine (DTMA) ligand. Each iron is additionally coordinated by one carbon monoxide and one cyanide.

An intriguing clue to H-cluster assembly was provided when analysis of insertional mutants revealed that two putative radical S-adenosyl-L-methionine (SAM) enzymes were necessary for maturation of HydA.⁸ These radical SAM enzymes, HydE and HydG, both contain the CX₃CX₂C motif characteristic of this enzyme superfamily, and both have been shown to coordinate a [4Fe-4S] cluster at this motif.⁹ HydG utilizes radical SAM chemistry to cleave tyrosine to produce *p*-cresol, CO, and CN[−]; the diatomics are subsequently incorporated into the 2Fe-subcluster component of the H-cluster of HydA.^{10–13} The role of HydE is undefined and the topic of the current study. A third

protein required for HydA maturation, HydF, is a GTPase that binds iron–sulfur clusters and appears to serve as a scaffold or carrier for assembly and delivery of the 2Fe-subcluster of the H-cluster.^{14–17}

While biochemical and structural studies of HydE have confirmed its assignment as a radical SAM enzyme with a complete TIM barrel (β α)₈ structure, no direct insight into its role in maturation has yet been provided.^{9,18,19} Because synthesis of the diatomics is accomplished by HydG,^{11,12} and scaffolding of the 2Fe precursor is carried out by HydF,^{14,16,20} it is presumed that HydE functions in DTMA synthesis.^{21,22} While it was previously proposed that the DTMA bridge could be derived from tyrosine,¹⁰ a recent spectroscopic study of HydA matured in the presence of ¹⁵N-tyrosine ascribes the bridgehead atom of the DTMA bridge to a source other than tyrosine.²³ HydE was originally hypothesized to catalyze biotin synthase-like sulfur insertion chemistry;^{9,48} however, it has more recently been observed that HydE shares significant sequence similarity to methylornithine synthase (PylB).²⁴ This homologous relationship perhaps points to a different type of radical SAM reaction for HydE.²⁵ Sequence percent identities

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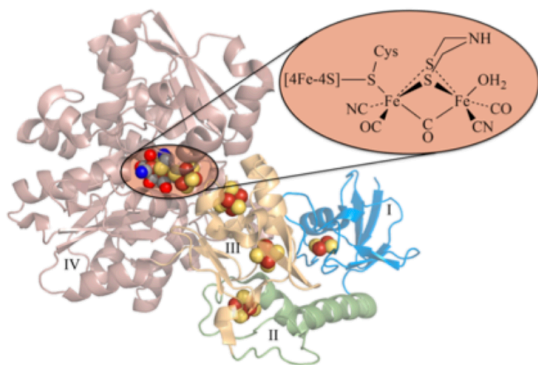


Figure 1. [FeFe]-hydrogenase crystal structure with H-cluster. The active site is located in domain IV common to all [FeFe]-hydrogenases. Atoms explicitly drawn have the following color scheme: iron-rust, sulfur-yellow, nitrogen-blue, oxygen-red, and carbon-gray. PDB ID: 3C8Y.

between HydE and related proteins have been previously described in the literature,²⁶ and a sequence alignment appears in Figure S1, Supporting Information.

In this study, we present data showing that select low-molecular weight thiol containing compounds significantly increase the rate of HydE catalyzed SAM cleavage and also promote incorporation of deuterium from D₂O into the SAM cleavage product 5'-deoxyadenosine (dAdoH). Molecular docking investigations place several of these thiols, including D-/L-cysteine, D-/L-homocysteine, mercaptopyruvate, γ -glutamylcysteine, and dithiothreitol, within the active site, such that the thiol hydrogens are positioned appropriately for direct abstraction by the dAdo• that would be generated upon reductive cleavage of SAM. The results help to define the chemical properties of the HydE substrate and indicate that it contains a thiol functional group that is located approximately 5 Å from the 5' position of SAM. These clues lead us to a proposed mechanism for synthesis of the DTMA bridge of the H-cluster involving small molecule lyase activity that is reminiscent of HydG chemistry. Together, our results shift the HydE paradigm away from that of BioB-like sulfur insertion chemistry and toward the premise that the DTMA sulfur atoms of the 2Fe subcluster are derived from HydE's substrate.

■ EXPERIMENTAL PROCEDURES

Cloning, Cell Growth, and Expression. The *Clostridium acetobutylicum* gene *hydE* was cloned into Novagen pET-23b vector utilizing the *Nde*I and *Xho*I restriction sites from a previously characterized *C. acetobutylicum* *hydE* construct.²⁰ Sequence verified constructs were transformed into Agilent Technologies BL21-CodonPlus(DE3)-RIL competent cells and used to express recombinant *C. acetobutylicum* HydE with a C-terminal poly-His tag. Cells were grown and harvested using procedures adapted from a previously published method.¹⁴ Cultures in phosphate buffered LB were grown to an OD₆₀₀ of 0.5 after which they were induced with 1 mM IPTG (final concentration), supplemented with 0.3 mM (NH₄)₂Fe(SO₄)₂·6H₂O, and incubated aerobically for 2.5 h. After room temperature equilibration, the cultures were sparged with N₂ at 4 °C for 14 h. The wet cell paste, pelleted by centrifugation, was flash frozen in liquid nitrogen and stored at −80 °C.

Purification and Reconstitution of HydE. All purification and reconstitution steps were carried out in an anaerobic Coy chamber (Grass Lakes, MI) containing a 2–4% hydrogen in nitrogen atmosphere in a 4 °C cold room. The stock buffer contained 25 mM HEPES pH 8.0, 0.5 M KCl, and 5% glycerol (w/v) unless otherwise specified. Cells were lysed using procedures similar to those previously described²⁰ with a lysis buffer cocktail containing the stock buffer supplemented with 10 mM imidazole, lysozyme, 1% Triton X-100, PMSF, DNase I, and RNase A. HydE protein in the clarified lysate was captured by Qiagen Ni-NTA resin in a gravity flow column. Bound protein was washed with 10 mM imidazole followed by 25 mM imidazole prior to elution with 250 mM imidazole all in the stock buffer. Colored fractions were pooled and then either dialyzed or buffer exchanged into the stock buffer to remove imidazole prior to flash freezing and storage at −80 °C.

Reconstitution of HydE was accomplished using previously published techniques.¹² Briefly, HydE was supplemented with 5 mM DTT, followed by a 6-fold excess of Na₂S·9H₂O and FeCl₃. This mixture was incubated for 2 h prior to centrifugation to remove FeS particulates; the clarified protein was then buffer exchanged with a Sephadex G25 resin column to remove adventitiously bound iron, sulfide, and DTT. The protein was concentrated using Amicon 10 kDa molecular weight cutoff filters (Millipore) and flash frozen in liquid N₂ for biochemical and spectroscopic characterization. Bradford assays were utilized for protein quantitation using a BSA standard solution. The iron content of purified HydE was determined using a ferrozine colorimetric assay.²⁷

Electron Paramagnetic Resonance (EPR) and UV–vis Spectroscopic Characterization. Samples were prepared in an MBRAUN chamber (O₂ ≤ 1 ppm) using reagents degassed on a Schlenk line. HydE samples for EPR characterization (200 μ L final volume) were prepared using two batches of reconstituted protein of 160 and 275 μ M concentrations with 7.6 ± 0.1 and 8.2 ± 0.2 irons per protein, respectively. Two methodologies were utilized for preparation of reduced HydE. The first employed incubation of as-reconstituted enzyme with 4 mM dithionite for 1 min prior to adding SAM; samples were rapidly mixed and then flash frozen in liquid N₂. The second technique relied on addition of 5-deazariboflavin (100 μ M final concentration) to as-reconstituted enzyme; samples were photoreduced when illuminated in the presence of 50 mM Tris for 1 h in an ice water bath by using a 500 W T3 halogen bulb.²⁸

Low temperature, continuous wave X-band spectra were recorded on a Bruker ER-200D-SRC spectrometer equipped with a liquid helium cryostat and temperature controller (Oxford instruments). EPR parameters, unless otherwise noted, were microwave frequency, 9.37 GHz; temperature, 12 K; power, 1 mW; time constant/conversion time, 20.48 ms; receiver gain, 1 × 10⁴; modulation frequency, 100 kHz; and modulation amplitude, 10 G. Experimental spectra were baseline adjusted using OriginPro (Version 8.5) and reported g-values were assigned by simulation using the EasySpin 4.5.0 software program.²⁹

The UV–vis absorption spectroscopy of proteins and small molecules was carried out on samples in 1.4 mL anaerobic cuvettes (Spectrocell) using a Varian Cary 6000i UV–vis/near IR spectrophotometer.

SAM Cleavage Assays. All enzymatic assays were conducted under anaerobic conditions in an MBRAUN glovebox (O₂ ≤ 1 ppm). SAM was prepared enzymatically

using crude SAM synthetase lysate by a previously described method.³⁰ HydE (25 μ M final concentration) was incubated with 0.5 mM (final concentration) SAM and putative substrates (2.5 mM final concentration). Reactions were initiated with addition of sodium dithionite (2 mM final concentration) and at select time points assay aliquots (45 μ L) were quenched by addition of 400 mM acetic acid, pH 4.5 (5 μ L). Quenched aliquots were incubated on ice for approximately 1 h prior to centrifugation for 10 min at 4 °C to remove precipitated protein.

Quantitation of 5'-deoxyadenosine (dAdoH) was accomplished with an Agilent Technology 1100 series HPLC. Mobile phase solvent A was H₂O, while solvent B was acetonitrile; both solvents contained 0.1% acetic acid. Assay supernatants (10 μ L) were injected onto a Phenomenex Kinetex Su PFP 100A column equilibrated in 2.0% solvent B. The column was washed with 2.0% solvent B for 3.3 min, and then a linear gradient was initiated up to 60% solvent B at 11 min; dAdoH eluted at 6.8 min as determined by HPLC of a standard solution and was observed as a sharp absorbance peak at 254 nm. Analysis was performed using peak integration by ChemStation software, and unknowns were compared to a dAdoH standard curve for quantitation.

Analysis of Deuterium Incorporation into dAdoH and SAM. In order to probe incorporation of substrate-derived deuterium into either dAdoH or SAM during HydE catalysis, a series of assays (Figure S2) were constructed to monitor the isotopic mass distributions of dAdoH and SAM. These assays were conducted in a manner similar to that described above, but reaction time point aliquots (20 μ L) were first quenched with 60 μ L of acetone, then centrifuged to remove precipitated protein, and further diluted 5-fold by addition of 50:50 acetone/H₂O prior to LC/MS injection. For assays to examine H atom abstraction from solvent exchangeable positions on putative substrate molecules, assays were run in D₂O buffer that was prepared by two sequential rounds of the stock H₂O buffer desiccated on a Schlenk line subsequently rehydrated in D₂O (99.8% D). L-Cysteine-2,3,3-*d*₃ purchased from Cambridge Isotope Laboratories was 98% D.

Following injection of sample (2 μ L), assay components were separated using an Agilent Technologies 1200 series HPLC with normal phase chromatography on a Cogent Diamond Hydride 100A 4 μ m 150 $\times$ 2.1 mm column. Mobile phases A and B were water and acetonitrile, respectively, and both contained 0.1% TFA. The loaded column was washed with 100% solvent B for 1 min, and solutes eluted during a linear gradient from 100% to 50% solvent B over the next 5 min. Mass spectrometry was performed downstream with an Agilent 6538 UHD Accurate-Mass QTOF LC/MS in positive mode. Extracted ion chromatograms of *m/z* values 252.106 (3.5 min) and 399.140 (6.5 min) principally corresponded to the dAdoH + H⁺ and SAM + H⁺, respectively. Agilent Qualitative Analysis B.04.00 of the MassHunter software suite was used to determine the isotopic distributions of dAdoH and SAM. Deuteration of dAdoH did not affect the retention time in the column as the 252, 253, and 254 *m/z* peaks temporally overlaid in the mass chromatogram. After initially locating the dAdoH + H⁺ signals from an extracted ion chromatogram (EIC) at 252.108 *m/z*, the bins (1 s) surrounding the center of the EIC peak with quantifiable isotopic peaks containing at least 252 and 253 *m/z* were selected for quantitation. The 252, 253, and 254 *m/z* peak areas were quantitated using the raw total ion chromatogram at the selected retention time range. The relative

incorporation of deuterium was calculated by dividing the 253 *m/z* area with that of the 252 *m/z* area. The isotopic ratio of dAdoH synthesized from natural abundance reagents used for standard curves (H₂O) was 0.121 $\pm$ 0.002 and is very close to the theoretical ratio of 0.130. Isotopic distributions of SAM and dAdoH with an additional proton were calculated using an isotopic distribution calculator from Scientific Instrument Services (sisweb.com).

Molecular Docking Experiments. Molecular docking was performed with Autodock 4.2.³¹ Ligand coordinates were prepared using Avogadro.³² The structure of HydE from *Thermotoga maritima* was obtained from Protein Data Bank (PDB ID: 3IIZ). HydE was prepared for docking by removing all bound water molecules and ligands with the exception of S-adenosyl-L-methionine and FeS clusters. Polar hydrogens were added using Autodock Tools.³¹ "Blind docking" of the various ligands was performed against HydE using a box that enclosed the solvent accessible area of the barrel. The size of the grid box was 60 Å $\times$ 60 Å $\times$ 60 Å with center *x*, *y*, *z* at 45, 3, 18. A Lamarckian genetic algorithm was used to generate 100 bound conformations for the ligand. All other settings were kept as default values.

Construction of Sequence Similarity and Genomic Context Networks. Sequence similarity networks were obtained from the Structure-Function Linkage Database (SFLD).³³ A cartoon was manually generated depicting the subgroup context in which HydE family sequences and those of its most similar reaction families are found.

Genomic context networks, representing an orthogonal approach to substrate docking or inference from HydE homologous proteins, were employed to study the genomic context of these enzymes. Since there are many cases in which HydE enzymes are encoded within a gene neighborhood comprising other components of the H-cluster biosynthetic pathway (*hydAFG*), other conserved gene neighbors may encode enzymes also involved in the pathway and potentially biochemically associated with HydE catalysis. To this end, a genomic context network approach was utilized, similar to the one described in Zhao et al.³⁴ Each of the 563 HydE family enzymes was mapped onto its corresponding genome, and the 10 proteins encoded upstream and downstream were gathered (the ENA database³⁵) creating a set of ~7000 gene neighbors. A sequence similarity network of all these proteins was then created using Pythoscape.³⁶ All proteins were subsequently annotated based on data retrieved from UniProtKB,³⁷ and the minimal pairwise similarity for similarity inclusion was determined by sampling and inspecting different thresholds, with the aim of finding the best compromise between network clusters and functional annotation. The optimal threshold of pairwise BLAST E-values was set to be 1e⁻²⁰. Upon ranking the different clusters in the network, the largest clusters displaying the widest phylogenetic distribution were analyzed to provide clues about the mechanism of HydE.

RESULTS

Spectroscopic Characterization of HydE. Anaerobically purified *C. acetobutylicum* HydE typically contains 2–4 irons per protein. Substoichiometric cluster loading is typical for radical SAM enzymes,²⁵ and therefore we introduced a chemical reconstitution step prior to further characterization. Reconstitution of recombinant HydE with exogenous iron and sulfide under reducing conditions caused the intensity of the LMCT features at 420 nm to increase 2.2-fold (Figure 2).

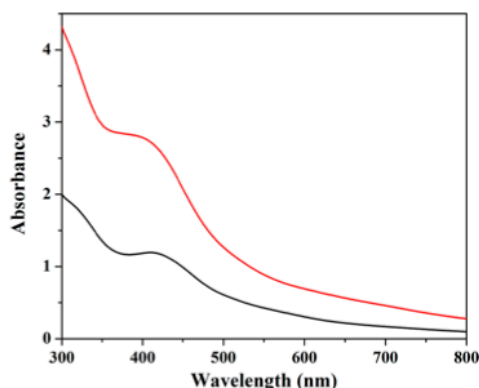


Figure 2. UV-vis spectra of *C. acetobutylicum* HydE as-purified (black) and following chemical reconstitution (red). Protein concentrations adjusted to 83.4 μM . As-purified and reconstituted protein contained 3.7 ± 0.1 and 8.6 ± 0.1 Fe/protein, respectively.

Using published extinction coefficients at 410 nm³⁸ and 420 nm^{39,40} for proteins with [4Fe-4S] clusters (15 000–17 000 ($\text{M}^{-1} \text{cm}^{-1}$) per [4Fe-4S] cluster), the iron loading for as-purified *C. acetobutylicum* HydE was calculated to be 3.5 Fe/protein, in good agreement with the value of 3.7 Fe/protein obtained from the colorimetric iron assay. Likewise, after reconstitution the iron numbers determined using either published extinction coefficients or results of the colorimetric iron assay were in excellent agreement (7.9 Fe/protein and 8.6 Fe/protein, respectively). Three independent purification and reconstitution events of HydE used for turnover assays showed that the enzyme averaged 8.1 ± 0.5 Fe/protein. On the basis of the presence of eight cysteine residues in the primary sequence, this iron number is indicative of full loading of two [4Fe-4S] clusters (Figure 2).

Low temperature EPR spectroscopy shows that as-reconstituted HydE exhibits a nearly isotropic signal that both temperature relaxation profiles (Figure S3C) and simulations (Figure 3A) show arises from two independent signals centered at $g = 2.01$ and $g = 2.00$ (composite 0.02 spin/protein). The first of these signals likely arises from a [3Fe-4S]⁺ cluster given its fast temperature relaxation profile.⁴¹ We assigned the second signal to that of a [2Fe-2S]⁺ cluster, given its maximum signal intensity at around 30 K.¹⁴ Both these cluster types are likely present due to either partial [4Fe-4S] cluster degradation or incomplete reconstitution (Figure S3C). In addition to the signals in the $g \approx 2$ region, broad field spectra (Figure S3A) of as-reconstituted protein show the presence of a minor spectral feature at $g = 4.3$ arising from high spin ferric iron adventitiously bound to the protein.

Reduction of as-reconstituted HydE with either sodium dithionite or 5-deazariboflavin (DRF) resulted in EPR spectra characteristic of reduced [4Fe-4S]⁺ clusters accounting for 0.53 and 0.69 spins/protein, respectively (Figures 3B and S3B). Treatment of reduced HydE with SAM resulted in an intensification of the reduced signals (to 1.2 and 0.87 spins/protein for dithionite (Figure 3C) and DRF (Figure S3B), respectively). Temperature relaxation profiles for reduced HydE EPR signals both in the absence and presence of SAM show fast relaxation typical of [4Fe-4S]⁺ clusters (Figure S4). Moreover, power dependence profiles reveal that the signals are largely resistant to saturation, consistent with their assignment

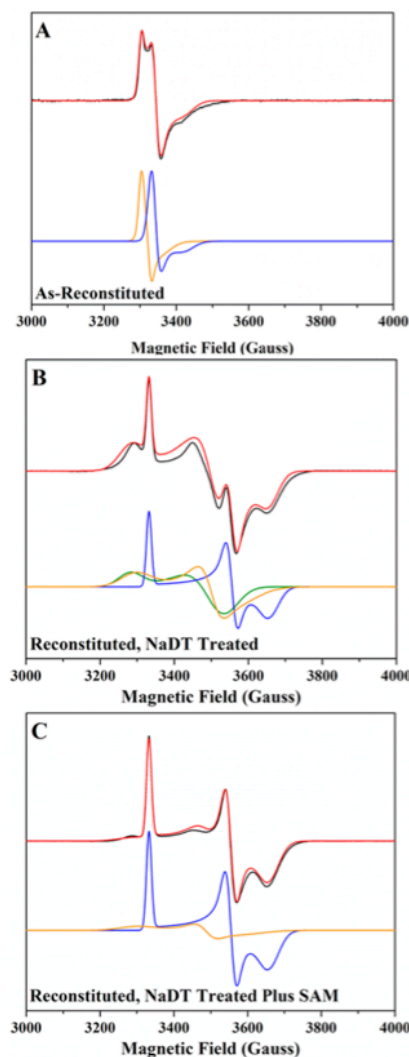


Figure 3. Low temperature EPR spectra of *C. acetobutylicum* HydE. (A) As-reconstituted enzyme. (B) Dithionite reduced enzyme. (C) Dithionite reduced enzyme plus exogenous SAM. All spectra were collected at 12 K with 275 μM chemically reconstituted HydE containing 7.6 ± 0.1 Fe/protein. In all panels, the experimental data traces appear in black and are overlaid with the composite simulation traces (shown in red, see below). In panel A, the simulated traces for cluster 1 and cluster 2 are depicted in orange and blue, respectively. In panel B, the simulated trace for the N-terminal cluster appears in orange; the simulated trace for the C-terminal cluster is shown in green; the blue simulated trace represents the SAM coordinated form of the N-terminal cluster. In panel C, the blue trace again represents the SAM coordinated form of the N-terminal cluster, while the orange trace depicts the C-terminal cluster.

to [4Fe-4S]⁺ clusters (Figure S4).⁴² Simulations of experimental data show that the EPR spectra for reduced samples are a composite of a three spin system that assign to SAM-bound and unbound N-terminal [4Fe-4S]⁺ clusters, in addition to a C-terminal [4Fe-4S]⁺ component (Figure 3B). The simulations

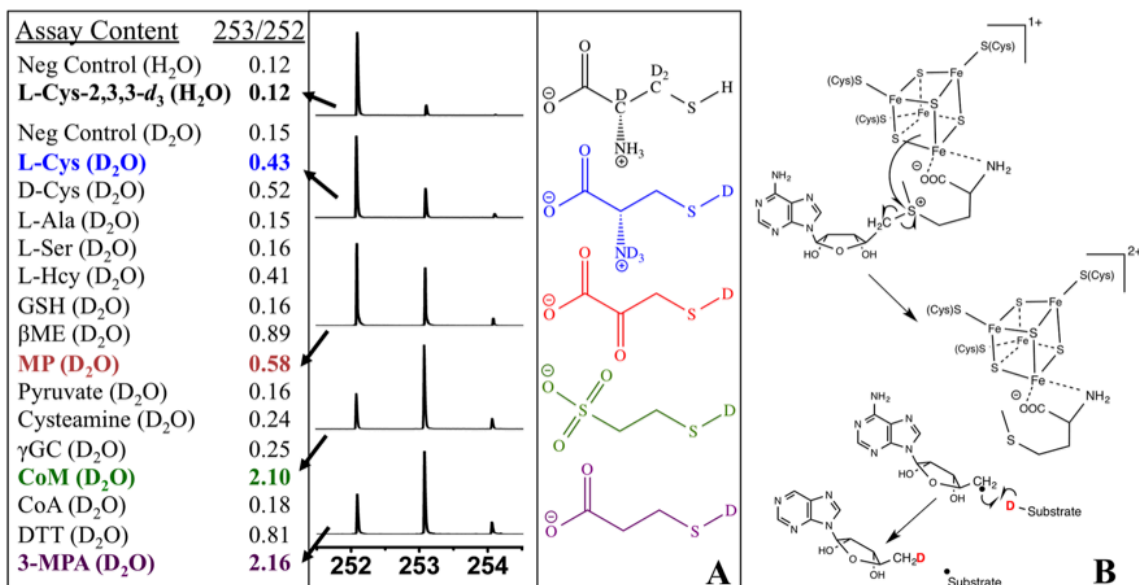


Figure 4. (A) Results of analysis for deuterium incorporation into product 5'-deoxyadenosine after assay of HydE with a range of small molecules in D₂O buffer. Mass spectroscopic result for dAdo(H/D) from selected assays are shown on the right, and the mass peak ratios are shown on left. (B) Initial steps in a radical SAM reaction that can lead to D atom incorporation into dAdoH.

reveal that the cluster signals attributed to the SAM-unbound states exhibit similar *g* values (*g* = 2.04, 1.92, 1.90 and *g* = 2.03, 1.91, 1.87); the *g*-values for the SAM-bound (presumably N-terminal) cluster underscore the perturbations induced by SAM coordination (*g* = 2.01, 1.88, 1.83). Moreover, simulations demonstrate that when exogenous SAM is added to reduced HydE, the resulting experimental spectra are well fit to a two spin system consisting of the SAM coordinated N-terminal [4Fe-4S]⁺ cluster and the C-terminal [4Fe-4S]⁺ cluster (Figure 3C). Table S1 lists the *g*-values obtained from spectral simulations for the data presented in Figure 3.

SAM Copurifies with HydE. Both dithionite-treated and photoreduced samples of HydE (Figures 3 and S3B) have spectra that can be simulated using a combination of unbound [4Fe-4S]⁺ and SAM coordinated [4Fe-4S]⁺ cluster signals, even in the absence of added SAM. On the basis of normalization and spectral subtraction of the SAM-bound component from the spectrum of dithionite-reduced *C. acetobutylicum* HydE, approximately 25% of purified HydE contains SAM bound. In support of this observation, a SAM cleavage assay using 25 μM as-reconstituted HydE was performed in the absence of exogenous SAM and in the presence of coenzyme M; results showed that the amount of dAdoH reached a maximum concentration of 3.5 μM after 30 min. This indicates that at least 14% of the protein copurifies with SAM. This is likely a low estimate, as abortive cleavage of SAM is also known to produce methylthioadenosine (MTA), a product that was not quantified in these studies.²⁸ Additionally, the enzyme stock that was assayed underwent chemical reconstitution and gel filtration after purification, which may have removed some of the SAM that copurified with HydE. Similarities exist between the unbound [4Fe-4S]⁺ cluster signal we observe herein and the axial signal reported for reconstituted, reduced *Thermatoga maritima* HydE, although the latter exhibited low and high field shoulders.⁹ While Rubach et al. attributed these shoulders to an

accessory FeS cluster,⁹ our analysis of *C. acetobutylicum* HydE suggests they instead likely arose from the coordination of SAM to the N-terminal, radical SAM cluster.

Substrate Derived Deuterium Atom Incorporation into Deoxyadenosine. Given the observation that [FeFe]-hydrogenase maturation could be stimulated upon addition of L-cysteine,⁴³ we explored the possibility that this molecule may be a substrate for HydE by using deuterium-labeled cysteine and monitoring for D atom incorporation into dAdoH and SAM. Deuterium atom incorporation into dAdoH was monitored by LC/MS by quantifying the ratio of peaks at *m/z* = 253 and 252; the ratio for dAdoH produced by HydE in the absence of cysteine in H₂O containing buffer was calculated to be 0.121 ± 0.002. In the presence of L-cysteine-2,3,3-*d*₃ (Figures 4A and S2), the 253/252 ratio was 0.120 ± 0.001, indicating that dAdo• did not abstract from either deuterated position. Assays were also performed in D₂O to test if a solvent exchangeable hydrogen on L-cysteine was abstracted by dAdo•; results from several assays show that the 253:252 peak ratios for L-cysteine increase from ~0.15 (no cysteine) to 0.43 ± 0.06 in the presence of cysteine (Figures 4A and S2), demonstrating that dAdo• abstracts a solvent-exchangeable hydrogen. While this H atom abstraction could be occurring from a solvent-exchangeable position on the protein itself, the fact that appreciable D incorporation into 5'-deoxyadenosine is observed only in the presence of cysteine argues that the abstraction is occurring from cysteine.

Similar experiments were carried out with L-Ser, L-Ala, D-Cys, and L-homocysteine (L-Hcy) in D₂O and revealed the importance of the thiol functionality. L-Ser and L-Ala lack the thiol but retain the amino and carboxylate functionalities; assays performed in their presence led to no detectable deuterium incorporation. Assays conducted with D-Cys and L-Hcy, on the other hand, resulted in similar D incorporation as observed with L-Cys (Figures 4A and S2). Mercaptopyruvate, glutathione

(GSH), γ -L-glutamyl-L-cysteine (biosynthetic precursor of glutathione), 3-mercaptopyruvic acid (3-MPA), and coenzyme M were also examined as they all have possible biological significance and could be used to probe the size of the active site. Cysteamine, dithiothreitol (DTT), and β -mercaptoethanol (β ME), while not biologically relevant, were also assayed to further investigate how charge plays a role in substrate binding. Other small molecules were also tested and are included in the summary that follows. From this set of experiments, the small molecules can be roughly divided into four groups based on their effect on D atom incorporation from D₂O into dAdoH. L-Ala, L-Ser, GSH, pyruvate, and coenzyme A had a negligible stimulatory effect on D atom incorporation into dAdoH. Cysteamine and γ -L-glutamyl-L-cysteine (γ GC) stimulated very low level D incorporation, while L-homocysteine, L-cysteine, D-cysteine, mercaptopyruvate, DTT, and β ME produced moderate incorporation of D into dAdoH. Coenzyme M and 3-mercaptopyruvic acid produced the highest quantities of D atom incorporation, with the dAdoH 2S3/2S2 ratios measured at 2.10 ± 0.33 and 2.16 ± 0.12 , respectively. The isotopic *m/z* ratio (400/399) for SAM remained unchanged from natural abundance in all assays, indicating that the deuterated dAdoH was not subsequently converted to SAM (data not shown).

Deoxyadenosine Production in the Presence of Select Small Molecules. In the absence of exogenous small molecules but in the presence of reducing agent, we calculated that *C. acetobutylicum* HydE catalyzes uncoupled SAM cleavage to produce dAdoH on the order of 1 turnover per HydE per hour, similar to a previously reported rate for *T. maritima* HydE.⁹ All 20 common amino acids, pyruvate, and fumarate were screened for effects upon HydE dAdoH production. From these initial screens, only cysteine had any effect on SAM cleavage. Several mercaptans that stimulated D atom incorporation into dAdoH were screened for their ability to increase dAdoH production (Figure 5). Stimulation of dAdoH production concurrent with D atom incorporation is consistent with known radical SAM enzymes in the presence of substrates and substrate analogues.^{10,44} L-Cysteine (3-fold increase),

mercaptopyruvate (4-fold increase), coenzyme M (8-fold increase), and 3-mercaptopyruvic acid (9-fold increase) all stimulate dAdoH production (Figure 5). Increased rates of dAdoH production for the thiols were consistent over 5.5 h in assays, after which SAM became the limiting reagent in the reaction.

Molecular Docking Studies of Putative HydE Substrates and Analogues. The X-ray crystal structure from *T. maritima* HydE (PDB ID: 3IIZ) was utilized to perform in silico docking studies with the small molecules shown in Figure S2. The AutoDock software suite was selected for these studies given its precedence for predicting substrate and ligand binding sites.^{45–47} All molecules containing either a carboxylate or sulfonate functional group were found to dock within the HydE active site adjacent to SAM with higher binding affinities than anywhere else inside or outside of the protein (Figure 6). The docked structures show that the small molecules are bound within a pocket comprising Arg159, the backbone nitrogens of Thr269 and Ala270, and the N3 nitrogen from the adenosyl group of SAM; these residues appear to play a critical role in stabilizing molecules with carboxylate/sulfonate groups. Moreover, the salt bridge formed by the guanidinium group of Arg159 and the negatively charged functional groups of the docked molecules appears to help position the thiols of the small molecules at distances (<5 Å) amenable to H/D abstraction by the deoxyadenosyl radical (Figure 4B).¹³ The binding energies for several small molecules are tabulated in Figure 6. The opposite side of the putative substrate binding pocket contains the amide functional group of Gln107; this amide group is positioned only 4.2 Å from the 5' carbon of SAM. As the in silico modeling results show, this short distance would allow for hydrogen bonding between Gln107 and the solvent exchangeable, thiol hydrogen at a distance <4 Å (Figure 6). In essence, Gln107 and the binding pocket that includes Arg159 appear to act as substrate handles for a variety of low molecular mass thiols, positioning these molecules in close proximity to SAM and thereby explaining the stimulatory effects in SAM cleavage and D atom incorporation into dAdoH reported herein.

Radical SAM Sequence Similarity Networks. Sequence similarity together with the similarity between the presumed dithiol product of HydE and the thiophane product of BioB led to the initial hypothesis that HydE could be involved in a sulfur insertion reaction during H-cluster biosynthesis.⁴⁸ Recent biochemical and crystallographic investigations into other structurally similar enzymes such as methylornithine synthase (PylB) and HydG leave room for a wide range of potential HydE chemistries.^{13,25} The high degree of structural parallels between HydE and PylB (Figure 7B) reveals the overlap in the global protein armature and also the prominent similarities in the two active site regions (Figure 7B).²⁴

One additional tool to gain insight into the HydE substrate and reaction mechanism is analysis of Sequence Similarity Networks. The Structure-Function Linkage Database (SFLD) has identified nearly 100 thousand putative radical SAM enzyme sequences that may be categorized by their primary amino acid sequence.³³ Comparable sequences that likely exhibit the same function are grouped into nodes and adjacent nodes may be both structurally and functionally similar. As Figure 7A highlights, the other characterized radical SAM enzymes closest to HydE include PylB, HmdB, HydG, ThiH, NosL/NocL (3-Me), and BioB enzymes that are either predicted to or have been confirmed to contain complete

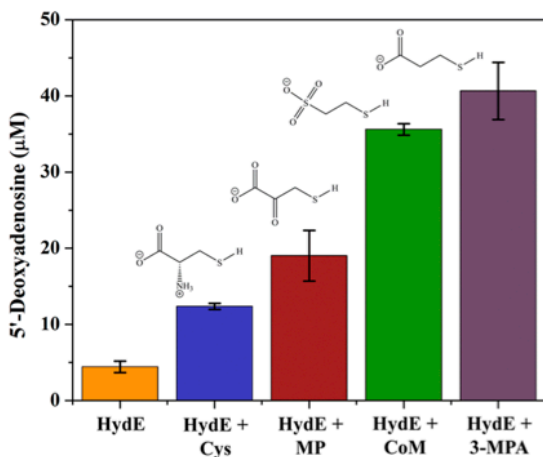


Figure 5. Stimulation of 5'-deoxyadenosine production by addition of L-Cys, mercaptopyruvate, and coenzyme M (2.5 mM) to HydE (25 μM) and SAM (500 μM). Assays were quenched after 30 min of reaction.

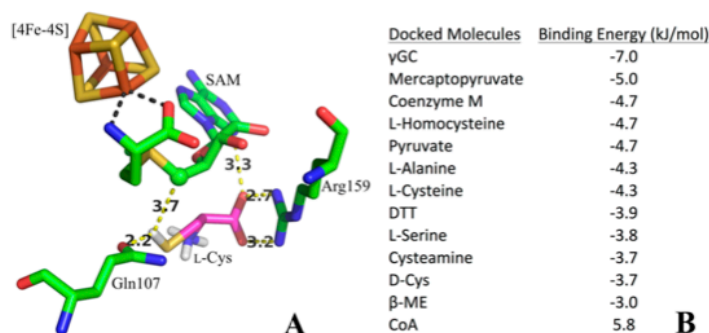


Figure 6. (A) Active site of *T. maritima* HydE with [4Fe-4S] cluster, SAM, Arg159, Gln107, and L-Cys from in silico modeling. L-Cysteine in purple represents the location of the other thiols all in their lowest energy binding conformation. The deoxyadenosyl radical's unpaired electron that abstracts the H/D atom from substrate is generated on the 5' carbon of SAM (green sphere). This carbon is at distances to the sulfur atom of the mercaptans comparable to those of other radical SAM enzymes with crystal structures solved with SAM and substrates.²⁵ All distances are in Å. Color scheme unless otherwise noted: carbon (green), oxygen (red), nitrogen (blue), iron (orange), and sulfur (yellow). PDB ID: 3IIZ. (B) Relative binding energies of molecules from in silico docking experiments.

TIM barrels ($\beta\alpha$)₈ and have small substrates that are less than 250 Da.²⁵

Genome Context Analysis. On the basis of genome context networks, gene clusters in Figures 8 and S5 represent the most conserved genes that are encoded near HydE enzymes from diverse phylogenetic origins. The largest and most phylogenetically diverse clusters turned out to be enriched with functions that typify HydF, HydG, and TM1266 proteins (plurality vote using GO biological process annotation⁴⁹), which are the known components of the H-cluster biosynthesis gene neighborhood; the genome context network captures other relevant pathway members, and we can accordingly have confidence that this analysis may offer clues about the HydE mechanism. The next clusters of highest rank are the ones annotated as AspA enzymes, cysteine synthase, Cys/Met PLP-dependent enzymes, and histidine kinases. The conservation of these HydE gene neighbors further supports the hypotheses involving thiols in HydE's catalytic reaction.

DISCUSSION

HydE Cluster Characteristics. Iron analysis of reconstituted *C. acetobutylicum* HydE, UV-vis extinction coefficients, and EPR temperature relaxation and power saturation characteristics indicate that the protein used herein contains two fully loaded [4Fe-4S] clusters. A recent crystal structure revealed a second [4Fe-4S] cluster in reconstituted *T. maritima* HydE,⁵⁰ consistent with our findings for the *C. acetobutylicum* protein. One cluster binds the radical SAM motif (CX₃CX₂C) in the N-terminal region of the protein and is absolutely conserved in HydE. The accessory CX_nCX_mC motif is only partially conserved, and substitutions at these three cysteines do not impair [FeFe]-hydrogenase activation.¹⁸ The second cluster of HydE likely plays no direct role in H-cluster biosynthesis. Given the high lability of the auxiliary cluster, as judged by the oxidation products observed in *T. maritima* HydE crystal structures,⁵⁰ this cluster may act as a sacrificial anode by reacting with external oxidants before they can damage the catalytically essential radical SAM [4Fe-4S] cluster.

Putative HydE Substrates Define the Active Site. Despite multiple solved X-ray crystal structures, insight into the substrate and reaction mechanism of HydE remains limited.^{18,19,50} When assaying purified HydE with the 20

common amino acids, we observed a stimulatory effect on SAM cleavage/dAdoH production with L-cysteine. Stimulation of SAM cleavage to produce dAdoH upon addition of substrate is a common characteristic of radical SAM enzymes and has been used, for example, to identify tyrosine as the substrate for HydG.^{10,25} The location of H atom abstraction from putative substrates can be identified through use of selectively deuterated molecules. While L-cysteine-2,3,3-*d*₃ did not change the isotopic ratio of dAdoH (252/253 ratio) from that of natural abundance, addition of L-cysteine to HydE assays in D₂O did result in a shift in the dAdoH isotopic ratio. Interestingly, when testing a battery of small molecules, two trends emerge. First, the thiol functional group appears to be important for observing deuterium incorporation into dAdoH. Second, there is an upper limit on the size of thiol that can produce altered isotope ratios, presumably due to a size limit for fitting in to the HydE active site. Coenzyme A, glutathione, and γ-L-glutamyl-L-cysteine show little to no increase in dAdoH 253/252 isotopic ratio, suggesting that the natural substrate of HydE is smaller than these molecules. In addition to the size limitation and thiol functionality, a negative or partial negative charge appears to be important, as cysteamine has only a marginal effect despite its small size and thiol functional group.

Coenzyme M shows the largest stimulatory effect on both dAdoH production and D atom incorporation into dAdoH; however coenzyme M cannot be the natural substrate of HydE because lysate activation experiments have suggested that the HydE substrate is a ubiquitous small molecule,^{20,43,51,52} and only a limited collection of organisms have been identified as capable of synthesizing coenzyme M.⁵³ The observed reactivity with coenzyme M does however help define the size requirement and overall charge associated with the natural substrate. Taken together, our results support a hypothesis in which the natural substrate of HydE likely contains a functional group with partial or full negative charge at a distance of approximately 5 Å from a thiol. Also, given the nature of the effector molecules tested coupled to the in silico docking results, the substrate is likely the size of a typical amino acid.

Potential Role of SAM in HydE Catalysis. The results reported herein provide evidence that *C. acetobutylicum* HydE purifies with a significant amount of SAM bound. The reduced purified enzyme exhibits two different EPR signals, both of

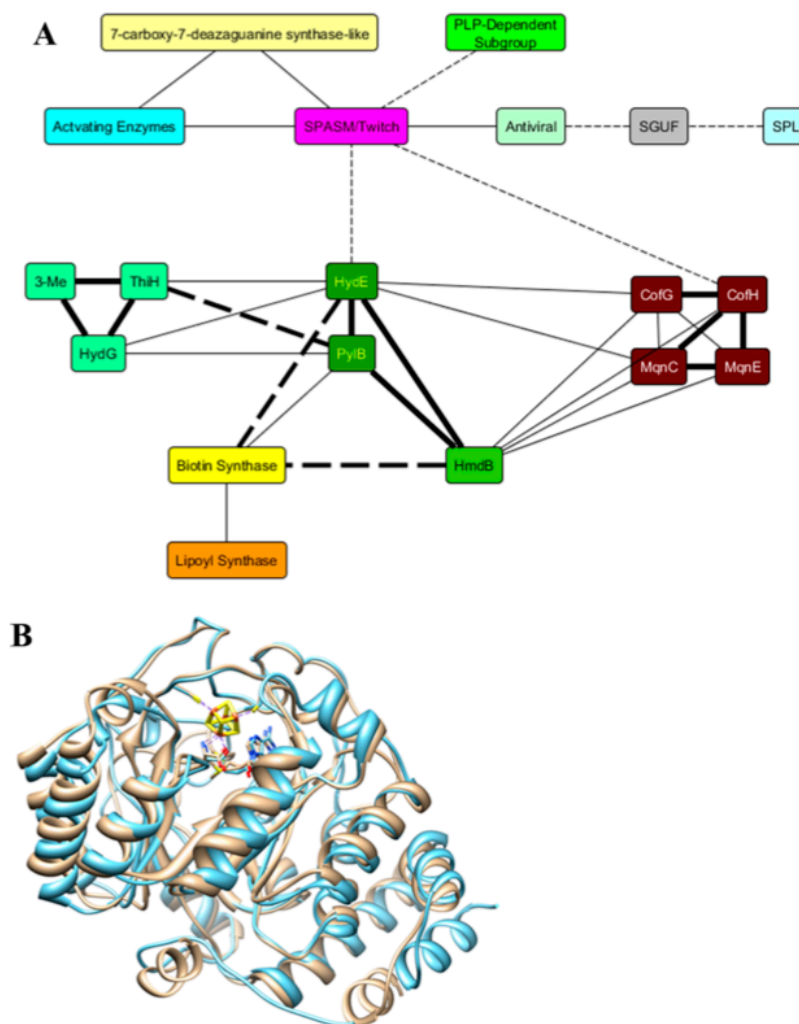


Figure 7. (A) Cartoon network representing the relationship of HydE family sequences to other well characterized subgroups and families described in the SFLD. The thick lines represent a higher degree of relatedness than thin lines. The unbroken lines along with the same colored boxes represent membership of the same subgroup. The thin, broken lines represent a more distant relationship. No information is encoded by the lengths of the edges. The “Antiviral” node includes Viperin, 3-Me includes NosL/NocL the tryptophan lyases, and SGUF denotes a subgroup of radical SAM enzymes with unknown function. (B) Overlay of PyIB (PDB ID: 3T7V) shown in tan and HydE (PDB ID: 3CIW) shown in cyan crystal structures.

which appear to arise from $[4\text{Fe-4S}]^+$ clusters; one of these signals overlays the signal observed for the SAM-bound state, suggesting that it is due to SAM that copurified with the enzyme. In addition, SAM cleavage assays in the absence of added SAM demonstrate the presence of SAM in the purified protein. The copurification of SAM, and by inference its high binding affinity, would be consistent with SAM serving as a cofactor rather than a cosubstrate during HydE catalysis. Alternatively, a high k_{on} rate for SAM binding could also be indicative of a mechanism utilizing 2 equiv of SAM, where rapid displacement of SAM cleavage products by SAM is required. Interestingly, other structurally similar radical SAM enzymes (BioB and LipA) also require multiple equivalents of SAM to accomplish their chemical reactions.^{54,55}

Mechanistic Insights into HydE Catalysis. If cysteine was the substrate for HydE, H atom abstraction from the thiol would yield a thiyl radical that might subsequently undergo $\text{C}\alpha\text{--C}\beta$ bond cleavage in a manner similar to the proposed mechanism for HydG.^{56–58} Heterolytic cleavage of cysteine’s $\text{C}\alpha\text{--C}\beta$ bond would then produce a dehydroglycine fragment that would hydrolyze to glyoxylate; no glyoxylate is observed, however, in samples from SAM cleavage assays in the presence of cysteine (Figure S6). In the event of homolytic $\text{C}\alpha\text{--C}\beta$ bond cleavage of cysteine, the reaction products would be thioformaldehyde ($\text{S}=\text{CH}_2$) and a glycy radical; we have not yet been able to detect a glycy radical nor its quenched product in assays. Following this latter possible reaction, two thioformaldehyde units could be joined by ammonia in a manner reminiscent of the *in vitro* synthesis of the DTMA

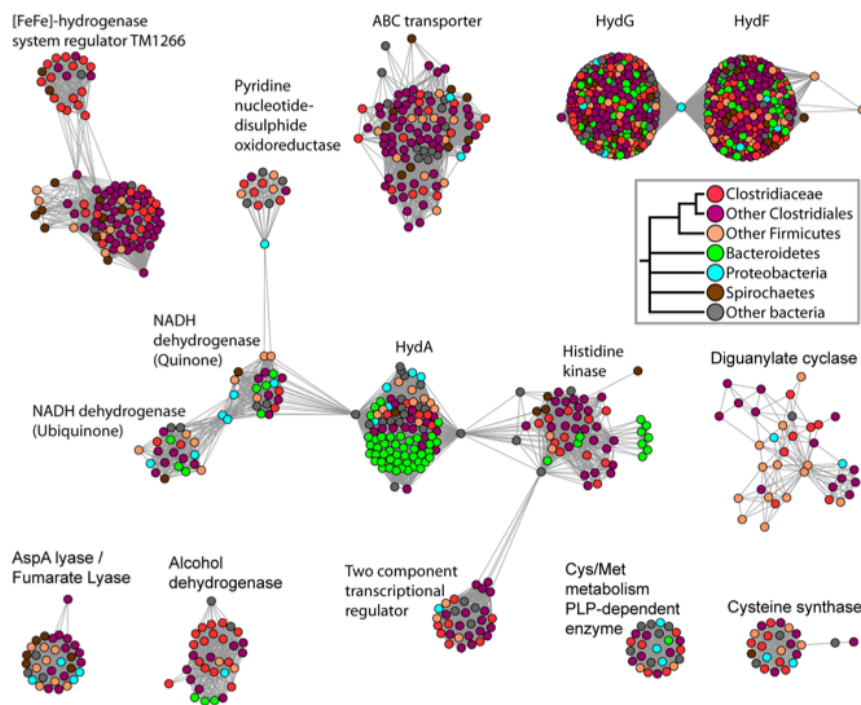


Figure 8. Abridged genomic context network for HydE enzymes. Circles represent genomic neighbors within 10 genes upstream/downstream of the 563 HydE enzymes as classified by the SFLD. Connections represent BLAST pairwise sequence similarity with an E-value more significant than $1e^{-20}$. The 1054 proteins are clustered into sets of similar sequences that are visually identifiable. Only clusters that could be assigned with function are shown and accompanied by the relevant titles. Colors represent the phylogenetic origin of the organisms in which enzymes are encoded, as shown in the legend. Clusters are roughly ordered by size and heterogeneous phylogenetic origin (from upper left to lower right). The largest and most phylogenetically diverse clusters are enriched with functions that typify the H cluster biosynthesis pathways, such as HydA, HydF, HydG, and TM1266 proteins, strengthening our confidence in the ability of this method to capture biologically relevant signals.

bridge from the condensation of formaldehyde and ammonia.⁵⁹ The highly conserved region in the HydE structure that was found to bind thiocyanate could act as a second active site for this condensation to produce the DTMA bridge or as a temporary holding site for a DTMA precursor.¹⁸ We have examined the effects of ammonia addition during HydE activity assays and have not observed stimulated dAdoH production (data not shown); this result is not unexpected given that ammonia may be reacting with thioformaldehyde at the bottom of the TIM barrel, some 13 Å away from the radical SAM cluster. Products of thiol lyase activity or intermediates in DTMA synthesis have not yet been detected by HPLC, LC/MS, or spectroscopic methods, due presumably to the inherent difficulty in detecting these small, and in some cases reactive, molecules.

Intriguingly, the gene *aspA* that codes for aspartate ammonia-lyase is often associated within the *hydAEFG* operon (Figures 8 and S5) and could help increase the local concentration of ammonia during HydE catalysis. In addition to *aspA*, two other clusters of genomic neighbors that are involved in cysteine synthesis and metabolism are also often found near HydE in phylogenetically diverse organisms (Figures 8 and S5). These two enzyme clusters are both PLP-dependent, which may explain PLP's stimulatory effect observed in [FeFe]-hydrogenase maturation assays.⁶⁰ Thioformaldehyde likely has a very short lifetime in a biological environment given its susceptibility

to nucleophilic attack;⁶¹ however, positioning ammonia (the putative source of the bridgehead atom) in the active site by the conserved carboxyl group of Glu58 and the side chain carbonyl group of Gln107 (*T. maritima* HydE numbering)¹⁸ may promote the reaction with thioformaldehyde. The precedence for radical SAM enzymes generating small highly reactive molecules has been clearly made with HydG,^{11,12} ThiH,⁶² NocL/NosL,^{58,63} and NifB.⁶⁴

Another potential candidate for the natural substrate of HydE is mercaptopyruvate. While mercaptopyruvate likely does not accumulate intracellularly as does cysteine,⁶⁵ the enzymes cysteine transaminase and aspartate transaminase (both PLP-dependent enzymes) catalyze the production of mercaptopyruvate from cysteine.⁶⁶ This observation again provides a rational explanation for the reported stimulation of hydrogenase activity 10-fold with addition of PLP to cellular lysate assays.⁶⁰ Given the dramatic stimulation of SAM cleavage and D atom incorporation by 3-mercaptopropionic acid, the potential for 3-MPA serving as a HydE substrate is also under investigation.

The radical SAM cartoon network (Figure 7A) shows that HydE is most closely related with PylB, HmdB, and BioB. It also shows similarity to CofG/CofH, MqnC/MqnE, and HydG/ThiH/3-Me; this network is a reflection of the degree of sequence and structural conservation among these enzymes and may translate mechanistically to HydE. Among these

enzyme groups, it is intriguing that the CofG/CofH and HydG/ThiH/3-Me systems are involved in tyrosine and tryptophan α -C β bond cleavage events with the subsequent generation of glycine-like intermediate species.²⁵ Moreover, PylB has been proposed to generate a glycy radical intermediate during conversion of lysine to methylornithine.²⁴ While the connections between HydE and MqnC/MqnE and HmdB are less clear, in part due to ambiguities regarding reactions catalyzed,²⁵ the striking similarity between PylB and HydE (Figure 7B) suggests the likelihood of mechanistic commonalities that may include similar substrates or intermediates. The structure of BioB also very closely resembles that of HydE; however, BioB's sulfur insertion reaction depends upon the presence of a labile sulfur source from an auxiliary [2Fe-2S] cluster located ~5 Å from the substrate dethiobiotin.^{18,54,67} Although HydE can have an auxiliary cluster, evidence indicates that this cluster is not essential for HydE function, arguing against BioB-like sulfur insertion chemistry. Collectively, these observations have led us to hypothesize that HydE DTMA synthesis may involve α -C β bond cleavage of a thiol containing molecule like cysteine to generate the reactive intermediate thioformaldehyde.

CONCLUDING REMARKS

The observed effects of select low molecular mass thiols during HydE assays provide experimental insights into the putative mechanism and role of this enzyme during synthesis of the bridging DTMA ligand of the H-cluster. While this allows us to put together a more detailed description of how the H-cluster is built, several important issues must first be resolved before a final picture can be achieved. The recent report of CN⁻ and CO-ligated iron species present on HydG during turnover, and the tracing of these to the 2Fe subcluster in HydA,⁶⁸ may suggest the product of HydE catalysis could link together two of the Fe(CO)₂CN units to accomplish 2Fe subcluster formation on HydF. The high binding affinity between HydE and HydF,⁶⁹ together with their existence as a *hydEF* fusion in some organisms,⁸ suggests that HydE and HydF work closely together during H-cluster maturation. The current work provides important insights into the substrate of HydE and a potential mechanism for DTMA synthesis and will provide a critical foundation for further illumination of H-cluster assembly.

ASSOCIATED CONTENT

Supporting Information

D atom incorporation into dAdoH and putative substrate structures, additional EPR spectra that include temperature relaxation and power saturation data, table of simulated *g*-values, and genomic context network diagram. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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ABBREVIATIONS

β ME, β -mercaptoethanol; CoA, coenzyme A; CoM, coenzyme M; dAdo•, 5'-deoxyadenosyl radical; dAdoH, 5'-deoxyadenosine; DRF, 5-deazariboflavin; DTT, dithiothreitol; DTMA, dithiomethylamine; EPR, electron paramagnetic resonance; GSH, glutathione; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HPLC, high pressure liquid chromatography; IPTG, isopropyl β -D-1-thiogalactopyranoside; LC/MS, liquid chromatography mass spectrometry; L-Hcy, L-homocysteine; LMCT, ligand to metal charge transfer; MP, mercaptopyruvate; MTA, methylthioadenosine; PLP, pyridoxal-phosphate; PMSF, phenylmethylsulfonyl fluoride; SAM, S-adenosyl-L-methionine; SFLD, Structure Function Linkage Database; TIM, triosephosphosphate isomerase; γ GC, γ -L-glutamyl-L-cysteine; 3-MPA, 3-mercaptopropionic acid

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Supporting information for:

[FeFe]-Hydrogenase Maturation: Insights into the role HydE plays in dithiomethylamine biosynthesis.

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1r30_chain_A1:22-346/1-369 1 .....MTOREILEKL..... 10
4jy9_chain_A1:1-148 1 .....MIQEMALDEFD 11
3t7v_chain_A1:1-350 1 MGS SHHHHHSS SGLVPROSHMTQNSQAMTSHAMTODFVLPELEDVRAEAATVD.....T.RAV..... 57
4r33_chain_B1:19-400/1-420 1 .....SRAA.V.....AL.A.....LWED.....SIG 84

1r30_chain_A1:22-346/1-369 1 .....GSSHHHHHDYDIPTTENLYFQGS AHRPR.WT..LSQ.V...TEL...FE.....KP 42
4jy9_chain_A1:1-148 11 E...R...R.E.....F..T.REVLK...E.ALSINDR.G..F 31
3t7v_chain_A1:1-350 12 SLG.DK.VI.EGY.Q.....LT..D..NDL...RTLLESKEG... 39
4r33_chain_B1:19-400/1-420 38 L.A.LA.EGEEPAE.....SRAA.V.....AL.A.....LWED.....SIG 84

1r30_chain_A1:22-346/1-369 43 LLDLLEFEEQQVHRQHFDPR.QVQVSTLLSIKTGAQPEDKCYGQSSRYKTGLEAERLMEVEQVLSARKAK.A.A. 113
4jy9_chain_A1:1-148 32 NEALFKLADEIRRYVVG.D.EVHIRAIEFNSVGRKNCLYCDLRRDNK.NLYR.YR.MTPEEIVRARLAVQF.. 99
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4jy9_chain_A1:1-148 100 G.AKTIVLQSOEDPYY.....MPD.VISDIVEE.IKMGV.AVTLSEOWPREYYEKWEAGA.. 153
3t7v_chain_A1:1-350 106 AGFHMVDLTMOEDPYY.....DPNRF.V.ELVQIVKEELO...L.PIMISPOLMDNATLLKAREKGA.. 163
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4r33_chain_B1:19-400/1-420 285 VAHGDH..L.RSROATADLSWPRMRPAMKTRD...TTRVQDD...DYLNLMSVVAFTCEQRILVLT.R.E..P 345

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1r30_chain_A1:22-346/1-369 360 TDEYYNAAAL 369
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3t7v_chain_A1:1-350 .....
4r33_chain_B1:19-400/1-420 .....

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Figure S1: Structure-based multiple alignment of HydE homologs. Sequence alignment derived from superposition of high resolution structures of proteins most similar to HydE, keyed to the sequence similarity network shown in Figure 7. Sequences are labeled by PDB accession: 4JY9, HydE; 1R30, BioB; 3T7V, PylB; 4R33, 3-Me (NosL). The SAM binding motif common to all of these proteins is shown in the boxed section. Positions at which >80% of the residues are identical in all four sequences are shaded. The structure-based sequence alignment was generated using high resolution structures liganded with the SAM moiety. These were superposed using the Match Align tool from the Chimera visualization software⁽¹⁾ and a sequence alignment based on that superposition produced.

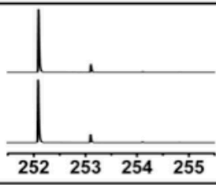
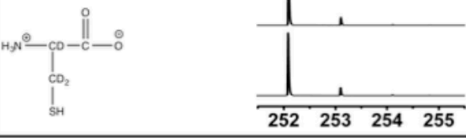
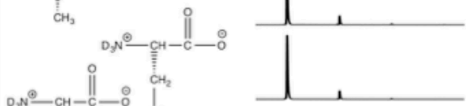
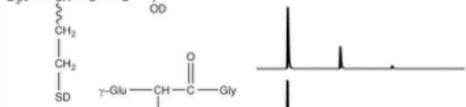
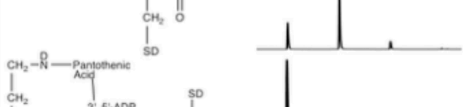
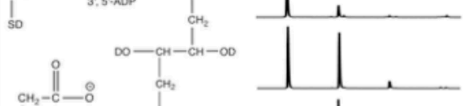
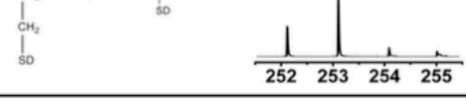
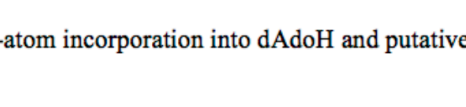
Assay Content	5'-Deoxyadenosine (dAdo) m/z Profile	D-atom Incorporation	253/252 Abund (%)
Neg. Control (H ₂ O)		No	0.12 +/- 0.00
L-Cys-2,3,3-d ₃		No	0.12 +/- 0.00
Neg. Control (D ₂ O)		No	0.15 +/- 0.01
L-Cys (D ₂ O)		Yes	0.43 +/- 0.06
D-Cys (D ₂ O)		Yes	0.52 +/- 0.03
L-Ala (D ₂ O)		No	0.15 +/- 0.01
L-Ser (D ₂ O)		No	0.16 +/- 0.02
L-Homocys (D ₂ O)		Yes	0.41 +/- 0.05
GSH (D ₂ O)		No	0.16 +/- 0.01
β-ME (D ₂ O)		Yes	0.89 +/- 0.27
MP (D ₂ O)		Yes	0.58 +/- 0.11
Pyruvate (D ₂ O)		No	0.16 +/- 0.02
Cysteamine (D ₂ O)		Maybe	0.24 +/- 0.05
γGC (D ₂ O)		Maybe	0.25 +/- 0.01
CoM (D ₂ O)		Yes	2.10 +/- 0.33
CoA (D ₂ O)		No	0.18 +/- 0.01
DTT (D ₂ O)		Yes	0.81 +/- 0.12
3-MPA (D ₂ O)		Yes	2.16 +/- 0.12

Figure S2: D-atom incorporation into dAdoH and putative substrate structures.

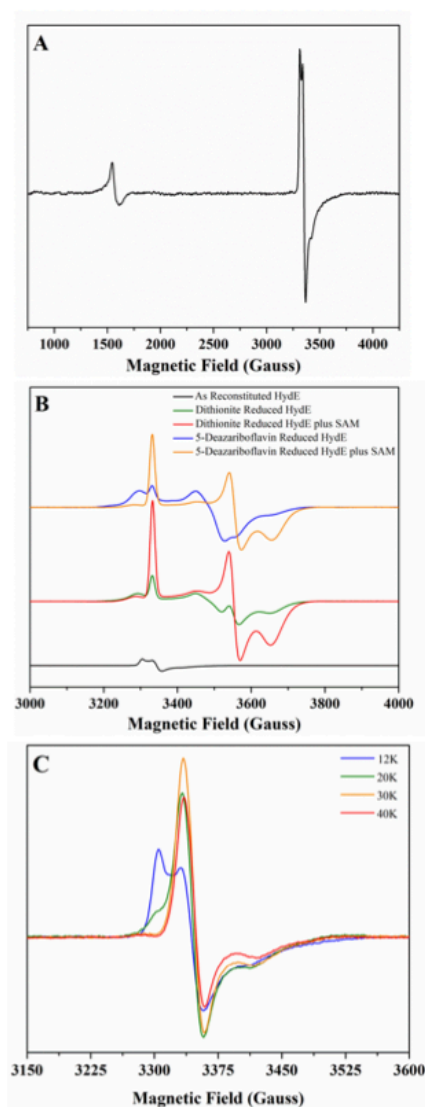


Figure S3: *Clostridium acetobutylicum* HydE spectra collected at 12 K unless otherwise noted. A. Broad field EPR spectrum of as reconstituted HydE with a very minor spectral feature at $g = 4.3$ due to high spin Fe^{3+} not specifically bound. B. (Top) 5-deazariboflavin photoreduced with (orange) and without (blue) exogenous SAM spectra. (Middle) Sodium dithionite reduced with (red) and without (green) exogenous SAM spectra. (Bottom) as reconstituted protein. The dithionite reduced HydE preparation has a much larger N-terminal cluster-SAM signal as photoreduction for 1 hr at 0 °C is likely sufficient for abortive cleavage of SAM that copurifies with HydE. C. Temperature profile of as reconstituted HydE. The low field signal observed at 12 K (blue trace) rapidly decreases upon increase in temperature. The high field signal exhibits a maximum signal intensity at 30 K (orange trace) and clearly has slower temperature relaxation properties relative to the low field signal.

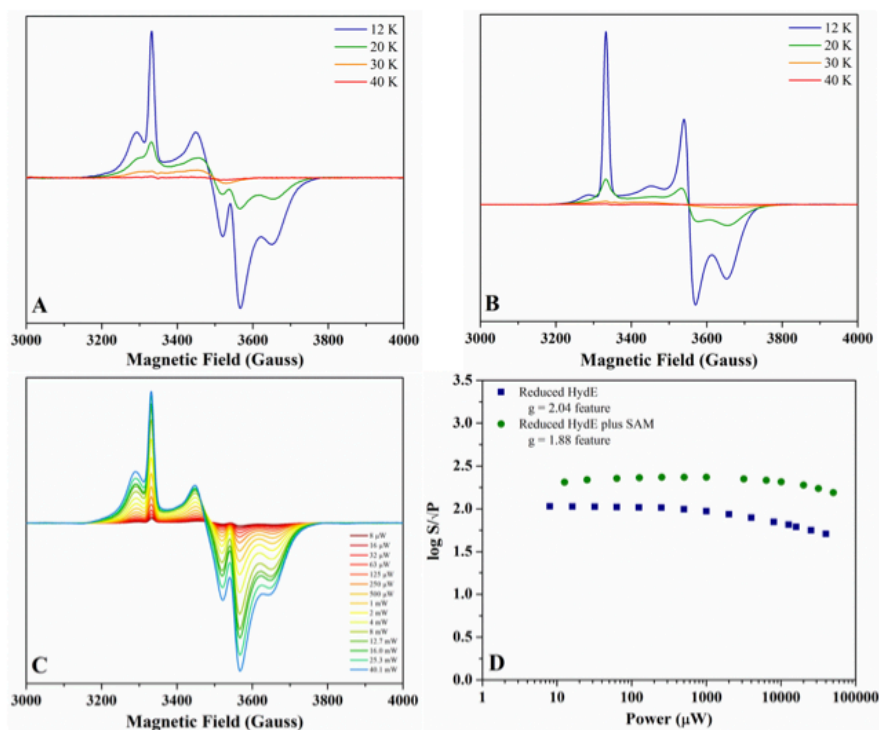


Figure S4: EPR spectra of dithionite reduced HydE without (Panel A) and with (Panel B) addition of exogenous SAM. The signals from the [4Fe-4S] clusters quickly decrease in intensity upon increase of temperature from 12 K (blue) to 40 K (red). Panel C: Dithionite reduced HydE EPR signals from 8 μ W (red trace) to 40.1 mW (blue trace). Panel D: Power saturation profile for $g = 1.88$ feature (reduced HydE plus SAM, green circles) and $g = 2.04$ feature (reduced HydE, blue squares).

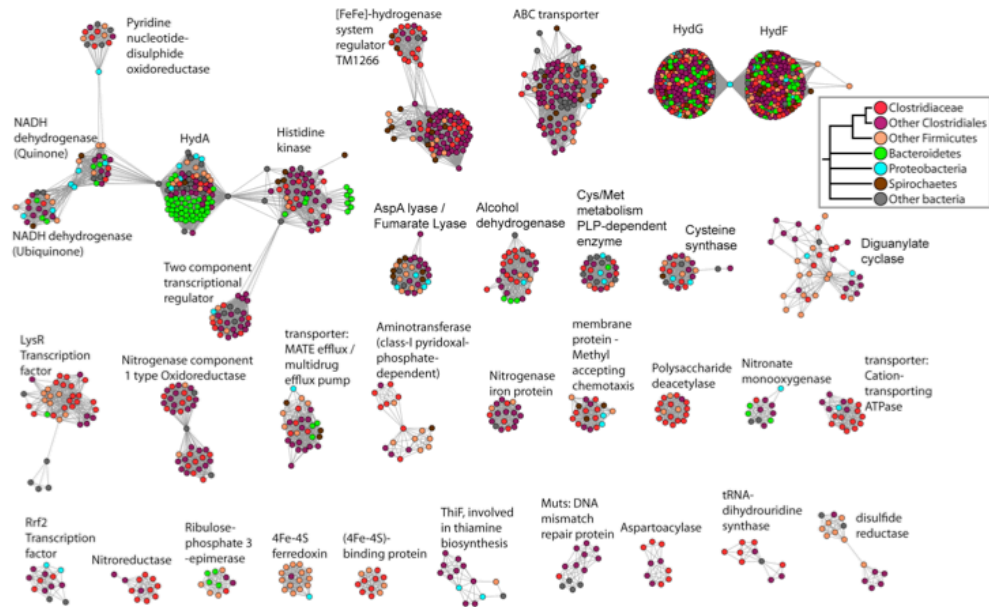


Figure S5: Larger genomic context network for HydE enzymes. Circles represent genomic neighbors within 10 genes upstream/downstream of the 563 HydE enzymes as classified by the Structural Function Linkage Database. Connections represent BLAST pairwise sequence similarity with E-value more significant than $1e-20$. The 1,390 proteins are clustered into sets of similar sequences that are visually identifiable. Only clusters that could be assigned with function are shown and accompanied with the relevant titles. Colors represent the phylogenetic origin of the organisms in which they are encoded, as shown in the legend. Clusters are roughly ordered by size and heterogeneous phylogenetic origin (from upper left to lower right). The largest and most phylogenetically diverse clusters are enriched with functions that typify the H cluster biosynthesis pathways, such as HydA, HydF, HydG and TM1266 proteins, strengthening our confidence in the ability of this method to capture biologically relevant signals.

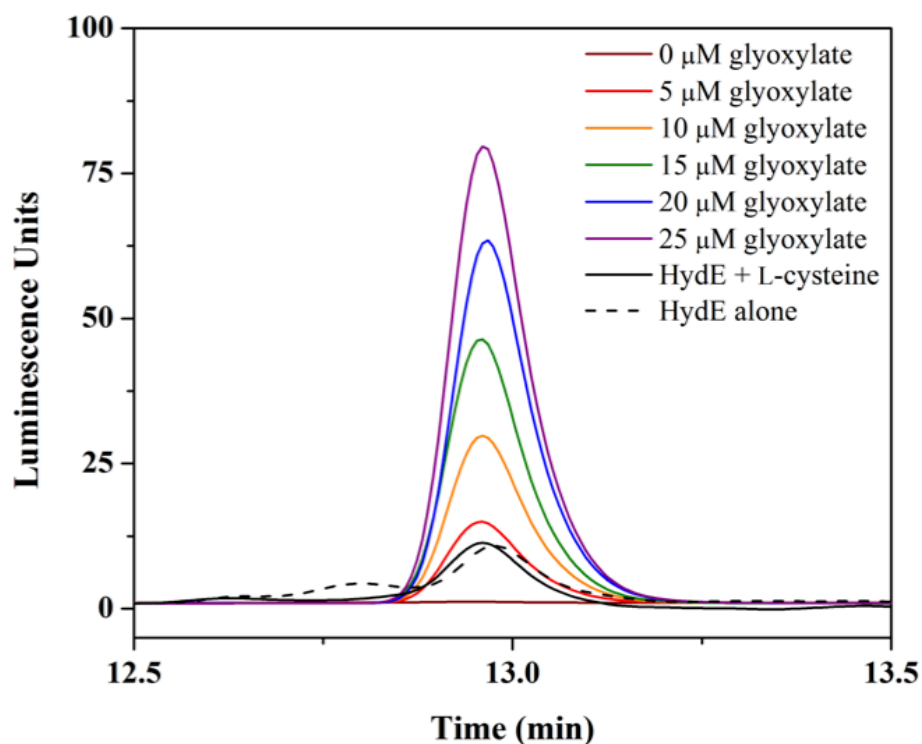


Figure S6: Glyoxylate standard titration curve and HydE SAM cleavage assay with cysteine. Enzymatic production of dehydroglycine (DHG) is quantitated via derivatization of glyoxylate (the hydrolysis product of DHG) to 2-quinoxalinol via reaction with *o*-phenylene diamine, as previously described.^(2,3) Briefly, detection of 2-quinoxalinol ($R_T = \sim 13$ min) is accomplished via HPLC wherein derivatized samples are applied to a Phenomenex® Luna® C18 reverse phase column connected in line with a fluorimeter set at 340 nm excitation and 420 nm emission wavelengths. Following sample injection, the column is washed with 95% 100 mM NH_4HCO_3 H_2O (solvent A) for 5 min, followed by a gradient to 60% with ACN (solvent B) over 15 min; the gradient is subsequently increased to 100% solvent B over 1 min, held isocratically for 4 min before re-equilibration in 95% solvent A. Quantitation of any DHG that could be enzymatically formed (see main text) is accomplished by using a synthetic solution of glyoxylic acid (0.8 mM) that is used to make a standard 2-quinoxalinol curve prepared in parallel with enzymatic assay aliquots. The data shown above represent a linear response for the standard curve. Low levels (< 5 μM) of glyoxylate appear to be present in assay aliquots of either HydE alone (black dashed line) or when HydE is assayed in the presence of cysteine (black solid line). Both assay aliquots are from reaction mixtures containing 25 μM HydE and represent 60 minute time points at 37 °C from a standard SAM cleavage assay; see main text for further experimental details.

Table S1: EPR characterization of as reconstituted and dithionite reduced HydE samples.

	g ₁ -value	g ₁ -strain	g ₂ -value	g ₂ -strain	g ₃ -value	g ₃ -strain
As-Reconstituted						
Cluster 1	2.0265	0.0091	2.0143	0.0104	1.9885	0.0419
Cluster 2	2.0066	0.0128	2.0041	0.0199	1.9554	0.0369
N-Terminal Reduced						
N-terminal	2.0397	0.0460	1.9208	0.0584	1.8970	0.0503
N-terminal-SAM	2.0096	0.0094	1.8830	0.0155	1.8308	0.0308
C-terminal	2.0317	0.0567	1.9104	0.0303	1.8719	0.0728
N-Terminal Reduced, SAM Bound						
N-terminal	2.0384	0.0302	1.9186	0.0642	1.8962	0.0772
N-terminal-SAM	2.0103	0.0095	1.8844	0.0152	1.8309	0.0323
C-terminal	2.0319	0.0645	1.9191	0.0280	1.8648	0.0784

Supporting Information References

1. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF chimera - A visualization system for exploratory research and analysis, *J Comput Chem* 25, 1605-1612.
2. Driesener, R. C., Challand, M. R., McGlynn, S. E., Shepard, E. M., Boyd, E. S., Broderick, J. B., Peters, J. W., and Roach, P. L. (2010) [FeFe]-hydrogenase cyanide ligands derived from S-adenosylmethionine-dependent cleavage of tyrosine, *Angewandte Chemie* 49, 1687-1690.
3. Driesener, R. C., Duffus, B. R., Shepard, E. M., Bruzas, I. R., Duschene, K. S., Coleman, N. J. R., Marrison, A. P. G., Salvadori, E., Kay, C. W. M., Peters, J. W., Broderick, J. B., and Roach, P. L. (2013) Biochemical and Kinetic Characterization of Radical S-Adenosyl-L-methionine Enzyme HydG, *Biochemistry* 52, 8696-8707.

CHAPTER 5

REACTION MECHANISM OF [FeFe]-HYDROGENASE
MATURATION ENZYME HydEAbstract

The three unique gene products HydE, HydF, and HydG are necessary for the maturation of the active site H-cluster of [FeFe]-hydrogenase. HydE and HydG are radical SAM enzymes that catalyze chemical transformations by H-atom abstraction and synthesize the nonproteinaceous ligands of the H-cluster. While HydG supplies the diatomic ligands, HydE likely generates the dithiomethylamine (DTMA) group that bridges the 2Fe subcluster at the heart of the H-cluster. A recent multidisciplinary study led to the proposal that HydE uses low molecular mass thiols to generate two units of thioformaldehyde that condense with ammonia producing the DTMA bridge.⁽¹⁾ Here we present our findings of mercaptopyruvate lyase activity by HydE under turnover conditions and expand our hypothesis to include the key residues in turnover and synthesis of the DTMA fragment in the lower part of the TIM barrel of HydE.

Introduction

While it was recognized early that hydrogen containing molecules were at the core of energy cycling in ecosystems, it was not until 1931 that it was shown molecular hydrogen could serve as an energy source for select bacterial isolates and the term hydrogenase was officially coined.⁽²⁾ The following several decades of research revealed

a tremendous diversity among hydrogenases including oxygen-sensitivity, intracellular location, metallic content, and electron transfer partners.⁽³⁾ While biochemical, spectroscopic, and analytical investigations helped classify hydrogenases into roughly three groups, termed [FeFe]-, [NiFe]-, and [Fe]-only hydrogenase, it was not until 1998 that Peters et al. gave the world a look at the non-proteinaceous component of the active site of a hydrogenase enzyme.⁽⁴⁾ The exact stoichiometry and identity of the unusual ligands of the active site of [FeFe]-hydrogenase, termed the H-cluster, was deciphered over the next thirteen years by several research groups.⁽⁵⁻⁷⁾ In total, the H-cluster contains a [4Fe-4S] cluster coordinated by four cysteines, one of which bridges a uniquely decorated 2Fe subcluster. The DTMA bridge, and a single carbon monoxide bridge the two irons. Additionally, both irons are coordinated by a carbon monoxide and cyanide ligand. Recently we proposed the DTMA bridge was synthesized from two thioformaldehyde fragments produced by HydE lyase activity on a low molecular weight thiol, perhaps cysteine or mercaptopyruvate.⁽¹⁾ Our current work supports this hypothesis given our observed HydE mercaptopyruvate lyase activity. Other results indicate that the synthesis of the DTMA bridge may occur in a second area of HydE in a stepwise fashion prior to delivery to HydF.

Materials and Methods

Growth, Purification, and Reconstitution of HydE

A detailed description of the cloning including the primers used to build the pET23b-*C.a.*HydE construct used to express *Clostridium acetobutylicum* HydE can be

found in Chapter 3. The overexpression of the C-terminally 6x-His tagged protein has been described in Chapters 2 and 4.⁽¹⁾ The HydE protein was purified using immobilized metal ion affinity chromatography by a method described in Chapters 2 and 4 and yielded brown colored protein judged >95% pure by SDS-PAGE with less than stoichiometric iron for a protein possessing two [4Fe-4S] clusters (Figure 5.1).^(1, 8, 9)

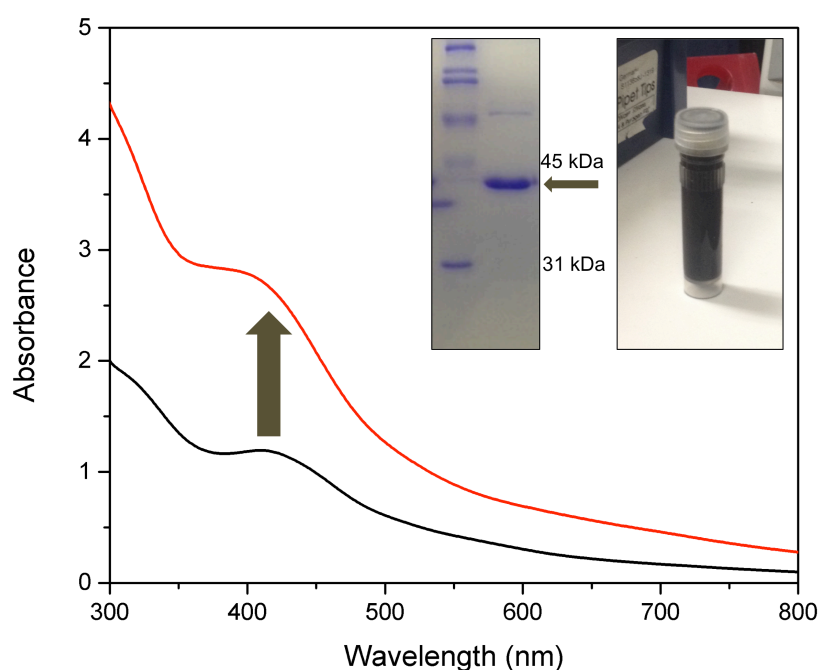


Figure 5.1. UV-vis spectroscopy of pre and post reconstituted HydE (83 μ M). The arrow denotes the increase in the 420 nm due to the loading of FeS clusters upon reconstitution. Left embedded image is SDS-PAGE gel representing the relative purity of HydE following purification. Right embedded image shows the intense brown color of reconstituted HydE.

The protein was reconstituted with exogenous iron and sulfide under reducing conditions yielding protein with 8.1 ± 0.5 Fe/protein over three separate purifications and reconstitutions of HydE. UV-vis absorption spectroscopy of HydE and *S*-adenosylmethionine (SAM) employed a Cary 60 spectrophotometer in a 1.4 mL

anaerobic cuvette. The dark brown reconstituted protein shared a marked increase in absorbance at 420 nm (Figure 5.1) indicative of increased occupancy of FeS clusters in the purified protein after removal of adventitiously bound iron.⁽¹⁰⁻¹²⁾

Preparation of HydE Variants

For the generation of the *C. acetobutylicum* HydE variants the Agilent Technologies QuikChange Lighting Multi Site-Directed Mutagenesis Kit was employed. The manufacture's recommendations were used with 0.5 μ L of the QuikSolution per 25 μ L of reaction and the previously described pET23b-*C.a.*HydE template (Chapter 3). The mutagenic primers used with the specific base substitutions bolded and underlines were

GAGTACAGAAAATCTGGAGCTGATAGATATTTAATAG**C**CAATAGAAACAACA
GATAAAG and

CAGAAAATCTGGAGCTGATAGATATTTAATAA**A**AATAGAAACAACAGATAA
AGAACTG for the R159A and R159K variants, respectively. The mutagenesis protocol produces single stranded plasmids that were transformed into Agilent XL-10 Gold Ultracompetent cells. The sequence verified constructs were transformed into the overexpression *E. coli* strain BL21(DE3)-*RIL*.

The overexpression, purification, and reconstitution steps were performed anaerobically in a manner similar to the wild-type *C. acetobutylicum* HydE previously described in Chapters 2 and 4. The purified variants possessed 9.8 ± 0.5 and 9.1 ± 0.4 Fe/protein for R159A and R159K, respectively.

Electron Paramagnetic Resonance Spectroscopy

All EPR samples were prepared in a strictly anaerobic environment maintained by a MBRAUN chamber ($O_2 \leq 1$ ppm). Solutions were prepared using buffer and water degassed on a Schlenk line by three cycles of 10 minutes of vacuum followed by backfill with nitrogen. The water mass lost from the buffers by evaporation was replaced with degassed water. Given the potential for interference with the high concentration of potassium ions (500 mM) with the putative cation binding pocket, the *C. acetobutylicum* HydE protein was buffer exchanged into 25 mM Tris-HCl pH 8.0 removing most KCl and glycerol. The protein was stable in this buffer after dialysis but rapidly precipitated after a single freeze/thaw. A saturated thallium (I) chloride solution stock was prepared to approximately 10 mM. The 250 μ L (final volume) EPR samples were prepared with 100 μ M HydE, 1 mM *S*-adenosylmethionine (SAM), ~5 mM TlCl, and 3 mM sodium dithionite. X-band, CW spectra were obtained using a Bruker ER-200D-SRC spectrometer with a liquid helium cryostat and Oxford Instruments temperature controller. EPR spectra were collected with the following parameters unless noted otherwise: 1 mW power, 20.48 ms time constant/conversion time, 1×10^4 receiver gain, 100 kHz modulation frequency, 12-16 K temperature, and a modulation amplitude of 10 G. OriginPro (Version 8.5) was used to baseline correct spectra and EasySpin 4.5.0 was used to assign *g*-values.⁽¹³⁾

HydE Turnover Assays

C. acetobutylicum wild type and R159A/K variant reconstituted proteins were assayed for SAM cleavage and 5'-deoxyadenosine (dAdo) production rates in the presence of 25 μ M HydE, 0.5 mM SAM, 2.5 mM thiol, and 2 mM sodium dithionite in the 25 mM HEPES pH 8.0, 500 mM KCl, and 5% glycerol (w/v) buffer. The assays were incubated at 37 °C for 30 minutes following the addition of sodium dithionite and were quenched by addition of 10% (v/v) 400 mM acetic acid buffer pH 4.5. The samples were placed on ice for about 1 hr followed by centrifugation for 10 min at 4 °C to remove the precipitated protein.

The quantities of dAdo produced were measured by reverse phase chromatography on an Agilent Technologies 1100 series HPLC using a Phenomenex Kinetix 5 μ PFP 100A column by a method that has previously been described.⁽¹⁾ The mobile phases were (A) water and (B) acetonitrile with 0.1% acetic acid. In short, 10 μ L of the reaction supernatant was loaded on a 2% B equilibrated column and washed with 2% B for 3.3 min. A linear gradient run up to 60% B over 11 min caused dAdo to elute at 6.8 min. The dAdo ($\lambda_{\text{max}} = 254$ nm) concentration was determined using a standard curve.

Production and Detection of Glyoxylate

Aliquots from dAdo production/SAM cleavage assays explained above were subjected to a derivatization method in order to detect any glyoxylate as previously described with minor modifications.^(14, 15) In addition to mercaptopyruvate and the full assay complement, controls without substrate, with cysteine, and without HydE were

tested. The supernatant (10 μ L) from 30 min quenched reaction aliquots were diluted 5-fold with 40 μ L of assay buffer, acidified by addition of 100 μ L of 500 mM HCl, and derivatized with 50 μ L of 10 mg/mL *o*-phenylenediamine. The solution was incubated at 95 °C for 10 min prior to addition of 120 μ L 1.25 M NaOH. A standard curve with 0 to 25 μ M glyoxylate was prepared for quantification using glyoxylic acid monohydrate from Sigma-Aldrich. The samples were flash frozen with liquid nitrogen and thawed just prior to HPLC-FLD analysis.

A Luna 5u 100A Phenomenex C18 column was used on an Agilent Technologies 1100 series HPLC linked to an Agilent Infinity 1260 fluorescence detector for separation and quantitation of the derivatized glyoxylate product 2-quinoxalinol. The HPLC solvents were (A) 100 mM NH_4HCO_3 in water and (B) acetonitrile. The 35 min HPLC-FLD run included a 5 min wash with 5% B followed by a linear gradient to 60% B over 15 minutes. The column was then washed with 100% B for 5 min and the column was equilibrated with 5% B for 10 min prior to the following injection. The fluorescence detector monitored a 420 nm emission during excitation at 340 nm. A peak corresponding to the derivatized glyoxylate standard 2-quinoxalinol eluted mid-gradient at 15 minutes.

Assays investigating the lyase activity of HydE on L-cysteine and L-homocysteine required modifications of the standard turnover assays described in the previous section. Given the interference of KCl and glycerol with the LC-MS signal, HydE was buffer exchanged into a 25 mM NH_4HCO_3 pH 8.0 buffer. Testing the hypothesis that HydE may convert L-homocysteine and L-cysteine to L-alanine and

glycine, respectively a normal phase chromatography separation prior to analysis by mass spectrometry was employed. Given the lower quantitation threshold for these amino acids than derivatized glyoxylate with HPLC-FLD the assay components' concentrations were increased to 100 μ M HydE, 300 μ M SAM, 10 mM substrates, and 3 mM NaDT. The reactions were allowed to react for 2 hrs at 37 °C prior to being quenched with an equal volume of acetone. The precipitated protein was removed by centrifugation at 14K rpm at 4 °C prior to LC-MS analysis. A volume of 2 μ L of the supernatant was injected onto a Cogent Diamond Hydride 100A 4 μ m 150 x 2.1 mm normal phase column equilibrated in 80% solvent B (acetonitrile) and 20% solvent A (water) both containing 0.1% trifluoroacetic acid and washed for 1 min at this ratio. A linear gradient from 80% down to 50% B of 5 min was sufficient to elute both glycine and alanine at around 1.8 min into the gradient. The column was washed at 50% B for 2.5 min and reequilibrated into 80% B for 1.5 min prior to injection of the following sample. Turnover assays were designed given the detection limit of 20 μ M alanine and glycine with a 2 μ L injection.

Mapping Conserved Residues and Internal Volume of HydE

Several topographic and bioinformatics investigations were made using the *Thermotoga maritima* HydE crystal structure solution PDB ID: 3IIZ.⁽⁸⁾ To map the void space within HydE, 3IIZ was uploaded to the CASTp server⁽¹⁶⁾ generating a .pyc file that could be opened in the software suite UCSF Chimera.⁽¹⁷⁾ A 1.4 Å space filling sphere size was chosen to model the solvent exposed and interior cavities of HydE.

To measure the relative conservation of individual HydE residues and map them to the crystal structure a Basic Local Alignment Search Tool (BLAST) search was conducted to identify and retrieve the top 500 sequences similar to *T. maritima* HydE. The BLAST result was associated with the *T. maritima* HydE crystal structure solution in UCSF Chimera allowing the selection of residues by their percent conservation.

Results

HydE R159 Variants Slow SAM Cleavage

The substrate binding pocket lies at the top of a large internal cavity inside the complete $(\beta\alpha)_8$ TIM barrel fold of HydE.⁽⁸⁾ The active site houses SAM at the top of the barrel and a channel extends to the bottom of the barrel. Several conserved residues that are speculated to be involved in substrate binding and product delivery line the interior of the barrel. The residue R159 is strictly conserved and likely binds an anionic moiety of HydE's substrate given that two chloride ions were crystallized in the protein in a manner similar to a carboxylic acid (Figure 5.2). The HydE variants R159A and R159K exhibited a much slower rate of dAdo production (20% and 40% of WT activity, respectively) in the absence of potential cosubstrates (Figure 5.2). The relative trend in stimulation of SAM cleavage by cysteine (Cys), mercaptopyruvate (MP), and coenzyme M (CoM) was similar to that observed in the WT enzyme, although in all cases the variants showed reduced rates.⁽¹⁾ These results indicate that R159 plays an important role in coordination of the substrate for HydE but other positively charged moieties may contribute to the anionic binding pocket.

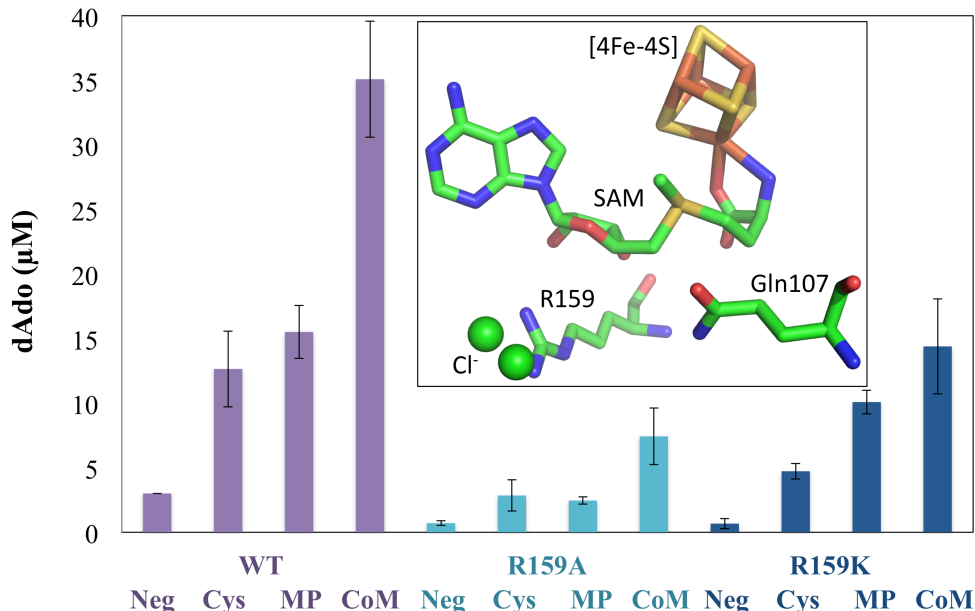


Figure 5.2. 5'-Deoxyadenosine produced by WT HydE (7.6 Fe/protein) and the R159A (9.8 Fe/protein) and R159K (9.1 Fe/protein) variants (25 μ M). SAM (0.5 mM) was present in all assays, and for each protein a negative control and putative substrates (2.5 mM) were tested. The inserted figure from PDB ID: 3IIZ shows the relative location of R159A, the chloride ions, SAM, and the [4Fe-4S] cluster in the active site of *Thermotoga maritima* HydE. Labeling is as follows: carbon (green), nitrogen (blue), oxygen (red), sulfur (yellow), iron (orange), and chlorine spheres (green).

HydE Mercaptopyruvate Lyase Activity

Mercaptopyruvate, L-cysteine, and L-homocysteine were all screened as substrates for HydE and investigations were made to find the potential products of catalysis. The hypothesis that HydE could catalyze H-atom abstraction from mercaptopyruvate, L-cysteine, and L-homocysteine to produce thioformaldehyde and glyoxylate, glycine, and L-alanine, respectively, was tested by looking for these fragments following turnover (Figure 5.3). Neither glycine nor L-alanine was observed by mass spectrometry in the assays of L-cysteine and L-homocysteine. The detection of these small polar molecules, however, is difficult and their empirically determined detection limits of ~ 20 μ M may have not allowed for observation. In contrast, the assay

of mercaptopyruvate turnover by HydE revealed levels of glyoxylate that were significantly increased over background (Figure 5.4). Approximately two molecules of glyoxylate per HydE after 30 min were produced in the presence of mercaptopyruvate upon incubation. These results support the hypothesis that a small molecular weight thiol, in particular mercaptopyruvate, serves as the substrate for HydE.

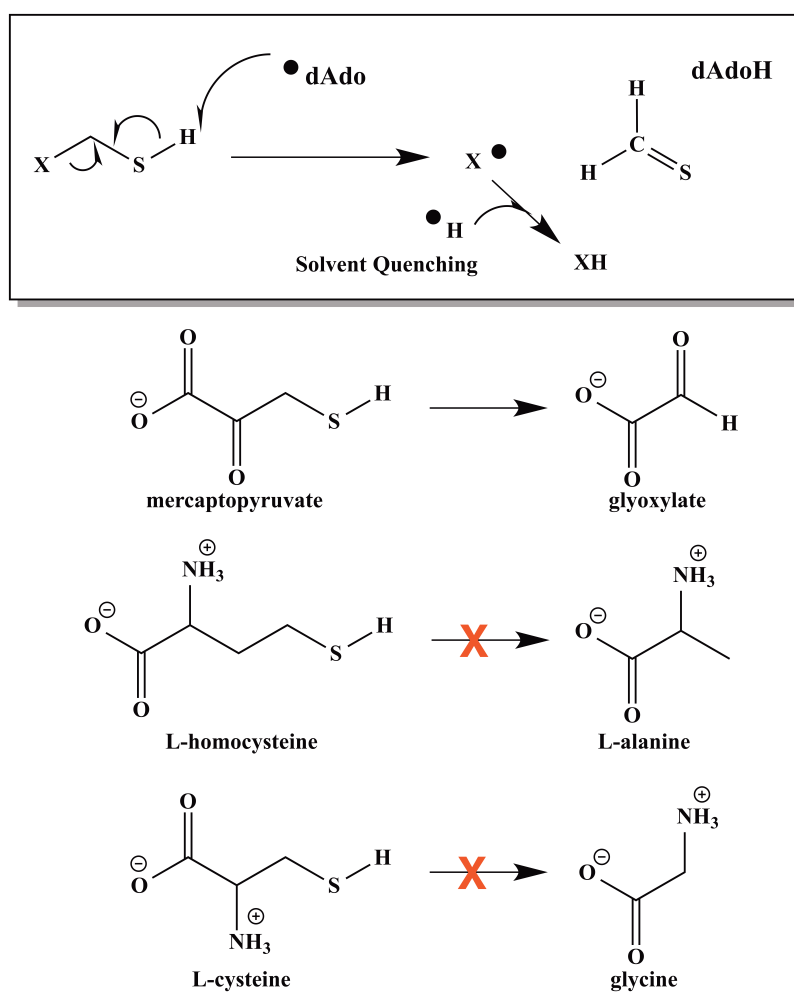


Figure 5.3. Boxed Area: the proposed mechanism for H-atom abstraction by HydE to produce thioformaldehyde (S=CH₂), 5'-deoxyadenosine (dAdoH), and a fragment. The potential substrates tested, and their predicted products, are shown below the box. Only the product from mercaptopyruvate lyase activity was observed.

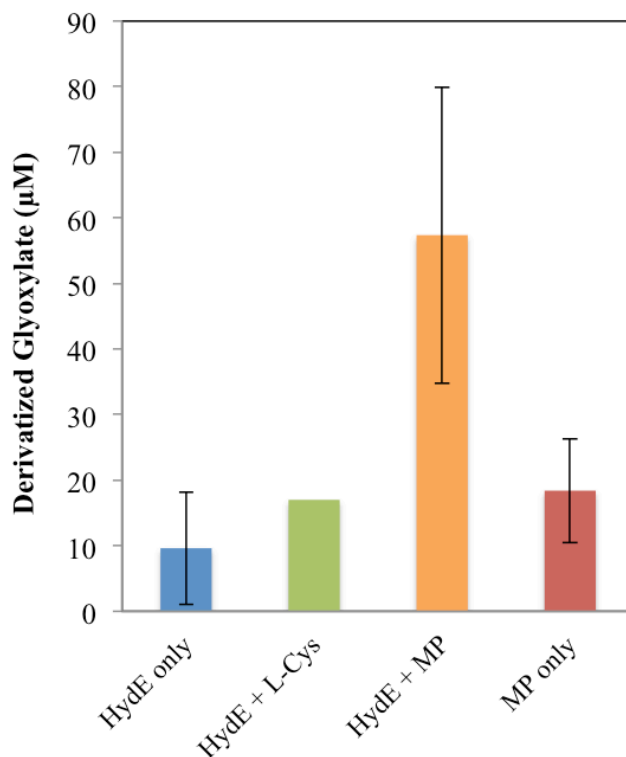


Figure 5.4. HydE production of glyoxylate. The production of glyoxylate after 30 min of addition of mercaptopyruvate (MP)/L-Cys (2.5 mM) to HydE with 7.6 Fe/protein (25 μM) with SAM (500 μM) and sodium dithionite (2 mM) under turnover conditions. HydE only, HydE + L-Cys (n = 1), and HydE + mercaptopyruvate (MP) all served as negative controls.

Thallium Nuclear Spin Interaction with FeS Cluster Signal

Both stable isotopes ^{203}Tl and ^{205}Tl possess $I = \frac{1}{2}$ nuclear spin; when these bind close to a paramagnetic center, splitting of the EPR signals into doublets can be observed.⁽¹⁸⁾ The superhyperfine interaction of thallium with paramagnetic clusters makes it a powerful tool to probe the distances of monovalent ion binding sites to EPR active paramagnetic centers.^(18, 19) Upon addition of thallium salts to HydE with or without SAM, the EPR signal was dramatically modified (Figure 5.5). The broadening observed in the features is most likely due to peak splitting caused by interaction of the

nuclear spin of the thallium with the unpaired electrons of the $[4\text{Fe-4S}]^+$ cluster. The most intense signal corresponding to the N-terminal cluster with SAM bound was the easiest feature to model. The splitting of ± 23 gauss from the parent 3500 Gauss signal decreased the parent signal by 50%, with the overall spectrum appearing to be the result of an overlap of two $[4\text{Fe-4S}]^+$ signals, only one of which is close to TI^+ (Figure 5.5B). These observations are indicative of a monovalent cation binding site near to the N-terminal radical SAM cluster. We postulated that this cation binding site could serve as a site for binding ammonium for use in synthesizing the DTMA bridge by reaction with thioformaldehyde. In order to probe the possibility of ammonium binding to the cation binding site, we carried out a competition experiment to determine whether ammonium can displace thallium(I) from this site. Upon addition of ammonium to 100 μM HyDE buffer exchanged into 100 mM Tris- NO_3^- pH 8.0, 1 mM SAM, 1 mM TlCl , and 3 mM sodium dithionite the EPR signal ($g = 2.01, 1.88, 1.83$) sharpened indicating that thallium was being displaced (Figure 5.5). Although relatively high concentrations of ammonium were required to achieve this displacement, thallium's persistence and resistance to substitution of potassium has previously been noted for other metalloenzymes.^(18, 19) Further spectroscopic investigations including ENDOR spectroscopy could give us a more accurate distance of the TI^+ to the paramagnetic $[4\text{Fe-4S}]^+$ cluster.

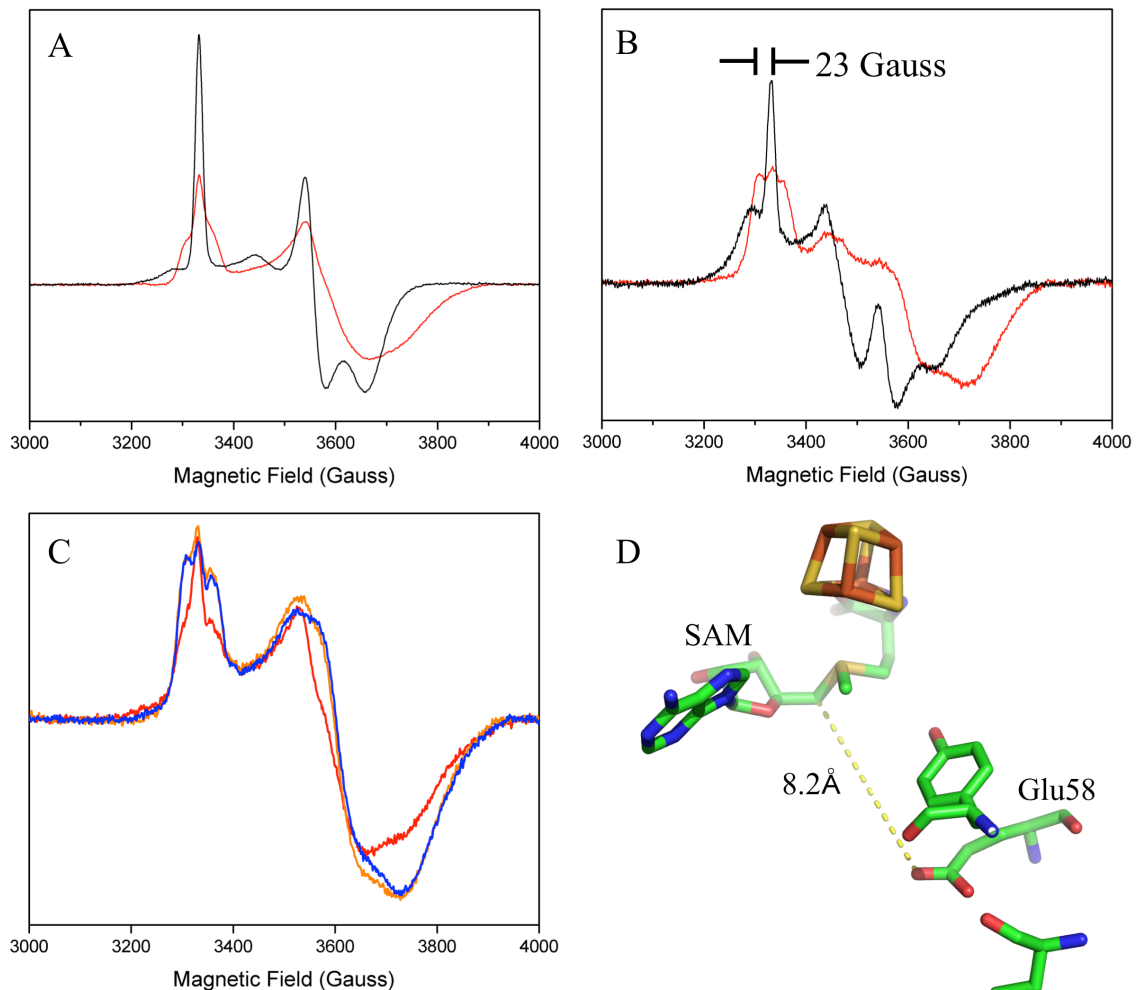


Figure 5.5. *C. acetobutylicum* HydE with addition of TI^+ . A. EPR spectra of reduced HydE with 7.6 Fe/protein (black trace) (100 μM) and reduced HydE with addition of TI^+ (red trace) (5 mM). B. Same as in panel A but with addition of exogenous SAM (1 mM). C. Titration with 0.5 mM (blue trace), 5.0 mM (yellow trace), and 20 mM (red trace) ammonium into reduced HydE (100 μM), SAM (1 mM), and TI^+ (1 mM). The EPR parameters for A-C are described in the methods section. D. Distance of 5' carbon of SAM to putative cation binding site in HydE (PDB ID: 3HIZ).

Discussion

Putative Active Site Residues

While PylB⁽²⁰⁾ and NosL,⁽²¹⁾ two radical SAM enzymes similar to HydE (Chapters 1 and 4), have published crystal structures with substrate/product bound, HydE has never

been crystallized with a substrate molecule as its identity is unknown. Our recent publication (Chapter 4) reported a multidisciplinary approach that implicated low molecular mass thiols as candidates for HydE's substrate.⁽¹⁾ *In silico* docking studies indicate that these thiols have an anionic moiety that can salt bridge with HydE Arg159 (*T. maritima* and *C. acetobutylicum* numbering). In addition, the crystal structure was found to contain two chloride ions occupying roughly the space of a carboxyl group of an undefined substrate.⁽⁸⁾ In the work described in this chapter, this arginine was mutated conservatively to a lysine and non-conservatively to an alanine. While neither of these residues likely affect the binding of SAM, a mutation that effects substrate binding of radical SAM enzymes would likely lower SAM cleavage rates. We hypothesized that the R159K variant protein would have much lower SAM cleavage/5'-deoxyadenosine production rates and the R159A variant would have little or no activity.⁽¹⁹⁾ Our results show that on average across the board R159K had 40% of WT SAM cleavage activity, while the R159A exhibited approximately 20% of WT activity. While the SAM cleavage rates were affected less than we might have expected, further investigation into the active site revealed that there are other positive moieties that likely contribute to the total anionic binding pocket; these include the backbone nitrogens from Thr269 and Ala270 (*T. maritima* numbering) located <3.5 Å from the cocrystallized chloride ions (Figure 5.6). In addition, the oxygen on the Tyr306 phenol group is only 3.5 Å away, perhaps with the hydrogen aiming towards the anion binding pocket acting as a H-bond donor to the negatively charged moiety of the substrate. These additional positive moieties may be sufficient to stabilize the thiols in the active site albeit at a compromised affinity. The

decrease in the SAM cleavage rates in the absence of thiols may be the result of changes in the electronic environment of the [4Fe-4S] cluster and perturb either the reduction of the cluster or the transfer of the electron to the positively charged sulfonium of SAM.

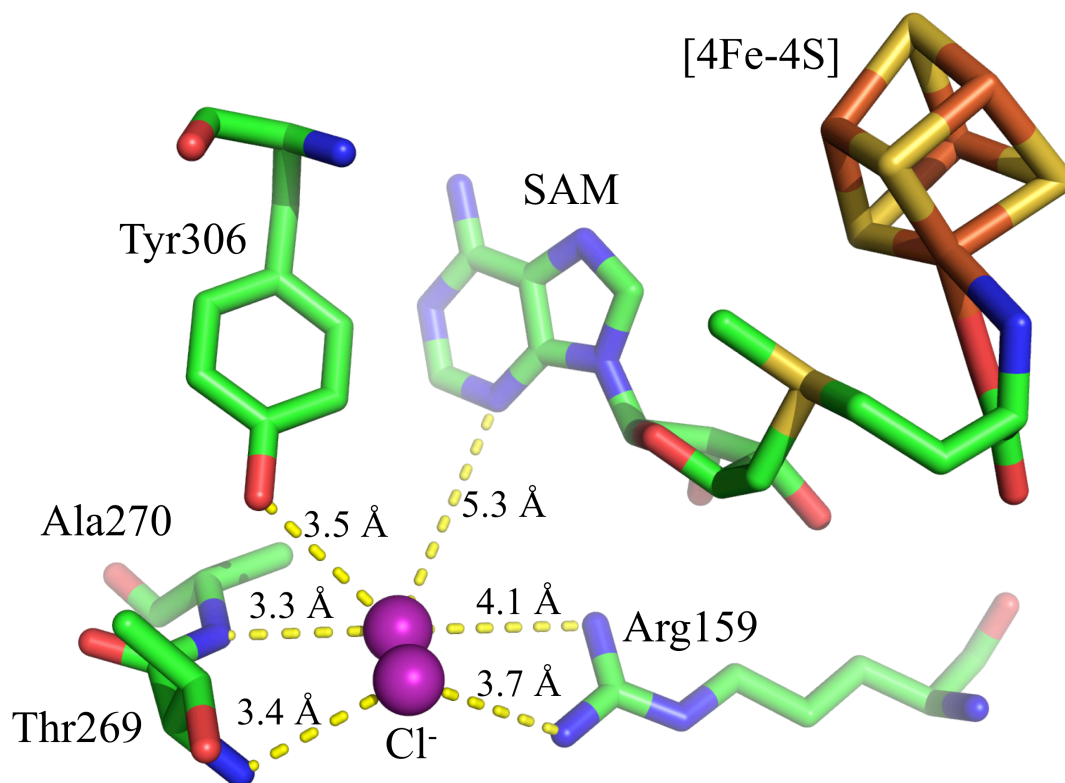


Figure 5.6. Active site anion binding pocket of *T. maritima* HydE (PDB ID: 3IIZ) with residues highlighted that likely contribute to the positively charged pocket. Labeling is as follows: carbon (green), nitrogen (blue), oxygen (red), sulfur (yellow), iron (orange), and chlorine spheres (purple).

Mechanism for Lyase Activity

We recently proposed that the synthesis of the dithiomethylamine (DTMA) bridge of the H-cluster might form via the result of HydE generating highly reactive thioformaldehyde units from a low molecular mass mercaptan, such as cysteine or mercaptopyruvate.⁽¹⁾ The observation that glyoxylate is produced enzymatically by

HydE in the presence of mercaptopyruvate supports the mechanism shown in Figure 5.4. Detection of the thioformaldehyde product itself is challenging due to its high reactivity.⁽²²⁾ Two successive HydE turnover events may produce two thioformaldehyde molecules that would condense with ammonia/ammonium in the interior of the TIM barrel to produce the dithiomethylamine bridge, in a manner reminiscent of the *in vitro* synthesis of H-cluster analogs (Figure 5.7).⁽²³⁾

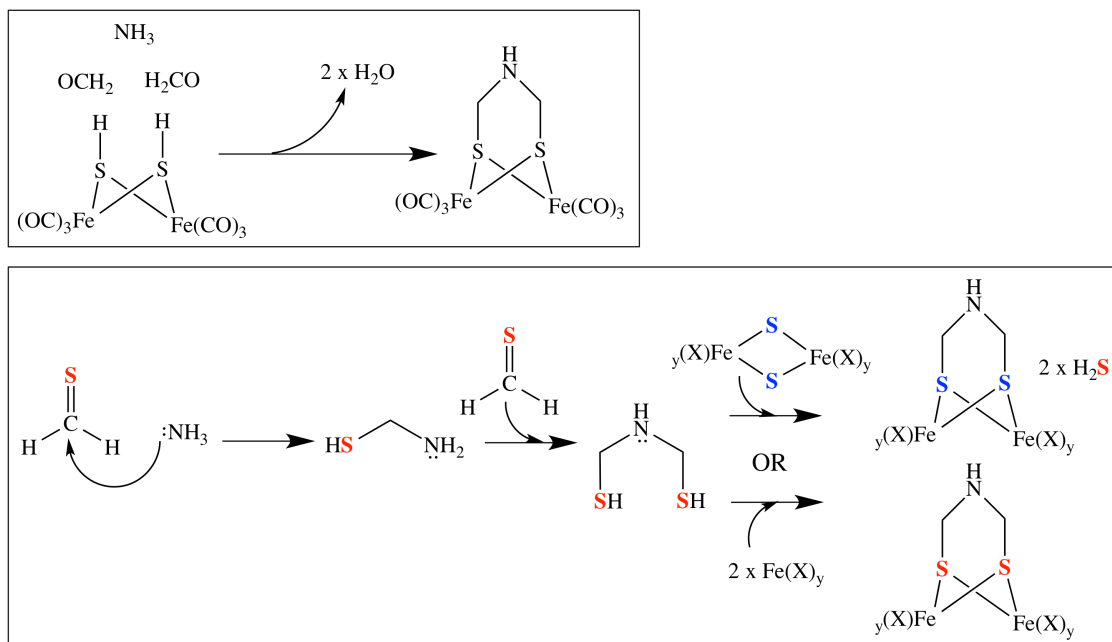


Figure 5.7. Upper Box: *In vitro* synthesis of H-cluster analogs by condensation of formaldehyde and ammonia on a 2Fe scaffold.⁽²³⁾ Lower Box: Proposed *in vivo* synthesis of DTMA bridge from thioformaldehyde condensation with ammonia. DTMA could subsequently form the H-cluster by joining two iron-dithiolate species proposed by Kuchenreuther et al.⁽²⁴⁾ (bottom) or by alkylation of an existing [2Fe-2S] cluster presumably bound to HydF⁽²⁵⁾ (top).

The observation that glyoxylate is enzymatically produced under HydE turnover conditions when mercaptopyruvate is present supports the hypothesis that a low molecular mass thiol is acted upon by HydE. It also favors the two hypotheses that lyase

activity on the substrate produces thioformaldehyde and the *in vivo* mechanism for synthesis of the DTMA bridge occurs in a manner similar as shown in Figure 5.7.

EPR investigations with HydE in the presence of Tl^{1+} provide additional support for this mechanism by pointing to an ammonium binding pocket in the active site. The EPR spectra show strong evidence of interaction of the nuclear spin of thallium with the paramagnetic $[4\text{Fe-4S}]^+$ cluster both in the presence and absence of SAM (Figure 5.3A and B). Further, the effects of the thallium nuclear coupling are diminished in the presence of ammonium ion (Figure 5.5C), indicating that ammonium can displace thallium from this binding site. The proposed site for binding the thallium or ammonium is the negatively charged pocket near ($\sim 8 \text{ \AA}$) the active site of HydE (Figure 5.5D), which could support the ammonium ion used for synthesis of the DTMA bridge. In the *T. maritima* HydE crystal structure PDB ID: 3IIZ, this negatively charged pocket is roughly $10 \text{ \AA}$ from the SAM binding N-terminal cluster which is likely too far to observe thallium coupling by EPR, but could still be close enough to bind a substrate ammonium.⁽¹⁸⁾ The affinity for ammonium appears to be less than that of thallium given the relative concentrations of these cations in the titration experiments (Figure 5.5C); however, intracellular concentrations of ammonium in some bacteria have been found to be as high as 138 mM , which may explain the apparent low affinity for ammonium.⁽²⁶⁾ Also, thallium has been reported to have a greater affinity for cation binding sites than the appropriate biologically relevant cation as it is difficult to displace.⁽¹⁸⁾

Key C-terminal Active Site Residues

Conservation analysis of the amino acid sequence of homologous proteins often reveals residues important to ligand binding, protein-protein interactions, structural stability, and active site catalysis.^(27, 28) Alignment of the top 500 most similar sequences to *T. maritima* HydE can be used to highlight the most conserved residues. This method is especially powerful when the conservation filters are applied to a crystal structure solution and residues of relative conservation are highlighted. Given the possibility for sequencing errors or misalignments of the amino acid sequences, we defined residues with 99.3% or greater conservation as highly conserved.

The tertiary structure for all known radical SAM enzymes is that of a complete or partial TIM barrel.^(29, 30) The TIM barrel is composed of alternating $\beta\alpha$ units that fold to create an environment that largely excludes the bulk solvent shielding the active site radical from side reactions. The purple “cast” of the interior space (Figure 5.8B) in HydE is composed of 1.4 Å spheres occupying a volume of 1744.8 Å³. A large portion of the upper chamber of this interior space is occupied by the [4Fe-4S] cluster and SAM cofactors as represented in Figure 5.8A. The most highly conserved residues ($\geq 99.3\%$) of HydE are depicted as sticks in Figure 5.8A, C, and D. A majority of these highly conserved residues are located in the central portion of the structure lining the interior of the TIM barrel. The amino acids that bind the SAM and the [4Fe-4S] cluster cofactors are highly conserved, chiefly being the CX₃CX₂C motif and Asp161 (*T. maritima* HydE numbering).

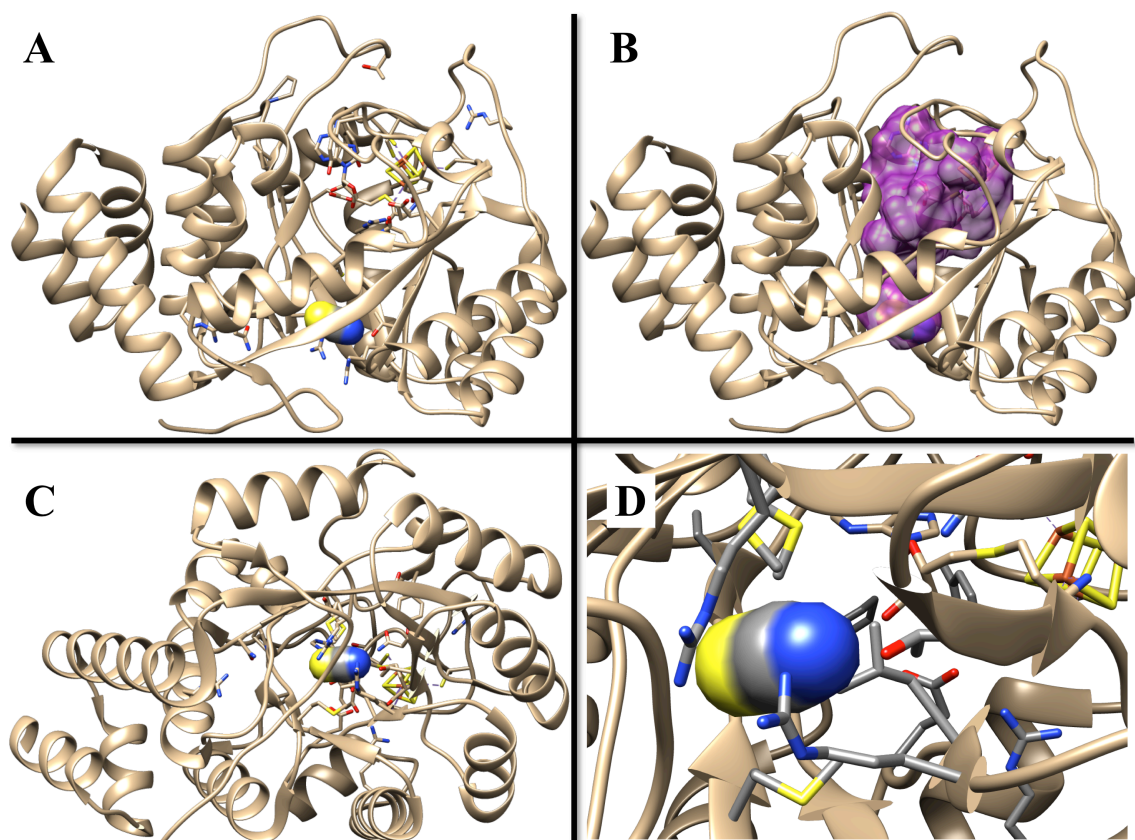


Figure 5.8. HydE Crystal Structures from PDB ID: 3IIZ and 3CIX. A. Side view of cartoon structures with 99.3% and higher conserved residues, thiocyanate (SCN⁻), SAM, and [4Fe-4S] cluster are explicitly shown. B. Side view of cartoon structure with interior volume represented as a purple cast made by 1.4 Å spheres. C. Bottom view of Figure 5.8A. D. Close up view of highly conserved residues surrounding thiocyanate. An alternate view of this site is given in Figure 5.9. The color-coding is tan and gray for carbon, blue for nitrogen, red for oxygen, yellow for sulfur, and brown for iron.

The “bottom” of the interior of the TIM barrel of HydE was found to bind thiocyanate with high affinity in a small molecule soak of HydE crystals.⁽⁸⁾ While the relevance of this thiocyanate binding is yet unknown, it is quite interesting that the residues that surround thiocyanate are highly conserved (Figures 5.8A, C, and D). Figure 5.8D and Figure 5.9 are representations of the highly conserved residues at the bottom of the HydE TIM barrel. Six of the highly conserved residues can be divided into two

nearly symmetrical triads with a methionine, arginine, and threonine. The arginines of the triads are solvent accessible and may be involved in a gating mechanism for the delivery of the DTMA bridge, presumably to HydF.⁽³¹⁾ Also, the symmetry of the residues (Figure 5.9) at the lower access point of HydE may serve to stabilize the symmetrical product or an intermediate in DTMA synthesis (Figure 5.7).

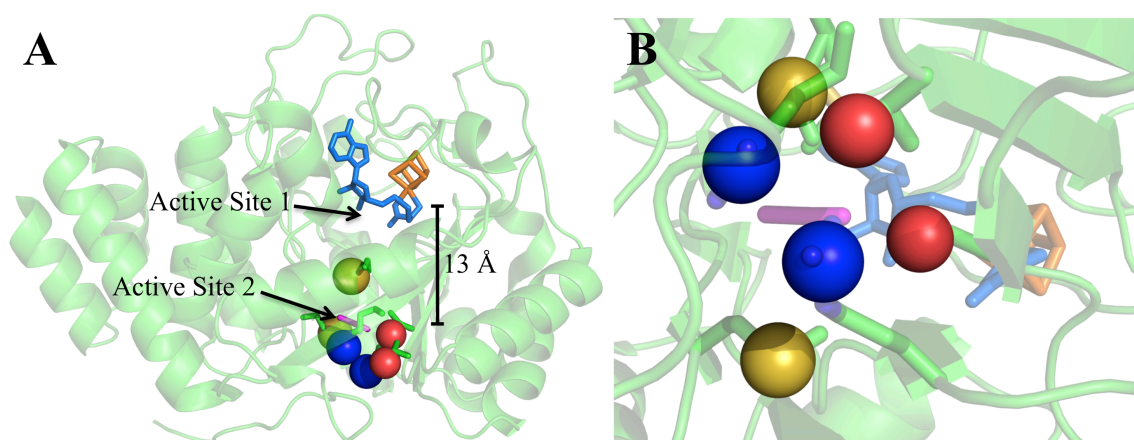


Figure 5.9. *T. maritima* HydE crystal structure PDB ID: 3CIX side (A) and bottom zoomed in (B) views. The tertiary structure is in semitransparent green. SAM, thiocyanate, and the [4Fe-4S] cluster are colored light blue, magenta, and orange, respectively. Six residues that can be divided into two semisymmetrical conserved triads at the bottom of the TIM barrel. The triads consist of Met224/Arg155/Thr134 and Met291/Arg54/Thr103. The residue Thr103 has a low occurrence of the conservative mutation to a serine. The sulfurs have multiple conformations but the spheres shown encompass both. Yellow spheres represent the sulfurs in the methionines. Blue spheres represent the central carbons of the guanidinium groups of the arginine residues. Red spheres represent the oxygens of the alcohol groups of the threonine residues.

Conclusions

Building on the multidisciplinary study that proposed the substrate for HydE is a low molecular mass thiol,⁽¹⁾ several further key observations have been made that add to this hypothesis and provide additional insight into the second active site of HydE where thioformaldehyde condenses with ammonia. Using sequence alignment to identify the

residues with the highest level of conservation, it was revealed that two triads of Arg/Met/Thr might be the site of the deliverable DTMA bridge to HydF. The symmetry of these two residue triads hints at a HydE product staging area of the presumably symmetrical DTMA precursor. Through mutagenesis of the anionic binding pocket near the site of radical catalysis we showed a marked decrease in SAM cleavage supporting the hypothesis that this pocket is important to substrate binding. While several thiols have been shown to stimulate production of 5'-deoxyadenosine from SAM cleavage and mediate incorporation of deuterium from the solvent exchangeable thiols to the 5' carbon of 5'-deoxyadenosine,⁽¹⁾ we observed a marked stimulation of production of a potential product (glyoxylate) only in the presence of mercaptopyruvate (Figure 5.4). The mercaptopyruvate lyase activity we observed by HydE is the first report of product formation by HydE. All these findings support the hypotheses that (i.) HydE's substrate is mercaptopyruvate, (ii.) the DTMA bridge precursor is formed by the condensation of ammonia and two lyase fragments, and (iii.) the lower active site area serves as the location of DTMA precursor synthesis and the antechamber for delivery to HydF. Further investigations with the other maturation partners HydG and HydF will refine the details of transfer of the DTMA bridge during the construction of the H-cluster.

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CHAPTER 6

[FeFe]-HYDROGENASE MATURATION

Contribution of Authors and Co-Authors

Manuscripts in Chapters 1, 6, 7

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Contributions: Researched the literature, compiled co-authors' contributions, and prepared the manuscript and figures.

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Contributions: Compiled literature and prepared parts of an early draft of the manuscript.

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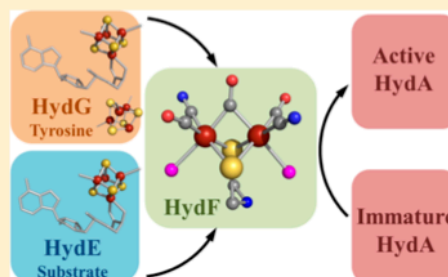
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[FeFe]-Hydrogenase Maturation

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ABSTRACT: Hydrogenases are metalloenzymes that catalyze the reversible reduction of protons at unusual metal centers. This Current Topic discusses recent advances in elucidating the steps involved in the biosynthesis of the complex metal cluster at the [FeFe]-hydrogenase (HydA) active site, known as the H-cluster. The H-cluster is composed of a 2Fe subcluster that is anchored within the active site by a bridging cysteine thiolate to a [4Fe-4S] cubane. The 2Fe subcluster contains carbon monoxide, cyanide, and bridging dithiolate ligands. H-cluster biosynthesis is now understood to occur stepwise; standard iron–sulfur cluster assembly machinery builds the [4Fe-4S] cubane of the H-cluster, while three specific maturase enzymes known as HydE, HydF, and HydG assemble the 2Fe subcluster. HydE and HydG are both radical S-adenosylmethionine enzymes that interact with an iron–sulfur cluster binding GTPase scaffold, HydF, during the construction of the 2Fe subcluster moiety. In an unprecedented biochemical reaction, HydG cleaves tyrosine and decomposes the resulting dehydroglycine into carbon monoxide and cyanide ligands. The role of HydE in the biosynthetic pathway remains undefined, although it is hypothesized to be critical for the synthesis of the bridging dithiolate. HydF is the site where the complete 2Fe subcluster is formed and ultimately delivered to the immature hydrogenase protein in the final step of [FeFe]-hydrogenase maturation. This work addresses the roles of and interactions among HydE, HydF, HydG, and HydA in the formation of the mature [FeFe]-hydrogenase.



Hydrogenases are metalloenzymes that perform essential metabolic roles in many microbial communities by catalyzing the consumption and production of dihydrogen (H_2).^{1,2} Their physiological role is either coupling hydrogen oxidation to anabolic processes or recycling reduced electron carriers during fermentation via the reduction of protons.³ Although numerous enzymes catalyze reactions that use H_2 as a substrate or cosubstrate or generate H_2 as a product or coproduct, only two classes of hydrogenases catalyze the reversible interconversion of H_2 to protons and electrons; these enzyme classes have been termed the [NiFe]- and [FeFe]-hydrogenases on the basis of the metal composition of their respective active site metal centers.⁴ The [NiFe]-hydrogenases are more commonly associated with H_2 oxidation under physiological conditions and contain a heterobimetallic active site with a Ni ion bridged to an Fe ion through two thiol groups supplied by cysteine side chains. Two additional cysteine thiols coordinate the nickel, and the iron is further coordinated by a carbon monoxide and two cyanide ligands.⁵ The presence of these diatomic ligands to Fe was largely unanticipated; in fact, when the first structure of a hydrogenase enzyme was determined in 1995 by the Fontecilla-Camps group, electron density indicating nonprotein ligands bound to the Fe ion was tentatively assigned as arising from water molecules.⁶ Only later when investigators examined the properties of carbon monoxide inhibition through infrared spectroscopy were the nonprotein ligands assigned carbon monoxide and cyanide.⁷ Soon thereafter, the presence of carbon monoxide and cyanide

ligands was more generally identified as being an intrinsic property of [NiFe]-hydrogenases.^{8,9}

When the first structures of [FeFe]-hydrogenases were determined in the late 1990s, it was anticipated that the active site metal centers, like those of the [NiFe]-hydrogenases, possessed carbon monoxide and cyanide ligands, although there were ambiguities concerning the number and arrangement.^{10,11} Two independent groups tentatively assigned five diatomic ligands to the active site and revealed a metal center consisting of a 2Fe subcluster, with one bridging and two terminal diatomics bound to each Fe ion. The assignment of the number of carbon monoxide and cyanide ligands was based on infrared (IR) studies.⁸ Two terminal diatomic ligands were observed in the structures to be directly hydrogen bonded to protein groups and were thus assigned as the cyanide ligands.¹¹ The dithiolate ligand is also a unique feature of the [FeFe]-hydrogenases; however, its composition has eluded direct determination for more than a decade.^{12–14}

In the initial structural work, there was a consensus that the ligand was a nonprotein dithiolate, i.e., a covalent linkage between two sulfur groups, but the structural work did not allow differentiation among C, N, and O at the bridgehead position.¹⁰ The Fontecilla-Camps group originally assigned the ligand as propane dithiolate but later revised their assignment

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to dithiomethylamine on the basis of mechanistic considerations that the bridgehead atom was poised to participate as a proton donor–acceptor group.^{11,12} This was an insightful and attractive proposal; however, it lacked experimental support. A number of subsequent studies involving computational work examined different scenarios, including propanedithiol, dithiomethylamine, and dithiomethyl ether, with mixed results.^{13,15,16} Essentially amine and ether functionalities at this position gave comparable agreement with the crystal structures, but the amine was more attractive as a potential proton donor and/or acceptor. Spectroscopic work did ultimately provide evidence of the presence of a nitrogen at this position.^{17,18} Although the composition has still not been determined by direct analysis of the enzyme matured *in vivo*, reconstitution studies involving hydrogenase activation with 2Fe subcluster synthetic mimics have provided strong evidence that the ligand is dithiomethylamine,^{19,20} removing ambiguities about the composition and architecture of the H-cluster (Figure 1).

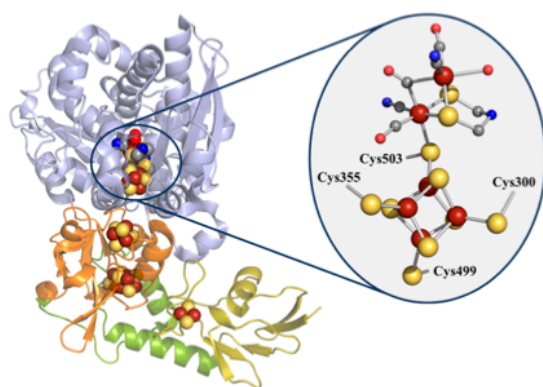


Figure 1. X-ray crystal structure of *Clostridium pasteurianum* I (CpI) [FeFe]-hydrogenase (PDB entry 3C8Y). CpI HydA (left) is composed of four domains. The catalytic domain containing the active site H-cluster (magnification, right) is colored light blue. Accessory iron–sulfur cluster domains are colored orange, green, and olive. Protein-bound iron–sulfur clusters are depicted as spheres. Color scheme: Fe, red; S, yellow; C, gray; N, blue; O, red.

Although active site diatomic ligands are common features of the hydrogenases, [NiFe]- and [FeFe]-hydrogenases are not evolutionarily related.^{21–24} [FeFe]-hydrogenases are found in several lower eukaryotes (algae and protists), and a wide range of bacteria but not cyanobacteria or archaea, whereas the [NiFe]-hydrogenases are present in cyanobacteria, archaea, and bacteria, but not in eukaryotes.^{4,24} The protein most closely related to [FeFe]-hydrogenases is Narf or Nar1, a protein found only in eukaryotes that has a role in cytoplasmic iron–sulfur cluster biosynthesis and repair.²⁵ [NiFe]-hydrogenases are most closely related to subunits of Nuo or respiratory complex I.²⁶ Given the unique nature of the diatomic ligands, one might characterize hydrogenases as one of the most profound cases of convergent evolution.²⁷

Further support for the convergent evolution of hydrogenases is the observation that [NiFe]- and [FeFe]-hydrogenase diatomic ligands are generated by completely different biochemical mechanisms. [NiFe]-hydrogenase maturation, including the synthesis of the unique diatomic ligands, involves a suite of maturase enzymes encoded by *hypABCDEFG*.²¹ The

maturation scheme proposed by Watanabe et al. provides a working model of how the six maturase enzymes operate.²⁸ HypE and HypF interact stoichiometrically with a K_D of 400 nM in their combined role of cyanide synthesis from carbamoyl phosphate and subsequent transfer to an Fe moiety likely bound to HypCD.^{29–31} HypD serves as a scaffold for the synthesis of the Fe–(CN)₂CO unit.³² A recent spectroscopic investigation revealed that HypC bound CO₂, implicating this enzyme in the synthesis of the carbon monoxide ligand.³³ HypA (and its homologues) with HypB are required for insertion of the Ni ion into the immature hydrogenase protein *in vivo*, which is likely to follow the transfer of the decorated Fe atom to the immature hydrogenase.^{28,34} [FeFe]-hydrogenase maturation requires the involvement of only three maturation proteins. Unlike the system employed for the production of the [NiFe]-hydrogenase active site, the critical steps during H-cluster biosynthesis involve radical S-adenosyl-L-methionine (SAM)-based chemical transformations. Radical SAM enzymes reductively cleave SAM into methionine and a 5'-deoxyadenosyl radical species (dAdo•) in a process coupled to abstraction of a H atom from substrate.³⁵ This Current Topic focuses on the experimental results in the [FeFe]-hydrogenase system and progress toward a pathway for the biological assembly of the H-cluster.

■ IDENTIFICATION OF THE [FEFE]-HYDROGENASE MATURATION MACHINERY

Early efforts to heterologously overexpress HydA in *Escherichia coli* were plagued by the production of an inactive enzyme,^{36,37} demonstrating the inability of *E. coli* to biosynthesize the H-cluster without specific maturases. It was not until 2004 that three hydrogenase accessory genes denoted *hydE*, *hydF*, and *hydG* were identified that when coexpressed with *hydA* could support the heterologous expression of active hydrogenase (Figure 2A).³⁸ It was further shown that hydrogenase activity was not observed in strains expressing HydA that lacked either *hydE*, *hydF*, or *hydG* (Figure 2C).³⁹ Importantly, the inactive enzyme obtained through expression of HydA alone in *E. coli* (HydA^{ΔEFG}) could be activated by the *in vitro* addition of an *E. coli* lysate containing HydE, HydF, and HydG coexpressed together (Figure 2B); this result demonstrated that H-cluster assembly was achieved through the actions of these three enzymes with ubiquitous substrate(s) present in the cellular lysate.²² Hydrogenase activity was, however, not observed when lysate mixtures of the maturases expressed either singly or in varying combinations in independent cell lines were added to HydA^{ΔEFG}.²² Because the substrates for H-cluster ligand synthesis were unknown at this time and not added to the *in vitro* activation assay, the results were interpreted to indicate that a preformed cluster intermediate was synthesized by HydE, HydF, and HydG that could be transferred to HydA to effect activation.

Sequence annotation of the maturase enzymes identified HydE and HydG as belonging to the radical SAM superfamily of enzymes because of the canonical CX₃CX₂C motif.^{38–41} HydF, on the other hand, contains the N-terminal Walker A P-loop and Walker B Mg²⁺ binding motifs associated with small Ras GTPases, as well as several putative C-terminal metal binding residues that indicate this enzyme binds iron–sulfur (FeS) clusters. The work of King et al. demonstrated that the CX₃CX₂C motifs in HydE and HydG, and the FeS cluster and GTP binding regions of HydF, were all essential to [FeFe]-hydrogenase activation.³⁹ These findings led us to develop a

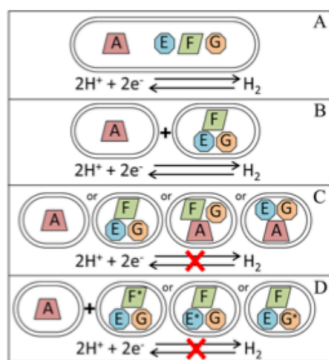


Figure 2. Whole cell extract hydrogenase activation results for the *Clostridium acetobutylicum* maturases. (A) HydA^{ΔEFG} coexpression yields hydrogenase activity. (B) HydA expressed singly can be activated by coexpressed HydEFG. (C) No hydrogenase activity is obtained in the absence of either one maturase enzyme or all maturase enzymes. (D) HydE, HydF, or HydG variant proteins coexpressed with their wild-type partners cannot activate HydA.³⁹ The asterisk denotes variant proteins. In the case of HydF, the variants contain point mutations of residues in either the P-loop or FeS cluster binding motifs. For HydE and HydG, point mutations of the conserved CX₃CX₂C residues, as well as the residues of the accessory C-terminal cluster motif in HydG, all proved to be deleterious to hydrogenase maturation.

hypothetical pathway for H-cluster assembly in which HydE and HydG synthesized the 2Fe subcluster on the scaffold HydF through the modification of a [2Fe-2S] cluster; alkylation of the sulfide groups was proposed to occur in a first step followed by the decomposition of an amino acid radical into CO and CN⁻.⁴² Moreover, we hypothesized that HydE, HydF, and HydG were solely directed at 2Fe subcluster biosynthesis, whereas standard FeS cluster assembly machinery (either the ISC or SUF systems) manufactured the [4Fe-4S] cubane of the H-cluster.⁴²

■ HYDA EXPRESSED WITHOUT MATURASES BINDS THE [4FE-4S] CUBANE OF THE H-CLUSTER

[FeFe]-hydrogenase enzymes all contain a common active site domain that houses the H-cluster (Figure 1) but vary in the

number and arrangement of the accessory FeS clusters involved in the transfer of an electron to and from the active site during catalysis.² [FeFe]-hydrogenase from the green algae *Chlamydomonas reinhardtii* (Cr) has become the focus of many biochemical and spectroscopic studies because it lacks accessory FeS clusters, thereby simplifying characterization.^{43–50} Analysis of Cr HydA^{ΔEFG} overexpressed in *E. coli* revealed UV–visible, EPR, and Mössbauer spectroscopic features typical of [4Fe-4S]^{2+/+} clusters.⁴⁷ While neither the as-purified states nor the metal free states of the Cr HydA^{ΔEFG} enzyme were active, hydrogenase activity was observed when either the as-purified or chemically reconstituted form was incubated with an *E. coli* cell extract containing HydE, HydF, and HydG (Figure 2B). HydA^{ΔEFG} in which the [4Fe-4S] cluster had been removed by iron chelation could not be activated in the presence of the HydE/HydF/HydG extract unless the [4Fe-4S] cluster was first in place. These results demonstrated the requirement for preassembly of the [4Fe-4S] cubane component of the H-cluster, which is presumably synthesized by either the ISC or SUF systems *in vivo*, prior to activation by the hydrogenase maturation enzymes.⁴⁷ Further support for this assembly mechanism has come from NRVs and EPR studies with *Clostridium pasteurianum* 1 (CpI) HydA.⁵¹ Kuchenreuther and co-workers have reported that when ⁵⁶Fe-loaded HydA^{ΔEFG} is activated *in vitro* with ⁵⁷Fe-labeled HydE, HydF, and HydG lysates enriched with exogenous ⁵⁷Fe, the ⁵⁷Fe isotope became associated with the 2Fe subcluster and not the [4Fe-4S] cubane of the H-cluster.⁵¹ Together, these results clearly demonstrate that the hydrogenase maturation machinery is directed at the synthesis of only the 2Fe subcluster and not the entire 6Fe H-cluster.

Comparison of the X-ray crystal structure of the active site domains of Cr HydA^{ΔEFG} (PDB entry 3LX4) (Figure 3A) with that of CpI HydA provided insight into the mechanism for cluster insertion and the maturation process.⁵⁰ HydA^{ΔEFG} possesses an electropositive channel possibly providing favorable electrostatic interactions with the presumably negatively charged 2Fe subcluster fragment. Furthermore, as this channel is lined with water molecules leading to the active site, perhaps the 2Fe subcluster insertion reaction is entropically driven.² In CpI HydA, the channel is occupied by two loop regions conserved across HydA proteins that presumably rearrange upon 2Fe subcluster insertion enclosing the active site, and yielding HydA activation (Figure 3B).^{2,50,52} The

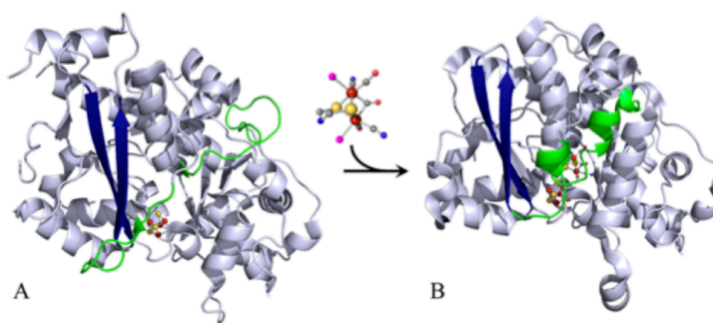


Figure 3. Insertion of the 2Fe subcluster into HydA. (A) The immature Cr HydA (PDB entry 3LX4) contains the [4Fe-4S] cubane of the H-cluster. (B) The holo CpI HydA (PDB entry 3C8Y) includes the active site H-cluster. The accessory FeS cluster domains have been omitted for the sake of clarity. The two β -strands in both structures are colored deep blue to emphasize structural similarities. The loop region that moves during 2Fe subcluster insertion is colored green. Color scheme: Fe, rust; S, yellow; C, gray; N, blue; O, red; undefined, magenta (see the legend of Figure 5).

protein surface of HydA at the channel opening contains residues Arg315, Glu320, Asp325, Lys328, Lys433, Gln437, and Lys449, which may facilitate binding interactions with HydF during the cluster insertion process.⁵⁰

■ HYDF AS A SCAFFOLD OR CARRIER FOR THE 2FE SUBCLUSTER

HydA^{ΔEFG} activation experiments demonstrate that hydrogenase activity is observed when the enzyme is mixed with an extract of *E. coli* expressing HydE, HydF, and HydG in concert (Figure 2B).^{22,47} Hydrogen evolution assays performed with HydA^{ΔEFG} and *Clostridium acetobutylicum* (*Ca*) HydE, HydF, or HydG purified proteins from an extract of *E. coli* expressing all three maturases (HydE^{EG}, HydF^{EG}, and HydG^{EG}) showed that only HydF^{EG} can support activation.⁵³ Importantly, activation occurs without the addition of any exogenous small molecules or potential precursors of nonprotein ligands, suggesting HydF^{EG} harbors an H-cluster 2Fe subcluster precursor that effects maturation of [FeFe]-hydrogenase, potentially in a manner analogous to the scaffold-directed biosynthesis of the nitrogenase FeMo cofactor.⁵⁴ The results supported a model in which HydF acts as either a scaffold or carrier protein for the H-cluster precursor moiety that is synthesized through the actions of HydE and HydG.

HydF Iron–Sulfur Cluster States. Chemically reconstituted and dithionite-reduced *Thermotoga maritima* (*Tm*) HydF expressed singly (HydF^{ΔEG}) exhibits an axial EPR signal with *g* values of 2.045 and 1.904, indicative of the presence of FeS clusters.⁵⁵ Several additional studies have now probed FeS cluster binding in heterologously and homologously expressed HydF from *Ca*, *Thermotoga neopolitana* (*Tn*), and *Shewanella oneidensis* (*So*).^{56–61} In our work, UV–visible spectra of the anaerobically purified, heterologously expressed *Ca* HydF^{EG} and HydF^{ΔEG} proteins exhibit clear differences in the ligand to metal charge transfer features of bound FeS clusters; the greater absorbance in the ~575 nm region of HydF^{ΔEG} samples was proposed to be indicative of [2Fe-2S]²⁺ clusters.⁵³ Low-temperature EPR studies of as-purified *Ca* HydF^{EG} and HydF^{ΔEG} samples revealed nearly isotropic signals centered at a *g* value of 2.00 typical of [3Fe-4S]⁺ clusters. Following photoreduction with 5-deazariboflavin, the [3Fe-4S]⁺ cluster signals in both HydF^{EG} and HydF^{ΔEG} were replaced by identical axial signals that displayed fast temperature relaxation properties assigned to [4Fe-4S]⁺ clusters with *g* values of 2.05, 1.89, and 1.86.^{56,59} Importantly, a more prominent overlapping axial signal with *g* values of 2.01, 2.00, and 1.96 was present in reduced HydF^{ΔEG} that was found to have much slower temperature relaxation properties; these characteristics together with the observations from the UV–visible spectroscopy support the assignment of this signal as originating from a [2Fe-2S]⁺ cluster.^{56,59} Similar spectra have now also been reported by Berto et al. and Albertini et al. for dithionite-reduced samples of *Tn* and *Ca* HydF^{ΔEG}; although the authors suggested that the overlapping *g* ≈ 2.0 centered signal arose from a radical in the proximity of the [4Fe-4S]⁺ cluster, the source of such a radical is neither examined nor discussed in this work.^{61,62}

In a third independent study, variable-temperature EPR data collected with dithionite-reduced *So* HydF^{ΔEG} exhibited only a strong axial signal attributed to [4Fe-4S]⁺ clusters, with no evidence of an overlapping *g* ≈ 2 centered signal;⁶⁰ these authors suggested that our photoreduced samples may have had remnant [3Fe-4S]⁺ cluster species or that the signal we have

assigned as originating from a [2Fe-2S]⁺ cluster could be the result of a protein-associated radical.⁶⁰ We find the former suggestion to be unlikely as [3Fe-4S]⁺ clusters are typically rapidly reduced even with much milder reductants; further, our preparations of *Ca* HydF continue to show a *g* ≈ 2.0 signal typical of [3Fe-4S]⁺ clusters in their as-purified state, with photoreduction resulting in the two overlapping axial signals with the distinct *g* values that we initially reported.^{56,59} Our results are also in line with the results observed for variant and wild-type forms of *Ca* and *Tn* HydF for which a similar overlapping *g* ≈ 2.0 signal has been reported.^{61,62} We have proposed that the [2Fe-2S] site could be a precursor for the 2Fe subcluster that is modified by the addition of nonprotein ligands catalyzed by HydE and HydG.^{54,56}

FTIR analysis of as-purified *Ca* HydF^{EG} revealed Fe–CO (1940 and 1881 cm^{−1}) and Fe–CN[−] (2046 and 2027 cm^{−1}) vibrations (Figure 4), whereas characterization of HydF^{ΔEG}

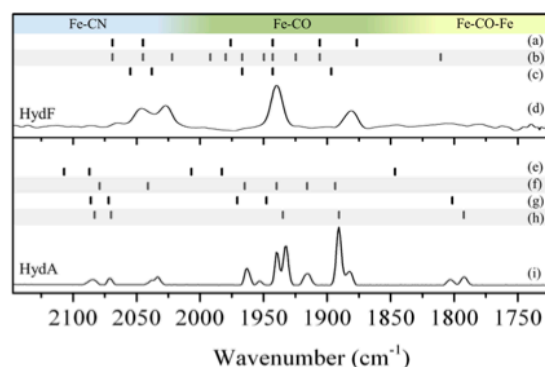


Figure 4. Summary of published HydF and HydA FTIR data. The top panel illustrates FTIR bands reported for the iron–diatomic species observed in HydF, while the bottom panel depicts the FTIR bands for the H-cluster in HydA. The HydF panel includes data from (a) nonreduced *Ca* HydF^{EG},⁵⁷ (b) reduced *Ca* HydF^{EG},⁵⁷ and (c) *Tm* HydF hybrid protein made with a biomimetic 2Fe subcluster mimic containing the dithiomethylamine bridge¹⁹ and (d) a spectrum of as-isolated *Ca* HydF^{EG}.⁵⁶ Vibrational bands for the H-cluster include data for (e) as-isolated *Dd*,¹² (f) reduced *Dd*,¹² (g) as-isolated *Cp*,⁶³ and (h) reduced *CrHydA1*⁴⁸ and (i) a spectrum of *CrHydA1* reduced with exogenous H₂.⁶⁵ For a more thorough summary of FTIR bands associated with the H-cluster, see ref 66.

demonstrated that no iron-bound diatomic species were present.⁵⁶ Czech et al. performed similar spectroscopic characterization of *Ca* HydF^{EG} that was homologously overexpressed, and their FTIR spectra showed multiple vibrational features assigned to Fe–CN[−], Fe–CO, and Fe–CO–Fe species (Figure 4).⁵⁷ Moreover, an additional high-field component in the EPR spectrum coupled to the slower relaxation of this signal was attributed by the authors to arise from “a cluster which contains three or less irons”.⁵⁷ Comparative XAS studies of active *Cr* HydA1 and *Ca* HydF enzymes and their inactive counterparts (HydA^{ΔEFG} and HydF^{ΔEG}, respectively) did provide evidence of both [4Fe-4S] and 2Fe species associated with HydF^{ΔEG} and also demonstrated that the *n*Fe–*n*S–*n*O/*N* species present on HydF^{EG} were similar to those measured for the H-cluster in active HydA.⁵⁸ Together, these data provided strong support for a 2Fe subcluster H-cluster precursor bound by HydF that

resembles the 2Fe subcluster of the [FeFe]-hydrogenase (Figures 5 and 6). Although the FTIR vibrational modes

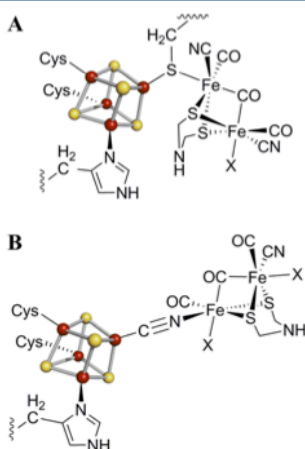


Figure 5. Hypothetical coordination environments for the 2Fe subcluster bound to *C. acetobutylicum* HydF. Cysteine and histidine ligands are presumed to be derived from both monomeric subunits, as FeS clusters are likely bound at the dimeric interface. Evidence of coordination of the histidine nitrogen to the [4Fe-4S] cubane has been provided by spectroscopic studies for *Ca* HydF (see the text). HydF proteins from *Tm* and *Tn* appear to have an exchangeable coordination site to the [4Fe-4S] cubane, and accordingly, the histidine in the representations given above could be replaced by an X for unknown. (A) Bridging cysteine thiolate coordination as proposed in ref 58. (B) Shared cyanide coordination as has been observed for 2Fe subcluster mimics bound to HydF.¹⁹ The mechanism of cluster transfer for panel B would require cyanide linkage isomerism. Cluster transfer involving the cysteine thiolate coordination in panel A would appear to involve a ligand exchange mechanism. The [4Fe-4S] cluster in both panels is depicted as a ball and stick diagram with iron ions colored rust and sulfide ions colored yellow. We have attempted to preserve the octahedral environment of the Fe ions in these species and accordingly use the X in panels A and B to represent unknown coordination of the 2Fe subcluster when bound to HydF, possibly being either protein- or solvent-derived in nature. In Figures 3, 6, and 8, we utilize magenta spheres to illustrate this concept.

observed for HydF^{EG} preparations were slightly different from those reported for the H-cluster itself (Figure 4),^{8,63–66} given the sensitivity of these modes to the electron richness of the metal and the polarity of the ligand environment, the small differences in the Fe–CN[−] and Fe–CO vibrational modes likely reflect variations in the 2Fe subcluster environment between HydA and HydF.

It was recently demonstrated that 2Fe subcluster synthetic analogues each containing four terminally bound CO ligands, two terminally bound CN[−] ligands, and either propane-dithiolate, dithiomethylamine, or dithiomethyl ether as a bridging dithiolate ligand can be loaded into chemically reconstituted *Tm* HydF and then delivered to HydA^{ΔEFG}; in the case of the dithiomethylamine-containing species, the result is maximally active [FeFe]-hydrogenase.¹⁹ Spectroscopic analysis indicated that when these cluster mimics are bound to HydF, one of the cyanide ligands forms a bridge to the [4Fe-4S] cubane (Figure 5B).¹⁹ Further studies have now demonstrated that *in vitro* activation of HydA^{ΔEFG} with the 2Fe subcluster mimics can occur in a manner that is completely

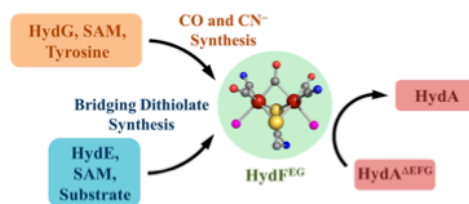


Figure 6. Hypothetical maturation scheme for H-cluster biosynthesis. HydG utilizes SAM and tyrosine to catalyze the formation of CO and CN[−] species. HydE uses SAM and an unidentified substrate to presumably synthesize the bridging dithiolate moiety. HydF likely acts as a scaffold protein during biosynthesis, and following interaction with HydE and HydG, HydF transiently binds a 2Fe subcluster precursor that resembles that of the H-cluster (see Figure 5; the [4Fe-4S] cubane bound by HydF is not shown for the sake of clarity). [FeFe]-hydrogenase maturation is then accomplished through the transfer of the 2Fe subcluster from HydF to HydA^{ΔEFG}. Three possible scenarios for the delivery of CO and CN[−] from HydG to HydF include (a) delivery of diatomics as free ligands to an alkylated [2Fe] cluster intermediate bound to HydF, (b) HydG-derived Fe–(CO)CN species displacing a placeholder [2Fe-2S] cluster bound to HydF, or (c) insertion of HydG-derived Fe–(CO)CN group(s) into a vacant cavity in the scaffold protein. In cases b and c, it is plausible that two Fe–(CO)CN groups are joined together on HydF via the delivery of the dithiomethylamine ligand. Color scheme: Fe, rust; S, yellow; C, gray; N, blue; O, red; undefined, magenta (see the legend of Figure 5).

independent of HydF.²⁰ Following the unimpeded integration of the mimic into the hydrogenase active site, free CO can be detected in assays, indicating that upon incorporation into hydrogenase the fourth CO ligand of the analogue is released into solution. While it has been proposed that this fourth CO ligand could be present in HydF during *in vivo* maturation, no experimental evidence has been reported to support this hypothesis.^{20,66}

The production of a 2Fe subcluster intermediate with four CO and two CN[−] ligands on HydF would require four distinct HydG turnover events (see Radical SAM Chemistry and Diatomic Ligand Synthesis). Although the results of the mimetic-based activation involving clusters with this diatomic ligand stoichiometry are intriguing, it might be premature to conclude that a cluster with such a composition is an intermediate in H-cluster biosynthesis.⁶⁶ The biological production of such an intermediate would be associated with the release of diatomic ligands (both CO and CN[−]) in the cell, and while this is possible, it does seem unlikely. It has been observed that purified HydF^{EG} exhibits low levels of hydrogenase activity, with <1% of the activity of the CpI HydA enzyme,⁶⁷ and it was reported that the HydF containing the dithiomethylamine-ligated 2Fe subcluster analogue did not produce any measurable hydrogenase activity,¹⁹ suggesting that possible differences exist.

Additional work involving HydA^{ΔEFG} activation experiments involving a cell free protein synthesis system reported that only reaction mixtures containing HydE and HydG or HydF and HydG resulted in low levels of hydrogenase activity, suggesting HydG was the only maturase whose presence was absolutely required. Moreover, they reported 100% hydrogenase activation could be obtained when clarified cell lysate mixtures were used that lacked HydF, although their results also showed that expression of HydA in a background of only HydE and HydG or HydF and HydG did not support the production of active HydA.⁶⁰ The latter results are in line with previous observations

that showed that all three maturases are required for heterologous expression of active hydrogenase in *E. coli*, and that even site specific amino acid substitutions of the maturases can prevent the synthesis of active [FeFe]-hydrogenase (Figure 2).^{22,39,53} Clearly, the differences between the results from the *in vitro* cell free synthesis system suggesting a nonessential role for HydF and those from *in vivo* studies supporting an essential role for HydF need to be resolved.

HydF Structure. Purified HydF proteins from both *Ca* and *Tn* heterologously overexpressed in *E. coli* exist as a mixture of dimers and tetramers, and both forms appear to coordinate FeS clusters given their visible absorbance spectra.^{56,59,68} Structural studies suggested that the purified dimer species is converted to a metal free tetramer form during the crystallization process, stabilized by an interdimer disulfide bond (Figure 7).⁶⁸ Each subunit can be divided into GTPase, FeS cluster binding, and dimerization domains.⁶⁸

Although the metal free HydF structure does not provide any direct insights into FeS composition or ligation, the putative cluster binding residues in the CXHX_{46–53}HCXXC motif from two subunits are observed to approach one another at the dimer interface in the tetrameric structure (Figure 7). This localization of two sets of putative cluster ligands could explain

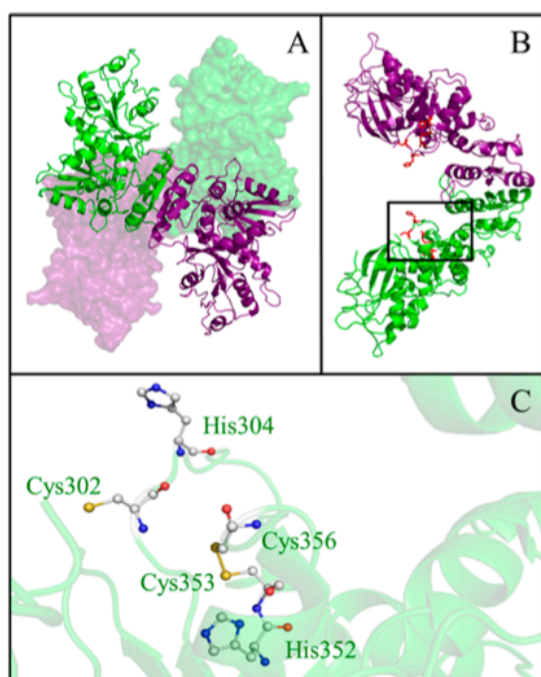


Figure 7. HydF structure. (A) Tetramer. The tetramer is a dimer of homodimers; here one dimer is a cartoon representation (front), and one dimer is a surface representation (back). Within the dimer, one monomer is green and the other purple. (B) Dimer. This image demonstrates the alignment of the FeS domains in the subunits of the dimer and the exposure of the GTPase domains at the far ends of the dimer. One monomer is colored green and one purple, and the cluster binding residues are colored red. (C) This expanded view of the box within panel B illustrates the putative residues associated with cluster binding. The residue numbers are for *Tn* HydF (PDB entry 3QQ5). Color scheme: S, yellow; C, gray; N, blue; O, red.

how the limited number of Cys and His ligands could coordinate multiple clusters and/or cluster types during 2Fe subcluster biosynthesis. Ligands are also located in loop regions in a cleft that presumably would allow for substantial degrees of freedom for cluster rearrangement. The dimer observed in solution is likely to exist with both the GTP binding environment and FeS cluster(s) site exposed to solvent, potentially providing a mechanism for facilitating the transfer of the 2Fe subcluster to HydA^{ΔEFG}. We propose that dimeric HydF is the form involved in activation of HydA (Figure 8).

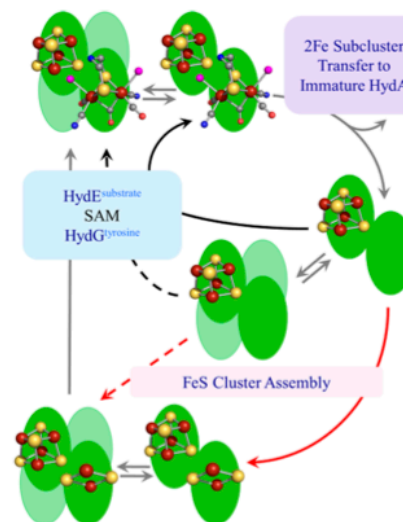


Figure 8. Hypothetical scheme detailing the proposed roles of the different oligomeric forms of HydF in H-cluster biosynthesis. Four pathways are depicted in this representation of putative cluster assembly on HydF. Two pathways involve assembly of the [2Fe-2S] cluster precursor on HydF by the endogenous iron-sulfur cluster machinery (ISC/SUF) of the cell (red pathways). Two pathways (colored black) rely on the delivery of an Fe-(CO)₂CN species (see Figure 6) from HydG to HydF as precursor units of the full 2Fe subcluster. In either of these two pathways, either the HydF dimer (—) or the HydF tetramer (---) could interact with HydE and HydG. An additional possibility that is not depicted would involve formation of the decorated 2Fe subcluster on the HydF dimer from a standard [2Fe-2S] cluster, in a pathway that does not involve tetramer formation prior to the interaction with HydE and HydG. Gray arrows indicate the overlap of two or more pathways. The number of clusters per subunit is unknown, and we are presenting only one possibility associated with the interface between subunits within a single dimer. Color scheme: Fe, rust; S, yellow; C, gray; N, blue; O, red; unknown, magenta (see the legend of Figure 5); HydF dimer, green or light green.

HydF Amino Acid Substitution Studies. HydF contains several putative FeS cluster binding ligands in its C-terminal region, including Cys304, His306, His352, Cys353, and Cys356 (*Ca* numbering) (Figure 7). Substitutions of either Cys304 or Cys356 had significant effects on FeS cluster integrity, whereas substitutions of either Cys353 or His306 resulted in only moderately deleterious effects on FeS cluster binding.⁵⁹ HYSCORE spectroscopy has suggested the coordination of nitrogen to the [4Fe-4S] cluster of wild-type *Ca* HydF,⁵⁷ and site-directed mutagenic studies have provided additional insight into FeS cluster ligation environments. Costantini and co-

workers have examined variants having either His306 or His352 substituted with Ala using both EPR and HYSCORE spectroscopic techniques and provided evidence that His352 is the fourth ligand to the [4Fe-4S] cluster in the *Ca* protein (Figure 5).⁶¹ When HydF was expressed together with HydE and HydG, all substitutions except those at position His306 yielded HydF protein that was unable to activate HydA^{ΔEFG}, although the His306 variants exhibited significantly reduced activity.⁵⁹ Further work with amino acid-substituted variants of *Tn* HydF is in line with the results summarized above for *Ca* HydF, namely, that the three Cys residues are required for [4Fe-4S] cluster coordination.^{61,62} The structural, biochemical, and spectroscopic data together suggest a model in which FeS cluster coordination occurs at the dimer interface. Interestingly, no evidence of His-based nitrogen coordination was obtained in the HYSCORE spectra of either *Tm*^{55,69} or *Tn* HydF,⁶¹ and recent characterization of *Tn* HydF suggests that solvent-derived oxygen may be a ligand, leading to the conclusion that the fourth coordination site might be occupied by a labile or exchangeable ligand.⁶² Further, results with *Tm* HydF show that both exogenous imidazole and His from the protein affinity tag can coordinate the [4Fe-4S] cluster in this enzyme, in line with the overall plasticity of cluster coordination.⁶⁹ Although significant effects on cluster coordination were not observed with substitution of His306 or His352, these residues were shown to be important for activation of HydA^{ΔEFG}, and it is possible that these residues may stabilize the 2Fe subcluster species through hydrogen bonding interactions.

GTPase Functionality and Maturase Interactions.

Initial annotation of the amino acid sequences of HydF revealed the presence of a canonical Walker A nucleotide binding motif, and GTP hydrolysis activity was subsequently shown.^{55,56} The importance of HydF GTPase activity in hydrogenase maturation was demonstrated through site-directed mutagenic studies: HydF variants in which conserved *Ca* Walker P-loop motif residues Gly19, Gly24, and Ser26 were substituted with Ala were shown to be incapable of supporting the heterologous expression of active [FeFe]-hydrogenase in *E. coli* (Figure 2D).³⁹

HydF GTP hydrolysis rates vary by as much as 40-fold in the presence of different monovalent cations, thus identifying HydF as belonging to a subclass of GTPase enzymes in which the alkali metal ion replaces the function of the "Arg finger" of GTPase activating proteins.⁵⁶ Additionally, both metal free and chemically reconstituted forms of *Ca* HydF displayed similar k_{cat} values for GTP hydrolysis, relative to that of the as-purified enzyme; this suggests that the GTPase active site and the FeS cluster site(s) are distinct from one another.^{56,68}

Interestingly, GTP was neither required nor provided stimulation for *in vitro* activation of HydA^{ΔEFG} by HydF^{EG}, indicating that GTP binding is not required for the 2Fe subcluster transfer and insertion step of maturation.⁵⁶ Because GTP binding or hydrolysis was not directly linked to the transfer of the 2Fe subcluster to HydA^{ΔEFG}, it was hypothesized that it may be involved in prior steps during maturation involving HydE and/or HydG.⁵⁶ Assays in which HydF was incubated in the presence of either HydE or HydG revealed that GTP hydrolysis rates were stimulated by ~1.5-fold relative to that with HydF alone.⁵⁶ Additional evidence of protein–protein interactions between HydF and HydE and HydG has been reported in purification trials of the maturase proteins from coexpression cell lines.³³ In addition, surface plasmon resonance studies indicate that HydE binds to HydF with a K_D

(9.19×10^{-8} M), which is an order of magnitude lower than that for binding of HydG to HydF (1.31×10^{-6} M).⁷⁰ Competition experiments indicate that HydF can interact with only a single radical SAM enzyme at a time (Figure 6), and interactions appear to occur in a manner independent of the GTPase functionality, although doping GTP over either HydE–HydF or HydG–HydF complexes resulted in increased dissociation rates.⁷⁰

RADICAL SAM CHEMISTRY AND DIATOMIC LIGAND SYNTHESIS

Tyrosine as the Source of Carbon Monoxide and Cyanide. Among the members of the radical SAM enzyme superfamily, HydG is most closely related to ThiH, which catalyzes the degradation of Tyr into *p*-cresol and dehydroglycine (DHG), the latter of which is utilized during thiamine biosynthesis.^{71–73} In addition to the radical SAM CX₃CX₂C motif located in the N-terminal domain that is conserved in ThiH, HydG contains an accessory CX₂CX₂C motif within an ~90-amino acid C-terminal domain (Figure 9).³⁸ Importantly,

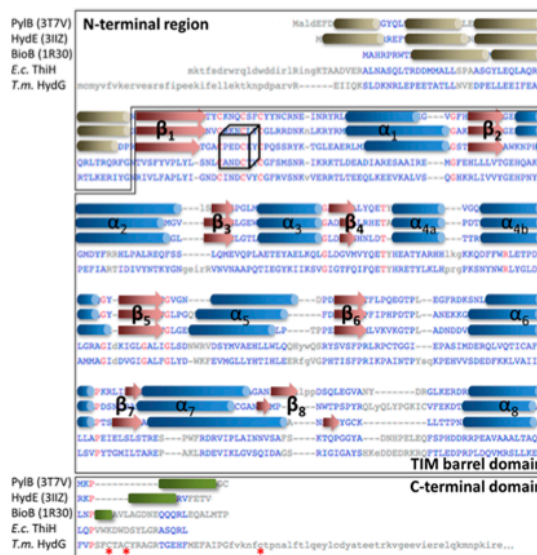


Figure 9. Sequence- and structure-based alignment of radical SAM enzymes with (βα)₈ TIM barrels. Residues colored red are strictly conserved. Cylinders represent α-helices (blue within the TIM barrel and brown outside). Red arrows are β-strands of the TIM barrel. The location of the canonical CX₃CX₂C motif is signified by a cube. Green bars within boxed C-terminal regions represent residues that may cover the bottom of the barrel. Red asterisks denote cysteine residues that bind the C-terminal [4Fe-4S] cluster.

Cys to Ser substitutions in both the radical SAM and C-terminal domain motifs of HydG had significant impacts on the heterologous expression of active [FeFe]-hydrogenase in *E. coli* (Figure 2D).³⁹

Given the close relationship between HydG and ThiH, Tyr was screened as a potential substrate for HydG and shown to increase rates of SAM cleavage and produce *p*-cresol as a reaction product.⁷⁴ Additionally, exogenously added Tyr was shown to enhance *in vitro* HydA^{ΔEFG} maturation, whereas no stimulation was observed with other Tyr analogues that lacked

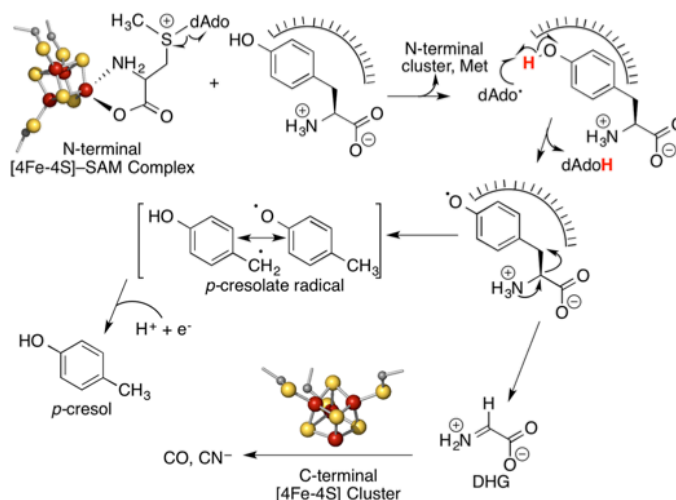


Figure 10. Hypothetical scheme detailing HydG's tyrosine lyase activity in the production of the diatomic ligands. Tyrosine is proposed to bind near the SAM-bound N-terminal [4Fe-4S] cluster. Following the reductive cleavage of SAM and the generation of dAdo•, the initial events of heterolytic tyrosine cleavage, including formation of the *p*-cresolate radical, presumably occur within the core TIM barrel structure. The product dehydroglycine is then converted into CO and CN⁻ by components of the C-terminal domain and/or accessory FeS cluster. Although the mechanism of decomposition of dehydroglycine into CO and CN⁻ is currently unresolved, it may occur through direct coordination to the C-terminal FeS cluster prior to breaking down into a [4Fe-4S]–cyano/carbonyl complex; this possibility is in line with results that provide evidence of coordination of a tyrosine-derived fragment to the C-terminal [4Fe-4S] cluster.^{83,88} Color scheme: Fe, rust; S, yellow.

a *p*-hydroxyl group.⁷⁵ Collectively, the results support a mechanism in which HydG catalyzes the reductive cleavage of SAM to generate the 5'-deoxyadenosyl radical, which then abstracts an H atom from the phenolic position of tyrosine, in a manner similar to that proposed to occur in ThiH (Figure 10).^{76,77} Such a mechanism readily accounts for the formation of *p*-cresol, but the inability to detect other HydG-based reaction products led to the proposal that HydG might be involved in the production of the dithiomethylamine ligand.⁷⁴

Ultimately, turnover experiments performed with chemically reconstituted HydG in the presence of tyrosine revealed the formation of cyanide, which was trapped as the fluorescent 1-cyanobenz[*f*]isoidole (CBI) adduct and quantified using liquid chromatography–mass spectrometry techniques.⁷⁸ The cyanide adduct was concomitantly generated along with 5'-deoxyadenosine and *p*-cresol in near 1:1:1 stoichiometric quantities; assays conducted with uniformly labeled [U-¹³C,¹⁵N]tyrosine gave rise to a CBI compound with a mass increase of 2 *m/z* units, signifying incorporation of the [¹⁵N]amino and [¹³C]- α -carbon groups of tyrosine. Subsequent turnover experiments performed with HydG and tyrosine in the presence of deoxyhemoglobin (deoxyHb; $\lambda_{\text{max}} = 430$ nm) revealed the isosbestic formation of carboxyhemoglobin (HbCO; $\lambda_{\text{max}} = 419$ nm) that initially occurred via burst phase kinetics.⁷⁹ Assays conducted in the presence of [U-¹³C,¹⁵N]tyrosine led to the detection of an FTIR vibrational feature at 1907 cm⁻¹ characteristic of Hb¹³CO, thereby validating the HydG-catalyzed synthesis of CO from tyrosine. Rate constants for the generation of CBI ($k_{\text{cat}} = 20 \times 10^{-4}$ s⁻¹ at 37 °C) and HbCO ($k_{\text{cat}} = 11.4 \times 10^{-4}$ s⁻¹ at 30 °C) species were quite comparable and implied that the diatomics originated from the same intermediate molecule.^{78,79}

The detection and quantification of the production of CN⁻ and CO by HydG were important discoveries but still left unanswered questions regarding the catalytic mechanism. For

example, the amount of HbCO quantitated in assays is substoichiometric to the levels of 5'-deoxyadenosine and *p*-cresol that are formed over the same time course.⁷⁹ In addition, the presence of three CO and two CN⁻ groups in the H-cluster raises the possibility that the third equivalent of CO could potentially be derived from an alternative substrate because the degradation of Tyr would yield *p*-cresol, CO, and CN⁻ in expected 1:1:1 ratios (Figure 10). However, these issues were resolved in a series of HydA^{ΔEFG} maturation experiments using stable isotopically labeled Tyr species, confirming that all five H-cluster diatomic ligands are derived from tyrosine.⁶⁷

Iron–Sulfur Cluster States and Diatomic Ligand Biosynthesis. As mentioned in the previous sections, both FeS cluster motifs present in the HydG sequence are critical for active [FeFe]-hydrogenase expression,³⁹ and formation of CN⁻ and CO *in vitro* required the chemical reconstitution of as-purified HydG with Fe²⁺ and S²⁻.^{78,79} Temperature-dependent EPR analysis of the photoreduced, as-purified *Ca* HydG showed both fast and slow relaxing signals, and these were attributed to a mixture of [4Fe-4S]⁺ and [2Fe-2S]⁺ clusters.⁷⁹ The chemically reconstituted form of HydG, on the other hand, clearly contained only fast relaxing [4Fe-4S]⁺ cluster signals.⁷⁹ On the basis of the presence of both N- and C-terminal three-cysteine motifs in HydG, the spectroscopic data suggested that HydG coordinated two site-differentiated [4Fe-4S] clusters in its active form. Spectral simulations substantiated this proposal by showing that two distinct axial signals were present in the photoreduced enzyme state when SAM was present; it was proposed that SAM bound the N-terminal radical SAM cluster, but not the C-terminal cluster.⁷⁹

Fontecilla-Camps and co-workers were the first to explore the role of the C-terminal [4Fe-4S] cluster via site-directed mutagenesis studies.^{80,81} In their initial work, Nicolet et al. produced two variant *Ca* HydG proteins, one in which C-terminal cluster ligands Cys386 and Cys389 were substituted

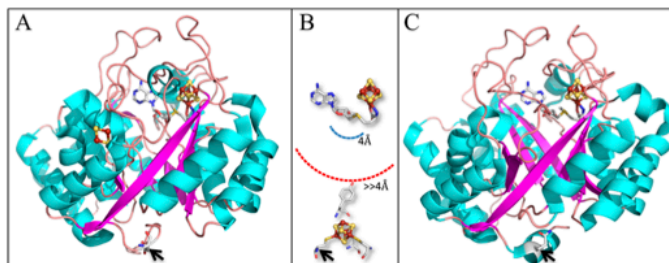


Figure 11. HydG structure-based template. (A) *Tm* HydE (PDB entry 3IIZ). (B) HydG cluster locations based on a homology model with respect to HydE and PylB crystal structures. The black arrows point to the location of the Ca residues homologous to Cys386 (Ca numbering) in the $CX_2CX_{22}C$ cluster binding motif in the C-terminal domain of HydG. Tyrosine is modeled to scale at the hypothesized location at the C-terminal cluster. This presumably places the phenolic oxygen at a distance greater (red dashed line) than the average (blue dashed line) for H atom abstraction events within radical SAM enzymes (4.0 ± 0.4 Å). (C) *Mb* PylB (PDB entry 3T7V).

with Ser and the other in which the entire C-terminal FeS cluster binding domain was deleted (Δ CTD).⁸⁰ The Cys386- and Cys389-substituted HydG did not generate any CO but was able to produce significant amounts of CN^- , yielding ~50% of the amount measured in the native enzyme. HydG Δ CTD did not produce any measurable CO or CN^- but did demonstrate low levels of tyrosine lyase activity (2% of that of the wild type enzyme).^{80,81}

We have recently provided new insights into the roles of the two $[4Fe-4S]$ clusters in *Ca* HydG in a comparative study in which the spectroscopic and kinetic properties of C-terminal variants were analyzed together with variants that had substitutions in the N-terminal or radical SAM cluster ligands.⁸² EPR spectroscopy demonstrated the presence of $[4Fe-4S]^+$ clusters in both the Δ CTD and radical SAM cluster variants upon photoreduction. Simulations of the EPR signatures for these two variants indicated these clusters displayed g values similar to what was observed in native HydG.⁷⁹ Moreover, no signal perturbations were observed with the addition of SAM to the radical SAM cluster variant, whereas addition of SAM to the Δ CTD variant results in a mixture of SAM-bound (73% of signal contributions) and unbound cluster states. These results suggest the C-terminal cluster cannot substitute for the N-terminal cluster with regard to SAM binding even though both clusters are site-differentiated. Collectively, the data allowed us to confidently assign g values for the N-terminal cluster in both SAM-bound and unbound states, as well as the C-terminal cluster, and these g value designations were used to accurately simulate the spectral signals of native HydG in the absence and presence of SAM.⁸²

Recently, EPR spectral characterization of native *So* HydG revealed the presence of both $S = 1/2$ $[4Fe-4S]^+$ signals in the $g \sim 2$ region along with low-field signals, with the latter interpreted as arising from a high-spin FeS cluster (possibly linear $[3Fe-4S]^+$ in nature) residing in the C-terminal accessory site. In this work, it was reported that addition of tyrosine to the reduced enzyme resulted in a large decrease in the signal intensity of the high-spin FeS cluster that was concomitant with the appearance of a small signal in the $g \sim 2$ region.⁸³ The large decrease in signal intensity was suggested to occur as a result of tyrosine-initiated cannibalization, resulting in the formation of the C-terminal $[4Fe-4S]^+$ cluster to which tyrosine was proposed to coordinate in N/O bidentate fashion.⁸³ We have not observed similar results in our work, and we do not see any evidence of the coordination of tyrosine to the accessory cluster in HydG in experiments performed on native and N-terminal

variant HydG proteins. In both cases, only very subtle spectral perturbations were observed in the presence of tyrosine, indicating that either tyrosine does not coordinate the C-terminal cluster or its coordination does not result in the significant alteration of g values.⁸² Previous spectroscopic studies of ThiH prepared in the presence of tyrosine either with or without SAM also do not show significant perturbations in FeS cluster EPR spectra.⁸⁴

To gain further insight into tyrosine binding, we measured $K_{M\text{ Tyr}}$ values for the native protein, a cysteine to serine variant (Cys386Ser), and Δ CTD HydG; $K_{M\text{ Tyr}}$ values increased from 0.3 mM in the native enzyme to 1.6 mM in Cys386Ser to 10.6 mM in Δ CTD, while $K_{M\text{ SAM}}$ values were roughly equivalent among these three forms.⁸² Importantly, similar p -cresol formation rates for Δ CTD ($3.0 \times 10^{-3} \text{ s}^{-1}$), Cys386Ser ($4.3 \times 10^{-3} \text{ s}^{-1}$), and native ($4.4 \times 10^{-3} \text{ s}^{-1}$) enzymes coupled with substantial amounts of p -cresol formation (representing multiple turnover events within a 60 min assay period) were observed in assays. Thus, despite an apparent loss of binding affinity, these results indicate that catalytic steps can be rescued at higher substrate concentrations and the rates of radical formation and tyrosine cleavage are largely unaffected by mild to extreme alterations to the C-terminal domain and to its FeS cluster binding residues.⁸² The effects on $K_{M\text{ Tyr}}$ however, demonstrate that the C-terminal domain and its associated cluster provide important (though not essential) binding determinants for the substrate tyrosine.

While the HydG tyrosine lyase activity that yields p -cresol appears to occur within the TIM barrel, as previously suggested by Fontecilla-Camps and co-workers,^{74,81} our results and those of others indicate that the accessory C-terminal domain and its FeS cluster play a critical role in formation of the diatomic ligands.^{80,82,83} Our expanded characterization of variant HydG proteins has shown that Cys386Ser HydG generates 2.8 molar equiv of CN^- and 0 equiv of CO, while Δ CTD HydG does not generate measurable equivalents of either CO or CN^- ,⁸² results that are in line with those of Nicolet et al.⁸⁰ Importantly, the decrease in CN^- : p -cresol ratios for Δ CTD and Cys386Ser in our work could be fully ascribed to stimulated glyoxylate levels.^{78,82} Glyoxylate formation occurs through hydrolysis of DHG, the latter of which is hydrolytically unstable under *in vitro* assay conditions and is observed to form in equal amounts with p -cresol in the ThiH system.⁷³ The fact that glyoxylate is produced in the cases of Cys386Ser and Δ CTD HydG proteins points toward a heterolytic $Ca-C\beta$ bond cleavage mechanism

for tyrosine, with the subsequent formation of dehydroglycine (Figure 10).

Confirmation of the heterolytic cleavage mechanism has been provided by rapid freeze-quench EPR experiments designed to trap intermediates during catalysis.⁸³ Turnover samples showed production of an FeS-based feature, as well as a transient $g = 2$ radical signal that disappeared with longer reaction times. HYSCORE and ENDOR spectral analysis of assays performed with uniformly labeled [¹³C,¹⁵N]tyrosine revealed evidence of both ¹⁵N and ¹³C coupling interactions with the FeS-based feature, indicating that either tyrosine or a tyrosine-derived species coordinates the C-terminal cluster.⁸³ The identity of the $g = 2$ radical was probed using various tyrosine isotopologs, and the pattern of hyperfine couplings was found to be consistent with a *p*-cresolate radical that would form upon heterolytic Cα–Cβ tyrosine bond cleavage.

While the observations made from the spectroscopic and kinetic studies of HydG described above clearly provide evidence of the involvement of both [4Fe-4S] clusters in catalysis, with each cluster serving discrete roles in the mechanism, we propose that tyrosine does not bind to the C-terminal cluster of HydG as part of its mechanism. This line of reasoning originates from the body of knowledge of substrate location relative to SAM binding in other radical SAM enzymes, and the biochemical evidence that we have from studies of ThiH (Figure 11).^{73,77,84} Sequence alignments of *Tm* HydG with *Tm* HydE, *Methanosarcina barkeri* (*Mb*) PylB, and *E. coli* BioB show that all four enzymes have complete (βα)₈ TIM barrels (Figure 9).⁸⁵ The alignment also reveals that the CX₂CX₂₂C-containing domain of HydG begins immediately after the last α-helix, as part of the environment covering the bottom lateral opening of the core TIM barrel structure. This suggests that the accessory [4Fe-4S] cluster bound to HydG caps the bottom of the TIM barrel where it is poised to bind a tyrosine-derived fragment.^{74,81,85} The predicted HydG structure also supports this putative location for the C-terminal domain and cluster (Figure 11); the comparisons between the sequences of HydG and the known X-ray structures of HydE, PylB, and BioB indicate that the accessory [4Fe-4S] cluster would lie near the bottom of the TIM barrel roughly 26 Å from the N-terminal radical SAM cluster. This distance would appear to be too large to allow for abstraction of a H atom from tyrosine by the 5'-deoxyadenosyl radical generated at the radical SAM cluster (Figure 11). As a frame of reference, for known radical SAM structures in which SAM is cocrystallized along with either substrate or product molecules, distances between the 5'-C of SAM and the abstractable H atom fall within the range of 4.0 ± 0.4 Å.^{85,86} For MoaA, an enzyme that utilizes an accessory [4Fe-4S] cluster to activate its GTP substrate, the distance between the site-differentiated iron ions of the two clusters is only 17.1 Å with GTP and SAM substrates bound directly between them (PDB entry 2FB3).⁸⁷

Along these lines, we propose that tyrosine binds in the N-terminal domain, and upon heterolytic cleavage, the DHG moiety travels to the bottom of the barrel where subsequent reactions occur (Figures 10 and 11). Not only is this structural model consistent with that proposed previously,⁸¹ but it fully acquiesces with the results that ΔCTD HydG catalytically resembles ThiH.^{81,82} It is intriguing that in ThiH, one potential mechanism that has been put forth regarding the controlled synthesis of DHG from tyrosine, in a manner that precludes its hydrolysis to glyoxylate and ensures its incorporation into the thiazole carboxylate, includes it binding at the interface between

ThiH and ThiG.⁷⁷ Along these lines, we propose that the fate of DHG in HydG is similarly controlled by the presence of the accessory domain and suggest that the ¹⁵N and ¹³C coupling interactions observed by Kuchenreuther et al. represent a fragment of tyrosine, possibly DHG, bound to the C-terminal cluster.⁸³

Stopped-flow FTIR studies have provided some insight into the fate of DHG.^{83,88} Experiments performed in the presence of myoglobin show that native *So* HydG forms both CO and CN[−] within 20 s of turnover initiation.⁸³ Similar assays performed with the Cys394Ser/Cys397Ser variant form of HydG showed only formation of CN[−] at longer time points, leading the authors to conclude that the CN[−] produced was not enzymatically relevant and that the accessory cluster is essential for the on-pathway formation of both diatomic species.⁸³ Additional work revealed the formation of two different Fe-bound diatomic complexes associated with the C-terminal cluster that develop under turnover conditions.⁸⁸ The first species is assigned as complex A and is composed of an Fe–CO/CN[−]–[3Fe-4S] unit whose formation precedes that of complex B, presumably an [Fe(II)(CO₂CN)]⁺–[3Fe-4S] moiety.⁸⁸ The time-dependent decay of the *p*-cresolate radical occurs on a time scale similar to that of the formation of complex A, which is then converted to complex B following the consumption of a second molecule of tyrosine; the formation of complex B at this stage is proposed to occur concomitantly with the release of CN[−]. Intriguingly, enzyme-derived CO was exogenously detected via binding to myoglobin only at longer time periods in these experiments, leading the authors to suggest that release of free CO by HydG is off-pathway,⁸⁸ a result that is in direct conflict with the rapid, burst phase production of CO in *Ca* HydG assays.⁷⁹ The fate of the HydG-derived Fe(CO₂)CN unit has been traced by *in vitro* activation experiments performed with HydA, HydE, HydF, and ⁵⁷Fe-loaded HydG; the resulting ENDOR spectrum for HydA demonstrates that the HydG-based organometallic product ultimately is incorporated into HydA as the 2Fe subcluster.⁸⁸

The observation of the formation of Fe–diatomic species formed on HydG that are subsequently transferred to HydA⁸⁸ provides the impetus for revisiting aspects of our proposed overall mechanism of H-cluster biosynthesis and the specific role and function of HydF.^{52,54} Clearly, the presence of the Fe–diatomic species on HydG that can be tracked to the functional hydrogenase precludes modification of Fe at a [2Fe-2S] site on HydF, as we have proposed.⁵⁶ However, in our work, we have yet to observe Fe–diatomic species on HydG and readily observe the production of free diatomic ligands in solution in native HydG activity assays, with the immediate, burst phase production of CO, suggesting that these do not initially bind to iron.^{78,79,82} Moreover, the formation of Fe–diatomic species on HydG does not preclude a role for a [2Fe-2S] cluster bound to HydF, as it may act as a placeholder given the propensity the site must have to bind metal cluster intermediates, and biogenesis could proceed via replacement of Fe at this site (Figure 6). Given the range of observations from different research groups, and the ambiguity of the cluster composition and coordination of HydF in different states, it is premature to eliminate the possible functions and roles that have been proposed for HydF in H-cluster maturation (Figures 6 and 8).

There are differences observed in the characterization of both HydG and HydF that are at least intuitively contradictory. One experimental variable is the microbial sources of enzymes used by various research groups; however, there is no sound basis to

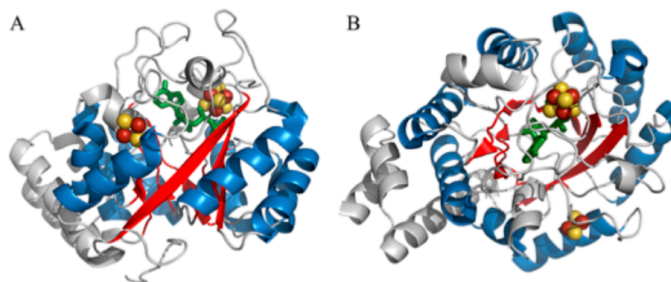


Figure 12. *Tm* HydE structure (PDB entry 3IIZ): (A) side view and (B) top view. Helices and strands in the TIM barrel are colored blue and red, respectively. SAM is colored green and shown in stick format.

anticipate that the mechanism of H-cluster biosynthesis would change as a function of microbial source. Experimental approaches also vary among the research groups working in the area; however, it is unclear how these assorted approaches would lead to the significantly different results observed. An imperative needs to be placed on resolving the reported differences, to improve our fundamental understanding of the complicated H-cluster assembly and [FeFe]-hydrogenase maturation process.

■ HYDE AND THE SYNTHESIS OF THE DITHIOMETHYLAMINE LIGAND?

An Unknown Substrate and Mechanism. The demonstration that HydG synthesizes CO and CN[−], coupled with HydF's role as a scaffold/carrier protein, suggests that HydE functions in the synthesis of the bridging dithiomethylamine ligand (Figure 6).^{52,54} As of the date of this writing, the HydE substrate and product still remain to be identified. Given that hydrogenase activation can readily be accomplished using *E. coli* cell free lysates in which *hydE*, *hydF*, and *hydG* from *Ca* are coexpressed (Figure 2), it is likely that HydE acts on a common metabolite.^{22,39,47,75} It should be noted, however, that the observation of partial *in vitro* HydA^{ΔEFG} activation in the absence of HydE and HydF has been reported and interpreted to mean that HydE plays no part in the synthesis of the dithiomethylamine ligand (and that HydF may not scaffold 2Fe subcluster assembly).⁶⁰ The alternative hypothesis put forth by Swartz and co-workers instead involved the construction of an Fe–CO/CN[−] complex resembling the 2Fe subcluster within HydG that could also include the dithiomethylamine bridge formed via a tyrosine-derived fragment in a manner reminiscent of that initially proposed by Pilet et al.^{60,74} Swartz and co-workers even invoked a yet unidentified protein in dithiomethylamine biosynthesis and relegated the role of HydE in H-cluster maturation to that of a chaperone protein, assisting in cluster translocation of the HydG cofactor among HydG, HydF, and HydA^{ΔEFG}.⁶⁰ However, given the unique and sometimes difficult chemistries catalyzed by radical SAM enzymes,³⁵ as well as the early experimental work of King and Posewitz demonstrating all three Hyd accessory proteins play essential roles in hydrogenase maturation (Figure 2),^{38,39} we strongly favor a hypothesis that invokes a critical role for HydE in H-cluster maturation related to dithiomethylamine production (Figure 6).

HydE Structure and Iron–Sulfur Cluster States. Preliminary biochemical characterization of chemically reconstituted *Tm* HydE showed that the enzyme could coordinate two S = 1/2 [4Fe-4S]⁺ clusters in the reduced state and could

also nonproductively cleave SAM at a rate of 1 mol of deoxyadenosine per mole of HydE per hour.⁴¹ For a number of years, HydE was frequently annotated as biotin synthase (BioB) because of the high degree of sequence similarity between these proteins and the presence of auxiliary FeS clusters.⁸⁹ Consequently, this led to proposals involving HydE's role in catalyzing a sulfur insertion reaction during 2Fe subcluster biosynthesis using an FeS cluster-derived sulfide akin to BioB-related chemistry,⁴² yet unlike BioB, the radical SAM and auxiliary clusters of HydE (when the latter exist) are located on opposite sides of the TIM barrel at a distance of >21 Å.⁸⁹ This distance suggests that the sulfide ions of the accessory cluster are not utilized in a BioB-like insertion into the substrate.⁹⁰ Moreover, the cluster occupancy of this site is quite variable depending on protein preparation, and the cysteines used to bind the accessory cluster (Cys311, Cys319, and Cys322) are only partially conserved in known HydE sequences. Lastly, these cysteines can be successfully mutated without any deleterious effects on hydrogenase activation, suggesting that this cluster plays no essential role in the maturation process.⁸⁹

Several crystal structures of HydE have been determined and show the complete (β_α)₈ TIM barrel fold of the enzyme (Figure 12).^{89,91,92} Intriguingly, Nicolet et al. observed a high affinity of the enzyme for methionine and 5'-deoxyadenosine products and suggested that HydE may utilize SAM as a cofactor and not a cosubstrate.⁹¹ The HydE structure revealed a large internal electropositive cavity spanning the full length of the TIM barrel that contained three distinct anion binding sites.⁸⁹ Thiocyanate bound with high affinity in the third site located at the bottom of the barrel. While it is still unknown why HydE would bind SCN[−] with such high affinity, the presence of such small anions could reflect a pathway by which the substrate reacts at the top of the barrel near the radical SAM cluster then the product migrates down to the bottom of the barrel for transfer to another protein.^{89,93} Lastly, *in silico* docking studies suggested that the putative substrate binding pocket could accommodate a small molecule containing a carboxylate group and a partial positive charge. Despite the extensive structural work done with HydE, no real insight into its role in hydrogenase maturation has been revealed.

While HydE was originally reported to have a structure significantly similar to that of BioB,⁸⁹ suggesting synthesis of the dithiolate ligand by radical SAM catalyzed sulfur insertion into an alkyl substrate, more recent sequence and structure analysis has shown that HydE shares a greater resemblance to methylornithine synthase (PylB) (Figure 11A,C).⁹⁴ PylB is a radical SAM enzyme composed of a full (β_α)₈ TIM barrel that catalyzes the isomerization of L-lysine to L-methylornithine in

the biosynthetic pathway of pyrrolysine, the 22nd naturally encoded amino acid.⁹⁴ DALI (distance alignment matrix method) analysis revealed that among all X-ray crystal structures, PylB was most similar to HydE and BioB, being 29.0 and 20.6% similar, respectively. Moreover, when PylB was superimposed with these structures, an rmsd of 1.3 Å was obtained with HydE, relative to an rmsd value of 1.8 Å with BioB. The overlay of the HydE and PylB crystal structures (PylB, PDB entry 3T7V; HydE, PDB entry 3CIW) shows comparable SAM and putative substrate binding pockets, potentially pointing to mechanistic similarities for these enzymes.⁹⁴ HydE has a much higher binding affinity for HydF than HydG does, and the two radical SAM proteins do not appear to interact with one another.⁷⁰ These data speak to the tightly controlled process that appears to exist during 2Fe subcluster biosynthesis and suggest that HydE may catalyze the first step in this reaction, delivering its product to HydF.^{42,54} Further support for this hypothesis comes from the observation that in nature HydE and HydF exist as a fused *hydEF* gene in certain organisms.⁹⁵

CONCLUSIONS

While many details of the H-cluster biosynthetic scheme still remain to be elucidated, the findings made in recent years have been imperative in clarifying many aspects of the maturation pathway and in illuminating some of the outstanding experimental questions that will undoubtedly guide future research. Some prominent research frontiers for the immediate future include resolving issues related to HydG's mechanism for the breakdown of dehydroglycine into the diatomic ligands, identifying HydE's substrate and a mechanism for incorporating the dithiolate ligand into the 2Fe subcluster, and defining HydF's role in H-cluster biosynthesis, including the nature of its function as a scaffold or carrier as well as the role of GTP binding and hydrolysis. The biochemical characterization of the steps involved in [FeFe]-hydrogenase maturation, including the description of H-cluster intermediate species, will surely continue to uncover the mysteries of nature's most prolific hydrogen catalyst.

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Notes

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ABBREVIATIONS

Ca, *C. acetobutylicum*; CBI, 1-cyanobenz[*f*]isoindole; ΔCTD, HydG mutant with a truncated C-terminal domain; *Cp*, *C. pasteurianum*; CpI, [FeFe]-hydrogenase I from *C. pasteurianum*; *Cr*, *Ch. reinhardtii*; DALI, distance alignment matrix method; dAdo*, S'-deoxyadenosyl radical; deoxyHb, deoxyhemoglobin; DHG, dehydroglycine; ENDOR, electron nuclear double-

resonance spectroscopy; EPR, electron paramagnetic resonance spectroscopy; FeS, iron-sulfur; FTIR, Fourier transform infrared spectroscopy; GTP, guanosine triphosphate; HbCO, carboxyhemoglobin; HydA, [FeFe]-hydrogenase; HydA^{ΔEFG}, HydA expressed without HydE, HydF, and HydG maturases; HydA^{EFG}, HydA coexpressed with HydE, HydF, and HydG maturases; HydF^{ΔEG}, maturase protein HydF expressed without HydE and HydG; HydF^{EFG}, maturase protein HydF coexpressed with HydE and HydG; HYSCORE, hyperfine sublevel correlation spectroscopy; ISC, iron-sulfur cluster assembly machinery; Δ*iscR*, deletion of the ISC repressor gene; NRVs, nuclear resonance vibrational spectroscopy; PDB, Protein Data Bank; rmsd, root-mean-square deviation; SAM, S-adenosyl-L-methionine; *So*, *S. oneidensis*; SUF, sulfur mobilization assembly machinery; ThiH, tyrosine lyase involved in thiamine biosynthesis; TIM barrel, triosephosphatase-like tertiary fold; *Tm*, *T. maritima*; *Tn*, *T. neopolitana*; *Tt*, *Thermoanaerobacter tengcongensis*; WT, wild type; XAS, X-ray absorption spectroscopy.

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CHAPTER 7

INVESTIGATING THE ACTIVE SITES OF HydG

Introduction

In a study of insertional *Chlamydomonas reinhardtii* mutants, three gene products, HydE, HydF, and HydG, were found necessary to mature the structural protein HydA also known as [FeFe]-hydrogenase.⁽¹⁾ Examination of HydE and HydG amino acid sequences found they both contain CX₃CX₂C motifs indicative of radical SAM enzymes.⁽¹⁾ Further biochemical and spectroscopic studies confirmed that they were radical SAM enzymes and revealed much more about HydG's role in the maturation of HydA.⁽²⁾ HydG contains two FeS clusters intimately involved in the breakdown of tyrosine into the CO and CN⁻ ligands that decorate the active site H-cluster of HydA.⁽³⁻⁵⁾ The reaction catalyzed by the HydG is thought to be initiated by electron transfer from the reduced N-terminal [4Fe-4S] cluster to *S*-adenosylmethionine to produce the highly reactive deoxyadenosyl radical intermediate. The work described in this chapter was designed to probe subsequent steps in catalysis. Our results support a mechanism in which this deoxyadenosyl radical abstracts an H-atom from the amine group of tyrosine bound near the N-terminal cluster, with this substrate radical undergoing homolytic cleavage to generate dehydroglycine and a *p*-hydroxybenzylic radical. Data is also presented that point to key residues in the HydG's active site that are required for substrate binding and catalysis. Finally, EPR spectra are presented that are interpreted as

the C-terminal cluster being a site differentiated [4Fe-4S] cluster that is likely involved in the subsequent production of CO and/or CN⁻.

Materials and Methods

Mutagenesis of Active Site Residues in *C. acetobutylicum* HydG

Several *Clostridium acetobutylicum* *hydG* mutant constructs were generated using previously prepared pCDFDuet-*hydG* constructs by two different mutagenesis methods.⁽⁶⁾ For the generation of the *C. acetobutylicum* HydG S338V and S338D variants the Agilent Technologies QuikChange Lightning Multi Site-Directed Mutagenesis Kit was employed. The manufacturer's recommendations were employed with 0.5 µL of the QuikSolution per 25 µL of reaction and the previously mentioned pCDFDuet-*hydG* template. The mutagenic primers used for the S338V and S338D were:
GTGCTAGAACCACCAACTATTTGAGATATACCGAGTTCAAGTACGC and
GTGCTAGAACCACCATCTATTTGAGATATACCGAGTTCAAGTACGC,
respectively. This technique produced single stranded plasmids that were transformed into Agilent XL-10 Gold Ultracompetent cells. The sequence verified constructs were then transformed into the overexpression *E. coli* strain BL21(DE3)-*RIL*.

The constructs for the HydG variants S338A, Q360E, and Y91F were created from the same WT construct, but by using the Gibson cloning mutagenesis technique.⁽⁷⁾ We employed this technique to join three DNA fragments with complementary ends using a three enzyme cocktail to produce a finished construct coding for the desired variant protein. The WT construct was linearized and the HydG gene was removed by

double digestion with the New England Biolabs restriction enzymes BamHI and HindIII in NEBuffer #2 according to the manufacturer's instructions. The enzymes were then permanently heat-inactivated by incubation at 80 °C for 20 min. The linearized pCDFDuet (Fragment 1) was subsequently used for all three variants prepared employing the Gibson technique. The second and third fragments for each mutant were prepared using the PCR conditions listed in Figure S2 and the two sets of relevant primers listed in Figure S1. The PCR fragments and linearized template were assembled in the triple enzyme and reagent cocktail that included a polymerase, ligase, and 5'-exonuclease (Figure S2) by incubating the reaction at 50 °C for 60 min. The assembled construct was immediately transformed into the *E. coli* strain Nova Blue chemically competent cells according to the manufacturer's directions. Constructs were sequence verified for the correct insert prior to a new transformation and overexpression, the protocols for which are as described in Chapter 2.

The two additional *C. acetobutylicum* HydG variants were obtained by utilizing previously constructed plasmids. The first was the HydG Δ CTD variant in which the C-terminal domain is truncated so that only the radical SAM [4Fe-4S] cluster is present.⁽⁴⁾ The second HydG variant, termed HydG-NTM, had C96A, C100A, and C103A mutations which effectively removed the radical SAM binding domain.⁽⁴⁾

Growth, Purification, and Reconstitution of HydG

Protein for biochemical and spectroscopic characterization was produced for each variant as described in Chapter 2. The only deviation from the protocol was the

expression of the HydG variants HydG- Δ CTD and HydG-NTM in which only half of the exogenous Fe^{3+} and S^{2-} were added during reconstitution (only a 3-fold excess over protein) given the variant proteins could bind only a single [4Fe-4S] cluster. All subsequent protein purification, reconstitution, and assays were carried out using a buffer that contained 500 mM KCl, 25 μM HEPES pH 8.0, and 5% glycerol unless otherwise described.

EPR of Rapidly Quenched HydG Turnover Samples

To capture a radical intermediate that HydG transiently produces during enzymatic turnover, samples were prepared by rapidly mixing two reaction components and flash freezing the reaction for analysis by EPR. Volumes of 125 μL of component A (200 μM HydG, 6 mM L-tyrosine, 6 mM *S*-adenosylmethionine, and buffer) and component B (6 mM sodium dithionite and buffer) were injected through separate syringes into quartz EPR tubes. Both WT HydG (6.5 Fe/protein) and HydG Δ CTD (2.4 Fe/protein) samples were made. The mixed components were allowed to incubate for 5-30 seconds after which they were flash frozen in liquid propane. Collection of EPR spectra, baseline correction, and *g*-value assignments were conducted as previously described in Chapters 4 and 5.

EPR of Cyanide Treated HydG-NTM

HydG-NTM, the variant without the three cysteine motif that binds the radical SAM [4Fe-4S] cluster, was treated with cyanide in a manner similar to that published by Roach and coworkers.⁽⁸⁾ Two 200 μL EPR samples were made with 150 μM HydG-

NTM and 10 mM sodium dithionite in the standard buffer with and without 3 mM KCN.

The samples were flash frozen in liquid nitrogen and EPR spectra were collected and analyzed in a manner described in Chapters 4 and 5.

HydG Turnover Assays and Product Quantitation

Two sets of HydG turnover assays were conducted while probing the substrate specificity and binding pocket of HydG. In the first set, L-tyrosine analogs (1 mM) including N-methyl-L-tyrosine, O-methyl-L-tyrosine, 5-fluoro-L-phenylalanine, 4-amino-L-phenylalanine, phenylalanine, and tryptophan (Figure 7.1) were incubated with *C. acetobutylicum* HydG (40 μ M), SAM (1 mM), and sodium dithionite (5 mM) in a 50 mM HEPES pH 8.0, 300 mM KCl, and 5% glycerol buffer for 30 min at 37 °C in an MBraun anaerobic chamber (≤ 1 PPM O₂). The assay samples were removed and the reaction was stopped by the addition of one part (v/v) 1 M acetate buffer pH 4.5 to nine parts (v/v) assay solution. The acidified assay mixture was cooled on ice for an hour and then centrifuged for 10 min at 4 °C to remove the precipitated protein. The small molecules in the supernatant were analyzed by HPLC as described in Chapter 5, and in addition the production of *p*-cresol was monitored by absorbance at 280 nm and comparison to a set of *p*-cresol standards.

A second set of turnover assays were conducted with the suite of HydG S338 variants. The HydG S338A/S338V/S338D variants all overexpressed well and when reconstituted contained 6.9, 7.5, and 6.7 Fe/protein, respectively. These iron-loading values were in good agreement with the WT HydG protein (6.5 Fe/protein) that was used

as a control. These mutations were selected to i) test the hypothesis proposed in a recent paper that HydG's initial H-atom abstraction occurs at the amine nitrogen of substrate tyrosine⁽⁹⁾ and ii) engineer the active site of HydG to accommodate alternate amino acids as substrates.

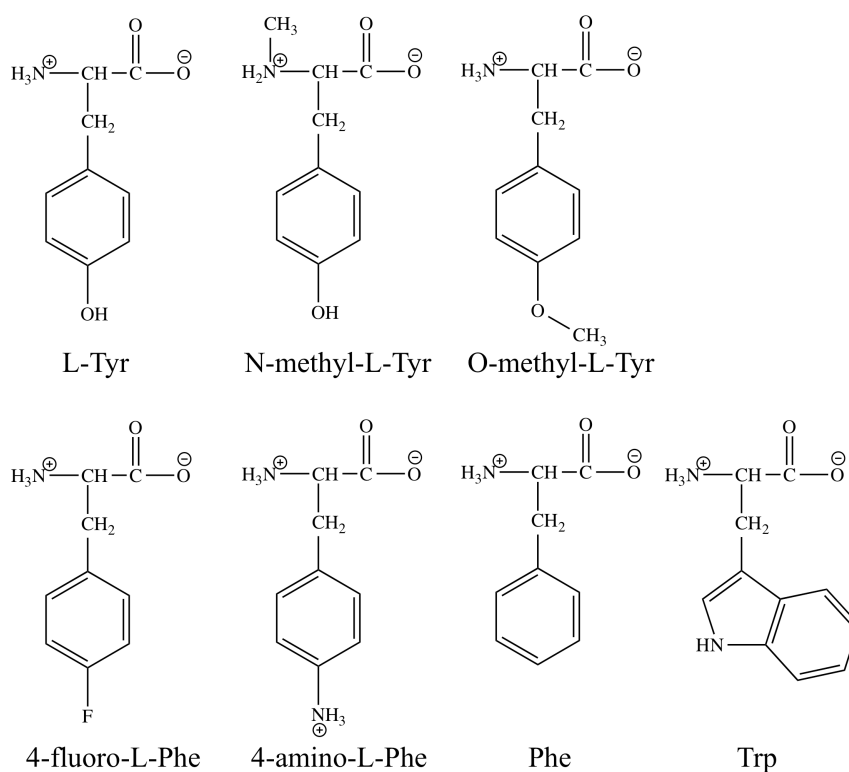


Figure 7.1. Tyrosine analogs used in HydG turnover assays.

To test the variant proteins' SAM cleavage rates, and therefore their functionality, turnover assays were conducted with 40 μ M protein, 1 mM SAM, 1 mM substrate, and 5 mM sodium dithionite in 25 mM HEPES pH 8.0, 500 mM KCl, and 5% glycerol buffer. The substrates that were used in the assays with the WT and each variant protein were L-tyrosine, L-phenylalanine, and L-histidine. After incubation at 37 °C for 2 hr, the reaction was quenched and the protein was destabilized by a 10% addition of 1 M acetate

buffer of pH 4.5. The precipitated protein was then removed by centrifugation and the supernatants were analyzed by HPLC as described above.

Results

Capturing a Radical Intermediate of the Tyrosine Lyase HydG

By using a hand-mixing and rapid-freezing technique, we were able to generate the *p*-hydroxybenzylic radical species previously reported for the *S. oneidensis* HydG⁽¹⁰⁾ in our *C. acetobutylicum* HydG. Interestingly, the EPR signal we observe for this radical intermediate is of considerably higher intensity than that initially reported by Kuchenreuther et al.⁽¹⁰⁾ By preincubation of HydG with both SAM and L-tyrosine, rather than just L-tyrosine as done by Kuchenreuther et al., a radical signal of greater intensity could be observed as early as 5 sec, which is the limit of our mixing and flash freezing technique. An EPR spectrum of this radical intermediate is shown in Figure 7.2; the radical signal is isotropic and centered at $g = 2$ while the contributions of the C-terminal cluster and the N-terminal-SAM bound cluster can also be observed.⁽⁴⁾ We have also generated this intermediate using the HydG Δ CTD variant that lacks the C-terminal domain and its associated iron-sulfur cluster. In Figure 7.2, the lower set of three EPR spectra for HydG Δ CTD under turnover conditions indicate that the kinetics of tyrosine lyase activity for the truncated variant protein may be slower than for the WT protein since the intermediate radical signal ($g = 2.0$) is of much lower intensity over all recorded time periods. The low field region of the EPR spectra for HydG shown in Figure 7.3 under turnover conditions, represented in Figure 7.3, is clearly devoid of signals other

than a minor feature at $g = 4.3$ that is due to adventitiously bound high spin ferric iron commonly referred to as “junk iron.” Britt and coworkers have observed additional spectral features around $g = 4.3$ that they have in separate papers attributed to both paramagnetic linear $[3\text{Fe-4S}]$ or $[5\text{Fe-5S}]$ clusters.^(8, 10)

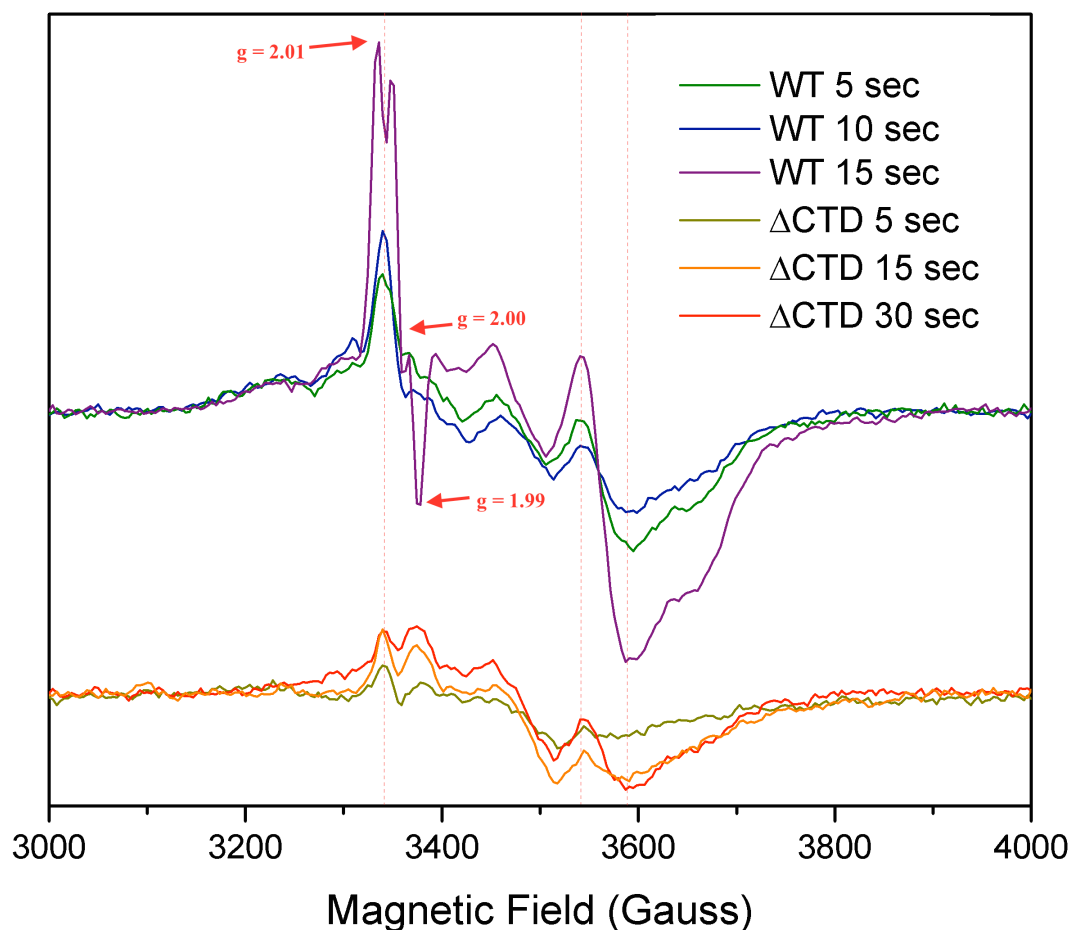


Figure 7.2. EPR spectra during turnover showing the generation of the *p*-hydroxybenzylic radical by HydG. Top three spectra: WT HydG (100 μM) turnover of tyrosine (3 mM) with SAM (3 mM) initiated by addition of sodium dithionite (3mM) and flash frozen in liquid propane at 5 sec (green), 10 sec (blue), and 15 sec (purple) after dithionite addition. Bottom three spectra: HydG ΔCTD (100 mM) turnover of tyrosine (3 mM) with SAM (3 mM) initiated by addition of sodium dithionite and flash frozen in liquid propane at 5 sec (dark yellow), 15 sec (orange), and 30 sec (red) after dithionite addition. The EPR spectra parameters were microwave frequency 9.37 GHz; temperature 14 $\pm$ 2K; power, 1 mW; time constant and conversion time, 20.48 ms; receiver gain, 1×10^4 ; modulation frequency, 100 kHz; and modulation amplitude, 10 G.

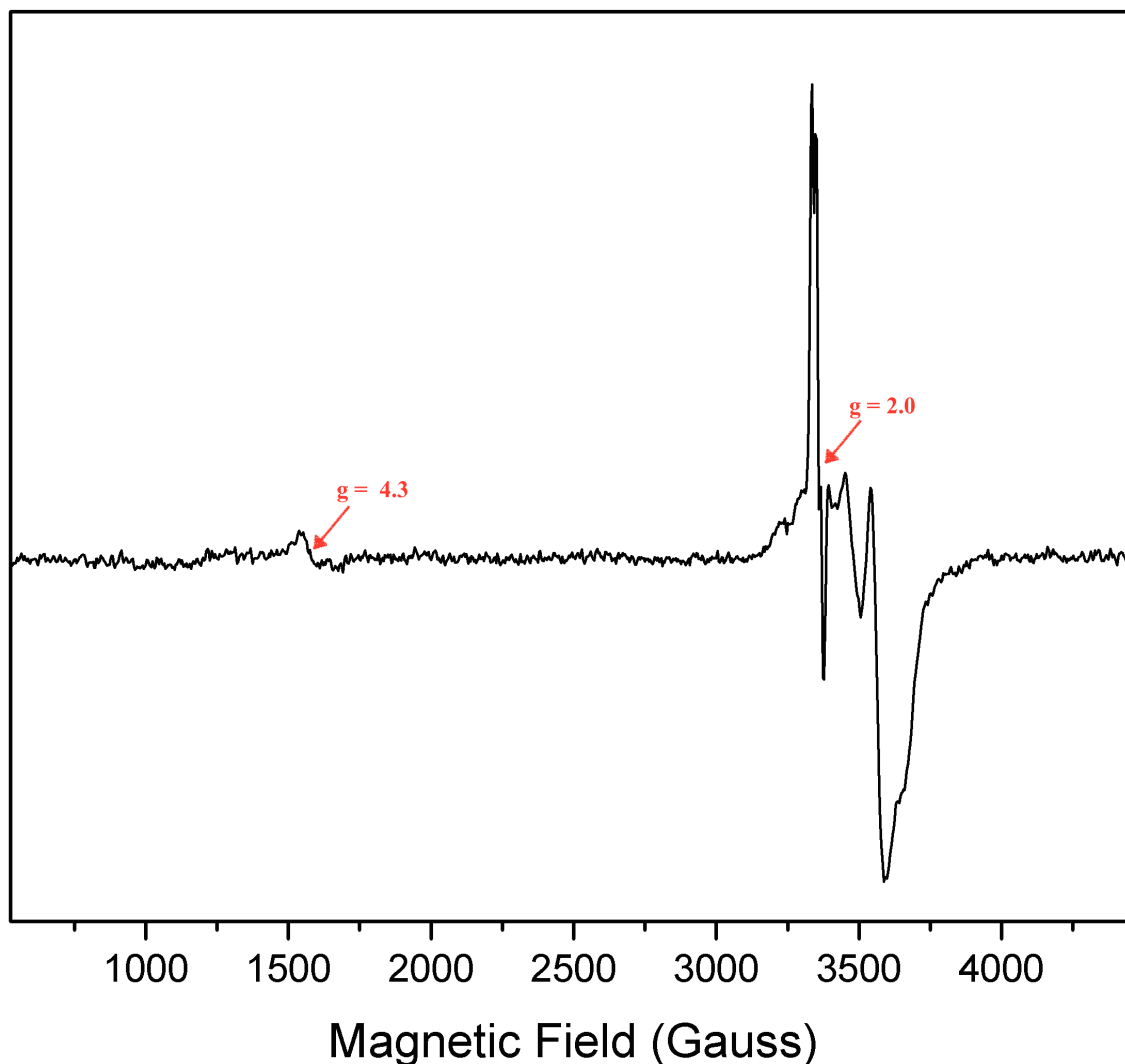


Figure 7.3. Broad field X-band EPR spectrum of HydG under turnover conditions. The $g = 4.3$ signal is from adventitiously bound high spin ferric iron. The relatively sharp feature centered at $g = 2.0$ is from the organic substrate based radical generated by HydG from tyrosine. The broad signals have previously been assigned to the $[4\text{Fe-4S}]^{1+}$ clusters of HydG.⁽⁴⁾ This spectrum is from the same sample used to generate the purple spectrum (WT 15 sec) in Figure 7.2 using EPR data collection parameters described in Figure 7.2.

Substrate Promiscuity of HydG

A recent X-ray crystal structure solution of the HydG homolog, NosL, gave new insight into the first step of the lyase activity for each of these enzymes. Previously, it

was proposed that tyrosine cleavage began by the abstraction of the phenolic hydrogen of tyrosine.⁽⁹⁾ Interestingly, in the NosL structure substrate, tryptophan, was bound with its amine moiety positioned near the 5'-C of SAM where the deoxyadenosyl radical is generated upon SAM cleavage. This strongly suggested that the amino moiety of tryptophan would be the location of H-atom abstraction. Due to the strong similarity between NosL and HydG, one could infer that the site of H-atom abstraction by HydG is also the amino group of the substrate tyrosine. In order to examine this hypothesis with HydG, the rates of enzymatic production of 5'-deoxyadenosine and *p*-cresol were measured after a 30 min incubation of WT HydG with several tyrosine analogs in turnover assays (Figures 7.1 and 7.4).

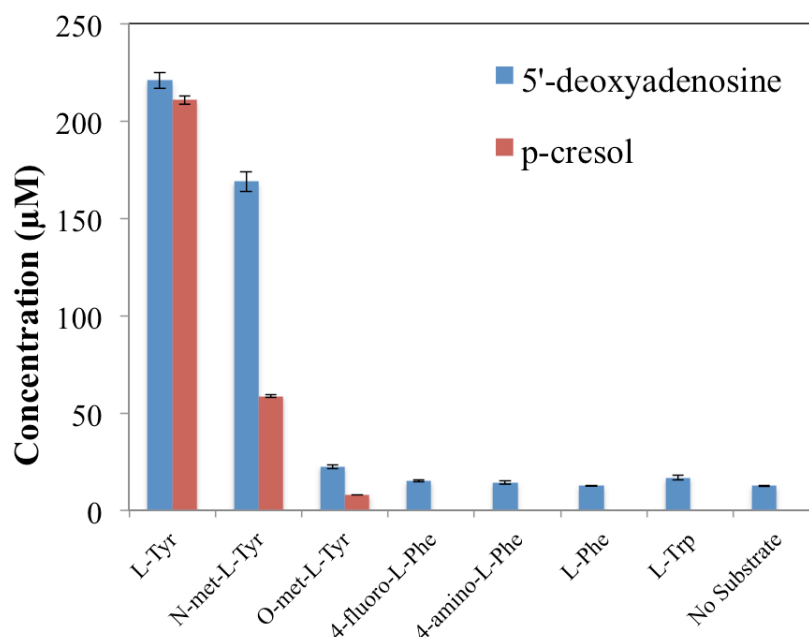


Figure 7.4. 5-Deoxyadenosine and *p*-cresol produced by HydG with substrate analogs. HydG (40 μM), SAM (1 mM), substrate analog (1 mM), and sodium dithionite (5 mM) were incubated at 37 °C for 30 min. The blue and red bars represent 5'-deoxyadenosine and *p*-cresol production, respectively. The error bars are +/- 1 standard deviation for injection replicates.

Most of these analogs retain the amino moiety but have alternate R-groups. WT HydG incubated with L-Tyr produced roughly 10 dAdo and *p*-cresol molecules per hour. When HydG was incubated with the analogs 4-fluoro-L-Phe, 4-amino-L-Phe, L-Phe, or L-Trp the production of dAdo was the same as the no-substrate controls (0.7 dAdo per hour). N-methyl-L-Tyr did stimulate dAdo production to 75% of L-Tyr but much of the SAM cleavage that occurred appeared to be uncoupled given that *p*-cresol production was only 25% that of L-Tyr. While O-methyl-L-Tyr appeared to increase SAM cleavage and produced *p*-cresol, this was later found to be due to L-Tyr contamination from the manufacturer (98% pure). Based on these results, the hydroxyl group on tyrosine appears to have a critical role in substrate specificity as well handling the radical intermediate during catalysis. If H-atom abstraction occurred with phenylalanine a benzylic radical would be produced. This radical would not have the hydrogen bond handle and would likely lead to side reactions and damage the enzyme.

While two crystal structures of HydG have recently been published, neither have been solved with substrate in the active site.^(8, 11) Given the high degree of conservation between amino acid sequences of NosL and HydG that comprise the core TIM barrels, the residues found in the active site of NosL structure could be used to determine those of HydG's active site. The residue S338 (*C. acetobutylicum* HydG numbering) that corresponds to S340 (*Streptomyces actuosus* NosL numbering) appears to be involved in hydrogen bonding to the polar moieties of the R-groups of the substrates tyrosine and tryptophan, respectively. Through mutating this residue we hoped to reduce the substrate specificity of HydG previously described. The variant proteins S338A and S338V

removed the polar moiety that is believed to hydrogen bond to the hydroxyl group of tyrosine. By creation of a relatively hydrophobic pocket it was thought that the alternate substrate phenylalanine could bind and react in a manner similar to tyrosine in WT HydG. HydG S338D was made as the replacement of the serine with a negatively charged residue, which may allow histidine to occupy the active site in place of tyrosine. Interestingly, S338A and S338V variant proteins did not exhibit even low level unproductive SAM cleavage, as no appreciable dAdo was produced (data not shown). Also it was discovered that, S338D cleaved SAM producing dAdo at the low rate observed by the WT enzyme in the absence of substrate. The addition of tyrosine did not increase the rate of SAM cleavage with any of the variants as typical for WT enzyme, indicating that this residue is paramount for substrate recognition.

EPR Spectroscopy of HydG-NTM

Roach and coworkers recently proposed the auxiliary cluster of HydG is a [5Fe-5S] cluster and suggested the fifth iron binds CO and CN⁻ ligands produced by tyrosine lyase activity of HydG.⁽⁸⁾ As evidence, they assigned a low field $g = 4.3$ centered signal to the [5Fe-5S] cluster (spectra in caption on lower left). They found that upon treatment with exogenous cyanide the $g = 4.3$ centered signal was abolished concomitant with the appearance of a $S = \frac{1}{2}$ FeS cluster signal centered at $g = 2$, which they interpreted as the substitution of the fifth iron and sulfide by cyanide. On the other hand we observe a $S = \frac{1}{2}$ FeS cluster signal centered at $g = 2$ that is changed upon the addition of exogenous cyanide but never observe the signal Roach and coworkers attributed to the [5Fe-5S] cluster. As with the turnover WT HydG spectra, the only signal in the $g = 4.3$ region is

from adventitiously bound iron and the signals reported by Roach and coworkers are not observed.

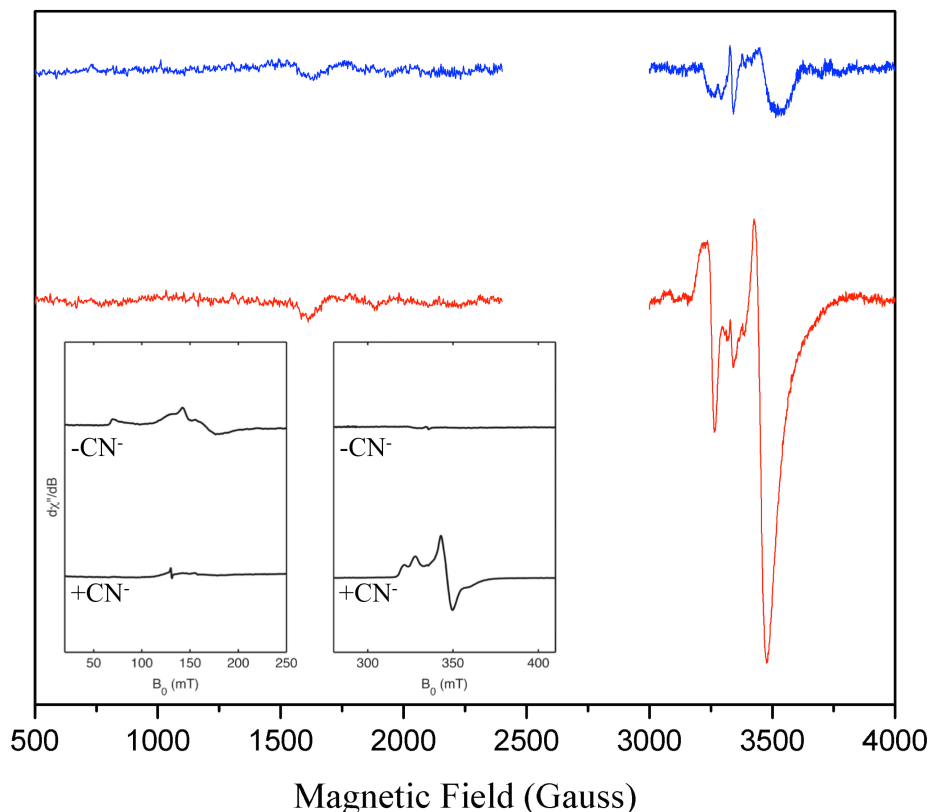


Figure 7.5. EPR spectra of *C. acetobutylicum* HydG-NTM treated with cyanide. HydG (150 μ M) reduced with sodium dithionite (10 mM)(top blue trace) and dithionite reduced with cyanide (3 mM)(bottom red trace). Roach and coworkers⁽⁸⁾ (Figure S5) published the *S. oneidensis* HydG-NTM EPR spectra shown as insets in lower left. The EPR spectra parameters were microwave frequency 9.38 GHz; temperature 14 $\pm$ 2K; power, 1 mW; time constant and conversion time, 40.96 ms; receiver gain, 1×10^4 ; modulation frequency, 100 kHz; and modulation amplitude, 10 G.

Discussion

The Tyrosine Lyase Active Site

Multiple investigations have demonstrated that HydG uses radical SAM chemistry to cleave tyrosine and produce the carbon monoxide and cyanide ligands of the

H-cluster of [FeFe]-hydrogenase.^(3, 5, 12, 13) Recently, multiple hypotheses, some conflicting, have been proposed regarding the mechanisms for tyrosine lyase activity, diatomic synthesis, and transfer of the diatomics to HydF.^(8-10, 14, 15) Britt and coworkers observed a radical signal produced by *S. oneidensis* HydG during turnover with tyrosine that they assigned to a *p*-hydroxybenzylic radical.⁽¹⁰⁾ This radical signal has been reproduced by our lab with the *C. acetobutylicum* HydG (Figures 7.2 and 7.3). Britt proposed that this radical was generated at the C-terminal cluster similar to MoaA⁽¹⁶⁾ where the substrate binds to a [4Fe-4S] cluster positioning it for H-atom abstraction by the deoxyadenosyl radical produced near the N-terminal [4Fe-4S] cluster.

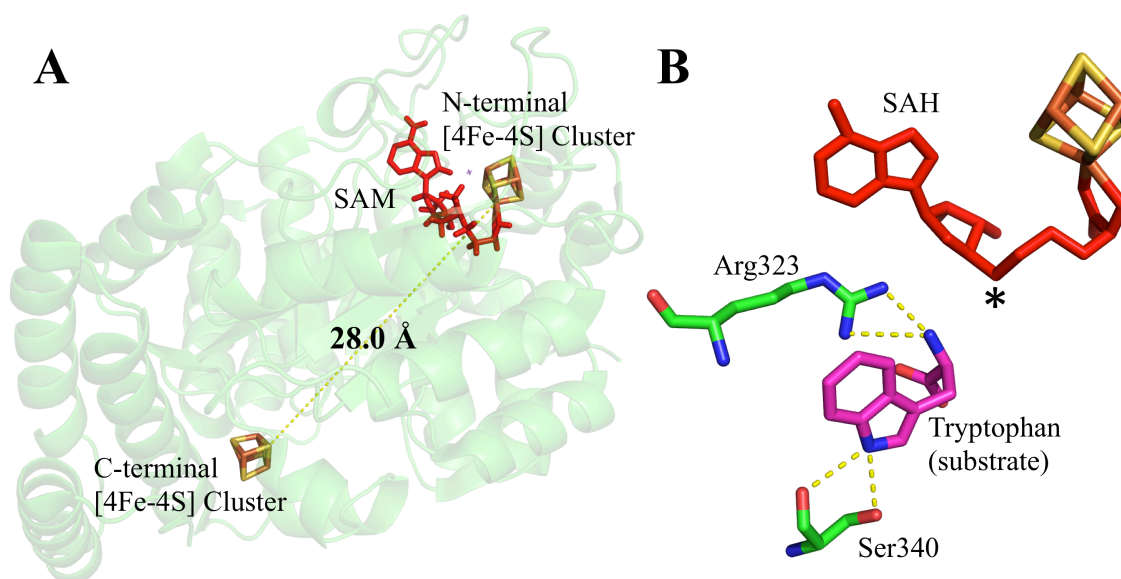


Figure 7.6. A. Crystal Structure of *Thermoanaerobacter italicus* HydG (PDB ID: 4WCX) displaying the distance of 28.0 Å between the two [4Fe-4S] clusters. B. The active site of tryptophan lyase, NosL, (PDB ID: 4R33) contains the substrate tryptophan (carbons drawn in purple). The asterisk denotes where the deoxyadenosyl radical is generated and H-atom abstraction likely occurs. SAM and S-adenosylhomocysteine (SAH) are colored red. Color scheme unless otherwise noted: carbon (green), oxygen (red), nitrogen (blue), sulfur (yellow), and iron (orange).

Given the close alignment of HydG with other radical SAM enzymes with full TIM barrels (HydE, PylB, and BioB) we predicted (prior to the publication of the HydG crystal structure) that the location of the two [4Fe-4S] clusters and the distance between them (26 Å).^(2, 17) We concluded that at this distance H-atom abstraction would not be possible if tyrosine were bound at the C-terminal cluster. The Roach lab (PDB ID: 4WCX) recently published a crystal structure solution in which the two [4Fe-4S] clusters (Figure 7.6A) are 28.0 Å apart, corroborating our conclusions drawn from the homology model investigations and refuting Britt's supposition that tyrosine coordinates the C-terminal cluster.

While neither group^(8, 11) crystallized HydG with tyrosine in the active site, significant insight into this active site and the binding of the substrate has been garnered from the structural solution (PDB ID: 4R33) of the homolog NosL (Figure 7.6B).⁽⁹⁾ The positioning of tryptophan, NosL's substrate, relative to the location that the deoxyadenosyl radical is generated strongly suggests that H-atom abstraction occurs at the amine moiety rather than at the phenolic hydrogen of tyrosine, as previously suggested.^(2, 10) To test this hypothesis a series of tyrosine analogs were screened in turnover assays for stimulation of SAM cleavage by HydG. The five analogs (Figures 7.1 and 7.4) that had the hydroxyl group removed from the phenyl group of tyrosine were unable to stimulate production of deoxyadenosine via SAM cleavage. Surprisingly, N-methyl-L-Tyr was still able to stimulate SAM cleavage albeit at a much lower rate than the natural substrate Tyr. However, ~60% of the SAM cleavage was off-pathway as production of *p*-cresol from tyrosine was not stoichiometric with dAdo production. Of all

the tyrosine analogs tested the only N-methyl-L-Tyr was able to stimulate 5'-deoxyadenosine production. This is likely because it retained the hydroxyl group that H-bonds to S338 aiding in orientation and recognition of the substrate in the substrate binding pocket. These findings in conjunction with the NosL crystal structure solution indicate that this active site is carefully tuned and that S338 is critical for substrate binding and cleavage. This new proposed mechanism (Figure 7.7) was further supported by the lack of SAM cleavage activity in the HydG variants S338A, S338V, and S338D.

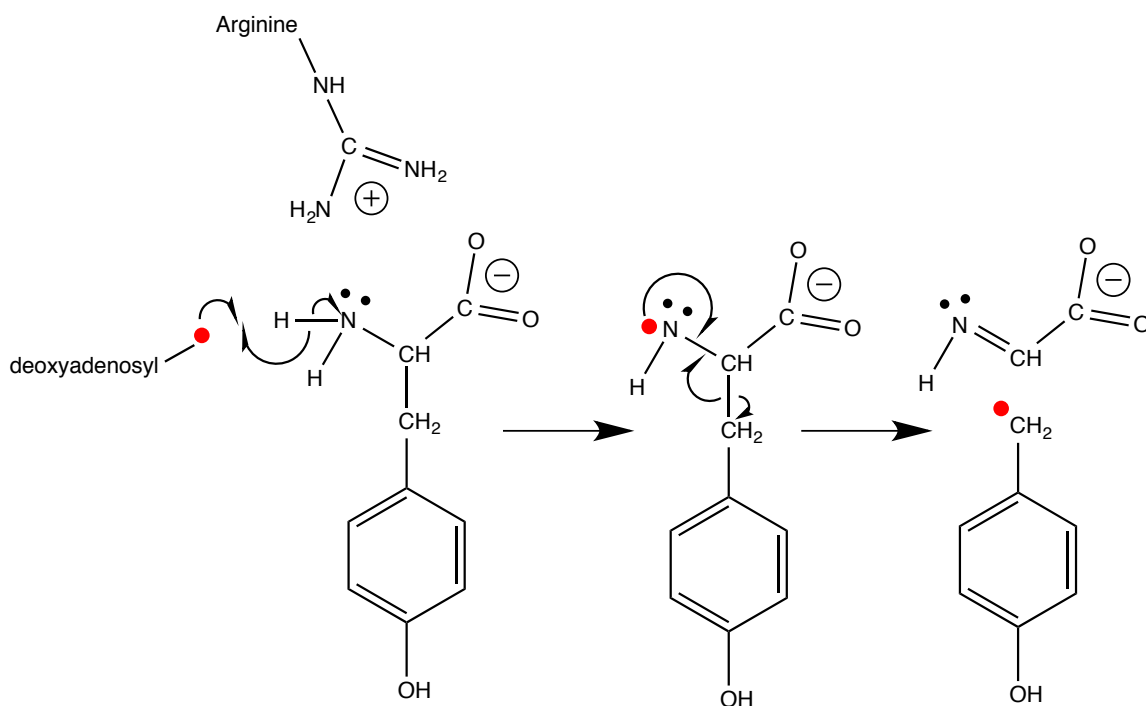


Figure 7.7. Proposed HydG tyrosine lyase mechanism. The close proximity of tyrosine's amine in the active site near Arg320 would suggest the amine is deprotonated for catalysis. Figure 7.6B is the NosL structure that is used to model substrate binding in the HydG active site. Following H-atom abstraction from the amine position, the tyrosine radical intermediate undergoes C α -C β bond cleavage producing dehydroglycine and the *p*-hydroxybenzylic radical.

Characterization of the Auxiliary Cluster of HydG

The crystal structure of HydG confirmed the presence of a complete $(\beta\alpha)_8$ TIM barrel containing a [4Fe-4S] cluster at the N-terminal portion of the protein and a separate domain at the C-terminal end that houses a second cluster.⁽⁸⁾ The C-terminal cluster has been speculated to be a [5Fe-5S] moiety given its presence in only one of the subunits of the HydG dimer crystal structure and a low field EPR signal that was assigned to a $S = 5/2$ signal from this unique cluster ($g = 9.5, 4.7, 4.1, \text{ and } 3.7$).⁽⁸⁾ It should be noted that chemical reconstitution, as was performed on the protein that was crystalized by Roach and coworkers, can lead to artifacts that appear in crystal structures.⁽¹⁸⁾ The ambiguous coordination environment of the fifth iron including a sulfide bridge to the C-terminal cluster, a nonprotein derived free amino acid, two waters, and a histidinyll group make the assignment of the [5Fe-5S] cluster as being biologically relevant suspect. They also report that upon treatment of HydG with cyanide the low field signal disappears, which they speculate is due to the displacement of the fifth iron and sulfur with cyanide.⁽⁸⁾ We feel this result is suspect for two main reasons. First, none of the broad field EPR spectra of WT and variant HydG collected by the Broderick lab have exhibited any low field spectral features other than a $g = 4.3$ junk iron signal. Given that the *C. acetobutylicum* enzyme used in the Broderick lab is active in producing 5'-deoxyadenosine, *p*-cresol, CO, and CN^- as well as forming appreciable quantities of *p*-hydroxybenzylic radical, the presence of the low field signal reported by Britt and coworkers does not appear to correlate with active enzyme. Secondly, linear $[\text{3Fe-4S}]^+$ clusters have a precedence in literature for having signals in the $g = 4.3$ range as originally mentioned by Britt and

coworkers in an earlier publication.^(19, 20) Given these conflicting results amongst labs investigating HydG, future investigations are needed in elucidating the complex series of reactions occurring at HydG's multiple active sites. The spectral features of HydG's C-terminal cluster appear to be very different between research labs; whether or not Roach's [5Fe-5S] cluster⁽⁸⁾ is artifactual or mechanistically relevant is an important question and further research is required.

Conclusions

Prior to the publication of the HydG crystal structures we predicted Britt and coworkers⁽¹⁰⁾ claim that tyrosine directly coordinated to the C-terminal cluster was unlikely. Using PylB and HydE as models, we found that the distance between HydG's clusters placed tyrosine too far from the active site for H-atom abstraction.⁽²⁾ Our mutagenesis studies and alternate substrate assays support the hypothesis that tyrosine's the N-terminal active site of HydG is homologous to that of NosL. This strongly implies that tyrosine lyase activity is initiated by H-atom abstraction at the amine position of tyrosine leading to production of dehydroglycine and the *p*-hydroxybenzylic radical as we have hypothesized.

The auxiliary cluster of HydG has recently been proposed to be a [5Fe-5S] cluster composed of a cubic [4Fe-4S] cluster with an additional iron ion bridged by a single sulfide, based on a crystal structure of exogenously reconstituted HydG and a low field EPR signal.⁽⁸⁾ No HydG samples prepared in the Broderick lab exhibit such an EPR signal, despite being catalytically active, suggesting that whatever give rise to the low

field signal is not functionally relevant. While it is generally accepted that CO and CN⁻ are derived from tyrosine by HydG's radical SAM chemistry, a few important mechanistic details need to be resolved. The first key question is the mechanism of the diatomic ligands from the break down of the intermediate dehydroglycine. This question is intimately linked to the role of the C-terminal cluster. Lastly, the delivery mechanism of the diatomics to HydF as either free species or as iron-diatomic species upon cannibalization of the fifth iron is paramount to the HydG/HydF interaction in the construction of the 2Fe subcluster of [FeFe]-hydrogenase.

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APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 7

S338A

Up Stream Flanking Primer	CCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAG
Down Stream Flanking Primer	CGTGTACAATACGATTACTTTCTGTTCG
Mutant Sense Primer	GGTATATCTCAAATAGCTGGTGGTTCTAGCACAAGTGTTG
Mutant Antisense Primer	CTAGAACCACCAGCTATTTGAGATATACCGAGTTCAAGTACGC

Q360E

Up Stream Flanking Primer	CCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAG
Down Stream Flanking Primer	CGTGTACAATACGATTACTTTCTGTTCG
Mutant Sense Primer	GAAGATAACTCTTCAGAATTCGAAGTCAATGATAACCGTACTCT
Mutant Antisense Primer	TGACTTCGAATTCTGAAGAGTTATCTTCTTCAGGTTCAAGT

Y91F

Up Stream Flanking Primer	CCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAG
Down Stream Flanking Primer	CGTGTACAATACGATTACTTTCTGTTCG
Mutant Sense Primer	GTTTGCTCCTCTTTTCTTTCAAACACTGTGTAAACGGATGT
Mutant Antisense Primer	TAGTTTGAAAGAAAAAGAGGAGCAAACATTACTATACGATTACC

Figure S1. Primers used for Gibson assembly mutagenesis of HydG variants S338A, Q360E, and Y91F.

A		B			
PCR For Mutagenesis Fragments		Thermocycling Conditions			
Component	Volume	Step	Temp (°C)	Time (sec)	Cycles
5X Phusion HF/GC Buffer	2.0 µL	Initial Denaturation	98	30	1
10 mM dNTPs	0.4 µL				
10 µM Primer 1	1.0 µL	Denaturation	98	10	
10 µM Primer 2	1.0 µL	Annealing	66	20	30
WT HydG Template	(250 ng)	Extending	72	20-30	
DMSO	0.6 µL				
Phusion DNA Polymerase	0.2 µL	Final Extension	72	7	1
Water	up to 20 µL				
C		D			
Gibson Reagent Mix		Gibson Enzyme Cocktail			
Component	Volume	Component	Volume		
1 M Tris-HCl pH 7.5	3 mL	Gibson Reagent Mix	384 µL		
2 M Magnesium Chloride	150 µL	T5 Exonuclease (10 U/µL)	0.64 µL		
100 mM dATP	60 µL	Phusion DNA Polymerase (2 U/µL)	20 µL		
100 mM dTTP	60 µL	<i>Taq</i> DNA Ligase (40 U/µL)	160 µL		
100 mM dGTP	60 µL	Water	635 µL		
100 mM dCTP	60 µL				
1 M DTT	300 µL				
PEG-8000	1.5 g				
100 mM NAD	300 µL				
Water	up to 6 mL				
E					
Gibson Assembly Reaction					
Component	Volume				
Gibson Enzyme Cocktail	15 µL				
Linear pCDFDuet	0.25 pmol				
Upstream PCR Fragment	0.25 pmol				
Downstream PCR Fragment	0.25 pmol				
Water	up to 20 µL				

Figure S2. Gibson assembly cloning components and reaction conditions. A. Components for amplification of mutagenic fragments from WT template. B. Thermocycling conditions for amplification of mutagenic fragments. C. Gibson cloning assembly reagents. D. List of enzyme and reagents for Gibson assembly cocktail. E. Gibson assembly reaction components.