

OXIDATION OF HUMAN NITROSYLHEMOGLOBIN MONITORED BY UV-VIS
AND EPR SPECTROSCOPIES: DETECTION OF PRODUCTS AND
INTERMEDIATES

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2005

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ABBREVIATIONS

NO – nitric oxide

NO_2^- – nitrite

NO_3^- – nitrate

NO^+ – nitrosonium ion

Hb – human hemoglobin A_o

oxyHb – oxyhemoglobin, $\text{HbFe}^{\text{II}}\text{O}_2$

deoxyHb – deoxyhemoglobin, HbFe^{II}

metHb – methemoglobin, HbFe^{III}

HbNO – nitrosylhemoglobin,

$\text{HbFe}^{\text{II}}\text{NO}$ – iron(II) nitrosylhemoglobin

$\text{HbFe}^{\text{III}}\text{NO}$ – iron(III) nitrosylhemoglobin

$\text{Hb}(\text{NO})_4$ – fully nitrosylated hemoglobin ($\text{Hb}(\text{Fe}^{\text{II}}\text{NO})_4$)

SNO-Hb – S-nitrosohemoglobin

αNO – α heme bound to NO

$\alpha_5\text{NO}$ – α heme bound to NO with proximal imidazole bond broken

βNO – β heme bound to NO

$\text{K}_3\text{Fe}(\text{CN})_6$ – potassium ferricyanide

EPR – electron paramagnetic resonance

EDRF – endothelium-derived relaxing factor

ABSTRACT

Once viewed only as a toxic free radical, nitric oxide (NO) has been established as an essential and ubiquitous signaling and regulatory molecule in biological systems. Notably, NO was identified as the endothelium-derived relaxing factor (EDRF) in the blood. NO is capable of complex redox chemistry and interaction with a host of protein families. Among these proteins is hemoglobin (Hb) which can interact with NO at the level of the heme and can bind NO at Cys93 on its β subunit to form S-nitrosylated Hb (SNO-Hb). NO bound as SNO-Hb is chemically labile and thus preserves bioavailability of NO. However, when NO reacts with oxyHb or deoxyHb NO bioavailability is quenched by conversion to nitrate or by tightly binding the heme, respectively. Therefore, the question is raised as to how NO can be EDRF in the presence of such high Hb concentrations in the blood. One way NO availability can be preserved is by exploiting the redox chemistries of both Hb and NO.

Human Hb(NO)₄ oxidation by K₃Fe(CN)₆ was studied and products and intermediates were identified by UV-Vis and EPR spectroscopies. Periodically, samples were withdrawn from the reaction mixture for nitrosylation product and/or EPR analysis. Reaction spectra converted to heme species concentration vs. time plots through least-squares fitting of five basis spectra. These data were then utilized to generate a *de minimis* model of the oxidation reaction. We demonstrate that the oxidation of Hb(NO)₄ by K₃Fe(CN)₆ 1) leads to the production of SNO-Hb, 2) occurs preferentially at the β heme, and 3) proceeds through an HbFe^{III}NO intermediate.

INTRODUCTION

The Hemoglobin-Nitric Oxide Interaction

Prior to the 1990s, nitric oxide (NO) was seen as a highly reactive free radical that was toxic to biological systems. Indeed, large doses of NO can be cytotoxic, and NO is associated with inflammation, sepsis, and shock (1). However, with substantial research revealing various essential roles for NO in biology, NO was named “Molecule of the Year” in 1992 by *Science* magazine (2). Throughout the early 90s, NO was implicated in processes such as microbial defense, endocrine secretion, fertilization, neurotransmission, and regulation of vascular tone (3,4).

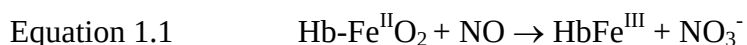
How does NO, a small and seemingly simple molecule, perform so many different functions in vivo? NO is a metal-binding neutral free radical with one unpaired electron. The unpaired electron can be removed (oxidative process) to form the nitrosonium ion (NO^+), or an additional electron can be added (reductive process) to generate the nitroxyl anion (NO^-). These three redox-related states afford NO diverse reactivity in various pathways throughout biological systems (5).

NO was the first gas discovered as a signaling molecule, and its identification as the endothelium-derived relaxing factor (EDRF) (6), an endogenous vasodilator, earned Furchgott, Ignarro, and Murad the Nobel Prize in Medicine in 1998. The action of NO in the cardiovascular system is particularly intriguing, especially from a pharmacological perspective. NO is produced from L-arginine by constitutively expressed endothelial NO-synthase (NOS) and secreted into the lumen of blood vessels (7). This extracellular

NO may then participate in a variety of cardiovascular activities including activation of guanylate cyclase that induces smooth muscle relaxation and consequently vessel dilation (8).

Release of NO into the blood exposes this relatively reactive molecule to a number of reaction partners including hemoglobin (Hb), which reacts with a variety of diatomic gases including O₂ and NO. The Hb protein is composed of four subunits – two α and two β – each with an iron-containing heme prosthetic group. The equatorial positions of the heme iron are occupied by four nitrogens of the porphyrin ring. One axial is occupied by a histidine residue of the protein chain (“proximal” side of heme). The last axial position (“distal” side) is free to bind various ligands including O₂ and NO. The heme iron may be in the “reduced” Fe^{II} or “oxidized” Fe^{III} state and is capable of dynamic redox chemistry. In pooled blood, approximately one percent of hemes are oxidized, forming metHb (HbFe^{III}), while the majority of heme irons are reduced and bound to O₂ (oxyHb, HbFe^{II}O₂) (9).

Because oxyHb constitutes the majority of Hb in the blood, researchers believed that the interaction of NO with Hb in the vasculature led predominately to the formation of nitrate (NO₃⁻) and consequently the elimination of NO bioactivity (Equation 1.1).



In addition to this reaction, the reaction of deoxyHb (HbFe^{II}) with NO to form nitrosylHb (HbFe^{II}NO) is regarded as essentially irreversible because of the large association ($\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$) and relatively small dissociation ($\sim 10^{-5} \text{ s}^{-1}$) constants of this reaction at pH 7.0 and 20°C (Equation 1.2) (10).



Therefore, it seemed that NO was either trapped or chemically inactivated by the heme. Consequently, Hb was viewed as a sink for NO bioavailability, and the question emerged, “How is the biological activity of NO preserved in the presence of high concentrations of Hb?”

NO bioactivity in the presence of Hb is preserved by three mechanisms. First, compartmentalization of Hb in the erythrocyte slows the NO reaction with Hb, as shown by Lancaster and coworkers (11,12). Second, Liao and coworkers have demonstrated that due to the flow of blood, the tendency of erythrocytes is to remain in the center of the vessels, avoiding the endothelial walls where NO is produced by eNOS (12). Finally, NO chemistry that forms thionitrites may be the most influential bioactivity-preserving mechanism. Formed via S-nitrosylation, thionitrites (RSNOs) exhibit some of the most potent vasodilatory effects and are the most abundant bioactive NO compounds in the blood. These include low molecular weight nitrosothiols such as derivatives of cysteine and glutathione and larger S-nitrosylated proteins such as S-nitrosalbumin (13). Hb was added to the list of proteins capable of S-nitrosylation in 1996 (14).

Heme Redox Coupling to NO Chemistry

The understanding of Hb-NO biology has been illuminated by the discovery of Hb reactions that maintain NO bioavailability – reactions that produce an S-nitrosylated species of Hb (SNO-Hb) or nitrite. Nitrite is considered a bioavailable form of NO because 1) nitrite can dilate blood vessels, 2) compared to NO, nitrite is relatively long

lived in solution, 3) in physiological acidic conditions, such as ischemia, acidified nitrite can nitrosate thiols, and 4) nitrite can be reduced by HbFe^{II} to yield $\text{HbFe}^{\text{II}}\text{NO}$ (15). Though $\text{HbFe}^{\text{II}}\text{NO}$ has been historically viewed as a dead end for NO biology, a description of how this NO sink can be rescued through redox is detailed below.

The SNO-Hb species is formed when NO reacts with Hb at the level of the protein chain; it represents a post-translational modification of cysteine residues that affects a number of proteins both in the blood and throughout the body (16). In SNO-Hb, the NO group binds to a highly conserved cysteine residue on the β globin chain (Cys- β 93) allowing each Hb tetramer to accommodate two SNO moieties. The NO bound as SNO-Hb is chemically labile. It is available for release as NO or via transnitrosation to form other nitrosothiols or nitrite.

Notably, the formation and elimination of SNO-Hb is coupled to heme redox. Luchsinger *et al.* (2003) demonstrated a connectivity between heme and Hb thiol through redox chemistry in the process of reductive nitrosylation. In experiments where metHb (fully oxidized Hb) was exposed to NO or an NO releasing agent, the heme was reduced to HbFe^{II} while an electrophilic NO^+ species was formed that was able to react with solvent OH^- or Cys- β 93 (Equation 1.3-Equation 1.5).



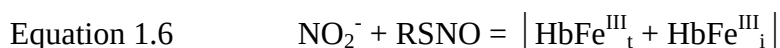
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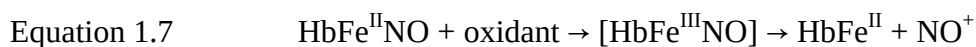
or



This scheme predicts that the combined nitrite and RSNO formation equals the amount of HbFe^{III} reacted (Equation 1.6).



In Equation 1.6, the right side of the equation represents the amount of reduced hemes calculated as reacted HbFe^{III} at a given time point less the initial HbFe^{III} (present before addition of NO). The products were formed through a proposed $\text{HbFe}^{\text{III}}\text{NO}$ intermediate that is relatively short-lived because the dissociation rate of NO from the oxidized heme is substantially faster than that of the $\text{HbFe}^{\text{II}}\text{NO}$ species. The electronic structure of this intermediate can be considered as formally $\text{HbFe}^{\text{II}}\text{NO}^+$ allowing for the release of the nitrosonium ion from the heme. Similar products are obtained upon oxidation of fully nitrosylated hemoglobin ($\text{Hb}(\text{NO})_4$), suggesting that the same intermediate species is formed.



This generic reaction represents a rescue for the $\text{HbFe}^{\text{II}}\text{NO}$ sink mentioned above and is thus of great significance. Ultimately, oxidation of $\text{HbFe}^{\text{II}}\text{NO}$ to $\text{HbFe}^{\text{III}}\text{NO}$ increases the off rate of NO from the heme, which leads to the formation of species that preserve bioavailability. Indeed, $\text{Hb}(\text{NO})_4$ oxidation experiments performed by Luchsinger *et al.* led to the formation of either or both nitrite and SNO-Hb (17,18). The reaction product formed, however, depended on the nature of the oxidant used. More specifically, of the oxidants used (H_2O_2 , KO_2 , $\text{K}_3\text{Fe}(\text{CN})_6$, multiple cytochromes, and air oxidation), only the

mild oxidant $\text{K}_3\text{Fe}(\text{CN})_6$ (reduction potential = 0.36 V) produced the S-nitrosylated product. Oxidation with strong oxidants such as H_2O_2 and KO_2 formed only nitrite while air oxidation formed neither SNO-Hb nor nitrite – presumably yielding only nitrate. Therefore, it appears that three different types of oxidation reactions of $\text{Hb}(\text{NO})_4$ may occur as classified by the reaction products: 1) air oxidation (nitrate), 2) strong oxidation (nitrite only), and 3) mild oxidation with oxidants such as $\text{K}_3\text{Fe}(\text{CN})_6$ (nitrite and SNO-Hb).

S-Nitrosylation is Coupled to Quaternary Structure

S-nitrosylation of Hb has been shown to be dependent on the quaternary structure of the protein. Fully ligated Hb is said to be in the R state conformation while deoxyHb assumes the T state structure. SNO-Hb is favored in R state when Cys- β 93 is buried within the protein (

Figure 1.1). Upon $\text{R} \rightarrow \text{T}$ transition, the cysteine residue is shifted such that it is more solvent-exposed and the SNO moiety is destabilized. Stamler and co-workers have demonstrated in vivo that, though the total number of nitrosylated species ($\text{HbFe}^{\text{II}}\text{NO}$ and SNO-Hb) remains relatively constant among venous and arterial samples, the balance of these species is shifted in favor of SNO-Hb in arterial blood and $\text{HbFe}^{\text{II}}\text{NO}$ in venous blood. Therefore, it appears that in vivo SNO-Hb is sensitive to changes in O_{sat} . This aspect of Hb biology presents an attractive potential mechanism for vasodilation controlled by Hb: Hb may act as both a sensor of changes in O_{sat} (well understood $\text{R} \rightarrow \text{T}$ transition) and a switch (NO release from SNO-Hb) to initiate smooth muscle relaxation. Therefore, NO may, in fact, be considered a third respiratory ligand of Hb.

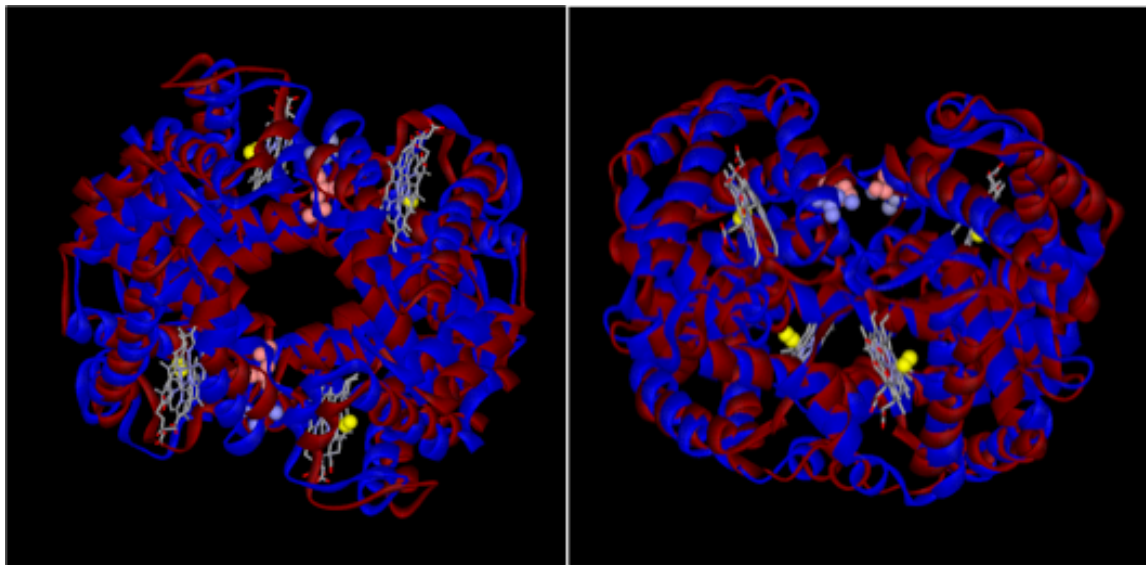


Figure 1.1 Structural alignment of R and T state hemoglobin. Crystal structures 1A3N (deoxyHb, blue) and 1BUW (S-nitroso-nitrosylHb, red) were obtained from the Protein Data Bank (UMass) and aligned at the heme irons using Discovery Studio® ViewerPro (Accelrys®). The protein chains are represented as ribbons while specific atoms are drawn as ball and stick: heme nitrosyls (yellow), Cys-β93 of T state deoxyHb (light blue), and Cys-β93-SNO of R state nitrosylHb (light red). Note the slight shift from buried to more solvent-exposed upon transition from R state to T state: left panel, R state Cys-β93-SNO is oriented toward the interior of the protein while T state Cys-β93 is more exterior; right panel, R state Cys-β93-SNO is tucked into the protein chains while T state Cys-β93 is oriented away from the protein chain.

Delimitation of Thesis Work

This thesis describes an investigation of three types of oxidation reactions of human Hb(NO)_4 employing various oxidants. Reaction progression was followed by UV-Vis spectroscopy and heme and non-heme NO products were analyzed by EPR and Griess/Saville assays, respectively. The raw UV-Vis reaction spectra were decomposed using a linear least-squares fitting program from which concentration vs. time plots were constructed. These kinetic data were utilized to generate a *de minimis* mechanistic model that simulates the data with excellent agreement. The rate constants used in this model

also agree well with published rate constants. Most significantly, this work further characterizes the production of SNO-Hb and demonstrates the intermediacy of $\text{HbFe}^{\text{III}}\text{NO}$ in the oxidation of $\text{Hb}(\text{NO})_4$ by excess $\text{K}_3\text{Fe}(\text{CN})_6$.

MATERIALS AND METHODS

Oxidation Reactions of Human Nitrosylhemoglobin

Formation of Nitrosylhemoglobin

Human hemoglobin A₀ was obtained from Apex Bioscience (Research Triangle Park, NC) and deoxygenated by ultra-high-purity argon (UHP Ar, Midwest Industries, Bozeman, MT) with gentle vortexing over five cycles of positive pressure (no exit needle) and purge (exit needle) for approximately 30 min in septa-capped 15-mL conical tubes. Concentrated solutions of sodium dithionite (BDH) and sodium nitrite (Sigma[®]) were prepared by adding deoxygenated phosphate buffered saline (PBS, 11.978 mM total phosphate) to deoxygenated, septa-capped microcentrifuge tubes containing either sodium dithionite or sodium nitrite. Deoxygenated sodium dithionite followed by deoxygenated sodium nitrite solution were added to the deoxygenated Hb samples via Ar-purged Hamilton GASTIGHT[®] syringes. The Hb became fully nitrosylated (Hb(NO)₄) after approximately one minute of gentle mixing and was passed, without delay, over 3-mL Bakerbond SPE[™] filtration columns containing Sephadex[™] G-25 Fine equilibrated with PBS, pH 7.4, to remove excess dithionite and nitrite. A maximum volume of 250 uL Hb was added to each column. Columns were spun for two minutes on an IEC centrifuge at 3100 rpm using a 221 rotor. Following filtration, solutions were iced, diluted to approximately 1.2 mM heme, capped with septa, and purged once with UHP Ar. Samples were stored on ice and under positive Ar pressure for a maximum of 45 min before used in experiments.

Potassium Ferricyanide Preparation

Potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$, Sigma[®]) solutions of 20 and 200 mM were prepared with nanopure H_2O (Nanopure Diamond[™], Barnstead) and stored on ice covered in foil. Solutions were deoxygenated as required (before reactions under deoxy conditions) and delivered via Hamilton GASTIGHT[®] syringes to $\text{Hb}(\text{NO})_4$ reaction solutions.

Hydrogen Peroxide Solution Preparation

Hydrogen peroxide (H_2O_2) 35% solution (Acros Organics) was diluted to 100 mM with nanopure H_2O and stored on ice until use.

Spectroscopy

UV-Vis Spectroscopy/Reactions

Oxidation reactions were monitored by UV-Vis spectroscopy performed on a Cary 300 Bio spectrophotometer (Varian, Inc.). Hb solutions were placed in 1 mm FUV quartz cuvettes (Spectrocell, Inc., Orelan, PA) that were previously capped and deoxygenated with UHP Ar for experiments performed under anaerobic conditions. Cuvettes were left uncapped for reactions performed under oxygenating conditions. Reactions were performed in PBS at pH 7.4 unless otherwise indicated. All scans taken were in the range of 450-700 nm, and initial scans were taken before the addition of oxidant. The appropriate amount of oxidant to add was calculated by determining the concentration of $\text{Hb}(\text{NO})_4$ from this initial scan using $A_{544\text{nm}} (A_{572\text{nm}}) = 11.4 \text{ mM}^{-1} \text{ cm}^{-1}$

(19). In $\text{K}_3\text{Fe}(\text{CN})_6$ experiments, scans from 400-700 nm were taken five minutes apart (451.128 nm/min scan rate, 1-nm steps). In H_2O_2 experiments, scans were taken every minute using the previously stated settings. The time between mixing and the first scan after oxidant addition was recorded and added to the scan time upon spectral analysis. Reactions were allowed to proceed until approximately 50% oxidation.

EPR spectroscopy

Samples of 50-75 μL were taken at approximately 25, 50, and 75% oxidation from select reactions and transferred to 4-mm inner diameter fused silica tubes and frozen by plunging into liquid N_2 . Samples remained frozen at 76 K until and during spectroscopy performed with a Varian E-9 EPR spectrophotometer. Two different field range scans were performed on each sample:

- 1) Field Center = 3270.00 G, Range = 400.0 G, Time Constant = 0.128, Scan Time = 2 min, Modulation Amplitude = 3.20 G, Microwave Frequency = 9.3442, Microwave Power = 10 mW.
- 2) Field Center = 2100.00 G, Range = 4000.0 G, Time Constant = 0.128, Scan Time = 4 min, Modulation Amplitude = 5.00 G, Microwave Frequency = 9.3442, Microwave Power = 10 mW.

Deviations from these settings are noted where appropriate. Scan (1) settings allow for the detection of $\text{Hb-Fe}^{\text{II}}\text{NO}$ species while conditions of (2) are suitable for the detection of the $\text{Hb-Fe}^{\text{III}}$ signature.

Quantification of Nitrite and S-nitrosylhemoglobin formation

Reaction mixtures were tested for the presence of nitrite and S-nitrosylhemoglobin (SNO-Hb) by the Griess and Saville methods, respectively, as previously reported by us, using a Cary 1E UV-Vis spectrophotometer both before addition of oxidant and at various time points during the course of the reaction. Samples were tested either with or without elution over PBS-equilibration SephadexTM G-25 allowing for elimination of nitrite, unreacted ferricyanide, and reacted ferrocyanide from the protein solution. Therefore, Saville chromophore detection from hemoglobin eluted from the column confirms the absence or presence of SNO-Hb.

The Griess and Saville reactions are depicted in Figure 2.1.

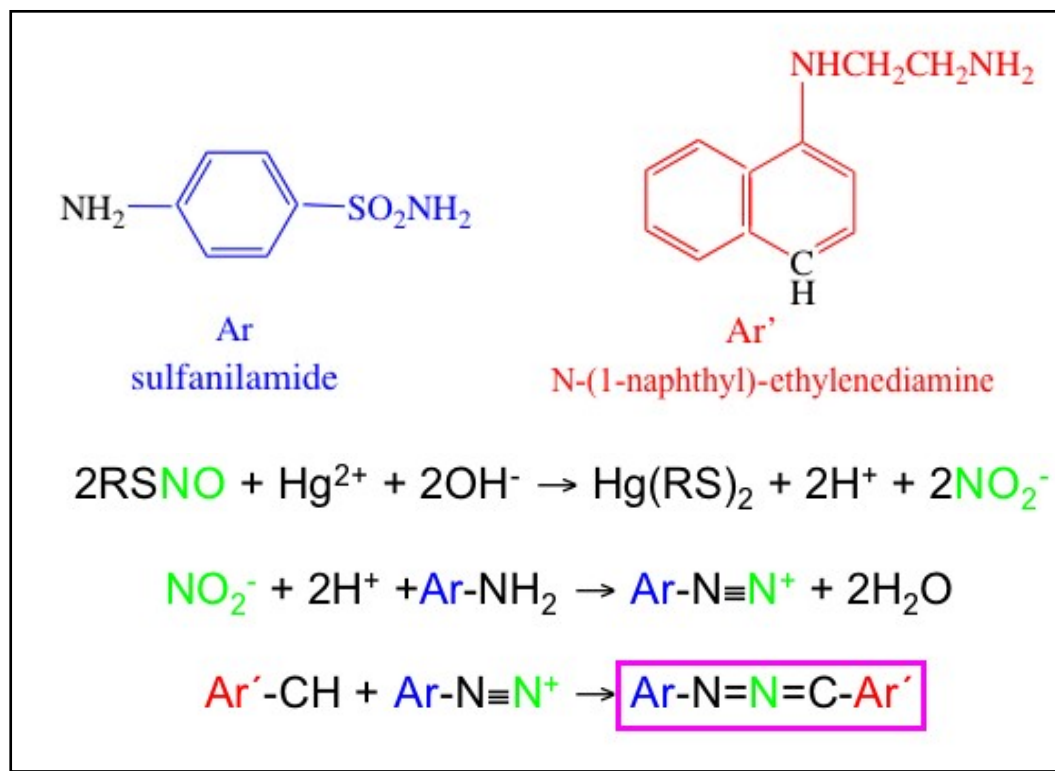


Figure 2.1 Griess and Saville reactions (20).

The Griess and Saville reaction mixtures are created as depicted in Table 2.1.

Sample (species detected)	Hb (20 uL)	SAA (333 uL)	SAA/Hg ²⁺ (333 uL)	NNED or HCl (313 uL)	HCl (333 uL)
<i>Blank (Hb)</i>	+	+	-	HCl	+
<i>Griess (nitrite)</i>	+	+	-	NNED	+
<i>Saville (SNO)</i>	+	-	+	NNED	+

Table 2.1 Preparation scheme for Griess and Saville assays. Hb = aliquot from the reaction cuvette, SAA = p-aminobenzene-sulfonamide (Sigma[®]), 1% solution, SAA/Hg²⁺ = SAA above with 0.05% mercuric chloride (HgCl₂, Sigma[®]), NNED = n-(1-naphthyl)ethylenediamine dihydrochloride (Sigma[®]), 0.02% solution, HCl (Fisher) = 0.5 M solution

All solutions were mixed and analyzed in 1-cm path length semimicro disposable plastic cuvettes (Fisherbrand[™]). Absorbance was measured at 540 nm and the concentration of nitrite or SNO-Hb was determined using $A_{540} = 50,000 \text{ M}^{-1} \text{ cm}^{-1}$ (20). In the Griess assay, the *Blank* serves as the reference cuvette in Cary 1E double beam UV-Vis spectrometer (Varian). Therefore, the spectrum resulting from Hb is automatically subtracted. In the Saville assay, the *Griess* serves as the reference. Therefore, the absorbance resulting from nitrite in solution is automatically subtracted allowing detection of the mercury-labile S-nitrosylated species. Differences in the concentrations of Hb in each cuvette resulted in slightly skewed baselines which were accounted for in determination of nitrite or SNO-Hb concentration.

Analyses

Spectral Analysis and Decomposition

UV-Vis spectra were imported into Kaleidagraph[™] Version 3.5, plotted, derivatized, and smoothed using the embedded functions. Analyzing derivative spectra enables better distinction between canonical spectra with similar shapes. The smoothed

derivative spectra were then imported into a least-squares analysis program in Mathematica[®] 5.1 where they were fit as linear combinations of five basis spectra derived from the five possible species of heme: $\text{HbFe}^{\text{II}}\text{O}_2$, HbFe^{II} , HbFe^{III} , $\text{HbFe}^{\text{II}}\text{NO}$, $\text{HbFe}^{\text{III}}\text{NO}$ (Appendix A). This program is equipped to constrain values as needed; however, the data included in this text were fit without constraint, except as noted. Decomposed UV-Vis spectra were converted to time course data manually in Kaleidagraph[™] Version 3.5 and plotted.

Kinetic Analysis

Time course data generated as detailed above were imported into a Gepasi 3.30 [<http://www.gepasi.org/gepasi.html>] program and fit for rate constants and/or initial concentrations. Detailed description of the reactions and parameters used in the model is included in Chapter 3.

RESULTS

Oxidation of Hb(NO)₄ by K₃Fe(CN)₆ Leads to SNO-Hb FormationAnaerobic Conditions

Human Hb(NO)₄ was oxidized by K₃Fe(CN)₆ under anaerobic conditions and monitored by UV-Vis spectroscopy over the course of each reaction. A representative set of spectra are included in Figure 3.1. A reaction time course was generated after decomposition of the raw spectra into linear combination of five Hb species using a least-squares fitting program (Figure 3.1). As expected, HbFe^{II}NO is the majority species at the beginning of the reaction which is converted stoichiometrically to HbFe^{III} upon oxidation by K₃Fe(CN)₆.

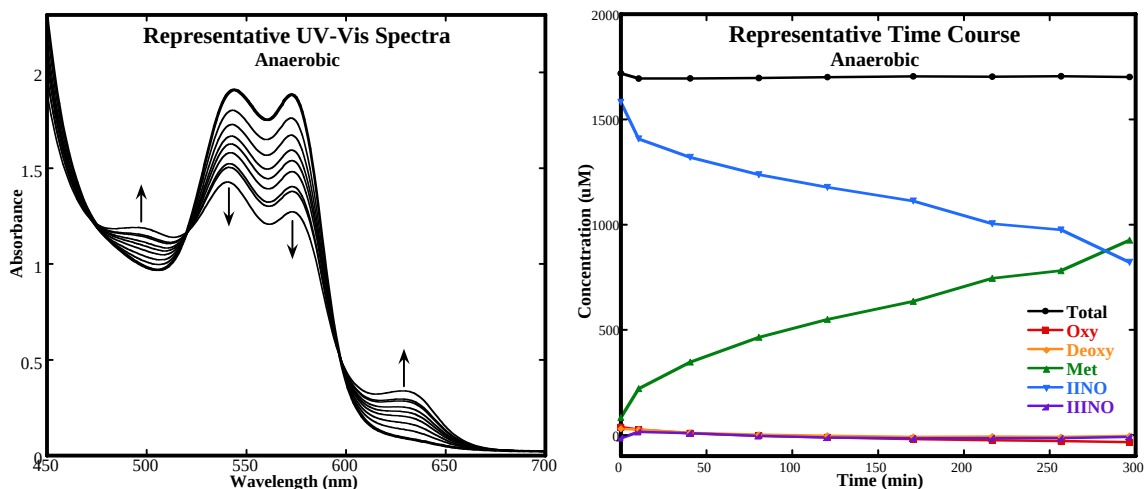


Figure 3.1 Representative UV-Vis spectra and corresponding kinetic profile: anaerobic conditions, 1:1 heme:K₃Fe(CN)₆. The spectra on the left correspond to the time points indicated on the right. The bold line represents Hb(NO)₄ before addition of K₃Fe(CN)₆. (Left) the UV-Vis spectra shown were selected from a set of scans taken every ten minutes. Reaction progression of Hb(NO)₄ oxidation is indicated by decreases in absorbance at approximately 544 and 572 nm (HbFe^{II}NO A_{max}) with concomitant increases at approximately 630 and 500 nm (HbFe^{III} A_{max}). (Right) Points are deconvoluted data. Lines connecting points are a guide to the eye. The initial change in total heme concentration is due to the dilution that occurs upon addition of K₃Fe(CN)₆. The sharp rise in HbFe^{III} at the end of the time course may indicate that a small amount of O₂ was introduced into the sample upon extraction of an aliquot for Griess/Saville assay or EPR analysis.

A representative plot of the fit generated by spectral decomposition by linear least squares analysis is given in Figure 3.2.

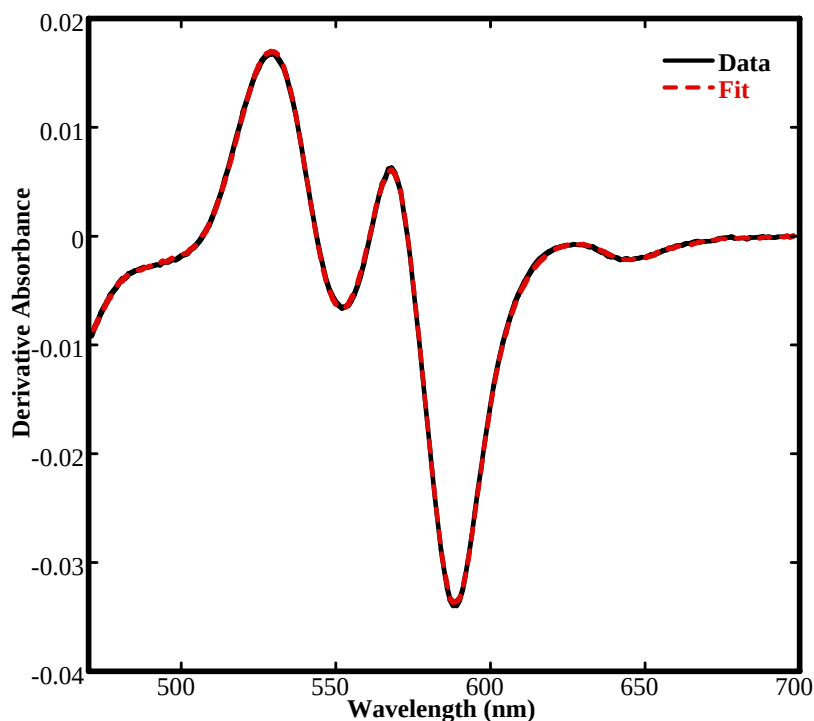


Figure 3.2 Representative plot of fit generated by least squares analysis. The data and fit are displayed in derivative mode.

Periodically during each reaction, aliquots of the reaction mixture were extracted for for analysis of non-heme (Griess/Saville) and heme (EPR) NO product formation. The Griess/Saville data obtained from the reaction depicted in Figure 3.1 are provided in Figure 3.3. The differences observed in baseline for each Griess/Saville sample are corrected for before determining the concentration of nitrite or SNO-Hb present. In the Griess assay of column contents, a portion of the column material remained suspended in the liquid, resulting in an overall positive baseline shift. This is similar to the baseline shift observed when bubbles distort the absorbance of a sample. In addition to this upward shift in baseline, the Saville assays display a diagonally shifted spectrum. This is

strictly due to the variability in heme concentration from one cuvette to the next. The Hb spectrum in the strongly acidic conditions of the Griess/Saville assays absorbs more strongly at 450 nm than at 700 nm where its absorbance is essentially zero. Therefore, if the reference cell has a lower heme concentration than the sample cell, for example, the observed spectrum will be shifted up at 450 nm.

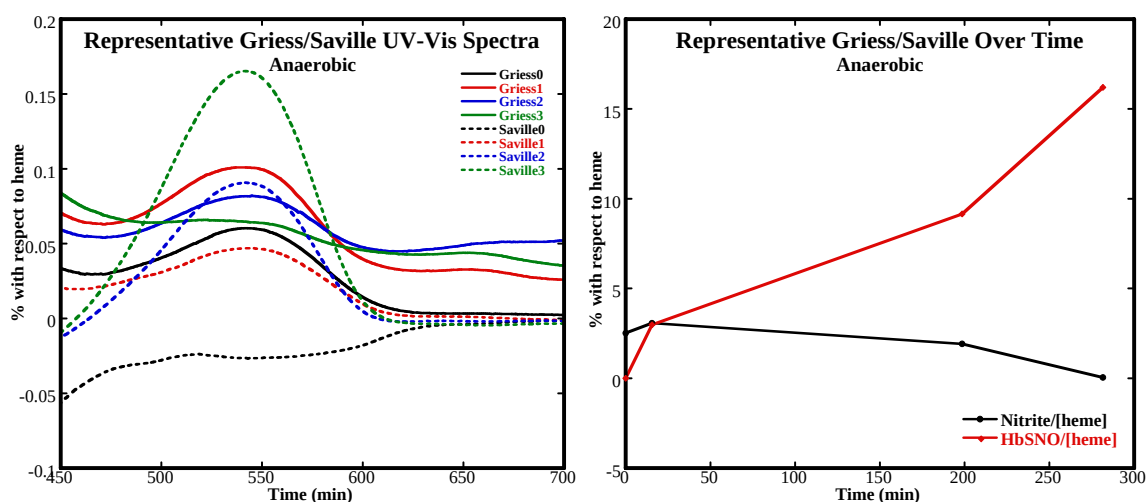


Figure 3.3 Representative Griess/Saville UV-Vis spectra and calculated concentrations observed for oxidation reactions performed in anaerobic conditions. (Left) Original Griess and Saville assay spectra taken from the reaction mixture at the indicated time points. (Right) Nitrite and SNO-Hb concentrations were calculated from the original spectra after correction for baseline offset. Concentrations are presented as a percent of total heme concentration to facilitate comparison to other conditions.

It is important to note that the nitrite concentrations generated from the Griess assay should be considered a guide to but not a quantitative measure of the amount of nitrite generated over the course of the reaction. Three major factors prevent accurate quantification of nitrite in these reactions. First, the column was required not only to be

sure SNO-Hb was measured (see Chapter 2) but also to eliminate $\text{K}_3\text{Fe}(\text{CN})_6$ from the Hb mixture. It was observed that upon addition of Hb with $\text{K}_3\text{Fe}(\text{CN})_6$ to the Griess assay, a large amount of nitrite was detected – much larger than the concentration of hemes oxidized. In fact, if the Griess reagents were incubated with excess ferricyanide prior to addition of fresh $\text{Hb}(\text{NO})_4$, nitrite was nearly quantitatively measured. Therefore, size exclusion columns were used at each Griess/Saville measurement time point to ensure separation of $\text{K}_3\text{Fe}(\text{CN})_6$ from Hb. Nitrite cannot be absolutely determined from the Griess assay because the volume of column material utilized varies from assay to assay. Finally, both nitrite and $\text{K}_3\text{Fe}(\text{CN})_6$ likely absorb into the G-25 beads and, thus, are potentially unavailable for complexing to form the chromophore.

Aerobic Conditions

Oxidation reactions of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$ were performed under aerobic conditions in which the reaction mixture was allowed to equilibrate with atmospheric O_2 . Reaction mixtures monitored with UV-Vis spectroscopy (Figure 3.4) and were periodically mixed upon extraction of aliquots for Griess/Saville and EPR analyses. As seen in anaerobic conditions, spectral decomposition reveals the stoichiometric conversion of $\text{HbFe}^{\text{II}}\text{NO}$ to HbFe^{III} with minimal contribution from all other heme species.

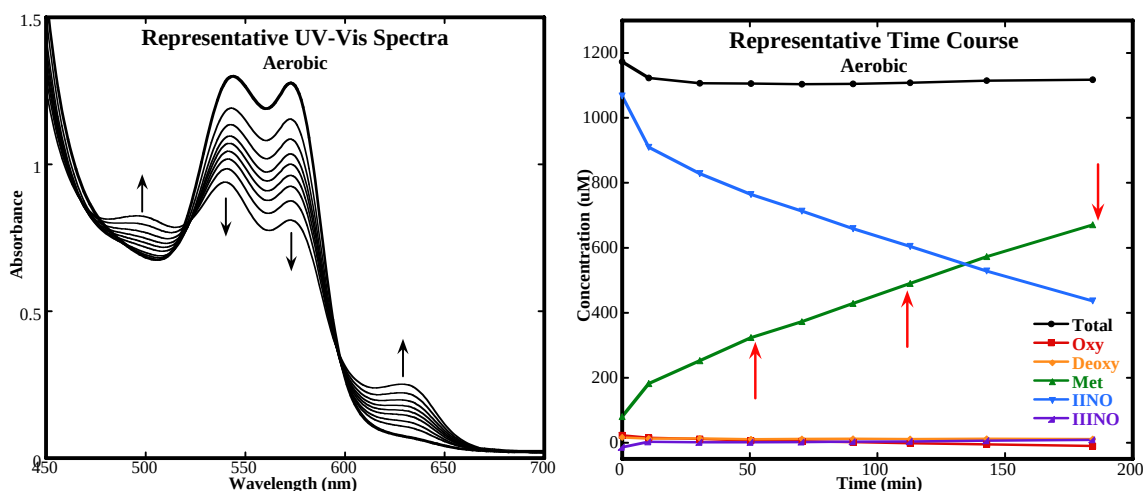


Figure 3.4 Representative time course UV-Vis and corresponding kinetic profile: aerobic conditions, 1:1 heme: $\text{K}_3\text{Fe(CN)}_6$. The spectra on the left correspond to the time points indicated on the right. The bold line represents Hb(NO)_4 before addition of $\text{K}_3\text{Fe(CN)}_6$. (Left) the UV-Vis spectra shown were selected from a set of scans taken every ten minutes. Reaction progression of Hb(NO)_4 oxidation is indicated by decreases in absorbance at approximately 544 and 572 nm ($\text{HbFe}^{\text{II}}\text{NO}$ A_{max}) with concomitant increases at approximately 630 and 500 nm (HbFe^{III} A_{max}). (Right) Points are decomposed data; lines connecting points are a guide to the eye. The initial change in total heme concentration is due to the dilution that occurs upon addition of $\text{K}_3\text{Fe(CN)}_6$.

It is clear that oxidation of Hb(NO)_4 under aerobic conditions proceeds at a faster rate than the same reaction in the absence of O_2 . Aerobic conditions allowed the selected reaction to reach 50% oxidation at approximately 140 min while the anaerobic oxidation reaction of similar total heme concentration and heme: $\text{K}_3\text{Fe(CN)}_6$ ratio required nearly 280 min to reach 50% oxidation.

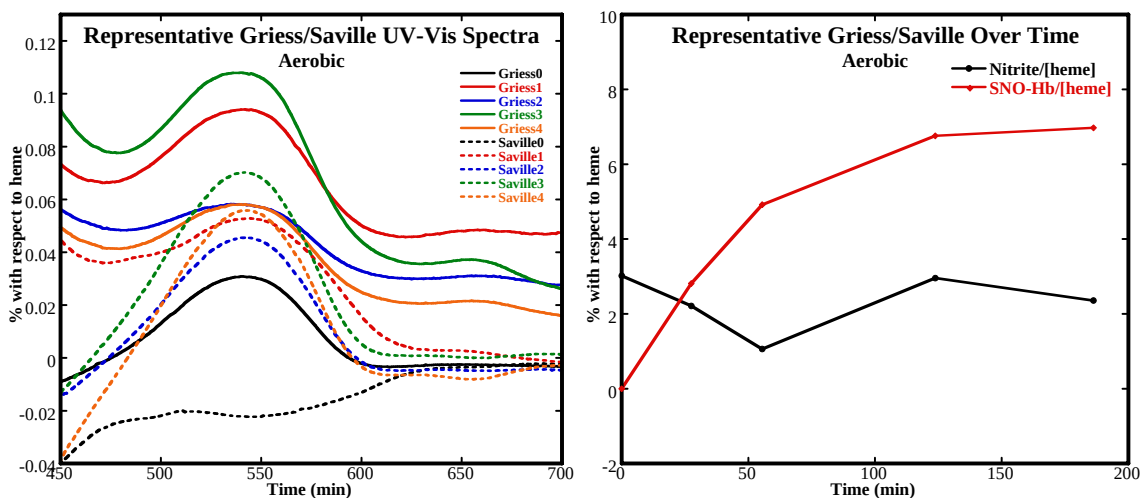


Figure 3.5 Representative Griess/Saville UV-Vis spectra and calculated concentrations observed for oxidation reactions performed in aerobic conditions. (Left) Original Griess and Saville assay spectra taken from the reaction mixture at the indicated time points. (Right) Nitrite and SNO-Hb concentrations were calculated from the original spectra after correction for baseline offset. Concentrations are presented as a percent of total heme concentration to facilitate comparison to other conditions.

Comparison of Aerobic and Anaerobic Conditions

In addition to the observation that the aerobic oxidation of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$ is faster than anaerobic, there are notable differences in the production of non-heme NO products. It is apparent that formation of SNO-Hb per heme oxidized is higher in anaerobic than aerobic conditions at a heme: $\text{K}_3\text{Fe}(\text{CN})_6$ ratio of one (Figure 3.6).

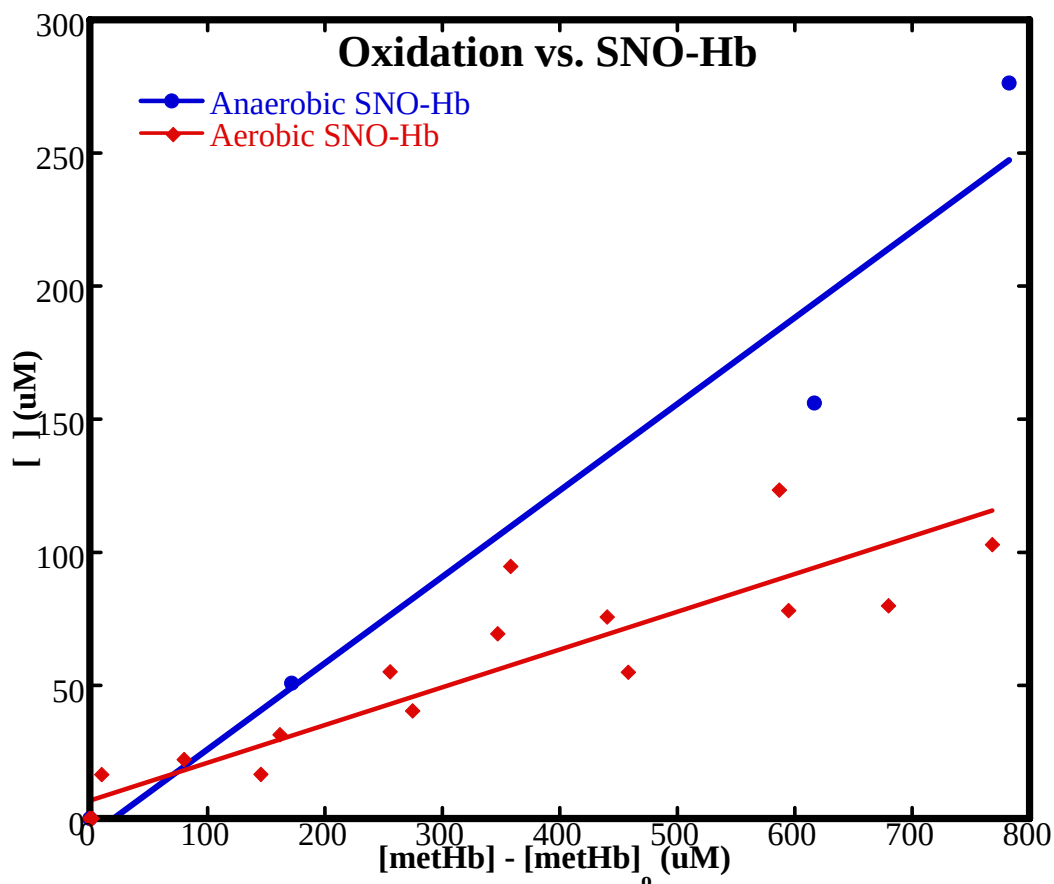


Figure 3.6 SNO-Hb formed per heme during oxidation reactions of Hb(NO)_4 performed under anaerobic and aerobic conditions. Aerobic data points represent four different Hb(NO)_4 oxidation reactions. Linear regressions were generated in Kaleidagraph™ 3.5 using the embedded function. The slope of the linear fit of the data from anaerobic conditions (0.324) is approximately twice that calculated for aerobic (0.142) SNO-Hb formation.

Oxidation reactions of Hb(NO)_4 by $\text{K}_3\text{Fe(CN)}_6$ consistently generated both nitrite and SNO over the heme: $\text{K}_3\text{Fe(CN)}_6$ ratios tested. Though nitrite data was not absolutely quantified, the increase in SNO-Hb production with increasing oxidation supports the hypothesis that SNO-Hb (and nitrite) should correlate with HbFe^{III} formation (Equation

1.6). However, if Equation 1.6 were strictly followed, the slope of the regression lines in Figure 3.6 would be near unity.

Collectively, these results demonstrate the unique production of SNO-Hb by $\text{K}_3\text{Fe}(\text{CN})_6$ mild oxidation. Oxidation of $\text{Hb}(\text{NO})_4$ by H_2O_2 generated only nitrite (data not shown) while simple air oxidation generated neither nitrite nor SNO-Hb but presumably only nitrate (data not shown).

$\text{Hb}(\text{NO})_4$ Oxidation by $\text{K}_3\text{Fe}(\text{CN})_6$ Proceeds Through $\text{HbFe}^{\text{III}}\text{NO}$

Evidence for $\text{HbFe}^{\text{III}}\text{NO}$ Intermediacy: pH 7.4

No evidence for the formation of $\text{HbFe}^{\text{III}}\text{NO}$ was observed in the reactions depicted in Figure 3.1 and Figure 3.4 where heme: $\text{K}_3\text{Fe}(\text{CN})_6$ ratios of 1:1 were employed. Experiments with limiting $\text{K}_3\text{Fe}(\text{CN})_6$ (2:1 heme: $\text{K}_3\text{Fe}(\text{CN})_6$) were consistent with this observation (data not shown). However, upon addition of $\text{K}_3\text{Fe}(\text{CN})_6$ in 10-fold excess over heme, significant evidence for $\text{HbFe}^{\text{III}}\text{NO}$ formation arises (Figure 3.7). The use of excess $\text{K}_3\text{Fe}(\text{CN})_6$ significantly increases the rate of oxidation of $\text{Hb}(\text{NO})_4$ and allows detection by UV-Vis spectroscopy of $\text{HbFe}^{\text{III}}\text{NO}$. Though not readily apparent by simple inspection of the UV-Vis spectra in Figure 3.7, the formation of an $\text{HbFe}^{\text{III}}\text{NO}$ intermediate induces a slight red shift of the spectral peaks between 500 and 600 nm; this change is detected through fitting. The decomposition reveals the formation of approximately six percent $\text{HbFe}^{\text{III}}\text{NO}$ with respect to the total heme concentration.

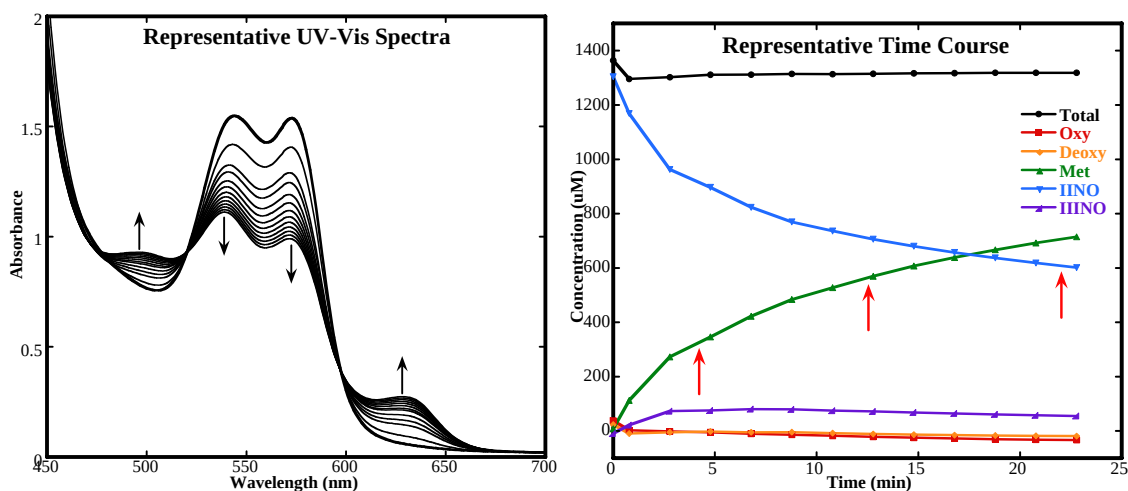


Figure 3.7 UV-Vis spectra and corresponding time course generated from oxidation of Hb(NO)_4 with excess $\text{K}_3\text{Fe(CN)}_6$ under anaerobic conditions. The spectra on the left correspond to the time points indicated on the right. (Left) The bold line represents Hb(NO)_4 before addition of $\text{K}_3\text{Fe(CN)}_6$ and arrows indicate the absorbance changes associated with oxidation. The formation of $\text{HbFe}^{\text{III}}\text{NO}$ is not observable by inspection. (Right) Upon spectral decomposition, generation of $\text{HbFe}^{\text{III}}\text{NO}$ is apparent. Points are deconvoluted data; lines connecting points are a guide to the eye. Red arrows indicate time points at which aliquots were extracted and frozen for EPR analysis.

Oxidation of Hb(NO)_4 by excess $\text{K}_3\text{Fe(CN)}_6$ (10-fold over heme) was also performed under aerobic conditions (Figure 3.8). The rate of reaction and distribution of heme species observed are similar among anaerobic and aerobic conditions. Therefore, the presence of the large excess of $\text{K}_3\text{Fe(CN)}_6$ likely masks any potential effect of O_2 oxidation.

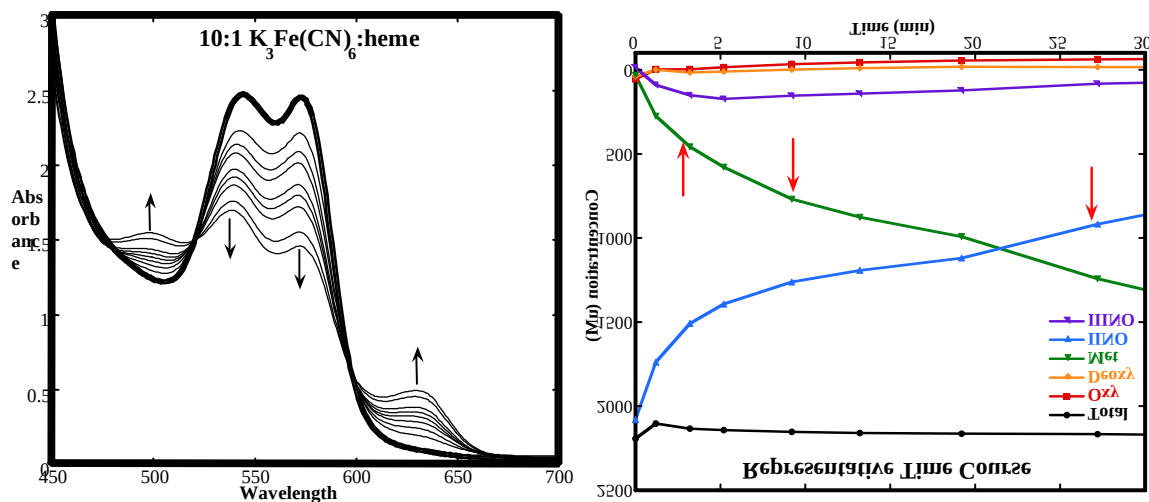


Figure 3.8 UV-Vis spectra and corresponding time course generated from oxidation of $Hb(NO)_4$ with excess $K_3Fe(CN)_6$ under aerobic conditions at pH 7.4. The spectra on the left correspond to the time points indicated on the right. (Left) The bold line represents $Hb(NO)_4$ before addition of $K_3Fe(CN)_6$ and arrows indicate the absorbance changes associated with oxidation. The formation of $HbFe^{III}NO$ is not observable by inspection. (Right) Upon spectral decomposition, generation of $HbFe^{III}NO$ through $Hb(NO)_4$ oxidation by $K_3Fe(CN)_6$ is apparent (purple). Points are deconvoluted data; lines connecting points are a guide to the eye. Red arrows indicate time points at which aliquots were extracted and frozen for EPR analysis.

Evidence for $HbFe^{III}NO$ Intermediacy: pH 6.0

Though $HbFe^{III}NO$ was formed under the conditions of the reactions depicted in Figure 3.7 and Figure 3.8, the maximum concentration of this intermediate in both reactions was approximately six percent of the total heme concentration. Because $HbFe^{III}NO$ was formed at such a low percentage, the reaction was repeated at pH 6.0 to stabilize the $HbFe^{III}NO$ intermediate to ensure that $HbFe^{III}NO$ was real and not attributable to error (21) (Figure 3.9).

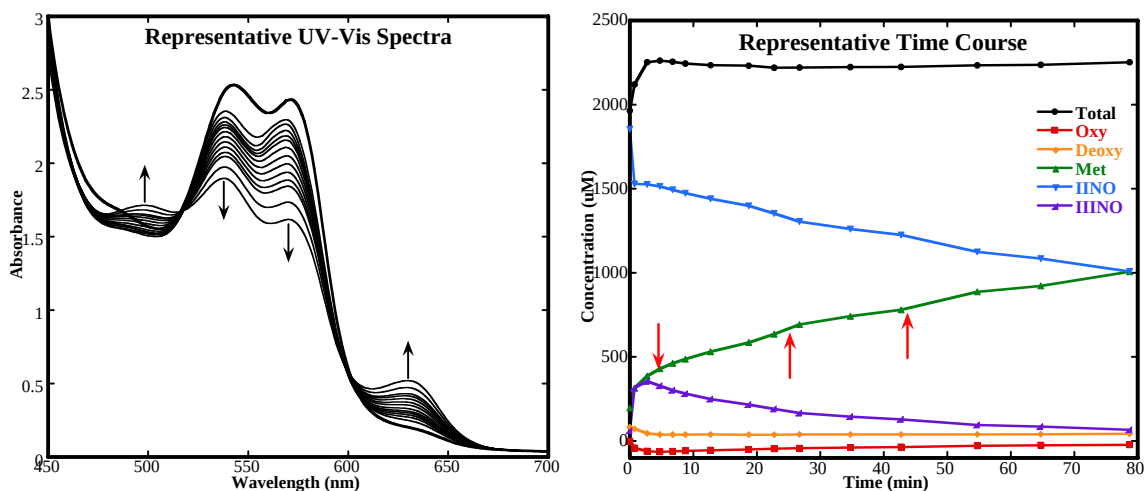


Figure 3.9 UV-Vis spectra and corresponding time course obtained from oxidation of Hb(NO)₄ with excess K₃Fe(CN)₆ under anaerobic conditions at pH 6.0. The spectra on the left correspond to the time points indicated on the right. (Left) The bold line represents Hb(NO)₄ before addition of K₃Fe(CN)₆. An immediate red shift of the HbFe^{II}NO peaks is evident just after K₃Fe(CN)₆ addition. The dramatic decrease in the region below 500 nm is due to 1) the dilution of Hb and 2) the small absorbance in this region of HbFe^{III}NO as compared to HbFe^{II}NO (see Appendix A). (Right) Least-squares analysis indicates the rapid formation of HbFe^{III}NO (purple) to a maximum of approximately 16% of the total heme concentration within the first five minutes of reaction. Points are deconvoluted data; lines connecting points are a guide to the eye. Red arrows indicate time points at which aliquots were extracted for EPR analysis.

Indeed, moving to lower pH stabilized the formation of HbFe^{III}NO to a maximum of approximately 16% of the total heme concentration. At this time point, HbFe^{II}NO has decreased by approximately 500 μM while 100 μM HbFe^{III} and 400 μM HbFe^{III}NO were generated. Due to the rate of HbFe^{III}NO formation, it is possible that the maximum HbFe^{III}NO may have been higher than that determined from UV-Vis because scans were taken every two minutes and the maximum HbFe^{III}NO concentration may have been missed.

EPR Analysis of HemeNO Identity

EPR was utilized to probe the identity of nitrosylated and oxidized hemes over the course of the reactions. In oxidation reactions of Hb(NO)_4 there are up to four EPR-detectable heme species present: $\text{HbFe}^{\text{II}}\text{NO}$ α heme (αNO), $\text{HbFe}^{\text{II}}\text{NO}$ β heme (βNO), $\text{HbFe}^{\text{II}}\text{NO}$ 5-coordinate α heme ($\alpha_5\text{NO}$), and HbFe^{III} (Figure 3.10). EPR silent species that may also be formed include HbFe^{II} , $\text{HbFe}^{\text{II}}\text{O}_2$, and notably $\text{HbFe}^{\text{III}}\text{NO}$. Before the addition of $\text{K}_3\text{Fe(CN)}_6$, Hb(NO)_4 has a characteristic EPR spectrum that is a combination of 50% αNO heme and 50% βNO . Upon oxidation by $\text{K}_3\text{Fe(CN)}_6$ in anaerobic or aerobic conditions, the overall character of nitrosylhemes changes throughout the reaction progression.

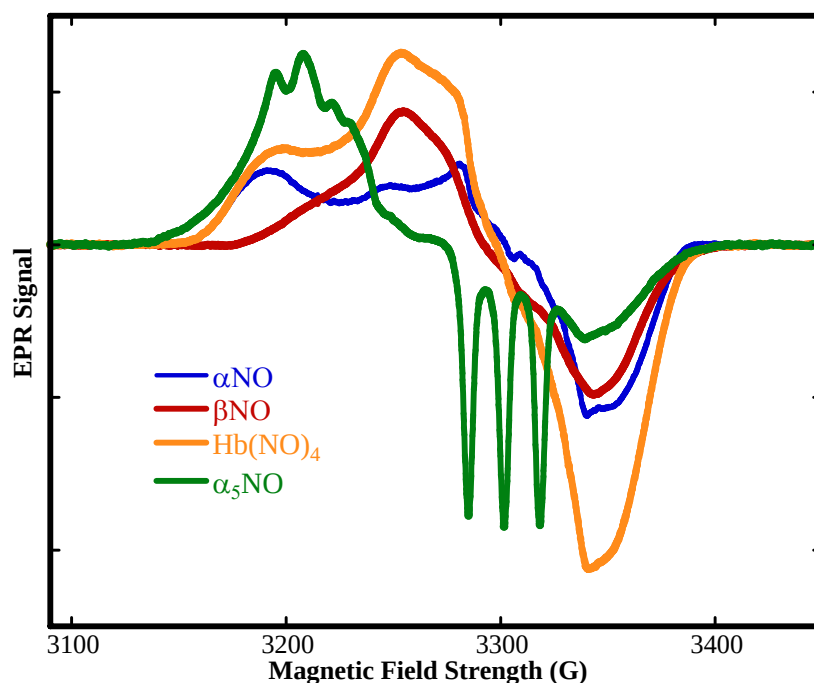


Figure 3.10 EPR spectra of Hb(NO)_4 , isolated subunits, and $\alpha_5\text{NO}$. The Hb(NO)_4 spectrum can be considered the addition of isolated βNO and αNO . The $\alpha_5\text{NO}$ spectrum is distinct with its three narrow components (resulting from hyperfine interactions).

β Hemes Are Preferentially Oxidized by $K_3Fe(CN)_6$

EPR spectra generated from oxidation of $Hb(NO)_4$ by $K_3Fe(CN)_6$ strongly suggest that $K_3Fe(CN)_6$ preferentially oxidizes β NO hemes. Both anaerobic and aerobic oxidations of $Hb(NO)_4$ by $K_3Fe(CN)_6$ in PBS buffer at pH 7.4 show a decrease in β NO contribution with reaction progression (Figure 3.11). In addition, Luchsinger and Singel have demonstrated the preference of O_2 oxidation for β NO hemes in PBS at pH 7.4 (unpublished results). Therefore, the similarity in heme-NO character among anaerobic and aerobic conditions reflects the fact that both $K_3Fe(CN)_6$ and O_2 preferentially oxidize the β hemes of $Hb(NO)_4$.

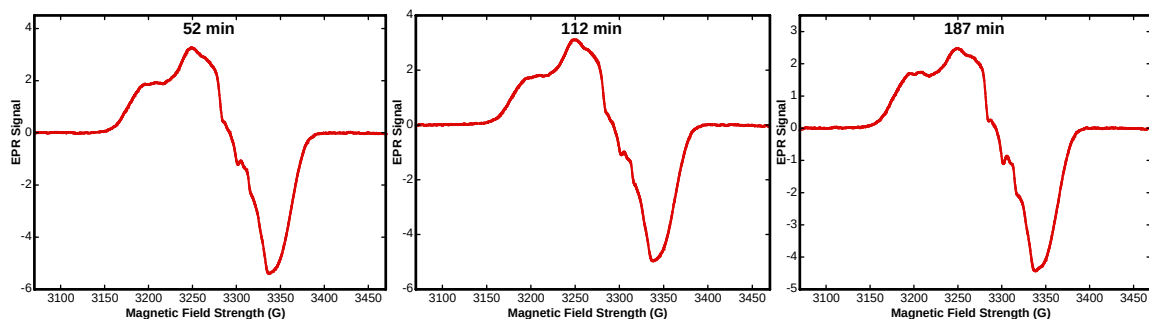


Figure 3.11 EPR spectra demonstrate preferential oxidation of β NO by $K_3Fe(CN)_6$. (1:1 heme: $K_3Fe(CN)_6$) These data correspond to the time points indicated by red arrows in Figure 3.4. Over the course of the reaction (left to right), the β spectral component diminishes, and the spectrum obtained above 50% oxidation is dominated by α NO. EPR signal decreases over time due to the loss of total hemeNO.

The preference for β heme oxidation is even more dramatic when excess $K_3Fe(CN)_6$ is employed (Figure 3.12). Again, in both aerobic and anaerobic conditions, oxidation occurs preferentially at β NO hemes. This is indicated by the rapid formation of

a strongly α NO signal (3 min). However, over time, the spectrum slowly shifts toward β NO indicating that α NOs gradually become oxidized. Thus, the EPR data from experiments with excess $\text{K}_3\text{Fe}(\text{CN})_6$ may suggest that the oxidation reaction proceeds through an initial fast step in which β NO is oxidized followed by a slower step of α NO oxidation. These results are further discussed in Chapter 4.

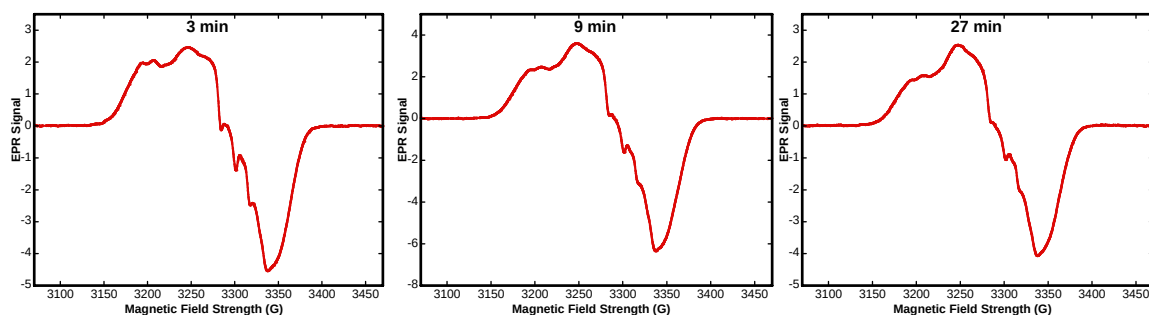


Figure 3.12 Preferential oxidation of β NO by excess $\text{K}_3\text{Fe}(\text{CN})_6$. (Aerobic conditions) These data correspond to the time points indicated by red arrows in Figure 3.8. Initially, there is a majority of α NO as β hemes have been oxidized to either HbFe^{III} (observed at lower field strength) or $\text{HbFe}^{\text{III}}\text{NO}$ (EPR silent). Over the course of the reaction (left to right), the β NO component strengthens with respect to α NO present. This may indicate slow oxidation of the α NO hemes.

The UV-Vis spectral observation of the formation of $\text{HbFe}^{\text{III}}\text{NO}$ during oxidation of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$ is supported by EPR data generated from oxidation at pH 6.0 (Figure 3.13). We were unable to detect any contribution from β NO in the samples removed from the reaction mixture for EPR analysis. Given the reaction, this strongly α NO spectrum could only be achieved from the oxidation of essentially all of the β NO to HbFe^{III} or $\text{HbFe}^{\text{III}}\text{NO}$. It is evident from the UV-Vis decomposition that only about 25% of the original $\text{HbFe}^{\text{II}}\text{NO}$ has been consumed. Therefore, β NO contribution to the spectrum

theoretically should be 25% if βNO is preferentially oxidized by $\text{K}_3\text{Fe}(\text{CN})_6$ as seen at pH 7.4. The deviation from this hypothesis may be attributable to a change in reaction dynamics associated with a reduction in pH. Acidic pH favors the presence of T state Hb as evidenced by the increase in $\alpha_5\text{NO}$ spectral contribution. However, the lack of mass balance observed by EPR of the oxidation of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$ at pH 6.0 warrants additional research.

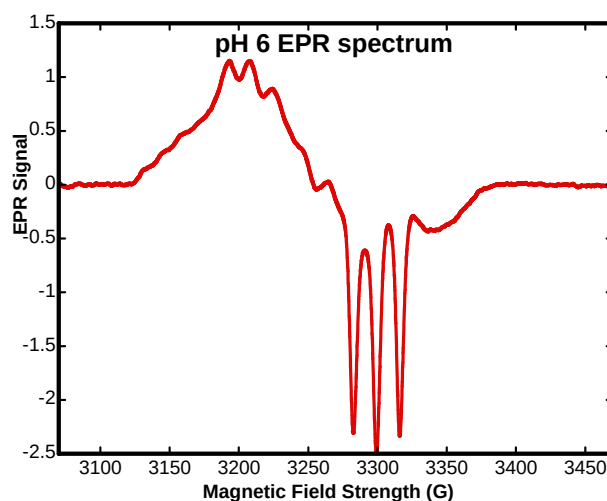


Figure 3.13 EPR spectrum of anaerobic $\text{Hb}(\text{NO})_4$ oxidation at pH 6.0 by $\text{K}_3\text{Fe}(\text{CN})_6$. The corresponding UV-Vis and time course are shown in Figure 3.9. This sample that produced this spectrum was frozen approximately 5 min after initiation of reaction by addition of $\text{K}_3\text{Fe}(\text{CN})_6$. The EPR spectrum did not change to a detectable degree over 44 min of the reaction.

Kinetic Analysis of the Oxidation of $\text{Hb}(\text{NO})_4$

A mechanistic model was developed in Gepasi and fit to the time courses generated from least-squares fitting. Though at low $\text{K}_3\text{Fe}(\text{CN})_6$ concentration it appears

as if only two heme species are at work, excess $\text{K}_3\text{Fe}(\text{CN})_6$ reveals the formation of an $\text{HbFe}^{\text{III}}\text{NO}$ intermediate.

	Reaction	Fit or Fixed	Rate Constant
R1a	$\text{HbFe}^{\text{II}}\text{NOa} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}\text{NO}$	Fit	$3.31 \times 10^{-9} (\mu\text{M}^{-1} \text{s}^{-1})$
R1b	$\text{HbFe}^{\text{II}}\text{NOb} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}\text{NO}$	Fit	$0.00171 (\mu\text{M}^{-1} \text{s}^{-1})$
R2	$\text{HbFe}^{\text{III}}\text{NO} \rightarrow \text{HbFe}^{\text{II}} + \text{nitrosation products}$	Fit	$9.43 \times 10^{-4} (\text{s}^{-1})$
R3	$\text{HbFe}^{\text{II}} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}$	Fit	$0.237 (\mu\text{M}^{-1} \text{s}^{-1})$
R4	$\text{HbFe}^{\text{II}}\text{NO} = \text{HbFe}^{\text{II}} + \text{NO}$	Fixed	Forward = $1 \times 10^{-5} (\text{s}^{-1})$ Reverse = $1 \times 10^6 (\mu\text{M}^{-1} \text{s}^{-1})$
R5	$\text{HbFe}^{\text{III}} + \text{NO} = \text{HbFe}^{\text{III}}\text{NO}$	Fixed	Forward = $1 \times 10^4 (\mu\text{M}^{-1} \text{s}^{-1})$ Reverse = $150 (\text{s}^{-1})$

Table 3.1 Reactions included in Gepasi model. Kinetic types are all “Mass Action” reversible (=) or irreversible ($\rightarrow$). Rate constants are representative of all fits and were generated using the data from pH 6.0 experiments.

A representative plot of the fit generated by this model is provided in Figure 3.13.

The model presented in

Table 3.1 suits aerobic, anaerobic, and low pH conditions. All rate constants are reasonable and match published constants where available. Notably, Reaction 2 is equivalent to the second step of the reductive nitrosylation scheme (Equation 1.3) whose published rate constant is $0.0012 \pm 0.0001 \text{ s}^{-1}$ in PBS to $0.0027 \pm 0.0001 \text{ s}^{-1}$ in HEPES (17). Upon conversion of the rate constant calculated in the model, we obtain remarkable agreement: $k_2 = 9.43 \times 10^{-4} \text{ s}^{-1}$. It should be noted that only the rate of R2 is well determined by the data. All other rate constants have little effect on the fit: they only need to be consistently larger or smaller than R2.

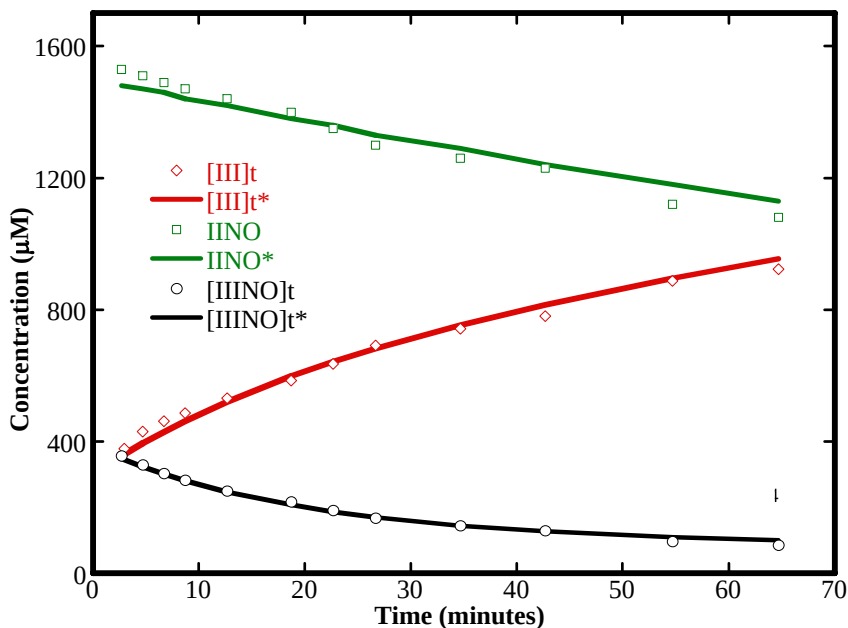


Figure 3.14 Data and fit from kinetic model for Hb(NO)_4 oxidation by excess $\text{K}_3\text{Fe(CN)}_6$ at pH 6.0. Data are represented as points while the line is the fit generated by Gepasi modeling. The reactions used for this model are given in Table 2.1.

In this *de minimis* model, the hemes are assumed to react independently in the uptake and release of NO (R4, R5), the oxidation of naked hemes by $\text{K}_3\text{Fe(CN)}_6$ (R3) and the reaction of $\text{HbFe}^{\text{III}}\text{NO}$ to yield $\text{HbFe}^{\text{II}}\text{NO}$ and nitrosation product (R2). On the other hand, a distinction is incorporated into the model in the reaction of $\text{HbFe}^{\text{II}}\text{NO}$ with $\text{K}_3\text{Fe(CN)}_6$ (R1a, R2b). At a qualitative level, the data show a rapid oxidation (R1b) by excess $\text{K}_3\text{Fe(CN)}_6$ of only 25% of $\text{HbFe}^{\text{II}}\text{NO}$. The remaining $\text{HbFe}^{\text{II}}\text{NO}$ s are oxidized, at a much slower rate.

In modeling the data obtained at pH 7.4, there was no need for the fast reaction of $\text{HbFe}^{\text{II}}\text{NO}$ with $\text{K}_3\text{Fe(CN)}_6$. This reaction (R1b) was eliminated from the model as our goal was to create the simplest set of reactions that was able to accommodate the data. A representative fit is provided in Figure 3.15.

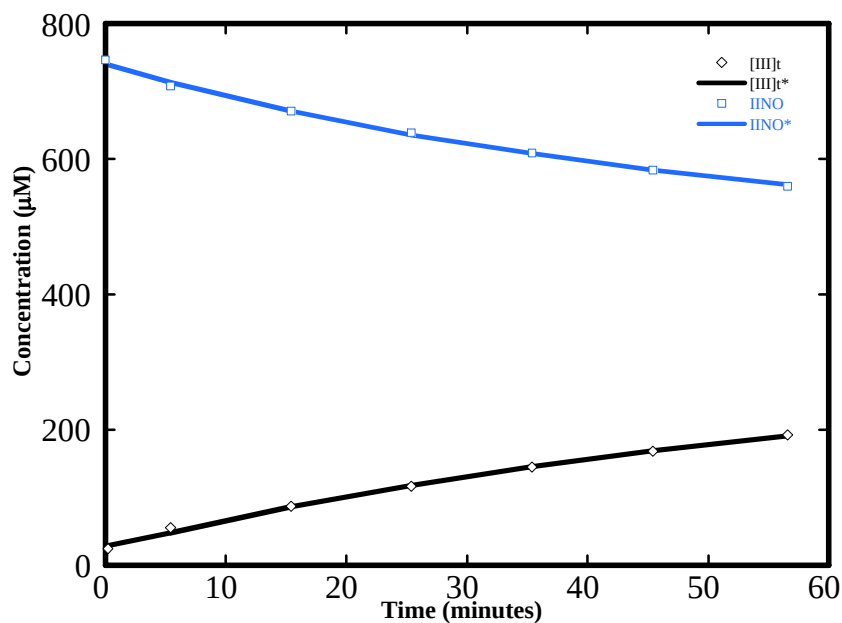


Figure 3.15 Data and fit from kinetic model for Hb(NO)₄ oxidation by limiting K₃Fe(CN)₆ under anaerobic conditions. Data are represented as points while the line is the fit generated by Gepasi modeling. The reactions used for this model are given in

Table 3.2.

	Reaction	Fit or Fixed	Rate Constant
R1	$\text{HbFe}^{\text{II}}\text{NO} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}\text{NO}$	Fit	$2.16 \times 10^{-7} (\mu\text{M}^{-1} \text{s}^{-1})$
R2	$\text{HbFe}^{\text{III}}\text{NO} \rightarrow \text{HbFe}^{\text{II}} + \text{nitrosation products}$	Fit	$0.0137 (\text{s}^{-1})$
R3	$\text{HbFe}^{\text{II}} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}$	Fit	$0.835 (\mu\text{M}^{-1} \text{s}^{-1})$
R4	$\text{HbFe}^{\text{II}}\text{NO} = \text{HbFe}^{\text{II}} + \text{NO}$	Fixed	Forward = $10^{-5} (\text{s}^{-1})$ Reverse = $10^6 (\mu\text{M}^{-1} \text{s}^{-1})$
R5	$\text{HbFe}^{\text{III}} + \text{NO} = \text{HbFe}^{\text{III}}\text{NO}$	Fixed	Forward = $10^4 (\mu\text{M}^{-1} \text{s}^{-1})$ Reverse = $150 (\text{s}^{-1})$

Table 3.2 Model equations and representative fit parameters for oxidation of Hb(NO)₄ by limiting K₃Fe(CN)₆ under anaerobic conditions.

For aerobic experiments at 1:1 heme:K₃Fe(CN)₆, we employed a reaction to account for the contribution from O₂ oxidation (Figure 3.16). This model demonstrates

that there are two distinct oxidation reactions occurring – $\text{K}_3\text{Fe}(\text{CN})_6$ and O_2 oxidation – with no interaction between the two.

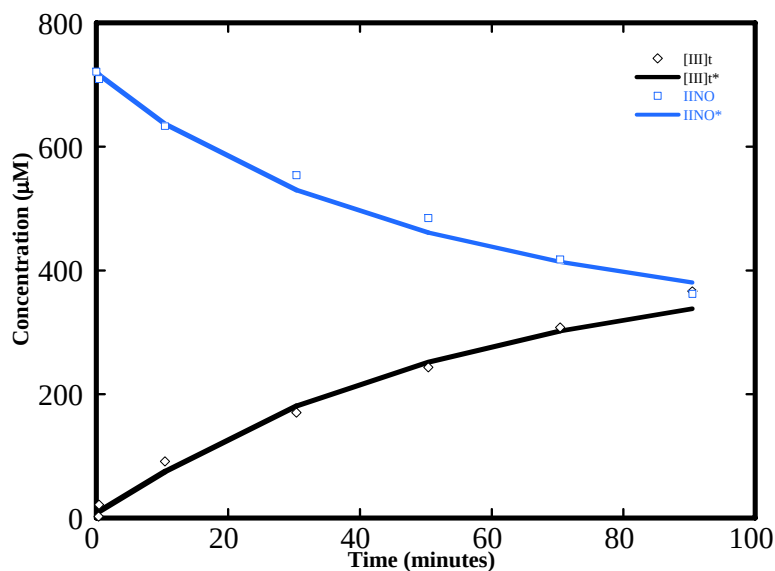


Figure 3.16 Data and fit from kinetic model for $\text{Hb}(\text{NO})_4$ oxidation by limiting $\text{K}_3\text{Fe}(\text{CN})_6$ under aerobic conditions. Data are represented as points while the line is the fit generated by Gepasi modeling. The reactions used for this model are given in Table 3.3.

	Reaction	Fit or Fixed	Rate Constant
R1	$\text{HbFe}^{\text{II}}\text{NO} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}\text{NO}$	Fit	$3.55 \times 10^{-7} (\mu\text{M}^{-1} \text{s}^{-1})$
R2	$\text{HbFe}^{\text{III}}\text{NO} \rightarrow \text{HbFe}^{\text{II}} + \text{nitrosation products}$	Fit	$0.0108 (\text{s}^{-1})$
R3	$\text{HbFe}^{\text{II}} + \text{K}_3\text{Fe}(\text{CN})_6 \rightarrow \text{HbFe}^{\text{III}}$	Fast	$0.357 (\mu\text{M}^{-1} \text{s}^{-1})$
R4	$\text{HbFe}^{\text{II}}\text{NO} = \text{HbFe}^{\text{II}} + \text{NO}$	Fixed	Forward = $10^{-5} (\text{s}^{-1})$ Reverse = $10^6 (\mu\text{M}^{-1} \text{s}^{-1})$
R5	$\text{HbFe}^{\text{III}} + \text{NO} = \text{HbFe}^{\text{III}}\text{NO}$	Fixed	Forward = $10^4 (\mu\text{M}^{-1} \text{s}^{-1})$ Reverse = $150 (\text{s}^{-1})$
R6	$\text{HbFe}^{\text{II}}\text{NO} + \text{O}_2 \rightarrow \text{HbFe}^{\text{III}} + \text{NO}_3^-$	Fit	$2.92 \times 10^{-7} (\mu\text{M}^{-1} \text{s}^{-1})$

Table 3.3 Model reactions and representative fit parameters for oxidation of $\text{Hb}(\text{NO})_4$ by limiting $\text{K}_3\text{Fe}(\text{CN})_6$ under aerobic conditions. R6 has been added to account for the contribution from air oxidation of $\text{Hb}(\text{NO})_4$.

DISCUSSION

Oxidation of Hb(NO)₄ by K₃Fe(CN)₆ Leads to SNO-Hb Formation

SNO-Hb production from the oxidation of Hb(NO)₄ by K₃Fe(CN)₆ confirms the connectivity between redox activity at the heme and thiol chemistry at Cys-β93. Mild oxidation by K₃Fe(CN)₆ is unique in its ability to form SNO-Hb – neither strong (H₂O₂) nor air (O₂) oxidation show evidence for induction of S-nitrosylation. This suggests that each type of oxidation employs a different mechanism as discussed below.

Difficulty in quantification of nitrite in the presence of K₃Fe(CN)₆ provides an intriguing possibility for quantification of HbFeNO. Due to the large spike in nitrite concentration upon addition of Hb(NO)₄ to Griess and Saville reagents incubated with K₃Fe(CN)₆, it appears that K₃Fe(CN)₆ may liberate NO from the heme after the protein denatures and the heme is no longer housed in the protein pocket. The mechanism for this action is not clear, but K₃Fe(CN)₆ coupled Griess/Saville assays may provide an additional way to quantify total HbFeNO concentration. The micromolar sensitivity of the Griess/Saville assays could provide accurate analysis of small HbFeNO concentrations that could potentially be masked by other majority species in UV-Vis spectroscopy. Further experimentation is needed in order to determine if K₃Fe(CN)₆ is able to generate nitrite from HbFeNO in stoichiometric amounts and, if so, to determine the appropriate reaction conditions for quantification of HbFeNO.

SNO-Hb generation, though correlated with HbFe^{III} production, did not constitute 50% of the non-heme NO products in any of the reactions. Reductive nitrosylation

experiments demonstrated equimolar production of SNO-Hb and nitrite from the reduction of HbFe^{III} by NO. Therefore, for each $\Delta\text{HbFe}^{\text{III}}$, we expect an equimolar distribution of SNO-Hb and nitrite, and the slope of the lines in Figure 3.6 should be 0.5.

To rationalize these results, we must first comment on the inability to absolutely quantify nitrite production. It is possible that under the reaction conditions, the nitrosonium ion preferentially reacts with OH^- in solution to produce nitrite. This is supported by the fact that nitrite and the Griess reagents were likely absorbed by and isolated in the column material, giving an artificially low nitrite concentration. Therefore, even though SNO-Hb production levels appear low, undetected nitrite production could satisfy mass balance of Equation 1.6. Additional experiments are required to understand how nitrite and the Griess/Saville reagents (SAA , Hg^{2+} , and NED) interact with the column.

Finally, higher SNO-Hb production per HbFe^{III} made under anaerobic conditions likely reflects the competition between $\text{K}_3\text{Fe}(\text{CN})_6$ and O_2 oxidation in aerobic conditions. Under aerobic conditions, air oxidation is responsible for a significant fraction of HbFe^{III} formed. This is supported by the fact that the reaction proceeds approximately twice as fast in aerobic conditions than when O_2 oxidation is eliminated. Therefore, hemes oxidized by O_2 will not form nitrite or SNO-Hb, and will, therefore, lower the ratio of SNO-Hb produced per oxidized heme. Taking this into consideration, the slopes in Figure 3.6 become approximately equal. Therefore, we reason that there is no detectable difference between anaerobic and aerobic conditions in the efficiency of SNO-Hb formation upon oxidation of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$.

K₃Fe(CN)₆ Oxidation of Hb(NO)₄: Kinetics and Mechanism

The mechanism for O₂ oxidation is well understood and involves a rate-limiting step of NO dissociation from the heme (22, 23). After dissociation, O₂ rapidly binds the heme which is immediately oxidized by NO to yield HbFe^{III} and nitrate thereby eliminating the bioavailability of NO (Equation 4.1).



This mechanism cannot account for SNO-Hb formation via oxidation by K₃Fe(CN)₆. SNO-Hb is formed by the reaction scheme provided in Equation 1.3 through Equation 1.5, thus we generated a *de minimis* model for K₃Fe(CN)₆ oxidation that involves mechanism of K₃Fe(CN)₆ oxidation must involve the generation of the nitrosonium ion (NO⁺) and subsequent SNO-Hb or nitrite products (R2) as depicted in Equation 1.3-Equation 1.5.

Together the UV-Vis and EPR data provide strong evidence for the generation of an HbFe^{III}NO intermediate. In an attempt to stabilize the observed HbFe^{III}NO intermediate, we performed the experiment at low pH and revealed the involvement of two distinct reactions of HbFe^{II}NO with K₃Fe(CN)₆: one fast and one slow (R1b and R1a, respectively). The need for both of these reactions may reflect 1) variable reactivities dependent on heme ligation. The reaction performed at pH 6.0 began with a substantial amount of initial HbFe^{III}, whereas all other reactions started with minimal (<5% total heme) initial HbFe^{III}. This may poise the Hb sample for fast oxidation, for instance, if the addition of one Fe^{III} heme per Hb tetramer fundamentally alters the

reactivity of the rest of the hemes. 2) A two-rate reaction with $\text{K}_3\text{Fe}(\text{CN})_6$ may reflect dissociation of heme from the protein. We see evidence for free heme in solution at the low field end of the EPR spectrum (Figure 3.13). These free hemes may react more quickly than those housed in the heme pocket and may account for the $\text{HbFe}^{\text{III}}\text{NO}$ formation.

The kinetic data from experiments performed at pH 7.4 can be modeled by using just the slow rate (R1b) $\text{HbFe}^{\text{II}}\text{NO}$ reaction with $\text{K}_3\text{Fe}(\text{CN})_6$. The rate constant for this step is within a factor of π for all experiments modeled – including that performed at pH 6.0. This simple *de minimis* model, therefore, is suitable in fitting the data obtained from the oxidation of $\text{Hb}(\text{NO})_4$ by $\text{K}_3\text{Fe}(\text{CN})_6$.

CONCLUSION

Results presented in this work demonstrate the formation of SNO-Hb through oxidation of human Hb(NO)₄ by K₃Fe(CN)₆, substantiating the connectivity between redox at the heme and thiol chemistry, thereby rescuing the bioavailability of NO. Furthermore, we have demonstrated by UV-Vis and EPR spectroscopies that this reaction occurs through an HbFe^{III}NO intermediate. Finally, we have developed a *de minimis* kinetic model with a calculated rate constant that agrees with published results.

APPENDICES

APPENDIX A

SPECTRAL DECOMPOSITION PROGRAM

Canonical Spectra Used to Fit Experimental Data

The spectra used for least-squares fitting analysis are shown in Figure 6.1. Derivative spectra are utilized in the fitting program to avoid mathematical difficulties associated with very similar spectra.

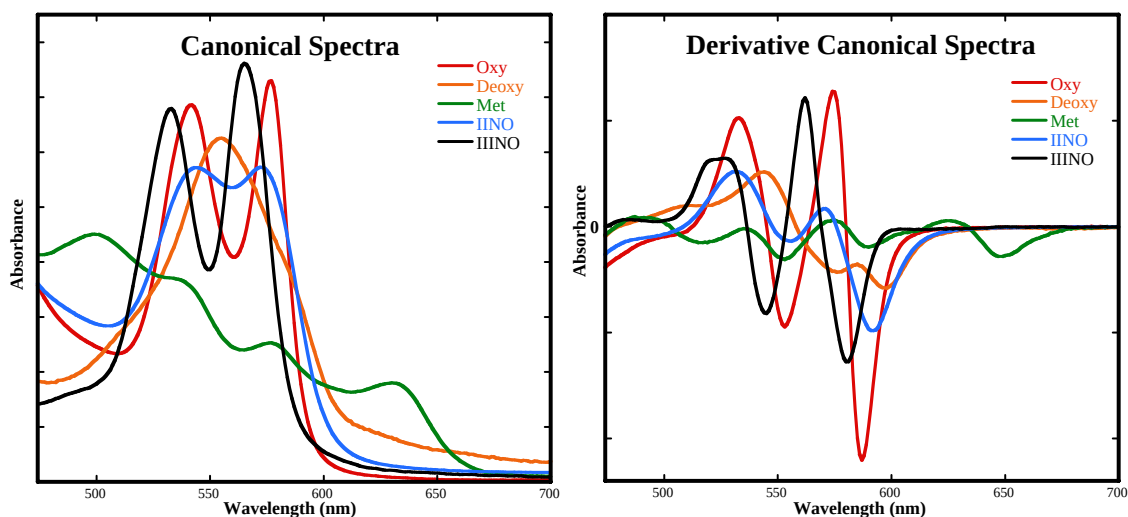


Figure 6.1 Canonical (basis) spectra for 5-component least-squares fitting analysis. The original UV-Vis spectra are shown in the left panel. Derivative spectra are shown in the right panel.

Least-Squares Fitting Program

The following is the least-squares fitting program in Mathematica[®] 5.1 used to deconvolve experimental spectra into linear combinations of five basis Hb species spectra: oxyHb, deoxyHb, metHb, HbNO, HbFe^{III}NO. By analyzing spectra taken over the course of a reaction, we generate a kinetic profile of the disappearance of reactant Hb species and the appearance of product Hb species. Red text in all capital letters are directions for which file to import.

```

<<LinearAlgebra`MatrixManipulation`
<<Graphics`MultipleListPlot`
<<Graphics`Colors`
<<Statistics`
A1=ReadList["INSERT DATA DERIVATIVE SPECTRUM TEXT FILE HERE", Number];
A=A1;
Dim = Dimensions[A]
DIM = Dim[[1]]
W = IdentityMatrix[DIM];
exp=0;

total heme;
W[[DIM-5,DIM-5]] = 10^(exp); oxy W[[DIM-4,DIM-4]] = 10^(exp); deoxy; W[[DIM-3,DIM-3]] = 10^(exp);
W[[DIM-2,DIM-2]] = 10^(exp); W[[DIM-1,DIM-1]] = 10^(exp); W[[DIM,DIM]] = 10^(exp);
EPS=Import["INSERT DERIVATIVE BASIS SPECTRA HERE", "Table"];
Dimensions[EPS]
SPE=Transpose[EPS];
g=SPE.W.EPS;
Dimensions[g]
G=Inverse[g];
c=G.SPE.W.A;
Dimensions[c];

Q=Part[c,1]*100000000;
R=Part[c,2]*100000000;
S=Part[c,3]*100000000;
T=Part[c,4]*100000000;
U=Part[c,5]*100000000;
V = Part [ c , 6 ] * 1 ;

Z = Q + R + S + T+U ;
q=Q/Z*100;
r=R/Z*100;
s=S/Z*100;
t=T/Z*100;
u=U/Z*100;
ABSTH=EPS.c;
DELTA=A-ABSTH;
Fdelta=DELTA/A;
WSQDEV=DELTA.W.DELTA
WRMSDEV=(WSQDEV/(DIM))^(1/2);

Sd11=100*(WSQDEV*G[[1,1]])^(1/2);
Sd22=100*(WSQDEV*G[[2,2]])^(1/2);
Sd33=100*(WSQDEV*G[[3,3]])^(1/2);
Sd44=100*(WSQDEV*G[[4,4]])^(1/2);
Sd55=10000*(WSQDEV*G[[5,5]])^(1/2);
Sdtot = (((Sd11)^2+(Sd22)^2+(Sd33)^2+(Sd44)^2)^(1/2)+(Sd55)^2)^(1/2) ;
Sdp1=100*(((Sd11)^2+(q*Sdtot/100)^2)^(1/2))/Z;
Sdp2=100*(((Sd22)^2+(r*Sdtot/100)^2)^(1/2))/Z;
Sdp3=100*(((Sd33)^2+(s*Sdtot/100)^2)^(1/2))/Z;
Sdp4=100*(((Sd44)^2+(t*Sdtot/100)^2)^(1/2))/Z;
Sdp5=100*(((Sd55)^2+(u*Sdtot/100)^2)^(1/2))/Z;
Sdptot = (((Sdp1)^2+(Sdp2)^2+(Sdp3)^2+(Sdp4)^2)^(1/2)+(Sdp5)^2)^(1/2) ;
Sdptot% = 100*Sdtot/Z ;

SIM=ColumnForm[ABSTH];
Type [ "mM Total"Z , "mMoxy"Q , "mMdeoxy"R , "mMmet"S , "mMHbNO"T,"uMFeIIINO"U ]
Type [ "±"Sdtot , "±"Sd11 , "±"Sd22 , "±"Sd33 , "±"Sd44 , "±"Sd55 ]
Type["WSQDEV" WSQDEV,"WRMSDEV" WRMSDEV]
Type [ "%oxy" q , "%deoxy" r , "%met" s , "%HbNO" t ,"%FeIIINO" u , "tot100%" ]
Type [ "±"Sdp1"% , "±"Sdp2"% , "±"Sdp3"% , "±"Sdp4"% , "±"Sdp5"% , "±"Sdptot"% ]
DELTAPLOT=ListPlot[DELTA];
DELTAPLOT=ListPlot[Fdelta];
DELTAPLOT=ListPlot[A];
DELTAPLOT=ListPlot[ABSTH];

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s, "%HbNO" t, "%FeIIINO" u, "sdTotal±" Sdtot, "uMoxy±" Sd11, "uMdeoxy±" Sd22, "uMmet±"
Sd33, "uMHbNO±" Sd44, "uMFeIIINO±" Sd55, "%Total±"Sdptot, "%oxy±" Sdp1, "%deoxy±" Sdp2, "%met±"
Sdp3, "%HbNO±" Sdp4, "%FeIIINO±" Sdp5, "offset" V, "WSQDEV" WSQDEV, "WRMSDEV"
WRMSDEV, Eigenvalues[G], Eigenvectors[G] , SIM } , "TABLE" ] ;

```

APPENDIX B

ASSESSMENTS OF THE CHEMISTRY AND VASODILATORY ACTIVITY OF
NITRITE WITH HEMOGLOBIN UNDER PHYSIOLOGICALLY RELEVANT
CONDITIONS (24)

My contribution to this published work included analysis of the reaction of HbFe^{II} with nitrite by least-squares fitting.



Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions

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Received 15 October 2004; received in revised form 22 November 2004; accepted 17 December 2004

Abstract

Hypoxic vasodilation involves detection of the oxygen content of blood by a sensor, which rapidly transduces this signal into vasodilatory bioactivity. Current perspectives on the molecular mechanism of this function hold that hemoglobin (Hb) operates as both oxygen sensor and a condition-responsive NO reactor that regulates the dispensing of bioactivity through release of the NO group from the β -cys93 S-nitroso derivative of Hb, SNO-Hb. A common path to the formation of SNO-Hb involves oxidative transfer of the NO-group from heme to thiol. We have previously reported that the reaction of nitrite with deoxy-Hb, which furnishes heme-Fe(II)NO, represents one attractive route for the formation of SNO-Hb. Recent literature, however, posits that the nitrite-reductase reaction of Hb might produce physiological vasodilatory effects through NO that evades trapping on heme-Fe(II) and may be stored before release as Fe(III)NO. In this article, we briefly review current perspectives in NO biology on the nitrite-reductase reaction of Hb. We report *in vitro* spectroscopic (UV/Vis, EPR) studies that are difficult to reconcile with suggestions that this reaction either generates a heme-Fe(III)NO reservoir or significantly liberates NO. We further show in bioassay experiments that combinations of nitrite and deoxy-Hb – under conditions that suppress SNO-Hb formation – exhibit no direct vasodilatory activity. These results help underscore the differences between physiological, RBC-regulated, hypoxic vasodilation versus pharmacological effects of exogenous nitrite.

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Keywords: Nitric oxide; Nitrite; S-Nitrosohemoglobin; Red blood cells; Hypoxic vasodilation

1. Introduction

1.1. Nitrite bioactivity and metabolism

The biological activity of the nitrite anion has been subject to scientific inquiry for over a century. Early

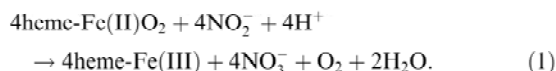
studies – recently reviewed by Marsh and Marsh [1] – were focused on the vasodilatory effects of nitrite, together with various organic and inorganic nitrites, nitrates, and nitro compounds. From a pharmacological standpoint, nitrite was found to be orders of magnitude less potent than the most bioactive substances [2,3]; accordingly interest in nitrite as a pharmacological vasodilator waned. Nitrite also attracted attention from toxicologists, who were interested in its capacity to oxidize hemoglobin [4,5], and, in a distinct research thrust, in

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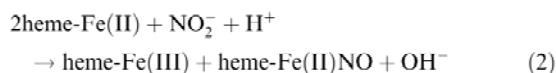
E-mail address: STAML001@mc.duke.edu (J.S. Stamler).

its capacity to be activated, for example in the gut (pH $\approx$ 1), to form nitroso compounds – some of which, for example, nitrosamines, posed a potential health risk [6]. At first glance these three areas of research would seem to have little, besides nitrite itself, connecting them. A more perceptive vision has emerged over the last 20 years, however, owing to advances in our understanding of the salutary biochemistry of nitric oxide. This new perspective supports the idea that reactions of nitrite under mildly acidic milieu – as found in airway lining fluid, on cell surfaces or ischemic tissues e.g. [51] – and with metalloproteins bearing redox active metals – can support chemical pathways that lead to the formation of bioactive nitroso-compounds, which may play a role in vascular physiology or pathophysiology.

In a 1996 review of the metabolic fate of NO and related *N*-oxides, Kelm and Yoshida [7] provided the entry level perspective on endogenous nitrite reactions. Nitrite was viewed as a marker of NOS (nitric oxide synthase) activity, being derived, via a series of reactions in oxygenated aqueous media, from the NOS product, NO. Nitrite was viewed as a metabolic dead-end; it was expected to be deactivated, primarily, by oxy-Hb mediated conversion to nitrate with accompanying formation of met-Hb:



Additionally, it could be expected that the nitrite-reductase reaction with deoxy-Hb [8] could occur, principally in venous blood, and furnish heme-Fe(II)NO – a species considered, at the outset of interest in NO biology, to represent a vestige of scavenged NO, and, like nitrate, to be a dead-end of NO bioactivity



This reaction (Eq. (2)) is thought to release NO from oxidized heme irons and through subsequent capture of the NO on vacant ferrous hemes, furnishes the heme-Fe(II)NO product. The kinetics of reaction (Eq. (1)) are complex, entailing an initial lag-phase, followed by rapid auto-catalytic product formation [9–11]. The kinetics of the relatively slow reaction (Eq. (2)) of nitrite with deoxy-Hb was examined by Doyle et al. [8], who determined the reaction to follow a simple second order rate law with specific rate constant $2.69 \text{ M}^{-1} \text{ s}^{-1}$ at 25°C and pH 7 [8].

The issue of nitrite metabolism was reconsidered in a paper by Gladwin et al., in 2000, where a *physiological* role for nitrite in vasodilation was suggested [12]. The primary basis for this suggestion was the observation of arterial-venous gradients in nitrite concentrations. The existence of such gradients was supported by earlier work of Kelm [13,14], but later questioned by Lauer

et al. [3]. Gladwin et al. [12] interpreted the putative gradient as an indication of nitrite delivery to tissues; nitrite effects were envisioned not as acting within the red blood cells, but as acting directly with target tissue, using for example acid milieu or enzymes such as xanthine oxidase to effect the conversion of nitrite to bioactive NO. By analogy to oxygen gradients, it might be conceived that decreased levels of nitrite in venous vs. arterial blood indicate a consumption of nitrite – and a corresponding generation of NO bioactivity – during the arterial-venous transit. The soundness of this interpretation relies on the similarity of the architecture of sources and sinks for oxygen versus nitrite. The overview of Kelm and Yoshida [7], however, highlights the dissimilarity: both nitrite production and consumption are dispersed throughout the cardiovascular circuit. With nitrite viewed as a marker of NOS activity, and with NOS activity greater in artery versus vein, the observed nitrite gradient would have been better understood as reflecting an arterial-venous drop in nitrite production. (On the basis of subsequent work by us [15] such a gradient might be presently understood to reflect both lower production and greater consumption of nitrite in veins (see below).) In the aftermath of this work [12], the relative vasodilatory efficacies of nitrite and other NO-compounds were re-examined [3] and the low specific potency of nitrite was reaffirmed.

1.2. Nitrite-reductase activity in NO biology

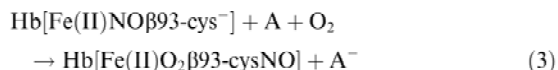
The possible importance of Hb's nitrite-reductase activity (Eq. (1)) in generating NO was underscored in a series of papers over the last decade by Reutov et al. [16–18]. They argued that the reaction of nitrite with deoxy-Hb may represent a source of NO bioactivity and may be the anaerobic counterpart of the oxygen-dependent production of NO by NOS. They have indeed suggested that more NO may be generated through this pathway than through NOS, particularly in low O_2 milieu. However, no explanation was provided in their papers for how the NO might avoid inactivation by oxy-Hb or escape heme-Fe(II); accordingly, these ideas were not incorporated into existing perspectives of NO biology.

The first potential problem that would need to be addressed in the Reutov model is the competition between the reaction of nitrite with deoxy-Hb (Eq. (2)) and oxy-Hb (Eq. (1)); the latter reaction would lead to a biologically inactive nitrate product. At nitrite:Hb ratios approaching the physiological situation, however, this reaction actually progresses very slowly compared with the NO-forming, nitrite-reductase reaction (Eq. (2)), presumably owing to lengthening of the lag phase of the oxy-Hb reaction when nitrite levels are low [15]. We have reported experimental observations in the course of Hb oxygenation/deoxygenation cycles, consistent with the

idea that the reaction with deoxy-Hb predominates over the reaction with oxy-Hb at nitrite levels approaching the physiological situation [15,19].

A more formidable problem is posed by avid uptake and tight-binding of NO to ferrous hemes: how could NO exert bioactivity? This fundamental problem has been of considerable interest to us in the context of our studies of the physiological biochemistry of red blood cell (RBC) regulation of blood flow. We have recently extensively reviewed this work [20]. In brief, the fundamental idea is that Hb in the RBC acts alternatively to store, arm, or dispense NO bioactivity in response to ambient oxygen levels. Hb thus participates in the matching of blood flow to metabolic oxygen demand – the classic physiological response of hypoxic vasodilation. In this picture, NO-bioactivity is stored, for example, as heme-Fe(II)NO and armed by conversion to a nitrosothiol, poised in an unreactive state; the “dispensing” is triggered by Hb allostery, which activates the SNO moiety for transfer/release of the NO group. Vasodilatory activity is meted out in response to metabolic oxygen-deficits, which are sensed and transduced, respectively, by the oxygen-binding and allosteric functions of Hb. The pivotal vasodilatory compound is SNO-Hb (T-state/high-spin) – a vasodilator of demonstrated high potency [21–23]. The release of NO bioactivity from SNO-Hb is linked to the transitions of Hb from its oxy- to deoxy- quaternary structures. SNO-Hb formation can be achieved through transnitrosation (e.g., from low molecular weight thiols) or via reactions with NO and other NO donor species [24] that are typically coupled to oxidative transfer of NO from the heme to the thiol.

A recent report from our laboratory on routes of SNO-Hb synthesis is especially noteworthy in the present context [15]. In a study of routes to SNO-Hb formation that involve coupling of heme-iron and NO redox chemistry, we reported the formation of SNO-Hb upon reaction of deoxy-Hb with nitrite (nitrite-reductase activity), followed by oxygenation to form the R-state of Hb, which stabilizes the nitrosothiol (Eq. (3)) [19]. The displacement of NO from Hb-Fe(II)NO formed by nitrite reduction had been briefly reported by us earlier [19]. Because of the lag between nitrite reduction and oxygenation, this reaction presumably involves oxidative transfer of the NO group from ferrous heme to thiol:



in which the electron acceptor, A, may be an oxidized heme, another metal ion, oxygen, or other, organic acceptors [20]. Reaction of heme-Fe(III)NO to furnish nitrosothiol and a remnant ferrous heme was also reported by us [15].

The reaction (Eq. (3)) may involve heme-Fe(III)/heme-Fe(II)NO hybrids that form in the nitrite reaction with deoxy-Hb (Eq. (2)) [20]. The production of SNO-Hb accompanying exposure of Hb to nitrite has since been observed by two other research groups [25]; its formation in the aftermath of the oxygenation of Hb Fe(II)NO species has also been observed in several laboratories [26,27].

This research on SNO-Hb synthesis provides a solution to the problem of bio-utilization of NO stored as heme-Fe(II)NO. Moreover, while a nitrite-reduction pathway is one of many ways to furnish heme-Fe(II)NO in vivo, it nonetheless provides a means by which the nitrite-reduction reaction of hemoglobin is coupled to the formation of a nitroso compound, SNO-Hb, which exhibits potent vasodilatory activity. It thus solves the formidable obstacle faced by Reutov. In addition, it links the three facets of nitrite biochemistry first studied – and does so within the context of an established physiological function – namely hypoxic vasodilation: the furnishing of heme-Fe(II)NO via the nitrite-reductase activity would occur in the veins and formation of SNO-Hb would be coupled to oxygenation in the lungs. RBCs replete in SNO-Hb dilate blood vessels at low $p\text{O}_2$ characteristic of tissues.

1.3. Reactivity versus bioactivity of heme-FeNO

Rifkind and co-workers [28] recently found that NO could be coaxed from solutions of deoxy-Hb and nitrite by argon sparging. They suggested that NO produced via reaction (Eq. (2)), has the possibility, at least in principle, of avoiding capture by ferrous (FeII) hemes, and thus directly exerting bioactivity. They further made the intriguing suggestion that nitrite taken up by deoxy-Hb is rapidly and extensively (in minutes) converted to an heme-Fe(III)NO species, which forms an NO-reservoir for relatively slow (tens of minutes) NO release. The basis of this idea was their observation – by means of a multi-step chemical assay – that nitrite disappeared more quickly than the heme-Fe(II)NO species was detected. Cosby et al. [25] at nearly the same time reported on infused nitrite-derived bioactivity (in tissues treated with NO synthase inhibitors), which they hypothesized to be linked to the nitrite-reductase reaction of deoxy-Hb. Like Rifkind and co-workers [28], these authors remarked on the intermediacy of heme-Fe(III)NO in this reaction in vitro. They also reported detecting in vivo the formation of heme-Fe(II)NO and SNO-Hb, which accompanied the infused nitrite-induced increase in blood flow following NO synthase inhibition. Nitrite – and S-nitrosothiols (SNOs) generated from it – have direct vasodilatory effects in tissues, and NO synthase inhibition will likely potentiate the bioactivity of nitrite by lowering the pH in the tissues thereby promoting formation of bioactive SNOs. Cosby

et al. [25] made some suggestion that free NO escaped hemoglobin in the RBCs to dilate blood vessels. The supporting in vitro evidence, however, involved entraining NO in a helium gas stream sparging an RBC solution.

In this article, we present results of in vitro studies on nitrite reactions with deoxy-Hb, in which the reaction is monitored by UV/Vis (Ultra-violet/visible) and EPR (electron paramagnetic resonance) spectroscopy. We probe specifically for the putative accumulation [28] of a heme-Fe(III)NO intermediate in advance of the generation of the heme-Fe(II)NO and met-Hb products – a species previously characterized by us in the study of the reductive nitrosylation of met-Hb [15]. Our results show that the met-heme and heme-Fe(II)NO products of the reaction are produced at rates that accurately mirror the disappearance of the deoxy-Hb reactant, with no discernible build-up of a heme-Fe(III)NO reservoir. More generally, our results show that if there is a more rapid nitrite-depleting reaction step that precedes transformation to products, it does not involve the heme irons in a manner that perturbs their spectra. These results are difficult to reconcile with the idea that heme-Fe(III)NO accumulates [28], or that NO itself significantly evades capture by ferrous hemes.

Additionally, we provide experimental results that assess the vasoactivity of nitrite/Hb under conditions that do not allow for SNO-Hb accumulation. In such bioassays, we find no vasodilatory activity attributable to nitrite/Hb. Coupled with the results of our in vitro studies, these experiments, in effect, confirm the results of multiple, previous studies that show the products of the reaction – in particular heme-Fe(II)NO and/or NO in the presence of deoxy-Hb or oxy-Hb – to be inactive as vasodilatory substances. Hb constricts blood vessels under conditions relevant to Cosby et al. [25]. Our results suggest that the in vitro effects observed in studies of Cosby et al. [25] can be ascribed to direct vasodilatory effects of hypoxia and inositol hexaphosphate (IHP), which they added in some studies. The in vivo vasodilation by infused nitrite (in the aftermath of NOS-inhibition [25]) is most likely mediated by *S*-nitrosothiols that form in the blood and/or by direct effects of nitrite-derived NO bioactivity on blood vessels.

2. Experiments and methods

Solutions of human hemoglobin A₀ (Apex Bioscience, Research Triangle Park, NC) were prepared in 100 mM Hepes, pH 7.4, as described previously [29]. Hb was deoxygenated by gentle vortexing in an ultra-high purity argon atmosphere. Reactions were conducted by delivering aliquots of similarly buffered, degassed solutions of sodium nitrite to deoxy-Hb solutions at [heme]/[nitrite]₀ ratios varying nominally

from 1.5 to 5; [nitrite]₀ is the initial concentration of the added sodium nitrite. The protein concentration was kept at ~250–300 μM in all spectroscopic experiments. The sample handling procedures during and after deoxygenation admitted small initial levels of met-Hb and oxy-Hb – typically ~6% and 3%, respectively. A very slight positive pressure of Argon was maintained on the solution during the reaction in order to mitigate introduction of atmospheric oxygen. (Occasional leakage was readily discerned by the concomitant rise of met-Hb and oxy-Hb levels; data beyond the point of such leakage was ignored in analysis.) Reaction progress was monitored by UV/Vis spectroscopy. Least-squares fitting methods similar to those described by us previously [15,29] were used to determine, from the UV/Vis spectra, the concentrations of deoxy-, oxy-, met-, Fe(II)-NO-, and Fe(III)NO-Hb in the sample at the time of each acquisition. Concentrations of met-Hb were corrected for the amounts of met-Hb and oxy-Hb initially present in the sample, as the latter species disappeared in these experiments, presumably via reaction (Eq. (1)) and/or the oxy-Hb reaction with NO [29]. For Fe(II)-NO-Hb, we employed spectra of authentic samples of Hb(NO)₄ and Hb[α(NO)₂β(deoxy)₂] (B.P. Luchsinger, D.J. Singel, unpublished results) [30] to determine the amounts of six-coordinate, and five-coordinate heme-Fe(II)NO complexes, respectively [27]. The particular methods that we use to improve the performance of the spectral decomposition have been noted previously. These methods include: fitting of numerically differentiated data; constraining the adjustable parameters to non-negative values; and non-uniform weighting of the data. Robust measures of, for example, met-Hb levels are facilitated by attaching extra weight to the spectral region ~647 nm, where, in the derivative spectrum, the signal is dominated by met-Hb. We estimate uncertainties in the determination of the concentrations from the variation of the concentrations estimates in multiple fits obtained when random noise – of amplitude approximately equal to the root-mean-squared deviation in the best fit – is added to the data. Typical uncertainties are tens of μM. At selected time points, samples of the reacting mixture were taken and were frozen by immersion in liquid nitrogen for characterization by EPR spectroscopy [31–34]. EPR spectra were recorded with a Varian E-109 X-band spectrometer that has been modified by the addition of a field-control unit obtained through the University of Denver [35]. Samples were immersed in liquid nitrogen during the EPR measurements. EPR spectra of the samples generally included high- and low-spin met-Hb components [36], as well as features assignable to Fe(II)NO-Hb. The latter spectra were examined for three components, reflective of NO occupying alternatively α- or β-subunits (with 6-fold coordination) [37–39], or the α-subunits with 5-fold coordination. The spectrum of the latter species is

characterized by a sharp triplet hyperfine structure [37]. The EPR spectra thus provide a check on the UV/Vis analysis of the coordination numbers of heme-Fe(II)NO species.

Organ chamber bioassay experiments were performed as previously described [19,22]. Rabbit aortic rings were mounted in a tissue bath through which a gas mixture was bubbled. The mixture consisted of 5% CO₂, ~2–20% O₂, with the balance N₂. Vessels were pre-contracted to 2 gm tension with phenylephrine. The response of the vessel tension to Hb, nitrite and IHP (inositol hexaphosphate) added in various combinations was monitored.

3. Results and discussion

3.1. *In vitro* reactions

Exemplary UV/Vis spectra recorded during the reaction of a slight stoichiometric excess of nitrite with deoxy-Hb are shown in Fig. 1 (left), together with the concentration versus time profiles obtained by decomposition of the spectra (right). Similar data were obtained in five experiments conducted at heme:nitrite ratios varying from 5 to 1.5. Several features of this reaction are readily discerned by inspection of these data. First, the profiles reveal that the disappearance of the deoxy-Hb reactant and the appearance of the met-Hb and Fe(II)NO-Hb products occur at very similar rates. The time-scale of the reaction is similar to the slow evolution toward Fe(II)NO observed by Rif-

kind and co-workers [28]; there is no evidence in the UV/Vis spectra for any substantially faster process that precedes heme-Fe(II)NO formation. Second, there is no evidence at any point in the profiles for heme-Fe(III)-NO. Whatever chemical reactions may be involved in the fast process reported by Rifkind and co-workers [28], they do not lead to the appearance a spectrum attributable to this species intermediate, nor do they lead to perturbations of the deoxy-Hb spectrum. It is thus unlikely that this process involves direct interactions between nitrite and the heme irons.

The only intermediate species, whose concentration grows then ebbs, in the profiles is the five-coordinate Fe(II)NO-Hb. This species would be expected to be found when Fe(II)NO-Hb is present in the α -subunits of Hb in its T quaternary state (with, principally, unliganded, reduced heme-irons). As the reaction progresses, however, the hemes are occupied or oxidized and the protein switches to its R quaternary state where the Fe(II)NO-Hb species are six-coordinate. The EPR spectra displayed in Fig. 2 show a progression in the amplitude of the triplet hyperfine structure characteristic of five-coordinate ferrous nitrosyl Hb that broadly qualitatively corroborates the analysis of the UV/Vis spectra. The detection of this minority, intermediate species analysis gives a sense of the overall quality of the analysis and thus provides a further check on the overall soundness of the analysis of the UV/Vis spectra – in particular the capability to detect an intermediate with a relatively small concentration.

Finally, the met- and iron(II) nitrosyl-Hb products are not formed in precisely equal amounts, commensu-

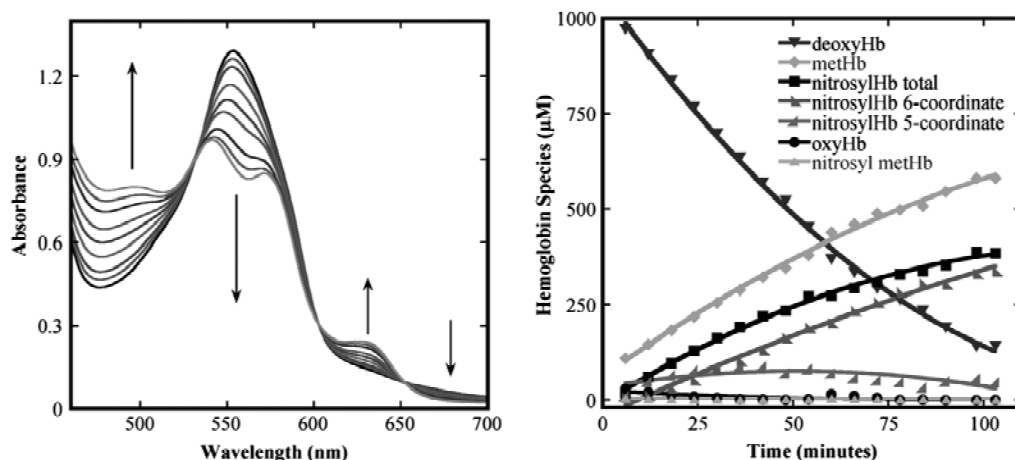


Fig. 1. Kinetics of nitrite reaction with deoxy-Hb in aqueous solution ~1 mM in heme and 0.67 mM nitrite, 100 mM Hepes, pH 7.4, 150 mM chloride. (Left) Time series of visible spectra of the reacting solution. The depicted spectra were initiated at the following time intervals after mixing: 6, 12, 18, 30, 42, 54, 72, 80, 108 min. The vertical arrows situated at various wavelengths indicate the direction of the trend in absorbance with time at the respective wavelength. (Right) Concentration versus time profiles for the indicated Hb compounds. Concentrations at each depicted point were determined by least-squares analysis of the corresponding visible absorption spectra, the smooth curves are a guide to the eye.

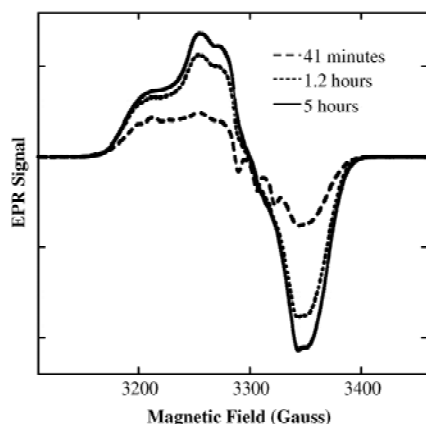


Fig. 2. EPR spectra of samples drawn from the reaction mixture of Fig. 1, at the indicated time intervals: 41 min (dashed line); 1.2 h (dotted line); 5 h (solid line). EPR spectra of samples at 76 K were obtained at 9.29 GHz, with ~ 10 mW incident power and field modulation of 10 G at 100 kHz. The external field was scanned over a range of 400 G in 2 min with a detection time constant of 0.128 s. The spectra obtained are of Fe(II)NO-Hb, and show the triplet hyperfine structure characteristic of five-coordinate (α -subunit) Fe(II)NO-Hb species only at the earliest time; at the later times the spectrum is six-coordinate Fe(II)NO-Hb on (α -subunit and β -subunits).

rate with the stoichiometry of (Eq. (2)). Instead, met-Hb is formed in some excess over Fe(II)NO-Hb. This behavior was previously investigated by Doyle et al. [8], who attributed it to NO-induced oxidation of ferrous hemes. Working with nitrite in greater than 10-fold excess over heme, this group observed the formation of met-Hb over Fe(II)NO-Hb in the ratio of $\sim 2.6:1$, with essentially no pH dependence over the pH range 6–8. We obtain lower ratios, but observed some variation, from ~ 1.1 to 1.6, roughly following the heme:nitrite ratios (linear correlation coefficient $R = 0.72$, $n = 5$). The departure of this ratio from the 1:1, as predicted by (Eq. (2)), may stem from NO-assisted heme-Fe(II) oxidation [8,29] or from the formation of products other than heme-Fe(II)NO (e.g. SNO-Hb). It has also been suggested to us (T. Yonetani, personal communication) that met-Hb formation during exposure of ostensibly deoxy-Hb solutions to NO may stem from the presence of trace oxygen in the solution. Although a similar effect might be at work in this reaction, we found no correlation between the product ratios and initial oxy-Hb or met-Hb levels (linear correlation coefficient, $R = 0.3$ and 0.2, respectively, $n = 5$).

Within experimental error, the only persistent species in the reaction medium are residual unreacted deoxy-Hb, Fe(II)NO-Hb and met-Hb; the production of the latter two matches the loss of the reactants, deoxy-Hb and nitrite. We explored the idea that some NO might simply escape the putative heme-Fe(III)NO intermedi-

ate and furnish only the met-Hb, not the Fe(II)NO-Hb product, by examining the correlation between the initial nitrite levels and the consumption of deoxy-Hb. According to (Eq. (2)), each reacted nitrite consumes two deoxy-hemes; in the limit of complete escape of NO only one deoxy-heme would be consumed. In the subset of experiments in which the nitrite reagent was limiting, we found an excellent correlation between initial nitrite and *half* of the deoxy-Hb consumed (slope = 0.97 ± 0.07 , $R = 0.96$, $n = 4$). The observation of NO liberation in experiments by Cosby et al. [25] and Rifkind and co-workers [28] involved subjecting the samples to an inert gas sparge – a common tool for degassing biological samples.

The production of excess met-Hb precludes a global kinetic analysis of the concentration vs. time profiles according to the simple reaction of (Eq. (2)). Nevertheless we can put our observations regarding the similarity of the rates of the of reactant loss and product formation on a more quantitative basis, by comparing the rates observed over the course of the reaction, for all of the kinetic trials. This comparison is shown in Fig. 3, where the rates of formation of the met-Hb and Fe(II)NO-Hb products are plotted versus the rate of deoxy-Hb disappearance. The correlation underscores the contemporaneous nature of the production and loss processes, and lack of an intermediate reservoir involving a heme-species.

The precision of these experiments does not eliminate entirely the possibility that NO products other than

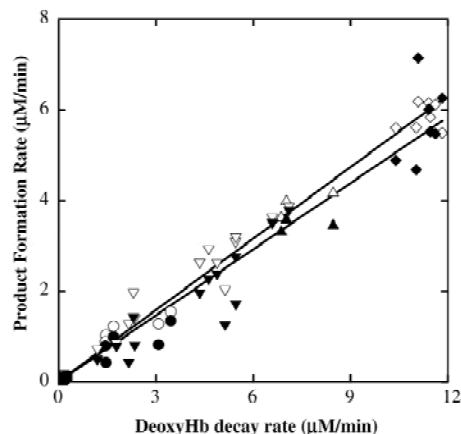


Fig. 3. Comparison of rates of deoxy-Hb disappearance and Fe(II)NO-Hb and met-Hb appearance. Rates are measured directly from the tangents to the concentration versus time profiles (Fig. 1, left), in five trials with heme:NO ratios of 1.5:1 (◆), 2:1 (■), 3:1 (▼), 3:1 (▲), and 5:1 (●). Open symbols pertain to met-Hb production; closed symbols to Fe(II)NO production. The lines are least-squares best-fit lines with slopes of 0.53 ± 0.01 ($R = 0.99$, open symbols) and 0.49 ± 0.01 ($R = 0.97$, closed symbols). Throughout the reaction the rates, balanced by the stoichiometry of (Eq. (1)), are equivalent.

heme-Fe(II)NO are formed in the reaction. They do show, however, that under our conditions any such leakage is too small to detect. This result, together with the slowness of the reaction, is very difficult to reconcile with the idea that this reaction produces prompt vasodilation during arterial-venous transit (lasting seconds or less). Some reservoir that collects these products for controlled release on subsequent transits would seem to be required. The idea of Rifkind and co-workers [28], that heme-Fe(III)NO could serve as this reservoir, however, is not consistent with our results. Moreover, there is no foundation for the idea that release of bioactivity from this species is controlled. Our original report [15,19] that the nitrite-reductase reaction of Hb provides a route to SNO-Hb synthesis, solves this problem. To the extent that the nitrite-reductase activity of Hb is an important source of the NO-group in the red blood cell, bioactivation by conversion of heme-iron NO species to SNO-Hb, generates a reservoir that can dispense the NO-group in an oxygen-dependent, Hb-allosterically controlled fashion.

3.2. Bioassay experiments

At physiological concentrations found in the blood ($<1 \mu\text{M}$), nitrite alone shows no vasoactivity in bioassays (e.g. see Fig. 4). We conducted experiments to test for the possibility that vasodilatory activity could be produced through bioactivation of nitrite (100 nM –

$1 \mu\text{M}$) by Hb (5 – $20 \mu\text{M}$) at low partial pressure of oxygen ($p\text{O}_2$) ($\sim 2\% \text{ O}_2$, a value selected to mirror the conditions of Cosby et al. [25]). In Fig. 4, we show bioassay data obtained with a Hb extract from red blood cells. Nitrite alone had no effect on vessel tension in the bioassay, while the hemoglobin alone potently constricted blood vessels. This constriction was observed, with no further change in the tension, when both Hb and nitrite were combined in the bath – independent of the order of addition of the compounds (Fig. 4, and data not shown). The addition of nitrite following hemoglobin, which induces vasoconstriction by sequestering endogenous NO, simulates the conditions in Cosby who added NOS inhibitors in their experiments. Nitrite had no effect on vessel tone under these conditions. Cosby et al. [25] reported that the allosteric effector inositol hexaphosphate (IHP, $100 \mu\text{M}$) potentiated the vasodilatory activity of nitrite in the presence of Hb by as much as 10,000-fold. This result is particularly striking, inasmuch as, if attributed to reacted nitrite, it implies a potency greater than NO per se. In experiments on purified hemoglobin, we found that IHP slightly attenuates the constriction induced by either hemoglobin or Hb/nitrite under hypoxia (Fig. 5). That is, the effect of IHP was independent of nitrite. Analogous results were obtained with lower ($5 \mu\text{M}$) Hb concentrations. In control experiments, IHP itself was observed to relax blood vessels independently of Hb/nitrite, with its vasodilatory activity potentiated at low $p\text{O}_2$ (Fig. 5). Collectively, these results indicate that un-

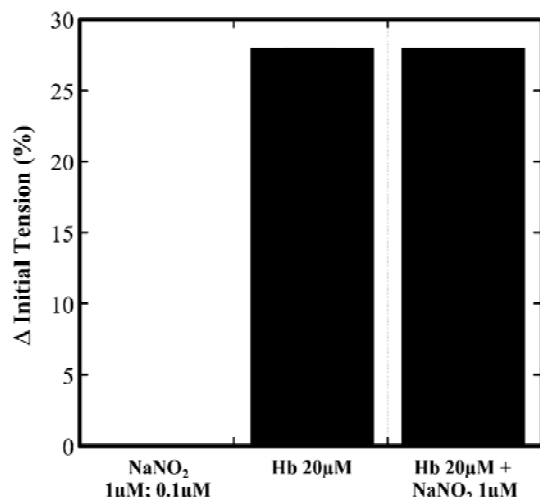


Fig. 4. Effects of Hb (partially purified from RBCs) and NaNO₂ on vessel tone. Rabbit aortic rings were pre-contracted to 2 gm tension in 2.8% O₂. Percentage increase in tone (contraction of blood vessels) relative to initial tone under the indicated additions of Hb and/or NaNO₂ is tabulated. Identical results were obtained when Hb was added alternatively before or after nitrite. NaNO₂ alone did not alter baseline tone either at 0.1 or 1 μM.

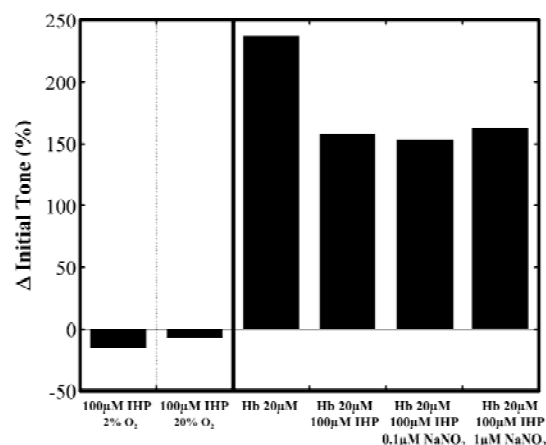


Fig. 5. Effects of Hb A₀, NaNO₂ and IHP on vessel tone. Rabbit aortic rings were pre-contracted to 2 gm tension in 2% O₂. Control experiments with IHP alone were conducted in atmospheres of 2% and 20% O₂. Percentage increases in tone relative to basal tone (contraction) under the indicated additions of Hb and/or NaNO₂ are tabulated in the right side of the figure. The negative values represent % decrease in initial tone (relaxations). The effects of IHP alone can be compared only qualitatively to those in the presence of Hb as the initial tone is substantially increased by Hb.

der physiologically relevant conditions – heme excess over nitrite and low oxygen tensions/saturations – the interaction of nitrite and deoxy-Hb does not involve or produce any species that exhibits direct vasodilatory activity. While IHP does induce relaxation, in an oxygen-dependent fashion, it does so independently of both Hb and nitrite. The addition of a vasodilator such as IHP to vessels that are already relaxing to hypoxia would enhance relaxation, as observed by Cosby et al. [25], but this relaxation is not connected to Hb or Hb allostery.

These results are difficult to reconcile with the idea that the nitrite-reductase activity of deoxy-Hb, without the intermediate formation of a vasodilator such as SNO-Hb, can produce vasodilation [25]. To extend these results, we exposed RBCs to nitrite under conditions that variably suppress or enable production of SNOs, and found that vasodilatory activity is correlated directly with SNO-Hb content (to be published). Taken as a whole, our work [15,19,20,40] points to a pivotal transformation accompanying oxygenation, in the aftermath of any nitrite-reductase activity, that converts heme-iron NO to nitrosothiols, and thus arms the protein for vasodilatory activity.

3.3. Physiological context of the nitrite-reductase activity of hemoglobin

How can this perspective on nitrite reduction by deoxyHb and the specific results presented here be reconciled with the reports of Cosby et al. [25] and Rifkind and co-workers [28]? We suggest that, in a broad sense, we have in fact obtained the same salient results. Nitrite has not been shown to alter the rate or extent of relaxation by RBCs or hemoglobin in any study (under either static or monotonically declining oxygen tension). These are the signature requirements of NO-mediated relaxations. The vasodilation observed in Cosby et al. [25] at low pO_2 exhibited a half-time of hundreds of seconds. Slow relaxations of this type (over many tens to hundreds of seconds) are characteristic of hypoxia per se, not of NO-induced relaxations. Slow relaxations are also incompatible with hypoxic vasodilation in vivo, where arterial-venous transit times are a few seconds or less. Further, the in vivo effects of nitrite infusion (in the aftermath of NOS-inhibition) are a pharmacological effect that does not show the requisite features of hypoxic vasodilation (see below). It will be interesting to elucidate whether this vasodilation by supplemented nitrite is mediated through direct effects of nitrite-derived NO bioactivity, in particular by NO/SNO in the tissues and/or through SNOs that form in the blood, for example, through acidification that likely accompanies the use of NOS inhibitors, or through processing of hemoglobin (i.e. SNO-Hb formation), as we have previously described [20].

In closing, we add a few cautionary notes pertinent to the study of reactions of NO-reagents in bioassays under hypoxic conditions. In organ chamber bioassays, the standard protocol for assessment of vasoactivity involves a pre-constriction of blood vessels to a preset initial tone. With this control, comparative vasodilatory activity can be gauged directly from the changes in tension. Allowing the initial tension to vary from experiment to experiment greatly complicates the analysis, given the non-linearity of the vessel's elastic response. Data presented simply as a percentage of variable initial tone [25] are not interpretable. Similarly, the conventional approach to assess effects of hypoxia, involves comparing responses at selected, fixed pO_2 s. Comparison of relaxations in vessels that are exposed to progressive declines in pO_2 [25], again should be avoided, inasmuch as the rate of decline is difficult to control adequately. More importantly, it is not the rate of decline in chamber pO_2 that determines the rate and extent of relaxation, but rather the pO_2 in the muscle interior – a quantity whose connection to chamber pO_2 varies from strip to strip (as a function of muscle thickness, adventitial content, deoxygenation rate, etc.). Without core pO_2 measurements, the bioassay method recently introduced [25] does not provide reliably interpretable data.

We also comment on the recent confusion in the NO literature surrounding the term hypoxic vasodilation. This term has a specific physiological definition in vivo; it does not simply refer to any arbitrary mechanism by which blood vessels might dilate at low pO_2 . In assessing effects of nitrite, in particular, it is important to distinguish the classical response of hypoxic vasodilation from infused nitrite-mediated increases in blood flow [25] that are associated with *increases* in venous O_2 saturation. In hypoxic vasodilation, increased blood flow is accompanied by *decreased* venous O_2 saturation [20,41–43], and should not be conflated with pharmacological vasodilation in which venous O_2 saturation rises. NOS inhibition, which acutely depletes endogenous nitrite [3,44], does not block exercise induced hypoxic vasodilation [25,45]; it may, however, sensitize tissues to nitrite, through multiple routes, for example, by lowering the pH of tissues. Nitrite infusion does not increase exercise induced hypoxic vasodilation [25,40]. Hypoxic vasodilation by native RBCs does not require addition of exogenous nitrite [19,46]. Each of these responses militates against a direct role for nitrite in physical hypoxic vasodilation.

Collectively, our bioassay results are consistent with multiple previous studies which indicate that the products expected when deoxy-Hb reacts with nitrite are unreactive [47–50] and do not relax blood vessels, unless production of SNO is enabled. Further, it is unlikely that quantities of nitrite sufficient to enable vasodilation can enter the RBC, or enough NO be formed and diffuse out of the RBC within the time that blood transits the arteriolar microcirculation.

4. Conclusions

The molecular basis of hypoxic vasodilation in vivo – that is, vasodilation that is coupled to and reflected in decreases in venous oxygen saturation of Hb [20,40] – involves detection of the oxygen content of blood by a sensor which rapidly transduces this signal into vasodilatory bioactivity [20]. Within the SNO-Hb paradigm, Hb functions as the sensor and as a condition-responsive reactor that regulates the dispensing of bioactivity through parsimonious net-release of the NO group from SNO-Hb. The reaction of nitrite with deoxy-Hb represents an attractive, though not an exclusive pathway for the formation of SNO-Hb [20]. This picture resolves the obstacles that led to the discounting of ideas by Reutov and Sorokina [17], who first suggested a potential significance of the nitrite-reductase activity of Hb in NO biology. The idea reprised in very recent literature [25,28] – that this “catalytic” pathway acts independently of SNO-formation in hypoxic vasodilation restores the obstacles to prominence; moreover the physiological signature of hypoxic vasodilation is absent. In this article, we demonstrated that the idea that NO is directly released from the Hb from a rapidly formed Fe(III)NO reservoir cannot be reconciled with direct spectroscopic measurements. We further showed with bioassay, that combinations of nitrite and deoxy-Hb – under conditions favoring production of Fe(II)NO (heme $\gg$ nitrite) – exhibit no direct vasodilatory activity. Our results help underscore the differences between physiological hypoxic vasodilation by RBCs versus pharmacological vasodilation by exogenous nitrite.

5. Abbreviations

Hb	human hemoglobin
SNO	S-nitrosothiol
SNO-Hb	S-nitroso derivative of human hemoglobin nitrosylated at β -cys93
oxy-Hb	oxyhemoglobin
met-Hb	methemoglobin
deoxy-Hb	deoxyhemoglobin
RBC	red blood cell
IHP	inositol hexaphosphate
NOS	nitric oxide synthase
UV/Vis	ultraviolet–visible
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Acknowledgments

This work was supported by the National Institutes of Health (HL52529, HL59130, and HL66179-02), the

ALS Association, and the National Science Foundation (MCB S 00981228).

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