The New Chemical Biology of Nitrite Reactions with Hemoglobin: R-State Catalysis, Oxidative Denitrosylation, and Nitrite Reductase/Anhydrase

MARK T. GLADWIN,*,†,‡ ROZALINA GRUBINA,†,§ AND
MICHAEL P. DOYLE⊥

†Pulmonary and Vascular Medicine Branch, National Heart Lung and Blood
Institute, National Institutes of Health, Bethesda, Maryland 20892, ‡Critical Care
Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland
20892, §Howard Hughes Medical Institute−National Institutes of Health Research
Scholars Program, Bethesda, Maryland 20814, ⊥Department of Chemistry and
Biochemistry, University of Maryland, College Park, Maryland 20742

CONSPECTUS

Because of their critical biological roles, hemoglobin and myoglobin are among the most extensively studied proteins in human history, while nitrite tops the list of most-studied small molecules. And although the reactions between them have been examined for more than 140 years, a series of unusual and critical allosterically modulated reactions have only recently been characterized. In this Account, we review three novel metal- and nitrite-catalyzed reaction pathways in the context of historical studies of nitrite and hemoglobin chemistry and attempt to place them in the biological framework of hypoxic signaling.

Haldane first described the reaction between nitrite and deoxymyoglobin, forming iron-nitrosylated myoglobin, in his analysis of the meat-curing process more than a century ago.

The reaction of nitrous acid with deoxyhemoglobin to form nitric oxide (NO) and methemoglobin was more fully characterized by Brooks in 1937, while the mechanism and unusual behavior of this reaction were further detailed by Doyle and colleagues in 1981. During the past decade, multiple physiological studies have surprisingly revealed that nitrite represents a biological reservoir of NO that can regulate hypoxic vasodilation, cellular respiration, and signaling. Importantly, chemical analysis of this new biology suggests a vital role for deoxyhemoglobin- and deoxymyoglobin-dependent nitrite reduction in these processes.

The use of UV−vis deconvolution and electron paramagnetic resonance (EPR) spectroscopy, in addition to refined gas-phase chemiluminescent NO detection, has led to the discovery of three novel and unexpected chemistries between nitrite and deoxyhemoglobin that may contribute to and facilitate hypoxic NO generation and signaling. First, R-state, or allosteric, autocatalysis of nitrite reduction increases the rate of NO generation by deoxyhemoglobin and results in maximal NO production at approximately 50% hemoglobin oxygen saturation, which is physiologically associated with greatest NO-dependent vasodilation. Second, oxidative denitrosylation of the iron−nitrosyl product formed in the deoxyhemoglobin−nitrite reaction allows for NO formation and release in a partially oxygenated environment. Finally, the deoxyhemoglobin−nitrite reaction participates in a nitrite reductase/anhydrase redox cycle that catalyzes the anaerobic conversion of two molecules of nitrite into dinitrogen trioxide (N_2O_3). N_2O_3 may then nitrosate proteins, diffuse across hydrophobic erythrocyte membrane channels such as aquaphorin or Rh, or reconstitute NO via homolysis to NO and $NO_2^•$. Importantly, the nitrite reductase/anhydrase redox pathway also represents a novel mechanism of both anaerobic and metal-catalyzed N_2O_3 formation and S-nitrosation and may thus play a vital role in NO-dependent signaling in a hypoxic and heme-rich environment. We consider how these reactions may contribute to physiological and pathological hypoxic signaling.
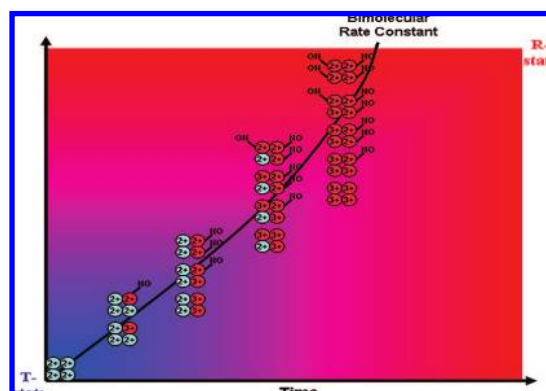
Historical Overview: The Reaction of Nitrite and Hemoglobin in the Absence of Oxygen

The reaction of sodium nitrite with oxyhemoglobin was first described almost 140 years ago by Gamgee.[1] John Haldane then reported the reaction of nitrite with deoxyhemoglobin, which generated iron−nitrosyl-hemoglobin, in his manuscript "The Red Colour of Salted Meat" examining the chemistry of meat curing.[2]

On cutting into pieces of uncooked salted meat, obtained from the butcher, I noticed that the colour of the exposed surface was bright red wherever the salt had penetrated, but slowly became dull on exposure to the air. On the other hand, if the salt had not thoroughly penetrated to the centre of the piece the exposed surface had at first the dull bluish tint of reduced haemoglobin, which soon changed into the red of oxyhaemoglobin on exposure to the air.

On extracting the freshly exposed salted part with water the red pigment was found to be quite soluble, and to give a spectrum not altered by warming the solution with ammonium sulphide, and possessing two absorption bands at about the position of the oxyhaemoglobin bands, but not nearly so well defined. This spectrum was found to be identical with that of nitric oxide haemoglobin...and the behaviour of the pigment in all other respects showed that it was nothing else but pure NO-haemoglobin. This substance, which was discovered by Herman in 1869, has hitherto not attracted much attention, as it was supposed only to occur as a laboratory product.[2]

Haldane concluded that nitrite was formed inside the raw meat via the reduction of nitre (nitrate) and that "NO-haemoglobin is formed by the action of nitrite on haemoglobin in the absence of oxygen, and in the presence of reducing agents."[2] This is the first known account describing deoxyhemoglobin-mediated reduction of nitrite to NO, leading to the formation of iron−nitrosyl-hemoglobin.

In 1937, Brooks presented his detailed analysis of the deoxyhemoglobin−nitrite reaction just as we might describe it today:

Much work has been done on the action of nitrite on haemoglobin, but there is little agreement on the course of the reaction. In the presence of a reducing agent Haldane (1901) detected only the formation of NO-haemoglobin (the compound formed by the action of nitric oxide on haemoglobin); in the presence of oxygen a mixture of methaemoglobin and NO-haemoglobin was apparently obtained (cf. Haurowitz 1924; Meier 1925).[3]

Brooks then examined the anaerobic reaction of nitrite and deoxyhemoglobin in the presence and absence of reducing agents and characterized the product yields and pH dependence. He reported that 1 mol of protonated nitrite (HNO_2; nitrous acid) and 2 mol of ferrous deoxyhemoglobin ($HbFe^{2+}$) generate 1 mol of methemoglobin ($HbFe^{3+}$) and 1 mol of iron−nitrosyl-hemoglobin ($HbFe^{2+}$−NO). He concluded that nitrous acid reacts with deoxyhemoglobin to form NO and methemoglobin (eq 1). The NO then binds a second deoxyhemoglobin to form iron−nitrosyl-hemoglobin (eq 2).

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^- \quad (1)$$

$$NO + HbFe^{2+} \rightarrow HbFe^{2+}-NO \quad (2)$$
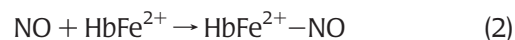
However, a number of unusual features of this reaction remained unsolved. For example, inositol hexaphosphate (IHP) was shown to significantly decrease the rate of hemoglobin oxidation by nitrite.[4,5] Because IHP lowers hemoglobin oxygen affinity, this result was interpreted as an indication that deoxyhemoglobin oxidation by nitrite was favored by higher oxygen affinity states of hemoglobin.[4,5]

In 1981, Doyle and colleagues revisited the deoxyhemoglobin−nitrite reaction and identified additional unusual properties of nitrite reduction. While the reaction appeared to follow a second-order rate law, with a rate constant (k_{obs}) of 2.69 M^{-1} s^{-1} at 25 °C and pH 7.0, the product yields were variable, with typical ratios of 28% iron−nitrosyl-hemoglobin and 72% methemoglobin (1:2.57).[6] These product yields did not follow the equimolar stoichiometry observed by Brooks and predicted by eqs 1 and 2.[3] Indeed, while some recent studies reproduced the 1:1 iron−nitrosyl-hemoglobin to methemoglobin product ratio,[7,8] others continue to report variable yields, with higher concentrations of methemoglobin than iron−nitrosyl-hemoglobin that are inconsistent between experiments.[9,10]

While Doyle et al. confirmed that reaction progress was linearly dependent on hydrogen ion in the pH range of 6.0−8.0, the observed order of proton dependence was 0.88 rather than the predicted 1.0.[6] The authors interpreted this unexpected deviation to imply that nitrous acid may not be the sole reactant with deoxyhemoglobin, suggestive of additional proton effects on hemoglobin allosteric conformations. These results were later confirmed and attributed to the redox Bohr effect on nitrite reduction.[7]

The use of least-squares analysis of spectroscopic experiments has recently permitted significantly higher accuracy in the identification and quantification of heme species. Such analysis of the deoxyhemoglobin−nitrite reaction has con-
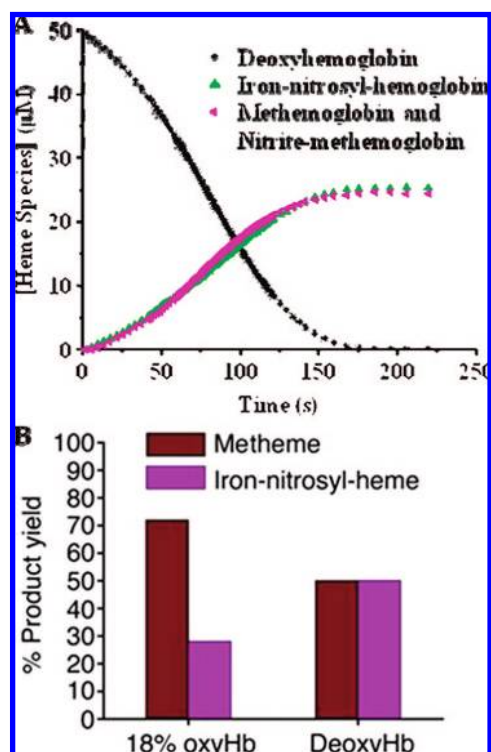
FIGURE 1. The sigmoidal reaction of nitrite with deoxyhemoglobin. (A) Spectrophotometric analysis of the strictly anaerobic reaction of deoxyhemoglobin (50 μM) with nitrite (10 mM) at pH 7.4 and 37 °C. Note that equimolar quantities of methemoglobin and iron−nitrosyl-hemoglobin are produced. Concentrations of heme species were determined by least-squares spectral deconvolution analysis. Reproduced with permission from ref 11. Copyright 2007 American Society for Biochemistry and Molecular Biology. (B) Introduction of oxygen leak (18% oxyhemoglobin) results in lower yields of iron−nitrosyl-hemoglobin than methemoglobin, as shown by spectrophotometrically monitoring the formation of ferric hemes at 630 nm. Reproduced with permission from ref 7. Copyright 2005 American Society for Clinical Investigation.

firmed that under purely anaerobic conditions, it generates equimolar quantities of methemoglobin and iron−nitrosyl-hemoglobin (Figure 1A).[7,11] Huang and colleagues also discerned four unique properties of this reaction.[7] (1) Under truly anaerobic conditions, when excess nitrite is reacted with 99.9% deoxyhemoglobin, the reaction deviates from a second-order rate law and appears to exhibit zero-order kinetics in terms of deoxyheme concentration, such that the reaction rate remains constant as deoxyheme is consumed (Figure 1B). (2) Closer inspection of the instantaneous rate reveals that the reaction rate is sigmoidal, with initial slow (lag), middle fast, and terminal slow phases (Figure 1A). (3) In the presence of an oxygen leak, the reaction appears to exhibit second-order dependence and its rate increases dramatically (Figure 1B). (4) Product yields under strictly anaerobic conditions conform to eqs 1 and 2 (50% methemoglobin and 50% iron−nitrosyl-hemoglobin, as observed by Brooks; Figure 1A,B), while oxy-

gen leak results in higher product yield ratios (70% methemoglobin and 30% iron−nitrosyl-hemoglobin, as observed by Doyle; Figure 1B).

Only during the last two years has it become clear that all of these unusual properties—the effect of IHP on reaction rate, the unusual proton dependence, the variable product yield stoichiometry, the deviation from second-order kinetics, and the effect of oxygen on reaction kinetics—are all due to the allosteric nature of the hemoglobin reaction with nitrite. Nitrite thus reacts not with a single static molecule of deoxyhemoglobin but rather with the many allosterically different conformational states of the deoxyhemoglobin tetramer. These observations paved the way toward the discovery of other surprising and novel chemistries of this storied reaction.

R-State or Allosteric Autocatalysis

When nitrite reacts with the deoxyhemoglobin tetramer as shown in Figure 1A, deviation from second-order kinetics occurs because products of this reaction, methemoglobin and iron−nitrosyl-hemoglobin stabilize reacting tetramers in the R-state and thus enhance heme reactivity (Figure 2A). While oxygen and carbon monoxide are classical ligands that promote the R, high-oxygen affinity conformation of hemoglobin, NO ligation and heme oxidation to methemoglobin are also R-state stabilizers (Figure 2B).[12] R-state hemoglobin has a low heme redox potential, analogous to that of myoglobin,[13,14] which favors an equilibrium distribution of electrons to nitrite and increases the reactivity of nitrite with the unliganded ferrous hemes.[7] The bimolecular rate constant for the reaction of nitrite with R-state hemoglobin (6 M^{-1} s^{-1}) is significantly higher than that with T-state hemoglobin (0.03 M^{-1} s^{-1}). There are therefore two *true* rate constants involved in the deoxyhemoglobin−nitrite reaction: one of R-state hemes and another of T-state hemes. While these true rate constants remain unchanged, the apparent *global* rate constant, determined by the ratio of T- and R-state hemes participating in the reaction, changes as the allosteric conformation of the reactive hemes changes. As the reaction of nitrite with deoxyhemoglobin progresses, newly formed ferric and iron-nitrosylated hemes stabilize the hemoglobin tetramer in the R-state (Figure 2B) and increase the reactivity of its remaining free ferrous hemes with nitrite (Figure 2A). This was confirmed by experiments using hemoglobin encased in a solid gel, locking it into specific allosteric configurations, which demonstrated a graded reaction rate spectrum that directly correlated with the extent of R-state stabilization imposed on the hemoglobin tetramer.[15]
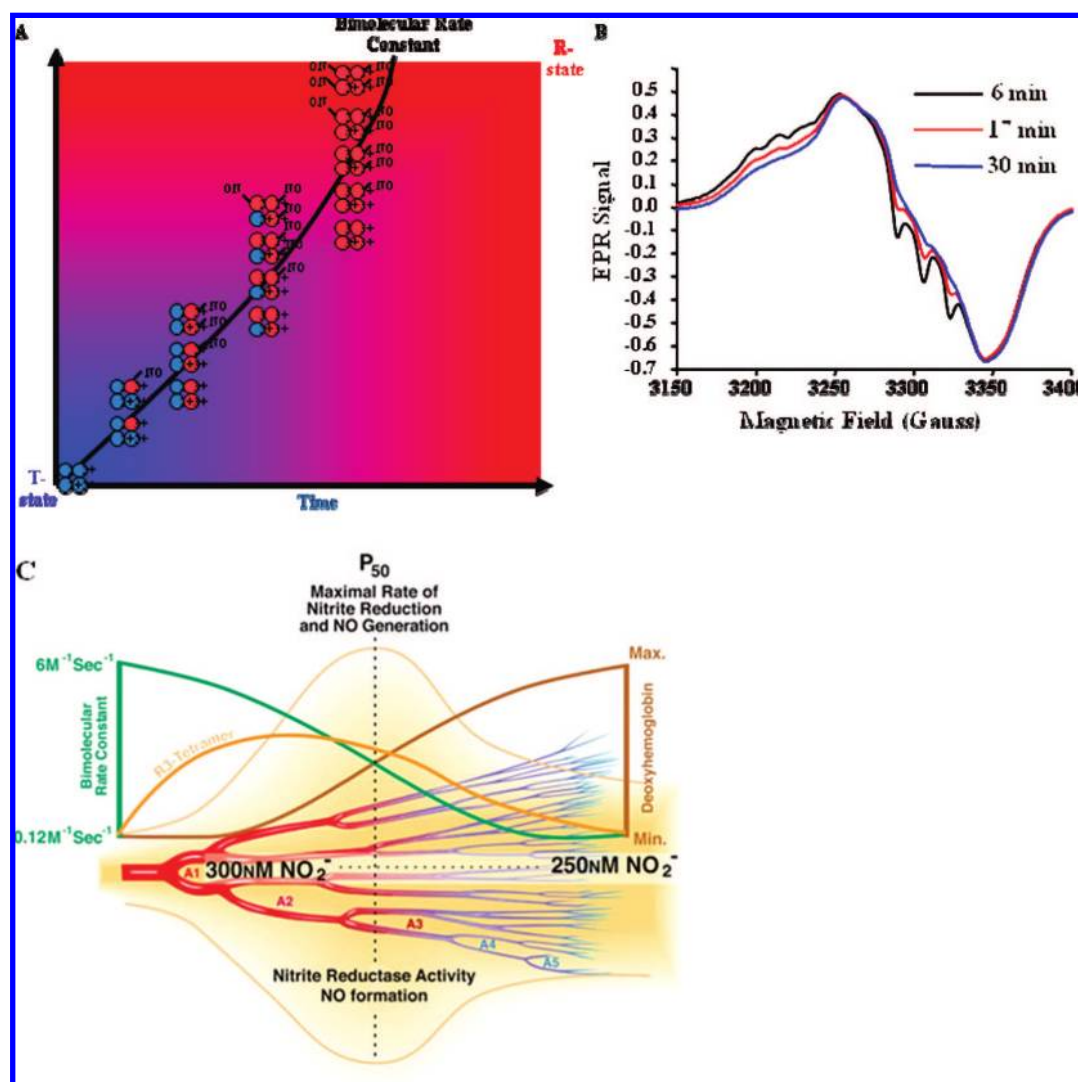
FIGURE 2. Allosteric autocatalysis in the deoxyhemoglobin–nitrite reaction. (A) Tetrameric T-state deoxyhemoglobin (2^+) reduces nitrite to NO, generating a ferric (3^+) and an iron-nitrosylated (2^+-NO) heme, on the same or different Hb tetramers, which stabilize the tetramer(s) in the R-state. Increasing R-state character is associated with a higher bimolecular rate constant and greater nitrite reductase activity. As a result, ferrous deoxy-hemes on these R-state stabilized tetramers react with nitrite faster than those on T-state stabilized tetramers and exponentially propagate nitrite reduction, NO generation, and further R-state stabilization. (B) Confirmation of the progressive T-to-R allosteric shift by EPR analysis of the deoxyhemoglobin (100 μM) reaction with nitrite (2.5 mM) at 6 min (initial), 17 min (intermediate), and 30 min (final) after initiation of the reaction. EPR spectra showed transition of the iron–nitrosyl-heme from the five-coordinate T-state (with characteristic hyperfine splitting) to the six-coordinate R-state heme geometry. Reproduced with permission from ref 7. Copyright 2005 American Society for Clinical Investigation. (C) The hemoglobin oxygen saturation gradient along the A1 to A5 arterioles suggests a nitrite reductase gradient that favors nitrite reduction at the anatomical position of R_3 tetramers (40–60% hemoglobin oxygen saturation). Reproduced with permission from ref 57. Copyright 2006 The American Physiological Society.

The deoxyhemoglobin–nitrite reaction is therefore an *allosteric autocatalytic* reaction, in which the reaction rate is accelerated by a conformational change imposed on the reacting species byproduct of that reaction. By favoring the R-state configuration, ferric and iron-nitrosylated hemes increase the bimolecular rate constant for subsequent reactions of remaining unliganded ferrous hemes, which will reduce nitrite faster and further propagate the R-state stabilizing methemoglobins

and iron–nitrosyl-hemoglobins (Figure 2A). These predictions were confirmed by modeling experiments.[16]

Importantly, because reaction rate is a product of the bimolecular rate constant and reactant (deoxyhemoglobin) concentration, the observed rate of nitrite reduction changes throughout the reaction in a sigmoidal fashion. It is initially slow (because all hemoglobin is in the T-state), peaks midway through the reaction (hemes are still available for nitrite

binding, but the tetramer is stabilized in the R-state by newly formed ferric and iron-nitrosylated ferrous hemes), and ultimately slows down again (all unliganded ferrous hemes are consumed, Figure 1A).

Proton Dependence: Nitrous Acid and the Redox Bohr Effect

This chemistry elegantly explains the 0.88 order of proton dependence observed by Doyle and colleagues, which was an unexpected deviation from the ideal value of 1.0 predicted by Brooks' original reaction mechanism.[3,6] There are actually two opposing effects of protons on the reaction: they promote the formation of nitrous acid (eq 1), thereby facilitating the reaction of deoxyhemoglobin with nitrite, and simultaneously stabilize hemoglobin in the T-state. The latter effect, called the redox or oxidation Bohr effect, slows the deoxyhemoglobin—nitrite reaction by lowering the heme redox potential and "dampening" allosteric autocatalysis.[7,17−20] Because the magnitude of the oxidation Bohr effect is smaller than that of increased nitrous acid formation, the resulting proton dependence is 0.88.

Physiological Implications of R-State Catalysis

Importantly, the chemistry described above has been derived using pseudo-first order conditions of high (millimolar) nitrite and low (micromolar) heme concentrations, with methemoglobin and iron—nitrosyl-hemoglobin acting as R-state stabilizers. *In vivo*, however, the relative concentrations of species are reversed, with 300 nM nitrite reacting with 20 mM hemoglobin in the red cell and the allosteric state of hemoglobin tetramers modulated by oxygen ligation rather than by iron-nitrosylated and ferric hemes generated in the deoxyhemoglobin—nitrite reaction. The bimolecular rate constant is therefore maximal in oxygenated arterial blood, while reactant (deoxyheme) concentration is maximal in venous blood (Figure 2C). The rate of nitrite reduction will be highest at approximately 50% oxygen saturation, or the intrinsic hemoglobin P_{50}, and the most effective tetrameric nitrite reductase will be the R-state hemoglobin that rapidly deoxygenates during arterial-to-capillary transit. This molecule would transition through R_4 (R-state with four oxygens bound), R_3, R_2, T_2 (T-state with two oxygens bound), and ultimately T_1 tetramers, with R_3 and R_2 tetramers being the most effective nitrite reductases. This chemistry has been confirmed experimentally, as nitrite reduction and NO signaling are greatest in systems subjected to rapid deoxygenation in the presence of oxyhemoglobin and nitrite.[21,22] NO generation and NO-mediated signaling,[7,22] including vasodilation[22−26] and inhibition of mitochondrial respiration,[22] are all maximized when hemoglobin reaches approximately 50% oxygen saturation.

Oxidative Denitrosylation

In the presence of deoxygenating hemoglobin, myoglobin, and erythrocytes, nitrite has been shown to facilitate NO-dependent signaling, including gas-phase NO generation,[7,22] tissue cGMP accumulation,[22] vasodilation,[22−26] and inhibition of mitochondrial cytochrome C oxidase activity.[22,27] However, the mechanism of NO escape from rapid scavenging by ferrous hemes inside and outside the erythrocyte subsequent to nitrite reduction remained a mystery.[28,29] Because NO reacts with both deoxy- and oxyhemoglobin extremely fast, with bimolecular rates in the range of $10^7−10^8$ M^{-1} s^{-1},[30−38] modeling calculations have shown that only 0.1 pM NO could be present outside a red cell at steady state, even at supraphysiological nitrite levels, unless mechanisms exist to limit these scavenging reactions.[8] So how does NO leave the red cell and perform multiple physiological signaling functions?

The answer lies with the important fact that under physiological conditions nitrite does not react with anaerobic deoxyhemoglobin, but rather with a partially oxygenated solution of oxy- and deoxyhemoglobin. The reaction of nitrite with oxyhemoglobin is a complex autocatalytic process with radical-mediated chain reactions and branching steps that ultimately oxidizes nitrite to nitrate and oxyhemoglobin to methemoglobin.[39,40] Although the mechanism of this reaction is not yet fully understood, it involves autocatalytic intermediates, including nitrogen dioxide ($NO_2^•$) and ferryl-hemoglobin ($Fe^{IV}=O$),[41−44] which give the oxyhemoglobin—nitrite reaction its unique kinetic profile, with an early lag (slow) phase and a rapid autocatalytic (propagation) phase.

We recently examined the reactions of nitrite with oxy- and deoxyhemoglobin at a wide range of oxygen partial pressures and found that they run in parallel and, surprisingly, interact.[11] As the fractional oxygen saturation of hemoglobin falls, the lag phase of the oxyhemoglobin—nitrite reaction is prolonged (autocatalysis is delayed) and the maximal reaction rate attained during the propagation phase is decreased.[11] This inhibitory effect of deoxyhemoglobin on the oxyhemoglobin—nitrite reaction is dose-dependent, such that at progressively lower oxygen partial pressures the oxyhemoglobin—nitrite reaction autocatalytic rate decreases further and occurs later in the course of the reaction. In contrast, because oxygenation stabilizes unliganded hemes in the R-state and lowers their redox potential, the rate of the

deoxyhemoglobin—nitrite reaction is maintained at its maximal level throughout the reaction, thereby increasing the overall rate of nitrite reduction.[11] As a result, the reductive deoxyhemoglobin—nitrite reaction dominates at low oxygen saturations, the oxidative oxyhemoglobin—nitrite reaction dominates at high oxygen saturations, and the two reactions proceed with comparable efficiencies when approximately 50% of hemoglobin is oxygenated.[11] At physiologically low hemoglobin oxygen saturations, nitrite will thus preferentially react with deoxyhemoglobin to generate NO, rather than be inactivated by oxyhemoglobin to the physiologically inert nitrate.

NO generated by deoxyhemoglobin in a partially oxygenated environment is not irreversibly trapped by adjacent deoxyhemes as an iron—nitrosyl as previously thought. Rather, immediately after the oxyhemoglobin—nitrite reaction enters its propagation phase, iron—nitrosyl-hemoglobin produced in the deoxyhemoglobin—nitrite reaction is rapidly and quantitatively depleted. Moreover, consumption of the iron—nitrosyl, as measured by UV—vis absorption spectroscopy and EPR, precisely correlates with the release of NO into the gas phase.[11] Mechanistically, these observations suggest that iron—nitrosyl-hemoglobin is consumed via the oxidation of the ferrous iron—nitrosyl-heme to the ferric iron—nitrosyl-heme, most likely by nitrogen dioxide (produced during oxyhemoglobin—nitrite reaction autocatalysis), in a process called oxidative denitrosylation. Because NO has low binding affinity for methemoglobin,[45] the ferric iron—nitrosyl-heme easily releases NO into the gas phase. Moreover, since nitrogen dioxide promotes oxy-reaction autocatalysis, its consumption interferes with autocatalysis, dampens the propagation phase, and directs nitrite through reductive pathways to NO.

The ability of deoxyhemoglobin to reduce nitrite to NO and release it upon oxygenation is demonstrated in Figure 3A—D. Rapid oxygenation of the anaerobic deoxyhemoglobin—nitrite reaction results in quantitative oxygenation of unreacted deoxyhemes and initiation of the oxyhemoglobin—nitrite reaction. Once the oxy-reaction autocatalytic intermediates (including nitrogen dioxide) accumulate and the reaction enters the propagation phase (signified by a peak in ferrylhemoglobin), the iron—nitrosyl produced during the anaerobic phase is oxidized and NO is released. Importantly, NO release occurs as a direct consequence of the oxyhemoglobin—nitrite reaction and is not observed with the T-to-R allosteric shift of iron—nitrosyl-hemoglobin alone (Figure 3E).

Anaerobic N_2O_3 Generation by the Catalytic Nitrite Reductase/Anhydrase Chemistry of Deoxyhemoglobin

The paradox of experimentally observed NO generation and release despite robust NO scavenging reactions inside the erythrocyte has led investigators to probe the deoxyhemoglobin—nitrite reaction for possible stable intermediate NO_x species that could avoid heme autocapture. Such hypothesized intermediates would include NO^+ (which could nitrosate thiols to form *S*-nitrosothiols), N_2O_3, $NO_2^\bullet$, HNO_2, and H_2NO_2.

Rifkind and colleagues have recently reported an EPR-silent NO-modified hemoglobin intermediate that they detected by reductive chemiluminescence.[9,46] They hypothesized that this intermediate is NO—methemoglobin (Fe^{3+}—NO), stabilized by electron delocalization between the NO, heme, and possibly β-cysteine 93, which could ultimately release NO or nitrosate thiol for red cell export. Luschinger and colleagues also found evidence of a hemoglobin-bound intermediate that liberated NO upon photolysis and, based on deconvolution analysis, suggested that this species was NO—methemoglobin.[28,47] However, while NO—methemoglobin is certainly a transient intermediate in the nitrite reductase reaction (eq 1), kinetic analysis indicates that this species would not be stable in the presence of high deoxyheme concentrations, because NO is released from methemoglobin at a rate of approximately 1 s^{-1}, while its on-rate for deoxyheme is $(2-6) \times 10^7$ M^{-1} s^{-1}.

In collaboration with Kim-Shapiro and colleagues, we carefully examined the reaction of nitrite and deoxyhemoglobin using least-squares deconvolution with five reference species: deoxyhemoglobin, iron—nitrosyl-hemoglobin, methemoglobin, nitrite—methemoglobin, and NO—methemoglobin.[8] Our analyses showed that nitrite—methemoglobin, and not NO—methemoglobin, was formed, yet it was not included in deconvolution analysis performed by Luschinger and colleagues.[28,47] During our attempt to confirm these results by EPR quantification of the low field, high spin $g = 6$ spectra characteristic of the ferric heme, we were surprised to find that nitrite—methemoglobin detected by UV—vis spectroscopy was not seen by EPR (Figure 4).[8] Indeed, addition of nitrite to methemoglobin resulted in silencing of the ferric EPR signal with high affinity and in a pH-dependent manner (faster at lower pH). We had thus identified an intermediate that possessed all characteristics described by the Rifkind group; it was nitrite—methemoglobin.
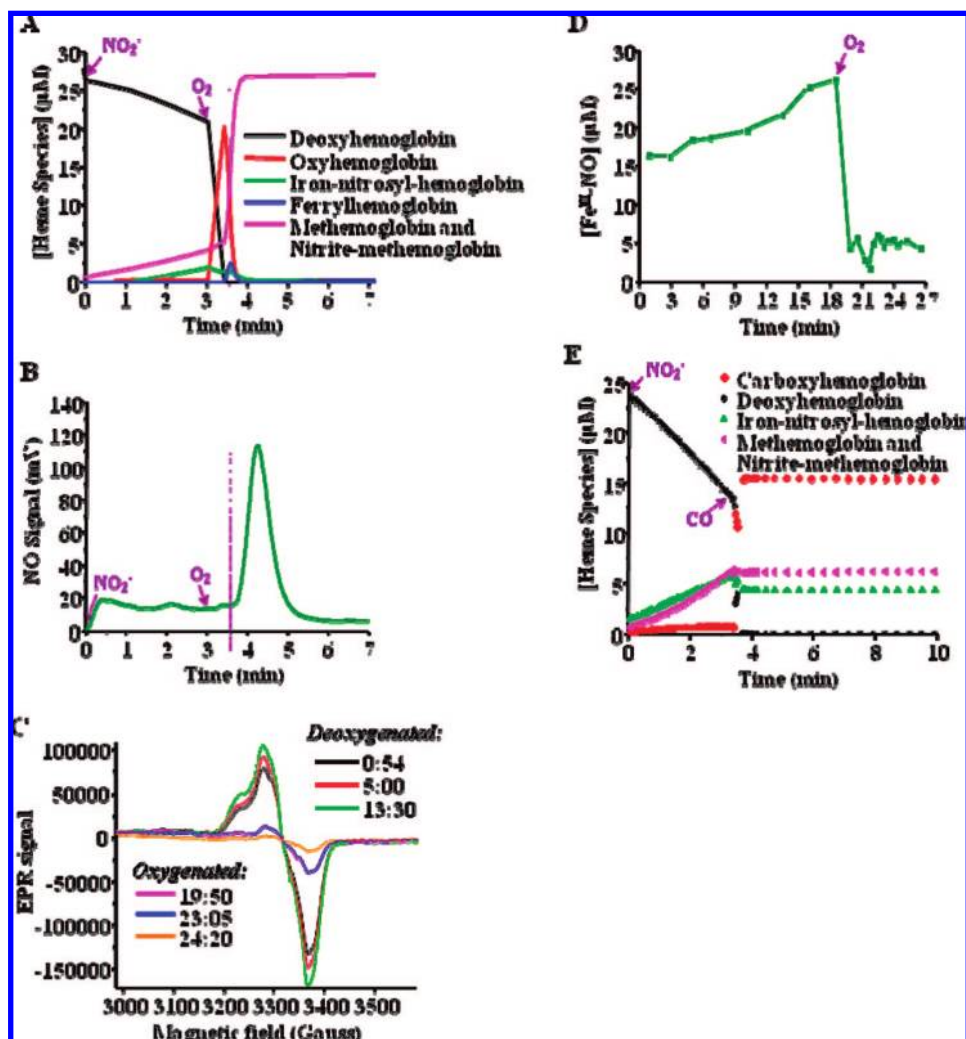
FIGURE 3. Oxidative denitrosylation: NO is released from iron−nitrosyl-hemoglobin during a hemoglobin oxygenation cycle. Reaction of deoxyhemoglobin (25 μM) with nitrite (5 mM) proceeded to partial completion and until oxygenation at 3 min to initiate the aerobic reaction of nitrite with remaining ferrous hemes. Reaction progress was monitored in parallel by (A) absorption spectroscopy and (B) the nitric oxide analyzer; dotted line indicates oxy-reaction autocatalysis. (C, D) Generation and consumption of iron−nitrosyl-hemoglobin was confirmed EPR spectroscopy in an analogous reaction of deoxyhemoglobin (80 μM) with nitrite (1 mM) that was oxygenated after 19 min. (E) There is no consumption of iron−nitrosyl-hemoglobin associated with the T-to-R allosteric shift induced by exposure of reaction from panel A to CO (at 3 min 27 s) in the absence of oxyhemoglobin−nitrite reaction autocatalysis. Reproduced with permission from ref 11. Copyright 2007 American Society for Biochemistry and Molecular Biology.

Nitrite silencing of the $g = 6$ EPR spectrum has been reported previously in bacterial nitrite and sulfite reductases and in model porphyrin systems.[48−50] Such an effect could occur secondary to electron delocalization from the nitrite to the ferric heme, which would produce an intermediate species with ferrous−nitrogen dioxide character (eq 3).

$$NO_2^- + HbFe^{3+} \rightarrow HbFe^{3+}-NO_2^- \leftrightarrow [HbFe^{2+}-NO_2^\bullet] \quad (3)$$
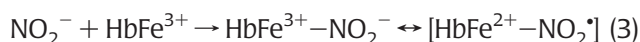
Indeed, molecular orbital modeling studies of the methemoglobin−nitrite complex demonstrated that the high electron affinity of the ferric heme in NO−methemoglobin can induce such electron delocalization.[8] Density functional theory (DFT) studies show that nitrite can bind ferric hemes at either the oxygen ("O−nitrito") or nitrogen ("nitro") atom, which has been confirmed by crystallographic studies of nitrite−myoglobin performed by the Richter-Addo laboratory.[51] The O-bound form of nitrite−methemoglobin favors electron delocalization from the nitrite to the extremely electron-hungry ferric heme and leads to the formation of the ferrous nitrogen dioxide-like species ($Fe^{II}-NO_2^\bullet$).[8] Rapid fluctuation between these states would explain the EPR silence of nitrite−methemoglobin.

Because the reaction between nitrogen dioxide and NO is diffusion-limited (10^9-10^{10} M^{-1} s^{-1}), NO produced in the deoxyhemoglobin−nitrite reaction could react with the ferrous nitrogen dioxide-like intermediate at a rate that poten-
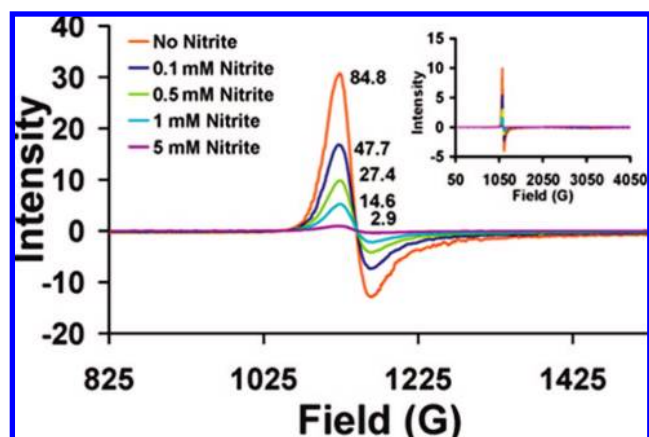
FIGURE 4. Addition of nitrite to methemoglobin leads to a rapid and concentration-dependent silencing of the metheme EPR signal. A range of nitrite concentrations (0, 0.1, 0.5, 1, and 5 mM) was added to methemoglobin (84.8 μM) at pH 7.4, and the low-field EPR was measured at 4 K. The concentrations of remaining methemoglobin, measured by double integration of the methemoglobin signal, are indicated adjacent to each tracing. No low spin methemoglobin EPR signals at lower g values were detected (inset). Modified with permission from ref 8. Copyright 2007 Nature Publishing Group.

tially competes with NO scavenging by other heme groups (10^7 M^{-1} s^{-1}). This reaction would generate N_2O_3 and ferrous hemoglobin (eq 4).

$$[\text{HbFe}^{2+}-\text{NO}_2{}^{\bullet}] + \text{NO} \rightarrow \text{HbFe}^{2+} + \text{N}_2\text{O}_3 \qquad (4)$$
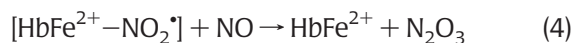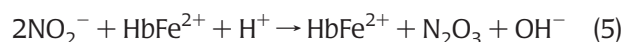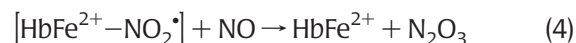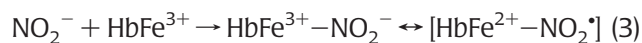
The proposed NO-catalyzed reduction of nitrite−methemoglobin is reminiscent of a similar reaction described by Fernandez and Ford, in which nitrite was shown to accelerate the rate of NO−methemoglobin (Fe^{3+}−NO) reduction to ferrous hemoglobin (Fe^{2+}) and N_2O_3 by approximately 4-fold.[52] However, while this mechanism accounts for the reduction of methemoglobin and generation of N_2O_3, it is slower than what has been observed experimentally[53,54] and undeniably too slow to compete with parallel NO scavenging reactions.
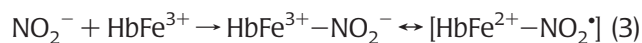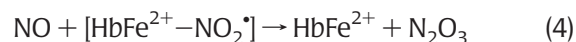
We considered that the high concentrations of NO used in studies of reductive nitrosylation by Fernandez and Ford could have inhibited our proposed reaction (eq 4) by competing with nitrite for binding to ferric hemes (methemoglobin has 25-fold higher affinity for NO than for nitrite) and precluding the formation of the nitrogen dioxide-like intermediate. Thus, when low concentrations of NO are added to a solution of nitrite and methemoglobin, NO preferentially reacts with nitrite−methemoglobin rather than with unbound methemoglobin. Consistent with this hypothesis, the k_{obs} of methemoglobin reduction by NO to form iron−nitrosyl-hemoglobin (reductive nitrosylation) actually increases as NO concentra-

tion is decreased.[8] Similarly, the rate of *S*-nitrosothiol formation in this reaction increases with decreasing NO concentrations.[8]

Importantly, when examined in the context of other anaerobic hemoglobin reactions with nitrite (eqs 1, 3, and 4), this chemistry suggests that deoxyhemoglobin acts as an enzymatic nitrite anhydrase that catalyzes the dehydration of two molecules of nitrite into N_2O_3 (eq 5).

$$\text{NO}_2{}^- + \text{HbFe}^{2+} + \text{H}^+ \rightarrow \text{NO} + \text{HbFe}^{3+} + \text{OH}^- \qquad (1)$$

$$\text{NO}_2{}^- + \text{HbFe}^{3+} \rightarrow \text{HbFe}^{3+}-\text{NO}_2{}^- \leftrightarrow [\text{HbFe}^{2+}-\text{NO}_2{}^{\bullet}] \quad (3)$$

$$[\text{HbFe}^{2+}-\text{NO}_2{}^{\bullet}] + \text{NO} \rightarrow \text{HbFe}^{2+} + \text{N}_2\text{O}_3 \qquad (4)$$

$$2\text{NO}_2{}^- + \text{HbFe}^{2+} + \text{H}^+ \rightarrow \text{HbFe}^{2+} + \text{N}_2\text{O}_3 + \text{OH}^- \qquad (5)$$

Because this reaction redox cycles, low concentrations of NO added to nitrite−methemoglobin catalyze the production of more NO and N_2O_3. When NO reacts with nitrite−methemoglobin (eq 4), it not only generates N_2O_3, which nitrosates one thiol and produces one nitrite (eq 6), but also reduces methemoglobin to deoxyhemoglobin, which can now react with the nitrite to generate a second NO (eq 1).

$$\text{NO} + [\text{HbFe}^{2+}-\text{NO}_2{}^{\bullet}] \rightarrow \text{HbFe}^{2+} + \text{N}_2\text{O}_3 \qquad (4)$$

$$\text{N}_2\text{O}_3 + \text{RSH} \rightarrow \text{RS}-\text{NO} + \text{NO}_2{}^- \qquad (6)$$

$$\text{NO}_2{}^- + \text{HbFe}^{2+} + \text{H}^+ \rightarrow \text{NO} + \text{HbFe}^{3+} + \text{OH}^- \qquad (1)$$

$$\text{NO}_2{}^- + \text{HbFe}^{3+} \rightarrow \text{HbFe}^{3+}-\text{NO}_2{}^- \leftrightarrow [\text{HbFe}^{2+}-\text{NO}_2{}^{\bullet}] \quad (3)$$

In the presence of excess nitrite, both NO and deoxyhemoglobin are catalytic, which potentially allows for amplification of NO signaling (eq 7).

$$\text{NO}_2{}^- + \text{H}^+ + \text{RSH} \xrightarrow{\text{HbFe}^{2+}} \text{RSNO} + \text{OH}^- \qquad (7)$$
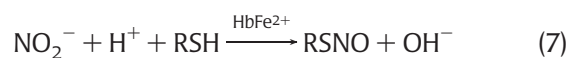
Notably, this catalytic nitrite anhydrase hemoglobin chemistry has remained "invisible" to scientists for over 100 years. It was not detected by UV−vis absorption spectroscopy in part because the ferrous heme redox cycles and is not consumed in the process, such that there is no net change in the composition of heme species over time. The key reactant, nitrite−methemoglobin, is EPR-silent, which precluded its detection or identification using that technique. Moreover, the high concentrations of NO typically used in reductive nitrosylation studies interfered with nitrite binding to methemoglobin and thus inhibited nitrite reduc-
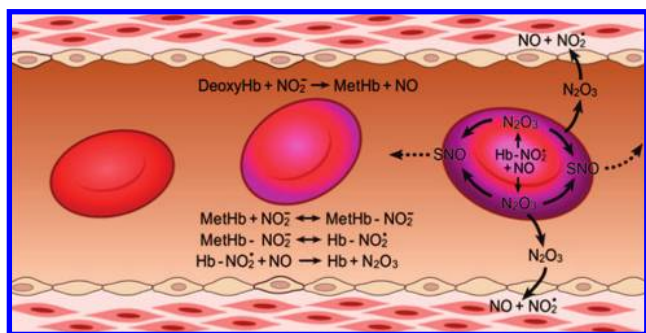
FIGURE 5. A putative nitrite reductase metabolon located within the red cell lipid raft composed of deoxy- and methemoglobin, an anion exchange protein (to import nitrite into the cell), carbonic anhydrase (to generate protons from CO_2 and water), aquaporin, and Rh channels[55] would effectively localize the NO-generating deoxyhemoglobin−nitrite reaction, the autocatalytic oxyhemoglobin−nitrite reaction necessary to oxidize iron−nitrosyl-hemoglobin and release free NO, and nitrite−methemoglobin to facilitate N_2O_3 formation near highly hydrophobic channels at the membrane. Reproduced with permission from ref 8. Copyright 2007 Nature Publishing Group.

tase/anhydrase chemistry. Finally, N_2O_3 is a highly unstable gas molecule that is not easily measured unless specifically trapped.

N_2O_3 is an ideal candidate for facilitation of NO export from the erythrocyte. It is highly lipid soluble and could diffuse across hydrophobic membrane channels such as aquaporin or Rh. N_2O_3 is also more stable in the red cell than NO, with a lifetime of 1 ms compared with 0.2 μs for NO, which enables N_2O_3 to diffuse for approximately 1 μm. Red blood cells have a flat biconcave disk morphology, with a flat surface area that is less than 2 μm thick; this provides a large area for N_2O_3 diffusional escape. After escaping from hemoglobin, the red cell, or both, N_2O_3 can homolyze to NO and $NO_2^\bullet$, reconstituting and liberating NO. Because it is the cell's primary nitrosating species, N_2O_3 could also nitrosate membrane proteins and L-cysteine, which has been shown to be transported through the LAT membrane transporter (thus facilitating NO escape). The physiologic efficiency of nitrite reduction and NO release will be greater *in vivo* if the effective concentrations of the reactants, that is, nitrite, proton, deoxyhemoglobin, and methemoglobin, are maximized at the erythrocyte submembrane in a nitrite reductase metabolome (Figure 5).

Conclusions

On the heels of over a century of rich experimental research into the chemical nature of nitrite reactions with hemoglobin, recent studies have revealed fundamental novel metal- and nitrite-catalyzed reaction pathways that limit iron−nitrosyl-hemoglobin accumulation within the erythrocyte and generate free NO, N_2O_3, and nitrosothiol. These reactions are likely to contribute to nitrite and hemeglobin dependent hypoxic vasodilation and signaling, open the door to study of nitrite delocalization intermediates with other ferric heme systems, and suggest a possible signaling role for nitrite reduction by myoglobin, cytoglobin, and neuroglobin.

BIOGRAPHICAL INFORMATION

Mark Gladwin received his M.D. from the University of Miami Honors Program in Medical Education in 1991. After completing his internship and chief residency at the Oregon Health Sciences University in Portland, Oregon, he joined the NIH in 1995 as a critical care fellow in the Clinical Center. After a one-year clinical fellowship in pulmonary medicine at the University of Washington in Seattle, he returned for a research fellowship at the Critical Care Medicine Department, CC, under the mentorship of James Shelhamer, Frederick Ognibene, Alan Schechter, and Richard Cannon. He is currently Chief of the Pulmonary and Vascular Medicine Branch within the NHLBI, NIH. The clinical research activities of the Branch are programmatically linked to the CC Critical Care Medicine Department. In August of 2008, Dr. Gladwin will become the Division Chief of the Pulmonary, Allergy, and Critical Care Medicine Department at the University of Pittsburgh and will lead the newly formed Hemostasis and Vascular Biology Research Institute.

Rozalina Grubina is a fourth year medical student at the Johns Hopkins School of Medicine and a former Howard Hughes Medical Institute−National Institutes of Health (HHMI−-NIH) Research Scholar. She will be beginning residency training in Internal Medicine in July 2009.

Michael Doyle received his B.S. (College of St. Thomas, 1964) and Ph.D. (Iowa State University, 1968) degrees in chemistry. Following a postdoctoral engagement at the University of Illinois at Chicago Circle, he joined the faculty at Hope College in 1968 and was appointed the first Kenneth Herrick Professor in 1982. In 1984, he moved to Trinity University as the Dr. D. R. Semmes Distinguished Professor of Chemistry, and in 1997, he moved to Tucson, AZ, as Vice President, then President, of Research Corporation and Professor of Chemistry at the University of Arizona. He moved to the University of Maryland in 2003, where he is Professor and Chair of the Department of Chemistry and Biochemistry. His research has focused on the chemistry of nitrogen compounds and catalysis.

REFERENCES

1 Gamgee, A. Researches on the Blood. On the Action of Nitrites on Blood. *Phil. Trans. R. Soc. London* **1868**, *158*, 589–625.

2 Haldane, J. The Red Colour of Salted Meat. *J. Hyg.* **1901**, *1*, 115–122.

3 Brooks, J. The Action of Nitrite on Haemoglobin in the Absence of Oxygen. *Proc. R. Soc. Med.* **1937**, *123*, 368–382.

4 Tomoda, A.; Matsukawa, S.; Takeshita, M.; Yoneyama, Y. Effect of Inositol Hexaphosphate on Hemoglobin Oxidation by Nitrite and Ferricyanide. *Biochem. Biophys. Res. Commun.* **1977**, *74*, 1469–1474.

5 Tomoda, A.; Yoneyama, Y. On the Relationship of Hemoglobin Oxidation with the Conformation of Hemoglobin. *Experientia* **1979**, *35*, 15–16.

6 Doyle, M. P.; Pickering, R. A.; DeWeert, T. M.; Hoekstra, J. W.; Pater, D. Kinetics and Mechanism of the Oxidation of Human Deoxyhemoglobin by Nitrites. *J. Biol. Chem.* **1981**, *256*, 12393–12398.

7 Huang, Z.; Shiva, S.; Kim-Shapiro, D. B.; Patel, R. P.; Ringwood, L. A.; Irby, C. E.; Huang, K. T.; Ho, C.; Hogg, N.; Schechter, A. N.; Gladwin, M. T. Enzymatic Function of Hemoglobin as a Nitrite Reductase That Produces NO under Allosteric Control. *J. Clin. Invest.* **2005**, *115*, 2099–2107.

8 Basu, S.; Grubina, R.; Huang, J.; Conradie, J.; Huang, Z.; Jeffers, A.; Jiang, A.; He, X.; Azarov, I.; Seibert, R.; Mehta, A.; Patel, R.; King, S. B.; Hogg, N.; Ghosh, A.; Gladwin, M. T.; Kim-Shapiro, D. B. Catalytic Generation of N₂O₃ by a Concerted Nitrite Reductase/Anhydrase Activity of Hemoglobin. *Nat. Chem. Biol.* **2007**, *3*, 785–794.

9 Nagababu, E.; Ramasamy, S.; Abernethy, D. R.; Rifkind, J. M. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J. Biol. Chem.* **2003**, *278*, 46349–46356.

10 Angelo, M.; Singel, D. J.; Stamler, J. S. An S-Nitrosothiol (SNO) Synthase Function of Hemoglobin That Utilizes Nitrite as a Substrate. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8366–8371.

11 Grubina, R.; Huang, Z.; Shiva, S.; Joshi, M. S.; Azarov, I.; Basu, S.; Ringwood, L. A.; Jiang, A.; Hogg, N.; Kim-Shapiro, D. B.; Gladwin, M. T. Concerted Nitric Oxide Formation and Release from the Simultaneous Reactions of Nitrite with Deoxy- and Oxyhemoglobin. *J. Biol. Chem.* **2007**, *282*, 12916–12927.

12 Bunn, H. F.; Forger, B. G. *Hemoglobin: Molecular, Genetic, and Clinical Aspects*; W.B. Saunders Company: Philadelphia, PA, 1984.

13 Taboy, C. H.; Faulkner, K. M.; Kraiter, D.; Bonaventura, C.; Crumbliss, A. L. Concentration-Dependent Effects of Anions on the Anaerobic Oxidation of Hemoglobin and Myoglobin. *J. Biol. Chem.* **2000**, *275*, 39048–39054.

14 Taboy, C. H.; Bonaventura, C.; Crumbliss, A. L. Anaerobic Oxidations of Myoglobin and Hemoglobin by Spectroelectrochemistry. *Methods Enzymol.* **2002**, *353*, 187–209.

15 Roche, C. J.; Dantsker, D.; Samuni, U.; Friedman, J. M. Nitrite Reductase Activity of Sol-Gel-Encapsulated Deoxyhemoglobin. Influence of Quaternary and Tertiary Structure. *J. Biol. Chem.* **2006**, *281*, 36874–36882.

16 Huang, K. T.; Keszler, A.; Patel, N.; Patel, R. P.; Gladwin, M. T.; Kim-Shapiro, D. B.; Hogg, N. The Reaction between Nitrite and Deoxyhemoglobin. Reassessment of Reaction Kinetics and Stoichiometry. *J. Biol. Chem.* **2005**, *280*, 31126–31131.

17 Brunori, M.; Wyman, J.; Antonini, E.; Rossi-Fanelli, A. Studies on the Oxidation-Reduction Potentials of Heme Proteins. V. The Oxidation Bohr Effect in Normal Human Hemoglobin and Human Hemoglobin Digested with Carboxypeptidase A. *J. Biol. Chem.* **1965**, *240*, 3317–3324.

18 Hoffman, B. M.; Bull, C. Linearity of the Hemoglobin Oxidation Bohr Effect. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 800–803.

19 Antonini, E.; Wyman, J.; Brunori, M.; Taylor, J. F.; Rossi-Fanelli, A.; Caputo, A. Studies on the Oxidation-Reduction Potentials of Heme Proteins. I. Human Hemoglobin. *J. Biol. Chem.* **1964**, *239*, 907–912.

20 Taylor, J. F.; Hastings, A. B. Oxidation-Reduction Potentials of the Methemoglobin-Hemoglobin System. *J. Biol. Chem.* **1939**, *131*, 649–662.

21 Blood, A. B.; Power, G. G. In Vitro and in Vivo Kinetic Handling of Nitrite in Blood: Effects of Varying Hemoglobin Oxygen Saturation. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *293*, H1508–H1517.

22 Crawford, J. H.; Isbell, T. S.; Huang, Z.; Shiva, S.; Chacko, B. K.; Schechter, A. N.; Darley-Usmar, V. M.; Kerby, J. D.; Lang, J. D., Jr.; Kraus, D.; Ho, C.; Gladwin, M. T.; Patel, R. P. Hypoxia, Red Blood Cells, and Nitrite Regulate NO-Dependent Hypoxic Vasodilation. *Blood* **2006**, *107*, 566–574.

23 Cosby, K.; Partovi, K. S.; Crawford, J. H.; Patel, R. P.; Reiter, C. D.; Martyr, S.; Yang, B. K.; Waclawiw, M. A.; Zalos, G.; Xu, X.; Huang, K. T.; Shields, H.; Kim-Shapiro, D. B.; Schechter, A. N.; Cannon, R. O., 3rd; Gladwin, M. T. Nitrite Reduction to Nitric Oxide by Deoxyhemoglobin Vasodilates the Human Circulation. *Nat. Med.* **2003**, *9*, 1498–1505.

24 Hunter, C. J.; Dejam, A.; Blood, A. B.; Shields, H.; Kim-Shapiro, D. B.; Machado, R. F.; Tarekegn, S.; Mulla, N.; Hopper, A. O.; Schechter, A. N.; Power, G. G.; Gladwin, M. T. Inhaled Nebulized Nitrite Is a Hypoxia-Sensitive NO-Dependent Selective Pulmonary Vasodilator. *Nat. Med.* **2004**, *10*, 1122–1127.

25 Kozlov, A. V.; Costantino, G.; Sobhian, B.; Szalay, L.; Umar, F.; Nohl, H.; Bahrami, S.; Redl, H. Mechanisms of Vasodilatation Induced by Nitrite Instillation in Intestinal Lumen: Possible Role of Hemoglobin. *Antioxid Redox Signaling* **2005**, *7*, 515–521.

26 Tsuchiya, K.; Kanematsu, Y.; Yoshizumi, M.; Ohnishi, H.; Kirima, K.; Izawa, Y.; Shikishima, M.; Ishida, T.; Kondo, S.; Kagami, S.; Takiguchi, Y.; Tamaki, T. Nitrite is an Alternative Source of NO in Vivo. *Am. J. Physiol. Heart Circ. Physiol.* **2005**, *288*, H2163–H2170.

27 Shiva, S.; Huang, Z.; Grubina, R.; Sun, J.; Ringwood, L. A.; MacArthur, P. H.; Xu, X.; Murphy, E.; Darley-Usmar, V. M.; Gladwin, M. T. Deoxymyoglobin Is a Nitrite Reductase That Generates Nitric Oxide and Regulates Mitochondrial Respiration. *Circ. Res.* **2007**, *100*, 654–661.

28 Luchsinger, B. P.; Rich, E. N.; Yan, Y.; Williams, E. M.; Stamler, J. S.; Singel, D. J. Assessments of the Chemistry and Vasodilatory Activity of Nitrite with Hemoglobin under Physiologically Relevant Conditions. *J. Inorg. Biochem.* **2005**, *99*, 912–921.

29 Singel, D. J.; Stamler, J. S. Chemical Physiology of Blood Flow Regulation by Red Blood Cells: The Role of Nitric Oxide and S-Nitrosohemoglobin. *Annu. Rev. Physiol.* **2005**, *67*, 99–145.

30 Eich, R. F.; Li, T. S.; Lemon, D. D.; Doherty, D. H.; Curry, S. R.; Aitken, J. F.; Mathews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N.; Olson, J. S. Mechanism of NO-Induced Oxidation of Myoglobin and Hemoglobin. *Biochemistry* **1996**, *35*, 6976–6983.

31 Herold, S.; Exner, M.; Nauser, T. Kinetic and Mechanistic Studies of the NO*-Mediated Oxidation of Oxymyoglobin and Oxyhemoglobin. *Biochemistry* **2001**, *40*, 3385–3395.

32 Cassoly, R.; Gibson, Q. H. Conformation, Co-operativity and Ligand-Binding in Human Hemoglobin. *J. Mol. Biol.* **1975**, *91*, 301–313.

33 Morris, R. J.; Gibson, Q. H. The Role of Diffusion in Limiting the Rate of Ligand-Binding to Hemoglobin. *J. Biol. Chem.* **1980**, *255*, 8050–8053.

34 Huang, Z.; Hearne, L.; Irby, C. E.; King, S. B.; Ballas, S. K.; Kim-Shapiro, D. B. Kinetics of Increased Deformability of Deoxygenated Sickle Cells upon Oxygenation. *Biophys. J.* **2003**, *85*, 2374–2383.

35 Kim-Shapiro, D. B. Hemoglobin-Nitric Oxide Cooperativity: Is NO the Third Respiratory Ligand. *Free Radical Biol. Med.* **2004**, *36*, 402–412.

36 Gladwin, M. T.; Shelhamer, J. H.; Schechter, A. N.; Pease-Fye, M. E.; Waclawiw, M. A.; Panza, J. A.; Ognibene, F. P.; Cannon, R. O. Role of Circulating Nitrite and S-Nitrosohemoglobin in the Regulation of Regional Blood Flow in Humans. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11482–11487.

37 Doyle, M. P.; Hoekstra, J. W. Oxidation of Nitrogen Oxides by Bound Dioxygen in Hemoproteins. *J. Inorg. Biochem.* **1981**, *14*, 351–358.

38 Doyle, M. P.; Pickering, R. A.; Cook, B. R. Oxidation of Oxymyoglobin by Nitric Oxide through Dissociation from Cobalt Nitrosyls. *J. Inorg. Biochem.* **1983**, *19*, 329–338.

39 Kim-Shapiro, D. B.; Gladwin, M. T.; Patel, R. P.; Hogg, N. The Reaction between Nitrite and Hemoglobin: The Role of Nitrite in Hemoglobin-Mediated Hypoxic Vasodilation. *J. Inorg. Biochem.* **2005**, *99*, 237–246.

40 Kosaka, H.; Imaizumi, K.; Tyuma, I. Mechanism of Autocatalytic Oxidation of Oxyhemoglobin by Nitrite. An Intermediate Detected by Electron Spin Resonance. *Biochim. Biophys. Acta* **1982**, *702*, 237–241.

41 Wallace, W. J.; Caughey, W. S. Mechanism for the Autoxidation of Hemoglobin by Phenols, Nitrite and "Oxidant" Drugs. Peroxide Formation by One Electron Donation to Bound Dioxygen. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 561–567.

42 Lissi, E. Autocatalytic Oxidation of Hemoglobin by Nitrite: A Possible Mechanism. *Free Radical Biol. Med.* **1998**, *24*, 1535–1536.

43 Doyle, M. P.; Pickering, R. A.; Dykstra, R. L.; Nelson, C. L.; Boyer, R. F. Involvement of Peroxide and Superoxide in the Oxidation of Hemoglobin by Nitrite. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 127–32.

44 Doyle, M. P.; Herman, J. G.; Dykstra, R. L. Autocatalytic Oxidation of Hemoglobin Induced by Nitrite: Activation and Chemical Inhibition. *J. Free Radicals Biol. Med.* **1985**, *1*, 145–153.

45 Ford, P. C.; Fernandez, B. O.; Lim, M. D. Mechanisms of Reductive Nitrosylation in Iron and Copper Models Relevant to Biological Systems. *Chem. Rev.* **2005**, *105*, 2439–55.

46 Nagababu, E.; Ramasamy, S.; Rifkind, J. M. S-Nitrosohemoglobin: A Mechanism for Its Formation in Conjunction with Nitrite Reduction by Deoxyhemoglobin. *Nitric Oxide* **2006**, *15*, 20–29.

47 Luchsinger, B. P.; Rich, E. N.; Gow, A. J.; Williams, E. M.; Stamler, J. S.; Singel, D. J. Routes to S-Nitroso-hemoglobin Formation with Heme Redox and Preferential Reactivity in the Beta Subunits. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 461–6.

48 Oshea, S. K.; Wang, W.; Wade, R. S.; Castro, C. E. Selective Oxygen Transfers with Iron(III) Porphyrin Nitrite. *J. Org. Chem.* **1996**, *61*, 6388–6395.

49 Castro, C. E.; Oshea, S. K. Activation of Nitrite Ion by Iron(III) Porphyrins - Stoichiometric Oxygen-Transfer to Carbon, Nitrogen, Phosphorus, and Sulfur. *J. Org. Chem.* **1995**, *60*, 1922–1923.

50 Day, E. P.; Peterson, J.; Bonvoisin, J. J.; Young, L. J.; Wilkerson, J. O.; Siegel, L. M. Magnetization of the Sulfite and Nitrite Complexes of Oxidized Sulfite and Nitrite Reductases: EPR Silent Spin S = 1/2 States. *Biochemistry* **1988**, *27*, 2126–2132.

51 Copeland, D. M.; Soares, A. S.; West, A. H.; Richter-Addo, G. B. Crystal Structures of the Nitrite and Nitric Oxide Complexes of Horse Heart Myoglobin. *J. Inorg. Biochem.* **2006**, *100*, 1413–1425.

52 Fernandez, B. O.; Ford, P. C. Nitrite Catalyzes Ferriheme Protein Reductive Nitrosylation. *J. Am. Chem. Soc.* **2003**, *125*, 10510–10511.

53 Han, T. H.; Hyduke, D. R.; Vaughn, M. W.; Fukuto, J. M.; Liao, J. C. Nitric Oxide Reaction with Red Blood Cells and Hemoglobin under Heterogeneous Conditions. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7763–7768.

54 Nagababu, E.; Ramasamy, S.; Rifkind, J. M. S-Nitrosohemoglobin: A Mechanism for Its Formation in Conjunction with Nitrite Reduction by Deoxyhemoglobin. *Nitric Oxide* **2006**, *15*, 20–29.

55 Gladwin, M. T.; Crawford, J. H.; Patel, R. P. The Biochemistry of Nitric Oxide, Nitrite, And Hemoglobin: Role in Blood Flow Regulation. *Free Radical Biol. Med.* **2004**, *36*, 707–717.

56 Gladwin, M. T.; Raat, N. J.; Shiva, S.; Dezfulian, C.; Hogg, N.; Kim-Shapio, D. B.; Patel, R. P. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *291*, H2026–H2035.