Intermediates Detected by Visible Spectroscopy during the Reaction of Nitrite with Deoxyhemoglobin: The Effect of Nitrite Concentration and Diphosphoglycerate†

Enika Nagababu, Somasundaram Ramasamy, and Joseph M. Rifkind*

Molecular Dynamics Section, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, Maryland 21224

Received February 21, 2007; Revised Manuscript Received July 18, 2007

ABSTRACT: The reaction of nitrite with deoxyhemoglobin (deoxyHb) results in the reduction of nitrite to NO, which binds unreacted deoxyHb forming Fe(II)-nitrosylhemoglobin (Hb(II)NO). The tight binding of NO to deoxyHb is, however, inconsistent with reports implicating this reaction with hypoxic vasodilation. This dilemma is resolved by the demonstration that metastable intermediates are formed in the course of the reaction of nitrite with deoxyHb. The level of intermediates is quantitated by the excess deoxyHb consumed over the concentrations of the final products formed. The dominant intermediate has a spectrum that does not correspond to that of Hb(III)NO formed when NO reacts with methemoglobin (MetHb), but is similar to metHb resulting in the spectroscopic determinations of elevated levels of metHb. It is a delocalized species involving the heme iron, the NO, and perhaps the β-93 thiol. The putative role for red cell reacted nitrite on vasodilation is associated with reactions involving the intermediate. (1) The intermediate is less stable with a 10-fold excess of nitrite and is not detected with a 100-fold excess of nitrite. This observation is attributed to the reaction of nitrite with the intermediate producing N_2O_3. (2) The release of NO quantitated by the formation of Hb(II)NO is regulated by changes in the distal heme pocket as shown by the 4.5-fold decrease in the rate constant in the presence of 2,3-diphosphoglycerate. The regulated release of NO or N_2O_3 as well as the formation of the *S*-nitroso derivative of hemoglobin, which has also been reported to be formed from the intermediates generated during nitrite reduction, should be associated with any hypoxic vasodilation attributed to the RBC.

The interest in a role for the red cell in transporting NO[1] to the vasculature began with the 1996 seminal paper by Stamler and collaborators (*1*) proposing a transfer of NO from the heme to the β-93 cysteine producing an *S*-nitroso derivative (SNOHb) and the subsequent conformationally linked release of the thiol NO. The 2003 studies by Rifkind (*2*) and Gladwin (*3*) and their collaborators proposed an alternative mechanism that involved the reduction of nitrite, formed by the oxidation of NO, back to NO by a reaction with deoxyHb. This mechanism overcame the conceptual difficulties (*4*) involved in transferring tightly bound NO from Fe(II) to the cysteine. The reduction of nitrite by deoxyhemoglobin (deoxyHb or Hb(II)) to produce nitric oxide was originally studied by Brooks (*5*) and subsequently by Doyle (*6*). The potential physiological role for this reaction was actually originally proposed by Reutov (*7*, *8*). Nitrite reduction, as proposed by Doyle et al. (*6*), involves the proton assisted reduction to NO (eq 1). Because of the high affinity of NO for deoxyHb, the released NO will in the absence of oxygen rapidly bind to unreacted deoxyHb producing nitrosylhemoglobin (Hb(II)NO) (eq 2).

$$\text{Hb(II)} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Hb(III)} + \text{NO} + \text{OH}^- \quad (1)$$

$$\text{Hb(II)} + \text{NO} \rightarrow \text{Hb(II)NO} \quad (2)$$

The rapid reaction of released NO with both deoxyHb, as well as oxyhemoglobin (oxyHb) (*9*), seems to prevent the formation of a pool of potentially bioactive NO in the red cell that can be released under the proper conditions. This is unlike the SNOHb hypothesis, where, once the NO reacts with the thiol, it no longer reacts with deoxyHb or oxyHb.

An intermediate with NO^+ associated with the Fe(II) heme that is in equilibrium with the Fe(III) heme with NO bound (Hb(III)NO) has been reported (*10*) to take place both during nitrite reduction by heme cd_1 and when NO binds to Hb(III). While such an intermediate would be expected to be formed during the reduction of nitrite by deoxyHb, it was assumed in the Doyle analysis that the reaction proceeds with the formation of Hb(III) and Hb(II)NO (both of which are detected by visible spectroscopy) without any significant concentration of the intermediates.

The initial studies by the Rifkind group comparing electron paramagnetic resonance (EPR) for the determination of Hb(II)NO and chemiluminescence for total heme associated NO products (*2*) suggested that the elevated chemiluminescence

† This research was supported by the Intramural Research Program of the NIH, National Institute on Aging.

* Address correspondence to this author. Tel: 410-558-8168. Fax: 410-558-8397. E-mail: rifkindj@grc.nia.nih.gov.

[1] Abbreviations: deoxyHb, deoxyhemoglobin; Hb(II), deoxyhemoglobin; EPR, electron paramagnetic resonance; NO, nitric oxide; RBC, red blood cell; Hb(II)NO, nitrosylhemoglobin; NO_2^-, nitrite; Hb(III)NO_2^-, methemoglobin nitrite; Hb(III)NO, nitrosylmethemoglobin; metHb, methemoglobin; Hb(III), methemoglobin; Hb(II)NO^+, nitrosonium complex of hemoglobin; oxyHb, oxyhemoglobin; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; CO, carbon monoxide; Hb(II)CO, carbonmonoxyhemoglobin.

10.1021/bi700364e
Published on Web 09/20/2007

signal implies that some of the intermediates formed during nitrite reduction may accumulate providing a pool of potentially bioactive NO.

$$\text{Hb(II)} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{intermediates} \rightarrow \text{Hb(III)} + \text{NO} + \text{OH}^- \quad (3)$$

Questions have been raised about this chemiluminescence data. The chemiluminescence data indicated an appreciable reaction of deoxyHb with nitrite within minutes even with a 4-fold excess of hemoglobin. While a rapid reaction of nitrite with deoxyHb is also seen by photolysis (*11*), the reaction of nitrite with deoxyHb followed by visible spectroscopy is much slower (*6*, *12*−*15*).

In the chemiluminescence assay the sample incubated at neutral pH is injected into a purge vessel at low pH to release the NO. Despite the observation that there is a dependence on the time of incubation at neutral pH (*2*) that indicates some form of nitrite interaction with hemoglobin at neutral pH, the discrepancy between the chemiluminescence and the visible spectroscopy suggests that at least part of the additional reaction obtained by chemiluminescence is generated during the analysis when the sample is injected into a highly acidic solution. These reservations are supported by the known enhancement of the nitrite reaction at low pH (*6*) and the finding that most of the added nitrite at neutral pH can be separated from hemoglobin by filtration (*16*).

Low pH determinations required for the chemiluminescence assay can, therefore, not be used to quantitatively determine the intermediates formed during the reaction of nitrite with deoxyHb. Neutral pH spectroscopic analysis should be able to quantitate any stable intermediates formed during the reduction of nitrite by deoxyHb. However, the identification of the spectrum of the intermediate(s) requires a complex multicomponent spectral analysis involving deoxyHb, Hb(II)NO, metHb, metHb with nitrite bound and one or more intermediates, all of which absorb in the same visible spectral region. The spectrum of metHb, which may be related to some of theses intermediates, involves multiple bands (*17*) and is sensitive to pH, heme pocket instability which produces a range of hemichromes (*18*) and the binding of a wide range of ligands including nitrite and NO. The metHb related complexes are, therefore, the most difficult components to accurately quantitate.

Our earlier studies (*2*) have demonstrated the Hb(III)NO nature of the intermediate by showing that the intermediate is converted to Hb(III) when flushed with argon. On the basis of these results, other investigators have included the known visible spectrum of Hb(III)NO (*19*) in their analysis of the spectral changes during the reaction of nitrite with deoxyHb. The dominant spectra for the intermediate(s) formed during the reduction of nitrite by deoxyHb, however, need not coincide with the reported spectrum for Hb(III)NO (*19*) obtained when NO is reacted with Hb(III). The intermediate formed during nitrite reduction is a delocalized species with the electron shared between NO, the iron, and perhaps the β-93 thiol (*20*). Although a similar intermediate is formed subsequent to the binding of NO to Hb(III) (*10*); it has not been established that this intermediate coincides with the spectroscopic species formed immediately after the addition of NO to Hb(III) (*21*, *19*).

In this paper we have investigated the spectral changes that take place both during the reaction of nitrite with deoxyHb and during the reaction of NO with metHb. We have used the overall reaction of nitrite with deoxyHb (*6*) and the effect of intermediates on the stoichiometry of this

$$2\text{Hb} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Hb(III)} + \text{Hb(II)NO} + \text{OH}^- \quad (4)$$

reaction (eq 3) to quantitate the level of intermediates. The concentration of intermediates is given by

$$[\text{intermediates}] = [\text{decrease in deoxyHb}] - 2[\text{Hb(II)NO}] \quad (5)$$

This analysis depends only on the well-defined spectra of deoxyHb and nitrosylHb and does not depend on a complex and uncertain determination of the spectra of the intermediate(s) formed. It does, however, neglect effects due to side reactions not included in eqs 3 and 4.

This analysis provides evidence for the formation of intermediate(s) that account for 15−20% of the initial deoxyHb. Theses levels are less than implied by the chemiluminescence data, but indicate the formation of a pool of NO that is protected from being quenched by reacting with deoxyHb or oxyHb. We have also demonstrated that the spectroscopic Hb(III)NO initially formed when NO reacts with Hb(III) is a transient species and not the dominant intermediate formed during the reaction.

By comparing the reaction at different nitrite concentrations, the role of the nucleophilic reaction of nitrite with the intermediate is established (*22*). The effect of 2,3-diphosphoglycerate (DPG) on the formation of the intermediates is also delineated.

MATERIALS AND METHODS

DPG was obtained from Sigma Chemical Company. Hemoglobin was prepared from fresh RBCs as described earlier (*2*). Hemoglobin in 50 mM NaCl and 4 mM phosphate buffer, pH 7.4 was deoxygenated in an anaerobic Coy glove box. The glove box uses hydrogen and a palladium catalyst to remove any residual oxygen. The oxygen level when used is <1 ppm. The sample was placed in a septum sealed cuvette in the glove box and then removed for spectral analysis. Multicomponent fitting of the deoxyHb sample obtained by this procedure did not detect any oxyHb, and no oxyHb accumulation was detected during the course of the experiment. The total hemoglobin concentrations in the samples were ~100 μM. Stock solutions of nitrite and DPG in the same buffer were also deoxygenated in the glove box and transferred to septum sealed cuvettes. The reaction was initiated by using a gastight syringe to add nitrite to the sealed cuvette containing hemoglobin.

The spectra of hemoglobin were recorded from 490 to 640 nm on a Perkin-Elmer Lambda 35 spectrophotometer for 35 min at a 1:1 and 10:1 molar ratio of nitrite to heme. For experiments with a 100-fold excess of nitrite the reaction was much faster and was only followed for ~10 min. For some experiments DPG was added 10 min before the initiation of the reaction with nitrite.

Spectral Analysis. Parent spectra of deoxyHb, metHb, Hb(II)NO, oxyHb, and nitrite bound metHb were prepared. The series of spectra obtained in any experiment were

analyzed using a least-squares multicomponent fitting program (Perkin-Elmer Spectrum QuantC v 4.51) including these 5 spectra. Since no oxyHb was detected, the spectra were also fit without including the oxyHb parent spectrum.

RESULTS AND DISCUSSION

Spectroscopic Evidence for the Buildup of Intermediates during the Reduction of Nitrite by DeoxyHb. A number of studies have recently been published investigating the reaction of nitrite with deoxyHb (*2*, *12*−*16*, *20*, *23*). A comparison of different experiments, however, needs to recognize that the reaction is very sensitive to experimental conditions. The pH plays a crucial role with the reaction faster at low pH due to the protonation of the nitrite (*6*), which facilitates binding. Almost all studies, except for the early studies of Doyle (*6*), which investigated the effect of pH, have been performed in the region of pH 7.4.

Recent studies indicate that the reaction is affected by the hemoglobin quaternary conformation (*24*). The reaction should, therefore, be sensitive to ionic strength and buffer systems although these effects have not been investigated in any organized fashion and in some cases the buffer and ionic conditions are not mentioned (*15*).

The nitrite:heme ratio used in any experiment is an important factor that must be considered in comparing results from different groups. Doyle (*6*) performed his experiments with 9−25-fold excess of nitrite. The Huang paper (*13*) looks at a range of ratios, but emphasizes the studies at a 5-fold excess of nitrite. Stamler and collaborators in many of their studies involving SNOHb (*25*−*29*) have stressed the importance of a very large excess of hemoglobin to mimic the physiological levels of NO. However, in the visible spectroscopy studies involving nitrite (*14*, *15*), they have not used more than a 10-fold heme excess and have investigated the differences between an excess of heme and an excess of nitrite. In our earlier studies (*2*) comparing the chemiluminescence and the EPR, we used a 4-fold excess of heme. However, in this study we start with equivalent concentrations of heme and nitrite to optimize the concentration of the products formed and further investigate the effect of a large excess of nitrite (see below).

Figure 1 shows the 21.24 s repetitive spectra obtained when 99.65 μM deoxyHb was reacted with 99.65 μM nitrite in 50 mM NaCl and 4 mM phosphate, pH 7.4. These spectra have good isosbestic points similar to the spectroscopic data reported earlier for this reaction (*6*, *13*, *14*). Good isosbestic points are supportive of a single reaction with no appreciable levels of intermediates. However, the time dependent changes in the concentrations (Figure 2) obtained, from the multicomponent fit of the spectra, are inconsistent with a reaction without intermediates.

The overall reaction (eq 4), without any intermediates, corresponds to two molecules of Hb(II) producing one molecule of Hb(III) and one molecule of Hb(II)NO. Because of the difficulties involved in quantifying the total metHb, and the requirement that the Hb(II)NO be equal to the total metHb formed, eq 4 without intermediates predicts that the consumption of deoxyHb be twice the formation of Hb(II)NO. An excess consumption of deoxyHb can be attributed to the formation of intermediates (eqs 3 and 5). While most investigators have noted the fact that the formation of Hb(III) > Hb(II)NO (see below), most studies have not quantitatively compared the consumption of deoxyHb with the formation of Hb(II)NO. It is, however, this data which provides the most direct evidence for intermediates.

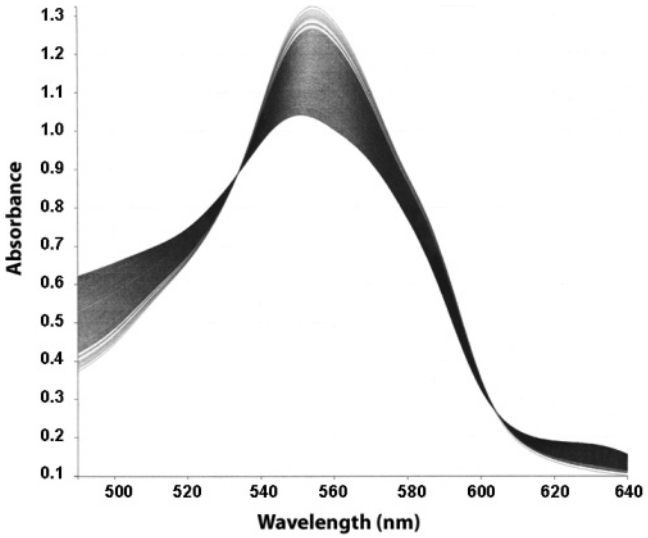


FIGURE 1: Series of spectra obtained during the reaction of an equimolar concentration of nitrite with deoxyHb. 99.65 μM deoxyHb was reacted with 99.65 μM nitrite in 50 mM NaCl and 4 mM phosphate buffer, pH 7.4 at 22 °C. Spectra were scanned at a rate of 960 nm/min with repetitive scans initiated every 21.24 s.

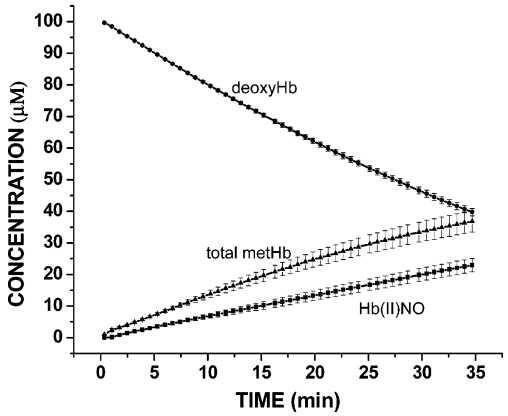


FIGURE 2: The consumption of deoxyHb and the formation of the reaction products as a function of time during the reaction of a 1:1 molar ratio of nitrite and deoxyHb under the same conditions as for Figure 1. (●) deoxyHb; (■) Hb(II)NO; (▲) total metHb (Hb(III) + Hb(III)NO_2^-).

The plot (Figure 2) of the decrease in deoxyHb together with a plot of Hb(II)NO and total metHb (Hb(III) + Hb(III)NO_2^-) shows that 60.4 μM of deoxyHb is consumed in 35 min during which time only 23.15 μM of Hb(II)NO is formed. The 2.6-fold greater consumption of deoxyHb than the formation of Hb(II)NO is inconsistent with the model assuming no intermediate(s). With intermediates being formed, some of the deoxyHb will be converted into intermediates (eq 3) and the consumption of deoxyHb will be greater than the concentration of Hb(II)NO + metHb or twice the concentration of Hb(II)NO. The comparison of the consumption of deoxyHb and the formation of Hb(II)NO not only establishes that intermediate(s) are formed, but makes it possible to calculate the time dependent formation of intermediate(s) (eq 5), which is shown in Figure 3.

An additional inconsistency with eq 4 noted in Figure 2 is that the total metHb > Hb(II)NO even though eq 4 predicts

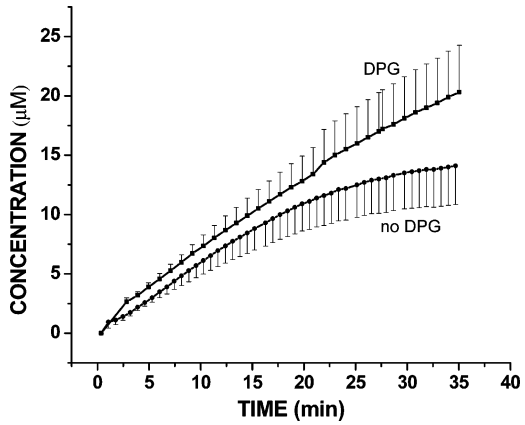


FIGURE 3: Time course for the formation of intermediates as determined from the [deoxyHb consumed] − 2[Hb(II)NO]. (●) without DPG; (■) in the presence of 1 mM DPG.

equal concentrations of both of these products. This discrepancy cannot be attributed directly to the formation of intermediate(s), since even with intermediate(s) both Hb(III) and Hb(II)NO are formed when NO is released from the intermediate (eq 4). Almost all investigators (*6*, *13*−*15*) that have looked at this reaction find that Hb(III) > Hb(II)NO. Doyle et al. (*6*) obtained higher levels of Hb(III) than Hb(II)NO (72% Hb(III) and 28% Hb(II)NO) when they followed the nitrite reaction at pH 7.0. They investigated several possibilities and suggested the oxidation of deoxyHb by the NO dimer, $(NO)_2$. The decomposition of Hb(II)NO to form NO^- and Hb(III) (*30*) can also contribute to this discrepancy, although these side reactions are not expected to be efficient enough to account for the appreciable excess Hb(III) generally detected.

A recent investigation of the excess Hb(III) produced during the reaction of nitrite with deoxyHb (*13*) found much closer agreement when including the nitrite complex with Hb(III) for carefully deoxygenated samples. They also demonstrated that the presence of low concentrations of oxygen will increase the relative values of total Hb(III), presumably due to the reaction of nitrite with oxyHb, and suggested that the discrepancy was attributed to oxygen contamination. While it is difficult to completely rule out a contamination of oxygen, most investigators have made every effort to eliminate oxygen contamination (*6*, *14*).

In our studies, we also included the nitrite complex of Hb(III) and eliminated the possibility of oxygen contamination by using an anaerobic Coy glove box containing hydrogen and a palladium catalyst to react with any oxygen resulting in <1 ppm oxygen in the glove box. Our spectral fitting of the samples during the 35 min reaction time did not indicate any accumulation of oxyHb, ruling out the leakage of oxygen into the sealed cuvette. Furthermore, the finding that using identical methods for experiments with a large excess of nitrite (see below) did not result in values of Hb(III) greater than those of Hb(II)NO rules out the possibility of oxygen leaking into the sample during the addition of nitrite.

In the paper by Huang et al. (*13*), EPR was used to support their contention that there was a 1:1 correspondence of Hb(III) and Hb(II)NO. In this analysis the ratio of Hb(II)NO:Hb(III) of 0.9 ± 0.2 (mean ± SE) is consistent with the 1:1 relationship, but the errors are too great to be able to conclude that equal concentrations of Hb(III) and Hb(II)NO are actually formed. The only spectroscopic data shown in this paper was obtained with a 5-fold excess of nitrite. Our results (see below) show that with an excess of nitrite the intermediates are less stable because of the reaction of nitrite with the intermediate producing N_2O_3. This side reaction can explain the correspondence of the concentrations of Hb(III) and Hb(II)NO in their spectral experiments.

An alternative explanation for [total metHb] > [Hb(II)NO] or a ratio of [total Hb(III)]:[Hb(II)NO] > 1 involves intermediate species with spectra more similar to those of Hb(III) or the Hb(III)nitrite complex than Hb(II)NO. An attempt to fit using multicomponent analysis without including spectra for the intermediate(s) would, therefore, result in higher values of total Hb(III) than expected.

Spectral Hb(III)NO and the Spectrum of the Intermediate. The Hb(III)NO character of the intermediate was demonstrated by its conversion to Hb(III) when flushed with argon (*2*). This observation led some investigators (*12*) to assume that the spectrum of the nitrite reduced intermediate should coincide with the reported spectrum of Hb(III)NO (*19*). Partially because of the difficulties involved in fitting with a number of similar spectra, in many cases (*13*, *14*) no Hb(III)NO was detected, and it was concluded that no significant concentration of intermediates is present.

The difficulties in establishing spectral intermediates for this reaction are indicated by recent results by Stamler and collaborators. Initially in a paper (*14*) at 1 mM heme and 0.67 mM nitrite they were not able to detect any Hb(III)NO, although they did find Hb(III) > Hb(II)NO and the Hb(II)NO after 100 min was clearly less than twice the consumption of deoxyHb. A more careful analysis by the same group (*15*) was, however, able to detect Hb(III)NO. The Hb(III)NO levels and its stability depended on the ratio of nitrite to heme. In the presence of excess heme (nitrite:heme = 0.3) the Hb(III)NO rose to a plateau, while with an excess of nitrite (nitrite:heme = 1.6) the Hb(III)NO reached a peak in the middle of the reaction (see below) consistent with a secondary reaction involving this intermediate.

In these experiments the maximum concentration of the Hb(III)NO intermediate corresponded to about 5% of the hemoglobin or nitrite, whichever was limiting. This can be compared with our analysis, which results in 15−20% of the hemoglobin found as intermediates. Our experiments with a 1:1 mole ratio fall between the two experiments performed in the Angelo paper, corresponding to a nitrite to heme ratio of 1.6:1 and 0.3:1. Thus, while some of our observed intermediates can be attributed to Hb(III)NO, other species seem to be present.

In order to understand the presence of intermediate spectral species other than Hb(III)NO during the nitrite reaction, we directly studied the reaction of NO with Hb(III), which produces Hb(III)NO with a well-defined visible spectrum (Figure 4).

This reaction results in reductive nitrosylation (*19*, *31*) thought to involve the partial transfer of electrons from NO to the heme producing the same delocalized species proposed (*16*) as an intermediate in the reaction of nitrite with deoxyHb.

$$\text{Hb(III)} + \text{NO} \leftrightarrow \text{Hb(III)NO} \rightarrow \text{“Hb(II)NO}^+ \leftrightarrow \text{Hb(III)NO”} \quad (6)$$

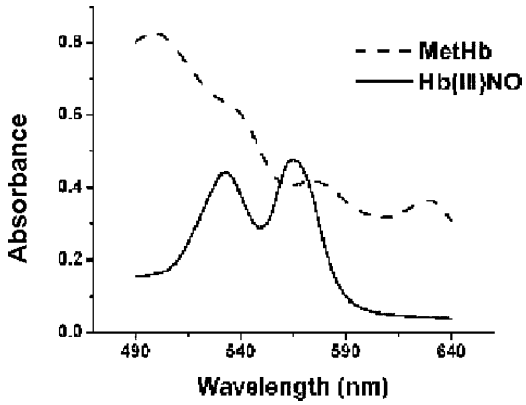


FIGURE 4: Visible spectrum of Hb(III)NO formed 30 s after the addition of 89 μM NO to 89 μM Hb(III). At this concentration only 41% of the Hb(III) reacted with NO producing 36.5 μM Hb-(III)NO. The Hb(III)NO spectrum shown (—) was obtained by subtracting the 52.5 μM unreacted Hb(III) from the observed spectrum. Also shown is the spectrum of 89 μM Hb(III) (– – –).

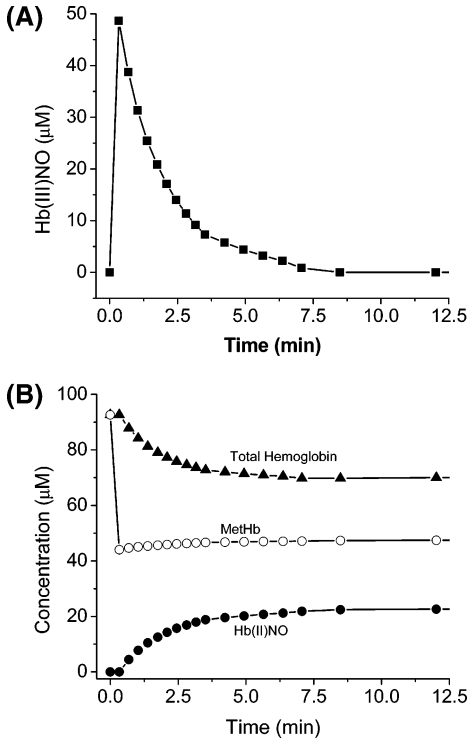


FIGURE 5: Time course for the reaction of a 92.7 μM metHb with 92.7 μM NO. (A) The time course for the formation and decay of Hb(III)NO (■). (B) The time course for the disappearance of metHb (○); the formation of Hb(II)NO (●); and the total hemoglobin (▲) calculated from the determination of concentrations of metHb, Hb-(III)NO, and Hb(II)NO. The total calculated hemoglobin is included to show that species other than those included in the figure must be forming.

It is this intermediate which reacts with another molecule of NO (*19*) resulting in the NO complex with reduced Fe(II) heme.

$$\text{“Hb(II)NO}^{+} \leftrightarrow \text{Hb(III)NO”} + \text{NO} \rightarrow \text{Hb(II)NO} + \text{NO}^{+} \quad (7)$$

It is generally assumed (*12*) that the reported Hb(III)NO spectrum is the spectrum of the delocalized intermediate (eq 6). In Figure 5 it is shown that the reaction forming spectroscopic Hb(III)NO occurs within 30 s. However, this

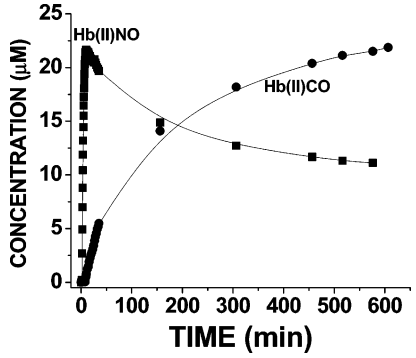


FIGURE 6: Time course for the reaction of a 90.9 μM metHb saturated with 1 atm of CO and then reacted with 90.9 μM NO. Even with the CO the initial reaction is similar to that shown in Figure 5. This is followed by a slow reaction involving the formation of Hb(II)CO (●) and the partial displacement of NO from Hb(II)NO (■).

Table 1: EPR Analysis for the Reaction of 1:1 NO with MetHb

sample	concentration (μM)
initial MetHb concentration	579.0
MetHb after 5 min	5.3
Hb(II)NO after 5 min	211.5
undetected hemoglobin after 5 min	362.2

reaction is followed by a second reaction, which corresponds to the consumption of the entire spectral component corresponding to Hb(III)NO, with a half-time of <2 min. While Hb(II)NO is formed during this second phase of the reaction, it accounts for less than half of the hemoglobin that was originally Hb(III)NO (Figure 5). When this reaction levels off, only ~75% of the total hemoglobin is accounted for. Although SNOHb is formed during this reaction (*20*, *25*), the relatively high 25% discrepancy requires that the presence of other intermediate species be considered.

To confirm the presence of species other than metHb and Hb(II)NO after the spectroscopic Hb(III)NO is no longer present, we used EPR which can directly detect both of these species. EPR can quantitate metHb based on the $g = 6$ high spin metHb band, and Hb(II)NO can be detected by its established spectrum (*32*) in the region of $g = 2$. As shown in Table 1, 5 min after NO saturated buffer corresponding to 579 μM of NO reacted with 579 μM of metHb, only 5.34 μM of unreacted metHb was detected and only 211.5 μM of Hb(II)NO had been formed. Thus, 62.6% of the hemoglobin is not accounted for. These results require that during the reaction of NO with metHb additional species are present other than metHb, spectral Hb(III)NO, and Hb(II)NO. The difference between the EPR experiment (Table 1) and the visible spectroscopy experiment (Figure 5) with less residual metHb and a higher percentage of unaccounted hemoglobin for the EPR experiment is due to the higher concentration of the reactants resulting in more of the added NO binding to the metHb and proportionally less free NO. Less free NO will reduce the formation of SNOHb (*20*) and limit the secondary NO reaction required for the formation of Hb(II)-NO (eq 7).

To further confirm the presence of an intermediate distinct from the spectroscopic Hb(III)NO, the visible spectroscopy experiment was performed in the presence of a large excess of carbon monoxide (Figure 6). The slow reaction of carbon monoxide to produce Hb(II)CO (Figure 6) does not involve

a direct reaction of CO with Hb(III), since carbon monoxide does not react with Fe(III). The decrease in Hb(II)NO corresponding to the formation of Hb(II)CO is attributed to the very large excess of CO, which can displace NO from the Fe(II) heme. However, as shown in Figure 6, the concentration of Hb(II)CO formed is more than twice that formed as a result of displacing NO from Hb(II)NO. This is attributed to a reaction with the delocalized intermediate.

$$\text{“Hb(II)NO}^+ \leftrightarrow \text{Hb(III)NO”} + \text{CO} \rightarrow \text{Hb(II)CO} + \text{NO}^+ \quad (8)$$

The nitrosonium cation released rapidly hydrolyzes in the presence of water producing nitrite.

$$\text{NO}^+ + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HNO}_2 \leftrightarrow 2\text{H}^+ + \text{NO}_2^- \quad (9)$$

The relatively slow (half time of 1−2 min) conversion of the Hb(III)NO spectral component to the more stable delocalized species (eq 6) can be explained by a globin rearrangement required to facilitate the different heme geometry associated with the partial Fe(II) nature of the delocalized intermediate. This rearrangement is expected to alter the visible spectrum. The spectrum of Hb(III)NO with two well-defined bands between 600 and 500 nm consistent with a low spin heme complex is thus not observed for the delocalized intermediate with characteristics of Fe(III) and Fe(II).

This change also alters kinetics for the release of NO. The Fe(III)NO has a reported NO off rate in the region of 1 s^{-1}, (*33*), while the delocalization stabilizes the complex resulting in a much slower rate of dissociation (*31*). In the same way that the binding of NO to Hb(III) goes through the initial Hb(III)NO complex and then the delocalized intermediate, the release of NO (but not NO^+) from the intermediate should involve the Hb(III)NO species. It is this species that is observed by Angelo et al. (*15*). However, as indicated by the comparison with our results, the other delocalized intermediate is present at a higher concentration than the Hb(III)NO. This is unlike the situation when NO is added to Hb(III) and the initial dominant species obtained is Hb(III)NO because of the relatively high initial NO concentration and the relatively slow (Figure 5) formation of the delocalized intermediate.

Combining the data on nitrite reduction by deoxyHb and the reductive nitrosylation when NO is reacted with Hb(III), the presence of a significant buildup of a delocalized species is established. The relative stability of this intermediate is indicated by the slow reaction with CO. This intermediate is, therefore, able to retain NO in a form which is not trapped as Hb(II)NO.

The Effect of Excess Nitrite on the Reduction of Nitrite by DeoxyHb. The data shown in Figure 2 for a 1:1 molar ratio of nitrite and deoxyHb indicates that deoxyHb is consumed faster than twice the formation of Hb(II)NO (Δ[deoxyHb]/Δ[Hb(II)NO] > 2) indicative of the formation of metastable intermediate(s) (eq 1). At the same time the higher level of total metHb than Hb(II)NO (Δ[(total metHb)]/Δ[Hb(II)NO] indicates that the intermediates are spectrally more similar to metHb than Hb(II)NO. Using the final points obtained after 35 min these two observations result in the ratios Δ[deoxyHb]/Δ[Hb(II)NO] = 2.61 and Δ[(total metHb)]/Δ[Hb(II)NO] = 1.60 for a 1:1 molar ratio of nitrite and deoxyHb (Table 2). However, with a 10-fold excess of nitrite both ratios decreased and with a 100-fold excess of nitrite the values of 2.09 for Δ[deoxyHb]/Δ[Hb(II)NO] and 1.006 for Δ[(total metHb)]/Δ[Hb(II)NO] are within error of the prediction for no intermediates.

Table 2: Analysis of Species Concentrations That Require Intermediates

sample	ΔdeoxyHb/ΔHb(II)NO	Δtotal metHb/ΔHb(II)NO
1:1 nitrite:Hb no DPG	2.61	1.60
10:1 nitrite:Hb no DPG	2.434	1.35
100:1 nitrite:Hb no DPG	2.09	1.01
1:1 nitrite:Hb DPG	4.52	3.68
100:1 nitrite:Hb DPG	2.00	0.623

All of these experiments were performed in exactly the same way, using our anaerobic Coy glove box followed by the injection of nitrite into the sample using a gastight syringe. Furthermore, any leakage of oxygen into the cuvette is ruled out by no observed increase in oxyHb during the course of the reaction. The nitrite dependence of these results (Table 2) can, therefore, not be attributed to the preferential leakage of oxygen into the sample with low nitrite concentrations or any other experimental artifact.

The dependence on nitrite concentration instead indicates that the intermediates are destabilized at elevated levels of nitrite. Angelo et al. (*15*) also found that the intermediate, which in their analysis was Hb(III)NO, was also less stable with an excess of nitrite. This was attributed to the transfer of NO or NO^+ directly from the Hb(III)NO intermediate to the β-93 thiol producing SNOHb when the quaternary conformation is shifted from T to R at elevated nitrite levels which increase the formation of Hb(III) and Hb(II)NO.

We have also reported the formation of SNOHb during the reduction of nitrite by deoxyHb and much more efficiently when NO reacts with Hb(III). We have attributed this reaction not to spectral Hb(III)NO, but the delocalized species which is thought to have the electron distributed between the iron, the NO, and the β-93 thiol.

$$\text{“:S−Hb(III)NO} \leftrightarrow \text{:S−Hb(II)NO}^+ \leftrightarrow \bullet\text{S−Hb(II)NO”} \rightarrow \text{ONS−Hb(II)} \quad (10)$$

The large excess of nitrite required to completely eliminate the intermediates suggests that in addition to a shift in quaternary conformation a secondary reaction with nitrite must be involved. This finding is consistent with the properties of the delocalized intermediate formed during nitrite reduction. The same intermediate formed during reductive nitrosylation of Hb(III) (see above) has been shown (*22*) to slowly react with nitrite producing N_2O_3.

$$\text{“Hb(II)NO}^+ \leftrightarrow \text{Hb(III)NO”} + \text{NO}_2^- \rightarrow \text{Hb(II)} + \text{N}_2\text{O}_3 \quad (11)$$

The regenerated Hb(II) will react with an additional molecule of nitrite when excess nitrite is present. During each cycle, the intermediate can either release NO (eq 1) producing Hb(II)NO and metHb or react with nitrite (eq 11) producing N_2O_3, which can react with thiols producing S-nitrosated thiols.

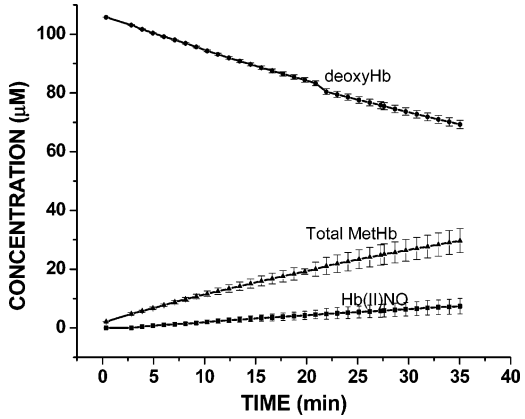


FIGURE 7: The consumption of deoxyHb and the formation of the reaction products as a function of time during the reaction of a 1:1 molar ratio of nitrite and deoxyHb in the presence of 1 mM DPG. 105.8 μM deoxyHb was reacted with 105.8 μM nitrite in 50 mM NaCl and 4 mM phosphate buffer, pH 7.4 at 22 °C. (●) deoxyHb; (■) Hb(II)NO; (▲) total metHb (Hb(III) + Hb(III)NO_2^-).

The instability of the intermediate in the presence of excess nitrite explains why investigators (*13*) using excess nitrite have had difficulties detecting the delocalized intermediate.

The Effect of 2,3-Diphosphoglycerate (DPG) on the Reaction of Nitrite with DeoxyHb. RBCs contain 0.2 mM DPG, which stabilizes the T-state by binding preferentially to T-state hemoglobin (*34*). However, it has been shown that DPG not only stabilizes the T-state but also alters the conformation of T-state hemoglobin (*35*). Recent data suggest that binding by DPG or other anions along the dyad axis between β-chains affects the interaction between subunits (*36*, *37*). Furthermore, DPG binding has been shown to alter interactions in the heme pocket. In an earlier report (*38*), we showed that inositol hexaphosphate (IHP), which has an effect similar to that of DPG, very appreciably alters the reaction of nitrite with deoxyHb. In order to determine what effect DPG has on the reaction of deoxyHb with nitrite and the formation of the intermediates, the spectral changes that take place with a 1:1 molar ratio of nitrite:hemoglobin were also studied in the presence of 1 mM DPG.

Figure 7 shows the consumption of deoxyHb, the formation of Hb(II)NO, and the formation of total metHb for the reaction of 105.8 μM deoxyHb with 105.8 μM nitrite in the presence of 1 mM DPG. Comparing Figure 7 with Figure 2 it is apparent that DPG slows down the consumption of deoxyHb as well as the formation of the end product Hb(II)NO. The observation that the formation of Hb(II)NO with DPG decreases to a greater extent than the corresponding decrease in the consumption of deoxyHb resulting in a higher value for Δ[deoxyHb]/Δ[Hb(II)NO] as well as higher values of Δ[(total metHb)]/Δ[Hb(II)NO] (Table 2) is consistent with higher levels of the intermediate(s) in the presence of DPG. This is shown in Figure 3 where the time dependent formation of the intermediate(s) formed with and without DPG is compared. Thus at the end of 35 min the concentration of intermediate(s) is 20.3 μM with DPG as compared to 14.1 μM without DPG. This effect is even more pronounced when we consider that with DPG only 36.5 μM deoxyHb has reacted while 60.4 μM deoxyHb reacts in the absence of DPG. These results indicate that, although the initial rate for the reaction of nitrite with deoxyHb (the formation of the intermediate) in the presence of DPG decreases, the rate for the release of NO from the delocalized intermediate decreases to a greater extent.

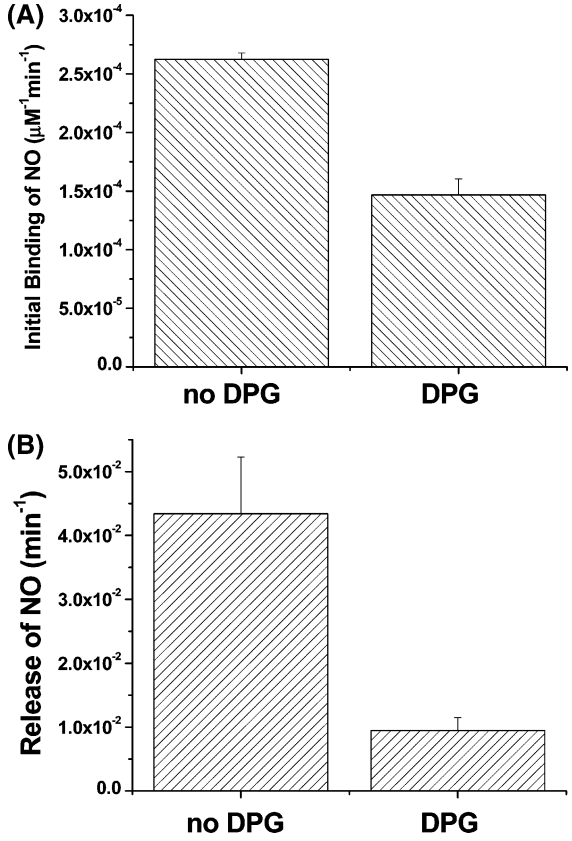


FIGURE 8: Comparison of rate constants with and without DPG. (A) Rate constants for the initial binding of nitrite to deoxyHb. (B) Rate constants for the release of NO from the delocalized intermediate.

Rate Constants for the Formation of Intermediates and the Release of NO from the Intermediates. The effect of DPG on the formation of intermediates and the release of NO from the intermediate can be quantitated from the time dependence of the reaction given in Figures 2, 3, and 7. From the time dependence for the consumption of deoxyHb (Figures 2 and 7) rate constants for the formation of the intermediate(s) can be calculated. Since we have shown that the consumption of deoxyHb does not correspond to the formation of Hb(II)NO (see above), the initial consumption of deoxyHb corresponds to the initial species formed, i.e., the intermediates (eq 3). Therefore, an apparent rate constant for the formation of the intermediate(s) (k_1) is given by the expression

$$-\{d[Hb(II)]/dt\}_0 = k_1[Hb(II)]_0[NO_2^-]_0 \qquad (12)$$

The initial slope was obtained from the plots of the consumption of deoxyHb shown in Figures 2 and 7, and the initial values of nitrite and hemoglobin are known. Using eq 12, there was a significant decrease ($p < 0.001$) in the value of k_1 by 44% from $2.6 \pm 0.1 \times 10^{-4}$ μM^{-1} min^{-1} without DPG to $1.5 \pm 0.1 \times 10^{-4}$ μM^{-1} min^{-1} with DPG (Figure 8A) quantitating the decrease in the interaction of nitrite with deoxyHb in the presence of DPG.

The formation of Hb(II)NO during the nitrite reaction is determined by the rate for the release of NO from the delocalized intermediate,

$$\text{“Hb(II)NO}^{+} \leftrightarrow \text{H(III)NO”} \rightarrow \text{Hb(III)} + \text{NO} \quad (13)$$

which then very rapidly reacts with deoxygenated hemoglobin chains producing Hb(II)NO (eq 2).

The rate constant for the release of NO from the delocalized intermediate was determined from eq 14 using the final slope of the plot of Hb(II)NO as a function of time and the final concentration of intermediates after 35 min.

$$\{d[\text{Hb(II)NO}]/dt\}_{\text{final}} = k_2[\text{delocalized intermediate}]_{\text{final}} \quad (14)$$

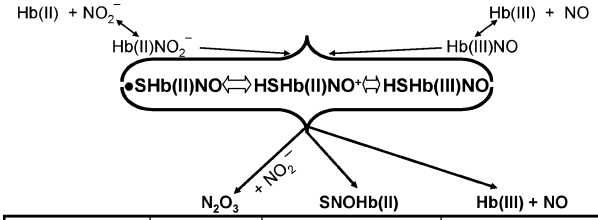
This rate constant decreased significantly ($p < 0.001$) by 78% from $4.3 \pm 0.9 \times 10^{-2}$ min^{-1} without DPG to $1.0 \pm 0.2 \times 10^{-2}$ min^{-1} with DPG (Figure 8B). The larger decrease in the rate for the release of NO than for the initial formation of intermediate(s) is consistent with the data of Table 2 and Figure 3, which indicates that DPG increases the concentration of the intermediates formed.

The influence of DPG on these rate constants can be attributed to impaired dynamic interactions across the $\alpha_1\beta_1$ interface (*39*) as a result of DPG binding in the region of the interface. This damping of dynamic interactions results in reduced heme pocket flexibility (*40*, *41*). Heme pocket flexibility facilitates interactions within the heme pocket involving the distal histidine. Two types of interactions with the distal histidine have been described in the literature. The first involves stabilizing the bound ligand by a hydrogen bond. Such a bond occurs in the α-chain (*42*) and the *β*-chain (*43*) of oxyHb and has been postulated to occur with metHb ligands (*44*). A role for the distal histidine in facilitating the binding of nitrite to deoxyHb is particularly important because of the negative charge contained on nitrite. With the heme cd_1 nitrite reductase (*45*), two histidines are involved in the uptake of nitrite. Any restriction on histidine flexibility, as expected with DPG, will therefore impair the initial reaction of nitrite with deoxyHb and the formation of the intermediate(s).

The second role of the histidine that has been studied involves an interaction of the histidine with the heme iron instead of the bound ligand. This reaction is responsible for the formation of reversible hemo(i)chromes for both deoxyHb (*40*) and metHb (*18*). In partially oxygenated hemoglobin this same reaction has been postulated as being involved in the autoxidation of hemoglobin where the distal histidine displaces the bound oxygen resulting in hemoglobin oxidation and the release of superoxide (*46*, *47*). In the nitrite reaction an analogous reaction with the delocalized intermediate would release NO from the intermediate.

These results further indicate that the buildup of intermediates reported in the absence of DPG with purified hemoglobin is expected to be even more pronounced in the RBCs, which contain DPG.

The Significance of a Red Cell Pool of Potentially Bioactive NO. We have demonstrated the accumulation of a pool of NO associated with hemoglobin following the reaction of nitrite with deoxyHb that is not deactivated by reacting with deoxyHb or oxyHb. The presence of such a pool of NO in the red cell should be coupled to the putative physiological significance for the reduction of nitrite to NO by deoxygenated hemoglobin chains. An appreciation of how this pool of NO can regulate vasodilation requires an understanding of the processes that release potentially bioactive form(s) of NO from this intermediate and the pathways for the release of these species from the red blood cell (Figure 9).



Reactions Involving the Delocalized Intermediate	Reaction with excess nitrite	Translocation of NO from heme to thiol facilitated by T to R conformation	Release of NO facilitated by interaction of distal histidine with heme Fe
Potential Transport of NO reactivity from the RBC	N_2O_3 can react with thiols and also diffuse directly out of the RBC	Membrane interactions result in nitrosation of membrane thiols and eventual RBC release.	NO released by Hb associated with the membrane will result in the release of NO from RBCs

FIGURE 9: Scheme showing the different pathways for the release of potential bioactivity from the delocalized intermediate formed when nitrite reacts with deoxyHb or NO reacts with metHb. The reactions involved are shown, and pathways for the release of bioactivity from the RBC are indicated.

Our results indicate two such processes. (1) In the presence of excess nitrite a reaction of nitrite with the intermediate produces N_2O_3. This molecule has been shown to react with thiols and can produce low molecular weight *S*-nitrosothiols and SNOHb. In addition it has been suggested that N_2O_3 being much more stable than NO and also hydrophobic may diffuse across the membrane and out of the RBC providing vasoactivity (*48*). (2) We have quantitated the direct release of NO from the intermediate by following the formation of Hb(II)NO. As demonstrated by the 4.5-fold decrease in this rate constant with DPG, this process is regulated by the configuration of distal heme pocket and presumably facilitated by the same nucleophilic interaction of the distal histidine with the heme iron that releases superoxide from oxyHb (*46*). Although NO released in the cytoplasm of the red cell will immediately be quenched by reacting with deoxyHb or oxyHb, the release of NO from hemoglobin bound to the membrane can escape from the red cell. This possibility is supported by preliminary results (*49*) indicating that the release of NO from the intermediate is enhanced for T-state hemoglobin known to bind to the membrane (*50*). This pathway out of the red cell is analogous to the observation that reactive oxygen species are released from hypoxic red cells (*51*) despite the presence of cytoplasmic enzymes that should quench any ROS generated.

In addition to these processes, it is necessary to consider the formation of SNOHb during nitrite reduction by deoxyHb. A transfer of NO from the intermediate(s) formed to the *β*-93 cysteine forming SNOHb when the sample is oxygenated has been demonstrated (*15*). Even in the absence of oxygen the thiyl radical properties of the delocalized intermediate (Figure 9) has been linked to the formation of SNOHb (*20*). This process is thought to be particularly important when free NO is available to react with the thiyl radical. The potential for the formation of SNOHb from the intermediate is further demonstrated by the SNOHb produced

when metHb reacts with NO (*20*, *25*). This reaction generates the same intermediate (Figure 9) and efficiently produced SNOHb.

The intermediates, thus, play a central role in the release of NO bioactivity from the red blood cell to the vasculature irrespective of which pathway or combination of pathways is involved.

ACKNOWLEDGMENT

Surya Bhamidipaty is acknowledged for help in analyzing the spectral data.

REFERENCES

1. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) S-Nitrosohaemoglobin: a dynamic activity of blood involved in vascular control, *Nature 380*, 221−226.
2. Nagababu, E., Ramasamy, S., Abernethy, D. R., and Rifkind, J. M. (2003) Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction, *J. Biol. Chem. 278*, 46349−46356.
3. 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, and Gladwin, M. T. (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, *Nat. Med. 9*, 1498−1505.
4. Xu, X., Cho, M., Spencer, N. Y., Patel, N., Huang, Z., Shields, H., King, S. B., Gladwin, M. T., Hogg, N., and Kim-Shapiro, D. B. (2003) Measurements of nitric oxide on the heme iron and beta-93 thiol of human hemoglobin during cycles of oxygenation and deoxygenation, *Proc. Natl. Acad. Sci. U.S.A. 100*, 11303−11308.
5. Brooks, J. (1937) *Proc. R. Soc. London, Ser. B: Biol Sci. 123*, 368−382.
6. Doyle, M. P., Pickering, R. A., DeWeert, T. M., Hoekst.ra, J. W., and Pater, D. (1981) Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites, *J. Biol. Chem. 256*, 12393−12398.
7. Reutov, V. P., Sorokina, E. G., and Kaiushin, L. P. (1994) [The nitric oxide cycle in mammals and nitrite reducing activity of heme-containing proteins], *Vopr. Med. Khim. 40*, 31−35.
8. Reutov, V. P. (2002) Nitric oxide cycle in mammals and the cyclicity principle, *Biochemistry (Moscow) 67*, 293−311.
9. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin, *Biochemistry 35*, 6976−6983.
10. Wang, Y., and Averill, B. A. (1960) Direct Observation by FTIR Spectroscopy of the Ferrous Heme-NO+ Intermediate in Reduction of Nitrite by a Dissimilatory Heme cd1 Nitrite Reductas, *J. Am. Chem. Soc 118*, 3972−3973.
11. Hausladen, A., Rafikov, R., Angelo, M., Singel, D. J., Nudler, E., and Stamler, J. S. (2007) Assessment of nitric oxide signals by triiodide chemiluminescence, *Proc. Natl. Acad. Sci. U.S.A. 104*, 2157−2162.
12. Kim-Shapiro, D. B., Gladwin, M. T., Patel, R. P., and Hogg, N. (2005) The reaction between nitrite and hemoglobin: the role of nitrite in hemoglobin-mediated hypoxic vasodilation, *J. Inorg. Biochem. 99*, 237−246.
13. Huang, K. T., Keszler, A., Patel, N., Patel, R. P., Gladwin, M. T., Kim-Shapiro, D. B., and Hogg, N. (2005) The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry, *J. Biol. Chem. 280*, 31126−31131.
14. Luchsinger, B. P., Rich, E. N., Yan, Y., Williams, E. M., Stamler, J. S., and Singel, D. J. (2005) Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions, *J. Inorg. Biochem. 99*, 912−921.
15. Angelo, M., Singel, D. J., and Stamler, J. S. (2006) An S-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate, *Proc. Natl. Acad. Sci. U.S.A. 103*, 8366−8371.
16. Rifkind, J. M., Nagababu, E., and Ramasamy, S. (2006) Nitric oxide redox reactions and red cell biology, *Antioxid. Redox. Signal 8*, 1193−1203.
17. Brunelle, J. A., Degtiarov, A. M., Moran, R. F., and Race, L. A. (1996) Simultaneous measurement of total hemoglobin and its derivatives in blood using CO-oximeters: analytical principles; their application in selecting analytical wavelengths and reference methods; a comparison of the results of the choices made, *Scand. J. Clin. Lab. Invest. Suppl. 224*, 47−69.
18. Levy, A., Kuppusamy, P., and Rifkind, J. M. (1990) Multiple heme pocket subconformations of methemoglobin associated with distal histidine interactions, *Biochemistry 29*, 9311−9316.
19. Addison, A. W., and Stephanos, J. J. (1986) Nitrosyliron(III) hemoglobin: autoreduction and spectroscopy, *Biochemistry 25*, 4104−4113.
20. Nagababu, E., Ramasamy, S., and Rifkind, J. M. (2006) S-nitrosohemoglobin: a mechanism for its formation in conjunction with nitrite reduction by deoxyhemoglobin, *Nitric Oxide 15*, 20−29.
21. Rifkind, J. M. (2005) S-Nitrosohemoglobin formation: a mechanism involving electron delocalization in the heme pocket, *Biophys. J. 88*, 391A-part 392 suppl.
22. Fernandez, B. O., and Ford, P. C. (2003) Nitrite catalyzes ferriheme protein reductive nitrosylation, *J. Am. Chem. Soc. 125*, 10510−10511.
23. 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., and Patel, R. P. (2006) Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation, *Blood 107*, 566−574.
24. 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., and Gladwin, M. T. (2005) Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control, *J. Clin. Invest. 115*, 2099−2107.
25. Luchsinger, B. P., Rich, E. N., Gow, A. J., Williams, E. M., Stamler, J. S., and Singel, D. J. (2003) Routes to S-nitroso-hemoglobin formation with heme redox and preferential reactivity in the beta subunits, *Proc. Natl. Acad. Sci. U.S.A. 100*, 461−466.
26. McMahon, T. J., Pawloski, J. R., Hess, D. T., Piantadosi, C. A., Luchsinger, B. P., Singel, D. J., and Stamler, J. S. (2003) S-nitrosohemoglobin is distinguished from other nitrosovasodilators by unique oxygen-dependent responses that support an allosteric mechanism of action, *Blood 102*, 410−411; author reply 412−413.
27. Pawloski, J. R., and Stamler, J. S. (2002) Nitric oxide in RBCs, *Transfusion 42*, 1603−1609.
28. Singel, D. J., and Stamler, J. S. (2005) Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin, *Annu. Rev. Physiol. 67*, 99−145.
29. Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., and Piantadosi, C. A. (1997) Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient, *Science 276*, 2034−2037.
30. Gow, A. J., and Stamler, J. S. (1998) Reactions between nitric oxide and haemoglobin under physiological conditions, *Nature 391*, 169−173.
31. Hoshino, M., Maeda, M., Konishi, R., Seki, H., and Ford, P. C. (1996) *J. Am. Chem. Soc. 118*, 5702−5707.
32. Bemski, G. (1997) Contribution of Electron Paramagnetic Resonance to the studies of hemoglobin: the nitrosylhemoglobin system, *Mol. Biol. Rep. 24*, 263−269.
33. Sharma, V. S., Traylor, T. G., Gardiner, R., and Mizukami, H. (1987) Reaction of nitric oxide with heme proteins and model compounds of hemoglobin, *Biochemistry 26*, 3837−3843.
34. Arnone, A. (1972) X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin, *Nature 237*, 146−149.
35. Minton, A. P., and Imai, K. (1974) The three-state model: a minimal allosteric description of homotropic and heterotropic effects in the binding of ligands to hemoglobin, *Proc. Natl. Acad. Sci. U.S.A. 71*, 1418−1421.
36. Yonetani, T., Park, S. I., Tsuneshige, A., Imai, K., and Kanaori, K. (2002) Global allostery model of hemoglobin. Modulation of O(2) affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors, *J. Biol. Chem. 277*, 34508−34520.
37. Tsuneshige, A., Kanaori, K., Samuni, U., Danstker, D., Friedman, J. M., Neya, S., Giangiacomo, L., and Yonetani, T. (2004) Semihemoglobins, high oxygen affinity dimeric forms of human

hemoglobin respond efficiently to allosteric effectors without forming tetramers, *J. Biol. Chem. 279*, 48959−48967.
38. Jia, Y., Ramasamy, S., Wood, F., Alayash, A. I., and Rifkind, J. M. (2004) Cross-linking with O-raffinose lowers oxygen affinity and stabilizes haemoglobin in a non-cooperative T-state conformation, *Biochem. J. 384*, 367−375.
39. Ramadas, N., and Rifkind, J. M. (1999) Molecular dynamics of human methemoglobin: the transmission of conformational information between subunits in an alpha beta dimer, *Biophys. J. 76*, 1796−1811.
40. Levy, A., and Rifkind, J. M. (1985) Low-temperature formation of a distal histidine complex in hemoglobin: a probe for heme pocket flexibility, *Biochemistry 24*, 6050−6054.
41. Levy, A., Sharma, V. S., Zhang, L., and Rifkind, J. M. (1992) A new mode for heme-heme interactions in hemoglobin associated with distal perturbations, *Biophys. J. 61*, 750−755.
42. Shaanan, B. (1983) Structure of human oxyhaemoglobin at 2.1 Å resolution, *J. Mol. Biol. 171*, 31−59.
43. Lukin, J. A., Simplaceanu, V., Zou, M., Ho, N. T., and Ho, C. (2000) NMR reveals hydrogen bonds between oxygen and distal histidines in oxyhemoglobin, *Proc. Natl. Acad. Sci. U.S.A. 97*, 10354−10358.
44. Asher, S. A., Adams, M. L., and Schuster, T. M. (1981) Resonance Raman and absorption spectroscopic detection of distal histidine−fluoride interactions in human methemoglobin fluoride and sperm whale metmyoglobin fluoride: measurements of distal histidine ionization constants, *Biochemistry 20*, 3339−3346.
45. Ranghino, G., Scorza, E., Sjogren, T., Williams, P. A., Ricci, M., and Hajdu, J. (2000) Quantum mechanical interpretation of nitrite reduction by cytochrome cd1 nitrite reductase from Paracoccus pantotrophus, *Biochemistry 39*, 10958−10966.
46. Balagopalakrishna, C., Manoharan, P. T., Abugo, O. O., and Rifkind, J. M. (1996) Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions, *Biochemistry 35*, 6393−6398.
47. Abugo, O. O., and Rifkind, J. M. (1994) Oxidation of hemoglobin and the enhancement produced by nitroblue tetrazolium, *J. Biol. Chem. 269*, 24845−24853.
48. Robinson, J. M., and Lancaster, J. R., Jr. (2005) Hemoglobin-mediated, hypoxia-induced vasodilation via nitric oxide: mechanism(s) and physiologic versus pathophysiologic relevance, *Am. J. Respir. Cell Mol. Biol. 32*, 257−261.
49. Rifkind, J. M., Ramasamy, S., and Nagababu, E. (2007) Conformationally regulated uptake of nitrite by deoxyhemoglobin and release of the NO produced, *Biophys. J.* 383a−384a.
50. Tsuneshige, A., Imai, K., and Tyuma, I. (1987) The binding of hemoglobin to red cell membrane lowers its oxygen affinity, *J. Biochem. (Tokyo) 101*, 695−704.
51. Rifkind, J. M., Zhang, L., Heim, J. M., and Levy, A. (1988) The role of hemoglobin in generating oxyradicals, *Basic Life Sci. 49*, 157−162.

BI700364E