

A New Paramagnetic Intermediate Formed during the Reaction of Nitrite with Deoxyhemoglobin

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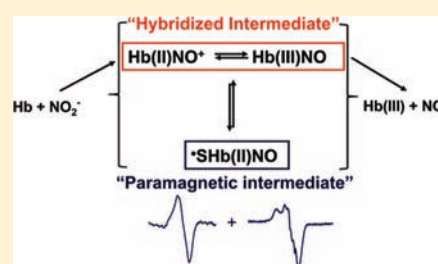
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 Supporting Information

ABSTRACT: The reduction of nitrite by deoxygenated hemoglobin chains has been implicated in red cell-induced vasodilation, although the mechanism for this process has not been established. We have previously demonstrated that the reaction of nitrite with deoxyhemoglobin produces a hybrid intermediate with properties of Hb(II)NO⁺ and Hb(III)NO that builds up during the reaction retaining potential NO bioactivity. To explain the unexpected stability of this intermediate, which prevents NO release from the Hb(III)NO component, we had implicated the transfer of an electron from the β -93 thiol to NO⁺ producing \cdot SHb(II)NO. To determine if this species is formed and to characterize its properties, we have investigated the electron paramagnetic resonance (EPR) changes taking place during the nitrite reaction.

The EPR effects of blocking the thiol group with *N*-ethyl-maleimide and using carboxypeptidase-A to stabilize the R-quaternary conformation have demonstrated that \cdot SHb(II)NO is formed and that it has the EPR spectrum expected for NO bound to the heme in the β -chain plus that of a thiyl radical. This new NO-related paramagnetic species is in equilibrium with the hybrid intermediate "Hb(II)NO⁺ \leftrightarrow Hb(III)NO", thereby further inhibiting the release of NO from Hb(III)NO. The formation of an NO-related paramagnetic species other than the tightly bound NO in Hb(II)NO was also confirmed by a decrease in the EPR signal by -20 °C incubation, which shifts the equilibrium back to the "Hb(II)NO⁺ \leftrightarrow Hb(III)NO" intermediate. This previously unrecognized NO hemoglobin species explains the stability of the intermediates and the buildup of a pool of potentially bioactive NO during nitrite reduction. It also provides a pathway for the formation of β -93 cysteine *S*-nitrosylated hemoglobin [SNOHb:*S*-nitrosohemoglobin], which has been shown to induce vasodilation, by a rapid radical–radical reaction of any free NO with the thiyl radical of this new paramagnetic intermediate.



INTRODUCTION

Nitric oxide (NO), also known as the endothelium-derived relaxing factor (EDRF), is an important signaling molecule involved in cardiovascular homeostasis.^{1–3} Endogenous NO is derived from both enzymatic and nonenzymatic sources in and near the vasculature. The primary enzymatic source of NO synthesis in the vasculature is from endothelial nitric oxide synthase (eNOS), which catalyzes an oxygen dependent oxidation of *L*-arginine to generate NO and *L*-citrulline.⁴ Once formed, NO can diffuse into the vascular smooth muscle cells, activate guanylyl cyclase, and result in vasodilation.⁵ However, NO's bioactivity is readily quenched within the circulation (NO lifetime <0.1 s in blood)⁶ in large part due to reactions of NO with RBCs. In RBCs, NO reacts with oxygenated hemoglobin [oxyHb, Hb(II)O₂] to form methemoglobin [metHb, Hb(III)] and nitrate, whereas it binds to deoxygenated hemoglobin [deoxyHb, Hb(II)] to form iron–nitrosyl hemoglobin [Hb(II)NO].^{7,8} These reactions eliminate and trap NO, respectively, thereby limiting the amount of NO available for vasodilation.

A role for bypassing the difficulty associated with the reactions of NO with oxyHb and deoxyHb and providing a mechanism whereby the RBC plays a role in delivering NO to the vasculature was originally proposed by Stamler and collaborators.⁹ They proposed the transfer of NO when hemoglobin is oxygenated from the heme to the β -93 cysteine producing *S*-nitrosylated hemoglobin [SNOHb]. Once reacted with the thiol, the NO is not scavenged by the heme and can be transferred to other thiols by a transnitrosylation reaction. It was subsequently demonstrated¹⁰ that during deoxygenation the NO is transferred to a membrane thiol group,⁹ providing a pathway for transport out of the RBC. A number of studies have suggested that this SNOHb hypothesis plays a major role in hypoxic vasodilation that regulates the flow of blood through the microcirculation.^{9–13} *S*-Nitrosothiols, which can originate in part from SNOHb, have been linked to many cellular functions.^{11,12} The importance of nitrosothiols has recently been corroborated by studies involving

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an *S*-nitrosogluthathione reductase, which regulates the level of *S*-nitrosothiols.¹³

Although the importance of *S*-nitrosothiols is established, the significance of Hb *S*-nitrosylation is controversial. The transfer of NO from the heme to the thiol requires an oxidation process,¹⁴ and it is not clear how this occurs. Furthermore, the detailed mechanism for the transfer of this pool of SNO's to the vasculature has not been fully explained. An alternative mechanism proposed for the transport of NO bioactivity by the RBC involves the reduction of nitrite back to NO.^{15,16} In plasma, nanomolar levels of nitrite are present that are thought to at least partially have formed due to oxidation of NO released from the endothelium.^{17–23} The reduction of this nitrite back to NO by deoxyHb has been proposed as a mechanism for maintaining NO bioactivity and a source for hypoxic vasodilation under various physiological and pathological conditions.^{16,24,25} However, the export of NO bioactivity from RBCs requires that the NO formed during nitrite reduction not be immediately scavenged by Hb.

One mechanism to accomplish this involves the formation of SNOHb,^{26,27} which produces a pool of NO that does not react with the heme iron. Basu et al.²⁸ recently proposed an alternative mechanism where nitrite binds to the oxidized hemoglobin (metHb) formed during the reduction of nitrite and that the reaction of the NO formed with this complex produces N₂O₃ that can diffuse out of the cell and/or react with thiols. The difficulty with this mechanism is that it uses the final products formed by the reaction (metHb and NO) and requires that the NO produced react with the low levels of nitrite reacted metHb instead of the readily available oxyHb and deoxyHb. We have recently proposed a mechanism where the nitrite reaction enhances the hypoxic release of ATP, which can stimulate eNOS, thereby producing NO.²⁹ The release of ATP provides a potential pathway for RBC nitrite induced vasodilation that does not involve the release of NO from RBCs.

We have also previously proposed the formation of an NO-containing intermediate, characterized as a hybridized species with properties of Hb(II)NO⁺ and Hb(III)NO, as a potential source of bioactive NO that can accumulate in RBCs.³⁰ This intermediate was found to be unexpectedly stable with ~25% of the nitrite retained in this form 60 min after the reaction of nitrite with deoxyHb at a 4:1 heme:nitrite molar ratio.³¹ To help understand this stability in the presence of an excess of hemoglobin, the potential involvement of the β -93 thiol group was suggested.²⁷ To further investigate the potential role of the β -93 thiol group, we have compared the nitrite reaction with deoxyHb under hypoxic conditions with and without *N*-ethyl-maleimide (NEM), a thiol group blocking agent. A quantitative comparison of both reactions by EPR indicates that in addition to the hybridized species with properties of Hb(II)NO⁺ and Hb(III)NO, an additional paramagnetic intermediate with EPR properties of both Hb(II)NO and a thyl radical (\cdot SHb(II)NO) is detected. Spectral analysis indicates that this additional intermediate is responsible for the overall stability of the hybridized intermediate regulating the dissociation of NO from the Hb(III)NO component.

Combining the current data, which provide a mechanism for the formation of a pool of bioactive NO in the RBC, with recent data showing that nitrite reacted hemoglobin has a high affinity for the membrane²⁹ suggests a potential mechanism, whereby the release of NO from nitrite reacted hemoglobin on the membrane can be released from the RBC. This hypothesis is

supported by a recent study that demonstrated the enhanced release of NO from hemoglobin when nitrite reacted Hb binds to the membrane.³² Further studies are required to determine if this actually provides a pathway for the release of NO from RBCs.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma-Aldrich unless otherwise mentioned.

Solution Preparations. All hemoglobin solutions were prepared in 50 mM NaCl and 4 mM phosphate buffer (PBS), pH 7.4.

DeoxyHb Preparation. Human hemoglobin was purified from fresh hemolysate by gel filtration using a Sephadex G-100 column equilibrated with PBS, pH 7.4 at 4 °C. A 1 mM hemoglobin solution was deoxygenated in an anaerobic Coy glovebox until the visible spectrum of Hb corresponded to that of deoxygenated Hb. A 0.1 cm path length cuvette was used for spectroscopic measurements of the 1 mM Hb solutions.

Semi-Hb Preparation. α -CO and β -CO semi-Hbs (dimers of the form $\alpha(\text{hemeCO})\beta(\text{apo})$ and $\alpha(\text{apo})\beta(\text{hemeCO})$, respectively) were prepared as previously described.³³ The CO semi-Hbs were purified by gel filtration through a G-25 Sephadex column equilibrated with PBS, pH 7.4. The CO was removed by strong illumination of the samples, and deoxygenation was performed by passing argon gas through septum sealed cuvettes containing the semi-Hb solutions until the visible spectra of each semi-Hb corresponded to that of deoxygenated Hb.

Nitrite Preparation. A 10 mM nitrite solution was prepared by dissolving NaNO₂⁻ in 10 mL of argon-saturated PBS, pH 7.4.

***N*-Ethylmaleimide (NEM) Preparation.** A 100 mM NEM solution was prepared by dissolving NEM in 10 mL of PBS, pH 7.4. NEM was reacted with oxygenated Hb at a 10:1, NEM:heme ratio for 30 min. NEM treated oxyHb was then deoxygenated in an anaerobic Coy globe box as previously described.

Preparation of Carboxypeptidase A (CPA) Reacted Hb. Hemoglobin was incubated with CPA obtained from Sigma Chemical Co., St. Louis, MO for 2 h at 37 °C in 0.1 M Tris-HCl buffer, pH 8.0 and then passed through a Sephadex G-100 column to remove the enzyme.³⁴ This reaction cleaves the terminal histidine and tyrosine on the β -chains stabilizing the R-state, even when hemoglobin is fully deoxygenated.

Azide Preparation. A 100 mM azide solution was prepared by dissolving azide in 10 mL of argon-saturated PBS, pH 7.4. Azide was reacted with deoxyHb or nitrite reacted deoxyHb at a 1:1, azide:heme ratio for 5 min.

Hb(II)NO Preparation. NO gas (2.5%) was purified, by passing through concentrated NaOH (5 M). Small amounts of this purified gas were added to a septum sealed 100 μ M deoxyHb solution to produce Hb(II)NO as confirmed by visible spectroscopy. The Hb(II)NO was then quickly passed down a Sephadex G-25 column anaerobically to remove unreacted NO. The final concentration of Hb(II)NO was determined by visible spectroscopy using the millimolar extinction coefficient for Hb(II)NO of 11.4 at 544 nm. The Hb(II)NO standard solution (~44 μ M) was flushed with inert gas and stored in the freezer at -150 °C. α -NO and β -NO semi-Hbs were also prepared in the same manner.

MetHb Preparation. Purified human hemoglobin (~1.5 mM) was oxidized with a 50 mM solution of K₃Fe(CN)₆ (50 μ L/mL) for 5 min. K₃Fe(CN)₆ was removed from the metHb solution by gel filtration using a Sephadex G-25 column equilibrated with PBS, pH 7.4. The final concentration of metHb was determined by visible spectroscopy using the millimolar extinction coefficient for metHb of 4.4 at 631 nm. The metHb standard solution (~0.75 mM) was stored in the freezer at -150 °C.

Nitrite Reduction by DeoxyHb. All experiments were carried out inside an anaerobic Coy glovebox. 1 mM deoxyHb or deoxyHb pretreated

with NEM, CPA, or CPA plus NEM was reacted with 0.25 mM nitrite in PBS, pH 7.4 for up to 60 min at room temperature (22 °C).

EPR Measurements. EPR spectra were measured using a Bruker EMX-61A spectrometer with an Oxford continuous flow ESR-900 cryogenic unit. An Oxford ITC 502 temperature controller was used to maintain the sample temperature in the cavity at 10 K with an Oxford VC41 gas flow controller to regulate the liquid helium flow through the cryostat. EPR spectra were recorded in the region of 500–4500 G with a sweep time of 335.54 s, a time constant of 80.42 ms, 2 mW power, and 100 kHz modulation frequency. All EPR samples were stored and measured in 4 mm clear fused quartz EPR tubes (707 SQ250M-WILMAD). All EPR measurements were analyzed with Bruker WINEPR software version 2.22 revision 10.

Determination of Hb(II)NO Standards by EPR. The Hb(II)NO standard solution (see above) was diluted accordingly with deoxygenated 3× diluted PBS, pH 7.4 to make 0.4 mL Hb(II)NO standards of 44, 33, 22, 11, and 5.5 μM . The 0.4 mL aliquots were transferred anaerobically into EPR tubes and frozen by submerging the tubes into liquid nitrogen. The samples were stored in the freezer at $-150\text{ }^\circ\text{C}$ until recorded. The EPR spectrum of each Hb(II)NO standard was recorded, and the region from 3150 to 3550 gauss was integrated. A standard calibration curve for Hb(II)NO was produced by plotting the concentration of Hb(II)NO versus the double integration obtained for each standard.

Determination of methHb Standards by EPR. The Hb(III) standard solution (see above) was diluted accordingly with deoxygenated 3× diluted PBS, pH 7.4 to make 0.4 mL Hb(III) standards of 260, 130, 65, and 33 μM . The 0.4 mL aliquots were transferred anaerobically into the EPR tubes and frozen by submerging the tubes into liquid nitrogen. The samples were stored in the freezer at $-150\text{ }^\circ\text{C}$ until recorded. The EPR spectrum of each methHb standard was recorded, and the region from 900 to 1500 gauss was integrated. A standard calibration curve for Hb(III) was produced by plotting the concentration of Hb(III) versus the double integration obtained for each standard.

Determination of methHb and Heme–NO Species Formed during the Reaction of deoxyHb with Nitrite by EPR. 0.3 mL aliquots of the reaction mixtures of nitrite with one of the deoxyHb samples (see above) were removed at various time intervals and transferred into EPR tubes and immediately frozen by submerging into liquid nitrogen. The samples were stored in the freezer at $-150\text{ }^\circ\text{C}$ until recorded. The EPR spectrum of each reaction mixture was recorded in the region from 900 to 3550 gauss. From the corrected spectra (see data analysis), the concentrations of Hb(II)NO and Hb(III) were determined during the nitrite reaction using the Hb(II)NO and Hb(III) standard curves, respectively.

Low Temperature Incubation of EPR Samples. EPR tubes corresponding to the 1, 15, and 60 min reaction mixtures for non-NEM and NEM treated deoxyHb reacted with nitrite, prepared under anaerobic conditions and frozen in liquid nitrogen, were incubated at $-20\text{ }^\circ\text{C}$ for 1, 5, and 10 min inside a temperature controlled cryobath (Neslab) set at $-20\text{ }^\circ\text{C}$. After each incubation, the EPR spectrum was recorded under the experimental conditions described above.

Data Analysis. All EPR data were analyzed with the Bruker WINEPR software (version 2.22 revision 10). Mathematical calculations were carried out in Microsoft Office Excel 2007.

Correction of EPR Spectra. During the reduction of nitrite by deoxyHb, both methHb and Hb(II)NO species are detected by EPR. MethHb has signals at both $g = 6$ and the $g = 2$ regions, while the Hb(II)NO spectrum is limited to the $g = 2$ region. To quantify the amount of Hb(II)NO formed during the reaction, it was necessary to subtract the contribution of methHb to the spectrum. This was carried out by first determining the methHb concentration in the sample from the $g = 6$ region and subtracting the signal in the $g = 2$ region associated with

that concentration of methHb from the spectrum. Spectral subtractions were carried out using the algebra/subtraction function of WINEPR.

Calculated Spectral Contributions to the EPR Spectra of Each Reaction Mixture. The spectral contributions to the EPR spectra recorded at various time intervals for the reactions of nitrite with deoxyHb in the absence and presence of NEM, as well as the spectra obtained after $-20\text{ }^\circ\text{C}$ incubations, were calculated by the addition of varying fractions of one or more of five EPR parent spectra. (1) The EPR spectrum of R-state β chain heme-NO (R-state Hb(II) β NO) was obtained using semihemoglobin, which is a dimer with one α globin and a β heme³⁵ where NO is bound to the β heme. (2) The spectrum of fully nitrosylated R-state Hb(II)NO that has NO bound to both α and β heme chains. (3,4) T-state α heme-NO (T-state Hb(II) α NO) and T-state β heme-NO (T-state Hb(II) β NO) spectra obtained from Ni-hybrids with added IHP to stabilize the T-state.³⁶ (5) The spectrum of a thiyl radical species was obtained by subtracting the EPR spectra of nitrite reacted CPA/NEM treated deoxyHb where no radical intermediate can form from nitrite reacted CPA deoxyHb where the intermediates are stabilized.

Calculated DI versus Experimental DI. As a means to compare whether the spectrum calculated for each reaction mixture represents a good fit for the experimental spectrum observed, the double integration (DI) of the signal in the $g = 2$ region obtained by both methods was compared. The DI value of the signal in the $g = 2$ region for each experimental spectra was obtained using the integration function of WINEPR. For the calculated spectra, the DI values of each of the parent spectra that make up the signal were also calculated using WINEPR and multiplied by the spectral fraction of that component. The summation of the DI values of the individual components that make up each spectrum was compared to its corresponding DI experimental value.

RESULTS AND DISCUSSION

At neutral pH, the binding of nitrite to Fe(II) hemoglobin is expected to be negligible. However, a number of studies have shown that the nitrite reacts with deoxyHb.^{7,16,30,37,38} This reaction has also been shown to occur in RBCs even at relatively low nitrite concentrations.^{15,29} The final products formed during the reaction of nitrite with deoxygenated hemoglobin subunits are methHb and NO.⁷ In the presence of excess Hb, any NO formed rapidly reacts with deoxyHb and oxyHb.³⁷ The high affinity rapid reaction with the Fe(II) of deoxyHb forms Hb(II)NO, which essentially forms an irreversible bond between the Fe(II) and the NO. The reaction with oxyHb oxidizes the heme forming nitrate with no bioactivity.

Our studies indicate that in the presence of an excess of Hb and in the absence of oxygen, within 60 min all of the nitrite was consumed when 250 μM nitrite reacted with 1 mM deoxyHb. Most of this nitrite that reacts with deoxyHb remains associated with the hemoglobin. However, only about 25% of the total nitrite is released as NO and is subsequently reacted with deoxyHb to form Hb(II)NO. About 50% of the total nitrite is associated with the hemoglobin in this unreduced form, because a strong reducing agent such as ascorbate is required to convert it to NO, and it does not react with sulfanilamide, a nitrite complexing agent in acidic media. The slow nitrite reaction observed is consistent with a low affinity of nitrite for the deoxy-heme. However, the initial binding must trigger a protein rearrangement, presumably involving hydrogen bonding of the nitrite in the heme pocket that enhances the affinity. The remaining 25% of the nitrite is retained as a hybridized species with properties of Hb(II)NO⁺ and Hb(III)NO.³¹ This hybridized complex releases NO when azide binds to the Hb(III)NO

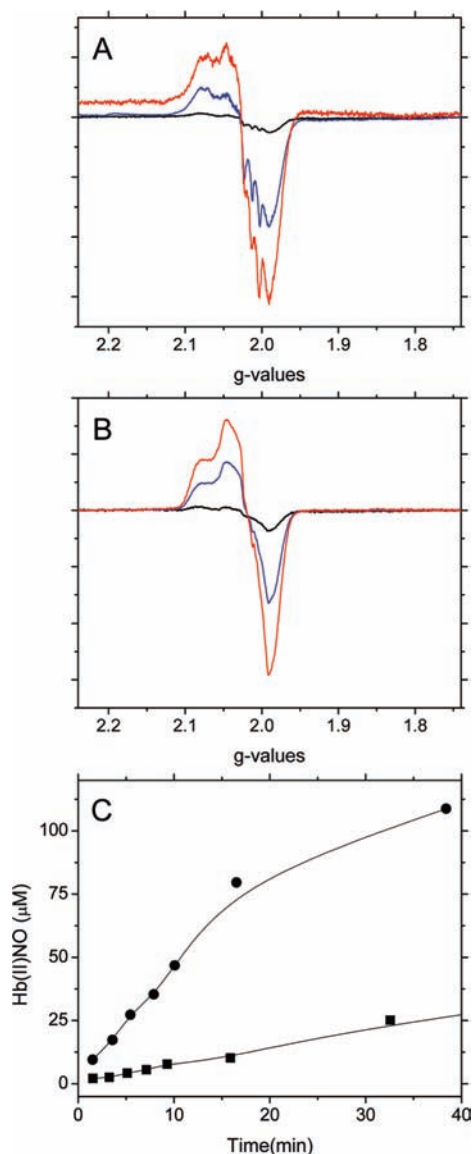


Figure 1. The X-band EPR spectra of Hb(II)NO formed as a function of reaction time when (A) 1 mM deoxyHb or (B) 1 mM NEM treated deoxyHb are both reacted with 0.25 mM nitrite under anaerobic conditions (black, 1 min; blue, 15 min; red, 60 min). (C) Total Hb(II)NO concentration as a function of reaction time for non-NEM (■) and NEM treated deoxyHb (●) reacted with nitrite. EPR spectra were recorded at 10 K.

component and is stable in the presence of excess hemoglobin. The potential added stability of this hybridized intermediate associated with the transfer of an electron from the NO^+ to the β -93 thiol group of Hb was proposed on the basis of studies directed at the formation of SNOHb during the nitrite reaction.^{27,39} By retaining nitrite in the reduced form, this complex has potential bioactivity.

Blocking the β -93 Thiol of Hb Increases the Rate for the Formation of Hb(II)NO. To further investigate the contribution of the β -93 thiol group to the stability of the intermediates, the effect of blocking the thiol group with NEM was investigated by EPR. Figure 1A and B, respectively, shows the EPR spectra of the heme–NO complex(es) formed when deoxyHb and NEM treated deoxyHb react with nitrite, at various time intervals. Figure 1C is

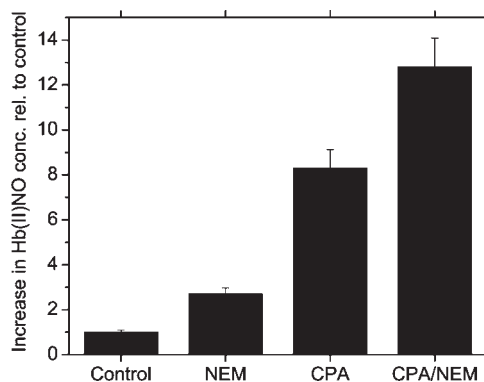
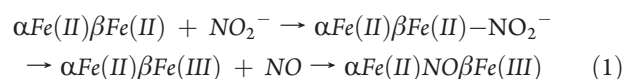


Figure 2. Relative increase in Hb(II)NO concentration relative to control for deoxyHb (control), NEM treated, CPA treated, and CPA and NEM treated deoxyHb reacted with nitrite for 60 min (heme:nitrite ratio is 4:1).

a comparison of the time-dependent formation of Hb(II)NO determined by double integration of its EPR signal in the $g = 2$ region for non-NEM and NEM treated deoxyHb reacted with nitrite. As indicated in Figure 1C, the formation of the Hb(II)NO spectrum is appreciably faster when the thiol group of Hb is blocked by NEM. The dissociation of NO from the hybridized intermediate “Hb(II)NO⁺ ↔ Hb(III)NO” required to form Hb(II)NO must therefore be faster when NEM reacts with the thiol group.

The faster rate for NEM reacted hemoglobin can be attributed to the fact that NEM stabilizes the R-state of Hb,⁴⁰ which has been shown to increase the rate for nitrite reduction.^{15,37} The stabilization of the R-state of Hb is indicated by the elimination of the T-state α -chain hyperfine lines⁴¹ of the Hb(II)NO EPR spectra seen in Figure 1A when compared to the EPR spectra of NEM treated deoxyHb reacted with nitrite seen in Figure 1B. Even though the nitrite reduction is taking place predominantly on the β -chains that have the active thiol group, the formation of α -chain Hb(II)NO is attributed to the released NO that reacts with both α and β chains (eq 1).



In addition to the conformational change, we wanted to determine if there is a direct effect due to the elimination of the free β -93 thiol group. To delineate a potential role of the β -93 thiol group in stabilizing the intermediate(s), it was necessary to convert T-state deoxyHb into an R-state deoxyHb known to have a higher rate for nitrite reduction. For this purpose, Hb was reacted with CPA. CPA cleaves the terminal tyrosine and histidine residues of the β -chain eliminating the cross-links that stabilize the T-state of Hb and produces R-state Hb even when the hemoglobin is fully deoxygenated.^{34,42} By investigating the effect of NEM blockage of the thiol groups for CPA treated hemoglobin, the effect of the free thiol on the nitrite reaction of R-state hemoglobin was determined. As shown in Figure 2, NEM increases the formation of Hb(II)NO by 2.7 times when compared to the control (untreated deoxyHb). CPA treatment of deoxyHb further increases Hb(II)NO formation to 8.3 times that of the control. However, even for CPA treated Hb, which is already in the R-state, NEM further increases the production of Hb(II)NO to 12.8 times higher when compared to the control. Although structures of different R-states have been reported,^{43,44}

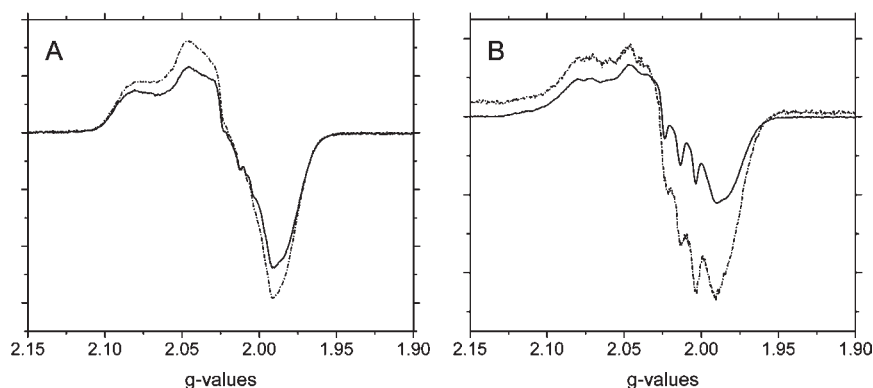


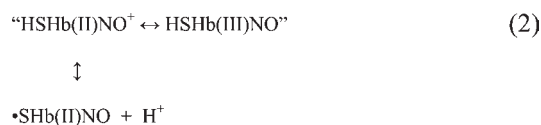
Figure 3. The X-band EPR spectra in the $g = 2$ region for (A) 1 mM NEM treated deoxyHb and (B) 1 mM deoxyHb both reacted with 0.25 mM nitrite for 60 min (—) and then incubated at $-20\text{ }^{\circ}\text{C}$ for 10 min (---). EPR spectra were recorded at 10 K.

we have assumed that the increased formation of Hb(II)NO when the β -93 thiol groups on CPA reacted hemoglobin react with NEM is primarily due to blocked thiol groups and not to a shift in the specific R-state of hemoglobin that may further increase the rate for the nitrite reaction. This is consistent with the predicted structural changes associated with CPA and NEM. CPA removes the terminal tyrosine and histidine of the β -chain, while reactions involving the β -93 cysteine have been reported to displace these residues.^{45–47} This can be contrasted with the structure of R2 hemoglobin where the 2 β -chain terminal histidines stack against one another.⁴³

A New Paramagnetic Species That Is Not Hb(II)NO and Requires the β -93 Thiol. The stabilization of the hybridized intermediate(s) by the free thiol group and the resultant decrease in the rate for NO release suggest the involvement of the β -93 thiol group. Because this thiol group is $\sim 9\text{ \AA}$ from the NO in the R-state and 13 \AA from the NO in the T-state,⁴⁸ it most likely involves a distinct complex that is in equilibrium with the hybridized intermediate “Hb(II)NO⁺ \leftrightarrow Hb(III)NO”.

In our previous studies on the autoxidation of hemoglobin, we have used subzero incubation studies to study the intermediates formed during the reaction.^{49,50} This is based on the concept that in the temperature range from below freezing until about $-65\text{ }^{\circ}\text{C}$, reactions involving the access of ligands into the heme pocket are expected to be very slow. However, subtle conformational changes necessary for the redistribution of species can take place, which will be driven by the decrease in temperature that favors the more stable species. On the basis of these earlier studies, we investigated the effect of $-20\text{ }^{\circ}\text{C}$ incubations on the reaction products present during the nitrite reaction. Figure 3 shows the changes in signal intensity in the $g = 2$ region of the EPR spectra produced after 60 min of the nitrite reaction with (A) NEM and (B) non-NEM treated deoxyHb incubated at $-20\text{ }^{\circ}\text{C}$ for 10 min. A small 12% decrease in signal intensity that is considered within experimental error is observed for NEM treated deoxyHb reacted with nitrite after low temperature incubation. However, for non-NEM treated deoxyHb reacted with nitrite, there is a much more significant 32% decrease in the intensity of this signal after low temperature incubation, which is generally attributed to Hb(II)NO. Hb(II)NO is known to be a very stable complex particularly at low temperatures, in the absence of oxygen, and no change in its intensity is expected to be observed. Instead, the observed decrease in signal intensity for Hb(II)NO with a free thiol group suggests the presence of a less stable intermediate species with an EPR signal. Yet both

Hb(II)NO⁺ and Hb(III)NO, the components of the hybridized intermediate, are EPR silent. In our earlier discussion of the contribution of the thiol group to the stability of the hybridized intermediate, the potential transfer of an electron from the thiol to the nitrosonium cation of this intermediate is suggested, resulting in a new intermediate species, $\cdot\text{SHb(II)NO}$ (eq 2).



Although we have both the thiol radical and the NO on the same molecule in $\cdot\text{SHb(II)NO}$, unlike the NO and the Fe(III) in Hb(III)NO, they are not directly linked and are separated by about $9\text{--}13\text{ \AA}$, and, therefore, its Hb(II)NO EPR signal may still be detected. For this new intermediate ($\cdot\text{SHb(II)NO}$), the thiol radical can accept an electron from the heme–NO and reform the hybridized intermediate with no EPR signal. This EPR signal is, therefore, much more labile than that of Hb(II)NO and can decrease in intensity during low temperature incubation. Therefore, the observed decrease in EPR signal during the $-20\text{ }^{\circ}\text{C}$ incubation can be attributed to an electron transfer process, which results in a thermodynamically driven shift in equilibrium between the paramagnetic $\cdot\text{SHb(II)NO}$ intermediate, which is perhaps less stable at low temperature, and the diamagnetic hybridized “Hb(II)NO⁺ \leftrightarrow Hb(III)NO” intermediate.

The decrease in the EPR signal can also be explained by the transfer of NO from the paramagnetic intermediate $\cdot\text{SHb(II)NO}$ to the thiol radical producing SNOHb(II). This was previously proposed²⁷ to explain the formation of SNOHb during the nitrite reaction.

The EPR Spectrum of the Paramagnetic Intermediate. The destabilization of the EPR signal in the $g = 2$ region during low temperature incubation at $-20\text{ }^{\circ}\text{C}$ (Figure 3) has established the presence of a new paramagnetic intermediate ($\cdot\text{SHb(II)NO}$) formed during the room temperature nitrite reaction that is not formed with NEM treated deoxyHb. The spectrum due to this intermediate could, however, not be determined from the decrease in signal at low temperature (Figure 3B), because of the presence of T-state hemoglobin and a possible shift in Hb’s quaternary conformation during incubation, which alters the EPR spectrum of Hb(II)NO. Therefore, to study the EPR spectra of this new paramagnetic intermediate, we compared the EPR spectra of CPA and CPA/NEM treated deoxyHb reacted with

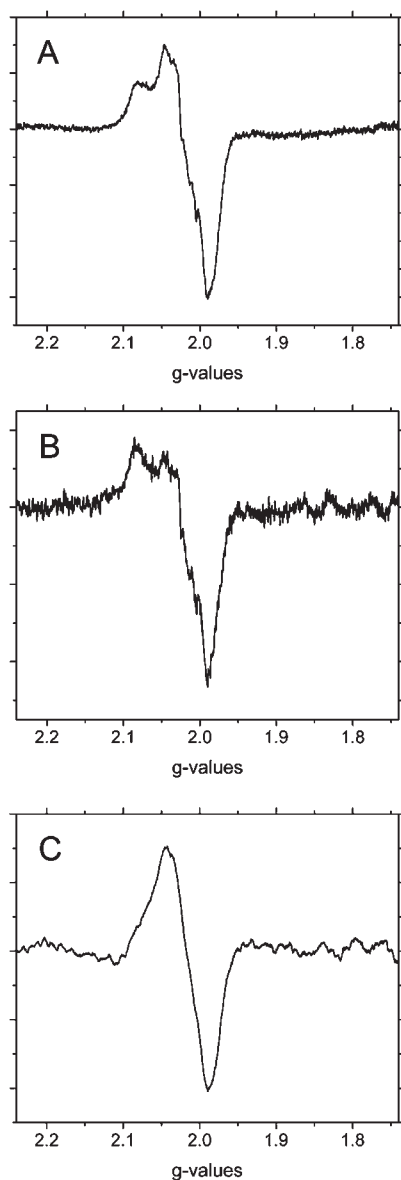


Figure 4. The X-band EPR spectra in the $g = 2$ region for (A) CPA treated deoxyHb and (B) CPA/NEM treated deoxyHb both reacted with nitrite for 60 min (heme:nitrite ratio is 4:1). (C) The difference EPR spectrum obtained by subtracting the CPA/NEM spectra (B) from the CPA spectra (A) after normalization for the intensity differences. EPR spectra were recorded at 10 K.

nitrite, which eliminate any spectral contribution from the T-state quaternary conformation. This comparison is valid even if CPA and NEM reacted CPA exist in different R-states, because the dominant conformational effects on Hb(II)NO involve the T-state, where rupture of the proximal histidine bond in the α -chain produces a spectrum with three sharp nitrogen hyperfine lines. In this way, we were able to compare the spectral differences between nitrite reacted R-state deoxyHb with a free thiol group and a blocked thiol group, respectively.

Figure 4 shows the EPR spectra of nitrite reacted (A) CPA and (B) CPA/NEM treated deoxyHb. Because both of these treated hemoglobins are in the R quaternary state, negligible nitrogen hyperfine lines attributed to T-state hemoglobin are observed (compare with Figure 1A where T-state hyperfine lines are

observed). There are, however, subtle differences observed between the two spectra. Because the Hb concentration and the amount of Hb(II)NO formed during the reactions are not the same, the intensities of the signals are not expected to be the same. To compare these spectra, the intensities were, therefore, normalized, and the CPA/NEM spectrum was subtracted from the CPA spectrum. The spectrum obtained by this subtraction shown in Figure 4C corresponds to an isotropic line in the $g = 2.015$ region with a line width of ~ 90 gauss. We attribute this signal to that of a thiyl radical.

Other potential protein radicals involving oxygen, nitrogen, or carbon would have g -values in the region of 2.003, much closer to the free electron with $g = 2.0023$. For a single crystal of a thiyl radical, $g = 2.03$ has been reported.⁵¹ For sulfur-based radicals, increased spin orbital coupling results in higher g -values with a wide range of values^{51,52} because the g -values are very sensitive to conformations, the environment of the sulfur, and the polarity of the region.⁵³ For a thiyl radical in a protein environment, values in the region of 2.01 have been reported.⁵⁴ The isotropic somewhat broadened signal is expected for a freely rotating thiyl radical within the sulfhydryl group from a cysteine in close proximity to the Fe(II)-heme-NO.⁵⁰ Our $g = 2.015$ value is, therefore, consistent with its designation as a thiyl radical. This analysis indicates that during nitrite reduction by deoxyHb with a free β -thiol group, a new intermediate is formed that involves a thiyl radical.

An alternative designation of the radical species detected could be of a thionitroxide radical that has been reported to be associated with the formation of SNOHb.^{55,56} The formation of the thionitroxide radical would require that the NO/NO⁺ be transferred from the heme to the thiol. However, the low temperature incubation (Figure 3) indicates that the intermediate must include in addition to the radical the spectrum of an Hb(II)NO component. This observation is, therefore, consistent with the formation of the putative \cdot SHb(II)NO intermediate, but inconsistent with the formation of the thionitroxide radical.

To quantitate the heme-NO contribution to this paramagnetic intermediate, we must differentiate between Hb(II)NO formed by the NO released from the hybridized intermediate and the Hb(II)NO that is part of the radical intermediate. The major difference between the two processes involves the relative contribution of α and β chains. The paramagnetic intermediate is only formed on β chains, while the Hb(II)NO formed by the release of NO from the hybridized intermediate involves both chains (eq 1). Because we do not know the relative affinity of this NO with the two types of chains, we used the CPA/NEM reaction to provide a measure for the relative reactivity of α and β chains in the R-quaternary conformation. Although the concentration of Hb(II)NO is different for the CPA and CPA/NEM reaction mixtures, the ratio of β/α chain Hb(II)NO component obtained for the CPA/NEM reaction mixture would indicate the fraction of β -chain Hb(II)NO formed in the CPA sample due to the release of NO from the hybridized intermediate, and any excess β -chain Hb(II)NO would be associated with the paramagnetic intermediate (eq 3).

$$\begin{aligned} \text{Hb(II)NO}_{\text{rad.-inter.}} &= [\text{Hb(II)}\beta\text{NO}_{\text{CPA}}] - [\text{Hb(II)}\alpha\text{NO}_{\text{CPA}}] \\ &\times [\text{Hb(II)}\beta\text{NO}_{\text{CPA-NEM}}/\text{Hb(II)}\alpha\text{NO}_{\text{CPA-NEM}}] \end{aligned} \quad (3)$$

Spectral fractions of α -NO semi-Hb (Figure 5A) and β -NO semi-Hb (Figure 5B) were used to fit the EPR spectra of nitrite reacted CPA and CPA/NEM treated deoxyHb. Although the

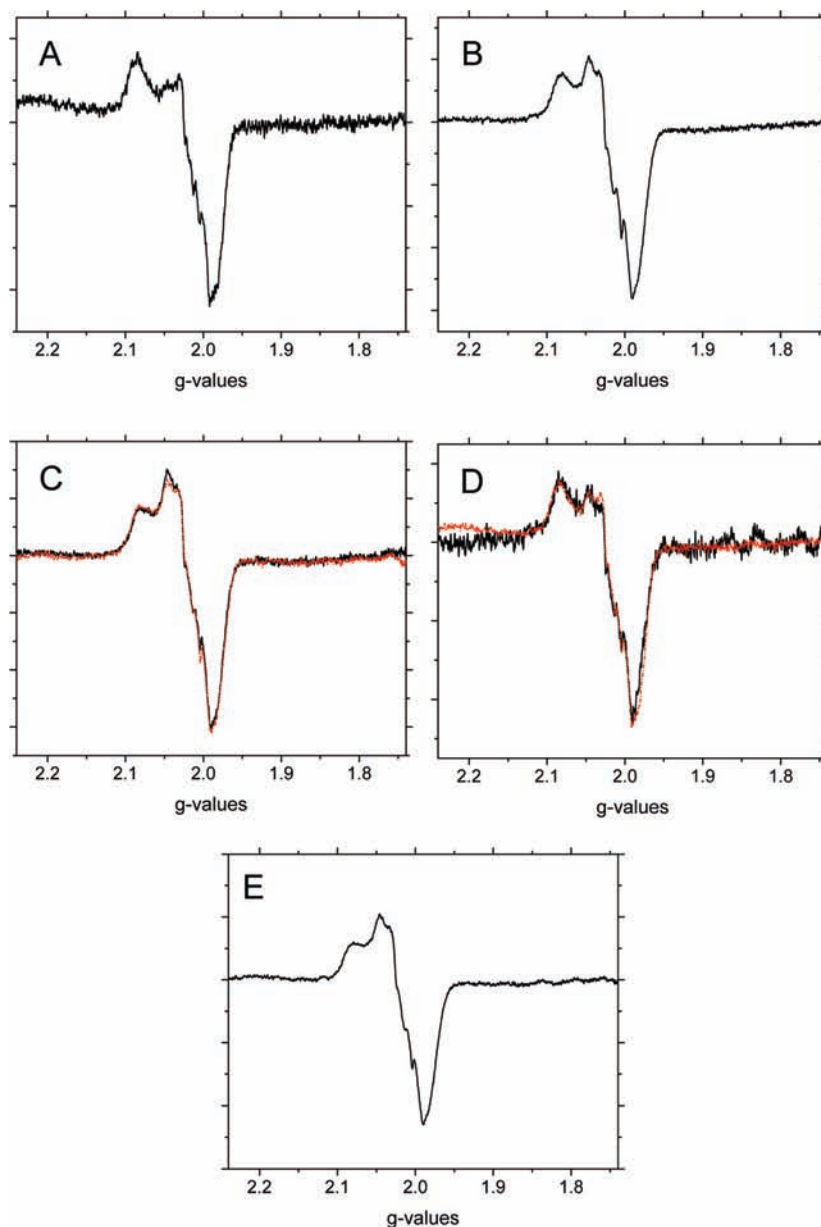


Figure 5. The X-band EPR spectra in the $g = 2$ region for (A) R-state Hb(II) α NO from semihemoglobin, and (B) R-state Hb(II) β NO from semihemoglobin. Spectral fits of nitrite reacted (C) CPA treated deoxyHb and (D) CPA/NEM treated deoxyHb at 60 min (heme:nitrite ratio is 4:1) (black, observed spectra; red, calculated spectra). [These spectra were fitted using fractions of R-state Hb(II) β NO (A) and R-state Hb(II) α NO (B) and the thiyl radical species (Figure 4C).] (E) The paramagnetic intermediate spectrum calculated from a summation of the thiyl radical spectrum (Figure 4C) and 0.43 times the R-state Hb(II) β NO spectrum (B) (see text). EPR spectra were recorded at 10 K. Calculated DI versus experimental DI: CPA calculated DI, 1.19×10^7 ; experimental DI, 1.17×10^7 ; % error, 2.0%; CPA/NEM calculated DI, 1.81×10^7 ; experimental DI, 1.72×10^7 ; % error, 5.0%.

concentrations of these semihemoglobins were not the same, the comparison of the β/α ratio for CPA hemoglobin and NEM reacted CPA hemoglobin is valid. The spectral fits are shown in Figure 5C and D for the CPA and CPA/NEM reaction mixtures, respectively. For the CPA/NEM sample, the fraction of Hb(II) α NO is 0.35 and the fraction of Hb(II) β NO is 0.1 corresponding to a β/α ratio of 0.29. For the CPA sample, the fraction of Hb(II) α NO is 0.25, and for Hb(II) β NO it is 0.5. Even though the total concentration of Hb(II)NO for the CPA sample is greater than that for the CPA–NEM sample (see above), the relative concentrations of Hb(II) α NO and Hb(II) β NO formed

by NO released from the hybridized intermediate should be the same for both. Therefore, eq 3 can be used to correct the fraction of Hb(II) β NO in the CPA sample for the amount formed by NO released from the hybridized intermediate resulting in a fraction of 0.07 and therefore a 0.43 fraction for Hb(II) β NO associated with the thiyl radical (Hb(II)NO_{rad-inter.}). By adding a 0.43 fraction of the R-state Hb(II) β NO spectrum (Figure 5B) plus the thiyl radical spectrum from Figure 4C, which is present in the CPA sample, we obtain the spectrum of the paramagnetic intermediate shown in Figure 5E. This figure represents a spectrum of the paramagnetic intermediate that involves both a thiyl radical

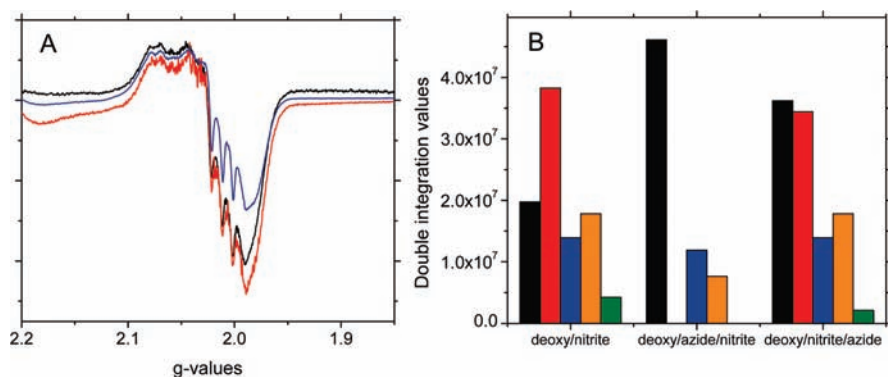
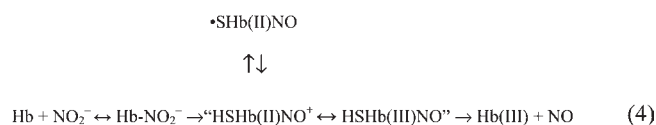


Figure 6. The X-band EPR spectra in the $g = 2$ region for (A) 1 mM deoxyHb reacted with (black) 0.25 mM nitrite, (blue) 1 mM azide before 0.25 mM nitrite addition, and (red) 1 mM azide after 0.25 mM nitrite addition for 60 min. (B) The spectral components that make up the calculated EPR spectra of (A) (black, R-state Hb(II)NO; red, R-state Hb(II) β NO; blue, T-state Hb(II) α NO; orange, T-state Hb(II) β NO; and green, thiyl radical). [Azide to heme ratio was 1:1. Note: Azide has EPR signals at $g = 2.84, 2.20,$ and 1.69 . These signals do not interact with the heme-NO signal at $g = 2.0$.] Calculated DI versus experimental DI: deoxy/nitrite calculated DI, 9.40×10^7 ; experimental DI, 8.38×10^7 ; % error, 12.2%; deoxy/azide/nitrite calculated DI, 6.57×10^7 ; experimental DI, 5.87×10^7 ; % error, 11.8%; deoxy/nitrite/azide calculated DI, 1.04×10^8 ; experimental DI, 1.07×10^8 ; % error, 9.72%.

and an R-state β heme-NO as expected for \cdot SHb(II)NO formed by the transfer of an electron from the β -93 thiol to Hb(II)NO $^+$.

Relationship between the Hybridized Intermediate and the Paramagnetic Intermediate. Our analysis of the spectra obtained when nitrite reacts with CPA treated hemoglobin with and without the thiol group blocked by NEM indicates the formation of a thiyl radical. To demonstrate that the thiyl radical containing paramagnetic intermediate \cdot SHb(II)NO is formed from the hybridized intermediate “Hb(II)NO $^+$ \leftrightarrow Hb(III)NO” (eq 2), we investigated the effect of adding azide to deoxyHb before and after reacting with nitrite. Azide has a high binding affinity to Fe(III) 57 and reacts with the Hb(III)NO component of the hybrid, destabilizing the hybridized intermediate and accelerating the release NO to bind to free hemes. 31 Figure 6A shows the EPR spectra of deoxyHb reacted with nitrite for 60 min, and those of deoxyHb reacted with azide before or after nitrite also for 60 min. The EPR intensity in the presence of azide before nitrite addition is significantly decreased. The analysis of the changes in the components of the spectrum for azide added before nitrite (Figure 6B) shows that this decrease is due to the complete elimination of R-state Hb(II) β NO and the thiyl radical, both of which are integral parts of the paramagnetic intermediate. At the same time, there is an increase in R-state Hb(II)NO consistent with the release of NO from the hybridized intermediate. With azide added before the nitrite, it is available to react with the hybridized intermediate as it is formed, and therefore no paramagnetic intermediate can form. However, when the azide is added after the nitrite has already reacted with the hemoglobin and the paramagnetic intermediate has already formed, the presence of azide for 5 min has only minor effects on the spectrum (Figure 6A). The analysis of the changes in the components of the spectrum for azide added after nitrite (Figure 6B) nevertheless indicates a 50% decrease in the thiyl radical and \sim 50% increase in R-state Hb(II)NO. These changes are consistent with an interaction of azide with the Hb(III)NO component of the hybridized intermediate, which will shift the equilibrium from the radical intermediate to the hybridized intermediate as NO is released from the hybridized intermediate forming Hb(II)NO. The results of adding azide both before and after the nitrite, therefore, support the proposal that the paramagnetic intermediate is formed subsequent to the hybridized

intermediate by the transfer of an electron from the thiol to the NO $^+$ (eq 4).



Thus, the release of NO from the Hb(III)NO component disrupts the hybridized species, which shifts the equilibrium away from the paramagnetic intermediate inhibiting its formation and resulting in the elimination of the paramagnetic intermediate signal from the heme-NO EPR spectrum.

Analysis of Spectral Changes That Take Place during the Nitrite Reaction. We were able to follow the changes in this new paramagnetic intermediate together with other heme(II)NO species formed during the nitrite reaction at room temperature. For this analysis, we included the spectra for T-state Hb(II) α NO and Hb(II) β NO, fully nitrosylated R-state Hb(II)NO, as well as R-state Hb(II) β NO and the thiyl radical, which are indicative of the new paramagnetic intermediate (\cdot SHb(II)NO). Using these spectra, we were able to fit all of the observed spectra shown in Figure 1A and B (see Supporting Information Figure 1). In the absence of NEM, analysis of the concentration of the different components indicates a gradual and significant increase in the paramagnetic intermediate as a function of reaction time as a result of the increase in the components corresponding to R-state Hb(II) β NO (Figure 7Ai) and the thiyl radical signal (Figure 7Aii). On the basis of the predicted intensity of heme-NO associated with the thiyl radical (Figure 5C), only 68% of the total heme-NO observed after 60 min of reaction time is due to Hb(II)NO. On the basis of this analysis, previously reported data 38 on the reaction of nitrite with deoxyHb overestimate the formation of Hb(II)NO.

For NEM treated deoxyHb reacted with nitrite (Figure 7B), no T-state heme-NO is observed, and no thiyl radical is observed. We do, however, observe a much higher increase in the component corresponding to fully nitrosylated R-state Hb(II)NO (\sim 2.25 times higher than non-NEM deoxyHb), which is consistent with a faster release of NO from Hb(III)NO when the paramagnetic intermediate cannot be formed. We also observe a gradual increase in R-state Hb(II) β NO, which is less

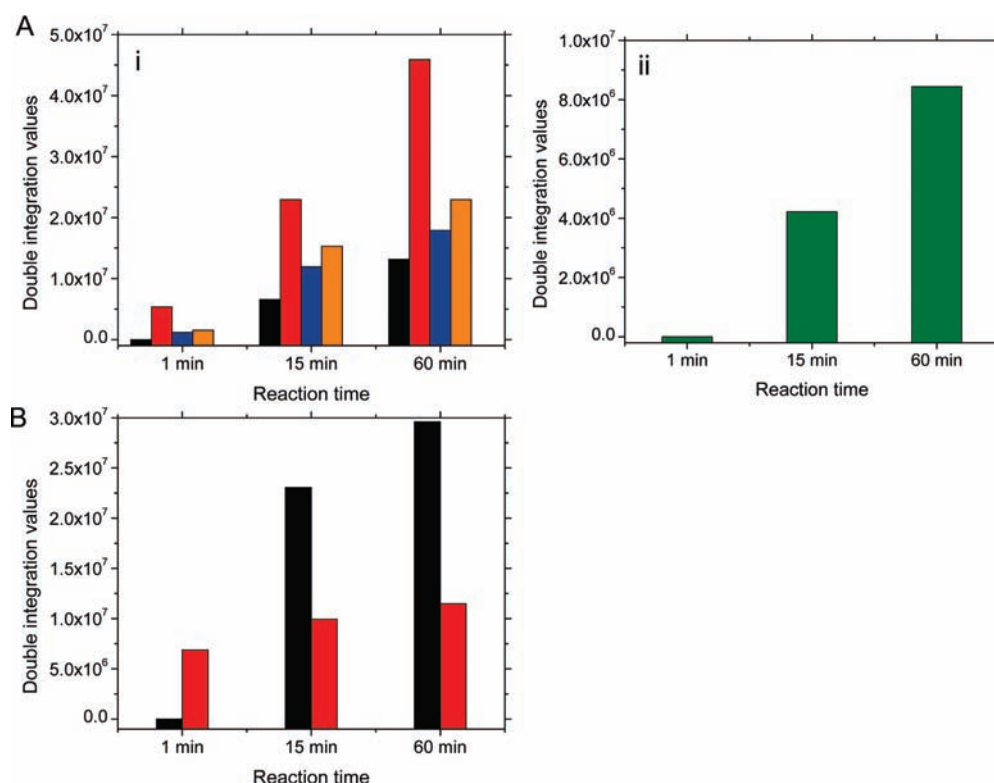


Figure 7. The spectral components that make up the calculated EPR spectra of the signal in the $g = 2$ region when (A) 1 mM deoxyHb and 0.25 mM nitrite are reacted for 1, 15, and 60 min; and (B) 1 mM NEM treated deoxyHb and 0.25 mM nitrite are reacted for 1, 15, and 60 min (black, R-state Hb(II)NO; red, R-state Hb(II) β NO; blue, T-state Hb(II) α NO; orange, T-state Hb(II) β NO; green, thiyl radical). Calculated DI versus experimental DI: (A) 1 min calculated DI, 8.08×10^6 ; experimental DI, 1.057×10^7 ; % error, 23.3%; 15 min calculated DI, 6.10×10^7 ; experimental DI, 7.01×10^7 ; % error, 13.0%; 60 min calculated DI, 1.08×10^8 ; experimental DI, 9.30×10^7 ; % error, 16.6%; (B) 1 min calculated DI, 6.89×10^6 ; experimental DI, 7.31×10^6 ; % error, 5.83%; 15 min calculated DI, 3.30×10^7 ; experimental DI, 3.75×10^7 ; % error, 11.9%; 60 min calculated DI, 4.11×10^7 ; experimental DI, 4.32×10^7 ; % error, 4.85%.

pronounced (3 times less) than the increase observed for non-NEM deoxyHb. This can be attributed to the preferred β -chain binding of NO released from the hybridized intermediate.

In the presence of NEM, the thiol group necessary for the formation of the paramagnetic intermediate is blocked, and therefore the thiyl radical is not formed. This data confirm the formation of the thiyl radical intermediate (\cdot SHb(II)NO) during the reaction of nitrite with deoxyHb and its role in slowing the release of NO from Hb(III)NO to form the reaction product Hb(II)NO.

Analysis of the Spectral Changes Taking Place during Low Temperature Incubation. The 10 min low temperature incubation results, after a 60 min reaction time, shown in Figure 3B indicate that the characteristic signal around $g = 2$ corresponding to the heme–NO species formed during nitrite reduction by deoxyHb decreases. This heme–NO signal is indicative of both R and T-state Hb(II)NO and the paramagnetic intermediate, \cdot SHb(II)NO, as was shown in Figure 7A. To analyze the nature of the spectral changes that take place during low temperature incubations, we have analyzed the spectra of nitrite reacted deoxyHb reacted for 1, 15, and 60 min that have been incubated at low temperature (-20°C) for 0, 1, 5, and 10 min (Figure 8).

As shown in Figure 8, there are qualitative differences between the results obtained during low temperature incubation for different reaction times. Samples for which the nitrite reaction has only proceeded for 1 min show an increase in the signal intensity in the $g = 2$ region (Figure 8A). However, for the 15 min

and to a greater extent the 60 min samples, the signal intensity in the $g = 2$ region decreases (Figures 8B,C). On the basis of the analysis of the spectral components at each reaction time (Figure 8D–F), it is clear that the dominant components responsible for both the increased intensity at 1 min and the decrease in intensity at 15 and 60 min correspond to the thiyl radical and the R-state Hb(II) β NO components that are associated with the paramagnetic intermediate, \cdot SHb(II)NO.

After a 1 min of reaction, when very little reaction has taken place, a major fraction of the nitrite is associated with the intermediates that have not yet formed paramagnetic species (either the Hb(II)NO formed when NO is released from Hb(III)NO or the paramagnetic intermediate). As the reaction proceeds during low temperature incubation, in addition to the formation of R-state Hb(II)NO associated with the release of NO, we see an increase in the paramagnetic intermediate (Figure 8D). Therefore, the low temperature incubation after 1 min facilitates the formation of the equilibrium distribution between the two intermediates (eq 4), such that the initial formation of the hybridized intermediate is followed by a slower formation of the paramagnetic intermediate. The shift from the EPR silent “Hb(II)NO ‡ \leftrightarrow Hb(III)NO” to the EPR active (\cdot SHb(II)NO) intermediate results in an increase in the EPR signal intensity.

After 15 min of reaction time, an appreciable amount of heme–NO species has formed, which include R-state Hb(II)NO, T-state Hb(II)NO, and both the hybridized intermediate

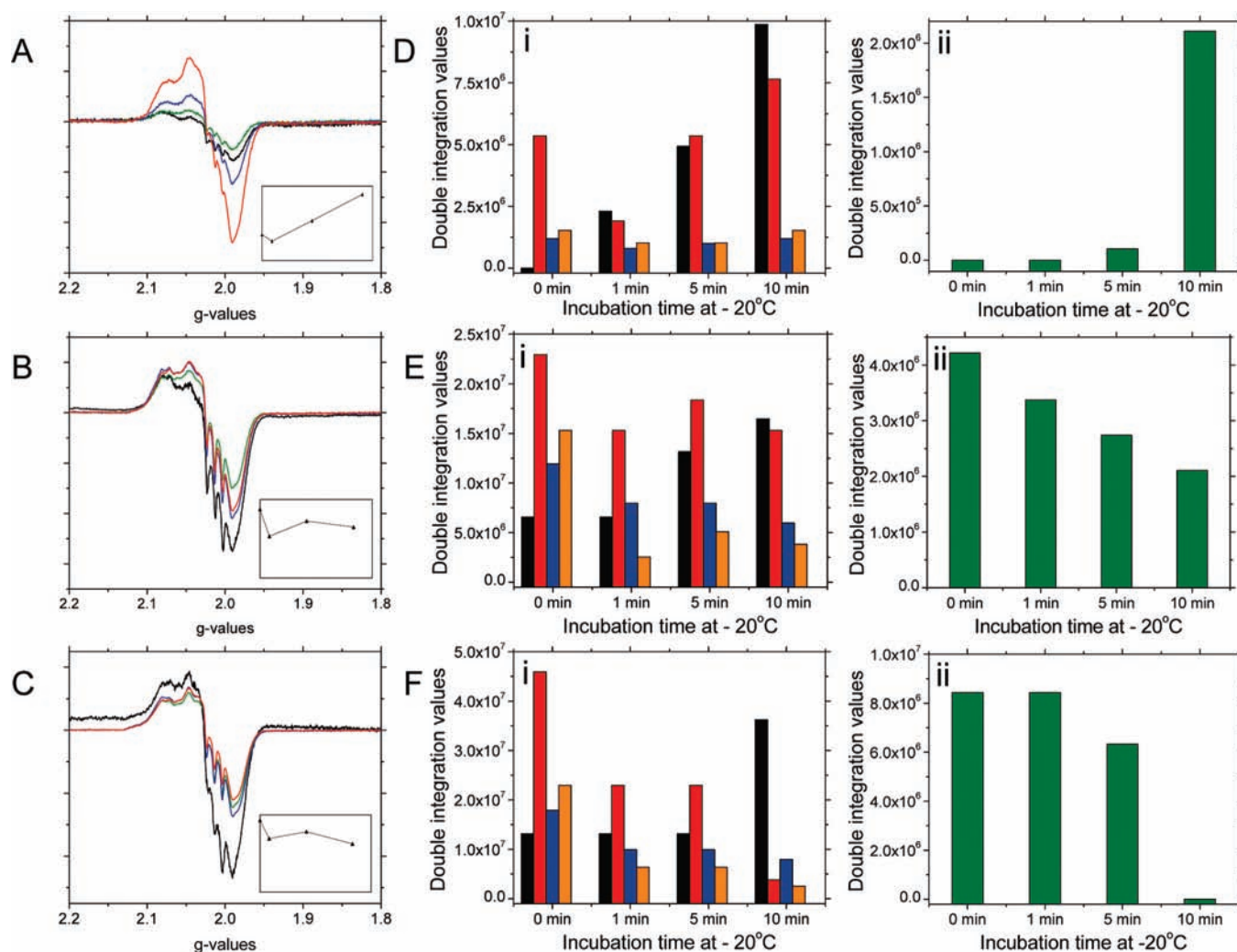


Figure 8. The X-band EPR spectra in the $g = 2$ region for a 1 mM deoxyHb and 0.25 mM nitrite mixture reacted for (A) 1 min; (B) 15 min; (C) 60 min (black), incubated at $-20\text{ }^{\circ}\text{C}$ for 1 (green); 5 (blue); 10 (red) min. [Insets represent the change in signal intensity in the $g = 2$ region versus incubation time.] The changes in the spectral components as a function of incubation time at $-20\text{ }^{\circ}\text{C}$ for the corresponding calculated EPR spectra for the 1 min (D); 15 min (E); and 60 min (F) reaction mixtures (black, R-state Hb(II)NO; red, R-state Hb(II)βNO; blue, T-state Hb(II)αNO; orange, T-state Hb(II)βNO; green, thiyl radical). Calculated DI versus experimental DI: (D) 1 min reaction time, 1 min incubation, calculated DI, 6.03×10^6 ; experimental DI, 7.85×10^6 ; % error, 23.1%; 5 min incubation, calculated DI, 1.24×10^7 ; experimental DI, 1.60×10^7 ; % error, 22.6%; 10 min incubation, calculated DI, 2.24×10^7 ; experimental DI, 2.67×10^7 ; % error, 16.2%; (E) 15 min reaction time, 1 min incubation, calculated DI, 3.58×10^7 ; experimental DI, 4.25×10^7 ; % error, 15.9%; 5 min incubation, calculated DI, 4.73×10^7 ; experimental DI, 5.80×10^7 ; % error, 18.4%; 10 min incubation, calculated DI, 4.37×10^7 ; experimental DI, 5.20×10^7 ; % error, 16.0%; (F) 60 min reaction time, 1 min incubation, calculated DI, 6.09×10^7 ; experimental DI, 6.93×10^7 ; % error, 12.2%; 5 min incubation, calculated DI, 5.88×10^7 ; experimental DI, 7.84×10^7 ; % error, 25.0%; 10 min incubation, calculated DI, 5.05×10^7 ; experimental DI, 6.28×10^7 ; % error, 19.5%.

($\text{Hb(II)NO}^+ \leftrightarrow \text{Hb(III)NO}$) and the paramagnetic intermediate ($\cdot\text{SHb(II)NO}$). Under these conditions, a small decrease in signal intensity in the $g = 2$ region is attributed to the low temperature destabilization of the paramagnetic intermediate indicated by the observed decrease in thiyl radical and R-state Hb(II)βNO components (Figure 8E). This effect is, however, less pronounced than for the 60 min nitrite-reacted sample because after 15 min the hybridized intermediate is still being formed at room temperature and the equilibrium distribution between the hybridized and paramagnetic intermediates (eq 4) has not been reached.

After a 60 min reaction time when the formation of the hybridized intermediate has leveled off,³¹ the equilibrium distribution of these intermediates is attained. Under these conditions, during the low

temperature incubation, we observe a significant decrease in both R-state Hb(II)βNO and the thiyl radical components, which are attributed to the destabilization of the paramagnetic intermediate relative to the hybridized intermediate. This result shows an apparent decrease in heme–NO species, which does not involve the highly stable Hb(II)NO, but instead involves a transfer of an electron from the NO of $\cdot\text{SHb(II)NO}$ to the thiyl resulting in the formation of the HSHb(II)NO^+ component of the hybridized intermediate. The transfer of NO from the heme of the paramagnetic intermediate ($\cdot\text{SHb(II)NO}$) to the thiyl radical producing SNOHb(II) can also contribute to the observed decrease in the EPR intensity. However, in the deoxygenated T-state hemoglobin,^{58,59} the formation of SNOHb should be minimal, because of T-state destabilization of SNOHb.

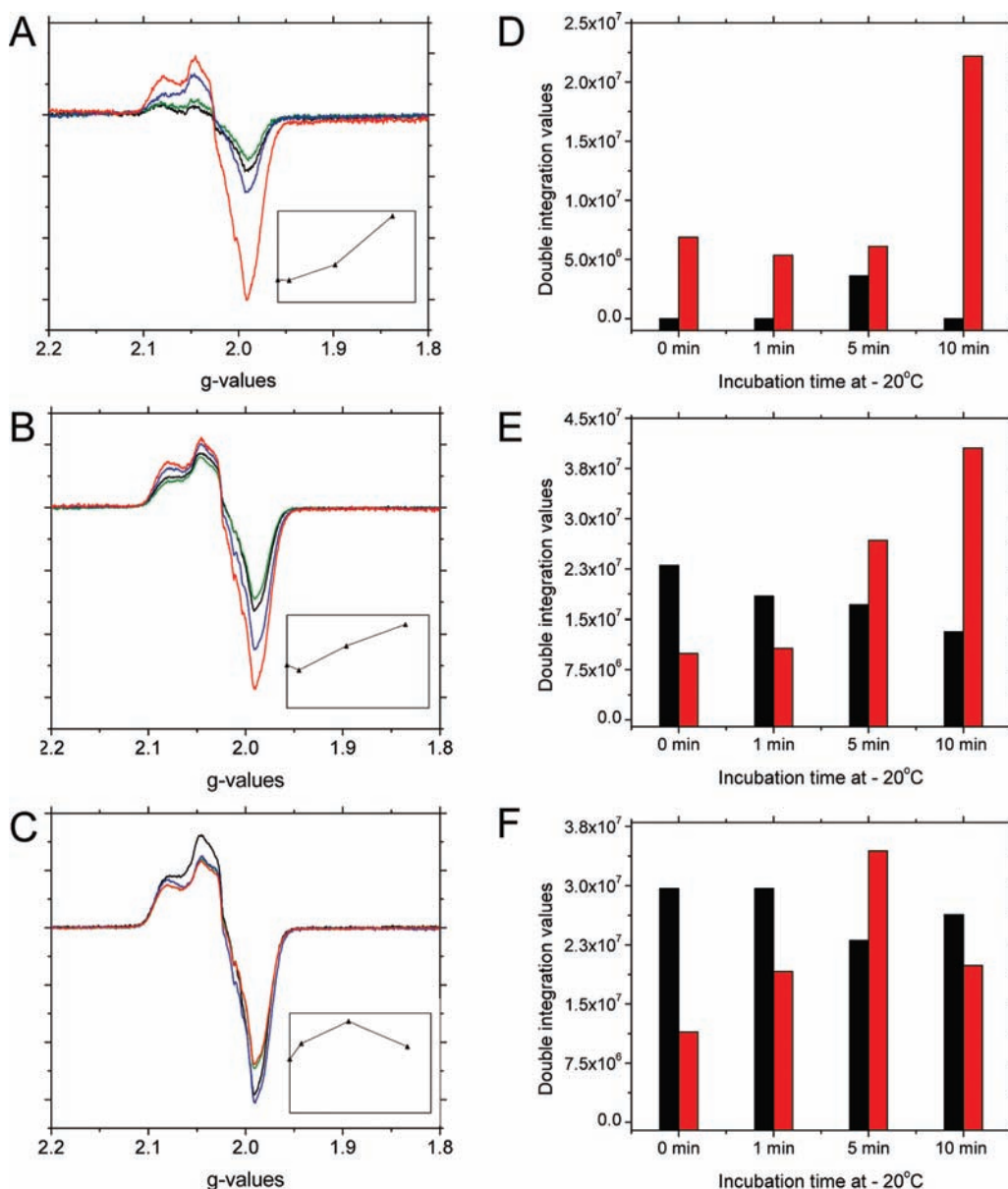


Figure 9. The X-band EPR spectra in the $g = 2$ region for a 1 mM NEM treated deoxyHb and 0.25 mM nitrite mixture reacted for (A) 1 min; (B) 15 min; (C) 60 min (black), incubated at -20°C for 1 (green); 5 (blue); 10 (red) min. [Insets represent the change in signal intensity in the $g = 2$ region versus incubation time.] The changes in the spectral components as a function of incubation time at -20°C for the corresponding calculated EPR spectra for the 1 min (D); 15 min (E); and 60 min (F) reaction mixtures (black, R-state Hb(II)NO; red, R-state Hb(II) β NO). Calculated DI versus experimental DI: (D) 1 min reaction time, 1 min incubation, calculated DI, 5.36×10^6 ; experimental DI, 7.31×10^6 ; % error, 25.7%; 5 min incubation, calculated DI, 9.74×10^6 ; experimental DI, 1.23×10^6 ; % error, 20.7%; 10 min incubation, calculated DI, 2.22×10^7 ; experimental DI, 2.84×10^7 ; % error, 21.7%; (E) 15 min reaction time, 1 min incubation, calculated DI, 2.91×10^7 ; experimental DI, 3.28×10^7 ; % error, 11.1%; 5 min incubation, calculated DI, 4.39×10^7 ; experimental DI, 5.38×10^7 ; % error, 18.4%; 10 min incubation, calculated DI, 5.37×10^7 ; experimental DI, 7.35×10^7 ; % error, 25.9%; (F) 60 min reaction time, 1 min incubation, calculated DI, 4.88×10^7 ; experimental DI, 5.56×10^7 ; % error, 4.85%; 5 min incubation, calculated DI, 5.75×10^7 ; experimental DI, 7.34×10^7 ; % error, 21.7%; 10 min incubation, calculated DI, 4.62×10^7 ; experimental DI, 5.31×10^7 ; % error, 13.0%.

The dominant changes occurring during the -20°C incubation involve the redistribution between the intermediates. This can be attributed to relatively minor conformational changes, which favor electronic rearrangements that cause a shift between the intermediates. However, at this low temperature, appreciable globin rearrangements can also take place, although at a reduced rate. The increase in the R-state Hb(II)NO involving both chains and the decrease in the T-state Hb(II)NO species imply that the

low temperature stabilizes the R-quaternary state relative to the T-quaternary state. Because we have previously shown that the release of NO is enhanced in the T-state quaternary conformation,⁶⁰ the low temperature stabilization of the R quaternary conformation would inhibit the release of NO from hemoglobin.

For NEM treated deoxyHb reacted with nitrite (Figure 9), low temperature incubations at -20°C reveal that for the 1 min reaction mixture (Figure 9A) the intensity of the EPR signal in

the $g = 2$ region increases as a function of incubation time, such that after 10 min incubation, the signal has tripled in intensity. Spectral fitting of this signal reveals that the intensity increase is correlated to a growth in R-state Hb(II) β NO (Figure 9D). For the 15 min reaction mixture, the total intensity changes for the signal in the $g = 2$ region (Figure 9B) also show an increase in intensity, such that by the end of 10 min incubation, the signal has doubled in intensity. Spectral fitting of this signal reveals that the intensity increase is also correlated to a growth in R-state Hb(II) β NO (Figure 9E). For the reaction mixture at 60 min, low temperature incubations at -20°C decrease the intensity of the signal in the $g = 2$ region by approximately 12% (Figure 9C); this decrease is within experimental error, and therefore it is not considered to be significant. Spectral fitting of the signal in the $g = 2$ region reveals that both R-state Hb(II)NO and R-state Hb(II) β NO do not significantly change as a function of incubation time at low temperature (Figure 9F).

For NEM treated deoxyHb reacted with nitrite, the only spectral change observed during low temperature incubation at -20°C is the increase in R-state Hb(II) β NO. This increase in Hb(II)NO must be associated with the relative instability of the hybridized intermediate. Because the paramagnetic intermediate $\cdot\text{SHb(II)NO}$ is not formed with NEM treated deoxyHb, the observed increase in intensity in the $g = 2$ region must be due to the binding of NO released from the "Hb(III)NO" component of the hybrid intermediate to reduced Fe(II) subunits. Thus, although diffusion through the globin required for the binding of NO to reduced subunits increasing Hb(II)NO is retarded with the increased levels of released NO, we still observed the formation of Hb(II)NO. The preferred formation of R-state Hb(II) β NO during low temperature incubation (Figure 9D) suggests that limited globin fluctuations at low temperature inhibit the transfer of NO from a β -chain to an α -chain to a greater extent than that between β -chains. The increased release of NO from the hybridized intermediate for NEM treated deoxyHb is also supported by an increase in the formation of metHb (results not shown).

The low temperature incubation studies thus indicate that the paramagnetic intermediate $\cdot\text{SHb(II)NO}$ is produced subsequent to the hybridized intermediate, by the transfer of an electron from the thiol to the NO^+ group of the hybridized intermediate. It also demonstrates that the reduction in temperature stabilizes the hybridized intermediate relative to the paramagnetic intermediate.

CONCLUSIONS

The finding that a paramagnetic intermediate is formed during the reaction of nitrite and deoxyHb has important implications. The general assumption that Hb(II)NO measures high affinity binding to an Fe(II) heme that can no longer be released is refuted by the formation of a paramagnetic Hb(II)NO species with a nearby thiyl radical ($\cdot\text{SHb(II)NO}$). This paramagnetic signal can readily be lost by the transfer of an electron back to the thiyl resulting in the reformation of the hybridized intermediate, which has no EPR signal. This newly characterized paramagnetic intermediate $\cdot\text{SHb(II)NO}$ explains the long-term stability of our earlier reported hybridized intermediate "Hb(II)NO $^+$ \leftrightarrow Hb(III)NO".

The slow reaction of nitrite with deoxyHb would seem to be incompatible with the physiological requirement of a rapid source of NO. The retention of this pool of bioactive intermediates

in the RBC provides a source of nitrite that is already reduced and can potentially be rapidly released from the RBC.

The actual mechanism involved in RBC mediated vasodilation is controversial. However, the accumulation of this pool of potentially bioactive NO in the RBC should have important ramifications for the delivery of NO to the vasculature irrespective of the particular mechanism involved. The involvement of a thiyl radical provides a pathway for the formation of SNOHb. S-Nitrosothiols that can be released from RBCs in conjunction with the formation of SNOHb are known to be vasodilators.

There are, however, a number of studies that have addressed a role for the reduction of nitrite to NO by hemoglobin. Whether NO generated by hemoglobin is important physiologically or only when dealing with pharmacological levels of nitrite, a satisfactory mechanism for the release of NO from RBCs needs to be elucidated. The pool of bioactive NO characterized in this Article, which does not spontaneously react with oxyHb or deoxyHb, is a crucial first step required for the release of NO from RBCs. This pool of bioactive NO must release its NO on the membrane for it to escape scavenging by hemoglobin. In an earlier publication, we have actually demonstrated that nitrite reacted hemoglobin has an unusually high affinity for the membrane,²⁹ and we have further demonstrated the enhanced release of NO from hemoglobin when nitrite reacted Hb binds to the membrane.³² NO released from hemoglobin bound to the membrane can potentially diffuse out of the cell without being scavenged by hemoglobin.

S-Nitrosothiols released from the RBC are relatively stable in plasma and can interact with thiols on the vasculature. For NO released from the RBC to be transferred to the vasculature, we are investigating (1) plasma components that can reversibly bind NO and (2) the direct transfer of NO from the RBC to the vasculature facilitated by transient adherence of RBCs to the vasculature. Further studies are, however, required to determine if, in addition to the transfer of S-nitrosothiols, the transfer of NO from RBCs to the vasculature provides an alternative pathway for the transfer of NO bioactivity.

ASSOCIATED CONTENT

S Supporting Information. Observed and calculated X-band EPR spectra in the $g = 2$ region for deoxyHb and NEM-treated deoxyHb reacted with nitrite at room temperature and incubated at -20°C for various times. Complete refs 24 and 28. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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