Reversible and Irreversible Hemichrome Generation by the Oxygenation of Nitrosylmyoglobin†

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ABSTRACT: The repeated oxygenation/reduction/nitrosylation of nitrosylmyoglobin produces low-spin ferric heme hemichromes which have been characterized by electron spin resonance spectroscopy. The predominant myoglobin hemichrome is a chemically reversible dihistidyl complex identified by the *g* values 1.53, 2.21, and 2.97. Also present is a low-spin ferric hydroxide derivative which is represented by the *g* values 1.83, 2.18, and 2.59. The formation of these species goes undetected by UV−vis spectroscopy, but the oxygenation of myoglobin to metmyoglobin is correlated with complete conversion of nitric oxide to nitrate which is released following a clear induction period. These results are interpreted in terms of the intermediates generated during the MbNO oxygenation reaction.

The oxidation of ferrous hemoglobin (Hb)[1] and myoglobin (Mb) to the high-spin (d^5, $S = {}^5/_2$) ferric state reduces the oxygen transport and storage capacities of these heme proteins. One pathway for generating metHb or metMb, which are the ferric forms, is an autoxidation process which produces superoxide as the other product (*1*)

$$[Fe^{II}O_2 \leftrightarrow Fe^{III}O_2^-] \quad (1)$$

$$Fe^{III}O_2^- + H_2O \rightarrow Fe^{III}(H_2O) + O_2^- \quad (2)$$

Although methemoglobin (*2*) and metmyoglobin (*3*) reductases are present to regenerate the ferrous heme, electron spin resonance spectroscopy (ESR) has shown that a small percentage of the ferric hemes spontaneously form a low-spin ($S = {}^1/_2$) adduct with either hydroxide or the nitrogen of the distal histidine. In the latter case, a dihistidyl complex is observed (*4*). ESR spectroscopy has been an indispensable tool for determining these structural changes which imply denaturation of the tertiary structure to bring the distal histidine within the coordination sphere of the iron. Collectively called hemichromes, these ferric derivatives are readily reduced back to the ferrous state in vivo by the enzymatic reductase system but can proceed to an irreversibly denatured state. The irreversibly denatured hemoglobin molecules are precipitated and sequestered as Heinz bodies attached to the red blood cell membrane for catabolism in the spleen (*5*). Thus, one of the key factors that determine the formation of irreversible hemichromes is the initial oxidation of the ferrous heme. Therefore, the mechanisms of heme oxidation are of great physiological significance.

In addition to the autoxidation pathway, there are several known routes whereby the met, deoxy, or oxy forms of heme proteins can react with NO which would cause the eventual oxidation of the heme center. In the first case, termed reductive nitrosylation, two molecules of NO are needed to produce $HbFe^{II}NO$ from $HbFe^{III}$ (*6*) (eqs 3−5)

$$NO + HbFe^{III} \rightarrow HbFe^{III}NO \quad (3)$$

$$HbFe^{III}NO + 2OH^- \rightarrow HbFe^{II} + ONO^- + H_2O \quad (4)$$

$$HbFe^{II} + NO \rightarrow HbFe^{II}NO \quad (5)$$

HbNO is also formed by the rapid ($k_6 = 2.5 \times 10^7\ M^{-1}\ s^{-1}$) reaction of NO and deoxyHb (*7*).

$$deoxyHbFe^{II} + NO \rightarrow HbFe^{II}NO \quad (6)$$

Oxygenation of nitrosylheme proteins to yield the ferric derivative and nitrate ($k_7 = 2.32 \times 10^{-4}\ M^{-1}\ s^{-1}$) proceeds at a vastly reduced rate compared to the reaction of NO and oxyheme proteins (*8*).

$$MbFe^{II}NO + O_2 \rightarrow MbFe^{III} + NO_3^- \quad (7)$$

The rate of reaction of NO with oxyhemoglobin ($k_8 = 3.7 \times 10^7\ M^{-1}\ s^{-1}$) has been suggested to produce a peroxynitrite as a precursor to the observed nitrate end product (*9*, *10*).

$$HbFe^{II}O_2 + NO \rightarrow HbFe^{III} + NO_3^- \quad (8)$$

These in vitro kinetic studies have a new found biological relevance as hemoglobin (*11*) and myoglobin (*12*) have both been shown to bind NO (*13*, *14*) in vivo. The source of NO

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[1] Abbreviations: NO, nitric oxide; NOS, NO synthase; Hb, hemoglobin; Mb, myoglobin; HbNO, nitrosylhemoglobin; MbNO, nitrosylmyoglobin; ESR, electron spin resonance; metMb, ferric myoglobin; metHb, ferric hemoglobin; spermineNONOate, 1,3-propanediamine, *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]; Tris, tris(hydroxymethyl)aminomethane.

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can be derived from the oxidation of L-arginine by constitutive nitric oxide synthase, NOS, in the vascular endothelial tissue (*15*), from inducible NOS in activated white blood cells (*16*), or from NO donors such as *S*-nitrosoglutathione and *S*-nitrososerum albumin (*17*) and pro drugs such as nitroglycerin (*18*) and sodium nitroprusside (*19*). Indeed, it has been recently demonstrated with ESR spectroscopy that MbNO is formed in cardiac tissue during cardioplegic ischemia (*12*). Subsequent reperfusion with oxygenated hemoglobin will lead to the oxidation of myoglobin as described in eq 7. It has been suggested that the redox cycling of myoglobin during ischemia reperfusion can be a significant factor linked to myocardial infarction tissue damage (*20*).

Although it has been proposed that myoglobin (*21*) and NO (*22*, *23*) might provide a cardioprotective effect in ischemia reperfusion, the oxygenation of MbNO may augment the tissue damage associated with reperfusion. The oxygenation reaction produces a ferric heme which may accumulate and interrupt the flow of oxygen to the affected tissue. Also, the oxidation of the iron is the first step on the path toward irreversible hemichrome formation. Thus, there exists the possibility that the cycling of $MbFe^{II}NO$ to $MbFe^{III}H_2O$ by introduction of oxygen could contribute to the overall deleterious effects observed during reperfusion. In this paper, we present ESR evidence for the formation of a low-spin Fe^{III} dihistidyl hemichrome generated from repeated nitrosylation/oxygenation cycling of MbNO.

MATERIALS AND METHODS

Horse heart myoglobin was obtained from Sigma and used without further purification. Sodium nitrite and sodium dithionite were supplied by Aldrich. Spermine was purchased from Aldrich, and spermineNONOate was synthesized as described elsewhere ($\epsilon = 8.5\ mM^{-1}\ cm^{-1}$ at $\lambda_{max} = 252$ nm) (*24*). A 10 mM Tris/HCl (pH 7.0) buffer was used for all protein solutions. Protein concentrations were determined by UV−visible spectroscopy from the millimolar extinction coefficients of metMb ($\epsilon = 10.2\ mM^{-1}\ cm^{-1}$ at $\lambda_{max} = 502$ nm) and MbNO ($\epsilon = 10.5\ mM^{-1}\ cm^{-1}$ at $\lambda_{max} = 576$ nm) (*25*).

Synthesis of Nitrosylmyoglobin. Two methods for deoxygenating the buffers and all solutions were employed in these experiments. The first protocol involved three cycles of a freeze, evacuate, thaw, and purge with nitrogen, as described previously, to rigorously deoxygenate the solution (*11*). Bubbling nitrogen gas through the solution for 30 min was an alternative method for deoxygenating these solutions.

Three methods were employed for introducing NO to the ferrous protein. This was done to ascertain the relative effects each procedure might have upon the protein.

Method A. To an anaerobic protein solution was added a stoichiometric amount of a 0.1 M anaerobic nitrite solution with a gastight microliter syringe. This was followed immediately by a similar injection of an anaerobic 0.1 M sodium dithionite solution. There was a nearly instantaneous color change from dark brown to the bright red nitrosyl complex. A typical synthesis is carried out by dissolving 50 mg of metMb in 0.005 L of the Tris/HCl buffer. This solution was transferred to a Schlenk flask and deoxygenated. An injection of 30 μL of a 0.1 M anaerobic solution of sodium nitrite is then added followed immediately (≈5 s) by 32 μL of a 0.1 M anaerobic solution of sodium dithionite.

Method B. As a control for nitrite-mediated hemichrome formation in solution, spermineNONOate was used as an NO donor compound. Synthesis in this case involved adding 5 mg of spermineNONOate to an anaerobic solution of deoxymyoglobin. Excess NO was purged with nitrogen. However, the rate of spermineNONOate decompostion is relatively slow ($k = 3 \times 10^{-4}\ s^{-1}$ at 37 °C and pH 7.4), and residual production of NO might contaminate the sample after a nitrogen purge and oxygenation (*24*).

Method C. A third alternative for preparing MbNO is introduction of NO gas directly into a solution of deoxygenated myoglobin. Excess NO was then removed by bubbling nitrogen for 20 min. Traces of nitrogen dioxide were removed from the NO stream by passing the gas through a bed of KOH pellets. In this way, the aqueous reaction of nitric oxide and oxygen to generate nitrite is averted.

It has been reported that nitrite incubated with ferric hemoglobin can generate several hemichromes (*4*). To demonstrate that nitrite is not the source of the observed hemichromes in our experiments, a 50 mg sample of deoxygenated metMb in 5 mL of 0.01 M Tris/HCl was injected with 30 μL of 0.1 M sodium nitrite. An ESR sample was taken and immediately (≈20 s) frozen in liquid nitrogen. The total time from mixing to freezing was 20 s. To the remaining solution was added an appropriate amount of dithionite to produce nitrosylmyoglobin. The ESR spectra of these samples were then compared.

Oxygenation of MbNO. After synthesis of MbNO, pure oxygen was gently bubbled through the solution for 30 s. The Schlenk flask, with the rubber septum still attached to ensure a headspace of oxygen, was then immersed in a constant-temperature bath set at 37 °C for 1 h (Fisher Isotemp model 9105). At this point, an ESR sample can be extracted from the flask and frozen in liquid nitrogen. The remainder of the solution then undergoes the deoxygenation process to start another cycle.

Electron Spin Resonance Spectroscopy. ESR spectra were obtained on a Bruker EMX X-band spectrometer equipped with an Oxford liquid helium flow cryostat. Experimental parameters are described in the figure captions.

Nitrate and Nitrite Determination. A nitrate selective electrode from Cole Parmer (serial number E-27502-30) was used to follow the formation of nitrate at 25 °C generated from the oxygenation of 1 mM MbNO in 0.01 M phosphate buffer (pH 7.0). The ionic strength was adjusted with 2 M ammonium sulfate to give a final concentration of 0.12 M. The electrode was calibrated against standard nitrate solutions in the same buffer at pH 7.0. Nitrite concentrations were determined by diazo coupling with the Greiss reagent and determination of the absorption at 546 nm (*26*).

RESULTS

Detection of Myoglobin-Derived Hemichromes. Figure 1 depicts the ESR results after the cyclic oxygenation of MbNO. Sigma metMb is used for a control at time 0 (Figure 1A). There is a typical high-field $g_{||}$ signal as well as a small amount of a low-spin ferric hydroxide which is characterized by g values of 1.83, 2.18, and 2.59 (*27*). Notably, after two

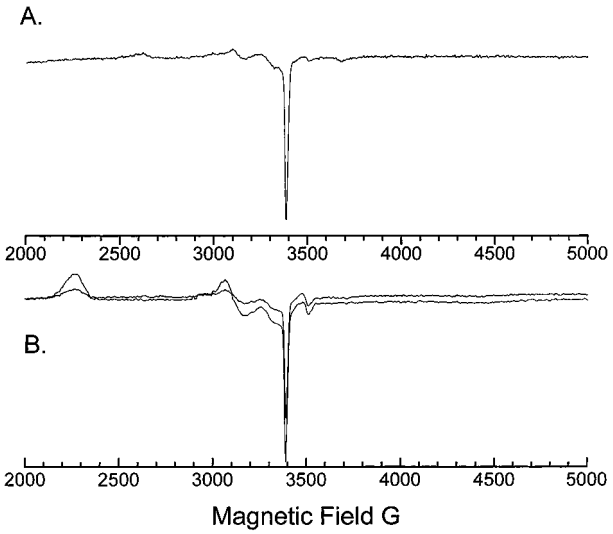


FIGURE 1: Hemichrome formation from the repetitive oxygenation/reduction/nitrosylation of MbNO. (A) Metmyoglobin control sample which was incubated at 37 °C following a 20 min purge with nitrogen. Some hydroxy hemichrome is indicated by *g* values of 1.83, 2.18, and 2.59. (B) An overlay of metmyoglobin after two and five nitrosylation/oxygenation cycles is shown. Spectrometer parameters were as follows: field setting, 3500 G; sweep width, 3000 G; receiver gain, 7×10^4; modulation amplitude, 10 G; microwave power, 2 mW; time constant, 82 ms; frequency, 9.464 GHz; and temperature, 9 K. The receiver gain was 1×10^5 in the experiments whose results are depicted in panel A.

Table 1: High-Field ESR *g* Values for Low-Spin Ferric Myoglobin and Porphyrin Complexes

sample composition	g_x	g_y	g_z	ref
metmyoglobin hydroxide	1.83	2.15	2.60	*32*
bis-imidazole heme	1.51	2.24	3.02	*30*
bis-nitro porphinato iron(III)	1.57	2.52	2.72	*44*
metMb and imidazole	1.51	2.22	2.92	*31*
metMb and NO_2^-	1.53	2.21	3.02	this work
metMb after five cycles	1.52	2.21	2.97	this work

nitrosylation/oxygenation cycles using method A (Figure 1B), another hemichrome appears which is distinguished by *g* values of 1.55, 2.21, and 2.97. This has been identified as a dihistidyl complex and will be addressed in the Discussion. After five nitrosylation/oxygenation cycles (Figure 1B), the signal intensity has increased as seen in the overlay. In Table 1 are summarized some of the experimental and known *g* values for various heme protein ferric complexes and hemichromes.

Nitrate Formation from the Oxygenation of MbNO. Two methods were employed to follow the nitrogen-containing byproducts from the oxygenation of MbNO. In the first experiment, the nitrite levels were determined by the Greiss reagent at the end of the reaction. These results, summarized in Table 2, illustrate that under a variety of conditions the level of nitrite produced is consistently within ±0.05%, or within the error of the experiment, for there being no nitrite formed. To demonstrate that the main reaction product is nitrate, we employed a nitrate sensitive electrode, low temperatures, and relatively high concentrations of protein, to follow not only the level of nitrate formed but also its time course. The result for a representative experiment is shown in Figure 2. There is a clear induction period for the formation of nitrate in solution, and this is followed by a rapid rise in its concentration, followed by a leveling off at

Table 2: Nitrate−Nitrite Partioning in MbNO Oxygenation

$[MbNO]_i$ (M)	$[NO_3^-]_f$ (M)	% conversion	$[NO_2^-]_f$ (M)[a]	% conversion	ref
0.001	0.001	100		–	this work
0.00208	0.00197	95		–	*8*
0.48		99.6[b]	$(1.9 \pm 0.5) \times 10^{-6}$	0.4	this work

[a] Nitrite analysis was preformed by the Greiss test (*26*) and is the value of three determinations. [b] Calculated value.

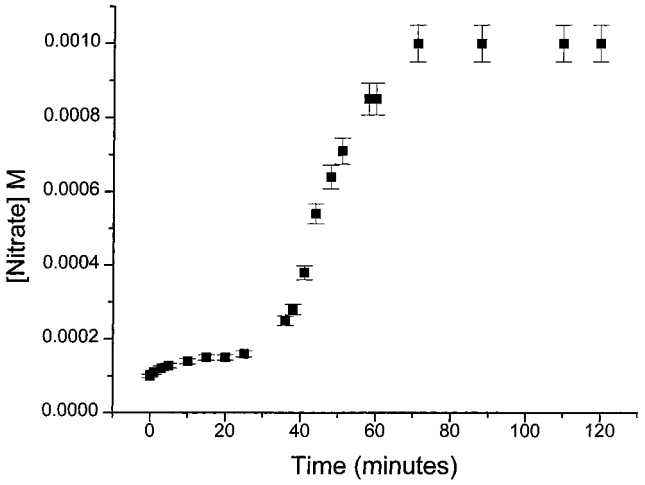


FIGURE 2: Time course for nitrate formation during the oxygenation reaction of MbNO. The conditions were as follows: 5 °C, 0.01 M phosphate buffer, pH 7.0, atmospheric oxygen, and [MbNO] of 0.9 mM.

a concentration which corresponds to complete conversion of the nitric oxide in MbNO to nitrate.

Denaturing Effects of Nitrite. A series of control experiments were performed to eliminate the possibility that nitrite is the source of hemichrome formation. The classic method of Hb hemichrome generation involves a 2 min incubation of Hb with an isotonic solution of nitrite (*4*). As is shown in Figure 3A, a 0.35 mM metMb solution mixed with an excess of nitrite (0.1 M) yields a myoglobin hemichrome. When a stoichiometric amount of nitrite was added to a metmyoglobin solution, followed by rapid freezing in liquid nitrogen, a reduced amount of hemichrome is observed in Figure 3B.

Hemichromes Observed from Different Sources of NO. It is known that the dithionite or ascorbate reduction of a nitrite/metMb mixture rapidly yields MbNO (*25*, *28*, *29*). Thus, in the presence of a slight excess of a reductant, there should be little free nitrite in solution and there will be little possibility that the nitrite/metMb solution can lead to hemichrome formation. A method for bypassing introduction of nitrite into the solution is desirable. One approach is to bubble authentic NO gas into the anaerobic protein solution followed by exhaustive nitrogen purging. This technique circumvents the presence of nitrite in the solution from the reaction of excess NO and dissolved oxygen. In Figure 4A, there is a characteristic ferric hemichrome after five nitrosylation/oxygenation cycles when this technique is used. Importantly, upon addition of dithionite, this hemichrome can form the nitrosyl adduct (Figure 4B), demonstrating that the hemichrome formation is reversible upon chemical reduction. These data support the observations realized using method A. Any hemichrome formed due to initial reaction

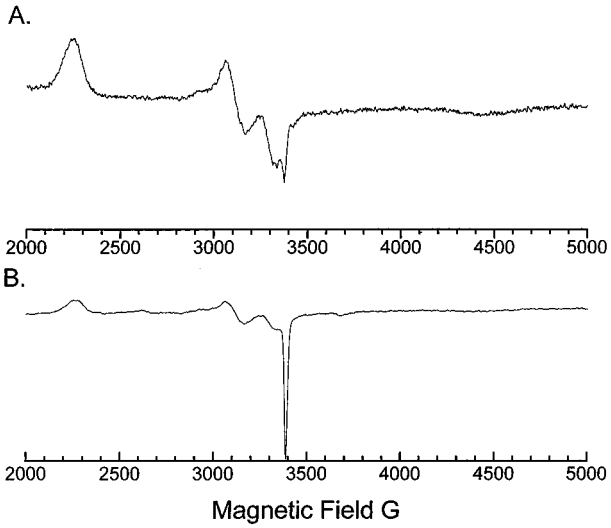


FIGURE 3: Effect of nitrite on metMb. In panel A, the concentrations of metMb and nitrite were 0.35 mM and 0.1 M, respectively. Whereas in panel B, the concentrations were 0.35 mM for both reactants. Spectrometer parameters were as follows: field setting, 3500 G; sweep width, 3000 G; receiver gain, 1×10^5; modulation amplitude, 10 G; microwave power, 2 mW; time constant, 82 ms; frequency, 9.464 GHz; and temperature, 10 K. The receiver gain was 2×10^4 in the experiments whose results are depicted in panel A.

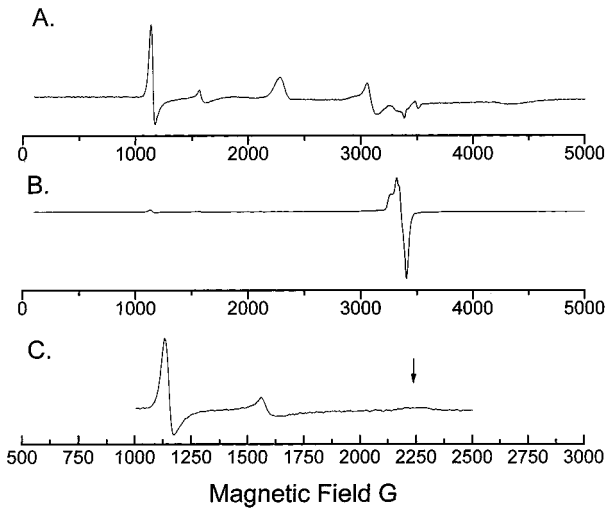


FIGURE 4: Cyclic oxygenation/reduction/nitrosylation of MbNO with authentic NO gas. Trace A shows a strong low-spin iron signature with g values at 1.55, 2.21, and 3.00. After deoxygenation, the sample in panel A was renitrosylated as demonstrated in panel B. Notice the complete loss of hemichrome peaks. An arrow in trace C indicates a $g = 3.02$ peak observed in an exploded view of the nitrosyl adduct in panel B. Spectrometer parameters were as follows: field setting, 2600 G; sweep width, 5000 G; receiver gain, 8×10^4; modulation amplitude, 10 G; microwave power, 2 mW; time constant, 164 ms; frequency, 9.464 GHz; and temperature, 10 K. The receiver gain was 1×10^4 in the experiments whose results are depicted in panel B.

with nitrite will be reduced to the ferrous state. Furthermore, the hemichromes observed from the oxygenation of MbNO are due to that reaction and not to excess nitrite. An indication that some irreversible hemichromes might be produced by this reaction is shown in Figure 4C. A weak feature at $g = 3.02$ is consistent with the presence of a low-spin hemichrome that cannot be chemically reduced.

Also noteworthy is the intensity of the low-spin species generated using different methods of NO introduction. Since

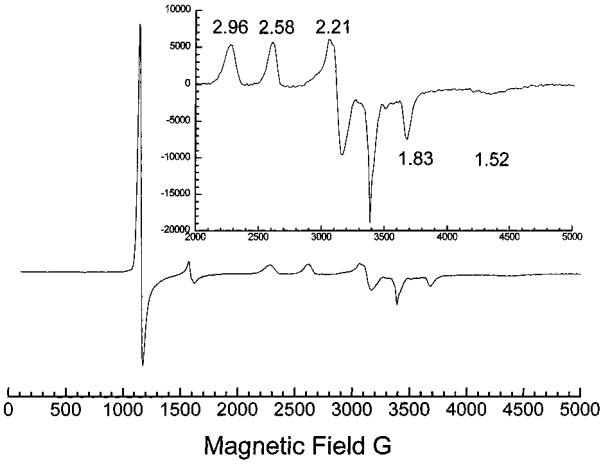


FIGURE 5: Cyclic oxygenation/reduction/nitrosylation of MbNO with spermineNONOate. The inset clearly illustrates the presence of two hemichromes. Spectrometer parameters were as follows: field setting, 2600 G; sweep width, 5000 G; receiver gain, 6.3×10^4; modulation amplitude, 10 G; microwave power, 2 mW; time constant, 164 ms; frequency, 9.464 GHz; and temperature, 10 K.

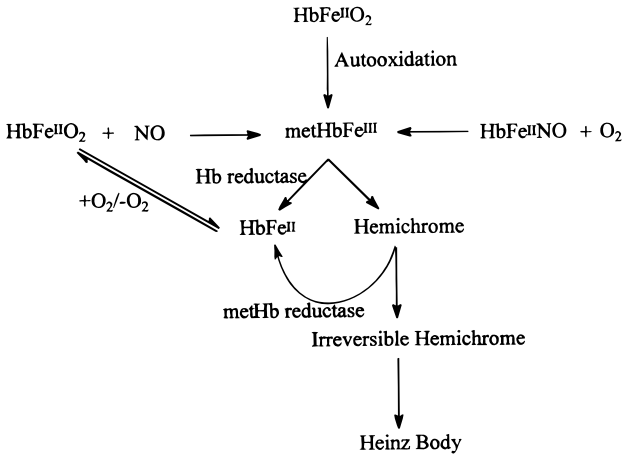
ESR is only probing the number of paramagnetic species in the system, then, provided that everything else is equal, the signal intensity will increase as the number of spin active species increases. The low-spin ferric signal intensity is much greater when NO gas (method B) was introduced to the system than when stoichiometric amounts of reagent were used (method A). This suggests that such a bolus addition of NO gas is inherently denaturing to the protein and causes tertiary structural changes in the protein. These changes are manifested in a large population of dihistidyl hemichromes. The same is true for bolus additions of nitrite (Figure 3A).

The results for the NO donor spermineNONOate are shown in Figure 5. Remarkably, there appear to be nearly equal populations of dihistidyl and hydroxide hemichromes present after three nitrosylation/oxygenation cycles. The peak intensities for these NONOate-derived absorptions are much greater than the intensities of the peaks observed using method A and are of the same magnitude of the peaks observed using method B. Notably, the distinct ESR spectrum representing two separate hemichromes is unique for this NO donor compound as compared to those obtained with the other methods of NO introduction.

DISCUSSION

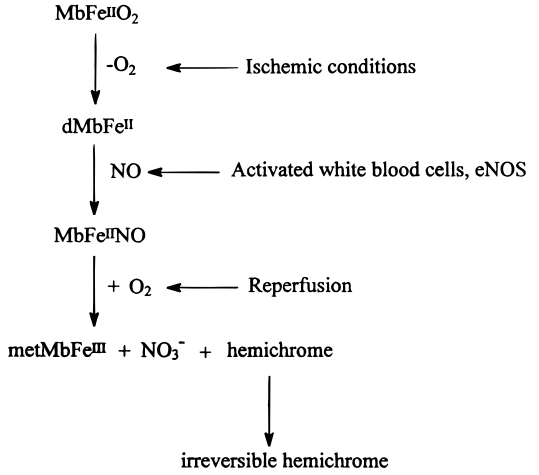
In this study, we present ESR evidence of a novel reaction mechanism for producing a low-spin ferric myoglobin dihistidyl hemichrome. Specifically, the oxygenation of MbNO disturbs the tertiary structure of the protein in such a way that coordination of the distal histidine to the iron heme center is allowed. Although numerous synthetic porphyrins have been studied (*30*), observation of myoglobin hemichromes has been hitherto limited to excess additions of nitrogenous bases (*31*) and sulfmyoglobin derivatives (*32*), and the description of a protozoan myoglobin hemichrome from *Paramecium caudatum* that is readily formed from the autoxidation process (*33*). However, our modeling of this in vivo process has demonstrated that myoglobin hemichromes can be detected with ESR spectroscopy, and their importance in heart disease may be as significant as that of hemoglobin hemichromes in blood abnormalities.

Scheme 1: Mechanisms for the Generation of Methemoglobin and, Ultimately, Hemichrome Formation[a]



[a] Myoglobin can be substituted in this scheme for hemoglobin.

Scheme 2: In Vivo Generation of Nitrosylmyoglobin and Subsequent Reactions



Initially, the accumulation of hemichromes in red blood cells had been linked to hemoglobin synthesis disorders, thalassemias, whereby the resulting protein is more prone to oxidation and less stable to denaturation (*34*, *35*). The initial step in hemichrome generation is the oxidation of the ferrous iron to the high-spin aquo met form (*27*). This occurs in a pH equilibrium with the low-spin hydroxide-bound form called O hemichrome (*4*, *36*). Via mechanisms that are not well understood, O hemichrome can spontaneously transform into H hemichrome. H hemichromes are best described as a dihistidyl iron complex which result from tertiary structural changes in the protein which allow the distal histidine to coordinate to the iron. A recent crystal structure of an invertebrate hemoglobin hemichrome (*37*) and the Fe K-edge X-ray absorption near-edge structure (XANES) spectra of two irreversible human hemoglobin hemichromes (*38*) confirm this structural deduction.

Although both of these Hb hemichromes, O and H, can be reduced to the ferrous state and complete functionality is regained, over time the protein structure can be significantly disrupted, making this process irreversible. These irreversible hemichromes are designated B hemichromes and are precipitated by the red blood cell in the form of Heinz bodies for future catabolism (*4*, *39*).

Thus, the focus of hemichrome research has been driven by various blood pathologies involving hemoglobin, whereas myoglobin hemichrome has escaped extensive scrutiny. This is surprising since the tertiary structure, function, and chemistry of myoglobin are very similar to those of hemoglobin (*25*). Considering that in early air pollution studies HbNO had been observed in the blood (*40*), it has only been recently demonstrated that MbNO is formed during cardiac ischemia (*12*). Since the oxygenation of a nitrosyl heme protein contributes to the ferric population of that protein, then the oxygenation of HbNO and MbNO could lead to hemichrome formation. In Scheme 1, the biochemical pathways connecting metHb and hemichromes are outlined. By extension, a similar scheme can be proposed for myoglobin, but it is unknown whether myoglobin hemichromes are precipitated and localized in a cellular feature similar to a Heinz body.

In vivo synthesis of MbNO during cardiac ischemia can be envisioned to occur with several sources of NO. As shown in Scheme 2, oxygenated myoglobin will release oxygen under ischemic conditions. As the oxygen tension drops in the tissue, several biochemical events are initiated. One process is the production of NO by eNOS which dilates the blood vessels and bring oxygenated blood to the tissue (*41*). The other process activates white blood cells which migrate to the ischemic tissue area and release NO (*42*). No matter the source, NO will rapidly bind to the deoxymyoglobin to form MbNO. In an anaerobic environment, MbNO is very stable. But, upon reperfusion and reintroduction of oxygen, myoglobin is oxidized to the ferric state and nitrate is produced. The redox cycling of myoglobin has been implicated in cardiac tissue damage during ischemia reperfusion via oxidation by hydrogen peroxide (*20*, *43*). The oxygenation of MbNO may contribute to this process by increasing the population of hemichromes.

Optically, the cycled metMb is indistinguishable from noncycled metMb, whereas differences in high-spin and low-spin ferric protein populations can be readily distinguished by ESR spectroscopy. The hemichrome formed from the redox cycling of MbNO is consistent with a dihistidyl ferric heme center. In addition, the ESR g values of the hemichrome are significantly different from the g values obtained from a structurally characterized picket fence iron porphyrin dinitro complex (*44*). These observations suggest that the dihistidyl assignment for the hemichrome, rather than a ferric nitro complex, is proper.

Thus, a chemically reversible hemichrome is formed, and it is easily reduced to the ferrous state. In subsequent renitrosylation, only the MbNO ESR spectrum is observed. There is no indication of a low-spin reversible or irreversible hemichrome in the MbNO spectra. Only after five cycles is there a weak $g = 3.02$ signal which is not reduced and may be indicative of an irreversible hemichrome. Therefore, upon oxygenation and oxidation of the heme, the observed dihistidyl hemichrome must be due to the reaction of MbNO and oxygen and not nitrite.

Nitrate is the sole oxynitrogen anion product from the oxygenation of MbNO, and its release follows an induction period following addition of oxygen to the nitrosylated protein. Among the mechanistic consequences of this observation is the fact that an initial dissociation of nitric oxide from MbNO can be ruled out because of the product

distribution; if free nitric oxide and oxygen react under these conditions, then nitrite will constitute one-half of the NO oxygenation product (*45*). Essentially no nitrite is observed under these conditions. Other groups have also found exclusive nitrate formation from these reactions, but since the reactions were performed at higher temperatures the induction period is smaller. Although this was present in their results, Andersen and Skibsted did not comment on it (*8*). The second consequence is that the addition of oxygen to nitric oxide must occur while the NO is bound to the metal or otherwise associated with the protein. Either way, the iron will promote its conversion specifically to nitrate, a process which also results in concomitant formation of metmyoglobin. Efficient catalytic conversion of peroxynitrite to nitrate has recently been described for ferric porphyrins. Since nitrite is not an end product, and nitrate does not lead to hemichrome formation, hemichrome formation in this system must be mediated by one of the MbNO oxygenation intermediates. Among the possible reactive intermediates are nitrogen dioxide, dinitrogen trioxide, peroxynitrite, and the pernitrous radical. Ascribing a specific role to any of these species will require careful further investigation. We note that the time course and products described herein must be explained by any scheme invoked to rationalize this chemistry. Some of the difficulties in this can be highlighted by pointing out that Alayash et al. recently described tyrosine nitration in hemoglobins treated with bolus peroxynitrite (*46*). But on its own, the presence or absence of tyrosine nitration in these myoglobin hemichromes may be insufficient evidence to demonstrate a role for peroxynitrite.

Of the three methods used in this study to synthesize MbNO, the use of stoichiometric amounts of reagents denatures less protein than the other techniques. Given the rapid rate with which nitrite reacts with myoglobin [4.7×10^2 M^{-1} s^{-1} at 22 °C and pH 7.0 (*48*)], it is best to immediately add a reductant to avoid protein denaturation. Likewise, introduction of NO gas is deleterious to the protein. A better method would be to add aliquots from an NO-saturated anaerobic buffer solution. In this way, known amounts of NO will be introduced to the protein. Also, care in the choice of a NO donor compound is advised. SpermineNONOate had adverse effects on the protein which might be ascribed to residual decomposition to NO and consequent nitrite formation upon introduction of oxygen. Considering that there are a multitude of methods for generating NO in vitro, reduction of *S*-nitrosothiols or sodium nitroprusside, oxidation of either Piloty's acid ($PhSO_2NH_2$-OH) or Angeli's salt ($Na_2N_2O_3$), or decomposition of diethylaminediazenium diolate (NONOate), etc., it is critical to understand the specific mechanisms by which these reagents lead to heme nitrosylation and the other reactive intermediates generated when they decompose.

In conclusion, we have demonstrated the formation of a dihistidyl hemichrome from the oxygenation of MbNO. Repeated cycling of this process denatures the protein as seen by the increase in the population of hemichromes. A comparison of different nitrosylating methods has shown that the best approach to MbNO synthesis is the use of stoichiometric amounts of reagents. Alternative methods of introducing NO produced denaturing effects on the protein. Research is in progress to examine different methods of MbNO synthesis as well as hemichrome formation from the reaction of NO with oxyheme proteins.

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