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Original Contribution

Immuno-spin trapping of hemoglobin and myoglobin radicals derived from nitrite-mediated oxidation

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Abstract

The reaction of nitrite with hemoglobin has become of increasing interest due to the realization that plasma nitrite may act as an NO congener that is activated by interaction with red blood cells. Using a combination of spectrophotometry, immuno-spin trapping, and EPR, we have examined the formation of radicals during the oxidation of oxyhemoglobin (oxyHb) and oxymyoglobin (oxyMb) by inorganic nitrite. The proposed intermediacy of ferryl species during this oxidation was confirmed by spectrophotometry using multiple linear regression analysis of kinetic data. Using EPR/spin trapping, a protein radical was observed in the case of oxyMb, but not oxyHb, and was inhibited by catalase. When DMPO spin trapping was combined with Western blot analysis using an anti-DMPO–nitrone antibody, globin/DMPO adducts of both oxyHb and oxyMb were detected, and their formation was inhibited by catalase. Catalase effects confirm the intermediacy of hydrogen peroxide as a heme oxidant in this system. Spectrophotometric kinetic studies revealed that the presence of DMPO elongated the lag phase and decreased the maximal rate of oxidation of both oxyHb and oxyMb, which suggests that the globin radical plays an active role in the mechanism of autocatalysis. Interestingly, the oxidation of oxyHb or oxyMb by nitrite, but not by hydrogen peroxide, produced a diffusible radical that was able to generate spin adducts on a bystander protein. This indicates that the oxidation of oxyhemeproteins by nitrite may cause more widespread oxidative damage than the corresponding oxidation by hydrogen peroxide. The immuno-spin trapping technique represents an important new development for the study of the range and extent of protein oxidation by free radicals and oxidants.

Keywords: Hemoglobin; Myoglobin; Nitrite; Ferryl heme; Globin radical; Immuno-spin trapping; EPR; Free radicals

The reactions of nitrite with blood components have recently become of increasing interest with the growing realization that plasma nitrite may represent a physiological NO congener and that NO generated via nitrite reduction could play a role in hypoxic vasodilation and as a therapeutic intervention [1–3]. The characteristic modifications in the absorption spectra of blood after its interaction with a solution of nitrite salts were first investigated in the middle of the 19th century [4]. The changes in optical properties were attributed to oxidation of hemoglobin (oxyHb) and the formation of metHb. At the same time, a decline in respiratory functions of the blood was also observed. Since this time, the influence of nitrite on hemoglobin has been the sporadic focus of research for many years, due mainly to the methemoglobinemia associated with nitrite toxicity [5–7], but also, more recently, because nitrite, present in the bloodstream at ~500 nM concentration [8], is thought to be an end-product metabolite of nitric oxide, and the oxidation of nitrite to nitrate has been thought to occur mainly via reaction with hemoglobin. Interestingly, plasma nitrite seems to track more closely to nitric oxide synthase activity than plasma nitrate [8].

As early as 1945, the aerobic oxidation of oxyHb by nitrite to form metHb was observed to be a multistep reaction, beginning with a slow induction period followed by a more rapid oxidation [9]. Since that time many efforts have been

Abbreviations: oxyHb, hemoglobin; metHb, methemoglobin; ferrylHb, ferryl hemoglobin; oxyMb, myoglobin; metMb, metmyoglobin; ferrylMb, ferryl myoglobin; UV-Vis, ultraviolet-visible; EPR, electron paramagnetic resonance spectroscopy; DTPA, diethylenetriamine pentaacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPOX, 5,5-dimethylpirrolidone-(2)-oxy(1).

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made to determine the possible reaction routes, but despite several suggestions, certain steps still remain unclear (reviewed in [1]). Intermediacy of hydrogen peroxide was presumed [6], because added catalase slowed down the reaction. Involvement of superoxide was also hypothesized based on the inhibiting effect of superoxide dismutase [10,11]. Kosaka et al. [12] attempted to unify these observations and described the oxidation as a chain reaction in which nitrogen dioxide radical acts as a chain-propagating species, whereas metHb and nitrate ion are the end-products. The intermediates in most proposed mechanisms for the reaction between nitrite and oxygenated heme proteins are the ferryl oxidation state of the heme protein and a protein radical. Direct electron paramagnetic evidence for the generation of the protein radical has been published [12], but there is little direct evidence for ferryl heme formation.

In this study, we have examined the formation of a ferryl heme and protein free radical intermediates in the reaction between nitrite and oxygenated heme proteins using direct electron paramagnetic resonance (EPR) spectroscopy, EPR spin trapping, immunoblotting against the protein/DMPO adduct [13], and direct UV–visible spectrophotometry. We show that a ferryl oxidation state of the heme is generated and decays during nitrite-mediated oxidation of oxyMb, and the protein free radical that is formed can be trapped and detected by EPR spectroscopy and immunoblotting. In the case of oxyHb, the trapped radical could not be observed by EPR spectroscopy, but could still be detected by immunoblotting techniques. In addition, we show that nitrite-dependent oxidation, in contrast to hydrogen peroxide, generates protein radicals on bystander proteins, indicating that protein radical formation occurs, at least in part, through the formation of diffusible oxidants.

Materials and methods

Materials

Horse heart myoglobin, bovine liver catalase, sodium nitrite, hydrogen peroxide, sodium dithionite, sodium chloride, diethylenetriamine pentaacetic acid (DTPA), tris(hydroxymethyl)aminomethane (Tris), glycine, casein blocking buffer, bovine serum albumin (BSA), sodium bicarbonate, *N*-ethylmaleimide (NEM), and DMPO were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). DMPO was further purified according to a published method [14] with slight modification [15]. Dithiothreitol (DTT), methanol, K_2HPO_4, and KH_2PO_4 were purchased from Fisher Scientific (Fairlawn, NJ, USA). Laemmli sampling buffer, Tris PAGel, electrophoresis buffer, 0.45-μm pure nitrocellulose membrane, and Tween 20 were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Human oxyHb was a gift from Apex Biosciences, Inc. (Research Triangle Park, NC, USA). Polyclonal rabbit anti-DMPO–nitrone adduct antiserum (primary antibody) was prepared as described elsewhere [13]. Goat anti-rabbit IgG alkaline phosphatase conjugate (secondary antibody) was obtained from Pierce Chemical Co. (Rockford, IL, USA). CDP-chemiluminescence substrate and Star Nitro Block II chemiluminescence enhancer were purchased from Applied Biosystems (Foster City, CA, USA). Hyperfilm ECL chemiluminescence film was an Amersham Bioscience Corp. (Piscataway, NJ, USA) product.

Preparation of hemoglobin and myoglobin derivatives

OxyMb was obtained from metmyoglobin (3 mM) with addition of excess sodium dithionate, followed by separation on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.4) containing 1 mM DTPA.

FerrylMb and ferrylHb were prepared by treating the corresponding oxyferrous species with 2- and 10-fold molar excess of hydrogen peroxide, respectively [16,17]. Concentrations of Hb, Mb, and their derivatives were determined spectrophotometrically, using published extinction coefficients [18]. Heme protein concentration is given in terms of total heme for both myoglobin and hemoglobin.

Spectrophotometric measurements

Reactants were mixed in a 1-cm path-length quartz cuvette in 0.1 M phosphate buffer containing 1 mM DTPA, pH 7.4. Spectra were recorded every 15 s between 500 and 700 nm using a Hewlett–Packard 8453 diode array spectrophotometer at room temperature. The kinetics of oxy-, met-, and ferrylHb/Mb were assessed by multiple linear regression analysis, using spectra of standard species.

EPR spectroscopy

Direct EPR spectra were recorded from the reaction of 1.8 mM oxyHb and 1.8 mM $NaNO_2$. Reactants were placed into a quartz EPR tube (3-mm i.d.) and frozen with liquid nitrogen. Four spectra were accumulated at 77 K using the following spectrometer settings: microwave power, 10 mW; modulation amplitude, 2 G; time constant, 64 ms; scan range, 100 G.

For spin trapping experiments, reactants were mixed with 50 mM DMPO in 0.1 M phosphate buffer containing 1 mM DTPA, pH 7.4, and immediately transferred into a 50-μl volume quartz capillary. Experiments were carried out at room temperature using a Bruker EMX EPR spectrometer with the following parameters: microwave power, 20 mW; modulation amplitude, 2 G; time constant, 10.24 ms; scan time, 2.56 s. In each experiment, 100 individual spectra were recorded consecutively. No signal averaging was performed; noise reduction and spectrum reconstructions were completed with singular value decomposition of all detected spectra [15]. Spectral simulations were performed using the WinSim program obtained from NIEH/NIH (Research Triangle Park, Durham, NC) [19].

Western blot analysis

Hemoglobin or myoglobin (50 μM), DMPO (50 mM), and, in certain cases, catalase (0.1 mg/ml, 21 kU/ml) or

BSA (0.1 mg/ml) were incubated with hydrogen peroxide (50 μM) or $NaNO_2$ (1 mM) in 0.1 M phosphate buffer (pH 7.4, containing 1 mM DTPA) for 1 h at room temperature. The reaction mixture was diluted twofold with Laemmli sampling buffer; DTT (10 mM final concentration) was added and incubated at 80°C for 10 min. Samples (1.2 μg protein) were subjected to SDS–PAGE (4–15% acrylamide gradient) and transferred onto a 0.45-μm nitrocellulose membrane for 2 h at 40 mV in freshly prepared transfer buffer (25 mM Tris/146 mM glycine/10% methanol). The membrane was blocked using a solution of 2.5% casein/BSA in 100 mM $NaHCO_3$ buffer (pH 9.6) overnight, washed three times with washing buffer (0.5% casein/BSA in 20 mM Tris/0.8% NaCl/0.1% Tween 20, pH 7.6), and incubated for 90 min with the primary anti-DMPO nitrone antibody (1:5000 in washing buffer). After three washes, the membrane was incubated for 60 min with the secondary antibody (1:5000 in washing buffer) and washed three times, and the antibody–antigen complexes were detected with enhanced chemiluminescence by exposure to high-performance chemiluminescence film. Band intensities were determined by densitometric scanning (AlphaImager 2000 software).

Statistics

A two-tailed Student *t* test was used for determining the significance; $p \leq 0.05$ was considered statistically significant.

Results

UV-Vis spectroscopy

Nitrite-mediated oxidation of oxygenated heme proteins can conveniently be followed spectrophotometrically by observing changes in the visible spectrum between 500 and 700 nm. Fig. 1A shows the spectral changes recorded during the nitrite-induced oxidation of oxyMb. The formation of metMb can clearly be observed by the development of an absorption band at 630 nm; however, the fact that there is no sharp isosbestic point between 590 and 620 nm indicates the presence of an intermediate species. Analyses of spectra revealed that the intermediate species resembled ferryl myoglobin, and the spectral changes were consistent with the formation and decay of a ferrylMb intermediate. Multiple linear regression analysis of this reaction using pure basis spectra of oxyMb, metMb, and ferrylMb (Fig. 1B) is shown in Fig. 1C, and residuals of this fit are shown in Fig. 1D. As can be seen, the ferryl oxidation state forms and decays during the time course of the reaction, and its formation seems to precede metMb formation. Similar data were obtained from the reaction between nitrite and oxyHb (data not shown). Although the reaction was slower, no sharp isosbestic points were observed, consistent with intermediate ferrylHb formation.

The effect of DMPO on the time course of nitrite-induced oxyHb/oxyMb oxidation was examined. As shown in Fig. 2, addition of DMPO inhibited the oxidation of both oxyMb and

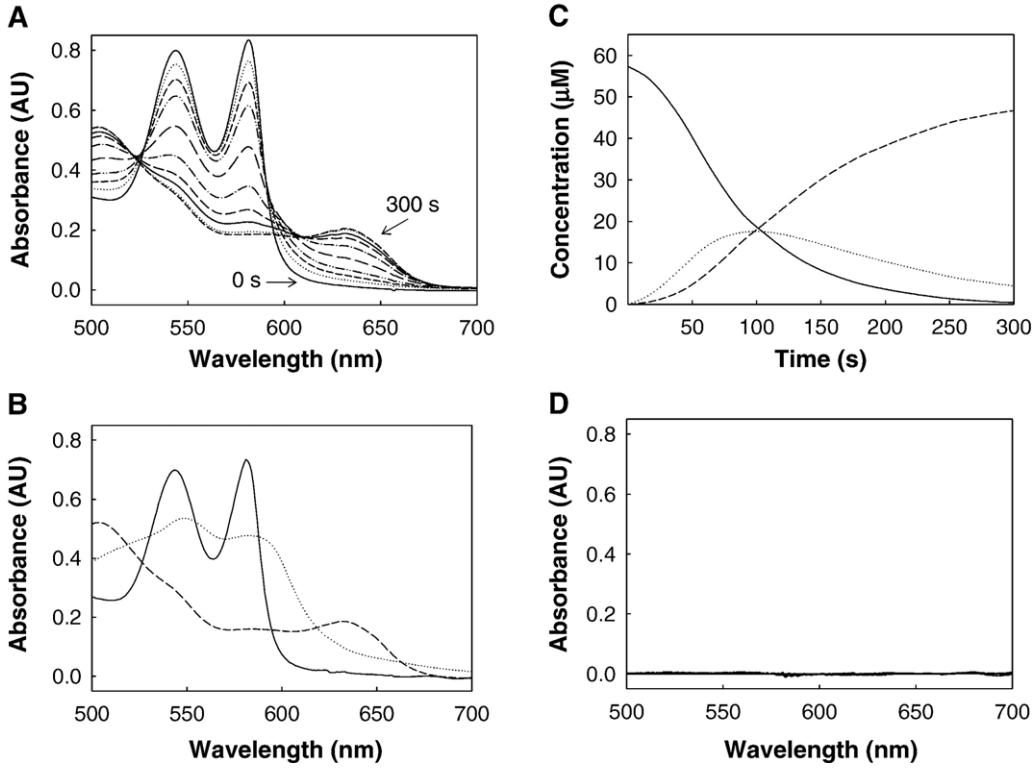


Fig. 1. Spectrophotometric investigations of nitrite-mediated oxidation of myoglobin. (A) UV-Vis spectra recorded in the oxidation of 57 μM oxyMb with 1 mM $NaNO_2$. Arrows indicate the initial point and the endpoint of the experiment. (B) Basis spectra used for linear regression: oxyMb (solid line), ferrylMb (dotted line), and metMb (dashed line). (C) The time courses of oxyMb (solid line), ferrylMb (dotted line), and metMb (dashed line) species in experiment (A). (D) Residual after subtraction of reconstructed spectra (B, C) from the original ones (A).

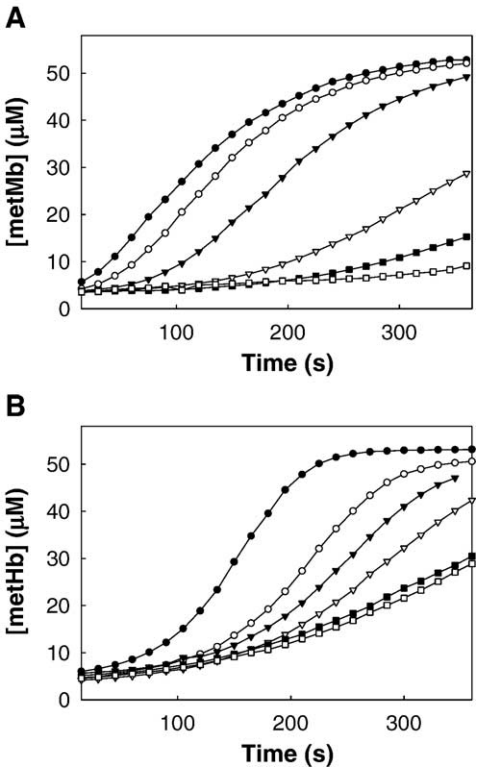


Fig. 2. Formation kinetics of metMb/metHb during nitrite-mediated oxidation. (A) 50 μM oxyMb or (B) 50 μM oxyHb was mixed with 1 mM $NaNO_2$ in the presence of different DMPO concentrations (●, 0 mM; ○, 10 mM; ▼, 25 mM; ▽, 50 mM; ■, 100 mM; and □, 200 mM).

oxyHb by both increasing the induction period and diminishing the maximal rate of oxidation as a function of DMPO concentration. The inhibitory effect of DMPO was more pronounced with oxyMb than with oxyHb.

Direct EPR spectroscopy

Similar to earlier investigations [12], we examined the EPR spectra recorded in a mixture of 1.8 mM oxyHb and 1.8 mM sodium nitrite at liquid nitrogen temperature (Fig. 3). The shape and the width of the spectrum at $g = 2.004$ were identical to those previously published by Kosaka et al. [12]. Decreasing the nitrite concentration to 0.2 mM resulted in a less intense spectrum, and when using 20 μM nitrite, no signal was detected (data not shown). A small, relatively broad signal at $g = 2.025$ was also observed, but this feature remains to be identified.

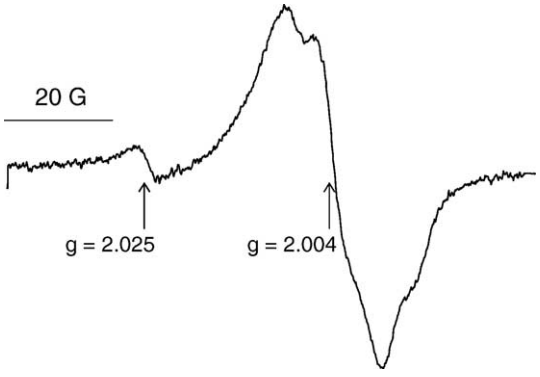


Fig. 3. Electron paramagnetic resonance spectrum of radicals produced in nitrite-mediated oxidation of hemoglobin. Mixture of 1.8 mM oxyHb and 1.8 mM $NaNO_2$ was frozen immediately in liquid nitrogen. Spectrum was recorded at 77 K.

EPR spin trapping of globin radicals

As a positive control, we first confirmed spin adduct formation from the reaction of heme proteins with hydrogen peroxide. MetMb (0.5 mM) or oxyMb (0.5 mM) was incubated with H_2O_2 (2 mM) in the presence of DMPO (50 mM). The EPR spectrum of the spin adduct using metMb (Fig. 4A) was found to be identical with those of published reports [20,21], and a similar spectrum was observed from the H_2O_2-mediated oxidation of oxyMb (see Fig. 5C). Interestingly, when using oxyHb (0.5 mM) under similar conditions, a broader spectrum was recorded (Fig. 4B).

It has been previously shown that the reaction between nitrite and oxyHb generates a protein radical that can be trapped with both DMPO and 2-methyl-2-nitrosopropane when both hemoglobin and nitrite were present at several millimolar concentration [22]. We investigated if trapped radicals could be

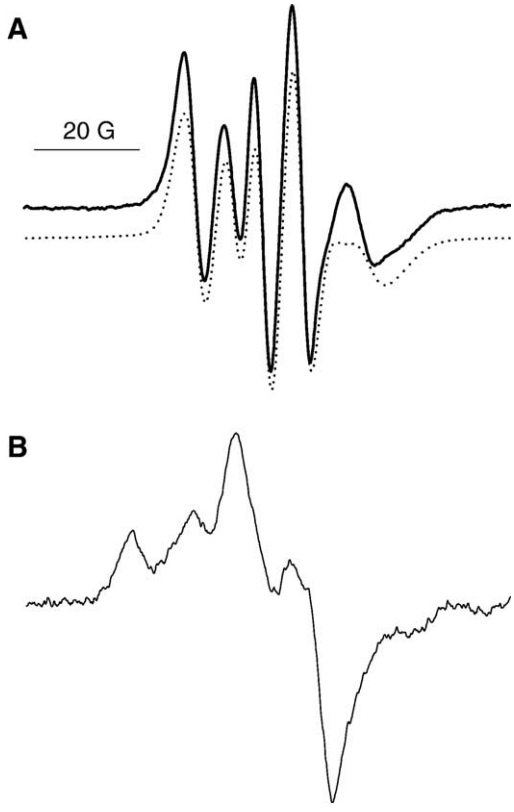


Fig. 4. Electron paramagnetic resonance/spin trapping studies on globin radical produced in hydrogen peroxide-mediated oxidation of myoglobin and hemoglobin. (A) Experimental (solid line) and simulated (dotted line) EPR spectra of protein adduct detected in oxidation of 500 μM metMb with 2 mM H_2O_2 in the presence of 50 mM DMPO. Simulation was completed with WinSim software. $a^N = 13.85$ G, $a^H\beta = 7.87$ G; line widths 2.83, 2.29, and 5.28 G. (B) EPR spectrum detected in oxidation of 500 μM oxyHb with 2 mM H_2O_2 in the presence of 50 mM DMPO.

observed at lower concentrations of reagents. As shown in Fig. 5A, the most prominent feature observed during the oxidation of more diluted (50 μM) oxyHb by nitrite was a sharp seven-line spectrum assigned as 5,5-dimethylpirrolidone-(2)-oxy(1) radical (DMPOX). No discernable spin adduct of hemoglobin was observed. In the case of oxyMb, the DMPOX signal was again present (Fig. 5B), but in this case an underlying protein radical was also observed (Fig. 5B). Subtraction of a simulated spectrum of DMPOX from Fig. 5B revealed the presence of the protein adduct (Fig. 5C). The EPR spectrum of the protein adduct was identical to that observed during the oxidation of oxyMb by hydrogen peroxide. In the presence of catalase all studied systems were EPR silent (not shown).

Immuno-spin trapping of globin radical

As only the unstable DMPO–nitroxide spin adduct is EPR-sensitive, we examined if we could use the immuno-spin trapping technique to detect the presence of DMPO–nitrone associated with the protein. Whereas the nitroxide concentration will be a steady-state level due to formation and decay, the nitrone should accumulate during the course of the reaction. Hemoglobin or myoglobin (50 μM) was co-incubated with an oxidizing agent (50 μM H_2O_2 or 1 mM $NaNO_2$) and 50 mM DMPO for an hour at ambient temperature and then subjected to Western analysis using an anti-DMPO nitrone antibody.

Fig. 6 shows a representative Western blot (top) and the average of relative band intensities (bottom, mean ± SE, n = 3 for metMb- and oxyMb-containing samples and n = 6 for oxyHb-containing samples) when metMb, oxyMb, or oxyHb was oxidized with H_2O_2 [13,23,24] or $NaNO_2$ in the presence of DMPO. Upon incubation of either metMb or oxyMb with hydrogen peroxide, a band characteristic of myoglobin was detected at ~15 kDa. The band density was reduced to control levels in the presence of catalase. In order to overcome the possible competition between catalase and methemoglobin [25], we used a relatively high catalase concentration. The oxidation of oxyMb by $NaNO_2$ resulted in the generation of a similar immunologically detectable DMPO adduct by a process inhibitable by catalase. OxyHb

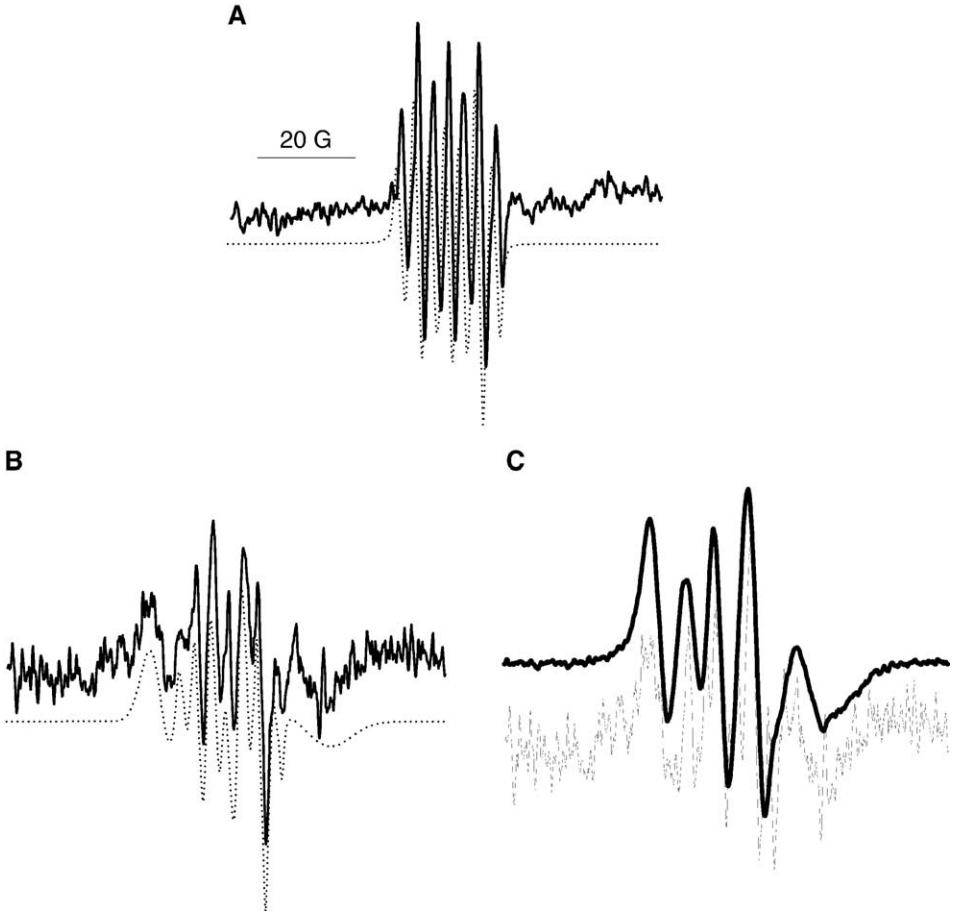


Fig. 5. Electron paramagnetic resonance/spin trapping studies on globin radical produced during nitrite-mediated oxidation of myoglobin and hemoglobin. (A) Experimental (solid line) and simulated (dotted line) EPR spectra of DMPOX radical detected during incubation of 50 μM oxyHb with 1 mM $NaNO_2$ and 50 mM DMPO. Simulation parameters: a^N = 7.06 G, a^H = 3.93 G. (B) Experimental (solid line) and simulated (dotted line) EPR spectra of radicals detected during incubation of 50 μM oxyMb with 1 mM $NaNO_2$ and 50 mM DMPO system. According to the simulation, spectra consisted of 85% protein radical adduct and 15% DMPOX. The simulation parameters for the protein radical are given in the legend to Fig. 4. (C) Lower spectrum: Experimental spectrum from B after subtraction of the simulated DMPOX signal. Upper spectrum: The protein radical adduct obtained during incubation of 500 μM oxyMb with 2 mM H_2O_2 and 50 mM DMPO.

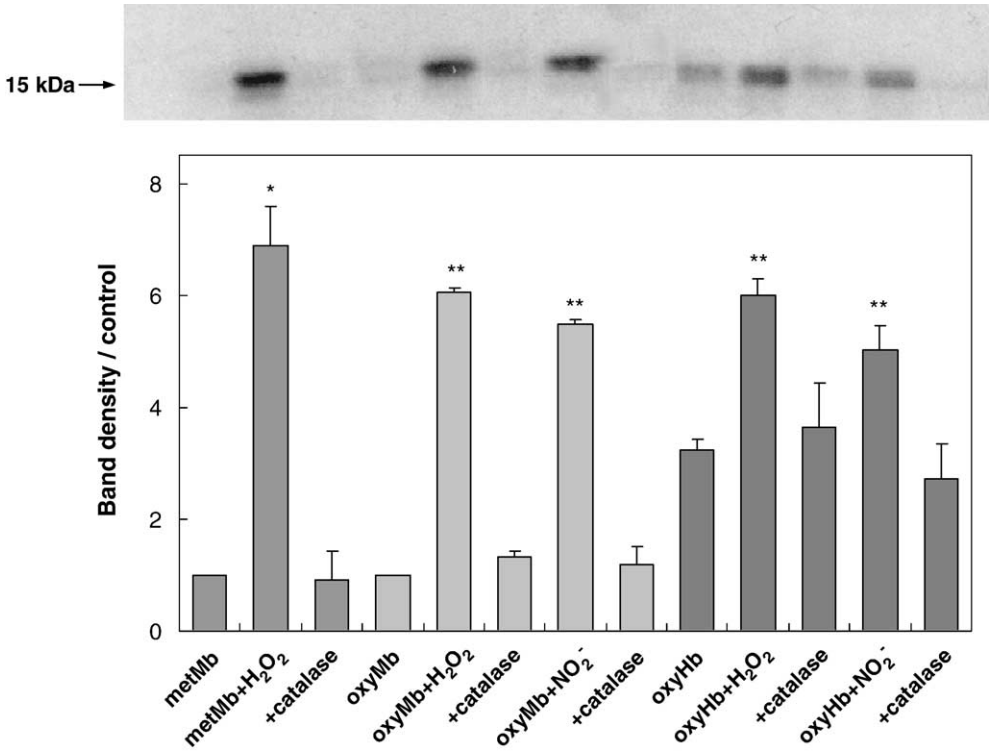


Fig. 6. Immunoblotting assay of globin radical produced in hydrogen peroxide- and nitrite-mediated oxidation of hemoglobin and myoglobin. Top: A characteristic Western blot (1.2 μg protein loaded). Concentrations: metMb, oxyMb, oxyHb, 50 μM; H_2O_2, 50 μM; $NaNO_2$, 1 mM; catalase, 21 kU/ml; and DMPO, 50 mM. OxyHb concentration was calculated by heme. Bottom: Band intensities compared to untreated control metMb (with densitometric scanning) from multiple experiments (means ± SE; $n = 3$ with metMb and oxyMb; $n = 6$ with oxyHb; $^*p < 0.05$, $^{**}p < 0.005$).

behaved in a similar way upon reaction with hydrogen peroxide or $NaNO_2$, forming immunoreactive proteins. Band densities were significantly higher in H_2O_2/$NaNO_2$-treated samples vs untreated controls. Catalase reduced the signal intensities to control levels. In the case of hemoglobin, we observed a much more dense protein band in control samples compared to myoglobin. The intensity of the basal Hb–DMPO band did not change when catalase was added or if protein cysteinyl sulfhydryls were blocked with NEM (not shown).

To examine if the effects of catalase on signal intensity were due to the nonspecific action of protein, we examined the effect of an identical amount (on a milligram basis) of BSA. As shown (Fig. 7), added BSA did not affect the intensity of the heme protein nitrone adduct band after oxidation with either hydrogen peroxide or nitrite. However, after nitrite, but not hydrogen peroxide treatment, a pronounced band appeared at the molecular mass of BSA (Fig. 7 for oxyMb, oxyHb not shown). This observation suggests that the nitrite/heme protein reaction is able to oxidatively modify bystander proteins.

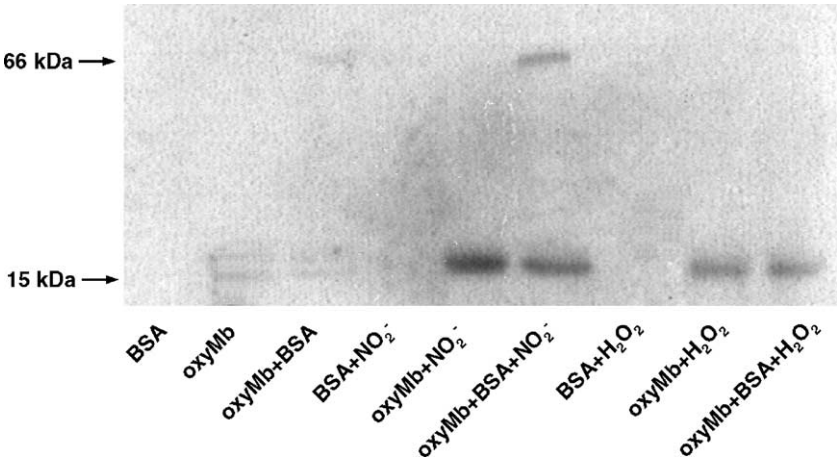


Fig. 7. Immunoblotting assay of nitrite- and hydrogen peroxide-mediated oxidation of oxyMb in the presence of BSA. Concentrations: oxyMb, 50 μM; H_2O_2, 50 μM; $NaNO_2$, 1 mM; BSA, 0.1 mg/ml; and DMPO, 50 mM. 1.2 μg protein was loaded into each lane.

Discussion

The nitrite-mediated oxidation of hemoglobin and myoglobin under oxygenated conditions has been reported to occur via a free radical chain reaction (Eqs. (1)–(6)) [12],

$$\text{oxyHb} + \text{NO}_2^- \rightarrow \text{metHb} + {}^\bullet\text{NO}_2 + \text{H}_2\text{O}_2, \quad (1)$$

$$\text{metHb} + \text{H}_2\text{O}_2 \rightarrow {}^\bullet\text{ferrylHb} + \text{H}_2\text{O}, \quad (2)$$

$${}^\bullet\text{NO}_2 + \text{oxyHb} \rightarrow \text{metHb} + \text{NO}_2^- + \text{O}_2, \quad (3)$$

$${}^\bullet\text{ferrylHb} + \text{NO}_2^- \rightarrow \text{ferrylHb} + {}^\bullet\text{NO}_2, \quad (4)$$

$$\text{ferrylHb} + \text{NO}_2^- \rightarrow \text{metHb} + {}^\bullet\text{NO}_2, \quad (5)$$

$$2\,{}^\bullet\text{NO}_2 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^-, \quad (6)$$

which occurs via formation of H_2O_2 (Eq. (1)). Interaction of metHb and hydrogen peroxide produced during the initiation step yields a ferryl heme species and a globin free radical (Eq. (2)). Lissi [26] criticized the above mechanism, as it did not explain the observed autocatalytic kinetics, and proposed a chain-branching reaction in which oxyHb oxidizes nitrogen dioxide into nitrate anion, reforming hydrogen peroxide (Eq. (7)):

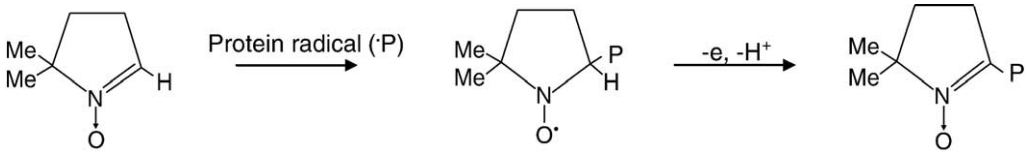
$$\text{oxyHb} + {}^\bullet\text{NO}_2 \rightarrow \text{NO}_3^- + \text{metHb} + \text{H}_2\text{O}_2. \quad (7)$$

We spectrophotometrically followed the kinetics of hemoglobin and myoglobin oxidation by nitrite and, using multiple linear regression, observed the formation and decay of an intermediate ferryl form in both cases. Similar kinetic curves for the formation and decay of ferryl species were recorded in hydrogen peroxide-mediated oxidation of sperm whale myoglobin [27]. These authors found that the generation rate and the stability of ferrylMb were dictated by the amino acid environment of the heme.

We have detected a transient free radical during the initial slow period by direct EPR at liquid nitrogen temperature at g = 2.004, in agreement with previous investigations [12,28]. A similar spectrum has also been observed in hydrogen peroxide-mediated oxidation of both metHb and metMb [29]. Later findings suggested that the first location of free radical on the globin is most probably the phenylalanine or histidine close to the porphyrin ring, then a migration occurs onto a tyrosine residue [30].

For immunological detection of protein free radical, the protein radical must be trapped with DMPO before Western analysis. To confirm that DMPO was able to trap the intermediate radical, we examined DMPO spin adduct formation during the reaction between nitrite and oxygenated heme proteins. In addition, we also examined the effect of DMPO on the kinetics of nitrite-dependent heme protein oxidation. We detected the spin adduct of oxyMb–protein radical and DMPO during nitrite-mediated oxidation. A similar adduct was not observed with oxyHb under the conditions used here; however, a spin adduct has been reported when much higher concentrations of hemoglobin were employed [22]. At low protein concentration the dominating spectrum was attributed to DMPOX radical. At the same time, according to spectrophotometric experiments, DMPO strongly inhibited the nitrite-induced oxidation of oxyHb or oxyMb. The inhibition was seen both in an elongated lag period and in a decrease of maximal oxidation rate. These findings seem to support the hypothesis [31] that DMPO is not exclusively a spin trap of globin radicals, but it is also able to reduce ferryl heme. However, thermodynamic considerations have indicated that this reaction is unfavorable [24], and we cannot exclude the possibility that the DMPOX formation from DMPO occurred as a result of ferryl-mediated oxidation of traces of hydroxylamine contaminant although the DMPO was extensively purified. According to the Kosaka/Lissi scheme (Eqs. (1)–(7)), efficient scavenging of the ferryl radical will be enough to prevent the reaction from entering the autocatalytic phase as the mechanism reverts from a branched to a linear chain reaction.

The immuno-spin trapping method, recognizing the accumulated nitrone-adduct end-product, rather than the steady-state concentration of spin adduct, enables improved detection of protein radicals. As can be seen in Scheme 1, in spin trapping, a covalent bond is formed between DMPO and the protein, resulting in the formation of an unstable nitroxide adduct that can be detected by EPR spectroscopy. A major route of nitroxide decay involves oxidation to the corresponding nitrone, leaving the covalent bond between the protein and the pyrroline ring intact. In the case of the reaction of nitrite with oxyHb, the spin-trapped radical could not be detected by EPR, but a robust increase in band density was observed by Western analysis. The strong band at the molecular mass of hemoglobin monomer and myoglobin (15 kDa) was suppressed by catalase, indicating that H_2O_2 is an intermediate in nitrite-dependent protein radical formation. The globin-centered radical of myoglobin has been earlier identified as a phenoxyl on tyrosine-103 in H_2O_2-induced oxidation with DMPO spin trapping/EPR [20,32]. Tryptophan-14 radical is also produced and can be oxidized into a peroxyl radical [33]. According to the latest mass spectral investigations



Scheme 1. Spin trapping of protein radical and formation of protein–DMPO nitrone.

[34], oxidation of hemoglobin can lead to radical formation on cysteine-93 in the β chain and on Tyr-42, Tyr-24, and histidine-20 on the α chain. Unpaired electron delocalization between the two latter sites was proposed.

Although hydrogen peroxide-mediated oxidation did not result in the formation of nitrone adducts of BSA, nitrone adducts of this bystander protein were formed during nitrite-mediated oxidation. This implies that the nitrite reaction generates oxidative intermediates that can diffuse away from their point of formation. The most likely candidate for this diffusible oxidant is nitrogen dioxide that may react with protein tyrosyl, and other residues, forming protein radicals that can be trapped with DMPO. As nitrogen dioxide is a reactive free radical, it is possible that the nitrite/oxyHb reaction in vivo would lead to protein and lipid oxidation and the depletion of endogenous antioxidants.

In summary, using UV-Vis spectroscopy and EPR/spin trapping, we characterized the oxyHb/oxyMb substrates, the transient heme protein species, and metHb/metMb end-products in nitrite-induced oxidation of oxyHb/oxyMb. By scavenging the globin radical intermediate, DMPO suppresses nitrite-mediated oxidation. Using the immuno-spin trapping approach, we demonstrated that nitrite-mediated formation of heme protein radicals depends on the intermediacy of hydrogen peroxide. This technique was revealed to be more sensitive than EPR/spin trapping and, because of its specificity against the EPR-silent protein–nitrone, may serve as a powerful tool to investigate the formation of globin radicals and also radical formation on bystander proteins in vivo.

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