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Reduction of Ferrylmyoglobin by the Spin Trap *N-tert*-butyl-α-phenylnitrone (PBN) in Aqueous Solution and During Freezing

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The hypervalent muscle pigment ferrylmyoglobin, formed by activation of metmyoglobin by hydrogen peroxide, was found to be reduced in a second-order reaction by *N-tert*-butyl-α-phenylnitrone (PBN, often used as a spin trap). In acidic aqueous solution at ambient temperature, the reduction is relatively slow ($\Delta H^{\ddagger} = 65 \pm 2\,\mathrm{kJ \cdot mol^{-1}}$ and $\Delta S^{\ddagger} = -54 \pm 7\,\mathrm{J \cdot mol^{-1}\,K^{-1}}$ for pH = 5.6), but phase transitions during freezing of the buffered solutions accelerates the reaction between ferrylmyoglobin and PBN. In these heterogenous systems at low temperature (but not when ice-formation was inhibited by glycerol), a PBN-derived radical intermediate was detected by ESR-spectroscopy, identified as a nitroxyl radical by a parallel nitrogen hyperfine coupling constant of 31.8 G, and from microwave power saturation behavior concluded not to be located in the heme-cleft of the protein. The acceleration of the reaction is most likely caused by a lowering of the pH during the freezing of the buffered solutions whereby ferrylmyoglobin becomes more oxidizing.

Keywords: Ferrylmyoglobin, ESR, PBN, freezing, radical intermediates, spin trapping, phase transitions

INTRODUCTION

Metmyoglobin (MbFe(III)) reacts with hydrogen peroxide in a reaction that generates the hypervalent species perferrylmyoglobin ($^{\bullet+}$MbFe(IV)=O) and ferrylmyoglobin (MbFe(IV)=O).[1,2] Both species contain iron in the oxidation state +4 with an oxo group as the sixth ligand, and $^{\bullet+}$MbFe(IV)=O furthermore contains a radical centered on the protein moiety.[3–6] The protein-based radical of $^{\bullet+}$MbFe(IV)=O is believed to be located on a tryptophan residue and to react rapidly with molecular oxygen yielding a peroxyl radical,[7,8] although other studies seem to show that the radical may also be centered on other amino acids or that a distribution exists between different radicals.[9–14] $^{\bullet+}$MbFe(IV)=O is unstable and decays in the course of a few minutes by an unknown pathway to yield MbFe(IV)=O.[10,15]

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MbFe(IV)=O is more stable and transforms to MbFe(III) by a specific acid catalyzed reaction, known as its autoreduction to yield a product that often is described as "slightly modified MbFe(III)".[16–19]

$^{\bullet+}$MbFe(IV)=O and MbFe(IV)=O are able to oxidize a number of compounds present in the living cell or present in or added to meat products, and they are believed to participate in the initiation of lipid oxidation.[20,21] Hypervalent myoglobin may be regarded as a potential danger to muscle tissue under oxidative stress especially in the light of the high concentration of myoglobin in certain (oxidative) muscles. However, their formation might also be seen as part of an antioxidative defense analogous to the peroxidase reaction cycle, in which hydrogen peroxide is removed at the expense of antioxidants, resulting in the formation of relatively harmless radicals.[22]

Many studies have been performed in order to understand the nature and the role of the hypervalent myoglobin species in muscle tissue, often with the use of low-temperature ESR, where samples are frozen in liquid nitrogen to prevent further reaction of short-lived radicals; other studies have employed the spin trapping technique in order to detect short-lived radicals derived from hypervalent myoglobin as stable spin adducts. Myoglobin has been reported to affect studies with the spin traps DMPO (5,5-dimethyl-1-pyrroline-1-oxide) and TMPO (3,3,5,5-tetramethyl-1-pyrroline-1-oxide), partly because MbFe(IV)=O is able to oxidize the DMPO and TMPO hydroxyl radical adducts, and for studies involving myoglobin the use of the spin trapping agent *N-tert*-butyl-α-phenylnitrone (PBN) is recommended instead of DMPO or TMPO since no interference by MbFe(IV)=O was observed at pH 7.4.[23,24]

Recently the effect of mildly acidic pH values on the autoreduction of MbFe(IV)=O was investigated by low-temperature ESR,[25] and two radicals were detected as intermediates in the reaction: The first radical gave rise to a broad signal at $g = 2.005$, and it was detected at pH values throughout the studied range of pH from 5 to 7. The second radical was seen as a sharp signal at $g = 2.003$ and was only detected in samples of pH lower than 6.5, which is also the pH below which specific acid catalysis of the autoreduction takes place.[19] The first radical was suggested to be formed in an intramolecular rate-determining electron-transfer in MbFe(IV)=O, and the second radical was suggested to be formed in a proton-assisted electron transfer that may initiate the specific acid-catalyzed autoreduction. Both radicals were detected in amounts that correlated with the immediate concentration of MbFe(IV)=O. However, it was also found that the process of rapid freezing with liquid nitrogen during the preparation of samples for the ESR measurements affected the course of the autoreduction reaction, since the detected amounts of radicals depended on the type of buffer used – most likely because of a change in pH or a change in myoglobin concentration due to phase separations, a change in protein conformation due to low temperatures, or a combination hereof.

In the present investigation the reactions of MbFe(IV)=O were studied by UV–VIS and ESR-spectroscopy with use of the spin-trap PBN in order to further characterize radical intermediates. PBN is demonstrated to be slowly oxidized by MbFe(IV)=O at room temperature, and the oxidation of PBN by MbFe(IV)=O is found to be advanced in mixtures of the two reagents frozen shortly after their preparation, further suggesting an effect of freezing on the course of the reaction, which seems of interest for understanding the role of myoglobin as a prooxidant during freezing of tissue or meat products.

MATERIALS AND METHODS

Materials

PBN was from Molecular Probes Europe BV (Leiden, Holland) and 4-POBN (α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone) was from Aldrich

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Chemical Co. (Milw., WI, USA). MbFe(IV)=O was synthesized in aqueous phosphate buffer solution from MbFe(III) (horse heart, type III) from Sigma Chemical CO. (St. Louis, MO, USA), purified on a Sephadex G25 column (Pharmacia Biotech AB, Uppsala, Sweden) and hydrogen peroxide (35% from Riedel-de Häen, Seelze, Germany) as previously described.[25]

ESR-Spectroscopy

A Bruker ECS-106 spectrometer equipped with an ER 4103 TM cavity and an ER 4112 continuous flow liquid nitrogen cryostat were used (Bruker, Rheinstetten, Germany). All spectra were measured in X-band mode at 200 K and with modulation frequency and amplitude equal to 100 kHz and 1.0 G, respectively.

Cold Baths

The following liquids and mixtures were used for the freezing of ESR samples at different temperatures: 249 K: ethanol-bath kept in a freezer at that temperature; 230 K: slurry made from liquid nitrogen and acetonitrile, 193 K: mixture of dry ice and acetone; 77 K: liquid nitrogen.

UV–VIS Absorption Measurements

An HP8453 diode array spectrophotometer equipped with a thermostated cell holder (Hewlett-Packard, Palo Alto, CA, USA) was used for determination of the MbFe(III) concentration,[26] for determination of the concentration of MbFe(IV)=O in mixture with MbFe(III),[27] and for kinetic studies of the reaction between MbFe(IV)=O and PBN (580 nm) at 27.7°C, while a Cary 3 UV–VIS spectrophotometer equipped with a thermostated cell holder (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia) was used for kinetic studies at 14.3°C and 21.2°C (20 mM phosphate buffer, ionic strength 0.16 in all kinetic experiments). Spectra of thawed solutions of MbFe(III), hydrogen peroxide and PBN were recorded using the Cary 3 UV–VIS spectrophotometer after centrifugation at 20 000g for 2 min in an Ole Dich Centrifuge (Ole Dich, Rødovre, Denmark).

RESULTS

Reaction of Ferrylmyoglobin with PBN in Solution

PBN accelerated the transformation of MbFe(IV)=O to MbFe(III). The reduction of MbFe(IV)=O by PBN was studied at pH = 5.6. The kinetic trace obtained by UV–VIS absorption spectroscopy at 580 nm showed that the transformation was first order in MbFe(IV)=O. The pseudo-first-order rate constant for reduction of MbFe(IV)=O to MbFe(III) measured under pseudo-first-order conditions with large excess of PBN followed the expression: $k_{obs} = k_0 + k_2 \cdot [\text{PBN}]$, where k_0 and k_2 are the rate constants of the autoreduction and the direct reduction of MbFe(IV)=O by PBN, respectively. Interpolation from Arrhenius plots as shown for k_2 in Figure 1 gave $k_2 = 0.0449 \pm 0.0008\ \text{M}^{-1} \cdot \text{s}^{-1}$ and $k_0 = (8.50 \pm 0.28) \times 10^{-4}\ \text{s}^{-1}$ at 25°C. The value of k_0 is in agreement with the rate previously reported for the MbFe(IV)=O autoreduction,[19] confirming that the reduction under these conditions takes place by a parallel autoreduction and direct reduction by PBN. The apparent activation parameters for pH = 5.6 were found to be $\Delta H^{\ddagger} = 65 \pm 2\ \text{kJ} \cdot \text{mol}^{-1}$ and $\Delta S^{\ddagger} = -54 \pm 7\ \text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ from the temperature dependence of k_2 (Figure 1).

The reaction between PBN and MbFe(IV)=O was found to proceed very slowly at pH = 7.4 and it was furthermore complicated by the denaturation and precipitation of the myoglobin species. The kinetics of the reaction was consequently studied only under slightly acidic conditions.

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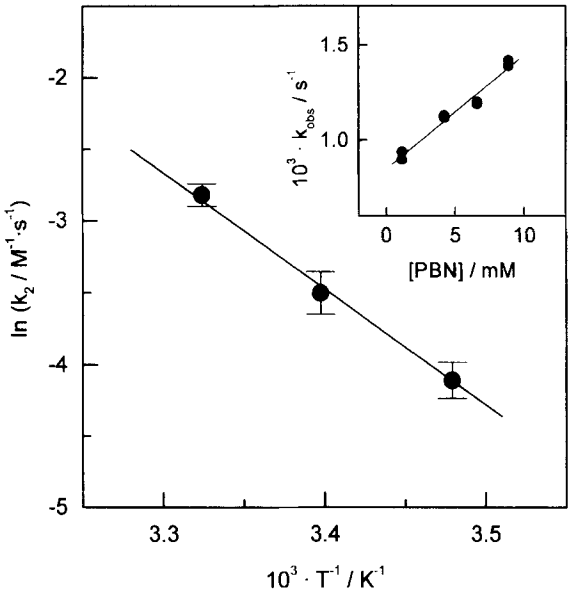

FIGURE 1 Arrhenius plot of the second-order rate constant k_2 for the reduction of MbFe(IV)=O by the spin-trap PBN in aqueous solution at pH = 5.6 and ionic strength 0.16 (NaCl). Inset: Observed pseudo-first-order rate constants as function of PBN concentration at 27.7°C for parallel autoreduction (k_0) and direct reduction (k_2); full line: $k_{obs} = k_0 + k_2 \cdot$ [PBN].

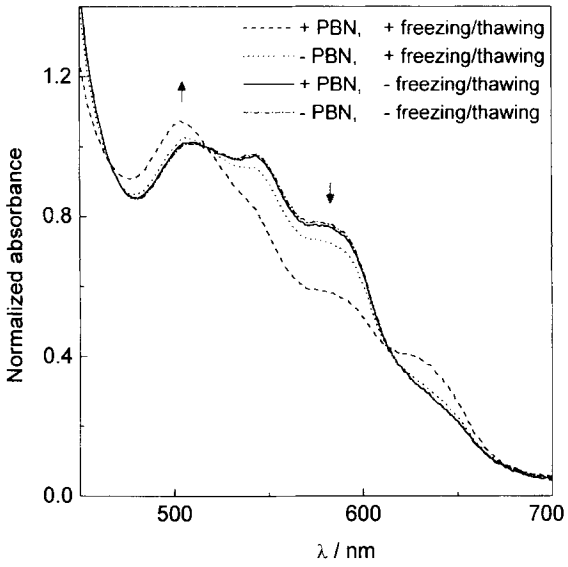

FIGURE 2 Visible spectra showing the effect of freezing and thawing solutions of MbFe(IV)=O. PBN solution or water was mixed with a 10 min old equimolar solution of MbFe(III) and hydrogen peroxide (pH 7.6). Phosphate buffer was added after 1 min giving pH = 5.5, [PBN] = 9.8 mM, [myoglobin] = 178 μM, [phosphate] = 30 mM and I = 0.16. Each solution (1.5 ml) was frozen in liquid nitrogen 1 min after addition of the buffer. After 2 min in liquid nitrogen, the frozen samples were thawed in cold water for 4 min, and the precipitate removed by centrifugation, and all samples were diluted with 5 mM phosphate buffer pH 7.6. Spectra of all four samples, recorded 14–16 min after PBN had been added, were normalized for comparison using the MbFe(III)/MbFe(IV)=O isosbestic point (517.5 nm) because of the difference in myoglobin concentrations in frozen and nonfrozen samples caused by precipitation (160 μM in nonfrozen samples and 80 and 150 μM in frozen samples with and without PBN, respectively).

Reaction of Ferrylmyoglobin with PBN During Freezing

The autoreduction of MbFe(IV)=O has been demonstrated to be accelerated during freezing of slightly acidified solutions of MbFe(IV)=O.[25] PBN is able to trap short-lived radicals as stable radical adducts, and the presence of PBN during the freezing therefore makes it possible to obtain further mechanistic information about the autoreduction at the low temperatures both from changes in rate and from ESR spectra of the trapped radicals.

The effect of PBN during the freezing of MbFe(IV)=O solutions was examined in a set of experiments, where an equimolar mixture of MbFe(III) and hydrogen peroxide after 10 min of reaction to ensure both the complete transformation into MbFe(IV)=O and the complete decay of $^{\bullet+}$MbFe(IV)=O was incubated at neutral pH with either PBN or water (control) for 1 min before acidification by addition of phosphate buffer (final pH = 5.5). One set of samples (with and without PBN), that was taken from the acidified solutions, was frozen in liquid nitrogen and subsequently thawed at room temperature, while another set was kept at room temperature. UV–VIS-spectra of all samples were recorded 15 min after the addition of PBN solution or of water as control (Figure 2). The presence of PBN had a negligible effect on the degree of conversion of MbFe(IV)=O to MbFe(III) in samples that had been kept at room temperature as may

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be seen from the spectra in Figure 2, confirming that the reaction between PBN and MbFe(IV)=O proceeds slowly at room temperature. The conversion of MbFe(IV)=O to MbFe(III) in the frozen and rethawed solution without PBN was on the other hand found to be more advanced, and the conversion was almost completed in the frozen and rethawed solution containing PBN. These results show that the process of freezing and thawing accelerates both the autoreduction of MbFe(IV)=O as well as the reaction between MbFe(IV)=O and PBN.

The influence of acidification and freezing upon the reactions between MbFe(IV)=O and PBN was further studied by ESR, since this technique makes it possible to detect paramagnetic reaction intermediates and products trapped in the frozen samples. A MbFe(IV)=O solution was made by incubating a 10 min old equimolar mixture of MbFe(III) and hydrogen peroxide (pH = 7.3) with PBN for 90 s at ambient temperature before freezing it in liquid nitrogen. The ESR spectrum of the frozen sample showed a weak broad ESR signal ($g = 2.005$) at 200 K shown for a total myoglobin concentration of 352 μM in Figure 3(a). Dilution of the MbFe(IV)=O–PBN mixture with buffer resulting in a myoglobin concentration equal to 235 μM and pH = 7.3 had no effect on the signal except for a lower intensity (Figure 3(b)). In contrast, dilution with an acidic buffer giving a final pH = 5.7 prior to freezing gave a frozen solution ESR spectrum with an intense anisotropic signal (Figure 3(c)). The spectrum is characteristic of an immobilized nitroxyl radical, i.e. it has the features of a powder spectrum of a radical with pseudo axial symmetry and a parallel nitrogen hyperfine coupling constant, $A_{\parallel}^{N}$, equal to 31.8 G.[15,28,29] The value of $A_{\parallel}^{N}$ is in agreement with a PBN-derived radical with the structure similar to a spin trapped radical adduct, **I**, whereas it is too large to originate from a PBN radical cation, **II**, which could have been formed by a direct one-electron transfer oxidation of PBN.[28]

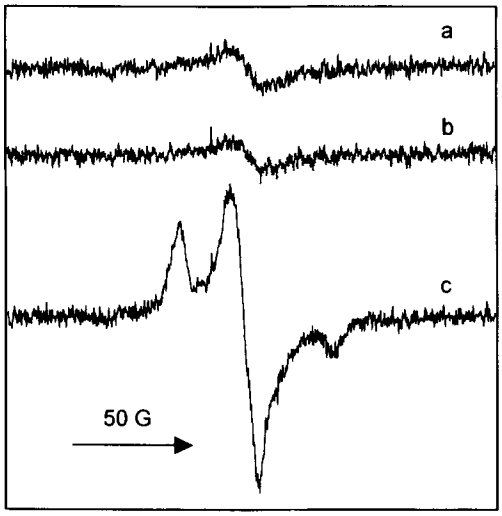
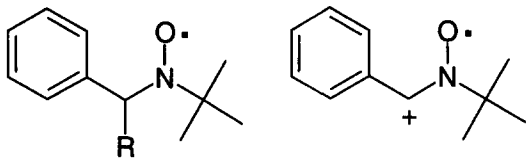


FIGURE 3 ESR spectra of frozen reaction mixtures of MbFe(IV)=O and PBN. (a) PBN was added 10 min after addition of hydrogen peroxide to MbFe(III), and a portion of the solution was frozen after 1 min 34 s; [myoglobin] = 352 μM, [PBN] = 15 mM, pH = 7.3. (b) After incubation 1 min with PBN, an aliquot of the solution in (a) was mixed with buffer giving pH = 7.3 and frozen after 34 s; [myoglobin] = 235 μM, [PBN] = 10 mM. (c) Like (b), except the added buffer gave pH = 5.7. The spectra were recorded at 200 K.



I **II**

Freezing acidified solutions of MbFe(IV)=O (pH below 7) has been shown to generate a radical with a sharp isotropic $g = 2.003$ ESR-signal, which otherwise has only been observed under anaerobic conditions at pH = 7.4.[25] The signal was not observed when PBN was added to the solutions in the present study, and PBN seems either to prevent the formation of the radical or the observed PBN-derived radical is a product of a reaction between the radical and PBN.

The PBN-derived radical, **I**, was not stable at room temperature. Only an extremely weak ESR-signal could be detected in the reaction mixtures

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of MbFe(IV)=O and PBN at room temperature after addition of the acidic buffer without freezing. It was likewise not possible to detect any ESR-signals at room temperature after thawing of the frozen samples, and subsequent freezing of the thawed samples in liquid nitrogen did not regenerate the ESR-signals at 200 K.

In a similar experiment the spin-trap 4-POBN was added to a 10 min old solution of MbFe(IV)=O before lowering the pH to 5.5 by addition of an acidic buffer solution and subsequent freezing in liquid nitrogen. The ESR spectrum of the frozen solution consisted of a very weak $g = 2.003$ signal, and the absence of the nitroxyl radical ESR spectrum indicated that 4-POBN in contrast to PBN is unable to either trap radicals or undergo any reactions with MbFe(IV)=O.

$^{\bullet+}$MbFe(IV)=O, which is formed immediately after mixing MbFe(III) with hydrogen peroxide, contains a protein-based radical and the possibility that this radical could be involved in the formation of the PBN-derived radical was further investigated by a series of experiments. Mixing MbFe(III) with hydrogen peroxide at neutral pH and quickly freezing it in liquid nitrogen has been reported to give an ESR spectrum with a low field line at $g = 2.03$. This ESR line has been assigned to an immobilized peroxyl radical located in the protein moiety.[15,30,31] Mixing the reagents at pH = 5.5 rather than at neutral pH considerably diminished the intensity of the ESR-signals observed after freezing.[25] The presence of PBN during the mixing of MbFe(III) and hydrogen peroxide resulted in the formation of the PBN-derived radical at both pH = 7.1 and 5.5 (Figure 4(a) and (b)), however, a $g = 2.03$ peroxyl line was also observed at pH = 7.1. Adding PBN to the reaction mixture after both the activation of MbFe(III) by hydrogen peroxide at pH = 7.3 and the lowering of the pH to 5.5 rather than having PBN present during the initial reaction between MbFe(III) and hydrogen peroxide gave only a slight increase in the intensity of the ESR-signals of the frozen samples, but had

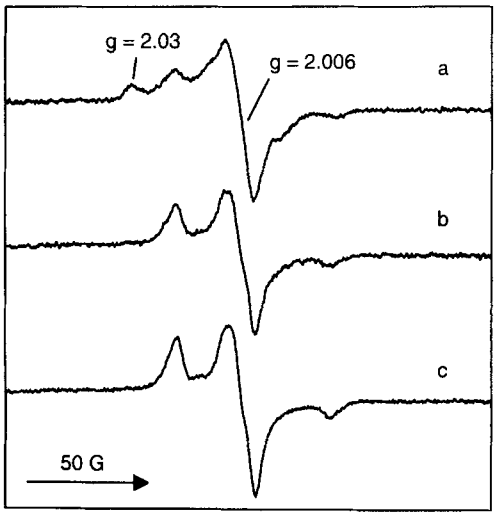

FIGURE 4 ESR spectra of reaction mixtures frozen 20 s after the addition of buffered solutions of hydrogen peroxide (final concentration 318 μM) to MbFe(III) (final concentration 318 μM). (a) PBN (10 mM) was present during the mixing; final pH = 7.1. (b) PBN (10 mM) was present during the mixing; final pH = 5.5. (c) MbFe(III) was incubated with hydrogen peroxide 15 min at pH = 7.3 before lowering the pH to 5.5. PBN (10 mM) was added to the mixture 150 s after the acidification and the sample was frozen 30 s later. The spectra were recorded at 200 K.

otherwise no effect on the appearance of the ESR spectra despite the completely different order of mixing the reagents (Figure 4(b) and (c)). The similarity between the two ESR spectra shows that PBN does not seem to interfere with the formation of the radical in $^{\bullet+}$MbFe(IV)=O, and $^{\bullet+}$MbFe(IV)=O, on the other hand, does not seem to affect the appearance of the ESR spectra of the frozen solution.

Furthermore, two experiments were performed where MbFe(III) and hydrogen peroxide were mixed at pH = 7.3. In one experiment PBN was added to the solution after 30 s while it was added after 10 min of reaction between MbFe(III) and hydrogen peroxide in the other (Figure 5). The pH of both solutions were then lowered to 5.3 10 min 30 s after the addition of hydrogen peroxide. The intensities of the ESR-signals of the PBN-derived radicals were virtually the same for mixtures of MbFe(III) and hydrogen peroxide irrespective of whether the PBN had been added

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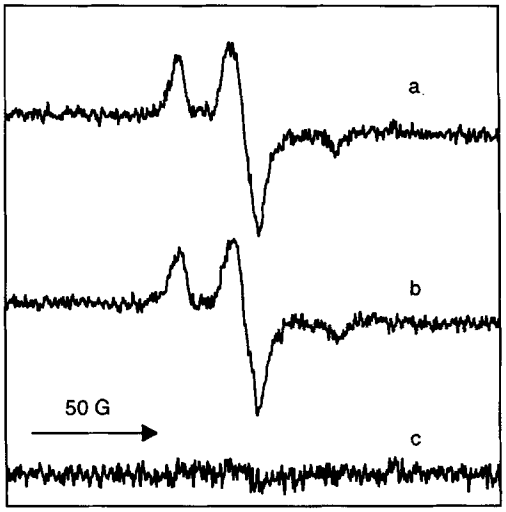

FIGURE 5 ESR spectra observed on addition of PBN at different times after the incubation of MbFe(III) with hydrogen peroxide. MbFe(III) was mixed with an equimolar amount of hydrogen peroxide at pH = 7.3. PBN was added either (a) 30 s or (b) 10 min later and both solutions were acidified by addition of a phthalate buffer 10 min 30 s after the mixing of MbFe(III) and hydrogen peroxide and were frozen in liquid nitrogen 1 min later. The final concentrations were: [myoglobin] = 58 μM and [PBN] = 6 mM and pH = 5.1. (c) Difference spectrum between (a) and (b).

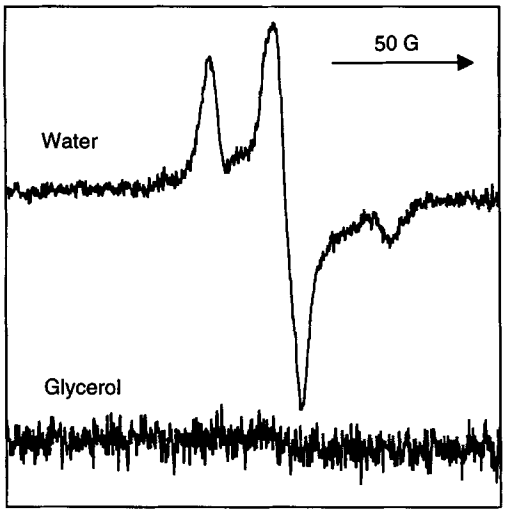

FIGURE 6 ESR spectra showing the effect of glycerol on the reaction between MbFe(IV)=O and PBN. MbFe(III) was mixed with an equimolar amount of hydrogen peroxide and subsequently with PBN, and to this mixture was added 10 min later either a glycerol buffer solution resulting in a total glycerol concentration of 50% vol., or a diluted buffer solution to give the same volume and final concentrations. Both samples were frozen in liquid nitrogen 30 s later. The final concentrations were: [myoglobin] = 117 μM, [PBN] = 5 mM, pH = 5.7 and ionic strength = 0.08. While the glycerol containing sample stayed homogeneous, the aqueous sample showed ice-formation.

either 30 s or 10 min after the addition of hydrogen peroxide, indicating that PBN does not react with the protein-derived $^{\bullet+}$MbFe(IV)=O radicals, since this type of intermediates decay within a few minutes and could not have been present in the sample to which PBN was added 10 min after the addition of hydrogen peroxide.[15,32]

The intensity of the ESR-signal was independent of the length of time from mixing the reagents until the freezing of the mixture, which in one experiment was varied from 25 s to 17 min without any notable effect on the ESR spectra.

These experiments demonstrate that the formation of the PBN-derived radical that is observed in the frozen solutions is independent of the order and timing of mixing the reactants before the freezing as long as the composition of the reaction mixtures is the same before the freezing. The observed PBN-derived radicals must consequently be formed during the freezing and not as a result of reactions occurring during the preparation of the reaction mixtures at ambient temperature.

No ESR-signals could be detected at 200 K when glycerol was added to the solutions (50% vol.) in order to avoid phase separations during the cooling of the sample in liquid nitrogen (Figure 6). A control experiment where an equivalent amount of water was added instead of glycerol gave the usual ESR-signal from the PBN-derived radical, proving that the absence of the signals in the glycerol experiment was not merely caused by a dilution effect. This set of experiments indicates that the phase separations that take place during freezing of the aqueous solutions are necessary for the formation of the PBN-derived radicals. An alternative explanation of the results is that glycerol is able to reduce MbFe(IV)=O and thereby prevent the formation of the PBN-derived radicals. This hypothesis was tested by an experiment where solutions of

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MbFe(IV)=O in either a 50% glycerol-water buffer at pH = 5.1 or in an aqueous buffer at pH = 5.1 were frozen and thawed. The UV–VIS spectra of the two thawed solutions were identical, demonstrating that the stability of MbFe(IV)=O was unaffected by the presence of glycerol during the freezing and thawing.

Further support for the formation of the PBN-derived radicals during freezing as a result of the phase transitions was obtained by using buffers that behave differently with respect to pH-changes during freezing. The intensities of the ESR spectra of the frozen samples increased as the reaction mixtures of MbFe(IV)=O and PBN were made more and more acidic, whereas the features of the ESR-signals and thus the type of PBN-derived radicals that was formed were unaffected (Figure 7). The intensity of the ESR-signal was found to depend on the type of buffer that was used for the acidification. Phosphate buffers gave more intense signals at a given pH value than phthalate buffers, which on the other hand gave larger signals than acetate buffers (data not shown). A similar pH and buffer dependence was observed when acidified solutions of MbFe(IV)=O were frozen without PBN, and the behavior can again be explained by the lowering of the pH that takes place during freezing of phosphate buffered solutions due to the precipitation of the monohydrogenphosphate ion as the sodium salt.[25]

Freezing the samples at different temperatures also affected the intensity of the ESR-signal from the PBN-derived radicals. The samples were frozen in cold baths with different temperatures for 200 s and were then transferred directly to the ESR cavity, where the temperature was kept at 200 K during the recording of all the ESR spectra (Figure 8). The intensities were found to increase with the temperature at which the samples were frozen. The highest intensity was reached at 249 K, whereas freezing at 264 K gave a lower intensity than for freezing at 249 K. However, the sample was not completely frozen at 264 K in contrast to the lower temperatures, and it remained a slurry

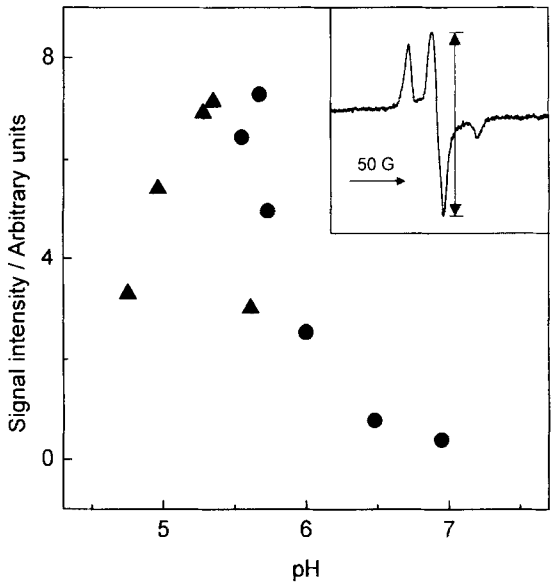

FIGURE 7 The pH dependence of the intensities of the ESR-signal from the PBN adduct observed in frozen mixtures of MbFe(III) incubated with hydrogen peroxide (1 : 1). PBN was added 15 min later and the pH of the solutions was lowered by addition of phthalate (triangles) or phosphate buffer (circles) 1 min after addition of PBN, and ESR samples were frozen 30 s later in liquid nitrogen; [myoglobin] = 145 μM, [PBN] = 9.9 mM. Inset: ESR spectrum measured from the solution with final pH = 5.3 (phthalate buffer) recorded at 200 K.

until it was transferred to the cavity where it became a solid. The increase of intensities seems to be the result of the slower rate of freezing as the temperature of the cold baths are increased resulting in a longer period of time where the system is only partly frozen and where the reactants are able to diffuse and react. The higher rates of reactions caused by the higher temperatures will also contribute to the higher intensities. However, the PBN-derived radicals are not stable in fluid solution as shown for ambient temperature (*vide supra*) and the decay of these compounds may have contributed to the lower amounts that was detected when the solution was cooled to 264 K and only partly frozen. In addition, the pH may not have decreased as much in the partly frozen solution as at the other lower temperatures where the samples had completely solidified. An additional peak was observed in the ESR spectra

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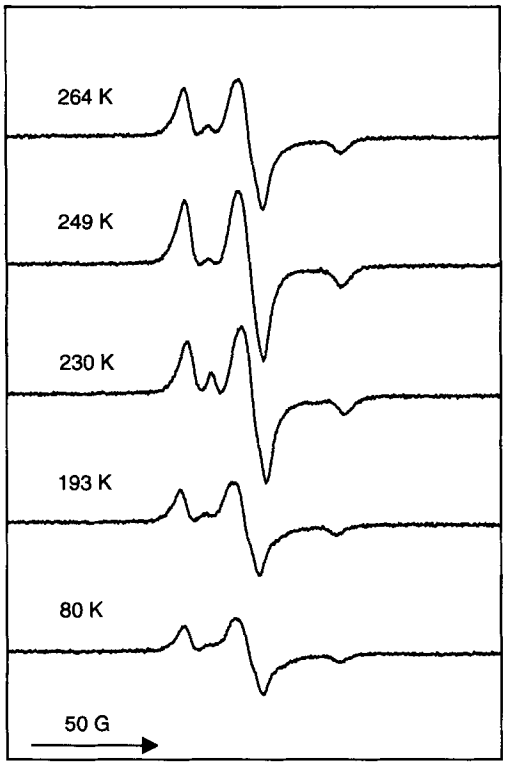

FIGURE 8 Effect of freezing temperature on ESR signal of PBN adduct in frozen mixtures of MbFe(IV)=O and PBN. MbFe(III) was mixed with an equimolar amount of hydrogen peroxide and left at room temperature and pH = 7.2 for 10 min before addition of PBN. After 1 min, pH was lowered to 5.8 by addition of phosphate buffer, and ESR samples were frozen 30 s later in cold baths with different temperatures. The frozen samples were transferred to the cavity after 200 s, and the spectra were recorded at 200 K. The final concentrations were: [myoglobin] = 235 μM and [PBN] = 10 mM.

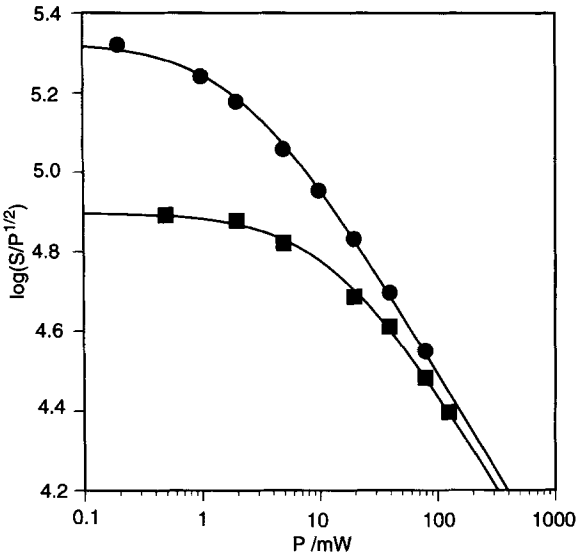

FIGURE 9 Microwave power saturation of ESR signals of frozen MbFe(IV)=O reaction mixtures. Normalized heights of ESR-signals obtained at different microwave powers for frozen solutions at $T = 200$ K. Circles: PBN adduct signal. MbFe(III) (209 μM) and an equimolar amount of hydrogen peroxide were allowed to react 10 min at pH = 7.5 before addition of 20 mM PBN. pH was lowered to 5.7 after 1 min and the solution was frozen after 30 s in liquid nitrogen. Squares: Sharp signal at $g = 2.003$. Myoglobin (195 μM) and an equimolar amount of hydrogen peroxide were allowed to react for 10 min at pH = 7.3 before pH was lowered to 5.8, and the solution was frozen 30 s later. The solid lines are calculated from Equation (1) (see text).

at a magnetic field approximately 15 G lower than the central peak. The origin of this signal is unknown, but the intensity of the peak reached a maximum in solutions frozen at 230 K which indicates that it may arise from an intermediate that is present only at this range of temperatures.

Saturation Experiments

The microwave saturation of the ESR signal from the PBN-derived radical was studied by monitoring the amplitude of the ESR signals as a function of the microwave power at 200 K. The sharp isotropic $g = 2.003$ signal that is observed in frozen samples of acidified MbFe(IV)=O solutions without PBN was also studied.[25] A plot of $\log(S/\sqrt{P})$, where S is the amplitude of the signals, against the microwave power, P, showed that the two signals were saturated at different levels of the microwave power (Figure 9). Both sets of data could be fitted to Equation (1), where $P_{1/2}$ is a saturation parameter, a is a constant that depends on the intensity of the unsaturated signal, and b is equal to 1 in the case of an inhomogenous broadening mechanism as in the present case:[33]

$$\log(S/\sqrt{P}) = a - \frac{b}{2} \cdot \log\left(1 + \frac{P}{P_{1/2}}\right). \qquad (1)$$

The parameter $P_{1/2}$ was found to be equal to 2 and 14 mW for the signal from the PBN-derived

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and the sharp $g = 2.003$ signal, respectively. This difference in the saturation behavior makes it unlikely that the PBN-derived radical, which is easily saturated, is closer to the iron-heme center than the $g = 2.003$ radical.

DISCUSSION

ESR studies involving $^{\bullet+}$MbFe(IV)=O have shown that it is able to oxidize proteins, amino acids and naturally occurring antioxidants, thereby transferring its radical character to the target molecule.[34–36] MbFe(IV)=O is one oxidation equivalent below $^{\bullet+}$MbFe(IV)=O and is usually not considered a radical, although a weak broad signal can be measured from solutions of MbFe(IV)=O as old as several hours by low-temperature ESR at neutral pH, together with a sharp signal at lower pH values.[25,37] The species that gives rise to the broad ESR signal ($g = 2.005$) seems to be a radical intermediate in a bimolecular autoreduction of MbFe(IV)=O:[13,25]

$$\text{MbFe(IV)=O} \rightleftharpoons {}^{\bullet+}\text{MbFe(III)}-\text{O}^{-} \qquad (2)$$

The intramolecular electron transfer from the protein to the iron center is further promoted by acid

$$\text{MbFe(IV)=O} + \text{H}^{+} \rightleftharpoons {}^{\bullet+}\text{MbFe(III)}-\text{OH} \qquad (3)$$

and $^{\bullet+}$MbFe(III)–OH may be an intermediate in the acid-catalyzed autoreduction and in the acid-promoted oxidation of other substrates.

We have in the present study demonstrated that MbFe(IV)=O in acidic solution reacts with the spin-trap PBN to yield a PBN-derived radical. At least two possibilities exist for this reaction: (i) direct trapping of a MbFe(IV)=O radical by PBN yielding a genuine radical adduct. It is in this respect noteworthy that the different results obtained with PBN and POBN may be a result of their different abilities to enter hydrophobic environments. POBN is considerably less hydrophobic than PBN and only the latter may thus be able to trap radicals located in hydrophobic parts of myoglobin.[38] (ii) PBN is oxidized by MbFe(IV)=O by one-electron transfer to a radical cation, **II**, that subsequently adds to nucleophiles that may be present in the vicinity yielding a compound similar to a genuine spin adduct, **I**, so-called "inverted spin trapping".[39,40] This mechanism may be less likely than direct spin trapping since no ESR-signals were observed when POBN was used instead of PBN, even though POBN is slightly easier to oxidize than PBN.[40] However, the latter mechanism cannot completely be ruled out since the hydrophilic nature of POBN may prevent it from entering the hydrophobic parts of myoglobin where an electron transfer could be limited to take place. The PBN-derived radical must in either case be located further away from the Fe-atom of myoglobin than the radical that gives rise to the sharp ESR-signal in the absence of PBN as judged by the microwave saturation behavior of the radical. It is thus unlikely that the PBN-derived radical is located in the heme-containing cleft of myoglobin.

Very similar activation parameters have been found for the reduction of MbFe(IV)=O in neutral solution by highly different substrates such as β-lactoglobulin, ascorbate, and thiocyanate, which has been explained by a common rate-determining intramolecular electron transfer taking place in the protein part of myoglobin (forward reaction of (2)) prior to the reaction with the substrate.[41] For reduction of MbFe(IV)=O by thiocyanate, a radical intermediate appears to be stabilized by binding of a thiocyanate ion in a charge transfer complex.[41] The reduction of MbFe(IV)=O by PBN was too slow to be studied reliably at neutral pH at room temperature, but lowering the pH accelerated the reaction, presumably as a consequence of the acid-catalyzed intramolecular electron transfer (3). Formation of the PBN-derived radical from reaction of MbFe(IV)=O with PBN was affected by the process of freezing as was also

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seen for autoreduction of MbFe(IV)=O.[25] This effect is probably important at acidic pH only and is most likely caused by the lowering of the pH of phosphate buffers due to freezing, an effect which is known to be much smaller at neutral pH than at lower pH-values.[42] UV–VIS spectroscopy and magnetic circular dichroism of the acidic form of MbFe(IV)=O at low pH in low-temperature glasses have shown that under these conditions it closely resembles horseradish peroxidase Compound II and the higher oxidation states of other peroxidases, confirming its oxidative capacity.[43,44]

Notably, the pH of meat is in the range 5.4–7.4,[45] and myoglobin is present in concentrations up to 7 mg/g wet weight.[46] For such food systems, proteins and lipids are possible substrates that may undergo similar oxidations as PBN with MbFe(IV)=O, and surprisingly long-lived protein radicals have indeed been detected following reduction of MbFe(IV)=O by proteins.[34]

Under experimental conditions, ferrylhemoglobin has been detected in frozen samples of whole blood as a reaction product of methemoglobin and hydrogen peroxide, both formed from forced autoxidation of oxyhemoglobin.[47,48] It is possible that generation of hypervalent myoglobin by similar reactions upon freezing of meat, coupled with enhanced reactivity due to the freezing process as demonstrated here for oxidation of PBN by MbFe(IV)=O, could lead to oxidative stress in frozen meat and meat products. However, such speculation needs further investigation in order to be confirmed, but it is clear that addition of phosphates to certain meat products could enhance such effects by introducing pH-changes and phase transitions at low temperature, which could lead to oxidative cross-linking of proteins.

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