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Acid-catalysed Autoreduction of Ferrylmyoglobin in Aqueous Solution Studied by Freeze Quenching and ESR Spectroscopy

MAIKEN V. KRÖGER-OHLSEN, MOGENS L. ANDERSEN and LEIF H. SKIBSTED*

Food Chemistry, Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

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Decay of the hypervalent muscle pigment ferrylmyoglobin, formed by activation of metmyoglobin by hydrogen peroxide, was found, when studied by a combination of ESR and UV/VIS spectroscopy in aqueous solution at physiological pH, to proceed by parallel second- and first-order kinetics. At pH below 6.5 a sharp ESR signal ($g = 2.003$) with an increasing intensity for decreasing pH were observed in solutions frozen in liquid nitrogen, and a broad signal ($g = 2.005$) was seen throughout the studied pH range also in frozen solutions. The $g = 2.005$ signal is suggested to arise from an intermediate formed in an intramolecular rate-determining electron-transfer in ferrylmyoglobin, whereas the $g = 2.003$ signal is caused by a radical formed in a proton-assisted electron-transfer initiating the specific acid-catalysed autoreduction.

Keywords: Ferrylmyoglobin, autoreduction, ESR, freezing, protein radicals, pH-dependence

INTRODUCTION

The reaction between metmyoglobin (MbFe(III)) and hydrogen peroxide generates the two hypervalent species perferrylmyoglobin ($^{\bullet}$MbFe(IV)=O) and ferrylmyoglobin (MbFe(IV)=O).[1–3] They both retain one oxidation equivalent from hydrogen peroxide at the Fe(IV)=O centre, and in perferrylmyoglobin the second oxidation equivalent is present as a protein-based radical. Perferrylmyoglobin decays to ferrylmyoglobin in about 30 s by a mechanism that is still unresolved,[4] and ferrylmyoglobin decays to metmyoglobin by a slower autoreduction reaction in which the protein moiety becomes slightly modified.[5–7] Autoreduction of ferrylmyoglobin is specific acid-catalysed and has at 25°C a half-life of 2.5 min at pH 5 compared to a half-life of 75 min at pH 7.[7] The autoreduction involves oxidation of amino acid residues in the protein and crosslinking between the porphyrin ring and the protein moiety, although the exact pathways have not been unravelled.[5,6,8,9] Fenwick *et al.* found a surprisingly slow rate of intramolecular electron-transfer to the oxoferryl

* Corresponding author. Tel.: +4535283221. Fax: +4535283344. E-mail: ls@kvl.dk.

group in ferrylmyoglobin and protonization of the ferryl oxygen seems to assist the electron-transfer.[10]

Protein-based radicals have been detected by ESR upon activation of metmyoglobin with peroxides. Tyrosine phenoxyl radicals have been identified at room temperature,[11–14] and spin trapping experiments have indicated the formation of radicals centred on tryptophan,[15] tyrosine,[4,16] and valine.[16] Freeze quenching of reaction mixtures has been used to trap short-lived radicals that are present immediately after the activation of metmyoglobin with hydrogen peroxide. This approach has led to observation of peroxyl radicals in the frozen samples by ESR as a peak with $g = 2.03$,[17–19] and it has been suggested that the peroxyl radical is formed upon addition of oxygen to a radical centred on the C-3 carbon atom of tryptophan.[20] The existence of peroxyl radicals has also been linked to a weak signal with $g = 2.014$ which was observed in a room temperature continuous-flow study.[14] A sharp isotropic signal with $g = 2.003$ has been observed in frozen samples obtained under anaerobic conditions and it has been assigned to the precursor of the peroxyl radical.[19] The $g = 2.03$ and the $g = 2.003$ lines have been observed with recombinant sperm whale myoglobins where all the tyrosines were absent, suggesting that tyrosine radicals are not the source of these ESR signals.[21]

The oxidative behaviour of myoglobin *in vivo* has been the main impetus of the majority of ESR studies and pH of the reaction mixtures has therefore usually been around the physiological value of 7.4. We have undertaken the present ESR studies in order to extend these studies to the lower pH values that are relevant to meat products in which myoglobin may play a role in lipid oxidation. In order to mimic conditions prevailing during the post-mortem pH-decrease in meat, phosphate buffers were used and compared to phthalate buffers which has a different freezing behaviour.

MATERIALS AND METHODS

Materials

Metmyoglobin (MbFe(III), horse heart, type III) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). H_2O_2 (35%) was from Riedel-de Haën (Seelze, Germany). HCl (0.0200 M) was obtained from Bie & Berntsen Laboratory (Bie & Berntsen A/S, Rødovre, Denmark). All other reagents (analytical grade) were from Merck (Darmstadt, Germany). Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA, USA).

Synthesis of Ferrylmyoglobin

MbFe(III) dissolved in aqueous 5 mM sodium phosphate buffer 0.15 M NaCl (pH 7.5) was purified by elution on a Sephadex G25 column (40 × 25 cm) (Pharmacia Biotech AB, Uppsala, Sweden), and the concentration of the purified MbFe(III) was determined spectrophotometrically after appropriate dilution. MbFe(III) was allowed to react at room temperature with hydrogen peroxide added at a molar ratio of 1 : 1 using a 7.6 mM stock solution. Unless otherwise stated, the reaction mixture was left at room temperature for 10 min in order to allow the decay of perferrylmyoglobin before further use. Prior to freezing in liquid nitrogen, the total concentrations of buffers were 33 mM and the ionic strength was 0.16 (adjusted with NaCl).

pH Measurements

pH was measured relative to concentration standards (0.0100 and 0.00100 M HCl, ionic strength 0.16 adjusted with NaCl), employing the definition $pH = -\log [H^+]$. pH was measured with a Hamilton 640.238-100 combination glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) connected to a Metrohm 713 pH-meter (Metrohm, Herisau, Switzerland).

ESR Spectroscopy

The ESR measurements were carried out using a Bruker ECS-106 spectrometer equipped with a Bruker ER 4103 TM cavity (Bruker, Rheinstetten, Germany). The temperature of the ESR cell inside the cavity was controlled by a Bruker ER 4112 HV continuous-flow liquid nitrogen cryostat. A flat quartz aqueous cell (Wilmad Glass, Buena, NJ, USA) was used for the room temperature experiments. The microwave frequency was measured with a HP 5350B Microwave Frequency Counter (Hewlett-Packard, Palo Alto, CA, USA). The modulation frequency and amplitude were 100 kHz and 1.0 G, respectively.

UV/VIS Absorption Measurements

A HP 8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA) was used for all measurements. The concentration of MbFe(III) was determined using $\varepsilon_{525} = 7700\,M^{-1}\cdot cm^{-1}$,[22] and the concentration of MbFe(IV)=O was calculated according to Miller *et al.*[23] In the kinetic experiments, MbFe(III) was mixed with hydrogen peroxide (1 : 1) and allowed to react for 3 min before collection of data. In the initial-rate determining experiments, the concentration of MbFe(IV)=O was varied between 17 and 130 μM (25.0°C, pH = 7.37, $I = 0.16$, 0.02 M phosphate buffer), and data were collected with intervals of 20 and 30 s in two experiments. In experiments with different amounts of MbFe(III) added to MbFe(IV)=O, [MbFe(IV)=O] was 20 μM and the added amount of MbFe(III) varied between 5 and 60 μM (25.0°C, pH = 7.36, $I = 0.16$, 0.02 M phosphate buffer).

RESULTS

Kinetic Experiments

Autoreduction of ferrylmyoglobin at pH = 7.4 is a slow process associated with subsequent protein denaturation and finally precipitation, and in contrast to the specific acid catalysed autoreduction which takes place at lower pH values,[7] the reaction was found not to follow first-order kinetics as evidenced by a poor fit to a first-order expression and by the fact the fitted first-order rate constants consistently were found to depend on the initial concentration of ferrylmyoglobin (data not shown). From measurements of the initial reaction rate made during the first 5–8 min of the reaction, the reaction order was found to be 1.57 ± 0.03 (Figure 1). This is in agreement with the autoreduction reaction proceeding as a second-order reaction in parallel with a first-order reaction, the latter becoming more important as the concentration of ferrylmyoglobin decrease during the reaction. Changing the concentration of metmyoglobin in the presence of ferrylmyoglobin did not influence the rate of ferrylmyoglobin reduction (Figure 2, inset). The first-order rate constant, k_1, was found to be $(1.2 \pm 0.1) \cdot 10^{-5}\,s^{-1}$

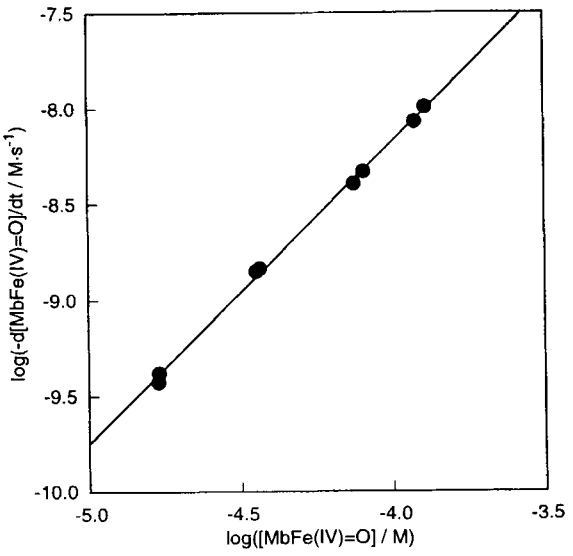

FIGURE 1 A double-logarithmic plot of the initial reaction rate of ferrylmyoglobin autoreduction, d[MbFe(IV)=O]/dt, versus the initial ferrylmyoglobin concentration (25.0°C, pH = 7.37, $I = 0.16$, 0.02 M phosphate buffer). The slope of the linear regression line is 1.57 ± 0.03, indicating a mixed reaction order with respect to ferrylmyoglobin.

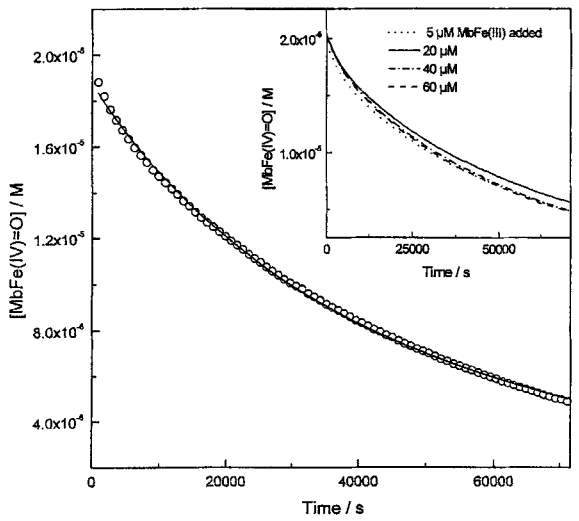

FIGURE 2 Autoreduction of ferrylmyoglobin at 25.0°C, pH = 7.36, $I = 0.16$ (○). Full line: Fit of the expression $[MbFe(IV)=O]_t = k_1 \cdot [MbFe(IV)=O]_0 \cdot \exp(-k_1 \cdot t)/(k_1 + 2 \cdot k_2 \cdot [MbFe(IV)=O]_0 \cdot (1-\exp(-k_1 \cdot t)))$ corresponding to a first-order reaction in parallel with a second-order reaction, giving $k_1 = (1.2 \pm 0.1) \cdot 10^{-5}\,s^{-1}$ and $k_2 = 0.34 \pm 0.01\,M^{-1} \cdot s^{-1}$. Insert: Autoreduction of ferrylmyoglobin in presence of different amounts of metmyoglobin (25.0°C, pH = 7.36, $I = 0.16$, 0.02 M phosphate buffer).

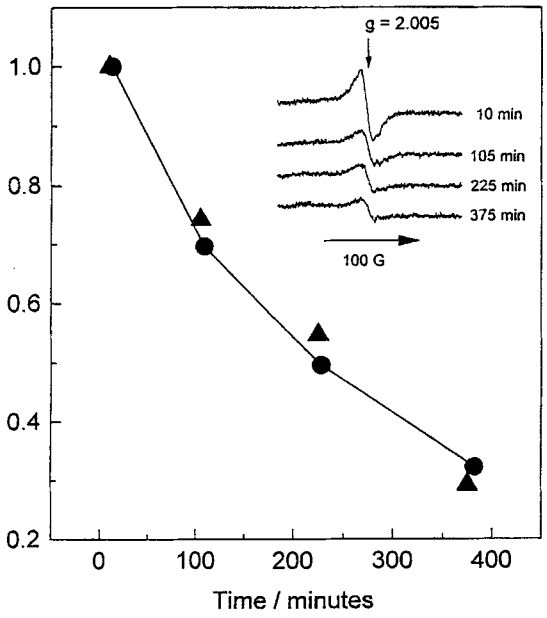

FIGURE 3 The time dependence of (●) the UV/VIS absorption at 580 nm of a 850 µM solution of myoglobin after addition of an equimolar amount of hydrogen peroxide to metmyoglobin at room temperature and pH = 7.09 (5 mM sodium phosphate buffer), and (▲) the double-integrated area of ESR spectra obtained at 200 K of aliquots taken from the same solution and immediately frozen in liquid nitrogen. The initial points (obtained 10 min after the addition of hydrogen peroxide) of both sets of data have been normalised to unity. The initial UV/VIS absorption corresponded to 75% of the myoglobin being present in the form of MbFe(IV)=O, as calculated according to Miller *et al.*[23] The ESR spectra recorded at $T = 200$ K are shown in the insert.

and the second-order rate constant, k_2, was equal to $0.33 \pm 0.01\,M^{-1} \cdot s^{-1}$ as determined from fits of Eq. (1) to data recorded at 25.0°C, pH = 7.36, $I = 0.16$ (Figure 2):[24]

$$[MbFe(IV)=O]_t = k_1 \cdot [MbFe(IV)=O]_0 \cdot \exp(-k_1 t)/\{k_1 + 2k_2 \cdot [MbFe(IV)=O]_0 \cdot (1 - \exp(-k_1 t))\} \quad (1)$$

ESR Experiments

An aqueous solution of metmyoglobin was mixed with an equivalent amount of hydrogen peroxide at pH = 7.09 and left at room temperature for 10 min before the solution was frozen in liquid nitrogen. The ESR spectrum was subsequently recorded at 200 K and a broad signal ($g = 2.005$, approximately 14 G peak to peak width) was observed (Figure 3, insert). Similar ESR-spectra, which are believed to be due to a long lived myoglobin protein radical, have previously been reported.[18,25] Albeit weak, the ESR signal could be detected in frozen samples of mixtures of metmyoglobin and hydrogen peroxide that had been left at room temperature for as long as 6 h before freezing. Furthermore, an experiment where the ferrylmyoglobin concentration in a mixture of metmyoglobin and hydrogen peroxide was followed with time by monitoring the UV/VIS absorption at 580 nm, and where aliquots were taken out from the same solution at different intervals and immediately frozen in liquid nitrogen, showed that the ferrylmyoglobin concentration and the intensity of $g = 2.005$ ESR signal from the frozen samples decreased in parallel with time (Figure 3).

A small ESR peak at $g = 2.03$ and a sharp ESR signal with $g = 2.003$ (5 G peak to peak width) on top of the broad $g = 2.005$ signal appeared when a ferrylmyoglobin solution (generated from metmyoglobin that had been allowed to react with hydrogen peroxide for 10 min in a neutral phosphate buffer solution (pH = 7.1) at room temperature) was acidified and subsequently frozen in liquid nitrogen (Figure 4). The acidification was achieved by mixing the ferrylmyoglobin solution with a 30 mM acidic buffer resulting in a total concentration of buffers of 33 mM. The same ESR signals were also observed upon acidifying and freezing a 6-h old mixture of metmyoglobin and hydrogen peroxide that had been kept at pH = 7.1 at room temperature, although, in this experiment the intensities of the signals were weak. Increasing the time between the acidification and the freezing of the ferrylmyoglobin solution led to a decrease in the intensity of the $g = 2.003$ ESR signal (Figure 4).

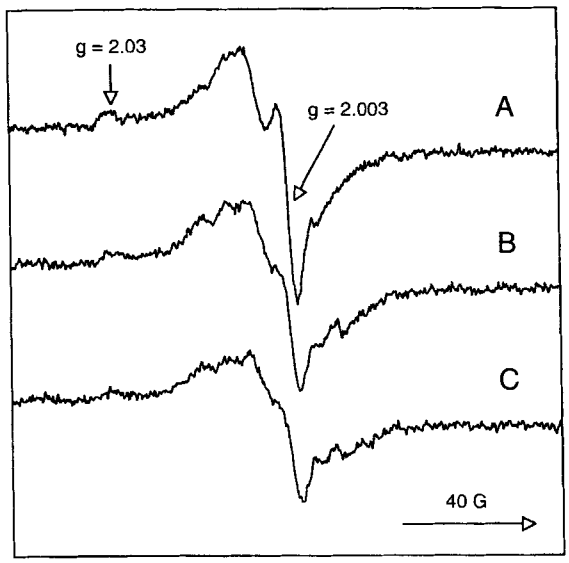

FIGURE 4 ESR spectra obtained at 200 K of frozen samples of acidified ferrylmyoglobin solutions. Equimolar amounts of metmyoglobin (570 μM) and hydrogen peroxide were mixed in a phosphate buffer with pH = 7.1 at room temperature. The pH was changed to 5.5 after 10 min by addition of an acidic phosphate buffer resulting in a total phosphate buffer concentration equal to 30 mM. Samples were taken from the acidified solution and frozen in liquid nitrogen after 30 s (A), 300 s (B) and 720 s (C).

The intensities of both signals were not only dependent on the final pH of the solution, but also on the type of buffer used for the acidification. Phosphate and phthalate buffers both gave rise to an increase in the intensity of $g = 2.003$ signal as the pH of the final solutions was made more acidic. However, a discontinuity was observed in the height of the signal when switching from phthalate to phosphate buffers (cf. Figure 5). The height of the broad signal at $g = 2.005$ increased by lowering the final pH in the case of phosphate buffers, but decreased when phthalate buffers were used to lower the pH. However, only a small discontinuity was observed in the pH for the phthalate buffers compared to the phosphate buffers.

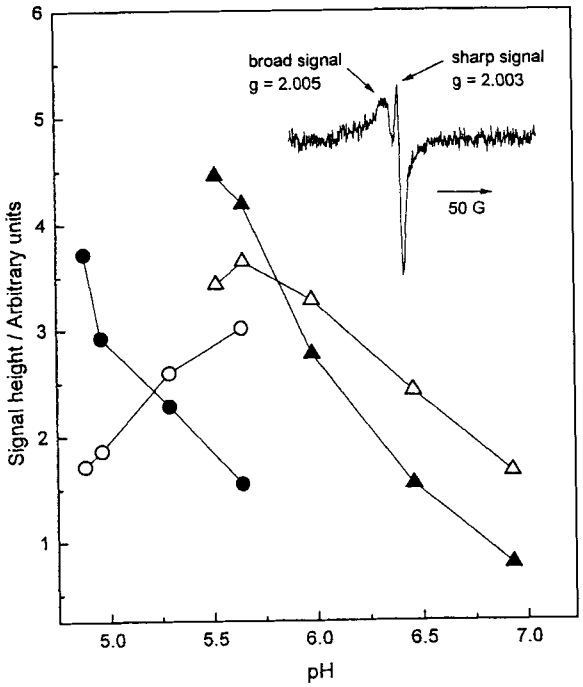

FIGURE 5 pH dependence of signal heights measured in acidified and frozen solutions of ferrylmyoglobin. Myoglobin (548 μM) and an equimolar amount of hydrogen peroxide were incubated at room temperature and pH = 7.07 for 15 min before addition of phthalate (circles) or phosphate buffers (triangles) and frozen 20 s later. The pH values were measured in the final solutions at room temperature. Open symbols: broad signal ($g = 2.005$), closed symbols: sharp signal ($g = 2.003$). Insert: ESR spectrum measured from the solution with final pH = 4.88 recorded at 200 K.

No ESR signals could be observed in acidified solutions of ferrylmyoglobin when the ESR measurements were carried out at room temperature even after only 90 s after addition of acidic phosphate buffer to the ferrylmyoglobin solution resulting in a final pH = 5.57, for which the half-life of ferrylmyoglobin is 14 min.[7] However, both the broad $g = 2.005$ signal and the sharp $g = 2.003$ signal were observed at 200 K in samples taken from the same solution and frozen in liquid nitrogen 80 s after the beginning of the recording of the room temperature ESR spectrum.

Mixing metmyoglobin and hydrogen peroxide at pH = 7.1 followed by freezing in liquid nitrogen after 20 s gave an ESR spectrum (Figure 5) similar to what has been observed previously with a $g = 2.03$ line normally assigned to the presence of a peroxyl radical.[18–20,25] Activation of myoglobin with hydrogen peroxide at pH = 5.5, followed by immediate freezing resulted in an overall decrease in the intensity of the ESR spectrum compared to pH = 7.1, but a weak shoulder at $g = 2.003$ appeared on top of the $g = 2.008$ signal (Figure 6). A weak $g = 2.03$ line was also present.

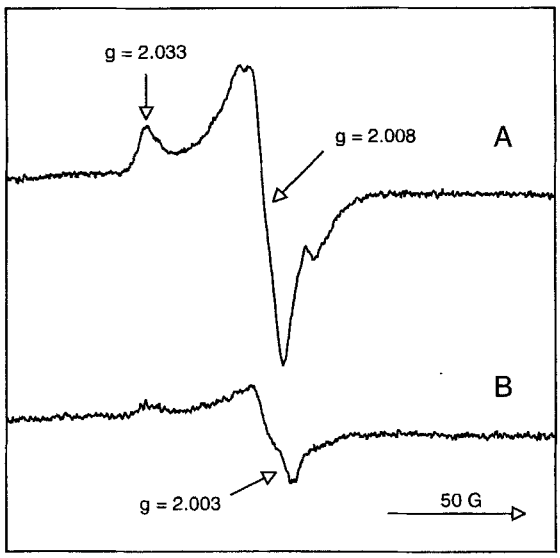

FIGURE 6 ESR spectra of reaction mixtures frozen in liquid nitrogen 20 s after mixing equimolar amounts of metmyoglobin (318 μM) and hydrogen peroxide. The pH of the metmyoglobin solution before mixing was 7.1, and the hydrogen peroxide was added as phosphate buffered solutions resulting in pH of the final reaction mixture of 7.1 (A) and 5.5 (B). The spectra were recorded at $T = 200$ K, microwave frequency: 9.403 GHz.

DISCUSSION

Ferrylmyoglobin is unstable and reacts in a slow process which has been termed "autoreduction" leading to the formation of "slightly modified metmyoglobin".[5,7,26] The reaction order equal to 1.57 in ferrylmyoglobin, as was determined in the present study from the initial rates of autoreduction at pH = 7.4, indicates that the reaction takes place via parallel reaction-paths, that are first- and second-order in ferrylmyoglobin. Further support for such parallel first- and second-order reactions during the autoreduction of ferrylmyoglobin in neutral solution has also been reported by Giulivi and Cadenas, who found that the oxoferryl UV/VIS absorption decayed by pseudo-first-order kinetics with a rate constant equal to $2.2 \cdot 10^{-5}\,s^{-1}$ at pH = 7.4 and 25°C, while a long lived broad ESR signal with $g = 2.0048$ observed at room temperature after the activation of high concentrations (up to 20 mM) of metmyoglobin with hydrogen peroxide, decayed by a reaction that was second-order in the radical concentration with a rate constant equal to $1.7\,M^{-1} \cdot s^{-1}$.[14] The long-lived radical was assigned to an aromatic amino acid radical (phenylalanine or histidine) in close proximity to the heme group. It is noteworthy in this respect, that a weak relatively stable radical ($g = 2.0056$) has also been observed in a flow system upon mixing hydrogen peroxide with modified metmyoglobin in which all the tyrosine residues had been acetylated, whereas the unmodified metmyoglobin gave the ESR signal of a short-lived tyrosine radical.[27]

Freezing the ferrylmyoglobin solutions at pH = 7.4 generated a weak broad ESR signal at $g = 2.005$. The intensity of this signal decreased between the frozen samples with the same rate as ferrylmyoglobin in the solution at the time of freezing, an observation which strongly suggests

that ferrylmyoglobin is the precursor of the radical that gives rise to the $g = 2.005$ signal. Although ferrylmyoglobin itself is not a free radical, a weak $g = 2.005$ ESR signal from solutions of ferrylmyoglobin has been observed up to 90 min after the reaction of MbFe(III) with hydrogen peroxide,[18,25] and it was possible in the present investigation to detect the signal for up to 6 h after the activation of myoglobin at pH = 7.09. It seems likely that this radical and the room temperature long-lived radical reported by Giulivi and Cadenas[14] are identical, based on their similar g-values and the fact, that both radicals can be detected surprisingly long time after the activation of metmyoglobin with hydrogen peroxide.

Lowering the pH of the ferrylmyoglobin solutions to 5.5 followed by immediate freezing in liquid nitrogen gave rise to a sharp $g = 2.003$ signal and a weak signal at $g = 2.03$, where the latter signal is a typical indication of the presence of a peroxyl radical. The observation of the $g = 2.003$ signal is remarkable, since a similar sharp isotropic signal with an identical g-value has only been observed when samples of metmyoglobin were freeze-quenched within microseconds after the activation with hydrogen peroxide at pH = 7.4, or when the activation was performed under anaerobic conditions. The signal has been assigned to a tertiary carbon-centered radical, most likely at the C-3 carbon of the indole ring in tryptophan-14, and these C-centered radicals have been shown to decay quickly in the presence of oxygen yielding peroxyl radicals, as indicated by the development of the $g = 2.03$ ESR lines.[15,19,20] Both the $g = 2.003$ and 2.03 signals are usually observed immediately after the activation of myoglobin with hydrogen peroxide and have therefore been associated with perferrylmyoglobin.[17,19,28]

The intensity of the $g = 2.003$ signal decreased as the time between the acidification of the solutions of ferrylmyoglobin and the freezing was increased. The first-order rate constant for the autoreduction of ferrylmyoglobin is $\sim 10^{-3}$ s at pH = 5.5 (25°C) resulting in a half-life of ~ 700 s.[7] This value agrees excellently with approximately 50% loss in the intensity of the $g = 2.003$ signal that was observed by increasing the time from 30 to 720 s between the acidification and the freezing in the experiment of Figure 4.

It seems possible that the $g = 2.003$ signal is due to an intermediate C-centered radical formed during the acid catalysed first-order decay of ferrylmyoglobin and that lowering the pH of the solutions leads to an increased rate of formation and thus a higher steady state concentration of the radical at room temperature. But such radicals are expected to be quenched by oxygen under aerobic conditions as in the present study preventing their detection by ESR.[19] It is therefore likely that a conformational change of the myoglobin–protein structure takes place at low pH, which makes the amino acid radical that gives rise to this signal less accessible to oxygen and thus hinders the formation of peroxyl radicals. Lower diffusion rates of oxygen in the solid state matrix may further hamper the transformation of the C-centered radical to a peroxyl radical.

The intensity of the $g = 2.003$ signal were found to increase upon lowering pH of the solution, and the effect was strongly dependent on the type of buffer used for the acidification. This behaviour is not unexpected, since the pH and the salt concentration of phosphate buffers that contain sodium, potassium, and chloride ions are known to change during freezing. Initially, pure ice precipitates leading to an increase in the salt concentration, followed by precipitation of $Na_2HPO_4 \cdot 12H_2O$ resulting in a significant lowering of the pH.[29–31] It is unknown whether ferrylmyoglobin precipitates before the buffer is completely frozen or remains trapped inside amorphous domains with high salt concentrations formed during the rapid liquid nitrogen cooling, but it seems likely that in both cases conformational changes will take place as it has been reported for carbonyl-myoglobin frozen in phosphate buffers.[32] The pH of phthalate buffers are on the other hand not expected to change significantly during freezing.[33]

However, stabilisation of the $g = 2.003$ radical by conformational changes of the protein structure at low pH at room temperature may not be the only factor that leads to a high intensity of the ESR signal in the frozen samples. Activation of metmyoglobin with hydrogen peroxide at pH = 5.5 at room temperature followed by freezing after 20 s gave only a very weak $g = 2.003$ signal (Figure 6), even though the radical is known to be formed during the activation process (*vide supra*). Furthermore, the dependency of the observed intensity of the $g = 2.003$ signal on the buffer type which is a result of their different freezing behaviour, indicates that some or all of the observed radicals must be formed at the low temperatures when the solution freezes. Formation of protein-based free radicals of ferrylhemoglobin induced by freezing has been reported by Svistunenko *et al.* who found that a fluctuating amount of the globin-based free radicals could be detected by ESR upon freezing human blood samples.[34] The fluctuations in concentrations were explained by the formation of hydrogen peroxide upon dismutation of superoxide released from oxyhemoglobin during the phase separations that take place during the freezing. It is unlikely that a similar mechanism explains our results, since (i) no oxymyoglobin was present in the metmyoglobin solutions used for the present experiments, and (ii) the concentration of hydrogen peroxide is expected to be negligible since it will be quenched by the metmyoglobin always present when equimolar amounts of hydrogen peroxide and metmyoglobin are mixed.[6,35]

Our results altogether suggest that the autoreduction of ferrylmyoglobin at pH = 7.4 proceeds along two parallel pathways, one resulting in first-order and the other in second-order kinetics. The broad $g = 2.005$ ESR signal most likely arise from an intermediate that is part of the bimolecular pathway in which one molecule of ferrylmyoglobin appears to oxidise another molecule to a species (two-electron-oxidised-MbFe(III)) in which the protein moiety has undergone a two electron oxidation, Eq. (2). The latter myoglobin species is very likely either a precursor to or it may be identical to the "slightly modified metmyoglobin".[5,14]

$$2\text{MbFe(IV)=O} + 2\text{H}^+ \rightarrow \text{MbFe(III)} + \text{two-electron-oxidised-MbFe(III)} + \text{H}_2\text{O} \quad (2)$$

The rate constant for this second-order reaction, $0.33\,\text{M}^{-1}\cdot\text{s}^{-1}$ at 25°C and pH = 7.4, is similar to that for reduction of ferrylmyoglobin by β-lactoglobulin ($1.24\,\text{M}^{-1}\cdot\text{s}^{-1}$),[36] and ascorbate ($2.7 \pm 0.8\,\text{M}^{-1}\cdot\text{s}^{-1}$),[37] and to the second-order rate constant for the decay of a ferrylmyoglobin amino acid radical ($1.7\,\text{M}^{-1}\cdot\text{s}^{-1}$).[14] A common intramolecular reaction in ferrylmyoglobin may be rate-determining. Second-order kinetics will be observed provided that the reverse reaction of Eq. (3) is fast and competing with a fast reaction with the reductant, Eq. (4).

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

$${}^{\bullet+}\text{MbFe(III)}-\text{O}^- + \text{reductant} + 2\text{H}^+ \rightarrow \text{MbFe(III)} + \text{oxidised reductant} + \text{H}_2\text{O} \quad (4)$$

While ${}^{\bullet+}\text{MbFe(III)}-\text{O}^-$ or an intermediate derived hereof is seen as the broad $g = 2.005$ ESR signal, the sharp $g = 2.003$ ESR signal seen at lower pH could be an intermediate in a specific acid-catalysed process in agreement with the findings of Fenwick *et al.*, Eq. (5):[10]

$$\text{H}^+ + \text{MbFe(IV)=O} \rightarrow {}^{\bullet+}\text{MbFe(III)}-\text{OH} \quad (5)$$

As for the first-order reaction at neutral pH, apparently an intramolecular reaction for ${}^{\bullet+}\text{MbFe(III)}-\text{O}^-$ or an intermediate derived hereof competes with the reaction of Eq. (4) to yield the oxidatively modified metmyoglobin. In conclusion, we have detected certain radicals as intermediates in both the non-catalysed and the acid-catalysed autoreduction of ferrylmyoglobin, which may help to further elucidate the detailed mechanism for this reaction that is important during oxidative stress.

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