Prooxidative Activity of Myoglobin Species in Linoleic Acid Emulsions

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Initiation of lipid peroxidation in oil-in-water linoleic acid emulsions catalyzed by metmyoglobin (MbFe(III)), metmyoglobin/oxymyoglobin (MbFe(III)/MbFe(II)O_2), ferrylmyoglobin (MbFe(IV)═O), and perferrylmyoglobin (•MbFe(IV)═O) was investigated in the pH area of interest for meat and meat products (pH 5.5−6.5). MbFe(III), MbFe(IV), and MbFe(III)/MbFe(II)O_2 showed similar lipid hydroperoxide dependent catalytic efficiency in initiating lipid peroxidation. The presence of lipid hydroperoxides, in heme protein-initiated lipid peroxidation, was found to be the crucial factor rather than the redox state of heme proteins. In contrast, •MbFe(IV)═O, formed in situ by reaction of MbFe(III) with H_2O_2, was an ineffective catalyst, as it undergoes intramolecular electron transfer resulting in heme globin cross-linkage to yield a green iron(III) heme pigment rather than reacting with the lipids.

Keywords: *Metmyoglobin; ferrylmyoglobin; perferrylmyoglobin; heme-protein degradation; lipid peroxidation*

INTRODUCTION

Lipid peroxidation is responsible for quality deterioration of many foods and development of rancidity often limits shelf-life (Labuza, 1971; Sanders, 1989). Oxidation of unsaturated fatty acids involves complex free radical processes, and the exact nature of the catalysts initiating lipid oxidation, as well as the reaction mechanisms involved, has yet to be fully elucidated. Initiation of lipid oxidation in muscle food has been attributed to free metal ions (Kanner *et al.*, 1988a,b; Rhee, 1988) and to heme proteins such as myoglobin (Lui and Watts, 1970; Kanner and Harel, 1985a). Autoxidation of the bright red meat pigment oxymyoglobin (MbFe(II)O_2), present at the surface of fresh meat, results in the formation of metmyogobin (MbFe(III)) and superoxide which rapidly dismutate to H_2O_2 and oxygen (Satoh and Shikama, 1981). Studies have shown that autoxidation of the meat pigments and lipid peroxidation are coupled phenomena (Greene *et al.*, 1971; Benedict *et al.*, 1975; Love, 1983). Johns *et al.* (1989) provided evidences for a role for MbFe(III) as oxidation catalyst and suggested that a balance between ferric and ferrous forms of myoglobin is necessary to cause rapid oxidation, a suggestion further supported by findings of Minotti *et al.* (1992). Verma *et al.* (1985) found no evidence for a role of the ferrous heme species or inorganic iron as prooxidants in meat or meat-model emulsion systems, at the concentration found in meat, while MbFe(III) was found to be a powerful catalyst of lipid oxidation. In contrast, Love and Pearson (1974) and Tichivangana and Morrissey (1985) demonstrated only little catalytic activity for MbFe(III) when added to water-extracted muscle fibers.

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Alternatively, it has been suggested that H_2O_2 formed during MbFe(II)O_2 autoxidation may be necessary for MbFe(III) to be an effective catalyst (Kanner and Harel, 1985a,b), which was recently supported by the results of Chan *et al.* (1996). H_2O_2 activated MbFe(III) has received increasing attention due to the generation of a short lived protein radical, perferrylmyoglobin (•MbFe(IV)═O), which is rapidly reduced to a more stable species, ferrylmyoglobin (MbFe(IV)═O). Both •MbFe(IV)═O and MbFe(IV)═O are hypervalent iron compounds and strong oxidants in biological systems (Newman *et al.*, 1991) or in meat systems (Kanner and Harel, 1985a; Rhee *et al.*, 1987). As such, they are capable of initiating lipid peroxidation and causing protein cross-linkage (Rice *et al.*, 1983; Østdal *et al.*, 1996). However, Xu *et al.* (1990) observed that the generation of •MbFe(IV)═O was completely inhibited at low pH values (pH 5.0), under which conditions a decrease in the prooxidative activity of H_2O_2-activated MbFe(III) also was observed by Kanner and Harel (1985a). The exact nature of coupling between the redox processes of the pigments and initiation of lipid peroxidation may, thus, be concluded as still being unknown.

In the present study, oil-in-water linoleic acid emulsion was used as a model system and the prooxidative activities of MbFe(III), the MbFe(III)/MbFe(II)O_2 redox couple, MbFe(IV)═O, and •MbFe(IV)═O were investigated for the pH range of interest for meats (pH 5.5−6.5) in order to identify major heme-based lipid peroxidation catalysts in meat and meat products.

MATERIALS AND METHODS

Chemicals. Equine metmyoglobin (Type III), linoleic acid, chelating resin (Chelex-100), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 13(*S*)-[9*Z*,11*E*]-hydroperoxyoctadecadienoic acid (13-[*S*]-HPODE), and Tween 20 were obtained from Sigma (St. Louis, MO), and 9(*S*)-[10*E*,12*Z*]-hydroperoxyoctadecadienoic (9[*S*]-HPODE) from Cayman Chemical Co. (Ann Arbor, MI). Analytical grade chemicals and double-deionized water were used throughout. All buffers were passed through a chelating resin column to remove any free metal ions (Dunn *et al.*, 1980).

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Preparation of Linoleic Acid Emulsions. Linoleic acid emulsions were prepared in a 10 mL volumetric flask by mixing Tween 20 (0.012 g) and linoleic acid (21 mM) with phosphate buffer (5.0 mM, pH 6.5) as described by Mikkelsen *et al.* (1992). The pH was adjusted to approximately 9.0 in order to provide the highest possible stability of the emulsion. Fresh emulsions were prepared daily.

Preparation of Myoglobin Species. Metmyoglobin, MbFe(III), was dissolved in 5.0 mM phosphate (pH 6.50) or acetate (pH 4.50) buffer. The solution was centrifuged (20000*g*, 10 min), passed through a chelating resin column (Dunn *et al.*, 1980), diluted to 0.2 or 0.4 mM using $\epsilon_{525} = 7700$ L mol^{-1} cm^{-1} (Andersen and Skibsted, 1992), and stored until use at 5 °C in the dark. Oxymyoglobin, MbFe(II)O_2, was prepared by adding sodium dithionite to an MbFe(III) solution and purified onto a mixed-bed ion exchange column (Dowex 50W-X8, 100−200 mesh, plus Dowex 1X8, 20−50 mesh) (Andersen *et al.*, 1988). The MbFe(III)/MbFe(II)O_2 ratio was determined using the formula of Puppo and Halliwell (1988). MbFe(IV)═O was prepared by mixing equal volumes of 0.4 mM MbFe(III) in phosphate buffer (pH 6.5) with 1.2 mM H_2O_2 and allowed to react for 10 min, in order to ensure complete conversion of MbFe(III) to MbFe(IV)═O. The green pigment GMbFe(III) was prepared by mixing 500 μL of a 0.4 mM MbFe(III) solution in acetate buffer (pH 4.5) with 125 μL of H_2O_2 (4.8 mM) and adjusting the volume to 1.00 mL with acetate buffer. The mixture was allowed to react for a minimum of 10 min in order to ensure complete conversion to GMbFe(III) (Fox *et al.*, 1974).

Reduction of Linoleic Acid Hydroperoxides. Linoleic acid (250 mg) was dissolved in 20 mL of a 1:1 mixture of ethanol and borax buffer (pH 8.8). The pH was adjusted to approximately 9.0, and $NaBH_4$ (100 mg) was added. After 1 h of continuous stirring at room temperature, the mixture was diluted with deionized water (10 mL) and the pH was lowered to approximately 3.0 with HCl (2 M). The mixture was extracted with 2 × 20 mL of diethyl ether, washed with a saturated solution of bicarbonate, dried with $MgSO_4$, and evaporated under a steam of N_2 (Iacazio *et al.*, 1990). The hydroperoxide free linoleic acid obtained was used immediately to prepare linoleic acid emulsions as described above.

Reactions of Linoleic Acid with Myoglobin Species. Initiation of lipid peroxidation by MbFe(III) was studied by mixing linoleic acid emulsion (0.42 mM) and MbFe(III) (4 μM) in air-saturated phosphate buffer (0.15 M, pH 5.5) incubated in a temperature-controlled waterbath at 25.0 °C. Initiation of oxidation by MbFe(IV)═O was studied using the same procedure, substituting MbFe(IV)═O for MbFe(III). Experiments with perferrylmyoglobin, •MbFe(IV)═O, were carried out by adding MbFe(III) (4 μM) into air-saturated phosphate buffer (0.15 M, pH 5.5) incubated at 25.0 °C and containing 0.42 mM linoleic acid emulsion and 12 μM H_2O_2. Experiments with hydroperoxide free linoleic acid emulsions were carried out by the same procedure for each of the three myoglobin species. In order to monitor spectral changes of the myoglobins in the visible range, experiments were performed as described previously using a 10-fold increase in both the linoleic acid concentration and the heme protein concentration. Spectra ($450 \leq \lambda \leq 700$ nm) were recorded using a Cary-3 (Varian, Victoria, Australia). Linoleic acid emulsions used were not linoleic acid hydroperoxide free unless specified.

Determination of Conjugated Dienes. The relative concentrations of conjugated dienes were determined during reaction after extraction of 1.00 mL samples of the linoleic acid emulsion mixtures with 4.00 mL of cold diethyl ether after 2, 5, and 10 min of incubation of the reaction mixture in a temperature-controlled waterbath at 25.0 °C. The ether phase was collected and evaporated under a steam of N_2. The residue was dissolved in 1.00 mL of ethanol and the absorption spectrum was recorded ($200 \leq \lambda \leq 300$ nm) with an HP 8452 UV−vis diode array spectrophotometer (Hewlett Packard Co., Palo Alto, CA). The relative amount of conjugated dienes was calculated for each sample by reading the zero crossing value after calculation of the second derivative at 234 nm (Corongiu and Milia, 1983). Additionally, formation of conjugated dienes was followed from absorption spectra (200−300 nm) calculated using the calibration model recently described by Baron *et al.* (1997).

Determination of Lipid Hydroperoxides. Linoleic acid hydroperoxide concentrations were determined after 2, 5, and 10 min of incubation of the reaction mixture in a temperature controlled waterbath at 25.0 °C. Samples of 1.00 mL were extracted with 4.00 mL of ice-cold diethyl ether. The ether phase was collected and evaporated under N_2, and the residue was dissolved in 250 μL of ethanol. Aliquots (20 μL) were separated on a C-18 column (ODS Hypersil) equilibrated with acetonitrile:water (65:35) with a flow of 1.00 mL/min, using an HPLC system (HP series 1050 system), equipped with an HP diode array detector 1040M (Hewlett Packard Co., Palo Alto, CA). Linoleic acid hydroperoxide was quantified as 9[*S*]-HPODE or 13[*S*]-HPODE using a standard curve of integrated areas of absorbance at 234 nm.

Heme Protein Degradation. The reaction mixtures [linoleic acid, 0.42 mM, and MbFe(III) or MbFe(IV)═O, 4 μM, or MbFe(III)/H_2O_2, 4 and 12 μM, respectively, in phosphate buffer (0.15M, pH = 5.50)] were prepared directly in a 1 cm quartz cuvette and placed in a temperature controlled cell compartment at 25.0 °C of the HP 8452 UV−vis diode array spectrophotometer. Spectra ($190 \leq \lambda \leq 700$ nm) were recorded immediately and at time intervals of 1 min for 10 min, and their second derivatives were used to determine the relative heme protein degradation at 410 nm for MbFe(III) and •MbFe(IV)═O, and at 424 nm for MbFe(IV)═O.

Electron Spin Resonance Spectroscopy (ESR). Freeze quench ESR experiments were performed using reaction mixtures identical to those used for visible spectroscopy. Reaction mixtures were immediately transferred into ESR capillary tubes (Wilmad Glass, Buena, NJ) and frozen in liquid nitrogen after 30 s of reaction. Capillary tubes were subsequently placed within a Bruker 4103 TM/9216 rectangular cavity equipped with a capillary Dewar (Bruker, Karlsruhe, Germany). The temperature was kept constant at 150 K with a flow of nitrogen gas directly into the quartz Dewar. The magnetic field was modulated with a frequency of 100 kHz using 5 gauss field modulation amplitude, 2 mW microwave power, and receiver gain of 2.5×10^5. Additionally, spin-trap ESR experiments were performed using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in a concentration of 200 μM as spin-trap. The sample was transferred to a flat ESR cell, and ESR spectra were obtained at ambient temperature after 60 s of incubation. The magnetic field was modulated with a frequency of 100 kHz using 1 gauss field modulation amplitude, 20 mW microwave power, and receiver gain of 2.5×10^4.

Oxygen Consumption. Oxygen consumption measurements were performed as described by Mikkelsen *et al.* (1992). The reaction mixture consisted of 100 μL of linoleic acid emulsion (21 mM), 100 μL of solution of myoglobin species [0.2 mM MbFe(III) or GMbFe(III)], and 4.8 mL of air-saturated phosphate buffer (0.15 M, pH 5.50) incubated at 25.0 °C. Solutions of MbFe(III)/MbFe(II)O_2 mixtures were added to 100 μL of linoleic acid emulsion (21 mM) with air-saturated phosphate buffer (0.15 M, pH 5.90) to yield a final volume of 5.00 mL. The MbFe(III)/MbFe(II)O_2 ratios were varied, whereas the total concentration of heme protein was maintained constant and equal to 1.0 μM. Immediately after mixing, the sample was injected into a 70 μL temperature-controlled (25.0 °C) measuring cell (Chemiware, Viby J, Denmark) with no headspace. The relative oxygen consumption was measured with a Clark electrode (Radiometer, Copenhagen, Denmark) connected to a multichannel analyzer. The electrode was calibrated with air-saturated buffer incubated in a temperature-controlled waterbath at 25.0 °C, and the relative oxygen concentration was recorded at time intervals of 5 s for 15 min.

RESULTS

Both metmyoglobin, MbFe(III), and ferrylmyoglobin, MbFe(IV)═O, induced lipid peroxidation in the oil-in-water linoleic acid emulsion used as model system, as demonstrated by determination of conjugated dienes (cd)

Table 1. Lipid Peroxidation in Oil-in-Water Linoleic Acid Emulsion Initiated with Metmyoglobin (MbFe(III)), Ferrylmyoglobin (MbFe(IV)=O), or Perferrylmyogobin (•MbFe(IV)=O) at 25.0 °C, As Followed for up to 10 min by Analysis of Conjugated Dienes and Linoleic Acid Hydroperoxides

time (min)	conjugated dienes[a]			[LOOH][b] (ng/μL)		
	MbFe(III)	MbFe(IV)=O	•MbFe(IV)=O	MbFe(III)	MbFe(IV)=O	•MbFe(IV)=O
0	10 ± 3	10 ± 3	10 ± 3	0.18 ± 0.03	0.18 ± 0.03	0.18 ± 0.03
2	16 ± 3	17 ± 3	19 ± 2	0.48 ± 0.01	0.52 ± 0.07	0.14 ± 0.01
5	28 ± 2	25 ± 4	16 ± 2	1.2 ± 0.2	1.06 ± 0.16	0.31 ± 0.2
10	42 ± 0	40 ± 4	17 ± 0.4	1.2 ± 0.3	0.89 ± 0.01	0.40 ± 0.1

[a] Relative concentration of conjugated dienes determined after ether extraction of the reaction mixture by spectrophotometry using the 2nd-derivative spectra at 234 nm. [b] Linoleic acid hydroperoxide determination after ether extraction as the sum of 13[*S*]-and 9[*S*]-HPODE.

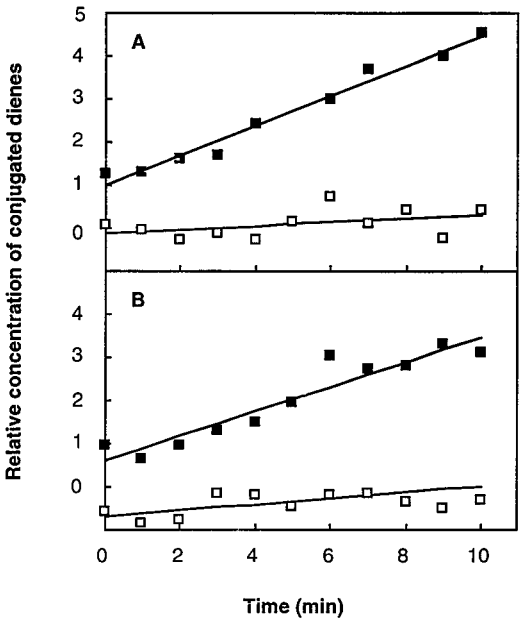

Figure 1. Formation of conjugated dienes in oil-in-water linoleic acid emulsion (21 mM) as initiated by (0.2 mM) MbFe(III) (A) or by MbFe(IV)=O (B), followed for 10 min by direct second-derivative multiwavelength UV spectroscopy ($200 \leq \lambda \leq 300$ nm) in the presence (■) and in the absence (□) of linoleic acid hydroperoxides (pH 5.5, 25.0 °C).

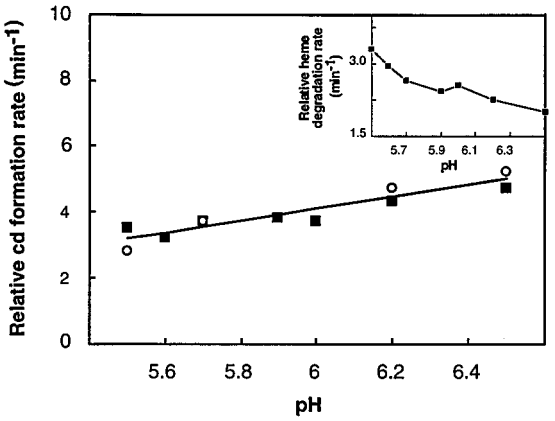

Figure 2. Relative rate of formation of conjugated dienes at different pH values during linoleic acid peroxidation initiated by (0.2 mM) MbFe(III) (■) or MbFe(IV)=O (○) at 25.0 °C for 10 min. Inset: Relative heme degradation rate, determined from Soret band absorption (410 nm) measured as second-derivative spectra, during MbFe(III)-initiated linoleic acid peroxidation at different pH values.

in extracted linoleic acid (Table 1). No significant difference in cd formation was observed between experiments with either MbFe(III) or MbFe(IV)=O as the initiator. In contrast, •MbFe(IV)=O did not initiate lipid peroxidation in the linoleic acid emulsion as evaluated from cd formation, even after 10 min of incubation (Table 1). Simultaneous HPLC analysis of lipid hydroperoxides formed during peroxidation of linoleic acid confirmed these results (Table 1). Initiation of lipid peroxidation with •MbFe(IV)=O did not result in any noticeable hydroperoxide formation, whereas addition of either MbFe(III) or MbFe(IV)=O to linoleic acid emulsions resulted in a pronounced and rather similar formation of lipid hydroperoxides. Direct second derivative multiwavelength UV absorption spectroscopy (Baron *et al.*, 1997) used on the heterogeneous reaction mixture clearly demonstrated that lipid hydroperoxides are essential for initiation of lipid peroxidation by MbFe(III) or MbFe(IV)=O, as no cd were formed when linoleic acid hydroperoxide free emulsions were used (Figure 1). The prooxidative activity of MbFe(III) and MbFe(IV)=O in the oil-in-water linoleic acid emulsion in the presence of lipid hydroperoxides was also evaluated at different pH values (pH 5.5−6.5) of interest for meat and meat products using the above-mentioned direct method for determination of conjugated dienes. Both MbFe(III) and MbFe(IV)=O were found to be slightly more efficient as peroxidation initiators in lipid hydroperoxide containing linoleic acid emulsions at increasing pH as can be seen from the data presented in Figure 2. Moreover, the insert in Figure 2 shows the concomitant decrease in the Soret band absorption of the MbFe(III) initiator for conditions of varying pH after 10 min of the reaction.

To obtain information on the redox state of myoglobin during lipid peroxidation and of the integrity of the heme protein complex, changes in the absorption in the visible range (450−700 nm) and in the Soret band absorption (410 or 424 nm) were followed simultaneously for each of the heme proteins. A dramatic decrease in the Soret band absorption (410 nm) was observed for MbFe(III) when added to the linoleic acid emulsions in the presence of lipid hydroperoxides, while no changes were observed in emulsions in the absence of lipid hydroperoxides (Figure 3A). Addition of MbFe(IV)=O to linoleic acid emulsions resulted in a slight decrease in the characteristic Soret absorption of ferrylmyoglobin (424 nm) independent of the presence of lipid hydroperoxides (Figure 3C). As observed for MbFe(III), a dramatic decrease in the absorbance at 410 nm occurred when •MbFe(IV)=O was tested as initiating species, but in contrast to MbFe(III), the decrease for •MbFe(IV)=O was independent of the presence of lipid hydroperoxides (Figure 3B). The spectral changes in the visible range recorded 1 min after incubation at 25.0 °C (Figure 4) showed that addition of MbFe(III) or MbFe(IV)=O to linoleic acid emulsions with lipid hydroperoxides present did not change the characteristic spectra of MbFe(III) and MbFe(IV)=O. However, addition of MbFe(III)/H_2O_2 to linoleic acid emulsions did not result in the characteristic expected spectrum of H_2O_2-activated myoglobin species but resulted in a spectrum with a maximum at 589 nm, and the solution

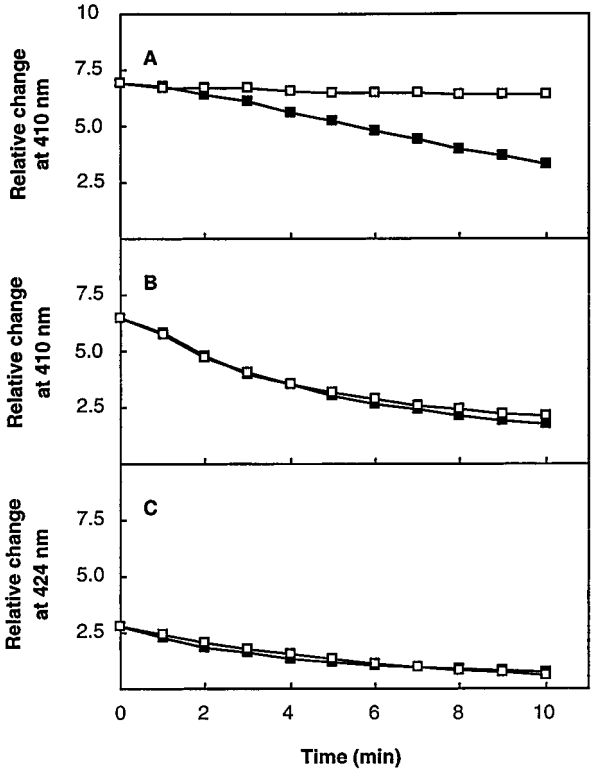

Figure 3. Decrease in Soret band absorption determined from second-derivative spectra of myoglobin species after addition of 0.2 mM (A) MbFe(III), (B) •MbFe(IV)═O, and (C) MbFe(IV)═O to oil-in-water linoleic acid emulsions with (■) and without (□) the presence of lipid hydroperoxides (pH 5.5, 25.0 °C). Each point is the mean of three determinations.

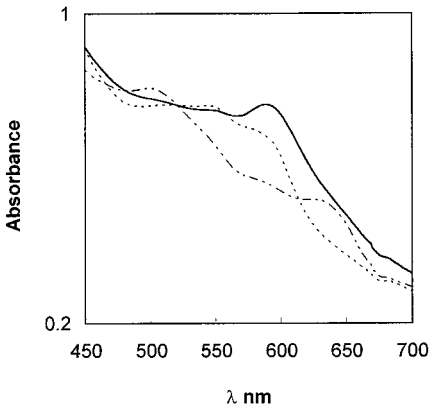

Figure 4. Visible spectra of reaction mixtures 1 min after addition of MbFe(III) (- - – - -), MbFe(III)/H_2O_2 (—), or MbFe(IV)═O (- - -) to linoleic acid emulsions with lipid hydroperoxides present.

had a green color (GMbFe(III)) similar to that of the heme compound described by Fox *et al.* (1974). The prooxidative activity of the green pigment, GMbFe(III), was compared to that for MbFe(III) by measuring oxygen consumption in linoleic acid emulsions. Addition of GMbFe(III) to linoleic acid emulsions did not result in any significant oxygen consumption compared to MbFe(III) during 15 min incubation at 25.0 °C, which is shown for a typical experiment in Figure 5.

Freeze quench and spin-trap ESR spectroscopy were used to identify the myoglobin radicals appearing during the initial interaction between heme protein species and linoleic acid or linoleic acid hydroperoxides in the oil-in-water emulsions. Freeze quench ESR spectra of the reaction mixture 30 s after addition of the heme protein

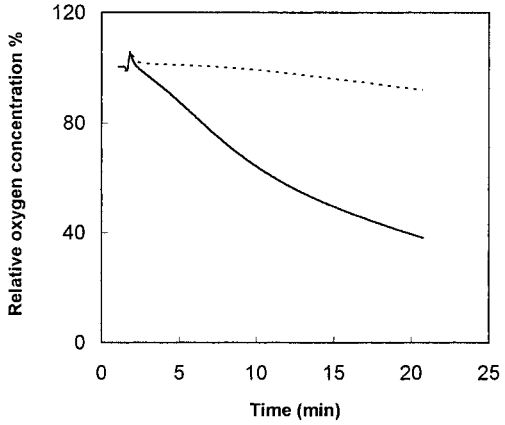

Figure 5. Oxygen consumption versus time after addition of 0.2 mM MbFe(III) (—) or GMbFe(III) (- - -) to oil-in-water linoleic acid emulsion (pH 5.5, 25.0 °C).

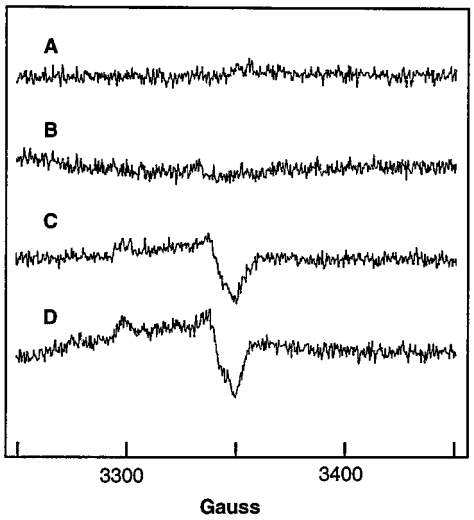

Figure 6. Freeze quench ESR spectra after 30 s incubation for (A) MbFe(III) initiated linoleic acid peroxidation, (B) MbFe(IV)═O-initiated linoleic acid peroxidation, (C) •MbFe(IV)═O-initiated linoleic acid peroxidation, and for (D) •MbFe(IV)═O without linoleic acid.

to the linoleic acid emulsions are presented in Figure 6. The mixture of MbFe(III) and MbFe(IV)═O did not show any ESR signals, while the addition of MbFe(III)/H_2O_2 to the reaction mixture resulted in the characteristic ESR spectrum of the •MbFe(IV)═O species (Davies, 1988), both in the presence and in the absence of linoleic acid emulsions. A weak ESR signal was recorded using spin-trap ESR, when MbFe(IV)═O was added to the linoleic acid emulsions, whereas for •MbFe(IV)═O no spin adducts were detectable with the resolution of our instrumentation (results not shown) as also reported by Xu *et al.* (1990).

Finally, the effect of the ferric and ferrous state of myoglobin on initial lipid peroxidation in the oil-in-water linoleic acid emulsions was investigated for different MbFe(III)/MbFe(II)O_2 ratios by oxygen consumption measurements (Figure 7). Oxygen consumption was only slightly enhanced by an increase in the MbFe(III) concentration.

DISCUSSION

Many different myoglobin species have been suspected to catalyze lipid peroxidation in muscle foods, but recent interest has been focusing on H_2O_2-activated myoglobin which has been believed to be a primary

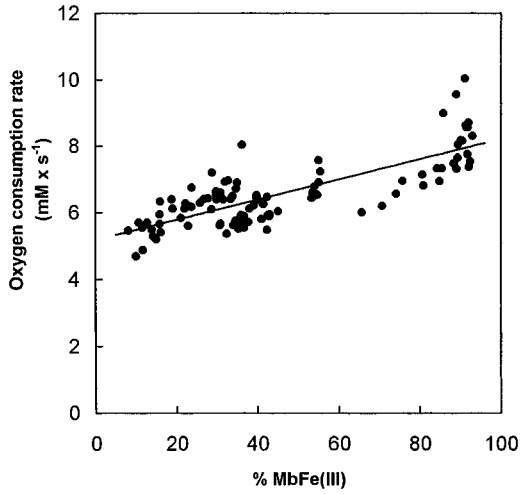

Figure 7. Initial rate of oxygen consumption after initiation of peroxidation in an oil-in-water linoleic acid emulsion with MbFe(III) (%) in a mixture of MbFe(III)/MbFe(II)O_2 with the total pigment concentration equal to 1 μM (pH 5.9, 25.0 °C).

catalyst (Kanner and Harel, 1985a,b). H_2O_2 activates myoglobin through formation of hypervalent states of iron, such as in MbFe(IV)═O and •MbFe(IV)═O. Both forms are powerful oxidants, capable of oxidizing other food constituents (Kanner and Harel, 1985a,b; Bhoite-Salomon *et al.*, 1992; Mikkelsen and Skibsted, 1995; Kröger-Ohlsen and Skibsted, 1997). However, somewhat conflicting results regarding the prooxidative activity of the hypervalent forms of myoglobins towards lipids have prompted further studies in order to elucidate the basic mechanisms of lipid oxidation in muscle based food. Both MbFe(III) and MbFe(IV)═O were found to be potent initiators of lipid peroxidation in the oil-in-water linoleic acid model system used provided that lipid hydroperoxides were present. In contrast, •MbFe(IV)═O was not found to initiate lipid peroxidation under similar conditions. The observation that myoglobin only initiated peroxidation in the presence of lipid hydroperoxides supports the early theory of heme protein catalysis as driven by hydroperoxides (Tappel, 1955; Machii *et al.*, 1995; Van der Zee *et al.*; 1996). Moreover, for both MbFe(III) and MbFe(IV)═O the catalytic lipid peroxidation activity increased significantly and very similarly by increasing the pH within the range found in meats (Figure 2). This is in agreement with the findings of Lui (1970) and Johns *et al.* (1989) but in contrast to the results obtained previously by Mikkelsen *et al.* (1992) using the same model system that was utilized in the present study. The better spectrophotometric analysis of conjugated dienes developed for turbid solutions by Baron *et al.* (1997) provided the necessary correction, especially at low pH, without which conditions cd will otherwise be overestimated, as was the case in the previous study (Mikkelsen *et al.*, 1992).

MbFe(III)-induced lipid peroxidation was found to occur with simultaneous heme protein degradation (Table 1, Figure 3A). Similar correlations between heme degradation and lipid oxidation have previously been reported (Galaris *et al.*, 1988; Sage *et al.*, 1991; Mikkelsen *et al.*, 1992). Heme protein denaturation was indicated by a decrease in the characteristic Soret band also for MbFe(IV)═O in the linoleic acid emulsions (Figure 3C). Notably, this spectral change proceeded both in the presence and in the absence of hydroperoxides in the emulsions, and can accordingly not solely be ascribed to effect on the protein from lipid peroxidation. Autoreduction of MbFe(IV)═O to MbFe(III) is known to be relatively fast in the pH region of relevance for meat (Mikkelsen and Skibsted, 1995), as also evidenced by a blue shift in the Soret band (424−410 nm) during incubation in the linoleic acid emulsions (data not shown). However, the increase in absorbance at 410 nm during incubation of MbFe(IV)═O was more pronounced in the absence of lipid hydroperoxides. This latter information is in strong support of a concomitant lipid peroxidation and heme protein denaturation or degeneration in the presence of lipid hydroperoxides. However, it still remains to be shown whether MbFe(IV)═O as such can initiate lipid peroxidation as a result of reduction to MbFe(III), or whether it is a result of the subsequent activity of MbFe(III). If MbFe(III) was the catalytic species upon addition of MbFe(IV)═O to the oil-in-water linoleic acid emulsions, a lag phase would be expected, but both MbFe(III)- and MbFe(IV)═O-initiated lipid peroxidation proceeded at the same rate (Figures 1 and 2). Thus, it appears safe to conclude that MbFe(IV)═O as such, rather than MbFe(III), formed by autoreduction of MbFe(IV)═O, is the initiator in lipid hydroperoxide dependent lipid peroxidation in the oil/water emulsion.

In contrast, •MbFe(IV)═O, the transient protein radical formed by reaction of MbFe(III) with H_2O_2 in the reaction mixture independent of the presence of linoleic acid, had no effect on the formation of cd or lipid hydroperoxides (Table 1). Earlier investigations using a semiquantitative ESR spin-trap method have shown a significant decrease in the spin adduct ESR signal of perferrylmyoglobin the presence of linoleic acid (Davies, 1990), an observation which was taken as an indication of a rapid reaction with linoleic acid leading to chain reactions characteristic of lipid peroxidation. In contrast, our data indicate only a moderate decrease in the amount of pigment radicals formed in the presence of linoleic acid (Figure 4), which notably is in accordance with the lack of formation of cd. MbFe(III)/H_2O_2 is apparently not an initiator of lipid peroxidation in oil-in-water linoleic acid emulsions, despite that a steady decrease in the Soret band (410 nm) indicating major changes in the heme−protein interaction in the heme cavity was observed both in the presence and in the absence of hydroperoxides. Moreover, visible spectra of MbFe(III)/H_2O_2 mixtures added to oil-in-water linoleic acid emulsions had an absorption maximum at 589 nm rather than at 548 and 588 nm as characteristic for H_2O_2 activated myoglobin. The observed spectrum with λ_{max} = 589 nm is consistent with the spectrum of the myoglobin species first described by Fox *et al.* (1974), formed during acidic reaction conditions and also detected as a reaction product during oxidative modification of myoglobin (Tajima and Shikama, 1993). This myoglobin species has recently been characterized as an iron(III) myoglobin derivative in which a cross-linkage of the intact heme moiety to an aromatic amino acid of the globin has occurred (Osawa and Korzekwa, 1991). The porphyrin cation radical initially formed during the reaction of H_2O_2 with MbFe(III) (Rao *et al.*, 1994) thus appears preferentially to react with an amino acid in an intramolecular reaction, resulting in cross-linkage between the heme moiety and the globin, rather than reacting with linoleic acid under the conditions present in oil-in-water linoleic acid emulsions. Such a preference for intramolecular electron transfer may, moreover, explain why only minor lipid oxidation was

observed in a similar model system at pH 5.0 upon addition of MbFe(III)/H_2O_2 to the reaction mixture (Kanner and Harel, 1985a).

As mentioned earlier, autoxidation of MbFe(II)O_2 to MbFe(III) results in the formation of superoxide that rapidly dismutates to hydrogen peroxide and oxygen (Misra and Fridovich, 1972). Hydrogen peroxides may subsequently react either with MbFe(III) or MbFe(II)-O_2 resulting in •MbFe(IV)═O and MbFe(IV)═O, respectively, and both of these hypervalent iron compounds may initiate lipid peroxidation under the proper conditions (Kanner and Harel, 1985b). Any factors that favor oxidation of MbFe(II)O_2 to MbFe(III) should accordingly result in the activation of heme compounds, initiating lipid peroxidation (Srinivasan *et al.*,1996). Low pH values enhance oxidation of MbFe(II)O_2 (Andersen *et al.*, 1988), a reaction which in combination with the metmyoglobin reductase cycle of MbFe(II)O_2/MbFe(III) results in continuous formation of H_2O_2 through disproportionation of superoxide under the conditions found in meat (Mikkelsen and Skibsted, 1992). Low pH should accordingly increase the rate of lipid peroxidation. Moreover, it has normally been agreed that iron(III) heme pigments are more catalytic than iron(II) heme pigments in lipid oxidation (Verma *et al.*, 1985; Mikkelsen and Skibsted, 1992). However, in the oil-in-water linoleic acid emulsions, MbFe(II)O_2 is found to be only slightly less catalytically active in the initiation of lipid peroxidation compared to MbFe(III) (Figure 7), suggesting that the redox state is less critical for the prooxidative activity of myoglobin derivatives found in fresh meat. Furthermore, •MbFe(IV)═O seems to form an exception, at least in oil-in-water linoleic acid emulsions, in which this oxidized heme species was found to convert to a non-catalytic heme species rather than exhibit strong catalytic activity. The latter may also explain the small decrease in catalytic activity of MbFe(II)O_2/MbFe(III) mixture with increasing MbFe(II)O_2 content, due to the concomitant formation during oxidation of the non-catalytic and denatured heme species GMbFe(III).

In conclusion, the present study using oil-in-water linoleic acid emulsions shows that (i) •MbFe(IV)═O cannot be expected to be an initiator of lipid peroxidation in meats in agreement with the finding of Xu *et al.* (1990); (ii) MbFe(II)O_2, MbFe(III), and MbFe(IV)═O must be expected to have approximately equal catalytic activities in lipid hydroperoxide dependent lipid peroxidation; and (iii) the presence of lipid peroxides is the most crucial factor in heme protein-initiated lipid peroxidation in meat, as was also found for other biological systems (Hemler and Lands, 1980; Hogg *et al.*, 1994). Future research regarding optimization of oxidative stability of muscle-based food should accordingly concentrate on factors controlling formation of lipid hydroperoxides rather than on the redox state of heme proteins, both pre- and post-slaughter and also during subsequent processing.

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