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Concentration Effects in Myoglobin-Catalyzed Peroxidation of Linoleate

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The concentration of the free fatty acid anion linoleate was found to be important for the pro-oxidative activity of metmyoglobin, MbFe(III), and for mixtures of metmyoglobin and hydrogen peroxide, MbFe(III)/H_2O_2, to yield perferrylmyoglobin, •MbFe(IV)═O, whereas for ferrylmyoglobin, MbFe(IV)═O, no concentration effect was noted as studied in linoleate emulsions (pH 7.4 and 25 °C). Determination of conjugated dienes using second-derivative absorption spectroscopy, changes in Soret band absorbance, and spin-trapping ESR spectroscopy with α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (POBN) as the spin trap were used to evaluate the pro-oxidative activity of myoglobins. At a linoleate (LA)/heme protein (HP) ratio of 100, no MbFe(III)-induced linoleate peroxidation was observed, as MbFe(III) was converted to its non-pro-oxidative low-spin derivative, hemichrome, independently of the presence of H_2O_2. At higher LA/HP ratios, linoleate peroxidation was initiated by the addition of MbFe(III), both in the presence and in the absence of H_2O_2. This proceeded with denaturation of MbFe(III), as followed by changes in Soret absorption band, which most probably release or expose the heme group to the environment and thereby permit hematin-induced lipid peroxidation. The obtained results show that the mechanism by which MbFe(IV)═O initiates linoleate peroxidation is different from MbFe(III)- and MbFe(III)/H_2O_2-initiated linoleate peroxidation. The shift in mechanism between heme protein cleavage of lipid hydroperoxides and hematin-induced lipid peroxidation is discussed in relation to oxidative progress in biological systems and muscle-based foods.

KEYWORDS: Metmyoglobin; ferrylmyoglobin; perferrylmyoglobin; hematin; heme degradation; lipid peroxidation

INTRODUCTION

Myoglobin and hemoglobin are the most abundant heme proteins in living organisms and have been demonstrated to initiate oxidative damage in biological tissue and muscle-based foods. However, the mechanism responsible for heme protein catalyzed lipid peroxidation still is a subject of controversy (*1*, *2*). Under physiological conditions, most heme proteins have been reported to be non-pro-oxidative (*3*−*5*). It has been proposed that heme protein must be activated by hydrogen peroxide or by lipid hydroperoxides to be an effective catalyst of oxidative processes (*3*, *4*, *6*). Hydrogen peroxide activation of metmyoglobin, MbFe(III), results in the generation of a protein radical, perferrylmyoglobin, •MbFe(IV)═O, that is rapidly converted to the more stable ferrylmyoglobin species, MbFe(IV)═O. Both of these hypervalent heme pigments effectively oxidize a variety of biological components (*7*−*9*), but again the ability of the individual heme proteins to initiate lipid oxidation in vivo and in muscle-based foods is controversial (*1*, *10*−*13*). Recent findings indicate that high oxidation state myoglobin species •MbFe(IV)═O and MbFe(IV)═O are produced in vivo (*14*, *15*), and they have, moreover, been suggested as major pro-oxidative candidates in muscle-based foods (*16*, *17*). This makes it obvious to include these heme proteins in the investigation of the role of individual myoglobins in oxidative deterioration of muscle-based foods.

Additionally, the lipid to heme protein ratio has been demonstrated to be an important factor affecting the pro-oxidative activity of heme proteins (*18*−*20*). At high linoleate (LA)/heme protein (HP) ratios it has been shown that heme proteins possess strong pro-oxidative activity, whereas, at lower LA/HP ratios, heme proteins become ineffective initiators of lipid oxidation (*20*−*22*). Immediately after slaughter, the amount of free fatty acids in muscle is low, but it increases slowly during storage, reaching ∼1% in 5−7 days (*23*, *24*) and being most pronounced in oxidative muscles (*24*). Moreover, during processing, for example, dry-curing, intramuscular lipids are

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subjected to an intensive lipolysis resulting in a free fatty acid content of 8−20% of total lipids in muscle (*25*−*29*). Variability in the amount of free fatty acid in muscle-based foods might also have contributed to conflicting reports regarding the abilities of heme protein to initiate lipid oxidaton in muscle foods (*30*−*33*). The mechanism responsible for the inhibition of lipid peroxidation at low LA/HP ratios (≤100) has recently been clarified for physiological conditions, and it has been demonstrated that fatty acid anions binds reversibly to MbFe(III), resulting in a spin transition, to yield the low-spin metmyoglobin derivative, hemichrome, which, additionally, was found not to be pro-oxidative (*34*). However, the ability of heme proteins to initiate lipid peroxidation at higher LA/HP ratios has yet to be clarified. The fatty acid to heme protein ratio is a factor that has somehow been overlooked, or neglected, in the ongoing discussion concerning heme protein initiated lipid oxidation but which deserves further attention.

In the present study, the pro-oxidative activity of MbFe(III), of MbFe(III)/H_2O_2 to yield •MbFe(IV)═O, and of MbFe(IV)═O was investigated in a simple lipid model system to investigate the effect of an increasing LA/HP ratio and to contribute to the understanding of the mechanisms responsible for heme protein initiated lipid oxidation in muscle-based foods.

MATERIALS AND METHODS

Chemicals. Equine metmyoglobin (type III), linoleic acid, chelating resin (Chelex-100), α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (POBN), and Tween 20 were obtained from Sigma (St. Louis, MO). Analytical grade Na_2HPO_4 and NaH_2PO_4 from Merck (Darmstadt, Germany) were used to prepare all buffers. Other chemicals were of analytical grade, and water was purified on a Millipore Q-plus purification train (Millipore, MA). All buffers were passed through a Chelex-100 chelating resin column to remove any free metal ions (*35*).

Preparation of Linoleate Emulsions. Linoleate emulsions were prepared in a 10 mL volumetric flask by mixing Tween 20 (0.012 g) and linoleate (60 mg) with phosphate buffer (5.0 mM, pH 6.5) as described by Mikkelsen et al. (*5*). The pH was adjusted to ∼9.0 with 1.0 M NaOH to ensure stability of the emulsion. Fresh emulsions were, however, prepared daily.

Preparation of Myoglobin Species. Metmyoglobin, MbFe(III), was dissolved in 5.0 mM phosphate buffer (pH 6.50). The solution was centrifuged (20000*g*, 10 min), passed through a chelating resin column (*35*), diluted to 0.2 or 0.4 mM using $\epsilon_{525} = 7700\ L \cdot mol^{-1} \cdot cm^{-1}$ (*36*), and stored at 5 °C in the dark until use. 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, which was allowed to react for 10 min, to ensure complete conversion of MbFe(III) to MbFe(IV)═O.

Reactions of Linoleate with Myoglobin Species. Initiation of lipid peroxidation by MbFe(III) was studied by adding MbFe(III) stock solution to linoleate emulsions diluted in air-saturated phosphate buffer (0.15 M, pH 7.4) to yield final linoleate concentrations of 0.42, 0.84, or 1.26 mM and a final MbFe(III) concentration of 4 μM and followed by incubation in a temperature-controlled water bath 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 MbFe(III)/H_2O_2 were carried out by adding stock solution of MbFe(III) to yield a final concentration of 4 μM into air-saturated phosphate buffer (0.15 M, pH 7.4), incubated at 25.0 °C and containing a linoleate emulsion corresponding to 0.42, 0.84, or 1.26 mM and 12 μM H_2O_2.

HPLC analysis of the reaction mixture before addition of the different myoglobin species did not reveal the presence of lipid hydroperoxides (detection limit = 0.5 μM). However, a catalytic amount of lipid hydroperoxides was believed to be present in the reaction mixture before addition of the different myoglobin initiating species, as has been shown in a previous study using the identical model system (*38*).

Determination of Conjugated Diene and Heme Protein Degradation. The different reaction mixtures [0.42, 0.84, or 1.26 mM linoleate and 4 μM MbFe(III) or 4 μM MbFe(IV)═O, or MbFe(III)/H_2O_2 4 and 12 μM, respectively, in phosphate buffer, 0.15 M, pH 7.4] were prepared directly in a 1 cm quartz cuvette and placed in an HP 8452 UV−vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) temperature-controlled cell compartment at 25.0 °C. Spectra ($190 \le \lambda \le 700$ nm) were recorded immediately and at time intervals of 1 min for 30 min and used to determine the relative amount of conjugated dienes by reading the second derivatives at 244 nm (*37*, *38*).

Heme protein degradation was determined by reading the absorbance in the Soret absorption band at 410 nm for MbFe(III) and MbFe(III)/H_2O_2 and at 424 nm for MbFe(IV)═O.

Electron Spin Resonance Spectroscopy (ESR). Spin trap ESR experiments were performed using reaction mixtures identical to those used for determination of conjugated dienes and heme protein degradation, using a 5-fold higher concentration of both lipid and myoglobins. POBN, in a final concentration of 4 mM, was used as spin trap as it is known to react more rapidly with carbon-centered free radicals and to give a more stable adduct than any other nitroso spin trap. After 2 min, and 120 min of incubation of the different reaction mixtures in a temperature-controlled water bath at 25.0 °C, samples were transferred to a flat ESR cell (Wilmad Glass, Bueno, NJ) and subsequently placed within a Bruker 4103 TM/9601 rectangular cavity (Bruker, Karlruhe, Germany). ESR spectra were obtained immediately at ambient temperature. The magnetic field was modulated with a frequency of 100 kHz using 1 G field modulation amplitude, 20 mW microwave power, and receiver gain of 2.5×10^4.

Table 1. Lipid Peroxidation in Oil-in-Water Linoleate Emulsions Initiated with Metmyoglobin, MbFe(III), or Metmyoglobin and Hydrogen Peroxide, MbFe(III)/H_2O_2, To Yield Perferrylmyoglobin, •MbFe(IV)═O, and Ferrylmyoglobin, MbFe(IV)═O, in the Presence of 100, 200, or 300 Times Excess Linoleate, Followed for 30 min by Second-Derviative Absorption Spectroscopy of Conjugated Dienes at 244 nm[a]

	time (min)	LA/HP ratio 100	200	300
MbFe(III)	0	0 ± 0.5	0 ± 1.7	0 ± 0.5
	10	4.3 ± 0	6.0 ± 1.0	28.7 ± 1.5
	30	4.3 ± 0	38.0 ± 9	34.7 ± 2.3
MbFe(III)/H_2O_2	0	0 ± 0.5	0 ± 1.7	0 ± 0.7
	10	1.7 ± 1.1	23 ± 14.0	35.5 ± 3.5
	30	2.7 ± 1.1	38.5 ± 2.5	36.0 ± 1.4
•MbFe(IV)═O	0	0 ± 0.5	0 ± 1.7	0 ± 2.8
	10	44.7 ± 0.5	30.7 ± 4.0	29.1 ± 5.1
	30	26.7 ± 1.0	27.7 ± 0.5	28.5 ± 2.5

[a] Each value is the mean of three determinations.

RESULTS

Linoleic acid peroxidation as initiated by MbFe(III), by MbFe(III)/H_2O_2, to yield •MbFe(IV)═O, and by MbFe(IV)═O was followed by the increase in conjugated dienes using second-derivative absorption spectroscopy for up to 30 min in the emulsion system where at least at the oil-in-water interface, the oxidation substrate will be present as the linoleate anion. MbFe(IV)═O was found to be able to initiate lipid oxidation irrespective of the LA/HP ratio (100, 200, or 300) as seen in **Table 1**. In contrast, the pro-oxidative activity of MbFe(III) and MbFe(III)/H_2O_2 to yield MbFe(IV)═O was, in this system, dependent on the LA/HP ratio. At an LA/HP ratio of 100, MbFe(III) and MbFe(III)/H_2O_2 were not able to initiate lipid oxidation, whereas at an LA/HP ratio of 200 or above, both MbFe(III) and MbFe(IIII)/H_2O_2 were able to induce lipid oxidation as effectively as MbFe(IV)═O, as deduced from the significant formation of conjugated dienes after 30 min of reaction (**Table 1**). Moreover, combination of MbFe(III) and H_2O_2 appears to initiate peroxidation earlier than MbFe(III) alone, an observation that may indicate mechanistic differences.

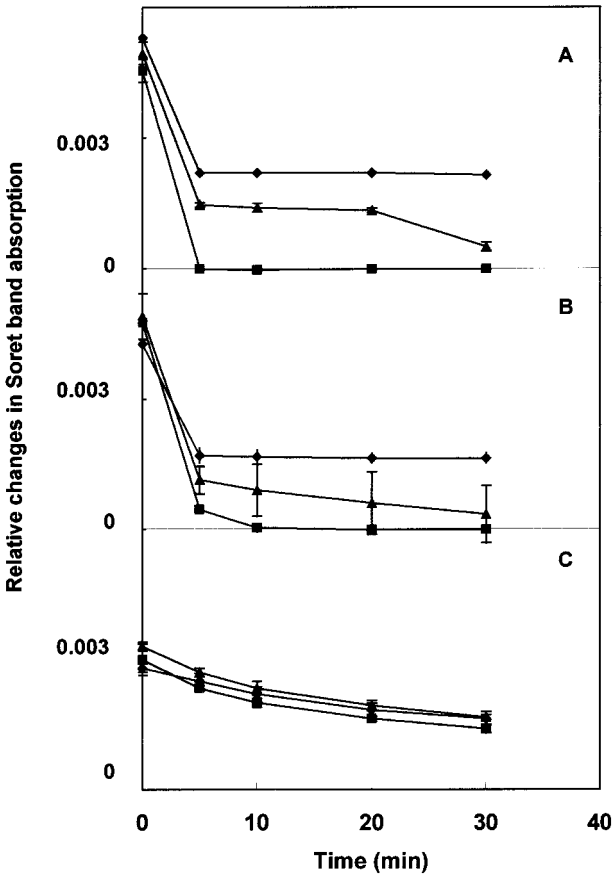

Figure 1. Changes in Soret band absorption of myoglobin followed as second derivative after addition of myoglobin to a final concentration of 4 μM: (A) MbFe(III) followed at 410 nm; (B) MbFe(III)/H_2O_2 in a ratio 1:3 to yield •MbFe(IV)═O and followed at 410 nm; (C) MbFe(IV)═O followed at 424 nm, to oil-in-water linoleate emulsions in phosphate buffer (pH 7.4; 25 °C) with a final linoleate concentration of 0.42, 0.84, or 1.26 mM and corresponding to a lipid/heme protein ratio equal to 100 (◆), 200 (▲) or 300 (■), respectively. Each point is the mean of three determinations.

Simultaneous to the development of lipid oxidation, changes in the Soret absorption band of the different heme proteins [410 nm for MbFe(III) and MbFe(III)/H_2O_2 or 424 nm for MbFe(IV)═O] were followed spectrophotometrically also using second-derivative absorption spectroscopy. Upon incubation of both MbFe(III) and MbFe(III)/H_2O_2 with linoleate emulsions a drastic decrease in the Soret absorption was observed already after 5 min of incubation, independently of the LA/HP ratio (**Figure 1A**,**B**). At high LA/HP ratios (200 and 300), lipid peroxidation induced by MbFe(III) or MbFe(III)/H_2O_2 was accompanied by a nearly complete, or even total, disappearance of the Soret absorption band, indicating destruction of the heme protein during incubation (**Figure 1A**,**B**). In contrast, incubation of MbFe(IV)═O with linoleate emulsions did not result in significant decrease in the characteristic Soret absorption band, irrespective of the LA/HP ratios (**Figure 1C**). Spin trap ESR spectroscopy performed for the different myoglobins and different LA/HP ratios revealed that already after 2 min of incubation only MbFe(IV)═O, but not MbFe(III) or MbFe(III)/H_2O_2 to yield •MbFe(IV)═O, was the only myoglobin species able to induce a significant formation of free radical independently of the LA/HP ratios (**Figure 2**). Moreover, for MbFe(IV)═O, formation of free radical was much more pronounced at a high LA/HP ratio (200 and 300). Notably, after 2 h of incubation the same pattern as produced by free radical

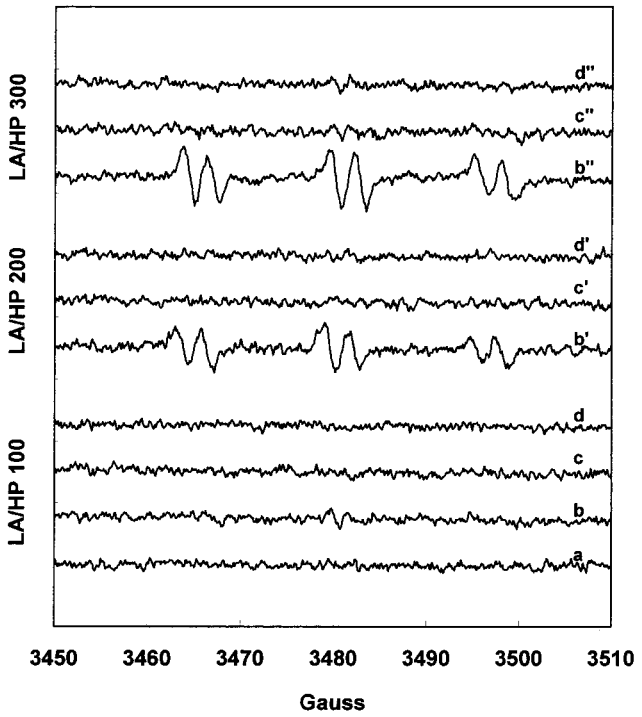

Figure 2. Spin trap ESR spectra obtained after a 2 min incubation of 4.0 mM POBN with (a) phosphate buffer or (b) MbFe(IV)═O, (c) MbFe(III)/H_2O_2 in a ratio 1:3, to yield MbFe(IV)═O, or (d) MbFe(III) to a final concentration 20 μM, in linoleate emulsions and with a lipid/heme protein ratio of 100 (pH 7.4; 25 °C). For spectra b′, c′, and d′ conditions were identical to those used for spectra b–d, respectively, except that the lipid/heme protein ratio was 200. For spectra b″, c″, and d″ conditions were identical to those used for spectra b–d, respectively, except that the lipid/heme protein ratio was 300.

formation was observed in the emulsion systems with added MbFe(III)/H_2O_2 as for those with MbFe(IV)═O added directly (**Figure 3**). Moreover, addition of MbFe(III)/H_2O_2 induced free radical formation at an LA/HP ratio of 200 in contrast to MbFe(III) alone (**Figure 3**), an observation in agreement with the delay in lipid oxidation observed under the same conditions (**Table 1**).

Finally, at an LA/HP ratio of 300, both MbFe(III) and MbFe(III)/H_2O_2 were able to induce free radical formation, as deduced from the ESR spectra (**Figure 3b″**,**c″**,**d″**), again in agreement with the pronounced lipid peroxidation found in these systems.

DISCUSSION

The pro-oxidative activity of heme proteins toward polyunsaturated lipids has been found to be strongly dependent on the lipid-to-heme ratio (*18*−*22*). In the absence of preformed lipid hydroperoxides, it has been proposed that heme proteins become pro-oxidative only after interaction with hydrogen peroxides, under which conditions reaction between MbFe(III) and hydrogen peroxide generates high oxidation state myoglobin species, perferrylmyoglobin and ferrylmyoglobin (*3*, *4*). Although these hypervalent myoglobins are strongly oxidant, their abilities to initiate lipid oxidation are controversial (*1*, *10*−*12*).

In agreement with earlier findings (*9*, *12*), MbFe(IV)═O was found in the present study to be a strong pro-oxidant and to be able to initiate lipid peroxidation. In addition, linoleate oxidation initiated by MbFe(IV)═O was found to occur with only minor simultaneous heme protein degradation, as monitored by changes

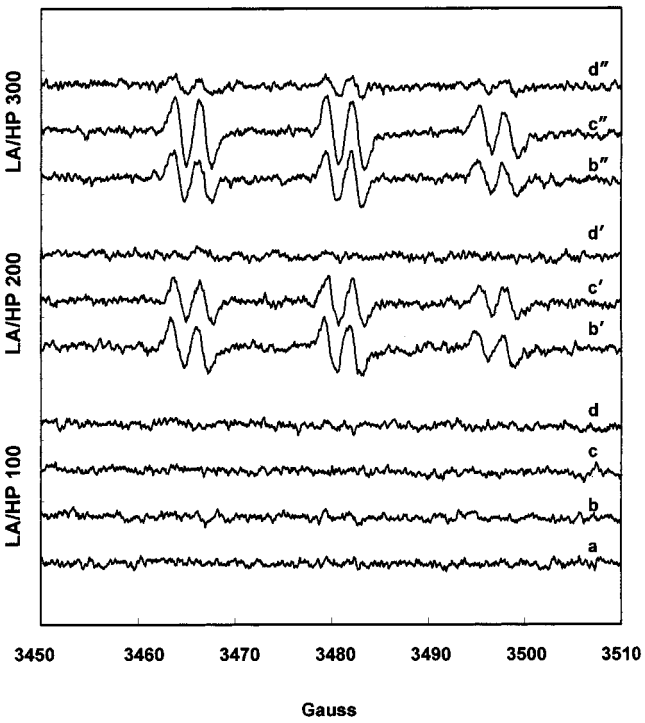

Figure 3. Spin trap ESR spectra obtained after a 2 h incubation of 4.0 mM POBN with (a) phosphate buffer or (b) MbFe(IV)═O, (c) MbFe(III)/H_2O_2 in a ratio 1:3, to yield MbFe(IV)═O, or (d) MbFe(III) to a final concentration 20 μM, in linoleate emulsions and with a lipid/heme protein ratio of 100 (pH 7.4; 25 °C). For spectra b′, c′, and d′ conditions were identical to those used for spectra b–d, respectively, except that the lipid/heme protein ratio was 200. For spectra b″, c″, and d″ conditions were identical to those used for spectra b–d, respectively, except that the lipid/heme protein ratio was 300.

in the Soret absorption band, known to be very sensitive to detachment of the porphyrin moiety from the globin (**Figure 1C**). Linoleate peroxidation induced by MbFe(IV)═O and the heme protein degradation that occurred during incubation were, however, unaffected by the LA/HP ratio, indicating that the mechanism responsible for the pro-oxidative activity of MbFe(IV)═O is independent of the LA/HP ratio. Additionally, reduction of preformed lipid hydroperoxides did not affect the prooxidative activity of MbFe(IV)═O (data not shown), and at physiological pH the prooxidative activity of MbFe(IV)═O is believed to be independent of the presence of preformed lipid hydroperoxides.

Depending on the LA/HP ratio, MbFe(III) was either non-oxidative or pro-oxidative in agreement with previous studies (*18*, *22*). At a low LA/HP ratio (100), MbFe(III) was an ineffective pro-oxidant (**Table 1**; **Figures 2** and **3**). Kendrik and Watts (*18*) suggested that binding of fatty acids to heme proteins simply reduces the amount of free fatty acids accessible to the heme group, thereby preventing oxidation of fatty acids by heme proteins. However, it has recently been shown that association of free fatty acid anions with MbFe(III) converts this species to its non-pro-oxidative low-spin derivative, hemichrome (*34*). The conversion of MbFe(III) to hemichrome appears to be reversible and to proceed via combined electrostatic and hydrophobic interactions between fatty acid anions and the heme proteins. This reversible binding induces conformational changes in the heme protein, resulting in further binding of the distal histidine to the heme iron blocking the access of fatty acids or their anions to the heme center. The rapid decay in the Soret absorption upon addition of MbFe(III) to linoleic acid and the shift to longer wavelength of the Soret absorption maximum (from 410 to 412 nm), observed a few minutes after mixing, confirm such a conversion of MbFe(III) to hemichrome also under these conditions (*34*, *39*, *40*).

Addition of MbFe(III) to linoleate emulsions containing H_2O_2 did not result in formation of •MbFe(IV)═O as is seen in homogeneous solution. This lack of reaction is readily explained by the recent findings which show that for mixtures of fatty acid anions and hydrogen peroxides, MbFe(III) preferentially binds the fatty acid anions generating hemichrome, rather than reacting with H_2O_2, to generate •MbFe(IV)═O (*34*). Because hemichrome cannot be activated by hydrogen peroxide to high oxidation state myoglobin species (*34*, *41*), combinations of MbFe(III) and H_2O_2, at an LA/HP ratio of ~100, result in the formation of hemichrome rather than •MbFe(IV)═O. The situation encountered with MbFe(III) plus H_2O_2 under these conditions is therefore very similar to that with MbFe(III) alone. The presence of a significant amount of hydrogen peroxide (12 μM) in the reaction mixture did not result in MbFe(III)-initiated lipid peroxidation; therefore, we argue that a low peroxide level cannot explain the non-pro-oxidative activity of MbFe(III), as previously reported in a similar study (*38*).

At an LA/HP ratio of 200, MbFe(III) exhibited pro-oxidative activity, although a relatively long lag period was observed before lipid peroxidation proceeded. Therefore, this ratio seems to be critical for the pro-oxidative activity of MbFe(III). In agreement with our findings, Kendrik and Watts (*18*) reported that maximum pro-oxidative activity for MbFe(III) was obtained at an LA/HP ratio of 250. At a higher LA/HP ratio of 300, MbFe(III) initiated the oxidation of linoleate without any noticeable lag period. These results cannot be explained on the basis of increasing amount of peroxides with increasing linoleate concentrations. Similar experiments performed with an oxidized linoleate sample or a reduced linoleate, in which lipid hydroperoxides are expected to be high and low, respectively, gave identical results (not shown). We suggest that at these higher LA/HP ratios of 200 and 300, a loss of integrity of the heme protein occurs as deduced from the complete loss of the Soret absorption band, and other mechanisms for the pro-oxidative activity of MbFe(III) have to be considered (**Figure 1**). The relatively long lag period observed for an LA/HP ratio of 200 indicates that the loss of the integrity of the heme protein proceeds through conformational or other structural changes and is a relatively slow process initiated by binding of fatty acid anions to the MbFe(III) molecule. It is urgent to emphasize that this dynamic process slowly destabilizes the heme protein, resulting in complete loss of its integrity and, therefore, the pro-oxidative activity of MbFe(III) at high LA/HP ratios cannot be compared directly to metal ion or hematin-induced peroxidation. The relatively long lag period observed in our experiments is believed to be the consequence of the slow but continuos denaturation of MbFe(III) with subsequent exposure of the heme group rather than due to a low level of hydroperoxide present in the reaction mixture.

Similarly, MbFe(III)/H_2O_2 was found to induce oxidation of linoleate at high LA/HP ratios (200 and 300), via similar degradation of the heme protein (**Figure 1**). However, hydrogen peroxide appeared to enhance the pro-oxidative activity of MbFe(III) as the lag period at an LA/HP ratio of 200 decreased compared to the system without hydrogen peroxide.

The denaturation of MbFe(III) at high LA/HP ratios is in contrast to the minor heme protein denaturation observed during MbFe(IV)═O incubation with linoleate emulsions. Similarly, under acidic conditions MbFe(III) has been reported to induce

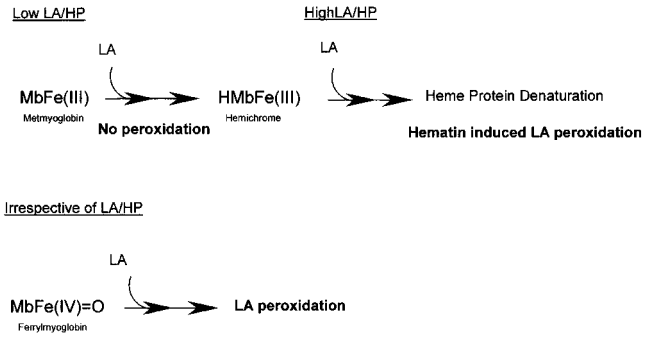

Figure 4. Schematic representation of the two different mechanisms proposed for heme protein (HP) induced linoleic acidic peroxidation (LA) as a function of the different LA/HP ratios.

lipid peroxidation without any drastic heme protein denaturation (*38*). This clearly demonstrates that the different myoglobins are able to initiate lipid oxidation via different mechanisms, depending on the conditions, such as pH and free fatty acid concentration. Its seems that the presence of high amounts of fatty acid relative to heme protein concentration results in physical changes in the MbFe(III) molecule with subsequent exposure of the heme group to the lipid environment. Therefore, lipid oxidation is probably induced by hematin rather than by the MbFe(III) itself. This hypothesis is supported by recent findings showing that hemoglobin interacts with liposomes, resulting in either hemichrome formation or dissociation of the heme from the globin transferred to the lipid environment (*40*, *42*). The heme group itself is very hydrophobic and can associate with membranal lipid (*43*), where it can effectively initiate lipid peroxidation (*44*). Moreover, heme proteins have been reported to have affinity for negatively charged surface lipids such as phophatidylserine, supporting the proposed interaction between heme proteins and fatty acid anions (*45*). The affinity of heme and hemoglobin to the lipid constituents of membranes may be explained by the catabolism of heme proteins, by which the heme group is transferred to the phospolipid membrane, removed and transported to the liver by serum albumin, and degraded (*14*).

Combined, these results suggest that the presence of high concentrations of fatty acids promotes physical changes and denaturation of heme proteins such as MbFe(III), as part of a mechanism that may be significant both in vivo and in muscle-based foods with high content of free fatty acids. In this process, the heme group is exposed to the lipid environment, and hematin can effectively induce lipid peroxidation. Moreover, hematin has been shown to be a more efficient catalyst of lipid oxidation in the presence of peroxides possibly accounting for the observed enhanced pro-oxidative activity of MbFe(III)/H_2O_2, compared to MbFe(III) alone (*46*). Such a hematin-induced lipid oxidation mechanism is new, differing completely from the well-described catalytic activity of heme proteins, where lipid oxidation is primarily initiated by binding of lipid hydroperoxide in the heme cavity, for example, for MbFe(III)-initiated lipid oxidation under more acidic conditions (*38*) and as outlined in **Figure 4**.

The present findings reveal (i) that irrespective of the lipid/heme ratio, MbFe(IV)═O is a strong pro-oxidant able to initiate lipid oxidation, (ii) that MbFe(III), in the presence of moderate amounts of fatty acid anions, is not a pro-oxidant as it is converted to hemichrome, and (iii) that high lipid concentrations destabilize the MbFe(III) molecule and result in lipid oxidation by a mechanism involving direct exposure of the heme group to the lipids. This clearly indicates that myoglobins are able to initiate lipid oxidation by at least two different mechanisms: (i) catalytic cleavage of lipid hydroperoxides in the heme crevice or (ii) initial denaturation of the heme protein structure, resulting in exposure of the heme, making subsequent hematin-induced peroxidation possible.

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