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Myoglobin Species with Enhanced Prooxidative Activity Is Formed during Mild Proteolysis by Pepsin

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Pepsin proteolysis at pH ~4 resulted in a lowering of the (pseudo)peroxidase activity of metmyoglobin both at physiological pH and at meat pH, as measured by a peroxidase assay with H_2O_2 and ABTS as substrates. In contrast, the mildly proteolyzed myoglobin had a strongly enhanced prooxidative effect on lipid oxidation in an oil in water methyl linoleate emulsion compared to native metmyoglobin, as evidenced by rates of oxygen depletion. More severe proteolysis of metmyoglobin at lower pH values near the optimum for pepsin did not result in a similar enhancement of prooxidative activity. The mildly proteolyzed metmyoglobin had spectral characteristics in agreement with a relative stabilization of the iron(II) state. On the basis of the observed effects of metal chelators, of lipophilic and hydrophilic peroxides and of radical scavengers on oxygen depletion rates, it is suggested that the increased prooxidative effect is due to radicals formed by cleavage of lipid peroxides by iron(II)/iron(III) cycling of a heme pigment with affinity for the lipid/water interface.

KEYWORDS: Lipid oxidation; myoglobin catalysis; proteolysis

INTRODUCTION

The muscle heme pigment myoglobin is known to have prooxidative properties (*1*, *2*), and current research is focused on a mechanistic understanding of the reactions by which this prooxidative activity is exerted. While heme pigments are less catalytically active at normal physiological conditions, other conditions seem to increase their prooxidative activity, and it is fairly well established that the reactivity of myoglobin and hemoglobin is enhanced by low pH (*3*−*5*), as found in e.g. meat products, in the digestive tract, and under certain pathological in vivo conditions.

In addition to the effect of decreased pH, other factors such as oxidative modifications, proteolysis, and salt, acid, and heat treatments may influence the prooxidative activity of heme pigments. Enhanced prooxidative effects of myoglobin or hemoglobin have thus been observed upon heating (*6*), treatment with acid (*7*), and reactions with lipid oxidation products such as 4-hydroxy-*trans*-2-nonenal (HNE) (*8*). In these cases, a conformational change of the heme proteins was suggested to increase the access to the catalytic iron center and hence the prooxidative activity.

Conditions of proteolysis are found during post mortem tenderization processes in meat and in the digestive tract. Recent studies pay attention to the significance for human health of oxidative processes in the digestive tract, which proceed upon ingestion of various types of meals (*9*−*11*). Extensive oxidation of dietary lipids may occur during the digestive process, where the conditions are clearly different from other in vivo conditions, and it seems that less well absorbed antioxidants play an important role in the digestive tract (*10*). Nevertheless, the digestive tract may be damaged by accelerated oxidation processes; additionally, the occurrence of arteriosclerosis has been linked to absorption of lipid oxidation products (*12*, *13*).

Proteolysis of cytochrome *c* with pepsin yields the so-called microperoxidase-11, a small heme-binding peptide, which contains 11 amino acid residues and has peroxidase activity (*14*−*16*). Myoglobin is more resistant to proteolytic digestion than cytochrome *c* (*17*), but a heme-binding fragment was obtained by Schwarzinger et al. (*18*) upon pepsin proteolysis of sperm whale apo-myoglobin. This micro-myoglobin consisted of 77 amino acid residues but has not been further characterized with respect to oxygen binding and redox activity. We speculate whether such products of myoglobin proteolysis, which retain their heme binding properties, may be active in oxidation processes and even possess significant prooxidative activity. Accordingly, proteolysis processes in meat and in the digestive tract should be viewed as having the potential of enhancing the prooxidative effect of heme pigments.

On the basis of the above considerations, we initiated experiments of myoglobin proteolysis, and we here report our initial findings that some pepsin-proteolyzed myoglobin solutions exert a remarkably high prooxidative activity.

MATERIALS AND METHODS

Chemicals. Pepsin from porcine stomach mucosa (pepsin A; EC 3.4.23.1, 871 units/mg protein), methyl linoleate, polyoxyethylenesorbitane monolaurate (Tween 20), horse heart metmyoglobin (MbFe(III)),

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hematin, trolox, α-tocopherol, cumene hydroperoxide, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid, diammonium salt) (ABTS), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were from Sigma (St. Louis, MO). Sodium dithionite ($Na_2S_2O_4 \cdot xH_2O$) was from Bie & Berntsen Laboratory (Bie & Berntsen A/S, Rødovre, Denmark). Methanol was from Lab-Scan (Dublin, Ireland), and hydrogen peroxide (H_2O_2) and all other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

Proteolysis of Myoglobin. Samples (1.40 mL) of approximately 1 mM metmyoglobin dissolved in purified H_2O were mixed with diluted HCl of various concentrations (1:1 v/v) and incubated with pepsin (dissolved in 50 mM acetate buffer, pH 4.5) at 37.0 °C. The myoglobin-to-pepsin ratio was approximately 90:1 (w/w), and the concentrations of the diluted HCl solutions were chosen to obtain different pH values during proteolysis. Immediately after 15 min of incubation, equal volumes of 0.60 M $NaHCO_3$ and 0.10 M NaOH were added to the samples to adjust the pH to neutral and thereby inhibit further proteolysis of myoglobin. During the proteolysis procedure, myoglobin was diluted by a factor of 3.63 with respect to the initial (approximately 1 mM) myoglobin solution. Exact concentrations of initial myoglobin solutions were determined spectrophotometrically, $\epsilon_{525\,nm} = 7700\ M^{-1}\ cm^{-1}$ (*19*), and for further experiments with the proteolyzed samples their concentrations were calculated in terms of the initial content of myoglobin by use of the actual dilution factor. HCl-treated myoglobin samples were obtained by the same procedure as described above, except that aliquots of acetate buffer was added to the samples instead of pepsin solution. For all samples, pH was measured immediately after pepsin digestion and again after readjustment of pH to neutral conditions. A Me6.0224.100 combination glass microelectrode connected to a Methrom 713 pH-meter (Methrom, Herisau, Switzerland) was used.

SE-HPLC Analysis of Myoglobin Samples. Size exclusion HPLC (SE-HPLC) was started 15 min after pressure release, and prior to injection the samples were diluted to 56.2 μM (1.0 mg/mL) with the mobile phase, which consisted of 50 mM MES buffer (pH 6.1). The HPLC equipment consisted of a Waters 600 E multisolvent system, a Waters 700 satelite wisp injector, and a Waters H90 programmable multiwavelength detector (Waters, Division of Millipore, Milford, MA), and the columns used were a TSK Gel G 2000 SWXL column (300 mm × 6 mm i.d.) and a TSK Gel G 2000 SWXL guard column (40 mm × 6 mm i.d.) (Tosohaas GmbH, Stuttgart, Germany). Twenty microliters of each sample was injected, and the components were eluted at a flow rate of 0.8 mL/min and detected at 220 nm. Total elution time was 25 min.

Oxygen Consumption Measurements in Methyl Linoleate Emulsions. Methyl linoleate emulsions were made by evaporating a methanol solution of methyl linoleate and Tween 20 by use of a flow of nitrogen, and subsequently adding 1.95 mL of air-saturated 0.10 mM MES buffer (pH 5.8). To the emulsions were then added immediately 25 μL of initiator and eventually 25 μL of inhibitor, and the emulsions were transferred to a thermostated cell (25.0 °C) equipped with a micro-cathode Clark oxygen electrode (Sable Systems, Henderson, NV), measuring the oxygen concentration every 0.8 s by a ReadOX-4 oxygen analyzer (Sable Systems). A two-point calibration [air-saturated water (25.0 °C) and N_2-saturated water (25.0 °C, and added $Na_2S_2O_4$ to remove residual oxygen)] was used to calibrate the electrode and the oxygen analyzer before use. In all experiments, concentrations in the reaction samples were 7.09 mM methyl linoleate and 5.1 mg/mL Tween 20, whereas initiators and inhibitors were diluted appropriately with 0.10 mM MES buffer (pH 5.8) before addition to obtain the desired concentrations. In experiments where linear oxygen consumption curves ($d(\%O_2)/dt$) were obtained as lipid oxidation progressed in the methyl linoleate emulsion, oxygen depletion rates ($\mu M \cdot s^{-1}$) were calculated from their slopes (α) by

$$v(O_2) = -\alpha\,[O_2]_{initial} \times 10^6/100 \qquad (1)$$

where water air-saturated at 25.0 °C, $[O_2]_{initial} = 2.6 \times 10^{-4}$ M, corresponds to the 100% calibration point. When nonlinear oxygen consumption curves were obtained, only qualitative comparisons of different experiments were made. For each oxygen depletion experiment, the pH was measured in excess reaction sample.

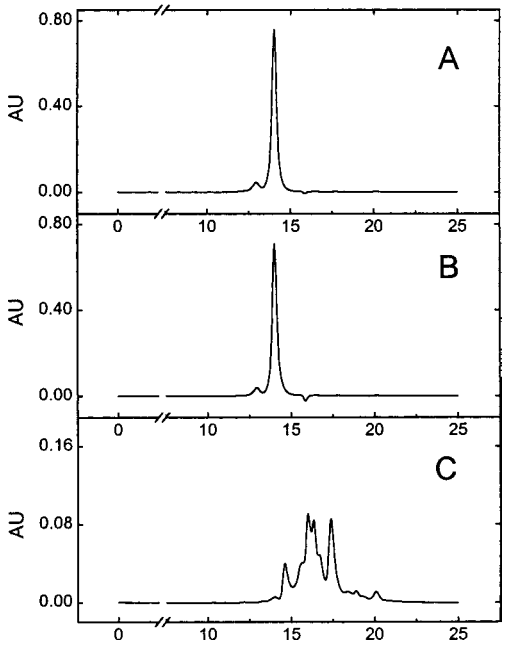

Figure 1. SE-HPLC profiles of MbFe(III) samples either HCl-treated at pH 3.95 (B) or subjected to pepsin proteolysis at pH 3.92 (C). The samples were treated for 15 min at 37 °C, and for comparison a profile of untreated MbFe(III), i.e., dissolved at neutral pH, is included (A). Before injection, samples were diluted with 50 mM MES buffer (pH 6.1) to approximately 56.2 μM (1.0 mg/mL, as estimated on the basis of the initial MbFe(III) concentration of the individual sample). The largest molecular size compounds elute first on this type of column.

Assay for Peroxidase Activity of Myoglobin Samples. The activity for one-electron oxidation of ABTS at pH 7.4 or 5.8 was measured for MbFe(III) in the presence of H_2O_2 at 25.0 °C by the use of an HP8453 UV−vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). Formation of the $ABTS^{\bullet+}$ radical was followed by absorbance readings between 400 and 800 nm with time intervals of 8 s, and values for $d[ABTS^{\bullet+}]/dt$ were determined from the increase in absorbance versus time at 734 nm ($\epsilon_{ABTS^{\bullet+},734\,nm} = 1.5 \times 10^4\ M^{-1} \cdot cm^{-1}$ (*20*)). The final reaction volumes were 2.00 mL and contained 0.5 μM MbFe(III), 1.0 mM ABTS, and variable $[H_2O_2]$ (0.025−0.850 mM) in aqueous 50 mM MES or phosophate buffer.

RESULTS

Incubation of MbFe(III) solutions with pepsin at different pH values resulted in proteolytic digestion of myoglobin, as may be seen from the SE-HPLC profile of a MbFe(III) solution pepsin-digested at pH 3.92 (**Figure 1C**), in comparison with the SE profiles for nontreated MbFe(III) and for MbFe(III) incubated at pH 3.95 with HCl (**Figure 1A,B**). Incubation with pepsin at even lower pH values resulted in more pronounced degradation of myoglobin (not shown), in agreement with an optimum at pH ∼2 for the proteolytic activity of pepsin (*21*). Incubations with pepsin at neutral pH values likewise did not result in proteolytic degradation of myoglobin.

The color of Mb samples proteolyzed at pH values in the range of 3.5−4.3 changed remarkably when the pH was adjusted back to neutral values following proteolysis. These samples had a brighter red appearance than the typical dark brown MbFe-(III) solutions, as also illustrated by the visible absorption spectra shown in **Figure 2**. A comparison of the absorption spectra reveals that at least some heme protein structure of myoglobin is conserved upon proteolysis at pH 3.92. In contrast, the more

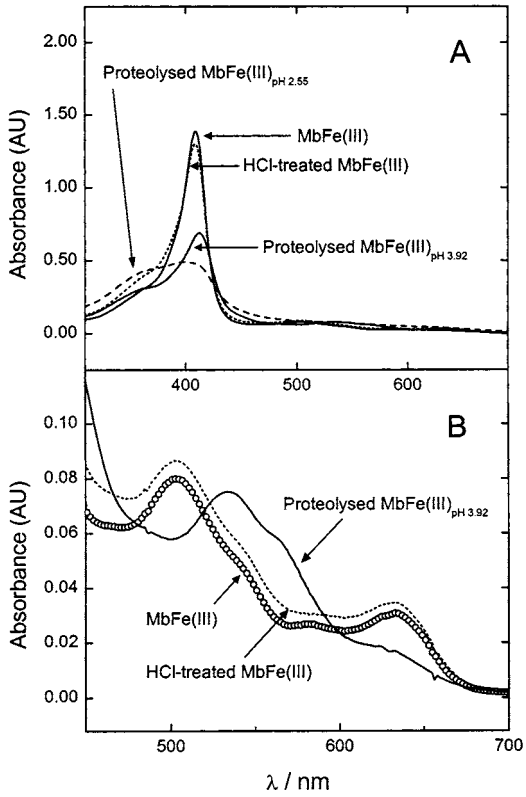

Figure 2. Absorption spectra (A, Soret band; B, α- and β-bands) in aqueous 0.10 M MES buffer solutions (pH 5.8, 20 °C) of 8 μM myoglobin samples either HCl-treated at pH 3.95 or subjected to pepsin proteolysis at pH 2.55 (only in A) or at pH 3.92. For comparison a spectrum of untreated MbFe(III), i.e., dissolved at neutral pH, is included. Concentrations of the samples are estimated on the basis of the initial MbFe(III) concentration, i.e., before HCl or pepsin treatment.

extensively proteolyzed Mb sample (at pH 2.55) had an indistinct absorption spectrum with loss of the typical myoglobin absorption bands, and the flat Soret band of this sample (see **Figure 2A**) is indicative of dimerization or polymerization of released heme iron (*22*).

The prooxidative activity of the various Mb samples was tested in methyl linoleate emulsions, and addition of Mb samples proteolyzed in the pH range 3.5−4.3 turned out to cause a much faster rate of oxygen depletion than the other Mb samples did. The oxygen consumption curves in **Figure 3** illustrate this enhancement of prooxidative activity, and the blank curves (containing no myoglobin) also included in **Figure 3** show that the prooxidative activity in the samples was not due to a presence of small impurities of metal ions in solvent or reagents as oxidation catalysts. A detailed comparison of oxygen depletion rates obtained upon addition of the Mb samples to the methyl linoleate emulsions is presented in Table 1. The rates of oxygen depletion fell into three groups: (i) the slowest rates were obtained for emulsions with non-proteolyzed MbFe(III) added; (ii) intermediate rates were obtained for emulsions with either hematin or MbFe(III) proteolyzed at pH 2.21 and 2.55 added; and (iii) remarkably high rates were obtained for emulsions with MbFe(III) proteolyzed at pH 3.5−4.3 added. Thus, proteolysis of MbFe(III) with pepsin at pH 3.5−4.3 apparently resulted in a strong prooxidative activity. Due to the presence in pepsin of amino acid residues with reducing properties, an antioxidative activity of the enzyme is a possibility. However, as the proteolyzed samples are even more prooxidative relative to the non-proteolyzed samples (without pepsin added), an antioxidative activity of pepsin may only have somewhat counteracted the high difference in the oxygen depletion rates between proteolyzed and non-proteolyzed samples.

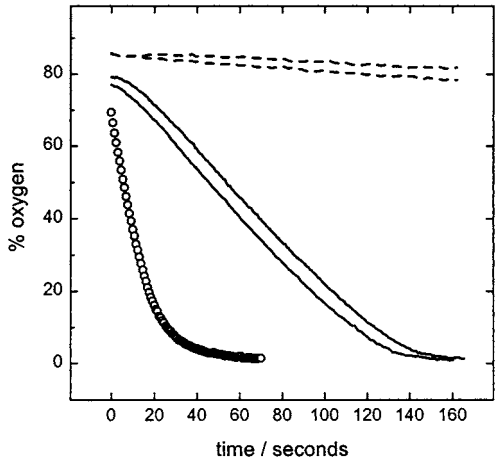

Figure 3. Examples of oxygen consumption curves as a measure of oxidation in methyl linoleate emulsions (pH 5.8, 25 °C) in the presence of untreated MbFe(III) (full lines, two experiments) or MbFe(III) proteolyzed by pepsin at pH 3.92 for 15 min at 37.0 °C (circles). Dotted lines are blank samples, i.e., pure methyl linoleate emulsion (pH 5.8, 25 °C). See Table 1 for comparison of oxygen consumption rates calculated on the basis of all experimental curves.

Table 1. Average Oxygen Depletion Rates (±Standard Error) in Methyl Linoleate Emulsions (pH 5.8, 25 °C) in the Presence of Initiators or Inhibitors of Oxidation (Concentrations of Initiators, 0.80 μM; Concentrations of Proteolyzed MbFe(III) and HCl-Treated MbFe(III) Are Estimated on the Basis of Their Initial MbFe(III) Concentration; See Experimental Section for Further Details of HCl Treatment and Proteolysis)

initiator/inhibitor	oxygen depletion rate, μM $O_2 \cdot s^{-1}$	no. of expts
MbFe(III)	1.76 ± 0.19^a	17
proteolyzed MbFe(III), pH 2.21	2.91 ± 0.46^b	6
proteolyzed MbFe(III), pH 2.55	3.25^b	2[e]
proteolyzed MbFe(III), pH 3.52	7.53 ± 0.36^c	4
proteolyzed MbFe(III), pH 3.82	7.62 ± 0.46^c	7
proteolyzed MbFe(III), pH 3.92	7.06 ± 0.92^c	14
proteolyzed MbFe(III), pH 4.22	7.15 ± 0.60^c	3
HCl-treated MbFe(III), pH 3.95	1.84 ± 0.17^a	3
hematin	3.27 ± 0.70^b	8
MbFe(III) + EDTA, 0.10 mM	1.77^a	2[e]
MbFe(III) + ascorbate, 0.19 mM	1.94^a	2[e]
MbFe(III) + ascorbate, 1.90 mM	1.11^d	2[e]
proteolyzed MbFe(III), pH 3.92 + EDTA, 0.10 mM	6.69^c	2[e]
proteolyzed MbFe(III), pH 3.92 + ascorbate, 0.19 mM	7.40^c	1
proteolyzed MbFe(III), pH 3.92 + ascorbate, 1.90 mM	3.39^b	2[e]

[a−d] *Oxygen consumption rates bearing different superscripts are significantly different* ($p < 0.001$). [e] Two independent determinations were performed for this sample type, and the oxygen depletion rate is therefore given as the average value only.

To investigate further the observed enhancements of prooxidative activity, different compounds known to affect free radical processes were added to the methyl linoleate emulsions. The effects of these compounds on oxygen consumption in emulsions to which was added either nontreated MbFe(III) or proteolyzed MbFe(III) at pH 3.92 were explored in order to understand why an enhanced prooxidative activity was seen upon pepsin proteolysis.

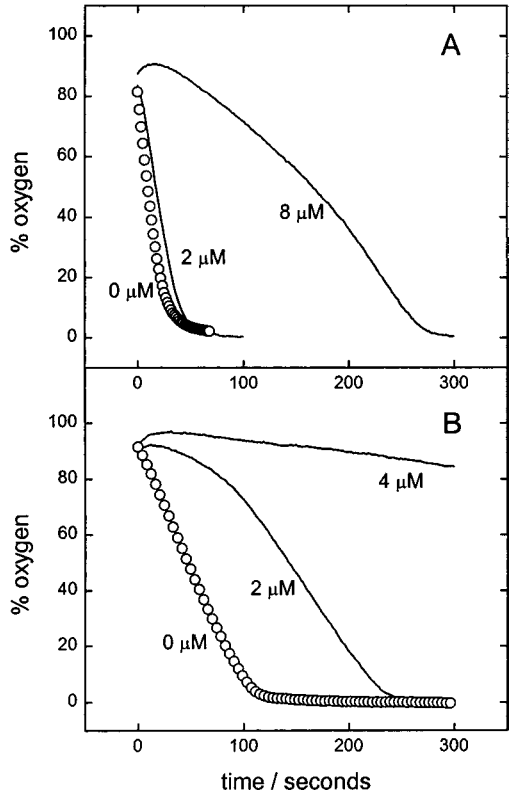

Figure 4. Qualitative comparison of the effect of Trolox on oxygen consumption in methyl linoleate emulsions (pH 5.8, 25 °C) with either MbFe(III) proteolyzed by pepsin at pH 3.92 (A) or untreated MbFe(III) (B) added. Trolox concentrations were as indicated, and the myoglobin concentration was 0.8 μM in all reaction mixtures.

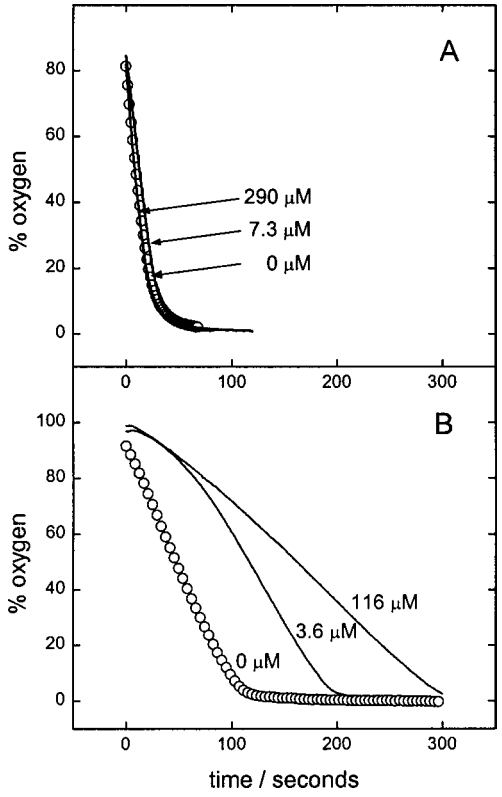

Figure 5. Qualitative comparison of the effect of α-tocopherol on oxygen consumption in methyl linoleate emulsions (pH 5.8, 25 °C) with either MbFe(III) proteolyzed by pepsin at pH 3.92 (A) or untreated MbFe(III) (B) added. α-Tocopherol concentrations were as indicated, and the myoglobin concentration was 0.8 μM in all reaction mixtures.

The water-soluble antioxidants ascorbate (see Table 1) and Trolox (see **Figure 4**) had an inhibiting effect on oxygen consumption in the methyl linoleate emulsions when added in sufficiently high concentrations. For the lipid-soluble α-tocopherol, however, much higher concentrations were needed to delay the rate of oxygen depletion (see **Figure 5**), and for emulsions with proteolyzed MbFe(III) at pH 3.92, no inhibiting effect was observed at all, even upon addition of α-tocopherol to a concentration of 290 μM.

Addition of EDTA did not affect the rate of oxygen depletion in the methyl linoleate emulsions (see Table 1), suggesting that non-heme iron, eventually released during myoglobin proteolysis, does not influence the oxidation process in the emulsions. Neither was observed any prooxidative effect of addition of non-heme ferro-ions (added in the form of freshly prepared solutions of $NH_4Fe(SO_4)_2$), as evidenced from their inability to induce a measurable consumption of oxygen in the methyl linoleate emulsions [$v(O_2) = 0.08 \pm 0.02$ $\mu M \cdot s^{-1}$ ($n = 3$), not different from the rate of oxygen depletion in pure methyl linoleate emulsions].

One hypothesis often discussed in relation to the prooxidative behavior of myoglobin is the formation of hypervalent myoglobin species upon reactions between hydroperoxides and MbFe(III) or MbFe(II) (*4*, *10*). Accordingly, we investigated the effect of pepsin proteolysis on the peroxidase activity of myoglobin by means of a classical peroxidase assay using H_2O_2 and ABTS as substrates. As can be seen in **Figure 6**, the pH dependence of the peroxidase activity was similar for the proteolyzed Mb samples and for the nontreated MbFe(III), cf. **Figure 6A,B**. However, the peroxidase activity of MbFe(III) was lowered by pepsin proteolysis, and the decrease in activity followed the extent of proteolysis, with proteolyzed MbFe(III) at pH 2.21 having less peroxidase activity than proteolyzed MbFe(III) at pH 3.92. Thus, the observed higher prooxidative activity of proteolyzed MbFe(III) at pH 3.92 was not reflected by a higher peroxidase activity when measured with H_2O_2 and ABTS as substrates.

In agreement with the results in **Figure 6**, we found that addition of H_2O_2 to the methyl linoleate emulsions increased the rate of oxygen depletion if nontreated MbFe(III) was present (**Figure 7**), whereas no detectable increase in oxygen depletion rates was observed following addition of H_2O_2 to emulsions with proteolyzed MbFe(III) at pH 3.92. However, as can also be seen in **Figure 7**, very different oxygen depletion curves emerged after addition of the more hydrophobic cumene hydroperoxide to the methyl linoleate emulsions instead of H_2O_2, indicating that the effect of myoglobin peroxidase activity is dependent on the type of peroxide, which is reduced during redox cycling.

DISCUSSION

The high rates of oxygen depletion obtained in methyl linoleate emulsions by adding solutions of mildly pepsin-proteolyzed myoglobin indicate that myoglobin fragments with high prooxidative activity are formed under conditions of proteolysis.

It seems reasonable to assume that some of the digestion products of pepsin proteolysis in the pH range 3.5−4.3 are polypeptide fragments, which retain their heme-binding properties and which have a higher prooxidative activity than both native MbFe(III) and hematin. These heme-binding myoglobin fragments are further degraded by more extensive proteolysis

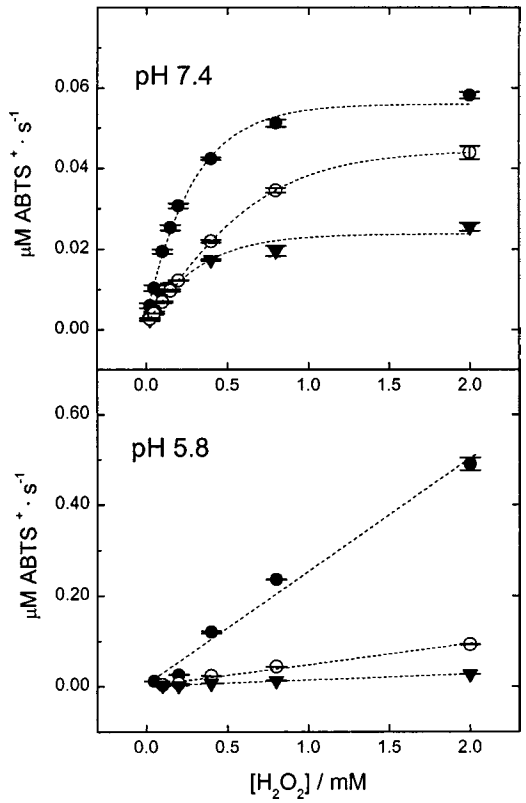

Figure 6. Rate of formation of ABTS•+ as a function of $[H_2O_2]$ at 20.0 °C in reaction mixtures of 0.5 μM myoglobin sample (based on initial MbFe(III) concentrations), 1.0 mM ABTS, and 0.025–2.0 mM H_2O_2 in aqueous 50 mM phosphate buffer (pH 7.4) or MES buffer (pH 5.8). ●, untreated MbFe(III); ○, MbFe(III) proteolyzed at pH 3.82 with pepsin; ▼, MbFe(III) proteolyzed at pH 2.21 with pepsin.

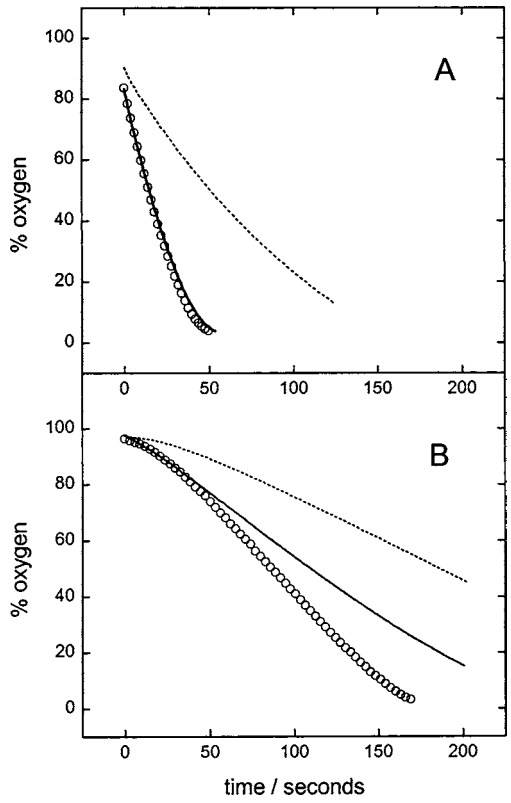

Figure 7. Effect of H_2O_2 (0.08 mM) or cumene hydroperoxide (0.8 mM) on oxygen consumption in methyl linoleate emulsions (pH 5.8, 25 °C) in the presence of 0.80 μM MbFe(III) proteolyzed by pepsin at pH 3.92 for 15 min at 37.0 °C (A) or 0.80 μM nontreated MbFe(III) (B). Average curves ($n = 2$) are shown. Circles, H_2O_2 addition; dotted lines, cumene hydroperoxide addition; full lines, no addition.

(at lower pH), and they apparently lose their ability to bind heme iron, since proteolyzed MbFe(III) at pH 2.55 had a prooxidative activity comparable with that of hematin. The finding that non-heme iron does not accelerate oxygen consumption in the investigated system is in accordance with previous observations (*6*) and excludes a Fenton-like mechanism where non-heme iron originating from myoglobin is responsible for the observed prooxidative effects.

For native MbFe(III), its reaction with lipid hydroperoxides is believed to accelerate oxidation processes and to occur in either one- or two-electron-transfer reactions:

$$\text{MbFe(III)} + \text{LOOH} \rightarrow \text{MbFe(IV)=O} + \text{LO}^{\bullet} + \text{H}^{+} \quad (2)$$

$$\text{MbFe(III)} + \text{LOOH} \rightarrow {}^{\bullet}\text{MbFe(IV)=O} + \text{LOH} \quad (3)$$

For the one-electron-transfer reaction (eq 2), a lipid alkoxy radical is directly formed, whereas in the two-electron-transfer reaction (eq 3), which is the first reaction of a peroxidase mechanism, the protein radical perferrylmyoglobin (•MbFe(IV)=O) is formed. The prooxidative effect of eq 3 depends on the nature of the compounds to be oxidized by the very reactive •MbFe(IV)=O.

It is possible that reactions such as those in eqs 2 and 3 account for the accelerating effect of myoglobin species on the oxygen consumption in methyl linoleate emulsions. This consideration is based on the observed, and somewhat surprising, inhibiting effect of adding cumene hydroperoxide to the emulsions. Cumene hydroperoxide, which is lipid-soluble, is known to react with native MbFe(III) in reaction types similar to those of eqs 2 and 3, with the two-electron-transfer reaction dominating (*23*). We speculate that reactions between myoglobin species (or hematin) and cumene hydroperoxide in the methyl linoleate emulsions may have occurred at the expense of similar reactions with LOOH, and that the reaction products obtained hereby are less oxidizing. Notably, cumene hydroperoxide, when the reaction is a one-electron-transfer process, forms an alkoxyl radical only as a reaction intermediate, which is instantaneously transformed into acetophenone and a methyl radical (*24*), which is less catalytic in lipid oxidation. When the reaction is a two-electron-transfer process, •MbFe(IV)=O and cumyl alcohol are reaction products (*23*), and according to the observed inhibiting effect of cumene hydroperoxide addition, it seems that •MbFe(IV)=O is rather inefficient in initiating lipid oxidation in the emulsions, relative to alkoxyl radicals formed from lipid hydroperoxides.

A shift in the ratio between one- and two-electron-transfer reactions in favor of eq 2 for the proteolyzed myoglobin compared to the native myoglobin may explain the higher prooxidative activity observed following proteolysis. In agreement with an increasing importance of one-electron transfer for the proteolyzed myoglobin, a decrease in peroxidase activity was observed upon proteolysis, since it depends on formation of •MbFe(IV)=O. The increased prooxidative activity following mild proteolysis of myoglobin may thus mainly rely on a more extensive formation of lipid alkoxyl radicals in reactions with lipid hydroperoxides.

The effect on oxygen consumption observed when H_2O_2 was added to the emulsions indicates that the location of formed radicals in the emulsion is important, since the radicals formed

upon reaction of myoglobin species with H_2O_2, in contrast to cumene hydroperoxide, will be located in the water phase. Adding H_2O_2 to methyl linoleate emulsions containing native MbFe(III) only slightly increased the oxygen consumption, whereas no effect of H_2O_2 additon was seen in emulsions containing proteolyzed myoglobin fragments. This difference may be due to the lower peroxidase activity of proteolyzed myoglobin, but it is also likely that the more apolar proteolyzed myoglobin fragments are located closer to the surface of the methyl linoleate droplets, and hence are less available for reactions with H_2O_2. Such a difference in location may also explain why proteolyzed myoglobin fragments exhibited a higher prooxidative activity, as LOOH cleavage should be favored by an increased accessibility of the peroxide and the heme iron.

The different absorption characteristics observed for the native and proteolyzed myoglobin solutions indicate that the proteolyzed myoglobin fragments are more stable in their reduced state (as oxygenated or deoxygenated heme−Fe(II) species) than native myoglobin (see **Figure 2B**). This led to another suggestion for the observed prooxidative effect of proteolyzed myoglobin, as reactions involving the Fe(II) state might be more prooxidative in the methyl linoleate emulsion. Hence, the breakdown of LOOH may occur in a Fenton-like mechanism:

$$\text{MbFe(II)} + \text{LOOH} \rightarrow \text{MbFe(III)} + \text{LO}^{\bullet} + \text{OH}^{-} \quad (4)$$

The prooxidative activity in lipid emulsions of the native form of $MbFe(II)O_2$ has previously been shown to be slightly lower that the prooxidative activity of native MbFe(III) (*25*, *26*). However, the recent observation that hemoglobins with decreased oxygen affinity are very prooxidative (*4*) suggests that deoxyheme proteins are important prooxidants, and if the proteolyzed myoglobin fragments have a lower oxygen affinity than native myoglobin, a significant prooxidative role of their reduced state can be expected.

The indications obtained in the present study that proteolytic digestion of myoglobin may result in heme-binding fragments with higher prooxidative activity than native myoglobin deserve further attention. A preparative fractionation of the proteins in pepsin-proteolyzed myoglobin samples will allow exact identification and characterization of prooxidative myoglobin fragments. Further, prooxidative effects of myoglobin proteolyzed by other enzymes (also with relevance for meat products) should be explored. In this respect, it is interesting to note that thermal denaturation and binding of phenols have been shown to increase the susceptibility of myoglobin to proteolytic digestion (*17*, *27*), pointing at the possibility that proteolysis of myoglobin may occur also at conditions less extreme than those found under gastric digestion.

Heme iron catalysis of lipid oxidation may occur by several mechanisms. The mechanism originally suggested by Tappel (*28*) involves formation of a lipid peroxyl anion complex with a heme iron center, which decomposes into a lipid alkoxyl radical and further transfers a lipid into a carbon-centered radical without any formal change in the iron oxidation state. More recently, both a heme iron(II)/iron(III) mechanism, comparable to the well-known Fenton mechanism for simple iron salts, and a heme iron(III)/iron(IV) mechanism have been described (*1*, *2*). Both of these catalytic cycles are based on one-electron-transfer processes, in contrast to the pseudoperoxidase mechanism, which depends on initial two-electron transfer. The mildly proteolyzed metmyoglobin clearly does not operate through a peroxidase mechanism, since the native pigment was found to have higher peroxidase activity. The modified pigment rather exerts its catalytic activity through a relative stabilizing of the iron(II) state and through an iron(II)/iron(III) cycling mechanism. The insensitivity to the water-soluble antioxidants Trolox and ascorbate in low concentrations favors this mechanism over an iron(III)/iron(IV) mechanism, since hypervalent iron will be efficiently reduced by ascorbate and Trolox. The lack of effect of α-tocopherol on the prooxidative activity is understandable, as α-tocopherol will be located in the lipid phase of the emulsion.

In conclusion, the enhanced prooxidative effect of myoglobin upon mild proteolysis merely depends on an iron(II)/iron(III) pigment couple, which due to proteolysis has an increased affinity for the lipid/water interface.

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