ARTICLES

Effect of Heat Denaturation on the Pro-oxidative Activity of Metmyoglobin in Linoleic Acid Emulsions

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To reveal the effect of heat denaturation on the pro-oxidative activity of MMb, the thermal denaturation of metmyoglobin (MMb) was investigated using differential scanning calorimetry (DSC) and UV−vis spectrometry in combination with an investigation of the pro-oxidative activity of heat-denatured MMb in linoleic acid emulsions, as registered by oxygen consumption. Thermograms of MMb solutions obtained using DSC and progress curves of the absorbance changes at 290, 409, and 540 nm during heating showed that temperatures immediately below thermal denaturation of MMb-induced structural changes in the heme protein which subsequently increased the pro-oxidative activity of the molecule. In contrast, temperatures above the denaturation temperature of MMb decreased the pro-oxidative activity of the resulting species compared to native MMb. The decrease in pro-oxidative activity was found to be related to hemichrome formation. Moreover, the amount of free iron released during heat denaturation of MMb was measured and found to be of an order where it would not be expected to play any significant role as an overall pro-oxidant in the initial phase of lipid peroxidation in the presence of other pro-oxidative species formed during heat denaturation of MMb. The latter could be concluded from a comparable study of the catalytic activity of free iron(II), hemin, MMb, and heat-denatured MMb in linoleic acid emulsions. Finally, heat treatment of MMb and the pro-oxidative activity of resulting species are discussed with regard to possible influence on oxidative stability of meat.

Keywords: *Myoglobin; heat-denatured myoglobin; lipid peroxidation; pro-oxidative species; free iron*

INTRODUCTION

The characteristic off-flavor, which rapidly develops in cooked meat upon chilled storage, and was first described by Tims and Watts (1958) in terms of warmed-over flavor (WOF) has attained much attention during recent years. In general WOF is caused by autoxidation of phospholipids (St. Angelo et al., 1987; Wilson et al., 1976; Igene and Pearson, 1979), resulting in formation of secondary oxidation products, which decrease the characteristic fresh meat aroma and increase off-aroma, resulting in sensory notes such as “cardboard” and “painty” (St. Angelo et al., 1987, 1990).

Although much effort has been devoted to lipid oxidation in meat, the relative importance of nonheme iron versus heme pigments in oxidation is not clear (Love, 1987; Fox and Benedict, 1987; Rhee, 1988). The prevailing mechanism proposed for lipid peroxidation in cooked meat has been that the nonheme iron released from meat pigments by the heating procedure is responsible for initiating lipid oxidation (Sato and Hegarty, 1971; Chen et al., 1984; Pearson and Gray, 1983), although more recent studies suggest the possibility of heme pigments being directly involved in the catalysis (Harel and Kanner, 1985; Rhee et al., 1987; Johns et al., 1989)

Heat denaturation of the heme proteins catalase and peroxidase induce up to a 22-fold increase in nonenzymatic lipid oxidation activity (Erikson et al., 1971). This increase in catalytic activity was explained by heat-induced conformation changes in the protein structure of the enzymes, leading to increased exposure of the heme group to surrounding lipid molecules. An equal role of heat-denatured heme pigments in the catalysis of lipid peroxidation in cooked meat has been proposed (Godber and Arganosa, 1985) but never studied in any detail, even though the catalytic site in these, according to the general catalytic mechanism of heme protein induced lipid peroxidation (Tappel, 1955), is equivalent to those present in the heme proteins investigated by Erikson and co-workers (1971, 1973). Any heat-induced change in meat pigments which increases the exposure of the heme to the surroundings should likewise enhance lipid peroxidation in meat.

The objectives of the present study were (1) to determine whether heat denaturation of metmyoglobin (MMb) changes the pro-oxidative activity of the heme pigment in a linoleic acid emulsion system, (2) to compare the pro-oxidative activity of heme iron and nonheme iron in the same model system, and (3) to

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determine the amount of nonheme iron released during heat denaturation of MMb.

MATERIALS AND METHODS

Chemicals. Horse heart MMb (Type III), linoleic acid, Tween 20, and bovine hemin (Type I) were obtained from Sigma. MMb was dissolved in 0.15 M HPO_4^{2-} buffer (pH 5.53) and kept at 4 °C for no more than 3 days before use. Hemin was dissolved in 1 M NaOH and stored as a 0.2 mM stock solution in 0.1 M NaOH. All other chemicals used were of analytical grade, and double deionized water was used throughout.

Differential Scanning Calorimetry (DSC). Samples of 10% MMb in 0.15 M HPO_4^{2-} buffer (pH 5.53) were weighed (≈15 mg) into DSC pans for volatile compounds (Perkin-Elmer, Germany), hermetically sealed, and placed in the sample cell of a TA-Instruments DSC 10 differential scanning calorimeter (TA Instruments, U.S.). A 0.15 M HPO_4^{2-} buffer (pH 5.53) was used as a reference. The DSC sample cell was equilibrated at 30 °C and heated with a gradient of 5 °C/min to 135 °C or heated to 80 °C (5 °C/min) with a subsequent holding time of 40 min. Data were analyzed using the Thermal Analyst 2000 program (TA Instruments, U.S.). The temperature at which protein denaturation occurred, T_D, was taken as the extrapolated onset temperature of the endothermic peak, and the peak temperature, i.e., the temperature at which maximum heat absorption occurred, T_p, was also recorded.

MMb solutions used in the investigation of pro-oxidative activity were heated to the predetermined temperature as described above and immediately cooled by placing the pan in a small beaker placed in ice.

UV−Vis Spectrometry. Samples of appropriately diluted MMb solutions (0.15 M HPO_4^{2-} buffer, pH 5.53) were placed in the thermoblock of a CARY 13, UV−vis spectrophotometer (Varian, U.S.). The thermoblock was equilibrated at 25 °C and programmed at a heating rate of 5 °C/min. The absorbance was recorded at 290, 409, and 540 nm, respectively. The chosen wavelengths represent the tryptophan band (290 nm)—including the contribution from the tyrosine—and the phenylalanine band, the Soret band (409 nm), and the heme-ligand band (540 nm) in the myoglobin molecule.

MMb solutions used in the investigation of pro-oxidative activity were heated to the predetermined temperature in the thermoblock and subsequently placed in a cold water bath (5 °C) to obtain rapid cooling.

Preparation of Linoleic Acid Emulsions. Emulsions of linoleic acid were made as previously described by Mikkelsen et al. (1992), with 60 mg of linoleic acid (0.21 mmol) being added dropwise to a 10 mL volumetric flask containing 12 mg of Tween 20 dissolved in 1 mL of 5 mM HPO_4^{2-} buffer (pH 5.8). Then 3.5 mL of buffer was added and the solution was vigorously stirred to make a fine emulsion, to which 400 μL of 2 M NaOH and 2 mL of buffer were added during continuous stirring until the solution appeared homogeneous. The resulting emulsion was adjusted to a pH between 8.9 and 9.1 with 2 M HCl in order to provide a clear and stable emulsion, and the flask was filled to volume with the buffer. Emulsions were prepared immediately before use and used within the same day.

Measurement of Pro-oxidative Effect in Linoleic Acid Emulsions. The pro-oxidative effect of the tested pro-oxidants was determined by oxygen consumption measurements in linoleic acid emulsions. Oxygen consumption was measured using a Clark electrode (Radiometer, Copenhagen, Denmark) in connection with a multichannel analyzer and a data collecting system as described by Mikkelsen et al. (1992). The electrode was calibrated with air-saturated buffer (0.15 M HPO_4^{2-}, pH 5.53) held at 25 °C. The relative oxygen concentration was recorded at 5 s intervals.

The assay consisted of 100 μL of 0.20 mM MMb, hemin, or $Fe(II)Cl_2$, unless stated otherwise, 100 μL of linoleic acid emulsion, and 4.8 mL of thermostated (25°C) air-saturated buffer (0.15 M HPO_4^{2-}, pH 5.53). Immediately after mixing, a sample from this assay emulsion was injected into a thermostatically controlled 70 μL cell (Chemiware, Aabyhoej, Denmark) with no headspace. The pro-oxidative effects were determined using the slopes of the tangent at zero time from the oxygen consumption versus time in combination with the oxygen concentration in an air-saturated aqueous solution at 25 °C (2.7×10^{-4} M), resulting in an initial oxygen consumption rate $V_{O_2} = [-\text{slope}(2.7 \times 10^{-4})/100]$ μM s^{-1}.

Effect of Different Heating Temperatures on Release of Free Iron from MMb in Aqueous Solutions. Cuvettes containing 0.2 mM MMb solution (0.15 M HPO_4^{2-}, pH 5.53) were heated to predetermined temperatures (25, 60, 70, 75, 80, 85, and 90 °C) in the thermoblock of a CARY 13 UV−vis spectrophotometer (Varian, U.S.) at a heating rate of 5 °C/min or heated to 80.5 °C (5 °C/min) with a subsequent holding time of 40 min. MMb solutions were subsequently cooled in an ice bath before samples were withdrawn for determination of total iron, heme iron, and free iron.

Iron Determinations. *Total Iron*. Three milliliters of 0.2 mM heated MMb solutions were added to 7.5 mL of concentrated nitric acid in Erlenmeyer flasks. Each flask was left to pre-digest at room temperature overnight. The flasks were placed on a hot plate until dry. Hydrogen peroxide−sulfuric acid reagent (Hatch et al., 1985), containing peroxymonosulfuric acid, was added in 1 mL aliquots to each sample until clear. The flasks were left on the hot plate until all peroxide was evaporated (approximately 10 min) and the white vapors of sulfuric acid became evident. After cooling, the digest was quantitatively transferred and appropriately diluted in 0.01 M HCl. The Ferrozine method was used as described by Carter (1971) and Stookey (1970), except that the final mix was 1 mL of sample, 1 mL of 1% ascorbic acid, 1 mL of 20% ammonium acetate, 1 mL of 1 mM ferrozine, and 1 mL of water. Concentrations were obtained using a standard curve constructed from known concentrations of iron (0−2 ppm).

Nonheme Iron. Three milliliters of 0.2 mM heated MMb solutions were added to 0.1 mL of sodium nitrite and extraction solution in Teflon-sealed screw-cap centrifuge tubes. The extraction solution was 7.5 mL of a 1:1 mixture of 40% trichloroacetic acid and 6 M HCl (Torrence and Bothwell, 1968; Schricker et al., 1982). The tubes were placed in a hot water bath (80 °C) overnight. After cooling, the mixtures were centrifuged at 2000g for 10 min, and the supernatants were passed through 20 μm filters. The iron concentrations of the filtrates were determined with ferrozine as described above.

Heme Iron. Hemin was determined using the acidified acetone extraction method of Hornsey (1956) with minor modifications. Three milliliters of heated 0.2 mM MMb solutions were added to 12 mL of acetone and 0.30 mL of 6 M HCl. Samples were placed at room temperature for 1 h and subsequently filtered. The absorbance of the filtrate at 640 nm was measured, and the heme iron in the sample was calculated using a molar extinction coefficient of 4800 M^{-1} cm^{-1} at 640 nm for hemin (Hornsey, 1956).

Statistical Analyses. Statistical differences in the iron fractions from MMb solutions heated to different end temperatures (Table 2) were tested using Student's t-test.

RESULTS

Thermal Characteristics of Metmyoglobin Solutions. Figure 1A shows a typical DSC thermogram of 10% MMb solutions upon heating from 30 to 135 °C at a constant rate (5 °C/min). Up to 60 °C, only small exothermic changes were registered, which most probably are due to small conformation changes in the protein structure. Above 60 °C, the heme protein began to denature, as seen by the drastic endothermic change with T_D at 65.0 °C and T_p at 70.08 °C. A secondary endothermic process was also noticed with T_D at 112.5 °C and T_p at 122.40 °C. Figure 1B shows a typical thermogram of 10% MMb solutions heated at a constant

Table 1. Pro-oxidative Activity, as Measured as Oxygen Consumption (μM/S), of Unheated and Heated Free Iron, Hemin, and Metmyoglobin at Different Concentrations in Linoleic Acid Emulsions[a]

	0.5 μM	1.0 μM	2 μM	4 μM	8 μM
			Nonheated Pro-oxidant		
$Fe(II)Cl_2$			0.028 ± 0.00002	0.049 ± 0.0065	0.045 ± 0.0028
hemin	0.200 ± 0.0028	0.203 ± 0.0044	0.172 ± 0.0023	0.079 ± 0.0059	
metmyoglobin	0.155[b] ± 0.0119	0.237[c] ± 0.0037	0.275 ± 0.0024	0.290 ± 0.0078	0.246 ± 0.0131
			Heat-Treated Pro-oxidant[d]		
$Fe(II)Cl_2$			0.036 ± 0.0058	0.037 ± 0.0122	0.020 ± 0.0014
hemin	0.187 ± 0.0012	0.226 ± 0.0007	0.227 ± 0.0015	0.167 ± 0.0216	
metmyoglobin	0.039[b] ± 0.0050	0.088[c] ± 0.0013	0.114 ± 0.0039	0.155 ± 0.0033	0.182 ± 0.0021

[a] Each number represents the mean of three independent determinations ± standard deviation. [b] Concentration ≡ 0.4 μM. [c] Concentration ≡ 1.2 μM. [d] Heated to 80 °C at a rate of 5 °C/min.

Table 2. Effect of Heating on Free Iron, Heme Iron, and Total Iron in 0.2 mM Solutions of Metmyoglobin[a]

end temperature, °C[b]	free iron, ppm	heme iron, ppm	total iron, ppm
25	0.46 ± 0.12 (100)[c]	10.83 ± 0.56 (100)	11.01 ± 0.88 (100)
60	0.57 ± 0.11 (124)	10.95 ± 0.78 (101)	10.82 ± 0.65 (98)
70	0.46 ± 0.11 (100)	9.94 ± 0.84 (92)	11.09 ± 0.69 (100)
75	0.45 ± 0.10 (98)	8.43 ± 0.93 (78)	10.67 ± 0.80 (97)
80	0.50 ± 0.11 (109)	8.54 ± 0.39* (79)	10.97 ± 0.84 (100)
85	0.48 ± 0.10 (104)	9.66 ± 0.56 (89)	10.81 ± 0.80 (98)
90	0.50 ± 0.07 (109)	9.27 ± 0.28* (85)	12.23 ± 0.42 (111)
80.5[d]	0.96 ± 0.21*** (209)	8.66 ± 0.34* (80)	9.90 ± 0.80* (90)

[a] Each number represent the mean of three independent determinations made in triplicate ± standard deviation. *, **, *** indicate values significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, relative to unheated sample (25 °C). [b] Heating rate: 5 °C/min. [c] Numbers in brackets represent the number in percent in relation to untreated sample within each column. [d] Kept at this temperature for 40 min.

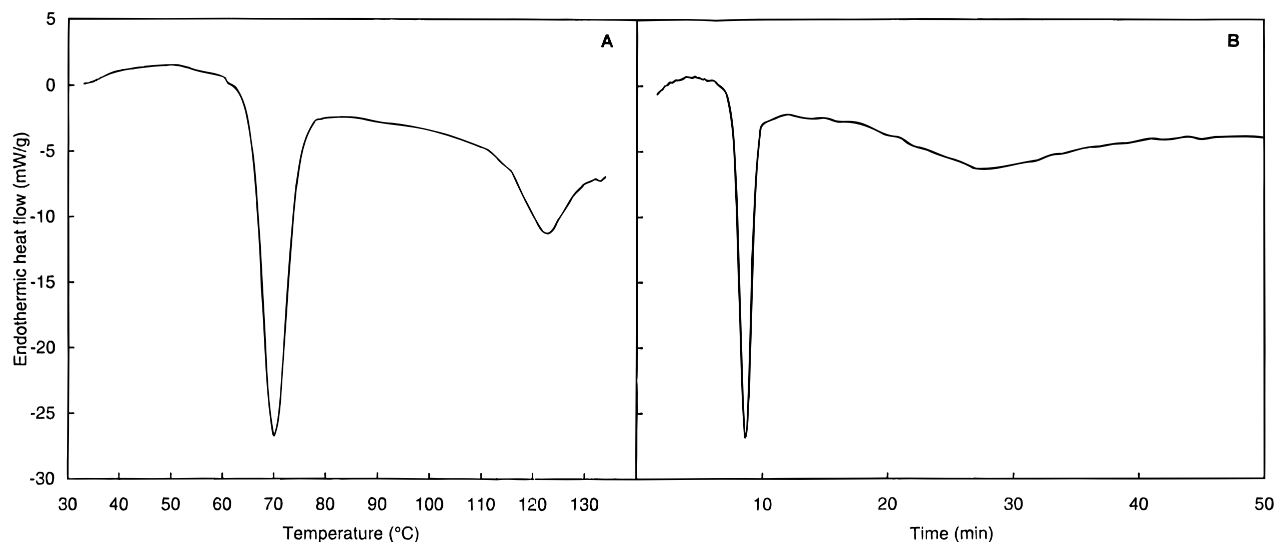


Figure 1. Thermograms obtained during heat denaturation of 10% MMb solutions (pH 5.5). (A) Thermogram obtained at a heating rate of 5 °C/min. The presented thermogram is the sum of four independent measurements. (B) Thermogram obtained at a heating rate of 5 °C/min to 80 °C with a subsequent holding time of 40 min at 80 °C. The presented thermogram is the sum of two independent measurements.

heating rate (5 °C/min) from 30 to 80 °C, and subsequently held at 80 °C for 40 min. An endothermic process was registered with a maximum after 8.6 min, while a secondary, less pronounced endothermic process appeared at approximately 18 min with a maximum at 28 min.

Figure 2 shows the typical spectral changes of 40 μM solutions of MMb at 290 and 540 nm and of a 4 μM solution of MMb at 409 nm during heating from 25 to 85 °C at a constant rate (5 °C/min).

At *290 nm*, an initial constant decrease in the absorbance was registered up to approximately 63 °C. This was followed by a drastic increase in absorbance up to approximately 77 °C, where the absorbance became steady (Figure 2, insert). Above 85 °C precipitation was registered (decreasing absorbance with high noise to signal ratio and formation of precipitate).

At *409 nm*, up to approximately 70 °C, the absorbance decreased slightly. At higher temperatures the absorbance decreased dramatically. Above 85 °C precipitation was observed.

At *540 nm,* a very small increase in the absorbance was seen up to approximately 64−65 °C. At higher temperatures a large increase in absorbance occurred up to approximately 79 °C, where the absorbance became steady (Figure 2, insert). Above 85 °C precipitation was registered.

Pro-oxidative Effect of Heat-Treated MMb. Both 10% MMb solutions heated in the *DSC* sample cell to specific temperatures and 0.2 mM MMb solutions

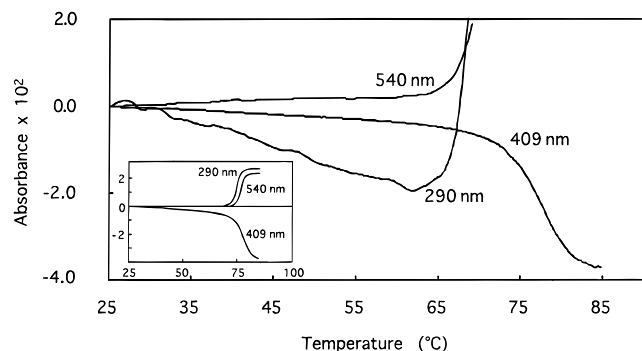


Figure 2. Absorbance changes of 40 mM MMb solutions (pH 5.5) at 290 and 540 nm and a 4 mM MMb solution (pH 5.5) at 409 nm with a heating rate of 5 °C/min. Presented absorbance changes are means of at least three individually absorbance scannings.

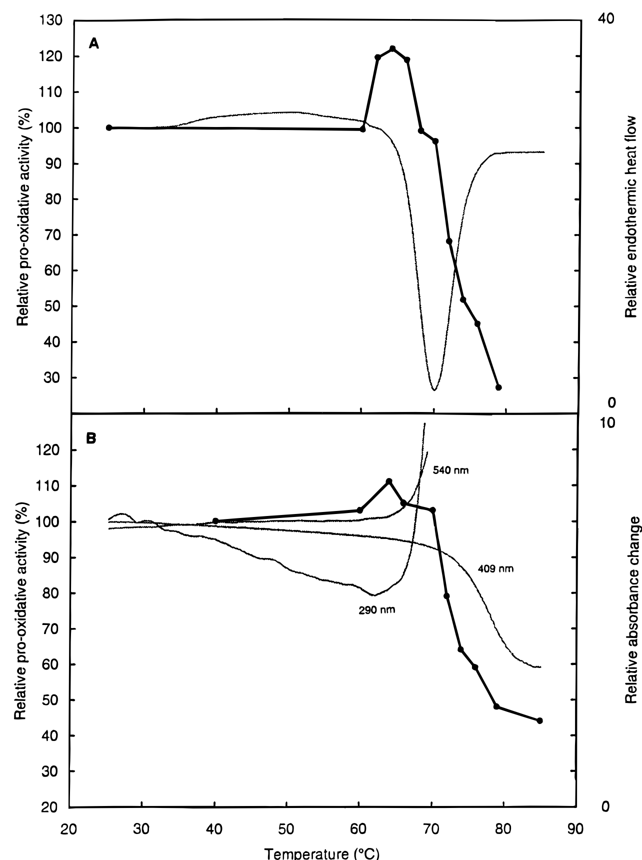


Figure 3. (A) Pro-oxidative activity of MMb solutions (pH 5.5) heated to specific temperatures with a heating rate of 5 °C/min in a DSC sample cell (–●–) and the contemporary thermogram obtained during heat denaturation of a 10% MMb. (B) Pro-oxidative activity of MMb solutions (pH 5.5) heated to specific temperatures with a heating rate of 5 °C/min in a thermoblock (–●–) and contemporary relative absorbance changes at 290, 409, and 540 nm. Determinations of pro-oxidative activity represent means of at least two independent measurements. Deviations between determinations did not exceed 10% at any time.

heated in the thermoblock of the spectrophotometer were subsequently tested for pro-oxidative activity in the linoleic acid emulsion system. Figure 3A shows the relative change in pro-oxidative activity of heat-treated MMb as a function of temperature. For clarity, a typical DSC thermogram during MMb denaturation is included in the figure. No change in the pro-oxidative activity of MMb was observed below 60 °C. Above 60 °C an increase in the pro-oxidative activity was observed, with a maximum (121%) at approximately 64 °C. Around 70 °C the pro-oxidative activity reached the level of unheated MMb, and at higher temperatures a drastic decrease in the pro-oxidative activity was observed. Figure 3B also displays the influence of heat on the pro-oxidative activity of MMb, but after the heating to distinct temperatures in the thermoblock of the spectrophotometer. For clarity, the typical absorbance changes in MMb solutions at 290, 409, and 540 nm during heating are also displayed in the figure. As also observed for samples heated in the DSC sample cell, no change in the pro-oxidative activity of MMb was registered below 60 °C. In the temperature area between 60 and 70 °C an increase in pro-oxidative activity was seen with a maximum at approximately 64 °C (111%). This merged with an initial increase in absorbance at 290 nm. Finally, a drastic decrease in pro-oxidative activity was observed above 70 °C, which was coincident with increasing absorbance at 540 nm.

Effect of Heat Exposure on the Pro-oxidative Activity of Iron and Iron Complexes. Table 1 shows the pro-oxidative activity in the linoleic acid emulsion system of different concentrations of Fe(II), hemin, and MMb with and without previous heating to 80 °C (5 °C/min). The pro-oxidative activity of free iron (Fe(II)) was scarcely dependent on heat treatment at the concentrations tested. In contrast MMb showed effect on both heat treatment and concentration. The pro-oxidative activity of unheated MMb increased with increasing concentration up to 4 μM, while a decrease in the pro-oxidative activity was observed at higher concentrations. Upon heating of MMb a pronounced decrease in the pro-oxidative activity was observed at all concentrations tested, but in contrast to unheated MMb, the pro-oxidative activity increased with increasing concentration for all concentrations tested. The pro-oxidative activity of hemin was only affected to a limited extent by the given heat treatment. The pro-oxidative activity of hemin above 1–2 μM decreased both with and without previous heat exposure even though the effect of the heat exposed hemin was less pronounced.

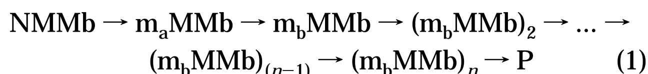
Influence of Heat Exposure on Iron Fractions in Aqueous MMb Solutions. Table 2 shows the effect of heating to different end temperatures on the amount of free iron, heme iron, and total iron in 0.2 mM MMb solutions. As expected, no significant change in total iron was observed. Neither did the amount of free iron seem to be affected by the different heating treatments, with the exception of a holding time of 40 min at a relatively high temperature (80.5 °C), which increased the amount of free iron by a factor of 2. The observed changes in heme iron were rather limited, with a tendency toward a minor loss of heme iron at temperatures at 70 °C and above, i.e., after heat denaturation of MMb. Prolonged heating at 80.5 °C did not result in any pronounced loss of heme iron compared to heating to end temperatures above 70 °C without subsequent heating, if heme iron is calculated as the percentage of total iron in the experiment with prolonged heating at 80.5 °C.

DISCUSSION

Heme pigments have been suggested to initiate lipid peroxidation during heating of meats with subsequent formation of WOF during cold storage (Rhee et al., 1987; Johns et al., 1989). However, heat treatment of meats has also been shown to release iron from heme compounds (Schricker and Miller, 1983; Chen et al., 1984;

Han et al., 1993), which has given rise to the hypothesis that free iron is the primary catalyst in oxidative processes resulting in WOF (Sato and Hegarty, 1971; Igene et al., 1979; Rhee et al., 1987). Moreover, formation of WOF in meats has been shown to be dependent on the time−temperature profile (Smith et al., 1987; Mielche and Bertelsen, 1993). This has been explained by a delicate balance between thermal acceleration of oxidative processes and formation of antioxidative Maillard reaction products (Zipser and Watts, 1961; Sato et al., 1973). Until now no work has focused on the possible effect of heating on the activity of the pro-oxidative species in meats, although some heme proteins in meats have shown drastic changes in their pro-oxidative activity during heat denaturation (Eriksson et al., 1971, 1973).

Present DSC data obtained with 10% MMb solutions (pH 5.53), $T_p = 70.1$ °C, are in agreement with previous results of Ledward (1978), who found an endothermic peak at 71.9 °C during thermal analysis of 10% MMb (pH 6.0), if the difference in pH in the two experiments is taken into consideration. Present UV−vis spectral data obtained during heating of horse heart MMb also correspond to the results of Award and Deranleau (1968), performed with sperm whale myoglobin. According to the most simple interpretation of the progress curves at 290 , 409, and 540 nm, a mechanism involving an initial conformational disturbance in the region of the heme group, followed by unfolding of the helical regions and a subsequent sequence of polymerization steps leading to precipitation, might best describe the heat denaturation process of horse heart MMb, as was also found to be the case for sperm whale myoglobin during heat denaturation by Award and Deranleau (1968):

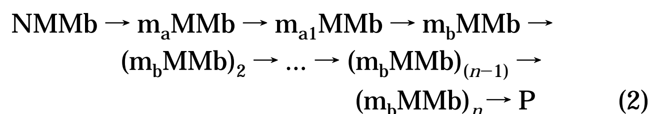


$$\mathrm{NMMb} \rightarrow \mathrm{m_aMMb} \rightarrow \mathrm{m_bMMb} \rightarrow (\mathrm{m_bMMb})_2 \rightarrow \ldots \rightarrow (\mathrm{m_bMMb})_{(n-1)} \rightarrow (\mathrm{m_bMMb})_n \rightarrow \mathrm{P} \qquad (1)$$

where NMMb represents native MMb, m_aMMb and m_b-MMb modified intermediate species, and P precipitate. A phase of polymerization prior to appearance of precipitate seems most applicable because at pH 5.5 there would be sufficient number of ionic groups to hold the modified monomer in solution.

The observed effect on the pro-oxidative activity of MMb during heating indicates changes in the heme cavity which modify the exposure of the catalytic heme group to the surrounding lipid hydroperoxides. The data obtained by DSC and UV−vis spectroscopy compared with the pro-oxidative activity of MMb during heating show that temperatures around T_D (65.0 °C) coincident with increasing absorbance at 290 nm (Figure 1 and 2) enhance the pro-oxidative activity of heated MMb. This indicates a modification of the tryptophan environment in the molecule (Award and Deranleau, 1968; Gratzer, 1978) and hereby a change in the protein structure, which exposes the heme to the surroundings and thereby increases pro-oxidative activity of the heated MMb as also was observed during heating of catalase and horseradish peroxidase by Eriksson et al. (1971, 1973). The relatively limited increase in pro-oxidative activity of MMb (20%) upon heating to maximal activity compared to catalase (∼6-fold increase) and horseradish peroxidase (∼22-fold increase) (Eriksson et al., 1971) might be explained by a more favorable exposure of the heme group in native MMb for catalytic activity compared to native catalase and horseradish peroxidase. Above the maximal temperature of transition, which coincides with an initial increase in absorbance at 540 nm, the catalytic heme group becomes less exposed to surroundings, as deduced from the drastic decrease in pro-oxidative activity of the heat-exposed MMb. The fact that this decrease in catalytic activity occurs simultaneously with a drastic absorbance increase at 540 nm indicates a connection between the beginning of hemichrome formation and catalytic activity of the heat exposed heme protein. Hemichrome formation, which most likely happens via binding of the distal histidine in the heme cavity to the sixth coordination site of the heme group under the given conditions must be expected to mask the catalytic heme group by blocking its access to the surrounding lipid hydroperoxides. Hemichrome has previously been shown to have reduced catalytic activity in a similar lipid emulsion system (Galaris et al., 1990)

The above interpretation of the structural changes vs pro-oxidative activity of MMb during heat treatment suggests that the reaction scheme for heat denaturation of MMb progresses through at least one more phase than those already proposed by Award and Deranleau (1968):



$$\mathrm{NMMb} \rightarrow \mathrm{m_aMMb} \rightarrow \mathrm{m_{a1}MMb} \rightarrow \mathrm{m_bMMb} \rightarrow (\mathrm{m_bMMb})_2 \rightarrow \ldots \rightarrow (\mathrm{m_bMMb})_{(n-1)} \rightarrow (\mathrm{m_bMMb})_n \rightarrow \mathrm{P} \qquad (2)$$

where NMMb, m_aMMb, m_bMMb, and P represents the stages shown in eq 1 and m_{a1}MMb represents the intermediate species with enhanced pro-oxidative activity. Furthermore, the obtained spectral data indicate that the intermediate species, m_bMMb, equals hemichrome.

The second endothermic transition obtained during thermal denaturation of MMb with a T_D of approximately 112.5 °C (Figure 1A) might correspond to the poorly defined endothermic change which appears after the primary denaturation upon continuous heating to 80 °C (Figure 1B). This has not been studied in further detail, but it might, however, reflect further denaturation of precipitated MMb resulting in release of the heme group from the protein. Taking the well-known pro-oxidative activity of hemin (Tappel, 1955) into consideration, such a release of hemin during thorough heat treatment of MMb solutions might explain the increased pro-oxidative activity of these solutions in a model system similar to those used in the present study (Mikkelsen et al., 1992). However, more experiments are needed to confirm this.

The comparative study of the pro-oxidative activity of free iron(II), hemin, and MMb with and without heat treatment (Table 1) confirms that free iron(II) only has negligible pro-oxidative activity at the concentrations relevant to meat from the present data and that no change in pro-oxidative activity of free iron(II) occurs upon heating. In contrast, both hemin and MMb show considerable pro-oxidative activity, even though high concentrations of both hemin and MMb show reduced pro-oxidative activity. This is a well-known phenomenon for several heme compounds (Lewis and Wills, 1963; Kendrick and Watts, 1969). In agreement with above results the pro-oxidative activity of MMb decreased drastically upon heat treatment, while hemin only was affected insignificantly. It is noteworthy that, even though the pro-oxidative activity of MMb decreased upon heat treatment, the catalytic activity of heat-

denatured MMb still exceeded the pro-oxidative activity of free iron(II) at concentrations relevant in meat and meat products.

Heat exposure of aqueous MMb solutions to different end temperatures had a surprisingly small effect on the measured iron fractions. Only an insignificant loss was observed in the amount of heme iron, while heat exposure at the tested end temperatures scarcely affected the amount of free iron. Even rather severe heat treatment (40 min at 80 °C) only doubled the amount of free iron and did not measurably decrease the heme iron fraction, as determined from the amount of total iron. A doubling in the amount of free iron due to thorough heat treatment did not cause any significant increase in free iron(II)-induced lipid peroxidation in the present model system, while the pro-oxidative activity of heat-denatured MMb did not decrease significantly at an equal heat treatment (Table 1). Provided these events also take place in meats during heating, the pro-oxidative activity in heat-treated meat must mainly be expected to arise from heat-denatured MMb.

In conclusion, the present study shows that it is the delicate balance between native MMb, heat-modified MMb with enhanced pro-oxidative activity, hemichrome, and hemin formed during heat treatment that determines, which heme complex is the dominant catalyst in lipid peroxidation during heating in the present model system. Moreover, heme complexes are much stronger pro-oxidants than free iron in the present model system. This indicates that a composition of heme species with different catalytic activity also will be present during heating of meats and that any of these to different extent dependent on specific catalytic activity can initiate pro-oxidative reactions during heating and subsequent storage of meat rather than free iron due to low catalytic concentration of free iron. Obtained data can moreover contribute to the discussion why heat inactivation of antioxidant enzymes in meat cannot explain heat-induced lipid oxidation reactions in muscle (Lee et al., 1996) and why development in secondary oxidation products must be expected to increase rapidly in stored meat which have been cooked to 70 °C or greater (Mei et al., 1994). Finally, limited lipid peroxidation in meat observed after heat treatment to high temperatures (Karastogiannidou and Ryley, 1994) might reflect that hemichrome is the prevalent heme complex during such processing conditions. This has to be taken into consideration, together with formation of antioxidative Maillard reaction products, in the evaluation of the oxidative stability of heat-processed meats.

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