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Myoglobin Oxidation in a Model System as Affected by Nonheme Iron and Iron Chelating Agents

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A model system was used to study the effect of nonheme iron on myoglobin oxidation at pH 5.6 and pH 7.2 at 23 °C. The addition of ferrous iron significantly ($p < 0.05$) increased the rate of myoglobin oxidation in the absence of lipid, demonstrating that iron promoted myoglobin oxidation independent of the effect of lipid oxidation. The addition of the type II, iron chelating antioxidants sodium tripolyphosphate (at pH 7.2) or milk mineral (at pH 5.6) negated the effect of added iron, slowing oxidation of myoglobin. A clear concentration dependence was seen for iron-stimulated myoglobin oxidation, based on both spectral and visual evidence. Further investigation is needed to determine the possible role for nonheme ferrous iron on myoglobin oxidation in vivo or in meat.

KEYWORDS: Myoglobin oxidation; nonheme iron; milk mineral

INTRODUCTION

Several studies have demonstrated a marked color-stabilizing effect of iron chelators in raw ground beef or beef model systems. Oxalate (*1*), phytic acid (*2*), and sodium pyro- and polyphosphate (*3*) were all found to inhibit metmyoglobin (MetMb) formation and lipid oxidation during storage. More recently, milk mineral (MM; *4*), a whey-derived compound consisting primarily of colloidal calcium phosphate particles, was also found to preserve red color and prevent lipid oxidation in fresh ground beef. It was further demonstrated that MM has strong iron chelating properties (*5*, *6*). The mechanism by which color is preserved has not been determined, but it is possible that the observed effect is a function of the removal of "free" iron from the system.

It is well-established that iron indirectly stimulates myoglobin (Mb) oxidation via lipid oxidation catalyzed by redox active iron (*7*−*9*). Current work regarding the effect of lipid oxidation on Mb stability follows two lines of thinking. (i) It has been proposed that aldehydes, byproducts from lipid oxidation, are capable of affecting the Mb molecule itself (*8*, *10*). Binding sites for 4-hydroxy-2-nonenal were recently identified by Alderton et al. (*11*). It was suggested that the resulting change to the tertiary structure of Mb opens the heme cleft, allowing oxidizing species easier access. (ii) Monahan et al. (*12*), however, suggested that the effect of lipid oxidation on Mb results in part from the consumption of O_2 and the concomitant lowering of its partial pressure (pO_2). This would agree with previous observations regarding the protective effect of high and very low pO_2 against Mb oxidation (*13*).

Several different pathways for Mb oxidation in the absence of lipid have been proposed. These include the dissociation of superoxide ($O_2^{\bullet-}$) from MbO_2 (*14*), anion-mediated electron transfer (*15*), nucleophilic displacement of oxygen from MbO_2 (*16*, *17*), oxidation of dMb by oxygen-derived species such as H_2O_2 and hydroxyl radicals ($OH^{\bullet-}$) (*18*, *19*), or a combination of two or more of the above factors (*9*, *20*). Most proposed models for Mb oxidation do not include a defined role for nonheme iron, although a few have attempted to provide for some contribution, suggesting that some oxidation of MbO_2 to MetMb is caused by Fe^{3+}. Gorelik and Kanner (*9*) included this pathway as one of several contributing to the overall oxidation of Mb but questioned the extent of its contribution.

The aim of this study was to demonstrate that ferrous iron can stimulate Mb oxidation, independent of the effect of lipid oxidation and its byproducts. To examine the effect of iron removal, two phosphate type iron chelators [sodium tripolyphosphate (STPP) and MM] were chosen. Two pH levels were used, representative of physiological (pH 7.2) and postmortem muscle (pH 5.6), since Mb stability is known to decrease with increasing acidity (*15*, *16*, *21*, *22*).

MATERIALS AND METHODS

Materials. Ferrous chloride ($FeCl_2$) was obtained from JT Baker (Phillipsburg, NJ). STPP, Tris(hydroxymethyl)aminomethane HCl (Tris), and 2-(4-morpholine)ethanesulfone acid (MES) were obtained from Fisher Scientific (Fairlawn, NJ). Lyophilized horse skeletal Mb (95−100% purity), sodium dithionite, and bathophenanthroline were obtained from Sigma Scientific (St. Louis, MO). Sephadex G-25 Fine beads were obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Soy phosphatadylcholine (SPC) was obtained from Avanti Polar Lipids (Alabaster, AL). MM was obtained from Glanbia Nutritionals (Twin Falls, ID).

Reagent and Buffer Preparation. $FeCl_2$ solution was prepared at a concentration of 0.1 mg/mL in 0.1 N HCl. Bathophenanthroline solution was prepared at a concentration of 0.13 mg/mL in a 5% ethanol/95% hexane mixture. To reduce potential competition for iron between

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Table 1. Model System Formulations[a]

model system	component: buffer[b] (mL)	MbO_2 (mL)	$FeCl_2$ (μL)	chelator[c] (mg)	SPC[d] (mg)
1-control	1.25	1.25			(5)
2-control + Fe	1.20	1.25	50		(5)
3-MM	1.25	1.25		5	(5)
4-MM + Fe	1.20	1.25	50	5	(5)
5-STPP	1.25	1.25		5	(5)
6-STPP + Fe	1.20	1.25	50	5	(5)

[a] Lipid-free and lipid-containing systems were formulated identically, with the exception of the lipid component. [b] Buffer used was either 0.04 M 2-(4-morpholine)ethanesulfone acid at pH 5.6 or 0.1 M Tris(hydroxymethyl)aminomethane HCl at pH 7.2. [c] Chelator used was either MM or STPP, as designated in the model system name. [d] SPC was used only in lipid-containing systems. MbO_2 = 0.1 mM MbO_2; $FeCl_2$ = 0.1 mg/mL in 0.1 N HCl.

Table 2. Means Pooled Over Time for $\%MbO_2$ Remaining in MS and LS Model Systems at pH 5.6[a]

pH 5.6	$\%MbO_2$ remaining in MS (lipid-free)	$\%MbO_2$ remaining in LS (2 mg lipid/mL)	p value (MS vs LS)
1-control	61.7 ± 20.8 B,C,D	50.7 ± 18.3 A,B	NS
2-control + Fe	45.4 ± 16.2 D	42.2 ± 14.6 B	NS
3-MM	82.1 ± 17.2 A	53.5 ± 19.5 A	$p = 0.0003$
4-MM + Fe	54.2 ± 18.1 C,D	46.1 ± 19.2 A,B	NS
5-STP	73.6 ± 16.5 A,B	45.4 ± 15.9 A,B	$p = 0.0003$
6-STP + Fe	63.3 ± 17.6 B,C	49.5 ± 16.9 A,B	NS

[a] Values represent means ± standard deviations. Values sharing letters within (but not between) columns are not significantly different ($p > 0.05$). p Values are shown for lipid-free vs lipid-containing systems with the same formulation. NS, no significant difference.

buffers and test compounds, nitrogen-based buffers (Tris and MES) were used. These buffers also slowed the rate of iron autoxidation [and the subsequent formation of oxidizing species such as hydrogen peroxide (H_2O_2)] as compared to oxygen-based buffers (*23*). MES buffer solution (0.04 M) was prepared in distilled water and adjusted to a final pH of 5.6 using a concentrated sodium hydroxide solution. Two portions of Tris buffer (0.1 M) were prepared as described for MES, with final pH values of 7.2 and 8.0. Residual iron was then removed from buffers using a bathophenanthroline extraction (*24*). Briefly, 100 mL of buffer was extracted three times with 10 mL aliquots of bathophenanthroline solution, using a separatory funnel. The extracted buffer was heated to approximately 95 °C while being stirred rapidly to remove residual ethanol. The buffer was cooled, and the final volume adjusted back to 100 mL with distilled, deionized water (DDI; <1 mL).

Iron Content of Mb and Phospholipid. Because Mb and lipid could not be purified of contaminating iron using the bathophenanthroline extraction, their total iron content was determined. Samples were dry ashed in triplicate and then assayed for iron using the Ferrozine procedure (*25*). The ratio of moles of Mb to moles of iron was 1:0.994, which was very close to the expected ratio of 1:1. Therefore, it was concluded that the horse skeletal Mb was not contaminated with extraneous iron. However, the SPC contained 12 μg iron/g. This contamination was considered in terms of model system design and subsequent interpretation of results.

Generation of Oxymyoglobin (MbO_2) Stock Solutions. A concentrated solution of horse skeletal Mb (~20 mg/mL) was prepared in DDI water (*21*). Because precipitation was observed when sodium dithionite crystals were added directly, the Mb was reduced by adding 100 μL of a 40 mg/mL dithionite solution (*26*). The Mb solution was agitated gently until the characteristic purplish color of deoxymyoglobin (dMb) was observed. To remove excess dithionite, the dMb solution was passed through a 4 cm Sephadex G25 column and eluted with Tris buffer (pH 8.0). dMb was converted to oxymyoglobin (MbO_2) by swirling and gently bubbling air through the solution with a Pasteur pipet. Conversion to MbO_2 was confirmed spectrophotometrically, based on the presence of the characteristic MbO_2 peaks at 545 and 580 nm (*27*). The concentration of the MbO_2 stock solution was adjusted to ~0.1 mM with the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2). Fresh MbO_2 stock was generated for each experiment and replicate. The total Mb concentration (MbO_2 + dMb + MetMb) of each individual stock was determined using a millimolar extinction coefficient of 7.6 at the isobestic point of 525 nm (*27*). The actual concentration of each model system solution was calculated as one-half of the corresponding stock concentration, because the MbO_2 stock was diluted 1:1 in all cases (**Table 1**).

Table 3. Means Pooled over Time for $\%MbO_2$ Remaining in MS and LS Model Systems at pH 7.2[a]

pH 7.2	$\%MbO_2$ remaining in MS (lipid-free)	$\%MbO_2$ remaining in LS (2 mg lipid/mL)	p value (MS vs LS)
1-control	77.7 ± 13.6 C	66.5 ± 23.8 A,B	$p = 0.01$
2-control + Fe	64.3 ± 16.7 D	58.7 ± 20.4 B	NS
3-MM	87.5 ± 12.9 A	68.6 ± 22.7 A,B	$p < 0.0001$
4-MM + Fe	80.3 ± 11.4 B,C	63.5 ± 21.2 A,B	$p < 0.0001$
5-STP	91.1 ± 7.6 A	70.2 ± 23.6 A	$p < 0.0001$
6-STP + Fe	86.1 ± 7.7 A,B	65.2 ± 21.5 A,B	$p < 0.0001$

[a] Values represent means ± standard deviations. Values sharing letters within (but not between) columns are not significantly different ($p > 0.05$). p Values are shown for lipid-free vs lipid-containing systems with the same formulation. NS, no significant difference.

Preparation of Mb Model System Samples. Model systems (MS) were prepared in the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2) using a combination of MbO_2, $FeCl_2$, and/or antioxidant (**Table 1**) to observe the effect of nonheme iron on the conversion of MbO_2 to MetMb in the absence of lipid. Lipid-containing model systems (LS) were prepared in the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2) using a combination of MbO_2, SPC, $FeCl_2$, and/or antioxidant to examine the effect in the presence of lipid. Systems were designed to provide the following concentrations: MbO_2, 50 μM; Fe, 35 μM (where the ratio of Mb to added iron was equal to reported concentrations of Mb and Fe in beef muscle; *21*, *28*); SP, 2 mg/mL; and antioxidant (MM or STPP), 2 mg/mL. Systems were prepared in disposable 3.5 mL spectrophotometer cuvettes, covered with disposable cuvette caps, and inverted 10 times prior to running the initial spectral scan (400−650 nm), using a Shimadzu UV2100U spectrophotometer (Shimadzu Corporation, Columbia, MD). Scans were repeated for each system at 15, 30, 45, and 60 min and at 1 and 2 days. Samples were held at room temperature (23 °C), as previous reports indicated that the effect of temperature on Mb oxidation was minimal between 10 and 20 °C (*22*). Five complete replicates were performed at each pH.

Preparation of Samples for Visual and Spectral Examination. Samples and control were prepared using the same formulation as for model systems 1−4 (**Table 1**) at pH 5.6 only, using 0.2 mM MbO_2 stock. Representative spectra (400−650 nm) were obtained at 0.5 min, 1 h, and 1 day. Digital photographs of the cuvettes were taken at 1 h and 1 day. Samples were held at room temperature (23 °C).

Preparation of Samples for Measurement of Iron Concentration Effects. Samples were prepared as for model system 2 (**Table 1**) at pH 5.6 only, but the iron concentration was varied from 35, 180, and 350 μM added iron. Control (0 μM added iron) consisted of equal parts MbO_2 and MES. Additional control samples were prepared containing 2 mg/mL MM or STPP, with no added iron. Representative spectra (400−650 nm) were obtained every 5 min for 1 h, for a total of 13 scans per sample.

Calculation of $\%MbO_2$ Remaining. Absorbance values were corrected to account for turbidity in samples resulting from the addition of MM and/or SPC. The total Mb concentration (MbO_2 + dMb + MetMb) can be determined using a millimolar extinction coefficient of 7.6 at the isobestic point of 525 nm (*27*). Because the concentration of Mb added to each model system was known (KC), a turbidity correction factor was calculated based upon the measured absorbance at 525 nm. The KC was used to estimate what the absorbance would be in the absence of turbidity (where $KC \times 7.6 = A_{525}$). The calculated

Table 4. Means for %MbO_2 Remaining at Selected Time Points in Model Systems at pH 5.6[a]

	%MbO_2 remaining			
samples at pH 5.6	initial (0.5 min)	15 min	60 min	1 day
		lipid-free model systems		
1-control	81.2 ± 7.6 A,B	73.5 ± 10.9 A,B,C	69.3 ± 10.7 A,B	36.4 ± 4.4* B,C
2-control + Fe	73.3 ± 5.9 A,B	54.0 ± 4.6* C	41.7 ± 6.8 C	27.1 ± 9.0 C
3-MM	93.9 ± 12.9 A	89.7 ± 10.6 A	89.1 ± 12.6 A	65.8 ± 7.7* A
4-MM + Fe	66.7 ± 18.5 B	61.3 ± 16.9 B,C	59.6 ± 16.5 B,C	38.3 ± 5.5* B,C
5-STP	84.5 ± 12.1 A,B	82.4 ± 12.1 A,B	80.0 ± 11.5 A,B	57.9 ± 6.5* A,B
6-STP + Fe	79.5 ± 12.1 A,B	72.8 ± 10.7 A,B,C	68.1 ± 7.9 A,B	44.3 ± 8.9* A,B,C
		lipid-containing model systems		
1-control	69.5 ± 10.6 NS	61.9 ± 8.9 NS	55.7 ± 8.8 A,B	24.9 ± 6.7* NS
2-control + Fe	62.4 ± 12.6 NS	51.7 ± 8.8 NS	37.3 ± 9.5 B	25.1 ± 4.7* NS
3-MM	72.8 ± 15.7 NS	62.4 ± 10.6 NS	58.0 ± 9.9* A	27.9 ± 7.3 NS
4-MM + Fe	59.2 ± 22.5 NS	54.8 ± 18.5 NS	50.1 ± 15.2 A,B	28.0 ± 8.2* NS
5-STP	55.5 ± 19.2 NS	50.9 ± 12.2 NS	50.8 ± 14.2 A,B	29.6 ± 10.3* NS
6-STP + Fe	63.4 ± 16.1 NS	55.7 ± 11.2 NS	55.3 ± 13.9 A,B	28.6 ± 6.2* NS

[a] Values represent means ± standard deviations. Values sharing letters within (but not between) column groupings are not significantly different ($p > 0.05$). Asterisk (*) denotes first significantly different value ($p < 0.05$) vs initial reading ($t = 0.5$) for the given system (within rows). NS, no significant difference.

Table 5. Means for %MbO_2 Remaining at Selected Time Points in Model Systems at pH 7.2[a]

	%MbO_2 remaining			
samples at pH 7.2	initial (0.5 min)	15 min	60 min	1 day
		lipid-free model systems		
1-control	97.6 ± 4.9 A	85.5 ± 3.7* A,B	79.3 ± 3.8 B	64.4 ± 3.1 B,C
2-control + Fe	86.2 ± 3.9 B	72.9 ± 4.2* C	67.6 ± 3.9 B	46.4 ± 12.3 D
3-MM	105.7 ± 10.3 A	94.0 ± 2.9* A,B	88.9 ± 2.3 A	77.3 ± 4.1 A,B
4-MM + Fe	96.3 ± 7.0 A,B	84.1 ± 1.2* B,C	82.8 ± 3.6 A	69.5 ± 3.4 B,C
5-STP	99.1 ± 5.3 A	95.9 ± 4.7 A	93.6 ± 4.7 A	84.8 ± 6.0* A
6-STP + Fe	94.7 ± 5.2 A,B	90.6 ± 4.3 A,B	86.7 ± 4.8 A	79.7 ± 4.7* A,B
		lipid-containing model systems		
1-control	87.5 ± 12.9 NS	79.9 ± 8.7 NS	77.0 ± 5.4 NS	33.2 ± 14.0* NS
2-control + Fe	81.5 ± 16.2 NS	70.1 ± 8.8 NS	67.7 ± 7.4 NS	30.2 ± 11.6* NS
3-MM	90.0 ± 10.8 NS	81.1 ± 11.4 NS	75.2 ± 12.9 NS	39.2 ± 10.9* NS
4-MM + Fe	84.0 ± 14.0 NS	72.9 ± 11.5 NS	70.4 ± 8.5 NS	35.4 ± 9.3* NS
5-STP	84.9 ± 14.0 NS	87.0 ± 12.0 NS	78.3 ± 15.4 NS	37.7 ± 7.3* NS
6-STP + Fe	83.9 ± 13.2 NS	76.3 ± 9.8 NS	71.5 ± 10.9 NS	34.4 ± 9.7* NS

[a] Values represent means ± standard deviations. Values sharing letters within (but not between) column groupings are not significantly different ($p > 0.05$). Asterisk (*) denotes first significantly different value ($p < 0.05$) vs initial reading ($t = 0.5$) for the given system (within rows). NS, no significant difference.

A_{525} was subtracted from the measured A_{525}, and the difference was used to correct spectra for turbidity.

Because only relative loss of MbO_2 was of interest during the time course of the experiment, actual MbO_2 concentrations were not calculated. The ratio of the sum of absorbance at 545 and 580 nm (characteristic features of MbO_2) to the absorbance at 505 nm [a characteristic feature of MetMb (*29*)] was monitored in each model system and compared to the initial reading of the corresponding MbO_2 stock (to account for background absorbance at 505 nm):

$$MbO_2\text{:met ratio} = \frac{A_{545} + A_{580}}{A_{505}}$$

$$\%\ MbO_2\ \text{remaining} = \frac{\text{sample } MbO_2\text{:met ratio}}{\text{stock } MbO_2\text{:met ratio}} \times 100$$

Data Analysis. For model system values, %MbO_2 remaining was evaluated as a repeated measures design. The proc mixed function in SAS version 9.0 (SAS Institute, Inc., Cary, NC) was used with an autoregressive moving average structure. Statistical significance was identified at the 95% confidence level, and posthoc means comparisons were made based on p values obtained using the Tukey−Kramer adjustment. For data obtained from the effect of iron concentration, %MbO_2 remaining was plotted vs time for each iron concentration.

RESULTS

Model Systems. For lipid-free systems at pH 5.6, significantly more MbO_2 was oxidized to MetMb over the 2 day time course of the experiment in the iron-added system (system 2) than in the systems containing an iron chelator (systems 3 and 5; **Table 2**). At pH 7.2, the difference was even more pronounced, since the iron-added system exhibited more MbO_2 oxidation than any other system, including the control (**Table 3**). Thus, addition of an iron chelator minimized conversion of MbO_2 to MetMb, regardless of pH. While the differences were not as pronounced in lipid systems, MM minimized Mb oxidation at pH 5.6 (system 3; **Table 2**), and STPP minimized Mb oxidation at pH 7.2 (system 5; **Table 3**). At pH 7.2, the addition of iron without a chelator promoted Mb oxidation to the same degree as lipid alone (**Table 3**).

At pH 5.6 (**Table 4**), significant oxidation occurred in the iron-added sample after 15 min, as compared to its initial reading (time = 0.5 min). In other model system formulations, Mb oxidation was not significantly increased until 1 day incubation time. By 15 min, the iron-added system (system 2) had significantly more MbO_2 oxidation than either system containing a chelator (systems 3 and 5). This difference became more pronounced as time passed. After 1 day, the MM sample (system

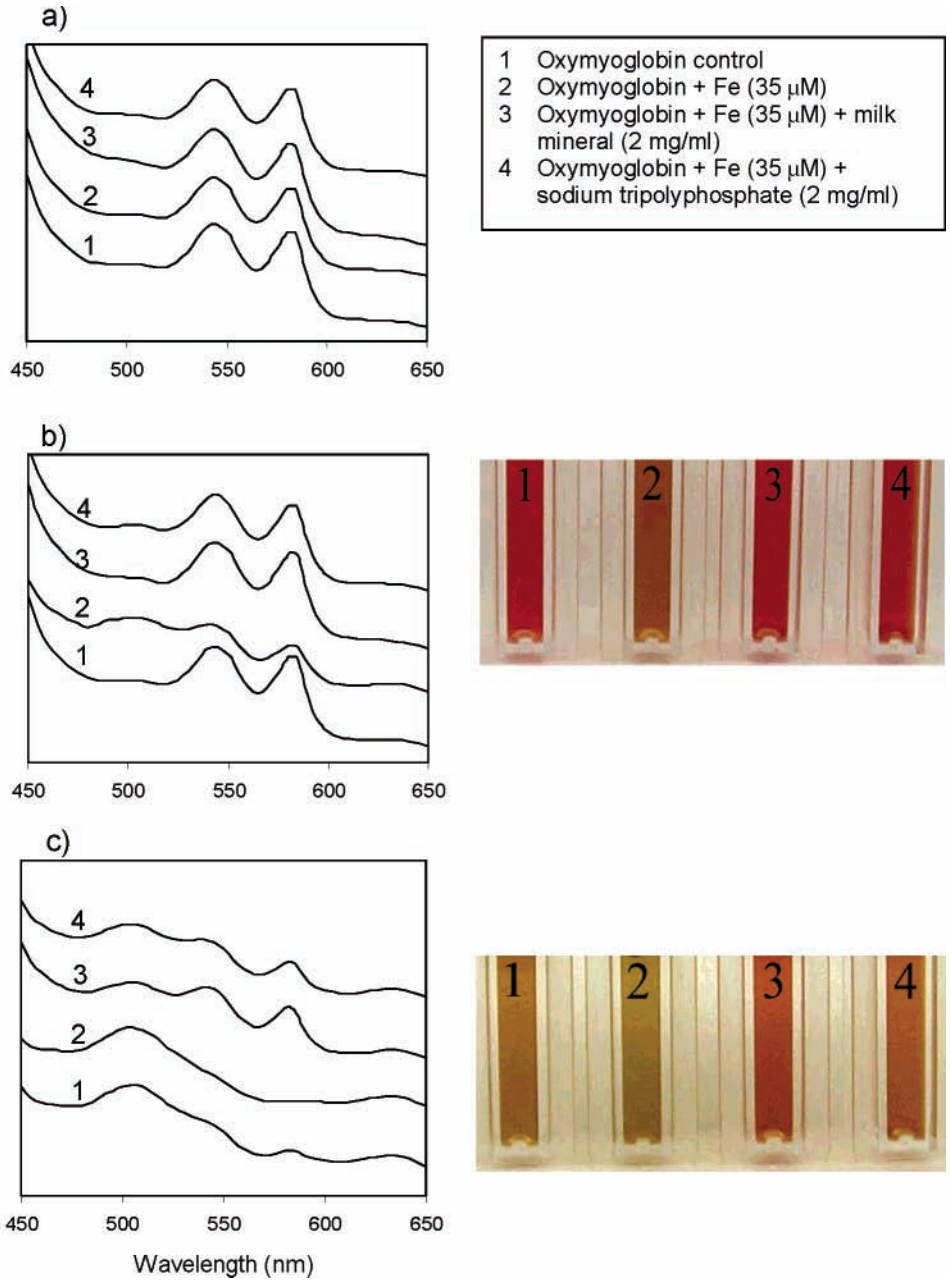

Figure 1. Spectral and visual evidence of Mb oxidation by nonheme iron after (**a**) 0.5 min, (**b**) 1 h, and (**c**) 1 day. Samples were held at room temperature (23 °C) at pH 5.6.

3) had more MbO_2 remaining than both the control (system 1) and the iron-added sample (system 2).

In lipid systems at pH 5.6 (**Table 4**), the MM system (system 3) exhibited a significant conversion of MbO_2 to MetMb by 60 min, while other systems did not change significantly in MbO_2 content until day one. However, the MM sample was less oxidized at 60 min than the iron-added sample.

At pH 7.2, the addition of STPP (with or without additional iron) delayed the onset of Mb oxidation (**Table 5**). Although other samples differed significantly from the initial readings by 15 min, samples containing STPP did not differ until 1 day. No significant difference existed, however, between samples containing only MM or STPP at any time point. The addition of either chelator preserved MbO_2 vs the control and the iron-added samples after 60 min; the effect was more pronounced after 1 day. In lipid systems, MbO_2 values did not differ from initial readings until 1 day had passed (**Table 5**).

Spectral and Visual Changes as Affected by Added Iron. Initially (0.5 min), spectra for all samples show distinct peaks at 545 and 580 nm, characteristic of MbO_2 (**Figure 1**). After 1 h, the sample containing 50 μM added iron (without chelator) was noticeably more brown, with lower absorbance values at 545 and 580 nm. As absorbance values at 545 and 580 nm decreased, new peaks appeared at 505 and 630 nm, indicative of a conversion from MbO_2 (bright red) to MetMb (brown). After 1 day, spectra indicated a shift to MetMb in all samples. This shift was more prominent, however, in the control and iron-added samples. Visually, the samples with added MM or STPP retained more red color than the control or iron-added samples. Distinct MbO_2 peaks were still visible in the spectra of both samples containing an iron chelator (MM or STPP).

Effect of Iron Concentration. MbO_2 oxidation appears to occur more rapidly with the addition of iron, in a concentration-dependent manner (**Figure 2**). After 60 min, in the MM sample

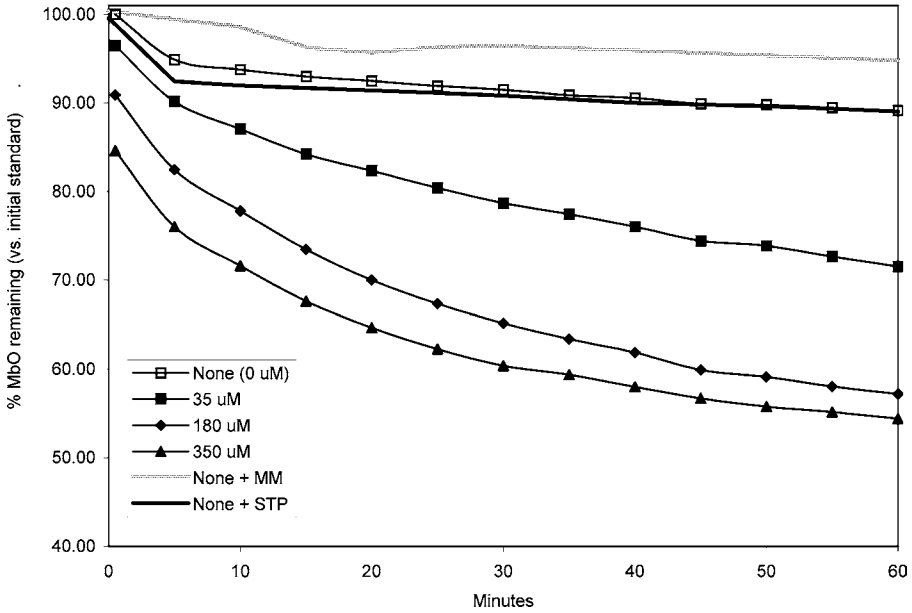

Figure 2. Effect of added iron concentration on Mb oxidation at pH 5.6. %MbO_2 remaining was calculated as the ratio of the MbO_2 peaks to the MetMb peak divided by the same ratio in the initial stock solution: %MbO_2 remaining = $[(A_{545} + A_{580})/(A_{505})$ in sample$]/[(A_{545} + A_{580})/(A_{505})$ in stock solution].

only, 5% of the initial MbO_2 had oxidized, while in both the control and the STPP samples, 11% was oxidized. The oxidation of MbO_2 in the control sample suggests that a trace level of unbound iron may have been present in the Mb stock, since this loss was not seen in either the MM or the STPP control. In samples with 35, 180, or 350 μM added iron, 18, 43, and 46% of the MbO_2 originally present, respectively, was converted to MetMb.

DISCUSSION

The addition of unchelated, nonheme ferrous iron had a significant effect on the rate at which MbO_2 was converted to MetMb in this experiment. In some systems containing an iron chelator, MbO_2 oxidation was prevented for over 1 h. However, all samples eventually exhibited some degree of Mb oxidation. The source of this background oxidation was not discernible based on these experiments. In lipid systems, the oxidation of MbO_2 was most likely due to the presence of lipid oxidation byproducts. Nonenal, a product of lipid oxidation, has been shown to bind to certain histidine residues in Mb, altering tertiary structure and potentially opening the heme cleft (*11*). This conformational change increases the accessibility of oxidant species to the heme iron.

The observed stimulatory effect of iron on Mb oxidation in the absence of lipid is in disagreement with the findings of Gorelik and Kanner (*9*). Ferric iron was used in their study, and no difference in oxidation was seen between the Mb control and the sample with added ferric iron in lipid free systems. It has been suggested that for iron to exhibit a catalytic effect on the oxidation of biological molecules, it must be redox active (i.e., both ferric and ferrous forms must be present; *38*). In the case of ferrous iron, both species would be present in aerobic systems as a result of autoxidation. Ferric iron, however, requires the addition of a compound capable of reducing it to its ferrous form. In the Gorelik and Kanner study (*9*), ascorbic acid was used as the reductant. Ascorbic acid minimized Mb oxidation in their study, both with and without the addition of iron. This result was most likely due to the addition of ascorbic acid in excess, reducing all ferric iron to the ferrous form, thus preventing redox cycling.

Although the exact manner in which ferrous iron promoted Mb oxidation in this study cannot be determined based on the current results, several possibilities exist. Castro (*30*) reported that, in anaerobic systems, ferric iron did not react with MbO_2, while ferrous iron accelerated the rate of dissociation of O_2. Because it is generally accepted that dMb is the oxidized species (*15*, *21*), nonheme ferrous iron may hasten Mb oxidation through this action, although the extent to which Castro's results apply to aerobic systems is not known.

Ferrous iron is also known to participate in various reactions resulting in the generation of oxidizing species. For example, H_2O_2 reacts with Fe^{2+} to produce $OH^{\bullet -}$ through the Fenton reaction. H_2O_2 may also be generated as a byproduct of iron autoxidation. Although these species are potent oxidants, the extent of their contribution to Mb oxidation has been questioned. Wazawa et al. (*18*) reported the rate at which H_2O_2 reacts with dMb was only 1 order of magnitude higher than that for its reaction with MetMb (1.3×10^7 and 1.6×10^6 h^{-1} M^{-1}, respectively). In contrast, $OH^{\bullet -}$ reacts with biomolecules rapidly and relatively indiscriminately (*31*, *32*). Although it is undoubtedly capable of oxidizing the heme iron of Mb, its overall contribution may be limited due to the logistics of transporting such a reactive species from its generation site.

While the ability of H_2O_2 and $OH^{\bullet -}$ to initiate biological oxidations cannot be denied, it has been suggested that a more important initiator is an iron−oxygen complex, such as the ferryl ($Fe^{2+}-O$) or perferryl ($Fe^{2+}-O_2$) ion (*33*−*35*). In a spin trapping study using dimethyl sulfoxide, ethanol, or glucose as subtrates, Qian and Buettner (*36*) demonstrated that iron−oxygen complexes, formed through "Fe^{2+} + O_2 chemistry", were the primary initiators of biological oxidation reactions. Evidence for the existence of such a complex in aqueous solution has also been suggested by Hochstein (*37*). This complex could partipate in the formation of an $Fe^{2+}-OO-Fe^{2+}$ bridge, which has been proposed as an intermediate step in Fe^{2+} autoxidation (*38*) and was detected in free porphyrin ring oxidation (*39*). Additional studies are needed to determine the possible existence of iron−oxygen intermediate species in the oxidation of aerobic Mb model systems.

In conclusion, oxygen-based chelators such as phosphate

stabilize iron in the ferric state, while nitrogen-based chelators stabilize ferrous iron (*40*). In the absence of an efficient reducing system, any phosphate-chelated iron would remain in the nonreactive ferric state, preventing its participation in any of the above-mentioned mechanisms. This explains the effectiveness of the type II iron-binding antioxidants used in this and previous studies (*1*−*4*). Regardless of the mechanism by which nonheme iron stimulates Mb oxidation, the effect has been demonstrated in this study and should be considered when discussing Mb oxidation pathways. Further investigation is needed to determine the possible role for nonheme ferrous iron on Mb oxidation in vivo or in meat.

ABBREVIATIONS USED

MetMb, metmyoglobin; MM, milk mineral; Mb, myoglobin; pO_2, partial pressure of oxygen; STPP, sodium tripolyphosphate; Tris, Tris(hydroxymethyl) aminomethane HCl buffer; MES, 2-(4-morpholine)ethanesulfone acid buffer; SPC, soy phosphatadylcholine; DDI, distilled, deionized water; dMb, deoxymyoglobin; MbO_2, oxymyoglobin; MS, myoglobin model system; LS, lipid model system; $O_2^{\bullet-}$, superoxide radical; $OH^{\bullet-}$, hydroxyl radical.

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