Oxymyoglobin Oxidation and Membranal Lipid Peroxidation Initiated by Iron Redox Cycle

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Oxymyoglobin is the main pigment in muscle tissues, responsible for the bright red color of fresh meat. Oxidation of the heme iron from the ferrous to the ferric metmyoglobin produces the brownish color that consumers find undesirable in fresh meat. The aim of this study was to elucidate the mechanism of oxymyoglobin oxidation in muscle tissues by using a model system containing oxymyoglobin and muscle membranes oxidized by an iron redox cycle. Oxidation of oxymyoglobin was determined from the decrease in absorption of the solution measured by a spectrophotometer at 582 nm. Lipid peroxidation was determined by accumulation of TBARS and conjugated dienes. The higher rates of oxidation of oxymyoglobin (20 μM) and lipid oxidation were achieved by using ferric iron and ascorbic acid at concentrations of 50 and 200 μM, respectively. Increasing the concentration of ascorbic acid to 2000 μM switched its effect to antioxidative. Increasing the concentration of oxymyoglobin from 20 to 80 μM inhibited lipid peroxidation by >90% and partially prevented oxymyoglobin oxidation.

Keywords: *Oxidation; oxymyoglobin; lipids; membranes; iron; ascorbic acid*

INTRODUCTION

Transition metals with their labile d-electron system are well suited to catalyzing redox reactions. Iron is an important catalyst in biological systems. The presence in tissues of a small labile pool of non-protein, non-heme iron provides "free" iron at micromolecular concentrations. This pool of "free iron", as distinct from ferritin, is catalytically active and participates in reactions involved in the production of oxygen species (*1*−*3*). The labile iron pool is the chelatable portion of the intracellular iron (*4*−*6*).

The amount of free iron in muscle tissues is in the range of 5−10 μM, but during ischimia and storage at low temperature it can rise to a concentration of ≥50 μM (*7*−*10*). Storage of turkey muscle at 4 °C increased the concentration of free iron from 15 >100 μM (*8*).

Ascorbic acid is a well-known reducing compound found at various concentrations in animal tissues. In turkey muscle, reducing compounds were found at a level of ∼3 mg of ascorbic acid equiv/100 g of fresh weight or at a concentration of ∼150 μM, 80% of which was found to have been ascorbic acid (*11*). Both free iron ions and ascorbic acid were found to act as the main catalyzers of lipid peroxidation in muscle foods (*13*); they act through a mechanism that produces ferrous iron, $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, ROOH, and lipid oxyradicals (*11*−*13*).

Oxymyoglobin is the main pigment in muscle tissues that is responsible for the bright red color of fresh meats. Oxidation of the heme iron from the ferrous form (bright red) to the ferric metmyoglobin produces a brownish color, which consumers find undesirable in fresh meat (*14*). It is well-known that in healthy living muscle, metmyoglobin does not accumulate because an active metmyoglobin-reducing enzyme system efficiently converts it to deoxymyoglobin (*15*).

Oxymyoglobin and oxyhemoglobin are not stable pigments, and autoxidation results in the formation of met heme proteins and superoxide radicals ($O_2^{\bullet-}$), which dismutases to produce hydrogen peroxide (H_2O_2) (*3*, *16*−*21*). During autoxidation, metmyoglobin and methemoglobin can be activated by H_2O_2 to a ferryl-heme protein, which, in the presence of membranes, initiates lipid peroxidation (*22*). The involvement of peroxides and free radicals in the reaction system can enhance the oxidation of the oxyheme form, resulting in a breakdown of the heme ring and liberating free iron (*23*, *24*).

Several authors have postulated that oxymyoglobin and lipid peroxidations in muscle tissues are interrelated (*25*−*27*).

Free radicals generated during lipid peroxidation promote the accumulation of metmyoglobin (*22*, *25*, *27*, *28*).

Autoxidation products from oxyheme proteins, the iron redox cycle system, and lipid peroxidation can all affect the stability of oxyhemes. Most recently, the use of hemichrome accumulation from the oxidation of heme proteins has been recommended as a method for the evaluation of tissue oxidation and autoxidation in vivo (*28*, *29*).

The aim of the present study was to elucidate the mechanism of oxymyoglobin oxidation in muscle tissues by the use of a model system containing oxymyoglobin and muscle membranes oxidized by an iron redox cycle.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis), ascorbic acid, and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Myoglobin type I from bovines, bovine serum albumin, sodium dithionite, 2-thiobarbituric acid, and L-histidine free-base were obtained from Sigma Chemical Co.

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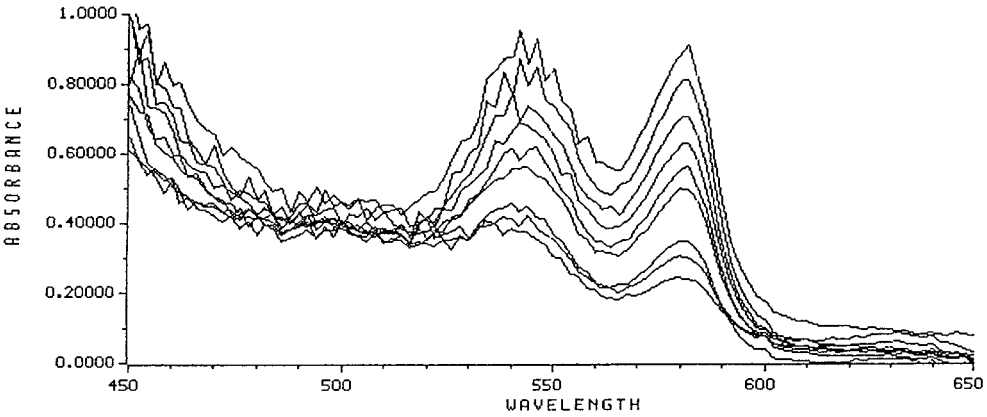

Figure 1. Oxymyoglobin (20 μM) oxidation by the microsomal model system containing ascorbic acid (200 μM) and ferric chloride (50 μM). Each line denotes 15 min of incubation.

(St. Louis, MO). Sephadex G-15 medium was obtained from Pharmacia Biotech (Uppsala, Sweden). Ferric chloride was obtained from Mallinckrodt Chemical Works (St. Louis, MO).

Methods. Myoglobin, treated with Chelex-100 and neutralized to pH 7.0, was separated from low molecular weight compounds on a column of Sephadex G-15. Oxymyoglobin was prepared by reducing 1 mL of metmyoglobin (1 mM) with dithionite (3 mg) under aerobic conditions and purifying by gel filtration on a column of Sephadex G-15, using a buffer of 0.01 M phosphate, pH 8.0, for elution (*30*, *31*). Oxymyoglobin concentration was calculated using an extinction coefficient $E_{582} = 15.1\ M^{-1}\ cm^{-1}$.

The microsomal fraction was isolated from muscle tissues as described previously (*22*). Protein determination was conducted according to the modified Lowry procedure (*32*), with BSA as a standard. Thiobarbituric acid reactive substances (TBARS) were determined according to the method of Bidlack et al. (*33*). The results are reported as nanomoles of malondialdehyde (MDA) per milligram of protein, using a molar extinction coefficient $E_{532} = 1.56 \times 10^5$.

The accumulation of conjugated dienes produced during lipid peroxidation was monitored by the increase in A_{233} (*34*).

Oxidation of oxymyoglobin was determined from the decrease in absorption of 582 nm in the extract solution, according to a method developed in our laboratory (*35*). To reduce the turbidity effect, an appropriate number of layers of Parafilm (usually one) was used as a background to subtract some of the absorbency caused by the turbidity of the samples. The samples were scanned from 450 to 650 nm, and the change in absorption at 582 nm was calculated (Figure 1). The results are means of triplicates; in the figures, each bar (I) denotes the standard deviation.

RESULTS

Oxymyoglobin and Membranal Lipid Peroxidation As Affected by the Reagents Included in the Model System. The complete model system contained microsomes (1 mg/mL), oxymyoglobin (20 μM), ferric iron (50 μM), and ascorbic acid (200 μM) at pH 6.5; it was incubated at 37 °C. Incubation of oxymyoglobin in this model system, for 120 min, oxidized 91.2% of the pigment (Figure 1 and Table 1) and gave maximum peroxidation of membranal lipid. Removing ascorbic acid and iron from the system decreased oxidation of oxymyoglobin by 60%. The greatest decrease in oxymyoglobin oxidation was obtained upon removing the microsomes and free iron ions from the system. Membranal lipid peroxidation in this model system was mostly affected by the iron redox cycle system containing free iron and ascorbic acid. Removal of oxymyoglobin from the system decreased lipid peroxidation by 28% (Table 1). Oxymyoglobin in the model system without microsomes was relatively more stable, and its stability increased in the presence of ascorbic acid.

Table 1. Oxymyoglobin Oxidation and Lipid Peroxidation in Microsomal Model System

treatment	oxymyoglobin oxidation, %	TBARS, nmol of MDA/mg of protein
Mic + MbO_2 + AA + Fe	91.2a	26.8b
Mic + AA + Fe		36.0a
Mic + AA		15.0c
Mic + Fe		5.2d
Mic + MbO_2 + AA	46.6b	12.0c
Mic + MbO_2 + Fe	50.3b	6.9d
Mic MbO_2	36.9c	1.0e
MbO_2 + AA + Fe	14.7e	
MbO_2 + AA	12.5e	
MbO_2 + Fe	29.8d	
MbO_2	25.0d	

[a] The full system contained the following: microsomes, Mic (1 mg/mL); oxymyoglobin, MbO_2 (20 μM); $FeCl_3$ (50 μM); ascorbic acid (AA; 200 μM); 0.05 M acetate buffer, pH 6.5, incubated at 37 °C for 120 min. Data are means of three experiments and two determinations of each sample ($p < 0.001$ significantly different between a, b, etc.).

Effect of Iron, Ascorbic Acid, and Oxymyoglobin Concentration on Oxymyoglobin and Lipid Oxidation. Oxymyoglobin and lipid oxidation were strongly affected by the concentration of free iron in the system. Both oxymyoglobin oxidation and lipid peroxidation increased significantly with the addition of ferric ions up to 50 μM. This result was expected.

The rate of oxidation was monitored either by TBARS or by accumulation of conjugated dienes (Figures 2 and 3). A pro-oxidant effect was obtained by addition of ascorbic acid up to a concentration of 500 μM and in the presence of 50 μM of ferric chloride. Increasing the concentration of ascorbic acid to 2000 μM switched its effect to antioxidative (Figure 4).

The effects on lipid peroxidation and oxymyoglobin oxidation are presented in Figure 5. Increasing the concentration of oxymyoglobin in the system did not change the absolute amount of oxymyoglobin oxidized, which was ~8−10 μM in 30 min. However, it very significantly affected lipid peroxidation: increasing the concentration of oxymyoglobin from 20 to 80 μM inhibited lipid peroxidation by >90%.

The stability of oxymyoglobin was very much affected by the hydrogen peroxide concentration (Figure 6): increasing it from 5 to 50 μM enhanced oxymyoglobin oxidation and lipid peroxidation by 5−8-fold, respec-

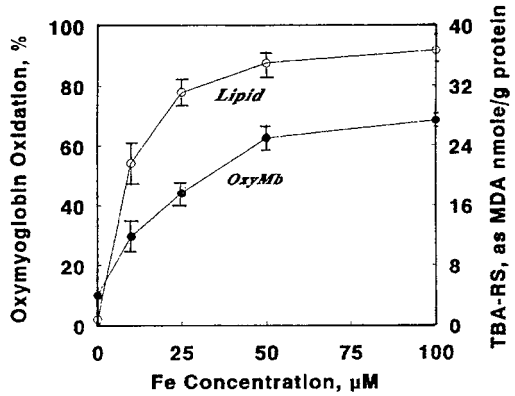

Figure 2. Oxymyoglobin oxidation and lipid peroxidation, detected by TBARS, as affected by ferric chloride concentration (model system, see Figure 1), 200 μM ascorbic acid, and reaction time of 30 min.

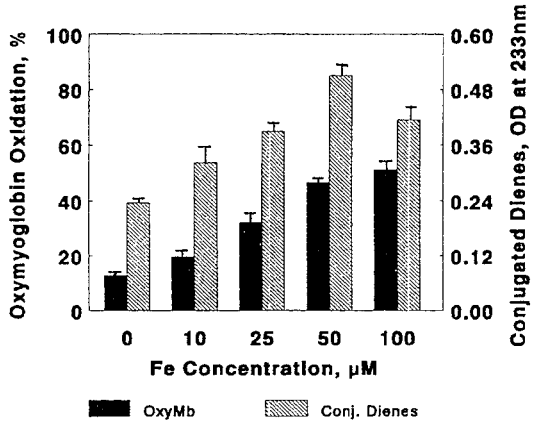

Figure 3. Oxymyoglobin oxidation and lipid peroxidation, detected by diene accumulation, as affected by ferric chloride concentration (model system, see Figure 1), 200 μM ascorbic acid, and reaction time of 30 min.

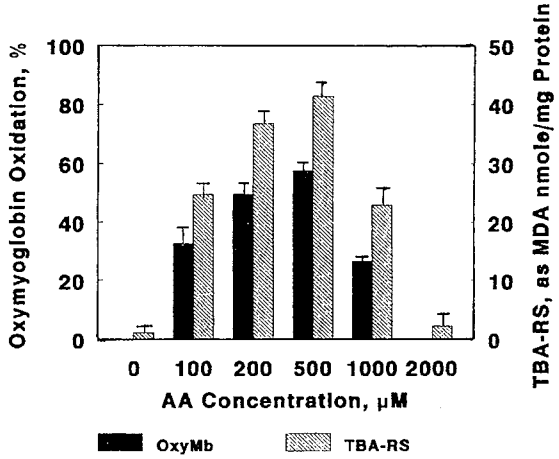

Figure 4. Oxymyoglobin oxidation and lipid peroxidation as affected by ascorbic acid concentration (model system, see Figure 1), ferric chloride (50 μM), and reaction time of 30 min.

tively. Correlations between oxymyoglobin oxidation and membranal peroxidations are presented in Figure 7.

DISCUSSION

The process of autoxidation of oxymyoglobin has been described by many authors (*16*, *19*−*22*). During this reaction $O_2^{\bullet -}$, H_2O_2, and ferryl radical are generated. In our model system only ∼25% of the oxidation of

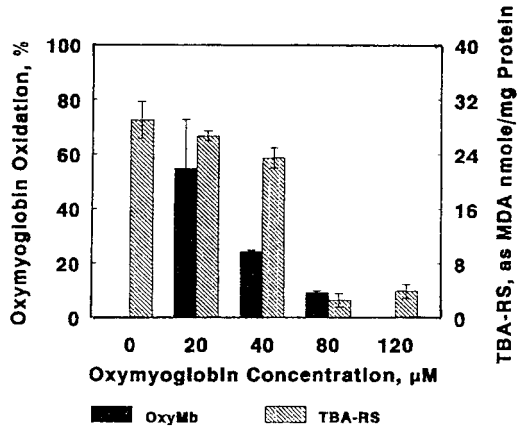

Figure 5. Oxymyoglobin oxidation and lipid peroxidation as affected by myoglobin concentration (model system, see Figure 1) and incubation time of 30 min.

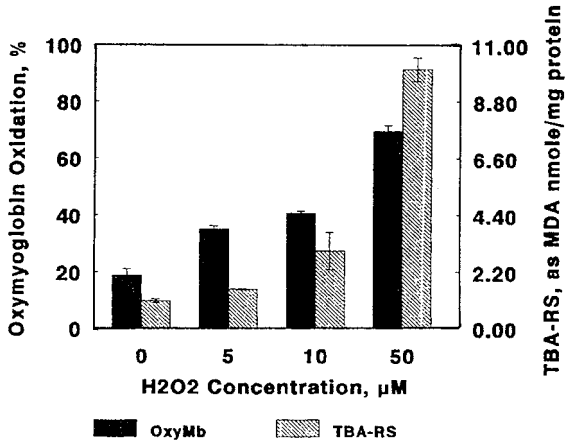

Figure 6. Effect of hydrogen peroxide concentration on oxymyoglobin and lipid oxidation (model system, see Figure 1), the reaction time being 15 min.

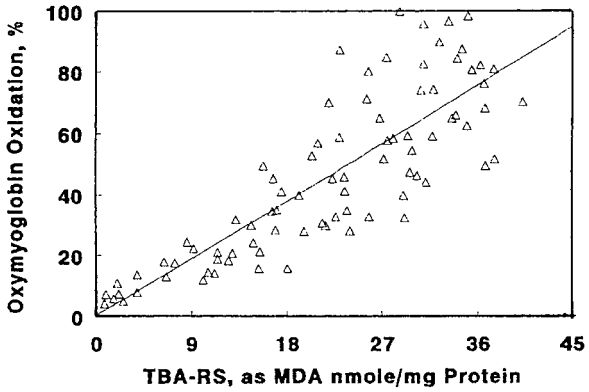

Figure 7. Correlation between oxymyoglobin and lipid oxidation.

oxymyoglobin could be associated with autoxidation. The addition of ascorbic acid to oxymyoglobin diminished its autoxidation by 50%, most probably by interacting with the superoxide and ferryl radical generated during autoxidation and in part by reduction of metmyoglobin to the reduced form. The addition of ferric chloride to the system containing oxymyoglobin and ascorbic acid increased the oxidation by only ∼2%, which is not a significant effect. The addition of microsomes to our model system containing oxymyoglobin, ascorbic acid, and ferric chloride increased oxymyoglobin oxidation by almost 4-fold, demonstrating that lipid

peroxidation strongly affected the stability of oxymyoglobin. The effect of membranal lipid peroxidation on oxymyoglobin oxidation has been studied by several authors (*25*−*27*, *36*).

In our model system, most of the membranal lipid peroxidation was effected by the iron redox cycle in the presence of ascorbic acid: the membrane lipid peroxidation was reduced by 97% in the absence of iron ascorbate (Table 1).

The iron redox cycle, in the presence of ascorbic acid, produces $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$ (*37*−*41*), species that could initiate membrane lipid peroxidation. Once hydroperoxides are generated, lipid peroxidation is propagated by ferrous ions present in the model system, as shown by the following reactions:

$$Fe^{3} + AH_2 \rightarrow Fe^{2+} + AH^{\bullet} \quad (1)$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \quad (2)$$

$$AH^{\cdot} + O_2^{\bullet -} \xrightarrow{H^+} A + H_2O_2 \quad (3)$$

$$O_2^{\bullet -} + O^{\bullet -}{}_2 \xrightarrow{2H^+} H_2O_2 + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad (5)$$

$$RH + HO^{\bullet} \xrightarrow{O_2} ROO^{\bullet} + H_2O \quad (6)$$

$$RH + ROO^{\bullet} \rightarrow ROOH + R^{\bullet} \quad (7)$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + HO^{-} \quad (8)$$

We consider that the H_2O_2, hydroperoxides, and lipid radicals generated during the process strongly stimulated the oxidation of oxymyoglobin. The interaction of oxymyoglobin with H_2O_2, hydroperoxides, or lipid free radicals seems to induce a transfer of an electron from the iron heme, heme, or amino acid to the free radical or peroxide. Such a transfer could destabilize oxymyoglobin, generating metmyoglobin and oxygen (*42*).

The concentration of free iron was found to affect both lipid peroxidation and oxymyoglobin oxidation significantly. Both reactions increased very significantly up to a ferric iron concentration of 50 μM. Ferrous ion seems to play a significant role in generating superoxide and hydrogen peroxide (reactions 2−4). Hydroperoxides are decomposed by ferrous ions (reaction 8) to free radicals, which strongly accelerate the oxidation reaction.

Ascorbic acid plays a double role in this model system. At low concentrations of up to 500 μM its effect is prooxidative, engaging the "iron redox cycle-dependent" oxidation of lipid peroxidation and oxymyoglobin oxidation (reactions 1 and 3). However, as is well-known (*43*−*48*), at higher concentrations, ascorbic acid works antioxidatively, preventing not only lipid peroxidation but also oxymyoglobin oxidation. At a concentration of 2 mM, ascorbic acid not only reduces ferric ions to the ferrous form but also interacts with free radicals such as $HO^{\bullet}$, $RO^{\bullet}$, and $ROO^{\bullet}$, so preventing the propagation of oxidation. At this concentration ascorbic acid might reduce in part metmyoglobin to oxymyoglobin.

Oxymyoglobin at a concentration of 20 μM slightly decreased membranal lipid peroxidation. However, at a higher concentration of 80−120 μM, lipid peroxidation was inhibited almost completely. These results resemble our previous findings on the inhibition by hemoglobin and myoglobin of hydroxyl radicals generated in a model system of an "iron redox" cycle (*30*).

In the present model system, which also contained membranal lipids and hydroperoxides, oxymyoglobin decomposed not only H_2O_2 but also hydroperoxides to species that convert the apoprotein to nonradical products by a process of autoreduction. This activity inhibits membranal lipid peroxidation and prevents an increase in oxymyoglobin oxidation. Data on the concentration-dependent activity of heme proteins have been reported by several authors (*22*, *49*−*51*).

On the basis of our present data and those from the literature, the following comprehensive reaction for oxidation of oxymyoglobin has been drawn up:

$$Mb{-}Fe^{2+}{-}O_2 \leftrightarrows Mb - Fe^{2+}O_2 \quad (9)$$

$$Mb{-}Fe^{2+} + O_2 \rightarrow Mb{-}Fe^{3+} + O_2^{\bullet -} \quad (10)$$

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{2H^+} H_2O_2 + O_2 \quad (11)$$

$$Mb{-}Fe^{3+} + H_2O_2 \rightarrow {}^{\bullet}Mb{-}Fe^{4+}{=}O + H_2O \quad (12)$$

$$Mb{-}Fe^{2+} + H_2O_2 \rightarrow Mb{-}Fe^{4+}{=}O + H_2O \quad (13)$$

$$Mb{-}Fe^{2+} + ROOH \rightarrow Mb{-}Fe^{3+} + RO^{\bullet} + HO^{-} \quad (14)$$

$$Mb{-}Fe^{2+} + RO^{\bullet} \rightarrow Mb{-}Fe^{3+} + RO^{-} \quad (15)$$

$$Mb{-}Fe^{2+}{-}O_2 + P{-}Fe^{4+}{=}O \xrightarrow{H^+} 2Mb{-}Fe^{3+} + HO^{-} + O_2 \quad (16)$$

$$Mb{-}Fe^{2+} - O_2 + Fe^{3+} \rightarrow Mb{-}Fe^{3+} + Fe^{2+} + O_2 \quad (17)$$

It is known also that free iron such as ferric ion can oxidize oxymyoglobin by reaction 17; however, this reaction was not significant in our model system.

In conclusion, the iron redox cycle, together with ascorbic acid, which is the main initiator of lipid peroxidation in muscle foods (*11*, *13*), greatly affects oxymyoglobin oxidation. Both reactions are partially inhibited by decreasing the amount of free iron or by greatly increasing the concentrations of ascorbic acid and oxymyoglobin. Part of the oxidation of oxymyoglobin can be attributed to autoxidation, which is mostly effected by H_2O_2 and ferryl formation, and the remainder by lipid peroxidation and, especially, by the presence of hydroperoxides and lipid free radicals.

ABBREVIATIONS USED

$MbFe^{2+}{-}O_2$, oxymyoglobin; $MbFe^{2+}$, deoxymyoglobin; $MbFe^{3+}$, methmyoglobin; $^{\bullet}Mb\text{-}Fe^{4+}{=}O$, oxymyoglobin ferryl cation radical; $Mb\text{-}Fe^{4+}{=}O$, myoglobin ferryl; AH2, ascorbic acid; SOD, superoxide dismutase.

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