JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Heating Oxymyoglobin and Metmyoglobin on the Oxidation of Muscle Microsomes

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Myoglobin (Mb) and its iron have been proposed to be major prooxidants in cooked meats. To understand the mechanisms and differentiate between the prooxidant and antioxidant potential of oxymyoglobin (OxyMb) and metmyoglobin (MetMb), their prooxidant activity, iron content, solubility, free radical scavenging activity, and iron binding capacity were determined as a function of thermal processing. The ability of native and heat denatured OxyMb and MetMb to promote the oxidation of muscle microsomes was different. MetMb promoted lipid oxidation in both its native and denatured states. Conversely, OxyMb became antioxidative when the protein was heated to temperatures ≥75 °C. The increased antioxidant activity of heat denatured OxyMb was likely due to a decrease in its prooxidative activity due to its loss of solubility. These data show that the impact on oxidative reactions of Mb is the result of the balance between its antioxidant and prooxidant activities.

KEYWORDS: Oxymyoglobin; metmyoglobin; lipid oxidation; meat; antioxidant; iron

INTRODUCTION

Myoglobin (Mb) is the primary pigment responsible for the red meat color of stock animals (*1, 2*). The protein consists of a globin and a nonprotein heme group composed of a central iron atom covalently bonded with four nitrogen atoms of the porphyrin ring structure. One of the remaining iron-binding positions is occupied by a proximal histidine residue of the Mb and several ligands can be present in the sixth position. *In vivo*, Mb is commonly found in the ferrous form and the sixth position can be vacant, that is, deoxymyoglobin, or have oxygen as a ligand, that is, oxymyoglobin (OxyMb). This latter oxygenated form of Mb is the main analogue in muscle foods that contributes to the red color associated with fresh meat. However, OxyMb can change color to brown because of the formation of metmyoglobin (MetMb) when ferrous iron is oxidized to ferric.

Ferrous and ferric heme compounds and other nonheme iron sources have been reported to initiate or catalyze lipid oxidation, although the mechanism in raw and cooked meats is not completely understood (*2*−*6*). Both OxyMb and MetMb can yield ferrylmyoglobin (also known as activated MetMb) in the presence of hydrogen peroxide or lipid hydroperoxides and are reported to be initiators of lipid oxidation in raw meat (*7*−*11*). Upon cooking, muscle foods become more prone to oxidative rancidity. One hypothesis for the rapid development of rancidity in cooked muscle foods is that heme degradation compounds coming from native Mb are highly prooxidative since Mb can deliver active iron into systems such as cell membranes where they can efficiently promote oxidation (*12*). For example, denaturation of Mb and hemoglobin can release lipid-soluble hematin, which has been reported to promote lipid oxidation (*13*−*15*). In addition, iron can be released from hemeproteins during heating, and several researchers have suggested that this released iron is a major factor in promoting lipid oxidation in cooked meats (*7, 16*−*21*). However, some researchers believe that free iron originating from Mb is not an important promoter of lipid oxidation in cooked meat because the cleavage of the porphyrin ring is difficult (*12, 22*). In addition, upon release iron has been proposed to bind to different compounds such as the denatured Mb itself (*12, 22*) as well as other denatured proteins (*23*) and low molecular weight molecules (*6*). Binding of iron to proteins and low molecular weight compounds can reduce its prooxidative activity.

In addition to the ability of thermal processing to increase the activity of prooxidants in muscle foods, heating can also inactivate antioxidant enzymes such as catalase and glutathione peroxidase that help protect against oxidation (*24*−*26*). Conversely, proteins that have not been biologically designed to control oxidative reactions can inhibit lipid oxidation through their ability to chelate metals and scavenge free radicals (*27*). Denaturation of proteins can increase their ability to inhibit lipid oxidation by increasing the solvent exposure of antioxidative amino acids that would normally be located in the core of the native protein structure (*28*). Therefore, it is possible that thermal

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10.1021/jf8009848 CCC: $40.75
Published on Web 09/25/2008

treatments could increase the prooxidant activity of some proteins while increasing the antioxidant activity of others. It is important to understand the prooxidant−antioxidant balance of proteins such as Mb in cooked muscle foods in order to develop strategies to effectively inhibit oxidative rancidity. Therefore, the objective of this work was to determine how heat denaturation of Mb changes its prooxidative activity and antioxidative properties in a muscle microsome membrane model system.

MATERIAL AND METHODS

Reagents and Standards. Horse skeletal muscle Mb, fluorescein sodium salt, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), L-ascorbic acid, ferrous sulfate heptahydrate, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), nitrilotriacetate (NTA), hydroxylamine hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), cumene hydroperoxide, barium chloride, ammonium thyocianate, and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) were from Sigma-Aldrich Co. (St. Louis, MO). Potassium chloride, sodium nitrite, sodium phosphate dibasic and monobasic, hydrochloric acid, ammonium acetate, and sodium hydrosulfite were obtained from Fisher-Scientific (Pittsburgh, PA). Trichloroacetic (TCA) anhydride, ferric chloride, and acetone were from Acros Organics (Fair Lawn, NJ). The chemicals used for the Lowry method (*29*) were of ACS grade.

Preparation of Microsomes. Pork tenderloin muscle microsomes were isolated according to the method of Brannan and Decker (*30*). Frozen pork was diced into approximately 0.5 mm cubes and then chopped in a stainless steel blender for 1 min, and the resulting paste (25 g) was homogenized in 90 mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, in a tissuemizer (20000 rpm; Tekmar, Cincinnati, OH) for 2 min, followed by centrifugation for 30 min at 10000*g* at 4 °C (Sorvall Ultra 80, DuPont, Wilmington, DE). The resulting supernatant was ultracentrifuged for 60 min at 100000*g* to pellet insoluble muscle components including the microsomes. Myofibrillar proteins were then solubilized from the pellet in 0.6 M KCl/25 mM phosphate buffer, pH 7.2, and a microsome-containing pellet was isolated by centrifugation for 60 min at 100000*g*. Isolated microsomes were standardized to 30 mg of protein/mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, and stored at −80 °C until use. Protein in the microsomal fraction was determined by using the method of Lowry et al. (*29*).

Preparation of OxyMb and MetMb. Commercial MetMb standard was dissolved in 50 mM sodium phosphate buffer (pH 5.6) kept at 4 °C at the required concentrations. OxyMb was prepared from a stock solution of MetMb, which was chemically reduced by mixing with sodium hydrosulfite (0.1 mg/mg MetMb), carefully shaken, and kept on ice 5 min. Then, excess sodium hydrosulfite was removed by passing through a Bio-Rad Econopak 10 DG disposable desalting column (Bio-Rad Laboratories, Hercules, CA; exclusion limit 6000 Da) as follows. Mb solution (3 mL) was layered onto the column and allowed to pass into the column bed. Then, 4 mL of phosphate buffer was passed into the stationary phase to elute the Mb out of the column. Desalted Mb was kept on ice and deoxymyoglobin was oxygenated by gentle shaking. OxyMb solutions were made fresh, and all buffered solutions were kept at 4 °C. The absorption spectra of MetMb and OxyMb were obtained using a Shimadzu UV-2101PC model UV−visible scanning spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD), and the total concentrations and yields of these solutions were calculated with the isobestic point at 525 nm using the modified Krzywicki equations described elsewhere (*31*).

Only those solutions with 90% or higher yields in OxyMb or MetMb were used for analyses. Test tubes containing aliquots of the freshly prepared OxyMb and MetMb solutions were capped and placed in a water bath set at 25 °C, and the water bath was set to 95 °C. When the temperature of the water bath reached 45, 60, 75, 85, and 95 °C (this latter temperature was reached after approximately 1 h and 20 min), tubes were immediately taken out and kept on ice. Samples were used the same day as preparation except for samples used for antioxidant capacity and heme and nonheme iron analysis, which were flushed with nitrogen and stored at −80 °C until use.

Determination of Lipid Oxidation. Equal volumes of microsomes (10 mg protein/mL) adjusted to pH 5.6 and native or heated Mb solutions (0.30 mM) dissolved in 50 mM phosphate buffer (pH 5.6) were mixed and incubated for different periods at 30 °C in a water bath under gentle agitation.

Lipid hydroperoxides were determined at each incubation time by adding 1 mL of 30% TCA dissolved in methanol containing 0.3% BHT to 2 mL of the above mixture of microsomes and Mb. In addition, two different blanks consisting of microsomes and buffer or Mb and buffer were prepared. Blanks and samples were vortexed and centrifuged for 2 min at 1750*g*, and 2 mL of the supernatants was taken and mixed with 30 μL of an solution containing 2 M ammonium thiocyanate and 72 mM ferrous ion (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). Samples were then immediately vortexed, and absorbance was determined after 30 min at 510 nm. Results were expressed as μmols of cumene hydroperoxide equivalents/g of microsomal protein and were calculated by means of a standard calibration curve prepared from cumene hydroperoxide.

Secondary oxidation products were monitored by measuring thiobarbituric acid reactive substances (TBARS) by means of a modified procedure described elsewhere (*32*). In addition to the samples, three different blanks were prepared including buffer without Mb, buffer with native Mb, and buffer with Mb heated at 95 °C solutions. Briefly, at each incubation time, 1 mL of the samples were mixed with 1 mL of a TBA solution containing 20% TCA, 0.5% TBA, 0.2% EDTA, and 30 mM HCl in screw-capped tubes. Immediately afterward, 30 μL of 3% BHT was added, and the tubes were then closed and vortexed. Samples were then heated in a boiling water bath for 15 min, cooled at room temperature, and centrifuged at 1600*g* for 20 min. The absorbance of the supernatant was measured at 532 nm, and the results were reported as μmol of malondialdehyde (MDA) per g of microsomal protein. Concentrations were determined from a MDA standard curve produced from TEP.

UV−Visible Spectrophotometry of Heated Mb. Appropriately diluted Mb samples (20 μM Mb using 50 mM phosphate buffer, pH 5.6), were placed in the thermoblock of an Ultrospec 3000 Pro model spectrophotometer (Biochrom Ltd., Cambridge, UK). The thermoblock was equilibrated at 25 °C and programmed at a heating rate of 0.8 °C/min up to 97 °C. The absorbance was recorded at 290 nm, to monitor changes in tryptophan (Trp) absorbance (*12*).

Protein Solubility. The solubility of Mb and heated Mb was determined after centrifugation of samples at 1750*g* for 10 min and then determining the protein content of the supernatant by the Lowry method (*29*).

Iron Content. The heme and nonheme iron were determined in either the whole solution containing 0.15 mM native Mb or heated Mb, or in the soluble fraction of these samples, which were obtained by collecting the supernatant after centrifugation at 1750*g* for 10 min. Heme concentrations were determined using the acidified acetone extraction method of Hornsey (*33*) with slight modifications. Five hundred microliters of the samples were added to 2.5 mL of acetone and 125 μL of 3 N HCl. After 1 h at room temperature, samples were centrifuged at 1750*g* for 20 min. The absorbance of this supernantant was measured, and heme content was calculated using a molar extinction coefficient of 4800 M^{-1} cm^{-1} at 640 nm for the resulting chlorohemin.

A slightly modified method described by Rhee and Ziprin (*34*) was used to measure nonheme iron. One hundred microliters of sodium nitrite (0.16% w/v) and 1.5 mL of extraction solution (6 N HCl plus 40% trichloroacetic acid in equal volumes) were added to screw-cap tubes containing 500 μL of Mb samples. The tubes were closed, mixed, and incubated in a water bath at 65 °C for 20 h. After cooling, the mixtures were centrifuged at 1750*g* for 10 min, and the supernatants were passed through 0.45 μm filters. To 1 mL of each filtrate, 1 mL of 0.8% ascorbic acid was added, and the samples were allowed to stand for 15 min. Then, 1 mL of 16% ammonium acetate and 1 mL of 0.8 mM ferrozine were added, and the absorbance at 562 nm was measured after 10 min. Concentrations were obtained using a standard curve from 0 to 2 mg of iron/L made with ferrous sulfate heptahydrate.

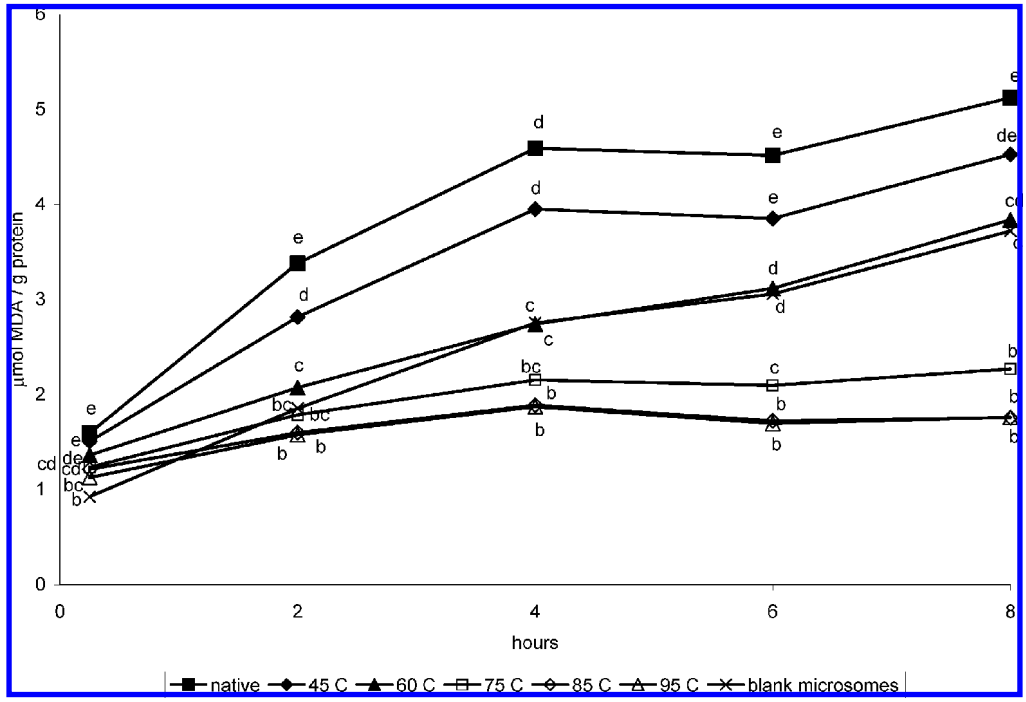

Figure 1. Effect of oxymyoglobin (OxyMb) (final concentration 0.15 mM) heated at different temperatures on the formation of thiobarbituric acid reactive substances (TBARS; μmols MDA/g protein) in the presence of muscle microsomes (final concentration 5 mg/mL) at 30 °C for different incubation times. Blank microsomes correspond to samples with microsomes but no myoglobin (Mb). Blanks made with native OxyMb or OxyMb heated at 95 °C were equal, did not change over time, and were significantly lower than samples containing microsomes (data not shown). For each incubation time, values corresponding to a certain variable with different letters differ significantly ($P \leq 0.05$).

Iron Binding Capacity. The ability of Mb and heated Mb to bind iron was determined using a modified method of Lin et al. (*35*). A solution of ferric iron chelated to nitrilotriacetate (NTA) was prepared by mixing 1 volume of 0.5 M $FeCl_3$ (in 0.05 M HCl) with 2 volumes of 0.5 M NTA in water. Fe-NTA was added to 0.05 M HEPES buffer (pH 7.0) so that the final concentration of $FeCl_3$ was 1.0 mM. Mb or heated Mb (8 mL of 0.075 mM in 50 mM phosphate buffer, pH 5.6) was placed inside a dialysis bag (Spectrum Laboratories Inc., Rancho Dominguez, CA; Spectra/Por 3 membrane, 500 molecular weight cutoff) and incubated at 4 °C in 1000 mL of the Fe-NTA buffer mixture for 24 h (*35*). A protein-precipitating solution was prepared with hydroxylamine hydrochloride (0.72 M), TCA (0.61 M), and 100 mL of 12 N HCl. To measure protein-bound iron, protein samples removed from the dialysis bags (2 mL) were mixed with 1 mL of the protein precipitating solution and incubated at room temperature overnight. Samples were then centrifuged at 1750*g* for 10 min. The resulting supernatants (750 μL) containing iron released from the protein were mixed with 2 mL of ammonium acetate buffer (10% w/v, pH 10) followed by 0.5 mL of 9.0 mM ferrozine. Absorbance was determined at 562 nm after 1 h. The iron concentration was determined using a standard curve prepared from $FeCl_3$.

Oxygen Radical Absorbance Capacity (ORAC). A 500 mM solution of AAPH in 75 mM potassium phosphate buffer at pH 7.0 was prepared for each experiment and kept on ice. Fluorescein was dissolved to a concentration of 50 nM in phosphate buffer containing 0.1 mM EDTA before each set of experiments. For each run, fluorescein was equilibrated to 37 °C in a water bath for 15 min. Reagents were added in the order of native Mb or heated Mb in 75 mM phosphate buffer (pH 7.0), fluorescein, and AAPH at a final concentration of 6 μM, 45 nM, and 20 mM, respectively. Fluorescence was recorded from 0 to 50 min every 10 min by taking 4 mL aliquots in which 40 μL of 500 mM ascorbic acid was added to stop the reaction followed by centrifugation for 10 min at 1750*g*. The fluorescence (excitation = 493 nm; emission = 515 nm; Hitachi F-2000 flourometer, Tokyo, Japan) of the supernatants was measured at 37 °C. The relative radical absorbance capacity values of the Mb or heated Mb were calculated using the area under the curve (AUC), which was calculated as follows: AUC = $1 + F_1/F_0 + F_2/F_0 + \ldots + F_n/F_0$, where F_0 is the initial fluorescence reading at 0 min, and F_i is the fluorescence reading at time i.

Calculation of Solvent Accessible Surface Areas of Selected Amino Acid Residues. The solvent accessible surface areas of tryptophan, histidine, phenylalanine, methionine, and tyrosine residues in MetMb and OxyMb were calculated using the GETAREA web-based application (*36*) using a probe radius of 1.4 Å. Surface area comparisons were based on the reported protein structures of sperm whale MetMb (PDB entry 1JP6) and OxyMb (PDB entry 1MBO), as determined by X-ray diffraction. Amino acid residues were considered to be buried within their respective protein's hydrophobic core if the ratio of their side-chain surface area to random coil value was less than 20%. Residues were considered to be fully solvent accessible if that same ratio was 50% or higher. The random coil value of a given residue X is the average solvent accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations (*36*). In general and for the purposes of this work, solvent exposure was considered to increase as the side-chain surface area to random coil ratio increased.

Statistical Analyses. All samples were measured in triplicate. Various analyses were carried out as described elsewhere (*37*). Briefly, a 1-way ANOVA was used to determine whether the heating temperature affected protein solubility, antioxidant capacity, and iron content in the different fractions of each Mb. By taking into consideration each incubation time, a 1-way ANOVA was carried out to study whether the heating temperatures affected the pro-oxidant activity of OxyMb and MetMb. A 1-way ANOVA was used to determine whether differences exist among the iron binding capacities of the selected Mb treatments. In all cases, a $P \leq 0.05$ was considered significant, and Scheffé's test was used to separate means.

RESULTS

Impact of OxyMb and MetMb on Oxidation of Muscle Microsomes. The ability of OxyMb to promote the formation of TBARS in muscle microsomes was highest for the unheated

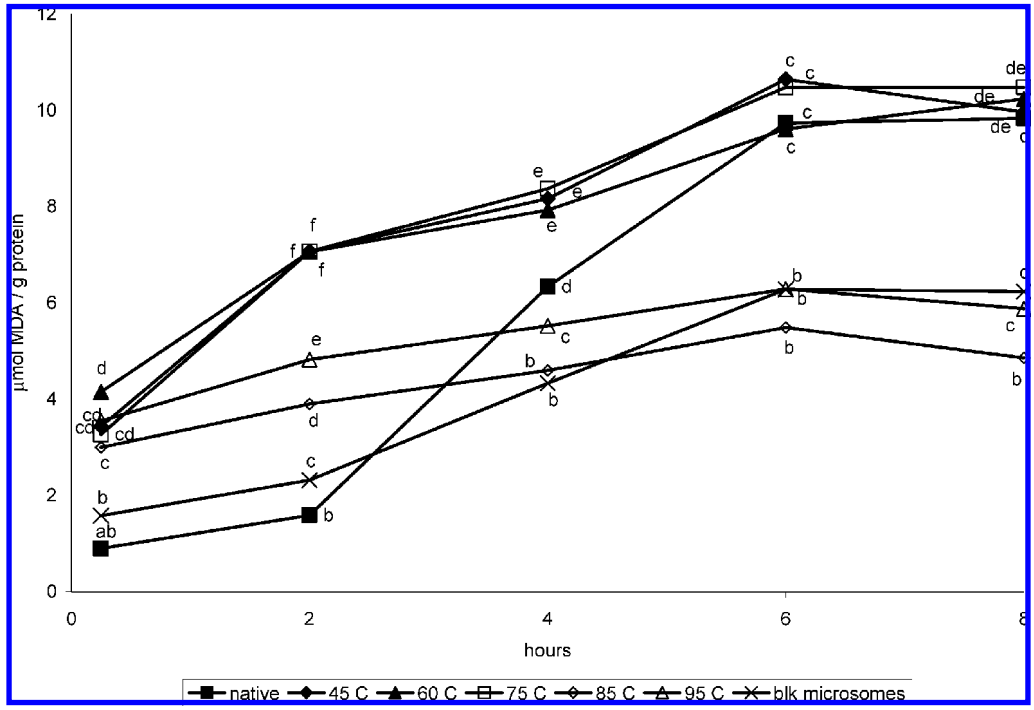

Figure 2. Effect of metmyoglobin (MetMb) (final concentration 0.15 mM) heated at different temperatures on the formation of thiobarbituric acid reactive substances (TBARS; μmols MDA/g protein) in the presence of muscle microsomes (final concentration 5 mg/mL) at 30 °C for different incubation times. Blank microsomes correspond to samples with microsomes but no myoglobin (Mb). Blanks made with native MetMb or MetMb heated at 95 °C were equal, did not change over time, and were significantly lower than samples containing microsomes (data not shown). For each incubation time, values corresponding to a certain variable with different letters differ significantly ($P \leq 0.05$).

protein (**Figure 1**) and decreased with increasing heating temperatures. The 60 °C heated OxyMb did not increase TBARS formation rates compared to the microsomes in the absence of Mb. At heat treatments ≥75 °C, the OxyMb inhibited lipid oxidation with TBARS, being less than the microsome control without added Mb. For unheated metMb, TBARS concentrations were greater than the no Mb microsome control after 4 h of incubation (**Figure 2**). Heating the MetMb from 45−75 °C increased prooxidant activity with an increase in TBARS concentrations being detected at the earliest sampling time. The formation rate of TBARS by MetMb heated from 45−75 °C was similar. Samples with MetMb heated at 85 and 95 °C initially had TBARS higher than the no Mb control, but TBARS formation only increased slowly with time with TBARS concentrations eventually becoming less than or equal to the no Mb control after 8 h of incubation. EDTA was not able to inhibit lipid oxidation by native or MetMb heated to 95 °C suggesting that free iron was not responsible for the promotion of TBARS formation (data not shown). No TBARS were detected in samples containing OxyMb or MetMb either in their native or denatured (95 °C) state (data not shown).

Hydroperoxides were detected in some of the samples containing OxyMb (**Figure 3A**). This can be explained by the autoxidation of OxyMb, which generates superoxide anion that can dismutate to hydrogen peroxide. Hydroperoxide concentrations in OxyMb samples with microsomes decreased faster than OxyMb alone. Hydroperoxide concentrations also decreased as a consequence of heating OxyMb. Hydroperoxide concentrations were much lower in samples with MetMb (**Figure 3B**), which is likely due to the absence of oxygen on the Mb that is required to form hydrogen peroxide. Lack of an increase in lipid hydroperoxide concentrations could also be due to the presence of transition metal-based prooxidants in the model system. These metal-based prooxidants can cause rapid hydroperoxide decomposition, preventing hydroperoxide accumulation (*25*). Under these conditions, hydroperoxide concentrations remain low while their decomposition leads to an increase in secondary lipid oxidation products such as TBARS (**Figures 1** and **2**).

Changes in Protein Properties Due to Thermal Processing. Conformational changes in OxyMb and MetMb started to occur at temperatures above 55 °C as determined by an increase in Trp absorbance at 290 nm (**Figure 5**). Trp absorbance increased rapidly above 75 °C indicating that the majority of protein unfolding occurs at these temperatures (*1, 38*). At temperatures above 83−85 °C, Trp absorbance decreases. These results were in agreement with those previously reported in MetMb (*12*). Once the protein denatures and hydrophobic amino acids such as Trp are exposed, protein aggregation occurs, thus decreasing Trp absorbance. Protein aggregation would also lead to precipitation of the Mb. The solubility of both OxyMb and MetMb decreased dramatically at temperatures ≥85 °C (**Table 1**) indicating that protein denaturation and aggregation are occurring at these temperatures.

Alterations in Heme and Iron As a Result of Thermal Processing. Measurement of the heme iron provides information on the concentration of intact porphyrin groups regardless of whether or not the heme is bound to the protein. As shown in **Table 1**, the thermal treatments used did not statistically ($p \geq 0.05$) alter total heme concentrations for both MetMb and OxyMb, suggesting that the heme group was not destroyed by heating. However, after centrifugation, the water-soluble heme concentration decreased with 4% of the total heme remaining in solution after heating at 95 °C for both OxyMb and MetMb. The decrease in water soluble heme concentrations were most likely due to a loss of Mb solubility since the amount of protein remaining in solution was 5 and 3% for OxyMb and MetMb, respectively, after heating at 95 °C.

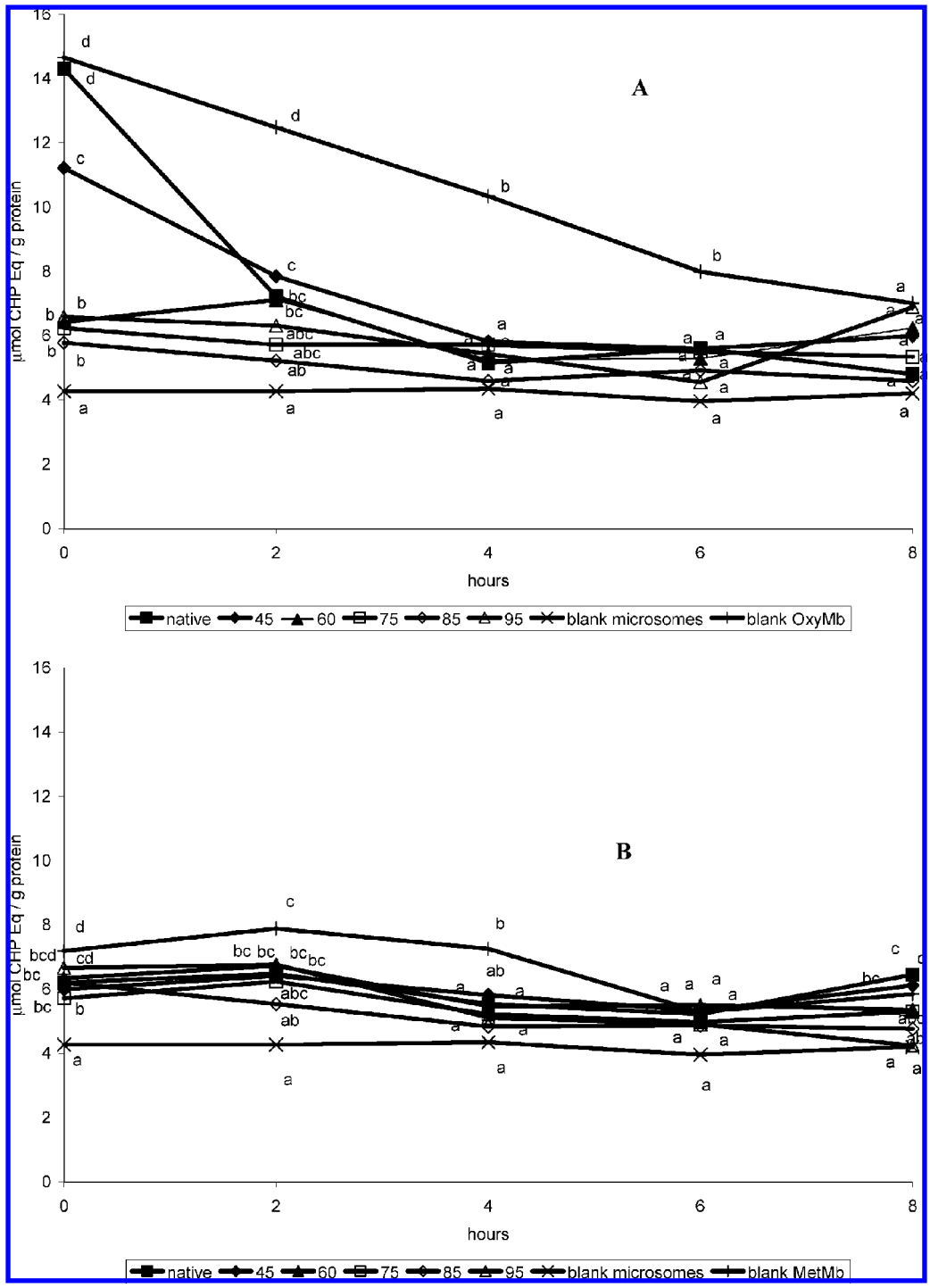

Figure 3. Concentrations of hydroperoxides (μmol of cumene hydroperoxide/g protein) in the presence of 0.15 mM oxymyoglobin (OxyMb) (**A**) or metmyoglobin (MetMb) (**B**) heated at different temperatures. Sample were with or without (blank OxyMb and blank MetMb) muscle microsomes (final concentration 5 mg/mL) and were held at 30 °C for different incubation times. Blank microsomes correspond to samples with microsomes but no myoglobin (Mb). For each incubation time, values corresponding to a certain variable with different letters differ significantly ($P \leq 0.05$).

Despite the fact that thermal treatments have also been postulated to cause an increase in nonheme iron in cooked meat samples (*20, 39*), total nonheme iron content did not increase during heating, indicating that iron is not released from Mb in solution (**Table 1**). These results are in agreement with the reported relatively high stability of the heme group to thermal treatments (*12, 23*). A decrease in water-soluble nonheme iron with increasing temperatures occurred in a manner similar to the loss of protein solubility. This decrease in water soluble nonheme iron suggested that the iron was bound to Mb and thus removed upon precipitation of the protein. At 95 °C, water soluble nonheme iron concentrations were higher for MetMb (63%) than OxyMb (31%), suggesting that OxyMb was able to bind and remove more nonheme iron from the solution.

Antioxidant Capacity of OxyMb and MetMb. Proteins can inhibit lipid oxidation reaction through their ability to chelate prooxidant metals and scavenge free radicals (*27*). The ORAC test can measure the ability of a protein to scavenge peroxyl radicals. Native OxyMb had radical scavenging activity over 2.5-fold higher than MetMb suggesting that its protein conformation resulted in a larger number of free radical scavenging amino acids exposed to the aqueous phase where they could

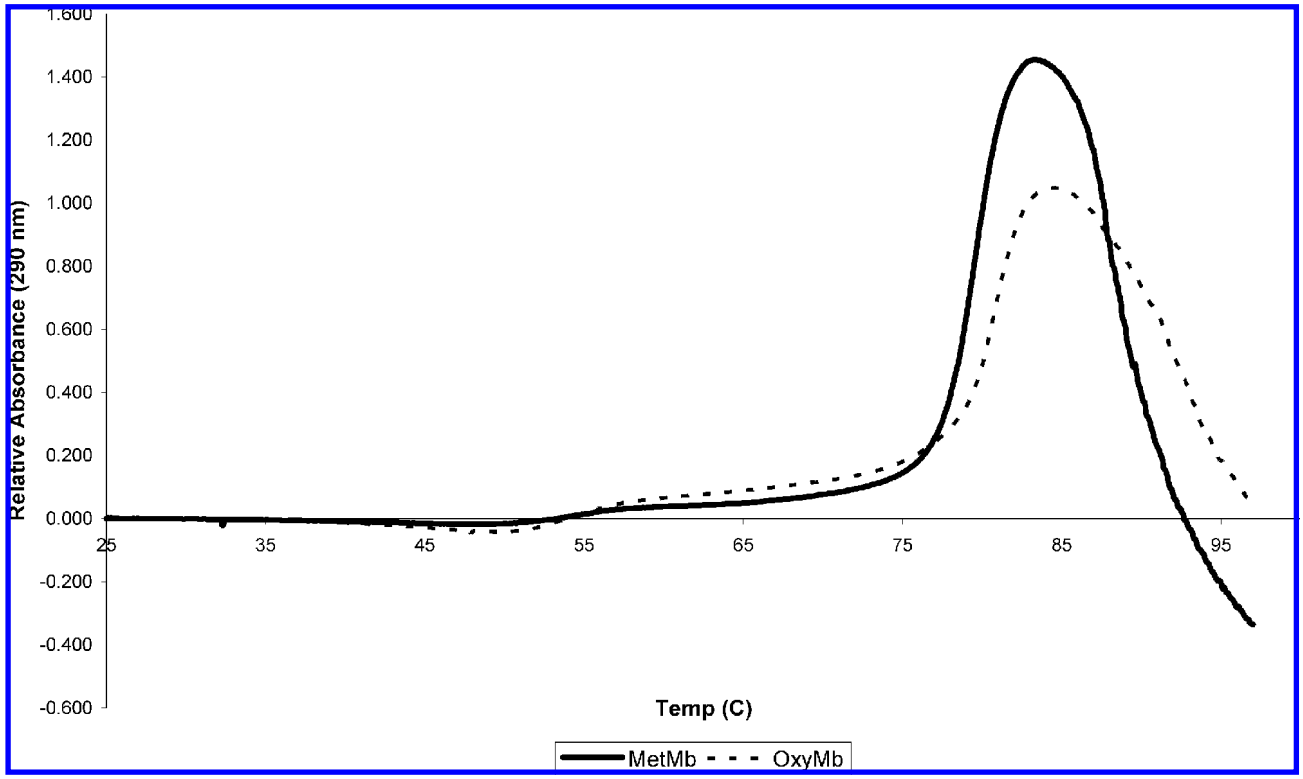

Figure 4. Absorbance changes of oxymyoglobin (OxyMb) and metmyoglobin (MetMb) solutions (pH 5.6) at 290 nm with a heating rate of 0.8 °C/min.

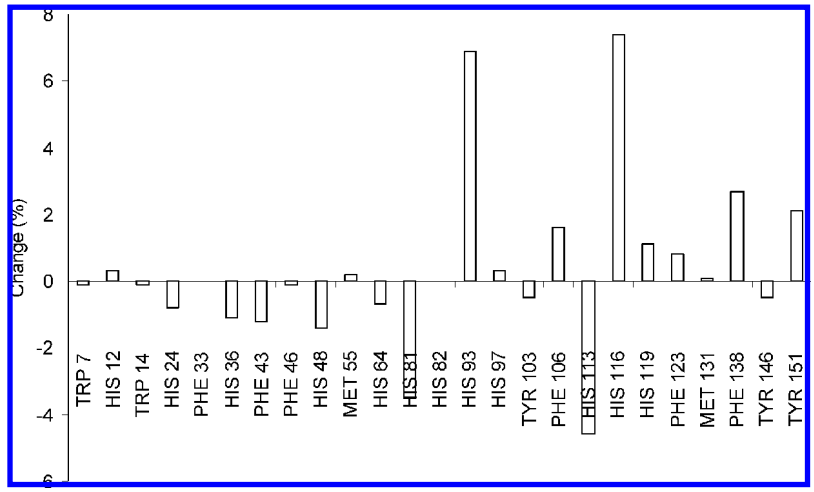

Figure 5. Changes in the solvent accessibility of oxidatively labile amino acid residues (methionine (MET), histidine (HIS), phenylalanine (PHE), tryptophan (TRP), tyrosine (TYR)) in sperm whale oxymyoglobin (OxyMb) and metmyoglobin (MetMb) expressed as a percent change in relation to MetMb.

interact with the water-soluble peroxyl radicals generated by AAPH (**Table 1**). Differences in the free radical scavenging of native OxyMb and MetMb could be due to differences in the native conformation of MetMb and OxyMb. Very little data exist on the conformation of OxyMb with the exception of sperm whale Mb. **Figure 6** shows how the surface exposure of antioxidant amino acids including Trp, His, Phe, Met, and Tyr are different between sperm whale MetMb and OxyMb. Overall, there is a greater exposure of antioxidant amino acids in native OxyMb than MetMb, which could help to explain the greater free radical scavenging activity of OxyMb. While differences existed in the free radical scavenging activity of native MetMb and OxyMb, at heating temperatures ≥75 °C, ORAC values become similar for both proteins. This is unlike proteins such as for β-lactoglobulin where heating will cause exposure of amino acids that can scavenge free radicals (*28*).

Native MetMb had a higher iron binding than native OxyMb (**Figure 6**). The ability of Mb to bind iron could also alter as a function of heating temperatures since heat denaturations could alter the physical location and therefore ability of metal chelating amino acid to interact with metals. Upon heating, the iron binding capacity of MetMb did not change, while OxyMb was able to bind more iron as the temperature of the thermal treatment increased. OxyMb heated at 95 °C had a higher iron binding capacity than MetMb heated to the same temperature.

DISCUSSION

Both OxyMb and MetMb have been reported to promote lipid oxidation (for review see ref *2*). The mechanisms by which these two proteins promote oxidation can be quite different. The prooxidant activity of MetMb has been suggested to be highly

Table 1. Effect of Heating Oxymyoglobin (OxyMb) and Metmyoglobin (MetMb) on Protein Solubility, Radical Scavenging Capacity, and Heme and Nonheme Content in Either the Whole Sample or in the Supernatant after Centrifugation[a]

heating temperature (C)	protein solubility (μg/mL)	radical scavenging capacity[b]	heme in whole sample (mg/L)	heme in the supernatant (mg/L)	nonheme in whole sample (mg/L)	nonheme in the supernatant (mg/L)
			OxyMb			
native	2930 a	2.72 a	8.4	8.0 a	1.22	1.36 a
45	2800 a	2.69 a	9.3	7.5 a	1.25	1.28 a
60	2600 b	1.36 b	8.7	8.4 a	1.23	1.16 ab
75	2350 c	0.74 c	8.8	7.2 a	1.14	0.86 b
85	230 d	0.74 c	7.1	0.5 b	1.07	0.35 c
95	140 d	0.67 c	6.7	0.3 b	1.18	0.42 c
SEM[c]	13	0.035	0.23	0.18	0.038	0.028
			MetMb			
native	2850 a	1.07 a	7.0	6.8 a	0.97	0.98 a
45	2770 a	0.95 ab	6.9	6.7 a	1.00	1.21 a
60	2860 a	0.71 ab	6.9	6.6 a	1.20	1.15 a
75	2590 a	0.61 b	7.0	6.4 a	1.14	1.04 a
85	160 b	0.79 ab	5.8	0.9 b	1.28	0.60 b
95	90 b	0.76 ab	6.8	0.3 b	1.24	0.61 b
SEM	26	0.030	0.26	0.21	0.044	0.028

[a] Values correspond to means obtained from a ANOVA ($n = 3$). Means corresponding to a certain variable with different letters differ significantly ($P \leq 0.05$). [b] Radical scavenging capacity was calculated using the area under the curve of the oxygen radical absorbance capacity (ORAC) assay. [c] Standard error of the global mean.

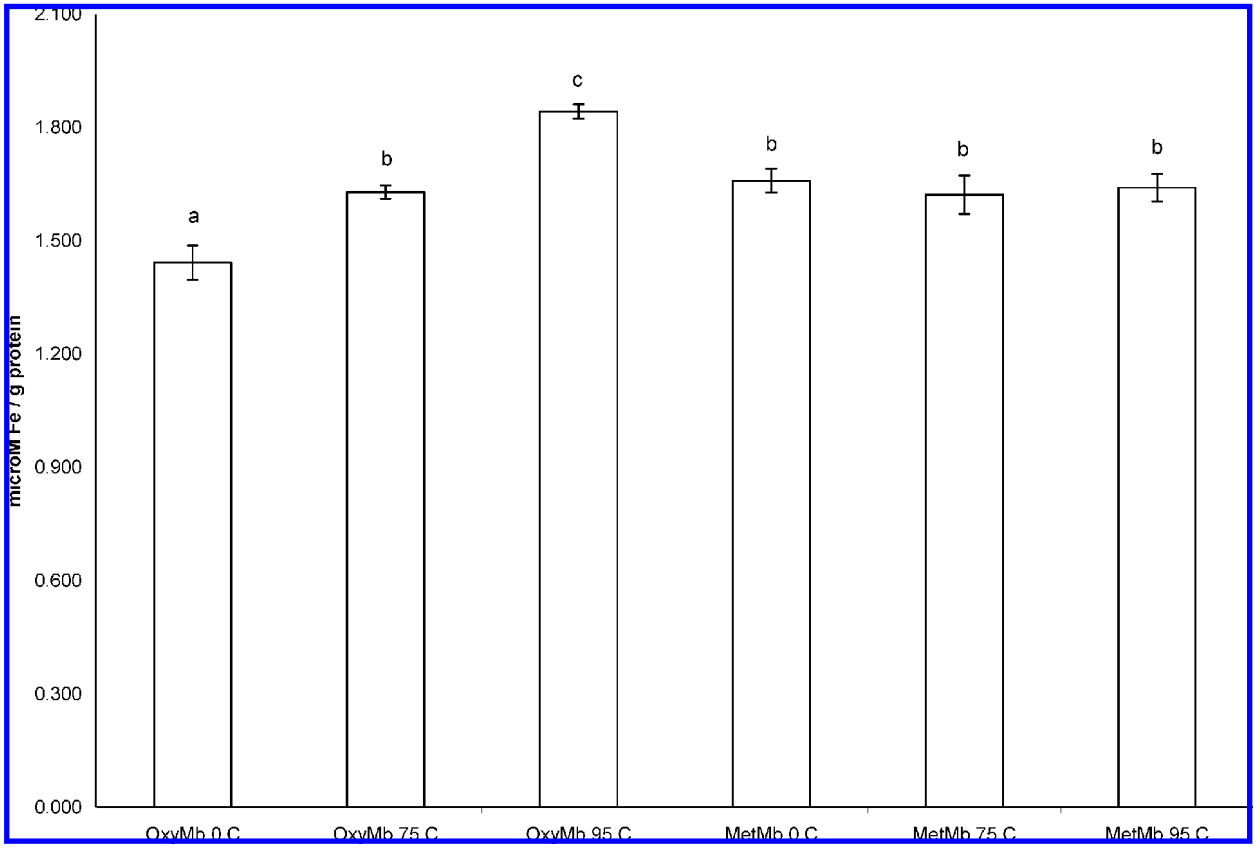

Figure 6. Ability of oxymyoglobin (OxyMb) and metmyoglobin (MetMb), and either unheated or heated to 75 or 95 °C to bind iron following incubation for 24 h at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

dependent on the presence of hydroperoxides since MetMb can decompose hydroperoxides into free radicals and hydroperoxides can convert MetMb into an activated species that can promote lipid oxidation (*2*). The mechanism of the prooxidant activity of OxyMb is less clear but may actually involve MetMb that is formed from the oxidation of OxyMb. In this proposed mechanism, OxyMb releases superoxide anion to form MetMb, and the superoxide anion dismutases to hydrogen peroxide (*2, 8, 40*). The resulting MetMb can then interact with the hydrogen peroxide to form activated MetMb or decompose hydroperoxides into free radicals that can both oxidize lipids (*2, 9*). In our model system, native MetMb did not promote TBARS formation until 4 h of incubation. This could be due to the low levels of hydroperoxide found in the muscle microsome that could limit the activation of MetMb or the ability of MetMb to rapidly generate free radicals. However, TBARS formation increased more rapidly in the presence of OxyMb (after 2 h of incubation), which could be due to the hydrogen peroxide arising from the release of superoxide anion from OxyMb resulting in the formation hydroperoxides that could interact with the resulting MetMb to produce ferrylmyoglobin. Thus, ferrylmyoglobin could also be responsible for promoting TBARS formation since

other authors reported that ferrylmyoglobin increased TBARS values in muscle membrane systems after only 30 min (*8, 10*).

The prooxidant activity of MetMb and OxyMb differed greatly when the proteins were subjected to thermal processing, even though their conformational changes (as determined by Trp absorbance) and solubility profiles as a function of temperature were similar. In the case of OxyMb, the heat treatments decreased prooxidative activity compared to that of the native protein to the point where the heated OxyMb was antioxidative when heated to temperatures ≥75 °C. In relation to MetMb, it is possible that heating treatments caused conformational changes, allowing this Mb to be very efficient at decomposing hydroperoxides resulting in the formation of free radicals and secondary lipid oxidation products. This could explain why MetMb, even at the earliest incubation period, became more prooxidative when heated to temperatures <85 °C. These results are similar to previous work that reported increased prooxidative activities of MetMb at moderate thermal treatments, which subsequently decrease in oxidation with higher heating temperatures (*12, 41, 42*). The decrease in the prooxidative activity of both MetMb and OxyMb above 85 °C could be due to its loss of solubility and thus ability to interact with the muscle microsomes.

Recent research has shown that proteins and peptides can be important antioxidants in foods due to their ability to scavenge free radicals and chelate prooxidative metals (*27, 28, 43*). The ability of OxyMb and MetMb to scavenge free radicals was found to be different in their native and heat denatured forms. The free radical scavenging activity of OxyMb was greater than that of MetMb at temperatures ≤60 °C (**Table 1**). For amino acid side chains in proteins to be able to scavenge free radicals, they must be at least partially exposed to the surface of the protein where they can interact with free radicals (*27*). Thus, the difference in free radical scavenging activity between native MetMb and OxyMb could be due to the increased solvent exposure of antioxidative amino acids in OxyMb especially in the helices at the terminal end (most notably everything after His93) of the polypeptide (**Figure 6**). The higher exposure of antioxidative amino acids, which could scavenge free radicals, could also help explain why heating OxyMb to temperatures up to 60 °C did not increase its prooxidant activity (**Figure 2**).

Overall, the prooxidant activity of both OxyMb and MetMb decreased at heating temperatures ≥85 °C (**Figures 1** and **2**). One reason for this decrease in prooxidant activity is the loss of protein solubility (**Table 1**), which would decrease the ability of the heme groups in OxyMb and MetMb to interact with the microsomes. While MetMb heated to ≥85 °C simply lost its prooxidant acitivity, OxyMb became antioxidative. This difference could be due to the increased iron chelating capacity of heated OxyMb (**Figure 5**), which would allow it to bind and inactivate iron, thus decreasing lipid oxidation rates.

The results of these studies show that Mb has the potential to both promote and inhibit lipid oxidation. Mb has the potential to promote lipid oxidation through the ability of the heme group to produce free radicals through interactions with hydroperoxides and through the heat induced release of prooxidative iron. However, the amino acids in Mb can also inhibit oxidative reactions by chelating prooxidant metals and scavenging free radicals. Therefore, the net effect of Mb on lipid oxidation is a result of the balance between its antioxidant and prooxidant activity. In MetMb, the balance lies predominantly toward prooxidant activity since MetMb promoted lipid oxidation in both its native and denatured states. In OxyMb, the balance was tipped toward prooxidant activity in the protein's native state but was reversed to antioxidative when the protein was denatured. The increased antioxidant activity of denatured OxyMb seemed to be due to its decrease in prooxidative activity because of its loss of solubility as well as its increase and antioxidant activity due to its increased ability to bind prooxidative metals such as iron. These results indicate that when investigating the overall role of a protein on lipid oxidation both its prooxidant and antioxidant activities should be considered.

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Received for review March 28, 2008. Revised manuscript received August 1, 2008. Accepted August 19, 2008. We thank the Fulbright Commission in Spain and the Secretary of State for Universities and Research of the Spanish Ministry of Education and Science for their financial support.

JF8009848