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Pro-oxidative Characteristics of Trout Hemoglobin and Myoglobin: A Role for Released Heme in Oxidation of Lipids

MARK P. RICHARDS,* MARK A. DETTMANN, AND ERIC W. GRUNWALD

Meat Science and Muscle Biology Laboratory, Department of Animal Sciences, University of Wisconsin—Madison, 1805 Linden Drive West, Madison, Wisconsin 53706

The molecular mass of trout myoglobin was 16017 Da based on electrospray ionization mass spectrometry. A Root effect (low oxygen affinity at pH 6.3) was determined in trout hemoglobin but not myoglobin. At pH 6.3, myoglobin autoxidized more rapidly (3.5-fold) as compared to anodic hemoglobin. Anodic hemoglobin was a better catalyst of lipid oxidation in washed cod muscle as compared to myoglobin at pH 6.3. This suggested that some process other than met heme protein formation was the rate-limiting step in lipid oxidation processes. Heme loss rates were determined using the apomyoglobin mutant H64Y prepared from sperm whale. Anodic hemoglobin released its heme group much more rapidly than myoglobin. In comparisons of anodic and cathodic hemoglobins, heme loss rate better predicted the onset of lipid oxidation than autoxidation rate. These studies collectively suggest that heme dissociation has a primary role in the ability of different heme proteins to promote lipid oxidation processes.

KEYWORDS: Heme proteins; lipid oxidation; autoxidation; blood; quality; browning; muscle foods

INTRODUCTION

The heme proteins hemoglobin (Hb) and myoglobin (Mb) are potent catalysts of lipid oxidation in muscle tissue after death. Lipid oxidation processes negatively affect food quality primarily by promoting off-odors and off-flavors (*1*). Heme proteins also provide the color of muscle. Reduced, oxygenated heme proteins result in a bright red color while oxidized pigments are brown (met heme proteins). Fully reduced, deoxygenated pigments appear purple at high concentrations. Oxygenated and deoxygenated heme proteins are in the ferrous form in which the iron atom within the heme ring is in the +2 oxidation state. The iron atom within the heme of met forms is in the +3 oxidation state. The conversion of heme proteins from the reduced to met form is a process called heme protein autoxidation.

Hb is a tetrameric molecule with allosteric oxygen-binding properties while Mb is a monomer without allosterism. Typically, the Hb tetramer is comprised of two α-subunits and two β-subunits. Hb and Mb possess one heme group per globin. Hb is a blood protein while Mb is an intracellular protein in muscle cells.

Dark muscle from Atlantic mackerel contained roughly equal amounts of Hb and Mb on a weight basis (*2*). The only detectable heme pigment in breast muscle from bled chicken broilers was Hb while Mb was undetectable; in the thigh muscle, 86% of the total heme protein was Hb on a weight basis (*3*). In sockeye salmon, there was no significant difference in Hb levels estimated in whole muscle from bled and unbled fish (*4*), which suggests that bleeding removed little Hb from the muscle. From three different reports, Hb made up on average 9, 20, and 32% of the total heme protein in commercial beef muscles such as *Longissiumus* and *Psoas* (*5*−*7*). Another report found that in the *Bicep femoris* of Holstein calves there was 95% Hb and 5% Mb while in trout light muscle the percentage of Hb to Mb was 99.6 to 0.4% (*8*). The increased percentage of Mb in beef tissue as compared to birds and fish was related to muscle fiber diameter (*8*). Muscle fibers of fish and birds are smaller than bovine species. The smaller diameter allows better diffusion of oxygen from capillaries into muscle cells; this makes intracellular Mb less needed.

Little research has been done comparing the oxidation characteristics of Hb and Mb. Equine metMb reacted more rapidly with hydrogen peroxide than bovine metHb resulting in the formation of a ferryl heme protein species (Fe^{4+}) (*9*). Components including ascorbate, uric acid, glutathione, and β-carotene were rapidly oxidized by ferrylMb whereas ferrylHb oxidized the components very slowly (*10*, *11*). Hb and Mb promoted DNA oxidation similarly (*12*).

Four different types of Hb have been isolated from blood of rainbow trout that are distinguishable based on electrophoretic mobility. Components I, II, and III are cathodic Hbs, and component IV is classified as an anodic Hb. Component IV represents approximately 65% of the total Hb pool with the remainder consisting of the cathodic components (*13*). The objective of our work was to compare the pro-oxidative characteristics of trout Hb and trout Mb.

* To whom correspondence should be addressed. Tel: 608- 262-1792. Fax: 608-265-3110. E-mail: mprichards@ansci.wisc.edu.

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MATERIALS AND METHODS

Materials. Hearts from rainbow trout (*Onchorhynchus mykiss*) were obtained from Rushing Waters Inc. (Palmyra, WI). Blood from rainbow trout used in the preparation of trout Hbs was obtained from fish located at Rushing Waters or the Water Science and Engineering Laboratory at the University of Wisconsin−Madison. Atlantic cod (*Gadus morhua*) fillets were obtained by overnight delivery via air transport from Boston, MA. Fillets used were considered of excellent quality based on odors that ranged from sealike (very fresh) to minimal overall odor. Sensory panelists familiar with seafood deterioration made this assessment. The sperm whale Mb mutant (H64Y) in pUC 19 vector was obtained from Dr. John Olson at Rice University (Houston, TX). Sodium heparin, sodium ascorbate, streptomycin sulfate, 2-thiobarbituric acid, ferrous sulfate, barium chloride, ammonium thiocyanate, tris[hydroxymethyl]-aminomethane, bovine Hb, and ethyl 3-aminobenzoate were obtained from Sigma Chemical A/S (St. Louis, MO). Trichloroacetic acid was obtained from Fisher Scientific (Fairlawn, NJ).

Preparation of Washed Cod Muscle. Washed cod was prepared from three to four fillets as soon as fish arrived at the laboratory. All dark muscle was removed. The light muscles were ground in a KS M90 mincer (Kitchen Aid Inc., St. Joseph, MI) (plate diameter, 5 mm). The mince was washed once in distilled deionized water at a 1:3 mince-to-water ratio (w:w) by stirring with a plastic rod for 2 min. Subsequently, the mixture was allowed to stand for 15 min before dewatering with two layers of cotton cheesecloth. The mince was then mixed with 50 mM sodium phosphate buffer (pH 6.3) at the same 1:3 ratio. This phosphate wash was repeated, and the mince−buffer mixture was homogenized (rheostat set to 30) using a Polytron Type PT 10/35 (Brinkmann Instruments, Westbury, NY). It was allowed to stand for 15 min and finally centrifuged (15000*g* for 20 min at 4 °C) using a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA)**.** The resulting pellet was then used as the washed cod muscle.

Preparation of Anodic and Cathodic Trout Hb. Blood was removed from the caudal vein via syringe after exposure of rainbow trout to aminobenzoic acid ethyl ester anesthetic (0.5 g/L water). Blood was collected from six rainbow trout. Three separate Hb preparations were examined in each analysis. Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, were added to heparinized blood (30 units heparin per mL of blood drawn) and centrifuged (700*g* for 10 min at 4 °C) in a Beckman J-6B centrifuge (Beckman Instruments Inc.). After the plasma was removed, the red blood cells were washed by suspending three times in 10 volumes of the above buffer (*14*). Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation (28000*g* for 15 min at 4 °C) using a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc.)**.** Hemolysate was transferred to 50 mM Tris, pH 8.6, buffer using PD-10 columns (Bio-Rad, Hercules, CA) and simultaneously separated from low molecular mass components (<10 kDa). Columns (20 mL, 1.5 cm diameter) (Bio-Rad) containing degassed DEAE anion exchange chromatography resin were equilibrated with 20 mM Tris buffer (pH 8.6). Initially, cathodic Hbs were eluted with 20 mM Tris, pH 8.6. Subsequently, anodic Hb was washed out using 20 mM Tris buffer (pH 8.6) containing 0.5 M NaCl (*15*). Finally, both anodic and cathodic Hbs were dialyzed extensively (4 °C) to exchange buffer that was 1 mM Tris buffer (pH 8.0). Hb solutions were stored at −80 °C prior to use.

Preparation of Trout Mb. Mb was prepared according to the method of Trout and Gutzke (*16*) with some modifications. Approximately 50 hearts were used for each Mb preparation. Three separate Mb preparations were examined in these studies. Cardiac tissue free of fat and connective tissue was frozen in liquid nitrogen. The tissue was pulverized with a mortar and pestle. The powdered tissue was then transferred to a Dounce Homogenizer. A homogenate was then obtained in 50 mM Tris and 1 mM disodium ethylenediamine tetraacetate (EDTA) (pH 8.0). The homogenate was centrifuged at 10000*g* for 10 min to remove myofibrillar proteins. The supernatant was removed and filtered through Whatman 4 filter paper. The pH was adjusted to 8.0 using 1 N NaOH. Solid ammonium sulfate was then added to the supernatant to produce 75% ammonium sulfate saturation (0.476 g/mL). Gentle stirring was done for 30 min to dissolve ammonium sulfate. The suspension remained undisturbed for 30 min while proteins precipitated. The suspension was then centrifuged at 18000*g* for 20 min. The supernatant was removed and filtered through Whatman 4 filter paper. Ammonium sulfate was then added to the filtrate to achieve 100% saturation (0.244 g/mL). The suspension was allowed to remain undisturbed for 30−60 min and then centrifuged at 20000*g* for 60 min. If a pellet was not formed, centrifugation at 25000*g* for 10 min was done. The pellet was resuspended in 5 mM Tris-HCl buffer, pH 8.5 (2 volumes pellet:1 volume buffer). The suspension was centrifuged at 13000 rpm for 5 min and filtered through a low-protein-binding 0.45 μm syringe filter. The suspension was then dialyzed against 5 mM Tris, pH 8.50, using 12000−14000 molecular weight cutoff membrane. The dialysis buffer was changed twice, allowing protein to dialyze overnight with the last buffer change.

For Sephadex G-100 chromatography, columns (300 mm × 20 mm) were washed using two column volumes of running buffer (5 mM Tris-HCl, pH 8.5) at a flow rate of 1 mL/min. pH and conductivity were adjusted to 8.50 and 150 μM HO, respectively. Five milliliters of crude Mb extract was added to a Sephadex G-100 fine grade column. The extract was resolved using 5 mM Tris-HCl, pH 8.5 (flow rate = 60 mL/h, 1 mL/min). One milliliter fractions were collected. Hb eluted between 30 and 40 min, and Mb eluted between 50 and 70 min.

For gradient elution with anion exchange chromatography on FPLC, a column (100 mm × 26 mm) loaded with DEAE 52 was washed with 2 column volumes of buffer A (5 mM Tris-HCl, pH 8.5) at a flow rate of 1 mL/min. The column was then washed with 1 column volume of buffer A followed by a linear gradient starting with 0% buffer B (5 mM Tris-HCl and 0.5 M NaCl, pH 8.5) and achieving 100% buffer B after 8 column volumes. Absorbance was monitored at 280 nm. Colored fractions that eluted were assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 15% gel. Mb fractions were then dialyzed extensively against 1 mM Tris, pH 8.0, and the Mb was concentrated to 100 μM using ultrafiltration.

SDS−PAGE Electrophoresis. Proteins in different fractions were characterized by the electrophoresis procedure described by Laemelli (*17*).

Quantifying Heme Protein Concentration. The method of Brown (*18*) was used to quantify Hb concentration. This involves reduction of the diluted heme protein solution in 50 mM Tris, pH 8.0, with a few crystals of dithionite followed by bubbling with carbon monoxide gas (99.3% purity). The peak absorbance between 440 and 400 nm is then recorded. A standard curve was prepared using bovine Hb.

Mass Spectrometry. A C4 zip-tip (Millipore, Billerica, MA) was used to desalt the protein solution. Samples were analyzed by electrospray ionization mass spectrometry (ESI-MS) on an API365 Triple quadrupole (Applied Biosystems, Foster City, CA). Masses between 300 and 2000 *m*/*z* were measured (*19*).

Measuring the Relative Oxygenation of Hb and Mb. Solutions containing Hb or Mb were scanned from 630 to 500 nm using a double-beam spectrophotometer model UV-2401 (PC) (Shimadzu Instruments, Inc., Columbia, MD). The blank contained only buffer. The absorbance at the peak (575 nm) minus the absorbance at the valley (560 nm) was calculated. Larger differences indicated that the Hb was more highly oxygenated (*20*). These experiments were run at atmospheric conditions.

Measuring Heme Protein Autoxidation. Heme protein solutions were diluted into 20 mM sodium phosphate buffer (pH 6.3) and stored at 4 °C. Samples were stored in polystyrene cuvettes. Spectra were obtained at regular time intervals between 700 and 500 nm using the UV-2401 spectrophotometer. The percentage of methemoglobin was calculated according to the equations of Benesch et al. (*21*). The slopes obtained during storage were used to determine the relative rates of heme protein autoxidation. For autoxidation studies, samples contained 3 mmol superoxide dismutase and catalase per mole of heme to remove any superoxide and hydrogen peroxide that was produced during incubation.

Preparation of Heme Loss Reagent (H64Y). Heme loss can be determined using the sperm whale Mb mutant, which has a tyrosine (Y) substitution for histidine (H) at the 64 position of the protein (*22*). The gene coding for H64Y Mb was subcloned from the pUC 19 plasmid to the pET 28 plasmid (Novagen, Madison, WI). The pET 28 plasmid is preferable to pUC 19 because larger amounts of protein can be

expressed from pET 28. The subcloning procedure entailed amplification of the mutant gene via PCR, digestion of the amplified gene and pET 28 plasmid with restriction endonucleases *Nco*I and *Bpu*1102I (Fermentas Inc., Hanover, MD), and ligation of the amplified gene with pET 28 DNA to form the final construct. Successful subcloning was confirmed by dideoxy sequencing at the University of Wisconsin Biotechnology Center (Madison, WI). The construct was then used to transform *Escherichia coli* BL21-CodonPlus (DE3)-RP host cells (Stratagene, La Jolla, CA) via the heat shock method provided with the cells. H64Y Mb was then expressed in the host *E. coli* cells using a 12 L culture vessel (Nalge Nunc International, Rochester, NY) and Terrific Broth (TB) adjusted to pH 7 as the culture medium. All culture media contained 30 μg/mL kanamycin and 47.6 μg/mL chloramphenicol. To perform the expression, a single colony of transformed host *E. coli* was transferred from an LB agar plate to a 40 mL volume of TB and incubated for 12 h in a 37 °C shaker. The 40 mL culture was then transferred to a 320 mL volume of TB and incubated for 4 h in a 37 °C shaker to produce a starter culture. The starter culture was then added to the TB containing 2 mL of 1 M $FeCl_3$ and 50 μL antifoam (Sigma) per liter, and the culture (12 L) was incubated at 37 °C. During incubation, the culture was bubbled with compressed air at 2−4 psi and mixed at 250 rpm with a motorized impeller. When OD_{600} of the culture reached approximately 2.0, isopropyl-D-thiogalactopyranoside was added to 1 mM to induce expression of the H64Y Mb. During induction, hemin chloride was added to the culture to 4 mg/L. After 4 h of induction, cell paste containing the expressed H64Y Mb was obtained by centrifuging the culture at 2000*g* for 15 min. The paste was frozen at −80 °C. This was followed by thawing and overnight lysis of the cells at 4 °C (*23*). The lysis buffer consisted of 50 mM Tris base, 1 mM disodium EDTA dihydrate, 0.5 mM dithiothreitol, 1 mM toluenesulfonyl chloride, 40 U/mL DNAse I, 3 U/mL RNAse A, and 78800 U/mL lysozyme and was adjusted to pH 6 (Sigma). H64Y Mb was then purified from the lysate via ammonium sulfate precipitation and anion and cation exchange chromatography as described previously (*24*).

Measuring Heme Loss. The heme of H64Y was removed with methyl ethyl ketone to form a pale, yellowish globin (*25*). Two milliliters of the H64Y Mb, diluted to approximately 0.5 mM (heme basis) in 1 mM tris, pH 8, was placed in a glass test tube on ice. Ice cold 0.1 M HCl was added to the Mb two drops at a time with mixing and monitoring of pH after each addition until the pH reached 2.2. Two milliliters of ice cold methyl ethyl ketone was then added to the tube, and the tube was mixed vigorously for 5 s. The tube was incubated on ice for 1 min to allow separation of aqueous and organic layers. A Pasteur pipet was used to separate the lower pale yellowish globin layer from the upper dark organic layer. The globin layer was then dialyzed overnight against 3 L of 10 mM sodium phosphate, pH 7, using a Slide-A-Lyzer 10K MWCO dialysis cassette (Pierce, Rockford, IL). This globin with the tyrosine substitution has a strong affinity for heme. Thus, heme that is released from the experimental heme proteins is gathered by the globin. When released heme binds to the mutant globin, a green color results that has a strong absorbance at 600 nm (*22*). Heme loss was measured during 4 °C storage, and samples were stored in quartz cuvettes.

Addition of Heme Proteins to Washed Cod Muscle. An appropriate volume of the Hb stock (around 400 μM Hb) was added to washed cod muscle so that a final concentration of 6 μmol Hb/kg tissue was obtained (24 μmol heme/kg). Samples were stirred with a plastic spatula for 3 min to distribute the heme protein. This level of heme protein was selected since it is near the range of heme protein levels found in light muscle of trout (*26*). Streptomycin sulfate (200 ppm) was added to inhibit microbial growth during storage. Samples were stored in amber glass bottles with screw-top lids (30 mL capacity) during 2 °C storage. The pH was adjusted if necessary by addition of 1 M NaOH or 1 M HCl. To measure the pH, around 0.5 g of sample was diluted in 10 vol of distilled deionized water and homogenized, and readings were recorded using an Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA). The final pH and moisture content of the samples were pH 6.3 and 90%, respectively. It is critical to maintain or adjust all samples at a uniform pH since trout Hb-mediated lipid oxidation in washed cod is sensitive to pH (*27*).

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a modified procedure of Buege and Aust (*28*). Fifty percent trichloroacetic acid (TCA) containing 1.3% TBA was heated to 65 °C on the day of use to dissolve the TBA. The TCA−TBA mixture was added to samples at a 1:10 weight:volume ratio and incubated for 1 h at 65 °C. After the mixture was cooled in a cold room for 1 h and centrifuged (10000*g* for 3.5 min), the absorbance of the supernatant at 532 nm was measured. A standard curve was constructed using tetraethoxypropane.

Statistical Evaluations. A general linear model of the SAS system was used to analyze data from storage studies in which lipid oxidation and heme loss were assessed (*29*). All of the data that was used to compose the figures within were used to determine if there were significant differences between the heme proteins being compared. Three separate preparations of each heme protein were examined and used as the source of replication at each time point assayed. For heme protein autoxidation studies, the slope obtained from each heme protein preparation was determined (expressed as change in percent met heme protein with increasing storage time). The slopes obtained were then used in a *t*-test to determine significant differences between the heme proteins being compared ($n = 3$ for each heme protein).

RESULTS

Mb was purified from cardiac tissue of rainbow trout by ammonium sulfate precipitation, size exclusion chromatography, and anion exchange chromatography. Gel electrophoresis of the preparation under denaturing conditions indicated the presence of a single polypeptide band (**Figure 1**). Gel electrophoresis of trout hemolysate prepared from erythrocytes indicated the presence of a large band and a smaller band that was weakly separated (**Figure 1**). The large band likely represents the major Hb present in trout blood (anodic component IV) while the smaller band represents the three cathodic Hbs that have been previously described (*13*).

ESI-MS was used to characterize the molecular mass of components present in the Mb preparation. A peak at 616 Da was detected, which represents the heme group (data not shown). A major peak at 15401 Da was obtained, which represents the globin of trout Mb (**Figure 2**). Two smaller peaks at 15439 and 15481 Da were also observed (**Figure 2**). Acetylation (42 Da) is common in fish heme proteins (*30*). This likely explains the two smaller peaks observed indicating one and two acetylations, respectively. The mass spectra of trout hemolysate containing cathodic (components I−III) and anodic (component IV) Hbs are shown in **Figure 3** to illustrate mass spectra characteristics of a sample containing multiple Hbs. Again, patterns of acetylation can be observed.

Comparisons of Trout Mb and Anodic Trout Hb. The relative oxygenation of Mb and anodic Hb were determined at pH 6.3 and pH 8.0. Anodic Hb was chosen for comparison to Mb because it represents the primary Hb (around 65% of total Hb by weight) (*13*). The concentration of each heme protein was 20 μM on a heme basis. No detectable met heme protein was present in the heme proteins based on the lack of an absorbance peak at 630 nm. At pH 8.0, the oxygenation of each heme protein was nearly equivalent (**Table 1**). However, at pH 6.3, anodic Hb had approximately a 4.5-fold greater content of deoxyheme protein as compared to Mb. The autoxidation rate of Mb and anodic Hb was also determined at pH 6.3 during 4 °C storage. The autoxidation rate of Mb was approximately 3.5 times greater than anodic Hb ($p < 0.001$) (**Table 2**).

The ability of trout Mb and anodic Hb to promote lipid oxidation in washed cod muscle was determined at pH 6.3 during 2 °C storage. The heme protein concentration was 24 μmol heme/kg washed cod, and the heme proteins were added to the substrate in the reduced state (i.e., there was negligible

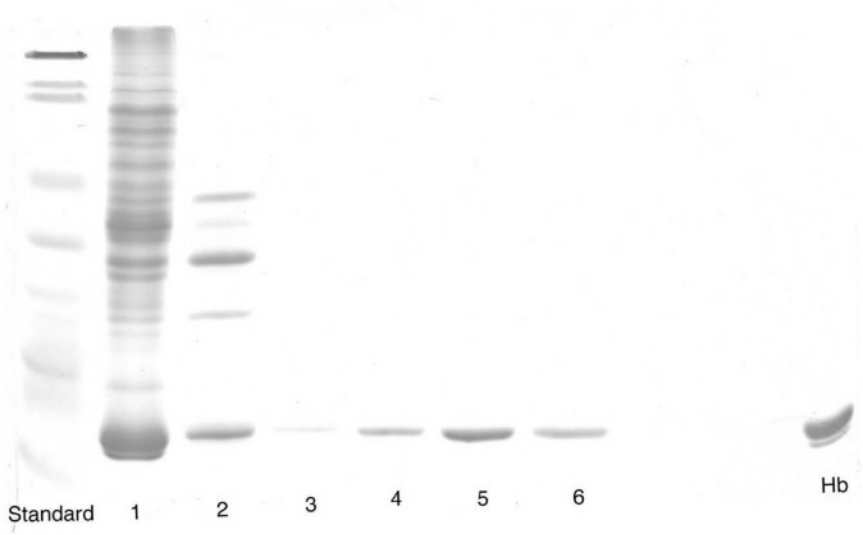

Figure 1. SDS–PAGE electrophoretic polypeptides obtained in the purification of Mb from trout heart tissue. Lane 1, heart tissue homogenate; lane 2, postammonium sulfate precipitation; lane 3, postgel filtration; lanes 4–6, postanion exchange chromatography; Hb polypeptides observed from trout hemolysate.

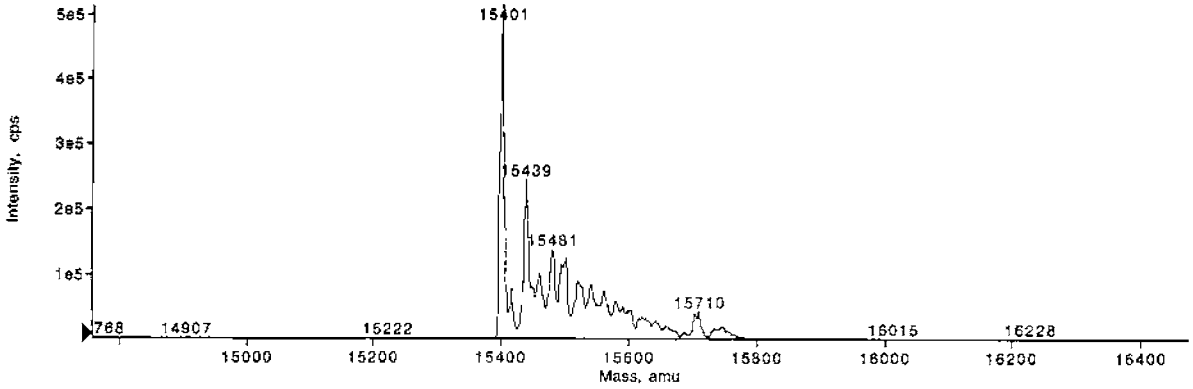

Figure 2. Deconvoluted mass spectra of trout Mb prepared from heart tissue obtained by ESI-MS analysis.

amounts of met heme protein present at the time of addition). Anodic Hb was a more potent promoter of lipid oxidation in washed cod as compared to Mb based on TBARS values during 6 days of storage at 2 °C ($p < 0.05$) (**Figure 4**). The rate of TBARS formation was 6.7 times faster in the anodic Hb over the initial 1.5 days of storage.

The time courses for hemin dissociation from Mb and anodic trout Hb using the H64Y heme loss reagent during 4 °C storage were determined. The met form of each heme protein was examined to remove any confounding effects due to the different rates of autoxidation between the two heme proteins. Heme dissociated from anodic Hb more rapidly than from Mb ($p < 0.05$) (4.8-fold over the initial 3 h of storage) (**Figure 5**).

Comparisons of Cathodic Hb and Anodic Hb. The autoxidation rate of cathodic Hb and anodic Hb was determined at pH 6.3 and a concentration of 80 μM on a heme basis. The autoxidation rate of anodic Hb was 1.4 times greater ($p < 0.05$) than cathodic Hb (**Table 3**). The time courses for hemin dissociation from cathodic and anodic Hb using the H64Y heme loss reagent during 4 °C storage were determined. The met form of each heme protein was examined. Heme dissociated from anodic Hb more rapidly than from cathodic Hb ($p < 0.05$) (15-fold over the initial 3 h of storage) (**Figure 6**).

DISCUSSION

The oxidative characteristics of trout Mb were unusual based on the fact that trout Mb autoxidized faster but was a weaker promoter of lipid oxidation as compared to anodic Hb (**Table 2** and **Figure 4**). Typically, a heme protein that autoxidizes rapidly is an especially potent promoter of lipid oxidation. This is because once the protein is converted from the ferrous to the met form its reactive heme group becomes around 60 times more loosely anchored in the globin, which favors hemin-mediated lipid oxidation (*31*, *32*). Another reason is that met heme protein can react with hydrogen peroxide or lipid peroxides to form the ferryl protein cation radical that is capable of initiating lipid oxidation (*9*). We decided to measure heme dissociation from trout Mb and anodic Hb to better understand the poor ability of trout Mb to stimulate lipid oxidation despite its rapid rate of autoxidation. Heme dissociated much slower from metMb as compared to anodic metHb (**Figure 5**). Anodic Hb developed TBARS 6.7-fold faster than Mb and had a 4.8-fold greater rate of heme dissociation as compared to Mb (**Figures 4** and **5**). This suggests that heme dissociation has a primary role in the ability of different heme proteins to promote lipid oxidation processes. Although trout Mb is rapidly converted to the met form, its high heme affinity caused it to be a poor promoter of lipid oxidation as compared to anodic trout Hb, which has a slower rate of autoxidation but a much greater rate of heme dissociation.

It has been reported that apoMb from sperm whale has an affinity for hemin, which is around 2000 times greater than that of isolated subunits of human Hb and 50 times greater than

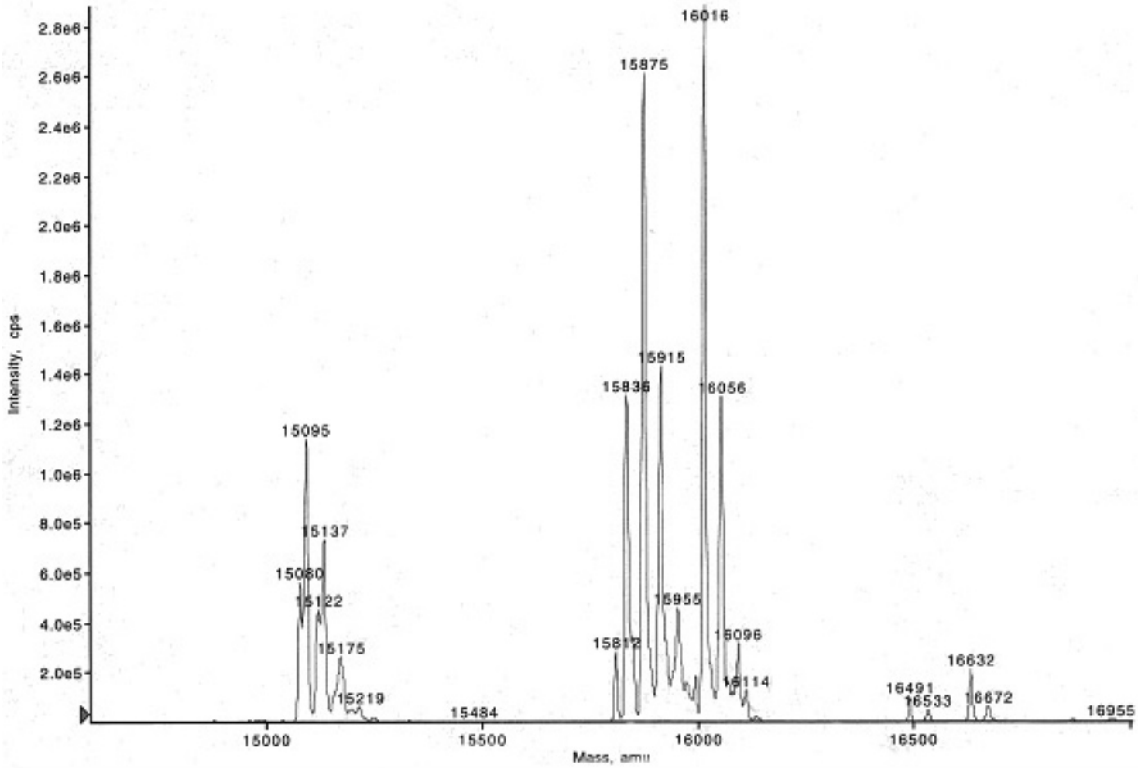

Figure 3. Deconvoluted mass spectra of trout hemolysate prepared from trout blood obtained by ESI-MS analysis.

Table 1. Relative Oxygenation of Trout Mb and Anodic Trout Hb at pH 6.3 and pH 8.0[a]

	relative oxygenation absorbance (peak at 576 nm and valley at 560 nm)	
heme protein	pH 6.3	pH 8.0
Mb	0.051 ± 0.002	0.054 ± 0.003
anodic Hb	0.011 ± 0.002	0.062 ± 0.003

[a] The heme protein concentration was 20 μM (heme basis).

Table 2. Relative Rates of Autoxidation of Trout Mb and Anodic Trout Hb during 3 Days of Storage (4 °C, pH 6.3)[a]

heme protein	relative rate of heme protein autoxidation
Mb	1.00 ± 0.12[b]
anodic Hb	0.28 ± 0.07

[a] The heme protein concentration was 20 μM on a heme basis (20 μM Mb and 5 μM Hb). Relative rates of heme protein autoxidation were based on the slopes obtained, expressed as the change in percent met heme protein with time. Solutions were buffered in 20 mM sodium phosphate and contained 3 mmol superoxide dismutase and catalase per mol of heme. The standard deviation in slope determinations is expressed. Samples were stored as protein solutions. [b] Significant difference between trout Mb and cathodic Hb ($p < 0.001$).

that of tetrameric human Hb (*33*). We did not determine the monomer−dimer−tetramer equilibrium of trout Hbs in our studies. It would seem a large majority of the trout Hbs used in our study were in the tetrameric form considering that subunit formation in trout Hb has been found to occur at concentrations at or below 0.15 μM heme at pH 6.3 (*34*) and the concentration of heme used in our studies was between 20 and 80 μM. The small amount of monomer and dimer present however should not be ignored. The rapid rate of heme release from the minor subunit population could provide a critical mass of released heme.

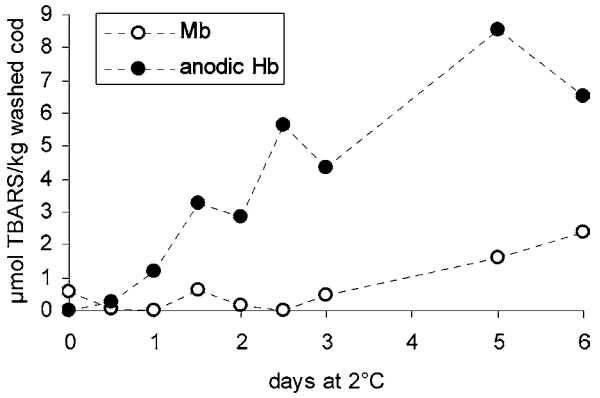

Figure 4. TBARS values obtained during 2 °C storage of washed cod muscle containing either trout Mb or anodic trout Hb (pH 6.3). The heme protein concentration was 24 μmol heme/kg washed cod.

We had previously found that anodic Hb promoted the oxidation of washed cod muscle lipids more rapidly than cathodic Hb (14.1-fold difference in rate of TBARS formation during 1.5 days storage) (*36*). Consistent with this finding was our finding in the current study of a greater rate of heme dissociation from anodic Hb as compared to cathodic Hb (15.0-fold during 3 h of storage) (**Figure 6**). Anodic Hb autoxidized only 1.4 times faster than cathodic Hb (**Table 3**). This indicated that autoxidation rate had little effect on the lipid oxidation rate. This is further evidence that heme affinity is a major contributing factor in the ability of different heme proteins to stimulate lipid oxidation. Other researchers have also found that anodic Hb was a better catalyst of lipid oxidation than cathodic Hb (*37*). These researchers utilized a model system consisting of liposomes prepared from egg lecithin and added organic hydroperoxides (pH 7.4 at 37 °C storage) (*37*).

Previously, it was reported that ferryl Mb was a more active oxidant of various biomolecules as compared to ferryl Hb (*35*). Our results showed that trout Mb was a weaker promoter of

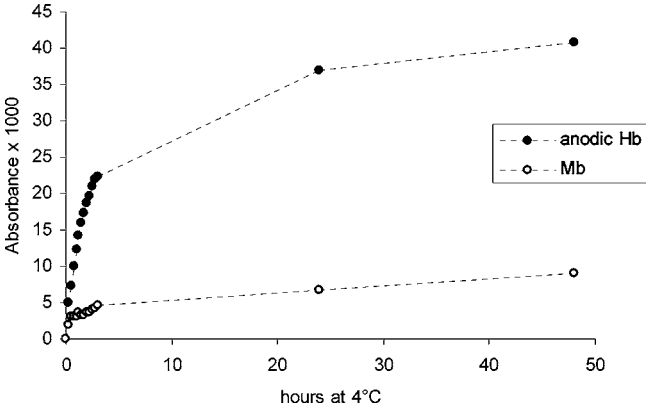

Figure 5. Time courses for hemin dissociation from trout Mb and anodic trout Hb using the H64Y heme loss reagent during 4 °C storage. Absorbance at 600 nm minus absorbance at 700 nm (due to any turbidity) was determined at each represented time point. The pH was 6.3. The heme protein concentration was 10 μM (heme basis). The globin concentration was 40 μM. The phosphate and sucrose concentrations were 152 and 456 μM, respectively.

Table 3. Relative Rates of Autoxidation of Anodic and Cathodic Hb during 8 Days of Storage (4 °C, pH 6.3)[a]

heme protein	relative rate of heme protein autoxidation
anodic Hb	1.00 ± 0.04[b]
cathodic Hb	0.73 ± 0.04

[a] The heme protein concentration was 80 μM on a heme basis (20 μM Hb). Relative rates of heme protein autoxidation were based on the slopes obtained, expressed as the change in percent met heme protein with time. Solutions were buffered in 20 mM sodium phosphate and contained 3 mmol superoxide dismutase and catalase per mol of heme. The standard deviation in slope determinations is expressed. Samples were stored as protein solutions. [b] Significant difference between anodic Hb and cathodic Hb ($p < 0.05$).

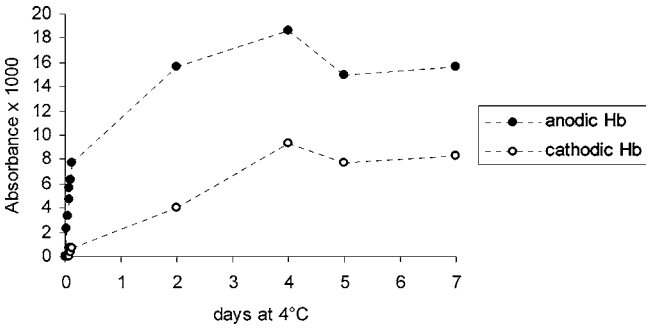

Figure 6. Time courses for hemin dissociation from anodic and cathodic trout Hb using the H64Y heme loss reagent during 4 °C storage. Absorbance at 600 nm minus absorbance at 700 nm (due to any turbidity) was determined at each represented time point. The pH was 6.3. The heme protein concentration was 10 μM (heme basis). The globin concentration was 40 μM. The phosphate and sucrose concentrations were 152 and 456 μM, respectively.

lipid oxidation than anodic Hb (**Figure 4**). If in fact ferryl Mb is a better oxidant than ferryl Hb, our results suggests that ferryl heme protein had a minor role in oxidation of lipids in washed cod muscle.

The ability of iron atoms that are released from heme proteins to stimulate lipid oxidation processes must also be considered. Heme was previously found to breakdown lipid hydroperoxides 40 and 430 times more rapidly than Fe^{2+} and Fe^{3+}, respectively (*38*). It has been reported that release of iron from heme was not responsible for the ability of heme to oxidize microsomal lipids (*39*). Heme was shown to be a more potent catalyst of lipid peroxidation in red blood cell membranes than nonheme iron (*40*). It was also shown that an excess of EDTA did not inhibit trout Hb-mediated lipid oxidation in washed cod, which suggested that released iron was not pro-oxidative (*41*).

For heme protein autoxidation studies, the slope obtained from each heme protein preparation was determined during storage. The slopes obtained were then used in a *t*-test to determine significant differences ($n = 3$ for each heme protein). The slopes obtained were also used to determine the relative rates of heme protein autoxidation. In comparisons of trout Mb and trout anodic Hb, 3 days of storage was used to calculate the relative rate of heme protein autoxidation whereas 8 days of storage was used in the comparison of anodic Hb and cathodic Hb. This was because trout Mb autoxidized very rapidly so that a linear response was not obtained beyond day 3. Thus, data were obtained at four time points over 3 days for the trout Mb vs anodic Hb comparison while data were obtained in the anodic Hb vs cathodic Hb comparison at nine time points over 8 days.

At pH 6.3, the deoxy heme protein content was higher in anodic Hb as compared to Mb (**Table 1**). This is reasonable considering that Root and Bohr effects occur in Hbs but not Mbs (*42*). The Bohr effect is a decrease in oxygen affinity as pH decreases from 7.4 to around 6.5. Further loss of oxygen affinity as pH decreases below 6.5 is termed a Root effect. Considering that trout Mb has a relatively high oxygen affinity as compared to anodic Hb, it would be expected that trout Mb would be more resistant to autoxidation (e.g., browning). This is because the rate of heme protein autoxidation has been found to reach a maximum at approximately half oxygen saturation (*43*). Binding of a sixth ligand (e.g., oxygen) to the iron atom in the heme ring causes the iron to be in a low spin state and thus resistant to oxidation as compared to unliganded heme protein (e.g., deoxygenated heme protein) in which the iron atom is in a high spin state that is susceptible to oxidation (*44*). Nevertheless, trout Mb was more susceptible to autoxidation than the poorly oxygenated anodic Hb (**Table 2**). This indicates that some mechanism other than oxygen affinity causes trout Mb to have a high rate of autoxidation. The rapid autoxidation of trout Mb probably does not involve increased access of solvent water to the heme crevice (*45*). This is because facile entry of water into the crevice also accelerates heme loss (*46*); yet, trout Mb had a relatively slow rate of heme dissociation (**Figure 5**).

One possible explanation for the rapid autoxidation rate of trout Mb despite high oxygen affinity involves the hydrogen-bonding distance between the distal histidine of trout Mb and the liganded O_2. Human Hb β-chain is more resistant to autoxidation than human Hb α-chain, which has been attributed to the proximity of the distal histidine to bound O_2 (*47*). The α-chain has its distal histidine 2.7 Å from bound O_2 providing hydrogen bonding and hence a "proton relay mechanism" that facilitates autoxidation. A distance of 3.2 Å exists in the β-chain, which is believed to prevent the hydrogen bonding and thus inhibit the proton relay mechanism, slowing autoxidation (*48*). Trout Mb at pH 6.3 may exist in a conformation that has an ideal distance between liganded O_2 and its distal histidine to facilitate rapid autoxidation due to a proton relay mechanism.

Future work should examine the role of heme dissociation in lipid oxidation processes that occur in muscle foods. The hypothesis that Hbs are more adept at oxidizing lipids due to ease of heme dissocation while Mbs are more sensitive to autoxidation (e.g., browning) due to an optimal hydrogen-bonding distance between the bound oxygen and the distal

histidine should also be further explored. The wide range of Hb:Mb ratios previously reported for beef muscle (*5*−*8*) warrants a reexamination into the relative amounts of these heme proteins that are present in the postmortem tissue, keeping in mind that Hb subunit formation occurs under conditions of extraction (e.g., dilution), which can cause Mb determinations to be errantly high. These subunits are also extremely prone to heme release, which will result in decreased recovery of the Hb present in the tissue.

ABBREVIATIONS USED

TBARS, thiobarbituric acid reactive substances; EDTA, disodium ethylenediamine tetraacetate; Hb, hemoglobin; Mb, myoglobin.

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LITERATURE CITED

(1) Kanner, J. Oxidative processes in meat and meat products: Quality implications. *Meat Sci.* **1994**, *36*, 169−189.

(2) Richards, M. P.; Hultin, H. O. Contributions of blood and blood components to lipid oxidation in fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 555−564.

(3) Kranen, R. W.; van Kuppevelt, T. H.; Goedhart, H. A.; Veerkamp, C. H.; Lambooy, E.; Veerkamp, J. H. Hemoglobin and myoglobin content in muscle of broiler chickens. *Poult. Sci.* **1999**, *78*, 467−476.

(4) Porter, P. J.; Kennish, J. M.; Kramer, D. E. The effects of exsanguination of sockeye salmon on the changes of lipid composition during frozen storage. In *Seafood Science and Technology*; Bligh, E. G., Ed.; Fishing News Books: Oxford, 1992; pp 76−83.

(5) Oellingrath, I. M.; Iverson, A.; Skreds, G. Quantitative determination of myoglobin and haemoglobin in beef by high-performance liquid chromatography. *Meat Sci.* **1990**, *28*, 313−320.

(6) Han, D.; McMillin, K. W.; Godber, J. S. Hemoglobin, myoglobin, and total pigments in beef and chicken muscles: Chromatographic determination. *J. Food Sci.* **1994**, *59*, 1279−1282.

(7) Fleming, H. P.; Blumer, T. N.; Craig, H. B. Quantitative estimation of myoglobin and hemoglobin in beef muscle extracts. *J. Anim. Sci.* **1960**, *19*, 1164−1171.

(8) O'Brien, P.; Shen, H.; McCutcheon, L.; O'Grady, M.; Byrne, P.; Ferguson, H.; Mirsalimi, M.; Julian, R.; Sargeant, J.; Tremblay, R.; Blackwell, T. Rapid, simple and sensitive microassay for skeletal and cardiac muscle myoglobin and hemoglobin: Use in various animals indicates functional role of myohemoproteins. *Mol. Cell. Biochem.* **1992**, *112*, 45−52.

(9) Kanner, J.; Harel, S. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. *Arch. Biochem. Biophys.* **1985**, *237*, 314−321.

(10) Giulivi, C.; Davies, K. J. Hydrogen peroxide-mediated ferrylhemoglobin generation in vitro and in red blood cells. *Methods Enzymol.* **1994**, *231*, 490−496.

(11) Galaris, D.; Cadenas, E.; Hochstein, P.; Mira, D.; Sevanian, A. Redox cycling of myoglobin and ascorbate: A potential protective mechanism against oxidative reperfusion injury in muscle. *Arch. Biochem. Biophys.* **1989**, *273*, 497−504.

(12) Mugweru, A.; J. Y.; Rusling, J. F. Comparison of hemoglobin and myoglobin for in situ metabolite generation in chemical toxicity sensors using a metallopolymer catalyst for DNA damage detection. *Electrochemistry* **2004**, *16*, 1132−1138.

(13) Binotti, I.; Giovenco, S.; Giardina, B.; Antonini, E.; Brunori, M.; Wyman, J. Studies on the functional properties of fish hemoglobins. II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. *Arch. Biochem. Biophys.* **1971**, *142*, 274−280.

(14) Fyhn, U. E.; Fyhn, H. J.; Davis, B. J.; Powers, D. A.; Fink, W. L.; Garlick, R. L. Hemoglobin heterogenity in amazonian fishes. *Comp. Biochem. Physiol.* **1979**, *62A*, 39−66.

(15) Dodson, G. G.; Richard, V. R.; Tolley, S. P.; Waller, D. A.; Weber, R. E. Crystallization of the rainbow trout (Salmo gairdneri) haemoglobin IV. *J. Mol. Biol.* **1990**, *211*, 691−692.

(16) Trout, G. R.; Gutzke, D. A. A simple, rapid preparative method for isolating and purifying oxymyoglobin. *Meat Sci.* **1996**, *43*, 1−13.

(17) Laemelli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophase T4. *Nature* **1970**, *227*, 680−685.

(18) Brown, W. D. Chromatography of myoglobin on diethylaminoethyl cellulose columns. *J. Biol. Chem.* **1961**, *236*, 2238−2240.

(19) Moll, T. S.; Harms, A. C.; Elfarra, A. A. A comprehensive structural analysis of hemoglobin adducts formed after in vitro exposure of erythrocytes to butadiene monoxide. *Chem. Res. Toxicol.* **2000**, *13*, 1103−1113.

(20) Pelster, B.; Weber, R. E. The physiology of the Root effect. In *Advances in Comparative and Environmental Physiology*; Gilles, R., Ed.; Springer-Verlag: Berlin, 1991; pp 51−77.

(21) Benesch, R. E.; Benesch, R.; Yung, S. Equations for the spectrophotometric analysis of hemoglobin mixtures. *Anal. Biochem.* **1973**, *55*, 245−248.

(22) Hargrove, M. S.; Singleton, E. W.; Quillin, M. L.; Ortiz, L. A.; Phillips, G. N., Jr.; Olson, J. S.; Mathews, A. J. His64(E7)→Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. *J. Biol. Chem.* **1994**, *269*, 4207−4214.

(23) Springer, B. A.; Sligar, S. G. High-level expression of sperm whale myoglobin in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8961−8965.

(24) Carver, T. E.; Brantley, R. E., Jr.; Singleton, E. W.; Arduini, R. M.; Quillin, M. L.; Phillips, G. N., Jr.; Olson, J. S. A novel site-directed mutant of myoglobin with an unusually high O2 affinity and low autoxidation rate. *J. Biol. Chem.* **1992**, *267*, 14443−14450.

(25) Ascoli, F.; Fanelli, M. R.; Antonini, E. Preparation and properties of apohemoglobin and reconstituted hemoglobins. *Methods Enzymol.* **1981**, *76*, 72−87.

(26) Gingerich, W. H.; Pityer, R. A.; Rach, J. J. Whole body and tissue blood volumes of two strains of rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **1990**, *97A*, 615−620.

(27) Richards, M. P.; Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: A possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141−3147.

(28) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. In *Methods in Enzymology*; Fleischer, S., Packer, L., Eds.; Academic Press: New York, 1978; p 302.

(29) Cody, R. P.; Smith, J. K. *Applied Statistics and SAS Programming Language*, 4th ed.; Prentice Hall: Upper Saddle River, NJ, 1997.

(30) Jolly, J. K.; Taketa, F. Biosynthesis of rainbow trout (Salmo gairdnerii) hemoglobins in vitro. *Comp. Biochem. Physiol. B* **1984**, *79*, 537−540.

(31) Hargrove, M. S.; Olson, J. S. The stability of holomyoglobin is determined by heme affinity. *Biochemistry* **1996**, *35*, 11310−11318.

(32) Tappel, A. L. Unsaturated lipid oxidation catalyzed by hematin compounds. *J. Biol. Chem.* **1955**, *217*, 721−733.

(33) Hargrove, M. S.; Whitaker, T.; Olson, J. S.; Vali, R. J.; Mathews, A. J. Quaternary structure regulates hemin dissociation from human hemoglobin. *J. Biol. Chem.* **1997**, *272*, 17385−17389.

(34) Manning, L. R.; Jenkins, W. T.; Hess, J. R.; Vandegriff, K.; Winslow, R. M.; Manning, J. M. Subunit dissociations in natural and recombinant hemoglobins. *Protein Sci.* **1996**, *5*, 775−781.

(35) Everse, J.; Hsia, N. The toxicities of native and modified hemoglobins. *Free Radical Biol. Med.* **1997**, *22*, 1075−1099.

(36) Richards, M. P.; Østdal, H.; Andersen, H. J. Deoxyhemoglobin-mediated lipid oxidation in washed fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 1278−1283.

(37) Fedeli, D.; Tiano, L.; Gabbianelli, R.; Caulini, G. C.; Wozniak, M.; Falcioni, G. Hemoglobin components from trout (Salmo irideus): Determination of their peroxidative activity. *Comp. Biochem. Physiol. B* **2001**, *130*, 559−564.

(38) O'Brien, P. J. Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds, and nucleophiles. *Can. J. Biochem.* **1969**, *47*, 485−492.

(39) Vincent, S. H.; Grady, R. W.; Shaklai, N.; Snider, J. M.; Muller-Eberhard, U. The influence of heme-binding proteins in heme-catalyzed oxidations. *Arch. Biochem. Biophys.* **1988**, *265*, 539−550.

(40) Chiu, D. T.; van den Berg, J.; Kuypers, F. A.; Hung, I. J.; Wei, J. S.; Liu, T. Z. Correlation of membrane lipid peroxidation with oxidation of hemoglobin variants: possibly related to the rates of hemin release. *Free Radical Biol. Med.* **1996**, *21*, 89−95.

(41) Richards, M. P.; Li, R. Effects of released iron, lipid peroxides and ascorbate in trout hemoglobin-mediated lipid oxidation of washed cod muscle. *J. Agric. Food Chem.* **2004**, *52*, 4323−4329.

(42) Stryer, L. Oxygen-transporting proteins: Myoglobin and hemoglobin. *Biochemistry*; W. H. Freeman and Co.: New York, 1988; pp 143−176.

(43) Brown, W. C.; Mebine, L. B. Autoxidation of oxymyoglobins. *J. Biol. Chem.* **1969**, *244*, 6696−6701.

(44) Livingston, D. J.; Brown, D. W. The chemistry of myoglobin and its reactions. *Food Technol.* **1981**, *35*, 244−252.

(45) Brantley, R. E.; Smerdon, S. J.; Wilkinson, A. J.; Singleton, E. W.; Olson, J. S. The mechanism of autoxidation of myoglobin. *J. Biol. Chem.* **1993**, *268*, 6995−7010.

(46) Liong, E. C.; Dou, Y.; Scott, E. E.; Olson, J. S.; Phillips, G. N., Jr. Waterproofing the heme pocket. *J. Biol. Chem.* **2001**, *276*, 9093−9100.

(47) Shaanan, B. The iron−oxygen bond in human oxyhaemoglobin. *Nature* **1982**, *296*, 683−684.

(48) Shikama, K.; Matsuoka, A. Human haemoglobin: a new paradigm for oxygen binding involving two types of alphabeta contacts. *Eur. J. Biochem.* **2003**, *270*, 4041−4051.

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