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Mechanisms of Heme Protein-Mediated Lipid Oxidation Using Hemoglobin and Myoglobin Variants in Raw and Heated Washed Muscle

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The hemoglobin variant rHb 0.1, which possesses a decreased ability to form subunits, stimulated lipid oxidation in washed fish muscle less effectively as compared to wild-type hemoglobin (rHb 0.0). This could be due to the lower hemin affinity and more rapid autoxidation rate of subunits as compared to tetramers. To differentiate between hemin affinity and autoxidation effects, ferrous V68T Mb was compared to ferrous wild-type myoglobin (WT Mb). WT Mb has a more rapid hemin loss rate (25-fold) than does V68T, while V68T autoxidized more rapidly than did WT Mb (60-fold). Ferrous WT Mb promoted TBARS and lipid peroxide formation more rapidly than did ferrous V68T ($p < 0.01$). This indicated hemin loss rate was more critical in determining onset of lipid oxidation as compared to autoxidation rate. Hemin alone was capable of stimulating lipid oxidation. Albumin enhanced the ability of hemin to promote lipid oxidation. MetMb promoted lipid oxidation more effectively than did ferrous Mb, which could be due to the lower hemin affinity of metMb as compared to that of ferrous Mb. EDTA, an iron chelator, had no effect on the rate or extent of lipid oxidation mediated by Mb in the cooked system. Variants with a 975-fold range of hemin affinities promoted lipid oxidation with equivalent efficacy in cooked washed cod contrary to results in uncooked washed cod. The cooking temperatures apparently denature the globin and release hemin reactant to such an extent that the impact of hemin affinity on lipid oxidation observed in the raw state is negated in the cooked state. These studies collectively suggest released hemin is of primary importance in promoting lipid oxidation in raw and cooked washed fish muscle.

KEYWORDS: Quality deterioration; rancidity; lipid oxidation; browning; autoxidation; mutagenesis; mutants, muscle foods; blood; poultry; beef; pork; fish

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in raw and cooked muscle foods manifested through discoloration, loss of nutritional value, and development of off-odors and off-flavors (*1*). The heme proteins hemoglobin (Hb) and myoglobin (Mb) are effective promoters of lipid oxidation (*2*). Myoglobin consists of a globin portion plus a porphyrin heme, the latter containing an iron atom coordinated inside the heme ring. Hemoglobin is made up of four polypeptide chains with each chain containing one heme group. Heme is the nomenclature used to describe the porphyrin ring containing ferrous (Fe^{2+}) iron, while hemin describes the porphyrin ring containing ferric (Fe^{3+}) iron. Ferrous Hb and Mb are typically either liganded with O_2 or no ligand is present (e.g., deoxyMb).

The process by which ferrous Mb (or Hb) is converted to ferric metMb is called autoxidation. Superoxide anion radical ($O_2^{\bullet -}$) or $^{\bullet}OOH$ is liberated in this process depending on whether deoxy or oxy heme protein undergoes autoxidation (*3*). $O_2^{\bullet -}$ and $^{\bullet}OOH$ can readily be converted to hydrogen peroxide (H_2O_2), which enhances the ability of heme proteins to promote lipid oxidation.

An important consideration as to the role of hemoglobin as a promoter of lipid oxidation is the residual Hb content in muscle tissue, especially after bleeding. The only detectable heme pigment in breast muscle from bled broilers was Hb, while Mb was undetectable (*4*). In the dark muscle of those broilers, 86% of the total heme protein was Hb on a weight basis (1.2 mol of Hb to 1.0 mol of Mb). In sockeye salmon, there was no significant difference in Hb levels estimated in whole muscle from bled and unbled fish, which suggests bleeding removed little Hb from the muscle (*5*). Thus, it appears that residual Hb is present in muscle foods and has the potential to contribute to lipid oxidation reactions.

There are numerous potential mechanisms by which Hb and Mb can promote lipid oxidation in muscle foods. MetHb or MetMb react with H_2O_2 or lipid hydroperoxides to generate ferryl heme protein radicals, which can abstract hydrogen from

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polyunsaturated fatty acids and hence initiate lipid oxidation (*6*). Alternatively, displaced hemin or released iron can stimulate lipid oxidation (*7*, *8*).

The problem in understanding the pathway by which heme proteins promote lipid oxidation is that heme protein autoxidation, ferryl radical formation, heme dissociation, heme destruction, and iron release can all occur in a very short time sequence and simultaneously so that the most relevant step related to lipid oxidation is obscured. Site-directed mutagenesis of sperm whale myoglobin can be used to investigate the mechanisms of heme protein-mediated lipid oxidation. Site-directed mutagenesis involves altering the Mb or Hb gene so that alternative amino acids result in the expressed protein as compared to the wild-type protein. Physical and chemical properties of the heme protein such as subunit formation, oxygen affinity, autoxidation rate, heme affinity, and heme destruction can be modulated by this amino acid substitution approach (*3*, *9*−*12*). Our objective was to use various sperm whale Mb and human Hb variants to investigate the mechanisms by which these heme proteins promote lipid oxidation in muscle food systems.

MATERIALS AND METHODS

Chemicals. The recombinant human hemoglobins were supplied by Dr. John Olson as protein solutions (Rice University, Houston, TX). Toluene sulfonyl chloride, dithiothreitol, disodium EDTA dihydrate, DNAse I, RNAse A, lysozyme, ferric chloride, hemin chloride, chloramphenicol, streptomycin sulfate, bovine hemoglobin, bovine serum albumin, ferrous sulfate, antifoam, barium chloride, ammonium thiocyanate, and sucrose were obtained from Sigma Chemical A/S (St. Louis, MO). Agar, tryptone, yeast extract, kanamycin, chloroform, methanol, methyl ethyl ketone, and tris [hydroxymethyl] aminomethane (Tris) were obtained from Fisher scientific (Pittsburgh, PA). Isopropyl-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, WI). All other chemicals used were analytical grade. Distilled, deionized water was used for the preparation of all solutions, substrates, and experiments.

Myoglobins and Hemoglobins Examined. All heme proteins were prepared using recombinant technology. Wild-type human Hb (rHb 0.0) was compared to human Hb (rHb 0.1), which is genetically cross-linked to prevent tetrameric Hb from dissociating into subunits (dimers and monomers) (*9*). Wild-type sperm whale Mb (WT Mb) was used as the model heme protein to which Mb variants were compared. V68T in which the native valine residue is substituted with threonine has an exceptionally high hemin affinity and rapid autoxidation rate as compared to WT Mb (*3*, *11*). H97A in which the native histidine residue is substituted with alanine has an exceptionally low hemin affinity as compared to WT Mb (*11*).

Preparation of Recombinant Myoglobins. Genes coding for the recombinant myoglobins was supplied by Dr. John Olson (Rice University Houston, TX) and subcloned from the pUC 19 plasmid to the pET 28 plasmid (Novagen Madison, WI). The subcloning procedure entailed amplification of the mutant genes via PCR, digestion of the amplified genes and pET 28 plasmid with restriction endonucleases *Nco*I and *Bpu*1102I (Fermentas Inc., Hanover, MD), and ligation of the amplified genes with pET 28 DNA to form the final constructs. Successful subcloning was confirmed by dideoxy sequencing at the University of Wisconsin Biotechnology Center (Madison, WI). The constructs were then used to transform *E. coli* BL21-CodonPlus (DE3)-RP host cells (Stratagene, La Jolla, CA) via the heat shock method provided with the cells. Recombinant myoglobins were then expressed in the host *E. coli* cells using a 12 L culture vessel (Cat#2602-0110, Nalge Nunc Int., Rochester, NY) and Terrific Broth (TB) adjusted to pH 7 as the culture medium. All culture media contained 30 μg/mL kanamycin and 50 μg/mL chloramphenicol. To perform the expression, a single colony of transformed host *E. coli* was transferred from an LB agar plate to a 12 mL volume of TB and incubated 14−16 h in a 37 °C shaker. The 12 mL culture was then transferred to a 360 mL volume of TB and incubated for 4 h in a 37 °C shaker to produce a starter culture. The starter was added to 12 L of 37 °C TB containing 2 mL of 1 M $FeCl_3$ and 50 μL of antifoam per liter, and the culture was incubated in the 12 L vessel. 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, IPTG was added to 1 mM to induce expression of the Mb. During induction, hemin chloride was added to the culture to 4 mg/L. After 4 h of induction, cell paste containing the expressed 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. The lysis buffer (*13*) consisted of 50 mM Tris base, 1 mM disodium EDTA dihydrate, 0.5 mM dithiothreitol, 1 mM toluene sulfonyl chloride, 40 U/mL DNAse I, 3 U/mL RNAse A, and 78 800 U/mL lysozyme, and was adjusted to pH 6. Myoglobins were then purified from the lysate via ammonium sulfate precipitation, and anion and cation exchange chromatography as described previously (*10*, *14*). Finally, the myoglobins were concentrated to 0.5−1.0 mM (heme basis) and snap frozen in liquid nitrogen and stored either in liquid nitrogen or at −80 °C.

Preparation of Washed Cod Muscle. Cod fish (*Gadus morhua*) fillets, determined to be fresh based on odor and appearance, were obtained from The Seafood Center (Madison, WI). Fillets were trimmed to remove all bones and dark tissue, and cut into small pieces. The pieces were ground using a Kitchen Aid, Inc. (St. Joseph, MI) KSM90WW household mixer equipped with grinding apparatus (5 mm plate diameter). Weight of ground cod muscle was determined. Muscle was washed by combining in a 3:1 ratio with cold distilled, deionized water and mixing for 2 min with a heavy glass rod. After settling for 15 min, muscle was collected and dewatered using a fiberglass screen. Next, using cold 50 mM sodium phosphate buffer pH 6.3 in place of the water, muscle was washed, dewatered, and washed a final time. Immediately after the final wash, muscle slurry was homogenized for approximately 3 min with a Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY) until it was of a fibrous consistency. The pH of the muscle slurry was checked and determined to be between 6.27 and 6.30. Muscle was collected in approximately 50 g pellets by centrifuging 25 min at 15 263*g*, and was then stored at −80 °C in vacuum sealed plastic bags. All washing, dewatering, and centrifugation steps were performed at 4 °C.

Quantification of Heme Protein Concentration. A heme protein solution was diluted in 50 mM Tris pH 8.0 (final volume 1.5 mL). Approximately 1 mg of dithionite was dissolved in the diluted solution. The solution was then bubbled with carbon monoxide gas (Badger Welding, Madison, WI) for 30 s. The solution was scanned from 440 to 400 nm in a model UV-2401 dual beam spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD) using 50 mM Tris pH 8.0 as the reference (*15*). Calculation of the concentration of heme protein on a heme basis was performed using the peak absorbance occurring near 420 nm and a standard curve generated from bovine hemoglobin.

Preparation of Free Hemin and Albumin Solutions. A 5 mM free hemin stock solution was prepared by dissolving solid hemin in 0.1 N NaOH. A bovine serum albumin (BSA) stock solution was prepared by carefully dissolving solid BSA in 50 mM sodium phosphate pH 5.7 buffer. Working solutions containing the free hemin and BSA in varying proportions were then prepared using the stock solutions and 50 mM sodium phosphate pH 5.7 as the diluent and were incubated at room temperature for 30 min prior to their use in lipid oxidation experiments. The stocks and working solutions were prepared fresh, protected from light, and only used on the day of preparation.

Heme Protein Autoxidation. Loss of absorbance at 581 nm was used to assess the rate of myoglobin autoxidation (*3*). The existence of ferrous myoglobin spectra was verified for each heme protein prior to initiating the experiment. This ensured that metmyoglobin was not present as a contaminant in the ferrous myoglobin samples. Igor Pro software (WaveMetrics Inc., Portland, OR) was used to calculate autoxidation rates (k_{ox}) by curve fitting using the linear function. The half-time ($t_{1/2}$) was calculated using the following equation: $t_{1/2} = \ln(2)/k_{ox}$.

Adding Heme Proteins to Washed Cod Muscle. Prepared washed cod mince was thawed overnight at 4 °C. The next day the mince was transferred to a plastic beaker on ice and mixed for 10−15 min with a plastic spatula to break up the pieces of mince. To further break up the

mince, the washed cod was ground for three 3-s pulses at the medium 15 setting in a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC). The pH of the muscle was then adjusted to 5.7 by addition of either 1 N HCL or 1 N NaOH and mixing on ice for 10−15 min. Tissue was then added to an amber reaction vial. Water was added to the vial so that final moisture content would be 90%, and streptomycin sulfate was added so the final concentration would be 200 ppm. The contents of the vial were mixed with a plastic spatula for 2 min. Sperm whale Mb mutant or free heme solution of interest was then added and mixed for 3 min with a plastic spatula so the final concentration on a heme basis was 40 μmol/kg tissue. The total heme pigment in muscles from chicken, pork, beef, trout, and mackerel ranged from 8.3 to 974 μmol/kg on a heme basis (*16*, *17*). The concentration of 40 μmol/kg washed cod was chosen for our studies because it is in the range found in post mortem muscle and a substantial amount of lipid oxidation occurred in washed cod at this concentration after 1 or 2 days of 2 °C storage at post mortem pH values using the wild-type sperm whale myoglobin. Zero-time samples were taken, and reaction vessels were stored on ice for the duration of the experiment. Cooked reactions with and without 2 mM EDTA were exposed to an internal temperature of 80 °C prior to addition of heat labile streptomycin, taking zero-time samples, and storing on ice. The pH of the reaction was measured by adding water to 10× the weight of a sample of the reaction, homogenizing the mixture for 20 s using a Polytron Type PTA 20/2 W probe (Brinkmann Instruments), and inserting a pH probe into the homogenate. Conversion to metMb was done by adding potassium ferricyanide at a 3-fold excess (heme basis) and incubating on ice for 1 h. Ferricyanide was removed using DG-10 desalting columns (Bio-Rad, Hercules, CA).

Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to the modified method of Buege and Aust (*18*). On the day of analysis, a solution of 50% trichloroacetic acid (TCA) with 1.3% thiobarbituric acid (TBA) was prepared by mixing and heating to 65 °C to dissolve solutes. A 1.2 mL volume of the TCA−TBA reagent was then added to each washed cod muscle sample (90−150 mg), and samples were mixed via inversion and heated at 65 °C for 60 min. After heating, the samples were cooled at 4 °C for 60 min. Samples were then centrifuged at 1600*g* for 5 min. Absorbances of supernatants were read at 532 nm. Absorbances at 650 nm were also read and subtracted from 532 nm values to account for any turbidity. A standard curve was constructed using tetraethoxypropane, and concentrations of TBARS in samples were expressed as μmol TBARS/kg tissue.

Determination of Lipid Peroxides. A 5 mL volume of cold chloroform:methanol (1:1) was added to a 300−500 mg muscle sample. Sample was homogenized in the solvent for 20 s using a Polytron Type PTA 20/2 W probe (Brinkmann Instruments), and homogenate was transferred to a screw-cap glass centrifuge tube. The Polytron probe was then rinsed for 12 s with another 5 mL of the solvent, which was also added to the centrifuge tube. A 3.08 mL volume of cold 0.5% NaCl was added to the centrifuge tube, and the contents of tube were vortexed for 30 s. The mixture in the centrifuge tube was then centrifuged at 1800*g* for 6 min at 4 °C. Using a glass syringe, a 2 mL volume of the lower chloroform layer was removed from the centrifuged sample and transferred to a clean glass screw-cap tube. A 1.33 mL volume of chloroform:methanol (1:1) was added to the 2 mL sample to begin the analysis for lipid hydroperoxides (*19*). Next, 25 μL of 3.94 M ammonium thiocyanate and 25 μL of 18 mM iron(II) chloride were added to the tube, vortexing 4 s after each addition. The sample was then incubated at room temperature for 20 min, and the absorbance was read at 500 nm. A standard curve was constructed using cumene hydroperoxide, and the concentration of lipid hydroperoxides in the sample was expressed as μmol lipid hydroperoxides/kg muscle.

Cooking Procedure. Amber bottles (30 mL capacity) containing around 15 g of washed cod and added heme protein were placed in a water bath held at 88 °C. Bottles were removed from the water bath when the internal temperature of the tissue reached 80 °C (*20*). Bottles were then quickly transferred to an ice bath for rapid cooling prior to 2 °C storage.

Statistical Evaluations. A MIXED procedure of the SAS system was used to analyze data from the storage studies (*21*). Means were separated using the p-diff test. For each treatment, two or three separate reactions were examined during storage. Because a subsample was removed from each reaction vessel at each time point, repeated measures were used. Analysis of repeated measures data requires special attention to the covariance structure due to the sequential nature of the data on each experimental unit (*22*). The mixed model allows the capability to address the issue directly by modeling the covariance structure. The variation between separate vessels within a treatment is specified by a "random" statement in the model. The covariation within a vessel is specified by a "repeated" statement in the model. The covariance structure that was fitted in the model was the spatial power matrix. This was used because the distance between each time point assayed was in some cases not equivalent. Standard deviations are reported.

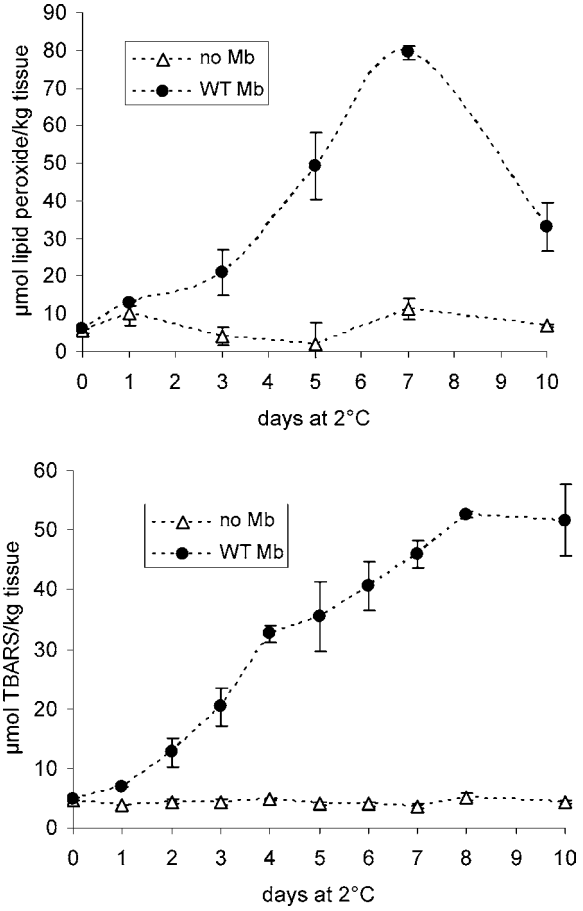

Figure 1. Ability of recombinant wild-type sperm whale Mb to promote lipid peroxide and thiobarbituric acid reactive substances (TBARS) formation in washed cod muscle at pH 5.7. The Mb concentration on a heme basis was 40 μmol/kg tissue. Mb was added in the ferrous form.

RESULTS

The ability of different hemoglobin (Hb) and myoglobin (Mb) variants to promote lipid oxidation was assessed in washed cod muscle. Washing removes aqueous antioxidants and prooxidants. The remaining washed tissue contains myofibrillar proteins and membrane phospholipids. The pH of the washed cod was adjusted to pH 5.7 so to be in the range of typical post mortem muscle tissue (pH 5.1−6.8). Formation of lipid peroxides and thiobarbituric acid reactive substances (TBARS) was used as indicators of lipid oxidation. In the absence of added heme protein, lipid peroxide and TBARS formation was negligible during 10 days of storage at 2 °C (**Figure 1**). Recombinant wild-type sperm whale myoglobin (WT Mb) in the ferrous state was also added to washed cod at a concentration of 40 μmol/kg tissue. The wild-type designation indicates that the

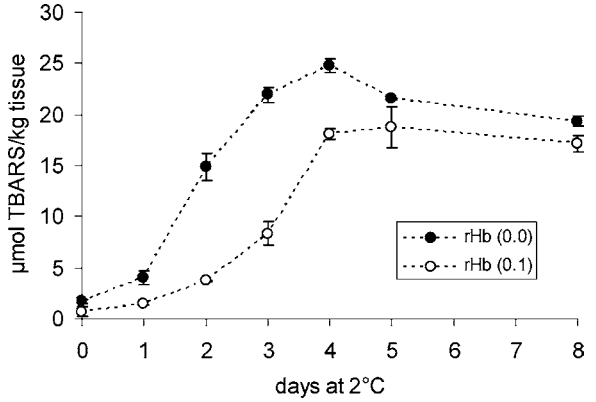

Figure 2. Ability of recombinant wild-type human Hb (rHb 0.0) and cross-linked human Hb (rHb 0.1) to promote thiobarbituric acid reactive substances (TBARS) formation in washed cod muscle (pH 5.7). Heme proteins were added in the ferrous form. Hb was added on a heme basis at a concentration of 40 μmol/kg tissue.

Table 1. Autoxidation Rate (k_{ox}) and Half-Time ($t_{1/2}$) for Wild-Type Sperm Whale Myoglobin and V68T (25 μM, Heme Basis) during 2 °C Storage at pH 5.7[a]

myoglobin	k_{ox} (h^{-1})	$t_{1/2}$ (h)
WT Mb	0.009 ± 0.001	73.9 ± 4.9
V68T	0.603 ± 0.089	1.2 ± 0.2

[a] k_{ox} is equivalent to the probability of decay of a single molecule to the ferric form in an hour. $t_{1/2}$ is the time required for one-half of the molecules to oxidize. V68T indicates that the valine residue in native sperm whale myoglobin has been substituted with a threonine residue.

native composition of sperm whale myoglobin is maintained (*13*). In the presence of WT Mb, lipid peroxide formation was slow from day 0 to day 3 followed by a rapid rate between days 3 and 7, which was followed by a decrease in lipid peroxides by 10 days of storage (**Figure 1**). There was a short lag phase prior to TBARS formation that was followed by a steady increase in TBARS between days 1 and 8, reaching a maximum value around day 8 (**Figure 1**). This indicated WT Mb effectively promoted lipid oxidation in washed cod muscle.

Lipid oxidation mediated by wild-type human Hb (rHb 0.0) and a genetically cross-linked Hb (rHb 0.1) was assessed. The cross-linking stabilizes the tetramer against dimer and monomer formation by fusion of two α subunit genes into a single gene (*9*). The cross-link is one glycine between the N-terminus of one α chain and the C-terminus of the other. The comparison was done because we hypothesized that rHb 0.1 would be a weaker promoter of lipid oxidation than rHb 0.0 due to the fact that Hb subunits are more prone to autoxidation and hemin release than tetramers (*9*, *23*). The ferrous form of each Hb was examined at pH 5.7 in washed cod muscle during 2 °C storage. TBARS formation due to rHb 0.0 occurred more rapidly as compared to that due to rHb 0.1 ($p < 0.001$) (**Figure 2**). It is noteworthy that decreasing hemoglobin concentration, which occurs when erythrocytes lyse, and decreasing pH, which occurs post mortem, increase subunit formation (*24*, *25*).

Because Hb subunit formation increases both hemin loss and metHb formation, we wished to examine the relative roles of hemin loss as compared to met formation in lipid oxidation processes. This can be accomplished by selecting variants with opposing propensities to release hemin and be converted to met heme protein (e.g., Mb autoxidation). Our results at pH 5.7 and 2 °C indicated that V68T autoxidized 60 times faster than WT Mb (**Table 1**). V68T is indicative of substituting threonine at

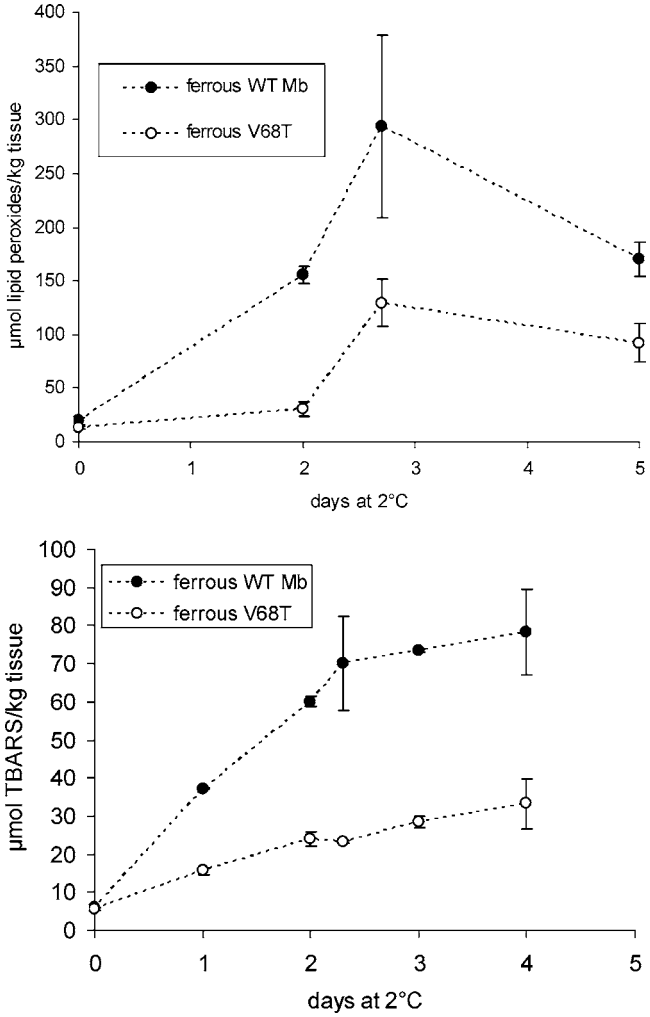

Figure 3. Ability of recombinant wild-type sperm whale Mb and V68T to promote lipid peroxide and thiobarbituric acid reactive substances (TBARS) formation in washed cod muscle (pH 5.7). Heme proteins were added in the ferrous form. Each heme protein was added on a heme basis at a concentration of 40 μmol/kg tissue.

the 68th site where valine is found in WT Mb. WT Mb has a 25-fold lower hemin affinity than V68T (*11*). Thus, if autoxidation rate is critical to the onset of lipid oxidation, then ferrous V68T should promote lipid oxidation more effectively than ferrous WT Mb. Conversely, if hemin loss is more critical to onset of lipid oxidation, then ferrous WT Mb should promote lipid oxidation more effectively as compared to ferrous V68T. Ferrous WT Mb promoted lipid oxidation more effectively than ferrous V68T based on lipid peroxide ($p < 0.05$) and TBARS formation in washed cod ($p < 0.01$) (**Figure 3**).

The ability of metMb and ferrous Mb to promote lipid oxidation in washed cod was examined during 2 °C storage. The reactive oxygen species $O_2^{\bullet -}$ and $^{\bullet}OOH$ form during autoxidation of ferrous Mb, while metMb has lower hemin affinity as compared to ferrous Mb. WT sperm whale Mb was used for the comparison between the reduced and oxidized forms of the heme protein. MetMb caused a more rapid formation of lipid peroxides as compared to ferrous Mb during 2 °C storage ($p < 0.01$) (**Figure 4**). MetMb stimulated formation of TBARS to occur more rapidly and to a greater extent than ferrous Mb ($p < 0.01$) (**Figure 4**).

Because hemin that dissociated from Mb appeared to be critical in promoting lipid oxidation, addition of hemin to washed cod was examined. Hemin and hemin associated with

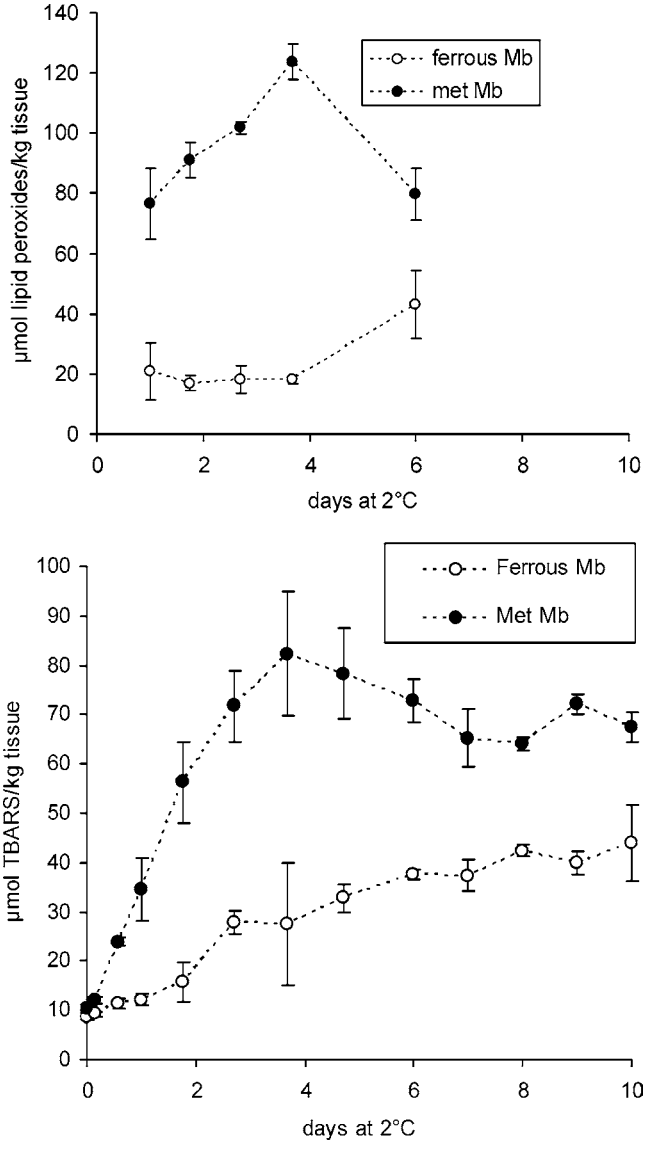

Figure 4. Ability of the ferrous and met forms of recombinant wild-type sperm whale Mb to promote lipid peroxide and thiobarbituric acid reactive substances (TBARS) formation in washed cod muscle (pH 5.7). Each heme protein was added on a heme basis at a concentration of 40 μmol/kg tissue.

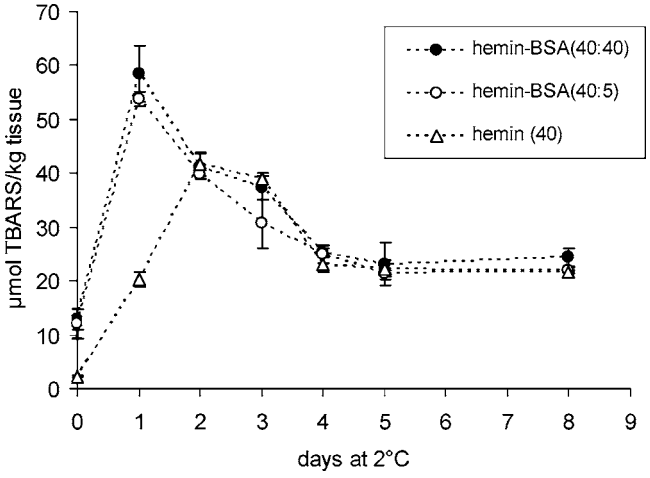

Figure 5. Ability of hemin and hemin in the presence of bovine serum albumin to promote thiobarbituric acid reactive substances (TBARS) formation in washed cod muscle (pH 5.7). Hemin was added on a heme basis at a concentration of 40 μmol/kg tissue. Albumin was added at either a 1:1 or an 8:1 (hemin:albumin) ratio.

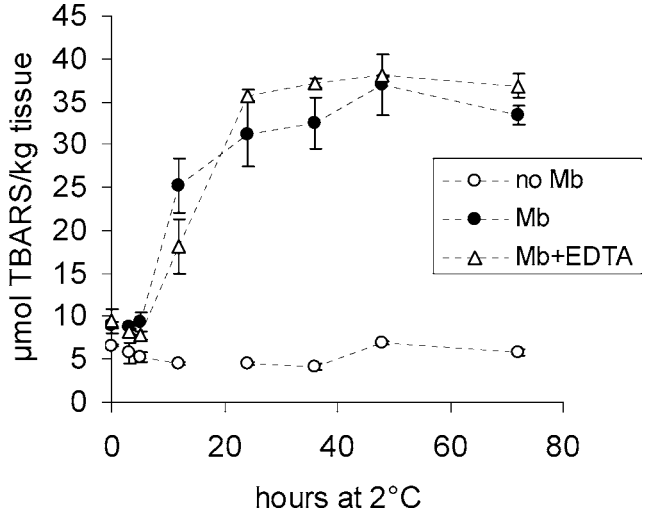

Figure 6. Effect of WT Mb and EDTA (2 mM) on thiobarbituric acid reactive substances (TBARS) formation in washed cod that was cooked and then stored at 2 °C (pH 5.7). Mb was added on a heme basis at a concentration of 40 μmol/kg tissue.

bovine serum albumin were added to washed cod prior to determining lipid oxidation products during 2 °C storage. Hemin was added at 40 μmol per kg tissue. Albumin was added at either a 1:1 or an 8:1 (hemin:albumin) ratio. Hemin effectively stimulated TBARS formation, reaching a maximum after 2 days of storage (**Figure 5**). The addition of albumin to hemin increased the onset of TBARS formation as compared to hemin alone during the storage period ($p < 0.001$). A hemin to albumin ratio of 1:1 (40 μmol/kg each) slightly but significantly increased TBARS values as compared to the ratio of 8:1 (40 μmol hemin/kg and 5 μmol albumin/kg) ($p < 0.05$) (**Figure 5**).

The ability of WT Mb and Mb variants to stimulate lipid oxidation in cooked washed cod was also examined. WT Mb was added to washed cod and then immediately cooked to an internal temperature of 80 °C. After being cooked, samples were cooled in ice and then stored at 2 °C. In the absence of WT Mb, TBARS values remained low during 3 days of 2 °C storage (**Figure 6**). In the presence of WT Mb, TBARS values rapidly increased and reached a plateau after around 1 day (**Figure 6**). The ability of EDTA at a concentration of 2 mM to alter the rate of lipid oxidation was determined in cooked washed cod muscle containing added WT Mb. EDTA will chelate iron that is liberated from Mb due to storage or the heating process itself. EDTA had little effect on the overall pattern of Mb-mediated TBARS formation during storage at 2 °C (**Figure 6**). Lipid peroxide content was also determined after 24 h storage at 2 °C. Addition of EDTA did not significantly affect Mb-mediated lipid peroxide formation (**Table 2**). Lipid peroxide values were around 8 times less in cooked washed cod without added Mb as compared to washed cod containing added Mb (**Table 2**).

The myoglobin variants H97A, V68T, and WT Mb were each separately added to washed cod muscle followed immediately by a cooking step. These variants were chosen because they exhibit a 975-fold range of hemin affinities (*11*, *26*). The met forms of each heme protein were examined so that any variation in autoxidation rates did not confound the effect of hemin affinity on lipid oxidation. After being cooked, samples were

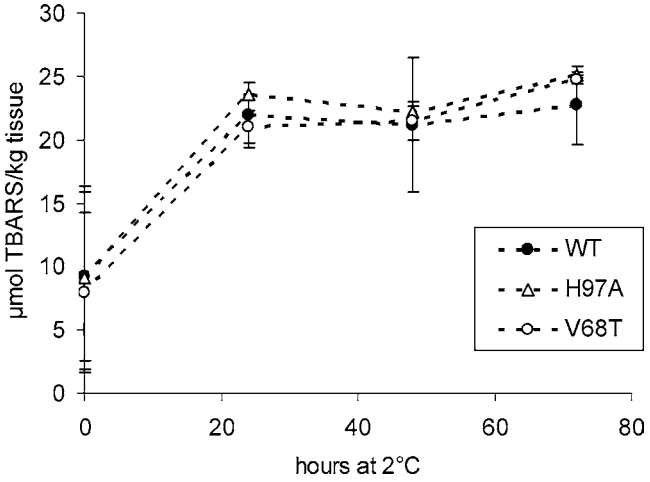

Figure 7. Effect of H97A, V68T, and WT Mb on thiobarbituric acid reactive substances (TBARS) formation in washed cod that was cooked and then stored at 2 °C (pH 5.7). Each Mb variant was added on a heme basis at a concentration of 40 μmol/kg tissue.

Table 2. Effect of Myoglobin and EDTA on Lipid Peroxide Formation in Washed Cod That Was Cooked and Then Stored 24 h at 2 °C[a]

cooked sample	lipid peroxides μmol/kg tissue
washed cod	10.13 ± 1.81a
washed cod + Mb	87.83 ± 1.38b
washed cod + Mb + EDTA	82.22 ± 13.64b

[a] Myoglobin (40 μmol/kg tissue) and EDTA (2 mM) were added to washed cod prior to the thermal treatment. Similar letters in a column indicate no significant difference between treatments.

cooled in ice and then stored at 2 °C. No significant differences in TBARS values among the three heme proteins were observed during 2 °C storage (**Figure 7**).

DISCUSSION

Inhibiting Hb subunit formation by genetically cross-linking the molecule decreased Hb-mediated lipid oxidation in washed cod muscle (**Figure 2**). This can be attributed to the fact that Hb subunits (e.g., monomers and dimers) undergo autoxidation more rapidly than tetramers. Hemoglobin dimers were found to autoxidize 16 times faster as compared to tetramers (*23*). Autoxidation can accelerate lipid oxidation processes due to the formation of superoxide anion radical ($O_2^{\bullet-}$) and met heme protein via the autoxidation process. $O_2^{\bullet-}$ dismutates to H_2O_2, which reacts with met heme protein to form a ferryl radical species capable of initiating lipid oxidation (*27*). Another reason that cross-linking of Hb decreased rates of lipid oxidation could be due to the fact that hemin is released more rapidly from subunits as compared to tetramers. The monomeric forms of isolated α and β chains lose hemin 30−40 times more rapidly as compared to the corresponding subunits in a tetramer (*9*). Hemin has been implicated as a potent promoter of lipid oxidation primarily through the decomposition of preformed lipid hydroperoxides to form free radicals capable of abstracting hydrogen atoms from fatty acids and hence initiate lipid oxidation (*7*, *28*).

To differentiate the roles of heme protein autoxidation and hemin affinity on lipid oxidation processes, WT Mb and V68T were compared in the ferrous oxidation state. V68T undergoes autoxidation faster than does WT Mb (60-fold difference), while WT Mb releases hemin more readily than does V68T (25-fold difference). The fact that WT Mb promoted lipid oxidation in washed cod more effectively than did V68T (**Figure 3**) indicated that hemin affinity was a rate-limiting step in lipid oxidation processes, while heme protein autoxidation had less of a role.

The fact that V68T autoxidized 60 times faster as compared to WT Mb (**Table 1**) is partly due to the fact that oxygen affinity is lowered 17-fold when threonine is substituted for valine (*29*). Valine is apolar so there is minimal interaction of this residue with liganded O_2. However, the −OH group of threonine at the same site provides electron density, which disrupts the ability of the electron-dense O_2 to coordinate to the heme iron (**Figure 8**). Lower O_2 affinity increases the content of deoxyMb present as compared to oxygenated myoglobin. Coordination of H_2O to deoxyMb is an intermediate that leads to metMb formation and $O_2^{\bullet-}$ production. This deoxyMb-mediated autoxidation is discouraged in the presence of oxygenated myoglobin (*3*).

The met form of V68T has an unusually high hemin affinity (*11*). This is due to the ability of threonine to hydrogen bond with water that is coordinated to the ferric iron atom within metMb (**Figure 9**). The threonine residue also hydrogen bonds with the distal histidine and the carbonyl oxygen of the distal histidine (*29*). These multiple hydrogen bonds stabilize the hemin group within the globin. The WT Mb containing valine (E11) at site 68 cannot hydrogen bond, and thus the hemin moiety is more prone to dissociation (**Figure 9**). Previously, it was shown that Mb variants with low hemin affinity (e.g., H97A) were especially potent promoters of lipid oxidation in washed cod, while Mb variants with high hemin affinity (e.g., V68T) were relatively weak promoters of lipid oxidation (*30*). WT Mb had intermediate hemin affinity and an intermediate ability to oxidize washed cod lipids. The transfer of hemin reactant from the globin to lipid substrates has been postulated as a potential key step in the mechanism by which heme proteins oxidize lipid substrates (*31*−*37*). Our results further substantiate this pathway involving dissociated hemin.

Superoxide anion radical ($O_2^{\bullet-}$) that is produced during conversion of ferrous Mb to met Mb during autoxidation has the potential to increase the ability of Mb to oxidize lipids. This is because the $O_2^{\bullet-}$ produced can dismutate to H_2O_2, a prooxidant. However, the met form of WT Mb promoted lipid oxidation more effectively than ferrous WT Mb in washed cod (**Figure 4**). Heme is anchored in ferrous Mb around 60 times more strongly as compared to met Mb (*38*). This can explain why met Mb was more effective at stimulating lipid oxidation in washed cod than ferrous Mb in that hemin can be more readily delivered into membrane phospholipids from the met form of the heme protein. A considerable amount of time needs to elapse for ferrous WT Mb to be converted to met Mb (**Table 1**). Based on the half-time shown, it would take around 6 days at our experimental temperature and pH for full conversion from ferrous to met WT Mb. In liposomes, ferrous Mb promoted lipid oxidation more effectively than did metMb (*39*). This suggests the mechanism by which lipid oxidation proceeds in liposomes as compared to that in washed cod muscle is different. It has been suggested that hemin must bind to the protein portion of lipoproteins prior to oxidizing the lipid of the lipoprotein particle (*34*). Washed cod contains myofibrillar and membrane proteins that may provide a docking site for hemin, while liposomes are devoid of protein.

Once hemin is released from the heme protein, either hemin or iron released from hemin can promote lipid oxidation processes. Previously, it was shown that a myoglobin mutant sensitive to heme destruction was a weaker promoter of lipid oxidation than WT Mb, which suggests released iron due to

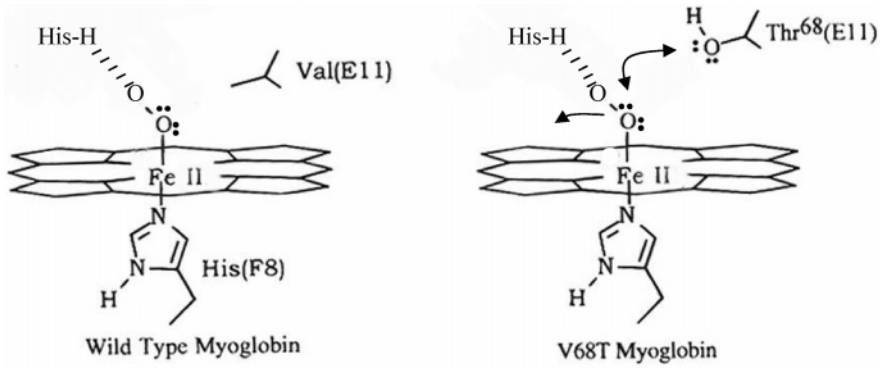

Figure 8. Coordination of O_2 within the heme crevice of wild-type and V68T ferrous myoglobin. Hydrogen bonding occurs between the distal histidine and liganded O_2. The native valine at the 68th site is electrostatically neutral, while threonine at the 68th site provides electron density that decreases oxygen affinity. The histidine below the heme ring is the proximal (F8) histidine.

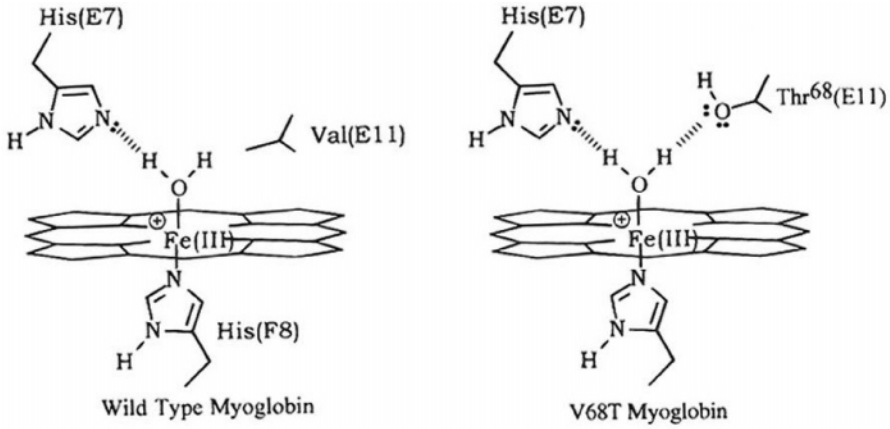

Figure 9. Coordination of water within the heme crevice of wild-type and V68T ferric met myoglobin. Hydrogen bonding occurs between the distal histidine and the liganded water molecule coordinated with the iron atom within the heme crevice. Threonine at the 68th site also hydrogen bonds with liganded water, which increases hemin affinity. The native valine at site 68 does not hydrogen bond.

destruction of the heme ring decreases lipid oxidation (*30*). This is reasonable if, in fact, hemin is the primary promoter of lipid oxidation processes.

Hemin alone effectively promoted lipid oxidation in washed cod (**Figure 5**). Albumin is known to form weak complexes with heme (*40*). It has been shown that albumin increased the ability of hemin to oxidize rat liver microsomes (*37*). The onset of hemin-mediated lipid oxidation in our studies was also accelerated in the presence of albumin (**Figure 5**). These results suggest that albumin acts as a shuttle to deliver hemin into lipid phases, which increases the ability of hemin to oxidized lipids. The globins of Mb and Hb chains likely also act as hemin shuttles although to different degrees. Mb was a weaker promoter of lipid oxidation in low-density lipoproteins (LDL) as compared to Hb, which was attributed to the greater ability of Hb to transfer hemin into the LDL particle (*34*).

Heating will rapidly convert ferrous heme protein to met heme protein based on the brown appearance of cooked meat. Heating also unfolds the globin of heme proteins, resulting in denaturation of the globin. Unfolding of deoxymyoglobin was found to disrupt the heme iron−proximal histidine bond (*38*). Thus, heating should facilitate release of hemin from the globin. Two possible mechanisms by which heme protein-mediated lipid oxidation occurs in cooked tissue involve released hemin and iron released from the hemin. Iron released from hemin will be designated as low molecular weight iron. EDTA can either inhibit or accelerate low molecular weight iron-mediated lipid oxidation (*41*, *42*). EDTA had little effect on the ability of WT Mb to oxidize washed cod lipids that were exposed to the heating treatment prior to 2 °C storage (**Figure 6**, **Table 2**). This suggests that released hemin, and not released iron from hemin, promoted lipid oxidation in the cooked washed cod system. It must also be noted that EDTA should only modulate the reactivity of released iron that is present in the aqueous phase because EDTA is a hydrophilic chelator.

Because high hemin affinity drastically decreased lipid oxidation in raw washed cod using met V68T as compared to met H97A and WT Mb (*30*), it was of interest to determine if met V68T would also be a weak promoter of lipid oxidation in cooked tissue. In cooked washed cod, H97A, V68T, and WT Mb promoted lipid oxidation with equivalent efficacy despite the 975-fold difference in hemin affinity among the three myoglobins (*11*) (**Figure 7**). Heating apparently denatures the globin and releases hemin reactant to such an extent that the impact of hemin affinity on lipid oxidation observed in the raw state is negated in the cooked state.

Berisha et al. (*43*) showed that heating myoglobin solutions to 100 °C released only 1.42% of the iron from horse heart myoglobin, which suggested that released iron had a small role in the amount of lipid oxidation observed. Ohshima et al. (*44*) examined the ability of iron chloride and sperm whale Mb to promote TBARS in washed mackerel white muscle that received a cooking treatment. On an iron basis, myoglobin was around 6 times more pro-oxidative than FeCl (ferrous and ferric forms). These authors also found that EDTA did not inhibit TBARS formation mediated by the myoglobin.

The pro-oxidative character of metmyoglobin in linoleic acid micelles increased at temperatures immediately below the denaturation temperature of metmyoglobin (*45*). This could be attributed to the weakening of the hemin−globin linkage. Above the denaturation temperature, Mb became less pro-oxidative, which was attributed to hemichrome formation. Hemichrome is formed when a nitrogen base covalently bonds to the heme group within the globin. The additional covalent bond decreases hemin release and reactivity of the heme iron. These authors further stated that the amount of iron released due to heating (10−20%) was insignificant in promoting lipid oxidation based on further experimentation using free iron (Fe^{2+}), hemin, and metMb.

It has also been shown that ionic ferrous iron (Fe^{2+}) promoted lipid oxidation in cooked washed muscle fibers more effectively than did horse heart metMb (*46*). The iron was added at 5 mg inorganic salt/kg, which is around 41 μM iron assuming $FeCl_2$ was used. If 10−20% of the iron is liberated from WT Mb in our studies due to heating, then only 4−8 μM low molecular weight iron was available for reaction. The lower amount of iron in our study may explain why released iron from Mb did not appear to be pro-oxidative in our cooked washed cod system based on the inability of EDTA to alter lipid oxidation kinetics (**Figure 6**). In addition, ferrous (Fe^{2+}) iron was added in the aforementioned study, while released iron from metMb, which occurs after cooking, is likely in the less reactive ferric state (Fe^{3+}). Fe^{2+} decomposes lipid hydroperoxides around 10 times faster than does Fe^{3+} (*47*).

The diffusate from a beef muscle extract promoted lipid oxidation more effectively than did the dialysate when each fraction was added to washed muscle fibers and heated (*48*). The dialysate will contain hemoglobin and myoglobin. The pro-oxidative activity in the diffusate was attributed to low levels of ascorbate activating the non-heme iron that was present. In our washed cod model system containing added heme proteins, no ascorbate was added, and some of the potential pools of iron from a beef muscle extract (e.g., ferritin and transferrin) were not present. In another study, the dialysate from a mackerel muscle extract promoted lipid oxidation more effectively than the diffusate when each fraction was added to washed fish muscle fibers and heated (*49*).

Although no direct comparisons were made, the extent of lipid oxidation mediated by SW Mb appeared to be greater in the unheated washed cod (**Figures 1**, **3**, and **4**) as compared to that in the heated washed cod (**Figures 6** and **7**). Maximal TBARS values in the unheated system ranged from 50 to 90 μmol/kg, while in the heated system the range was around 25−40 μmol/kg. It may be that heating rapidly dissociates hemin, which promotes lipid oxidation, albeit less effectively than when the globin chains can deliver hemin into the lipid phase. Consistent with this is the fact that direct addition of hemin to unheated washed cod caused maximal TBARS values of 40 μmol/kg tissue, and adding hemin in the presence of albumin, a protein with weak binding affinity for hemin, increased the maximal TBARS value to 60 umol/kg tissue (**Figure 5**). Hemichrome formation due to denaturation of the heme protein by heating could also decrease the extent of lipid oxidation (*45*).

In conclusion, inhibiting autoxidation of Mb and Hb decreases lipid oxidation in washed cod due to the fact that hemin is more strongly bound to the globin in ferrous heme proteins as compared to the met forms. Moreover, hemin release appears to be the rate-limiting step in heme protein-mediated lipid oxidation due to the fact that the ferrous form of the Mb variant with high hemin affinity (V68T) was a weaker promoter of lipid oxidation as compared to ferrous WT Mb despite V68T autoxidizing 60 times faster than WT Mb. Hemoglobin is especially capable of promoting lipid oxidation due to the formation of subunits that have lower hemin affinity as compared to Hb tetramers and myoglobins (*9*, *36*). The relatively acidic pH of post mortem tissue enhances subunit formation as compared to higher physiological pH values. Dilution that occurs upon lysis of erythrocytes also favors subunit formation. Released hemin is more capable of stimulating lipid oxidation in the presence of proteins such as albumin or apoglobin. The globins may act as a shuttle to deliver hemin into lipid phases. Cooking loosens the hemin−globin linkage, allowing hemin-mediated lipid oxidation to proceed. This may partly explain why precooked muscle foods develop rancidity rapidly after cooking (*50*). Release of iron from heme proteins due to heme destruction will decrease rates of lipid oxidation due to the destruction of hemin reactant (*30*, *51*). The inability of excess EDTA to inhibit Mb-mediated lipid oxidation in cooked washed cod suggests released iron due to heating does not stimulate lipid oxidation. Collectively, these studies suggest that hemin is of primary importance in the onset of lipid oxidation in raw and cooked washed fish muscle. Future work should examine strategies to inhibit hemin-mediated lipid oxidation in muscle food systems. This may be accomplished by maintaining the heme group in the ferrous state or when this is not possible preventing hemin reactant from incorporating into lipid phases.

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