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Studies with Myoglobin Variants Indicate that Released Hemin Is the Primary Promoter of Lipid Oxidation in Washed Fish Muscle

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Variants of sperm whale myoglobin (Mb) were used to assess the mechanism of heme protein-mediated lipid oxidation in washed cod muscle. A myoglobin variant with high hemin affinity (V68T) was an exceptionally poor promoter of lipid oxidation, while a Mb variant with low hemin affinity (H97A) was a potent promoter of lipid oxidation. V68T releases hemin slowly due to the ability of threonine to hydrogen bond with coordinated water and the distal histidine within the heme crevice. H97A rapidly releases hemin because the relatively small alanine residue creates a channel for water to easily enter the heme crevice which weakens the covalent linkage of hemin to the proximal histidine. A variant sensitive to heme degradation (L29F/H64Q) was a weaker promoter of lipid oxidation compared to wild-type Mb. This suggests that degrading the heme ring and releasing iron decreased the ability of Mb to promote lipid oxidation. Free radicals resulting from hemin-mediated decomposition of lipid hydroperoxides have the capacity to propagate lipid oxidation and degrade hemin catalyst. This may explain why heme proteins behave as reactants rather than "catalysts" of lipid oxidation in washed cod. Collectively these studies strongly suggest that released hemin is the critical entity that drives heme protein-mediated lipid oxidation in washed fish muscle.

KEYWORDS: Hemoglobin;myoglobin; rancidity; blood; quality deterioration; muscle food; heme; fish; pork; beef; turkey; free iron; heme degradation; mutagenesis

INTRODUCTION

Lipid oxidation is a primary cause of quality deterioration in muscle foods (*1*). There is still little known about the actual pathway of lipid oxidation, but the heme proteins hemoglobin (Hb) and myoglobin (Mb) are likely contributors (*2*, *3*). 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. There are numerous potential mechanisms by which Hb and Mb can promote lipid oxidation. Each heme protein autoxidizes to the met form which reacts with H_2O_2 or lipid peroxides to generate ferryl heme protein radicals that can abstract a hydrogen atom from polyunsaturated fatty acids and hence initiate lipid oxidation (*4*). Alternatively, displaced hemin or released iron can stimulate lipid oxidation (*5*, *6*).

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 so that the most relevant step related to lipid oxidation is obscured. Site-directed mutagenesis of sperm whale myoglobin has been used to better understand the mechanisms by which heme proteins are pro-oxidative in an effort to develop a stable blood substitute that can bind and release oxygen in vivo (*7*). Site-directed mutagenesis involves altering the myoglobin gene so that alternative amino acids result in the expressed protein compared to the native (wild type) protein. Properties of the heme protein such as oxygen affinity, autoxidation rate, heme affinity, and heme destruction can be modulated by this amino acid substitution approach (*8*−*11*). Our objective was to use various sperm whale myoglobin variants to investigate the mechanisms by which heme proteins promote lipid oxidation in muscle food systems.

MATERIALS AND METHODS

Chemicals. Toluenesulfonyl chloride, dithiothreitol, disodium ethylenediaminetetraacetic acid (EDTA) dihydrate, DNAse I, RNAse A, lysozyme, ferric chloride, hemin chloride, chloramphenicol, streptomycin sulfate, bovine hemoglobin, 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)

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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.

Preparation of Recombinant Myoglobins. Genes coding for the recombinant myoglobins were 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 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 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 *Escherichia coli* (*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 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 then added to the bioreactor (12 L reaction volume, 37 °C) containing 2 mL of 1 M $FeCl_3$ and 50 μL antifoam/L. 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 (*12*) 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 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 (*8*, *13*). 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). Muscle was washed by combining in a 1:3 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. Then, 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 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 15263*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. Myoglobin was diluted in 50 mM Tris, pH 8.0. Approximately 1 mg of dithionite was dissolved in the diluted myoglobin solution. The solution was then bubbled with carbon monoxide gas (Badger Welding, Madison, WI) for 30 s. The myoglobin solution was then 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 (*14*). Calculation of the concentration of myoglobin on a heme basis was performed using the peak absorbance occurring near 420 nm and a standard curve generated from bovine hemoglobin.

Addition of Mb Mutants to Washed Cod Muscle. 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. Washed cod muscle was then added to an amber bottle (30 mL capacity). Water was added so that final moisture content was 90%. Streptomycin sulfate (200 ppm) was added to inhibit microbial growth. Contents were mixed with a plastic spatula for 2 min. The myoglobin of interest was then added to the mixture and mixed for 3 min with a plastic spatula so that the final concentration on a heme basis was 40 μmol/kg washed cod. 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 (*15*, *16*). 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 (WT) sperm whale myoglobin. Samples were held at 2 °C during storage. pH was measured by adding water to 10× the weight of a sample, 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. In some cases the met form of Mb was added to washed cod. 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).

Determination of Thiobarbituric Acid Reactive Substances. Thiobarbituric acid reactive substances (TBARS) were determined according to the modified method of Buege and Aust (*17*). 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 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. A standard curve was constructed using tetraethoxypropane and concentrations of TBARS in samples were expressed as μmol of TBARS/(kg of washed cod).

Determination of Lipid Peroxides. A 5 mL volume of cold chloroform:methanol (1:1) was added to a 300−500 mg muscle sample. The 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 the 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 peroxides (*18*). Then 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 absorbance read at 500 nm. A standard curve was constructed using cumene hydroperoxide, and the concentration of lipid peroxides in the sample was expressed as μmol of lipid peroxides/(kg of muscle).

Heme Destruction Assay. Ferrous sperm whale myoglobin mutants were used in the heme destruction assay. Cuvettes were set up in a model UV-2401 dual beam spectrophotometer (Shimadzu Scientific Instruments Inc.) using a refrigerated six-cell adaptor. First, 10 mM sodium phosphate, pH 5.7, buffer was added to each cuvette. Then diluted H_2O_2 (freshly prepared) was added so the final concentration would be 25 μM. Finally, the sperm whale myoglobin of interest was added to a concentration of 10 μM, with mixing by pipetting immediately after each addition of the myoglobin. Samples were then

scanned from 700 to 400 nm every 5 min for the initial 3.5 h of the experiment and then every 12−24 h afterward. The reference contained only 10 mM sodium phosphate, pH 5.7, buffer. Destruction of the heme group was monitored by a reduction in absorbance at the Soret band (400−430 nm) and increases in absorbance around 670 and 355 nm which are indicative of the formation of heme breakdown products including biliverdin (*19*). Once scission of the heme ring occurs, the iron formerly within the heme ring is released.

Measuring Hemin Loss. The heme of H64Y was removed with methyl ethyl ketone to form a pale, yellowish apoglobin (*20*). A 2 mL aliquot 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 pH reached 2.2. A 2 mL aliquot 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 apoglobin layer from the upper dark organic layer. The apoglobin 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 apoglobin with the tyrosine substitution has a strong affinity for hemin. Thus, hemin that is released from the experimental heme proteins is gathered by the apoglobin. When released hemin from the experimental heme proteins binds to the H64Y apoglobin, a chromophore results that has a strong absorbance at 600 nm (*21*). The experimental heme proteins (i.e. myoglobins without tyrosine at position 64) do not have substantial absorbance at 600 nm, so interference from their spectra does not occur. Hemin loss was measured during 2 °C storage. Samples containing both the H64Y apoglobin and the experimental heme proteins were stored in quartz cuvettes. Igor Pro software (WaveMetrics Inc., Portland, OR) was used to calculate rates of hemin loss by curve fitting using the exponential function. Units expressed along the ordinate axis are optical density values.

Statistical Evaluations. A MIXED procedure of the SAS system was used to analyze data from the storage studies (*22*). Means were separated using the p-diff test. For each treatment, two or three separate reactions were examined during storage. Since 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 (*23*). 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 the 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 since the distance between each time point assayed was in some cases not equivalent.

RESULTS

Hemin loss from H97A and V68T was examined at pH 6.3 during 2 °C storage. H97A indicates that the 97th histidine residue present in wild-type sperm whale myoglobin was substituted with an alanine residue. Similarly valine residue 68 was mutated to threonine. Each variant was converted to the met form prior to conducting the assay. This was done so that the effect of differences in hemin affinity on lipid oxidation could be examined exclusively. If the ferrous forms of each mutant were examined, then differences in the rate of met formation (e.g. myoglobin autoxidation) between V68T and H97A could obscure the effect of hemin affinity differences. Varying the rate of met formation should affect the rate of lipid oxidation because ferrous heme proteins can promote lipid oxidation less or more effectively compared to met heme proteins (*24*, *25*). Negligible hemin release from V68T was detected during 14 days storage at 2 °C, while rapid hemin loss from H97A was observed (**Figure 1**). Hemin release from H97A

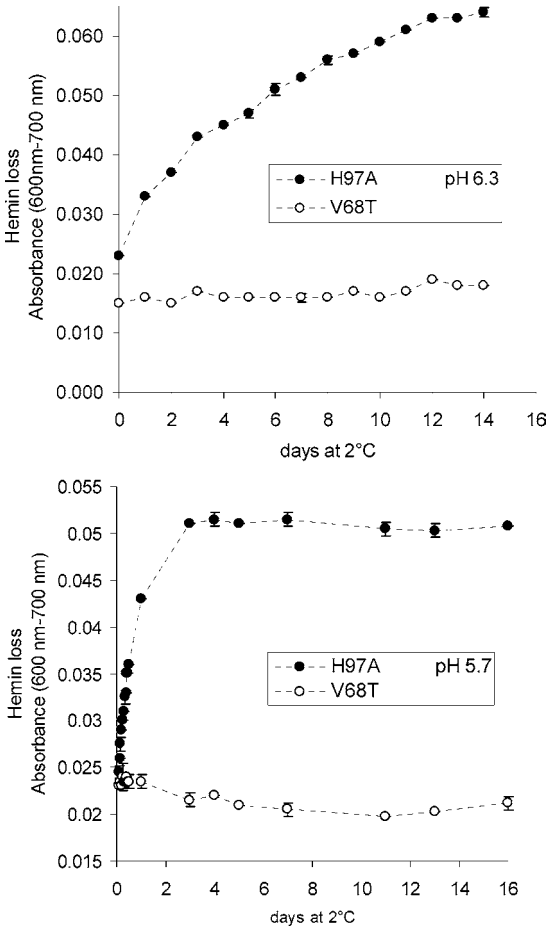

Figure 1. Time courses for hemin dissociation from met H97A and met V68T using the H64Y heme loss reagent during 2 °C storage. Absorbance at 600 nm minus absorbance at 700 nm was determined at each represented time point. pH was 6.3 (top panel) and pH 5.7 (bottom panel). The heme protein concentration was 10 μM (heme basis). Globin concentration was 40 μM. Phosphate and sucrose concentrations were 152 and 456 μM, respectively.

appeared to reach a maximum at around day 12 of storage. At pH 5.7, hemin release from V68T was not detected during 16 days storage at 2 °C (**Figure 1**). Hemin release rate from H97A at pH 5.7 was rapid, reaching a maximum by day 3 of storage. Rates of hemin loss could not be calculated for V68T at pH 6.3 and 5.7 due to the fact that hemin loss was not detectable during the storage period at either pH value. Hemin loss rates from H97A during 2 °C storage was 0.15 day^{-1} at pH 6.3 and 1.43 day^{-1} at pH 5.7. Literature values for relative hemin loss rates from met H97A and met V68T were 39 and 0.04 h^{-1}, respectively, at pH 5 and 37 °C (*10*, *26*). Thus, these variants were good candidates to test the role of hemin affinity in lipid oxidation processes.

Washed cod muscle was used as the substrate for lipid oxidation. Thiobarbituric acid reactive substances were used as an indicator of lipid oxidation products. H97A caused formation of TBARS to occur in washed cod more rapidly than the V68T variant at pH 5.7 ($p < 0.01$) (**Figure 2**). V68T caused almost no TBARS formation during 8 days of storage at 2 °C. H97A developed TBARS as soon as day 1 of storage. The extent of TBARS reached around 90 μmol of TBARS/(kg of tissue) due to H97A compared to 3 μmol of TBARS/(kg of tissue) from V68T. Lipid peroxides were used as another indicator of lipid oxidation (**Figure 3**). H97A was a more potent promoter of lipid peroxide formation compared to V68T based on both the

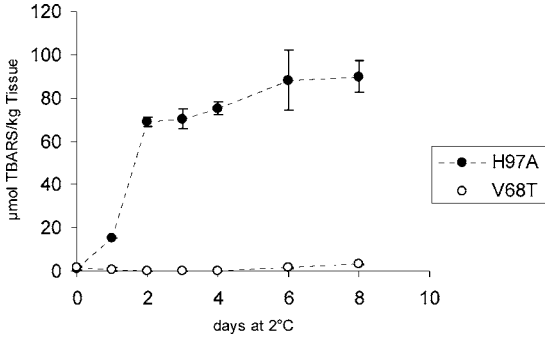

Figure 2. Thiobarbituric acid reactive substances (TBARS) development due to the met forms of H97A and V68T (40 μM) in washed cod muscle (pH 5.7) during 2 °C storage.

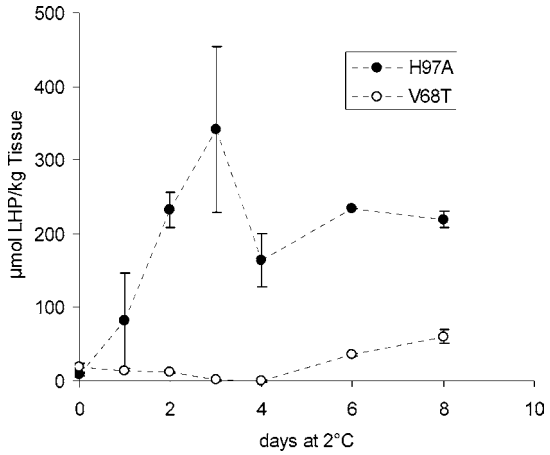

Figure 3. Lipid peroxide development due to the met forms of H97A and V68T (40 μM) in washed cod muscle (pH 5.7) during 2 °C storage.

rate and extent of lipid peroxide formation during 2 °C storage ($p < 0.01$). These data indicate that the myoglobin mutant with high heme affinity was an exceptionally poor promoter of lipid oxidation, while the mutant with low heme affinity was a potent promoter of lipid oxidation.

To further substantiate the role of hemin loss in lipid oxidation processes, the ability of wild-type myoglobin to oxidize washed cod lipids was examined in addition to H97A and V68T. Wild-type myoglobin has an intermediate rate of hemin loss compared to H97A and V68T (*10*). Thus it was expected that WT Mb would have an intermediate ability to oxidize lipids in washed cod muscle. H97A promoted TBARS formation more effectively than WT Mb ($p < 0.05$), while WT Mb promoted TBARS more effectively than V68T ($p < 0.01$) (**Figure 4**).

Another parameter that could either accelerate or slow lipid oxidation processes is destruction of the heme ring. If indeed intact hemin is the active form of myoglobin that promotes oxidation of lipids, heme destruction would inhibit lipid oxidation. On the other hand, heme destruction liberates iron from the heme ring (*27*, *28*). If the released iron is pro-oxidative then heme destruction should accelerate lipid oxidation processes. Wild-type sperm whale myoglobin and the double mutant L29F/H64Q, which have different susceptibilities to heme destruction in the presence of hydrogen peroxide (H_2O_2), were examined. H_2O_2 is produced during post-mortem storage of muscle tissue (*29*) and thus is relevant as a component in the washed cod muscle model system. The double mutant is noted for being especially sensitive to heme destruction in the presence of H_2O_2 compared to wild-type sperm whale myoglobin at a Mb:H_2O_2 ratio of 1:2.5 (*11*). Our measurements in aqueous solutions indicated that ferrous L29F/H64Q underwent rapid

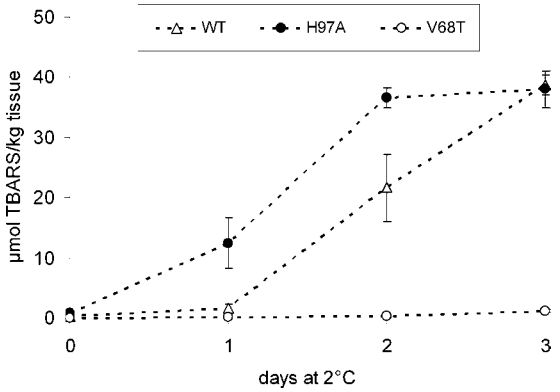

Figure 4. Ability of H97A, V68T, and wild-type Mb (40 μM, pH 5.7) to oxidize washed cod lipids based on TBARS formation during 2 °C storage. Each heme protein was in the met form prior to addition to washed cod.

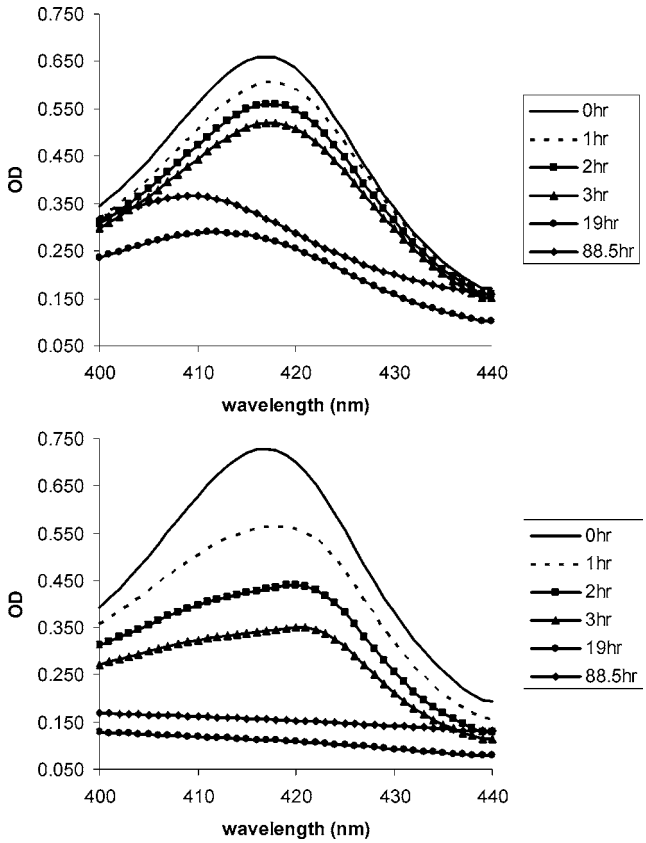

Figure 5. Heme destruction of wild-type sperm whale myoglobin (top panel) and L29F/H64Q (bottom panel) at pH 5.7 (25 μM H_2O_2 and 10 μM Mb) during 2 °C storage. Each heme protein was in the ferrous state upon addition to the buffer.

heme destruction compared to WT Mb during 2 °C storage at pH 5.7 in the presence of H_2O_2 based on a decrease in absorbance in the Soret band. (**Figure 5**).

Further evidence of heme destruction can be seen by examining spectra from 700 to 300 nm. The double mutant in the presence of H_2O_2 exhibits peaks at 674 and 350 nm, while WT Mb mutant does not after 2 days of storage at 2 °C (**Figure 6**). These peaks are evidence of biliverdin production which results due to destruction of the heme ring (*30*).

The ability of ferrous WT Mb and L29F/H64Q to oxidize lipids in washed cod was also assessed. Ferrous WT Mb in the presence of H_2O_2 caused formation of TBARS to occur more rapidly in washed cod compared to the ferrous L29F/H64Q double mutant ($p < 0.01$) (**Figure 7**). By day 3, there was

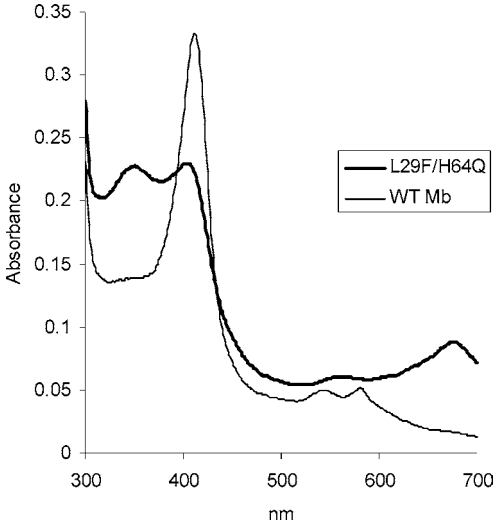

Figure 6. Absorbance spectra of wild-type sperm whale myoglobin and L29F/H64Q at pH 5.7 (25 μM H_2O_2 and 10 μM Mb) after 2 days of 2 °C storage. Each heme protein was in the ferrous state upon addition to the buffer.

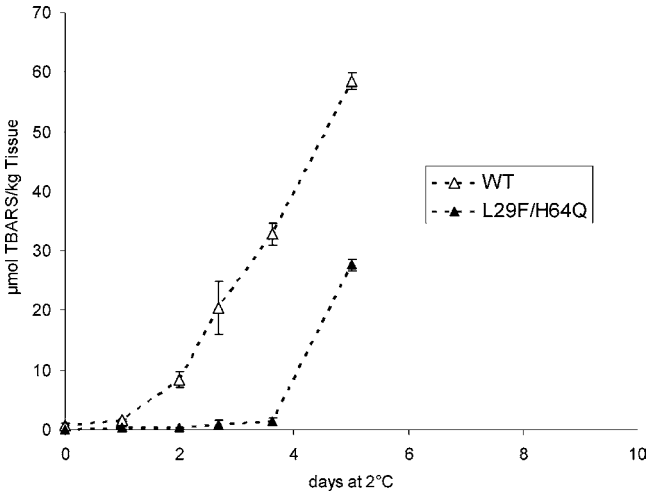

Figure 7. TBARS formation in washed cod muscle containing wild-type sperm whale myoglobin and L29F/H64Q, pH 5.7 (100 μM H_2O_2 and 40 μM Mb), during 2 °C storage. Each heme protein was in the ferrous state upon addition to the washed cod.

extensive TBARS formation resulting from WT Mb whereas almost no TBARS formation occurred due to the double mutant. By day 5, substantial amounts of TBARS formation was observed in both heme proteins, albeit greater in WT Mb.

The ability of WT Mb to promote lipid oxidation was assessed at pH 5.7 and 6.3 during 2 °C storage (**Figure 8**). The ferrous form of the myoglobin was examined. Lipid oxidation occurred more rapidly at pH 5.7 compared to pH 6.3 ($p < 0.05$).

DISCUSSION

These studies indicated that a myoglobin variant with high hemin affinity (V68T) was an exceptionally poor promoter of lipid oxidation while a Mb variant with low hemin affinity (H97A) was a potent promoter of lipid oxidation (**Figures 1**−**4**). The high hemin affinity of V68T is due to the ability of threonine to hydrogen bond with liganded H_2O, the imidazole group of the distal histidine and the main chain carbonyl group of the distal histidine (*31*). The native valine cannot hydrogen bond due to its nonpolar character (**Figure 9**). Anchoring the

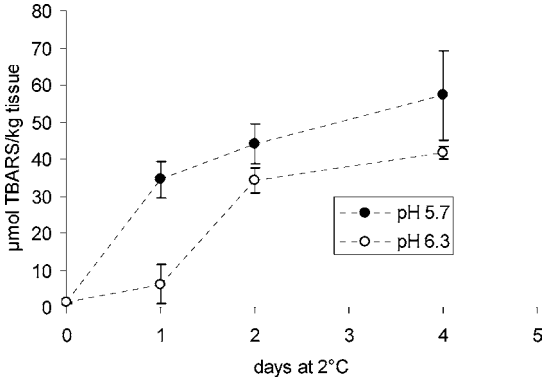

Figure 8. Effect of pH on TBARS formation in washed cod containing wild-type sperm whale myoglobin during 2 °C storage.

hemin group in the globin due to the multiple hydrogen bonds in V68T effectively prevented myoglobin-mediated lipid oxidation in washed cod muscle. The low hemin affinity of H97A compared to wild-type myoglobin is due to the small size of the alanine residue which allows water to enter the heme crevice at a rapid rate compared to the more bulky histidine residue in wild-type myoglobin (*32*). Hydration weakens the covalent bond between the proximal His^{93} (F8) and Fe^{3+} that is located within the porphyrin ring (**Figure 9**).

Once hemin is liberated from the globin, potential promoters of lipid oxidation are the intact hemin molecule or iron that may be subsequently released from hemin upon destruction of the heme ring. Iron liberated from the heme protein decreased the rate of lipid oxidation in washed cod. This is based on the fact that L29F/H64Q was a weaker promoter of lipid oxidation as compared to wild-type Mb (**Figure 7**) and L29F/H64Q was more susceptible to heme destruction than WT Mb (**Figures 5 and 6**).

Based on these findings, the mechanism of Mb-mediated lipid oxidation begins to be elucidated. First of all, hemin dissociates from the globin during storage of post-mortem muscle. Hemin concentrations in mackerel light muscle increased around 3-fold during iced storage (*33*). Following release of hemin from the globin, hemin is proposed to intercalate within phospholipid membranes due to hydrophobic attractions. Also the propionate groups of hemin can bond with phospholipid headgroup amines by electrostatic interactions (*34*). Preformed lipid hydroperoxides have been determined in phospholipid membranes in vivo (*35*). Hemin can react with lipid hydroperoxide to form alkoxyl radical and ferryl−hydroxo complex (reaction 1) (*36*). Ferryl−hydroxo complex can react with another lipid hydroperoxide to form a peroxyl radical and regenerate hemin (reaction 2):

$$\text{hemin}(3+) + \text{LOOH} \rightarrow \text{LO}\bullet + \text{hemin}(4+)-\text{OH} \quad \text{(reaction 1)}$$

$$\text{hemin}(4+)-\text{OH} + \text{LOOH} \rightarrow \text{LOO}\bullet + \text{hemin}(3+) + \text{H}_2\text{O} \quad \text{(reaction 2)}$$

Alkoxyl and peroxyl radicals are capable of abstracting a hydrogen atom from a polyunsaturated fatty acid which will stimulate the lipid oxidation processes. Other mechanisms by which alkoxyl, peroxyl, alkyl, and hemin radicals form from reaction of hemin with lipid hydroperoxides have been described (*5*, *37*).

Previously it was shown that 0.07, 0.56, 2.0, and 6.4 μM hemoglobin added to washed cod caused the extent of TBARS formation during storage to be around 2.3, 8.4, 29, and 70 μmol/(kg of tissue), respectively (*16*). This indicated that hemoglobin

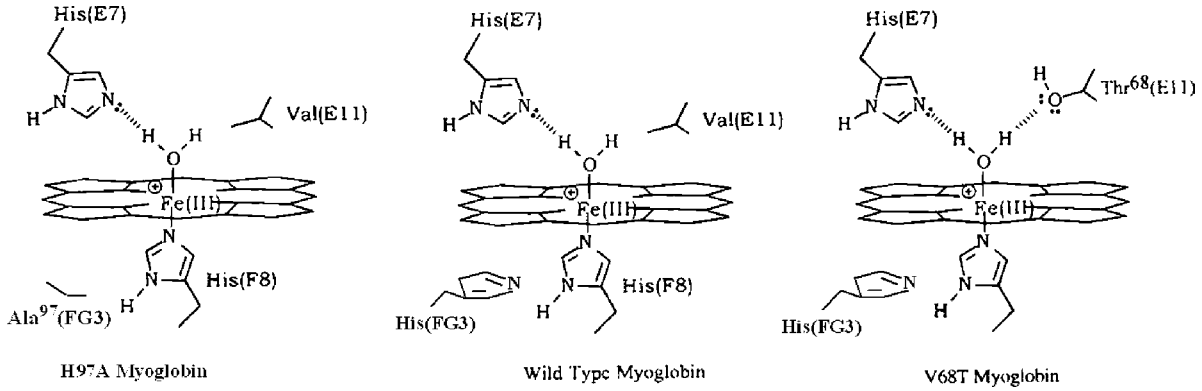

Figure 9. Effects of V68T and H97A substitutions on hemin affinity. Hydrogen bonding of coordinated water to distal histidine stabilizes hemin in WT Mb. In V68T, the distal histidine and threonine hydrogen bond with coordinated water to increase hemin affinity. In H97A, substitution with the smaller alanine increases access of water to the heme crevice, which weakens the covalent bond between the proximal histidine and the iron atom within the hemin ring.

was acting as a reactant, not as a true "catalyst" of lipid oxidation. This may be because a portion of alkoxyl and peroxyl radicals resulting from hemin-mediated decomposition of lipid hydroperoxides degrade hemin, while the remaining portion of free radicals stimulate a finite amount of lipid oxidation depending on the quantity of hemin present. Reactions 1 and 2 above indicate a catalytic cycle that would be disrupted by destruction of the heme ring. Alkoxyl and peroxyl radicals formed by hemin-mediated decomposition of lipid hydroperoxides have been implicated in heme degradation (*38*).

Our results using a Mb variant sensitive to heme destruction support the theory that heme destruction decreases the ability of Mb to promote lipid oxidation (**Figure 7**). It may seem odd that increasing the pool of released iron through heme destruction would decrease lipid oxidation. Iron is known to effectively stimulate lipid oxidation processes. It may be that heme degradation resulting from the reaction of L29F/H64Q with H_2O_2 caused the released iron to remain in the aqueous phase preventing reaction of iron with lipid components. Further, chelating agents such as ADP have been found to enhance the ability of iron to promote lipid oxidation (*39*). Our washed cod system was devoid of ADP. However, when hemin intercalates into phospholipid membranes and reacts with preformed lipid hydroperoxides, the production of alkoxyl and peroxyl radicals will destroy the heme ring which can deliver iron into the membrane. Thus the ability of iron delivered by hemin into lipid phases must also be considered.

Nearly all the iron in hemin was released as lipid oxidation progressed in lipoproteins exposed to hemin (*40*). These authors also found that adding iron to the lipoprotein preparation did not stimulate lipid oxidation. From these results it was suggested that iron released from hemin due to interaction with lipid hydroperoxides promoted lipid oxidation in lipoproteins because the iron was likely delivered by hemin into hydrophobic compartments that are inaccessible to externally added free iron or hydrophilic iron chelates. An oil-soluble metal chelator would separate effects due to released iron compared to hemin. Unfortunately, oil-soluble metal chelators currently available (e.g. lazaroids) have free radical scavenging capacities that should also scavenge free radicals derived from hemin-mediated lipid oxidation.

To resolve the potential roles of hemin compared to iron delivered by hemin into lipid phases, the mechanism of iron-mediated lipid oxidation should be considered. Adding iron and iron-chelates to washed cod does not promote lipid oxidation (*41*) which may be due to failure to incorporate the iron into the lipid phase. Using sarcoplasmic reticulum from cod as substrate, added ferrous iron (Fe^{2+}) promoted lipid oxidation while ferric iron (Fe^{3+}) was not effective (*42*). Hemin which contains ferric iron will deliver Fe^{3+} into the membrane. Further, it was found that increasing concentrations of Fe^{2+} increased the extent of lipid oxidation in sarcoplasmic reticulum but also increased the lag phase prior to lipid oxidation, which was attributed to the ability of Fe^{2+} to reduce oxidized tocopherol in the membrane (*42*). Increasing concentrations of Hb in washed cod did not increase the lag phase prior to lipid oxidation (*16*). These results suggest that iron which is released from hemoglobin or myoglobin is not responsible for lipid oxidation in washed cod; rather lipid oxidation is mediated by hemin.

Using low-density lipoprotein (LDL) as substrate, lipid oxidation by hemoglobin was believed to occur by hemin displacement from methemoglobin (*43*). These researchers found that hemin transfer from metHb to LDL was inhibited more effectively by haptoglobin 1−1 compared to haptoglobin 2−2, which explained why haptoglobin 1−1 more effectively inhibited hemoglobin-mediated lipid oxidation.

Hemin has also been implicated in erythrocyte lysis (*44*). Erythrocyte lysis is likely a critical step in promoting lipid oxidation in muscle foods for a number of reasons. First, hemoglobin is diluted upon lysis which accelerates hemin release. Dilution causes the formation of dimers and monomers from the tetrameric hemoglobin (*45*). Subunits are known to have lower heme affinity than tetramers (*46*). Second, lysis physically causes decompartmentalization of the red blood cell so that hemoglobin can interact with various membrane lipids in the tissue that were segregated prior to lysis. Third, the concentration of ATP is around 25 times higher in fresh muscle compared to whole blood (*47*, *48*). Since breakdown of ATP post-mortem decreases pH, the pH will be higher in the blood fractions. Hence there will be a decrease in pH when Hb leaves the cocoon of the erythrocyte and then mixes with muscle components. Decreasing pH accelerates Hb subunit formation, met heme protein formation, and hemin loss (*10*, *49*, *50*) (**Figure 1**). Decreasing pH also accelerated lipid oxidation in washed cod mediated by sperm whale Mb (**Figure 8**). Previously it was shown that lysis of erythrocytes accelerated hemoglobin-mediated lipid oxidation in washed cod muscle (*16*). Physical processes during food manufacturing (e.g. grinding and mincing) will further facilitate rupture of capillaries and erythrocytes which will promote hemin-mediated lipid oxidation.

Inhibiting autoxidation of the ferrous heme protein is a viable approach to inhibit hemin-mediated lipid oxidation. This is

because heme is anchored in the globin of ferrous heme protein approximately 60 times stronger compared to met heme protein (*26*). Ascorbate, glutathione, and enzymic systems present in the erythrocyte reduce met hemoglobin to ferrous hemoglobin (*51*). Hemopexin, a heme binding protein, inhibited heme and Hb-mediated lipid oxidation in lipoproteins (*52*). The proposed mechanism was conversion of oxyHb to ferrylHb followed by a comproportionation reaction yielding metHb from which hemin was transferred to hemopexin. Hemopexin also effectively inhibited hemin-mediated lipid oxidation in microsomes (*53*). Heme oxygenase cleaves the heme ring to form biliverdin, iron, and carbon monoxide (*54*). The ability of heme oxygenase to protect against oxidative damage caused by hemin has been reported (*55*).

Loss of hemin from the myoglobins in our studies was measured in aqueous solutions that did not contain lipids (**Figure 1**). Hemin loss is likely to occur more rapidly in washed cod than in solution. This is because a strong binding of hemin to lipid vesicles was demonstrated by the failure of hemin to interact with globin in the presence of phosphatidylserine vesicles (*56*). A method to measure hemin release in the presence of lipids is based on a modified Langmuir trough lipid monolayer technique (*57*). In this system surface pressure in the monolayer increases as hemin is released from oxidized hemoglobin. Using Hb A, S, F, and E, it was found that there was an increase in membrane lipid peroxidation with increasing hemin release rate (Hb E > F > S > A). Hemoglobin S and E are hemoglobin variants found in humans, while Hb A and F represent typical adult and fetal hemoglobin, respectively. Hb S and E have been associated with sickle cell anemia and β-thalassemia pathologies, respectively.

Our studies utilized sperm whale myoglobin variants as the experimental heme proteins. It should be noted that both myoglobin and hemoglobin are present in muscle tissues. Hemoglobin is more prevalent than myoglobin in most muscles from birds and fish (*58*, *59*). This is relevant because hemoglobin has been shown to have lower hemin affinity compared to myoglobin (*46*, *60*). Thus muscles rich in hemoglobin compared to myoglobin should be more susceptible to lipid oxidation due to the lower hemin affinity of hemoglobins.

Based on the above findings, our proposed pathway for Mb-mediated lipid oxidation in washed cod muscle is illustrated in **Figure 10**. Hemin release from Mb is a rate-limiting step based on the fact that mutants with high hemin affinity were weak promoters of lipid oxidation compared to mutants with low hemin affinity. Hemin release from metMb occurs more rapidly at reduced pH presumably due to increased protonation of the proximal His^{93} at acidic conditions, which weakens the linkage of the globin to the heme iron (*10*). Decreasing pH accelerates Mb-mediated lipid oxidation in washed cod due to not only more rapid hemin loss at acidic conditions but also ferrous Mb autoxidizes to metMb faster at reduced pH which weakens the heme-globin linkage. Hemin release will be accelerated in the event that access of water to the heme crevice is enhanced as demonstrated by H97A. Hydration weakens the globin-hemin linkage. If water can be stabilized in the heme crevice by hydrogen bonding, heme release is inhibited as demonstrated by V68T. Once released, hemin can react with preformed lipid hydroperoxides to form a wide array of lipophilic free radicals. These radicals not only promote lipid oxidation by abstracting hydrogen atoms from polyunsaturated fatty acids but also deactivate hemin by degrading the heme ring. The ability of these radicals to destroy the heme ring causes hemin to act as a reactant rather than a "catalyst" of lipid oxidation in washed cod.

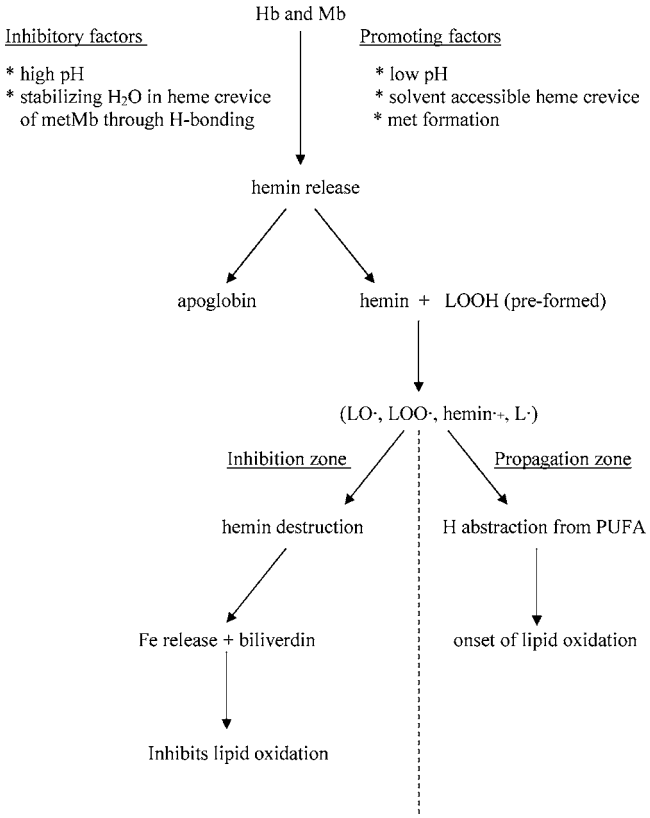

Figure 10. Proposed pathway for Mb-mediated lipid oxidation in washed cod muscle.

Although iron released from myoglobin decreased the ability of the heme protein to promote lipid oxidation, iron delivered by hemin into lipid phases should be further explored. In our studies, H_2O_2 caused the heme destruction which may have released iron into the aqueous phase. An oil-soluble metal chelator without the ability to scavenge free radicals generated by hemin-mediated decomposition of lipid hydroperoxides could be used to definitively differentiate between hemin action and that of iron delivered by hemin into lipid phases. In any event, our studies strongly suggest that released hemin is the critical entity that drives heme protein-mediated lipid oxidation in washed fish muscle. Future work should examine inexpensive strategies to either inhibit release of hemin from the globin or prevent hemin from intercalating within membrane phospholipids of muscle foods.

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