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Phenylalanine Substitution at Site B10 (L29F) Inhibits Metmyoglobin Formation and Myoglobin-Mediated Lipid Oxidation in Washed Fish Muscle: Mechanistic Implications

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Wild-type sperm whale myoglobin (WT Mb) and L29F mutant were used to examine the effect of metMb formation rate on Mb-mediated lipid oxidation in washed cod muscle. MetMb formation was 15-fold slower in L29F compared to WT Mb at 2 °C (pH 5.7). The electrostatic interaction of bound O_2 and the partial positive edge of the phenyl ring of phenylalanine^{29} inhibits deoxyMb-mediated metMb formation and may displace protons that promote oxyMb oxidation. Ferrous L29F was a poor promoter of lipid oxidation in washed cod during extended storage, whereas ferrous WT Mb oxidized the substrate readily. This diminishes a role for free radicals produced by the reaction of oxyMb with peroxides. MetL29F was an effective promoter of lipid oxidation. Mb mutants with low hemin affinity (H97A) were better promoters of microsomal lipid oxidation than mutants with higher hemin affinity (WT Mb and V68T). The much higher heme affinity of oxyMb compared to metMb partly explains the poor ability of ferrous L29F to oxidize lipids in washed cod at post-mortem pH during 2 °C storage. The relative roles of cross-linked Mb and hypervalent Mb species to promote lipid oxidation in washed cod at post-mortem pH are discussed.

KEYWORDS: Quality deterioration; rancidity; hemoglobin; autooxidation; heme dissociation; hemin; lipid oxidation

INTRODUCTION

The heme proteins myoglobin (Mb) and hemoglobin (Hb) are capable of promoting lipid oxidation during storage of muscle foods. Formation of sufficient amounts of lipid oxidation products results in off-odors and off-flavors (e.g., rancidity in raw muscle and warmed-over flavor in cooked products). The mechanism by which Mb and Hb promote lipid oxidation in muscle tissue remains poorly understood. This is due to the fact that multiple forms of each heme protein are present simultaneously during storage. For instance, oxyMb and deoxyMb concentrations are being depleted as metMb concentrations increase during storage. Furthermore, ferryl Mb and cross-linked Mb formation occurs during storage as well as release of the hemin porphyrin moiety (*1*). Release of iron atoms from the porphyrin also occurs as well as hemichrome formation during storage (*2*). Delineating the effect of each potentially pro-oxidative form of Hb and Mb can be addressed through the use of site-directed mutagenesis in which the Mb or Hb gene is manipulated so that alternative amino acids are present when the protein is expressed and purified from a bacterial host (*3*). For example, heme destruction that releases iron atoms from the porphyrin ring occurs relatively rapidly in the Mb mutant L29F/H64Q when hydrogen peroxide is present (*4*). In this case, the leucine at position 29 is substituted with phenylalanine and the distal histidine at position 64 is substituted with glutamine. This mutant poorly promoted lipid oxidation in washed cod muscle containing added hydrogen peroxide, which suggested that heme destruction and the resulting iron atoms that were released (in the aqueous phase) were poorly reactive in the washed cod matrix (*5*).

Examining a Mb mutant with a slow autooxidation rate (e.g., slow rate of metMb formation) would be useful because the reaction by which oxyHb reacted with preformed lipid hydroperoxides (LOOH) to promote lipid oxidation via formation of alkoxyl radical has been described (eq 1) (*6*).

$$\mathrm{LOOH} + \mathrm{O_2Hb{-}Fe^{(2+)}} \rightarrow \mathrm{LO^{\bullet}} + \mathrm{OH^-} + \mathrm{O_2} + \mathrm{MetHb{-}Fe^{(3+)}} \quad (1)$$

Mb mutants that remain in the oxy form for a relatively long period of time will have more of an opportunity to react with LOOH in washed cod muscle as described in eq 1. Other lines of research point to the pro-oxidative capacity of metMb. MetMb reacts with LOOH producing alkoxyl radical (*7*) and ferryl protein radical (*8*), each of which can abstract hydrogen atoms from polyunsaturated fatty acids to further propagate lipid oxidation. The hemin affinity of metMb is around 60-fold lower than the heme affinity in reduced forms of Mb (oxyMb and deoxyMb) (*9*). This is relevant because hemin released from the

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globin is a potent promoter of lipid oxidation, producing alkoxyl and peroxyl radicals (eqs 2 and 3) (*10*).

$$\mathrm{LOOH} + \mathrm{hemin}^{(3+)} \rightarrow \mathrm{LO}^{\bullet} + \mathrm{hemin}^{(4+)}\text{–OH} \qquad (2)$$

$$\mathrm{LOOH} + \mathrm{hemin}^{(4+)}\text{–OH} \rightarrow \mathrm{LOO}^{\bullet} + \mathrm{hemin}^{(3+)} \qquad (3)$$

Previously it was shown that Mb mutants with low hemin affinity were better promoters of lipid oxidation in washed cod at pH 5.7 compared to Mb mutants with high hemin affinity (*5*). Our primary objective was to examine the relative importance of oxyMb compared to metMb-mediated lipid oxidation using Mb mutants that have widely varying metMb formation rates. We have also further examined the ability of Mb mutants with widely varying hemin loss rates to promote lipid oxidation at postmortem pH values.

MATERIALS AND METHODS

Chemicals. Toluene sulfonyl chloride, dithiothreitol, disodium 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) was obtained from Promega (Madison, WI). All other chemicals used were of 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 Prof. 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 *Escherichia 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 (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 for 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 of antifoam per liter. 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 (*3*) 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 78800 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 (*11*, *12*). Finally, the myoglobins were concentrated to 0.5–1.0 mM (heme basis), snap frozen in liquid nitrogen, and stored either in liquid nitrogen or at −80 °C.

MetMb Formation. Loss of absorbance at 581 nm was used to assess the rate of myoglobin autooxidation (*13*). 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 autooxidation rates (k_{ox}) using the linear function. The half-time ($3t_{1/2}$) was calculated using the following equation: $t_{1/2} = \ln(2)/k_{ox}$.

Preparation of Washed Cod Muscle. Cod fish (*Gadus morhua*) fillets, determined to be fresh on the basis of 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 KitchenAid, Inc. (St. Joseph, MI) KSM90WW household mixer equipped with a 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. 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 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 for 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.

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 the 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 the 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 was then added and mixed for 3 min with a plastic spatula so the final concentration on heme basis was 40 μmol/kg of tissue. Zero-time samples were taken, and reaction vessels were stored on ice for the duration of the experiment.

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to the modified method of Buege and Aust (*14*). 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. Around 100 mg of sample was added to 1.2 mL of the reagent. After heating at 65 °C for 40 min, the samples were cooled at 4 °C for 60 min. Samples were then centrifuged at 1600*g* for 5 min. Absorbances of supernatants were measured at 532 nm. A standard curve was constructed using tetraethoxypropane, and concentrations of TBARS in samples were expressed as micromoles of TBARS per kilogram of reaction solution or per kilogram of washed cod.

Determination of Lipid Peroxides. A 5 mL volume of cold chloroform/methanol (1:1) was added to a 200 μL microsome sample in a glass test tube. Sample was vortexed in the solvent for 15 s and was transferred to a screw-cap glass centrifuge tube. The test tube was then rinsed by vortexing 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 (*15*). Then 25 μL of 3.94 M ammonium thiocyanate and 25 μL of 18 mM iron(II) chloride were added to the tube, with 4 s of vortexing after each addition. The samples were 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 sample was expressed as micromoles of lipid hydroperoxides per kilogram of reaction solution.

Preparation of Trout Microsomes. The method of microsome preparation was adapted from that of McDonald et al. (*16*). Whole rainbow trout (*Oncorhynchus mykiss*), immobilized by blunt cranial trauma, were obtained from the University of Wisconsin—Madison (Water Science and Engineering Laboratory) and stored on ice at 4 °C overnight. The following day, the trout were filleted and cut into small pieces. A 135 g amount of the pieces was combined with 540 mL of 0.12 M KCl−5 mM histidine, pH 7.3, buffer and blended for three 10 s pulses in a Waring Commercial Blender (Waring Commercial, New Hartford, CT). Blendate was then homogenized for three 45 s pulses with a Polytron type PT 10/35 probe (Brinkmann Instruments). Homogenate was centrifuged at 21400*g* for 30 min at 4 °C. The supernatant was recovered and filtered through four layers of cheesecloth. Filtered supernatant was centrifuged at 105000*g* for 60 min at 4 °C. Pellets were resuspended in 0.6 M KCl−5 mM histidine, pH 7.3, buffer for a total of 60 mL of crude microsome preparation. Crude preparation was centrifuged at 105000*g* for 60 min at 4 °C. Pellets were resuspended in 0.12 M KCl−5 mM histidine, pH 7.3, buffer using a 15 mL Wheaton dounce tissue grinder with a loose pestle (Wheaton Science Products, Millville, NJ) for a volume of 26 mL of final microsome preparation. The preparation was aliquoted in 1 mL volumes, snap frozen in liquid nitrogen, and stored at −80 °C.

Lipid Oxidation in Microsomes. Protein concentration of microsomes was determined using a modified Lowry method (*17*). A final microsomal protein concentration of 2.43 mg/mL was used. The sperm whale Mb mutant of interest was examined at a final concentration of 10 μM Mb. Reactions were incubated at 37 °C (pH 5.7) in 25 mM sodium phosphate buffer and 20 mM NaCl containing 1 mM histidine.

Statistical Evaluations. A MIXED procedure of the SAS system was used to analyze data from the storage studies (*18*). Means were separated using the *p*-diff test. For each treatment, 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 (*19*).

RESULTS

The myoglobin concentration chosen for the lipid oxidation studies was 40 μmol/kg of tissue (wet weight). This concentration is in the range of heme pigment concentrations found in post-mortem muscle. The total heme pigment in muscles from chicken, pork, beef, trout, and mackerel ranged from 8.3 to 974 μmol/kg of tissue (heme basis) (*20*, *21*). Previous studies indicated that 40 μmol of Mb/kg of tissue caused a substantial amount of lipid oxidation to occur in washed cod muscle after 1−2 days of 2 °C storage at pH 5.7 when using wild-type sperm whale myoglobin (WT Mb) (*5*). This allows for the determination of antioxidative or pro-oxidative abilities of various Mb mutants in the washed cod relative to WT Mb.

L29F was previously reported to autooxidize 10-fold more slowly compared to WT Mb during storage at 37 °C and pH 7.0 (*13*). In L29F the leucine residue at position 29 (the 10th residue along the B-helix) has been replaced with a phenylalanine residue. We wished to measure the metMb formation rate of WT Mb and L29F at conditions present in post-mortem muscle tissue. The L29F mutant autooxidized 15-fold more slowly compared to WT Mb at 2 °C and pH 5.7 (**Table 1**). Samples contained 3 mmol of superoxide dismutase and catalase per mole of heme to remove any superoxide and hydrogen peroxide that was produced during incubation, which can confound metMb spectra.

The fact that L29F remains in the reduced state much longer relative to WT Mb presented an opportunity to investigate the role of metMb formation rate on Mb-mediated lipid oxidation. The storage period was 10 days at 2 °C (pH 5.7). Lipid peroxides were measured periodically during storage as an indicator of primary lipid oxidation products. Lipid peroxide formation was negligible in washed cod that did not contain added Mb (**Figure 1**). Ferrous L29F was not capable of stimulating lipid peroxide formation in washed cod during the entire storage period (**Figure 1**). Ferrous WT Mb, on the other hand, was capable of stimulating lipid peroxide formation after only 1 day of storage. Lipid peroxide values reached a maximum value around day 7 and then declined on the 10th day of storage (**Figure 1**).

Table 1. Autooxidation Rate (k_{ox}) and Half-Time ($t_{1/2}$) for Wild-Type Sperm Whale Myoglobin and L29F (25 μM, Heme Basis) during 2 °C Storage at pH 5.7[a]

myoglobin	k_{ox} (day^{-1})	$t_{1/2}$ (days)
WT Mb	0.076 ± 0.005	9.1 ± 0.6
L29F	0.005 ± 0.001	137.5 ± 11

[a] k_{ox} is equivalent to the probability of decay of a single molecule to the ferric form in 1 day. $t_{1/2}$ is the time required for half of the molecules to oxidize. L29F indicates that the leucine residue in native sperm whale myoglobin has been substituted with a phenylalanine residue.

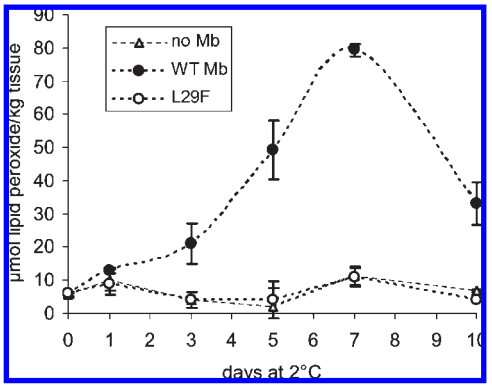

Figure 1. Ability of ferrous L29F and ferrous WT Mb (40 μM, pH 5.7) to oxidize washed cod lipids based on lipid peroxide formation during 2 °C storage. Each heme protein was in the reduced, ferrous form prior to addition to washed cod.

TBARS were also measured during 10 days of storage as an indicator of secondary lipid oxidation products. TBARS formation was negligible in washed cod that did not contain added Mb (**Figure 2**). TBARS values increased only slightly in samples containing added ferrous L29F during the 10 day storage period (**Figure 2**). The maximal TBARS values in samples containing ferrous L29F were < 10 μmol/kg of washed cod throughout the storage period. However, ferrous WT Mb effectively stimulated the formation of TBARS during storage in a roughly linear fashion over the first 8 days of storage, reaching a maximal TBARS level of around 55 by day 8 (**Figure 2**).

Visual observations were made when sampling for lipid oxidation products during the 10 day storage period, indicating that L29F samples remained a reddish hue throughout the 10 day storage period. This indicated that little oxidation of the pigment occurred during storage. WT Mb samples, on the other hand, had almost no detectable red color after only 2 days of storage.

The ability of WT Mb and L29F in their met forms to promote lipid oxidation in washed cod was also examined at pH 5.7. Almost no lag phase and a rapid onset of TBARS formation were observed when metWT Mb and metL29F were added separately to washed cod muscle (**Figure 3**). Lipid oxidation due to metWT Mb was slightly more rapid compared to that due to metL29F.

Previously it was shown that lipid oxidation in washed cod was more rapid in Mb mutants with low hemin affinity compared to

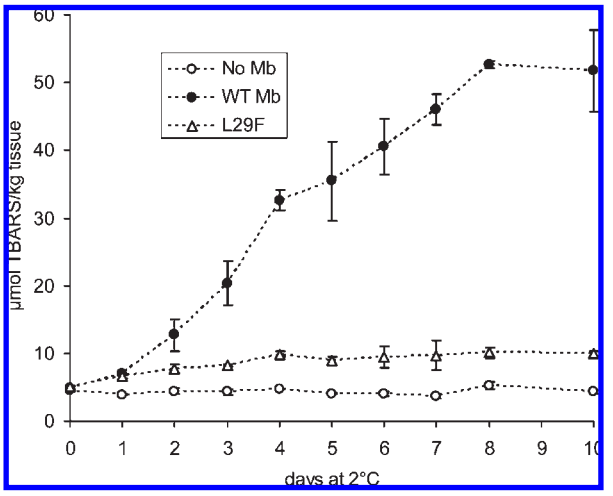

Figure 2. Ability of ferrous L29F and ferrous WT Mb (40 μM, pH 5.7) to oxidize washed cod lipids based on thiobarbituric acid reactive substances (TBARS) formation during 2 °C storage. Each heme protein was in the reduced, ferrous form prior to addition to washed cod.

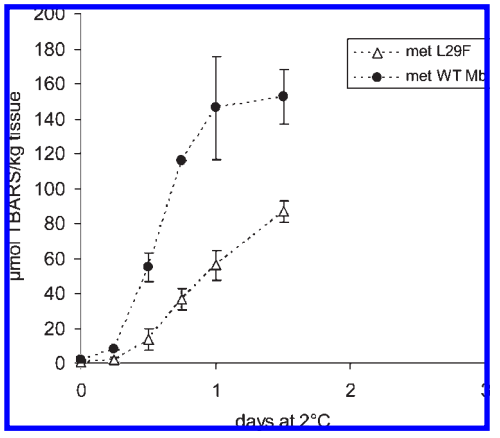

Figure 3. Ability of metL29F and metWT Mb (40 μM, pH 5.7) to oxidize washed cod lipids based on thiobarbituric acid reactive substances (TBARS) formation during 2 °C storage.

mutants with higher hemin affinity (*5*). We wished to examine these same mutants in trout microsomes, a more highly purified lipid substrate. H97A and V68T were compared to WT Mb. Literature values for relative hemin loss rates from metH97A and metV68T were 39 and 0.04 h^{-1}, respectively, at pH 5 and 37 °C, whereas WT Mb has an intermediate hemin affinity (1.0 h^{-1}) (*22*, *23*). We have shown that H97A released nearly all of its hemin at pH 5.7 and 2 °C within 3 days (0.06 h^{-1}), whereas no detectable hemin release from V68T was detected during 16 days of storage (*5*). Thus, H97A, V68T, and WT Mb are good candidates to test the role of hemin affinity in microsomal lipid oxidation. 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 Mb was converted to the met form prior to addition to the microsomes and measurement of lipid oxidation products during storage. 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 autooxidation) between the Mbs could obscure the effect of hemin affinity differences. Within 1 h, H97A caused TBARS values to reach 28 μM,

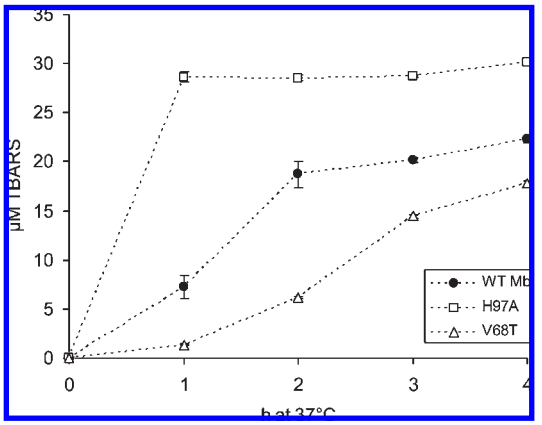

Figure 4. Ability of metH97A, metV68T, and metWT Mb (10 μM, pH 5.7) to oxidize microsomal lipids based on TBARS formation during 37 °C storage.

whereas V68T and WT Mb caused TBARS values to reach 2 and 7 μM, respectively, in the first 60 min (**Figure 4**). TBARS values were significantly higher in H97A compared to WT, and WT values were significantly greater than V68T ($P < 0.05$). These data indicate that the Mb mutants with elevated hemin affinity were poor promoters of lipid oxidation, whereas mutants with low hemin affinity were relatively potent promoters of microsomal lipid oxidation.

DISCUSSION

This work demonstrates that inhibiting metMb formation essentially prevented Mb-mediated lipid oxidation in washed cod muscle at pH 5.7 during 2 °C storage. This was accomplished using the L29F mutant, which has a 15-fold slower autooxidation rate compared to WT Mb at pH 5.7. The remarkable inability of ferrous L29F to promote lipid oxidation can be partly attributed to the 60-fold higher porphyrin affinity of ferrous Mb compared to oxidized Mb (*9*, *22*). Using Mb mutants with various hemin affinities, retention of the hemin moiety in the globin was shown to effectively inhibit Mb-mediated lipid oxidation in washed cod muscle (*5*) and trout microsomes (**Figure 4**). Furthermore, in the presence of phospholipid vesicles, detachment of the hemin moiety from human metHb occurred around 10-fold faster compared to CN-metHb or oxyHb (*24*). This is evidence of a weakened linkage between porphyrin and globin upon oxidation of the pigment in the presence of phospholipid.

Ferrous L29F remained in the reduced state during long-term storage primarily due to its very high oxygen affinity. DeoxyMb-mediated autooxidation will not occur when O_2 is liganded (*13*). The high oxygen affinity of L29F is due to the ability of the phenyl ring of phenylalanine to stabilize O_2 that is liganded to the iron atom of the heme and which hydrogen bonds to the distal histidine (**Figure 5**). Conversely, the native leucine residue at site 29 in WT Mb does not interact with liganded O_2 (**Figure 5**). These characteristics of L29F suggest that oxyMb-mediated decomposition of preformed lipid hydroperoxides as described previously (*6*) did not contribute to Mb-mediated lipid oxidation in washed cod.

The large size and partial positive edge of the phenyl ring can also repel hydronium ions away from the heme crevice by steric and electrostatic forces. This will inhibit protonation of bound O_2 that results in formation of metMb and the neutral superoxide radical ($^{\bullet}$OOH) (*13*). The phenylalanine substitution may also sterically block access of water to the binding site of deoxyMb. Water is a reactant in deoxyMb-mediated metMb formation (*13*).

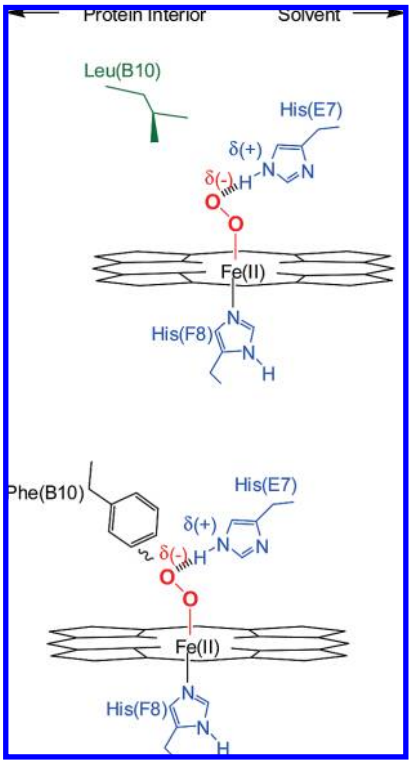

Figure 5. Selected residues present in the heme crevice of myoglobin. The B10 residue of WT Mb contains leucine, which does not stabilize O_2 that is liganded to the iron atom of the heme group. Phenylalanine at site B10 (L29F) stabilizes liganded O_2, which decreases rates of metMb formation.

The met forms of L29F and WT Mb were potent promoters of lipid oxidation in washed cod muscle (**Figure 3**), indicating that superoxide radicals that dissociate from ferrous forms of Mb during autooxidation were not necessary for lipid oxidation to occur in washed cod muscle. Rather, it is likely that the preformed lipid hydroperoxides (LOOH) present in the washed cod provide the necessary amount of reactant to allow Mb to promote lipid oxidation. Previously, at pH 6.6, ferrous trout Hb promoted lipid oxidation in linoleic acid micelles more effectively compared to metHb when the LOOH concentration was 3.4 μM; however, at 7.9 μM LOOH, both metHb and ferrous Hb oxidized linoleic acid micelles similarly (*25*). It may be that at low LOOH levels, the peroxide equivalents from dissociated superoxide are necessary to stimulate heme protein-mediated lipid oxidation.

The mechanism by which metMb promotes lipid oxidation in washed cod cannot be definitively determined at this point; however, there is compelling evidence that hemin affinity is a major factor when other studies are taken into consideration. Lipid oxidation rates in low-density lipoproteins (LDL) increased as heme affinity decreased in comparative studies of hemoglobin (low heme affinity), myoglobin (intermediate), and horseradish peroxidase (high heme affinity) (*26*). Low hemin affinity was also correlated with increased rates of lipid oxidation when variant forms of human hemoglobin were examined (*27*). Other studies further describe the ability of released hemin from Hb to stimulate lipid oxidation in various substrates including LDL (*28*).

The released hemin is ideally suited to intercalate into membrane phospholipids. The propionate groups of hemin orient toward the phospholipid head groups, whereas the esterified fatty acyl chains of the phospholipid orient toward the hydrophobic portion of the hemin ring (*29*). Hemin can then react with preformed LOOH to form a wide array of radicals capable of stimulating the formation of lipid oxidation products (*30*). These same radicals have the ability to cause hemin destruction that liberates iron atoms as potential reactants in the immediate vicinity of the cellular membrane phospholipids. Balla et al. (*31*) showed that hemin degraded and promoted lipid oxidation in LDL, whereas $FeSO_4$ or $FeCl_3$ did not promote lipid oxidation. This suggested the need for a hydrophobic moiety, in this case hemin, to deliver iron to the lipid substrate.

Upon oxidation, L29F was converted from a remarkably nonreactive Mb to a potent promoter of lipid oxidation (**Figure 3**). This indicates that Mb oxidation is a critical step that allows Mbs to promote lipid oxidation in washed cod. The porphyrin moiety was reported to be released only from oxidized met protein but not from the reduced protein (*32*). Another study describes porphyrin as being much more loosely attached in metHb compared to oxyHb (*33*). The collective results reported herein suggest that the ability of Mb to promote lipid oxidation involves release of hemin from oxidized Mb, but other mechanisms should also be considered.

The released hemin mechanism is at odds with the ability of cross-linked Mb to promote lipid oxidation (*34*). In this case, there is a covalent linkage between the heme moiety and the globin that is not present in the native Mb. The cross-linked Mb forms upon reaction with H_2O_2 and was found to promote lipid oxidation more effectively than non-cross-linked Mb at pH 7.4 in LDL particles (*34*). The pH of the system is likely critical in the estimation of the relative effects of released hemin compared to cross-linked Mb-mediated lipid oxidation. Hemin affinity of metWT sperm whale Mb was 14-fold higher at pH 7.0 compared to pH 5.5 (*35*). We suggest that at lower pH values (e.g., postmortem pH), the released hemin-mediated pathway should dominate.

Another potential mechanism by which Mb can promote lipid oxidation in washed cod is reaction of metMb with preformed lipid hydroperoxides or hydrogen peroxide to form a high-valent (oxo-ferryl) species of Mb that can abstract hydrogen atoms from polyunsaturated fatty acids and hence facilitate lipid oxidation (*8*). It should be kept in mind that ferrylMb reverts spontaneously to metMb (*36*). FerrylMb reacts with oxyMb to produce metMb (*37*). Hydrogen peroxide also converts ferrylMb to metMb (*38*). Thus, the transitory formation of ferrylMb has the potential to be a minor contributor to lipid oxidation in washed cod at pH 5.7; rather, the importance of ferrylMb may be that it is one of the sources of metMb that releases hemin. Future work should characterize ferrylMb as a reactant in washed cod muscle. However, the ability of ferrylMb to promote lipid oxidation requires the heme moiety to remain within the globin, which is contrary to reports showing that Mbs with low hemin affinity were better promoters of lipid oxidation in washed cod at pH 5.7 compared to those with higher hemin affinity (*5*) (**Figure 4**).

It has been described that hemoglobin docks to apoB protein in LDL prior to delivering hemin into the phospholipid portion of the particle, which begins a series of reactions that stimulate the onset of lipid oxidation (*26*). Future work should investigate interactions between heme proteins and protein binding sites as well as lipid sites in washed muscle fibers to better understand mechanisms by which Mb (and hemoglobin) stimulates lipid oxidation.

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LITERATURE CITED

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