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Site E14 in Hemoglobins and Myoglobins: A Key Residue That Affects Hemin Loss and Lipid Oxidation Capacity

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ABSTRACT: Fish hemoglobins (Hbs) frequently contain glycine at site E14 while mammalian Hbs contain larger residues (e.g., alanine and serine). These differences were examined by creating structural variants at E14 using recombinant bovine myoglobin (Mb) as a model heme protein that contains alanine at E14. The Ala(E14)Gly mutation increased k_{ox} and hemin loss 3-fold and 45-fold, respectively. Glycine at E14 creates a channel for solvent to enter the heme crevice, which enhances autoxidation and hemin loss rates. Hydration of the proximal heme pocket facilitates hemin loss because protonation of the proximal histidine weakens the linkage of the imidazole group to the iron atom of the hemin moiety. Ala(E14)Gly promoted lipid oxidation in washed fish muscle more rapidly during iced storage compared to wild type Mb at pH 5.7. This suggested that the rapid hemin loss from Ala(E14)Gly accelerated lipid oxidation. Ala(E14)Ser and Ala(E14)Val had little effect on k_{ox} but somewhat accelerated net hemin loss. These studies suggest that enhanced access of solvent to the heme crevice of many fish Hbs at site E14 facilitates rapid hemin loss and moderately accelerates autoxidation. This likely is part of the reason fish Hbs promote lipid oxidation much more effectively compared to mammalian Hbs.

KEYWORDS: *heme protein, heme crevice, mechanisms of lipid oxidation, site-directed mutagenesis, quality of meat and fish*

INTRODUCTION

Lipid oxidation in muscle foods results in discoloration, off-flavors, and off-odors.[1] The particularly high susceptibility of fish muscle to lipid oxidation is often attributed to elevated levels of polyunsaturated fatty acids in fish compared to terrestrial animals.[2] Differing abilities of fish, avian, and mammalian hemoglobins (Hbs) to promote lipid oxidation should also be considered since substantial quantities of residual Hb have been determined in beef, pork, poultry, and fish muscle.[3] Fish Hbs promoted lipid oxidation in washed muscle much more effectively compared to beef and poultry Hbs.[4,5] It was suggested that rapid autoxidation and rapid hemin loss in fish Hbs could account for the fact that fish Hbs promoted lipid oxidation much more rapidly compared to bovine Hb.[5]

Autooxidation occurs when the ferrous iron atom (Fe^{2+}) in the heme ring of Hb and Mb is converted to the met state (Fe^{3+}). Ferrous porphyrin is termed "heme" while oxidized porphyrin is "hemin". Autooxidation is a critical step in the onset of lipid oxidation because metHb and metMb formation weakens the porphyrin−globin linkage, causing hemin loss.[6,7] Subsequently, released hemin decomposes preformed lipid hydroperoxides into free radicals which readily stimulate the oxidation of lipids.[8] MetMb and metHb formation also facilitates formation of ferryl forms of heme proteins that can initiate lipid oxidation.[9]

Accelerating hemin loss from sperm whale Mb was previously accomplished by substituting histidine at site FG3 with alanine.[10] FG3 indicates the third residue in the random coil between helix F and G (Figure 1). The smaller alanine creates a channel into the proximal heme cavity. Hydration of the proximal cavity facilitates protonation of the proximal histidine, which weakens the hemin−globin linkage.[11] His-

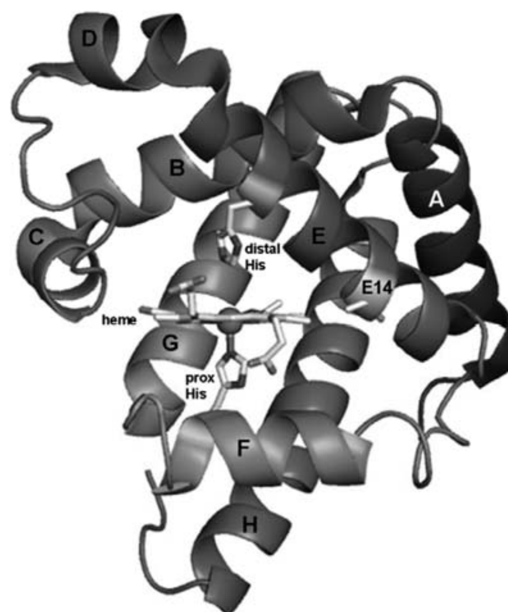

Figure 1. A representation of myoglobin is shown. Each helix is labeled (A−H). The heme group, site E14 (alanine), site E7 (distal histidine), and site F8 (proximal histidine) are shown in stick representation. Image is derived from PDB 1MBO[32] using PyMOL software. E14 is residue 71 in native bovine Mb and residue 72 in recombinant bovine Mb due to the initiator methionine residue.

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(FG3)Ala promoted lipid oxidation in washed muscle more rapidly compared to the wild type Mb.[10] These results suggested that hemin which dissociates from the globin is especially effective at facilitating lipid oxidation in washed fish muscle.

Primary structure along the CD turn and the E-helix differs between fish and mammalian Hbs at CD3, E10, and E11.[12] These amino acid residue differences partly explained the enhanced ability of the fish Hbs to promote lipid oxidation based on mutations that were made in sperm whale Mb. For example, mammalian Hbs contain valine at site E11 while perch Hb contains isoleucine at E11. Modifying mammalian Mb to resemble the fish Hbs with the Val(E11)Ile mutation increased the autoxidation rate 15-fold.[13] The differing amino acids at CD3, E10, and E11 create spatial disruptions in the protein structure of the fish Hbs. This decreases oxidative stability by introducing steric hindrance to heme-bound ligands, facilitating solvent access to the heme pocket, and decreasing the extent of bonding that anchors the heme group to the globin.[12]

Amino acid differences were also observed at site E14 when comparing fish and mammalian Hbs. E14 is the 14th residue along the E-helix which is adjacent to the heme ring (Figure 1). Many fish Hbs contain a small amino acid (glycine) at site E14 while bovine Hb contains larger residues (alanine in α chains and serine in β chains). A larger gap for solvent entry into the heme pocket at site E14 was noted in perch and trout IV Hb compared to bovine Hb based on crystal structures.[12] We decided to conduct site-directed mutagenesis studies in bovine Mb to investigate the relative abilities of E14(Gly), E14(Ser), and E14(Val) to undergo autoxidation and hemin loss compared to wild type bovine Mb that contains E14(Ala). The ability of the Ala(E14)Gly Mb mutant to promote lipid oxidation compared to wild type Mb was also determined.

MATERIALS AND METHODS

Materials. DNase I, RNase A, lysozyme, ferric chloride, hemin chloride, toluenesulfonyl chloride, chloramphenicol, potassium ferricyanide, maleic acid, superoxide dismutase, catalase, streptomycin sulfate, antifoam 204, thiobarbituric acid, and sucrose were obtained from Sigma Chemical A/S (St. Louis, MO). Kanamycin, tryptone, yeast extract, sodium chloride, glycerol, potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate monobasic, sodium phosphate dibasic, agar, disodium EDTA, methanol, methyl ethyl ketone, Tris base, Bis-Tris, and trichloroacetic acid were obtained from Fisher scientific (Pittsburgh, PA). Isopropyl-D-thiogalactopyranoside (IPTG) and dithiothreitol were obtained from Promega (Madison,WI). All other chemicals and reagents were analytical grade. Fresh cod fish (*Gadus morhua*) fillets were obtained via overnight transport from the Seafood Center (Madison, WI).

Mutagenesis at E14 and Protein Purification of Bovine Mb. pET28a(+) plasmid (Novagen, Madison, WI) containing the gene of wild type bovine Mb was constructed by the services from Geneart (Regensburg, Germany). The fragment of gene bovine Mb was assembled from synthetic oligonucleotides or PCR products, and cloned into pET28a(+) (Kan^R) using *Nco*I and *Xho*I restriction sites. In order to obtain mutant bovine myoglobin derivatives at E14, a QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was applied. The mutagenic oligonucleotide primers were synthesized individually according to the desired mutation (in boldface).

A(E14)G: (5′-CGTGCTGACC**GGG**CTGGGCGGCA-3′)
A(E14)G-antisense: (5′-TGCCGC**CCC**AGCCCGGTCAG-CACG-3′)
A(E14)S: (5′-CCGTGCTGACC**TCG**CTGGGCGGC-3′)
A(E14)S-antisense: (5′-GCCGCCC**AGC**GAGGTCAG-CACGG-3′)
A(E14)V: (5′-CGTGCTGACC**GTG**CTGGGCGGCA-3′)
A(E14)V-antisense: (5′-TGCCGCCCAG**CAC**GGTCAG-CACG-3′)

Mutant strand synthesis PCR was conducted for 18 cycles each consisting of 20 s at 95 °C for denaturation, 10 s at 60 °C for annealing, and 170 s at 68 °C for extension. The mutated plasmids were sequenced to confirm the desired mutation. The verified plasmids were transformed into *Escherichia coli* host BL21(DE3) for protein expression (Novagen, Madison, WI) and Novablue (Novagen, Madison, WI) for storage.

Recombinant bovine Mbs (wild type and E14 derivatives) were expressed in the host *E. coli* cells BL21(DE3) using a 10 L fermentation 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. 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 LB and incubated overnight 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 fermentation vessel (10 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 aerated with compressed air at 25 L/min 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 consisted of 20 mM Tris base, 50 mM NaCl, 1 mM disodium EDTA, 0.5 mM dithiothreitol, 1 mM toluene sulfonyl chloride, 40 U/mL DNase I, 3 U/mL RNase A, and 78800 U/mL lysozyme. Mbs were then purified from the lysate via salting out with ammonium sulfate precipitation, and cation exchange chromatography using 50 mL of DEAE-FF and 10 mL of monoQ (GE Healthcare, Piscataway, NJ) with equilibration buffer (10 mM Tris pH 8.50) and elution buffer (10 mM Tris, 75 mM NaCl, pH 8.50). Twenty column volumes (200 mL) were required for sufficient separation in the monoQ step. Finally, the Mbs were concentrated, frozen instantly in liquid nitrogen, and stored at −80 °C. The expression and purification of sperm whale myoglobin H64Y followed the above procedures with the following modifications: 30 μg/mL kanamycin and 50 μg/mL chloramphenicol were added into all cultures. CM column (GE Healthcare, Piscataway, NJ) was utilized instead of monoQ as the final step of purification.[10]

Preparation of metMb. Potassium ferricyanide (4 mol/mol heme) was added to bovine Mb solution. After incubation on ice for 1−2 h, protein was passed through the HiTrap desalting column 5 × 5 mL (GE Healthcare, Piscataway, NJ) equilibrated with 10 mM Tris, pH 8.0 to remove excess oxidant. Ratio of OD_{280}/OD_{262} was used to assess the residual potassium ferricyanide (successful ratio should be greater than 1.1).

Preparation of apoMb. The heme of H64Y Mb was removed using the methyl ethyl ketone method to form the apoglobin.[14] A 1 mL aliquot of 1 mM H64Y Mb was added to 1 mL of 200 mM maleic acid buffer at pH 2.2. The mixture was incubated on ice for 10 min to which 2 mL of ice-cold methyl ethyl ketone was added and mixed vigorously for 10 s. The tube was placed on ice until the separation of the lower golden aqueous phase and the upper dark organic phase. The apoglobin H64Y Mb was removed and desalted using a HiTrap 5 × 5 mL desalting column equilibrated with 150 mM Bis-Tris (at pH 5.7 and 6.3) or 150 mM Tris (at pH 8.0) containing 450 mM sucrose. The apoH64Y Mb was then quantified at 40 μM using the ε_{280nm} of 15.2 mM^{-1} cm^{-1}.

Determining Hemin Loss and Autoxidation Rates. Hemin loss rates were determined by mixing the apo H64Y sperm whale Mb with holo metMb derivatives (pH 5.7). The met H64Y Mb has a strong

absorbance at 600 nm and is used in hemin loss rate calculations due to the unique absorbance of the mutant Mb.[15] 4-fold concentration of apo H64Y Mb was added to the holo Mb. Changes in absorbance at 600 nm were measured over time, and increases in turbidity at 700 nm were subtracted from each subsequent reading at 600 nm due to gradual precipitation of the apo H64Y Mb.

Igor Pro software (WaveMetrics Inc., Portland, OR) was used to calculate rates of hemin loss with the following assumptions: (i) In the presence of excessive apo H64Y Mb, all the free hemin released from tested heme protein is acquired by apo H64Y Mb. (ii) Denaturation to apoglobin can be neglected. With these assumptions, hemin transfer between tested met heme protein and apo H64Y sperm whale Mb is measured by

$$\mathrm{PH} \underset{k_{\mathrm{H}}}{\overset{k_{-\mathrm{H}}}{\rightleftharpoons}} \mathrm{YH} + \mathrm{P} \tag{1}$$

PH is the test Mb (holo form), YH is the H64Y Mb (holo form), and P is the test Mb (apo form). k_{-H} is hemin loss rate from the test holo Mb and k_H is hemin association rate back to the test apo Mb. If the hemin transfer reaction is relatively slow, the normalized decay curve with time will follow a single exponential function and an apparent hemin transfer rate of

$$k_{obs} = k_{-H} + 2k_H[\mathrm{YH}]_{eq} \tag{2}$$

The equilibrium constant for hemin loss reaction is

$$K_{-H} = [\mathrm{YH}]_{eq}{}^2/([\mathrm{PH}]_{total} - [\mathrm{YH}]_{eq}) = k_{-H}/k_H \tag{3}$$

Hemin loss from met perch hemoglobin at the same temperature was set as reference assay to determine $[\mathrm{PH}]_{total}$ because of its extraordinary ability to release hemin comparing with other heme proteins. From eqs 2 and 3, hemin loss rate k_{-H} and other kinetic parameters are derived.

Autooxidation rates were determined using 80 μM Mb in 200 mM Bis-Tris (pH 5.7). Samples also contained 3 mmol of superoxide dismutase and catalase per mol of heme to remove any superoxide and hydrogen peroxide formation that occurs during metHb formation. Spectra were obtained at regular time intervals between 700 and 390 nm using the UV-2401 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD). The rate of metMb formation was determined using previously determined equations from the absorbance at 630, 576, and 560 nm.[16]

Preparation of Washed Cod Muscle. Fresh cod fillets were trimmed to remove all bones and dark tissue, cut into small pieces, and ground using a Kitchen Aid, Inc. (St. Joseph, MI), KSM90WW household mixer equipped with grinding apparatus (5 mm plate diameter). Ground muscle was washed by combining in a 1:3 ratio with cold 50 mM sodium phosphate buffer (pH 6.3) and mixing for 2 min with glass rod. After settling for 15 min, muscle was collected and dewatered using fiberglass mesh (~2 mm diameter). The washing operation was repeated twice. Immediately after the final wash, the muscle slurry was homogenized for 3.5 min with a Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY) until it was of a fibrous consistency. pH of muscle slurry was checked and determined to be between 6.30 and 6.40. Muscle was collected in approximately 50 g pellets by centrifuging 25 min at 15263g, and was then stored at −80 °C in vacuum sealed plastic bags. All washing, dewatering, and centrifugation steps were performed at 4 °C.

Addition of Heme Proteins to Washed Cod Muscle. Washed cod mince (thawed overnight at 4 °C) was crumbled into pieces by mixing for 10 min with a plastic spatula on ice. To further break up the mince, the washed cod was ground for three 5 s bursts 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 1 N HCl and mixing on ice for 10−15 min. Tissue was then added to a specific well of 12-well polystyrene plate (Corning, NY) placed on ice. Water was added to the well so that final moisture content would be 90%, and streptomycin sulfate was added to final concentration 200 ppm in order to prevent microbial growth during experiment. The contents of the well were mixed with a plastic spatula for 2 min. Experimental heme proteins were separately added and mixed for 3 min with a plastic spatula to a final Mb concentration of 40 μmol/kg tissue. Zero-time samples were taken, and reaction vessels were stored on ice for the duration of the experiment.

Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a 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 for 1 h. Tissue sample (80−120 mg) was added to 1.2 mL of the reagent. After heating at 65 °C for 60 min, the samples were cooled at 4 °C for 60 min. Samples were then centrifuged at 16000g for 5 min. Absorbances of supernatants were measured at 532−650 nm minus 650 nm. A standard curve was constructed using tetraethoxypropane, and concentrations of TBARS in samples were expressed as μmol of TBARS/kg of washed muscle.

Statistical Analysis. Igor Pro software (WaveMetrics Inc., Portland, OR) was used to calculate rates of autoxidation and hemin loss by data fitting. Each study was replicated at least twice, and samples were analyzed in duplicate or triplicate. Data was analyzed using the SAS system (version 9.2). The experimental design for autoxidation and hemin loss was a one-way (type of Mb) analysis of variance using the MIXED procedure. Least squares means were generated, and the differences among treatments were significant at $p < 0.05$ using the p-diff test. A MIXED procedure was used for the storage studies in which 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.

RESULTS

Effect of Mutations at E14 on Autoxidation Rates. Autooxidation rates (k_{ox}) in wild type Mb and three Mb mutants were determined at pH 5.7 and 37 °C. Wild type bovine Mb contains alanine at site E14 which provides a representation of the α chains of bovine Hb. The β chains of bovine Hb contain serine. Thus the Ala(E14)Ser Mb mutant is used as a representation of the bovine Hb β chain. There was no significant difference in k_{ox} when comparing wild type Mb and Ala(E14)Ser (Table 1). The Ala(E14)Gly mutant was used as a representation of the α and β chains of perch Hb and the β chain of trout IV Hb. Substituting alanine at site E14 in bovine Mb with glycine increased k_{ox} 3-fold ($P < 0.05$) (Table 1). It was considered possible that substituting valine, a residue slightly larger than alanine, at site E14 in bovine Mb would decrease k_{ox} by better sterically sealing the gap for solvent entry to the heme crevice. The k_{ox} rate was significantly slower in Ala(E14)Val compared to wild type Mb, a difference of about 2-fold.

Table 1. Autooxidation Rates (k_{ox}) of Wild Type Bovine Mb and E14 Mutants at pH 5.7 and 37 °C[a]

Mb	k_{ox} (h^{-1})
wild type	0.45 ± 0.15 b
A(E14)G	1.23 ± 0.08 a
A(E14)S	0.40 ± 0.01 bc
A(E14)V	0.25 ± 0.02 c

[a]Mb concentration was 80 μM. Same letters in a column indicate no significant difference. Superoxide dismutase and catalase were added at 3 mmol/mol heme.

Effect of Mutations at E14 on Hemin Dissociation and Association Rates. Hemin dissociation (k_{-H}) and association (k_H) rates in wild type Mb and the three Mb mutants were determined at pH 5.7 and 37 °C. This temperature and pH

were chosen to have the assay reach equilibrium considering that Mbs have higher hemin affinity compared to hemoglobins.[18] Hemin loss occurs more rapidly at increasing temperatures and lower values of pH.[15] Mbs contain Ser or Thr at F7, which dramatically increases hemin affinity compared to Leu at site F7 in Hbs. The Ser(F7)Leu Mb mutant released hemin 10−20-fold faster compared to wild type Mb.[19] Ser(F7) interacts with the heme-7-propionate stabilizing hemin in the globin whereas Leu(F7) does not form a contact with the propionate group. Mbs contain His(FG3), which also forms a contact with the heme-7-propionate, whereas Leu(FG3) in Hbs cannot form a contact.[11]

Wild type Mb, Ala(E14)Gly, and Ala(E14)Val had similar hemin association rates (k_H), but k_H was around 10-fold higher in Ala(E14)Ser compared to wild type Mb (Table 2). The hemin dissociation rate (k_{-H}) was nearly 20-fold greater in Ala(E14)Ser. This resulted in an equilibrium hemin dissociation constant (K_{-H}) that was approximately 2-fold higher in Ala(E14)Ser compared to wild type Mb (Table 2). K_{-H} of Ala(E14)Val was 5-fold higher compared to wild type Mb, indicating substitution of E14(Ala) with valine lowered hemin affinity. K_{-H} of Ala(E14)Gly was 45-fold higher compared to wild type Mb. This suggests that the glycine residues present at E14 in fish Hbs facilitate hemin loss while the larger residues at E14 in mammalian Hbs act to retain the porphyrin moiety.

Table 2. Dissociation Rate, Association Rate, and the Equilibrium Hemin Dissociation Rate Constant for Wild Type Bovine Mb and E14 Mutants (pH 5.7 and 37 °C)[a]

protein	k_{-H} (h^{-1})	k_H (μM^{-1} h^{-1})	K_{-H} (μM)
wild type	0.003 ± 0.0003 d	0.08 ± 0.02 b	0.04 ± 0.007 d
A(E14)G	0.08 ± 0.004 a	0.05 ± 0.002 b	1.79 ± 0.016 a
A(E14)S	0.06 ± 0.002 b	0.68 ± 0.08 a	0.10 ± 0.01 c
A(E14)V	0.013 ± 0.005 c	0.06 ± 0.02 b	0.20 ± 0.01 b

[a] K_{-H} is the equilibrium constant of hemin loss reaction derived from k_{-H}/k_H. Hemin dissociaton values were measured at 10 μM metMb and 40 μM apoH64Y reagent. Same letters in a column indicate no significant difference.

Effect of Mutations at E14 of Bovine Myoglobin (Mb) on Lipid Oxidation. The ability of wild type Mb and Ala(E14)Gly to promote lipid oxidation in washed cod muscle was examined at pH 5.7 during iced storage. Thiobarbituric acid reactive substances (TBARS) were used as an indicator of lipid oxidation. The met forms of each Mb were examined since Ala(E14)Gly Mb autooxidized faster than wild type Mb (Table 1). We wished to exclusively assess the effect of rapid hemin loss from Ala(E14)Gly on lipid oxidation which would be confounded if the oxyMbs with differing autoxidation rates were examined. Ala(E14)Gly stimulated lipid oxidation more rapidly compared to wild type metMb ($p < 0.05$) (Figure 2). After 1 day of storage TBARS values were around 15-fold greater in Ala(E14)Gly compared to wild type Mb.

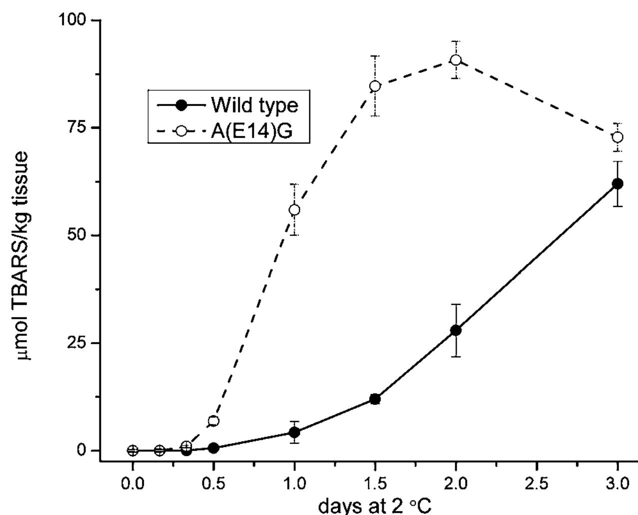

Figure 2. Thiobarbituric acid reactive substances (TBARS) values in washed cod muscle containing added wild type bovine Mb (met form) and Ala(E14)Gly bovine Mb (met form). The Mb concentration was 40 μmol per kg of tissue. pH was 5.7.

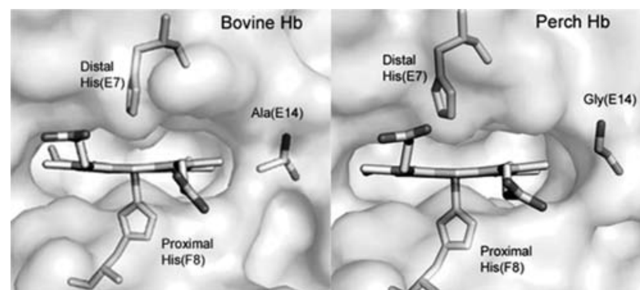

Figure 3. There is a larger gap for solvent to enter the heme crevice in perch Hb compared to bovine Hb due to the smaller glycine at E14 in perch Hb compared to the larger alanine at E14 in the bovine Hb. The heme, distal histidine, proximal histidine, and E14 are shown in stick representation. A space-filled model with shadowing is shown to illustrate the contours of the heme crevice around the heme. One of the α chains from each Hb is shown. The PDB structures 2QSP and 3BJ1[12] were used to prepare the image shown using PyMOL software.

■ DISCUSSION

A large number of fish species contain glycine at site E14 of their Hb chains while mammalian Hbs have larger residues at E14 (e.g., alanine and serine). Crystallographic studies indicated a larger channel for solvent to enter the heme crevice in perch and trout IV Hb containing glycine at E14 compared to bovine Hb.[12] An illustration of the larger gap at E14 in perch Hb compared to bovine Hb is shown (Figure 3). Enhanced access of solvent to the proximal histidine (F8) will enhance protonation of F8, which results in dissociation of the porphyrin moiety from the globin. Protonation of His(F8) weakens the bond between the imidazole group and the iron atom of the heme group. Bovine Mb contains alanine at site E14. Mutating E14(Ala) to glycine was done to simulate the heme crevice environment of the fish Hbs at site E14 and to determine the effect of this substitution on hemin affinity. Hemin affinity was 45-fold lower in Ala(E14)Gly compared to wild type Mb, which suggests the rapid hemin loss observed in the fish Hbs compared to mammalian Hbs is partly due to the unique presence of E14(Gly) in some of the fish Hb chains. Thus it appears that having glycine at E14 facilitates entry of solvent into the proximal pocket of the fish Hbs, which dramatically decreases hemin affinity. In addition, glycine replacement is also deleterious to the stability of the resultant apohemoglobin either by promoting structural entropy[20] or by producing a large change in helix propensity within the globin.[21] Decreased stability of apohemoglobin will accelerate the hemin loss reaction irreversibly.

It also appears that having the smaller glycine at E14 increases access of protons to the *distal* pocket. Enhanced access of protons will accelerate autoxidation. The protonation of ε-N of the distal histidine (E7) disrupts hydrogen bonding between E7 and O_2 that is liganded to the iron atom of the ferrous heme in the globin (Figure 1). Protons can then more easily bind to liganded O_2, resulting in the dissociation of neutral superoxide radical ($HO_2^{\bullet}$) and the formation of oxidized, ferric metMb.[13] The k_{ox} rate was 3-fold higher in Ala(E14)Gly compared to wild type Mb that contains the larger residue at site E14. This suggests that Gly(E14) in fish Hbs accelerates k_{ox} by increasing access of protons to the distal heme pocket compared to Ala(E14) found in mammalian Hbs.

In addition to steric effects at E14, the ability of relatively hydrophobic side chains to suppress proton mobility should be considered. Fish Hbs containing E14(Gly) lack a hydrophobic side chain whereas the alanine residue in most mammalian Hb α chains is relatively hydrophobic due to the methyl group. Suppression of proton mobility by "hydrophobic hydration" was noted previously.[22] Suppression of proton mobility by E14(Ala) may contribute to excluding solvent from the heme crevice so that relatively high hemin affinity is observed in Hbs and Mbs that contain hydrophobic residues at E14 (e.g., alanine).

Hemin loss and k_{ox} from perch Hb was more rapid compared to trout IV Hb.[12] This can be partly attributed to the fact that perch Hb contains E14(Gly) in both its α and β chains while trout IV Hb contains E14(Gly) only in the β chain.[12] It is interesting to note that trout I Hb contains E14(Ala) in its α and β chains.[23] Hemin loss was slower in trout I Hb compared to trout IV Hb.[24]

In humans, the β Hb chains that contain E14(Ser) lose hemin more rapidly compared to the α Hb chains that contain E14(Ala).[25] Consistent with this, the Ala(E14)Ser Mb mutant had a higher hemin dissociation rate compared to wild type Mb containing Ala at E14 (Table 2). It may be that the polar portion of serine attracts solvent to the heme crevice (relative to alanine) causing an increase in hemin dissociation rate. The increased hemin association rate of Ala(E14)Ser (Table 2) is more difficult to explain but may involve the ability of the polar serine side chain to attract the polar regions of the porphyrin moiety, facilitating acquisition of displaced hemin back into the heme cavity of the globin. The ability of side chain polarity to affect water mobility in the proximal heme crevice of sperm whale Mb mutants is complex. Replacing apolar with highly polar side chains in the proximal heme pocket has been shown to immobilize water molecules within the heme cavity of sperm whale Mb.[26]

Ala(E14)Val slightly decreased k_{ox} but had an accelerating effect on hemin loss. It was thought that the somewhat larger and apolar E14(Val) would be of appropriate size and apolar character to optimally exclude solvent from the heme crevice and thereby decrease k_{ox} and hemin loss rates. The mass of valine is 27 Da greater than that of alanine. Apparently this increase in size either disrupted the E-helix or slightly displaced the heme group, both events favoring hemin loss.

Hemoglobin from a cold water fish (*Coryphaenoides armatus variabilis*) had a more rapid autoxidation rate (23-fold) compared to Hb from a warm water fish (*Paralabrax nebulifer*).[27] Elevated environmental temperatures may exert stronger evolutionary pressure for animals to have slowly autooxidizing Hbs. Hb autoxidation is accelerated at elevated temperature, and the resulting metHb cannot transport O_2 needed for respiration. The amino acid sequence of Hb from sand bass (*P. nebulifer)* and the abyssal grenadier (*C. armatus variabilis)* is not available.

There are multiple pathways by which Hb and Mb can promote lipid oxidation. These include (i) the reactivity of ferryl heme protein species, (ii) hemin that dissociates from the globin, and (iii) iron atoms that are released from hemin. The fact that Ala(E14)Gly had a 45-fold lower hemin affinity and stimulated lipid oxidation in washed muscle more rapidly than wild type Mb suggests that hemin dissociation from the globin is an efficient mechanism by which lipid oxidation occurs in washed cod muscle. It was previously shown that a Mb mutant prone to heme destruction and subsequent release of iron atoms weakly promoted lipid oxidation in washed cod compared to wild type Mb.[10] Hemin loss is much greater in Hbs compared to Mbs.[18,25] The relatively high hemin affinity of Mb might suggest ferryl Mb catalyst is the primary mechanism that facilitates Mb-mediated lipid oxidation. At the same time, hemin loss from bighead carp Mb became elevated when the pH was decreased from 6.0 to 5.5 during 4 °C storage.[28] Thus pH may be a switch that can convert the mechanism of Mb-mediated lipid oxidation from ferryl Mb to hemin-mediated.

In conclusion, replacement of alanine at E14 with smaller residues creates a larger channel that enhances access of solvent to the heme group. This enhances protonation of E7(His) and liganded O_2 to accelerate autoxidation and F8(His) protonation to accelerate hemin loss. E14(Ala) is particularly suited to prevent hydration of the heme crevice by providing the proper degree of steric hindrance and hydrophobicity to repel protons. It is revealed that glycine at E14 in fish Hbs likely contributes to the rapid autoxidation, hemin loss, and lipid oxidation capacity of fish Hbs compared to mammalian Hbs. With regard to Mbs, most fish Mbs contain E14(Lys) while E14(Gln) has been characterized in most avian Mbs.[29,30] This is in contrast to E14(Ala) in most mammalian Mbs. It has been shown that tuna Mb autooxidizes faster than turkey Mb, which in turn autooxidizes faster than mammalian Mb.[31] Future work should examine relative hemin loss rates from fish, avian, and mammalian Mbs as well as Ala(E14)Lys and Ala(E14)Gln Mb mutants to investigate the effect of E14 variation on pro-oxidative characteristics of the different myoglobins.

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Notes

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ABBREVIATIONS USED

Mb, myoglobin; Hb, hemoglobin

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