Effect of 4-hydroxy-2-nonenal on myoglobin-mediated lipid oxidation when varying histidine content and heme affinity

Abstract

The compound 4-hydroxy-2-nonenal (HNE) dissolved in water was examined to remove potential effects of using ethanol to solubilize the aldehyde such as altering protein structure or redox properties of myoglobin (Mb). HNE became covalently bound to sperm whale Mb at up to five sites based on ESI-MS analysis. Adducted Mb promoted lipid oxidation in washed muscle more effectively than non-adducted Mb. Alkylation of P88H/Q152H Mb with HNE accelerated metMb formation and subsequent lipid oxidation. P88H/Q152H Mb exposed to HNE enhanced lipid oxidation compared to wild-type Mb exposed to HNE. Results using H97A Mb suggested that the combination of HNE and low heme affinity facilitated rapid decomposition of preformed lipid hydroperoxides to secondary lipid oxidation products. HNE and HHE (4-hydroxy-2-hexenal) facilitated Mb-mediated lipid oxidation similarly. The potential mechanisms by which Mb binding of α,β -unsaturated aldehydes affect Mb oxidation and the onset of lipid oxidation are discussed.

KEYWORDS: myoglobin, lipid oxidation, mutant, redox stability, HNE, protoporphyrin IX

Short title: Effect of HNE on lipid oxidation due to wild-type Mb and Mb mutants

1. INTRODUCTION

The compound 4-hydroxy-2-nonenal (HNE) is a hydroxyalkenal product resulting from the oxidation of linoleic and arachidonic acids (Esterbauer, Schaur, & Zollner, 1991). HNE readily forms a covalent bond with histidine residues of myoglobin (Mb) by Michael addition (Bolgar & Gaskell, 1996). The term 'alkylation' has been used to describe covalent binding of HNE to histidine residues in Mb. Alkylation of Mb by HNE increased the oxidation rates of equine, bovine, and porcine Mbs, as well as lipid oxidation rates in model systems of muscle lipids (Alderton, Faustman, Liebler, & Hill, 2003; Faustman, Liebler, McClure, & Sun, 1999; S. Lee, Phillips, Liebler, & Faustman, 2003; Lynch & Faustman, 2000; Suman, Faustman, Stamer, & Liebler, 2006, 2007). These authors suggested that alkylation of Mb by HNE results in the disruption of the spatial arrangement of amino acid residues of Mb, which could result in the observed increases in Mb oxidation rate and the enhanced pro-oxidative activity toward lipid substrates.

Bovine Mb contains four additional histidine residues compared to porcine Mb which may explain the greater ability of HNE to accelerate oxidation in bovine Mb compared to porcine Mb (Suman, Faustman, Stamer, & Liebler, 2007). Porcine Mb has proline at position 88 and glutamine at position 152, whereas bovine Mb has histidine at each of these positions. Mutagenesis of sperm whale Mb can be performed such that the proline and glutamine residues at sites 88 and 152 are replaced with histidines, yielding the P88H/Q152H mutant. Mutagenesis allows the effect of amino acid variation at site 88 and 152 to be directly assessed in regard to HNE-mediated Mb oxidation and the ability of HNE to accelerate Mb-mediated lipid oxidation. Position 88 (site F3) is the third amino acid along the F-helix which is near the proximal histidine (site F8) that anchors the globin to the protoporphyrin moiety (**Figure 1**). Position 152 (site HC3) is the third amino acid in the random coil after the H-helix (**Figure 1**).

Free HNE has been quantified in pork, beef, sardine, yellowfin tuna, and coho salmon muscle with concentrations ranging from 0.07 to 28 $\mu\text{mol/kg}$ tissue (Sakai, Kuwazuru, Yamauchi, & Uchida, 1995; Sakai, Munasinghe, Kashimura, Sugamoto, & Kawahara, 2004; Sakai, Yamauchi, Kuwazuru, & Gotoh, 1998). The association of HNE with inflammation and other disease states has been reviewed (Esterbauer, Schaur, & Zollner, 1991; Poli, Schaur, Siems, & Leonarduzzi, 2008). The main objective of this research was to vary the histidine content and heme affinity of Mb in order to better understand the role of these variables on HNE-induced oxidation of Mb and lipids.

2. MATERIALS AND METHODS

2.1 Site-directed mutagenesis for P88H/Q152H swMb. The gene coding for P88H/Q152H swMb was prepared from the recombinant WT swMb gene using the QuikChange® multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were obtained from Integrated DNA Technologies (IDT) (Coralville, IA). Primers were designed with the assistance of the OligoAnalyzer software application provided by IDT.

2.2 Subcloning of recombinant sperm whale Mb genes. Genes coding for wild-type (WT) and mutants of recombinant sperm whale Mb were prepared as described previously (Grunwald & Richards, 2006).

2.3 Expression of recombinant sperm whale Mb. Expression of WT and mutants of sperm whale Mb were prepared as described previously (Grunwald & Richards, 2006). A modification was made to the indicated method such that air flow into the fermentation reaction was increased to 25 L/minute. This increased final protein yield several fold.

2.4 Purification of recombinant sperm whale Mb. Recombinant WT and mutant sperm whale Mb was purified as described previously using cation and anion exchange chromatography steps (Grunwald & Richards, 2006).

2.5 Anaerobic reduction of recombinant sperm whale Mb. A 2 ml volume of recombinant swMb (~1 mM) was desalted using an Econo-Pac® 10DG (BioRad, Hercules, CA) desalting column equilibrated with 1 mM tris pH 8.0. The desalted Mb was transferred to a glass vial with septum closure. A small quantity (2-3 mg) of sodium hydrosulfite (dithionite) was added to a second glass vial with a septum closure. In order to create an anaerobic condition, both vials were flushed with nitrogen gas while incubating on ice for at least 10 minutes. To begin the reduction, a gas-tight glass syringe was used to transfer the degassed Mb to the vial containing the dithionite. Development of a dark purple color indicated reduction of the Mb to the deoxy form. The reduction mixture was incubated on ice for 15 minutes with periodic swirling. To remove the dithionite and convert the deoxyMb to oxyMb, the reduction mixture was desalted using another BioRad Econo-Pac® 10DG desalting column equilibrated with 1 mM tris pH 8.0 saturated with oxygen gas. The headspace of the column was also flushed with oxygen gas. Successful reduction of the Mb was confirmed spectrophotometrically by scanning from 700 nm to 200 nm range and observing the peaks at 543, 581, and 418 nm indicative of oxyMb (Antoni & Brunoni, 1971).

2.6 Replacement of ethanol HNE solvent with water. The aldehyde 4-hydroxy-2-nonenal (HNE) (Cayman Chemical, Ann Arbor, MI) was procured as a 1 mg/100ml solution in 100% ethanol. To replace the ethanol with water, the ethanol was first evaporated under a gentle stream of nitrogen gas while incubating on ice. After confirmation (visual and odor) of the

removal of the ethanol and presence of the remaining neat HNE oil, 1 ml of ice cold nitrogen flushed deionized water was added to the HNE. The HNE was dissolved by vortexing vigorously for 1 minute. Spectrophotometric confirmation of the dissolution of the HNE in the water was made by observing an HNE peak at 221 nm. This method was used to replace the ethanol solvent with deionized water for 4-hydroxy-2-hexenal (HHE) (Cayman Chemical, Ann Arbor, MI) as well.

2.7 Alkylation of Mb by aldehydes and measurement of autooxidation (%MetMb

formation). Unless otherwise noted, alkylation and control reactions were prepared by combining to the indicated final concentrations of purified ferrous Mb (0.015 mM), aqueous HNE (0.105 mM), and Bis-Tris (195 mM) in a final 1.1 ml volume in sealed spectrophotometric cuvettes. Unless noted, reactions were incubated at 4°C, pH 5.7 and autooxidation was monitored spectrophotometrically over time. For indicated experiments, aqueous HHE (4-hydroxy-2-hexenal) was used as the alkylating agent. Alkylation reactions prepared for the purpose of addition to washed cod muscle were prepared in a scaled-up format such that the final concentrations of the components were as follows: ferrous Mb (0.225 mM heme basis), bis-tris (98 mM), and aqueous HNE (1.6 mM). This was done so that when the desalted alkylation reactions were added to washed cod, a final Mb concentration of 40 µmol/kg washed cod was achieved. Autooxidation of swMb was measured spectrophotometrically during the alkylation reaction using optical densities at 503, 525, 557, and 582 nm, and the calculations of (Tang, Faustman, & Hoagland, 2004).

2.8 Mass spectrometry analysis. Volumes of 100 µl were taken from alkylation and control reactions prior to use in lipid oxidation studies and snap-frozen in liquid nitrogen and stored at -80°C until analysis at the Mass Spectrometry Facility of the University of Wisconsin-Madison

Biotechnology Center. The preparation of alkylated Mb for mass spectrometry was as follows: A 30 µl volume of the thawed alkylation reaction was precipitated with 470 µl ice cold 100% acetone. The pellet was collected by microfuging at 16,100g for five minutes. The pellet was then washed twice, first with ice cold 100% acetone, then 80% ice cold acetone, with 2 minute spins in between washes. A third wash was performed with 100% methanol. The pellet was then dissolved in a mixture of 5 µl neat formic acid, 20 µl water, and 20 µl methanol. A 5-10 µl quantity of the dissolved pellet solution was then injected into the ESI-QTRAP mass spectrometer. The instrumentation and parameters for the Mb analysis using ESI-QTRAP (electrospray ionization quadrupole ion trap) methodology were as follows: Analyses were carried out on 3200 QTRAP tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Turbo V™ spray source. The sample was injected as a 10ul aliquot at 30ul/ml from the 4.6mm diameter auto-syringe delivery system (Harvard Apparatus, Holliston, Massachusetts) in 50:50% methanol:water. The following instrumental parameters were used to generate the most optimum protonated ions $[M+H^+]$ in positive mode: ion spray voltage (IS), 5.5 kV; curtain gas (CUR), 20 psi; nebulizer gas (GS1), 20 psi; turbo gas (GS2), 20psi; turbo gas temperature (TEM), 250°C; interface heater (ihe), ON; declustering potential (DP), 40eV; entrance potential (EP), 10eV; detector (CEM), 2,400; dwell time, 2,000 msec; pause time, 5.0 msec. Acquired data was processed using Analyst 1.4.2 software (Applied Biosystems, Foster City, CA) to deconvolute parent mass observed in the range from 100 to 1700 amu (atomic mass units).

2.9 Preparation of washed cod muscle. Washed cod muscle was prepared as described previously (Grunwald & Richards, 2006). Washed cod contains lipids and myofibrillar proteins which is preferable to microsomes or liposomes that lack myofibrillar proteins.

2.10 Addition of alkylated Mb to washed cod muscle. Alkylation reactions were desalted prior to addition to washed cod to remove unreacted aldehydes using an Econo-Pac® 10DG desalting column equilibrated with 205 mM bis tris, pH 5.6. Desalted alkylation reactions were then mixed into washed cod muscle such that the final concentration of Mb after addition to washed cod was 40 µmol/kg washed cod. Unless otherwise noted, washed cod reactions were performed at pH 5.6, contained 200 ppm streptomycin sulfate, had a moisture content of 90%, and were stored on ice. Mb was examined at 40 µmol/kg washed cod as this concentration generated substantial quantities of lipid oxidation products during the timeframe of iced storage.

2.11 Analysis of thiobarbituric reactive substances (TBARS). Samples collected for TBARS analysis were processed as described previously (Grunwald & Richards, 2006). TBARS are an indicator of secondary lipid oxidation products that react with thiobarbituric acid reagent resulting in a chromophore with maximal optical density at 532 nm.

2.12 Analysis of lipid hydroperoxides (LHP). Samples collected for LHP analysis were processed as described previously (Grunwald & Richards, 2006). LHP are primary lipid oxidation products that react with ferrous iron chloride reagent. The resulting ferric ions bind to ammonium thiocyanate reagent causing formation of the Fe-thiocyanate complex with maximal optical density at 500 nm.

2.13 Statistical analysis. A typical experiment involved addition of washed cod muscle to four separate vessels. A specific Mb treatment (treatment #1) was then added to two of the vessels. A different Mb treatment (treatment #2) was added to the remaining two vessels. This represents two independent reactions per treatment. Each vessel was sampled and measured

for TBARS and LHP at various time points during storage. The MIXED procedure of the SAS[®] system was used for statistical analysis of the data (Littell, Milliken, Stroup, & Wolfinger, 2000). This model is able to account for fixed effects (e.g. treatment, time, interaction between treatment and time) and random effects (e.g. variation between vessels of a treatment at specific time points of measurement). Additionally, the covariation between measurements obtained from a specific vessel of a treatment over time is addressed by including a “repeated” statement. Due to the inconsistent interval between time points of measurement, the covariation was structured using a spatial power matrix. The least squares means procedure was used to calculate means for the fixed (main) effects. The p-diff option was used in these calculations in order to make comparisons between means of the fixed effects. A level of significance of $p < 0.05$ was the criteria used to establish differences between means. If necessary, experiments were repeated.

2.14 Chemicals. All chemicals used were of analytical grade. Oxygen gas was of “Chemically Pure” grade. Nitrogen gas was of “Industrial” grade (Airgas Inc., Radnor Township, PA). Water was of “Ultrapure” quality (18.2 MΩ•cm).

2.15 Graphics. All graphics were created using PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

3. RESULTS

3.1 Effects of alkylation by aldehydes on oxidative reactions of myoglobin

Wild-type (WT) Mb (0.225 mM heme basis) was alkylated by aqueous HNE (1.6 mM) at pH 5.7 and 4°C. ESI-MS indicated that adduction of up to 5 HNE molecules to WT swMb occurred during the alkylation reaction. After incubation for 78 hours and removal of unreacted aldehydes by desalting, the alkylated protein solutions were mixed into washed cod such that final concentrations of Mb were 40 µmol/kg washed cod. Measurement of TBARS formation during iced storage at pH 5.7 indicated that alkylation of WT swMb by HNE increased the ability of WT swMb to promote lipid oxidation in washed cod compared to WT swMb treated with water control ($p = 0.009$) (**Figure 2A**). Increases in lipid hydroperoxides (LHP) measured during iced storage emphasized the increase in ability of WT swMb alkylated by HNE to promote lipid oxidation in washed cod ($p = 0.018$) (**Figure 2B**). Measurement of metMb formation during HNE alkylation showed no statistical increase in Mb oxidation rate compared to WT swMb treated with water control ($p = 0.224$) (**Figure 2C**).

P88H/Q152H (0.225 mM heme basis) was alkylated by aqueous HNE (1.6 mM) at pH 5.7 and 4°C. Alkylation of P88H/Q152H increased the Mb oxidation rate compared to P88H/Q152H treated with water control ($p < 0.05$ for hours 7 through 76) (**Figure 3A**). ESI-MS indicated that adduction of up to 5 HNE molecules to P88H/Q152H occurred during the alkylation reaction. After incubation for 76 hours and removal of unreacted aldehydes by desalting, the alkylation reactions were mixed into washed cod such that final concentrations of Mb were 40 µmol/kg washed cod. TBARS increased more rapidly during iced storage in alkylated P88H/Q152H compared to non-alkylated (**Figure 3B**). Lipid hydroperoxides (LHP) measured during iced storage also increased more rapidly in alkylated P88H/Q152 compared to non-alkylated (**Figure 3C**).

To comparatively investigate the effect of histidines at positions 88 and 152 on the oxidative characteristics of Mb, the effects of WT Mb and P88H/Q152H (0.225 mM heme basis) alkylated by HNE (1.6 mM) at pH 5.6 and 4°C were compared. The incubation period with HNE

was 90 hours for this comparison whereas the previous incubations were 76 hours for WT Mb (Figure 2) and 78 hours for P88H/Q152H (Figure 3). ESI-MS indicated that alkylation of up to 4 HNE molecules to P88H/Q152H and 6 HNE molecules to WT Mb occurred during the alkylation reaction. The longer incubation period may explain the differing quantities of adductions observed for WT Mb and P88H/Q152H. After removal of unreacted HNE using PD-10 gel filtration columns, the alkylation reactions were mixed into washed cod such that final concentrations of Mb were 40 μ mol/kg washed cod. Alkylated WT Mb and alkylated P88H/Q152H each caused rapid TBARS formation yet the extent of TBARS formation was greater for treated P88H/Q152H in washed cod (**Figure 4A**). Measurement of metMb formation during HNE alkylation indicated no enhancement in Mb oxidation rate of HNE alkylated P88H/Q152H Mb compared to HNE alkylated WT Mb ($p = 0.291$) (**Figure 4B**). HNE alkylated P88H/Q152H Mb showed a significantly greater zero time metMb percentage than HNE alkylated WT Mb ($p = 0.003$).

H97A is a mutant of Mb in which the histidine residue at position 97 (site FG3) is substituted by an alanine. Site FG3 is the third amino acid between the F- and G-helix (**Figure 1A**). Hargrove et al (1996) demonstrated a 39-fold decreased heme affinity of H97A compared to WT Mb. The decreased heme affinity of H97A compared to WT swMb increased the ability of H97A to promote lipid oxidation compared to WT Mb (Grunwald & Richards, 2006). In order to further understand the mechanism through which HNE modulates the prooxidative character of Mb, and the relevance of heme affinity in this mechanism, the effect of alkylation by HNE on H97A swMb was investigated. H97A swMb (0.225 mM heme basis) was alkylated by aqueous HNE (1.6 mM) at pH 5.7 and 4°C. ESI-MS indicated that adduction of up to 3 HNE molecules to H97A swMb occurred during the alkylation reaction. The rapid heme loss from H97A decreased solubility of the heme protein which may explain the relatively low quantity of adductions relative to WT Mb. After incubation for 71 hours and removal of unreacted

aldehydes by desalting, the alkylation reactions were mixed into washed cod such that final concentrations of Mb were 40 $\mu\text{mol/kg}$ washed cod. Alkylation of H97A increased the rate of formation of TBARS during the early stages of storage yet the extent of TBARS formation was diminished by alkylation (**Figure 5A**). Alkylation of H97A decreased the formation of lipid peroxides during storage (**Figure 5B**). Measurement of percent metMb formation during HNE alkylation showed no increase in Mb oxidation rate compared to H97A Mb treated with the water control ($p = 0.109$) (**Figure 5C**).

An additional study was conducted with HNE and H97A to confirm the unexpected result from the first trial. The results showed again that HNE accelerated TBARS formation early in storage while decreasing the extent of TBARS formation later in storage (**Figure 5D**), decreased lipid peroxides (**Figure 5E**) and did not statistically affect the rate of Mb oxidation (**Figure 5F**). It should be noted that the initial lipid peroxide values in washed cod were 35-60 $\mu\text{mol/kg}$ for added WT Mb (**Figure 2B**) compared to 100-175 $\mu\text{mol/kg}$ for added H97A (**Figure 5B and 5E**). The initial TBARS values in washed cod were less than 1 $\mu\text{mol/kg}$ for added WT Mb (**Figure 2B**) compared to 4-7 $\mu\text{mol/kg}$ for added H97A (**Figure 5A and 5D**). These findings indicate that H97A promoted initial formation of primary and secondary lipid oxidation products more rapidly upon addition to washed cod compared to WT Mb.

Part of the mechanism through which aldehydic products of PUFA decomposition affect the prooxidative character of Mb may be related to an alteration in the hydrophobic character of the Mb molecule through alkylation by those aldehydes. Consequently, a comparison was made between HNE, a nine carbon aldehyde possessing a five carbon acyl tail, and 4-hydroxy-2-hexenal (HHE), a six carbon aldehyde possessing a two carbon acyl tail. WT Mb (0.225 mM heme basis) was alkylated either by HNE (1.6 mM) or HHE (1.6 mM) at pH 5.6 and 4°C. Measurement of percent metMb formation during HNE and HHE alkylation at a 7:1 ratio of aldehyde to Mb (heme basis) showed no difference between the two aldehyde treatments for

accelerating Mb oxidation ($p = 0.736$) (**Figure 6A**). ESI-MS indicated that adduction to Mb of up to 5 HNE molecules and up to 6 HHE molecules occurred during the alkylation reaction. After incubation for 88 hours and removal of unreacted aldehydes by desalting, the alkylation reactions were mixed into washed cod such that final concentrations of Mb were 40 $\mu\text{mol/kg}$ washed cod. Measurement of TBARS formation during iced storage at pH 5.7 suggested that alkylation by HNE or HHE at a 7:1 ratio of aldehyde to Mb did not differently affect the ability of Mb to promote lipid oxidation in washed cod that contains lipids primarily as phospholipids with lesser amounts of triacylglycerols and free fatty acids ($p = 0.641$) (**Figure 6B**).

4. DISCUSSION

4-Hydroxy-2-nonenal (HNE) dissolved in water was examined to remove potential confounding effects due to using ethanol to solubilize the aldehyde, a standard approach previously used by many investigators (see Introduction section). Superoxide radicals are generated as reduced Mb is oxidized to metMb (Brantley, Smerdon, Wilkinson, Singleton, & Olson, 1993). These superoxide radicals readily dismutate to H_2O_2 that reacts with deoxyMb and metMb to form ferrylMb and perferrylMb, respectively (Carlsen, Mller, & Skibsted, 2005). SOD and CAT can be added during Mb autoxidation studies to remove H_2O_2 so that metMb can be measured spectrophotometrically without interference from ferryl Mb species. SOD and CAT were not used in our study due to the concern that added HNE could alkylate the added enzymes. The ability of ethanol to reduce ferrylMb to oxyMb with the production of acetaldehyde has been described (Harada, Tamura, & Yamazaki, 1986). Thus, added ethanol used to solubilize HNE can compromise the metMb determination. In addition, ethanol carrier can

298 disrupt structural attributes of Mb which is undesired when examining the prooxidative
299 characteristics of Mb in a lipid model.

300 HNE delivered via aqueous carrier became covalently bound to wild type (WT) Mb at 4
301 to 6 sites on the molecule based on ESI-MS. Two sites of adduction by HNE were determined
302 previously in WT Mb when ethanol carrier was utilized (Tatiborworntham, Faustman, Yin,
303 Ramanathan, Mancini, Suman, et al., 2012). It may be that using water as a carrier increases
304 the ability of HNE to form covalent adducts with Mb. Added HNE in water carrier did not
305 significantly increase metMb formation when examining WT Mb relative to non-HNE containing
306 controls. Previously, added HNE increased metMb formation in WT Mb when ethanol was used
307 as a carrier (Tatiborworntham, et al., 2012). This suggests that the type of carrier may affect
308 HNE-mediated oxidation of Mb.

309 After removal of unreacted HNE, adducted WT Mb promoted lipid oxidation more
310 effectively than non-adducted WT Mb. This may be the result of an increase in the hydrophobic
311 character of Mb that was adducted with HNE, displaying five carbon fatty acyl tails at each site
312 of adduction. The increased hydrophobicity increases the potential for interaction of the
313 adducted Mb with phospholipid substrates considering that adding a lipid tail to Mb caused Mb
314 to associate with a model phospholipid bilayer membrane (Hamachi, Nakamura, Fujita, &
315 Kunitake, 1993). Further support for this comes from the fact that Mb alkylated at a 14:1 ratio of
316 HNE to Mb demonstrated a greater ability to promote lipid oxidation in washed cod compared to
317 a 7:1 ratio of HNE (data not shown). It may be that alkylation at a higher aldehyde to Mb ratio
318 was more effective in increasing the hydrophobicity of Mb, thereby increasing the efficiency of
319 interaction of Mb with the lipid substrate, and thus the ability to promote lipid oxidation. Each
320 adduction can also disrupt interactions between amino acids of the globin and the porphyrin
321 moiety to accelerate lipid oxidation as will be described later in the discussion.

Alkylation of P88H/Q152H with HNE accelerated metMb formation compared to control reactions without added HNE (**Figure 3A**). The accelerated autooxidation of P88H/Q152H may be a consequence of decreased stabilization of the heme group by the F-helix containing His 88 adducts. It has been shown that His 88 was adducted by HNE in P88H/Q152H (Tatibaborworntham, et al., 2012). Three residues of the F helix stabilize the heme group (**Figure 1B**). Ser 92 (F7) stabilizes the heme-7-propionate through hydrogen bonding, Leu 89 (F4) stabilizes the δ meso bridge of the heme ring through hydrophobic interactions, and His 93 (F8) covalently binds the iron atom of the heme (Hargrove, Wilkinson, & Olson, 1996; Peterson, Friedman, Chien, & Sligar, 1998). There is also a hydrogen bond between Ser 92 and His 93. During Mb folding, the proline residue at position 88 (F3) is responsible for maintaining a flexible random coil-like character of the F region prior to heme incorporation into the apoprotein (Picotti, Marabotti, Negro, Musi, Spolaore, Zambonin, et al., 2004). Subsequent to heme incorporation, the F region adopts helical character and imparts a more compact aspect to the holoprotein. Furthermore, it was observed that replacement of the proline residue at position 88 (F3) with alanine in WT apo swMb significantly changed the structural character of the F region, introducing a higher percentage of helical character (Picotti, et al., 2004). Previous work by Chakrabartty, Kortemme, and Baldwin (1994) indicated that histidine is an intermediate helix-forming residue compared to proline (breaker) and alanine (former). It is possible then that replacement of Pro88 with His could alter the folding process, resulting in a holoprotein displaying greater solvent access to the heme crevice compared to WT swMb. This would permit increased access of the hydrophobic HNE molecule to the heme crevice, where it may interfere with the hydrophobic stabilization of the heme by residue Leu 89. HNE likely adducts to an amino acid in the protein when there is an adjacent hydrophobic core in which the 5 carbon acyl tail can reside. In this case, the hydrophobic character of the region between Leu89 and the δ meso bridge of the heme ring may serve as the core (**Figure 1B**). As a consequence

of the acyl tail seeking this core, the interaction between Leu 89 and the heme ring may be disrupted or weakened, leading to destabilization of the heme ring, and finally leading to weakening of the hydrogen bond between His64 and the oxygen ligand bound to the iron atom, which increases autooxidation (Brantley, Smerdon, Wilkinson, Singleton, & Olson, 1993). In addition to His 88 being adducted in P88H/Q152H, it was shown that His 152 is one of the residues adducted by HNE (Tatijaborworntham, et al., 2012). It is possible that further rearrangement of hydrophobic residues in the region where the ends of the F, G, and H helices are adjacent to each other may occur as a result of the acyl tail of the adducted HNE at His-152 (site HC3) seeking a hydrophobic core (**Figure 1A**). This reinforces the suggestion that the observed increase in Mb autooxidation is the result of HNE interference with heme stabilization.

Alkylation of P88H/Q152H with HNE accelerated lipid oxidation compared to non-adducted P88H/Q152H. The ability of metMb to react with peroxides generating ferryl Mb oxidants should depend on structural aspects of the distal heme pocket. The ability of HNE to adduct at His88 and associate near the δ meso bridge of the heme ring could alter the distal heme pocket so that the heme pocket is more readily accessible to peroxides. In addition, heme affinity relies on 16 amino acid contacts of the globin with the porphyrin ring in Mb (Hargrove, Wilkinson, & Olson, 1996). Four of these sites, Leu89, Ser92, Ala71 and Thr67 are in the vicinity of HNE adduction at His88 (**Figure 1B**) and the latter is likely to disrupt some of these contacts favoring lowered heme affinity. The ability of decreased heme affinity to enhance Mb-mediated lipid oxidation in washed cod due to disruption of the contact at Ala71 has been described (Cai & Richards, 2012).

WT Mb exposed to HNE was compared to P88H/Q152H exposed to HNE to directly examine the effect of additional histidines on oxidative capacity of WT Mb exposed to HNE. The greater extent of TBARS formation (without enhanced metMb formation) in the double mutant

treatment indicated that the two additional histidine residues had a stronger impact on HNE-facilitated lipid oxidation than HNE-mediated metMb formation (**Figure 4**).

HNE accelerated the *initial* phase of thiobarbituric acid reactive substances (TBARS) formation when utilizing H97A (site FG3) yet the *extent* of TBARS formation was greater in H97A not exposed to HNE. Replacement of His97 with the smaller alanine residue increases access of solvent to the proximal heme pocket which increased heme dissociation 39-fold, presumably due to enhanced protonation of the proximal histidine (Hargrove, Wilkinson, & Olson, 1996). H97A was previously shown to be uniquely adducted at His24 and His82 compared to WT Mb (Tatiborworntham, et al., 2012). Adduction of HNE at these sites may disrupt the tertiary structure of the protein leading to destabilization and acceleration of heme loss considering that adduction by HNE was shown to increase heme loss from H97A (Tatiborworntham, et al., 2012). Our assessments using pyMOL software showed His82 to be 11.2 Å from the heme moiety of WT Mb (pdb 1MBO) and the length of HNE adduct in fatty acid binding protein was 8.8 Å (pdb 3JS1) which suggests adducted HNE at His82 could disrupt the orientation of the heme moiety. Thus, the increased lipid oxidation demonstrated by HNE adducted H97A compared to non-adducted H97A may be due, in part, to increased heme loss from H97A adducted with HNE. The greater *extent* of TBARS in H97A not exposed to HNE may be due to HNE adduction causing such rapid heme loss that released heme decomposes preformed lipid hydroperoxides (LOOH) to produce an abundance of lipid radicals that degrade the heme ring so that the extent of heme-mediated TBARS formation was suppressed. The ability of lipid radicals resulting from LOOH breakdown to degrade the heme ring has been described (Nagababu & Rifkind, 2004). The amount of degraded heme ring in the washed muscle was not measured for these studies but such methods are available (S. K. Lee, Tatiborworntham, Grunwald, & Richards, 2015). The decreased lipid peroxides in adducted

395 H97A compared to non-adducted HNE is consistent with HNE facilitating heme loss that leads
396 to breakdown of LOOH to lipid radicals. Overall these results suggest that the combination of
397 HNE and low heme affinity facilitated rapid decomposition of LOOH to lipid radicals that
398 promoted lipid oxidation and degraded the heme ring.

399 It must also be recognized that interpretation of TBARS values at later stages of storage
400 after extensive lipid oxidation has occurred, should be done with caution. An alternative
401 explanation would be that HNE binding to H97A caused a relatively rapid formation of
402 secondary lipid oxidation products that readily became bound to myofibrillar proteins of washed
403 cod during the later stages of storage, rather than being available to react with TBA reagent to
404 generate TBARS. This scenario could occur without a requirement of increased heme
405 degradation occurring in the H97A+HNE treatment.

406 HNE and HHE (4-hydroxy-2-hexenal) facilitated Mb oxidation and Mb-mediated lipid
407 oxidation similarly. It appears that when the hydrophobicities of the aldehydes differ by three
408 CH₂ units, any enhancement of hydrophobicity imparted by HNE does not differ enough to
409 significantly affect the prooxidative character of the Mb molecule in washed cod. The degree of
410 alkylation by each aldehyde should also be considered. ESI-MS indicated one additional adduct
411 by the smaller HHE so that the net pro-oxidative effect towards Mb was about the same when
412 comparing HHE and HNE. It was shown that alkylation of horse heart Mb by aldehydes varying
413 in length from 6 carbons through 10 carbons all enhanced TBARS formation in liposomes
414 approximately the same amount compared to control (Lynch & Faustman, 2000).

415 The hypothesis that increased histidine content of bovine Mb (compared to porcine Mb)
416 increases the ability of HNE to exacerbate oxidative properties of Mb has been examined using
417 site-directed mutagenesis in which mutated sperm whale Mb containing histidine at site 88 and
418 152 (bovine-like) is compared to the wild-type sperm whale Mb that contains proline and

glutamine at those respective sites (porcine-like). Our findings do establish that His⁸⁸ and His¹⁵² increases metMb formation and lipid oxidation capacity while the presence of Pro⁸⁸ and Gln¹⁵² protects Mb from oxidative effects of HNE.

5. Conclusion

Additional histidine residues in the primary structure of Mb increased HNE-mediated lipid oxidation. Adduction of histidine residues in Mb by HNE likely increased Mb-mediated lipid oxidation by disrupting contacts between the heme moiety and neighboring amino acids of the globin. Future work should examine crystal structures of Mb adducted with HNE in order to better elucidate the mechanisms by which HNE increases oxidative characteristics of the heme protein. Investigating the ability of HNE to enhance formation of ferryl- and perferryl Mb is also warranted.

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References

- Alderton, A. L., Faustman, C., Liebler, D. C., & Hill, D. W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry*, 42, 4398-4405.
- Antoni, E., & Brunoni, M. (1971). *Hemoglobin and myoglobin in their reactions with ligands*. Amsterdam, The Netherlands: North-Holland Publ. Co.

444 Bolgar, M. S., & Gaskell, S. J. (1996). Determination of the sites of 4-hydroxy-2-nonenal
 445 adduction to proteins by electrospray tandem mass spectrometry. *Analytical*
 446 *Biochemistry*, 68, 2325-2330.

447 Brantley, R. E., Smerdon, S. J., Wilkinson, A. J., Singleton, E. W., & Olson, J. S. (1993). The
 448 mechanism of autooxidation of myoglobin. *Journal of Biological Chemistry*, 268, 6995-
 449 7010.

450 Cai, H., & Richards, M. P. (2012). Site E14 in hemoglobins and myoglobins: A key residue that
 451 affects heme loss and lipid oxidation capacity *Journal of Agriculture and Food*
 452 *Chemistry*, 60, 7729-7734.

453 Carlsen, C. U., Mller, J. K. S., & Skibsted, L. H. (2005). Heme-iron in lipid oxidation-A Review.
 454 *Coordination Chemistry Reviews*, 249, 485-498.

455 Chakrabarty, A., Kortemme, T., & Baldwin, R. L. (1994). Helix propensities of the amino acids
 456 measured in alanine-based peptides without helix-stabilizing side-chain interactions.
 457 *Protein Science*, 3, 843-852.

458 Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-
 459 hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology &*
 460 *Medicine*, 11, 81-128.

461 Faustman, C., Liebler, D. C., McClure, T. D., & Sun, Q. (1999). alpha,beta-unsaturated
 462 aldehydes accelerate oxymyoglobin oxidation. *Journal of Agriculture and Food*
 463 *Chemistry*, 47, 3140-3144.

464 Grunwald, E. W., & Richards, M. P. (2006). Studies with myoglobin variants indicate that
 465 released heme is the primary promoter of lipid oxidation in washed fish muscle. *Journal*
 466 *of Agriculture and Food Chemistry*, 54, 4452-4460.

467 Hamachi, I., Nakamura, K., Fujita, A., & Kunitake, T. (1993). Anisotropic incorporation of lipid-
 468 anchored myoglobin into a phospholipid bilayer membrane. *Journal of the American Oil*
 469 *Chemist Society*, 115, 4966-4970.

470 Harada, K., Tamura, M., & Yamazaki, I. (1986). The 2-electron reduction of sperm whale ferryl
 471 myoglobin by ethanol. *Journal of Biochemistry*, 100, 499-504.

472 Hargrove, M. S., Wilkinson, A. J., & Olson, J. S. (1996). Structural factors governing heme
 473 dissociation from metmyoglobin. *Biochemistry*, 35, 11300-11309.

474 Lee, S., Phillips, A. L., Liebler, D. C., & Faustman, C. (2003). Porcine oxymyoglobin and lipid
 475 oxidation in vitro. *Meat Science*, 63, 241-247.

476 Lee, S. K., Tatiyaborworntham, N., Grunwald, E. W., & Richards, M. P. (2015). Myoglobin and
 477 haemoglobin-mediated lipid oxidation in washed muscle: Observations on crosslinking,
 478 ferryl formation, porphyrin degradation, and haemin loss rate. *Food Chemistry*, 167, 258-
 479 263.

480 Littell, R. C., Milliken, G. A., Stroup, W. W., & Wolfinger, R. D. (2000). Analysis of repeated
 481 measures data. In *SAS system for mixed models* 4th ed., (pp. 87-134). Cary, NC: SAS
 482 Institute Inc.

483 Lynch, M. P., & Faustman, C. (2000). Effect of aldehyde lipid oxidation products on myoglobin.
 484 *Journal of Agriculture and Food Chemistry*, 48, 600-604.

485 Nagababu, E., & Rifkind, J. M. (2004). Heme degradation by reactive oxygen species.
 486 *Antioxidant and Redox Signaling*, 6, 967-978.

487 Peterson, E. S., Friedman, J. M., Chien, E. Y., & Sligar, S. G. (1998). Functional implications of
 488 the proximal hydrogen-bonding network in myoglobin: a resonance Raman and kinetic
 489 study of Leu89, Ser92, His97, and F-helix swap mutants. *Biochemistry*, 37, 12301-
 490 12319.

491 Phillips, S. E. (1980). Structure and refinement of oxymyoglobin at 1.6 Å resolution. *Journal of*
 492 *Molecular Biology*, 142, 531-554.

493 Picotti, P., Marabotti, A., Negro, A., Musi, V., Spolaore, B., Zambonin, M., & Fontana, A. (2004).
 494 Modulation of the structural integrity of helix F in apomyoglobin by single amino acid
 495 replacements. *Protein Science*, 13, 1572-1585.

496 Poli, G., Schaur, R. J., Siems, W. G., & Leonarduzzi, G. (2008). 4-hydroxynonenal: a membrane
 497 lipid oxidation product of medicinal interest. *Medicinal Research Reviews*, 28, 569-631.

498 Sakai, T., Kuwazuru, S., Yamauchi, K., & Uchida, K. (1995). A lipid peroxidation-derived
 499 aldehyde, 4-hydroxy-2-nonenal and omega 6 fatty acids contents in meats. *Bioscience*
 500 *Biotechnology and Biochemistry*, 59, 1379-1380.

501 Sakai, T., Munasinghe, D. M., Kashimura, M., Sugamoto, K., & Kawahara, S. (2004). Effect of
 502 NaCl on lipid peroxidation-derived aldehyde, 4-hydroxy-2-nonenal formation in minced
 503 pork and beef. *Meat Science* 66, 789-792.

504 Sakai, T., Yamauchi, K., Kuwazuru, S., & Gotoh, N. (1998). Relationships between 4-hydroxy-2-
 505 nonenal, 2-thiobarbituric acid reactive substances and n-6 polyunsaturated fatty acids in
 506 refrigerated and frozen pork. *Bioscience Biotechnology and Biochemistry*, 62, 2028-
 507 2029.

- Suman, S. P., Faustman, C., Stamer, S. L., & Liebler, D. C. (2006). Redox instability induced by 4-hydroxy-2-nonenal in porcine and bovine myoglobins at pH 5.6 and 4 degrees C. *Journal of Agriculture and Food Chemistry*, 54, 3402-3408.
- Suman, S. P., Faustman, C., Stamer, S. L., & Liebler, D. C. (2007). Proteomics of lipid oxidation-induced oxidation of porcine and bovine oxymyoglobins. *Proteomics*, 7, 628-640.
- Tang, J., Faustman, C., & Hoagland, T. A. (2004). Krzywicki Revisited: Equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts. *Journal of Food Science*, 69, C717-C720.
- Tatiyaborworntham, N., Faustman, C., Yin, S., Ramanathan, R., Mancini, R. A., Suman, S. P., Beach, C. M., Maheswarappa, N. B., Grunwald, E. W., & Richards, M. P. (2012). Redox instability and heme loss of mutant sperm whale myoglobins induced by 4-hydroxynonenal in vitro. *Journal of Agriculture and Food Chemistry*. 60, 8473-8483

List of Figures

Figure 1. (A) Image of sperm whale myoglobin with site FG3 (histidine⁹⁷), F3 (proline⁸⁸), and HC3 (glutamine¹⁵²) shown in stick representation. The distal histidine (site E7) and proximal histidine (site F8) are shown above and below the heme ring, respectively, with the heme group (protoporphyrin IX) also shown in stick representation. The E-, F-, G-, and H-helix are labeled. Image was constructed in pymol using PDB 1MBO (Phillips, 1980). **(B)** Illustration of amino acid residues along the E- and F-helix that have bonding contacts with the heme moiety which can be disrupted upon adduction by HNE at site F3. Thr⁶⁷(site E10), Ala⁷¹(site E14), Pro⁸⁸(site F3), Leu⁸⁹(site F4), Ser⁹²(site F7), His⁹³(site F8) and the heme moiety of WT sperm whale Mb are shown in stick representation. Proline⁸⁸ is shown to illustrate where the histidine is located in bovine Mb and the P88H/Q152H Mb mutant, which can become adducted with HNE.

Figure 2. Effect of alkylation of wild-type sperm whale Mb (WT Mb) by aqueous HNE on TBARS **(A)** and lipid peroxides **(B)** in washed cod muscle during iced storage at pH 5.7. Unreacted HNE was removed by desalting before addition to washed cod muscle. Mb concentration was 40 µmo/kg washed cod. Error bars fall within the symbols at some time

points. **(C)** MetMb formation during reaction of Mb (0.225 mM) and aqueous HNE (1.6 mM) for 78 hr at 4°C and pH 5.6 compared to control reactions without HNE.

Figure 3. **(A)** MetMb formation during reaction of P88H/Q152H (0.225 mM) and aqueous HNE (1.6 mM) for 76 hr at 4°C and pH 5.7 compared to control reactions without HNE. Unreacted HNE was removed by desalting before addition to washed cod muscle. Effect of alkylation of P88H/Q152H by aqueous HNE on TBARS **(B)** and lipid peroxides **(C)** in washed cod muscle during iced storage at pH 5.7. Mb concentration was 40 µmo/kg washed cod. Error bars fall within the symbols at some time points.

Figure 4. **(A)** Effect of alkylation of WT Mb and P88H/Q152 Mb by aqueous HNE on TBARS in washed cod muscle during iced storage at pH 5.6. Unreacted HNE was removed by desalting before addition to washed cod muscle. Mb concentration was 40 µmo/kg washed cod. Error bars fall within the symbols at some time points. **(B)** MetMb formation during reaction of Mb (0.225 mM) and aqueous HNE (1.6 mM) for 90 hr at 4°C and pH 5.6 compared to control reactions without HNE. Asterix indicates $p < 0.05$ at that time point.

Figure 5. Effect of alkylation of H97A Mb by aqueous HNE on TBARS **(A and D)** and lipid peroxides **(B and E)** in washed cod muscle during iced storage at pH 5.6. Unreacted HNE was removed by desalting before addition to washed cod muscle. Mb concentration was 40 µmo/kg washed cod. Error bars fall within the symbols at some time points. **(C and F)** MetMb formation during reaction of Mb (0.225 mM) and aqueous HNE (1.6 mM) for 72 hr at 4°C and pH 5.6 compared to control reactions without HNE. Asterix indicates $p < 0.05$ at that time point.

Figure 6. (A) Effect of alkylation of WT sw Mb by aqueous HNE and HHE on TBARS in washed cod muscle during iced storage at pH 5.6. Unreacted HNE and HHE were removed by desalting before addition to washed cod muscle. Mb concentration was 40 $\mu\text{mo/kg}$ washed cod. Error bars fall within the symbols at some time points. **(B)** MetMb formation during reaction of Mb (0.225 mM) and aqueous HNE or HHE (1.6 mM) for 88 hr at 4°C and pH 5.6 compared to control reactions without HNE.

A

B

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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606 **Figure 6**

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