JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Redox Instability and Hemin Loss of Mutant Sperm Whale Myoglobins Induced by 4-Hydroxynonenal in Vitro

Nantawat Tatiyaborworntham,[†] Cameron Faustman,*[,†] Shuang Yin,[†] Ranjith Ramanathan,[†] Richard A. Mancini,[†] Surendranath P. Suman,[§] Carol M. Beach,[⊗] Naveena B. Maheswarappa,[⊥] Eric W. Grunwald,[#] and Mark P. Richards[#]

[†]Department of Animal Science, University of Connecticut, Storrs, Connecticut 06269, United States

[§]Department of Animal and Food Sciences and [⊗]Proteomics Core Facility, University of Kentucky, Lexington, Kentucky 40546, United States

[⊥]National Research Centre on Meat, Chengicherla Hyderabad, Andhra Pradesh 500039, India

[#]Meat Science and Muscle Biology Laboratory, Department of Animal Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: The effects of 4-hydroxy-2-nonenal (HNE) on redox stability of Oxy- and Deoxy- wild-type (WT) and recombinant sperm whale myoglobins (P88H/Q152H, L29F, H97A, and H64F) and hemin loss from Met-myoglobin (Mb) were investigated. HNE induced greater redox instability in WT and mutant Mbs compared to controls ($p < 0.05$). The extent of HNE-induced OxyMb oxidation was lesser in L29F ($p < 0.05$) and greater in H97A and P88H/Q152H than in WT ($p < 0.05$). H64F DeoxyMb was more redox stable than WT DeoxyMb in the presence of HNE ($p < 0.05$). HNE alkylation occurred exclusively on histidine residues, and histidine 48 was alkylated in all sperm whale myoglobins. HNE alkylation accelerated the protoporphyrin moiety loss only in H97A. Met- forms of WT and L29F but not Deoxy- or Oxy- forms released hemin during storage. Primary structure strongly influenced Mb redox stability in the presence of reactive secondary lipid oxidation products.

KEYWORDS: *myoglobin, mutant, hemin, redox stability, HNE*

INTRODUCTION

Myoglobin (Mb) is a sarcoplasmic protein pigment in meat that is responsible for color. During storage, the cherry-red color of fresh red meat turns brown with the autoxidation of ferrous oxymyoglobin (OxyMb) to ferric metmyoglobin (MetMb). Under low oxygen partial pressure conditions (e.g., vacuum-packed meat; deep portions of post-mortem muscles), ferrous Mb is generally present in the deoxygenated form (DeoxyMb) and has a purple color. Exposure of DeoxyMb to oxygen results in cherry-red OxyMb. The redox stability of ferrous Mbs can be impaired by many factors including reactive aldehydes derived from lipid oxidation.[1−4]

In fresh meat, the oxidation of lipids and ferrous Mbs appears to be positively correlated.[5−7] Vitamin E-supplemented beef demonstrated slowed formation of thiobarbituric reactive substances and improved OxyMb stability.[7−9] 4-Hydroxy-2-nonenal (HNE) is an α,β-unsaturated aldehyde derived from the oxidation of ω-6 polyunsaturated fatty acids (e.g., linoleic acid), which are present abundantly in membrane phospholipids of post-mortem muscle. The double bond between carbons 2 and 3 of HNE is located in the vicinity of the carbonyl group and hydroxyl group at carbon 4 acting as electron-withdrawing forces that induce polarization of the double bond of HNE, contributing to its highly electrophilic nature.[10−13] Studies have demonstrated the electrophilicity of HNE toward nucleophilic amino acid side chains of cysteine, lysine, and histidine.[11,13] To date, sites of HNE alkylation via Michael addition have been identified exclusively on histidine residues of equine,[2] bovine,[14] porcine,[15,16] yellowfin tuna,[17] and chicken[18] Mbs. HNE alkylation of proteins can cause local or potentially global unfolding of Mb structure.[14] Changes in the structure of Mb, especially at the hydrophobic heme pocket, could predispose ferrous heme to solvent exposure and oxidation.[19]

Suman et al.[16] hypothesized that a greater number of histidine residues and their locations in the primary sequence of bovine Mb (13 histidine residues) contributed to its greater vulnerability to alkylation by HNE relative to porcine Mb (9 histidine residues). Histidine residues present on the protein surface should be more accessible than those buried within the macromolecule, and HNE alkylation of the proximal (HIS 93) and distal (HIS 64) histidine residues could destabilize heme−globin interactions directly and enhance oxidation. Suman and co-workers[16] reported that the number of HNE adducts was proportional to the number of histidine residues. Liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) revealed that HIS 88 and 152 were preferentially alkylated by HNE in bovine Mb; however, these residues are not present in porcine Mb. The observation suggested that bovine Mb could be more susceptible to HNE-

Received: April 29, 2012
Revised: August 3, 2012
Accepted: August 9, 2012
Published: August 9, 2012

enhanced OxyMb oxidation than porcine Mb because these two locations were readily accessible to HNE.

Myoglobins from several red meat-producing species share considerable homology in their primary sequences.[20] Small variations in their primary sequences, especially those in close proximity to the heme pocket, could be expected to alter the autoxidation rate,[21,22] heme affinity,[23] and structural stability.[24,25] Joseph et al.[20] reported that the primary sequences of cattle (*Bos taurus*) and bison (*Bison bison*) Mbs are identical, and their thermal stability, autoxidation rate, and HNE-induced oxidation rate tested in vitro were not statistically different. Mutant Mbs provide a logical model for investigating the effects of amino acid substitutions on autoxidation and redox stability in the presence of lipid breakdown products. Sperm whale Mb has been studied extensively, and recombinant sperm whale Mbs have been employed to elucidate the interactions of amino acid residues present in the vicinity of the heme moiety on Mb redox stability.[26−30] Richards et al.[31] studied the effect of sperm whale OxyMb oxidation rate on its ability to promote lipid oxidation in washed cod muscle minces using Mb mutant L29F (leucine 29 replaced by phenylalanine). L29F exhibited greater redox stability and was less effective at promoting lipid oxidation than wild-type Mb (WT). Moreover, they used H97A (histidine 97 substituted by alanine), a mutant in which hemin (ferriprotoporphyrin IX) is bound more weakly than in WT, to test the effect of hemin release on lipid breakdown and found that greater hemin dissociation of H97A led to greater lipid oxidation compared to WT. Tang et al.[30] substituted phenylalanine with a larger hydrophobic side chain for the distal histidine 64 in H64F (histidine 64 replaced by phenylalanine) and showed that it could block solvent entry and slow heme (ferroprotoporphyrin IX) release rate in the presence of guanidinium chloride or at low pH.

Understanding the mechanisms by which HNE alkylation affects Mb stability is important for comprehending oxidation interactions between lipids and proteins, in general, and Mb, specifically. The use of mutant Mbs could provide insight into the effects of primary protein structure on HNE alkylation. The objectives of this study were (i) to investigate the effect of HNE on redox stability of WT and mutant sperm whale OxyMbs and (ii) to characterize the extent and sites of HNE alkylation in these Mbs.

MATERIALS AND METHODS

Chemicals. Agar, tryptone, yeast extract, sodium citrate and kanamycin were purchased from Fisher Scientific (Pittsburgh, PA, USA). HNE was obtained from Cayman Chemical (Ann Arbor, MI, USA). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), dithiothreitol, disodium ethylenediaminetetraacetic acid (EDTA) dihydrate, lysozyme, ferric chloride ($FeCl_3$), chloramphenicol, sodium hydrosulfite, DNase I, RNase A, toluene sulfonyl chloride, hemin chloride, antifoam, α-cyano-4-hydroxycinnamic acid, and horse heart Mb were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Promega (Madison, WI, USA). All other chemicals used were of analytical grade.

Wild-Type and Mutant Sperm Whale Myoglobins. Besides WT, a new double-mutant sperm whale Mb, P88H/Q152H (proline 88 and glutamine 152 both substituted by histidine residues), was expressed and used to investigate the effect of histidine quantity on Mb susceptibility to HNE alkylation. L29F,[29,31] H97A,[23,32,33] and H64F[29,30] were also included in this study.

The identities of mutant sperm whale Mbs are based on their position in the primary sequence of the native sperm whale Mb. However, compared with native sperm whale Mb (153 amino acid residues), recombinant sperm whale Mbs (154 amino acid residues) contain one additional methionine residue at the N-terminus.[29] Thus, the actual positions of the amino acid residues in mutant sperm whale Mbs are shifted forward by one position.

WT and mutant (P88H/Q152H, L29F, H97A, and H64F) sperm whale Mbs were prepared at the Meat Science and Muscle Biology Laboratory, University of Wisconsin—Madison, as described by Grunwald and Richards[23,33] and Richards et al.[31] Mutant Mb genes were prepared by site-directed mutagenesis using the WT sperm whale Mb gene on the pET-28 plasmid (Novagen, Madison, WI, USA) as template. The mutagenesis was confirmed by dideoxy sequencing at the University of Wisconsin Biotechnology Center (Madison, WI, USA). The constructed plasmid was then transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RP host cells (Stratagene, La Jolla, CA, USA) via heat shock. Recombinant Mbs were expressed in host *E. coli* cells using a 12 L culture vessel (Nalge Nunc International, Rochester, NY, USA) 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. The expression was performed by transferring a single colony of the transformed *E. coli* to 12 mL of TB and incubating for 14−16 h in a 37 °C shaker. The 12 mL culture was transferred into 360 mL of TB and incubated for 4 h in a 37 °C shaker to produce a starter culture. The starter was 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 25 L/min and mixed at 250 rpm with a motorized impeller. When the OD_{600} of the culture reached approximately 2.0, IPTG was added to 1 mM to induce expression of the recombinant Mbs. During induction, hemin chloride was added to the culture to 4 mg/L. After 4 h of induction, cell paste containing the expressed Mbs was obtained by centrifuging the culture at 2000*g* for 15 min. The paste was frozen at −80 °C, followed by thawing and overnight lysis of the cells at 4 °C. The lysis buffer[34] contained 50 mM Tris base, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM toluenesulfonyl chloride, 40 U/mL DNase I, 3 U/mL RNase A, and 78800 U/mL lysozyme, adjusted to pH 6. The obtained Mbs were purified from the lysate via ammonium sulfate precipitation and anion and cation exchange chromatography as described previously.[28,35] Purified Mbs were concentrated to 0.5−1.0 mM, snap frozen in liquid nitrogen, and stored either in liquid nitrogen or at −80 °C.

Oxymyoglobin Preparation. Wild-type and mutant (P88H/Q152H, L29F, and H97A) sperm whale Mb protein pellets were thawed in an ice bucket and resuspended in 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.4. OxyMb solutions were prepared by sodium hydrosulfite-mediated reduction using a molar ratio (Mb/sodium hydrosulfite) of 1:10.[36] Residual hydrosulfite was removed by passing the Mb solutions through PD-10 desalting columns (Sephadex G25, 0.15% Kathon preservative CG, GE Health Care, Uppsala, Sweden) precalibrated with 50 mM sodium citrate buffer, pH 5.6. The reduced Mb was converted to OxyMb upon exposure to dissolved oxygen in the column buffer and to atmospheric oxygen while swirling. OxyMb concentration was calculated using a millimolar extinction coefficient at 525 nm (E = 7.6 cm^{-1} mM^{-1}).[37,38] The percentage of OxyMb was determined spectrophotometrically according to the method of Tang et al.[39]

Deoxymyoglobin Preparation. DeoxyMb solutions of WT and H64F Mbs were prepared anaerobically in an airtight glovebag (Sigma Chemical Co.). Nitrogen gas was of ultralow oxygen grade (Airgas East, Cheshire, CT, USA). The glovebag was connected to an aspirator to remove all air and followed by nitrogen flushing. The cycle of removing air and flushing with nitrogen was repeated three times (3 volumes) until the measurable oxygen partial pressure (pO_2) became approximately 3 mmHg as measured by a model 6600 headspace oxygen/carbon dioxide analyzer (Illinois Instruments, Chicago, IL, USA). A 50 mM sodium citrate buffer, pH 5.6, was warmed to 40 °C, degassed, and allowed to cool in the glovebag. Myoglobin (0.08 mM; 5 mL) was chemically reduced by sodium hydrosulfite (0.1 mM; 0.4 mL) and immediately passed through a PD-10 desalting column pretreated with 2.5 mL of 0.1 mM sodium hydrosulfite to remove dissolved oxygen in the column and further calibrated by the degassed

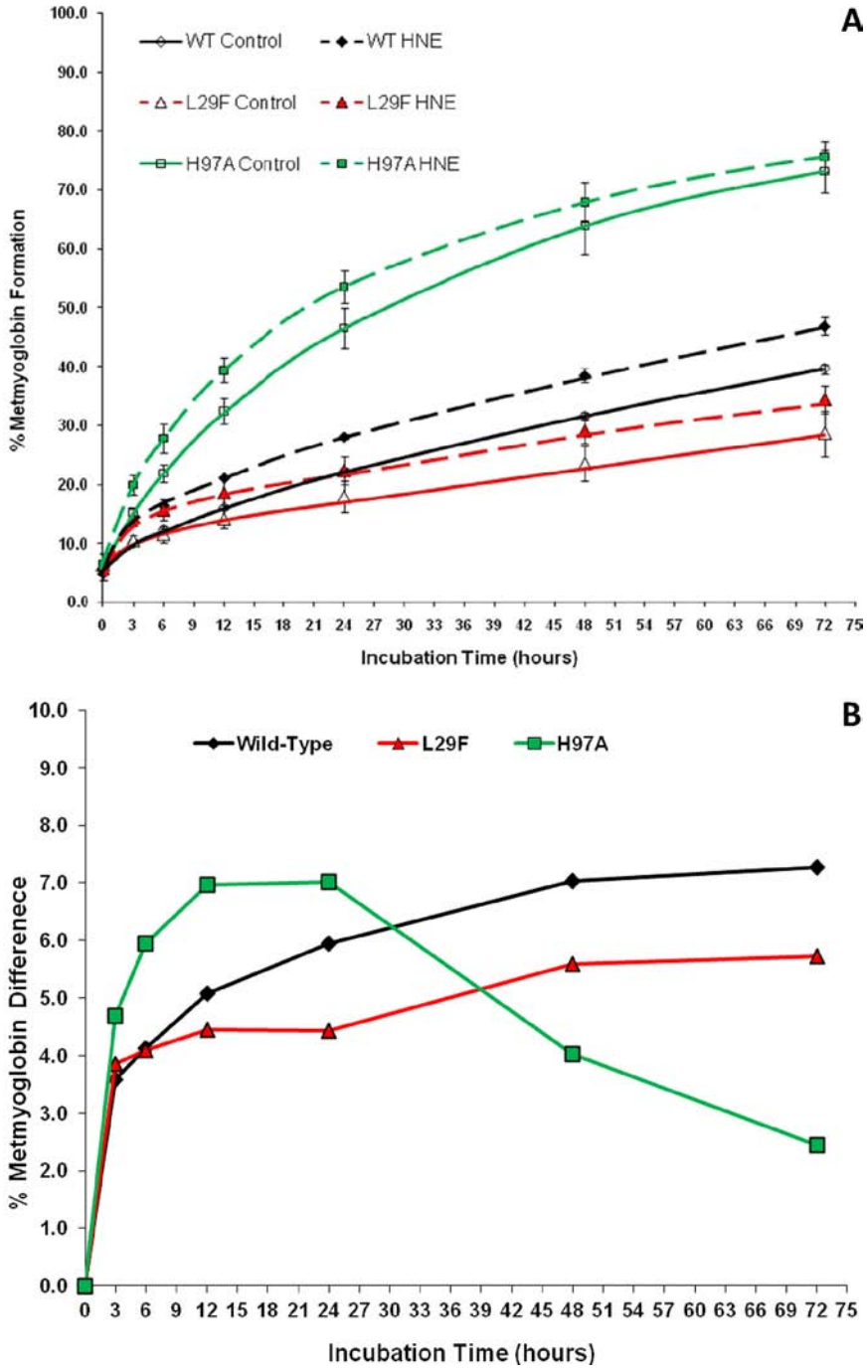

Figure 1. Effect of HNE (0.42 mM) on OxyMb oxidation of mutant sperm whale myoglobins (0.06 mM) at pH 5.6, 4 °C: (A) %MetMb formation; (B) %MetMb difference between HNE-treated myoglobin and their respective controls.

buffer for 30 min to remove residual sodium hydrosulfite. The eluent from the column was collected into a screw-capped quartz cuvette and flushed with nitrogen. Cuvettes were firmly capped immediately following nitrogen flushing. The concentration of the prepared DeoxyMb was calculated using a millimolar extinction coefficient at 525 nm (E = 7.6 cm^{-1} mM^{-1}).[38,39] The percentage of DeoxyMb was determined spectrophotometrically according to the method of Tang et al.[39]

Hemin Loss from Mixtures of OxyMb and MetMb. Preparation of hemin loss reagent and the hemin loss assay followed the procedure of Grunwald and Richards.[23] A modification was needed because the Mb solutions examined were mixtures of OxyMb and MetMb. MetMb at the initiation of hemin loss was 51−85% depending on the mutant under examination and whether the Mb was alkylated with HNE or not (i.e., control). The oxidation of OxyMb to MetMb is accompanied by increased absorbance at 600 nm, which obscures detection of hemin transfer from Mb to the apoH64Y/V68F. HoloH64Y/V68F (containing bound hemin) has a characteristic absorbance at 600 nm that is used to quantify hemin transfer. Thus, the blank used in spectrophotometric analysis contained the partially oxidized Mb, sucrose, and buffer to account for this interference.

Reaction with HNE. Freshly prepared ferrous Mb solutions (OxyMb or DeoxyMb; 0.06 mM) were incubated with HNE (0.42 mM) at pH 5.6 and 4 °C in screw-capped polypropylene conical tubes (30 × 115 mm) for OxyMb or in screw-capped quartz cuvettes for DeoxyMb. The selected concentration of Mb reflected that reported in pork[40] and was adopted to optimize the limited supply of the mutant Mbs and to be consistent with that used by Suman et al.[16] The molar

ratio of OxyMb to HNE was maintained at 1:7, and ethanol was delivered to controls at volumes equivalent to that used for delivering HNE[2] (approximately 8 μL of ethanol/mL of Mb). All experiments were performed at 4 °C, pH 5.6.

For the experiment with WT, L29F, and H97A OxyMbs, the reaction with HNE occurred over 72 h and the MetMb formation was measured at 0, 3, 6, 12, 24, 48, and 72 h. For the experiment with WT and P88H/Q152H OxyMbs, MetMb formation was determined at 0, 3, 6, 12, 18, and 24 h; the reaction was continued to 72 h to secure samples for mass spectrometry analysis. MetMb formation was determined at 0, 1, 2, 3, 4, 6, 8, 12, 18, and 24 h for WT and H64F DeoxyMbs. Myoglobin solutions were scanned spectrophotometrically from 650 to 400 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu, Columbia, MD, USA). MetMb formation was calculated according to the equation given in ref 39. The %MetMb difference was calculated as the difference in %MetMb between HNE-treated samples and their respective controls and expressed as $\%MetMb_{HNE} - \%MetMb_{Control}$. The %Relative MetMb formation was the difference in %MetMb at each time point relative to the initial value at 0 time and expressed as $\%MetMb_t - \%MetMb_{t=0}$.

To prepare samples for mass spectrometry analysis, 1 mL of sample was taken from the reaction mixture (at 24 and 72 h for OxyMb experiments or at 24 h for DeoxyMb experiment) and passed through a PD-10 desalting column pretreated with deionized water to remove unreacted HNE. The 1 mL eluent was collected into two separate (0.5 mL each) capped cryogenic storage vials (Fisher Scientific) and stored at −80 °C until further analysis.

Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) Mass Spectrometry and MALDI-TOF-TOF Tandem Mass Spectrometry (MS/MS). Mass spectrometric analyses were performed at the University of Kentucky, Center for Structural Biology Protein Core Facility. Intact proteins were analyzed by MALDI-TOF mass spectrometry, and masses were measured in the linear mode (500 shots/spectrum) using saturated α-cyano-4-hydroxycinnamic acid as the matrix. Myoglobin (1−5 μL) was digested in 10 mM ammonium bicarbonate + 80% acetonitrile containing 2 ng/μL proteomic grade trypsin (Sigma Chemical Co.) at 37 °C for 1 h. Acetonitrile (CH_3CN) was removed by vacuum centrifugation for 10 min, and the peptides were desalted and concentrated by solid-phase extraction using a 0.1−10 μL pipet tip (Sarstedt, Newton, NC, USA) packed with approximately 1 mm Empore C-18 (3M, St. Paul, MN, USA). Peptides were eluted in 5 μL of 50% CH_3CN and 0.1% trifluoroacetic acid. Desalted peptide extracts were spotted onto an Opti-TOF 384 well insert (Applied Biosystems, Foster City, CA, USA) with 0.3 μL of 5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/50% 0.1% trifluoroacetic acid and made 10 mM in ammonium dihydrogen phosphate. Crystallized samples were washed with cold 0.1% trifluoroacetic acid and analyzed in a 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems/MDS Sciex, Foster City, CA). An initial MALDI-MS spectrum was acquired for each spot (400 laser shots per spectrum). Peaks with a signal-to-noise ratio of >20 were selected for MS/MS analysis (1000 shots per spectrum) by postsource decay or by collision-induced dissociation using air at a pressure of 6.67×10^{-5} N/m^2. Data acquired were manually inspected by comparing theoretical b- and y-ion series (generated by MS-Product web program) with the obtained spectra.

Statistical Analysis. The experimental design was a three-way (mutant Mb type × treatment × incubation time) randomized complete block design in which each treatment had three replications. The block was defined as each replication that was prepared within 1 h followed by the next replications except for DeoxyMb studies, which were repeated each day (block = day). The obtained data were analyzed using the Proc Mixed procedure of SAS (version 9.1, SAS Institute, Inc., Cary, NC, USA). Least-squares means were generated, and the differences among treatments were significant at $p < 0.05$ using least significant differences (LSD).

RESULTS AND DISCUSSION

Effects of HNE on Autoxidation of WT and Mb Mutants. The effect of HNE on MetMb formation in WT, L29F, and H97A Mbs is presented in Figure 1A. OxyMb autoxidation followed the order H97A > WT > L29F ($p < 0.05$), and HNE enhanced MetMb formation in all Mbs ($p < 0.05$). However, the relative susceptibilities of the mutant Mbs differed (Figure 1B). Within the first 24 h of incubation, the effect of HNE on redox stability of OxyMbs was greatest in H97A, followed by WT and L29F (H97A > WT > L29F; $p < 0.05$). After 24 h, controls and HNE-treated samples of H97A oxidized readily, and their difference in %MetMb became smaller. This was presumably due to the weaker hemin affinity of H97A, compared with WT,[31] that renders H97A more redox-unstable even in the absence of HNE. The relatively small methyl group of alanine was expected to be less effective in preventing hydration of the hydrophobic heme pocket compared to the imidazole group of histidine. The access of water to the heme pocket can interrupt hydrophobic interactions and weaken the coordinate bond between the imidazole side chain of the proximal histidine (HIS 93) and heme iron.[23,41] As a result, the globin may become slightly unfolded and histidine residues that are normally buried within the heme pocket are exposed to HNE and subject to alkylation, which would lead to enhanced heme oxidation.

Richards et al.[31] demonstrated that L29F was 10-fold more redox-stable than its WT counterpart. The authors suggested that the presence of a larger hydrophobic side chain prevents hydration of the hydrophobic heme pocket, a process that would destabilize globin−heme interactions and lead to heme iron oxidation. In addition, the electron delocalization of the phenyl ring of phenylalanine 29 in L29F can stabilize the bound oxygen, whereas the alkyl side chain of LEU 29 in WT cannot.[31] Our results in Figure 1 confirmed this suggestion as L29F was the most redox stable of the OxyMbs investigated both in the presence and in the absence of HNE.

Despite many attempts to accomplish a relatively low initial %MetMb for P88H/Q152H, the initial %MetMb level for this mutant could not be achieved below 30%. Thus, WT OxyMb was prepared so as to achieve an initial %MetMb as close to that of P88H/Q152H as possible. However, the initial %MetMb values of these Mbs still differed by 10% (Figure 2A). Hence, relative %MetMb ($\%MetMb_t - \%MetMb_{t=0}$) was calculated and is presented in Figure 2B. It is important to note that P88H/Q152H had an autoxidation rate comparable to that of WT ($p > 0.05$). The double mutation present in P88H/Q152H, in which histidine residues were substituted at positions 88 and 152, appeared to be more affected by HNE than WT following 18 h of incubation (Figure 2A,C). Turbidity was observed after 24 h of incubation, and this solubility problem has been noted in native hemoglobin solutions treated with HNE.[42] Samples for mass spectrometry were acquired at 72 h to provide for comparisons of alkylation with the other mutants. The insoluble particles that contained Mb and caused turbidity were removed by centrifugation.

As the partial pressure of oxygen decreases, the autoxidation rate of Mb increases and reaches its maxima when approximately 50% of Mb remains in the oxygenated form (pO_2 = 5 mmHg);[43] when oxygen is completely absent, 100% of the Mb is in the deoxygenated form.[29] In this study, oxygen (pO_2 = 3 mmHg) could not be eliminated completely from the glovebag or the headspace of the capped quartz cuvettes, and

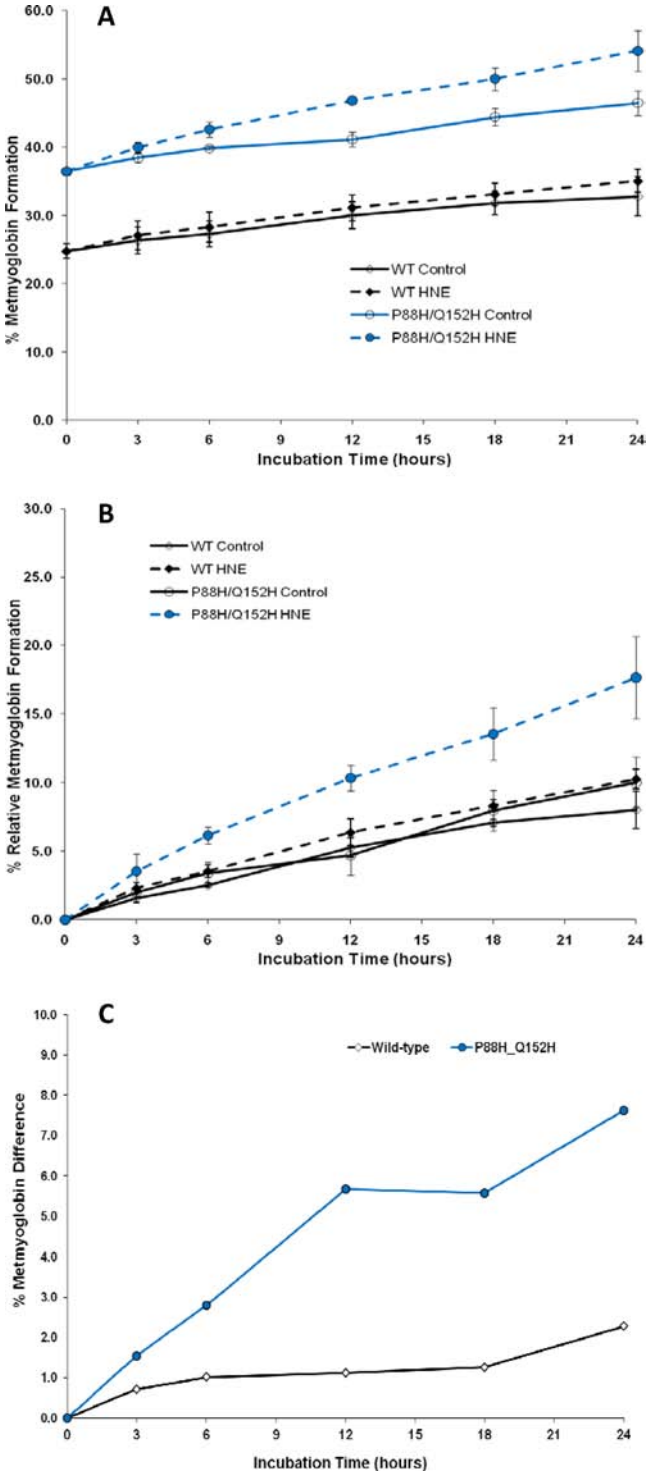

Figure 2. Effect of HNE (0.42 mM) on OxyMb oxidation of P88H/Q152H and WT sperm whale myoglobins (0.06 mM) at pH 5.6, 4 °C: (A) %MetMb formation; (B) %Relative MetMb difference between HNE-treated P88H/Q152H and WT sperm whale myoglobins and their respective controls; (C) %MetMb difference between HNE-treated P88H/Q152H and WT sperm whale myoglobins and their respective controls.

this provided an opportunity for heme oxidation. The heme moiety of DeoxyMb lacks a bound ligand at the sixth coordination site, resulting in decreased stability of the heme because of its high spin electrons, which leads to more rapid oxidation than OxyMb.[19] To compare the effects of HNE on oxidation rates of OxyMb and DeoxyMb, equine Mb was used as a model, and the results are presented in Figure 3. To the

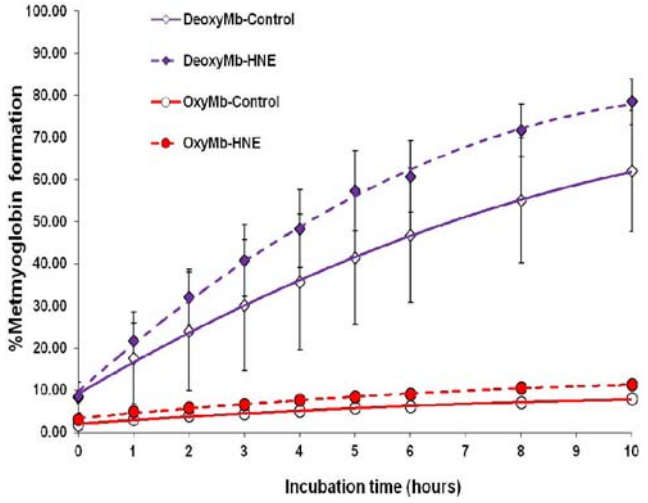

Figure 3. Effect of HNE (0.42 mM) on oxidation of equine DeoxyMb (0.06 mM; pO_2 = 3.00 mmHg) and OxyMb (0.06 mM; pO_2 = 166.44 mmHg) at pH 5.6 and 4 °C.

best of our knowledge, the present study is the first to demonstrate the effect of HNE on oxidation of DeoxyMb. DeoxyMb oxidized more rapidly than OxyMb, as previously reported,[19,43] and appeared to be more adversely affected by HNE. Lack of an oxygen ligand allows water to enter the hydrophobic heme pocket more readily and weaken the heme–globin linkage. H64F is a mutant Mb in which HIS 64 is replaced by the larger and more hydrophobic side chain of phenylalanine expected to prevent hydration of the heme pocket.[30] As expected, this mutant appeared to be more stable than WT under the same oxygen tension (pO_2 = 3 mmHg) (Figure 4). The bulkier hydrophobic side chain of phenylalanine was expected to stabilize the heme moiety and prevent HNE from entering the pocket, resulting in a less adverse effect of HNE on H64F DeoxyMb stability.

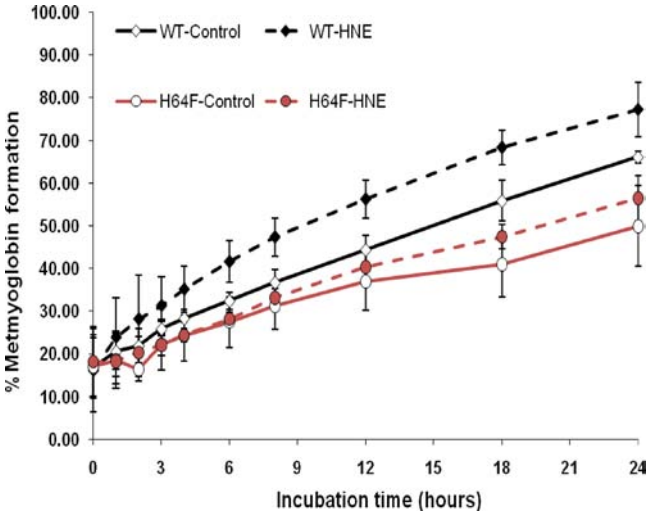

Figure 4. Effect of HNE (0.42 mM) on oxidation of DeoxyMb of H64F and WT sperm whale myoglobins (0.06 mM) at pH 5.6, 4 °C, and pO_2 = 3.00 mmHg.

dx.doi.org/10.1021/jf301770p | J. Agric. Food Chem. 2012, 60, 8473–8483

Sites of HNE Adducts in WT and Mb Mutants. Table 1 summarizes the number of HNE adducts observed in WT and mutant sperm whale Mbs obtained from MALDI-TOF mass spectrometry. The numbers of peaks representing HNE-adducted WT and P88H/Q152H Mbs were not different despite more histidine residues in the mutant. There were two peaks in L29F at both 24 and 72 h of incubation. H97A was similarly alkylated as mono- and di-adducts with HNE, but the peaks corresponding to HNE-alkylated Mb were relatively low in abundance (data not shown). The number of peaks present in WT appeared to be greater than in H64F as expected from the hypothesis that the replacement of HIS 64 by PHE 64 leads to greater hydrophobicity in the heme pocket with associated exclusion of water and decreased access of HNE to histidine residues buried within.

Table 1. Number of HNE Adducts on WT and Mutant Sperm Whale Myoglobins Determined by MALDI-TOF Mass Spectrometry

		incubation period	
myoglobin	form of myoglobin	24 h	72 h
wild-type (WT)	OxyMb	1	2
P88H/Q152H	OxyMb	1	2
L29F	OxyMb	2	2
H97A	OxyMb	2	2
H64F	DeoxyMb	2	N/A[a]
WT	DeoxyMb	1	N/A[a]

[a]The incubation of deoxy-WT and H64F with HNE was not performed at 72.

The results from MALDI-TOF mass spectrometry are not sufficient to indicate which specific histidine residues are alkylated by HNE. For example, mono-HNE adducts identified by MS could represent a population of monoadducts that formed at all of the accessible histidine residues. It is also possible that one or more residues are preferentially alkylated because of their relative location in the protein's tertiary structure. Therefore, trypsinized Mb samples were analyzed using MALDI-TOF-TOF tandem mass spectrometry due to its high throughput and sensitivity in both peptides and intact proteins up to 12 kDa.[44] The sites of HNE alkylation in the WT and mutant sperm whale Mbs after 72 h of incubation determined by tandem mass spectrometry are presented in Tables 2−7, which are found in the Supporting Information. Immonium ions (indicated in Figure 5) are ion products from secondary fragmentation of the protonated peptide during low-energy collision-induced dissociation (CID). Their general structures can be represented as $RCH{=}NH^{2+}$ with R being the amino acid side chain. Because the immonium ion is derived from an amino acid residue, it can be a useful marker for covalent modification of an amino acid residue.[45,46] Therefore, the immonium ion with the mass of 266 Da (110 Da of histidine immonium ion + 156 Da of HNE) indicates the Michael addition of HNE on histidine residues. HIS 48 was the only histidine residue that was alkylated in all sperm whale Mbs after 24 h (data not shown) and 72 h of incubation (Supporting Information). The location of HIS 48 on the loop connecting two helices may be highly accessible to HNE molecules. Moreover, at pH 5.6, >50% of HIS 48 (pK_a = 5.3−5.5) should be in the unprotonated form, which is available for the reaction with HNE.[47,48] However, this histidine residue has not been reported as HNE alkylation sites in either bovine or porcine Mbs.[14−16] Mono- and di-HNE adducts were observed in WT incubated with HNE for 72 h (Table 1). However, only HIS 48 was identified as the alkylation site of HNE in WT (Supporting Information, Table 2). It is possible that di-HNE-alkylated WT Mb aggregated, which is thermodynamically favored by

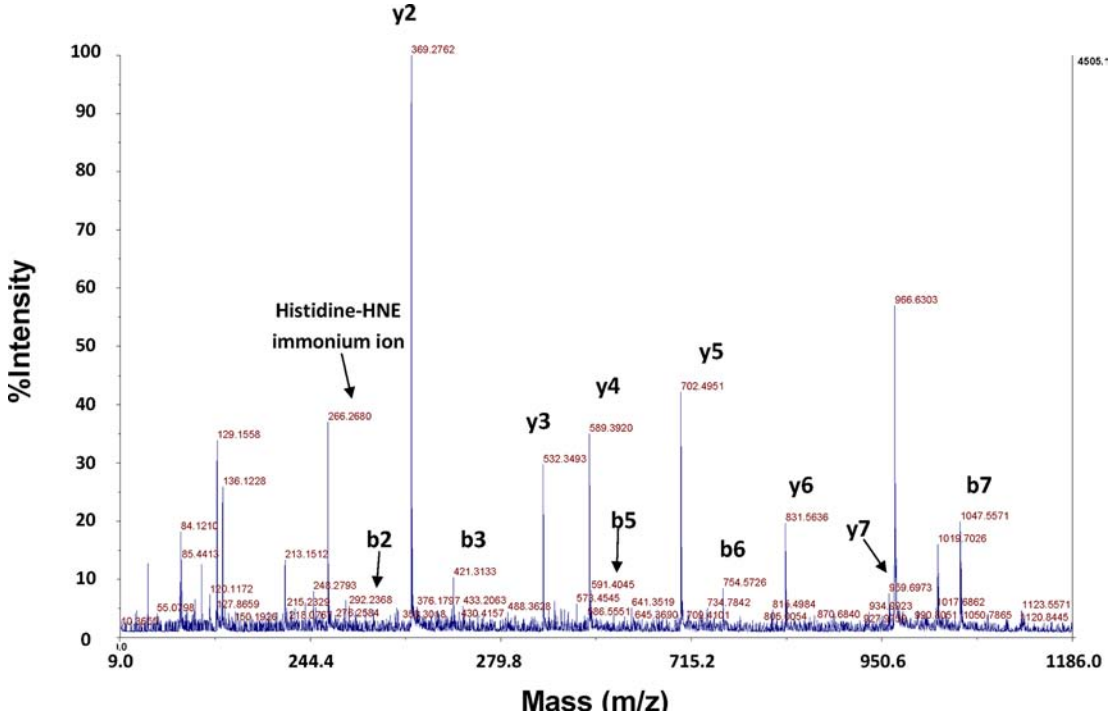

Figure 5. MS/MS spectrum of b- and y-ion series from the tryptic-digested peptide 147−154 of P88H/Q152 (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6 for 72 h. The immonium ion indicates HNE-alkylated histidine residues.

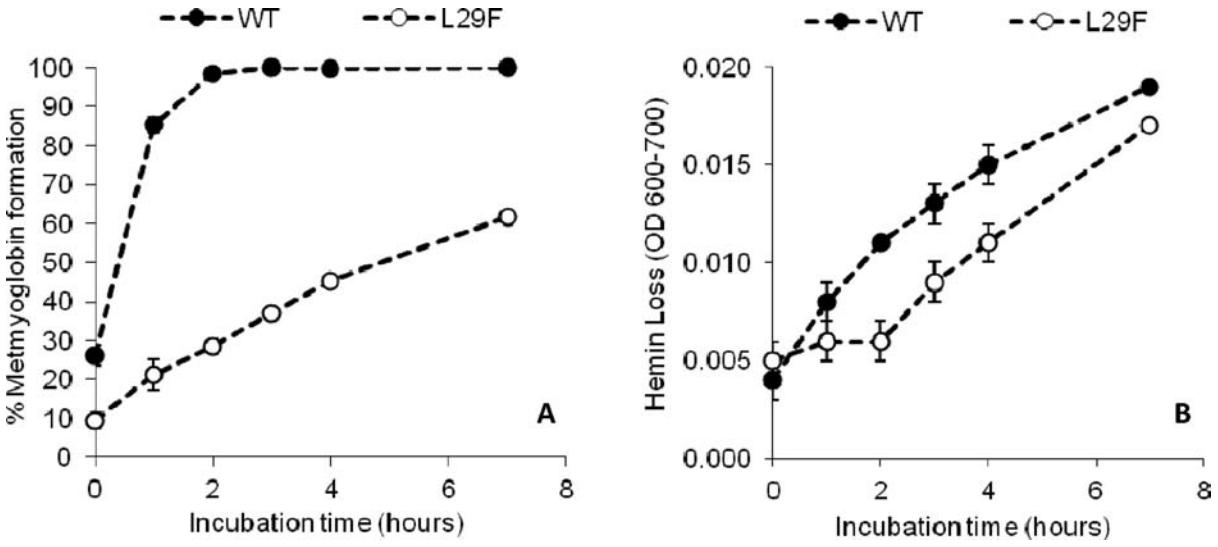

Figure 6. (A) MetMb formation and (B) dissociation of the porphyrin group from WT and L29F during storage at 28 °C at pH 5.6. The porphyrin loss reaction consisted of 0.010 mM Mb, 0.040 mM apoH64Y/V68F (hemin capturing reagent), 559 mM sucrose, and 186 mM Bis-Tris. The reaction to blank absorbance at 600 nm due to MetMb formation during the hemin loss assay was prepared as above but without added apo-reagent.

hydrophobic interactions, and formed aggregates through intra- and intermolecular cross-linking.[42] Formation of aggregates would inhibit flow through the matrix of the desalting column during the removal of unreacted HNE. However, we did not observe precipitation of HNE-treated Mbs in this study, and it remains unclear why we were not able to identify the residue alkylated in diadducts.

The substituted histidine residues at positions 88 and 152 appeared to be vulnerable sites for HNE binding in P88H/Q152H (Figure 5; Supporting Information, Table 3). In addition to HIS 48, these two histidine mutations were alkylated by HNE. This result is in agreement with the result in bovine Mb in which adducts were identified in HIS 88 and 152.[15,16] Alderton et al.[14] reported HNE adducts on HIS 152 but not HIS 88 of bovine Mb. The increased number of histidine residues in the double mutant and their alkylation by HNE likely provide a logical explanation for the faster OxyMb oxidation rate observed in this mutant when compared with WT.

At 72 h of incubation, HIS 12 of L29F was also alkylated by HNE (Supporting Information, Table 4). HIS 12 is uniquely present in the primary sequence of sperm whale Mb, whereas asparagine is conserved to this 12th amino acid in the primary sequences of other mammalian Mbs including bovine and porcine Mbs.[49] HIS 12 in L29F is located on the opposite side of the protein from the heme moiety, and the HNE adduct at this location might be less effective in compromising the redox stability of L29F than on histidine residues in the vicinity of the heme moiety.

There was evidence for HNE modification of HIS 24 and 82 of H97A (Supporting Information, Table 5). Because the peptide 80–97 contains three histidine residues (HIS 81, HIS 82, and HIS 93), alkylation of one histidine residue might prohibit the other histidine residue located nearby from being alkylated by HNE due to steric hindrance. In Table 5 of the Supporting Information, an HNE adduct on HIS 82 was confirmed due to the 156 Da mass shift of b4 and y15 ions. However, there was no evidence for an HNE adduct on HIS 81 due to the absence of the mass shift in the b3 ion. Interestingly, despite being in close proximity to each other, the pK_a of HIS 81 is 6.5–6.6, whereas the pK_a of HIS 82 is estimated to be <5.0.[47,48] The difference in the ionization state at pH 5.6 can partly explain the preference of HNE for HIS 82. HNE alkylation of the proximal HIS 93 or distal HIS 64 of H97A could not be confirmed in our study. This observation differs from that of Alderton et al.,[14] who reported the formation of HNE adducts on both histidine residues of bovine Mb at pH 7.4 and 37 °C. In addition to variations in the primary sequences between bovine and sperm whale Mbs, the differences in pH (7.4 vs 5.6) and incubation temperature (4 vs 37 °C) can also contribute to the different HNE alkylation profiles. The protonated imidazole group of histidine at pH <6.0 is less likely attacked by HNE, and the reaction is favored at elevated temperatures.[14,50] The formation of an HNE adduct at HIS 82, which is located within the vicinity of the heme pocket, could further disrupt the heme stability of H97A.

After 24 h of incubation with HNE, DeoxyMb H64F contained mono-HNE adducts (Table 1), whereas diadducts were identified on WT (Table 1). This could be attributed to H64F containing fewer histidine residues than WT as a result of the mutation of HIS 64 to PHE 64. Moreover, the large hydrophobic phenyl side chain of PHE 64 could affect the heme redox stability of DeoxyMb H64F by blocking access of solvent as well as HNE into the heme pocket.[30] Hence, the observed greater redox stability and fewer HNE adducts observed in H64F than WT can be explained by the combined effects of the lesser histidine number and the presence of phenylalanine 64. The MS/MS data of both WT (Supporting Information, Table 6) and H64F (Supporting Information, Table 7) DeoxyMbs confirmed only a single HNE adduct site at HIS 48.

Redox Instability, HNE Alkylation, and Hemin Loss from Mbs. It is generally accepted that ferrous Mbs (OxyMb and DeoxyMb) have much greater affinity for the porphyrin moiety than MetMb, yet studies demonstrating this are limited. To address this, MetMb formation and porphyrin dissociation from the globin were examined simultaneously in WT and L29F, as L29F oxidizes to MetMb more slowly. The term porphyrin is used here because nomenclature dictates that reduced Mbs contain heme and oxidized Mbs contain hemin.

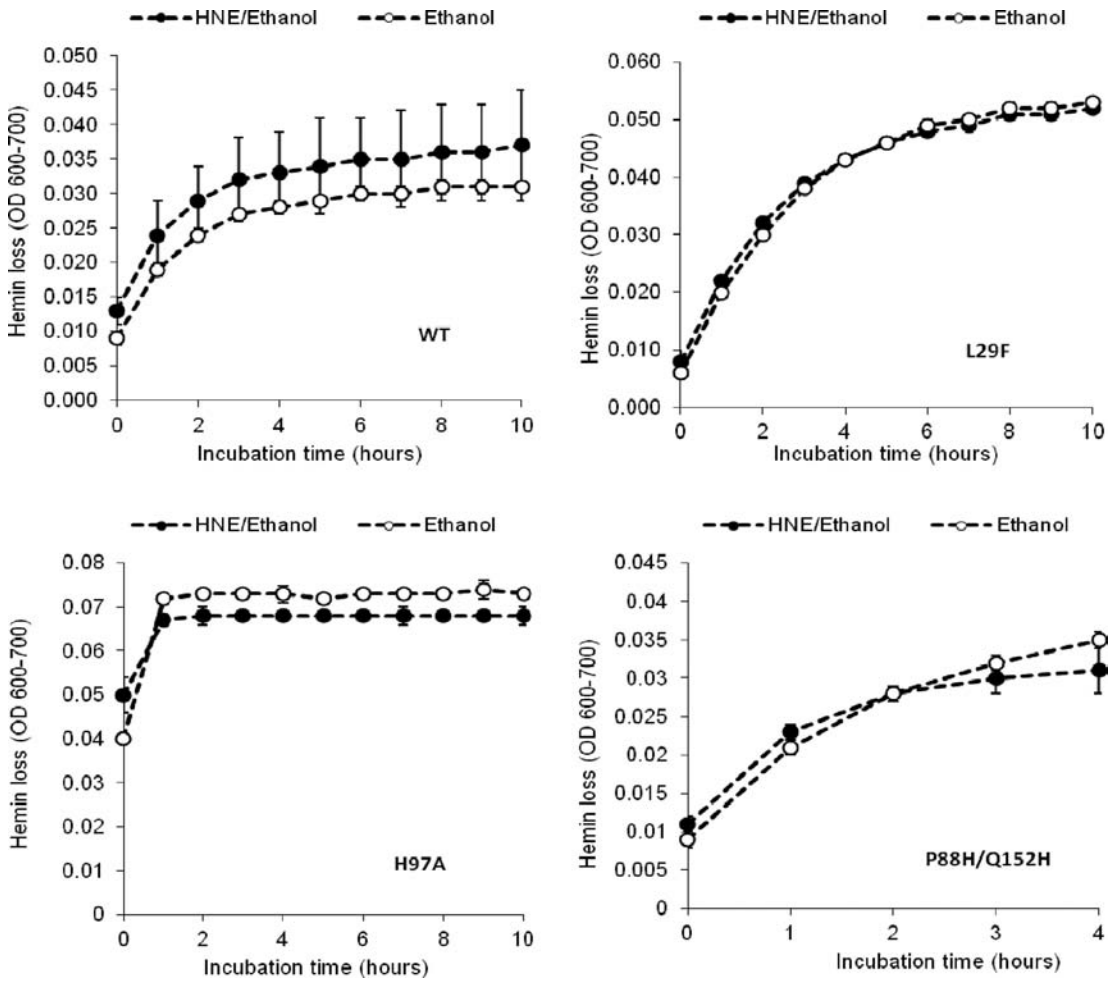

Figure 7. Effect of HNE on hemin loss from WT, L29F, H97A, and P88H/Q152H during incubation at pH 5.6 and 37 °C. Each Mb was incubated at pH 5.6 and 4 °C for 72 h in 124 mM ethanol in the absence and presence of a 7-fold excess of HNE. Mbs reacted with HNE were desalted prior to hemin loss measurement. The hemin loss reaction consisted of 0.010 mM Mb, 0.040 mM apoH64Y/V68F (hemin capturing reagent), 446−484 mM sucrose, and 149−161 mM Bis-Tris. The reaction to blank absorbance at 600 nm due to MetMb formation during the assay was prepared as above but without added apo-reagent.

As expected, MetMb formation was more rapid in WT than in L29F at pH 5.6 and 28 °C (Figure 6A). After 1 h, there was 20% MetMb in L29F compared to 85% in WT. Porphyrin dissociation was more rapid from WT compared to L29F ($p < 0.05$; Figure 6B). Porphyrin dissociation was not detected from L29F until approximately 30% MetMb formation occurred (Figure 6). These findings suggest that hemin was transferred from MetMb readily, whereas OxyMb retained its heme moiety. Thus, the tendency of WT to lose porphyrin more quickly than L29F was apparently due to slower formation of MetMb in L29F.

Considering the more rapid hemin loss from WT (rapidly autoxidizing) compared to L29F (slowly autoxidizing), we would expect HNE to accelerate hemin loss because of its ability to hasten MetMb formation. However, hemin loss using the apo-reagent cannot be assessed during alkylation because HNE will alkylate the apo-reagent, which is present in excess of the experimental Mb. With this in mind, the approach taken was to alkylate Mb, remove nonreacted HNE, and then assess hemin loss. This approach of first alkylating with HNE and subsequently, rather than concurrently, assessing hemin loss unfortunately caused high levels of MetMb at the initiation of the hemin loss assay for control and HNE-treated samples. This presented an abundance of releasable hemin from both treatments to the apo-reagent so that increased hemin loss due to HNE was unlikely to be observed simply on the basis of a slightly elevated percentage of MetMb in the alkylated samples. Despite these limitations, hemin loss from WT, L29F, H97A, and P88H/Q152H, with and without alkylation by HNE, was determined at pH 5.6 during 37 °C storage. It seemed logical to consider the possibility that covalent protein modification by HNE could alter the secondary and tertiary structures of Mb, which in turn would provide a mechanism for enhanced hemin release from MetMb. The %MetMb was, on average, 7% greater in alkylated Mbs (55.8−84.7%) compared to nonalkylated Mbs (51.0−76.2%) at the initiation of hemin loss measurement. Hemin loss from WT increased rapidly within 2 h and reached a plateau after 3 h of incubation (Figure 7). Roughly 13% of the hemin in WT MetMb transferred to the apo-reagent on the basis of extinction coefficients for holoH64Y/V68F. Alkylation of WT with HNE did not increase hemin loss ($p < 0.05$). Hemin loss from L29F increased during the initial 6 h of incubation, and alkylation did not accelerate hemin loss. Alkylation did not accelerate hemin loss from P88H/Q152H, and the kinetics of hemin loss were similar to those of WT and L29F (Figure 7). Hemin loss from H97A was

rapid and more extensive compared to WT, L29F, and P88H/Q152H (Figure 7). This can be explained in part by the lack of hydrogen bonding between Ala97 and the heme-7-proionate that normally occurs with HIS 97. At zero time, hemin loss was greater from alkylated H97A compared to nonalkylated H97A ($p < 0.05$). Hemin loss from H97A was also examined at 2 °C and pH 5.6 because hemin loss from H97A was very rapid at 37 °C, which limited examination of alkylation effects on hemin loss. HNE accelerated hemin loss from H97A during 2 °C storage at all time points except at time zero (Figure 8).

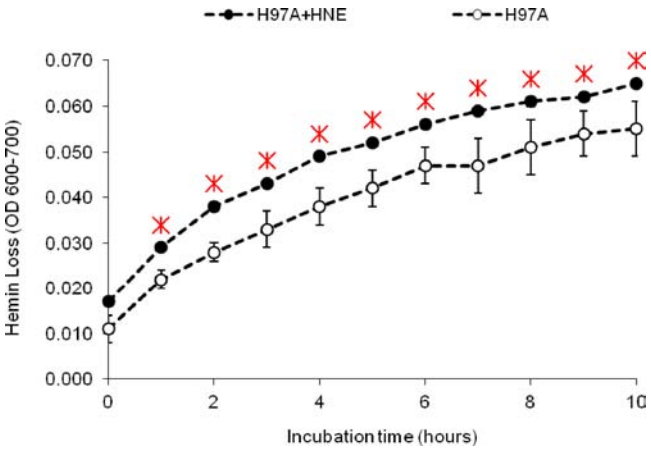

Figure 8. Effect of alkylation by aqueous HNE on hemin loss from H97A during iced storage (2 °C). Hemin loss reactions (1 mL volume) contained 40 μM apo H64Y/V68F sperm whale Mb and 10 μM H97A previously reacted with HNE or water. Buffer conditions were 0.5 M sucrose and 0.2 M Bis-Tris, 0.2 mM Tris, pH 5.6. The previous alkylation reactions (1.1 mL volume) consisted of 0.225 mM H97A sperm whale Mb, 1.6 mM aqueous HNE or water, 97 mM Bis-Tris, and 0.3 mM Tris. The reactions were allowed to proceed at 4 °C, pH 5.6, for 122 h before desalting to remove unreacted HNE. Asterisks indicate $p < 0.05$. Error bars fall within symbols for s data points.

It should be noted that HIS 97 is optimally located to stabilize the heme moiety within the globin of WT, L29F, and P88H/Q152H by electrostatic and hydrogen bonding.[51] These interactions apparently stabilized hemin within the globin so that alkylations at HIS 48 in WT, HIS 12 and HIS 48 in L29F, and HIS 48, HIS 88, and HIS 152 in P88Q/Q152H were not sufficiently disruptive to affect hemin affinity under the conditions examined. H97A, on the other hand, has more weakly anchored heme within the globin due to the ALA 97 substitution. Taken together, the alkylations at HIS 24, HIS 48, and HIS 82 of H97A and the lack of stabilization of the heme ring at site 97 may explain the elevated hemin loss in H97A exposed to HNE (Figure 8). These findings may have relevance in regard to HNE effects in hemoglobins, which lack residues to stabilize the heme in the globin at the site analogous to 97 (FG3) in Mbs. A more open structure in H97A would explain the observation that sites 82 and 24 were alkylated in H97A but not in the other Mbs (Figure 9). It is interesting that site 12 was alkylated in L29F but not in WT, H97A, or P88H/Q152H (Figure 9). A hydrogen bond between HIS 12 and ASN 122 was detected in L29F but not WT Mbs using PROPKA software (PDB 2G14 versus 110M). This hydrogen bond might increase the probability of alkylation at HIS 12.

Relevance of In Vitro Results to Muscle Foods. Dietary vitamin E supplementation inhibits both lipid and Mb oxidations in beef.[6,9,52−54] However, elevated α-tocopherol levels in pork do not have enhanced meat color stability to the same relative degree despite the observed delay of lipid oxidation.[55−57] Similar results were observed in thigh meat from vitamin E-supplemented chickens.[58] Recently, Yin and coworkers[59] reported that mammalian OxyMb redox instability induced by HNE was greater in Mbs with 12 ± 1 histidine residues (horse, cattle, sheep, and deer) than in Mbs with 9 histidine residues (chicken, turkey, and pig). Their observation further supports the suggestion that greater histidine residue numbers in the primary sequence lead to greater vulnerability to HNE.

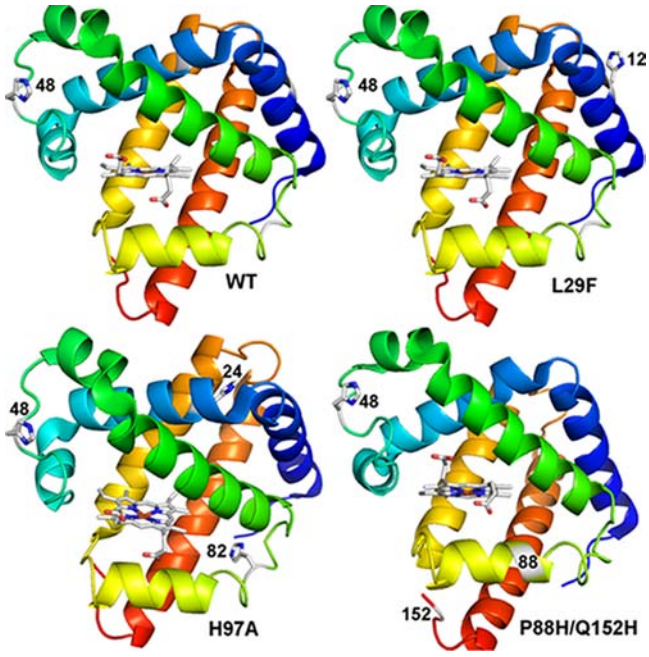

Figure 9. Site(s) of histidine alkylation by HNE are shown for WT (one site), L29F (two sites), H97A (three sites), and P88H/Q152H (three sites). The site of alkylation in H64F was that observed for WT. The structures shown were created from PDB file 2MGJ using PyMOL software. The histidine residues shown are only to provide their approximate location in a generic Mb that was not exposed to HNE. Histidine side chains are not shown at sites 88 and 152 because a crystal structure for a myoglobin with His at those sites (e.g., bovine Mb) was not available. The sites of the histidine residues are based on their positions in the amino acid sequence of the native sperm whale myoglobin. Their locations in WT and mutant sperm whale Mbs are shifted forward by one position due to the presence of a methionine residue at the N-terminus of the amino acid sequence.

Our observations provide a potential explanation for the observation that porcine Mb is less affected by HNE alkylation than bovine Mb.[15,16] The absence of histidine residues at specific locations on the primary sequences of porcine and chicken Mbs but not bovine Mb prevents HNE from covalently binding to them. The presence of HIS 88 and HIS 152 predisposed the P88H/Q152H mutant Mb to be at greater risk of alkylation by HNE, and this was confirmed by our observations (Figure 2). Our observation with this double mutant supports the suggestion by Suman and colleagues[16] that bovine Mb oxidation was more susceptible to the effects of HNE than porcine Mb because it contained four more histidine residues available for alkylation by HNE.

The primary structure of Mb affects its autoxidation rate and susceptibility to HNE. An increased number of histidine

residues at certain locations (e.g., 88 and 152) can be detrimental to the redox stability of Mb in the presence of this secondary product of lipid oxidation. The large hydrophobic side chain of PHE 29 that substitutes for LEU 29 can delay autoxidation as well as HNE-induced oxidation of L29F OxyMb. The substitution of ALA 97 for HIS 97 may allow hydration of the heme pocket and leave a channel for HNE to covalently alkylate the histidine residues buried within. HNE accelerated hemin loss from H97A but not the other Mbs examined. The large hydrophobic side chain of PHE 64 stabilizes the redox stability of DeoxyMb by preventing hydration of the heme pocket and possibly access by HNE. This study demonstrated that the effect of primary sequence on Mb redox stability and hemin release can be exacerbated by the presence of secondary lipid oxidation products.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables 2−7. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (860) 486-2919. Fax: (860) 486-4643. E-mail: Cameron.faustman@uconn.edu.

Funding

This project was supported by National Research Initiative Grant 2007-35503-18482 from the USDA Cooperative State Research, Education, and Extension Service Improving Food Quality and Value Program. We thank the Royal Thai government for financial support for N.T.'s scholarship.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Mass spectrometric analysis was performed at the University of Kentucky, Center for Structural Biology Protein Core Facility. This core facility is supported in part by funds from NIH National Center for Research Resources (NCRR) Grant P20 RR020171.

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