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Redox Instability Induced by 4-Hydroxy-2-nonenal in Porcine and Bovine Myoglobins at pH 5.6 and 4 °C

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Myoglobin (Mb) redox stability affects meat color and is compromised by lipid oxidation products such as 4-hydroxy-2-nonenal (HNE). Pork lipids are generally more unsaturated and would be expected to oxidize readily and produce more oxidation products than beef. Supranutritional supplementation of vitamin E improves Mb redox stability of beef but not pork. The present study investigated HNE-induced redox instability in porcine and bovine myoglobins at 4 °C and pH 5.6. Oxymyoglobin (OxyMb) was incubated with HNE (0.075 mM porcine OxyMb + 0.5 mM HNE; 0.15 mM bovine OxyMb + 1.0 mM HNE). In porcine Mb, only monoadducts formed via Michael addition were detected after 72 h, whereas in bovine Mb both mono- and diadducts were identified. LC-MS-MS identified four histidine residues (His 36, 81, 88, and 152) of bovine Mb that were readily adducted by HNE, whereas in porcine Mb only two histidine residues (His 24 and 36) were adducted. These results suggested that the primary structure of bovine Mb predisposes it to greater nucleophilic attack by HNE and subsequent adduction than is suffered by porcine Mb.

KEYWORDS: Myoglobin; lipid oxidation; 4-hydroxy-2-nonenal; mass spectrometry

INTRODUCTION

Lipid oxidation generates a variety of secondary oxidation products, for example, aldehydes, that react readily with other biological molecules in cellular systems. These secondary products are responsible for off-flavors and off-odors associated with rancidity in muscle foods (*1*). The aldehyde products, when compared to free radicals, are more stable and readily diffuse into the cellular environment, where they can exert toxicological effects (*2*). Chan et al. (*3*) utilized several aldehydes, known to be secondary products of meat lipid oxidation, to demonstrate destabilizing effects of these on oxymyoglobin (OxyMb) redox stability. Of the aldehydes tested, monounsaturated aldehydes were found to be most effective. When different aldehydes were incubated with equine OxyMb, metmyoglobin (MetMb) formation was greater in the presence of unsaturated aldehydes than with their saturated counterparts of the same carbon chain length (*4*). α,*β*-Unsaturated aldehydes are products of lipid oxidation and are very reactive to many macromolecules and proteins (*5*); this has led researchers to suggest that adducted proteins could be used as markers for oxidative stress (*6*). 4-Hydroxy-2-nonenal (HNE), an α,*β*-unsaturated aldehyde formed by the oxidation of *ω*-6 polyunsaturated fatty acids (*7*), is reactive toward proteins and has substantial toxicological and biological activity (*2*). HNE exhibits a high reactivity toward thiol and amino groups; it binds with histidine residues in insulin (*8*), with lysine in glucose-6-phosphate dehydrogenase (*9*), and with arginine in cytochrome *c* (*10*). Through covalent modifications of amino acids, HNE has been reported to inactivate several proteins and enzymes including glucose-6-phosphate dehydrogenase (*9*), pyruvate dehydrogenase (*11*), cathepsin B (*12*), insulin (*8*), and cytochrome *c* oxidase (*13*, *14*). Volkel et al. (*15*) suggested that HNE-adducted glutathione, or its secondary metabolites, could be used as a reliable biomarker of oxidative stress-induced lipid peroxidation.

HNE has been detected in fresh meat from pigs, cattle, and fish (*16*−*18*) and in smoked pork (*19*) and proposed as a marker to assess the quality of muscle foods containing significant amounts of polyunsaturated fatty acids (*16*). HNE accelerated equine OxyMb oxidation in vitro, and covalent modification of equine OxyMb by HNE was demonstrated by Faustman et al. (*4*). Bolgar and Gaskell (*20*) presented evidence for the reaction of HNE with histidine residues in equine apomyoglobin via Michael addition. Histidine residues were the major sites for HNE adduction in bovine heart cytochrome *c* oxidase (*21*). These findings, along with the results from a study by Alderton et al. (*22*) on HNE-induced redox instability in bovine OxyMb, demonstrated that histidine was the sole amino acid candidate for HNE adduction in myoglobin (Mb).

Discoloration and myoglobin oxidation in meat are governed by a variety of factors including pH, temperature, partial pressure of oxygen, light, and microbial growth. These factors, along with the presence/absence of antioxidants/prooxidants, also

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affect lipid stability. Lipid oxidation and concomitant discoloration of beef have been minimized by dietary supplementation of vitamin E (*23*). Specifically, vitamin E (α-tocopherol) protects highly oxidizable polyunsaturated fatty acids in cell membranes from peroxidation by oxygen free radicals (*24*). However, in pork obtained from vitamin E-supplemented pigs, a lipid-stabilizing but no color-stabilizing effect was observed (*25*−*29*). Interestingly, porcine muscle generally contains a greater proportion of unsaturated fatty acids than beef, and it is reasonable to hypothesize that pork lipids would undergo lipid oxidation and produce secondary products more readily than beef. HNE is formed by the oxidation of ω-6 polyunsaturated fatty acids such as linoleic acid and arachidonic acid. The levels of these fatty acids are significantly greater in pork compared to beef (*30*). Hence, the formation and yield of HNE are anticipated to be greater in pork than in beef. In turn, this would be expected to result in decreased OxyMb (color) stability in pork, which would be readily mediated by α-tocopherol. However, findings that vitamin E-supplemented pork did not demonstrate consistently improved pork color stability from α-tocopherol supplementation led us to consider the possibility that the redox stability of porcine Mb differs from that of bovine Mb as a function of its susceptibility to lipid oxidation.

The primary sequence of a protein determines its tertiary structure, which in turn dictates the potential interaction(s) of the protein with other ligands and reactants. Sequence analysis of myoglobin from different meat-producing livestock species reveals that porcine myoglobin contains 9 histidine residues, whereas bovine myoglobin contains 13 residues (*31*). Alderton et al. (*22*) identified six nucleophilic histidine (His) residues, namely, His 24, 64, 93, 116, 119, and 152, in bovine myoglobin that were readily adducted with HNE at pH 7.4 and 37 °C. These included the proximal (His 93) and distal (His 64) histidines associated with the heme group, which could be the basis for the observed redox instability caused by HNE. Comparing the primary sequences of porcine and bovine myoglobins shows that of the six susceptible His residues in bovine myoglobin, His 116 and 152 are absent in porcine myoglobin.

The effect of HNE on redox stability of porcine Mb (*32*) and bovine Mb (*22*) was investigated at pH 7.4 and 37 °C. However, a comparison of the redox destabilizing effect of HNE on porcine and bovine Mb at pH 5.6 and 4 °C would elucidate a better understanding about how the two different proteins interact with lipid oxidation products at a storage temperature and pH value typical of meat. The present study was carried out to compare HNE-induced redox instability in porcine and bovine Mb and to identify the sites of HNE adduction at a storage temperature and pH value typical of meat.

MATERIALS AND METHODS

Materials and Chemicals. Sephacryl 200HR, ammonium bicarbonate, ammonium sulfate, Tris-HCl, EDTA, sodium hydrosulfite, sodium citrate, ethanol, acetonitrile, sodium borohydride, formic acid, sinapinic acid, and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI), PD-10 columns were obtained from Pharmacia (Piscataway, NJ), and sequence grade modified porcine trypsin was obtained from Promega (Madison, WI). All chemicals were of reagent grade or greater purity.

Mb Isolation and Purification. Bovine and porcine hearts were obtained locally within 0.5 h of exsanguination, placed on ice, and transported to the laboratory. Mb was purified via ammonium sulfate precipitation and gel filtration chromatography according to the method of Faustman and Phillips (*33*). Briefly, cardiac muscle was homogenized in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 4 °C) and centrifuged at 5000*g* for 10 min. The supernatant was brought to 70% ammonium sulfate saturation and centrifuged at 18000*g* for 20 min. The resulting supernatant was saturated with ammonium sulfate (100%) and centrifuged at 20000*g* for 1 h. The precipitate was resuspended in homogenization buffer and dialyzed (3 × 40 volumes) against 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, at 4 °C for 24 h. Mb was separated from hemoglobin using a Sephacryl 200HR gel filtration column (2.5 × 100 cm). The elution buffer contained 5 mM Tris-HCl, 1 mM EDTA, at pH 8.0 and 4 °C, and the flow rate was 60 mL/h.

OxyMb Preparation. OxyMb was prepared by hydrosulfite-mediated reduction of purified Mb. The residual dithionite was removed, and the pH of the Mb solution was adjusted to 5.6 by dialysis (3 × 40 volumes) against 50 mM sodium citrate (pH 5.6) buffer at 4 °C.

Reaction with HNE. OxyMb was incubated with HNE (0.075 mM porcine OxyMb + 0.5 mM HNE; 0.15 mM bovine OxyMb + 1.0 mM HNE 1.0 mM) at 4 °C and pH 5.6. For each species, the concentrations of OxyMb used reflected the concentration in beef and pork muscles in situ. However, the molar ratio of Mb to HNE was maintained at 1:7 for both species. Controls consisted of OxyMb plus a volume of ethanol equivalent to that used to deliver the aldehyde to treatment mixtures. Samples were scanned spectrophotometrically at specific incubation times from 650 to 500 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD). MetMb formation was calculated according to the method of Tang et al. (*34*). Samples (0.5 mL) were removed from the reaction assays at 0, 24, 48, and 72 h, passed through a PD-10 desalting column to remove unreacted HNE, and frozen at −80 °C for subsequent analysis.

Matrix-Assisted Laser Desorption Ionization−Time of Flight Mass Spectrometry (MALDI-TOF MS) of Mb−HNE Adducts. Native and HNE-treated myoglobins were analyzed by MALDI-TOF MS to detect changes in the total mass resulting from HNE adduction. Briefly, 1 μL of sample was mixed with 1 μL of freshly prepared 1% solution of sinapinic acid in acetonitrile/0.1% aqueous trifluoroacetic acid (60:40 v/v); 0.5 μL of the mixture was spotted on MALDI target plates, spread uniformly, and allowed to dry. The spots were treated with 1 μL of 0.1% aqueous trifluoroacetic acid to remove buffer salts prior to MALDI-TOF MS analysis. Protein molecular ions were analyzed in a linear, positive ion mode using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) set at an acceleration voltage of 25 kV. Each spot was analyzed a minimum of three times, accumulating spectra composed of 250 laser shots per analysis. The resulting spectra were analyzed by Data Explorer (Applied Biosystems, Foster City, CA), noise-smoothed, baseline-corrected, and mass-calibrated using an external standard of equine apomyoglobin.

Mb Peptide Preparation. Control and treated samples were subjected to tryptic digestion in spin filters (*35*). Briefly, ∼100 μg of protein sample was added to the upper chamber of a spin filter with a 10000 molecular weight cutoff membrane, washed with double-distilled water, and resuspended in ammonium bicarbonate buffer (pH 8.0). Prior to tryptic digestion, proteins were reduced with 200 mM sodium borohydride to stabilize potential lysine−HNE adducts for subsequent detection. The samples were digested with mass spectrometry grade modified porcine trypsin (Promega) to yield peptides for mass spectral analysis. Trypsin was added to achieve a ratio of 1:50 of trypsin to protein. The mixture was incubated at 37 °C for 18−24 h. Tryptic peptides were collected by centrifugation through the filter at 4500*g*, and the filtrate was acidified to a pH of <3.0 using concentrated formic acid to stop the enzyme activity. The concentration of peptides was adjusted to 0.6 μg/mL in 0.1% formic acid prior to tandem mass spectrometry (MS-MS).

Liquid Chromatography−Tandem Mass Spectrometry (LC-MS-MS). MS-MS of adducted peptides was accomplished using electrospray ionization mass spectrometry (ESI-MS). LC-MS-MS analyses were done on a ThermoFinnigan LTQ linear ion trap instrument equipped with a microelectrospray source (Thermo Electron, San Jose, CA), a ThermoFinnigan Surveyor HPLC, and an autosampler (Thermo Electron). Before MS-MS analyses, peptides were separated by reverse phase liquid chromatography on an 11-cm fused silica capillary column (100 μm internal diameter) packed with C-18 resin (Column Engineering, Ontario, CA) and eluted first with water/acetonitrile/formic acid

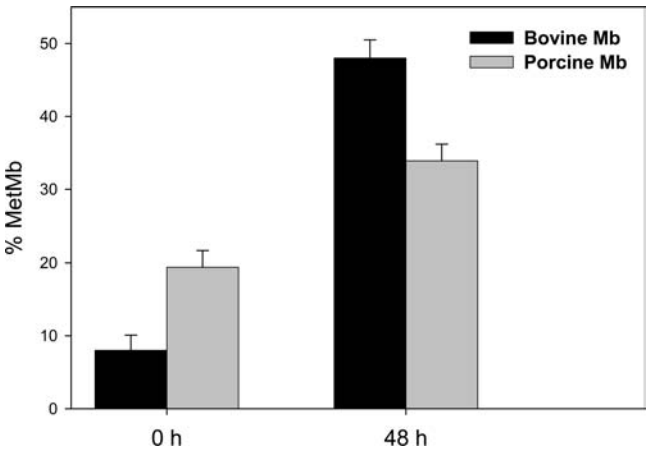

Figure 1. MetMb formation in bovine and porcine oxymyoglobins incubated with HNE at pH 5.6 and 4 °C for 48 h. Standard error bars are indicated. Results for bovine Mb are adopted from Alderton et al. (*22*).

(99:1:0.1, v/v/v) for 3 min at a flow rate of 140 μL/min. A linear gradient then increased acetonitrile to 28% by 33 min and to 80% by 40 min and decreased acetonitrile to 1% by 52 min. A 2 μL injection volume was used, and eluted peptides entered the LTQ mass spectrometer directly where collision-induced dissociation (CID) spectra were obtained using helium as the collision gas at 35 eV of collision energy. The MS-MS spectra were acquired in data-dependent scanning mode with one full MS scan followed by four MS-MS scans on the first four most intense ions with dynamic exclusion of previously selected precursors for a period of 1 min. MS-MS spectra were matched to database sequences with TurboSequest (Thermo Electron). Mb and Mb−HNE adducts were subsequently detected and confirmed using the protein database search software SEQUEST (*36*, *37*), SALSA (*38*−*40*), and the P-Mod (*41*) algorithms for ion series-based identification of MS-MS spectra corresponding to modified Mb sequences.

RESULTS AND DISCUSSION

The effect of HNE incubation on MetMb formation in bovine and porcine oxymyoglobins is presented in **Figure 1**. MetMb formation was greater in bovine Mb than in porcine Mb following 48 h of incubation at pH 5.6 and 4 °C. This suggests that bovine Mb is more susceptible to lipid-oxidation-induced oxidation than porcine Mb at typical meat storage conditions.

MALDI-TOF analysis of the HNE-adducted and control myoglobins revealed that prior to incubation with HNE the primary mass ion peaks present were equivalent to the mass of porcine Mb (16956 Da) and bovine Mb (16940 Da). However, following 48 h of incubation with HNE, a relatively small peak of mass 17114 Da appeared in addition to the native porcine Mb, and it became prominent after 72 h (**Figure 2**). This corresponded to a monoadduct of HNE with porcine Mb and was 158 Da greater than the mass of porcine Mb; a mass shift equal to the molecular weight of HNE. In bovine samples, a monoadduct peak (17097 Da) was prominent after 24 h of incubation, and a diadduct peak (17254 Da) apparent at 24 h was prominent by 72 h. The unadducted, monoadduct, and diadduct peaks were separated by a mass shift of 157 Da, and these results indicated that HNE adducts were formed by Michael addition. If the adduct had resulted from Schiff base formation, a molecular mass addition of 140 Da would have been expected as 1 mol of water is lost per mol of HNE−Mb in the reaction.

An average increase in mass of 158 Da resulted from HNE adduction. However, a positive mass shift of 156 Da, the molecular weight of HNE, by adduction was reported earlier (*20*, *21*). The difference of 2 Da (158 vs 156) was likely due to the reducing environment that we used to stabilize potential lysine adducts for subsequent MS analysis. It is possible that protonation of HNE-adducted HIS might have occurred and resulted in a 2 Da addition to the adduct's molecular mass.

After 72 h of incubation with HNE, porcine Mb formed only monoadducts, and their abundance, indicated by relative peak height, was considerably less than unadducted Mb (**Figure 2**). In bovine Mb, mono- and diadducts were present after 72 h of incubation with HNE, and the intensity of the monoadduct peak

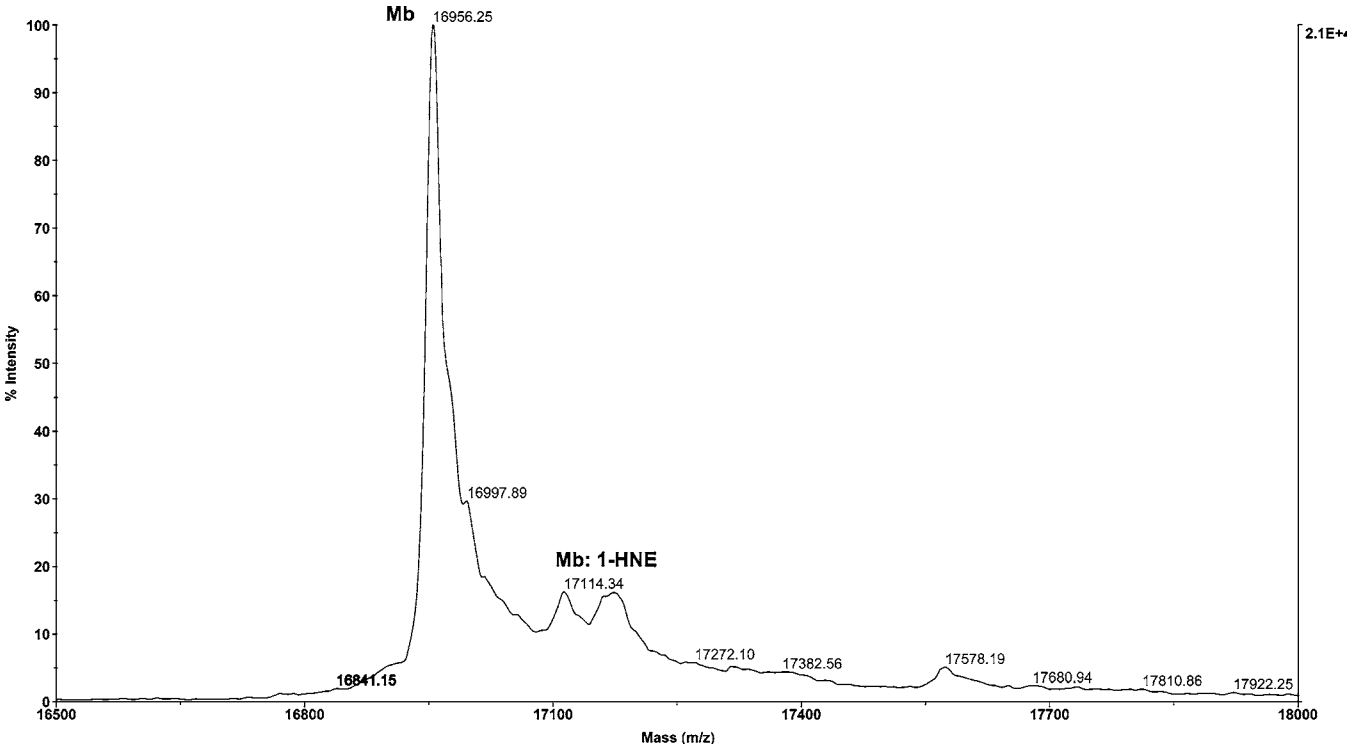

Figure 2. MALDI-TOF MS spectrum of porcine oxymyoglobin (0.075 mM) following reaction with HNE (0.5 mM) at pH 5.6 and 4 °C for 72 h.

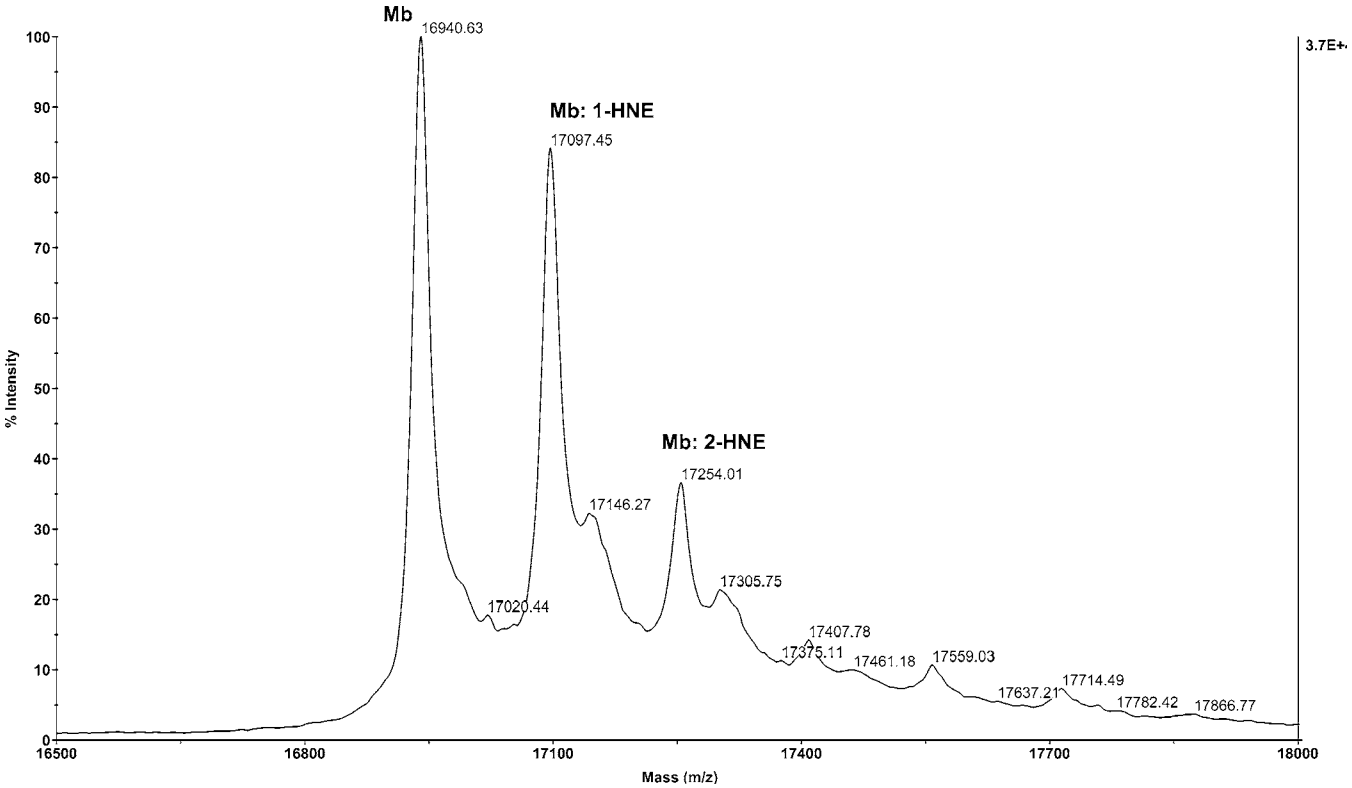

Figure 3. MALDI-TOF MS spectrum of bovine oxymyoglobin (0.15 mM) following reaction with HNE (1.0 mM) at pH 5.6 and 4 °C for 72 h.

was similar to that of unadducted Mb, indicating its similar relative abundance (**Figure 3**). Earlier studies showed that histidine residues were the major sites for HNE adduction in bovine OxyMb (*22*). Porcine Mb contains 9 His residues, whereas bovine Mb contains 13 His residues (*31*). The four fewer His residues in porcine Mb would provide fewer sites for HNE adduction, and this might have rendered porcine Mb less susceptible to nucleophilic attack by HNE. Our results for bovine Mb were similar to those observed by Alderton et al. (*22*) in which up to three HNE molecules were found to be covalently bound per bovine Mb molecule following incubation at pH 7.4 and 37 °C. Lee et al. (*32*) observed a diadduct in porcine Mb, albeit at much less relative abundance than the monoadduct at pH 7.4 and 37 °C. Our failure to observe a diadduct is likely due to the lower pH and incubation temperature that we used.

MALDI-TOF MS of Mb−HNE cannot identify specific amino acid sites of HNE adduction in proteins. For example, a given mono-HNE adduct mass ion peak may represent several different Mb−HNE monoadducts, each with HNE bound to a potentially different amino acid residue. The nucleophilic amino acids cysteine, lysine, and histidine, as well as the protein N-terminal amine, would be expected to be most reactive (*8*, *20*). Mammalian Mb lacks cysteine residues (*31*), and the primary nucleophilic candidates thus would be histidine and lysine. Studies on the redox instability of bovine OxyMb in relation to HNE showed that histidine was the sole amino acid adducted by HNE (*22*).

To determine the specific amino acid site(s) of HNE adduction, Mb incubated with and without (control) HNE was subsequently digested with trypsin to obtain peptides of appropriate size for LC-MS-MS. The MS-MS data were analyzed with SEQUEST (*36*, *37*), SALSA (*38*−*40*), and P-Mod (*41*) as described previously to identify spectra corresponding to unadducted and adducted Mb peptides. This approach identified four nucleophilic histidine residues confirmed to be adducted with HNE in bovine Mb, whereas only two HIS residues were adducted in porcine Mb. His 36, 81, 88, and 152 were the sites of adduction in bovine Mb, and each had a mass addition equivalent to that of HNE (158 Da). **Table 1** summarizes the *b*- and *y*-series ions for the four bovine Mb peptides identified with HNE adducts. However, in porcine Mb His 24 and 36 were adducted with HNE, the mass shift being 158 Da. The *b*- and *y*-series ions for the two porcine Mb peptides with HNE adductions are summarized in **Table 2**. Sodium borohydride reduction of Mb prior to trypsin treatment was performed in an attempt to stabilize potential lysine/HNE Schiff base adducts for subsequent detection; however, no lysine modifications were identified by MS-MS in these samples.

LC-MS-MS spectra at 24 and 48 h indicated that His 88 and 36 were more readily adducted by HNE in bovine Mb, whereas His 36 was the site of early adduction in porcine Mb. This result is interesting because His 88 is located close to the proximal histidine (His 93), which is bound to the heme moiety within the tertiary structure of Mb (*42*). The presence of an HNE adduct on His 88 appeared to compromise the redox stability, and this might have resulted from potential interference with His 93. His 36 is the common site of HNE adduction in both porcine and bovine Mb. Because His 36 lies in close proximity to the heme group in the tertiary structure (*42*), its modification by HNE might alter the protein structure around the heme cleft sufficiently to affect redox stability.

Our results differ slightly from those of Alderton et al. (*22*) and Lee et al. (*32*). Alderton et al. (*22*) reported that six His residues in bovine Mb, namely, His 24, 64, 93, 116, 119, and 152, were adducted by HNE at pH 7.4, whereas we found only four (His 36, 81, 88, and 152). Lee et al. (*32*) confirmed four histidine residues in porcine Mb (His 24, 64, 81, and 119) to be adducted with HNE at pH 7.4 and 37 °C. The differences in observations could be due to differences in pH (pH 7.4 vs 5.6)

Table 1. MS-MS Spectral Features of Unadducted and HNE-Adducted Bovine Myoglobin Peptides

peptide position[a]	peptide sequence[b]	modification and mass shift	precursor *m*/*z*	*b* and *y* ions identified[c]
32–42	LFTGHPETLEK	unadducted	636.63	*b* ions: 261.06 (*b*2), 362.29 (*b*3), 419.24 (*b*4), 556.31 (*b*5), 653.67 (*b*6), 782.41 (*b*7), 883.49 (*b*8), 996.46 (*b*9), 1125.56 (*b*10) *y* ions: 276.20 (*y*2), 389.34 (*y*3), 490.36 (*y*4), 618.64 (*y*5), 716.43 (*y*6), 853.47 (*y*7), 910.53 (*y*8), 1011.49 (*y*9), 1158.72 (*y*10)
32–42	LFTG**H****PETLEK	HNE, +158.0	715.33	*b* ions: 259.29 (*b*2), 364.59 (*b*3), **811.42** (***b*6**), **940.65** (***b*7**), **1154.87** (***b*9**), **1283.57** (***b*10**) *y* ions: 276.04 (*y*2), 387.98 (*y*3), 490.52 (*y*4), 619.50 (*y*5), **1011.51** (***y*7**), **1068.35** (***y*8**), **1170.30** (***y*9**)
80–87	GHHEAEVK	unadducted	453.69	*b* ions: 195.11 (*b*2), 332.27 (*b*3), 461.27 (*b*4), 532.32 (*b*5), 661.37 (*b*6), 760.42 (*b*7) *y* ions: 246.26 (*y*2), 375.57 (*y*3), 446.57 (*y*4), 575.34 (*y*5), 712.37 (*y*6)
80–87	G**H****HEAEVK	HNE, +158.1	532.75	*b* ions: **353.25** (***b*2**), **490.96** (***b*3**), **619.41** (***b*4**), **690.46** (***b*5**), **819.40** (***b*6**), **918.57** (***b*7**) *y* ions: 246.21 (*y*2), 446.26 (*y*4), 575.47 (*y*5), 712.56 (*y*6)
88–96	HLAESHANK	unadducted	504.03	*b* ions: 251.22 (*b*2), 322.29 (*b*3), 451.21 (*b*4), 538.26 (*b*5), 675.34 (*b*6), 746.37 (*b*7), 860.46 (*b*8) *y* ions: 147.17 (*y*1), 261.19 (*y*2), 332.29 (*y*3), 469.36 (*y*4), 556.30 (*y*5), 685.25 (*y*6), 756.41 (*y*7), 869.54 (*y*8)
88–96	**H****LAESHANK	HNE, +158.1	582.78	*b* ions: **296.16** (***b*1**), **409.35** (***b*2**), **480.46** (***b*3**), **609.40** (***b*4**), **696.30** (***b*5**), **833.44** (***b*6**), **904.50** (***b*7**), **1018.48** (***b*8**) *y* ions: 261.23 (*y*2), 332.26 (*y*3), 469.21 (*y*4), 556.26 (*y*5), 685.40 (*y*6), 756.42 (*y*7), 869.54 (*y*8)
148–153	VLGFHG	unadducted	631.45	*b* ions: 214.17 (*b*2), 270.21 (*b*3), 417.21 (*b*4), 555.31 (*b*5) *y* ions: 213.11 (*y*2), 361.23 (*y*3), 418.23 (*y*4), 532.49 (*y*5)
148–153	VLGF**H****G	HNE, +158.4	787.67	*b* ions: 269.26 (*b*3),**712.47** (***b*5**) *y* ions: **371.40** (***y*2**), **518.39** (***y*3**), **575.41** (***y*4**), **688.71** (***y*5**)

[a] Amino acid positions in the bovine myoglobin target peptide. [b] Amino acid sequence in the bovine myoglobin target peptide. HNE-adducted histidine residue listed in boldface. [c] The peptide bond between amide nitrogen and carbonyl oxygen is cleaved to form a "*b* ion" and a "*y* ion". A *b* ion is the fragment in which the positive charge is retained at the N-terminal portion of the original peptide ion; a *y* ion is the fragment in which the charge is retained at the C terminus of the original peptide ion. Observed signals assigned as *b* or *y* ions are listed. Ions containing an adduct moiety are mass-shifted with respect to the corresponding ions in unmodified peptides and are listed in boldface.

Table 2. MS-MS Spectral Features of Unadducted and HNE-Adducted Porcine Myoglobin Peptides

peptide position[a]	peptide sequence[b]	modification and mass shift	precursor *m*/*z*	*b* and *y* ions identified[c]
17–31	VEADVAGHGQEVLIR	unadducted	796.86	*b* ions: 229.10 (*b*2), 300.07 (*b*3), 415.23 (*b*4), 514.19 (*b*5), 585.32 (*b*6), 642.33 (*b*7), 779.29 (*b*8), 836.42 (*b*9), 964.28 (*b*10), 1093.61 (*b*11), 1192.59 (*b*12), 1305.50 (*b*13), 1418.70 (*b*14) *y* ions: 288.34 (*y*2), 401.44 (*y*3), 500.40 (*y*4), 629.61 (*y*5), 757.49 (*y*6), 814.53 (*y*7), 951.51 (*y*8), 1008.49 (*y*9), 1079.55 (*y*10), 1178.82 (*y*11), 1294.73 (*y*12), 1364.66 (*y*13)
17–31	VEADVAG**H****GQEVLIR	HNE, +158.8	876.28	*b* ions: 416.34 (*b*4), 586.22 (*b*6), **938.46** (***b*8**), **994.47** (***b*9**), **1123.30** (***b*10**), **1251.85** (***b*11**), **1350.76** (***b*12**), **1463.79** (***b*13**), **1577.83** (***b*14**) *y* ions: 401.21 (*y*3), 500.40 (*y*4), 629.51 (*y*5), 757.55 (*y*6), 814.49 (*y*7), **1109.69** (***y*8**), **1166.55** (***y*9**), **1237.64** (***y*10**), **1337.71** (***y*12**)
35–42	GHPETLEK	unadducted	455.83	*b* ions: 195.05 (*b*2), 292.23 (*b*3), 421.32 (*b*4), 522.76 (*b*5), 635.52 (*b*6), 764.34 (*b*7) *y* ions: 147.14 (*y*1), 276.12 (*y*2), 389.38 (*y*3), 490.30 (*y*4), 619.49 (*y*5), 716.39 (*y*6)
35–42	G**H****PETLEK	HNE, +158.0	534.7	*b* ions: **353.25** (***b*2**), **450.22** (***b*3**), **579.38** (***b*4**), **793.31** (***b*6**), **922.53** (***b*7**) *y* ions: 276.35 (*y*2), 389.16 (*y*3), 490.36 (*y*4), 716.55 (*y*6)

[a] Amino acid positions in the porcine myoglobin target peptide. [b] Amino acid sequence in the porcine myoglobin target peptide. HNE-adducted histidine residue listed in boldface. [c] The peptide bond between amide nitrogen and carbonyl oxygen is cleaved to form a "*b* ion" and a "*y* ion". A *b* ion is the fragment in which the positive charge is retained at the N-terminal portion of the original peptide ion; a *y* ion is the fragment in which the charge is retained at the C terminus of the original peptide ion. Observed signals assigned as *b* or *y* ions are listed. Ions containing an adduct moiety are mass-shifted with respect to the corresponding ions in unmodified peptides and are listed in boldface.

and temperatures of incubation (37 vs 4 °C). The measured redox destabilizing effect of HNE on OxyMb was greater at pH 7.4 than at 5.6 (*22*, *32*). At pH 5.6 it is likely that adduct formation would be lower than at pH 7.4, because at pH 5.6 a greater proportion of ionizable groups would be charged and therefore less reactive with HNE.

HNE, a secondary product of lipid oxidation, reacted with and adducted to bovine and porcine myoglobins via covalent modification of multiple histidine residues. The results of the present study suggested that bovine Mb is more susceptible to nucleophilic attack and subsequent adduction by HNE than porcine Mb at typical meat storage conditions. This could explain the basis for the relatively lower response of pork color to vitamin E when compared to beef.

ABBREVIATIONS USED

Mb, myoglobin; OxyMb, oxymyoglobin; MetMb, metmyoglobin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris-[hydroxymethyl]aminomethane hydrochloride; HNE, 4-hydroxy-2-nonenal; His, histidine; MS, mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization−time of flight mass spectrometry; MS-MS, tandem mass spectrometry; LC-MS-MS, liquid chromatography−tandem mass spectrometry; ESI-MS, electrospray ionization mass spectrometry.

LITERATURE CITED

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