α,β-Unsaturated Aldehydes Accelerate Oxymyoglobin Oxidation

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This study investigates the potential basis for enhancement of oxymyoglobin (OxyMb) oxidation by lipid oxidation products. Aldehydes known to be formed as secondary lipid oxidation products were combined with OxyMb in aqueous solution at 37 °C and pH 7.4. Metmyoglobin (MetMb) formation was greater in the presence of α,β-unsaturated aldehydes than their saturated counterparts of equivalent carbon chain length. Additionally, increasing chain length from hexenal through nonenal resulted in increased MetMb formation ($P < 0.05$). Electrospray ionization mass spectrometry (ESI-MS) revealed that OxyMb incubated with 4-hydroxynonenal (HNE) at pH 7.4 at 37 °C yielded myoglobin molecules adducted with one to three molecules of HNE from 0.5 to 2 h of incubation, respectively. A prooxidant effect of HNE was noted at pH 7.4 but was not apparent at pH 5.6 when compared to the control ($P < 0.05$). This appeared to be due to rapid OxyMb autoxidation at this pH compared to pH 7.4. ESI-MS demonstrated that adduction of HNE to OxyMb occurred at pH 5.6. This research demonstrates that α,β-unsaturated aldehydes accelerate OxyMb oxidation and appear to do so via covalent attachment.

Keywords: *Oxymyoglobin; metmyoglobin; aldehydes; hydroxynonenal*

INTRODUCTION

The red to brown color change that occurs in fresh beef during retail display results from oxidation of ferrous oxymyoglobin (OxyMb) to ferric metmyoglobin (MetMb). The process of lipid oxidation is one of a variety of factors that influence the rate at which this occurs (Faustman and Cassens, 1990). Heme proteins are capable of binding to membranes (Szebeni et al., 1988) and fatty acids (Gotz et al., 1994), and this proximity would enhance the likelihood of interaction with products released as a consequence of lipid oxidation. Haurowitz et al. (1941) were among the first investigators to demonstrate that lipid oxidation products could be deleterious toward heme proteins. OxyMb oxidation appears to be linked with that of lipids in meat (Greene, 1969; Greene et al., 1971); as concentrations of the lipid soluble antioxidant α-tocopherol are increased in beef, the rates of lipid oxidation and OxyMb oxidation are decreased (Faustman et al., 1989). Additionally, OxyMb stability in liposome and microsome models is improved with elevated concentrations of α-tocopherol (Yin and Faustman, 1993; Yin et al., 1994).

Chan et al. (1997) attempted to better understand the potential basis for this oxidative interaction. Incubation of OxyMb in dialysis sacs (MW cutoff 500 Da) placed in solutions of fresh and oxidized liposomes revealed that thiobarbituric acid reactive substances (TBARS) were measurable within the dialysis sacs after 5 h at 30 °C. The concentration of TBARS increased with the extent of oxidation of the lipid solution in which the sacs were incubated. Historically, investigators of meat lipid oxidation have focused on the production of malonaldehyde. However, several aldehyde products can be formed from membrane lipids (Esterbauer et al., 1982). Chan et al. (1997) utilized several aldehydes, known to be secondary lipid oxidation products of meat lipid oxidation, to demonstrate prooxidative activity toward oxymyoglobin. Of the aldehydes tested, the α,β-unsaturated aldehydes nonenal and heptenal were found to be especially prooxidative.

It has been reported that α,β-unsaturated aldehydes are very reactive toward protein (Witz, 1989). Several of these are produced from fatty acids typically found in beef products (Esterbauer, 1982). Among these, 4-hydroxynonenal (HNE) has received the most attention (Esterbauer et al., 1991). HNE has been reported to inactivate protein sulfhydryls (Esterbauer et al., 1991), insulin (Uchida and Stadtman, 1992), and glucose-6-phosphate dehydrogenase (Szweda et al., 1993). It appears to increase in concentration during perfusion of cardiac tissue and increases dramatically during postischemic reperfusion injury (Blasig et al., 1995). The presence of HNE has been documented in beef and pork at concentrations of 14−150 and 1−152 nmol/g, respectively (Sakai et al., 1995).

In our attempts to better understand the oxidative interaction between lipids and OxyMb, our working hypothesis has been that secondary lipid oxidation products are more polar than their parent compounds and could diffuse from membranes into the surrounding sarcoplasm. Many of these compounds have reactive groups that would allow them to interact with sarcoplasmic constituents including myoglobin. The objective of this study was to determine the relative reactivities of different aldehyde oxidation products and assess the ability of HNE to react with myoglobin.

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MATERIALS AND METHODS

Materials. Horse heart myoglobin and sodium hydrosulfite were from Sigma Chemical Co. (St. Louis, MO). PD-10 columns were from Pharmacia Inc. (Piscataway, NJ). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI); all other aldehydes were purchased from Aldrich Chemical Co. (Milwaukee, WI). HNE is a cytotoxic and mutagenic compound that should be handled appropriately to minimize exposure (Bruenner et al., 1995).

OxyMb Preparation. OxyMb was prepared by hydrosulfite-mediated reduction of commercial MetMb (Brown and Mebine, 1969), and residual hydrosulfite was removed by dialysis (3 × 10 volumes) against sodium phosphate (pH 7.4) or sodium citrate (pH 5.6) buffer.

Reaction with Aldehydes and MetMb Formation. The reactions (3 mL total volume) were carried out in capped tubes (16 × 100) at 37 °C and contained 0.15 mM OxyMb and 1 mM aldehyde. Controls consisted of OxyMb plus a volume of ethanol (15 μL/mL) equivalent to that used to deliver the aldehydes. Reaction assays were scanned spectrophotometrically from 650 to 450 nm, and MetMb formation was calculated according to the method of Krzywicki (1982).

Preparation of HNE−Mb Adducts. OxyMb (0.15 mM) and HNE (1.0 mM) were combined in a capped test tube (16 × 100) and incubated at 37 °C. At desired time points, 1-mL aliquots were removed from the assay mixture and passed over a PD-10 desalting column to remove unreacted HNE. The colored solution was collected and heme removed from myoglobin by precipitation with ice-cold acidic acetone (Fanelli et al., 1958). This was necessary to avoid iron-based interference with formation of ions by electrospray. For each 1 volume of OxyMb, 25 volumes of acidic acetone (3 mL of 2 N HCl/L of acetone) was added with stirring for 20 min. Following centrifugation at 2000*g*, the supernatant was discarded and the heme-free protein precipitate dissolved in a minimum volume of distilled water.

Electrospray Ionization Mass Spectrometry (ESI-MS) of Mb−HNE Adducts. ESI-MS analyses were done on a Finnigan MAT TSQ7000 instrument (San Jose, CA) equipped with a standard Finnigan atmospheric pressure ionization and electrospray probe. Data acquisition and analysis were performed on a DEC 3000 alpha work station. Protein samples (~0.8 mg/mL) were diluted 1:10 with MeOH/H_2O/acetic acid (1:1:0.05) and infused into the instrument at 3 μL/min. Spectral deconvolution was done with Finnigan Bioworks software.

Statistical Analysis. Data for triplicate samples were analyzed by analysis of variance (ANOVA) and computed by using the SAS General Linear Model (GLM) procedure (SAS Institute Inc., 1985). Differences among means were determined by the least significance difference test.

RESULTS AND DISCUSSION

The effect of different saturated and unsaturated aldehydes on MetMb formation is presented in Figure 1. During 5 h of incubation at 37 °C and pH 7.4, the formation of MetMb was greater in the presence of α,β-unsaturated aldehydes than their saturated counterparts of equivalent carbon chain length ($P < 0.05$). Additionally, prooxidative behavior of the α,β-unsaturated aldehydes increased as chain length increased from six to nine carbons. Agerbo et al. (1992) reported a similar result for inactivation of glucose-6-phosphatase by aldehydes associated with fish oil oxidation. The prooxidative effect of HNE was similar to that of nonenal (results not shown).

To understand the potential basis for the prooxidative effect of the α,β-unsaturated aldehydes, HNE was incubated with OxyMb during 2 h of incubation at 37 °C and pH 7.4. ESI-MS revealed that HNE became adducted to OxyMb. Deconvoluted spectra of the myoglobin−HNE adduct mixture are presented in Figure 2. At the start of incubation, the only detectable species was that of OxyMb indicated by the peak at mass 16952 equivalent to that of horse heart myoglobin without heme (Figure 2A). Following 0.5 h of incubation, a second peak was evident, which represented a molecule with a mass of 17108 (Figure 2B). This peak was 156 Da greater than that of oxymyoglobin alone, a mass difference equivalent to the molecular weight of HNE. This indicated that HNE had bound covalently to OxyMb. A total of four peaks representing myoglobin and three different myoglobin−HNE species was present after 2 h of incubation (Figure 2C). Each succeeding peak represented the adduction of an additional HNE molecule to OxyMb and had an increased mass of 156 Da (within experimental accuracy) over that of the preceding peak. Thus, after this incubation period, some OxyMb remained unadducted, while other molecules contained one, two, or three adducts of HNE. These spectra do not provide any information as to the site(s) of adduction. The single HNE adduct indicated in Figure 2B could represent several different myglobin−HNE monoadducts, each with a single HNE bound to a potentially different amino acid.

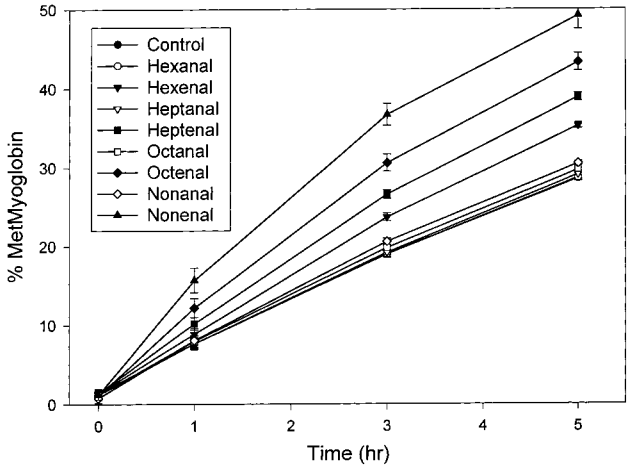

Figure 1. MetMb formation in assays of OxyMb and saturated and unsaturated aldehydes during incubation at pH 7.4 and 37 °C. Standard error bars are indicated.

Bruenner et al. (1995) reported that one to four molecules of HNE were adducted per hemoglobin α-chain when a 46-fold molar excess of HNE was incubated with this protein at 37 °C for 3 h. They also reacted HNE with β-lactoglobulin B and found that >99% of the modification of both proteins was due to Michael addition with only traces of Schiff base formation. Deconvoluted ESI-MS spectra of adducted proteins showed the presence of one or more peaks each sequentially higher by 156 Da. Our results suggest that HNE adducts are formed by Michael addition (Figure 2B,C). Schiff base formation is a condensation reaction between a carbonyl and primary amino group in which a water molecule is lost. Covalent attachment of aldehydes by this mechanism would be expected to yield adducts with increased mass of 138 Da, a result that we did not observe. In addition, OxyMb oxidation in the presence of saturated aldehydes was not different from controls (Figure 1), and this observation would also support a Michael addition reaction.

In general, the amino acids that are the best candidates as nucleophiles for Michael addition are cysteine, histidine, and lysine(Uchida and Stadtman, 1992; Bol-

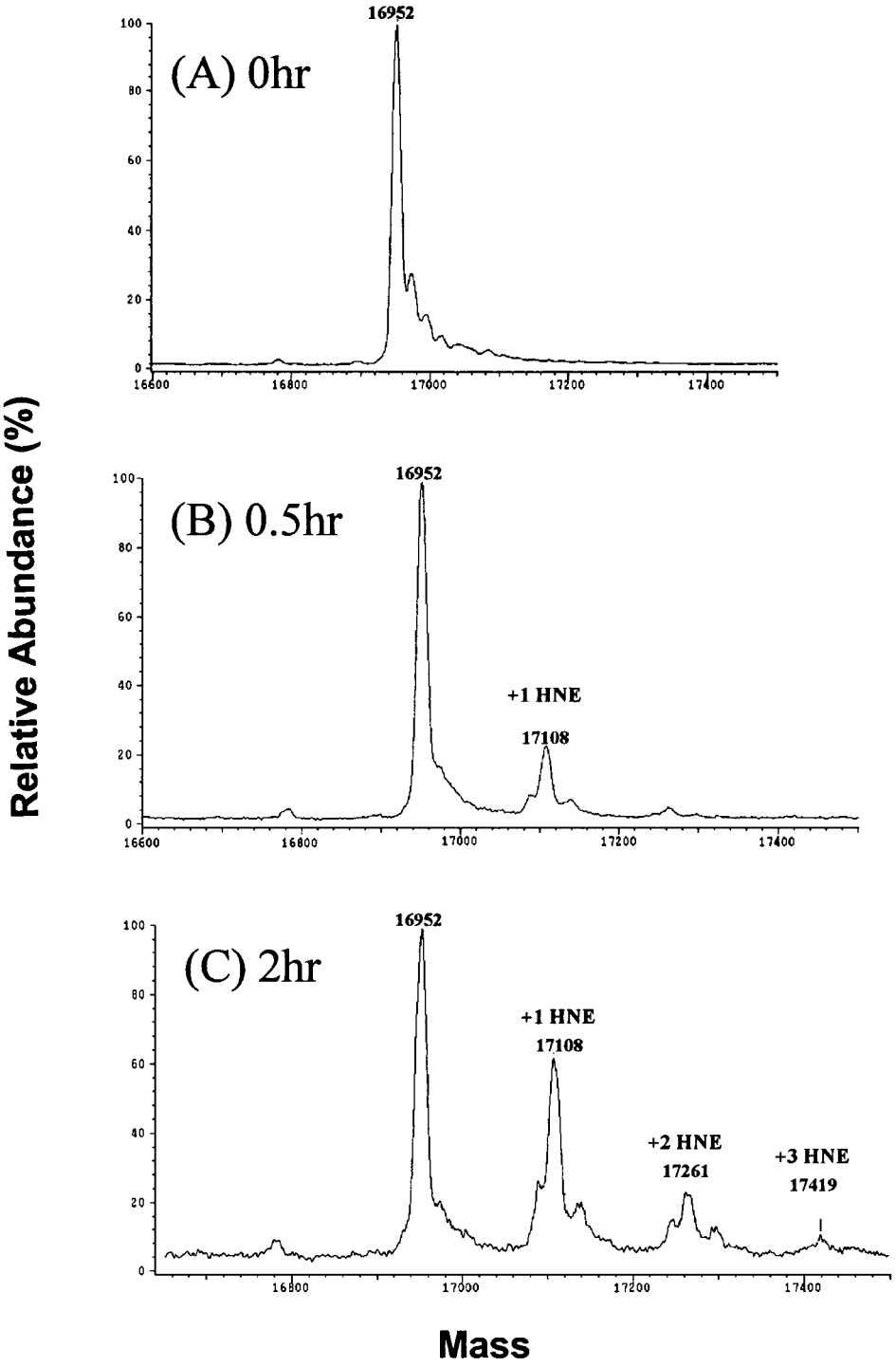

Figure 2. Deconvoluted ESI-MS spectra for OxyMb after reaction with HNE at (A) 0, (B) 0.5, and (C) 2 h of incubation at pH 7.4 and 37 °C.

gar and Gaskell, 1996). Although the sulfhydryl of cysteine is the most nucleophilic of these, mammalian myoglobins lack cysteine residues (Livingston and Brown, 1981). The site of HNE adduction on OxyMb was not investigated in our study. Bolgar and Gaskell (1996) performed a related study and used a 33-fold molar excess of HNE to react with apomyoglobin for 2 h at 37 °C. A range of 3−10 HNE adducts per molecule of protein was found, and ESI-MS and tandem MS were used to show that Michael addition of HNE was solely to histidine residues. Uchida and Stadtman (1992) used insulin to demonstrate that HNE can adduct histidine residues. Reaction of HNE and 2-nonenal with *N*-acetylhistidine revealed adduction to the imidazole nitrogen atoms of histidine. Interestingly, subsequent work by Szweda et al. (1993) provided support that Michael addition of HNE to glucose-6-phosphate dehydrogenase appeared to be at lysine residues and resulted in a 70% loss in enzyme activity.

The pH of beef is ∼5.6, and experiments were carried out to determine the effect of HNE and pH on OxyMb oxidation. A comparison of control and HNE treatments at pH 7.4 and 5.6 is presented in Figure 3. During incubation at 37 °C, HNE exerted a prooxidant effect when compared to the control at pH 7.4 ($P < 0.05$). Oxidation of OxyMb was faster at pH 5.6 than at 7.4 ($P < 0.05$). However, there did not appear to be any prooxidant effect of HNE at pH 5.6 compared to the

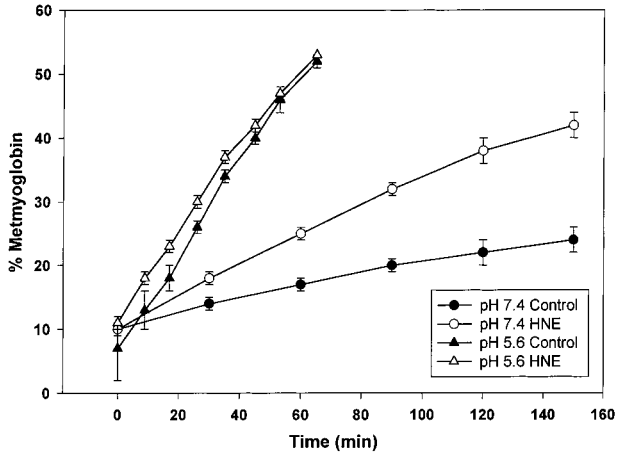

Figure 3. MetMb formation in assays of OxyMb with HNE and pH 5.6 and 7.4 incubated at 37 °C.

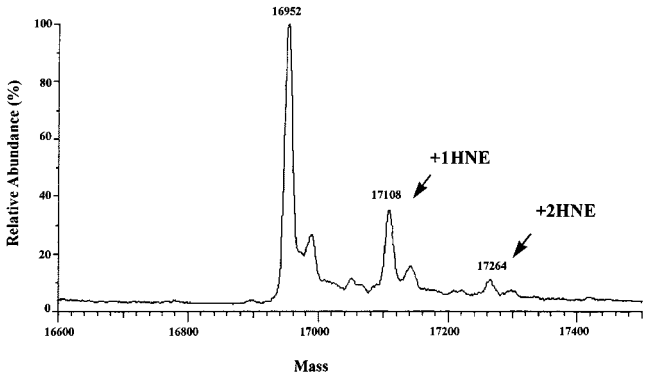

Figure 4. Deconvoluted ESI-MS spectra for OxyMb following reaction with HNE at 2 h incubation at pH 5.6 and 37 °C.

control. Autoxidation of OxyMb is enhanced at lower pH values (Gotoh and Shikama, 1974) and could have proceeded sufficiently rapidly to prevent observation of any prooxidant effect of HNE adducts. Bolgar and Gaskell (1996) determined that HNE adduction of apomyoglobin at pH 7.4 was exclusively at histidine residues. However, in our investigation at pH 5.6, histidine residues would be expected to be partially charged (Barrick et al., 1994) compared to pH 7.4, and this would reduce their ability to serve as nucleophiles for reaction with HNE. Thus, OxyMb was reacted with HNE for 2 h at pH 5.6 and 37 °C, and the products were analyzed by ESI-MS. The deconvoluted spectrum for this experiment is presented in Figure 4. It is clear that adduction by HNE did occur and that there were two peaks in addition to that of unadducted OxyMb representing one and two adducts of HNE per molecule of OxyMb. We believe that the rapid autoxidation of OxyMb at pH 5.6 rendered the effects of HNE irrelevant at that pH. In support of this contention is the observation that whereas at 0.5 h of incubation at pH 7.4 there was only a single adduct evident (Figure 2B), the prooxidant effect of HNE relative to that of the control was significant ($P < 0.05$; Figure 3).

The prooxidative effect of α,β-unsaturated aldehydes toward OxyMb provides a potential mechanism for the observed oxidative interaction between lipids and myoglobin. The impetus for this work was the observed oxidative interaction between myoglobin and lipids in meat. It is not possible to extrapolate our present results directly to this complex food. Our results may be more relevant for muscle than meat given the pH dependence of the HNE effect. It is possible that HNE-induced oxidation could be differentiated from autoxidation at pH 5.6 at temperatures lower than those used in the present work. Adduction of α,β-unsaturated aldehydes to OxyMb would be expected to alter the tertiary structure of this protein and predispose it to greater oxidation. We are currently pursuing identification of the specific sites on OxyMb to which aldehydes adduct and are attempting to ascertain whether myoglobin adduction occurs in meat.

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