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# Effects of 4-Hydroxy-2-Nonenal on Mutant Sperm Whale Myoglobins

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**Effects of 4-Hydroxy-2-Nonenal on Mutant Sperm Whale Myoglobins**

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# Chapter I

# Introduction

The appearance of fresh meat is essential for consumer acceptance. Discolored meat is usually rejected by consumers who judge meat color as a sign of wholesomeness. In fresh meat, lipid oxidation and myoglobin oxidation are common biochemical reactions. Loss of desirable cherry-red color along with formation of off-odor and off-flavor leads to decreased sensorial quality. A positive correlation between these two reactions suggests an interaction effect that exacerbates each reaction (Greene, 1969; Faustman et al., 1989b). Many studies suggest that secondary lipid oxidation products, including unsaturated aldehydes and ketones, have an adverse effect on redox stability of oxymyoglobin (OxyMb) (Chan et al., 1997a; Faustman et al., 1999; Lynch and Faustman, 2000; Maheswarappa et al., 2009), and that oxidized myoglobin is capable of promoting lipid oxidation (Chan et al., 1997b; Grunwald and Richards, 2006a; Richards et al., 2005; Richards et al., 2009). Therefore, mechanistic interaction between secondary lipid oxidation products, in particular 4-hydroxy-2-nonenal (4-HNE), and OxyMb oxidation needs to be elucidated in order to potentially curtail undesirable quality changes in meat.

Interestingly, the effect of HNE, a model secondary product of lipid oxidation, on the redox stability of myoglobin appears to be species-dependent. According to previous reports, HNE alkylation has been exclusively identified on histidine residues of equine (Faustman, et al., 1999), bovine (Alderton et al., 2003), porcine (Suman et al., 2007), yellowfin tuna (Lee et al., 2003a), and chicken and turkey (Naveena et al., 2010) myoglobins. Suman et al. (2007) proposed that the different histidine numbers between bovine (13 histidine

residues) and porcine (9 histidine residues) myoglobins was responsible for their different relative susceptibilities to HNE alkylation. Hence, variations in the number and location of histidine residues in the primary sequences of myoglobins from different animal species can lead to different susceptibilities towards HNE alkylation and associated redox instabilities and color changes.

The ability to apply recombinant technology to the production of mutant myoglobins provides an opportunity for investigation of the mechanistic bases of these reactions. Recombinant sperm whale myoglobins have been used extensively for studying the mechanisms of OxyMb oxidation (Braunstein et al., 1988; Egeberg et al., 1990; Carver et al, 1992; Brantley et al., 1993; Tang et al., 1998) and myoglobin-mediated lipid oxidation (Grunwald and Richards, 2006ab, Richards et al., 2009). Mutant sperm whale myoglobins with additional and/or substituted histidine residues provide appropriate models for testing hypotheses that focus on the effects/roles of amino acids in biochemical reactions/interactions. Also, the effects of oxidation rate and heme affinity of myoglobin towards 4-HNE modification and their ability to mediate lipid oxidation can be studied using mutant myoglobins.

Meat systems consist of a variety of components (e.g. non-heme iron, copper, metmyoglobin reductase, endogenous reducing agents etc.) that can either impair or improve the stabilities of myoglobin and lipids. Therefore, lipid system models are widely used for *in vitro* studies of the interactions between lipid and myoglobin oxidation in meat. Liposomes and microsomes are commonly employed as lipid model systems with different advantages (Pietrzak and Miller,

1989; Yin and Faustman, 1993; Yin and Faustman, 1994; Chan et al., 1996; Chan et al., 1997ab; Lynch and Faustman, 2000; Lee et al., 2003b). Microsomes are fragmented cellular and subcellular membranes isolated from muscles or liver using sequential centrifugation (Guengerich, 1977). Due to their origins, they contain lipids and naturally occurring pro-oxidants and antioxidants, which approximate cell membrane lipids of meat. Liposomes are manually constructed lipid multi-layers that have been used extensively as lipid membrane models (Pietrzak and Miller, 1989; Yin and Faustman, 1993; Chan et al., 1997b; Lynch and Faustman, 2000). The composition of phospholipids (PL); fatty acid profile; and the presence, type and concentration of antioxidants can be manipulated.

The aim of this study was to investigate the effect of HNE on the redox stability and pro-oxidative activity of OxyMb with variations in primary sequences.

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# Chapter II

# Literature Review

## 1. Myoglobin chemistry and meat color

Meat color is one of the most important quality traits that substantially influences consumers' purchasing decisions. Generally, meat with bright red color is preferred as it represents freshness. Discolored meat is generally misjudged as bacterially deteriorated; this change is also a chemical process. Discoloration of meat results in lost value due to price cuts and in some cases, discards. Losses have been estimated at $1 billion dollars annually for the US meat industry (Smith et al., 2000).

Myoglobin, a monomeric sarcoplasmic protein pigment present in muscle, functions biologically as an oxygen resource for the oxidative respiration of cells and is primarily responsible for meat color. Hemoglobin and cytochrome C are pigment proteins also present in meat, but their concentrations are relatively low compared to myoglobin, especially for hemoglobin if bleeding at slaughter is done properly. Therefore, myoglobin content is considered to dictate the color of meat. Variation in myoglobin content depends on species, age, diet, sex, muscle, and physical activity of animals (Hedrick et al., 1994). Myoglobin is more abundant in beef than pork, chicken, and fish, which are paler in color. Veal color is lighter than beef because calves are fed with iron-restricted diets and myoglobin content is less in young animals. Dark muscles, which are metabolically oxidative, contain greater amounts of myoglobin than white glycolytic muscles. In addition, myoglobin is abundant in active muscle tissues which require significant oxygen for their high respiration rates.

Myoglobin is a globular protein consisting of 153 amino acid residues and a prosthetic iron-containing heme group that resides in the hydrophobic heme pocket of myoglobin. This protects the ferrous heme iron ($Fe^{2+}$) from oxidation to ferric iron ($Fe^{3+}$) and thus preserves the oxygen binding ability of the heme iron (Livingston and Brown, 1981). Heme is a porphyrin ring with an iron atom located at the center by coordinating with 4 nitrogen atoms of the ring. On one side of the ring's plane is the fifth coordinate bond between the imidazole side chain of the proximal histidine residue (histidine 93) of the globin sequence and the heme iron. The sixth coordinate bond of heme iron is available for ligands. The distal histidine (histidine 64) interacts with the ligand and influences the heme-ligand interaction and redox stability of myoglobin.

Myoglobin exists in one of three forms depending on the redox state of heme iron and the type of ligand. When heme iron is in a reduced or ferrous form, the myoglobin is purple in color and called deoxymyoglobin (DeoxyMb); the sixth coordination site of heme is vacant. Upon binding of an oxygen molecule to heme $Fe^{2+}$, myoglobin turns bright red and is called oxymyoglobin (OxyMb). This process is known as "blooming" (e.g., when vacuum-packaged meat is exposed to air). When ferrous heme iron reversibly oxidizes to ferric iron, the oxidized form of myoglobin is brown and called metmyoglobin (MetMb); a water molecule is bound to the heme.

### 1.1. Myoglobin autoxidation

In live animals, the spontaneous oxidation of ferrous myoglobin into its ferric form is detrimental to cell viability. However, muscle tissues possess

various protective mechanisms such as metmyoglobin reductase, ascorbic acid, and cytochrome C to reduce the oxidized pigments (Brown and Snyder, 1969; Hagler et al., 1979; Taylor and Hochstein, 1982; Livingston et al., 1985). Post-mortem meat, on the contrary, loses some of these antioxidant mechanisms. Thus, discoloration of meat is inevitable during storage.

Color stability of fresh meat depends on several intrinsic (age of animal, meat species, muscle type, pH) and extrinsic factors (temperature, oxygen partial pressure ($pO_2$), light, packaging, microbial growth, lipid oxidation, presence of antioxidants/pro-oxidants) (Faustman and Cassen, 1990). Essentially, pH, temperature, and $pO_2$ are major influences on myoglobin autoxidation in meat.

Redox stability of heme proteins is usually disrupted at either acidic or basic conditions. Conformational changes in the tertiary structure of proteins can potentially expose the prosthetic heme to the solvent (Livingston and Brown, 1981; Shikama and Matsuoka, 1986) and facilitate heme-proximal histidine bond cleavage accelerated by hydration of the heme pocket (Tang et al., 1998). Additionally, heme affinity of MetMb is weakened when the proximal histidine is protonated (Hargrove et al., 1996). Bound oxygen is usually in resonance forms with heme iron. In one of the resonant forms, ferric superoxide, the outer oxygen atom is negatively charged and becomes a proton acceptor, and the ferrous iron oxidizes to ferric iron (Giddings, 1977). Protonation of bound oxygen accelerates superoxide anion formation (Livingston and Brown, 1981). The role of the distal histidine (histidine 64 which interacts with the bound oxygen by hydrogen bonding) in a proton-catalyzed OxyMb autoxidation was proposed by Shikama

and Matsuoka (1986). They observed that OxyMb from *Aphysia kurodai* which lacks the distal histidine oxidized at a lower rate than sperm whale (SW) OxyMb over an acidic pH range (pH 4 – 7). However, Brantley and co-workers (1993) used mutant SW myoglobins to show that the proposed proton-relay mechanism of the distal histidine might not be significant. They reported that substitutions of the distal histidine with non-polar (valine) or non-ionizable polar (glutamine) amino acids did not affect the pH-dependence of OxyMb autoxidation. On the other hand, greater solubility of heme in basic solutions favors heme-globin dissociation (Sogbien et al., 2000), and released heme is more likely to oxidize than anchored heme (Cox and Nelson, 2008). However, this effect is less likely to occur at the low post-mortem pH of meat.

Generally, elevated temperature accelerates endothermic chemical reactions by increasing kinetic energy of the system. OxyMb autoxidation is temperature-dependent with an approximate $Q_{10}$ value (the increase in reaction rate when temperature increases by 10°C) of 5.0 (Brown and Mebine, 1969). Although myoglobin is fairly stable at a broad temperature range, elevated temperature can induce partial unfolding of the protein and expose heme to the solvent and/or favor heme-globin dissociation (George and Stratmann, 1954). Furthermore, the combined effects of low pH and high temperature may exacerbate the loss of heme iron redox stability. The equilibrium fraction of ferric superoxide in oxygen-heme iron resonance forms becomes dominant at greater temperature (Giddings, 1977). Additionally, the availability of oxygen becomes limited at elevated temperatures as oxygen solubility decreases, and oxygen

association/dissociation equilibrium is shifted towards DeoxyMb which is less redox stable than OxyMb (Giddings, 1977).

Under atmospheric $pO_2$, ferrous myoglobin is saturated by oxygen resulting in very little heme protein in the deoxygenated form. The first-order rate constant of ferrous myoglobin autoxidation increases with decreased $pO_2$. The rate constant reaches maximum at its half-saturation ($P_{50}$) of 1 mmHg, while the rate constant is generally independent of oxygen level when $pO_2$ is greater than 30 mmHg (George and Stratmann, 1952). Under very low $pO_2$ (0-1 mmHg), the rate constant is proportional to $pO_2$. Activation energy required for autoxidation of OxyMb under high $pO_2$ (720 mmHg) is greater than that under low $pO_2$ (4 mmHg) implying that the additional energy is essential for breaking the connection between heme iron and oxygen (George and Stratmann, 1952).

In contrast to OxyMb which is a 6-coordinated complex with an oxygen molecule, deoxygenated ferrous myoglobin (DeoxyMb) is a 5-coordinated complex without a ligand. Lack of a ligand leads to a greater spin state of the heme ferrous iron electron that is more likely to oxidize in the presence of oxygen compared to that in OxyMb which is in a lower spin state (Livingston and Brown, 1981). Therefore, DeoxyMb is less stable than OxyMb.

The autoxidation of OxyMb to MetMb also results in superoxide anion which rapidly dismutates to hydrogen peroxide (Tajima and Shikama, 1987; Wazawa et al., 1992). Under air-saturated conditions, MetMb can decompose the formed hydrogen peroxide via its pseudo-peroxidase activity as hypervalent myoglobin (Fe(IV) or FerrylMb) (Alayash et al, 1999). On the other hand, when

oxygen is scarce, DeoxyMb becomes a preferred target of hydrogen peroxide through FerrylMb formation, which readily oxidizes DeoxyMb yielding 2 moles of MetMb (Yusa and Shikama, 1987; Wazawa et al., 1992). Interestingly, the heme moiety of hemoglobin and myoglobin is vulnerable to breakdown by the formed hydrogen peroxide. Free, non-heme ferrous iron has been reported to induce OxyMb oxidation (Allen and Cornforth, 2006).

### 1.2. Preparation of myoglobin forms for in vitro study

Due to the complexity of *in vivo* studies, many investigations of myoglobin properties are performed *in vitro*. Myoglobins from many animal species, unless commercially available, have been obtained by sequential purification from fresh meat. Normally, the myoglobin obtained is a mixture of ferrous and ferric forms. Therefore, OxyMb preparations are achieved by hydrosulfite (sodium dithionite)-mediated reduction of the isolated myoglobins (Brown and Mebine, 1969), and residual hydrosulfite is removed by dialysis or desalting columns. Reduced myoglobin (DeoxyMb) is oxygenated upon exposure to dissolved oxygen. Brantley and colleagues (1993) demonstrated that the chemical reduction of MetMb using a stoichiometric quantity or a slight excess of hydrosulfite did not affect autoxidation rates of the prepared OxyMb compared to OxyMb controls without dithionite treatment.

The preparation of deoxygenated heme-containing proteins, particularly DeoxyMb, requires anaerobic conditions. Due to cooperativity between subunits of hemoglobin, its affinity towards oxygen can be affected by pH. At a lower pH, hemoglobin becomes less oxygenated. This phenomenon is called the Bohr

effect (Benesch and Benesch, 1961). However, unlike hemoglobin, myoglobin does not show a Bohr effect because it contains only one polypeptide chain. Stoichiometric addition of hydrosulfite can be one of the options to reduce MetMb since hydrosulfite can remove dissolved oxygen rather than interact directly with OxyMb (Antonini and Brunori, 1971). However, the oxidized products remaining in the DeoxyMb solution compromise the redox stability of DeoxyMb by side reactions (Dalziel and O'Brian, 1957). Hydrosulfite-mediated reduction of MetMb under $N_2$ gas atmospheres in an anaerobic chamber can overcome the exposure of DeoxyMb to oxygen (Antonini and Brunori, 1971).

### 1.3. Measurement of myoglobin forms

Spectrophotometry is the most convenient method to analyze myoglobin species in solution. In the visible range of 400 to 650 nm, a myoglobin spectrum exhibits an isobestic point at 525 nm, the wavelength at which all three species of myoglobin share the same molar extinction coefficient (Bowen, 1949; Krzywicki, 1982). Thus, measuring absorbance at 525 nm can be used to estimate the myoglobin concentration of a mixed myoglobin solution (Stewart et al., 1965; Krzywicki, 1982).

To estimate proportions of oxy-, deoxy- and metmyoglobin in a myoglobin solution, a set of equations reported by Tang et al. (2004) can be used. These equations are formulated based on those originally published by Krzywicki (1982) using millimolar extinction coefficients of myoglobin at specific wavelength maxima for each of the myoglobin forms: Total myoglobin (525 nm), MetMb (503 nm), DeoxyMb (557 nm), and OxyMb (582 nm).

## 2. Mutant myoglobins

Mutant or recombinant myoglobins are synthetic proteins designed for investigating the effects of one or more amino acid residues on redox stability. The recombinant myoglobins have been studied extensively since they provide advantages for: 1) manipulation of amino acid residues and 2) investigating effects on designated properties of myoglobin such as the redox-stability, oxygen affinity, heme affinity, and heme destruction. Recombinant proteins are produced by the molecular biological technique, site-directed mutagenesis (Hutchison et al., 1978).

Site-directed mutagenesis is used to create mutations (deletion, insertion, substitution) at specific sites in DNA fragments using synthetic oligonucleotides that can form a duplex with a single-stranded DNA template despite mismatch that lead to mutation (Carter, 1986; Primrose and Twyman, 2006; Cox and Nelson, 2008). Normally, a plasmid vector is used to carry mutated genes into *Escherichia coli*, from which the protein coded by the gene will be expressed (Springer and Sligar, 1987; Primrose and Twyman, 2006). Gene sequences without any mutation are known as wild-types (WT). This technique has become important for several studies of structure-function relationships of proteins and the creation of proteins with novel properties (Primrose and Twyman, 2006).

Sperm whale (SW) myoglobin was the first protein to have its three-dimensional structure revealed by X-ray crystallography by Kendrew and colleagues (1958), and its primary sequence has been elucidated (Edmundson, 1965). Recombinant WT sperm whale myoglobin shares the same amino acid

sequence with the native protein, except that the WT myoglobin contains 154 amino acid residues (compared to 153 residues) with one extra methionine residue at the N-terminal end, and aspartate 122 is substituted for by asparagine (Tang et al., 1998). However, the autoxidation rates and X-ray crystallographic tertiary structure of the native and WT sperm whale proteins are similar (Brantley et al., 1993). Recombinant wild-type and mutant myoglobins have been constructed by site-directed mutagenesis to elucidate mechanisms of myoglobin autoxidation (Braunstein et al., 1988; Egeberg et al., 1990; Carver et al, 1992; Brantley et al., 1993; Tang et al., 1998) and myoglobin-mediated lipid oxidation (Grunwald and Richards, 2006ab, Richards et al., 2009).

## 3. Lipid oxidation in muscle foods

Lipids are fats and oils that serve as carriers of fat-soluble vitamins, aroma precursors and that contribute to the textures of foods. Oxidation of lipids can be either non-enzymatic (e.g. metal ions, such as $Fe^{3+}$ or $Cu^{2+}$) or enzymatic (e.g. lipoxygenase). Generally, lipid oxidation deteriorates the quality of food products. However, in certain kinds of food, such as fermented cheeses, lipid oxidation may be desirable for flavor development. The process is undesirable in fresh or cooked meat products due to loss of sensory characteristics and essential fatty acids. Saturated fatty acids are more stable against oxidation than their unsaturated counterparts. Polyunsaturated fatty acids, such as linoleic acid ($18:2\Delta^{9, 12}$), linolenic acid ($18:3\Delta^{9, 12, 15}$), and arachidonic acid ($20:4\Delta^{5, 8, 11, 14}$), are

found extensively in phospholipids of cell membranes to maintain fluidity. These fatty acids contain double bonds which are prone to oxidation.

Physiologically, lipids are used as a primary energy source via β-oxidation in the aerobic respiration of mitochondria. Reactive oxygen species may be generated and must be eliminated to prevent oxidative damage. Therefore, muscle tissues contain several enzymatic (superoxide dismutase, catalase glutathione oxidase) and non-enzymatic (α-tocopherol, vitamin C, glutathione, carotenoids) antioxidant systems for protection (Valko et al., 2006).

During post-mortem storage of meat, lipid soluble antioxidants, in particular α-tocopherol present in cell membranes, become critical to retard lipid oxidation and are generally more effective than water-soluble antioxidants (Niki, 1987). Endogenous vitamin E inhibited the formation of thiobarbituric reactive substances (TBARS) better than exogenous vitamin E blended manually into ground beef (Mitsumoto et al., 1993). Elevated concentrations of α-tocopherol found in the cell membranes of vitamin E-supplemented animals minimize lipid breakdown more effectively than exogenous vitamin E which may only superficially contact muscle lipids and form micelles due to its hydrophobicity (Liu et al., 1995).

A positive correlation between lipid oxidation and discoloration of meat has been reported in many studies (Greene, 1969; Faustman et al., 1989b). The supplementation of vitamin E in feed improved color stability of beef (Faustman et al., 1989ab; Liu et al., 1995; Liu et al, 1996; Faustman et al., 1998) and lamb (Wulf et al., 1995; Turner et al., 2002). In contrast, pork containing elevated

vitamin E contents generally did not show significant differences from controls though lipid oxidation was delayed (Cannon et al., 1996; Phillips et al., 2001; Guo et al., 2006). However, Lanari et al. (1995) was able to demonstrate the enhanced surface color stability of pork from pigs fed 198 and 207 mg α-tocopheryl acetate/kg diet which led to α-tocopherol contents of 6.91 and 7.88 μg α-tocopherol/g meat, respectively.

### 3.1. Effects of heme proteins on lipid oxidation

Lipid oxidation progresses at a fairly slow rate in the absence of catalysts or initiators. In meat, hemoglobin and myoglobin are two major heme-containing proteins that can enhance the propagation of lipid oxidation more effectively than inorganic non-heme iron (Johns et al., 1989; Baron and Andersen, 2002).

As noted previously, the autoxidation of OxyMb yields MetMb and superoxide anion which immediately converts to hydrogen peroxide via spontaneous dismutation. Harel and Kanner (1985a) reported the accumulation of endogenous hydrogen peroxide in ground turkey dark muscle tissues incubated at pH 5.6 and 60°C. At this temperature, most enzymes would become denatured, and hydrogen peroxide formed by enzymatic reactions (e.g., glucose oxidase) would be minimal. Moreover, OxyMb autoxidation is faster at this elevated temperature contributing to an accumulation of hydrogen peroxide. Chan et al. (1997b) demonstrated that MetMb and TBARS formation significantly decreased when OxyMb-liposomes were incubated in the presence of catalase or catalase and superoxide dismutase (SOD), while SOD alone did not affect OxyMb and lipid oxidation. This study suggested that the effect of superoxide

anions on lipid and OxyMb oxidation might be minimal compared to hydrogen peroxide. In the presence of ferrous or ferric iron, hydrogen peroxide decomposes to hydroxyl radicals which are potent oxidants (Tachiev et al., 2000). Additionally, hydrogen peroxide produced from a glucose oxidase/glucose system activated MetMb via the formation of hypervalent FerrylMb which abstracts electrons directly from double bonds of polyunsaturated fatty acids in the cell membrane (Harel and Kanner, 1985b). Thus, one of the pathways that myoglobin can initiate lipid oxidation is via the formation of hydrogen peroxide-activated MetMb.

Another possible mechanism of heme-mediated lipid oxidation is through the breakdown of preformed lipid hydroperoxides into alkoxy radicals and hydroxyl radicals by ferric heme (hemin) of MetMb and intermediate hypervalent FerrylMb formation (Baron and Andersen, 2002). Hence, the propagation step of lipid oxidation takes place progressively. Additionally, the formed radicals can also abstract hydrogen atoms from unsaturated fatty acids. The role of MetMb was confirmed by Richards et al. (2009) using mutant myoglobins with different redox stabilities. The results showed that a mutant myoglobin with greater redox stability (i.e. L29F – leucine 29 replaced by phenylalanine) was a poorer lipid oxidation initiator compared to WT myoglobin. The difference in MetMb formation rates could explain the effectiveness of the mutant myoglobins as lipid oxidation promoters. Among heme-containing proteins, methemoglobin (MetHb) is an excellent promoter of preformed lipid hydroperoxide breakdown followed by cytochrome c, oxyhemoglobin (OxyHb) and myoglobin (O'Brien, 1969).

Since ferrous heme has approximately 60-fold more affinity to globin than ferric heme (Hargrove et al., 1996; Tang et al., 1998), heme released from heme-containing proteins is believed to stimulate lipid peroxidation. At acidic pH, the proximal histidine can become protonated, and cleavage of the heme-histidine bond is likely to occur (Antonini and Brunori, 1971). Richards et al. (2005) compared the effect of released heme from trout anodic hemoglobin (i.e., a fraction of fish hemolysates with low isoelectric points ($\leq$ 8) that has the greatest anionic mobility towards anode in starch gel electrophoresis and shows a large Bohr effect (Giovenco et al., 1970; Binotti et al., 1971)) and myoglobin on lipid oxidation in washed cod muscle. Even though myoglobin autoxidized faster than anodic hemoglobin, anodic hemoglobin was a better lipid oxidation promoter. Using sperm whale apomyoglobin mutant H64Y (histidine 64 replaced by tyrosine) which has a high affinity for free heme moieties, they found that anodic hemoglobin lost its heme more rapidly than myoglobin. It could be concluded that the difference in the rate of heme dissociation from heme-containing proteins led to their ability to differentially enhance lipid oxidation (i.e. faster loss equals greater lipid oxidation enhancement). Richards et al. (2009) used mutant SW myoglobins with different heme affinities to test the hypothesis that released heme plays an important role in initiating lipid oxidation. They demonstrated that ferric H97A (histidine 97 replaced by alanine) which has a low heme affinity was the most effective in accelerating TBARS formation followed by WT and V68T (valine 68 substituted for by threonine) which more strongly bind to the heme moiety.

It is unclear which redox form of heme proteins is most pro-oxidant. Chan et al. (1997b) demonstrated that OxyMb was more pro-oxidant towards lipids than MetMb using liposomes. This was attributed to the combined action of hydrogen peroxide and MetMb (caused by OxyMb autoxidation) rather than MetMb alone. In contrast, Grunwald and Richards (2006a) reported that MetMb was more effective than ferrous myoglobins at mediating lipid oxidation. Richards and co-workers (2002) demonstrated that deoxyhemoglobin (DeoxyHb) oxidized faster and was more pro-oxidative than OxyHb in washed cod muscle. This is in agreement with Pietrzak and Miller (1989) who demonstrated that DeoxyHb was the greatest lipid oxidation inducer followed by OxyHb and MetHb in egg lecithin liposome models. Deoxygenated heme proteins are 5-coordinated complexes without ligands bound to them. This makes them less stable due to the high spin state electron of heme ferrous iron that is more likely to oxidize (Livingston and Brown, 1981). Autoxidation of DeoxyHb results in MetHb and superoxide anion, which is capable of initiating lipid oxidation. Additionally, the structure of deoxygenated hemoglobin is less compact than its oxygenated counterpart (Richards et al., 2002), and this allows access of fatty acids or preformed hydroperoxides to the heme pocket and stimulation of lipid oxidation (Rao et al., 1994).

Heme ring destruction by hydrogen peroxide could release free iron into solution and accelerate lipid oxidation (Nagababu et al., 2003). Released $Fe^{2+}$ and $Fe^{3+}$ were found to be 40- and 430-fold less effective, respectively, in promoting decomposition of lipid peroxides when compared to free hemin at pH

5.5 and 23°C (O'Brien, 1969). Moreover, unlike non-heme iron, heme-mediated lipid oxidation could not be inhibited by the presence of chelators such as EDTA (Richards and Li, 2004). A ferrous double mutant sperm whale myoglobin, L29F/H64Q (leucine 29 replaced by phenylalanine and distal histidine 64 replaced by glutamine), which is more sensitive to heme destruction in the presence of hydrogen peroxide than WT, was a poorer lipid oxidation initiator than ferrous WT in the presence of hydrogen peroxide in washed cod models (Grunwald and Richards, 2006b). The hydrophobicity of the heme moiety facilitates interaction with lipids, such as phospholipids, where lipid peroxidation occurs.

The effects of heme protein-mediated lipid oxidation are enhanced at low pH. At acidic conditions, the bond between the proximal histidine (His 93) residue and the heme iron might be weakened owing to protonation of the former resulting in increased concentration of released heme (Antonini and Brunori, 1971). Furthermore, the rate of ferric heme protein formation from ferrous heme proteins becomes greater at low pH, and oxidized heme proteins have lower heme affinity than their ferrous counterparts (Hargrove et al., 1996; Tang et al., 1998).

Bou et al. (2008) reported that the ability of OxyMb to promote lipid oxidation in pork microsomes was diminished upon denaturation by pre-heat treatment at temperatures greater than 75°C while MetMb could stimulate lipid oxidation in either native or unfolded forms. The unfolding of OxyMb was found to expose a greater proportion of antioxidant side chains of amino acids, normally

buried within the hydrophobic core of the native globin, than that of MetMb. Normally, cysteine, tyrosine and tryptophan are well-known for their antioxidant capacity (Meucci and Mele, 1997). Sulfhydryl groups of cysteine act as reducing agents, and phenyl rings of tyrosine and tryptophan act as radical scavengers. However, since non-human mammalian myoglobins lack cysteines, tyrosine and tryptophan are more likely responsible for the antioxidant activity of heated myoglobin.

### 3.2. Effects of lipid oxidation on redox instability of heme proteins

Besides pH, temperature, and oxygen partial pressure, redox destabilization of ferrous myoglobins can be caused by lipid oxidation and its products.

During initiation of lipid oxidation, an oxygen molecule is added into an alkyl radical to form a peroxide radical. Hence, oxygen uptake indicates the extent of the initiation step. O'Grady et al. (2001) proposed that oxygen consumption leads to decreased oxygen partial pressure and subsequent oxidation of DeoxyMb becomes dominant. This hypothesis was confirmed by Monahan et al. (2005) reporting that rates of OxyMb oxidation increased remarkably in stirred bovine muscle homogenates without oxygen bubbling compared to oxygen-bubbled samples and controls (without stirring or bubbling). This might apply to the myoglobin present in the interior of meat due to limited oxygen penetration, but surface myoglobin is mostly OxyMb due to oxygen exposure. Antioxidants such as α-tocopherol can limit oxygen consumption by lipid oxidation and thus preserve OxyMb. The authors concluded that lipid oxidation and OxyMb oxidation

may not always be positively related as lipid oxidation promotion of myoglobin was not observed in this study.

Primary products of lipid oxidation are hydroperoxides. OxyMb promotes breakdown of lipid hydroperoxides to alkoxy radicals. Simultaneously, myoglobin is converted into FerrylMb, which is reduced back to MetMb upon reaction with another lipid hydroperoxide to form a peroxy radical (Baron and Andersen, 2002). Under ultimate meat pH conditions (pH 5.5-5.8), the rates of FerrylMb formation and reduction are enhanced owing to acid-catalyzed mechanisms (Baron and Andersen, 2002).

Aldehydes and ketones are secondary products of lipid oxidation that are more volatile and that can yield rancid off-flavors. Moreover, they are more water-soluble than their parent lipid molecules. It is possible that these compounds enter the sarcoplasm where myoglobin resides and destabilize it. Among various conjugated carbonyls in which the carbon-carbon double bond is in the vicinity of the carbonyl group, aldehydes were most reactive towards glutathione, followed in order by ketones, esters, amides and carboxylates (Esterbauer et al., 1975; Esterbauer et al., 1991). It is important to note that decreases in electron-withdrawing effect of the carbonyl group lowers the reactivity of the secondary compounds.

Incubation of myoglobin with saturated or unsaturated aldehydes accelerated metmyoglobin formation (Chan et al., 1997a; Faustman et al., 1999; Lynch and Faustman, 2000; Maheswarappa et al., 2009). Carbon length and degree of unsaturation in the carbon chain of aldehydes or ketones determine

their effect towards heme oxidation (Faustman et al., 1999). More specifically, greater carbon number of these products increased metmyoglobin formation. Also, α,β-unsaturated aldehydes in which the carbon-carbon double bond is located next to the carbonyl group are highly electrophilic. They can readily react with nucleophilic side chains of amino acid residues in proteins.

**4. 4-Hydroxy-2-nonenal (HNE)**

HNE is a secondary oxidation product of n-6 unsaturated fatty acids especially linoleic and arachidonic acids which abound in cell membranes. HNE is an amphiphilic molecule and is soluble in either aqueous of hydrophobic solvents (Poli et al., 2008). HNE is one of the most extensively investigated lipid oxidation products due to its high reactivity towards proteins. The double bond between carbon 2 and carbon 3 of HNE is located in the vicinity of the carbonyl group and hydroxyl group at carbon 4. The hydroxyl and carbonyl groups can act as electron-withdrawing groups that induce polarization of the double bond of HNE (Esterbauer et al., 1975; Uchida and Stadtman, 1992; Grimsrud et al., 2008; Poli et al., 2008). This makes HNE highly electrophilic and likely to react with nucleophilic side chains of amino acid residues. The sulfhydryl group of cysteine, imidazole group of histidine and ε-amino group of lysine are susceptible to modification by these reactive molecules (Uchida and Stadtman, 1992; Bolgar and Gaskell, 1996).

Due to its ability to rapidly and covalently react with nucleophilic side chains of protein amino acids, HNE may not be a good indicator for lipid

oxidation because the measured amount reflects only free measurable HNE and will not include bound HNE. Still, HNE has been investigated extensively, especially for its adverse health effects and as a biomarker of oxidative stress. HNE-protein adducts can be a marker of lipid oxidation during liver injury (Poli et al., 2008). Cytotoxicity of HNE is due to covalent binding of HNE onto proteins, especially enzymes, which can lead to loss of activity due to induced conformational changes in tertiary structure (e.g. glyceraldehyde-3-phosphate dehydrogenase (Uchida and Stadtman, 1993). Incubation of insulin, a polypeptide containing no free sulfhydryl groups, with HNE led to HNE adduction on histidine residues (Uchida and Stadtman, 1992).

### 4.1. Reactions with proteins

Uchida and Stadtman (1992) revealed the modification of histidine residues of several proteins, including insulin, glyceraldehyde-3-phosphate dehydrogenase and bovine serum albumin. Interestingly, lysine residues rather than histidine residues of glucose-6-phosphate dehydrogenase from *L. mesenteroides* were preferentially alkylated by HNE.

Several studies have shown that incubation of myoglobins with HNE leads to greater heme protein oxidation rates (Faustman et al., 1999; Alderton et al., 2003; Lee et al., 2003b; Suman et al., 2006; Suman et al., 2007; Joseph et al., 2010; Naveena et al., 2010). It was hypothesized that HNE adducts can cause a slight shift in globin structure that increases exposure of heme and compromises redox stability of ferrous myoglobin. Alderton and colleagues (2003) used differential scanning calorimetry and showed a decrease in the

melting temperature ($T_m$) as myoglobin was destabilized by HNE alkylation. Moreover, the conformational change in structure could make myoglobin less recognizable for MetMb reductase from beef liver extract (Lynch and Faustman, 2000). Heme exposure also enhances heme-mediated lipid oxidation by increasing access of heme to lipids in liposome and microsome models (Lynch and Faustman, 2000). Furthermore, inter- and intra-molecular cross-linking between the carbonyl group of HNE and amino acid side chains (i.e. ε-amino group of lysine) can also disrupt protein folding and stability (Bruenner et al., 1995; Grimsrud et al., 2008).

A physiological pH of 7.4 is more effective for HNE adduction than lower pH (e.g. postmortem pH of 5.6) (Faustman et al. 1999; Alderton et al., 2003; Joseph et al., 2009). Protonation of imidazole groups of histidine and ε-amino groups of lysine at low pH (e.g. pH 5.6 of meat) results in charges to these side chains which would reduce their ability to serve as reactive nucleophiles for HNE. High temperature can increase kinetic energy to the system as well as induce conformational changes to myoglobin structure that could expose amino acid residues (particularly histidine, and lysine) typically buried inside the proteins, leading to greater access of HNE to these nucleophilic residues.

Lynch et al. (2001) reported the *in vitro* and *in vivo* formation of HNE as a lipid-derived product, detected by reverse-phase HPLC, during incubation of 1,2-dilinoleoyl-phosphatidylcholine liposomes and beef liver microsomes in the presence of OxyMb. In the same study, the concentration of the formed HNE was greater in the control than the α-tocopherol treatment. Moreover, HNE adducts

were also identified by using a polyclonal HNE:histidine antibody (Lynch et al., 2001).

Since HNE is a breakdown product of ω-6 unsaturated fatty acids, the contradicting effect of α-tocopherol on beef and pork color could be partially explained by differences in unsaturated fatty acid profiles among these red meats. Pork contains greater proportions of ω-6 unsaturated fatty acids (e.g., linoleic acid, arachidonic acid) than beef and lamb (Enser et al., 1996). It is possible that greater amounts of HNE could be produced in pork than beef.

### 4.2. Detection of HNE alkylation

HNE forms covalent adducts on proteins which can be identified by various approaches: chemical methods (e.g. 2,4-dinitrophenylhydrazine derivatization), immunoassay methods with two-dimensional gels, or mass spectrometry (MS).

Using polyclonal antibodies, Lynch et al. (2001) showed lipid-derived aldehyde adducts of HNE, malondialdehyde, hexenal, heptenal, octenal and nonenal, both *in vitro* and in soluble fractions of beef. Uchida and Stadtman (1992) reported that a shift in wavelength maximum from 224 nm (free HNE) to 217 nm (N-acetylhistidine:HNE adducts) indicated saturation of an aliphatic double bond. This observation suggested that the double bond between carbon 2 and 3, rather than the carbonyl group, acts as an electrophile attacking nucleophilic amino acid side chains. Mass-spectrometry has become a powerful analytical tool to detect HNE alkylation and has revealed a 156 Da increase in the molecular weight of myoglobins from horse (Faustman, et al., 1999), cattle

(Alderton et al., 2003), yellowfin tuna (Lee et al., 2003a), pig (Suman et al., 2007), and chicken and turkey (Naveena et al., 2010). The results suggest that HNE adducts in myoglobin occur by Michael addition rather than Schiff's base formation. Since Schiff's base formation is a dehydration reaction, it is expected that the increase in molecular weight would be 138 Da due to loss of a water molecule (MW = 18 Da). HNE adducts formed by Schiff's base reaction may be less stable than adducts formed by Michael addition since Schiff's base reactions may reverse in the presence of water. Michael addition leaves the carbonyl group of HNE intact and available to react with 2,4-dinitrophenylhydrazine (DPNH) to form a hydrazone (Esterbauer et al., 1991; Carini et al., 2004). However, Uchida and Stadtman (1992) reported that the formation of a hemiacetal (a stabilized form of HNE adducts that prevents the dissociation of HNE Michael adducts in acidic conditions (Orioli et al., 2007) between the carbonyl moiety and a hydroxyl group at carbon 4 could occur and prevent the reaction with DPNH. When 2-nonenal, which lacks a hydroxyl group to form a hemiacetal, was used instead of HNE, the adducts formed hydrazones with DPNH (Uchida and Stadtman, 1992). However, Nadkarni and Sayre (1995) argued that the DPNH reactivity of Michael HNE adducts could be retained despite hemiacetal arrangement, and Fenaille et al. (2002) demonstrated that reactivity of HNE and 2-nonenal towards DPNH were comparable.

HNE adducts were detected in low-density lipoprotein apoB-100 (mg/ml) incubated with tritium-labeled HNE (mg/ml) (Annangudi et al., 2008). The authors reported that the number of HNE adducts exceeded the number of reactive

nucleophilic amino acid residues. Multi-adducts of HNE on the same amino acid residue in a domino-like manner were revealed by using N-acetyl-histidine incubated with HNE and the adducts were detected by mass spectrometry (Annangudi et al., 2008).

To investigate the amino acid residue sites of HNE alkylation on proteins, tandem mass spectrometry (MS/MS) has been employed (Bolgar and Gaskell, 1996; Fenaille et al., 2002; Alderton et al., 2003; Liu et al., 2003; Annangudi et al., 2008). Proteins of interest are digested by proteolytic enzymes (i.e. trypsin) with subsequent tandem MS analyses of peptide ions. The digested peptides are separated by liquid chromatography and subjected to tandem MS which consists of two-stage mass analysis. The first mass analysis is performed to separate peptide ions of interest. The selected ions are then subjected to collision-induced dissociation (CID) into fragments with an inert gas such as helium or argon. In the second mass analysis, fragmented ions are analyzed and mass-to-charge ratios are deconvoluted into mass spectra. The interpretation of acquired spectra requires database searches to match the sequence of the peptide with known sequences of proteins (Liebler, 2002; Tabb and Yates, 2006).

Non-human mammalian myoglobins lack cysteine residues (Antonini and Brunori, 1971; Livingston and Brown, 1981). Hence, histidine and lysine residues are the primary candidates for alkylation. Nonetheless, when myoglobin, regardless of species, is exposed to HNE at a 1:7 molar ratio, histidine rather than lysine has been alkylated by HNE (Alderton et al., 2003; Suman et al., 2006; Suman et al., 2007; Naveena et al., 2010). Bolgar and Gaskell (1996) showed a

maximum of 10 adducts of HNE per molecule of horse apomyoglobin, which has 10 histidine residues, by incubating with a 33-fold molar excess of HNE. They were unable to detect HNE-modified lysine. However, these results do not exclude the possibility of HNE:lysine adducts because this type of adduct could be labile to the conditions encountered in mass spectrometry analysis. Thus, samples have to be treated by sodium borohydride-mediated reduction to stabilize any possible HNE:lysine adducts formed by a Schiff's base reaction between the carbonyl group of HNE and the ε-amino side chain of lysine (Esterbauer et al., 1975; Suman et al., 2007). It is possible that the variation in histidine residue number in amino acid sequences of myoglobin from different animal species dictates the susceptibility of OxyMb towards secondary products of lipid oxidation. Bovine myoglobin consists of 13 histidine residues while porcine myoglobin has only 9 residues, and it has been hypothesized that this is the basis bovine OxyMb being more affected by lipid oxidation than porcine OxyMb. Suman and co-workers (2006) demonstrated that bovine myoglobin incubated with HNE at a 1:7 molar ratio (pH 5.6 for 4°C for 72 h) oxidized faster than porcine myoglobin. Mass spectrometry analysis revealed that bovine myoglobin contained mono- and di-adducts, while only mono-adducts were identified in porcine myoglobin. LC-MS-MS analysis revealed that 4 histidine residues (His 36, 81, 88, and 152) of bovine myoglobin were alkylated, whereas 2 histidines (His 24 and 36) of porcine myoglobin were covalently modified with HNE (Suman et al., 2006). In short, differences in primary sequence (i.e. the number and location of histidine residues) of myoglobin might partially explain

the finding that vitamin E-supplemented feed could inhibit lipid oxidation but not OxyMb oxidation in pork.

## 5. Lipid system models

To simplify the study of factors involved with lipid oxidation in meat, lipid system models are widely used. There are several lipid models, each of which provides different advantages suitable for various types of experiments. These included liposomes, microsomes, lipid dialysis sacs, and washed muscle minces.

### 5.1. Liposomes

Liposomes are manually constructed lipid multi-layers that have been used extensively as lipid membrane models (Pietrzak and Miller, 1989; Yin and Faustman, 1993; Chan et al., 1997b; Lynch and Faustman, 2000). The advantage of using liposomes is that the composition of phospholipids (PL); fatty acid profile; and the presence, type and concentration of antioxidants can be manipulated. OxyMb-liposomes can be formed by adding OxyMb solution into precast lipid films and shaking with subsequent myoglobin encapsulation.

Yin and Faustman (1993) reported the influence of fatty acid composition and PL polar head group on OxyMb and PL oxidation. Phosphatidylserine (PS) had the greatest effect on PL oxidation followed by phosphatidylethanolamine (PE) and phosphatidylcholine (PC) even though PE contained greater proportions of PUFA than PC and PS. The OxyMb-PL oxidation followed the same trend as PL oxidation. Both PL and OxyMb-PL oxidations increased with the number of double bonds and length of fatty acids. At pH 5.6, PC and PE have

a zero net charge, while the net charge of PS is -1. The negative charge of PS is then considered to be responsible for greater PL oxidation.

### 5.2. Microsomes

Microsomes are fragmented cellular and subcellular membranes isolated from muscles or liver using sequential centrifugation (Guengerich, 1977). Due to their origin, they contain lipids and naturally occurring pro-oxidants and antioxidants, which approximate cell membrane lipids of meat. Microsomes are more complex than liposomes and considered more relevant to the *in vivo* condition (Faustman and Wang, 2000). The naturally occurring α-tocopherol content in membranal microsomes can vary from animal to animal. Hence, the concentrations of endogenous α-tocopherol in microsomes have to be determined and potentially standardized since microsomes isolated from vitamin E-supplemented animals usually contain greater vitamin E content and are more stable against lipid oxidation (Chan et al., 1996; Lee et al., 2003b). Microsomes isolated from beef muscles with elevated α-tocopherol content slowed TBARS formation and OxyMb oxidation relative to controls (Chan et al., 1996). Low α-tocopherol and greater proportions of unsaturated fatty acid contents of tuna microsomes compared to beef and pork microsomes contributed to significant TBARS values and OxyMb oxidation (Yin and Faustman, 1994).

### 5.3. Lipid dialysis sacs

Products of lipid oxidation are smaller and more water-soluble that their parent compounds and facilitate interaction with myoglobin in the aqueous phase of the sarcoplasm. Dialysis membranes can act as barriers preventing direct

contact between myoglobin and physical lipids. This permits study to test the hypothesis that small, water-soluble lipid oxidation breakdown-derived products can cross the dialysis membrane and interact with OxyMb causing accelerated MetMb formation.

Chan et al. (1997a) observed that immersing dialysis sacs (molecular weight cut-off (MWCO) = 500 Da) containing OxyMb in oxidized liposomes resulted in increased TBARS in the dialysis sacs and accelerated MetMb formation compared to dialysis sacs submerged in either buffer or freshly-prepared liposomes. The increase in TBARS within the dialysis tubing confirmed that lipid-derived products can diffuse inwards across the membranes and destabilize OxyMb located inside the dialysis tube. In related work, Chan et al. (1997b) used dialysis tubing with a MWCO of 10,000 Da to study the effect of hydrogen peroxide produced from OxyMb autoxidation on lipid oxidation. It was hypothesized that dialysis membranes would allow hydrogen peroxide generated from OxyMb autoxidation to enter the dialysis sacs containing liposomes. Since, myoglobin could not cross the membrane, the interference of hydrogen hydroxide-activated MetMb with liposome oxidation could be prevented. However, the effect of hydrogen peroxide on oxidation of liposome was not significant in the study because hydrogen peroxide could react with other compounds, such as OxyMb and MetMb, in the reaction assay before entering the dialysis sacs.

## 6. Summary and objectives

The appearance of fresh meat is essential for consumer acceptance. A positive correlation between lipid and OxyMb oxidation suggests an interaction effect that exacerbates each reaction. Many studies suggest that secondary lipid oxidation products, including unsaturated aldehydes and ketones, have an adverse effect on redox stability of OxyMb, and that oxidized myoglobin is capable of promoting lipid oxidation. Therefore, mechanistic interaction between secondary lipid oxidation products, in particular HNE, and OxyMb oxidation needs to be elucidated in order to better understand and potentially curtail the undesirable changes in meat. It has been revealed by tandem MS that histidine residues of myoglobin are exclusively alkylated by HNE. Variation in the number and location of histidine residues in primary sequences of myoglobin from different animal species can lead to different vulnerabilities of these proteins to HNE attack. Recombinant technology of mutant myoglobin production provides an opportunity for investigation of mechanistic basis of these reactions as it can be used to produce mutant myoglobins with variation in number/position of histidine residues, while the rest of the sequence shares similarity with WT myoglobin. Also, the effects of oxidation rate and heme affinity of myoglobin towards HNE modification and subsequently their ability to mediate lipid oxidation can be studied using mutant myoglobins.

The primary objectives of this thesis research were to

1) To evaluate the effect of 4-hydroxy-2-nonenal (HNE) on redox stability of mutant sperm whale myoglobins,
2) To characterize the sites of HNE adducts on mutant sperm whale myoglobins, and
3) To investigate the interrelationship between WT and mutant OxyMbs and lipid oxidation using lipid model systems.

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# Chapter III

## Redox Instability of Mutant Sperm Whale Myoglobins Induced by 4-Hydroxy-nonenal *in vitro*.

**Redox Instability of Mutant Sperm Whale Myoglobins Induced by 4-Hydroxy-nonenal *in vitro*.**

## 1. Abstract

Wild-type (WT) and recombinant sperm whale myoglobins (P88H/Q152H, L29F, H97A and H64F) were used as models for investigating the effects of 4-hydroxy-nonenal (HNE), a secondary lipid oxidation product, on redox stability of deoxymyoglobin (DeoxyMb) and oxymyoglobin (OxyMb). We observed that HNE induced greater redox instability in WT and mutant myoglobins compared to their respective controls ($p<0.05$). The extent of HNE-induced OxyMb oxidation was lesser in L29F than in WT ($p<0.05$), while it was greater in H97A and P88H/Q152H than WT ($p<0.05$). Also, H64F DeoxyMb was more redox stable than WT DeoxyMb in the presence of HNE ($p<0.05$). The Michael addition of HNE was found exclusively on histidine residues of myoglobin. Based on MALDI-TOF-TOF tandem mass spectrometry, histidine 48 (His 48) appeared to be a preferred site of HNE alkylation as HNE adducts at this residue were identified in all sperm whale myoglobins tested. The histidine residues substituted at position 88 (proline) and 152 (glutamine) in P88H/Q152H also were covalently modified by HNE, suggesting that the increase in histidine residues at certain locations was concomitant with more covalent HNE-alkylation and induced redox instability of myoglobin. This study underlines the significance of the primary sequence of myoglobin for influencing the redox stability and susceptibility of myoglobin to reactive secondary lipid oxidation products.

## 2. Introduction

Myoglobin is a sarcoplasmic protein in meat that is responsible for color. During storage, the cherry-red color of fresh 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 postmortem muscles), ferrous myoglobin 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 myoglobins can be impaired by many factors including reactive aldehydes derived from lipid oxidation (1-4).

In fresh meat, the oxidation of lipids and ferrous myoglobin is positively correlated (5-7). Vitamin E-supplemented beef demonstrated slowed formation of thiobarbituric reactive substances and improved color 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. 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). Many studies have demonstrated the electrophilicity of HNE towards nucleophilic amino acid side chains of cysteine, lysine, and histidine (11, 13). Sites of HNE alkylation via Michael addition are exclusively on histidine residues of equine (2), bovine (15), porcine (16, 17), yellowfin tuna (18), and chicken (19) myoglobins. HNE

alkylation of proteins can cause local or potentially global unfolding of myoglobin structure (15). Changes in the structure of myoglobin, especially at the hydrophobic heme pocket, could predispose ferrous heme to solvent exposure and to oxidation (20).

Suman et al. (17) hypothesized that a greater number of histidine residues and their locations in the primary sequence of bovine myoglobin (13 histidine residues) could increase its vulnerability to alkylation by HNE relative to porcine myoglobin which contains fewer histidine residues (i.e., 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 histidine (HIS 64) residues could destabilize heme-globin interactions directly and enhance oxidation. Suman and co-workers (17) 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 myoglobin; however, these residues are not present in porcine myoglobin. The observation suggested that bovine myoglobin could be more susceptible to HNE-enhanced OxyMb oxidation than porcine myoglobin because these 2 locations were readily accessible to HNE.

Myoglobins from several red meat-producing species share considerable homology in their primary sequences (e.g. ranging from 97.4% to 100% among ruminant species such as bison, cattle, sheep, and goat (21)). Small variations in their primary sequences, especially those in close proximity to the heme pocket

could be expected to alter autoxidation rate (22, 23), heme affinity (24), and structural stability (25, 26). Joseph et al. (21) reported that the primary sequences of cattle and bison (*Bison bison*) myoglobins are identical, and their thermal stability, autoxidation rate and HNE-induced oxidation rate tested *in vitro* were not statistically different. Mutant myoglobins 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 myoglobin has been studied extensively and recombinant sperm whale myoglobins have been employed to elucidate the interactions of amino acid residues present in the vicinity of the heme moiety and their significance on myoglobin redox stability (27-31). Richards et al. (32) studied the effect of sperm whale OxyMb oxidation rate on its ability to promote lipid oxidation in washed cod muscle minces using myoglobin mutant L29F (leucine 29 replaced by phenylalanine). L29F exhibited greater redox stability and was less effective at promoting lipid oxidation than wild-type myoglobin (WT). Moreover, they used H97A (histidine 97 substituted for by alanine), a mutant in which heme is bound more weakly than in WT, to test the effect of heme release on lipid breakdown and found that greater heme dissociation of H97A led to greater lipid oxidation compared to WT. Tang et al. (31) demonstrated that substitution of phenylalanine with a larger hydrophobic side chain for the distal histidine 64 in H64F (histidine 64 replaced by phenylalanine) could block solvent entry and slow heme release rate.

Understanding the basic mechanisms of how HNE affects myoglobin stability is important for comprehending oxidation interactions between lipids and

proteins in general, and myoglobin, specifically. The use of mutant myoglobins could provide insight into the effects of primary protein structure on HNE alkylation. The objectives of this study were (1) to investigate the effect of HNE on redox stability of wild-type (WT) and mutant sperm whale OxyMbs, and (2) to characterize the extent and sites of HNE alkylation in these myoglobins.

## 3. Materials and methods

### Wild-type and mutant sperm whale myoglobins

Besides WT, a new double mutant sperm whale myoglobin, P88H/Q152H (proline 88 and glutamine 152 both substituted for by histidine residues), was expressed and used to investigate the effect of histidine quantity in myoglobin on susceptibility to HNE alkylation. L29F (30, 32), H97A (24, 33, 34) and H64F (30, 31) were also included in this study. A comparison of the mutated sites of WT and mutant sperm whale myoglobins is presented in Table 1.

The identities of mutant sperm whale myoglobins are based on their position in the primary sequence of the native sperm whale myoglobin. For example, L29F means the 29$^{th}$ amino acid from the N-terminus of the native sperm whale myoglobin, which is leucine, is replaced by alanine. However, compared to native sperm whale myoglobin, recombinant sperm whale myoglobins contain one additional amino acid residue of methionine at the N-terminus (30).Thus, LEU 29 in the native sperm whale myoglobin is actually the 30$^{th}$ amino acid from the N-terminus of L29F and the proximal HIS 64 is at position 65 of WT and mutant sperm whale myoglobins.

The preparations of WT and mutant (P88H/Q152H, L29F, H97A and H64F) sperm whale myoglobins were performed at the Meat Science and Muscle Biology Laboratory, University of Wisconsin Madison. The procedures have been described in Grunwald and Richards (24, 34) and Richards et al. (32). Mutant myoglobin genes were prepared by site-directed mutagenesis using the WT sperm whale myoglobin gene on the pET 28 plasmid as template. The mutagenesis was confirmed by dideoxy sequencing at the University of Wisconsin Biotechnology Center (Madison, WI). The constructed plasmid was then transformed into *E. coli* BL21-CodonPlus® (DE3)-RP host cells (Stratagene, La Jolla, CA) via heat shock method. The recombinant myoglobins were expressed in the host *E. coli* cells using a 12-liter culture vessel (Nalge Nunc International, Rochester, NY) and Terrific Broth (TB) adjusted to pH 7 as the culture medium. All culture media contained 30 µg/ml kanamycin and 50 µg/ml chloramphenicol. The expression was performed by transferring a single colony of the transformed *E. coli* to 12 ml TB and incubating for 14-16 h in a 37°C shaker. The 12 ml culture was transferred into 360 ml 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 iron (III) chloride ($FeCl_3$) and 50 µl antifoam per liter. During incubation, the culture was bubbled with compressed air at 2-4 psi and mixed at 250 rpm with a motorized impeller. When the $OD_{600}$ of the culture reached approximately 2.0, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM to induce expression of the recombinant myoglobins. During induction, hemin chloride was added to the

culture to 4 mg/L. After 4 h of induction, cell paste containing the expressed myoglobins was obtained by centrifuging the culture at 2000 x 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 (35) containing 50 mM Tris base, 1 mM disodium EDTA dehydrate, 0.5 mM dithiothreitol, 1 mM toluene sulfonyl chloride, 40 U/ml DNAse I, 3 U/ml RNAse A, and 78800 U/ml lysozyme, adjusted to pH 6. The obtained myoglobins were purified from the lysate via ammonium sulfate precipitation, and anion and cation exchange chromatography as described previously (29, 36). Finally, the purified myoglobins were concentrated to 0.5 to 1.0 mM (heme basis), 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 myoglobin protein pellets were stored at –80°C. Prior to use, they were thawed in an ice bucket and re-suspended 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 (myoglobin: sodium hydrosulfite) of 1:10 (37). Residual hydrosulfite was removed by passing the myoglobin solutions through PD-10 desalting columns (Sephadex G25, 0.15% Kathon preservative CG, GE Health Care, Sweden) pre-calibrated with 50 mM sodium citrate buffer, pH 5.6. Myoglobin was converted to OxyMb upon exposure to dissolved oxygen in the column buffer and to atmospheric oxygen. The concentration of the prepared OxyMb was calculated using a millimolar extinction coefficient at 525 nm (E = 7.6

$cm^{-1}$ $mM^{-1}$) (38, 39). The percentage of OxyMb was determined spectrophotometrically according to Tang et al. (40) based on absorbance values recorded at 503, 525, 557 and 582 nm.

**Deoxymyglobin preparation**

DeoxyMb solutions of WT and H64F were prepared anaerobically in an air-tight glove bag (Sigma Chem. Co.). Nitrogen gas was of ultra low oxygen grade (Airgas East, Cheshire, CT). The glove bag 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 for 3 times (3 volumes) until the measurable oxygen reached $pO_2$ of 3 mmHg measured by a Model 6600 headspace oxygen/carbon dioxide analyzer (Illinois Instruments, Chicago, IL). A 50 mM sodium citrate buffer, pH 5.6, was warmed to 40°C, degassed and allowed to cool in the glove bag. 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 pre-treated with 2.5 ml of 0.1 mM sodium hydrosulfite to remove dissolved oxygen in the column, followed by passing the degassed 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 Tang et al. (40).

**Reaction with HNE**

Freshly-prepared ferrous myoglobin 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 x 115 mm) for OxyMb or in screw-capped quartz cuvettes for DeoxyMb. The selected concentration of myoglobin reflected that reported in pork (41) and was adopted to optimize the limited supply of the mutant myoglobins, and to be consistent with that used by Suman et al. (17). The molar ratio of OxyMb to HNE was maintained at 1:7 (2), and ethanol was delivered to controls at volumes equivalent to that used for delivering HNE (approximately 8 µl ethanol/ml of myoglobin).

All experiments were performed at 4°C, pH 5.6. For the OxyMb experiment of WT, L29F, and H97A, the reaction was run for 72 h and MetMb formation was measured at 0, 3, 6, 12, 24, 48, and 72 h. For the OxyMb experiment of WT and P88H/Q152H, the MetMb formation was determined at 0, 3, 6, 12, 18, and 24 h. However, the reaction continued until 72 h for mass spectrometry. For DeoxyMb experiment of WT and H64F, MetMb formation was determined at 0, 1, 2, 3, 4, 6, 8, 12, 18, and 24 h. Myoglobin solutions were scanned spectrophotometrically from 650 to 400 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu, Columbia, MD). MetMb formation was calculated according to (40). %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 difference 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 aliquot 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 pre-treated with deionized water to remove unreacted HNE. The 1 ml eluent was collected into 2 separated (0.5 ml each) capped cryogenic storage vials (Fisher Scientific, Pittsburgh, PA) 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**

Mass spectrometric analyses were performed at the University of Kentucky, Center for Structural Biology Protein Core Facility. MALDI-TOF mass spectrometry was applied to intact proteins, and masses were measured in the linear mode (500 shots/spectrum) using saturated alpha-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 Chem Co. St. Louis MO) at 37$^{o}$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 pipette tip (Sarstedt, Newton NC) packed with approximately 1 mm Empore C-18 (3M, St. Paul, MN). Peptides were eluted in 5 µl 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) with 0.3 µl 5 mg/ml alpha-cyano-4-

hydroxycinnamic acid (Aldrich, St. Louis MO) 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 greater than 20 were selected for MS/MS analysis (1000 shots per spectrum) by post-source decay or by collision-induced dissociation using air at a pressure of $6.67 \times 10^{-5}$ $N/m^2$. 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 3-way (mutant myoglobin type x treatment x incubation time) randomized complete block design in which each treatment had 3 replications. Block was defined as each replication that was prepared within 1 hr 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). Least square means were generated, and the differences among treatments were significant at $p<0.05$ using least significant differences (LSD).

## 4. Results and Discussion

MetMb formation in WT and mutant sperm whale myoglobins (L29F and H97A) as affected by the absence/presence of HNE is presented in Figure 1. The rates of OxyMb autoxidation followed the order H97A>WT>L29F ($p<0.05$), and HNE increased MetMb formation in all mutants ($p<0.05$). However, the relative susceptibility of the mutant myoglobins differed. Within the first 24 hrs of incubation, the effect of HNE on redox stability of mutant OxyMbs was greatest in H97A, followed by WT and L29F (H97A>WT>L29F; $p<0.05$) (Figure 2). After 24 hrs, controls and HNE-treated samples of H97A oxidized readily and their difference in %MetMb became smaller. This is presumably due to the weaker heme affinity of H97A, compared to WT (32) 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 with the imidazole group of histidine. The access of water to the heme crevice can interrupt hydrophobic interactions and weaken the coordinate bond between the imidazole side chain of the proximal histidine (HIS 93) and heme iron (24, 42). As a result, the globin becomes 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. (32) 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 which 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 while the alkyl side chain of leucine 29 in WT cannot (32). Our results (Figures 1 and 2) confirmed this as L29F was the most redox stable of the OxyMbs investigated both in the presence and absence of HNE.

Despite many attempts to accomplish a relatively low initial % MetMb for P88H/Q152H, initial %MetMb levels 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 myoglobins still differed by 10% (Figure 3). Hence, relative %MetMb ($\%MetMb_t$ - $\%MetMb_{t=0}$) was calculated and is presented in Figure 4. It is important to note that P88H/Q152H had an autoxidation rate comparable to 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 affected by HNE to a greater extent when compared to WT following 18 hrs of incubation (Figures 3 and 5). Turbidity was observed after 24 h of incubation and this has been noted occasionally in hemoglobin solutions exposed to high relative concentrations of HNE (43). Samples for mass spectrometry were acquired at 72 hr in order to provide for comparisons of alkylation with the other mutants. The insoluble particles that contained myoglobin and caused turbidity were removed by centrifugation.

As the partial pressure of oxygen decreases, the autoxidation rate of myoglobin increases and reaches its maxima when approximately 50% of myoglobin remains in the oxygenated form ($pO_2$ = 5 mmHg) (44); when oxygen is completely absent, 100% of the myoglobin is in the deoxygenated form (30). In this study, oxygen ($pO_2$ = 3 mmHg) could not be eliminated completely from the glove bag or the headspace of the capped quartz cuvettes and this provided an opportunity for heme iron oxidation. The heme moiety of DeoxyMb lacks a bound ligand resulting in decreased stability of the heme because of its high spin electrons and tends to oxidize faster than OxyMb (20). The effects of HNE on oxidation rates of equine OxyMb and DeoxyMb are presented in Figure 6. DeoxyMb oxidized faster than OxyMb as previously reported (20, 44) and appeared to be adversely affected by HNE to a greater extent. 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 myoglobin in which HIS 64 is replaced by the larger and more hydrophobic side chain of phenylalanine expected to prevent hydration of the heme pocket (31). As expected, this mutant appeared to be more stable than WT under the same oxygen tension ($pO_2$ = 3 mmHg) (Figure 7). The bulkier hydrophobic side chain of phenylalanine was expected to stabilize the heme moiety and prevent HNE from entering into the pocket resulting in a less adverse effect of HNE on H64F DeoxyMb stability. The greater rate of heme oxidation causes accumulation of MetMb to which the heme moiety is weakly bound (relative to ferrous myoglobins) (31, 42). The weakened

heme-globin interactions could create space for HNE to attack histidine residues buried within (42).

The mass-transformed spectra of WT and mutant sperm whale myoglobins are presented in Figures 8 (WT), 9 (P88H/Q152H), 10 (L29F), 11 (H97A), and 12 (WT and H64F in the DeoxyMb form). In each figure, the first peak represents the unmodified myoglobin, while the other peaks with a mass increase of 156 Da correspond to HNE-alkylated myoglobin species. Table 2 summarizes the number of HNE adducts observed in WT and mutant sperm whale myoglobins. The numbers of peaks representing HNE-containing myoglobin of WT (Figure 8) and P88H/Q152H (Figure 9) myoglobins were not different despite more histidine residues in the mutant. Figure 10 presents the spectra of L29F. Unexpectedly, the spectra indicate that there were more peaks in L29F than WT, indicating more HNE adducts compared to WT. H97A (Figure 11) appears to be alkylated with more HNE but the peaks corresponding to HNE-alkylated myoglobin are relatively low in abundant. The molecular basis for the unexpected peak at 17818 Da with a 240 Da increase in mass is unknown but could have obscured potential adjacent expected peaks for HNE-alkylated myoglobin peaks (i.e., 17724 Da (H97A + 3 HNE); 17884 Da (H97A + 4 HNE)) (Figure 11B). Mass-transformed spectra of deoxy-WT and H64F are presented in Figures 12A and 12B, respectively. 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.

The number of HNE adducts identified by MALDI-TOF mass spectrometry does not reveal which specific histidine residues are alkylated by HNE. The presence of mono-HNE adduct could be at various histidine residues. It is interesting to see which histidine is the most accessible for HNE alkylation. For this purpose, the 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 (45). The sites of HNE alkylation in the WT and mutant sperm whale myoglobins determined by tandem mass spectrometry are presented in Tables 3 to 7. Immonium ions (indicated in Figures 13 and 14) 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. Since the immonium ion is derived from an amino acid residue, it can be a useful marker for covalent modification of an amino acid residue (46, 47). 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. MALDI-TOF mass spectrometry revealed multiple adducts in samples at 24 h and 72 h of incubation; HIS 48 was the only histidine residue that was shown to be alkylated in all samples. There has been no evidence of this histidine being alkylated by HNE in either bovine or porcine myoglobins (15-17). Mono- and di-HNE adducts were observed in WT incubated with HNE for 72 h (Figure 8B).

However, only HIS 48 was identified as the alkylation site of HNE in WT (Table 3). It was expected that the sites of alkylation should be equal to or greater than the number of HNE molecules on a myoglobin molecule if HNE molecules bind to histidine residues on a 1:1 molar basis. Extensively HNE-alkylated myoglobin tends to aggregate, which is thermodynamically favored by hydrophobic interactions, and form intra- and intermolecular cross-linking, resulting in precipitation out of the aqueous phase (43). Some of the aggregates might not be able to flow through the matrix of the desalting column during the removal of unreacted HNE. Moreover, the low pH condition (e.g. pH 5.6) does not favor the alkylation of HNE because the imidazole group of histidine residues is protonated and becomes less nucleophilic at low pH (2, 15, 48). In addition to peptide ion quantity, ionization efficiency of peptides is important for the detection of different peptides in mass spectrometry (17). HNE alkylation on histidine residues could prevent protonation and ionization the imidazole groups. Taken together, the relative low abundance of HNE-containing peptides from samples at 24 h of incubation was not sufficient to generate enough ions to give prominent peaks in the MS/MS results.

In addition to histidine 48, the substituted histidine residues at positions 88 and 152 appeared to be vulnerable sites for HNE binding in P88H/Q152H (Figures 13 and 11, Table 4). The HIS 88 and 152 were alkylated in bovine myoglobin (16, 17) whereas Alderton et al. (15) reported HNE adducts on HIS 152 but not HIS 88 of bovine myoglobin. The increased number of histidine

residues in the double mutant and their alkylation by HNE likely explains the faster OxyMb oxidation rate of this mutant when compared to WT.

At 72 h of incubation, besides HIS 48, HIS 12 of L29F was also alkylated by HNE (Table 5). HIS 12 is uniquely present in the primary sequence of sperm whale myoglobin, while asparagine is conserved to this 12$^{th}$ amino acid in the primary sequences of other mammalian myoglobins including bovine and porcine myoglobins (49). Despite HNE alkylation of HIS 12 in L29F, this histidine is located on the opposite side of the heme moiety and the HNE adduct on 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 an evidence for HNE modification of HIS 24 and 82 of H97A (Table 6). Since the peptide 80-97 contains 3 histidine residues (HIS 81, HIS 82 and HIS 93), alkylation of one histidine residue might prohibit the other histidine residue located nearby from being attacked by HNE due to the steric hindrance. In Table 6, 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 b3 ion. HNE alkylation of the proximal HIS 93 or distal HIS 64 of H97A could not be confirmed in our study. This observation differs from Alderton et al. (15) that reported the formation of HNE adduct on both histidine residues of bovine myoglobin at pH 7.4 and 37$^{\circ}$C. Besides variations in the primary sequences of bovine and sperm whale myoglobin, the differences in pH (7.4 vs 5.6) and incubation temperature (4$^{\circ}$C vs 37$^{\circ}$C) can also contribute to the different HNE alkylation profiles. The protonated imidazole group of histidine

at pH less than 6.0 is less likely attacked by HNE, and the reaction is favored at elevated temperatures (15, 50). The attachment of HNE at HIS 82, which is located within the vicinity of the heme pocket, could further disrupt heme stability of H97A. As mentioned above regarding the unexpected 240 Da increase in mass of H97A (Figure 11B), we suspected the Schiff's base alkylation of HNE on lysine residues. The sample was treated with sodium borohydride-mediated reduction to stabilize possible Schiff's base HNE adducts on lysine residues (10, 17). However, we could not identify alkylation of lysine residues. The immonium ions of HNE-alkylated alkylated lysine with the expected mass of 239 Da (101 Da of lysine immonium ion+156 Da of HNE–18 Da of water) or 222 Da (84 Da of lysine immonium ion with loss of ammonium+156 Da of HNE–18 Da of water) were not observed.

After 72 h of incubation with HNE, DeoxyMb H64F contained mono-HNE adducts (Figure 12B), while up to 2 molecules of HNE were present on WT (Figure 12A). This could be attributed to H64F containing fewer histidine residues than WT as a result of to the mutation of HIS 64 to PHE 64. Moreover, the large hydrophobic phenyl side chain of PHE 64 could affect the heme redox and linkage stability of DeoxyMb H64F by blocking access of solvent as well as HNE into the heme pocket (31). Hence, the observed greater redox stability and fewer HNE adducts in H64F compared to WT can be explained by the combined effects of the fewer histidine number and the presence of phenylalanine 64. The MS/MS data of both WT (Table 7) and H64F (Table 8) in DeoxyMb form confirmed only a single HNE adduct site at HIS 48.

Dietary vitamin E supplementation inhibits lipid and myoglobin oxidations in beef (6, 9, 51-53). However, elevated α-tocopherol levels in pork did not enhance meat color stability to the same relative degree despite the fact that lipid oxidation was delayed (54-56). Similar results also observed in vitamin-E-supplemented chicken thighs that were on display (8$^{o}$C for 5 days) or in refrigerator (4$^{o}$C for 9 days) (57). Recently, Yin and co-workers (58) reported that the OxyMb redox instability induced by HNE was greater in myoglobins with 12±1 histidine residues (horse, cattle, sheep and deer) than in myoglobins with 9 histidine residues (chicken, turkey and pig). Their observation explains that greater histidine numbers in the primary sequence lead to greater vulnerability towards HNE.

Our observations provide a potential explanation for the observation that porcine myoglobin is less affected by HNE alkylation than bovine myoglobin (16, 17). The absence of histidine residues at specific locations on the primary sequence of porcine and chicken myoglobins prevents HNE from covalently binding whereas it could bind in bovine myoglobin. The presence of HIS 88 and 152 predisposed the P88H/Q152H mutant myoglobin to be a target of HNE alkylation and this was confirmed by our results (Figures 3 to 5). The observation with the double mutant supports the suggestion by Suman and colleagues (17) that bovine myoglobin oxidation is more susceptible to the effects of HNE than porcine myoglobin because it contained 4 more histidine residues that were alkylated by HNE than porcine myoglobin.

## 5. Conclusion

The primary structure of myoglobin affects its autoxidation rate and susceptibility to HNE. The increase of histidine number at certain locations (e.g., HIS 88 and 152) can be detrimental to the redox stability of myoglobin in the presence of HNE. The large hydrophobic side chain of PHE 29 that substitutes for LEU 29 can delay autoxidation as well as HNE-induced oxidation of OxyMb. The presence of ALA 97 instead of HIS 97 might allow hydration of the heme pocket and leave a channel for HNE to covalently alkylate the histidine residues buried within. The large hydrophobic side chain of PHE 64 stabilizes the redox stability of DeoxyMb by preventing hydration of the heme pocket and possibly entrance of HNE. According to results from tandem mass spectrometry, HIS 48 of WT and mutant sperm whale myoglobins appeared to be a preferred site of HNE alkylation as HNE-modified HIS 48 was present in all mutant myoglobins at 24 and 72 h. The MS/MS data showed the evidence that both mutated histidine residues in P88H/Q152H were alkylated by HNE. This study demonstrated that the effect of primary sequence on myoglobin redox stability can be exacerbated by the presence of secondary lipid oxidation products.

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## Figure Captions

**Figure 1** The effect of HNE (0.42 mM) on oxidation of mutant sperm whale OxyMbs (0.06 mM) at pH 5.6, 4°C.

**Figure 2** %MetMb difference between HNE-treated myoglobin and their respective controls at pH 5.6, 4°C.

**Figure 3** The effect of HNE (0.42 mM) on oxidation of P88H/Q152H and WT sperm whale OxyMbs (0.06 mM) at pH 5.6, 4°C.

**Figure 4** % Relative MetMb difference between HNE-treated P88H/Q152H and WT sperm whale myoglobins and their respective controls at pH 5.6, 4°C.

**Figure 5** %MetMb difference between HNE-treated P88H/Q152H and WT sperm whale myoglobins and their respective controls at pH 5.6, 4°C.

**Figure 6** The 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, 4°C.

**Figure 7** The effect of HNE (0.42 mM) on oxidation of H64F and WT sperm whale DeoxyMbs (0.06 mM) at pH 5.6, 4°C and $pO_2$ = 3.00 mmHg.

**Figure 8** Mass-transformed spectra of WT OxyMb (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6, 4°C for (A) 24 h and (B) 72 h.

**Figure 9** Mass-transformed spectra of P88H/Q152H OxyMb (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6, 4°C for (A) 24 h and (B) 72 h.

**Figure 10** Mass-transformed spectra of L29F OxyMb (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6, 4°C for (A) 24 h and (B) 72 h.

**Figure 11** Mass-transformed spectra of H97A OxyMb (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6, 4°C for (A) 24 h and (B) 72 h.

**Figure 12** Mass-transformed spectra of (A) WT and (B) H64F DeoxyMb (0.06 mM) incubated with HNE (0.42 mM) at pH 5.6, 4°C and $pO_2$ = 3.00 mmHg for 24 h.

**Figure 13** MS/MS spectrum of b- and y-ion series from the tryptic-digested peptide 189-97 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 14** 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 1**

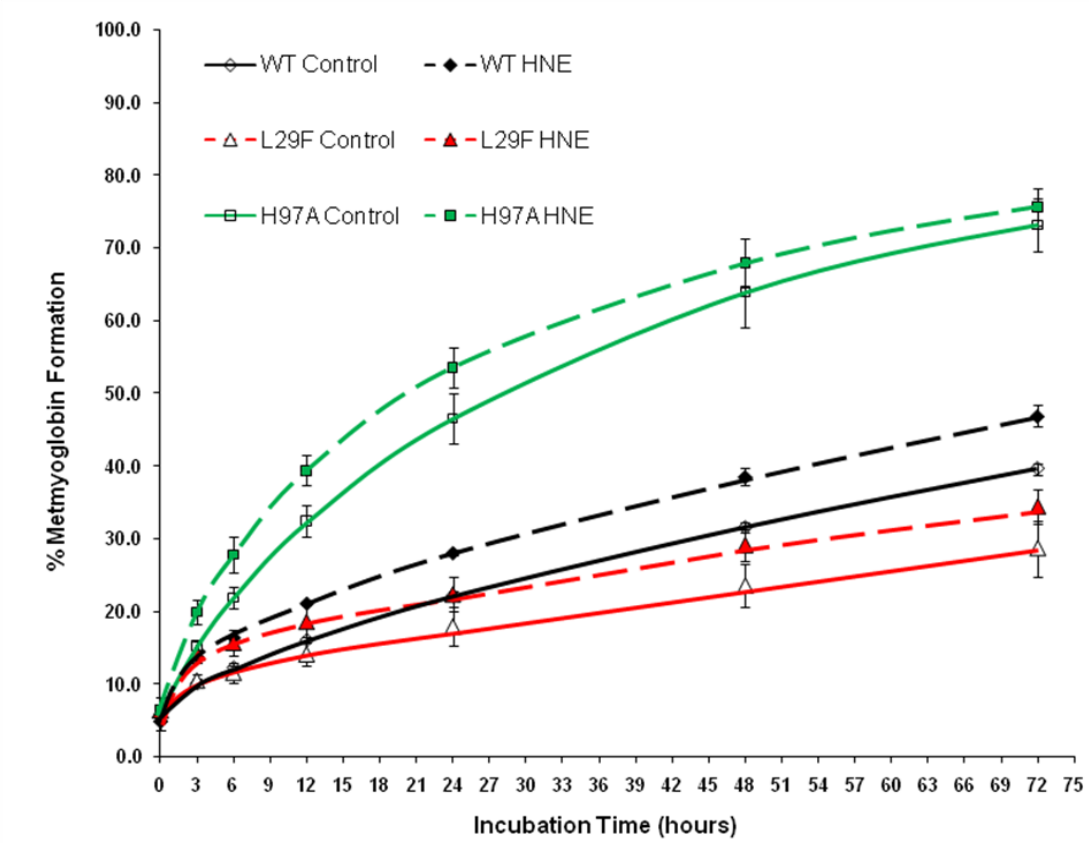
WT Control
WT HNE
L29F Control
L29F HNE
H97A Control
H97A HNE
% Metmyoglobin Formation
Incubation Time (hours)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69 72 75

**Figure 2**

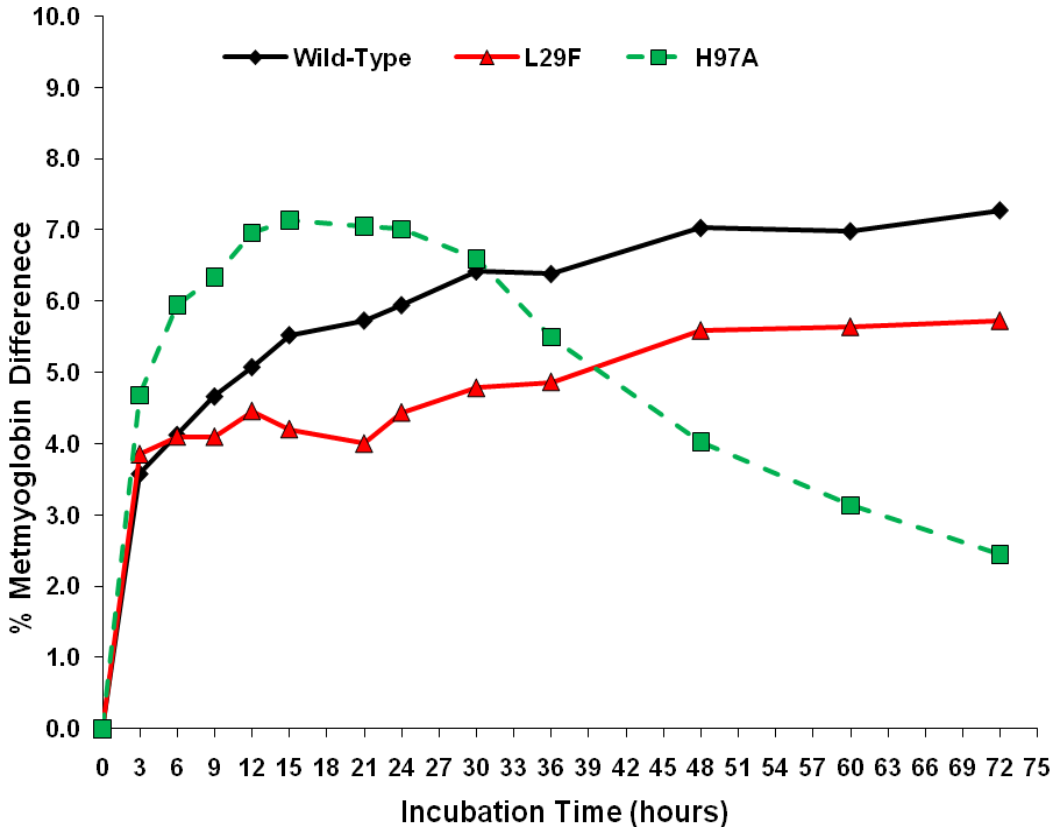
Wild-Type
L29F
H97A
% Metmyoglobin Differenece
Incubation Time (hours)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69 72 75

**Figure 3**

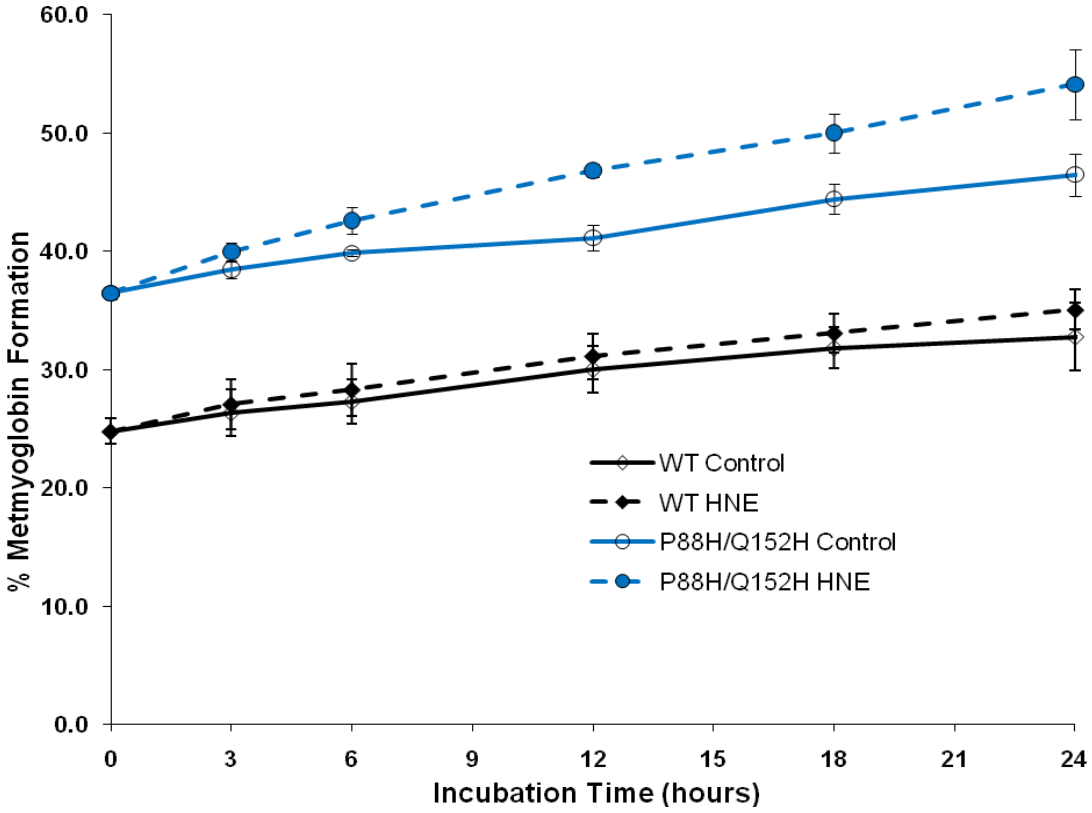
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Metmyoglobin Formation
0
3
6
9
12
15
18
21
24
Incubation Time (hours)
WT Control
WT HNE
P88H/Q152H Control
P88H/Q152H HNE

**Figure 4**

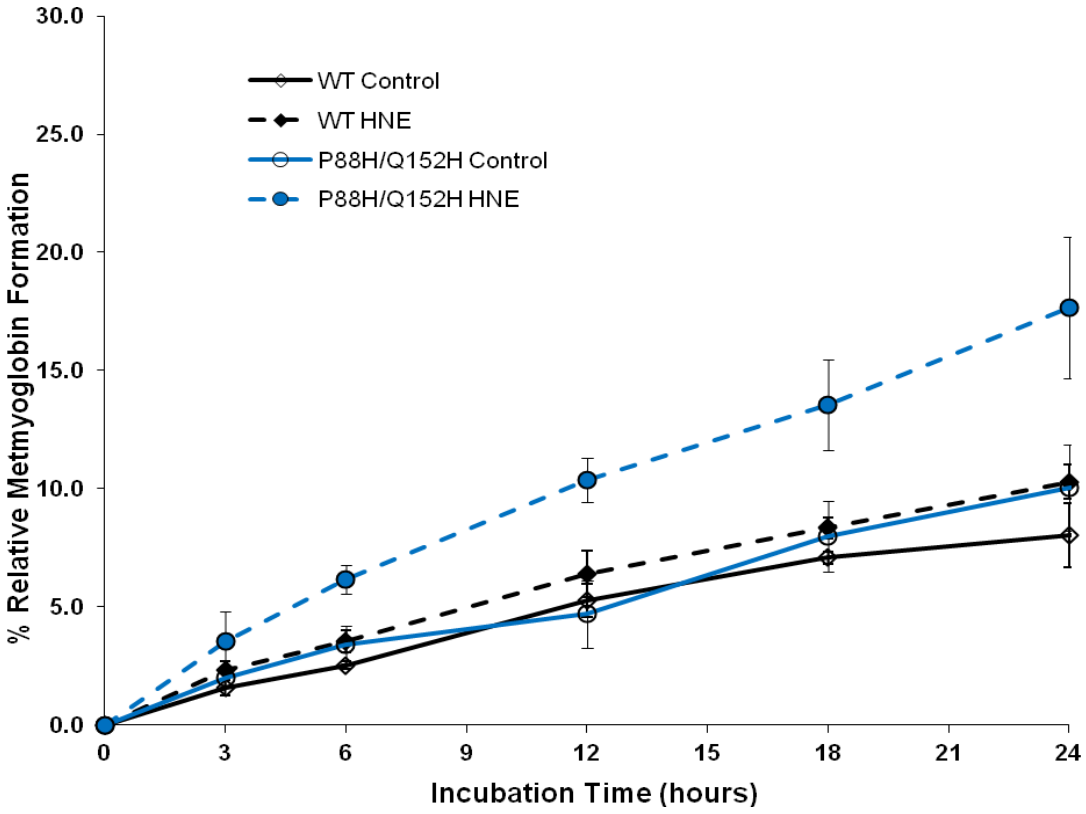
30.0
25.0
20.0
15.0
10.0
5.0
0.0
% Relative Metmyoglobin Formation
WT Control
WT HNE
P88H/Q152H Control
P88H/Q152H HNE
0
3
6
9
12
15
18
21
24
Incubation Time (hours)

**Figure 5**

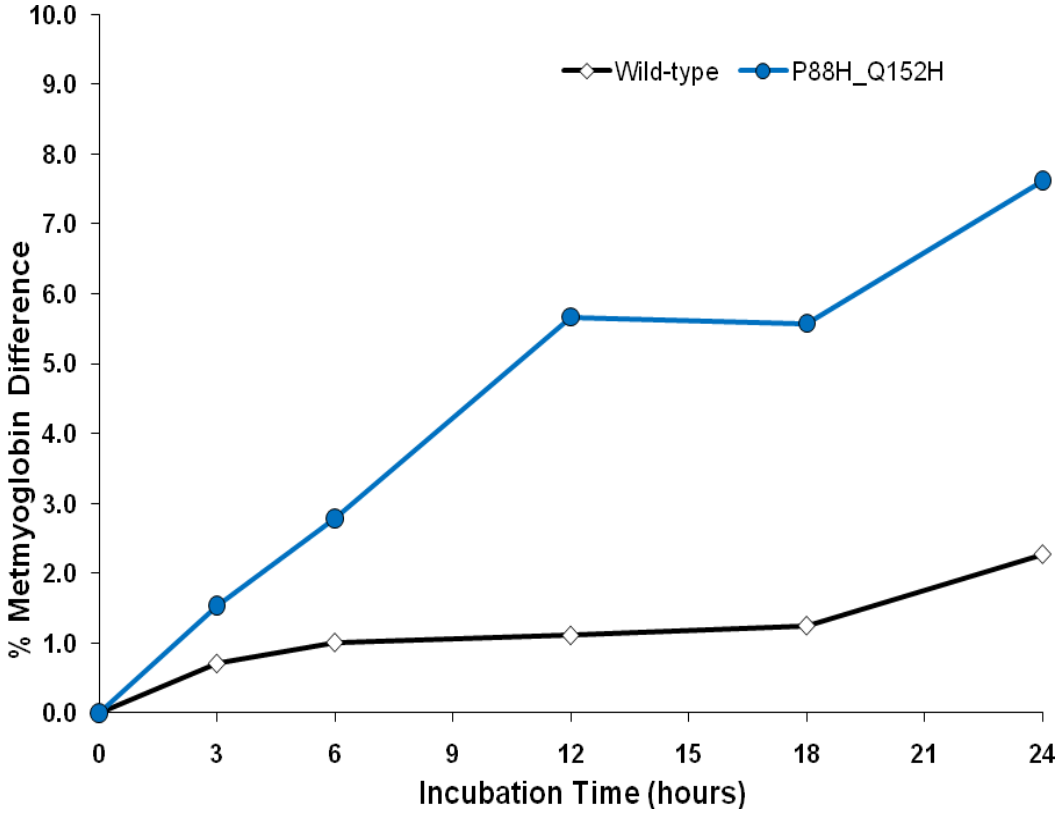
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
% Metmyoglobin Difference
Wild-type
P88H_Q152H
0
3
6
9
12
15
18
21
24
Incubation Time (hours)

**Figure 6**

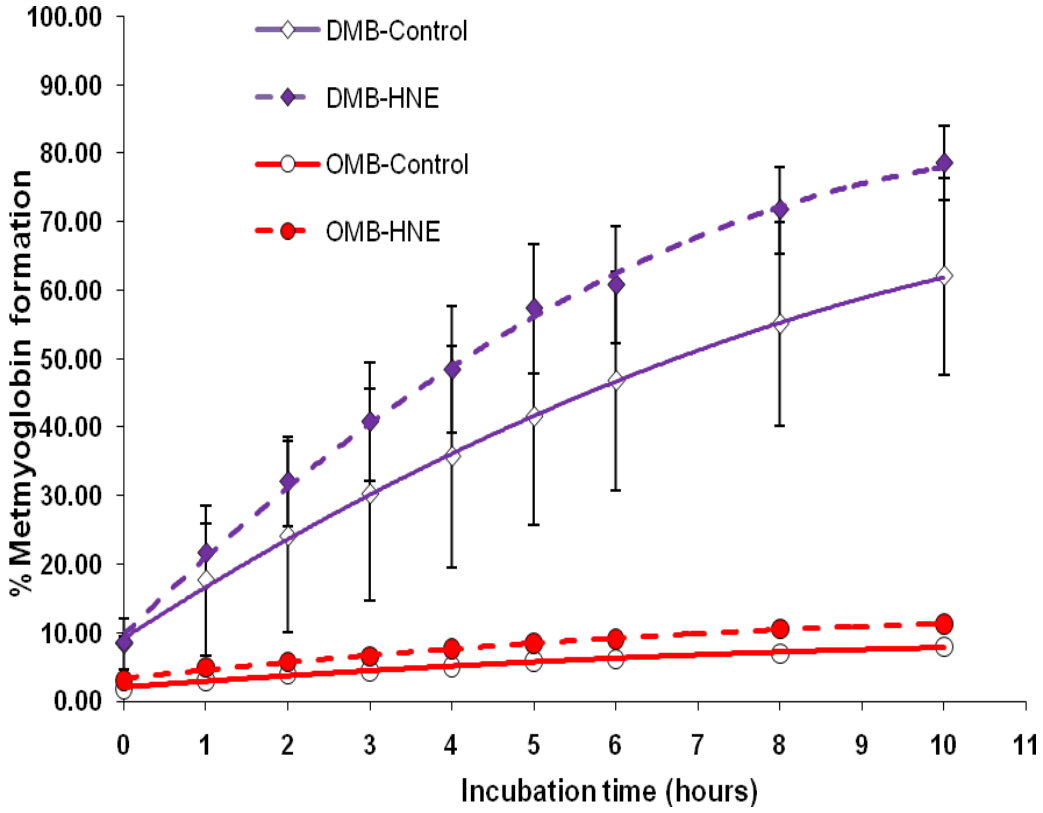
DMB-Control
DMB-HNE
OMB-Control
OMB-HNE
% Metmyoglobin formation
Incubation time (hours)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
1
2
3
4
5
6
7
8
9
10
11

**Figure 7**

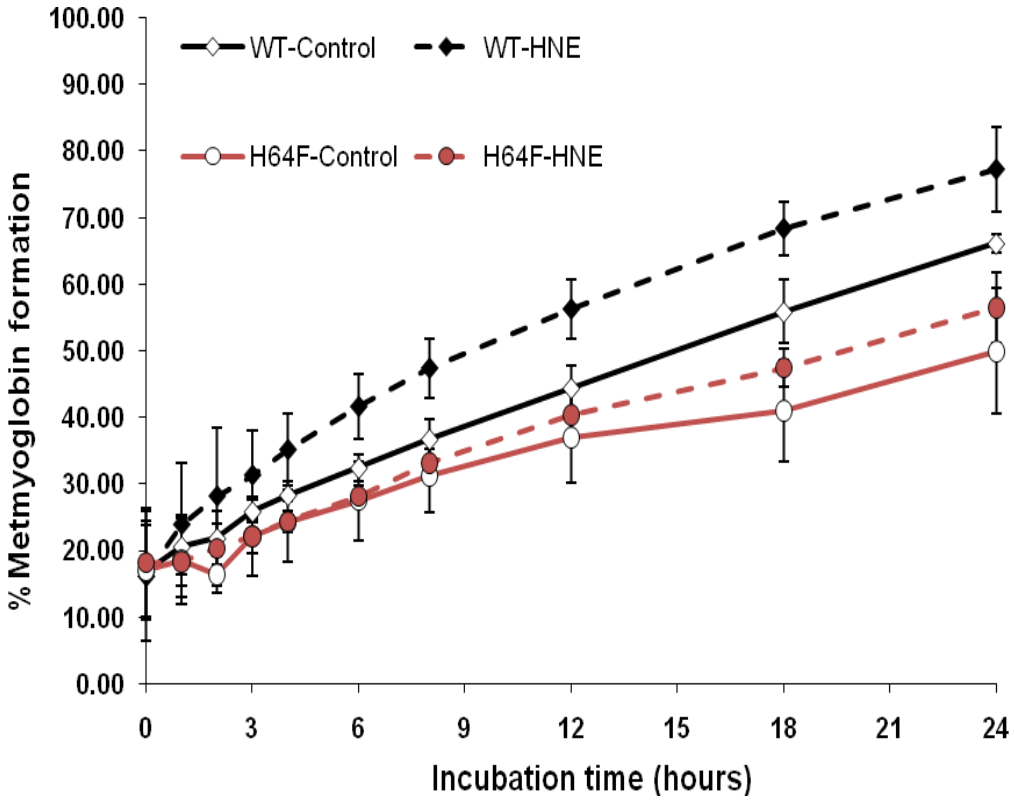
WT-Control
WT-HNE
H64F-Control
H64F-HNE
% Metmyoglobin formation
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
3
6
9
12
15
18
21
24
Incubation time (hours)

**Figure 8**

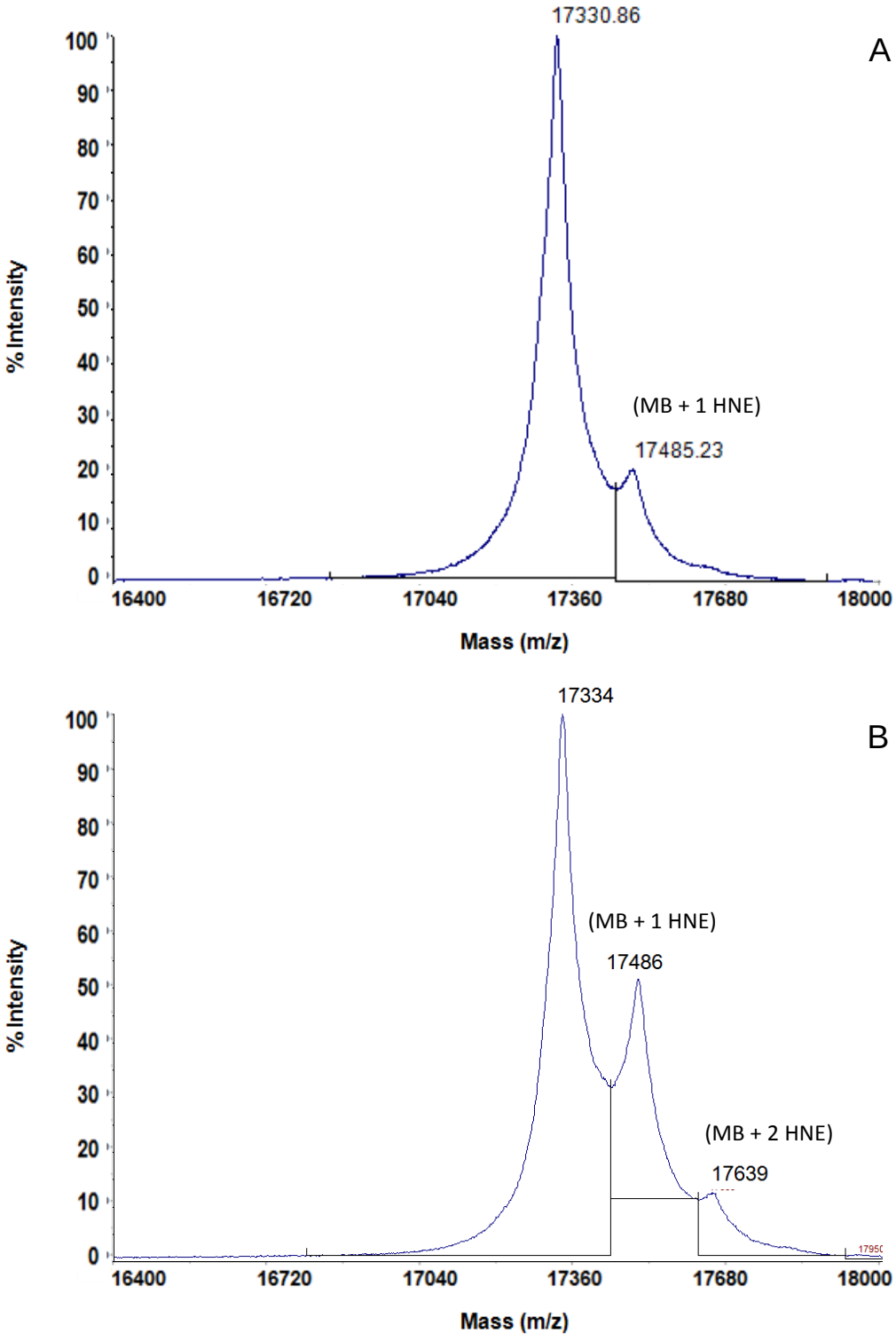
17330.86
A
100
90
80
70
60
50
40
30
20
10
0
% Intensity
(MB + 1 HNE)
17485.23
16400
16720
17040
17360
17680
18000
Mass (m/z)
17334
B
(MB + 1 HNE)
17486
(MB + 2 HNE)
17639
Mass (m/z)

**Figure 9**

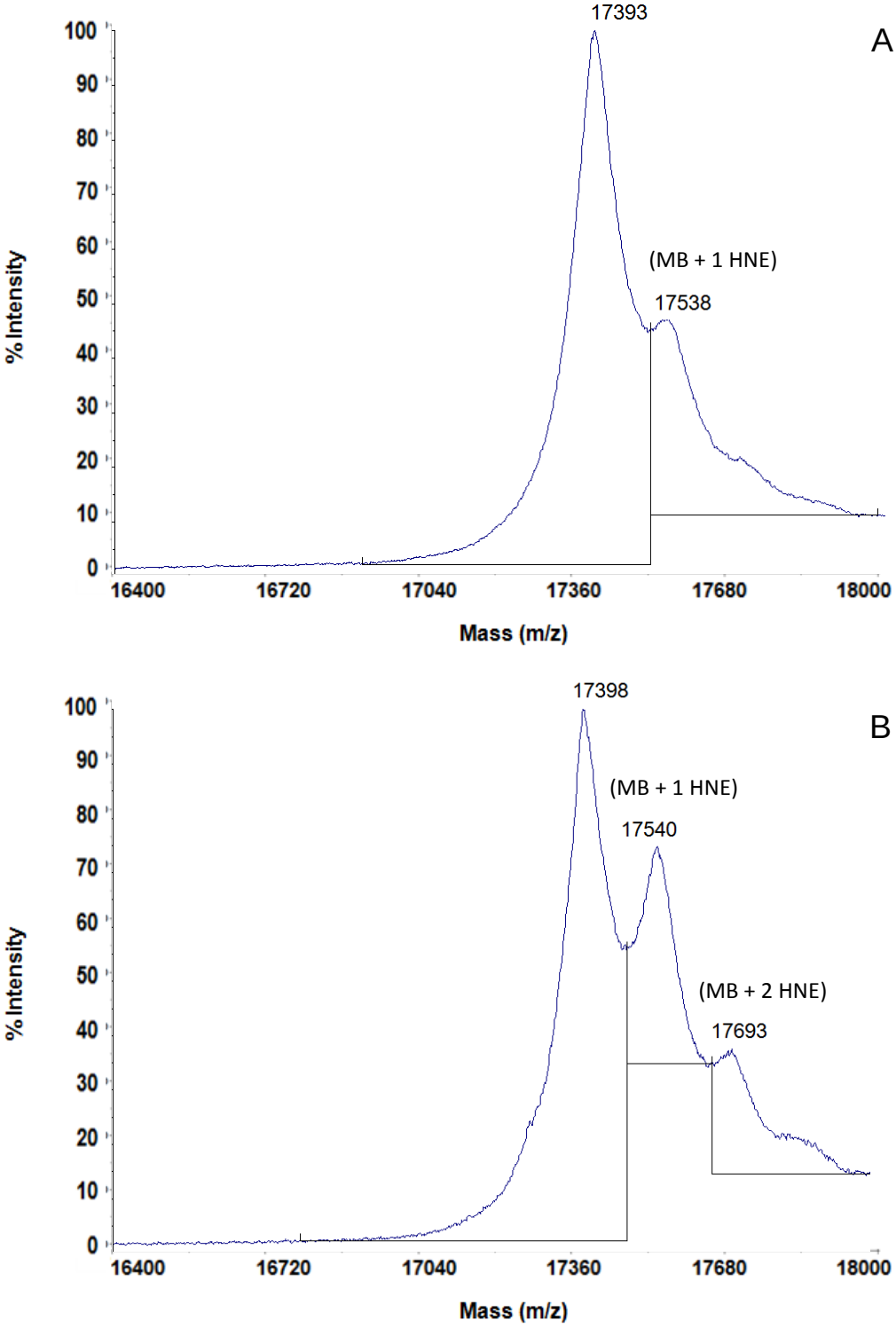
A
17393
(MB + 1 HNE)
17538
% Intensity
Mass (m/z)
B
17398
(MB + 1 HNE)
17540
(MB + 2 HNE)
17693
% Intensity
Mass (m/z)

**Figure 10**

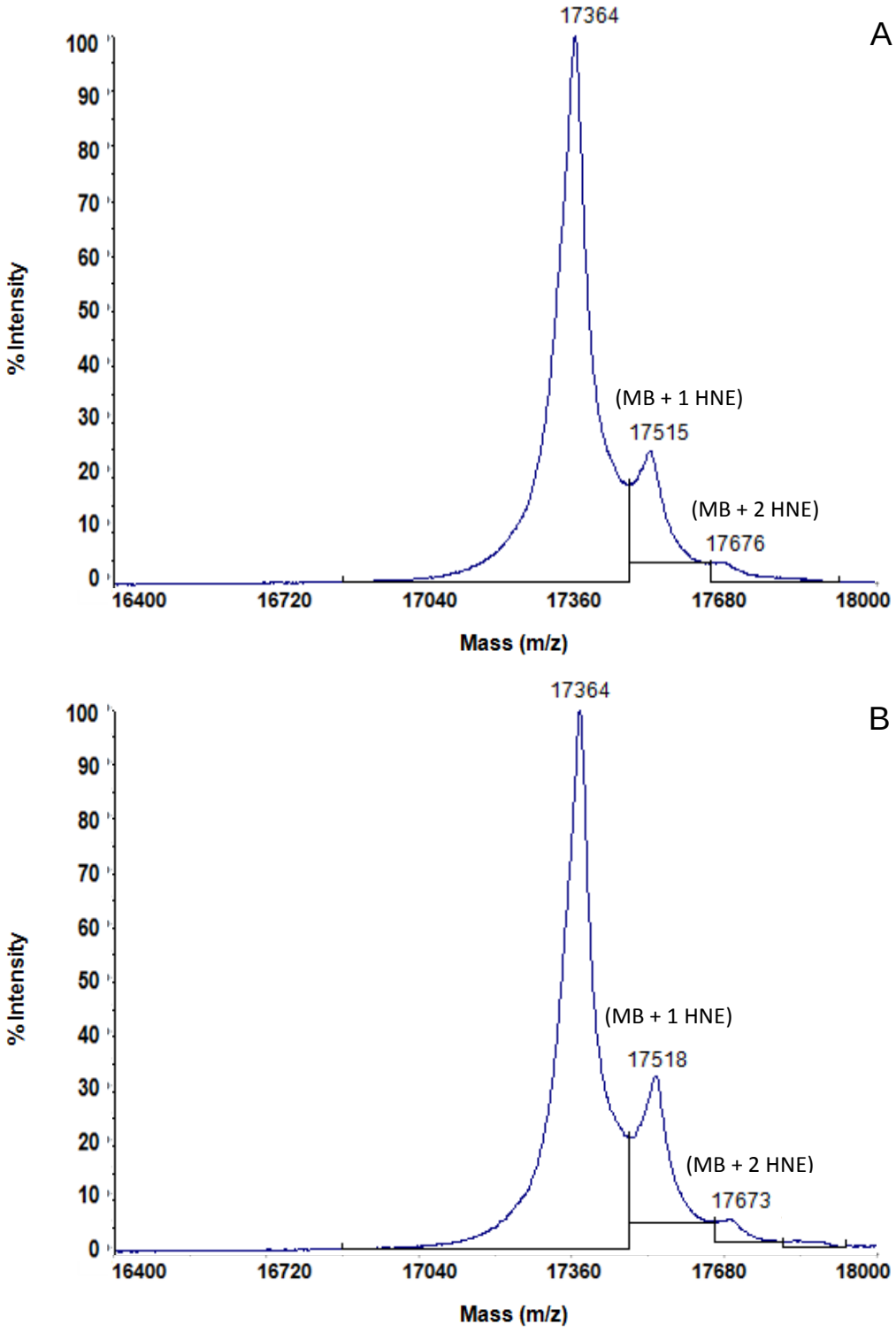
A
17364
100
90
80
70
60
50
40
30
20
10
0
% Intensity
(MB + 1 HNE)
17515
(MB + 2 HNE)
17676
16400
16720
17040
17360
17680
18000
Mass (m/z)
B
17364
100
90
80
70
60
50
40
30
20
10
0
% Intensity
(MB + 1 HNE)
17518
(MB + 2 HNE)
17673
16400
16720
17040
17360
17680
18000
Mass (m/z)

**Figure 11**

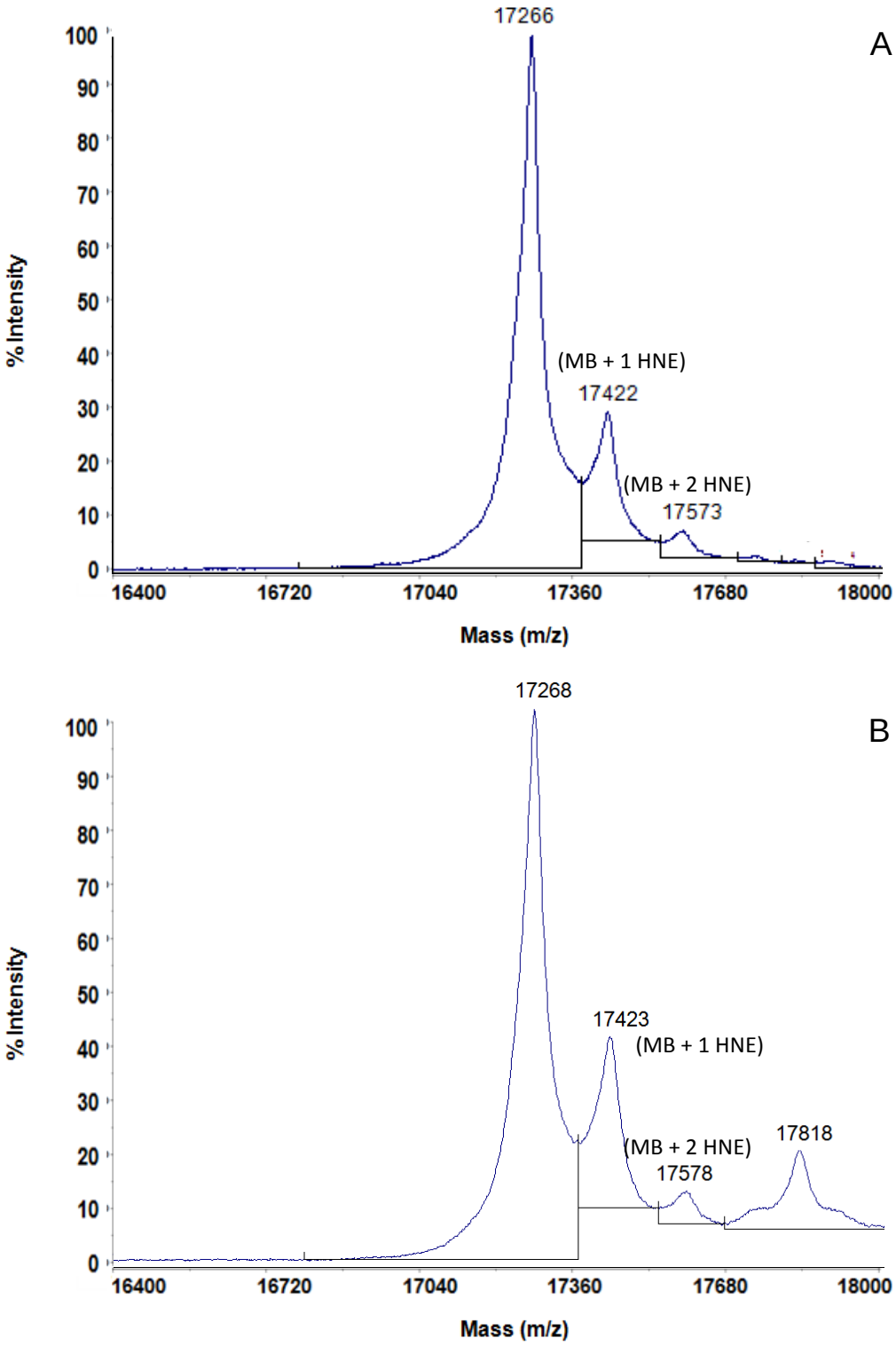
A
17266
(MB + 1 HNE)
17422
(MB + 2 HNE)
17573
% Intensity
Mass (m/z)
B
17268
17423
(MB + 1 HNE)
(MB + 2 HNE)
17578
17818
% Intensity
Mass (m/z)

**Figure 12**

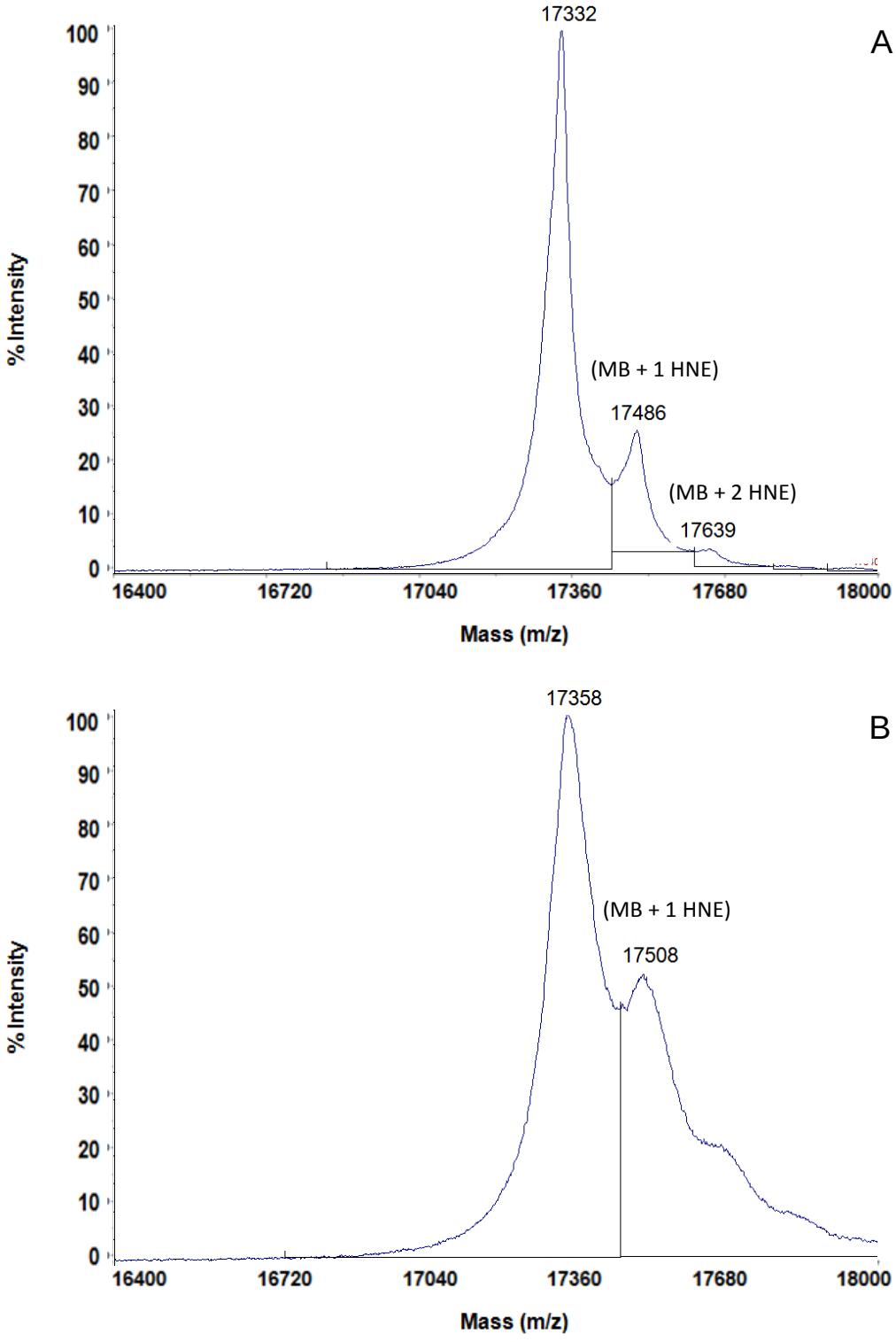
A
17332
(MB + 1 HNE)
17486
(MB + 2 HNE)
17639
% Intensity
Mass (m/z)
B
17358
(MB + 1 HNE)
17508
% Intensity
Mass (m/z)

**Figure 13**

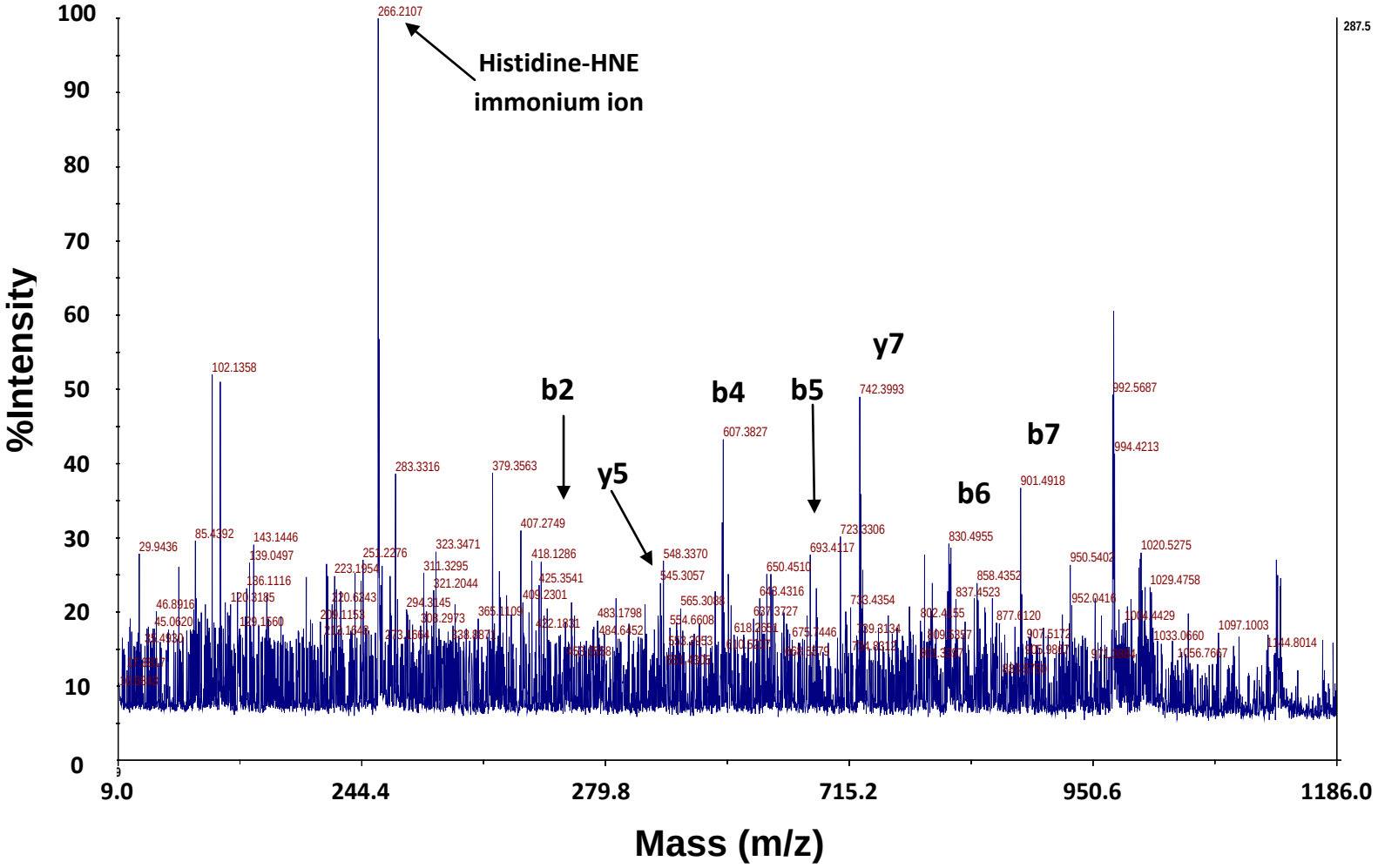
Histidine-HNE
immonium ion
b2
y5
b4
b5
y7
b6
b7
%Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
9.0
244.4
279.8
715.2
950.6
1186.0
287.5
266.2107
102.1358
283.3316
379.3563
407.2749
418.1286
548.3370
607.3827
742.3993
693.4117
723.3306
830.4955
901.4918
992.5687
994.4213
1020.5275
1029.4758
1097.1003
1144.8014

**Figure 14**

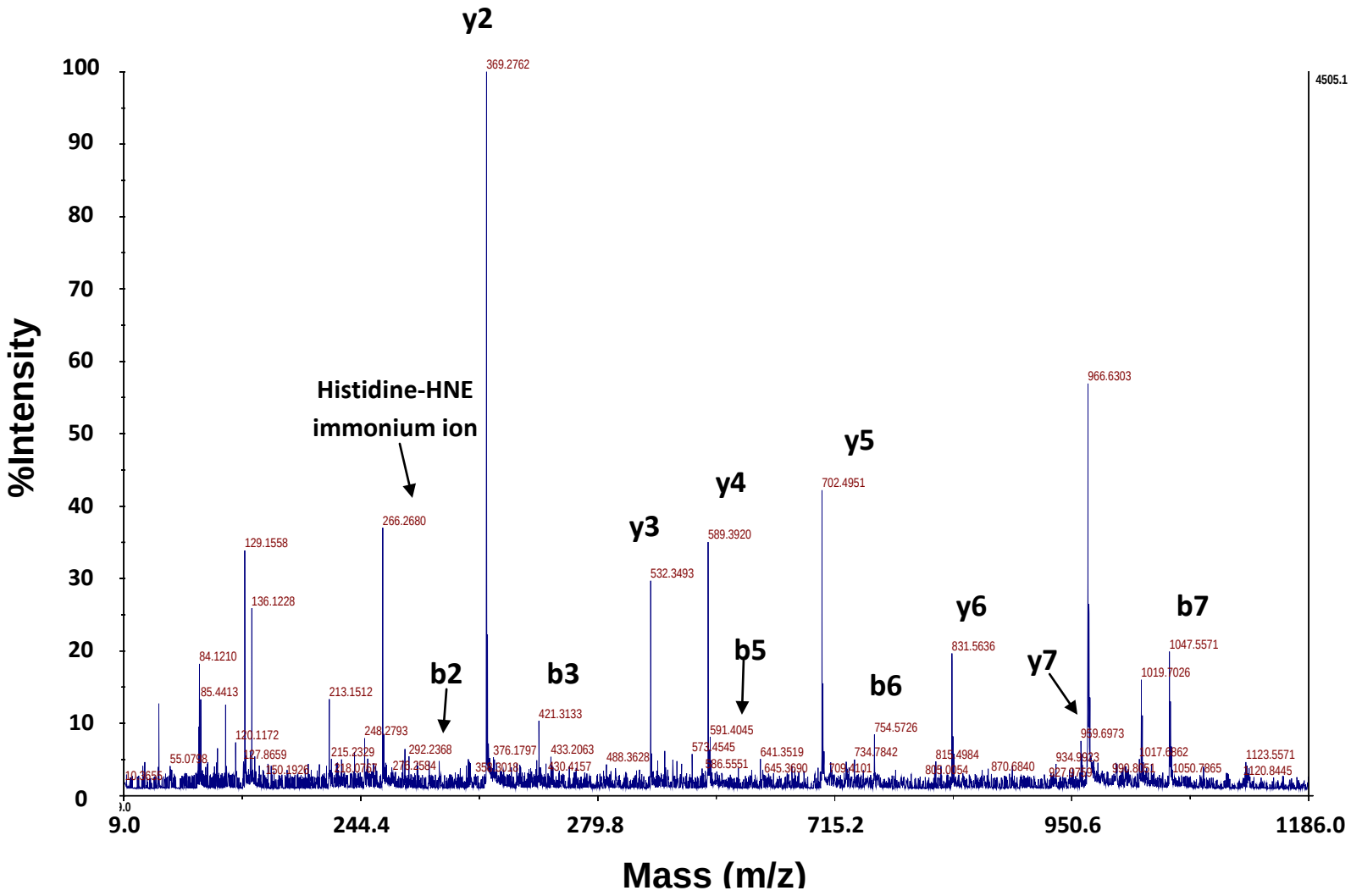
y2
369.2762
4505.1
100
90
80
70
60
50
40
30
20
10
0
%Intensity
Histidine-HNE
immonium ion
266.2680
y3
532.3493
y4
589.3920
y5
702.4951
y6
831.5636
b7
1047.5571
966.6303
b2
b3
421.3133
b5
591.4045
b6
754.5726
y7
959.6973
129.1558
136.1228
84.1210
85.4413
213.1512
120.1172
248.2793
215.2329
292.2368
55.0798
127.8659
160.1926
376.1797
433.2063
488.3628
573.4545
586.5551
641.3519
645.3690
734.7842
815.4984
870.6840
934.9923
1017.6362
1019.7026
1050.7865
1123.5571
9.0
244.4
279.8
715.2
950.6
1186.0
Mass (m/z)

**Table 1** Comparisons of the characteristics of WT and mutant sperm whale myoglobins.

| Mb | Mutation site | |
|---|---|---|
| | **Native** | **Mutant** |
| **Wild-type (WT)** | None | None |
| **P88H/Q152H** | Proline 88 (PRO 88)<br>Glutamine 152 (GLN 152) | Histidine 88 (HIS 88)<br>Histidine 152 (HIS 152) |
| **L29F** | Leucine 29 (LEU 29) | Phenylalanine 29 (PHE 29) |
| **H97A** | Histidine 97 (HIS 97) | Alanine 97 (ALA 97) |
| **H64F** | Histidine 64 (HIS 64) | Phenylalanine 64 (PHE 64) |

**Table 2** The number of HNE adducts on WT and mutant sperm whale myoglobins from MALDI-TOF mass spectrometry.

| Myoglobin | Form of Myoglobin | Incubation Period | |
|---|---|---|---|
| | | 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 | - |
| **WT** | DeoxyMb | 1 | - |

**Table 3** MS/MS spectral features of unalkylated and HNE-alkylated WT sperm whale Mb peptides.

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass indicating the HNE-adduct on histidine residue.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

**Table 4** MS/MS spectral features of unalkylated and -alkylated P88H/Q152H sperm whale Mb peptides

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |
| **89-97** | **H*** LAQSHATK | 1148.642 (+HNE) | b-ions: **407.265 (b2), 478.302 (b3), 606.361 (b4), 693.393 (b5), 830.452 (b6), 901.489 (b7), 1002.537 (b8)** y-ions: 147.113 (y1), 248.161 (y2), 319.198 (y3), 456.257 (y4) 543.289 (y5), 671.347 (y6), 742.384 (y7), 855.468 (y8) |
| **89-97** | HLAQSHATK | 992.527 | b-ions: 251.150 (b2), 322.187 (b3), 450.246 (b4), 537.278 (b5), 674.337 (b6), 745.374 (b7), 846.422 (b8) y-ions: 147.113 (y1), 248.161 (y2), 319.198 (y3), 456.257 (y4), 543.289 (y5), 671.347 (y6), 742.384 (y7), 855.468 (y8) |

**Table 4** MS/MS spectral features of unalkylated and -alkylated P88H/Q152 sperm whale Mb peptides (continue).

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **147-154** | YKELGY**H*** G | 1122.583 (+HNE) | b-ions: 292.166 (b2), 421.209 (b3), 534.293 (b4), 591.314 (b5), 754.378 (b6), **1047.552 (b7)**<br>y-ions: 76.040 (y1), **369.214 (y2), 532.277 (y3), 589.299 (y4), 702.383 (y5), 831.425 (y6), 959.520 (y7)** |
| **147-154** | YKELGYHG | 966.486 | b-ions: 292.166 (b2), 421.209 (b3), 534.293 (b4), 591.314 (b5), 754.378 (b6), 891.436(b7)<br>y-ions: 76.040 (y1), 231.099 (y2), 376.162 (y3), 433.184 (y4), 546.268 (y5), 675.310 (y6), 803.405 (y7) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass indicating the HNE-adduct on histidine residue.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

**Table 5** MS/MS spectral features of unalkylated and -alkylated L29F sperm whale Mb peptides.

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **1-17** | MVLSEGEWQLVL**H*** VWAK | 2181. 178 (+HNE) | b-ions: 231.117 (b2), 344.201 (b3), 431.233 (b4), 560.275 (b5), 617.297 (b6), 746.339 (b7), 932.419 (b8), 1060.477 (b9), 1173.561 (b10), 1272.630 (b11), 1385.714 (b12), **1697.929 (b13), 1777.956 (b14), 1964.036 (b15), 2035.073 (b16)** y-ions: 147.113 (y1), 218.150 (y2), 404.230 (y3), 503.298 (y4), **796.472 (y5), 909.556 (y6), 1008.625 (y7), 1121.709 (y8), 1249.767 (y9), 1435.847 (y10), 1564.889 (y11), 1621.911 (y12), 1750.953 (y13), 1837.985 (y14), 1951.069 (y15), 2050.138 (y16)** |
| **1-17** | MVLSEGEWQLVLHVWAK | 2025.063 | b-ions: 231.117 (b2), 344.201 (b3), 431.233 (b4), 560.275 (b5), 617.297 (b6), 746.339 (b7), 932.419 (b8), 1060.477 (b9), 1173.561 (b10), 1272.630 (b11), 1385.714 (b12), 1522.773 (b13), 1640.882 (b14), 1807.921 (b15), 1878.958 (b16) y-ions: 147.113 (y1), 218.150 (y2), 404.230 (y3), 503.298 (y4), 640.357 (y5), 753.441 (y6), 852.510 (y7), 965.594 (y8), 1093.652 (y9), 1279.732 (y10),), 1408.774 (y11), 1465.796 (y12), 1594.838 (y13), 1681.870 (y14), 1794.954 (y15), 1894.023 (y16) |

**Table 5** MS/MS spectral features of unalkylated and -alkylated L29F sperm whale Mb peptides (Continue).

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass indicating the HNE-adduct on histidine residue.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

**Table 6** MS/MS spectral features of unalkylated and -alkylated H97A sperm whale Mb peptides.

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **18-32** | VEADVAG**H*** GQDILIR | 1748.954 (+HNE) | b-ions: 229.118 (b2), 300.155 (b3), 415.182 (b4), 514.251 (b5), **585.288 (b6), 642.309 (b7), 935.483 (b8), 992.505 (b9), 1120.563 (b10), 1235.590 (b11), 1348.674 (b12), 1461.758 (b13), 1574.842 (b14)** y-ions: 175.119 (y1), 288.203 (y2), 401.287 (y3), 514.371(y4), **629.398 (y5), 757.457 (y6), 814.478 (y7), 1107.652 (y8), 1164.674 (y9), 1235.7101 (y10), 1334.779 (y11), 1449.806 (y12) 1520.843 (y13), 1649.886 (y14)** |
| **18-32** | VEADVAGHGQDILIR | 1592.839 | b-ions: 229.118 (b2), 300.155 (b3), 415.182 (b4), 514.251 (b5), 585.288 (b6), 642.309 (b7), 779.368 (b8), 836.390 (b9), 964.448 (b10), 1079.475 (b11), 1192.559 (b12), 1305.643 (b13), 1418.727 (b14) y-ions: 175.119 (y1), 288.203 (y2), 401.287 (y3), 514.371 (y4), 629.398 (y5), 757.457 (y6), 814.478 (y7), 951.537 (y8), 1008.559 (y9), 1079.596 (y10), 1178.664 (y11), 1293.691 (y12), 1364.728 (y13), 1493.771 (y14) |
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |

**Table 6** MS/MS spectral features of unalkylated and -alkylated H97A sperm whale Mb peptides (Continue).

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **80-97** | KGH**H*** EAELKPL AQSHATK | 2138.172 (+HNE) | b-ions: 186.124 (b2), 323.183 (b3), **616.357 (b4), 745.399 (b5), 816.436 (b6), 945.479 (b7), 1058.563 (b8),1186.658 (b9), 1283.711 (b10), 1396.795 (b11), 1467.832 (b12), 1595.890 (b13),1682.922 (b14), 1819.981 (b15), 1891.018 (b16), 1992.066 (b17)**<br>y-ions: 147.113 (y1), 248.161 (y2), 319.198 (y3), 456.257 (y4), 543.289 (y5), 671.347 (y6), 742.384 (y7), 855.468 (y8), 952.521 (y9), 1080.616 (y10), 1193.700 (y11), 1322.743 (y12), 1393.780 (y13), 1522.822 (y14), **1815.996 (y15), 1953.055 (y16), 2010.077 (y17)** |
| **80-97** | KGHHEAELKPL AQSHATK | 1982.057 | b-ions: 186.124 (b2), 323.183 (b3), 460.242 (b4), 589.284 (b5), 660.321 (b6), 789.364 (b7), 902.448 (b8), 1030.543 (b9), 1127.596 (b10), 1240.680 (b11), 1311.717 (b12), 1439.775 (b13), 1526.807 (b14), 1663.866 (b15),1734.903 (b16), 1835.951 (b17)<br>y-ions: 147.113 (y1), 248.161 (y2), 319.198 (y3), 456.257 (y4), 543.289 (y5), 671.347 (y6), 742.384 (y7), 855.468 (y8), 952.521 (y9), 1080.616 (y10), 1193.700 (y11), 1322.743 (y12), 1393.780 (y13), 1522.822 (y14),1659.881 (y15), 1796.940 (y16) 1853.962 (y17) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass indicating the HNE-adduct on histidine residue.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

**Table 7** MS/MS spectral features of unalkylated and HNE-alkylated deoxy-WT sperm whale Mb peptides.

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass indicating the HNE-adduct on histidine residue.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

**Table 8** MS/MS spectral features of unalkylated and HNE-alkylated deoxy-H64F sperm whale Mb peptides.

| Peptide position[1)] | Peptide sequence | Precursor (m/z) | b-and y-ions[2) and 3)] |
|---|---|---|---|
| **44-51** | FDRFK**H*** LK | 1246.731 (+HNE) | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), **987.542 (b6), 1100.626 (b7)** y-ions: 147.113 (y1), 260.197 (y2), **553.371 (y3), 681.466 (y4), 828.535 (y5), 984.636 (y6), 1099.663 (y7)** |
| **44-51** | FDRFKHLK | 1090.616 | b-ions: 263.103 (b2), 419.204 (b3), 566.273 (b4), 694.368 (b5), 831.427 (b6), 944.511 (b7) y-ions: 147.113 (y1), 260.197 (y2), 397.256 (y3), 525.351 (y4), 672.420 (y5), 828.521 (y6), 943.548 (y7) |

1) The number of peptide position reflects the location of amino acid residues in WT and mutant sperm whale myoglobins that contain an additional methionine residue at the N-terminus. Hence, the numbers are shifted by 1 position.

2) The bolded b- and y-ions have a 156-Da increase of mass.

3) The underlined b- and y-ions were observed in the MS/MS spectrum.

# Chapter IV

# The effects of 4-hydroxy-2-nonenal on oxymyoglobin oxidation and its pro-oxidative activity in lipid model systems.

**The effects of 4-hydroxy-2-nonenal on oxymyoglobin oxidation and its pro-oxidative activity in lipid model systems.**

## 1. Abstract

In this study, the increased redox instability of HNE-alkylated Oxymyoglobin (OxyMb) and its ability to facilitate oxidation in liposome and microsome models were investigated. Also, the effect of lipid oxidation products on OxyMb oxidation was studied using a dialysis sac model. Wild-type (WT) and mutant sperm whale OxyMbs (P88H/Q152H, L29F, and H97A) alkylated with HNE allowed recombinant OxyMbs to oxidize faster ($p<0.05$) and promote better lipid oxidation ($p<0.05$) than controls. The magnitude by which HNE increased OxyMb oxidation and lipid oxidation were correlated with the extent of alkylation (tri-adducts in H97A and P88H/Q15H, di-adducts in WT, mono adduct in L29F). P88H/Q152H and H97A were more susceptible to HNE alkylation and more potent promoters of lipid oxidation than WT ($p<0.05$). OxyMb of L29F, on the other hand, remained stable in the oxygenated reduced form, and was least effective at stimulating lipid oxidation. Small lipid oxidation products were able to diffuse across dialysis membranes (molecular weight cut-off (MWCO) = 500 Da) and induce redox instability of OxyMb. OxyMbs of P88H/Q152H, WT and L29F oxidized faster in the presence of oxidized liposomes than in the buffers (controls) ($p<0.05$). L29F was more resistant to the adverse effect of the lipid oxidation products than P88H/Q152H and WT. In conclusion, our results suggested that HNE alkylation induces myoglobin redox instability and enhances

the capacity for OxyMb to facilitate lipid oxidation. However, the effect of HNE alkylation varies upon the intrinsic properties of myoglobin (e.g. redox stability, heme affinity, susceptibility to HNE alkylation etc) which can be altered by changing selected amino acid residues of myoglobin.

Key words: Mutant sperm whale myoglobin, 4-hydroxy-nonenal, lipid oxidation, liposomes, microsomes, lipid dialysis sacs

## 2. Introduction

In fresh meat, the oxidation of lipids and ferrous myoglobin has a positive correlation (Greene, 1969; Faustman et al., 1989b; Faustman et al., 2010). Despite improvement of lipid and color stability in beef (Faustman et al., 1989ab; Mitsumoto et al., 1993), dietary vitamin E delayed the thiobarbituric reactive substances (TBARS) formation in pork during storage without an impact on color stability (Cannon et al., 1996; Phillips et al., 2001; Guo et al., 2006). Lee and colleagues (2003b) also observed similar results using microsomes obtained from vitamin E-supplemented pigs as *in vitro* models and suggested that the interactions between lipid and myoglobin oxidation is species-dependent.

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 meat. Many studies have demonstrated the electrophilicity of HNE towards nucleophilic amino acid side chains of cysteine, lysine, and histidine (Uchida and Stadtman, 1992; Bolgar and Gaskell, 1996). HNE has been shown to induce redox instability of myoglobin

(Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2007) and this has been attributed to alkylation-induced partial unfolding of the heme protein (Alderton et al., 2003). A more “open” structure of myoglobin, especially at the hydrophobic heme pocket, predisposes ferrous heme to solvent exposure and to ready oxidation (Livingston and Brown, 1981). To date, the sites of HNE alkylation have been reported to be exclusively on histidine residues of equine (Faustman et al., 1999), bovine (Alderton et al., 2003), porcine (Suman et al., 2007), yellowfin tuna (Lee et al., 2003a), and chicken and turkey (Naveena et al., 2010) myoglobins. Suman et al. (2006) hypothesized that a greater number of histidine residues and their locations in the primary sequence of bovine myoglobin (13 histidine residues) could increase its vulnerability to alkylation by HNE relative to porcine myoglobin which contains fewer histidine residues (i.e., 9 histidine residues). Recently, Yin and co-workers (2011) compared the susceptibility of HNE alkylation among myoglobins from 7 meat-producing animal species. They observed that the OxyMb redox instability induced by HNE was greater in myoglobins with 12±1 histidine residues (horse, cattle, sheep and deer) than in myoglobins with 9 histidine residues (chicken, turkey and pig). However, the redox stability of myoglobin from these animal species *per se* also differed in that study. For example, chicken and turkey myoglobins tended to oxidize faster than bovine myoglobin in the absence of HNE. These studies concluded that the enhanced OxyMb oxidation was due to covalent attachment of HNE to histidine residues in myoglobin. However, the possibility that HNE could mediate redox instability of OxyMb through other mechanisms cannot be

excluded. For example, the unbound HNE could possibly induce conformational change in the folding of myoglobin by creating a hydrophobic environment surrounding myoglobin.

Lynch and Faustman (2000) demonstrated that horse heart metmyoglobin (MetMb) that had been exposed to HNE induced more TBARS formation in liposome and microsome models than controls. The pro-oxidative effect increased with the incubation time of MetMb with HNE. Their results also suggested that MetMb pre-incubated with HNE was a poorer substrate for enzymatic MetMb reduction. However, the effects of HNE alkylation on redox instability and pro-oxidative activity of OxyMb was not reported.

Wild-type (WT) and mutant sperm whale myoglobins whose primary sequences can be manipulated for desirable characteristics (e.g. heme affinity, oxidation rate) provide logical models for studying the oxidation of OxyMb and its association with lipid oxidation. Richards et al. (2009) demonstrated that an enhanced redox stability of L29F due to the substitution of leucine 29 with phenylalanine decreased its pro-oxidative activity compared to WT. The fact that L29F stays in the ferrous form longer than WT provides a good opportunity to investigate the effect of OxyMb autoxidation on its susceptibility to lipid oxidation products as well as its pro-oxidant effect on lipid oxidation. Also, heme affinity of myoglobin can be altered by changing certain amino acid residues that interact with the heme moiety. H97A (histidine 97 replaced by alanine) showed a significant reduction in heme affinity and promoted lipid oxidation in washed fish muscle (Grunwald and Richards, 2006ab; Richards et al., 2009). Suman and co-

workers (2006 and 2007) reported that histidines 88 and 152 of bovine myoglobin were accessible for HNE alkylation. In contrast, the amino acid residues at the same positions of porcine myoglobin are proline and glutamine, respectively. A novel mutant sperm whale myoglobin whose $88^{th}$ (proline) and $152^{nd}$ (glutamine) amino acid residues were replaced by 2 histidines demonstrated that both histidine residues were accessible for HNE alkylation (Figures 13 and 14, Table 4 in Chapter 3). Therefore, P88H/Q152H should be useful for investigating the susceptibility of myoglobin with an altered number of histidine residues towards HNE.

Products of lipid oxidation such as aldehydes and ketones are relatively smaller and more water-soluble than their parent lipids. Their increased water solubility facilitates the interaction with myoglobin in the aqueous phase of the sarcoplasm. Dialysis membranes can act as physical barriers preventing direct contact between myoglobin and lipids (Chan et al., 1997). This permits study of the hypothesis that small, water-soluble lipid oxidation-derived products can cross the dialysis membrane and interact with OxyMb causing accelerated MetMb formation.

The objectives of this study were to 1) investigate the ability of HNE alkylation to decrease the redox stability and the pro-oxidative potential of OxyMb on liposome and microsome models and 2) of lipid oxidation products to diffuse across dialysis membranes and mediate WT and mutant OxyMb oxidation.

## 3. Materials and methods

### 3.1 Reagents

Disodium ethylenediaminetetraacetic acid (EDTA) dihydrate, DNAse I, RNAse A, lysozyme, ferric chloride ($FeCl_3$), hemin chloride, chloramphenicol, 2,4-dinitrophenylhydrazine (DNPH), cholesterol, dihexadecyl phosphate and antifoam were obtained from Sigma Chemical A/S (St. Louis, MO). Agar, kanamycin, methanol, sodium citrate, tris-(hydroxymethyl) aminomethane (Tris), methylene chloride and sodium hydrosulfite were obtained from Fisher Scientific (Pittsburgh, PA). Isopropyl-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, WI). 4-hydroxy-2-nonenal (HNE) was obtained from Cayman Chemical (Ann Arbor, MI). Phosphatidylcholine was purchased from Avanti Polar Lipid, Inc. (Alabaster, AL). All other chemicals used were analytical grade. Distilled, deionized water was used for the preparation of all solutions, substrates, and experiments.

### 3.2 Wild-type and mutant myoglobins

Wild-type (WT) and mutant (P88H/Q152H, L29F, and H97A) sperm whale myoglobins were synthesized using site-directed mutagenesis as previously described (Grunwald and Richards, 2006ab, Richards et al., 2009). P88H/Q152H was prepared using WT sperm whale myoglobin as previously described (See Chapter 3).

### 3.3 Pre-incubation of the recombinant myoglobins with HNE

Metmyoglobin solutions (0.09 mM) were pre-incubated with HNE (0.63 mM) for 2 h at pH 5.6 and 37°C in a screw-capped polypropylene conical tube

(30 x 115 mm). However, the molar ratio between MetMb and HNE was maintained at 1:7 (Faustman, et al., 1999). Ethanol was delivered to controls at equivalent volumes to HNE (approximately 10 µL/ml of myoglobin). After incubation, the residual unreacted HNE was removed by passing the pre-incubated myoglobin solutions pre-calibrated PD-10 desalting columns (Sephadex G25, 0.15% Kathon preservative CG, GE Health Care, Sweden).

### 3.4 Electrospray ionization (ESI)-mass spectrometry

Samples from WT and mutant sperm whale myoglobins previously incubated with HNE were prepared for ESI-mass spectrometry as previously described in Maheswarappa et al. (2009) and Naveena et al. (2010). A 150-µl sample was mixed with 150 µl of aqueous methanol/distilled water (1:1) with 0.1% acetic acid to enhance protonation of the myoglobin samples. The mass spectrometry analysis was performed on an electrospray ionization-triple-quadrupole (ESI-Q-TOF) mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada). To confirm HNE adducts on the myoglobins, the obtained raw data were transformed into a true mass scale in the mass range between 10,000 and 60,000 daltons (Da) and obtained through the instrumentation software. The peaks with multiple 156 Da mass increases from the unmodified myoglobin peak represent HNE-alkylated myoglobin species.

### 3.5 Preparation of oxymyoglobin

Oxymyoglobins were prepared by sodium hydrosulfite-mediated reduction of the HNE-pretreated MetMbs using a molar ratio (myoglobin:hydrosulfite) of 1:10 to completely reduce heme iron to the ferrous form. The residual

hydrosulfite was removed by PD-10 desalting columns. The reduced myoglobin was converted to OxyMb upon exposure to dissolved oxygen in the column, and atmospheric oxygen with gentle swirling. The concentration of the obtained OxyMb was calculated using a millimolar extinction coefficient at 525 nm (E = 7.6 $cm^{-1}$ $mM^{-1}$). The percentages of myoglobin species in the prepared OxyMb were determined as described in 3.9.

HNE-alkylated and unmodified recombinant myoglobins converted to more than 90% OxyMb except for P88H/Q152H which never achieved more than approximately 70 to 80% OxyMb.

**3.6 Preparation of microsomes and incubation with OxyMb**

Microsomes (pH 5.6) were prepared from 3 ovine livers (n=3) according to Guengerich (1977) with modifications. Briefly, 100 g of liver tissues with visible fat trimmed were placed in ice cold 1.15% KCl. Three volumes of Buffer A (100 mM Tris acetate, pH 7.4; 100 mM KCl; 1 mM EDTA; 20 µM butylated hydroxytoluene (BHT)) was added to one volume of liver. The mixture was minced using a homogenizer for approximately 60 seconds. The homogenate was then transferred to screw-capped tubes and centrifuged at 27,000 x g for 30 min at 4°C. The supernatants were collected and centrifuged at 35,000 rpm for 60 min. The supernatant was discarded and replaced with an equivalent volume of Buffer B (100 mM potassium pyrophosphate, pH 7.4; 1 mM EDTA; 20 µM BHT). The pellets were dislodged by vortex shaking. The homogenate was centrifuged at 35,000 rpm for 60 min. The supernatant was discarded and replaced with a 0.5 volume of Buffer C (10 MM Tris acetate, pH 7.4; 1 mM EDTA;

20% glycerol; 100 µM phenylmethylsulfonyl fluoride (PMSF)). The pellets were dislodged by shaking the tubes on the vortex mixer, pooled (5 ml), and stored at -80°C. Microsomal protein contents were determined using the BCA protein assay.

Freshly-prepared OxyMb solutions were added to the prepared ovine liver microsomes (Yin and Faustman, 1994; Yin et al., 2011). The final concentrations of the microsomal protein and OxyMb in assays were adjusted to 1 mg/ml (Kanner et al., 1986) and 0.06 mM, respectively. The selected concentration of OxyMb was to optimize the very limited supply of mutant myoglobins. The reaction took place in a 15-ml screw-capped polypropylene conical tube (30 x 115 mm) incubated at 25°C for 0, 3.0, 6.0 and 9.0 h. At each time point, 1.0 ml of the reaction solution was removed and scanned spectrophotometrically. One-half of the sample was subjected to the thiobarbituric reactive substances (TBARs) assay. The remaining solution was reacted with DNPH for determination of protein carbonyls.

### 3.7 Preparation of OxyMb:liposomes

Liposomes were prepared as described by Yin and Faustman (1993). Phosphatidylcholine (15 mg), cholesterol (6 mg), and dihexadecyl phosphate (1.5 mg) were dissolved in 2.5 ml of methylene chloride/methanol (2:1) and placed in a 100-ml round bottom flask. The solvents were removed by a rotary evaporator at 40°C for 4 min. The flask with lipid film formed was immediately flushed with nitrogen gas, capped, and kept in the cooler (4°C). A 5-ml aliquot of the freshly prepared OxyMb samples (0.06 mM) was added into the flask with approximately

1 mg of glass beads (1-mm diameter). OxyMb:liposome micelles were formed by shaking on a rotary flask shaker at the speed of 60 rpm for 10 min. The resulting OxyMb:liposome micelles were placed into a 15-ml screw-capped polypropylene conical tube (30 x 115 mm) and incubated at 25°C for 0, 0.5, 1.0, 1.5, 2.0 and 3.0 h. At each time point, 1.0 ml of OxyMb:liposome was sampled and scanned spectrophotometrically. One-half of this sample was subjected to thiobarbituric reactive substances (TBARs) analysis.

### 3.8 Lipid dialysis sac experiment

Prior to the experiment, liposomes were prepared as described above and allowed to oxidize in a water bath at 37°C (pH 5.6) for 5 days. TBARS were determined for the extent of lipid oxidation in the liposomes. A 3.0-ml aliquot of 0.06 mM OxyMbs was separately placed into 3 dialysis membrane tubes (1 ml each) (diameter = 1.6 cm; molecular weight cut-off (MWCO) = 500 Da from Fisher Scientific, Inc. (Springfield, NJ)) and immersed in either oxidized liposome aqueous solutions (30 ml) or 50 mM citrate buffer pH 5.6 containing 1.0 mM EDTA (30 ml). The reaction assays were incubated in a water bath set at 25°C for 0, 3.0 and 6.0 h on a magnetic stirrer to enhance diffusion of lipid oxidation products across the dialysis membrane. At each time point, a 1.0-ml myoglobin sample was removed from the dialysis sac and analyzed for MetMb formation.

### 3.9 Determination of metmyoglobin formation

The formation of MetMb was measured since it reflects the oxidation of OxyMb. In the microsome and liposome experiments, samples were scanned spectrophotometrically from 500 to 600 nm using an integrated sphere

(Shimadzu, Columbia, MD). Metmyoglobin formation was calculated based on absorbance at 503, 525, 557 and 582 (Tang et al., 2004). For the lipid dialysis sac experiment, samples were scanned spectrophotometrically from 500 to 600 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu, Columbia, MD).

### 3.10 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to Schmedes and Holmer (1989). Samples of 0.5 ml were combined with 1.0 ml 20% trichloroacetic acid and centrifuged at 1400 x g for 10 min. One ml of the supernatants was reacted with 1.0 ml of 20mM thiobarbituric acid. The aliquots were incubated at 25°C for 20 h. The absorbance at 532 nm was measured against blanks using a Shimadzu UV-2101PC spectrophotometer (Shimadzu, Columbia, MD). TBARS were reported as $A_{532nm}$.

### 3.11 Determination of protein carbonyls

Samples from the reaction mixture were assayed for protein carbonyls as hydrazone derivatives from reaction with DNPH as described by Reznick and Packer (1994) with some modifications. A 0.5-ml aliquot of sample was added to 1 ml of 10 mM DNPH in 2.5 M HCl and incubated in darkness at 25°C for 1 h. Samples were vortexed briefly and 5 ml of 20% TCA (w/v) solution was added to each sample with subsequent incubation in ice for 10 min. The samples were centrifuged using a tabletop centrifuge at 15,000 x g for 5 min and supernatants were discarded. The pellets were re-washed using 4 ml of 10% TCA while being

broken using a pipet tip followed by centrifugation and discard of the supernatant. Next, the pellets were subject to 3 cycles of washing with 4 ml of ethanol-ethyl acetate (1:1) (v/v) to remove unreacted DPNH and centrifuging. The resulting pellets were re-suspended in 2 ml of 6 M guanidine chloride and incubated for 10 min at 37°C. Insoluble particles were removed by centrifugation. The absorbance values of samples were then measured spectrophotometrically at 370 nm and the protein carbonyl values expressed as $A_{370nm.}$

### 3.12 Statistical analysis

The experimental design of all the experiments was a 3-way (mutant myoglobin type x treatment x incubation time) completely randomized design with three independent replicates (n=3). The obtained data were analyzed using the Mixed procedure with the repeated option of SAS (Version 9.1, SAS Institute Inc. Cary, NC, USA). %MetMb formation, protein carbonyls, TBARS values were measured repeatedly at specific time points. Least square means were generated, and the differences among treatments were significant at $p<0.05$ using least significant differences (LSD).

## 4. Results

We hypothesized that HNE-alkylation of myoglobin would decrease redox stability and facilitate lipid oxidation. Since WT and mutant myoglobins were obtained in the ferric form, they were incubated with HNE before conversion to OxyMb to avoid repeated chemical reduction of the myoglobins. Moreover, the enhanced HNE alkylation was expected since the conformational stability of ferric

myoglobin is less than that of ferrous myoglobin (Hargrove and Olsen, 1996; Sepe et al., 2005).

Mass spectra for control and HNE-treated WT, P88H/Q152H, L29F and H97A myoglobins are presented in Figures 15, 16, 17 and 18, respectively. The peaks denoting relative increases of 156-Da relative to the molecular weight of the unmodified myoglobin represent mono-, di-, and poly-HNE adducts on myoglobin molecules (Faustman et al, 1999) and demonstrated that mutants were alkylated. Similar results for myoglobins from other species have been previously reported (Faustman, et al. 1999; Alderton et al., 2003; Suman et al., 2006; Suman et al., 2007; Lee et al., 2003a; and Naveena et al., 2010). WT myoglobin was alkylated by up to 2 HNE molecules (Figure 15). Tri-HNE adducts were observed in P88H/Q152H, of which proline 88 and glutamine 152 were substituted with 2 histidine residues (Figure 16). Moreover, the peak intensity of P88H/Q152 appears to be greater than that of WT. A mono-HNE adduct was detected in L29F (Figure 17). H97A was found to be alkylated by 3 HNE molecules with relatively small relative abundance of the peak corresponding to tri-HNE adducts (Figure 18).

When incubated with ovine liver microsomes, WT and mutant sperm whale OxyMbs, obtained by pre-incubation of MetMb with HNE and chemically reduced, oxidized faster than controls but the extent of total oxidation was myoglobin-type dependent ($p<0.05$; Figure 19). The greater extent of HNE alkylation (tri-adducts of P88H/Q152 vs. di-adducts of WT) is consistent with the observed increased redox instability of P88H/Q152H compared to WT. The HNE-

pretreated/reduced L29F OxyMb oxidized more readily than its control. However, the rates of MetMb formation of L29F appeared relatively slower than the other recombinant myoglobins. The insignificant effect of HNE on redox stability of H97A relative to its respective control could be the result of substitution of alanine for histidine 97 that allows hydration of the hydrophobic heme pocket (Grunwald and Richards, 2006ab; Richards et al., 2009) and which might dominate the effect of HNE. Under acidic conditions, the hydration of the heme pocket facilitates protonation of the bound oxygen, which accelerates heme iron autoxidation (Livingston and Brown, 1981).

TBARS formation in microsomes increased during incubation with mutant myoglobins ($p<0.05$ Figure 20) and was greater in HNE-pretreated/reduced WT and P88H/Q152H than their respective controls ($p<0.05$; Figure 20). HNE-treated P88H/Q152H showed the greatest TBARS formation compared to other samples, which might be related to greater MetMb formation of this mutant due to the greater degree of HNE alkylation that it demonstrated. Interestingly, and despite showing the greatest MetMb formation, H97A did not induce TBARS formation as expected (Figure 20). Possible explanations could be related to the potential for molecular aggregation driven by hydrophobic interaction among HNE molecules attached on H97A followed by precipitation out of the aqueous phase. TBARS values presented in this study for microsomal lipid oxidation were less than those reported by Richards et al. (2009). In that study, microsomes from washed cod muscle were used and these would be expected to contain greater concentrations of polyunsaturated fatty acids.

Protein carbonyl formation is presented in Figure 21. After 9 h of incubation, the $A_{370nm}$ values were greater in HNE-pretreated/reduced myoglobins than controls. Microsomes incubated with the HNE-pretreated/reduced P88H/Q152H contained the greatest concentration of protein carbonyls ($p<0.05$) while wild-type and L29F controls had the lowest values ($p<0.05$). It's important to note that HNE molecules adducted on myoglobin have a carbonyl that could react with DPNH in the same way protein-affiliated carbonyl compounds (Nadkarni and Sayre, 1995). Thus, results are confounded by this possibility, the extent of which could not be controlled for since the specific concentration of protein-bound HNE could not be quantified. Additionally, the observed presence of insoluble particles after pellets were re-suspended in guanidine hydrochloride indicated the probability of incomplete recovery of DNPH derivatives and thus the variations in absorbance at 370 nm ($A_{370nm}$). The differences in the absorbance at 370 nm between initial (time = 0 h) and 9 hours incubation are presented in Figure 22. Increases in absorbance at 370 nm were observed in all samples except for HNE-pretreated/reduced P88H/Q152H which demonstrated decreased $A_{370nm}$ whereas its respective control had increased $A_{370nm}$. Moreover, both control and HNE-pretreated/reduced L29F samples also had greater increases in absorbance at 370 nm than WT samples. These observations were not consistent with MetMb formation or TBARS.

Trends for MetMb formation in liposomes were similar to results obtained in microsomes. MetMb formed at faster rates in HNE-pretreated/reduced WT and mutant OxyMbs than controls ($p<0.05$; Figure 23). The difference in the initial

%MetMb formation (time = 0 h) among the recombinant OxyMb:liposomes could be due to OxyMb oxidation that occurred even during formation of OxyMb:liposomes. P88H/Q152H oxidized fastest followed by WT and L29F, respectively ($P<0.05$). TBARS (Figure 24) shared a similar trend for MetMb formation. Liposomes incubated with P88H/Q152H also showed the greatest TBARS values followed by WT and L29F, respectively ($p<0.05$). Pre-incubation with HNE enabled the recombinant myoglobins to promote lipid oxidation more effectively than their respective controls ($p<0.05$). The effect of HNE on the ability to promote TBARS formation was greater in P88H/Q152H than in WT and L29F ($p<0.05$). This could be related to the extents to which these recombinant myoglobins were alkylated by HNE (tri-adducts in P88H/Q152H, di-adducts in WT, and mono-adduct in L29F).

Lipid oxidation products formed from the oxidation of aqueous liposomes at pH 5.6 and 25°C for 5 days were able to diffuse across a dialysis membrane with MWCO = 500 Da. Evidence for this is provided by the presence of TBARS values inside the dialysis tubes after 9 h of incubation with the oxidized lipids compared with when incubating with the buffer controls ($p<0.05$; Figure 25). There was no significant difference in the diffusion of TBARS across the dialysis tube among the myoglobin samples incubated with the oxidized lipids ($p>0.05$; Figure 25). Redox instability of OxyMbs inside the dialysis tubes was enhanced as noted by the increased MetMb formation observed (Figure 26). P88H/Q152H seemed to be the most sensitive towards TBARS among the recombinant myoglobins, which is evidenced by the 8% to 9% enhanced MetMb formation of

the recombinant OxyMb incubated with oxidized lipids compared to controls incubated with citrate buffer (Figure 26). In contrast, an approximate 5 to 6% increase in MetMb formation was observed in WT OxyMb incubated with oxidized lipids. L29F appeared to be the most redox stable and least affected by TBARS from oxidized lipids with approximately 2 to 3% more MetMb.

## 5. Discussion

Previously, Lynch and Faustman (2000) demonstrated the HNE-enhanced pro-oxidative nature of horse (heart) MetMb. The pH and temperature condition used in that study was pH 7.2 and 37°C. A pH value of 5.6 was chosen for our study based on the postmortem pH of bovine muscles. At pH 5.6, histidine residues are expected to remain partially protonated compared to pH 7.4 (Barrick et al., 1994). The nucleophilic ability of the imidazole groups of histidine residues would be expected to be muted with greater positive charge at lower pH. The extent of HNE alkylation of myoglobin was reported to be greater at pH 7.4 than pH 5.6 for equine and bovine myoglobins (Faustman et al., 1999; Alderton et al., 2003). Moreover, the elevated temperature can accelerate lipid oxidation even at the neutral pH of 7.2. The effect of HNE alkylation on heme redox instability becomes less significant at pH 5.6 and 37°C due to enhanced OxyMb autoxidation under combined low pH and elevated temperature conditions (Gotoh and Shikama, 1974; Alderton et al., 2003). Therefore, a milder incubation condition (pH 5.6 and 25°C) was chosen for microsome and liposome models.

Previous studies demonstrated the effect of HNE on OxyMb redox stability. However, since myoglobin was incubated in the presence of HNE, the possibility that redox stability of OxyMb could be compromised by unbound HNE via other mechanisms cannot be excluded. Our results (Figures 19 and 23) suggested that HNE could increase OxyMb oxidation by covalent modification of myoglobin. In the absence of unbound HNE, the recombinant myoglobins that had been incubated with HNE and chemically-reduced subsequently oxidized faster than controls ($p<0.05$). The binding of HNE molecules to histidine residues could be expected to induce partial unfolding of myoglobin tertiary structure (Alderton et al., 2003). Spatial changes of myoglobin folding could expose the heme moiety to the external hydrophilic environment in which the redox stability of the ferrous ($Fe^{2+}$) heme might become impaired.

The oxidation of OxyMb results in MetMb and superoxide anion. The former product was reported to promote homolytic scission of preformed lipid hydroperoxides via formation of a hypervalent intermediate ferryl myoglobin (Baron and Andersen, 2002). The latter, on the other hand, spontaneously undergoes dismutation and forms hydrogen peroxide, which is a strong oxidant (Tajima and Shikama, 1987; Wazawa et al., 1992). Moreover, MetMb can be activated by hydrogen peroxide and initiate electron abstraction from polyunsaturated fatty acids (Harel and Kanner, 1985). The heme affinity of MetMb was reported to be approximately 60-fold lower than that of OxyMb (Hargrove et al., 1996; Tang et al., 1998). Low heme affinity of myoglobin is associated to its enhanced ability to promote lipid oxidation (Grunwald and

Richards, 2006b). The released heme was expected to embed itself in phospholipids due to hydrophobic interactions (Chiu et al., 1996). The close proximity to lipids increases the chance for the ferric ($Fe^{3+}$) heme moiety to promote lipid oxidation. The rate of heme dissociation from heme proteins (myoglobin and hemoglobin) was found to influence its ability to promote lipid oxidation (Richards et al., 2005; Grunwald and Richards, 2006ab; Richards et al., 2009).

The number of histidine residues and their relative location in the primary sequence of myoglobin appear to determine the relative susceptibility of the heme protein to covalent modification by secondary lipid oxidation products. Suman et al. (2007) reported that bovine myoglobin (13 histidine residues) was more susceptible to HNE alkylation and HNE-mediated OxyMb oxidation than porcine myoglobin (9 histidine residues). Histidines 88 and 152 of bovine myoglobin, which are absent in porcine myoglobin, were both alkylated by HNE. Our previous result showed that HNE adducts were found at both substituted histidines 88 and 152 of P88H/Q152 (Figures 13 and 14, Table 4 in Chapter 3). The results in this study also indicate that the increase in histidine residues in the primary sequence predisposes P88H/Q152H to greater HNE alkylation via Michael addition (Figure 16) compared to WT (Figure 15). The phenyl ring of phenylalanine 29 of L29F is bulky and hydrophobic, which could help prevent hydration of the heme pocket (Carver et al., 1992; Richards et al., 2009) and possibly sterically block HNE from alkylating the histidine residues buried within. However, histidine residues on the surface remain accessible. On the other

hand, the replacement of histidine 97 with an alanine with a relatively smaller side chain might allow hydration of the hydrophobic heme pocket and destabilize the heme-globin interactions (Hargrove et al., 1996; Tang et al., 1998; Liong et al., 2001; Grunwald and Richards, 2006b). The presence of the polar solvent in the heme pocket may weaken the hydrophobic interactions that stabilize the tertiary structure of myoglobin (Livingston and Brown, 198; Hargrove et al., 1996). As a result, histidine residues buried within the hydrophobic pocket, particularly proximal histidine 64 and distal histidine 93 that interact with the heme moiety and the bound ligand, respectively, could become more accessible to HNE molecules. The HNE alkylation on these two histidines should, in principle, exert a greater negative effect on heme affinity and redox stability than the HNE alkylation on other histidine residues.

The deleterious effects of HNE on stability of OxyMb and lipids were more obvious in liposomes than in microsomes. This could be due to the fact that the microsomes models were more complex than the liposome models. Microsomes consisted of phospholipids, lipid soluble antioxidants, microsomal proteins etc. The endogenous vitamin E present in lipid membranes of microsomes could delay oxidation of lipids. Moreover, the microsomal proteins could provide side chains of certain amino acid residues, which are cysteine, histidine, methionine, tryptophan, tyrosine, and proline, that possess radical scavenging activity (Elias et al., 2008). In addition, OxyMb incorporated into multilamellar liposomes (Yin and Faustman, 1993) would be expected to be in close contact with membrane lipids. Recently, Yin et al. (2011) reported that HNE-pre-incubation/reduction of

myoglobin failed to induce redox instability and pro-oxidative activity of the myoglobins from multiple meat-producing species in ovine liver microsomes. They explained the effect of HNE was obscured by microsome-derived lipid oxidation products.

The effect of small lipid oxidation products of egg yolk phosphatidylcholine on OxyMb redox stability observed in our study was similar to the previous report by Chan et al. (1997). Secondary products of lipid oxidation especially unsaturated aldehydes (e.g. HNE) are highly reactive towards nucleophilic side chains of cysteines, histidines and lysines (Uchida and Stadtman, 1992; Bolgar and Gaskell, 1996). These low molecular weight products are relatively smaller and more hydrophilic than their parent lipids. The increased solubility allows them to diffuse into the aqueous phase and interact with water-soluble proteins, including myoglobin.

The redox stability and oxygen affinity of L29F were reported to be 10-fold and 15-fold, respectively, greater than in WT sperm whale myoglobin (Carver et al., 1992; Brantley et al., 1993). The remarkable redox stability of L29F OxyMb could be due to the stabilizing effect of the large hydrophobic side chain of phenylalanine 29. The phenyl side chain helps strengthen the binding of $O_2$ to the heme moiety and keeps hydronium ions away from the heme crevice by its partially induced positive charge and steric hindrance (Carver et al., 1992). Protonation of the $O_2$ ligand accelerates heme oxidation and superoxide anion formation (Livingston and Brown, 1981), whereas protonation of distal histidine weakens the coordinate bond between the imidazole group and the heme iron

(Hargrove et al., 1996). The ferrous L29F previously exhibited greater redox stability than WT and therefore was a less potent lipid oxidation initiator in washed cod muscles (Richards et al., 2009). In our study, HNE could enhance redox instability and pro-oxidative activity of L29F to a lesser extent compared to WT and other mutant myoglobins. These observations were obvious in the liposome models (Figure 24) which could be related to the degree of HNE alkylation of L29F (mono-adduct) compared to WT (di-adducts) and P88H/Q152H (tri-adducts) (Figures 15 to 17).

The imidazole group of HIS 97 was previously shown to form a hydrogen bond with the heme-7-propionate group and act as a barrier against salvation of the heme pocket (Liong et al., 2001). The porphyrin ring is therefore stabilized within the globin protein. The aforementioned stabilizing effects of histidine 97 are diminished when this histidine residue is substituted with a smaller alanine residue by creating a channel for solvent to hydrate the heme crevice. Lack of hydrogen bonding with the heme-7-propionate group and the hydrophobic environment exposes heme and the proximal histidine to the solvent and contributes to decreased heme affinity and redox stability (Liong et al., 2001; Grunwald and Richards, 2006b). This provides an opportunity for HNE to enter and modify histidine residues within the heme crevice. The heme affinity of H97A might be further compromised by extensive HNE alkylation that might induce conformational change in the folding of myoglobin. Despite its excellent pro-oxidative activity in previous studies mentioned above, intermolecular aggregation and precipitation of multiple HNE adducts on H97A driven by

hydrophobic interaction might have minimized or prevented the interaction with lipids. A similar phenomenon was described by Bruenner et al. (1995) when they observed insoluble particles of hemoglobin heavily modified by HNE.

## 6. Conclusions

The variation in the primary sequence of myoglobin could result in differences in autoxidation rate, heme affinity, and/or susceptibility to nucleophilic attack by reactive unsaturated aldehydes derived from lipid oxidation. The ability of HNE to enhance pro-oxidative activity of myoglobin was related to the extent to which it modified myoglobin. Increased MetMb formation induced by HNE adducts was concomitant with formation of TBARS in ovine liver microsomes and liposomes. Small lipid oxidation products diffused across dialysis membranes (MWCO = 500 Da) and accelerated OxyMb oxidation. Substitutions of 2 histidine residues for the native phenylalanine 88 and glutamine 152 residues decreased P88H/Q152 redox stability and enhanced its pro-oxidative activity due to additional sites for HNE alkylation. The enhanced redox stability of L29F, owing to the hydrophobic side chain of phenylalanine 29, withstood HNE-induced OxyMb oxidation and pro-oxidative activity to a certain level. The low heme affinity of H97A and effect of HNE alkylation led to significant redox instability and pro-oxidative activity towards lipids.

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## Figure captions

**Figure 15** Mass-transformed spectrum of WT sperm whale MetMb (0.09 mM) incubated with HNE (0.63 mM) at pH 5.6 and 37°C for 2 h.

**Figure 16** Mass-transformed spectrum of P88H/Q152H sperm whale MetMb (0.09 mM) incubated with HNE (0.63 mM) at pH 5.6 and 37°C for 2 h.

**Figure 17** Mass-transformed spectrum of L29F sperm whale MetMb (0.09 mM) incubated with HNE (0.63 mM) at pH 5.6 and 37°C for 2 h.

**Figure 18** Mass-transformed spectrum of H97A sperm whale MetMb (0.09 mM) incubated with HNE (0.63 mM) at pH 5.6 and 37 °C for 2 h.

**Figure 19** MetMb formation of HNE-pretreated/reduced and control WT, P88H/Q152H, L29F. and H97A OxyMbs (0.06 mM) incubated with ovine liver microsomes (1 mg microsomal protein/ml) at pH 5.6 and 25°C.

**Figure 20** TBARS ($A_{532}$) of HNE-pretreated/reduced and control WT, P88H/Q152H, L29F, and H97A sperm whale OxyMbs (0.06 mM) incubated with ovine liver microsomes (1 mg microsomal protein/ml) at pH 5.6 and 25°C.

**Figure 21** Protein carbonyls expressed as hydrazine derivatives ($A_{370}$) of HNE-pretreated/reduced and control WT, P88H/Q152H, L29F, and H97A sperm whale OxyMbs (0.06 mM) incubated with ovine liver microsomes (1 mg microsomal protein/ml) at pH 5.6 and 25°C.

**Figure 22** Differences in $A_{370}$ between time 0 and 9 hr of incubation at pH5.6, 25°C, for HNE-pretreated/reduced and control WT, P88H/Q152H, L29F, and H97A sperm whale OxyMbs (0.06 mM) incubated with ovine liver microsome (1 mg microsomal protein/ml).

**Figure 23** MetMb formation in HNE-pretreated/reduced and control WT, P88H/Q152H, and L29F sperm whale OxyMbs (0.06 mM) incubated with phosphatidylcholine liposomes at pH 5.6 and 25°C.

**Figure 24** TBARS formation ($A_{532}$) of HNE-pretreated/reduced and control WT, P88H/Q152H, and L29F sperm whale OxyMbs (0.06 mM) incubated with phosphatidylcholine liposomes at pH 5.6 and 25°C.

**Figure 25** TBARS values of aqueous samples removed from dialysis sacs containing WT, P88H/Q152H, and L29F sperm whale OxyMbs (0.06 mM) at 0 and 6 h incubation in oxidized liposomes at pH 5.6 and 25°C for 5 days.

**Figure 26** MetMb formation of WT, P88H/Q152H, and L29F sperm whale OxyMbs (0.06 mM) sampled from dialysis sacs submersed in 50 mM citrate buffer during incubation at pH 5.6 and 25°C.

**Figure 15**

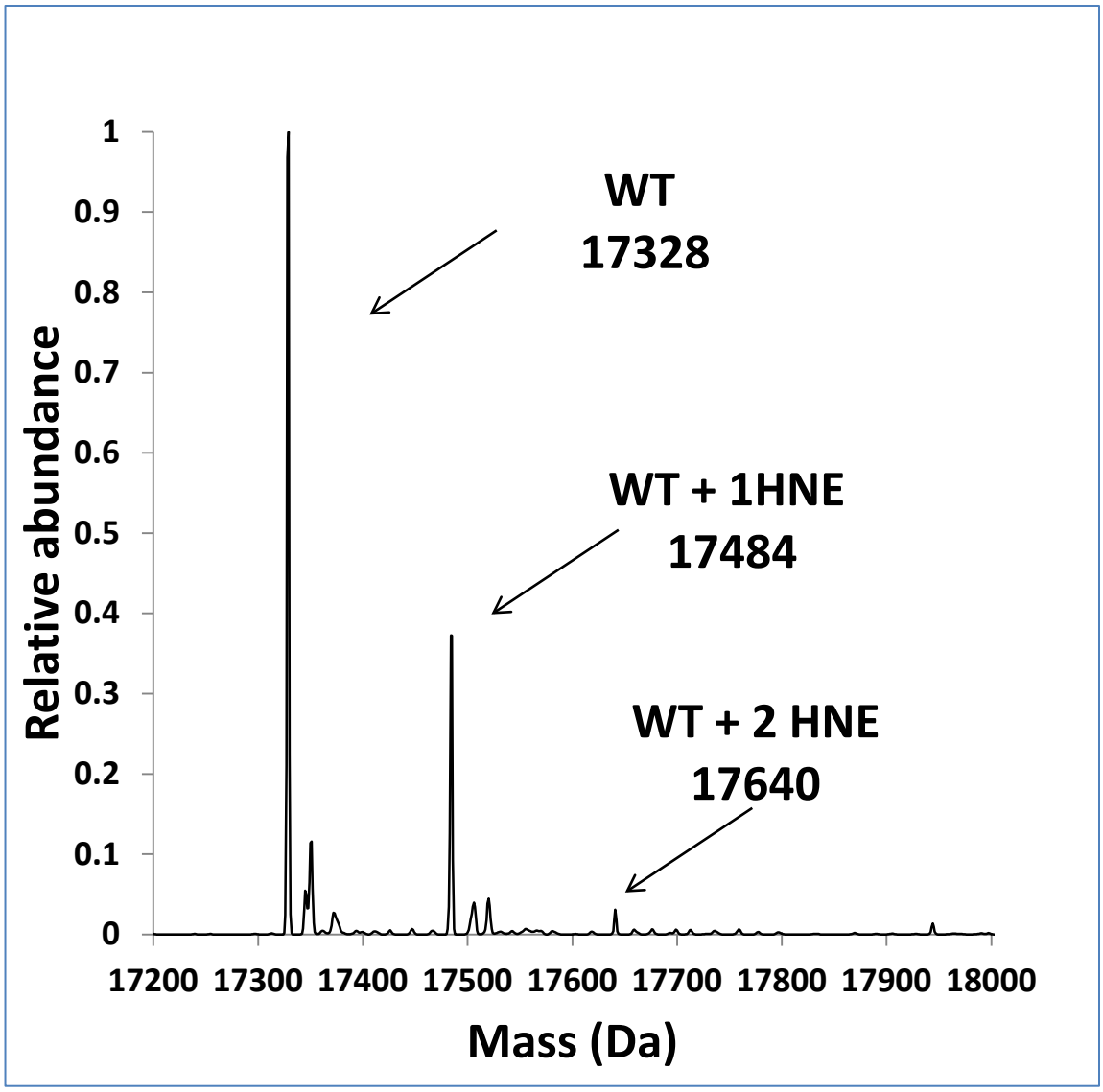

Relative abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
17328
WT + 1HNE
17484
WT + 2 HNE
17640
17200 17300 17400 17500 17600 17700 17800 17900 18000
Mass (Da)

**Figure 16**

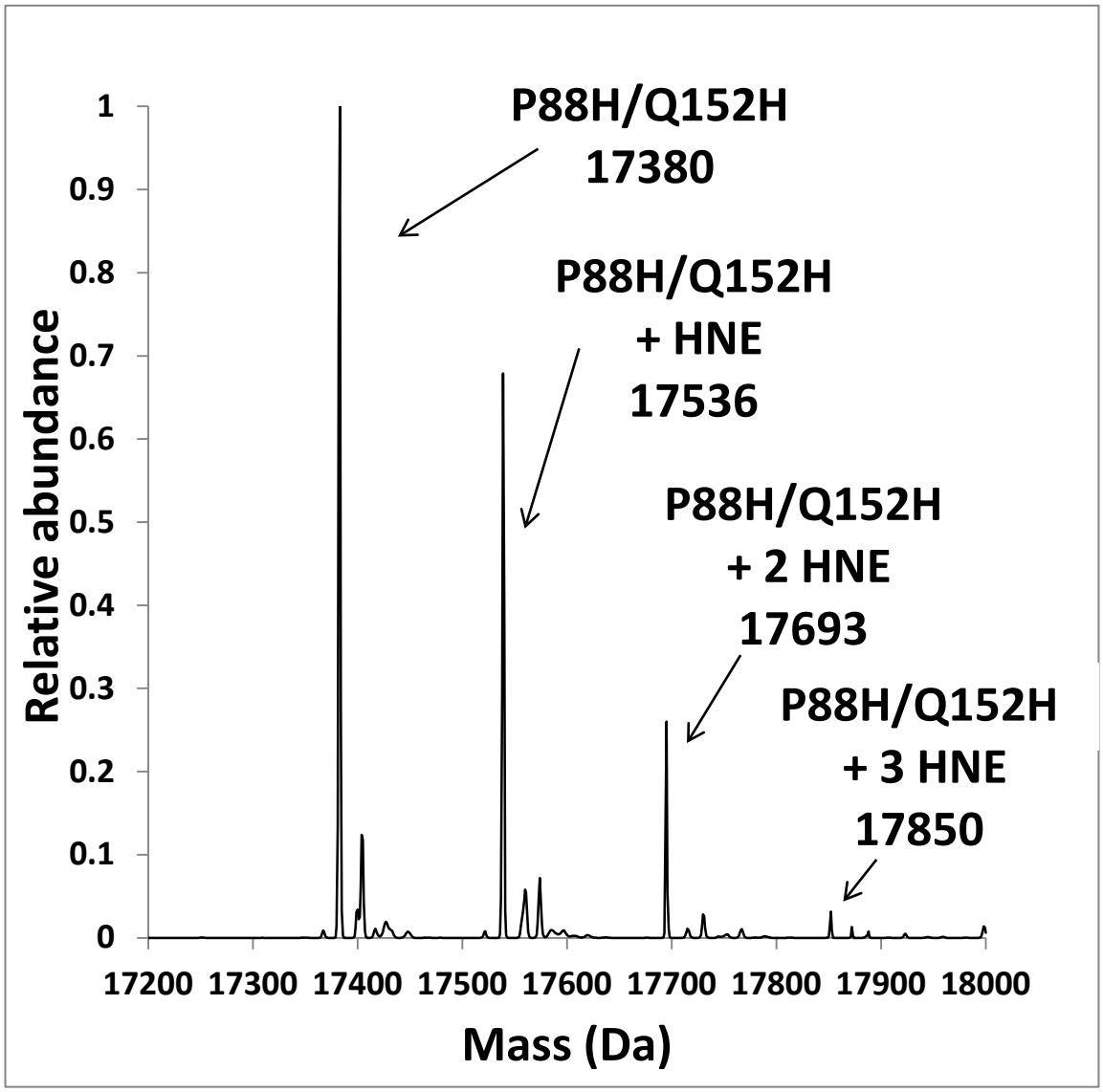

P88H/Q152H
17380
P88H/Q152H
+ HNE
17536
P88H/Q152H
+ 2 HNE
17693
P88H/Q152H
+ 3 HNE
17850
Relative abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
17200 17300 17400 17500 17600 17700 17800 17900 18000
Mass (Da)

**Figure 17**

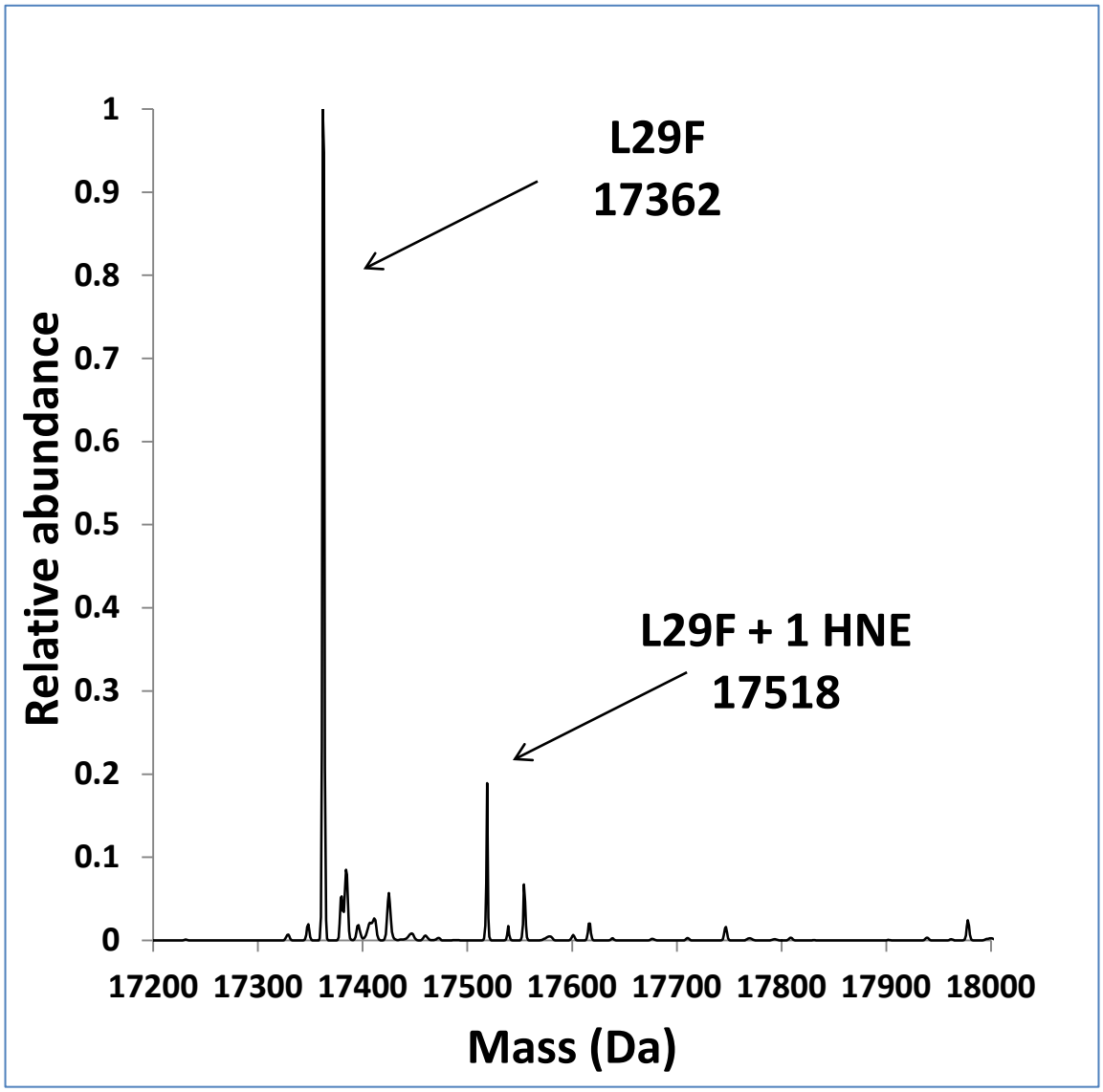

L29F
17362
L29F + 1 HNE
17518
Relative abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
17200 17300 17400 17500 17600 17700 17800 17900 18000
Mass (Da)

**Figure 18**

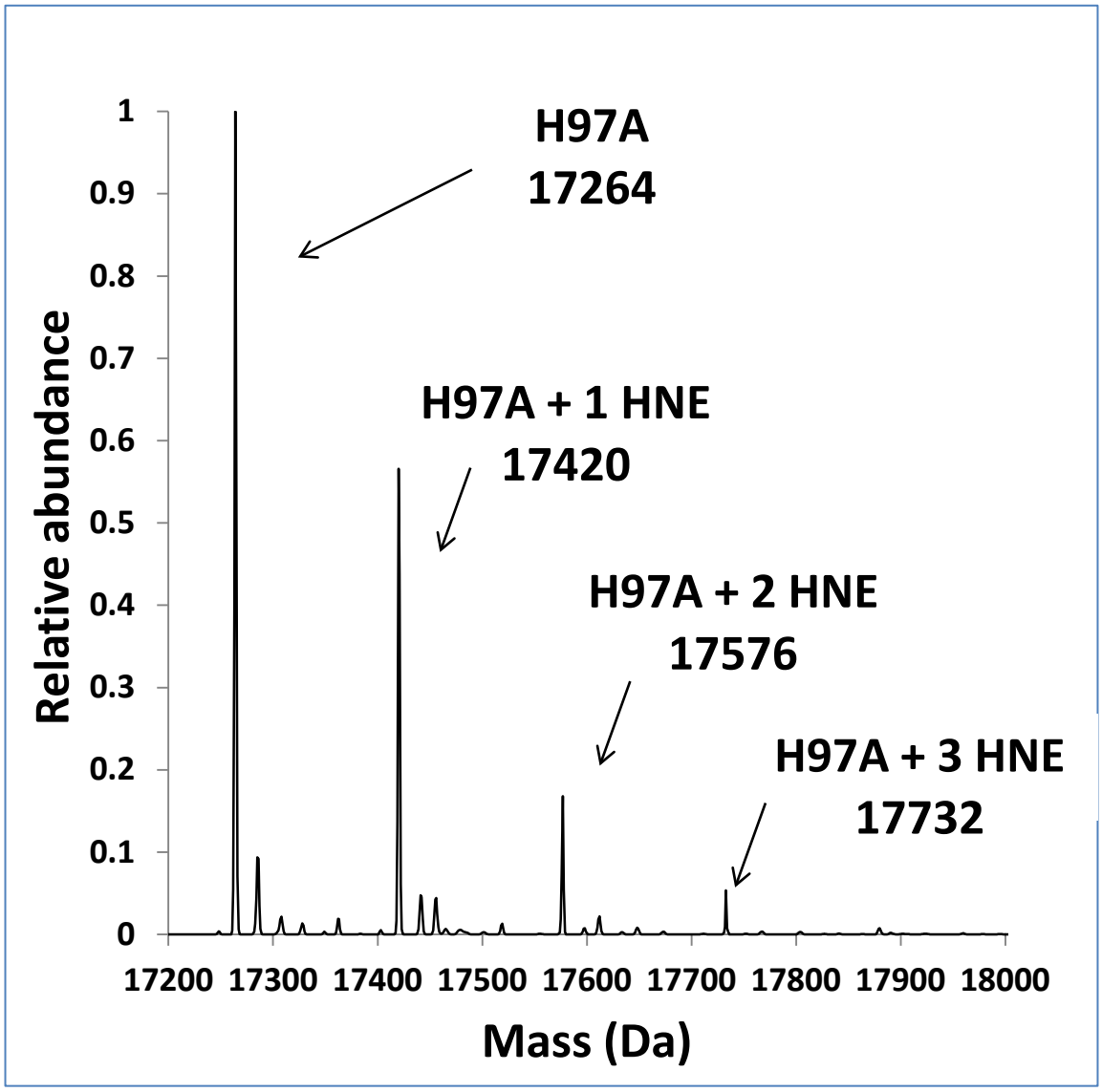

H97A
17264
H97A + 1 HNE
17420
H97A + 2 HNE
17576
H97A + 3 HNE
17732
Relative abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
17200 17300 17400 17500 17600 17700 17800 17900 18000
Mass (Da)

**Figure 19**

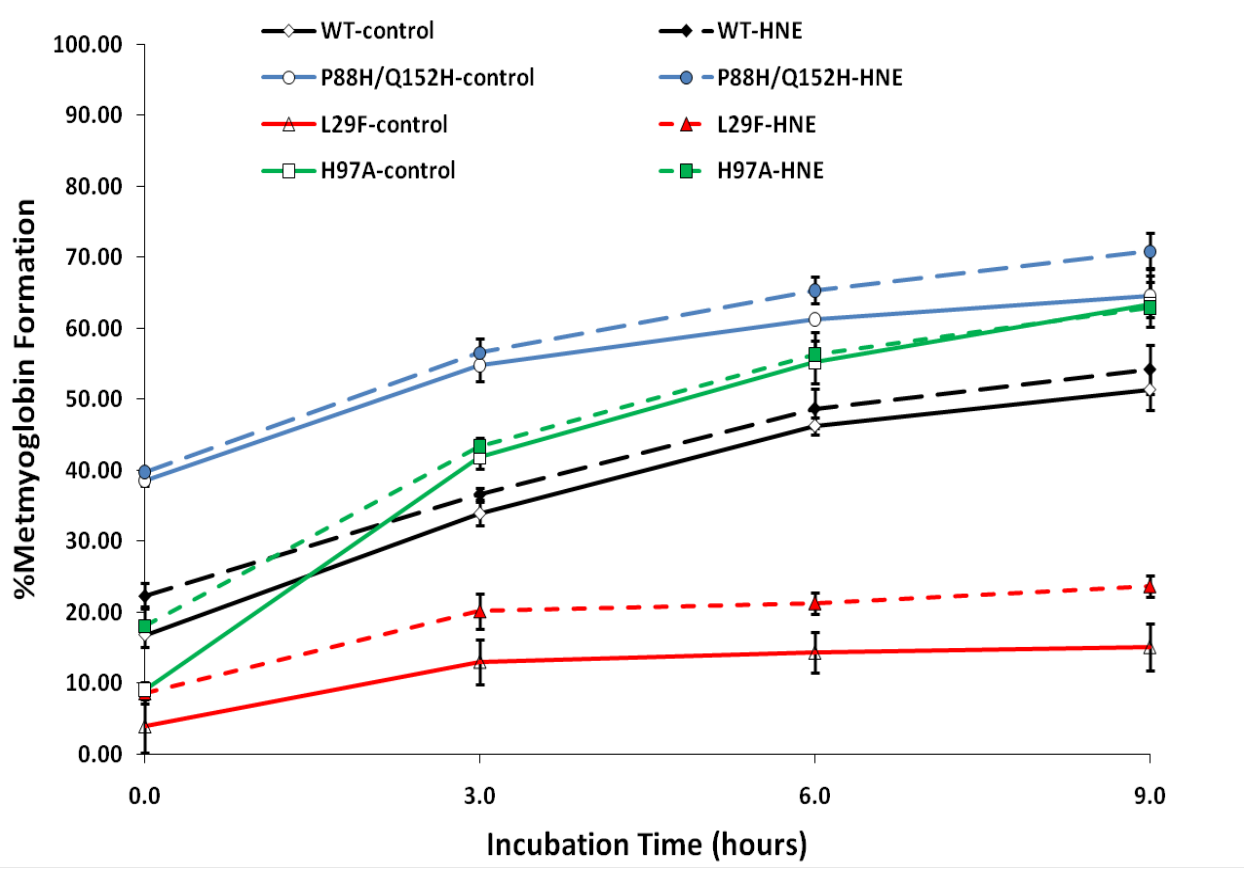

WT-control
WT-HNE
P88H/Q152H-control
P88H/Q152H-HNE
L29F-control
L29F-HNE
H97A-control
H97A-HNE
%Metmyoglobin Formation
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.0
3.0
6.0
9.0
Incubation Time (hours)

**Figure 20**

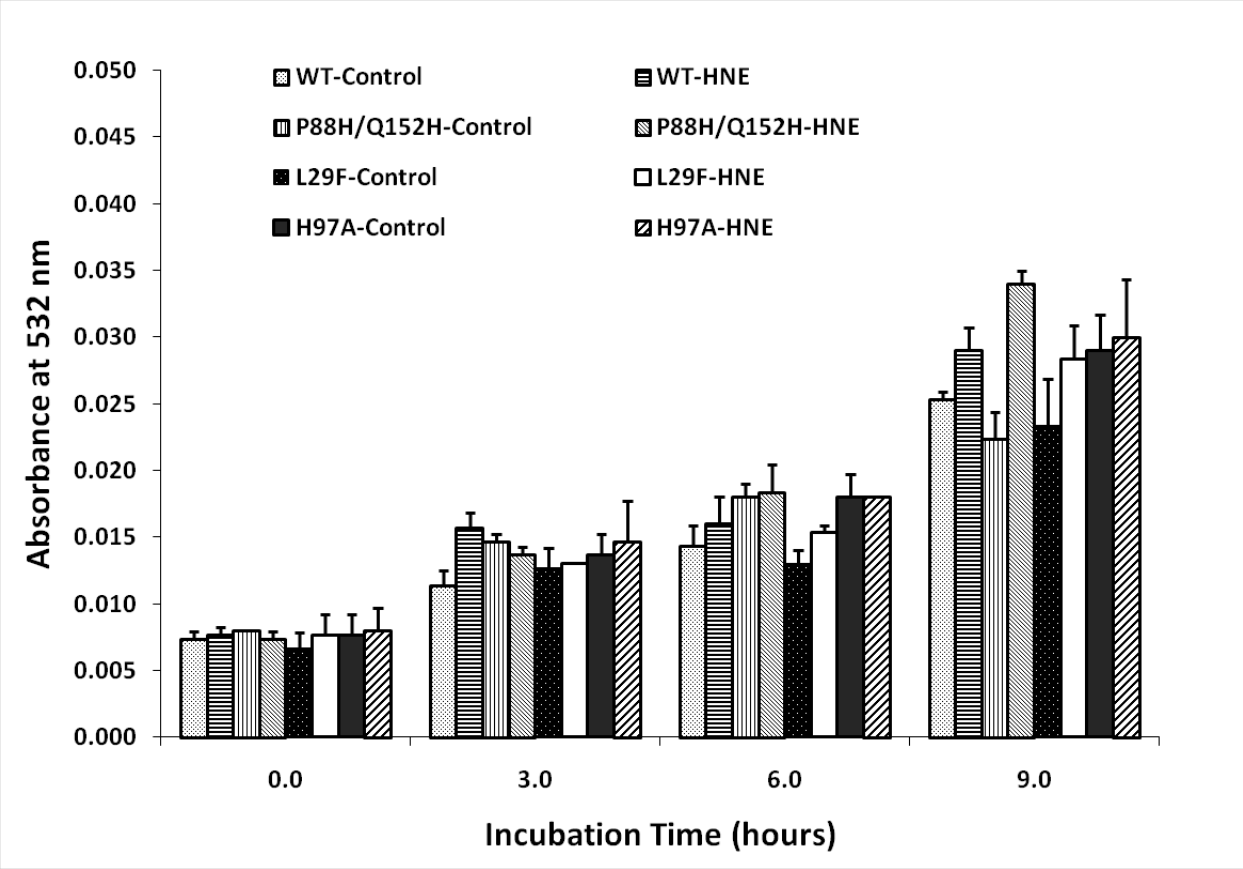

WT-Control
WT-HNE
P88H/Q152H-Control
P88H/Q152H-HNE
L29F-Control
L29F-HNE
H97A-Control
H97A-HNE
Absorbance at 532 nm
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.0
3.0
6.0
9.0
Incubation Time (hours)

**Figure 21**

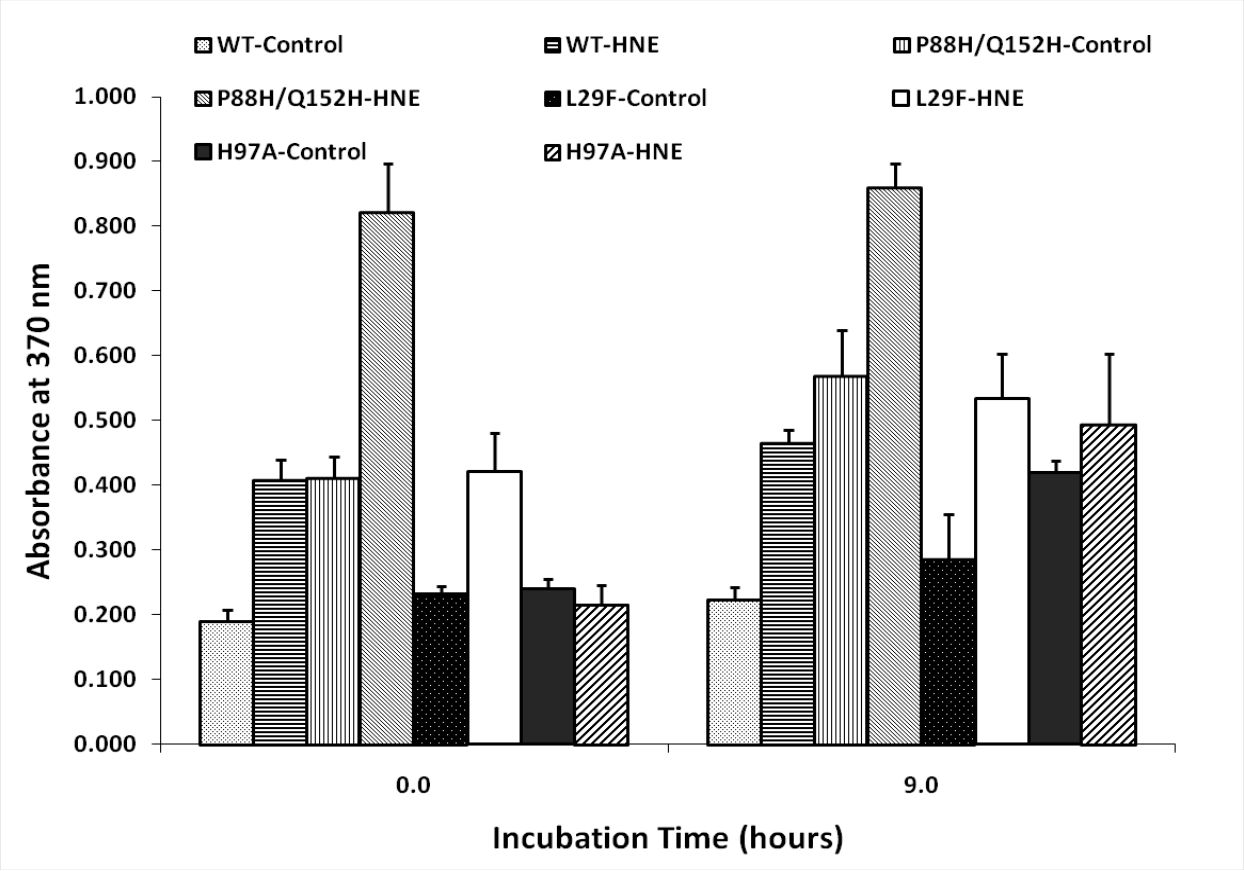

WT-Control
WT-HNE
P88H/Q152H-Control
P88H/Q152H-HNE
L29F-Control
L29F-HNE
H97A-Control
H97A-HNE
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Absorbance at 370 nm
0.0
9.0
Incubation Time (hours)

**Figure 22**

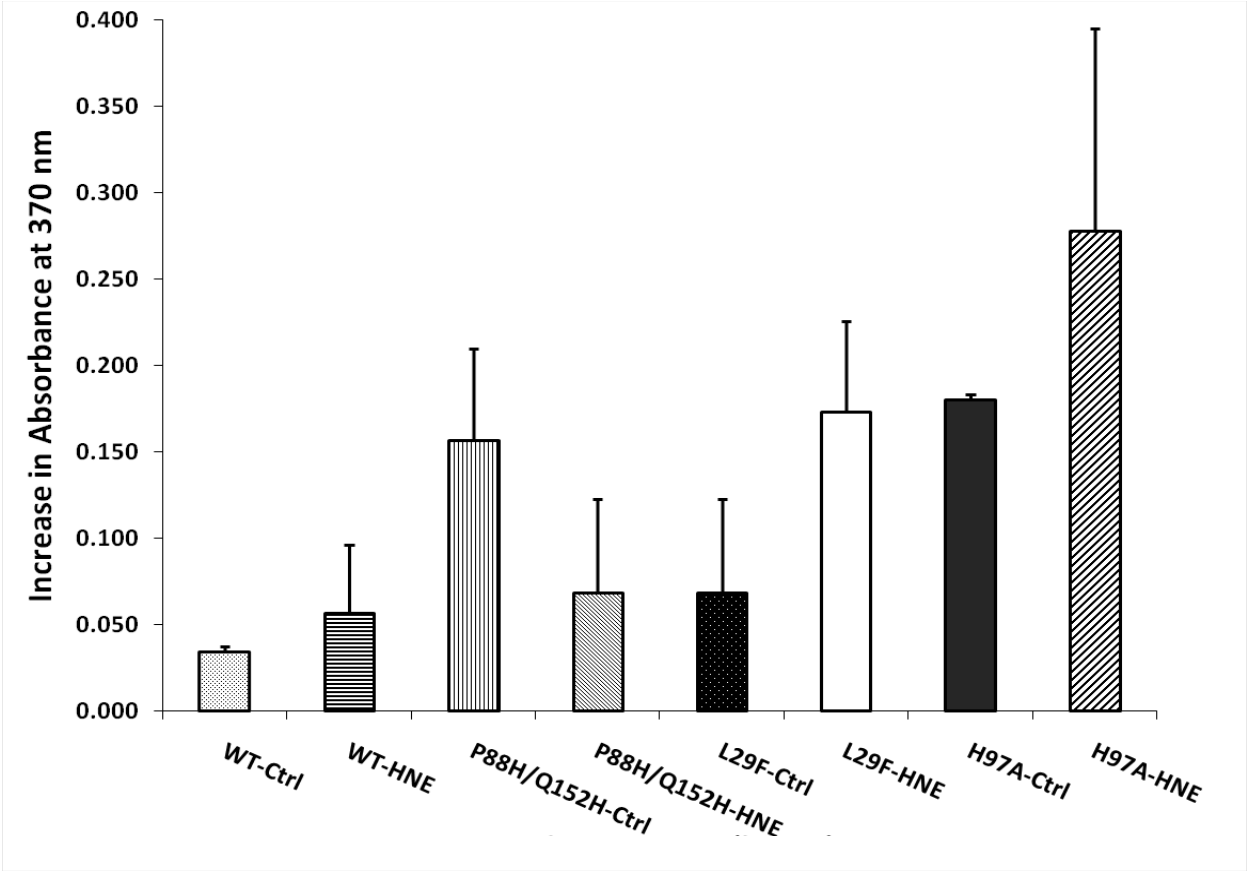

Increase in Absorbance at 370 nm
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
WT-Ctrl
WT-HNE
P88H/Q152H-Ctrl
P88H/Q152H-HNE
L29F-Ctrl
L29F-HNE
H97A-Ctrl
H97A-HNE

**Figure 23**

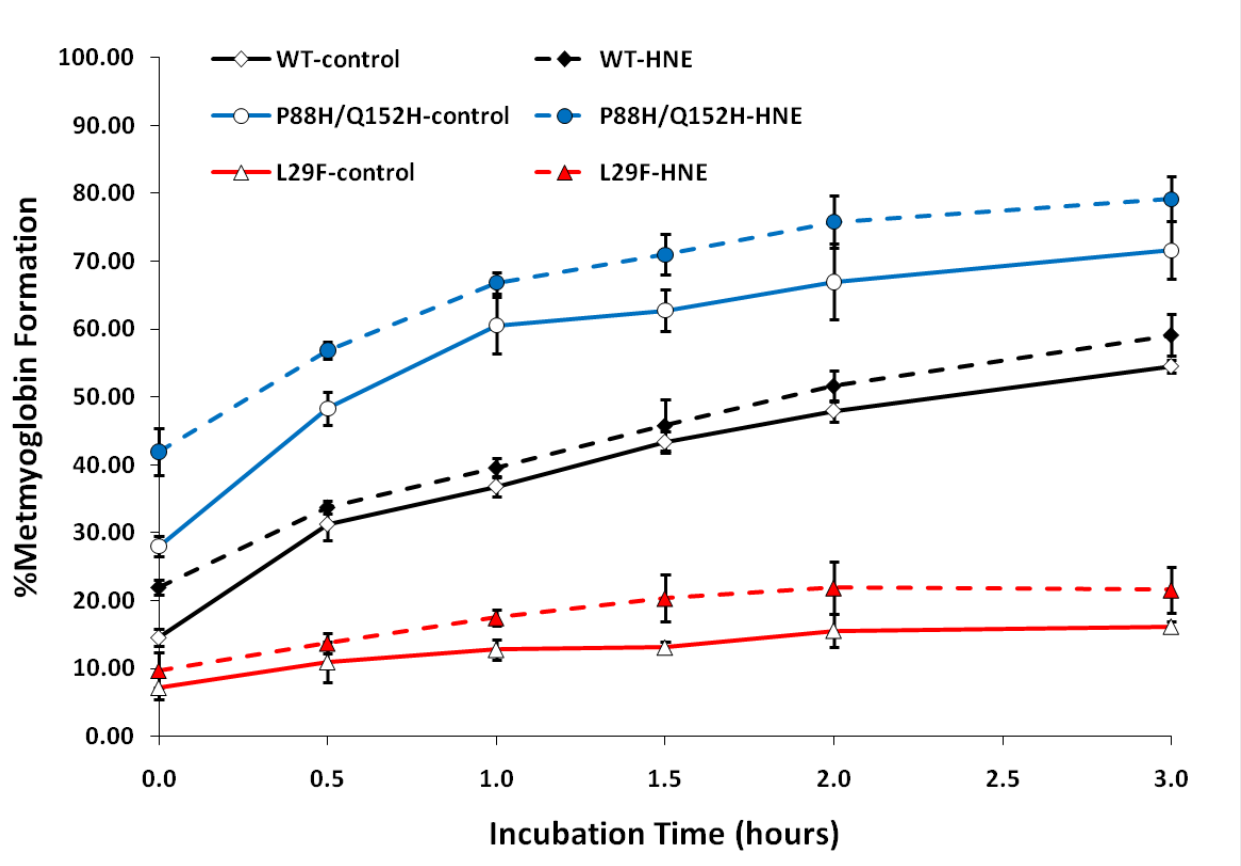

WT-control
WT-HNE
P88H/Q152H-control
P88H/Q152H-HNE
L29F-control
L29F-HNE
%Metmyoglobin Formation
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Incubation Time (hours)

**Figure 24**

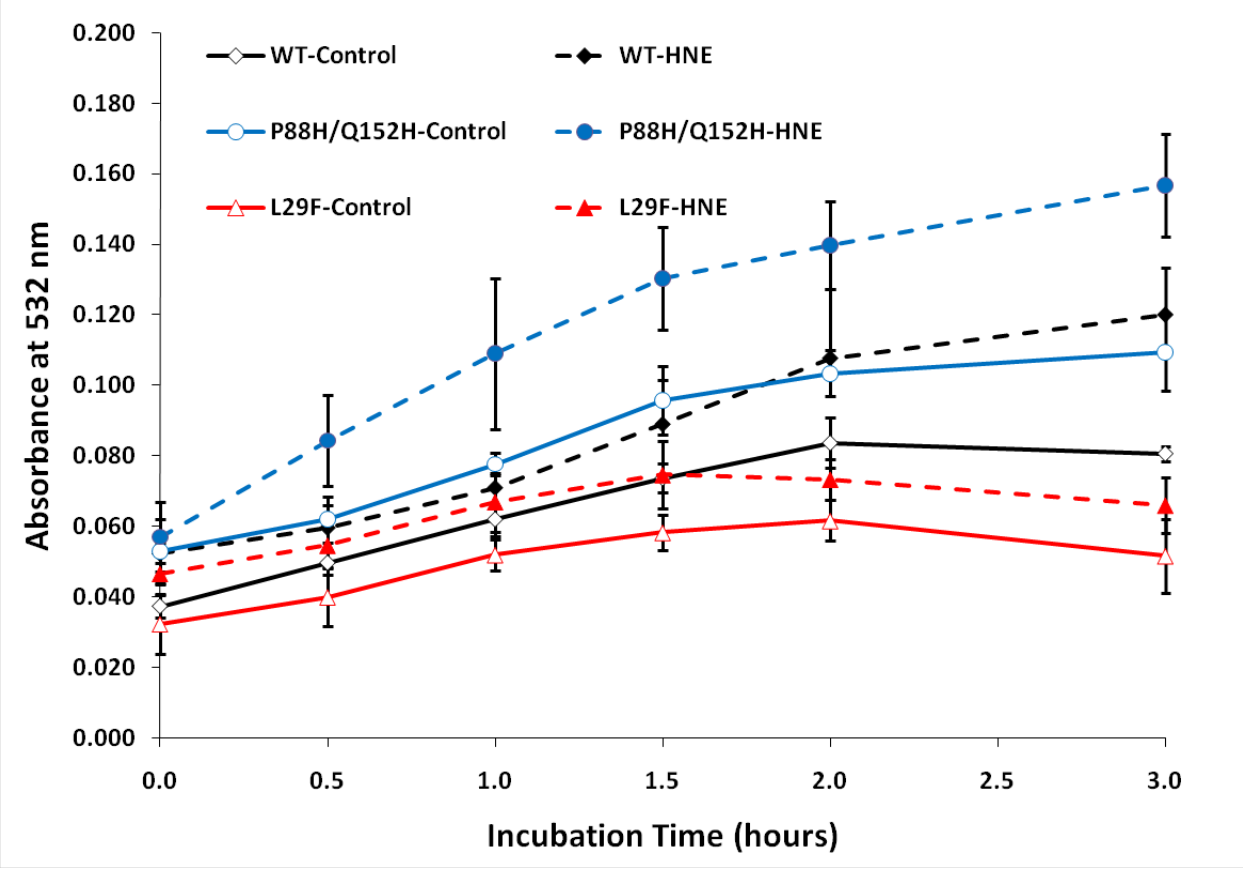

WT-Control
WT-HNE
P88H/Q152H-Control
P88H/Q152H-HNE
L29F-Control
L29F-HNE
Absorbance at 532 nm
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Incubation Time (hours)

**Figure 25**

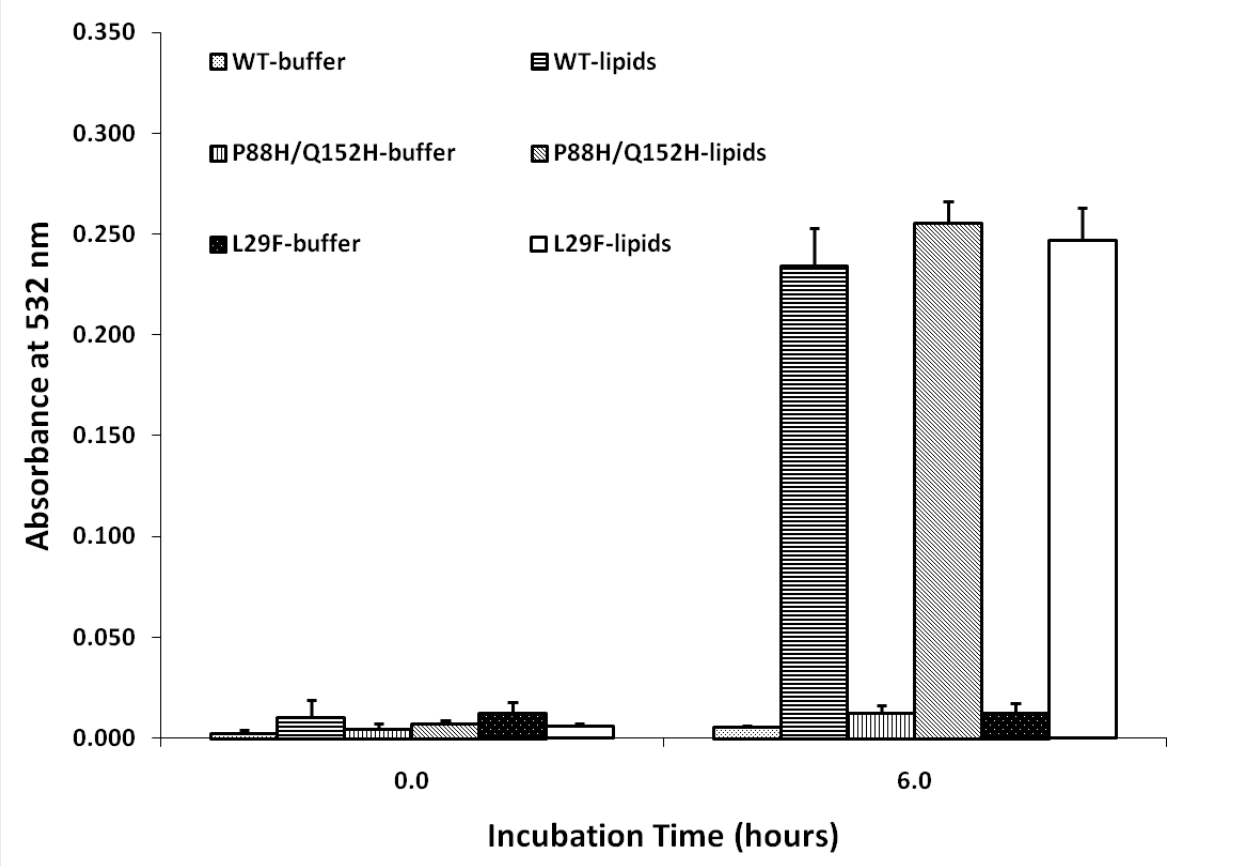
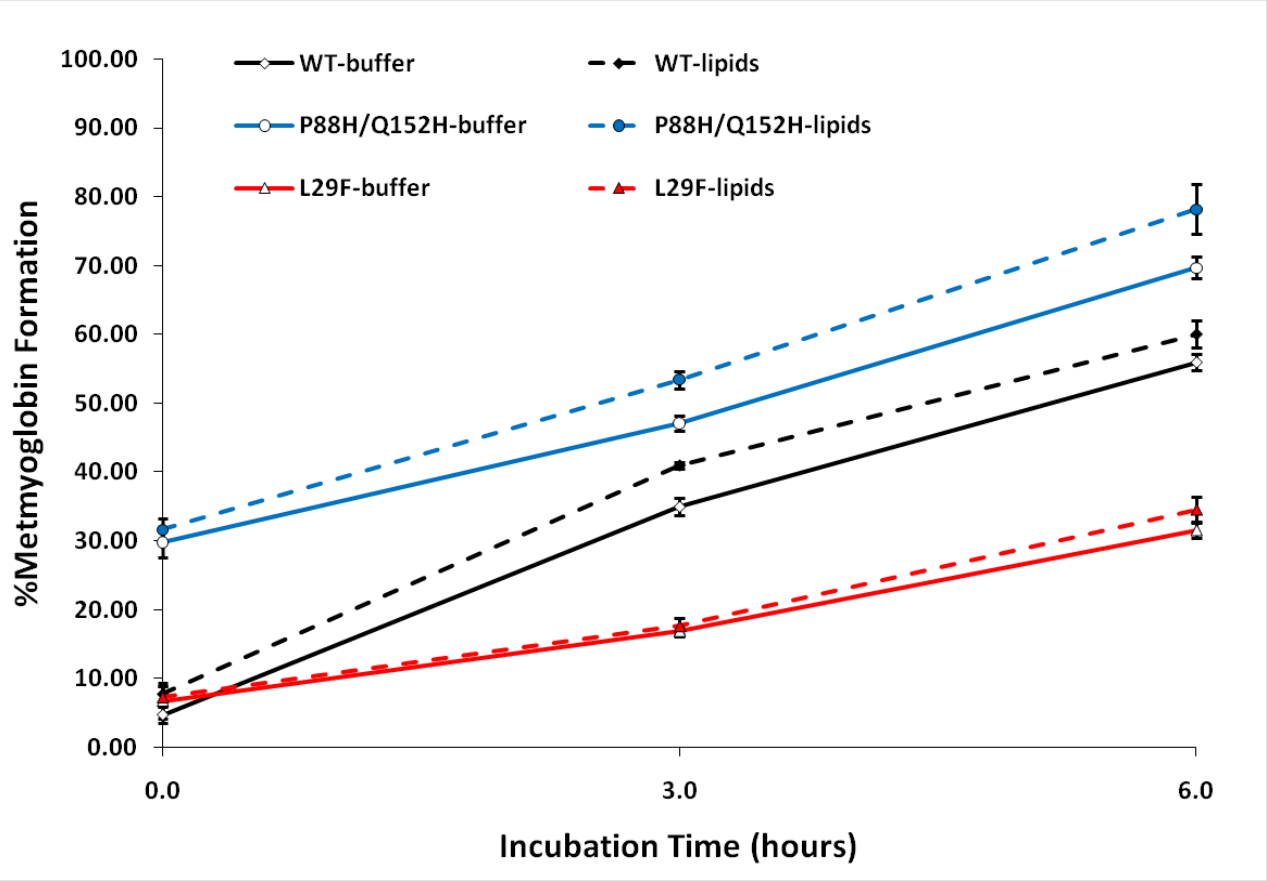

0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Absorbance at 532 nm
WT-buffer
WT-lipids
P88H/Q152H-buffer
P88H/Q152H-lipids
L29F-buffer
L29F-lipids
0.0
6.0
Incubation Time (hours)

**Figure 26**


WT-buffer
WT-lipids
P88H/Q152H-buffer
P88H/Q152H-lipids
L29F-buffer
L29F-lipids
%Metmyoglobin Formation
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.0
3.0
6.0
Incubation Time (hours)

# Chapter V

# Summary

In fresh meat, myoglobin and lipid oxidation have been shown to be interrelated in the manner that oxidation of one component exacerbates the oxidation of the other. 4-hydroxy-2-nonenal (HNE), a secondary lipid oxidation product, is highly reactive towards nucleophilic amino acid residues. HNE has been shown to accelerate OxyMb oxidation by causing conformational changes in myoglobin tertiary structure upon its alkylation solely on histidine residues via Michael addition. The different histidine numbers between bovine (13 histidine residues) and porcine (9 histidine residues) myoglobin were previously suggested to be responsible for their different susceptibilities toward HNE.

We hypothesized that intrinsic variations in the primary sequence of myoglobin, in particular the number of histidine residues, could contribute to different susceptibilities of myoglobin to covalent attachment by HNE. In order to control for the number and identity of non-histidine amino acids, a single species' myoglobin (i.e., sperm whale) was used. WT and 4 recombinant sperm whale myoglobins (P88H/Q152H, L29F, H97A, and H64F) were used in this study. Specific substitution of one or more amino acid residues can cause dramatic changes in the properties of myoglobin (e.g. redox stability, heme affinity, oxygen binding ability, thermal stability).

The intrinsic properties of the recombinant myoglobins predisposed them to HNE alkylation differently. OxyMbs of P88H/Q152H and H97A oxidized faster than WT in the presence of HNE at 4°C, pH 5.6. In contrast, redox stability of L29F OxyMb appeared to be less affected by HNE than WT. Under low oxygen partial pressure ($pO_2$ = 3 mmHg), the redox-destabilizing effect of HNE was

greater in WT than in H64F. The sites of HNE adducts on myoglobin were identified using tandem mass spectrometry. Our results revealed that histidine 48 was the most vulnerable to HNE alkylation after incubation with HNE for 72 h at 4°C, pH 5.6. This is supported by this particular histidine residue being alkylated by HNE in every recombinant myoglobin. P88H/Q152H, with its native proline 88 and glutamine 152 replaced by 2 histidine residues, became a better substrate for HNE alkylation. Both of these substituted histidines were alkylated by HNE molecules in addition to histidine 48. Two histidine residues (histidines 12 and 48) were alkylated by HNE. In H97A, HNE adducts were identified on histidines 24, 49, and 82. However, histidine 48 was the only histidine residue that was alkylated by HNE after DeoxyMbs of WT and H64F were incubated with HNE at 4°C, pH 5.6 for 24 h. These results suggested that variations in the primary sequence could result in an increased (P88H/Q152H and H97A) or decreased (L29F and H64F) redox-destabilizing effect of HNE on myoglobin. In particular, the increase in histidine number at certain locations of the primary sequence led to greater redox instability of P88H/Q152.

The ability of HNE to induce redox instability and pro-oxidative activity of myoglobin was investigated using lipid model systems. MetMbs of the recombinant myoglobins (WT, P88H/Q152H, L29F, and H97A) were incubated with HNE at 37°C, pH 5.6 for 2 h, followed by hydrosulfite-mediated reduction and aeration. The HNE-pretreated/reduced OxyMb showed greater redox instability and pro-oxidant activity than control OxyMb. ESI-mass spectrometry revealed that three HNE adducts were identified in P88H/Q152H and H97A,

while di- and mono-adducts were found in WT and L29F, respectively. When incubated with ovine liver microsomes at 25°C, pH 5.6 for 9 h, HNE-preincubated/reduced myoglobins oxidized faster than their respective controls but the extent of total oxidation was myoglobin-type dependent. The HNE-pretreated/reduced P88H/Q152H also induced TBARS formation readily. In liposome:Oxymb models, MetMb formation and TBARS formation of HNE-pretreated/reduced recombinant myoglobins followed the order: P88H/Q152H > WT > L29F. HNE alkylation enhanced OxyMb oxidation and the ability of myoglobin to promote lipid oxidation in ovine liver microsomes and liposomes.

In lipid dialysis sac experiments, OxyMbs of WT, P88H/Q152H and L29F were placed into dialysis sacs (MWCO = 500 Da) and submersed in either buffers (controls) or liposomes previously allowed to oxidize at 37°C, pH 5.6 for 5 days. Lipid oxidation products (expressed as TBARS) were able to diffuse across the dialysis membranes and interact with the OxyMbs inside. Redox instability was enhanced when OxyMbs were exposed to the oxidized liposomes compared with controls. The redox stability of P88H/Q152H was more susceptible to lipid oxidation products than the redox stability of WT. These observations implied that small lipid oxidation products (< 500 Da) were more water-soluble and able to decrease redox stability of the recombinant OxyMbs.

In conclusion, the susceptibility towards lipid oxidation products, in particular HNE, depends on the primary sequence of myoglobin. Even though the primary (and thus tertiary) structure of myoglobin is highly conserved, the amino acid sequence does vary from species to species, which can result in different

intrinsic properties (e.g. autoxidation rate, heme affinity etc.). An increase in histidine number predisposes myoglobin to greater HNE alkylation and impaired redox stability of the heme iron. Other changes in particular amino acid residues involved in the hydrophobic heme pocket can either improve or diminish heme affinity, redox stability of the heme iron and susceptibility towards HNE. HNE adducts are assumed to cause slight spatial changes of myoglobin tertiary structure, especially at the heme pocket, that result in exposure of the heme moiety to the external environment. The exposed heme tends to oxidize more readily and become a greater lipid oxidation promoter due to greater chances for interaction with membrane lipid substrates.