Meat and Muscle Biology™

Myoglobin and Hemoglobin: Discoloration, Lipid Oxidation, and Solvent Access to the Heme Pocket[a]

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Abstract: Conversion of the heme iron in myoglobin (Mb) and hemoglobin (Hb) from Fe^{2+} to Fe^{3+} is a critical step that causes quality deterioration—such as discoloration and generation of oxidative species, including dissociated heme, that oxidize lipids and proteins—in muscle foods. Increased solvent access to the heme pocket has been proposed to cause oxidation of the heme iron and decrease heme affinity for the globin, although empirical results are lacking. This review introduces plasma-induced modification of biomolecules (PLIMB) as an approach to modify amino acids of Mb and Hb and thereby assess solvent access to the heme pocket. After PLIMB, liquid chromatography tandem mass spectrometry peptide analysis and a user-friendly, software platform are used to quantify modified amino acid side chains of the heme proteins. Our findings indicate that PLIMB➔liquid chromatography-tandem mass spectrometry provides a platform to measure solvent access to portions of the heme pocket environment. Evaluation of PLIMB under additional conditions (e.g., different pH values) can differentiate the role of solvent access to the heme pocket relative to the "outer-sphere" mechanism of heme protein oxidation and the ability of hydrogen bonding to stabilize heme within metHb. Some aspects of heme protein-mediated lipid oxidation that occur at low O_2 partial pressures are discussed in relationship to solvent access to the heme pocket. Other approaches to study mechanisms of discoloration and lipid oxidation related to Mb/Hb oxidation and heme loss from metHb are also discussed.

Key words: protein footprinting, heme protein oxidation, oxidative rancidity, bovine, trout
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Introduction

There are numerous reviews that describe the various pathways by which myoglobin (Mb) and hemoglobin (Hb) undergo oxidation themselves and cause oxidation of other biomolecules (Alayash et al., 2001; Carlsen et al., 2005; Wilson and Reeder, 2022). Yet some aspects of the underlying mechanisms involved remain poorly understood. This Proceedings paper will focus on aspects of metmyoglobin (metMb) and methemoglobin (metHb) formation as well as dissociation of hemin from metHb. The heme moiety is an amphiphilic porphyrin that resides within the single "heme pocket" of Mb (Figure 1) and the 4 heme pockets of Hb, noting that Mb is a monomer and Hb is a tetramer.

The abbreviation HP, for heme protein, will be used from this point forward when referring to Mb and

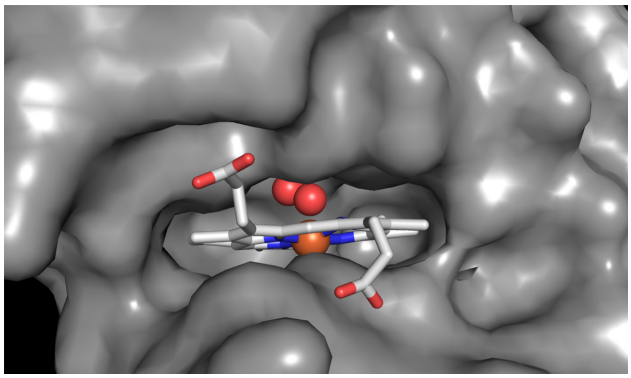

Figure 1. Space-filled (surface) view of sperm whale oxyMb illustrating the contours of the heme pocket. PDB 1MBO (Phillips, 1980) was used to prepare the Mb structure shown. Mb, myoglobin; 1MBO, file name of the structure; PDB, Protein Data Bank.

Hb collectively. The term met heme protein (metHP) indicates a one-electron oxidation of HP. OxyHP and deoxyHP contain Fe^{2+}-protoporphyrin IX, termed "heme," whereas metHP contains Fe^{3+}-protoporphyrin IX termed "hemin." There also are hypervalent ferryl forms of HP that contain Fe^{4+}-protoporphyrin IX. OxyHP and deoxyHP are described as in the reduced state, noting the reduced state of the iron atom within the heme moiety (Fe^{2+}-protoporphyrin IX).

Oxidation of reduced HP forms (e.g., oxyMb and deoxyMb) to metHP causes raw muscle to change from red to brown color, which is a sensory cue that consumers use to discard and avoid procurement of raw muscle foods. Off-odors and off-flavors from oxidative rancidity is the other major cause of quality deterioration in muscle foods. Both Mb and Hb have the potential to incur oxidative rancidity. The use of an Hb-specific inhibitor suggested that Hb was the primary oxidant in highly macerated trout muscle (Cai et al., 2013). Mb and Hb concentrations in beef, pork, poultry, and fish have been summarized, in which Mb was more abundant than Hb on a weight basis in beef, pork, turkey, and yellowfin tuna muscle whereas Hb was more abundant in muscle from chicken, trout, mackerel, and bluefin tuna (Wu et al., 2022).

During auto-oxidation of Mb (as oxyMb and deoxyMb) to metMb, 2.5 mol of O_2 was consumed per mol of metMb formed, which suggested that the hydrogen donor groups (e.g., tyrosine and tryptophan) on the protein molecule were involved (George and Stratmann, 1952b). In other words, some oxidation of tyrosine and tryptophan may occur as metMb formation takes place. MetMb formation occurs most rapidly at approximately 1.4 mm Hg partial pressure of O_2 (PO_2) and then decreases to a constant value above 30 mm Hg (George and Stratmann, 1952a). This implicates the ability of deoxyMb to react with O_2 to facilitate metMb formation as described previously (Brantley et al., 1993) because at low PO_2 there are large quantities of deoxyMb to react with available O_2. The ability of metMb formation to occur rapidly at low PO_2 is likely relevant in discoloration that occurs in incompletely vacuum-packaged raw meat. There also was increased metMb formation at low PO_2 with decreasing pH (George and Stratmann, 1954). The proton-mediated formation of metMb from oxyMb during auto-oxidation has also been described (Brantley et al., 1993). These findings encompass the difficulty in separating the relative contributions of deoxyMb-mediated metMb formation (with O_2 as a co-reactant) from those of proton-mediated oxidation of oxyMb. The proton-mediated auto-oxidation of oxyMb has been described to involve protonation of liganded O_2 within the heme pocket of oxyMb (Brantley et al., 1993). We have begun to examine access of small molecules to the heme pocket of Mb and Hb using plasma-induced modification of biomolecules (PLIMB). A schematic of the PLIMB set-up that delivers plasma to the HP solution is shown (Figure 2). The plasma generates hydroxyl radicals ($^{\bullet}OH$) that lead to covalent modification of amino acid side chains of a protein that are encountered by the free radical (Blatz et al., 2020). $^{\bullet}OH$ is similar in size to a proton (H_3O^+) so that increased modification of heme pocket residues by $^{\bullet}OH$ may affect access of protons to the heme pocket. Other approaches incorporate

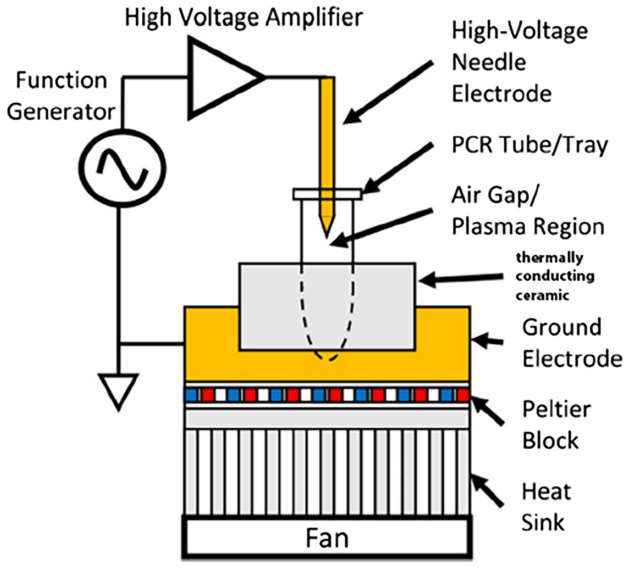

Figure 2. Illustration of set-up for PLIMB. A DBD from the plasma is used to generate hydroxyl radicals (Blatz et al., 2020). Figure shown is a modified version of a figure from Blatz et al. (2020). DBD, dielectric barrier discharge; PCR, polymerase chain reaction; PLIMB, plasma-induced modification of biomolecules.

Fe^{2+} and H_2O_2 to generate •OH which causes unwanted reactivity with the HP of interest, whereas PLIMB does not require iron and H_2O_2 to generate •OH.

Assessing Solvent Access to the Heme Pocket in Relation to MetHP Formation and Heme Dissociation

A first assessment is to measure PLIMB-induced modification of amino acids that are near the surface of the HP. Methionine residues are particularly reactive with •OH and a good surface residue to quantify. It must be confirmed that pH is maintained during PLIMB because plasma discharge in water without buffer has been found to decrease pH (Shainsky et al., 2012). Adequate buffering is needed. When investigating the mechanism of metHP formation, the time of PLIMB exposure is limited because longer exposure times will lead to elevated metHP concentration. High amounts of metHP formation due to PLIMB would obscure solvent access measurements to the reduced forms of the HP. It is necessary that the reduced forms of HP (e.g., deoxyMb and oxyMb) are mostly present during PLIMB exposures to assess the mechanism of metHP formation. Bovine Mb has a methionine residue near the surface of the molecule on the D-helix. The ability of •OH generated by plasma discharge to convert methionine to its sulfoxide has been described (Xu and Chance, 2007). The modification caused a mass shift of 16 Da. This methionine is the 55th amino acid along the polypeptide chain of bovine Mb as well as the 5th residue along the D-helix and thus can be termed Met^{55} or Met(D5). Met(D5) is preferred considering that, when different Mb and Hb are being compared, this location on the helix is more precise relative to the numbering nomenclature. For example, site D5 in bovine Hb β-chain is the 53rd amino acid of that polypeptide chain. A finding that plasma discharge results in a dose-dependent modification of the surface methionine provides confidence that the •OH abundance from PLIMB reaches a reasonable threshold to measure modifications to the accessible sites of the heme pocket. Amino acid side chains other than methionine that have elevated reactivity with •OH include tryptophan, phenylalanine, tyrosine, and histidine (Xu and Chance, 2007).

One approach that we have found to extend the time of PLIMB exposure and still maintain a limited amount of metHP formation is to manually adjust pH to 8.0 immediately after each PLIMB exposure. The increased exposure time allows for elevated concentrations of •OH to be exposed to the heme pocket and thereby better incur modifications to measure solvent access to the heme pocket. For example, up to 3 s of plasma exposure could be achieved with the pH adjustment of bovine Mb to maintain low metMb formation, whereas 1 s was the maximal exposure without pH adjustment. Ensuring that the HP undergoes minimal changes in secondary structure during PLIMB is assessed by circular dichroism (Zentz et al., 1994). Solvent access measurements with the most value in relationship to the mechanism of metHP formation require that the native structure be maintained. The mechanism by which the covalent binding of 4-hydroxy-2-nonenal to Mb increases metMb formation remains poorly understood. 4-hydroxy-2-nonenal binding to Mb may cause a change in structure that either increases solvent entering the heme crevice to facilitate the inner sphere mechanism of metMb formation (proton-mediated oxidation of oxyMb) or has little effect on solvent entering the heme crevice to favor the outer sphere mechanism of metMb formation (bimolecular reaction of deoxyMb with O_2). PLIMB represents an approach to investigate these different mechanisms of metMb formation.

PLIMB can also be utilized to examine solvent access to different metHb. Heme affinity is noted to be higher in bovine metHb compared with trout IV metHb (Aranda IV et al., 2009). Most of the amino acid residues that make up the heme pocket are identical when bovine Hb is being compared with that of trout IV (16 out of 17 residues in α-chain and 12 out of 17 in β-chain). This allows a reasonable species comparison, more so in α-chain. Released hemin from metHb readily promotes lipid oxidation by decomposition of lipid hydroperoxides to free radicals (Van der Zee et al., 1996). Hemin release from metHb and metMb variants has been described to effectively oxidize lipids (Balla et al., 1991; Chiu et al., 1996; Miller et al., 1996; Grunwald and Richards, 2006). The relationship between heme affinity and solvent access to the heme pocket is worthy of investigation, in that enhanced proton access to the heme pocket may facilitate heme release due to protonation of His(F8), which forms a pseudo-covalent bond with the heme moiety (Hargrove et al., 1996). The ability of bonding between the heme moiety and site E10 to retain heme within metHb represents an alternative pathway by which heme affinity is elevated in mammalian Hb (Aranda IV et al., 2009). Heme loss from Mb and Hb is also noted to occur more rapidly at lower pH values. Thus, measuring solvent access to the heme pocket

has the potential to elucidate the causal mechanisms by which fish metHb release their heme moiety more readily than mammalian metHb as well as the relationship between pH and loss of heme from the globins. Exposure of the metHb to the plasma for time periods that caused any detectable loss of secondary structure is to be avoided. Solvent access measurements with the most value in relationship to the mechanism of hemin release from metHP require that the native structure be maintained as much as possible. Our initial findings indicate that site CE3 and C7 were differentially modified by PLIMB in bovine metHb compared with trout IV metHb, providing insight that solvent access to portions of the heme pocket environment can be measured using PLIMB.

A key aspect of assigning modifications to specific residues of Mb and Hb involves firstly preparing peptides of each HP after PLIMB using proteolytic enzyme (trypsin/LysC). The peptides are then examined using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Minkoff et al., 2017). A user-friendly software program, Byos (Protein Metrics, Cupertino, CA) is used to identify and quantify percentage modification for a given site of Hb and Mb. Peptides and the site of modification may be determined using the software based upon tandem mass spectrometry (MS2) fragmentation (determined by a peptide spectrum match score) along with inspection of MS1 isotope plots and extracted ion chromatograms (XIC) (Kassa et al., 2021). In cases in which multiple MS2 plots are obtained, the one chosen is the one with the best combination of i) acceptable mass accuracy from the isotope plots, ii) anticipated intensity of b- and y-ions for the fragmentation type and sequence particularly for residues near the site of modification, and iii) precision regarding the place from which the MS2 was extracted based on the location of indicator dot(s) being within or near the peak in the XIC plot.

The percentage modification of a given residue (such as F in ALELFR) can be calculated by normalizing the XIC area under the curve values for peptides that contain the modified residue, along with peptides that also contain the F where that residue is unmodified. These may include fully unmodified peptides, or peptides that are otherwise modified at a different site, but still contain an unmodified F.

An example of the XIC plot (generated from one of the isotopes in the MS1 spectra) and the MS2 spectra for a selected modified peptide is shown (Figure 3), along with its corresponding wild type (or unmodified peptide). The retention time of this modified peptide differs from the corresponding unmodified peptide in the XIC plots (Figure 3). The modified peptide elutes earlier by reverse phase chromatography owing to the increased polarity of the peptide caused by the modification (e.g., hydroxylation of the phenylalanine [F] residue). In cases in which the retention time of the modified and unmodified peptide are similar, this may indicate an "in source" oxidation artifact of a wild type peptide generated in the ionization source of the mass spectrometer after elution from the LC column. Byos software identifies a peptide based on its MS2 fragmentation. The LC-MS/MS analysis software makes an initial assignment of where a modification takes place on a peptide. Then, for validation of the assignment of modification to F in the ALELFR peptide, a y2-ion or b5-ion ion peak that "shifts" in mass (m/z) relative to the native peptide provides confirmation that modification is at F since these 2 ions surround F in the peptide (Figure 3). Increased peak height for a given ion further provides confidence of correct assignment.

Some critical parameters of PLIMB that affect the quantity of hydroxyl radicals generated include height of needle spacing above the liquid surface, applied frequency, peak applied voltage, and waveshape type (Blatz et al., 2020). PLIMB more specifically involves dielectric barrier discharges. It was shown that, as the peak voltage applied was increased, the number of breakdowns in a single period increased (Blatz et al., 2020). It should be noted that additional reactive oxygen species and reactive nitrogen species may be generated by dielectric barrier discharge in air atmosphere. The possible radical species include the nitrogen dioxide radical ($^{\bullet}NO_2$), the superoxide anion ($^{-}O_2$), the superoxide anion radical ($^{\bullet-}O_2$), the neutral superoxide radical ($^{\bullet}OOH$), and hydrogen peroxide (H_2O_2) (Ryu et al., 2013).

Oxidative Potential of DeoxyMb and DeoxyHb

The ability of reduced PO_2 to accelerate metMb and metHb formation has been recognized (Brantley et al., 1993; Balagopalakrishna et al., 1996). This has been demonstrated in beef at low PO_2 (Ledward, 1970). For reduced Mb in air atmosphere, 99% has been reported to be oxyMb and the remainder deoxyMb (George and Stratmann, 1952b). For reduced Hb in air atmosphere, the oxygen affinity of Hb decreases more in fish and avians compared with mammals, especially as pH values are decreased from physiological to that of postmortem muscle (Richards

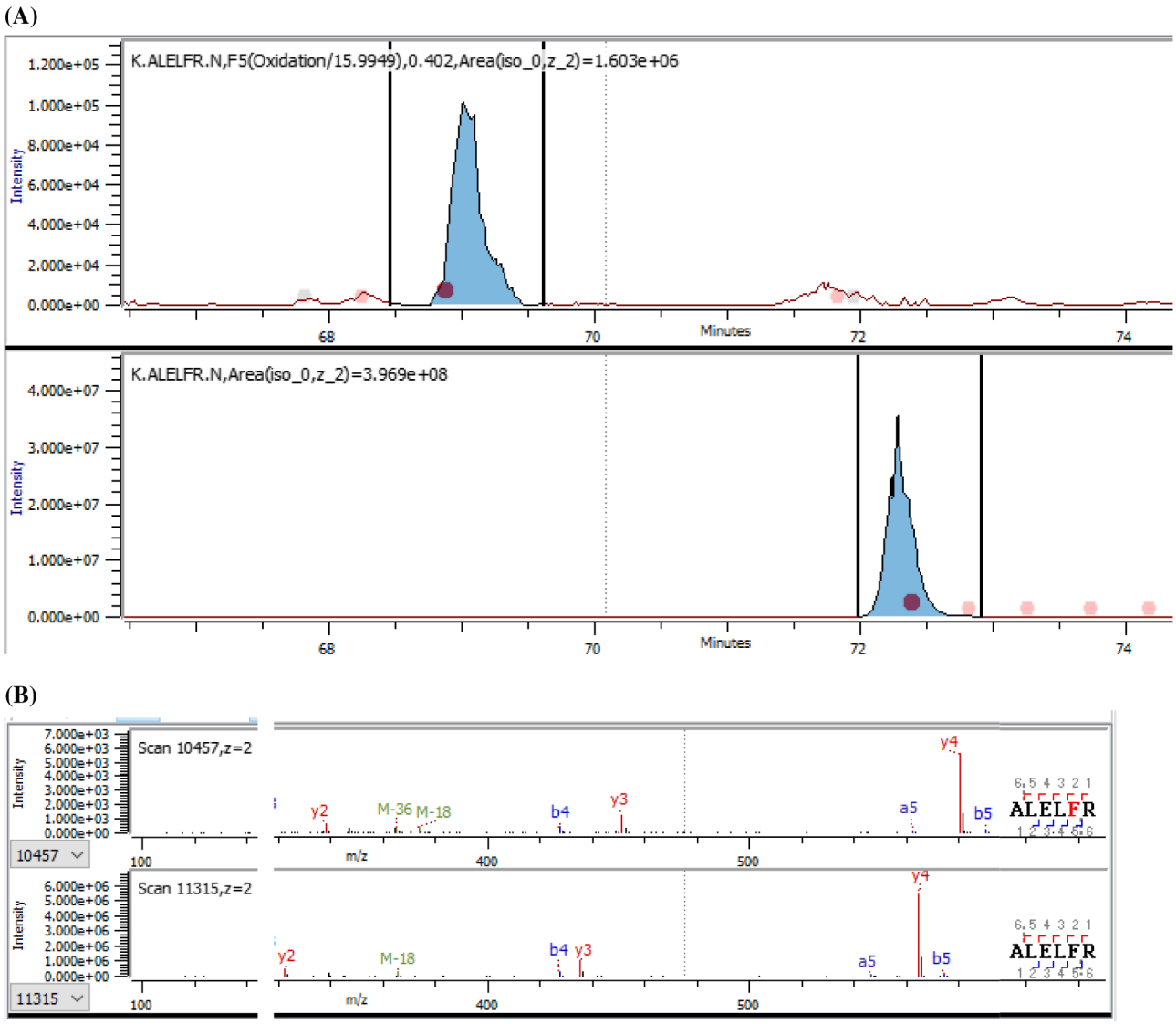

Figure 3. (A) The XIC plots for modified peptide (ALELFR) and the corresponding non-modified peptide below for bovine Mb exposed to PLIMB. (B) MS2 spectra generated from modified and unmodified ALELFR. In the spectra for the modified peptide (top) the y2 and b5 ion peaks shifts in mass (m/z) relative to the native peptide, which provides confirmation that modification is at F since these two ions surround F in the peptide. Protein Metrics software (Cupertino, CA) was used to prepare the XIC plots from the MS1 spectra and the ion plots from the MS2 spectra. Mb, myoglobin; MS, mass spectrometry; PLIMB, plasma-induced modification of biomolecules; XIC, extracted ion chromatogram.

et al., 2002). Therefore, there will be elevated deoxyHb in fish and avian Hb in even air atmosphere. There was increased metHb formation in air atmosphere at pH 7.3 when fish Hb was being compared with mammalian Hb, supporting a role for deoxyHb to facilitate metHb formation (Jensen, 2001). There also was increased metHb formation in air atmosphere at pH 5.7 and 6.3 when fish Hb were being compared with mammalian Hb (Aranda IV et al., 2009). These findings highlight the abilities of deoxyMb and deoxyHb to facilitate metMb and metHb formation, respectively. For reduced Mb, low PO_2 will be needed to have elevated amounts of deoxyMb while deoxyHb will be elevated in fish Hb even in air atmospheres, an effect that becomes more pronounced as pH decreases due to the Root effect in some fish Hb (Pelster and Weber, 1991).

There also are findings that suggest that deoxyHb facilitates lipid oxidation as follows. The ability of deoxyHb (and deoxyMb) to promote lipid oxidation is relevant in the context of low O_2 environments that exist in raw muscle tissue, particularly in certain packaging conditions. In addition, the pro-oxidative potential of the deoxygenated forms of Hb and Mb are often overlooked. The relative ability of deoxyHb, oxyHb, and metHb to promote lipid oxidation was examined

using mammalian Hb and egg liposomes as a model system (Pietrzak and Miller, 1989). Liposomes are composed of phospholipids that form spherical bilayers. It should be emphasized that phospholipids have been found to be the main class of lipid that oxidizes in raw and cooked muscle tissue relative to other lipid classes such as triacylglycerols and free fatty acids (Igene and Pearson, 1979; Igene et al., 1980; Wu and Sheldon, 1988; Gandemer and Meynier, 1995; Undeland et al., 2002). By measuring metHb formation and O_2 consumption during lipid oxidation in closed vessels, the relative ability of deoxyHb, oxyHb, and metHb to promote lipid oxidation was modeled. The O_2 consumption increases as lipid oxidation occurs and informs about the concentrations of oxyHb and deoxyHb relative to metHb, noting the known oxygen affinity of the Hb at changing PO_2. Unexpectedly, deoxyHb was shown to facilitate lipid oxidation more effectively than oxyHb and metHb using the numerical integration technique (Pietrzak and Miller, 1989). This indicates that a better understanding of deoxyHb (and possibly deoxyMb) to oxidize lipids is needed. Another study, in washed muscle, demonstrated that adenosine triphosphate (ATP) increased the ability of a cationic Hb to oxidize the phospholipids of the matrix (Richards et al., 2002). ATP lowered the ability of the Hb to bind O_2 so that the concentration of deoxyHb was increased. The ability of deoxyHb to react with O_2 and facilitate metHb formation is one oxidative property to consider as well as whether the deoxyHb is an oxidant itself. ATP likely binds Hb in a manner analogous to that of 2,3-bisphosphoglycerate to lower Hb oxygen affinity. Future work should further evaluate the ability of deoxyHb and deoxyMb to promote lipid oxidation in a role unrelated to accelerated metHP formation due to deoxyHP reactant.

Conclusions

Entry of solvated protons into the heme pocket is one mechanism by which oxyHP may be converted to metHP. Alternatively, deoxyHP can react with O_2 to form metHP by a mechanism that is considered to not require solvent access to the heme pocket. In other words, dissolved O_2 in the solvent does not need to enter the heme pocket to react with deoxyHP to form metHP. Our current studies show that PLIMB in combination with LC-MS/MS measures solvent access to some regions of the heme pocket environment of Mb and Hb. Approaches that allow for longer plasma exposure times prior to metHP formation and/or loss of native structure should allow better assessment of solvent access to the heme pocket. Varying environmental conditions, such as pH and the gas atmosphere, may better inform about the ability of PLIMB to i) differentiate the role of oxyHP from deoxyHP in relationhip to metHP formation and ii) better inform about solvent access–related aspects of heme loss from metHP. The existing literature suggests that deoxyHP may cause lipid oxidation by a mechanism other than enhanced metHP formation that occurs as the amount of deoxyHP reactant is elevated.

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