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Forum Review

The Radical and Redox Chemistry of Myoglobin and Hemoglobin: From *In Vitro* Studies to Human Pathology

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ABSTRACT

Recent research has shown that myoglobin and hemoglobin play important roles in the pathology of certain disease states, such as renal dysfunction following rhabdomyolysis and vasospasm following subarachnoid hemorrhages. These pathologies are linked to the interaction of peroxides with heme proteins to initiate oxidative reactions, including generation of powerful vasoactive molecules (the isoprostanes) from free and membrane-bound lipids. This review focuses on the peroxide-induced formation of radicals, their assignment to specific protein residues, and the pseudoperoxidase and prooxidant activities of the heme proteins. The discovery of heme to protein cross-linked forms of myoglobin and hemoglobin *in vivo*, definitive markers of the participation of these heme proteins in oxidative reactions, and the recent results from heme oxygenase knockout/knockin animal model studies, indicate that higher oxidation states (ferryl) of heme proteins and their associated radicals play a major role in the mechanisms of pathology. *Antioxid. Redox Signal*. 6, 954–966.

RESPIRATORY HEME PROTEINS AS PROOXIDANT ENZYMES

THE NORMAL FUNCTION OF MYOGLOBIN (Mb) and hemoglobin (Hb) is to bind oxygen reversibly and, it now appears, to play an important role in the metabolism and transport of nitric oxide. Under certain conditions, however, these heme proteins, particularly when in their ferric (Fe^{3+}) states, can also exhibit a "rogue" enzymatic activity and participate in a variety of redox-linked reactions, including oxidation of lipid molecules and consumption of lipid hydroperoxides. These oxidation reactions can contribute to the pathogenesis of various disease conditions, such as those following ischemia/reperfusion injuries or myolytic or hemolytic events, where the heme protein is released from its normal reductant/antioxidant-rich cellular environment. It is widely held that the major causative agent of lipid oxidation following a myolytic or hemolytic event is not the heme proteins, but rather the iron that is released as a result of heme breakdown, either from peroxide-induced heme degradation or from protease and heme oxygenase activity. "Free" iron, in the presence of reducing agents that render it ferrous, can react with hydrogen peroxide (H_2O_2) to form the highly reactive hydroxyl radical ($OH^{\bullet}$) that, in turn, can abstract a hydrogen atom from an organic molecule and initiate a radical chain reaction leading to large-scale oxidation. This reaction with peroxide is often termed Fenton chemistry and may be written thus:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (1)$$

In addition to this reaction, there is an accumulating body of evidence that suggests that the heme proteins themselves, in either their native or oxidatively modified forms, also play a central role in the mechanism of oxidative damage *in vivo* and hence to the pathology associated with many disease states.

The mechanisms through which Mb and Hb induce oxidation of lipids and the relative efficiencies of the processes have been extensively studied *in vitro*. Although respiratory heme

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proteins, in either the ferrous or ferric (met) oxidation states, cannot, in themselves, initiate lipid oxidation reactions, they can enter a higher oxidation state (the ferryl state) that is able to do this (71, 73). Peroxides are required to generate the higher oxidation states of Mb or Hb and hence "kick-start" a lipid oxidation cascade reaction (*c.f.* Fenton chemistry). Such peroxides can be generated from the autooxidation of oxyHb by dismutation of dissociated superoxide, or released from activated phagocytes. Crucially, however, unlike free iron, lipid oxidation reactions catalyzed by heme proteins are not solely dependent on H_2O_2 and can also be driven by endogenous lipid hydroperoxides (27, 31, 32, 61).

In this article, we will review the reactions of Mb and Hb with peroxides, emphasizing the roles of the ferric to ferryl transition and of the accompanying radicals that are formed when this transition is induced by peroxides. We will also review the evidence that these reactions occur *in vivo* and finally consider the part played by these reactions in pathogenesis.

THE REACTION BETWEEN RESPIRATORY HEME PROTEINS AND H_2O_2

It has been known for more than 100 years that H_2O_2 reacts with Hb in its oxidized (Fe^{3+}) state (41). The change of color, brown to red, in the methemoglobin (metHb) solution on addition of H_2O_2 can be seen by eye. It was later apparent that the change of color is caused by oxidation of the heme iron to the ferryl ($Fe^{4+} = O^{2-} \equiv [FeO]^{2+}$) state. The reaction is similar for two related heme proteins, ferric (met) Hb (metHb) and ferric (met) Mb (metMb). Whereas formation of ferryl heme requires removal of one electron from ferric heme, reduction of H_2O_2 to water requires two electrons. The second electron comes from oxidizing the protein and results, at least in part, in the formation of a free radical located on an amino acid residue of the globin (25, 39, 40) that may be detected by electron paramagnetic resonance (EPR) spectroscopy (24). This reaction may be depicted as:

$$P\text{-}Fe^{3+} + H_2O_2 \rightarrow P^{\bullet+}\ Fe^{4+} = O^{2-} + H_2O \qquad (2)$$

P denotes the site that donated an electron (the porphyrin or protein) to form the radical cation. Subsequent migration and deprotonation yield a neutral radical (often termed R$^\bullet$) observed by EPR spectroscopy (see below).

The ferrous (deoxy) protein may also react with H_2O_2 to yield the ferryl form but in this case an additional electron is not required and thus no primary radical is formed. In fact, reaction of the ferrous proteins with peroxide is difficult to achieve and the reaction rapidly becomes complex. This is because the ferryl form decays, either through a disproportionation reaction with ferrous heme or through autoreduction, to reform ferric heme that now preferentially reacts with the remaining peroxide before this is consumed.

Where precisely the neutral radical (R$^\bullet$) is located on the protein remains a matter of discussion. This uncertainty stems, in part, from the variation in the behavior that Mbs and Hbs display in their reactions with peroxide. For example, (a) the nature of the radical formed depends on the respiratory protein used, *i.e.*, Mb, Hb, the origin of species, etc.; (b) in most cases, more than one type of radical is formed; (c) the yield of the radicals varies enormously from protein to protein. A further problem relates to the total yield of radicals as observed by EPR spectroscopy. Although Eq. 2 indicates that for each ferryl heme formed a radical cation is generated, in fact, the neutral radicals observed amount to only a few percent (up to a maximum of ~20%) of this value (see also Table 1). The majority of the radicals migrate away from the heme and are either quenched through chemical processes within the protein or pass into bulk solution where termination reactions take place. Current research is directed toward determining the nature and location of the observed neutral radicals and how radicals migrate from the primary site of their formation to these residues.

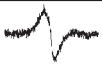
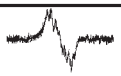
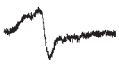
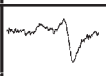
ASSIGNMENT OF THE FREE RADICALS OF Mb AND Hb

There are three ways that EPR spectroscopy has been used to study the radicals formed in heme proteins upon reaction with peroxide. The first approach involves freezing heme protein solutions at specific times after addition of H_2O_2 and recording their low-temperature EPR spectra. In the second approach, the EPR spectrum is recorded at room temperature (liquid phase) and in real time after peroxide addition. When using this direct method for detecting the free radicals, it is not always possible to record an EPR spectrum with a reasonable signal/noise ratio within the lifetime of the radical, as the radicals are usually short-lived. To overcome this problem, a third approach is sometimes used, namely spin trapping the unstable radicals. The spin trap adducts are more stable than the primary radicals, although the interpretation of the EPR spectra becomes more difficult and is not always free from ambiguities.

The first attempt to assign the radical(s) formed in the metMb/H_2O_2 and metHb/H_2O_2 systems was undertaken in 1967 (40). The EPR parameters of the protein radical(s) (*i.e.* the low-temperature EPR line width, *g*-factor, power saturation behavior, etc.) were compared with those of radicals formed in individual amino acids or short peptides. The authors postulated that the radical was first formed on a phenylalanine or a histidine residue and then rapidly transferred to a tyrosine. They also stressed that the rate of oxidation of the aromatic amino acids is greater than that of the aliphatic amino acids and is greatest for tyrosine and tryptophan (40). However, for technical reasons, the precise analysis of the EPR line shapes was not possible at that time.

We now know that when a respiratory heme protein reacts with a peroxide, two kinds of free radical are generally seen in the low-temperature EPR spectrum. One of these has a very specific $g \approx 2.03$ band characteristic of a peroxyl radical (ROO$^\bullet$). This band had been repeatedly reported in the EPR spectra for such systems since the pioneering work by Kelso-King and Winfield, although not recognized as a component of the ROO• radical (12, 39, 40, 95, 96). Kelso-King and Winfield did note, however, that "oxygenation of free radicals occurs to some extent" (12, 39, 40, 95, 96), implying interaction of the free radicals with oxygen. Interestingly, the low-temperature EPR spectra of various peroxyl radicals, with clear 2.03 bands,

TABLE 1. FREE RADICALS FORMED IN HEME PROTEINS UNDER PEROXIDE TREATMENT

Protein	Temperature	ROO• EPR spectrum	ROO• Radical concentration (μM)	Tyr radical (singlet/quintet) EPR spectrum	Tyr radical (singlet/quintet) Radical concentration (μM)	Tyr radical (septet) EPR spectrum	Tyr radical (septet) Radical concentration (μM)
HH Mb	10 K	 (25)	1.20 ROO-I	 (32)	0.10	 Unpublished data	0.70
	Room temperature	 Unpublished data	No data	Not found Unpublished data	No data	 Unpublished data	No data
SW Mb	10 K	 (25)	0.28 ROO-I	 (32)	1.57	Not found Unpublished data	<0.04
	Room temperature	Not found (31)	No data	 (31)	No data	Not found (31)	No data
HbA	10 K	 (25)	0.52 ROO-I+ROO-II	 (32)	3.38	Not found Unpublished data	<0.04
	Room temperature	Not found (32)	No data	 (32)	No data	Not found Unpublished data	No data
Lb	10 K	No data	No data	No data	No data	No data	No data
	Room temperature	Not found (34)	No data	 (34)	No data	Not found (34)	No data

When the radical concentrations are indicated (for the low-temperature experiments), both protein and peroxide initial concentrations were 100 μ*M* and the samples were frozen ~1 min after mixing. ROO•, peroxyl radical; ROO-I and ROO-II, two types of peroxyl radical.

have been appearing in many publications regularly since the 1960s (6, 7, 35, 36, 63, 75, 76, 81). It was, however, only in 1989, that the radical with the 2.03 band in a heme/peroxide system was assigned to a peroxyl radical for the first time (62) and confirmed as such more recently (80, 82).

FORMATION OF PEROXYL RADICALS

It was shown by use of $^{17}O_2$ that labeled peroxyl radicals are formed in metMb on addition of H_2O_2 (38), implying that the mechanism involves the reaction of dioxygen with a protein radical. This radical can be spin trapped in the horse heart (HH) metMb/H_2O_2 system and was shown to be centered on C3 of the indole ring of a tryptophan residue (28). The addition of the spin trap inhibited oxygen uptake in this system, indicating that the peroxyl radical seen at low temperature on reacting HH metMb with H_2O_2 was on this tryptophan residue (28). This was confirmed using wild-type recombinant sperm whale (SW) Mb ^{13}C-labeled at the C3 atom of the indole ring (16). It has been shown that Trp14 (and not Trp7, the only other tryptophan in SW and HH Mb) is responsible for formation of the peroxyl radical with O_2 attached to the C3 atom of indole ring. It has been noted that Trp14 is coplanar with the heme, whereas Trp7 is almost orthogonal, which might be the reason why Trp14 undergoes a redox reaction with heme more easily than Trp7 (16).

The formation of peroxyl radicals was also observed when human metHb was treated with H_2O_2 (82). The metHb/H_2O_2 system forms two types of peroxyl radical, ROO-I and ROO-II, with different kinetics and pH dependences of formation. The ROO-I isoform is identical to the peroxyl radical seen in the Mbs (80). The yield of ROO-I depends critically on the nature of the protein: it constitutes 81% of all radical species seen in HH metMb at 30 s after mixing with H_2O_2 (100 μ*M* heme/100 μ*M* H_2O_2, pH 7.6), whereas for SW metMb and metHb only 18 and 3%, respectively, are formed. Interestingly, the capacity of the three proteins to oxidize styrene (55) seems to correlate with this quantitative characteristic of the ROO-I radical (80), which indicates that the Trp peroxyl radical might play an important role in the mechanism of oxidation of some substrates.

The HH metMb/H_2O_2 system is characterized by the highest relative yield of the peroxyl radicals (80). This is probably why this system is the only one for which peroxyl radicals have been detected in the liquid phase at room temperature (29), when the sensitivity of the EPR spectrometer is lower. The fact that the EPR signal was still anisotropic at room temperature indicates that the Trp peroxyl radical experiences

slow molecular motion rotating with the relatively large Mb molecule and not relative to it.

THE NONPEROXYL RADICALS IN Mb

The quintet and septet signals seen in SW and HH Mbs; comparison of spectra taken in the liquid phase and at low temperature

Liquid-phase EPR detection of the radicals in real time has proven to be very instructive in determining the nature of the nonperoxyl radicals. An investigation of the liquid-phase spectra of metMb in the reaction with ethyl hydroperoxide (EtOOH) was first undertaken in 1989 (45). SW Mb, which is characterized by an extra Tyr (Tyr151) (replaced by Phe151 in HH Mb), exhibited a five-component spectrum, whereas HH Mb treated with EtOOH showed a seven-component spectrum. It was shown that K_2IrCl_6 can oxidize these proteins in their apo forms with the production of the same five- and seven-component spectra as seen in the EtOOH-treated native SW and HH Mbs (45). Tetranitromethane (TNM) treatment of proteins results in tyrosine residues being lost through nitration. Miki *et al.* (45) used TNM under conditions in which SW Mb lost one Tyr residue and HH Mb did not suffer any loss. Addition of EtOOH to TNM-treated SW metMb resulted in the formation of a seven-component EPR spectrum (the septet), typical for the HH metMb/EtOOH system and in contrast to what is observed using the native SW metMb (45). This observation provided the first hard evidence for the involvement of Tyr in the reaction of SW metMb with peroxides. The authors attributed the five-component spectrum to the Tyr151 radical. This spectrum has now been simulated using parameters that take into account the rotational conformation of the phenoxyl ring of Tyr151 as determined from the crystal structure of SW Mb (85). It has also been shown that the resolution of the components in the liquid-phase five-component spectrum is lost when the system is frozen, resulting in a singlet EPR signal in the low-temperature EPR spectra (85). A similar singlet (in addition to the seven-component spectrum), although with a much lower yield, was also detected in the HH metMb/H_2O_2 system at low temperature, indicating that a Tyr radical similar to that in SW Mb might be forming in this system (85).

Miki *et al.* speculated that the seven-component signal seen in the HH metMb/EtOOH system originates from another tyrosine, either Tyr103 or Tyr146 or a mixture of the two (45). The same seven-component spectrum was subsequently reported for the HH metMb/H_2O_2 system and also interpreted as a tyrosyl radical (13). As the signal was not observed in the iodinated HH Mb (molar ratio KI_3/metMb = 6:1), the author concluded that iodination occurred at the only tyrosine residue (Tyr103) exposed to solution and, therefore, the seven-component EPR spectrum was attributed to this site (13). This tentative assignment was later confirmed (29) by showing that the seven-component spectrum could be simulated as a spectrum of a tyrosyl radical. It was demonstrated that the EPR line shape (seven components and an overall width of ~40 G), unusual for Tyr radicals, can be simulated if the rotational conformation of the phenoxyl ring in a tyrosyl radical is such that the two methylene protons occupy practically opposite positions with respect to the ring plane. In fact, the crystal structure of HH Mb shows that Tyr103 is exactly in such a conformation (29).

LEGHEMOGLOBIN (Lb)

A free radical, detectable in the liquid phase, was also found in metleghemoglobin (metLb) immediately after mixing with H_2O_2 or with other peroxides (14). Lb is a monomeric heme protein that binds oxygen with high affinity and is found in root nodules of legumes where nitrogen fixation takes place. This protein has homology in sequence and structure with mammalian Mbs. The EPR spectrum of the radical has five components (14), as in SW Mb, but the overall line shape was different. The EPR spectrum of the SW Mb/EtOOH system was assigned to a Tyr151 radical (45, 85). Lb, however, does not possess Tyr151. Instead, it has three tyrosines at the positions 25, 30, and 132 (21) (the last of the residues is numbered 133 in reference 20). The authors attributed the radical seen in the reaction to a tyrosine (possibly Tyr132) phenoxyl radical (14). The simulation of the Lb spectrum has confirmed that, of the three tyrosine residues, only Tyr133 is in the conformation that might give rise to the experimentally observed EPR spectrum (85).

LIQUID PHASE SIGNAL IN Hb, RELEVANCE TO THE LOW-TEMPERATURE SPECTRA

The first direct detection of the radical in the liquid phase for the metHb/H_2O_2 system was reported in 1975 (77). The authors were not able to identify the nature of the radical, but pointed out that it was a slowly tumbling radical residing on a residue of metHb. A similar spectrum was reported for the metHb/H_2O_2 system (44), and although the lack of resolution of the hyperfine structure made interpretation difficult, the authors assigned this to a tyrosine-derived phenoxyl radical.

In 2002, the room-temperature EPR spectrum of the metHb/H_2O_2 system was further investigated (85). It was shown that the five-component asymmetrical EPR line shape of the radical is identical to that of the "dark" EPR signal of photosystem II, known to originate from a Tyr radical. It was also shown that the poorly resolved hyperfine structure in the liquid-phase spectrum is practically lost, when the metHb/H_2O_2 system is frozen, showing that the Tyr radical that gives a five-component spectrum at room temperature displays a singlet EPR line at low temperatures (85). This finding has important implications because it allows the free radical found in frozen blood (Fig. 1) to be assigned to a tyrosine residue of Hb (83, 84). Furthermore, it shows that *in vivo* this radical is generated by the reaction of metHb with endogenously formed H_2O_2, probably produced by autooxidation of oxyHb, forming superoxide anion that subsequently dismutates. However, an alternative assignment of the radical seen in frozen blood, namely, superoxide formed in the heme pocket, has been reported (4).

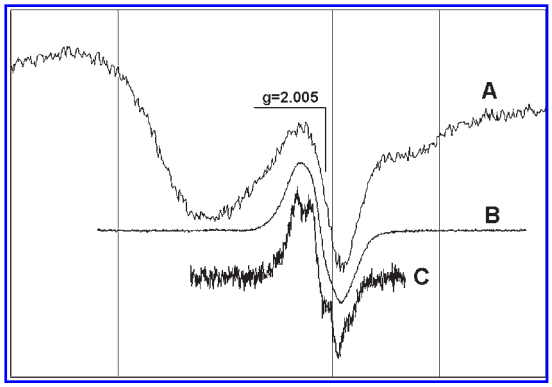


FIG. 1. The free radical EPR signals in human blood (A) and in the metHbA + H_2O_2 system, pH 7, measured at 10 K (B) and at room temperature (C). The *g* factor of tyrosyl radical is indicated. The grid lines are drawn at a 50 G interval.

SUMMARY OF THE NATURE OF THE RADICALS SEEN IN Mb AND Hb

The data reviewed above are summarized in Table 1. In this table, we see that under standard reaction conditions human Hb is characterized by the greatest total yield of free radicals. A Tyr radical that shows a singlet line in the low-temperature spectra and a five-component anisotropic signal at room temperature constitutes the major component. There are also two different peroxyl radicals in the metHb H_2O_2 system, ROO-I and ROO-II, probably both formed on Trp residues.

The EPR spectra in the SW Mb/H_2O_2 system are similar to those of Hb: they comprise a Tyr151 radical (singlet at low temperature, five-component at room temperature) and a peroxyl Trp14 radical (identical to the ROO-I isoform in Hb). It is worth noting that the line shape of the five-component signals in the liquid phase is not the same in SW Mb and Hb: both originate from Tyr radicals, but the environment and conformation of the radical in the two proteins are different.

The room-temperature EPR spectrum of the metLb/peroxide system is characterized by a five-component EPR signal, which is different from both such quintets in SW Mb and Hb. This EPR signal is attributable to Tyr133 in Lb. The low-temperature EPR spectra of the metLb/peroxide system are not available.

The HH Mb/H_2O_2 system is characterized by a Trp14 peroxyl radical (the major species) and by two Tyr radicals, with a seven-component EPR signal (from Tyr103) and with a singlet (detectable at low concentrations at low temperatures) from the only other Tyr146. The seven-component spectrum was only seen in HH Mb.

INTRA- AND INTERMOLECULAR RADICAL TRANSFER

Once formed, radicals migrate within the protein, as shown by reference to the SW/H_2O_2 system. In this system, two radicals are formed, namely, the peroxyl radical on Trp14 and a radical located on Tyr151 that exhibits a singlet EPR spectrum at low temperatures and a quintet at room temperature (see Table 1). Unexpectedly, if Tyr103 is removed by mutation (Tyr103Phe), the Tyr151 radical singlet can no longer be detected (85). This result is best explained by proposing that Tyr103 is on the pathway through which the radical migrates from its site of formation (the heme/porphyrin) to Tyr151. Removal of Tyr103 stops the radical reaching Tyr151, where it is normally found. Examination of the structure of the protein suggests that the pathway from Tyr103 to Tyr151 possibly includes Tyr146.

Other evidence for radical migration has been provided by the HH Mb/H_2O_2 system. This system is characterized by three different EPR signals: a peroxyl radical signal (Trp14), a singlet (Tyr146), and a septet (Tyr103) (see Table 1). When apoMb (heme-free protein that does not itself react with peroxide) was added to native metMb prior to H_2O_2 addition an increase in the Tyr146 radical concentration and a corresponding decrease in the Tyr103 radical concentration were observed (86). The higher the concentration of added apoprotein, the greater were these changes. These results show that there is transfer of radical from Tyr103 to Tyr146, the protein concentration dependence implying this is from Tyr103 in one Mb molecule to the Tyr146 in another. However, it is difficult to see how this mechanism accounts for such preferential and specific transfer of the radical to the Tyr146. Rather one might expect an intermolecular mechanism to transfer the radical to any one of the sites in the apoprotein that is able to act as acceptor. An alternative suggestion is that the radical transfer is intramolecular, but triggered by protein collision. In other words, collision of a Mb molecule bearing a Tyr103 radical with another Mb molecule promotes an *intramolecular* transfer of the radical site to Tyr146.

PROOXIDANT AND PSEUDOPEROXIDASE REACTIONS OF Mb AND Hb

Both the ferryl heme and protein-based radical (R$^\bullet$) can initiate lipid oxidation by abstraction of a hydrogen atom (LH, Eqs. 3 and 4).

$$P\text{-}Fe^{4+}\text{-}OH^- + LH \rightarrow P\text{-}Fe^{3+}\text{-}H_2O + L^\bullet \quad (3)$$

$$R^\bullet + LH \rightarrow RH + L^\bullet \quad (4)$$

The reactivity of the ferryl form is, however, very pH-sensitive (70), being much greater at low pH values. We have proposed, therefore, that it is the protonated form of the oxyferryl species (P-Fe^{4+}-OH^-), rather than the unprotonated form (Fe^{4+}=O^{2-}) that is the active species, noting that the protonated form may be considered equivalent to a ferric iron plus a hydroxyl (or porphyrin/protein) radical (65), *i.e.*, [P-Fe^{4+}-OH^-] ≡ [P-Fe^{3+} $OH^\bullet$] ≡ [P-Fe^{3+} H_2O + Porphyrin$^\bullet$].

The pK for the protonation of ferryl heme is very low, and we have estimated this to be around pH 3.5. However, even at pH values well above this, where the protonated form is only poorly populated, Mb and Hb retain significant pseudoperoxidase and prooxidant activities. This is because the protonated ferryl form has such high intrinsic activity that it dominates the reactivity even when present at low concentration. This view is consistent with the observed pH dependences of lipid hydroperoxide consumption by Mb (65), oxidation of low-

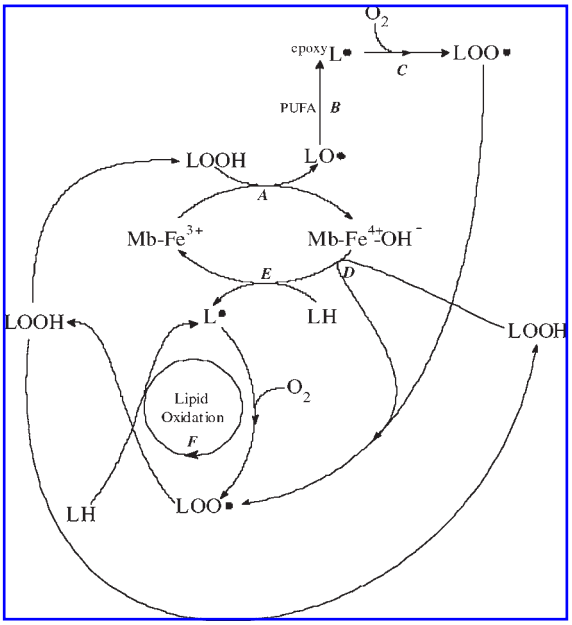
density lipoproteins (LDL) by Mb (70), and isoprostane formation from LDL by Mb (47). All these pH profiles are remarkably similar to the pH dependence of the rate constant for ferryl autoreduction, itself dependent on the protonation state of the oxyferryl form (65).

Once the protonated ferryl species has abstracted a proton from the lipid to form a lipid radical, a chain reaction is triggered. In the presence of oxygen and an absence of antioxidants, these lipid radicals can induce a cascade reaction in which an autocatalytic cycle generates further lipid oxidation products (Eqs. 5 and 6).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (5)$$

$$LH + LOO^{\bullet} \rightarrow L^{\bullet} + LOOH \quad (6)$$

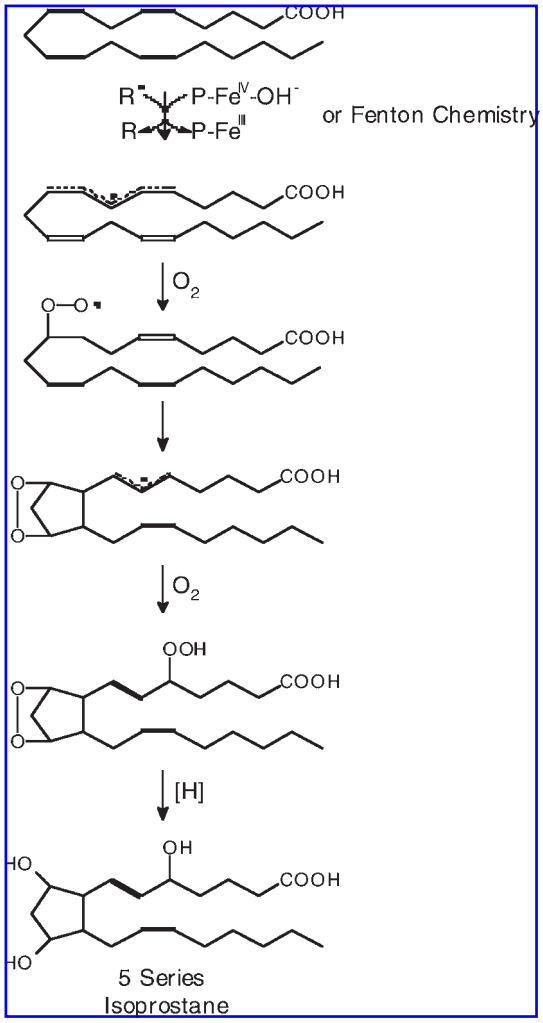
Lipid hydroperoxides that result from this cascade can reenter the reaction, oxidizing ferrous or ferric Mb/Hb in one-electron steps to generate the state once more. The overall scheme for the prooxidant and pseudoperoxidase activity of Mb is presented in Scheme 1 (64).



SCHEME 1. Prooxidant and pseudoperoxidase reactions of ferric and ferryl MB. In addition to the reactions with H_2O_2, Mb or Hb will catalyze a variety of prooxidant and pseudoperoxidase reactions with lipids and lipid hydroperoxides. Lipid hydroperoxide (LOOH) will react with ferric Mb to generate ferryl and a lipid alkoxyl radical (LO•, *A*). This alkoxyl radical, if formed near a *cis-cis* pentadiene system like that contained in polyunsaturated fatty acids (PUFA) such as arachidonate, linoleate, or docasahexenate, will rearrange to form an epoxy-alkyl radical ($^{epoxy}L^{\bullet}$, *B*) (1, 60, 94). Oxygen will then react with $^{epoxy}L^{\bullet}$ to form a lipid peroxyl radical (LOO•, *C*). Ferryl heme will react with lipid hydroperoxides to generate ferric heme and lipid peroxyl radicals (*D*) or will oxidize lipids to generate lipid alkyl radicals (L•, *E*). The lipid peroxyl radicals will react with further lipids and oxygen to form a cycle of oxidation resulting in generation of lipid hydroperoxides (*F*), as well as a variety of other compounds such as the isoprostanes.

ISOPROSTANES

Polyunsaturated fatty acid side groups of lipid membranes such as arachidonate (eicosatetraenoic acid) are particularly vulnerable to free radical-mediated oxidation due to their diene structure. The formation of a relatively stable lipid-based radical can lead to rearrangement of lipid conjugation, resulting in the formation of a variety of complex oxidation products. This includes the generation of a class of potent vasoconstrictor molecules, the F_2-isoprostanes (50). These prostaglandin-like molecules are derived from the oxidation of arachidonic acid side chains of membrane phospholipids [Scheme 2, (68)]. Although the exact mechanism of formation is unknown, both free transition metals and Mb and Hb



SCHEME 2. Formation of isoprostanes from arachidonic acid. Free transition metals and ferryl Mb or ferryl Hb (P-Fe^{IV}-OH^-) can initiate isoprostrane formation from arachidonate (5,8,11,14 all-*cis* eicosatetraenoic acid). Oxygen addition and rearrangement lead to isoprostane formation. The position of initial oxidation determines the product. The pathway to generate five series isoprostanes is shown, but 8, 12, and 15 series can also be generated depending on site of first oxygen addition (50, 68).

can mediate lipid oxidation reactions that generate isoprostanes. Isoprostanes have been found in high quantities in the urine of patients suffering from renal dysfunction as a result of muscle trauma injuries (rhabdomyolysis), as well as increased concentrations found in animal models of rhabdomyolysis (33, 34, 47, 53). Isoprostanes have been identified esterified to the tissue lipids following oxidative injury to rats, implying that isoprostanes are initially formed *in situ* with later release by phospholipases (51). Isoprostane formation has been directly implicated in the pathogenesis of acute renal failure following rhabdomyolysis (47) and delayed vasospasm following subarachnoid hemorrhage (72), as well as a variety of other disorders, including ischemic reperfusion injuries, smoking, Alzheimer's disease, Huntington disease, and selenium deficiency [for a more detailed list, see Roberts and Morrow (68)]. As a result of this research, the formation of isoprostanes is now used as the most reliable index of oxidative stress *in vivo* (68).

Recently, oxidation products similar to isoprostanes have been identified in the brain. These "neuroprostanes" are derived from the oxidation of docosahexenoic acid, a polyunsaturated phospholipid side chain commonly found in the brain (69). Like isoprostanes, the formation of neuroprostanes is thought to be generated by a free radical mechanism of lipid oxidation. Neuroprostanes and neuroketals, other lipid oxidation products of the same oxidation pathway, have been linked to Alzheimer's disease and other neurodegenerative diseases (8, 9, 15). However, the precise mechanism of their formation, and any possible link to heme proteins or free transition metals, have yet to be established.

HEME TO PROTEIN CROSS-LINKED Mb AND Hb

Providing evidence that heme proteins are involved in oxidative reactions *in vivo* presents considerable difficulties as the ferryl oxidation state is transient and thus difficult to identify and measure quantitatively. Nevertheless, ferryl Mb has been identified in isolated ischemic rat hearts using addition of sodium sulfide (2). Sodium sulfide reacts with ferryl heme to give a distinctive spectral band with absorbance maximum at 617 nm (26, 92). However, the sulfur–heme complex is not particularly stable, and the toxicity of sulfides makes this method unsuitable for identifying ferryl heme proteins *in vivo*.

An alternative stable and definitive marker for the previous presence of the ferryl oxidation state is provided by heme to protein cross-linked Mb (Mb-X) and Hb (Hb-X), in which a covalent bond has been formed between the heme and the globin moieties (11). This covalent cross-link is generated when the protonated ferryl heme in Mb or Hb reacts with the protein-based radical that accompanies its formation (Eq. 2) before this has the opportunity to migrate away from the vicinity of the heme (see Fig. 2) (67). The Hb-X or Mb-X is very stable and possesses optical characteristics that distin-

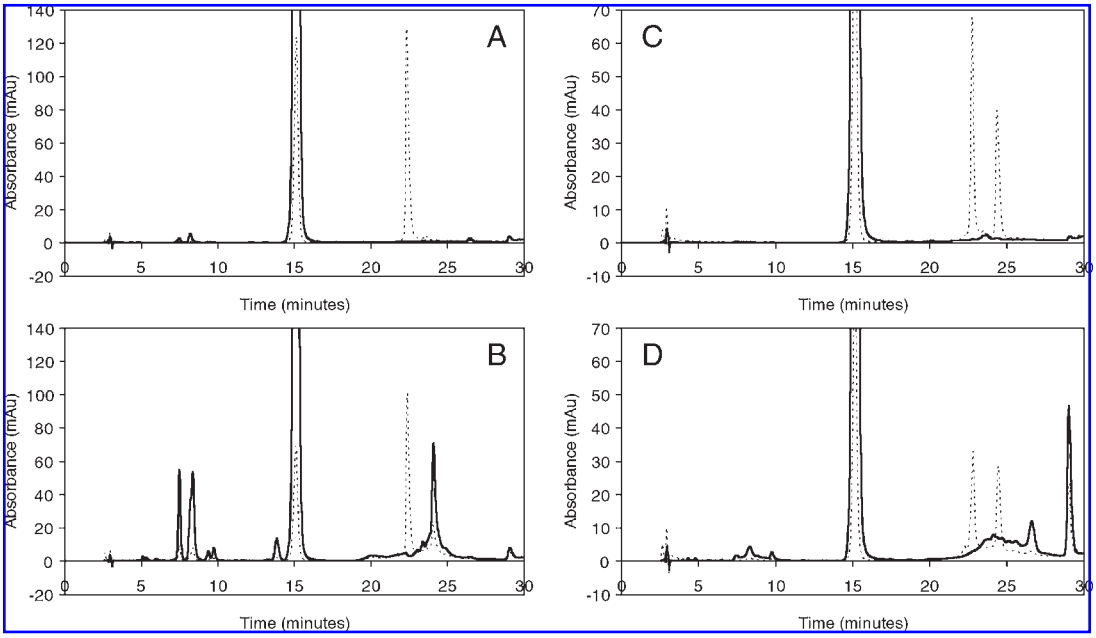


FIG. 2. Reverse-phase HPLC of HH Mb and human Hb before and after the reaction with H_2O_2 Ferric Mb (100 μ*M*, **A**) was analyzed on a Zorbax 300SB C3 column using solvents acidified with 0.1% trifluoroacetic acid. Absorbances (mAu) were monitored at 400 nm (———) and 280 nm (-------). Under these conditions, the heme and protein components elute separately, with heme eluting at 15.2 min and apoMb at 22.3 min. Following the reaction with H_2O_2 [(300 μ*M* at pH 5.0 in 25 m*M* sodium acetate + 25 μ*M* diethylenetriaminepentaacetic acid (DTPA)], Mb exhibits the presence of damaged heme groups eluting between 5 and 14.5 min, as well as heme to protein cross-linked forms eluting between 19.5 and 30 mins **(B)**. Substituting Hb for Mb produces similar results with apoHb separating into its two subunits, β eluting at 22.8 min and α eluting at 24.4 min **(C)**. Heme to protein cross-linked Hb elutes between 21 and 30 min **(D)**.

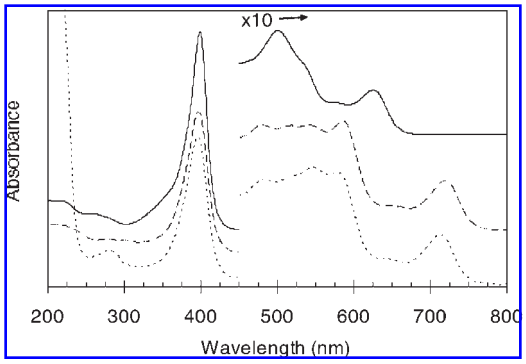


FIG. 3. Spectral properties of unmodified heme (upper, solid line), oxidatively modified 'free' heme (middle, dashed line), and heme protein cross-linked species (lower, dotted line). Spectra were taken from the chromatogram in Fig. 2B and offset for clarity. The upper spectrum is of the unmodified heme (15.2-min component); the middle spectrum is from the 8.4-min component and is of an oxidatively modified "free" heme, the structure of which has been identified (79). The lower spectrum is of heme to protein cross-linked Mb (23.6-min component) and is virtually identical to the spectrum of oxidatively modified free heme plus a protein component.

guish it from the native protein (Fig. 3) (67). As such, these cross-linked proteins are definitive markers for the previous presence of the high, ferryl, oxidation state and hence a marker of previous history of the oxidative activity of these heme proteins *in vivo* (34, 66). The presence of the cross-linked form of Mb and Hb, predicted to occur as a by-product of the involvement of these heme proteins in oxidative reactions, has recently been identified in a number of disease states, implicating Mb and Hb in the mechanisms of their pathology.

The study of oxidatively modified heme proteins including the cross-linked forms has a long history. In the early 1950s, George and Irvine described the changes in the optical characteristics of ferric Mb in the presence of peroxides *in vitro* (22). In the absence of reducing agents and at high pH values a transient, but relatively stable [$t_{½} \approx 15$ h at pH 7.4 increasing to $t_{½} > 960$ h at pH 10 (65)] red species formed and was suspected to be (and later confirmed as) the ferryl oxidation state ($[Fe^{4+} = O^{2-}]^{2+}$) (23, 39). At low values of pH (pH ~5), the ferryl oxidation state is unstable and highly reactive and rapidly decays [$t_{½} \approx 3$ min (65)]. Under these conditions, however, a stable green species forms, and this was interpreted by George and Irvine to be the result of oxidative attack on the porphyrin ring inducing chemical modification, but without ring fission. In 1974, Fox *et al.* noted that this green species could not be removed from the protein by standard acid–solvent extraction (19). This method disrupts the iron-histidine bond and unfolds the protein, allowing the extraction of the hydrophobic heme group into a solvent such as acetone or butanone (19). This implied that, in the modified green species, the heme was covalently bound to the protein, a hypothesis that was later confirmed (11).

A reverse-phase HPLC technique has been developed by Osawa and Korzekwa to identify and quantify more easily Mb-X formed *in vitro* on peroxide addition to Mb (56). We have further developed this HPLC technique and shown in Fig. 2 a set of typical chromatograms. Figure 2A and C shows the chromatograms obtained from reverse-phase HPLC of native Mb and Hb, respectively. These were monitored at 280 nm, where both heme and protein absorb, and at 400 nm, where only heme absorbs. Due to the acid nature of the HPLC solvents (pH ~2), the heme and protein components elute separately. In this figure, heme (heme B) elutes at 15.2 min for both Mb and Hb, whereas the protein components elute as apoproteins at 22.3 min for apoMb and 22.8 and 24.4 min for the apoHb β subunit and α subunit, respectively. After incubation with three molar equivalents of H_2O_2 at pH 5.0 (Fig. 2B for Mb, Fig. 2D for Hb), both Mb and Hb undergo extensive oxidative changes. Both show considerably decreased amounts of unmodified heme (15.2 min) in conjunction with the formation of damaged hemes that are not covalently bound to the protein (eluting between 5 and 14.5 min). Sugiyama *et al*. showed that two of these oxidatively modified hemes have altered optical properties due to disruption of the conjugation of one of the pyrrole rings (79). This is similar in structure to the "chlorin" hemes of *d*-type cytochromes (*e.g., E. coli* cytochrome *bd* oxidase), giving the heme a green color. The spectral properties of Mb-X (Fig. 3) are virtually identical to the spectral properties of these *d* type hemes (plus a protein component), suggesting a similar modification to the pyrrole ring conjugation (67). However, no such spectral perturbation is predicted from the structure proposed by Catalano *et al.* (11).

Formation of the heme to protein cross-linked species requires the participation of both the ferryl heme and the initial protein-based radical (11). However the pH dependence of the yield of the cross-linked form indicates that it is the protonated ferryl form that is the active species (67). The pH dependence of Mb-X formation is remarkably similar to the pH dependence of both the prooxidant and pseudoperoxidase activities of Mb (as discussed above). This suggests that heme to protein cross-linking is linked to the same underlying mechanism as the enhanced prooxidant and pseudoperoxidase activities, *i.e.*, protonation of the oxyferryl heme.

The amino acid that is proposed to link covalently to the heme (Tyr103) has yet to be confirmed in this role. Preliminary experiments conducted in our laboratory with tyrosine mutants of recombinant SW Mb show that this tyrosine is not necessary for heme to protein cross-linking. Whether Tyr103 is the residue that cross-links to the heme in the wild-type protein is not presently known; what is clear is that another residue may fulfill this role when Tyr103 is removed.

One property of Mb-X that is biologically significant is that it is markedly more prooxidant than the native protein, oxidizing human fibroblast cells and LDL up to five times more rapidly (57, 58, 91). This is illustrated in Fig. 4, where a liposome model membrane is oxidized by heme to protein cross-linked Mb-X much more rapidly than native Mb. This enhanced toxicity may play an important role in the initiation of the severe complications that can arise in certain disease states, a role that native Mb, because it is a poorer oxidant may not be able to play so effectively. The prooxidant activity of Hb is significantly greater than that of Mb, approaching that of Mb-X, but no additional increase in this activity is observed on formation of Hb-X.

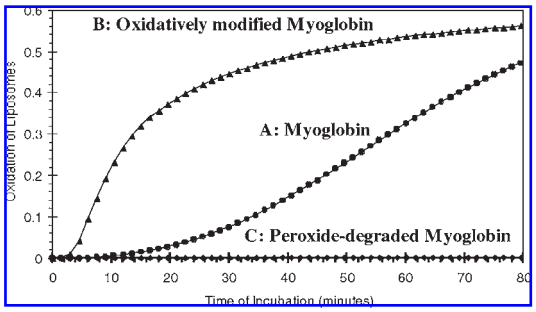


FIG. 4. Peroxide-induced modifications to Mb enhances its ability to oxidize liposomes. Three samples of ferric Mb (100 μ*M*) were reacted with H_2O_2 (0 μ*M*, 300 μ*M*, and 10,000 μ*M*) in 25 m*M* sodium acetate buffer, pH 5. Remaining peroxide was removed with catalase (10 n*M*) before aliquots of each Mb sample was diluted to 1 μ*M* in 25 m*M* sodium phosphate, pH 7.4, and 25 μ*M* DTPA and reacted with lethicin liposomes (200 μg/ml). Oxidation of liposomes was monitored optically at 234 nm for increases in lipid conjugation [$\epsilon_{234\,nm} = 2.5 \times 10^4$ $M^{-1}cm^{-1}$ (18)]. The Mb without peroxide incubation (A, ●) induced liposome oxidation after a lag period of ~20 min. The Mb that was prereacted with 3× excess peroxide contained ~25% of the heme to protein cross-linked form and oxidized the liposomes faster and with a lower lag period (B, ▲). Peroxide-degraded Mb (C, ♦) showed no appreciable liposome oxidation. The presence of DTPA in all samples prevented oxidation of liposomes by free iron.

HEME PROTEINS AND OXIDATIVE STRESS IN DISEASES

Although the catalytic activity of Mb and Hb in oxidizing substrates has long been demonstrated *in vitro* and *ex vivo*, finding evidence that these reactions occur *in vivo* has proven difficult. Mb-X and Hb-X, however, provide a specific marker for the previous presence of the ferryl state of the heme protein. Recently, Mb-X and Hb-X have been identified in two disease states. Although this finding does not formally prove that these heme proteins are the major cause of oxidative stress in these conditions, it does, in our view, strongly implicate them in the mechanism of pathology.

Rhabdomyolysis is the term used to describe the breakdown of striated muscle and can be caused by a variety of insults, including crush injury, alcohol and drug abuse, hypothermia and hyperthermia, and strenuous exercise. Following muscle damage, Mb is found in the kidney renal tubules, associated with tubular necrosis and intense renal vasoconstriction (3, 42, 89). The release of free Hb or Mb into the bloodstream can lead to the scavenging of nitric oxide, an endogenous vasodilator, causing elevation of arterial pressure (5, 93). High levels of isoprostanes are found in the kidney and urine, indicating a possible mechanism of pathology involving lipid oxidation (47, 87). The high levels of isoprostanes cause vasoconstriction and hence oxygen depletion and acidosis, ultimately leading to acute renal failure. Rhabdomyolysis accounts for 7% of cases of acute renal failure in the U.S. (10). The appearance of oxidized lipids in the form of isoprostanes is accompanied by the presence of high concentrations of Mb released from the muscle and filtered through the kidney. The question arises whether the isoprostanes are formed by the catalytic action of Mb. Although this is difficult to answer, what can be stated unambiguously is that the Mb, post filtration, reacts with peroxides and is oxidized to the ferryl state, and thus is capable of forming the isoprostanes from lipid. One can be certain that Mb has entered the ferryl state because within the urine is found a high concentration of Mb-X. Figure 5A illustrates this finding.

Bleeding into the cerebrospinal fluid from a brain hemorrhage, such as caused by trauma, aneurysm, or alveolar malformations, leads to Hb contamination of the cerebrospinal fluid. Such a brain hemorrhage is life-threatening with a 12% mortality rate from the initial hemorrhage before hospitalization (74). The mortality rate 1 month from a diagnosed hemorrhage is ~40–45%, with the initial hemorrhage and subsequent rehemorrhaging being a major source of mortality (17). However, about one third of patients that survive to receive treatment, usually in the form of surgical repair of the damaged vessels, often develop delayed vasospasm causing ischemia. The mechanisms behind delayed vasospasm in patients suffering from subarachnoid hemorrhage have been recently reexamined. It is now believed free radical-mediated isoprostane formation is an important factor in the development of vasospasm (54, 72). This being the case, it is of signifi-

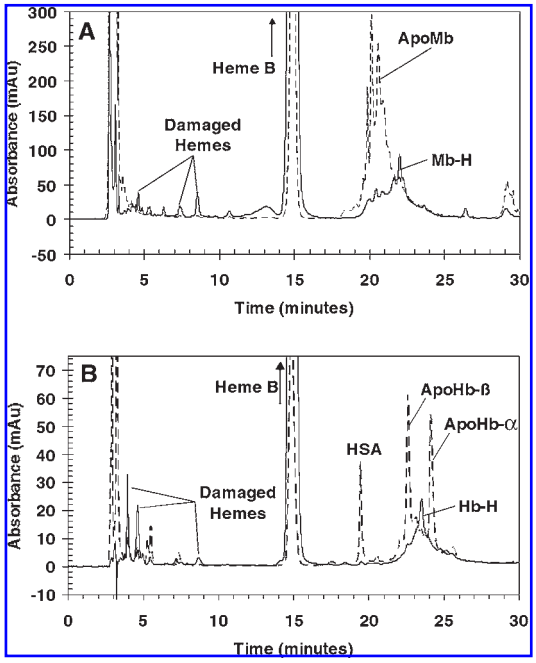


FIG. 5. Reverse-phase HPLC of heme to protein cross-linked heme proteins in rat urine following rhabdomyolysis (A) and in human cerebrospinal fluid following a subarachnoid hemorrhage (B). Urine from a rat following glycerol-induced rhabdomyolysis (A) or cerebrospinal fluid from human patients following an aneurysmal subarachnoid hemorrhage (B, taken from reference 63) were analyzed by reverse-phase HPLC as described in Fig. 2. Absorbances (mAu) were monitored at 400 nm (———) and 280nm (-------). In both cases, unmodified and oxidatively modified hemes are present, as well as the apoproteins Mb and Hb α and β subunits. Mb-X (A) and Hb-X (B) provide definitive evidence that these heme proteins have been involved in oxidative reactions *in vivo*. HSA, human serum albumin.

cance that the Hb recovered from the spinal fluid contains a large fraction that is in the form of Hb-X (see Fig. 5B). This provides unequivocal evidence that the Hb in this compartment had passed through the ferryl state and thus possibly caused the lipid oxidation and isoprostane formation.

Both heme proteins and free transition metals are able to initiate lipid oxidation reactions *in vivo*. The importance of free iron in many disease conditions, such as iron overload resulting from treatment of thalassemia, is well reported. There is confusion, however, as to the precise mechanism of oxidative damage when heme proteins are present. Free iron can be derived from the heme proteins themselves, especially from the ferrous form of the protein (52). As such, the relative importance of heme protein versus free iron in the pathogenesis of many diseases following hemolytic or myolytic events is currently under debate. There is accumulating evidence that the heme proteins are a major driving force for lipid oxidation reactions, particularly following rhabdomyolysis and subarachnoid hemorrhaging. Data showing that iron chelators such as desferrioxamine ameliorate such conditions do not generally take into consideration the diverse properties of such iron chelators, such as radical scavenging (30, 37, 48), inhibition of peroxidases (48), and their ability to act as reducing agents (46, 78, 88). These are all properties that can impact on oxidative stress independent of their capacity as iron chelators. It has been reported that the ferric, but not ferrous, Hb releases its heme to be incorporated into endothelial cells, intensifying oxidant injury (90). Injured endothelial cells respond by induction of the heme-degrading enzyme heme oxygenase, releasing free iron, bilirubin, and carbon monoxide. Bilirubin is an antioxidant (43), and carbon monoxide has reported vasodilator and cytoprotective properties (49, 59). Ferritin, increased synthesis of which normally accompanies heme oxygenase expression, sequesters the prooxidant free iron, thus detoxifying it.

The study of knockout heme oxygenase mice given a myolytic shock shows that these are unable easily to survive rhabdomyolysis (53), unlike their wild-type counterparts. Urinary levels of plasma creatinine, used as a marker of renal function, increased in both knock-out and wild-type mice following induction of rhabdomyolysis. However, although the creatinine levels recovered over a period of a few days in wild-type mice, the knockout mice exhibited ever increasing levels until death intervened. Consistent effects are observed in heme oxygenase knockin rats with subarachnoid hemorrhage. These rats are able to cope much better than wild-type rats with the influx of heme protein into the spinal fluid (54). As heme oxygenase metabolizes heme, releasing free iron, these studies imply that heme proteins are active agents for lipid oxidation in both rhabdomyolysis and subarachnoid hemorrhage.

The heme oxygenase knockin and knockout studies, together with the presence of Mb-X and Hb-X *in vivo*, suggest that respiratory heme proteins can play an important role in the pathology of complications following rhabdomyolysis and subarachnoid hemorrhage. The ability of these heme proteins to generate isoprostanes leads to vasoconstriction and oxygen depletion, resulting in acidosis. This lowering of pH enhances the prooxidant and pseudoperoxidase activities of the native and oxidatively modified forms of Mb and Hb, leading to increased isoprostane generation and hence forming a vicious cycle of oxidative damage. Indeed, one treatment for renal dysfunction following rhabdomyolysis is to raise the pH of the blood/urine (10, 47, 97). It is likely that these oxidative processes prevail whenever a heme protein is isolated from its normal reductant/antioxidant-rich cellular environment. This is a major consideration for the development of cell-free Hb as blood substitutes.

Although we believe the evidence supports the proposition that respiratory heme proteins act to catalyze lipid oxidation this way, that is not so say that free iron is unimportant in this context. It is often difficult to distinguish the roles played by free iron and by heme as degradation of the latter, which always accompanies its oxidative reactions, gives rise to the former. In this review, we draw attention to the sometimes neglected prooxidant and pseudoperoxidase activities of respiratory heme proteins while remaining aware of the prooxidant effects of free iron.

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ABBREVIATIONS

DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; EtOOH, ethyl hydroperoxide; Hb, hemoglobin; Hb-X, heme to protein cross-linked form of Hb; HH, horse heart; H_2O_2, hydrogen peroxide; Lb, leghemoglobin; LDL, low density lipoproteins; Mb, myoglobin; Mb-X, heme to protein cross-linked form of Mb; metHb, methemoglobin; metLb, metleghemoglobin; metMb, metmyoglobin; SW, sperm whale; TNM, tetranitromethane.

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