Characteristics and Mechanism of Formation of Peroxide-Induced Heme to Protein Cross-Linking in Myoglobin†

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ABSTRACT: At acidic pH values heme−protein cross-linked myoglobin (Mb−H) forms as a product of a peroxide-induced ferric−ferryl redox cycle. There is evidence that this molecule acts as a marker for heme−protein-induced oxidative stress in vivo and may exacerbate the severity of oxidative damage due to its enhanced prooxidant and pseudoperoxidatic activities. Therefore, an understanding of its properties and mechanism of formation may be important in understanding the association between heme−proteins and oxidative stress. Although the mechanism of formation of heme−protein cross-linked myoglobin is thought to involve a protein radical (possibly a tyrosine) and the ferryl heme, we show that this hypothesis needs revising. We provide evidence that in addition to a protein-based radical the *protonated* form of the oxoferryl heme, known to be highly reactive and radical-like in nature, is required to initiate cross-linking. This revised mechanism involves radical/radical termination rather than attack of a single radical onto the porphyrin ring. This proposal better explains the pH dependence of cross-linking and may, in part, explain the therapeutic effectiveness of increasing the pH on myoglobin-induced oxidative stress, e.g., therapy for rhabdomyolysis-associated renal dysfunction.

Recently, interest has grown regarding the implications for the formation, in vivo, of the heme−protein cross-link species (Mb−H)[1] that results from the reaction of myoglobin with peroxides. Early studies of the reaction between Mb and H_2O_2 (*1*−*6*) showed that this resulted in the production of two derivatives of myoglobin, identifiable from visual inspection by their distinctive colors. A red species is transiently formed from metMb and H_2O_2 at high pH values. This red species was interpreted to be the ferryl oxidation state of Mb ($[Fe^{4+}{=}O^{2-}]^{2+}$) (*1*), an assignment later confirmed (*5*). When metMb reacts with H_2O_2 at acidic pH values, a stable green species is formed. This green compound was tentatively identified as a species formed from substitution on the porphyrin ring without ring fission (*1*). However, the specific nature of the structures were unknown. Fox et al. noted in 1974 that a proportion of the peroxide-induced green myoglobin species was resistant to acid−solvent extraction, a method that removes the heme group from the protein by disrupting the iron−histidine ligation. Therefore, the green species was identified as a heme group covalently bound to the protein (*7*). It was not until Catalano et al. examined the green compound in 1989 that this covalent association was confirmed (*8*). The mechanism of formation of this heme−protein cross-linked species (Mb−H) was conjectured to involve the second oxidizing equivalent of hydrogen peroxide forming, among other things, a tyrosine radical that reacts with the heme periphery to form a covalent bond. This form of cross-linking is specific to peroxide-induced ferric−ferryl redox cycling and may be important in the mechanisms of oxidative stress in vivo. Cross-linking the heme to the protein can also be induced by $BrCCl_3$ but, in this case, covalently linking the heme via the proximal histidine (*9*−*11*).

Formation of Mb−H is a specific marker for peroxide interaction with Mb as it is stable both in vitro (*7*, *8*) and in vivo (*12*); it is, therefore, a potentially useful marker for selective oxidation processes. Recent studies of rhabdomyolysis using an animal model have identified Mb−H excreted in the urine of the animals (rats) used (*13*). Human patients suffering from rhabdomyolysis also excrete measurable concentrations of Mb−H in their urine (*12*). Significantly, Mb−H itself is reported to possess potent peroxidative activity. Investigations into the toxicological effects of Mb−H by Osawa and co-workers have shown Mb−H to be a far more powerful oxidant than native Mb. Mb−H can increase NADH oxidation 32-fold (*14*), increase human fibroblast cell death 5-fold (*15*), and enhance LDL oxidation (measured by formation of thiobarbituric acid reactive substances, TBARS) 5-fold (*16*) with respect to native Mb.

The accumulated evidence points to Mb−H being a potentially important species, a study of which may advance understanding of the nature of oxidative stress involving respiratory heme−protein. Despite this, the experimental data regarding the properties of Mb−H are limited. The mechanism of formation described by Catalano et al. is, as the authors state, "not unambiguous"; e.g., the pH profile of the extent of Mb−H formation remains unexplained. Further-

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[1] Abbreviations: Mb, myoglobin; Mb−H, heme to protein cross-linked myoglobin.

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more, its prooxidant activity in comparison to that of native Mb may be important in understanding the mechanisms involved in conditions such as rhabdomyolysis and the pathophysiology of other oxidative stress disorders. We have, therefore, conducted a series of in vitro experiments to determine the characteristics of this heme−protein cross-linked species and to provide a clearer understanding of the nature of this molecule and the mechanism of its production.

MATERIALS AND METHODS

Equine myoglobin and 30% hydrogen peroxide were purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, U.K.). Butanone, HPLC grade acetonitrile, was purchased from BDH (Poole, Dorset, U.K.), and trifluoroacetic acid was from Fischer (Loughborough, Leicestershire, U.K.).

Metmyoglobin and oxymyoglobin were prepared as previously described (*17*) and used on the day of preparation.

Reverse-Phase HPLC. The reverse-phase HPLC was adapted from the method by Osawa and Korzekwa (*14*). Samples were analyzed on an Agilent HP1100 HPLC fitted with a diode array spectrophotometer. The column used was a Zorbax StableBond 300 C3 250 mm × 4.6 mm fitted with a 12 mm × 4.6 mm guard column. Solvents were (A) 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA. The gradient was initially 35% solvent B, stable for 10 min, increasing to 37% solvent B over 5 min. This was increased to 40% solvent B over 1 min and then to 43% solvent B over 10 min. The flow rate was 1 mL min^{-1}, and the temperature was 25 °C.

Acid−Butanone Extraction of Heme−Protein Cross-Linked Species. Noncovalently bound heme was removed from the globin by a method adapted from Catalano et al. (*8*). MetMb (500 μM unless otherwise stated) was reacted with H_2O_2 (3 times molar excess) in 0.1 M sodium acetate buffer, pH 5.0, containing 100 μM DTPA. After 1 h the sample was acidified to around pH 2 with 5 M HCl. Butanone (approximately 0.75 volume) was added and the solution vortex mixed. The sample was centrifuged for 30 s at 2000*g* and the organic layer containing noncovalently bound heme removed. The butanone extraction step was repeated until the butanone layer was colorless. The heme−protein cross-linked/apomyoglobin mixture was dialyzed against deionized water for 18 h with two changes of water. The mixture was then dialyzed with 1 mM sodium acetate buffer, pH 5, for a further 2 h. The concentration of Mb−H was determined by reverse-phase HPLC by integrating the area beneath the chromatogram measured at 400 nm identified as the Mb−H component. This area was converted to concentration by a comparison with a standard sample of Mb−H, the concentration of which was previously measured spectroscopically using $\epsilon_{408nm} = 76$ mM^{-1} cm^{-1} (*8*), taking into account light scattering caused by contaminating apomyoglobin.

HPLC Injector Program. An HPLC injector program was used to examine the initial time course of formation of oxidatively modified myoglobin. MetMb (7.9 μL, 316 μM, 100 μM final) was drawn into the HPLC injector module after 1 μL of buffer (25 mM sodium acetate, pH 5.0, containing 100 μM DTPA). The injector needle was washed, and a further 13.1 μL of buffer was drawn to the injector. The injector needle was washed again, and H_2O_2 (2.0 μL, 6.25 mM, 500 μM final) was added to the injector module. After a final wash 1 μL buffer was added to give a total injector volume of 25 μL. The sample in the injector sample loop was mixed twice and injected onto the HPLC column after a programmed period of time.

Electron Paramagnetic Resonance Measurements. The electron paramagnetic resonance (EPR) spectra were recorded using a Bruker EMX EPR spectrometer equipped with an Oxford Instruments liquid helium system and a spherical high-quality Bruker resonator SP970. Wilmad SQ EPR tubes were used for EPR samples. Tubes containing myoglobin solutions or water (blanks) were quickly frozen in methanol kept on dry ice. Once frozen, samples were transferred to liquid nitrogen where they were stored prior to making measurements. The EPR spectra were measured at 10 K, modulation frequency 100 kHz, modulation amplitude 4 G, microwave frequency 9.468 GHz, microwave power 3.18 mW, spectra sweep rate 24 G/s, and time constant 82 ms. The EPR spectra of the blank samples (frozen water) were measured and subtracted from the EPR spectra of the protein samples to eliminate the baseline caused by the resonator's walls, quartz insert, or the EPR quartz tube. The *g*-values were obtained using the built-in microwave frequency counter and a 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder standard, the *g*-value for which is $g = 2.0037 \pm 0.0002$ (*18*).

RESULTS AND DISCUSSION

Isolation of the Cross-Linked Heme Species. It is difficult to follow the formation of Mb−H by optical spectroscopy due to interference from other oxidatively modified hemes that are not covalently bound to the protein, yet have very similar spectral properties to those of the heme to protein cross-linked form of myoglobin. Procedures such as acid−butanone extraction remove noncovalently bound heme; however, they do not separate Mb−H from apomyoglobin (apoMb). Reverse-phase HPLC is the preferred method for studying the reaction between Mb and peroxides and for monitoring the progress of Mb−H formation (*8*, *14*). This method separates the components of the reaction, i.e., Mb−H, apoMb, unmodified "free" heme (i.e., not covalently bound to protein), and other oxidatively modified free hemes. Figure 1A shows an example of a chromatogram of unreacted metMb where, due to the acid solvent conditions (∼pH 2), unmodified heme (iron protoporphyrin IX, heme B) and the apoprotein elute separately. The chromatogram of metMb that has reacted with H_2O_2 (1:1 ratio, Figure 1B) shows the separation of the components into oxidatively modified free hemes (7−14 min elution times), unmodified heme B (14.6 min elution time), apoprotein (22.0 min elution time), and Mb−H (23.6 min elution time). While this Mb−H peak is very distinct under the conditions described, some Mb−H is spread in a broad band eluting between 19 and 24.5 min. This broadening is more apparent when higher concentrations of peroxides are used. Second derivative spectroscopy of these heme species shows the typical tryptophan and tyrosine bands, indicating the presence of protein, thus confirming these as heme−protein cross-linked species (data not shown). This heterogeneity possibly arises from oxidative damage to the protein affecting the retention time of Mb−H on the column but may also reflect different ways of cross-linking the heme to the protein.

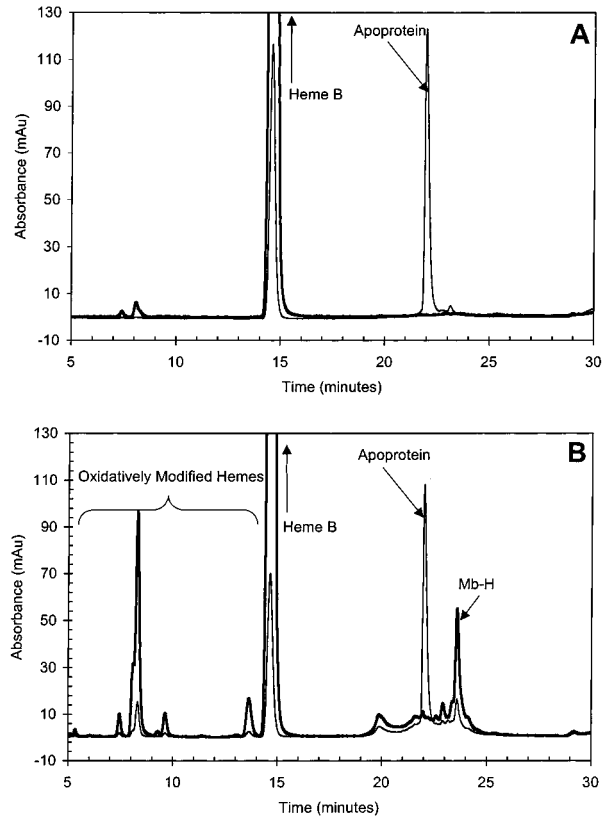


FIGURE 1: HPLC of myoglobin before and after reaction with hydrogen peroxide. (A) HPLC chromatogram of metMb (100 μM). (B) Heme and protein oxidation products from the reaction between metMb (100 μM) and H_2O_2 (100 μM) at pH 5. See Materials and Methods for HPLC conditions. Thin line: absorbance measured at 280 nm. Thick line: absorbance measured at 400 nm.

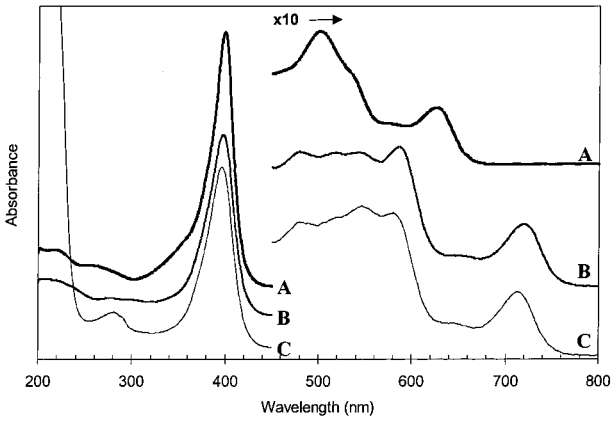


FIGURE 2: Spectral properties of unmodified heme (A, thick line), oxidatively modified free heme (B, intermediate line), and the heme−protein cross-linked species (C, thin line). Spectra were taken from the chromatogram in Figure 1B and offset for clarity. Spectrum A is from the 14.6 min eluant (heme B), spectrum B from the 8.4 min eluant, and spectrum C from the 23.6 min eluant.

Spectral Properties of Heme−Protein Cross-Linked Species. All of the heme species separated by reverse-phase HPLC fall into one of three distinct spectral categories (Figure 2).

Category I includes those that have peak maxima at 398, 502, and 630 nm, identical to those of unmodified heme B (Figure 2A). Species displaying these spectral features elute at 7.4, 8.1, and 14.6 min, the former two being minor components (<0.5% compared to heme B). All are present before the reaction with peroxide and are depleted on peroxide addition.

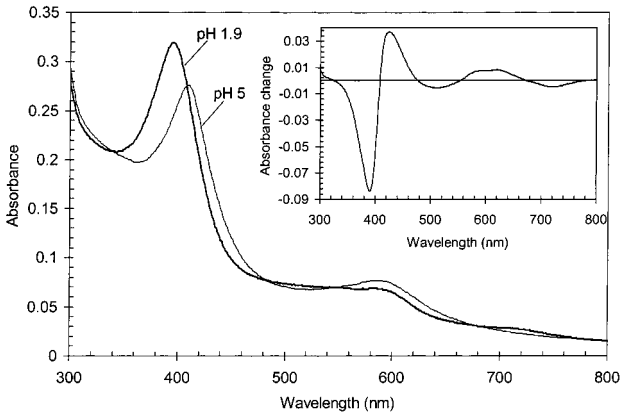


FIGURE 3: Spectrum of acid−butanone prepared heme−protein cross-linked species at pH 1.9 and 5. MetMb (100 μM) was reacted with H_2O_2 (100 μM). Noncovalent bound heme was removed by acid−butanone treatment to leave Mb−H and apoMb. Contaminating butanone was removed by dialysis. At pH 1.9 (very similar to the pH of the HPLC solvents) the spectrum was similar to that exhibited from the reversed-phase HPLC elution (Figure 2). Increasing the pH to 5 red shifted the Soret peak from 398 to 408 nm. The visible spectrum showed removal of the 720 nm band and a change in the 500−600 nm region to a broad single band at 590 nm. Small additions of butanone did not significantly affect the spectrum. Inset: difference spectrum between pH 5 and pH 1.9.

Category II includes those that have peak maxima at 398, 482, 520, 546, 580, and 720 nm (Figure 2B). Species displaying these features elute at 7.5, 8.4, 9.3, 9.7, and 13.6 min (plus numerous more minor peaks). All of these species arise from the reaction with peroxide and do not coelute with any protein components.

Category III includes those showing the presence of a protein component through their enhanced absorbance at 280 nm and below 240 nm (i.e., Mb−H, Figure 2C) eluted around 23.6 min. These species show slight variations in the position and intensity of the bands between 480 and 720 nm compared to other oxidatively modified hemes (II).

The acidic pH of the HPLC eluant shifts the Soret peak of unmodified heme B from 408 to 398 nm, while the visible bands are largely unaffected. Changing the pH of the Mb from pH 1.9 to pH 5 returns the Soret band to 408 nm (data not shown). Changing the pH of acid−butanone-purified Mb−H from pH 1.9 to pH 5 significantly changes the spectrum in both the Soret and visible regions (Figure 3). Increasing the pH shifts the Soret peak from 398 to 408 nm and, unlike native Mb, also significantly changes the visible spectrum to a single broad band with a peak maximum around 589 nm, identical to that reported by Fox et al. (*7*). The acid-induced changes in the spectrum of Mb−H are reversible. We note that the contaminating apomyoglobin component produces optical scattering, making concentration determination by this method more difficult than by HPLC.

EPR Spectrum of Cross-Linked Myoglobin. Figure 4 shows the EPR spectra (10 K) of metMb before reaction with H_2O_2 (Figure 4A) and 1 h after an equimolar amount of peroxide was added to the protein (Figure 4B). At first sight, only the intensity of the high-spin heme signal appears different: it is approximately 1.54 times smaller in the sample that has reacted with peroxide. However, further differences are

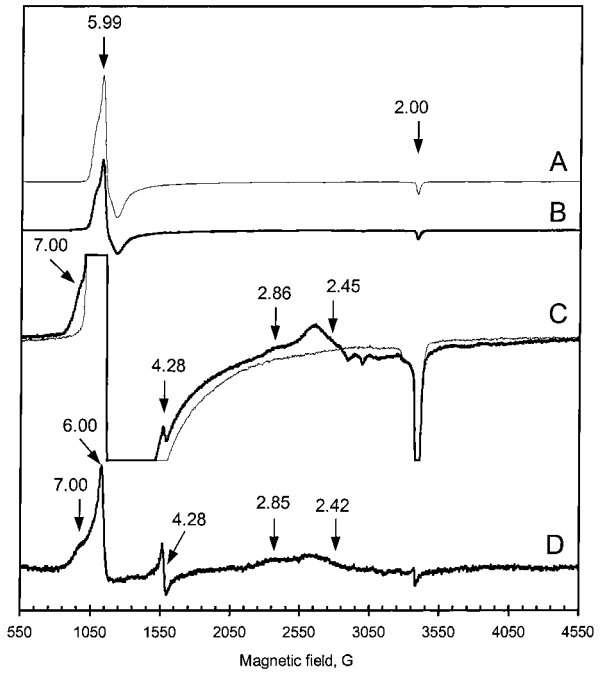


FIGURE 4: EPR spectra of metmyoglobin before and after reaction with hydrogen peroxide and of the cross-linked myoglobin. (A) MetMb (500 μM). (B) MetMb (500 μM) + H_2O_2 (500 μM); the sample was frozen 1 h after mixing. Both samples were at pH 5 (50 mM sodium acetate containing 100 μM DTPA). (C) (A) and (B), each multiplied by a factor of 40 and overlaid [thin line is 40(A) and thick line is 40(B)]. (D) Mb−H purified from 950 μM metMb reacted with 2.85 mM H_2O_2 for 1 h and noncovalently bound heme removed (see Materials and Methods). The *g*-factors are indicated with arrows. Spectrum D was recorded at an 80-fold gain compared to spectra A and B.

revealed when the spectra are enlarged (40-fold, Figure 4C). The $g = 4.28$ signal from the rhombic non-heme iron appears, indicating some heme degradation. Also, a wing at $g = 7$ can be seen in the protein that has reacted with the peroxide, indicating a structural change to the high-spin ferric heme. A signal appears at $g = 2.45$ (with a possible component of the same species at $g = 2.86$) that might be a characteristic of a new low-spin ferric heme form. When these features are compared to the spectrum of the sample of Mb−H (Figure 4D), purified as described in Materials and Methods, it becomes clear that these new high-spin and low-spin signals seen in the protein that has reacted with H_2O_2 are characteristic of Mb−H. The EPR spectrum of Mb−H has not been previously reported. However, the EPR spectrum of peroxide-induced heme−protein cross-linked leghemoglobin has been reported and shows a single $g = 4.3$ signal (*19*). This signal was assigned to a "high-spin iron-(III) in a very distorted, or degraded heme environment". Given that the EPR spectrum of Mb−H is very different from the leghemoglobin EPR spectrum, it is probable that the structure of the reported purified heme−protein cross-linked leghemoglobin differs substantially from that of horse heart Mb−H.

The Extent of Formation of Heme−Protein Cross-Linked Myoglobin Is Dependent on Peroxide Concentration and the Initial Oxidation State of Myoglobin. Figure 5 shows the extent of Mb−H production as a function of H_2O_2 concentration for both metMb and oxyferrous Mb. With Mb in either of these valance states, increasing the peroxide concentrations increased the quantity of Mb−H produced. However, above

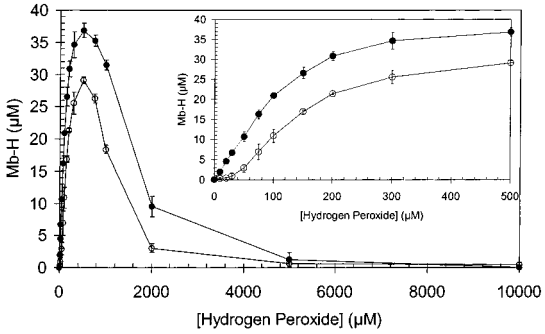


FIGURE 5: Effect of hydrogen peroxide concentration on the extent of formation of heme−protein cross-linking from metmyoglobin and oxymyoglobin. MetMb (●) or oxyMb (○) (100 μM) was reacted with hydrogen peroxide at pH 5.0 (0.1 M sodium acetate containing 100 μM DTPA). The reaction was allowed to stand for 2 h before HPLC analysis. See Materials and Methods for HPLC conditions. The inset shows an expansion of the first section of the graph ($n = 3$).

500 μM H_2O_2 the final concentration of Mb−H decreased, as a result of heme degradation rather than lower production of Mb−H. This degradation of heme may be followed by the appearance of fluorescent heme degradation products (*20*) (data not shown). A maximum of 37% of the metMb was converted to Mb−H under the conditions of the experiment, accounting for 53% of the altered heme. Using ^{14}C-heme studies, Osawa et al. found that at pH 4.7 up to 88% of the oxidatively altered heme was covalently bound to the protein (*15*).

Unlike metMb, the addition of small, substoichiometric amounts of H_2O_2 to oxyMb does not induce Mb−H production (Figure 5, inset). This may be understood from examination of the equations describing the reaction between Mb and H_2O_2. Ferrous Mb (Mb−Fe^{2+}), from which the oxygen has dissociated, reacts with H_2O_2, a two-electron acceptor, to form ferrylMb (Mb−Fe^{4+}=O^{2-}):

$$\text{Mb}-\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow [\text{Mb}-\text{Fe}^{4+}{=}\text{O}^{2-}]^{2+} + \text{H}_2\text{O} \quad (1)$$

Ferric Mb (metMb, Mb−Fe^{3+}) reacts with H_2O_2 to form the ferryl species and a protein cation radical species ($P^{\bullet+}$):

$$\text{Mb}-\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow [\text{Mb}-\text{Fe}^{4+}{=}\text{O}^{2-}]^{2+} + \text{P}^{\bullet+} + \text{H}_2\text{O} \quad (2)$$

As described previously, the radical partially resides on an amino acid residue, proposed by Catalano et al. to be tyrosine 103 (*8*), and this protein radical initiates Mb−H formation. As the reaction between ferrous Mb with H_2O_2 does not produce a protein radical (eq 1), Mb−H cannot be generated. If both ferryl Mb and ferrous Mb are present, then these may rapidly react together to form two molecules of metMb:

$$[\text{Mb}-\text{Fe}^{4+}{=}\text{O}^{2-}]^{2+} + \text{Mb}-\text{Fe}^{2+} + \text{H}^{+} \rightarrow 2\text{Mb}-\text{Fe}^{3+} + \text{OH}^{-} \quad (3)$$

The reaction of H_2O_2 with metMb is faster than with oxyMb due to the presence of oxygen ligated to the heme iron, and thus H_2O_2 will preferentially react with metMb (eq 3) rather than with oxyMb. Initially, therefore, peroxide is consumed to form metMb from the oxy derivative, thus

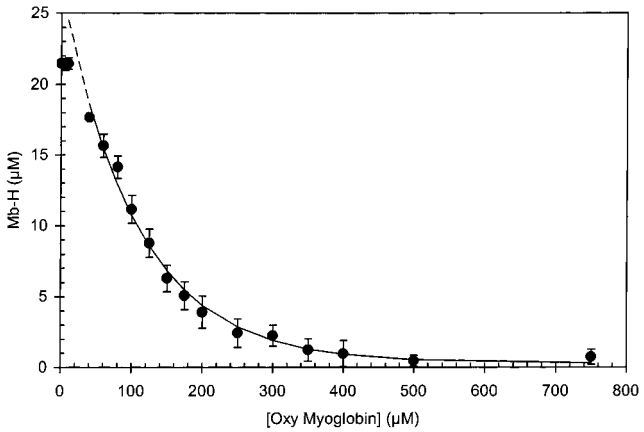


FIGURE 6: Peroxide-induced heme−protein cross-linking from metmyoglobin is inhibited in the presence of oxymyoglobin. MetMb (100 μM) was reacted with H_2O_2 (100 μM) at pH 5 (25 mM sodium acetate containing 100 μM DTPA) in the presence of oxyMb (0−750 μM). Cross-linking was inhibited by the presence of concentrations of oxyMb >30 μM. Samples were analyzed after 1 h incubation by reverse-phase HPLC ($n = 3$).

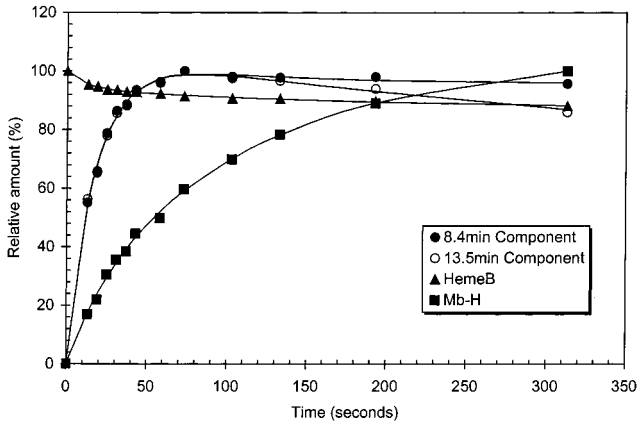


FIGURE 7: Time course of the formation of heme−protein cross-linked myoglobin, heme B degradation, and formation of other oxidatively modified heme species. MetMb (100 μM) and H_2O_2 were mixed and injected onto a C3 HPLC column as a function of time using an automated HPLC injector program (see Materials and Methods). Heme B decreases due to degradation and conversion by peroxide. Oxidatively modified hemes not covalently bound to the protein result from peroxide interactions (8.4 and 13.5 min elution times). These free hemes develop within 50 s, and one begins to decay within the time frame of the experiment. Heme−protein cross-linked myoglobin (Mb−H) forms more slowly compared to the other oxidative modified free hemes.

explaining the initial plateau in Figure 5 (inset) in which peroxide is consumed but no Mb−H is formed.

The effect of adding increasing amounts of oxyMb to the metMb−peroxide reaction at pH 5 is presented in Figure 6. Higher concentrations of oxyMb inhibit the formation of Mb−H. As described above, inhibition of Mb−H formation by oxyMb is expected as increasing concentrations of oxyMb, under conditions of restricted peroxide, limit the amount of peroxide available to react with metMb. This, therefore, decreases protein radical formation and hence Mb−H formation. At low concentrations of oxyMb the reaction of metMb with H_2O_2, being much faster than oxyMb with H_2O_2, is not significantly affected. This creates the initial plateau discernible in Figure 6 at low concentrations of oxyMb (<30 μM). This initial plateau is abolished when concentrations of oxyMb are sufficient to compete with the metMb for the peroxide.

The Mechanism of Heme−Protein Cross-Linking Is Different from That of Other Oxidative Modifications to the Heme. Figure 7 shows the time course for the formation of Mb−H compared to the degradation or modification of heme B and the formation of two oxidatively modified major free heme species (from 8.4 and 13.5 min elution times; see Figure 1). These free hemes are not covalently attached to the protein and are thus separated from the protein by HPLC. An HPLC injector program was used to mix the myoglobin, buffer, and peroxide in the injector needle loop, which was injected onto the column after a number of preprogrammed times. The rate of formation of Mb−H is slower than that of the other oxidatively modified heme species (~5 times slower), and Mb−H continues to form as one of the species (13.5 min) begins to decay. Two oxidatively modified free hemes were examined and identified by Sugiyama et al. (*21*). One species was identified as a chlorin product formed from the saturation of the double bond on one of the pyrrole rings of the heme, probably resulting from radical damage. Water was found to add to a cationic site of the first chlorin product to form a second product that mirrored the decrease of the first product over a time course of several hours. Therefore, at least one of the oxidatively modified heme species shown in Figure 7 is not that of either the chlorins previously examined. Our HPLC technique has resolved more heme species than were previously reported (*21*). The time courses of the formation of the chlorin hemes examined are clearly different from that of Mb−H formation and are different again from the slow ($t_{1/2}$ several hours) formation of the hydration product reported by Sugiyama et al. These results suggest that the mechanism of formation of Mb−H is different from the other oxidatively modified hemes that result from radical damage. However, as Mb−H is redox active, we cannot entirely exclude the possibility that Mb−H and probably also the other oxidatively modified heme−proteins have significant rates of decay that perturb the time courses seen in Figure 7. In this case the rates of formation are only apparently different.

Heme−Protein Cross-Linking Is pH Dependent. The reaction of metMb with H_2O_2 can be divided into two phases: (a) the formation of the ferryl species (eq 2) and (b) its subsequent decay through reduction/autoreduction. The rate of ferryl formation has been shown to be strongly dependent upon the initial ligation state of Mb (*17*). The rate of ferryl formation is approximately constant over the pH range of 4.5−7, decreasing at higher pH values, as hydroxymetMb (Mb−Fe^{3+}−OH) replaces aquo-metMb (Mb−Fe^{3+}•••H_2O) [pK 8.93 (*22*)]. However, the rate of ferryl autoreduction has been shown to very pH sensitive over the pH range of 4.5−7 (*17*). The results of adding a 1 molar equiv of H_2O_2 to metMb at a number of pH values are presented in Figure 8. The extent of Mb−H formation is pH dependent, increasing considerably at acidic pH values. Once formed, Mb−H is very stable (*7*). The pH profile for Mb−H formation is remarkably similar to the pH profiles for the (i) rate of metMb-catalyzed consumption of the lipid hydroperoxide 13L-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid (13-HPODE) (*23*), (ii) extent of Mb-catalyzed formation of F_2-isoprostanes from low-density lipoproteins (*13*), and (iii) rate of ferrylMb autoreduction (*17*).

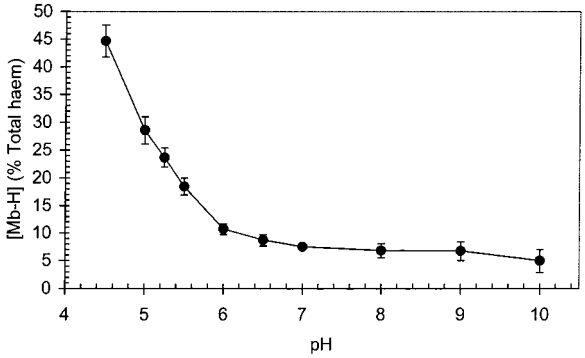


FIGURE 8: Effect of pH on heme−protein cross-linked species formation. MetMb (100 μM) was reacted with H_2O_2 (100 μM) at various pH values. Buffers: pH 4.5−5.5, sodium acetate; pH 6−8, sodium phosphate; pH 9 and 10, sodium tetraborate. All buffers were 0.1 M containing 100 μM DTPA. After 24 h, the samples were analyzed by reverse-phase HPLC ($n = 3$).

We propose that this similarity reflects the underlying reactivity of a protonated form of ferryl Mb. The enhancement of the rate constants for the processes described above reflects the increased population of this protonated species at lower pH values (*13*, *17*). This observation of the pH dependency has not been incorporated into the previously described mechanism for Mb−H formation. An alternative view is that protein conformational changes, induced by acidic conditions, may affect the extent of Mb−H formation. Such pH-induced protein conformational changes affect the reactivity of H_2O_2 consumption by another heme protein horseradish peroxidase (*24*, *25*). However, we have previously shown that the rate of ferryl decay and the rate of HPODE consumption by both Mb and protein-free hemin are very similar (*17*) and these, in turn, are very similar to the pH profile of Mb−H formation (Figure 8). These observations suggest that the effect of protein conformation on the extent of Mb−H formation is minor.

An earlier hypothesis describing the mechanism of formation of Mb−H suggests that the ferryl heme reacts with a protein radical harbored on a tyrosine residue (Tyr_{103}, equine) (*8*). The data presented above indicate that this mechanism requires modification to explain why cross-linking best

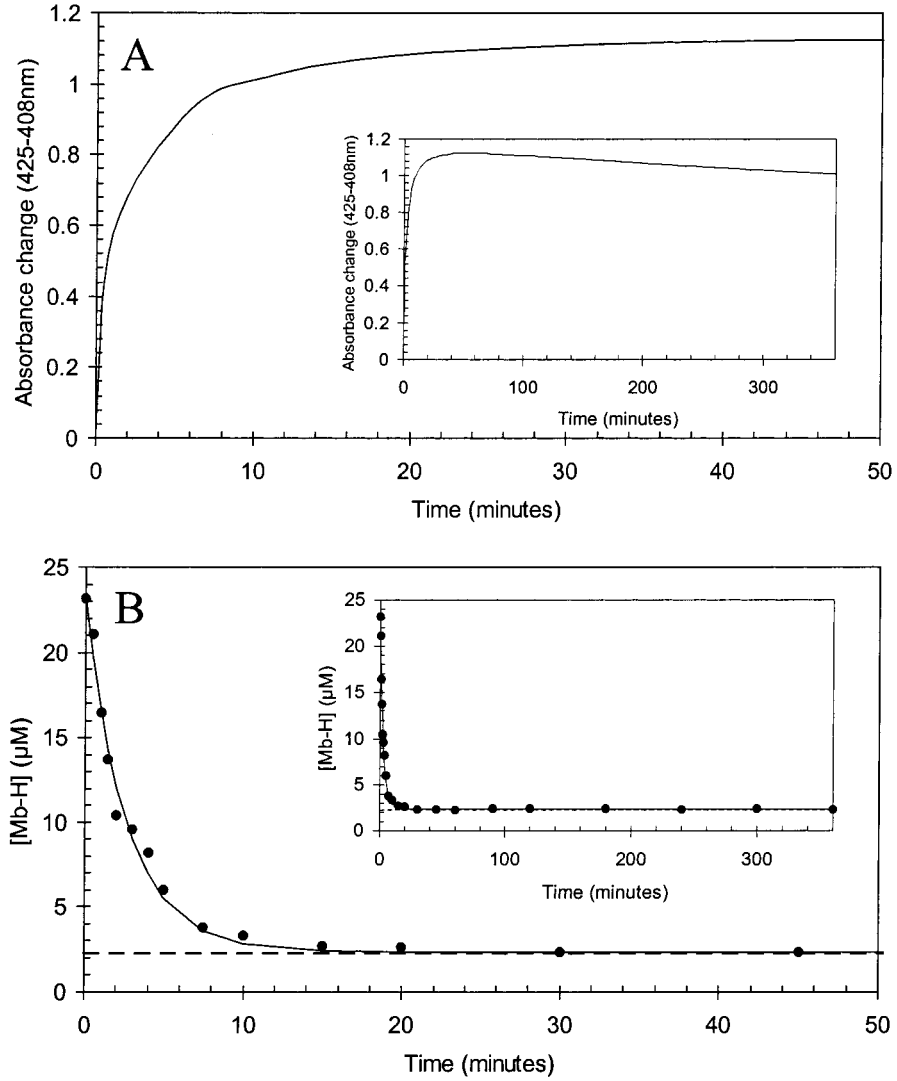
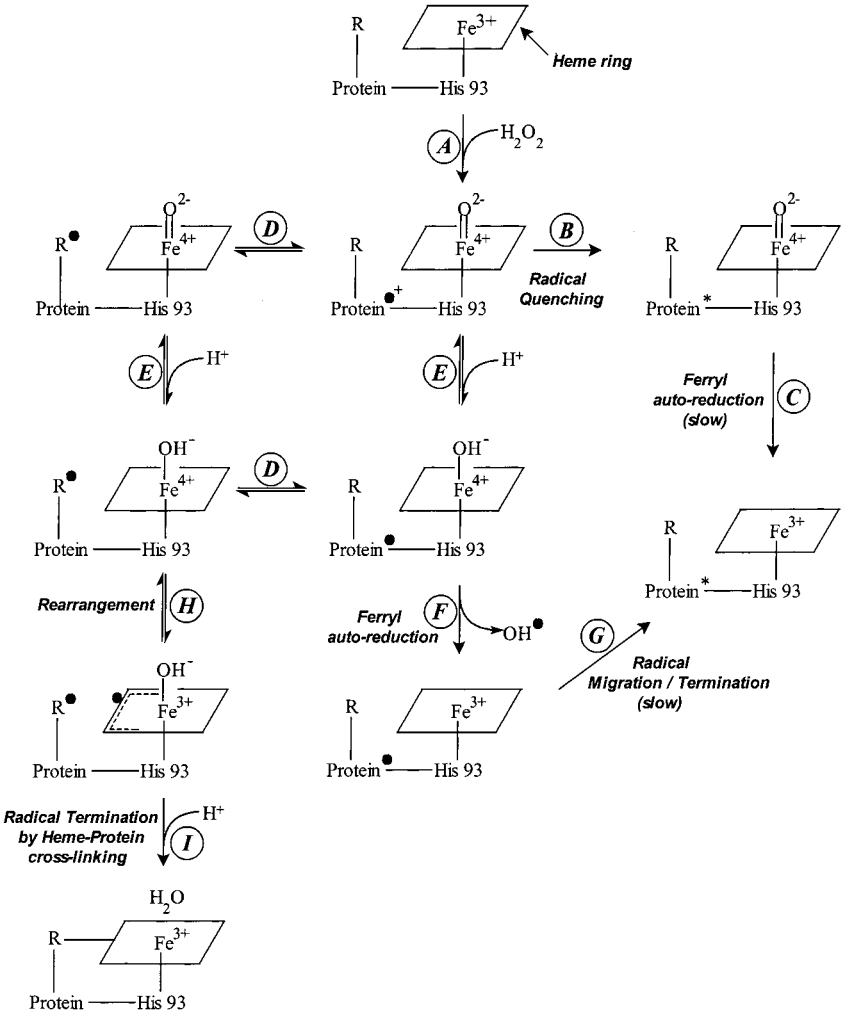


FIGURE 9: Time course of heme−protein cross-link formation as measured by pH jump. (A) Reaction of metMb (160 μM) and H_2O_2 (160 μM) at pH 10 (10 mM sodium teraborate and 200 μM DTPA) showing the formation (~150 μM) and slow decay of ferrylMb as followed optically at 425 and 408 nm using an optical cell with path length of 0.1 cm. (B) At various times aliquots from (A) were diluted to 100 μM Mb with 0.5 M sodium acetate buffer (pH 5.0) to give a final pH of 5.2. This was allowed to stand for 30 min, and Mb−H concentration was measured by reverse-phase HPLC. The dashed line represents the concentration of Mb−H formation for the control at pH 10. Both insets show data over the full range of the time course of experiment.

Scheme 1: Mechanism of Heme−Protein Cross-Linking in Myoglobin[a]



[a] Hydrogen peroxide reacts with ferric (met) Mb to produce the ferryl species and a protein cation radical (step A). The charge of this radical will be quickly lost through deprotonation. At high pH the ferryl species is stable, but no Mb−H is formed. The protein radical has time to quench before the ferrylMb eventually autoreduces (steps B and C). The radical can migrate to an amino acid residue [R, reportedly Tyr_{103}, equine (*8*)] close to the porphyrin ring (step D) where, if the nature of the amino acid allows, the charge maybe lost via deprotonation. Protonation of the ferryl oxygen at low pH (step E) destabilizes the ferryl species, allowing the ferryl to decay (autoreduction, steps F and G). Alternatively, the heme ring, being partially radical in nature, reacts with the radical on the nearby amino acid side chain, resulting in termination of both radical species and forming a protein−porphyrin covalent bond (steps H and I). Heme−protein cross-linking will only occur if the protein radical *and* protonated oxoferryl species are present.

occurs at low pH values. At pH values above 6 the ferryl species can be formed and is stable, yet no significant cross-linking occurs. To further elucidate the mechanism of Mb−H formation, we have conducted a series of "pH-jump" experiments described below.

A Protein Radical and a Ferryl Heme Are Necessary but Not Sufficient To Form Heme−Protein Cross-Linked Myoglobin. FerrylMb was formed at pH 10 by addition of a stoichiometric quantity of H_2O_2 to metMb (Figure 9A). At this high pH the formation of ferrylMb is slower than that observed at more acidic pH values and produces relatively small amounts of Mb−H (Figure 8). At pH 10 the ferryl form is stable, decaying slowly (Figure 9A, inset), allowing migration and termination of the protein radicals. These radicals can be detected by EPR spectroscopy and shown to decay while the ferryl form persists (*26*, *27*). Thus at pH 10, although an oxoferryl heme species is produced, and, initially, a protein radical, there is very little reaction between these to form Mb−H (Figures 8 and 9). We conclude, therefore, that although ferryl and radical species may be necessary for cross-linking, they are not in themselves sufficient.

The effect of a pH jump from 10 to 5.2 is shown in Figure 9B, where the extent of Mb−H formation is displayed as a function of the time of incubation at pH 10 prior to rapidly lowering the pH. For short incubation times at pH 10 the concentration of Mb−H formed was much larger than seen in control incubations at pH 10. At longer times, dropping the pH to 5.2 exponentially decreased the production of Mb−H from 23.2 to 2.3 μM ($t_{1/2} \approx 1.75$ min; Figure 9B, inset), even though the ferryl form was close to being fully populated (Figure 9A, inset). This confirms that the ferryl species, even at pH 5.2, where it is partially protonated, is insufficient to cause heme to protein cross-linking. The decrease in Mb−H formation closely follows but does not exactly mirror the formation of ferrylMb at pH 10. A simple explanation for this behavior is that the extent of Mb−H formation when the pH is dropped largely reflects the amount

of remaining H_2O_2 in solution and the concentration of radicals generated at pH 10. The differences between the time course of ferryl formation at pH 10 and the decrease in the concentration of Mb−H formed when the pH is dropped may be due to the persistence of the various protein radicals generated at pH 10. At pH 8 and above these have half-lives of ~5 min (*28*). It is not currently known which protein radicals may participate in Mb−H formation.

Taken together, these data imply that cross-linking only occurs when Mb possesses a *protonated* oxoferryl heme and a protein radical. These data suggest that previous mechanisms, describing Mb−H formation, are incomplete and a revised mechanism of Mb−H formation is required to provide explanations for the above findings.

Mechanism of Heme−Protein Cross-Linking. The importance of a protein radical species in the mechanism of Mb−H formation has been discussed previously (*8*). Substituting oxyMb at concentrations high compared to peroxide concentrations inhibits the production of Mb−H by preventing protein radical formation (Figures 5 and 6). This supports the earlier findings that the protein radical is essential in the mechanism of Mb−H formation. It is, however, clear from the pH profile of Mb−H production (Figure 8) and pH-jump experiments (Figure 9) that the previously published mechanism of Mb−H formation neglects the requirement for a low-pH form of ferryl Mb.

We have shown previously (*17*) that the protonated form of ferrylMb ($Fe^{4+}-OH^-$) has an enhanced pseudoperoxidase activity. The unprotonated ferryl species ($Fe^{4+}=O^{2-}$) was included in the original mechanism of Mb−H formation (*8*) to act as an electron sink for the radical attack on the porphyrin ring. We show that this unprotonated ferryl species induces very little Mb−H (Figures 8 and 9). The protonated ferryl species is an essential component in the mechanism of Mb−H formation with both the protonated ferryl species and the protein radical required to produce Mb−H.

Scheme 1 shows a mechanism of Mb−H formation based on the data presented above, and the various steps are now discussed in sequence. Step A: From our data and from numerous previous studies the reaction between the two-electron oxidant H_2O_2 and metMb forms a protein cation radical and a ferryl species. The positive charge of this radical will be quickly lost through deprotonation (e.g., $Tyr + P^{\bullet+} \rightarrow Tyr^{\bullet} + P + H^+$). Steps B and C*:* The species generated in step A decays through radical quenching and electron donation, yielding the ferric protein and no Mb−H. This pathway dominates at high pH values and in the presence of high concentrations of radical scavengers and reductants. Damage to the heme and protein can irreversibly alter their physical properties (e.g., optical), especially after numerous ferric−ferryl redox cycles (damaged heme−protein collectively referred to as protein*). Step D: The protein-based radical migrates (either from the heme or, here, the protein) onto an amino acid residue [possibly tyrosine 103 (*8*)]. Step E: Protonation of the oxoferryl species to form $Fe^{4+}-OH^-$. We have previously proposed that the *protonated* form of ferrylMb may be considered "radical-like" in nature, this ferryl species being electronically equivalent to a ferric species plus a radical [e.g., $Fe^{3+}\cdots OH^{\bullet}$ or porphyrin$^{\bullet}$ $Fe^{3+}OH^-$ (*17*)]. It is this radical-like nature of the protonated species that is the key to the mechanism of both enhanced autoreduction (step F and subsequently step G) and the formation of Mb−H (steps H and I). Step F: MetMb quickly regenerates, reflecting the intrinsic instability of the protonated ferryl form. This pathway results in no Mb−H production and protein damage (protein*). If the protonated ferryl-heme moiety can be considered as radical-like and is in the vicinity of a protein radical [e.g., Tyr_{103} (*8*), step H], then termination of both the protein radical and the ferryl-derived pseudoradical is the preferred pathway. The result of the radical termination between the protein radical and the protonated ferryl-heme pseudoradical is the formation of a covalent bond between the protein and the porphyrin ring (step I). The mechanism shown in Scheme 1, while being similar to that previously proposed, has the advantage that it better explains the pH dependence of cross-linking and that it involves radical/radical termination rather than an attack of a single radical onto the porphyrin ring.

In summary, we have provided strong evidence that the formation of Mb−H requires not only a protein radical but also the *protonated* oxoferryl Mb species. Protonated ferrylMb is a highly active form that may promote radical termination between the heme ring and the protein radical. Prevention of ferryl protonation by increasing the pH, or quenching the protein radical, is an effective way to prevent Mb−H formation. The in vivo consequences of these findings are yet to be explored, but we point out that "alkalinization" treatment used to ameliorate the effects of rhabdomyolytic acute renal failure (*13*) should inhibit Mb-induced oxidative stress by preventing protonation of the ferryl species. This would have two effects: first, the prooxidant and peroxidatic activities of the native Mb species are decreased, as discussed in earlier publications (*13*, *17*), and second, the formation of cytotoxic derivatives of Mb, namely, Mb−H, would be substantially decreased.

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