Reaction of Hydrogen Peroxide with Ferrylhemoglobin: Superoxide Production and Heme Degradation

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ABSTRACT: The reaction of Fe(II) hemoglobin (Hb) but not Fe(III) hemoglobin (metHb) with hydrogen peroxide results in degradation of the heme moiety. The observation that heme degradation was inhibited by compounds, which react with ferrylHb such as sodium sulfide, and peroxidase substrates (ABTS and *o*-dianisidine), demonstrates that ferrylHb formation is required for heme degradation. A reaction involving hydrogen peroxide and ferrylHb was demonstrated by the finding that heme degradation was inihibited by the addition of catalase which removed hydrogen peroxide even after the maximal level of ferrylHb was reached. The reaction of hydrogen peroxide with ferrylHb to produce heme degradation products was shown by electron paramagnetic resonance to involve the one-electron oxidation of hydrogen peroxide to the oxygen free radical, superoxide. The inhibition by sodium sulfide of both superoxide production and the formation of fluorescent heme degradation products links superoxide production with heme degradation. The inability to produce heme degradation products by the reaction of metHb with hydrogen peroxide was explained by the fact that hydrogen peroxide reacting with oxoferrylHb undergoes a two-electron oxidation, producing oxygen instead of superoxide. This reaction does not produce heme degradation, but is responsible for the catalytic removal of hydrogen peroxide. The rapid consumption of hydrogen peroxide as a result of the metHb formed as an intermediate during the reaction of reduced hemoglobin with hydrogen peroxide was shown to limit the extent of heme degradation.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species involved in the propagation of cellular injury during various pathophysiological conditions (*1*, *2*). The concentration of H_2O_2 in normal human plasma is 4−5 μM (*3*) and increases under inflammatory conditions (*4*). It has also been shown that the toxicity of H_2O_2 is enhanced in the presence of hemoglobin (*5*−*7*). Potential toxicity associated with the reaction of hemoglobin with H_2O_2 can be found both in the red cell and in the extracellular meilleiu when hemoglobin is released from damaged red cells (*8*−*10*).

The formation of H_2O_2 in the red cell is associated with autoxidation of oxyhemoglobin (*11*). Autoxidation produces superoxide, most of which reacts with superoxide dismutase, producing H_2O_2 and oxygen. Most of the H_2O_2 is eliminated by catalase and glutathione peroxidase, resulting in a steady state concentration of H_2O_2 in the red blood cell of approximately 2×10^{-10} M (*12*). It has been shown (*13*) that about 3% of hemoglobin undergoes autoxidation in a 24 h period, resulting in a large flux of reactive oxygen species. Despite the antioxidant defense system, which minimizes the steady state levels of these reactive oxygen species, oxidative stress associated with reactions in the red cell involving these species needs to be considered (*8*, *14*, *15*).

The dramatic increase in the rate of autoxidation under hypoxic conditions (*16*−*19*) implies that red cell oxidative stress is most prevalent at the reduced oxygen pressures normally attained within the microcirculation. The physiological relevance of these processes within the red cell is suggested by the finding that, despite the extensive red cell antioxidant system, membrane oxidation by reactive oxygen species has been shown to take place as the cell ages. This process is thought to be involved in the mechanism for sequestration of senescent red cells (*8*, *20*).

Hemolysis is observed in diseases such as thrombotic thrombocytopenic purpura and disseminated intravascular coagulation (*21*). Several reports have shown the toxic effects of free hemoglobin (*22*) in the brain (*23*), the eye (*24*), the central nervous system (*25*), and the kidney (*26*). It has been shown that iron accumulates in atherosclerotic lesions (*27*) in a catalytically active form (*28*). The source of this iron was thought to be hemoglobin released from damaged red cells at sites of vascular turbulence (*29*) or in hemorrhagic atheromatous plaques (*30*). The relatively rapid oxidation of extracellular hemoglobin facilitates release of heme, which is rapidly incorporated into the plasma membrane of endothelial cells and releases its iron (*31*, *32*). In response to these pro-oxidants, endothelial cells induce heme oxygenase (*33*) and the iron binding protein, ferritin (*34*).

The reaction of hydrogen peroxide with Fe(II) hemoglobin (oxyHb and deoxyHb) and Fe(III) hemoglobin (metHb) results in the formation of ferrylhemoglobin (ferrylHb) and oxoferrylhemoglobin (oxoferrylHb), respectively (*35*, *36*). Both are strong oxidizing agents and are considered the putative source for cellular and tissue damage (*37*−*41*). We

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have recently demonstrated the formation of fluorescent heme degradation products with the concomitant release of iron during the reaction of H_2O_2 with oxyHb, but not with metHb (*42*). This reaction is thought to play a major role in the toxicity of hemoglobin.

In this paper, we have investigated the mechanism for heme degradation during the reaction of oxyHb with H_2O_2. Using visible absorption, fluorescence, and low-temperature electron paramagnetic resonance studies, it has been established that degradation involves the reaction of an additional hydrogen peroxide molecule with ferrylHb to produce metHb and the $O_2^{\bullet -}$ radical anion. This radical produced in the heme pocket is ideally located to react with the porphyrin entity, resulting in heme degradation and the release of iron. These heme degradation products and iron are potential sources for oxidative damage in red cells and endothelial cells.

MATERIALS AND METHODS

Materials. Reagent grade 30% hydrogen peroxide was obtained from Fisher Scientific Co. The concentration of hydrogen peroxide was determined spectrophotometrically by absorption at 240 nm. Sodium sulfide, *o*-dianisidine (3,3-dimethoxybenzidine), 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), desferrioxamine, diethylenetriaminepentaacetic acid (DTPA), quinine sulfate, *p*-hydroxyphenylacetic acid, ferrozine disulfonate, horseradish peroxidase, protoporphyrin IX, ferriprotoporphyrin IX, and NADPH were obtained from Sigma Chemical Co. 18-Crown-6 was obtained from Aldrich Chemical Co. Hemoglobin was prepared from fresh blood as described previously (*42*).

Measurement of Fluorescence during the Reaction of Hydrogen Peroxide with Hemoglobin. Fluorescence measurements were made using a Perkin-Elmer fluorescence spectrophotometer (model 650-40) with a Hitachi 057 recorder. Two fluorescent bands were measured with emission wavelengths of 465 nm (excitation wavelength of 321 nm) and 525 nm (excitation wavelength of 460 nm) during the reaction of oxyHb (50 μM) and hydrogen peroxide (0.5mM) in 50 mM potassium phosphate buffer (pH 7.4). The concentration of fluorescent products was determined from the maximal fluorescence obtained during the reaction of heme with a large excess of hydrogen peroxide (H_2O_2/heme ratio of 2000). It was assumed that this maximal fluorescence corresponded to a concentration of fluorescent products equal to the heme concentration.

Effect of Various Treatments on the Time Course for the Formation of Fluorescent Products. The involvement of the heme iron was demonstrated by comparing the results obtained for oxyHb with results obtained for deoxyhemoglobin and carboxyhemoglobin. Deoxyhemoglobin was prepared by stirring (Helma CUV-O model 333 stirrer) the solution in an anaerobic cell (Aminco), while flushing the gas phase above the solution with nitrogen. This process was continued until the oxyHb was completely converted to deoxyhemoglobin, as determined by visible spectroscopy. Flushing with CO until the oxyHb was completely converted to carboxyhemoglobin produced carboxyhemoglobin. The reaction of hydrogen peroxide in the absence of oxygen was performed using the Aminco anaerobic cell assembly, which includes a plunger with a 50 μL perforated plastic vessel. A solution of H_2O_2 was placed in the plunger prior to changing the liganded state of the hemoglobin. The reaction with H_2O_2 was initiated by depressing the plunger.

The effect of sodium sulfide, ABTS, *o*-dianisidine, desferrioxamine, DTPA, EDTA, mannitol, benzoic acid, dimethyl sulfoxide, and thiourea on the time course of the formation of fluorescent products was also studied. These agents were added to hemoglobin 15 min before initiating the reaction with H_2O_2.

The effect of H_2O_2 removal during the time course of the formation of fluorescent products was studied by adding 200 units of catalase 1.0, 2.0, 3.0, and 4.0 min after the reaction started.

Formation of Fluorescent Products during the Reaction of Potassium Superoxide with Ferriprotoporphyrin and Protoporphyrin. Potassium superoxide was dissolved anaerobically in dimethyl sulfoxide (DMSO) containing crown ether (*43*). Ferriprotoporphyrin and protoporphyrin were dissolved in DMSO. Ferriprotoporphyrin (50 μM) or protoporphyrin (50 μM) was incubated with potassium superoxide (0.1 mM) in dimethyl sulfoxide (DMSO) at room temperature for 60 min. After the incubation period, the emission spectra of two fluorescent products with excitation at 321 and 460 nm were assessed.

Oxidation of Oxyhemoglobin by Hydrogen Peroxide. The oxidation of oxyHb by H_2O_2 in potassium phosphate buffer (pH 7.4) at room temperature was assessed by repetitive scanning of a visible spectrum from 490 to 640 nm on a Perkin-Elmer Lambda 6 UV−visible spectrophotometer. For this purpose, the Aminco anaerobic cell with a plunger for rapid mixing (see above) was used for mixing H_2O_2 with oxyHb. A solution of 50 μM Hb in 50 mM potassium phosphate buffer (pH 7.4) was placed in the cell, and 50 μL of concentrated H_2O_2 was placed in the plunger assembly to produce a final H_2O_2 concentration of 0.5 mM. The scan speed was set at 1500 nm/min. Depressing the plunger rapidly mixing the H_2O_2 with oxyHb started the reaction, and repetitive scans at 18 s intervals were taken for 3.0 min. After that period, scans were taken at 60 s time intervals for 30 min. Similar experiments were performed at the same concentration of oxyHb and varying concentrations of H_2O_2 (0.125, 0.25, 1.0, and 2.0 mM).

Spectral Analysis. The concentration of ferrylhemoglobin at each time point was determined by multicomponent fitting of each spectrum to a linear combination of the known spectra of various hemoglobin species. For this purpose, the oxyHb and deoxyHb spectra were determined from the spectra of fully oxygenated and fully deoxygenated hemoglobin. The ferrylHb spectrum was obtained 90 s after the addition of a 20-fold excess of H_2O_2 to fully deoxygenated Hb. OxoferrylHb obtained by the reaction of hydrogen peroxide with metHb was found to have the same spectrum as ferrylHb. The high-spin metHb was prepared by addition of a molar excess of ferricyanide to oxyHb at pH 7.0. This spectrum was assumed to be the same if superoxide was in the heme pocket, but not coordinated with the iron (see eq 2 below). Methemoglobin hydroxide was obtained by adjusting the pH of metHb to 8.5. Both high-spin methemoglobin and methemoglobinn hydroxide are required to fit the methemoglobin present in our experiments performed at pH 7.4. A minor improvement of the fit that resulted in small alterations in the concentrations of ferrylhemoglobin was

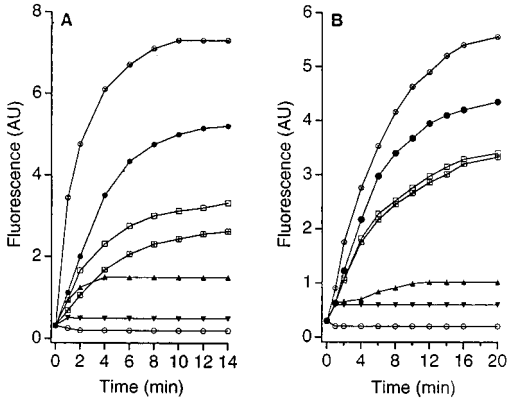

FIGURE 1: Effect of various treatments on the time course of fluorescence formation. The assay mixture contained hemoglobin (50 μM) and H_2O_2 (0.5 mM) in 50 mM potassium phosphate buffer (pH 7.4) in a total volume of 2 mL. The reaction was performed at 22 °C: (●) no treatment, (⊕) anaerobic conditions, (▼) CO pretreatment, (□) desferrioxamine (0.2 mM), (⊞) thiourea (5.0 mM), (▲) Na_2S (5.0 mM), and (○) ABTS (5.0 mM). (A) Fluorescence at an excitation wavelength of 321 nm and an emission wavelength of 465 nm. (B) Fluorescence at an excitation wavelength of 460 nm and an emission wavelength of 525 nm.

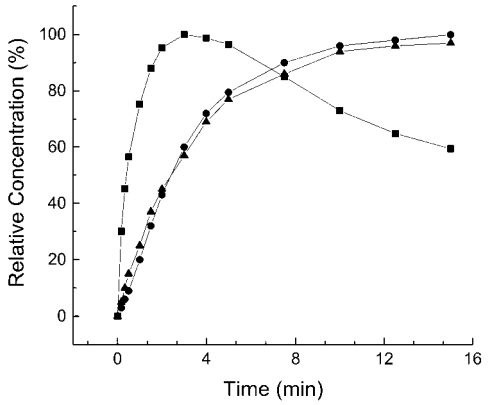

FIGURE 2: Comparison between the formation of ferrylHb and fluorescent products and H_2O_2 consumption as a function of time. The reaction conditions are those described in the legend of Figure 1. To compare the result for ferrylHb, heme degradation products, and H_2O_2, the data were normalized in the following way. For ferrylHb and degradation products, the results at each time point were normalized relative to the maximum change observed within the 15 min time frame. For hydrogen peroxide, the concentration of H_2O_2 remaining at any time was subtracted from the initial value, and this drop in H_2O_2 concentration was normalized relative to the total amount of added H_2O_2: (■) ferrylHb, (●) fluorescence (excitation at 321 nm and emission at 465 nm), and (▲) H_2O_2 consumption.

obtained by including the spectrum of a low-spin denatured hemichrome prepared by adding 0.5 mM salicylate to 50 μM metHb.

Consumption of Hydrogen Peroxide. The hydrogen peroxide consumption during the reaction of oxyHb with H_2O_2 both in the presence and in the absence of cyanide was assessed spectrofluorometrically by the formation of a fluorescent dimer of *p*-hydroxyphenylacetic acid (excitation at 310 nm and emission at 415 nm) produced during the horseradish peroxidase-catalyzed reaction (*44*, *45*).

Oxygen Release. The release of oxygen during the reaction of oxyHb and H_2O_2 in the presence and absence of cyanide was assessed using a YSI model 5300 Biological Oxygen Monitor (Yellow Spring Instruments). The oxygen electrode was calibrated using the oxygen-consuming reaction of phenylhydrazine-HCl and ferricyanide, according to the method of Misra and Fridovich (*46*). Oxyhemoglobin in phosphate buffer was completely deoxygeneted in the instrument sample chamber by passing nitrogen gas. The reaction was started with the addition of a small volume of deoxygenated H_2O_2 using a gastight syringe.

Electron Paramagnetic Resonance (EPR). Samples were prepared for EPR studies by mixing oxyHb or metHb (450 μM) with 4.5 mM H_2O_2 in 4 mm quartz tubes (Wilmad) and freezing the samples by submerging them in liquid nitrogen at various time intervals. Samples were stored at 77 K until spectra were recorded. The EPR spectra were measured using an IBM ER-200D-SRC spectrophotometer with 100 kHz modulation. An Air Products model LTD-3-110 liquid transfer Heli-Tran cryogenic unit with an ADP-E temperature controller was used to maintain the temperature at 8−10 K.

RESULTS

Time-Dependent Production of Fluorescent Products and the Factors That Influence the Production of Fluorescent Products. Figure 1 shows the gradual increase in the concentration of fluorescent products, which levels off at 10 min for the 465 nm fluorescent product (excitation at 321 nm) and at >20 min for the 525 nm fluorescent product (excitation of 460 nm). The maximal levels of fluorescence formed at a 10-fold excess of H_2O_2 corresponds to 1.9 and 3.0% of the hemes degraded for the 465 nm emission product and the 525 nm emission product, respectively. The effect of deoxygenation, the binding of carbon monoxide to the heme and prior addition of various reagents, on the extent of fluorescent product formation is also shown in this figure. Deoxygenation enhanced the intensity of fluorescence by about 35 and 25% for the 465 nm emission band and the 525 nm emission band, respectively. The binding of carbon monoxide to Hb resulted in inhibition of about 95% of the fluorescence in both bands. Reagents which react with ferrylHb were inhibited by the formation of both fluorescent products. Thus, sodium sulfide inhibited about 75% of the fluorescence intensity of both bands, while peroxidase substrates such as ABTS and *o*-dianisidine completely suppressed the formation of both fluorescent products.

The results with •OH radical scavengers and iron chelators were inconsistent (Figure 1). Thus, thiourea, a putative •OH radical quencher, suppressed 36 and 22% of the fluorescence intensity of the 465 nm emission band and the 525 nm emission band, respectively, while the •OH radical scavengers mannitol, benzoic acid, and dimethyl sulfoxide had no significant effect on the formation of fluorescent bands. Desferrioxamine, an iron chelator, inhibited about 50 and 23% of the fluorescence intensity with emission at 465 nm and emission at 525 nm, respectively, while DTPA and EDTA, two other iron chelators, had no effect on the intensity of the fluorescent bands. This inconsistency implies that •OH radicals and free iron are not necessarily involved in the formation of heme degradation products. The finding that the addition of ferrous sulfate had no effect on the intensity of the fluorescent bands also supports the lack of an effect of free iron. Superoxide dismutase also had no effect on the intensity of either fluorescent band (data not shown).

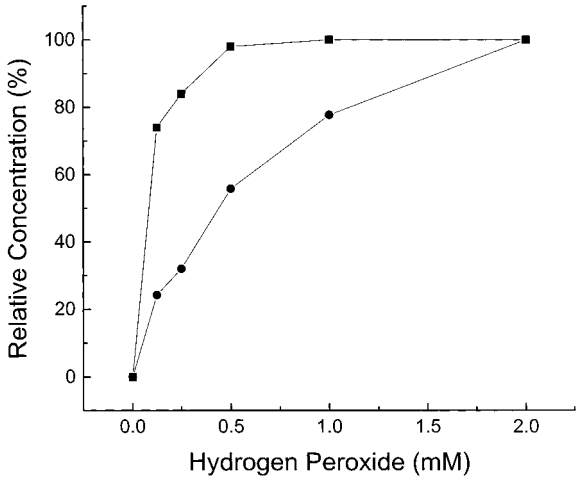

FIGURE 3: Comparison between the formation of ferrylHb and fluorescent products as a function of H_2O_2 concentration. The hemoglobin concentration, pH, and buffer are those described in the legend of Figure 1. At each H_2O_2 concentration, the maximal concentration of ferrylHb was determined. For the heme degradation products, the concentration of fluorescent product was determined from the 465 nm fluorescence (excitation at 321 nm) for 30 min. To compare results obtained for the addition of different concentrations of hydrogen peroxide, the concentrations obtained at each H_2O_2 concentration are normalized relative to the values obtained at 2 mM H_2O_2: (■) ferrylHb and (●) fluorescence (excitation at 321 nm and emission at 465 nm).

Dependence of Fluorescent Product Formation on the Concentration of FerrylHb and Hydrogen Peroxide. As shown in Figure 2, the maximal amount of ferrylhemoglobin was reached within 3 min when a 10-fold excess of H_2O_2 reacts with oxyHb. To compare the ferrylHb formation with the production of fluorescent heme degradation products, the concentrations of fluorescent products were normalized to the maximal levels obtained within the 15 min time course. A comparison of the curve for fluorescent intensity with that obtained for the formation of ferrylHb indicates that at the point where the production of ferrylHb levels off (3 min) only 57% of the maximal concentration of fluorescent products has taken place. The observation that a major fraction of the heme degradation exists after the maximal level of ferrylHb has been reached indicates that the degradation is not a direct result of the formation of ferrylHb, but instead involves a subsequent reaction of ferrylHb. The percentage of H_2O_2 consumption is shown in the same figure. The correspondence between the formation of fluorescent products and the remaining H_2O_2, with the rate for the formation of fluorescent products approaching zero when all the H_2O_2 is used up, implicates H_2O_2 in the production of fluorescent products from ferrylHb.

The involvement of H_2O_2 in the formation of fluorescent products from ferrylHb was confirmed by the effect of varying the concentration of H_2O_2 shown in Figure 3. To be able to compare results obtained for a wide range of H_2O_2 concentrations, the levels of ferrylHb and fluorescent products that were formed were normalized to the maximal levels reached at 2 mM H_2O_2. Though the hemoglobin was completely converted to ferryhemoglobin at 0.5 mM H_2O_2, the extent of formation of fluorescent products increased with increasing H_2O_2 concentration over the entire concentration range.

Effect of the Addition of Catalase during the Reaction of Oxyhemoglobin with H_2O_2. To establish the role of H_2O_2 on the formation of fluorescent products, catalase was used to quench the residual H_2O_2 present 1.0, 2.0, 3.0, and 4.0 min after the reaction had been started. For each time point, the addition of catalase slowed the subsequent formation of fluorescent products with a partial inhibition of the final level of fluorescent products (Figure 4A,B). In a description of the results shown in this figure, the effect of catalase will be normalized relative to the level of fluorescent intensity obtained after 20 min without the addition of catalase.

The addition of catalase 1.0 min after the H_2O_2 had been added, prior to maximal ferrylHb formation (Figure 2), resulted in the continued formation of fluorescent products. For the 465 nm emission band, the fluorescence increased from 27 to 43% of the maximal fluorescence, and for the 525 nm emission band, the fluorescence increased from 11.5 to 38% of the maximal fluorescence. Similarly, the addition of catalase 2.0 min after the reaction started resulted in the fluorescence from the 465 nm emission band increasing from 46 to 58% of the maximal fluorescence and for the 525 nm emission band from 23 to 64% of the maximal fluorescence. The addition of catalase 3 min after the reaction began, at which time the maximal level of ferrylHb has already been formed (Figure 3), resulted in an increase in the fluorescence of the 465 nm emission band from 60 to 68% of the maximal fluorescence and of the 525 nm emission band from 34 to 78% of the maximal fluorescence. Similarly, the addition of catalase 4.0 min after the H_2O_2 had been added, which is after complete ferrylHb formation, still inhibited maximal fluorescence of the 465 nm band and 525 nm band by 21 and 14%, respectively. These results indicate that the quenching of H_2O_2 after maximal ferrylHb has been formed inhibits the formation of heme degradation products. Heme degradation, therefore, requires a reaction of H_2O_2 with ferrylHb. Furthermore, the production of heme degradation products (although to a lesser extent) after the removal of H_2O_2 by catalase indicates that the reaction of ferrylHb with H_2O_2 does not directly produce the heme degradation products. The degradation products must instead be produced as a result of reactive species formed during the reaction of ferrylHb with H_2O_2.

Hydrogen Peroxide Consumption. Figure 5 shows the consumption of H_2O_2 for the reaction of 50 μM oxyHb and 0.5 mM H_2O_2. Although the concentration of H_2O_2 was 10 times more than that of oxyHb, it was completely consumed within 15 min. The time course for the consumption of H_2O_2 is in fact the same even with an appreciably larger excess of H_2O_2 (data not shown). The consumption of these large excesses of H_2O_2 indicates that H_2O_2 was consumed by other processes in addition to the reactions of hydrogen peroxide with oxyHb and ferrylHb. To investigate the nature of these additional reactions, we have added cyanide to the reaction mixture. The extent of consumption of H_2O_2 was very significantly reduced in the presence of cyanide (Figure 5). Thus, in the presence of cyanide, which strongly binds to metHb, only about 15% of the 500 μM H_2O_2 was consumed in 15 min. The 75 μM H_2O_2 consumed can be accounted for by the initial reaction of oxyHb forming ferrylHb and the partial reaction of ferrylHb with additional H_2O_2. The 425 μM H_2O_2, which is not consumed in the presence of cyanide, can be attributed to reactions of H_2O_2 involving

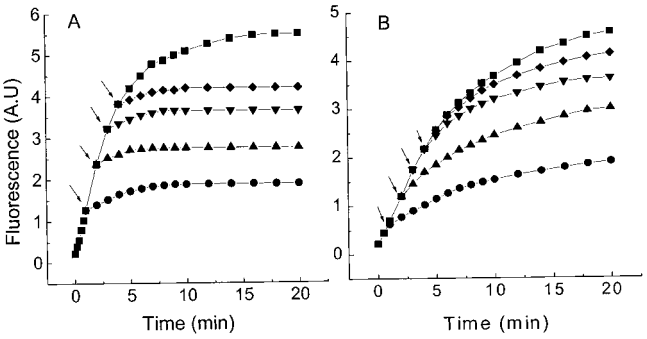

FIGURE 4: Effect of catalase on the time course of fluorescence formation. The reaction conditions were the same as those described in the legend of Figure 1. Catalase (200 units) was added at the indicated time points: (●) 1, (▲) 2, (▼) 3, and (◆) 4 min. During the progress of the reaction, the fluorescence was measured as a function of time: (A) excitation at 321 nm and emission at 465 nm and (B) excitation at 460 nm and emission at 525 nm.

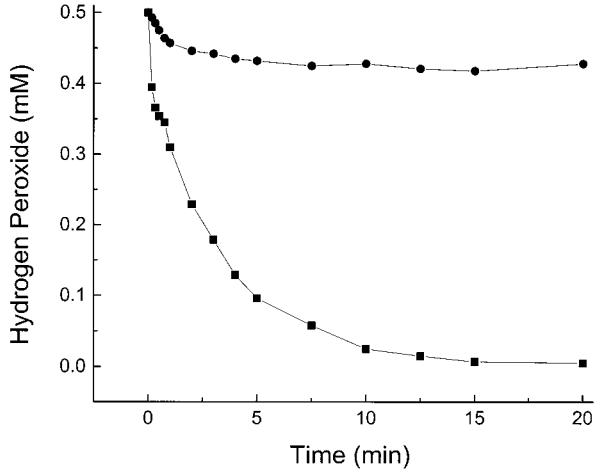

FIGURE 5: Consumption of hydrogen peroxide during the reaction with hemoglobin in the presence and absence of cyanide. The reaction mixture contained 50 μM oxyHb and 0.5 mM H_2O_2 in potassium phosphate buffer (pH 7.4). Aliquots of the reaction mixture were taken at different time intervals for the determination of H_2O_2 concentration as mentioned in Materials and Methods. Sodium cyanide was added to the incubation mixture 15 min prior to the addition of H_2O_2: (■) without cyanide and (●) with cyanide.

metHb (see below).

Oxygen Evolution. Figure 6 shows the evolution of oxygen during the reaction of oxyHb with H_2O_2. A gradual release of oxygen continued for 20 min, with negligible amounts liberated after longer periods of time. During this time, 175 nmol of oxygen was liberated. In the presence of cyanide, a dramatic decrease in the amount of oxygen that was released was observed. These results indicate that nearly all of the oxygen that is generated is associated with reactions involving metHb.

Fluorescent Bands Produced by Reactions with Ferriprotoporphyrin and Protoporphyrin. Figure 7 shows the emission spectra of ferriprotoporphyrin and protoporphyrin at an excitation of 321 nm (Figure 7A,C) and an excitation of 460 nm (Figure 7B,D) resulting from the reaction with potassium superoxide associated with crown ether in DMSO. The fluorescent spectra of ferriprotoporphrin (Figure 7A,B) are similar to those reported (*42*) for the reaction of H_2O_2 with ferriprotoporphyrin and oxyHb. The reaction with superoxide, however, takes place at an appreciably lower concentration than the reaction of ferriprotoporphyrin with H_2O_2. In

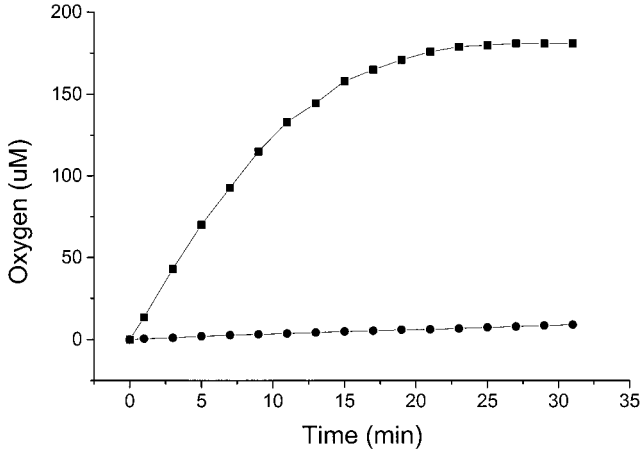

FIGURE 6: Oxygen generation during the reaction of oxyHb with H_2O_2 in the presence and absence of cyanide. The reaction mixture was the same as that described in the legend of Figure 5. The oxyHb was completely deoxygenated by nitrogen gas prior to the determination. Cyanide was added to the reaction mixture 15 min prior to the addition of hydrogen peroxide, using a gastight syringe. The release of oxygen was monitored as a function of time: (■) without cyanide and (●) with cyanide.

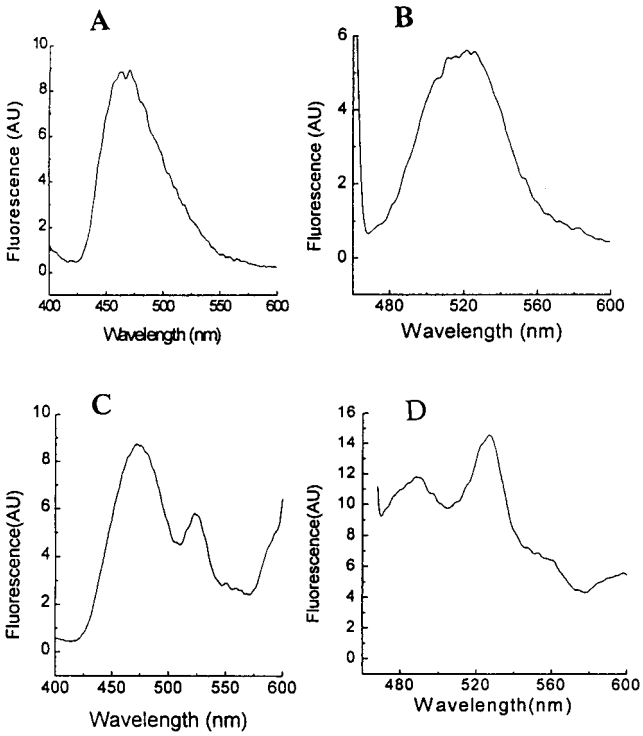

FIGURE 7: Fluorescent emission spectra produced during the reaction of potassium superoxide with ferriprotoporphyrin or protoporphyrin. Ferriprotoporphyrin or protoporphyrin (50 μM) was incubated with 100 μM potassium superoxide in dimethyl superoxide for 60 min at room temperature. (A) Emission spectra of ferriprotoporphyrin at an excitation wavelength of 321 nm. (B) Emission spectra of ferrirotoporphyrin at an excitation wavelength of 460 nm. (C) Emission spectra of protoporphrin at an excitation wavelength of 321 nm. (D) Emission spectra of protoporphyrin at an excitation wavelength of 460 nm.

addition, two fluorescent bands were observed during the reaction of protoporphyrin with potassium superoxide (Figure 7C,D). The reaction of H_2O_2 with protoporphyrin did not produce any fluorescent products (data not shown). These results establish that superoxide does react with porphyrins to produce heme degradation products. The greater reactivity

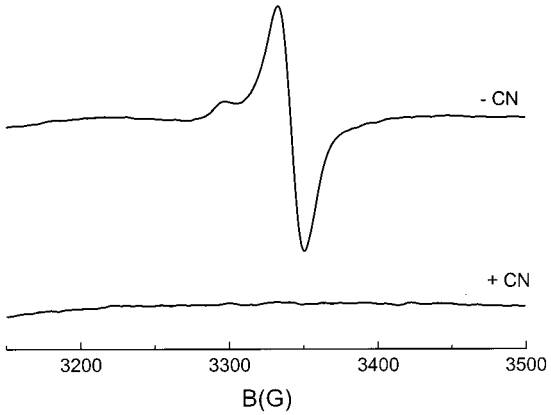

FIGURE 8: Free radical production during the reaction of metHb with hydrogen peroxide and the effect of cyanide. The EPR spectrum determined at 10 K for a sample quenched into liquid nitrogen 30 s after initiation of the reaction between metHb (0.4 mM) and hydrogen peroxide (4.5 mM) in 50 mM potassium phosphate buffer. For the spectrum obtained in the presence of cyanide, sodium cyanide (5.0 mM) was added 15 min prior to initiation of the reaction with H_2O_2.

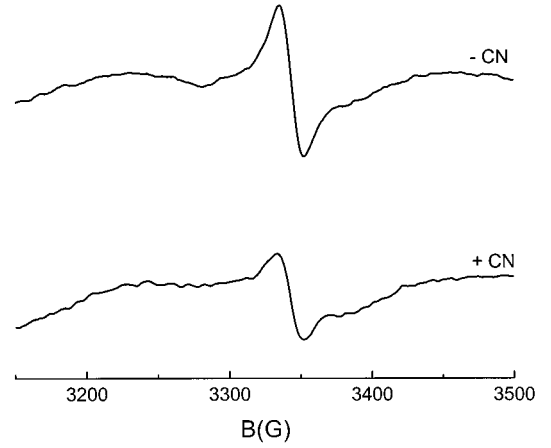

FIGURE 9: Free radical production during the reaction of oxyHb with hydrogen peroxide and the effect of sodium cyanide (5.0 mM). The reaction mixture contained 0.45 mM oxyHb, 4.5 mM H_2O_2, and 5 mM sodium cyanide in 50 mM potassium phosphate buffer (pH 7.4). The EPR spectrum was determined at 10 K on a sample quenched into liquid nitrogen 30 s after the reaction was initiated by the addition of H_2O_2.

of superoxide compared to that previously reported (*42*) for H_2O_2 suggests that superoxide may be the reactive species responsible for heme degradation. Ligation of iron to porphyrin is presumably essential for H_2O_2-mediated heme degradation, but not for superoxide-mediated heme degradation.

Electron Paramagnetic Resonance (EPR). The addition of H_2O_2 to metHb(III) produces a free radical signal in the region of 3200 G (Figure 8). The free radical signal was much more intense than the $g = 2$ signal found as a trough in this region of the metHb spectrum (*47*). This signal has been attributed to the production of the protein radical associated with oxoferrylHb (*48*). The reaction of Fe(II) hemoglobins (deoxyHb or oxyHb) with H_2O_2 to produce ferrylHb does not produce a globin radical. Nevertheless, the EPR in the 3200 G region indicates the formation of a free radical signal (Figure 9), which has, however, an intensity much lower than that produced by metHb.

Since metHb is produced as an intermediate during the reaction of H_2O_2 with Fe(II) hemoglobins (see below), it was necessary to eliminate any contribution of metHb to the free radical signal shown in Figure 9. Cyanide, which rapidly binds to the Fe(III) heme iron, dramatically inhibits reactions of hydrogen peroxide with metHb as indicated by Figures 5 and 6. Cyanide is, therefore, expected to minimize the formation of oxoferrylHb and the protein radical. As shown in Figure 8, the presence of cyanide prevented the formation of any detectable radical during the reaction of H_2O_2 with metHb. However, as shown in Figure 9, even in the presence of cyanide, a free radical is produced during the reaction of oxyHb with H_2O_2. The 45% decrease in the magnitude of the free radical signal can be attributed to the metHb, which is formed during the reaction. Complete elimination of the free radical signal produced during the reaction of oxyHb with H_2O_2 was, however, observed when Na_2S was added to the reaction mixture (data not shown). A reaction involving ferrylHb is, therefore, shown to be responsible for the formation of these free radicals.

DISCUSSION

The Reaction of Ferrylhemoglobin with Hydrogen Peroxide To Produce Superoxide Is Required for Heme Degradation. Our previous studies (*42*) have shown that the heme moiety is partially degraded during the reaction of H_2O_2 with oxyHb, but not metHb. The heme degradation products during this reaction were identified by their characteristic fluorescence (*42*). We have now established the reactions responsible for heme degradation.

Ferrylhemoglobin. The requirement for ferrylHb is indicated by the dramatic inhibition (Figure 1) of the formation of fluorescent products, i.e., heme degradation, when ferrylHb reacts with peroxidase substrates or sodium sulfide. Thus, the peroxidase substrates ABTS and *o*-dianisidine, which reduce ferrylHb to metHb, completely inhibit the formation of fluorescent products, and sodium sulfide, which reacts with ferrylHb to produce sulfhemoglobin, results in a 75% inhibition of fluorescent product formation. These results indicate that heme degradation involves reactions of ferrylhemoglobin and not the primary reaction of hydrogen peroxide with hemoglobin.

Although the reaction of hemoglobin with H_2O_2 to form ferrylHb

$$\mathrm{HbFe(II)O_2 + H_2O_2 \rightarrow HbFe(IV){=}O + H_2O + O_2} \quad (1)$$

has been extensively studied (*36*, *38*), and the Fe(IV) heme proteins (ferryl and oxoferryl) are considered to be highly reactive pro-oxidants (*37*−*41*, *49*), their potential reaction with hydrogen peroxide has not been considered.

Reaction of Hydrogen Peroxide with Ferrylhemoglobin. A reaction between ferrylHb and H_2O_2 that is associated with heme degradation is unambiguously indicated by the results given in Figures 2−4. Thus, Figure 2 shows that fluorescent products are continuously made as long as H_2O_2 is still present in solution (even after maximal levels of ferrylHb are formed). Figure 3 shows that higher levels of fluorescent products are produced by increased concentrations of H_2O_2, even though the elevated H_2O_2 level has no effect on the maximal level of ferrylHb. Finally, quenching of unreacted H_2O_2 by catalase, even after the maximal level of ferrylHb

has been formed (Figure 2), inhibits the subsequent formation of heme degradation products (Figure 4). These studies for the first time establish that H_2O_2 directly reacts with ferrylHb.

A Role for Superoxide. The formation of fluorescent products, even after catalase is added to the reaction mixture of oxyHb and H_2O_2 (Figure 4), indicates that heme degradation does not result directly from the reaction of H_2O_2 with ferrylHb, but involves reactive species generated during this reaction. Electron paramagnetic resonance was used to identify the reactive species formed when H_2O_2 reacts with ferrylHb (Figures 8 and 9).

To detect these reactive species, it was necessary to prevent the formation of the protein radicals formed when H_2O_2 reacts with metHb (Figure 8). This was accomplished by using cyanide to react with metHb and minimize any further reaction of metHb with H_2O_2 (Figures 5, 8, and 9). The decrease in the extent of hydrogen peroxide consumption in the presence of cyanide (Figure 5) indicates that the reaction of hydrogen peroxide with metHb is dramatically inhibited by the binding of cyanide to the Fe(III) heme of hemoglobin. The EPR spectrum of metHb reacted with hydrogen peroxide in the presence of cyanide shows that the protein radical formed when hydrogen peroxide reacts with metHb is no longer observed (Figure 8).

The residual free radical signal obtained when cyanide is added to the reaction mixture of oxyHb and H_2O_2 (Figure 9) is, therefore, due to radicals produced during the reaction of H_2O_2 and ferrylHb. The involvement of ferrylHb in the production of this free radical is established by the complete inhibition when Na_2S was added. The similarity of the EPR parameters for the signal detected during the reaction of H_2O_2 with hemoglobin (Figure 9) and those previously reported (*19*) for superoxide generated in the heme pocket suggests that the free radical superoxide is formed during the one-electron oxidation of hydrogen peroxide.

$$\mathrm{HbFe(IV){=}O} + \mathrm{H_2O_2} \rightarrow \mathrm{HbFe(III)} + \mathrm{O_2^{\bullet -}} + \mathrm{H_2O} \quad (2)$$

FerrylHb being a strong oxidizing agent abstracts an electron from H_2O_2 and is reduced to methemoglobin, while the H_2O_2 is oxidized to superoxide. This reaction can be understood in relation to the known peroxidase activity of Hb, which is analogous to that of horseradish peroxidase. In the classical peroxidase reaction, H_2O_2 withdraws two electrons from the enzyme, producing intermediate compound **1**. This compound withdraws one electron from the substrate, forming compound **2**. Compound **2** then withdraws another electron from the substrate to produce the original enzyme and product (*50*). FerrylHb, the heme iron being one oxidizing equivalent above that of metHb, corresponds to peroxidase compound **2**, and can withdraw one electron from the substrate. In the absence of substrates, ferrylHb would oxidize H_2O_2 to produce superoxide and metHb (reaction 2).

Peroxidases have also been shown (*51*) to react with cyanide, producing cyanide radicals. In fact, metHb, consistent with its peroxidase activity, has been shown by spin trapping to produce cyanide radicals when it reacts with hydrogen peroxide in the presence of cyanide. Appreciably lower levels of cyanide radicals are produced with metHb than with lactoperoxidase (*51*). However, in our experiment, we detect no EPR signal (Figure 8) when hydrogen peroxide reacts with metHb in the presence of cyanide. This observation suggests that if cyanide radicals are produced they have a relaxation time that is too short to be directly detected in the temperature range used in our studies. FerrylHb (compound **2**) would be a poorer oxidizing agent for cyanide than the oxyferryl (compound **1**) produced with metHb (*51*), and any cyanide radicals that formed would not be detected under our experimental conditions. A contribution of cyanide radicals to the EPR signals observed in Figure 9 can, therefore, be ruled out.

Giulivi and Davies (*52*) have also considered the possibility of reaction 2. They, however, have dismissed this reaction in favor a process whereby metHb is produced from ferrylHb via a comproportionation reaction involving the reduction of the ferryl by a tyrosine residue. The resultant tyrosine radical is then reduced by another Fe(II) heme to yield a second oxidized heme. They, however, did not perform EPR studies and could, therefore, not directly detect the superoxide. They were also not using heme degradation as an end point and could, therefore, not perform the time-dependent, concentration-dependent, and catalase experiments, which clearly establish a reaction of H_2O_2 with ferrylHb. They do have evidence for comproportionation under their experimental conditions where low levels of H_2O_2 are continuously being generated by glucose oxidase. Under our experimental conditions involving a large excess of H_2O_2 added in one bolus, the comproportionation reaction is less favored. In our experiments, non-H_2O_2-generated reduction of ferrylHb to met, which may be related to their comproportionation reaction, is only a minor reaction.

Superoxide produced in the hydrophobic heme pocket is not only at the right location to react with porphyrin, but because of the aprotic environment of the heme pocket, the superoxide exhibits greater reactivity (*53*). Enhanced reactivity can also be attributed to the protonation of the superoxide producing the perhydroxyl radical ($^{\bullet}$OOH). The perhydroxyl radical is a strong oxidizing agent known to initiate lipid peroxidation (*54*). The reaction involving superoxide in the heme pocket also explains the observation that superoxide dismutase, which cannot enter the heme pocket, does not inhibit fluorescent product formation.

These results demonstrate the importance of generating free radicals at the proper site. Thus, although superoxide, and even the perhydroxyl radical, is much less reactive than the hydroxyl radical, the results in Figure 1 indicate that the $^{\bullet}$OH radical does not contribute to H_2O_2-generated heme degradation. Thus, no effects on the formation of fluorescent products (Figure 1) were observed when, prior to the addition of H_2O_2, the sample was treated with ferrous sulfate (a Fenton reagent), iron-complexing agents DTPA and EDTA, or $^{\bullet}$OH radical scavengers such as mannitol, benzoic acid, and dimethyl sulfoxide. The partial inhibition of fluorescent product formation by thiourea and desferrioxamine (Figure 1) can be attributed to nonspecific effects of these reagents (*55*−*58*).

Our results on degradation of both protoporphyrin and ferriprotoporphyrin by superoxide (Figure 7) are consistent with the reported (*59*) observation that superoxide generated by xanthine oxidase and xanthine reacts with tetrapyrole rings of heme and, thereby, bleaches heme proteins. These results confirm the proposed role for superoxide in heme degradation (eq 2). The superoxide generated in the heme pocket oxidizes

the tetrapyrole rings, leading to the degradation of heme, the release of iron, and the formation of two fluorescent products (excitation at 321 nm and emission at 465 nm) and (excitation at 460 nm and emission at 525 nm).

These results establish the reaction pathway for the formation of heme degradation products in the course of the reaction of hemoglobin with hydrogen peroxide.

$$\text{Hb(II)} + H_2O_2 \rightarrow \text{HbFe(IV)=O} + H_2O_2 \rightarrow \text{HbFe(III)} + O_2^{\bullet -} \rightarrow \text{heme degradation} \quad (3)$$

Competitive Reactions That Limit the Level of Heme Degradation. While this scheme provides the basis for heme degradation, it does not explain the low level of heme degradation products that are formed. Since each of the steps in this scheme is for the most part irreversible, the level of heme degradation products should continue to increase with time, and for an excess of H_2O_2, the concentration of these products should approach that of the starting hemoglobin concentration. We, however, find that, even with a 10-fold excess of H_2O_2, only 4.9% of the hemes are degraded after 15 min, when the formation of fluorescent products has leveled off.

To explain the actual amount of heme degradation products, it is necessary to consider alternative reactions involving various products generated prior to heme degradation.

$$\begin{array}{l} \text{Hb(II)} + H_2O_2 \rightarrow \text{HbFe(IV)=O} + H_2O_2 \rightarrow \\ \qquad\qquad\qquad\qquad \downarrow \qquad\quad \downarrow \qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad \text{Fe(III)Hb} + O_2^{\bullet -} \rightarrow \text{heme degradation} \\ \qquad\qquad\qquad\qquad\quad \downarrow \qquad\quad \downarrow \end{array}$$

Vertical arrows in the scheme shown above indicate the alternative reactions, which limit the efficiency of heme degradation. Superoxide produced in the heme pocket via reaction 2 can also reduce (*60*) the oxidized Fe(III)Hb and/or leak out of the heme pocket prior to reacting with the heme (*61*). Heme degradation will also be affected by any competing reactions, which use up the hydrogen peroxide required for both the formation of ferrylHb and the reaction of ferrylHb to produce superoxide. The results shown in Figures 5 and 8 show that cyanide inhibits most of the hydrogen peroxide consumption by binding to the Fe(III) heme, preventing the reactions of metHb with hydrogen peroxide.

$$\text{HbFe(III)} + H_2O_2 \rightarrow {}^{\bullet}\text{HbFe(IV)=O} + H_2O \quad (4)$$

OxoferrylHb is two oxidizing equivalents above metHb. It is, therefore, equivalent to peroxidase compound **1**, except that the second oxidizing equivalent exists on the globin instead of the porphyrin. In the absence of substrates, oxoferrylHb will also react with H_2O_2. Although the first step in this reaction may generate superoxide (*62*), this superoxide rapidly reacts with the one-electron-reduced oxoferryl to produce metHb and oxygen. The lifetime of this transient superoxide is much shorter than that produced by the reaction of ferrylHb, and no fluorescent heme degradation is observed. Therefore, metHb in the absence of substrates has catalase type activity in which one molecule of H_2O_2 serves as a two-electron acceptor, and another H_2O_2 molecule serves as a two-electron donor producing an oxygen molecule. The consumption of hydrogen peroxide

$${}^{\bullet}\text{HbFe(IV)=O} + H_2O_2 \rightarrow \text{HbFe(III)} + O_2 + H_2O \quad (5)$$

via reactions 4 and 5 plays a dominant role in limiting the reaction of ferrylhemoglobin with hydrogen peroxide and, thereby, heme degradation.

The oxygen generation during the reaction of hydrogen peroxide with oxyHb (Figure 6) in the absence of added cyanide is evidence for reaction 5. A reaction of the oxoferrylHb with H_2O_2 is also supported by the observation that the globin free radical (Figure 8) has a lower intensity with a larger excess of H_2O_2 (results not shown). A faster decay of the globin radical in the presence of excess H_2O_2 has been previously reported, but has not been explained (*48*, *49*).

Unlike the reactions involving HbFe(II), which utilize only two molecules of H_2O_2 (reactions 1 and 2), the reaction of HbFe(III) with H_2O_2 (reactions 4 and 5) regenerates Fe(III) and will, therefore, continue to use up H_2O_2, providing the basis for the catalase-like activity of Fe(III) heme proteins (*63*).

The competition between oxyHb (reaction 1), metHb (reaction 4), ferrylHb (reaction 2), and oxoferrylHb (reaction 5) for H_2O_2 limits the level of heme degradation products, as well as the maximal level of ferrylHb produced. A 10-fold excess of H_2O_2 is, therefore, necessary for complete conversion of hemoglobin to ferrylHb.

An additional factor, which may contribute to the concentration of ferrylHb formed, is the spontaneous decay of ferrylHb to metHb. While our studies emphasize the importance of the reaction between hydrogen peroxide and ferrylHb, most investigators consider some form of spontaneous decay to be the primary mechanism for the elimination of Fe(IV) species (*38*, *52*, *65*). We have also found that after the addition of catalase the ferrylHb is slowly converted to a methemoglobin (results not shown). The metHb produced during this decay requires an extra electron, presumably from the globin. The disproportionation reaction (*52*) takes an electron from amino acid side chains of the globin. Evidence has also been reported for cross-linking between the heme and a tyrosine residue of the globin molecule during the reaction with H_2O_2 (*66*, *67*).

MetHb is generally considered more toxic than the reduced form of hemoglobin (*31*). Our results, however, indicate that toxic effects derived from the Fe(II) hemoglobin need to be considered. This prediction is consistent with the reported degradation of the heme moiety (*68*, *69*) under reducing conditions, which was inhibited by catalase. It is also of interest to note that Fe(II) hemoglobin in the presence of H_2O_2 has been implicated in lipid peroxidation (*70*) and deoxyribose degradation (*6*).

In conclusion, the ferrylHb formed by the reaction of hemoglobin with hydrogen peroxide reacts with another molecule of hydrogen peroxide to produce superoxide in the heme pocket. This superoxide is responsible for heme degradation. The oxoferrylHb formed by the reaction of methemoglobin with hydrogen peroxide produces oxygen instead of superoxide, and no heme degradation takes place. The production of these heme degradation products and free iron may be responsible for the toxicity associated with reduced hemoglobin.

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