Inhibition of Methemoglobin Formation from Purified Oxyhemoglobin by Superoxide Dismutase†

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ABSTRACT: To test further the hypothesis that O_2^- can mediate the formation of methemoglobin from oxyhemoglobin, the formation of methemoglobin from chromatographically purified oxyhemoglobin was studied in the same photochemical O_2^- generating system used to study methemoglobin formation in red cell lysates (Lynch, R. E., Lee, G. R., and Cartwright, G. E. (1976), *J. Biol. Chem. 251,* 1015). The results differed from those in lysates in that the product of the reaction in solutions of purified oxyhemoglobin was not methemoglobin and superoxide dismutase did not inhibit the reaction. In addition, the absorbance loss at the 577-nm maximum of oxyhemoglobin was faster than in lysates. In the presence of catalase the absorbance change at 577 nm slowed, methemoglobin became the product, and superoxide dismutase again inhibited the reaction. Hydrogen peroxide, when present in concentrations greater than 94 μM, a concentration exceeded in the photochemical system, produced spectral changes in oxyhemoglobin solutions like those observed in the photochemical system. Thus, H_2O_2, in high concentrations, could react with oxyhemoglobin faster than did O_2^-, and possibly in competition with O_2^-, to yield products other than methemoglobin. In the presence of catalase this reaction could be abolished, thereby allowing the slower reaction of O_2^- with oxyhemoglobin to be detected. The hypothesis that O_2^- can mediate the formation of methemoglobin from oxyhemoglobin was also examined in experiments which attempted to explain the inhibitory effect of superoxide dismutase in other ways. Superoxide dismutase did not inhibit the inactivation of catalase nor did catalase prevent the inactivation of superoxide dismutase. Singlet oxygen scavengers, dimethylfuran and histidine, failed to inhibit methemoglobin formation in lysates. Superoxide dismutase inhibited rather than accelerated the "reverse" reaction (methemoglobin → oxyhemoglobin), suggesting that O_2^- can convert oxyhemoglobin to methemoglobin and back to oxyhemoglobin, thereby acting catalytically as a superoxide dismutase.

We have reported that superoxide dismutase inhibited the formation of methemoglobin from oxyhemoglobin in oxygenated solutions of photoreduced riboflavin (Lynch et al., 1976). We proposed that methemoglobin was formed when O_2^- reduced the heme bound oxygen by one electron leading to the dissociation of methemoglobin and H_2O_2:

$$\underset{\text{(oxyHb)}}{HbFe^{3+}\text{-}O_2^-} + O_2^- + 2H^+ \rightarrow \underset{\text{(metHb)}}{HbFe^{3+}} + O_2 + H_2O_2$$

It seemed possible that superoxide dismutase inhibited the reaction in vivo, thereby helping to maintain hemoglobin in a functional state.

Because these experiments were performed in erythrocyte lysates, O_2^- might have acted indirectly by affecting the activity of some constituent of the lysate. Consequently, we reinvestigated methemoglobin formation in solutions of hemoglobin purified by ion exchange chromatography. We now present the different results in the more purified system. The removal of catalase from the lysate during the purification of hemoglobin is shown to account for the differences between the results in the lysate and those in the purified system and an explanation for the catalase effect is advanced. Because superoxide dismutase would inhibit reactions mediated not only by O_2^- but also by species derived from it, the role of $O_2(^1\Delta g)$ is examined by testing the effects of scavengers of $O_2(^1\Delta g)$. The results of experiments testing hypotheses that explain the effect of superoxide dismutase in various alternate ways are also presented. The results of one of these in which superoxide dismutase is shown to inhibit the "reverse" reaction (methemoglobin → oxyhemoglobin) provide an explanation for the weak superoxide dismutase activity of hemoglobin.

Experimental Procedure

Reagents. Bovine erythrocyte superoxide dismutase (Truett) and bovine catalase (Boehringer) were each found to be virtually free of contamination by the other (Lynch et al., 1976). Before use catalase was dialyzed to remove the thymol preservative.

Preparation of Hemoglobin Solutions. Pig red cells were sedimented from either citrated or heparinized blood by centrifugation and washed three times in 0.15 M NaCl. The buffy coat was aspirated with each wash. Packed, washed red cells were lysed in 25 vol of distilled, deionized water. The ghosts were sedimented by centrifugation at 17 000*g* for 20 min at 4 °C. After adjustment of the pH to 6.8 when necessary, the supernatant was applied to a carboxymethyl-cellulose column (CM52, Whatman) previously equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed with 5 mM potassium phosphate buffer (pH 6.8) until the effluent was colorless. The adsorbed hemoglobin was eluted with 1 M potassium phosphate (pH 6.8). Neither superoxide dismutase nor catalase was detectable in the concentrated hemoglobin solution so eluted. Hemoglobin prepared in this manner is referred to hereafter as the homoglobin solution to distinguish it from the lysate.

Methemoglobin was prepared by reaction of this carboxymethyl-cellulose eluate with an excess of $K_3Fe(CN)_6$. The ferri- and ferrocyanide were separated from the methemoglobin by gel filtration chromatography in 1.0 M potassium

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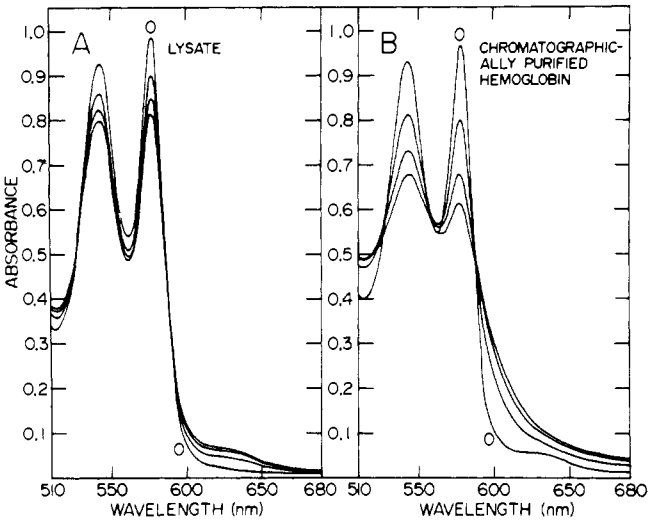

FIGURE 1: The spectral changes in a lysate (A) and in a solution of chromatographically purified hemoglobin (B) in the photochemical system. A lysate and the hemoglobin solution obtained therefrom were subjected to the flux of photochemically generated superoxide anions at pH 6.8, 60 mM EDTA as described under Experimental Procedure. At 3-min intervals the light was interrupted, the O_2 inlet was removed, and the visible absorption spectrum was recorded with a Cary 118C spectrophotometer. 0 indicates initial spectrum.

phosphate buffer (pH 6.8) on Sephadex G-25 (Pharmacia). The Prussian blue reaction for ferrocyanide was negative in fractions containing methemoglobin after precipitation with 7.5% trichloroacetic acid.

Fractionation of Nonhemoglobin Proteins on DEAE[1]*-cellulose.* Hemolysates, free of stroma, were prepared as described above, the supernatant fluid was diluted further with 4 vol of distilled water, and the pH was adjusted to 7 with potassium hydroxide. The diluted lysate was applied to a 2.5 × 22 cm column of DEAE-cellulose (Whatman DE-52) equilibrated with 0.003 M potassium phosphate buffer (pH 7.0) (Haut et al., 1964). After washing the column with 134 mL of 0.003 M potassium phosphate buffer (pH 7) the nonhemoglobin proteins were eluted with 500 mL of a linear concentration gradient of potassium phosphate buffer (pH 7) (0.003–0.5 M).

Assays. Superoxide dismutase was assayed by its inhibitory effect on the superoxide-mediated reduction of cytochrome *c* (McCord and Fridovich, 1969). Superoxide for this assay was generated by the enzymic activity of xanthine oxidase with xanthine as substrate. Catalase was assayed by determining the rate of decrease in absorbance at 240 nm of 22.5 mM hydrogen peroxide in 0.1 M phosphate buffer (pH 7) at 25 °C (Beers and Sizer, 1952). Catalase was also assayed by determining the rate of change in hydrogen peroxide concentration in solutions of 4.2 mM hydrogen peroxide–0.01 M phosphate buffer (pH 6.8) by titration with 0.002 M potassium permanganate (Takahara et al., 1960). Hydrogen peroxide concentrations were determined by measuring the conversion by lactoperoxidase of 4.6 mM *p*-phenylenediamine to its colored product(s) at 525 nm in 0.1 M phosphate buffer at room temperature. Standard curves were linear in the range of 10–300 μM. The absorbance at 525 nm per mol of H_2O_2 per cm was 4100 in a representative standard curve. The hydrogen peroxide concentration in the concentrated stock solution (nominally 30%) was assayed by absorbance at 240 nm at pH 7 in a Cary 118C recording spectrophotometer and calculations were based on a molar extinction coefficient of 43.6 cm^{-1} (Noble and Gibson, 1970) and verified by permanganate titration.

Photochemical Superoxide Generating System. Solutions of 53 μM riboflavin, 20–100 mM EDTA, 0.1 M phosphate buffer (pH 6.8 or 7) and various concentrations of hemoglobin were illuminated by a 750-W tungsten lamp. The light passed through a 448-nm interference filter before striking the oxygen-bubbled solutions in quartz cuvettes in a reflective aluminum cuvette holder at 20 °C (Lynch et al., 1976).

Results

Differences between the Reactions Observed in Lysates and in Solutions of Chromatographically Purified Hemoglobin. When 15 μM hemoglobin solutions were illuminated in the photochemical system at pH 6.8 with 60 mM EDTA, the spectral changes (Figure 1) were different from those seen with lysates. The rate of decrease in absorbance at 577 nm was much greater than in the lysate under the same conditions. Although a 630-nm absorbance maximum was present at zero time in hemoglobin solutions, representing 10% methemoglobin formed spontaneously, this peak was not preserved in the product(s) of the photochemical reaction, indicating that the products did not include significant amounts of methemoglobin. Superoxide dismutase in a concentration (12.5 μg/mL) at which methemoglobin formation in the lysates from which the hemoglobin was purified was inhibited by 85%, produced no inhibition of the absorbance change in solutions of purified hemoglobin.

Effects of Erythrocyte Proteins Other Than Hemoglobin. A pig red cell lysate was chromatographed on DEAE-cellulose. After hemoglobin, five peaks absorbing at 280 nm eluted from the column. The fractions comprising each peak were combined, yielding five pools. Aliquots from each pool were tested for their effects on the spectral changes exhibited by purified oxyhemoglobin in the photochemical system. The first peak to emerge after the gradient commenced contained a component which restored to the reaction of the hemoglobin solution the characteristics of the reaction in the lysate. The rate of absorbance change at 577 nm was reduced, the 630-nm absorbance maximum of methemoglobin appeared, and superoxide dismutase inhibited methemoglobin formation. Over 90% of the catalase activity recovered was found in this peak. None of the other peaks had these effects. The effects of crystalline bovine liver catalase on methemoglobin formation from chromatographically purified oxyhemoglobin were also examined. Hemoglobin solutions were illuminated in the photochemical system at pH 6.8 with 60 mM EDTA in the presence and absence of catalase (12.5 μg/mL). The beef liver catalase, like the catalase from porcine erythrocytes, caused the purified oxyhemoglobin to behave as it had in lysates.

Mechanism of the Catalase Effect. To determine whether H_2O_2 could mimic the effect of the photochemical system on purified oxyhemoglobin, H_2O_2 in varying concentrations was added to aerobic solutions of hemoglobin. The rate of absorbance change at 577 nm increased with the H_2O_2 concentration from 18.7 through 93.7 μM (Table I), and throughout this range the 630-nm absorption maximum of methemoglobin was observed. At concentrations of H_2O_2 greater than 93.7 μM the 630-nm absorbance peak of methemoglobin was lost but the rate of absorbance loss at 577 nm increased still further, yielding products with a spectrum like that of the products of purified oxyhemoglobin in the photochemical system.

It remained to be determined whether sufficient H_2O_2 to account for the changes observed was present in the photo-

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Effect of Hydrogen Peroxide on the Visible Absorption Spectrum of Oxyhemoglobin.[a]

$[H_2O_2]$ (μM)	A_{577} change in 3 min	Methemoglobin formed
18.7	0.069	+
28.2	0.104	+
37.6	0.135	+
56.5	0.202	+
75.3	0.212	+
93.7	0.260	+/−
113	0.290	−
188	0.314	−
226	0.317	−
377	0.344	−

[a] Hemoglobin solutions (15 μM) were incubated at ambient temperature with H_2O_2 at the concentrations indicated in 0.1 M phosphate buffer (pH 7). The visible absorption spectrum was recorded after 3 min. When an absorbance maximum at 630 nm was observed, methemoglobin was judged to be present.

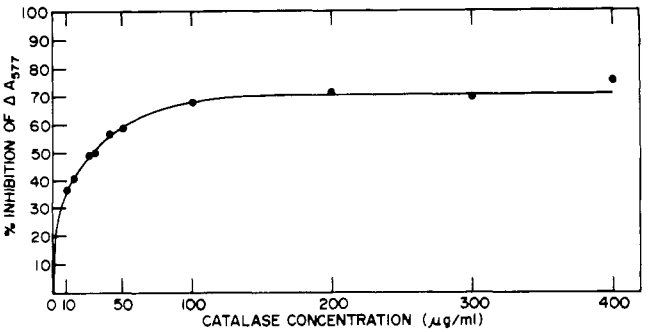

FIGURE 2: The effect of catalase concentration on the rate of absorbance change at 577 nm of chromatographically purified hemoglobin in the photochemical system. Hemoglobin solutions (15 μM) were subjected to the flux of photochemically generated superoxide anions at pH 7.0, 20 mM EDTA. After 6 min the light beam was interrupted, the oxygen inlet was removed, and the visible absorption spectrum was recorded. The loss of absorbance in 6 min of illumination in the absence of catalase was the uninhibited rate.

chemical system. The H_2O_2 concentration in aliquots from the complete, illuminated, oxygen-bubbled, photochemical system was found to reach 730 μM after 9 min of illumination in the presence of chromatographically purified oxyhemoglobin. Thus, at the high concentrations reached in the photochemical system, H_2O_2 appeared to react with oxyhemoglobin faster than did O_2^-, yielding nonmethemoglobin products, whereas in the presence of catalase the slower, O_2^--dependent conversion of oxyhemoglobin to methemoglobin could be observed.

The observation that superoxide dismutase inhibited methemoglobin formation only in the presence of catalase might be explained by assuming that superoxide dismutase acted to protect catalase from inactivation by O_2^- and that catalase in turn prevented methemoglobin formation. If this were correct the addition of a sufficient concentration of catalase alone should completely inhibit methemoglobin formation. This was not observed (Figure 2). As the catalase concentration in the photochemical system increased, the rate of the oxyhemoglobin absorbance change at 577 nm decreased until maximum inhibition of about 70% was observed at a catalase concentration of 100 μg/mL. At this concentration the addition of superoxide dismutase, 1.5 μg/mL, inhibited methemoglobin formation completely. Since at this level catalase inhibition was at a maximum, the superoxide dismutase effect could not be accounted for by any protective effects on catalase.

It was also possible that catalase was required for superoxide dismutase to have an effect because in the absence of catalase H_2O_2 inactivated superoxide dismutase. Solutions of superoxide dismutase (15 units/mL), 53 μM riboflavin, 0.1 M phosphate buffer (pH 7), and 0.1 M EDTA were illuminated and oxygenated at 20 °C in the presence and absence of catalase. At intervals aliquots were removed for superoxide dismutase assay. No inactivation of superoxide dismutase could be detected and catalase had no effect on superoxide dismutase activity.

Studies of the Possible Roles of Other Active Oxygen Species. Superoxide has been reported (Pederson and Aust, 1975; Kellogg and Fridovich, 1975; Beauchamp and Fridovich, 1970) to react with H_2O_2, forming both OH· and $O_2(^1\Delta g)$. Therefore, a process inhibited by superoxide dismutase may be caused by OH· or $O_2(^1\Delta g)$ rather than by O_2^-. Mannitol, a scavenger of hydroxyl radicals, did not inhibit methemoglobin formation in lysates (Lynch et al., 1976). The effects of the $O_2(^1\Delta g)$ scavengers, 1 mM 2,5-dimethylfuran and 5 mM histidine, on the rates of methemoglobin formation in lysates were tested. Neither inhibited methemoglobin formation in the photochemical system at pH 7 with 60 mM EDTA.

The Effects of Superoxide Dismutase on the Conversion of Methemoglobin to Oxyhemoglobin. Another way in which superoxide dismutase could appear to inhibit methemoglobin formation is by accelerating the "reverse" reaction, the conversion of methemoglobin or oxyhemoglobin. Massey et al. (1971) have shown that the autoxidation of reduced riboflavin is retarded by superoxide dismutase, implicating O_2^- in the reaction. Thus, superoxide dismutase would be expected to increase the steady-state concentration of reduced riboflavin in the photochemical system. If reduced riboflavin could convert methemoglobin to oxyhemoglobin, superoxide dismutase, by increasing the reduced riboflavin concentration, could appear to slow the conversion of oxyhemoglobin to methemoglobin.

The ability of reduced riboflavin to convert methemoglobin to oxyhemoglobin was tested in an anaerobic Thunberg cuvette. When an anaerobic solution of reduced riboflavin was mixed with methemoglobin the spectrum of deoxyhemoglobin was obtained (Figure 3). The subsequent admission of air gave the spectrum of oxyhemoglobin. Reduced riboflavin can thus reduce methemoglobin to deoxyhemoglobin which, in the presence of oxygen, yields oxyhemoglobin.

The effect of superoxide dismutase on the conversion of methemoglobin to oxyhemoglobin in the photochemical system was tested. Contrary to what might have been expected from the considerations outlined above, the rate of oxyhemoglobin formation from methemoglobin was greater in the absence than in the presence of superoxide dismutase (Figure 4).

Discussion

These studies demonstrate that the spectral changes of purified oxyhemoglobin in our photochemical system differ in at least three respects from methemoglobin formation in lysates. The rate of absorbance loss at 577 nm is greater in the purified system, the product (s) is not methemoglobin, and superoxide dismutase does not inhibit the reaction. The differences are attributable to the absence of catalase in the purified hemoglobin solution since the addition either of crystalline beef liver catalase or of a catalase-rich chromatographic fraction from red cell lysates abolished the differences.

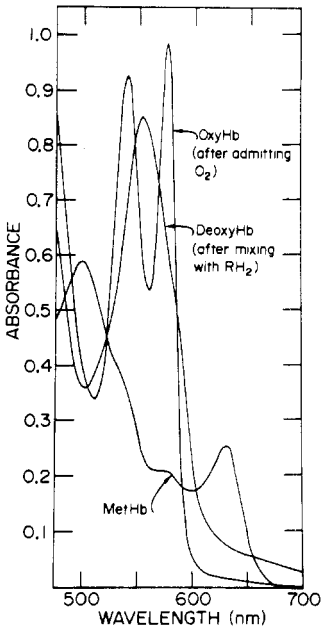

FIGURE 3: The effect of photoreduced riboflavin on the visible absorption spectrum of methemoglobin. A Thunberg cuvette with methemoglobin in the side arm and 10 mM EDTA, 0.1 M phosphate buffer (pH 7), and 53 μM riboflavin (all final concentrations after mixing) in the main compartment was subjected to six cycles of evacuation-N_2 admission. The main compartment was illuminated until the spectrum of reduced riboflavin was obtained. The side-arm contents were mixed thoroughly with the contents of the main compartment and the visible absorption spectrum was recorded, ending where riboflavin absorbance commenced. Air was admitted and the visible absorption spectrum recorded again.

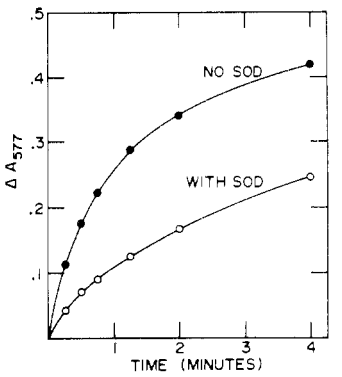

FIGURE 4: The effect of superoxide dismutase on the rate of conversion of methemoglobin to oxyhemoglobin. Two solutions which were identical except for the presence or absence of superoxide dismutase (6.7 μg/mL) were illuminated and bubbled with O_2 at 20 °C as described in the Experimental Procedure. Both solutions contained 15 μM hemoglobin, 0.1 M phosphate buffer (pH 7), 53 μM riboflavin, 66.7 μg/mL catalase, and 20 mM EDTA. At intervals the cuvettes were removed from the light, the oxygen inlets were removed, and the visible absorption spectra were recorded.

Catalase prevented the reaction of H_2O_2 at high concentration with oxyhemoglobin to form products other than methemoglobin. Such a reaction could be demonstrated by the exposure of oxyhemoglobin to H_2O_2 in concentrations greater than 94 μM, a concentration exceeded in the photochemical system. However, even though the spectral changes of purified hemoglobin solutions were caused by the predominant reaction with H_2O_2, if O_2^- and H_2O_2 acted independently, their effects should be additive. Why then was no inhibition by superoxide dismutase evident? The simplest answer is that H_2O_2 eliminated the O_2^- dependent reaction. For example, hydrogen peroxide may compete with O_2^- for a common site of action, possibly the heme crevice in oxyhemoglobin.

Evidence against some other explanations for the catalase requirement was obtained. Catalase did not prevent inactivation of superoxide dismutase nor did superoxide dismutase prevent inactivation of catalase. However, we cannot exclude the possibility that catalase prevents a reaction in which H_2O_2 decreases the concentration of O_2^-, such as the Haber-Weiss reaction (Haber and Weiss, 1934) or the methemoglobin-catalyzed peroxidation of O_2^-.

$$2H^+ + H_2O_2 + 2O_2^- \rightarrow 2H_2O + 2O_2$$

We previously suggested that superoxide dismutase prevented methemoglobin formation by inhibiting a reaction in which O_2^- reduced heme-bound oxygen in oxyhemoglobin leading to the dissociation of methemoglobin and H_2O_2. Alternate explanations were considered in the present study. Inhibition of methemoglobin formation by a Mn superoxide dismutase, which appears to lack peroxidatic activity, suggests that the effect of the Cu-Zn superoxide dismutase is not peroxidatic. The conditions in which the inhibitory effect of superoxide dismutase is seen further support this conclusion. Under these conditions, low peroxide concentrations due to the presence of catalase, high oxygen concentrations from oxygen bubbling through the solution, low enzyme concentrations, and neutral pH, the peroxidatic activity of the Cu-Zn enzyme is very unlikely (Hodgson and Fridovich, 1975). Also considered was the possibility that other activated oxygen species mediate methemoglobin formation. Mannitol, a scavenger of hydroxyl radicals, does not inhibit (Lynch et al., 1976), and, in the present study, two scavengers of $O_2(^1\Delta g)$ were similarly ineffective.

The possibility that the inhibitory effect of superoxide dismutase represented an acceleration of the "reverse" (methemoglobin → oxyhemoglobin), rather than inhibition of the "forward" reaction, initially seemed attractive, especially after the ability of reduced riboflavin to reduce methemoglobin to deoxyhemoglobin was shown. However, inhibition of the conversion of oxyhemoglobin to methemoglobin in lysates (Lynch et al., 1976) was apparent in the earliest phases of the change, at a time when the "reverse" reaction should make no contribution to the rate of the "forward" reaction. Furthermore, in the present study superoxide dismutase inhibited rather than accelerated the conversion of methemoglobin to oxyhemoglobin. These data support the observations of Winterbourn et al. (Winterbourn et al., 1976; Sutton et al., 1976) who, using other means of generating superoxide, demonstrated inhibition by superoxide dismutase of both the "forward" and "reverse" reactions. We propose that superoxide dismutase inhibits the conversion of methemoglobin to oxyhemoglobin in the oxygen-bubbled photochemical system because oxygen competes successfully with hemoglobin for reduced riboflavin and, in the absence of superoxide dismutase, the O_2^- so produced reacts with methemoglobin to yield oxyhemoglobin. In the presence of superoxide dismutase less O_2^- remains to react with methemoglobin while O_2 continues to oxidize reduced riboflavin, diminishing the concentration of reduced riboflavin and blunting its effect.

These observations and those of Winterbourn et al. (1976) provide an explanation for the apparent superoxide dismutase activity of hemoglobin (Kovacs and Matkovics, 1975; Beauchamp and Fridovich, 1971). When hemoglobin-containing samples are subjected to electrophoresis on polyacrylamide gels and stained for superoxide dismutase activity by the method of Beauchamp and Fridovich (1971), a weak achromatic zone indicating superoxide dismutase activity appears in the hemoglobin-containing region of the gel, a region quite far from

the position to which superoxide dismutase itself migrates. Indeed, the effect of the cyclic conversion of oxyhemoglobin to methemoglobin and back to oxyhemoglobin by O_2^- constitues catalytic dismutation of O_2

$$2H^+ + O_2^- + \underset{\text{(oxyHb)}}{HbFe^{3+}\text{-}O_2^-} \rightarrow \underset{\text{(metHb)}}{HbFe^{3+}} + O_2 + H_2O_2$$

$$k = (4 \pm 1) \times 10^3\ M^{-1}\ s^{-1}\ \text{(Sutton et al., 1976)}$$

$$\underset{\text{(metHb)}}{HbFe^{3+}} + O_2^- \rightarrow \underset{\text{(oxyHb)}}{HbFe^{3+}\text{-}O_2^-}$$

$$k = (5.7 \pm 1.1) \times 10^3\ M^{-1}\ s^{-1}\ \text{(Sutton et al., 1976)}$$

$$\text{sum:} \quad 2H^+ + 2O_2^- \rightarrow O_2 + H_2O_2$$

Thus, hemoglobin does exhibit superoxide dismutase activity but it is six orders of magnitude less active than the authentic superoxide dismutase for which the rate constant is 2×10^9 $M^{-1}\ s^{-1}$ (Klug et al., 1972; Rotilio et al., 1972). The superoxide dismutase activity of hemoglobin is clearly not great enough to obviate the need for additional superoxide dismutase in our experiments.

Our results differ in certain respects from those of Goldberg and Stern (1977) who found that methemoglobin and a green pigment were formed in red cells exposed to the autoxidizable compound, dihydroxy fumarate. The formation of the green pigment, and, presumably, methemoglobin, was inhibited by catalase, superoxide dismutase and carbon monoxide. In accord with our observations the green pigment was not seen in lysates. Two important differences between the studies of Goldberg and Stern (1977) in intact cells and our investigations in solutions of purified hemoglobin should be noted. In whole cells there are multiple pathways to deal with activated oxygen species, complicating interpretation of the data. Catalase, superoxide dismutase, glutathione, glutathione peroxidase, methemoglobin reductases, and vitamin E may all affect the results. Secondly, the use of autoxidizable compounds as sources of O_2^- is problematical. An inhibitory effect of superoxide dismutase may mean that O_2^- mediates the reaction or that O_2^- is a necessary reactant in the autoxidation but not a mediator of methemoglobin formation. Indeed, in the case of dihydroxy fumarate, superoxide dismutase has been shown to inhibit the autoxidation (Kellogg and Fridovich, 1977; Halliwell, 1977). Therefore, the actual mediator of the formation of methemoglobin and of the green pigment may have been an autoxidation product of dihydroxy fumarate or the H_2O_2 generated. In the present work some of these difficulties were circumvented by studying chromatographically purified hemoglobin in an O_2^- generating system designed to minimize the direct effects of the generator, photoreduced riboflavin.

The data obtained provide further support for the hypothesis that O_2^- can mediate the formation of methemoglobin from oxyhemoglobin. It appears likely that superoxide dismutase would function in the red cell in vivo to prevent the formation of methemoglobin but the importance of this action in the economy of the red cell remains to be determined.

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References

Beauchamp, C., and Fridovich, I. (1970), *J. Biol. Chem. 245,* 4641.

Beauchamp, C., and Fridovich, I. (1971), *Anal. Biochem. 44,* 276.

Beers, R. F., Jr., and Sizer, I. W. (1952), *J. Biol. Chem. 195,* 133.

Goldberg, B., and Stern, A. (1977), *Arch. Biochem. Biophys. 178,* 218.

Haber, F., and Weiss, J. (1934), *Proc. R. Soc. London, Ser. A 147,* 332.

Halliwell, B. (1977), *Biochem. J. 163,* 441.

Haut, A., Cartwright, G. E., and Wintrobe, M. M. (1964), *J. Lab. Clin. Med. 63,* 279.

Hodgson, E. K., and Fridovich, I. (1975), *Biochemistry 14,* 5299.

Kellogg, E. W., III, and Fridovich, I. (1975), *J. Biol. Chem. 250,* 8812.

Kellogg, E. W., III, and Fridovich, I. (1977), *J. Biol. Chem.* (in press).

Klug, D., Rabani, J., and Fridovich, I. (1972), *J. Biol. Chem. 247,* 4839.

Kovacs, K., and Matkovics, B. (1975), *Enzymes, 3rd Ed., 20,* 1.

Lynch, R. E., Lee, G. R., and Cartwright, G. E. (1976), *J. Biol. Chem. 251,* 1015.

Massey, V., Palmer, G., and Ballou, D. (1971), in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., Eds.) pp 25–43, University Park Press, Baltimore, Md.

McCord, J. M., Boyle, J. A., Day, E. D., Jr., Rizzolo, L. J., and Salin, M. L. (1977), in *Proceedings of the First EMBO Workshop on Superoxide and Superoxide Dismutase* (Michelson, A. M., Fridovich, I., and McCord, J. M., Eds.) (in press).

McCord, J. M., and Fridovich, I. (1969), *J. Biol. Chem. 244,* 6049.

Noble, R. W., and Gibson, Q. H. (1970), *J. Biol. Chem. 245,* 2409.

Pederson, T. C., and Aust, S. D. (1975), *Biochim. Biophys. Acta 385,* 232.

Rotilio, G., Bray, R. C., and Fielden, E. M. (1972), *Biochim. Biophys. Acta 268,* 605.

Sutton, H. C., Roberts, P. B., and Winterbourn, C. C. (1976), *Biochem. J. 155,* 503.

Takahara, S., Hamilton, H. B., Neel, J. V., Kobara, T. Y., Ogura, Y., and Nishimura, E. T. (1960), *J. Clin. Invest. 39,* 610.

Winterbourn, C. C., McGrath, B. M., and Carrell, R. W. (1976), *Biochem. J. 155,* 493.