Histidine at the Active Site of Superoxide Dismutase†

Henry J. Forman,‡ Herbert J. Evans,§ Robert L. Hill, and Irwin Fridovich*

ABSTRACT: Superoxide dismutase, which catalyzes the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, at rates which approach the diffusion limit, appears to be an important component of the defenses which have evolved to deal with the toxicity of oxygen. This enzyme contains Cu^{2+} and Zn^{2+}. The apoenzyme is inactive and Cu^{2+} can restore activity. Analysis of the superhyperfine lines of the epr (Rotilio *et al.* (1971), *Biochemistry 10*, 616) spectrum suggests that the ligand field of the Cu^{2+}, in this enzyme, contains three nitrogenous groups. Chemical modifications of specific residues have been applied in an attempt to identify these nitrogenous ligands. The apoenzyme was inactivated by exposure to light in the presence of a photosensitizing dye. This photoinactivation was paralleled by a decrease in histidine content, whereas other amino acid residues, including cysteine and methionine, remained unchanged. The holoenzyme was completely resistant to photosensitized inactivation and to photosensitized oxidation of histidine residues, under otherwise identical conditions. The apoenzyme was protected by stoichiometric amounts of either Cu^{2+} or Zn^{2+}, whereas Fe^{2+}, Mn^{2+}, or Ni^{2+} was ineffective, even when added in tenfold excess. The photooxidized apoenzyme had lost not only its ability to be reactivated by Cu^{2+} but also its capacity for binding Cu^{2+} with high affinity. Electrophoresis of the apoenzyme, after varying degrees of photoinactivation, demonstrated a progressive decrease in the amount of reconstitutable material and a concomitant increase in modified forms which could not be reactivated with Cu^{2+}. There was no indication of modified but reconstitutable material. Diazotized sulfanilamide, selected because of its preferential reactivity toward histidine residues, was similarly found to derivatize and inactivate the apoenzyme, while having no effect on the holoenzyme. Cu^{2+}, and to a lesser extent Zn^{2+}, protected the enzyme against derivatization and inactivation by this reagent. The change in absorbance, associated with the reaction of the apoenzyme with diazotized sulfanilamide, was consistent with the derivatization of 3.6 histidine residues/subunit of enzyme. Zn^{2+} in large excess was unable to prevent reactivation of the apoenzyme by Cu^{2+}.

The chemical functionalities which are essential for the activity of enzymes have frequently been identified through the application of reagents which preferentially attack specific chemical groupings (Cohen, 1968; Glazer, 1970). In such studies, a loss of activity which correlates with modification of a residue has usually been taken as evidence of the importance of that residue for the catalytic function of the enzyme under study. If the reactive residue is protected and the inactivation prevented by substrate, substrate analog or prosthetic group then the residue may be part of the active site of the enzyme. Histidine and tryptophan have been found to be quite susceptible to photosensitized oxidation, whereas tyrosine, cysteine, and methionine are much less reactive and the other amino acid residues found in proteins are resistant (Weil and Buchert, 1951; Weil *et al.*, 1952, 1953). Martinez-Carrion *et al.* (1967) have used photosensitized oxidation to establish the importance of specific histidine residues for the catalytic action of aspartic aminotransferase, and in this case histidine was the only residue significantly attacked.

Diazotized sulfanilamide or sulfanilic acid couples with the imidazole ring of histidine to give colored products whose absorbance can be used to quantitate histidine in solutions (Macpherson, 1942) or to localize histidine-containing peptides on paper chromatograms (Baldridge and Lewis, 1953). A similar reagent, diazonium-1*H*-tetrazole, has been used to derivatize histidine residues in proteins (Horinishi *et al.*, 1964) and to distinguish between the heme-linked and the free histidines of cytochrome *c* (Horinishi *et al.*, 1965).

In the present investigation, photosensitized oxidation and coupling with a diazonium compound are used to probe the histidine residues of bovine superoxide dismutase. The results, which demonstrated that histidine residues were susceptible to attack in the apoenzyme but not in the holoenzyme and that Cu^{2+} or Zn^{2+} conferred immunity upon the histidine residues of the apoenzyme, while only Cu^{2+} restored its activity, are described and discussed below.

Materials and Methods

Methylene Blue and cytochrome *c* type III were obtained from the Sigma Chemical Co. and Rose Bengal was obtained from the Allied Chemical Co. Superoxide dismutase was prepared from bovine erythrocytes and was freed of its metal prosthetic groups as previously described (McCord and Fridovich, 1969) except that bound EDTA was removed from the apoenzyme by dialysis against changes of 0.05 M sodium acetate–0.10 M NaCl (pH 3.8) for 5 days followed by dialysis against the same buffer, without the NaCl, for an additional day. These dialyses were performed at 5°.

The copper and zinc content of the holo and apo superoxide dismutase was determined by Mr. Dennis Winge, through the use of a Perkin-Elmer Model 303 atomic absorption spectrometer. Superoxide dismutase was assayed in terms of its ability to inhibit the superoxide-dependent reduction of cytochrome *c* by xanthine oxidase (McCord and Fridovich, 1969). The apoenzyme was assayed by exposure to 5×10^{-4} M $CuSO_4$ for a few minutes prior to dilution into the assay mixture (McCord and Fridovich, 1969). The dilution of the apoenzyme plus Cu^{2+} mixture into the assay medium was 300- to 3000-fold, so that the concentration of Cu^{2+}, in the final assay

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received October 25, 1972.* This work was supported by Grants GM-10287 and HE-06400 from the National Institutes of Health, Bethesda, Md.

‡ Postdoctoral Fellow of the Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md.

§ Postdoctoral Fellow of the Institute of Child Health and Human Development of the National Institutes of Health, Bethesda, Md.

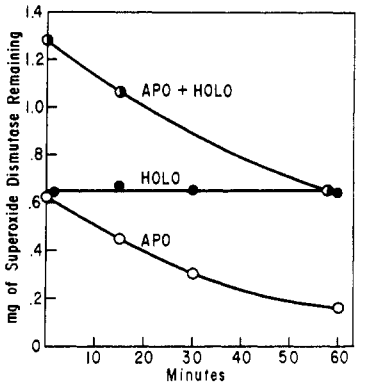

FIGURE 1: Photoinactivation of superoxide dismutase sensitized by Methylene Blue. Reaction mixtures containing 6.1×10^{-5} M Methylene Blue, 0.05 M potassium phosphate, and 0.62 mg of apoenzyme (○) or 0.62 mg of holoenzyme (●) or 0.62 mg of apoenzyme plus 0.62 mg of holoenzyme (◑) in a total volume of 3.0 ml, at pH 7.8 and at 35°, were exposed, at a distance of 30 cm, to the light of a 200-W incandescent lamp. These mixtures were agitated in a water bath at 35° throughout the period of illumination and aliquots were removed at intervals for assay of the residual superoxide dismutase activity. Residual apoenzyme was assayed after reconstitution with excess Cu^{2+} as described under Materials and Methods.

TABLE I: Amino Acid Analyses of Apo Superoxide Dismutase before and after Photooxidation.[a]

Amino Acid	Residues/Subunit			
	Before	10 min	30 min	60 min
Histidine	7.7	6.50	4.5	2.2
Alanine	11.8	12.0	11.9	12.0
Methionine	1.1	1.1	1.1	1.0
Tyrosine	1.3	1.4	1.3	1.2
Phenylalanine	4.8	4.7	4.7	4.6
Leucine[b]	10.0	10.0	10.0	10.0
Half-cystine	2.8	2.5	2.9	3.0

[a] Superoxide dismutase and the corresponding apoprotein were photooxidized for 1 hr under the conditions described in Figure 2. Aliquots (0.28 mg of holoenzyme and 0.22 mg of apoenzyme) were then subjected to acid hydrolysis and amino acid analysis, as described under Methods. [b] All data normalized on the basis of a leucine content of 10 residues/subunit. All other amino acid residues although not listed in this table were unchanged by photooxidation.

medium, was no greater than 1.67×10^{-6} M. Since the assay medium contained 1×10^{-4} M EDTA (McCord and Fridovich, 1969), this amount of free copper did not interfere with the assay procedure. Photosensitized oxidation was performed by exposing reaction mixtures to a 200-W incandescent lamp at the indicated distance. These photooxidations were performed in 25-ml erlenmeyer flasks, which were agitated in a Dubnoff incubator, at the specified temperature. The depth of liquid in these flasks was approximately 5 mm. Amino acid analyses were performed with a Beckman-Spinco Model 120 automatic amino acid analyzer after hydrolysis of the protein samples for 24 hr at 110° in 6.0 N HCl, in Pyrex vials sealed *in vacuo* (Moore and Stein, 1963; Spackman *et al.*, 1958).

Sulfhydryl groups were estimated by applying the method of Ellman (1959) to protein samples dissolved in 6.0 M guanidinium chloride–3×10^{-3} M EDTA–0.1 M potassium phosphate (pH 8.0). Methionine was assayed by use of the methods of Harris and Hill (1969). Thus the protein samples, in 6.0 M guanidinium chloride and at pH 5.0, were treated with 0.03 M iodoacetate for 15 hr in the dark. This treatment quantitatively alkylated methionine but would have no effect on methionine sulfoxide, which might be present in the photooxidized protein. After addition of mercaptoethanol, to consume unreacted iodacetate, the samples were lyophilized and then oxidized with performic acid. This would have converted any unalkylated methionine derivatives, such as the sulfoxide, to the methionine sulfone. Acid hydrolysis and amino acid analysis were then applied to quantitate such methionine sulfone as might be present. The presence of methionine sulfone in the photooxidized samples would have been evidence for photooxidation of methionine residues. Electrophoresis on polyacrylamide gel was performed according to Davis (1964). Superoxide dismutase was localized on gel electrophoretograms as previously described (Beauchamp and Fridovich, 1971).

Results

Photosensitized Oxidation of Superoxide Dismutase. When apo superoxide dismutase was exposed to light in the presence of 6.1×10^{-5} M Methylene Blue and 0.05 M potassium phosphate, at pH 7.8 and 35°, it's ability to regain activity in the presence of Cu^{2+} was progressively destroyed. The dye and the light were both essential for this inactivation. Under identical conditions, the holo superoxide dismutase was completely unaffected. The holoenzyme, while itself resistant to photoinactivation, did not prevent the photoinactivation of admixed apoenzyme. Similarly, the residual superoxide dismutase activity of apoenzyme, which was due to incomplete removal of copper during the resolution procedure, was completely resistant to photoinactivation. These results, which demonstrate that holoenzyme did not confer resistance upon apoenzyme present in the same solution, are illustrated in Figure 1.

Complete amino acid analyses of photooxidized enzymes demonstrated that photoinactivation of the apoenzyme was associated with a destruction only of histidine residues. These results are presented in Table I. The sulfhydryl titer of the apoenzyme was 1.7/molecule before photoinactivation, 1.8/molecule after 67% photoinactivation, and 1.4/molecule after 100% photoinactivation. Assays which would have detected oxidation of methionine residues indicated that this had not occurred to any perceptible degree, even after 80% photoinactivation. See Materials and Methods for details. Tryptophan is a residue which may be susceptible to photooxidation (Weil and Buchert, 1951; Weil *et al.*, 1952, 1953) but need not be considered in this case because bovine superoxide dismutase does not contain tryptophan.

Comparable analyses of the holoenzyme demonstrated that it was entirely unchanged by similar exposure to light in the presence of the dye. Figure 2 presents the rate of loss of histidine residues as a function of time of photosensitized oxidation. It is apparent that the holoenzyme was entirely unaffected (line 1) whereas the apoenzyme suffered a progressive loss of histidine residues (line 2). The rate of loss of activity by the apoenzyme was also measured under identical conditions and complete photoinactivation first occurred at 30 min, at which time 3.6 histidine residues/subunit had been destroyed.

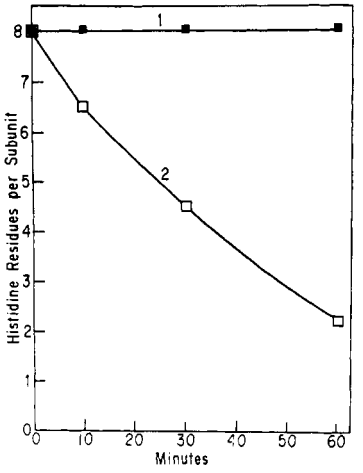

FIGURE 2: Photosensitized oxidation of histidine residues in apo superoxide dismutase. Reaction mixtures contained 6.3×10^{-5} M Methylene Blue, 0.05 M potassium phosphate, and either 16 mg/ml of holoenzyme (line 1) or 11.4 mg/ml of apoenzyme (curve 2) at pH 7.8 and 25° and were exposed to a 200-W tungsten lamp at a distance of 10 cm. Aliquots were removed at intervals and analyzed for residual histidine.

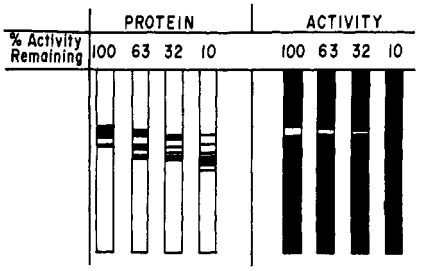

FIGURE 3: Effects of photoinactivation on electrophoretic mobility in polyacrylamide gel. Apo superoxide dismutase at 23 mg/ml was incubated in 1.5×10^{-5} M Methylene Blue–0.02 M potassium phosphate (pH 7.8, 25°) and was illuminated as in Figure 2. Aliquots were removed at intervals, treated with 10 Cu^{2+}/subunit and 10 Zn^{2+}/subunit, and then assayed for activity and analyzed by polyacrylamide gel electrophoresis. The gels which were stained for protein contained 125 μg of superoxide dismutase per gel whereas those stained for activity contained 5 μg/gel.

Histidine residues did continue to suffer photooxidation after complete inactivation had occurred.

Does photosensitized inactivation occur in an all-or-none fashion with respect to any single molecule of the apoenzyme? In an attempt to answer this question apoenzyme was photoinactivated to varying degrees. Cu^{2+} and Zn^{2+} were then added to affect reconstitution and samples were taken for electrophoresis on polyacrylamide gels. These gels were then stained for protein and for superoxide dismutase activity. As shown in Figure 3 there was a progressive diminution in the amount of the native and active species and a concomitant appearance of inactive protein species of different electrophoretic mobilities. At no time did an active species of altered mobility appear. It appears, on this basis, that the photoinactivation was effectively an all-or-none process.

Photoinactivation was also associated with loss of the ability of the apoenzyme to bind Cu^{2+} or Zn^{2+}. Thus apoenzyme, which had been photoinactivated, was exposed to a tenfold excess of Cu^{2+} in the absence and in the presence of a comparable amount of Zn^{2+}. It was then dialyzed against a neutral phosphate buffer containing 1 mM EDTA for 1 hr and then against changes of water over a period of 48 hr. All of this was done at 4°. At the end of this dialysis the enzyme was assayed for Cu^{2+} and for Zn^{2+}. There was no detectable Cu^{2+} and only 0.25–0.30 Zn^{2+}/subunit. Apoenzyme, which was not photoinactivated, bound 1 Cu^{2+} and 1 Zn^{2+} per subunit after similar treatment.

When the holoenzyme was photooxidized, at pH 10.2 for 150 min, it was found to remain fully active, as it had at pH 7.8. However, at this higher pH one histidine residue per subunit was destroyed. It appears that raising the pH from 7.8 to 10.2 exposed a nonessential histidine residue of superoxide dismutase to photosensitized oxidation.

Effects of EDTA on Photooxidation. When 1×10^{-4} M EDTA was present in the photooxidizing reaction mixture, both holoenzyme and apoenzyme were inactivated. Control experiments demonstrated that both light and the dye were essential for this inactivation of holo superoxide dismutase. EDTA could be replaced by tetramethylethylenediamine but not by histidine. Thus 5×10^{-3} M histidine did not sensitize holoenzyme toward photoinactivation. It is possible that EDTA or tetramethylethylenediamine were able to abstract Cu^{2+} and Zn^{2+} from superoxide dismutase, under the influence of light plus Methylene Blue, or that substances generated by the photooxidation of EDTA or of tetramethylethylenediamine were responsible for this phenomenon. These possibilities were not further explored.

Protection of the Apoenzyme by Cu^{2+} or Zn^{2+}. Apo superoxide dismutase was protected against photosensitized inactivation by Cu^{2+} or Zn^{2+}. Indeed 1 equiv/subunit of either of these metal cations afforded virtually complete protection. These results are illustrated in Figure 4. These experiments were repeated with Rose Bengal replacing Methylene Blue as the photosensitizer and identical results were obtained.

Several other transition metals were tested for their abilities to protect the apoenzyme against photooxidation and were found to be completely ineffective. Thus mixtures containing 55 μg/ml of apo superoxide dismutase, 9.5×10^{-6} M Methylene Blue, 0.05 potassium phosphate, and 10 equiv/enzyme subunit of Fe^{2+}, Mn^{2+}, or Ni^{2+} were exposed to photooxidation under conditions described in Figure 4. None of these metals afforded any detectable protection against photoinactivation of the apoenzyme. There was evidently a great deal of specificity involved in the protection afforded the apoenzyme by Cu^{2+} or by Zn^{2+}.

It was considered possible that the protective effects of Cu^{2+} or of Zn^{2+} related to an action on the photosensitizing dye or on singlet oxygen (White and Harding, 1965; Wasserman *et al.*, 1968), rather than to a direct action on the apo superoxide dismutase. This was explored by testing the effects of 3.3×10^{-3} M Cu^{2+} or of 3.3×10^{-3} M Zn^{2+} on the rate of photooxidation of 3.3×10^{-4} histidine in the presence of 3.1×10^{-5} Methylene Blue. These metal cations were entirely without effect on the rate of photosensitized oxidation in this model system, thus minimizing the aforementioned possibilities.

Effect of Diazotized Sulfanilamide. Sulfanilamide was diazotized by mixing equal volumes of 1% sulfanilamide in 1.0 N HCl with 5% $NaNO_2$. Aliquots of apo superoxide dismutase containing 14.3 mg of protein in 0.3 ml, with and without the addition of 1 μl of 0.1 M $CuSO_4$, were treated with 0.6 ml of the diazotized sulfanilamide and after incubation for 3 min at room temperature, 2.0 ml of 20% Na_2CO_3 was added to each. The apoenzyme turned bright orange, whereas the Cu^{2+}-reconstituted enzyme did not exhibit any change in color. This procedure was varied without affecting the results. Thus,

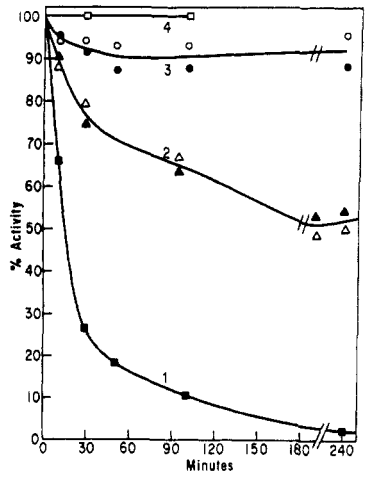

FIGURE 4: Effects of Cu^{2+} and of Zn^{2+} on the photosensitized inactivation of apo superoxide dismutase. Reaction mixtures contained 55 μg/ml of apo superoxide dismutase, 9.5×10^{-6} M Methylene Blue, 0.025 M potassium phosphate, and the following amounts of Cu^{2+} or of Zn^{2+}: line 1 = none, line 2 = 0.5 equiv/subunit, line 3 = 1.0 equiv/subunit, and line 4 = 1.5 equiv/subunit at pH 7.8 and at 25°. Illumination was by a 200-W incandescent lamp at a distance of 25 cm. Samples were withdrawn at intervals and assayed for residual superoxide dismutase activity after reconstitution with excess Cu^{2+}, as described under Materials and Methods.

TABLE II: Effect of Cu^{2+} and of Zn^{2+} on Rate of Coupling of Diazotized Sulfanilamide with Histidine.[a]

Metal–Histidine	Absorbancy/min	Free His/His–Metal Complex
0	0.185	∞
1 Cu^{2+}	0.130	1.73
4 Cu^{2+}	0.052	0.34
10 Cu^{2+}	0.043	0.03
10 Zn^{2+}	0.180	1600

[a] 1 ml of 1% sulfanilamide, in 1.0 N HCl, was mixed with 1 ml of 5% $NaNO_2$. After 5 min at 25° 4 ml of 20% Na_2CO_3 was added. 2.5 ml of this mixture was then added to 0.275 ml of 9.1×10^{-5} M histidine containing the indicated amounts of Cu^{2+} or Zn^{2+} and the coupling reaction was followed at 500 nm. The rate of the coupling of the diazotized sulfanilamide with the imidazole ring of histidine is here presented as a function of the ratio free histidine/complexed histidine. The ratios were calculated from published binding constants (Hallman *et al.*, 1971).

in some instances the diazotized sulfanilamide, which was at pH 1.5, was neutralized by adding the sodium carbonate, which raised the pH to 11.5, and was then mixed with the apoenzyme or with the Cu^{2+}-reconstituted enzyme. In other experiments the reaction mixture, containing diazotized sulfanilamide, was adjusted to pH 7.8 by careful addition of Na_2CO_3, under the glass electrode, before the enzyme was added. The enzymes were then freed of small molecules by dialysis against cold 0.05 M potassium phosphate, 0.5% imidazole at pH 7.8 for 3 days at 4°, followed by dialysis against 0.05 M potassium phosphate (pH 7.8) for 18 hr. The apoenzyme retained the orange color generated by the diazo coupling reaction and was completely devoid of the superoxide dismutase activity which could ordinarily be elicited by restoration of Cu^{2+}. In contrast the apoenzyme, which had been treated with Cu^{2+} prior to exposure to the diazosulfanilamide, exhibited no orange color and was fully active. Diazotized sulfanilamide was reacted with 1.79×10^{-5} M apoenzyme, 1.67×10^{-5} M histidine, or 1.67×10^{-5} M tyrosine and the resultant absorption spectra, recorded against a reagent blank, are shown in Figure 5. The diazo coupling product obtained from the apoenzyme was similar to those obtained from histidine or tyrosine. The color yield from tyrosine was, however, small and given that the enzyme contains only one tyrosine per subunit (Keele *et al.*, 1971), whereas it contains eight residues of histidine/subunit, it is clear that histidine is very likely to be the reactive residue in the apoenzyme. If we assume that the chromogen generated from histidine and from the apoenzyme had the same absorption coefficient, we may calculate that 3.6 residues of histidine/subunit of apo superoxide dismutase were available for coupling with diazosulfanilamide. The holoenzyme gave no color and we may conclude that its histidine residues were unavailable for coupling with the diazo reagent. Cu^{2+}, when present at a level of 1 equiv/subunit, completely protected the apoenzyme against attack by the diazo reagent. Zn^{2+} was less effective and when present at 10 equiv of Zn^{2+}/subunit it slowed but did not entirely prevent the coupling of the diazo reagent with the apoenzyme. In control experiments Cu^{2+} and Zn^{2+} were tested for their effects on the coupling of free histidine with the diazo reagent and Cu^{2+} at 10 equiv/histidine slowed the reaction whereas Zn^{2+} was without effect. Table II presents some of these data.

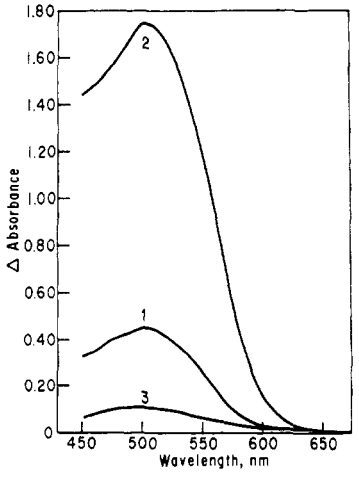

FIGURE 5: Absorption spectra of the chromophores resulting from coupling diazotized sulfanilamide with histidine (line 1), apo superoxide dismutase (line 2), or tyrosine (line 3). 25 μl of 2×10^{-3} M histidine (line 1), 1.07×10^{-3} M apo superoxide dismutase (line 2), or 2×10^{-3} M tyrosine (line 3) was treated at 25° with 0.5 ml of 1% sulfanilamide in 1.0 N HCl followed immediately by 0.5 ml of 5% $NaNO_2$. After a delay of 5 min, 1.97 ml of 20% Na_2CO_3 was added and the color was allowed to develop for 5 min at 25°. Spectra were recorded against a reagent blank.

Reactivation of the Apoenzyme by Cu^{2+}. Apo superoxide dismutase was prepared by dialysis against EDTA at pH 3.8. The activity of this apoenzyme could be restored by Cu^{2+} (McCord and Fridovich, 1969) but amounts of Cu^{2+} in excess of one equivalent per subunit appeared to be required for full restoration of activity. This seems to be due to a tendency of the apoenzyme to bind EDTA. Thus, when apo-

enzyme was exhaustively dialyzed against changes of NaCl-containing acetate buffer at pH 3.8, as described under Methods, and was then titrated with Cu^{2+}, 90% of its original activity was restored by 1 Cu^{2+}/subunit. Zn^{2+} did not restore activity to the apoenzyme and even when present at 800 Zn^{2+}/subunit, it did not interfere with the ability of Cu^{2+} to restore activity.

Discussion

Apo superoxide dismutase was inactivated by photosensitized oxidation or by coupling with diazotized sulfanilamide, whereas the holoenzyme was completely unreactive. Analytical data indicated that only histidine residues were susceptible to attack by these reagents in the apoenzyme and that there were no detectable changes in the holoenzyme. Cu^{2+} or Zn^{2+} protected the apoenzyme against photooxidation and Cu^{2+} and to a lesser extent Zn^{2+} protected the apoenzyme against diazosulfanilamide. Nevertheless Cu^{2+} alone restored full activity to the apoenzyme and large excesses of Zn^{2+} failed to influence this reactivation by Cu^{2+}. These results can be accommodated by a model which places the Cu^{2+} and the Zn^{2+} binding site in close proximity on the enzyme. This model also requires that the Cu^{2+} binding site have no significant affinity for Zn^{2+}, so that Zn^{2+} could not compete with Cu^{2+} for this active site and thus prevent activation. Because 3.6 histidine residues/subunit of apoenzyme, out of a total of 8 such residues (Keele *et al.*, 1971), were susceptible to coupling with the diazo reagent, it is tempting to speculate that the Cu^{2+} binding site is composed of 3 or 4 imidazole groups. The superhyperfine structure of the electron paramagnetic resonance (epr) spectrum of the superoxide dismutase has indeed been interpretated as indicating that the ligand field of the Cu^{2+} contains three to four nitrogenous groups (Rotilio *et al.*, 1971). Fee and Gaber (1972), on the basis of the effects of anionic ligands on the spectrometric properties of superoxide dismutase, have recently proposed that the Zn^{2+} and Cu^{2+} may be situated in close proximity, perhaps as a ligand-bridged bimetal complex. With the specific Cu^{2+} and Zn^{2+} binding sites in close proximity, it is reasonable to propose that the binding of Zn^{2+} was able to affect the reactivity of the imidazole groups, which presumably form the liganding groups for Cu^{2+}, without detectably interfering with the binding of Cu^{2+}.

It has been proposed (Jori *et al.*, 1969, 1971; Jori and Cauzzo, 1970) that paramagnetic metals protect against photosensitized oxidation by virtue of their ability to capture electrons or to facilitate conversions of excited state species to the corresponding ground states. This cannot be their mechanism of protection, in the present case, because Zn^{2+}, which is diamagnetic, was also capable of protecting the apoenzyme against photosensitized inactivation and because neither Cu^{2+} nor Zn^{2+} protected free histidine against photooxidation. Cu^{2+} did protect free histidine against reaction with diazotized sulfanilamide and this protection correlated with the degree of association of Cu^{2+} with the histidine, as shown in Table II. This observation merits comment because Cu^{2+} is thought (Sarkar and Wigfield, 1967) to ligate with the α-amino and carboxyl groups of free histidine, rather than with the imidazole ring. Electrostatic and steric factors could be invoked to explain the decreased reactivity, towards the diazo reagent, of the imidazole ring of the Cu^{2+}–histidine complex.

The experiments in which superoxide dismutase was exposed to diazotized sulfanilamide were performed under a variety of conditions, including those in which the enzyme was briefly exposed to dilute HCl. The observation that the holoenzyme lost no activity under these conditions suggested two possibilities. Either superoxide dismutase is stable to low pH or it is capable of rapid renaturation after such exposure. This was explored. Incubation of superoxide dismutase in 0.5 M HCl for 5 min at 25°, followed by dilution into neutral phosphate buffer and assay, indicated retention of 86% of the original activity. In contrast, a comparable exposure to 0.33 M HCl–0.0033 M EDTA prior to dilution and assay resulted in complete inactivation. Exposure of the enzyme to neutral EDTA solutions did not cause diminution of activity. It follows that superoxide dismutase unfolds in dilute HCl and releases its metal components and that, in the absence of a chelating agent, this denaturation is very rapidly reversed upon neutralization of the acid.

References

Baldridge, R. C., and Lewis, H. B. (1953), *J. Biol. Chem. 202*, 169.

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

Cohen, L. A. (1968), *Annu. Rev. Biochem. 37*, 695.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fee, J. A., and Gaber, B. P. (1972), *J. Biol. Chem. 247*, 60.

Glazer, A. N. (1970), *Annu. Rev. Biochem. 39*, 101.

Hallman, P. S., Perrin, D. D., and Watt, A. E. (1971), *Biochem. J. 121*, 549.

Harris, C. M., and Hill, R. L. (1969), *J. Biol. Chem. 244*, 2195.

Horinishi, H., Hachimori, Y., Kurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 86*, 477.

Horinishi, H., Kurihara, K., and Shibata, K. (1965), *Arch. Biochem. Biophys. 111*, 520.

Jori, G., and Cauzzo, G. (1970), *Photochem. Photobiol. 12*, 231.

Jori, G., Galiazzo, G., and Scoffone, E. (1969), *Biochemistry 8*, 2868.

Jori, G., Gennari, G., Galiazzo, G., and Scoffone, E. (1971), *Biochim. Biophys. Acta 236*, 749.

Keele, B. B., Jr., McCord, J. M., and Fridovich, I. (1971), *J. Biol. Chem. 246*, 2875.

Macpherson, H. T. (1942), *Biochem. J. 36*, 59.

Martinez-Carrion, M., Turano, C., Riva, F., and Fasella, P. (1967), *J. Biol. Chem. 242*, 1426.

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

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Rotilio, G., Finazzi, A., Calabrese, L., Bossa, F., Guerrieri, P., and Mondovi, B. (1971), *Biochemistry 10*, 616.

Sarkar, B., and Wigfield, J. (1967), *J. Biol. Chem. 242*, 5572.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Wasserman, K., Stiller, M., and Floyd, B. (1968), *Tetrahedron Lett. 1968*, 3277.

Weil, L., and Buchert, A. R. (1951), *Arch. Biochem. Biophys. 34*, 1.

Weil, L., Buchert, A. R., and Maher, J. (1952), *Arch. Biochem. Biophys. 40*, 245.

Weil, L., James, S., and Buchert, A. R. (1953), *Arch. Biochem. Biophys. 46*, 266.

White, E. H., and Harding, M. J. C. (1965), *Photochem. Photobiol. 4*, 1129.