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Potentiometric Titrations and Oxidation–Reduction Potentials of Manganese and Copper–Zinc Superoxide Dismutases†

Glen D. Lawrence and Donald T. Sawyer*

ABSTRACT: Bovine erythrocyte superoxide dismutase and two manganese-containing superoxide dismutases have been reduced by the indirect coulometric titration method with methylviologen as the mediator–titrant. On the basis of the titration data the manganese-containing superoxide dismutases contain 1 g-atom of metal per mol of enzyme (dimer). $E^{0\prime}$ = +0.31 V for the enzyme from *Escherichia coli* which exhibits a complicated pH dependence above neutral pH. The *Bacillus stearothermophilus* manganese-containing enzyme has an $E^{0\prime}$ = +0.26 V and ΔE_m/pH is 50 mV. Bovine erythrocyte superoxide dismutase exhibits anomalous behavior in the coulometric titration curves, which is indicative of two nonequivalent copper centers in the enzyme. Addition of $K_3Fe(CN)_6$ or K_2IrCl_6 to the enzyme solution, prior to coulometric titration, indicates that these anions bind preferentially to one of the copper centers.

Although superoxide dismutases have been the subject of numerous studies in the past several years, the details of the oxidation–reduction chemistry for the enzymatic process are not understood (Fee, 1977). There is general agreement that all three classes of the enzyme (Cu/Zn-, Mn-, and Fe-containing SOD)[1] have a common mechanistic pathway for the catalysis of the disproportionation of superoxide ion ($O_2^{-}\cdot$), as shown by eq 1 and 2. However, the thermodynamics and

$$E_A + O_2^{-}\cdot \rightarrow E_B + O_2 \quad (1)$$

$$E_B + O_2^{-}\cdot + 2H^+ \rightarrow E_A + H_2O_2 \quad (2)$$

mechanisms for the individual electron transfer steps of the enzymatic process have not been determined (Fee, 1977).

The X-ray crystal structure of bovine Cu/ZnSOD has been determined to 3-Å resolution (Richardson et al., 1975a,b). For this enzyme the catalytic cycle has been postulated from pulse radiolytic investigations to be a ping-pong type mechanism (Klug et al., 1972; Klug-Roth et al., 1973), with the copper moiety undergoing alternate oxidation and reduction by $O_2^{-}\cdot$. The mechanism is complicated by the fact that H_2O_2 (one of the products of the reaction) causes the Cu(II) sites of the enzyme to be reduced (Klug-Roth et al., 1973; Rotilio et al., 1973; Bray et al., 1974). This poses a dilemma if one considers the nearly diffusion controlled rate of $O_2^{-}\cdot$ reduction by the enzyme and the redox potentials for the Cu(II)/Cu(I) and $O_2^{-}\cdot/H_2O_2$ couples. Fee & DiCorleto (1973) have determined that the Cu(II)/Cu(I) redox potential ($E^{0\prime}$) of BESOD has a value of +0.42 V vs. NHE. The potential for the $O_2^{-}\cdot/H_2O_2$ couple has been estimated to be +0.87 V (Fee & Valentine, 1977), a value much too positive to effect the direct reduction of the Cu(II) in the enzyme by H_2O_2.

The results of a detailed kinetic investigation of bovine SOD

† From the Department of Chemistry, University of California, Riverside, California 92521. *Received July 17, 1978; revised manuscript received February 22, 1979.* Supported by the U.S. Public Health Service, National Institutes of Health, under Grant No. GM 22761.

[1] Abbreviations used: BESOD, bovine erythrocyte superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; FeSOD, iron-containing superoxide dismutase; MV^{2+}, methylviologen dication; $MV^{+}\cdot$, methylviologen cation radical; EPR, electron paramagnetic resonance; cyt *c*, horse heart cytochrome *c*; DCIP, 2,6-dichlorophenolindophenol.

0006-2960/79/0418-3045$01.00/0

have led to the conclusion that only half of the copper sites of the enzyme are functional in catalysis (Fielden et al., 1974). However, a more recent paper indicates that the enzyme may be contaminated with a nonfunctional modification that has similar EPR properties (Cockle & Bray, 1977).

Pulse radiolysis investigations of MnSOD from *Escherichia coli* (Pick et al., 1974) and *Bacillus stearothermophilus* (McAdam et al., 1977a) and of FeSOD from *Photobacterium lieognathi* (Lavelle et al., 1977) indicate that a similar mechanism is in effect for all of the superoxide dismutases. MnSOD and FeSOD also are reduced by H_2O_2, but the MnSOD is not inactivated by H_2O_2 in contrast to the Cu/Zn and Fe enzymes (McAdam et al., 1977b).

The present investigation has utilized the indirect coulometric titration method for the determination of the redox properties of superoxide dismutases in order to gain greater insight into the structures and mechanisms for this group of enzymes. The results provide a more complete measure of the redox stoichiometry and thermodynamics for the MnSOD enzymes and further demonstrate the anomalous properties associated with the bovine Cu/ZnSOD enzyme.

Experimental

The electrochemical cell consisted of a Brinkman Model EA875-5 glass cell with a plastic cell top. Different combinations of a four-electrode assembly were used, and the best reproducibility was obtained with a pyrolytic graphite working electrode with ca. 3-cm^2 surface area for coulometry, a Pt-wire auxiliary electrode separated from the bulk solution by a fine porosity fritted disk, a 1-mm diameter Pt-inlay measuring electrode, and a Ag|AgCl (in saturated KCl) cracked-bead reference electrode with a potential of +199 mV vs. NHE at 25 °C. Glassy carbon and pyrolytic graphite measuring electrodes were also used in titrations of BESOD and gave the same results as obtained with a Pt electrode. Gold measuring electrodes were found to be unsatisfactory in these titrations. A Pt-foil working electrode was used on occasion, but the success of titrations was dependent on the history of the electrode, especially with BESOD. A Ag|AgCl (in aqueous Me_4NCl) cracked-bead reference electrode adjusted to 0.000 V vs. SCE was used in some titrations but this electrode had a potential of +30 mV vs. SCE in phosphate buffers (due to an apparent junction potential).

The coulometric titrations were performed with a Princeton Applied Research Corp. Model 173 potentiostat/galvanostat that included a Model 179 digital coulometer or with a potentiostat/amperostat that was constructed by use of Philbrick solid-state operational amplifiers. The measuring and reference electrodes were connected to a Corning Model 12 pH meter, and the titration curves were recorded on a Sargent Model SR recorder interfaced with the pH meter. Titrations were generally performed in a N_2-atmosphere glovebox to avoid any interference by O_2 or in an airtight electrochemical cell under an Ar atmosphere.

Each titration used a 5.0-mL sample solution that contained an appropriate buffer and 2–6 mg (1.5–5.5 mM) of methylviologen as the titrant for the reduction. The methylviologen cation radical, which was produced electrochemically, reacted rapidly with the superoxide dismutases and was reoxidized at a rate much faster than it was produced, until the protein became completely reduced. The protein sample solutions (including cyt *c*) also contained a redox mediator (at a concentration less than 10% of the protein concentration) for the potentiometric indicating electrode. The buffered solutions were deaerated with Ar before the addition of protein to minimize the amount of dissolved oxygen in the solutions prior to their introduction into the glovebox.

Bovine erythrocyte superoxide dismutase and cytochrome *c* (Type VI) were purchased from Sigma (St. Louis, MO) and used without further purification. Highly purified BESOD was obtained from Dr. J. V. Bannister, University of Malta, Msida, Malta, in lyophilized form and dissolved in the appropriate buffer before titration. The concentration of BESOD in all samples was determined by the absorbance at 680 nm by using ϵ_{680} = 300 M^{-1} cm^{-1}. Manganese superoxide dismutase from *B. stearothermophilus* was a gift of the late Dr. J. I. Harris, Medical Research Council, Cambridge, England, and was obtained as the lyophilized protein and used without further purification. The concentration of *B. stearothermophilus* MnSOD was determined spectrophotometrically by using $A_{280}^{1\%}$ = 13.2 (McAdam et al., 1977a). MnSOD from *E. coli* B was the generous gift of Dennis Ose and Professor I. Fridovich, Duke University, Durham, NC, and was obtained as the ammonium sulfate solution. The *E. coli* enzyme was brought into the buffer solutions by ultrafiltration on an Amicon P-10 filter with 10 volumes of buffer containing 5 × 10^{-4} M EDTA to remove free metal ions, and then the solution was washed with 50 volumes of the appropriate buffer without EDTA. The concentration of MnSOD in the stock buffer solution was determined spectrophotometrically by using ϵ_{283} = 6.0 × 10^4 M^{-1} cm^{-1} (Pick et al., 1974). The stock solution was then diluted with deaerated buffer, and the pH was adjusted with HCl or NaOH.

Sedimentation velocity experiments were performed on a Beckman Model E analytical ultracentrifuge, and polyacrylamide gel electrophoresis was performed by the method of Beauchamp & Fridovich (1971).

Methylviologen was obtained from Mann Research Laboratories, New York, and 2,6-dichlorophenolindophenol was obtained from Sigma (St. Louis, MO); both were used without further purification. *p*-Benzoquinone was obtained from Matheson Coleman and Bell and was sublimed once before preparing stock solutions. All other chemicals were reagent grade and used without further purification.

Results

Spectrophotometric Studies of B. stearothermophilus and E. coli B MnSOD. The absorption maximum for *B. stearothermophilus* MnSOD (20 mg of lyophilized protein in 20 mL of 50 mM Tris-HCl buffer, pH 7.5) is at 460 nm [in contrast to the λ_{max} at 480 nm reported by McAdam et al. (1977a–c)] with ϵ_{460} = 705 M^{-1} cm^{-1}. The concentration of protein in stock solutions has been determined from $A_{280}^{1\%}$ = 13.2 (McAdam et al., 1977a). Aliquots of this stock solution have been added to the electrochemical cell under a N_2 atmosphere for titration.

After ultrafiltration the *E. coli* MnSOD solution (in 25 mM Tris–phosphate buffer, pH 7.5) yields an absorption spectrum similar to that reported by Keele et al. (1970). The concentration of the enzyme has been determined by use of a molar absorptivity value of 6.0 × 10^4 M^{-1} cm^{-1} at 283 nm (Pick et al., 1974). In turn, this has permitted evaluation of ϵ_{470} from the absorption spectrum. For our conditions ϵ_{470} = 520 M^{-1} cm^{-1}, in contrast to the value of 400 M^{-1} cm^{-1} that has been reported earlier (Keele et al., 1970). This greater value is consistent with the molar absorptivity values for other MnSOD's [*Streptococcus mutans*, ϵ_{473} = 280 M^{-1} cm^{-1} per subunit of dimer (Vance et al., 1972); mitochondrial, ϵ_{475} = 292 M^{-1} cm^{-1} per subunit of tetramer (Weisiger & Fridovich, 1973)].

Coulometric Titration of Cytochrome c and Redox Mediators. Coulometric titrations of 2,6-dichlorophenolindophenol (DCIP), *p*-benzoquinone (BQ), and cytochrome *c* (cyt *c*) with methylviologen as the titrant–mediator verify the

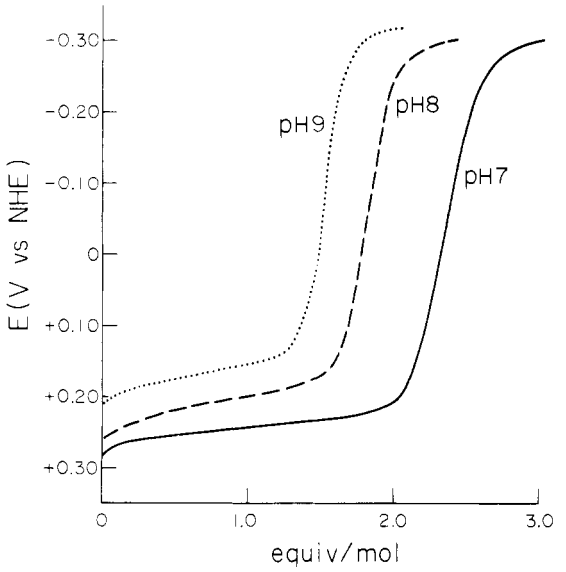


FIGURE 1: Coulometric titration curves for *B. stearothermophilus* MnSOD. All solutions contained 25 mM Tris–phosphate buffer, I = 0.15 with NaCl, 1.5–2.8 mM MV^{2+}, 2.6 μM DCIP, and 2.1 × 10^{-5} M *B. stearothermophilus* MnSOD. Titration current was 100 μA.

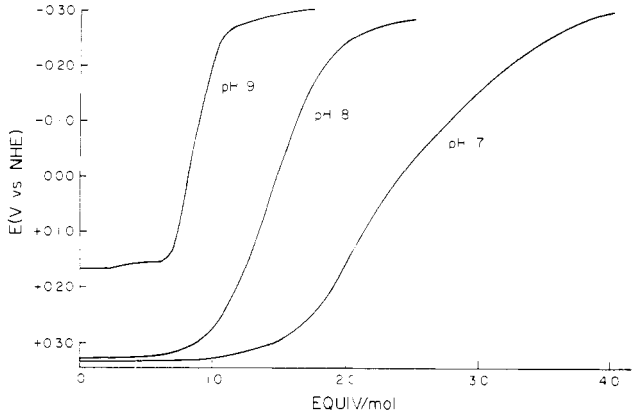


FIGURE 2: Coulometric titration curves for *E. coli* B MnSOD. All solutions contained 25 mM Tris–phosphate buffer, I = 0.15 with NaCl, 1.8–2.6 mM MV^{2+}, 2.0 μM DCIP, and 1.2 × 10^{-5} M MnSOD. Titration current was 50 μA.

validity of the indirect coulometric titration method. The midpoint potentials obtained for these species are +210, +280, and +265 mV vs. NHE for DCIP, BQ, and cyt *c*, respectively; all are in good agreement with the literature values (Loach, 1970).

Coulometric Titration of MnSOD. Coulometric titration curves for *B. stearothermophilus* and *E. coli* MnSOD are shown in Figures 1 and 2, respectively. In both cases the net reduction stoichiometry is greater than 1 equiv/mol of enzyme but less than 2 equiv/mol. The greater number of equivalents observed at lower pH values probably is due to the reduction of H^+ by the MV^+ radical (Shelepin & Ushakov, 1975). This reaction is sufficiently slow at pH 7 so as not to interfere significantly with the potential measurements, but at lower pH values the determination of the end point becomes difficult. The midpoint potentials for both the MnSOD enzymes and the BESOD enzymes from two sources are listed in Table I. The *B. stearothermophilus* MnSOD exhibits nearly Nernstian behavior for one proton coupled to the one-electron reduction: $\Delta E_m/pH \sim 50$ mV. The *E. coli* MnSOD, on the other hand, shows a peculiar dependence on pH. The midpoint potential for this enzyme changes little between pH 7.0 and pH 8.0, but exhibits a pronounced change between pH 8.0 and pH 9.0: ΔE_m = 140 mV.

Table I: Redox Potentials (V vs. NHE) for Manganese and Copper Superoxide Dismutases[a]

	E_m (V)			
pH	Sigma BESOD	Bannister BESOD	*B. stearothermophilus* MnSOD	*E. coli* MnSOD
7.0	+0.35	+0.28	+0.26	+0.31
7.5			+0.23	
8.0	+0.30	+0.21	+0.21	+0.32
8.5			+0.18	+0.29
9.0	+0.25		+0.16	+0.18

[a] Conditions are as described in Figures 1-3. E_m represents the midpoint potential from the reductive titration.

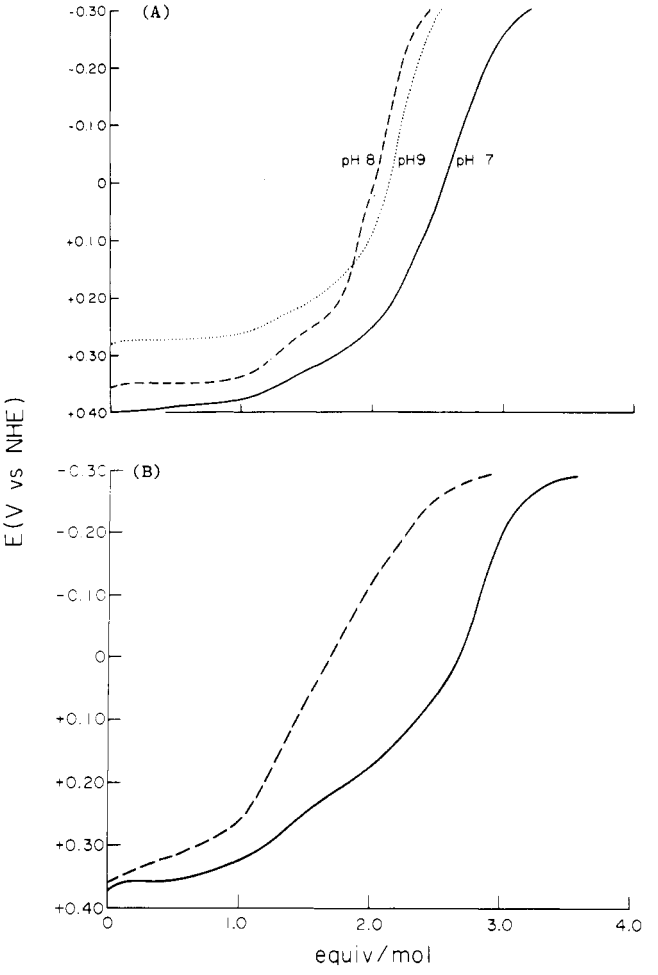


FIGURE 3: Coulometric titration curves for BESOD. All solutions contained 50 mM Tris–phosphate buffer, I = 0.15 with NaCl, 1.6–2.5 mM MV^{2+}, and 1.3 μM DCIP. Titration current was 100 μA. (A) (—) pH 7.0, 2.4 × 10^{-5} M Sigma BESOD; (---) pH 8.0, 2.6 × 10^{-5} M Sigma BESOD; (···) pH 9.0, 2.0 × 10^{-5} M Sigma BESOD. (B) pH 7.0: (—) 2.3 × 10^{-5} M Bannister BESOD, no oxidant added; (---) 1.8 × 10^{-5} M Bannister BESOD, with 7.2 μM K_2IrCl_6 added before titration.

Potentiometric Titration of BESOD. Typical coulometric titration curves for Sigma BESOD at three pH values are shown in Figure 3A. The titration curves indicate a large degree of anomalous behavior for this protein. At first glance, the two copper centers in BESOD appear to be titrated at different potentials because the break in the coulometric titration curves consistently occurred in the region of 1 equiv/mol of protein. Many preparations of BESOD have been shown to contain considerable amounts of inactive or altered protein [cf. Bray et al. (1974)], and polyacrylamide

gel electrophoresis proved the existence of at least three components in this lot of enzyme: one major and two minor components, each with SOD activity. However, sedimentation velocity experiments have confirmed that the enzyme migrates as a single band.

Higher purity BESOD (see Experimental) also exhibits a sedimentation velocity pattern characteristic of a homogeneous protein, although polyacrylamide gel electrophoresis shows two distinct bands: one major band and one minor band, both with SOD activity. The coulometric titration curve for this protein (Figure 3B) at pH 7.0 indicates an even greater degree of anomalous behavior than that observed with the Sigma protein. The end point is difficult to discern in these titrations, and the best estimate of the midpoint potential from the reductive titrations is +0.27 V vs. NHE. When the fully reduced protein is back titrated with $K_3Fe(CN)_6$, the midpoint potential is +0.40 V, which is in good agreement with the midpoint potential reported by Fee & DiCorleto (1973) using a similar method. The spectrophotometric results of the back-titration indicate that when 1 equiv of ferricyanide is added per g-atom of copper in the enzyme, only 70% of the absorbance at 680 nm for the enzyme is restored and 30% of the ferricyanide remains oxidized. The midpoint potential calculated from these results [by using $E^{0\prime}$ = +0.42 V for the hexacyanoferrate(III/II) couple under these conditions] also is +0.40 V, in good agreement with the potential observed with the Pt measuring electrode. Similar results are obtained when K_2IrCl_6 is used as an oxidant for the back-titration, although the midpoint potential that results from use of this oxidant is +0.25 V, far removed from the value that is obtained with ferricyanide as the oxidant.

The results indicate that anions may play an important role relative to the midpoint potential for BESOD. Addition of either $K_3Fe(CN)_6$ or K_2IrCl_6 to a solution of BESOD prior to the reductive titration resulted in nearly a stoichiometric loss of titratable copper in the oxidized enzyme up to 0.5 equiv of copper. Figure 3B illustrates the coulometric titration of BESOD in the presence of 0.4 equiv of K_2IrCl_6. Instead of the expected increase in reducing equivalents needed to reach the end point, the number of reducing equivalents is decreased by about 0.4 equiv. As the amount of K_2IrCl_6 is increased above 0.5 equiv/Cu, the number of titratable equivalents increases again. This provides strong support for the conclusion that the $IrCl_6^{2-}$ ion binds to only one of the copper centers and causes it to be nontitratable. Similar results are observed when ferricyanide is added to the BESOD solution prior to coulometric titration.

Studies with CN^- and H_2O_2. Addition of CN^- to oxidized BESOD gives a titration curve similar to that observed in solutions without added protein. This indicates that the Cu(II)–CN^- complex which forms (see Rotilio et al., 1972) is not reduced by the $MV^{+}\cdot$ radical.

Addition of H_2O_2 to either BESOD or MnSOD results in a complicated coulometric titration curve which is attributed to the species present in the solution after the interaction of H_2O_2 with these enzymes.

Discussion

The coulometric titration of MnSOD from either *E. coli* or *B. stearothermophilus* indicates that there probably is only one Mn(III) ion per molecule of enzyme (dimer), which is in agreement with other reports (Brock et al., 1976; Ose & Fridovich, 1976). The pH profile for the midpoint potential of the *Bacillus* enzyme indicates that there is a one-proton process associated with the one-electron reduction. The ΔE_m/pH ratio is approximately 50 mV between pH 7 and pH 9. The *E. coli* MnSOD, on the other hand, shows little or no pH dependence on E_m between pH 7 and pH 8 but exhibits a pronounced effect between pH 8 and pH 9. These results are consistent with the kinetic data for catalytic activity of the enzyme over this pH range (Forman & Fridovich, 1973). There is only a small change in the catalytic rate constant between pH 6.0 and pH 7.8, but the rate constant decreases by approximately one-third between pH 7.8 and pH 8.5. The pH dependence of the midpoint potential above pH 8 indicates that this is not a simple one-proton process.

BESOD exhibits more complicated coulometric titration curves, which may be attributed to several factors. (1) The copper centers of BESOD are nonequivalent or there is subunit interaction whereby the redox state of one of the copper centers affects the physical properties of the second copper site. (2) The anomalous behavior may be due to impurities in the sample or titration of the different-charge isomers which appear in the gel electrophoresis, although the more homogeneous enzyme exhibits the more erratic behavior. (3) The reduced protein may be affecting the electrode surface, resulting in an erroneous response of the measuring electrode; however, back-titration of the enzyme gives a good electrode response.

The first possibility is favored because a back-titration of the enzyme with ferricyanide or chloroiridate appears to alter the redox properties of only half of the copper sites. Addition of these oxidants to the enzyme also affects the coulometric titration in a manner that indicates that one of the copper sites is more susceptible to the influence of such anions. The results obtained in the present study clearly indicate that hexacyanoferrate is not the mediator or titrant of choice for the determination of the redox potential of BESOD and shed some doubt on the reliability of the E_m values that have been obtained with this ion as a titrant (Fee & DiCorleto, 1973). A positively charged organic radical, such as $MV^{+}\cdot$, appears to be a better mediator–titrant for the cupric ion in BESOD.

Previous work has demonstrated that CN^- binds to copper in SOD at a ratio of 1 mol/g-atom of metal (Rotilio et al., 1972). The cyanide binding causes inactivation of the enzyme which can be reversed by dialysis. The EPR and electronic absorption spectra confirm that the CN^- ion binds to the Cu(II) form of the enzyme. Because the coulometric titration results indicate that the $MV^{+}\cdot$ radical does not reduce the resultant Cu(II)–CN^- complex, its reduction potential must be quite negative ($E^{0\prime}_{MV}$ = −0.44 V) (Clark, 1960).

Finally, the H_2O_2 reduction of the Cu(II) site in bovine SOD poses thermodynamic and mechanistic problems. H_2O_2 is not a good one-electron reductant ($E^{0\prime}_{O_2^{-}\cdot/H_2O_2}$ = +0.87 V) (Fee & Valentine, 1977), and although it is a better two-electron reductant ($E^{0\prime}_{O_2/H_2O_2}$ = +0.27 V) (George, 1965), this is unlikely to be its function because the Cu centers of the protein function as one-electron oxidants. We favor a mechanism (eq 3–6) which invokes $O_2^{-}\cdot$ as the ultimate reducing agent.

$$ECu^{II} + H_2O_2 \rightleftharpoons ECu^{II}\text{–}O_2H + H^+ \quad (3)$$

$$ECu^{II}\text{–}O_2H + H_2O_2 \rightarrow ECu^{II}\text{–}O_2^{-}\cdot + \cdot OH + H_2O \quad (4)$$

$$ECu^{II}\text{–}O_2^{-}\cdot \rightarrow ECu^{I} + O_2 \quad (5)$$

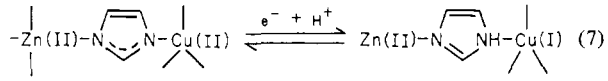
$$Pr(ImH) + \cdot OH \rightarrow Pr(ImH^{+}\cdot) + OH^- \quad (6)$$

Base-induced generation of $O_2^{-}\cdot$ and ·OH from H_2O_2 has been demonstrated in aprotic solvents (Roberts et al., 1978). A similar reaction is feasible at the active site of the enzyme with the ·OH that is produced being rapidly consumed by aromatic groups within the protein at the active site (viz.,

imidazole, vide infra). This in turn leaves the less reactive $O_2^{-}\cdot$ radical to reduce the Cu(II) site. That $O_2^{-}\cdot$ acts as the reductant is further supported by the fact that excess H_2O_2 does not effect complete reduction of the protein as indicated by some residual EPR signal and visible absorption (Rotilio et al., 1973). This probably is due to some enzyme turnover under these conditions.

A large excess of H_2O_2 (>10-fold) at pH >9.5 causes an irreversible inactivation of bovine SOD (Hodgson & Fridovich, 1975). The irreversible inactivation has been attributed to oxidation of an imidazole residue in the protein, probably at the active site (Bray et al., 1974). Alcohols and benzoate, which are scavengers of ·OH, are ineffective in preventing this inactivation. However, if the ·OH is indeed generated near the active site, its direct reactivity with the imidazole residues of the enzyme probably would prevent any inhibition by the exogenous ·OH scavengers.

In conclusion, the present results for BESOD are in agreement with the H^+ ion dependence that has been reported earlier (Fee & DiCorleto, 1973). However, the titration curves (Figure 3A) indicate two unequivalent Cu centers in the enzyme. The pH dependence of the midpoint potential for BESOD also is in agreement with the previously proposed mechanistic scheme (Hodgson & Fridovich, 1975; Fee, 1977; McAdam et al., 1977c) as shown in eq 7.



(7)

The pH dependence of the midpoint potential for *E. coli* MnSOD (Figure 2), on the other hand, indicates that a different mechanism is in effect. Apparently the reduction of the Mn(III) center in the enzyme is not accompanied by a redox-associated protonation–deprotonation. Proton relaxation rate studies for *E. coli* MnSOD indicate that a water molecule is bound to the Mn(III) in the oxidized enzyme (Villafranca et al., 1974). The kinetic data indicate that the water exchange rate is too slow to be involved catalytically and that the enzyme may function via an outer-sphere mechanism. The pulse radiolysis investigations of MnSOD from *B. stearothermophilus* (McAdam et al., 1977a,b) and from *E. coli* (Pick et al., 1974) lend support to a fast outer-sphere mechanism at low substrate concentrations; a slower inner-sphere mechanism may be important at high substrate concentrations (McAdam et al., 1977b).

If the outer-sphere mechanism is in effect at low $[O_2^{-}\cdot]$, the O_2^{2-} that is produced by the Mn(II) form of the enzyme is such a strong base it probably would deprotonate the Mn(III)-bound water to form HO_2^{-} and EMn(III)–OH. The absence of such a strong base under the conditions of the potentiometric titration experiments would preclude such a deprotonation of the oxidized enzyme. The unusual pH behavior of the MnSOD under alkaline conditions may be due to one or more deprotonation steps in the enzyme at a site removed from the metal, which cause a conformational change in the protein to give an alteration of the manganese coordination sphere.

The 50 mV/pH unit dependence of the *B. stearothermophilus* MnSOD midpoint potential indicates that the physical properties of the manganese center in this enzyme may be slightly different from the *E. coli* enzyme. Such a difference in the pH dependence of the midpoint potentials may be due to modest differences in the manganese microenvironment in these closely related proteins. An extended X-ray absorption fine structure investigation of the *B. stearothermophilus* MnSOD is currently underway at the Stanford Synchrotron Radiation Laboratory to gain additional insight into the coordination sphere of the manganese center in this enzyme.

Acknowledgments

The authors express their gratitude to the late Dr. J. I. Harris for supplying the *Bacillus stearothermophilus* MnSOD. We thank Dennis Ose and Professor I. Fridovich for the sample of *E. coli* B MnSOD. We also thank Professor Ning Pon of the Department of Biochemistry, University of California, Riverside, CA, for performing the sedimentation velocity and gel electrophoresis experiments and for his helpful discussions.

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Proteolytic Activity of Nerve Growth Factor: A Case of Autocatalytic Activation†

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ABSTRACT: Nerve growth factor is a highly specific protease that can convert plasminogen to plasmin and that can hydrolyze certain synthetic N-substituted arginine esters (e.g., N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAME); N. S. Orenstein et al. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497). Hydrolysis of TAME is characterized by a lag phase of lower velocity which precedes development of the steady-state maximal velocity. Kinetic analyses indicate that this behavior stems from autocatalytic activation of a nerve growth factor (NGF)-zymogen by NGF. As isolated from the mouse submandibular gland at high concentration, NGF is largely enzymically inactive. Upon high dilution, the protein undergoes autocatalytic activation with concomitant generation of full enzymic activity. The biologic significance of this unusual property of NGF is not clear, but it may serve to prevent expression of enzymic activity until the protein reaches its target cell(s) or until it recognizes its physiological substrate.

Recent studies have shown that crude extracts of male mouse submandibular glands contain multiple molecular forms of nerve growth factor (NGF)[1] (Young et al., 1978). In fact, by using a variety of chromatographic and electrophoretic procedures, at least six different forms of NGF can be observed. Of these six species, only one is stable in highly dilute solution; i.e., it does not dissociate into its constituent polypeptide chains. In contrast, all other forms of mouse salivary gland NGF are appreciably unstable in dilute solution. These observations led us to propose that the several unstable forms of the protein arise by proteolytic degradation of the single stable form—either in vivo or during the process of isolation in vitro (Young et al., 1978).

The single stable form of gland NGF (HMW-NGF) has been purified to homogeneity by chromatographic, ultracentrifugal, and electrophoretic criteria (Young et al., 1978). It has a molecular weight of 116000, and it contains as part of its quaternary structure a noncovalently linked subunit(s) which is immunochemically and electrophoretically indistinguishable from that of the smaller 2.5S form of NGF, first described by Bocchini & Angeletti (1969). Furthermore, the 116000 molecular weight stable form of the protein is the predominant species of NGF which is secreted in extraordinarily high concentrations in mouse saliva (Murphy et al., 1977a,b). Thus, it would appear that this is the principal form of NGF which is destined for export by the mouse submandibular gland.

One other form of high molecular weight salivary gland NGF has been described. In 1967, Varon et al. (1967) reported the isolation of an NGF species with a sedimentation coefficient of 7 S, from which a molecular weight of 140000 was estimated. These authors also reported that all of the NGF activity in gland homogenates is associated with the 7S species. We have been unable to confirm this result (Young et al., 1978). Furthermore, it is now known that, like the multiple unstable species of NGF present in gland extracts, 7S-NGF also is unstable and that it dissociates into its subunits at relatively high protein concentrations (Baker, 1975; Pantazis et al., 1977).

In another study, Greene et al. (1969) observed that 7S-NGF possessed esterase activity toward several N^{α}-substituted arginine and lysine ester substrates (e.g., TAME). This observation led us to ask whether the stable 116000 molecular form of gland NGF referred to above also exhibits esterase activity—and further, what protein substrates it might hydrolyze. In a recent study, we have shown that the stable form of NGF also hydrolyzes arginine and lysine esters and furthermore that it can convert plasminogen to plasmin with subsequent lysis of a fibrin clot (Orenstein et al., 1978). NGF-mediated fibrinolysis is strictly plasminogen dependent: no fibrin hydrolysis occurs in the absence of plasminogen. DFP inhibits the plasminogen activation reaction, and thus HMW-NGF appears to be a member of the general class of serine proteases. At the present time, plasminogen activation is the only known enzymic action of HMW-NGF upon a substrate of physiologic importance and of nonneural origin. Whether the reaction has any physiologic significance is not known.

In their study on the hydrolysis of arginine esters by 7S-NGF, Greene et al. (1969) observed that, when the protein was diluted from high to low concentration, the maximum catalytic velocity of the reaction was achieved only after a lag phase of lower velocity. Furthermore, predilution of the

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[1] Abbreviations used: NGF, nerve growth factor; HMW-NGF, high molecular weight form of NGF prepared as described by Young et al. (1978); EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphofluoridate; TAME, N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester.

0006-2960/79/0418-3050$01.00/0