Barber, M. J., Salerno, J. C., & Siegel, L. M. (1982b) *Biochemistry 21*, 1648–1656.
Bordas, J., Bray, R. C., Garner, C. D., Gutteridge, S., & Hasnain, S. S. (1980) *Biochem. J. 191*, 499–508.
Bray, R. C. (1975) *Enzymes, 3rd Ed. 12*, 299–419.
Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol. 51*, 107–165.
Cleere, W. F., & Coughlan, M. P. (1975) *Comp. Biochem. Physiol. B 50B*, 311–322.
Coughlan, M. P., Rajagopalan, K. V., & Handler, P. (1969) *J. Biol. Chem. 239*, 2027–2035.
Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188.
Gutteridge, S., Tanner, S. J., & Bray, R. C. (1978) *Biochem. J. 175*, 887–897.
Hille, R., Stewart, R., & Massey, V. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 862.
Johnson, J. L., & Rajagopalan, K. V. (1978) *Bioinorg. Chem. 8*, 439–444.
Kikuchi, T., Sugiura, Y., & Tanaka, H. (1982) *Inorg. Chim. Acta 66*, L5–L8.
Lowe, D. J. (1978) *Biochem. J. 171*, 649–651.
Lowe, D. J., & Bray, R. C. (1978) *Biochem. J. 169*, 471–479.
Lowe, D. J., Barber, M. J., Pawlik, R. T., & Bray, R. C. (1976) *Biochem. J. 155*, 81–85.
Massey, V., & Edmondson, D. (1970) *J. Biol. Chem. 245*, 6595–6598.
Massey, V., Komai, H., Palmer, G., & Elion, G. B. (1970) *J. Biol. Chem. 245*, 2837–2844.
Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974) *J. Biol. Chem. 249*, 4363–4382.
Peters, J. M., & Sanadi, D. R. (1961) *Arch. Biochem. Biophys. 93*, 312–313.
Pick, F. M., & Bray, R. C. (1969) *Biochem. J. 114*, 735–742.
Pick, F. M., McGartoll, M. A., & Bray, R. C. (1971) *Eur. J. Biochem. 18*, 65–72.
Rajagopalan, K. V., & Handler, P. (1964) *J. Biol. Chem. 239*, 2027–2035.
Rajagopalan, K. V., Fridovich, I., & Handler, P. (1962) *J. Biol. Chem. 237*, 922–928.
Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D., & Vincent, S. P. (1977) *Biochem. J. 167*, 593–600.

Potentiometric Titrations and Oxidation–Reduction Potentials of Several Iron Superoxide Dismutases†

William C. Barrette, Jr., Donald T. Sawyer,* James A. Fee, and Kozi Asada

ABSTRACT: The iron-containing superoxide dismutases for *Escherichia coli*, *Azotobacter vinelandii*, and *Pseudomonas ovalis* have been titrated with reduced benzylviologen (via in situ coulometric generation) in the presence of a potentiometric monitoring electrode. On the basis of the titration data the midpoint potentials (E_m), the pH dependence of the latter, and the electron stoichiometries for the three proteins have been determined; these results are compared with previous evaluations for Cu/Zn- and Mn-containing superoxide dismutases. At pH 7 the average value of E_m for this group of proteins is +0.26 ± 0.04 V vs. normal hydrogen electrode (NHE). The reduction potential of the Fe-containing protein from *E. coli* decreases by 0.06 V/pH unit from pH 6 to pH 9 but apparently is independent of pH from pH 9.5 to pH 10.8. Hence, the redox reaction for this protein (and, presumably, all of the Fe–proteins) obeys the equilibria

$$E_{ox} + H^+ + e^- \rightleftharpoons HE_{red}$$
$$(E_m)_{pH7} = +0.27 \text{ V vs. NHE} \qquad pH < 9$$
$$E_{ox} + e^- \rightleftharpoons E_{red}$$
$$(E_m)_{pH10} = +0.12 \text{ V vs. NHE} \qquad pH > 9$$

Superoxide dismutases are metalloproteins which catalyze the disproportionation of superoxide ion (McCord & Fridovich, 1969):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

There are three classes of superoxide dismutases as distinguished by the metal ion required for catalysis of reaction 1: Cu, Mn, and Fe. The proteins are isolated as either dimers or tetramers of identical subunits that have molecular weights of 16 000–20 000 with each subunit binding an active metal ion. In general, the Cu–protein, which contains a Zn^{2+} that is not necessary for catalysis (Fee, 1981; Valentine & Pantoliano, 1981), has been isolated from the cytosol of eucaryotic cells while Mn–proteins have been isolated from both pro- and eucaryotic cells. The iron-containing protein has not been observed in mammalian cells and is generally associated with procaryotes, notably anaerobes; however, an iron-containing protein recently has been found in plant tissues (Bridges & Salin, 1981). The distribution, function, and properties of these proteins have been widely discussed (cf. Bannister & Hill, 1980; Bannister & Bannister, 1980).

The iron-containing superoxide dismutases, which normally have two identical 20 000-dalton subunits with one iron per subunit (a four-subunit protein has been reported; Kusonose et al., 1976), exhibit unique spectral and annulation properties (Slykhouse & Fee, 1976; Asada et al., 1975; Fee et al., 1981a). The optical spectrum is characterized by a broad band near 350 nm (ϵ_{Fe} = 1850 M^{-1} cm^{-1}) that is attributed to a ligand-to-metal charge-transfer band. The relatively high energy for this transition indicates that tyrosine is not a ligand to the Fe(III) (Gaber et al., 1974). The EPR spectrum (g_{av} = 4.3) is characteristically rhombic and has been shown to possess unusual magnetic properties (Emptage, 1981). Azide and fluoride act as inhibitors (Misra & Fridovich, 1978; Fee et al., 1981a) of the dismutase activity while cyanide is not an inhibitor nor does it bind to the Fe(III) (Asada et al., 1975; Slykhouse & Fee, 1976). Hydrogen peroxide rapidly destroys the catalytic activity, and this process has been used to dis-

† From the Department of Chemistry, University of California, Riverside, California 92521 (W.C.B. and D.T.S.), Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109 (J.A.F.), and The Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan (K.A.). *Received August 23, 1982.* This work was supported by the National Institutes of Health–U.S. Public Health Service under Grants GM-22761 (D.T.S.) and GM-21519 (J.A.F.).

0006-2960/83/0422-0624$01.50/0

tinguish the iron dismutases from the manganese dismutases in crude cell extracts (Okada et al., 1979).

There appears to be a protic group associated with the Fe(III) of the *E. coli* protein. Thus, the association of azide, the spectral properties of the Fe(III) (Fee et al., 1981a), and the Michaelis constant for superoxide (Fee et al., 1981b) are all affected by pH in a similar fashion, which is consistent with an ionizing group with $pK_a \sim 9$. Proton relaxation measurements (Villafranca et al., 1974; Villafranca, 1976) indicate that H_2O may be a ligand to the metal, and the suggestion has been made that hydrolysis occurs at high pH (Fee et al., 1981b).

A widely discussed reaction scheme for catalysis of reaction 1 involves alternate reduction and oxidation of the metal between adjacent valence states:

$$O_2^- + P\text{–}M^{n+} \rightarrow P\text{–}M^{(n-1)+} + O_2 \quad (2)$$

$$O_2^- + P\text{–}M^{(n-1)+} \xrightarrow{H^+} P\text{–}M^{n+} + H_2O_2 \quad (3)$$

Thus, any detailed understanding of this catalysis must include information about the valence states available to the metal ion and the relevant reduction potentials. Some information of this type is available for the bovine erythrocytic Zn/Cu–protein (Fee & DiCorleto, 1973; Lawrence & Sawyer, 1979) and two bacterial Mn–proteins (Lawrence & Sawyer, 1979). The present study has been directed to the potentiometric titrations with reduced benzylviologen of the Fe-containing proteins of *Escherichia coli*, *Azotobacter vinelandii*, and *Pseudomonas ovalis*. In addition, the effect of solution pH on the midpoint potential for the Fe–protein from *E. coli* has been determined.

Experimental Procedures

The electrochemical cell that was used for the potentiometric titrations has been described previously (Lawrence & Sawyer, 1979). The reduced benzylviologen was produced by coulometric generation with an amperostat that is based on operational amplifiers (Goolsby & Sawyer, 1967). The potentiometric measuring and reference electrodes were connected to a Corning Model 12 pH meter, and the titration curves were recorded with a Sargent Model SR recorder that was interfaced with the pH meter.

A 5.0-mL sample solution that contains 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH adjusted with H_3PO_4) and 1.5 mM benzylviologen dichloride ($BVCl_2$) was used for each titration. The viologen cation radical ($BV^{+\cdot}$), which was produced by the coulometric reduction of $BVCl_2$, reacted rapidly with the oxidized proteins. Electron transfer from $BV^{+\cdot}$ to the proteins occurred at a much faster rate than its rate of generation. When the protein was fully reduced, the potential of the system sharply decreased at the equivalence point. Each sample solution also contained 1.3 μM dichloroindophenol (DCIP) as a redox mediator for the working electrode. Because DCIP appeared to interact with the protein or electrochemical system above pH 9.5, it was not used for these solutions. The buffered solutions were deaerated with Ar prior to the addition of proteins. The pH of each sample was measured before and after its titration. The blue-colored solutions of $BV^{+\cdot}$ were stable for at least 1 h after generation with this cell assembly in the absence of a protein sample. The coulometric titration system was validated regularly by the use of samples of ferricytochrome *c*. Over the course of the present investigation the average midpoint potential for such titrations was +0.265 ± 0.010 V vs. normal hydrogen electrode (NHE), a value consistent with those by several other methods (Eddowes & Hill, 1979).

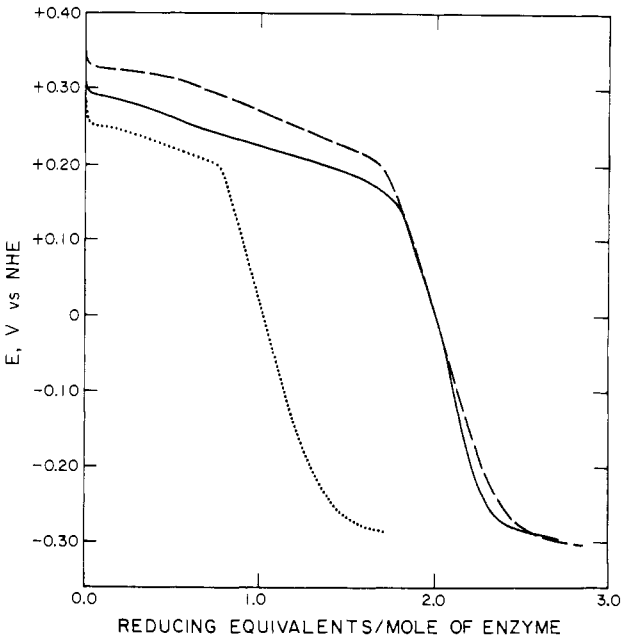

FIGURE 1: Potentiometric titration curves for three iron superoxide dismutases. All solutions contained 50 mM Tris–PO_4 buffer, 1.5 mM $BVCl_2$ (intermediate for the coulometric generation of the $BV^{+\cdot}$ titrant), and 1.3 μM DCIP. The ionic strength was adjusted to I = 0.15 with NaCl, and an electrolytic generation current of 100 μA was used. Each of the titration curves is at pH 7: (---) 2.5×10^{-5} M *E. coli* protein (T = 25 °C); (—) 2.12×10^{-5} *M A. vinelandii* protein (T = 25 °C); (···) 2.5×10^{-5} *M P. ovalis* protein (T = 0 °C).

Benzylviologen was obtained from Sigma (St. Louis, MO) as the dichloride, and dichloroindophenol was obtained from Aldrich (Milwaukee, WI). Both were used without further purification. The water for the titration solutions was prepared with a Millipore Milli-Q ultrapure water system. All other chemicals were used without further purification.

The iron-containing superoxide dismutase from *Azotobacter vinelandii* was isolated and shipped from Japan in a solution that was 94% saturated with ammonium sulfate. This protein has a molecular weight of 40 000 and is composed of two identical subunits with one atom of iron per subunit. Its concentration was determined spectrophotometrically with $A^{1\%}_{1cm}$ (350nm) = 0.905 and ϵ_{Fe} = 1810 M^{-1} cm^{-1} at 350 nm (S. Kanematsu and K. Asada, unpublished results) after dialysis against 50 mM Tris–PO_4 pH 7.0. The iron–protein from *Pseudomonas ovalis* was provided by Professor K. Suzuki and Dr. F. Yamakura, Juntendo University, Chiba, Japan, as a 3.85 mM protein solution in 4.3 mM sodium phosphate buffer, pH 7.0. This protein was titrated at 0 °C because of its thermal instability. The iron–protein from *E. coli* was isolated by the procedure of Slykhouse & Fee (1976) and shipped from Michigan as a frozen solution that contained 0.69 mM protein-bound iron and 1% glycerol. It was titrated as received.

Results

Typical potentiometric titration curves for the three iron proteins are illustrated by Figure 1. The net reduction stoichiometry for the *A. vinelandii* and the *E. coli* proteins is close to the expected 2.0 equiv/mol of enzyme [two Fe(III) per mol]. For the *P. ovalis* protein the reduction stoichiometry is approximately 1.0 equiv/mol, which is half the expected value. Apparently, the iron in this protein is extremely labile. For example, one sample of the purified protein that contained 1.4 equiv of Fe/mol of enzyme decreased to 1.0 equiv of Fe/mol upon dialysis with a chelating agent. The present

Table I: Midpoint Potentials [E_m (V) vs. NHE] from the Potentiometric Reduction by Reduced Benzylviologen of Several Fe, Cu/Zn, and Mn Superoxide Dismutase Proteins

	Fe			Cu/Zn[b,c]	Mn[c]	
pH	*E. coli*	*P. ovalis*[a]	*A. vinelandii*	bovine	*B. stearothermophilus*	*E. coli*
6.0	+0.33, +0.30					
6.5	+0.28					
7.0	+0.27, +0.29	+0.23	+0.23	+0.28, +0.40[d]	+0.26	+0.31
7.5	+0.23, +0.28				+0.23	
8.0	+0.21, +0.19	+0.20		+0.21	+0.21	+0.32
8.25	+0.18					
8.5	+0.17, +0.22				+0.18	+0.29
8.75	+0.15, +0.16					
9.0	+0.12	+0.19			+0.16	+0.18
9.25	+0.11					
9.5	+0.14					
9.75	+0.12					
10.0	+0.12					
10.8	+0.12, +0.13					

[a] Titrated at 0 °C due to the thermal instability of the protein. [b] Bovine erythrocytic (Bannister). [c] From Lawrence & Sawyer (1979). [d] From Fee & DiCorleto (1973). Determined by equilibration with the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple.

results are consistent with the conclusion (Yamakura, 1976) that this protein contains two iron atoms per molecule, but one is lost during purification. Because the *P. ovalis* protein is thermally unstable, its titration was carried out at 0 °C.

The titration curves for the three proteins are well-defined and exhibit a broad stable midpoint potential region ($E_{1/4} - E_{3/4} \simeq 60$ mV), close to the theoretical value of 56 mV. The reduction midpoint potentials (E_m) of these, as well as for several previously studied superoxide dismutases, are summarized in Table I.

Dependence of E_m on pH. Potentiometric titrations have been carried out with the iron–protein from *E. coli* for solution acidities from pH 6 to pH 10.8. The observed midpoint potentials are summarized in Table I and plotted in Figure 2. The reduction stoichiometry varies from 1.5 to approximately 3 equiv/mol of protein over the entire pH range, and all the titration curves exhibit Nernstian behavior except those above pH 10.0. From pH 6 to pH 9, E_m decreases with a slope of approximately 60 mV/pH. However, above pH 9 E_m appears to become independent of pH; an approximate pK_a of 9.4 ± 0.2 is estimated from the data. There is considerable scatter of the data in this pH region, which may be due in part to the decreased stability of the protein at high pH (J. A. Fee, unpublished observations). Nevertheless, the experiments have been repeated several times with different preparations of protein and are, thus, thought to represent a true behavior of the protein.

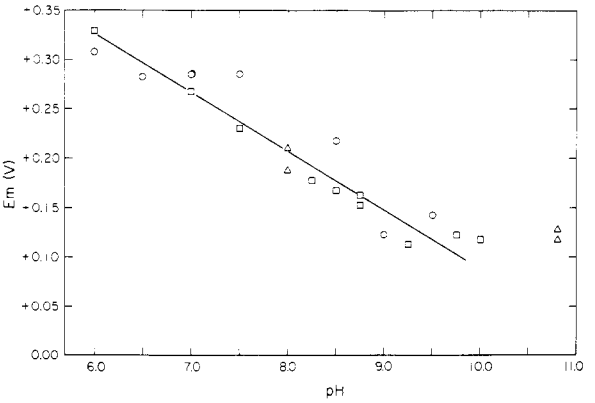

FIGURE 2: pH dependence of E_m values for *E. coli* Fe superoxide dismutase from pH 6.0 to pH 10.0. Solution conditions were the same as for Figure 1. The sample solutions at pH 10.8 did not contain DCIP. The line drawn through the data from pH 6.0 to pH 9.5 has a slope of −0.060 V/pH unit.

Discussion and Conclusions

The data of Table I show that the three bacterial Fe–proteins accept one electron per Fe(III) present and have an average E_m value of +0.27 ± 0.04 V vs. NHE at pH 7. Thus, there is no significant variation of the midpoint potential of these proteins isolated from the different bacteria. The positive value of E_m is significant in view of the fact that the EPR signal of the Fe–protein can be observed in whole cells of *A. vinelandii* and that the signal intensity varies with different states of metabolism (Davis et al., 1972; Shah et al., 1973; W. H. Orme-Johnson, personal communication). Because the cytosol is strongly reducing, due primarily to an abundance of thiols (Meister & Tate, 1976), the Fe(III) of the Fe–protein does not appear to be coupled to this major redox component of the cell. The significance of these observations is not presently clear.

Further examination of Table I confirms that all of the measured potentials for the several superoxide dismutases (Fe, Mn, and Cu) fall in the range from +0.23 to +0.40 V at pH 7.0. As discussed by Stein et al. (1979), for a metal ion to be active as a superoxide dismutase, its reduction potential must fall between the potentials for the two half-reactions of superoxide (O_2 at unit molarity).

$$O_2 + e^- \rightleftharpoons O_2^- \qquad E^{0\prime} = -0.16 \text{ V vs. NHE} \qquad (4)$$

$$O_2^- + e^- + 2H^+ \rightleftharpoons H_2O_2 \qquad E^{0\prime} = +0.89 \text{ V vs. NHE} \qquad (5)$$

Inasmuch as these electron-transfer processes are proportional to the free energy change associated with each step (Basolo & Pearson, 1967), an optimum catalyst might be expected to have a reduction potential midway between those for reactions 4 and 5; $(E^{0\prime}_4 + E^{0\prime}_5)/2 = +0.36$ V vs. NHE. While this condition appears to be met by the superoxide dismutases (Figure 3), this does not establish that elementary electron-transfer theory can be applied usefully to reactions 2 and 3. Thus, in the case of the Cu–protein, both reactions are diffusion controlled (Fielden et al., 1974), while the kinetic behavior of the Mn (McAdam et al., 1977) and the Fe dismutases is complicated by the apparent formation of protein–superoxo complexes. Hence, the nature of the rate-limiting process is not known. Nevertheless, the close similarity of the E_m values for the three classes of superoxide dismutase proteins is striking, as is their approximate agreement with the optimal value to facilitate O_2^- disproportionation. If the biological function of these proteins is other than superoxide dispro-

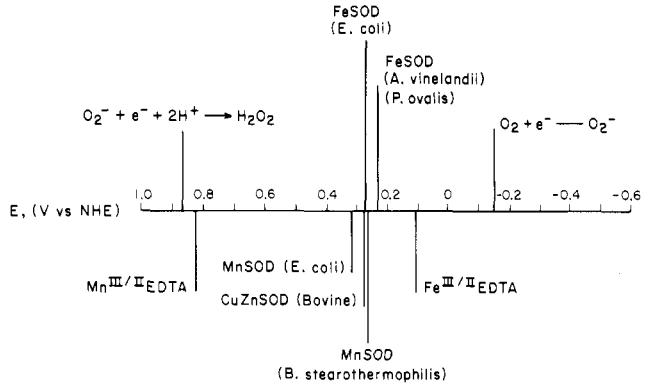

FIGURE 3: Formal reduction potentials ($E^{0\prime}$) for O_2 (unit molarity), O_2^-, and superoxide dismutase model complexes and midpoint potentials (E_m) for several dismutases in aqueous solution at pH 7.0. Values are from Lawrence & Sawyer (1979), Stein et al. (1979), and the present investigation.

portionation, this represents a remarkable coincidence of redox potentials for a diverse set of metals and peptides.

The pH dependence of E_m for the Fe–protein from *E. coli* indicates that the chemistry of the metal binding site is complicated. The data of Figure 2, for acidities below approximately pH 9, indicate that reduction of the Fe(III) center causes a proton to be bound by the protein; above pH 9 reduction occurs without concomitant proton binding. As with the Cu–protein (Fee & DiCorleto, 1973) a ligand bound to the Fe probably is responsible for this type of behavior

$$\overline{\text{L–Fe(III)}} + e^- + H^+ \rightleftharpoons \overline{\text{LH Fe(II)}} \quad (6)$$

$$\overline{\text{L–Fe(III)}} + e^- \rightleftharpoons \overline{\text{L–Fe(II)}} \quad (7)$$

with the apparent pK_a representing a competition of L for H^+ and Fe(II).

Previous studies have shown that the Fe(III) form of the protein ionizes with an apparent pK_a of 9.0 ± 0.3 so as to change the spectral properties of the Fe(III) and to decrease the association constant of Fe(III) for azide (Fee et al., 1981a). Rapid kinetic studies have shown that the Michaelis constant for O_2^- also is controlled by an ionizing group ($pK_a \sim 9$). These observations have been interpreted (Fee et al., 1981a) in terms of the hydrolysis of a bound water molecule

$$\text{P–Fe(III)–OH}_2 \rightleftharpoons \text{P–Fe(III)–OH}^- + H^+ \quad (8)$$

with $pK_a \sim 9$. However, the simultaneous occurrence of reactions 6–8 does not allow a rationalization of the high pH behavior of this protein; further work is required. Recently, three-dimensional structures of the *E. coli* (W. Stallings, T. B. Powers, K. Pattridge, J. A. Fee, and M. L. Ludwig, unpublished results) and *P. ovalis* (F. Yamakura, K. Suzuki, D. Ohmori, D. R. Ponzi, and G. A. Petsko, unpublished results) proteins have been solved to 3-Å resolution, which should aid in these efforts.

Acknowledgments

We thank Carol J. Nettleton for excellent assistance in the protein purification.

Registry No. Superoxide dismutase, 9054-89-1.

References

Asada, K., Yoshikawa, K., Takohoshi, M., Maeda, Y., & Enmanji, K. (1975) *J. Biol. Chem. 250*, 2801.

Bannister, W. H., & Bannister, J. V., Eds. (1980) *Dev. Biochem. 11B.*

Bannister, J. V., & Hill, H. A. O., Eds. (1980) *Dev. Biochem. 11A.*

Basolo, F., & Pearson, R. G. (1967) *Mechanisms of Inorganic Reactions*, 2nd ed., p 509, Wiley, New York.

Bridges, S. M., & Salin, M. L. (1981) *Plant Physiol. 68*, 275.

Davis, L. C., Shah, V. K., Brill, W. J., & Orme-Johnson, W. H. (1972) *Biochim. Biophys. Acta 256*, 512.

Eddowes, M. J., & Hill, H. A. O. (1979) *J. Am. Chem. Soc. 101*, 4461.

Emptage, M. H. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1798 (Abstr. No. 1488).

Fee, J. A. (1981) *Met. Ions Biol. Syst. 13*, 259.

Fee, J. A., & DiCorleto, P. E. (1973) *Biochemistry 12*, 4893.

Fee, J. A., McClune, G. J., Lees, A. C., Zidovetzki, R., & Pecht, I. (1981a) *Isr. J. Chem. 21*, 54.

Fee, J. A., McClune, G. J., O'Neill, P. O., & Fielden, E. M. (1981b) *Biochem. Biophys. Res. Commun. 100*, 377.

Fielden, E. M., Roberts, P. B., Gray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G., & Calabrese, L. (1974) *Biochem. J. 139*, 49.

Gaber, B. P., Miskowski, V., & Spiro, T. G. (1974) *J. Am. Chem. Soc. 96*, 6868.

Goolsby, A. D., & Sawyer, D. T. (1967) *Anal. Chem. 34*, 411.

Kusonose, E., Ichihara, K., Noda, Y., & Kusonose, M. (1976) *Biochem. J. 80*, 1343.

Lawrence, G. D., & Sawyer, D. T. (1979) *Biochemistry 18*, 3045.

McAdam, M. F., Fox, R. A., Lavelle, F., & Fielden, E. M. (1977) *Biochem. J. 165*, 71.

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

Meister, A., & Tate, S. S. (1976) *Annu. Rev. Biochem. 45*, 559.

Misra, H. P., & Fridovich, I. (1978) *Arch. Biochem. Biophys. 189*, 317.

Okado, S., Kanematsu, S., & Asada, K. (1979) *FEBS Lett. 103*, 106.

Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., & Brill, W. J. (1973) *Biochim. Biophys. Acta 292*, 246.

Slykhouse, T. O., & Fee, J. A. (1976) *J. Biol. Chem. 251*, 5472.

Stein, J., Fackler, J. P., Jr., McClune, G. J., Fee, J. A., & Chan, L. T. (1979) *Inorg. Chem. 18*, 3511.

Valentine, J. S., & Pantoliano, M. W. (1981) *Met. Ions Biol. 3*, 291.

Villafranca, J. J. (1976) *FEBS Lett. 62*, 230.

Villafranca, J. J., Yost, F. J., & Fridovich, I. (1974) *J. Biol. Chem. 249*, 3532.

Yamakura, F. (1976) *Biochim. Biophys. Acta 422*, 280.