Kinetics of Electron Transfer between Cytochrome *c* and Laccase†

Takeshi Sakurai

College of Liberal Arts and Sciences, Kanazawa University, Kanazawa, Ishikawa 920, Japan

Received February 28, 1992; Revised Manuscript Received June 25, 1992

ABSTRACT: Rate constants have been determined for the electron-transfer reactions between reduced horse heart cytochrome *c* and resting *Rhus vernicifera* laccase as a function of pH, ionic strength, and temperature. The second-order rate constant for the oxidation of reduced cytochrome *c* was determined to be $k = 125$ M^{-1} s^{-1} at 25 °C in 0.2 M phosphate buffer at pH 6.0 with the activation parameters $\Delta H^{\ddagger} = 16.2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 28.9$ J mol^{-1} K^{-1}. The rate constants increased with decreasing buffer concentration, indicating that electron transfer from cytochrome *c* to laccase is favored by the local electrostatic interaction ($Z_A Z_B = -0.9$ at pH 6 and -1.3 at pH 4.8) between the basic proteins with positive net charges. From the increase of the rate of electron transfer with decreasing pH, one of the driving forces of the reaction was suggested to be the difference in the redox potentials between the type 1 copper in laccase and the central iron in cytochrome *c*. Further, on addition of one hexametaphosphate anion per cytochrome *c* molecule, the rate of the electron transfer was increased, probably because the association of both proteins became more favorable.

Oxidoreduction reactions between metalloproteins have received special attention from both biological and mechanistic points of view (Kostić, 1991; McLendon, 1991; Therien et al., 1991). Respiration and photosynthetic chains are composed of a series of highly specific and directional electron-transfer reactions. Various factors which govern the rate of these processes are the distance and intervening medium (bonds) between redox centers, the orientation and redox potential difference (ΔE) between donor and acceptor, an appropriate association of the redox couple, protein structure dynamics coupled with electron transfer, etc. (Williams, 1990; Balton & Archer, 1991; Isied, 1991). Although electron transfer between inherent redox partners is naturally important, the reactions between metalloproteins which are not intrinsic redox partners are also significant to explore the driving force of the reaction and the origins of specificity and efficiency in macromolecular recognition. In line with this, the reactions between the heme proteins, cytochromes, and the blue copper protein plastocyanin have been examined by kinetic methods (Rosen & Pecht, 1976; Niwa et al., 1980; Corin et al., 1983; Augustin et al., 1984; Morand et al., 1989; Perrey et al., 1991; Roberts et al., 1991; Zhou et al., 1991). The reactions of these proteins with inorganic oxidants or reductants have also been studied in detail, aiming at the same goal (Rosenberg et al., 1976; Lappin et al., 1979; Farver & Pecht, 1981, 1989; Brunschwig et al., 1985; Pladziewicz et al., 1985; Gray, 1986; Tollin et al., 1986; Blake et al., 1991; Kyritsis et al., 1991; Navarro et al., 1991).

Laccase is a multi-copper oxidase which is widely distributed in plants and fungi (Reinhammar, 1984). The active site of laccase consists of a set of four copper ions classified according to its ESR feature: type 1 copper (blue copper), type 2 copper (nonblue copper), and a pair of type 3 coppers (ESR-nondetectable copper). A recent X-ray crystallographic study of another multi-copper oxidase, ascorbate oxidase, displayed that electrons enter into the protein molecule through the type 1 copper site and are transferred to the trinuclear center constructed with the type 2 and type 3 coppers (Messerschumidt et al., 1989). Much of the research on laccase has focused on defining the structure of its complex active site and the four-electron-reduction process of a dioxygen molecule at the trinuclear center (Reinhammar, 1984). Although kinetic studies have also frequently been performed, information about the binding site of urusiol, a derivative of catechol contained in lacquer latex, and information about the mechanism of how an electron is transferred from the substrate to the type 1 cooper site are still quite unsatisfactory (Holwerda & Gray, 1974; Nakamura, 1976). In this paper, we report the electron-transfer reaction between laccase and another metalloprotein for the first time. We selected cytochrome *c* as the electron donor to laccase, because its structure is known in detail, its properties have been thoroughly examined by spectroscopic and electrochemical methods, and its reactions with various redox reagents have been well examined by kinetic methods (Yamanaka, 1988; Moore & Pettigrew, 1990). These points are very favorable in studying how the two proteins associate and how intermolecular electron transfer occurs, because information on the protein structure of laccase is limited at present.

EXPERIMENTAL PROCEDURES

Chinese lacquer latex (*Rhus vernicifera*) was supplied by Takano and Co., Kanazawa, Japan. Laccase was purified according to the method of Reinhammar (1972) with minor modification ($A_{280}/A_{615} = 16$). Horse heart cytochrome *c* was purchased from Sigma Chemical Co. (type VI, 99% purity) and dialyzed against 0.2 M phosphate buffer (pH 6.0). Protein concentrations were determined on the basis of molar absorptivities, $\epsilon_{615} = 5800$ for laccase and $\epsilon_{550} = 27\,500$ for reduced cytochrome *c*, by using a JASCO Ubest-50 absorption spectrometer. Potassium phosphate buffer was used throughout experiments. All chemicals were of the highest quality available and were used without further purification. Water was deionized and distilled.

Cytochrome *c* was treated with a small amount of dithionite and was dialyzed against a buffer solution under N_2. Cytochrome *c* in the reduced form was quickly used for kinetic measurements in order to avoid its gradual autoxidation under air. Kinetic experiments were performed in a 1-cm path-

† This study was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan (03241210 and 04225211).

0006-2960/92/0431-9844$03.00/0

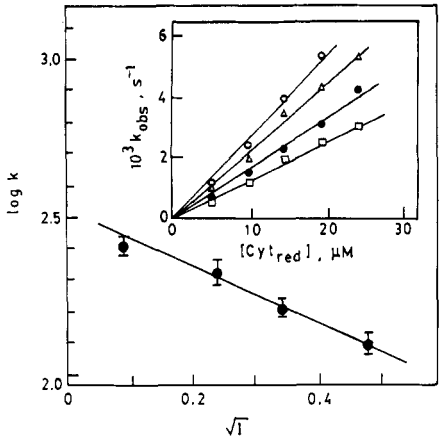

FIGURE 1: Plots of the second-order rate constants for cytochrome c oxidation versus the square root of the ionic strength (pH 6.0, 25 °C). Inset: Kinetics of electron transfer from reduced cytochrome c to laccase in 0.2 (□), 0.1 (●), 0.05 (△), and 0.008 M (○) phosphate buffer (laccase concentration was 4.88 μM).

length cell on an Otsuka Denshi MCPD-1000 spectrophotometer with diode array detector. Absorption spectra in the range 300–800 nm were monitored for 5 min at 1-s intervals, and the data of the absorbance change of reduced cytochrome c at 550 nm were stored in a desk-top computer. Pseudo-first-order decreases of reduced cytochrome c concentration were observed. The margins of error were approximately ±15%.

RESULTS

Effects of the Ionic Strength Dependence on Electron-Transfer Reactions between Cytochrome c and Laccase. For the kinetic measurements, a 1–5-fold excess of cytochrome c to laccase was used. The reason why more excess of reductant was not used is that the α band of reduced cytochrome c at 550 nm is so strong that the linearity of absorbance larger than 2 is not ensured. Four sets of reactions were performed at 25 °C in 0.2, 0.1, 0.05, and 0.008 M phosphate buffer at pH 6.0. First-order plots of the absorbance–time data were linear at the early stage of the reactions. The electron-transfer reaction with reduced cytochrome c was a second-order process

$$Cyt_{red} + Lac_{ox} \rightleftharpoons Cyt_{ox} + Lac_{red}$$

and the reaction rate could be obtained by the equation:

$$-d[Cyt_{red}]/dt = k_{obs}[Cyt_{red}] = k[Lac_{ox}][Cyt_{red}]$$

where k_{obs} is the pseudo-first-order rate constant and k is the second-order rate constant for the oxidation of reduced cytochrome c by laccase. The rate constants k_{obs} at pH 6 depending on the concentration of cytochrome c are shown in the inset of Figure 1. This figure displays that the rate of the reactions was increased with decreasing buffer concentration [linearity was not retained for the reactions in 0.008 M phosphate buffer with more than 10-fold excess of cytochrome c, probably because an association of proteins occurs in the low-concentration buffer (data not shown)]. According to Debye–Hückel's limiting law, the following simplified relation is retained for the reaction of the charged reactants (k, rate constants; μ, ionic strength; Z_A and Z_B, charges of reactants A and B):

$$\log k = 1.0182\sqrt{\mu} Z_A Z_B$$

This equation shows that the rate constant for a reaction depends on the ionic strength of the solution and the charges

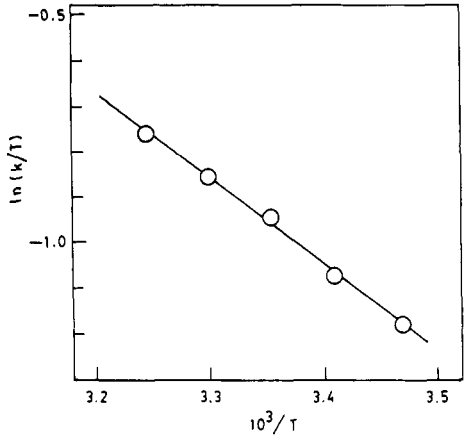

FIGURE 2: Eyring plots of the electron-transfer rate from reduced cytochrome c (24.4 μM) to laccase (4.88 μM) (25 °C, pH 6.0, 0.2 M phosphate buffer).

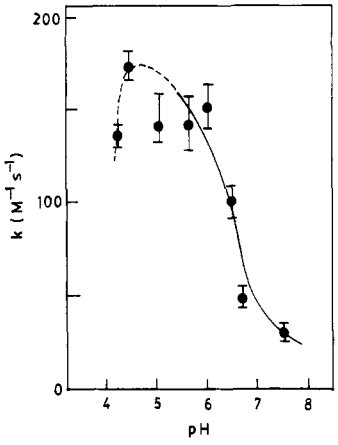

FIGURE 3: Effect of pH on the second-order rate of the oxidation of cytochrome c (24.4 μM) by laccase (4.88 μM) (25 °C, 0.2 M phosphate buffer).

of the reactants. Log values of the averaged second-order rates for a set of data at each buffer concentration were plotted against the square root of the ionic strength in Figure 1, where the ionic strengths were evaluated from the ionization constants of phosphoric acid (pK_{a1}, pK_{a2}, and pK_{a3} values are 2.12, 7.21, and 12.32, respectively). From the slope of the line in the figure, $Z_A Z_B = -0.9$ was obtained. This shows that the association of cytochrome c and laccase with an electrostatic interaction allows electron transfer although the net charges of both proteins are positive at pH 6.0 (+10 for cytochrome c and +30 for laccase). Further, $Z_A Z_B = -1.3$ was obtained at pH 4.8, at which condition the net charges of cytochrome c and laccase are +11 and +38, respectively (data not shown).

Effect of Temperature on Electron-Transfer Reactions between Cytochrome c and Laccase. Kinetic measurements have been performed at five different temperatures (15, 20, 25, 30, and 35 °C) in 0.2 M phosphate buffer at pH 6.0, and the linear Eyring plot was obtained in Figure 2. From the slope and intercept, the thermodynamic parameters $\Delta H^{\ddagger}$ = 16.2 kJ mol^{-1} and $\Delta S^{\ddagger}$ = 28.9 J mol^{-1} K^{-1} were determined.

Effect of pH on Electron-Transfer Reactions between Cytochrome c and Laccase. We investigated the pH dependence of the rate constants at 25 °C in 0.2 M phosphate buffer. The rate constant increased with decreasing pH from 7.5 to 5.5 but did not change profoundly between pH 4 and 5.5 as shown in Figure 3. As pH 4 was approached, the rate seemed to begin to decrease. However, we did not get precise data near pH 4, since this pH value does not biologically make sense and is even harmful for protein stability.

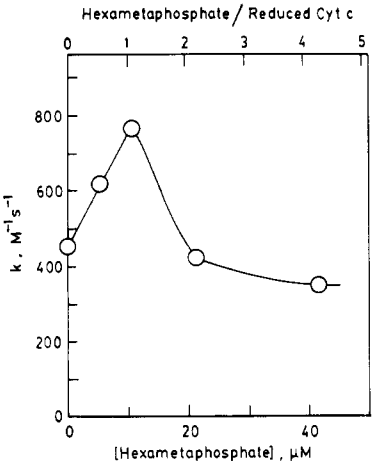

FIGURE 4: Effect of hexametaphosphate concentration on the second-order rate constant of the oxidation of cytochrome *c* (24.4 μM) by laccase (4.88 μM) (25 °C, pH 6.0, 4 mM phosphate buffer).

Effect of the Hexametaphosphate Anion on Electron-Transfer Reactions between Cytochrome c and Laccase. One or more hexametaphosphate anion(s) has been revealed to bind to the basic patch(es) of the cytochrome *c* surface surrounding the exposed heme edge (Concar et al., 1991a,b; Whitford et al., 1991). Since this polyanion is expected to affect the manner of the interaction between cytochrome *c* and laccase and accordingly is expected to affect the electron-transfer process, the experiments were performed by using cytochrome *c* which had been allowed to equilibrate previously with hexametaphosphate. Figure 4 shows that the presence of one hexametaphosphate ion per protein molecule accelerates the rate of electron transfer ca. 1.5 times. However, the electron-transfer process was inhibited on addition of more than one hexametaphosphate.

DISCUSSION

Electron-transfer reactions between cytochrome *c* or *f* and blue copper proteins such as plastocyanin, azurin, or stellacyanin have been reported to occur with a second-order rate constant as high as ca. 10^7 M^{-1} s^{-1} (Rosen & Pecht, 1976; Niwa et al., 1980; Corin et al., 1983; Augustin et al., 1984; Morand et al., 1989; Peerey et al., 1991; Roberts et al., 1991; Zhou et al., 1991). These results might not be unexpected, since the physiological function of both types of proteins is electron transfer. However, the biological function of laccase is essentially to oxidize urusiol, and its reaction is highly specific for phenol derivatives. This will be one of the reasons why no intermolecular electron-transfer reaction between laccase and another metalloprotein has been performed. However, although the electron-transfer rate is considerably slow as can be followed by using a conventional spectrophotometer (k = ca. 1×10^2 M^{-1} s^{-1}), the reaction between laccase and cytochrome *c* was actually found to occur.

Since the reactions which take place inside the laccase molecule, the intramolecular electron transfer between the copper centers and the four-electron reduction of dioxygen, occur much faster than the rate of intermolecular electron transfer from cytochrome *c* (Andréasson & Reinhammar, 1976, 1979; Goldgerg et al., 1980), the absorption feature of laccase did not change during the reaction. Although it is not clear which type of copper in laccase is involved in the entry of electrons in the reaction with cytochrome *c*, the type 1 copper site will be most probable. In the normal enzyme process, four electrons enter into the laccase molecule through this site and are transferred to the trinuclear center. Apparently, the oxidation of reduced cytochrome *c* proceeded at a rate much faster than that of the autoxidation under air.

The result of the ionic strength dependence (Figure 1) was unexpected, because both cytochrome *c* and laccase are basic proteins (p*I* values for cytochrome *c* and laccase are 10.5 and 8.6, respectively) having positive net charges within the pH range we used in our experiments (+10 at pH 6 and +11 at pH 4.8 for cytochrome *c*, and +30 at pH 6 and +38 at pH 4.8 for laccase). Therefore, it will be quite natural to expect that the collision of the positively charged molecules becomes more favorable with increasing ionic strength. Such an ionic strength dependent reaction between positively charged proteins was, for example, found for the electron-transfer reaction from ^{3}Zn–myoglobin to stellacyanin by flash photolysis (Tsukahara et al., 1992). However, the Debye–Hückel law showed that a weak, local electrostatic interaction is important (Z_AZ_B = –0.9 at pH 6 and –1.3 at pH 4.8) in the association which allows the electron transfer between cytochrome *c* and laccase (Figure 1). In line with this, the rate of electron transfer between positively charged cytochrome *c* and cytochrome *f* has been reported to be much slower (4×10^{-7} M^{-1} s^{-1}) (Wood, 1974) compared to the present result (ca. 1×10^2 M^{-1} s^{-1}). If we are permitted to speculate further, a negatively charged amino acid might be present near the substrate-binding site in laccase. The observed rather slow rate will not be that the thermodynamic driving force is not sufficient but will be that the intervening medium (bonds), especially that in laccase, or the orientation between redox centers is considerably unfavorable (vide infra).

The activation enthalpy (16.2 kJ mol^{-1}) obtained from Figure 2 was similar to those reported for the reactions between cytochrome *c* and plastocyanin (31 kJ mol^{-1}) (Augustin et al., 1984), cytochrome *f* and plastocyanin (35 kJ mol^{-1}) (Niwa et al., 1980), and cytochrome *c* and azurin (9.2 kJ mol^{-1}) (Augustin et al., 1984), indicating that the relatively low activation energy did not necessarily lead to a high reaction rate. Since the entropy term was positive, the free energy ($\Delta G^{\ddagger}$) value is considerably small (8.6 kJ mol^{-1} at 25 °C), and, accordingly, the cause for the relatively slow rate will be that the association of cytochrome *c* and laccase molecules is not very favorable. The positive activation entropy has been attributed to the reaction between the oppositely charged proteins (Segal & Sykes, 1974).

The reaction rate increased with decreasing pH from ca. 7.2 to ca. 5.5 but did not change prominently below pH ca. 5.5 (Figure 3). In line with this, the redox potential of the type 1 copper in laccase has been reported to change from ca. 380 mV to ca. 460 mV in harmony with the pH change from ca. 9 to 4 (Nakamura, 1958), while that of cytochrome *c* is almost independent of pH (250 mV) (Ikeshoji et al., 1989). Therefore, the thermodynamic driving force of the electron transfer between laccase and cytochrome *c* should increase with decreasing pH: the $\Delta E^{\circ}_{Cyt-Lac}$ value increases from 130 to 210 mV with decreasing pH. The increase in the second-order rate constant with decreasing pH from 7.2 to ca. 5.5 supports the idea that the difference in the redox potential between the donor and acceptor is a driving force of the reaction. Nevertheless, it does not necessarily seem to account fully for the data between pH 5.5 and 4.2. Alternatively, the acid–base equilibrium of a residue which has a pK_a value of ca. 6.5 might be concerned with the pH dependency. The plausible residue to give such the pK_a value is histidine. However, the imidazole group is protonated in the acidic form, while un-ionized in the basic form. This is contradictory to

the fact that the reaction between laccase and cytochrome *c* molecules is favored at weak acidic pHs than of neutral pH. The decrease of the reaction rate near pH 4 might reflect an electrostatic effect: the pK_a value of a carboxylic acid group in the side chain of glutamic acid or aspartic acid is ca. 4, and the group is negatively charged at a higher pH but is unionized at a lower pH. In addition to this effect near pH 4 and the redox potential difference between the donor and acceptor, it is probable that a structure change on the protein molecule(s) dependent on pH also affected the electron-transfer process, although it was not noticeable in the absorption, CD, and ESR spectra (Sakurai et al., 1990). The ionic strength dependence at pH 6 and 4.8 (the Z_AZ_B values were −0.9 and −1.3 at pH 6 and 4.8, respectively) indicates that the local electrostatic charge on the reactants also changes depending on the pH.

According to Williams et al. (Concar et al., 1991a,b; Whitford et al., 1991), hexametaphosphate binds close to lysines-13, -86, and -87 in cytochrome *c* (Concer et al., 1991a,b; Whitford et al., 1991), facilitating the approach of the two molecules by reducing intermolecular electrostatic repulsion. The prominent effect of this polyanion on the electron-transfer rate might suggest that the positively charged patch formed by the three lysine residues on cytochrome *c* surface is not the site to associate with laccase. The approach of cytochrome *c* toward laccase would have become easier in the presence of this polyanion.

We are trying to modify the charged residues in cytochrome *c* in order to get further information about the manner of the association of the two proteins. The study on ascorbate oxidase in the place of laccase indicated that this multi-copper oxidase shows similar behavior except that the activation enthalpy for the electron transfer is practically zero (unpublished data).

ACKNOWLEDGMENT

We are grateful to Dr. Keiichi Tsukahara of Nara Woman's University for helpful discussion.

REFERENCES

Andréasson, L.-E., & Reinhammar, B. (1976) *Biochim. Biophys. Acta 445*, 579–599.
Andréasson, L.-E., & Reinhammar, R. (1979) *Biochim. Biophys. Acta 568*, 145–156.
Augustin, M. A., Chapman, S. K., Davis, D. M., Walton, A. D., & Sykes, A. G. (1984) *J. Inorg. Biochem. 20*, 281–289.
Blake, R. C., II, White, K. J., & Shute, E. A. (1991) *Biochemistry 30*, 9443–9449.
Bolton, J. R., & Archer, M. D. (1991) in *Electron Transfer in Inorganic, Organic, and Biological Systems* (Bolton, J. R., Mataga, N., & McLendon, G., Eds.) pp 7–23, American Chemical Society, Washington, D.C.
Brunschwig, B. S., DeLaive, P. J., English, A. M., Goldberg, M., Gray, H. B., Mayo, S. L., & Sutin, N. (1985) *Inorg. Chem. 24*, 3743–3749.
Concar, D. W., Whitford, D., & Williams, R. J. P. (1991) *Eur. J. Biochem. 199*, 553–560, 569–574.
Corin, A. F., Bersohn, R., & Cole, P. E. (1983) *Biochemistry 22*, 2032–2038.
Farver, O., & Pecht, I. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4190–4193.
Farver, O., & Pecht, I. (1989) *FEBS Lett. 244*, 379–382.
Golberg, M., Farver, O., & Pecht, I. (1980) *J. Biol. Chem. 255*, 7353–7361.
Gray, H. B. (1986) *Chem. Soc. Rev. 15*, 17–30.
Holwerda, R. A., & Gray, H. B. (1974) *J. Am. Chem. Soc. 96*, 6008–6022.
Ikeshoji, T., Taniguchi, I., & Hawkridge, F. M. (1989) *J. Electroanal. Chem. 270*, 297–308.
Isied, S. S. (1991) *Met. Ions Biol. Syst. 27*, 1–56.
Kostić, N. M. (1991) *Met. Ions Biol. Syst. 27*, 129–182.
Kyritsis, P., Lundberg, L. G., Nordling, M., Vänngård, T., Young, S., Tomkinson, N. P., & Sykes, A. G. (1991) *J. Chem. Soc., Chem. Commun.*, 1441–1442.
Lappin, A. G., Segal, M. G., Weatherburn, D. C., Henderson, R. A., & Sykes, A. G. (1979) *J. Am. Chem. Soc. 101*, 2302–2306.
Messerschmidt, A., Rossi, A., Ledenstein, R., Huber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzelli, R., & Finazzi-Agrò, A. (1989) *J. Mol. Biol. 206*, 513–529.
Moore, G. R., & Pettigrew, G. W. (1990) *Cytochromes c*, Springer-Verlag, Berlin.
Morand, L. Z., Frame, M. K., Colvert, K. K., Johnson, D. A., Krogmann, D. W., & Davis, D. J. (1989) *Biochemistry 28*, 8039–8047.
McLendon, G. (1991) *Struct. Bonding 75*, 159–174.
Nakamura, T. (1958) *Biochim. Biophys. Acta 30*, 44–52.
Nakamura, T. (1976) in *Iron and Copper Proteins* (Yasunobu, K., Mower, H. F., & Hayaishi, O., Eds.) pp 408–423, Plenum, New York.
Navarro, J., De la Rosa, M. A., & Tollin, G. (1991) *Eur. J. Biochem. 199*, 239–243.
Niwa, S., Ishikawa, H., Nikai, S., & Takebe, T. (1980) *J. Biochem. 88*, 1177–1183.
Peerey, L. M., Brothers, H. M., II, Hazzard, J. T., Tollin, G., & Kostić, N. M. (1991) *Biochemistry 30*, 9297–9304.
Pladziewicz, J. R., Brenner, M. S., Rodeberg, D. A., & Likar, M. D. (1985) *Inorg. Chem. 24*, 1450–1453.
Reinhammar, B. (1970) *Biochim. Biophys. Acta 205*, 35–47.
Reinhammar, B. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 3, pp 1–35, CRC Press, Boca Raton, FL.
Roberts, V. A., Freeman, H. C., Olson, A. J., Tainer, J. A., & Getzoff, E. D. (1991) *J. Biol. Chem. 266*, 13431–13441.
Rosen, P., & Pecht, I. (1976) *Biochemistry 15*, 775–785.
Rosenberg, R. C., Wherland, S., Holwerda, R. A., & Gray, H. B. (1976) *J. Am. Chem. Soc. 98*, 6364–6368.
Sakurai, T., Suzuki, S., & Chikira, M. (1990) *J. Biochem. (Tokyo) 107*, 37–42.
Segal, M. G., & Sykes, A. G. (1874) *J. Am. Chem. Soc. 100*, 4585–4592.
Therien, M. J., Chang, J., Raphael, A. L., Bowler, B. E., & Gray, H. B. (1991) *Struct. Bonding 75*, 109–129.
Tollin, G., Cheddar, M. G., Getzoff, E. D., & Cusanovich, M. A. (1986) *Biochemistry 25*, 3363–3370.
Tsukahara, K., Asami, S., Okada, M., & Sakurai, T. (1992) *J. Am. Chem. Soc.* (submitted for publication).
Whitford, D., Concar, D. W., & Williams, R. J. P. (1991) *Eur. J. Biochem. 199*, 561–568.
Williams, R. J. P. (1990) in *Electron Transfer in Biology and the Solid State*, pp 1–23, American Chemical Society, Washington, D.C.
Wood, P. M. (1974) *Biochim. Biophys. Acta 357*, 370–379.
Yamanaka, T. (1988) in *Metalloproteins. Chemical Properties and Biological Effects* (Otsuka, S., & Yamanaka, T., Eds.) pp 139–153, Kodansha, Tokyo.
Zhou, J. S., & Kostić, N. M. (1991) *J. Am. Chem. Soc. 113*, 6067–6073, 7040–7042.