5 should both proceed with formation of a chromophore around 550 nm, so long as reprotonation is slow. The results shown in Figure 4B,C bear out this prediction. It would appear, therefore, that stereochemical specificity (cis vs. trans isomerism) in the enzyme active site can radically alter the kinetic course of the reactions described. Thus, a substrate is converted into a potential inactivator by virtue of increased lifetime and so greater availability for capture.

References

Baldwin, J., Haber, S., Hoslins, C., & Kruse, L. (1977) *J. Org. Chem. 42*, 1239.

Cromartie, T., Fisher, J., Kaczorowski, G., Laura, R., Marcotte, P., & Walsh, C. (1974) *J. Chem. Soc., Chem. Commun.*, 597.

Davis, L., & Metzler, D. (1972) *Enzymes, 3rd Ed. 7*, 33.

Esaki, N., Suzuki, T., Tanaka, H., Soda, K., & Rando, R. (1977) *FEBS Lett. 84*, 309.

Giovanelli, I., Ownes, L., & Mudd, S. (1971) *Biochim. Biophys. Acta 227*, 671.

Guggenheim, S., & Flavin, M. (1971) *J. Biol. Chem. 246*, 3562.

Heyl, D., Leez, E., Harris, S., & Fockers, K. (1951) *J. Am. Chem. Soc. 13*, 3430.

Johnston, M., Donovan, J., Marcotte, P., & Walsh, C. (1979a) *Biochemistry 18*, 1729.

Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, J., Soda, K., & Walsh, C. (1979b) *Biochemistry 18*, 4690.

Johnston, M., Raines, R., Walsh, C., & Firestone, R. (1980) *J. Am. Chem. Soc. 102*, 4241.

Karube, Y., & Matsushima, Y. (1976) *J. Am. Chem. Soc. 98*, 3725.

Karube, Y., & Matsushima, Y. (1977) *J. Am. Chem. Soc. 99*, 7356.

Marcotte, P., & Walsh, C. (1978) *Biochemistry 17*, 5620.

Miles, E. (1975) *Biochem. Biophys. Res. Commun. 66*, 94.

Orlowski, M., Rheingold, D., & Stanley, M. (1977) *J. Neurochem. 28*, 349.

Posner, B., & Flavin, M. (1972) *J. Biol. Chem. 247*, 6412.

Rando, R. (1974a) *Nature (London) 250*, 586.

Rando, R. (1974b) *Biochemistry 13*, 3859.

Rossi, A., & Schinz, H. (1948) *Helv. Chim. Acta 31*, 473.

Scannell, O., Preuss, D., Demny, T., Weiss, F., Williams, J., & Stemple, A. (1971) *J. Antibiot. 4*, 329.

Silverman, R., & Abeles, R. (1977) *Biochemistry 16*, 5515.

Soda, K. (1968) *Anal. Biochem. 25*, 228.

Soper, T. S., Manning, J. S., Marcotte, P., & Walsh, C. (1977) *J. Biol. Chem. 252*, 1571.

Tanaka, H., Esaki, N., & Soda, K. (1977) *Biochemistry 16*, 100.

Walsh, C., Johnston, M., Marcotte, P., & Wang, E. (1978a) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., & Koch-Weser, J., Eds.) p 177, Elsevier/North-Holland, Amsterdam.

Walsh, C., Cromartie, T., Marcotte, P., & Spencer, R. (1978b) *Methods Enzymol. 53*, 437.

Yorifugi, T., Ogata, K., & Soda, K. (1971a) *J. Biol. Chem. 246*, 5085.

Yorifugi, T., Misono, H., & Soda, K. (1971b) *J. Biol. Chem. 246*, 5093.

Spectroscopic Studies of Stellacyanin Derivatives†

David B. Knaff,* Claudia E. Harsh, and Robert A. Holwerda*

ABSTRACT: Two covalently modified derivatives of the apoprotein of the blue copper protein stellacyanin have been prepared. In one case, a dansyl group was linked to the cysteine at the copper binding site of apostellacyanin; in the other, a nitrophenol moiety has been attached to this same cysteine. Fluorescence yields and emission maxima of the dansylated protein and pK determinations of the nitrophenol group linked to the protein suggest that the solvent microenvironment at the copper binding site of apostellacyanin is quite similar to bulk water.

The blue copper protein stellacyanin is found in the latex of the Japanese lacquer tree *Rhus vernicifera* (Reinhammar, 1970). Stellacyanin contains 40% carbohydrate by weight (Bergman et al., 1977) and one type-1 copper atom per molecular weight of 20000 (Omura, 1961). The single polypeptide chain of 107 amino acid residues contains one cysteine (Cys-59), four histidines (His-32, -46, -92, and -100), and one cystine unit (linking Cys-87 and -93), but no methionine (Bergman et al., 1977). The absence of methionine distinguishes stellacyanin from two other blue copper proteins, plastocyanin (Colman et al., 1978) and azurin (Adman et al., 1978), whose X-ray crystal structures show two histidine nitrogen atoms, one methionine sulfur atom, and one cysteine sulfur atom in the first coordination sphere of copper.

Crystallographic results are not available for stellacyanin, but physical studies have provided some information about the copper site in this metalloprotein. Cobalt substitution (McMillin et al., 1974), X-ray photoelectron spectroscopy (Wurzbach et al., 1977), and chemical modification studies with organomercurials (Morpurgo et al., 1972) have provided good evidence for cysteine sulfur coordination in stellacyanin, and ^{1}H NMR results (Hill & Lee, 1979) suggest the presence of two histidine ligands. Cystine disulfide ligation has been suggested (Ferris et al., 1978; Hill & Lee, 1979), but the evidence in this case is not as strong as for the cysteine ligand. Absorption and circular dichroism spectra of stellacyanin have been successfully interpreted in terms of the d–d and charge-transfer transitions expected for copper(II) in a flattened tetrahedral coordination environment containing both

† From the Department of Chemistry, Texas Tech University, Lubbock, Texas 79409. *Received December 24, 1980.* This research was supported by grants from the Robert A. Welch Foundation (D-710 to D.B.K. and D-735 to R.A.H.).

0006-2960/81/0420-4333$01.25/0

N and S donor atoms (Solomon et al., 1976a). Spectroscopic studies of cobalt(II)–stellacyanin support the formulation of a distorted tetrahedral coordination environment (Solomon et al., 1976b).

In addition to its lack of methionine, stellacyanin differs from azurin and plastocyanin by having a much less positive standard reduction potential, +0.184 V, compared with +0.347–+0.390 V (*P. vulgaris* and *Chlorella* plastocyanin, respectively) and +0.330 V (*P. aeruginosa* azurin) (Sailasuta et al., 1979; Katoh, 1960) and a substantially higher redox reactivity in reactions with transition metal ion reductants and oxidants (Cummins & Gray, 1977; Holwerda et al., 1980). Both of these observations suggest that the type-1 copper site in stellacyanin has a comparatively hydrophilic environment and is readily accessible to the solvent and external redox agents (Sailasuta et al., 1979). We have attempted to directly measure the environment around the copper site by covalently attaching environment-sensitive chromophores to the single cysteine residue in the protein.

Materials and Methods

Stellacyanin was prepared from an acetone powder from *Rhus vernicifera* (Saito and Co., Ltd., Tokyo) according to standard procedures (Reinhammar, 1970). Apostellacyanin was prepared by dialysis against NaCN for 1 h (Morpurgo et al., 1972). To ensure that minimal denaturation had occurred during copper removal, an aliquot of apoprotein was tested for reconstitutability with $Cu(ClO_4)_2$ (Morpurgo et al., 1972). All preparations of apoprotein used were at least 90% reconstitutable. Stellacyanin concentration was determined from the absorbance at 604 nm of the oxidized protein by using an extinction coefficient of 4.08×10^3 M^{-1} cm^{-1} (Malmström et al., 1970). The concentration of apostellacyanin was determined either from the absorbance at 280 nm by using an extinction coefficient of 2.32×10^4 M^{-1} cm^{-1} (Morpurgo et al., 1972) or by measuring the protein concentration against a bovine serum albumin standard curve (Bradford, 1976). Free sulfhydryl groups were determined (Ellman, 1959) by assaying with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[1]

2-(Chloromercuri)-4-nitrophenol (CMNP) was prepared in 45% yield from 2-(acetatomercuri)-4-nitrophenol (McMurray & Trentham, 1969). Labeling of apostellacyanin by CMNP was accomplished by incubating the protein at a concentration of 1 mM in 0.1 M KOH with a 4-fold excess of CMNP for 10 min. The mixture was run through a Sephadex G-25 column and then dialyzed to remove any unreacted CMNP. The labeled protein, hereafter referred to as MNP-stellacyanin, contained 0.95 MNP residue per molecule. The protein concentration was determined as described above (Bradford, 1976), and the MNP concentration was determined from the absorbance of the nitrophenoxide anion at 405 nm by using an extinction coefficient of 1.74×10^4 M^{-1} cm^{-1} (McMurray & Trentham, 1969).

Apostellacyanin was dansylated by two methods. In one method, apostellacyanin was incubated with a 10-fold excess of *N,N′*-didansylcystine for 24 h at 4 °C under a nitrogen atmosphere (Wu & Stryer, 1972). The mixture was run through a Sephadex G-25 column, and fractions containing significant absorbance at 280 nm were pooled and dialyzed exhaustively against 0.1 M potassium phosphate buffer (pH 6.0). Apostellacyanin dansylated in this manner contained 0.95 dansyl group per molecule on the basis of the absorbance at 330 nm of the dansyl group and an extinction coefficient of 3.98×10^3 M^{-1} cm^{-1} (Cheung et al., 1971). In the other method, apostellacyanin was dansylated by using a 5-fold excess of *S*-mercuri-*N*-dansylcysteine (Leavis & Lehrer, 1974). After passage through Sephadex G-25 and dialysis to remove unbound probe, the modified protein was shown to contain 0.94 dansyl group per molecule.

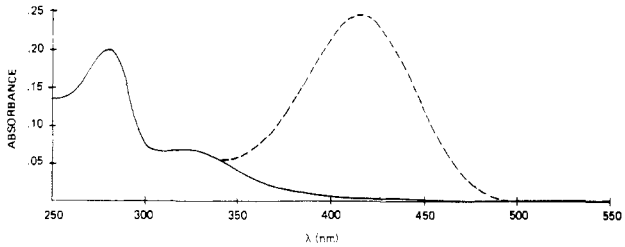

FIGURE 1: Absorbance spectra of MNP-apostellacyanin at acid and alkaline pH. The protein (8.6×10^{-6} M) was present in 0.1 M ionic strength potassium phosphate at pH 5.0 (—) or pH 10.0 (---). The spectrum of the protein at pH 10.0 was measured from 350 to 500 nm. The optical pathlength was 1 cm.

The p*K* values of MNP-stellacyanin and free CMNP were determined by dissolving the samples (chromophore concentration = 10 μM) in 0.1 M ionic strength potassium phosphate buffers ranging in pH from 4 to 10.5. Spectra of all samples were measured from 350 to 500 nm, and A_{405} was plotted as a function of pH. A nonlinear least-squares analysis of the curves was performed by using a modified Marquardt algorithm (Marquardt, 1963), with the extinction coefficients of the protonated and unprotonated nitrophenol and the acid dissociation constant as the parameters to be fitted.

Absorbance spectra were measured by using an Aminco DW-2a spectrophotometer. Corrected fluorescence emission and excitation spectra were obtained on a Perkin-Elmer Model MPF-44B spectrofluorometer equipped with a DCSU-2 corrected spectra accessory.

Results

Figure 1 shows the spectra of MNP-stellacyanin at pH 5.0 (where the MNP group is fully protonated) and at pH 10 (where the MNP group is fully ionized). The spectrum at pH 5.0 is that expected for the sum of the spectra of apostellacyanin (Morpurgo et al., 1972) and that of the protonated MNP group. There is little absorbance in the 400–500-nm region where native stellacyanin shows an absorbance band (λ_{max} = 453 nm), indicating that at least 94% of the copper had been removed during this apoprotein preparation. Figure 1 shows that at alkaline pH a prominent peak at 405 nm characteristic of the MNP anion appears. The peak at 323 nm characteristic of the protonated MNP disappears at alkaline pH, as would be expected (data not shown). The MNP group appeared to be covalently attached to apostellacyanin as it remained associated with the protein after passage through Sephadex G-25 and after prolonged dialysis. It was anticipated that the mercurial functional group would result in attachment of the MNP at the single cysteine residue of the copper binding site. Evidence to support this assignment comes from three observations: (1) MNP-stellacyanin was no longer reconstitutable with $Cu(ClO_4)_2$, (2) MNP-stellacyanin contained less than 0.05 detectable sulfhydryl group per molecule compared to the 0.98 sulfhydryl group measured with unlabeled apostellacyanin, and (3) native, copper-containing stellacyanin was not labeled with CMNP.

Figure 2 shows the results of pH titrations of the phenol group of MNP-stellacyanin and of free CMNP. p*K* values of 6.72 ± 0.23 and 6.49 ± 0.09, respectively, were obtained

[1] Abbreviations used: CMNP, 2-(chloromercuri)-4-nitrophenol; MNP, 2-mercuri-4-nitrophenol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

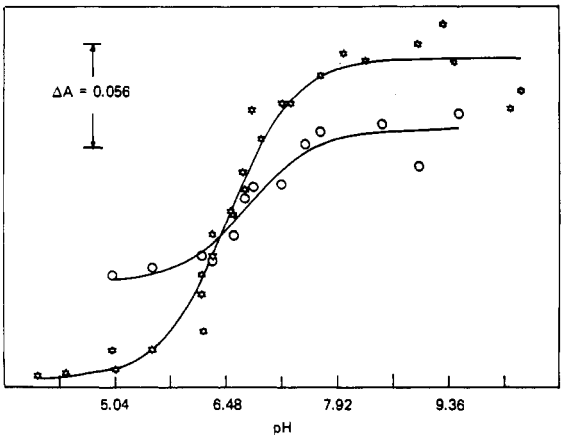

FIGURE 2: Effect of covalent linkage to apostellacyanin on the pK of MNP. pH titrations were performed by using solutions that were 1.0×10^{-5} M in MNP as described under Materials and Methods. Free CMNP (*); MNP-stellacyanin (O).

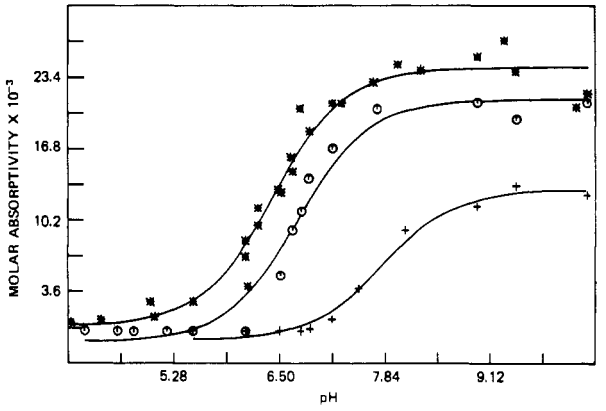

FIGURE 3: Effect of solvent composition on the pK of CMNP. pH titrations were performed as described under Materials and Methods. 0% (v/v) dioxane in water (*); 25% dioxane (O); 50% dioxane (+).

from the two titrations. Thus, the pK of an MNP group at the copper binding site of apostellacyanin is quite similar to that of free CMNP in aqueous solution. Figure 3 shows that the pK of CMNP varies with the polarity of the solvent, changing from 6.49 ± 0.09 in water to 6.79 ± 0.10 in a 3:1 (v/v) water–dioxane mixture and to 7.81 ± 0.10 in a 1:1 water–dioxane mixture. The results of all these titrations suggest that the microenvironment at the copper binding site is similar to that of the bulk aqueous phase. However, the pK of MNP is not a sufficiently sensitive function of solvent composition to allow an unambiguous characterization of the microenvironment at the copper binding site. (The pK of MNP-stellacyanin is within the experimental uncertainties of both MNP in pure water and 3:1 water–dioxane.) Thus, we turned to another environment-sensitive probe and took advantage of the known effect of solvent polarity on the fluorescence yield and emission maximum of the dansyl group (Chen, 1967).

As described under Materials and Methods, apostellacyanin could be covalently dansylated by either of two methods. The fluorescence emission and excitation spectra of apostellacyanin dansylated by didansylcystine and by *S*-mercuri-*N*-dansylcysteine were identical. As was the case described above for MNP-stellacyanin, the dansylated apostellacyanin preparations retained the dansyl group after prolonged dialysis, showed no detectable free sulfhydryl groups, and could not be reconstituted with $Cu(ClO_4)_2$. Furthermore, native, copper-containing stellacyanin could not be dansylated by either of the dansylating reagents. These results strongly suggest that the dansyl

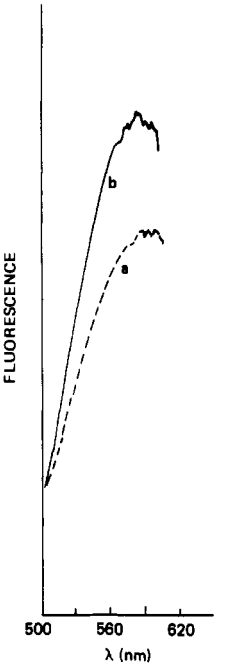

FIGURE 4: Emission spectra for dansyl derivatives. (a) Didansylcystine; (b) dansylapostellacyanin dansylated by using didansylcystine as described under Materials and Methods. The samples were dissolved in 0.1 M potassium phosphate buffer (pH 7.0) to give $A_{330} = 0.3$. The monochromater bandwidths at half-maximum were 2 nm for emission and 5 nm for excitation. Excitation wavelength = 330 nm. The instrumental gain was 3.3 times more sensitive for the dansylapostellacyanin sample.

group has been covalently attached to the single cysteine residue at the copper binding site. Figure 4 shows the fluorescence emission spectra for didansylcystine itself and dansylapostellacyanin. The fluorescence emission maxima for both samples are essentially identical. Furthermore, the emission maximum of the dansyl group in dansylapostellacyanin, 580 nm, is similar to that reported for a variety of dansyl derivatives in aqueous solution (Chen, 1967). Environments less polar than water shift the fluorescence emission maximum to markedly shorter wavelengths (Chen, 1967). The intensity of dansyl group fluorescence in dansylapostellacyanin, estimated by the peak height of the emission spectrum, appears to be somewhat lower than that for didansylcysteine itself. As decreases in solvent polarity produce substantial increases in dansyl fluorescence quantum yield (Chen, 1967), these results also support the conclusion that the environment at the copper binding site is very similar to that of bulk water.

Discussion

The studies of covalent modification of apostellacyanin described above support the earlier conclusions that the single cysteine residue in the protein contributes to the inner coordination sphere of the copper in stellacyanin. The pK measurements of MNP-stellacyanin are consistent with a microenvironment around this cysteine that does not differ appreciably from that of bulk water. There does appear to be a small difference in the pK of MNP-stellacyanin compared to that of free CMNP. However, this difference of 0.23 pH unit is less than the combined experimental uncertainties of the two pK determinations. If a difference exists, it may be due to factors other than microenvironment polarity, such as the presence of nearby hydrogen-bonding groups.

The fluorescence properties of the dansyl group in dansylapostellacyanin also indicate that the microenvironment at the copper binding site is indistinguishable from that of bulk water. Dansyl environments less polar than water would have pro-

duced significant blue shifts in emission maximum. For example, dansyl groups in glycerol emit at 553 nm, in ethanol at 529 nm, in dimethylformamide at 517 nm, and in dioxane at 500 nm (Chen, 1967). Caution must always be exercised in interpreting data obtained with modified proteins, as the possibility exists for conformational differences between the native and modified proteins. Nevertheless, the observations obtained in this study with three different stellacyanin derivatives all support the conclusion that the microenvironment at the copper binding site of stellacyanin is essentially identical with that of bulk water. Such a conclusion is in agreement with that reached previously from kinetic accessibility studies with transition metal ion oxidants and reductants (Cummins & Gray, 1977; Holwerda et al., 1980).

Acknowledgments

We thank Rebecca Moore and Dr. Roy Auerbach for assistance with the fluorescence measurements, Gary Gray for help in characterizing the dansylapostellacyanin, Dr. Roy Auerbach for the computer fitting of the pH titrations, and Professors Pill-Soon Song and G. Wilse Robinson for the generous use of their fluorescence facilities. Finally, we thank Dr. P. C. Leavis for his advice on the preparation of *S*-mercuri-*N*-dansylcysteine.

References

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35.
Bergman, C., Gandvik, E.-K., Nyman, P. O., & Strid, L. (1977) *Biochem. Biophys. Res. Commun. 77*, 1052.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Chen, R. F. (1967) *Arch. Biochem. Biophys. 120*, 609.
Cheung, H. C., Cooke, R., & Smith, L. (1971) *Arch. Biochem. Biophys. 142*, 333.
Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319.
Cummins, D., & Gray, H. B. (1977) *J. Am. Chem. Soc. 99*, 5158.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Ferris, N. S., Woodruff, W. H., Rorabacher, D. B., Jones, T. E., & Ochrymowyxz, L. A. (1978) *J. Am. Chem. Soc. 100*, 5932.
Hill, H. A. O., & Lee, W. K. (1979) *J. Inorg. Biochem. 11*, 101.
Holwerda, R. A., Knaff, D. B., Gray, H. B., Clemmer, J. D., Crowley, R., Smith, J. M., & Mauk, A. G. (1980) *J. Am. Chem. Soc. 102*, 1142.
Katoh, S. (1960) *Nature (London) 186*, 533.
Leavis, P. C., & Lehrer, S. S. (1974) *Biochemistry 13*, 3042.
Malmstrom, B. G., Reinhammar, B., & Vanngard, T. (1970) *Biochim. Biophys. Acta 205*, 48.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.
McMillin, D. R., Holwerda, R. A., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1339.
McMurray, C. H., & Trentham, D. R. (1969) *Biochem. J. 115*, 913.
Morpurgo, L., Finazzi-Argo, A., Rotilio, G., & Mondovi, B. (1972) *Biochim. Biophys. Acta 271*, 292.
Omura, T. (1961) *J. Biochem. (Tokyo) 50*, 394.
Reinhammar, B. (1970) *Biochim. Biophys. Acta 205*, 35.
Sailasuta, N., Anson, F. C., & Gray, H. B. (1979) *J. Am. Chem. Soc. 101*, 455.
Solomon, E. I., Hare, J. W., & Gray, H. B. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1389.
Solomon, E. I., Rawlings, J., McMillin, D. R., Stephens, P. J., & Gray, H. B. (1976b) *J. Am. Chem. Soc. 98*, 8046.
Wu, C.-W., & Stryer, L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1104.
Wurzbach, J., Grunthaner, P. J., Dooley, D. M., Gray, H. B., Grunthaner, F. J. Gay, R. R., & Solomon, E. I. (1977) *J. Am. Chem. Soc. 99*, 1257.

Reactivity of Cuprous Stellacyanin as a Quinone and Semiquinone Reductase†

Robert A. Holwerda,* David B. Knaff,* Gary O. Gray, and Claudia E. Harsh

ABSTRACT: The reactivity of cuprous stellacyanin as a quinone and semiquinone reductase has been examined. Rate constants (25.0 °C) measured for the oxidation of stellacyanin by 1,4-benzoquinone and benzosemiquinone are 2.3×10^4 M^{-1} s^{-1} ($\Delta H^* = 4.4$ kcal/mol, $\Delta S^* = -24$ eu) and 5.1×10^6 M^{-1} s^{-1}, respectively [pH 7.0, $I = 0.1$ M (phosphate)]. The agreement of these rate constants with those calculated on the basis of relative Marcus theory is discussed. Stellacyanin is more effective than laccase in quenching benzosemiquinone, suggesting that the physiological role of this metalloprotein is to regulate the concentration of free radicals generated through the laccase-catalyzed oxidation of phenols.

Stellacyanin, a blue copper protein isolated from the latex of the lacquer tree *Rhus vernicifera*, exhibits an electron-transfer reactivity distinctly different from those of other blue copper proteins, i.e., plastocyanin and azurin (Wherland & Gray, 1977). The understanding of rate data for blue copper protein redox reactions has been greatly facilitated by the use of relative Marcus theory for outer-sphere electron transfer (Holwerda et al., 1980). Apparent stellacyanin self-exchange (cuprous/cupric) electron-transfer rate constants derived from rate data for reactions with transition metal ion reductants and oxidants generally are quite similar, suggesting that the copper site is readily accessible to redox agents (Cummins & Gray, 1977). Spectroscopic studies of stellacyanin derivatives containing polarity probes at the active site (Knaff et al., 1981), and comparisons of blue copper protein reduction potentials

† From the Department of Chemistry, Texas Tech University, Lubbock, Texas 79409. *Received December 24, 1980.* R.A.H. and D.B.K. thank the Robert A. Welch Foundation (Grants D-735 and D-710) for their support of this research.

0006-2960/81/0420-4336$01.25/0