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.

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Reactivity of Cuprous Stellacyanin as a Quinone and Semiquinone Reductase†

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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

(Sailasuta et al., 1979) support this conclusion. An isokinetic relationship between $\Delta H^{\ddagger}$ (10.7 kcal/mol range) and $\Delta S^{\ddagger}$ (34-eu range) pertains in the oxidation of cuprous stellacyanin by cobalt(III) complexes of the form *cis*(*N*)-$[CoN_2O_4]^-$ (isokinetic temperature = 300 K), demonstrating that the activation requirements are highly flexible (Holwerda & Clemmer, 1979).

Unlike plastocyanin and azurin, whose respective roles in photosynthetic and bacterial electron transport are well understood, stellacyanin has no known physiological function (Holwerda et al., 1976). We present in this paper kinetic studies aimed at determining the reactivity of cuprous stellacyanin [St(I)] as a quinone and semiquinone reductase. Such a physiological role seems reasonable considering that St(I) is a much stronger reducing agent than other cuprous blue proteins [$E^{0\prime}$ = 0.184 V, compared with 0.347–0.390 V (*P. vulgaris* and *chlorella* plastocyanin) and 0.330 V (*P. aeruginosa* azurin)] (Sailasuta et al., 1979; Katoh, 1960). Ample thermodynamic driving force thus is available for the reduction of many quinones to the corresponding hydroquinones under physiological conditions; for the reduction of 1,4-benzoquinone to hydroquinone, $E^{0\prime}$ = 0.285 V, calculated from E^0 = 0.699 V (Latimer, 1952).

Laccase functions as a nonspecific one-electron phenol oxidase, generating aryloxy free radicals from phenols and polyphenols (Brown, 1967). These free-radical intermediates then react further to produce coupling products, quinones, or polymers (Brown, 1967). Our hypothesis, then, is that stellacyanin acts in concert with laccase, regulating the concentration of aryloxy free radicals and quinones produced from the oxidation of phenols by O_2.

Experimental Procedures

Reagents and Solution Preparation. Materials and methods used in preparing anaerobic solutions of cuprous stellacyanin for use in kinetics studies have been described previously (Yoneda & Holwerda, 1978). Practical grade 1,4-benzoquinone (BQ) was purified through vacuum sublimation, and reagent grade hydroquinone (H_2Q) (Eastman) was used as supplied. All solutions were prepared with triply distilled water and were made anaerobic by purging with chromous-scrubbed nitrogen for at least 30 min.

As 1,4-benzoquinone is light sensitive and unstable with respect to polymerization of pH 7, solutions of this oxidant were prepared in 1 mM HCl and stored in serum bottles wrapped with aluminum foil. These solutions were used within 8 h after their preparation; no change in the ultraviolet spectrum of benzoquinone (ϵ_{246} (max) = 2.18×10^4 M^{-1} cm^{-1}) (Baxendale et al., 1951) was observed over this interval. In most kinetic determinations, benzoquinone in 1 mM HCl was mixed (1:1) with stellacyanin buffered with sufficient NaH_2PO_4/Na_2HPO_4 to give a final pH and ionic strength of 7.0 and 0.1 M, respectively. A different method was used in studies of the oxidation of stellacyanin in solutions containing large initial concentrations of both benzoquinone and hydroquinone. Deoxygenated solutions of BQ (in 1 mM HCl) and H_2Q (buffered with sodium phosphate) were mixed 30 min before each kinetic determination, allowing sufficient time for the semiquinone (SQ) formation equilibrium (see below) to be established at pH 7.0, I = 0.1 M. Kinetic determinations then were made by mixing equilibrated BQ/H_2Q solutions with St(I) prepared in the same buffer.

Kinetic Measurements. Formation of cupric stellacyanin was followed at 604 nm on a Durrum D-110 stopped-flow spectrophotometer. Pseudo-first-order conditions for St(I) were employed, holding the metalloprotein concentration constant at ~10 μM and maintaining at least a 10-fold excess of benzoquinone. Anaerobic solutions were transferred to the stopped-flow apparatus through Teflon needles, and a minimum of 30 min was allowed for temperature equilibration to occur before kinetic runs were initiated. Observed first-order rate constants (k_{obsd}) were derived from the least-squares slopes of linear ln ($A_\infty - A_t$) vs. time plots. Reported values generally are the average of three determinations.

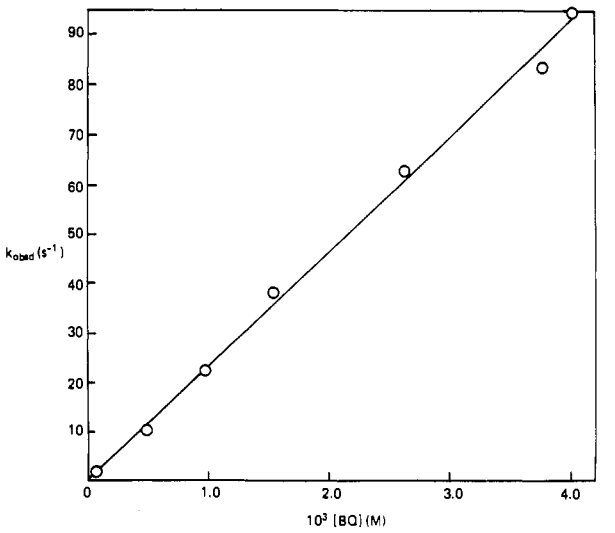

FIGURE 1: Dependence of k_{obsd} on the concentration of 1,4-benzoquinone; 25.0 °C, pH 7.0, I = 0.1 M.

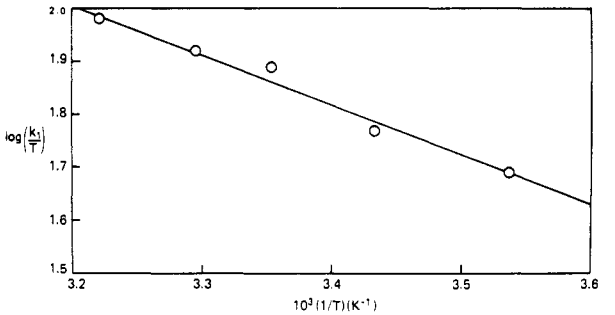

FIGURE 2: Eyring plot for the oxidation of stellacyanin by 1,4-benzoquinone; [BQ] = 9.73×10^{-4} M, pH 7.0, I = 0.1 M.

Instrumentation. UV–visible absorption spectra were acquired with an Aminco DW-2a spectrophotometer, and pH readings were obtained with a Brinkmann pH-103 meter.

Results

Plots of ln ($A_\infty - A_t$) vs. time were found to be linear throughout our kinetic studies of the oxidation of stellacyanin by benzoquinone (>90% of ΔA_{604}), indicating a first-order St(I) dependence. In the absence of added hydroquinone, the reaction is also first order in benzoquinone, as shown in Figure 1.

Thus, a plot of k_{obsd} vs. [BQ] is linear over a 58-fold concentration range [$(6.88 \times 10^{-5}) - (4.00 \times 10^{-3})$ M], yielding a second-order rate constant (k_1) of $(2.3 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (25.0 °C, pH 7.0, I = 0.1 M) and an intercept within experimental error of 0 (0.6 ± 1.6 s^{-1}). A study of the temperature dependence of k_1 was carried out by measuring k_{obsd} at five temperatures between 9.6 and 37.4 °C while holding [BQ] constant at 9.73×10^{-4} M. The results of this study, shown as an Eyring plot of log k_1/T vs. $1/T$ (Figure 2), provide a basis (Frost & Pearson, 1961) for calculating the activation enthalpy ($\Delta H^{\ddagger}$ = 4.4 ± 0.4 kcal/mol) and entropy ($\Delta S^{\ddagger}$ = −24 ± 2 eu). The effect of hydroquinone on k_{obsd} was determined by holding [BQ] constant (1.0×10^{-4} M) while varying [H_2Q]

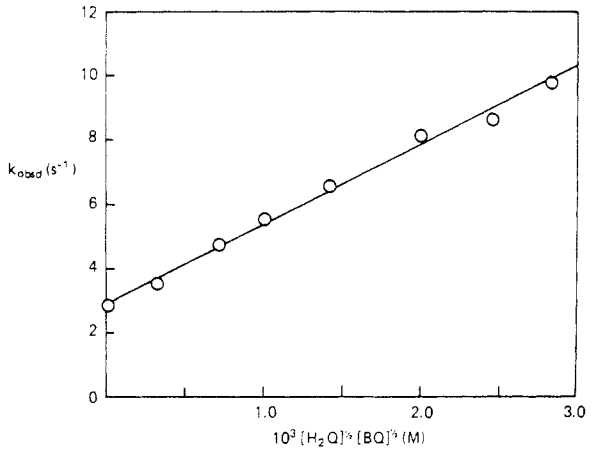

FIGURE 3: Plot illustrating the dependence of k_{obsd} on the concentration of 1,4-benzosemiquinone (proportional to $[H_2Q]^{1/2}[BQ]^{1/2}$); 25.0 °C, pH 7.0, I = 0.1 M.

[(0–8.0) × 10^{-2} M]. A modest 4-fold increase in k_{obsd} was observed over this interval at 25.0 °C. Figure 3 shows that a plot of k_{obsd} vs. $[H_2Q]^{1/2}[BQ]^{1/2}$ is linear with a positive intercept, demonstrating that the complete rate law (at constant pH = 7.0) for the oxidation of stellacyanin is

$$d[St(II)]/dt = k_{obsd}[St(I)] = (k_1[BQ] + k_2[BQ]^{1/2}[H_2Q]^{1/2})[St(I)] \quad (1)$$

The rate constant k_2, derived from the least-squares analysis of the data in Figure 3, is (2.5 ± 0.1) × 10^3 M^{-1} s^{-1}. The least-squares intercept of the k_{obsd} vs. $[BQ]^{1/2}[HQ]^{1/2}$ plot (2.9 ± 0.2 s^{-1}) agrees reasonably well with the value (2.3 s^{-1}) expected from eq 1, using the k_1 value derived from Figure 1.

Discussion

The rate law described in eq 1 is consistent with a mechanism involving the oxidation of cuprous stellacyanin by both benzoquinone and benzosemiquinone:

$$BQ + H_2Q \overset{K_{sq}}{\rightleftharpoons} 2SQ \quad \text{(fast)}$$

$$BQ + St(I) \xrightarrow{k} SQ + St(II)$$

$$SQ + St(I) \xrightarrow{k'} H_2Q + St(II) \quad (2)$$

When the first step is treated as a rapid preequilibrium (Bishop & Tong, 1965) and the relationship $[SQ] \ll [BQ]_0$ is used, the rate law implied by this mechanism is

$$d[St(II)]/dt = (k[BQ] + k'[SQ])[St(I)] = (k[BQ]_0 + k'K_{sq}^{1/2}[BQ]_0^{1/2}[H_2Q]_0^{1/2})[St(I)] \quad (3)$$

where $[BQ]_0$ and $[H_2Q]_0$ are the initial concentrations of benzoquinone and hydroquinone, respectively. On this basis, the relationships between experimental and theoretical rate constants are $k_1 = k$ and $k_2 = k'K_{sq}^{1/2}$. By estimations of K_{sq} at 2.35 × 10^{-7} (25 °C, pH 7) from data given by Bishop & Tong (1965), k' is calculated to be 5.1 × 10^6 M^{-1} s^{-1}. Comparing k' with k, it is seen that benzosemiquinone oxidizes cuprous stellacyanin 220 times faster than does benzoquinone.

A rate law quite similar to that reported here has been observed in the anaerobic reduction of the type-1 cupric site in *Rhus vernicifera* laccase (L(II)) by hydroquinone in the presence of large initial concentrations of benzoquinone (Holwerda & Gray, 1974):

$$-d[L(II)]/dt = (k_1[H_2Q] + k_2[BQ]^{1/2}[H_2Q]^{1/2})[L(II)] \quad (4)$$

Table 1: Marcus Theory Calculations for the Oxidation of Stellacyanin by Benzoquinone and Benzosemiquinone[a]

oxidant	k_{12} (M^{-1} s^{-1})	product	$E^{0\prime}$ (V)[b]	k_{12}^{calcd} (M^{-1} s^{-1})[h]
BQ	2.3 × 10^4	SQ^-	+0.089[c]	5 × 10^5[i]
		HSQ	+0.326[d]	5 × 10^7[i]
		H_2Q	+0.481[e]	5 × 10^8[j]
SQ^-	5.1 × 10^6	HQ^-	−0.102[f]	6 × 10^3[j]
		Q^{2-}	−0.776[g]	5 × 10^{-6}[j]

[a] 25.0 °C, pH 7.0, I = 0.1 M (sodium phosphate). [b] pH 7.0 reduction potential of the oxidant. [c] $E^{0\prime}(BQ + e^- \rightarrow SQ^-) = E^{0\prime}(BQ + 2H^+ + 2e^- \rightarrow H_2Q) + 0.0296 \log K_{sq}$ (pH 7); $E^{0\prime}(BQ + 2H^+ + 2e^- \rightarrow H_2Q) = 0.285$ V (Latimer, 1952); K_{sq} (pH 7) = 2.35 × 10^{-7} [estimated from data and equations given by Bishop & Tong (1965)]. [d] $E^{0\prime}(BQ + H^+ + e^- \rightarrow HSQ) = E^{0\prime}(BQ + e^- \rightarrow SQ^-) + 0.05916\ pK_a(HSQ)$; $K_a(HSQ)$, the acid ionization constant of protonated benzosemiquinone, is 1.0 × 10^{-4} (Willson, 1971). [e] $E^{0\prime}(SQ^- + 2H^+ + e^- \rightarrow H_2Q) = E^{0\prime}(BQ + 2H^+ + 2e^- \rightarrow H_2Q) - 0.0296 \log K_{sq}$ (pH 7). [f] $E^{0\prime}(SQ^- + H^+ + e^- \rightarrow HQ^-) = E^{0\prime}(SQ + 2H^+ + e^- \rightarrow H_2Q) - 0.05916\ pK_{a_1}(H_2Q)$; $pK_{a_1}(H_2Q)$, derived from the first ionization constant of hydroquinone, is 9.85 (Bishop & Tong, 1965). [g] $E^{0\prime}(SQ^- + e^- \rightarrow Q^{2-}) = E^{0\prime}(SQ^- + 2H^+ + e^- \rightarrow H_2Q) - 0.05916\ (pK_{a_1}(H_2Q) + pK_{a_2}(H_2Q))$; $pK_{a_2}(H_2Q)$ = 11.4 (Bishop & Tong, 1965). [h] Calculated from eq 5, as described in the text. $Z = 1 \times 10^{11}$ M^{-1} s^{-1}; $\Delta E^0 = E^{0\prime}$(oxidant) − $E^{0\prime}$[St(II)]. $E^{0\prime}$[St(II)] = 0.184 V (Sailasuta et al., 1979). k_{11} is taken to be 2 × 10^5 M^{-1} s^{-1} (see text). [i] k_{22} (benzoquinone/semiquinone) is estimated at 6.2 × 10^7 M^{-1} s^{-1} (Meisel & Fessenden, 1976). [j] k_{22} (semiquinone/hydroquinone) is estimated at 5 × 10^7 M^{-1} s^{-1} (Pelizzetti et al., 1976).

The rate constants k_1 and k_2 are 3.5 × 10^2 M^{-1} s^{-1} and 1.52 × 10^2 M^{-1} s^{-1}, respectively (26.1 °C, pH 7.0, I = 0.1 M) (Holwerda & Gray, 1974). When the k_2 pathway is assigned to the reduction of L(II) by benzosemiquinone, the specific second-order rate constant for this process may be estimated at 3.1 × 10^5 M^{-1} s^{-1} [$=k_2/K_{sq}^{1/2}$; revised from earlier value of 1.5 × 10^5 M^{-1} s^{-1} given by Holwerda & Gray (1974)]. While the reactions of benzosemiquinone with cuprous stellacyanin and laccase type-1 copper are both very fast, the former metalloprotein is substantially more reactive (by a factor of 17) at room temperature, pH 7.0. The rate constant of the St(I)–SQ^- reaction is, in fact, only 1 order of magnitude smaller than those for semiquinone disproportionation ($2k$ = 5.5 × 10^7 M^{-1} s^{-1}; pH 9.2, 1–3 M 2-propanol) (Rao & Hayon, 1973) and the SQ^-–BQ electron exchange reaction [k = 6.2 × 10^7 M^{-1} s^{-1}; pH 7 (5 mM phosphate), 5 M 2-propanol and 1 M acetone] (Meisel & Fessenden, 1976).

A comparison of experimental rate constants with those calculated on the basis of relative Marcus theory is presented in Table I. The Marcus relationship is

$$k_{12} = (k_{11}k_{22}K_{12}f)^{1/2}$$

$$\ln f = (\ln K_{12})^2/[4 \ln (k_{11}k_{22}/Z^2)] \quad (5)$$

in the case of weak-overlap outer-sphere reactions for which the influence of electrostatic work terms on cross-reaction (k_{12}) and self-exchange (k_{11} and k_{22}) rate constants may be neglected (Marcus, 1963). The bimolecular collision frequency of uncharged species in aqueous solution, Z, generally is taken to be 1 × 10^{11} M^{-1} s^{-1}, and K_{12} is the equilibrium constant of the electron-transfer process. The neglect of electrostatic work terms in the reactions of St(I) with BQ and SQ^- is reasonable, considering the insensitivity of stellacyanin redox rate constants to variations in ionic strength (Holwerda et al., 1980) and the fact that an uncharged species is present in both of the predominant oxidation half-reactions (BQ/SQ^- and SQ^-/H_2Q). Expressed in terms of ΔE^0, the standard cell potential of the

electron-transfer step (in volts), the Marcus relationship becomes

$$\log k_{12} = \tfrac{1}{2}[\log k_{11} + \log k_{22} + 16.9\Delta E^0 + (16.9\Delta E^0)^2/[4 \log (k_{11}k_{22}/Z^2)]] \quad (6)$$

While self-exchange rate constants (k_{22}) for benzoquinone/semiquinone and semiquinone/hydroquinone couples may be found in the literature (Table I), k_{11} for the St(II)/St(I) couple has not been measured directly. Since k_{11} values estimated from the Marcus relationship are relatively invariant for this metalloprotein [$(2 \pm 1) \times 10^5$ M^{-1} s^{-1}, 25.0 °C, derived from cross-reactions with $[Ru(NH_3)_5py]^{3+}$, $Fe(EDTA)^{2-}$, and $Co(phen)_3^{3+}$] (Cummins & Gray, 1977), it seems likely that this figure closely approximates the true self-exchange rate constant.

The predominant form of semiquinone at pH 7.0 is the radical anion SQ^- (pK_a(HSQ) = 4.0) (Willson, 1971). While the thermodynamic products of the one-electron reduction of BQ and SQ^- at pH 7.0 are SQ^- and H_2Q, respectively, these are not necessarily the immediate products of the rate-limiting electron-transfer step which determines the value of ΔE^0 (eq 6). We have, therefore, calculated cross-reaction rate constants (k_{12}^{calcd}) corresponding to the reduction of SQ^- to H_2Q, HQ^-, and Q^{2-} and for the reduction of BQ to both HSQ and SQ^-. The calculations set out in Table I make it clear that k_{12}^{calcd} is highly sensitive to the ionization state of the organic product. Indeed, k_{12}^{calcd} values for the reduction of SQ^- to Q^{2-}, HQ^-, and H_2Q span 14 orders of magnitude.

The rate constant calculated for the reduction of BQ to its most probable immediate product, SQ^-, is only in fair agreement with the experimental value, the latter being smaller by a factor of 20. Numerous uncertainties are propagated in obtaining k_{12}^{calcd}, but these alone cannot account for the substantial discrepancy between experimental and calculated rate constants. Nonadiabaticity resulting from extremely weak orbital overlap between the oxidant and reductant is thought to be responsible in the case of some metalloprotein redox reactions whose rates are smaller than predicted from the Marcus relationship (Wherland & Gray, 1977). Electron tunneling typically is associated with unusual activation parameters (very small $\Delta H^{\ddagger}$, large negative $\Delta S^{\ddagger}$), while those reported here are entirely consistent with expectations for a bimolecular, adiabatic process. A more likely explanation of the discrepancy between k_{12} and k_{12}^{calcd} is that the half-cell potential of the BQ/SQ^- couple (+0.089 V) used in calculating ΔE^0 is more positive than the value which actually pertains in the St(I)–BQ reaction. Benzoquinone is susceptible to attack by nucleophiles, including the side chains of lysine, cysteine, and tryosine residues (Brandt et al., 1975), forming addition products whose oxidizing strengths may be substantially smaller than that of free benzoquinone [i.e., for the 1:1 hydroxide–benzoquinone carbonyl addition product ($BQ{\cdot}OH^-$), $E^{0\prime}$ ($BQ{\cdot}OH^- + e^- \rightarrow SQ^- + OH^-$) = +0.034 V] [calculated from data reported by Bishop & Tong (1965)]. It should be noted, however, that no direct evidence has been obtained for such an interaction between BQ and St(I) prior to the electron-transfer step.

The rate constant of the St(I)–SQ^- reaction falls between those estimated from eq 6 on the basis of HQ^- and H_2Q as the immediate organic products. Examination of k_{12}^{calcd} values for the reduction of SQ^- to Q^{2-}, HQ^-, and H_2Q shows that the relationship

$$\log k_{12}^{calcd} = a + b(NP)^{1/2} \quad (7)$$

successfully correlates calculated rate constants with the number of protons accepted by BQ (NP) in the electron-transfer step. A linear least-squares fit of k_{12}^{calcd}, NP pairs to this empirical equation yields a correlation coefficient of 0.996 and values of −5.42 and 9.70 for the constants a and b, respectively. When NP is treated as an adjustable parameter, agreement between k_{12} and k_{12}^{calcd} is found when NP = 1.6. While the quantitative significance of this calculation is questionable, a nonintegral NP value may in fact be physically meaningful in the present context. Thus, NP reasonably could fall between 1 and 2 if SQ^- was reduced while one of its oxygen atoms was hydrogen bonded to a carbohydrate or amino acid proton near the copper site and the other oxygen atom was freely interacting with water molecules in the medium. In any case, the Marcus theory calculations on the St(I)–SQ^- reaction certainly are consistent with the observed rate constant.

While our kinetic results do not prove that cuprous stellacyanin functions as a quinone/semiquinone reductase, the high reactivity of the metalloprotein with these substrates provides some support for our hypothesis. The rates of these reactions are comparable to, but certainly do not exceed, those expected from relative Marcus theory. There is, therefore, no special stabilization of the precursor complexes formed between St(I) and BQ or SQ^-. Stellacyanin's substantial reactivity advantage over laccase in quenching benzosemiquinone suggests an important role for the former metalloprotein in regulating the concentration of free radicals generated by the latter.

Acknowledgments

We thank Vanessa Lum and Professor Harry B. Gray of the California Institute of Technology for a generous gift of stellacyanin.

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Nuclear Magnetic Resonance and Chemical Modification Studies of the Role of the Metal in Yeast Aldolase†

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ABSTRACT: The C-2 proton resonances of approximately six of the ten histidine residues per monomer of yeast aldolase are detected at 360 MHz. In the metal-free apoenzyme, all of these detected resonances shift upfield with increasing pH* (uncorrected pH meter reading in ^2H_2O solutions) between 6.5 and 8, in a manner typical for histidines. In the Zn^{2+}–metalloenzyme, 3 ± 1 of these proton signals remain downfield as a single resonance over a wide range of pH* values, indicating metal coordination of these imidazoles. This downfield signal is not detected in the paramagnetic Co^{2+}–aldolase complex. Chemical modification of the Zn^{2+}–enzyme by diethyl pyrocarbonate results in acylation of histidines as detected by an increase in absorbancy at 240 nm and loss of enzymatic activity, both occurring with the same second-order rate constant (70 M^{-1} min^{-1} at pH 6.0, 20 °C). Titrations with diethyl pyrocarbonate monitoring enzymatic activity, optical absorbancy at 240 nm, and 360-MHz NMR of the histidine C-2 protons indicate that all ten histidines react in parallel. Modification of Mn^{2+}-substituted aldolase with diethyl pyrocarbonate, monitoring the paramagnetic effect of the bound Mn^{2+} on $1/T_1$ of water protons, reveals a transient increase in $1/T_1$ which occurs during enzyme inactivation, presumably due to an increased accessibility of the bound Mn^{2+} to water. This is followed by a decrease in $1/T_1$ due to the dissociation of Mn^{2+} from the enzyme, as detected by EPR. These results provide independent evidence for binding of imidazole ligands to the metal. Thiomethylation with methyl methanethiosulfonate of approximately one of the five cysteine residues per monomer of Zn^{2+}–aldolase, Mn^{2+}–aldolase, or apoaldolase (followed by reconstitution with Zn^{2+}) results in loss of activity. Such modification of Mn^{2+}–aldolase does not alter the metal site as measured by $1/T_1$, nor does it cause the dissociation of Mn^{2+}, but it does prevent the binding of the substrate fructose diphosphate. These findings indicate that the essential thiol functions at the active site, not as a metal ligand but possibly as a general base. The present results are consistent with the coordination of the enzyme-bound metal by three imidazole ligands, and previous data indicate one rapidly exchanging water ligand [Smith, G. M., Mildvan, A. S., & Harper, E. T. (1980) *Biochemistry 19*, 1248]. If the coordination geometry is tetrahedral [Simpson, R. T., Kobes, R. D., Erbe, R. W., Rutter, W. J., & Vallee, B. L. (1971) *Biochemistry 10*, 2466], then all four of the metal ligands are identified. The results are also consistent with a metal-bound imidazole functioning to polarize the carbonyl group of dihydroxyacetone phosphate by hydrogen bonding.

Fructose-1,6-diphosphate aldolases catalyze the reversible condensation of dihydroxyacetone phosphate (DHAP)[1] and glyceraldehyde 3-phosphate to form fructose 1,6-diphosphate, an essential reaction in the pathways for fermentation and synthesis of hexoses. The enzyme is therefore widely distributed in nature. The fructose-1,6-diphosphate aldolases from lower organisms (class II aldolases) differ from those of higher plants and animals (class I aldolases) in that they do not employ a Schiff base intermediate (Rutter, 1964). The class II aldolases contain a metal ion, usually Zn^{2+}, at the active site (Warburg & Christian, 1943; Rutter, 1964). Yeast aldolase is a dimer of molecular weight 75 000–80 000 (Harris et al., 1969) and contains one essential metal site per subunit (Harris et al., 1969; Richards & Rutter, 1961; Mildvan et al., 1971). The zinc ion of yeast aldolase can be removed by high concentrations of EDTA to yield an inactive apoenzyme (Kobes et al., 1969). Activity can be recovered by the addition to the apoenzyme of stoichiometric amounts of Zn^{2+}, Co^{2+}, Mn^{2+}, or Fe^{2+}, though all ions other than Zn^{2+} yield a holoenzyme of lower activity (Kobes et al., 1969; Rutter, 1964).

Inhibition by low concentrations of chelating agents (Warburg & Christian, 1943; Rutter, 1964), optical spectroscopy with the Co^{2+}–enzyme (Simpson et al., 1971), and early distance measurements with the Mn^{2+}–enzyme (Mildvan et al., 1971) implicated the metal ion as a direct participant in catalysis although there was a 3-fold uncertainty in the metal–substrate distances (Mildvan et al., 1971). A mechanism based on this early evidence proposed that the metal ion catalyzed the condensation by polarizing the carbonyl group of DHAP via direct coordination to facilitate deprotonation

† From the Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received January 30, 1981.* Supported by National Institutes of Health Grants AM-13351, CA-06927, and RR-05539, National Science Foundation Grant PCM-79-23154, and an appropriation from the Commonwealth of Pennsylvania to The Institute for Cancer Research. Support for the computation was provided by National Institutes of Health Grant CA-22780. The 360-MHz studies were done at the Middle Atlantic Regional NMR Facility which is supported by National Institutes of Health Grant RR-542.

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[1] Abbreviations used: DEP, diethyl pyrocarbonate; MMTS, methyl methanethiosulfonate; FDP, fructose diphosphate; DHAP, dihydroxyacetone phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; Tris, tris(hydroxymethyl)aminomethane; pH*, uncorrected pH meter reading in ^2H_2O solutions; $NaDodSO_4$, sodium dodecyl sulfate.

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