Kinetic Studies of the Reduction of Blue Copper Proteins by $Fe(EDTA)^{2-}$

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Abstract: Kinetic studies of the anaerobic reduction of stellacyanin, plastocyanin, azurin, and laccase by $Fe(EDTA)^{2-}$ have been performed, giving second-order rate constants of 4.3×10^5 (25°, pH 7, $\mu = 0.5$ *M*), 8.2×10^4 (25°, pH 7, $\mu = 0.2$ *M*), 1.3×10^3 (25°, pH 7, $\mu = 0.2$ *M*), and 2.6×10^2 (25°, pH 7, $\mu = 0.5$ *M*) M^{-1} sec^{-1}; $\Delta H^{\ddagger}$ of 3, 2, 2, and 13 kcal/mol; and $\Delta S^{\ddagger}$ of −21, −29, −37, and −5 eu, respectively. The results are discussed in terms of two classes of mechanism. A normal outer sphere mechanism appears to operate in the reduction of azurin, plastocyanin, and stellacyanin, whereas laccase employs a pathway requiring specific protein activation (about 10 kcal/mol in $\Delta H^{\ddagger}$) to accept reductant. Relative protein self-exchange electron-transfer rates are calculated to decrease according to 2×10^{10} (stellacyanin) > 1×10^5 (plastocyanin) > 7×10^2 (azurin) > 1 (laccase).

Copper-containing proteins are found in a variety of biological electron-transfer systems. Of the different copper centers that have been classified, the most distinctive is the type 1, which gives proteins associated with it their characteristic blue color.[1] This work is concerned with the relative reactivity of the type 1 site in four proteins, stellacyanin, plastocyanin, azurin, and *Rhus* laccase. Plastocyanins are common components of chloroplasts, azurins are found in several bacteria, and stellacyanin and laccase may be isolated from the latex of the Japanese lacquer tree.[1] Plastocyanin, azurin, and stellacyanin are one-copper proteins and are thought to have simple electron-transfer functions. Laccase is a four-copper protein and is classed as a *p*-diphenol:O_2 oxidoreductase (E.C. 1.10.3.2).

Detailed kinetic comparisons of the four proteins have not been made previously. One problem that must be overcome is that reactions involving some of the most common reducing agents, including ferrocyanide, give complex kinetic behavior.[2] We have used $Fe(EDTA)^{2-}$ in the present investigation, because it has given simple pseudo-first-order kinetics under anaerobic conditions with horse heart ferricytochrome c.[3] Furthermore, this complex cleanly reduces all four blue proteins.

Experimental Section

Laccase and stellacyanin were prepared essentially by the method of Reinhammer.[4] The acetone powder from Japanese *Rhus vernicifera* lacquer was acquired from Saito and Co., Ltd., Tokyo. Laccase was purified to an A_{280}/A_{614} of 15.2–15.6. Stellacyanin was used with A_{280}/A_{604} of 5.60–5.75.

Plastocyanin from French bean (*Phaseolus vulgaris*) was purified by the method of Milne and Wells[5] to an A_{280}/A_{597} of 1.1–1.2.

Azurin from *Pseudomonas aeruginosa* was purified by the method of Ambler and Brown[6] as modified by Rosenberg.[7] The A_{625}/A_{280} ratio of the material used was 0.42–0.46.

Laccase, stellacyanin, and plastocyanin were shell frozen and stored under liquid nitrogen. Azurin was millipore-filtered and stored refrigerated in sterile vials in 0.05 *M*, pH 4.0 ammonium acetate buffer.

Reagent grade chemicals were used without further purification. Sephadex and Whatman ion exchangers and Sephadex gel filtration resins were used. Union Carbide dialysis membrane was boiled extensively to remove sulfur-containing impurities. Rubber serum caps were boiled in concentrated base before using.

The nitrogen gas used for deoxygenation of kinetic solutions was purified of oxidizing impurities by passage through two chromous scrubbing towers.

Kinetic Measurements and Data Analysis. All kinetic experiments were performed on a Durrum Model D-110 stopped-flow spectrometer. Solutions to be mixed were allowed at least 15 min at room temperature and 30 min at other temperatures to come to temperature equilibrium. Bath temperature was maintained ±0.2° with a Forma Scientific bath. Drive syringes with Viton O rings were used, and the valve block inlets were modified to accept gas-tight fittings for Teflon needles, through which deoxygenated solutions were drawn. Any solution which remained in the Teflon needle transfer lines for more than the time needed to transfer was not used, as these lines are oxygen permeable. Most data were collected on a 128 channel analog input buffer, collecting points at intervals of 1–9999 msec. Absorbance changes of 0.05 were routinely used. These data were then transferred to disk storage on the Caltech PDP-10 time-sharing system. Some data were taken as Polaroid pictures from a Tektronix 564B oscilloscope or, for slow runs, from Hewlett-Packard Model 7004B x-y recorder traces.

Laccase and stellacyanin solutions were prepared by first dialyzing against triply distilled water and then diluting to volume with precisely weighed amounts of phosphate buffer and ammonium sulfate to give the desired pH, ionic strength, and protein concentration. Plastocyanin and azurin were made up by dialyzing against the required buffer and then diluting with the same buffer to the desired protein concentration. Protein solutions were degassed by gentle bubbling with deoxygenated nitrogen, or by evacuation of the serum-capped bottles through a large needle, followed by storage under a stream of deoxygenated nitrogen. Laccase solutions were maintained cold, whereas the other proteins were left at room temperature for the duration of a day of kinetic experiments.

Ferrous EDTA solutions were made up by dissolving ferrous ammonium sulfate hexahydrate in deoxygenated water and then transferring an aliquot of this solution to a deoxygenated solution of disodium dihydrogen EDTA, phosphate buffer, and ammonium sulfate. The protons released from the EDTA were neutralized with sodium hydroxide and the pH of the resulting stock solution was determined with a Brinkmann pH meter using a combination electrode in the bubbler. This stock solution was then diluted into deoxygenated solutions of buffer and ammonium sulfate. All transfers were done with Hamilton gas-tight syringes. The concentration of EDTA was maintained in at least 20% excess over iron.

All data were analyzed as pseudo-first-order in protein with the reducing agent in 100–10,000-fold excess. Standard first-order plots were typically linear over at least 3 half-lives, and rate constants were taken from unweighted least-squares analysis. For the laccase data, an initial induction period had to be ignored. The origin of this induction period and a discussion of the rationale for ignoring it have been discussed at length elsewhere and will not be repeated here.[8] The wavelengths followed were 626, 614, 597, and 604 nm for azurin, laccase, plastocyanin, and stellacyanin, respectively.

For concentration and temperature dependence data, weighted least-squares analyses were done. Weighting factors for the concentration dependence fits were the square of the inverse of the standard deviation from the mean of the multiple determinations

Table I. Rate Constants

Protein	Dependence	$[Fe(EDTA)^{2-}]$, M	pH	μ, M	k
Stellacyanin	Reducing agent		6.9	0.5	$4.3 \times 10^5 M^{-1}$ sec^{-1}
	pH	5.0×10^{-4}	5.0	0.5	272 ± 10[a] sec^{-1}
		5.0×10^{-4}	7.8	0.5	209 ± 3 sec^{-1}
Plastocyanin	Reducing agent		6.9	0.2	8.2×10^4 (4)[b] M^{-1} sec^{-1}
	pH	2.0×10^{-4}	5.6	0.2	8.4 ± 0.2 sec^{-1}
		2.0×10^{-4}	7.4	0.2	12.2 ± 0.5 sec^{-1}
Azurin	Reducing agent		7.0	0.2	1.3×10^3 (0.6) M^{-1} sec^{-1}
Laccase	Reducing agent		6.9	0.5	2.6×10^2 (0.7) M^{-1} sec^{-1}
	pH	1.0×10^{-3}	4.9	0.5	0.295 ± 0.001 sec^{-1}
		1.0×10^{-3}	7.7	0.5	0.166 ± 0.001 sec^{-1}
	Ionic strength	1.0×10^{-3}	6.9	0.06	0.318 ± 0.007 sec^{-1}
			7.1	0.52	0.250 ± 0.012 sec^{-1}
	$[Fe(EDTA)^-]$[c]	1.0×10^{-3}	6.9	0.5	
	$4.96 \times 10^{-4} M$				0.315 ± 0.009 sec^{-1}
	$8.50 \times 10^{-3} M$				0.404 ± 0.036 sec^{-1}
	$[EDTA^{2-}]$	1.0×10^{-3}	6.9	0.5	
	$2.0 \times 10^{-4} M$				0.31 ± 0.02 sec^{-1}
	$5.0 \times 10^{-3} M$				0.37 ± 0.03 sec^{-1}

[a] The standard error of the mean of multiple determinations done on one filling of the drive syringes is designated as ±. [b] The numbers in parentheses quoted here are the standard deviations of the slope as given by the weighted least-squares analysis and are included as an indication of goodness of fit. [c] These values for free $Fe(EDTA)^-$ are corrected from the amounts actually added for the amount of oxo-bridged dimer at pH 7 [see H. J. Schugar, A. T. Hubbard, F. C. Anson, and H. B. Gray, *J. Am. Chem. Soc.*, 91, 71 (1969)].

Table II. Activation Parameters

Protein	$[Fe(EDTA)^{2-}]$, M	pH	μ, M	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu
Stellacyanin	5.0×10^{-4}	7.0	0.1	3.0 (0.003)[a]	−21 (0.1)
Plastocyanin	8.0×10^{-4}	7.0	0.2	1.9 (0.01)	−30 (0.02)
	4.0×10^{-4}	7.0	0.2	2.4 (0.005)	−28 (0.002)
Azurin	7.5×10^{-4}	7.0	0.2	2.4 (0.01)	−36 (0.04)
	2.0×10^{-3}	7.0	0.2	1.7 (0.01)	−38 (0.02)
Laccase	1.09×10^{-3}	7.0	0.1	13 (0.004)	−5.1 (0.001)

[a] The numbers in parentheses are the standard deviations of the slope or intercept (or quantities directly proportional to these standard deviations) as given by the weighted least-squares analysis and are included as a measure of goodness of fit.

done (usually three) from one filling of the drive syringes. For the temperature dependence data, the Eyring plots had the ln (k/T) values weighted as one over their standard deviations, with the standard deviations determined in the same way.[9]

Results and Discussion

Plots of k_{obsd} vs. $[Fe(EDTA)^{2-}]$ for all four blue proteins are shown in Figure 1. The second-order rate constants and certain other kinetic results are set out in Table I.[10] For *Rhus* laccase, rate variations upon changing pH, ionic strength, ferric EDTA, and excess EDTA are small, as are the pH dependences for stellacyanin, plastocyanin, and azurin. For example, the largest pH dependence is that for plastocyanin, where k_{obsd} varies by only a factor of 1.5 over the pH range 5.6–7.4. An interpretation of these small medium effects will be presented in a subsequent paper.[7]

It is apparent from the second-order rate constants that the four blue proteins span a large range of electron-transfer reactivity (laccase < azurin < plastocyanin < stellacyanin). Before any detailed analysis of this range can be made, however, the potentials of the various blue sites must be considered. Experimental values for the potentials are 328 mV for azurin (pH 6.4),[11] 415 mV for laccase type 1 copper (pH 7.5),[12] 350 mV for bean plastocyanin (pH 6.6),[13] and 184 mV for stellacyanin (pH 7.1).[12] The observed order of increasing reactivity, then, is strikingly different from that expected from consideration only of the thermodynamic driving force. Stated in another way, we may estimate that the rate constants for the self-exchange transfer reactions involving the blue copper centers in these proteins will differ by a great many orders of magnitude.

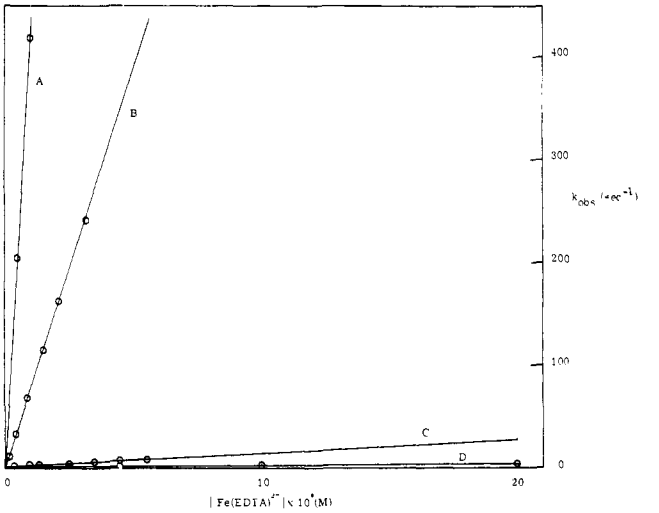

Figure 1. Dependence of k_{obsd} on the concentration of $Fe(EDTA)^{2-}$ for stellacyanin (A), plastocyanin (B), azurin (C), and laccase (D). Conditions are given in Table I.

Relative Marcus theory has been successful in correlating the rate constants of reactions of cytochrome c with various outer sphere oxidants and reductants.[14-17] Straightforward application of the theory here immediately gives estimates of the products of the self-exchange rates of the blue proteins and $Fe(EDTA)^{2-}/Fe(EDTA)^-$.[18] The relative protein self-exchange rates are then simply the ratios of these products, and the need to know the $Fe(EDTA)^{2-}/Fe(EDTA)^-$ exchange rate is eliminated. We therefore estimate that the relative self-exchange rates are 2×10^{10}, 1×10^5, 7×10^2, and 1 for stellacyanin, plastocyanin, azurin, and laccase, respectively.

Some indication as to the mechanistic origin of the wide range of electron-transfer reactivity can be found in the activation parameters for $Fe(EDTA)^{2-}$ reduction of the four proteins (Table II). Because of the possible differences in the kinetic systems with regard to pH and ionic strength dependences, and the inherent imprecision of activation parameters even in the best of cases, differences in $\Delta S^{\ddagger}$ of less than 10 eu (or $\Delta H^{\ddagger}$ of less than 5 kcal/mol) are difficult to interpret unambiguously in mechanistic terms. The difference that is clear from the activation parameter data is that three proteins, azurin, stellacyanin, and plastocyanin, ex-

hibit low, positive $\Delta H^{\ddagger}$ and large, negative $\Delta S^{\ddagger}$ values. In contrast, the $Fe(EDTA)^{2-}$ reduction of laccase is characterized by a much larger, positive $\Delta H^{\ddagger}$ and a near-zero $\Delta S^{\ddagger}$.

The activation parameters for the laccase reduction by $Fe(EDTA)^{2-}$ are consistent with results using other reductants.[2,8] Using hydroquinone as the reductant and referencing the activation parameters to the hydroquinone monoanion (HQ^-) as the active species, we find $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values of 10.7 kcal/mol and 2 eu at 614 nm and 11.6 kcal/mol and 8 eu at 330 nm.[10] The reduction mechanism of *Rhus* laccase blue copper by hydroquinone is thought to be dominated by a slow step involving protein conformational movement coupled to coordination of HQ^- at the type 2 (EPR detectable, nonblue) site;[8] the enzyme thus does not take advantage of the high inherent reactivity of type 1 copper by utilizing it to oxidize external electron sources rapidly. The relatively large activation enthalpy in the case of the $Fe(EDTA)^{2-}$ reduction of laccase as compared to the other blue proteins is further evidence for specific protein activation, probably by opening a pocket in the region of the type 2 copper. Evidence that $Fe(EDTA)^{2-}$ attacks in the same pocket as hydroquinone comes from inhibitor studies. Azide is known to inhibit laccase with hydroquinone as the reductant and to interact with type 2 copper;[8] this inhibition is strongly in evidence with $Fe(EDTA)^{2-}$ as the reductant also.[19] The model we propose for laccase reduction, then, requires attack in the vicinity of type 2 copper, with the requirement that the protein undergo a conformational change to accept the reductant and possibly to make the type 1 and type 3 (EPR nondetectable) coppers available for reduction.[8] Based on our observation of small $\Delta H^{\ddagger}$ values for type 1 reduction of one-copper proteins, we may assume that this protein activation amounts to approximately 10 kcal/mol.

Electron-transfer pathways to the other blue proteins studied here are best considered as purely outer sphere, with little requirement for protein or Franck–Condon activation. The latter is reasonable in terms of the suggested distorted tetrahedral structure for type 1 copper, which would make it intermediate between the preferred square-planar geometry of copper(II) and tetrahedral copper(I).[20] The activation enthalpy of 2–3 kcal/mol could easily be explained as the contribution from the reductant, as the reaction between $Fe(EDTA)^{2-}$ and $Fe(CyDTA)^-$ has a $\Delta H^{\ddagger}$ of 4 kcal/mol.[21] The reaction rate differences in the simple blue proteins, then, are controlled by entropy effects, with $\Delta S^{\ddagger}$ decreasing according to stellacyanin > plastocyanin > azurin. Origins of these differences in $\Delta S^{\ddagger}$ in the three single blue proteins can only be speculated on with the information presently available. The range of reactivity may reflect differences in the distance of the blue center to the protein surface, as a variety of physical measurements have shown the copper to be substantially buried in a hydrophobic environment.[22-25] Alternatively, there may be significant differences in solvation effects at the site of reduction in the proteins. In view of such uncertainties, we shall postpone any more detailed interpretation of the relative reactivities of type 1 copper proteins until much more kinetic and structural information is available.

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Supplementary Material Available. Complete tabulation of k_{obsd} values and calculations of activation parameters for the hydroquinone reduction of laccase will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th Street, N.W., Washington, D.C. 20036. Remit check or money order for $4.50 for photocopy or $2.50 for microfiche, referring to code number JACS-75-5260.

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(18) The self-exchange rate constant for $Fe(EDTA)^{2-}/Fe(EDTA)^-$ is not known, but based on the specific rate of the cross reaction between $Fe(EDTA)^{2-}$ and ferricytochrome c (2.6×10^4)[3] and a potential of 120 mV for the $Fe(EDTA)^{2-}/Fe(EDTA)^-$ couple [R. Belcher, D. Gibbons, and T. S. West, *Anal. Chim. Acta*, **12**, 107 (1955)], it may reasonably be expected to fall between 10^3 and 10^4 M^{-1} sec^{-1}. The protein self-exchange rate constants may then be estimated (pH 7, 25°): laccase, $0.7–7 \times 10^{-4}$; azurin, 0.05–0.5; plastocyanin, $0.9–9 \times 10^2$; stellacyanin, $0.2–2 \times 10^7$ M^{-1} sec^{-1}. It is of interest that NMR experiments have shown that the self-exchange rate constant for plastocyanin at 50° is much smaller than 4×10^3 M^{-1} sec^{-1} (J. K. Beattie, D. J. Fenson, H. C. Freeman, E. Woodcock, H. A. O. Hill, and A. M. Stokes, *Biochim. Biophys. Acta*, submitted for publication).
(19) The reduction of laccase type 1 copper by $Fe(EDTA)^{2-}$ (pH 6.0, phosphate, μ = 0.5 *M*, 25°) in the presence of varying concentrations of NaN_3 up to 0.15 *M* (equilibrated with the protein before reduction) gives complex kinetic behavior. Analysis of the kinetic data indicates that three laccase species undergo reduction.

$$\text{laccase} + N_3^- \overset{K_1}{\rightleftharpoons} \text{laccase}\cdot N_3^-$$

$$\text{laccase}\cdot N_3^- + N_3^- \overset{K_2}{\rightleftharpoons} \text{laccase}\cdot 2N_3^-$$

For reduction by 2.5 m*M* $Fe(EDTA)^{2-}$ the parameters are K_1, 5500 M^{-1}; k (laccase), 0.87 sec^{-1}; k (laccase·N_3^-), <0.005 sec^{-1}; k (laccase·$2N_3^-$), 0.065 sec^{-1}. The various reduction pathways remain first order in $[Fe(EDTA)^{2-}]$ at high NaN_3 concentrations (S. Wherland, unpublished results).
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