Kinetics Studies of the Oxidation of Blue Copper Proteins by Tris(1,10-phenanthroline)cobalt(III) Ions

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Abstract: A kinetics study of the oxidation of *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* stellacyanin by tris complexes of 1,10-phenanthroline and its 5-chloro, 5,6-dimethyl, 4,7-dimethyl, and 4,7-diphenyl-4′-sulfonate derivatives with Co(III) has been performed. The reactivity order at 25 °C (pH 7.0) is stellacyanin ($k = 1.80 \times 10^5$ M^{-1} s^{-1}, $\mu = 0.1$ M) > plastocyanin ($k = 4.87 \times 10^3$ M^{-1} s^{-1}, $\mu = 0.1$ M) > azurin ($k = 3.20 \times 10^3$ M^{-1} s^{-1}, $\mu = 0.2$ M) for $Co(phen)_3^{3+}$ as the oxidant. This order matches that found previously for the $Fe(EDTA)^{2-}$ reduction of the blue Cu(II) proteins. The electron transfer pathways from azurin(I) and plastocyanin(I) to $Co(phen)_3^{3+}$ are characterized by large enthalpic activation requirements of 14.3 and 14.0 kcal/mol coupled with favorable activation entropies amounting to 5 cal/(mol deg) in both cases. Reduced stellacyanin, on the other hand, prefers an oxidation mechanism for which $\Delta H^{\ddagger}$ is considerably smaller (6.1 kcal/mol) and $\Delta S^{\ddagger}$ is negative (−13 cal/(mol deg)). It is suggested that the activation parameters for electron transfer from reduced plastocyanin and azurin to $Co(phen)_3^{3+}$ may be accounted for in terms of oxidant-induced protein structural changes which expose active sites that are, by comparison with stellacyanin, inaccessible to reagent attack. Arguments based on blue protein reduction potentials, calculated protein self-exchange electron transfer rate constants, and isokinetic correlations among activation parameters for the series of cobalt(III) oxidants containing 1,10-phenanthroline derivatives, are presented in support of this hypothesis. Kinetic data for the oxidation of azurin by $Co(5,6\text{-}Me_2phen)_3^{3+}$ and $Co(4,7\text{-}Me_2phen)_3^{3+}$ provide confirmatory evidence for the existence of a difficult-to-oxidize azurin isomer.

Copper proteins containing only type 1 (or blue) sites function as electron carriers.[2] Although x-ray crystallographic results are not presently available for any of the blue proteins, detailed structural models are emerging for the type 1 copper site. The intense absorption near 600 nm characteristic of blue proteins has been assigned as a cysteine sulfur to copper(II) charge transfer transition, and a number of physical studies have provided evidence that the metal atom occupies a slightly flattened tetrahedral binding site.[3-5] It should also be noted that magnetic resonance[6-11] and fluorescence[12] measurements strongly suggest that in at least some blue proteins the copper atom lies within a solvent-inaccessible hydrophobic environment.

Kinetic parameters for electron transfer from the nonphysiological reductant $Fe(EDTA)^{2-}$ to blue copper proteins have recently been reported.[13,14] Large second-order rate constants and minimal activation enthalpies for the reduction of stellacyanin ($k = 4.3 \times 10^5$ M^{-1} s^{-1}, 25 °C, pH 6.9, $\mu = 0.5$ M; $\Delta H^{\ddagger} = 3.0$ kcal/mol, $\Delta S^{\ddagger} = -21$ cal/(mol deg), pH 7.0, $\mu = 0.1$ M), bean plastocyanin ($k = 8.2 \times 10^4$ M^{-1} s^{-1}, 25 °C, pH 6.9, $\mu = 0.2$ M; $\Delta H^{\ddagger} = 2.1$ kcal/mol, $\Delta S^{\ddagger} = -29$ cal/(mol deg)), and *Pseudomonas aeruginosa* azurin ($k = 1.3 \times 10^3$ M^{-1} s^{-1}, 25 °C, pH 7.0, $\mu = 0.2$ M; $\Delta H^{\ddagger} = 2.0$ kcal/mol, $\Delta S^{\ddagger} = -37$ cal/(mol deg)) have been interpreted as an indication that there is little activation requirement for either protein or inner-sphere rearrangement.[2,13] It has been suggested that the wide range of electron transfer reactivity of the blue copper proteins reflects variations in the accessibility of the active site to outer sphere contact with external redox agents, and that the large negative apparent entropies of activation may be attributable to some nonadiabaticity in the electron transfer from $Fe(EDTA)^{2-}$ to the Cu(II) centers.[2,14]

To explore the electron transfer reactivity of blue proteins further, we have investigated the oxidation of reduced stellacyanin, plastocyanin, and azurin by tris complexes of 1,10-phenanthroline and several of its derivatives with cobalt(III). By utilizing substitution in the 4, 5, 6, and 7 positions of the phenanthroline ring system, we have been able to evaluate the reactivity of the reduced blue proteins as influenced by systematic variations in the charge and molecular structure of the Co(III) oxidant complex.

Experimental Section

Reagent grade chemicals and deionized distilled water were used throughout. Nitrogen gas passed through two chromous scrubbing towers was used to deoxygenate kinetics solutions. *Rhus vernicifera* stellacyanin, bean plastocyanin, and *Pseudomonas aeruginosa* azurin were isolated and purified as previously described.[13,14]

Solutions of reduced plastocyanin and azurin were prepared by adding a 20-fold excess of $Fe(EDTA)^{2-}$ to deoxygenated, buffered solutions of the cupric proteins. The excess reductant was then removed by dialysis against deoxygenated buffer using a hollow fiber Dow beaker dialyzer obtained from Bio-Rad Laboratories. As reduced stellacyanin was readily oxidized by traces of O_2 which could not be excluded using this technique, solutions of cuprous stellacyanin were prepared by adding an equivalent amount of ascorbic acid to the cupric protein without removing the dehydroascorbate product. Dehydroascorbate was shown to have no influence on the rate of oxidation of stellacyanin(I) by $Co(phen)_3^{3+}$ in concentrations up to 1×10^{-4} M (tenfold molar excess over protein concentration).

The various oxidant solutions were prepared by standard methods.[15,16] Some kinetic runs with stellacyanin were performed using the perchlorate salt of $Co(phen)_3^{3+}$, prepared by the method of Schilt and Taylor.[17] Good agreement was found between observed rate constants obtained with the chloride and perchlorate salts. Sodium phosphate buffers were used throughout, and sodium chloride or ammonium sulfate was added to adjust the ionic strength to the desired level.

Kinetic data for the oxidation of stellacyanin were obtained by monitoring absorbance increases at 604 nm using a Durrum Model D-110 stopped flow spectrophotometer. Plastocyanin and azurin oxidations were monitored at 597 and 625 nm, respectively. Kinetic measurements were made under pseudo-first-order conditions by employing 10- to 100-fold excess of oxidant for protein concentrations of ca. 10 μM. Most data were assimilated in an analog to digital converter and transmitted directly to a PDP-10 computer for analysis. Some data were collected as photographs of absorbance-time traces on a Tektronix Model 564 B storage oscilloscope. Observed rate

Table I. Kinetic Parameters for the Oxidation of Blue Copper Proteins at 25 °C

Oxidizing agent	Stellacyanin[a] k, $M^{-1}\,s^{-1}$	Stellacyanin[a] $\Delta H^{\ddagger}$, kcal/mol	Stellacyanin[a] $\Delta S^{\ddagger}$, cal/(mol deg)	Plastocyanin[e] k, $M^{-1}\,s^{-1}$	Plastocyanin[e] $\Delta H^{\ddagger}$, kcal/mol	Plastocyanin[e] $\Delta S^{\ddagger}$, cal/(mol deg)	Azurin[f] k, $M^{-1}\,s^{-1}$	Azurin[f] $\Delta H^{\ddagger}$, kcal/mol	Azurin[f] $\Delta S^{\ddagger}$, cal/(mol deg)
$Co(phen)_3^{3+}$	$1.80\,(5) \times 10^5$	6.0 (2)	−13 (1)	$4.87\,(5) \times 10^3$	14.0 (5)	5 (1)	$3.20\,(5) \times 10^3$	14.3 (5)	5 (1)
	$1.8\,(1) \times 10^5$[b]	6.1 (2)	−14 (1)						
	$1.3\,(1) \times 10^5$[c]	5.9 (2)	−15 (1)						
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	$1.85\,(5) \times 10^4$	9.5 (2)	−7 (1)	$7.97\,(5) \times 10^3$	13.6 (5)	1 (1)	$1.54\,(5) \times 10^3$	11.6 (5)	−5 (1)
$Co(5\text{-}Cl\text{-}(phen))_3^{3+}$	*d*		−10 (1)	$6.96\,(5) \times 10^2$	10.9 (5)	−8 (1)	$4.21\,(5) \times 10^2$	8.9 (5)	−17 (1)
$Co[4,7\text{-}(PhSO_3)_2\text{-}phen]_3^{3-}$	$2.31\,(5) \times 10^6$	5.9 (2)		$2.59\,(5) \times 10^1$	7.8 (5)	−26 (1)	*d*		
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	*d*			*d*			$8.41\,(5) \times 10^1$	9.9 (5)	−17 (1)

[a] For pH 7.0 (phosphate), μ = 0.1 M (NaCl) unless otherwise specified. [b] μ = 0.5 M (phosphate). [c] pH 5.1 (acetate), μ = 0.5 M (acetate). [d] Experimental determinations were not made. [e] For pH 7.0 (phosphate), μ = 0.1 M ($(NH_4)_2SO_4$). [f] For pH 7.0 (phosphate), μ = 0.2 M (NaCl).

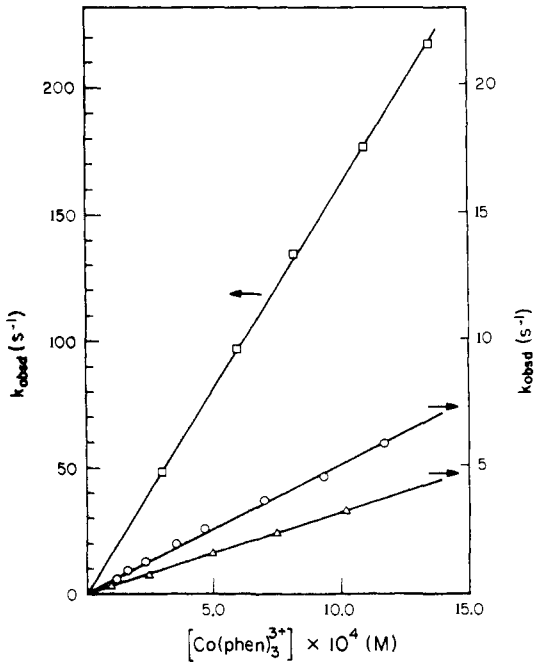

Figure 1. The dependences of observed rate constants on the concentration of $Co(phen)_3^{3+}$ at 25 °C, pH 7.0 (phosphate): stellacyanin, □ (μ = 0.5 M (phosphate)); plastocyanin, ○ (μ = 0.1 M ($(NH_4)_2SO_4$)); azurin, △ (μ = 0.2 M (NaCl)).

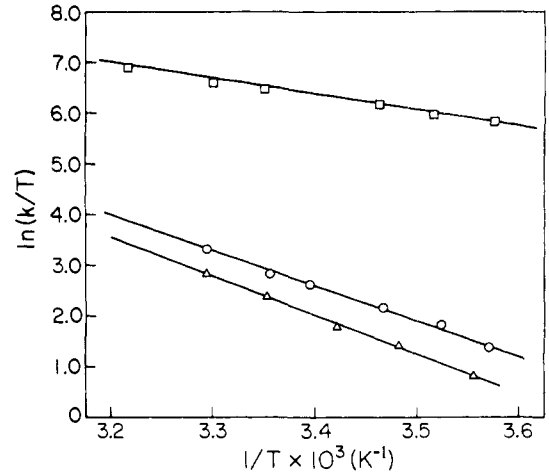

Figure 2. Eyring plots of the rate data for oxidations by $Co(phen)_3^{3+}$ at pH 7.0 (phosphate): stellacyanin, □ (μ = 0.5 M (phosphate)); plastocyanin, ○ (μ = 0.1 M ($(NH_4)_2SO_4$)); azurin, △ (μ = 0.2 M (NaCl)).

constants (k_{obsd}) were evaluated as usual from the least-squares slopes of log ($A_\infty - A_t$) vs. time plots.

All spectral data were acquired on a Cary 17 UV-visible spectrophotometer. Brinkman pH 101 and Ionalyzer Model 801 instruments were used to make pH measurements.

Results

I. Oxidation of Cuprous Stellacyanin, Plastocyanin, and Azurin by $Co(phen)_3^{3+}$. First-order plots of absorbance-time data for the oxidation of azurin(I), plastocyanin(I), and stellacyanin(I) by $Co(phen)_3^{3+}$ were found to be linear for greater than 90% of the reactions. The dependence of observed rate constants on $[Co(phen)_3^{3+}]$ is illustrated in Figure 1 for data collected at 25 °C (pH 7.0). In each case the experimental results are compatible with the rate law:

$$\frac{d[\text{type 1 Cu(II)}]}{dt} = k[\text{type 1 Cu(I)}][Co(phen)_3^{3+}]$$

over the tenfold range in oxidant concentration accessible in phosphate media. Second-order rate constants obtained from the least-squares slopes of k_{obsd} vs. $[Co(phen)_3^{3+}]$ plots are summarized in Table I, along with activation parameters derived from linear Eyring plots of ln (k/T) vs. $1/T$ (Figure 2). Rate parameters evaluated for stellacyanin in acetate (μ = 0.5 M, pH 5.1) and in phosphate (μ = 0.5 M, pH 7.0; μ = 0.1 M, pH 7.0) buffers are in good agreement.

Data describing the dependence of the oxidation rate of the proteins on ionic strength at pH 7.0 are assembled in Table II. The rates of oxidation of stellacyanin and azurin are observed to be nearly independent of ionic strength over the interval 0.05–0.5 M, whereas the plastocyanin oxidation rate decreases slightly with increasing ionic strength. Table II also contains results of a study of the pH dependence of the rate of oxidation of stellacyanin at an ionic strength of 0.5 M. Second-order rate constants for the oxidation of stellacyanin increase with increasing pH, but vary by less than a factor of 2 between pH 5 and 9. An abrupt increase in k from 1.8×10^5 to 2.5×10^5 $M^{-1}\,s^{-1}$ occurs between pH 7 and 8, taking the form of a titration curve with apparent pK of 7.7 ± 0.2.

II. Oxidations by Ring-Substituted Tris(1,10-phenanthroline)cobalt(III) Ions. The rate law observed for the oxidation of reduced blue proteins by $Co(phen)_3^{3+}$ was found to pertain

Table II

A. Ionic Strength Dependences of $Co(phen)_3^{3+}$ Rate Parameters[a]

Protein	μ (M)	k (M^{-1} s^{-1})	10^3 [$Co(phen)_3^{3+}$] (M)
Stellacyanin[b]	0.052	1.89×10^5	2.00
	0.194	1.89×10^5	2.00
	0.296	1.93×10^5	2.05
	0.390	1.92×10^5	1.50
	0.502	1.84×10^5	0.30-2.35
Plastocyanin[c]	0.05	2.49×10^0	0.56
	0.10	1.50×10^0	0.56
	0.20	8.11×10^{-1}	0.56
	0.30	5.95×10^{-1}	0.56
	0.50	4.75×10^{-1}	0.56
Azurin[c]	0.05	2.61×10^3	2.00
	0.08	2.20×10^3	2.00
	0.12	2.11×10^3	2.00
	0.16	2.11×10^3	2.00
	0.20	2.02×10^3	2.00
	0.05	2.43×10^3	1.00
	0.08	2.26×10^3	1.00
	0.12	2.08×10^3	1.00
	0.16	1.83×10^3	1.00
	0.20	1.89×10^3	1.00

B. pH Dependence of $Co(phen)_3^{3+}$ Rate Parameters

Protein	pH	k (M^{-1} s^{-1})	10^3 [$Co(phen)_3^{3+}$] (M)
Stellacyanin	5.00	1.89×10^5	2.10
		1.71×10^5	0.20
	6.04	1.83×10^5	2.05
		1.83×10^5	0.20
	6.44	1.98×10^5	2.05
		1.73×10^5	0.19
	7.46	1.99×10^5	1.80
		1.99×10^5	0.19
	7.98	2.54×10^5	1.95
		2.46×10^5	0.20
	8.53	2.46×10^5	0.34
		2.46×10^5	1.73
	9.02	2.46×10^5	1.08
		2.50×10^5	1.61

[a] All values are the mean of at least two separate determinations. [b] 25.3 °C, pH 7.0 (phosphate). [c] 25 °C, pH 7.0 (phosphate).

in most cases to the analogous reactions employing tris complexes of the 5-chloro, 4,7-dimethyl, 5,6-dimethyl, and 4,7-diphenyl-4′-sulfonate derivatives of 1,10-phenanthroline with Co(III) as oxidants. Kinetic parameters for oxidations by $Co(phen)_3^{3+}$ derivatives (Table I) exhibit striking variations in both second-order rate constants and activation parameters. Oxidation rate constants for the ring-substituted tris(1,10-phenanthroline)cobalt(III) ions are, with the exception of that for the stellacyanin(I)-$Co[4,7\text{-}(PhSO_3)_2\text{-}phen]_3^{3-}$ reaction, substantially smaller than the corresponding parameters for the unsubstituted oxidants.

The oxidation of azurin(I) by either $Co(5,6\text{-}Me_2phen)_3^{3+}$ or $Co(4,7\text{-}Me_2phen)_3^{3+}$ displays complicated kinetic behavior. Spectral changes at 625 nm are biphasic (Figure 3), with the faster phase accounting for about 65% of the observed absorbance change. The absorbance-time traces were successfully treated in terms of two parallel first-order reactions,[18] yielding observed rate constants for slow and fast rate processes. Plots of k_{obsd} (fast) against oxidant concentration are linear with near zero intercepts, consistent with the second-order rate expression given previously. Solutions of azurin(I) that showed biphasic oxidation traces with $Co(5,6\text{-}Me_2phen)_3^{3+}$ and $Co(4,7\text{-}Me_2phen)_3^{3+}$ were checked for possible contamination by performing several runs with $Co(phen)_3^{3+}$ as the oxidant. Monophasic kinetic behavior was still found for the unsubstituted oxidant, and the rate constants were in agreement with previous results. The second phases of the reactions were difficult to characterize because of their slowness and the small absorbance changes involved. For $Co(5,6\text{-}Me_2phen)_3^{3+}$ as the oxidant, an oxidant-independent k_{obsd} (slow) value of $(1.1 \pm 0.1) \times 10^{-2}$ s^{-1} (25 °C, pH 7.0, μ = 0.2 M) was found over the concentration range 1.0-10.0 $\times 10^{-4}$ M, with a $\Delta H^{\ddagger}$ of 4 ± 1 kcal/mol and a $\Delta S^{\ddagger}$ of −54 ± 2 cal/(mol deg). By contrast, observed rate constants for the slow phase of the oxidation of azurin by $Co(4,7\text{-}Me_2phen)_3^{3+}$ vary linearly with the oxidant concentration ($k = (4.2 \pm 0.4) \times 10^1$ M^{-1} s^{-1}; 25 °C, pH 7.0, μ = 0.2 M) over the range 5.0-20.0 $\times 10^{-4}$ M (Figure 4).

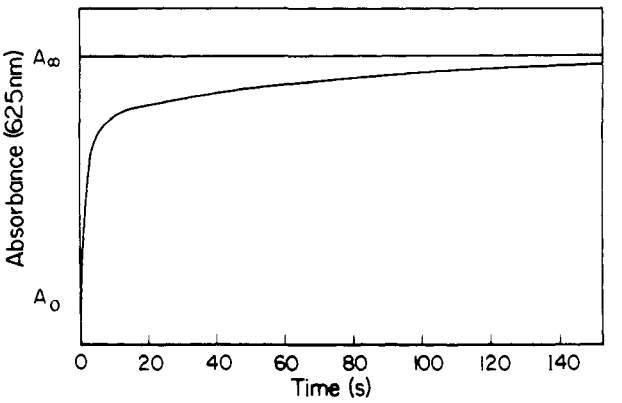

Figure 3. Absorbance vs. time data for the oxidation of azurin(I) by 2×10^{-4} M $Co(5,6\text{-}Me_2phen)_3^{3+}$ at 25 °C, pH 7.0 (phosphate), μ = 0.2 M (NaCl).

First-order analytical plots for the oxidation of azurin(I) by $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$ are linear for about 90% of the reaction. However, a plot of k_{obsd} vs. [$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$] reveals that the reaction is not first order with respect to the oxidant. The plot remains nonlinear even when an ionic strength of 1.0 M is used to limit electrostatic interactions between the oxidant and the metalloprotein. The data of Figure 5, which were taken at an ionic strength of 0.5 M, may be interpreted in terms of a mechanism involving rapid preequilibrium oxidant-protein complex formation followed by rate limiting intramolecular electron transfer:

$$\text{Ox(III)} + \text{Az(I)} \overset{K_p}{\rightleftharpoons} \text{Ox(III)} \cdots \text{Az(I)}$$

$$\text{Ox(III)} \cdots \text{Az(I)} \xrightarrow{k_2} \text{Ox(II)} \cdots \text{Az(II)}$$

$$\text{Ox(II)} \cdots \text{Az(II)} \xrightarrow{\text{fast}} \text{Ox(II)} + \text{Az(II)}$$

$$\frac{d[\text{Az(II)}]}{dt} = k_2[\text{Ox(III)} \cdots \text{Az(I)}] = \frac{k_2 K_p [\text{Ox(III)}]_{tot}[\text{Az(I)}]_{tot}}{1 + K_p[\text{Ox(III)}]_{tot}} = k_{obsd}[\text{Az(I)}]_{tot}$$

Reduced azurin and the cobalt(III) oxidant are represented, respectively, by Az(I) and Ox(III). A plot of k_{obsd}^{-1} vs. $[\text{Ox(III)}]_{tot}^{-1}$ is expected to be linear with slope $(k_2K_p)^{-1}$ and intercept k_2^{-1}. The experimental data may be fit well through such a plot (correlation coefficient of 0.999), yielding apparent values for the precursor complex formation constant K_p of (7.0 ± 0.1) × 10^2 M^{-1} and for k_2 of (3.7 ± 0.1) × 10^{-1} s^{-1}.

Discussion

Comparison of kinetic parameters for the oxidation of reduced stellacyanin, plastocyanin, and azurin by $Co(phen)_3^{3+}$ reveals that the latter two proteins have similar reactivities, whereas stellacyanin falls into a distinctly different class. The

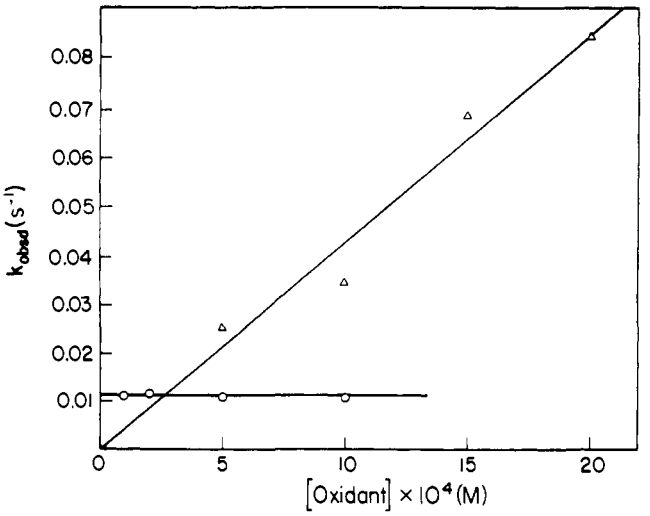

Figure 4. Plot of k_{obsd} vs. oxidant concentration for the slow phases of the reactions between azurin(I) and $Co(5,6\text{-}Me_2phen)_3^{3+}$ (○) and $Co(4,7\text{-}Me_2phen)_3^{3+}$ (△) at 25 °C, pH 7.0, μ = 0.2 M (NaCl).

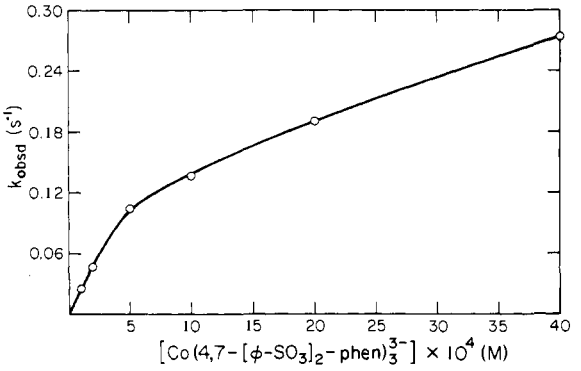

Figure 5. Plot of k_{obsd} vs. $[Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}]$ for the oxidation of azurin(I) at 25 °C, pH 7.0 (phosphate), μ = 0.5 M (NaCl).

room temperature reactivity sequence for $Co(phen)_3^{3+}$ oxidation is stellacyanin > plastocyanin > azurin, which matches that found[13] for the $Fe(EDTA)^{2-}$ reduction of the blue Cu(II) proteins. The azurin and plastocyanin oxidation pathways are characterized by abnormally large enthalpic activation requirements of ca. 14 kcal/mol, coupled with favorable entropies of activation amounting to 5 cal/(mol deg) in both cases. Stellacyanin, on the other hand, prefers a pathway for which $\Delta H^{\ddagger}$ is smaller by ca. 8 kcal/mol and $\Delta S^{\ddagger}$ is more negative by ca. 20 cal/(mol deg). Activation parameters obtained for the reaction between $Co(phen)_3^{3+}$ and a low molecular weight reductant, $Co(terpy)_2^{2+}$ ($\Delta H^{\ddagger}$ = 6.6 kcal/mol, $\Delta S^{\ddagger}$ = −24 cal/(mol deg); μ = 0.5 M= PH 7.0)[19] resemble those for the oxidation of stellacyanin much more closely than those for the oxidation of the other two blue proteins.

On the basis of the observed activation parameters we propose that the oxidation of stellacyanin by $Co(phen)_3^{3+}$ occurs by an adiabatic outer-sphere electron transfer mechanism. The entropy of activation (−13 cal/(mol deg)) is in good quantitative correspondence with expectations for the association contribution to $\Delta G^{\ddagger}$ resulting from the conversion of the separated reactants into a bimolecular collision complex.[20] Furthermore, the small difference in rate parameters for tris(1,10-phenanthroline)cobalt(III) and its 4,7-diphenyl-4′-sulfonate derivative suggests that electrostatic interactions between reduced stellacyanin and the external oxidant are of little importance in stabilizing the precursor complex for electron transfer. The insensitivity of the pH 7.0 oxidation rate to the charge carried by the oxidant is especially striking, as stellacyanin is a strongly basic metalloprotein (pI 9.86).[21] The fact that the stellacyanin oxidation rate varies very little over wide ranges in pH and ionic strength provides further support for the hypothesis that interactions between the oxidant and charged amino acid side chains are not an important feature of the electron transfer mechanism.

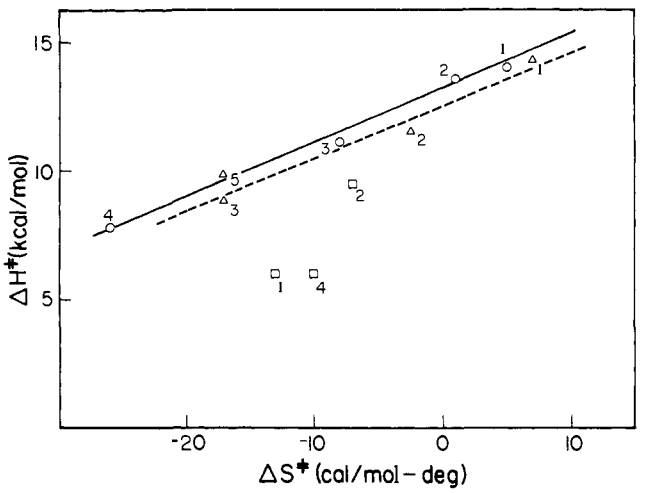

Figure 6. Compensation plot for the oxidation of blue copper proteins by tris(1,10-phenanthroline)cobalt(III) ions. Data from Table I: pH 7.0 (phosphate); stellacyanin, □; plastocyanin, ○, —; azurin, △, - - -. Oxidant key: 1, $Co(phen)_3^{3+}$; 2, $Co(5,6\text{-}Me_2phen)_3^{3+}$; 3, $Co(5\text{-}Cl(phen))_3^{3+}$; 4, $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$; 5, $Co(4,7\text{-}Me_2phen)_3^{3+}$.

The activation parameters for the oxidation of plastocyanin and azurin may be accounted for in terms of structural changes in the reduced proteins which expose type 1 copper sites that are, by comparison with stellacyanin, inaccessible to attack by external redox agents. No conclusions based on kinetic results may be drawn regarding the position of the redox centers in the crystallized proteins; rather, the crucial factor apparently is the ability of $Co(phen)_3^{3+}$ and its derivatives to influence the protein conformation in such a way that overlap is established between donor and acceptor redox orbitals. A requirement for both normal Franck-Condon and substantial conformational activation is consistent with the unusually high experimental $\Delta H^{\ddagger}$ values. The entropic driving force for specific protein activation perhaps could be linked to hydrophobic interactions between the phenanthroline ligands and nonpolar amino acid side chains, which result in the disordering of water molecules held in the outer coordination sphere of $Co(phen)_3^{3+}$ as well as those on a portion of the surface of the reduced proteins. Activation parameters for the reduction of parsley plastocyanin by cytochrome *f* ($\Delta H^{\ddagger}$ = 10.5 kcal/mol, $\Delta S^{\ddagger}$ = 11 cal/(mol deg), pH 7.0, μ 0.1 M)[22] and for electron transfer between the physiological redox partners cytochrome c_{551}(II) and azurin(II) ($\Delta H^{\ddagger}$ = 10.6 kcal/mol, $\Delta S^{\ddagger}$ = 8.6 cal/(mol deg); pH 7.0, μ = 0.05 M phosphate)[23] are remarkably similar to those for the reactions of azurin(I) and plastocyanin(I) with $Co(phen)_3^{3+}$. In accord with our proposal, Wood has accounted for the positive entropy of activation for the cytochrome *f*(II)-plastocyanin(II) reaction in terms of unfolding of the proteins and breakage of ordered water structure.[22]

Plots of $\Delta H^{\ddagger}$ against $\Delta S^{\ddagger}$ for the oxidation of blue proteins by the series of tris(1,10-phenanthroline)cobalt(III) ions (Figure 6) reveal that good isokinetic correlations[24] exist among the activation parameters for azurin and plastocyanin but not for stellacyanin. Thus $\Delta H^{\ddagger}$ varies linearly with $\Delta S^{\ddagger}$, yielding β values (slopes) of 217 K for azurin (correlation coefficient 0.981) and 206 K for plastocyanin (correlation coefficient 0.992). Compensation behavior has been documented for a number of protein unfolding or denaturation processes,[25,26] with β values typically falling somewhat higher

(250–320 K)[25] than those reported here. That linear compensation plots are found for the oxidation of plastocyanin and azurin strongly suggests that the mechanism by which each of the reduced proteins transfers an electron to $Co(phen)_3^{3+}$ is not substantially altered upon introduction of substituents into the 1,10-phenanthroline ring system. Rather it seems more likely that in each case variations in activation parameters may be accounted for in terms of substituent-induced perturbations on a common interaction mechanism.

Introduction of substituents into the phenanthroline ligands probably limits the distance of closest approach between the cobalt(III) oxidant and the type 1 Cu(I) site, forcing electron transfer to occur over a larger distance than is preferred by the unsubstituted oxidant. The conformational contribution to $\Delta H^{\ddagger}$ presumably will become smaller to the extent that ring substituents prevent close approach between the Cu(I) and Co(III) centers. At the same time, however, disordering of water molecules linked to extensive van der Waals attractions between a reduced protein and the phenanthroline ligands of the oxidant will occur to a lesser extent, causing $\Delta S^{\ddagger}$ to become less favorable.

Looked at in another way, the isokinetic relationships between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the oxidation of azurin and plastocyanin are consistent with the reaction acquiring nonadiabatic character to the extent that ring substituents block overlap between donor and acceptor redox orbitals on the two metal centers. Thus nonadiabaticity or tunneling is associated with minimal reorganizational activation requirements and large negative apparent entropies of activation, the latter a consequence of decreases in the transmission coefficient to values less than 1.[27] Nonadiabaticity is expected to become increasingly important as electron transfer is constrained to occur over larger and larger distances. Indeed, the values of both activation parameters consistently decrease with increasing size of the ring substituent. The correlation between the activation parameter trends and substituent size, however, also is compatible with an interpretation of the isokinetic relationships based solely on the extent of protein conformational rearrangement. Both factors may in fact contribute to the enthalpy–entropy compensation effect. On the basis of the data presently available, it is not possible to ascertain the relative importance of the two proposed substituent-induced perturbations.

The availability of only three points in the compensation plot for stellacyanin precludes our firmly ruling out the presence of an isokinetic relationship in this case. The available data, however, seemingly indicate that, although activation parameters are a sensitive function of the structure of the oxidant, they do not follow the systematic compensation pattern exhibited by the other two blue proteins. Thus it may be concluded that ring substituents have a much larger effect on the stellacyanin oxidation mechanism than on the related processes for azurin and plastocyanin. In accord with this reasoning are comparisons of the ratio of rate constants for oxidation by $Co(phen)_3^{3+}$ to that for oxidation by the 5,6-Me_2phen derivative. The ratio decreases in the order ten for stellacyanin, six for plastocyanin, and two for azurin. For plastocyanin or azurin the oxidation rate is less sensitive to substituent effects as the activation process appears to be primarily protein dependent. In addition, each protein apparently is able to compensate for steric hindrance by allowing electron transfer to occur over larger distances without drastically altering the overall activation free energy. Activation free energies and standard free energy changes for the redox reactions of interest are set out in Table III. Many of the derivatives exhibit activation free energies higher than those for $Co(phen)_3^{3+}$ by only about 1 kcal/mol, the maximum displacement being 3 kcal/mol for the $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$–plastocyanin(I) reaction. By contrast, variations in $\Delta H^{\ddagger}$ of over 6 kcal/mol and in $\Delta S^{\ddagger}$ of over 30 cal/(mol deg) may be noted in Table I.

As both plastocyanin[28] and azurin[29] are acidic metalloproteins with substantial net negative charges at pH 7.0, the possibility of electrostatic stabilization of precursor complexes for electron transfer to $Co(phen)_3^{3+}$ must be considered. Although the reactivity of the anionic 4,7-diphenyl-4′-sulfonate derivative with plastocyanin(I) is a factor of 200 lower than that of $Co(phen)_3^{3+}$, activation parameter comparisons cast some doubt on the assignment of this difference to electrostatic effects. A tendency toward rate saturation with increasing oxidant concentration is expected in the presence of attractive forces between redox partners which enhance the equilibrium constant for precursor complex formation, yet no such tendency is evident in rate data for the azurin(I)- or plastocyanin(I)–$Co(phen)_3^{3+}$ reactions. Quite to the contrary, deviations from a first-order oxidizing agent dependence were noted only in the case of the azurin(I)–$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$ reaction. Interestingly, rate data for electron transfer between azurin and various cytochromes support the hypothesis that this blue protein has a marked specificity for acid redox partners in spite of expected electrostatic repulsions between negatively charged species.[22] Additionally, Goldberg and Pecht have shown[30] that Az(I) and $Fe(CN)_6^{3-}$ form a precursor complex with a K_p of 6.1×10^2 M^{-1} and a k_{et} of 4.5×10^1 s^{-1} at 25 °C. The K_p values are surprisingly similar for Az(I)–$Fe(CN)_6^{3-}$ and Az(I)–$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$, but the intracomplex electron transfer rate is much faster in the former case, possibly owing to better overlap of $Fe(CN)_6^{3-}$ and Az(I) redox orbitals.

It has been suggested that an equilibrium exists between two forms of reduced azurin and that only one of these two forms can exchange electrons with cytochrome c_{551}.[31] The proposed mechanism for electron transfer between the physiological redox partners is:

$$\begin{array}{ccc} C(II) + Az(II) & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & C(III) + Az(I) \\ & & k_{23} \downarrow \uparrow k_{32} \\ & & Az(I)' \end{array}$$

where C(II) and C(III) stand for reduced and oxidized forms of cytochrome c_{551}, and Az(I)′ represents the form of azurin that is unable to participate directly in an electron transfer reaction with C(II). Wilson et al. have reported a value of 40 s^{-1} (20 °C, pH 7.0, 0.1 M phosphate)[31] for k_{32}, whereas Rosen and Pecht have given a value of 11 s^{-1} (25 °C, pH 7.0, 0.05 M phosphate).[23] No evidence for a redox inactive form of azurin was found in experiments with $Co(phen)_3^{3+}$ as the oxidant, but this is not surprising considering that k_{obsd} values were always much smaller than k_{32}, even assuming that the lower estimate for this parameter is correct. Under these circumstances the isomerization may be treated as a rapid preequilibrium and second-order kinetics will be found with $k_{obsd} = k'[Co(phen)_3^{3+}]/(1 + k_{23}/k_{32})$, where k' represents the second-order rate constant for the oxidation of azurin(I) by $Co(phen)_3^{3+}$. The value of k_{23}/k_{32} is in the range 1–2.[23,31]

The rate constant for the oxidant-independent slow phase found for the oxidation of azurin(I) by $Co(5,6\text{-}Me_2phen)_3^{3+}$ clearly does not correspond to k_{32}, as it is too small by three orders of magnitude. A possible explanation of the slow phase involves tight complexation between Az(I)′ and $Co(5,6\text{-}Me_2phen)_3^{3+}$ followed by slow intramolecular electron transfer from Cu(I) to Co(III). The complex-forming step is required to be competitive with Az(I)′ → Az(I) isomerization, as roughly 35% of the total oxidation of reduced azurin proceeds via the intramolecular reaction. Our observation of a slow phase is important in that it provides strong confirmatory evidence for the presence of a difficult-to-oxidize reduced azurin isomer. Presumably the Cu(I) in this isomer is so positioned that it cannot be oxidized very efficiently in a direct bimolec-

Table III. Free Energy Changes[a]

Protein	Oxidant	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta G°$ (kcal/mol)[b]
Stellacyanin	$Co(phen)_3^{3+}$	9.9	−4.3
	$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	11.6	−5.7
	$Co[4,7\text{-}(PhSO_3)_2\text{-}phen]_3^{3-}$	8.9	−3.4
Plastocyanin	$Co(phen)_3^{3+}$	12.5	−0.5
	$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	13.3	−1.8
	$Co(5\text{-}Clphen)_3^{3+}$	13.3	−1.6
	$Co[4,7\text{-}(PhSO_3)_2\text{-}phen]_3^{3-}$	15.6	0.5
Azurin	$Co(phen)_3^{3+}$	12.8	−1.0
	$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	13.1	−2.4
	$Co(5\text{-}Clphen)_3^{3+}$	14.0	−2.1
	$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	15.0	−0.3

[a] 25 °C, μ = 0.1 M, pH 7.0 (phosphate). [b] E (stellacyanin), 184 mV (pH 7.1, μ = 0.3 M), B. Reinhammar, *Biochem. Biophys. Acta*, **275**, 245 (1972); E (bean plastocyanin), 350 mV (pH 6.6), N. Sailasuta, unpublished results; E (*Pseudomonas aeruginosa* azurin), 328 mV (pH 6.4), T. Yamanaka in "The Biochemistry of Copper", J. Peisach, P. Aisen, and W. E. Blumberg, Ed., Academic Press, New York, N.Y., 1966, p 275; E ($Co(phen)_3^{3+}$), 370 mV, D. Cummins, unpublished results; E ($Co(5,6\text{-}Me_2phen)_3^{3+}$), 430 mV (0.05 M NaCl); E ($Co(4,7\text{-}Me_2phen)_3^{3+}$), 340 mV (0.05 M NaCl); E ($Co(5\text{-}Cl(phen))_3^{3+}$), 420 mV (0.05 M NaCl); E ($Co[4,7\text{-}(PhSO_3)_2\text{-}phen]_3^{3-}$), 330 mV (0.05 M NaCl), A. R. Bowen, unpublished results.

ular process by either cytochrome c_{551} or $Co(5,6\text{-}Me_2phen)_3^{3+}$. For small molecule oxidants containing highly hydrophobic ligands such as 5,6-Me_2phen, then, an alternative pathway could involve tight binding some distance away from the Cu(I) center. Electron transfer from Cu(I) to Co(III) in such a complex could easily have substantial nonadiabatic character, as suggested by the observed −54 cal/(mol deg) value for $\Delta S^{\ddagger}$.[32]

The kinetic results for the oxidation of azurin by $Co(4,7\text{-}Me_2phen)_3^{3+}$ provide an intriguing contrast with those for the 5,6-dimethyl analogue. Thus biphasic kinetics are found, suggesting that the oxidant effectively freezes the Az(I)′–Az(I) isomerization; interestingly, the slow phase no longer is oxidant concentration independent, but rather obeys a rate law first order in $[Co(4,7\text{-}Me_2phen)_3^{3+}]$. One mechanistic possibility is that electron transfer within a tight Az(I)′–$Co(4,7\text{-}Me_2phen)_3^{3+}$ complex is slow on the time scale of bimolecular oxidation of the complex by a second molecule of the oxidant. Electron transfer via an analogous mechanism in the Az(I)′–$Co(5,6\text{-}Me_2phen)_3^{3+}$ system evidently is prohibitively slow. On the basis of present evidence it may only be concluded that the Az(I)′ oxidation mechanism is highly sensitive to the nature and position of substituents in the phenanthroline ring system; further experimental work is planned to elucidate the factors controlling the reactivity of Az(I)′ with oxidizing agents.

It might be expected that differences in the kinetic accessibility of type 1 Cu(I) among the blue electron carriers would be reflected in some of the physical properties exhibited by the metal site. Very few differences may be noted among the optical or the EPR spectra, or the composition and geometry of the copper coordination environment.[2,4,33] However, a main point of distinction among the blue proteins lies in their reduction potentials (Table III). The potential for stellacyanin (184 mV) is comparable with that for the cupric/cuprous couple in water (153 mV),[34] whereas E^0 values for plastocyanin and azurin lie above 300 mV, indicative of a markedly

Table IV. Calculated Protein Self-Exchange Rate Constants[a]

Protein	$Fe(EDTA)^{2-}$ k_{12} (M^{-1} s^{-1})	$Fe(EDTA)^{2-}$ k_{11} (M^{-1} s^{-1})	$Co(phen)_3^{3+}$ k_{12} (M^{-1} s^{-1})	$Co(phen)_3^{3+}$ k_{11} (M^{-1} s^{-1})
Stellacyanin	1.0×10^6	3×10^6 [b]	1.8×10^5	5×10^5
Plastocyanin	5.3×10^4	1×10^1 [c]	4.9×10^3	2×10^5
Azurin	1.3×10^3	2×10^{-2} [c]	3.2×10^3 [d]	4×10^4

[a] For pH 7.0, μ = 0.1 M, 25 °C, except where noted otherwise. Protein and $Co(phen)_3^{3+}$ reduction potentials are given in Table III. For $Fe(EDTA)^{2-}$, E = 120 mV (G. Schwarzenbach and J. Heller, *Helv. Chim. Acta*, **34**, 576 (1951). k_{22} ($Co(phen)_3^{3+/2+}$) = 4.5×10^1 M^{-1} s^{-1}, k_{22} ($(Fe(EDTA)^{2-/-})$ = 3×10^4 M^{-1} s^{-1} (see S. Wherland and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2950 (1976). [b] From ref 13. [c] From ref 14. [d] For μ = 0.2 M.

enhanced driving force for reduction of Cu(II) to Cu(I) ($\Delta G°$ more negative by 3–4 kcal/mol). Kassner has proposed[35] that the standard reduction potentials of high potential cytochromes may be accounted for in terms of a local heme environment of low dielectric constant. Thus the ferriheme unit carries a formal charge of +1, causing it to develop a larger tendency for reduction to the electrically neutral ferroheme analogue as its immediate environment becomes more hydrophobic. An increase of 300 mV was noted between E^0 values for the reduction of a pyridine complex of ferrimesoheme dimethyl ester in water and in benzene solution.[35] Similar considerations may be applied toward understanding the potential differences among the blue proteins. Thus the relatively low reduction potential of stellacyanin may be attributable to the fact that its blue Cu(II) site lies in a comparatively polar environment near the protein surface, whereas the azurin and plastocyanin type 1 copper atoms presumably are buried deep within the hydrophobic interior of the polypeptide structure.

It has been shown recently that self-exchange electron transfer rate constants calculated on the basis of relative Marcus theory provide a useful framework for comparisons of the reactivity of metalloproteins with a variety of reductants and oxidants.[2,13,14] Three distinctly different reactivity types have been identified for azurin, with kinetic access to the blue copper center decreasing according to cytochrome c_{551}(II) ≫ cytochrome c(II) ≫ $Fe(EDTA)^{2-}$.[14] The reactions that give the highest predicted self-exchange rates frequently are associated with relatively favorable activation entropies, whereas the more forbidden reactions proceed with minimal $\Delta H^{\ddagger}$ but highly unfavorable $\Delta S^{\ddagger}$ values.[2,14]

The Marcus equation[36]

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\Delta E^0]$$

was employed to acquire calculated protein self-exchange rates based on the present data. The self-exchange rate constants for the metalloprotein and its redox partner are k_{11} and k_{22}, respectively, the cross reaction rate constant is k_{12}, and the cell potential for the redox reaction is ΔE^0. Table IV provides a comparison of k_{11} values calculated for stellacyanin, plastocyanin, and azurin on the basis of $Fe(EDTA)^{2-}$ reduction and $Co(phen)_3^{3+}$ oxidation rate constants.

It is immediately evident from the data in Table IV that the k_{11} estimates based on $Fe(EDTA)^{2-}$ and $Co(phen)_3^{3+}$ cross reactions agree closely in the case of stellacyanin, but differ by four and six orders of magnitude for plastocyanin and azurin, respectively. The former observation is consistent with the hypothesis that the type 1 copper atom in stellacyanin is readily accessible in aqueous solution to outer sphere contact with external redox agents and employs essentially similar mechanisms in donating and accepting an electron. As little in the way of protein conformational change appears to be

required for electron transfer in the case of stellacyanin, we expect the calculated k_{11} value of about 10^6 M^{-1} s^{-1} based on $Fe(EDTA)^{2-}$ to accord with the actual protein self-exchange rate constant, although no experimental measurement is available to test this point.

The wide variation in calculated k_{11} values for plastocyanin and azurin based on the two redox agents is entirely consistent with the view that the copper center in each of these proteins is buried.[37] It is apparent that $Fe(EDTA)^{2-}$ has particularly poor access to the blue copper in these proteins and is forced to employ electron transfer mechanisms characterized by substantial nonadiabaticity. The relatively large calculated k_{11} values for plastocyanin and azurin based on $Co(phen)_3^{3+}$, however, accord with our earlier proposal that this redox agent evidently is able to gain intimate contact with the type 1 Cu(I) site, presumably by a mechanism involving induced protein conformational change.

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