donors are imine nitrogens and was held constant at 0.0388 cm^{-1} for each complex. This assumption is based upon crystallographic evidence that the bond lengths remain relatively constant throughout the series. Thus, taking the sign of the hyperfine coupling constant to be negative,[21] the calculated Fermi contact parameters are as follows: [Cu(TC-3,3)], 0.369; [Cu(TC-4,4)], 0.322; [Cu(TC-4,5)], 0.285; [Cu(TC-5,5)], 0.264. From these values α^2 was calculated by eq 5 to be ~0.6. It is therefore apparent that the major factor involved in the attentuation of $A_\parallel$ in the Cu(II) tropocoronands is the Fermi contact interaction.

Ligand superhyperfine splitting is observed in the parallel and perpendicular region of the ESR spectrum of [Cu(TC-3,3)] and in the perpendicular region for the remaining complexes. In all cases, there are more than the expected nine (^{14}N ($I = 1$), 1), $2NI + 1$) ligand superhyperfine lines on $g_\perp$, possibly due to rhombic character in the spectra or to superhyperfine coupling from the hydrogen nuclei of the tropocoronand ligands.[23] Nine lines are observed in the parallel region of the [Cu(TC-3,3)] spectrum. The values of the superhyperfine coupling constants in all of the spectra fall in the 10–15-G range consistent with literature values for copper(II)–nitrogen donor ligand interactions.

Magnetic Susceptibility Measurements. The effective magnetic moments of the Cu(II) tropocoronands are independent of the extent of tetrahedral distortion. Since tetrahedral, d^9 metal complexes have a $^2T_2$ ground state, spin–orbit coupling is expected to contribute to the magnetic susceptibilities of such complexes, raising the value of the effective magnetic moments. The observed magnetic moments of the Cu(II) tropocoronand complexes, however, display little deviation from the spin-only value of 1.732 μ_B. This observation indicates that, although θ is as large as 61.3°, the orbital contribution to the magnetic moments is efficiently quenched by the ligands.

Concluding Remarks. The synthesis and structural characterization of the four mononuclear copper(II) tropocoronands has sharpened our understanding of the steric forces transmitted to the metal center. By packing an increasing number of methylene groups into the linker chains, the ligands distort the Cu(II) coordination geometry from planar toward tetrahedral along a D_2 (vs. a D_{2d}) symmetry path,[24] considering only the CuN_4 atom set. Unlike the corresponding nickel(II) tropocoronands,[2] this geometric transition is a smooth one and produces a corresponding set of changes in the optical and ESR spectroscopic properties of the copper(II) complexes. When the number of linker chain atoms becomes 6 or larger, the tropocoronand ligands become binucleating. The synthesis and properties of these interesting binuclear tropocoronand complexes are the subjects of separate reports.[13]

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Supplementary Material Available: Tables S1–S19, reporting heavy-atom thermal parameters, hydrogen atom positional and thermal parameters, bond lengths and angles, torsional angles, observed and calculated structure factors, and magnetic susceptibility data for the copper(II) tropocoronands (37 pages). Ordering information is given on any current masthead page.

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Contribution from the Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973, and Contribution No. 7144 from the Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena, California 91125

Kinetics and Mechanisms of Electron Transfer between Blue Copper Proteins and Electronically Excited Chromium and Ruthenium Polypyridine Complexes

BRUCE S. BRUNSCHWIG,[1] PATRICIA J. DeLAIVE,[2] ANN M. ENGLISH,[2,3] MICHEL GOLDBERG,[2] HARRY B. GRAY,*[2] STEPHEN L. MAYO,[2] and NORMAN SUTIN*[1]

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The kinetics of the quenching of the long-lived excited states of CrL_3^{3+} and RuL_3^{2+} complexes (L is 1,10-phenanthroline and 2,2′-bipyridine or substituted derivatives) by the copper proteins plastocyanin, azurin, and stellacyanin have been studied in aqueous solution. The rate constants for quenching by the Cu(I) proteins approach a limiting value of $\sim 10^6$ s^{-1} at high protein concentration. The kinetic behavior for plastocyanin is discussed in terms of a model in which the metal complex binds at a remote site 10–12 Å from the copper center. The model allows for electron transfer both from this remote site and by attack of the metal complex adjacent to the copper center. The results show that at low protein concentration the adjacent pathway is about 10 times faster than the remote pathway. The rate constant for the intramolecular electron transfer from the remote site is consistent with the value expected on the basis of theoretical calculations.

Introduction

The use of inorganic complexes to study the electron-transfer reactions of metalloproteins has been exploited in a number of laboratories.[4–7] In recent years increasing use has been made of electronically excited complexes.[8,9] The use of an oxidant (or reductant) that can be "instantaneously" generated by a laser pulse

(1) Brookhaven National Laboratory.
(2) California Institute of Technology.
(3) Present address: Department of Chemistry, Concordia University, Montreal, Quebec, Canada H3G 1M8.
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affords an opportunity for the study of reactions that are too fast to be observed with conventional mixing techniques. In addition, since the excited state can be a powerful oxidant or reductant, the electron-transfer kinetics can be studied under highly exergonic conditions. An early application[8,9] of this technique exploited the properties of tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$), which is both a very good reductant ($E° = -0.84$ V) and a moderately strong oxidant ($E° = +0.84$ V) in its excited state. Additional studies with the ruthenium(II) complex, as well as with tris(1,10-phenanthroline)chromium(III) ($Cr(phen)_3^{3+}$), which is a very strong oxidant in its excited state ($E° = +1.42$ V),[10] and with other CrL_3^{3+} complexes are described here.

Discussions of the kinetics of reactions between oxidation–reduction metalloproteins and inorganic redox agents often have centered on the role played by encounter or precursor complexes formed from the separated reactants.[5,6] Some studies suggest that the associative interaction between the protein (e.g., blue copper proteins, iron sulfur proteins, *c*-type cytochromes) and its redox partner is quite strong, with association constants up to 10^4 M^{-1}.[6] On the other hand, the results of the other studies[5] can be analyzed in terms of a bimolecular model requiring nothing more than a weak binding and reasonably close approach of the inorganic redox agent to the protein redox site.

The blue copper proteins plastocyanin, azurin, and stellacyanin studied in this work contain one copper atom per molecule. Plastocyanin and azurin function in the electron-transport chain of biological organisms.[11] The function of stellacyanin is unknown. In their oxidized (Cu(II)) state the copper proteins have an intense absorption in the visible region (~600 nm) and are colorless when the copper center is reduced (Cu(I)). The crystal structures of plastocyanin[12] (*Populus nigra, var. italacia*) and *Pseudomonas* azurin[13] have been determined and their molecular structures compared.[14] In general the copper atom is located at one end of the protein in a hydrophobic pocket formed by three loops in the polypeptide backbone. The residues near the pocket are highly evolutionarily conserved.[14] The copper is coordinated by two nitrogens (histidines) and two sulfurs (methionine and cysteine). The copper(II)–ligand bond lengths are ~2.1 Å except for Cu–S(Met), which is 2.9 Å.[15] The Cu(I) bond lengths for plastocyanin determined by X-ray crystallography depend on the pH of the solution from which the crystals were prepared. The variations in distance are interpreted as indicating two forms of the Cu(I) protein. The form that is assumed to be redox active has copper–nitrogen bond distances that are ~0.1 Å longer for the Cu(I) than for the Cu(II) protein. The low-pH (redox-inactive) form of the Cu(I) protein has one long copper–nitrogen bond (3.2 Å) and a short copper–sulfur bond (~2.5 Å).[15] Plastocyanin has a negative, acidic patch and a hydrophobic region on one side of the protein that is conserved and located 10–15 Å from the Cu atom. Azurin has no similar negative patch, but the same region has a conserved hydrophobic region.[15]

The tyrosine-83 region of plastocyanin has been shown to be a binding site for $Cr(phen)_3^{3+}$,[16,17] and a purpose of the present study was to determine whether this binding site opens up a pathway for (long-range) electron transfer from the excited phenanthroline or bipyridine complexes to the copper center, as has been proposed for the analogous $Co(phen)_3^{3+}$ reaction.[16] This pathway will be called the remote pathway in order to distinguish it from the adjacent pathway, which involves "direct" electron transfer at the copper site.

Experimental Section

Preparation and Purification of Materials. Plastocyanin (*Phaseolus vulgaris*) was isolated from the leaves of 6-week-old French bean plants and purified (absorbance ratio $A_{278}/A_{597} = 1.0$) by methods described by Milne and Wells;[18] azurin (*Pseudomonas aeruginosa*) was purified ($A_{280}/A_{625} = 1.9$) from the acetone extract by the method of Ambler,[19] and Japanese lacquer tree (*Rhus vernicifera*) stellacyanin was isolated ($A_{280}/A_{604} = 5.8$) from the acetone extract by the method of Reinhammar.[20] Commercial tris(2,2′-bipyridine)ruthenium(II) chloride was used as received from G. Frederick Smith Chemical Co. Tris(1,10-phenanthroline)chromium(III) perchlorate, tris(5-chloro-1,10-phenanthroline)chromium(III) perchlorate, and tris(4,7-dimethyl-1,10-phenanthroline)chromium(III) perchlorate were prepared according to literature methods.[21]

Preparation of Samples. All protein experiments were performed with use of phosphate buffer at pH 7.0 (Na_2HPO_4, 27 mM; NaH_2PO_4, 20 mM) and an ionic strength of 0.1 M in deionized water (conductivity $<7 \times 10^{-8}$ cm Ω^{-1}). The blue copper proteins were reduced by either of two methods; chemical reduction by sodium ascorbate followed by a 4-h dialysis against phosphate buffer to ensure that little or no excess ascorbate or dehydroascorbate was present in the final solutions or hydrogen reduction over platinum metal. These two procedures were performed under nitrogen or argon flushing to prevent air oxidation of the proteins. Stock solutions of the inorganic reagent were deaerated by nitrogen (or argon) flushing for at least 30 min prior to sample preparation in an anaerobic environment.

Spectral Measurements. All absorption spectra were recorded on a Cary 219 UV–visible recording spectrophotometer. Concentrations of the proteins in the final solutions were determined spectrophotochemically with use of the following data: plastocyanin $\lambda_{max} = 597$ nm ($\epsilon = 4500$ M^{-1} cm^{-1}),[18] azurin $\lambda_{max} = 625$ nm ($\epsilon = 5700$ M^{-1} cm^{-1}),[6e] and stellacyanin $\lambda_{max} = 604$ nm ($\epsilon = 4080$ M^{-1} cm^{-1}).[20] Emission spectra were recorded on a Perkin-Elmer Hitachi MPF-4 spectrofluorimeter.

Photochemical Experiments. The transient emission and absorption measurements were made with either a Phase-R D-1100 dye laser system (fwhm 0.5 μs) or Quanta Ray DC-R Nd:YAG (fwhm 8 ns) laser. In the former case the active dye was coumarin 440 with an output at ~440 nm. For absorption measurements the probe beam source (positioned at 90° to the laser beam) was a 150-W tungsten–halogen lamp filtered to remove light at wavelengths shorter than the monitoring wavelength. The probe beam (or the emission) passed through a Jarrell-Ash monochromator and was detected by a photomultiplier tube and preamplifier. The signal from the preamplifier was digitized and stored in a Biomation 8100 transient recorder. The data were then transferred to a PDP-11/23 computer, where the 2000 data points were averaged in sets of 10 (converted to change in absorbance when necessary) and fit by a nonlinear least-squares fitting routine to a first-order decay. For the Nd laser the emission was detected by a photomultiplier tube and digitized by a Biomation 6500. Thirty to sixty curves were summed to get the decay curve that was then analyzed on a MINC-11/03 computer. The measurements were performed at room temperature unless otherwise noted. The $Cr(phen)_3^{3+}$ emission was monitored at 730 nm and excited either at 440 nm or with the third harmonic of the Nd laser (353 nm). The $Ru(bpy)_3^{2+}$ was excited with the second harmonic of the Nd laser (532 nm) and the emission observed at 610 nm. The metal complex concentration was 2.5×10^{-5} M for $Ru(bpy)_3^{2+}$ and 3×10^{-5} M for $Cr(phen)_3^{3+}$; the protein concentration was varied from zero to 5×10^{-3} M.

The intensity of the emission from the polypyridine chromium(III) complexes was also measured as a function of protein concentration ($<10^{-4}$ M) on a spectrofluorimeter. Solutions of the protein and the chromium complex were excited at 350 nm, and the emission was monitored at the emission maximum of the complex (720–730 nm). Plots

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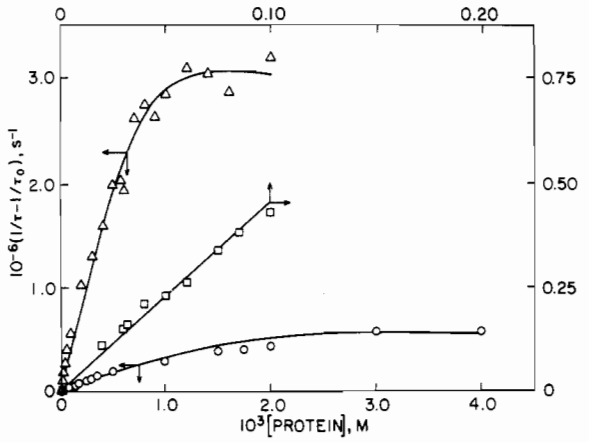


Figure 1. Plot of $1/\tau - 1/\tau_0$ vs. protein concentration for the reaction of reduced plastocyanin (Δ), azurin (□), and stellacyanin (○) with the excited state of $Cr(phen)_3^{3+}$ at pH 7.0, 0.1 M ionic strength, and 25 °C.

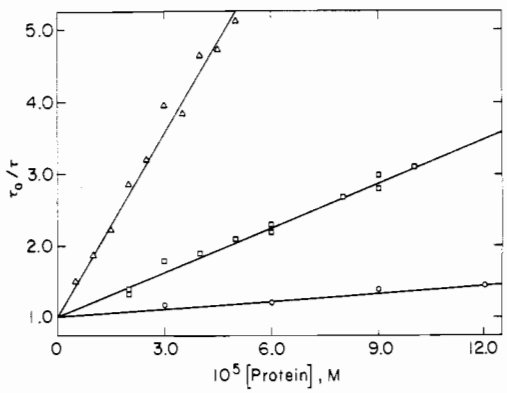


Figure 2. Plot of τ_0/τ vs. protein concentration for the reaction of oxidized plastocyanin (Δ), azurin (□), and stellacyanin (○) with the excited state of $Cr(phen)_3^{3+}$ at pH 7.0, 0.1 M ionic strength, and 25 °C.

Table I. Rate Constants for Reaction of the Oxidized Blue Copper Proteins with $*Cr(phen)_3^{3+}$, $Cr(phen)_3^{2+}$, and $*Ru(bpy)_3^{2+}$ at pH 7.0, 0.1 M Ionic Strength, and 25 °C

protein	sensitizer	$\Delta E°$, V	$10^{-8}k_q$, M^{-1} s^{-1}
plastocyanin(II)	$*Cr(phen)_3^{3+}$		6.0
	$Cr(phen)_3^{2+}$	0.64	23
	$*Ru(bpy)_3^{2+}$	1.20	42
azurin(II)	$*Cr(phen)_3^{3+}$		1.0
	$*Ru(bpy)_3^{2+}$	1.14	12
stellacyanin(II)	$*Cr(phen)_3^{3+}$		0.49
	$*Ru(bpy)_3^{2+}$	1.03	13

of I_0/I vs. protein concentration gave values of $k_q\tau_0$, where k_q is the quenching rate constant and τ_0 is the lifetime of the excited state in the absence of the metalloprotein.

Results

Quenching of the CrL_3^{3+} Excited State. Rate parameters ($1/\tau$) for quenching of the $Cr(phen)_3^{3+}$ emission by plastocyanin, azurin, and stellacyanin are presented in Supplementary Tables 1–3 and are plotted vs. protein concentration in Figure 1 ($1/\tau - 1/\tau_0$) for the reduced metalloproteins and in Figure 2 (τ_0/τ) for the oxidized metalloproteins. The quenching at low protein concentration was also studied by steady-state emission methods with similar results. The plots for the quenching by the reduced protein (Figure 1) were all similar with a "normal" region at low protein concentration, where $1/\tau - 1/\tau_0$ is proportional to the protein concentration, and a "plateau" region at higher concentration, where $1/\tau - 1/\tau_0$ is independent of protein concentration.[22] The Stern–Volmer plots for the quenching by the oxidized protein were all normal, with no departure from linearity up to 0.1 mM protein. The quenching rate constants are given in Table I. The effect of driving force on the rate constant for the quenching of the chromium(III) excited state by reduced azurin was studied by use of $Cr(5\text{-}Clphen)_3^{3+}$ ($E° = +1.53$ V)[21] or $Cr(4,7\text{-}(CH_3)_2phen)_3^{3+}$ ($E° = +1.23$ V)[21] instead of $Cr(phen)_3^{3+}$. The rate constants for these reactions agreed to within 10% with those found with $Cr(phen)_3^{3+}$.

The spectrum of a solution containing $Cr(phen)_3^{3+}$ and reduced plastocyanin, measured ~1 μs after the laser flash at high protein concentration, revealed approximately uniform bleaching from 500 to 700 nm. This is expected for production of Cu(II) protein and $Cr(phen)_3^{2+}$.[23] The rate constant for the back-reaction between the oxidized metalloprotein and $Cr(phen)_3^{2+}$ is 2.3×10^9 M^{-1} s^{-1} and is independent of monitoring wavelengths between 500 and 650 nm. The second-order fit of the bleaching at 597 nm is shown in Figure 3.

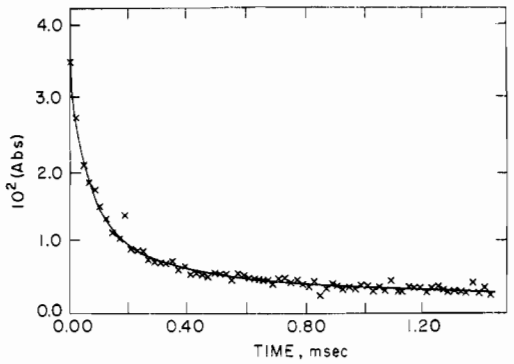


Figure 3. Decay of the transient absorption at 597 nm in a solution of reduced plastocyanin (2×10^{-3} M) and $Cr(phen)_3^{3+}$ (5×10^{-4} M). The solid line is the fit of the data points (×) to a second-order plot (pH 7.0, 0.1 M ionic strength, and 25 °C).

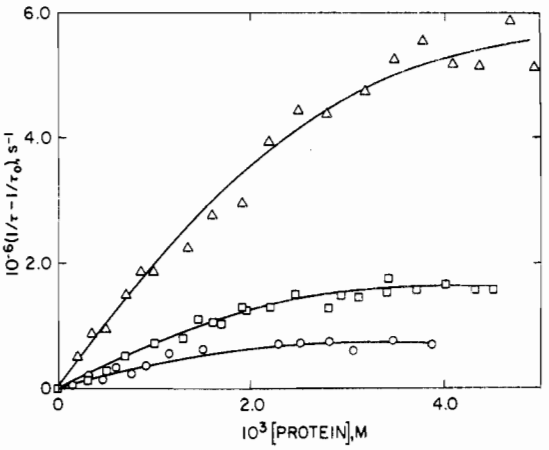


Figure 4. Plot of $1/\tau - 1/\tau_0$ vs. protein concentration for the reaction of reduced plastocyanin (Δ), azurin (□), and stellacyanin (○) with the excited state of $Ru(bpy)_3^{2+}$ at pH 7.0, 0.1 M ionic strength, and 25 °C.

Quenching of the $Ru(bpy)_3^{2+}$ Excited State. The $1/\tau - 1/\tau_0$ vs. protein concentration plots for the quenching of the $Ru(bpy)_3^{2+}$ emission by the three reduced copper proteins are shown in Figure 4. The plots are qualitatively similar to those for quenching of $Cr(phen)_3^{3+}$ emission and show a linear region at low metalloprotein concentrations and a plateau at higher concentrations. The results at low protein concentrations were reported previously.[9] The absorption spectra taken 5.0–160 μs after the laser flash for a solution of $Ru(bpy)_3^{2+}$ and reduced plastocyanin show the ab-

(22) (a) The data for azurin(I) quenching of the $Cr(phen)_3^{3+}$ excited state do not show limiting behavior for [Az(I)] ≤ 0.1 mM; however, data under slightly different conditions show the plateau region beginning at [Az(I)] ≈ 2 mM.[22b] Therefore, all the data for quenching by the reduced protein were treated in the same manner. (b) English, A.; DeLaive, P.; Gray, H. B., unpublished results.

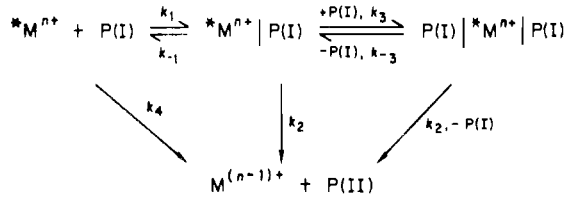
(23) (a) The production of equal concentrations of $Cr(phen)_3^{2+}$ and Cu(II) plastocyanin would produce a fairly uniform change in absorbance in the region monitored since $Cr(phen)_3^{2+}$ has an absorption maximum at 700 nm ($\epsilon \approx 2500$ M^{-1} cm^{-1}).[23b] (b) Serpone, N.; Jamieson, M. A.; Emmi, S. S.; Fouchi, P. G.; Mulazzani, Q. G.; Hoffman, M. Z. *J. Am. Chem. Soc.* **1981**, *103*, 1091.

Table II. Quenching of $*Cr(phen)_3^{3+}$ and $*Ru(bpy)_3^{2+}$ Emission by Blue Copper(I) Proteins at 22 °C

protein	complex	$-\Delta G°$,[a] V	$10^{-9}(k_1$ or $k_4)$,[b] M^{-1} s^{-1}	K_1, M^{-1}	$10^{-6}k_2$, s^{-1}	$10^{-3}K_3$, M^{-1}	k_{adj}/k_{rem}[c]
plastocyanin(I)	CrL_3^{3+}	1.06	3.5	250	2.5	4.2	7
plastocyanin(I)	RuL_3^{2+}	0.48	1.9	100	3.0	0.2	7
azurin(I)	CrL_3^{3+}	1.11	0.40	60	1.2[d]		7
azurin(I)	RuL_3^{2+}	0.53	0.65	40	1.2	2.3	14
stellacyanin(I)	CrL_3^{3+}	1.23	0.35	60	0.20	1.3	30
stellacyanin(I)	RuL_3^{2+}	0.65	0.40	40	0.20	1.4	51

[a] References 10, 21, and 37a. [b] The rate constants were calculated by fitting the data to eq 1 with $k_1 = k_4$ and K_1 ($=k_1/k_{-1}$) equal to the values given in the table. [c] The ratio of the adjacent to remote paths at low protein concentration calculated from $k_4(k_{-1} + k_2)/k_1k_2$. [d] Calculated from the initial slope of $1/\tau$ vs. protein concentration with the values of the other parameters given in the table.

Scheme I



sorbance changes expected for electron-transfer products. The spectrum shows a bleach at 430–450 nm due to the loss of $Ru(bpy)_3^{2+}$ and absorbance increases at 470–530 and 550–700 nm due to the formation of $Ru(bpy)_3^{+}$ and oxidized plastocyanin.[18,24]

The quenching of the $Ru(bpy)_3^{2+}$ emission by the oxidized copper proteins gave normal τ_0/τ plots up to 5 mM protein. No indication of rate saturation was observed. The quenching rate constants are included in Table I. Control experiments showed that the $Ru(bpy)_3^{2+}$ emission was not quenched by the apoproteins.

Discussion

It is necessary to consider energy-transfer as well as electron-transfer pathways for the quenching of the excited states of the metal complexes. Oxidation of the copper(II) proteins by the excited state of $Cr(phen)_3^{3+}$ is thermodynamically unfavorable; however, there is significant overlap between the proteins' absorption spectra and the chromium(III) emission and the observed quenching very probably proceeds by an energy-transfer pathway. By contrast, the quenching of the emission of the excited chromium(III) and ruthenium(II) complexes by the reduced metalloprotein can be ascribed to an electron-transfer mechanism since the transient spectra identify the reduced complex and the oxidized protein as the quenching products. An energy-transfer mechanism is not likely for these systems because there is no overlap between the absorption spectrum of the reduced protein (the reduced protein has no absorption between 400 and 800 nm) and the emission of the excited metal complex ($Cr(phen)_3^{3+}$, λ_{em} = 727 nm; $Ru(bpy)_3^{2+}$, λ_{em} = 607 nm).

We next consider adjacent and remote pathways for the electron-transfer quenching. These are shown in Scheme I, which also includes a 2:1 complex that may be formed at high protein concentrations. In this scheme $*M^{n+}|P(I)$ is a complex in which the excited inorganic complex $*M^{n+}$ is bound at a remote site from the copper. The formation constant of the 1:1 complex is K_1 ($=k_1/k_{-1}$), the formation constant of the 2:1 complex (formed between two reduced protein molecules and the inorganic complex) is K_3 ($=k_3/k_{-3}$), k_2 is the intramolecular (remote) electron-transfer rate constant, and k_4 is the bimolecular electron-transfer rate constant for adjacent attack, that is, for the pathway that involves close approach of $*M^{n+}$ to the copper(I) site. We assume that the ground and excited states of the inorganic complex bind with the same equilibrium constant to the metalloprotein and that the bound inorganic complexes have the same intrinsic lifetimes (τ_0) as the free complexes. The various equilibria between the proteins and the ground-state metal complexes are established prior to the laser flash. These equilibria are, of course, still maintained immediately after the excitation of the metal complex.

The above kinetic scheme is intrinsically multiphasic. In order to simplify the resulting kinetic expressions, we assume that the 2:1 complex is in equilibrium with the 1:1 complex. Under these conditions the differential equations for the above scheme can be solved to yield two (first-order) rate constants given by[25]

$$\theta_{1(2)} = \frac{S_1 + S_2}{2} + (-)\left[\frac{(S_1 - S_2)^2}{4} + \frac{k_1k_{-1}}{1 + K_3[P(I)]}\right]^{1/2} \quad (1)$$

where $\theta = 1/\tau - 1/\tau_0$, $S_1 = (k_1 + k_4)[P(I)]$, and $S_2 = k_2 + k_{-1}/(1 + K_3[P(I)])$. Trial values of the various parameters indicate that the negative root (θ_2) is the appropriate one under the conditions used. The low- and high-[protein] limits of this root are of interest: in the low-[protein] limit the value of θ_2 is given by

$$\theta_2 = k_4[P(I)] + \frac{k_1k_2[P(I)]}{k_{-1} + k_2} \quad (2)$$

and the quenching rate constant is directly proportional to the protein concentration. At high protein concentrations θ_2 is given by

$$\theta_2 = k_2 \quad (3)$$

and the rate constant is independent of protein concentration. In the intermediate region the rates have a complex dependence on protein concentration.

The first term on the right-hand side of eq 2 is the rate constant for adjacent attack while the second term is the effective rate constant for the remote electron-transfer pathway. If $k_4 \gg k_1k_2/(k_{-1} + k_2)$, then the adjacent-attack pathway predominates with the remote pathway being relatively slow.[26] On the other hand, if $k_1k_2/(k_{-1} + k_2) \gg k_4$, then the remote electron-transfer path predominates. At high protein concentration only the remote pathway is available for reaction (eq 3).

Not all five constants (k_1, K_1, k_2, K_3, and k_4) can be calculated from the four parameters in eq 1 without introducing additional assumptions. Unconstrained fits of the data indicated that k_4 is very large, and we shall assume that $k_4 = k_1$: this assumption seems reasonable since k_1 is a rate constant for diffusion and, because of the large driving force for the electron transfer and the high probability of electron transfer at the adjacent site, it is likely that k_4 is also diffusion controlled. In addition, we assume that K_1 is equal to 250 and 100 M^{-1} for the association of reduced plastocyanin with $Cr(phen)_3^{3+}$ and $Ru(bpy)_3^{2+}$, respectively, and

(24) Creutz, C.; Sutin, N. *J. Am. Chem. Soc.* **1976**, *98*, 6384.

(25) Bamford, C. H.; Tipper, C. F. H. "Comprehensive Chemical Kinetics"; Elsevier: New York, 1969; Vol. 2, p 31. Rybak, W.; Haim, A.; Netzel, T. L.; Sutin, N. *J. Phys. Chem.* **1981**, *85*, 2856.

(26) The limiting form of this case is the so-called "dead-end" mechanism. In fact, it may be advantageous to refer to it as an unproductive binding mechanism. Because the protein is present in excess, "dead-end" refers to the activity of the oxidant, not of the protein. It would be erroneous to assume that this mechanism necessarily means that $Cr(phen)_3^{3+}$ or $Ru(bpy)_3^{2+}$ inactivates the protein redox site. Nor does it imply photophysical or chemical inactivation of the redox agent by the protein. We only postulate that in this mechanism the oxidant enters a cul-de-sac; i.e., it is bound at a site sufficiently remote from the Cu center so that intramolecular electron transfer is slower than that along an alternative (bimolecular) pathway, which presumably involves encounter of $*M^{n+}$ very near the copper center (adjacent attack). At high protein concentrations, under saturation conditions, all the $*Cr(phen)_3^{3+}$ or $*Ru(bpy)_3^{2+}$ is bound at the remote site. Provided that the intramolecular electron-transfer rate is significantly slower than the effective dissociation rate, reaction takes place only when the oxidant dissociates from the remote site and then follows the fast bimolecular pathway.

to 40–60 M^{-1} for the other systems. The value assumed for the association constant for reduced plastocyanin with $Cr(phen)_3^{3+}$, while slightly larger than the value reported by Sykes et al.,[27] is more consistent with the present data.[28a] The larger values of K_1 for the plastocyanin reaction are consistent with the more negative charge on reduced plastocyanin (−10)[5d,29] compared with those on azurin (−2)[5d] and stellacyanin (0).[5d] These values are also consistent with the results of kinetic and binding studies that are described below.[6a,b,16,27,29]

The values of k_1, K_1, k_2, K_3, and k_4 for the $Cr(phen)_3^{3+}$ and $Ru(bpy)_3^{2+}$ data determined for Scheme I subject to the above constraints are given in Table II. The curves calculated with these constants are shown in Figures 1 and 2. The values of the quenching rate constants in the low-[protein] limit (eq 2) calculated by using the constants in Table II are within 25% of their measured values. The value of K_3 is very sensitive to the curvature in the $1/\tau - 1/\tau_0$ vs. protein concentration plots, and because of the scatter in the high-[protein] data, K_3 is the least well-determined parameter. The k_1 values seem consistent with diffusion-controlled rates when the steric factors for the reaction are considered. The larger values of k_1 for the plastocyanin reactions are expected on the basis of the larger charge products for these reactions (−30 and −20 for the $Cr(phen)_3^{3+}$ and $Ru(bpy)_3^{2+}$ reactions, respectively, compared with −6 and −4 for the azurin and zero for the stellacyanin reactions).[5d,29] Moreover, the k_1 value of 2×10^9 M^{-1} s^{-1} for the reaction of $*Ru(bpy)_3^{2+}$ with reduced plastocyanin is in excellent agreement with the value directly measured for the reaction of $Cr(phen)_3^{2+}$ with the oxidized protein (the reactants have similar charge products). Although the k_2 values are large, at low protein concentration the remote electron-transfer path is about 1 order of magnitude slower than the adjacent path, i.e., $k_1k_2/(k_{-1} + k_2) < k_4$ for the systems studied (Table II); this is a consequence of the fact that k_4 is comparable to k_1 while $k_2 \ll k_{-1}$.

The association of a second protein molecule renders more abrupt the transition from a roughly linear concentration dependence of the quenching lifetime to an almost concentration-independent region. Saturation is accelerated because the effective dissociation rate constant of the 1:1 complex is lowered by the binding of the additional protein molecule, $k'_{-1} = k_{-1}/(1 + K_3[P(I)])$. Note that a rate effect is only observed because the formation of a dimeric species is affected by the presence of the bound oxidant. If a protein dimer were formed in the absence of a redox partner, but with the same equilibrium constant (K_3), there would be no modulation effect. We conclude that the oxidant is somehow "sandwiched" between two protein molecules. It is also worth noting that the association of the second P(I) exhibits positive cooperativity for all proteins ($K_3 > K_1$).

Sykes et al.[6a,b,16,27] have reported kinetics for the oxidation of reduced plastocyanin and azurin by $Co(phen)_3^{3+}$. Their plots of the observed first-order rate constant vs. cobalt(III) concentration at pH 7.5 exhibit slight downward curvature for plastocyanin but none for azurin. They interpret this curvature in terms of complex formation at the remote site and derive $K_1 = 170$ and ≤ 40 M^{-1} for plastocyanin and azurin, respectively (ionic strength 0.1 M, pH 7.5).[28a] They also studied[27] the $Cr(phen)_3^{3+}$ inhibition of the $Co(phen)_3^{3+}$ oxidation of reduced plastocyanin and found $K_1 \approx 180$ M^{-1} for binding of the chromium(III) complex at pH 7.5. NMR studies clearly show that $Cr(phen)_3^{3+}$ binds to plastocyanin at or near Tyr-83, which is in the vicinity of the negative patch formed by the carboxylate groups of residues 42–45 (and residues 51 and 59).[16,17] This region is 10–15 Å from the copper site. Because $Co(phen)_3^{3+}$ is structurally very similar to $Cr(phen)_3^{3+}$ and because its reaction with reduced plastocyanin is blocked by the latter, $Co(phen)_3^{3+}$ almost certainly binds at the same site. This is consistent with the exothermic character of the precursor-complex formation ($\Delta H^\circ \simeq 10$ kcal mol^{-1}),[6b] which indicates that electrostatic factors dominate the interaction of $Co(phen)_3^{3+}$ with the reduced plastocyanin. It is also proposed that $Ru(bpy)_3^{2+}$ binds in the region of Tyr-83. Its association constant, though not known exactly, is certainly smaller than that for the phenanthroline complexes. Moreover, the oxidation of reduced plastocyanin by $Co(phen)_3^{3+}$ is inhibited by $Co(NH_3)_6^{3+}$, $Pt(NH_3)_6^{4+}$, and $(Co(NH_3)_5)_2NH_2^{5+}$, suggesting that all these complexes associate in the same region.[6c] Further support for the above interpretation is provided by the k_2 values. Since the magnitude of k_2 is governed primarily by the distance from the copper center to the binding site (see below), the similarity in the k_2 values for $Cr(phen)_3^{3+}$ and $Ru(bpy)_3^{2+}$ would indicate that both metal complexes bind at similar positions for a given protein.

The association constants of reduced plastocyanin with $Co(NH_3)_6^{3+}$, $Pt(NH_3)_6^{4+}$, and $(Co(NH_3)_5)_2NH_2^{5+}$ are large.[6c] However, the rate decreases observed even at high inhibitor concentration are only about twofold. The fact that the blocking is only partial suggests that more than one pathway is effective in the $Co(phen)_3^{3+}$ oxidation. The blocking agent can totally inhibit only the remote pathway by binding to the protein: the adjacent pathway can operate even when the blocking agent is present provided that the binding is at the remote site. The ratios of rate constants for the adjacent and remote pathways are significantly different for the $Co(phen)_3^{3+}$ and $*Cr(phen)_3^{3+}$ oxidations, ≤ 1 and ~ 7, respectively. For the $Co(phen)_3^{3+}$ reaction neither pathway has a rate constant close to the diffusion limit and the rate ratio reflects the relative association constants at the adjacent and remote sites, as well as the rate difference arising from the difference in distance between the metal complex and the copper center for the two paths. On the other hand, for the $Cr(phen)_3^{3+}$ reaction the adjacent pathway is diffusion-controlled and the rate ratio reflects only the relative magnitudes of k_{-1} and k_2.[28b]

Table III. Distances in the Computer-Generated Complex Formed between Plastocyanin and $Cr(phen)_3^{3+}$

	dist, Å
cysteine-84 S to nearest phen C	10.3
Cu center to nearest phen C	12.7
Cu to Cr	18.4
tyrosine-83 *o*-C to Cr	8.0, 8.7
tyrosine-83 *m*-C to Cr	9.4, 10.0
valine-40 Me C to Cr	8.5, 11.5

Binding Site for $Cr(phen)_3^{3+}$. As discussed in the Introduction, NMR studies of reduced plastocyanin have shown that the proton resonances of tyrosine-83 (as well as the methyl group resonances of an aliphatic side chain, possibly valine-40) are broadened by added $Cr(phen)_3^{3+}$. The former residue affords a particularly attractive binding site because of the possibility of hydrophobic interactions between the phenyl ring of the tyrosine-83 and a phenanthroline ring of the chromium complex.[30] In order to further examine this possibility, computer simulations of the docking of $Cr(phen)_3^{3+}$ with the tyrosine region of plastocyanin were performed.[31] The best-fit docked complex, obtained by

(27) Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 2302. Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 2297.

(28) (a) It should be recognized that parallel adjacent and remote pathways may also operate in the $Co(phen)_3^{3+}$ reaction. A least-squares analysis of the published data[6] in terms of the corresponding rate law gave an excellent fit with the $K_1 = 220 \pm 40$ M^{-1}. (b) For the $Co(phen)_3^{3+}$ oxidation $k_{adj}/k_{rem} \approx (K_{adj}/K_{rem}) \exp(-\beta(d_{adj} - d_{rem}))$, where we assume that ΔG^* is the same for the two pathways, $K_{rem} = K_1$, and K_{adj} is the association constant for the adjacent pathway. For the $*Cr(phen)_3^{3+}$ oxidation $k_{adj}/k_{rem} \approx 1 + k_{-1}/k_2$; see eq 4.

(29) Augustin, M. A.; Chapman, S. K.; Davies, D. M.; Watson, A. D.; Sykes, A. G. *J. Inorg. Biochem.* **1984**, *20*, 281.

(30) (a) Farver and Pecht have presented evidence that Cr^{2+} binds to oxidized plastocyanin at a residue near Tyr-83 prior to electron transfer.[30b] (b) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4190.

(31) (a) Computer graphics were done with the program BIOGRAF/1 (written by S. L. Mayo, B. D. Olafson, and W. A. Goddard, III) and a VAX 11/780-supported Evans and Sutherland P5300 interactive graphics terminal. The metal complex used as a structural model was $Co(phen)_3^{3+}$ (Niederhoffer, E. C.; Martell, A. E.; Rudolf, P.; Clearfield, A. *Cryst. Struct. Commun.* **1982**, *11*, 1951). (b) The surfaces of the protein and of the metal complex were calculated by using the program MS: Connolly, M. *Quant. Chem. Program Exchange Bull.* **1981**, *1*, 75.

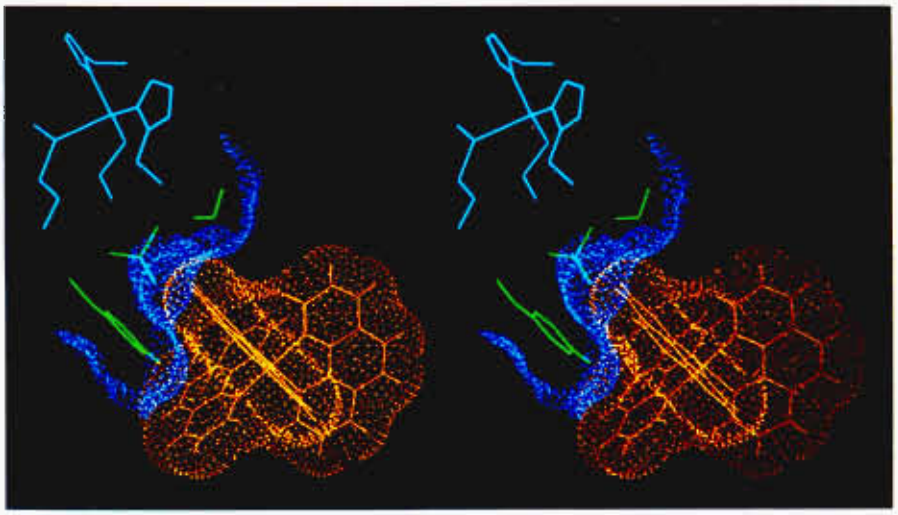


FIG 5

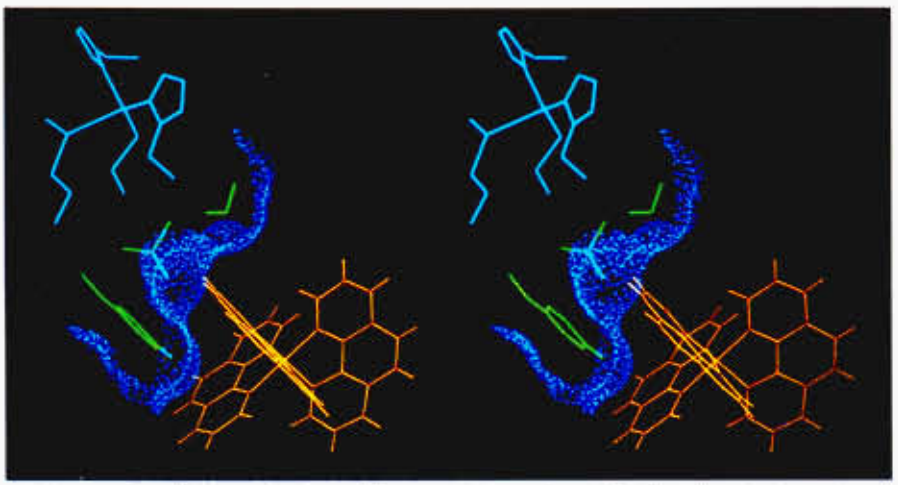


Figure 5. Computer-generated stereoviews of the complex formed between plastocyanin and $Cr(phen)_3^{3+}$. The lower view shows the copper atom with its four ligands (light blue), three nearby residues, valine-40, serine-85, tyrosine-83 (green), the docked $Cr(phen)_3^{3+}$ (orange), and the solvent-accessible surface (blue) of the atoms near tyrosine-83. The upper view also shows the van der Waals surface of $Cr(phen)_3^{3+}$ (orange).

rotation of the phenyl ring of the tyrosine, is shown in Figure 5. It is evident that very good overlap between the phenyl ring and a phenanthroline ligand of the complex can be achieved. Various distances determined from the computer-generated complex are presented in Table III. The closest lead-in atom to the copper site is the coordinated sulfur of cysteine-84. The distance from this sulfur atom to the nearest phenanthroline carbon of the bound $Cr(phen)_3^{3+}$ complex is 10.3 Å. This is the edge-to-edge electron-transfer distance for the remote pathway, and its implications for the electron-transfer rate are discussed next.

Rate Constant for the Remote Pathway. When nuclear tunneling can be neglected, the first-order rate constant for electron transfer can be expressed as

$$k_{el} = \kappa_{el}\nu_n \exp[-(\lambda + \Delta G^\circ)^2/4\lambda RT] \quad (4)$$

where κ_{el} is the electronic transmission coefficient, ν_n is the effective nuclear vibration frequency that destroys the activated complex configuration, λ is the vertical reorganization energy, and ΔG° is the free energy change for the reaction.[32-36] When the electronic coupling of the two redox sites is relatively strong, the electron transfer will be adiabatic: for such systems $\kappa_{el} \approx 1$ and $\nu_n \approx 10^{13}$ s^{-1}.[32,35,36] On the other hand, if the two redox sites are relatively far apart, as is the case for the remote pathway, then the coupling between the redox sites will be weak and the electron transfer will be nonadiabatic. The product $\kappa_{el}\nu_n$ is then independent of the frequency for nuclear motion and is given by[32-36]

$$\kappa_{el}\nu_n = 10^{13} \exp(-\beta d)\ s^{-1} \quad (5)$$

where d is the electron-transfer distance defined so that $\kappa_{el}\nu_n$ is equal to some nuclear vibration frequency, typically 10^{13} s^{-1}, when $d = 0$; i.e., the reaction is adiabatic when $d = 0$. The measured rate constants for the reactions of a reduced protein with $*Cr(phen)_3^{3+}$ or $*Ru(bpy)_3^{2+}$ have similar values despite the different driving forces for the reactions. This suggests that these reactions are essentially barrierless. In the barrierless regime $\lambda + \Delta G^\circ \approx 0$ so that $\lambda \approx 0.9$ eV[37] for the cross-reactions of the proteins. The vertical reorganization energies for the cross-reactions are given by $\lambda = (\lambda_{11} + \lambda_{22})/2$, where λ_{11} and λ_{22} are the vertical reorganization energies for the self-exchange reactions of the $*Cr(phen)_3^{3+/2+}$ (or $*Ru(bpy)_3^{2+/+}$) and protein(II/I) couples, respectively. The value of λ_{11} for $*Cr(phen)_3^{3+/2+}$ is 22 kcal mol^{-1}.[38] This yields a vertical reorganization energy for the proteins of ~20 kcal mol^{-1}. This value can be compared with the measured self-exchange rate constants of $\leq 2 \times 10^4$ (50 °C), 2×10^6 (50

(32) Sutin, N.; Brunschwig, B. S. *ACS Symp. Ser.* **1982**, *198*, 105.
(33) Hopfield, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *215*, 642; **1974**, *71*, 3640.
(34) Buhks, E.; Jortner, J. *FEBS Lett.* **1980**, *109*, 117.
(35) Sutin, N., *Prog. Inorg. Chem.* **1983**, *30*, 441.
(36) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.

(37) (a) The reduction potentials for the plastocyanin(II/I), azurin(II/I), and stellacyanin(II/I) couples are 0.36, 0.31, and 0.19 V, respectively.[37b] (b) Taniguchi, V. T.; Sailasuta-Scott, N.; Anson, F. C.; Gray, H. B. *Pure Appl. Chem.* **1980**, *52*, 2275.

°C), and 1.2×10^5 (20 °C) M^{-1} s^{-1} for plastocyanin, azurin, and stellacyanin, respectively. From the λ_{22} values we estimate that $S\kappa_{el}$, the product of the steric and electronic factors,[38] is $\sim 10^{-4}$ for the protein self-exchange reactions.

The electron-transfer distance for the remote pathway can be estimated from eq 4. Since these reactions are essentially barrierless,[39] the last term in eq 4 is close to unity and the rate constant for the remote pathway reduces to

$$k_{el} = 10^{13} \exp(-\beta d)\ s^{-1} \quad (6)$$

The value of β estimated from studies of electron transfer in frozen media and other systems[32,36,40] is ~ 1.2 Å^{-1}. Substitution of the experimental value of k_{el} (k_2) into eq 6 yields a value of 12 Å for d, the electron-transfer distance for the reaction of reduced plastocyanin with $*Cr(phen)_3^{3+}$ by the remote pathway. The closest distance from the edge of the phenanthroline ring to the "lead-in" atom to the copper site (the sulfur atom of cysteine-84), is ~ 10 Å (Table III), in reasonable agreement with this value. The difference of 2 Å (approximately 1 order of magnitude in the rate) may suggest that the frequency factor of 10^{13} s^{-1} is too large and/or that the estimated β is too low.[41] However, considering the complexity of the protein system and the assumptions involved, the agreement can be regarded as satisfactory.

Electron Transfer at the Adjacent Site. We next consider the value of the diffusion-controlled rate constant for electron transfer at the adjacent site. This rate constant can be calculated from

$$k_{diff} = (4\pi ND/1000) / \int_{r=\sigma_{12}}^{\infty} \exp[-w(r)/RT] r^2\, dr \quad (7)$$

$$w(r) = \frac{z_1 z_2 e^2}{D_s r}\left[\frac{\exp(B\sigma_1\mu^{1/2})}{1 + B\sigma_1\mu^{1/2}} + \frac{\exp(B\sigma_2\mu^{1/2})}{1 + B\sigma_2\mu^{1/2}}\right]\exp(-Br\mu^{1/2})$$

In these expressions D is the sum of the diffusion constants of the two reactants, $B = 0.329$ Å^{-1} for water at 25 °C, $\sigma_1 = a_1 + a_1'$, $\sigma_2 = a_2 + a_2'$, where a' is the radius of the dominant ion of opposite charge in the ionic atmosphere, and z_1 and z_2 are the charges of the reactants.[36] Substitution of $D = 2.70 \times 10^{-6}$ cm^2 s^{-1}, $a_1 = 15.8$ Å, $a_1' = 0.95$ Å, $z_1 = -10$, $a_2 = 7.0$ Å, $a_2' = 1.81$ Å, and $z_2 = +3$ gives $k_{diff} = 6 \times 10^9$ M^{-1} s^{-1}. This value is slightly larger than the observed value of k_1 for the reaction of reduced plastocyanin with $Cr(phen)_3^{3+}$ (3.5×10^9 M^{-1} s^{-1}) and may indicate that there is a rotational contribution to k_{diff} or, perhaps, that the reaction is activation controlled with the maximum value of the rate constant equal to $SK_A\kappa_{el}\nu_n$, where S is a steric factor.[35,36] The effect is small, and it is not possible to distinguish between these alternatives at this time.

Finally, for electron transfer at a remote site to successfully compete with adjacent electron transfer, it is necessary that the binding constant at the remote site be large enough to compensate for the larger electron-transfer distance. For $d - d' = 10$ Å, where d' is the (edge-to-edge) electron-transfer distance at the adjacent site, and $\beta = 1.2$ Å^{-1}, the binding constant at the remote site needs to be $\sim 10^5$ times larger than the binding constant at the adjacent site if the (bimolecular) remote path is to successfully compete with the (bimolecular) adjacent path. Although this may be the case for certain biological systems, it is unlikely to be the case for reactions of metalloproteins with most metal complexes. On the other hand, at high protein or metal complex concentrations the (intramolecular) remote path can be significant if the dissociation of the 1:1 complex is sufficiently slow. This is the case for the systems considered here.

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Registry No. $Ru(bpy)_3^{2+}$, 15158-62-0; $Cr(phen)_3^{3+}$, 15276-16-1; $Cr(5\text{-}Clphen)_3^{3+}$, 51194-62-8; $Cr(4,7\text{-}Me_2phen)_3^{3+}$, 51194-72-0; L-Tyr, 60-18-4.

Supplementary Material Available: Tables of reciprocal lifetimes of $*Cr(phen)_3^{3+}$ and $*Ru(bpy)_3^{2+}$ as a function of plastocyanin (Table S1), azurin (Table S2), and stellacyanin (Table S3) concentration (3 pages). Ordering information is given on any current masthead page.

(38) (a) The values of λ_{jj} are estimated with use of[35,36] $k_{jj} = SK_A\kappa_{el}\nu_n \exp(-\lambda_{jj}/4RT)$, where k_{jj} is the self-exchange rate constant, S is a steric factor, and K_A is the association constant for the reactants. The association constant can be calculated from $(4 \times 10^{-3})\pi N\sigma^2(\delta r) \exp(-w_{jj}/RT)$, where N is Avogadro's number, σ is the close-contact distance for the exchange reaction, δr is 0.8 Å, and w is the electrostatic work required to bring the two reactants together.[35] The self-exchange rate for the $*Cr(phen)_3^{3+/2+}$ couple has been estimated as $\sim 3 \times 10^8$ M^{-1} s^{-1} at 25 °C at 0.1 M ionic strength,[21] and the self-exchange rates for the plastocyanin(II/I), azurin(II/I), and stellacyanin(II/I) couples are $\leq 2 \times 10^4$, 2×10^6 (both at 50 °C), and 1.2×10^5 (at 20 °C) M^{-1} s^{-1}, respectively.[38b-d] The value of $S\kappa_{el}$ is unity for the $*Cr(phen)_3^{3+/2+}$ exchange and $\sim 10^{-4}$ for the protein self-exchanges. (b) Beattie, J. K.; Fensom, D. J.; Freeman, H. C.; Woodcock, E.; Hill, H. A. O.; Stokes, A. M. *Biochim. Biophys. Acta* **1975**, *405*, 109. (c) Canters, G. W.; Hill, H. A. O.; Kitchen, N. A.; Adman, E. T. *J. Magn. Reson.* **1984**, *57*, 1. (d) Dahlin, S.; Reinhammar, B.; Wilson, M. T. *Biochem. J.* **1984**, *218*, 609.

(39) In the $Co(phen)_3^{3+}$ reaction,[6a,b,16,27] by contrast, the rate-determining step may be the reorganization of the precursor complex: note the large activation energy for the $Co(phen)_3^{3+}$ reaction.

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(41) In this connection it is interesting to compare intramolecular electron-transfer rates in $(NH_3)_5Ru(His\text{-}33)^{2+}$–ferricytochrome c (Ru^{II}–Fe^{III}) and $(NH_3)_5Ru(His\text{-}83)^{2+}$–azurin ($Ru^{II}$–$Cu^{II}$): the rate constants are 30 and 2 s^{-1}, respectively.[42,43] The distance between the two redox sites is very similar for the two reactions (11.8 Å), and both reactions are temperature independent over a wide range. The $\Delta S°$ values for the two reactions are also very similar (−25.8 cal deg^{-1} mol^{-1} for Ru^{II}–Fe^{III} and −26.8 cal deg^{-1} mol^{-1} for Ru^{II}–Cu^{II}).[42-44]

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(43) Margilit, R.; Kostič, N. M.; Che, C.-M.; Blair, D. F.; Chiang, H.-J.; Pecht, I.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6554.

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