Electron Transfer in Ruthenium-Modified Plastocyanin

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Abstract: Reaction of *Scenedesmus obliquus* plastocyanin with excess $[Ru(trpy)(L)(H_2O)]^{2+}$ (trpy = 2,2′:6′,2″-terpyridine; L = 2,2′-bipyridine, 4,4′-$(CH_3)_2$-2,2′-bipyridine, 4,5,4′,5′-$(CH_3)_4$-2,2′-bipyridine) affords Ru(trpy)(L)(His59)Pc as the main product. These RuPc derivatives are luminescent with λ_{max}(emission) ∼ 650 nm and lifetimes of (Cu^+) in the range 110−140 ns. Photogenerated $*Ru^{2+}PcCu^{2+}$ is quenched by $*Ru^{2+} \rightarrow Cu^{2+}$ electron transfer (ET) to produce $Ru^{3+}PcCu^+$; intramolecular ET was monitored by transient absorption at 590 ($Cu^+ \rightarrow Cu^{2+}$) and 424 nm ($Ru^{3+} \rightarrow Ru^{2+}$). The Cu^+ to Ru^{3+} ET rate constants (k_{ET}) are as follows: 2.9(2) × 10^7 s^{-1} (L = bpy); 2.3(2) × 10^7 s^{-1} (L = dmbpy); and 1.9(2) × 10^7 s^{-1} (L = tmbpy). Activationless rates ($-\Delta G° \sim \lambda \sim 0.70-0.75$ eV) are consistent with coupling-limited tunneling through a β sheet at an estimated Cu−Ru distance of 15.6 Å (calcd $k_{ET} = 10^7$ s^{-1} for a tunneling decay constant of 1.1 $Å^{-1}$). Biphasic $Cu^+ \rightarrow Ru^{3+}$ ET kinetics ($k_{ET} > 10^7$ and ∼10^4 s^{-1}) were observed after flash-quench generation of $Ru^{3+}PcCu^+$ in acidic solutions. The slow phase kinetics are markedly temperature and pH dependent: the activation parameters ($\Delta H^‡ = 43.1$ kJ/mol; $\Delta S^‡ = -17$ J/(K·mol) for L = bpy) suggest that the trigonal low-pH form of Cu^+ reorganizes to the tetrahedral form prior to oxidation to the blue Cu^{2+} state.

Introduction

Plastocyanin is a small (10.5 kD) blue copper protein (cupredoxin) that functions as an electron carrier in photosynthesis. In eukaryotic organisms the protein is present as a solute in the lumen of the thylakoid of chloroplasts, where it mediates ET between cyt *f*, in the membrane bound cyt b_6/f complex, and $P700^+$ of photosystem I.[2,3]

The structure of poplar plastocyanin[4] is similar to those of plastocyanins from green algae [*Enteromorpha prolifera* (X-ray);[5] *Chlamydomonas reinhardtii* (X-ray);[6] *Scenedesmus obliquus* (NMR)[7]] and from the cyanobacterium *Anabaena variabilis* (NMR,[8] X-ray[9]); it is an eight-stranded, antiparallel β barrel with a single copper atom in distorted tetrahedral coordination. The copper ligands are two histidines (His37, His87), a cysteine (Cys84), and a methionine (Met92), the latter being weakly bound to the copper atom through S_δ (bond length ∼2.8 Å).[10]

The unique spectroscopic properties of blue copper include an LMCT [S(Cys) → Cu^{2+}] absorption at ∼600 nm ($\epsilon \sim 4700$ M^{-1} cm^{-1}) and a narrow parallel hyperfine EPR splitting pattern.[11−13] Plastocyanins have relatively high reduction potentials (340−370 mV vs NHE at pH 7).[14] The small $Cu^{2+/+}$ nuclear reorganization energy and the covalent nature of the Cu−S(Cys) bond facilitate long-range ET reactions with donor and acceptor molecules.[13,15−18]

Based on structural work, Guss and Freeman suggested that two regions on the protein surface could be involved in binding biological partners;[4,19] these are the hydrophobic area surround-

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(3) Abbreviations: ET, electron transfer; Pc, plastocyanin; *S.o.*, *Scenedesmus obliquus*; *A.v.*, *Anabaena variabilis*; cyt, cytochrome; P_i, phosphate; TRIS, tris(hydroxymethyl)aminomethane; trpy, 2,2′:6′,2″-terpyridine; bpy, 2,2′-bipyridine; dmbpy, 4,4′-$(CH_3)_2$-2,2′-bipyridine; tmbpy, 4,5,4′,5′-$(CH_3)_4$-2,2′-bipyridine; im, imidazole; MLCT, metal-to-ligand charge transfer; LMCT, ligand-to-metal charge transfer; NHE, normal hydrogen electrode; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry.

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ing the solvent-exposed His87 ligand, and the negative patch of aspartic and glutamic acid residues in the 42−45 and 59−61 regions that encircle the conserved Tyr83 residue. In its interactions with proteins in photosystem I, plastocyanin has been shown to utilize both its acidic and hydrophobic surfaces. The acidic patch associates with the PsaF subunit,[20] whereas the hydrophobic region interacts closer to $P700^+$ and is involved in ET.[21] Computations indicate that the acidic region is involved in docking plastocyanin and cyt *f* prior to rearrangement to a complex with an efficient heme to His87 tunneling pathway;[22] these calculations further suggest that $heme Fe^{2+}$ to Cu^{2+} ET through His87 is faster than a Tyr83-mediated reaction.[23] Interestingly, the residues that form the negative patch are not conserved in plastocyanins from cyanobacteria.[11]

There is evidence that the pH in the thylakoid lumen drops below pH 5 upon illumination.[24] This observation is relevant because the redox activity of plastocyanin is strongly influenced by pH; in acidic solutions, the $Cu^{2+/+}$ potential is high, owing to protonation of His87.[25] Structural studies have shown that there are two forms of the protonated, reduced protein (H^+-$PcCu^+$): in one, Cu moves 0.76 Å and the $Cu-S_\delta$(Met92) bond shortens from 2.9 to 2.6 Å to give a trigonal (Cys84, His37, Met92) Cu^+ center; in the second, rotation of $His87H^+$ around C_β-C_γ gives a conformational isomer in which $N_\delta H^+(87)$ H-bonds to a water molecule on the outside of the protein, and $N_\epsilon H$ (87) H-bonds to the Pro36 backbone carbonyl.[25] Although both $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidize reduced plastocyanin, it does not appear that H^+PcCu^+ participates in the reaction.[11,26,27] Selected hydrophobic redox partners can turn on electron flow from H^+PcCu^+. Indeed, *in vitro* experiments have shown that the reduced protein reacts with [($P700^+$)(chl a)] with optimal redox activity at pH ∼4.[28] Clearly, electron transfer from $PcCu^+$ to oxidants is highly regulated in acidic solution.

Some algal plastocyanins feature a unique exposed surface histidine at position 59, adjacent to the upper part of the acidic patch.[29] We have found that $[Ru(trpy)(L)(H_2O)]^{2+}$ (L = bpy, dmbpy, tmbpy)[3] reacts cleanly with *S. obliquus* plastocyanin to produce Ru(trpy)(L)(His59)Pc derivatives[30] that are suitable for photochemical ET investigations.[31] Interestingly, the oxidation of Cu^+ by photogenerated Ru^{3+} in RuH^+Pc is 3 orders of magnitude slower than the $Cu^+ \rightarrow Ru^{3+}$ reaction in the RuPc derivatives, owing to the large structural rearrangement that accompanies electron transfer. It is apparent that Ru-modified plastocyanin is a robust model system for in-depth experimental investigations of protein-gated electron flow.

Results and Discussion

Modified Proteins. The attachment of $Ru(bpy)_2(im)^{2+}$ to a protein histidine normally is performed in two steps: reaction of the protein with $[Ru(bpy)_2(H_2O)_2]^{2+}$ followed by incubation with excess (>100 mM) imidazole at pH 7 or higher for several days. Unfortunately, *S. obliquus* plastocyanin is not stable in solutions with high concentrations of imidazole and we were not able to obtain pure fractions of $Ru(bpy)_2(im)$(His59)Pc;[32] for this reason, we decided to investigate structurally analogous polypyridine complexes of the type $[Ru(trpy)(L)(im)]^{2+}$ (L = bpy, dmbpy, tmbpy). Although electronically excited $[Ru(trpy)(bpy)(X)]^{2+}$ (X = pyridine or cyanide) is very short-lived, the luminescent MLCT excited state of $[Ru(trpy)(bpy)(im)]^{2+}$ has a longer lifetime ($\tau \sim 20$ ns)[33] in aqueous solution at room temperature (and coordination of the $Ru(trpy)(L)^{2+}$ to His59 of *S. obliquus* plastocyanin enhances its emission intensity as well as its lifetime).

The electronic absorption spectra of the Ru(trpy)(L)(His59)-$PcCu^{2+}$ derivatives are shown in Figure 1. Each of these spectra matches closely the sum of the $[Ru(trpy)(L)(im)]^{2+}$ and copper protein spectra, thereby indicating in each case that Ru^{2+} is attached to the His59 imidazole and that the structure of the native site is unperturbed. The site of ruthenium modification was confirmed by peptide mapping. A representation of the structure of Ru(trpy)(bpy)(His59)Pc is shown in Figure 2.

Electron-Transfer Kinetics. The luminescence of *Ru(trpy)(L)(His59)$PcCu^{2+}$ ($\tau \sim 20$ ns) is quenched relative to that of the Ru-modified Cu^+ protein ($\tau \sim 120-140$ ns). Transient absorption measurements show that photoreduction (k_f) plays an important role in the deactivation of *Ru(trpy)(L)(His59)-$PcCu^{2+}$ (Figure 3; Scheme 1). The rates also were measured by a flash-quench method:[31,34] in this experiment the copper is reduced and $*Ru^{2+}$ reacts with an exogenous quencher, generating Ru^{3+}(trpy)(bpy)(His59)$PcCu^+$. After rapid intramolecular $Cu^+ \rightarrow Ru^{3+}$ ET to give Ru^{2+}(trpy)(bpy)(His59)$PcCu^{2+}$, the Cu^{2+} is rereduced by the reduced quencher (Scheme 2). The flash/quench ET rates at neutral and basic pH are the same within experimental error as the $Cu^+ \rightarrow Ru^{3+}$ rates measured by direct photoinduction. However, flash-quench experiments performed in the pH range 5.6−4.1 yielded an initial fast component of $>10^7$ s^{-1} followed by a slower phase of $\sim 10^4$ s^{-1} (Figure 4). Careful examination showed that the slower kinetics phase is markedly temperature and pH dependent. Scheme 2 cannot account for these observations, because at least

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(30) Other Ru-modified plastocyanins have been studied: $Ru(NH_3)_5$-(His59)Pc (from *A. variabilis* and *S. obliquus*);[29] several mutants (Tyr83His, Leu12His, Leu15His, Thr79His, Lys81His) of spinach plastocyanin have been modified with $Ru(bpy)_2(im)^{2+}$ [Sigfridsson, K.; Sundhal, M.; Bjerrum, M. J.; Hansson, Ö. *JBIC, J. Biol. Inorg. Chem.* **1996**, *1*, 405−414. Sigfridsson, K.; Ejdebäck, M.; Sundahl, M.; Hansson, Ö. *Arch. Biochem. Biophys.* **1998**, *351*, 197−206]. Also note that Ru(trpy)(bpy)-modified cyt *c* has been reported [Isied, S. S. In *Metal in Biological Systems*; Sigel, H., Sigel, A., Eds.; Dekker: New York, 1991; Vol. 27, pp 1−56].

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(32) The standard synthetic procedure[15,31] could not be employed because the protein was denatured by excess imidazole. Similar problems have been encountered in attempts to modify spinach plastocyanin.[30] Nonetheless, pure $Ru(bpy)_2(H_2O)$(His59)Pc is readily made, and partially im-ligated fractions also can be prepared.

(33) The complexes $[Ru(trpy)(L)(im)]^{2+}$ (L = bpy, tmbpy) display broad band emission spectra in aqueous solution, with $\lambda_{max} \sim 650$ nm; the luminescence decay lifetimes are 20 and 54 ns, respectively.

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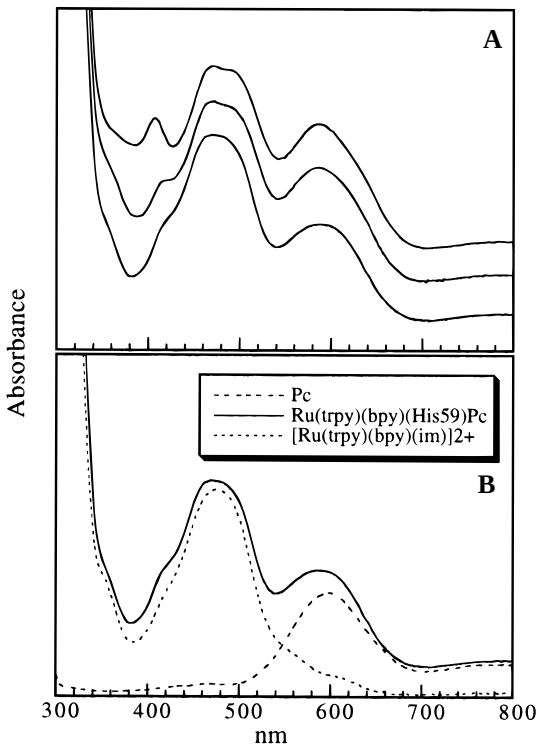

Figure 1. Absorption spectra (KP_i buffer, 100 mM, pH 7): (A) Ru(trpy)(L)(His59)$PcCu^{2+}$ (L = bpy (bottom), dmbpy (middle), tmbpy (top)); (B) $PcCu^{2+}$ (ϵ = 4500 M^{-1} cm^{-1} at 597 nm),[29] $[Ru(trpy)(bpy)(im)]^{2+}$ (ϵ = 8800 M^{-1} cm^{-1} at 478 nm), and Ru(trpy)(bpy)(His59)Pc.

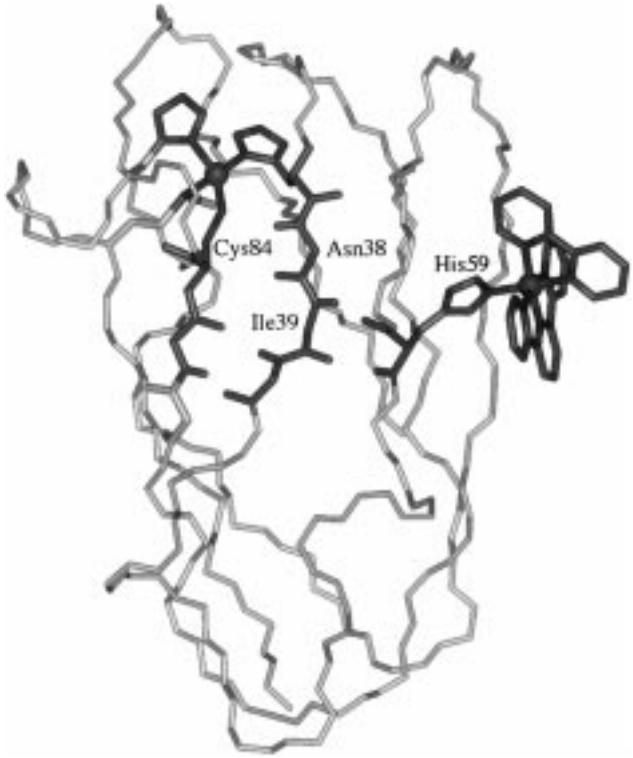

Figure 2. Structural model of Ru-modified *S. obliquus* plastocyanin. The copper center and attached Ru(trpy)(bpy) complex are highlighted along with segments of the protein involved in ET pathways [*R*(Cu−Ru) = 15.6 Å]. The shortest ET pathways involve hydrogen bonds directly to the copper center as well as between β strands. The Ru couples through the ligated imidazole to either the main chain amide or carbonyl of His59; at this point, there are two good pathways to the copper center. One includes the hydrogen bond between the His59 amide and the Ile39 carbonyl, the Ile39/Val40 peptide unit, and the hydrogen bond between the Val40 amide and the Phe83 carbonyl; from Phe83, the path goes through the main chain to Cys84. The second route utilizes the hydrogen bond between the His59 carbonyl and the Ile39 amide, then proceeds directly to the copper center by going through the main chain and the Asn38(amide)−Cys84(sulfur) hydrogen bond [*R*(N−S) = 2.63 Å].

two Cu^+ species must be present. The transient spectrum (Figure 4, inset) recorded in the visible region is identical with the absorption spectrum of oxidized plastocyanin. The same transient spectrum was recorded 75 μs and 1 ms after excitation (Figure 4, inset). The dependence of the slow phase on pH in the range 5.6−4.1 ($k_{obs} \sim [H^+]^{-1}$) can be analyzed in terms of a pK_a of 5.5, in agreement with the values obtained from NMR experiments.[11] The temperature dependence of the slow oxidation of Cu^+ in Ru(trpy)(L)(His59)Pc was measured in the range 2−37 °C (L = bpy, tmbpy). Analysis of the data for L = bpy yielded the following activation parameters: $\Delta H^{\ddagger}$ = 43.1 kJ/mol; $\Delta S^{\ddagger}$ = −17 J/(K·mol).

The $Cu^{2+/+}$ reduction potential of $Ru(NH_3)_5$(His59)Pc (from *S. obliquus*) is 392 mV vs NHE (100 mM NaP_i, pH 7.0).[35] The relatively high value (compared to that of the wildtype protein) is likely due to the influence of the positively charged Ru complex.[36] The $Ru^{3+/2+}$ potentials of $[Ru(trpy)(L)(im)]^{2+}$ complexes are 1.09 (L = bpy)[18] and 1.03 V (L = tmbpy) vs NHE; these potentials may be slightly higher in the protein derivatives. In any case, the $Cu^+ \rightarrow Ru^{3+}$ ET reactions are expected to be activationless ($-\Delta G^\circ \sim \lambda \sim 0.70-0.75$ eV).[31] Indeed, the rate of $Cu^+ \rightarrow Ru^{3+}$ ET in Ru(trpy)(tmbpy)(His59)Pc is independent of temperature in the range 2 to 37 °C. It follows that the small differences in rates in Ru(trpy)(L)(His59)Pc derivatives (2.9×10^7 s^{-1} for L = bpy; 1.9×10^7 s^{-1} for L = tmbpy) are likely due to slight variations in Cu−Ru electronic couplings, as found in related work on Ru-modifed azurin.[18] Coupling-limited rates in the $(2-3) \times 10^7$ s^{-1} range are in excellent agreement with expectation [k_{ET}(calcd) = 10^7 s^{-1}; *R* = 15.6 Å; decay constant = 1.1 $Å^{-1}$] for Cu to Ru tunneling in a β-sheet protein (Figure 2).[16]

In acidic solutions, the Cu^+ in the protein is partitioned between three-coordinate (H^+PcCu^+) and distorted tetrahedral ($PcCu^+$) structures (Scheme 3). The fast component of the biphasic kinetics corresponds to intramolecular oxidation of the $PcCu^+$ by the surface-bound Ru^{3+} (as in flash-quench experiments at higher pH or by direct photoinduction), whereas the slow phase is assigned to the oxidation of H^+PcCu^+.[37] Oxidation either could occur before[38] or, more likely, after the perturbed Cu^+ site undergoes a conformational rearrangement (Scheme 3). Evidence for conformational interconversion at low pH has been obtained in NMR experiments on *A. variabilis* plastocyanin, where a lifetime of 400 μs was found for the proton on the N_δ of His87.[39,40] The extensive nuclear reorga-

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(37) Interestingly, a high yield of transient Cu^+ is generated by direct photoinduction (Figure 3); yet, there is no evidence from the kinetics at acidic pH of any reaction other than coupling-limited $Cu^+ \rightarrow Ru^{3+}$ ET. It follows that the Cu^+ formed by photoinduction decays by ET to Ru^{3+} before any significant population rearranges to H^+PcCu^+.

(38) Although the reduction potential of $HPcCu^+$ is not known, it is expected to be very positive since the trigonal geometry strongly favors Cu^+. Thus the rate of direct oxidation of H^+PcCu^+ should depend strongly on the $Ru^{3+/2+}$ reduction potential. The rates we have measured for the slow phase are insensitive to the complexes used, although we did not examine a large driving-force range. Thus the results do not allow us conclusively to rule out an oxidation/rearrangement mechanism.

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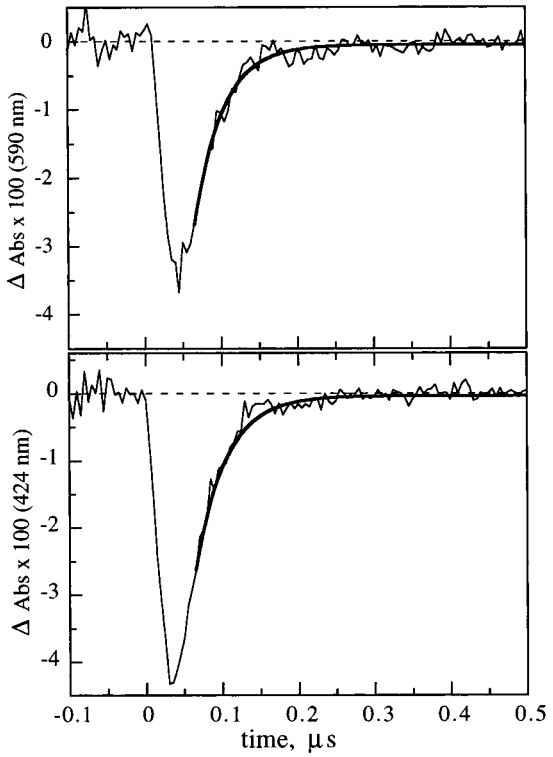
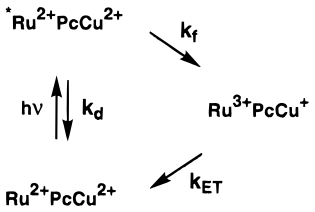
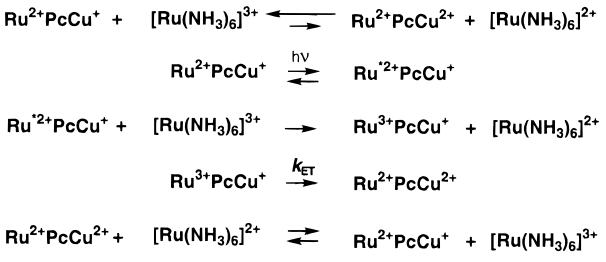


Figure 3. Transient absorption kinetics of a ~20 μM solution of Ru(trpy)(bpy)(His59)PcCu^{2+} in KP$_i$ buffer (100 mM, pH 7.4) at room temperature. (Top) Kinetics monitored at 590 nm (Cu^{2+}) and (bottom) 424 nm (Ru^{3+}). 424 nm is an isosbestic point for [Ru(trpy)(bpy)(im)]$^{2+}$ and its MLCT excited state; this wavelength was particularly useful in monitoring Cu^{+} → Ru^{3+} ET (*Ru^{2+} decays to Ru^{2+} at a similar rate). Cu^{2+} was monitored at 590 nm instead of 597 nm to avoid interferences due to emitted light (*Ru^{2+} emission). The heavier lines are best fits to a single-exponential function.

Scheme 1



Scheme 2



nization associated with the conformational change accounts for the 43 kJ/mol barrier in the gated Cu^{+} → Ru^{3+} ET reaction.

Experimental Section

Materials and Methods. Ligands trpy, bpy, and dmbpy were used as received from Aldrich; tmbpy was prepared according to a literature procedure.[41] RuCl$_3$·3H$_2$O, Ru(bpy)$_2$Cl$_2$·2H$_2$O, and [Ru(NH$_3$)$_6$]Cl$_3$ were obtained from Strem. Distilled water was further purified by passage through a Barnstead NANOpure system. Buffers were prepared by using reagent grade chemicals. Protein solutions were concentrated by using ultrafiltration units (stirred cells or centricon devices) containing YM10 or YM3 membranes (Amicon). Immobilized metal ion affinity chromatography (IMAC) and ion-exchange chromatography were performed by using pre-packed Chelating Superose 10/2 HR and Mono Q 5/5 HR columns attached to an FPLC system (Pharmacia). The following experimental conditions were used for IMAC: buffer A was 750 mM NaCl, 20 mM NaP$_i$, pH 7.2; buffer B was 750 mM NH$_4$Cl, 20 mM NaP$_i$, pH 7.2; the column was in the copper form. To eliminate nonspecifically bound copper a blank run was performed before running the sample. The buffers for anion-exchange were 25 mM TRIS/50 mM NaCl, pH 7.8 (A), and 25 mM TRIS/500 mM NaCl, pH 7.8 (B). Gel filtration was carried out on PD-10 columns prepacked with G-25 Sepharose (Pharmacia). Standard gravity cation-exchange chromatography was performed with S-Sepharose or SP-Sepharose resin (Pharmacia).

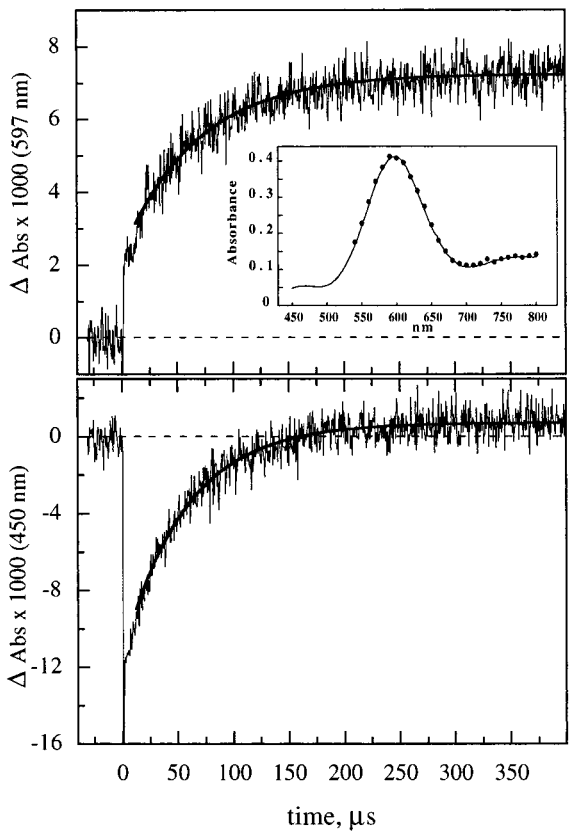


Figure 4. Transient absorption kinetics of a ~20 μM solution of Ru(trpy)(bpy)(His59)PcCu^{+} containing ~8 mM [Ru(NH$_3$)$_6$]Cl$_3$ (100 mM NaOAc, pH 4.78) at 24.9 °C. (Top) Kinetics monitored at 590 nm (Cu^{2+}) and (bottom) 450 nm (Ru^{3+}). The best fits to single-exponential functions (smooth lines) yielded 1.4 × 10^4 s^{-1} (Cu) and 1.7 × 10^4 s^{-1} (Ru). The initial fast component was omitted in the fitting procedure. Inset: Transient spectrum (dots) 1 ms after excitation, compared to the absorption spectrum of plastocyanin (solid line). Although the Cu^{2+} (LMCT) and Ru^{2+} (MLCT) bands overlap considerably (see Figure 1), there is only a small contribution to the transient due to Ru^{3+}. The increase in absorbance after ~200 μs may be due to the small absorption of Cu^{2+} at 450 nm.

Preparation of Complexes. Ru(trpy)Cl$_3$ and [Ru(trpy)(bpy)Cl]Cl·2.5H$_2$O were prepared by literature procedures.[42,43] The [Ru(trpy)(L)-

(40) A deprotonation time of <1 ms was estimated by direct electrochemical methods.[27]

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Scheme 3

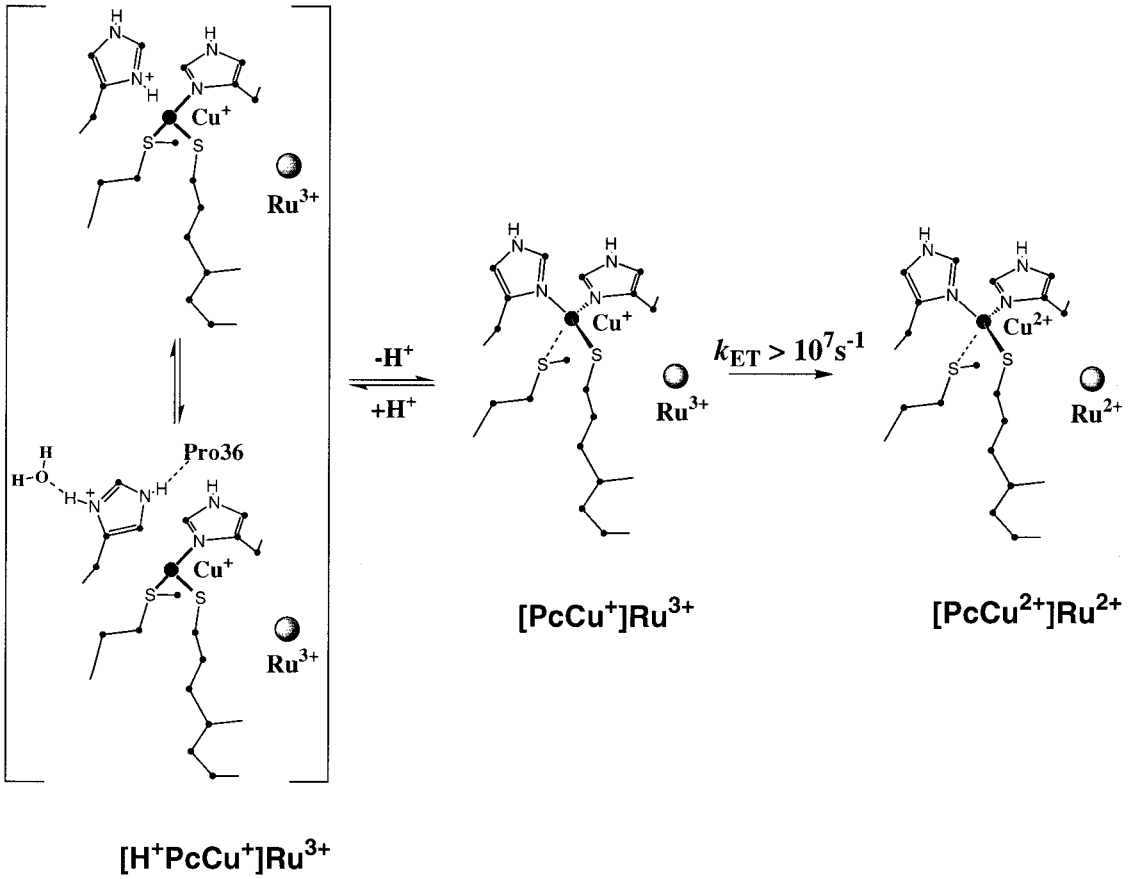

Table 1. [$Cu^+ \rightarrow Ru^{3+}$] ET Data for Modified Plastocyanin

protein	k_{ET} (s^{-1})	$-\Delta G°$ (eV)[a]	pH[b]	τ[c]
Ru(trpy)(bpy)(His59)Pc	$2.7(2) \times 10^7$	0.70(2)	7.4	(110, 10)
	$2.8(2) \times 10^7$	*e*	5.2	(*e*, 19)
	$[3.0(3) \times 10^4]$[d]		5.2	
Ru(trpy)(dmbpy)(His59)Pc	$2.4(2) \times 10^7$	*e*	7.4	(*e*, 25)
	$2.1(2) \times 10^7$	*e*	5.2	(136, 28)
	$[3.2(3) \times 10^4]$[d]		5.2	
Ru(trpy)(tmbpy)(His59)Pc	$1.9(2) \times 10^7$	0.64(2)	7.6	(*e*, 22)
	$2.0(2) \times 10^7$	*e*	5.2	(136, *e*)
	$[3.0(3) \times 10^4]$[d]		5.2	
$Ru(bpy)_2(im, H_2O)$(His59)Pc	$1.2(2) \times 10^7$	0.69(2)	7.6	(~150, *e*)

[a] $E°$ {$[Ru(NH_5)_5$(His59)$PcCu^{2+/+}] = 0.392$ V (pH 7.0, ref 35); $[Ru^{3+/2+}$(trpy)(bpy)(im)$] = 1.09$ V; $[Ru^{3+/2+}$(trpy)(dmbpy)(im)$] = e$; $[Ru^{3+/2+}$(trpy)(tmbpy)(im)$] = 1.03$ V; $[Ru^{3+/2+}(bpy)_2$(im)(His83)Az$] =$ 1.082 V (pH 7.0, ref 18)} vs NHE. [b] (pH 7.0−7.6 P_i buffer; pH 5.2 acetate buffer). [c] Luminescence decay lifetimes (ns) for reduced and oxidized derivatives. [d] Slow phase ($T = 295$ K). [e] Not measured.

Cl]Cl (L = dmbpy, tmbpy) analogues were obtained by the same method. These complexes were further purified by cation-exchange chromatography (elution was achieved by using a stepwise gradient of NaCl). The corresponding aqua ions were obtained by reacting aqueous solutions of the chloro complexes with $AgNO_3$. Care was exercised to remove the precipitated AgCl. $[Ru(bpy)_2CO_3]\cdot 4H_2O$ was obtained as described previously.[44,45] $[Ru(trpy)(L)(im)]^{2+}$ (L = bpy, dmbpy, tmbpy) was prepared by reacting $[Ru(trpy)(L)(Cl)]^+$ with a large excess of imidazole in aqueous solution at 60−80 °C and isolated by cation-exchange chromatography (using a stepwise gradient of $(NH_4)_2SO_4$). The $[Ru(trpy)(L)(im)]^{2+}$ (L = bpy, dmbpy, tmbpy) complexes were not obtained as solids. $[Ru(bpy)_2(im)(H_2O)](ClO_4)_2$ was prepared by reacting $[Ru(bpy)_2CO_3]\cdot 4H_2O$ and imidazole in a 1:1 ratio at room temperature with continuous stirring for several hours. $[Ru(bpy)_2(im)(H_2O)]^{2+}$ was separated from $[Ru(bpy)_2(im)_2]^{2+}$ [and other Ru(bpy) complexes] by means of cation-exchange chromatography (a stepwise gradient of $NaClO_4$ was used). $[Ru(bpy)_2(im)(H_2O)]^{2+}$ was eluted in a large volume and precipitated as a perchlorate salt by concentrating the solution at room temperature.

All the chromatographic steps for the complexes were performed on standard gravity columns. The columns were equilibrated with the proper counterions and washed with water before the complexes were loaded.

Protein Modification. *S. obliquus* plastocyanin**,** isolated as described previously,[46] was kindly provided by Dr. R. Powls. $Ru(bpy)_2(H_2O)$(His59)Pc was obtained by reacting plastocyanin (0.1−0.2 mM) with an equivalent amount of $[Ru(bpy)_2CO_3]\cdot 4H_2O$ in 250 mM carbonate buffer. The pH was adjusted to 7.6−7.8 and the reaction allowed to take place for 24 h at room temperature in a capped vial.[15] $Ru(bpy)_2(H_2O)$(His59)Pc was isolated by anion-exchange chromatography.

Ru(trpy)(L)(His59)Pc was prepared by reacting concentrated plastocyanin with an excess of $[Ru(trpy)(L)Cl]^+$ or $[Ru(trpy)(L)(H_2O)]^{2+}$ (L = bpy, dmbpy, tmbpy). In a typical experiment a concentrated solution of ruthenium complex (5−10 mM) in water was added to a ~0.2 mM solution of oxidized plastocyanin in low ionic strength P_i buffer (the pH after mixing was adjusted to 7.2−7.8) and reacted at room temperature for at least 24 h. The reaction mixture was then concentrated to a small volume (100−200 μL) and passed through a gel filtration column equilibrated with 750 mM NaCl/20 mM NaP_i at pH 7.2 (buffer A, IMAC). To eliminate nonspecifically bound ruthenium, the solution was kept at 4 °C for 24 h, concentrated, and purified (by gel filtration) again. The protein was then loaded on a 10/2 HR Chelating Superose column. Unreacted protein and some

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modified protein (ruthenated at amino acids other than histidines) were retained, whereas the protein modified at His59 was found in the breakthrough fractions.[47] The Ru(trpy)(L)(His59)Pc so obtained was relatively pure; it was purified further by anion-exchange chromatography. The yield of ruthenated plastocyanin was a function of the reaction time and the concentrations of ruthenium complex and protein. Increasing the temperature to nearly 30 °C increased the yields of Ru(trpy)(bpy)(His59)Pc without appreciably denaturing the protein. Plastocyanin was reacted with $[Ru(bpy)_2(im)(H_2O)]^{2+}$ by the same procedure in an attempt to prepared $Ru(bpy)_2(im)$(His59)Pc. We found that a mixture of $Ru(bpy)_2(im)$(His59)Pc and $Ru(bpy)_2(H_2O)$(His59)Pc was obtained. In each case unreacted plastocyanin was recovered, repurified, and reused.

Peptide mapping on Ru(trpy)(bpy)(His59)Pc was conducted as follows. Mass analysis by MALDI-TOF of intact Ru(trpy)(bpy)Pc was consistent with a single modification of the plastocyanin plus 490.23 amu corresponding to the Ru(trpy)(bpy) group. The measured $M + H^+$ average mass was 10 594.1 amu (the calculated average mass of apo plastocyanin plus the Ru(trpy)(bpy) group is 10 594.3 amu). A sample of Ru(trpy)(bpy)(His59)Pc was denatured by incubation with freshly deionized 8 M urea for 15 min at 65 °C prior to tryptic digestion. A single tryptic peptide with absorptions at 200 and 478 nm was found by HPLC, collected, and analyzed by MALDI-TOF mass spectrometry (data not shown). This peptide gave a measured mass of 5625.6 and corresponded to residues 25 to 73 plus the Ru(trpy)(bpy) group (calcd 5625.6 amu). The mass spectrum of this peptide showed an atypical isotopic cluster consistent with the presence of the ruthenium atom. This peptide was further cleaved, with endoAsp-N protease, yielding two peptides with masses 1030.7 and 2556.8. Both peptides contained the ruthenium complex as evidenced by the absorption at 478 nm and the characteristic isotopic pattern in their mass spectra. The 2556 mass fragment was sequenced by Edman degradation, giving the following partially digested peptide sequence: Asp55-Ala56-Leu57-Ser58-His59-Asp60-Asp61-Tyr62-Leu63-Asn64-Ala65-Pro66-Gly67-Glu68-Ser69-Tyr70-Thr71-Ala72-Lys73 (calcd mass 2556.4). Modification of His59 was inferred by the absence of a PTH-histidine signal at position 5 of this peptide. The smaller peptide corresponded to the sequence Asp55-Ala56-Leu57-Ser58-His59 + modifier (calcd mass 1030.8).

Spectroscopy and Kinetics. UV−visible absorption spectra were measured with a Hewlett-Packard 8452A Diode-Array spectrophotometer. The excitation source for the kinetics measurements (luminescence decay and transient absorption) was a dye laser (Lambda Physik FL3002; Coumarin 480, ∼25 ns pulse width, ∼1−2 mJ/pulse) pumped by a XeCl excimer laser (Lambda Physik LPX210i, 308 nm, ∼25 ns pulse width). Single wavelength transient absorption kinetics were obtained with use of a continuous-wave 75-W xenon arc lamp as a probe source. The probe light was passed through 385 and 530 nm colored-glass cutoff filters before reaching the sample for measurements at 424 and 590 nm, respectively. Probe light was dispersed by an Instruments SA 1680B double monochromator placed after the sample and detected with a photomultiplier tube. The transient signal was amplified by using either a 200-MHz quasi differential amplifier (for kinetics to ∼500 μs) or a DSP 1402E programmable amplifier (for kinetics ≥1 ms) and digitized by using a Tektronix RTD710 200-MS/s transient digitizer interfaced to a microcomputer. $[Ru(NH_3)_6]Cl_3$ was recrystallized according to a published procedure before use.[48] Solutions for laser experiments were deaerated with several pump/fill (Ar) cycles (Schlenk line) prior to use. Kinetics measurements at pH < 4.8 were conducted immediately after equilibrating reduced Ru-plastocyanin with the proper buffer and quencher. We could recover most of the protein within 1 h of exposure to the low pH. We have not checked the stability of reduced *S. obliquus* plastocyanin to pH <4.1 or to exposure times over 1 h.

Computer Modeling. Because the relevant coordinates from the NMR structure of *S. obliquus* plastocyanin[7] are not available, we have constructed a model of this protein. The *C. reinhardtii* plastocyanin X-ray structure[6] served as the starting point. All steps of model building were completed with the Biograf (version 3.21) simulations program, employing DreidingII force field parameters.[49] Sequence alignment was done as described previously.[6] The positions of all common heavy atoms of the main chain and side chains were maintained. Replacement of nonidentical side chains was achieved in one of two ways: (1) for similar chains, the appropriate chemical groups (i.e., −H, $-CH_3$, −OH) were either added to or deleted from the existing side chain, and (2) for dissimilar side chains, the new side chain added had the $\chi 1$ dihedral of the old one. Explicit hydrogens were added and POLARIS charges were assigned to this structure. The copper ion and four ligands (His37, Cys84, His87, Met92) were held fixed throughout all minimization steps. Five steps of steepest-descent minimizations, followed by 200 steps of conjugate-gradient minimization, yielded a structure having an overall rms force of ∼ 1.2 kJ/(mol·Å) and a C_α rms coordinate difference of 0.44 Å. To model the ruthenated protein, a Ru(trpy)(bpy) unit[50] was attached to the surface via the N_ϵ of His59. The His59 side chain dihedrals were adjusted to minimize steric contacts between surrounding side chains and the rings of the trpy and bpy ligands. This structure was minimized with all the atoms fixed except for those of the His59 side chain and the attached ruthenium complex. The final rms force for this structure was ∼1.5 kJ/(mol·Å).

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Supporting Information Available: Values of slow phase $Cu^+ \rightarrow Ru^{3+}$ rate constants for Ru(trpy)(bpy)(His59)Pc at different temperatures and pHs (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(47) We have used immobilized metal ion affinity chromatography (IMAC) to great advantage in our ruthenium-modification work. Proteins having surface histidines may coordinate to an immobilized metal ion (usually Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}) with different affinities depending on the number and particular microenvironment of the histidine residue(s) [Hemdan, E. S.; Zhao, Y.-J.; Sulkowski, E.; Porath, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1811−1815]; this ability is lost when, for instance, the histidine is derivatized with a metal complex. In general, metal complexes will not react specifically with histidine residues; however, the technique provides a fast and inexpensive way to ascertain whether a protein has actually been modified at a histidine. In addition to providing a convenient purification method, IMAC can be employed to probe the accessibility of surface histidines.

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