the cis amide conformer energetically accessible for binding and recognition by the receptor, then the tetrazole analogue should provide a useful probe of this role. On the other hand, the cis amide offers a unique arrangement of an adjacent hydrogen-bond donor and acceptor, which the tetrazole does not possess and whose steric bulk would prevent from assuming close proximity to the receptor. Activity of analogues with tetrazole replacement will provide strong evidence for the role of the cis amide in receptor recognition. Lack of activity will not exclude the cis amide from consideration, because of the differences pointed out above. Other cis amide surrogates such as the cis olefinic isostere would be useful to probe this question. Hann et al.[20] have reported synthetic difficulties in the preparation of peptides with the cis olefinic surrogate due to rearrangement from the cis-β,γ-unsaturated isomer to the more stable trans-α,β-unsaturated isomer.

Conclusion

Conditions for the preparation of tetrazole dipeptide analogues in which the amide bond is replaced by a 1,5-disubstituted tetrazole ring have been found that preserve the chiral integrity of the starting dipeptide. A comparison of the crystal structure of a diketopiperazine in which one of the cis amides has been replaced with a tetrazole ring demonstrates clearly the strong geometric similarity of these two functional groups. A method to assess the impact of the minor geometrical changes and the increase in steric bulk on the conformational ability of the tetrazole analogue to mimic those conformers accessible to the cis amide has been developed that shows 88% mimicry. The combination of a synthetic route to chirally pure products with the knowledge that the tetrazole isostere allows nearly complete sampling of the conformers available to the cis amide bond argues strongly for its use to probe the role of proline and other *N*-alkyl amino acids in molecular recognition.

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Preparation and Characterization of Two His-59 Ruthenium-Modified Algal Plastocyanins and an Unusually Small Rate Constant for Ruthenium(II) → Copper(II) Intramolecular Electron Transfer over ~12 Å

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Abstract: Plastocyanins from the algae *Anabaena variabilis* and *Scenedesmus obliquus* possess a single uncoordinated surface histidine at position 59. Procedures for Ru modification of this residue using $[Ru(NH_3)_5H_2O]^{2+}$ are described. The modification time required is strongly dependent on the net charges on the proteins, estimated as 1+ and 9- respectively for PCu^I at pH 7. The major product in each case has been characterized by ICP atomic emission spectroscopy (1:1 ratios of Cu to Ru). The His-59 residue of the Ru-modified products no longer reacts with diethyl pyrocarbonate (DEPC). Also the sharp ^1H NMR His-59 C_2H resonance at 8.2 ppm is lost due to paramagnetic line broadening by the adjacent Ru(III). The PCu^{II}/PCu^I reduction potentials remain essentially unchanged, and the PCu^{II} UV-vis spectrum is unperturbed by Ru modification, except for the additional shoulder at 300 nm due to the $[Ru(NH_3)_5His]^{3+}$ moiety. On pulse radiolysis using $CO_2^{\bullet-}$ to reduce $PCu^{II}Ru^{III}$ (pH 7, 20 °C) the behavior observed in both cases is very similar. Reduction is partitioned between the Cu(II) (72%) and Ru(III) (28%), rate constant 6.7×10^8 M^{-1} s^{-1}, yielding stable PCu^IRu^{III} and transient $PCu^{II}Ru^{II}$, respectively. The latter decays to PCu^IRu^{III} by intra- and/or intermolecular processes (k_1, k_2), which together constitute the second stage. For *A. variabilis*, $k_1 = 0.024 \pm 0.058$ s^{-1} and $k_2 = 1.2 \times 10^5$ M^{-1} s^{-1}; and for *S. obliquus*, $k_1 = 0.04 \pm 0.22$ s^{-1} and $k_2 = 3.3 \times 10^5$ M^{-1} s^{-1}. Therefore k_1 values are <0.082 and <0.26 s^{-1}, respectively, with zero values not excluded from this study. Modification of *Pseudomonas aeruginosa* azurin was also carried out by a procedure already described. From four pulse radiolysis runs, transient $ACu^{II}Ru^{II}$ gives $k_1 = 2.5 \pm 0.8$ s^{-1} (pH 7, 17 °C), with no significant competition from k_2, in satisfactory agreement with the flash photolysis value of 1.9 ± 0.4 s^{-1} from the Gray group. Donor-acceptor distances (~12 Å) and driving forces are similar for the $PCu^{II}Ru^{II}$ and $ACu^{II}Ru^{II}$ systems. Of particular interest is the very small k_1 for both Ru-modified plastocyanins, indicating that electron transfer from the His-59 site through to the Cu is not a favorable route. On the other hand when unattached $[Ru(NH_3)_5Im]^{2+}$ is the reductant, stopped-flow studies indicate $k_{et} > 5 \times 10^3$ s^{-1} for reduction from the acidic patch (42-44) region of *S. obliquus* PCu^{II}.

The complex $[Ru(NH_3)_5H_2O]^{2+}$ is an appropriate reagent for the modification of metalloproteins because of its affinity for accessible surface histidine residues. It has for example been attached to specific histidine residues on ribonuclease A,[1] lysozyme,[2] horse heart cytochrome *c*,[3] and *Pseudomonas aeruginosa*

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azurin.[4] Four singly modified sperm whale myoglobin derivatives in which the Ru label is attached to different histidine residues have also been isolated.[5] Because of their kinetic inertness in the Ru(II) and Ru(III) states, the modified proteins lend themselves to the study of intramolecular electron-transfer processes.[6,7] Plastocyanin has not so far been a part of these studies because the protein from the more readily available higher plant sources has only two histidine residues, both of which are coordinated to the Cu at the active site.[8] From sequence information a number of algal plastocyanins are known to have an uncoordinated surface histidine, and it is two of these plastocyanins[9,10] that provide the subject of the present study. More specifically, plastocyanin from the blue-green alga *Anabaena variabilis* and from the green alga *Scenedesmus obliquus* have a surface histidine at position 59. Both plastocyanins are of interest because in the case of *A. variabilis* the protein is basic (p*I* ~ 7.8) with an estimated PCu^{I} charge of 1+ at pH ~ 7 and *S. obliquus* is acidic (p*I* ~ 4.2) with an estimated PCu^{I} charge of 9−, in common with higher plant plastocyanins (±1). Of further interest are the two deletions in aligned sequences at positions 57 and 58 on *S. obliquus*, which results in a tightening of the peptide loop incorporating His-59.[11,12]

Experimental Section

Proteins. Procedures for growing and isolating plastocyanin from the blue-green alga *A. variabilis* (original sample from Sea Plantations Inc., Salem, MA)[13-15] and the green alga *S. obliquus*[16-18] have been described. Homogeneity of the purified *A. variabilis* plastocyanin, after passage down two C-50 cation-exchange Sephadex columns, was confirmed by chromatography on a Mono-S FPLC (Pharmacia) column in 20 mM acetate buffer at pH 5.2. Protein of absorbance (*A*) ratio $A_{278}/A_{597} \leq 1.3$ was used for modification. Plastocyanin from *S. obliquus* was purified as described,[18] to give protein with $A_{278}/A_{597} = 3.1$ or less, before use in modification procedures. Spinach plastocyanin (*Spinacea oleracea*) was isolated by the procedures used previously in this laboratory and purified to $A_{278}/A_{597} = 1.1 \pm 0.1$.[19a] The His-59 p$K_a$ values for the native plastocyanins in the PCu^{I} state, determined by NMR, are 7.3 (*A. variabilis*)[20] and 7.8 (*S. obliquus*).[18]

Complexes. The aquapentaammineruthenium(II) complex $[Ru(NH_3)_5H_2O](PF_6)_2$ was prepared[21] by a method similar to that of Sundberg et al.[22] and the composition confirmed by analysis. The imidazole Ru(II) complex, $[Ru(NH_3)_5Im]^{2+}$ was prepared by reduction of a solution of $[Ru(NH_3)_5H_2O](CF_3SO_3)_3$ (0.47 g) and imidazole (0.15 g; Sigma) in 0.10 M HCl (10 mL) with amalgamated zinc shot (~1.2 g) under argon. Reduction was allowed to proceed for 5 h in the dark, after which the solution was transferred by argon pressure through a glass wool filter into a flask containing a deaerated (argon) solution of NH_4PF_6 (1.5 g) in 2.5 mL of water. The pale yellow precipitate was filtered off, washed with methanol and a little ether, and dried under an argon stream overnight. It was possible to handle the dry solid in air for short periods. Storage was under argon. $[Ru(NH_3)_5Im](PF_6)_2$: yield 0.34 g (87%); UV [λ/nm (ϵ/M^{-1} cm^{-1})] 255 (2660), 280 sh (2580) [lit.[22] 255 (2800), 280 sh (2700)].

Table I. Products from the Reaction of *A. variabilis* Plastocyanin with $[Ru(NH_3)_5H_2O]^{2+}$ [$[PCu^{II}]$ = 0.48 mM, $[Ru(NH_3)_5H_2O]^{2+}$ = 21 mM, pH 7.5 (20 mM Hepes), Reaction Time 4.3 h]

band	% yield[a]	Ru/Cu	band	% yield[a]	Ru/Cu
1	40	no Ru	4	2	2.26[b]
2	11	1.00	5	4	
3	30	1.05			

[a] 21 mg of protein recovered following modification of 24 mg. [b] Determined for bands 4 and 5 combined.

Table II. Effect of Redox Cycling (RC) on Product Distribution for the *S. obliquus* Plastocyanin Ru Modification Reaction

band	% yield		Ru/Cu
	no RC[a]	RC[b]	
1	20	0	1.93
2	42	58	0.97
3	38	38	no Ru

[a] Modification conditions: $[PCu^{II}]$ = 0.20 mM, $[Ru(NH_3)_5H_2O]^{2+}$ = 11 mM, pH 7.5 (20 mM Hepes), reaction time 25 min, 20 mg of protein chromatographed. [b] Modification conditions: $[PCu^{II}]$ = 0.18 mM, $[Ru(NH_3)_5H_2O]^{2+}$ = 11 mM, pH 7.5 (20 mM Hepes), reaction time 20 min, 18.5 mg of protein chromatographed. Redox cycling as in the Experimental Section.

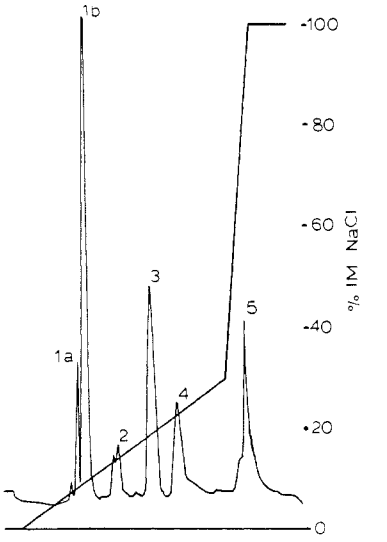

Figure 1. Elution profile by FPLC (absorbance monitored at 280 nm) of reaction mixture from Ru modification of *A. variabilis* plastocyanin. A Mono-S column in 20 mM acetate, pH 4.5, was used with a 0–30% linear gradient of 1 M NaCl. Labeled peaks: 1a, native PCu^{I}; 1b, native PCu^{II}. Other bands (2–5) as defined in the text. All the fractions except 1a were blue.

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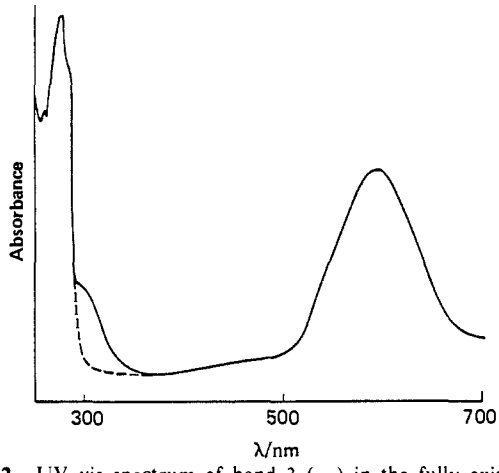

Figure 2. UV-vis spectrum of band 3 (—) in the fully oxidized *A. variabilis* $PCu^{II}Ru^{III}$ state compared with that of native PCu^{II} (- - -) at pH 7 (phosphate buffer). When the pH is increased to 9.5 (glycine buffer), the spectrum of band 3 changes, 300–450 nm, due to acid dissociation of the pyrrole nitrogen. The effect is similar to that reported for $[Ru(NH_3)_5(His)]^{3+}$, $pK_a = 8.7$.[24]

Modification Procedures. In a typical procedure *A. variabilis* plastocyanin PCu^{II} (28 mg, 0.48 mM) was treated with $[Ru(NH_3)_5H_2O]^{2+}$ (57 mg, 21 mM) in 10 mL of solution at pH 7.5 (20 mM Hepes buffer) for 4 h at room temperature, $I = 0.10$ M (NaCl). The same conditions were employed for *S. obliquus* PCu^{II} (17.5 mg, 0.18 mM) and $[Ru(NH_3)_5H_2O]^{2+}$ (38 mg, 11 mM), with a reaction time of 20 min only. In both cases the reaction was terminated by gel filtration on a Sephadex G25 column (3 × 25 cm), previously equilibrated with phosphate buffer (0.10 M, pH 7) under argon. The protein mixture obtained from the gel column was immediately oxidized with a small excess of $[Fe(CN)_6]^{3-}$ and the protein determined spectrophotometrically, assuming here and elsewhere that $\epsilon = 4500\ M^{-1}\ cm^{-1}$, at 597 nm.[23] The protein was then subjected to redox cycling. It was fully reduced with excess ascorbate and then left overnight at 4 °C after the ascorbate was removed by diafiltration. Under these conditions Ru(II) attached at other amino acid residues aquates and multiply modified *S. obliquus* protein is converted back to the singly modified derivative. Samples were subsequently reoxidized with $[Fe(CN)_6]^{3-}$ and diafiltered into the appropriate buffer for ion-exchange chromatography.

The *A. variabilis* plastocyanin mixture was loaded onto a CM52 cation-exchange column (1.5 × 8 cm) in 1 mM phosphate buffer, pH 7.0, and eluted with a 20–100 mM gradient of phosphate buffer, pH 7.0. The flow rate was 3 mL/min. Three blue bands were collected and concentrated by diafiltration. A small amount of protein that remained bound at the top of the column on completion of the gradient was eluted in 0.10 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. Two bands were resolved and collected as described above. Recovery of protein from the column was ≥90%. Relative yields of the five isolated bands are given in Table I, together with their Ru to Cu ratios. Small variations in reaction time, ionic strength, and protein concenIration did not dramatically affect the relative yields of the bands or alter their chromatographic properties. The product distribution in Table II is typical of a sample left in the reduced state for several hours before chromatography. If however the redox cycling was omitted and the protein mixture was chromatographed directly, the product distribution was weighted more toward the multiply modified species. The chromatographic separation described above was less satisfactory under these conditions due to some merging of protein bands during the 4-h elution period. This could be minimized by more rapid chromatographic separation (~20 min) using a Mono-S FPLC (Pharmacia) column. Protein was loaded in 20 mM acetate buffer, pH 4.5, and eluted in a linear gradient of 0–100% NaCl in this buffer. The elution profile is shown in Figure 1.

Recovery of *S. obliquus* plastocyanin following modification was 95%. Chromatographic separation of the oxidized protein mixture used a DE52 anion-exchange column, to which the modified (negatively charged in this case) protein is expected to bind less strongly than native protein. Elution in a 20–100 mM gradient of phosphate buffer, pH 7.4, resulted in the resolution of two bands if redox cycling had been performed before column loading or three bands if it had not. Bands were collected separately and concentrated by diafiltration. The relative yields of these bands and their corresponding Ru to Cu ratios are given in Table II.

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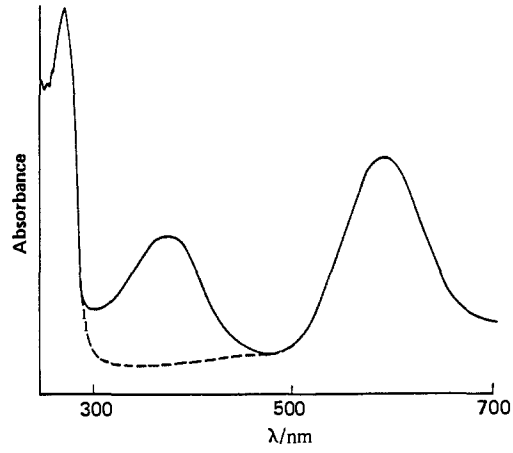
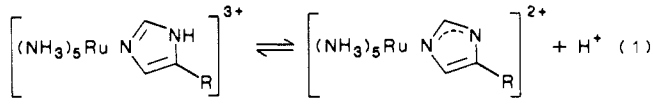


Figure 3. UV-vis spectrum of *A. variabilis* band 2 (—) in the fully oxidized $PCu^{II}Ru^{III}$ state compared with native PCu^{II} (- - -). The spectrum is independent of pH in the range 4.5–9.5.

Stability of Products. When fully oxidized *A. variabilis* and *S. obliquus* samples, $PCu^{II}Ru^{III}$, were rechromatographed after standing at 4 °C in 0.10 M phosphate (pH 7) for 48 h, some native PCu^{II} (<10%) was detected. Storage of a 5-mg sample of *A. variabilis* PCu^{II}/Ru^{III} (band 3) thawed and rechromatographed after 2 weeks at −20 °C contained no detectable native PCu^{II}. Observations on the *A. variabilis* $PCu^{II}Ru^{III}$ (band 2) suggested a similarly inert product.

UV-Visible Spectra. The *S. obliquus* $PCu^{II}Ru^{III}$ product and band 3 from the *A. variabilis* modification have similar spectra, illustrated for the latter in Figure 2. A new peak at 300 nm ($\epsilon \sim 2000\ M^{-1}\ cm^{-1}$) is a feature of both spectra that corresponds to that observed for $[Ru(NH_3)_5His]^{3+}$ at 303 nm ($\epsilon = 2100\ M^{-1}\ cm^{-1}$).[24] The peak at 597 nm remains unaltered, indicating little or no perturbation of the PCu^{II} active site as a result of modification. Both the 597- and 300-nm peaks disappear on ascorbate reduction to $PCu^{I}Ru^{II}$ with increased absorption at lower wavelengths. The pH dependence of the spectrum of *A. variabilis* $PCu^{II}Ru^{III}$ was investigated. At pH 9.5 in 20 mM glycine buffer the 300-nm peak decreases in intensity and a new peak at ~370 nm is observed. Readjustment to pH 7.0 gave the original spectrum, with a small (~10%) deterioration in purity. No change in spectrum was observed over the range pH 4.5–7.0. The changes observed are similar to those for $[Ru(NH_3)_5His]^{3+}$, which gives a displacement of the 300-nm peak to 365 nm ($\epsilon = 3350\ M^{-1}$), assigned as deprotonation of the pyrrole nitrogen of $[Ru(NH_3)_5His]^{3+}$ ($pK_a = 8.8$) (eq 1).[24] The pK_a of this deprotonation in free histidine is ~14.[22] The changes observed for *A. variabilis* $PCu^{II}Ru^{III}$ at pH 9.5 were somewhat less, suggesting a $pK_a > 8.8$.



Band 2 obtained for *A. variabilis* has a quite different spectrum (Figure 3) with a peak at 370 nm ($\epsilon \sim 2500\ M^{-1}\ cm^{-1}$) that is independent of pH in the range 4.5–9.5. Reduction by excess ascorbate resulted in loss of the 597-nm, but not the 370-nm, absorbance, indicating that the Ru(III) center is not reduced. Absorbance bands at 370 nm have been reported for *P. aeruginosa* azurin modified at His-83 to give *cis*-$[Ru(NH_3)_4(OH)His\text{-}83]$,[25] but the absence of pH effects on the UV-vis spectrum of band 2 does not support such an assignment in the present case. The spectroscopic properties of band 2 also seem to preclude the presence of a C-bound histidine complex.[22,24] The exact nature of this product therefore remains uncertain. Since no reaction is observed in the DEPC modification procedure (see below), it would appear that attachment is also at His-59. Loss of an NH_3 ligand and possibly further coordination to the peptide (or phosphate buffer[26]) may be occurring. This product is not considered further, and subsequent reference is made only to band 3 of the *A. variabilis* modification.

DEPC Modification. Absorbance changes observed on reacting *A. variabilis* native (44 μM) and Ru-modified protein (25 μM) with a 20-fold excess of diethyl pyrocarbonate (DEPC; Sigma) in 0.10 M

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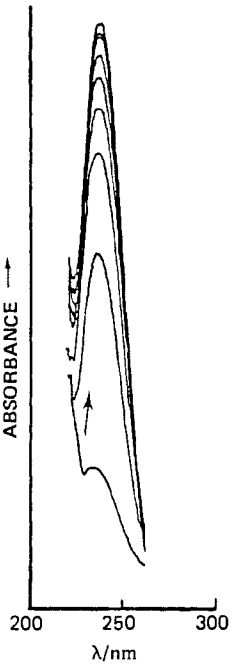

Figure 4. Increase in absorbance at ~238 nm on DEPC (diethyl pyrocarbonate) modification of *A. variabilis* plastocyanin. Spectra were recorded at 5-min intervals.

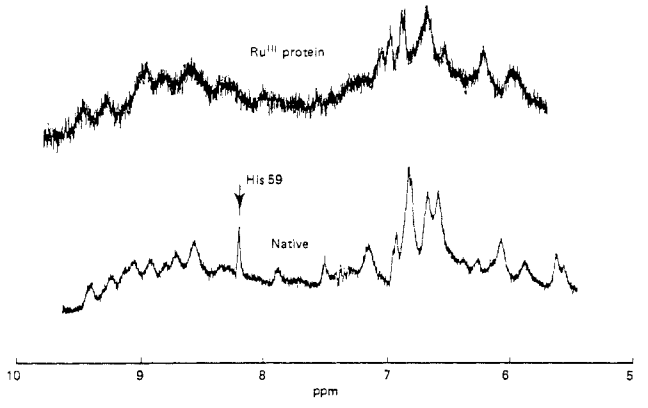

Figure 5. 300-MHz ^{1}H NMR spectra in the aromatic region for native *A. variabilis* PCu^{II} (lower spectrum) and the Ru(III)-modified protein (band 3).

phosphate (pH 7) at 25 °C are shown in Figure 4. The reaction is as in eq 2. Similar results were observed for *S. obliquus*. Spinach plastocyanin, which has no His-59, does not react with DEPC. After 40 min the extent of reaction with native *A. variabilis* and *S. obliquus* was ~84% based on the absorption coefficient for *N*-ethoxyimidazole at 240 nm of 3200 M^{-1} cm^{-1}.[27] For *N*-ethoxyhistidine in proteins a smaller value, 2900 M^{-1} cm^{-1}, has been reported,[28] and in the present work a value 2750 ± 100 M^{-1} cm^{-1} with a peak at 238 nm would seem appropriate. The Ru-modified proteins give little or no absorbance change at 238 nm consistent with Ru attachment at His-59, which prevents reaction with DEPC. The PCu^{II} spectrum at >280 nm was unaffected by DEPC modification.

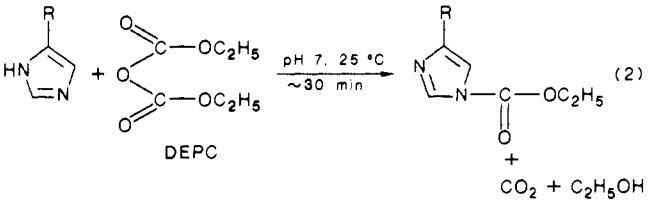

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Table III. Comparison of Reduction Potentials (25 °C) for Cu Active Site in Native and Ru-Modified Proteins, pH 7.0, I = 0.21 M [Errors in Parentheses]

protein	$E°$/mV (vs NHE) native	$E°$/mV (vs NHE) Ru modified	ref
spinach PCu^{II}/Ru^{I}	363 (2)		this work
A. variabilis PCu^{II}/Ru^{I}[a]	339 (2)[b]	340 (4)	this work
S. obliquus PCu^{II}/Ru^{I}[a]	363 (2)	385 (7)	this work
P. aeruginosa ACu^{II}/Ru^{I}[c]	308 (2)	320 (2)	4a

[a] Ru modified at His-59. [b] Value 342 mV determined by optical transparent thin-layer electrode (OTTLE) (J. A. Goodwin, this laboratory). [c] Ru modified at His-83.

NMR Spectra. Proton NMR spectra at 300 MHz were obtained for the fully oxidized protein in 0.10 M deuteriated phosphate buffer at pH 7.0 (no correction for the deuterium isotope effect is made). The aromatic regions of the ^{1}H NMR spectra of native and Ru-modified *A. variabilis* plastocyanin are compared in Figure 5. The most striking change due to modification is the disappearance of a sharp resonance at 8.25 ppm, which has been assigned previously to the imidazole C_2H proton of His-59.[22] The C_4H proton resonance should occur at 6.75 ppm at pH 7.0. However, the complex nature of the spectrum in this region prevented its assignment here.

Reduction Potentials. These were determined for native and Ru-modified plastocyanin by titration of the oxidized protein with $[Fe(CN)_6]^{4-}$. The separation of the $[Fe(CN)_6]^{3-/4-}$ (410 mV)[29] and $[Ru(NH_3)_5His]^{3+/2+}$ (80 mV)[30] reduction potentials is sufficient to ensure that negligible reduction to Ru(II) occurs. For *A. variabilis* plastocyanin six protein solutions were prepared containing 0.50 mL of fully oxidized protein in 0.10 M phosphate buffer, pH 7. Aliquots of 0, 0.03, 0.05, 0.20, 0.30, and 0.40 mL of buffered $[Fe(CN)_6]^{4-}$ (1.22 mM) were added by microsyringe, and the volume was adjusted to 1 mL in each case. For *S. obliquus* plastocyanin consecutive additions were made to one solution and corrections for the volume change made. The absorbance of the protein (53 μM) at 597 nm was measured after 5 min for equilibration. Since PCu^{II} is the only absorbing species at 597 nm (ϵ = 4500 M^{-1} cm^{-1}), equilibrated concentrations could be determined and K calculated for each solution. Hence, $E°$ values (Table III) were obtained. If an absorption coefficient for PCu^{II} of 5000 M^{-1} cm^{-1} rather than 4500 M^{-1} cm^{-1} is taken, the $E°$ value obtained increased by ~5 mV.

Other Ru Products. Brief mention is made here of the more extensively Ru-modified products (Ru/Cu ≈ 2/1), bands 4 and 5 (*A. variabilis*), and a single *S. obliquus* product. UV-vis spectra of the *S. obliquus* and band 5 *A. variabilis* products are like those of the singly modified products (band 3 for *A. variabilis*) but with the 300-nm peak more intense ($\epsilon \approx$ 2500 M^{-1} cm^{-1}). Band 4 gives a broad absorbance centered around 300 nm that is less intense ($\epsilon \approx$ 1000 M^{-1} cm^{-1}) than for the band 3 product. As already indicated, storage of these products in the reduced form leads to the formation of the singly Ru-modified products. Further characterization was not attempted.

Pulse Radiolysis. All solutions were made in triply distilled water with phosphate buffer (Analar salts). Experiments were carried out at the Cookridge Radiation Research Centre, using 1- or 5-cm light path cells and a beam of 2.5 MeV electrons. Pulse lengths were in the 0.2–1.2-μs range. Some data were also obtained at Paterson Laboratories, Christie Hospital, Manchester, using a 2.5- or 10-cm light path cell. The yields of reducing radical (R) for a given pulse were calculated from (3). The

$$[R] = V(SEC) \times S(SEC) \times G_R \quad (3)$$

secondary emission chamber voltage, V(SEC), was measured directly, and the sensitivity, S(SEC), obtained by thiocyanate dosimetry.[31] Here G_R is the radiation chemical yield of radicals per joule of energy absorbed by the system.

Pulse radiolysis was performed at 19 ± 1.5 °C on N_2O-saturated solutions containing the oxidized protein, either PCu^{II} or $PCu^{II}Ru^{III}$, in the concentration range 1–23 μM. Samples of Ru-modified protein were purified by column chromatography the day prior to kinetic experiments to remove any traces of native protein. They were then oxidized and exhaustively diafiltered into 0.10 M phosphate buffer generally at pH 7.0, but in some experiments (to check the effect of pH) at pH 6.2 or 7.8. The absorbance of solutions prior to pulsing confirmed that the protein was present as $PCu^{II}Ru^{III}$.

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Solutions also contained 0.10 M sodium formate to give formate radicals, $CO_2^{\bullet-}$, on pulsing. Formate radicals are generated as the sole (radical) product from a number of highly reactive primary radicals. Their reactivity while high (−2.0 V)[32] is lower than that of e^-_{aq} (−2.9 V),[33] and they are less likely to undergo spurious radical trapping at the protein surface. Moreover, they do not absorb at 597 nm (unlike e^-_{aq}). Several previous reports involving the reduction of metalloproteins by $CO_2^{\bullet-}$ have been reviewed by Buxton.[34] The ionic strengths were 0.31, 0.22, and 0.39 M, respectively, for pH 7, 6.2, and 7.8 runs. For reactions with the methyl viologen radical, $MV^{\bullet+}$, solutions contained initially 2 mM MV^{2+} (systematic name 1,1′-dimethyl-4,4′-bipyridylium ion). For both $CO_2^{\bullet-}$ and $MV^{\bullet+}$ radicals G_R is 7.38×10^{-7} mol J^{-1}.[32]

An estimate of the efficiency of the reduction was made by comparing the yield of $CO_2^{\bullet-}$ with the amount of protein reduced. At 23 μM $PCu^{II}Ru^{III}$ the efficiency was 80–90%, but this dropped to only ~10% for 2 μM $PCu^{II}Ru^{III}$. At lower protein concentrations the bimolecular decay of $CO_2^{\bullet-}$ occurs ($2CO_2^{\bullet-} \rightarrow C_2O_4^{2-}$, rate constant 7×10^8 M^{-1} s^{-1} at pH 7.0, I = 0.31 M).[35] Reduction efficiencies were, as far as could be ascertained, the same for both plastocyanins. With knowledge of these efficiencies the dose was adjusted so that the expected reduction of $PCu^{II}Ru^{III}$ was <20%. This constraint decreased the probability of double reduction to yield $PCu^{I}Ru^{II}$ and also ensured that $PCu^{II}Ru^{III}$ was in >10-fold excess over $PCu^{II}Ru^{II}$. Examples of doses: (i) 1-cm cell with 23 μM $PCu^{II}Ru^{III}$, S(SEC) = 23 Gy V^{-1}, V(SEC) 0.23 V, dose 5.3 Gy; (ii) 5-cm cell with 2 μM $PCu^{II}Ru^{III}$, S(SEC) 1.5 Gy V^{-1}, V(SEC) ≈ 3 V, dose 4.5 Gy. Absorbance (A) changes were calculated using (4),

$$\Delta A = \log (V_t/V_0) \quad (4)$$

where V is the photomultiplier voltage, and V_0 and V_t are the signals from the photomultiplier before the pulse and at time t after the pulse, respectively. Reduction of $PCu^{II}Ru^{III}$ was monitored at 597 nm. Light of wavelength below 550 nm was excluded by an OG550 filter. In the final analysis, only traces obtained from first pulses were considered in order to avoid complications arising from the buildup of reduced species.

A sample of Ru-modified *A. variabilis* plastocyanin partially reduced by pulse radiolysis at pH 7.0 was chromatographed on a Mono-S FPLC column in 20 mM acetate buffer, pH 5.2. The majority (>95%) of the protein eluted at 15–17% 1 M NaCl, consistent with retention of the Ru label during pulse radiolysis.

Stopped-Flow Studies. The rate constant for reaction of *S. obliquus* $PCu^{I}Ru^{II}$ (fully reduced) with $PCu^{II}Ru^{III}$ (fully oxidized) protein was determined at 17 °C under buffer conditions as in the pulse radiolysis experiments (I = 0.31 M, pH 7.0). Reactant concentrations were $PCu^{I}Ru^{II}$ (34 μM) and $PCu^{II}Ru^{III}$ (4 μM), giving a small (<0.035) absorbance change. Concentrations of air-sensitive $PCu^{I}Ru^{II}$ were moreover small for handling on the stopped flow. The $[Ru(NH_3)_5Im]^{2+}$ reduction of *A. variabilis* and *S. obliquus* PCu^{II} was also studied by this method.

Treatment of Data. Pulse radiolysis traces retrieved from disc file were subjected to first-order kinetic analysis. From absorbance changes, plots of $-\ln [(A_\infty - A_t)/(A_\infty - A_0)]$ against time were linear to at least 3 half-lives. Computed rate constants were extracted by weighted linear least-squares fitting procedures. In some cases (generally when reduction was >10%), a slight downward drift in A_∞ was observed. These traces were treated by the Guggenheim method,[36] taking a Δt of at least 2 half-lives. Rate constants were obtained by standard least-squares fitting procedures.

Results

***A. variabilis* Plastocyanin.** The $CO_2^{\bullet-}$ reduction of native PCu^{II} (23 μM) gave one kinetic stage only, rate constant $(8 \pm 2) \times 10^8$ M^{-1} s^{-1}. With $PCu^{II}Ru^{III}$ the combined rate constant obtained by monitoring the Cu(II) decay is $k_a = (6.7 \pm 1.5) \times 10^8$ M^{-1} s^{-1}. No further absorbance changes are observed for times up to 0.2 s (6 μM protein). However, on a longer time base (2 s/div for 6 μM protein) a slow decay is observed. These two stages have been illustrated previously.[37] If a sample was repeatedly pulsed, and each time allowed to reach a steady A_∞, then $PCu^{I}Ru^{III}$ accumulated. Under such conditions the fractional absorbance change for stage 1 to stages 1 and 2 combined, the selectivity factor F progressively decreased, as did the first-order rate constant (k_b) for stage 2. From first-pulse traces $F = 0.72 \pm 0.06$, and the dependence of k_b (Table IV) on $[PCu^{II}Ru^{III}]$ is as shown in Figure 6, which can be expressed as in (5). The intercept gives k_1 = 0.024 ± 0.058 s^{-1} and the slope $k_2 = (1.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1}. The first stage of reaction can therefore be expressed as in (6),

$$k_b = k_1 + k_2[PCu^{II}Ru^{III}] \quad (5)$$

$$CO_2^{\bullet-} + PCu^{II}Ru^{III} \xrightarrow{k_a} \begin{matrix} PCu^{I}Ru^{III}\ (72\%) \\ PCu^{II}Ru^{II}\ (28\%) \end{matrix} \quad (6)$$

with the second stage consisting of in-parallel first-order intramolecular (k_1) and second-order intermolecular (k_2) steps (7) and (8).

$$PCu^{II}Ru^{II} \xrightarrow{k_1} PCu^{I}Ru^{III} \quad (7)$$

$$PCu^{II}Ru^{II} + PCu^{II}Ru^{III} \xrightarrow{k_2} PCu^{II}Ru^{III} + PCu^{I}Ru^{III} \quad (8)$$

Two stages were also observed for runs at pH 6.2 and 7.8, and the fractional absorbance remained essentially unchanged. The dependence of k_b on $[PCu^{II}Ru^{III}]$ was not determined at these pH's, and the different ionic strengths pertaining make it difficult to assess whether there are any trends in data.

In the presence of methyl viologen, MV^{2+} (2 mM), all the $CO_2^{\bullet-}$ radicals are converted to $MV^{\bullet+}$. In contrast to $CO_2^{\bullet-}$, $MV^{\bullet+}$ is

Table IV. Rate Constants (~20 °C) for Reaction of *A. variabilis* $PCu^{II}Ru^{III}$ with $CO_2^{\bullet-}$, pH 7.0, I = 0.31 M

$10^6[PCu^{II}Ru^{III}]^a$/M	F^b	k_b/s^{-1}	$10^6[PCu^{II}Ru^{III}]^a$/M	F^b	k_b/s^{-1}
1.4	0.80	0.38	4.4	0.79	1.06[e]
1.4	0.74	0.16[c]	4.5	0.73	0.44
1.8	0.75	0.14	4.7	0.71	0.45
1.8	0.78	0.15	5.6	0.67	0.69
1.8	0.72	0.18	5.7	0.71	0.87
1.8	0.68	0.28	8.1	0.79	1.1
1.8	0.72	0.37	11.4	0.80	0.99
3.1	0.63	0.28	11.4	0.81	1.25
3.1	0.71	0.29	11.4	0.78	1.58
3.1	0.70	0.31	11.5	0.76	1.58
3.2	0.75	0.32	11.6	0.70	1.47
3.2	0.79	0.44	17.6	0.65	2.3
3.3	0.97	0.40[d]	18.7	0.62	2.5
3.4	0.62	0.60	20.9	0.63	2.0

[a] Concentration remaining after first pulse. [b] Ratio of absorbance change for first stage to that for first and second stages combined. [c] Average of two runs at pH 7.8. [d] Average of four runs at pH 6.2. [e] Average of four runs at pH 7.8.

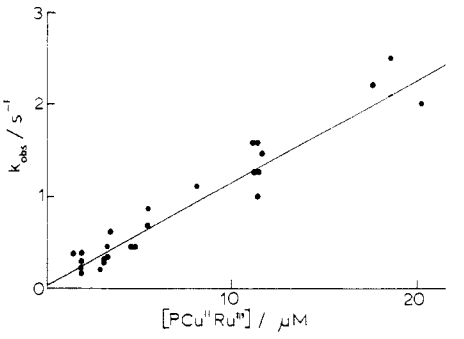

Figure 6. First-order rate constants k_b (~20 °C), for the second stage of reaction in pulse radiolysis experiments on *A. variabilis* band 3 Ru-modified plastocyanin. The rate constant is for the decay (597 nm) of $PCu^{II}Ru^{II}$ (generated in the first stage) by intermolecular (slope) and intramolecular (intercept) processes, pH 7.0 (0.10 M phosphate), I = 0.31 M.

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(33) Swallow, A. J. *Radiation Chemistry*; Longmans: New York, 1973.
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Table V. Rate Constants (~20 °C) for the Reaction of *S. obliquus* $PCu^{II}Ru^{II}$ with $CO_2^{\bullet-}$, pH 7.0, I = 0.31 M

$10^6[PCu^{II}Ru^{III}]^a$/M	F^b	k_b/s^{-1}	$10^6[PCu^{II}Ru^{III}]^a$/M	F^b	k_b/s^{-1}
0.6	0.76	0.20[c]	13.4	0.52	3.8[c]
1.1	0.76	0.40	15.1	0.60	4.0[c]
1.8	0.67	0.58	15.1	0.58	4.1[c]
1.8	0.71	0.76	15.1	0.81	4.4
7.6	0.77	3.0	15.1	0.81	4.6
8.1	0.80	2.5	15.1	0.72	4.9
8.2	0.74	3.3	22.5	0.73	7.7
13.0	0.71	4.3	22.7	0.80	7.9

[a]Concentration after first stage of pulse radiolysis. [b]Ratio of absorbance change in first stage to that of first and second stages combined. [c]Runs taken out in second analysis of data.

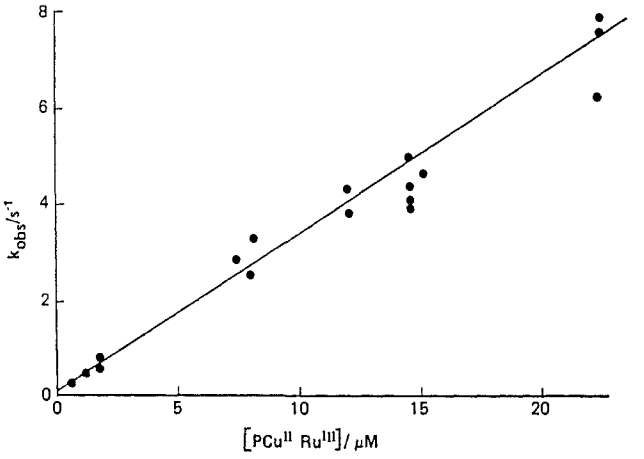

Figure 7. First-order rate constants k_b (~20 °C) for the second stage of reaction in pulse radiolysis experiments on Ru-modified *S. obliquus* plastocyanin. The rate constant is for the decay (597 nm) of $PCu^{II}Ru^{II}$ (generated in the first stage) by intermolecular (slope) and intramolecular (intercept) processes, pH 7.0 (0.10 M phosphate), I = 0.31 M.

relatively long-lived and should react quantitatively with $PCu^{II}Ru^{III}$. The yield of $MV^{\bullet+}$ ($G_R = 7.38 \times 10^{-7}$ mol J^{-1})[35] was restricted by controlling V(SEC) such that $[MV^{\bullet+}] \leq 5[PCu^{II}Ru^{III}]$. An essential difference is that $MV^{\bullet+}$ absorbs strongly at 597 nm ($\epsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}).[38] The rate constant for the first stage is 2.5×10^8 M^{-1} s^{-1}. The selectivity F is now ~0.9, and the small amplitude of the change for the second stage makes it very difficult to determine k_b with any precision.

***S. obliquus* Plastocyanin.** Native PCu^{II} is reduced by $CO_2^{\bullet-}$ in a single step, rate constant (7.8 ± 1.0) $\times 10^8$ M^{-1} s^{-1}, with no subsequent absorbance changes. With $PCu^{II}Ru^{III}$ a second stage was again observed on a slower time base, giving first-order rate constants k_b (Table V). A plot of k_b against $[PCu^{II}Ru^{III}]$ is shown in Figure 7, from which $k_1 = 0.04 \pm 0.22$ s^{-1} and $k_2 = (3.2 \pm 0.2) \times 10^5$ M^{-1} s^{-1}, with the selectivity factor 0.72 ± 0.08. By removing one point in which 40% reduction occurred and three points in which F lies >1.5 standard deviations from the mean F, the recalculated values are $F = 0.75 \pm 0.04$, $k_1 = 0.08 \pm 0.19$ s^{-1}, and $k_2 = (3.3 \pm 0.2) \times 10^5$ M^{-1} s^{-1}. Clearly the inclusion of these points does not affect the interpretation.

Absorbance–time traces for the stopped-flow study of the reaction of $PCu^{I}Ru^{II}$ (34 μM) with $PCu^{II}Ru^{III}$ (4 μM) were noisy (small-amplitude change) and gave slightly biphasic behavior (breakdown of pseudo-first-order conditions). Rate constants from four traces were 1.31, 1.70, 1.71, and 2.20 s^{-1}, giving 1.7 ± 0.3 s^{-1}, from which a second-order rate constant (5.1 ± 0.9) $\times 10^4$ M^{-1} s^{-1} is obtained. This compares with $k_2 = (3.4 + 0.2) \times 10^5$ M^{-1} s^{-1} from pulse radiolysis and suggests that the assignment of this process to the reaction of $PCu^{II}Ru^{II}$ with $PCu^{II}Ru^{III}$ is a reasonable one.

Reduction of PCu^{II} with $[Ru(NH_3)_5Im]^{2+}$. A stopped-flow study of the reaction of *A. variabilis* and *S. obliquus* PCu^{II} with $[Ru(NH_3)_5Im]^{2+}$ indicated rate constants too fast to follow at 8.7 °C with the complex in 10-fold excess of protein. With the complex and protein in equal concentrations (1.4×10^{-5} M) second-order rate constants of $>4 \times 10^6$ M^{-1} s^{-1} were determined at pH 7.8 (Tris-HCl), I = 0.10 M (NaCl). It was demonstrated from competitive inhibition studies with redox-inactive $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ that ~50% of the reaction with *S. obliquus* is at the acidic residues in the vicinity of Tyr-83, and when a value of 400 M^{-1} is assigned for the equilibrium constant K for prior association of the two reactants (which is likely to be high for a 2+ reactant) an estimate $k_{et} > 5 \times 10^3$ s^{-1} is obtained.[19b,c]

Reaction of Ru-Modified Azurin. A sample of *P. aeruginosa* azurin was modified by the procedure previously described.[4] The modification at His-83 was confirmed by absence of a DEPC reaction. With a similar pulse radiolysis procedure as above with $CO_2^{\bullet-}$ reductant, four runs were carried out and an intramolecular rate constant for Ru(II) → Cu(II) electron transfer of 2.5 ± 0.8 s^{-1} was obtained in satisfactory agreement with the value of 1.9 ± 0.4 s^{-1} reported from flash photolysis studies.[4b] The value of k_b (here k_1) was independent of repeated pulsing, in contrast to the Ru-modified plastocyanin studies.

Discussion

The $[Ru(NH_3)_5H_2O]^{2+}$ modification of *A. variabilis* plastocyanin is at least 1 order of magnitude slower than that for *S. obliquus*, consistent with the 1+ and 9– charges, respectively, on PCu^{I}. Yields of Ru-modified protein from (9) were ~85% and

$$PCu^{I}\text{His-59} + [Ru(NH_3)_5H_2O]^{2+} \rightarrow PCu^{I}\text{His-59}Ru(NH_3)_5 + H_2O \quad (9)$$

~95%, respectively. The pH was maintained at 7.5 so that formation of the low-pH form of PCu^{I} (with His-87 protonated) was negligible.[9,12b] The *A. variabilis* product pattern is more complicated with two 1:1 Cu to Ru products. The major product (band 3) was spectroscopically similar to the 1:1 product of the *S. obliquus* modification, both giving an absorbance peak at 300 nm ($\epsilon \approx 2000$ M^{-1} cm^{-1})[24] as is observed for $[Ru(NH_3)_5His]^{3+}$. Further characterization of these 1:1 products included the observation that (a) there is no His-59 modification by DEPC and (b) the presence of Ru(III) results in the loss of the ^{1}H NMR His-59 δ 8.2 signal due to paramagnetic line broadening.

The reduction potential of the PCu^{II}/PCu^{I} component in the two Ru-modified products has been determined. For *A. variabilis* the perturbation is so small as to be experimentally undetectable (<5 mV), while for *S. obliquus* a small (20-mV) increase was detected. In the cases of Ru-modified azurin (Table III),[4] cytochrome *c*,[5] and myoglobin,[6] marginally higher values are also observed as compared to the native protein. The Ru(III)/Ru(II) reduction potential is not as readily accessible by spectrophotometric titration, but for other modified proteins it has been shown to lie in the range 75–90 mV, compared to 80 mV for the $[Ru(NH_3)_5His]^{3+/2+}$ couple.[4-6]

Turning to the side products of the Ru modification reactions, the doubly modified derivatives were readily depleted to 1:1 products on redox cycling, indicating that at alternative modification sites the Ru(II) is more labile. The second site for modification clearly cannot be a further histidine, since the 597-nm peak is unperturbed, and His-37 and His-87 must therefore remain coordinated to the Cu. The identity of the second site(s) remains uncertain, and carboxylate residues cannot for example be ruled out[39] in view of the absorbance of the doubly modified products at 300 nm (absorption coefficient, M^{-1} cm^{-1}): *A. variabilis* band 4, 1000; *A. variabilis* band 5, 2500; *S. obliquus* band 1, 3000. Isolation of a second 1:1 product absorbing at 370 nm in the *A. variabilis* case (band 2) is of interest in light of similar observations reported by Matthews and co-workers from the Ru modification of ribonuclease A[1] and lysozyme.[2] In the latter case the broad ~350-nm band was reported to be insensitive to pH in the range 5–11. With ribonuclease A a component absorbing at 350 nm

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was detected at reaction times >12 h.

In the pulse radiolysis studies, reduction by the formate radical $CO_2^{\bullet-}$ is rapid for both *A. variabilis* and *S. obliquus* PCu^{II} plastocyanins, with rate constants close to 8×10^8 M^{-1} s^{-1} for unmodified protein and 7×10^8 M^{-1} s^{-1} for Ru(III)-modified protein (*A. variabilis* only). These values are consistent with the reaction of $CO_2^{\bullet-}$ at the uncharged hydrophobic north surface of the protein, since other surface regions of *A. variabilis* and *S. obliquus* have significantly different charge distributions.

Rapid reduction of $PCu^{II}Ru^{III}$ is partitioned between the Cu(II) and Ru(III) centers according to eq 6. The combined rate constant k_a in eq 6 is obtained by monitoring the Cu(II) reduction at 597 nm. Since PCu^{II}/PCu^{I} (~370 mV) and $[Ru(NH_3)_5His]^{3+/2+}$ (~80 mV)[3b] have different reduction potentials, $PCu^{II}Ru^{II}$ is thermodynamically unstable, and this can subsequently decay by intramolecular (first-order) and intermolecular (second-order) processes, k_1 and k_2, respectively, in eq 5, which together constitute the second stage of reaction (k_b).

For the reaction scheme proposed, the fractional absorbance change F, defined as the fraction of the total absorbance change occurring in the first stage, can be equated to the selectivity of the reducing radical $CO_2^{\bullet-}$ for Cu(II) over Ru(III). For both the Ru-modified proteins with $CO_2^{\bullet-}$ the selectivity is 0.72 ± 0.06. When $MV^{\bullet+}$ is the reductant for *A. variabilis* $PCu^{II}Ru^{III}$, the selectivity is ~0.9 in favor of the Cu site. In their studies on Ru-modified horse heart cytochrome *c* Isied et al.[3c] found a variation in selectivity for the heme iron of 0.65–0.05 using a range of reducing radicals, including $CO_2^{\bullet-}$ (~0.3). The minimum value obtained was for the polyhydroxylic pentaerythritol radical $C(CH_2OH)_3(\dot{C}HOH)$. The favorable selectivity for the $[Ru(NH_3)_5His]^{3+}$ center shown by the radical was ascribed to its hydrophobic nature. In the present study the unfavorable selectivity for $[Ru(NH_3)_5His]^{3+}$ using $MV^{\bullet+}$ (−450 mV)[38] can be ascribed to the positive charge on $MV^{\bullet+}$ and the aromaticity of $MV^{\bullet+}$, which is expected to favor reactions at the hydrophobic northern end of plastocyanin close to the active site.

For pulse radiolysis doses giving <20% reduction of $PCu^{II}Ru^{III}$, the amount of doubly reduced protein will be small (<5%). Taking into account the selectivity factor, it can be concluded that $[PCu^{II}Ru^{II}]$ is <7% of $[PCu^{II}Ru^{III}]$. Thus pseudo-first-order conditions apply in considering the reaction of $PCu^{II}Ru^{II}$ with $PCu^{II}Ru^{III}$. Plots of k_b against $[PCu^{II}Ru^{III}]$, illustrated for *A. variabilis* (Figure 6) and *S. obliquus* (Figure 7), reveal the relative contributions of the intramolecular (k_1) and intermolecular (k_2) processes (eq 5). Values of k_1 obtained for electron transfer from $[Ru(NH_3)_5His\text{-}59]^{2+}$ to the Cu(II) are 0.024 ± 0.058 s^{-1} in the case of *A. variabilis* and 0.04 ± 0.22 s^{-1} for *S. obliquus*. The magnitude of the errors indicates small values of k_1 that are at the most 0.08 and 0.26 s^{-1}, respectively. The error ranges clearly do not exclude the possibility that k_1 is zero.

Values of the intermolecular rate constant k_2, 1.2×10^5 M^{-1} s^{-1} for *A. variabilis* and 3.2×10^5 M^{-1} s^{-1} for *S. obliquus*, are well-defined. This process will be sensitive to any differences in driving force and electrostatic work terms for association of the protein–protein complexes. The ionic strength of the medium is quite high (I = 0.31 M), which will help to shield electrostatic interactions betwen the proteins. The close similarity in k_2 for the two plastocyanins is somewhat surprising but may be rationalized by considering the estimated net charges on the reacting species. For Ru-modified *A. variabilis* at pH 7 these are 4+/5+, and for *S. obliquus*, 7−/6−, with different charge distributions applying in the two cases. The driving force is probably larger by ~40 mV for the Cu site of *S. obliquus* than for *A. variabilis*, which would help to compensate for the presumably less favorable electrostatic work term. For Ru-modified *S. obliquus* the related rate constant determined by stopped-flow kinetics, 5×10^4 M^{-1} s^{-1} (17 °C), is 1 order of magnitude slower. Unless the oxidation state of the Cu significantly perturbs the reduction potential of the $[Ru(NH_3)_5His\text{-}59]^{3+/2+}$ couple, the driving force should be about the same for both reactions. While the disparity between these results is larger than expected, it should be remembered that the accuracy of the stopped-flow study was well below that normally considered reliable ($\Delta A \leq 0.05$). In addition, spectrophotometric methods could not be used to ensure that the reagent in excess, $PCu^{I}Ru^{II}$, was fully reduced. The stopped-flow experiment did serve to confirm that, in the concentration range 1–23 μM for Ru-modified protein, bimolecular protein–protein reactions are slow and require seconds rather than milliseconds for their completion.

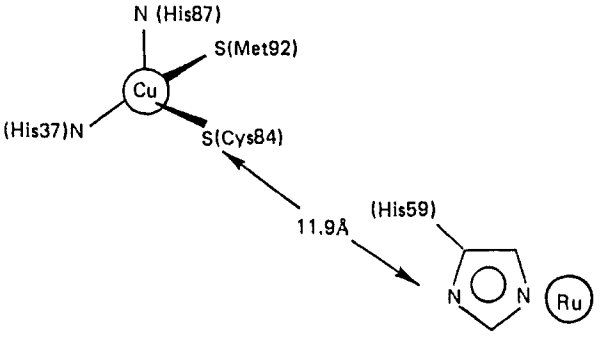

Figure 8. Representation of the edge-to-edge separation of the donor and acceptor sites in Ru-modified *A. variabilis* plastocyanin.

In summary, the direct one-electron reduction of $PCu^{II}Ru^{III}$ by $CO_2^{\bullet-}$ takes place mainly at the Cu(II), but a significant fraction (28 ± 8%) of reaction is at the Ru(III) center. The subsequent decay of $PCu^{II}Ru^{II}$ to $PCu^{I}Ru^{III}$ is dominated by an intermolecular process, and the rate constant for the intramolecular Ru(II) to Cu(II) electron-transfer process is small (<0.26 s^{-1}). This is a surprisingly low value with important implications regarding current understanding of such reactions.

The effect of donor–acceptor separation on rate constants for biological electron transfer is a subject of both theoretical[40] and experimental interest.[41,42] An equation relating distance d and rate constant k_{intra} has been proposed (10), where β is the

$$k_{intra} = k_0 \exp(-\beta d) \qquad (10)$$

transmission coefficient, a measure of the intrinsic coupling of donor and acceptor sites, and k_0 is the hypothetical rate constant at a van der Waals contact distance. The separation of the donor and acceptor sites in the two Ru-modified plastocyanins has been determined, where in both cases the shortest distance separating the delocalized metal centers is defined as N^{δ} (His-59) to S (Cys-84).

An estimate of this distance in *A. variabilis* has been provided with help from Professor Freeman's group in Sydney.[12c] The Glu-59 residue in the crystal structure of poplar plastocyanin was replaced by a histidine in the program FRODO, assuming an ideal geometry for the imidazole. In its ideal conformation the histidine is facing out into solution, and the separation distance is 11.9 Å. A closer approach on rotation of the His-59 is unlikely because of unfavorable contacts with Ser-85 (Glu-85 in *A. variabilis*). Although measured accurately, it is to be emphasized that the value obtained is only an approximation to the true donor–acceptor separation, since the possible effect of Ru attachment on local protein conformation has not been considered. In other Ru-modified proteins, such perturbation of the peptide chain conformation appears to be minimal.[3a,4] Another source of error that cannot be ruled out is the effect of local differences in peptide chain conformation between poplar and *A. variabilis* plastocyanins.[14] However, both sequence[43] and NMR[20,44-46] data suggest strong structural homologies for the whole plastocyanin family.

The comparable distance in *S. obliquus* plastocyanin has been estimated from a solution structure generated from the results

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Table VI. Comparison of Rate Constants[a] for Intramolecular Electron Transfer in Ru-Modified Metalloproteins

protein[b]	d/Å	$\Delta E°$[c]/mV	k/s^{-1}	ref
cytochrome *c* (horse heart)	11.8	180	30, 53	3b,c
azurin (*P. aeruginosa*)	11.8	240	1.9	4a
myoglobin (sperm whale)	13.3	−20	0.02	5b
plastocyanin (*A. variabilis*)	11.9	260	<0.08	this work
plastocyanin (*S. obliquus*)	10–12	290	<0.26	this work

[a] Temperature 25 °C, except for plastocyanin ~20 °C. [b] Ru attachment sites are His-33 for cytochrome *c*, His-83 for azurin, His-48 for myoglobin, and His-59 for plastocyanin. [c] Assuming an $E°$ = 80 mV for $[Ru(NH_3)_5His]^{3+/2+}$.

of 2D correlation NMR experiments.[11] The deletion of residues 57 and 58 slightly alters the peptide chain conformation, and as a result d is in the range 10–12 Å, with the most likely distance close to 10 Å. Therefore, a distance 10–12 Å encompasses not only both plastocyanins but also the Ru-modified metalloproteins cytochrome c[3a] and azurin,[4a] in which rate constants of 30–53[3b,c] and 1.9 s^{-1} [4b] have been observed for the intramolecular reoxidation of Ru(II). Clearly, distance alone is not rate limiting in the two plastocyanin cases.

As far as the energetics of electron transfer are concerned, both the reorganizational energy and driving force should be considered. Reorganizational energy, λ, can be split into inner- and outer-sphere contributions λ_i and λ_o.[40] The geometry at the Cu site is a compromise between preferred Cu(II) and Cu(I) geometries so that inner-sphere reorganization is minimized. UV–vis and $E°$ measurements indicate that this situation is essentially unchanged for Ru-modified plastocyanin. Also, since the Cu site is buried and contains no detectable solvent H_2O,[12] λ_o is expected to be small. Therefore, λ at the Cu site is not expected to be rate determining.

It is likely that the $[Ru(NH_3)_5His]^{3+/2+}$ environment in Ru-modified plastocyanin is closely analogous to that in other Ru-modified metalloproteins. Reduction potentials for $[Ru(NH_3)_5His]^{3+/2+}$ in Ru-modified cytochrome *c*, azurin, and myoglobin are all in the range 75–90 mV[4-6] and similar to that observed for unattached $[Ru(NH_3)_5His]^{3+,2+}$ (80 mV). The thermodynamic driving force for Ru(II) → Cu(II) intramolecular electron transfer in Ru-modified plastocyanin is not therefore expected to be rate limiting. Electron-transfer data for the systems under discussion are summarized in Table VI.[6] For myoglobin (which is not an electron-transport protein) reorganization of the high-spin heme Fe site is large due to the loss of H_2O on reduction of the Fe(III) state. This appears to retard substantially the Ru(II) → Fe(III) electron transfer.

The effect of intervening medium on electron transfer in metalloproteins is not easy to assess experimentally. Theoretical studies by Larsson[46] have suggested that any material positioned between the donor and acceptor sites will enhance the rate of electron transfer. The energy of some intervening structure elements (such as aromatic rings) may enhance electronic coupling between the donor and acceptor, as has recently been demonstrated in site-directed mutagenesis studies involving the reaction of mutant cytochrome *c* forms with Zn-substituted cytochrome *c* peroxidase,[47] where removal of aromatic residues in the electron-transfer path results in a decrease in the rate of 4 orders of magnitude.

In the case of plastocyanin one favorable route for electron transfer to and from Cu has been established,[8] involving a surface site in the vicinity of solvent exposed Tyr-83 (or Phe-83 for *S. obliquus*) and Asp-42. The donor–acceptor separation between the carboxylate of Asp-42 and the S atom of Cys-84 is estimated to be ~14 Å. For reactions such as the $[Co(phen)_3]^{3+}$ oxidation of PCu[I] in which saturation kinetics apply, rate constants (k_{et}) for electron transfer within the PCu[I], $[Co(phen)_3]^{3+}$ precursor complex have been determined and are 5.75 s^{-1} for plastocyanin from parsley, 23 s^{-1} for spinach, and 26 s^{-1} for *S. obliquus*.[8,18,19] These values are substantially bigger than k_1 despite a driving force of less than 20 mV and a less favorable self-exchange rate constant (45 M^{-1} s^{-1}) for the $[Co(phen)_3]^{3+/2+}$ couple.[48] Also as a part of this study we estimate that $k_{et} > 5 \times 10^3$ s^{-1} for the reaction of $[Ru(NH_3)_5Im]^{2+}$ with *A. variabilis* and *S. obliquus* PCu[II], indicating that unattached $[Ru(NH_3)_5Im]^{2+}$ is able to select a site to its advantage as compared to the His-59 site (which is in fact closer to the Cu). As for other Ru-modified proteins the histidine is most probably located at a physiologically irrelevant site. There are no aromatic residues between His-59 and the Cu, but such residues at positions 80 and 82 close to the Tyr-83/Phe-83 emphasize the availability of aromatic residues at (or close to) this site. This cannot be the only factor, however, since in the case of Ru-modified cytochrome *c* and azurin there are no intervening aromatic residues and quite favorable electron transfer is observed. In the case of plastocyanin we note that Tyr-83 (via Cys-84) and Asp-42 (via His-37) are linked directly to the Cu active site, generating a connecting channel that by providing low-lying orbitals may further assist electron transfer.

The results obtained in this study support the belief that quite specific spatial alignment of redox partners is required for electron transfer in the case of plastocyanin. Intramolecular (or intra-adduct) electron transfer from the Tyr-83/acidic patch region to Cu(II) is known to be physiologically relevant in the reduction of higher plant PCu[II] by its natural partner cytochrome *f*.[49] Clearly a favorable electron-transfer pathway is being utilized from this location.

The unexpectedly small rate constant for the Ru(II) → Cu(II) electron transfer in His-59-modified plastocyanin indicates that, at least in weakly coupled systems, the effect of intervening medium on long-distance electron transfer may be more significant than previously supposed. A recent study on the *cis*-$[Ru(NH_3)_4(Isn)His\text{-}33]$-modified cytochrome *c* (Isn = isonicotinamide), in which the cytochrome is now a stronger reductant than the Ru moiety, has shown that electron transfer is very much slower than in the reverse direction.[50] A gated mechanism involving redox-state-dependent conformational changes in the cytochrome *c* structure has been suggested.[51] However, a related study involving *cis*-$[(NH_3)_4Ru(py)His\text{-}48]$-modified myoglobin has demonstrated the reversibility of electron transfer in this system.[5a] Further studies on the effect of varying the Ru coordination sphere are of interest with plastocyanin.

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Registry No. $[Ru(NH_3)_5H_2O]^{2+}$, 21393-88-4; $[Ru(NH_3)_5Im]^{2+}$, 39003-94-6; $[Ru(NH_3)_5H_2O](CF_3SO_3)_3$, 53195-18-9; $CO_2^{\bullet-}$, 85540-96-1; imidazole, 288-32-4.

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