Ruthenium Bisbipyridine Complexes of Horse Heart Cytochrome *c*: Characterization and Comparative Intramolecular Electron-Transfer Rates Determined by Pulse Radiolysis and Flash Photolysis

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The reaction of $[Ru(bpy)_2L(H_2O)]^{2+}$ (bpy = 2,2′-bipyridine, L = imidazole, water) with reduced horse heart cytochrome *c* results in coordination of $[Ru^{II}(bpy)_2L]$ at the His 33 and His 26 sites. Coordination at the His 33 site gave a diastereomeric $[Ru^{II}(bpy)_2L]$-His-cyt *c*(II) mixture favoring the Λ-Ru form regardless of the substituent on the bipyridine ligands, while substitution at the more buried His 26 site gave an isomeric distribution that varies according to the substituent on the bipyridine ligands. The diastereomeric aquoproteins (L = H_2O) are distinguished by their redox potentials and their conversion to the corresponding fluorescent imidazole proteins. Intramolecular electron transfer between the reduced ruthenium bipyridine and cyt *c*(III) in $[Ru^{II}(bpy^{\bullet})(bpy)L]$-His33-cyt *c*(III) was determined by reductive pulse radiolysis using the aqueous electron as a reducing agent, $k_{ret} = (2.0 \pm 0.3) \times 10^5\ s^{-1}$, and k_{ret} is independent of the sixth ligand L = H_2O, imidazole. In addition, the rate constant for intramolecular electron transfer from cyt *c*(II) to the ruthenium(III) center in $[Ru^{III}(bpy)_2L]$-His33-cyt *c*(II) was determined by oxidative pulse radiolysis using azide and carbonate radicals. This rate is very sensitive to the nature of the sixth ligand. When L = H_2O, the intramolecular electron-transfer rate for the major diastereomer Λ-*cis*-$[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II) is $k = 1.1 \times 10^4\ s^{-1}$ and is independent of pH between 5.6 and 8.3. The minor Δ-*cis*-$[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II) isomer has pH-dependent electrochemistry and a lower rate of intramolecular electron transfer. Complete conversion from L = H_2O to L = imidazole is slow, requiring more than 7 days in 1 M imidazole. A lower limit ($k > 2 \times 10^6\ s^{-1}$) for the intramolecular electron-transfer rate constant in $[Ru^{III}(bpy)_2(L)]$-His33-cyt *c*(II), L = imidazole, could be obtained by pulse radiolysis in the absence of the slower reacting aquo species. This observation is in agreement with the value of $3 \times 10^6\ s^{-1}$ measured by flash photolysis. Earlier pulse radiolysis experiments primarily measured the aquoligated ruthenium protein, while the flash photolysis experiments measured the imidazole-ligated fraction because it is the only species oxidatively quenched in the photoinduced reactions. Intramolecular electron-transfer reactions for a new series of ruthenium bipyridine complexes, $[Ru(dabpy)_2L]$-His33-cyt *c* proteins (dabpy = 4,4′-diamino-2,2′-bipyridine) (L = imidazole, pyridine, isonicotinamide and pyrazine), proceed with lower driving force, resulting in slower rate constants amenable to measurement by oxidative pulse radiolysis. The electron-transfer rate constants for this series spanned a wide range of the Marcus log *k* vs Δ*G* plot.

Introduction

Cytochrome *c* proteins modified with ruthenium ammine and bipyridine complexes continue to provide a very important avenue for studying the dependence of intramolecular electron-transfer (IET) rates on driving force, distance, and electronic properties.[1−9] Ruthenium bipyridine complexes of the type $[Ru(bpy)_2L(H_2O)]^{2+}$ (bpy = 2,2′-bipyridine, L = imidazole, water) are more hydrophobic than ruthenium tetraammines and bind to both the His 33 and His 26 residues on horse heart cytochrome *c* (Hh cyt *c*).[4] Rates of IET in these ruthenium proteins were first studied using pulse radiolysis as a means of generating oxidized and reduced intermediates. In the reductive

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(1) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659.

(2) Isied, S. S. In *Electron Transfer in Biology and the Solid State*; Johnson, M. K., King, R. B., Kurtz, D. M., Kutal, C., Norton, M. L., Scott, R. A., Eds.; Advances in Chemistry Series 226; American Chemical Society: Washington, DC, 1990; pp 91−100.

(3) Isied, S. S. In *Electron-Transfer Reactions in Metalloproteins*; Sigel, H., Sigel, A., Eds.; Metals in Biological Systems 27; Marcel Dekker: New York, 1991; pp 1−56.

(4) Durham, B. D.; Pan, L. P.; Hahm, S.; Long, J.; Millett, F. In *Electron Transfer in Biology and the Solid State*; Johnson, M. K., King, R. B., Kurtz, D. M., Kutal, C., Norton, M. L., Scott, R. A., Eds.; Advances in Chemistry Series 226; American Chemical Society: Washington, DC, 1990; pp 180−193.

(5) Chang, I.-J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 7056−7057.

(6) Wuttke, D. S.; Bjerrum, M. J.; Winkler, J. R.; Gray, H. B. *Science* **1992**, *256*, 1007−1009.

(7) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369−379.

(8) Mines, G. A.; Bjerrum, M. J.; Hill, M. G.; Casimiro, D. R.; Chang, I.-J.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1996**, *118*, 1961−1965.

(9) Wright, J. L.; Wang, K.; Geren, L.; Saunders, A. J.; Pielak, G. J.; Durham, B.; Millett, F. In *Photochemistry and Radiation Chemistry: Complementary Methods for the Study of Electron Transfer*; Wishart, J. F., Nocera, D. G., Eds.; Advances in Chemistry Series 254; American Society Society: Washington, DC, 1998; pp 99−110.

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pulse radiolysis experiments, the aqueous electron, $e^-_{(aq)}$, was used as a reductant (eq 1a), while in the oxidative pulse radiolysis experiments, azide or carbonate radicals were used as oxidants (eq 2a). The intermediate generated in eq 1a was shown to undergo rapid intramolecular electron transfer from the reduced ligand-centered radical species (bpy•) to the heme of cyt *c*(III) ($k = 1.8 \times 10^5$ s^{-1}).[2]

$$[Ru^{II}(bpy)_2L]\text{-His33-cyt } c(III) + e^-_{(aq)} \rightarrow [Ru^{II}(bpy^{\bullet})(bpy)L]\text{-His33-cyt } c(III) \quad (1a)$$

$$[Ru^{II}(bpy^{\bullet})(bpy)L]\text{-His33-cyt } c(III) \rightarrow [Ru^{II}(bpy)_2L]\text{-His33-cyt } c(II) \quad (1b)$$

bpy• = one-electron reduced 2,2′-bypyridine;
L = imidazole, H_2O

In contrast to this, radiolytic oxidation of the $Ru^{II}Fe^{II}$ species (eq 2a) showed a relatively slow intramolecular electron-transfer rate constant (eq 2b, $k \approx 50$ s^{-1}, pH 8.3) from the cyt *c*(II) to the oxidized Ru(III) site.[2,3] This observed rate is unexpectedly slow, given the high driving force and the small reorganization energy for the reaction (eq 2).

$$[Ru^{II}(bpy)_2L]\text{-His33-cyt } c(II) + N_3^{\bullet}/CO_3^{\bullet -} \rightarrow [Ru^{III}(bpy)_2L]\text{-His33-cyt } c(II) + N_3^-/CO_3^{2-} \quad (2a)$$

$$[Ru^{III}(bpy)_2L]\text{-His33-cyt } c(II) \rightarrow [Ru^{II}(bpy)_2L]\text{-His33-cyt } c(III) \quad (2b)$$

L = imidazole, H_2O

In contrast to the pulse radiolysis experiments, flash photolysis methods (later developed for the generation of the intermediate in eq 2b) showed a faster reaction rate constant for eq 2, in line with theoretical expectation, $k = 3 \times 10^6$ s^{-1}, pH 7.[5,8] This large difference between the observed rates of electron transfer of the radical-oxidized and photooxidized $[Ru^{III}(bpy)_2L]$-His33-cyt *c*(II) intermediate prompted us to further investigate the nature of the species formed in eq 2 by the pulse radiolytic and flash photolytic oxidation techniques.

Recently, we reported on multiple stereoisomeric species resulting from the reaction of $[Ru^{II}(bpy)_2(L)(H_2O)]$ (L = imidazole, water) with Hh cyt *c*.[10] Some of the species identified are precursors to those in eq 2. In this paper we discuss the reaction chemistry of $[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt*c*(II) and $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(II)] and the different rates and driving forces for the electron-transfer reaction in eq 2. The effects of the protein on the imidazole substitution and aquation rates on ruthenium-modified cytochrome *c* are compared to imidazole substitution on the model complex $[Ru(bpy)_2(H_2O)(Im)]^{2+}$. Finally, intramolecular electron-transfer rate constants in a related series of ruthenium-modified proteins, $[Ru(dabpy)_2L]$-His33-cyt*c* (dabpy = 4,4′-diamino-2,2′-bipyridine, L = imidazole, pyridine, isonicotinamide, and pyrazine), were studied. The electron-donating groups on the 2,2′-bipyridine ligands in this series were expected to lower the driving force and decrease the rate constant of the reaction in eq 2b, making it more amenable to pulse radiolysis investigations. The studies presented here together with the earlier published work lead to a rational interpretation of the differences between the previous results of the pulse radiolysis and flash photolysis experiments.[2,3,5,8]

Experimental Section

Methods and Materials. All the chemicals were reagent grade and were used as received unless otherwise indicated. The 2,2′-bipyridine (bpy) and 4,4′-dimethyl-2,2′-bipyridine (dmbpy) were from Aldrich, and ruthenium trichloride hydrate was obtained from Matthey Bishop. Imidazole (Im) was recrystallized from water before use. The $Ru(bpy)_2Cl_2$,[11] $Ru(bpy)_2CO_3$,[12] $Ru(dmbpy)_2CO_3$,[13] and *cis*-$[Ru(bpy)_2(Im)(H_2O)](CF_3COO)_2$[14] complexes were prepared according to literature procedures.

Horse heart cytochrome *c* (type VI) (Sigma) was purified using literature procedures.[15] Protein solutions were concentrated by ultrafiltration with YM3 or YM10 membranes (Amicon). Gel filtration chromatography was done with G-25 Sephadex (Pharmacia). Protein purification was done using cation exchange chromatography on CM-52 Cellulose (Whatman) or Bio-Rex 70 resin (minus 400 mesh, BioRad) for the Ru protein separations.

$Ru(dabpy)_2Cl_2 \cdot xH_2O$ was prepared as follows. Amounts of 76 mg of $K_2[RuCl_5(H_2O)]$ (0.2 mmol), 74 mg of dabpy (0.4 mmol), and ~300 mg of LiCl were suspended in 12 mL of DMF solvent. The solution was refluxed for 1 h, ~50 mg of ascorbic acid was added, and the solution was refluxed for another 1 h. To the resulting red-purple solution, 30−40 mL of acetone was added to precipitate a red-purple solid. The solid was collected and dissolved in a minimum volume of methanol and reprecipitated by adding ethyl ether. A typical yield was 80 mg (~50%). The product was characterized by UV−vis absorption spectrum and electrochemistry.

Instruments and Techniques. UV−visible spectra were obtained on a Hewlett-Packard 8452A diode array spectrometer. HPLC was carried out using a Waters μ-Bondapak C-18 reverse phase column. Fluorescence emission spectra were measured in 1 cm quartz cells using a FluoroMax spectrofluorometer (SPEX Industries, Inc., Edison, NJ).

Cyclic voltammograms (CV) and Osteryoung square wave voltammograms (OSWV) were recorded using a BAS 100A electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN). Electrochemical experiments were carried out in a three-electrode glass cell containing a working electrode, a platinum wire auxiliary electrode and a saturated sodium chloride calomel reference electrode (all potentials are reported vs SSCE). Cyt *c* electrochemistry was carried out in a 50 mM potassium phosphate buffer solution, pH 7.0, at room temperature with a pyrolytic carbon working electrode; typical concentration is ~1 mM. Before each scan, the carbon working electrode was freshly polished with a 0.03 mm alumina slurry (Union Carbide, Indianapolis, IN). For OSWV a typical condition of 4 mV (step height), 25 mV (square-wave amplitude), and 5 Hz (frequency) was used. To accurately determine the peak separations of closely spaced OSWV waves for the ruthenium-modified cytochrome *c*, the digitized voltammograms were fitted using Igor Pro 3.0 (WaveMetrics, Inc., Lake Oswego, OR) on a Macintosh computer.[16]

Electron pulse radiolysis transient absorption experiments were carried out with the 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory as described previously.[17]

Preparation of $[Ru(bpy)_2(Im)]$-His33-cyt *c*. Two methods were used to prepare $[Ru(bpy)_2(Im)]$-His33-cyt *c*.

(10) Luo, J.; Wishart, J. F.; Isied, S. S. *J. Am. Chem. Soc.* **1998**, *120*, 12970−12971.
(11) Sullivan, B. P.; Salmon, D. J.; Meyer, T. J. *Inorg. Chem.* **1978**, *17*, 3334−3341.
(12) Johnson, E. C.; Sullivan, B. P.; Adeyemi, S. A.; Meyer, T. J. *Inorg. Chem.* **1978**, *17*, 2211−2215.
(13) Dobson, J. C.; Meyer, T. J. *Inorg. Chem.* **1988**, *27*, 3283−3291.
(14) Reddy, K. B.; Cho, M. P.; Wishart, J. F.; Emge, T. J.; Isied, S. S. *Inorg. Chem.* **1996**, *35*, 7241−7245.
(15) Sun, J.; Wishart, J. F.; Gardineer, M. B.; Cho, M. P.; Isied, S. S. *Inorg. Chem.* **1995**, *34*, 3301−3309.
(16) Sun, J.; Wishart, J. F.; van Eldik, R.; Shalders, R. D.; Swaddle, T. W. *J. Am. Chem. Soc.* **1995**, *117*, 2600−2605.
(17) Wishart, J. F.; Sun, J.; Cho, M. P.; Su, C.; Isied, S. S. *J. Phys. Chem. B* **1997**, *101*, 687−693.

In the first method, *cis*-$[Ru(bpy)_2(Im)(H_2O)](CF_3COO)_2$ was used as the starting material. To a degassed solution (2 mL) of ferrocytochrome *c* (4 mM) in 50 mM potassium phosphate solution (pH 7.0), 40 mg (0.05 mmol) of *cis*-$[Ru(bpy)_2(Im)(H_2O)](CF_3COO)_2$ was added, and the mixture was kept at room temperature in the dark for 3 days under anaerobic conditions. The solution was then oxidized with potassium ferricyanide and passed through a G-25 gel column (1 cm × 40 cm) to remove the excess reagent. The protein solution was then loaded on a Bio-Rex 70 cation exchange column (1.5 cm × 4 cm) and eluted with a linear gradient from 0 to 100% of 250 mM KCl in 100 mM potassium phosphate buffer. The Ru-modified species obtained from the column were concentrated and equilibrated with 50 mM potassium phosphate buffer (pH 7.0) by ultrafiltration. To ensure purity, the Ru-modified cyt *c* species was rechromatographed twice on a Bio-Rex 70 cation exchange column under the same conditions as above. The yield of this modified protein after repurification was ~3%. The UV−visible spectra of the modified proteins showed the presence of a single $Ru(bpy)_2$ species per cyt *c*, $OD_{292}/OD_{410} = 0.65$ for ferricytochrome *c* or $OD_{292}/OD_{416} = 0.55$ for ferrocytochrome *c*. The site of modification was determined by tryptic digestion of the modified protein followed by HPLC separation of the digested peptides and amino acid analysis of the Ru-containing peptide.[18] The purified Ru-modified cyt *c* was stored at −5 °C.

The second method used a minor modification of a published procedure.[4] An amount of 5 mg of $Ru(bpy)_2CO_3$ was added to 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ferrocytochrome *c*, and the solution was left at room temperature in the dark under an argon stream for 18−24 h. To obtain *cis*-$[Ru(bpy)_2(H_2O)]$-His33-cyt *c*, the reaction product was oxidized with ferricyanide and purified as above. To obtain $[Ru(bpy)_2(Im)]$-His33-cyt *c*, solid imidazole was added to make 1 M imidazole and the incubation was continued for 6 days. The separation and purification were the same as described above. Yield: ~15%. The *cis*-$[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* and the *cis*-$[Ru(dmbpy)_2(Im)]$-His33-cyt *c* were prepared from the $Ru(dmbpy)_2CO_3$ complex using similar procedures.

The *cis*-$[Ru(bpy)_2(H_2O)]$-His33-cyt *c* and *cis*-$[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* were prepared using the second method for $[Ru(bpy)_2(Im)]$-His33-cyt *c* described above, without the imidazole incubation step.

Modification of Horse Heart Cytochrome *c* with $[Ru(dabpy)_2(H_2O)_2]^{2+}$. The 3−4 mg $Ru(dabpy)_2Cl_2 \cdot xH_2O$, suspended in 1 mL of EtOH, was dissolved by heating. After it was cooled, several crystals of ascorbic acid were added to the purple ethanol solution. The ruthenium solution was then transferred into 3 mL of 50 mM potassium phosphate buffer solution containing 50 mg of ferrocytochrome *c*. The mixture was degassed by slowly bubbling with argon for 1 h, then kept in the dark for 1 day at room temperature under anaerobic conditions. The excess ruthenium reagent was removed by passing the mixture through a G-25 gel column (1 cm × 40 cm). The protein solution was then loaded on a Bio-Rex 70 cation exchange column (1.5 cm × 4 cm) and eluted with a linear gradient from 0 to 100% 250 mM KCl in 50 mM potassium phosphate buffer containing ca. 0.1 mM ascorbic acid. The Ru-modified species obtained from the column was concentrated and equilibrated with 50 mM potassium phosphate buffer (pH 7.0) by ultrafiltration. To ensure purity, the Ru-modified cyt *c* species was rechromatographed twice on a Bio-Rex 70 cation exchange column under the same conditions as above to give ~10% yield based on the absorption spectra.

Preparation of $[Ru(dabpy)_2L]$-His33-cyt *c* (L = Im, Py, Pz and isn). About 50 μM of $[Ru(dabpy)_2(H_2O)]$-His33-cyt *c* solution (50 mM potassium phosphate buffer, pH 7.0, in the presence of ~0.1 mM ascorbic acid) was incubated with 1 M L (except in the case of isn, 0.2 M) for 1 day in the dark at room temperature under anaerobic conditions. The excess ligand was removed by passing the mixture through a G-25 gel column. Purification was the same as that for $[(dabpy)_2Ru(H_2O)]$-His33-cyt *c*.

Determination of the Site of Modification. Imidazole solution was incubated with $[Ru(bpy)_2(OH_2)]$-cyt *c* to form the imidazole derivative, as described above, before tryptic digestion. This was carried out by incubating the reduced ruthenium protein in 1 M imidazole solution in phosphate buffers (pH 7.0) for ~5 days under dark, anaerobic conditions. The reduced protein was oxidized by ferricyanide, and the excess imidazole and ferricyanide reagents were removed by ultrafiltration. The tryptic digestion and peptide mapping were then carried out as described previously.[18−20]

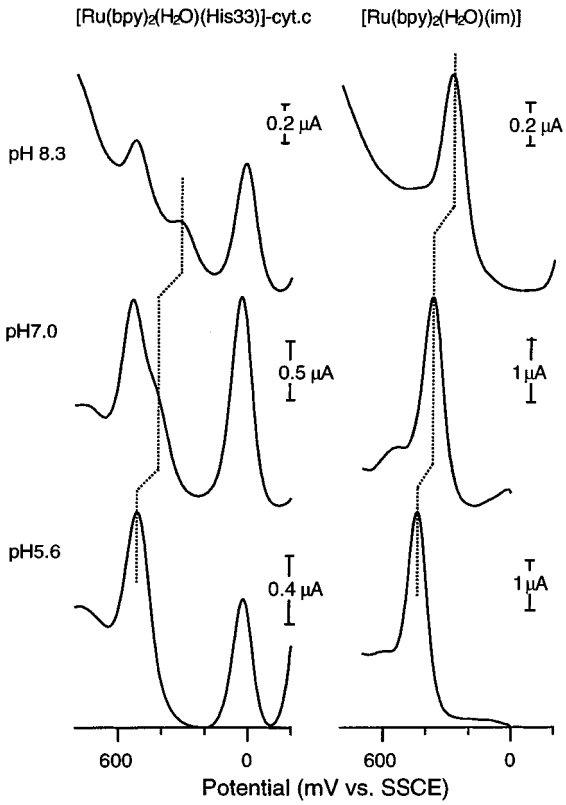


Figure 1. Comparison of the pH dependence of the voltammograms for $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* and $[Ru(bpy)_2(H_2O)(Im)]^{2+}$ (OSWV in 50 mM phosphate buffer, protein concentration of ~1 mM).

Results and Discussion

I. Ruthenium cyt *c* Modified at His 33 and His 26: Voltammetry, Kinetics of Substitution, and Preparative Comments. I.1. Voltammetry of $[Ru(L-L)_2(L)]$-His33-cyt *c* and $[Ru(L-L)_2(L)]$-His26-cyt *c* (L−L = bpy, dmbpy; L = Im, H_2O). Voltammograms of the ruthenium complexes $[Ru(bpy)_2(H_2O)_2]$ and $[Ru(bpy)_2(H_2O)Im]$ clearly show a pH dependence between pH 4 and pH 8 attributed to the redox-related ionization of the coordinated aquo ligand on these complexes. The reduction potentials of these ruthenium complexes change from 430 to 230 mV (vs SSCE) between pH 5.6 and pH 8.5 (eq 3) (Figure 1) (the pK_a of the coordinated water is ~5.6[13]).

$$[Ru^{II}(bpy)_2(Im)(H_2O)]^{2+} \rightleftharpoons [Ru^{III}(bpy)_2(Im)(OH^-)]^{2+} + e^- + H^+ \quad (3)$$

For $[Ru(bpy)_2(H_2O)]$-His33-cyt *c*, a major pH-independent band at 534 mV (highest current) and a minor pH-dependent band (which shifts from 534 to 311 mV between pH 5.6 and pH 8.3) appear in the Osteryoung square wave voltammograms

(18) Cho, M. Ph.D. Thesis, Rutgers University, Piscataway, NJ, 1989.

(19) Yocom, K. M.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Worosila, G.; Isied, S. S.; Bordignon, E.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7052−7055.

(20) Pan, L.-P.; Durham, B.; Wolinska, J.; Millett, F. *Biochemistry* **1988**, *27*, 7180−7184.

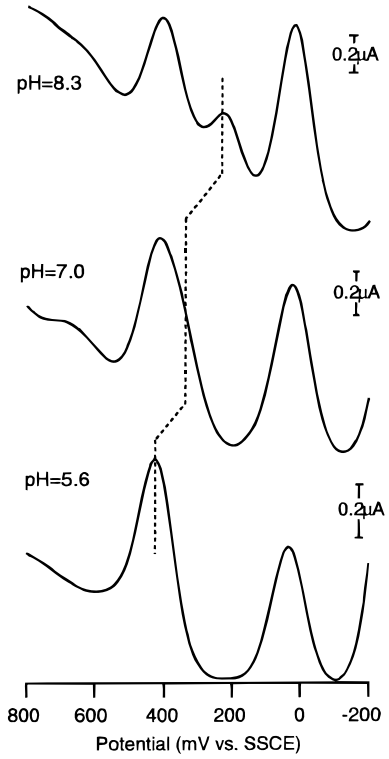


Figure 2. pH dependence of the voltammograms for $[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* (OSWV in 50 mM potassium phosphate buffer).

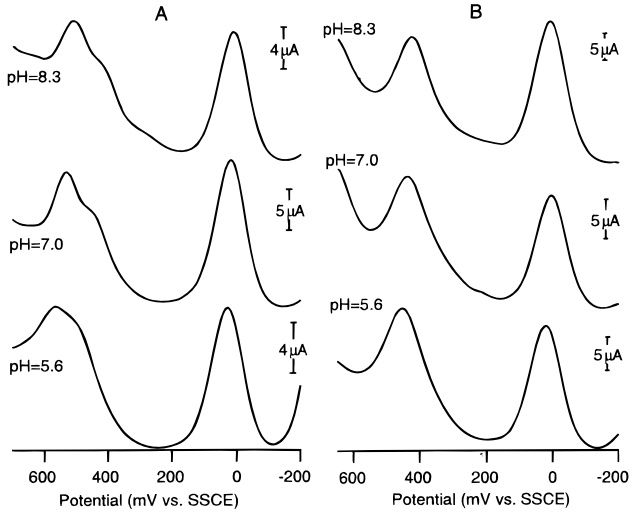


Figure 3. pH dependence of the voltammograms for $[Ru(bpy)_2(H_2O)]$-His26-cyt *c* (A) and $[Ru(dmbpy)_2(H_2O)]$-His26-cyt *c* (B) (OSWV in 50 mM potassium phosphate buffer).

(Figure 1). The voltammograms of ruthenium aquo proteins and the $[Ru^{II}(bpy)_2(H_2O)(Im)]^{2+}$ complex are compared at pH 5.6, 7.0, and 8.3 (Figure 1). For $[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c*, similar results at lower potentials were observed, as expected (Figure 2).

Unlike $[Ru(bpy)_2(H_2O)]$-His33-cyt *c*, $[Ru(bpy)_2(H_2O)]$-His26-cyt *c* shows two overlapping bands at 517 and 430 mV that do not change with pH (pH 5.6−8.5) (Figure 3A). The closely related $[Ru(dmbpy)_2(H_2O)]$-His26-cyt *c* shows one major pH-independent band at 402 mV (Figure 3B) (with a minor band at 344 mV that can be resolved by OSWV fitting techniques; see Table 1). The pH-independent voltammograms for three of the four ruthenium-modified proteins are in sharp contrast to

Table 1. CD, OSWV, and Substitution Kinetics for $[Ru^{II}(L-L)(H_2O)]$-His*X*-cyt *c* Diasteriomers

				$E^{\circ\prime}$	$k_{sub} \times 10^5$	isomer percentage (%)[c]		
X	L−L	isomer	pH	(mV)[a]	(s^{-1})[b]	CD	OSWV	kinetics
33	bpy	Λ	7.0	534	0.8(0.1)	67,	70,	79(24)
			8.3	513			68	
		Δ	7.0	427	2.6(1.9)	33	30	21(24)
			8.3	311			32	
	dmbpy	Λ	7.0	420	1.0(0.4)	65,	65	45(27)
			8.3	402			64	
		Δ	7.0	351	3.5(1.2)	35,	35	55(27)
			8.3	224			36	
26	bpy	Λ	7.0	430	3.1(0.8)	47,	45,	33(7)
		Δ	7.0	517	0.46(0.08)	53	55	64(7)
	dmbpy	Λ	7.0	344		31	16	
		Δ	7.0	402	0.61(0.06)	69	84	

[a] The redox potentials are obtained from the fitted OSWV data (see Experimental Section).[16,23] [b] The observed substitution rate of imidazole (1 M) on $[Ru^{II}(L-L)(H_2O)]$-His*X*-cyt *c*. [c] The percentage of the Λ and Δ isomers in the mixture calculated from CD $\Delta\epsilon_{294nm}$,[10] the area of the fitted OSWV, and the double-exponential kinetics analysis of imidazole substitution (the σ values are in parentheses). The values obtained from the substitution kinetics have large errors and are less reliable than those obtained directly from CD and voltammetry data.

that in the model ruthenium bipyridine complex $[Ru^{II}(bpy)_2(H_2O)(Im)]^{2+}$.

The ruthenium aquo proteins (seen as two voltammetric peaks) have been assigned to two diastereomeric forms.[10] For His 33 substituted proteins, stereoselectivity is favoring both Λ-*cis*-$[Ru(bpy)_2(H_2O)]$-His33-cyt *c* and Λ-*cis*-$[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* proteins. For His 26 substituted proteins, the stereoselectivity is largely dependent on the ligand size, favoring greater amounts of the Δ form for the larger complex $[Ru(dmbpy)_2(H_2O)_2]^{2+}$. The different voltammetric behavior observed for these forms is attributed to the different interactions of the coordinated waters in the Δ and Λ forms of $[Ru(L-L)_2(H_2O)]$-cyt *c* with different residues in the protein matrix. The pH-dependent voltammetry for Δ-*cis*-$[Ru(L-L)_2(H_2O)]$-His33-cyt *c* between pH 5.5 and pH 8.5 is consistent with the coordinated water in the Δ isomer pointing toward the solvent, while the water in the Λ isomer (which does not show a pH dependence) would be pointing toward the protein matrix where a hydrogen bond may stabilize the coordinated water against ionization. In agreement with this, the coordinated waters of the $[Ru(bpy)_2(H_2O)]$-His26-cyt *c* species (which are more buried in the protein) show no pH dependence. Substitution of imidazole on these ruthenium aquo proteins proceeds with retention of configuration[10] and eliminates the differences in the redox potentials between the Δ and Λ protein forms (Figure 4).

I.2. Substitution and Aquation of Imidazole in $[Ru(L-L)_2(H_2O)]$-His*X*-cyt *c* and *cis*-$[Ru(bpy)_2(Im)(H_2O)]^{2+}$ (L−L = bpy, dmbpy; *X* = 26, 33). The substitution and aquation of imidazole on $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* was monitored by the increase in fluorescence emission (λ_{ex} = 436 nm and $\lambda_{em,max}$ at 667 nm) of the product $[Ru(bpy)_2(Im)]$-His33-cyt *c* (Figure 5). The quantum yield of fluorescence for $[Ru(bpy)_2(Im)]$-His33-cyt *c* was found to be similar to that of a mixture made from a 1:1 molar ratio of $[Ru(bpy)_2(Im)_2]^{2+}$ to cyt *c* (Figure 5). The kinetics of substitution of imidazole onto $[Ru(bpy)_2(H_2O)]$-His26-cyt *c* fits two exponentials, consistent with substitution on two forms, while that for $[Ru(dmbpy)_2(H_2O)]$-His26-cyt *c* fits a single exponential, consistent with the presence of one major form (Figure 6). The substitution kinetics of Im on $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* and $[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* could be fit to either a single or a double exponential; however,

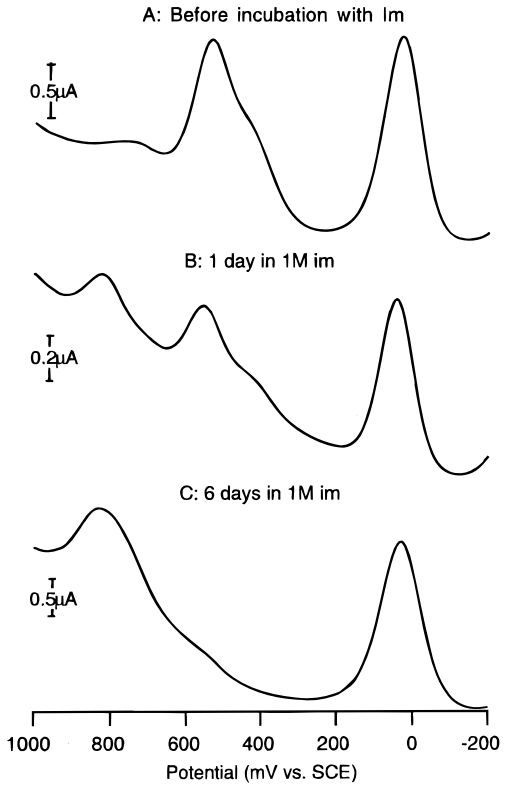


Figure 4. Voltammograms for the substitution of imidazole onto $[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c* (monitored by OSWV in 50 mM potassium phosphate buffer, pH 7.0).

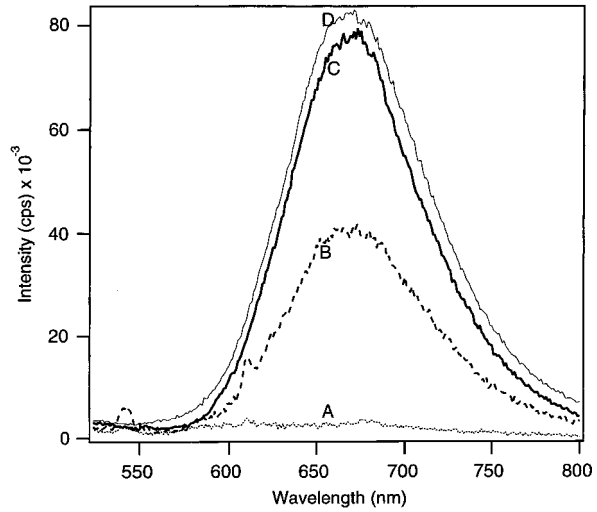


Figure 5. Fluorescence spectra for the substitution of imidazole on $[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c* (A, time 0; B, 1 day; and C, 6 days) (incubated with 1 M imidazole, 50 mM potassium phosphate solution pH 7.0). The fluorescence spectrum D is for a mixture of native cyt *c* and $[Ru(bpy)_2(Im)_2]^{2+}$ in a 1:1 molar ratio (50 mM potassium phosphate buffer, pH 7.0). The concentrations of Ru complex and cyt *c* were adjusted to 0.2 absorbance at λ_{ex} 436 nm for all the solutions. (Baseline was adjusted to zero intensity.)

the better fit to a double-exponential is consistent with substitution at two similarly accessible ruthenium centers (Table 1). The rate constant for imidazole aquation from $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c* was estimated from initial rate kinetics to be $\sim 5 \times 10^{-8}$ s^{-1}.

Overall, the substitution rate of imidazole onto $[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c* and the imidazole aquation rate from $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c* are found to be 2 orders of magnitude

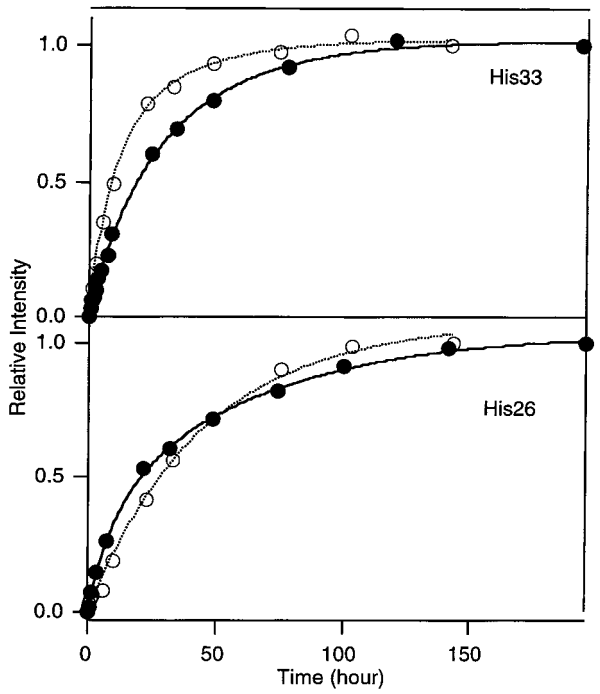


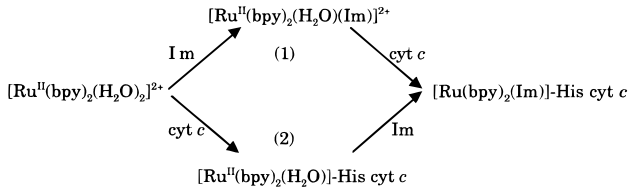
Figure 6. Kinetics of substitution of imidazole on $[Ru(bpy)_2(H_2O)]$-His*X*-cyt *c* (●) and $[Ru(dmbpy)_2(H_2O)]$-His*X*-cyt *c* (○) (1 M imidazole in 50 mM phosphate buffer, pH 7.0). The relative intensity (which is used for comparison) is normalized by taking the average fluorescence intensity between 658 and 666 nm divided by the maximum intensity of the fully formed imidazole complex.

slower than the corresponding values for the model complex $[Ru(bpy)_2(Im)(H_2O)]^{2+}$ (substitution and aquation rates are reported to be $k_{sub} = 7 \times 10^{-4}$ M^{-1} s^{-1} and $k_{aq} = 2.5 \times 10^{-6}$ s^{-1}, respectively).[14] The rate of substitution of imidazole onto the more surface-exposed His 39 residue of $[Ru^{II}(bpy)_2(H_2O)]$-His39-cyt *c*(II) from a similar yeast cytochrome *c* (Iso-2 from *Saccharomyces cerevisiae*) is only 4 times faster than that for $[Ru(bpy)_2(H_2O)]$-His33-cyt *c*,[21] and thus, substitution of imidazole on all of the ruthenium proteins investigated is at least an order of magnitude slower than the same reaction in the model complex $[Ru(bpy)_2(Im)(H_2O)]^{2+}$.

The substitution and aquation of imidazole onto $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* were also monitored electrochemically by the change in the amplitudes of the voltammetry waves of $[Ru(bpy)_2(Im)]$-His33-cyt *c* and $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* (Figure 4). The voltammetry and the fluorescence results both show that the reaction between $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* and imidazole is slow (requiring about 6 days to proceed to completion) at 1 M imidazole concentration.

I.3. Comments on the Preparation and Characterization of $[Ru(bpy)_2(Im)]$-His33-cyt *c*. Two methods were used to prepare $[Ru(bpy)_2(Im)]$-His33-cyt *c* (Scheme 1). In the first

Scheme 1



method *cis*-$[Ru(bpy)_2(Im)(H_2O)]^{2+}$ and reduced *Hh* cyt *c* were stirred for 3 days at 25 °C followed by ion exchange chroma-

(21) Luo, J.; Isied, S. S. Unpublished results.

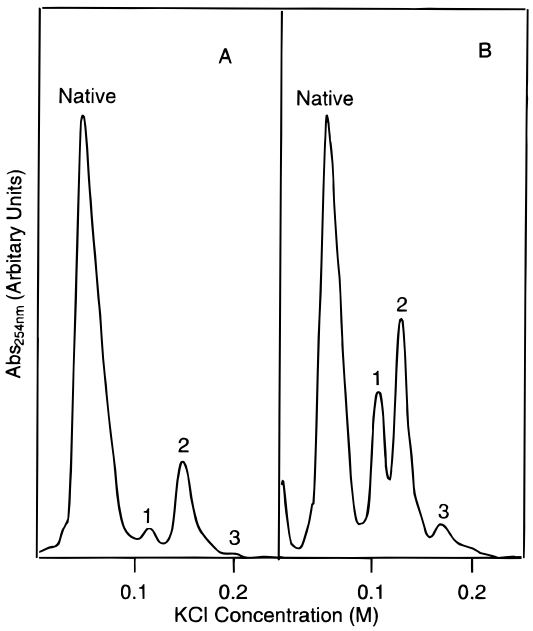


Figure 7. Elution profile for the separation of $[Ru^{II}(bpy)_2(L)]$-His-cyt *c* (L = Im, H_2O) derivatives prepared using $[Ru(bpy)_2(Im)(H_2O)]^{2+}$ (A) or $[Ru(bpy)_2(H_2O)_2]^{2+}$ (B) as the starting material on a Bio-Rex 70 cation exchange column.

tography to separate two species with a 1:1 ruthenium to cyt *c* ratio (Figure 7A).[18] The first band, identified as His 26 site modification, is not luminescent (but becomes luminescent after incubation with 1 M imidazole for hours). The second band, identified as $[Ru(bpy)_2(Im)]$-His33-cyt *c*, showed luminescence similar to that of the model compound *cis*-$[Ru(bpy)_2(Im)_2]^{2+}$.

In the second method for the preparation of $[Ru(bpy)_2(Im)]$-His33-cyt *c*, $[Ru^{II}(bpy)_2(H_2O)_2]$ (generated in situ from $[Ru^{II}(bpy)_2(CO_3)]$ was allowed to react with oxidized cyt *c* for 16 h. The product $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* was then separately converted to the $[Ru(bpy)_2(Im)]$-His33-cyt *c* by reaction with 1 M imidazole for 18 h.[4] This method, with minor modifications (incubating a mixture of ~2.5 mM $Ru(bpy)_2CO_3$ and 2 mM *reduced* cyt *c* for 16 h followed by reaction with 1 M imidazole for 1−6 days), was used throughout this study. Higher overall yields of $[Ru(bpy)_2(Im)]$-His33-cyt *c* product were obtained using this procedure. Finally using a 1:1 molar ratio of 2 mM $Ru(bpy)_2CO_3$ to *reduced* cyt *c* also resulted in more of the desired singly modified product and less of the highly charged multisubstituted species (Figure 7B).

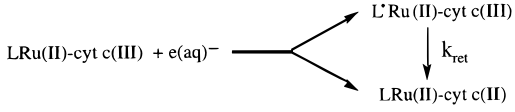
An important difference observed between the above two methods is that the band assigned to $[Ru(bpy)_2(Im)]$-His26-cyt *c* using *cis*-$[Ru(bpy)_2(Im)(H_2O)]^{2+}$ as a starting material is not fluorescent except when separately incubated with 1 M imidazole for days. In contrast, when starting with $Ru(bpy)_2CO_3$ and cytochrome *c* followed by addition of imidazole, both bands ($[Ru(bpy)_2(Im)]$-His26-cyt *c* and $[Ru(bpy)_2(Im)]$-His33-cyt *c* are fluorescent. When $[Ru^{II}(bpy)_2(Im)(H_2O)]^{2+}$ was used as the starting material (method 1), several reactions can occur on the time scale of the substitution of the ruthenium complex on the protein; these include the aquation of $[Ru^{II}(bpy)_2(Im)(H_2O)]^{2+}$ to $[Ru(bpy)_2(H_2O)_2]^{2+}$, which is known to react more quickly with the protein than $[Ru(bpy)_2(Im)(H_2O)]^{2+}$.[14] Both the aquo and the imidazole derivatives can then form with a ratio governed by their substitution rates and reaction time. The $[Ru(bpy)_2(Im)]$-His33-cyt *c* product obtained using this method was estimated (from the fluorescence spectra) to be 10% of the total $[Ru(bpy)_2(L)]$-His33-cyt *c* species.

The results presented here are consistent with slow formation and loss of imidazole from the $[Ru(bpy)_2(Im)]$-His33-cyt *c*. The $[Ru(bpy)_2(H_2O)]$-His33-cyt *c* species results primarily from the direct reaction of $[Ru(bpy)_2(H_2O)_2]^{2+}$ with cyt *c*. Substitution on His 26 of cyt *c* also occurs primarily from the diaquo $[Ru(bpy)_2(H_2O)_2]^{2+}$ (since the reaction with the $[Ru(bpy)_2(Im)(H_2O)]^{2+}$ is significantly slower).

The overall ratio of His 33 to His 26 modified protein (aquo and imidazole) is higher when starting with $[Ru(bpy)_2(Im)(H_2O)]^{2+}$ than when starting with $[Ru(bpy)_2(H_2O)_2]^{2+}$ (Figure 7A). These results point to a large discrimination factor favoring substitution at His 33 over His 26 for $[Ru(bpy)_2(Im)(H_2O)]^{2+}$ but only a modest factor for $[Ru(bpy)_2(H_2O)_2]^{2+}$ starting material.

Earlier samples of $[Ru(bpy)_2(Im)]$-His33-cyt *c* investigated prior to this work (from our lab and others) all contained varying amounts of the aquo species $[Ru(bpy)_2(H_2O)]$-His33-cyt *c*.[4,22] The imidazole and aquo species, $[Ru(bpy)_2(Im)]$-His33-cyt *c* and $[Ru(bpy)_2(H_2O)]$-His33-cyt *c*, could not be separated either by high-resolution cation exchange chromatography (Bio-Rex 70, −400 mesh)[23] or by fast protein liquid chromatography (FPLC).[22] However, the amount of $[Ru(bpy)_2(Im)]$-His33-cyt *c* in a given sample can be determined quantitatively, and the conversion of the aquo species to the imidazole-substituted species can be monitored using the electrochemical and isomer identification methods described above.

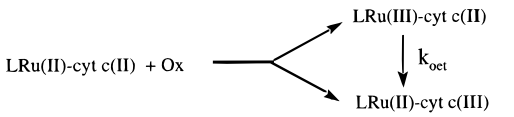
II. Kinetics of Intramolecular Electron Transfer. II.1. Electron Transfer in $[Ru^{II}(bpy^{\cdot})(bpy)(L)]$-His33-cyt *c*(III) by Reductive Pulse Radiolysis. The reduction of $[Ru^{II}(bpy)_2(L)]$-His33-cyt *c*(III) (L = Im, H_2O) by the aqueous electron e^-_{aq} via pulse radiolysis (eq 4) was carried out using 20−50 μM protein in 0.1 M *tert*-butyl alcohol solutions.



(4)

The reaction was monitored at 550 nm, where the reduction of cyt *c*(III) to cyt *c*(II) is accompanied by an absorbance increase, and at 504 nm, an isosbestic point of cytochrome *c* where the absorbance decrease is due to the reoxidation of the bipyridine-reduced ruthenium site to yield the $[Ru^{II}(bpy)_2(L)]$-His33-cyt c^{II} (L = Im, H_2O) (eqs 1 and 4). The rate determined at both wavelengths, $k_{ret} = (2.0 \pm 0.3) \times 10^5\ s^{-1}$, was independent of the sixth ligand L = H_2O, imidazole. These rates are in good agreement with those determined by flash photolysis for $[Ru^{II}(bpy)_2(Im)]$-His33-cyt c^{II} by reductive quenching techniques.[8]

II.2. Kinetics of Intramolecular Electron-Transfer Oxidation in $[Ru^{II}(bpy)_2(L)]$-His33-cyt *c*(II) (L = Im, H_2O). Oxidation of $[Ru^{II}(bpy)_2(L)]$-His33-cyt *c*(II) derivatives with one-electron radical oxidants $N_3^{\bullet}$ or $CO_3^{\bullet -}$ generated by pulse radiolysis (eqs 2a and 5) was studied between pH 5.6 and pH 8.3. The formation and decay of the $[Ru^{III}(bpy)_2(L)]$-His33-cyt *c*(II) intermediate was followed at 550 and 504 nm.



(5)

(22) Casimiro, D. R. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1994.
(23) Luo, J. Ph.D. Thesis, Rutgers, The State University of New Jersey, Piscataway, NJ, 1998.

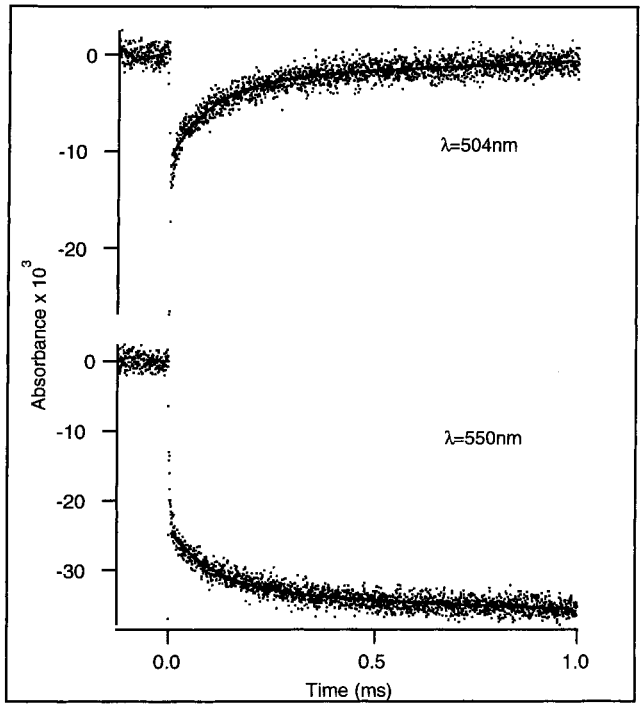


Figure 8. Intramolecular reduction of the Ru(III) site (504 nm) and oxidation of the heme(II) site (550 nm) in $[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II) as observed by transient absorption (in 100 mM NaN_3, 50 mM phosphate buffer, pH 7.0).

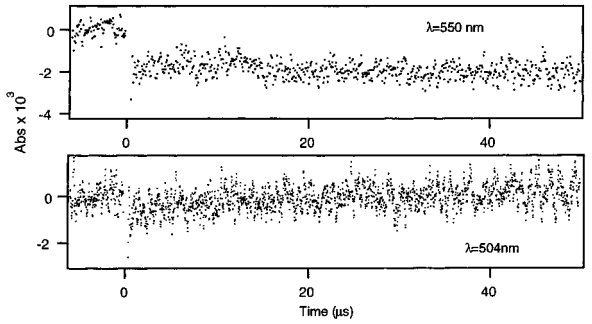


Figure 9. Intramolecular reduction of the Ru(III) site (504 nm) and oxidation of the heme(II) site (550 nm) in $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) as observed in the transient absorption changes (1.8 mM in 100 mM NaN_3, 50 mM phosphate buffer, pH 7.0, 1 mm path length). The small signals are due to the extremely short path lengths.

The absorbance vs time profiles of the electron-transfer reactions for $[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II) and $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) are shown in Figures 8 and 9. For $[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II), an intramolecular ET rate constant $k_{oet} = 1.1 \times 10^4\ s^{-1}$, independent of pH between pH 5.6 and pH 8.5, was determined. This rate corresponds to the ruthenium protein with a pH-independent voltammetry peak at 550 mV vs SSCE, which has been assigned to Λ-$[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c*(II).[10] No consistent rate was detectable for the pH-dependent minor component assigned to the Δ-$[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c*(II).[10] However, on the basis of its redox potential, its IET rate is expected to be at least 1 order of magnitude less than the Λ isomer at pH 7 (the rate may be even slower if additional reorganization energy associated with the water ligand exposure to solvent is included). At slower time scales a range of rates ($k \approx 20-100\ s^{-1}$) appeared (as previously reported)[2,3] that could be attributed to the minor Δ isomer above and/or intra- and intermolecular reactions involving Ru(III/IV) states formed during the pulse radiolysis experiment. Voltammograms of the ruthenium aquo protein and model complexes show that these Ru(III/IV) states are accessible (Figure 10). Similar but slower rates were observed for Δ-$[Ru(dmbpy)_2(H_2O)]$-His33-cyt *c* ($k_{oet} = 2.7 \times 10^3\ s^{-1}$) and a range of rates ($k \approx 5-20\ s^{-1}$) for the Λ isomer and/or the corresponding intra- and intermolecular reactions involving Ru(III/IV) states. The IET kinetics for both derivatives are summarized in Table 2.

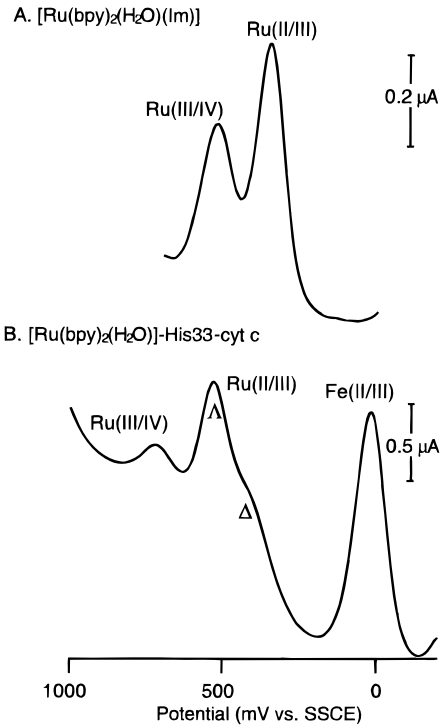


Figure 10. Voltammograms of the ruthenium aquo complex (III/IV) oxidation. (OSWV conditions: scan from negative to positive potential; square wave amplitude = 25 mV; step = 4 mV; frequency = 1 Hz (A) or 10 Hz (B)).

Table 2. Rates for Intramolecular Electron-Transfer from cyt *c* Heme to the Ruthenium Complex in $[Ru(L-L)_2L_1]$-His33-cyt *c*, 50 mM Potassium Phosphate Buffer, pH 7.0 (Radical Precursor Is NaN_3)

L−L	L_1	$-\Delta G°$ (eV)	k_{et} (s^{-1})
bpy	H_2O	0.50	$1.1(0.2) \times 10^4$
dmbpy	H_2O	0.39	$2.7(0.6) \times 10^3$
dabpy	H_2O	0.0	$1.8(0.4) \times 10$
	Im	0.18	$3.9(0.3) \times 10^3$
	Py	0.33	$4.3(0.3) \times 10^4$
	Isn	0.36	$8.9(0.8) \times 10^4$
	Pz	0.45	$2.0(0.1) \times 10^5$

After thorough incubation of the aquoruthenium protein derivatives with imidazole followed by purification, the $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(II) protein can now be studied by pulse radiolysis without interference. Pulse radiolysis experiments on bona fide $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(II) samples do not exhibit significant kinetic features in the range $10^2-10^4\ s^{-1}$ (Figure 9). Transient absorption kinetics using the azide radical to oxidize $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(II) (1.8 mM in a 0.1 cm cell) are consistent with an intramolecular electron-transfer reaction faster than $2 \times 10^6\ s^{-1}$ (Figure 9), as previously obtained for the same intermediate at much lower concentrations using laser flash photolysis.[5,8] The limiting factor for observing this fast reaction is the time required for the azide radical to

oxidize the $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(II). Even at the highest practical concentrations of ruthenium-modified proteins, the oxidation and decay of the electron-transfer precursor $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) occur on the same time scale.

Early pulse radiolysis and laser flash photolysis studies were performed on samples that contained a mixture of imidazole and aquo complexes. In the laser photolysis studies, the only ruthenium(III) intermediate formed is the product of oxidative quenching of $[Ru^{II}*(bpy)_2(Im)]$-cyt *c*(II) by $[Ru(NH_3)_6]^{3+}$ (since the aquoruthenium cyt *c* species present have very short excited-state lifetimes, they are not oxidatively quenched and do not interfere with the observed fast transient absorption profiles on the microsecond time scale). Thus, only the Fe(II)-to-Ru(III) intramolecular electron-transfer reaction in $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) was observed ($k = 3 \times 10^6$ s^{-1}) despite the presence of these other components.[5,8,22] In oxidative pulse radiolysis there is no selectivity between the L = H_2O and Im species of $[Ru(bpy)_2(L)]$-His33-cyt *c*. The L = H_2O and Im constituents contribute to the transient absorption profile roughly in proportion to their concentration, resulting in multiple electron-transfer reactions at different time scales.

II.3. Kinetics of Intramolecular Electron Transfer in $[Ru^{III}(dabpy)_2L]$-His33-cyt *c*(II). The ruthenium bipyridine cyt *c* derivatives reported to date cover a wide (>1 eV) range of values of the electron-transfer driving force. However, the observed electron transfer rates vary by only 1 order of magnitude, since all these reactions fall near the top of the Marcus curve describing the dependence of ET rates on driving force.[8] It is of interest to study these reactions at lower driving forces while still maintaining the features of the bipyridine ligands. The diamino bipyridine cytochrome *c* series $[Ru^{III}(dabpy)_2L]$-His33-cyt *c*(II) (L = H_2O, Im, Py, Isn, and Pz) proved to be useful for extending the range of driving force in the Marcus rate vs free energy diagram for eq 2.

The electron-transfer rates obtained were all in a range measurable by radiolytic oxidation with azide radical, whereas the short excited-state lifetimes of these complexes preclude measurement by oxidative quenching of their excited states. The IET rate constants determined for all the modified proteins are summarized in Table 2. A plot of IET rate constants vs driving force is presented in Figure 11, including the results reported by Mines et al.[8] for comparison. For a given driving force, the diamino series shows a slower IET rate than the other bipyridine series. A slightly larger reorganization energy for the reaction in eq 2 is expected because of the hydrophilic and electron-donating properties of the amino groups, making the dabpy ruthenium complexes more strongly solvated in aqueous solution and allowing redox modulation of the water−amino group interaction.

If we assume the same H_{DA} determined earlier ($H_{DA} = 0.095$ cm^{-1}) for these complexes (since the same number of bonds separate the donor from the acceptor), a reorganization energy of 0.95 eV is calculated for the IET in the $[Ru^{III}(dabpy)_2L]$-His33-cyt*c*(II) series compared to 0.74 eV for the more hydrophobic bipyridine series (Figure 11). In all the series studied, when L = H_2O, the rates for IET were slower than those found for other heterocyclic ligands (Figure 11). The bound water molecule increases the reorganization energy of the IET and therefore decreases the rate. If the same H_{DA} determined earlier ($H_{DA} = 0.095$ cm^{-1}) applies for these aquo complexes, a reorganization energy of 1.26 eV is calculated for these IET reactions, consistent with the slower observed rate.

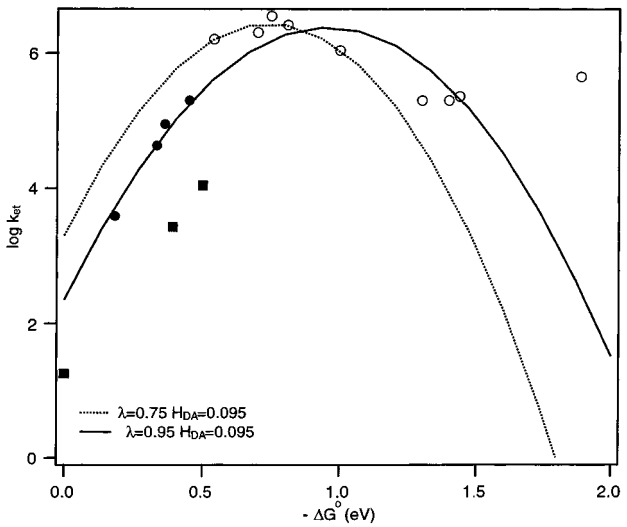


Figure 11. Driving force ($-\Delta G^{\circ}$) dependence of intramolecular rate constants in Ru(His 33)-cyt *c* plotted using the classical Marcus equation. The open circles are from the results from Mines et al., and the dotted line is the Marcus fit of their data yielding λ = 0.74 eV and $H_{DA} = 0.095$ cm^{-1} (ref 8). The filled circles are the results for $[Ru(dabpy)_2L]$-His33-cyt *c* (L = Im, Py, Isn, and Pz) obtained in this work, and the solid line is the Marcus fit of these data where H_{DA} was fixed at 0.095 cm^{-1} and λ was found to be 0.95 eV. The filled squares are the results for the ruthenium aquo modified proteins (Table 2).

Summary

The $[Ru^{II}(bpy)_2L]$-His33-cyt *c*(II) derivatives prepared by starting from either *cis*-$[Ru(bpy)_2(Im)(H_2O)]^{2+}$ or *cis*-$[Ru(bpy)_2CO_3]$ and imidazole were found to be mixtures of three ruthenium-modified protein species of the type $[Ru^{II}(bpy)_2L]$-His33-cyt *c*(II) (L = Im, H_2O). The aquo species all react slowly with imidazole (1 M for several days) to form $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*, which has a characteristic fluorescence spectra similar to $[Ru(bpy)_2(Im)_2]^{2+}$. Reaction of *cis*-$[Ru(bpy)_2(H_2O)_2]^{2+}$ with reduced cytochrome *c* results in the formation of a diasteriomeric $[Ru^{II}(bpy)_2(H_2O)]$-His33-cyt *c*(II) mixture favoring the Λ-Ru form. These diasteriomeric proteins are best distinguished by the redox potentials of their ruthenium sites but could not be chromatographically separated. Substitution and aquation of imidazole in these modified proteins is significantly slower (50−100 times) than similar processes in the model compound *cis*-$[Ru(bpy)_2(Im)(H_2O)]^{2+}$. The kinetics of intramolecular electron transfer for the imidazole and the aquo proteins were determined from reductive and oxidative pulse radiolysis. In reductive pulse radiolysis, the $[Ru^{II}(bpy)_2(Im)]$-His33-cyt *c*(III) was reduced with the aqueous electron and similar rates of reduction of cyt *c* from the intermediate $[Ru^{II}(bpy^{\bullet})(bpy)L]$-His33-cyt *c*(III) were observed, regardless of the nature of the sixth ligand L = Im, H_2O. By oxidative pulse radiolysis, intramolecular oxidation of cyt *c* in the intermediate $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) was too fast to measure ($k > 2 \times 10^6$ s^{-1}), even at extremely high ruthenium protein concentrations (ca. 1.8 mM), consistent with the flash photolysis results. The rate constant for intramolecular oxidation in the major aquo isomer of the Λ-$[Ru^{III}(bpy)_2(H_2O)]$-His33-cyt *c*(II) intermediate is $k = 1.1 \times 10^4$ s^{-1} and is pH-independent. The pH-dependent rate for the minor Δ isomer was more difficult to determine. Slower rate constants $k \approx 20-100$ s^{-1} were assigned to the Δ isomer and/or to intra- and intermolecular oxidation of cyt *c* by ruthenium (III) and/or (IV) aquo species.

While oxidative pulse radiolysis produced $[Ru^{III}(bpy)_2L]$-His33-cyt *c*(II) (L = Im, H_2O) intermediates from all the components present in solution, with rate constants ranging from 10^6 to 10^2 s^{-1}, flash photolysis from similarly prepared samples (flash quench techniques) showed only the intramolecular electron transfer for the $[Ru^{III}(bpy)_2(Im)]$-His33-cyt *c*(II) intermediate. This is because $[Ru^{II*}(bpy)_2(Im)]$-His33-cyt *c*(II) is the only intermediate formed in the photooxidation of the ruthenium protein (even in the presence of other aquoruthenium-modified proteins).

Pulse radiolysis was used to study intramolecular electron-transfer kinetics in a series of ruthenium bipyridine complexes with lower reaction driving forces. IET for $[Ru(dabpy)_2L]$-His33-cyt *c* proteins (dabpy = 4,4′-diamino-2,2′-bipyridine) (L = imidazole, pyridine, isonicotinamide, and pyrazine) provided a wide range of rates at different driving forces from which the reorganization energy for the intramolecular electron-transfer reaction was more accurately determined.

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