Substituted Tetraammineruthenium Cytochrome *c* Derivatives: Chemistry and Electron-Transfer Reactions

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Received December 9, 1994[⊗]

Horse-heart (hh) cytochrome *c*, modified at His-33, and *Candida krusei* (*Ck*) cytochrome *c*, modified at His-39, with a series of *cis*- and *trans*-[$L(NH_3)_4Ru^{III}$cyt *c*] derivatives, where L is isonicotinamide (isn) or pyridine (py), have been prepared and characterized. Rate constants for intramolecular electron transfer from the heme(II) to Ru(III) in the [$L(NH_3)_4Ru^{III}$cyt c^{II}] intermediates generated by oxidative pulse radiolysis of the fully reduced modified protein species were as follows (k (s^{-1}), $\Delta H^{\ddagger}$ (kcal mol^{-1}), $\Delta S^{\ddagger}$ (cal $deg^{-1}mol^{-1}$), $-\Delta G^{\circ}$ (eV)): 440, 7.3, −22, 0.18 for L = *trans*-isn (hh); 440, 6.2, −26, 0.18 for L = *cis*-isn (hh); 126, 8.8, −19, 0.11 for L = *trans*-py (hh); 220, 6.4, −27, 0.13 for L = *trans*-isn (*Ck*); 154, 2.3, −41, 0.18 for L = NH_3 (*Ck*). Relative differences in the rates are accounted for by variations in the driving force and reorganization energies in these ruthenium-modified proteins resulting from the nature of the ligands around the ruthenium center and from the different sites of modification on the cytochrome. The fully oxidized [$L(NH_3)_4Ru^{III}$cyt c^{III}] species undergo slow redox disproportionation reactions ($k = 35\ M^{-1}\ s^{-1}$, pH 7.0) which have been studied by optical and electrochemical methods. The Ru(IV) species thus created subsequently rearranges in an irreversible manner. In the presence of excess oxidant, all of the bound ruthenium is converted to the rearranged form. To avoid this problem, the [$L(NH_3)_4Ru^{III}$cyt c^{II}] intermediates for intramolecular electron-transfer studies were generated from the stable [$L(NH_3)_4Ru^{II}$cyt c^{II}] form.

Introduction

Electron-transfer proteins covalently modified with substitution-inert redox reagents have been used extensively to examine the factors which control the rates of electron transfer across protein matrices.[1−3] The most versatile of these reagents include ruthenium complexes of the type [$(NH_3)_5RuOH_2$], [$L(NH_3)_4Ru(OH_2)$] (L = pyridine derivative), and ruthenium polypyridine complexes, where the ruthenium center is selectively bound to histidine residues on the surface of the protein. One of the attractive features of these ruthenium complexes is that the nature of the ligands around the ruthenium center can be used to control the driving force, direction, and rate of electron transfer within the modified protein.

In this paper we report on the rate constants for intramolecular electron transfer in a series of *cis*- and *trans*-[$L(NH_3)_4Ru^{III}$cyt *c*] derivatives (cyt *c* is horse-heart (hh) or *Candida krusei* (*Ck*) cytochrome *c* and L is isonicotinamide (isn) or pyridine (py)). The rate constants were obtained by generating the [$L(NH_3)_4Ru^{III}$cyt c^{II}] intermediates from the [$L(NH_3)_4Ru^{II}$cyt c^{II}] proteins by oxidative pulse radiolysis.[4] The intramolecular electron-transfer kinetics of these modified proteins demonstrate that electron transfer from and to the heme is a reversible process governed by the reorganization energy, driving force, and location of the binding site of the ruthenium label in the protein matrix.

In addition to the expected intramolecular redox reactions that occur in the [$L(NH_3)_4Ru^{III}$]-modified proteins, we report here that the LRu^{III} center in these modified proteins also undergoes a bimolecular redox disproportionation reaction. Similar disproportionation reactions were previously reported to occur for $[(NH_3)_5Ru^{III}py]^{3+}$ in basic solution (pH > 8)[5a] and for $[((NH_3)_5Ru^{III})_2pyrazine]^{6+}$ in solutions as low as pH 4.[5b] In the case of [$L(NH_3)_4Ru^{III}$cyt *c*] (both *Ck* and hh cyt *c*) studied here, the disproportionation reaction has been observed under neutral and weakly acidic conditions (ca. pH ≥ 6). This disproportionation reaction was prevented from interfering with the normal intramolecular electron-transfer reaction ($LRu^{II/III}$ redox states) by keeping the modified proteins in the Ru^{II} form prior to the electron-transfer studies. The discovery of this disproportionation reaction led us to revise an earlier interpretation of a low rate constant for intramolecular electron transfer in [$L(NH_3)_4Ru^{III}$cyt c^{II}].[22] The effect of this disproportionation reaction on intramolecular electron transfer rate constants in ruthenium modified protein derivatives is discussed. In the previous work with [$(NH_3)_5Ru^{III}$cyt c^{III}] derivatives[1] evidence of this disproportionation reaction had not been observed even when the proteins are stored in the Ru^{III} state for a long time.

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[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1995.

(1) (a) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659. (b) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722. (c) Isied, S. *Prog. Inorg. Chem.* **1984**, *32*, 443. (d) Isied, S. S. In *Metal Ions in Biological Systems*; Sigel, H., Sigel, A., Eds.; Marcel Dekker: New York, 1991; Vol. 27, pp 1−56.

(2) (a) Yocom, K.; Shelton, J. B.; Schroeder, W. A.; Worosila, G.; Isied, S. S.; Bordignon, E.; Gray, H. B. *Proc. Natl. Acad. Sci U.S.A.* **1982**, *91*, 7052. (b) Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145. (c) Meade, T. J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 4353. (d) Therien, M. J.; Selman, M.; Gray, H. B.; Chang, I.-J.; Winkler, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 2420. (e) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369.

(3) (a) Osvath, P.; Salmon, G. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1988**, *110*, 7114. (b) Jackman, M. P.; Lim, M. C.; Sykes, A. G.; Salmon, G. A. *J. Chem. Soc., Dalton Trans.* **1988**, 2843. (c) Sykes, A. G. *Chem. Br.* **1988**, *24*, 551.

(4) Reference 1d, p 43.

(5) (a) Rudd, D. P.; Taube, H. *Inorg. Chem.* **1971**, *10*, 1543. (b) Creutz, C.; Taube, H. *J. Am. Chem. Soc.* **1973**, *95*, 1086.

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Experimental Section

Methods and Materials. All the chemicals were reagent grade unless otherwise indicated. Cation exchange chromatography was carried out using microgranular CM 52 Cellulose (Whatman) and Bio-Rex 70 (BioRad) (400 mesh). Ultrafiltration cells and YM5 and YM10 membranes were obtained from Amicon (Division of W. R. Grace).

Horse-heart cytochrome *c* (Type VI) was obtained from Sigma. *Candida krusei* cytochrome *c* was provided as a gift by Sankyo Chemical Co. (Japan). Both proteins were purified using minor modifications of literature procedures[6] (see below).

Instruments and Techniques. UV−visible spectra were obtained on a Hewlett-Packard 8452A diode array spectrometer. HPLC was done using a Waters 994 programmable diode array detector and an Autochrome M320 HPLC gradient workstation. Circular dichroism (CD) spectra were recorded using an Aviv Associates Model 60DS spectrometer with a programmable, thermoelectrically controlled cell holder. For the CD spectra, a 10 μM protein solution in 50 mM, pH 7.0, potassium phosphate buffer was used, and each spectrum was the average of three scans. CD spectra in the visible region (300−600 nm) were obtained using quartz cells with a 1 cm path length, while in the ultraviolet region (180−300 nm) cells with a 0.1 cm path length were used.

Cyclic voltammograms (CV), differential pulse voltammograms (DPV), and Osteryoung square wave voltammograms (OSWV) were recorded using either a BAS 100A or a BAS 100B electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN). A three-electrode configuration was used, consisting of a gold disk working electrode (MF 2014, Bioanalytical Systems), a platinum wire auxiliary electrode, and a saturated sodium chloride calomel reference electrode. The gold disk working electrode was polished with a 0.05 μm alumina slurry (Union Carbide, Indianapolis, IN). In all the electrochemical measurements for both the native and modified proteins, 4,4′-bipyridyl disulfide was used to modify the gold electrode surface,[7] and the cyt *c* concentrations were ~0.1−0.3 mM in 50 mM potassium phosphate (pH 7.0) solution. The formal potentials ($E^{\circ\prime}$) were determined from the average of reduction (E_{pc}) and oxidation (E_{pa}) peak potentials obtained in cyclic voltammetry experiments. Potentials vs the normal hydrogen electrode (NHE) are obtained by adding 236.0 mV to the potentials vs SSCE.[9]

Tryptic digestions of the modified proteins were carried out as previously described.[10] Ruthenium concentrations in the modified proteins were determined by directly coupled plasma atomic emission spectroscopy (DCP-AES) at either 372.8 or 349.9 nm using a Spectrometrics Spectrascan IIIB spectrometer.

Synthesis of the Metal Complexes. Pentaamminechlororuthenium(III) trifluoroacetate, $[(NH_3)_5RuCl](TFA)_2$, was prepared as follows: $[(NH_3)_5RuCl]Cl_2$ (340 mg; 1.16 mmol) was added to a solution of silver carbonate Ag_2CO_3 (320 mg; 1.16 mmol) in 2 mL of 4 M trifluoroacetic acid (TFA), and the mixture was stirred for 1 h. The resulting AgCl precipitate was filtered off and washed with a small amount of 4 M trifluoroacetic acid. The filtrate was concentrated to dryness by rotary evaporation, redissolved in water, and taken to dryness again. The solid was dissolved in 1 mL of ethanol, and 20 mL of ether was added to precipitate the yellow product. The product was collected by filtration, washed with ether, and dried in a desiccator *in vacuo* to yield 330 mg (63%).

trans-$[(NH_3)_4Ru(SO_2)Cl]Cl$[11] was prepared as described before. *trans*-$[(NH_3)_4Ru(HSO_3)_2]$, *trans*-$[(NH_3)_4Ru(isn)(SO_2)](CF_3SO_3)_2$, *trans*-$[(NH_3)_4Ru(isn)(SO_4)]Cl$, *trans*-$[(NH_3)_4Ru(py)(SO_4)]BF_4$, *trans*-$[(NH_3)_4Ru(im)L](TFA)_3$ (L = isn, py), and *trans*-$[(NH_3)_4Ru(isn)_2]Cl_3$ were prepared according to ref 12.

Potassium tris(oxalato)cobaltate, $K_3[Co(C_2O_4)_3]\cdot 3.5H_2O$, was prepared as in ref 13, and its spectrum, (ϵ_{425} = 228 M^{-1} cm^{-1} and ϵ_{605} = 174 M^{-1} cm^{-1}) agreed with literature values (ϵ_{425} = 230 M^{-1} cm^{-1} and ϵ_{605} = 175 M^{-1} cm^{-1}). Potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8]$, was prepared as described in ref 14 and then oxidized in acidic solution by $KMnO_4$. The Ag^+ salt was converted to $K_3[Mo(CN)_8]$ by addition of KCl.[14] Ammonium bis(dipicolinato)cobaltate(III), $(NH_4)[Co(dipic)_2]$, was prepared according to ref 15.

Purification of Cytochrome *c*. Horse-heart (hh) cytochrome *c* (Sigma, Type VI) (150 mg) or *Candida krusei* (*Ck*) cyt *c* (75 mg)[6] in ~3 mL of 50 mM potassium phosphate buffer, pH 7.0, was oxidized by the addition of several crystals of solid potassium ferricyanide and purified by cation exchange chromatography on a CM-52 cellulose column (1.4 × 20 cm) by eluting the major protein fraction with 80 mM pH 7.0 potassium phosphate buffer for hh cyt *c* or a gradient of 50−80 mM pH 7.0 potassium phosphate buffer for *Ck* cyt *c*.

Modification of *Candida krusei* Cytochrome *c* with $[(NH_3)_5Ru(OH_2)]^{2+}$. Purified *Candida krusei* (*Ck*) ferrocytochrome *c* (50 mg, 0.004 mmol) was equilibrated with 100 mM HEPES buffer (pH 7.0) or with 20 mM potassium phosphate buffer (pH 7.0), concentrated to ~5 mL by ultrafiltration (Amicon, YM-5 membrane), and stirred gently under argon for 30 min.

Separately, a degassed solution of $[(NH_3)_5RuCl](TFA)_2$ (26 mg; 0.06 mmol in ~2 mL of water) was reduced with zinc amalgam for 45 min. The bright yellow solution was then transferred by gastight syringe to the cyt *c* solution. The reaction was allowed to proceed for 45 min at room temperature with stirring under an argon atmosphere. The reaction mixture was then centrifuged for ~5 min, and the supernatant was added to 100 mL of 10 mM ascorbic acid in 50 mM potassium phosphate buffer (pH 7.0). The resulting solution was washed by ultrafiltration to remove the excess unreacted ruthenium complex and then washed with several volumes of 50 mM potassium phosphate buffer (pH 7.0) to remove the excess ascorbic acid and concentrated to ~2 mL.

The concentrated protein solution was oxidized with solid potassium ferricyanide (few crystals) and loaded onto a Bio-Rex 70 cation exchange column (1 × 5 cm) which was previously equilibrated with 100 mM potassium phosphate buffer (pH 7.0). Unreacted native *Ck* cyt *c* eluted first with 100 mM potassium phosphate buffer (pH 7.0). The modified protein was eluted with a linear gradient from 0 to 100% 0.25 M KCl in 100 mM potassium phosphate buffer (pH 7.0). The Ru-modified cyt *c* species collected were concentrated and equilibrated with 50 mM potassium phosphate buffer (pH 7.0) by ultrafiltration (Amicon, YM-5 membrane). To ensure purity, the major Ru-modified cyt *c* species was rechromatographed two more times on a Bio-Rex 70 cation exchange column under the same conditions described above. The yield of this major modified protein after repurification was 20−25%. The purified Ru-modified cyt *c* was stored at −5 °C.

Modification of Horse Heart or *Candida krusei* Cytochrome *c* with *trans*-$[(NH_3)_4Ru(isn)(OH_2)]^{2+}$. Purified cyt *c* (hh or *Ck*) (~120 mg; 0.01 mmol) in ~5 mL of 50 mM potassium phosphate buffer (pH 7.0) was reduced with excess solid ascorbic acid, washed with several volumes of the same buffer to remove the ascorbic acid, concentrated to ~3 mL by ultrafiltration (Amicon, YM-5 membrane), and degassed by stirring gently under an argon blanket for 30 min.

Separately, a degassed solution of *trans*-$[(NH_3)_4Ru(isn)(SO_4)]Cl\cdot 2H_2O$ (60 mg; 0.14 mmol) in ~4 mL of water was reduced by degassing for 45 min in the presence of fresh zinc amalgam, during which time the solution changed from pale yellow to blood red. This red solution was then transferred using a gastight syringe to the reduced cyt *c* solution, and the resulting solution was stirred under argon for 15 h (hh) or 10 h (*Ck*) while shielded from light. The solution was then centrifuged for ~5 min, and the supernatant was washed by ultrafiltration with ~100 mL of 10 mM ascorbic acid in 50 mM pH

(6) Brautigan, D. L.; Ferguson-Miller, S.; Margoliash, E. In *Methods in Enzymology*; Fleischer, S., Packer, L., Eds.; Academic Press: New York, 1978; Vol. III, p 128.
(7) Taniguchi, I.; Toyosama, K.; Yamaguchi, K. *J. Electroanal. Chem. Interfacial Electrochem.* **1982**, *140*, 187.
(8) Parry, E.; Osteryoung, R. *Anal. Chem.* **1965**, *37*, 1634.
(9) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications*; John Wiley & Sons: New York, 1980.
(10) Bechtold, R.; Gardineer, M. B.; Kazmi, A.; van Hemelryck, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800−3804.
(11) Vogt, L. H.; Katz, J. L.; Wiberley, S. E. *Inorg. Chem.* **1965**, *4*, 1157.
(12) Isied, S. S.; Taube, H. *Inorg. Chem.* **1976**, *15*, 3070.
(13) Bailar, J. C., Jr.; Jones, E. M. *Inorg. Synth.* **1939**, *1*, 37.
(14) Furman, N. H.; Miller, C. O. *Inorg. Synth.* **1950**, *3*, 160.
(15) Mauk, A. G.; Coyle, C. L.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1979**, *101*, 5054.

7.0 potassium phosphate buffer to remove any unreacted ruthenium complex, and then washed with several volumes of 50 mM pH 7.0 potassium phosphate buffer to remove the ascorbic acid and concentrated to ~2 mL.

The concentrated protein solution was loaded onto a Bio-Rex 70 cation exchange column (1 × 5 cm), previously equilibrated with 100 mM potassium phosphate buffer (pH 7.0). For hh cyt *c*, three protein bands were usually separated—unmodified native cyt *c*, singly modified cyt *c*, and highly charged species which remained bound near the top of the resin bed. For *Ck* cyt *c* both the His 39 and His 33 sites can be modified; thus four protein species usually resulted—native cyt *c*, singly and doubly modified cyt *c* species, and highly charged species which stayed near the top of the resin bed. For either hh or *Ck* cyt *c*, unreacted cyt *c* was eluted first, using 100 mM potassium phosphate buffer (pH 7.0). Then the modified protein (one species for hh and two species for *Ck*) was eluted using a linear gradient from 0 to 100% 0.25 M KCl in 100 mM potassium phosphate buffer (pH 7.0). For hh cyt *c*, this modified protein species was concentrated and equilibrated with 50 mM potassium phosphate buffer (pH 7.0) by ultrafiltration (Amicon, YM-5 membrane). For *Ck* cyt *c*, the singly and doubly Ru-modified cyt *c* bands were separately collected, concentrated, and washed with phosphate buffer using ultrafiltration. The modified protein species were rechromatographed two more times onto a Bio-Rex 70 cation exchange column under the same conditions as described above. Yields of modified hh cyt *c* varied from 7 to 15% after repurification. Yields of the major singly Ru-modified *Ck* cyt *c* species varied from 10 to 30% after repurification. The yield of the doubly Ru-modified *Ck* cyt *c* species was ~10%. The purified Ru-modified cyt *c* was stored at −5 °C.

The horse-heart cytochrome *c* complexes modified with [$L(NH_3)_4$Ru] where L = *cis*-py and *trans*-py were synthesized using a procedure similar to that for *cis*- and *trans*-[isnRu$(NH_3)_4$cyt *c* (hh)] described above.

Results and Discussion

Synthesis and Characterization of the Modified Proteins. The modification of *Candida krusei* cyt *c* with [$(NH_3)_5Ru^{III}$] at His 39 was performed using a slight variation of the published procedure for the hh cyt *c* species [$(NH_3)_5Ru^{III}$(His 33)cyt c^{III}(hh)].[1] The difference between the hh and *Ck* preparations is mainly in the time of the reaction (<1 h for *Ck* and 24−48 h for hh). The His 39 modification site is more accessible and therefore modifies faster. The shorter reaction time for *Ck* also minimizes the amount of doubly-modified protein. The [$(NH_3)_5$-Ru^{III}(His 39)cyt c^{III}(*Ck*)] derivative was characterized by UV−vis spectra, CD spectra, electrochemistry, and tryptic digestion as described earlier for the hh protein.[10]

The horse-heart cytochrome *c* complexes modified with [$L(NH_3)_4$Ru−] where L = *trans*-isn, *cis*-isn, *trans*-py, and the *Candida krusei* cytochrome *c* complex where L = *trans*-isn, were synthesized and stored in the LRu^{II}cyt c^{II} state ($L(NH_3)_4$-Ru is abbreviated as LRu throughout the paper).

The modified (hh and *Ck*) cytochrome *c* proteins were characterized in part by their UV−vis spectra. The presence of the absorption band at λ = 695 nm in each of the modified ferricytochromes corresponds to the Met 80−Fe^{III} LMCT.[16] The difference spectra between the unmodified cytochrome species and the corresponding LRu^{II}cyt *c* complexes showed additional absorption bands at λ = 478 and 480 nm in the [isnRu^{II}cyt *c* (hh)] and [isnRu^{II}cyt *c* (*Ck*)] species, respectively, corresponding to Ru^{II}-to-N-heterocycle LMCT bands. The CD spectra obtained for the ruthenium-modified cyt *c* derivatives were very similar to those for the native cyt *c* species. DCP-AES spectroscopy was used to measure the extent of ruthenium modification in several of the modified cytochromes (protein concentrations were determined by UV−vis absorbance measurements of the Soret band maximum). The ruthenium-to-iron ratios were as follows: [isnRu^{II}cyt *c* (hh)], 0.80; [isnRu^{II}cyt *c* (*Ck*)], 0.85; doubly-modified [$(isnRu^{II})_2$cyt *c* (*Ck*)], 1.86.

Peptide mapping of the tryptic digests for the [LRu^{II}cyt *c* (hh)] and [LRu^{II}cyt *c* (*Ck*)] derivatives was carried out as described in ref 10. The peptide fragment with the ruthenium label was isolated separately in each case and characterized by its spectra (λ_{max} = 485 nm for [isn$(NH_3)_4Ru^{II}$]) and by amino acid analysis. For [LRu^{II}cyt *c* (hh)], the peptide fragment containing the ruthenium complex was T7 (amino acids 28−38 = Asn (as Asp, 1.0), Thr (0.9), Gly (3.3), Leu (2.0), Phe (1.1), Lys (0.5), His (1.0), Arg (1.0), Pro not detectable). For [LRu^{II}cyt *c* (*Ck*)] and [$(NH_3)_5Ru^{III}$cyt *c* (*Ck*)], the peptide fragment containing the ruthenium complex was T6 (amino acids 39−54, for L = isn: Asp and Asn (2.1), Thr (1.0), Ser (2.1), Glu and Gln (2.3), Gly (2.2), Ala (1.6), Tyr (1.6), Lys (1.1), His (1.05), Arg (1.1)). Spectra of the ruthenium-modified tryptic digestion fragments for [$Ru(NH_3)_5$cyt *c* (*Ck*)], [LRu^{III}-cyt *c* (*Ck*)], and [LRu^{III}cyt *c* (hh)] (L = *trans*-isn) are contributed as supplementary material.

The ruthenium-modified proteins were studied in their reduced LRu^{II}cyt c^{II}, mixed-valence LRu^{II}cyt c^{III}, and oxidized LRu^{III}cyt c^{III} states. The reduced LRu^{II}cyt c^{II} state was generated from the mixed-valence state (the state which was used throughout the chromatographic separations) by the addition of excess ascorbic acid, followed by ultrafiltration, and handled under argon. The reduced cyt *c* solution was washed and concentrated before it was stored at −5 °C. The LRu^{II}cyt c^{III} complex can also be reversibly generated from the reduced state by oxidation with excess $K_3[Fe(CN)_6]$ ($E°$ = 0.41 V),[17] followed by chromatographic separation of the excess oxidant on a Bio-Gel P2 or Pharmacia Sephadex G-15 or G-25 column and concentration by ultrafiltration before storage. The LRu^{III}cyt c^{III} complex was prepared by oxidation of LRu^{II}cyt c^{II} or LRu^{II}-cyt c^{III} with $[Co(ox)_3]^{3-}$ ($E°$ = 0.57 V),[18a] $[Co(dipic)_2]^-$ ($E°$ = 0.747 V),[18a] *trans*-$[Ru(NH_3)_4(isn)_2]^{3+}$ ($E°$ = 0.70 V),[18b] or $[Mo(CN)_8]^{3-}$ ($E°$ = 0.725 V).[18a] These oxidants have sufficiently high potentials to oxidize the ruthenium sites. The oxidized LRu^{III}cyt c^{III} solution was washed with buffer, concentrated by ultrafiltration, and diluted to the desired concentration before use.

Reversible Electrochemistry of $LRu^{II/III}$cyt $c^{II/III}$. The LRu^{II}cyt c^{II} (and LRu^{II}cyt c^{III}) complexes exhibit reversible cyclic voltammetry (CV) and differential pulse voltammetry (DPV) in all cases (L = *trans*-isn, *cis*-isn, *trans*-py; cyt *c* = hh, *Ck*). The cyclic voltammogram for LRu^{II}cyt c^{II} (hh) (L = *trans*-isn) is shown in Figure 1, and the other derivatives (*Ck* cyt *c* or L = py, isn) show similar results. The redox potentials of the ruthenium sites and the heme sites for the different $LRu^{III/II}$cyt $c^{II/III}$ species are summarized in Table 1. The redox potentials of the ruthenium site in the $LRu^{III/II}$cyt *c* proteins are significantly higher than that for the model complex *trans*-$[(NH_3)_4Ru(isn)im]^{3+/2+}$ (where im = imidazole), with the ruthenium potential of $LRu^{III/II}$cyt *c* (*Ck*) (modified at His 39) 34 mV lower than that of $LRu^{III/II}$cyt *c* (hh) (modified at His 33). The same trend is observed for the redox potentials of the corresponding [$(NH_3)_5Ru^{III}$cyt c^{III}] derivatives and the model compound $[Ru(NH_3)_5(im)]^{3+/2+}$ (Table 1).

Kinetics of Intramolecular Electron Transfer. The LRu^{III}-cyt c^{II} electron-transfer intermediate can be generated either by

(16) Margoliash, E.; Schejter, A. *Adv. Protein Chem.* **1966**, *21*, 113.

(17) Butler, J.; Davies, D. M.; Sykes, A. G. *J. Inorg. Biochem.* **1981**, *15*, 41.

(18) (a) *Standard Potentials in Aqueous Solutions*; Bard, A. J., Parson, J., Eds.; Marcel Dekker: New York, 1985. (b) Silverman, M.; Isied, S. S. Unpublished work.

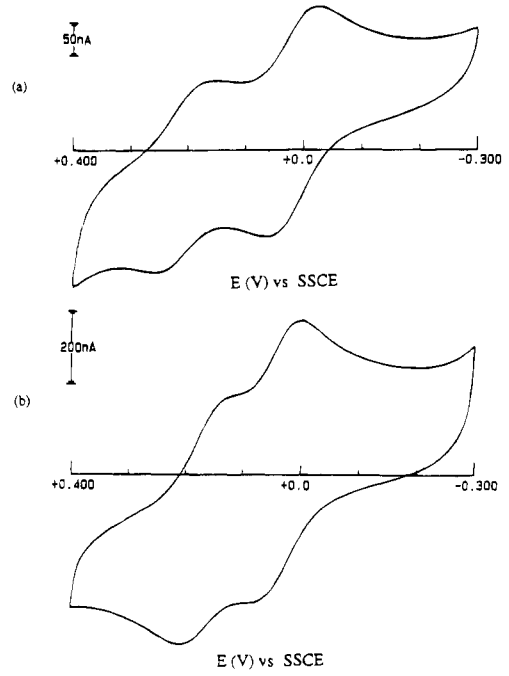


Figure 1. Cyclic voltammograms of (a) ~330 μM horse-heart *trans*-isn$(NH_3)_4$Ru-cyt *c* in 20 mM pH 7.0 phosphate buffer and 0.1 M $NaClO_4$ (scan rate 10 mV s^{-1}) and (b) ~200 μM *Candida krusei trans*-isn$(NH_3)_4$Ru-cyt *c* in 50 mM pH 7.0 phosphate buffer (scan rate 50 mV s^{-1}).

Table 1. Redox Potentials for Native Cytochromes, Ruthenium-Modified Cytochromes, and Ruthenium Model Complexes[a]

complex	$E^{\circ\prime}$, mV vs NHE	
	$Fe^{III/II}$	$Ru^{III/II}$
cyt *c* (hh)	259	
cyt *c* (*Ck*)	266	
$[(NH_3)_5$Ru-cyt *c* (hh)]	260	128
$[(NH_3)_5$Ru-cyt *c* (*Ck*)]	275	95
$[(NH_3)_5Ru(im)]^{3+/2+}$		71
trans-$[(NH_3)_4Ru(isn)]$-cyt *c* (hh)	253	434
trans-$[(NH_3)_4Ru(isn)]$-cyt *c* (*Ck*)	273	400
trans-$[(NH_3)_4Ru(isn)im]^{3+/2+}$		371
trans-$[(NH_3)_4Ru(pyridine)]$-cyt *c* (hh)	256[b]	370[b]

[a] Cyclic voltammetry unless otherwise noted. Averages of anodic and cathodic peak potentials. Conditions: 50 mM phosphate buffer, pH 7.0, 25 °C. [b] Differential pulse voltammetry. Corrected for 25 mV pulse amplitude ($E^{\circ\prime} = E_{peak} + \Delta E/2$).

oxidizing LRu^{II}cyt c^{II} with a one-electron oxidant (e.g., $CO_3^{\cdot-}$ or $N_3^{\cdot}$) or by reducing LRu^{III}cyt c^{III} with a one-electron reductant (e.g. $CO_2^{\cdot-}$). In order to prevent complications due to the disproportionation of ruthenium(III) in the LRu^{III}cyt c^{III} proteins (discussed in the next section), the LRu^{III}cyt c^{II} intermediate was formed by oxidation of LRu^{II}cyt c^{II} according to eq 1, where $Ox^{\cdot} = CO_3^{\cdot-}$ or $N_3^{\cdot}$.

$$LRu^{II}\text{cyt } c^{II} + Ox^{\cdot} \xrightarrow{k_{et}} LRu^{III}\text{cyt } c^{II} + Ox^{-} \quad (1)$$
$$LRu^{II}\text{cyt } c^{II} + Ox^{\cdot} \rightarrow LRu^{II}\text{cyt } c^{III} + Ox^{-}$$

The intramolecular electron-transfer rate constants were measured between pH 7 and 9 at λ = 550, 504, and 432 nm. The absorbance vs time profiles for the electron-transfer reaction for the different derivatives are illustrated in Figure 2. These profiles show the direct oxidation of the cyt *c*(II) (λ = 550 nm) and the Ru(II) (λ = 504 nm (L = isn), 432 nm (L = py)) to generate the kinetic intermediate LRu^{III}cyt c^{II}, which then

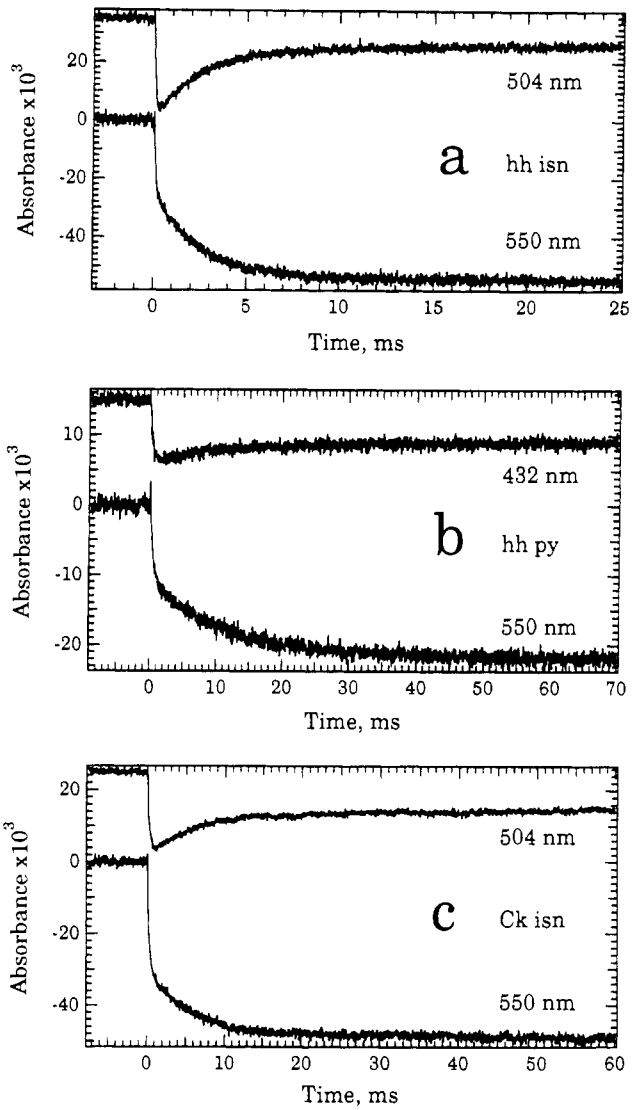


Figure 2. Absorbance vs time profiles at 25 °C for intramolecular electron transfer from heme (absorbance decrease at 550 nm) to Ru^{III} (recovery at 432 or 504 nm, cytochrome *c* isosbestic points where Ru^{II} absorbs) in *trans*-$L(NH_3)_4ru^{III}$cyt c^{II}: (a) horse-heart, L = isonicotinamide; (b) horse-heart, L = pyridine; (c) *Candida krusei*, L = isonicotinamide. The (Ru^{III},Fe^{II}) electron-transfer intermediates were created by the oxidation of the (Ru^{II},Fe^{II}) starting materials by azide radical ($N_3^{\cdot}$). Conditions: 4 μM protein, 50 mM pH 7.0 phosphate buffer, 1 mM NaN_3, N_2O-saturated solution.

undergoes intramolecular electron transfer to form the stable product LRu^{II}cyt c^{III}. For each protein derivative, the same rate constant for intramolecular electron transfer was obtained at each wavelength studied. The intramolecular electron-transfer rate constants measured for the Ru-modified proteins at different concentrations are given in Table 2. The intramolecular electron-transfer rate constants for the *trans*- and *cis*-LRu-cyt *c* (hh) (L = isn, py) and the activation parameters obtained by determination of the rate constants at different temperatures are given in Tables 3 and 4.

For $[(NH_3)_5$Ru-cyt *c* (hh)], the direction of electron transfer (k = 53 s^{-1})[1,2] is from the Ru^{II} bound at the surface His 33 to the heme group in the interior of the protein, whereas for isnRu^{III}cyt *c* (hh) the intramolecular electron transfer takes place from the heme to the LRu^{III} site (k = 440 s^{-1}, for both the *trans*- and *cis*-isnRu-cyt *c* derivatives). The similarity of the

(19) Sun, J.; Wishart, J. F.; Isied, S. S. *Inorg. Chem.*, in press.

Table 2. Concentration Dependence of Intramolecular Electron-Transfer Rate Constants in $(NH_3)_4Ru(L)$-Modified Cytochromes *c*[a,b]

derivative	concn, μM	k_{et}, s^{-1}
trans-[$(NH_3)_4$Ru(isn)]-cyt *c* (hh)	4	422 ± 35
	12	448 ± 25
	16	442 ± 25
cis-[$(NH_3)_4$Ru(isn)]-cyt *c* (hh)	1.0	472 ± 63
	2.6	457 ± 34
	5.2	437 ± 24
trans-[$(NH_3)_4$Ru(isn)]-cyt *c* (*Ck*)	4	217 ± 20
	8	221 ± 20
	12	222 ± 20
trans-[$(NH_3)_4$Ru(pyridine)]-cyt *c* (hh)	1.4	99 ± 9
	3.6	112 ± 6
	7.2	103 ± 7

[a] For reaction in eq 1. [b] Solution: 0.1 M $NaHCO_3$, 50 mM phosphate buffer, N_2O-saturated, pH 7.6–8.3.

Table 3. Temperature Dependence of Intramolecular Electron-Transfer Rate Constants in $(NH_3)_4Ru(L)$-Modified Cytochromes *c*[a]

derivative	*T*, K	k_{et}, s^{-1}
trans-[$(NH_3)_4$Ru(isn)]-cyt *c* (hh)[b] (4.0 μM, pH 7.0)	283.9	234 ± 7
	289.1	294 ± 15
	298.2	440 ± 20
	308.2	690 ± 30
	318.1	1040 ± 90
cis-[$(NH_3)_4$Ru(isn)]-cyt *c* (hh)[c] (5.2 μM, pH 8.3)	276.4	180 ± 17
	298.0	437 ± 24
	315.3	576 ± 53
trans-[$(NH_3)_4$Ru(isn)]-cyt *c* (*Ck*)[c] (8 μM, pH 7.4)	276.8	83 ± 7
	287.7	149 ± 5
	298.2	220 ± 20
	308.2	308 ± 12
trans-[$(NH_3)_4$Ru(pyridine)]-cyt *c* (hh)[b] (4.5 μM, pH 7.0)	282.2	49 ± 2
	288.1	74 ± 2
	298.2	126 ± 5
	308.2	202 ± 4
	318.3	335 ± 10

[a] For reaction in eq 1. [b] Solution: 1 mM NaN_3, 50 mM pH 7.0 phosphate buffer, N_2O-saturated. [c] Solution: 0.1 M $NaHCO_3$, 50 mM phosphate buffer, N_2O-saturated.

Table 4. Rate Constants and Activation Parameters for Intramolecular Electron Transfer in $(NH_3)_4Ru(L)$-Modified Cytochromes *c*

L	species	$-\Delta G°$, eV	k(25 °C), s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal deg^{-1} mol^{-1}
NH_3	hh	0.13	53 ± 2[a,c]	3.5 ± 0.1[a]	-39.0 ± 0.4[a]
		−0.13	0.40 ± 0.01[c,d]	12.5 ± 0.2[d]	−18.3 ± 0.7[d]
NH_3	*Ck*	0.18	154 ± 8[a,c]	2.3 ± 0.2	−41.0 ± 0.7
trans-py	hh	0.11	126 ± 5[b,e]	8.8 ± 0.1	−19.3 ± 0.3
trans-isn	hh	0.18	440 ± 23[b,e]	7.3 ± 0.1	−22.0 ± 0.4
cis-isn	hh	0.18	437 ± 24[b,f]	6.2 ± 0.3	−26 ± 1
trans-isn	Ck	0.13	220 ± 20[b,f]	6.4 ± 0.3	−27 ± 1

[a] Ru^{II}-to-Fe^{III} electron transfer; ref 1b. [b] Fe^{II}-to-Ru^{III} electron transfer for reaction in eq 1. [c] Solution: 50–100 mM Na formate, 50 mM pH 7.0 phosphate buffer, N_2O-saturated. [d] Reference 19. [e] Solution: 1 mM NaN_3, 50 mM pH 7.0 phosphate buffer, N_2O-saturated. [f] Solution: 0.1 M $NaHCO_3$, 50 mM phosphate buffer, N_2O-saturated, pH 8.3.

rate constants for the *trans*- and *cis*-isnRu-cyt *c* derivatives indicates that orientation and orbital symmetry differences in *trans*- and *cis*-isnRu-cyt *c*, to the extent that they exist in this pair of complexes, have no effect on the electron-transfer rate constants. The difference between the rate constants for the [$(NH_3)_5$Ru-cyt *c* (hh)] (k = 53 s^{-1}) and isnRu-cyt *c* (hh) (k = 440 s^{-1})[20] complexes can be ascribed to an increase in driving force in isnRu-cyt *c* (hh), along with a small decrease in reorganization energy of the ruthenium center.[19] The pyridine–Ru-cyt *c* (hh) derivative also behaves similarly to the isonicotinamide derivative but with a lower rate constant (k = 126 s^{-1}), reflecting the weaker driving force for the intramolecular electron-transfer reaction in this derivative. For [$(NH_3)_5$Ru-cyt *c* (*Ck*)] (modified at His 39), the driving force for Ru-to-heme electron transfer (at this modification site) is increased ($-\Delta G°$ = 0.18 eV), while that for the reverse direction in the LRu-cyt *c* (*Ck*) (L = *trans*-isn) is decreased ($-\Delta G°$ = 0.13 eV), resulting in nearly the same electron-transfer rate constants k = 154 and 220 s^{-1}, respectively (Table 4); i.e., the LRu-cyt *c* (*Ck*) (L = *trans*-isn) system has a weaker driving force but also a lower reorganization energy than [$(NH_3)_5$Ru-cyt *c* (*Ck*)].

Disproportionation of LRu^{III} in LRu^{III}cyt c^{III}. (a) Spectral Evidence. Oxidation of a pH 7.0 solution of LRu^{II}cyt c^{III} (L = *trans*-isn) (~10–20 μM) by 1 equiv of $[Co(ox)_3]^{3-}$ results in absorbance changes in the visible region (Figure 3 (bottom)). The initially formed LRu^{III}cyt c^{III} partially converts back to LRu^{II}cyt c^{III}, as evidenced by an absorbance increase at λ = 472 nm characteristic of the LRu^{II} MLCT transition (Figure 3 (top)). As much as 40–50% of the LRu^{II}cyt c^{III} can be recovered. The formation of LRu^{II}cyt *c* (~50%) from LRu^{III}-cyt *c* is analogous to the previously reported disproportionation of [$(NH_3)_5Ru^{III}$py] in basic solution,[5a] that of [$((NH_3)_5Ru^{III})_2$-pyrazine]$^{6+}$ at pH 4 or greater,[5b] and that of other ruthenium complexes.[21,25] In the [$(NH_3)_5Ru^{III}$py] case, the rate of disproportionation followed the rate law $-d[Ru(III)]/dt = k[Ru(III)]^2$, with second-order rate constants varying from ~0.3 to 3 M^{-1} s^{-1} (pH 8.3–10).[5a]

By analogy to the behavior of $[(NH_3)_5Ru^{III}py]^{3+}$, the absorbance change and the formation of 50% LRu^{II}cyt c^{III} (L = *trans*-isn) from the oxidation of LRu^{III}cyt c^{III} at pH 7 are attributed to the following disproportionation reaction:

$$2[LRu^{III}\text{cyt } c^{III}] \rightarrow [LRu^{II}\text{cyt } c^{III}] + [LRu^{IV}\text{cyt } c^{III}] \quad (2)$$

The disproportionation rate of LRu^{III}cyt *c* is concentration dependent, with an estimated rate constant of $2k$ = 35 M^{-1} s^{-1} at pH 7 (for horse-heart, estimated from absorbance vs time plots at 504 nm on freshly oxidized solutions). This is at least an order of magnitude faster than the analogous disproportionation reaction of $[(NH_3)_5Ru^{III}py]^{3+}$.[5a] Due to the second-order dependence in Ru^{III}-modified cytochrome *c*, reaction 2 occurs even faster when the protein solutions are concentrated after elution from chromatographic columns. This disproportionation occurs appreciably in both the hh and *Ck* species of LRu^{III}cyt c^{III} at lower pH values than is observed for the model compound

(20) Moreira, I.; Sun, J.; Cho, M. O.-K.; Wishart, J. F.; Isied, S. S. *J. Am. Chem. Soc.* **1994**, *116*, 8396.
(21) (a) Ilan, Y.; Taube, H. *Inorg. Chem.* **1983**, *22*, 1655. (b) *Inorg. Chem.* **1983**, *22*, 3144.
(22) Bechtold, R.; Kuehn, C.; Lepre, C.; Isied, S. S. *Nature (London)* **1986**, *322*, 286.
(23) Therien, M. J.; Bowler, B. E.; Selman, M. A.; Gray, H. B.; Chang, I.-J.; Winkler, J. R. In *Electron Transfer in Inorganic, Organic and Biological Systems*; Bolton, J. R., Mataga, N., McLendon, G., Eds.; Advances in Chemistry 228; American Chemical Society: Washington, DC, 1991; pp 191–200.
(24) (a) Lieber, C. M.; Karas, J. L.; Gray, H. B. *J. Am. Chem. Soc.* **1987**, *109*, 3778. (b) Karas, J. L.; Lieber, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 599.
(25) Disproportionation reactions of Ru^{III} to Ru^{II} and Ru^{IV} for a variety of ruthenium ammine complexes with unsaturated pyridine ligands and other ligands have been reported. See for example: (a) Ridd, M. J.; Keene, F. R. *J. Am. Chem. Soc.* **1981**, *103*, 5733. (b) Berhard, P.; Sargeson, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 597 and references therein.

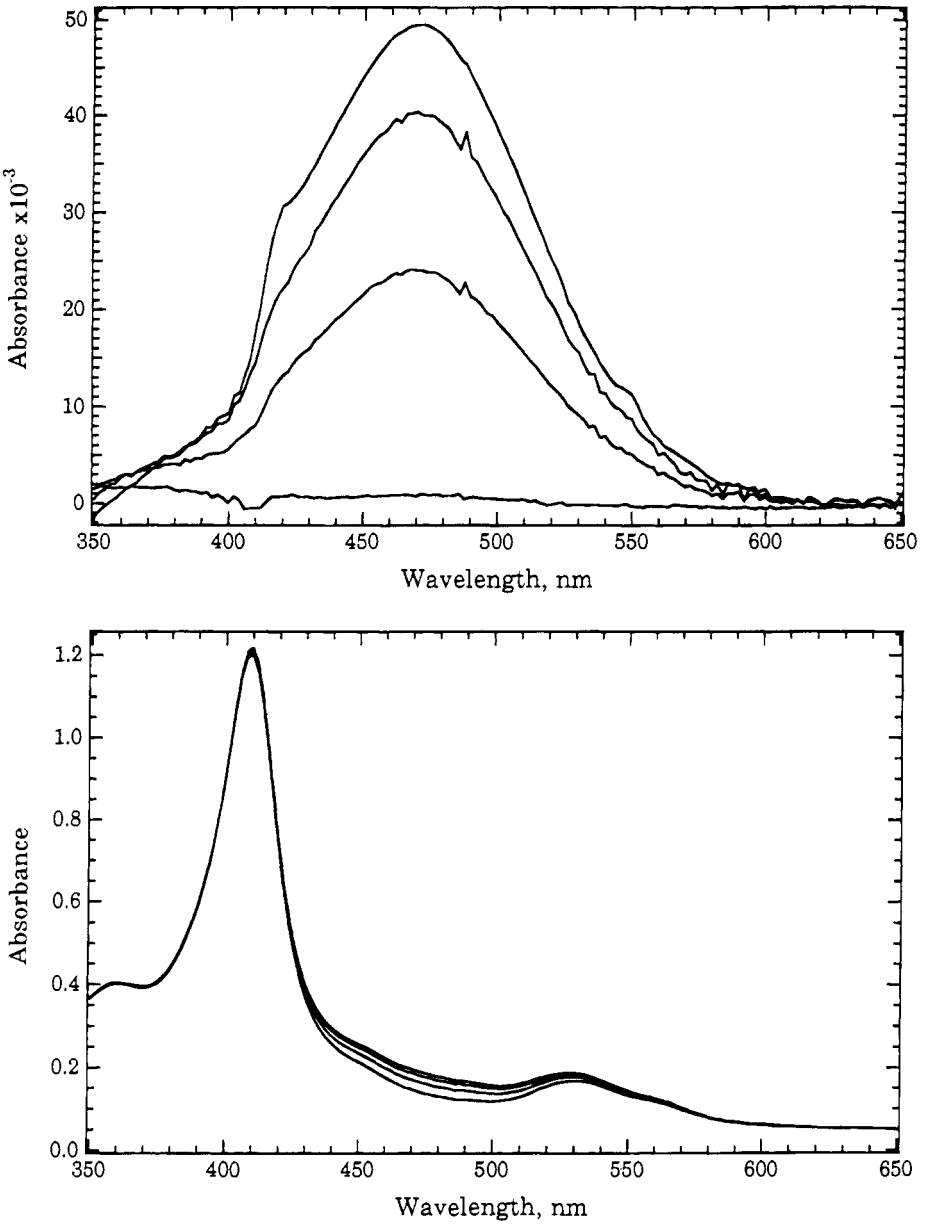


Figure 3. Bottom: Spectra of *trans*-isn$(NH_3)_4Ru^{III}$-cyt c^{III} immediately after oxidation of the (Ru^{II},Fe^{III}) form with 1 equiv of $[Co(ox)_3]^{3-}$. Spectra were recorded at 0, 60, 120, and 180 min after oxidation. Conditions: 12 μM ruthenated cyt *c*, 50 mM phosphate buffer, pH 7.0, solution purged with argon. Top: Difference spectra relative to the initial spectrum in the bottom part, showing production of the isn$(NH_3)_4Ru^{II}$– chromophore (λ_{max} = 472 nm) from the disproportionation of Ru^{III}.

trans-[isn$(NH_3)_4Ru^{III}$im]$^{3+}$. Keeping the modified protein in the Ru(II) state prevents this undesirable reaction.

The disproportionation reaction has not been observed for [$(NH_3)_5Ru^{III}$cyt c^{III}], where no π-acid ligand is present. In L$(NH_3)_4$Ru, the presence of a π-acid ligand shifts reaction 2 to the right because of the back-bonding stabilization of Ru^{II}.

(b) Electrochemical and Structural Evidence. The disproportionation of *trans*-[isnRu^{III}cyt *c* (hh)] was observed electrochemically after oxidation of the LRu^{II}cyt c^{III} species with excess $[Co(ox)_3]^{3-}$, followed by ultrafiltration to remove $[Co(ox)_3]^{3-}$ and concentrate the protein. After this oxidation, the electrochemical behavior observed is different from that seen for the LRu^{II}cyt *c* form of the protein. Similar observations were made over a pH range of 5.5–8.1. The differential pulse voltammograms of *trans*-[isnRu-cyt *c* (hh)], *trans*-[isnRu-cyt *c* (*Ck*)], and *cis*-[pyRu-cyt *c* (hh)] after oxidation with excess $[Co(ox)_3]^{3-}$ are shown in the lower half of Figure 4 and compared to the DPV of the LRu^{II}cyt *c* species (stored in the Ru(II) state) in the upper half of Figure 4. In the upper half of Figure 4, the two peaks correspond to the L$Ru^{III/II}$ and cyt $c^{III/II}$ redox waves. If the LRu-cyt *c* species are chemically oxidized to LRu^{III}cyt c^{III} and rapidly reduced, the DPV's shown in Figure 4a–c are also observed.

After sufficient time in the presence of excess oxidant, the redox peaks for the L$Ru^{II/III}$ couples are no longer present at the expected potentials (Figure 4d–f). For *trans*-[isnRu-cyt *c* (hh)] (Figure 4d) and for *cis*-[pyRu-cyt *c* (hh)] (Figure 4e), the disproportionation products have a redox couple which is close enough to the heme potential that the DPV shows only one peak. For *trans*-[isnRu-cyt *c* (hh)], comparison of the peak amplitudes before and after irreversible oxidation indicates that the peak in Figure 4d includes two nearly coincident, one-electron peaks, the peak for the Ru and that for the heme. This is further substantiated by examining the DPV for *trans*-[isnRu-cyt *c* (*Ck*)] (Figure 4f), which shows a newly-formed shoulder for a LRu couple at a slightly more negative potential than the heme site. For both the hh and *Ck trans*-[isnRu-cyt *c*] cases, Ru:Fe ratios

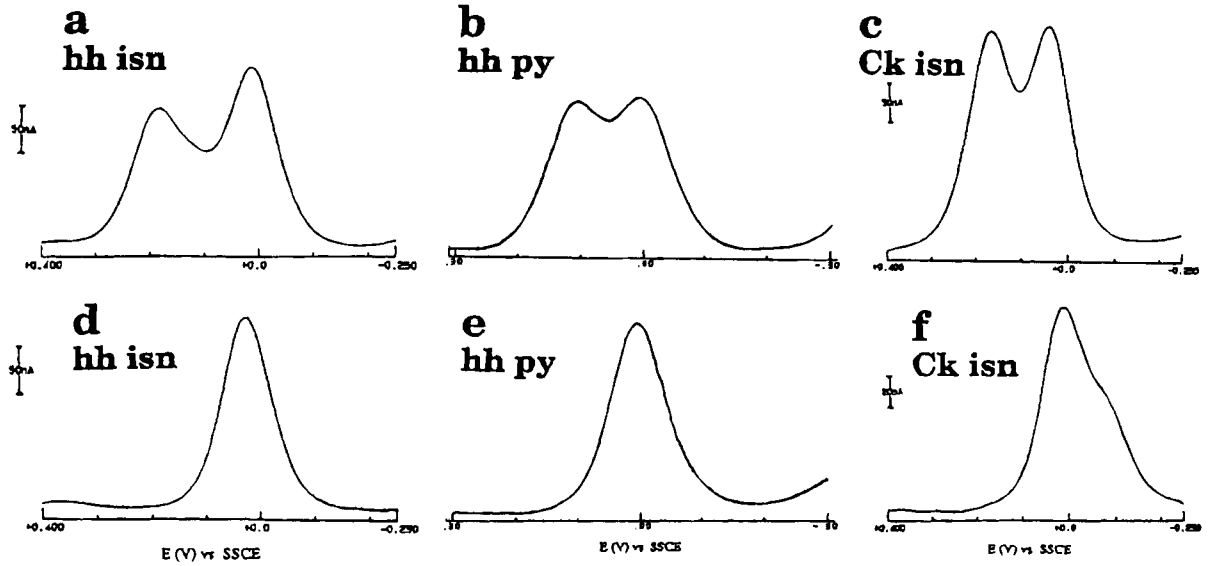


Figure 4. Differential pulse voltammograms of $L(NH_3)_4Ru^{II}$-modified cytochromes *c*, measured at a 4,4′-bipyridyl disulfide-treated gold disk electrode versus SSCE: (a, d) horse-heart, L = isonicotinamide; (b, e) horse-heart, L = pyridine; (c, f), *Candida krusei*, L = isonicotinamide. A comparison is shown for the DPV's of reduced, freshly-prepared modified proteins (a–c) with the DPV's of the same modified proteins (d–f) after exposure to excess $[Co(ox)_3]^{3-}$ and time to rearrange.

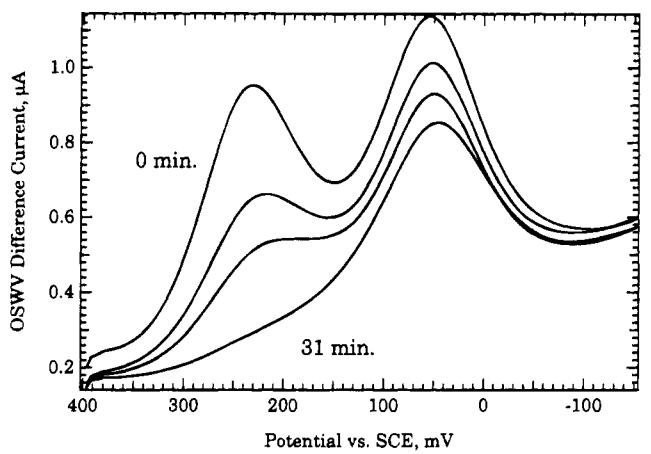
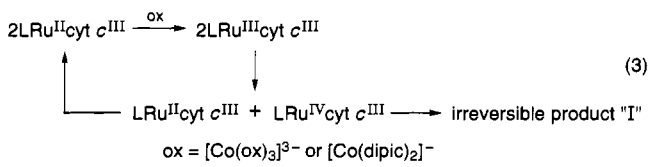


Figure 5. Osteryoung square wave voltammograms of the disproportionation of horse-heart *trans*-isn$(NH_3)_4Ru^{III}$-cyt *c*, taken at 0, 4, 9, and 31 min after oxidation with excess $[Co(dipic)_2]^-$. Solution: 118 μM protein, 0.6 mM $(NH_4)[Co(dipic)_2]$, 50 mM pH 7.0 phosphate buffer.

determined by DCP-AES were the same after irreversible oxidation as they were for the freshly-prepared samples.

The time course of reaction 2 for *trans*-[isnRu^{III}cyt c^{III}] in the presence of excess $[Co(dipic)_2]^-$ is shown in the set of Osteryoung square wave voltammograms (OSWV's) in Figure 5, where the redox peak on the left which corresponds to the $LRu^{II/III}$ couple of freshly oxidized LRu^{III}cyt c^{III} is found to disappear over time. For a 120 μm protein concentration in the presence of 5-fold excess oxidant, the $LRu^{II/III}$ redox couple disappeared completely within 30 min. At 4 and 9 min after oxidation, the OSWV scans showed that only a fraction of the unchanged $LRu^{II/III}$ redox couple is present. The oxidation of LRu^{II}cyt c^{III} in the presence of *excess* $[Co(ox)_3]^{3-}$ or $[Co(dipic)_2]^-$ can be described by eq 3. Oxidation of LRu^{II} is followed by



disproportionation as in eq 2; however, with excess oxidant the LRu^{II}cyt c^{III} formed from the disproportionation reaction is again oxidized by Co^{III} until all of the material is converted to LRu^{IV}-cyt c^{III}. If the reaction was stopped at that point by adding a reducing agent, the LRu^{II}cyt c^{III} complex could be regenerated by reduction and the $LRu^{III/II}$ couple would not disappear. Production of LRu^{IV}cyt c^{III} is followed by a reasonably rapid, irreversible chemical step to produce a species which shall be referred to as **I**.

The process of forming **I** may involve an ammine deprotonation, followed by ligand labilization forming an oxo, hydroxo, or nitrido species which can further irreversibly react with other ligands in the medium. Therefore, the rearranged product **I**, when reduced, will not convert back to the starting LRu^{II}cyt c^{III} complex. The redox process of the rearranged complex **I**, which nearly coincides with the heme couple, is assumed to be the reduction of Ru^{IV}.

Tryptic digestion of the protein after oxidation and concentration to form the rearranged product species can help to elucidate its nature. Comparison of the tryptic digestion HPLC chromatograms of horse-heart product **I** (L = *trans*-isn), LRu^{II}cyt c^{III} (hh, L = *trans*-isn), and horse-heart $(NH_3)_5Ru^{III}$cyt *c* shows marked differences (Figure 6). Whereas the $[(NH_3)_5Ru^{III}$cyt *c*] and LRu^{II}cyt c^{III} (L = *trans*-isn) HPLC elution profiles show only minimal differences from the native cyt *c* (other than the presence of the T7 fragment with the Ru complex), the HPLC elution profile of species **I** shows a very small amount of the peptide fragment containing the LRu group, plus a number of new high-molecular-weight species which elute after the heme peptide peak appears. These observations are consistent with a new LRu^{IV}cyt *c* species which may be cross-linking the protein at more than one site and interfering with the enzymatic hydrolysis. Similar reactivity of ruthenium complexes with peptide ligands has been reported earlier.[21]

Effect of the Disproportionation of LRu^{III} in LRu^{III}cyt c^{III} on the Electron-Transfer Kinetics. Earlier attempts to measure the rate constant for intramolecular electron transfer in LRu^{III}cyt c^{III} (L = isn) were carried out using a reductive method of pulse radiolysis to partially reduce the fully oxidized LRu^{III}cyt c^{III} complex according to eq 4.[22] This reductive method has been used successfully for the measurement of the rate constant for electron transfer from $(NH_3)_5Ru^{III}$ to the heme iron in $[(NH_3)_5Ru^{II}$cyt $c^{III}]$.[1b]

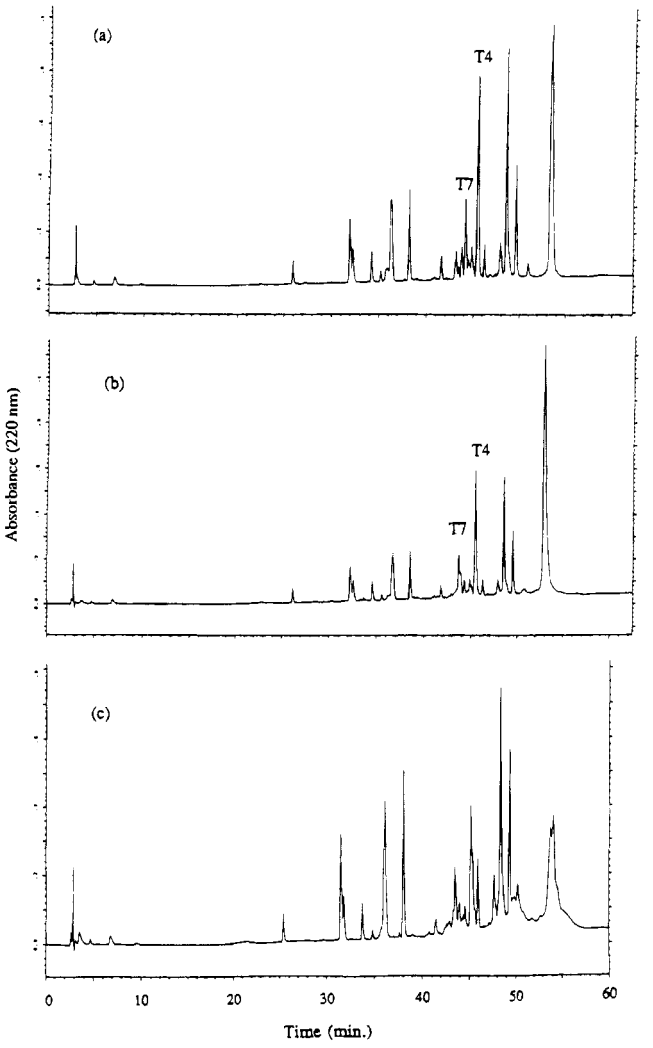


Figure 6. Reverse-phase HPLC elution profiles at 300 nm of the tryptic digestion products of (a) native horse-heart cytochrome *c*, (b) freshly-prepared *trans*-isn$(NH_3)_4Ru^{II}$-modified horse-heart cytochrome *c*, and (c) *trans*-isn$(NH_3)_4Ru^{II}$-modified horse-heart cytochrome *c* after exposure to excess $[Co(ox)_3]^{3-}$ until the rearrangement was complete.

$$LRu^{III}\text{cyt } c^{III} + CO_2^{\bullet} \begin{cases} \nearrow LRu^{III}\text{cyt } c^{II} + CO_2 \\ \downarrow k_{et} \\ \searrow LRu^{II}\text{cyt } c^{III} + CO_2 \end{cases} \quad (4)$$

When the same reductive method (eq 4) was used on the LRu^{III}cyt c^{III} (L = *cis*-isn, *trans*-isn, *trans*-py) derivatives, unexpectedly low rate constants of intramolecular electron transfer were observed (Figure 7). These slow reactions were previously interpreted to involve the $LRu^{II/III}$ couple in the modified cyt *c*, leading us to incorrectly conclude that rates of oxidation of cyt *c* were slower than rates of reduction of cyt *c* in these ruthenium derivatives.[22] This present study clearly indicates that the observed reactions involved the rearranged species **I** generated by the disproportionation of the LRu^{III}-modified proteins during the oxidation and concentration by ultrafiltration processes in preparation for the kinetic experiments. Consequently, the desired electron-transfer reaction, eq 4, could not be followed since the LRu^{III} complex was no longer present. Instead, as shown in Figure 7, the rapid direct reduction of the ruthenium and the heme sites by the $CO_2^{\bullet-}$ is followed by slow spectral changes at 550 nm (for the heme site) and at 504 and 432 nm (for the Ru sites) arising from nearly zero-driving-force electron-transfer processes from the LRu sites to the heme(III) site, rather than the expected electron transfer from

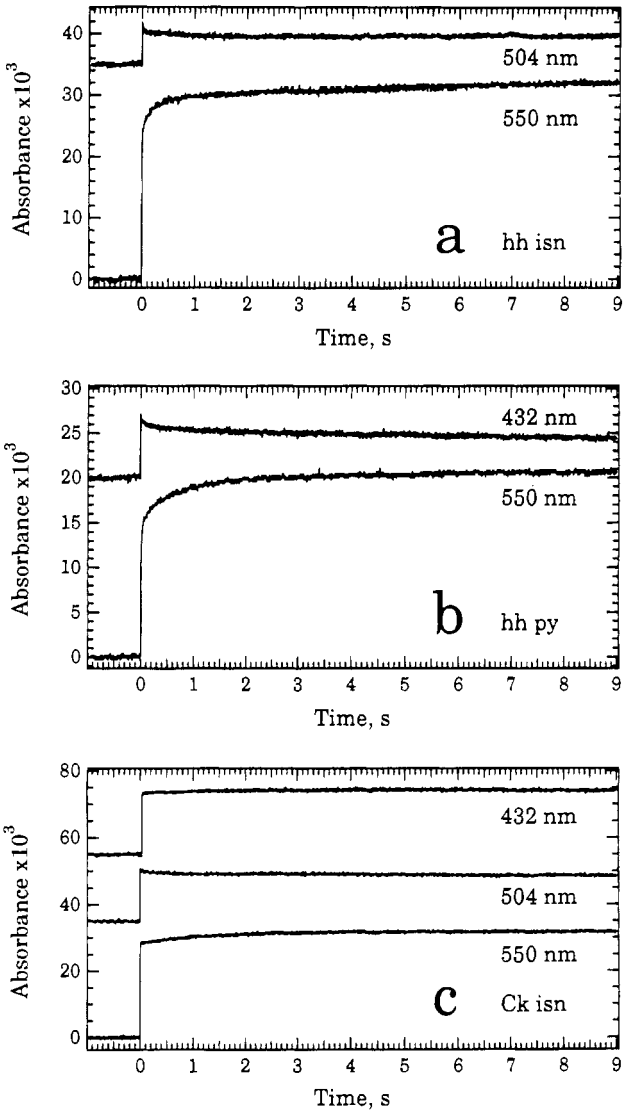


Figure 7. Absorbance vs time profiles at 25 °C of reductive pulse radiolysis of *trans*-L$(NH_3)_4Ru^{III}$cyt c^{III} samples which had been prepared by oxidation with excess oxidant (see Experimental Section) and concentrated by ultrafiltration: (a) horse-heart, L = isonicotinamide; (b) horse-heart, L = pyridine; (c) *Candida krusei*, L = isonicotinamide. Conditions: 4 μM protein, 100 mM sodium formate, 50 mM pH 7.0 phosphate buffer, N_2O-saturated solution.

the heme(II) site to the LRu^{III} sites as in Figure 2. (The fact that electron transfer is observed in the Ru-to-heme direction is due to the initial reduction step, which generates a nonequilibrium distribution in favor of reduced ruthenium.)

By understanding the nature of the disproportionation reaction, it is possible to define an experimental protocol to carry out the kinetic measurement given in eq 4. If oxidation of LRu^{II}-cyt c^{III} (L = isn, py) is quickly followed by dilution, the second-order dependence of the disproportionation reaction can be used to slow it long enough to perform kinetic measurements by reduction of LRu^{III}cyt c^{III} with $CO_2^{\bullet-}$ as in eq 4. Such experiments were performed on the horse-heart derivative with L = isn; however it was found that the initial reduction step favored the direct reduction of the ruthenium site over the heme site by a ratio of about 10:1, making it difficult to follow the kinetic intermediate, LRu^{III}cyt c^{II}. Although accurate kinetics were difficult to obtain using LRu^{III}cyt c^{II} generated by the reductive method, the fact that LRu^{II}cyt c^{III} (the unrearranged, thermodynamically stable product) could be generated indicates that this protocol succeeded in retarding the disproportionation of LRu^{III}cyt c^{III} long enough to do experiments on this species.

The preference for reduction of the isn$Ru^{III}(NH_3)_4$ site over the heme is significantly higher than observed in the case of $(NH_3)_5Ru^{III}$cyt c^{III},[1b] due to the stronger driving force and lower reorganization energy of isn$Ru(NH_3)_4$cyt *c*.

The significance of the disproportionation reaction reported here for substituted pyridine derivatives of ruthenium(III) complexes bound to cytochrome *c* goes beyond the specific examples cited above. This disproportionation reaction should be considered in any future kinetic studies of proteins modified with $LRu(NH_3)_4$ reagents. Stability and reversibility studies of all oxidation states of the ruthenium-modified protein over time should be carried out. Also previous reports on measurement of electron-transfer rate constants of ruthenium-modified proteins where $L(NH_3)_4Ru^{III}-$ (L = isn, py) complexes were used[2d,23,24] should be reinvestigated in light of the new disproportionation reaction reported in this study.

Summary. Ruthenium complexes of the type $[(NH_3)_4LRu-]$, where L = pyridine or isonicotinamide, can be attached to proteins as probes for studying long-range electron transfer. Facile intramolecular electron transfer can be observed in these systems if the ruthenium-modified protein complexes are isolated and purified while the ruthenium complex is in the reduced state. In the LRu^{III}cyt c^{III} state, disproportionation of the ruthenium complex occurs to form LRu^{II}cyt c^{III} and LRu^{IV}-cyt c^{III}. The Ru^{IV} complex subsequently undergoes an irreversible reaction to create a different protein-bound, redox-active species. The rate for the disproportionation reaction is significantly faster than that for the comparable small-molecule reaction.[5a] The protein environment seems to facilitate the process.

Tryptic digestion elution profiles of the LRu^{II}cyt c^{III} and rearranged LRu^{IV}cyt c^{III} complexes show pronounced differences consistent with the ligation of the rearranged LRu^{IV} complex to more than one protein site. The resulting rearranged LRu^{IV}-cyt *c* proteins also undergo intramolecular electron-transfer kinetics; however, differences in redox potential compared to that of the $LRu^{II/III}$cyt *c* form, changes in the ligands around the ruthenium center, and possibly different reorganization energies between the different oxidation states involved in the electron-transfer process will result in different reaction rates. The disproportionation reaction can be controlled by stoichiometric oxidation of the modified proteins followed by immediate dilution shortly before use. Under such conditions LRu^{III}cyt c^{III} solutions (~10 μM) are stable for as long as 1 h.

The results presented here are expected to be applicable also to other proteins modified with $L(NH_3)_4Ru^{III}$ for L = pyridine derivatives or other π-acid ligands where the disproportionation of the LRu^{III} center can complicate the study of intramolecular electron transfer.

Acknowledgment. The research was supported by the Center for Advanced Food Technology (CAFT) at Rutgers University, the National Science Foundation (Grant CHE 840552), and the Charles and Johanna Busch Foundation. The work performed at Brookhaven National Laboratory was supported under Contract DE-AC02-76CH00016 with the U.S. Department of Energy and supported by its Division of Chemical Sciences, Office of Basic Energy Sciences. The authors thank Dr. Harold Schwarz (BNL) for valuable assistance in the early phases of this study.

Supplementary Material Available: Spectra of (1) the T7 fraction from the HPLC chromatogram of the tryptic digestion products of horse-heart *trans*-[(isn)$(NH_3)_4Ru^{II}$cyt c^{III}] and (2) the T6 fraction from the HPLC chromatogram of the tryptic digestion products of *Candida krusei trans*-[(isn)$(NH_3)_4Ru^{II}$cyt c^{III}] (2 pages). Ordering information is given on any current masthead page.

IC941404T