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Directional Electron Transfer in Ruthenium-Modified Cytochrome c

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Studies of intramolecular oxidation and reduction in cytochrome c complexes covalently modified at the His-33 residue with a variety of ruthenium amine and ruthenium polypyridine complexes are presented. The redox potential of the ruthenium complexes vary over a potential range above and below the redox potential of the native cytochrome c. These studies show that the reduction of cytochrome c with these ruthenium complexes proceeds with a rate-limiting electron-transfer step that changes with the driving force of the reaction, as expected. Oxidation of cytochrome c proceeds with rates significantly lower than those expected on the basis of the driving force of the reaction. A mechanism to interpret the directional electron-transfer behavior of these ruthenium cytochrome c complexes on the basis of conformational changes of the reduced cytochrome c is described.

RAPID ELECTRON TRANSFER CAN BE OBSERVED OVER LONG DISTANCES (~10–20 Å) (*1–11*), as shown by studies on electron transfer with organic and inorganic donor–acceptor complexes. The factors that control the rate of electron transfer in these systems are driving force, reorganization energy, and distance and orientation.

Polypeptide Donor–Acceptor Complexes

In an attempt to gain further insight into these factors, we have synthesized and studied a series of bridged polyproline complexes (*12*) of the type $[(NH_3)_5Os\text{-}L\text{-}Ru(NH_3)_5]^{5+}$, where L (the ligand) is isn$(Pro)_n$, isn is the isonicotinyl group, and n = 0, 1, 2, 3, or 4 (Table I).

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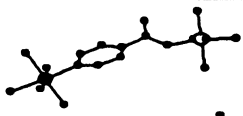
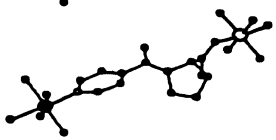
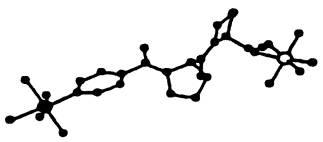
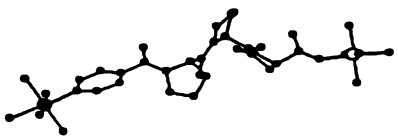
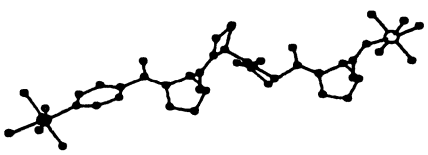
Table I. Rates and Distances of Intramolecular Electron Transfer in $[(NH_3)_5Os\text{-}L\text{-}Ru(NH_3)_5]^{5+}$

Complex	*Rate Constant, s^{-1}*	*Os–Ru Distance, Å*
	$\geq 5 \times 10^9$	9.0
	3.1×10^6	12.1–12.3
	3.7×10^4	14.4–15.1
	3.2×10^2	17.8–18.3
	~50	20.9–21.5

NOTE: L is isn(Pro)$_n$, isn is isonicotinyl group, and n = 0, 1, 2, 3, or 4.

The C- and N-terminal residues in Table I are derivatized with $[(NH_3)_5Ru^{II}\text{-}]$ and $[(NH_3)_5Os^{II}isn\text{-}]$, respectively. The redox potentials of the Os(II–III) couple and the Ru(II–III) couple are such that electron transfer in this series of complexes occurs from Os(II) → Ru(III) with a driving force of ~150 mV (*13*). In this series of complexes, the driving force (the difference in redox potential between the Ru(II–III) and the Os(II–III) couple) and the inner-sphere reorganization energy are kept constant, while the distance between the Os and Ru centers increases by 3.2 Å per proline. Under the conditions used to carry out these experiments (~0.1 M CF_3COOH), the proline oligomers are predominantly in the all-*trans* configuration and therefore act as a rigid spacer separating the metal ions (*12*).

The rate of intramolecular electron transfer in this series of molecules decreases by more than 8 orders of magnitude as proline residues are introduced. Analysis of the temperature dependence of these rate constants showed that the decrease in rate with distance in these molecules is attributed to both the increase in outer-sphere reorganization with distance and the decrease in electronic coupling between the donor and acceptor as proline residues are introduced (*12*, *13*).

Intramolecular electron transfer for the tetraproline complex occurs with a rate constant ~50 s^{-1} at a metal-to-metal distance of ~21 Å (*12*, *14*). Rapid electron transfer at these long distances is observed at very low driving forces. Hence, increasing the driving force or decreasing the outer-sphere reorganization energy is expected to yield rapid intramolecular electron-transfer rates at even longer distances. Extrapolation of these results indicates that intramolecular electron transfer will be observable at metal–metal separations of 30–40 Å in the millisecond time scale. We are currently pursuing this goal by synthesizing molecules that have 6–10 prolines separating the donor and acceptor metal ions.

Protein Donor–Acceptor Complexes

One of the techniques that has led to a new understanding of the mechanism of electron transfer in electron-transfer proteins is the use of the protein as a donor–acceptor complex by covalently attaching to these proteins a well-defined transition metal complex that binds to a specific amino acid site. Although this technique had been used to modify ribonuclease (*15*, *16*), the synthetic breakthrough in modifying cytochrome c occurred when the reaction of $[(NH_3)_5Ru(OH_2)]^{2+}$ with cyt c was carried out at high protein concentrations with high metal-to-protein molar ratios (*17*). Exhaustive characterization of this modified protein showed that the ruthenium is covalently bound to the His 33 side chain of cyt c and that the protein did not undergo any measurable perturbation as a result of the modification (*18–20*).

When the modified protein is prepared in the Ru(III)cyt c(III) oxidation state and then reduced with a variety of radicals generated by pulse radiolysis techniques (*21*, *22*), intramolecular electron transfer from the ruthenium site to the heme site occurs with a rate constant $k = 53\ s^{-1}$ (reduction potential, $E°$, for cyt c = 0.26 V and $E°$ for $[(NH_3)_5Ru^{II-III}(His)] = 0.10$ Vs. NHE) (*23*, *24*). The temperature dependence, concentration dependence, and pH dependence of this electron-transfer reaction were investigated. The results of this investigation showed that the rate of electron transfer is independent of concentration and moderately sensitive to temperature (enthalpy change $\Delta H^{\ddagger} \sim 3.5$ kcal M^{-1} and entropy change $\Delta S^{\ddagger} \sim -39$ eu). The electron-transfer reaction is independent of pH between pH 5 and 9, and then increases below pH 5 as the native conformation of the cyt c changes (*23*).

The observation of intramolecular electron transfer between the ruthenium site and the heme site occurring at distances of 12–15 Å (Figure 1) is extremely significant, because it represents the first observation of an intramolecular electron-transfer reaction within a ruthenium-modified electron-transfer protein. The magnitude of the rate constant (53 s^{-1}) is similar to the rate constants for other dynamical processes that are known to occur within the native cyt c protein. This finding led us to question whether the unimolecular rate observed is rate limiting in electron transfer (as in equa-

In Electron Transfer in Biology and the Solid State; Johnson, Michael K., et al.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1989.

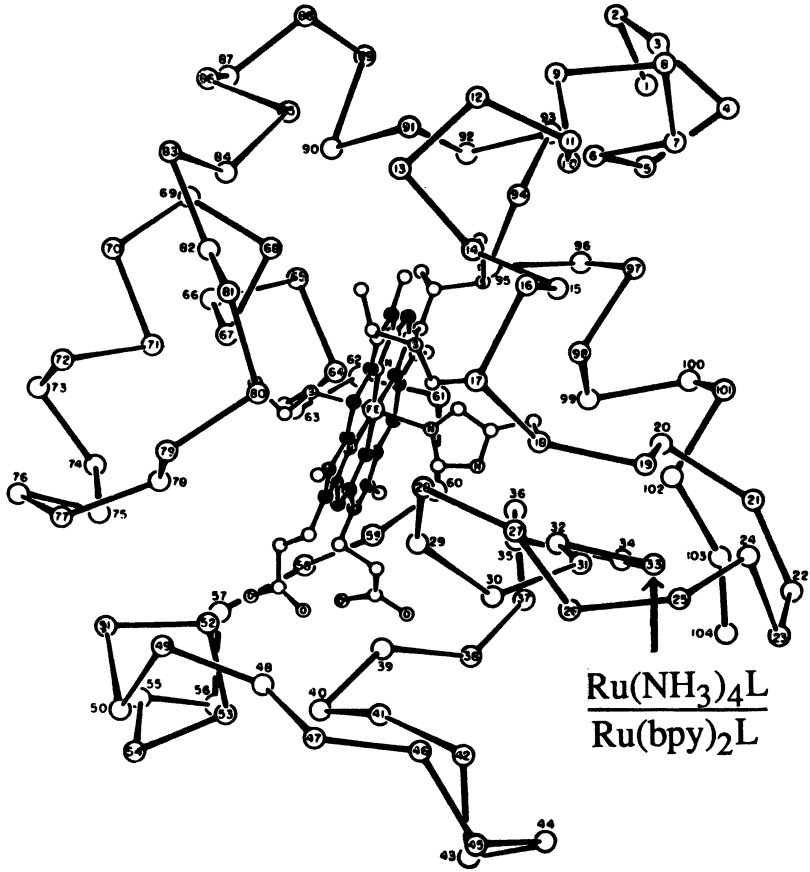


Figure 1. Ruthenium-modified cytochrome c, showing the relative position of the heme and the ruthenium sites.

tion 1) or in a protein-associated conformational change (as in equations 2a and 2b).

$$Ru^{II}cyt\ c^{III} \xrightarrow{k_{ET}} Ru^{III}cyt\ c^{II} \quad (1)$$

where k_{ET} is the rate constant for intramolecular electron transfer.

$$Ru^{II}cyt\ c^{III} \xrightarrow{k_{CC}} Ru^{II*}cyt\ c^{III} \quad (2a)$$

$$Ru^{II*}cyt\ c^{III} \xrightarrow{k_{CC}} Ru^{III}cyt\ c^{II}\ (fast) \quad (2b)$$

where k_{CC} is the rate constant for a protein conformational change.

To answer this question, we designed a series of related ruthenium molecules that are more oxidizing than cyt c and would therefore allow us

to reverse the direction of electron transfer in the modified cyt c. Thus, we could change the heme of cyt c from an electron acceptor to an electron donor. The rationale behind these experiments is rather simple. If the unimolecular rate observed is rate limiting in electron transfer, then oxidation as well as reduction should be observed within these ruthenium-modified proteins.

The remaining part of this chapter describes the results of these experiments and proposes a kinetic scheme for the electron-transfer reactions of ruthenium-modified cytochrome c.

Results

The ruthenium-modified proteins listed in Table II were prepared and purified by procedures similar to those published earlier (*19*, *23*, *25*). The complexes were characterized by difference visible spectra, circular dichroism, tryptic digestion, Ru–Fe analysis, cyclic voltammetry, and differential pulse polarography. Results of these characterization studies clearly showed that all the ruthenium complexes were bound to the His 33 site and that there was no measurable difference in the conformation of the modified and the native proteins. Table II lists the reduction potential of the ruthenium ammine site in the ruthenium-modified proteins. For the bipyridine series, the reduction potentials have been obtained only for the corresponding ruthenium complexes.

Figure 1 shows the structure of cyt c and the relative positions of the heme to the ruthenium-modified sites. Table II summarizes the rates of

Table II. Rates of Intramolecular Electron Transfer and Reduction Potential of Ruthenium–Cytochrome c Complexes

Ruthenium-Modified cyt c	E°, V	*Rate Constant, Intramolecular ET,* k, s^{-1}	*Direction of ET*
Native HH cytochrome c	0.26	—	—
c-[$(NH_3)_4$Ru(OH)]-(II/III)	−0.01	5×10^2	Ru → heme
[$(NH_3)_5$Ru]-(II/III)	0.13	55	Ru → heme
c-[$(NH_3)_4$Ru(py)]-(II/III)	0.36	2.0	Ru → heme
t-[$(NH_3)_4$Ru(py)]-(II/III)	0.37	1.5	Ru → heme
c-[$(NH_3)_4$Ru(isn)]-(II/III)	0.44	$<10^{-2}$	Heme → Ru
t-[$(NH_3)_4$Ru(isn)]-(II/III)	0.44	$<10^{-2}$	Heme → Ru
c-[$(NH_3)_4$Ru(Mepz)]-(II/I)	−0.02	6×10^2	Ru → heme
c-[$(NH_3)_4$Ru(Mepz)]-(II/III)	0.72	~1.5	Heme → Ru
[Ru(bpy)$_2$(py)]-(II/I)	−1.3	2.8×10^5	Ru → heme
[Ru(bpy)$_2$(im)]-(II/I)	−1.3	2.0×10^5	Ru → heme
[Ru(bpy)$_2$(py)]-(II/III)	1.1	40	Heme → Ru
[Ru(bpy)$_2$(im)]-(II/III)	1.0	55	Heme → Ru

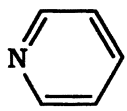
NOTE: $E°$ was taken versus the normal hydrogen electrode. ET is electron transfer.

intramolecular electron transfer for the reduction and the oxidation of cyt c by these ruthenium reagents. Chart I shows the ligands in the ruthenium reagents.

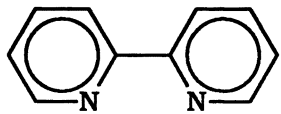
Discussion

Table II shows that the rate of reduction of cyt c can be changed by more than 5 orders of magnitude, depending on the redox potential and the reorganization energy of the ruthenium-modified protein. Two types of complexes coordinated to His 33 of cytochrome c can be identified. In the the first type, electron transfer takes place from (or to) a ruthenium t_{2g}-type orbital. This condition is true for all the oxidation reactions of cytochrome c by the different ruthenium complexes.

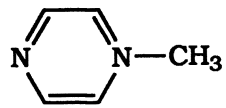
For the intramolecular reduction of cytochrome c, the reducing agent can be either a ruthenium t_{2g} metal-centered electron as in the $[(NH_3)_4Ru^{II}$-L] (L is *cis* or *trans* OH^-, NH_3, py, isn) complexes or a ligand-centered π^* orbital as in the *cis*-$[(NH_3)_4Ru^{II}(Mepz)]$ and the $[(bpy)_2Ru^{II}$-L] (L is py or im) complexes. The role of the ruthenium(II) attached to the cytochrome c is either as a reducing agent or as a covalent linker to hold the Mepz and bpy ligand in the proximity of the His 33 site. Thus, the distance for electron transfer from these organic radicals to the cytochrome closely resembles the distances for reduction from the ruthenium orbitals. The reorganization energy for electron transfer for the ligand-centered reductions could be different from the reorganization energy from the ruthenium-centered orbitals. This difference should be taken into account when comparing rates of reduction of cytochrome c from ruthenium-centered orbitals vs. ligand-centered orbitals.



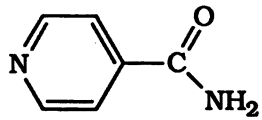
py: pyridine



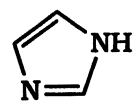
bpy: 2,2'-bipyridine



Mepz: methylpyrazinium



Isn: isonicotinamide



Im: imidazole

Chart I. Ligands in ruthenium reagents.

The oxidized ruthenium(III) form of the $[(NH_3)_4Ru\text{-}Mepz]^{3+}$ and the $[(bpy)_2Ru\text{-}L]^{3+}$ are intramolecularly reduced by cytochrome c to the corresponding ruthenium(II) species (a metal-centered orbital). In these cases, reorganizational energies of the ruthenium center are associated with the rate of electron transfer.

The novel property of the second type, the $[(NH_3)_4Ru^{II}\text{-}Mepz]$ and the $[(bpy)_2Ru^{II}\text{-}L]$ cytochrome c species, is that oxidation to the ruthenium(III) center can be observed, as well as reduction from the ligand-centered radicals. Thus both the oxidation and reduction of cytochrome c can be observed from the same inorganic modifier, even though it is metal-centered in one direction and ligand-centered in the other. Correction for the reorganizational energies between the metal center and the ligand center allows a direct comparison of oxidation and reduction at these similar distances.

Binuclear ruthenium complexes of the bridging pyrazine and bipyridine type can be used to observe metal-centered oxidation and reduction. Complexes of the type $[(NH_3)_5Os\text{-}LL\text{-}Ru(NH_3)_4\text{-}L\text{-}(OH_2)]^{4/5+}$ (where LL is pyrazine and L is 4,4′bpy) have multiple oxidation–reduction properties that will allow the oxidation and reduction of cytochrome c to metal-centered orbitals. In these cases, correction for reorganizational energies because of the origin of the orbital for the electron-transfer reaction will not be necessary.

"One-Direction" Electron Transfer

Table II shows that the rates of oxidation and reduction of cytochrome c by these covalently modified ruthenium complexes do not proceed in a simple reversible elementary step. The fact that the rate of intramolecular oxidation of *cis*- and *trans*-$[(NH_3)_4Ru^{II}\text{-}L]$ (L is isn) by cytochrome c is much slower than the reduction of cytochrome c with $[(NH_3)_5Ru^{II}\text{-}]$ indicates that more complex chemistry is associated with electron transfer in the protein. Association of this complexity with the protein rather than with the ruthenium label is inferred from the variety of ruthenium complexes that exhibit the same behavior. We interpreted this in 1986 as "directional electron transfer", where protein conformational states play a role in the intramolecular electron-transfer reaction (*24*).

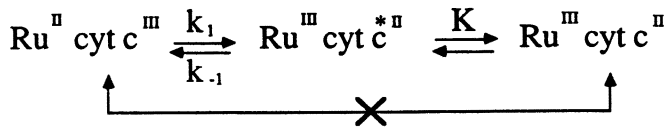
Subsequent to our work, a paper on "gated electron-transfer reactions" by Hoffman et al. (*26*) was published to interpret a related observation in their work on photoinduced electron transfer in protein electron-transfer complexes. Sutin et al. (*27*, in this volume) worked out the general theoretical formalisms for the many possible types of "directional electron transfer". This theoretical work opens up more avenues for designing new types of donor–acceptor complexes that exhibit directional electron transfer.

The chemistry of cytochrome c can be used to aid in the interpretation of this directional electron transfer in ruthenium-modified cytochrome c.

The reduction of cytochrome c with ruthenium reagents covalently attached to His 33 is dependent on the driving force of the reaction. Table II shows that this rate can be varied over 5 orders of magnitude by changes in the ruthenium complexes. On the other hand, the intramolecular oxidation of cytochrome c is 4–5 orders of magnitude slower than its reduction after correction for driving force and reorganizational energy. A mechanism to interpret these results is shown in Scheme I. Thus cytochrome c^{III} is reduced to an activated intermediate, cyt c^{*II}, which undergoes a conformational change to the stable form of cyt c^{II}. Therefore, in the reduction of cyt c we measure the rate of formation of this activated intermediate, k_1. For the oxidation of cytochrome c, the pre-equilibrium to form the same activated intermediate is required first. This requirement depresses the observed rate of intramolecular oxidation to k_{-1}/K_{eq}, and therefore we observe a significantly decreased intramolecular rate of oxidation for the cytochrome c. This mechanism is one of many that can be used to interpret the observed results. The attractiveness of this mechanism is its simplicity. Other mechanisms involving more intermediates and associated rate constants can also be used to interpret the results.

Another attractive feature of this mechanism is that it is very similar to the mechanisms proposed for electron transfer at solid electrodes. In the language of electrochemistry, the mechanism (Scheme I) is referred to as the EC mechanism (i.e., a chemical reaction [an equilibrium or conformational change, etc.] following the electron-transfer step in the reduction process).

Finally, it is of interest to define the molecular event that leads to this conformational change. Further work from NMR and time-dependent res-



Forward reaction

$$k_{obs} = k_1$$

Reverse reaction

$$k_{obs} = k_{-1}/K_{eq}$$

Scheme I. Directional electron-transfer mechanism.

onance Raman spectroscopy might shed light on these molecular events. The other important question that should be addressed is whether "one-direction electron transfer" can be observed from different sites of the protein surface to the heme and vice versa. Chemical modification of cytochrome c and site-directed mutagenesis experiments are required to generate specific binding sites in different regions of the cytochrome c so that experiments similar to the ones outlined in this chapter can be carried out. Comparison of the intramolecular oxidation and reduction properties of the heme site from different regions of the protein should provide answers to these questions.

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