Notes

Uphill Electron Transfer in Pentaammineruthenium(III)-Modified Ferrocytochrome c: Rates, Thermodynamics, and the Mediating Role of the Ruthenium Moiety

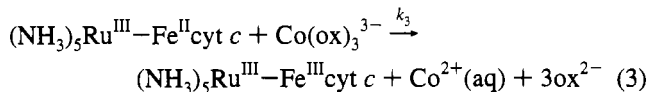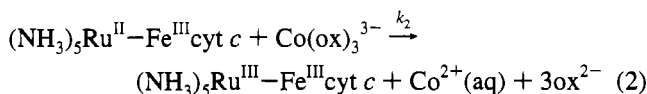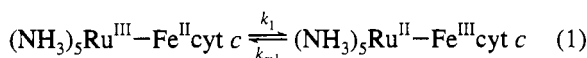Ji Sun,[†] James F. Wishart,*,[†] and Stephan S. Isied*,[‡]

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Electron transfer in partially-reduced $(NH_3)_5Ru$-modified horse-heart cytochrome c proceeds spontaneously from ruthenium(II) to iron(III) with a driving force of approximately 0.13 eV.[1,2] The rate of electron transfer has been measured by pulse radiolysis[1b] (53 ± 2 s^{-1}) and flash photolysis[2] (30 ± 3 s^{-1}). In this note we report the measurement of the rate and activation parameters of the reverse electron transfer process using sufficiently high concentrations of a selective oxidant.

The rate of oxidation of ferrocytochrome c by $Co^{III}(ox)_3^{3-}$ (ox = oxalate) in $\mu = 0.5$ M phosphate buffer (pH 7) is quite slow, 5.5 M^{-1} s^{-1},[3] for a driving force of 0.31 eV. The bimolecular rate has been shown to depend strongly on ionic strength; the value at $\mu = 0$ is 1750 M^{-1} s^{-1}.[4] However, in the course of our studies with ruthenium-modified cytochromes c we observed that the reaction of $Co(ox)_3^{3-}$ with ruthenium ammine complexes bound to cytochrome c is relatively facile, even at moderate ionic strengths.[5] We have used these facts to measure the rate of thermodynamically-unfavored iron-to-ruthenium electron transfer using the redox pre-equilibrium scheme in eqs 1–3. Reaction 1 represents the unfavorable equilibrium for reduction of the ruthenium center by intramo-

$$(NH_3)_5Ru^{III}-Fe^{II}cyt\ c \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (NH_3)_5Ru^{II}-Fe^{III}cyt\ c \quad (1)$$

$$(NH_3)_5Ru^{II}-Fe^{III}cyt\ c + Co(ox)_3^{3-} \overset{k_2}{\longrightarrow}$$
$$(NH_3)_5Ru^{III}-Fe^{III}cyt\ c + Co^{2+}(aq) + 3ox^{2-} \quad (2)$$

$$(NH_3)_5Ru^{III}-Fe^{II}cyt\ c + Co(ox)_3^{3-} \overset{k_3}{\longrightarrow}$$
$$(NH_3)_5Ru^{III}-Fe^{III}cyt\ c + Co^{2+}(aq) + 3ox^{2-} \quad (3)$$

equilibrium for reduction of the ruthenium center by intramo-

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(1) (a) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659–7661. (b) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722–1726.
(2) (a) Winkler, J. R.; Nocera, D. G.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1982**, *104*, 5798–5800. (b) Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145–5150.
(3) Holwerda, R. A.; Knaff, D. B.; Gray, H. B.; Clemmer, J. D.; Crowley, R.; Smith, J. M.; Mauk, A. G. *J. Am. Chem Soc.* **1980**, *102*, 1142–1145.
(4) Rush J. D.; Lan, J.; Koppenol, W. H. *J. Am. Chem Soc.* **1987**, *109*, 2679–2682.
(5) Sun, J. Ph.D. Dissertation, Rutgers, The State University of New Jersey, 1993.

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lecular electron transfer. Reaction 2 is the relatively rapid oxidation of Ru^{II} by $Co(ox)_3^{3-}$. Reaction 3 is the much slower oxidation of the heme center in Ru^{III}-modified ferrocytochrome c by $Co(ox)_3^{3-}$. The steady-state approximation can be applied to the above reaction scheme to obtain the following rate law for the disappearance of Fe^{II}cyt c:

$$k_{obs} = \frac{k_1 k_2 [Co(ox)_3^{3-}]}{k_{-1} + k_2 [Co(ox)_3^{3-}]} + k_3 [Co(ox)_3^{3-}] \quad (4)$$

If the concentration of $Co(ox)_3^{3-}$ is low enough, reaction 2 is rate limiting and the observed rate will be equal to $(K_1 k_2 + k_3)[Co(ox)_3^{3-}]$, where $K_1 = k_1/k_{-1}$. At higher $Co(ox)_3^{3-}$ concentrations, k_{obs} will equal $k_1 + k_3[Co(ox)_3^{3-}]$.

Electron transfer "assistance" through an unfavorable pre-equilibrium with a more reactive electronic isomer was demonstrated by Olabe and Haim[6] in the persulfate oxidation of $(NH_3)_5Ru^{III}-(1,2\text{-dipyridylethane})-Fe^{II}(CN)_5$. As in the present case, oxidation of the ruthenium center of the Ru^{II}–Fe^{III} isomer is much faster than direct oxidation of Fe^{II}. In the persulfate concentration range used in their study, the analog of reaction 2 was rate limiting and the observed bimolecular rate constant of 430 M^{-1} s^{-1} was the product of the equilibrium quotient (2.9×10^{-3}) and the ruthenium oxidation rate constant (1.5×10^5 M^{-1} s^{-1}). In contrast, the rate constant for persulfate oxidation of the corresponding mononuclear iron complex $Fe^{II}(CN)_5BPA^{3-}$ is only 0.10 M^{-1} s^{-1}.

The rate of pentaammineruthenium(III)-modified cytochrome c(II) oxidation was measured by stopped-flow kinetics[7] at 550 nm and 25 °C as a function of $Co(ox)_3^{3-}$ concentration over the range 4.9×10^{-5} M to 5 mM (see Figure 1 and supporting information). The observed rates follow the predictions of eqs 1–4, spanning from one limiting behavior to the other.[8] A nonlinear least-squares fit[9] of the data in Figure 1 to eq 4 results in the following rate constants: $k_1 = 0.404 \pm 0.001$ s^{-1}, $k_{-1} = 52$ s^{-1},[9] $k_2 = (1.02 \pm 0.01) \times 10^6$ M^{-1} s^{-1},[9] $k_3 = 23.9 \pm 0.5$ M^{-1} s^{-1}. The rate constant for uphill Fe-to-Ru intramolecular electron transfer, 0.40 s^{-1}, is consistent with the value estimated above from the reaction free energy and the previously reported[1b] rate in the spontaneous direction, 53 s^{-1}. The rate

(6) Olabe, J. A.; Haim, A. *Inorg. Chem.* **1989**, *28*, 3277–3278.
(7) Stopped-flow kinetic measurements were carried out using an Applied Photophysics DX.17MV/S stopped-flow spectrofluorimeter (Leatherhead, UK). Observed first-order rates were fit to the data using the software supplied with the instrument. All experiments were performed in 50 mM pH 7 phosphate buffer ($\mu = 0.1$ M). $(NH_3)_5Ru^{II}-Fe^{III}cyt$ c[7a] and $K_3[Co(ox)_3]\cdot3.5\ H_2O$[7b] were prepared by literature methods: (a) 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. (b). Bailar, J. C., Jr.; Jones, E. M. *Inorg. Synth.* **1939**, *1*, 37.
(8) An alternate mechanism which also explains the behavior of k_{obs} is the preequilibrium formation of a $[Co(ox)_3^{3-}\text{-Cyt}\ c]$ ion pair ($K = 2 \times 10^4$ M^{-1}), followed by first-order intracomplex electron transfer ($k = 0.4$ s^{-1}), plus an independent second-order term (k_3). This explanation is doubtful in the present case since the ion pair constant is unlikely to be as high as 2×10^4 M^{-1} in this medium and Ficke et al. have observed that there is no mechanistic evidence for strong binding in this system: Ficke, J. T.; Pladziewicz, J. R.; Sheu, E. C.; Lappin, A. G. *Inorg. Chem.* **1991**, *30*, 4282–4285.
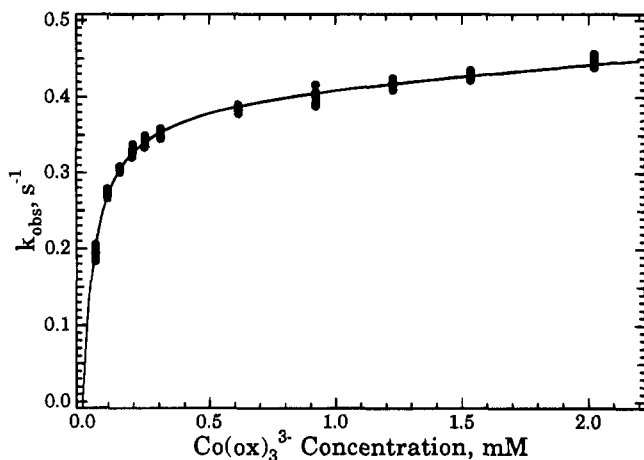(9) The data were plotted and fit to eq 4 using Igor 1.28 (WaveMetrics, Inc., Lake Oswego, OR). Rate constants k_{-1} and k_2 are highly correlated in this fitting procedure, such that error limits cannot be calculated independently. If the result of the unconstrained fit $k_{-1} = 52$ s^{-1} is held fixed, the standard deviation of k_2 is 1%.

Figure 1. Observed rates of oxidation of $(NH_3)_5Ru^{III}-Fe^{II}Cyt$ c at 25 °C as a function of $Co(ox)_3{}^{3-}$ concentration, showing the fit to the rate law given in eq 4 for $k_1 = 0.40$ s^{-1}, $k_{-1} = 53$ s^{-1}, $k_2 = 1.0 \times 10^6$ M^{-1} s^{-1}, and $k_3 = 24$ M^{-1} s^{-1}.
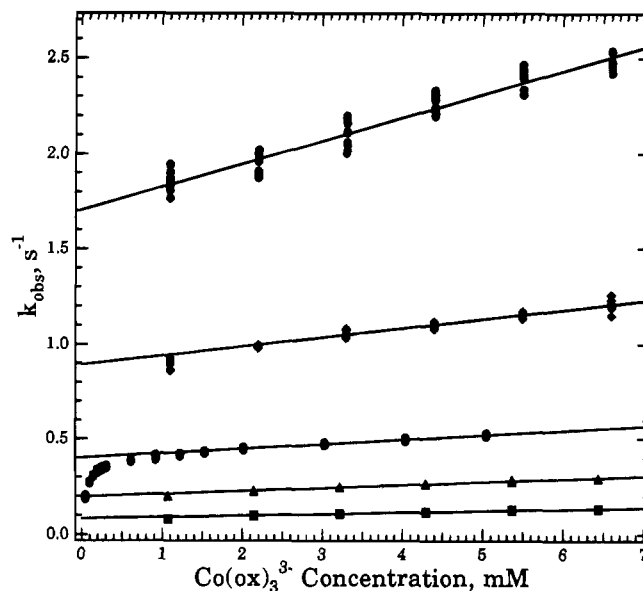
constant obtained for spontaneous (downhill) electron transfer, k_{-1}, agrees very well with the directly measured value under similar solution conditions.[1b] Using the observed rate constants, the equilibrium quotient for reaction 1 is 7.55×10^{-3} and the free energy change $\Delta G^\circ = +0.125$ eV, which agrees well with the direct electrochemical measurement.[1b]

The bimolecular rate constant for $Co(ox)_3{}^{3-}$ oxidation of the ruthenium center in $(NH_3)_5Ru^{II}-Fe^{III}cyt$ c, $k_2 = 1.0 \times 10^6$ M^{-1} s^{-1}, is consistent with other direct measurements carried out on $trans$-$(NH_3)_4$(isonicotinamide)$Ru^{II}-Fe^{III}cyt$ c after corrections are made for differences in driving force between the two protein derivatives.[10]

The rate constant for oxidation of ferrocytochrome c by $Co(ox)_3{}^{3-}$ was also measured under our solution conditions (50 mM pH 7 phosphate buffer) and found to be 10 M^{-1} s^{-1}, close to the value of k_3 (24 M^{-1} s^{-1}) obtained for $(NH_3)_5Ru^{III}$-modified ferrocytochrome c. It is reasonable to expect that the addition of three positive charges from the Ru^{III} center to the net $Fe^{II}cyt$ c charge[4] of $+6$ would increase the bimolecular rate constant for reaction with the $Co(ox)_3{}^{3-}$ trianion.[11]

In order to determine the activation parameters for k_1, reaction rates were measured at 5.0, 15.3, 35.1, and 45.0 °C at six $Co(ox)_3{}^{3-}$ concentrations between 1 and 7 mM. The results are plotted in Figure 2 and listed in the supporting information. The detailed concentration study performed at 25 °C indicates that the approximation $k_{obs} = k_1 + k_3[Co(ox)_3{}^{3-}]$ applies in this concentration range; therefore the data were subjected to linear fits.[12] The resulting values of k_1 and k_3 are given in Table 1, and the Eyring plot for iron(II)-to-ruthenium(III) electron transfer is shown in Figure 3. The enthalpy of activation for the uphill electron transfer process is 12.5 ± 0.2 kcal mol^{-1}, and $\Delta S^\ddagger = -18.3 \pm 0.7$ cal deg^{-1} mol^{-1}.

Since the activation parameters for both directions of the intramolecular electron transfer reaction are now known, the

(10) Sun, J.; Wishart, J. F.; Isied, S. S. Manuscript in preparation.
(11) The effect of a greater overall charge is moderated by the location of the ruthenium modification site on the side opposite the exposed heme edge where electron transfer occurs, which effectively decreases the net dipole moment of 300 D in the native protein which points at the heme-edge electron transfer site.[4] The largest determinant of the rates of these reactions is the ionic medium, which minimizes the effects of the electrostatic differences mentioned above.
(12) The data at each temperature were plotted and fit to a line using Igor 1.28.[8] In order to ensure that only the linear region of the concentration dependence was used, only data taken at concentrations ≥ 2 mM were used for the linear fit (≥ 3 mM for 5 and 15 °C). The points at lower concentrations can be used to judge the degree of curvature.

Figure 2. Observed rates of oxidation of $(NH_3)_5Ru^{III}-Fe^{II}Cyt$ c as a function of $Co(ox)_3{}^{3-}$ concentration at various temperatures (from bottom to top: 5.0, 15.3, 25.0, 35.1, 45.0 °C). Lines are fit as described in the text.

Table 1. Calculated Rate Constants and Activation Parameters for Intramolecular Iron(II)-to-Ruthenium(III) Electron Transfer (k_1) and Direct $Co(ox)_3{}^{3-}$ Oxidation of the Iron(II) Center (k_3) in Pentaammineruthenium-Modified Horse-Heart Cytochrome c^a

| T, °C	k_1, s^{-1}	k_3, M^{-1} s^{-1}
5.0	0.087 ± 0.001	7.4 ± 0.3
15.3	0.203 ± 0.003	14.2 ± 0.6
25.0	0.399 ± 0.002	23.7 ± 0.6
35.1	0.89 ± 0.01	48 ± 2
45.0	1.70 ± 0.02	122 ± 5
$\Delta H^\ddagger$, kcal mol^{-1}	12.5 ± 0.2	11 ± 1^b
$\Delta S^\ddagger$, cal mol^{-1} deg^{-1}	-18.3 ± 0.7	-14 ± 3^b

^{a} Conditions: 50 mM pH 7.0 sodium phosphate buffer. ^{b} Approximate values from a linear fit of the Eyring plot. The data set is curved, presumably due to temperature dependences of the ionic strength effects and of phosphate ion binding.
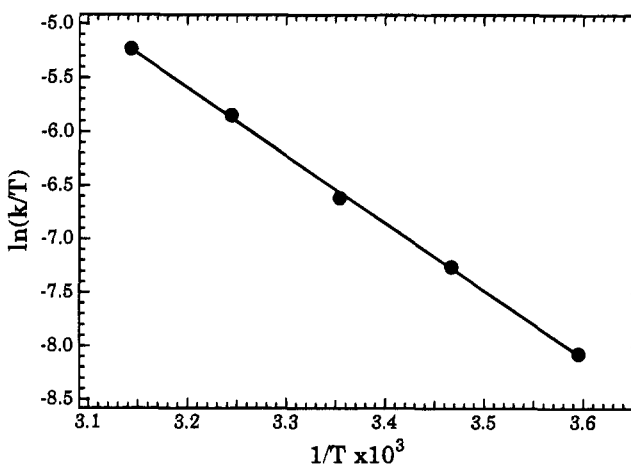
Figure 3. Eyring plot for intramolecular Fe^{II}-to-Ru^{III} electron transfer in pentaammineruthenium-modified horse-heart cytochrome c. $\Delta H^\ddagger = 12.5 \pm 0.2$ kcal mol^{-1}; $\Delta S^\ddagger = -18.3 \pm 0.7$ cal mol^{-1} deg^{-1}.

net reaction enthalpy and entropy can be calculated. Isied and co-workers[1b,13] obtained values of $\Delta H^\ddagger = 3.5 \pm 0.1$ kcal mol^{-1}

(13) The standard deviations were recalculated from the data in Table I of ref 1b.

and $\Delta S^{\ddagger} = -39.0 \pm 0.4$ cal $\deg^{-1}$ mol^{-1} for the downhill reaction by pulse radiolysis experiments performed in 0.1 M pH 7 phosphate buffer with 0.1 M sodium formate. Combining these values with the present results, one obtains $\Delta H^{\circ} = (3.5 \pm 0.1) - (12.5 \pm 0.2) = -9.0 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\circ} = (-39.0 \pm 0.4) - (-18.3 \pm 0.7) = -20.7 \pm 0.8$ cal $\deg^{-1}$ mol^{-1} in the spontaneous direction. The activation parameters for the flash photolysis experiments[2b] in pH 7 phosphate buffer ($\mu = 0.1$ M), as reported in the review of Winkler and Gray[14] ($\Delta H^{\ddagger} = 2.0 \pm 0.5$ kcal mol^{-1}, $\Delta S^{\ddagger} = -43 \pm 5$ cal $\deg^{-1}$ mol^{-1}) lead to similar values for the net reaction ($\Delta H^{\circ} = -10.5 \pm 0.5$ kcal mol^{-1}, $\Delta S^{\circ} = -25 \pm 5$ cal $\deg^{-1}$ mol^{-1}). We have performed nonisothermal differential pulse voltammetry[15] on pentaammineruthenium-modified cytochrome c over a range of 5–45 °C and obtained $\Delta H^{\circ} = -9.0 \pm 0.1$ kcal/mol and $\Delta S^{\circ} = -20.7 \pm 0.4$ cal $\deg^{-1}$ mol^{-1}, in excellent agreement with the measured activation parameters.

The results reported here complete the thermodynamic profile for the intramolecular electron transfer system in pentaammineruthenium-modified horse-heart cytochrome c. The system is entirely reversible and shows no evidence of conformational gating on the millisecond and longer time scales.[16] This is the first example of the direct measurement of intramolecular electron transfer rates in both directions between cytochrome c and a pendant redox group in the ground state. Rates in both directions had previously been obtained for pentaammineruthenium-modified sperm whale myoglobin where the equilib-

rium quotient is close to unity.[17] In the present case, however, $K_{eq} = 130$, and it is the selectivity of the oxidation at the ruthenium site which enables our observations.

The results reported here provide a clear example of how an artificial active site can be introduced into a protein to provide a new pathway for the communication between the interior of the protein and the surrounding medium and, in the examples shown here, increase the effective rate of heme oxidation. This type of approach has been earlier applied in efforts to activate electron transfer from glucose oxidase for the development of enzyme-based sensors.[18,19]

Supporting Information Available: A table of observed rates of oxidation of pentaammineruthenium(III)-modified horse-heart ferrocytochrome c by $Co(ox)_3{}^{3-}$ at several temperatures and $Co(ox)_3{}^{3-}$ concentrations (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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

(15) 4,4′-Bipyridyldisulfide-modified gold disk working electrode, 50 mM pH 7.0 sodium phosphate buffer: Sun, J.; Wishart, J. F.; Cho, M. P.; Isied, S. S. Manuscript in preparation.

(16) Earlier suggestions of directional gating in ruthenium-modified cytochrome c have been revised: Sun, J.; Wishart, J. F.; Gardineer, M. B.; Cho, M. P.; Isied, S. S. *Inorg. Chem.* **1995**, *34*, 3301–3309.

(17) (a) Crutchley, R. J.; Ellis, W. R., Jr.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002–5004. (b) Lieber, C. M.; Karas, J. L.; Gray, H. B. *J. Am. Chem. Soc.* **1987**, *109*, 3778–3779.

(18) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128.

(19) Badia, A.; Carlini, R.; Fernandez, A.; Battaglini, F.; Mikkelsen, S. R.; English, A. M. *J. Am. Chem Soc.* **1993**, *115*, 7053–7060.