

# High-Pressure Pulse-Radiolysis Study of Intramolecular and Intermolecular Reduction of Cytochrome *c* by Ruthenium(II) Ammine Complexes

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Volumes of activation for intra- and intermolecular electron transfer from ruthenium(II) ammine complexes to two types of cytochrome *c* were measured by pulse radiolysis using a portable, high-pressure optical cell. Electron-transfer rates were measured as a function of pressure from 1 to 1500 atm. The  $\Delta V^\ddagger$  values for intramolecular electron transfer in  $(\text{NH}_3)_5\text{Ru}^{\text{II}}\text{-(His33)}$  horse heart ferricytochrome *c* and  $(\text{NH}_3)_5\text{Ru}^{\text{II}}\text{-(His39)}$  *Candida krusei* ferricytochrome *c* are  $-17.7 \pm 0.9$  and  $-18.3 \pm 0.7$   $\text{cm}^3 \text{mol}^{-1}$ , respectively. For the intermolecular reaction between  $[\text{Ru}(\text{NH}_3)_6]^{2+}$  and horse heart ferricytochrome *c*,  $\Delta V^\ddagger$  is  $-15.6 \pm 0.6$   $\text{cm}^3 \text{mol}^{-1}$ . The results indicate that the electron-transfer transition state is significantly more compact than the reactant state in both the intramolecular and intermolecular cases. Most of this volume change can be accounted for by an increase in solvent electrostriction at the ruthenium center.

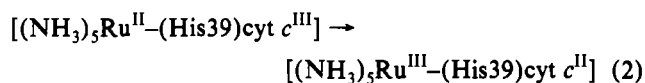
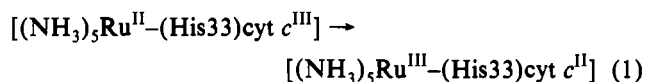
## Introduction

The interest in the understanding of electron-transfer reactions of transition metal complexes has increased significantly in recent years. This can partly be ascribed to an improved ability to describe such processes theoretically and to the application of new techniques with which additional experimental information has become available. It is in this respect that the effect of pressure on electron-transfer processes in inorganic and organometallic systems has received considerable attention in recent years.<sup>1–18</sup> Such studies have added a new dimension to the kinetic information available and enable a direct comparison of experimentally determined and theoretically calculated volumes of activation for such processes.

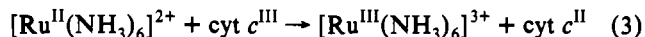
Several laboratories are presently investigating long-range electron-transfer processes in inorganic systems<sup>19</sup> and in proteins.<sup>20</sup> Particular interest has been paid to the nature of the electron-transfer pathway in these systems. Such processes can be studied through the application of flash-photolysis and pulse-radiolysis techniques.<sup>19,20</sup> Recent instrumental developments<sup>21–23</sup> have allowed application of these kinetic techniques at pressures up to

200 MPa (i.e., 2 kbar), such that the pressure dependence of long-range electron-transfer processes can now be investigated. This enables the determination of activation volumes for such processes and should contribute to our understanding of the electron-transfer mechanisms.

This article reports the first study of the effects of pressure on the rates of intramolecular electron-transfer reactions in metal complex-modified proteins. The rates of intramolecular electron transfer in pentaammineruthenium(III)-modified horse heart (eq 1) and *Candida krusei* (eq 2) cytochrome *c* were measured by pulse radiolysis using a high-pressure cell.



For purposes of comparison, the effect of pressure on the intermolecular reaction (eq 3) was also determined. These three processes exhibit a remarkable pressure acceleration which can be interpreted in terms of intrinsic and solvational effects.



## Experimental Section

**High-Pressure Pulse Radiolysis.** Electron pulse-radiolysis transient-adsorption experiments were carried out with the 2-MeV Van de Graaff accelerator at Brookhaven National Laboratory<sup>24</sup> using a PC-controlled,

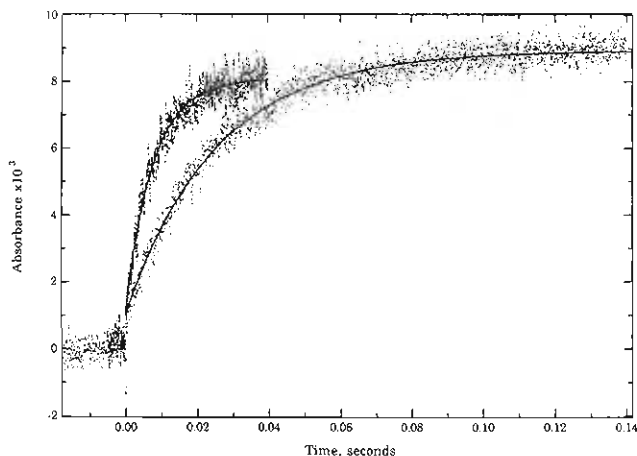
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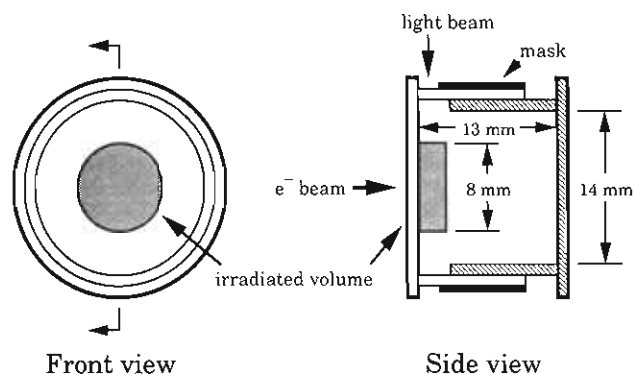
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**Figure 1.** Typical 550-nm transient absorption data for intramolecular electron transfer in (NH<sub>3</sub>)<sub>5</sub>Ru<sup>II</sup>-His33 horse heart cytochrome *c* at 1 atm (slower) and 1500 atm (faster), plotted on the same time scale.

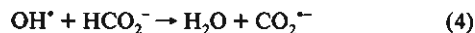


**Figure 2.** Schematic diagram of the pillbox sample cell showing the electron beam and light beam paths and the irradiated volume.

CAMAC-based data acquisition and control system. The transient absorption data, measured at 550 nm, were analyzed to single exponentials by nonlinear least-squares curve fitting to 2000 points. The reported rate error limits are the standard deviations for sets of 4–6 runs. Figure 1 shows typical kinetic traces at 1 and 1500 atm plotted on the same time axis.

For each pressure-dependence experiment a sample was placed in a pillbox quartz cell inside a thermostated, four-window high-pressure vessel.<sup>21</sup> The window of the vessel through which the 2-MeV electron beam penetrates was a 10-mm thick steel window in which 7 holes 2.3 mm in diameter and 9.4-mm deep were drilled in a hexagonal pattern within a circle of 8-mm diameter. In this way, a sufficient electron pulse for accurate experiments could penetrate the thinned-out sections of the window to irradiate the sample (Figure 2).<sup>25</sup> The solutions were stirred with the aid of a magnetic stirring bar within the high-pressure vessel between the kinetic measurements.

The aqueous solutions used in these experiments were N<sub>2</sub>O-saturated, 0.10 M sodium formate and 0.050 M phosphate buffer at pH 7.0. Under these conditions, the radiolytically generated hydroxyl radical reacts with the formate ion to produce the carbon dioxide radical anion.

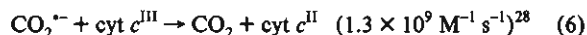
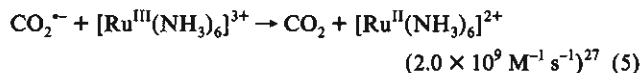


**Pressure Dependence of the Intermolecular Reaction.** Horse heart cytochrome *c* (Sigma, type VI) was purified according to literature procedures.<sup>26</sup> Hexaammineruthenium(III) chloride (Matthey Bishop) was recrystallized from 0.1 M HCl. For the pulse-radiolysis experiments, the concentration of purified native horse heart ferricytochrome *c* was 20.4 μM and that of [Ru(NH<sub>3</sub>)<sub>6</sub>]Cl<sub>3</sub> was 200 μM. Thus, reaction 5

**Table I.** Correction of the Observed Intermolecular Rate Constants for Depletion of Ferricytochrome *c*

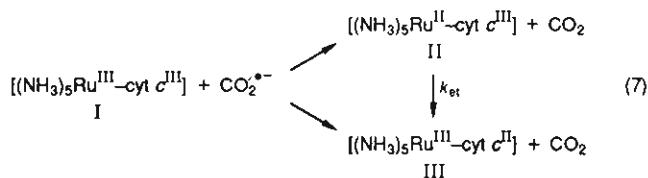
<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>
pressure, atm	<i>k</i> <sub>obs</sub> , s <sup>-1</sup>	tot. dose at middle pulse, μM	<i>C</i> -3.7 μM	fraction cyt <i>c</i> consumed	<i>k</i> <sub>corr</sub> , s <sup>-1</sup>
1	1.29 ± 0.02	3.7	0	0	1.29
500	1.66 ± 0.08	5.7	2.0	0.023	1.70
1000	2.16 ± 0.10	8.1	4.4	0.050	2.27
1500	3.14 ± 0.15	9.7	6.0	0.068	3.37
1000	2.27 ± 0.07	11.3	7.6	0.087	2.49
500	1.48 ± 0.05	14.6	10.9	0.124	1.69
1	1.08 ± 0.02	18.0	14.3	0.163	(1.29)

dominates reaction 6 and intermolecular electron transfer (eq 3) can be followed conveniently at 550 nm.



**Correction of the Observed Intermolecular Rates.** Because the entire pillbox cell volume is not evenly irradiated (see Figure 2), correction of an observed intermolecular rate for depletion of the limiting reagent (ferricytochrome *c*) over the course of the experiment is not as simple as subtracting the total applied dose from the original concentration (20.4 μM) to get the effective concentration for a given data set. The initial and final observed rates are used to normalize the corrections as shown in Table I. Columns *A* and *B* list the average observed rates at each pressure in sequence. Column *C* shows the total dose applied to the irradiated portion of the cell at the middle pulse for a given pressure set, and column *D* contains the total dose relative to the first data set (*D* = *C* - 3.7 μM). The total fraction of cytochrome *c* consumed for the final runs at 1 atm was determined by the ratio of the rate constants (1 - 1.08/1.29 = 0.163). The remaining entries in column *E* were obtained by the formula *E* = *D*(0.163/14.3). The corrected rate constants are then calculated: *F* = *B*/(1 - *E*).

**Pressure Dependence of the Intramolecular Reactions.** Pentaammineruthenium(III)-modified horse heart (hh) and *C. krusei* (Ck) ferricytochromes *c* were prepared according to literature procedures.<sup>28–30</sup> Modified protein concentrations were 14.6 and 12.5 μM, respectively, for hh and Ck. Under these conditions, the electron-transfer intermediate II is generated in ~70% yield from the reaction of ruthenium(III)-modified ferricytochrome with CO<sub>2</sub><sup>•-</sup>.



## Results and Discussion

Each rate constant reported in Table II is the mean of 4–6 individual kinetic measurements at the stated pressure. Independent measurements were made during pressure ascent and descent; these are indicated by separate table entries for a given pressure. As explained above, the intermolecular rates were corrected for depletion of ferricytochrome *c* during the experiment by normalization based on the accumulated radiation dose and the initial and final average observed rates at 1 atm.

Activation enthalpies, entropies, and volumes for the three reactions investigated here are shown in Table III. The inter- and intramolecular electron-transfer reactions are all significantly

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**Table II.** Intramolecular and Intermolecular Electron-Transfer Rates of Native and Pentaammineruthenium-Modified Cytochromes *c* as a Function of Pressure at 25 °C<sup>a</sup>

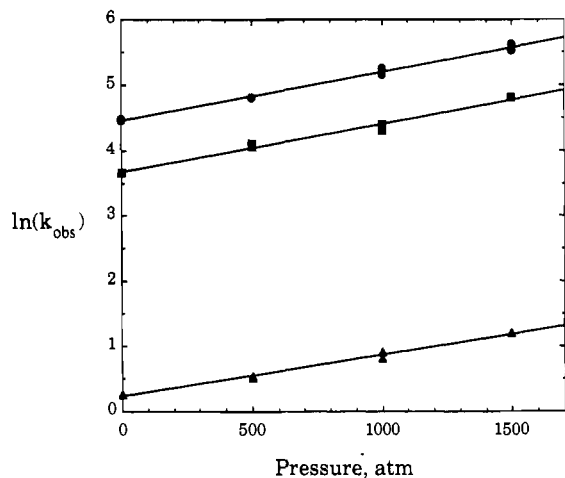
pressure, atm	electron-transfer rate		
	horse heart (NH <sub>3</sub> ) <sub>5</sub> Ru <sup>II</sup> -His33, s <sup>-1</sup>	<i>C. krusei</i> (NH <sub>3</sub> ) <sub>5</sub> Ru <sup>II</sup> -His39, s <sup>-1</sup>	intermolecular [Ru(NH <sub>3</sub> ) <sub>6</sub> ] <sup>2+</sup> + hh, M <sup>-1</sup> s <sup>-1</sup> <sup>b</sup>
1	39 ± 1	86 ± 1	6.3 × 10 <sup>4</sup>
	39 ± 1	88 ± 3	
500	58 ± 3	124 ± 3	8.3 × 10 <sup>4</sup>
	61 ± 1	124 ± 6	8.3 × 10 <sup>4</sup>
1000	74 ± 3	175 ± 3	11.1 × 10 <sup>4</sup>
	81 ± 4	192 ± 9	12.2 × 10 <sup>4</sup>
1500	124 ± 3	254 ± 16	16.5 × 10 <sup>4</sup>
		276 ± 23	

<sup>a</sup> Where two rates are given for a single pressure, the first was measured during pressure ascent and the second was measured on descent from 1500 atm. <sup>b</sup> Rates are corrected for depletion of ferricytochrome *c*. Error limits are 5%. See text.

**Table III.** Activation Parameters for Intramolecular and Intermolecular Electron Transfer in Native and Pentaammineruthenium-Modified Cytochromes *c*

reacn	$\Delta H^\ddagger$ , kcal mol <sup>-1</sup>	$\Delta S^\ddagger$ , cal deg <sup>-1</sup> mol <sup>-1</sup>	$\Delta V^\ddagger$ , cm <sup>3</sup> mol <sup>-1</sup>
horse heart (NH <sub>3</sub> ) <sub>5</sub> Ru <sup>II</sup> -His33	3.5 ± 0.2 <sup>a</sup>	-39 ± 1 <sup>a</sup>	-17.7 ± 0.9 <sup>d</sup>
<i>C. krusei</i> (NH <sub>3</sub> ) <sub>5</sub> Ru <sup>II</sup> -His39	2.3 ± 0.2 <sup>b</sup>	-41 ± 1 <sup>b</sup>	-18.3 ± 0.7 <sup>d</sup>
intermolecular [Ru(NH <sub>3</sub> ) <sub>6</sub> ] <sup>2+</sup> + hh cyt <i>c</i>	0.9 ± 0.2 <sup>c</sup>	-34 ± 1 <sup>c</sup>	-15.6 ± 0.6 <sup>d</sup>

<sup>a</sup> Reference 28. <sup>b</sup> Reference 29. <sup>c</sup> Reference 30. <sup>d</sup> This work.

**Figure 3.** Pressure dependence of the observed rates of intramolecular electron transfer in pentaammineruthenium-modified *C. krusei* (●) and horse heart (■) cytochrome *c* and the observed rate of intermolecular reduction of horse heart ferricytochrome *c* by Ru(NH<sub>3</sub>)<sub>6</sub><sup>2+</sup> (▲).

accelerated by pressure and exhibit similar volumes of activation between -16 and -18 cm<sup>3</sup> mol<sup>-1</sup> (Figure 3). In order to account for the observed volume collapse, the transition state for the electron-transfer process must be significantly more compact than the reactant state. The latter can arise from intrinsic (bond lengths and angles) and solvational (solvent electrostriction) volume contributions.<sup>1,2</sup>

The intermolecular, outer-sphere reduction of cytochrome *c* by Ru(NH<sub>3</sub>)<sub>6</sub><sup>2+</sup> may involve ion-pair formation, as suggested for the reduction of cytochrome *c* by Fe(CN)<sub>6</sub><sup>4-</sup> and Co(phen)<sub>3</sub><sup>2+</sup>.<sup>31</sup> The volume changes associated with such ion-pair formation were calculated to be +2.8 and -1.1 cm<sup>3</sup> mol<sup>-1</sup>, respectively, on the basis that the charge of ferricytochrome *c* is +7.5.<sup>31</sup> The difference in sign arises from a decrease or increase in electrostriction due to charge neutralization or charge concentration during ion-pair

formation, respectively. Since ion-pair formation between cytochrome *c* and Ru(NH<sub>3</sub>)<sub>6</sub><sup>2+</sup> will in general be very weak and is expected to be accompanied by a very small volume change, the experimentally observed  $\Delta V^\ddagger$  (for eq 3) arises mainly from the electron-transfer step itself. This is consistent with the observation that the measured intermolecular  $\Delta V^\ddagger$  is close to those obtained for the intramolecular electron-transfer reactions in the ruthenium-modified hh and Ck cytochromes *c*.

During the electron-transfer process cytochrome *c* is reduced and the overall charge decrease from +7.5 to +6.5.<sup>31,32</sup> However, the partial molar volume of cytochrome *c* upon reduction is estimated to be small (-5 to +5 cm<sup>3</sup> mol<sup>-1</sup>).<sup>33</sup> This means that the observed  $\Delta V^\ddagger$  mainly arises from effects involving the oxidation of Ru(II) to Ru(III). However, the contribution to  $\Delta V^\ddagger$  from intrinsic volume changes due to shortening of the Ru-N bond during oxidation is small ( $0 \geq \Delta V^\ddagger_{\text{int}} \geq -3.7$  cm<sup>3</sup> mol<sup>-1</sup>).<sup>34</sup>

Furthermore, this large volume collapse seems to be independent of whether the Ru center is covalently bonded to cytochrome *c* or just in close contact with cytochrome *c*. The bonding sites in Ru(His33)-cyt *c* (hh) and Ru(His39)-cyt *c* (Ck) are both on the outside surface of the protein<sup>35</sup> and ca. 18 Å (center-to-center) away from the heme (~13 Å imidazole to porphyrin).<sup>20c</sup> The contact ion-pair center-to-center distance is ca. 14 Å in the case of the intermolecular electron-transfer process.<sup>36</sup> The very similar observed activation volumes indicate that the observed volume decrease is associated with the oxidation of the Ru center, which is located on the outside of the protein surface of cytochrome *c* in the intramolecular and intermolecular reactions (eqs 1-3).

The above analysis leads to the conclusion that the observed volume collapse is caused by an increase in solvent electrostriction due to the increase in charge on the Ru center (2+ to 3+) on the surface of the protein. This interpretation agrees with the findings of earlier studies involving intermolecular electron-transfer reactions of cytochrome *c*. A volume of activation of -11 cm<sup>3</sup> mol<sup>-1</sup> was estimated for the reduction of cytochrome *c* by Co(phen)<sub>3</sub><sup>2+</sup>,<sup>31</sup> which is close to that found in this study. The measured volume of activation of +13 cm<sup>3</sup> mol<sup>-1</sup> for the reduction of horse heart cytochrome *c* by Fe(CN)<sub>6</sub><sup>4-</sup><sup>31</sup> can be accounted for in terms of a decrease in electrostriction during the oxidation of Fe(CN)<sub>6</sub><sup>4-</sup> to Fe(CN)<sub>6</sub><sup>3-</sup>. In addition, data for related Co(II/III) complexes<sup>14,37</sup> indicate that solvational processes make significant contributions to volume effects even when large intrinsic volume changes are present.

The significantly negative  $\Delta S^\ddagger$  values (see Table III) support the above hypothesis and correlate well with  $\Delta V^\ddagger$  data. However, the close agreement of the  $\Delta S^\ddagger$  values in the intramolecular and intermolecular cases is fortuitous due to the effects of nonadditivity in the former case and precursor complex formation in the latter.

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- (33) The estimated range stems from the uncertainty in  $\Delta \bar{V}$  for Fe(CN)<sub>6</sub><sup>3+/4+</sup> (due to counterion effects, etc.)<sup>33a</sup> when applied to the reaction volume obtained by Heremans.<sup>31,32</sup> (a) Tregloan, P. A. Personal communication.
- (34) Assuming a spherical geometry and van der Waals radii of 3.50 and 3.46 Å for the [Ru(NH<sub>3</sub>)<sub>6</sub>]<sup>2+</sup> and [Ru(NH<sub>3</sub>)<sub>6</sub>]<sup>3+</sup> ions,<sup>36a</sup> respectively, the intrinsic volume change upon oxidation is -3.7 cm<sup>3</sup> mol<sup>-1</sup>.
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**Conclusion**

This is the first reported investigation of the effect of pressure on rates of intramolecular electron transfer in metal complex-modified proteins. In the case of the ruthenium ammine-cytochrome *c* complexes studied here, the results indicate that the electron-transfer transition state is significantly more compact than the reactant state in both the intramolecular and intermolecular cases.

Comparison of the intermolecular result with previous intermolecular studies using different metal ions shows that the dominant contribution to the observed activation volume (and activation entropy) comes from an intrinsic volume collapse and increased electrostriction of water around the ruthenium center upon oxidation. The similarity of the intramolecular and intermolecular activation volumes and entropies may be due to the fact that the electron-transfer distances and the exposure of the metal complexes to solvent are similar in all three cases.

It will therefore be important in future experiments to investigate the differences in  $\Delta V^\ddagger$  between systems in which the redox centers are located at significantly different distances on the inside and outside of the protein shell. Further investigations of intermolecular and intramolecular *oxidations* of cytochrome *c* by ruthenium complexes would provide complementary mechanistic information.

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