Pressure Tuning Voltammetry. Reaction Volumes for Electron Transfer in Cytochrome *c* and Ruthenium-Modified Cytochromes *c*

J. Sun,[1a] J. F. Wishart,*[,1a] R. van Eldik,*[,1b] R. D. Shalders,[1c] and T. W. Swaddle*[,1c]

Contribution from the Department of Chemistry, Brookhaven National Laboratory, Upton, New York 11973, Institute for Inorganic Chemistry, University of Erlangen−Nürnberg, Egerlandstrasse 1, 91058 Erlangen, Germany, and Department of Chemistry, The University of Calgary, Calgary, Alberta, Canada T2N IN4

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Abstract: High-pressure differential pulse voltammetry and cyclic voltammetry were employed to determine the reaction volume associated with electron transfer in cytochrome *c* and a series of ruthenium-modified cytochromes *c*. The reduction of Cyt c^{III}, either in the native or ruthenium-modified form, is characterized by a reaction volume of -14.0 ± 0.5 cm^3 mol^{-1} when measured versus a Ag/AgCl, KCl(sat'd) reference electrode. A detailed study of the reference electrode system resulted in a value of -9.0 ± 0.6 cm^3 mol^{-1} for the contribution to $\Delta\bar{V}$ for the net reaction Cyt c^{III} + Ag(s) + Cl$^-$(aq) → Cyt c^{II} + AgCl(s) from the reference electrode components Ag(s), Cl$^-$(aq), and AgCl(s). It follows that the absolute molar volume of Cyt c^{III} exceeds that of Cyt c^{II} by only 5.0 ± 0.8 cm^3 mol^{-1} ($\mu = 0.1$ M, pH = 7), i.e. much less than the value of 24 cm^3 mol^{-1} reported in the recent literature. Reaction volumes for a series of intramolecular electron-transfer reactions of the type *trans*-$(NH_3)_4Ru^{III}$(L)-Cyt c^{II} → *trans*-$(NH_3)_4Ru^{II}$(L)-Cyt c^{III} were found to be 31.7 ± 1.2 (L = NH_3), 21.1 ± 1.0 (L = isonicotinamide), 23.3 ± 0.6 (L = pyridine), and 18.6 ± 0.4 cm^3 mol^{-1} (L = 3,5-lutidine). This volume increase is mainly assigned to a decrease in electrostriction during the reduction of the ruthenium center and can be correlated with the number of coordinated ammine ligands. It is concluded that cytochrome *c* undergoes only a small volume change during electron-transfer reactions.

Introduction

Several laboratories are presently involved in the study of kinetic and thermodynamic aspects of long-range electron-transfer reactions in proteins.[2] A common goal in many studies is to improve the insight into the role of the protein structure in determining the route of the electron-transfer process. In efforts to gain more mechanistic information, fast kinetic techniques such as stopped-flow, flash-photolysis and pulse-radiolysis are employed, and more recently these techniques have been extended to hydrostatic pressures up to 200 MPa.[3−5] The pressure dependence of such long-range electron-transfer reactions enables the determination of the activation volume and the construction of a volume profile for such reactions. Among the first systems studied using high-pressure kinetic techniques are the intramolecular and intermolecular reduction and oxidation of cytochrome *c* by ruthenium ammine complexes.[4,5] The results indicated that the volume changes associated with the electron-transfer reactions could be accounted for in terms of changes in electrostriction on the ruthenium ammine centers. No evidence for any significant volume changes on cytochrome *c* itself was found, which is in agreement with earlier findings.[6,7]

Electrochemical measurements at high pressure can be used to determine reaction volumes for electron-transfer processes from the pressure dependence of $\Delta G°$ and to supplement related data obtained from kinetic measurements.[8−12] In a recent study, Cruanes et al.[13] investigated the effect of pressure (up to 500 MPa) on the reduction potential of horse heart cytochrome *c* using cyclic voltammetry (CV). They concluded from their measurements that the observed reaction volume of -24 cm^3 mol^{-1} can be ascribed to the volume collapse associated with the reduction of cytochrome *c*. This conclusion is significantly

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(1) (a) Brookhaven National Laboratory. (b) University of Erlangen-Nürnberg. (c) University of Calgary.

(2) (a) Long-Range Electron Transfer in Biology *Struct. Bonding (Berlin)* **1991**, *75*. (b) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369. (c) Isied, S. S. In *Electron Transfer in Inorganic, Organic, and Biological Systems*; Bolton, J. R., Mataga, N., McLendon, G., Eds.; Advances in Chemistry Series; American Chemical Society: Washington, DC, 1991; Vol. 228, pp 229−245. (d) McLendon, G.; Hake, R. *Chem. Rev.* **1992**, *92*, 481−490. (e) Fenwick, C.; Marmor, S.; Govindaraju, K.; English, A. M.; Wishart, J. F.; Sun, J. *J. Am. Chem. Soc.* **1994**, *116*, 3169. (f) King, B. C.; Hawkridge, F. M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1992**, *114*, 10603. (g) Farver, O.; Skov, L. K.; Pascher, T.; Karlsson, B. G.; Nordling, M.; Lundberg, L. G.; Vänngard, T.; Pecht, I. *Biochemistry* **1993**, *32*, 7317. (h) Lloyd, E.; Tomkinson, N. P.; Salmon, G. A.; Sykes, A. G. *Biochim. Biophys. Acta* **1993**, *1202*, 113. (i) Willie, A.; Stayton, P.; Durham, B.; Sligar, S.; Millett, F. *Biochemistry* **1992**, *31*, 7237. (j) Conrad, D. W.; Zhang, H.; Stewart, D. E.; Scott, R. A. *J. Am. Chem. Soc.* **1992**, *114*, 9909. (k) Meyer, T. E.; Rivera, M.; Walker, F. A.; Mauk, M. R.; Mauk, A. G.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1993**, *32*, 622.

(3) (a) Spitzer, M; Gärtig, F.; van Eldik, R. *Rev. Sci. Instrum.* **1988**, *59*, 2092. (b) Wishart, J. F.; van Eldik, R. *Rev. Sci. Instrum.* **1992**, *63*, 3224. (c) van Eldik, R.; Gaede, W.; Wieland, S.; Kraft, J.; Spitzer, M.; Palmer, D. A. *Rev. Sci. Instrum.* **1993**, *64*, 1355.

(4) Wishart, J. F.; van Eldik, R.; Sun, J.; Su, C.; Isied, S. S. *Inorg. Chem.* **1992**, *31*, 3986.

(5) Bänsch, B.; Meier, M.; Martinez, P.; van Eldik, R.; Su, C.; Sun, J.; Isied, S. S.; Wishart, J. F. *Inorg. Chem.* **1994**, *3*, 4744.

(6) Heremans, K.; Bormans, M.; Snauwaert, J.; Vandersypen, H. *Faraday Discuss. Chem. Soc.* **1982**, *74*, 343.

(7) Heremans, K. In *Inorganic High Pressure Chemistry: Kinetics and Mechanisms*; Elsevier: Amsterdam, 1986; Chapter 7.

(8) Golas, J.; Drickamer, H. G.; Faulkner, L. R. *J. Phys. Chem.* **1991**, *95*, 10191.

(9) Cruañes, M. T.; Drickamer, H. G.; Faulkner, L. R. *J. Phys. Chem.* **1992**, *96*, 9888.

(10) Doine, H.; Whitcombe, T. W.; Swaddle, T. W. *Can. J. Chem.* **1992**, *70*, 81.

(11) Takagi, H.; Swaddle, T. W. *Inorg. Chem.* **1992**, *31*, 4669.

(12) Sachinidis, J.; Shalders, R. D.; Tregloan, P. A. *J. Electroanal. Chem.* **1992**, *327*, 219.

(13) Cruañes, M. T.; Rodgers, K. K.; Sligar, S. G. *J. Am. Chem. Soc.* **1992**, *114*, 9660.

0002-7863/95/1517-2600$09.00/0

at variance with those referred to above and encouraged us to perform a detailed study of the effect of pressure (up to 200 MPa) on the redox potential of cytochrome *c* and ruthenated cytochrome *c* using differential pulse voltammetry (DPV) as well as CV.

The results of this study clearly demonstrate that horse heart cytochrome *c* exhibits no major change in volume during electron transfer and that the observed volume changes can largely be accounted for in terms of processes that occur on the redox partner, i.e. the reference electrode and/or the ruthenium ammine complexes.

Experimental Section

Materials. Horse heart cytochrome *c* (Sigma, Type VI) was purified according to literature procedures.[14] Ruthenium ammine modified horse heart histidine 33 cytochromes *c* were prepared according to the procedures of Isied and Gray.[15-18] Pentaammine(isonicotinamide)-ruthenium(III) trifluoroacetate, $[Ru(NH_3)_5(isn)](CF_3COO)_3$, was prepared following a procedure similar to that used by Ford et al.[19] The cytochrome *c* concentration was in the range of 100−150 μM. The electrolyte buffer solutions (80 mM $NaClO_4$, 10 mM Tris, 10 mM NaN_3, 0.1 mM 4,4′-dipyridyl disulfide, pH 7) were prepared from NANOpure water and were selected to be the same as those used in the intra- and intermolecular electron-transfer kinetic experiments, except for the presence of the 4,4′-dipyridyl disulfide (see below).

Measurements. Details on the pressure vessel and Teflon sample cell have been reported elsewhere.[10] The Teflon sample cell was a miniaturized version (capacity 4.5 mL) of that previously described.[10] For the differential pulse voltammetric experiments reported here, a gold wire working electrode, a Pt wire counter electrode and a Ag/AgCl (saturated KCl) reference electrode were used. The gold wire of 0.5 mm diameter was manually polished with 0.05 μm alumina slurry and a Buehler polishing pad, rinsed with NANOpure water, and modified by dipping it into a 1 mM 4,4′-dipyridyl disulfide solution for about 10 min.[20] The electrode was finally rinsed with NANOpure water and the appropriate buffer solution before use. The addition of 4,4′-dipyridyl disulfide (DPDS) surface modifier to the buffer solution was to assure the activity of the gold working electrode throughout the duration of the experiments. DPDS is electrochemically inert, within the potential window concerned, and has been shown not to interact significantly with Cyt *c*.[20,21]

Differential pulse voltammetry (DPV) as well as CV was performed with the aid of an EG&G Princeton Applied Research Model 174A Polarographic Analyzer and Model RE 0074 X−Y recorder. Test solutions were introduced to the sample cell under a stream of N_2, after which the cell was immediately sealed with the movable piston. For all DPV measurements the scan rate was 2 mV/s and the modulation amplitude was 5 or 25 mV for native Cyt *c* (with identical pressure dependences) and 25 mV for ruthenated cytochromes *c*. Formal potentials were obtained from the differential pulse voltammograms by a peak fitting procedure described in the Appendix. The pressure vessel was immersed into a thermostat bath at 25.0 ± 0.1 °C, and the system was pressurized to 5 MPa for the first measurements. At least 1 h was allowed for temperature equilibration of the pressure vessel and test sample.[10] Voltammetric measurements were usually performed at pressure intervals of 50 MPa up to 200 MPa and down to 5 MPa again. Usually two such pressure cycles were measured for each investigated system, thus providing 4 sets of potential/pressure data. Between 20 and 30 min was allowed for temperature equilibration for a change in pressure of 50 MPa.

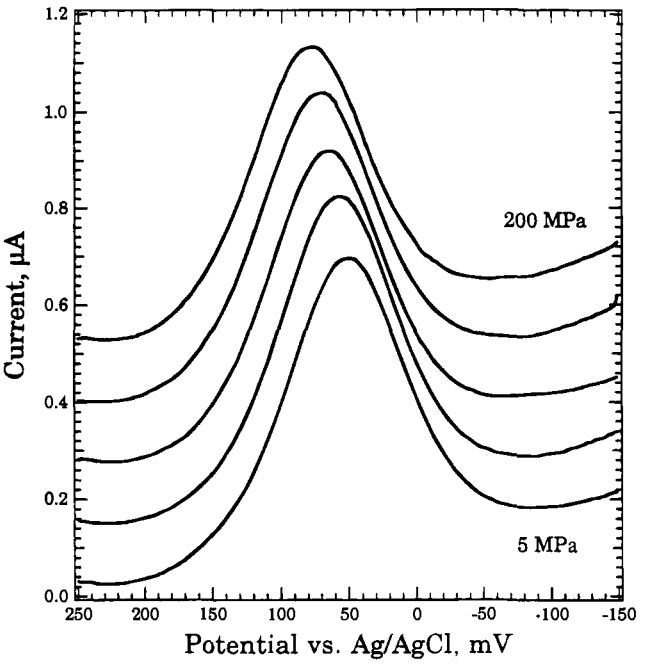

Figure 1. Differential pulse voltammograms of native horse heart cytochrome c as a function of pressure, recorded at 5, 50, 100, 150, and 200 MPa. The traces are offset by arbitrary amounts on the current scale to improve legibility.

CV measurements at ambient pressure were performed with the use of a BAS 100 B/W electrochemical analyzer under the same conditions as those selected in the literature.[13]

In another series of measurements the electrochemical cell was modified to contain two similarly constructed reference compartments. This modification was used to compare the effect of pressure on the Ag/AgCl, KCl(sat'd) electrode in reference to the $Ag/AgNO_3$(0.01 M), 1 M KNO_3 electrode. A 1 M KNO_3 solution was used as supporting electrolyte. The potential was measured directly with a Fluke 8300A voltmeter.

Results and Discussion

In our high pressure measurements, DPV[22,23] was generally used in preference to CV,[8-13,24] but the techniques are complementary, and independent series of CV and DPV measurements on native Cyt *c* gave pressure dependences of the redox potential that were identical within the experimental uncertainty.

The DPVs and CVs recorded for the reduction of native Cyt c^{III} show a gradual shift to higher potentials on increasing the pressure stepwise from 5 to 200 MPa (see Figure 1). This shift is fully reversible on reducing the pressure stepwise to 5 MPa, and DPV and CV data for independently-prepared but otherwise identical solutions are summarized in Figures S1 and 2. The slight curvature of these plots may be accommodated by a quadratic dependence of potential on pressure, giving $\Delta\bar{V} = -16.0 \pm 0.6$ and -17.3 ± 0.6 cm^3 mol^{-1} at zero pressure and $(\partial\Delta\bar{V}/\partial P)_T = 0.011 \pm 0.003$ and 0.017 ± 0.003 cm^3 mol^{-1} MPa^{-1} for the DPV and CV data, respectively, for reaction 1.

$$\text{Cyt } c^{III} + \text{Ag(s)} + \text{Cl}^- \rightarrow \text{Cyt } c^{II} + \text{AgCl(s)} \qquad (1)$$

This curvature, however, falls within the experimental uncertainty in the potential (±2 mV), and a simple linear fit is

(14) Brautigan, D. L.; Ferguson-Miller, S.; Margoliash, E. *Meth. Enzymol.* **1978**, *53*, 128.
(15) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722.
(16) Nocera, D. G.; Winkler, J. R.; Yocum, K. M.; Bordingnon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145.
(17) Bechtold, R.; Kuehn, C.; Lepre, C.; Isied, S. S. *Nature (London)* **1986**, *322*, 286.
(18) Sun, J. Ph.D. Thesis, Rutgers, The State University of New Jersey, 1993.
(19) Ford, P. C.; Rudd, De F. P.; Gaunder, R.; Taube, H. *J. Am. Chem. Soc.* **1968**, *90*, 1187.
(20) Taniguchi, I.; Toyosama, K.; Yamaguchi, K. *J. Electroanal. Chem.* **1982**, *140*, 187.
(21) Sagara, T.; Satake, I.; Murakami, H.; Akutsu, H.; Niki, K. *J. Electroanal. Chem.* **1991**, *301*, 285.
(22) Osteryoung, J. *J. Chem. Educ.* **1983**, *60*, 296.
(23) Mabbott, G. A. *J. Chem. Educ.* **1983**, *60*, 697.
(24) Rodgers, K. K.; Sligar, S. G. *J. Am. Chem. Soc.* **1991**, *113*, 9419.

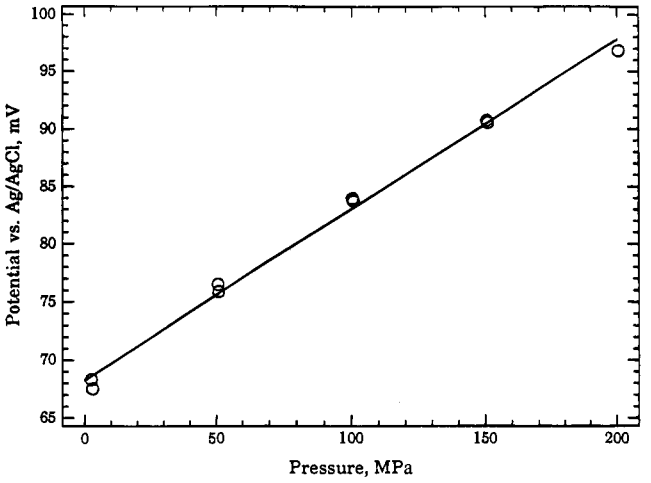

Figure 2. Formal potentials for native cytochrome *c* as a function of pressure, obtained by cyclic voltammetry.

adequate, giving $\Delta\bar{V} = -13.7 \pm 0.4$ (DPV) and -14.3 ± 0.4 (CV) cm^3 mol^{-1}. The average of these values, -14.0 ± 0.5 cm^3 mol^{-1}, is adopted as the mean $\Delta\bar{V}$ for reaction 1 over the experimental pressure range, so facilitating comparison with other reaction volume data calculated on the same basis of a linear pressure dependence of potential. Experiments without azide (0.09 M $NaClO_4$, 0.01 M Tris, 0.1 mM DPDS) gave the same $\Delta\bar{V}$ (-14.0 ± 1.0 cm^3 mol^{-1}) as the CV and DPV measurements with the azide present. The zero-pressure potentials in Figures S1 and 2 are in satisfactory agreement with those obtained in conventional DPV measurements at atmospheric pressure (285 mV vs NHE, or 63 mV vs Ag/AgCl/saturated KCl).

Cruañes et al.[13] used CV at a microelectrode referred to Ag/AgCl/0.1 M NaCl over the pressure range 0−500 MPa to obtain $\Delta\bar{V} = -27$ cm^3 mol^{-1} at zero pressure and $(\partial\Delta\bar{V}/\partial P)_T \approx 0.049$ cm^3 mol^{-1} MPa^{-1} for reaction 1 in 0.1 M NaCl containing L-cysteine as the electrode modifier. In an attempt to resolve the differences between these results and ours, we carried out CV measurements using the same solution and reference electrode electrolyte compositions as did Cruañes et al.[13] The CV peaks were less sharp, and this was traced to the use of L-cysteine rather than DPDS, but a linear fit represented the potential-pressure data to within ±2 mV, giving $\Delta\bar{V} = -18.0 \pm 0.9$ cm^3 mol^{-1} and a zero-pressure potential of −29 mV. Thus, the discrepancy between our measurements and those of Cruañes et al.[13] lies partly in a medium effect (0.1 M $NaClO_4$/NaN_3/DPDS vs 0.1 M NaCl/cysteine). To eliminate the possibility that the pressure dependence of the liquid junction potential in our system might contribute to this discrepancy, we measured the pressure dependence of the potential of a model couple, tris(1,10-phenanthroline)cobalt(III/II), in 0.1 M NaCl against (a) 0.1 M NaCl/AgCl/Ag and (b) saturated KCl/AgCl/Ag reference electrodes; the zero-pressure potentials were (a) 83.9 ± 0.2 and (b) 151.8 ± 2.3 mV, and the reaction volume was (a) +27.0 ± 1.0 and (b) +27.0 ± 1.7 cm^3 mol^{-1}. Thus, while a significant junction potential may exist, its pressure dependence is negligible.

DPV measurements on *trans*-Ru(NH_3)$_4$L-Cyt *c* (L = isn, NH_3) exhibited well-separated waves for the $Ru^{III/II}$ and Cyt $c^{III/II}$ couples (Figures 3 and S2). The effect of pressure on the latter gave $\Delta\bar{V} = -11.9 \pm 1.1$ (L = isn) and -12.1 ± 1.0 (L = NH_3) cm^3 mol^{-1} for the reduction of the Cyt *c* center relative to saturated KCl/AgCl/Ag. Thus, attachment of the Ru complexes had only a very small (+2 cm^3 mol^{-1}) effect on the redox volume profile of the protein itself.

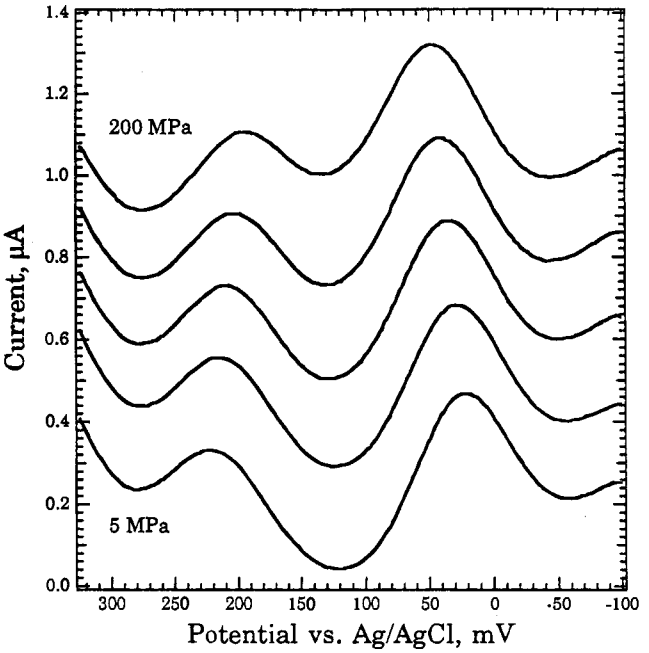

Figure 3. Differential pulse voltammograms of *trans*-(NH_3)$_4$Ru(isn)-Cyt *c* as a function of pressure, recorded at 5, 50, 100, 150, and 200 MPa. The traces are offset by arbitrary amounts on the current scale to improve legibility.

As already indicated above, we also used the DPV technique to study the effect of pressure on the reduction potentials of a series of ruthenium-modified cytochromes *trans*-(NH_3)$_4$-$Ru^{II/III}$(L)-Cyt $c^{II/III}$, where L = isonicotinamide (isn), NH_3, 3,5-lutidine (3,5-lut), and pyridine (py). Typical DPVs as a function of pressure are shown in Figures 3, S2, S3, and S4 for these derivatives, respectively. The purpose of these experiments was to estimate the net volume change for the intramolecular electron transfer reaction:

$$trans\text{-}(NH_3)_4Ru^{III}(L)\text{-Cyt } c^{II} \rightarrow trans\text{-}(NH_3)_4Ru^{II}(L)\text{-Cyt } c^{III} \quad (2)$$

This can be done by measuring the effect of pressure on the difference between the observed ruthenium and cytochrome potentials:

$$Ru^{III}\text{-Cyt } c^{III} + Ag(s) + Cl^- \rightarrow Ru^{II}\text{-Cyt } c^{III} + AgCl(s) \quad (E_{Ru};\ \Delta\bar{V}_{Ru}) \quad (3)$$

$$Ru^{II}\text{-Cyt } c^{III} + Ag(s) + Cl^- \rightarrow Ru^{II}\text{-Cyt } c^{II} + AgCl(s) \quad (E_{Fe};\ \Delta\bar{V}_{Fe}) \quad (4)$$

$$Ru^{III}\text{-Cyt } c^{III} + Ru^{II}\text{-Cyt } c^{II} \rightarrow 2Ru^{II}\text{-Cyt } c^{III} \quad (\Delta E = E_{Ru} - E_{Fe};\ \Delta\bar{V} = \Delta\bar{V}_{Ru} - \Delta\bar{V}_{Fe}) \quad (5)$$

If one assumes that the redox volume change of each couple is not perturbed by the oxidation state of the other couple (as we have already shown to be valid to a good approximation for the Cyt $c^{III/II}$ couple), the volume change for eq 2 can be taken to equal that for eq 5. Note also that by taking the direct difference ΔE between the redox couples we remove the effect of the reference electrode.

The $Ru^{II/III}$ and Cyt $c^{II/III}$ redox potentials for L = isn, py, and 3,5-lut in Figures 3, S3, and S4 move closer together (ΔE becomes smaller) with increasing pressure, whereas they move

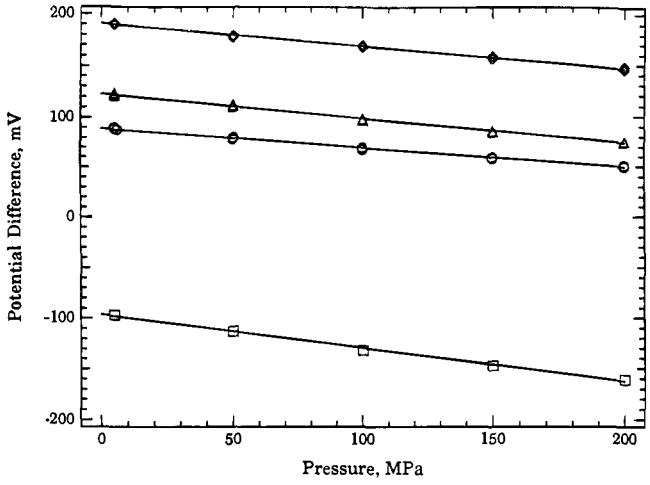

Figure 4. Measured differences between ruthenium and heme redox potentials for *trans*-[$(NH_3)_4$LRu]-modified cytochromes *c* as functions of pressure: (◇) L = isonicotinamide; (△) L = pyridine; (○) L = 3,5-lutidine; (□) L = NH_3.

Table 1. Summary of Available $\Delta\bar{V}$ Data for Intermolecular and Intramolecular Electron-Transfer Reactions between Ruthenium Ammine Complexes and Cytochrome *c* at 25 °C

reaction	method	$\Delta\bar{V}$, cm^3 mol^{-1}	ref
$Ru(NH_3)_5isn^{3+}$ + Cyt c^{II} ⇌ $Ru(NH_3)_5isn^{2+}$ + Cyt c^{III}	kinetic	+33 ± 3	5
	spectrophotometric	+31 ± 1	5
	electrochemical	+26.4 ± 0.9	*a*
$(NH_3)_5Ru^{III}$-Cyt c^{II} ⇌ $(NH_3)_5Ru^{II}$-Cyt c^{III}	electrochemical	+31.7 ± 1.2	*a*
trans-$(NH_3)_4(isn)Ru^{III}$-Cyt c^{II} ⇌ *trans*-$(NH_3)_4(isn)Ru^{II}$-Cyt c^{III}	electrochemical	+21.1 ± 1.0	*a*
trans-$(NH_3)_4(py)Ru^{III}$-Cyt c^{II} ⇌ *trans*-$(NH_3)_4(py)Ru^{II}$-Cyt c^{III}	electrochemical	+23.3 ± 0.6	*a*
trans-$(NH_3)_4$(3,5-Lut)Ru^{III}-Cyt c^{II} ⇌	spectrophotometric	+21.1 ± 0.8	*b*
trans-$(NH_3)_4$(3,5-Lut)Ru^{II}-Cyt c^{III}	electrochemical	+18.6 ± 0.4	*a*

[a] This work. [b] Unpublished results.

further apart (ΔE becomes larger) with increasing pressure for L = NH_3 in Figure S2. In all cases, the reduction potentials of the ruthenium complexes decrease with increasing pressure while that of cytochrome *c* increases as shown above. The effects of pressure on the observed ΔE's for eq 5, and by extension the driving forces for eq 2, are plotted in Figure 4 for all four systems, where each line represents the average of between 3 and 4 series of measurements.[25] The resulting $\Delta\bar{V}$ values are summarized in Table 1 along with the data available for these or related reactions in which different techniques were used to determine $\Delta\bar{V}$.

We also investigated one intermolecular electron-transfer reaction given in (6). As in the *intra*molecular isonicotinamide case, the reduction potentials of the two couples move closer together under pressure (see Figure S5). The results obtained

$$Ru(NH_3)_5isn^{3+} + \text{Cyt } c^{II} \rightarrow Ru(NH_3)_5isn^{2+} + \text{Cyt } c^{III} \quad (6)$$

for two series of experiments are summarized in Figure 5. The decrease in ΔE with increasing pressure results in a $\Delta\bar{V}$ value of +26.4 ± 0.9 cm^3 mol^{-1} for reaction 6. This result is in good agreement with the $\Delta\bar{V}$ values for the same reaction obtained from kinetic and spectrophotometric measurements under pressure[5] and clearly demonstrates the accuracy of the different methods.

(25) The values of ΔE were determined using the method reported in the Appendix.

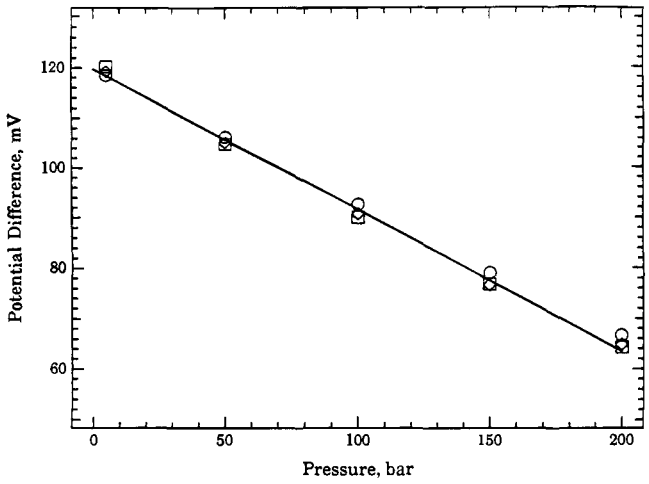

Figure 5. Differences between ruthenium and heme redox potentials for a solution containing 20 μM $Ru(NH_3)_5(isn)^{2+}$ and 100 μM cytochrome *c* as a function of pressure.

At this point we turn to a discussion of the observed reaction volumes for the investigated redox couples. For the Cyt *c* systems the overall reaction is given in (1), from which it follows that $\Delta\bar{V}$ can be expressed as in (7), i.e. as the sum of the

$$\Delta\bar{V} = \Delta\bar{V}(\text{Cyt } c^{III/II}) + \Delta\bar{V}(\text{Ag/AgCl}) \quad (7)$$

volume changes associated with the reduction and oxidation steps, respectively. In earlier reports it was argued on the basis of molar volume data that $\Delta\bar{V}$(Ag/AgCl) is 3.1 cm^3 mol^{-1}, or 0.1 cm^3 mol^{-1} if the purported molar volume of the electron is included,[9,13] such that the observed $\Delta\bar{V}$ represents the volume change associated with the reduction of Cyt c^{III}. However, we have good reasons to believe that the arguments are not correct, and that in fact $\Delta\bar{V}$(Ag/AgCl) is substantially negative and can account for most of the observed $\Delta\bar{V}$.

A comparison of available $\Delta\bar{V}$ data measured electrochemically, using both Ag/Ag^+ and Ag/AgCl as reference electrodes, shows remarkably close agreement. For instance, Swaddle and co-workers[10] report $\Delta\bar{V}$ values of +6.2 ± 0.5, +5.0 ± 0.3, and −38.3 ± 1.0 cm^3 mol^{-1} for the reduction of $Fe(phen)_3^{3+}$, $Fe(H_2O)_6^{3+}$, and $Fe(CN)_6^{3-}$, respectively, measured at a Pt electrode at 25 °C and 1 M ionic strength (0.28 M for $Fe(H_2O)_6^{3+}$) relative to Ag/AgCl, KCl(sat'd). Tregloan and co-workers[12,26,27] reported $\Delta\bar{V}$ values of +6.7 ± 1.0, +5.4 ± 0.8, and −37.5 ± 0.4 cm^3 mol^{-1} for the same reactions under similar conditions, respectively, but measured relative to $Ag/AgNO_3$, 1 M KNO_3. The good agreement between these values indicates that the reaction volumes associated with the oxidation at the reference electrode must be almost identical for the Ag/AgCl and Ag/Ag^+ systems.[28] The latter reference electrode was also used to measure $\Delta\bar{V}$ for the reduction of a series of differently charged low-spin Fe(III) complexes,[26,27] from which it could be concluded that the contribution to the observed volume changes from the reference electrode components Ag(s) and Ag^+(aq) is −11.9 ± 0.5 cm^3 mol^{-1} at 25 °C and 1.0 M ionic strength. Thus a similar volume change is expected for the

(26) Shalders, R. D. Ph.D. Thesis, University of Melbourne, 1992.
(27) Sachinidis, J. I.; Shalders, R. D.; Tregloan, P. A. *Inorg. Chem.* **1994**, *33*, 6180.
(28) The effect of pressure on the reduction potential of $Fe(CN)_6^{3-}$ in 0.1 M KCl at a Pt microelectrode relative to Ag(s)/AgCl(s), using CV and square wave voltammetry, was also reported in ref 8. However, the authors made an error in the calculation of the $\Delta\bar{V}$ data, the reported values should be 10 times larger. In the lower pressure range of their data,[8] $\Delta\bar{V}$ has a value of ca. −35 cm^3 mol^{-1}, which is close to that reported elsewhere.[10,12]

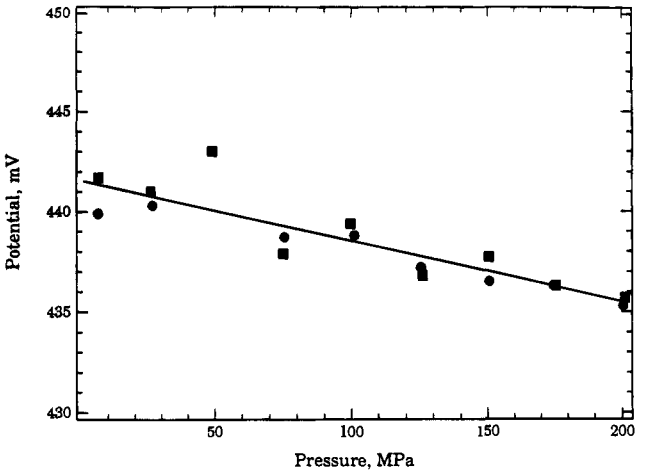

Figure 6. Observed potential for the electrochemical cell Ag/0.01 M $AgNO_3$, 1 M KNO_3//1 M KNO_3//KCl(sat'd)/AgCl/Ag as a function of pressure. Circles and squares denote two series of measurements.

oxidation of Ag(s) to AgCl(s) on the basis of the above reported $\Delta\bar{V}$ data using both reference systems.

In order to test this conclusion further, a modified electrochemical cell was used to measure the potential of the Ag/AgCl-(KCl sat'd) electrode relative to the Ag/Ag^+ electrode as a function of pressure. The electrochemical cell constructed from the Ag/AgCl(KCl sat'd) and $Ag/AgNO_3$(0.01 M), 1 M KNO_3 half-cells gave the potential readings summarized in Figure 6 as a function of pressure. A linear fit of these data resulted in a reaction volume of $+2.9 \pm 0.4$ cm^3 mol^{-1}, indicating that the two half-cells indeed exhibit very similar pressure dependences and associated volume changes. The half-cell and overall reactions are summarized in (8),

$$\begin{aligned} Ag^+ + e^- &\rightarrow Ag(s) \\ Ag(s) + Cl^- &\rightarrow AgCl(s) + e^- \\ \hline Ag^+ + Cl^- &\rightarrow AgCl(s) \end{aligned} \qquad (8)$$

from which it follows that $\Delta\bar{V} = \Delta\bar{V}(Ag^+/Ag) + \Delta\bar{V}(Ag/AgCl)$, such that $\Delta\bar{V}(Ag/AgCl) = \Delta\bar{V} - \Delta\bar{V}(Ag^+/Ag) = (+2.9 \pm 0.4) - (+11.9 \pm 0.5) = -9.0 \pm 0.6$ cm^3 mol^{-1}. When using partial molar volumes at 25 °C and infinite dilution,[29] $\Delta\bar{V}$ for reaction 5 results in $\Delta\bar{V} = V(AgCl) - V(Ag^+) - V(Cl^-) = 25.8 - (-0.7) - 17.8 = +8.7$ cm^3 mol^{-1}. This value is significantly more positive than the experimental value of $+2.9 \pm 0.4$ cm^3 mol^{-1} for this reaction, which is not surprising when the difference in ionic strength, i.e. infinite dilution versus saturated KCl, is taken into account. Thus molar volume calculations for such electrode reactions based on partial molar volume data can result in misleading $\Delta\bar{V}$ data.[9,13]

With the value of -9.0 ± 0.6 cm^3 mol^{-1} now available for the contribution to the reaction volume from the reference electrode components Ag(s), Cl^-(aq), and AgCl(s), the molar volume of Cyt c^{III} can be estimated to be 5.0 ± 0.8 cm^3 mol^{-1} larger than that of Cyt c^{II}. This value indicates that there is a slight compression of Cyt c during the reduction process, even though the overall charge is reduced from +7.5 to +6.5.[30] There are many reports in the literature that indicate a more compact structure for Cyt c^{II} compared with Cyt c^{III}.[31-34] However, the contraction of the protein results in the release of water molecules from the protein into the bulk, resulting in a small overall volume change.[35] Our present finding of -5.0 ± 0.8 cm^3 mol^{-1} is consistent with this and it is significantly less negative than the value of -24 cm^3 mol^{-1} assigned by Cruañes et al.[13] to the reduction of Cyt c^{III} (where the reference electrode was assumed to contribute -3 cm^3 mol^{-1} to the observed pressure dependence of the cytochrome c reduction potential), or the -18 cm^3 mol^{-1} value which one obtains from their experimental results and our value for the reference electrode. Our earlier studies already provided evidence that such a large volume change could not be the case.[4,5] The data from the present study corroborate the earlier studies and fit in well with volume data obtained from high-pressure spectrophotometric and kinetic data (see further Discussion).

As noted above, the discrepancy between our data for the reduction of native Cyt c and those of Cruañes et al.[13] resides partly in a medium effect of chloride vs perchlorate, which might be expected in view of the known strong interaction between chloride and Cyt c.[36] However, a further -9 cm^3 mol^{-1} in $\Delta\bar{V}$ and a 3- to 4-fold larger $(\partial\Delta\bar{V}/\partial P)_T$ in the microelectrode study remain unaccounted for. As these two quantities are correlated, and since the $\Delta\bar{V}$ values reported by Cruañes et al.[9] at 200–300 MPa are close to ours for the 0–200 MPa range, it is possible that systematic errors may exist in the low-pressure regime of the microelectrode experiments. This possibility is also suggested by the fact that $\Delta\bar{V}$ values reported by Cruañes et al.[9] for the reduction of $Co(bpy)_3^{3+}$ and $Fe(bpy)_3^{3+}$ are much larger in the lower pressure range than those found elsewhere,[26] although the data at higher pressures[9] do agree with the other published data.

We now turn to a discussion of the $\Delta\bar{V}$ data reported for the intra- and intermolecular electron-transfer reactions between ruthenium ammine complexes and cytochrome c. The results in Table 1 show that reduction of the ruthenium(III) ammine complexes is accompanied by a large volume increase, which can mainly be ascribed to desolvation effects on the ruthenium center. We have shown above that only minor volume changes are associated with the oxidation of the Cyt c center. Furthermore, the contribution of intrinsic volume changes, $\Delta\bar{V}_{intr}$, due to lengthening of the Ru–N bond during reduction of Ru(III) is small ($0 \leq \Delta\bar{V}_{intr} \leq 3.7$ cm^3 mol^{-1}).[4,37] One interesting observation which can be drawn from the results in Table 1 is the similarity of the net reaction volumes for the intermolecular pentaammine(isonicotinamide)ruthenium/cyt c system and the intramolecular pentaammineruthenium-cyt c system. The average volume increases in both cases are approximately 32 cm^3 mol^{-1} for electron transfer from Fe(II) to Ru(III). These values can also be compared to the reaction volume of 31 ± 2 cm^3 mol^{-1} calculated for the intermolecular hexaammineruthenium/cyt c system, which one obtains from our redox partial volume of cytochrome c and the value of $+28.8 \pm 0.6$ cm^3 mol^{-1} reported[26] for the reduction of $Ru(NH_3)_6^{3+}$ to $Ru(NH_3)_6^{2+}$ after appropriate correction for the contribution from the $Ag/AgNO_3$

(29) Millero, F. In *Water and Aqueous Solutions*; Horne, R. A., Ed.; Wiley-Interscience: New York, 1972; Chapter 13.
(30) Heremans, K.; Bormans, M.; Snauwaert, J.; Vandersypen, H. *Faraday Discuss. Chem. Soc.* **1982**, *74*, 343.
(31) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959.
(32) Feng, Y.; Roder, H.; Englander, S. W. *Biochemistry* **1990**, *29*, 3494.
(33) Liu, G.; Grygon, C. A.; Spiro, T. G. *Biochemistry* **1989**, *28*, 5046.
(34) Trewhella, J.; Carlson, V. A. P.; Curtis, E. H.; Heidorn, D. B. *Biochemistry* **1988**, *27*, 1121.
(35) Margalit, R.; Schejter, A. *Eur. J. Biochem.* 1973, *32*, 492.
(36) Gopal, D.; Wilson, G. S.; Earl, R. A.; Cusanovich, M. A. *J. Biol. Chem.* **1988**, *263*, 11652.
(37) This is based on the very similar van der Waals radii of 3.50 and 3.46 Å for the $Ru(NH_3)_6^{2+}$ and $Ru(NH_3)_6^{3+}$ ions, respectively. See: Sutin, N. In *Tunneling in Biological Systems*; Chance, B., De Vault, D. C., Frauenfelder, H., Marcus, R. A., Schrieffer, J. R., Sutin, N., Eds.; Academic Press: New York, 1979; p 201.

(0.01 M) reference electrode.[27] Clearly, the similarity of the reaction volume for the intramolecular pentaammineruthenium/cyt *c* system to those of the intermolecular cases must be the result of cancelling effects. The protein must certainly screen a significant solid angle of the ruthenium solvation sphere in the covalently-bound case, which would lessen the amount of solvent electrostriction. It is possible that the increased solvent polarization caused by the positively charged lysine groups on the surface of cytochrome *c* extends the influence of the electrostrictive effect around the bound ruthenium complex enough to compensate for the screening effect.

The effect of substitution by non-ammine ligands is shown in the intramolecular electron-transfer reactions of the tetraammineruthenium(L)/cyt *c* complexes, where the volume increase is around 22 $cm^3\ mol^{-1}$, only 70% of that observed for the pentaammineruthenium/cyt *c* system. In replacing a coordinated ammonia with a substituted pyridine, electrostriction may be reduced due to exclusion of the solvent from the volume occupied by the pyridine ligand, as well as the loss of hydrogen bonds which efficiently conduct the charge state of the metal ion to the surrounding water molecules. The possibility of effects from specific interaction between amino acid side chains and the coordinated metal complex must also be considered.

We conclude that solvational changes on the ruthenium center largely account for the observed reaction volumes in the systems we have studied, in line with arguments presented before[4,5] and independent of whether the electron-transfer process is intramolecular or intermolecular. Furthermore, the different techniques employed to determine $\Delta\bar{V}$ for redox reactions between ruthenium ammine complexes and cytochrome *c* result in reasonably consistent values. This could indicate that there are no serious deviations in terms of volume effects caused by the heterogeneous electron-transfer reactions involved in the electrochemical techniques employed.

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Appendix

Electrochemical Data Analysis. The high-pressure differential pulse voltammograms (δi vs E) in this study were originally recorded on chart paper with an X−Y recorder. Accurate potentials for single peaks and well-resolved pairs of peaks can be obtained directly from the chart paper; however, several of the systems investigated here have significantly overlapping peaks which must be fitted to obtain correct potentials for the individual couples. Consequently, for the sake of consistency all voltammograms were scanned with an Apple OneScanner (Apple Computer, Cupertino, CA) and Ofoto scanning software (Light Source, Inc., Larkspur, CA) and the scanned images were digitized automatically at 2 mV intervals using FlexiTrace software (Tree Star, Inc., San Carlos, CA).

The digitized voltammograms were fitted on a Macintosh computer using Igor (WaveMetrics, Inc., Lake Oswego, OR), and a version of the "Custom Peak Measurement and Fitting" demonstration experiment[38] which was adapted to use the theoretical DPV peak function:[39]

$$\delta i(E) = \{(nFAD_0^{1/2}C_0{}^*\pi^{-1/2}(\tau - \tau')^{-1/2})^*P_A(1 - \sigma^2)\}/ \{(\sigma + P_A)(1 + P_A)\} \quad \text{(A1)}$$

$$P_A = (D_0/D_r)^{1/2} \exp[(nF/RT)(E + \Delta E/2 - E^{o\prime})] \quad \text{(A2)}$$

$$\sigma = \exp(nF\delta E/2RT) \quad \text{(A3)}$$

The quantities grouped between the first set of parentheses in eq 1 can be treated as a constant for the purpose of peak fitting. The diffusion constants (D_o and D_r) are assumed to be the same for both oxidation states. The DPV pulse potential (ΔE) is 25 mV for these experiments. Peak amplitude and formal potential ($E^{o\prime}$) were used as parameters for peak fitting.

In test measurements using a BAS 100B Electrochemical Analyzer and a solution of $[(NH_3)_5Ru(3,5\text{-lutidine})]^{3+}$ at a polished gold electrode under the same solution conditions as the high-pressure work (but without dipyridyl disulfide), the theoretical DPV waveform agreed well with the experimental data, with a peak width (fwhm) of 92.7 mV. However, when the gold electrode was modified with 4,4′-dipyridyl disulfide, which is used as a mediator for the cytochrome couple, peak widths for the cytochrome and the ruthenium complex (measured separately) were wider than theoretical (~102 mV). The effect of this quasireversibility could be accurately simulated by empirical adjustment of *n* (the number of electrons for the redox process) from 1.0 to 0.9, which was used in all subsequent calculations.

Each experimental current/potential plot was simulated by the summation of one or two DPV waves as the case required, plus a polynomial of order 2 to 4 to model the background current. The peak fitting routine used a Simplex least-squares minimization to find the optimum peak amplitude and value of $E^{o\prime}$ for each wave and the background polynomial coefficients. All scans from a given experiment were modeled with the same-order polynomial. Even so, it was found that the measured difference potentials ($\Delta E^{o\prime} = E^{o\prime}_{Ru} - E^{o\prime}_{Cyt}$) were insensitive to the background polynomial order. For a given measurement on a system with two redox couples, changing the order of the background polynomial (i.e., 2nd vs 3rd vs 4th order) resulted in the same value of $\Delta E^{o\prime}$ to within 2 mV. Relative peak amplitudes were consistent for all measurements on the same solution at various pressures.

Supplementary Material Available: Figures S1−S5 reporting potential data and current−potential curves as a function of pressure for the investigated systems (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(38) Igor Technical Note No. 020-A; WaveMetrics, Inc.: Lake Oswego, OR. Also available by anonymous FTP from d31rz0.stanford.edu.

(39) Bard, A. J.; Faulkner, L *Electrochemical Methods*; John Wiley & Sons: New York, 1980; pp 193−4.