Kinetic and Thermodynamic Study of the Mechanism of Reversible Intermolecular Electron-Transfer Reactions Between Horse Heart Cytochrome *c* and a Series of Cobalt Imine Complexes

Martin Meier and Rudi van Eldik*

Abstract: The kinetics and thermodynamics of the reversible outer-sphere electron-transfer reactions between horse heart cytochrome $c^{II/III}$ and $[Co(phen)_3]^{3+/2+}$ and $[Co(bpy)_3]^{3+/2+}$ were studied in detail, in particular as a function of temperature and pressure. It was possible to construct a volume profile for both reactions from the pressure data. The transition state was found to be halfway between the reactant and product states on a volume basis in all studied systems. This is in agreement with the λ^+ parameter estimated from the Marcus theory. For all the systems investigated, the differences in the activation volumes are in good agreement with the reaction volumes determined from spectrophotometric and electrochemical measurements at elevated pressure, and from the difference in the partial molar volumes of the metal complexes. The activation and reaction volumes of the bipyridine system are significantly smaller than those of the corresponding phenanthroline and terpyridine systems. A detailed mechanistic analysis is presented. The results show that the different kinetic and thermodynamic techniques employed complement one another.

Keywords: cobalt · cytochrome *c* · electron transfer · kinetics · volume profile

Introduction

The interest in understanding the electron-transfer reactions of redox proteins has increased significantly over the past years.[1-3] Electron transfer takes place over long distances in these reactions. Since cytochrome *c* is one of the most thoroughly characterized electron-transfer proteins, there is significant interest in investigating the reactions of this metalloprotein from different viewpoints.[4-6] Currently, we are interested in outer-sphere electron-transfer reactions between cytochrome *c* and small, inorganic complexes with a low driving force. This permits us to analyse the kinetics of these processes in both directions. We are interested in the associated activation parameters ($\Delta H^{\neq}$, $\Delta S^{\neq}$, $\Delta V^{\neq}$) for such processes, and in particular, their pressure dependence. The application of high-pressure techniques in mechanistic studies of outer-sphere electron-transfer systems reveals further information on the intimate mechanism of these processes. The activation volumes thus obtained can be combined with the overall reaction volume, obtained from partial molar volume measurements, or electrochemical and spectrophotometric measurements as a function of pressure, to construct a volume profile for the overall process. Such a volume profile presents a detailed description of the chemical process in terms of volume changes along the reaction coordinate.[7-9]

We have previously reported[10, 11] a detailed volume profile analysis for the electron-transfer reactions between cytochrome *c* and a series of pentaamminepyridineruthenium complexes. In these studies we clearly showed that the main volume changes that occur during the redox reaction are caused by electrostriction effects on the metal-ammine complex, whereas cytochrome *c* itself showed only a small volume change during the redox process. This was further demonstrated by electrochemical measurements as a function of pressure,[12] from which it followed that the reduction of ferricytochrome *c* is accompanied by a small volume collapse of -5 ± 1 cm^3 mol^{-1}. The kinetic experiments[10, 11] indicated that the position of the transition state showed no significant trend in the low driving force range from -0.011 to -0.112 eV. The transition state was always located halfway between the reactant and the product states on a volume basis. This position was also unaffected by substituents on the pyridine ligand.[11] From the Marcus theory, the λ^+ parameter, which represents the location of the transition state relative to reactants and products along the reaction coordinate ($0 \le \lambda^{\neq} \le 1$),[10, 13] can be calculated. λ^+ could be estimated for low driving force systems using reorganisation energies published previously.[14] The calculated values of λ^+ for these systems were very close to 0.5 and in good agreement with experimental data. These were the first studies in which it was possible to compare the theoretical $\lambda^{\neq}$ value with experimental results in terms of activation and reaction volume data.[10, 11]

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It was suggested that the electron-transfer pathway in these reactions proceeds from the ruthenium centre by way of the pyridine ring to the exposed heme edge.[15] We concluded from our data[11] that the exposed heme edge is accessible, since no steric requirements for the different pyridine systems were observed. However, it was found that the electronic transmission coefficient was up to 10 times larger for complexes with pyridine ligands, since these can effectively penetrate the heme groove.[11]

We have now studied the reactions of $[Co(bpy)_3]^{3+/2+}$ and $[Co(phen)_3]^{3+/2+}$ (bpy = 2,2′-bipyridine, phen = 1,10-phenanthroline) with cytochrome $c^{II/III}$ as a function of temperature and pressure in both directions of electron transfer. The corresponding reaction between cytochrome c and $[Co(terpy)_2]^{3+/2+}$ (terpy = 2,2′:6′,2″-terpyridine) was studied previously.[16] The advantage of the cobalt complexes compared to the ruthenium complexes is their higher stability in aqueous solution. The ruthenium complexes used in the earlier studies[10, 11] are sensitive to oxygen and light, and the Ru^{III} complexes tend to disproportionate at pH 7. Furthermore, coordinated pyridine can be displaced if chloride or other potential ligands are present.[15] Another advantage of the cobalt complexes is the availability of X-ray data for all the complexes, which enables more quantitative calculations than in the case of the ruthenium complexes. In addition, these complexes can be isolated in both oxidation states so that partial molar volume measurements can be performed. Electrostriction effects (which control solvent reorganization) are predicted to be significantly different for the selected complexes than for the pentaammineruthenium complexes studied previously.[10, 11] The driving force for the reactions studied varies from 0.003 eV for the oxidation of the protein by $[Co(terpy)_2]^{3+}$ to −0.085 eV for the oxidation by $[Co(phen)_3]^{3+}$.[17] Therefore, the redox process can be studied in both directions.

The activation volumes for the reaction of $[Co(phen)_3]^{3+}$ with cytochrome $c^{II/III}$ were reported in an earlier study to be +8.5 and −11.5 $cm^3 mol^{-1}$ for the oxidation and the reduction of the protein, respectively.[17] An overall reaction volume of 20 $cm^3 mol^{-1}$ for the oxidation of the protein was reported.[17] Since it is known that the volume contribution from cytochrome c during this reaction is very small,[12] the reaction volume of +20 $cm^3 mol^{-1}$ should be assigned to the reduction of the $[Co(phen)_3]^{3+}$ complex.

However, this value differs significantly from the data reported for the $[Co(terpy)_2]^{3+}$ complex.[17] Therefore we repeated the kinetic measurements and also performed density measurements, as well as spectrophotometric and electrochemical measurements as a function of pressure, to obtain the overall reaction volume. On the basis of our results we can propose a detailed mechanism and clarify the apparent discrepancy.

Results and Discussion

General comments: The redox reactions of cytochrome c with several metal complexes have been studied extensively.[4, 5, 18, 19] The main topic of interest was the oxidation of the protein, because the relatively high driving force of these reactions results in an unfavourable equilibrium situation for the investigation of the reverse reaction, reduction of the protein. In our studies we selected systems with a relatively small driving force ($\Delta G^\circ \leq 0.12$ eV), that is, an equilibrium constant (K) relatively close to unity, which enabled us to follow the electron-transfer process in both directions.

In earlier studies[10, 11] we demonstrated that the second-order rate constants for the reaction of a series of complexes of the type $[Ru(NH_3)_5X]^{3+/2+}$ with cytochrome $c^{II/III}$ can be correlated with the overall equilibrium constant. Thus, it is possible to follow these reactions almost independently from each other, even if there is an unfavourable equilibrium situation for the reduction of the protein. The reaction scheme for such a reversible process involving the investigated cobalt complexes is given in Equation (1), where k_f and k_b are the second-order rate

$$\text{cyt } c^{II} + [CoL_3]^{3+} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{cyt } c^{III} + [CoL_3]^{2+} \quad (1)$$

constants for the forward and back reactions, respectively. The following abbreviations are used: L = phen (1,10-phenanthroline) and bpy (2,2′-bipyridine). The driving force for these reactions is −0.028 and −0.085 eV for the reduction of $[Co(bpy)_3]^{3+}$ and $[Co(phen)_3]^{3+}$, respectively. This means that we are, in terms of the Marcus theory, working in the low driving force regime. The driving force was calculated from the difference in the redox potentials of cytochrome c and the cobalt complex determined for the medium used in this study.

Kinetic Results: The forward and reverse reactions for both systems were studied as a function of temperature (20 to 40 °C) and pressure (0.1 to 150 MPa for the bpy and 0.1 to 100 MPa for the phen system) using stopped-flow techniques. The kinetic data are reported as Supplementary Material. The second-order rate constants and activation parameters are reported in Table 1. Figure 1 shows the plots of k_{obs} versus concentration of the complex for both the forward and back reactions (top and bottom, respectively). For the oxidation of the protein there is a very small intercept (Figure 1, top) whereas for the reduction (Figure 1, bottom) there is a larger intercept due to the influence of the reverse reaction. The second-order rate constants were determined from the slope of the k_{obs} versus complex concentration plots.

Under the selected conditions we obtained values of 582 ± 13 and $169 \pm 5\ M^{-1} s^{-1}$ for k_f and k_b respectively, for the reaction of cytochrome $c^{II/III}$ with $[Co(bpy)_3]^{3+/2+}$ (at 25 °C and 0.1 M ionic strength). For the corresponding reaction of the protein with $[Co(phen)_3]^{3+/2+}$, we obtained the rate constants 3750 ± 40 and $217 \pm 5\ M^{-1} s^{-1}$ for k_f and k_b, respectively. The reactions were found to be independent of pH in the range 6.5 to 7.5.

For the oxidation and reduction of the protein by $[Co(bpy)_3]^{2+/3+}$, the following activation parameters were obtained: $\Delta H^{\neq} = 28 \pm 1$ kJ mol^{-1}, $\Delta S^{\neq} = -107 \pm 5$ J $K^{-1} mol^{-1}$ and $\Delta H^{\neq} = 49.9 \pm 0.7$ kJ mol^{-1}, $\Delta S^{\neq} = -28 \pm 2$ J $K^{-1} mol^{-1}$, respectively. For the corresponding reactions of the protein with $[Co(phen)_3]^{3+/2+}$ we found: $\Delta H^{\neq} = 14 \pm 1$ kJ mol^{-1}, $\Delta S^{\neq} = -136 \pm 4$ J $K^{-1} mol^{-1}$ and $\Delta H^{\neq} = 44 \pm 3$ kJ mol^{-1}, $\Delta S^{\neq} = -28 \pm 9$ J $K^{-1} mol^{-1}$, respectively (Table 1).

The oxidation of the protein (forward reaction) is significantly decelerated, whereas the reverse reaction is significantly accelerated by increasing pressure. Under the conditions used, acti-

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Table 1. Summary of rate and activation parameters for the electron-transfer reaction between cytochrome *c* and ruthenium or cobalt complexes: cyt c^{II} + complex^{3+} ⇌ cyt c^{III} + complex^{2+}.

Reaction	k, $M^{-1}s^{-1}$ [a]	$\Delta H^{\neq}$, $kJ\,mol^{-1}$	$\Delta S^{\neq}$, $J\,K^{-1}mol^{-1}$	$\Delta V^{\neq}$, $cm^3\,mol^{-1}$	ΔV, $cm^3\,mol^{-1}$	$-\Delta G$, eV	K_E [b]	K_{TD} [b]	K_{KIN} [b]
$[Ru a_5 py]^{3+}$ + cyt c^{II} [c]	48620±1161	28±1	−64±5	+17.4±1.5	33.4±1.9 [e]	0.045	5.8	6.4±2.1	4.6±0.4
$[Ru a_5 py]^{3+}$ + cyt c^{II} [d]	5960	33.4	−58.5						
$[Ru a_5 py]^{2+}$ + cyt c^{III} [c]	10517±494	33±4	−59±13	−17.7±0.8	35.1±1.0 [f]				
$[Co(bpy)_3]^{3+}$ + cyt c^{II}	582±13	49.9±0.7	−28±2	12.5±0.9	21.8±0.7 [e]	0.028	3.0	3.3±0.4	3.4±0.3
$[Co(bpy)_3]^{2+}$ + cyt c^{III}	169±5	28±1	−107±5	−12.6±1.5	25.1±1.7 [f]				
					27.5±1.4 [g]				
$[Co(phen)_3]^{3+}$ + cyt c^{II}	3753±39	44±3	−28±9	17.0±0.9	37.9±2.0 [e]	0.085	32	20 ±3	17.3±0.6
$[Co(phen)_3]^{2+}$ + cyt c^{III}	217±5	14±1	−136±4	−16.2±1.0	34.2±1.7 [f]				
					35.4±2.0 [g]				
$[Co(terpy)_2]^{3+}$ + cyt c^{II}	1427±36	40±1	−47±4	18.4±1.2	33 ±3 [e]	−0.003	0.9	0.7±0.2	0.9±0.1
$[Co(terpy)_2]^{2+}$ + cyt c^{III}	1704±46	14±1	−136±4	−18.0±1.4	36 ±2 [f]				
					36.3±2.0 [g]				

[a] Reaction conditions: $T = 25\,^{\circ}C$, $\mu = 0.1\,M$, [cyt *c*] = $1 \times 10^{-5}\,M$, [Tris] = 0.05 M, $[LiClO_4]/[LiNO_3]$ = 0.05 M, pH = 7.1, λ = 550 nm. [b] Equilibrium constant for the oxidation of cytochrome (K_E calculated from the redox potential, K_{TD} from spectroscopic measurements, K_{KIN} from kinetic measurements). [c] Ref. [11]. [d] pH = 5.3, acetate, μ = 0.1 M, ref. [15]. [e] Reaction volume determined spectrophotometrically for the oxidation of cytochrome *c*. [f] Reaction volume determined kinetically for the oxidation of cytochrome *c*. [g] Reaction volume determined electrochemically, assuming cytochrome contribution is ≈0.

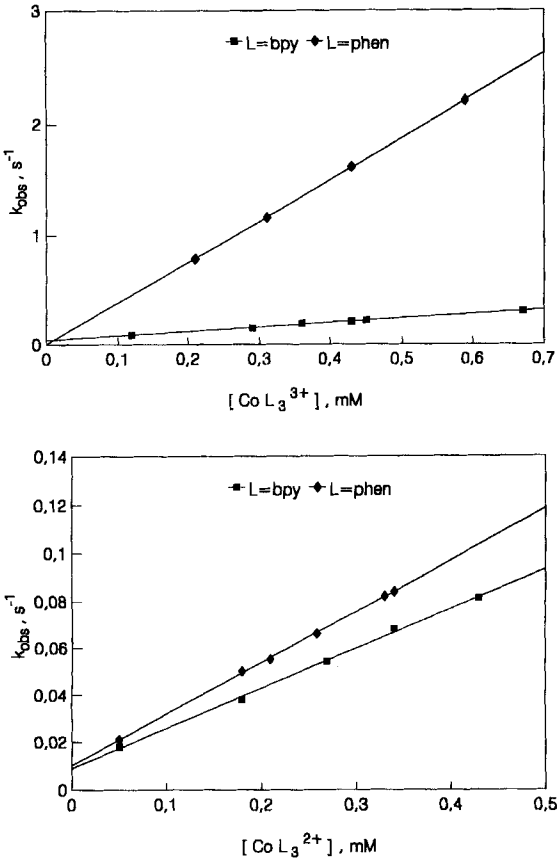

Fig. 1. Concentration dependence of k_{obs} for the oxidation (top) and reduction (bottom) of cyt $c^{II/III}$ by $[Co(bpy)_3]^{3+/2+}$ and $[Co(phen)_3]^{3+/2+}$. Experimental conditions: [cyt $c^{II/III}$] = 1.0×10^{-5} M, pH = 7.2, ionic strength = 0.1 M, [Tris] = 0.05 M, $[LiNO_3]$ = 0.05 M, λ = 550 nm, $T = 25.0\,^{\circ}C$.

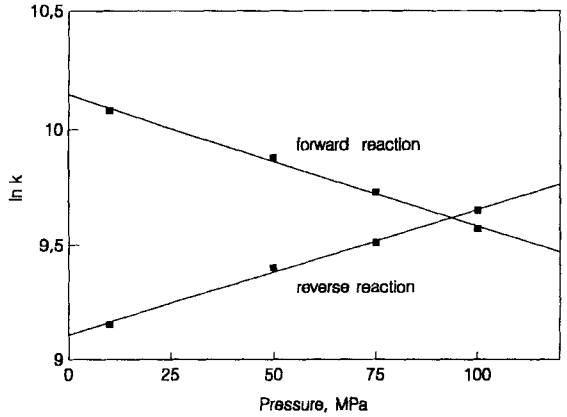

Fig. 2. Plot of ln *k* versus pressure for the reaction cyt c^{II} + $[Co(phen)_3]^{3+}$ ⇌ cyt c^{III} + $[Co(phen)_3]^{2+}$. For experimental conditions see Figure 1.

vation volumes of $+17.0 \pm 0.9$ and $-16.2 \pm 1.0\ cm^3\,mol^{-1}$ were found for the forward and reverse reactions, respectively, of the phen complex in Equation (1). These values were obtained from plots of ln *k* versus pressure. A typical example is given in Figure 2 for the reaction of the phen complex with cytochrome *c*. For the corresponding reactions of the bpy complex, activation volumes of $+12.5 \pm 0.9$ and $-12.6 \pm 1.5\ cm^3\,mol^{-1}$, respectively, were obtained.

Thermodynamic Results: The equilibrium constant for these processes can be calculated from the overall driving force, and they are 3.0 and 32 for the oxidation of cytochrome c^{II} by $[Co(bpy)_3]^{3+}$ and $[Co(phen)_3]^{3+}$, respectively.[18, 19] In addition, spectroscopic measurements were performed to estimate the equilibrium constants for these reactions. For both reactions we observed an increase in absorbance as a function of the cobalt complex concentration at 550 nm on mixing cytochrome c^{III} and cobalt(II) solutions. The difference in absorbance before and after mixing the solutions in a tandem cuvette can be used to estimate the overall equilibrium constant.[10, 11] This gave equilibrium constants of 3.3 ± 0.4 and 20 ± 3 for the reaction of the protein with $[Co(bpy)_3]^{3+}$ and $[Co(phen)_3]^{3+}$, respectively. These values are in good agreement with the equilibrium constants obtained from the kinetic data ($K = k_f/k_b$), viz. 3.4 ± 0.3 and 17.3 ± 0.6, respectively.

In order to confirm the activation volumes mentioned above, the overall reaction volume was determined from the pressure dependence of the equilibrium constant up to 150 MPa. In these

systems it is possible to adjust the concentrations in such a way that the UV/Vis spectrum showed characteristic spectral changes as a function of pressure (a typical example for the reaction of cytochrome $c^{II/III}$ with $[Co(phen)_3]^{3+/2+}$ is given in Figure 3). Comparison of the observed changes with the spectra

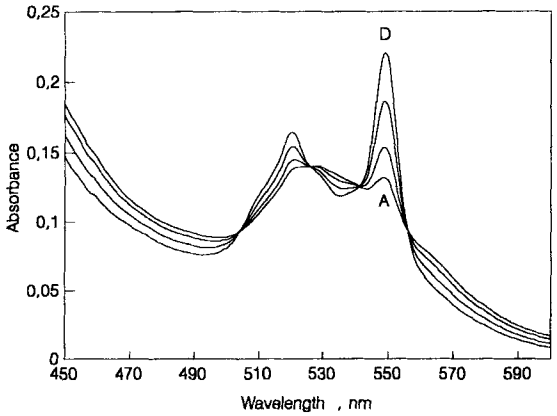

Fig. 3. UV/Vis spectra of an equilibrium mixture of cyt $c^{II/III}$ and $[Co(phen)_3]^{3+/2+}$ as a function of pressure. A = 5 MPa, B = 50 MPa, C = 100 MPa, D = 150 MPa. Experimental conditions: [cyt $c^{II/III}$] = 0.7×10^{-5} M, [$Co^{II/III}$] = 2.0×10^{-4} M, pH = 7.2, ionic strength = 0.1 M, [Tris] = 0.05 M, T = 25.0 °C.

of the reduced and oxidized protein, indicate that the equilibrium is shifted towards cytochrome c^{II} under pressure. The UV/Vis spectra of ferri- and ferrocytochrome c show no spectral changes as a function of pressure in the range 0–150 MPa. This is in agreement with earlier observations.[20] From the observed absorbance change as a function of pressure, the equilibrium constant can be calculated at each pressure. From the corresponding plot of ln K versus pressure the reaction volume can be determined from the slope (= $-\Delta V/RT$) (Figure 4). The spectroscopically determined reaction volumes are $+21.8\pm0.7$ and $+37.9\pm2.0$ cm^3 mol^{-1}, for L = bpy and phen, respectively.

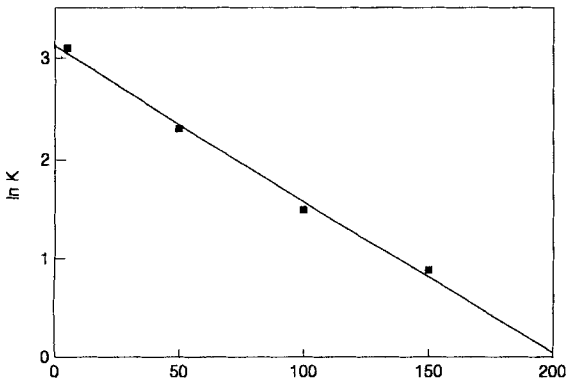

Fig. 4. Plot of ln K versus pressure for the reaction cyt c^{II} + $[Co(phen)_3]^{3+}$ ⇌ cyt c^{III} + $[Co(phen)_3]^{2+}$. For experimental conditions see Figure 3.

We also performed density measurements and determined the partial molar volumes of the cobalt complexes. By correcting for the volume contribution of the water molecules and anions present in the complexes, the volume of the cation could be calculated. The partial molar volumes of the anions were taken from the literature[21] on the basis of $V(H^+) = -4.5$ cm^3 mol^{-1}. The partial molar volumes of the complexes and cations are reported in Table 2. From these the expected volume contribution of the cobalt complexes during the redox reaction can be calculated to be 24.2 ± 2.4, 31.7 ± 2.2 and 37.9 ± 2.8 cm^3 mol^{-1} for the reduction of $[Co(bpy)_3]^{3+}$, $[Co(phen)_3]^{3+}$ and $[Co(terpy)_2]^{3+}$, respectively.

Table 2. Partial molar volume data determined from density measurements.

Compound	V, cm^3 mol^{-1} [a]	V_{cation}, cm^3 mol^{-1} [b]
$[Co(bpy)_3](ClO_4)_2$	461.1 ± 1.2	363.9 ± 1.2
$[Co(bpy)_3](ClO_4)_3\cdot2H_2O$	521.5 ± 2.1	339.7 ± 2.1
$[Co(terpy)_2]Br_2\cdot H_2O$	437.0 ± 1.9	360.7 ± 1.9
$[Co(terpy)_2](ClO_4)_3\cdot H_2O$	487.5 ± 2.1	322.8 ± 2.1
$[Co(phen)_3](ClO_4)_2\cdot H_2O$	484.1 ± 1.2	368.9 ± 1.2
$[Co(phen)_3](ClO_4)_3\cdot2H_2O$	519.1 ± 1.8	337.2 ± 1.8

[a] Average value from at least 5 measurements. [b] The following volumes were used: $V(ClO_4^-) = 48.9$ cm^3 mol^{-1}; $V(Br^-) = 29.2$ cm^3 mol^{-1}; $V(Cl^-) = 22.3$ cm^3 mol^{-1}; $V(H_2O) = 18$ cm^3 mol^{-1} ($V(H^+) = -4.5$ cm^3 mol^{-1}).

In addition, we determined the redox potentials of these complexes as a function of pressure using differential pulse voltammetry.[12] Typical voltammograms as a function of pressure for the $[Co(bpy)_3]^{2+/3+}$ system are given in Figure 5. The corresponding plot of E versus pressure is given in Figure 6. All plots

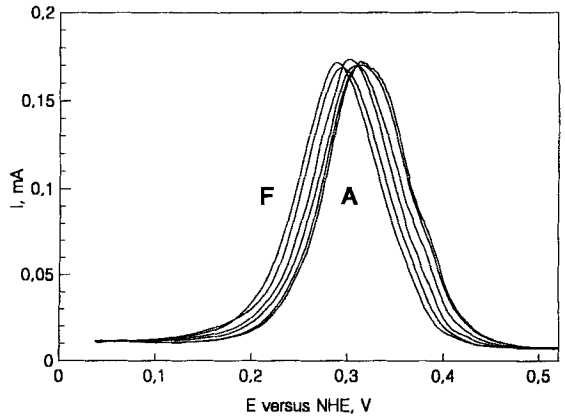

Fig. 5. Differential pulse voltammograms for the $[Co(bpy)_3]^{2+/3+}$ system recorded as a function of pressure. A = 10 MPa, B = 20 MPa, C = 50 MPa, D = 70 MPa, E = 110 MPa, F = 150 MPa.

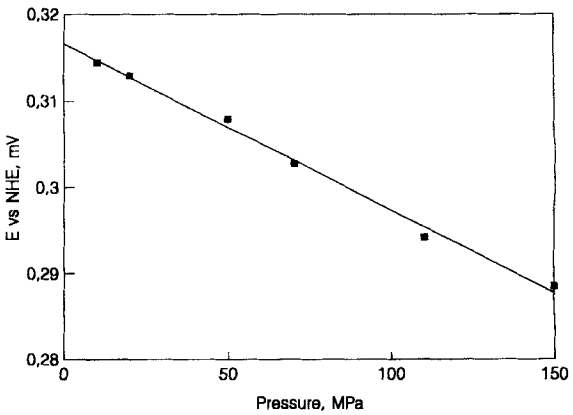

Fig. 6. Plot of E versus pressure for the $[Co(bpy)_3]^{2+/3+}$ system.

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were linear within the limits of experimental error. The reaction volume can be calculated from the slope of such plots (Supplementary Material). From these measurements we obtained reaction volumes of 18.5 ± 1.4, 26.4 ± 2.0 and $27.3 \pm 2\ cm^3\,mol^{-1}$ for the reduction of $[Co(bpy)_3]^{3+}$, $[Co(phen)_3]^{3+}$ and $[Co(terpy)_2]^{3+}$, respectively. We used a Ag/AgCl (sat'd KCl) reference electrode, which has previously been shown to contribute $\approx -9\ cm^3\,mol^{-1}$ to the overall reaction volume, so that the above values must be corrected.[12, 22] This results in values of 27.5 ± 1.4, 35.4 ± 2.0 and $36.3 \pm 2\ cm^3\,mol^{-1}$ for the reduction of $[Co(bpy)_3]^{3+}$, $[Co(phen)_3]^{3+}$ and $[Co(terpy)_2]^{3+}$, respectively. These values are in good agreement with those obtained from the partial molar volume data quoted above (see Table 3).

Table 3. Reaction volumes (in $cm^3\,mol^{-1}$) for the oxidation of cyt c^{II}, determined in different ways, in the overall reaction $[CoL]^{3+}$ + cyt $c^{II} \rightleftharpoons [CoL]^{2+}$ + cyt c^{III}.

System	ΔV_{kin}[a]	ΔV_{TD} [b]	ΔV_{PMV} [c]	ΔV_E [d]	average
$[Co(bpy)_3]^{3+/2+}$	25.1 ± 1.7	21.8 ± 0.7	24.2 ± 2.4	27.5 ± 1.4	24.6 ± 2.3
$[Co(phen)_3]^{3+/2+}$	34.2 ± 1.7	37.9 ± 2.0	31.7 ± 2.2	35.4 ± 2.0	34.8 ± 2.6
$[Co(terpy)_2]^{3+/2+}$	36.0 ± 2.0	33.0 ± 3.0	37.9 ± 2.8	36.3 ± 2.0	35.8 ± 2.0

[a] Kinetically determined value for the overall reaction between the cobalt complex and cytochrome *c* ($\Delta V = \Delta V_f^{\neq} - \Delta V_b^{\neq}$). [b] Spectrophotometrically determined value for the overall reaction between the cobalt complex and cytochrome *c*. [c] Difference in the partial molar volumes of the cobalt complex cations, neglecting the volume change on the protein (see Discussion). [d] Electrochemically determined value for the reduction of the cobalt complex cations, neglecting the volume change on the protein and corrected for the contribution from the reference electrode (see Discussion).

Mechanistic interpretation: For the investigated systems it was possible to correlate the equilibrium constants obtained from driving force and spectral measurements with those from kinetic experiments ($K = k_f/k_b$). This indicates that a good correlation between the kinetics and thermodynamics of these systems exists (compare data summarized in Table 1).

The activation parameters for the redox processes in both directions of reaction (1) showed the same systematic trends as observed in our earlier study of the reaction of cytochrome *c* with the pentaamminepyridineruthenium complexes.[10, 11] All activation entropies are significantly negative. However, the reduction of the protein is accompanied by a more negative activation entropy than that for the oxidation reaction. As shown earlier by Sutin,[23] in order to compare these values with other systems it is necessary to correct the entropies for the net reaction by using the correction term $\Delta S^{\circ}/2$. The corrected entropies are typical for an outer-sphere process between a metalloprotein and a metal complex. Due to a relatively large error in the absolute entropy value, which stems from the long extrapolation to the intercept ($1/T \approx 0$), we can only conclude that the negative activation entropy points to a highly structured transition state. This is different in the case of the activation and reaction volume data, since these are calculated from the slope of the plot of ln *k* or ln *K* versus pressure, respectively.

The volumes of activation clearly indicate that electrostriction effects play an important role in these processes. In all cases we observed a negative activation entropy for the oxidation of the protein, as mentioned above; however, the activation volume was positive (see Table 1). For the reverse step, both the activation entropy and the activation volume were negative. The activation volumes for the forward and back reactions can be combined to give the reaction volume for the overall process ($\Delta V = \Delta V_f^{\neq} - \Delta V_b^{\neq}$). For both systems we observed a good agreement between the reaction volume determined from spectral measurements as a function of pressure, and that calculated from the kinetic data.

A comparison of these volume data with the reaction volumes for the electrochemical reduction of the cobalt complexes and the difference in the partial molar volumes (Tables 1 and 3) clearly shows that the main volume changes observed in the redox reaction between the metal complexes and cytochrome *c* occur on the metal centre. The contribution arising from the oxidation/reduction of cytochrome *c* is so small that it can practically be ignored in the overall reaction volume.[12] This means, in terms of volume changes along the reaction coordinate, that the reaction volume for the oxidation of the protein is positive due to a decrease in solvent electrostriction and an increase in intrinsic volume during the reduction of the cobalt complex. Using the available volume data, it is possible to construct a volume profile which illustrates the volume changes during this redox process along the reaction coordinate. Figure 7 shows a typical volume profile for the reaction of cytochrome *c* with $[Co(phen)_3]^{3+/2+}$. The transition state is located exactly halfway between the reactant and product states on a volume basis, in agreement with the predicted λ^{+} value from the Marcus theory for a system with a low driving force.

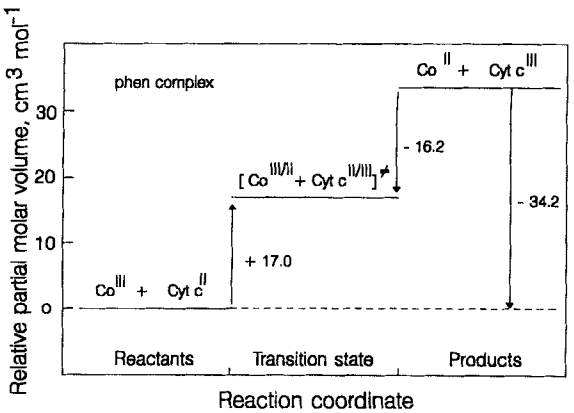

Fig. 7. Volume profile for the overall reaction cyt c^{II} + $[Co(phen)_3]^{3+} \rightleftharpoons$ cyt c^{III} + $[Co(phen)_3]^{2+}$.

We observed a volume increase for the forward step in reaction (1). This was caused by the reduction of Co^{III} to Co^{II}, which is accompanied by a decrease in solvent electrostriction. In the case of the ruthenium complexes, the volume changes could be attributed to solvation effects only, since the change in the metal to ligand bond length during the redox process is very small (ca. 0.01 Å) and can be neglected.[24] For the cobalt complexes, however, this contribution cannot be neglected. The phen and bpy complexes show a metal to ligand bond length change during the redox reactions of 0.2 Å for all bonds, whereas the terpy complex showed different metal to ligand bond length changes, probably due to the high-spin/low-spin equilibrium of the $[Co(terpy)_2]^{2+}$ complex. The average bond length change during the reaction is also 0.2 Å in this case.[16] It is interesting that

the ruthenium complexes show almost the same reaction volume during the redox process.[11, 12] Nevertheless, these two types of complex do behave differently due to the basic difference in the selected ligands. We conclude that the main volume changes in the case of the cobalt complexes arise from a combination of intrisic as well as solvation effects.

For the $[Co(bpy)_3]^{2+/3+}$ complexes we observe smaller volume effects compared with the phen and terpy complexes. This difference could be caused by the more flexible bpy ligand, since both pyridine rings are connected by a single C–C bond, which allows partial rotation of the pyridine rings and a twisted conformation in the metal complex in solution. These conformational changes are not possible to such an extent in the case of the phen and terpy ligands. From a comparison of the partial molar volumes of the terpy and bpy complexes, it follows that the Co^{II} complexes exhibit almost the same volume. However, the Co^{III} complex of bpy is significantly larger than the phen complex, which indicates that the electrostriction in the bpy complex is smaller. This difference can be attributed to electrostriction effects only, since the changes in the metal to ligand bond length are almost the same for all cobalt complexes. In the twisted conformation of the bpy complex, the solvent molecules are presumably shielded more from the Co^{III} centre and so result in a larger partial molar volume for this complex.

The reported volume profiles (see $\Delta V^{\neq}$ and ΔV data in Table 1) for the series of cobalt complexes studied to date are all very symmetric in terms of the volume changes associated with the forward and reverse reactions. The nature of the chelate ligands (bpy, terpy, phen) does not seem to have a marked influence on the efficiency of the electron-transfer process. We conclude that chelate penetration into the groove of the heme is very similar in all three complexes.

Comparison with literature data: The rate constant found in the present study for the oxidation of the protein by $[Co(phen)_3]^{3+}$ is larger by a factor of about 2.5 than that reported by Gray et al. ($1500\,M^{-1}s^{-1}$ at 25 °C, 0.1 M ionic strength).[25] In contrast to our investigations, they performed the measurements in a chloride medium using phosphate or tris buffer. They reported that the reaction is somewhat slower in phosphate than in tris buffer, which is in agreement with our observations. It is known that phosphate and chloride bind to cytochrome *c* and can result in a smaller net charge on the reactants,[26, 27] which may affect the rate accordingly. In order to check this difference in rate constants, we performed measurements in a chloride medium and were able to reproduce their data. This is in agreement with our results reported previously for the cytochrome $c^{II/III}/[Co(terpy)_2]^{3+/2+}$ system, where the rate constants for the reaction in both directions were about two times lower in a chloride than in a nitrate medium.[17] This clearly shows that reactions involving cytochrome *c* and positively charged metal complexes are not only dependent on the ionic strength but also on the medium. The rate constant is affected by the salt used to adjust the ionic strength and the type of buffer employed.

From a comparison of the corresponding activation parameters for this reaction studied in different media, it follows that the salt selected to adjust the ionic strength and the type of buffer employed affect the rate constants but not the activation parameters ($\Delta H^{\neq}$, $\Delta S^{\neq}$) to a significant extent. A similar finding was reported by Swaddle et al.[28] for the activation volume for the self-exchange reaction of $[Co(phen)_3]^{2+/3+}$ in nitrate and chloride media. We therefore intended to perform the measurements in a lithium perchlorate medium in order to minimize the effect of binding of the anion to ferrocytochrome *c*. Unfortunately, this was not possible because the solubility of the cobalt complexes in a perchlorate medium was too low, and we therefore used a nitrate medium. The activation parameters for the oxidation of the protein by $[Co(phen)_3]^{3+}$ determined in nitrate medium/tris buffer are in excellent agreement with the values reported by Gray and coworkers for a chloride medium at the same ionic strength.[25]

A comparison of our activation parameters for the cytochrome $c^{II/III}/[Co(phen)_3]^{3+/2+}$ with the values reported in the earlier study by Heremans et al. indicates some significant differences.[17] Heremans et al. reported values of +8.5 and $-11.5\,cm^3\,mol^{-1}$ for the oxidation and the reduction of the protein, respectively. A comparison of these results with our data indicates that both sets of data show the same trend, a transition state approximately halfway between the reactant and product states. We found a value of -16.2 ± 1.0 for the reduction of the protein, and a value of $+17.0\pm0.5\,cm^3\,mol^{-1}$ for the oxidation. Therefore, the results only differ in the absolute size of the volume effect. Heremans et al. performed their measurements in a Na_2SO_4 medium (ionic strength 0.2 M).[17] There is expected to be a stronger binding of the sulfate anion to the +6.5/ +7.5 charged protein and/or the +2/ + 3 charged cobalt complex than in the case of the nitrate ion. This can affect the net charge of the reactants and result in a smaller activation volume. From electrochemical measurements it is known that the volume contribution arising from cytochrome *c* strongly depends on the salt used to adjust the ionic strength.[12, 29] If the electrochemical measurements are performed in a perchlorate medium, the oxidation of the protein is accompanied by a small volume increase of $5\,cm^3\,mol^{-1}$. In a chloride medium the volume change is much larger than first reported by Sligar et al.[29] and later confirmed by our own work.[12] Thus a direct comparison is restricted to results from experiments performed in the same medium.

From the results reported in this and in an earlier paper[12] it is reasonable to assume that the reduction of the cobalt–phenanthroline complex is accompanied by a volume increase of ca. $34\,cm^3\,mol^{-1}$. In order to resolve the apparent discrepancy with Heremans' data,[17] we performed spectroscopic measurements as a function of pressure in a sulfate medium. The absorbance changes as a function of pressure in a sulfate/tris medium (ionic strength 0.2 M) could be used to calculate a reaction volume of $22.2\pm0.8\,cm^3\,mol^{-1}$ for the oxidation of the protein by the cobalt–phenanthroline complex. This value is in good agreement with the value of $20\,cm^3\,mol^{-1}$ reported by Heremans et al.,[17] indicating that the difference in the data must arise from the different salts used to adjust the ionic strength.

Very recently, after completion of this work, Tregloan et al.[30] reported a detailed analysis of the intrinsic and electrostriction volume effects associated with the reduction of a series of metal complexes observed with high-pressure cyclic voltammetry. They found that, in the case of the $[Co(phen)_3]^{3+/2+}$ and $[Co(bpy)_3]^{3+/2+}$ systems, about two-thirds

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of the overall reduction volume was associated with changes in electrostriction, whereas one-third was associated with intrinsic volume changes. These conclusions are in good agreement with arguments presented in this report.

Theoretical calculations: The reactions of cytochrome *c* with inorganic metal complexes are known to be of the outer-sphere type.[31, 32] The mechanism outlined in reaction (2) includes the precursor formation step (K_1) due to weak electrostatic interaction between the reactants that depends on the charge and the size of the reactants. This step is followed by the rate-determining electron-transfer step (k_{ET}) to form the successor complex, which dissociates in the final step (K_2).

$$[\text{cyt } c^{II}] + [CoL_3]^{3+} \rightleftharpoons [\text{cyt } c^{II} \cdot CoL_3^{3+}] \qquad K_1 \qquad (2a)$$

$$[\text{cyt } c^{II} \cdot CoL_3^{3+}] \longrightarrow [\text{cyt } c^{III} \cdot CoL_3^{2+}] \qquad k_{ET} \qquad (2b)$$

$$[\text{cyt } c^{III} \cdot CoL_3^{2+}] \rightleftharpoons \text{cyt } c^{III} + [CoL_3]^{2+} \qquad K_2 \qquad (2c)$$

$$k_{obs} = k_{ET} K_1 [CoL_3^{3+}] / \{1 + K_1 [CoL_3^{3+}]\} \qquad (3)$$

$$k_{obs} = k_{ET} K_1 [CoL_3^{3+}] \qquad (4)$$

The pseudo-first-order rate constant is given by Equation (3), which reduces to Equation (4) at low complex concentration and/or low K_1 values. This means that the second-order rate constant, determined from the slope of a linear plot of k_{obs} versus the cobalt complex concentration (see Figure 1), is equal to the product of the precursor equilibrium constant (K_1) and the electron-transfer rate constant (k_{ET}).

If the precursor formation constant (K_1) is large, its value can be determined from the saturation observed in the kinetic plot mentioned. For the system $[Fe(CN)_6]^{3-/4-}$/cytochrome $c^{II/III}$, a K_1 value of 285 M^{-1} was determined from NMR experiments.[19] This value is in very good agreement with the value estimated from the Fuoss equation,[33] indicating that this theoretical approach can be used for this type of reaction. In systems where the reactants have a positive charge, for example cytochrome $c^{II/III}$ and $[Co(phen)_3]^{3+/2+}$, the K_1 value is expected to be small and can only be estimated using the Fuoss equation [Eq. (5)]. In Equations (5) and (6), w_{ij} is the electrostatic

$$K_1 = 4/3\,\pi N_A \sigma_{12}^3 \exp(-w_{12}/RT) \qquad (5)$$

$$w_{ij} = z_i z_j e_o^2 N_A / 4\pi\varepsilon_o \varepsilon \sigma_{ij} (1 + \kappa\sigma_{ij}) \qquad (6)$$

work required to bring reactants and products together, z_i and z_j are the charges of the reacting ions, e_o is the electronic charge, ε_o the permittivity of vacuum, ε the bulk dielectric constant, σ_{ij} the contact distance of the ions ($\sigma_{ij} = r_i + r_j$) and κ the reciprocal Debye–Hückel length. For aqueous solutions at 25 °C, $\varepsilon = 78.5$ and $\kappa = 3.29\,\mu^{0.5}\,nm^{-1}$, with the ionic strength μ in M.[34, 35]

In order to calculate the precursor formation constant, rate constant and activation volume for the redox reactions between cytochrome $c^{II/III}$ and the metal complexes under the experimental conditions selected in this study, the following values were used: for $[Co(phen)_3]^{2+/3+}$: $E_{11}^{\circ} = 0.358$ V,[36] radii: 5.27/5.11 Å.[37] For $[Co(bpy)_3]^{2+/3+}$: $E_{11}^{\circ} = 0.301$ V,[36] radii: 5.24/5.13 Å.[37] For cytochrome $c^{II/III}$: $E_{22}^{\circ} = 0.273$ V,[10, 38] radii 16.6 Å,[39] charges +7.5/ + 6.5.[39]

Using Equations (5) and (6) we obtained values for the precursor formation constant K_1 of 3.6 M^{-1} and for the successor dissociation constant K_2 of 0.17 M at 25 °C and ionic strength 0.1 M. With this K_1 value it is obvious why $1 + K_1[CoL_3] \approx 1$, the assumption made in Equation (4). Furthermore, with these values it is possible to estimate the rate constant for the electron-transfer step within the precursor and successor complexes. For the reactions of the bpy complex, we calculated a k_{ET} value of 162 (forward reaction) and 29 s^{-1} (reverse reaction), and 1043 and 37 s^{-1} for the corresponding reactions of the phen complex, respectively.

The Marcus–Hush–Stranks–Swaddle relationships,[40–47] can be used to calculate the activation volume for these reactions. Earlier studies[10, 11] on the pentaamminepyridineruthenium complexes showed that the calculated value for both processes were too negative. We reported that for this type of reaction, the coulombic and Debye–Hückel terms compensate each other and only the contributions from solvent reorganisation $\Delta V_{SR}^{\pm}$ and the $\lambda^{\pm}\Delta V$ term determine the calculated value. Since the main volume changes occur on the metal complex and not on cytochrome *c*,[12] the real $\Delta V_{SR}^{\pm}$ value was expected to be smaller than the calculated one. For both reactions the calculated activation volumes are too negative (bpy: $\Delta V^{\pm}(k_f) = 8.8\ cm^3\,mol^{-1}$, $\Delta V^{\pm}(k_b) = -15.2\ cm^3\,mol^{-1}$; phen: $\Delta V^{\pm}(k_f) = 14.8\ cm^3\,mol^{-1}$, $\Delta V^{\pm}(k_b) = -21.1\ cm^3\,mol^{-1}$) and indicate an early transition state for this kind of process. This is not in agreement with the $\lambda^{\pm}$ value from the Marcus theory and from the experimental results, since the value should be 0.5 for a low driving force system. If the volume changes on cytochrome *c* are neglected, we can correct the $\Delta V_{SR}^{\pm}$ term to be only 24% (ratio of the radii of cytochrome *c* and the cobalt complexes) of the calculated value ($\Delta V_{SR}^{\pm} = -1.1\ cm^3\,mol^{-1}$). Using this correction, the experimental value is in excellent agreement with the theoretical value (bpy: $\Delta V^{\pm}(k_f)_{corr} = 12.3\ cm^3\,mol^{-1}$, $\Delta V^{\pm}(k_b)_{corr} = -11.7\ cm^3\,mol^{-1}$; phen: $\Delta V^{\pm}(k_f) = 18.3\ cm^3\,mol^{-1}$, $\Delta V^{\pm}(k_b)_{corr} = -17.7\ cm^3\,mol^{-1}$), indicating that our assumption is correct.

From our measurements, it follows that the contribution from cytochrome *c* is very small ($< 5\ cm^3\,mol^{-1}$) and can be neglected in the calculations. Very similar volume effects were obtained from kinetic, spectrophotometric, electrochemical and density measurements. This proves that the different methods complement one another and indicates an excellent agreement between the thermodynamic and kinetic parameters for these reactions.

Experimental Section

Materials: Horse heart cytochrome *c* (Sigma) solutions were prepared according to a standard procedure as described previously [10]. The concentration of these solutions was determined from the extinction coefficients ($\varepsilon = 27600\ M^{-1}cm^{-1}$ for ferrocytochrome *c* and $\varepsilon = 9100\ M^{-1}cm^{-1}$ at 550 nm for ferricytochrome *c*) [48]. All cobalt complexes were prepared and purified according to methods reported in the literature [49–52]. The chemical analyses, redox potentials and UV/Vis spectra were in good agreement with those reported in the literature [49–52]. All solutions were prepared with deionized, argon-saturated Millipore water. In this study we used 0.05 M Tris buffer (Sigma), pH 7.3, and 0.05 M $LiNO_3$ (Fluka) as reaction medium. All chemicals used were of analytical grade and were used without further purification. All solutions were kept under argon to avoid complications caused by dissolved oxygen. The solutions were transferred into the instruments using gas-tight Hamilton syringes.

Measurements: Hewlett Packard HP8452 and Cary1 (Varian) spectrophotometers were used for recording UV/Vis spectra at ambient and elevated pressure, respectively. The latter instrument was equipped with a high-pressure cell which enabled us to record spectra as a function of pressure up to 150 MPa [53]. The reactions were followed by monitoring the absorbance at 550 nm. The kinetic experiments were performed on a Durrum D110 stopped-flow and on a home-made high-pressure stopped-flow system [54]. All instruments were kept at constant temperature (within $\pm 0.1\,^{\circ}C$). The data were recorded on an IBM-compatible computer using Biologic software. The subsequent calculations were performed using Olis Kinfit Programs (Bogart, Georgia). Differential pulse voltammetry (DPV) as well as cyclic voltammetry (CV) were performed on a EG&G PAR 263 system. For all DPV measurements the scan rate was $5\,mV\,s^{-1}$. For the measurements at elevated pressure, we constructed a high-pressure cell according to information given in the literature [55]. For all DPV measurements a gold wire working electrode, a Pt wire counter electrode and a Ag/AgCl (sat'd. KCl) reference electrode were used [12]. All measurements were performed at $25.0 \pm 0.1\,^{\circ}C$.

The partial molar volumes were determined from density measurements using an Anton Paar (Graz, Austria) precision densitometer. The temperature of this instrument was held constant at $25.000 \pm 0.001\,^{\circ}C$ by a circulation bath, controlled by a Hewlett Packard precision thermometer.

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Supplementary material available from the authors: Tables with k_{obs} data as a function of concentration, temperature and pressure for the redox reactions of $[Co(bpy)_3]^{2+/3+}$ and $[Co(phen)_3]^{2+/3+}$ with cytochrome $c^{III/II}$ (4 pages). Table with redox potentials as a function of pressure for $[Co(bpy)_3]^{2+/3+}$, $[Co(phen)_3]^{2+/3+}$ and $[Co(terpy)_2]^{2+/3+}$ (1 page).

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