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Kinetics of the reduction of cytochrome *c* by $[Fe^{II}(edta)(H_2O)]^{2-}$: outer-sphere *vs.* inner-sphere electron transfer mechanisms †

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The reduction of horse heart cytochrome c^{III} by $[Fe^{II}(edta)(H_2O)]^{2-}$

$$[Fe^{II}(edta)(H_2O)]^{2-} + [\text{cytochrome } c^{III}]^{7+} \longrightarrow [Fe^{III}(edta)(H_2O)]^{-} + [\text{cytochrome } c^{II}]^{6+}$$

was reinvestigated as a function of all chemical and physical variables. Possible electrostatic interaction between the oppositely charged redox partners can assist the electron transfer process. Rate and activation parameters determined in the study are discussed in reference to those reported in the literature and compared with theoretical calculations based on the Marcus–Hush theory for outer-sphere electron transfer reactions. The reaction can also be interpreted in terms of an inner-sphere mechanism due to the presence of a very labile water ligand on $[Fe^{II}(edta)(H_2O)]^{2-}$. Experiments performed at elevated pressure reveal additional mechanistic information in terms of volume changes associated with the electron transfer process.

Introduction

The significant progress made in the understanding of electron transfer reactions is reflected by numerous monographs and review articles that have appeared during the last decade.[1–5] Redox processes of biologically active substances, for instance cytochrome *c* or myoglobin, deserve special attention. Several electron transfer reactions between transition metal complexes and such proteins were studied as intra- and intermolecular processes using different kinetic and thermodynamic techniques.[6–19]

In an earlier study of the electron transfer reaction between $[Fe^{II}(edta)(H_2O)]^{2-}$ and horse heart cytochrome c^{III}, the kinetic data were analysed in terms of an outer-sphere electron transfer mechanism for reaction (1).[20]

$$[Fe^{II}(edta)(H_2O)]^{2-} + [\text{cytochrome } c^{III}]^{7+} \longrightarrow [Fe^{III}(edta)(H_2O)]^{-} + [\text{cytochrome } c^{II}]^{6+} \quad (1)$$

In the present study the electron transfer process described by reaction (1), is also considered in terms of an inner-sphere mechanism, due to the presence of a very labile water molecule in the seven-coordinate $[Fe^{II}(edta)(H_2O)]^{2-}$ complex and the availability of suitable potential binding sites on the surface of the protein. In the case of either an intermolecular or intramolecular electron transfer process, it is very useful to study redox systems for which a kinetic separation of the precursor formation constant (outer-sphere or inner-sphere) and the subsequent electron transfer rate constant, *i.e.*, K and k_{et} in reaction (2), respectively, is possible.

Earlier studies in our laboratories demonstrated that through the selection of suitably charged redox partners, properties can be created to reach this goal for intermolecular redox processes that involve significant ion-pair formation.[21–24] In the case of an intramolecular process, the lability of one of the redox partners and the presence of a bridging ligand on the other redox partner will control the extent of precursor (bridged complex) formation. According to the rate law given in eqn. (3) for the reaction sequence in reaction scheme (2), independent of whether an outer-sphere or inner-sphere precursor is formed, the kinetic separation of K and k_{et} is in principle possible if K values are high enough, which in turn depends on the characteristics of the redox partners.

$$[Fe^{II}(edta)H_2O]^{2-} + \text{cyt } c^{III}]^{7+} \underset{\text{fast}}{\overset{K}{\rightleftharpoons}} \{[Fe^{II}(edta)H_2O]^{2-} \text{ - cyt } c^{III}]^{7+}\} \quad (2)$$

$$\downarrow k_{et}$$

$$\{[Fe^{III}(edta)H_2O]^{-} \text{ - cyt } c^{II}]^{6+}\}$$

$$\updownarrow \text{fast}$$

$$[Fe^{III}(edta)H_2O]^{-} + \text{cyt } c^{II}]^{6+}$$

$$k_{obs} = \frac{k_{et}[Fe^{II}(edta)^{2-}]}{1 + K[Fe^{II}(edta)^{2-}]} \quad (3)$$

Under such conditions saturation kinetics can be observed and the limiting observed rate constant is then given by $k_{obs} = k_{et}$, *i.e.* the kinetic parameters for the electron transfer process itself can be directly measured. This could in principle also be the case for the reaction between the positively charged cytochrome *c* and negatively charged metal complexes.

Along these lines we have recently studied several redox reactions involving cytochrome *c* and inert anionic redox partners. A good example is the reaction between cytochrome c^{II} and $[Co^{III}(Ox)_3]^{3-}$, where Ox = oxalate. In this case ion-pair (IP) formation is efficient and K_{IP} is therefore large enough, *viz*. $K_{IP} = 253 \pm 34\ M^{-1}$ at 298 K, to cause a significant deviation from linearity of the plot of k_{obs} *vs.* the complex concentration at low ionic strength.[25] Possible electrostatic interactions between reactant molecules results from the net positive charge of the reduced form of cytochrome *c* and the negative charge on the cobalt complex, *i.e.* +6.5 and −3, respectively.[25] In another reaction between cytochrome c^{II} and *trans*-bis(2-ethyl-2-hydroxybutanoato(2−))oxochromate(V), the complex has negatively charged donor centres, and the concentration dependence shows slight deviation from linearity at high concentrations of the chromium(V) species. In comparison to the trisoxalatocobalt(III) system, the ion-pair formation constant is significantly smaller, *viz.* $K_{IP} = 37 \pm 5\ M^{-1}$ at 288 K.[26]

† Electronic supplementary information (ESI) available: Figures showing a typical kinetic trace for the reduction of cytochrome *c* by $[Fe^{II}(edta)(H_2O)]^{2-}$ (Fig. S1), absorption spectra recorded during the reverse reaction (Fig. S2), a kinetic trace for the reverse reaction (Fig. S3), CVs recorded for $[Fe(edta)(H_2O)]^{-/2-}$ at ambient (Fig. S4) and at elevated pressure (Fig. S5), calculations for variations of possible charges on the redox partners (Table S2), and experimental data obtained in all the measurements (Tables S1 and S3). See http://www.rsc.org/suppdata/dt/b3/b301424j/

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In continuation of this work, we reinvestigated the title reaction that involves a 2− charged redox partner for cytochrome c^{III} in an effort to resolve the kinetic and thermodynamic parameters for the mechanism given in reaction (2) for the case that ion-pair formation is responsible for precursor formation. $[Fe^{II}(edta)(H_2O)]^{2-}$ is a hepta-coordinate complex with a pentagonal bipyramidal structure.[27] Two conformers are possible, *viz.* pentagonal bipyramidal and monocapped trigonal prismatic.[28] The edta chelate occupies six coordination sites around the metal centre, while the seventh position is available for a water molecule. The coordinated water molecule in $[Fe^{II}(edta)(H_2O)]^{2-}$ is very labile and enables rapid substitution of water by various ligands.[29] This high lability can therefore also lead to the effective formation of an inner-sphere precursor complex. In addition, we also studied the effect of hydrostatic pressure on the redox process, since such data have in the past added to our mechanistic understanding of such reactions.

Experimental

General remarks

All chemicals were of analytical reagent grade and used without further purification. Ultra pure water was used for the preparation of all solutions. Buffer solutions containing 0.05 M Tris (2-amino-2-(hydroxymethyl)-1,3-propanodiol, Sigma-Aldrich Chemicals) were used, and the ionic strength (I = 0.1 M) was adjusted by the addition of $LiNO_3$. Tris buffer was chosen since its acid dissociation constant is practically pressure independent.[30] The pH was adjusted with HCl. All experiments were performed under exclusion of air. Buffer solutions were deaerated (at least 1 min per ml of solution) with pure N_2. All solutions were kept under nitrogen atmosphere.

Materials and solutions

Horse heart cytochrome c^{III} (Sigma-Aldrich, Type VI) was used without further purification. To prepare horse heart cytochrome c^{II}, cytochrome c^{III} was first reduced by the addition of an excess of sodium dithionite (50 mM), then hexacyanoiron(II) (< 50 mM) was added, which was subsequently removed by repeated ultrafiltration through a 3000 Da molecular weight cut-off membrane (Amicon) with degassed ultra-pure water.[25] The buffered solutions of cytochrome $c^{II/III}$ were stored under anaerobic conditions and analysed spectrophotometrically. Characteristic UV-Vis bands were found for cytochrome c^{III} at 416 nm ($\varepsilon = 4.16 \times 10^5$ M^{-1} cm^{-1}) and 530 nm ($\varepsilon = 9.1 \times 10^3$ M^{-1} cm^{-1}), and for cytochrome c^{II} at 420 nm ($\varepsilon = 1.29 \times 10^5$ M^{-1} cm^{-1}) and 550 nm ($\varepsilon = 2.67 \times 10^4$ M^{-1} cm^{-1}).[31]

Aqueous solutions of $FeCl_2$, $FeCl_3$, $NaNO_3$ and Na_2H_2-edta·$2H_2O$ salts were deoxygenated under vacuum and bubbled with N_2. The (ethylenediamminetetraacetate)iron(II) complex ($[Fe^{II}(edta)(H_2O)]^{2-}$) was prepared in solution by a modification of the procedure described by Hodges.[20] $FeCl_2$ (Sigma-Aldrich) was added to an aqueous buffered deoxygenated solution. 20% excess of deoxygenated Na_2H_2edta was added to this solution. Colourless $[Fe^{II}(edta)(H_2O)]^{2-}$ solutions are extremely oxygen sensitive and rapidly oxidized to $[Fe^{III}$-$(edta)(H_2O)]^-$ (yellow). The ethylenediamminetetraacetate-iron(III) complex ($[Fe^{III}(edta)(H_2O)]^-$) was prepared by mixing $FeCl_3$ and Na_2H_2edta in the molar ratio 1 : 1.2 in a buffered aqueous solution. The yellow Fe(III) solution was stirred for some time. UV-Vis spectra were recorded to check the purity of the complex; absorption maxima for $[Fe^{III}(edta)(H_2O)]^-$ are observed at 342 (ε = 1080), 435 (ε = 820) and 633 nm ($\varepsilon = 130$ M^{-1} cm^{-1}).[32]

Spectroscopic and kinetic measurements

UV-Vis spectra were recorded on Shimadzu UV-2100 and Varian Cary G-5 spectrophotometers equipped with thermostated sample holders. UV-Vis rapid scan spectra were recorded in the wavelength range 390–650 nm on a J & M diode array detector connected to an Applied Photophysics SX-18 MV stopped-flow unit. The reduction of cyt c^{III} by $[Fe^{II}(edta)$-$(H_2O)]^{2-}$ was monitored at 550 nm. The kinetic measurements were performed on an Applied Photophysics SX-18 MV stopped-flow instrument at ambient pressure and on a home-made high pressure stopped-flow instrument at pressures up to 130 MPa.[33] Pseudo-first order conditions were achieved using an excess of the reductant. The reactants were thermostated prior to mixing (±0.1 °C). Rate constants were calculated as the mean of at least six reproducible kinetic runs. Kinetic traces were analysed with the OLIS KINFIT program.

Electrochemical measurements

Cyclovoltammetric measurements were performed with a classical three electrodes set-up, where a glassy carbon disk electrode (BAS) was used as the working electrode, a platinum wire electrode (BAS) as auxiliary electrode and Ag/AgCl (BAS) as the reference electrode. 0.1 M $NaClO_4$ (Merck) was used as a supporting electrolyte. All experiments were performed at room temperature. Electrochemical experiments under pressure were performed in a home made high pressure cell. A three-electrode system was employed for this purpose; a glassy carbon (Metrohm) working electrode, a platinum wire (Aldrich-Sigma) auxillary electrode, and an Ag/AgCl reference electrode. The latter was prepared as follows: the Ag wire covered with AgCl was placed in a shrink tube (BAS), filled with saturated KCl and closed carefully with a porous Vycor tip (BAS). The electrodes were placed in a Teflon cup, which was screwed onto the body of the electrochemical cell. The cell was kept under nitrogen for a few minutes. The oxygen-free cell was filled with the test solution containing the electrolyte (0.1 M $NaClO_4$), closed with a Teflon plunger and a screw. All cyclovoltammograms were recorded on an EG&G PAR model 263 instrument.

Results and discussion

The intermolecular electron transfer reaction (1) was studied under a nitrogen atmosphere and pseudo-first order conditions with the iron(II) complex in at least a ten-fold excess. All thermodynamic and kinetic data were estimated at two different pH values, *viz.* 7.0 and 7.4. Spectral changes accompanying this fast reaction, recorded on a rapid scan spectrophotometer, are presented in Fig. 1. There are five isosbestic points at 435, 505, 525, 545 and 560 nm. The characteristic absorption maximum for cytochrome c^{III} at 530 nm decays with time. New peaks typical for cytochrome c^{II} appear at 512 and 550 nm. Spectral changes observed in the range from 410 to 560 nm, *i.e.* within the Soret band, are related to configurational changes on the porphyrin system during electron transfer process.

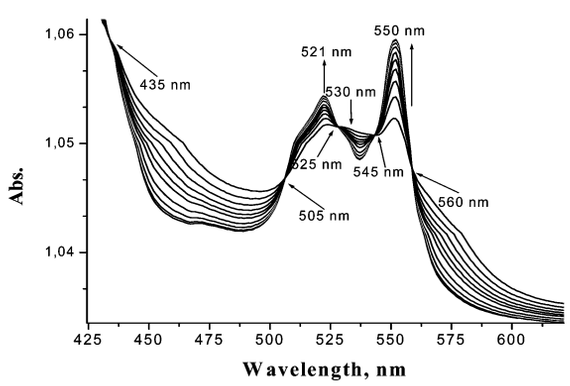

Fig. 1 Spectral changes recorded during the reduction of cytochrome c^{III} by $[Fe^{II}(edta)(H_2O)]^{2-}$. Experimental conditions: [cyt c^{III}] = 2×10^{-5} M, $[Fe^{II}(edta)(H_2O)^{2-}] = 8 \times 10^{-4}$ M, temp. = 25.0 °C, pH = 7.0, I = 0.1 M (Tris/$LiNO_3$), Δt = 6 s.

Kinetic traces for reaction (1) were recorded at 550 nm and fitted with a single exponential function; a typical example is shown in Fig. S1 (ESI †). All kinetic data are collected in Table S1 (ESI †). For this reaction the concentration dependencies (k_{obs} *vs.* $[Fe^{II}(edta)(H_2O)^{2-}]$ shown in Fig. 2) are straight lines and no significant curvature could be observed. The second-order rate constants for the forward reaction (k_{12}) calculated from the slopes of the lines k_{obs} *vs.* $[Fe^{II}(edta)(H_2O)^{2-}]$ in Fig. 2 are (2.2 ± 0.1) × 10^4 and (2.4 ± 0.1) × 10^4 M^{-1} s^{-1} at pH = 7.0 and 7.4, respectively. These values are in excellent agreement with those reported earlier by Hodges *et al.*, *viz.* $k_{12} = 2.57 \times 10^4$ M^{-1} s^{-1} (at 25.0 °C, $I = 0.1$ M, pH = 7.0).[20]

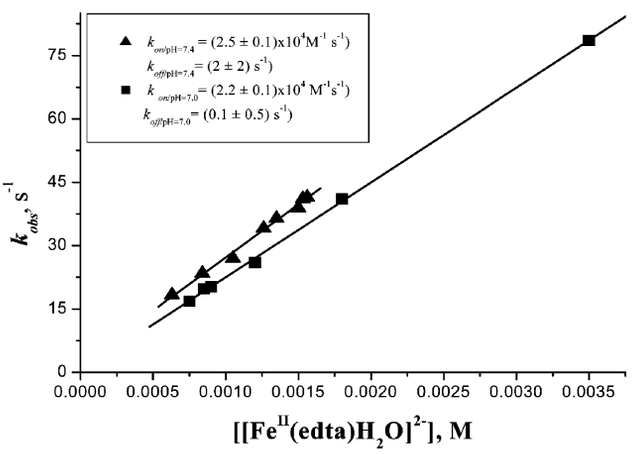

Fig. 2 Dependence of k_{obs} on the concentration of $[Fe^{II}(edta)(H_2O)]^{2-}$ for the reduction of cyt c^{III}. Experimental conditions: [cyt c^{III}] ≈ 2 × 10^{-5} M, temp. = 25.0 °C, $I = 0.1$ M (Tris–$LiNO_3$), $\lambda = 550$ nm.

The properties of $[Fe^{II}(edta)(H_2O)]^{2-}$ depend on pH due to formation of hydroxo and dimeric species at higher pH. In basic medium, deprotonation of the complex could affect the redox mechanism of the complex. For that reason, kinetic parameters were determined at two different pH values, *viz.* 7.0 and 7.4. Since no significant differences were observed, there is no reason to expect any interference of the hydroxo species under the selected experimental conditions.

The reverse reaction, *i.e.* formation of $[Fe^{II}(edta)(H_2O)]^{2-}$ and cyt c^{III}, is very slow as judged from the intercepts in Fig. 2, *viz.*, 0.1 ± 0.5 and 2 ± 2 s^{-1} at pH = 7.0 and 7.4, respectively. The large errors result from the extrapolation to $[Fe^{II}(edta)(H_2O)^{2-}] = 0$. Therefore, the reverse reaction was studied in a direct manner by treating cyt c^{II} with $[Fe^{III}(edta)(H_2O)]^{-}$. The accompanying UV-Vis spectral changes are illustrated in Fig. S2. The kinetic trace presented in Fig. S3 confirms a very slow reverse reaction within an overall reaction time of *ca.* 20 h. The estimated value of the rate constant is (5.0 ± 0.1) × 10^{-5} M^{-1} s^{-1} at pH = 7.0. The rate of the reverse reaction is therefore negligible as compared to the rate of the forward reaction.

For the investigated reaction the precursor formation constant (K) is indeed very small. Saturation of the observed rate constant at high $[Fe^{II}(edta)(H_2O)]^{2-}$ concentrations was not achieved such that $k_{obs} = k_{et}K[Fe^{II}(edta)^{2-}]$. Values of k_{et} and K could therefore not be separated on the basis of the kinetic data and the overall second order rate constant $k_{12} = k_{et}K$.

It is possible to predict values for k_{et} and K on the basis of the Marcus–Hush theory for intermolecular electron transfer processes (eqns (4)–(7)).[34–39]

$$K = 4/3\pi N_A \delta^3 \exp(-w_{12}/RT) \quad (4)$$

where

$$w_{12} = \frac{z_1 z_2 e_0^{\ 2} N_A}{4\pi\varepsilon_0\varepsilon_r\delta(1+\kappa\delta)} \quad (5)$$

The observed second order rate constant can be calculated from eqn. (6),

$$k_{12} = (k_{12}k_{11}k_{22}f_{12})^{1/2} W_{12} \quad (6)$$

and finally the electron transfer rate constant (k_{et}) can be calculated from eqn. (7).

$$k_{et} = k_{12}/K \quad (7)$$

For calculations under our experimental conditions the following values were used: $z_1 = +7.5$ (+3), $z_2 = -2$, r_1(cyt $c^{III/II}$) = 16.6 Å, $r_2([Fe^{II/III}(edta)(H_2O)]^{2-/-})$ = 4.00 Å,[40] $\varepsilon_1 = 78.5$, κ = 1.04 nm^{-1} ($\kappa = 3.29 \times I^{0.5}$ nm^{-1}, $I = 0.1$ M), $k_{11} = 3.5 \times 10^2$ M^{-1} s^{-1}, [41–43] $k_{22} = 4 \times 10^4$ M^{-1} s^{-1},[40] $k_{12} = \exp(nF\Delta E/RT)$, E_1° = 0.26 V,[44] E_2° = 0.12 V,[45,46] from which: $k_{12(calc)} = (0.96 \sim 5.55) \times 10^5$ M^{-1} s^{-1}, $K_{(calc)} = (42.7 \sim 115)$ M^{-1}, $k_{et(calc)} = (2247–4826)$ s^{-1}. The calculated value of the electron transfer rate constant is very large and could not be reached experimentally.

The experimental value of k_{12} equals 2.6×10^4 M^{-1} s^{-1} and is smaller than the range of theoretical values. It should be noted that in the case of reactants with large opposite charges, the overall value of the second-order rate constant (k_{12}) depends on the electrostatic correction term (W_{12}), and therefore the value taken for z_1z_2 in the calculations. For the studied reaction the maximum product of net charges, *viz.* −15, originates from +7.5 (oxidized protein) times −2 (iron complex). The effective charge does not need to be so large, especially in terms of the location of the active site on the protein. Possible variations of the effective charges taken into the calculations are compared in Table S2 (ESI †). Also the contact radii of the reactants should be taken into account. Here for simplicity r_1 and r_2 are assumed to be constant. Similar calculations were performed in earlier studies for reactions where both reactants had positive charges,[6,7,10] and also for systems where reagents had opposite charges.[25,26] The results in Table S2 clearly show that the theoretically calculated values for k_{12} decrease and reach the experimental value on reducing the effective charge on the protein surface that inteacts with the metal complex.

The value of z_1z_2 can also be obtained from the slope of log k_{obs} *vs.* $I^{1/2}$ (ionic strength dependence) as done in an earlier study of the reaction of cyt c^{III} with $[Fe^{II}(edta)]^{2-}$. The authors showed that the product of the effective ionic charges is indeed small, *viz.* −3.4.[20] If the overall charge on the reluctant is taken to be −2, the effective charge on cytochrome *c* is only +1.7. Thus, only a specific site of the metalloprote in participates in the electron transfer reaction, since only 4% of the heme edge is exposed to the solution, *i.e.* 0.06% of the overall protein.[2] The high overall positive charge of +7.5 originates from the hydrophobic side chains and is inhomogeneously distributed over the surface of cytochrome *c*. Thus it is very reasonable to conclude that the Fe(II) complex only experiences a fraction of the overall charge on the protein surface, *i.e.* localized interactions are responsible for the electron transfer process.

The thermal activation parameters ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) for the reaction were derived from the linear dependence of $\ln k_{12}$ on the reciprocal temperature (T^{-1}) illustrated in Fig. 3. The activation enthalpy is small, *viz.* 32 ± 1 kJ mol^{-1} and 26 ± 1 kJ mol^{-1} at pH = 7.4 and 7.0, respectively. This could be in agreement with data for other outer-sphere electron transfer reactions,[47] also with cytochrome *c*.[10,11,26] Significantly negative values for the activation entropy, *viz.* −107 ± 4 and −128 ± 4 J K^{-1} mol^{-1} at pH = 7.0 and 7.4, respectively, suggest a highly structured transition state. These $\Delta S^\ddagger$ values agree with data obtained for the reduction of cytochrome c^{III} by cobalt imine complexes.[11] The $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values determined in the low concentration range (second-order rate constant, *i.e.* Kk_{et}) for the oxidation of cytochrome c^{II} by the chromium(v) complex, showed similar behaviour.[26]

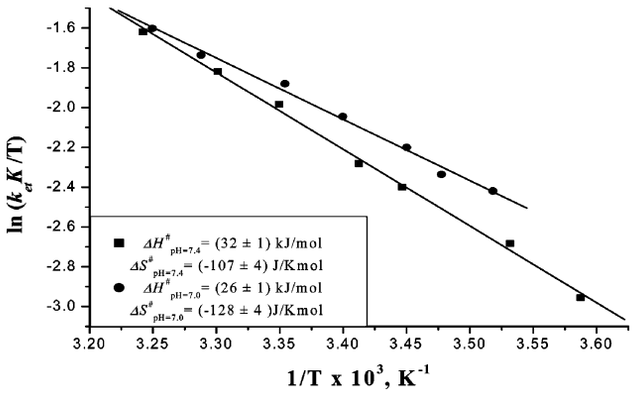

Fig. 3 Temperature dependence of the reduction of cyt c^{III} by $[Fe^{II}(edta)(H_2O)]^{2-}$. Experimental conditions: [cyt c^{III}] = 1×10^{-5} M, $[Fe^{II}(edta)(H_2O)^{2-}] = 1.2 \times 10^{-4}$ M, $I = 0.1$ M (Tris–$LiNO_3$), $\lambda = 550$ nm.

The activation enthalpy was also small, $\Delta H^{\ddagger} = 21 \pm 1$ kJ mol^{-1}, and the activation entropy was also significantly negative, $\Delta S^{\ddagger} = -80 \pm 2$ J K^{-1} mol^{-1}. The activation parameters for the reaction of cytochrome c with $[Fe^{II}(edta)(H_2O)]^{2-}$ can also be interpreted in terms of an inner-sphere electron transfer process as mentioned above. The presence of a labile water molecule in the $[Fe^{II}(edta)(H_2O)]^{2-}$ complex supports this suggestion. Moreover, the large negative activation entropy also supports the operation of an inner-sphere electron transfer process in the studied reaction.

The activation volume calculated from the plot of $\ln k_{obs}$ *vs.* pressure (Fig. 4) is negative with an average value of $\Delta V^{\ddagger} = -8 \pm 1$ cm^3 mol^{-1}. The reaction rate is not as strongly accelerated by pressure as in the case of reaction between cytochrome c^{III} and cobalt imine complexes.[11] In the latter case the negative activation volume originates from intrinsic and solvational volume contributions. Oxidation of the Co^{II} complex is accompanied by a spin change from high-spin Co^{II} $^4T(\pi^5\ \sigma^{*2})$ to low-spin Co^{III} $^1A(\sigma^{*6})$ and a shortening of the Co–N bond.[48,49] Furthermore, the electron transfer process is accompanied by a large increase in solvent electrostriction, which is reflected in a further volume decrease in the transition state.

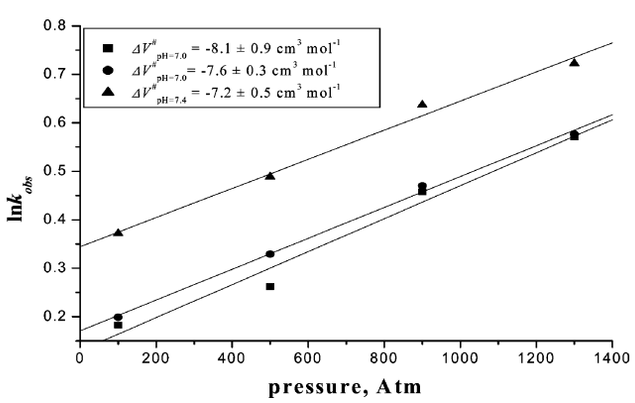

Fig. 4 Pressure dependence of the reduction of cyt c^{III} by $[Fe^{II}(edta)(H_2O)]^{2-}$. Experimental conditions: [cyt c^{III}] = 5×10^{-5} M, triangles – $[Fe^{II}(edta)(H_2O)^{2-}] = 8 \times 10^{-4}$ M, circles – $[Fe^{II}(edta)(H_2O)^{2-}] = 8 \times 10^{-4}$ M, squares – $[Fe^{II}(edta)(H_2O)^{2-}] = 9 \times 10^{-4}$ M, temp. = 25.0 °C, $I = 0.1$ M (Tris–$LiNO_3$).

The overall reaction volume for electron transfer between cytochrome c^{III} and $[Fe^{II}(edta)(H_2O)]^{2-}$, *viz.* $\Delta V = \Delta V_{Fe(II/III)} + \Delta V_{cyt(III/II)}$, can be predicted after separation of the redox reaction (eqn. (1)) into:

$$\text{cyt } c^{III} + e^- \longrightarrow \text{cyt } c^{II}, \quad \Delta V_{cyt(III/II)} \qquad (8)$$

$$[Fe^{II}(edta)]^{2-} \longrightarrow [Fe^{III}(edta)]^- + e^-, \quad \Delta V_{Fe(II/III)} \qquad (9)$$

The volume change on the cytochrome *c* surface (reaction (8)) estimated in earlier studies seems to be small, *viz.* $\Delta V_{cyt(III/II)} = -5$ cm^3 mol^{-1}.[11,50] For several electron transfer reactions between cyt *c* and various inorganic complexes, the overall reaction volume is controlled by volume changes on the redox partner and not by volume changes on the protein itself.[10,11,26]

The reaction volume for the $[Fe^{III/II}(edta)(H_2O)]^{2-/-}$ couple (reaction (9)), was determined from electrochemical measurements as a function of pressure. The $[Fe^{III/II}(edta)(H_2O)]^{-/2-}$ couple in buffered solution shows a reversible redox wave with $\Delta E = 64$ mV, Fig. S4 (ESI †), at ambient pressure. The measured standard potential equals −128 mV *vs.* Ag/AgCl, which is in good agreement with literature data.[29,46,51] The redox potential at elevated pressure, which was measured *vs.* an Ag/AgCl reference electrode for all quasi-reversible waves, increased with increasing pressure (Fig. S5, ESI †). The potential difference between the oxidation and reduction peaks was between 63 and 90 mV at a scan rate of 50 mV s^{-1}. All experimental data are collected in Table S3 (ESI †). From the slope of the plot of redox potential as a function of pressure (Fig. 5) for reaction (10) it was possible to calculate the reaction volume for the cell (eqn. (11)).

$$[Fe^{III}(edta)(H_2O)]^- + Ag(s) + Cl^- \longrightarrow [Fe^{II}(edta)(H_2O)]^{2-} + AgCl(s) \qquad (10)$$

$$\Delta V_{cell} = \Sigma V_{prod} - \Sigma V_{react} = \varphi([Fe^{II}(edta)(H_2O)]^{2-}) - \varphi([Fe^{II}(edta)(H_2O)]^-) + \varphi(AgCl(s)) - \varphi(Ag(s)) \qquad (11)$$

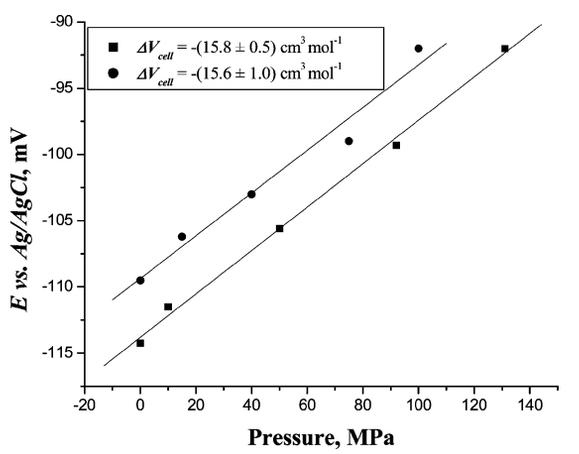

Fig. 5 Redox potential of the $[Fe^{III/II}(edta)(H_2O)]^{-/2-}$ couple as a function of pressure. Experimental conditions: $[[Fe^{III}(edta)(H_2O)]^-] = 5 \times 10^{-4}$ M, temp. = 25.0 °C, $I = 0.1$ M ($NaClO_4$), pH = 4.5 (acetate–acetic acid).

The estimated overall reaction volume includes contributions from the working and reference electrodes, $\Delta V_{cell} = \Delta V_{Fe(III/II)} + \Delta V_{ref} = -15.7 \pm 1.0$ cm^3 mol^{-1}. The reference electrode contributes to the overall reaction volume for which $\Delta V_{Ag/AgCl/sat.KCl} = -9.0 \pm 0.5$ cm^3 mol^{-1} according to data reported by Tregloan and Swaddle.[52,53] The reaction volume for the reduction of the $[Fe^{III}(edta)(H_2O)]^-$ complex in buffered solution was calculated from $\Delta V_{Fe(III/II)} = \Delta V_{cell} - \Delta V_{ref} = -6.7 \pm 1.0$ cm^3 mol^{-1}.

Differences in ΔV_{ref} for different reference electrodes are rather small. For instance, ΔV_{ref} for $Ag/AgNO_3$ is $\Delta V_{Ag/Ag^+} = -11.9 \pm 0.5$ cm^3 mol^{-1}, whereas for Ag/AgCl/sat. KCl, $\Delta V_{Ag/AgCl} = -9.0 \pm 0.5$ cm^3 mol^{-1}. Most important and decisive are changes in the redox potential of the metal complex in solution as a function of pressure. These changes can be separated into intrinsic (ΔV_{intr}) and electrostatic volume changes (ΔV_{elec}) as expressed by eqn. (12).[53]

$$\Delta V_{complex} = \Delta V_{intr} + \Delta V_{elec} \qquad (12)$$

Table 1 Kinetic and thermodynamic parameters for typical outer-sphere electron transfer reactions

Reaction	k/M^{-1} s^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}	$\Delta V^{\ddagger}$/cm^3 mol^{-1}	ΔV/cm^3 mol^{-1}
$[Ru(NH_3)_6]^{2+}$ + cyt c^{III}		3.8 ± 0.8	−142 ± 4	−15.6 ± 0.6	–
$[Ru(NH_3)_5(isn)]^{3+}$ + cyt c^{II}	11.5×10^4	21.8 ± 0.9	−75 ± 3	15.9 ± 0.7	–
$[Ru(NH_3)_5(isn)]^{2+}$ + cyt c^{III}	2.03×10^3	28 ± 3.7	−87 ± 12	−17.2 ± 1.5	–
$[Ru(NH_3)_5(lut)]^{3+}$ + cyt c^{II}	$(2.7 \pm 0.1) \times 10^4$	35.4 ± 0.3	−41 ± 1	16.9 ± 1.4	33.6 ± 1.7
$[Ru(NH_3)_5(lut)]^{2+}$ + cyt c^{III}	$(9.4 \pm 0.5) \times 10^3$	21 ± 1	−99 ± 5	−17.8 ± 1.6	34.7 ± 2.1
$[Ru(NH_3)_5(Etpy)]^{3+}$ + cyt c^{II}	$(2.7 \pm 0.1) \times 10^4$	29 ± 2	−61 ± 7	14.7 ± 1.8	26.9 ± 1.8
$[Ru(NH_3)_5(Etpy)]^{2+}$ + cyt c^{III}	$(9.2 \pm 0.1) \times 10^3$	25 ± 2	−86 ± 6	−14.9 ± 1.1	29.6 ± 1.4
$[Ru(NH_3)_5(py)]^{3+}$ + cyt c^{II}	$(4.9 \pm 0.1) \times 10^4$	28 ± 1	−64 ± 5	17.4 ± 1.5	33.4 ± 1.9
$[Ru(NH_3)_5(py)]^{2+}$ + cyt c^{III}	$(1.1 \pm 0.1) \times 10^4$	33 ± 4	−59 ± 13	−17.7 ± 0.8	35.1 ± 1.7
$[Co(bpy)_3]^{3+}$ + cyt c^{II}	582 ± 13	49.9 ± 0.9	−28 ± 2	12.5 ± 0.9	21.8 ± 0.7
$[Co(bpy)_3]^{2+}$ + cyt c^{III}	169 ± 5	28 ± 1	−107 ± 5	−12.6 ± 1.5	25.1 ± 1.7
$[Co(phen)_3]^{3+}$ + cyt c^{II}	3753 ± 39	44 ± 3	−28 ± 9	17.0 ± 0.9	37.9 ± 2.0
$[Co(phen)_3]^{2+}$ + cyt c^{III}	217 ± 5	14 ± 1	−136 ± 4	−16.2 ± 1.0	34.2 ± 1.7
$[Co(terpy)_3]^{3+}$ + cyt c^{II}	1427 ± 36	40 ± 1	−47 ± 4	18.4 ± 1.2	33 ± 3
$[Co(terpy)_3]^{2+}$ + cyt c^{III}	1704 ± 46	14 ± 1	−136 ± 4	−18.0 ± 1.4	36 ± 2

isn = Isonicotinamide, lut = luditine, Etpy = 4-ethylpyridine, py = pyridine.

The electrostatic changes in most of the studied systems are proportional to the difference in the square of the charges on the metal complexes.[53] The intrinsic changes were found to be very small for complexes with constant coordination number during the electron transfer process (*e.g.* $[Fe(CN)_6]^{3-/4-}$, $[Fe(CN)_4(bpy)]^{2-/-}$, $[Fe(CN)_4(phen)]^{2-/-}$).[53] However, for some complexes intrinsic volume changes contribute significantly to the overall reaction volume. The intrinsic volume changes could be calculated on the basis of the ionic radii of the small metal complexes.[54] These calculations are not suitable for larger coordination compounds, since the effective radii are smaller than the overall radii. Nevertheless, the intrinsic volume changes can be estimated for these redox couples, which are characterised by constant values of ΔV_{ref} and ΔV_{elec}. Therefore, ΔV_{intr} may be obtained directly from eqn. (13):

$$\Delta V_{intr} = \Delta V_{cell} - (\Delta V_{ref} + \Delta V_{elec}) \quad (13)$$

ΔV_{elec} can be calculated on the basis of estimations made by Tregloan *et al.*[52] For a number of $Fe^{III/II}$ systems with an overall charge of −1/−2 this value equals −15.5 cm^3 mol^{-1}; thus the intrinsic volume change is close to ΔV_{intr} = +9 ± 1 cm^3 mol^{-1} according to eqn. (13) for the $[Fe^{III/II}(edta)(H_2O)]^{-/2-}$ couple. There is enough evidence from the literature to conclude that the coordination number remains unchanged during this redox reaction.[29,55] On the basis of the predicted value of ΔV_{intr}, it can be concluded that the Fe(edta) system shows larger differences in the size of the oxidized and reduced forms than usually found for octahedral iron complexes.[28,52,56] Coupled to the predicted, significantly negative value for ΔV_{elec}, which may partially arise from inhomogeneously distributed charges on the edta ligand,[57] the overall reaction volume for the reduction of $[Fe^{III}(edta)(H_2O)]^-$, *viz.* −6.7 ± 1.0 cm^3 mol^{-1}, can be understood in terms of an intrinsic volume increase, balanced by a significantly larger volume decrease as a result of an increase in electrostriction on the edta chelate.

The overall reaction volume for reaction (1), *viz.* $\Delta V = \Delta V_{Fe(II/III)} + \Delta V_{cyt(III/II)}$, can now be estimated to be + 1.7 ± 1.0 cm^3 mol^{-1}. The small positive reaction value results from an overall volume *increase* due to the oxidation of $[Fe^{II}(edta)(H_2O)]^{2-}$ and a volume decrease due to the reduction of cyt c^{III}. Along with the measured activation volume of −8 ± 1 cm^3 mol^{-1}, a volume profile for reaction (1) can be constructed as presented in Fig. 6. The transition state in the studied reaction is characterised by a significantly smaller partial molar volume than either the reactant or product states. Thus reorganization of the redox partners to allow electron transfer in the transition state cannot account for the significant volume collapse on the basis of the overall reaction volume estimated for reaction (1). The significant volume decrease in the transition state must be associated with an effective precursor formation process, for which no kinetic evidence could be found. Nevertheless, the high lability of the $[Fe^{II}(edta)(H_2O)]^-$ complex could cause the effective formation of an inner-sphere precursor species with a nucleophile located on the surface of the protein, and so induce the electrontransfer process. This can then account for the compact nature of the transition state.

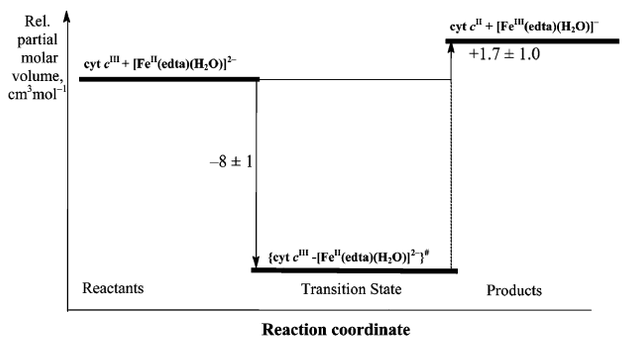

Fig. 6 Volume profile for the reduction of $[cyt\ c^{III}]^{7+}$ by $[Fe^{II}(edta)(H_2O)]^{2-}$.

Conclusions

The studied redox reaction (1) can in principle proceed *via* an inner-sphere or an outer-sphere electron transfer mechanism. In the case where only inert reactants participate in the redox reaction, an outer-sphere process will occur. Some of these systems are summarized in Table 1.[7,10,11] None of these systems has a labile coordinated water molecule as in $[Fe^{II}(edta)(H_2O)]^{2-}$. In this case rapid substitution of $[Fe^{II}(edta)(H_2O)]^{2-}$ by one of the nucleophilic residues of cytochrome *c* can occur and a bridge between the two redox centres can be formed. Therefore, the suggestion of an inner-sphere electron transfer mechanism for reaction (1) is reasonable and can account for the observed volume changes as seen from the overall volume profile in Fig. 6. Unfortunately, no evidence for such a bridged intermediate or other reaction products, was found that could prove this suggestion. Spectral changes recorded during the reaction only showed a clean, irreversible transformation of cyt c^{III} to cyt c^{II}. The high lability of the $[Fe^{III}(edta)(H_2O)]^-$ complex[57] will result in a rapid aquation of whatever oxidation product may be formed as a result of the transfer of a bridging group coupled to the electron transfer process. In such case the products of the inner- and outer-sphere processes are expected to be identical. Furthermore, the kinetic parameters also do not permit an unequivocal assignment of the reaction mechanism. Theoretical calculations of the rate constant according to Marcus-Hush theory for outer-sphere electron transfer are in

reasonable agreement with the obtained experimental values, which does not exclude any of the mechanism. The volume profile in Fig. 6 clearly supports the inner-sphere electron transfer mechanism.

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