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Kinetic studies on the redox reactions of *Clostridium pasteurianum* rubredoxin †

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Electron-transfer reactions (25 °C) of the single iron protein rubredoxin from *Clostridium pasteurianum* with three inorganic complexes as redox partners have been studied at pH 7.0, I = 0.100 M (NaCl). With $[Co(sep)]^{2+}$ (sep = sepulchrate = 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]icosane) (−300 mV) as reductant for the oxidised iron(III) form of rubredoxin (Rd^{III}) (−57 mV) a second-order rate law with $k = 7.5 \times 10^5$ M^{-1} s^{-1} has been determined. No dependence on pH 3.8–8.8 is observed. With $[Co(terpy)_2]^{3+}$ (terpy = 2,2′:6′,2″-terpyridine) (+260 mV) as oxidant for the Rd^{II} form saturation kinetic behaviour is obtained, with K = 470 ± 70 M^{-1} for association prior to electron transfer, k_{et} = 515 ± 65 s^{-1}. Again no dependence on pH, in this case the range 5.2–7.0, is observed. With $[Ru(NH_3)_6]^{3+}$ (+70 mV) as oxidant for the Rd^{II} form saturation kinetics is also observed with K = 4430 ± 240 M^{-1} and k_{et} = 240 ± 26 s^{-1}. No information is available concerning the site on rubredoxin at which these reactions occur, although it is noted that the Cys-9 and Cys-42 residues at the $Fe(Cys)_4$ active site are partially exposed to solvent.

Rubredoxins (Rd) are non-haem iron-containing proteins found in bacterial cells,[1-3] with 46 to 54 amino acids ($M_r \approx 6000$) and one iron atom per molecule.[4] The protein from *Clostridium pasteurianum* was the first to be isolated.[5] The structure determined from high-resolution X-ray crystallography[6] shows the Fe to be tetrahedrally co-ordinated by four cysteines at positions 6, 9, 39 and 42 in the sequence. There are four other rubredoxin crystal structures.[7-10] The molecule has a barrel-like arrangement made up of seven anti-parallel β-sheet sections, and a core of aromatic residues. The $Fe(Cys)_4$ unit is positioned at one end of the barrel, with the Fe ≈ 6 Å from the surface.[6] The protein is highly acidic with pI < 4.5. The two oxidation states Rd^{II} and Rd^{III} incorporate high-spin forms of Fe^{II} and Fe^{III} respectively.[11] Structural changes associated with the redox interconversion give an average Fe–S lengthening on reduction of 0.035 Å (X-ray crystallography),[10] and 0.06 Å [EXAFS (extended X-ray absorption fine structure) analysis].[12] Similar changes are observed for analogue systems.[13]

Kinetic properties associated with the Rd^{II} and Rd^{III} redox interconversion have received little attention, largely because the amounts of the proteins obtained from natural sources are small. The availability of larger amounts from an *Escherichia coli* overproducing strain make it possible to address this situation. In the present paper reactions with three well established substitution-inert inorganic complexes as redox partners are considered. We have recently determined by NMR methods the electron self-exchange rate constant for the Rd^{II}–Rd^{III} couple as 1.60×10^5 M^{-1} s^{-1} at 25 °C, I = 0.100 M (NaCl), and studied two protein–protein cross-reactions.[14] The electron-transfer properties of rubredoxin are of particular interest because it is a further example of a single metal active site co-ordinated by amino acids.[15]

Experimental

Rubredoxin

Recombinant *Clostridium pasteurianum* rubredoxin was generously provided by Dr. J.-M. Moulis. The protein was isolated from an overproducing *Escherichia coli* K38/pGP1-2/pTRD 1 strain according to a previously described procedure.[16,17] Purification of protein was by fast protein liquid chromatography (FPLC) using a mono-Q anion-exchange column and 20 mM tris(hydroxymethyl)aminoethane (Tris)–HCl buffer, pH 7.0, with a linear 0 to 1.0 M NaCl ionic strength gradient. The oxidised protein was eluted at 39% of 1.0 M NaCl, with UV/VIS absorption peak ratio A_{490}/A_{280} of 0.43. The UV/VIS absorption bands λ/nm (10^{-3} ε/M^{-1} cm^{-1}) for *C. pasteurianum* Rd^{III} are at 490 (8.85), 380 (10.8) and 280 (21.3), and at 333 (6.3), 311 (10.8) and 275 (24.8) for Rd^{II}.[5] The spectra are shown in Fig. 1. Protein Rd^{III} concentrations were determined from the absorbance at 490 nm. In order to obtain the reduced form, sodium dithionite was added to the protein solution in a Miller-Howe glove-box (O_2 < 2 ppm).

Buffers

The following buffers (20 mM) were used. At pH 3.8 and 4.5 sodium acetate trihydrate (pK_a = 4.88), 2-(morpholino)-ethane-sulfonic acid (Mes, pK_a = 6.10) with NaOH at pH 5.0–6.7, phosphate at pH 6.4, and Tris (pK_a = 8.08) with HCl added at pH 7.0–8.8. All buffers were from Sigma Chemical Co. Ltd. The pH of solutions were measured on a Radiometer PHM 62 pH meter calibrated using pH 7.0

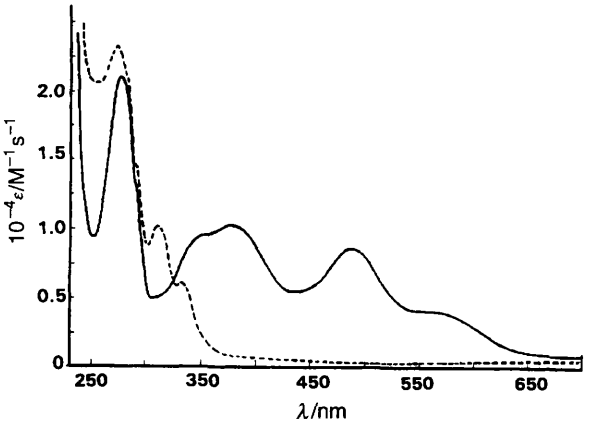

Fig. 1 The UV/VIS absorbance spectra for rubredoxin in the two oxidation states Rd^{III} (——) and Rd^{II} (– – –)[5]

† *Non-SI unit employed:* M = mol dm^{-3}.

buffer tablets, and/or a pH 4.0 colour-key standard (both from BDH).

Complexes

These were prepared and purified to known UV/VIS peak positions, λ/nm (ε/M^{-1} cm^{-1}), by procedures already described. The cobalt(III) complex $[Co(sep)]Cl_3 \cdot H_2O$ (sep = sepulchrate cage ligand, 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]icosane), 472 (109) and 340 (116), was prepared[18] by electrochemical reduction under air-free conditions. Bis(2,2′:6′,2″-terpyridine) cobalt(II) perchlorate, $[Co(terpy)_2][ClO_4]_2 \cdot H_2O$, 445 (1578) and 505 (1387), was prepared as described.[19] The oxidised form has UV/VIS peaks at 378 (4.6×10^4) and 336 (2.10×10^4). Hexaammineruthenium(III) chloride, $[Ru(NH_3)_6]Cl_3$, 276 (530) (Johnson Matthey), was further purified by recrystallisation.[20] Reduction potentials for the relevant couples[14,21–24] are listed in Table 1.

Kinetic studies

Absorbance changes are consistent with 1:1 stoichiometries in all cases. For kinetic runs the inorganic complex was in $\geq$10-fold excess of the protein. Absorbance changes of the rubredoxin at a fixed wavelength, 490 nm ($\Delta\varepsilon$ = 8850 M^{-1} cm^{-1}), were monitored on a Dionex D-110 stopped-flow spectrophotometer, Fig. 1. All runs were at 25.0 ± 0.1 °C and ionic strength I = 0.100 ± 0.001 M adjusted with NaCl. Contributions from the 20 mM buffer were taken into account. First-order rate constants, k_{obs}, were obtained directly from an OLIS software package interfaced to the stopped-flow. The favourable absorbance change enabled larger than normal k_{obs} to be determined. Unweighted least-squares fitting procedures were used.

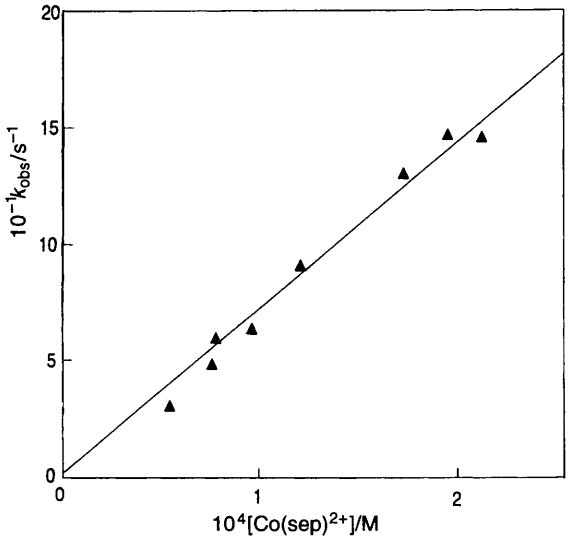

Fig. 2 Dependence of first-order rate constants k_{obs} (25 °C) for the $[Co(sep)]^{2+}$ reduction of Rd^{III} on reductant concentration (reactant in large excess), at pH 7.0, I = 0.100 M (NaCl)

Table 1 Reduction potentials $E^{\circ\prime}$ (25 °C) *vs.* the normal hydrogen electrode (NHE) for couples used

Couple	$E^{\circ\prime}$/mV	Ref.
Rd^{II}/Rd^{III}	−57*	4
$[Co(sep)]^{3+/2+}$	−300	21
$[Co(terpy)_2]^{3+/2+}$	+260	21, 22
$[Ru(NH_3)_6]^{3+/2+}$	+70	23

* In a recent study[14] using cyclic voltametry/square-wave voltammetry and promoter for reaction at a gold electrode a value of −81 mV was obtained at I = 0.100 M (NaCl), pH 7.0 (20 mM Tris-HCl).

Results

$[Co(sep)]^{2+}$ Reduction of Rd^{III}

At pH 7.0 first-order rate constants k_{obs}, Table 2, with $[Co(sep)]^{2+}$ in > 10-fold excess give a linear dependence on the concentration of reductant, Fig. 2. The rate law is therefore of the form (1), and from the slope of Fig. 2 the second-order

$$\text{Rate} = k[Co(sep)^{2+}][Rd^{III}] \quad (1)$$

rate constant $k = (7.5 \pm 0.3) \times 10^5$ M^{-1} s^{-1}. Values of k determined over a range pH 3.8–8.8, with three different $[Co(sep)]^{2+}$ concentrations (two- to three-fold variations) at each pH, gave no variation with pH, Fig. 3.

$[Co(terpy)_2]^{3+}$ Oxidation of Rd^{II}

First-order rate constants k_{obs}, Table 2, give a non-linear dependence on $[Co(terpy)_2]^{3+}$, the reactant in > 10-fold

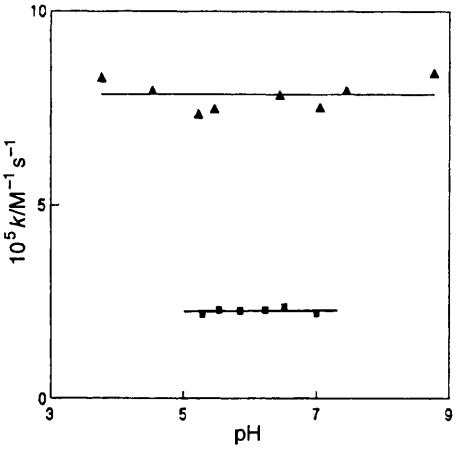

Fig. 3 Variation of second-order rate constants k (25 °C) for the $[Co(sep)]^{2+}$ reduction of Rd^{III} (▲) and the $[Co(terpy)_2]^{3+}$ oxidation of Rd^{II} (■) with pH, I = 0.100 M (NaCl)

Table 2 First-order rate constants k_{obs} (25 °C) for (*a*) the $[Co(sep)]^{2+}$ reduction of *C. pasteurianum* rubredoxin (Rd^{III}), together with data for (*b*) the $[Co(terpy)_2]^{3+}$ and (*c*) the $[Ru(NH_3)_6]^{3+}$ oxidations of rubredoxin (Rd^{II}); protein concentration (0.50–0.75) × 10^{-5} M, pH 7.0 (20 mM Tris-HCl), I = 0.100 M (NaCl)

(*a*) $[Co(sep)]^{2+}$ reduction

$10^4[Co(sep)^{2+}]/M$	k_{obs}/s^{-1}	$10^4[Co(sep)^{2+}]/M$	k_{obs}/s^{-1}
0.55	30	1.20	92
0.76	49	1.71	132
0.78	60	1.93	149
0.96	65	2.10	148
1.10	52	2.13	180

(*b*) $[Co(terpy)_2]^{3+}$ oxidation

$10^4[Co(terpy)_2{}^{3+}]/M$	k_{obs}/s^{-1}	$10^4[Co(terpy)_2{}^{3+}]/M$	k_{obs}/s^{-1}
2.04	43	6.67	125
2.73	61	7.30	130
2.99	61		
4.07	81	7.65	140
5.16	100	8.13	141
6.13	116	9.35	152
6.27	109		

(*c*) $[Ru(NH_3)_6]^{3+}$ oxidation

$10^4[Ru(NH_3)_6{}^{3+}]/M$	k_{obs}/s^{-1}	$10^4[Ru(NH_3)_6{}^{3+}]/M$	k_{obs}/s^{-1}
1.00	60	3.01	144
1.92	98	3.98	152
2.03	133	4.20	164
2.56	131	4.92	160
3.00	152	6.15	172

excess, Fig. 4. The behaviour observed is accounted for by the reactions (2) and (3), for which the expression (4) can be derived

$$Rd^{II} + \text{oxidant} \overset{K}{\rightleftharpoons} Rd^{II}, \text{oxidant} \quad (2)$$

$$Rd^{II}, \text{oxidant} \xrightarrow{k_{et}} \text{products} \quad (3)$$

$$k_{obs} = \frac{k_{et}K[\text{oxidant}]}{1 + K[\text{oxidant}]} \quad (4)$$

by mass balance. A plot of k_{obs}^{-1} *vs.* $[Co(terpy)_2^{3+}]^{-1}$ is linear, and the slope and intercept give $K = 470 \pm 70\ M^{-1}$ and $k_{et} = 515 \pm 65\ s^{-1}$ at pH 7.0. Variations in pH were carried out at the lower end of the range of $[Co(terpy)_2^{3+}]$ concentrations (2.2×10^{-4} M), when it can be assumed that $K[Co(terpy)_2^{3+}] \ll 1$ in equation (4). No dependence on pH was observed over the more restricted range pH 5.2–7.0, Fig. 3.

$[Ru(NH_3)_6]^{3+}$ Oxidation of Rd^{II}

Saturation kinetic behaviour is also observed for this reaction with $[Ru(NH_3)_6]^{3+}$ at levels >10-fold excess of the Rd^{II} protein, Fig. 5. Rate constants k_{obs} are listed in Table 2. The reactions (2) and (3) define K and k_{et}, and from the corresponding plot of k_{obs}^{-1} against $[Ru(NH_3)_6^{3+}]^{-1}$, $K = 4430 \pm 240\ M^{-1}$ and $k_{et} = 240 \pm 26\ s^{-1}$ at pH 7.0. A second-order rate constant obtained from the slope close to the origin, when $[Ru(NH_3)_6^{3+}]$ is small and $K[Ru(NH_3)_6^{3+}] \ll 1$, corresponds to $k_{et}K$. The value obtained is $1.09 \times 10^6\ M^{-1}\ s^{-1}$. The equilibrium constant K for reaction (5) can be calculated

$$Rd^{II} + [Ru(NH_3)_6]^{3+} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} Rd^{III} + [Ru(NH_3)_6]^{2+} \quad (5)$$

from the $\Delta E^{\circ\prime}$ of 153 mV and is 390. Hence with $k_f = 1.09 \times 10^6\ M^{-1}\ s^{-1}$ the rate constant k_b is $2800\ M^{-1}\ s^{-1}$. From the latter at $[Ru(NH_3)_6^{2+}] = 5.0 \times 10^{-4}$ M, assuming saturation kinetics do not apply, a first-order rate constant of $1.4\ s^{-1}$ is obtained. This compares with the value previously reported by Jacks *et al.*[24] of $55\ s^{-1}$.

Discussion

Rubredoxin is an acidic protein, with a charge balance at pH ≈7 for the Rd^{II} (−10) and Rd^{III} form (−9) determined from the amino acid (and active-site) composition, 1− for Asp and Glu residues and 1+ for Lys. These values indicate a high overall negative charge density for a 54 amino acid protein. The saturation kinetics observed with $[Co(terpy)_2]^{3+}$ and $[Ru(NH_3)_6]^{3+}$ as oxidants for the Rd^{II} form gives association constants (K) prior to first-order rate constants (k_{et}) for electron transfer within the adduct. The implication is that a negative patch on rubredoxin is involved. The association constants (M^{-1}), $[Co(terpy)_2]^{3+}$ (470) and $[Ru(NH_3)_6]^{3+}$ (4430), are determined in large part by the favourable electrostatics. The differences observed are larger than normal,[25] and can be attributed to the closer approach to the protein surface with NH_3 as opposed to the more bulky terpy ligands. In view of the smaller K, favourable interaction of the ligands of $[Co(terpy)_2]^{3+}$ with aromatic residues on the protein is not a part of our interpretation. With $[Co(sep)]^{2+}$ as a reductant for the Rd^{III} form, saturation kinetics is not observed, and the electrostatic component (K) is less. All three cationic redox partners may well use the same site on rubredoxin. One possibility is that this involves acidic residue(s) and is close to the $Fe(Cys)_4^-$ active site. The latter is near to the protein surface with two cysteinyl ligands (Cys-9 and Cys-42) protruding through the surface. The immediate surrounding surface is hydrophobic however, Fig. 6. We do not believe that

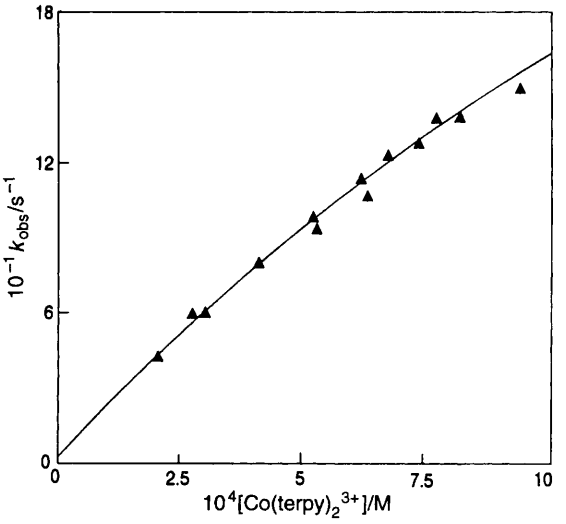

Fig. 4 Non-linear variation of first-order rate constants k_{obs} (25 °C) for the $[Co(terpy)_2]^{3+}$ oxidation of Rd^{II} with oxidant concentration (in large excess), at pH 7.0, $I = 0.100$ M (NaCl)

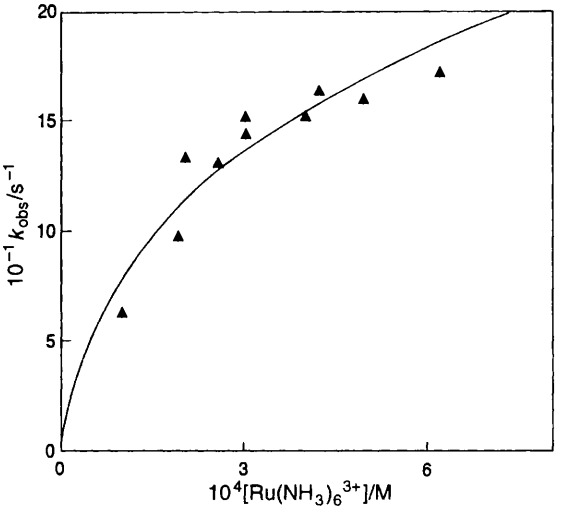

Fig. 5 Non-linear variation of first-order rate constants for the $[Ru(NH_3)_6]^{3+}$ oxidation of Rd^{II} with oxidant concentration (in large excess), at pH 7.0, $I = 0.100$ M (NaCl)

the effect of ionic strength on rate constants referred to by Jacks *et al.*[24] provides any further information regarding the site of reaction, because it has not yet been demonstrated convincingly whether such ionic strength effects stem from variations in the local or overall charge on the protein.

The $[Co(sep)]^{2+}$ reduction of Rd^{III} was studied at pH from 3.8 to 8.8 with no effect on rate constants. There are no His residues in *C. pasteurianum* rubredoxin, which would normally give protonation/deprotonation effects in this range. It can also be concluded that there are no Asp/Glu residues at or near to the reaction site on the surface of the Rd^{III} form undergoing protonation in the pH range investigated. Samples of this form retain their identity over the range of pH explored, with $Fe(Cys)_4^-$ remaining intact.[27] Over the less-extensive range pH 5.2–7.0 the $[Co(terpy)_2]^{3+}$ oxidation of Rd^{II} is also independent of pH.

Some attempt was made to study Cr^{III}-induced line-broadening effects as a means of determining the site or sites for interaction of cationic reactants on the surface of Rd^{III}. However the only amino acid assignments available to us were for the diamagnetic ^{113}Cd-substituted protein.[28] Since both wild type rubredoxin forms (Rd^{II} and Rd^{III}) are paramagnetic the interpretation is not straightforward, and no firm conclusions were possible.

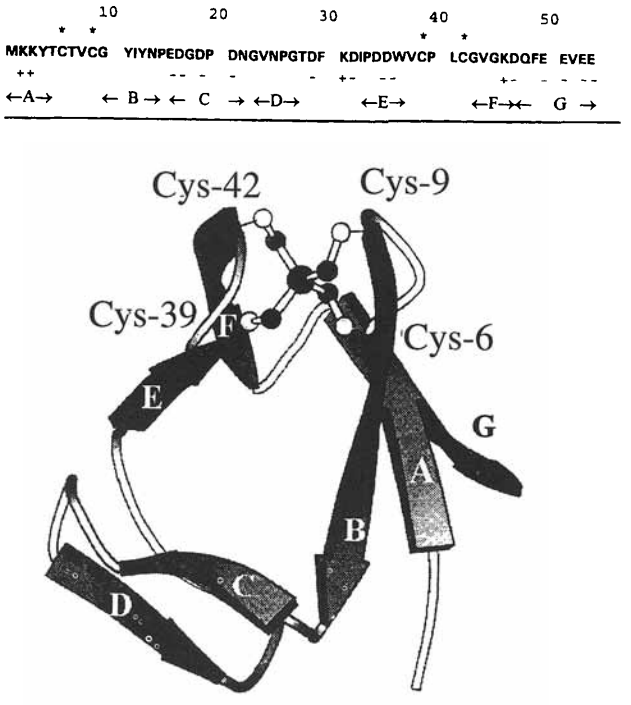

Fig. 6 The protein fold of *Clostridium pasteurianum* rubredoxin, and the amino acid sequence with co-ordinated cysteines (*) and +/− charged residues indicated[6a,26]

In the only previous kinetic study of rubredoxin Jacks *et al.*[24] studied the reductions of the Rd^{III} form with $[Cr(H_2O)_6]^{2+}$, $[V(H_2O)_6]^{2+}$ as well as $[Ru(NH_3)_6]^{2+}$. The ratio k_V/k_{Cr} of rate constants for the $[Cr(H_2O)_6]^{2+}$ (1.2×10^3 M^{-1} s^{-1}) and $[V(H_2O)_6]^{2+}$ (1.6×10^4 M^{-1} s^{-1}) reactions at pH 4.0, $I = 0.10$ M, falls within the range 10–60:1 for a number of different oxidants, which is theoretically accounted for by outer-sphere reaction paths. The rate constant reported for the thermodynamically unfavourable $[Ru(NH_3)_6]^{2+}$ reduction of Rd^{III} is however 34 times that inferred from the present studies on the $[Ru(NH_3)_6]^{3+}$ oxidation of Rd^{II}. The Jacks *et al.*[24] study was under equilibration conditions (27–60% completion), and using an integrated form of the rate law to evaluate rate constants. Saturation kinetic behaviour was not identified or allowed for.

Applicability of the Marcus equations (6) and (7) to these

$$k_{12} = (k_{11}k_{22}K_{12}f)^{\frac{1}{2}} \quad (6)$$

$$\log f = (\log K_{12})^2/4 \log (k_{11}k_{22}/Z^2) \quad (7)$$

studies was also investigated, where k_{12} is the rate constant for a cross-reaction having an equilibrium constant K_{12}, k_{11} and k_{22} are electron self-exchange rate constants for the relevant couples, and Z is the frequency factor 10^{11} M^{-1} s^{-1}. The approach was to calculate k_{22}, using experimentally determined k_{12} (K_{12} was calculated from $\Delta E^{\circ\prime}$), as well as previously determined k_{11} values for the inorganic couple involved. Since the site(s) for reaction have not been determined, and might well vary for different oxidants and reductants, no allowance for work terms was attempted. Values of k_{22} obtained for the different redox partners making use of self-exchange rate constants for $[Co(sep)]^{2+/3+}$ (5),[18] $[Co(terpy)_2]^{2+/3+}$ (400)[29] and $[Ru(NH_3)_6]^{2+/3+}$ (3.2×10^3 M^{-1} s^{-1}),[23] are in the range 10^3–10^7 M^{-1} s^{-1}. None of the values was in satisfactory agreement with the electron self-exchange rate constant of 1.6×10^5 M^{-1} s^{-1} determined by NMR measurements.[14] The value of $\approx 10^9$ M^{-1} s^{-1} recently used by Solomon and co-workers[30] from ref. 24 certainly does not apply.

Acknowledgements

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