Preparation and Intramolecular Electron-transfer Rate Constant for the Ruthenium-modified Selenium-substituted [4Fe–4Se] High-potential Protein from *Chromatium vinosum* and Related Studies†

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The Se-substituted [4Fe–4Se] form of the high-potential iron–sulfur protein from *Chromatium vinosum* (M_r ≈9500, 85 amino acids) has been prepared and Ru-modified (68% yield) at the single histidine residue His 42. The purified product gives an Fe:Ru ratio close to 4:1 consistent with a single stoichiometric modification. The His 42 of the Ru-modified protein no longer reacts with $O(CO_2Et)_2$ and the sharp ^1H NMR C_2H resonance for His 42 at δ 8.38 is no longer present due to paramagnetic line broadening by the attached Ru^{III}. The His 42 is bonded *via* Cys 43 to the active site, with an edge-to-edge distance close to 7.9 Å. Pulse-radiolysis reduction of the fully oxidised $Fe_4Se_4^{3+}$–Ru^{III} protein with e_{aq}^- gives the metastable product Fe_4Se^{3+}–Ru^{II}. A second slower stage, corresponding to conversion of the $Fe_4Se_4^{3+}$–Ru^{II} to $Fe_4Se_4^{2+}$–Ru^{III} is made up of intramolecular (1.30 s^{-1}) and intermolecular (2.65 × 10^5 M^{-1} s^{-1}) electron-transfer steps. The k_{intra} step is of interest alongside the earlier value (18 s^{-1}) for native [4Fe–4S] protein. The smaller reduction potential for the $Fe_4Se_4^{3+/2+}$ (285 mV) as compared to $Fe_4S_4^{3+/2+}$ (350 mV) couple is believed to be the major contributing factor to the slower intramolecular electron-transfer process. The Beratan–Onuchic pathways program has been used and indicates through-bond electron transfer from the Ru at His 42 to the cluster as the most favourable route. The rate constant at 25 °C (M^{-1} s^{-1}) for the $[Fe(CN)_6]^{3-}$ oxidation of the $Fe_4Se_4^{2+}$ protein has also been determined (4.5 × 10^3 M^{-1} s^{-1}), and is less than that for native $Fe_4S_4^{2+}$ protein (2.0 × 10^3 M^{-1} s^{-1}) at pH 7.0, I = 0.100 M (NaCl).

High-potential iron–sulfur protein (hipip) from the photosynthetic bacterium *Chromatium vinosum* is one of the most extensively characterised [4Fe–4S] proteins.[1,2] The X-ray crystal structure has been reported for both the oxidised and reduced oxidation states.[3,4] The protein is acidic, with isoelectric points pI 3.88 (oxidised) and 3.68 (reduced),[5] and from the amino-acid composition has an estimated charge of −3 for the oxidised form at pH ≈7.0.[6] It contains a single cubane-like Fe_4S_4 cluster with the iron atoms co-ordinated to four cysteinyl sulfurs of a single polypeptide chain of 85 amino acids (M_r ≈9500).[6] Structurally the active site has similar features to that of Fe_4S_4 in 2[4Fe–4S] ferredoxins.[7] However the reduction potential (all $E°$ values *vs.* normal hydrogen electrode, NHE) of hipip is 350 mV whereas that for the ferredoxins is around −400 mV. The three-state hypothesis of Carter[7] accounts for the different $Fe_4S_4^{3+/2+}$ (hipip) and $Fe_4S_4^{2+/+}$ (ferredoxin) redox changes which are observed. Recently the replacement of acid-labile sulfurs in the cube of *C. vinosum* hipip by selenium has been reported.[8,9] The substituted protein has been fully characterised, and its properties determined.[8,9] The UV/VIS spectrum of the Fe_4Se_4 analogue is very similar to that of the native protein, and the EPR and NMR spectra also have similar features.[8–10] However the reduction potential is 285 mV,[8] which is 65 mV less than that of native hipip under similar conditions.

A particular focus in the case of native hipip is the single histidine residue at His 42. This surface residue is bonded to Cys 43, the thiolate side chain of which is co-ordinated to an Fe of the active site. In a previous study the His 42 of native hipip was $Ru(NH_3)_5$-modified, and the rate constant for intramolecular electron transfer studied.[11] Here we report similar studies for the [4Fe–4Se] analogue.

† *Non-SI units employed:* M = mol dm^{-3}, eV ≈ 1.60 × 10^{-19} J.

Experimental

Many of the procedures relating to the ruthenium modification were as previously described.[11] The $[Ru(NH_3)_5(Him)]^{2+/3+}$ couple (Him = imidazole) of the Ru-modified hipip has been shown to have an $E°$ of 150 mV *vs.* NHE at pH 6.9 by direct cyclic voltammetry at a pyrolytic graphite 'edge' electrode.[12]

Preparation of Se-*Substituted hipip.*—Native *C. vinosum* hipip was first isolated and purified according to a recent procedure.[13] Replacement of the Fe_4S_4 active site by Fe_4Se_4 was then carried out as previously described.[8] Purification of the Se-substituted hipip product was carried out on a Mono-Q (HR 5/5) FPLC (Pharmacia) column in 20 mM Tris–HCl buffer [Tris = tris(hydroxymethyl)methylamine] at pH 7.50. Oxidized protein with an absorbance ratio $A_{283}/A_{480} \leqslant 2.70:1$ was used for modification.

Preparation of $[Ru(NH_3)_5(H_2O)]^{2+}$.—The pentaammineaquaruthenium(II) complex $[Ru(NH_3)_5(H_2O)][PF_6]_2$[14] was prepared by a modified procedure. The ruthenium(III) trifluoromethanesulfonate complex $[Ru(NH_3)_5(CF_3SO_3)][CF_3SO_3]_2$ was used in place of $[Ru(NH_3)_5Cl]Cl_2$ due to its greater solubility.[15] The complex $[Ru(NH_3)_5(H_2O)]^{2+}$ was obtained by reduction of $[Ru(NH_3)_5(CF_3SO_3)]^{2+}$ with Zn/Hg amalgam under an argon atmosphere. The product was stored under argon and used within 2 weeks of preparation.

Buffers.—The buffers Tris (pK_a 8.08), *N'*-(2-hydroxyethyl)-

piperazine-*N*-ethanesulfonic acid (hepes, pK_a 7.50) and 2-(morpholino)ethanesulfonic acid (mes, pK_a 6.10), all from Sigma Chemicals, were used at different stages. Non-radical-forming phosphate buffer, made up from dipotassium hydrogenphosphate and potassium dihydrogenphosphate (BDH, AnalaR), was used in the pulse-radiolysis experiments to maintain the pH at 7.0.

Procedure for Ruthenium Modification.—A sample of Se-substituted hipip (15 mg) in hepes buffer (20 mM, 4 cm^3) at pH 7.50 was treated with $[Ru(NH_3)_5(H_2O)][PF_6]_2$ (35 mg) in the same buffer (5 cm^3), I = 0.10 M (NaCl). The reaction was carried out under rigorous air-free conditions and terminated by gel filtration on a Sephadex G25 column (3.0 × 30.0 cm) after 50 min. The column was previously equilibrated with 0.10 M phosphate buffer (pH 7.0) under argon. The (unseparated) protein eluted was collected and oxidised with an excess of $[Fe(CN)_6]^{3-}$. This solution was then ultrafiltered with 20 mM Tris–HCl buffer at pH 7.50. Assuming that $\varepsilon = 1.6 \times 10^4$ M^{-1} cm^{-1} at 480 nm the total recovery was ≈80%.

The mixture of products was separated by FPLC (fast protein liquid chromatography) with a Mono-Q anion-exchange column (equilibrated in 20 mM Tris–HCl at pH 7.50). The main product did not absorb on the column, but eluted at 0% of a 1 M NaCl gradient with two other small fractions at 7–8 (unmodified) and 100% NaCl gradient (denatured product). The major fraction was exchanged into mes buffer (20 mM, ph 6.0), and subjected to further separation on a Mono-S cation-exchange column. Again the main fraction of singly modified product was eluted at 0% NaCl, with a small fraction of multiply modified product at 6% NaCl.

ICP Analyses.—Ratios of Fe:Ru were determined at the Johnson Matthey Technology Centre, Sunning Common, Reading, by inductively coupled plasma (ICP) atomic emission spectroscopy (Bausch and Lamb ARL 3580 spectrometer), and found to be 4.00:1.05 and 4.00:1.79 for the singly- and multiply-modified fractions respectively.

UV/VIS Spectra.—Charge-transfer bands in the spectra of the oxidised and reduced forms of the Se-substituted protein did not show any red shift and are similar to those of native hipip.[8] The spectrum of the fully oxidised $Fe_4Se_4^{3+}$–Ru^{III} protein was obtained by treating with an excess of $[Fe(CN)_6]^{3-}$ (reduction potential of 3 − /4 − couple 410 mV), and that of $Fe_4Se_4^{2+}$–Ru^{III} by reduction with an excess of $[Co(terpy)_2]^{2+}$, terpy = 2,2′:6′,2″-terpyridine (reduction potential of 2+/3+ couple 270 mV), both at pH 7.0 (0.1 M phosphate). In each case the excess of inorganic reagent was removed by Amicon filtration using either YM5 or YM3 membranes. The protein has strong absorbance in the UV region, and no contribution from the $[Ru(NH_3)_5(Him)]^{3+}$ centre could be detected at 300 nm (ε = 2100 M^{-1} cm^{-1}).[16]

depc Modification.—The UV/VIS scan spectra (25 °C) were recorded for the reaction of the oxidised form of the Se-substituted protein (69.9 μM), and its ruthenium derivative (42.0 μM), with a ≈25-fold excess of diethyl pyrocarbonate (diethyl oxydiformate) $O(CO_2Et)_2$ (depc, Sigma) at pH 7.0 (0.10 M phosphate). After 25 min the reaction with the Se-substituted protein was ≈95% complete based on the absorption of the *N*-ethoxyimidazole product at 240 nm (ε = 3200 M^{-1} cm^{-1}).[17] The Ru-modified derivative gave no absorbance change in the region of 240 nm. It could be concluded therefore that the ruthenium attachment was at His 42.

^1H *NMR Spectra.*—Proton NMR spectra (field 300 MHz) were obtained for the Se-substituted protein in the $Fe_4Se_4^{2+}$ state and the $Fe_4Se_4^{2+}$–Ru^{III} derivative (≈2 mM) in 0.10 M deuteriated phosphate buffer at pH 7.0 (not corrected for the deuterium isotope effect). Spectra were referenced to the internal water signal at δ 4.80. The previously assigned C_2H histidine resonance at δ 8.38[18] is no longer present in the case of the ruthenium(III) derivative as a result of line broadening by the paramagnetic Ru^{III} attached to His 42.

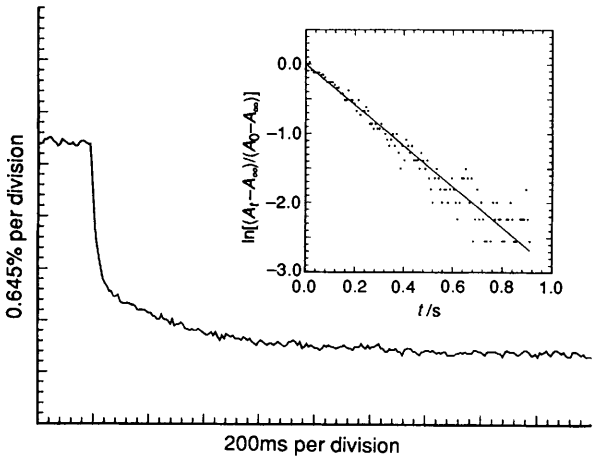

Fig. 1 Absorbance changes (monitored as voltage) at 480 nm for the second-stage decay following reaction of e_{aq}^- with $Fe_4Se_4^{3+}$–Ru^{III}. The inset shows the corresponding first-order plot

Pulse Radiolysis.—Experiments were carried out at the Cookridge Radiation Research Centre using standard procedures.[19] A reaction cell of 2.5 cm path length was used. The temperature was 19.0 ± 1.5 °C. The ionic strength was I = 0.100 M from the phosphate buffer (no NaCl added). As in previous studies[10] the hydrated electron e_{aq}^- was used as the reductant. The observed dose in each pulse was measured by a secondary emission monitor (SEC) which was calibrated by thiocyanate dosimetry.[20] Doses used were such that less than 10% of the protein was reduced by each pulse. Other radical species $^{\bullet}OH$ and $^{\bullet}H$ were removed by reaction with Bu^tOH (0.03 M) to give the relatively unreactive 2-hydroxy-2-methylpropyl radical.

Protein solutions were prepared in argon-saturated phosphate buffer at pH 7.0 (45 mM). Care was taken to avoid any denaturation or autoreduction of the protein during bubbling. Absorption changes associated with the reduction of $Fe_4Se_4^{3+}$ were monitored at 480 nm ($\Delta\varepsilon = 10^4$ M^{-1} cm^{-1}), and the decay of the e_{aq}^- could be monitored (check runs only) at 700 nm. Signals were recorded on a Gould 4072 100 MHz Transient Digitiser and were transferred to a DEC 11/73 computer for storage and processing. The pulse-radiolysis traces were treated for first-order kinetic behaviour using the program TREAT.[21] Plots of $-\ln[(A_t - A_\infty)/(A_0 - A_\infty)]$ against time were linear for at least three half-lives, Fig. 1.

Stopped-flow Studies.—The oxidation of $Fe_4Se_4^{2+}$ protein (1.32×10^{-5} M) with $[Fe(CN)_6]^{3-}$ [$(0.8–2.7) \times 10^{-4}$ M] at pH 7.0 (10^{-2} M phosphate), I = 0.100 M (NaCl), was monitored at 500 nm on a Dionex-D110 stopped-flow spectrophotometer. Rate constants (25.0 ± 0.1 °C) were obtained using fitting procedures and programs from OLIS (Bogart, GA, USA).

Results

Pulse Radiolysis.—The rate constant for the reaction of e_{aq}^- with the Se-substituted $Fe_4Se_4^{3+}$ protein was determined to be (3.3 ± 0.3) × 10^{10} M^{-1} s^{-1} and no further absorption changes at 480 nm were noted on time-scales up to 0.2 s per division. For the reaction of e_{aq}^- with the Ru-modified derivative, $Fe_4Se_4^{3+}$–Ru^{III}, the rate constant was (3.1 ± 0.2) × 10^{10} M^{-1} s^{-1} partitioned between reduction at the ruthenium (68%) and Fe_4Se_4 (32%) centres, equation (1). Subsequently the metastable

Table 1 Rate constants (19 °C) for decay of the metastable $Fe_4Se_4^{3+}$–Ru^{II} in pulse-radiolysis experiments at pH 7.0 (45 mM phosphate), I = 0.100 M, monitored at 480 nm

10^6[Protein]*/M	2.36	2.36	2.36	3.5	3.5	3.5	3.5	4.5	4.5	4.5
k_{obs}/s^{-1}	1.9	1.9	2.0	2.1	1.9	2.1	1.9	3.3	3.3	3.7
10^6[Protein]*/M	4.5	6.3	6.3	6.3	6.3	6.3	7.4	7.4	7.4	9.2
k_{obs}/s^{-1}	2.6	3.6	2.6	3.4	2.7	3.2	3.1	2.6	2.8	3.3
10^6[Protein]*/M	9.2	9.2	12.3	12.3	12.3					
k_{obs}/s^{-1}	2.8	2.9	4.6	4.7	4.6					

* Concentration of $Fe_4Se_4^{3+}$–Ru^{III} used.

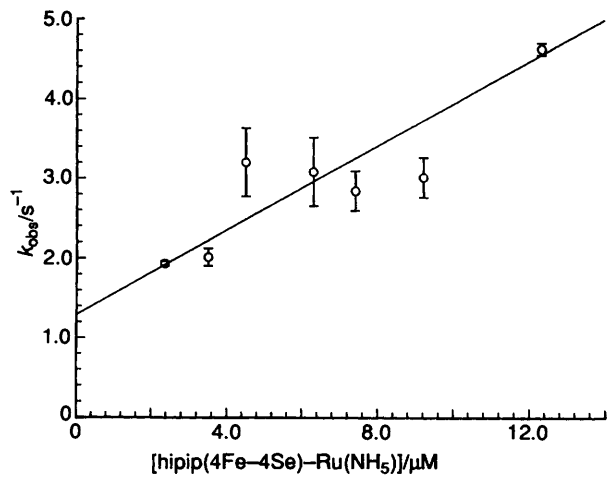

Fig. 2 The dependence of first-order rate constants k_{obs} (19 °C) for the second stage observed in the pulse radiolysis of $Fe_4Se_4^{3+}$–Ru^{III} on total protein concentration, pH 7.0, I = 0.100 M. The intercept and slope correspond to intra- and inter-molecular steps respectively, equations (2) and (3)

$$Fe_4Se_4^{3+}\text{–}Ru^{III} + e_{aq}^- \rightarrow \begin{cases} Fe_4Se_4^{2+}\text{–}Ru^{III} \text{ (stable)} \\ Fe_4Se_4^{3+}\text{–}Ru^{II} \text{ (metastable)} \end{cases} \quad (1)$$

form is transformed into the stable form and this process can be monitored on timebases of 0.2–0.5 s per division. The rate constants, k_{obs}, which are listed in Table 1, are dependent on the protein concentration which implicates intra- and inter-molecular electron-transfer contributions as defined in equations (2) and (3). Thus, k_{obs} can be expressed as in equation

$$Fe_4Se_4^{3+}\text{–}Ru^{II} \xrightarrow{k_{intra}} Fe_4Se_4^{2+}\text{–}Ru^{III} \quad (2)$$

$$Fe_4Se_4^{3+}\text{–}Ru^{II} + Fe_4Se_4^{3+}\text{–}Ru^{III} \xrightarrow{k_{inter}} Fe_4Se_4^{3+}\text{–}Ru^{III} + Fe_4Se_4^{2+}\text{–}Ru^{III} \quad (3)$$

(4), Fig. 2. A weighted linear-regression treatment gives k_{intra} = 1.30 ± 0.04 s^{-1} and k_{inter} = (2.65 ± 0.01) × 10^5 M^{-1} s^{-1}.

$$k_{obs} = k_{intra} + k_{inter}[Fe_4Se_4^{3+}\text{–}Ru^{III}] \quad (4)$$

Stopped-flow Studies.—Rate constants (25 °C) were determined at pH 7.0 for the oxidation of the $Fe_4Se_4^{2+}$ protein with $[Fe(CN)_6]^{3-}$, which was present in > 10-fold excess, equation (5). Reactions were monitored at 500 nm ($\Delta\varepsilon \approx 10^4$ M^{-1} cm^{-1}).

$$Fe_4Se_4^{2+} + [Fe(CN)_6]^{3-} \longrightarrow Fe_4Se_4^{3+} + [Fe(CN)_6]^{4-} \quad (5)$$

First-order kinetic plots were linear for up to four half-lives and gave rate constants k_{obs}, Table 2. A linear plot of k_{obs} *vs.* $[Fe(CN)_6]^{3-}$ was obtained, Fig. 3, the slope of which gives a second-order rate constant k_{Fe} = (4.5 ± 0.1) × 10^3 M^{-1} s^{-1}. This compares with the literature value for the $Fe_4S_4^{2+}$ protein, k_{Fe} = 2.0 × 10^3 M^{-1} s^{-1}, obtained under identical conditions, I = 0.100 M (NaCl).[22]

Table 2 First-order rate constants k_{obs} (25 °C) for the oxidation of Se-substituted $Fe_4Se_4^{2+}$ protein with $[Fe(CN)_6]^{3-}$ in 10^{-2} M phosphate (pH 7.0), I = 0.100 M (NaCl)

$10^4[Fe(CN)_6^{3-}]$/M	k_{obs}*/s^{-1}
0.82	0.41
1.24	0.58
1.65	0.76
2.04	0.93
2.59	1.112

* Average values obtained from at least six different traces.

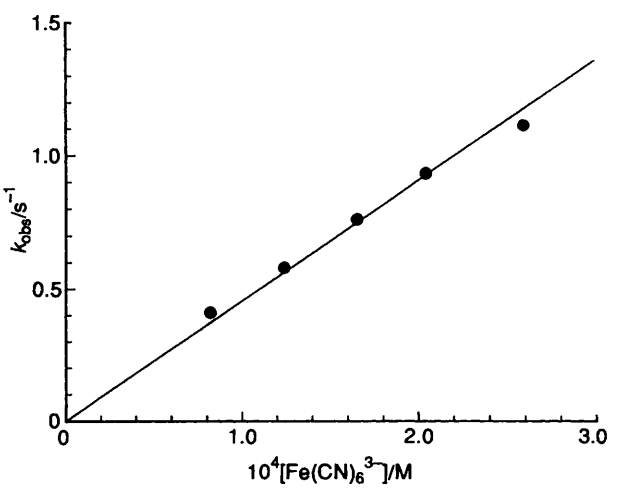

Fig. 3 The linear dependence of first-order rate constants k_{obs} (25 °C) for the $[Fe(CN)_6]^{3-}$ oxidation of the $Fe_4Se_4^{3+}$ form of Se-substituted *C. vinosum* hipip at pH 6.96 (10^{-2} M phosphate), I = 0.100 M (NaCl)

Discussion

The aim of the present work was to explore further the reactivity of hipip on replacing the Fe_4S_4 core by Fe_4Se_4. While there is no evidence for Fe/Se clusters in biology, selenium is known to have biological roles centring around the existence of selenocysteine.[23] Although studies on the replacement of S by Se in Fe/S clusters are now quite extensive,[8,24–28] the effect on electron-transfer reactivity has not so far been explored in any detail. Since the covalent radius of Se^{2-} (1.17 Å) is larger than that of S^{2-} (1.02 Å), and Se has different redox properties, its introduction could bring about significant changes. However the replacement of Fe_4S_4 by Fe_4Se_4 introduces only minor changes in *e.g.* spectroscopic properties, and major structural changes are not evident.[8,10]

The reaction of e_{aq}^- with the $Fe_4Se_4^{3+}$ protein, $k = 3.3 \times 10^{10}$ M^{-1} s^{-1}, is faster than that of native hipip, $k = 1.70 \times 10^{10}$ M^{-1} s^{-1}.[29] With the fully oxidised Se-substituted Ru-modified protein $Fe_4Se_4^{3+}$–Ru^{III} the first stage of the reaction with e_{aq}^- gives $k = 3.1 \times 10^{10}$ M^{-1} s^{-1}, with 68% reduction at Ru^{III} and 32% at Fe_4Se_4. The second stage is a combination of intramolecular (1.30 s^{-1}) and intermolecular (2.65×10^5 M^{-1} s^{-1}) reactions of the metastable product $Fe_4Se_4^{3+}$–Ru^{II}, equations (2) and (3). The rate constant for the intramolecular step compares with 18 s^{-1} observed under identical conditions in studies on the $Fe_4S_4^{3+}$–Ru^{III} protein. No intermolecular contribution was detected in the Fe_4S_4 study, which may be due to the larger k_{intra} and the fact that high protein concentrations were not used (the highest was 1.83 μM). In the present study a bimolecular step is apparent, Fig. 2. However we did not have unlimited amounts of protein because of preparative procedures involving selenium-substitution and then ruthenium modification, and were not able to carry out as many runs at the higher concentrations as we would have liked. A more critical inspection of Fig. 2 and deletion of the point at highest protein concentration suggests that k_{intra} may also be dominant for Fe_4Se_4 with k_{inter} making little or no contribution. If this is the case then k_{intra} will be somewhat larger than indicated and in the range 2.5 ± 0.5 s^{-1}. The latter is not however sufficiently different to affect any of the arguments and discussions to follow. If k_{inter} contributes with Fe_4Se_4 and not with Fe_4S_4 then it might appear that the larger seleniums at the active site facilitate the bimolecular step by bringing about greater cluster exposure.[30] This is supported by the rate constants with e_{aq}^-, and the self-exchange rate constants (see next paragraph).

Stopped-flow studies on the oxidation of the Se-substituted $Fe_4Se_4^{2+}$ protein with $[Fe(CN)_6]^{3-}$ give a rate constant k_{Fe} (25 °C) of 4.5×10^3 M^{-1} s^{-1}, which compares with the value for native hipip of 2.0×10^3 M^{-1} s^{-1} under identical conditions.[22] For the $[Fe(CN)_6]^{3-}$ reaction with $Fe_4Se_4^{2+}$ the driving force is 125 mV as compared to 60 mV for the corresponding $Fe_4S_4^{2+}$ reaction (pH ≈ 7). Rate constants for the reduction of $Fe_4Se_4^{3+}$ (36 M^{-1} s^{-1}) and $Fe_4S_4^{3+}$ protein (200 M^{-1} s^{-1}) with $[Fe(CN)_6]^{4-}$ can be calculated from the data given. The latter order (S > Se) is the same as for k_{intra}. Self-exchange rate constants for the Se-substituted protein (7.0×10^4 M^{-1} s^{-1}) and for the native hipip forms (1.7×10^4 M^{-1} s^{-1}) have been determined by ^1H NMR line-broadening experiments.[10]

Further understanding of the intramolecular electron-transfer step comes from the application of the Beratan–Onuchic pathways program.[31–33] Rate constants k_{et} for electron transfer between weakly coupled donor–acceptor sites in the protein can be written as in equation (6),[34] where T_{DA} is the tunnelling

$$k_{et} = 2\pi(T_{DA})^2(FC)/h \quad (6)$$

matrix element (electronic coupling) and (FC) is the Franck–Condon factor as defined in equation (7). The tunnelling matrix

$$(FC) = (4\pi\lambda RT)^{-\frac{1}{2}} \exp - [(\lambda + \Delta G^\circ)/4\lambda RT] \quad (7)$$

element T_{DA} strongly depends on the distance d separating the donor and acceptor sites, equation (8),[34,35] where β is a measure

$$T_{DA} \propto \exp(-\beta d) \quad (8)$$

of the intrinsic coupling capability of a particular donor–acceptor system. Thus k_{et} is expected to depend on the electronic coupling (T_{DA}), the driving force ($-\Delta G^\circ$), and the reorganisation energy (λ) for solvent dipoles and redox centre ligands. The Beratan–Onuchic program[31–33] is able to search for and identify the most favourable electron-transfer routes through the protein, and calculate the electronic coupling for each pathway. We are able to use this program knowing the crystal structure coordinates for *C. vinosum* hipip,[3,4] and an averaged β value of 1.2 $Å^{-1}$.[31] The best electron-transfer route from His 42 (C^γ of the imidazole) to Cys 43 (S^γ atom, Fe/S cluster ligand) involves the seven connecting covalent bonds, and has a maximum electronic coupling of 2.80×10^{-2}. The edge to edge distance so defined is 7.9 Å, or 13 Å through bond, from the Fe_4S_4 structure of [4Fe–4S] hipip,[3,4] and can be assumed to be about the same in the Fe_4Se_4 case.

Since the electron-transfer routes in Ru-modified [4Fe–4S] and [4Fe–4Se] proteins are most probably the same, similar electronic coupling might be expected in both cases. The self-exchange rate constants[10] also suggest that the two forms have similar electronic couplings with the surrounding polypeptide and a similar reorganisation energy is assumed therefore. The reorganisation energy (λ) for intramolecular electron transfer can be calculated from equations (9) and (10),[34,36] where ΔG_{11}^*

$$\Delta G_{12}^* = \frac{\Delta G_{11}^* + \Delta G_{22}^* + \Delta G_{12}^\circ}{2} + \frac{\Delta G_{12}^{\circ 2}}{8(\Delta G_{11}^* + \Delta G_{22}^*)} \quad (9)$$

$$\Delta G_{12}^* = \lambda/4 \quad (10)$$

and ΔG_{22}^* are the reorganisation energies for the $Fe_4S_4^{3+/2+}$ (12.9 kcal mol^{-1})[37] and closely related $[Ru(NH_3)_5(py)]^{3+/2+}$ (py = pyridine) (6.9 kcal mol^{-1})[38] self-exchange processes, ΔG_{12}° is the free-energy change for the intramolecular electron-transfer step (−3.8 kcal mol^{-1}), and ΔG_{12}^* is the reorganisation free energy (1 kcal = 4.2 kJ). From this treatment a value λ = 1.41 eV is obtained for the intramolecular electron-transfer process step.

A value of the rate constant for $Fe_4S_4^{3+}$–Ru^{II} intramolecular electron transfer has been calculated with reference to the corresponding reactions $Fe_4Se_4^{3+}$–Ru^{II} and cytochrome c(III)–Ru^{II} (often employed as a reference) using equation (11).[33]

$$\frac{k}{k_{ref}} = \frac{(T_{DA})^2}{(T_{DA})_{ref}^2} \cdot \frac{(FC)}{(FC)_{ref}} \quad (11)$$

Values of $-\Delta G^\circ$ are 0.165, 0.230 and 0.19 eV for the Fe_4Se_4 (hipip), Fe_4S_4 (hipip) and cytochrome c reactions respectively. The tunnelling matrix element $T_{DA} = 2.8 \times 10^{-2}$ and reorganisation energy of λ = 1.41 eV were used for Fe_4Se_4 (hipip) and Fe_4S_4 (hipip) whereas $T_{DA} = 2.83 \times 10^{-4}$ and λ = 1.2 eV for cytochrome c. With the Fe_4S_4 (hipip) reaction as reference the calculated value is 5.9 s^{-1} which, considering the approximations made, is in reasonable agreement with the experimental value of 1.3 s^{-1}. However with cytochrome c as reference the calculated rate constant of 2.4×10^4 s^{-1} is ≈10^4 times higher indicating fundamental differences. It is interesting to compare the intramolecular rate constants for the Ru-modified derivatives of 18 (Fe_4S_4)[11] and 1.3 s^{-1} (Fe_4Se_4) with those for Ru-modified cytochrome c at 30 or 53 s^{-1}.[39,40] The electron-transfer reaction for hipip is surprisingly slow in spite of the strong electronic coupling ($T_{DA} = 2.80 \times 10^{-2}$) as compared with cytochrome c ($T_{DA} = 2.83 \times 10^{-4}$). The small rate constants for hipip may be due to a significantly higher activation barrier for the intramolecular step. In other words, the calculated reorganisation energy (λ) may have been underestimated in this appraisal and a value of around 2 eV may be more appropriate.

Hence, despite the direct connection between the ruthenium probe and the cluster mediated by two amino acids, no privileged electron-transfer pathway seems to be involved in the His 42 region of the protein. Indeed, were the route under study of significance, and the same as is operative in the self-exchange process, the reactivity of the selenium derivative might have been expected to translate into a larger intramolecular rate constant for Ru-modified derivative. It has also been suggested from recent NMR results on hipip[41,42] that electron transfer may involve the pair of Fe atoms ligated by the Cys 43

and Cys 46 residues. Because of the low electron-transfer reactivity of the Ru-modified protein it would appear that one ligand in this pair (Cys 43) is unlikely to play a prominent role in the self-exchange and biological electron-transfer processes.

From the structure of *C. vinosum* hipip the Fe_4S_4 cluster is to some extent buried inside the protein matrix. One side of the cluster is however closer to the surface of the protein,[43] and the side chain of Cys 46 is part of this area. It has also been observed[44] that heterogeneity in the EPR and Mössbauer spectra recorded on frozen solutions could be explained by association of two hipip molecules bringing the clusters into relatively close proximity. Considering these proposals in the case of Ru–Se–hipip, one may imagine that reoxidation of Ru^{II} at the surface of the molecule can occur either by intramolecular electron transfer through a poor pathway (see above) or by reaction with a nearby molecule through a more efficient route. According to the previously proposed site of interaction,[43] the latter would involve Cys 46 in line with NMR results.[41,42] This view neither contradicts previous suggestions about the asymmetric involvement of the cysteine ligands in the redox transition,[8] nor the specific status of the Cys 46 inferred from the value of its Fe–S–C–C dihedral angle deduced from X-ray crystallographic data.[45]

In further work there is need to check the detailed mechanism of electron transfer in *C. vinosum* hipip with data from other Ru-modified forms of the protein. Since a single His only is present in the primary structure of hipip, such experiments will have to await the availability of recombinant hipip, and of suitable His-containing mutants. In conjunction with modifications in the protein sequence, comparative studies involving [4Fe–4S] and [4Fe–4Se] forms should provide useful information, as exemplified here with the native polypeptide chain.

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