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Electron transfer between multihaem cytochromes c_3 from *Desulfovibrio africanus*

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ABSTRACT

The tetrahaem type I cytochromes c_3 from *Desulfovibrionaceae* shuttle electrons from a periplasmic hydrogenase to transmembrane electron transfer complexes. In *D. africanus*, it is believed that the electrons are received by another tetrahaem cytochrome c_3, denoted type II, which is associated with the membrane complex. Thermodynamic measurements show that the type I cytochrome c_3 has the potential to transfer two electrons at a time. This study uses two-dimensional NMR to investigate the exchange of electrons between type I and type II cytochromes c_3 at equilibrium in intermediate stages of oxidation. The results indicate that the two proteins are physiological partners but that only single-electron transfers occur in solution.

1. Introduction

Cytochromes c_3 are a group of small (~15 kDa) and very stable globular tetrahaem proteins. The polypeptide chains comprise 102–118 amino acid residues and the four *c*-type haems have bis-histidinyl coordination. They are expressed by several members of the *Desulfovibrionaceae* family and, despite low homology in amino acid sequence [1], the general folding and the haem architecture are strictly conserved [2]. Furthermore, the soluble cytochromes c_3, called type I (TpIc_3), possess a conserved patch of positive charges close to haem IV, composed mostly of lysine residues, that is supposed to be the site of interaction with its partners [3]. They are present in large quantities in the periplasm and are proposed to act as mediators between periplasmic hydrogenases and transmembrane electron transfer complexes [2]. In the membrane, the electron acceptor is thought to be one of the multihaem cytochromes associated with the transmembrane complexes. The complexes in *Desulfovibrio africanus* and *D. vulgaris* include a tetrahaem cytochrome c_3, denoted type II (TpIIc_3), which are structurally similar to the periplasmic ones but lack the lysine patch [4]. Perhaps because of the absence of a lysine patch, TpIIc_3 shows low reactivity with hydrogenase, but the presence of TpIc_3 increases the rate of electron transfer between those two proteins [5]. Hence, it is proposed that TpIc_3 and TpIIc_3 are physiological partners, with TpIc_3 receiving electrons from hydrogenase and then delivering them to TpIIc_3 associated to the transmembrane complex. However, despite evidence of complex formation between TpIc_3 and TpIIc_3 [6], the two proteins have not been shown to interact functionally.

The thermodynamic properties of several TpIc_3 have been studied in detail [7]. In all cases, cooperative processes have been observed, namely positive homotropic cooperativity, where accepting one electron facilitates the acceptance of a second one. This led to speculation about the possibility of a two-electron transfer step (in which the two electrons are transferred within the lifetime of the encounter complex between the partner proteins) that would efficiently channel the electrons from hydrogenase to the membrane acceptor. TpIc_3 could transport several electrons acquired in separate collisions, but this would be less efficient. Although the proteins possess the necessary thermodynamic properties for the transfer of two electrons to be favourable [7], a two-electron transfer step has never been observed experimentally. Therefore, we sought to determine the nature of electron transfer between *D. africanus* TpIc_3 and the soluble *D. africanus* TpIIc_3.

The study of exchange processes by NMR has a long history [8] and formed one of the earliest applications of two-dimensional NMR [9]. The so-called EXSY experiment is essentially identical to the normal 2D-NOESY experiment where, during the mixing time (τ_m) between the second and third 90° pulse, magnetization may be transferred between the nuclei. If the exchange rate is slow with respect to the difference of the chemical shifts of the nuclei, a cross peak appears in the 2D-spectrum with an intensity proportional to the amount of exchange that occurred during τ_m. The peculiar advantage of NMR is that nuclei are frequency labelled in individual molecules so that exchange can be studied in systems at equilibrium.

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The EXSY method is not often applied to proteins because of the extensive overlap between peaks that occurs in complex spectra. However, the signals of the methyl substituents of haems in oxidized cytochromes are pushed out of the main envelope by interaction with the paramagnetic low-spin FeIII. Because the intramolecular electron exchange is fast in multihaem cytochromes but intermolecular exchange is typically slow, each haem methyl group in a protein molecule at a particular stage of oxidation gives rise to a single averaged signal. Since the tetrahaem cytochromes may release from 0 to 4 electrons, each methyl resonance has 5 possible frequencies in the macroscopic oxidation stages, which are labelled 0 to 4. In a solution of partially oxidized cytochrome, molecules at different stages of oxidation are in dynamic equilibrium, with intermolecular electron transfers occurring at a rate that is typically fast enough to generate cross peaks in two-dimensional exchange spectra but slow enough to cause minimal line broadening [10–12].

Individual exchange rate constants may be obtained from the initial rate of build-up of cross peak intensities with respect to the mixing time, τ_m, or, better, by non-linear fits to the full set of intensities. The matrix of intensities, **S**, in a two-dimensional exchange spectrum [9] of a system undergoing relatively slow exchange is given by

$$\mathbf{S} = exp(\mathbf{L}\tau_m)\mathbf{M}_0 \quad (1)$$

where $\mathbf{M}_0$ is a diagonal matrix containing the equilibrium populations, τ_m is the mixing time, and **L** is the exchange matrix

$$\mathbf{L} = \begin{pmatrix} -R_0 - \sum_j k_{j0} & k_{10} & \cdots & k_{40} \\ k_{01} & -R_1 - \sum_j k_{j1} & \cdots & \cdots \\ \cdots & \cdots & \cdots & \cdots \\ k_{04} & \cdots & \cdots & -R_4 - \sum_j k_{j4} \end{pmatrix} \quad (2)$$

in which k_{ij} are rate constants and R_i are longitudinal relaxation rates. However, because the forward and reverse fluxes are equal for a system at equilibrium, the matrix product $\mathbf{LM}_0$ is symmetric and Eq. (1) can be reformulated as

$$\mathbf{S} = \mathbf{M}_0^{1/2} exp\left(\mathbf{M}_0^{-1/2}\mathbf{L}\mathbf{M}_0^{1/2}\tau_m\right)\mathbf{M}_0^{1/2} \quad (3)$$

in which the exponent is a symmetric matrix.

In this work, we investigate the intermolecular electron transfer rates of TpIc_3 from *D. africanus* in intermediate stages of oxidation. Stages 0–3 are observed simultaneously, hence we must consider four populations, four relaxation rates, and six exchange rates in the analysis using Eq. (3). The exchange rates comprise three single electron transfers as well as possible two- and three-electron transfers. Adding catalytic quantities of TpIIc_3 then allows us to measure the potentially physiological electron transfer rates between the two cytochromes.

Even if only one electron could be transferred in each collision, cross peaks would appear in the NMR spectra that connect stages separated by two or more. These result from successive collisions and electron transfers occurring during the mixing period. A mechanism involving the transfer of two electrons within a single collisional event would lead to an excess of intensity in cross peaks connecting stages separated by two electrons. In this work, we compare the non-linear fits of the complete set of measured intensities to a model in which any number of electrons may be transferred, to the fit to a model in which only single electron transfers are allowed, in order to detect the effect of possible multi-electron transfers.

2. Materials and methods

2.1. Expression and purification

TpIc_3 was purified from *D. africanus* grown as previously described [5]. The soluble fraction resulting from French-press disruption was applied to a DEAE Sepharose Fast Flow ion exchange column (GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 7.6) and eluted with a salt gradient from 0 to 1 M NaCl prepared in the same buffer. The non-adsorbed fractions were collected and loaded onto a SP-sepharose ion exchange column (GE Healthcare) equilibrated and eluted as the previous step. Finally, fractions containing TpIc_3 were inserted onto a Superdex-75 gel-filtration column (GE Healthcare) equilibrated with 100 mM phosphate buffer (pH 7.6). The purification procedure was monitored by SDS-PAGE and UV–visible spectroscopy, with the pure protein presenting a purity index $(Abs_{552}^{red} - Abs_{570}^{red})/Abs_{280}^{ox}$ of 3.6.

Attempts to purify TpIIc_3 from the native organism were not successful. We therefore sought to produce recombinant protein. The DNA sequence coding for TpIIc_3 was amplified from the genomic DNA of *D. africanus* by PCR, using primers containing restriction sites for enzymes NotI and HindIII (Fwd: 5′-GCGGCCGCCCAGGAGGACATGACTC ATGTG-3′ Rev: 5′-AAGCTTCTAATTCTTCACGTGGCATTC-3′). This fragment was subsequently digested with NotI and HindIII and inserted into vector pVA203 [13] digested with the same enzymes, resulting in plasmid pDAC32. The protein was produced using *Escherichia coli* BL21 (DE3) cells co-transformed with plasmids pDAC32 and pEC86 encoding genes for cytochrome *c* maturation [14].

Cells were grown aerobically at 30 °C and 200 rpm in medium 2xYT containing 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol. After 9–10 h, expression was induced by the addition of 10 μM IPTG and the temperature was lowered to 25 °C. After 18–20 h cells were harvested by centrifugation and the periplasmic fraction was isolated as previously described [15]. The purification was performed by column chromatography in three steps: (i) ion exchange on a Q-Sepharose column (GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 7.6) and eluted with a salt gradient from 0 to 1 M NaCl prepared in the same buffer, (ii) gel-filtration on Superdex-75 column (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.6) + 100 mM NaCl, and (iii) ion exchange on a Resource-Q column (GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 7.6) and eluted as (i). The purification procedure was monitored by SDS-PAGE and UV–visible spectroscopy, with the purity index never going beyond 2.6. Despite having only one band in the SDS-PAGE gel, a homogeneous UV–visible spectrum, and the expected NMR spectrum in the reduced form, the NMR spectrum of the oxidised form shows a doubling of the most shifted peaks (Supplementary Material Fig. S1). We attribute this to a non-native form of TpIIc_3 in which the N-terminal glutamine is not cyclised. Expressing the protein more slowly slightly improved the yield of the native form but the two forms could not be separated and so all experiments were done with this heterogeneous material.

2.2. NMR sample preparation

Pure TpIc_3 was lyophilised and resuspended in ^2H_2O with 50 mM KCl and 0.05% NaN_3 to a final concentration of 1 mM. Partial reduction was achieved by the addition of small amounts of a sodium dithionite solution. Small amounts of a concentrated TpIIc_3 solution were added to a final concentration of 1%, 2% and 4% of that of TpIc_3. The pH of the sample was 7.6, measured after each addition. All manipulations were performed inside an anaerobic chamber.

2.3. NMR experiments

Proton NMR spectra of partially oxidised TpIc_3 in the presence of different amounts of TpIIc_3 (0–4%) were obtained in an 800 MHz Bruker Avance III spectrometer equipped with a TXI-Z 5 mm probe. Several 2D-exchange spectroscopy (EXSY) NMR data sets were collected at 298 K with different mixing times (0.25–128 ms in pseudo-random order). Each spectrum comprised 256 increments with 32 scans and 4096 points. The spectral width was 69.4 ppm in both dimensions, centred on the water signal. Crucially, the frequency of the pulses

was offset to the middle of the haem methyl signals to ensure even excitation.

2.4. Data analysis

NMR spectra were transformed, and phase and baseline corrected using Bruker Topspin 2.1 then integrated by lineshape fitting using UCSF Sparky 3.114. Peak intensities were fitted by Eq. (3) using GNU Octave 3.6.1. Fits were performed with rate constants for the exchange of two or more electrons set to zero as well as with the complete set of variables and the results were compared by F-tests.

3. Results

A typical 2D exchange spectrum is shown in Fig. 1, with assignments from [16]. Signals from haem methyl groups I, II and IV 18[1] are visible in oxidation stages 0–3 in each spectrum. There are no signals visible for haem III, which is largely reduced in stages 0–3 and only becomes oxidised in stage 4. This shows that the concentration of stage 4 is negligible; hence the analysis was restricted to stages 0–3.

Data for the seven most shifted methyl groups were fitted separately to Eq. (3) to estimate errors. In principle, different methyl groups might have different relaxation rates, R_i, but no significant differences in relaxation rates were found within each stage and the final analysis used the average of the intensities of corresponding peaks. The fitted data for pure TpIc_3, and TpIc_3 with 4% TpIIc_3 added, are shown in Fig. 2.

In each of the four samples, the fits to Eq. (3) showed that stage 2 was the most abundant species, forming about 50% of the total (see Table 1). Comparison with the proportions calculated from the thermodynamic parameters given in [16] shows that the solution potential was close to −280 mV in each case (Fig. 3). The fitted rates of two- or three-electron transfers were generally smaller than the uncertainties estimated by fitting the data for individual methyl groups, and an F-test showed a probability of 99.9%, 39.3%, 79.0% and 99.9% that these rates were in fact zero for exchange in the presence of 0, 1, 2, and 4% TpIIc_3, respectively. Overall, there is no evidence for the transfer of two or more electrons in a single encounter. Multi-electron exchanges were therefore neglected for the remainder of the analysis. However, cross peaks corresponding to the transfer of two or three electrons appear because of successive electron transfer events occurring during the mixing time.

Electron transfer may occur when two molecules in oxidation stages i and j collide:

$$C_i + C_j \rightarrow C_{i+1} + C_{j-1} \tag{4}$$

with the restrictions in the tetrahaem cytochrome that $i<4$ and $j>0$. The rate constant for this reaction can be predicted by Marcus theory [17] using the energies of the individual forms [16]:

$$k = Z \exp\left(\frac{-\left[\lambda - \left(e_i + e_j\right) + \left(e_{i+1} + e_{j-1}\right)\right]^2}{4\lambda RT}\right) \tag{5}$$

where λ is the reorganisation energy, which has the approximate value of 0.7 eV (0.7 F Jmol^{-1}) in this case [18]. The energies also determine the equilibrium concentrations of the forms,

$$[C_i] = [C_{tot}] \exp(-e_i/\mathrm{RT}) / \sum_j \exp(-e_i/\mathrm{RT}) \tag{6}$$

where $[C_{tot}]$ is the total concentration of cytochrome. Hence the reaction of Eq. (4) gives a flux from C_i to C_{i+1} (and equally from C_j to C_{j-1}) of $k[C_i][C_j]$. It is appropriate to use the concentrations of the

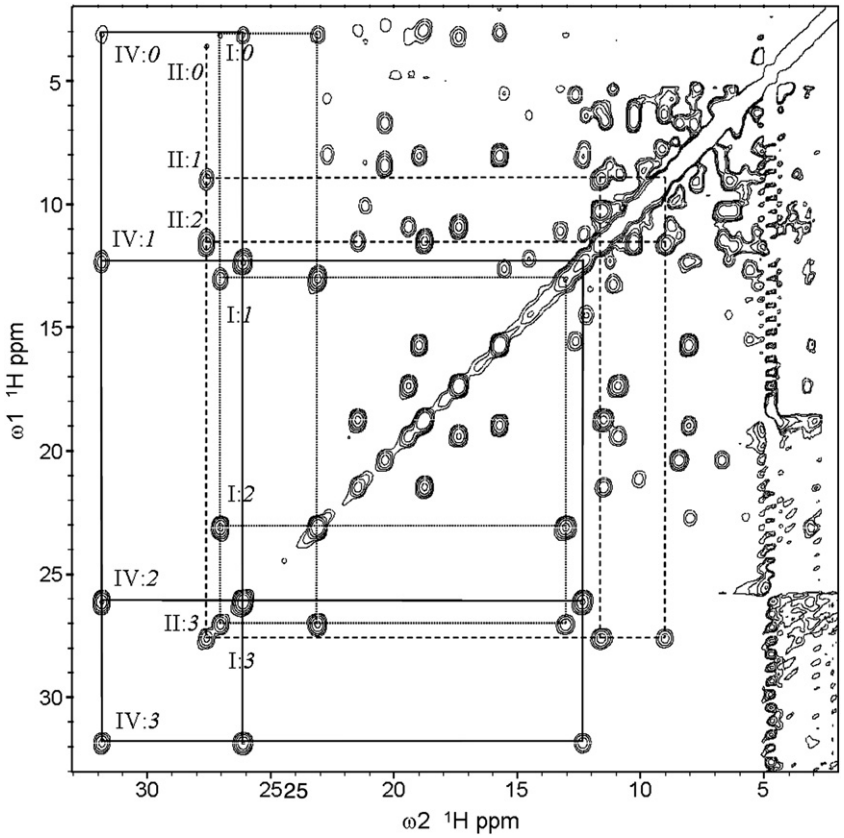

Fig 1. Part of the two-dimensional exchange spectrum of type I cytochrome c_3 from *D. africanus* with a mixing time of 8 ms. The three most shifted haem methyl resonances are labelled with the haem, indicated by a Roman numeral, and the oxidation stage in italic. In each case the cross peaks are connected by lines.

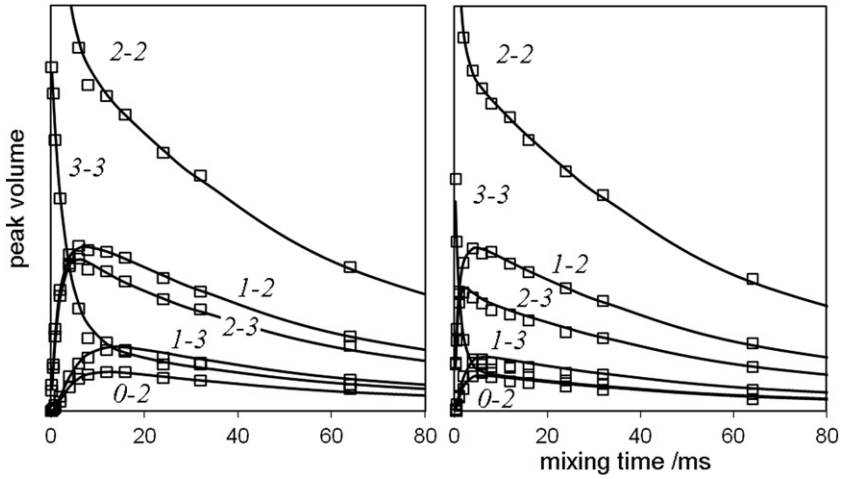

Fig. 2. Raw NMR data and fitted curves for electron transfer in type I cytochrome c_3 from pure *D. africanus* (left panel) and TpIc$_3$ with 4% TpIIc$_3$ (right panel). The italic numerals show the oxidation stages involved in each diagonal or cross peak. The curves were fitted using Eq. (3).

oxidation stages rather than those of the haem microstates because the geometry suggests that intramolecular electron exchange should be at least as fast as the intermolecular electron exchange. There is a complication insofar as TpIc$_3$ has an important protonation site, and the rate of proton transfer is likely to be much slower than that of electron transfer [12]. Therefore, protonated and deprotonated forms should be treated separately. This calculation, using the parameters at 298 K [16], pH 7.6 and a solution potential of −280 mV, assuming equal coupling in all collisions and adding terms from all possible combinations of oxidation stages, gives fluxes $C_0 \rightarrow C_1$, $C_1 \rightarrow C_2$, and $C_2 \rightarrow C_3$ in the ratio 1.0:2.7:2.4. The experimentally determined fluxes between oxidation stages are shown in Fig. 4. The uncertainties for the flux between stages 0 and 1 are greater because of the small population of stage 0 and the fact that cross peaks could only be measured on one side of the diagonal. However, in all cases, it is clear that the flux increases with an approximately linear dependence on the amount of TpIIc$_3$ added. The ratios of the fluxes determined for pure TpIc$_3$ are 1.0:2.9:2.5, which are in reasonable agreement with the ratios predicted by Marcus theory.

The collision frequency, Z in Eq (5), may be approximated by applying the Stokes–Einstein equation to diffusion control:

$$Z = 8RT/3\eta \qquad (7)$$

where the viscosity of water, η, is 0.89 cP at 298 K. The diffusion controlled fluxes in TpIc$_3$ are then calculated to be 770, 2080, and 1840 mMs^{-1}, about thirty times the observed values, as would be expected if not all collisions are able to lead to electron transfer.

Addition of small amounts of TpIIc$_3$ increases the flux between the oxidation stages of TpIc$_3$ molecules in an approximately linear fashion. Since the N-terminus is on the far side of a haem from the proposed binding site [6], the effect of the incomplete cyclisation of the terminal glutamine should be small. The concentrations of TpIIc$_3$ used here, a maximum of 40 μM, are too small for TpIIc$_3$ resonances to be observed directly and analysed, although its assignments are known [19]. However, electron transfer between TpIc$_3$ and TpIIc$_3$ adds to the fluxes between oxidation stages observed for TpIc$_3$. The effect on the flux between oxidation stages 0 and 1 is rather uncertain because of the low concentration of stage 0 and the proximity of signals to the residual water peak, but it agrees with the other values within experimental error: 4% TpIIc$_3$ increases the flux between the oxidation stages of TpIc$_3$ by a factor of 2.5. This implies that the rate of electron transfer between TpIc$_3$ and TpIIc$_3$ is about 37 times (i.e. 1.5/0.04) greater than that between TpIc$_3$ molecules. Allowing for the approximations in Eq. (7), this suggests that the reaction between TpIc$_3$ and TpIIc$_3$ is diffusion controlled.

Table 1
Mole fractions of the oxidation stages of type I cytochrome c_3 from *D. africanus* obtained by fitting NMR data to Eq. (3). The RMSD of the fits for individual methyl groups are given in parentheses.

	Stage *0*	Stage *1*	Stage *2*	Stage *3*
1 mM TpIc$_3$	0.06 (0.01)	0.24 (0.03)	0.48 (0.06)	0.22 (0.02)
+ 1% TpIIc$_3$	0.09 (0.02)	0.28 (0.03)	0.47 (0.04)	0.15 (0.02)
+ 2% TpIIc$_3$	0.09 (0.02)	0.28 (0.03)	0.47 (0.05)	0.16 (0.02)
+ 4% TpIIc$_3$	0.05 (0.01)	0.25 (0.02)	0.51 (0.05)	0.19 (0.02)

4. Conclusions

No evidence was found for two-electron transfers occurring in the lifetime of the TpIc$_3$ encounter complexes. However, electron transfer between TpIc$_3$ and TpIIc$_3$ was much faster than transfer between TpIc$_3$ molecules and essentially diffusion controlled. This is good evidence for the two cytochromes being physiological partners; it has already been shown that TpIc$_3$ is the likely partner of hydrogenase and that TpIIc$_3$ is not (5). The failure to detect two-electron transfers between TpIc$_3$ and TpIIc$_3$ does not eliminate the possibility that TpIc$_3$ serves to transport two electrons and protons from the hydrogenase to the

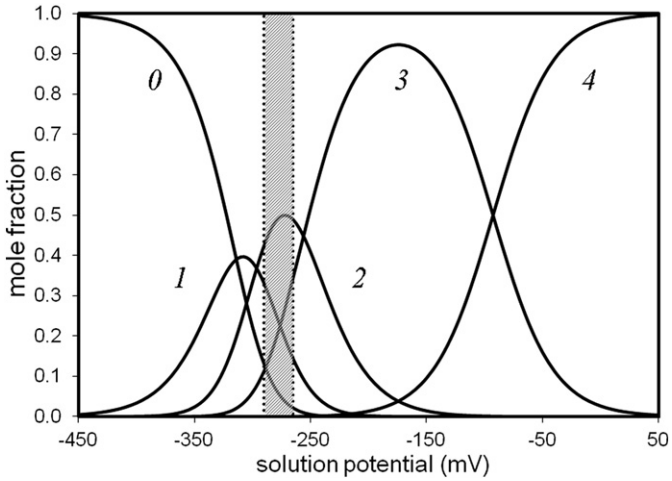

Fig. 3. Mole fractions of the five oxidation stages of type I cytochrome c_3 from *D. africanus* as a function of solution potential. The curves were calculated for pH 7.6 using parameters from [16]. The region of potentials of the NMR samples is indicated by a shaded band.

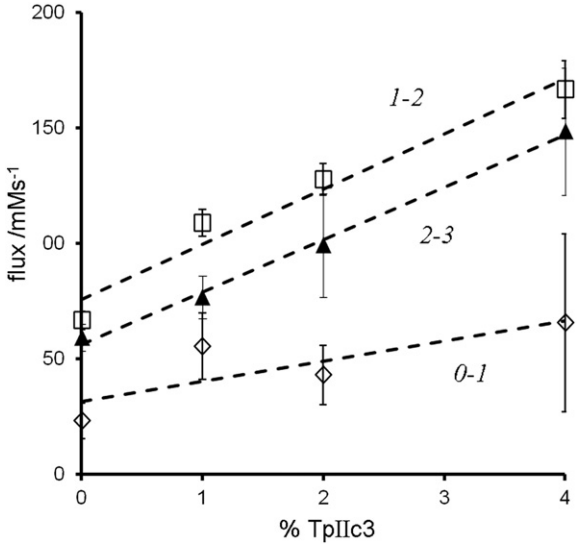

Fig. 4. Flux between oxidation stages 1 and 2 (open squares), stages 2 and 3 (filled triangles) and stages 0 and 1 (open diamonds). The dashed lines are linear fits to the fluxes as a function of added TpIIc$_3$, with error bars from the RMSD of fits for individual methyl groups.

membrane. When TpIIc$_3$ is associated with a membrane complex, electrons should be drained by the complex such that the TpIIc$_3$ is mainly in the fully oxidised form [16]. By contrast, there is only about 1% of TpIIc$_3$ oxidation stage 4 at the solution potential of about −280 mV used here. If a two-electron transfer occurs from TpIc$_3$ to fully oxidised TpIIc$_3$ then it would only be detectable in a non-equilibrium system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.01.014.

References

[1] V. Magro, L. Pieulle, N. Forget, B. Guigliarelli, Y. Petillot, E.C. Hatchikian, Further characterization of the two tetraheme cytochromes c_3 from *Desulfovibiro africanus*: nucleotide sequences, EPR spectroscopy and biological activity, Biochim. Biophys. Acta 1342 (1997) 149–163.
[2] P.M. Matias, I.A. Pereira, C.M. Soares, M.A. Carrondo, Sulphate respiration from hydrogen in *Desulfovibrio* bacteria: a structural biology overview, Prog. Biophys. Mol. Biol. 89 (2005) 292–329.
[3] P.M. Matias, C.M. Soares, L.M. Saraiva, R. Coelho, J. Morais, J. Le Gall, M.A. Carrondo, [NiFe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 27774: gene sequencing, three-dimensional structure determination and refinement at 1.8 A and modelling studies of its interaction with the tetrahaem cytochrome c_3, J. Biol. Inorg. Chem. 6 (2001) 63–81.
[4] S. Norager, P. Legrand, L. Pieulle, C. Hatchikian, M. Roth, Crystal structure of the oxidised and reduced acidic cytochrome c_3 from *Desulfovibrio africanus*, J. Mol. Biol. 290 (1999) 881–902.
[5] L. Pieulle, J. Haladjian, J. Bonicel, E.C. Hatchikian, Biochemical studies of the *c*-type cytochromes of the sulfate reducer *Desulfovibrio africanus*. Characterization of two tetraheme cytochromes c_3 with different specificity, Biochim. Biophys. Acta 1273 (1996) 51–61.
[6] L. Pieulle, X. Morelli, P. Gallice, E. Lojou, P. Barbier, M. Czjzek, P. Bianco, F. Guerlesquin, E.C. Hatchikian, The type I/type II cytochrome c(3) complex: an electron transfer link in the hydrogen-sulfate reduction pathway, J. Mol. Biol. 354 (2005) 73–90.
[7] D.L. Turner, T. Catarino, Homotropic and heterotropic interactions in cytochromes c_3 from sulphate reducing bacteria, FEBS Lett. 586 (2012) 494–503.
[8] R.A. Hoffman, S. Forsén, High resolution nuclear magnetic double and multiple resonance, Prog. Nucl. Magn. Reson. Spectrosc. 1 (1966) 15–204.
[9] J. Jeener, B.H. Meier, P. Bachmann, R.R. Ernst, Investigation of exchange processes by 2-dimensional NMR-spectroscopy, J. Chem. Phys. 71 (1979) 4546–4553.
[10] H. Santos, D.L. Turner, A.V. Xavier, Two-dimensional NMR-studies of electron-transfer in cytochrome c_3, J. Magn. Reson. 59 (1984) 177–180.
[11] C.A. Salgueiro, D.L. Turner, H. Santos, J. LeGall, A.V. Xavier, Assignment of the redox potentials to the four haems in *Desulfovibrio vulgaris* cytochrome c_3 by 2D-NMR, FEBS Lett. 314 (1992) 155–158.
[12] D.L. Turner, C.A. Salgueiro, T. Catarino, J. Legall, A.V. Xavier, NMR studies of cooperativity in the tetrahaem cytochrome c_3 from *Desulfovibrio vulgaris*, Eur. J. Biochem. 241 (1996) 723–731.
[13] P.R. Pokkuluri, Y.Y. Londer, N.E. Duke, J. Erickson, M. Pessanha, C.A. Salgueiro, M. Schiffer, Structure of a novel c_7-type three-heme cytochrome domain from a multidomain cytochrome *c* polymer, Protein Sci. 13 (2004) 1684–1692.
[14] E. Arslan, H. Schulz, R. Zufferey, P. Kunzler, L. Thony-Meyer, Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the cbb_3 oxidase in *Escherichia coli*, Biochem. Biophys. Res. Commun. 251 (1998) 744–747.
[15] P.O. Quintas, T. Catarino, S. Todorovic, D.L. Turner, Highly selective ligand binding by *Methylophilus methylotrophus* cytochrome *c*″, Biochemistry 50 (2011) 5624–5632.
[16] C.M. Paquete, P.M. Pereira, T. Catarino, D.L. Turner, R.O. Louro, A.V. Xavier, Functional properties of type I and type II cytochromes c_3 from *Desulfovibrio africanus*, Biochim. Biophys. Acta 1767 (2007) 178–188.
[17] R.A. Marcus, N. Sutin, Electron transfers in chemistry and biology, Biochim. Biophys. Acta 811 (1985) 265–322.
[18] M. Sulpizi, S. Raugei, J. VandeVondele, P. Carloni, M. Sprik, Calculation of redox properties: understanding short- and long-range effects in rubredoxin, J. Phys. Chem. B 111 (2007) 3969–3976.
[19] P.M. Pereira, I. Pacheco, D.L. Turner, R.O. Louro, Structure-function relationship in type II cytochrome c(3) from *Desulfovibrio africanus*: a novel function in a familiar heme core, J. Biol. Inorg. Chem. 7 (2002) 815–822.