NMR studies of cooperativity in the tetrahaem cytochrome \( c_3 \) from *Desulfovibrio vulgaris*

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The thermodynamic properties of the *Desulfovibrio vulgaris* (Hildenborough) tetrahaem cytochrome \( c_3 \) (Dvc\(_3\)) are rationalised by a model which involves both homotropic (\( e^-/e^- \)) and heterotropic (\( e^-/H^+ \)) cooperativity. The paramagnetic shifts of a methyl group from each haem of the Dvc\(_3\) have been determined in each stage of oxidation at several pH values by means of two-dimensional exchange NMR. The thermodynamic parameters are obtained by fitting the model to the NMR data and to redox titrations followed by visible spectroscopy. They show significant positive cooperativity between two of the haems whereas the remaining interactions appear to be largely electrostatic in origin. These parameters imply that the protein undergoes a proton-assisted two-electron transfer which can be used for energy transduction. Comparison with the crystal structure together with measurement of the kinetics of proton exchange suggest that the pH dependence is mediated by a charged residue(s) readily accessible to the solvent and close to haem I.

**Keywords:** cooperativity; energy transduction; multiheme cytochrome; NMR; redox-Bohr.

The functional cooperativity between different regions of some proteins [1] is a fundamental property to control and coordinate important chemical events in the living cell. Although the molecular basis for the fine regulation of several types of cooperativity mechanisms has been successfully established [2], little is known about the structural basis for electron-electron and electron/proton cooperativities and their role either in electron transfer or in energy transduction [3].

*Desulfovibrio* spp. cytochrome \( c_3 \) is a small (\( \approx 14 \) kDa), monomeric tetrahaem protein which exhibits cooperativity between the four haems and acid/base group(s): the haem redox potentials are pH dependent (redox-Bohr effect) and each haem redox potential is dependent on the oxidation state of the other three haems (redox interaction potentials) [4–6].

Due to its small size and the fact that the haems are diamagnetic in the reduced state and paramagnetic in the oxidised one, NMR is particularly well suited to characterise this protein from the structural and thermodynamic point of view [4, 7–15]. Furthermore, several X-ray structures are available for cytochromes \( c_3 \) from *Desulfovibrio* spp. [16–23].

The thermodynamic properties of cytochrome \( c_3 \) have been analysed by previous NMR studies [4, 5, 8, 15, 24]. In the first of these studies an NMR data set obtained at two discrete pH values for *Desulfovibrio gigas* cytochrome \( c_3 \) was used to calculate nine parameters (three relative microscopic redox potentials and six haem-haem redox interactions) for each pH value, independently treated. The redox interaction potentials were fixed according to the maximum concentration reached by the intermediate oxidation stages (defined according to the number of oxidised haems) in redox titrations followed by NMR [4]. Using the same NMR data set, a second study proposed a model with 21 parameters in which the four haem redox potentials and six haem-haem redox interaction potentials were assumed to depend on the state of protonation. In order to fix both the absolute redox potentials and haem redox interaction potentials, potentiometric titrations followed by visible spectroscopy were used [5]. However, the experimental data contain insufficient information to characterise accurately 21 independent parameters, giving only approximate results with errors which are difficult to quantify since grid searches were used. In fact, in the framework of the present model, the assumption that the haem-haem redox interaction potentials change on protonation is equivalent to introducing three-centre interactions, which we have shown to be unnecessary in a preliminary evaluation of this system [6].

The characterization of the thermodynamic properties of *Desulfovibrio vulgaris* (Miyazaki) cytochrome \( c_3 \) was made at a single pH [8]. In this case an arithmetical method was used to obtained four redox potentials and six haem-haem redox interactions using both NMR and electrochemical data. Recently, this analysis was repeated at three different pH values [15] using NMR assignments which had been extensively revised in the light of data from Dvc\(_3\) [11, 14]. Analysis of the redox behaviour either at a single pH or at a series of pH values treated independently is clearly insufficient to separate the haem-haem interactions from the redox-Bohr interactions since the \( pK_a \) changes between oxidation stages [4, 8]. Neglect of the redox-Bohr effect inevitably leads to the conclusion that the redox interaction potentials are themselves pH-dependent [4, 15]. The recent work [15] did not propose a mechanism to explain this dependence, but it is implicit that the protein must undergo a pH-dependent change in conformation. This is precisely the assumption un-

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**Abbreviations.** Dvc\(_3\), *Desulfovibrio vulgaris* (Hildenborough) cytochrome \( c_3 \); Dgc\(_3\), *Desulfovibrio gigas* cytochrome \( c_3 \), ROESY, rotating frame NOE spectroscopy.
MATERIALS AND METHODS

Sample preparation. Cytochrome $c_5$ from Desulfovibrio vulgaris (Hildenborough) was purified as previously described [10]. For NMR studies the protein was lyophilized and resuspended in 99.96% $^2$H$_2$O (Sigma Chemical Co.) to a final concentration of approximately 2 mM. The pH was adjusted with either NaOH or $^2$HCl and monitored using a glass electrode (Ingold Refill 9811) inserted directly into the NMR tubes (pH values are quoted as the meter reading uncorrected for the isotope effects). The fully reduced stage was achieved by addition of catalytic amounts of Desulfovibrio gigas hydrogenase in a hydrogen atmosphere (grade U from L’Air Liquide) [10]. The intermediate oxidation levels were obtained by first washing out the hydrogen from the reduced samples with argon (grade N46 from L’Air Liquide) and then adding controlled amounts of air into the NMR tube with a Hamilton syringe through serum caps [11]. Since the pH of the solution changes with the level of oxidation, because of the concomitant change of $pK_v$ values, the pH was measured under strict anaerobic conditions inside a glove box (MBraun MB 150-G1) with an argon atmosphere before and after each NMR experiment.

NMR experiments. All $^2$H-NMR spectra were obtained using a Bruker AMX-500 spectrometer operating at 500.13 MHz for protons. The probe temperature was kept at 24.3 ± 0.01°C using a Bruker Eurotherm Controller B-CU 05 together with B-VT2000 and BTO 2000 temperature controllers.

The pattern of reoxidation for each haem methyl group at each pH was observed either by NOESY or ROESY experiments acquired over a spectral width of 33.33 kHz in both dimensions, collecting 512($t_1$)×2048($t_2$) data points in the phase sensitive mode using the time-proportional phase incrementation method, and transformed to give 1024($t_1$)×2048($t_2$) real data points.

The NOESY spectra were accumulated with a mixing time of 25 ms and ROESY spectra performed with a pulse for spin lock of 10 ms as described previously [11], with presaturation of the water signal during the relaxation delay and also during the NOESY mixing time. Before transformation the data were multiplied by a gaussian function in $t_2$ and a cosine-squared function in $t_1$. Automatic base-plane correction was applied after Fourier transformation in each dimension.

The chemical shifts for each pH, relative to an internal sodium 3-trimethylsilyl-2,2,3,3-$^2$H$_2$ propionate reference, were obtained from one-dimensional saturation-transfer experiments with stable samples in which stages 4, 3, 2, and 1 coexist. The water signal was saturated for approximately 2 s followed by an irradiation time of 500 ms with the on- and off-resonance irradiation alternating after 16 signal-averaged transients. A total of 256 transients were accumulated for each free induction decay in 32 768 data points and Fourier-transformed to a digital resolution of approximately 0.5 Hz/point.

The linewidths of the haem methyl resonances in different stages of oxidation were obtained by fitting Lorentzian lineshapes to the NMR spectra. Normal spectra were used whenever possible, but if the resonance of interest was too heavily overlapped then simplified spectra were obtained by saturation transfer from a resolved resonance of the same methyl group in another stage of oxidation.

Visible spectroscopy experiments. Visible redox titrations of DvC$_5$ were performed as previously described [25] using about 2 μM protein solutions in 50 mM Tris/maleate or 50 mM Tris/HCl buffer. To ensure a good equilibrium between the redox centres and the electrode used to measure the solution potential, the following redox mediators were added to the protein solution, all at ≈1 μM final concentration, except for triquat.

Fig. 1. Electronic distribution scheme for a tetrahaem cytochrome with a proton-linked equilibrium showing the 32 possible microstates. The four inner circles represent the haems which can either be reduced (black) or oxidised (white) with the shaded circles representing the protonated (acidic A) forms and the open circles representing the deprotonated (basic B) forms. In DvC$_5$, fast intramolecular electron exchange and fast proton exchange is observed within each stage (defined by the number of oxidised haems). In order not to overcrowd the picture, only a few of the microstates have been chosen to show the scheme used for labelling their energies relative to the fully reduced and protonated form (see text). The term $G_E$ is the energy for deprotonation in stage 0, $G_A$, and $G_B$ are the energies of the microstates with one haem oxidised (stage 1) for the protonated and deprotonated forms, respectively, $G_{mix}$ and $G_{ox}$ are the energies of the microstates with haems $i$, $j$ and $k$ oxidised (stage 3), for the protonated and deprotonated forms, respectively.

derlying the two-state model used to treat data from DgC$_5$ [5]. We now show that it is not necessary to invoke any change in conformation in order to explain the pH dependence of the NMR spectra.

Instead, we developed a model which involves only two-centre interactions, so that the pH dependence is attributed solely to the interaction between ionising groups and each of the four haems, and thereby reduces the number of thermodynamic parameters from 21 to 15 (i.e. four redox potentials, six haem-haem redox interactions, four redox-Bohr interactions and one $pK_v$). Thus, the model used involves interactions among five charge centres: each of the four haems may be oxidised or reduced, giving 16 microstates, and for each of these microstates the group responsible for the redox-Bohr $pK_v$ may be protonated or deprotonated, leading to a total of 32 possible microstates (Fig. 1).

The NMR paramagnetic shifts of a methyl group from each of the four haems of DvC$_5$ in each of the four oxidation stages are reported and analysed as a function of pH. Both the pH range and the number of measurements have been increased considerably since a preliminary account of this work appeared in order to provide a stringent test of the simpler model [6].

Since intramolecular electron exchange is fast on the NMR timescale and intermolecular exchange is slow, the paramagnetic contribution to the chemical shifts of each haem methyl group of molecules in intermediate oxidation stages provides a measure of the midpoint redox potentials of the different haems [4, 8, 11, 12]. Thus, the thermodynamic parameters of DvC$_5$ were determined by fitting the measured paramagnetic shifts to the model developed. These parameters show that the cytochrome has the ability to couple proton transfer with a concerted two-electron transfer.
(0.25 μM): methylene blue ($E'_0 = +11$ mV), indigo tetrasulphonate ($E'_0 = -30$ mV), indigo trisulphonate ($E'_0 = -70$ mV), indigo disulphonate ($E'_0 = -110$ mV), 2-hydroxy-1,4-naphthoquinone ($E'_0 = -152$ mV), anthaquinone-2,7-disulphonate ($E'_0 = -182$ mV), anthaquinone-2-sulphonate ($E'_0 = -225$ mV), safranine O ($E'_0 = -280$ mV), neutral red ($E'_0 = -325$ mV), diquat ($E'_0 = -350$ mV), methyl viologen ($E'_0 = -440$ mV), and triquat ($E'_0 = -550$ mV). The redox titrations were performed at room temperature (25°C) in a quartz cuvette with stirring. The system was kept anaerobic by continuously flushing the cuvette with humidified argon N46. The protein was reduced in steps by adding a few microliters of a degassed 10 mM sodium dithionite solution. After each addition the redox potential was measured and the visible spectrum recorded. Solution potential measurements were made with a combined Pt, Ag/AgCl microelectrode previously calibrated against saturated quinhydrone solutions at pH 4.0 and 7.0, and all spectra were recorded with a spectral slit width of 0.5 nm in a Shimadzu UV-3100 spectrophotometer.

Since the contribution of the redox mediators to the absorbance at the peak ($λ = 552$ nm) is not constant throughout the titration, a correction was made by taking the absorbance at the two isosbestic points near to the peak ($λ = 542$ nm and $λ = 560$ nm) and subtracting the mean value from the absorbance of the peak. The data points representing the calculated reduced fraction ($A_{552} - A_{552,0} = (A_{552,red} - A_{552,0})$) were plotted against the solution redox potential (standard hydrogen electrode) for two titrations performed at different pH values.

RESULTS

Under the experimental conditions used, the intermolecular electron exchange is slow on the NMR timescale so that separate peaks are observed for each stage of oxidation, but it is fast enough for significant transfer to occur before the magnetisation has decayed so that exchange cross-peaks can be observed in NOESY and ROESY spectra. One methyl group from each haem was chosen on the basis of being situated far enough from the remaining haems for extrinsic paramagnetic shifts to be negligible [11]. Using NOESY spectra at intermediate oxidation levels such as those illustrated in Fig. 2, one haem methyl resonance for each haem was traced from the different oxidation stages back through each step of electron transfer to the chemical shifts of the fully reduced protein which have been assigned specifically [10, 11]. ROESY experiments (not shown) were used in order to distinguish between the NOE and exchange cross-peaks [11, 26]. The pH dependence of the methyl chemical shifts is shown in Fig. 3. At each pH a methyl group of any haem, m, in oxidation stage S, has a single peak at a position $\delta_{obs}^m$. This shift depends on the populations of the microstates in which that haem is oxidised (since there is fast intramolecular electron exchange within each stage) weight-averaged according to the populations of the acidic (A) and basic (B) forms (which are in fast proton exchange for that same stage) [5]:

$$\delta_{obs}^m = \left(\delta_A^m - \delta_B^m\right) \sum P_A^m S + \left(\delta_B^m - \delta_A^m\right) \sum P_B^m S + \delta_{B0}^m$$  

where $\delta_{B0}^m$ is the observed chemical shift of methyl m in the fully reduced protein (stage 0) and $\delta_A^m$ and $\delta_B^m$ are those observed in the fully oxidised (stage 4) protonated or deprotonated protein, respectively; $\sum P_A$, $\sum P_B$ are the sums over all the populations with haem m oxidised in stage S, protonated or deprotonated, respectively; and $\sum P_B$ is the sum over all the populations, protonated and deprotonated in stage S (Fig. 1).

The pH dependence of the paramagnetic shifts appears to be dominated by a single pKa for each stage which decreases from stage 0 to stage 4 (cf. Fig. 3). The shifts in the fully oxidised protein are weakly pH dependent, showing that the ionisation has a small effect on the pattern of electron delocalisation. However, since the pH dependence is much larger for the chemical shifts of some of the intermediate stages of oxidation, there must also be changes in the relative populations for each haem, cf. Eqn (1), which in turn implies different interactions between the four haems and the ionising group(s).

The presence of haem-haem redox cooperativity is also particularly noticeable in the case of the methyl of haem III which has a smaller paramagnetic shift in stage 2 than in stage 1 at neutral and high pH.

Modelling of the thermodynamic parameters. It is important to fit these experimental results into the framework of a model in order to characterise the pattern of interactions fully. The thermodynamic properties of the model system described in Fig. 1 will be determined completely by the standard free energies of each of the 32 microstates together with the solution potential and the pH. It is convenient to express these energies as sums of other parameters, represented by a term for each independent centre, plus a series of multi-centre interactions. The numbers of terms of each type are given by the row 1:5:10:10:5:1 from Pascal’s triangle in which the first single energy is simply the reference zero for the remaining terms. The next group contains the energies of the five independent centres, which are equivalent to the microscopic redox potentials of the four haems and the pKa of the ionisable group in the fully reduced and protonated protein. Then there are ten possible two-centre interactions, ten three-centre interactions, and so on. The total number of energy terms is of course 32, representing a complete description of the system. These terms are constant and independent of the solution potential or pH, ensuring that the thermodynamic description is complete and independent of any structural model, but unfortunately it is impractical to attempt to measure all 32 parameters. Since the qualitative features of the pH dependence discussed above clearly require the inclusion of two-centre interactions, we shall neglect the three-, four-, and five-centre terms in the first instance. This model therefore requires 15 energetic parameters, corresponding to four microscopic redox potentials and one pKa for the fully reduced state, plus the ten two-centre terms comprising the six redox and the four redox-Bohr interactions [6].

A complication arises because the solution potential of each NMR sample is unknown and so the relative intensities of the NMR signals from the different stages of oxidation provide no information about the absolute redox potentials. At each pH we have the paramagnetic shifts of the four methyl groups in stages 1–3 relative to the fully oxidised form, which give the averaged oxidation state for each haem in each stage. However, the fact that each oxidation step involves a single electron means that the relative shifts of any three methyl groups determine that of the fourth:

$$\sum_{m=1}^{4} \left(\delta_{obs}^m - \delta_{obs}^0\right) = S$$

where S is the oxidation stage, and so the NMR spectra provide a maximum of nine independent pieces of information at each pH. Four additional pieces of information are implicit in the macroscopic pKa observed for each stage. Thus, the relative shifts in the NMR spectra can only provide 13 independent pieces of information, but a total of 15 energetic parameters must be measured. We therefore set one haem redox potential and one haem-haem redox interaction arbitrarily to zero and determine all other energies relative to this, and subsequently fix the absolute energy scales for the redox potentials and the haem-
Fig. 2. NOESY spectra (mixing time 25 ms) of partially oxidised cytochrome c, at pH 5.3 and 6.0. Cross peaks resulting from intermolecular electron transfer are indicated for one methyl group from each haem in the four stages of oxidation (1, 2, 3 and 4). Above diagonals: methyls 2' CH,I (solid line) and 18' CH,II (dashed line). Below diagonals: methyls 12' CH, III (solid line) and 18' CH, IV (dashed line). The boxes indicate the cross peak for methyl 18' CH, I between stages 2 and 3. Note the large pH dependence of the chemical shift of this methyl in stages 2 and 3 and the larger line broadening observed for stage 2 at pH 6.0. Sections of one-dimensional spectra recorded immediately before each NOESY experiment are shown above the corresponding two-dimensional spectra.

Fig. 3. Chemical shifts of the haem methyl group resonances 2' CH,I, 18' CH,II, 12' CH, III, 18' CH, IV, of Dve₃. Roman numbers refer to the haems in order of attachment to the polypeptide sequence in the oxidation stages 1 (□), 2 (○), 3 (▽) and 4 (△), at different pH. The full lines represent the best fit (rmsd = 0.25 ppm) of the shifts using the pH-5.3–8.0 data set (filled symbols) to the model of five interacting centres.
haem interactions by reference to potentiometric titrations in which the total concentration of reduced haem is monitored by visible spectrophotometry (see below).

These parameters are sufficient to define the relative populations of the 32 microstates at any pH and solution potential and hence the four macroscopic redox potentials, \( E_s \) (\( S = 1, \ldots, 4 \)), i.e., the solution potential at which the sum of the populations in stage \( S \) equals the sum of the populations in stage \( S-1 \),

\[
\Sigma n_i = \Sigma n_{i-1}^{-1},
\]

as well as the five macroscopic \( pK_s \) values, i.e., the solution pH at which the sum over the protonated populations equals the sum of the deprotonated ones in stage \( S \),

\[
\Sigma P_i = \Sigma P_{i-1}.
\]

The energy of each of the 32 microstates relative to that of the reduced protonated protein is then given by a simple sum of the appropriate energies of the five independent centres and of the ten possible two-centre interactions, plus a term \(-SFE\) to account for the effect of the solution potential, \( E_s \), in oxidation stage \( S \), and another for the proton chemical potential, \(-2.3RT\) pH, added for the deprotonated forms. As a concrete example (cf. Fig. 1), the relative energy of the protonated microstate of stage 3 with haems \( i, j, k \) oxidised is:

\[
G_{ijk} = g_i + g_j + g_k + g_h + g_{ha} + g_{ha} - 3FE
\]

where \( g_i, g_j, \) and \( g_k \) are energies proportional to the individual haem redox potentials and \( g_h, g_{ha}, \) and \( g_{ha} \) are two-site redox interaction energies. In addition, the corresponding deprotonated microstate involves the ionisation energy of the fully reduced molecule, \( g_{ha} \), the proton chemical potential, and additional energy terms for the interactions between the oxidised haems and the ionised group:

\[
G_{ijk} = g_{ijk} + g_{ha} + g_{ha} + g_{ha} + g_{ha} - 2.3RT\ \text{pH}.
\]

Again, as an example, the NMR signals observed for stage 3 are the population weighted average over the eight microstates which have three haems oxidised. The relative populations are given by the Boltzmann distribution, for example, \( P_{ijk} = \exp (-G_{ijk} / RT) \), and the chemical shifts of the haem methyl groups \( m \) can then be calculated for any set of energetic parameters since their shifts in the fully oxidised protein are known (Eqn 1).

Note that the absolute solution potential is not needed for determining the chemical shifts since it makes the same contribution to the energy of each of the microstates within a given oxidation stage. The solution potential does determine the relative concentrations of the different stages, and hence would affect the chemical shifts if intermolecular electron transfer were fast, as is the case in cytochrome \( c_s \) from Desulfomicrobium baculatum [27]. However, intermolecular electron transfer is slow in DVC, and therefore the solution potential affects only the intensity of the NMR signals. Since intensity measurements from NMR spectra are generally less precise than chemical shifts and, anyway, is impossible to measure accurately the redox potentials of the NMR samples, it is convenient to use instead visible spectrophotometry redox titrations (with mediators) in order to establish the absolute scale for the energetic terms.

The differences between the observed chemical shifts and the pH-independent shifts of the methyl groups in the fully reduced protein were fitted to the model described above using the Marquartd method on the basis of accurate pH readings and experimental errors for each shift which are proportional to the linewidths. The 142 data points in the pH range 5.3–8.0, excluding the extremes of pH, were also fitted as a separate set in order to evaluate the effect of any additional ionisations which have \( pK_s \) values well outside the physiological pH range. The solid lines in Fig. 3 show the result of the latter fit.

![Fig. 4. Mole fraction of the total reduced haem determined by visible spectroscopy at pH 7.0 (○) and 8.0 (□) for various solution potentials. The full lines represent the best fit to the model of five interacting centres obtained by varying only the absolute values of the haem redox potentials and of the haem-haem interactions with all of the relative values fixed. The energy parameters used to calculate these curves are those corresponding to the second data set, listed in Table 1.](image)

The absolute values of the redox potentials and haem-haem interactions remain to be determined by fitting the results of redox titrations monitored by visible spectroscopy, as shown in Fig. 4. These two energy parameters are reflected first by the macroscopic redox potential of the fully reduced protein, \( E_s \), which is independent of the haem-haem interactions and therefore determines the absolute values for the macroscopic redox potentials of the four haems, and second by the redox potential of the last step, \( E_s \), which is shifted by three times any change in the base value against which all of the haem-haem interactions were referenced. This simple relationship arises because any change in the base value for the energies of the four redox centers (\( \delta g_i \)) or the haem-haem interaction energies (\( \delta g_{ha} \)) modifies the energy of each microstate in stage \( S \) by \( \delta G^s \), according to:

\[
\delta G^s = S \delta g_i + \frac{S(S-1)}{2} \delta g_{ha}.
\]

Hence, from the Nernst equation, the changes in the macroscopic redox potentials (\( \delta E_s \)) are given by:

\[
F \delta E_s = \frac{\delta G^s - \delta G^{s-1}}{\delta g_i + (S-1) \delta g_{ha}}.
\]

Thus, it is clear that the final adjustment of the absolute values of the reference energies has a purely linear effect on all of the thermodynamic parameters of the model.

Data from two visible redox titrations, at pH 7.0 and 8.0, were fitted simultaneously by varying only the two parameters described above, i.e., the absolute microscopic redox potential of one haem and the absolute value of one of the haem-haem redox interactions, with the relative redox potentials and relative redox interactions kept fixed. The \( pK^s \) and the redox-Bohr interactions between the haems and the ionised group(s) were also kept fixed at the values obtained by fitting the NMR data. The shape and pH dependence of the visible titration curve provides an additional qualitative test of the parameters obtained from the NMR data since this behaviour is completely determined by the ionisation energy of the group and its interactions with the four haems, which were fixed by fitting the chemical shifts. The results are plotted as solid lines in Fig. 4 and incorporated in the absolute values of the energy parameters which are given in Table 1. It should be noted that the agreement between the model simulations and the visible redox titration data is not perfect, especially at pH = 7, for reduced fractions above 0.8. The increase of the slope probably arises from lack of equilibrium in
Table 1. Energy parameters for the five interacting centres in Dve₃, obtained by fitting the model described in the text to the NMR (Fig. 3) and visible data (Fig. 4) using the pH 4.4–10.6 and 5.3–8.0 data sets. Diagonal elements (boldface) represent the energy for oxidising haems (i.e., reduction potentials in mV) and deprotonating the ionisable group in the fully reduced molecule. The off-diagonal elements represent the energy of redox interaction (meV) between the oxidised haems and between these and the deprotonated group. Note that the energy of each oxidised haem is lowered by deprotonation, as expected on electrostatic grounds, and the substantial negative redox interaction between haems I and II represents positive cooperativity. The standard errors are 5 meV for relative redox potentials and interactions, 5 meV for the absolute reference value for the potentials, and 3 meV for the absolute reference value for the interactions. The energy of each microstate is given by the sum of the relevant diagonal terms, $g_n$, and interaction energies, $g_i$ and $g_m$, as fully explained in the text. The $pK_a$ of the reduced protein is given by $g_n F/(2.3RT)$ and the $pK_a$ of the oxidised one is given by $\left( g_n + \sum_{i=1}^{4} g_i \right) F/(2.3RT)$, where $g_n$ is the diagonal term and $\sum_{i=1}^{4} g_i$ is the sum of the off-diagonal terms of the fifth column.

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the titrations for the low redox potential region. This effect is more obvious for higher reduced fractions at lower pH values because no additional time could be allowed for equilibration. This is because the reducing agent used, sodium dithionite, is not stable at low pH and so spontaneous reoxidation of the cytochrome becomes a problem.

It should be clear from this discussion and from the quality of the fits in Figs 3 and 4 that the model presented here uses the minimum number of parameters necessary to describe the data and that there is insufficient information to characterise any further parameters. Earlier analyses [4, 5] treated smaller data sets and yet introduced six more parameters than those used here. Inevitably, the values obtained were imprecise, though the errors were not easily quantifiable since the fits were obtained by grid searching. Nonetheless, this work supports the essential conclusion that the individual haem redox potentials are pH dependent, and that there are both positive and negative haem-haem interactions in cytochromes c₃ [4].

The additional parameters used previously [5] represent six out of the ten possible three-site interactions, namely those which involve two oxidised haems and the acidic group. This was based on a specific structural model in which protonation was assumed to induce a conformational change which in turn modified all of the haem-haem interactions. For the present set of data we have tested the effect of introducing three-site interactions and found that they have little effect on the one- or two-site parameters. The values obtained for the three-site interactions were not considered since they were poorly defined and yielded no qualitative improvement in the fit. Thus, without imposing any structural model, we find no evidence for any major change in conformation associated with protonation or with electron transfer. It is of course possible that there are subtle conformational changes, and these may become detectable by fitting the chemical shifts of all 16 haem methyl groups. However, this must await the determination of the magnetic susceptibility tensors for the haems in order to allow the inclusion of extrinsic paramagnetic shifts.

Furthermore, although the present thermodynamic model treats five distinct charge centres without making any assumptions about their spatial arrangement, the parameters obtained appear to be physically significant. This is discussed below in relation to the X-ray structure.

Significance of NMR line broadening. The linewidths of the haem methyl resonances in different stages of oxidation show considerable variation, both between different haems and between different stages of oxidation (Fig. 5). The intrinsic methyl linewidths should be dominated by paramagnetic relaxation, which will be roughly proportional to the degree of oxidation of each haem. Since the linewidths of these methyls in the fully oxidised protein all lie in the range 30–60 Hz [28], this cannot be the major source of line broadening. Additional line broadening may result from intermolecular electron exchange, which is slow on the NMR timescale, and this effect must contribute an equal amount to all of the linewidths in a given oxidation stage since it depends simply on the lifetime of a molecule between exchange events. However, although the effect of electron exchange may vary between stages of oxidation and also between samples, it cannot account for the observed differences between haems. Thus the variation between the linewidths of different haem methyls may be caused by acid/base equilibria with a timescale approaching the fast exchange limit. In the case of rapid two-site exchange, a single line would be observed for each methyl group, m, in each oxidation stage, S, with a residual line broadening given by [29]:

Fig. 5. The pH dependence of haem methyl resonance linewidths in oxidation stage 2 (upper plot) and stage 3 (lower plot) of Dve₃. The solid lines represent the fit obtained with the proton transfer rate constants $k_n$ and $k_{on}$ given in the text and an underlying linewidth of 133 Hz for all resonances in stage 2 and 93 Hz for all resonances in stage 3.
\[ A_{\text{w,ex}} = \frac{4n_{f}r_{0}^{2}(\frac{\chi_{H}^{2}}{k_{\text{BA}}})^{2}(\delta_{0}^{K_{S}} - \delta_{0}^{K_{S}})^{2}} \]

(7)

in which the two species are assumed to have identical intrinsic linewidths; \( \chi_{H}^{2} = \sum P_{n}^{K_{S}} \sum P_{n}^{K_{S}} \) and \( \chi_{H}^{2} = \sum P_{n}^{H_{0}} \sum P_{n}^{H_{0}} \) are the mole fractions for all the protonated and deprotonated microstates of stage \( S \), respectively; their chemical shifts, \( \delta_{0}^{K_{S}} \) and \( \delta_{0}^{K_{S}} \), are given in Hz; and \( k_{\text{BA}} \) is the pseudo-first-order rate constant for protonation. For the pH range used, the largest effects are observed in stages 2 and 3 (cf. Figs 2 and 5), where it can be seen that the maximum line broadening occurs near the \( pK_{a} \) of each stage. The approximation of Eqn (7) should be adequate for the methyl groups considered in this work though it would be less good for the much larger effects observed for methyl groups, such as \( \text{CH}_{3} \) of haem I in stage 2 when \( \text{pH} = pK_{a} \). The pH-dependent line broadening of that resonance can be seen clearly at pH 5.3 and pH 6.0 in the spectra of Fig. 2, together with that of \( \text{CH}_{3} \) of haem I which was used in this analysis. It is also clear that the pH-dependent line broadening is much larger than any variation in the underlying linewidth. Since the observed broadening of each methyl peak is clearly correlated with the magnitude of the pH dependence of its chemical shift (cf. Figs 3 and 5), the effect can be explained by a rapid acid/base equilibrium. If the protonation reactions are written for any stage \( S \):

\[
\begin{align*}
A^{-} + H_{2}O^{+} & \rightleftharpoons AH + H_{2}O \\
A^{-} + H_{2}O & \rightleftharpoons AH + OH^{-}
\end{align*}
\]

(8)

then the overall pseudo-first-order rate constant for protonation is given by

\[ k_{\text{BA}} = 10^{-pH} k_{H} + \left( \frac{K_{W}}{k_{H}} \right) k_{\text{OH}} \]

(9)

where \( K_{W} \) and \( K_{H} \) are the dissociation constants of water and of the ionisable group on the protein, \( k_{H} \) is the rate constant for protonation of the group in acid solution and \( k_{\text{OH}} \) is the rate constant for deprotonation of the group in basic solution [30, 31].

The NMR measurements reported here were made in \( ^{2}H_{2}O \) solutions and the pH values are given as uncorrected meter readings, pH*. This is not significant for fitting the NMR data together with the potentiometric titration data obtained in \( H_{2}O \) since the isotope effect on the \( pK_{a} \) values is roughly compensated by the behaviour of the glass electrode [32]. However, we now require the true deuteron concentration, which is obtained from the meter reading through \( p\text{H} = \text{pH}^{*} + 0.4 \), and the dissociation constant of \( ^{2}H_{2}O \), \( K_{W} = 1.12 \times 10^{-15} \text{ M}^{2} \) at 298 K [33].

As noted above, the intrinsic linewidths are similar for all of the exchange resonances, and the contribution from electron exchange should be identical for all of the resonances in a given oxidation stage. In a comparable situation encountered in ferricytochromes c' by LaMar et al. [31], the intrinsic linewidths of the acidic and basic forms were clearly different but the approximation \( k_{\text{OH}} = 0 \) was used. Since the four possible parameters (the intrinsic linewidths of the acidic and basic forms, \( k_{H} \) and \( k_{\text{OH}} \)) are strongly interacting, some approximation is desirable to avoid all four parameters being poorly defined. In the present case, the protein is low spin, \( S = +1/2 \), in the acidic and basic forms and the intrinsic linewidths of methyls in the intermediate stages are dominated by electron exchange. The approximation that the intrinsic linewidths are constant for all methyl groups is therefore appropriate. The measured linewidths were fitted to Eqn (7) using only three parameters for each stage: \( k_{H}, k_{\text{OH}} \), and a common intrinsic linewidth. The rate constants effectively determine the shape of the curve; and this shape is scaled by the differences in chemical shift of each resonance in the acidic and basic forms, cf. Eqn (7), which were fixed by the preceding analysis.

The results obtained for the methyls in stages 2 and 3 are shown by the solid lines in Fig. 5; there is no significant excess line broadening observed for these methyls either in stage 1 or 4. The fitted linewidths in stage 2 yielded the rate constants \( k_{H} = 2.22 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1} \) and \( k_{\text{OH}} = 3.92 \times 10^{9} \text{ M}^{-1} \text{ s}^{-1} \), which should be regarded only as order-of-magnitude estimates because of the approximations made. The broadening in stage 3 yielded \( k_{H} = 4.91 \times 10^{9} \text{ M}^{-1} \text{ s}^{-1} \) and \( k_{\text{OH}} = 2.55 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1} \), but these numbers are less reliable since the effects are smaller and the part of the curve at low pH is poorly defined.

Although these rate constants are only approximate, it is notable that they are at least an order of magnitude greater than the diffusion-controlled rates for deuteron self-exchange in \( ^{2}H_{2}O \) [34]. Such anomalously high rates have been attributed to the catalytic effect of species other than \( ^{2}H_{2}O^{+} \) and \( \text{OH}^{-} \) [35], although the mechanisms have not been fully characterized.

A full discussion of the rate constants measured in \( ^{2}H_{2}O \) must be deferred until the nature of the ionising group has been determined, but it is sufficient for our purposes simply to note that the exchange rates of the redox-Bohr proton(s) must be diffusion controlled. We therefore conclude that this group must be readily accessible to the solvent and is likely to be close to the protein surface.

**DISCUSSION**

There are significant deviations between the observed chemical shifts of the haem methyl groups and the theoretical curves in the regions below pH 5.4 and above pH 8 (Fig. 3). This is hardly surprising since the protein contains many groups which may ionise at extreme pH values and there must be some electrostatic interactions between such groups and the haems. The important question is whether the parameters obtained from a simple model which considers only a single ionisation process will be significantly distorted by such non-physiological effects. In fact, the differences between the parameters obtained by fitting data from the full pH range studied or by restricting the range by excluding the extremes (Table 1) are very small. There is a weak systematic tendency to decrease the energies of interaction between the haems and the ionisable groups and hence to reduce the range of \( pK \) values in the different stages, but the haem-haem interactions are remarkably insensitive to the distortions at extremes of pH. Thus, although additional ionisations undoubtedly do occur, the pH dependence of the NMR spectra in the physiological pH range is adequately described by interactions between the haems and a single ionisation process. An identical result is of course obtained if the observed interactions are the sum of interactions with more than one ionisable group which share the same \( pK \) and which do not interact with each other [5]. In fact, pH titrations performed in both the oxidised and reduced form show that two protons are involved in the redox-Bohr effect [3].

All of the interaction energies between the deprotonated group(s) and the oxidised haems are negative, which is consistent with a purely electrostatic effect, and the relative magnitudes indicate that this group(s) is most closely associated with haem I. The macroscopic \( pK \) values obtained by fitting the data in the pH range 5.3–8.0 are 7.4, 7.1, 6.4, 5.6 and 5.3 for oxidation stages 0–4, respectively. These observations, together with the redox-Bohr effect and the kinetic data, suggest that the dominant ionisable group is close to haem I. This would be consistent with the titration curve observed for the \( 1^{3} \text{CH}_{3} \) protons of haem I in the similar protein from *D. vulgaris* (Miyazaki) [15].
The haem-haem interaction energies obtained for the pairs I-IV, II-III, and II-IV, are comparable with their standard errors and therefore may not be significant. These haem pairs are found to have the largest iron–iron distances in the crystal structure: 1.77, 1.60, and 1.66 nm, respectively [21]. Significant positive interactions are found between oxidised haems I and III and between haems III and IV. This is consistent with the interactions being electrostatic and also with the positions of the haems in the three-dimensional structure since these haems have the shortest iron–iron distances at 1.11 and 1.19 nm, respectively. However, the largest interaction is found between haems I and II, which have an iron–iron distance of 1.24 nm, and it is negative. A negative interaction energy between two oxidised haems implies positive cooperativity, whereby oxidising one haem makes it easier to oxidise the other. This cannot be explained by simple electrostatics, thus implying a redox-linked conformational change. A mechanism could be envisaged in which a charged group which interacts with both haems changes its position when either haem is oxidised. This group would be relocated by some conformational change driven by the electrostatic interaction with the oxidised haem. Such a conformational change cannot be defined more precisely at this stage.

Alternatively, there could be some direct mechanical interaction propagated through the axial ligands to the two haem irons, which happen to include the consecutive residues His34 and His35, though preliminary studies of Dgc, indicated that an equivalent positive cooperativity is found between the second and the third haem to be oxidised [5] which have been assigned to haems II and III [12]. In principle, therefore, each microscopic state of the protein could have a slightly different equilibrium conformation and a different pattern of fixed interactions. However, differences between the microscopic states which make up each macroscopic oxidation stage are unlikely to be important since intramolecular electron exchange is extremely fast [4], giving the protein conformation insufficient time to respond to the redistribution of charge. Finally, although several of the interaction energies appear reasonable in terms of electrostatic interactions within the crystal structure, it should be emphasised that the model from which these values were obtained made no use whatsoever of any structural information.

CONCLUSIONS

This article presents the results of a careful study of the pH dependence of the NMR spectrum of Dvc, and rationalises these results by means of a simple model with five interacting centres: four haems and one acidic group. The model uses the minimum number of parameters necessary to describe the general behaviour of such a system and fitting these parameters to NMR spectra and visible redox titrations together leads to a unique solution. The simplified 15 parameter model used here not only gives an excellent fit to the data but also ensures that each parameter is well defined. Thus, the values of the parameters which were obtained by fitting the data reported in this work are statistically significant. Furthermore, they also appear to be meaningful with respect to the known structure of the protein.

The eventual inclusion of data from other resonances will require the extrinsic paramagnetic shifts generated by each haem to be taken into account. In future, the model will allow data for other protons to be included with proper allowance for the extrinsic shifts caused by dipolar interactions with other haems so that the dominant ionisable group can be located and any small redox-related changes in conformation can be characterised.

Finally, the thermodynamic parameters obtained by fitting the data in the pH range 5.3–8.0 (Table 1) have been used to calculate macroscopic redox potentials as a function of pH, and these are shown in Fig. 6. These curves show quite clearly that the macroscopic potentials of steps 2 and 3 (equilibrium between stages 1 and 2, and between 2 and 3, respectively) are reversed in the pH range 6.0–7.1. The protein is therefore driven to cycle between stages 1 and 3 in the physiological pH range, exchanging 2e− coupled with a proton transfer. Since the redox potential is modulated by proton transfer, a concerted 2e− exchange with a single partner would require the proton transfer rate to be significantly higher than the dissociation rate of the electron transfer complex. It is therefore significant that the rate of proton transfer in water approaches the diffusion limit since, although this is relatively slow near neutral pH, the effective rate could be much higher if the proton transfer occurred directly between the electron transfer partners.

Taken together, the thermodynamic parameters discussed above show that cytochrome c, has all the necessary properties to work as the charge separation device [36] necessary to achieve energy transduction [3].

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