Cooperativity between Electrons and Protons in a Monomeric Cytochrome c_3: The Importance of Mechano-Chemical Coupling for Energy Transduction

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To fully understand the structural bases for the mechanisms of biological energy transduction, it is essential to determine the microscopic thermodynamic parameters which describe the properties of each centre involved in the reactions, as well as its interactions with the others. These interactions between centres can then be interpreted in the light of structural features of the proteins. Redox titrations of cytochrome c$_3$ *from* Desulfovibrio desulfuricans *ATCC 27774 followed by NMR and visible spectroscopy were analysed by using an equilibrium thermodynamic model. The network of homotropic and heterotropic cooperativities results in the coupled transfer of electrons and protons under physiological conditions. The microscopic characterisation allows the identification of several pairs of centres for which there are clear conformational (non-Coulombic) contributions to their coupling energies, thus establishing the existence of localised redox- and acid–base-linked structural modifications in the protein (mechano-chemical coupling). The modulation of interactions between centres observed for this cytochrome may be an important general phenomenon and is discussed in the framework of its physiological function and of the current focus of energy transduction research.*

KEYWORDS:

cooperative phenomena · cytochromes · electrostatic interactions · heme proteins · NMR spectroscopy

1. Introduction

Current studies on proteins involved in energy transduction processes, such as cytochrome *c* oxidase and the bc_1 complex, reveal the importance of redox-linked acid–base equilibria,[1] also called redox-Bohr effects.[2] However, detailed studies of these large transmembrane complexes are difficult to perform and soluble systems of smaller molecular weight are coming under scrutiny in order to probe the relationship between the microscopic thermodynamic properties and the structural features.[3–6] The monomeric tetrahaem cytochromes c_3 from sulfate-reducing bacteria have been successfully used to develop analytical methods for the determination of the microscopic thermodynamic properties of proteins containing multiple redox and acid–base centres that display coupling between electrons and protons.[7–11] The presence of four closely packed haems together with pH-linked changes of the redox potentials in a soluble protein with ca. 15 kDa makes it a good target for experimental and theoretical studies.[12–14]

The free energy change associated with charge transfer to a redox centre can be described as a sum of several terms [Eq. (1)]:[15]

$$\Delta G_{\mathrm{redox}} = \Delta G_{\mathrm{cen}} + \Delta G_{\mathrm{el}} + \Delta G_{\mathrm{lig}} + \Delta G_{\mathrm{conf}} \qquad (1)$$

Equation (1) comprises bonding interactions (ΔG_{cen}), electrostatic interactions (ΔG_{el}), differences in ligand affinity (ΔG_{lig}) and conformational energy differences (ΔG_{conf}). The identification of the relative importance of these terms in controlling the reduction potentials of metalloproteins is an active field of research where both experimental[16–18] and theoretical approaches are being applied.[19–21] Experimentally, the problem is usually addressed by comparing closely related proteins or point mutants of a protein,[16, 18] which is problematic because replacing even a single residue can cause extensive changes in protein properties.[22] Here we consider a different approach. The

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microscopic thermodynamic characterisation of this multicentre protein allows the study of modifications in the ΔG_{redox} value of one centre when a single charge is introduced or removed from another functional centre of the same protein. The analysis of the microscopic thermodynamic properties of *Desulfovibrio desulfuricans* ATCC 27774 cytochrome c_3 (Ddc3) shows that substantial conformational terms contribute to the interactions between some pairs of centres. These effects are discussed with respect to their general importance in establishing the properties in multicentre proteins required to achieve the desired function, and in the particular case of this cytochrome, to perform a concerted two-electron step coupled to the transfer of protons.

2. Results

The NMR spectra of cytochrome c_3 display discrete signals for the five macroscopic oxidation stages corresponding to the fully oxidised state (stage 4), the states with three oxidised haems (stage 3), two oxidised haems (stage 2), one oxidised haem (stage 1) and the fully reduced state (stage 0).[10] The signals for each stage are resolved as a consequence of the slow intermolecular electron transfer and fast intramolecular electron exchange on the NMR time scale.[7] Under these conditions, the paramagnetic shift for each haem in each stage is determined by the relative fraction of oxidation of that haem in that stage. In addition, at the temperature used, the pH dependence of the signals at intermediate redox stages indicates fast equilibrium between the acid and base forms, although some coalesced peaks showed residual line broadening.[10]

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born in Porto (Portugal) in 1943, studied chemical engineering at Universidade Técnica de Lisboa and finished with a diploma degree in 1969. He obtained his doctorate at Oxford University in 1972 and did postdoctoral work with R. J. P. Williams to establish NMR spectroscopic methods to determine structures of biological macromolecules, resulting in the determination of the first three-dimensional structures in solution. He was appointed as Assistant Professor at Universidade Técnica de Lisboa in 1973 and became Associate (1974) and Full Professor of Biochemistry (1979) at Universidade Nova de Lisboa, where he was the Founding President of the Institute of Technology for Chemistry and Biology (1986 – 1998). He was Adjunct Professor of Biochemistry at the universities of Minnesota and Georgia. His major interest in research is to understand the role of the metal ions in metalloproteins and to develop spectroscopic methods for their structural characterisation to obtain structure – function relationships. He and his collaborators discovered and characterised several novel metal ion clusters in active sites of proteins, combining chemistry with structural and molecular biology. His recent work focuses on the response of anaerobic bacteria to oxygen stress, and on the structural basis for cooperativity in electron/proton coupling mechanisms involved in vectorial energy transduction processes.

The thermodynamic model used to analyse the experimental data was extended from that applied in previous studies on different cytochromes c_3[11, 13, 23] in order to incorporate one extra acid – base centre, which was necessary to fit the bell-shaped pH dependence of the NMR spectroscopic data. The chemical shifts observed for the paramagnetic NMR signals of methyl groups 18^1 of haems I, II and IV and methyl group 12^1 of haem III, together with the fit of the model are reported in Figure 1 (IUPAC – IUB nomenclature for the haem substituents is used, and the haems are labelled with Roman numerals according to the order of covalent attachment to the polypeptide chain). The entire data set is fitted well and, although the signals of methyl groups 18^1 of haems I and II in stage 1 appear to have larger deviations, this is a consequence of relatively large error bars resulting from linewidths typically greater than 1 ppm at half height. The order of oxidation of the four haems can be determined immediately from these plots, and at pH 8 it is the same as that reported previously at 275 K.[24] However, it is now clear that haems I and II change their order of oxidation twice in the experimental pH range, as can be appreciated from the marked bell-shaped pH dependence of their oxidised fractions at stage 1, which is caused by the combined effects of two different acid – base centres with different pK_a values. The presence of these two redox-linked acid – base centres is not readily apparent from the results of the redox titrations followed by visible spectroscopy alone (Figure 2). The thermodynamic parameters determined from the fit of the data are reported in

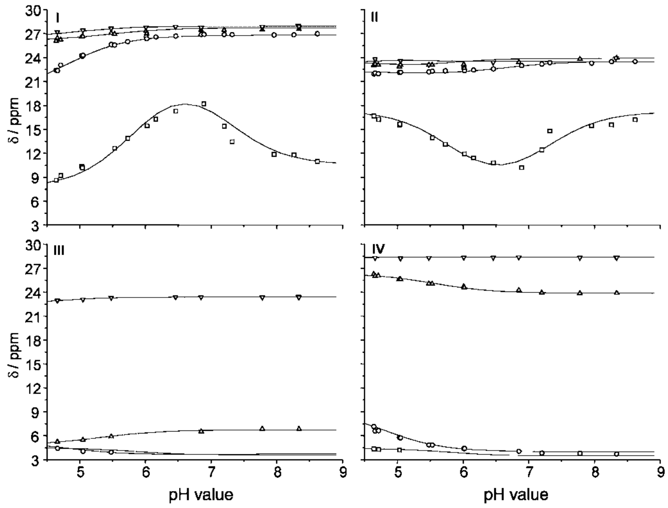

Figure 1. Paramagnetic chemical shifts of haem methyl groups M18^1 I, M18^1 II, M12^1 III, M18^1 IV at oxidation stages 1 (□), 2 (○), 3 (△) and 4 (▽). The continuous lines are calculated from the fit of the six-centre model to the experimental data.

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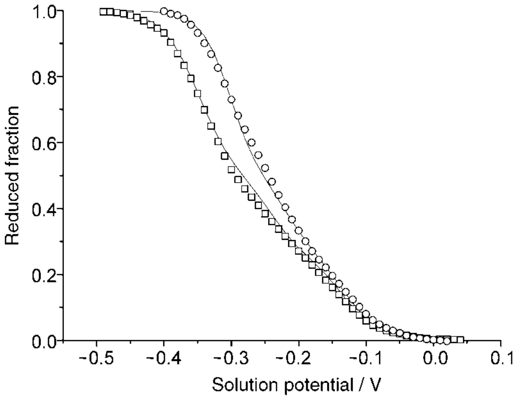

Figure 2. *Visible redox titration performed at 298 K and pH 6.3 (○) and 8.4 (□). The lines were calculated from the fit of the model to the experimental data using the parameters reported in Table 1.*

Table 1. Diagonal terms are the free energies of oxidation of the four haems (I–IV) and of deprotonation of the two acid–base centres (V and VI) in the fully reduced and protonated form of the cytochrome. Off-diagonal terms are the interaction energies between each pair of centres. These interaction energies indicate how the free energy of one centre in each pair is modified by oxidising or deprotonating the other. Since the reference state chosen is the fully reduced and protonated form, a negative interaction energy indicates positive cooperativity, that is, oxidation or protonation of one centre facilitates the oxidation or protonation of the other.

Table 1. *Thermodynamic parameters determined for Ddc3.*[a]

	Haem I	Haem II	Haem III	Haem IV	Centre V	Centre VI
Haem I	−237 (4)	2 (4)	48 (5)	3 (5)	−110 (15)	6 (13)
Haem II		−268 (4)	23 (7)	−7 (5)	−35 (7)	−59 (4)
Haem III			−207 (7)	60 (4)	11 (8)	−70 (7)
Haem IV				−195 (7)	20 (6)	−56 (4)
Centre VI					408 (17)	−13 (12)
Centre V						445 (17)

[a] The reference state is the fully reduced protonated form of the protein. Diagonal terms are the energies of oxidation (Haems I to IV) and of deprotonation (centres V and VI). Off-diagonal terms are the energies involved in the pairwise interactions. All values are reported in units of millielectronvolt (meV). The values in brackets are the standard errors associated with each parameter.

For each acid–base centre at each macroscopic redox stage, Table 2 reports the solution pH for which the protonated and deprotonated forms are equally populated, that is, the mid-point of the titration curve. These values were calculated iteratively from the thermodynamic parameters, since the existence of cooperativities in the intermediate redox stages modifies the pH dependence of the acid and base forms from a simple Henderson–Hasselbalch equation. These values differ from those determined at 283 K in the absence of salt as expected,[25, 26] since the equilibrium thermodynamic properties of tetrahaem cytochromes depend on ionic strength[27] and temperature.[13]

Table 2. *Macroscopic pK_a values of each acid–base centre defined by the model.*[a]

	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Centre V	7.1	5.9	4.8	5.0	5.0
Centre VI	7.6	7.1	6.6	5.7	4.6

[a] Values were calculated for each of the macroscopic stages of oxidation. Stage 0 corresponds to the fully reduced protein and stage 4 to the fully oxidised protein.

The microscopic thermodynamic data reported in Table 1 can also be used to determine the pH-dependent macroscopic redox potentials associated with the four sequential one-electron steps necessary to fully reduce or oxidise this protein (Figure 3).[10] The first and second reduction steps are separated by ca. 80 mV, whereas the last two steps, more negative by ca. 100 mV, have redox potentials separated by less than 30 mV throughout the pH range studied.

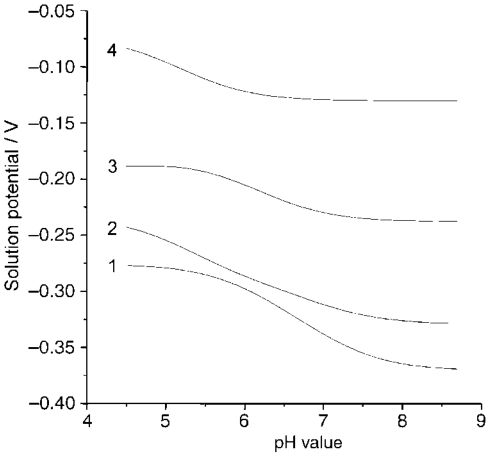

Figure 3. *The pH dependence of the macroscopic redox potentials of Ddc3 at 298 K, calculated from the parameters listed in Table 1. The lines, numbered 1 to 4, refer to the four sequential macroscopic steps of oxidation of the cytochrome.*

3. Discussion

According to Coulomb's law, which applies to a medium of uniform polarisability with a fixed separation of charges, the electrostatic interaction between charged centres is proportional to the inverse of the distance with a sign that depends only on the relative signs of the charges of the interacting particles, that is, repulsive coupling between pairs of haems (electrons) or of acid–base groups (protons), and attractive coupling between haems and acid–base groups. The latter effect is sometimes called the electroneutrality principle.[28] However, in the presence of conformational changes associated with the redox or acid–base transitions, such a simple correlation may not hold since changes in the distance between the interacting centres and in their environment introduce additional terms in the observed coupling which shall be referred to as "non-Coulombic effects".

Although the relative positions of the haems are known from the X-ray crystal structure,[29] the nature and position of the acid – base groups responsible for the redox-Bohr effect has yet to be determined unequivocally. Electrostatic calculations performed by using the crystal structure obtained at low pH as a basis have identified propionate 13 of haem I, propionate 13 of haem IV, and histidine 76 as likely candidates for the acid – base groups participating in the redox-Bohr effect in this protein.[26] These calculations are supported by the values determined for the heterotropic interaction energies (see Table 1). Indeed: i) A very large negative value was determined for the interaction energy between centre V and haem I, suggesting the assignment of propionate 13 of haem I to centre V; and ii) relatively large and similar negative interaction energies were obtained between centre VI and haems II, III and IV, which indicates that both propionate 13 of haem IV and histidine 76 are likely to contribute to the effect assigned to centre VI, since no single acid – base group is close to all these three haems. It should be recalled that negative values of the interaction energy in Table 1 indicate a positive cooperativity between the centres, and thus the values discussed above have the sign expected from Coulomb's law.

To analyse the coupling energies between the redox and acid – base centres further, the interaction energy between each pair of centres are plotted versus distance in Figure 4 by using the coordinates available from the crystal structure of the oxidised form of the protein.[29] Homotropic couplings (haem – haem and proton – proton) are plotted in Figure 4 A and heterotropic couplings (haem – proton) are plotted in Figure 4 B. Due to the repulsive nature of the former and the attractive nature of the latter, the scales of the two panels have opposite signs. The two solid lines were calculated for the distance dependence of charge – charge interactions by using Coulomb's law and values for the dielectric constant (ε) of 15 and 80, the consensus values for protein interior and bulk solvent, respectively.[18, 30] In Figure 4 B, a pair of points is plotted for each coupling involving centre VI, one using the coordinates of histidine 76 and the other using the coordinates of propionate 13 of haem IV. As noted above, both of these groups are likely to be involved in the interactions attributed to centre VI, but the lack of features in the NMR spectroscopic data indicates that their $\mathrm{p}K_\mathrm{a}$ values cannot be resolved. The two residues are expected to contribute quite different proportions to different interactions,[26] with one of the groups being dominant in each case. Thus, the true positions of the points in the figure should be lower in the energy scale since the plotted values are the observed totals. This analysis also supports the assignment of propionate 13 of haem I to the redox-Bohr effect associated with centre V, which shows a strong coupling with haem I and weaker couplings with the other haems.

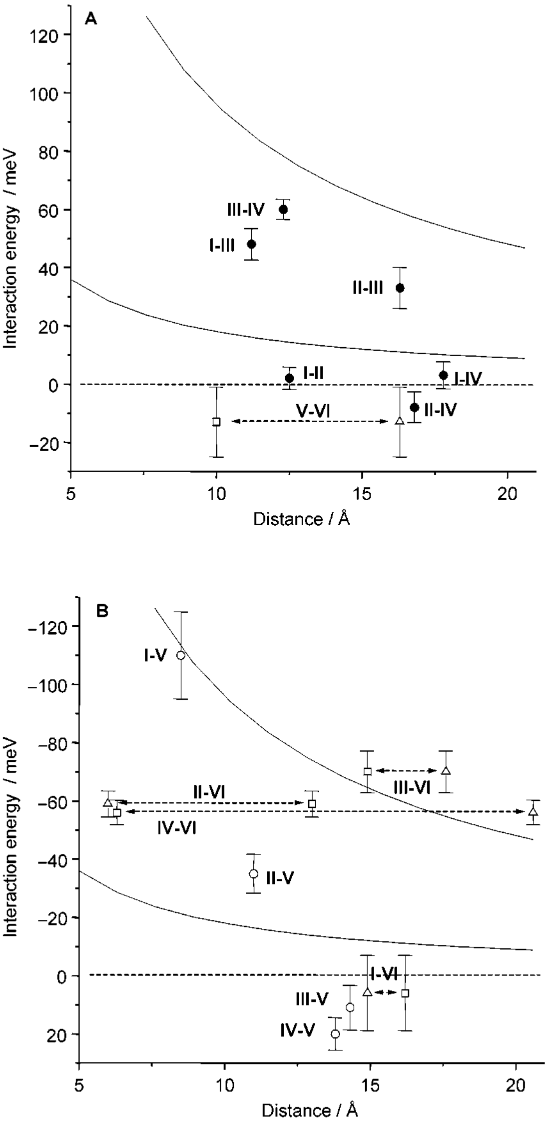

Figure 4. *Plot of interaction energies between pairs of centres versus distance. A: Homotropic interactions: Closed symbols refer to haem – haem interactions and open symbols refer to acid – base centre interactions. B: Heterotropic interactions. For centre VI, the two alternatives discussed in the text are plotted; distances to the average position of the carboxylate oxygen atoms of propionate 13 of haem IV are represented by squares, and distances to the ring N3 atom of histidine 76 as triangles. Interactions between the haems and centre V, assumed to be propionate 13 of haem I, are represented by circles. The vertical bars indicate the standard errors of the fitted parameters (see Table 1). The two curves in each panel were calculated by using Coulomb's law for effective dielectric constants* (ε) *of 80 (lower) and 15 (upper).*

The area defined by the two lines in each of the panels of Figure 4 should encompass the distance dependence for electrostatic couplings observed for a small molecule such as Ddc3, in which there is substantial solvent exposure of the centres. Part of the scatter in the data points may be due to local variations in the dielectric constant, but the existence of several points outside these boundaries indicates that the interactions in cytochrome c_3 are not described by Coulomb's law. In particular, some cases such as the interaction between haems I and II and between haems I and IV show an apparent dielectric constant larger than that of the solvent. Large values for the apparent dielectric constant have been predicted from electrostatic calculations by using the structure of cytochrome c_3,[12] and were also reported for other systems.[31] In the latter case, they were interpreted as resulting from the contribution of the overall protein dipole to the charge – charge interaction. However, for some interactions, such as that between haems II and IV, and the interaction between acid – base centre V and haems III and IV, the overall coupling takes a sign which is not even compatible with positive values for the effective dielectric constant considering a unique structure. These observations demonstrate that there are other contributions to the interactions between centres. Such contributions must involve modifications in the structure, including movement of charges, or rearrangement of hydrogen bond networks, all linked to the redox or acid – base transitions and will be referred to collectively as mechano-chemical couplings. These couplings add a non-Coulombic term to the overall interaction energy that cannot be predicted by electrostatic calculations based on one set of atomic coordinates. This effect is most clearly evident in the cases where the experimental sign of the coupling is reversed when the non-Coulombic term outweighs the ordinary electrostatic interaction. However, non-Coulombic terms may also enhance interactions, giving the appearance of unusually low effective dielectric constants.

As mentioned above, an electrostatic analysis based on a single structure of the protein such as those reported in the literature for cytochromes c_3[26] can neither predict the negative value for the coupling energy between haems II and IV, nor the positive coupling energies between centre VI and haem I and between centre V and both haems III and IV, since it does not account for the structural modifications associated with either acid – base or redox transitions.

On the basis of currently available structural data for the reduced and oxidised forms of other cytochromes c_3,[3, 5, 32] the mechano-chemical effects observed do not appear to involve changes of the overall folding of the polypeptide chain. However, a difference in the water content or side chain movements in the interior of the protein could account for the non-Coulombic contributions to the experimental data. Indeed, recent detailed NMR spectroscopic structural studies of the oxidised and reduced forms of cytochromes c_3 isolated from *D. vulgaris* (Hildenborough) and *D. gigas*,[3, 5] and X-ray crystallographic studies of *D. africanus*,[32] show that the redox-linked differences involve highly localised modifications, which may be a common situation.[33] However, the low sequence homology between the various cytochromes c_3 precludes the use of these structures as bases for discussing the origin of the conformational effects in Ddc3. This discussion must await a detailed structural characterisation of the oxidised and reduced forms of this protein, which is beyond the scope of the present article.

Structural modifications that reduce or reverse the electrostatic coupling between specific pairs of charged centres as observed in these data narrow the range of redox potentials and pH in which the transitions for the haems and acid – base centres occur. Furthermore, the structural modifications provide the necessary flexibility for maintaining or enhancing the electrostatic interaction between other coupled pairs, in particular heterotropic couplings, allowing the full set of centres to operate within physiologically accessible conditions of reduction potential and pH.

Table 3 lists the order of oxidation of the haems for the cytochromes c_3 that have been studied in detail. It indicates pairs of haems that display conformational effects in their coupling energies that favour positive cooperativity, as well as the haem with the largest redox-Bohr effect. Despite the structural similarities among the various cytochromes, the order of reduction for their haems is different. It is also apparent from Table 3 that in all cases there is cooperativity between two haems that favours a concerted two electron step, although it does not always involve either the same pair of haems in the structure, or in the relative order of oxidation. Moreover, haem I is always dominant in the redox-Bohr effect. These two features, common to all cytochromes c_3 so far characterised thermodynamically at the microscopic level, result in a concerted two-electron step, and electron/proton coupling, respectively. The resulting energy transduction performed by these soluble proteins was named "proton thrusting"[34] to distinguish it from the "proton-pumping" activity of transmembrane protein complexes. This coupled transfer of two electrons associated with proton transfer in the physiological pH range is fundamental for the ability of cytochrome c_3 to accelerate the oxidation of hydrogen by hydrogenase, with subsequent transfer of electrons to the membrane-associated nine-haem cytochrome.[35] These two proteins are the up- and downstream physiological partners of Ddc3 in the respiratory chain leading to the reduction of sulfate.[36–38]

Table 3. *Order of oxidation (from left to right) of the haems for different cytochromes* c_3.[a]

Protein	Order of oxidation of the haems		
Dvc3	III	II **I**	IV
Dgc3	**I**	II III	IV
Ddc3	II **I**	IV	III

[a] Roman numbers refer to the order of attachment of the haems to the polypeptide chain. Closely spaced numbers correspond to haems that have similar redox potentials and display a tendency towards positive cooperativity as a result of non-Coulombic effects. The number in bold indicates the haem with the strongest redox-Bohr effect. The order reported in this table refers to the midpoint of the titration curves for each haem, which includes contributions from the off-diagonal terms of Table 1 as well as the diagonal terms. Data refers to cytochromes isolated from *Desulfovibrio vulgaris* (Dvc3),[10] *D. gigas* (Dgc3)[13] and *D. desulfuricans* (Ddc3; this work).

4. Conclusion

The present study of cytochrome c_3 from *D. desulfuricans* attains a new level of complexity and biological relevance in understanding the thermodynamic properties of multicentre proteins. Although evidence for the participation of two protons in the redox-Bohr effect in one cytochrome c_3 had already been presented,[2] this is the first case for which the experimental data has allowed the parameters of two distinct acid – base centres to be resolved within the model. However, further structural data is required to distinguish whether the centres modelled correspond to the acid – base groups identified in the previous section or describe the combined effect of partial contributions from additional groups, as observed for the Bohr effect in haemoglobin.[39] Nevertheless, it is noteworthy that two acid – base centres with pK_a values in the physiological pH range are well resolved in a cytochrome that was isolated from an organism with a flexible bioenergetic metabolism, capable of using either sulfate or nitrate as terminal electron acceptors.[40] It is likely that the physiological pH value of the periplasm is different for the two types of respiration that produce sulfide and ammonia, respectively. The difference between the pK_a values associated with each of the centres broadens the total range of pH in which coupled transfer of electrons and protons is efficient, as can be observed in Figure 3. Thus, broadening the range of pH at which efficient proton thrusting can be achieved may be an adaptation to the variety of environmental conditions of growth of this organism, which was isolated from the rumen of sheep.[40]

A corollary of the adaptation of our thermodynamic model to this more complex protein is that similar adaptations may yield valuable analyses of other multicentre redox enzymes, either soluble, such as hydrogenase, xanthine oxidase, and nitrogenase, or membrane-bound such as cytochrome *c* oxidase. Structures are available in at least one state for some of these proteins, which could help in focusing the search for relevant structural modifications. Also, the thermodynamic properties of some of these systems have been studied for quite some time and a great variety of experimental results is available in the literature.[41] In this context, it was recently observed that cytochrome *c* oxidase from bovine heart mitochondria releases a proton upon reduction of haem *a*.[42] This observation defies an electrostatic interpretation since the uptake of a negative charge should increase the affinity for a positive charge and not the reverse. However, a substantial non-Coulombic contribution to the overall coupling energy, such as those reported in the present work, can account for this observation.[43]

The present work shows that mechano-chemical coupling arising from local redox-linked and/or acid–base-linked conformational changes can be an important factor in controlling the thermodynamic properties of proteins involved in energy-transducing processes. The results show a selective modulation of the electrostatic interactions that would be expected from Coulomb's law. This may prove to be a general phenomenon, whereby biological processes are controlled by modulating and channelling electrostatic forces.

Experimental Section

Sample purification: Cytochrome c_3 from *D. desulfuricans* ATCC 27774 (Ddc3) was obtained as previously described.[24]

NMR spectroscopy: Samples containing 1–2 mM of protein and 0.1 M potassium chloride in aqueous solution were poised at partially oxidised conditions and at different solution pH values using the procedures reported in the literature.[13] Measurements were performed in the pH range 4.6–8.6 (meter readings not corrected for the isotope effect).

NMR spectra were recorded in a Bruker DRX 500 spectrometer at 298 ± 0.1 K. NOESY experiments were collected with 1024 × 512 points, a 20-ms mixing time and an 800-ms water pre-saturation pulse. The 2D maps were zero-filled to 2048 × 1024 points and multiplied by cosine functions in both dimensions. The chemical shifts and the linewidths of the signals of one methyl group of each haem having a large paramagnetic shift were measured in all experiments. The positions of the four methyl group signals were followed throughout redox titrations of the protein by observation of the exchange cross-peaks between the different redox stages.[7]

Redox titrations followed by visible spectroscopy: The titrations were performed using a Shimadzu 1201 spectrophotometer placed inside an anaerobic glove box under an argon atmosphere. The cell holder was adapted to allow stirring of the sample by using a bar magnet and the temperature was maintained at 25 ± 0.2 °C by using an external circulating bath. Solution potentials were measured using a combined Pt/Ag/AgCl electrode, calibrated before each titration with freshly prepared saturated solutions of quinhydrone at pH 7 and 4 and checked at the end for stability. Experiments were performed at pH 6.3 and 8.4 using 0.1 M Tris-maleate buffer and ca. 2 mM of cytochrome. The following redox mediators were added to the sample to ensure good electrode response according to the recommendations reported in the literature:[44] anthraquinone-2,6-disulfonate, anthraquinone-2-sulfonate, indigo disulfonate, indigo trisulfonate, indigo tetrasulfonate, methyl viologen, neutral red, diquat, methylene blue, phenosafranine and safranine O. The final concentration of each mediator was 4–8 mM. Each titration consisted of a stepwise reduction using a solution of ca. 2 mM dithionite, followed by oxidation using a solution of ca. 2 mM ferricyanide, and the pH was checked for changes at the end. After each addition of titrant, ample time was allowed for a stable measurement of redox potential to be reached, and a spectrum of the sample was taken in the range 600–400 nm. The optical contribution of the mediators was subtracted by measuring the height of the peak at 552 nm relative to the straight line connecting the two isosbestic points that flank the strong α band of Ddc3. Three experiments were performed at each pH and the results averaged after linear interpolation of the data. No hysteresis between the reductive and oxidative parts of the curves was observed. Due to the very low redox potentials of the haems in cytochrome c_3, the end point of the titrations at the low potential end is not always clear, thus the signal amplitude at 552 nm was normalised approximately to give the overall fraction of reduced haems.

Modelling: The thermodynamic model previously presented[10] was extended to six charged and interacting centres to analyse the NMR spectroscopic and visible experimental data. It defines free energies of oxidation for each of the four haems and free energies of deprotonation for two acid–base centres, as well as the magnitude of the coupling energies between each pair of centres. The energies of oxidation and of deprotonation can be easily related to standard reduction potentials and pK_a values by using standard thermodynamic equations.[23] This parameterisation is sufficient to describe the experimental data, and the thermodynamic parameters were determined by simultaneous fit of the model to the NMR spectroscopic and visible data by using the Marquardt method.[45] Attempts to reduce the number of parameters resulted in a marked decrease in the goodness of the fit, and analysis of the covariance matrix reveals that all parameters are adequately defined by the available data. In addition to the thermodynamic parameters, the chemical shifts of the haem methyl groups in the fully oxidised molecule in the different protonation states and the normalisation of the visible titration curves were also optimised. The dipolar field generated by the unpaired electron in each oxidised haem was calculated separately as previously described[46] by using the ^{13}C NMR data reported in the literature[47] together with the crystal structure.[29] These data were used to correct the paramagnetic chemical shift of each haem methyl group for extrinsic effects resulting from the oxidation of the other haems in the protein.[11] The linewidths of the NMR signals at half-height were used to estimate the uncertainty of each measurement and the visible redox titrations were considered to have an uncertainty of ± 2% of the total amplitude of the reduced minus oxidised optical signal.

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