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Redox-Bohr effect in the nine haem cytochrome from *Desulfovibrio desulfuricans* 27774

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We dedicate this article to Helmut Siegel for his enormous contributions to Bioinorganic Chemistry (BIC) and, together with his wife Astrid, had an instrumental role in the construction of a friendly BIC community.

Abstract

Redox titrations followed by visible spectroscopy were performed at various pH values with the nine-haem cytochrome *c* from *Desulfovibrio desulfuricans* ATCC 27774 (*Dd*27774). Macroscopic midpoint reduction potentials of the haems and pK_a^{ox} and pK_a^{red} values of acid–base centres that interact with the redox centres were estimated by using a thermodynamic model, in which the haems are grouped in three sets, and two acid–base centres are considered. The results show that this cytochrome displays redox-Bohr effects in the physiological pH range and that electrostatic effects dominate the macroscopic interactions between the acid–base and redox centres.

Keywords: Nine-haem cytochome *c*; Redox-Bohr effect; Energy transduction; Electrostatic models; *Desulfovibrio desulfuricans*; Redox titrations

1. Introduction

Respiratory electron transfer chains leading to oxidative phosphorylation involve proteins containing multiple redox centres, where the redox transitions are coupled with acid–base reactions (redox-Bohr effect). This coupled transfer of electrons and protons is fundamental to energy conservation [1]. Systems involved in energy transduction processes are usually complex and membrane bound, such as those present in transmembrane respiratory complexes [2]. The detailed analysis of such complex systems is difficult and requires that good quality experimental data is obtained and that proper theoretical analysis of the results is performed.

A large variety of multi-haem *c*-type cytochromes is found in the periplasmic space of sulfate reducing bacteria (SRB) and a soluble monomeric tetrahaem cytochrome (cyt c_3) has been the focus of the most detailed studies [3,4]. A nine-haem cytochrome *c* (9Hcc), isolated from the SRB *Desulfovibrio desulfuricans* ATCC 27774 (*Dd* 27774) [5] is a 37.8 kDa monomeric protein [6] containing 292 aminoacid residues. The haems are all low-spin with bis-histidinyl axial coordination and are folded into two tetrahaem clusters similar to that in cyt c_3, with one extra haem located between them (Fig. 1) [7]. Its role in the bioenergetic metabolism of *Dd* 27774 is proposed to be analogous to that of the high molecular weight cytochrome *c* (Hmc) containing 16 haems found in the SRB *Desulfovibrio vulgaris* (Hildenborough) (*Dv*H) [7]. The reduction of the Hmc and 9Hcc by periplasmic hydrogenases occurs

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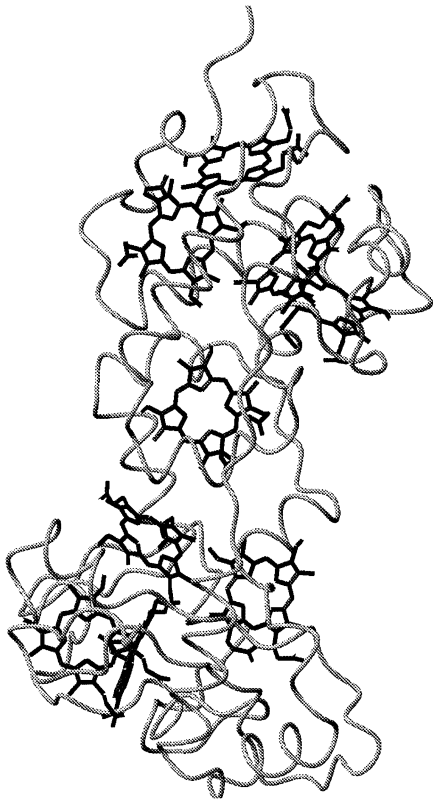

Fig. 1. Representation of the backbone and haems from the crystal structure of the 9Hcc (PDB code 1c9h). The C-terminus is at the top of the figure.

in vitro mediated by type I cyt c_3 (TpI-c_3), as the presence of catalytic amounts of the latter increases considerably the rate of reduction of the Hmc and 9Hcc [7,8]. The same catalytic effect was observed for the recently isolated type II cyt c_3 (TpII-c_3) from *Dv*H [9], a cytochrome that shares several important structural features with the 9Hcc. The *Dd* 27774 9Hcc can accept electrons from *Dd* 27774 cyt c_3 via specific docking [10]. These studies, indicating that the 9Hcc does not act as direct partner for hydrogenase, together with the fact that both the Hmc gene and the TpII-c_3 genes in *Dv*H and the 9Hcc gene in *Dd* are part of operons encoding membrane bound redox complexes, suggest that 9Hcc is part of an assembly of membrane associated proteins involved in transferring the electrons back to the cell interior [9,11,12]. Thus it may constitute a link between periplasmic hydrogen oxidation and cytoplasmic sulfate reduction in *Desulfovibrio* spp.

In the present article we report redox titrations of the 9Hcc followed by visible spectroscopy performed at various pH values in the range 6.3–8.4. The results are analysed using an adaptation of the thermodynamic model previously applied to cyt c_3 [13]. It is shown that the haems in the 9Hcc span a relatively wide range of redox potentials and display redox-Bohr effects.

2. Experimental

2.1. Cell material

D. desulfuricans ATCC 27774 cells were grown anaerobically at 37 °C in nitrate as previously described [5]. Cells were ruptured by two passages in the French press. The resulting extract was centrifuged at 11 300 × *g* at 4 °C for 1 h and the soluble extract was ultracentrifuged at 18 6000 × *g* for 90 min at 4 °C. The supernatant was used for the purification procedures described below.

2.2. Nine-haem cytochrome purification from soluble fraction

All purification steps were performed at pH 7.6 and 4 °C in an HPLC Amersham-Pharmacia biotec system unless otherwise stated. The supernatant fraction obtained was dialysed against 10 mM Tris–HCl for 24 h and loaded onto a DEAE-fast flow column (5 × 50 cm) equilibrated with 10 mM Tris–HCl. A continuous Tris–HCl gradient (0.01–0.4 M) was applied. The fraction eluted at 150–200 mM and containing the 9Hcc was then dialysed and applied to a Q-Sepharose (2.6 × 15 cm) with a continuous gradient (20 mM–1 M) of Tris–HCl. The 9Hcc was eluted between 150 and 200 mM. This fraction was then loaded into a hydroxylapatite column (3 × 20 cm, Bio-Rad) and the 9Hcc was eluted at 50–100 mM in a linear gradient of potassium phosphate buffer (5 mM–1 M). Finally, a Resource-Q column (6 ml) was used and the fraction eluted at 100–150 mM was loaded onto a Superdex 200 (2.6 × 60 cm) column equilibrated in 50 mM Tris–HCl buffer and 150 mM NaCl to obtain pure 9Hcc. The purity of the protein was revealed as a single band on a 12% SDS-PAGE and a purity index (A_{552}/A_{280}) around 2.4.

2.3. Visible spectroscopy experiments

Redox titrations were followed by visible spectroscopy, performed as previously described [14], at approximately 180 mM ionic strength in TRIS–maleate buffer at pH 6.3, 7.4, and 8.4, at 25 ± 1 °C. To achieve a proper electric contact between the protein and the electrode surface, a cocktail of redox mediators with concentrations of 5–9 μM was prepared for each pH following the recommendations in the literature [15] and using published tables of data [16,17]. At pH 6.3, indigo tetrasulfonate, indigo trisulfonate, anthraquinone-2,7-disulfonate, phenosafranine, anthraquinone-2-sulfonate, 2-hydroxy-1,4-naphthoquinone, safranine O, diquat, benzyl viologen, methyl viologen, neutral red were used. At pH 7.4, indigo disulfonate was added to the previous mixture. Phenosafranine and benzyl viologen

were removed and methylene blue was added in the titrations at pH 8.4. Dithionite is unstable in solutions of low pH and therefore, complete reduction of 9Hcc was more difficult at pH 6.3. Also, at lower pH the mediation is less effective and the mediator concentration was increased for the experiments at pH 7.4 and 6.3. Three experiments were performed at each pH in the reductive and oxidative directions to check for hysteresis and reproducibility, and the results of the three experiments at each pH were averaged. In order to check for possible effects resulting from specific binding of mediator molecules to the cytochrome, titrations were performed at different ratios of protein and mediator concentrations. These showed no modifications in the titration curves. Reduced fractions were calculated as described in the literature [14] using the α band maximum at 552 nm and the isosbestic points at 543 and 559 nm to subtract the optical contributions of the mediators.

3. Modelling

A complex redox protein such as 9Hcc, even if it behaves reversibly, cannot be analysed microscopically in terms of individual redox centres based solely on the data obtained by visible spectroscopy because the different centres cannot be distinguished. Furthermore, detailed analysis of such a system could require up to 512 independent thermodynamic parameters just to account for the redox transitions in the presence of pairwise haem–haem interactions. The multicentre model used in this work simplifies this situation and provides an acceptable theoretical approximation of the experimental data, allowing a macroscopic analysis and interpretation of the present results. The minimal model used considers three independent sets of redox groups and two acid–base centres. The properties of the haems within each of the three sets of redox groups are assumed to be identical, and each group interacts with only one of the acid–base centres. Thus, a total of eight macroscopic parameters are optimised in the fitting process. Although no attempt is made to associate the haems within each of the three sets of redox groups with specific groups in the structure, it seems natural to include an integral number of haems in each set. It was found that sets in the proportion 4:3:2 gave significantly improved fittings relative to 3:3:3. Thus the experimental data was fit with a model of ten linearly independent parameters, of which two were fixed by specifying the size of the sets and eight were optimised by the fitting program.

4. Results

4.1. Visible redox titrations

The fraction of reduced haem in 9Hcc is plotted in Fig. 2 as a function of the solution potential for the experiments performed at pH 6.3, 7.4 and 8.4. This shows that the reduction potentials are pH dependent and cover a wide range of values, as expected for a multiredox centre protein. No hysteresis was observed for the reduced fraction between the reductive and oxidative branches of the redox titrations in the experimental conditions used. This indicates that there are no slow-relaxing modifications in the protein structure associated with the redox transitions.

The multicentre model allows a good fit of the experimental data (Fig. 2(panel A)) with residuals lower than 2% of the total reduced fraction as reported in panel B of Fig. 2. The best solution associates two sets of redox centres (comprising four and two haems) with one acid–base centre, which has values for pK_a^{ox} and pK_a^{red} of 6.4 and 7.4, respectively, and one set of redox centres (comprising three haems) with a second acid–base centre, which has values of 7.3 and 7.9 for pK_a^{ox} and pK_a^{red}, respectively.

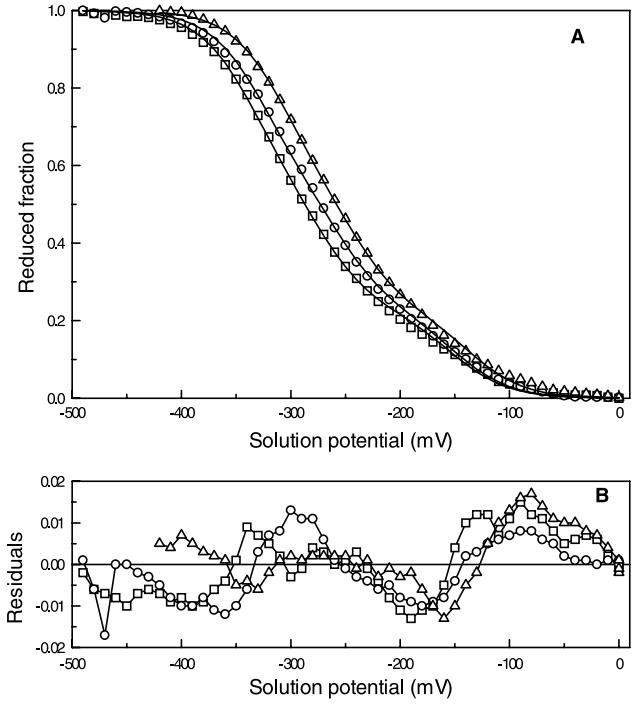

Fig. 2. (A) Redox titration of 9Hcc followed by visible spectroscopy performed at 25 °C and pH 6.3 (△), 7.4 (○), 8.4 (□). The lines correspond to the fitting of the multicentre thermodynamic model considering three groups of haems and two acid–base centres. (B) Residuals of the fitting of the thermodynamic model to the experimental data.

Table 1
Thermodynamic parameters for the model of five centres in *Dd*27774 9Hcc obtained by fitting the experimental data

E_1 (four haems)	−**338** (3)			−36 (4)	
E_2 (three haems)		−**274** (5)			−36 (5)
E_3 (two haems)			−**152** (3)	−25 (10)	
H_1				**438** (12)	
H_2					**466** (17)

Diagonal elements (bold-face) are the free energies (in meV) necessary to oxidise the redox centres or deprotonate the acid–base centres. The off-diagonal elements represent the energies of the redox-Bohr interactions between the redox centres and the acid–base centres. The pK_a values for each acid–base centre in the reduced protein are given by the diagonal element $\Delta G \times F/(2.3RT)$ and the pK_a values of the oxidised protein are given by $(\Delta G + \Sigma\ \Delta\Delta G) \times F/(2.3RT)$, where the diagonal term is summed to all off-diagonal terms in the respective column. Values in parentheses are the standard errors of the energies and interactions.

5. Discussion

The experimental procedures for performing redox titrations followed by visible spectroscopy are well established in the literature [15]. Redox proteins of low redox potentials with multiple centres present additional problems. In particular, the need to use mixtures of redox mediators that cover adequately the wide range of potentials in which the titration will take place creates problems of spectral interference since a set of mediators that do not absorb in the region of interest may not exist. Thus, although multihaem cytochromes are experimentally convenient systems due to their intense absorption bands, a proper analysis of their equilibrium redox properties cannot be made without subtracting this spectral interference in order to determine the fractional oxidation of the protein. Since some mediators are not reversible and others are light sensitive [15], their optical contribution may not be equal in the reductive and oxidative titrations. This can lead to an apparent hysteresis in the absence of a correction for the optical contribution of the mediators.

The data obtained show that no hysteresis is observed for the titration curves of 9Hcc from *Dd* 27774, indicating that in these experimental conditions the protein can cycle between the fully reduced and the fully oxidised states in a completely reversible way at the pH values probed. In contrast, redox titrations reported at pH 7.0 and 100 mM ionic strength for a 9Hcc isolated from another substrain of *D. desulfuricans*, (strain Essex) were considered to indicate the existence of hysteresis in that protein despite the large scatter in the data presented [18].

The results of the fit of all experimental data to the thermodynamic model are reported in panel A of Fig. 2. Previous reductive titrations obtained for 9Hcc from *Dd* 27774 [6] were performed under different experimental conditions from the present work and thus were not incorporated in the current analysis. Modelling two non-interacting acid–base centres instead of one is reasonable considering the size and structure of this molecule, in which it would be difficult to envisage a single acid–base group having sizeable interactions with all haems (see Fig. 1). Furthermore, when only one acid–base centre is modelled, reducing the number of fitted parameters by just one from eight to seven, the target function of the fitting is 30% worse. The thermodynamic parameters for each group of haems and the two macroscopic acid–base centres are reported in Table 1 as free energy (ΔG) for the redox and acid–base transitions and variations in these free energies ($\Delta\Delta G$) for the redox-Bohr interactions. In order to increase the detail of the present characterisation it would be necessary to apply a technique, such as NMR, that is capable of distinguishing the individual centres, as already demonstrated for several soluble tetrahaem cytochromes [13,14,19–21].

The shift of the redox titration curves to more negative solution potentials as the pH increases shows that the 9Hcc exhibits a redox-Bohr effect [2,22] in the physiological pH range. These interactions are of similar magnitude to those reported for tetrahaem cyt c_3 [13,14,20,21] and make 9Hcc a possible acceptor for both electrons and protons released by cyt c_3 in vivo.

The results indicate that the overall interactions between the redox and acid–base centres are predominantly of electrostatic nature in which the uptake or release of one positive charge facilitates thermodynamically the uptake or release of a negative charge. The data show that valuable insights into the details of energy transduction processes that involve coupled changes in the reduction potentials of functional sites, including redox dependent pK_a values of acid–base centres of the protein, may be obtained from the macroscopic analysis of these very complex cytochromes. The thermodynamic multicentre model has potential applications to the elucidation of the equilibrium properties not only in haem proteins but also in any protein with multiple interacting centres. The detailed physico-chemical knowledge of these thermodynamic properties is fundamental for analysing the kinetics of electron transfer in these complex systems [23] and thus obtain a functional understanding of energy transduction events.

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