Structural and functional characterization of cytochrome c_3 from *D. desulfuricans* ATCC 27774 by ^{1}H-NMR

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Abstract Cooperativity between redox and protonation centres is known to be crucial for the function of complex proteins, but it is often difficult to describe in terms of thermodynamic parameters. Cytochrome c_3 is a good model for these studies since, while retaining the overall complexity of larger systems, it is suitable for detailed crystallographic and spectroscopic studies. Assignment of the haem substituent NMR resonances, together with NMR redox titrations of cytochrome c_3 from *D. desulfuricans* ATCC 27774, was used to correlate relative redox potentials to specific haems in the structure: haem II ≅ haem I < haem IV < haem III. This order is different from that determined for the homologous proteins studied and in disagreement with that previously reported for this cytochrome (Morais, J., Palma, N., Frazão, C., Caldeira, J., LeGall, J., Moura, I., Moura, J.J.G. and Carrondo, M.A. (1995) Biochemistry 34, 12830–12841).

Key words: Cytochrome c_3; 2D-NMR; X-ray structure; Electron transfer

1. Introduction

Tetrahaem cytochrome c_3 was the first cytochrome to be identified in strict anaerobic non-photosynthetic bacteria [1]. It is a small periplasmic protein of about 14 kDa which is present in bacteria from the *Desulfovibrionaceae* family [2]. The haems are covalently bound to the polypeptide chain by two thioether bridges and bis-histidinyl axial coordination and have very low redox potentials. For the *D. gigas* and *D. vulgaris* cytochromes, the microscopic haem mid-point redox potentials were shown to be dependent on the redox state of the protein and pH [3–6], thus establishing a network of redox and redox-Bohr cooperativities. In the case of *D. vulgaris*, this was used to explain how cytochrome c_3 can act as a charge separation device for electrons and protons originating from the oxidation of molecular hydrogen via hydrogenase, leading to energy transduction [7].

Remarkably, although the structures available for this protein from different organisms show the same spatial organisation of the four-haem core [8–16], the amino acid sequence homology can be as low as 23% [17]. In contrast, 64% of the conserved residues are directly involved in the binding of the haems [18]. This, together with the preservation of the structural architecture of the four haems which suggests that it is crucial to the function, shows that cytochrome c_3 can be considered a distinct family of homologous proteins.

The low molecular weight of cytochrome c_3 makes it quite suitable for high resolution NMR structural studies since the haems act as intrinsic probes inside the protein [2,19]. The octahedral coordination of the iron favours the diamagnetic low-spin form (S = 0) in the reduced state (Fe^{2+}) and the NMR spectra have several resonances shifted by the ring currents of the haems. In this state, a complete specific assignment of the haem substituents is possible with subsequent cross assignment to the three-dimensional structure. In the oxidised form the low spin iron is paramagnetic (S = 1/2) giving rise to hyperfine shifts for the haems and neighbouring nuclei. When the intermolecular electron exchange is slow on the NMR time scale and the intramolecular electron exchange is fast [3,4], a separate set of NMR resonances is observed for the haem protons in each stage of oxidation (numbered according to the number of oxidised haems) with shifts which depend on the relative populations of each oxidised haem. This property is extremely useful since it can not only be used to determine the relative microscopic redox potentials for the different haems [3], but also provides the means to extend the structure-specific assignments from the spectra of the reduced form by the observation of chemical exchange connectivities between resonances of the same haem nucleus in the different stages of oxidation of the protein [3,4,20–22]. In multiredox-centre proteins, this is a crucial piece of information for understanding their mode of action since it links unequivocally specific structural features with the respective redox potentials. In the particular case of *D. desulfuricans* ATCC 27774 cytochrome c_3, the macroscopic haem redox potentials have already been determined by EPR (−380, −370, −260, −140 at pH = 8) [16], but unfortunately the assignments deduced for the ordered microscopic haem redox potentials as well as some of the reported midpoint redox potentials are wrong, as shown in this article.

2. Materials and methods

D. desulfuricans ATCC 27774 was grown in a medium containing nitrate as terminal electron acceptor, and cytochrome c_3 was purified as previously described [23]. The protein was dialysed overnight against deionised water at 4°C and lyophilised twice from ^2H_2O. The NMR sample was prepared by dissolving the powder in ^2H_2O to a concentration of about 5 mM. The pH (not corrected for the isotope effect) was adjusted with small amounts of ^2HCl and NaO^2H. The reduced and intermediate oxidation states of the protein were obtained as previously described [24].

The NMR spectra were recorded on a 500 MHz Bruker AMX-500 spectrometer equipped with an inverse detection 5 mm probe. The

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Abbreviations: NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; DSS, 4,4-dimethyl-4-silapentanesodium sulphonate; Mi, haem methyl numbered according to IUPAC-IUB nomenclature; Hi, haem proton substituents numbered according to IUPAC-IUB nomenclature

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NOESY spectra were measured with mixing times of 50, 100, and 400 ms in the reduced state at 25°C, 25 and 75 ms in the oxidised state at 25°C, 75 ms in the oxidised state at 2°C, and 25 ms in the intermediate state at 2°C. The TOCSY spectra were performed using the MLEV 17 spin lock sequence [25], with a field strength of 10 kHz and 40 ms mixing time. All experiments were obtained using the time proportional phase incrementation method [26], with water pre-saturation, and chemical shifts are presented in ppm relative to DSS, using formate (8.459 ppm) as an internal reference.

The pH of the sample in the reduced and intermediate stages of oxidation was measured inside a glove box with argon atmosphere, to avoid reoxidation. Calculated ring current shifts were obtained as previously described [24].

3. Results

The general strategy for the assignment of the haem resonances in the reduced form of multihaem cytochromes, using NOESY and TOCSY spectra, has been described elsewhere [24]. As in the case of the homologous cytochrome c_3 from *D. gigas* [21] several of the haem meso proton resonances overlap, thus requiring the identification of NOESY cross-peaks between haem substituents and specifically assigned aromatic residues to obtain the unambiguous assignment of the four self-consistent sets of haem resonances to each haem in the structure (Table 1). This assignment was confirmed by the observation of NOE cross-peaks (not shown) between resonances of substituent nuclei from different haems, in agreement with the X-ray structure, showing that the spatial arrangement of the haems in the crystal is maintained in solution. Furthermore, the calculated ring-current shifts for the aromatic and haem protons are in good agreement with the experimental data (Fig. 1).

Chemical exchange experiments using the NOESY pulse sequence allowed the extension of the reduced haem proton assignments to intermediate stages of oxidation and the fully oxidised state [20,27], simultaneously revealing the structural cross-assignment of the haems according to their relative redox potentials.

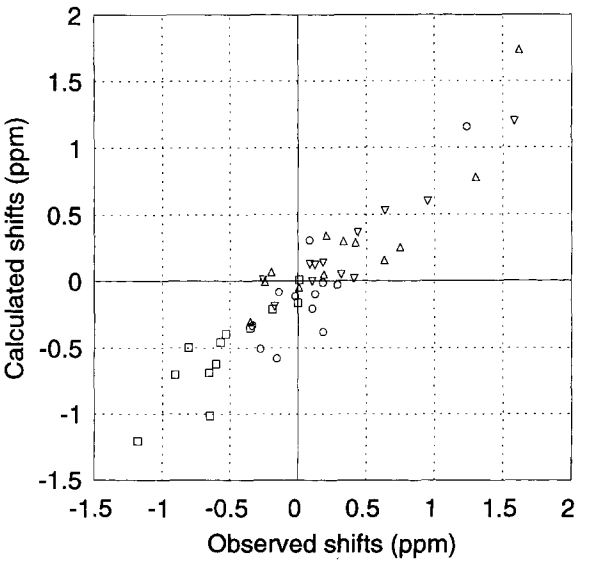

Fig. 1. Calculated haem proton ring current shifts versus observed deviation from reference values. △ haem I; ▽ haem II; □ haem III; ○ haem IV.

The correlations between stages 0, 1, and 2 identified the lower potential haems as haems I and II in the sequence (Fig. 2A), which become almost fully oxidised at stage 2. The chemical shifts are 15.09 and 29.00 for M18[1] I, 13.81 and 24.73 and for M7[1] II at oxidation stages 1 and 2, respectively. Since the paramagnetic shift of the haem substituent resonances is a measure of the extent of oxidation of that particular haem, these shifts demonstrate that haems I and II have very similar redox potentials at this pH, although haem II is slightly more negative than haem I (approximately 5 mV). This behaviour hinders the direct cross-assignment to the structure of the last two haems to oxidise (haems III and IV) because in the first two oxidation steps their resonances hardly move, staying in the very crowded region near their diamagnetic positions.

Table 1
Proton chemical shift of haem substituents and aromatic ring resonances in ppm

Haem substituent	Haem I chemical shift	Haem II chemical shift	Haem III chemical shift	Haem IV chemical shift
M2[1]	3.08	3.58	4.58	3.54
H3[1]	6.31	5.10	6.71	5.76
M3[2]	2.04	0.46	2.70	1.94
H5	9.48	8.65	9.89	9.10
M7[1]	3.20	2.96	3.98	3.32
H8[1]	5.31	5.97	6.59	6.34
M8[2]	0.42	1.92	2.85	0.81
H10	7.98	9.10	9.64	9.10
M12[1]	2.77	2.99	3.40	3.56
H15	9.65	9.18	9.28	9.64
M18[1]	3.22	3.10	3.59	3.28
H20	8.87	9.55	10.19	9.31

Aromatic residues	Ring substituents	Chemical shift
Tyr 43	2,6 H	5.38
	3,5 H	5.93
Tyr 65	2,6 H	6.05
	3,5 H	5.07
His 76	2 H	6.76
	4 H	3.98

The nomenclature is that proposed by the IUPAC-IUB [30].

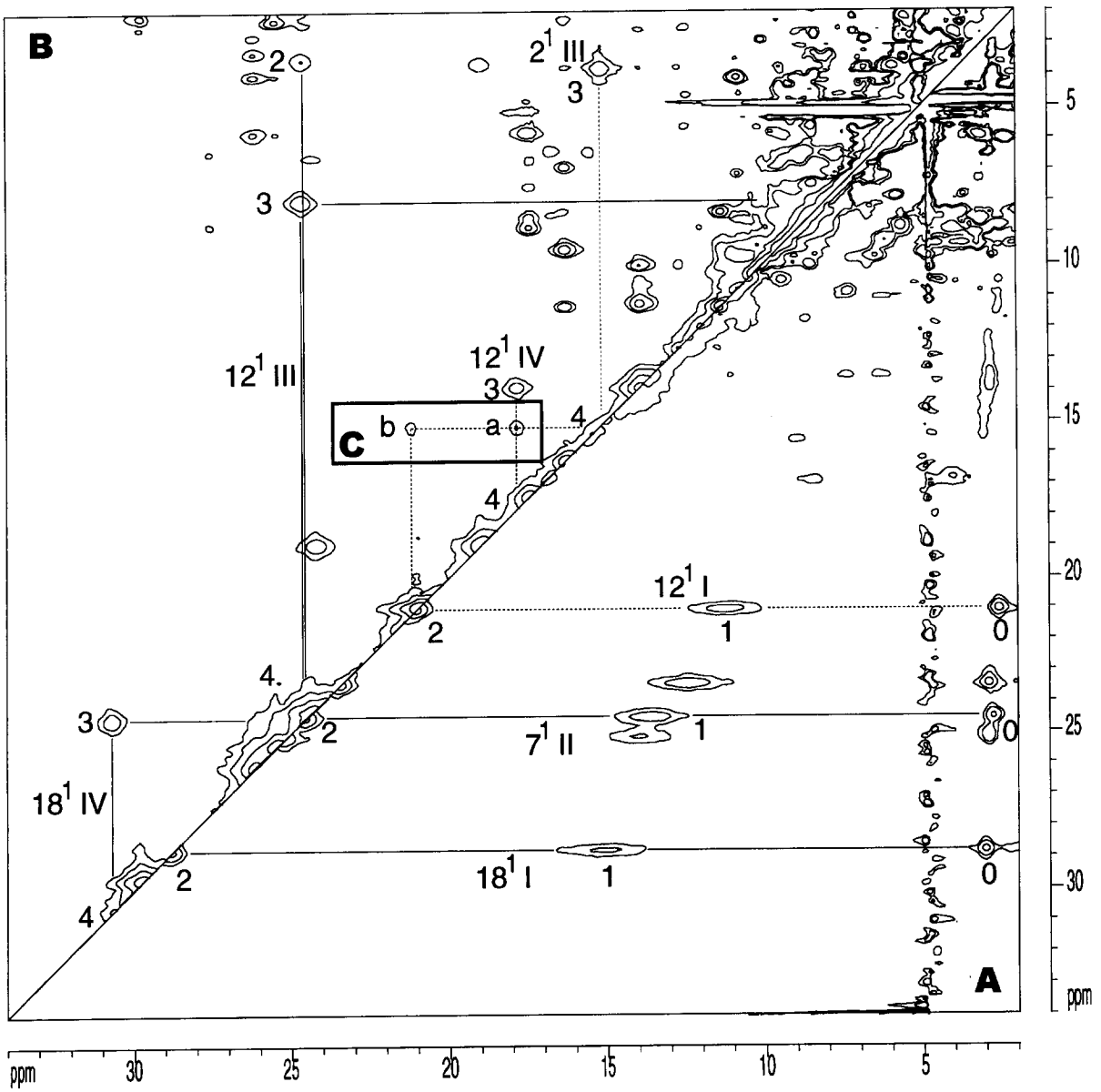

Fig. 2. NMR redox titrations of cytochrome c_3, with haems indicated by roman numbers and stages of oxidation indicated by arabic numbers connected by continuous lines. Dotted lines indicate the resonances used to specifically assign haems III and IV. A: Reoxidation up to stage 2 at pH = 8.1. B: Reoxidation from stage 2 to stage 4 at pH = 8.0. C: Detail of a 75 ms NOESY spectrum of the oxidised protein showing the cross-peaks: (**a**) $M2^1$ III–$M12^1$IV, and (**b**) $M12^1$ I–$M2^1$ III.

However, because they have quite different redox potentials, their signals can easily be distinguished by the oxidation pattern, as depicted in Fig. 2B.

The observation in the oxidised form of a strong NOESY cross-peak (signal **a** in Fig. 2C) between two methyls of the haems with the two highest redox potentials confirms this assignment since, as was pointed out earlier [4], according to the X-ray structure the shortest inter-haem methyl-methyl distance occurs between $M2^1$ III and $M12^1$ IV (at 2.6 Å) [16].

It remains to distinguish which is $M2^1$ III and which is $M12^1$ IV. This was easily done by observing the cross-peaks between $M2^1$ III and $M18^1$ III, and between $M12^1$ IV and $P13^1$ IV (not shown). This assignment was further confirmed by the observation of a weaker NOESY cross-peak (signal **b** in Fig. 2C) between $M12^1$ I and $M2^1$ III (at 4.1 Å) [16]. Thus, haem III was unequivocally assigned as that with the highest redox potential, with chemical shifts of 8.17 (stage 3) and 24.81 (stage 4) for $M12^1$, corresponding to approximately 22 and 100% oxidation, respectively. The $M18^1$ IV resonances appear at 3.87 (stage 2), 24.79 (stage 3), and 30.86 (stage 4), corresponding to oxidation fractions of 2, 78, and 100%, respectively.

The structural assignment of the redox potentials is now complete and establishes the order of oxidation for the haems with increasing mid-point redox potential as II and I, which have similar redox potentials at pH = 8, then IV and finally III, with values approximately 100 and 160 mV higher than the first ones, respectively.

A different assignment was reported in the literature [16] based on EPR difference spectra. However, it should be noticed that this difference was performed between spectra for

which the signals are overlapping and have different linewidths (Fig. 2 of [16]). But, even if the assignments were correct, it can easily be seen from Fig. 3 of the same reference that the second haem to reduce should be almost fully oxidised at −176 mV and hence would not be observable in the difference spectrum of their Fig. 2. As a result, the reported difference in midpoint redox potential for the last two haems to oxidise (120 mV) was considerably overestimated. In addition, the use of EPR studies together with differences in degree of haem exposure to solvent derived from the X-ray data to determine the structural cross-assignment of the redox potentials has already been shown to be unreliable [22].

4. Discussion

The agreement between NMR and crystallographic data shows that the overall spatial arrangement of the haems is maintained in solution, as in the case of the homologous proteins for which such information has been obtained. However, although this structural motif is conserved in all cytochromes c_3 so far studied, the pattern for the redox potentials of the haems is not. In the case of *D. desulfuricans* ATCC 27774 two haems have very low and similar redox potentials, and the other two potentials are much higher, by ca. 100 and 160 mV, respectively. Furthermore, the order of relative redox potentials in terms of the haem position in the three-dimensional structure is different from that observed for all the other cytochromes c_3 previously described. In fact, for the cytochromes c_3 from the other *Desulfovibrionaceae*, haem IV has the highest redox potential [4,20,21] and, although for *Dsm. baculatum* cytochrome c_3 haem III is also the less negative [22] and haem II has the lowest redox potential, the haems with similar redox potentials are I and IV [28].

These facts confirm the importance of the subtle fine-tuning of the redox potentials caused by the arrangement of the polypeptide backbone in these small but complex proteins. The correct assignment of the redox potentials to the structure is a prerequisite for understanding the functional involvement of the haems in the interaction of this protein with its redox partners, hydrogenase and transmembrane electron carrier protein complexes as proposed for *D. vulgaris* [7], and their participation in the energetic metabolism of sulfate reducing bacteria. However, it is still not easy to understand how the tetrahaem cytochromes c_3 can perform identical roles, having such different redox potential patterns for the haems. One possibility worth pursuing is that, since the intramolecular electron exchange is fast, the most important factors controlling their function should be those which determine the interactions within the electron transfer complex and define the point of entry and exit of the electrons.

A comprehensive understanding of the proton/electron and electron/electron interactions in these proteins should provide a useful model for the mechanism of functional cooperativities between redox and protonation centres which have been shown to be important in larger proteins, e.g. cytochrome *c* oxidase [7,29], for which precise measurements of the specific thermodynamic parameters are difficult to obtain.

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