REDOX STATES OF CYTOCHROME c_3 IN THE ABSENCE AND PRESENCE OF FERREDOXIN

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1. Introduction

The electron transfer chain from hydrogen to terminal reductase in the sulphate-reducing bacteria of *Desulphovibrio* species consists of the following pathway [1]:

$$H_2 \rightarrow \text{Hydrogenase} \rightarrow \text{Cytochrome } c_3 \rightarrow$$

$$\rightarrow \text{Ferredoxin} \cdots \rightarrow \begin{matrix} SO_4^{2-} \\ \downarrow \\ S^{2-} \end{matrix} .$$

Hydrogenase [2], cytochrome c_3 [3] and ferredoxin [4,5] from *Desulphovibrio gigas* have been purified. Physico-chemical studies of the isolated proteins of *Desulphovibrio* species are being carried out [6–12] allowing a better understanding of their role in the above pathway.

Further understanding of the mechanism of electron transfer can be obtained by the study, in vitro, of interactions between the components of the pathway. Proton nuclear magnetic resonance (NMR) spectroscopy has proved useful for structural investigations of both cytochrome c_3 [7,8] and ferredoxin [9] and many resonances have been assigned in their spectra. Selective perturbations of these resonances by protein–protein interactions contain structural information concerning the sites of interaction. In this paper we report a proton NMR study of the interaction between *D. gigas* cytochrome c_3 and the tetrameric form of *D. gigas* ferredoxin (FdII) [5]. FdII was chosen for this study as it is more efficient in the sulphite reduction pathway than the trimeric form, (FdI) [5]. Some of this work has recently been presented at a conference [13].

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2. Materials and methods

The four haem *D. gigas* cytochrome c_3 (mol. wt 13 000) and the tetrameric FdII (mol. wt 24 000) were purified as previously described [3,5]. Cytochrome c_3 was dialysed against distilled water at 4°C and lyophilized twice from D_2O. The exchangable protons of FdII were exchanged by simultaneous concentration and dialysis against D_2O at 4°C using a Diaflo ultrafilter, Amicon membrane UM 10 under a nitrogen pressure of 2 bar/cm^2. The purity index was $(A^{red}_{553} - A^{red}_{570})/A^{ox}_{280} = 3.0$ for cytochrome c_3 and

A_{280}/A_{415} = 1.85 for FdII. For the NMR experiments 3.5 mM solutions of cytochrome c_3 were prepared in deaerated D_2O. Reduction was accomplished with crystalline sodium dithionite, under argon to prevent autoxidation. The solutions of FdII (2.5 mM) were also deaerated under argon. pH variations were performed with NaOD and DCl. Uncorrected pH* meter readings are given.

The NMR spectra were recorded using a Bruker 270 MHz spectrometer equipped with an Oxford Instrument Co. magnet and a Nicolet 1085 computer in which mathematic manipulations were carried out [14] and a 100 MHz JEOL (JNM 100 PFT) spectrometer equipped with a JEOL 980A computer.

The temperature at which the spectra were obtained was 37 ± 0.5°C unless otherwise stated. Dioxan was used as internal standard but chemical shifts are quoted in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulphonate.

3. Results and discussion

The proton NMR spectra of the oxidized and reduced forms of *D. gigas* cytochrome c_3, over a spectral width of 40 ppm, are shown in Figure 1. The spectrum of the oxidized protein contains many resonances shifted out of the main envelope between 0 and 10 ppm which are absent in the spectrum of the reduced protein. In the low field region of the proton NMR spectrum of oxidized cytochrome c_3 (fig.1) 15 of the 16 expected contact shifted haem methyl resonances are observed between 9 ppm and 30 ppm together with several one and two proton peaks. Additionally there are some broad peaks between −1 ppm and −5 ppm. Most of these resonances are strongly temperature dependent, shifting back towards the diamagnetic protein envelope with increasing temperature, confirming that they are shifted by the paramagnetic haem centre. In the spectrum of the reduced protein, 16 one-proton peaks are observed in the region 8–11 ppm, corresponding to the four haem meso protons. The methyl region is more compact than in the spectrum of the oxidized protein, although some resonances are shifted upfield by about 2 ppm due to diamagnetic anisotropic effects.

D. gigas cytochrome c_3 is a small protein of 111 amino acid residues length (approx. 20 amino acid residues are required to cover each haem). This accounts for the spread of the usually more compact diamagnetic methyl region of the protein since it is expected that all the amino acid residues are under the influence of the pseudo contact and ring current fields of the haem groups.

Spectra of oxidized cytochrome c_3 were obtained

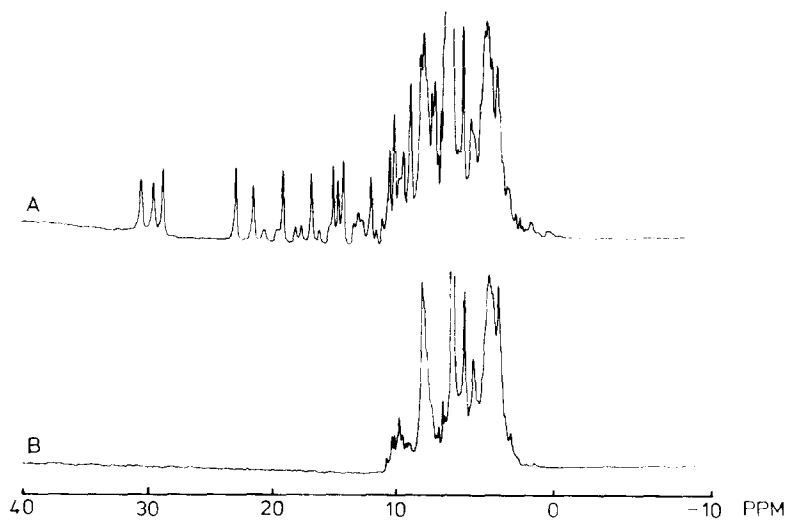

Fig.1. 270 MHz spectra of oxidized *D. gigas* cytochrome c_3 (A) and of reduced *D. gigas* cytochrome c_3 (B). Region downfield of HOD peak eight-times gain of upfield region.

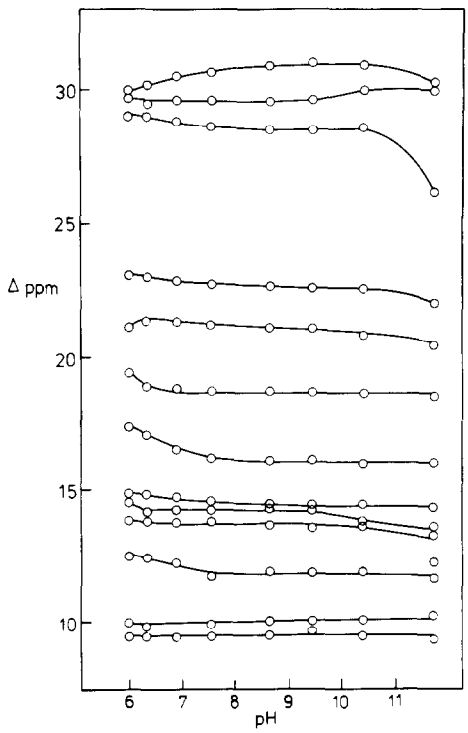

Fig.2. pH* titration curves of *D. gigas* cytochrome c_3. Spectra were recorded on a 100 MHz JEOL (JNM 100 PFT).

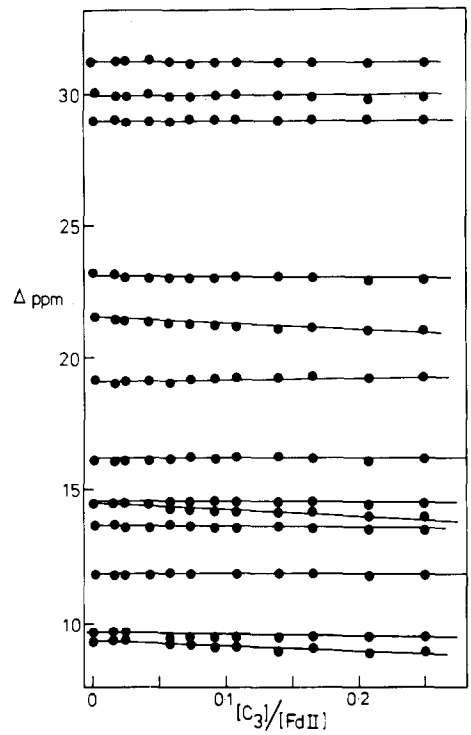

Fig.3. Titration of *D. gigas* cytochrome c_3 with FdII up to a molar ratio of 4:1, at pH 7.40. Spectra were recorded on a 100 MHz JEOL (JNM 100 PFT).

over the pH* range 5.8–11. A large number of the contact shifted resonances titrate with pH (fig.2). Below pH* 5.8 the protein precipitates. The titrating resonances shifted with a $pK_a < 6$ which is associated with ionisation of the haem propionic acid groups [15–17].

Shifts of the haem resonances of oxidized cytochrome c_3 induced by increasing amounts of FdII are given in fig.3. These shifts were obtained for unbuffered solutions. In the presence of 0.2 M phosphate buffer FdII produced no perturbation of the spectrum of oxidized cytochrome c_3. Comparison of the FdII shifts (fig.3) with pH shifts (fig.2) shows that the shifts observed on increasing FdII are not due to a variation of pH. Spectra of oxidized cytochrome c_3 and a 4:1 oxidized c_3/FdII mixture are given in fig.4. There is selective broadening of some of the haem methyl resonances by FdII.

The four haem centres of *Desulphovibrio vulgaris* cytochrome c_3 have been shown by electron para-

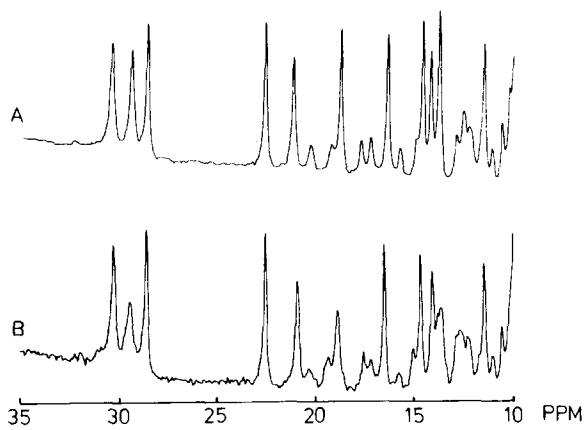

Fig.4. Low field region of the 270 MHz spectra of oxidized proteins: (A) *D. gigas* cytochrome c_3, nominal pH* 7.34; (B) *D. gigas* cytochrome c_3 plus FdII. Nominal pH* 7.00.

magnetic resonance (EPR) measurements [12], to be in non-equivalent sites and to have different redox potentials. The EPR measurements involved redox cycling by chemical reduction with sodium dithionite followed by reoxidation with controlled amounts of oxygen. Given the above spectroscopic data NMR may also be used to follow such a redox change. The number of observed haem methyl resonances in the region 5–30 ppm is related to be the number of oxidized haems at any degree of reoxidation. Progress of oxidation of the four haems can be followed by studying the relative intensities of these resonances during reoxidation.

Spectra at different reoxidation stages of cytochrome c_3 were obtained after mixing different amounts of oxidized and reduced forms of cytochrome c_3. The pattern of contact shifted haem resonances for different reoxidation stages is shown in fig.5. The spectrum obtained upon complete reoxidation of reduced cytochrome c_3 was identical to that observed before addition of sodium dithionite, indicating that the protein has undergone no irreversible change during the redox cycle.

The effect of electron exchange is complex. Many strongly shifted resonances in the spectrum of the oxidized protein are broadened on addition of less than 1% of the reduced protein, whilst others remain narrow and begin to shift. The haem meso resonances in the spectrum of reduced cytochrome c_3 are completely broadened upon addition of approximately 2% of oxidized cytochrome c_3. Some of the haem methyl resonances appear in the spectra of the reoxidizing protein before the remaining haem resonances (fig.5). Several of these resonances start shifting when the remaining heme methyl resonances appear. Thus, the major effects seen in this system are (1) the immediate effect of reoxidation on the resonances of some individual haem groups which is followed by (2) cooperative shift effects on neighbouring haem groups. During these steps inter- and intramolecular electron exchange affects the appearance of the haem resonances in different ways. Qualitative examination shows that some of the resonances are in each of the categories fast, intermediate and slow exchange [18]. At the present time this complicates the assignment of resonances to particular haem groups and prevents us from concluding definitely that a particular haem is the first to

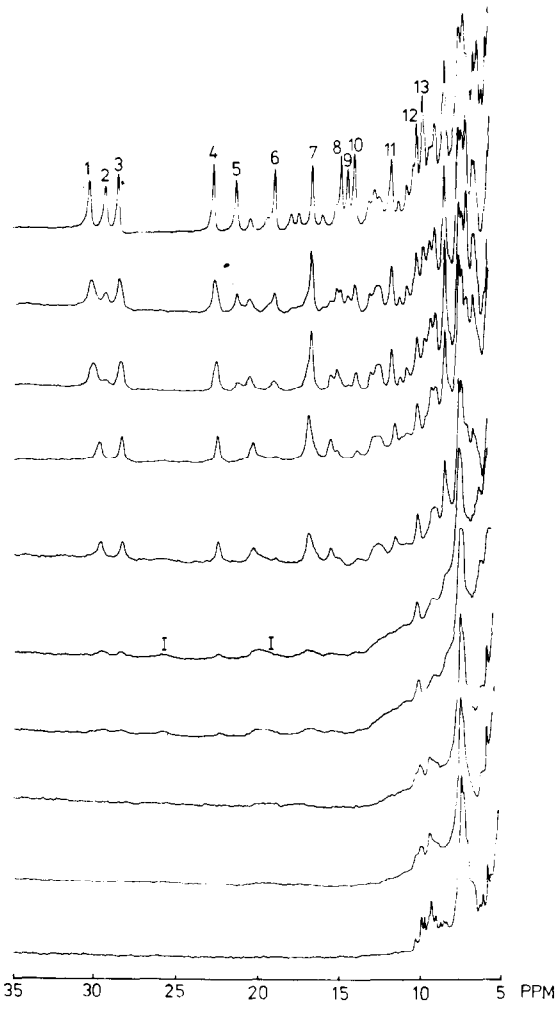

Fig.5. Reoxidation pattern obtained at 270 MHz of *D. gigas* cytochrome c_3.

reoxidize. Additionally, resonances arising from a distinct intermediate in the reoxidation of *D. gigas* cytochrome c_3 are present in the spectra shown in fig.5. This intermediate form will be discussed more fully in a later publication. Despite these difficulties we can unequivocally point to distinct differentiating effects between the oxidation equilibrium and electron exchange of the different haems and we can show that the patterns are different in the presence and absence of FdII.

Figure 6 represents an equivalent titration carried

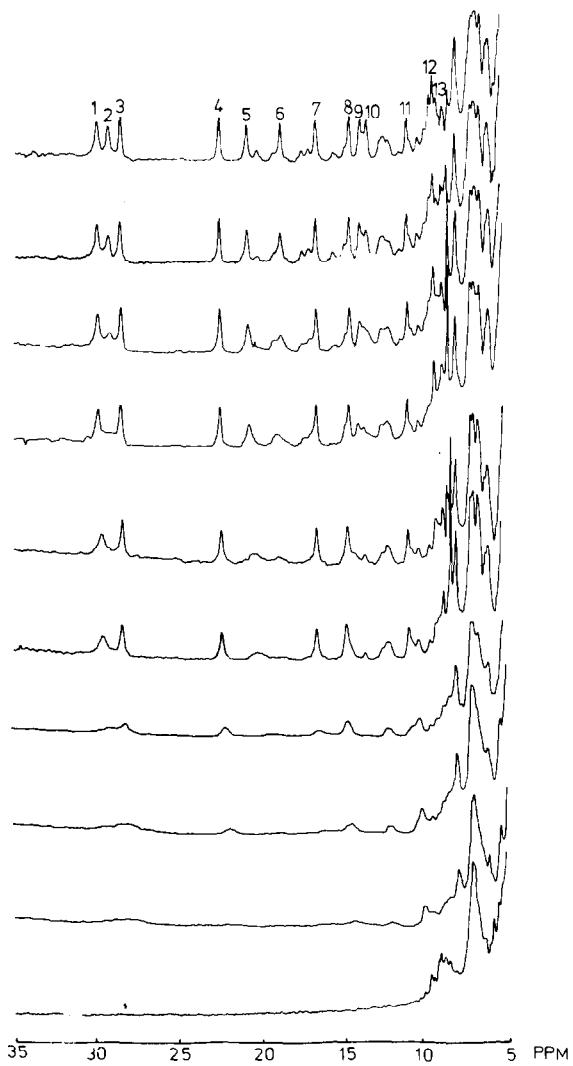

Fig.6. Reoxidation pattern obtained at 270 MHz of *D. gigas* cytochrome c_3 plus FdII.

out in the presence of FdII at a molar ratio of 4:1 cytochrome c_3: FdII. Spectra of oxidized and reduced cytochrome c_3 over the spectral range 0–10 ppm show that many resonances are considerably broadened upon addition of FdII. Gradual reoxidation of the reduced sample now gives a different pattern of appearance of haem methyl resonances (compare fig.6 with fig.5) suggesting that the electron exchange rates between the four haems are altered in the presence of ferredoxin. A final equilibrium with air destroys the cytochrome c_3/FdII complex probably due to the ionic products resulting from dithionite oxidation which must have a similar effect to phosphate ions.

In the absence of ferredoxin, methyl resonances 1,3,4 and 7 have the same relative rates of increase in intensity and these resonances appear first. In the presence of ferredoxin, resonances 3,4,7 and 8 appear first. Similarly, the relative appearance of methyl resonances 5 and 6 is affected by ferredoxin. The broad resonances present in the early stages of reoxidation of cytochrome c_3, marked I in fig.5, disappear when the methyl resonances 5 and 6 start to appear. This provides further evidence for the protein-protein interaction.

The mechanism of electron transfer between proteins has been discussed many times [19,20] and it has been an implicit assumption in these discussions that the specific interaction sites are present for the redox partners. The present study has shown that for cytochrome c_3 there is a specific interaction with FdII. Furthermore, the interaction between FdII and cytochrome c_3 is likely to be electrostatic in character, as shown by the competition with phosphate, in common with other electron transfer systems. A more detailed description of the cytochrome c_3/FdII interaction requires more specific assignments for the cytochrome c_3 resonances. These are now being carried out. The experiments described here demonstrate that NMR spectroscopy is capable of monitoring interactions between electron transfer proteins and we are now extending these studies to the interaction of other redox couples.

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