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Spectroscopic studies of *Rhodobacter capsulatus* cytochrome *c'* in the isolated state and in intact cells

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Ferricytochrome *c'* from *Rhodobacter capsulatus* was investigated by ^{1}H-NMR, EPR and optical spectroscopies. A haem-linked ionisation, occurring with a pK_a of 8.4 at 25°C, was observed and assigned to the ionisation of the axial histidine ligand by comparison with data for related proteins. At pH values below this pK_a the spin-state of the haem Fe^{3+} is shown to be a quantum mechanically admixed $S = 3/2, 5/2$ state. Above the pK_a the Fe^{3+} is high-spin. EPR studies of intact cells grown photoheterotrophically reveal that in situ cytochrome *c'* exists largely in the ferrous state. Upon the addition of $[Fe(CN)_6]^{3-}$ the protein becomes oxidised and EPR spectra reveal that the Fe^{3+} spin-state is a quantum mechanically admixed $S = 3/2, 5/2$ state. These data indicate that the unusual spin-state of ferricytochrome *c'* is not a consequence of changes to the protein on its isolation, as had been suggested previously. They also indicate that in situ cytochrome *c'* is located in an environment with a pH < 7.

Introduction

Cytochrome *c'* is a periplasmic class II cytochrome *c* widely distributed amongst different bacteria [1–3]. For example, it is present in many photosynthetic bacteria as well as aerobes, such as *Azotobacter vinelandii*, and facultative anaerobes, such as *Alcaligenes* spp. It was first isolated by Kamen and his colleagues who noted that the ferric protein had an unusual optical spectrum that was pH dependent over the range 7–9 [4–6]. Subsequently it was shown that other spectroscopic properties of the ferricytochrome, such as its EPR [7], and NMR [8–11] spectra, were unusual compared to those of other ferrihaemoproteins. Maltempo accounted for these differences by showing that the ferric spin-state of cytochrome *c'* could be described as a quantum-mechanically admixed $S = 5/2, 3/2$ state [7,12], whilst the spin-states of other cytochromes and globins are well-known to be simple high-spin ($S = 5/2$) or low-spin ($S = 1/2$) states ([13] and references therein). The pH dependence has been proposed to result from the ionisation of the axial histidine ligand to histidinate [14,15], which causes the iron spin-state to change to a pure $S = 5/2$ state. This proposal is consistent with the structure and all the reported spectroscopic data of cytochrome *c'*, as well as with structural and spectroscopic studies of the homologous His-Met coordinated cytochrome *b*562 of *Escherichia coli* [16] and the class IIb cytochromes *c* [13].

The wealth of spectroscopic and structural data on cytochrome *c'* is not matched by a corresponding body of biochemical data. In fact, the biochemical function of cytochrome *c'* is not known. It is not even clear whether the properties of the protein in vivo are the same as those of the isolated cytochrome *c'* [2,3]. Despite its distinctive spectrum whole-cell EPR studies reported by Corker and Sharpe [17] and Prince et al. [18] failed to identify clearly cytochrome *c'* in either *Rhodobacter capsulatus* or *Rhodospirillum rubrum*. With the subsequent discovery of low-spin analogues of cytochrome *c'* containing both histidine and methionine coordination of the iron [9,19], the proposal was made that cytochrome *c'* existed in the cell as a low-spin protein that underwent a transition to a high-spin protein upon isolation in some cases [9]. This proposal

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was consistent with the earlier suggestion of Kakuno et al. regarding a change in optical spectrum of cytochrome c' on isolation [20].

The question of whether cytochrome c' in situ is different from the isolated protein is the subject of the present paper. We here report the characterisation of *Rb. capsulatus* ferricytochrome c' by optical and EPR spectroscopies and show that the spin-state of the haem iron in the intact cell under oxidising conditions is the same as that for the isolated protein; namely, a quantum mechanically admixed $S = 5/2, 3/2$ spin-state at pH values below 8.

Materials and Methods

Rb. capsulatus strain 37b4 [21] was grown chemoheterotrophically on RCV medium [22] and low oxygen tensions in a 100 l fermenter. This generated approximately 600 g of cell paste. From this material the ferricytochrome c' was isolated and purified by the procedure described by Bartsch [1]. Its optical and EPR spectral characteristics at pH 7 were almost the same as those reported by Yoshimura et al. for *Rb. capsulatus* (B100) ferricytochrome c' [23].

Samples of ferricytochrome c' for NMR were prepared by exchanging the supporting H_2O buffer for ^2H_2O, containing 25 mM phosphate buffer at pH 7.4, with a Centricon device. The protein concentration was 1–2 mM. Samples of cytochrome c' for EPR were 0.5 mM in protein in a 25 mM phosphate solution or in a buffer cocktail consisting of 0.2 M Caps, 0.05 M Taps, 0.05 M Hepes, 0.05 M Pipes and 0.05 M Mes. Identical results were obtained with both types of samples. For experiments with intact cells, *Rb. capsulatus* 37b4 was grown phototrophically on RCV medium in completely filled 1 litre flat-sided bottles. Cells of *Rb. capsulatus* were pelleted by centrifugation at $10\,000 \times g$ for 20 min, resuspended in 0.1 M Hepes buffer at pH 7.4, and then spun again at $10\,000 \times g$ for 20 min and the excess buffer was then removed with a syringe.

The cell suspension was then transferred to an EPR tube with a syringe. In some samples 60 mM $K_3[Fe(CN)_6]$, or 60 mM tetramethyl-*p*-phenylenediamine (TMPD), in 0.1 M Hepes buffer at pH 7.4 was added dropwise to the cell suspension in the EPR tube. The sample was then shaken and immediately frozen.

All pH values given in the figure captions are direct meter readings uncorrected for any isotope effect. Thus pH* is used to indicate the meter reading for ^2H_2O solutions.

The ^{1}H NMR spectra were recorded with a JEOL GX-400 spectrometer. 1,4-dioxan was used as an internal reference but all chemical shifts are reported in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

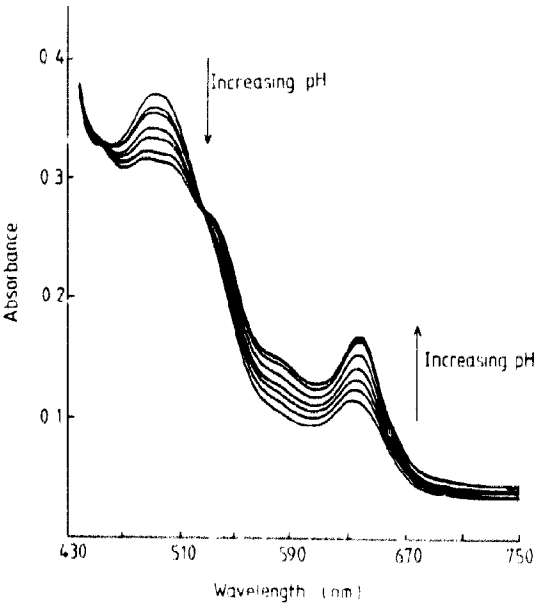

Fig. 1. UV-visible spectra of 9 μM ferricytochrome c' in 25 mM sodium phosphate, as a function of pH. The pH range of the spectra is 7–11.

The EPR spectra were obtained with a Bruker ER-200D spectrometer equipped with a Bruker ESP 1600 data system and Oxford Instruments ESR 9 cryostat. The spectra were recorded with a modulation amplitude of 9.78 G and microwave power of 2.02 mW. The optical spectra were measured with a Hitachi 557 dual beam spectrophotometer.

Results

Optical spectra of ferricytochrome c'

The UV-visible region of the spectrum of ferricytochrome c' over the wavelength range 430–750 nm and at various pH values is shown in Fig. 1. The general appearance of the spectrum and its pH dependence is typical of ferricytochromes c' [1–3]. One of the major distinguishing features is the increase in absorbance of the 638 nm band with increasing pH. This change is indicative of an increase in the high-spin character of the ferric spin-state [24,25] which is proposed to result from the ionisation of the histidine ligand to a histidinate [14,15]. The pK_a for the transition obtained from the optical spectra is 8.4 (Fig. 2).

NMR spectra of ferricytochrome c'

High-frequency regions of the ^{1}H-NMR spectra of ferricytochrome c' at 25°C and various pH* values are shown in Fig. 3. The general appearance of the spectrum strongly resembles that of *R. rubrum*, *R. molischianum* and *Chromatium vinosum* ferricytochromes c', in

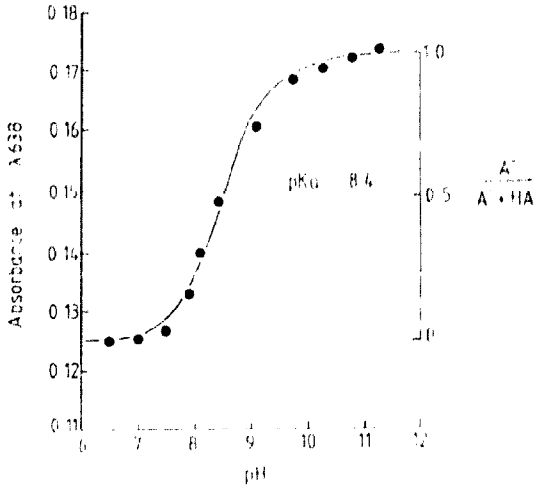

Fig. 2. The pH dependence of the 638 nm absorbance of ferricytochrome c'. The solid line is a theoretical curve for a single proton ionisation with a pK_a of 8·4. The solid circles are experimental data points.

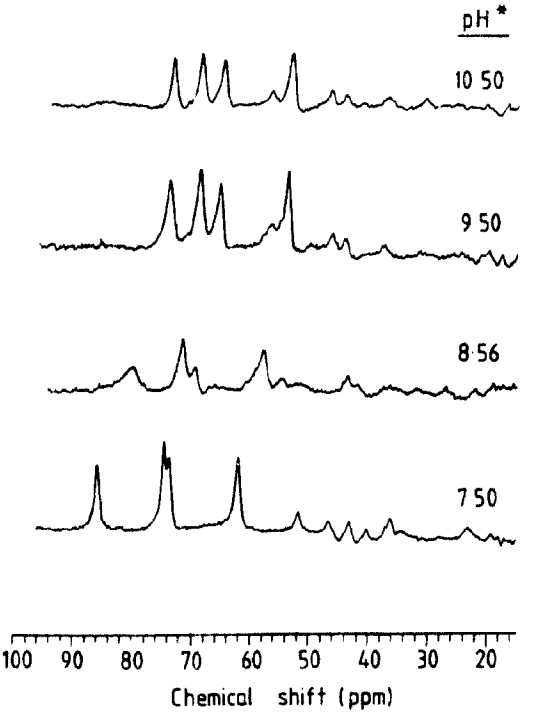

Fig. 3. High-frequency regions of the 400 MHz ^{1}H-NMR spectra of ferricytochrome c' at various pH* values. The spectra are the sum of 400 scans and measured with a spectral width of 60 000 Hz and 32 K data points, and with suppression of the solvent signal by a 0.4 s pre-irradiation pulse.

particular the presence of the three-protons intensity resonances between 60 and 80 ppm [8,9,11]. These arise from the haem methyls. The shift in these resonances as a function of pH* is consistent with a haem linked one-proton ionisation occurring with a pK_a of ~ 8.6. The line-broadening of the haem methyl resonances at pH* 8.6 indicates that the rate of proton association and dissociation at the pK_a is $\sim 10^3$ s^{-1}. The increase in linewidth of the haem methyl resonance at 88 ppm (pH* 7.5), 180 Hz, on-going from pH* 7.5 to pH* 10.5 is consistent with a change in the proportion of high-spin character of the Fe^{3+}, with the more alkaline protein being more high-spin [10].

EPR spectra of ferricytochrome c'

The EPR spectra of ferricytochrome c' at 10 K and pH 6.1 and 9.8 are shown in Fig. 4. At pH 9.8 the spectrum is typical of high-spin ferrihaem, the g values of 6.09, 5.80 and 1.99 arising from the lowest Kramers doublet of the $S = 5/2$ state. At pH 6.1 the spectrum

Fig. 4. X-band EPR spectra of ~ 1 mM ferricytochrome c' in deuterated buffer mixture (see Materials and Methods). (left) pH = 6.1; (right) pH = 9.8. Sample temperature = 10 K, microwave frequency = 9.3 GHz; gain = (left) $0.3 \cdot 10^{-3}$, (right) $4.0 \cdot 10^{-3}$.

has the appearance of a quantum mechanically admixed $S = 5/2, 3/2$ spin-state, the g values being 5.70, 4.97 and 1.99. The g_z signals are sensitive to the degree of mixing between the sextet and the quartet states such that $g_z = 6.0\ a^2 + 4.0\ b^2$ where a and b are the coefficients of the $S = 5/2$ and $3/2$ states contributing to the ground state. Analysis by the procedure developed by Maltempo [12] shows the admixed spin-state at pH 6.1 can be described as a mixture of ~ 60% $S = 5/2$ and ~ 40% $S = 3/2$ states.

The pH dependence of the EPR spectrum is shown in Fig. 5. The signals of the high-spin and intermediate-spin/high-spin species are in slow exchange and thus as one increases in intensity the other decreases.

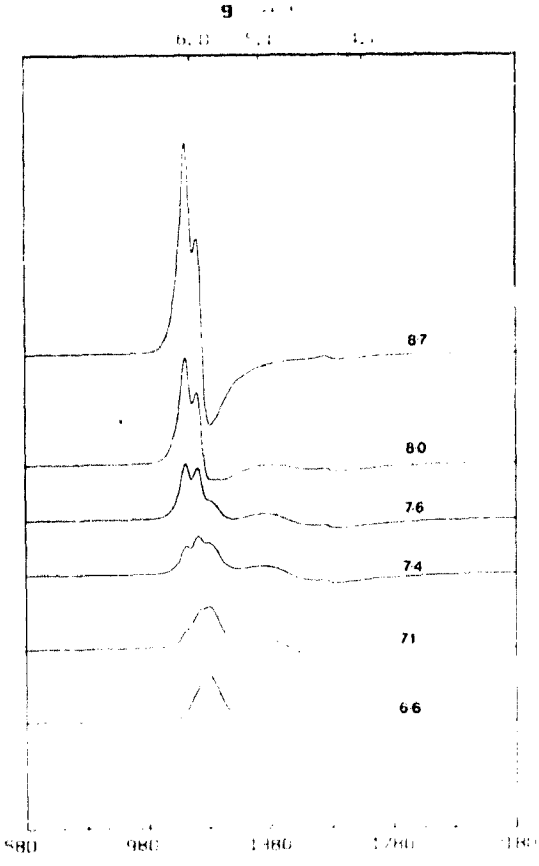

Fig. 5. pH dependence of the low-field region of the X-band EPR spectrum of ferricytochrome c'. The quoted pH values are from room temperature measurements. Samples were prepared at room temperature, using the buffer mixture described in Materials and Methods to achieve the desired pH value, and then frozen to 10 K for the EPR measurement. Protein concentration ~ 1 mM in deuterated zwitterionic buffer; sample temperature = 10 K; microwave frequency = 9.3 GHz; power = 2.02 mV; modulation amplitude = 9.78 G; gain = $6.3 \cdot 10^{-4}$ or $4.0 \cdot 10^{-4}$; spectra normalised for gain

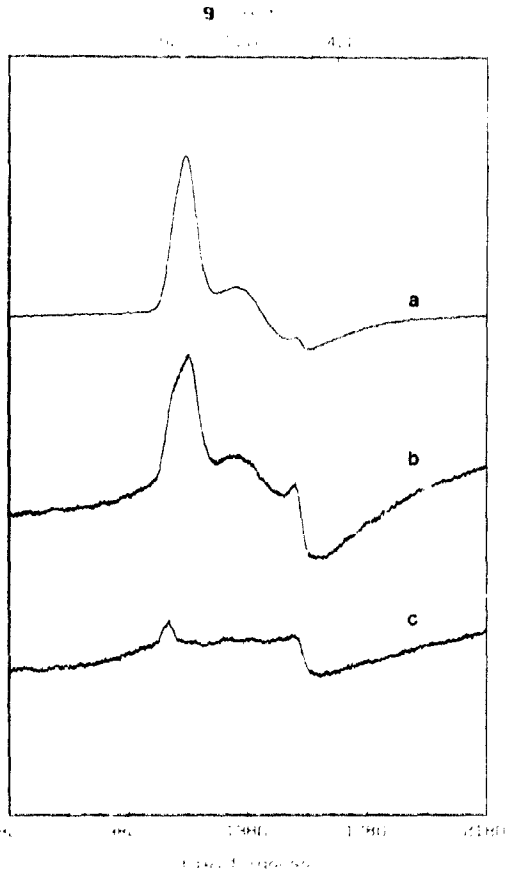

Fig. 6. Low field regions of the X-band EPR spectra of (a) *Rb. capsulatus* ferricytochrome c', pH 6.1, and intact *Rb. capsulatus* cells (c) before and (b) after addition of $K_4[Fe(CN)_6]$. Cells were suspended in 0.1 M Hepes buffer, pH 7.4. Sample temperature = 10 K; microwave frequency = 9.3 GHz; power = 2.02 mV; modulation amplitude = 9.78 G; gain = $6.3 \cdot 10^{-4}$.

The pK_a for the spectral change is ~ 7.8. The difference between this and the value obtained from the optical and NMR pH titrations probably arises from the sample temperature differences. This series of spectra are essential for the analysis of the whole cell EPR since the pH of the cytochrome c' environment in situ is not known.

EPR spectra of whole cells

The low-field region of the EPR spectrum of intact *Rb. capsulatus* cells suspended in a zwitterionic buffer at pH 7.4 is shown in Fig. 6c. The addition of $K_4[Fe(CN)_6]$ (Fig. 6b) or TMPD (not shown) causes the spectrum to change. The $g \sim 5.7$ and $g \sim 4.3$ signals increase in intensity and a broad feature at $g \sim 4.5$–5.2 becomes apparent. The broad feature and the $g \sim 5.7$ signal strongly resemble the spectrum of ferri-

cytochrome c' at pH 6.1 (compare Figs. 6b and a) and therefore we assign these features to cytochrome c'. The $g = 6.0$ shoulder is due to other high spin ferrihaems. By comparison with other systems the $g = 4.3$ signal is assigned to monomeric non-haem iron.

The appearance of the ferricytochrome c' spectrum in intact cells shows that the unusual electronic structure of the haem is maintained within the cell and is not a consequence of the protein being altered on isolation. Therefore, since the haem electronic structure is particularly sensitive to changes in the ligand field [12], which in turn depend on the protein structure, our data strongly suggest that the protein in situ has the same structure as the isolated protein.

Discussion

The function of cytochrome c' is not known but the data of Fig. 6 indicate two factors that may be important in future attempts to define its function. Firstly, the protein can exist in the cell largely in the ferrous form. This was shown by the EPR experiments on whole cells. Since cytochrome c' is autoxidisable it indicates that the cells were anaerobic under our conditions. Cytochrome c' is located in the periplasm of Gram-negative bacteria and this raises the question of how the ferrous form is generated in situ. One possible reductant is ubiquinol. Although this molecule is located in the cytoplasmic membrane it is possible that membrane-associated cytochrome c' could interact with this reductant. Cytochrome c' can be reduced by water-soluble duroquinol (A.G. McEwan, unpublished observations).

Secondly, the pH of the local environment is less than 7. Thus, the ionisation of the axial histidine is unlikely to have a major physiological significance unless it is perturbed in situ. Therefore the functional form of cytochrome c' will have a mono-His coordinated haem. This form of cytochrome c' has relatively slow ligand binding properties [2,26,27] and this, coupled with its relatively low redox potential of -10 to 130 mV [[13]] and references therein), makes it unlikely that cytochrome c' is a terminal oxidase. However, the question of whether cytochrome c' functions in electron transfer or in ligand binding remains open.

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