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pH DEPENDENCE OF THE REDOX POTENTIAL OF *PSEUDOMONAS AERUGINOSA* CYTOCHROME *c*-551

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Summary

The redox potential of *Ps. aeruginosa* cytochrome *c*-551 varies with pH between pH 5 and 8. The pH dependence can be analysed in terms of a pK_a of 6.2 in the oxidised form and a pK_a of 7.3 in the reduced form. The same pK_a values are also observed in NMR spectra of the two oxidation states and the pK_a of 7.3 is observed in titration of the visible absorption spectrum of the ferrocytochrome. From the NMR studies these pK_a values have been assigned to the ionisation of one of the haem propionic acid groups.

pH dependence of redox potential is of variable occurrence among cytochromes and the possible significance and basis of this variation is discussed.

Introduction

The *c*-type cytochromes are haemoproteins which function as components of a variety of electron transport systems [1]. The properties of their haem groups are defined both by the iron ligands provided by the protein and by the environment created by the folded peptide chain. One property of cytochromes which has recently generated much interest is the mid-point oxidation-reduction (redox) potential [2—8]. However the features of the protein environment important in determining the redox potential of the haem are poorly understood. One method of investigating this problem is to monitor the redox potential of a cytochrome as its haem environment is specifically perturbed.

Perturbations of the haem environment of a protein are usually reflected in its visible absorption spectrum, its nuclear magnetic resonance (NMR) spec-

trum and in its redox potential. For example, the 695 nm band of horse ferricytochrome *c* is lost with the same pK_a [9] as that observed for the redox potential [10] and for the loss of the methionine ligand methyl resonance in the NMR spectrum of the oxidised species [11]. In this case such structural changes are of interest from the point of view of detailed chemical explanation of the protein but presumably are not a part of the functional performance of the protein in the cell since the pK_a is about 9.2. However for a number of prokaryotic cytochromes *c* changes in redox potentials and spectral properties have been observed in the physiological pH range [3,6]. In this paper we describe the dependence of the redox potential of *Pseudomonas aeruginosa* cytochrome *c*-551 upon the hydrogen ion concentration in solution and correlate the redox potential with the ionisation state of particular functional groups from a study of the NMR spectra of the protein. *Pseudomonas* cytochrome *c*-551 is a small relative of eukaryotic respiratory cytochrome *c* and it is thought to perform a similar function in donating electrons to a terminal oxidase [12,13].

Materials and Methods

Pseudomonas aeruginosa was grown and the cytochrome prepared as described by Ambler and Wynn [14].

Mid-point oxidation-reduction potentials were determined by spectrophotometric examination at 25°C of cytochrome *c*-551 in ferrocyanide-ferricyanide solutions of known potential [3,15,16] and buffered as described in Fig. 1. pH titrations were carried out in cuvettes by the addition of 0.01 M NaOH or 0.1 M HCl. Near-infrared spectra were recorded on a Cary 15 spectrophotometer.

Samples of cytochrome *c*-551 were prepared for the NMR experiments and the NMR spectra were obtained as previously described [17]. The pH of solutions in the NMR experiments were monitored by a glass electrode (manufactured by Radiometer Ltd.) which was inserted directly into the NMR tubes. The pH was adjusted by the addition of small aliquots of concentrated NaO^2H or ^2HCl in ^2H_2O (all supplied by Merck. Sharp and Dohme Ltd.). pH values quoted for the NMR experiments are the direct meter readings and since they are not corrected for the small isotope effect [18] they are denoted by pH*. Derived pK values are denoted pK*. Chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

Results

1. Redox potential

The pH dependence of the mid-point oxidation reduction potential (E_m) of *Ps. aeruginosa* cytochrome *c*-551 is shown in Fig. 1. Inspection of the results using the methods of Clark [19] indicated the presence of one haem-linked ionisation in the oxidised form and one in the reduced form. The experimental results were fitted to a theoretical curve described by Equation 1:

$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{K_{red} + H^+}{K_{ox} + H^+} \quad (1)$$

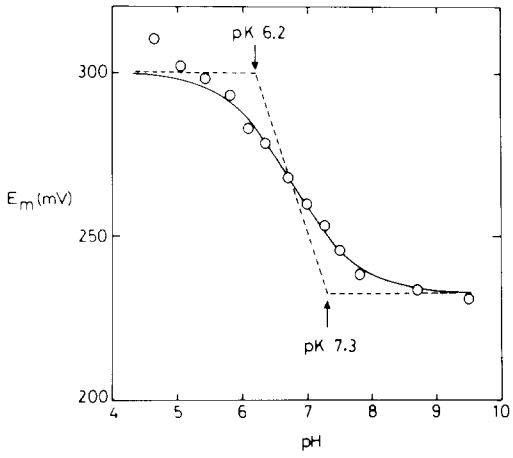

Fig. 1. pH dependence of the redox potential of *Ps. aeruginosa* cytochrome *c*-551. Experimental points (open circles) are for measurements made at a single poised potential at each pH value. Added potassium ferrocyanide was 0.5 mM, added potassium ferricyanide was 0.017 mM or zero; $T = 25°C$; the cytochrome concentration was approximately $5 \cdot 10^{-6}$ M; total ionic strength was 0.006—0.008 mol/l. Buffers were 1 mM acetic acid/sodium acetate (pH 4.5—5.8); 1 mM Na_2HPO_4-NaH_2PO_4 (pH 5.8—7.5); 1 mM Tris-HCl (pH 7.5—8.8); 1 mM glycine-NaOH (pH 8.8—9.5). The pH was measured before the addition of sodium dithionite to achieve complete reduction. The addition of the dithionite resulted in a lowering of the pH so that the completely reduced spectrum was always that of the acid form. The small difference in extinction coefficients between the acid and alkaline spectral species was therefore corrected for in the experiments above pH 6.5. The solid line is a theoretical curve for Equation 1.

where the ionisation constants K_{red} and K_{ox} were assigned the values $5 \cdot 10^{-8}$ M ($pK_a^{red} = 7.3$) and $6.3 \cdot 10^{-7}$ M ($pK_a^{ox} = 6.2$), respectively. The theoretical curve fits the experimental results except in the low pH region where a second ionisation in the reduced species may be occurring (this is not included in the analysis). The value of E_m at pH 6.5 (275 mV) is in reasonable agreement with a previously obtained value (286 mV) measured under very different conditions of ionic strength [20].

2. Visible/near infra-red spectrum

The visible/near infra-red spectrum of ferricytochrome *c*-551 was virtually unchanged between pH 4.4 and pH 10.1, only very small changes being observed around 560 nm. These changes have not been analysed in difference spectra of concentrated solutions. The near infra-red band was lost above pH 10.1.

These data are consistent with previous studies [21]. Thus there is no spectroscopic pK_a for ferricytochrome *c*-551 coincident with the pK_a of 6.2 observed in the redox potential studies.

Spectrophotometric titration of ferrocytochrome *c*-551 is shown in Fig. 2. There is a shift in the α-peak maximum from 551 nm to 553 nm with increasing pH and the α-peak becomes asymmetrical. This spectroscopic change was analysed in terms of a single pK_a of 7.2 (inset, Fig. 2).

3. Nuclear magnetic resonance spectra

Previous NMR studies of cytochrome *c*-551 have included assignments

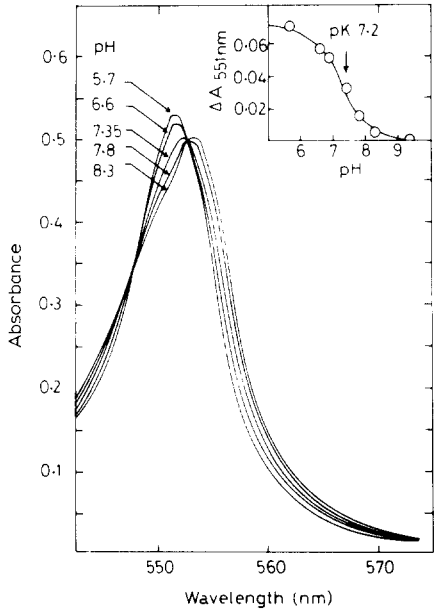

Fig. 2. pH dependence of the α-band of *Ps. aeruginosa* ferrocytochrome *c*-551. The pH of an unbuffered solution of ferrocytochrome *c*-551 in 10 mM NaCl containing 0.5 mM sodium ascorbate was adjusted with 0.1 M NaOH and the spectrum recorded after each addition. The inset shows the loss in absorbance at 551 nm with increasing pH (open circles) and the solid line is a theoretical curve for a pK_a of 7.2.

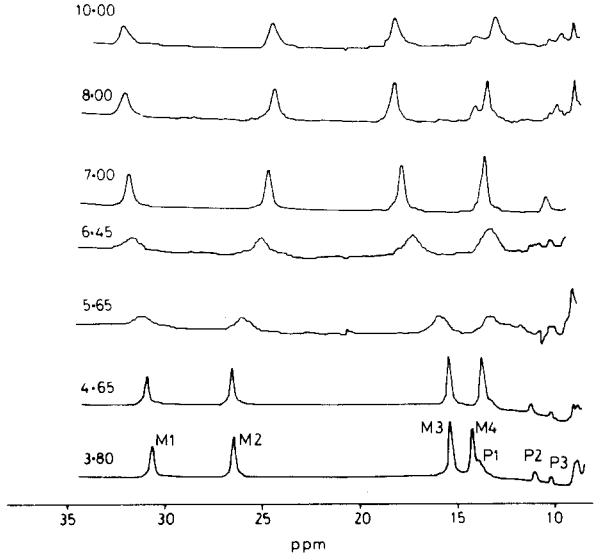

Fig. 3. pH dependence of the NMR spectrum of *Ps. aeruginosa* ferricytochrome *c*-551. The pH of a solution of ferrocytochrome *c*-551 in ^2H_2O was adjusted with concentrated NaO^2H and ^2HCl and the spectrum recorded at 27°C after each addition. Only the downfield region of the spectra are shown. The pH values given are uncorrected meter readings. The resonance designations M_1, P_1, etc. are defined in the text.

for resonances of the haem group and axial ligands [22,23] and for resonances of other amino acid side chains [17]. Those assignments are used in the present work.

(a) Assignment of P_1 and P_2. The assignment of resonances P_1 and P_2 (Fig. 3) were made using double-resonance techniques. Irradiation of P_1 with a gated pulse produced a small intensity decrease of P_2. Similarly irradiation of P_2 caused the intensity of P_1 to decrease. No such intensity effects were observed for any resonance in difference spectra obtained with irradiation of resonance P_3. The intensity effects involving P_1 and P_2 arise from a negative nuclear Overhauser enhancement indicating that P_1 and P_2 arise from the two geminal protons of a methylene group. From their position in the spectrum these two one-proton intensity resonances can be identified as resonances of the β-CH_2 protons of the haem propionate substituents (β relative to the carboxyl group) [24—26] or the β-CH_2 protons of the histidine ligand to the iron [27]. Later in this paper we will use the pH dependence of resonances P_1 and P_2 to complete their assignment.

(b) pH dependence. Nuclear magnetic resonance (NMR) spectra of ferrocytochrome *c*-551 in ^2H_2O were obtained over the pH* range 4.0 to 10.8 at 27°C. The solutions were either unbuffered or contained 0.05 M sodium phosphate buffer. The protein concentration was 5 mM.

TABLE I

pH DEPENDENCE OF SELECTED RESONANCES OF CYTOCHROME *c*-551

Proton [a]	Chemical shift in [b]		pK_a^*
	Low pH form	High pH form	
A. Ferrocytochrome *c*-551			
Haem meso γ	9.37	9.47	7.2
Methionine 61 ϵ-CH_3	—2.91	—2.85	
Methionine 61 γ-CH	—3.49	—3.47	
Methionine 61 β-CH	—2.73	—2.83	
Methionine 61 γ-CH	—0.88	—0.83	
Methionine 61 β-CH	—0.48	—0.57	
B. Ferricytochrome *c*-551			
Methionine 61 ϵ-CH_3	—17.0	—16.0	Complex
Methionine 61 γ-CH	—8.1	—8.5	6
Haem methyl 1 (M_2)	26.4	24.3	6
Haem methyl 3 (M_4)	14.2	13.1	Complex
Haem methyl 5 (M_1)	30.6	31.9	Complex
Haem methyl 8 (M_3)	15.3	18.0	6
Propionate β-CH (P_1)	13.8	9.6	6
Propionate β-CH (P_2)	10.9	13.9	6

[a] Assignments taken from Refs. 17, 22, 23, and from this work for P_1 and P_2. The designation M_1, P_1 etc. refer to the resonances given in Fig. 3.

[b] The chemical shifts are for low pH and high pH forms of ferrocytochrome at pH 4.2 and 10.5 respectively and the low pH and high pH forms of ferricytochrome *c* at pH 3.8 and pH 8.7 respectively.

[c] The pK_a^* values for ferricytochrome *c* were difficult to determine precisely. This was partly because many of the strongly shifted resonances are also broadened and partly because some of the resonances appeared to have a linear dependence upon the pH (Fig. 4). pK_a^* values are derived from uncorrected pH readings.

The spectrum of ferrocytochrome *c*-551 was little perturbed over the above pH range; few resonances shifted and those that did so shifted by $\leqslant 0.1$ ppm. Three ionisations were observed with pK_a^* values of <4.4, 7.2 and >9.4. Most notable of the resonances which shifted were those of methionine-61 and those of the phenylalanine-34 which shifted with the pK_a^* of 7.2. The shifts for the former set of resonances are given in Table I, and the data for the latter set will be given in a subsequent paper concerned with further NMR assignments and comparison of related proteins (Ambler, Moore, Pettigrew and Williams; Manuscript in preparation). Resonances of the haem thioether substituents and three of the haem meso protons were unaffected by pH. The resonance of haem meso γ shifted downfield with increasing pH (Table I).

NMR spectra of ferricytochrome *c*-551 were obtained over the pH* range 3.5 to 10.5 at 27°C. Sample conditions were the same as for ferrocytochrome *c*-551. It is convenient because of the large chemical shifts experienced by protons close to the iron to discuss the spectrum of ferricytochrome *c*-551 in

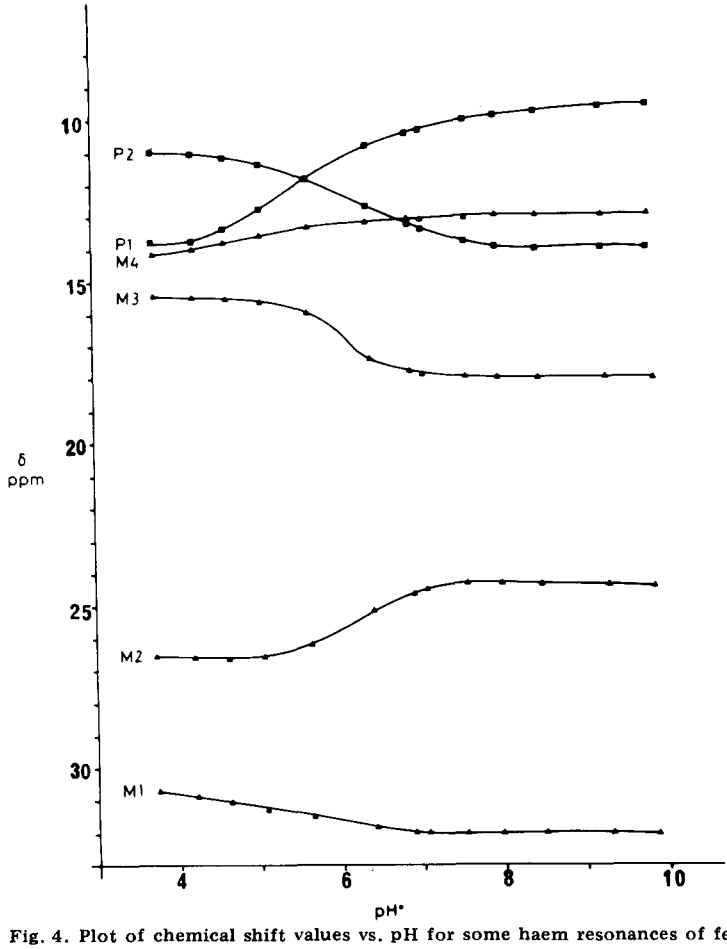

Fig. 4. Plot of chemical shift values vs. pH for some haem resonances of ferricytochrome *c*. Quoted pH values are uncorrected meter readings.

two parts; firstly resonances of the haem group and axial ligands of the iron, and secondly all other resonances.

The most striking pH dependence was exhibited by resonances of the haem group. In Fig. 3 are given regions of the spectra of ferricytochrome *c*-551 obtained at various pH* values. It is notable that not only are the chemical shift values of the haem resonances perturbed by variation in pH but also their linewidths. The chemical shift variations for the resonances of Fig. 3 are presented in the graph of Fig. 4 and summarised in Table I. All the identified haem resonances shifted though some, such as P_3 and the α-meso resonance, were only slightly affected. The most affected resonances were P_1 and P_2 which shifted by +4.2 ppm and −3.0 ppm respectively over the pH* range 3.8 to 8.7.

The pK_a^* for the principle ionisation affecting the haem resonances was calculated to be approximately 6. This pK_a^* could not be precisely determined because the large variation in linewidths made measurements of chemical shift values difficult, and because some of the haem resonances have a complex pH dependence (Fig. 4) and do not titrate with a single well-defined pK^*. The variations in linewidths are associated with the $pK_a^* = 6$ ionisation; linewidths were at a maximum at approximately pH 6.0 and much narrower at pH* 4.0 and pH* 8.0 (Fig. 3).

The only identified resonances of amino acid ligands of ferricytochrome *c*-551 are those of methionine-61 and these were affected by pH in a similar fashion to the haem methyl resonances; they were both shifted and broadened although to a lesser extent than the haem resonances (Table I). With increasing pH above pH 8.9 the intensity of the methionine-61 resonances of native ferricytochrome *c*-551 decreased and by pH* 10.6 were no longer observable.

A number of resonances of amino acids close to the haem group but not bound to the iron shifted with pH. The largest of these shifts was for the resonances of tyrosine-27, one of which shifted by 0.2 ppm between pH* 4.0 and pH* 9.0 with a pK_a^* of 6.0. The overall impression however was similar to that obtained for ferrocytochrome *c*-551; few resonances of groups not bound to the iron were perturbed. Additionally, ionisations with pK^* values of >9.2 and <4.4 were observed.

Discussion

Our central observation is that the redox potential of *Ps. aeruginosa* cytochrome *c*-551 is pH dependent in the pH range 5.0 to 9.5. We interpret this pH dependence as due to two ionisations affecting the redox potential, one in the oxidised form with a pK_a of 6.2 and the other in the reduced form with a pK_a of 7.3. Increasing pH over this pH range results in a 60 mV decrease in the redox potential (Fig. 1).

We tested this proposal by studying the spectroscopic properties of the two oxidation states. Both the visible/near infra-red absorption spectrum (Fig. 2) and the NMR spectrum of ferrocytochrome *c*-551 (Table I) showed changes with pH consistent with an ionisation with pK_a near 7.3. The shift in the α-peak of the visible absorption spectrum is a feature not found for any other characterised low-spin *c*-type cytochrome. This perturbation of haem environment is also reflected in the NMR spectrum of the reduced species but the

resonance shifts involved are neither extensive nor large indicating that the protein conformation is little affected.

In the case of the ferricytochrome no ionisation was reflected in the visible/near infra-red absorption spectrum corresponding to the pK_a of 6.2 proposed for the redox potential studies. In the NMR spectrum of this oxidation state, however, several resonances experienced pronounced shifts with pK_a^* near 6.2. Thus the analysis of the pH dependence of the redox potential in terms of ionisations in the oxidised form of pK_a 6.2 and in the reduced form of pK_a 7.3 is supported by these spectroscopic results.

These pH dependent properties of cytochrome *c*-551 may have implications for other studies. (a) We have noted [5] the relationship between the redox potentials of a series of cytochromes *c* and the chemical shift values of their methionine ligand methyl resonances. This relationship predicts that with decreasing redox potential the chemical shift should become more negative: the methyl resonance moves upfield. The small downfield shift of the methyl resonance of methionine-61 of ferrocytochrome *c*-551 with increasing pH (Table I) is contrary to the prediction indicating that the Fe-S bond length is not the sole factor in determining the redox potentials. (b) The X-ray crystallographic analysis was carried out on crystals grown from solutions of below pH 6 and thus presumably represents the form of the molecule with unionised propionic acid. (c) In the kinetic studies of the reaction of azurin and cytochrome *c*-551 from *Ps. aeruginosa* [28,29] a biphasic pattern was obtained during temperature jump relaxation studies monitored at pH 7. The fast rate was dependent on azurin concentration and is consistent with bimolecular collision but the slower rate was independent of azurin concentration and may reflect relaxation to a new equilibrium defined by the ionisation in the reduced from of pK_a 7.3. The pertubation of the equilibrium might be due either to a shift in this pK_a with temperature or to a small pH change with temperature.

We now consider the nature of the ionising group. In ferricytochrome *c*-551 the resonances of the haem group and axial ligands experience large shifts caused by the unpaired electron of the paramagnetic ferrihaem. The large pH dependent shifts of the haem resonances indicate that the ionisable group is close to the haem. There are two possible interpretations: the ionisable group may be the single haem-bound histidine residue or it may be a haem propionate (we can discount the possibilities that the ionisable group is an amino acid carboxyl group or the N-terminal amino group situated close to the haem on the basis of the X-ray crystallographic structure [30]).

The possibility that it is the histidine ligand can be discounted for two reasons. Firstly, ionisation of the histidine ligand should result in a large structural change since a protonated imidazole cannot function as a ligand. But all the spectroscopic evidence points to, at most, only a small change in structure accompanying ionisation. Secondly, the apparent pK_a for the ionisation of a haem-bound histidine is less than 3 [31—33]. There are no other histidine residues in the protein.

The remaining possibility is that the ionisable group is a haem propionate. Not only is this consistent with the NMR data but also the unperturbed apparent pK_a of haem propionate groups are generally in the region 5—6 [34].

The analogous pH dependent shift of haem resonances of cytochrome *b*-5 has been ascribed to ionisation of a haem propionate substituent and the mechanisms giving rise to the chemical shift variations discussed [26].

The pH dependent behaviour of other haem resonances can be analysed in terms of this single pK_a. The extreme broadening of these resonances close to the pK_a is due to exchange-broadening caused by a relatively slow proton 'on-off' rate. From the extent of line-broadening this rate was calculated to be approximately 10^4 s^{-1}.

We assume that the ionisable group of ferrocytochrome *c*-551 with pK_a of 7.3 is the same propionate that ionises in the oxidised species. We have no direct evidence for this as the haem propionic acid resonances have not been identified in the NMR spectrum of the ferrocytochrome. However the only haem resonance of the ferrocytochrome observed to shift with variation in pH was that of the meso γ-CH proton, one of the closest haem protons to the propionic acids. Also it seems unlikely that different groups in cytochrome *c*-551 ionise in the different oxidation states. It is not possible at present to identify which of the two haem propionic acids is the ionisable group.

At pH values between pK_a^{ox} and pK_a^{red} purified cytochrome *c*-551 acts as a proton as well as an electron donor ($Cyt._{red} \rightleftharpoons Cyt._{ox.} + H^+ + e^-$). In view of the importance of proton carriers in the various schemes of energy coupled electron transfer [35,36] it would be of interest to determine whether the redox potential in situ is also pH dependent. Also the electron transfer rate depends upon the redox potentials of both electron donor and acceptor and a change in the redox potential of one would lead to an altered rate. The 'tunneling' theory of the electron transport process [4] requires that the redox potentials of the donor and acceptor be closely matched. Thus cytochrome *c*-551 has the capability to function as a control mechanism, especially as the proton 'on-off' rate is so slow.

Ionisable haem propionic acids with pK_a values between 5 and 7 have been identified by nmr studies of cytochrome *b*-5 [26] and cytochrome *c*-3 [37] and proposed as an explanation for the pH dependence of the redox potential of certain cytochromes *c*-2 [3,6]. However, in situ, the redox potentials of those cytochromes *c*-2 studied are not pH dependent [38], unlike several of the *b*-type cytochromes of both mitochondrial and photosynthetic electron transport chains [39—42].

The reason for the internal location of the propionic acid groups in the *c*-type cytochromes in comparison to their exterior position in proteins containing protohaem IX is not understood. It may be that for a protein such as mitochondrial cytochrome *c* with a very conserved and pH-independent redox potential [10] it is important that the propionic acids be stabilised by hydrogen-bonding and a hydrophobic environment so that the properties of the haem may not be perturbed by their ionisation. Here we have a possible explanation for the redox potential differences between mitochondrial cytochromes *c* and *Ps. aeruginosa* cytochrome *c*-551. The most striking difference in tertiary structure between the two is that cytochrome *c*-551 lacks a large loop of chain at the bottom of the haem crevice relative to mitochondrial cytochrome *c* [30]. It is from this region of the chain that several of the stabilising hydrogen bonds are donated and although cytochrome *c*-551 appears to partially

compensate for this lack by a swinging down of the left side of the molecule it seems likely that the propionic acids of cytochrome *c*-551 are more exposed and less hydrogen-bonded than in cytochrome *c*.

In the absence of in situ redox potential determinations we do not know whether the pH dependence of redox potential for the purified cytochrome has functional significance. It may be that all *c*-type cytochromes attempt to shield themselves from the effects on redox potential of an ionising propionic acid and some are simply more successful at this than others.

For the ones that are less successful, such as cytochrome *c*-551 this may be an acceptable failure in evolutionary terms or it may be that, in the cell, cytochrome *c*-551 binds to other electron transport components and that this binding achieves a stabilisation of the haem propionic acids in the way intramolecular stabilisation is achieved in the mitochondrial cytochrome *c*.

Addendum

After the studies reported in this paper were completed similar NMR studies on the oxidised protein were reported by Chao et al [43]. We are in agreement with them concerning the identification of a haem linked ionisation with pK_a^{ox} of 6 as a propionate group. Our studies have been briefly communicated elsewhere [44,13].

Acknowledgements

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