Biochimica et Biophysica Acta, 764 (1984) 331–342
Elsevier

BBA 41465

CHARACTERISATION OF IONISATIONS THAT INFLUENCE THE REDOX POTENTIAL OF MITOCHONDRIAL CYTOCHROME *c* AND PHOTOSYNTHETIC BACTERIAL CYTOCHROMES c_2

GEOFFREY R. MOORE [a], DAVID E. HARRIS [b], FIONA A. LEITCH [c] and GRAHAM W. PETTIGREW [c]

[a] *The Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR,* [b] *Laboratory of Molecular Biophysics, University of Oxford, South Parks Road, Oxford OX1 3PS and* [c] *Department of Biochemistry, Royal Dick School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH (U.K.)*

(Received August 12th, 1983)

Key words: Cytochrome c; Redox potential; Ionization; NMR

Several cytochromes c_2 from the Rhodospirillaceae show a pH dependence of redox potential in the physiological pH range which can be described by equations involving an ionisation in the oxidised form (pK_o) and one in the reduced form (pK_r). These cytochromes fall into one of two groups according to the degree of separation of pK_o and pK_r. In group A, represented here by the *Rhodomicrobium vannielii* cytochrome c_2, the separation is approx. one pH unit and the ionisation is that of a haem propionic acid. Members of this group are unique among both cytochromes c_2 and mitochondrial cytochromes *c* in lacking the conserved residue Arg-38. We propose that the role of Arg-38 is to lower the pK of the nearby propionic acid, so that it lies out of the physiological pH range. Substitution of this residue by an uncharged amino acid leads to a raised pK for the propionic acid. In group B, represented here by *Rhodopseudomonas viridis* cytochrome c_2, the separation between pK_o and pK_r is approx. 0.4 pH unit and the ionisable group is a histidine at position 39. This was established by NMR spectroscopy and confirmed by chemical modification. Only a few other members of the cytochrome c_2/mitochondrial cytochrome *c* family have a histidine at this position and of these, both *Crithidia* cytochrome *c*-557 and yeast cytochrome *c* were found to have a pH-dependent redox potential similar to that of *Rps. viridis* cytochrome c_2. Using Coulomb's law, it was found that the energy required to separate pK_o and pK_r could be accounted for by simple electrostatic interactions between the haem iron and the ionisable group.

Introduction

The cytochromes c_2 of photosynthetic bacteria are a family of small monomeric and monohaem proteins that are structurally similar to the mitochondrial cytochrome *c* family. Ambler et al. [1] have classed the two families together on the basis of the similarities in their amino-acid sequences, and by these criteria some cytochromes c_2 more closely resemble mitochondrial cytochrome *c* than they do other cytochromes c_2. X-ray crystallographic and NMR spectroscopic studies of members of both cytochrome families confirm that their structures, and their haem environments in particular, are very similar [2–5]. There is also a marked functional similarity. Mitochondrial cytochrome *c* transfers electrons from cytochrome c_1 to cytochrome oxidase in the respiratory chain [6], a function also performed by some cytochromes c_2 [7,8]. Cytochrome c_2 has also been shown to act as the immediate electron donor to photo-oxidised bacteriochlorophyll in some bacteria [8].

Despite the structural and functional similarities, the two cytochrome families differ in one fundamental property: their redox potentials. Whereas in the pH range 5–8 the redox potentials

of mitochondrial cytochromes *c* are 260 ± 20 mV and are generally considered to be pH-independent [9], those of the cytochromes c_2 vary from 290 to 400 mV and some are markedly pH-dependent [10,11].

Pettigrew et al. [11] distinguished two patterns of pH dependence: one involving a single pK in the oxidised form, which correlated with loss of the 695 nm band of that form, and the other involving three pK values, one in the reduced form and two in the oxidised form in the sequence pK_{o1}, pK_r and pK_{o2}. pK_{o2} involved loss of the 695 nm band, indicating that the iron coordination was changed. The degree of separation of pK_{o1} and pK_r defined the appearance of the redox potential-pH curves obtained in the physiological pH range and they varied from the very pronounced pH dependence of *Rhodomicrobium vannielii* cytochrome c_2 ($pK_{o1} = 6.3$; $pK_r = 7.4$) to the slight pH dependence of *Rhodopseudomonas viridis* cytochrome c_2 ($pK_{o1} = 6.7$; $pK_r = 7.1$).

The present paper investigates the nature of the ionisations affecting the haem in these cytochromes and proposes a structural basis for their variable effects on redox potential. We also comment on the results of Prince and Bashford [12], who suggested such ionisations are not physiologically relevant because, they proposed, binding of the cytochrome c_2 to the bioenergetic membrane suppresses a conformation change coupled to the ionisation, thus leading to pH independence of redox potential.

Materials and Methods

Preparation of cytochromes

Cells of *Rm. vannielii* (ATCC 17100), *Rps. viridis* (NTHC 133) and *Rhodopseudomonas capsulata* SL (ATCC 11166) were grown as described by De Klerk et al. [13] in the laboratory of Professor M.D. Kamen (University of California at San Diego). The cytochromes c_2 from these organisms were purified according to the procedures of Bartsch [14] and Meyer [15]. Cytochrome *c*-557 from *Crithidia oncopelti* and cytochrome *c* from *Saccharomyces cerevisiae* (DCL, Kirkliston, West Lothian, U.K.) were purified in described by Pettigrew et al. [16] and cytochrome c_2 from *Rhodopseudomonas globiformis* was a kind gift from Dr. T.E. Meyer (University of California, San Diego).

N^{ϵ}-Acetimidylated fragments of horse cytochrome *c* were prepared as described by Harris and Offord [17] and Harris [18]. Complexes composed of residues (1–38) + (39–104) and (1–37) + (39–104) were formed by mixing solutions of the two fragments to the point where no further change was observed in the Soret peak [18].

Redox potential determinations

Single point measurements in the presence of known concentrations of ferri- and ferrocyanide were made as previously described [10]. The midpoint potential for ferri-ferrocyanide was taken from Fig. 1 of Ref. 19 according to the conditions of total ionic strength. The redox state of the cytochrome was determined by spectrophotometry in the region of the α-band on a Cary 219 spectrophotometer. In a few cases, full oxidative titrations were performed as described in the caption to Fig. 8.

The complexes formed using fragments of horse cytochrome *c* were autoxidisable and had midpoint potentials out of the useful range of the ferri-ferrocyanide couple. For these systems three-point reductive titrations were performed in an anaerobic cuvette constantly bubbled with argon. The Fe-EDTA couple was used as a redox buffer and to mediate electron transfer between the cytochrome and the platinum pin of a combination Pt-Ag|AgCl electrode (Russell pH Ltd., Auchtermuchty, U.K.) inserted into the cuvette. Stirring was achieved by a Teflon-coated 'cell stirrer' (Bel-Art), following a stirring magnet fitted in the sample compartment of a Unicam SP1800 spectrophotometer. The cuvette contained 0.05 M sodium acetate, sodium phosphate, Tris-HCl or glycine-NaOH buffers, 0.5 mM ferric ammonium sulphate and 10 mM EDTA. The cytochrome complexes (5 μM) were progressively reduced in the cuvette by the addition of small volumes of 0.5 mM ferrous ammonium sulphate, and the state of reduction and the ambient potential were recorded after each addition. Complete reduction was finally achieved by addition of a few crystals of sodium dithionite after opening the cuvette and measuring the pH. The standard potential of the Ag|AgCl reference was taken as 198 mV [20] and this was periodically checked using the Fe-EDTA couple

(E_m = 110 mV at pH 5; Ref. 21). Data plotted according to the Nernst equation gave slopes close to 60 mV except for a few points near pH 9. This may be a consequence of the known instability of the Fe-EDTA chelate at high pH [22].

pH-jump stopped-flow kinetics

The progress of cytochrome reduction after rapid mixing of an unbuffered cytochrome solution and a buffer containing ferrocyanide was monitored at 550 nm in an Applied Photophysics stopped-flow spectrophotometer. The pH of the solution after mixing was measured.

Modification of histidine

Conditions based on those of Miles [23] were used. Diethylpyrocarbonate (Sigma) was diluted in acetonitrile and the concentration of active reagent was determined by the absorbance change produced in a 10 mM imidazole solution ($\epsilon_{230} = 3$ $mM^{-1} \cdot cm^{-1}$). Five equal aliquots of reagent were added at approximately hourly intervals to a solution of 45 μM cytochrome in 20 mM sodium phosphate pH 7.0 to give an excess of 14 mol per mol cytochrome and a final concentration of acetonitrile of 1%. The progress of reaction was followed by difference spectroscopy at 238 nm (equal volumes of acetonitrile being added to the reference cuvette). Once a level of histidine modification of 1 mol per mol had been achieved ($\Delta\epsilon_{238}$ = 3.2 $mM^{-1} \cdot cm^{-1}$), the cytochrome was passed through a column of Sephadex G-25 (fine) equilibrated in 10 mM NaCl/0.5 mM sodium phosphate (pH 7.0).

NMR spectroscopy

Cytochrome samples were prepared for NMR spectroscopy by passage through Sephadex G-25 packed into a Pasteur pipette and equilibrated in 10 mM NaCl/0.5 mM sodium phosphate (pH 7.0) in ^2H_2O (Merck, Sharp and Dohme). Cytochrome concentrations were in the range 2–3 mM. The pH values of solutions in the NMR experiments were monitored by a glass electrode (Radiometer) which was inserted directly into the NMR tubes. The pH was adjusted by addition of small aliquots of concentrated NaO^2H or ^2HCl in ^2H_2O. pH values quoted for NMR experiments are direct meter readings, and since they are not corrected for any isotope effect, they are denoted pH*.

^{1}HNMR spectra were obtained using a Brucker 270 MHz or Brucker 300 MHz spectrometer. Chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate. 1,4-Dioxan was used as the internal reference.

Results

Subdivision of the cytochrome c_2 on the basis of pH dependence of redox potential

On the basis of NMR experiments described later, we can distinguish three subdivisions of the cytochromes c_2 which we call groups A, B and C. Group A includes only *Rm. vannielii* and *Rhodospirillum rubrum* cytochromes c_2. Their pH dependence of redox potential (Fig. 1) can be described in terms of two ionisations in the oxidised protein (pK_{O1} and pK_{O2}) and one ionisation in the reduced protein (pK_r). The relevant equation (Ref. 10), derived using the methods of Clark [24], is:

$$E_m = \tilde{E} + \frac{RT}{nF} \ln \left(\frac{[H^+]^2 + K_r[H^+]}{[H^+]^2 + K_{O1}[H^+] + K_{O1}K_{O2}} \right) \quad (1)$$

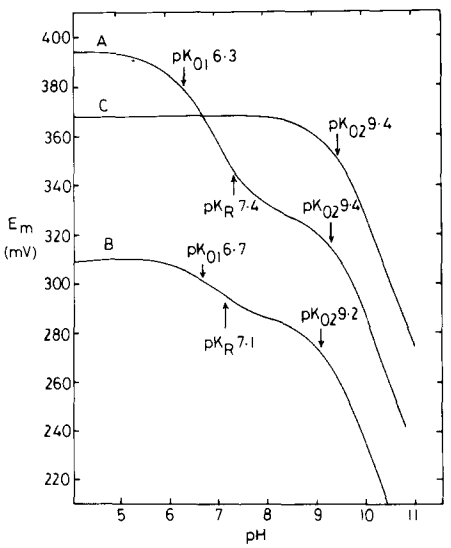

Fig. 1. The pH dependence of the redox potentials of (A) *Rm. vannielii* cytochrome c_2; (B) *Rps. viridis* cytochrome c_2; (C) *Rps. capsulata* cytochrome c_2. The lines are theoretical curves required to fit the experimental data published in Refs. 10 and 11. Curves A and B are defined by Eqn. 1 with the pK values shown and curve C is defined by Eqn. 2.

These cytochromes are characterised by having an asparagine or glutamine in place of Arg-38 [1] and by having a large separation of pK_{O1} and pK_r (approx. 1 pH unit).

Group B includes, amongst others, *Rps. viridis* cytochrome c_2. Their pH dependence of redox potential can also be described by the three-ionisation model and Eqn. 1 (Fig. 1), but pK_{O1} and pK_r are separated by approx. 0.5 pH unit only. Cytochromes of this group contain Arg-38, and at least one non-liganded histidine at the rear of the molecule [1–3].

Group C is represented here by *Rps. capsulata* cytochrome c_2 and horse cytochrome *c*. These exhibit a simple pH dependence of redox potential (Fig. 1) influenced only by pK_{O2} which is common to all cytochromes *c*. The relevant equation is:

$$E_m = \tilde{E} + \frac{RT}{nF}\ln\left(\frac{[H^+]}{[H^+]+K_{O2}}\right) \qquad (2)$$

Cytochromes of this group contain Arg-38.

Group A – structural basis for pH dependence of redox potential

NMR studies of Rm. vannielii cytochrome c_2. The pH dependence of the ^{1}H-NMR spectra of the ferri- and ferrocytochrome were examined. The spectrum of the ferricytochrome (Fig. 2) exhibits the distinctively downfield-shifted resonances arising from the haem substitutent groups found for all low-spin *c*-type cytochromes [4,25,26]. Three of these are methyl resonances (M1–M3). M1 was shown by double-resonance techniques to come from haem methyl-8, in agreement with the horse cytochrome *c* pattern [25]. The fourth haem methyl was not resolved. M1 and M2 shifted with pH with a pK_a of 6.3 (Fig. 3).

Three single proton resonances (P1–P3) were also resolved in the downfield region of the spectrum (Fig. 2). These arise from either the histidine ligand [27] or haem propionates [28]. One-proton intensity resonances in this region have been rigorously assigned to the β-CH_2 protons of the haem propionate substituents of *Pseudomonas* cytochromes *c*-551 [21] and horse cytochrome *c* (Moore, G.R. and Williams, G., unpublished data), and we similarly assign P2 and P3 to a haem propionate in *Rm. vannielii* cytochrome c_2 for the following reasons. Firstly, these single proton resonances occur in the same spectral region as those of horse cytochrome *c* and *Pseudomonas* cytochromes *c*-551 [26,28,29] with the extreme downfield shift indicating that the group involved must be part of the haem or its protein ligands. Secondly, in the *Pseudomonas* cytochromes, propionate resonances shift with pH to the same extent as P3 [26,30]. Thirdly, the pH dependence of the haem methyl resonances of the two cytochromes *c* are similar. In contrast, in systems (such as bisimidazole ferric porphyrins [31] and *Escherichia coli* cytochrome *b*-562; see Moore, G.R., Mathews, F.S. and Williams, R.J.P., unpublished data) where it is known that imidazole ligand ionisations occur, all the downfield-shifted haem and ligand resonances shift upfield as the imidazole ionises to imidazolate, with many of the resonances shifting by approx. 10 ppm.

The methyl resonance of the methionine ligand of *Rm. vannielii* ferricytochrome c_2 experiences a small shift with pH which can be approximately fitted to a curve with pK_a of 6.3 (Fig. 3), in

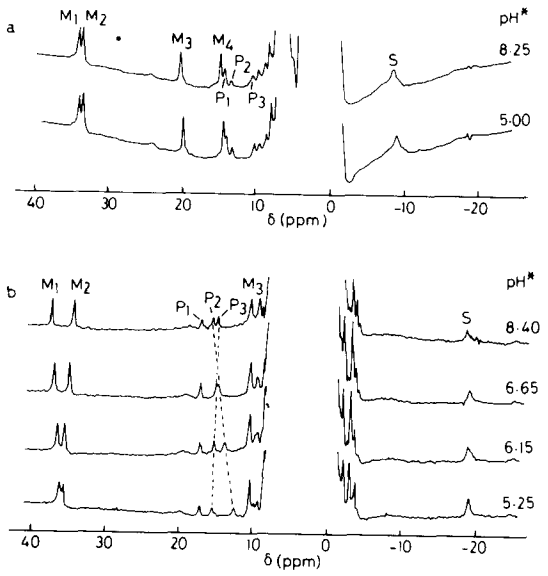

Fig. 2. 270 MHz·^{1}H NMR spectra of (a) *Rps. capsulata* ferricytochrome c_2 and (b) *Rm. vannielii* ferricytochrome c_2. The cytochromes are approx. 3 mM in ^2H_2O at 27°C and pH* was adjusted by the addition of ^{2}HCl or NaO^{2}H. The resonance nomenclature is: S, the methionine ligand methyl resonance; M1–M4, the haem methyl resonances; P1–P3, single-proton resonances that are proposed to come from haem propionates.

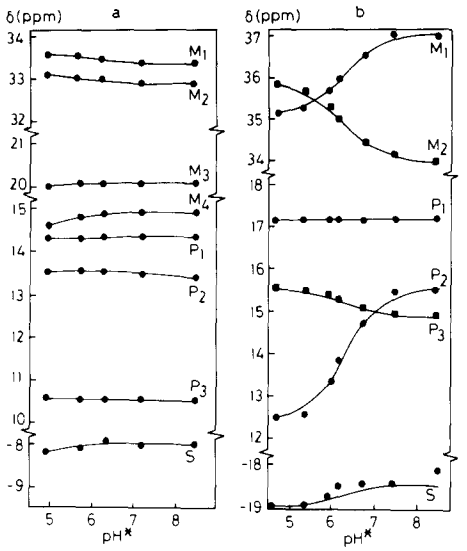

Fig. 3. The pH* dependence at 27°C of the chemical shifts of selected resonances of (a) *Rps. capsulata* ferricytochrome c_2 and (b) *Rm. vannielii* ferricytochrome c_2. The curves of M1, M2, P2, P3 and S in (b) are theoretical curves for an ionisation with pK_a of 6.3.

agreement with the pK_a observed for resonances M1, M2, P2 and P3. The continued presence of this methionine resonance at pH 8 demonstrates that the coordination structure of the iron remains intact in the pH region in which pK_{O1} and pK_r lie. Also, the sharpness of the haem resonances throughout the titration (linewidths of approx. 30 Hz) shows that the rates associated with the ionisation are not lower than $10^4\ s^{-1}$, which precludes any associated conformational change involving the iron coordination or large movements of the main chain and amino acid side-chains.

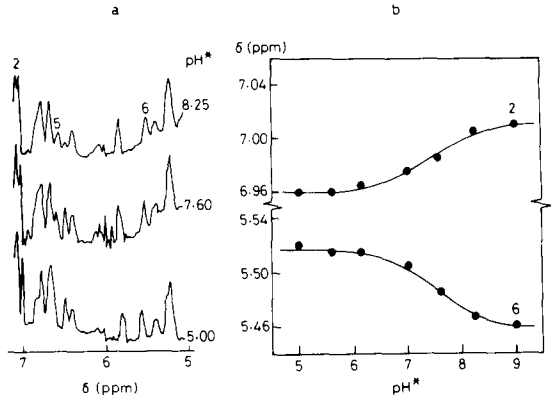

Fig. 4. The pH* dependence of resonances of Trp-59 of *Rm. vannielii* ferrocytochrome c_2. (a) Parts of the aromatic regions of the resolution enhanced 300 MHz·^{1}H-NMR spectra of approx. 2 mM ferrocytochrome c_2 in ^2H_2O at 27°C. Resonances marked 2, 5 and 6 come from the C-2, C-5 and C-6 protons, respectively of Trp-59. (b) The pH* dependence of the C-2 and C-6 resonances of Trp-59 at 27°C. The dots (●) are experimental points and the lines are theoretical curves drawn with a pK_a of 7.4.

The pH dependence of *Rm. vannielii* ferrocytochrome c_2 in ^2H_2O at 27°C was studied over the pH* range from 5.0–9.0. None of the resolved haem resonances (which included the haem methyl-8 resonance at 2.01 ppm) or the methionine ligand resonances shifted. However, the C-2, C-5 and C-6 resonances of the single tryptophan, which were identified by standard methods [5], were found to shift with pH* with a pK_a of 7.4 (Fig. 4). The tryptophan C-7 resonance did not shift and the C-4 resonance was not resolved. A number of unassigned resonances shifted with the pK_a of 7.4, but these shifts were less than 0.05 ppm. As with the ferricytochrome, these small changes in the spectrum indicate that the pK_a of 7.4 is not associated with a large conformational change.

We propose that the pK_a is due to the ionisation of the inner haem propionic acid (propionate-7) for the following reasons. Firstly, we have argued that the pK_a of 6.3 which perturbs the spectrum of ferricytochrome c_2 is due to a haem propionate, and a very similar pattern of pK_o and pK_r has been ascribed to the ionisation of a propionic acid in *P. aeruginosa* cytochrome *c*-551 [26,30]. Secondly, from the X-ray crystal structures of tuna cytochrome *c* [3] and *Rsp. rubrum* cytochrome c_2 [2], it is known that Trp-59 is hydrogen-bonded to propionate-7. Thus, the resonances of Trp-59 would be likely to be affected by ionisation of the propionic acid.

Kinetics of reduction of Rm. vannielii ferricytochrome c_2 in pH-jump experiments. Solutions of *R. vannielii* ferricytochrome c_2 ($2 \cdot 10^{-5}$ M) and ferricyanide (10^{-4} M) at pH 5 were rapidly mixed with buffers ($5 \cdot 10^{-3}$ M) containing ferrocyanide ($5 \cdot 10^{-4}$ M). For final pH values below 8.5, the kinetics of reduction were fast and monophasic (Fig. 5B), while above pH 8.5 kinetics of reduction were biphasic, with an initial rapid reduction followed by a much slower oxidation (Fig. 5C). Mid-

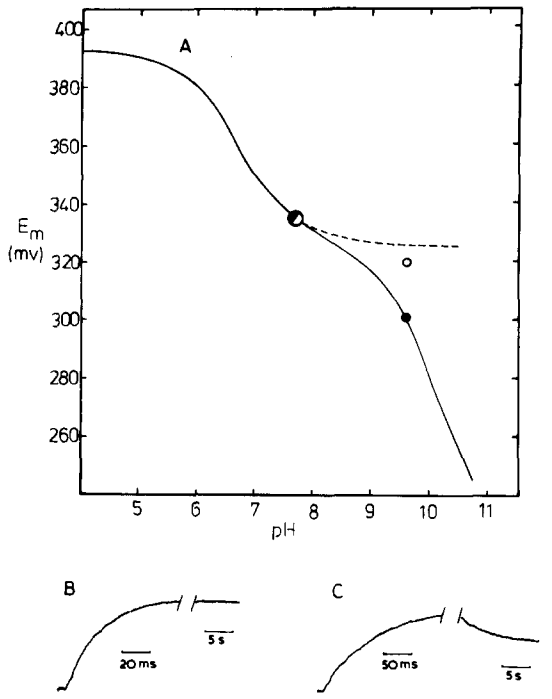

Fig. 5. The redox potential of *Rm. vannielii* cytochrome c_2, measured after the fast and slow phases of ferrocyanide reduction after pH jump. (A) ●, midpoint potential after completion of the slow phase of ferrocyanide reduction; ○, midpoint potential after completion of the fast phase of ferrocyanide reduction. The solid line is defined by Eqn. 1 and the parameters $pK_{O1} = 6.3$, $pK_r = 7.4$, $pK_{O2} = 9.4$ and $\tilde{E} = 392$ mV. The broken line is the extension of the theoretical curve for $pK_{O1} = 6.3$ and $pK_r = 7.4$ in the absence of pK_{O2}. (B) Ferricytochrome c_2 ($2 \cdot 10^{-5}$ M) and ferricyanide (10^{-4} M) in unbuffered solution at pH 5 was rapidly mixed with ferrocyanide ($5 \cdot 10^{-4}$ M) in $5 \cdot 10^{-3}$ M phosphate buffer to give a final pH of 7.7 and the progress of reduction monitored on an oscilloscope. (C) Ferricytochrome c_2 ($2 \cdot 10^{-5}$ M) and ferricyanide (10^{-4} M) in unbuffered solution at pH 5 was rapidly mixed with ferrocyanide ($5 \cdot 10^{-4}$ M) in 10^{-4} M glycine-NaOH buffer to give a final pH of 9.6. The progress of reduction was monitored on an oscilloscope.

point redox potentials calculated at the end of the fast phase and at the end of the slow phase are shown in Fig. 5A.

These results demonstrate that at pH values greater than 8.5, *Rm. vannielii* cytochrome c_2 undergoes the same type of ionisation coupled to a slow conformational equilibrium (with an apparent $pK = pK_{O2}$) that has been proposed for horse cytochrome *c* [32] and *Rps. sphaeroides* cytochrome c_2 [12]. However, the kinetic results of Fig. 5 and the results of the NMR spectroscopy show that at pH values below 8.5, the pH-dependent changes in redox potential do not involve a slow conformation change of the protein.

Group B – structural basis for pH dependence of redox potential

NMR studies of Rps. viridis cytochrome c_2. The pH* dependence of *Rps. viridis* ferri- and ferrocytochrome c_2 in ^2H_2O at 27°C was studied in the pH* range 4.4–9.2. None of the assigned aromatic resonances, resolved haem resonances or methionine ligand resonances were shifted in either redox state. In the ferricytochrome c_2 spectrum, a singlet resonance from the C-4 proton of the sole unliganded histidine (His-39) shifted with a pK_a of

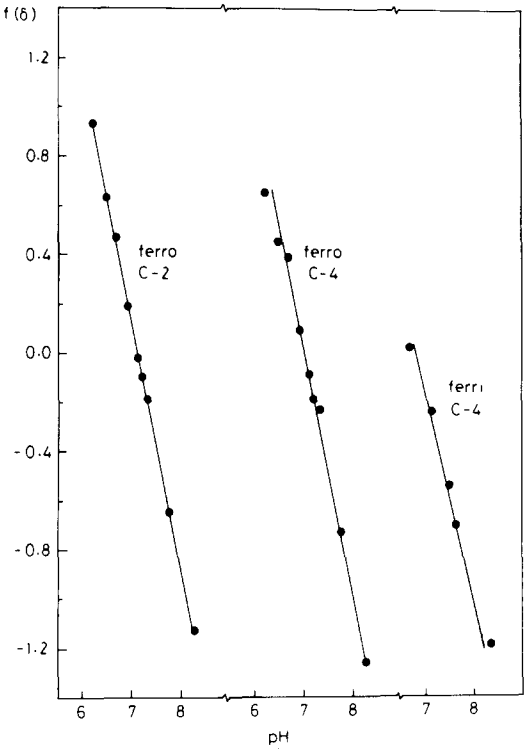

Fig. 6. Hill plot of the pH* dependence of resonances of His-39 of *Rps. viridis* cytochrome c_2 at 27°C. ($f(\delta)$ is $\log\{(\delta - \delta_A)/(\delta_{HA} - \delta)\}$, where δ is the chemical shift at a given pH*, δ_{HA} the chemical shift of the fully protonated species and δ_A the chemical shift of the fully deprotonated species. The solid lines are best fits through the experimental data (●) and yield for the ferrocytochrome, C-2 $pK_a = 7.10$ ($n = 1$) and C-4 $pK_a = 7.05$ ($n = 1$), and for the ferricytochrome, C-4 $pK_a = 6.8$ ($n = 0.9$).

approx. 6.8 (Fig. 6). In the ferrocytochrome c_2 spectrum, both the C-2 and C-4 proton resonances of His-39 were identified and found to titrate with a pK_a of 7.1 (Fig. 6). The NMR data show that the haem propionates do not ionise over the pH* range studied.

Modification of His-39 of Rps. viridis cytochrome c_2 Diethylpyrocarbonate (final concentration, 225 μM) was gradually added to *Rps. viridis* cytochrome c_2 (45 μM). The change in absorbance at 238 nm indicated that 1 mol of histidine per mol of cytochrome had been modified [23]. After removal of excess reagent and breakdown products, the redox potential of the modified cytochrome was determined between pH 5 and pH 9. It was found to be essentially independent of pH, in contrast to the pattern observed for the unmodified protein (Fig. 7). The modification does not result in major structural changes in the cytochrome, since both the redox potential at alkaline pH and the absorbance at 695 nm were almost unchanged.

pH dependence of redox potential of C. oncopelti cytochrome c-557 and S. cerevisiae iso-1 cytochrome c. Among the mitochondrial cytochrome *c* bacterial cytochrome c_2 family, only *Rps. globiformis* cytochrome c_2 and the cytochromes *c* from *C. oncopelti, S. cerevisiae* and *Candida krusei* are like *Rps. viridis* cytochrome c_2 in having a histidine at position 39. The pH dependence of redox potential of *C. oncopelti* cytochrome *c*-557 and *S. cerevisiae* cytochrome *c* is shown in Fig. 8 and compared with that for horse cytochrome *c*.

The data for horse cytochrome *c* can be accurately fitted by a theoretical curve with a single pK_o of 8.75 in the oxidised form as described by Eqn. 2. We propose that the data for *C. oncopelti* cytochrome *c*-557 and *S. cerevisiae* cytochrome *c* deviate significantly from the curve described by this simple equation (broken line of Fig. 8b and 8c) and are fitted best by Eqn. 1 with $pK_{O1} = 6.6$ and $pK_r = 7.0$ in the case of the *Crithidia* cytochrome *c*, and $pK_{O1} = 6.9$ and $pK_r = 7.3$ for the *Saccharomyces* cytochrome. pK_{O2} values are given in the figure caption.

We were unable to determine the pH dependence of redox potential for *Rps. globiformis* cytochrome c_2 because of the very slow equilibration of this cytochrome with the ferri-ferrocyanide couple. Measurements at pH 7 suggest that the midpoint potential may be more positive than 450 mV.

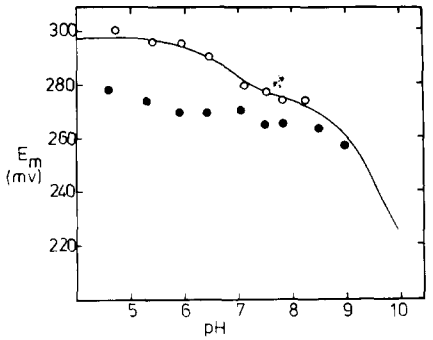

Fig. 7. The effect of histidine modification on the pH dependence of redox potential of *Rps. viridis* cytochrome c_2. ○, single-point determinations of midpoint potential of native cytochrome in the presence of 0.5 mM ferrocyanide and 0.017 mM ferricyanide. Buffers were 2 mM in acetate, phosphate, Tris or borate. The solid line is a theoretical curve described by Eqn. 1 with the parameters $pK_{O1} = 6.7$, $pK_r = 7.1$ and $pK_{O2} = 9.2$, which gave the best fit to the published experimental results for *Rps. viridis* cytochrome c_2. $\bar{E}$ was here taken as 297 mV (309 mV gave the best fit to the original results). ●, single point determinations of midpoint potential of cytochrome proposed to be modified at His-39. Conditions were as above.

Group C – the pH independence of redox potential of Rps. capsulata cytochrome c_2 *and horse cytochrome c*

NMR studies of Rps. capsulata cytochrome c_2. The ^1H NMR spectrum of *Rps. capsulata* ferricytochrome c_2 was studied at different pH* values in ^2H_2O at 27°C. Although some of the haem methyl resonances shifted in the pH* range 5.0–9.0, the effects were very much smaller than those observed for the haem methyls of *Rm. vannielii* ferricytochrome c_2 (Fig. 2 and 3). Also, the single proton resonances, P1–P3, some (or all) of which came from haem propionic acids, did not shift with pH*. Thus, the haem propionate groups of *Rps. capsulata* cytochrome c_2 do not ionise in the pH* range 5.0–9.0.

The pH dependence of redox potential of horse cytochrome c cleaved at Arg-38. In view of the emphasis we place on the role of Arg-38 in modulating the ionisation of haem propionate-7 (see Discussion) we studied two of the two-fragment complexes of horse cytochrome *c* in which the

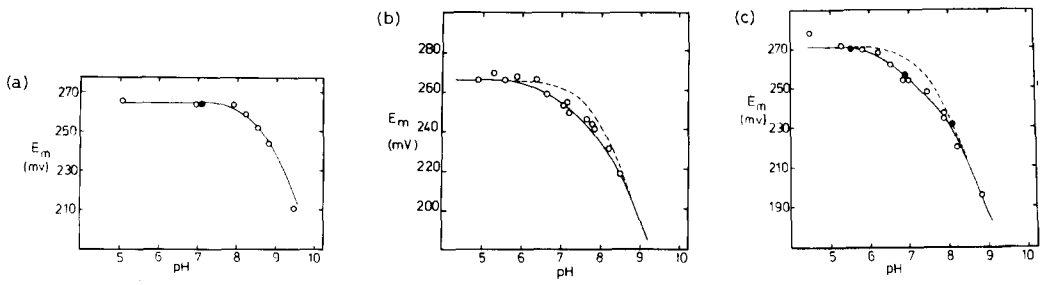

Fig. 8. The pH dependence of redox potential of (a) horse cytochrome *c*; (b) *S. cerevisiae* cytochrome *c*; (c) *C. oncopelti* cytochrome *c*-557. ○, single-point determinations of midpoint potential. In all cases, ferrocyanide was 0.5 mM. In (a) ferricyanide was 0.004 mM, and in (b) and (c) it was 0.0083 mM. Cytochromes were $2 \cdot 10^{-6}$ M and buffers were 2 mM acetate, phosphate, Tris or borate. ●, full oxidative titrations in the presence of 0.5 mM ferrocyanide and varying concentrations of ferricyanide. The cytochromes were reduced with 50 mM ascorbate and excess reductant removed by gel filtration. Thus the titrations started with fully reduced cytochrome. The solid line in (a) is a theoretical curve described by Eqn. 2 with $pK_{O2} = 8.75$ and $\tilde{E} = 265$ mV. The solid line in (b) is a theoretical curve described by Eqn. 1 with $pK_{O1} = 6.9$, $pK_r = 7.3$, $pK_{O2} = 8.3$ and $\tilde{E} = 266$ mV. The broken line in (b) is for a single ionisation with $pK_{O2} = 7.85$. The solid line in (c) is a theoretical curve described by Eqn. 1 with $pK_{O1} = 6.6$, $pK_r = 7.0$, $pK_{O2} = 8.0$ and $\tilde{E} = 271$ mV. The broken line in (c) is for a single ionisation with $pK_{O2} = 7.55$.

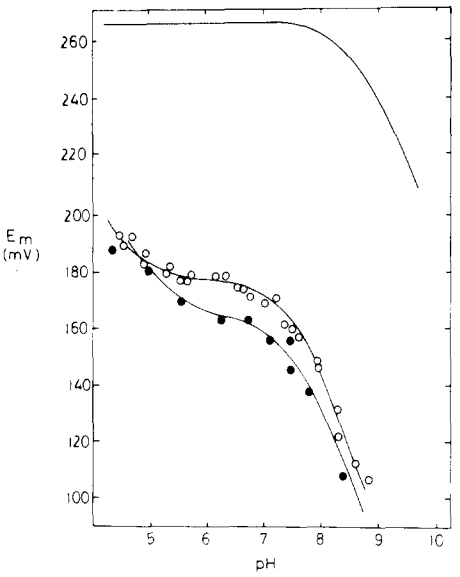

Fig. 9. The effect of removal of Arg-38 on the pH dependence of redox potential of horse cytochrome *c*. The experimental points (○ and ●) were obtained from three-point titration data, using 0.5 mM ferric ammonium sulphate in 10 mM EDTA as the mediator and 0.1 M ferrous ammonium sulphate as the titrant in the presence of a Pt-Ag|AgCl electrode in an argon-flushed cuvette. Buffers were 0.1 M in acetate, Tris or glycine and 0.05 M in phosphate. Cytochromes were 10^{-5} M. ○, horse cytochrome *c* (1–38·39–104). The line is a theoretical curve according to Eqn. 3 with $pK_r = 4.3$ and $pK_o = 7.6$. ●, horse cytochrome *c* (1–37·39–104). The line is a theoretical curve according to Eqn. 3 with $pK_r = 4.9$ and $pK_o = 7.6$. The top curve is taken from Fig. 8a for unmodified horse cytochrome *c*.

Arg-38–Lys-39 bond is selectively cleaved; the parent complex (1–38 · 39–104) and the complex in which the exposed C-terminal Arg-38 had been removed by carboxypeptidase B (1–37 · 39–104). Both of these systems were autoxidisable and redox titrations were performed anaerobically in the presence of a Pt-Ag|AgCl combination electrode and the Fe-EDTA couple. The results are shown in Fig. 9 and were analysed according to the equation:

$$E_m = \tilde{E} + \frac{RT}{nF} \ln\left(\frac{[H^+]^2 + K_r[H^+]}{[H^+] + K_o}\right) \qquad (3)$$

which defines the theoretical curves. A similar pattern of pH dependence was obtained in both cases, with $pK_r = 4.3$ and $pK_o = 7.6$ for (1–38 · 39–104) and $pK_r = 4.9$ and $pK_o = 7.6$ for (1–37 · 39–104).

pH titration of the ferricytochromes was performed and followed spectroscopically between 500 and 750 nm. The 695 nm bands, indicative of methionine coordination to the iron [29,33,34], were present and were lost with a p*K* of approx 7.8 in (1–38 · 39–104) and approx. 7.9 in (1–37 · 39–104). The ionisations with pK_r may be the haem propionate-7 ionisations.

Discussion

The ionisation of a haem propionic acid in cytochrome c_2

The redox potential of *Rm. vannielii* cytochrome c_2 was influenced by an ionisation in the oxidised protein, with $pK_{O1} = 6.3$, and in the reduced protein, with $pK_r = 7.4$ (Fig. 1). These pK values are in addition to the pK_{O2} of 9.4 in the oxidised protein [10], which involves loss of the methionine-iron coordination and is found in all cytochromes c_2, although at different pH values [11]. A simple interpretation of pK_{O1} and pK_r is that they represent the ionisation of the same group but with the proton dissociation being influenced by the redox state of the haem [10]. NMR spectroscopy of the ferricytochrome has allowed direct identification of a haem propionate as the ionising group with pK_a of 6.3. In the spectrum of the ferrocytochrome c_2, the haem propionate resonances were not resolved but resonances of Trp-59 shifted with a pK_a of 7.4. Because the corresponding tryptophan is hydrogen-bonded to the inner haem propionate (propionate-7) in *R. rubrum* cytochrome c_2 [2] and tuna cytochrome *c* [3], we conclude that its resonances reflect the propionate ionising with a pK_a of 7.4 in *Rm. vannielii* ferrocytochrome c_2. Thus, we propose that the $pK_{O1} = 6.3$ and $pK_r = 7.4$ deduced [10] from the pH dependence of redox potential are due to the ionisation of the inner haem propionic acid with the proton being lost more readily from the ferricytochrome c_2 due to the electrostatic influence of the ferric haem. In the pH range in which these ionisations occur, the methionine-iron bond remains intact. This pattern of pK_{o1} and pK_r is similar to that found for the *Pseudomonas* cytochromes *c*-551 [26,30].

The propionic acid NMR resonances of *Rps. viridis* and *Rps. capsulata* cytochromes c_2 do not shift with pH* in the range 5.0–9.0 and in this respect they resemble those of horse cytochrome *c* [29,35]. On the basis of the X-ray crystallographic structure of tuna cytochrome *c* [3], Arg-38 lies close enough to the inner haem propionate to form a hydrogen bond or salt link. Only *R. rubrum* and *Rm. vannielii* cytochromes c_2 of the mitochondrial cytochrome *c*-bacterial cytochrome c_2 family lack this residue; they have an asparagine or glutamine instead. Therefore, we propose that the function of Arg-38 is to interact with the haem propionate in such a way that the propionate does not ionise in the physiological pH range. This could be achieved in two ways: (i) the inner haem propionate is maintained in the unionised state by the hydrophobic interior of the protein and the extensive set of interactions in which it is involved; (ii) the positively charged Arg-38 stabilises the ionised form and thereby lowers the pK_a. According to this view, Gln-38 of *Rm. vannielii* cytochrome c_2 could not act in the same way and, as a consequence, the propionate pK_a values are in the range 6–8. Thus, the second possibility seems most likely.

Cleavage of the Arg-38–Lys-39 bond in horse cytochrome *c* and subsequent removal of Arg-38 yields a protein with the methionine-iron bond intact at pH 7 but with a midpoint potential 90 mV more negative than that of the unmodified cytochrome (Fig. 9). We propose that this effect is due to an increase in the relative stabilisation of the oxidised form of the modified protein by the unneutralised negative charge of propionate-7. It is interesting that simple cleavage of the arginyllysyl peptide bond without removal of the arginine yields a protein with a very similar pattern of pH dependence of redox potential to the system with the arginine removed. Possibly, the appearance of a new α-carboxylate close to the guanidino group interferes with the ability of the latter to interact with the haem, propionate, thus giving rise to similar effects on the redox potential as the removal of the arginine itself. Consistent with this view is the conspicuous absence of acidic amino acids on the surface of cytochromes *c* and cytochromes c_2 close to Arg-38. In the loop of residues 30–42 in the 67 mitochondrial cytochrome sequences given in the compilation of Dickerson and Timkovich [36], and in the 14 cytochrome c_2 sequences given by Ambler et al. [1], acidic amino side-chains are found only three times and all three cases occur in *R. rubrum* and *Rm. vannielii* cytochromes c_2.

The effect of histidine ionisation on the redox potential of cytochromes c

In the case of *Rps. viridis* cytochrome c_2, the haem propionates have been shown not to ionise in the pH* range 5.0–9.0 and yet the redox poten-

tial is influenced by a group with a $pK_a = 6.7$ in the oxidised form and a $pK_a = 7.1$ in the reduced form (Fig. 1). NMR spectroscopy identified this group as His-39 (Fig. 6). After modification of this histidine by diethylpyrocarbonate, the redox potential becomes independent of pH and has a value close to that of the unmodified protein at pH 8 (Fig. 7). The more positive value of the midpoint potential at acid pH for the native protein is consistent with an electrostatic effect resulting in destabilisation of the ferric cytochrome relative to the ferrous cytochrome due to the protonated histidine, a destabilisation not possible in the modified protein until very low pH values because the pK_a of *N*-ethoxyformylhistidine is 3.4 [37].

Our study of *Rps. viridis* cytochrome c_2 led us to predict that the few members of the cytochrome c_2-mitochondrial cytochrome *c* family that contained His-39 would show a similar pH dependence of redox potential. This was demonstrated for *C. oncopelti* cytochrome *c*-557 and *S. cerevisiae* cytochrome *c* (Fig. 8) and it is consistent with previous NMR studies showing that His-39 of *S. cerevisiae* cytochrome *c* ionises with a pK_a of 6.8 in the ferric protein and a pK_a of 7.2 in the ferrous protein [38]. It seems likely that similar histidine effects occur in the pH dependence of redox potential of *Rps. palustris* and *Rps. sphaeroides* cytochrome c_2 where the separation of pK_o and pK_r is 0.5 and 0.3 pH units, respectively [10,11]. However, in these cytochromes histidine does not occur at position 39 but at positions 53 and 63, and at position 92, respectively [1].

There are three possible explanations for the histidine effects which we cannot yet distinguish. Firstly, the ionisation of a histidine may be directly influenced by the redox state of the iron and may in turn perturb the redox potential by the same mechanism of electrostatic interaction that we found for the haem propionate. Secondly, a protonated histidine may contribute to the shielding of the propionate charge and deprotonation would therefore result in a less positive redox potential. Thirdly, the small conformational change which occurs at the rear of the cytochrome molecule [3,39] (and which may be triggered by changes in the electrostatic relay $Fe(III)–CO_2^- –Arg\text{-}38^+$ on reduction) may result in a difference in the environment of histidines in this region of the protein in the two redox states.

Energies of interaction between the haem iron and ionisable groups

Using Coulomb's law:

$$E = \frac{1347\, q_1 \cdot q_2}{\epsilon \cdot D} \qquad (4)$$

where q_1 and q_2 are the charges on two groups, D is the distance between them, ϵ is the effective dielectric constant and E is the energy of interaction, it is possible to assess the effect that an ionising propionic acid would have on the redox potential [40]. The actual fall in redox-potential associated with this ionisation in *Rm. vannielii* cytochrome c_2 is 65 mV, which is equivalent to $-6.27\ kJ \cdot mol^{-1}$ (Table IA). Energies of interaction of this size can be obtained from Eqn. 4 if the product $q_1 \cdot q_2$ is taken as -1 (i.e., $+1$ charge on the iron and -1 on the propionate), D taken as 10 Å and ϵ taken as 20 (Table IB). Since $q_1 \cdot q_2$ and ϵ are unknown quantities which may be impossible to determine accurately, this calculation does not provide a definitive answer, but it does serve to

TABLE IA

ENERGIES OF INTERACTION BETWEEN THE HAEM IRON AND IONISABLE GROUPS

Cytochrome	ΔpK	ΔE_M (mV)	ΔG ($kJ \cdot mol^{-1}$)	Ionisable group
Rm. vanielli cytochrome c_2	1.1	65	−6.27	haem propionate-7
Rps. viridis cytochrome c_2	0.4	23	−2.22	His-39

TABLE IB

ENERGY OF INTERACTION CALCULATED FROM EQN. 4

$q_1 \cdot q_2$	ϵ	D (Å)	E ($kJ \cdot mol^{-1}$)
−1	20	10	−6.73

demonstrate that energies of the appropriate size can be produced by the type of interaction proposed. The energies associated with histidine ionisation are smaller (Table IA) and in view of our uncertainty as to the detailed mechanism operating in these cases, it is difficult to decide on the cause of this smaller effect. However, it is probable that the effective dielectric constant between the iron and a surface histidine will be larger than that between the iron and an internal propionate (judging from the position of Lys-39 of tuna cytochrome *c*, His-39 is approx. 18 Å from the iron which with $\epsilon = 20$, gives an E of $-3.75\ kJ \cdot mol^{-1}$). It is interesting to note that the fall in redox potential (90 mV) observed on removal of Arg-38 from horse cytochrome *c* (Fig. 9) is comparable to the fall observed on propionic acid ionisation in *R. vannielii* cytochrome c_2 (65 mV) (Fig. 1). This is consistent with the suggested role for Arg-38 in neutralising the negative charge of the propionate in the interior of the unmodified cytochrome.

Biological aspects of haem-linked ionisations

The pH dependence of the redox potential of purified *Rps. sphaeroides* cytochrome c_2 at alkaline pH (pK_{O2}) [10] was not observed for the cytochrome attached to the chromatophore membrane [41]. Nor was pH-dependent behaviour observed if the redox potential was estimated after a rapid phase of reduction of the cytochrome [12]. Brandt et al. [32] have shown for horse cytochrome *c* that the apparent pK of 9 could be viewed as due to an ionisation with a pK of 11 coupled to a conformation change with a pK of 2, and in applying this analysis to *Rps. sphaeroides* cytochrome *c*, Prince and Bashford [21] have argued that the conformation change in this cytochrome is suppressed upon attachment of the cytochrome to the chromatophore membrane and that the redox potential is consequently independent of pH between 7–11. These authors thus concluded that pH-dependent effects of redox potential of purified cytochromes c_2 were not relevant to the physiological situation. We accept their elegant analysis of the nature of the apparent pK_o near 9, which is observed in all cytochromes and involves loss of methionine coordination. However, we have shown by NMR spectroscopy and by kinetics of reduction that pK_{O1} and pK_r of *Rm. vannielii* cytochrome c_2 do not involve a slow conformational change and are thus different in nature from pK_{O2}. The question as to whether pK_{o1} and pK_r operate in situ thus remains open.

There is much interest at present in how electron transport may drive vectorial proton translocation across a bioenergetic membrane [42–45]. Both mitochondrial cytochrome oxidase and cytochrome *c* reductase have been proposed to be proton pumps in which electron transfer at the redox centre is coupled via the intervening polypeptide chain to deprotonation events in an analogous way to the Bohr effects in haemoglobin. Although we do not suggest that the soluble periplasmic cytochromes considered here could act in such a transmembrane role, they may serve as useful scalar models for how the more complex systems work. We propose that simple electrostatic considerations will result in an imposed redox-state-dependent separation of pK values of a group close to the redox centre and that this will give rise to proton-coupled electron transfer in the intervening pH range. Such a mechanism avoids the kinetic problems associated with schemes requiring slow conformation changes, and for systems in which the effective dielectric constant is low, which is probably the case for intrinsic membrane proteins, it provides substantial interaction energies over a large distance. The question of how the proton gate operates remains open [44,45]. It is difficult to envisage a mechanism whereby proton translocation against a proton gradient is controlled entirely by electrostatic terms and a small conformational change, which may involve no more than the orientation of a few amino-acid side-chains or a helix, may be required.

Acknowledgements

We are indebted to Prof. M.D. Kamen and Drs. R.G. Bartsch and T.E. Meyer for providing the facilities and expertise for the growth of *Rm. vannieli, Rps. viridis* and *Rps. capsulata* S.L. G.W.P. thanks the Wellcome Trust and the SERC for financial support and G.R.M. thanks the Science and Engineering Research Council for an Advanced Fellowship. F.A.L. acknowledges an SERC studentship.

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