Fourier-transform infra-red studies of the alkaline isomerization of mitochondrial cytochrome *c* and the ionization of carboxylic acids

Peter TONGE,*† Geoffrey R. MOORE‡§ and Christopher W. WHARTON*

*Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K., and ‡School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The alkaline transitions of tuna and horse ferricytochromes *c* and the trifluoroacetyl-lysine derivative of horse ferricytochrome *c* have been studied by Fourier-transform (FT) i.r. spectroscopy. The spectral perturbations resulting from the transition have been interpreted by reference to FT i.r. data on simple carboxylic-acid-containing compounds and a bacterial cytochrome *c*₅₅₁ in which a haem propionate ionizes without causing a significant conformational change. The analysis strongly suggests that ionization of a haem propionate of mitochondrial cytochrome *c* triggers the alkaline conformation change.

INTRODUCTION

The pH-dependence of mitochondrial cytochrome *c* has been studied by a wide variety of spectroscopic techniques including magnetic-resonance methods (Gupta & Koenig, 1971; Lambeth *et al.*, 1973; Brautigan *et al.*, 1977; Morishima *et al.*, 1977; Gadsby *et al.*, 1987), optical methods (Theorell & Åkesson, 1941; Greenwood & Wilson, 1971; Gadsby *et al.*, 1987) and Raman spectroscopy (Kitigawa *et al.*, 1977; Uno *et al.*, 1984). All these methods show that ferricytochrome *c* undergoes a change in ligation with a *pK* of ~9 which depends on temperature and pH. This transition has been shown to be a complex process involving an ionization with a *pK*_a of 11 coupled to a conformational change with a *pK*_c of -2 (Brandt *et al.*, 1966; Davis *et al.*, 1974). In the present paper we report a FT i.r. study of this transition which indicates that a carboxylic acid group may be involved.

In the preceding paper (Hartshorn & Moore, 1989) we demonstrated that one of the haem propionic acid substituents of cytochrome *c* has a *pK*_a of < 4.5 and the other has a *pK*_a of > 9. In view of the possibility that the propionic acid with a high *pK*_a might be involved in the transition, we studied various carboxylic acids and a haemoprotein, *Pseudomonas aeruginosa* cytochrome *c*₅₅₁, in which a haem propionic acid ionization occurs with a *pK*_a of 6.2 (Leitch *et al.*, 1984), as model compounds.

MATERIALS AND METHODS

Materials

Tuna (type XI) and horse (type VI) cytochromes *c* were obtained from Sigma (Poole, Dorset, U.K.) and before use passed through an ion-exchange column as previously described (Eley *et al.*, 1982).

Pseudomonas aeruginosa cytochrome *c*₅₅₁ was a gift of Professor C. Greenwood (School of Biological Sciences,

University of East Anglia, Norwich, U.K.). Trifluoroacetylated cytochrome *c* was prepared by the method of Fanger & Harbury (1965): ¹⁹F n.m.r. of the product using a JEOL FX-100 spectrometer showed all 19 lysine residues were modified. Cyanoferricytochrome *c* was prepared by the addition of a 20% excess of KCN to a solution of ferricytochrome *c*.

²H₂O was obtained from Aldrich (Gillingham, Dorset, U.K.) and NaO²H and ²HCl from Merck, Sharpe and Dohme (Montreal, Quebec, Canada).

All other reagents were obtained from Sigma, Aldrich or BDH (Poole, Dorset, U.K.), and were of the highest available quality.

Adjustment of pH

The solution pH values were adjusted by the addition of small quantities of HCl or ²HCl and NaOH or NaO²H. pH values were measured with a Radiometer electrode (type MWL) or a Russell electrode (type CMAWL). All quoted values for ²H₂O solutions, designated by pH*, are the direct meter readings uncorrected for any small isotope effects (Kalinichenko, 1976).

Samples for i.r. spectroscopy

Protein samples were dissolved in ²H₂O to give a final concentration in the range of 10–32 µg/ml and the pH* adjusted to 8.0. The sample was then incubated in a water bath at 40 °C for 60–90 min to promote ¹H–²H exchange. After cooling to room temperature the samples were clarified when necessary by centrifugation and divided into 200 µl aliquots. The pH* of each aliquot was then adjusted to the required value. Samples of ferrocyclochrome *c* were prepared by the addition of sodium ascorbate immediately before the adjustment of pH*. The sample volumes were kept the same within each series of samples by the addition of ²H₂O. Total additions amounted to 10–20 µl.

Abbreviations used: FT, Fourier transform; *pK*_a, negative logarithm of an acid dissociation constant; *pK*_c, negative logarithm of an equilibrium constant for a protein conformational change.

† Permanent address: Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1N 6R5.

§ To whom correspondence and reprint requests should be sent.

The low- M_r carboxylic-acid-containing compounds were studied at a concentration of 0.1 M in $^2\text{H}_2\text{O}$.

Titration of carboxy groups by means of i.r. difference spectroscopy

Protonated and unprotonated carboxy groups of simple compounds and proteins usually absorb in the i.r. with frequencies near to 1710 cm^{-1} and 1570 cm^{-1} respectively. The intensities of these two bands may be monitored over a range of pH* in order to determine the dissociation constant(s) of the carboxy groups. It is necessary to substitute $^2\text{H}_2\text{O}$ for H_2O in order that the strong 1640 cm^{-1} bending frequency of water is eliminated; contaminant water equilibrates with $^2\text{H}_2\text{O}$ to give ^2HOH , which has a strong bending frequency at $\sim 1450\text{ cm}^{-1}$, nearly coincident with the N-D stretch of amides. The amide II N-H stretch, which occurs near to 1540 cm^{-1} in protic media, is eliminated by prior exchange of the protein amide protons with deuterium. Exchange is achieved by incubating the protein with $^2\text{H}_2\text{O}$ under reversible, partially unfolding, conditions.

It is particularly important that the baselines of the i.r. spectra are reasonably flat in the regions in which difference spectroscopic features are to be observed. Since the amide I absorption, which primarily arises from carbonyl stretch near 1640 cm^{-1} , is very strong, subtractions or dual-beam differences may be unreliable in this region. It is usually expected that pH*-dependent changes in both the protonated carboxy region ($\sim 1710\text{ cm}^{-1}$) and the carboxylate region ($\sim 1570\text{ cm}^{-1}$) will be observed as a result of pH* titration; this provides a useful check that a carboxy group is indeed undergoing ionization rather than some other ionizing group. This is certainly the case with simple carboxylic acids, lysozyme (Timasheff & Rupley, 1972; Timasheff *et al.*, 1973) and chymotrypsin (Koepppe & Stroud, 1976). The frequencies shown by the protonated amino-acid side-chain carboxy groups of these proteins are sufficiently clear of the

amide I absorption that they give good pH-dependent bands in difference spectra, even though they occur as shoulders in the raw spectra.

I.r. spectroscopy

A Nicolet MX-1 FT i.r. spectrometer equipped with a demountable CaF_2 cell with a 0.077 mm path length was used. Each sample was scanned 64 times with a total acquisition time of approx. 2 min. Difference spectra were obtained by using the spectrum of the most acidic solution as the reference and a subtraction multiplication factor of 1.0.

For a particular titrating band, the spectral regions for integrated intensities were determined by inspection. Band intensities were then calculated as a fraction of the largest intensity and fitted to the equation:

$$Y = \frac{K}{[\text{H}^+] + K}$$

where Y is the fraction of a group ionized and K is the equilibrium constant. Curve-fitting was carried out with a non-linear least-squares program supplied by Dr. A. Cornish-Bowden (Department of Biochemistry, University of Birmingham, Birmingham, U.K.).

RESULTS

pH-dependence of i.r. spectra of carboxylic acids

I.r. spectroscopic frequencies of a number of relatively simple carboxylic-acid-containing compounds were measured in $^2\text{H}_2\text{O}$. Bands due to the carboxy-group-carbonyl stretch were found in the range $1658\text{--}1727\text{ cm}^{-1}$ for the carboxylic acid forms, and in the range $1548\text{--}1572\text{ cm}^{-1}$ for the carboxylate forms. The results are summarized in Table 1.

Table 1. I.r. frequencies of cytochromes c and model-compound carboxy group stretch vibrations

Material	Frequencies (cm^{-1})		
	COO^2H	Half-ionized or zwitterion	COO^-
<i>P. aeruginosa</i> * ferricytochrome c_{551}			1571
Tuna ferricytochrome c			1565
Trifluoroacetylated* horse ferricytochrome c			1572
Lysine†	1727	1614 (COO^-)‡	1572
Benzoic acid†	1684		1548
Benzoic acid§	1720		
<i>o</i> -Hydroxybenzoic acid§	1680		
<i>p</i> -Hydroxybenzoic acid§	1716		
α -Resorcylic acid† (<i>oo'</i> -dihydroxybenzoic acid)	1658		
Acetic acid†	1710		1561
Fumaric acid† (<i>trans</i> -butenedioic acid)	1697	1695 (COO^2H) 1575 (COO^-)	1569
Maleic acid† (<i>cis</i> -butenedioic acid)	1704	1693 (COO^2H) 1580 (COO^-)	1564

* See the Materials and methods section and Figure legends.

† Spectra of 0.1 M solutions measured in $^2\text{H}_2\text{O}$, titrated with ^2HCl and NaO^2H .

‡ See Nakamoto *et al.* (1963) and Sawyer & Tackett (1963).

§ Spectra of dioxan solutions (Flett, 1951).

Fig. 1. FT i.r. difference spectra of *P. aeruginosa* ferricytochrome c_{551}

Cytochrome c_{551} ($32 \mu\text{g}/\mu\text{l}$) in $^2\text{H}_2\text{O}$ was scanned 64 times in a 0.07 mm-pathlength cell with CaF_2 windows. To obtain each difference spectrum, a reference spectrum of cytochrome c_{551} at pH^*5 was subtracted. The arrows indicate the limits for the intensity integration procedure. The pH^* values of the sample spectra were: 1, 5.5; 2, 6.15; 3, 6.6; 4, 7.0; 5, 7.8.

pH-dependence of i.r. difference spectra of cytochrome c_{551}

The pH^* -dependence of the i.r. difference spectra for ferricytochrome c_{551} is shown in Fig. 1. A clearly defined pH^* -dependent feature is seen at 1571 cm^{-1} , which is indicative of carboxylate anion formation as the pH^* becomes more alkaline relative to the reference. However, assignment of this pH^* -dependent difference feature to carboxylic acid ionization is uncertain, since the reciprocal pH^* -dependent feature expected in the region of 1710 cm^{-1} , which would represent loss of the protonated form as the system becomes more alkaline relative to the reference, is not seen. A pH^* -dependent feature centred upon 1657 cm^{-1} is seen (Fig. 1), but this is at a frequency that is unusually low for protonated carboxy groups (Timasheff & Rupley, 1972). Also, neither the difference absorbance values nor the integrated intensities relative to a pH^* value of 5 gave a satisfactory fit to the equation for ionization of a single prototropic series (see the Materials and methods section). The reason for the failure to observe a pH^* -dependent feature which clearly corresponds to the protonated carboxy group at the expected frequency has been examined by using model compounds, and is discussed below.

Fig. 2 shows the pH^* -dependence of the integrated intensity of the 1571 cm^{-1} band. The data give a good fit to a line calculated for a pK_a^* value of 6.14 ± 0.11 . This value is in excellent agreement with the value of 6.2 found for the pK_a of haem propionate 7 of ferricytochrome c_{551} (Leitch *et al.*, 1984). Thus although the protonated carboxy group has not been observed by i.r., the coincidence of the expected and observed pK_a values provides confidence that the ionization of haem propionate-7 is seen in this experiment. It is notable that, as the pH^* difference is increased, the amide I band is perturbed. This is considered further below.

pH-dependence of i.r. difference spectrum of native mitochondrial cytochrome *c*

The pH^* -dependence of the i.r. difference spectrum of tuna ferricytochrome *c* is shown in Fig. 3. The feature at 1565 cm^{-1} is indicative of carboxylate anion formation. As with cytochrome c_{551} (Fig. 1) a corresponding pH^* -dependent absorption at 1710 cm^{-1} , arising from the loss of the carboxylic acid species, is not observed. The absorbance of the difference band at 1565 cm^{-1} increases with increasing pH^* and gives a good fit to a curve calculated for an ionization with a pK^* of 9.35 (Fig. 4). This value agrees well with previous measurements of the alkaline isomerization of cytochrome *c* (see the Introduction).

The i.r. spectrum of tuna ferrocycytochrome *c* over the range $1500\text{--}1700 \text{ cm}^{-1}$ is independent of pH^* over the pH^* range 6.9–12.9.

Spectroscopic studies of horse *N*-trifluoroacetyl-lysine ferricytochrome *c*

As first reported by Stellwagen *et al.* (1975) the optical spectrum of trifluoroacetylated ferricytochrome *c* was found to show a pH-dependence that is consistent with the ionization of the protein causing a transition from a low-spin iron electronic state to a high-spin state. The change in absorbance at 605 nm is consistent with a pK_a of 10.2 for this transition (Fig. 5).

The optical spectrum at pH values > 11 was time-dependent. A slow decrease in absorbance at 605 nm occurred; after 16 min, the absorbance at 605 nm decreased from 1.306 to 0.270 for a $6 \times 10^{-5} \text{ M}$ solution. To minimize the effects of this, all sample manipulations were carried out rapidly, once the pH or pH^* value was adjusted, with freshly prepared solutions. The measured pK of 10.2 agrees well with the value of 9.9 reported by Stellwagen *et al.* (1975).

Fig. 2. Ionization of a carboxy group of *P. aeruginosa* ferricytochrome c_{551} monitored by i.r. difference spectroscopy

Y represents the fraction of ionized carboxy group determined from the absorbance difference, integrated between 1552 cm^{-1} and 1592 cm^{-1} , relative to $\text{pH}^* 5.0$. The continuous line is a theoretical curve calculated for a pK^* of 6.14 ± 0.1 . (Other details are as in Fig. 1 and the Materials and methods section.)

Fig. 4. Ionization of a carboxy group of tuna ferricytochrome c monitored by i.r. difference spectroscopy

Y represents the fraction of ionized carboxy group determined from the absorbance difference, integrated between 1550 and 1593 cm^{-1} , relative to $\text{pH}^* 8$. The continuous line is a theoretical curve calculated for a pK^* of 9.35 ± 0.07 .

Fig. 3. FT i.r. difference spectra of (a) tuna ferricytochrome c (44 mg/ml in $^2\text{H}_2\text{O}$) and (b) horse trifluoroacetylated ferricytochrome c (28 mg/ml in $^2\text{H}_2\text{O}$)

(a) —, $\text{pH}^* 8.8-7.6$; -----, $\text{pH}^* 12.1-7.6$. (b) —, $\text{pH}^* 8.7-8.0$; -----, $\text{pH}^* 11.3-8.0$.

Fig. 5. Alkaline isomerization of trifluoroacetylated ferricytochrome c monitored by the A_{605}

The continuous line is a theoretical curve calculated for a pK of 10.2.

The time-dependent absorbance change is consistent with the high-spin protein reverting to a low-spin form, perhaps due to autoreduction of the iron and subsequent ligation by methionine, or to hydrolysis of substituted lysine residues (Fanger & Harbury, 1965) followed by lysine ligation.

The i.r. difference spectra of trifluoroacetylated ferricytochrome c obtained on variation of pH^* at

Fig. 6. Ionization of a carboxy group of trifluoroacetylated ferricytochrome *c* monitored by i.r. difference spectroscopy

Y represents the fraction of ionized carboxy group determined from the absorbance difference, integrated between 1552 and 1592 cm^{-1} , relative to pH^* 7.6. The continuous line is a theoretical curve calculated for a pK^* of 10.36 ± 0.15 .

alkaline pH^* values are similar to the analogous spectra of native ferricytochrome *c*, but shifted to higher pH^* (Fig. 3). A plot of *Y* determined from the integrated intensity between 1552 and 1592 cm^{-1} gives a pK^* of 10.36 (Fig. 6). This agrees well with the pK obtained for the optical titration (Fig. 5).

I.r. difference spectrum between native and cyanoferricytochrome *c*

The i.r. difference spectrum between horse ferricytochrome *c* and horse cyanoferricytochrome *c* at pH^* 7 does not contain a feature around 1560 cm^{-1} .

DISCUSSION

Assignment of the pH^* -induced i.r. difference spectra of mitochondrial ferricytochromes *c*

There are two features in the difference spectra of mitochondrial ferricytochrome *c* to be assigned: the change in the region 1620–1680 cm^{-1} and the change at $\sim 1570 \text{ cm}^{-1}$ (Fig. 3).

The region 1620–1680 cm^{-1} is the amide I region which contains contributions from the amide carbonyl groups. The observed changes do not titrate normally for a simple ionization, and therefore they probably arise, at least in part, from perturbations of the amide I band. The large absorbance of this band in the raw spectra makes it difficult to completely remove by difference; for example, the absorbance in the raw spectra used to generate Fig. 3 is 1.07, whereas the absorbance on the difference spectra themselves is < 0.02 .

The pH^* -dependent band at $\sim 1570 \text{ cm}^{-1}$ titrates

normally for a one-proton ionization (Figs. 4 and 6). The observed pK^* values, 9.35 for the native protein and 10.36 for the trifluoroacetylated protein, are coincident with the pK values of the alkaline isomerization, which involves a significant conformation change (Davis *et al.*, 1974). The question arises as to whether the i.r. difference at $\sim 1570 \text{ cm}^{-1}$ is a reflection of the conformation change or of the ionization itself. Cyanide binding to ferricytochrome *c* causes a conformation change similar to that produced by high pH (Sutin & Yandall, 1972; Moore *et al.*, 1982), and since the i.r. difference spectrum resulting from cyanide binding does not contain a feature at $\sim 1570 \text{ cm}^{-1}$, we assign this band to the ionizing group. This is supported by the difference spectrum of ferricytochrome c_{551} (Fig. 1), which contains a similar band that appears at lower values of pH^* . This protein does not undergo a significant conformation change over the appropriate pH range (Leitch *et al.*, 1984).

Although proteins contain various types of ionizable groups we attribute the band at $\sim 1570 \text{ cm}^{-1}$ to the asymmetric carbonyl stretch of a carboxylate anion. The chief reasons for this are as follows.

1. There is a haem propionic acid substituent of mitochondrial cytochrome *c* with a $\text{pK} > 9$ (Hartshorn & Moore, 1989).

2. A haem propionic acid substituent of *P. aeruginosa* ferricytochrome c_{551} has been shown by n.m.r. measurements to titrate with a pK^* of 6.2, the same as the 1570 cm^{-1} band in i.r. difference spectra of this protein (Fig. 2).

3. Carboxylate anions normally have i.r. absorbances at about 1570 cm^{-1} (Table 1).

However, owing to our failure to observe a pH-dependent feature near 1710 cm^{-1} in the spectra of cytochromes, which would arise from the un-ionized form of the carboxylic acid, the assignment of the pH^* -dependent feature at 1570 cm^{-1} to carboxy-group ionization is less secure than is desirable. In order to investigate why the un-ionized form of the cytochrome carboxylic acid does not have an absorbance near 1710 cm^{-1} , we have determined the i.r. spectra of a range of carboxylic-acid-containing compounds in the neutral and anionic forms (Table 1). The data of Table 1 are consistent with previous i.r. studies of carboxylic acids (Flett, 1951; Nakamoto *et al.*, 1963; Sawyer & Tackett, 1963; Dunn & McDonald, 1969).

In the case of lysine it is seen that the frequency-dependence of the carboxy group passes through two stages as the molecule is progressively deprotonated. In this relatively simple molecule the protonated carboxy group is seen at 1727 cm^{-1} , an unusually high frequency (cf. acetic acid in $^2\text{H}_2\text{O}$ at 1710 cm^{-1}) owing to the proximity of positive charge. For a similar reason the carboxylate anion has the unusually high frequency of 1614 cm^{-1} in the zwitterion (cf. acetate in $^2\text{H}_2\text{O}$ at 1561 cm^{-1}). As the pH^* is increased such that the positive charges on the molecule are neutralized, this band shifts to 1572 cm^{-1} . It is thus clear that neighbouring groups can have a dramatic effect upon carboxy-group frequencies.

The effect of neighbouring positive charge on carboxylic acid-carbonyl stretching frequencies has also been examined by Sawyer & Tackett (1963) and Nakamoto *et al.* (1963). These authors have studied the i.r. spectra in aqueous solutions of EDTA and have shown that the carboxy-carbonyl stretch has a frequency

of 1730–1750 cm^{-1} when the adjacent nitrogen atom is protonated. On deprotonation of the nitrogen atom the frequency assumes the 'normal' value of 1710 cm^{-1} . Similarly the carboxylate–carbonyl stretch is seen at 1620 cm^{-1} in the presence of adjacent positively charged nitrogen; this moves to $\sim 1570 \text{ cm}^{-1}$ on deprotonation of the nitrogen atom.

The most important influence on the cytochrome carboxy group is likely to be hydrogen-bonding involving neighbouring hydroxy groups and amides. (Only one propionic acid of *P. aeruginosa* cytochrome c_{551} , and one of horse cytochrome, have short-range interactions with positively charged groups.) The model compounds show that hydrogen-bonding can lead to a decrease in the carbonyl frequency, ν_{COOH} (Table 1). This is clearly demonstrated by the hydroxybenzoic acids in dioxan. The electronic effects of *o* and *p* substitution are similar, so the difference in ν_{COOH} is probably due to intramolecular hydrogen-bonding in *o*-hydroxybenzoic acid (Flett, 1951). Similarly the difference between fumaric and maleic acids is probably due largely to intramolecular hydrogen-bonding in fumaric acid.

In circumstances where the protonated carboxy group can act as a strong hydrogen-bond donor, it is predicted that the carbonyl frequency should decrease and, assuming the carboxylate group can also hydrogen-bond strongly, ν_{COO^-} should increase (Koeppel & Stroud, 1976).

If the frequency shifts observed for the neutral forms of the carboxylates in the model compounds, under conditions when hydrogen-bonding may occur, were repeated with the cytochromes, the absorbance of the neutral groups corresponding to the 1570 cm^{-1} absorbance of the anionic species would be shifted into the region of the amide I band. Thus our failure to observe pH*-dependent bands at about 1710 cm^{-1} for the cytochromes may be due to strong hydrogen-bonding between the COOH group and group(s) in the protein. In support of this hypothesis, X-ray structures show that the haem propionates interact with a variety of polar groups in the protein (Takano & Dickerson, 1981; Matsuura *et al.*, 1982).

In view of the foregoing it is surprising that the frequencies of the carboxylate anions are observed at the normal position of $\sim 1570 \text{ cm}^{-1}$. However, the model compounds indicate that this stretching vibration is not as sensitive to neighbouring-group effects as the vibration of the corresponding protonated species (Table 1).

Another possible explanation for the appearance at high pH* of the 1570 cm^{-1} band in difference spectra of mitochondrial cytochromes is that it arises from an amino acid carboxylate anion which normally interacts with a lysine residue and which is perturbed when the lysine residue is neutralized. Thus the band could be the result of a frequency shift rather than a generation of carboxylate anion absorbance *de novo*. In the presence of charged lysine the frequency is likely to be such that the band would be obscured by the amide I band. The experiment with trifluoroacetylated cytochrome *c* was carried out to investigate this possibility.

Trifluoroacetylation of lysine neutralizes its positive charge so that the surface carboxylate groups will not be involved in short-range charge neutralization interactions with protein side chains. The modification does not significantly perturb the structure, as judged by spectroscopic methods (Stellwagen *et al.*, 1975; Boswell *et al.*,

1983) or particular functional properties, such as the redox potential (Holwerda *et al.*, 1978). The similarity in i.r. difference spectra between the native and modified proteins (Fig. 3) supports the view that the appearance of the 1570 cm^{-1} band is due to carboxylic acid ionization.

Alkaline isomerization of ferricytochrome *c*

Horse ferricytochrome *c* undergoes a transition to a low-spin globular form at alkaline pH. This transition involves the ionization of a group with a pK_a of 11 coupled to a slow conformational change with a pK_c of -2 according to the scheme (Davis *et al.*, 1974):

where Cyt^{3+} is the normal conformer of ferricytochrome *c* and ${}^*\text{Cyt}^{3+}$ is the alkaline conformer. The conformational change involves the displacement of the methionine ligand and its replacement by another strong field ligand, possibly lysine (Gadsby *et al.*, 1987). This conformational change displaces the equilibrium to give an overall apparent pK of ~ 9 when measured by equilibrium methods (Brandt *et al.*, 1966; Davis *et al.*, 1974). Other mitochondrial cytochromes undergo a similar transition with an apparent pK in the range 7.5–9.0. Bacterial cytochromes c_2 also isomerize at alkaline pH, with apparent pK values in the range 7.5–9.0 (Pettigrew *et al.*, 1978), but the alkaline denaturation of *P. aeruginosa* ferricytochrome c_{551} appears to take a different course. In this case the methionine ligand is lost with a $\text{pK} > 11$, in a transition that does not involve a slow conformational change (Vinogradov, 1970; G. R. Moore, unpublished work).

The nature of the ionizing group that triggers the transition of mitochondrial cytochrome *c* has not been established. A variety of groups have been proposed to fulfil this role, including the histidine ligand (Gadsby *et al.*, 1987), buried water (Takano & Dickerson, 1981) and a lysine or tyrosine residue (Davis *et al.*, 1974). The data presented here strongly suggest that a group not previously considered, namely a haem propionic acid, is the triggering group. If our assignment of the 1570 cm^{-1} band in the FT i.r. spectra of tuna ferricytochrome *c* is correct, then the observed pK^* of this acid (Fig. 4) is the same as the pK of the alkaline transition. The increased pK^* of the trifluoroacetylated protein (Fig. 6) is consistent with this proposal, because in this protein the pK of the alkaline transition is also increased (Fig. 5), perhaps because of a change in pK_c .

This proposal is consistent with the observation that cytochrome c_{551} does not undergo an analogous transition because other than the well-characterized ionization of haem propionate-7, which does not cause a significant conformational change, the i.r. studies failed to identify a carboxylic acid ionization over a wide pH* range.

pK_a values of haem propionic acid substituents of mitochondrial cytochrome *c*

The i.r. studies are consistent with the denaturation-induced proton-uptake experiments of the preceding paper (Hartshorn & Moore, 1989) in identifying only one haem propionate pK at high pH.

If the ionization of this group does trigger the conformational change of ferricytochrome *c*, then its pK_a of 11 is one of the highest measured for a carboxylic acid in a protein. The corresponding pK_a of ferrocyclochrome *c*

appears to be even higher, since no i.r. spectral features recognizable as those from a titrating carboxylate were observed for this protein over the pH* range 6.0–12.9. The alternative explanation, that the corresponding acid has a low pK_a in ferrocycytochrome *c*, is inconsistent with the reported pH-dependence of the redox potential (Brandt *et al.*, 1966; Pettigrew *et al.*, 1978).

An increase in the pK_a of the haem propionic acid on reduction of the iron is expected because of the removal of the electrostatic influence of the ferrihaem charge, which stabilizes the anionic form of the acid (Leitch *et al.*, 1984). On the basis of work with cytochrome *c*₅₅₁ (Rogers *et al.*, 1985) we estimate that the haem propionate pK_a of ferrocycytochrome *c* should be at least one pH unit higher than the corresponding pK_a of ferricytochrome *c*.

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