Biochem. J. (1989) 258, 595–598 (Printed in Great Britain)

A denaturation-induced proton-uptake study of horse ferricytochrome *c*

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The observation that 6 M-urea denatures horse ferricytochrome *c* in the pH range 4–6, but not horse ferrocytochrome *c*, has been exploited to determine the denaturation-induced proton uptake of ferricytochrome *c*. This is related to the pK_a values of ionizable groups buried within the native protein. The data indicate that one of the haem propionic acid substituents of ferricytochrome *c* has a pK_a of < 4.5, whereas the other has a pK_a of > 9.

INTRODUCTION

Mitochondrial cytochrome *c* is a monohaem protein of 103–113 amino acids. *X*-ray structures of the cytochromes from tuna (Takano & Dickerson, 1981), bonito (Tanaka *et al.*, 1975), rice (Ochi *et al.*, 1983) and yeast (Louie *et al.*, 1987) reveal a common structure in which the haem is completely enfolded by the polypeptide with the haem propionic acid substituents shielded from the bulk solvent. The ionization state of these acids in the native protein has not been firmly established, and this was the aim of the present study.

^{1}H-n.m.r. (Ångström *et al.*, 1982) and FT i.r. [the following paper (Tonge *et al.*, 1989)] studies demonstrate that the haem propionic acids do not ionize over the pH range 4.5–9.4. Outside this range, ferricytochrome *c* denatures (Theorell & Åkesson, 1941), and so spectroscopic methods are not a definitive approach. A wide range of other methods, both theoretical and experimental, have been used to investigate this problem, with conflicting results. Thus it has been claimed that both propionic acid substituents have high pK_a values (Koppenol & Margoliash, 1982), both have low pK_a values (Paleus, 1954; Marini *et al.*, 1981) and one has a high pK_a and the other a low pK_a (Moore, 1983; Moore *et al.*, 1984*b*). It has even been suggested that the pK_a of one of the acids depends upon whether the protein is titrated from acid pH to alkaline pH or vice versa (Shaw & Hartzell, 1976).

The method reported in the present paper is a modified form of the experiment used by Fersht & Sperling (1973) to determine the ionization state of Asp-102 of chymotrypsin. Namely, the proton uptake of ferricytochrome *c* as it unwinds is taken to be an indicator of the ionization state of buried groups. The results indicate that one of the haem propionic acids has a low pK_a and the other a high pK_a.

MATERIALS AND METHODS

Horse (type VI) cytochrome *c* was obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.) and treated before use as described by Eley *et al.* (1982). All other reagents were obtained from BDH Limited (Poole, Dorset, U.K.) or the Sigma Chemical Co. and were of the highest grade obtainable.

N.m.r. spectra were measured with a Bruker WH-300 spectrometer, and fluorescence spectra with an Aminco–Bowman spectrofluorimeter. Fluorescence spectra of cytochrome *c* were obtained as described by Tsong (1974).

pH titrations of the n.m.r. spectra of aspartate, glutamate, *N*-acetyl-lysine and histidine were carried out in the presence or absence of 8 M-urea. The measured p*K* values are given in Table 2 (below).

Determination of the proton uptake of horse ferricytochrome *c* on denaturation at 21 °C

Within the pH range 4–6, 1 ml samples of a 10^{-2} M solution of ferricytochrome *c* were mixed with 4 ml of water and the pH adjusted to the required value with concentrated NaOH or HCl. The pH of a 15 ml sample of 8 M-urea was adjusted to the same value and the urea solution mixed with the appropriate cytochrome solution. The final concentration of the protein was 5×10^{-4} M with 6 M-urea. The mixture was stirred thoroughly and the pH change noted. The pH was then restored to its initial value by the addition of known volumes of standardized HCl or NaOH. Within the pH range 6–8, a similar procedure was carried out to yield mixtures of 5×10^{-4} M-ferricytochrome *c* and 8 M-urea.

Control experiments were carried out with ferrocytochrome *c* in a manner similar to that described above. Before the initial pH adjustment of the ferricytochrome solution, a reducing agent was added under argon. All subsequent operations with the ferrocytochrome were carried out under argon. Both solid sodium dithionite and a 0.4 M solution of ascorbic acid were used to reduce ferricytochrome *c*. When ascorbic acid was used, a stoichiometric amount was added to avoid complications from the titration of the excess acid. Ascorbic acid was used in the pH range 4–6. The difference in titration between the ferric and ferrous proteins directly measures the proton uptake on denaturation of the ferricytochrome *c* (see the Results and discussion for a justification of this). The results are presented in Fig. 1 (below).

Abbreviation used: FT, Fourier transform.
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RESULTS AND DISCUSSION

Spectroscopic indicators of urea-induced denaturation

^{1}H n.m.r. spectra (not shown) of 5×10^{-4} M-cytochrome *c* at pH 4 clearly demonstrate that 6 M-urea unfolds ferricytochrome *c* but does not significantly affect ferrocytochrome *c*. The spectrum of ferricytochrome *c* in 6 M-urea resembles the simulated random-coil spectrum (Moore *et al.*, 1984*a*). At pH 7, n.m.r. spectra indicate that ferricytochrome *c* is not completely unfolded by 8 M-urea.

Fluorescence measurements of 10^{-6} M-cytochrome *c* indicates that urea denatures ferricytochrome *c* to a greater extent than ferrocytochrome *c* (Table 1). The relative fluorescence intensity of Trp-59 at pH 7 is similar to that reported by other workers, who have also used viscosity measurements and c.d. spectra to show the protein is largely unfolded under these conditions. Fluorescence of ferrocytochrome *c* was much less affected by the presence of urea, consistent with its structure remaining largely intact. The relative fluorescence intensity of Trp-59 at pH 8 suggests that some denaturation occurs, but considerably less than for ferricytochrome *c*.

Table 1. Relative fluorescence intensities* of horse cytochrome *c* at 25 °C

Conditions	Relative intensity	Other reported values
Ferricytochrome		
pH 4 (acetate buffer)	5.0	
+6 M-urea	70.0	
pH 6 (phosphate buffer)	4.0	
+6 M-urea	59.5	
pH 7 (phosphate buffer)		
+8 M-urea	64.3	
+6 M-urea (Tris/HCl)		58 (Myer *et al.*, 1979)
(+9 M-urea)		65 (Tsong, 1974)
pH 8 (phosphate buffer)		
+8 M-urea	71.0	
pH 9.2 (borate buffer)		
+8 M-urea	29.2	
Ferrocytochrome		
pH 4 (acetate buffer)	3.5	
+6 M-urea	5.2	
pH 6 (phosphate buffer)		
+6 M-urea	9.7	
pH 8 (phosphate buffer)		
+8 M-urea	16.0	

* Relative to the fluorescence intensity of 2×10^{-6} M free tryptophan. The fluorescence was measured at 350 nm after excitation at 280 nm.

Table 2. p*K* values for amino acid side chains at 25 °C

Amino acid	p*K* in water	p*K* in 8 M-urea
Glutamic acid	4.07	4.58
Aspartic acid	3.90	4.12
Histidine	6.04	6.50
Lysine	10.79	10.50

^{1}H n.m.r. spectra of cytochrome *c* over a range of protein concentrations, and at constant pH and urea concentration, show that denaturation of ferricytochrome *c* is suppressed as the protein concentration is increased (A. P. Boswell & G. R. Moore, unpublished work). Therefore the n.m.r. experiments probably give a truer indication of the extent of denaturation in the proton-uptake experiments.

Denaturation-induced proton uptake of horse ferricytochrome *c*

Titration curves of ferricytochrome *c* and ferrocytochrome *c* in urea are shown in Fig. 1 as a plot of relative proton uptake (mol of H^+ added/mol of protein), ΔH^+_o and ΔH^+_r, against pH. The curves reflect the change in p*K* values for the ionizable groups on going from H_2O to 6 M- or 8 M-urea in water. The p*K* values of aspartate, glutamate, histidine and *N*-acetyl-lysine are perturbed by urea (Table 2).

Titration curves of cytochrome *c* in water have been reported by several workers, and theoretical fits to the experimental data have been made (Paleus, 1954; Shaw & Hartzell, 1976; Marini *et al.*, 1981). However, different workers produce fits with different parameters, and the general procedures for analysing such data to yield the number of ionizable groups and their p*K* values appear to be unsatisfactory. Therefore these methods were not adopted for the present analysis.

The titration data in the denaturation experiment are presented as the difference, $\Delta H^+_o - \Delta H^+_r$, plotted against pH in Fig. 1. This procedure, which eliminates systematic errors and simplifies the analysis, assumes that ferrocytochrome *c* is not denatured under the conditions of the experiment and that the ionizable groups on the surface of the protein have similar p*K* values in the native forms and denatured form. The difference, $\Delta H^+_o - \Delta H^+_r$, should then reflect the ionizations of groups which have abnormal pK_a values in ferricytochrome *c*.

There are three groups expected to contribute to the difference curve: His-26, which has a p*K* of < 3.2 in native cytochrome *c* (Cohen *et al.*, 1974; Moore & Williams, 1980), and the two haem propionic acid substituents which do not ionize in the native protein over the pH range 4.5–9.4 (Gupta & Koenig, 1971; Ångstrom *et al.*, 1982). These p*K* indications were obtained from n.m.r. studies and from redox-potential measurements as a function of pH (Moore *et al.*, 1984*b*). All of the lysine p*K* values are > 9 (Boshard & Zürrer, 1980), and there is no indication that the amino acid carboxylates have anomalous p*K* values. All of these groups are on the protein surface exposed to solvent (Takano & Dickerson, 1981) and therefore are expected to have normal p*K* values. Fitting of pH titration curves of ferricytochrome *c* are consistent with this because, despite the differences referred to above, all of the reported data show that the number of ionizable groups at pH < 5 are greater than the number of amino acid carboxylates (Paleus, 1954; Shaw & Hartzell, 1976; Marini *et al.*, 1981). Therefore the assumption in the present paper that only His-26 and the haem propionic acid substituents have anomalous p*K* values that will be substantially affected by denaturing ferricytochrome *c* has support from a variety of studies.

A difference curve adjusted for the ionization of His-26 (Fig. 1) was obtained assuming His-26 has a p*K* of 3 in the native protein and 6.5 in the urea-denatured

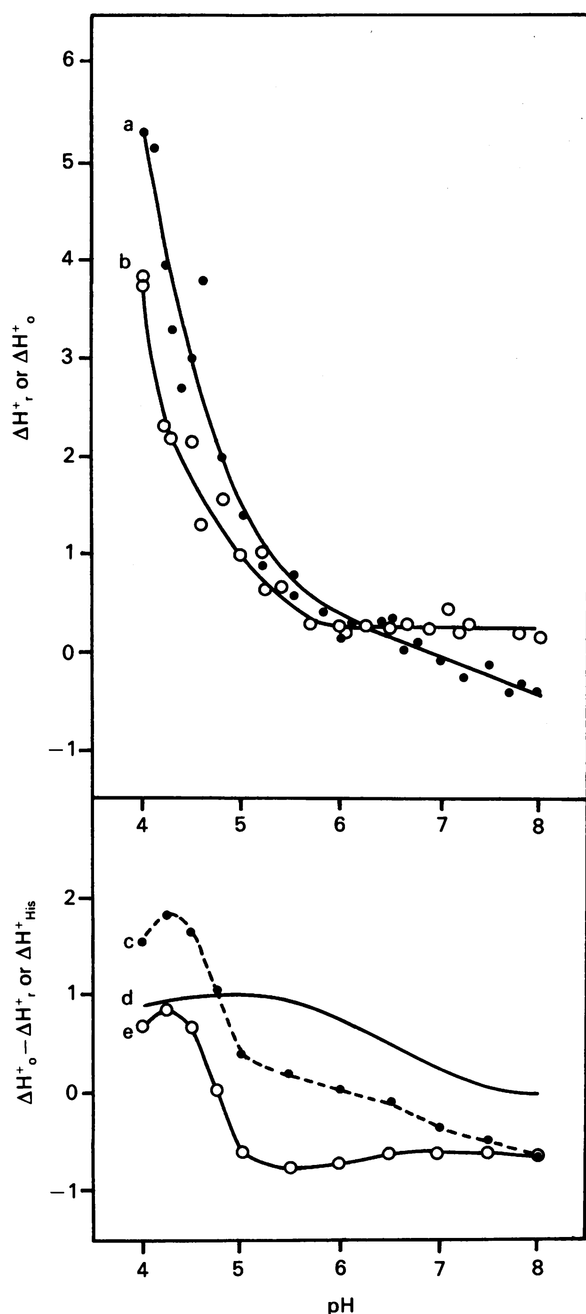

Fig. 1. Denaturation-induced proton uptake of cytochrome *c*

Curve a, proton uptake of ferricytochrome *c* (ΔH^+_o, ●); curve b, proton uptake of ferrocytochrome *c* (ΔH^+_r, ○); curve c, difference $\Delta H^+_o - \Delta H^+_r$ (------); curve d, calculated contribution to ΔH^+_o from His-26 (ΔH^+_{His}) assuming its pK_a in the native protein (pK_o) is 3 and in the urea-denatured protein is 6.5 (——) (taking $pK_o < 3$ does not significantly affect the curve); curve e, the difference ($\Delta H^+_o - \Delta H^+_r$) corrected for the change in protonation of His-26 (○).

protein (Table 2). This curve forms the basis of the following analysis of the ionization states of the haem propionic acid substituents in native cytochrome *c*.

Three simulated curves based on different models for the haem propionate ionizations are given in Fig. 2, together with the observed difference curve modified for

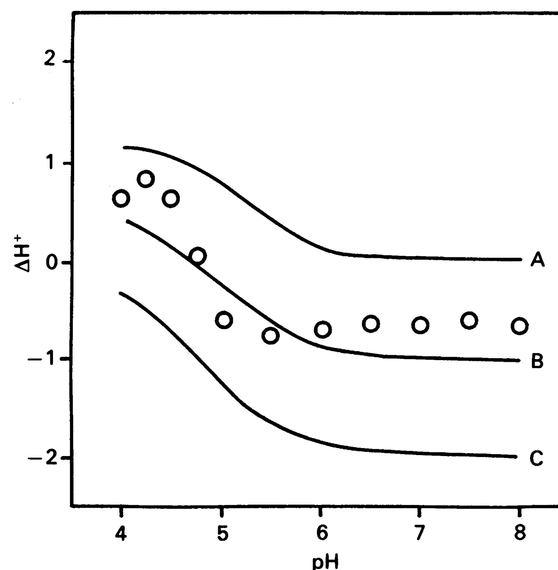

Fig. 2. Calculated denaturation-induced proton-uptake curves for horse ferricytochrome *c*

These curves reflect only the protonation states of haem propionates-6 and -7. In the following, pK_o represents the pK_a of the group in native ferricytochrome *c* and pK^i the corresponding pK_a of the urea-denatured protein. Curve A, one haem propionate with $pK_o = 3.5$ and $pK^i = 4.5$, the other with $pK_o = 3.5$ and $pK^i = 5.0$; curve B, one haem propionate with $pK_o = 3.5$ and $pK^i = 4.5$, the other with $pK_o = 9.4$ and $pK^i = 5.0$; curve C, one haem propionate with $pK_o = 9.4$ and $pK^i = 4.5$, the other with $pK_o = 9.4$ and $pK^i = 5.0$; the open circles are the points from curve e in Fig. 1.

the ionization of His-26. In all three calculated curves the pK_a values of the haem propionic acids of denatured cytochrome *c* have been taken to be 4.5 and 5.0. These values have not been determined experimentally; they have been obtained from theoretical considerations and from measurements on related systems (Phillips, 1960). For the purpose of comparing the experimental and theoretical curves, it is useful to consider the pH ranges 4–6 and 6–8 separately.

Over the pH range 4–6 there is reasonable agreement between model B (one propionate with a pK of > 9 and the other with a pK of < 3.5) and the experimental data. Model A (both propionates have low pK values) and model C (both propionates have high pK values) do not fit the experimental data at any pH. Over this pH range the ferricytochrome *c* is fully denatured by urea, whereas the ferrocytochrome *c* is largely unaffected by it; thus the main condition for the experiment to be valid is met.

Over the pH range 6–8 none of the theoretical models fit the data (Fig. 2). The indication from n.m.r. that the ferric protein is not completely denatured by 8 M-urea over this pH range suggests that $\Delta H^+_o - \Delta H^+_r$ is less than expected. Taking this into account the data are more consistent with model B than with either of models A or C.

An improved fit between model B and the experimental data could be obtained by taking different values for the intrinsic haem propionic acid pK_a values and the cytochrome *c* haem propionic low pK_a. However, this would not be justified without additional supporting data.

Haem propionic acid pK_a values

The data reported in the present paper support a model in which one haem propionic acid substituent has a pK_a of < 4.5 and the other has a pK_a of > 9. The *X*-ray structures show both substituents are involved in an extensive series of contacts with neighbouring polar groups, including, in the case of haem propionate-7, Arg-38. Haem propionate-6 does not have a short-range interaction with a positively charged group and therefore substituent-7 appears to be the most likely to have a low pK_a.

The possible importance of the haem propionic acid ionization states for the redox-linked conformation change of cytochrome *c* have been discussed by Moore (1983) and for the alkaline isomerization of ferricytochrome *c* by Tonge *et al.* (1989) (the following paper).

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Received 20 June 1988/19 September 1988; accepted 3 October 1988

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