Biochem. J. (1983) **213**, 679–686
Printed in Great Britain

Ionization of tyrosine and lysine residues in native and modified horse cytochrome *c*

Andrew P. BOSWELL,* Geoffrey R. MOORE,*‖ Robert J. P. WILLIAMS,* David E. HARRIS,†¶ Carmichael J. A. WALLACE,‡ Stephen BOCIECK§ and David WELTI§

**Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K.,*
†*Department of Molecular Biophysics, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.,*
‡*Department de Biochimie Medicale, Université de Génève, 9 Rue Michel Server, 1211 Génève 4, Switzerland, and* §*Unilever Research, Colworth Laboratory, Unilever Ltd., Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.*

(*Received 2 March 1983/Accepted 19 May 1983*)

^{1}H-n.m.r. and ^{13}C-n.m.r. spectroscopy of horse cytochrome *c* and ^{1}H-n.m.r. spectroscopy of the lysine-modified proteins N^{ϵ}-acetimidyl-, N^{ϵ}-amidino-, N^{ϵ}-trifluoroacetyl- and N^{ϵ}-maleyl-cytochrome *c* have shown that, although the lysine modifications do not greatly perturb the protein structure at pH 7 and 27°C, at higher temperature or at alkaline pH some parts of the structure are markedly perturbed. At pH 7 and 27°C the region of the protein about Ile-57 is affected in all the modified proteins, though not all to the same degree. N^{ϵ}-Maleylation most seriously affects the protein structure, and the fully maleylated protein is readily unfolded. At 27°C all four of the tyrosine residues of native horse cytochrome *c* have pK_a values above 11, but in N^{ϵ}-acetimidyl-cytochrome *c* the pK_a of one tyrosine residue is 10.2.

The amino acid sequence of horse cytochrome *c* contains four tyrosine residues and 19 lysine residues (Margoliash *et al.*, 1961), many of which are conserved throughout a wide range of eukaryotic cytochromes *c* (Dayhoff, 1968). The tyrosine residues play important roles in stabilizing the native conformation of cytochrome *c* (Sokolovsky *et al.*, 1970; Pal *et al.*, 1975; Dickerson & Timkovich, 1975; Boon, 1981), and where they are replaced it is always by phenylalanine (Dayhoff, 1968). Some of the lysine residues play important roles in binding the physiological reaction partners of cytochrome *c* (Smith *et al.*, 1977; Ferguson-Miller *et al.*, 1978; Pettigrew, 1978; Rieder & Bosshard, 1980), and some are important to the stability of the protein (Smith & Millett, 1980; Osheroff *et al.*, 1980; Falk *et al.*, 1981). In the present paper we report a ^{13}C-n.m.r. study of the tyrosine residues of horse ferrocytochrome *c* and a ^{1}H-n.m.r. study of derivatives of horse cytochrome *c* in which some or all of the lysine residues have been modified. The proteins studied are, with the approximate total charge of each ferrocytochrome *c* at pH 7 indicated in parentheses: native horse cytochrome *c* (+8); N^{ϵ}-amidino-cytochrome *c* (+8); N^{ϵ}-acetimidyl-cytochrome *c* (+8); N^{ϵ}-trifluoroacetyl-cytochrome *c* (−11); N^{ϵ}-maleyl-cytochrome *c* (−30); N^{ϵ}-acetimidyl-[Hse^{65}]cytochrome *c* (+8); (N^{ϵ}-acetimidyl-lysine)$_{11}$(1–65)-(lysine)$_8$(66–104)-[Hse^{65}]-cytochrome *c* (+8) [i.e. a molecule that has been reconstituted semi-synthetically from a fragment (1–65) in which the lysine residues have been modified and a fragment (66–104) in which they have not]; (lysine)$_{11}$-(1–65)-(N^{ϵ}-acetimidyl-lysine)$_8$-(66–104)-[Hse^{65}]cytochrome *c* (+8).

The paper describes structural perturbations accompanying modification of lysine residues, and shows how the ionization of certain groups affects the structure of ferrocytochrome *c* at alkaline values of pH.

Abbreviation used: Hse, homoserine.

‖ To whom correspondence should be addressed.

¶ Present address: Department of Chemistry, University of Indiana, Bloomington, IN 47405, U.S.A.

Materials and methods

All reagents used in this work, except those listed below, were obtained from BDH Chemicals (Poole, Dorset, U.K.) and were of the highest obtainable purity.

Horse heart cytochrome *c* (type III and type VI) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.); *O*-methylisourea hydrogen sulphate

was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.); *S*-ethyl trifluorothioacetate was from Eastman Chemical Co. (Kodak Ltd., Kirby, Liverpool, U.K.); O_2-free Ar was from Air Products (Bracknell, Berks., U.K.); ^2H_2O (99.8%) was from Merck, Sharpe and Dohme (Montreal, Que., Canada); NaO^2H (40% in ^2H_2O, isotopic purity 99.0%) and ^2HCl (35% in ^2H_2O, isotopic purity 99.6%) were from CIBA (Duxford, Cambridge, U.K.).

N^{ϵ}-Acetimidyl-cytochrome *c* was prepared by the reaction of methyl acetimidate hydrochloride with cytochrome *c* (type III) by the method of Wallace & Offord (1979). The modified protein was filtered on a column (1.5cm×30cm) of Sephadex G-50 (fine grade) in 40mM-potassium phosphate buffer, pH 7.2, and purified by cation-exchange chromatography with SP-(sulphopropyl-)Sephadex C-25 to remove the side products of the acetimidation (Di Marchi *et al.*, 1978). The N^{ϵ}-acetimidyl-lysine content of the purified product was shown by time-course hydrolysis and extrapolation to zero time to be >98% of the expected value. The N^{ϵ}-acetimidyl derivatives of [Hse^{65}]cytochrome *c* were prepared by the method of Wallace & Offord (1979). N^{ϵ}-Amidino-cytochrome *c* was prepared in accordance with published procedures (Hettinger & Harbury, 1964; Stellwagen *et al.*, 1977) by the reaction of *O*-methylisourea hydrogen sulphate with cytochrome *c* (type III). The modified protein was purified in the same manner as N^{ϵ}-acetimidyl-cytochrome *c*. Amino acid analysis of the major product indicated that the reaction was >98% complete. N^{ϵ}-Trifluoroacetyl-cytochrome *c* was prepared by the method of Fanger & Harbury (1965), and N^{ϵ}-maleyl-cytochrome *c* by the method of Pettigrew *et al.* (1976). Amino acid analysis of these derivatives after dinitrophenylation showed that the reaction had proceeded to >95% for trifluoroacetylation and to >99% for maleylation.

Samples of native cytochrome *c* (type VI) were prepared for n.m.r. as previously described (Eley *et al.*, 1982). Samples of the modified proteins were prepared for n.m.r. by using an Amicon ultrafiltration cell fitted with a UM10 membrane. Four cycles of concentration and dilution with ^2H_2O at pH 7 were conducted. The final sample volumes were 0.4ml. The quoted pH values are uncorrected for the isotope effect (Kalinichenko, 1976).

The ^{1}H-n.m.r. spectra were obtained with a Bruker 270MHz spectrometer, and the ^{13}C-n.m.r. spectra were obtained with a Bruker WP-200 spectrometer. Resolution-enhanced n.m.r. spectra were obtained by using the convolution difference procedure (Campbell *et al.*, 1973). Chemical shifts are quoted in parts per million (p.p.m.) downfield from the methyl resonance of 2,2-dimethyl 2-silapentane-5-sulphonate.

Results and discussion

^{13}C-n.m.r. spectroscopy of horse ferrocytochrome c

The aromatic regions of ^{13}C-n.m.r. spectra of horse ferrocytochrome *c* in ^2H_2O at 30°C and at various pH values are given in Fig. 1. The marked similarity of the spectra agrees with previous work that shows ferrocytochrome *c* does not denature until about pH 12 (Theorell & Åkeson, 1941; Dickerson & Timkovich, 1975).

The resonances of major concern in the present work are the four peaks of one carbon intensity each marked 1 to 4. These arise from the C-4 carbon atoms of the four tyrosine residues (Oldfield & Allerhand, 1973; Oldfield *et al.*, 1975). Over the pH range 8.3–11.15 peaks 1, 3 and 4 are independent of pH, whereas peak 2 shifts downfield by 0.3 p.p.m.

The purpose of the ^{13}C-n.m.r. pH titration was to determine whether any of the tyrosine residues of ferrocytochrome *c* ionized with a pK_a below 11. It was necessary to confirm the spectrophotometric titration reported by Stellwagen (1964), which indicated that the tyrosine pK_a values are above 11, since Oldfield *et al.* (1975) implied that one of the tyrosine residues of ferrocytochrome *c* began to be

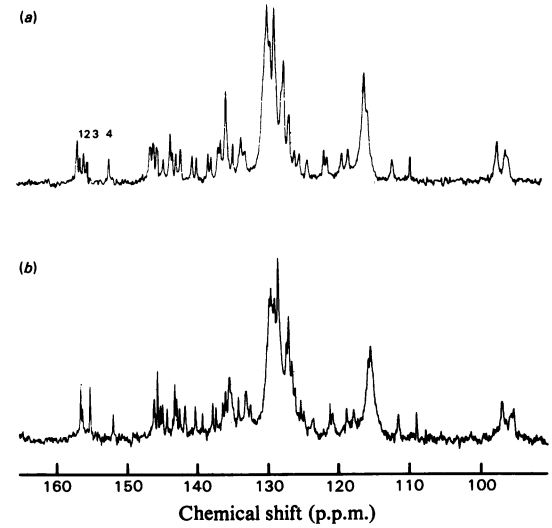

Fig. 1. *50.32MHz ^{13}C-n.m.r. spectra of horse ferrocytochrome c (10mM) in ^2H_2O at 30°C at (a) pH 11.15 and (b) pH 8.30*

The spectra were obtained with a 15mm probe requiring 5ml of sample. The spectra are proton-decoupled and are the sum of between 25000 and 30000 scans each. They were obtained with a pulse width of 20μs (90° pulse), an acquisition time of 0.27s and a recycle delay of 0.73s. The peaks marked 1 to 4 are from the C-4 carbon atoms of the four tyrosine residues.

deprotonated at pH approx. 9.4, and they did not extend their ^{13}C-n.m.r. pH titration beyond pH 9.4. Since on ionization the C-4 resonance of tyrosine shifts downfield by approx. 10 p.p.m. (Oldfield *et al.*, 1975), and since none of the peaks 1 to 4 exhibits such a shift (Fig. 1), it can be concluded that the four tyrosine residues of ferrocytochrome *c* have pK_a values higher than 11. This conclusion is in agreement with the work of Stellwagen (1964), and it is important for work described previously (Gupta & Koenig, 1971; Moore & Williams, 1980*c*) and investigated further in the present paper. Namely, what is the origin of the small pH-induced conformational change that affects a particular region of the surface of ferrocytochrome *c*? Clearly, the origin cannot be the ionization of a tyrosine residue.

^1H-n.m.r. spectroscopy of modified horse cytochrome c

Convolution difference ^1H-n.m.r. spectra of horse ferrocytochrome *c* and its N^{ϵ}-acetimidyl, N^{ϵ}-amidino, N^{ϵ}-trifluoroacetyl and N^{ϵ}-maleyl derivatives, all in ^2H_2O at pH 7 and 57°C, are given in Fig. 2. The resemblance between the spectra is striking. The small differences arise from the following two sources. (*a*) Additional non-exchangeable protons have been added to two of the modified cytochromes. These are the 19 N^{ϵ}-acetimidyl methyl groups and the 19 maleyl groups. They give rise to 19 singlet resonances between 2.0 p.p.m. and 2.3 p.p.m. (Fig. 2) and 38 one-proton doublet resonances between 5.5 p.p.m. and 6.5 p.p.m. (Fig. 2) respectively. In both cases all the protons have been accounted for in the n.m.r. spectra, confirming that the modifications have gone to completion. There is a main band of 13 resonances centred on the average chemical shift, with three resonances shifted upfield and three downfield of the central band. (*b*) Some resonances of native cytochrome *c* are shifted by the modifications. Among the largest shifts are those of the lysine ϵ-CH_2 resonances. These resonances overlap at approx. 3 p.p.m. in the spectrum of ferrocytochrome *c* and shift downfield on modification (Fig. 2). In each case the group of ϵ-CH_2 resonances cover a range of approx. 0.2 p.p.m. to 0.4 p.p.m. The shifts caused by modifying the lysine residues are also approx. 0.2 p.p.m. to 0.4 p.p.m. The other resonance to receive a large shift (varying from

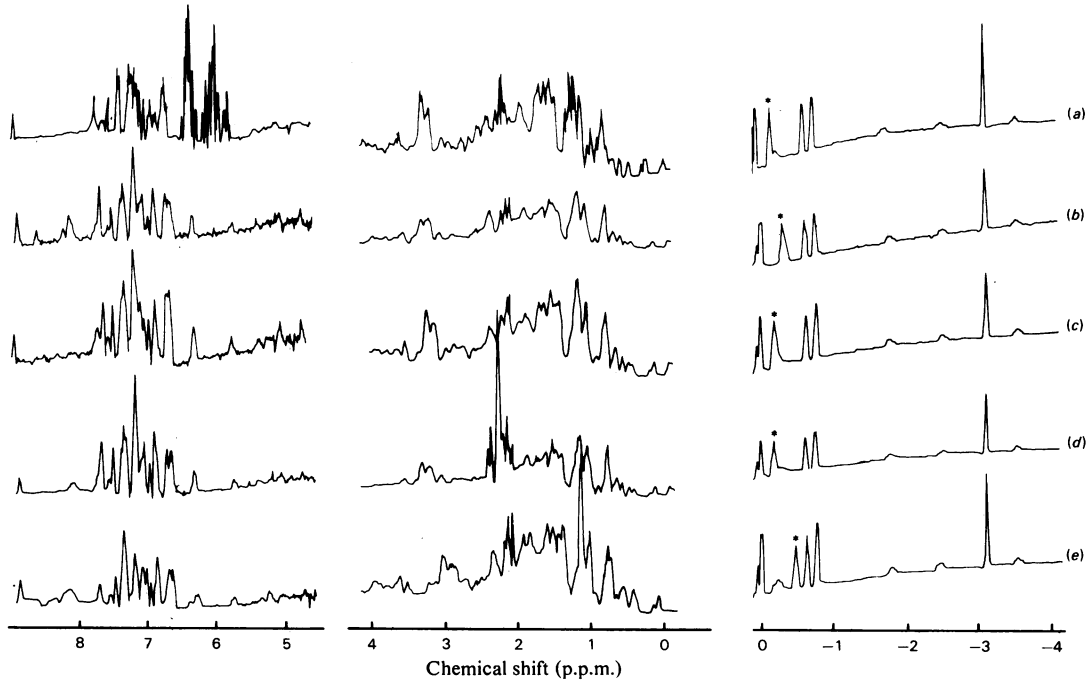

Fig. 2. *Convolution difference ^1H-n.m.r. spectra of (a) N^{ϵ}-maleyl-ferrocytochrome c, (b) N^{ϵ}-trifluoroacetyl-ferrocytochrome c, (c) N^{ϵ}-amidino-ferrocytochrome c, (d) N^{ϵ}-acetimidyl-ferrocytochrome c and (e) native horse ferrocytochrome c*

The spectra were obtained with samples of 3 mM protein in ^2H_2O at 57°C at pH 7. δ-CH_3 resonances of Ile-57 are indicated by *.

0.20 p.p.m. to 0.35 p.p.m.) is that of the Ile-57 δ-CH_3 (Fig. 2). Shifts of all other resolved resonances are less than 0.05 p.p.m. The spectral profiles between 1.0 p.p.m. and 1.5 p.p.m. are different for the various proteins (Fig. 2), a reflection of the small changes in the environments of surface methyl groups.

A major difference between the spectrum of N^{ϵ}-maleyl-ferrocytochrome *c* and that of the other proteins is the presence in the former of an additional pair of coupled doublet resonances at 6.85 p.p.m. and 7.15 p.p.m., which arise from a tyrosine residue (Fig. 2). These resonances are not observed at 37°C (see below).

Most of the resolved and assigned resonances of ferrocytochrome *c* (Redfield & Gupta, 1971; McDonald & Phillips, 1973; Keller & Wüthrich, 1978, 1981; Moore & Williams, 1980*a*,*b*; Boswell *et al.*, 1980*a*) belong to interior residues (Dickerson & Timkovich, 1975), and these are largely unaffected by the modifications, showing that the tertiary structure of ferrocytochrome *c* is not much perturbed. However, the fact that the δ-CH_3 resonance of Ile-57 is greatly shifted (Fig. 2) shows that there is a structural change accompanying modification. This resonance is shifted from −0.43 p.p.m. in native ferrocytochrome *c* to −0.23 p.p.m., −0.13 p.p.m., −0.13 p.p.m. and −0.08 p.p.m. in N^{ϵ}-trifluoroacetyl-ferrocytochrome *c*, N^{ϵ}-acetimidyl-ferrocytochrome *c*, N^{ϵ}-amidino-ferrocytochrome *c* and N^{ϵ}-maleyl-ferrocytochrome *c* respectively, all at pH 7 and 57°C. Despite the shifts, the Ile-57 δ-CH_3 resonances of the modified proteins maintain a similar temperature-dependence to that of the native protein.

Ile-57 is a residue on the surface of the protein close to both Tyr-74 and Trp-59, and Trp-59 is an internal residue close to the haem (Takano *et al.*, 1977). The relative orientations of these groups causes resonanes of Trp-59 and the δ-CH_3 of Ile-57 to be shifted upfield (Moore & Williams, 1980*b*,*c*), and only slight changes in the relative orientations of the groups (haem, Ile-57, Trp-59 and Tyr-74) are required to cause resonances to be perturbed. Since the resonances of Trp-59 and Tyr-74 are not significantly perturbed by the modification, whereas the δ-CH_3 resonance of Ile-57 is, it must be Ile-57 itself that is displaced.

^{1}H-n.m.r. spectra of native and modified horse ferricytochrome *c* were obtained and analysed in the same manner as those of the ferrocytochromes. Some of these spectra have been given elsewhere (Boswell *et al.*, 1980*b*). As with the ferrocytochromes, there are many similarities between the spectra of the ferricytochromes.

The ferricytochromes were not studied to the same extent as the ferrocytochromes because of the complications caused by paramagnetism. Resonances of the haem group and axial ligands, which experience large contact and pseudo-contact shifts, were markedly affected by the modifications (Table 1; and Boswell *et al.*, 1980*b*). The analogous shifts to these resonances in four singly modified [4-carboxy-2,6-dinitrophenyl-lysine]ferricytochromes *c* have been described (Falk *et al.*, 1981), and the shifts shown to stem from a change in the angular direction of the electronic *g*-tensor, probably resulting from a movement of the S–CH_3 group of Met-80. The shifts to some of the haem and axial ligand resonances of the derivatives studied in the present work are not as large as some of those for the single-site modification (Table 1), presumably because the substituting groups are not the same and possibly because perturbation at different sites might compensate each other.

Table 1. *Comparison of chemical shifts of the hyperfine shifted resonances for different lysine-modified ferricytochromes c and native horse ferricytochrome c*

Chemical shifts were measured at pH 7 and 25°C for native cytochrome *c* and its fully lysine-modified derivatives, and at pH 7 and 27°C for the specifically lysine-modified 4-carboxy-2,6-dinitrophenol (CDNP) derivatives. Assignments of chemical groups are taken from Redfield & Gupta (1971) and Keller & Wüthrich (1978). The unassigned CH resonances are from either the haem propionate side chains or the β-CH protons of His-18. A positive sign indicates a downfield shift. Abbreviation: N.D., not determined.

Chemical group	Chemical shift in native protein (p.p.m.)	[Modified Lys]$_{19}$cytochrome *c**				[CDNP-Lys]cytochrome *c*†			
		a	g	t	m	13	27	60	72
Haem-CH_3-8	34.8	0	0	−0.1	−0.4	−0.24	−0.08	+0.06	−0.53
Haem-CH_3-3	32.0	+0.1	+0.1	+0.1	−0.4	+0.17	−0.04	0	−0.36
Haem-CH_3-5	10.1	+0.7	+0.8	+0.8	+0.85	+0.02	0	+0.04	0
CH	19.0	−1.0	−1.0	−1.1	−1.5	N.D.	N.D.	N.D.	N.D.
CH	14.3	0	0	0	0	N.D.	N.D.	N.D.	N.D.
CH	11.5	+0.4	+0.4	+0.4	+0.4	N.D.	N.D.	N.D.	N.D.
Met-80 CH_3	−23.8	+0.2	+0.2	+0.2	+0.1	+0.30	+0.06	+0.05	−0.29

* Key: a, N^{ϵ}-acetimidylated; g, N^{ϵ}-amidinated; t, N^{ϵ}-trifluoroacetylated; m, N^{ϵ}-maleylated.

† Results for the CDNP derivatives taken from Falk *et al.* (1981).

pH-dependence of N^{ϵ}-acetimidyl-ferrocytochrome c

In Fig. 3 are shown convolution difference spectra of N^{ϵ}-acetimidyl-ferrocytochrome *c* at various pH values between 8 and 11.3 at 27°C. Three features of the spectra are of interest. (*a*) The spectra are little affected by increasing pH other than by a marked deterioration in the signal/noise ratio, which probably results from protein aggregation and denaturation at high pH. (*b*) A pair of resonances in the aromatic region shift from approx. 6.9 p.p.m. and approx. 7.15 p.p.m. at pH 8 to approx. 6.6 p.p.m. and approx. 7.0 p.p.m. at pH 11.3 with a pK_a of 10.2. These resonances are due to a tyrosine residue. (*c*) The resonance of the Ile-57 δ-CH_3 group is pH-independent over the whole pH range. This is a notable difference from native ferrocytochrome *c*, where this resonance is pH-dependent (Fig. 4). The corresponding resonance of N^{ϵ}-acetimidyl-[Hse^{65}]-ferrocytochrome *c* is also pH-independent.

The spectra in Fig. 3 show that over the pH range 8–11 N^{ϵ}-acetimidyl-ferrocytochrome *c* remains in a conformation similar to the native one, although there are two minor structural perturbations. The finding that one of the tyrosine residues of N^{ϵ}-acetimidyl-ferrocytochrome *c* ionizes with a pK_a of 10.2 is a significant difference between the modified and native proteins. This residue cannot be Tyr-74, since resonances of Tyr-74 are present in the spectra at their normal positions (Fig. 3). Nor is it likely to be Tyr-67, since this residue is an internal group close to Met-80 and the haem group, and resonances of these groups are pH-independent. Therefore the resonances must be due to either Tyr-48 or Tyr-97; further assignment is not yet possible. Nevertheless the appearance of tyrosine resonances with chemical shifts similar to those of free tyrosine indicates that part of the structure of N^{ϵ}-acetimidyl-ferrocytochrome *c* is different from that of native ferrocytochrome *c* at alkaline pH.

The loss of the pH-dependence of the Ile-57 resonance (Fig. 4) implies that the ionization of a lysine residue causes the pH-dependence. N^{ϵ}-Acetimidylation of lysine shifts the lysine pK_a from 10.5 to 12.7 (G. R. Moore & C. J. A. Wallace,

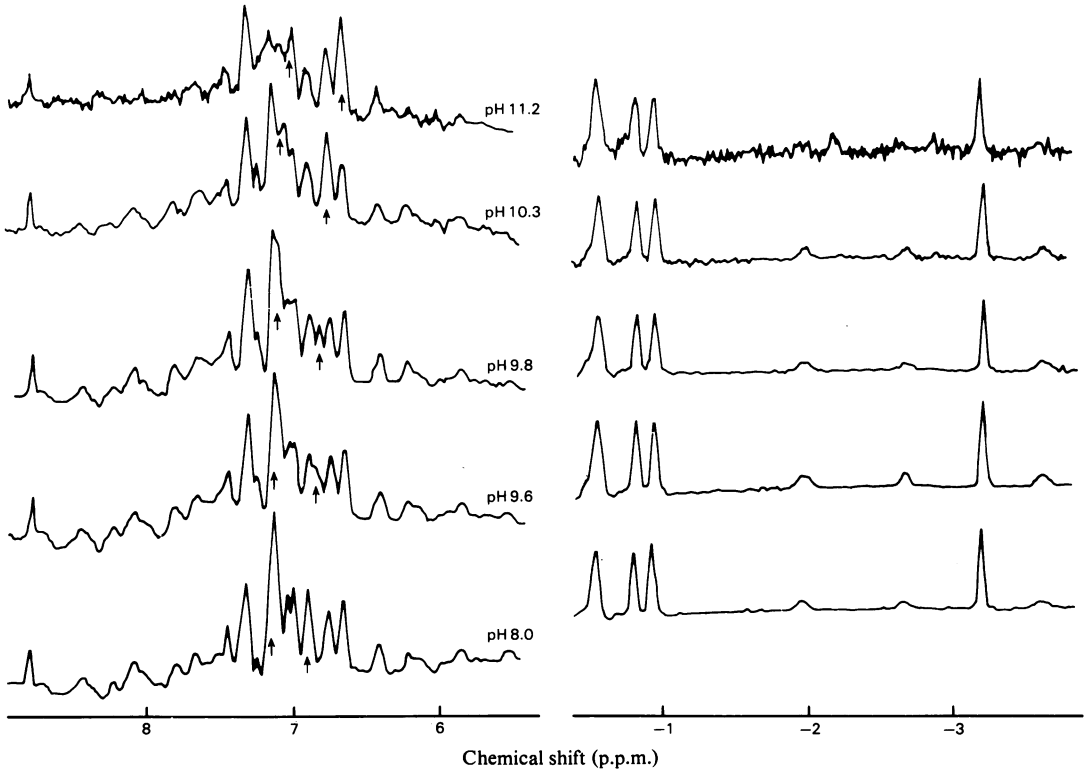

Fig. 3. *Convolution difference ^1H-n.m.r. spectra of N^{ϵ}-acetimidyl-ferrocytochrome c (3 mM) in ^2H_2O and at various pH values at 27°C*

The titrating resonances of a tyrosine residue are indicated by ↑.

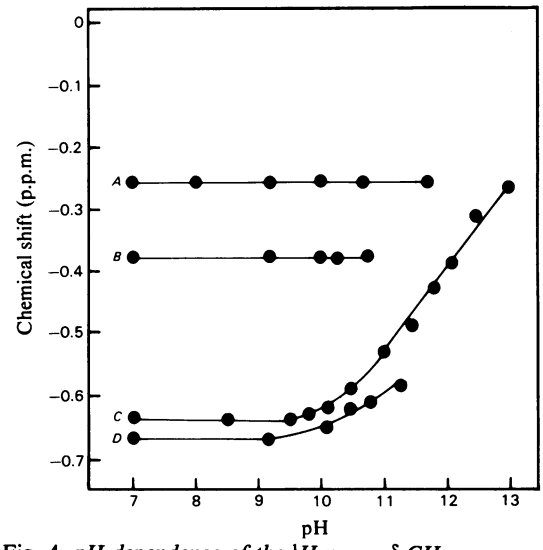

Fig. 4. *pH-dependence of the ^1H-n.m.r. δ-CH_3 resonance of Ile-57 at 27°C for (A) N^{ϵ}-acetimidyl-ferrocytochrome c, (B) (N^{ϵ}-acetimidyl-lysine)$_{11}$ (1–65)-(lysine)$_8$(66–104)-[Hse^{65}]ferrocytochrome c, (C) native horse ferrocytochrome c and (D) (lysine)$_{11}$(1–65)-(N^{ϵ}-acetimidyl-lysine)$_8$(66–104)-[Hse^{65}]ferrocytochrome c*

unpublished work), and either the shift in pK_a, or a small change in conformation, causes the loss of the Ile-57 pH-dependence.

The pH-dependence of two other derivatives of horse ferrocytochrome *c* was studied. The derivatives were (N^{ϵ}-acetimidyl-lysine)$_{11}$(1–65)-(lysine)$_8$(66–104)-[Hse^{65}]ferrocytochrome *c* (called aA · BC) and (lysine$_{11}$(1–65) - (N^{ϵ} - acetimidyl - lysine)$_8$(66–104) - [Hse^{65}]ferrocytochrome *c* (called A · aBC), and the intention was to identify the lysine residue(s) responsible for the pH-dependent shift of Ile-57 of native ferrocytochrome *c*. The data are presented in Fig. 4. The Ile-57 resonance of aA · BC is pH-independent, whereas that of A · aBC is pH-dependent. These findings locate the ionizing residue or residues as being among the 11 lysine residues in the first 65 residues of horse cytochrome *c*. In this portion of the protein only three lysine residues are close to the Ile-57/Trp-59/Tyr-74 triad: Lys-39, Lys-55 and Lys-60. Presumably, one or more of these residues is responsible for the pH-dependence of Ile-57.

The importance of the region of the protein about Ile-57 to the function of cytochrome *c* is not known. It is the region of protein that undergoes the largest

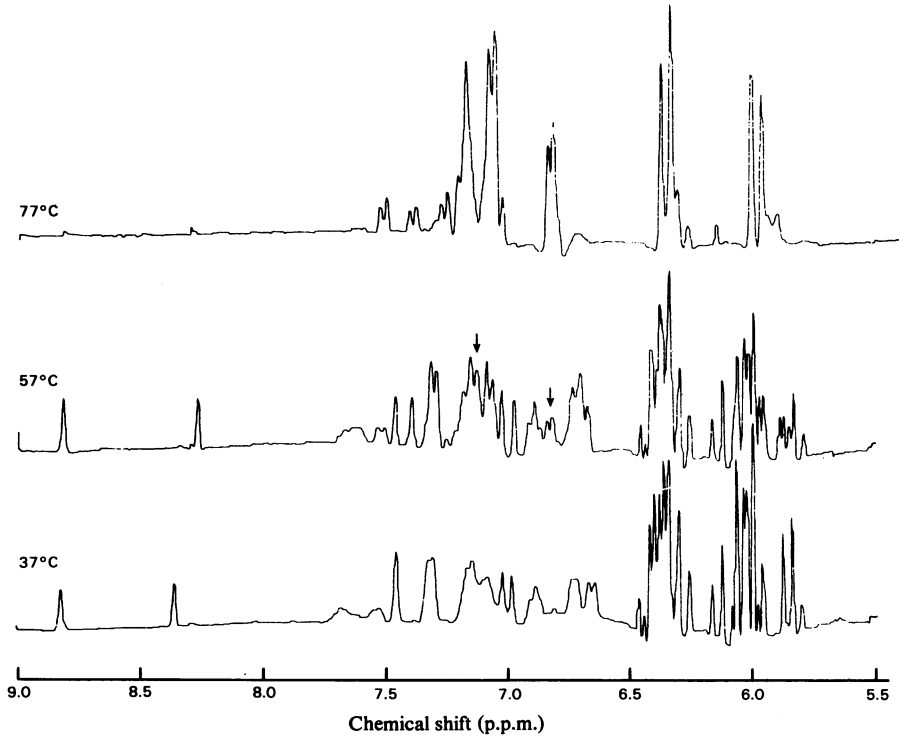

Fig. 5. *Convolution difference ^1H-n.m.r. spectra of N^{ϵ}-maleyl-ferrocytochrome c (5 mM) in ^2H_2O at pH 7 and at various temperatures*

A pair of tyrosine doublet resonances in the spectrum at 57°C are marked by ↓.

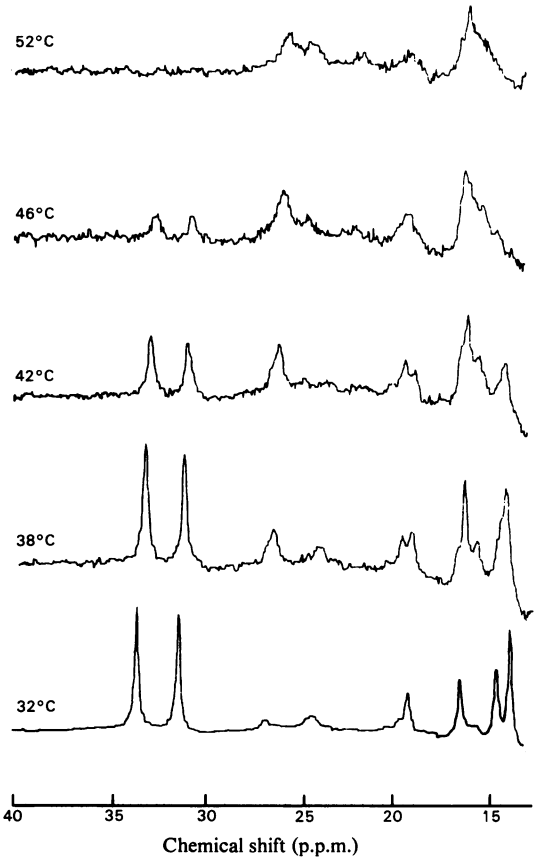

Fig. 6. *^{1}H-n.m.r. spectra of N^ϵ-maleyl-ferricytochrome c (5 mM) in ^2H_2O at pH 7 at various temperatures*

change in conformation on oxidation-state change (Moore & Williams, 1980*c*; Bosshard & Zürrer, 1980; Takano & Dickerson, 1981), yet it is on the opposite side of the protein to the oxidase–reductase interaction site(s) (Rieder & Bosshard, 1980; Takano *et al.*, 1977). The accompanying paper (Robinson *et al.*, 1983) describes the extent of the structural change with variation in solution conditions.

Temperature-dependence of N^ϵ-maleyl-cytochrome c

In Fig. 5 are shown regions of the convolution difference ^{1}H-n.m.r. spectra of N^ϵ-maleyl-ferrocytochrome *c* in ^2H_2O at pH 7 at 37°C, 57°C and 77°C. The spectrum at 37°C is very similar to that of native ferrocytochrome *c*, but at 57°C an additional pair of coupled doublet resonances are present at 7.15 and 6.85 p.p.m. These additional resonances arise from a tyrosine residue. At 77°C the spectrum is that of a random-coil protein; the resonances of the maleyl groups have collapsed to two 19 proton intensity doublets, and the rest of the aromatic region resembles the superimposed spectra of tyrosine, phenylalanine and tryptophan.

On heating of the protein, the additional tyrosine resonances increase in intensity from zero at 30°C to two-protons intensity at 67°C. At 67°C the remainder of the spectrum closely resembles that of native ferrocytochrome *c* at 67°C, although there are perturbations to unassigned resonances in the aliphatic region of the spectrum. The transition to a random-coil protein, which is complete at 77°C, occurs with a mid-point of approx. 72°C. At 30 min after cooling of the sample from 77°C to 57°C, the n.m.r. spectrum is a superimposition of the 'normal' and random-coil spectra at about equal intensities. At 3 days after cooling to room temperature the n.m.r. spectrum at 57°C is identical with the spectrum obtained before heating to 77°C. Thus there is hysteresis in the refolding of N^ϵ-maleyl-ferrocytochrome *c*.

A reviewer has suggested that hysteresis might be connected with aggregation of the unfolded protein at high temperatures. It is suggested that disaggregation is a slower process than refolding at low temperatures.

The presence of tyrosine resonances at the chemical-shift positions for the free amino acid (i.e. chemical shift unperturbed by secondary effects caused by protein folding) immediately before unfolding of N^ϵ-maleyl-ferrocytochrome *c* is similar to the presence of tyrosine resonances in the spectrum of N^ϵ-acetimidyl-ferrocytochrome *c* at high pH (Fig. 3). In both cases the resonances must come from Tyr-48 or Tyr-97; and their appearance in the spectra (Figs. 3 and 5) indicates that there is local unfolding of the protein before complete unfolding.

The low-field regions of spectra of 5 mM-N^ϵ-maleyl-ferricytochrome *c* in ^2H_2O at pH 7 and various temperatures are given in Fig. 6. At 32°C the spectrum is similar to that of native ferricytochrome *c*, although some of the haem resonances are shifted (Table 1). With increasing temperature resonances of the haem group and axial ligands (at −24 p.p.m.) decrease in intensity, and a new set of resonances (at 26 p.p.m. and 19.5 p.p.m.) increase in intensity. The spectrum at 42°C is a superimposition of those of the two forms of N^ϵ-maleyl-ferricytochrome *c* at about equal intensities. Above 50°C the high-temperature form is further modified, and at 57°C the spectrum is that of a random-coil protein. Thus the temperature-induced denaturation of N^ϵ-maleyl-ferricytochrome *c* passes through a low-spin intermediate that lacks Met-80 as a sixth ligand. This intermediate may be the alkaline isomer (state IV) of ferricytochrome *c*. This isomer has haem methyl resonances at 23.8 p.p.m. and 21.3 p.p.m. (Gupta & Koenig, 1971) and is an intermediate in the thermal denaturation of horse

ferricytochrome *c* at pH 7 (Ångström *et al.*, 1982). The sixth ligand in this state is not known (Pettigrew *et al.*, 1976; C. J. A. Wallace, unpublished work).

The denaturation of N^{ϵ}-maleyl-ferricytochrome *c* has been extensively studied by Schejter *et al.* (1979), who demonstrated that at 25°C in dilute buffer a 0.26mM solution of the protein lacked the 695nm band, and thus lacked the normal Met-80 ligation, though it remained in a low-spin state. The difference between the denaturation temperature that we observe and that found previously is attributable to the difference in protein concentration (Schejter *et al.*, 1979).

We thank the Science and Engineering Research Council, the Medical Research Council, The Royal Society and the Fonds National Suisse de la Recherche Scientifique for financial support. G. R. M. thanks the Science and Engineering Research Council for an Advanced Fellowship. R. J. P. W. is a member of the Oxford Enzyme Group.

References

Ångström, J., Moore, G. R. & Williams, R. J. P. (1982) *Biochim. Biophys. Acta* **703**, 87–94

Boon, P. J. (1981) Ph.D. Thesis, University of Nijmegen

Bosshard, H. R. & Zürrer, M. (1980) *J. Biol. Chem.* **255**, 6694–6699

Boswell, A. P., Moore, G. R., Williams, R. J. P., Chien, J. C. W. & Dickinson, L. C. (1980*a*) *J. Inorg. Biochem.* **13**, 347–352

Boswell, A. P., McClune, G. J., Moore, G. R., Williams, R. J. P., Pettigrew, G. W., Inubishi, T., Yonetani, T. & Harris, D. E. (1980*b*) *Biochem. Soc. Trans.* **8**, 637–638

Campbell, I. D., Dobson, C. M., Williams, R. J. P. & Xavier, A. V. (1973) *J. Magn. Reson.* **11**, 172–181

Dayhoff, M. O. (1968) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring

Dickerson, R. E. & Timkovich, R. (1975) *Enzymes 3rd Ed.* **11**, 397–547

Di Marchi, R. D., Garner, W. H., Wang, C.-C., Hanania, G. I. H. & Gurd, F. R. N. (1978) *Biochemistry* **17**, 2822–2829

Eley, C. G. S., Moore, G. R., Williams, R. J. P., Neupert, W., Boon, P. J., Brinckhof, H., Nivard, R. J. F. & Tesser, G. I. (1982) *Biochem. J.* **205**, 153–165

Falk, K. E., Jovall, P. Å. & Ångström, J. (1981) *Biochem. J.* **193**, 1021–1024

Fanger, M. W. & Harbury, H. A. (1965) *Biochemistry* **4**, 2541–2545

Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1978) *J. Biol. Chem.* **253**, 149–159

Gupta, R. K. & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1134–1143

Hettinger, T. P. & Harbury, H. A. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1469–1476

Kalinichenko, P. (1976) *Stud. Biophys.* **58**, 235–240

Keller, R. M. & Wüthrich, K. (1978) *Biochim. Biophys. Acta* **533**, 195–208

Keller, R. M. & Wüthrich, K. (1981) *Biochim. Biophys. Acta* **668**, 307–320

Margoliash, E., Smith, E. L., Kreil, G. & Tuppy, H. (1961) *Nature (London)* **192**, 1125–127

McDonald, C. C. & Phillips, W. D. (1973) *Biochemistry* **12**, 3170–3186

Moore, G. R., & Williams, R. J. P. (1980*a*) *Eur. J. Biochem.* **103**, 493–502

Moore, G. R. & Williams, R. J. P. (1980*b*) *Eur. J. Biochem.* **103**, 503–512

Moore, G. R. & Williams, R. J. P. (1980*c*) *Eur. J. Biochem.* **103**, 513–521

Oldfield, E. & Allerhand, A. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3531–3535

Oldfield, E., Norton, R. S. & Allerhand, A. (1975) *J. Biol. Chem.* **250**, 6381–6402

Osheroff, N., Borden, D., Koppenol, W. H. & Margoliash, E. (1980) *J. Biol. Chem.* **255**, 1689–1697

Pal, P. K., Verma, B. & Myer, Y. P. (1975) *Biochemistry* **14**, 4325–4334

Pettigrew, G. W. (1978) *FEBS Lett.* **86**, 14–16

Pettigrew, G. W., Aviram, I. & Schejter, A. (1976) *Biochem. Biophys. Res. Commun.* **68**, 807–813

Redfield, A. G. & Gupta, R. K. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 405–411

Rieder, R. & Bosshard, H. R. (1980) *J. Biol. Chem.* **255**, 4732–4739

Robinson, M. N., Boswell, A. P., Huang, Z.-X., Eley, C. G. S. & Moore, G. R. (1983) *Biochem. J.* **213**, 687–700

Schejter, A., Zuckerman, M. & Aviram, I. (1979) *J. Biol. Chem.* **254**, 7042–7046

Smith, H. T. & Millett, F. (1980) *Biochemistry* **19**, 1117–1120

Smith, H. T., Staudenmayer, N. & Millett, F. (1977) *Biochemistry* **16**, 4971–4978

Sokolovsky, M., Aviram, I. & Schejter, A. (1970) *Biochemistry* **9**, 5113–5122

Stellwagen, E. (1964) *Biochemistry*, 919–923

Stellwagen, E., Smith, L. M., Cass, R., Ledger, R. & Wilgus, H. (1977) *Biochemistry* **16**, 3672–3679

Takano, T. & Dickerson, R. E. (1981) *J. Mol. Biol.* **153**, 95–115

Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R. & Dickerson, R. E. (1977) *J. Biol. Chem.* **252**, 776–785

Theorell, H. & Åkeson, A. (1941) *J. Am. Chem. Soc.* **63**, 1804–1827

Wallace, C. J. A. & Offord, R. E. (1979) *Biochem. J.* **179**, 169–182