Biochem. J. (1982) **205**, 153–165
Printed in Great Britain

Structural role of the tyrosine residues of cytochrome *c*

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(*Received 27 January 1982/Accepted 7 April 1982*)

The tertiary structures of horse, tuna, *Neurospora crassa*, horse [Hse^{65},Leu^{67}]- and horse [Hse^{65},Leu^{74}]-cytochromes *c* were studied with high-resolution ^{1}H n.m.r. spectroscopy. The amino acid sequences of these proteins differ at position 46, which is occupied by phenylalanine in the horse proteins but by tyrosine in the remaining two, and at positions 67, 74 and 97, which are all occupied by tyrosine residues in horse and tuna cytochrome *c* but in the other proteins are substituted by phenylalanine or leucine, though there is only one such substitution per protein. The various aromatic-amino-acid substitutions do not seriously affect the protein structure.

Anfinsen (1973) has shown that the tertiary structure of a protein is determined by its amino acid sequence, yet many proteins are able to accommodate a larger number of substitutions while retaining their biochemical activities. The effect upon a structure of single or multiple amino acid substitutions must depend upon the nature of the amino acid(s) concerned. Thus the question is posed, how does a protein structure respond to the modification of amino acid residues, be they either natural substitutions or synthetic modifications?

The application of X-ray-crystallographic techniques has produced detailed models for the structures of many proteins and, with these structures as a guide, n.m.r. spectroscopy can be gainfully employed to investigate the structural effects of protein modification. At the same time, n.m.r. spectroscopy has the advantage that dynamic characteristics of the structures can be readily investigated. For these reasons we have been studying cytochrome *c* in solution by n.m.r. spectroscopy.

Horse cytochrome *c* consists of 104 amino acids, four of which are tyrosine: residues 48, 67, 74 and 97 (Margoliash *et al.*, 1961). These residues play important roles in stabilizing the conformation of cytochrome *c* (Sokolovsky *et al.*, 1970; Pal *et al.*, 1975), and when they are replaced in other native eukaryotic cytochromes, it is always by phenylalanine (Dayhoff, 1968; Dickerson & Timkovich, 1975). Although tyrosine residues have been implicated directly in the function of cytochrome *c*, as free-radical intermediates in electron transfer (Dickerson *et al.*, 1972), the data now available argue strongly for their possessing a purely structural role (Dickerson & Timkovich, 1975; Boon *et al.*, 1979*a*). In the present paper a ^{1}H n.m.r. study of the structural role of the tyrosine residues of cytochrome *c* is reported.

^{1}H n.m.r. investigations of tyrosine residues are complicated by the presence of side-chain mobility, which, however, provides a measure of the protein dynamics. The tyrosine (and phenylalanine) side chain flips between equivalent orientations about its Cβ–Cγ bond (Fig. 1) (Campbell *et al.*, 1975; Wüthrich & Wagner, 1975). Where the flipping is fast the two *ortho* protons become equivalent, as do the two *meta* protons, and an AA'BB' spectrum

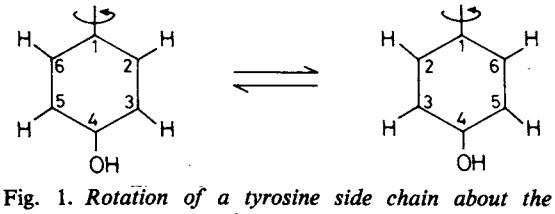

Fig. 1. *Rotation of a tyrosine side chain about the Cβ—Cγ bond*

Abbreviation used: n.o.e., nuclear Overhauser enhancement; Hse, homoserine.

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0306-3275/82/070153-13$01.50/1

occurs. With slow flipping an ABCD spectrum results, and at intermediate rates of flipping the resonances are too broad to be observed. In the spectrum of horse ferrocytochrome *c* at pH 7 and 27°C, resonances of only one tyrosine residue are observed as an AA'BB' spectrum (Moore & Williams, 1980*a*). It is for this reason that ^{13}C n.m.r. has been used to investigate the pH-dependence of the tyrosine residues of ferrocytochrome *c* (Oldfield *et al.*, 1975; S. Bocieck, D. Welti, G. R. Moore & R. J. P. Williams, unpublished results). Since the non-protonated carbon atoms are on the C_2 symmetry axis their resonances are unaffected by rotation about the Cβ–Cγ bond and thus are readily observed.

Previous ^1H n.m.r. studies of cytochrome *c* have led to the preliminary assignment of resonances of only two tyrosine residues (Moore & Williams, 1980*a*; Perkins, 1980; Keller & Wüthrich, 1981). In the present paper we report the assignment of ^1H n.m.r. signals from each of the four tyrosine residues of horse ferrocytochrome *c* and then go on to discuss the structural role of tyrosine with reference to *Neurospora crassa* cytochrome *c* [in which Tyr-97 is replaced by Phe-97 (Heller & Smith, 1966; Lederer & Simon, 1974)], horse [Hse^{65},Leu^{67}]-cytochrome *c* (in which Tyr-67 is replaced by Leu-67) and horse [Hse^{65},Leu^{74}]cytochrome *c* (in which Tyr-74 is replaced by Leu-74).

Materials and methods

Horse (type VI) and tuna (type XI) cytochromes *c* were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). *N. crassa* cytochrome *c* was isolated by the procedure of Korb & Neupert (1978). The preparation of horse [Hse^{65},Leu^{67}]- and [Hse^{65},Leu^{74}]-cytochromes *c* are described by Boon (1981).

$K_3[Fe(CN)_6]$ and $Na_2S_2O_4$ were purchased from BDH Chemicals (Poole, Dorset, U.K.). Oxygen-free argon was obtained from Air Products (Bracknell, Berks., U.K.), ^2H_2O (99.8%) was from Merck, Sharp and Dohme (Montreal, Que., Canada), and 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.).

Samples were prepared for n.m.r. by the following procedure: cytochrome *c* was dissolved in 5mM-NaH_2PO_4/Na_2HPO_4 buffer, pH 7, and a small excess of $K_3[Fe(CN)_6]$ added to ensure complete oxidation. After ~30min the solution was applied to a column (30cm × 2cm) packed with Whatman CM-cellulose (CM-23) that had been previously equilibrated with the phosphate buffer. The column was washed with 150ml of phosphate buffer to remove impurities. The ferricytochrome *c* was then eluted with 1M-NaCl as a single, sharp band. The eluate was de-salted by ultrafiltration and subsequently freeze-dried at pH 7. N.m.r. samples of ferricytochrome *c* were prepared by dissolving weighed amounts of freeze-dried cytochrome *c* in known volumes of ^2H_2O and the pH was adjusted to the required value by addition of small portions of concentrated NaO^2H and ^2HCl. Samples of ferrocytochrome *c* were prepared by the addition of solid $Na_2S_2O_4$ to solutions of ferricytochrome *c* before final adjustment of the pH. All samples of ferrocytochrome *c* were prepared in glassware flushed with argon. Where required, N-H protons were exchanged for deuterons by heating solutions of ferricytochrome *c* in ^2H_2O at pH 8 for about 2h at 45°C. Quoted pH values are direct meter readings uncorrected for the small isotope effect (Kalinichenko, 1976).

The n.m.r. spectra were recorded with either a Bruker 270MHz spectrometer or a Bruker 300MHz spectrometer. Resolution enhancement (Ernst, 1966; Campbell *et al.*, 1973), saturation-transfer (Campbell *et al.*, 1976) and n.o.e. experiments (Boswell *et al.*, 1980) were performed as previously described. Resonance assignments for *N. crassa* cytochrome *c* and the modified horse cytochromes *c* were obtained in an analogous manner to those of horse and tuna cytochromes *c* (Moore & Williams, 1980*a*,*b*,*d*). 1,4-Dioxan was used as an internal standard, but all chemical shifts are quoted in p.p.m. downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

Results and discussion

Assignment of tyrosine resonances of ferrocytochrome c

Previous ^1H n.m.r. work includes the tentative assignment of one set of resonances to Tyr-74 (Moore & Williams, 1980*a*) and a further set to either Tyr-48 (Moore & Williams, 1980*a*) or Tyr-97 (Perkins, 1980; Keller & Wüthrich, 1981). The Tyr-74 assignment is confirmed by the lack of the assigned resonances from the spectra of [Hse^{65},Leu^{74}]cytochrome *c* (see below). The assignment of the second set of resonances to Tyr-97 is confirmed by comparison of *N. crassa* cytochrome *c* with horse cytochrome *c* and by observation of n.o.e. in horse cytochrome *c*. Assignment of further resonances to Tyr-48 and Tyr-67 is made from n.o.e. measurements on horse cytochrome *c*. The resonance assignments are summarized in Table 1; the details of the assignment procedure are given below.

(*a*) *Tyr-97.* In Fig. 2 are shown the aromatic regions of the spectrum of horse ferrocytochrome *c* in ^2H_2O, at pH 5.5 and 95°C, and various n.o.e. difference spectra obtained from the irradiation of assigned resonances. The difference spectrum of Fig. 2(*b*) was obtained upon irradiation of the tyrosine

Table 1. *Tyrosine resonances of ferrocytochromes c at pH 7*
Abbreviation used: n.d., not determined.

		Chemical shift (p.p.m.)			
		Horse ferrocytochrome *c*		*N. crassa* ferrocytochrome *c*	
Assignment	Spectrum ...	ABCD	AA'BB'	ABCD	AA'BB'
Tyr-48		5.27 6.43	5.75	5.30 6.34	n.d.
Tyr-67		n.d. n.d. n.d. n.d.	5.13 6.73	n.d. n.d. n.d. n.d.	n.d. n.d.
Tyr-74					
ortho		—	7.21	—	7.29
meta		—	6.61	—	6.69
Tyr-97		7.17 6.60	6.78	— —	— —
		6.73 5.55	6.18	— —	— —

resonance at 6.18 p.p.m. A decrease in intensity is observed for the coupled tyrosine resonance at 6.78 p.p.m. and for resonances at 6.7 p.p.m. and 7.1 p.p.m. The latter two resonances are due to Phe-10 (Boswell *et al.*, 1980; Keller & Wüthrich, 1981). The n.o.e. is critically dependent upon the distance between nuclei (Noggle & Schirmer, 1971) and this experiment assigns the tyrosine resonances to a residue close to Phe-10. Only Tyr-97 is sufficiently close to Phe-10 (Takano *et al.*, 1977) and thus the resonances at 6.18 p.p.m. and 6.78 p.p.m. are assigned to Tyr-97.

The tyrosine resonances at 6.18 p.p.m. and 6.78 p.p.m. are only observed at high temperature (Moore & Williams, 1980*a*). With decreasing temperature they broaden until they are no longer observable, and at room temperature they are replaced by four resonances, each of one proton intensity (Campbell *et al.*, 1976). This is the kind of behaviour expected of a tyrosine residue whose motion about its Cβ–Cγ bond is restricted. Resonances with the same properties at similar chemical shifts are not present in the spectrum of *N. crassa* ferrocytochrome *c* over the temperature range 4–75°C, thus confirming the assignment to Tyr-97, since *N. crassa* cytochrome *c* contains Tyr-48, Tyr-67 and Tyr-74, but not Tyr-97 (Heller & Smith, 1966; Lederer & Simon, 1974). Keller & Wüthrich (1981) have independently assigned these four peaks to Tyr-97 by using two-dimensional n.m.r. techniques.

(*b*) *Tyr-67*. The spectra presented in Fig. 3 are of horse ferrocytochrome *c* in ^2H_2O at pH 6.5. With increasing temperature from 57 to 93°C (Fig. 3*a*), pronounced changes in the spectra occur at ~6.7 p.p.m. and at 5.13 p.p.m. Some of the changes at ~6.7 p.p.m. are due to a shift of the *meta* resonance of Tyr-74 and to a decrease in linewidth of the *meta* resonance of Phe-10 (Moore & Williams, 1980*c*). The change at 5.13 p.p.m. has not previously been described. At 93°C (Fig. 3*b*) the peak at 5.13 p.p.m. is observed to be a slightly broadened doublet coupled to a sharp doublet at 6.73 p.p.m. These resonances arise from Tyr-48 or Tyr-67. Their further assignment to Tyr-67 follows a series of n.o.e. measurements made at 95°C and which are presented in Figs. 2(*c*)–2(*e*).

Irradiation of the Met-80 SCH_3 resonance (−3.25 p.p.m.; McDonald *et al.*, 1969) at 95°C causes resonances of Phe-82 (6.7 p.p.m. and 7.4 p.p.m.) and the aromatic resonances at 6.73 p.p.m. and 5.13 p.p.m. to appear in the difference spectrum (Fig. 2*e*). This difference spectrum should be compared with the one obtained for the same irradiation at 30°C (Fig. 1 of Boswell *et al.*, 1980). In that difference spectrum the only aromatic amino acid resonances are those of Phe-82. Consideration of the X-ray structure (Takano *et al.*, 1977) shows that Phe-82 and Tyr-67 are the closest aromatic amino acids to Met-80 (the relative distances of some residues from Met-80 are illustrated by Fig. 4, which is a co-ordinate plot taken from the X-ray structure). Thus the coupled resonances at 5.13 p.p.m. and 6.73 p.p.m. observed at 93°C are assigned to Tyr-67.

(*c*) *Tyr-48*. All the *ortho*/*meta* resonances of the four phenylalanine residues and three of the four tyrosine residues have been assigned at 95°C as AA'BB' spectra. The only tyrosine resonances not identified as an AA'BB' pattern are those of Tyr-48.

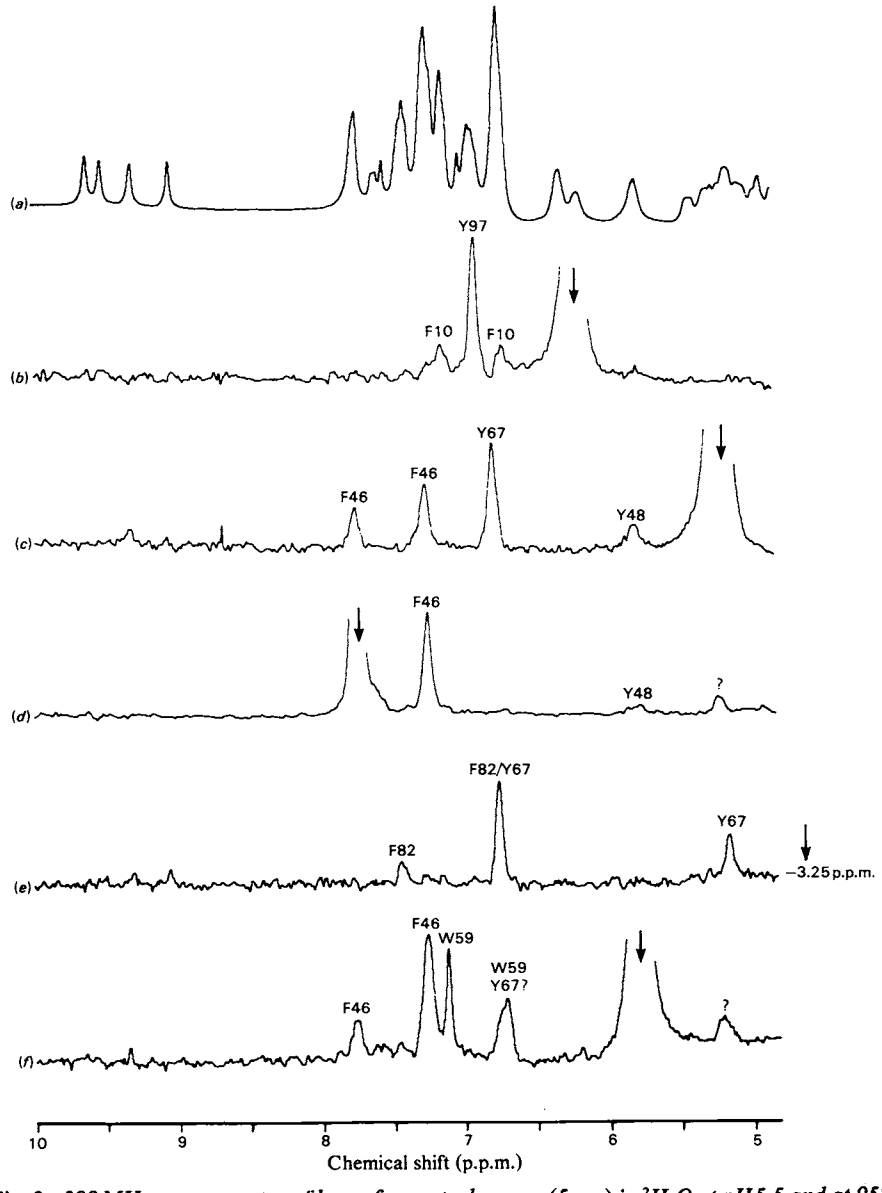

Fig. 2. *300 MHz n.m.r. spectra of horse ferrocytochrome c (5 mM) in ^2H_2O at pH 5.5 and at 95°C*
(*a*) is the normal spectrum and (*b*)–(*f*) are n.o.e. difference spectra obtained upon irradiation at the indicated positions (↓) for 0.5 s before acquisition. The spectra were obtained with a stationary sample. Resonances of groups affected by the irradiation pulse are indicated by F10 (Phe-10), F46 (Phe-46), Y48 (Tyr-48), W59 (Trp-59), Y67 (Tyr-67), F82 (Phe-82) and Y97 (Tyr-97). The resonance at 5.18 p.p.m. marked by ? is unassigned.

Thus any further aromatic resonances identified at 95°C must, by elimination, arise from Tyr-48.

An additional, previously unassigned, aromatic resonance is observed at 5.75 p.p.m. in the spectrum of horse ferrocytochrome *c* at 95°C. It overlaps with the C-6 proton resonance of Trp-59 (Moore & Williams, 1980*a*; Moore, 1978). A decrease in intensity at 5.75 p.p.m. with decreasing temperature can be clearly seen in Fig. 1 of Campbell *et al.* (1976) and less clearly in Fig. 3(*a*) of the present paper. The additional resonance at 95°C is from Tyr-48, and although it is entering the fast-exchange region (AA'BB' spectrum), it is still too broad to exhibit coupling.

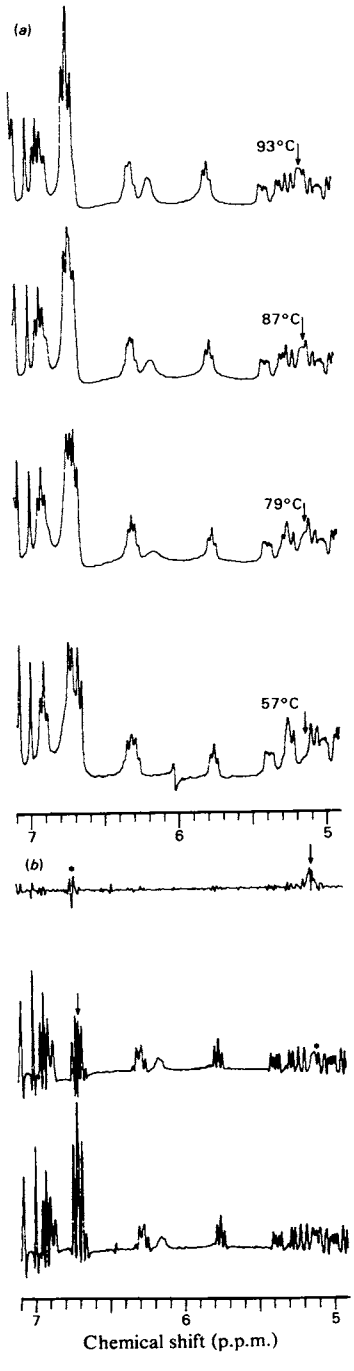

Fig. 3. *300 MHz n.m.r. spectra of horse ferrocytochrome c (5 mM) in ^2H_2O at pH 6.5 and at (a) various temperatures and (b) 93°C*

(*a*) Temperature-dependence of a region of the conventional n.m.r. spectrum. The arrow at 5.13 p.p.m. indicates a resonance whose linewidth is markedly temperature-dependent (see the text). (*b*) Resolution-enhanced spectra showing spin-decoupling of the peaks at 5.13 p.p.m. and 6.73 p.p.m.

The difference spectrum of Fig. 2(*d*) illustrates the n.o.e. difference spectrum obtained upon irradiation of the *ortho* and *para* resonances of Phe-46 at 95°C. Peaks appear in the difference spectrum at 7.23 p.p.m. (Phe-46), 5.75 p.p.m. (Tyr-48) and 5.18 p.p.m. (?). The resonance of haem methyl-5 at 3.6 p.p.m. also appears (result not shown). The resonance at 5.75 p.p.m. assigned to Tyr-48 does not appear in the difference spectrum when the Met-80 SCH_3 resonance is irradiated (Fig. 2*e*) nor when the *para* resonance of Phe-46 is irradiated at temperatures below 40°C. Irradiation at 5.75 p.p.m. at 95°C produces the n.o.e. difference spectrum shown in Fig. 2(*f*). Peaks appear at 7.74 p.p.m. and 7.23 p.p.m. (Phe-46), 5.18 p.p.m. (?) and 7.09 p.p.m. and 6.69 p.p.m. (Trp-59). The Met-80 SCH_3 resonance does not appear in the difference spectrum. The observed n.o.e. between resonances of Phe-46 and the resonance at 5.75 p.p.m. confirms the assignment of the latter to Tyr-48, since Phe-46 and Tyr-48 are close together (Fig. 3). Assignment of the peak at 5.18 p.p.m. is uncertain. Its position close to the Tyr-67 resonance at 5.13 p.p.m. prohibits selective n.o.e. experiments. Thus irradiation at ~5.15 p.p.m. results in peaks appearing in the n.o.e. difference spectrum (Fig. 2*c*) at 9.32 p.p.m. (haem *meso* α), 7.74 p.p.m. and 7.23 p.p.m. (Phe-46), 6.74 p.p.m. (Tyr-67), 5.75 p.p.m. (Tyr-48) and −3.25 p.p.m. (Met-80; result not shown). The haem *meso* α resonance appears because the CH resonance of thioether bridge-2 at 5.25 p.p.m. (Keller & Wüthrich, 1978) is affected by the irradiation pulse.

In Fig. 5 are shown the aromatic regions of spectra of horse ferrocytochrome *c* in ^2H_2O at pH 5.25 and 15°C. The normal spectrum and difference spectra obtained from irradiation of slowly exchanging aromatic groups (ABCD spectra) are shown. The resonances indicated by F belong to Phe-46 (Table 2*b*) and the resonances marked by Y belong to another aromatic group. An analogous set of Y-resonances are present in the spectrum of *N. crassa* ferrocytochrome *c* at temperatures below 15°C (Table 1). The *N. crassa* resonances were located in n.o.e. difference spectra resulting from irradiation of resonances of Tyr-46. The only residues the Y-resonances can belong to are Tyr-48 and Tyr-67, since resonances of the Tyr-74 and Tyr-97 have been assigned at this temperature. The n.o.e. from Phe-46 assigns the Y-resonances to Tyr-48.

Structural studies of tyrosine in cytochrome c

The main approach to the determination of structures of proteins in solution is based upon the

↓ indicates the irradiated resonance and * indicates the decoupled resonance. The top trace is a spin-decoupling difference spectrum.

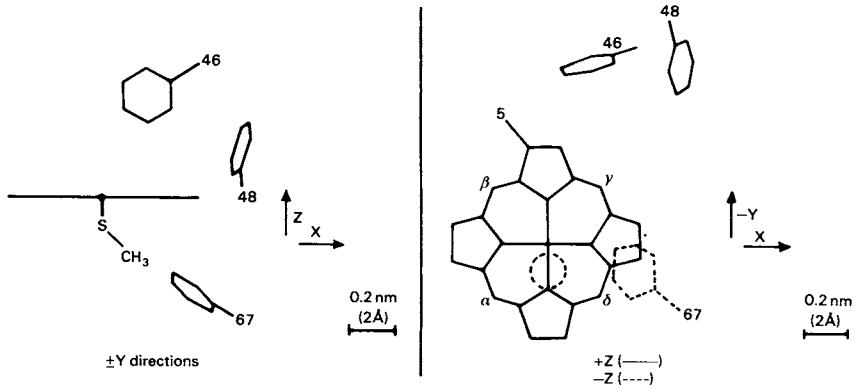

Fig. 4. *Relative orientations of Phe-46 (46), Tyr-48 (48), Tyr-67 (67), Met-80 and the haem group*
The diagram was constructed from co-ordinates of the X-ray structure of tuna cytochrome *c* (Takano *et al.*, 1977). The dotted circle is the Met-80 methyl group.

observation of n.o.e. between assigned resonances. Since we have used n.o.e. to assign some of the tyrosine resonances of ferrocytochrome *c*, we cannot now use them to compare the solution and crystal structures. However, it is pertinent to note that n.o.e. involving Tyr-74 and Tyr-97 (assigned by chemical modification and sequence comparison) are consistent with the X-ray structure (Takano *et al.*, 1977; Boswell *et al.*, 1980; Keller & Wüthrich, 1981).

A second approach to the determination of protein structures is the interpretation of conformation-dependent shifts (the difference in chemical shift of a resonance between its value in simple peptides and its value in the protein). This is not such a good structural probe as n.o.e. because the computational procedure for predicting such shifts is not entirely satisfactory (Perkins, 1981; Clayden, 1981). However, although the calculation of conformation-dependent shifts *a priori* is not yet well-defined, they can be satisfactorily used to compare related proteins. This comparison is done by using resonances that are subject to a reasonably large conformation-dependent shift (about 0.8 p.p.m.); these are the resonances most sensitive to a change in conformation. A difference in conformation-dependent shift between corresponding resonances of two proteins of $\leqslant$15% is taken to indicate, at most, only a minor change in structure. By this criterion the modified proteins studied in this work have similar structures to native cytochrome *c*.

Neurospora crassa cytochrome c

N. crassa cytochrome *c* differs from horse cytochrome *c* in 42 amino acids (Margoliash *et al.*, 1961; Heller & Smith, 1966; Lederer & Simon, 1974). The substitutions involving tyrosine residues are: Phe-46 (horse)→Tyr-46 (*N. crassa*) and Tyr-97 (horse)→Phe-97 (*N. crassa*). Despite the large number of substitutions, the n.m.r. spectra of *N. crassa* cytochrome *c* are very similar to those of horse and tuna cytochrome *c*. An illustration of this is the n.m.r. spectrum of *N. crassa* ferrocytochrome *c* in ^2H_2O at pH 6.5 and 47°C given in Fig. 6. This should be compared with the spectrum of horse ferrocytochrome *c* in Fig. 7 and with the spectra given in Moore & Williams (1980*a*,*b*,*c*,*d*). Resonance assignments for horse and *N. crassa* cytochromes *c* are given in Table 2. The correspondence of chemical shifts for resonances of conserved residues indicates that horse and *N. crassa* cytochromes *c* have the same protein fold.

The substitution of Tyr-46 for phenylalanine has been previously studied by the comparison of horse and tuna cytochromes *c* (Moore & Williams, 1980*d*). Comparison of the conformation-dependent shifts of the *ortho* resonances of the different residues at positions 46 (Table 2*b*) indicates that their environments are similar although conformational differences are apparent from the shifts of the *meta* resonances. The motion of these groups about their $C\beta$–$C\gamma$ bonds (Fig. 1) is similar and in all cases the motion is hindered.

Phe-97 of *N. crassa* cytochrome *c* cannot be compared directly with Tyr-97 of horse cytochrome *c* because its resonances have not yet been assigned. However, the perturbing effects of the substitution are negligible, since resonances of the neighbouring residues Phe-10 and Ala-15 have similar properties to those of horse cytochrome *c*; their chemical shifts are only slightly different (Table 2*a*) and the motions of the Phe-10 residues of both cytochromes *c* are hindered.

Thus the substitution of phenylalanine for tyrosine at position 97 has only a minor perturbing effect on the cytochrome structure, while the substitution

Table 2. *(a) ^1H n.m.r. comparison of N. crassa cytochrome c with horse cytochrome c and (b) conformation-dependent shifts of resonances of residue 46*

	Chemical shift (p.p.m.)			
	Ferrocytochrome *c*		Ferricytochrome *c*	
(*a*) Assignment	Horse	*N. crassa*	Horse	*N. crassa*
Haem *meso*				
α	9.32	9.39	n.d.*	n.d.
β	9.59	9.70	n.d.	n.d.
γ	9.62	9.66	n.d.	n.d.
δ	9.04	9.06	n.d.	n.d.
Met-80				
SCH_3	−3.28	−3.23	−24.6	−22.4
His-18	0.13	0.20	n.d.	n.d.
Ala-15				
αCH	n.d.	n.d.	5.98	5.94
CH_3	n.d.	n.d.	2.21	2.29
Leu-32				
CH_3	−.076	−0.83	n.d.	n.d.
CH_3	−0.60	−0.66	n.d.	n.d.
Ile-57				
δCH_3	−0.49	−0.44	−0.36	−0.27
Phe-10				
ortho	7.10	7.30	n.d.	n.d.
meta	6.70	6.83	n.d.	n.d.
para	6.29	6.18	8.40	8.56
Phe-82				
ortho	6.71	6.72	6.15	6.27
meta	7.40	7.40	6.15	6.27
Trp-59				
C-2	6.99	6.99	6.85	6.85
C-4	7.58	7.68	7.36	7.50
C-5	6.68	6.71	6.53	6.58
C-6	5.76	5.74	6.33	6.35
C-7	7.07	7.10	7.58	7.56
Tyr-74				
ortho	7.22	7.29	7.66	7.76
meta	6.64	6.69	6.81	6.93

(*b*) Species	Assignment	Chemical shift†	Conformation-dependent shift‡
Horse	Phe-46		
	ortho	8.01	0.65
	ortho	7.53	0.19
	meta	7.53	0.19
	meta	6.92	−0.42
	para	7.76	0.42
Tuna	Tyr-46		
	ortho	8.02	0.87
	ortho	7.49	0.34
N. crassa	Tyr-46		
	ortho	8.04	0.89
	ortho	7.62	0.47
	meta	7.49	0.63
	meta	7.29	0.43

* Abbreviation used: n.d., not determined.

† Chemical shifts measured at pH 7 and 47°C for all resonances, excepting those of Ala-15, Ile-57 and Met-80 (ferricytochromes), which were measured at 27°C, and those of residues 46, which were measured at 15°C.

‡ A conformation-dependent shift is the difference in chemical shift of a resonance between its value in simple peptides and its value in the protein.

at position 46 produces larger conformational differences. These have not yet been defined.

[*Hse*^{65},*Leu*^{74}]*Cytochrome c*

The semi-synthetic procedure used to replace Tyr-74 of horse cytochrome *c* with leucine involves

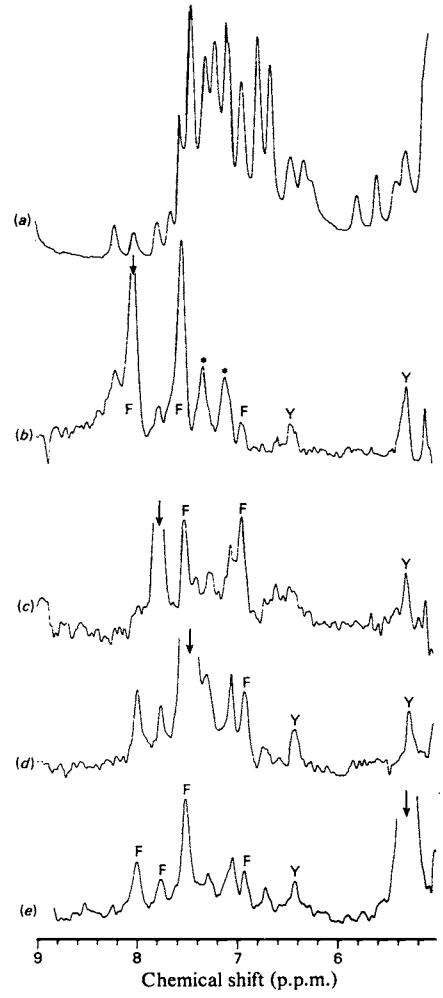

Fig. 5. *300 MHz n.m.r. spectra of horse ferrocytochrome c (100 mM) in ^2H_2O at pH 5.3 and 15°C*

(*a*) is the normal spectrum and (*b*)–(*e*) are n.o.e. difference spectra obtained upon irradiation at the indicated positions (↓) for 0.5 s before acquisition. Resonances marked Y are from Tyr-48, and resonances marked by F are from Phe-46. Resonances marked by * at 7.08 p.p.m. and 7.32 p.p.m. are unassigned; they may come from Tyr-48.

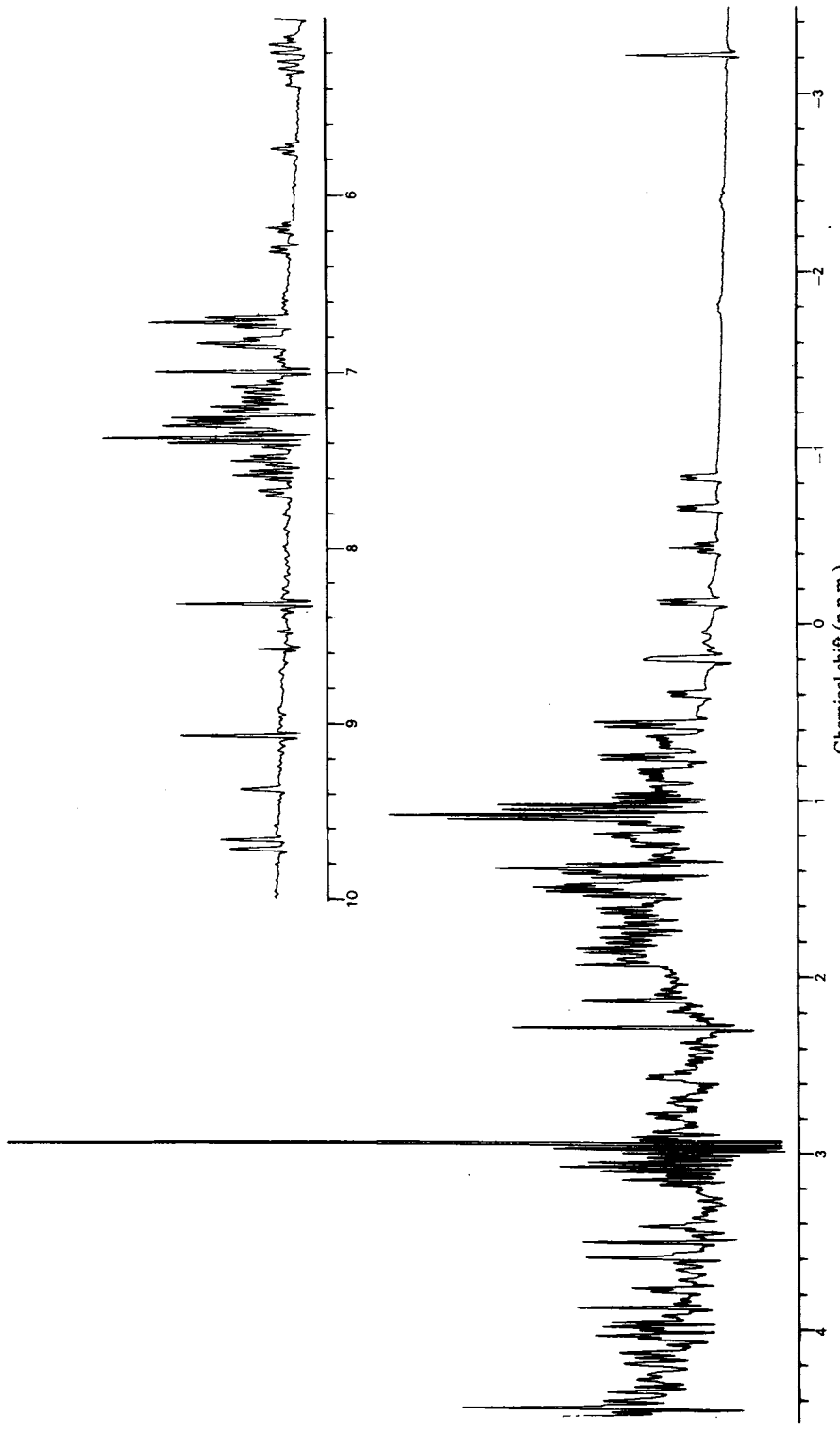

Fig. 6. *300 MHz resolution-enhanced n.m.r. spectrum of N. crassa ferrocytochrome c (3 mM) in ^2H_2O at pH 6.5 and 47°C*

the replacement of Met-65 by homoserine (Boon *et al.*, 1979*a*,*b*). However, the latter modification does not greatly perturb the structure of the protein; only small perturbations of the n.m.r. spectra occur (Boswell *et al.*, 1981) and the protein is as active in biological assays as native cytochrome *c* (Boon *et al.*, 1979*a*; Wallace & Offord, 1979).

Two regions of the convolution difference n.m.r. spectrum of [Hse^{65},Leu^{74}]cytochrome *c* in ^2H_2O at pH 7 and 27°C are given in Fig. 7 with the corresponding regions of the spectrum of native horse ferrocytochrome *c*. There is a striking resemblance between the spectra best exemplified by a comparison of the chemical shifts of conformationally sensitive resonances. This comparison is given in Table 3. With few exceptions the resonances tabulated are those suffering sizeable conformation-dependent shifts and, again with few exceptions, there is a marked similarity in chemical shifts. This indicates that the structure of cytochrome *c* is not greatly perturbed by the replacement of Tyr-74 with leucine. However, there are some differences between spectra of the two proteins.

It is notable that the resonances of Tyr-74 present in the spectrum of native ferrocytochrome *c* (indicated by Y in Fig. 7) are missing from the spectrum of [Hse^{65},Leu^{74}]ferrocytochrome *c*. The slightly broadened resonance at 6.61 p.p.m. of Fig. 7(*b*) is the *meta* resonance of Phe-10 slightly shifted from its position in the spectrum of ferrocytochrome *c* (Table 3); with increasing temperature it sharpens and can be decoupled by irradiation of the *para* resonance at 6.26 p.p.m. Other notable features of these spectra are that the resonances of Trp-59 (marked by W) and Ile-57 (marked by I) are shifted upon modification (Fig. 7 and Table 3). In addition, the resonance of haem methyl-9 is shifted from 2.22 p.p.m. (Redfield & Gupta, 1971) to 2.13 p.p.m. This is the only haem resonance to be significantly shifted.

The X-ray structure of tuna ferrocytochrome *c* (Takano *et al.*, 1977) shows that the haem group, Ile-57, Trp-59 and Tyr-74 are all relatively close; the co-ordinate plot of Fig. 8 illustrates the spatial relationships. In the spectrum of native ferrocytochrome *c*, the haem methyl-8 resonance is perturbed by Trp-59, resonances of Trp-59 by the haem group, Tyr-74 and Tyr-67, resonances of Tyr-74 by Trp-59 and Tyr-67, and the δCH_3 resonance of Ile-57 by Tyr-74 and Trp-59 (Redfield & Gupta, 1971; Moore & Williams, 1980*a*,*b*,*c*; Perkins, 1980). With the replacement of Tyr-74 by leucine a ring-current centre is replaced by a non-aromatic group, resulting in the conformational-dependent shifts of the remaining nuclei being changed, even though their spatial relationships may be unperturbed. It is clear from the magnitude of the shifts that, if there is a conformational change accompanying the modification, it is only a small change. The temperature-dependence of the δCH_3 resonance of Ile-57 provides further support for this conclusion. In native ferrocytochrome *c* this resonance shifts 0.33 p.p.m. downfield with increasing temperature over the range 27–77°C, whereas in the modified protein it shifts 0.06 p.p.m. downfield over the same

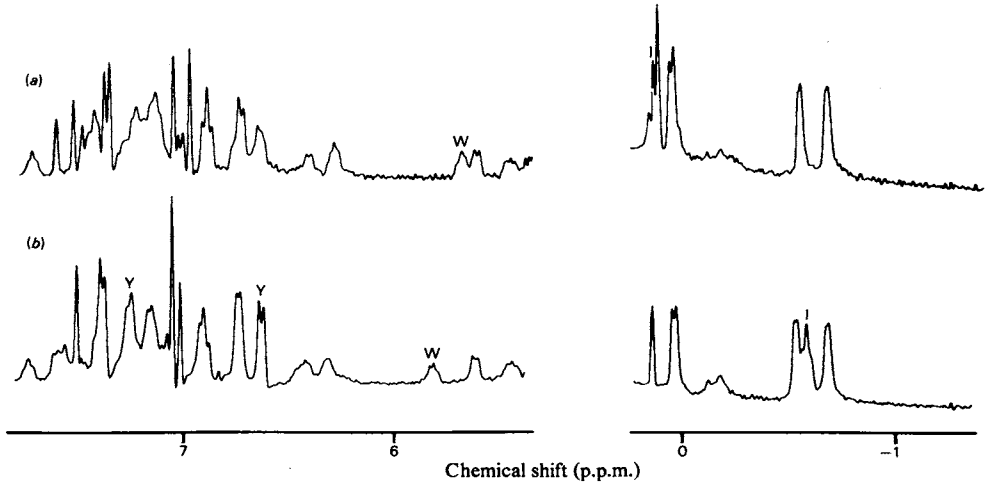

Fig. 7. *Regions of the 300 MHz resolution-enhanced n.m.r. spectra*

(*a*) Horse ferrocytochrome *c* (5 mM) in ^2H_2O at pH 7, and 27°C and (*b*) horse [Hse^{65},Leu^{74}]ferrocytochrome *c* (5 mM) in ^2H_2O at pH 7 and 27°C. Resonances of Tyr-74, Trp-59 and Ile-57 are indicated by Y, W and I respectively.

Table 3. *^1H n.m.r. comparison of modified ferrocytochrome c with the native protein*

Chemical-shift values are given in p.p.m. at 57°C and at pH 6.8 (^2H-labelled 0.1 M-phosphate buffer) for the modified proteins, and at 57°C and pH 7 (unbuffered) for the native protein, for all resonances other than that of Tyr^{97}, which was measured at 27°C.

Assignment	Cytochrome *c* ...	δ (p.p.m.) Horse	[Hse^{65}]-	[Hse^{65}, Leu^{67}]-	[Hse^{65}, Leu^{74}]-
Haem *meso*					
α		9.32	9.32	9.23	9.36
β		9.59	9.59	9.46	9.57
γ		9.62	9.61	9.42	9.59
δ		9.04	9.03	8.79	9.02
His-18					
C-2		0.13	0.12	0.02	0.10
C-4		0.50	n.d.	n.d.	0.47
Met-80	SCH_3	−3.28	−3.28	−3.14	−3.29
Leu-32					
	CH_3	−0.76	−0.76	−0.77	−0.75
	CH_3	−0.60	−0.60	−0.62	−0.61
Trp-59					
C-2		6.99	6.98	n.d.	6.95
C-4		7.58	7.57	n.d.	7.46
C-5		6.68	6.70	n.d.	6.61
C-6		5.76	5.78	n.d.	5.59
C-7		7.07	7.10	n.d.	7.04
Phe-10					
ortho		7.10	7.11	7.11	7.12
meta		6.70	6.70	6.68	6.61
para		6.29	6.26	6.25	6.26
Phe-46	*para*	7.75	7.75	7.75	7.75
Phe-82					
ortho		6.71	6.72	6.83	6.70
meta		7.40	7.39	7.38	7.42
Tyr-97		5.55	5.55	5.57	5.56
Tyr-74					
ortho		7.22	7.21	6.99	—
meta		6.64	6.61	6.71	—
Ile-57	δCH_3	−0.43	−0.48	−0.43	0.15

range of temperature. Much of the difference in temperature-induced shift between the Ile-57 resonances of the two proteins is due to their different conformation-dependent shifts; 1.50 p.p.m. for native ferrocytochrome *c* and 0.78 p.p.m. for [Hse^{65},Leu^{74}]ferrocytochrome *c* at pH 7 and 27°C. In both proteins the resonances of haem methyl-8 and Trp-59 are independent of temperature over the range 27–77°C.

Thus the substitution of leucine for tyrosine at position 74 does not seriously perturb the structure of cytochrome *c*.

[Hse^{65},Leu^{67}]Cytochrome c

Owing to the limited availability of material, the study of [Hse^{65},Leu^{67}]cytochrome *c* was less complete than that of [Hse^{65},Leu^{74}]cytochrome *c*. However, it was sufficiently detailed to establish that the replacement of Tyr-67 by leucine does not greatly perturb the structure of ferrocytochrome *c*.

The spectrum of [Hse^{65},Leu^{67}] ferrocytochrome *c* given in Fig. 9 is clearly identifiable as a spectrum of ferrocytochrome *c* with a native-like structure (compare with Fig. 7). Resonance assignments obtained for this protein are given in Table 3.

No assignments have been obtained for Trp-59. The main reason for this is that the resonance of the C-6 proton has been perturbed from its position in the spectrum of native ferrocytochrome *c*; there are no resonances in the spectrum of Fig. 9 between 5.3 p.p.m. and 6.2 p.p.m. However, resonances of Tyr-74 and Ile-57 have been assigned (Table 3). The Tyr-74 resonances are slightly shifted, but the δCH_3 resonance of Ile-57 is unperturbed. These data fit well with the shifts observed for the analogous resonances of [Hse^{65},Leu^{74}]cytochrome *c* described

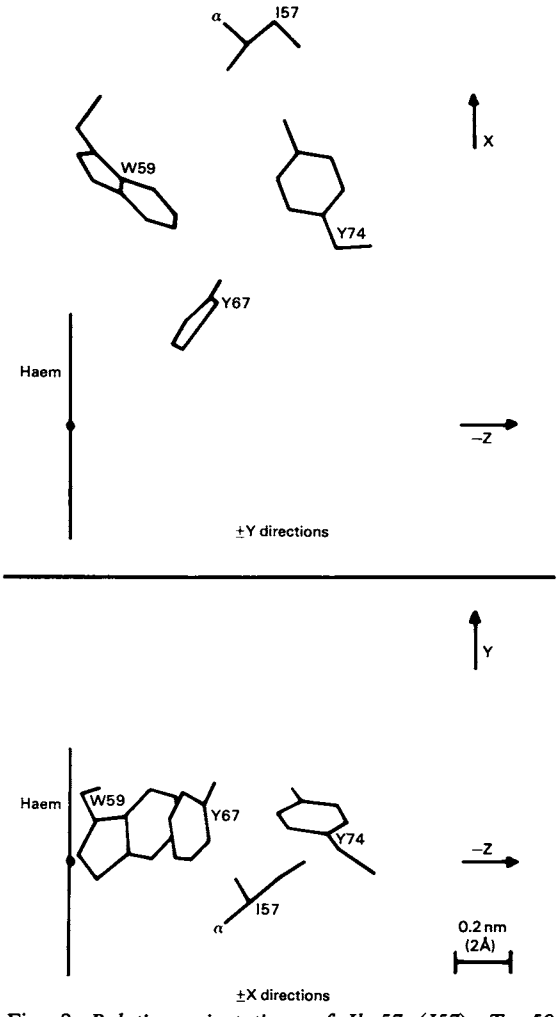

Fig. 8. *Relative orientations of Ile-57 (I57), Trp-59 (W59), Tyr-67* (Y67) *and Tyr-74 (Y74)*
The diagram was constructed from co-ordinates of the X-ray structure of tuna cytochrome *c* (Takano *et al.*, 1977).

in the previous section. With the removal of the Tyr-67 ring-current the resonances of Trp-59 and Tyr-74 would be expected to be perturbed, whereas those of Ile-57 would not (Fig. 8). This does not mean that the replacement of Tyr-67 by leucine does not produce any conformational changes, only that such changes are very small. An indication that a change has occurred is the small shifts of the *ortho* resonance of Phe-82, a resonance that should not be perturbed by the ring current of Tyr-67. A similar slight conformation change is indicated for the [Hse^{65},Leu^{74}]cytochrome *c* where the *meta* resonance of Phe-10 is slightly perturbed by the removal of Tyr-74. Phe-10 is not expected to be perturbed by the ring current of Tyr-74.

Table 4. *Conformation-dependent shifts (p.p.m.) of Tyr-67 and Leu-67*
Chemical shifts were measured at 93°C for Tyr-67 and at 27°C for Leu-67.

	Chemical shift	Primary chemical shift	Conformation-dependent shift
Tyr-67			
ortho	6.73	7.15	−0.42
meta	5.13	6.86	−1.73
Leu-67			
CH_3	0.33	0.94	−0.61
CH_3	−0.22	0.94	−1.16

The two additional methyl resonances in the spectrum of [Hse^{65},Leu^{67}]ferrocytochrome *c* (marked L in Fig. 9) at 0.33 p.p.m. and −0.22 p.p.m. come from Leu-67. In Table 4 their conformation-dependent shifts are compared with those of Tyr-67 of native ferrocytochrome *c*. The similarity in shifts is compatible with the two residues occupying similar spatial positions with respect to the haem group, Trp-59 and Tyr-74. The proximity of Tyr-67 to Met-80 shown by the X-ray structure (Takano *et al.*, 1977) provides the explanation of the downfield shift of the Met-80 SCH_3 resonance of [Hse^{65},Leu^{67}]ferrocytochrome *c* (Table 3). The main source of the Met-80 conformation-dependent shift is the haem group, but Tyr-67 shifts the SCH_3 resonance by a small amount (Perkins, 1980).

Thus the replacement of Tyr-67 by leucine causes only minor perturbations to the structure of cytochrome *c*.

Structural role of tyrosine residues of cytochrome c

The maintained tertiary structure of [Hse^{65},Leu^{67}]cytochrome *c* and [Hse^{65},Leu^{74}]cytochrome *c* is in excellent agreement with the observation that their activities are not much different from that of native cytochrome *c* (Boon *et al.*, 1979*a*; Boon, 1981). One notable feature of the modified proteins is that their stabilities towards denaturing conditions (e.g. temperature) are lower than that of native cytochrome *c* (Boon, 1981). Thus although leucine can fill the role of tyrosine under physiological conditions, outside of these conditions it is not so satisfactory. These observations must be considered together with the dynamic properties of the protein.

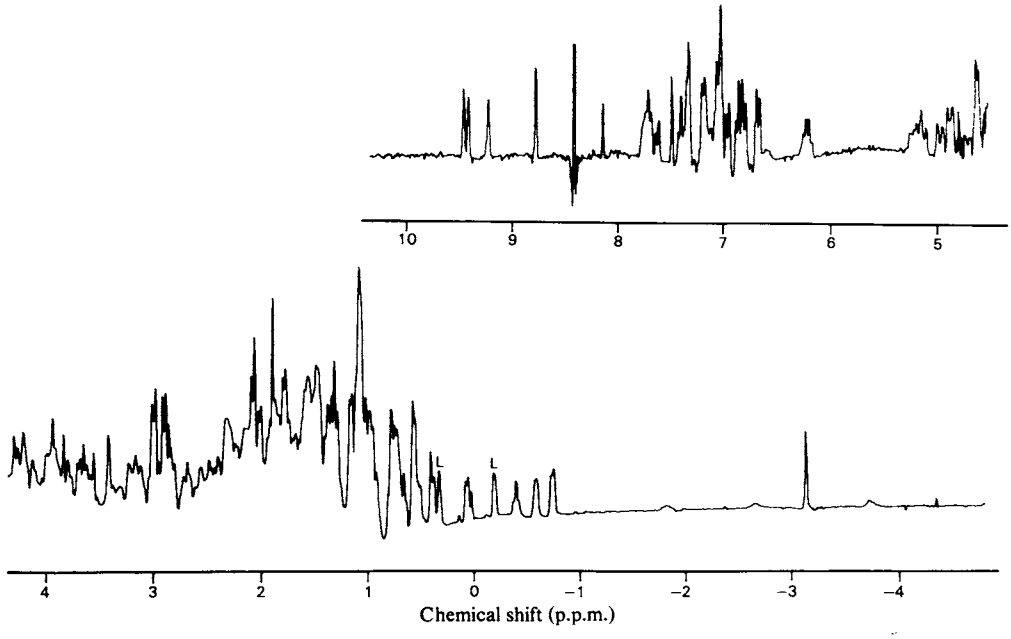

Fig. 9. *270MHz resolution-enhanced n.m.r. spectrum of horse [Hse^{65},Leu^{67}]cytochrome c (5mM) in ^2H_2O at pH6 and 57°C*

Resonances of Leu-67 are indicated by L. The intense resonance at 8.4p.p.m. is from an impurity, most probably formate.

The motion of many aromatic groups of cytochrome *c* is hindered. The activation energy for the flip of Phe-46 and Tyr-97 of horse and tuna ferrocytochromes *c* has been determined to be in the range 101 ± 4–$120 \pm 8\,kJ \cdot mol^{-1}$ (Campbell *et al.*, 1976; Moore & Williams, 1980). The requirement for high temperature to reveal and so assign resonances of Tyr-48 and Tyr-67, and also resonances of Phe-10 (Moore & Williams, 1980*a*; Boswell *et al.*, 1980), indicates that the motion of these groups, too, is hindered. Thus the tyrosine and phenylalanine residues of cytochrome *c* can be divided into two groups: those whose motion is not restricted (Tyr-74, Phe-36 and Phe-82) and those whose motion is restricted (Tyr-48, Tyr-67, Tyr-97, Phe-10 and Phe-46). This correlates rather well with the packing seen in the X-ray structure (Takano *et al.*, 1977). Phe-10 and Tyr-97 are very close together, as are Phe-46 and Tyr-48, and Tyr-67 and the haem group. Thus two aromatic rings adjacent to each other produce a rigid local structure. They could act as structural anchors around which the protein folds.

In addition to the relatively rigid parts of cytochrome *c* around residues 10, 97, 46, 48 and 67, there are relatively flexible parts of the protein. These are around Phe-82, Tyr-74 and Ile-57, and possibly Phe-36. The degree of flexibility of these regions and their importance to the function of cytochrome *c* is not yet known. The region about Phe-82 is particularly interesting, since this residue forms part of the haem crevice (Takano *et al.*, 1977) and is in the centre of the oxidase interaction site (Ferguson-Miller *et al.*, 1978). It may be that the flexibility of the protein about Phe-82 is associated with conformational relaxation during electron transfer.

Conclusions

The structures of *N. crassa* cytochrome *c*, [Hse^{85},Leu^{67}]cytochrome *c* (horse) and [Hse^{65}, Leu^{74}]cytochrome *c* (horse) are very similar to those of tuna and horse cytochromes *c*. Replacement of Tyr-74 by leucine, Tyr-67 by leucine or Tyr-97 by phenylalanine does not significantly perturb the structure of cytochrome *c*.

We thank the Science and Engineering Research Council (S.E.R.C.), the Medical Research Council and the Royal Society for financial support. G. R. M. thanks the S.E.R.C. for an Advanced Fellowship. This is a contribution from the Oxford Enzyme Group.

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