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The conformation of eukaryotic cytochrome *c* around residues 39, 57, 59 and 74

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^{1}H-n.m.r. studies of horse, tuna, *Candida krusei* and *Saccharomyces cerevisiae* cytochromes *c* showed that each of the proteins contains a similar cluster of residues at the bottom of the protein that assists in shielding the haem from the solvent. The relative positions of the residues forming these clusters vary continuously with temperature, and they change with the change in protein redox state. This conformational heterogeneity is discussed with reference to the conformational flexibility of cytochrome *c* around residues 57, 59 and 74. Spectroscopic measurements of pK_a values for Lys-55 (horse and tuna cytochromes *c*) and His-33 and His-39 (*C. krusei* and *S. cerevisiae* cytochromes *c*) are in excellent agreement with expectations based on chemical-modification studies of horse cytochrome *c*. [Bosshard & Zürrer (1980) *J. Biol. Chem.* **255**, 6694–6699] and on the X-ray-crystallographic structure of tuna cytochrome *c* [Takano & Dickerson (1981) *J. Mol. Biol.* **153**, 79–94, 95–115].

The observation that many properties of ferricytochrome *c* are different from those of ferrocytochrome *c* (Margoliash & Schejter, 1966; Dickerson & Timkovich, 1975) has led to a detailed investigation of the oxidation-state-dependence of the structure of cytochrome *c*. After a period of uncertainty, when the results of different X-ray-crystallographic studies suggested either very different structures (Takano *et al.*, 1971, 1973) or identical structures (Mandel *et al.*, 1977; Takano *et al.*, 1977), it is now known that there is a small structural difference (Moore *et al.*, 1980*a*; Takano & Dickerson, 1981*a*,*b*). In order to describe the conformation change fully it is necessary to define the structures of cytochrome *c* in greater detail.

The results of previous ^{1}H-n.m.r. spectroscopic (Moore & Williams, 1980*d*), chemical-modification (Bosshard & Zürrer, 1980) and X-ray-crystallographic studies (Takano & Dickerson, 1980, 1981*a*,*b*) have shown that the region of the protein around residue 57 is one of the regions most affected by the conformation change. In the present paper we report a detailed ^{1}H-n.m.r. study of the conformation of different eukaryotic cytochromes *c* around residue 57. In all animal cytochromes *c* and in *Neurospora crassa* cytochrome *c* this residue is isoleucine (Margoliash *et al.*, 1961; Kreil, 1963, 1965; Heller & Smith, 1966; Lederer & Simon, 1974; Dickerson & Timkovich, 1975), whereas in *Candida krusei* and *Saccharomyces cerevisiae* cytochromes *c* it is valine (Yaoi, 1967; Narita & Titani, 1968, 1969; Lederer *et al.*, 1972; Lederer, 1972). The sequence identity varies from 98% to 50%. Though in the present paper we describe only minor conformational differences within a very stable overall structure, it should be kept in mind that biochemical systems readily distinguish one cytochrome from another (Urbanski & Margoliash, 1977; Margoliash, 1980).

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

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Materials and methods

Horse (type VI), tuna (type XI), cow (type IV), dog (type XVIII), rabbit (type XV), pigeon (type XIII), rat (type XX), *Saccharomyces cerevisiae* iso-1 (type VIII) and *Candida krusei* (type VII) cytochromes *c* were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Guanaco, lamprey and kangaroo cytochromes *c* were gifts from Dr. R. Wever (University of Amsterdam, Amsterdam, The Netherlands), and *Neurospora crassa* cytochrome *c* was a gift from Professor W.

Neupert (Georg-August University, Göttingen, Germany).

Samples were prepared for n.m.r. as previously described (Eley *et al.*, 1982). Quoted pH values are direct meter readings uncorrected for the small isotope effect (Kalinichenko, 1976). The n.m.r. spectra were recorded with a Bruker 270MHz spectrometer, a Bruker WH-300 spectrometer, or a 470MHz spectrometer equipped with an Oxford Instruments Co. magnet, a Nicolet 1180 computer and 293A pulse controller. Resolution enhancement (Ernst, 1966; Campbell *et al.*, 1973), saturation-transfer (Redfield & Gupta, 1971) and nuclear-Overhauser-enhancement (n.o.e.) experiments (Boswell *et al.*, 1980*a*) were performed as previously described. 1,4-Dioxan was used as an internal standard, but all 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

The n.m.r. experiments reported in the present paper are based on previous n.m.r. work that has led to the assignment of a wide range of resonances of horse and tuna cytochromes *c* (McDonald & Phillips, 1973; Keller & Wüthrich, 1978, 1981; Boswell *et al.*, 1980*a*; Moore & Williams, 1980*a*,*b*,*e*; Moore *et al.*, 1980*a*,*b*; Eley *et al.*, 1982). The principal assignments used are those of His-39, Ile-57 or Val-57, Trp-59 and Tyr-74. The n.m.r. experiments were designed to assign resonances of these groups, and to monitor their response to variations in solution conditions.

N.m.r. spectroscopy of horse cytochrome c

Horse cytochrome *c* contains Lys-39, Ile-57, Trp-59 and Tyr-74 (Margoliash *et al.*, 1961). Aromatic resonances of Trp-59 and Tyr-74 and some resonances of Ile-57 have been previously assigned (Moore & Williams, 1980*a*,*b*). Further resonance assignments of Ile-57 are presented below.

In Fig. 1 are shown regions of the n.o.e. difference spectra of horse ferrocytochrome *c* in ^2H_2O at pH5.3 at various temperatures between 27°C and 67°C, which result from irradiation of the temperature-dependent triplet resonance of Ile-57 at −0.43p.p.m. (57°C). Between 27°C and 47°C the Ile-57 resonance overlaps with a methyl resonance of Leu-32. At 77°C irradiation of the Leu-32 resonance at −0.60p.p.m., which no longer overlaps an Ile-57 resonance, resulted in the peaks marked by * in Fig. 1 appearing in the n.o.e. difference spectrum. These peaks are due to the haem group, Phe-10, His-18 and Val-20, all of which are relatively close to Leu-32 and far from Ile-57 (Takano *et al.*, 1977). Their appearance in the difference spectra in Fig. 1 results from the overlap of the methyl resonances of Ile-57 and Leu-32, and consequently with increasing temperature their intensity decreases. The remaining resonances in the spectra in Fig. 1 arise from the irradiation of the δ-CH_3 resonance of Ile-57. The resonances in the region 8p.p.m. to 5.5p.p.m. are from Tyr-74 and Trp-59 (Table 1). With the exception of resonances at approx. 0.5p.p.m. and approx. 0.9p.p.m., which are the two γ-CH proton resonances of Ile-57, the resonances in the region 2.5p.p.m. to 0.0p.p.m. have not been previously assigned.

Irradiation of the broad resonance at approx. 1.8p.p.m. (57°C) resulted in the spin-decoupling of a doublet resonance at 0.72p.p.m. No other methyl resonance decoupled. These two peaks are assigned to the CH–CH_3 spin-system of an isoleucine residue (Campbell *et al.*, 1975), and specifically to Ile-57. Other spin-decoupling experiments showed that the doublet methyl resonances at 1.4p.p.m. and 0.9p.p.m. (Fig. 1) are coupled to one-proton resonances at 4.54p.p.m. and 4.46p.p.m. (27°C) respectively. Therefore these resonances must arise from alanine or threonine residues. Their further assignment to a specific amino acid is not certain, but candidate groups are Thr-40 and Thr-63.

Analogous double-resonance experiments have led to the assignment of the β-CH and γ-CH_3 resonances of Ile-57 of horse ferricytochrome *c* (Table 2).

Integration of the resonances in the n.o.e. difference spectra in Fig. 1 that arise from irradiation of the Ile-57 δ-CH_3 resonance shows that, whereas with increasing temperature the n.o.e. to resonances of Trp-59 rapidly decreases, the n.o.e. to the *meta* resonance of Tyr-74 falls off to a much smaller extent. Also, the n.o.e. to the methyl resonances are little affected by temperature. The decrease in n.o.e. between Ile-57 and Trp-59 could originate from a change in the average distance between the two groups or from a change in the correlation time governing the n.o.e. (Noggle & Schirmer, 1971). Since the correlation time will most probably be the same for both Trp-59 and Tyr-74, the decrease in n.o.e. for Trp-59 must be due to a change in geometry. The temperature-dependence of the n.o.e. from groups in this region of the protein whose geometries are fixed (Tyr-74 *m* to Tyr-74 *o* and Trp-59 C-6 to Trp-59 C-5 and C-7) also shows that a variation in correlation times is unlikely to account for the diminished Ile-57–Trp-59 n.o.e.

With increasing temperature the chemical shifts of the Ile-57 methyl resonances vary (Fig. 2), with the δ-CH_3 resonance shifting downfield and the γ-CH_3 resonance shifting upfield. Over the temperature range 27–97°C resonances of Tyr-74 are slightly temperature-dependent, the *meta* resonance shifting

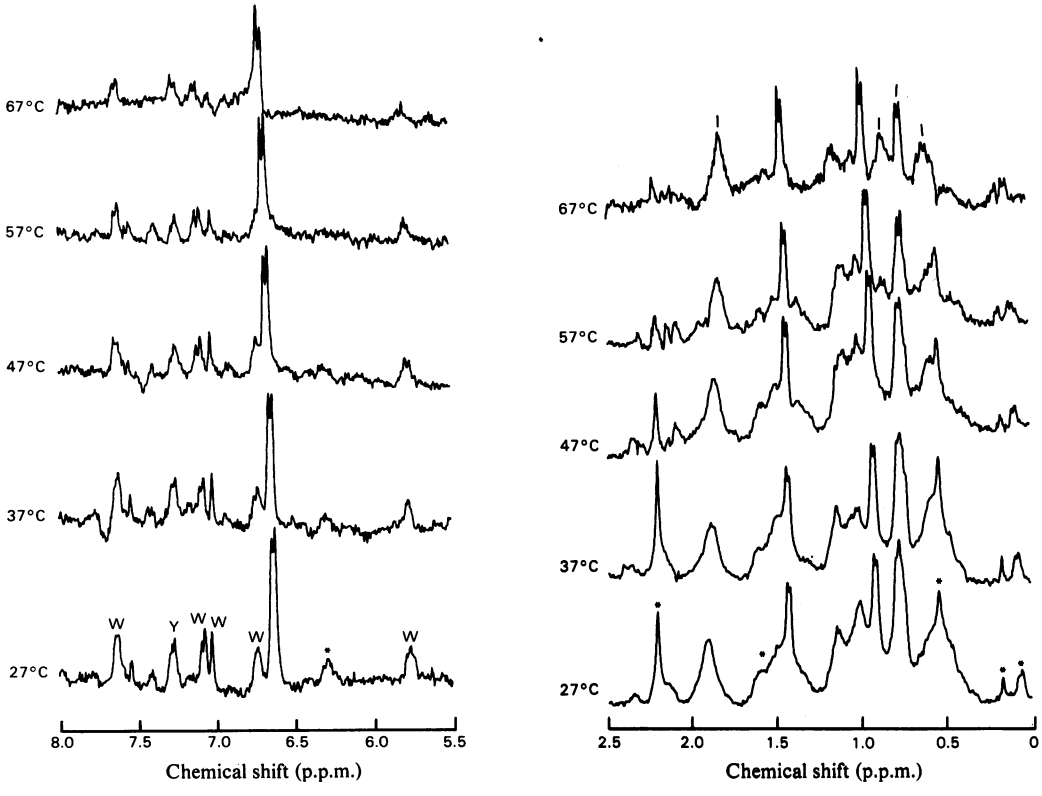


Fig. 1. *300 MHz n.o.e. difference spectra of horse ferrocytochrome c (5 mM) in ^2H_2O at pH 5.3 obtained on irradiation of the Ile-57 δ-CH_3 resonance at <0 p.p.m. (not shown) for 0.5 s before acquisition at various temperatures*
Resonances of Trp-59, Tyr-74 and Ile-57 are indicated by W, Y and I respectively. The resonances indicated by * result from irradiation of an Leu-32 resonance that overlaps with the Ile-57 resonance at some temperatures (see the text).

Table 1. *Comparison of chemical shifts of resonances of Tyr-74 and Trp-59 of eukaryotic ferrocytochromes c at pH 7 at 27°C*

	Chemical shift (p.p.m.)						
	Tyr-74		Trp-59				
Cytochrome *c*	*o*	*m*	C-2	C-4	C-5	C-6	C-7
Horse	7.21	6.61	6.99	7.58	6.68	5.76	7.07
Tuna	7.22	6.67	7.02	7.58	6.72	5.76	7.07
N. crassa	7.30	6.70	6.99	7.68	6.61	5.74	7.07
S. cerevisiae	7.25	6.66	6.93	7.54	6.75	5.74	7.18
C. krusei	7.10	6.80	6.92	7.28	6.85	5.69	7.26

downfield by 0.08 p.p.m., but resonances of Trp-59 are largely temperature-independent, the largest shift being that for the C-6 proton, which shifts downfield by 0.03 p.p.m. (Moore & Williams, 1980*c*). Taken together with the n.o.e. data, these data indicate that the average position of the Ile-57 side chain within the protein changes with increasing temperature.

N.m.r. spectroscopy of tuna cytochrome c

Tuna cytochrome *c* contains Lys-39, Ile-57, Trp-59 and Tyr-74 (Kreil, 1963, 1965). Resonances

Table 2. *Comparison of chemical shifts of resonances of Ile-57 and Val-57 of eukaryotic cytochromes c at pH 7*
Abbreviations: N.D., not determined; c.d.s., conformation-dependent shift. A c.d.s. is the difference in chemical shift of a resonance between its value in simple peptides and its value in the protein. The values for valine and isoleucine in p.p.m. in simple peptides are (Bundi & Wüthrich, 1979): valine: α, 4.18; β, 2.13; γ, 0.97 and 0.94; isoleucine: β, 1.89; γ-CH_2, 1.48 and 1.19; γ-CH_3, 0.94; δ, 0.89. The furthest downfield methyl resonances of Val-57 of the yeast proteins were taken to be the 0.97 p.p.m. resonances.

		Ferrocytochrome *c*			Ferricytochrome *c*		
Cytochrome *c* (residue)	Proton	δ (p.p.m.)	C.d.s. (p.p.m.)	Temp. (°C)	δ (p.p.m.)	C.d.s. (p.p.m.)	Temp. (°C)
Horse (Ile-57)	β-CH	1.92	0.03	27	1.91	0.02	27
	γ-CH_3	0.81	−0.17	27	0.88	−0.15	27
	γ-CH	0.52	N.D.	67	N.D.		
	γ-CH	0.90	N.D.	67	N.D.		
	δ-CH_3	−0.61	−1.50	27	−0.38	−1.27	27
Tuna (Ile-57)	β-CH	1.8	−0.08	27	1.75	−0.13	27
	γ-CH_3	0.73	−0.21	27	0.79	−0.15	27
	δ-CH_3	−0.56	−1.45	27	−0.26	−1.15	27
Rat (Ile-57)	δ-CH_3	−0.67	−1.56	27	−0.44	−1.33	27
Rabbit (Ile-57)	δ-CH_3	−0.68	−1.57	27	−0.44	−1.33	27
Guanaco (Ile-57)	δ-CH_3	−0.66	−1.55	27	−0.45	−1.34	27
N. crassa (Ile-57)	β-CH	1.89	0.00	27	N.D.		
	γ-CH_3	0.76	−0.18	27	N.D.		
	δ-CH_3	−0.55	−1.44	27	−0.27	−1.16	27
S. cerevisiae (Val-57)	α-CH	3.74	−0.44	47	N.D.		
	β-CH	1.03	−0.10	47	N.D.		
	γ-CH_3	0.45	−1.52	27	N.D.		
	γ-CH_3	−0.24	−1.18	27	N.D.		
C. krusei (Val-57)	β-CH	0.57	−1.56	27	0.88	−1.25	27
	γ-CH_3	0.63	−0.34	27	0.80	−0.17	27
	γ-CH_3	−0.18	−1.12	27	−0.08	−1.02	27

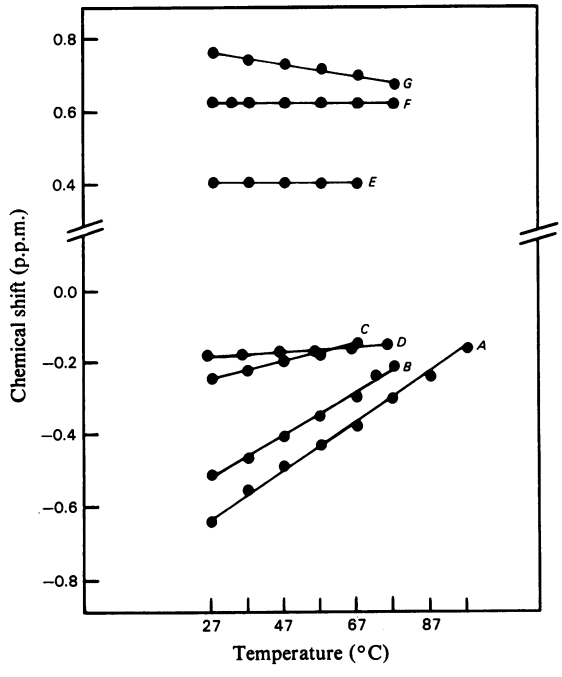


Fig. 2. *Temperature-dependence of methyl resonances of ferrocytochromes c in ^2H_2O at pH 7*
A, Ile-57 δ-CH_3, horse; *B*, Ile-57 δ-CH_3, *N. crassa*; *C*, Val-57 γCH_3, *S. cerevisiae*; *D*, Val-57 γ-CH_3, *C. krusei*; *E*, Val-57 γ-CH_3, *S. cerevisiae*; *F*, Val-57 γ-CH_3, *C. krusei*; *G*, Ile-57 δ-CH_3, horse.

of Trp-59 and Tyr-74 have been previously assigned and resonances of Ile-57 were assigned in an analogous manner to the corresponding resonances of horse cytochrome *c*. The assignments are summarized in Tables 1 and 2.

The pH-dependence of the Ile-57 δ-CH_3 and the Tyr-74 *m* resonances of tuna ferrocytochrome *c* at 27°C over the range pH 7–12.2 is shown in Figs. 3 and 4. With increasing pH the resonances shift downfield. Most of the ferrocytochrome *c* resonances, including those of Trp-59, are pH-independent until the protein denatures at approx. pH 13. N.o.e. experiments at pH 7 and pH 11, both at 27°C, have identified the γ-CH_3 resonance of Ile-57 at 0.73 p.p.m. and 0.63 p.p.m. respectively.

The spectra of tuna ferrocytochrome *c* given in Fig. 4 show that as the Ile-57 δ-CH_3 resonance shifts

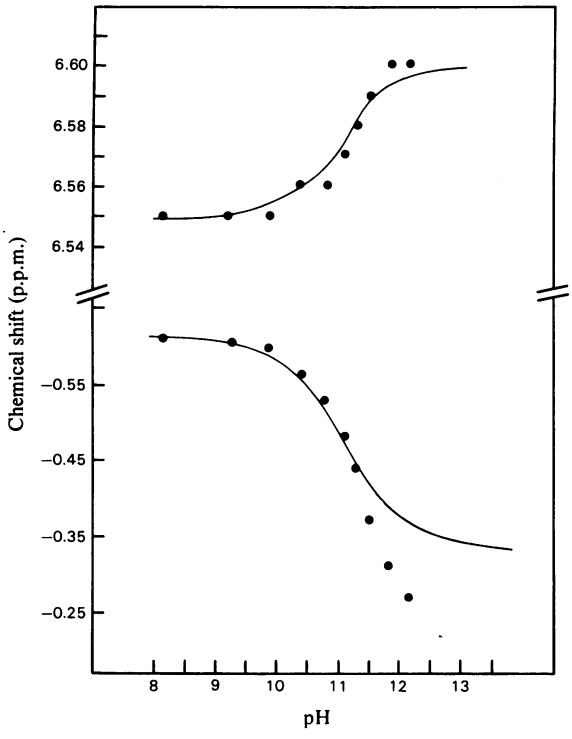


Fig. 3. *pH-dependence of the meta resonance of Tyr-74 (top) and the δ-CH_3 resonance of Ile-57 (bottom) of tuna ferrocytochrome c in ^2H_2O at 27°C*

The continuous lines are theoretical curves drawn for a one-proton ionization with a pK_a of 11.15. The ● symbols represent experimental points.

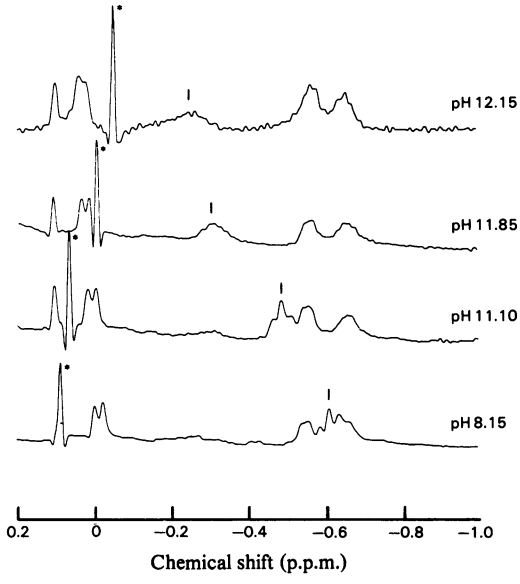


Fig. 4. *Regions of the 300MHz resolution-enhanced n.m.r. spectra of tuna ferrocytochrome c (4mM) in ^2H_2O at various pH values at 27°C*

The δ-CH_3 resonance of Ile-57 is indicated by I. The singlet resonance indicated by * is from an impurity.

at high pH its linewidth increases. This is a specific effect to this resonance, since the linewidths of other resonances, including those of Tyr-74, are not perturbed. Such variations in linewidths are common where a structure possesses conformational heterogeneity with appreciable interchange of molecules from one conformation to another (Jardetzky & Roberts, 1981).

The pH-dependence of the Ile-57 resonances of tuna ferrocytochrome *c* is similar both to the temperature-dependence of the Ile-57 resonances of tuna and horse ferrocytochromes (Moore & Williams, 1980*c*,*e*) and to the pH-dependence of the Ile-57 δ-CH_3 resonance of horse ferrocytochrome *c* (Boswell *et al.*, 1983); with increasing pH or temperature, below the denaturation point, the δ-CH_3 resonance shifts downfield and the γ-CH_3 resonance shifts upfield. A large increase in linewidth only occurs for the pH-dependence. The cause of the pH-induced chemical-shift variation is the ionization of a lysine residue (Boswell *et al.*, 1983). This produces a similar change in conformation to that produced by a variation in temperature: the position of Ile-57 with respect to the surrounding amino acid side chains is perturbed.

N.m.r. spectroscopy of C. krusei cytochrome c

C. krusei cytochrome *c* contains His-39, Val-57, Trp-59 and Tyr-74 (Narita & Titani, 1968; Lederer, 1972). Resonances of Trp-59 and Tyr-74 (Table 1) were identified by standard methods (Moore & Williams, 1980*a*). Resonances of Val-57 were identified by a series of double-resonance experiments (Table 2). The initial assumption in this series of experiments was that the side chain of Val-57 occupied a similar position with respect to the remainder of the protein as does that of Ile-57 of horse and tuna cytochromes *c*. The success of the assignment procedure affirms the validity of the assumption.

In the region between 0.5p.p.m. and −2.0p.p.m. of the spectrum of *C. krusei* ferrocytochrome *c* in ^2H_2O at pH7 at 47°C there are seven doublet resonances (Fig. 5*a*). Gated irradiation of the resolved triplet at 5.69p.p.m. belonging to the C-6 proton of Trp-59 produced an n.o.e. to two methyl groups, one at 0.63p.p.m. and the other at −0.18p.p.m. (Fig. 5*c*). Irradiation of the methyl resonance at −0.18p.p.m. produced n.o.e. effects to resonances of Tyr-74 and Trp-59 (not shown) and an n.o.e. to the methyl resonance at 0.63p.p.m. (Fig.

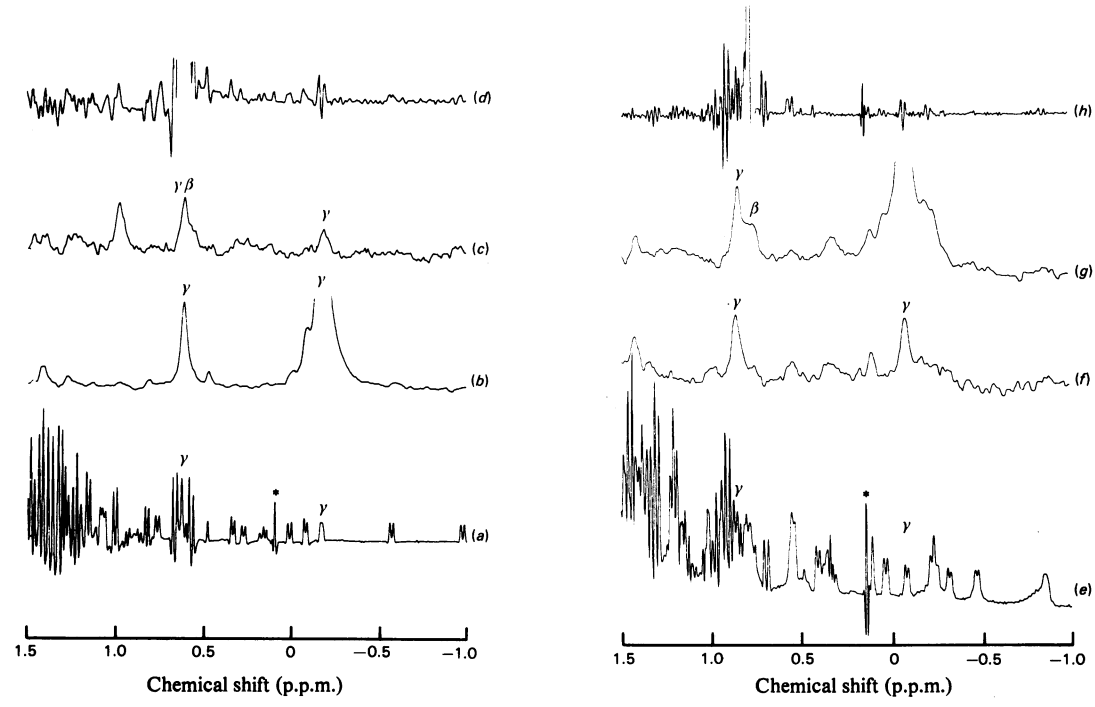
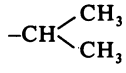


Fig. 5. *300 MHz n.m.r. spectra of C. krusei ferrocytochrome c (a–d) and C. krusei ferricytochrome c (e–h) (5 mM) in ^2H_2O at pH 7*

(*a*) Resolution-enhanced spectrum at 47°C; (*b*) and (*c*) n.o.e. difference spectra resulting from irradiation of (*b*) the γ-CH_3 resonance of Val-57 at −0.18 p.p.m., for 0.5 s before acquisition, and (*c*) the C-6 responance of Trp-59 at 5.69 p.p.m. (not shown); (*d*) resolution-enhanced spin-decoupling difference spectrum at 47°C showing decoupling of the peak at −0.18 p.p.m. resulting from irradiation of the β-CH resonance of Val-57 at 0.57 p.p.m.; (*e*) resolution-enhanced spectrum at 27°C; (*f*) and (*g*) n.o.e. difference spectra resulting from irradiation of (*f*) the *meta* resonance of Tyr-74 at 7.07 p.p.m. (not shown) and (*g*) the γ-CH_3 resonance of Val-57 at −0.08 p.p.m. for 0.5 s before acquisition; (*h*) resolution-enhanced spin-decoupling difference spectrum at 27°C showing decoupling of the peak at −0.08 p.p.m. resulting from irradiation of the β-CH resonance of Val-57 at 0.80 p.p.m. The γ-CH_3 and β-CH resonances of Val-57 are indicated by γ and β respectively. The singlet resonance indicated by * is from an impurity.

5*b*). Spin-decoupling (Fig. 5*d*) and lineshape analysis of the peak at 0.63 p.p.m. (Fig. 5*a*) established that the resonances at 0.57 p.p.m., 0.63 p.p.m. and −0.18 p.p.m. were from a



spin-system, and therefore they are assigned to a valine or leucine residue close to Trp-59 and Tyr-74. Only Val-57 is expected to be in such a position, and thus they are assigned to Val-57. Attempts to assign the Val-57 resonances of ferricytochrome *c* by saturation transfer from the reduced protein failed, because the chemical shifts of the corresponding resonances were too similar. However, the upfield methyl resonances of Val-57 were cross-assigned in a mixture of ferricytochrome *c* and ferrocytochrome *c* under conditions where there was relatively rapid electron exchange (M. N. Robinson, unpublished work). Assignment of the methyl resonance of ferricytochrome *c* at −0.08 p.p.m. to Val-57 was confirmed by the n.o.e. resulting from irradiation of the Tyr-74 resonance at 7.07 p.p.m. (Fig. 5*f*). Subsequent spin-decoupling experiments involving the methyl resonance at −0.08 p.p.m. only clearly identify a CH–CH_3 spin-system. This is because the β-CH resonance and one of the methyl resonances almost overlap at approx. 0.84 p.p.m.: this can be clearly seen in the difference spectrum in Fig. 5(*g*). The assignments to Val-57 are supported by the assignment of resonances of seven of the remaining eight valine and leucine spin-systems (M. N. Robinson, unpublished work).

The methyl resonance of Val-57 of *C. krusei* ferrocytochrome *c* at −0.18 p.p.m. is slightly temperature-dependent at pH 7, whereas the resonance

at 0.63 p.p.m. is temperature-independent (Fig. 2). The methyl resonances of ferrocytochrome *c* are pH-independent between pH 5.3 and pH 11.3 at 27°C.

With increasing pH from pH 5 to pH 8 at 27°C two one-proton intensity singlet resonances at approx. 8.7 p.p.m. (pH 5) in the spectra of *C. krusei* ferricytochrome *c* and ferrocytochrome *c* shifted upfield. These resonances are from the C-2 protons of His-33 and His-39. The other histidine residues, His-18 and His-26, do not titrate with pH (McDonald & Phillips, 1973; Cohen *et al.*, 1974; Dobson *et al.*, 1975). Analysis of the pH-induced shifts show that one histidine residue of ferrocytochrome *c* titrates with a pK_a of 7.3 ± 0.05 and that one histidine residue of ferricytochrome *c* titrates with a pK_a of 6.65 ± 0.05. It was not possible to determine all the pK_a values, because there was extensive broadening of the histidine resonances throughout the titration and this prevented measurements of chemical shifts. However, Cohen & Hayes (1974) have reported that His-33 and His-39 of *C. krusei* ferricytochrome *c* titrate with pK_a values of 6.74 and 6.56; extensive line-broadening was not a problem in their study. The titration of *S. cerevisiae* cytochrome *c* reported in the following subsection was more satisfactory.

N.m.r. spectroscopy of S. cerevisiae iso-1 cytochrome c

S. cerevisiae iso-1 cytochrome *c* contains His-39, Val-57, Trp-59 and Tyr-74 (Yaoi, 1967; Narita & Titani, 1969; Lederer *et al.*, 1972). Resonances of Val-57, Trp-59 and Tyr-74 of the ferrous protein were assigned by the procedures already described (Tables 1 and 2). N.o.e. were observed between resonances of Ile-57, Trp-59 and Tyr-74. The methyl resonance of Val-57 at −0.19 p.p.m. (47°C) was temperature-dependent at pH 7, whereas the resonance at 0.45 p.p.m. was temperature-independent (Fig. 2). Resonances of Trp-59 were independent of temperature over the same temperature range, but the *meta* resonance of Tyr-74 shifted downfield by 0.04 p.p.m. Resonances of Val-57, Trp-59 and Tyr-74 were pH-independent over the range pH 5–11 at 27°C.

Variation in pH over the range pH 4–11 for *S. cerevisiae* ferrocytochrome *c* and pH 5–8.5 for *S. cerevisiae* ferricytochrome *c* resulted in a number of one-proton singlet resonances shifting. These shifts were due to titration of the side chains of His-33 and His-39. As with *C. krusei* cytochrome *c*, there was line-broadening for some of the singlet resonances throughout the titration, but unlike *C. krusei* cytochrome *c* it was possible to follow resonances and obtain accurate chemical shifts. The graph in Fig. 6 illustrates the magnitude of shift for two resonances of *S. cerevisiae* ferrocytochrome *c*, which remained reasonably sharp throughout the titration. The two residues titrate with pK_a values of 6.7 ± 0.05 and 7.2 ± 0.05. Resonance linewidths for one histidine residue were unchanged on oxidation of the protein, but the resonance linewidths of the second histidine residue were markedly perturbed. However, it was possible to estimate the pK_a by determining chemical shifts at the acidic and the basic ends of the titration. Extensive line-broadening occurred at pH values of $pK_a \pm 0.5$ pH unit. It

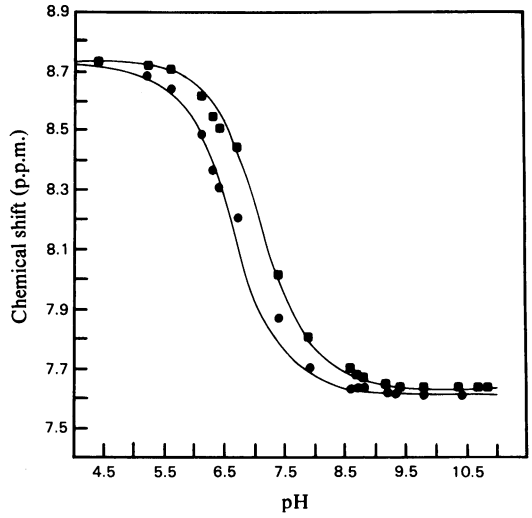


Fig. 6. *pH-dependence of the C-2 resonances of His-33* (●) *and His-39* (■) *of S. cerevisiae iso-1 ferrocytochrome c in ^2H_2O at 27°C*

The continuous lines are theoretical curves drawn for one-proton ionizations with pK_a values of 6.7 (lower) and 7.2 (upper). The ● and ■ symbols represent experimental points.

Table 3. *pK_a values of histidine residues of cytochromes c at 25–27°C*

Key to references: a, Dobson *et al.* (1975); b, Cohen *et al.* (1974); c, Cohen & Hayes (1974); d, present work. Abbreviation: N.D., not determined.

Cytochrome *c* residue	pK_a Ferrocytochrome *c*	pK_a Ferricytochrome *c*	Reference
Horse			
His-33	6.5 ± 0.05	6.4 ± 0.05	a
	6.54 ± 0.02	6.41 ± 0.02	b
C. krusei			
His-33	N.D.	6.56 ± 0.09	c
His-39	7.3 ± 0.05	6.65 ± 0.05	d
		6.74 ± 0.01	c
S. cerevisiae			
His-33	6.7 ± 0.05	6.8 ± 0.05	d
His-39	7.2 ± 0.05	6.7 ± 0.2	d

proved to be impossible to locate and titrate all of the histidine C-4 resonances.

The histidine pK_a values for horse, *C. krusei* and *S. cerevisiae* cytochromes *c* are summarized in Table 3. The data allow the specific assignment of pK_a values of the yeast cytochromes to His-33 and His-39 by comparison with horse cytochrome *c*, which does not contain His-39. Despite the problems in determining precise pK_a values for the yeast cytochromes, the data clearly show that there is an oxidation-state-dependence to the pK_a of His-39. This reflects a difference in the environment of the group in the two oxidation states, a difference that is probably linked to the redox conformation change.

N.m.r. spectroscopy of other eukaryotic cytochromes c

Nine other cytochromes *c* were investigated (see the list given in the Materials and methods section), though to a more limited extent than those already described. They all contained Lys-39, Ile-57, Trp-59 and Tyr-74 (Dickerson & Timkovich, 1975). Resonances were assigned without any difficulty (Tables 1, 2 and 4). The data show that the general behaviour of resonances of Ile-57 is maintained throughout a range of different proteins varying in three to 41 amino acid residues out of 104. Some small differences in chemical shifts (Tables 2 and 4) do exist as a result of amino acid substitutions in the region of the protein around Ile-57. These differences are discussed further below.

Resonances of Ile-57 were temperature-dependent, with similar properties to the Ile-57 resonances of horse cytochrome *c*. The pH-dependence of the Ile-57 δ-CH_3 resonances of lamprey and *N. crassa* ferrocytochromes *c* was determined because these proteins have amino acid substitutions involving ionizable groups that may affect Ile-57. The resonance of lamprey ferrocytochrome *c* was pH-independent over the range pH 3.6–7 at 27°C, and the resonance of *N. crassa* ferrocytochrome *c* shifted downfield with increasing pH above pH 9.5 at 27°C to the same extent as the corresponding resonance of tuna ferrocytochrome *c*.

In spectra of horse ferrocytochrome at pH below 4.2 the δ-CH_3 resonance of Ile-57 is pH-dependent, and it shifts downfield with decreasing pH (Moore & Williams, 1980*c*). This shift was suggested to be due to the ionization of Glu-66, which is close to Tyr-74 and Ile-57 (Takano & Dickerson, 1981*a*,*b*). The lack of pH-dependence at low pH of resonances of Ile-57 of lamprey ferrocytochrome *c*, which contains valine in place of Glu-66, supports the suggestion that Glu-66 influences Ile-57 of horse cytochrome *c*.

In *N. crassa* cytochrome *c* residue 72 is trimethyl-lysine (Heller & Smith, 1966; Lederer & Simon, 1974), and, since the Ile-57 δ-CH_3 resonance has the same pH-dependent properties as does horse and tuna cytochromes *c* at high pH, the ionization of Lys-72, which is near to Tyr-74, is not the cause of the Ile-57 pH-dependence.

Structure of eukaryotic cytochromes c

Most discussions of the amino acid sequence variation of eukaryotic cytochromes assume that the tertiary structures of the different proteins are the same and that the only differences are in the disposition of side chains, some of which are conserved and some substituted (Margoliash, 1980). Putting together all the available structural data (Dickerson & Timkovich, 1975; Moore & Williams, 1980*e*,*f*; Shelnutt *et al.*, 1981) indicates that this may not be true and that, in addition to local side-chain changes, there may be small co-operative conformational changes over large distances. Thus each particular protein needs to be investigated independently.

Table 4. *Comparison of chemical shifts of resonances of the Ile-57 δ-CH_3 groups of various cytochromes c at pH 5.3 at 57°C*

The amino acid sequences are taken from the compilation made by Dickerson & Timkovich (1975). The one-letter amino acid code is used: n, asparagine; i, isoleucine; t, threonine; w, tryptophan; k, lysine; e, glutamic acid; g, glycine; d, aspartic acid; q, glutamine; s, serine; v, valine. Upper-case letters indicate that the particular residue is conserved throughout the cytochromes given in the Table.

	Chemical shift (p.p.m.)		Sequence	Changes from sequence
Cytochrome *c*	Ferrocytochrome *c*	Ferricytochrome *c*	54-57-58-59-60-61-62	of horse protein
Horse	−0.43	−0.18	n I t W k e e	0
Cow	−0.49	−0.25		3
Dog	−0.48	−0.25		6
Guanaco	−0.49	−0.25	n I t W g e e/d	4
Rabbit	−0.49	−0.29		6
Pigeon	−0.48	−0.27		11
Kangaroo	−0.49	−0.27	n I i W n q e	7
Tuna	−0.34	−0.07	s I v W d e n	19
N. crassa	−0.32	−0.03	q I t W d e n	40

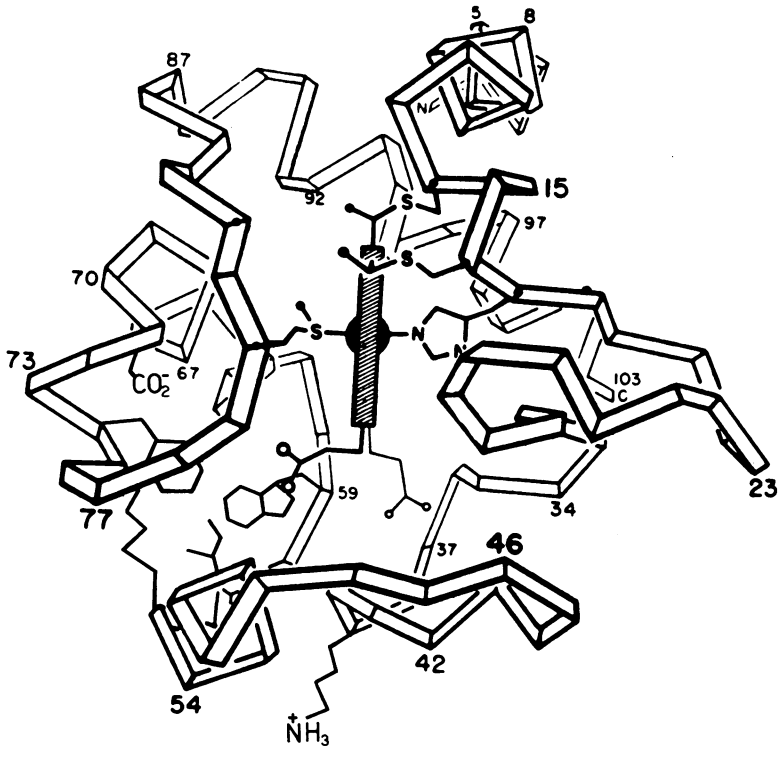


Fig. 7. *Ribbon diagram of the chain folding of tuna ferrocytochrome c showing the relative positions of the side chains of Lys-39, Lys-55, Ile-57, Trp-59, Glu-66 and Tyr-74*

The proteins studied in the present work fall into two classes: those containing Lys-39 and Ile-57 and those containing His-39 and Val-57. The ribbon diagram of the chain folding of tuna cytochrome *c* given in Fig. 7 illustrates the relationship of Lys-39 to Ile-57. The loop of residues containing Lys-39 and the loop of residues containing Ile-57 are close together and form the bottom of the cytochrome molecule. Fig. 7 also illustrates the relationship between Ile-57, Trp-59 and Tyr-74 (a different view of this structural unit is given in Fig. 8 of Eley *et al.*, 1982). Trp-59 and Tyr-74 are conserved throughout the proteins studied in the present work, and, although the chemical shifts of their resonances vary (Table 1), the similarity in the shifts indicates that they are involved in similar structural units. Even for the most different protein, *C. krusei* ferrocytochrome *c*, the large upfield shifts of the C-5 and C-6 proton resonances of Trp-59 show that this group has a similar environment to the Trp-59 residues of the other cytochromes: the unperturbed chemical shifts of the C-5 and C-6 proton resonances of tryptophan are 7.17 p.p.m. and 7.24 p.p.m. (Bundi & Wüthrich, 1979). It is not clear why *C. krusei* cytochrome *c* is so different from the others, although the data for *S. cerevisiae* cytochrome *c* show that it is not simply due to the replacement of Ile-57 by Val-57.

There are two further n.m.r. probes of the 57/59/74 structural unit. Firstly, there are n.o.e. between resonances of the three groups, and, secondly, the δ-CH_3 resonance of Ile-57 is shifted upfield. The chemical shifts of the Ile-57 δ-CH_3 resonances of the ferrocytochromes *c* at pH 7 at 27°C are in the range −0.55 p.p.m. to −0.68 p.p.m., whereas the chemical shifts of the corresponding γ-CH_3 resonances are in the range 0.73 p.p.m. to 0.81 p.p.m. These chemical shifts correspond to a conformation-dependent shift (the shift on a resonance that results from removing its group from a random polypeptide and placing it in a structured protein) of −1.44 p.p.m. to −1.57 p.p.m. for the δ-CH_3 resonance, and 0.17 p.p.m. to 0.21 p.p.m. for the γ-CH_3 resonance. On the basis of these probes, all the proteins that contain Ile-57 have very similar structures for the 57/59/74 triad; differences in internuclear distances are at most 0.05 nm. Also, by these criteria, *C. krusei* and *S. cerevisiae* cytochromes *c* contain an analogous structural unit of Val-57, Trp-59 and Tyr-74. The conformation-

dependent shifts of the valine methyl resonances were −1.12 p.p.m. and −0.34 p.p.m., and −1.18 p.p.m. and −0.52 p.p.m., for *C. krusei* and *S. cerevisiae* ferrocytochromes *c* respectively, both proteins at pH 7 at 27°C. The similarities in structure were also confirmed by the similarities in properties. Methyl resonances of the different valine and isoleucine side chains were temperature-dependent and all the δ-CH_3 resonances of the different Ile-57 residues were pH-dependent at high pH. Taken together with the small differences in chemical shifts resulting from amino acid substitutions (Tables 1, 2 and 4), these properties demonstrate that this region of the protein structure is particularly sensitive to the nature of neighbouring amino acid side chains. This accounts for the perturbations resulting from modification of lysine residues (Boswell *et al.*, 1983), and for the perturbations resulting from cleavage of the Arg-38–Lys-39 peptide bond (D. E. Harris & G. R. Moore, unpublished work). Cleavage of this bond produces a protein with an activity about 50% of that of native cytochrome *c* in assays with depleted mitochondria (Harris & Offord, 1977). Its 57/59/74 triad is seriously perturbed; in fact, there is no direct evidence for its presence in the modified protein.

Conformational heterogeneity involving Ile/Val-57

The temperature- and pH-dependencies of Ile-57 and Val-57 are completely reversible as long as the protein has not been converted into an unstructured form. To explain the dependencies, one of two types of conformational perturbations must be occurring. Either there is a gradual change in conformation with changing temperature and pH, or there are different conformations of cytochrome *c* co-existing in solution with molecules rapidly interconverting between one conformation and another. The latter kind of conformational heterogeneity seems more plausible, especially in view of the line broadening of the Ile-57 δ-CH_3 resonance of ferrocytochrome *c* at high pH (Fig. 4).

A number of mechanisms could give rise to the n.m.r.-spectral perturbations. There could be either independent movement of Ile-57, independent movement of Trp-59, independent movement of Tyr-74, or co-operative movement of two or more of these groups. A number of pieces of evidence point conclusively to the movement of Ile-57, as follows. (*a*) Resonances of Trp-59 of ferrocytochrome *c* are temperature- and pH-independent (Moore & Williams, 1980*c*). Therefore Trp-59, which is close to the haem group, does not move. (*b*) In spectra of semi-synthetic [Hse^{65},Leu^{74}]cytochrome *c* (horse) (in which Tyr-74 is replaced by leucine), resonances of Trp-59 are temperature-independent, whereas the δ-CH_3 resonance of Ile-57 is temperature-dependent (Eley *et al.*, 1982). There are indications that there is a small movement of Tyr-74. Tyr-74 flips about its $C_{(\beta)}$–$C_{(\gamma)}$ bond (Eley *et al.*, 1982), and there is a small temperature- and pH-dependence to the Tyr-74 resonances (Figs. 2 and 4). However, the shifts are too small to interpret accurately.

Since the δ-CH_3 resonance of Ile-57 shifts downfield (decreasing conformation-dependent shift) with increasing temperature and increasing pH, whereas the γ-CH_3 resonance moves upfied (increasing conformation-dependent shift), the simplest model to explain the data is that Ile-57 rotates or flips about its $C_{(\alpha)}$–$C_{(\beta)}$ bond (Fig. 8). This results in the γ-CH_3 group moving into the ring-current field of Trp-59 and Tyr-74, while the δ-CH_3 group moves out of it. Schemes involving rapid fluctuations of the peptide chain can also be envisaged, but there is no spectroscopic evidence to favour such schemes over the simpler rotation scheme. Probably both processes occur simultaneously. Val-57 of the yeast proteins probably undergoes a similar temperature-induced conformation change, although the smaller shifts (Fig. 2) indicate that it is not as extensive a change as that of the other cytochromes.

The pH-dependence of Ile-57 of horse ferrocytochrome *c* is due to the deprotonation of a lysine residue, either Lys-39, Lys-55 or Lys-60 (Boswell *et al.*, 1983). Since tuna cytochrome *c* contains asparagine in place of the lysine residue at position 60 (Kreil, 1963, 1965), and since the pH-dependence of its Ile-57 is very similar to that of horse cytochrome *c*, the responsible group must be either Lys-39 or Lys-55. One of the intentions behind studying the yeast cytochromes was to identify the responsible group positively. Neither of the yeast cytochromes contains Lys-39 (they contain His-39), and, although *S. cerevisiae* iso-1 cytochrome *c* contains Lys-55, *C. krusei* cytochrome *c* contains Ala-55. Since in neither protein are resonances of Val-57 pH-dependent, we conclude that either: (*a*) Lys-39 is responsible for the pH-dependence in horse and tuna cytochromes *c*, although His-39 does not perturb Val-57 of the yeast proteins, or (*b*) Lys-55 is responsible in horse and tuna cytochromes *c*, but does not perturb Val-57 of *S. cerevisiae* cytochrome *c*. The latter interpretation is correct, as is shown below.

Bosshard & Zürrer (1980) have measured the pK_a values of different lysine residues of horse ferricytochrome *c* and horse ferrocytochrome *c*, and they find for Lys-39 $pK_a^{ox.} = 9.30$ and $pK_a^{red.} = 9.53$, whereas for Lys-53 and Lys-55 together $pK_a^{ox.} = 10.70$ and $pK_a^{red.} = 11.13$. The titration of the *meta* resonance of Tyr-74 of tuna ferrocytochrome *c* is due to the ionization of a group with pK_a 11.15 ± 0.1 (Fig. 3), and this group therefore must be Lys-55. It is particularly noteworthy that two very different techniques agree so well on the value of an ionization constant. There is also good agreement between the

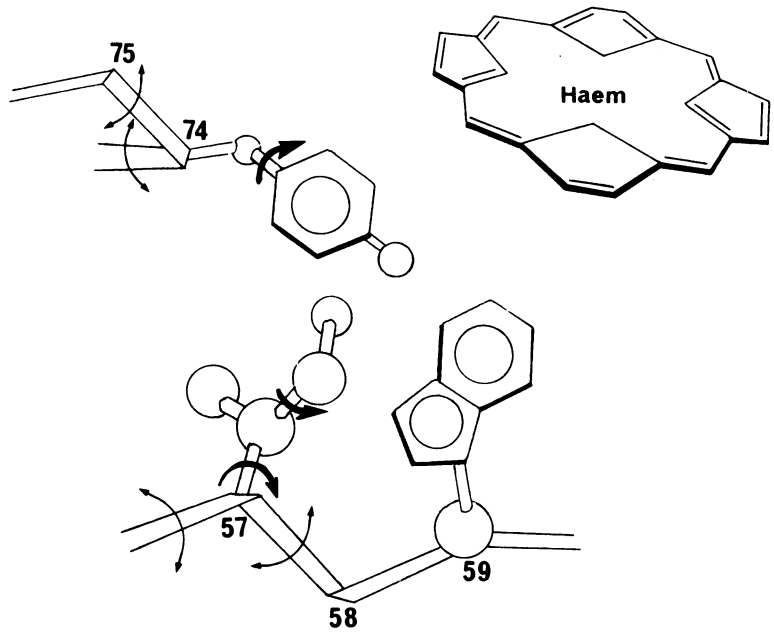


Fig. 8. *Flexibility of cytochrome c around Ile-57*

Both Ile-57 and Tyr-74 rotate or flip about their $C_{(\beta)}$–$C_{(\gamma)}$ bonds and Ile-57 rotates or flips about its $C_{(\alpha)}$–$C_{(\beta)}$ bond (heavy single arrows), and the peptide backbone may also be flexible (double arrows). The γ-CH_3 of Ile-57 moves further into the interior as the δ-CH_3 moves out of it.

above data and the X-ray-crystallographic structure of tuna cytochrome *c* (Takano & Dickerson, 1981*a*), which shows that the Lys-55 $-\overset{+}{N}H_3$ group is hydrogen-bonded to the carbonyl group of Tyr-74.

The Ile-57 δ-CH_3 pH titration is not due to a simple one-proton ionization, nor to an *n*-protons ionization with $\delta_{HA} = -0.56$ p.p.m. and $\delta_{A^-} =$ 0.89 p.p.m. (the unperturbed chemical shift). However, the initial step of the titration does fit a one-proton ionization with a pK_a of 11.15 ± 0.1 (Fig. 3). Thus it seems likely that, as Lys-55 is deprotonated and Tyr-74 perturbed, the side chain of Ile-57 rapidly interchanges between different conformations. Above the pK_a of Lys-55, Tyr-74 is no longer directly affected by changing pH, but Ile-57 is directly affected and it remains so until the protein unwinds. The sensitivity of Ile-57 is also reflected by other methods of unwinding ferrocytochrome *c*, such as methanol-induced denaturation (Boswell *et al.*, 1980*b*), which perturbs the region around Ile-57 before any other region of the protein. The pH-independence of Val-57 of *S. cerevisiae* ferrocytochrome *c* may be due to a higher pK_a for Lys-55 or to a change in conformation that removes the lysine $-\overset{+}{N}H_3$ away from Tyr-74. It is relevant to note that *S. cerevisiae* cytochrome *c* has amino acid substitutions at a number of positions in this region of the protein that are not found in animal cytochromes (Dickerson & Timkovich, 1975).

Oxidation-state-linked conformation change

Cytochrome *c* undergoes a small conformation change with change in oxidation state (Margoliash & Schejter, 1966; Moore *et al.*, 1980*a*; Boswell *et al.*, 1980*a*; Bosshard & Zürrer, 1980; Takano & Dickerson, 1981*a*,*b*). Many parts of the protein are involved, although the exact details are not known. X-ray-crystallographic (Takano & Dickerson, 1981*a*,*b*), n.m.r.-spectroscopic (Moore *et al.*, 1980*a*) and chemical-reactivity studies (Bosshard & Zürrer, 1980) are in agreement that the change is small and largely confined to the surface of the molecule, and that one of the main regions of change is around the bottom of the molecule that includes the 50s loop and parts of the 30s-and-40s loop (Fig. 7). The conformation change in this region of the protein may be another manifestation of the conformational heterogeneity already described. However, there are subtle differences between the oxidation-state-dependent conformation change and the conformation change induced by temperature and pH.

The data reported in the present paper extend earlier work (Moore *et al.*, 1980*a*) that could only

define the conformation change affecting Ile-57 as a movement of one or more of the groups Trp-59, Tyr-74 and Ile-57 away from each other, resulting in a smaller conformation-dependent shift for the δ-CH_3 resonance of Ile-57. From comparison of n.o.e. in spectra of ferricytochrome *c* and ferrocytochrome *c*, and from analysis of conformation-dependent shifts, the movement can now be described as mainly a movement of Ile-57 such that the δ-CH_3 resonance moves away from the aromatic rings of Trp-59 and Tyr-74 without the γ-CH_3 group moving closer towards them. The movement of the δ-CH_3 methyl group is approx. 0.05 nm. This movement is not simply a rotation of the Ile-57 side chain about its $C_{(\alpha)}$–$C_{(\beta)}$ bond (Fig. 8), since this would result in there being a large oxidation-state-dependence to the chemical shift of the γ-CH_3 resonance, and this is not observed (Table 2). Probably it is a movement of the peptide chain bearing Ile-57, as is indicated by the X-ray-crystallographic studies (Takano & Dickerson, 1981*a*,*b*).

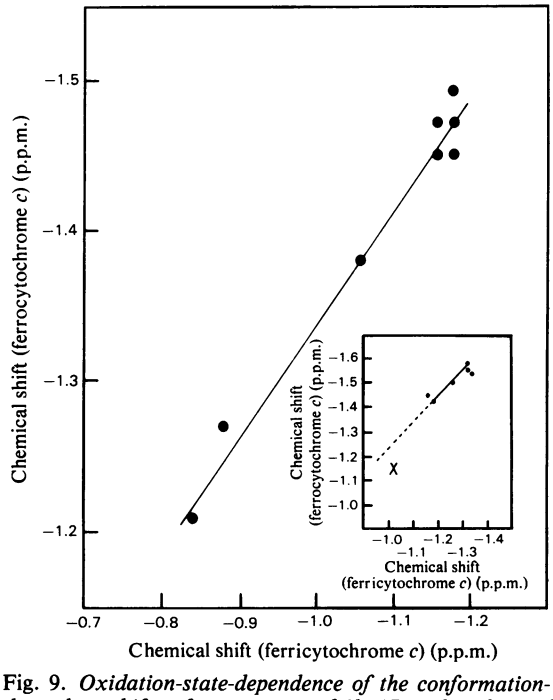


Fig. 9. *Oxidation-state-dependence of the conformation-dependent shifts of resonances of Ile-57 and Val-57 of various cytochromes c*

The data plotted in the main graph are for δ-CH_3 resonances of Ile-57 at pH 5.3 at 57°C. They are taken from Table 3 and from Moore & Williams (1980*f*). The inset is a similar plot for δ-CH_3 resonances of Ile-57 (●) and for the upfield-shifted CH_3 resonance of Val-57 of *C. krusei* cytochrome *c* (×), all at pH 7 and at 27°C. The data are taken from Table 2.

Trp-59 is also involved in the conformation change (Moore *et al.*, 1980*a*; Takano & Dickerson, 1981*a*,*b*); it is hydrogen-bonded to a haem propionate group, and changes its orientation with respect to the haem group.

The precise relationship of the Ile-57 δ-CH_3 group to Trp-59 and Tyr-74 depends on the surrounding amino acid residues (Tables 2 and 4) (Moore & Williams, 1980*f*; Boswell *et al.*, 1983). The oxidation-state-linked conformation change preserves the sequence-induced change, and there is a linear relationship between the conformation-dependent shift experienced by a resonance of ferrocytochrome *c* and its corresponding resonance of ferricytochrome *c* (Fig. 9). This relationship indicates that the magnitude of the conformation change is a conserved feature of the cytochrome *c* structure, even when 40% of the amino acid sequence is changed. When the yeast cytochromes are considered, it is clear that the conformation change in this region is maintained even when about 50% of the amino acids have been changed, including many in the conformation-change region itself. Val-57 of *C. krusei* and *S. cerevisiae* cytochromes *c* undergo a similar small change in conformation to that of Ile-57 (Fig. 9), and the change in pK_a of His-39 (Table 4), with the pK_a slightly lower for ferricytochrome *c* than for ferrocytochrome *c*, is in excellent agreement with the data of Bosshard & Zürrer (1980) for the pK_a of Lys-39 of horse cytochrome *c*.

Biochemical implications of the conformational heterogeneity

The biochemical implications of the conformational heterogeneity around residues 39 and 57 are not clear. This reflects a general problem with interpretation of structural data: because a structure possesses anomalous properties does not mean that the anomalous properties have direct biochemical significance. However, there are indications that the loops of residues forming the base of cytochrome *c* (Fig. 7) influence the binding of cytochrome *c* to other macromolecules.

There are many studies of the interaction between cytochrome *c* and its redox partners (Rieder & Bosshard, 1980; Koppenol & Margoliash, 1982). These studies have relied heavily on the use of chemically modified proteins, and they clearly identify the interaction site as being the exposed haem edge. This is far from the region of the protein that includes residues 39 and 57 (Takano *et al.*, 1977), yet there are reports indicating that amino acid substitutions and chemical modifications in the region including residues 39 and 57 do affect the kinetics of the reactions between cytochrome *c* and its redox partners (Margoliash, 1980; Koppenol & Margoliash, 1982). The molecular bases for these

effects are not known, though they have been ascribed to changes in dipole moment (Koppenol & Margoliash, 1982), which is a co-operative vectorial term critically dependent on conformation. Other chemical-modification studies have implicated the region around Met-65, which is 1.4nm from Ile-57 (Takano *et al.*, 1977), in membrane binding of cytochrome *c* (Vanderkooi *et al.*, 1973).

Cytochrome *c* has been used as a model system to study protein antigenicity, and one of its most antigenic sites has been shown to be the region of the protein around Ile-57. Previous studies have shown that differences in structure around Ile-57 that are detectable by immunochemical techniques (Urbanski & Margoliash, 1977) are also detectable by n.m.r., with the n.m.r. probe being the chemical shift of the δ-CH_3 resonance of Ile-57 (Moore & Williams, 1980*f*). An immunochemical response that is directed towards the region including Ile-57 has been detected for mouse cytochrome *c* when injected into rabbits (Urbanski & Margoliash, 1977), and thus it was of particular interest to compare rabbit cytochrome *c* with rat cytochrome *c*, which has the same amino acid sequence as mouse cytochrome *c* (Carlson *et al.*, 1977). No differences in structure between the proteins in the regions around Ile-57 were detected by n.m.r., in keeping with the amino acid sequences, which show there are conserved amino acids around Ile-57 (there are a total of only two amino acid differences between the two proteins). Therefore the immunochemical response may be an example of self-recognition, since the structure it is directed against is the same as the equivalent host structure.

At present it is difficult to assess the importance of the conformational heterogeneity around Ile-57 with respect to the binding of cytochrome *c* to its redox partners, membranes and antibodies. Measurement of the rates of the motions depicted in Fig. 8 should helpt to clarify this.

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References

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., Moore, G. R. & Williams, R. J. P. (1980*b*) *Biochem. Soc. Trans.* **8**, 638–639

Boswell, A. P., Moore, G. R., Williams, R. J. P., Harris, D. E., Wallace, C. J. A., Bocieck, S. & Welti, D. (1983) *Biochem. J.* **213**, 679–686

Bundi, A. & Wüthrich, K. (1979) *Biopolymers* **18**, 285–297

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

Campbell, I. D., Dobson, C. M. & Williams, R. J. P. (1975) *Proc. R. Soc. London Ser. A* **345**, 23–40

Carlson, S. S., Mross, G. A., Wilson, A. C., Mead, R. T., Wolin, L. D., Bowers, S. F., Foley, N. T., Muijsers, A. O. & Margoliash, E. (1977) *Biochemistry* **16**, 1437–1442

Cohen, J. S. & Hayes, M. B. (1974) *J. Biol. Chem.* **249**, 5472–5477

Cohen, J. S., Fisher, W. R. & Schechter, A. N. (1974) *J. Biol. Chem.* **249**, 1113–1118

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

Dobson, C. M., Moore, G. R. & Williams, R. J. P. (1975) *FEBS Lett.* **51**, 60–65

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

Ernst, R. R. (1966) *Adv. Magn. Reson.* **2**, 1–135

Harris, D. E. & Offord, R. E. (1977) *Biochem. J.* **161**, 21–25

Heller, J. & Smith, E. L. (1966) *J. Biol. Chem.* **241**, 3165–3180

Jardetzky, O. & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, pp. 115–123, Academic Press, New York

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

Koppenol, W. H. & Margoliash, E. (1982) *J. Biol. Chem.* **257**, 4426–4437

Kreil, G. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **334**, 154–166

Kreil, G. (1965) *Hoppe-Seyler's Z. Physiol. Chem.* **340**, 86–87

Lederer, F. (1972) *Eur. J. Biochem.* **31**, 144–147

Lederer, F. & Simon, A. M. (1974) *Biochem. Biophys. Res. Commun.* **56**, 317–323

Lederer, F., Simon, A. M. & Verdière, J. (1972) *Biochem. Biophys. Res. Commun.* **47**, 55–58

Mandel, N., Mandel, G., Trus, B. L., Rosenberg, J., Carlson, G. & Dickerson, R. E. (1977) *J. Biol. Chem.* **252**, 4619–4636

Margoliash, E. (1980) in *The Evolution of Protein Structure and Function* (Sigman, D. S. & Brazier, M. A. B., eds.), pp. 299–321, Academic Press, New York

Margoliash, E. & Schejter, A. (1966) *Adv. Protein Chem.* **21**, 113–287

Margoliash, E., Smith, E. L., Kreil, G. & Tuppy, H. (1961) *Nature (London)* **192**, 1125–1127

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
Moore, G. R. & Williams, R. J. P. (1980*d*) *Eur. J. Biochem.* **103**, 523–532
Moore, G. R. & Williams, R. J. P. (1980*e*) *Eur. J. Biochem.* **103**, 533–541
Moore, G. R. & Williams, R. J. P. (1980*f*) *Eur. J. Biochem.* **103**, 543–550
Moore, G. R., Williams, R. J. P., Chien, J. C. W. & Dickinson, L. C. (1980*a*) *J. Inorg. Biochem.* **12**, 1–15
Moore, G. R., De Aguiar, A. B. V. P., Pluck, N. D. & Williams, R. J. P. (1980*b*) in *Biochemical and Medical Aspects of Tryptophan Metabolism* (Hayaishi, O., Ishimura, Y. & Kido, R., eds.), pp. 83–94, Elsevier, Amsterdam
Narita, K. & Titani, K. (1968) *J. Biochem.* (*Tokyo*) **63**, 226–241
Narita, K. & Titani, K. (1969) *J. Biochem.* (*Tokyo*) **65**, 259–267
Noggle, J. H. & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect*, pp. 76–95, Academic Press, New York
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
Shelnutt, J. A., Rousseau, D. L., Dethmers, J. K. & Margoliash, E. (1981) *Biochemistry* **20**, 6485–6497
Takano, T. & Dickerson, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6371–6375
Takano, T. & Dickerson, R. E. (1981*a*) *J. Mol. Biol.* **153**, 79–94
Takano, T. & Dickerson, R. E. (1981*b*) *J. Mol. Biol.* **153**, 95–115
Takano, T., Swanson, R., Kallai, O. B. & Dickerson, R. E. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 397–403
Takano, T., Kallai, O. B., Swanson, R. & Dickerson, R. E. (1973) *J. Biol. Chem.* **248**, 5234–5255
Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R. & Dickerson, R. E. (1977) *J. Biol. Chem.* **252**, 776–785
Urbanski, G. J. & Margoliash, E. (1977) *J. Immunol.* **118**, 1170–1180
Vanderkooi, J., Erećinska, M. & Chance, B. (1973) *Arch. Biochem. Biophys.* **157**, 531–540
Yaoi, Y. (1967) *J. Biochem.* (*Tokyo*) **61**, 54–58