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^{1}H NMR Studies of Eukaryotic Cytochrome *c*

Resonance Assignments and Iron-Hexacyanide-Mediated Electron Exchange

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^{1}H NMR resonance assignments in the spectra of horse, tuna, *Neurospora crassa* and *Candida krusei* cytochromes *c* are described. Assignments have been made using NMR double-resonance techniques in conjunction with electron-exchange experiments, spectral comparison of related proteins, and consideration of the X-ray structure of tuna cytochrome *c*. Resonances arising from 11 residues of horse cytochrome *c* have been assigned.

Well-resolved ^{1}H NMR spectra of both eukaryotic ferricytochrome *c* and eukaryotic ferrocytochrome *c* have been reported together with many resonance assignments [1–5]. One of the aims of this work is that resonances be specifically assigned and correlated between spectra of the oxidised and reduced species.

Resonance correlations can be obtained from spectra of mixtures of the oxidised and reduced forms of cytochrome *c* [6]. In cases of rapid electron exchange the chemical shift value of a resonance is a weighted average of its respective chemical shift values for ferricytochrome *c* and for ferrocytochrome *c*, whilst in slow electron exchange the spectrum of a mixture is a superimposition of spectra of the individual species. In the first case resonance correlations can be obtained by monitoring resonance chemical shifts as a function of the relative concentration of oxidised and reduced forms present, and in the second case correlations can be obtained with saturation-transfer techniques [7]. A third exchange category is intermediate exchange, in which resonance linewidths are drastically perturbed as a function of the relative concentrations of oxidised and reduced species. In this exchange region correlation of resonances is not a simple matter.

The electron self-exchange rate of horse cytochrome *c* places this system into slow exchange for ^{1}H NMR [8]; many resonance correlations have been obtained using saturation-transfer. Other correlations have been suggested based on spectral perturbations caused by variation in pH and the addition of paramagnetic ions. These correlations need to be confirmed.

The first aim of the present study was to increase the electron exchange rates of eukaryotic cytochrome *c* to place resonances in the fast exchange region. This was achieved by using a procedure recommended by Stellwagen and Shulman [9] involving the addition of iron hexacyanides to solutions of cytochrome *c* at elevated temperature. The second aim of the study was to define the iron hexacyanide binding sites on cytochrome *c*. These are reported in the following paper [10] although a number of resonance assignments required for that study are described in the present paper.

Abbreviations. NOE, Nuclear Overhauser Enhancement; Me_3Lys, N^{ϵ}-trimethyl-lysine.

MATERIALS AND METHODS

Horse (type VI), tuna (type XI) and *Candida krusei* (type VIII) cytochromes *c* were obtained from the Sigma Chemical Co. *Neurospora crassa* cytochrome *c* was prepared as previously described [11]. Kangaroo cytochrome *c* was a gift from Dr R. Wever (University of Amsterdam). The proteins were prepared for NMR as previously described [12]. Quoted pH values are meter readings uncorrected for the isotope effect.

The redox titrations were carried out as follows. Spectra were recorded of a solution of 5 mM horse ferricytochrome *c* with 5 mM $K_3[Fe(CN)_6]$ in ^2H_2O at pH 7 both before and after the additions of aliquots of a solution of 0.2 M sodium ascorbate in ^2H_2O at pH 7. The titrations were carried out at 57 °C and continued until spectra of the fully reduced species were obtained.

The NMR spectra were obtained using a Bruker 270-MHz spectrometer, a Bruker WH-300 spectrometer or a 470-MHz spectrometer equipped with an Oxford Instrument Co. magnet and Nicolet software. Resolution enhancement was obtained by convolution difference [13] or by Gaussian multiplication [14]. Spin-decoupling and saturation-transfer were carried out as previously described [2, 3]. Nuclear Overhauser Enhancements (NOEs) were observed following gated irradiation of particular resonances for 0.5–1.5 s. NOE difference spectra were recorded as previously described [4]. Chemical shift values are quoted downfield of the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

RESULTS AND DISCUSSION

Redox Titration of Horse Cytochrome c

Various regions of the convolution difference spectra of horse cytochrome *c* during a redox titration from the fully oxidised to the fully reduced species at pH 7 and 57 °C are given in Fig. 1; only five of the twelve spectra are shown. The resonance correlations obtained from these spectra are

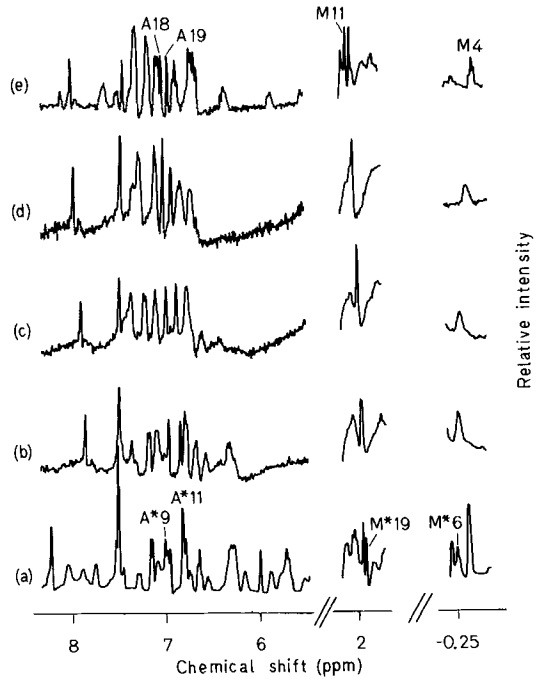

Fig. 1. *Regions of the 270-MHz convolution difference NMR spectra of 5 mM horse cytochrome* c *in* ^2H_2O *at 57 °C and pH 7 ± 0.2, and with 5 mM* $K_3[Fe(CN)_6]$ *and varying concentrations of ascorbic acid.* (a–e) are respectively, 0%, 20%, 30%, 60% and 100% ferrocytochrome *c*. M4 and M*6 are the resonances of the δCH_3 group of Ile-57, M11 and M*19 are the resonances of the CH_3 group of Met-65, A18 and A*9 are the C-4 resonances of His-26 and A19 and A*11 are the C-2 resonances of Trp-59

Table 1. *Correlation of resonances of horse cytochrome* c
Chemical shift values were measured at pH 5.25 and 57 °C. Assignments are from [2–5, 15]. Methods were (A) correlation from exchange in the presence of $[Fe(CN)_6]^{3-}$, (B) correlation by saturation-transfer in the absence of $[Fe(CN)_6]^{3-}$ and (C) correlation from the presence of spin-spin coupling with resonances correlated by A or B

Ferri-cytochrome *c*		Ferro-cytochrome *c*		Assignment	Method
desig-nation	chemi-cal shift	desig-nation	chemi-cal shift		
	ppm		ppm		
A*1	8.62	A1	8.55	His-33 C-2	A
A*3	7.69	A6	7.55	His-33 C-4	A
A*4	7.62	A8	7.52	His-26 C-2	A
A*9	7.01	A18	7.06	His-26 C-4	A
A*11	6.86	A19	6.99	Trp-59 C-2	A
A*6	7.57	A16	7.10	Trp-59 C-7	B
A*16	6.31	A30	5.74	Trp-59 C-6	B
A*15	6.54	A24	6.71	Trp-59 C-5	A
A*7	7.37	A5	7.60	Trp-59 C-4	A
A*5	7.62	A11	7.22	Tyr-74 *ortho*	B
A*12	6.82	A26	6.64	Tyr-74 *meta*	A
A*18	6.2	A23	6.71	Phe-82[a] *ortho*	B
A*18	6.2	A10	7.40	Phe-82[a] *meta*	B
A*8	7.23	A9	7.40	Phe-36 *ortho*	A
A*14	6.64	A20	6.89	Phe-36 *meta*	A
A*13	6.76	A15	7.1	Phe-36 *para*	C
M*6	−0.18	M4	−0.43	Ile-57 δCH_3	A
M*19	1.94	M11	2.11	Met-65 CH_3	A
M*20	1.97	M10	2.07	*N*-acetyl CH_3	A

[a] In a previous papers of this series [2] these resonances were incorrectly assigned to Phe-10. The correct assignment is Phe-82 [4, 5].

summarised in Table 1. The resonance nomenclature scheme previously described [2–4] is used for continuity.

Under the conditions of the spectra of Fig. 1 many resonances are in fast exchange; for example, the five singlet resonances of His-26, His-33 and Trp-59. These resonances were used to calibrate a graph of chemical shift versus mole fraction of ferricytochrome *c* and the graph used to correlate resonances which overlapped with others during the titration.

Resonance correlation were obtained from the redox titration for the following groups: N-terminal acetyl, His-26, Phe-36, Ile-57, Trp-59 and Met-65. The corresponding resonances for these groups are separated by < 0.3 ppm. For corresponding resonances with a larger chemical shift difference there was severe exchange broadening throughout the titration which made it impossible directly to obtain their correlation. However for such resonances the larger chemical shift difference allows saturation transfer techniques to be used [2, 3].

^1H *NMR Assignments of Eukaryotic Cytochrome* c

In this section are reported new assignments in the spectra of horse, tuna, *Candida krusei* and *Neurospora crassa* cytochromes *c*. These assignments are required to describe the interaction of $[Fe(CN)_6]^{3-}$ with cytochrome *c*, which is given in the following paper [10]. The assignments follow normal procedure [2, 4]: resolution enhancement and double resonance are used to identify particular spin systems; spectral and sequence comparisons of related cytochromes, and Nuclear Overhauser Enhancements in conjunction with consideration of the crystal structure of tuna cytochrome *c* [16] are used to make specific assignments.

The cytochromes *c* from *C. krusei* and *N. crassa* are particularly useful for the assignment of resonances of eukaryotic cytochrome *c* since they contain many amino acid substitutions involving residues conserved in most other eukaryotic cytochromes *c* [17]. Despite the amino acid substitutions the spectra of *N. crassa* and *C. krusei* cytochromes *c* are very similar to those of other eukaryotic cytochromes (Fig. 2; also see Fig. 6 of [12].

His-26

The aromatic region of the spectrum of horse ferricytochrome *c* (Fig. 2) contains three singlets which are independent of pH over the pH range 4.5–8. Two of these come from His-26 and one from Trp-59. *N. crassa* cytochrome *c* contains asparagine in place of His-26 which leads to the assignment of the singlet resonances of horse ferricytochrome *c* as follows: His-26, 7.61 ppm and 6.98 ppm; Trp-59 6.86 ppm at 25 °C.

Phe-82

Complete assignment of the aromatic resonances of Phe-82 of horse and tuna ferricytochromes *c* was obtained from spin-decoupling and NOE experiments (Fig. 3). Previous work [2, 4] only described the assignment of the *ortho* and *meta*

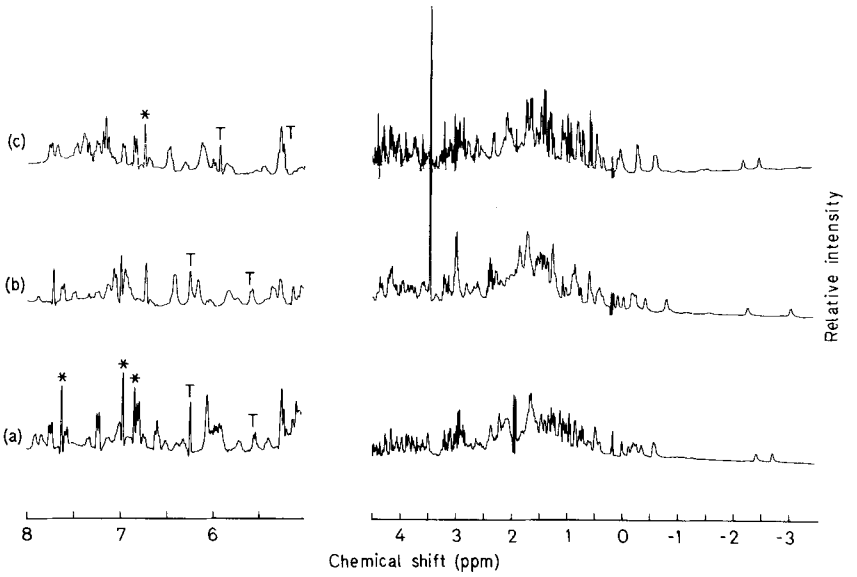

Fig. 2. *The 300-MHz resolution-enhanced NMR spectra of (a) horse ferricytochrome* c, *3 mM in* ^2H_2O *at pH 7 and 27 °C. (b)* C. krusei *ferricytochrome* c, *3 mM in* ^2H_2O *at pH 7.4 and 27 °C, (c)* N. crassa *ferricytochrome* c, *4 mM in* ^2H_2O *at pH 7.3 and 27 °C.* The singlet resonances of His-26 and Trp-59 of horse and *N. crassa* ferricytochromes *c* are indicated by *, and resonances of Thr-19 by T. The intense singlet resonances at 3.4 ppm in the spectra of *C. krusei* and *N. crassa* ferricytochromes *c* are from Me_3Lys-72

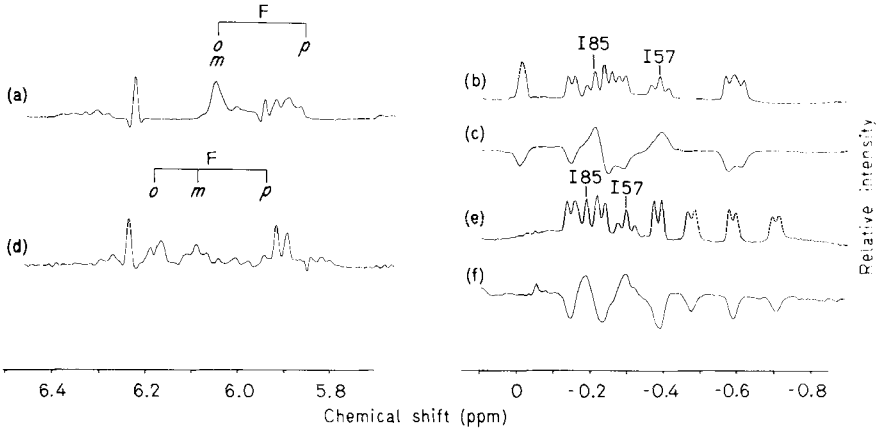

Fig. 3. *Regions of 300-MHz NMR spectra of (a–c) horse ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.3 and 27 °C; (d–f) tuna ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.6 and 27 °C.* (a), (b), (d) and (e) are regions of resolution-enhanced spectra and (c) and (f) are regions of Carr-Purcell spectra obtained with a 90°-τ-180°-τ pulse sequence with τ = 60 ms. Resonances of Phe-82, Ile-57 and Ile-85 are indicated by F, I57 and I85 respectively

resonances of horse ferricytochrome *c*. At 25 °C the *ortho*, *meta* and *para* resonances of Phe-82 of tuna ferricytochrome *c* are separately resolved whilst the corresponding *ortho* and *meta* resonances of horse ferricytochrome *c* overlap (Fig. 3a and 3d)

Ile-85

Of the eight three-proton-intensity resonances resolved in the resolution-enhanced spectra of horse and tuna ferricytochromes at 25 °C with chemical shifts between 0.1 ppm and −1.0 ppm two are triplets (Fig. 3) and must therefore arise from the δCH_3 groups of isoleucine residues. The furthest upfield triplet has been assigned to Ile-57 [3]. The assignment of the second triplet to Ile-85 is described below.

Irradiation of resonances of Phe-82 (Fig. 4) and the haem thioether-2 methyl group [18] in spectra of ferricytochrome *c* result in an NOE to the triplet methyl resonances at −0.20 ppm (horse) and −0.16 ppm (tuna). The triplet resonance therefore arises from an isoleucine residue which is close in space to both Phe-82 and the haem thioether-2 methyl. Examination of the crystal structure [16] shows that only Ile-85 is in such a position.

Spectral comparisons of closely related cytochromes *c* support this assignment. In Table 2 are given the sequence positions of the isoleucine residues of a variety of cytochromes *c*. In the spectrum of *C. krusei* ferricytochrome *c* upfield of 0.4 ppm there are no triplet methyl resonances (Fig. 2). The comparison of *C. krusei*, horse and tuna cytochromes *c* restricts the assignment to either Ile-81 or Ile-85. The crystal structure and NOE show it to be Ile-85.

Val-20

In a previous paper [3] the methyl resonances of horse ferrocytochrome *c* at 0.02 ppm and 0.43 ppm were shown to arise from a valine or leucine residue with the tentative assignment of Val-20 suggested. This assignment is confirmed

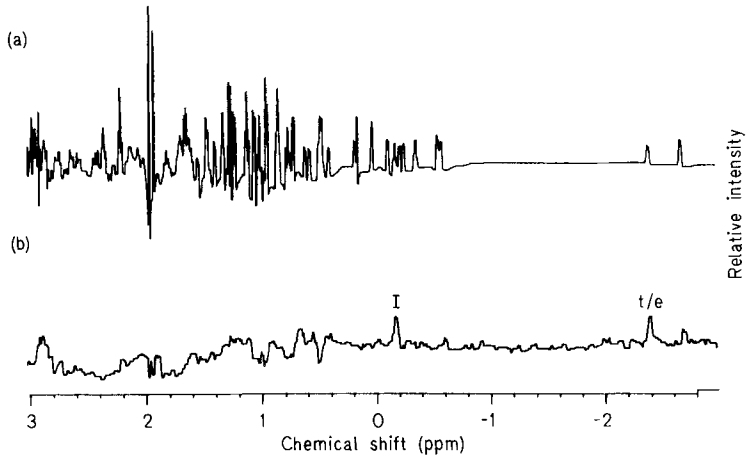

Fig. 4. *470-MHz NMR spectra of horse ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.3 and 25 °C.* (a) Resolution-enhanced spectrum; (b) NOE difference spectrum resulting from saturation of the Phe-82 resonance at 6.05 ppm for 1.0 s prior to application of the observation pulse. Resonances of Ile-85(I) and thioether-2 (t/e) are indicated

Table 2. *Sequence positions of isoleucine residues*
The amino acid sequences are taken from the compilation of Dickerson and Timkovich [17]

Protein	Sequence position							
	9	20	35	57	75	81	85	95
Kangaroo	I	–	I	I	I	I	I	I
Horse	I	–	–	I	I	I	I	I
Tuna	–	–	–	I	I	I	I	–
C. krusei	–	I	I	–	I	–	–	–

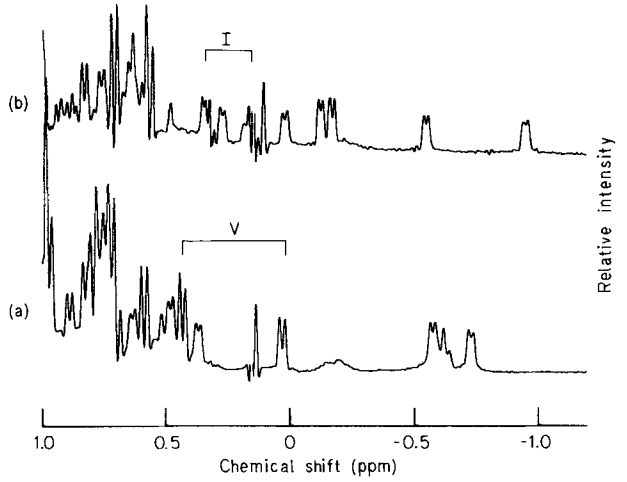

Fig. 5. *Resolution-enhanced 300-MHz NMR spectra of (a) horse ferricytochrome* c, *5 mM in* ^2H_2O *at pH 6.8 and 27 °C; (b)* C. krusei *ferricytochrome* c; *5 mM in* ^2H_2O *at pH 6.2 and 27 °C.* Methyl resonances of Val-20 and Ile-20 are indicated by V and I respectively

by the observation that the spectrum of *C. krusei* ferrocytochrome *c* has these two doublet resonances replaced by a doublet and a triplet resonance (Fig. 5). NOE and spin-decoupling experiments have shown that the triplet at 0.20 ppm and the doublet at 0.39 ppm arise from the same residue. The doublet is coupled to a resonance at 0.79 ppm (47 °C). These three resonances are clearly due to the δCH_3, γCH_3 and βCH protons, respectively, of an isoleucine residue. Consideration of the amino acid sequences of various cytochromes *c* (Table 2) leads to their firm assignment to Ile-20 since the spectrum of kangaroo ferrocytochrome *c* contains an analogous set of doublet resonances to those of horse ferrocytochrome *c*.

Thr-28 (Horse); Val-28 (Tuna)

NOEs were observed on a few aliphatic resonances of horse and tuna ferricytochromes *c* as a consequence of the irradiation of the resonance of haem methyl-5 [18] at ≈ 10 ppm at 25 °C (Fig. 6). The $CH-CH_3$ spin system of an alanine or threonine residue of horse ferricytochrome *c* and the $CH_3-CH-CH_3$ spin system of a valine or leucine residue of tuna ferricytochrome *c* were identified, by spin-decoupling, and by the observation of an NOE between the two methyl groups of the valine or leucine.

Horse	$CH-CH_3$
	3.07, −0.01 ppm
Tuna	$CH_3-CH-CH_3$
	0.74, 1.11, −0.46 ppm.

The NOEs indicate that these two spin systems are from corresponding residues. The only relevant amino acid substitutions are at positions 28 and 58, which are threonine in horse cytochrome *c* and valine in tuna cytochrome *c* [17]. Consideration of the crystal structure [16] shows the assignment to be residue 28 since only Val-28 is close to haem methyl-5 (≈ 0.4 nm) and there are no other valine residues within 1.4 nm of it.

Thr-19

A common feature of the spectra of eukaryotic ferricytochromes *c* is the presence of a one-proton-intensity singlet resonance at ≈ 6.2 ppm (Fig. 2). This resonance is from an αCH proton whose α-β coupling constant is small [3]. Gated irradiation of the singlet resonance at 6.27 ppm of tuna ferricytochrome *c* at 25 °C results in NOEs to a one-proton quartet resonance at 5.52 ppm and its coupled methyl reso-

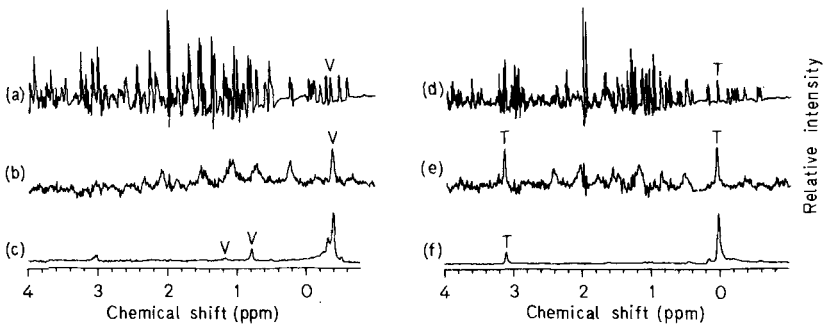

Fig. 6. *470-MHz NMR spectra of (a–c) tuna ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.6 and 25 °C; (d–f) horse ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.6 and 25 °C.* (a) and (d) are resolution-enhanced spectra; (b) and (e) are the NOE difference spectra resulting from saturation of the resonance of haem methyl-5 at ≈10 ppm for 1.5 s prior to application of the observation pulse; (c) is the NOE difference spectrum resulting from saturation of the CH_3 resonance of Val-28 at −0.46 ppm for 1.5 s prior to application of the observation pulse; (f) is the NOE difference spectrum resulting from saturation of the CH_3 resonance of Thr-28 at −0.01 ppm for 1.0 s prior to application of the observation pulse. Resonances of Val-28 of tuna ferricytochrome *c* and Thr-28 of horse ferricytochrome *c* are indicated by V and T respectively. The CH_3 resonances of thioether-4 occurs at ≈3.1 ppm for horse [18] and tuna ferricytochromes *c*. These resonances may be affected by saturation of resonances of Val-28 and Thr-28 (c) and (f). The βCH resonance of Thr-28 overlaps with the CH_3 resonance of thioether-4

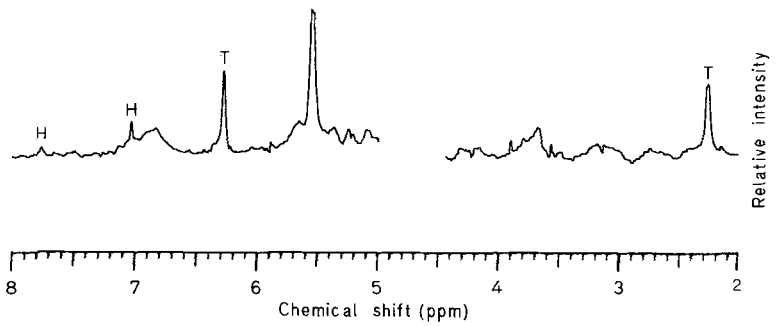

Fig. 7. *300-MHz NOE difference NMR spectrum of tuna ferricytochrome* c, *8 mM in* ^2H_2O *at pH 5.6 and 27 °C, resulting from saturation of the βCH resonance of Thr-19 at 5.52 ppm for 2.0 s prior to application of the observation pulse.* Resonances of Thr-19 and His-26 that experience NOEs are indicated by T and H respectively

nance at 2.25 ppm. These three resonances therefore arise from a threonine residue. Their secondary assignment depends upon the observation of an NOE between the βCH resonance and the C-2 and C-4 proton resonances of His-26 (Fig. 7).

The only threonine residues which are present in all the cytochromes studied are Thr-19, Thr-49 and Thr-78 [17]. The crystal structure [16] shows that of these only Thr-19 is sufficiently close to His-26 to produce an NOE. Therefore the resonances of horse ferricytochrome *c* at 6.23 ppm, 5.55 ppm and 2.24 ppm at 25 °C are assigned to the αCH, βCH and γCH_3 protons of Thr-19. Further evidence for this assignment is obtained from the observation that these resonances are shifted in the spectrum of *N. crassa* ferricytochrome *c* at 25 °C to 5.92 ppm, 5.27 ppm and 2.06 ppm, respectively. This cytochrome is unique amongst the proteins studied in that it contains asparagine rather than histidine at position 26.

Since resonances of Thr-19 of horse ferricytochrome *c* broadened to the extent that they could not be observed during the redox titration (Fig. 1), their correlation with resonances of ferrocytochrome *c* was achieved using saturation-transfer. Only the βCH and γCH_3 resonances have been correlated. In Fig. 8 is shown one of the difference spectra obtained for this correlation. Gated irradiation of the βCH resonance of Thr-19 (ferricytochrome *c*) results in NOEs to the α-CH and γCH_3 resonances of ferricytochrome *c* and, following electron-exchange, saturation-transfer to the βCH resonance and NOE to the γCH_3 resonance of ferrocyto-

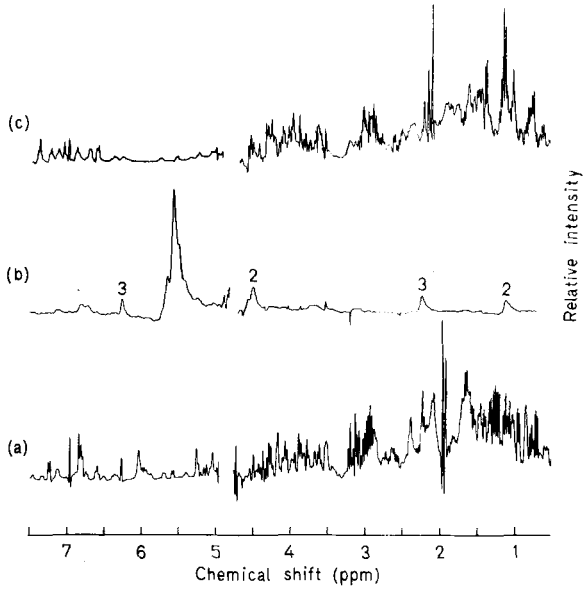

Fig. 8. *300-MHz NMR spectra of horse cytochrome* c *in* ^2H_2O *at pH 6.2 and 25 °C.* (a) Resolution-enhanced spectrum of 4 mM ferricytochrome *c*; (b) difference spectrum resulting from saturation of the β-CH resonance of Thr-19 of ferricytochrome *c* at 5.52 ppm for 0.5 s prior to observation of a mixture of 4 mM ferricytochrome *c* and 4 mM ferrocytochrome *c*; (c) resolution-enhanced spectrum of 4 mM ferrocytochrome *c*. NOEs and saturation-transfer to resonances of Thr-19 in ferricytochrome *c* and ferrocytochrome *c* are indicated by 3 and 2 respectively

Table 3. *^1H NMR assignments for cytochrome c at pH7 and 25°C*
n.d. = not determined; – indicates that the particular protons are not present, the residue has been replaced by another amino acid

Cytochrome	Proton	Chemical shift in			
		horse	tuna	*N. crassa*	*C. krusei*
		ppm			
Ferricyto-chromes *c*	Thr-19				
	α	6.23	6.27	5.92	6.25
	β	5.55	5.52	5.27	5.57
	γ	2.24	2.25	2.06	2.33
	His-26				
	C-2	7.61	7.74	–	n.d.
	C-4	6.98	6.99	–	n.d.
	Thr-28				
	β	3.07		–	–
	γ	−0.01	–	–	–
	Val-28				
	β	–	1.11	–	n.d.
	γ	–	0.74	–	n.d.
	γ	–	−0.46	–	n.d.
	Me_3Lys-72				
	εCH_3	–	–	3.44	3.43
	Phe-82				
	o	6.05	6.19	6.12	6.19
	m	6.05	6.10	6.12	6.19
	p	5.89	5.95	n.d.	5.86
	Ile-85				
	δ	−0.20	−0.16	–	–
Ferrocyto-chromes *c*	Thr-19				
	β	4.49	n.d.	n.d.	n.d.
	γ	1.10	n.d.	n.d.	n.d.
	Val-20				
	β	1.56[a]	1.6[a]	–	–
	γ	0.02	0.04	–	–
	γ	0.43	0.54	–	–
	Ile-20				
	β	–	–	n.d.	0.79[b]
	γCH_3	–	–	n.d.	0.39
	δ	–	–	–	0.20
	His-26				
	C-2	7.52	7.61	–	n.d.
	C-4	7.06	7.08	–	n.d.
	Me_3Lys-72				
	εCH_3	–	–	2.96	2.94
	Phe-82				
	o	6.71	6.73	6.72	6.77
	m	7.40	7.41	7.40	7.43
	p	7.2	n.d.	n.d.	n.d.

[a] These resonances were assigned at 67 °C.
[b] This resonance was assigned at 47 °C.

chrome *c*. Spin-spin coupling between the βCH resonance at 4.49 ppm and the γCH_3 resonance at 1.10 ppm of ferrocytochrome *c* was confirmed by spin-decoupling.

Me_3Lys-72

N. crassa and *C. krusei* cytochromes *c* contain trimethyllysine at position 72 whereas horse and tuna cytochromes *c* contain Lys-72 [17]. The nine-proton-intensity singlet resonance resulting from Me_3Lys-72 is clearly observable in the NMR spectra (Fig. 2).

CONCLUSION

Resonance assignments for a variety of residues of eukaryotic cytochrome *c* have been obtained and many correlations between the two oxidation states established. The techniques of $[Fe(CN)_6]^{3-}$-mediated electron exchange at high temperature and saturation-transfer at room temperature satisfactorily complement each other. The resonance assignments are summarised in Table 3. They can be used as structural probes and as probes for the reactions of cytochrome *c*. In the following paper they are used to probe the reaction of cytochrome *c* with $[Fe(CN)_6]^{3-}$.

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