ASSIGNMENT OF AROMATIC AMINO ACID PMR RESONANCES OF HORSE FERRICYTOCHROME *C*.

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1. Introduction

Interest in cytochrome *c*, an electron-transfer protein functioning in mitochondrial redox chains, lies in an appreciation of the role of the protein fold and the amino acid side chains in electron-transfer processes. Such dynamic steps of a reaction cannot be followed by X-ray crystallographic methods and fast spectroscopic analysis must therefore be used. By virtue of its low mol. wt (13 500) and possession of a low-spin heme group cytochrome *c* is suitable for study by proton magnetic resonance (PMR) spectroscopy. So far only contact shifted resonances of the heme group [1] and the methionine ligand of the iron [2], some protein resonances, shifted by ring-current and pseudo-contact effects [3], some exchangeable NH protons [4] and the resonances of a titratable histidine [5] residue have been studied. We wish to investigate the solution structure of cytochrome *c* in much greater detail by new PMR procedures, to compare features of the solution structure with the crystal structure and then to examine dynamic processes relevant to electron transfer. To this end we need to resolve and assign as many proton resonances as possible. The techniques used will be those shown to be successful for the study of lysozyme [6].

Initially it will be shown that a resolution enhancement technique, convolution difference [7], applied to the Fourier transform spectrum of ferricytochrome *c* allows resonances to be more easily resolved. Next, assignment to particular types of amino acids is made based upon the now observable multiplet structure and upon spin decoupling experiments. Assignment of some proton resonances to particular amino acids in the sequence will then be made from (i) a comparison of the PMR spectra of cytochromes *c* from different sources, (ii) knowledge of the location of particular amino acids with respect to aromatic groups and the porphyrin (from X-ray crystallographic studies [8]), and (iii) the effects of diamagnetic (e.g. H^+) and paramagnetic (e.g. Pr^{3+}) extrinsic probes. In this report we discuss the application of the above procedures to the aromatic region only of the PMR spectra of horse and tuna ferricytochrome *c*. The experimental work has shown that not all of the aromatic amino-acids lend themselves to this analysis due to the line widths of their resonances.

2. Materials and methods

The PMR spectra were recorded using a Bruker 270 MHz spectrometer with an Oxford Instrument Co. magnet. The instrument has an internal field-frequency lock. Free induction decays resulting from a 270 MHz pulse were collected in a Nicolet 1085 computer in which mathematical manipulations were carried out. Spin decoupling was performed as previously described [9].

Horse cytochrome *c* (Grade VI) and tuna cytochrome *c* were obtained from Sigma Chemical Co. The proteins were dialyzed at pH 3.0 against distilled water at 4°C to remove bound ions and then lyophilized from D_2O. Solutions of $PrCl_3$ were prepared as previously described [7].

For the PMR experiments 5 mM solutions of cytochrome *c* were prepared. The pH was adjusted to 8.0 ± 0.2 with NaOD and the solutions immersed in water at 60°C for 5 min to exchange the NH protons for deuterions. After cooling to room temperature.

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To some solutions 30 mM $PrCl_3$ was added. The nominal pH of the cytochrome *c* solutions was adjusted to pH 5.25 ± 0.1 with NaOD and DCl.

The temperature at which the spectra were obtained was 57 ± 0.5°C. Acetone and dioxan were used as internal standards but all shifts are quoted in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulphonate.

3. Results and discussion

Horse cytochrome *c* contains 1 tryptophan, 3 histidine, 4 tyrosine and 4 phenylalanine residues [14]. There is therefore a total of 47 aromatic protons to locate and assign. Histidine 18 is the fifth iron ligand and its resonances in the PMR spectrum of ferricytochrome *c* are expected to be shifted out of the aromatic region (10 to 5.5 ppm) of the protein spectrum, probably downfield. Tuna cytochrome *c* contains 2 tryptophan, 2 histidine, 5 tyrosine and 3 phenylalanine residues [14], a total of 49 aromatic protons. Again, the resonances of histidine 18 are not expected to be present in the aromatic region of the PMR spectrum of tuna ferricytochrome *c*. The resemblance between the aromatic regions of the PMR spectra of horse ferricytochrome *c* (fig.1) and tuna ferricytochrome *c* (fig.2) indicates that the two proteins have similar structures in solution and the comparisons between the two spectra, for assignment purposes, are therefore meaningful. Their structures in the solid state are known to be isomorphous [14].

In fig.1, the normal and the convolution difference (CD) [7] spectra of horse ferricytochrome *c* in D_2O at pH = 5.25 and 57°C are shown between 11 and 5.5 ppm. The multiplet structure, which can only just be seen in the normal spectrum, is well resolved in the CD spectrum for the many sharp resonances. In addition there are two classes of broad resonances which can be seen in the spectrum. One of these in the region 8–12 ppm is only seen in the normal spectrum. It is known that by taking a CD spectrum very broad resonances are relatively reduced in area [7]. Two broad resonances in the normal spectrum, at 10.6 ppm and 10.1 ppm have been assigned [3], by their dependence on pH and temperature, to contact shifted resonances of the heme group. We assign the other broad resonances between 7.8 and 8.8 ppm to solvent exchanging

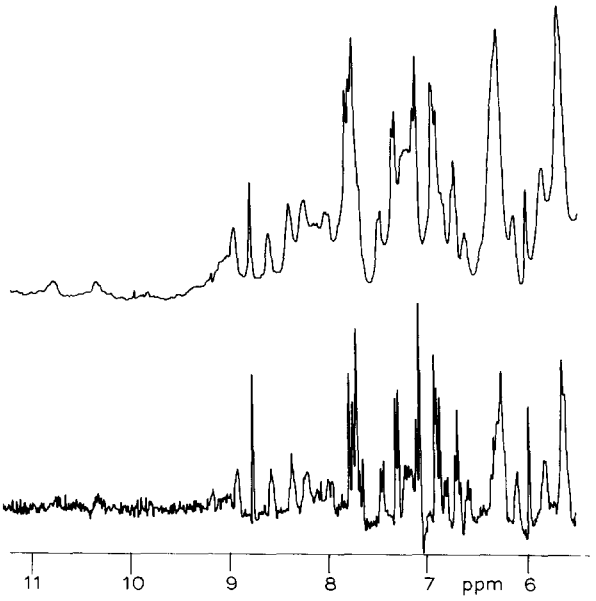

Fig.1. Conventional and C.D. spectra of horse ferricytochrome *c* in D_2O at pH 5.25 and 57°C.

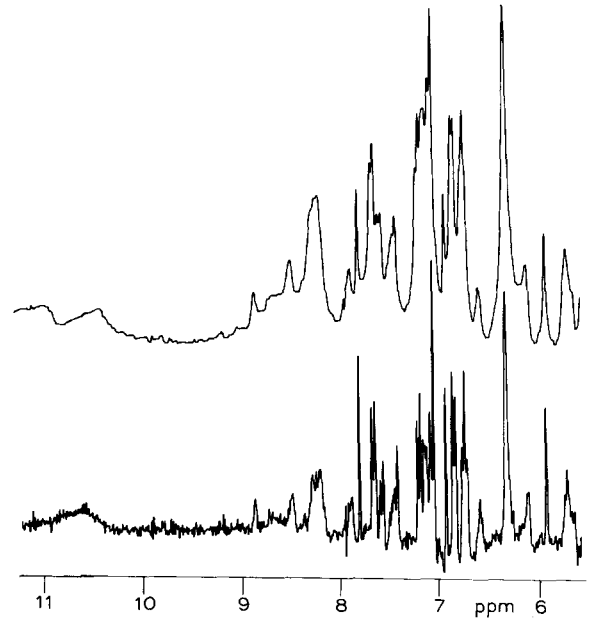

Fig.2. Conventional and C.D. spectra of tuna ferricytochrome *c* in D_2O at pH 5.25 and 57°C.

> NH protons since after 5 hr in the above conditions these resonances disappear. This leaves the second class of several broad resonances which are in the aromatic region below 7 ppm of both the normal and CD spectra. We return to these resonances later.

Amongst the sharp resonances there are 5 singlets due to single protons between 6.8 and 8.6 ppm in the spectrum of horse ferricytochrome *c*. A singlet resonance in this region can only arise from a C-2 or C-4 proton of a histidine residue or a C-2 proton of a tryptophan residue. The singlets at 8.62 ppm and 7.69 ppm shift with pH with the same pK value of 6.4. Thus, they arise from the protons of a histidine residue. Tuna cytochrome *c* possesses a tryptophan residue in place of histidine 33 and in the PMR spectrum of tuna ferricytochrome *c* the two titratable singlet histidine resonances are missing. The resonances at 8.62 ppm and 7.69 ppm are therefore assigned to the C-2 and C-4 protons of His 33 respectively [5]. The other three singlets, which arise from tryptophan 59 and histidine 26, are not in their normal positions (8.75, 7.45 and 7.25 ppm for histidine C-2 and C-4 respectively and tryptophan C-2 protons at pH 5.25 [10]) and so must be subject to ring current and/or pseudo contact shifts. They are not titratable. It is therefore not possible to assign unambiguously these resonances as yet.

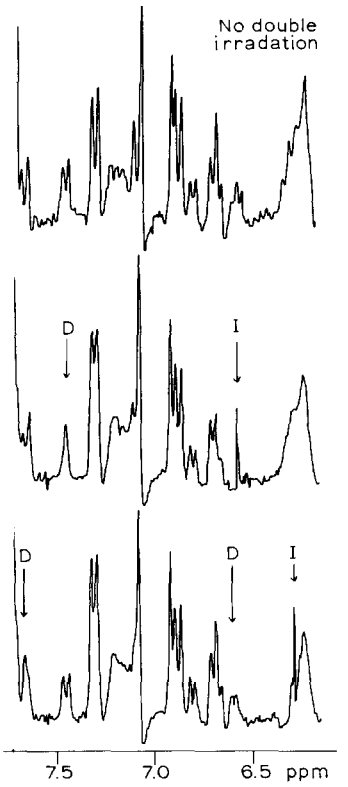

Fig.3. C.D. spectra of horse ferricytochrome *c*; decoupling of tryptophan resonances. I is position of irradiation. D are decoupled peaks.

Table 1
Assignment of the PMR resonances in the aromatic spectra of horse and tuna ferricytochrome *c*

Assignment	Horse ferricyto-chrome *c*	Tuna ferricyto-chrome *c*
His 33 C-2 proton	8.62	His 33 not present
His 33 C-4 proton	7.69	,,
His 26 C-2 or C-4, or Trp 59 C-2	7.61	7.76
His 26 C-2 or C-4, or Trp 59 C-2	7.00	7.04
His 26 C-2 or C-4, or Trp 59 C-2	6.86	6.94
Trp 59 C_4 or C_7 proton	7.57	7.53
Trp 59 C_5 or C_6 proton	6.31	6.33
Trp 59 C_6 or C_5 proton	6.54	6.58
Trp 59 C_7 or C_4 proton	7.37	7.40
Tyr 74 or 97 *o* or *m* protons	7.64	7.60
Tyr 74 or 97 *o* or *m* protons	6.82	6.83
Phe 10 or 36 ortho protons	7.23	7.17
Phe 10 or 36 meta protons	6.64	6.73
Phe 10 or 36 para proton	6.76	6.83

Resonances at 7.37, 6.54, 6.31 and 7.57 ppm were shown to arise from tryptophan 59 by spin decoupling (fig.3). The triplet at 6.31 ppm was separated prior to decoupling from the overlapping resonances by the addition of the paramagnetic lanthanide cation, Pr (III), which shifted this resonance down-field. Final assignment cannot be made at present but the C-4 and C-7 proton resonances of tryptophan 59 are at 7.57 and 7.37 ppm while the C-5 and C-6 resonances are at 6.31 and 6.54 ppm. (table 1). Tryptophan 59 of tuna ferricytochrome *c* has been assigned by a similar procedure as have some of the protons of tryptophan 33.

Further assignment proceeds by allocating other resonances to types of aminoacids but not yet to their positions in the sequence. The four ring protons of several tyrosine residues have been seen in a number of proteins as two two-proton coupled doublets [9]. No other aromatic residue can give this type of spectrum. By systematic double irradiation experiments with horse ferricytochrome *c* we have observed a two-proton doublet at 7.64 ppm coupled to a two proton doublet at 6.82 ppm. We therefore identify these resonances as belonging to a tyrosine residue although we cannot assign them conclusively to either the ortho or meta protons. Both doublets are shifted from their normal position (7.20 for meta protons and 6.89 for ortho protons [10]).

Close to and partially overlapping the doublet at 6.82 ppm is a one proton triplet at 6.76 ppm. This triplet can be separated from the tyrosine doublet by adding Pr (III) which shifts the doublet downfield. The one-proton triplet could arise from within a tryptophan or phenylalanine residue but as we have assigned all protons of the only tryptophan (59) it must arise from a phenylalanine. We have identified the other protons of this phenyalanine by double irradiation experiments (fig.4). The two-proton doublet at 7.23 is coupled to the two-proton triplet at 6.64 ppm, which is also coupled to the one-proton triplet at 6.76 ppm. These resonances arise from the ortho meta and para protons of a phenylanine residue respectively.

Decoupling experiments also link together the remainder of the sharp resonances, i.e. a two-proton doublet at 7.1 ppm with a resonance at 6.2 ppm but it is not yet possible to say whether they belong to a phenylalanine or tyrosine residue. However, all the *sharp* resonances of the horse ferricytochrome *c* PMR spectrum in the aromatic region have now been described.

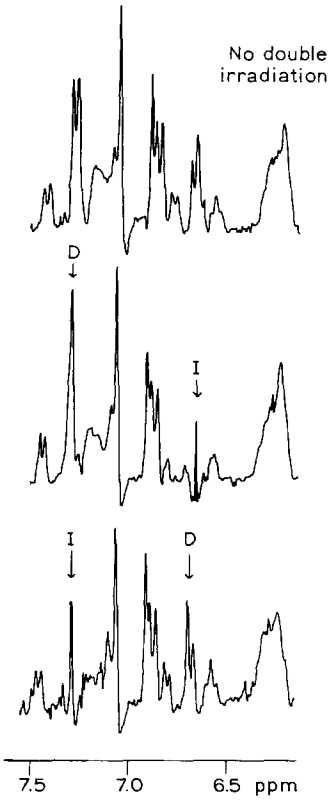

Fig.4. C.D. spectra of horse ferricytochrome *c*; decoupling of phenylalanine resonances. I is position of irradiation. D are decoupled peaks.

A number of resonances close to 7.3 ppm in the PMR spectrum of horse ferricytochrome *c* exhibit no observable multiplet structure. Amongst them there are three one proton singlets which could arise from non-exchangeable NH protons shifted upfield by ring-current or pseudo-contact shifts or which could belong to a tyrosine or phenylalanine residue in slow exchange between different orientations.

We return now to the assignment in the sequence of the tyrosine (7.64 and 6.82 ppm) and phenylalanine (ortho 7.23 ppm, meta 6.64 ppm and para 6.76 pmm) residues which have been identified. It is not possible to make such assignments from a consideration of their chemical shifts alone. Their resonances are all shifted slightly from their primary resonance positions (for tyrosine ortho 6.89 ppm and meta 7.20 ppm; for phenylalanine ortho 7.35 ppm and

meta and para 7.40 ppm [10]). From estimates of ring current shifts (table 2) it is clear that the internal tyrosine residues (No. 67 and 48) and phenylalanine residues (No. 82 and 46) PMR resonances should be shifted by up to 2.0 ppm. It is therefore likely that the resonances observed belong to either tyrosine 74 or 97 and phenylalanine 10 or 36.

All of these residues are on the surface of the molecule. Tuna cytochrome *c* differs from horse cytochrome *c* in possessing a tyrosine as residue 46 in place of a phenylalanine residue [14]. This indicates that the phenylalanine and tyrosine observed in the PMR spectrum of horse and tuna ferricytochrome *c* are not phenylalanine 46 or tyrosine 46 which is an internal residue. Extrinsic shift and relaxation probes are now being used for the full assignment of these proton resonances.

At this stage all the sharp resonances in the aromatic spectrum of horse cytochrome *c* have been assigned to types of amino acids. Before passing to the broad resonances we wish to draw attention to a feature of the spectra given by the resonances assigned to tyrosine and phenylalanine residues which is in marked contrast with the spectra of tryptophan residues. Each tyrosine observed gives rise to two two-proton doublets while the phenylalanine observed gives a one-proton triplet, a two-proton triplet and a two-proton doublet. Thus the two ortho and two meta protons are equivalent in all spectra of these residues which have been resolved and assigned.

Equivalence of the two ortho and equivalence of the two meta protons of tyrosine residues has been observed previously in NMR spectra of lysozyme [9], bacterial ferredoxin [11] and trypsin inhibitor protein [12]. These equivalences can be accounted for by a 'flip' mechanism [9]; the aromatic rings flipping from one orientation to another with a frequency $\geq 10^4$ per sec. Assuming this explanation to be correct segments of the cytochrome *c* molecule must be able to undergo small breathing fluctuations. In the light of what follows it should be remembered that these tyrosine and phenylalanine residues are probably on the surface of the molecule as mentioned above.

We turn now to the remaining part of the aromatic region of the spectra and its relationship to the number of aromatic groups in the sequence. First we note that approximately the same number of sharp resonances are seen in the spectra of horse and tuna

Table 2
Resonance shifts for tyrosine and phenylalanine proton resonances of horse ferricytochrome *c*

Residue	Shift from primary position	Source of shift
Phenylalanine 10	0.5 ppm upfield	tyrosine 97
Phenylalanine 36	None – no aromatic group nearer than 7 Å	
Phenylalanine 46	2.0 ppm upfield	tyrosine 48
	Also upfield ring current shift and downfield pseudo contact shift	heme
Phenylalanine 82*	Downfield pseudocontact shift and upfield ring current shift	heme
Tyrosine 48	2.0 ppm upfield	phenylalanine 46
	Also upfield ring current shift and downfield pseudo contact shift	heme
Tyrosine 67	Large downfield pseudo contact and upfield ring current shifts	heme
Tyrosine 74	No aromatic groups nearer than 7 Å	
Tyrosine 97	0.5 ppm upfield	phenylalanine 10

Ring current shifts arising from tyrosine and phenylalanine residues determined by data in [15].

* Phenylalanine 82 is in the heme crevice lying close to the heme group [16] and not out of the heme crevice as previously reported.

ferricytochrome *c* and that these resonances all have a line width <10Hz with well resolved multiplet structure. However the total number of protons represented by these sharp resonances is far less than the total expected number of aromatic protons. Horse ferricytochrome *c* contains 47 protons belonging to aromatic amino acids. We see the resonances of only 21–24 as sharp peaks and therefore we need to find 21–24 further aromatic proton resonances. This applies to both cytochromes under study.

By analogy with the ^{13}C spectra of horse cytochrome *c* [13] only the resonances of histidine 18 and tyrosine 67 could be shifted outside the region 5.5 to 10 ppm. Thus the resonances of some 17–20 aromatic protons are still to be recognised. The resonances of these protons could be extremely *broad*, so that are not seen, or they could be the *broad* peaks in the region 5.5 to 6.5 ppm in fig.1. In either case the broadening cannot be due to the proximity of the heme to these aromatic protons as the resonances of Trp 59, which is 7–8 Å from the iron, are sharp. The broadening must therefore be connected with molecular motion. Such broadening would occur if, for example, flipping of these residues was not as rapid as that of the aromatic residues on the surface.

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