ASSIGNMENT OF AROMATIC AMINO ACID PMR RESONANCES OF HORSE FERROCYTOCHROME C

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1. Introduction

Cytochrome *C*, an electron-transfer protein functioning in mitochondrial redox chains, has been intensively studied by X-ray crystallographic methods. Structures for horse ferricytochrome *C* [1], tuna ferrocytochrome *C* [2], and bonito ferrocytochrome *C* [3] are now available whilst the structure determination of tuna ferricytochrome *C* is in progress [4]. Comparisons of these structures reveals a number of differences in conformation, even for bonito and tuna ferrocytochrome *C*, which cannot be ascribed simply to species differences or oxidation state change [5]. Bonito and tuna cytochrome *C* differ in only two residues out of 103 [4]. It could be that the conformation differences are due to differences in space group of their crystal forms.

We wish to investigate the solution structure of cytochrome *C* by proton nuclear magnetic resonance (PMR) spectroscopy in order to discover the relationship between crystal and solution structures and especially to determine the extent of the conformation change upon oxidation/reduction of cytochrome *C*. As a prelude to probing the PMR spectra of cytochrome *C* with lanthanide ions we need to resolve and assign as many resonances as possible. The general approach for assigning the resonances of aromatic amino-acids involves pH titration of histidine (his) resonances, and resolution enhancement of the PMR spectrum followed by spin-decoupling of the coupled resonances. We have recently applied these techniques to the PMR spectra of horse and tuna ferricytochrome *C* and identified the resonances arising from the C–H protons of 2 his, 1 tryptophan (trp) and other aromatic groups of 3 tyrosines (tyr) or phenylalanines (phe) residues [6]. However, several of the resonances showed unusual dynamic properties. In this paper we report the results of a similar study with horse ferrocytochrome *C* and discuss the results in light of the X-ray structure of cytochrome *C*.

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 [7].

Horse cytochrome *C* (Grade VI) was obtained from Sigma Chemical Co. The protein was dialysed at pH 3.0 against distilled water at 4°C to remove bound ions, reduced with a 2-fold excess of ascorbate and dialyzed at pH 5.0 against distilled water at 4°C to remove the dehydroascorbate and excess ascorbate. The protein was then lyophilized from D_2O.

For the PMR experiments 5 mM solutions of ferrocytochrome *C* in D_2O were prepared and kept under argon to prevent autoxidation. The pH was adjusted to 8.0 ± 0.2 with NaOD and the solution immersed in H_2O at 60°C for 10 min. to exchange the NH protons for deuterions. After cooling to room temperature the nominal pH of the cytochrome *C* solutions was adjusted to pH 5.25 ± 0.1 with DCl.

The temperature at which the spectra were obtained was 57 ± 0.5°C unless otherwise mentioned. Acetone and dioxan were used as internal standards but all

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shifts are quoted in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulphonate. The integrity of the protein conformation was checked after experiments in which high temperatures were used by remeasuring the pmr spectrum at the standard temperature.

3. Results and discussion

Proton resonances occurring in the spectral region 11–5.5 ppm of diamagnetic heme protein arise from C–H protons of his, trp, tyr and phe amino-acid residues, some protons of the heme group and N–H protons. N–H protons can often be exchanged for deuterions, as was done for the experiments described here, which leads to a considerable simplification of the PMR spectrum. In addition 'non-aromatic' C–H protons may be shifted downfield by ring current shifts into this region. For horse ferrocytochrome *C* there do not appear to be any resonances attributable to such protons, see below. The meso C–H protons of the heme have been assigned by comparison to model heme compounds [8]. These are the four one-proton singlets between 9.7–9.0 ppm. We are therefore left with the task of assigning the PMR resonances arising from the 3 his, 1 trp, 4 tyr and 4 phe residues of horse cytochrome *C*. His 18 is the fifth iron ligand and its resonances will be subject to a large upfield ring current shift from the heme group. Similarly the resonances of tyr 67, which is placed near the heme group [1,2], might well be expected to be shifted upfield out of the region under study. Of the remaining aromatic protons the largest upfield shifted resonances are expected to be shifted no further than to 5.5 ppm. Thus, we expect to observe the resonances of 2 his, 1 trp, 3 tyr and 4 phe residues in the region 11.0–5.5 ppm.

For the assignment of aromatic aminoacid proton resonances it is necessary to identify families of resonances as in fig.1. The his resonances can often be identified by pH titration over the range pH 5–8 whilst the coupled resonances are identified by spin-decoupling techniques.

In fig.2 the normal and convolution difference spectra (CDS) [9] of horse ferrocytochrome in D_2O at pH 5.25 and 57°C are shown. Assignments can

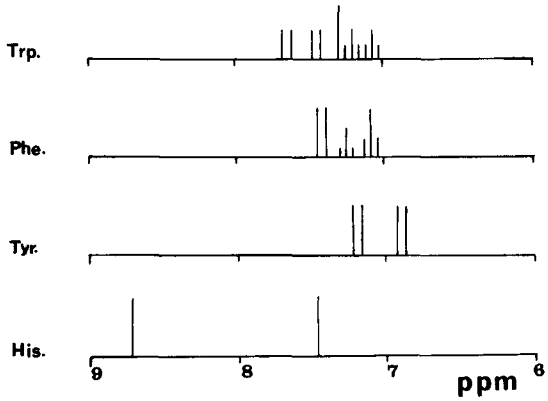

Fig.1. Resonance patterns for the aromatic C–H protons of amino-acids.

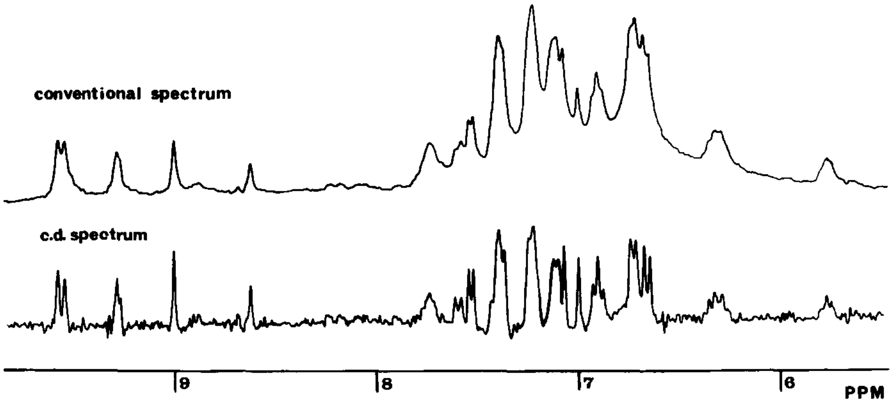

Fig.2. Conventional and C-D-spectra of horse ferrocytochrome *C* in D_2O at pH 5.25 and 57°C.

readily be made based on the multiplet structure observable in the CDS. Cohen and Hayes [10] have identified the resonances of a titrating his residue (8.55 and 7.55 ppm) and assigned them to the C-2 and C-4 protons of his 33 by comparison of the spectrum of horse ferrocytochrome *C* with that of tuna ferrocytochrome *C* which possesses a trp in place of his 33. The remaining three singlets (7.52, 7.06 and 6.99 ppm) can be assigned to the C-2 and C-4 protons of his 26 and the C-2 proton of trp 59 although it is not yet possible to assign them individually.

The coupled resonances of trp 59 were identified by spin decoupling-difference spectra [7] (fig.3). The C-4 and C-7 proton resonances are at 7.60 and 7.07 ppm whilst the C-5 and C-6 proton resonances are at 6.68 and 5.74 ppm (table 1). Finally, the resonances of 3 tyr/phe residues have been identified (table 1). Spin decoupling-difference spectra were again necessary for these assignments (fig.4). The tyr/phe resonances (table 1) are only slightly shifted from their primary positions (fig.1), and so probably arise from three of the surface residues tyr 74 and 97, and phe 10 and 36. These resonances are not expected to be subject to a large ring-current shift from the X-ray structure.

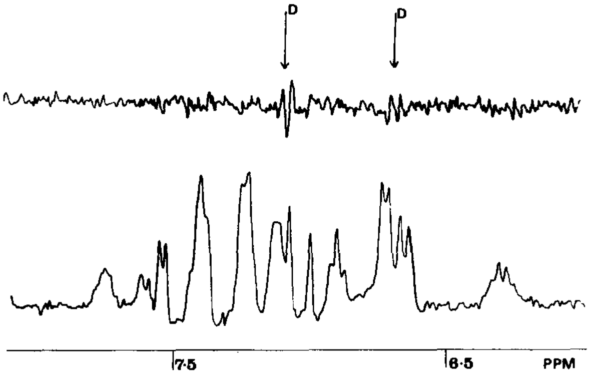

Fig.3. Decoupling of tryptophan resonances. (a) Difference spectrum between spectrum obtained without decoupling irradiation and spectrum obtained with decoupling irradiation at 5.60 ppm. (b) C-D-spectrum of horse ferrocytochrome *C*. D are the decoupled peaks.

All the sharp resonances in the aromatic region of the horse ferrocytochrome *C* PMR spectrum (fig.2) have now been assigned to particular types of protons. All the his and trp resonances which we should observe are in fact seen but only the resonances of 3 tyr/phe

Table 1
Assignment of resonances in the aromatic region of the PMR spectrum of horse ferrocytochrome *C*.

Assignment	Chemical shift (ppm)
Meso C–H protons of the heme groups 8	9.61, 9.58, 9.31 and 9.02
His 33 C-2 10	8.55
His 33 C-4 10	7.55
His 26 C-2 or C-4, or trp 59 C-2	7.52
His 26 C-2 or C-4, or trp 59 C-2	7.06
His 26 C-2 or C-4, or trp 59 C-2	6.99
Trp 59 C-4 or C-7	7.60
Trp 59 C-5 or C-6	6.68
Trp 59 C-6 or C-5	5.74
Trp 59 C-7 or C-4	7.07
Phe 10 or 36 ortho	7.40
Phe 10 or 36 meta	6.89
Phe 10 or 36 para	7.12
Tyr 74 or 97 ortho or meta	6.64
Tyr 74 or 97 meta or ortho	7.22
Tyr or Phe 2 proton doublet	6.71
Tyr or Phe coupled peak	7.40

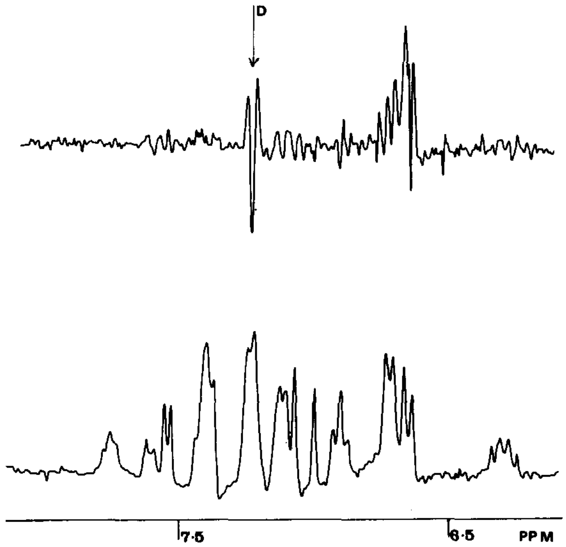

Fig.4. Decoupling of tyrosine resonances. (a) Difference spectrum between spectrum obtained without decoupling irradiation and spectrum obtained with decoupling irradiation at 6.64 ppm. (b) C-D-spectrum of horse ferrocytochrome *C*. D is the decoupled peak.

groups can be seen as sharp peaks. This is the same situation as for horse and tuna ferricytochrome *C* [6]. The only mechanism whereby specific resonances can be broadened in a diamagnetic molecule is by motional processes. The exact equivalence of the tyr and phe pairs of ortho and meta protons seen as sharp resonances can be understood on the basis of a 180° flip of the aromatic group about the C1-C4 axis [7]. If the rate of flipping were not fast with respect to the chemical shift, difference between the two environments (say of two ortho protons) exchange broadening of the resonances could occur. The conclusion that exchange broadening accounts for the broad resonances in the spectra can be checked by changing the temperature.

Spectra of horse ferrocytochrome *C* have been obtained between 5°C and 95°C. At low temperatures (< 25°C) a number of peaks in the aromatic region of the spectrum merge and broaden out so that they are not observable, whilst at higher temperatures (> 75°C) some broad peaks appear and sharpen up with an increase in temperature. The peak at 7.75 ppm in the spectrum of fig.2 is clearly identifiable as a doublet at 95°C. At 57°C this resonance is broad and cannot be shown to be coupled to any other resonance whereas at 95°C it is a sharp doublet and coupled to a doublet at 7.30 ppm. Thus these resonances belong to a tyrosine. In addition to these changes in linewidth a number of resonances change their chemical shift with temperature. Clearly some change in conformation accompanies the change in temperature but the effects of temperature on the linewidths of some of the aromatic resonances may also be connected with the problem of exchange broadening. There can be no gross change in heme coordination as the methyl group resonance of methionine is not altered from its position over the whole temperature range.

The chemical shifts of the benzenoid proton resonances of the trp 59 group in both oxidation states of cytochrome *C*, and the chemical shifts of the trp benzenoid proton resonances in L-tryptophanyl-L-alanine [11,12] are given in table 2. It is not possible from these data to correlate a resonance in the reduced state with its counterpart in the oxidised state or to assign the resonances to specific trp protons but two points can be made. Firstly, ring current calculations using the X-ray co-ordinates show that in ferrocytochro-

Table 2
Chemical shift (ppm) of the benzenoid proton resonances of trp

Assignment	Horse ferricyto-chrome *C*	Horse ferrocyto-chrome *C*	L-tryptophanyl-L-alanine	
C-4	7.57	7.60	7.68	(12)
C-7	7.37	7.07	7.54	(11)
C-5	6.54	6.68	7.20	(11)
C-6	6.31	5.74	7.28	(11)

me *C* the benzenoid proton resonances of trp 59 should be subject to a downfield shift [8]. In fact, they are subject to a large upfield shift which indicates that either the X-ray co-ordinates or the ring current calculations are in error. Secondly, there is a shift in the trp resonances upon reduction. This is not due to the trp protons being subject to a pseudocontact shift in ferricytochrome *C* because the trp resonances are temperature independent. Thus, a conformation change involving trp 59, and/or aromatic groups around trp 59, occurs upon reduction. The exact nature of this conformation change can only be understood after a much more detailed assignment using probe methods.

Acknowledgements

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