STRUCTURAL STUDIES BY PROTON NMR OF CYTOCHROME *C*-557 FROM *CRITHIDIA ONCOPELTI*

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1. Introduction

A vast amount of information has been accumulated about the amino acid sequences of cytochromes *c* from different species [1]. In addition to their phylogenetic importance, these data are of great interest for the investigation of structure–function relations in cytochrome *c*. From the primary structures of the proteins of over 30 species it had been inferred that the amino acids involved in the fixation of the heme group, i.e. Cys-14, Cys-17, His-18, and Met-80 [2–7] are among the invariant residues required for the proper cytochrome *c* function. More recently two cytochromes *c* from protozoans were found to have Cys-14 replaced by Ala, yet otherwise to be homologous with other eukaryotic cytochromes *c* [8–10]. the heme in these proteins is probably covalently linked with the remaining Cys-17, whereas the second vinyl side chain is free [8]. It is of course of much interest to investigate how this apparently important variation affects other properties of the proteins. In this paper the proton NMR features of cytochrome *c*-557 from *Crithidia oncopelti* are reported and compared with those of other cytochromes *c*.

Proton NMR spectroscopy has in recent years been found to be a suitable technique for structural studies of cytochromes *c* [4, 5, 7, 11, 12], e.g. in the identification of methionine as the sixth ligand of the heme iron [4, 5, 7]. Outstanding NMR spectral features are conserved in cytochromes *c* from different species both in the reduced [12] and oxidized [5] states. For both oxidation states of the protein this implies that characteristic features of the molecular conformations are maintained. In the oxidized cytochromes *c* the NMR spectra show further that the electronic structure of heme *c* is very nearly identical in all the different species studied [5, 13]. Since the typical heme electronic structure and the cytochrome *c* function are simultaneously lost in several chemically modified proteins [14], it was of particular interest to study the effects of the alanine at position 14 in place of the heme binding cysteinyl residue in cytochrome *c*-557 [8] on the hyperfine-shifted heme resonances. From this several previous resonance assignments in the spectra of ferricytochromes *c* could then be confirmed.

2. Materials and methods

Cytochrome *c*-557 from *Crithidia oncopelti* has been isolated and purified as described earlier [8]. For the NMR measurements a solution of the protein in 0.05 M deuterated phosphate buffer was prepared by repeated ultrafiltration. The protein concentration was approx. 8×10^{-3} M, and the pH was 7.2 as measured with a combination glass electrode without correction for deuterium isotope effects. Reduction of the protein was achieved by adding solid disodium dithionite to the solution of ferricytochrome *c*-557.

High resolution proton NMR spectra were recorded on a Varian HR-220 spectrometer equipped with a standard Varian variable temperature control unit. The signal: noise ratio was in some experiments improved by data accumulation in a Varian 1024 computer of average transients. Chemical shifts are expressed in parts per million (ppm) from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), where shifts to low field are assigned negative values.

3. Results

Fig. 1 shows the proton NMR spectrum of ferricytochrome *c*-557, where the heme iron is in the paramagnetic low spin ferric (Fe^{3+}, $S = 1/2$) state. The following spectral features, which are typical for ferricytochromes *c* [5, 15] are readily recognized: there is a pair of methyl resonances between −30 and −35 ppm, and a methyl resonance and two single proton resonances between 20 and 25 ppm, where the line at 20.5 ppm (fig. 1) was found from its intensity to consist of a methyl and a one-proton resonance. These resonances

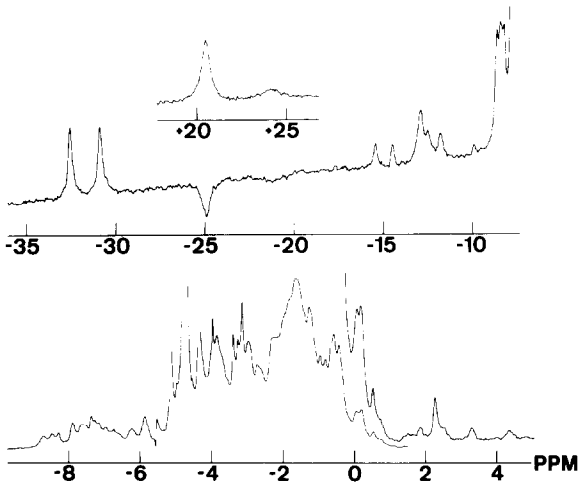

Fig. 1. 220 MHz proton NMR spectrum of approx. 0.008 M ferricytochrome *c*-557 in 0.05 M deuterated phosphate buffer (pH 7.6) at 30°C. The different spectral regions are represented with different horizontal and vertical scales. The strong lines between −4 and −6 ppm are the resonances of HDO and its first and second spinning side bands. The lines between 20 and 25 ppm appear also in the lowfield region of the first side band spectrum as inverted center band resonances.

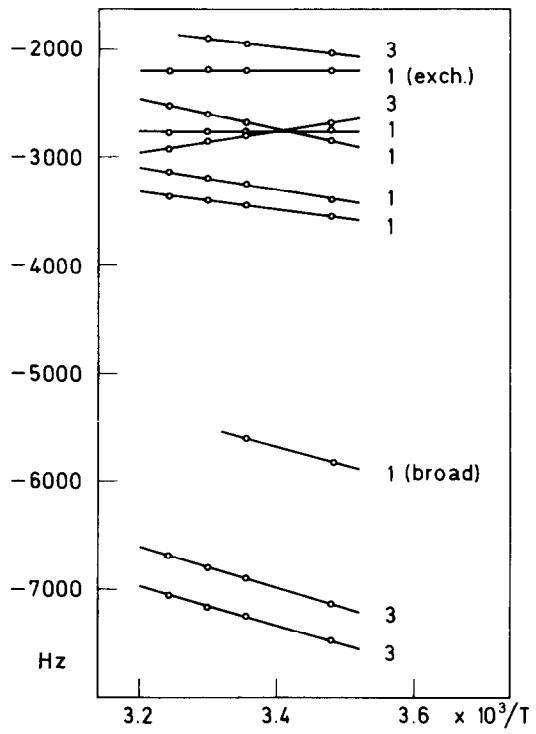

Fig. 2. Dependence on the reciprocal of temperature of the proton resonances between −9 and −35 ppm in ferricytochrome *c*-557. The number of protons corresponding to the resonance intensities are given on the right hand side. The 4 resonances with intensity 3 protons are assigned to the heme ring methyls. The intensity of the line at −10 ppm, which shows no temperature dependence, was found to decrease with time in the D_2O solution, and hence is assigned to a slowly exchanging proton.

had previously been assigned to two heme ring methyls and to the methyl group and the γ-methylene protons of the axial methionine [4, 5, 7], respectively. In addition, from the resonance positions, intensities, line-widths, and dependences on temperature (fig. 2) a one-to-one correspondence is also found for all the lines between −10 and −25 ppm in ferricytochrome *c*-557 and the vertebrate proteins (table 1). Fig. 2 also shows that the inverse temperature dependence of one of the ring methyl resonances, which had previously been noticed in ferricytochromes *c* [5], is also present in cytochrome *c*-557.

The different amino acid compositions and primary

Table 1

Prominent resonances of the heme group and the axial ligands of the heme iron in the NMR spectra of ferricytochrome *c* from guanaco and ferricytochrome *c*-557 at 30°C.

Chemical shift cyt. *c* guanaco*	(ppm from DSS) Cyt. *c*-557	Resonance assignment	Comments
−34.5	−32.5	Heme ring methyl [5]	
−31.8	−30.8	Heme ring methyl [5]	
−10.2	−12.9	Heme ring methyl [7]	'Inverse' temperature dependence
− 7.2	− 8.6	Heme ring methyl [7]	
−23.4	−24.9	His-18 ring C [7]	Very broad, coincides in the spectrum of *c*-557 at 30°C with the center band resonance of the Met-80 methyl resonance of the Met-80 methyl
−18.3	−15.5	Propionic acid side chain**	
−14.1	−14.5	...	These three lines show a similar temperature dependence
−12.1	−11.8	...	
−11.5	−12.5	...	Very small temperature dependence
+23.7	+20.5	Met-80 methyl and one Met-8 γ-proton [5]	
+27.6	+23.7	Second Met-80 γ-proton [7]	

* Resonance positions for ferricytochromes *c* from other vertebrates are essentially identical to those in the guanaco protein.
** C.C. McDonald and W.D. Phillips, private communication.

structurers of cytochrome *c*-557 [8] and other cytochromes *c* [1] are of course manifested in the spectral region from −10 to 0 ppm, where most of the resonances of the polypeptide chain occur. In the ferric proteins, outstanding spectra differences include

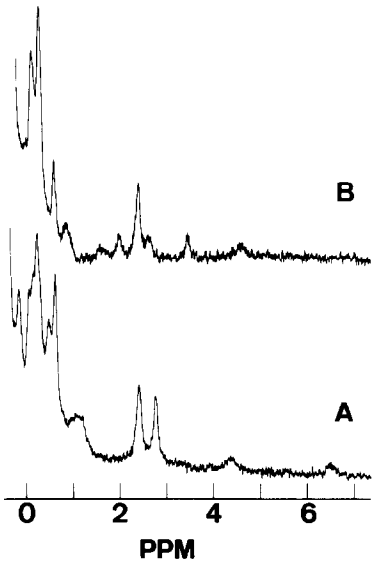

Fig. 3. Spectral region between 0 and 7 ppm of the 220 MHz proton NMR spectra at 25°C of ferricytochrome *c* from turkey (A) and ferricytochrome *c*-557 (B). The spectrum of ferricytochrome *c* from turkey is representative for the NMR spectra of all the vertebrate ferricytochromes *c* studied so far [5].

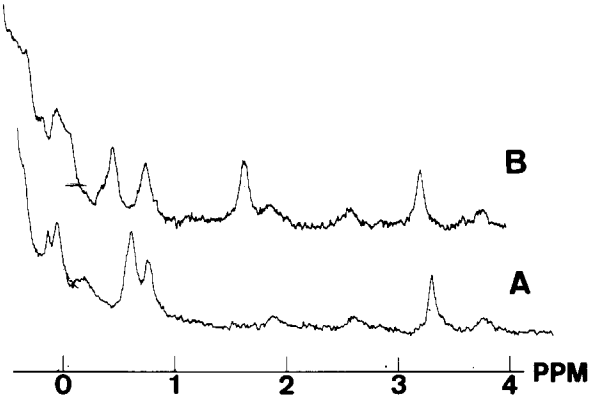

Fig. 4. High field part of the 220 MHz proton NMR spectra at 35°C of ferrocytochrome *c* from guanaco (A) and ferrocytochrome *c*-557 (B). The spectrum of ferrocytochrome *c* from guanaco is representative for the NMR spectra of all the vertebrate ferrocytochromes *c* studied so far [12].

further the methyl resonance at 2.7 ppm and the one-proton resonance at 6.5 ppm in the spectrum of vertebrate cytochromes *c* (fig. 3A), which are missing in the spectrum of cytochrome *c*-557, where there appear instead two, one-proton resonances at 2.6 ppm and 3.4 ppm (fig. 3B).

The NMR spectrum of the reduced diamagnetic cytochrome *c*-557 is shown in fig. 4 together with that of a vertebrate species, ile. guanaco. The spectral features which are known to arise from the methionyl coordination to the heme iron, i.e. a methyl resonance around 3 ppm and three, one-proton resonances between 1.5 and 4 ppm [5] are also present in the spectrum of cytochrome *c*-557. Furthermore, both spectra contain three additional methyl resonances at high field from DSS, of which one at 0.75 ppm is in exactly the same position in the two species. It should also be pointed out that none of the high field methyl lines in ferrocytochrome *c*-557 shows as large a temperature dependence as one of the methyl resonances at approx. 0.7 ppm in the guanaco protein (see fig. 6 of ref. [5]).

4. Discussion

In low spin ferric heme proteins (Fe^{3+}, $S = 1/2$), shifts of proton NMR lines to positions outside the normal spectral region from −10 to 0 ppm are mainly due to interactions with the unpaired electron on the heme iron [13, 15, 16]. These hyperfine shifts may be the sum of contact [17] and a pseudocontact term [18] or a pseudocontact term alone, depending on whether or not unpaired spin density from the iron is transferred to that particular proton [13]. We calculated the pseudocontact shifts [19] for the molecular geonetry given by the 2.8 Å resolution X-ray data of horse ferricytochrome *c* [6], and the g-tensor determined by single crystal EPR measurements in the same protein [20] for every proton in the molecule. This calculation showed that of all the amino acid residues in ferricytochrome *c*, only those involved in the binding of the heme group are to be expected to have some proton resonances outside the spectral region from −10 to +2 ppm. Therefore the resonances outside of this spectral range appear to come from protons of the heme group, the thioether bridges, and the axial ligands, and these may in principle all be shifted also by contact interactions. The contact shifts are directly related to the unpaired spin density distribution in the heme group [21]. The close agreement between corresponding ones of these largely hyperfine-shofted lines in ferricytochrome *c*-557 and the proteins from vertebrates (table 1) then indicates that the electronic structures of the hemes are very similar. The small differences in the resonance positions (table 1) are of the same order of magnitude as those between the spectra of vertebrate cytochromes *c* and ferricytochrome *c* from *Candida crusei* [5]. Hence it would appear that the loss of one of the thioether bridges in cytochrome *c*-557 does not markedly change the spin density distribution in the heme and the axial ligands. This is not realy surprising if one considers the earlier NMR data on low spin ferric hemes and hemoproteins which indicated that different 2,4-substituents of the heme group produced only limited changes in the NMR spectra [22]. On the other hand the spectral similarities outlined in table 1 would hardly be comprehensible unless one assumes that the interactions between the heme group and the polypeptide chain are very similar in the different proteins, e.g. the bond angle and distance of the axial ligands of the heme iron must be essentially identical in ferricytochrome *c*-557 and the vertebrate ferricytochromes *c*.

From what has been said up to this point, the spectral differences in the regions from 2 to 7 ppm (fig. 3) have to be related to the different covalent structures of the heme group and its links with the polypeptide chain in cytochrome *c*-557 and the vertebrate proteins. Hence the methyl resonance at 2.7 ppm and the one-proton line at 6.5 ppm in fig. 3A, which are missing in the spectrum of cytochrome *c*-557 (fig. 3B), arise most probably from the methyl group and the methine proton of the thioether bridge to Cys-14. In analogy to these resonance identifications, the methyl resonance at 2.4 ppm and the one-proton line at approx. 4.5 ppm, which occur in both spectra (fig. 3), are assigned to the thioether bridge to Cys-17. The data on ferricytochrome *c*-557 thus confirm the identification of the thioether bridge resonance which had previously been based on spin relaxation studies [7]. The new one-proton resonances at 2.6 and 3.4 ppm in ferricytochrome *c*-557 (fig. 3) are in similar positions as those of the vinyl methylene protons in low spin iron (III) protoporphyrin complexes [23]. Hence the spectrum in fig. 3B appears to support the earlier suggestion by Pettigrew [8] that the 2,4-sub-

stituents of the heme in cytochrome *c*-557 are a vinyl group and a cytochrome *c*-type thioether link with the polypeptide chain.

Below 15°C all the four ring methyl resonances of the heme appear as resolved lines in the low field region of the spectrum of ferricytochrome *c*-557 and hence reliable measurements of the temperature dependence could be made (fig. 2). This is in contrast to the vertebrate ferricytochromes *c* where one ring methyl line is at all accessible temperatures in the aromatic region of the spectrum [5, 7] . Fig. 2 shows that only one of the four ring methyls moves downfield with increasing temperature. This comes rather as a surprise. From considerations on the symmetry of the molecular orbital wave functions [5, 13], one might have anticipated that such an unusual temperature dependence, if it occurs at all, should be observed either for two or for all four of the ring methyl resonances. This point is under further investigation*.

In the reduced diamagnetic proteins all the high field resonances shown in fig. 4 correspond to protons which are sizeably affected by the local ring current fields of the heme and the aromatic amino acids [24]. In particular the methyl resonance at 3.3 ppm and the three, single-proton resonances at 1.9, 2.6 and 3.75 in the spectrum of vertebrate cytochromes *c* (fig. 4A) are known to arise from the axial methionyl residue and to be largely shifted by the ring current field of the heme [5, 12]. The corresponding resonances in the spectrum of ferrocytochrome *c*-557 (fig. 4B) are found at 3.2, 1.85, 2.55 and 3.75 ppm. The close agreement between the positions of the methionyl resonances in the two spectra of fig. 4 implies that the relative positions of the heme and the axial methionyl residue are nearly identical in the two proteins. The slightly smaller upfield shifts in ferrocytochrome *c*-557 (fig. 4) would correspond to an increase of the iron–sulfur bond length of at most several hundredths of a Å over that in the vertebrate protein.

The NMR data discussed so far and the sequence homology between cytochrome *c*-57 [8] and vertebrate cytochromes *c* [1] suggest that the polypeptide chain may be folded in much the same way. It is then likely that the three methyl resonances, which are at 0.6, 0.6 and 0.75 ppm in fig. 4A and at 0.4, 0.75 and 1.6 ppm in fig. 4B arise from corresponding amino acid residues. From the computation of ring current fields, on the basis of the 2.45-Å resolution X-ray data for tuna ferrocytochrome *c* [25], the most likely assignments are to the two methyl groups of Leu-32, which is located near the heme, and to one methyl group of Ile-57 which is near Try-59. In cytochrome *c*-557 the residue at position 57 is Val whereas Leu-32 and Try-59 are unvaried for all eukaryotic cytochromes *c* [1, 8–10].

In conclusion the present high resolution proton NMR studies show that the coordination of the heme iron in cytochrome *c*-557 is essentially identical to that in vertebrate cytochromes *c*, both in the reduced and the oxidized states. The close similarity extends also to the electronic states of the paramagnetic heme groups in the oxidized proteins. From these and earlier [14] observations we would like to predict that cytochrome *c*-557 should have the ability to restore the respiration of cytochrome *c* – depleted vertebrate mitochondria, although wther or not this restoration will be quantitative as it appears to be for other cytochromes *c* [26] can not be decided.

* It appears that the ring methyl resonance at approx. –7.2 ppm in vertebrate ferricytochromes *c* [7] shows a 'normal' Curie-type temperature dependence. However, because this line occurs in a crowded region of the spectrum, the results are not as unambiguous as in the case of cytochrome *c*-557.

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