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Two-dimensional ^{1}H NMR spectra of ferricytochrome c_{551} from *Pseudomonas aeruginosa*

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The full assignment of ^{1}H NMR signals of heme proton resonances of ferricytochrome c_{551} from *Pseudomonas aeruginosa* has been performed by means of 2D NMR experiments. This technique allows the complete and unequivocal assignment of all heme resonances, including methylene resonances of the propionic groups, directly implicated in the pH dependence of the redox properties of cytochrome c_{551}.

Cytochrome c_{551}; Two-dimensional nuclear magnetic resonance; Paramagnetic metalloprotein; *Pseudomonas aeruginosa*

1. INTRODUCTION

Cytochromes are electron transfer proteins containing the heme group. Cytochromes *c* are low spin diamagnetic in the reduced state and low spin with S = $^1/_2$ in the oxidized state. They have relatively small in size and their NMR studies have mostly relied on the full assignment of the reduced species and through saturation transfer techniques the assignment has been reached also on the oxidized species [1–4]. Horse heart cytochrome *c* has been mostly investigated [5–9] and recently ^{13}C studies have been reported [10]. On cytochrome c_{551} from *Pseudomonas aeruginosa* a first investigation on the diamagnetic species was reported in 1970 [11]. The assignment of methyls and meso protons of the heme was performed on the ferricytochrome through saturation transfer techniques [12]. On the other hand, a ^{13}C–H heteronuclear multiple quantum coherence study on the oxidized species has recently been reported with the assignment of some of the heme protons [13], although the main purpose of this work was to demonstrate the feasibility on HMQC for paramagnetic systems. We want to show here that with the help of the available X-ray structure [14] a more complete and consistent assignment can be reached through ^{1}H 2D NMR spectroscopy of the paramagnetic species. The signals of the heme protons and those of the protons to them connected are assigned.

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2. EXPERIMENTAL

Ferricytochrome c_{551} was isolated from *P. aeruginosa* [15] and purified by a previously reported procedure [16]. The purity of the protein was checked by measuring the ratio $(\varepsilon_{551} - \varepsilon_{570})/\varepsilon_{280}$ (where ε is the molar extinction coefficient for the reduced form) [16]. An excess of $K_3Fe(CN)_6$ was added prior to NMR experiments in order to ensure the complete oxidation of the protein. The NMR experiments were performed on a Bruker AMX 600 and on a Varian Unity 400. Phase sensitive NOESY experiments [17] were performed using from 30 to 70 ms of mixing time and from 150 to 400 ms of relaxation delay. COSY experiments were performed in the magnitude mode [18] 1024 × 191 data points matrix was acquired, using 1024 scans each experiment. In order to optimize the detection of scalar connectivities involving signals with 100 Hz line-width a 360 × 120 data points was zero filled to 1K × 1K and Fourier transformed. TOCSY experiment [19] over the region 12/−2 ppm was performed using a spin-locking period of 30 ms and a relaxation delay of 400 ms. A superWEFT [20] pulse sequence (180° − τ − 90° − AQ) with 9 ms of relaxation delay and 12 ms of τ delay was used to detect faster relaxing signals.

3. RESULTS AND DISCUSSION

The 600 MHz ^{1}H NMR spectrum of ferricytochrome c_{551} is shown in Fig. 1 and it is consistent with that previously reported [12]. A minor species is also observed (signals labeled with *X* in Fig. 1) indicating the presence of two different isomers of cytochrome c_{551}, in a 1:10 ratio. The existence of isomers has already been reported in the case of several cytochromes c_{551} from different sources [15,21,22]. From here on, we will only refer to the signals of the major isomer. The spectrum is similar in shape to the more known horse heart cytochrome *c* [5–9] although, in the latter, the most shifted methyls are 7^1 and 18^1 whereas in the present case are the 2^1 and the 12^1 (see inset of Fig. 1). The different methyl shift pattern is ascribed to a different chirality of the methionine axial ligand [23,24].

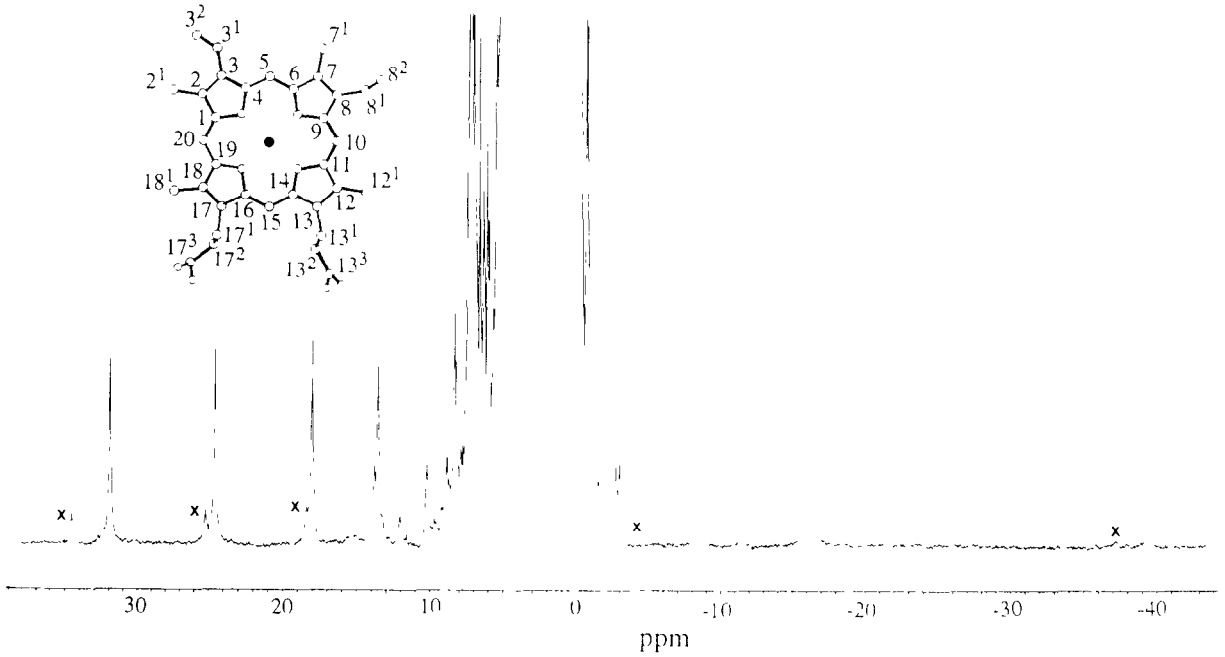

Fig. 1. 600 MHz ^{1}H NMR spectrum of ferricytochrome c_{551} from *P aeruginosa* in D_2O solution, pD 7.2, 303 K, 0.1 M phosphate buffer. A scheme of the prosthetic group is also reported, with the IUPAC-IUB nomenclature for a protoporphirin IX ring

Figs. 2 and 3 show the 600 MHz NOESY and COSY spectra, where the relevant cross peaks are labelled. The assignment of the heme proton resonances and of some relevant group close to the heme is reported in Table I. Such assignment, which is only partly consistent with previously reported assignments, is obtained by inspection of NOESY and COSY connectivities reported below.

Signals M_2 and M_3 give three NOESY cross peaks with the same protons (cross peaks 1–3 from M_2 and 4–6 from M_3). Their assignment to methyls 2^1 and 18^1 is therefore straightforward (see inset of Fig. 1). The observed cross peaks 1–6 connect the two methyls with the meso proton at position 20 (1,4) and with γ_1CH (2,5) and δCH_3 (3,6) of Ile^{48}. The complete assignment of signals of Ile^{48} can be performed by means of NOESY (7–14) and COSY (15–18) experiments. The different intensities and pattern of the connectivities between Ile^{48} and M_2 and M_3 signals immediately lead to the unequivocal assignment for these methyls. Indeed, 18^1 methyl is connected to γ_2-CH (19) while 2^1 methyl is connected to γCH_3 (20), according to what predicted from the X-ray structure.

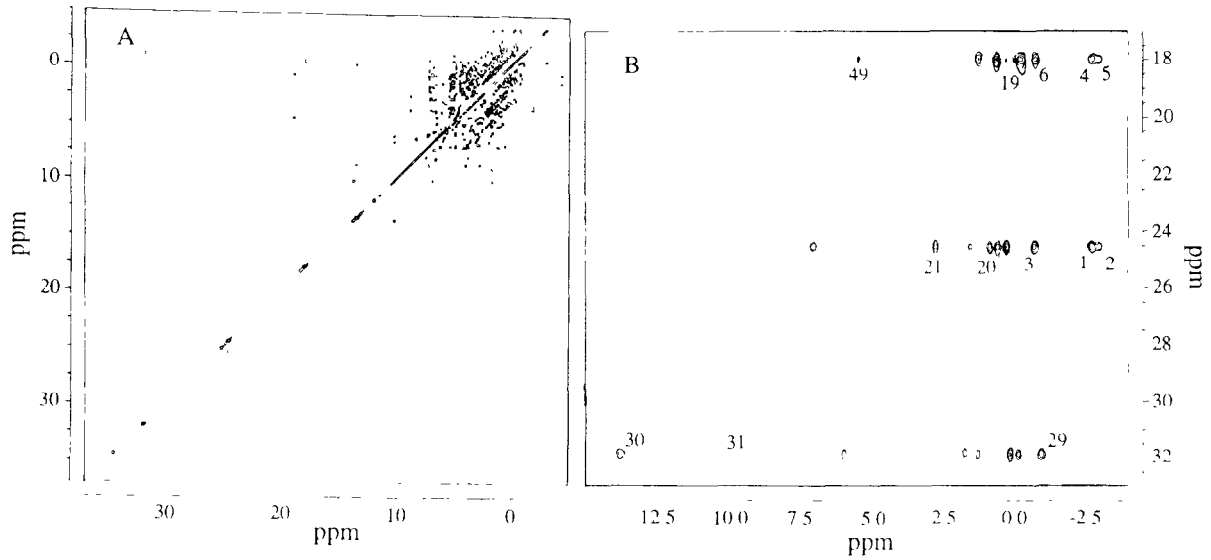

Fig. 2. 600 MHz NOESY experiment on ferricytochrome c_{551} from *P aeruginosa*. Experimental conditions as reported in the text. Panel B reports an expansion of the region 15/−4 ppm in F2 dimension and +33/+17 in F1 dimension

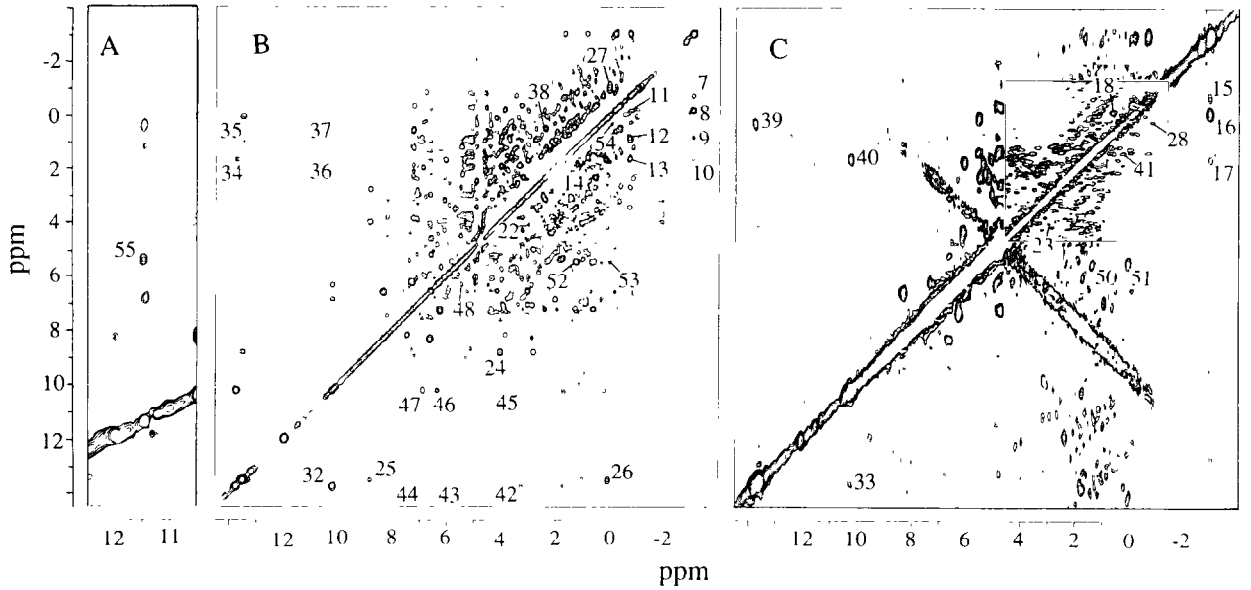

Fig. 3. (A) 600 MHz NOESY experiment in H_2O solution of ferricytochrome c_{551} from *P. aeruginosa*. The region shown in the figure allows to observe connectivities involving Trp^{56} $NH\varepsilon_1$ proton. (B) 600 MHz NOESY experiment on ferricytochrome c_{551} from *P aeruginosa*. The reported spectrum is an expansion of the spectrum in Fig. 2, reported to better appreciate cross peaks in the region 14/−4 ppm in comparison with the COSY experiment. (C) COSY experiment (magnitude mode) in the region 14/−4 ppm. The processing of the spectrum has been optimized to the detection of connectivities having 100 Hz of line-width. The inset of the figure shows a different data processing for the more crowded region of the aliphatic resonances.

Methyl at position 2^1 (M_2) gives also a very strong cross peak (21) with signal M_5, that gives NOESY and COSY with signal g (22,23). This signal gives a strong NOESY cross peak with the meso 5 signal (signal c, cross peak 24). This indicates that signals M_5 and g correspond to 3^2-CH_3 and 3^1-CH of the heme group, respectively. Signal M_4 gives a NOESY cross peak with meso 5 (25), indicating that this signal is the heme 7^1-CH_3. By exclusion, signal M_1 is assigned to 12^1-CH_3. Signal M_4 also gives a very strong cross peak with 8^2-CH_3 (26). Such signal gives NOESY and COSY cross peak (27,28) with signal n, suggesting that they correspond to 8^2-CH_3 and CH-8^1 of the heme group. NOESY experiments performed at 293 K, confirmed the M_4-$CH8^1$ connectivity and allowed us to detect a cross peak between signal CH-8^1 and meso 10 (data not shown).

Dipolar connectivities are observed between meso 10 and 12^1-CH_3 (29). The latter is connected with two signals (30,31) which, on their turn, are both scalarly and dipolarly coupled to each other (32,33) and are assigned to the 13^1-CH of propionic 13. The COSY spectrum reported in Fig. 3C has been optimized for the detection of connectivities among signals with large line-widths. The number of data points in both dimensions permits the detection of geminal and vicinal proton connectivities among signals of about 100 Hz of line-width (i.e. the observed line-width of methylene 13^1 signal). The observed COSY cross peaks were found to be consistent with what expected on the basis of density matrix simulations [25]. The vicinal connectivities of propionic moiety 13 are clearly observed in both NOESY (34–38) and COSY (39–41). Three strong dipolar connectivities are observed from both 13 methylene protons (42–47). Among these, the signal at 6.32 ppm is connected (48) to a signal at 5.45 ppm which, on its turn, is dipolarly coupled to 18^1-CH_3 (49). Signal at 5.45 ppm is therefore assigned to a methylene-17^{1a} proton. As in the case of propionic 13, the COSY experiment permits the detection of the 17^{1a}–17^{1b} (50) and 17^1–17^2 protons (51) connectivities, confirmed by the NOESY experiment (52–54).

An elegant confirmation of the assignment of the two propionic groups comes from the assignment of all the resonances of Trp^{56}. As only two Trp residues are present in the protein, they can be easily assigned by a series of 2D NMR experiments optimized to the detection of connectivities among diamagnetic signals (data not shown). Trp^{56} NH is connected to the 17^{1a} proton (55) while Trp^{56} $H\delta_1$ is connected to both 6 protons (44,47) and the same holds for Trp^{56} $H\delta_1$ (42,45). The assignment of the Trp signals is only partially in agreement with what previously reported [22].

Met^{61} protons show shorter relaxation times in comparison to the other protons due to their proximity to the metal ion. They are lost in the NOESY with 70 ms mixing time. The assignment of these protons is based on the observation of connectivities in the NOESY experiment with 30 ms mixing time (data not shown). No cross peaks relative to Met^{61} protons are observed in the COSY map.

Finally, an ^1H NMR experiment performed using superWEFT pulse sequence with very short delays be-

Table I

Assignment of the ^{1}H NMR signals of heme group proton resonances and of some other amino acid residues in cytochrome c_{551} from *Pseudomonas aeruginosa* at 303 K and pD 7.2

Signal	δ (ppm)	Assignment
M_1	+31.83[a]	methyl-12^1
M_2	+24.52[a]	methyl-2^1
M_3	+17.95[a]	methyl-18^1
M_4	+13.47[a]	methyl-7^1
M_5	+2.73[a]	methyl-3^2
M_6	+0.07[a]	methyl-8^2
M_7	−16.50[a]	Met^{61} εCH_3
a	+13 73	13^{1a}
b	+10.24	13^{1b}
c	+8 79[a]	meso 5
d	+7 03	Met^{61} Hβ_1
e	+6 32[a]	meso 15
f	+5.45	17^{1a}
g	+4.02[a]	methine 3^1
h	+1.70	13^{2a}
i	+1.22	17^{1b}
j	+0.28	17^{2a}
k	+0.15	13^{2b}
l	−0.02	17^{2b}
m	−0 99[a]	meso 10
n	−0.09[a]	methine 8^1
o	−1.49	Met^{61} Hβ_1
p	−2.75	meso 20
q	−8.46	Met^{61} Hγ_1
r	−39.79	Met^{61} Hγ_2
	+1.65	Ile^{48} Hβ
	+0.81	Ile^{48} γCH_3
	−0.18	Ile^{48} Hγ_2
	−0.76	Ile^{48} δCH_3
	−3.02	Ile^{48} Hγ_1
	+5.89	Gly^{24} Hα_1
	+2 78	Cys^{12} Hα
	+11.25[b]	Trp^{56} NHε_1
	+6 85	Trp^{56} Hδ_1
	+7.27	Trp^{56} Hζ_2
	+7.81	Trp^{56} Hη
	+7.62	Trp^{56} Hζ_3
	+7.35	Trp^{56} Hε_3

[a]Assignment already reported in [12b,13] and confirmed in this work.
[b]Observed in H_2O, pH 7.0.

tween pulses allowed us to detect broad, fast relaxing signals, at 11.4 and −6.1 ppm. They are tentatively assigned to the non-exchangeable ring protons of His^{16}.

The redox potential of this cytochrome changes with the pH, showing a pK_a value of 6.2 [26]. This pK_a has been associated to the deprotonation of the 17 propionic acid and its high value has been explained to be due to the existence of hydrogen bonds among this propionic acid and Trp^{56} and Arg^{47} [22]. The good agreement between the pK_a value for signals a and b and that of the redox potential, as well as the NOE observed between signal b and the Hδ_1 proton of Trp^{56} were lines of evidence to assign these signals to protons of the 17 propionic moiety [22]. These were reasonable assumptions since the 17^1 protons are near enough to Trp^{56} to give dipolar connectivities, as it has been observed in the Fe(II)-cytochrome [27]. In contrast with that, our present results unambiguously show that signals a and b correspond to the 13 propionic moiety, that it is also near to Trp^{56}. The greater isotropic shifts of the 13 propionic acid protons than those of the 17 propionic acid are probably due to the fact that the unpaired electronic spin density in the heme group is larger on the pyrrole III and pyrrole I groups than in the other pyrrole rings [23].

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