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Proton NMR studies of horse ferricytochrome *c*

Completion of the assignment of the well resolved hyperfine shifted resonances

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^{1}H NMR saturation transfer and nuclear Overhauser effect (NOE) measurements have been used together with two-dimensional spectra to complete the assignment of the well resolved hyperfine shifted resonances in the spectrum of horse ferricytochrome *c* and obtain their shifts in the reduced protein. New assignments include the β-CH_2 protons of Met-80, both ring protons of His-18, and the α-CH_2 of Gly-29 and δ-CH_2 of Pro-30, which resonate surprisingly far upfield despite the absence of any Fermi contact contribution to the shift.

Cytochrome *c*; Proton NMR; Hyperfine shift; Nuclear Overhauser effect; Saturation transfer; (Horse)

1. INTRODUCTION

Class I cytochromes *c* have been the subject of a large number of NMR studies, not only because of their important physiological role as electron transfer proteins, but often because of the attractive features in their spectra. In the reduced form, several resonances such as those of the heme meso protons or protons of an axial methionine ligand are shifted outside the main protein envelope by the large ring currents of the porphyrin. In the oxidized form, the paramagnetism of the iron causes heme and protein resonances to be spread over a wide range by Fermi contact and/or dipolar interactions [1]. The nature of the sixth ligand was determined in several cytochromes by analysis of these well resolved resonances [2]. More recently, the geometry of the coordination of the iron in a series of mitochondrial and bacterial *c*-type cytochromes was determined using methods which depend on the observation of well separated resonances [3]. The pH dependence of hyperfine shifted resonances has also been used to characterize ionizations which affect the redox properties of various cytochromes [4]. However, the most useful information to be extracted from these resonances is probably the characterization of the interaction between cytochromes and other electron transfer proteins which are potential partners in the electron transport chain. The strong overlap of resonances in the region 0–10 ppm in proton spectra of multiprotein complexes makes their use as indicators of the effects of binding extremely difficult, whereas the hyperfine shifted resonances can be monitored easily. Several studies of interactions between horse cytochrome *c* and other proteins have taken advantage of this, including the interactions with flavodoxin [5], cytochrome b_5 [6], cytochrome-*c* oxidase [7] and cytochrome-*c* peroxidase [8]. Detailed interpreta-

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tion of such data depends on the availability of firm assignments of resonances to specific protons in the cytochrome, and it is surprising that while horse cytochrome *c* has been the subject of NMR studies for twenty years, the assignment of the hyperfine shifted resonances remained incomplete.

We have recently become interested in the determination of Fermi contact and pseudocontact (dipolar) shifts in the proton and carbon-13 spectra of horse cytochrome *c* with the aim of obtaining a large enough data set to provide accurate information about possible redox linked conformational changes in solution and about the electronic structure of the heme *c*. All of the methyl carbon resonances, including those of the heme, and many methines have been specifically assigned in both oxidation states, and the proton assignment of these groups has been completed [9,10]. Here, we describe the completion of the assignment of the well resolved proton resonances in the spectrum of horse ferricytochrome *c* and correct some of the assignments reported previously.

2. EXPERIMENTAL

Horse cytochrome *c* (Type VI) was purchased from Sigma, lyophilised twice from D_2O and used without further purification. The amount of residual acetate in this batch was sufficiently small to cause no interference with our measurements. Samples were prepared for NMR by dissolving weighed quantities of protein in known volumes of D_2O and adjusting the pH by addition of 1 M NaOD or DCl. Partially reduced samples were obtained by adding small amounts of solid sodium dithionite. NMR experiments were performed using a Bruker AM300 spectrometer and chemical shifts were referenced to the Met-80 methyl of the reduced form at −3.28 ppm in mixed samples. Saturation transfer effects were measured from the difference of spectra obtained with 0.2 s preirradiation on and off resonance.

3. RESULTS AND DISCUSSION

The 300 MHz proton NMR spectrum of a solution of horse ferricytochrome *c* at 30°C is shown in fig.1. Sixteen resonances are clearly separated from the main envelope of overlapping signals at this temperature. The six signals of three-proton intensity, designated A–F, have been firmly assigned and will not be considered further here. This leaves five resonances from single protons, designated *a–e*, on the low field side of the envelope, and five to high field which are labelled

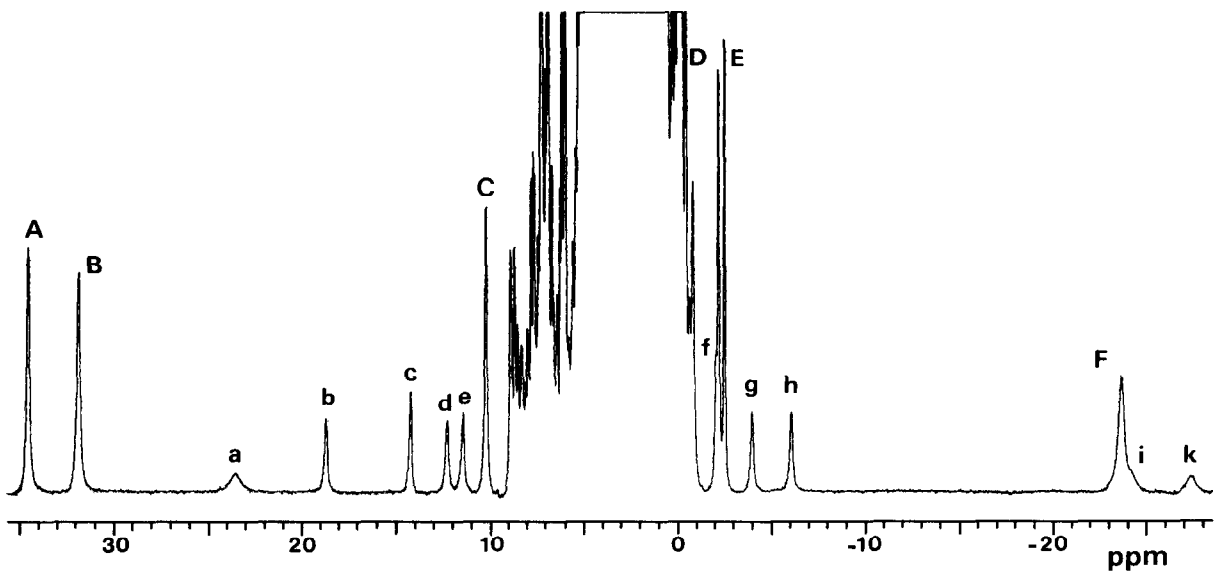

Fig.1. The 300 MHz proton NMR spectrum of 2 mM horse ferricytochrome *c* in D_2O at 30°C and pH 6.8 (uncorrected). The well resolved hyperfine shifted methyl groups are labelled A–F, and the single protons *a–i* and *k*.

f–i and *k*. Resonances in the spectrum of tuna cytochrome *c* which correspond to *b* and *e* have been assigned recently to the α-methylene protons of the heme propionate at position 7, and the resonance corresponding to *c* was assigned to one of the β-methylene protons of His-18 [11]. The assignments are expected to be identical in horse cytochrome *c* because of the close similarity between the NMR spectra of horse and tuna cytochromes. While this report was in preparation, Satterlee and Moench [12] published assignments for these resonances among others in the spectrum of horse cytochrome *c* which were based on the observation of NOEs among protons in the oxidised form. Our own studies of the effects of saturation transfer in mixed samples, with various ratios of the oxidised and reduced forms over a range of temperatures, confirm the expected assignments and identify the corresponding shifts in the reduced form, which are 3.61 and 4.16 ppm for resonances *b* and *e*, respectively (fig.2i). The effects of a two-step saturation transfer via NOE and electron transfer also allow the assignment of the propionate β-methylene protons in the oxidised

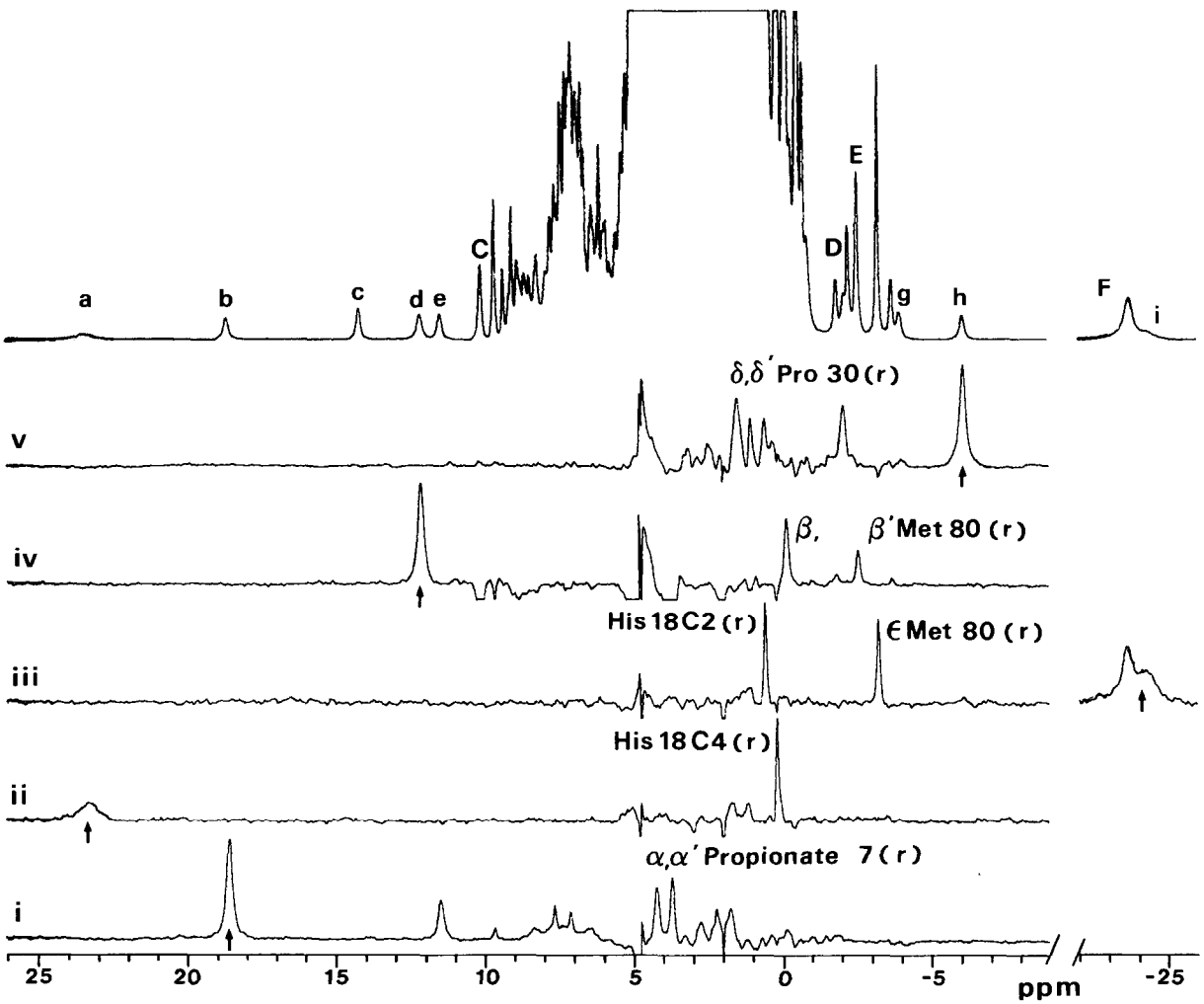

Fig.2. Saturation transfer experiments in a mixture of oxidised (40%) and reduced (60%) horse cytochrome *c* at 303 K and pH 6.8. Traces *i–v* are differences between spectra obtained with pre-irradiation at the positions arrowed, and off-resonance, and have their baselines corrected to minimise interference from residual HDO. The addition to trace iii covers the range −22 to −26 ppm and shows partial saturation of the Met-80 ϵ-CH_3 as well as the His-18 C2 proton.

form. Resonance *a* can be assigned specifically to the C4 (IUPAC C_5H) proton of His-18 since saturation is transferred strongly to a resonance at 0.14 ppm in the reduced form, as shown in fig.2ii, which has been assigned unambiguously [13,14]. A similar effect is observed between resonance *i* and a peak at 0.51 ppm in the reduced form, shown in fig.2iii. This resonance has been assigned to the C2 (IUPAC C_4H) proton of His-18 [13] and peak *i* is assigned accordingly, notwithstanding the fact that it was tentatively assigned to a γ proton of Met-80 in one of the earliest NMR studies of horse cytochrome *c* [15] and that the assignment has been accepted as correct since then [8,12,16]. Even so, our revision of the assignment of this resonance is in full agreement with predictions based on spectra of model compounds [17], in which the C4 proton resonance of an axial histidine ligand is shifted to low field with a similar shift of the C2 proton resonance to high field. This pattern is also observed in the cyanoferricytochrome complex, and in other ferric cyanide complexes of heme peroxidases [18].

Satterlee's recent work left resonance *d* unassigned [12], but fig.2iv illustrates a clear transfer of saturation from this peak to resonances of the reduced protein at −0.19 and −2.58 ppm, with weaker effects at −1.87 and −3.73 ppm. Since these resonances have been assigned firmly to β-CH_2 and γ-CH_2 protons of Met-80 [19], resonance *d* may be assigned to a β proton of Met-80 without difficulty. Saturation is also transferred to a peak at 3.0 ppm in the oxidised form with the intensity expected for a geminal proton, and we assign this to the second β proton. This is obviously in conflict with Satterlee's assignment of resonance *h*, and we offer an alternative interpretation of his results below. Resonance *h* has also been tentatively assigned to a heme meso proton [15], but COSY experiments reveal a scalar coupling between *f* and *h*, and a strong NOE shown in fig.2v suggests that *f* and *h* arise from geminal methylene protons. A similar relationship is observed between resonance *g* and a peak at −0.7 ppm. Smaller NOEs and COSY cross peaks at −0.3 and −0.9 ppm in the spectrum of the oxidised form demonstrate that *f* and *h* are part of a larger spin system. Furthermore, weak NOEs are observed for *f* and *h* when *g* is saturated, and at *g* and −0.7 ppm when *h* is saturated. Careful exclusion of the possibility of affecting neighbouring signals directly during the pre-irradiation demonstrates a close spatial relationship between these two pairs of protons.

Saturation of resonance *i* produces NOEs at *h* and *f* which were used to assign them to the Met-80 β-CH_2 on the basis of the misassignment of resonance *i* itself [12]. Rather, this places the spin system close to the C2 edge of His-18, and it is even possible to discern relayed NOE effects at −0.3 and −0.9 ppm in published spectra [12]. In mixed samples, saturation of resonance *i* leads to saturation of the His-18 C2 proton resonance at 0.51 ppm in the reduced form, as described above. A weak relayed NOE effect also appears at 1.00 ppm, which is the diamagnetic shift of the proton which gives rise to peak *h* in the oxidised form. The reduced form resonance which corresponds to peak *f* appears at 1.46 ppm, thus both protons exhibit large hyperfine shifts and it is easy to attribute this to a Fermi contact interaction with the unpaired electron, which is partially delocalised throughout the porphyrin and axial ligands. However, the X-ray structure of tuna cytochrome *c* [20] reveals that there is a δ proton of Pro-30 within 2.1 Å of the His-18 C2 proton. The relationship of these residues is illustrated in fig.3. Calculations based on these atomic coordinates

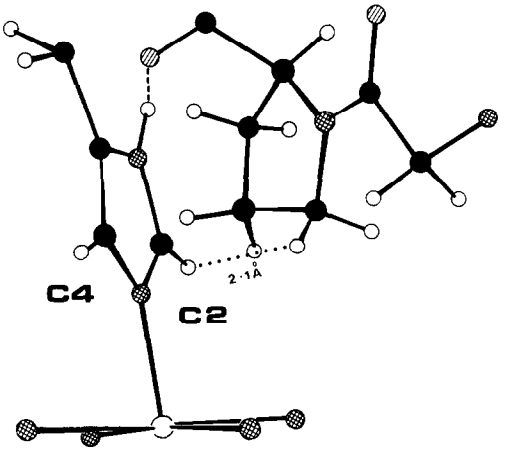

Fig.3. The residues His-18, Gly-29 and Pro-30 drawn from the X-ray coordinates of tuna ferricytochrome *c* [20]. The heme is represented by the iron and nitrogen ligands only. The His-18 C2 proton is 2.1 Å from the Pro-30δ (dotted line), and the hydrogen bond between the Pro-30 carbonyl and the His-18 NH is indicated by a dashed line.

predict a pure pseudocontact shift of −7 ppm for this δ proton and −4.4 ppm for its geminal partner, and the unusual shifts of the Pro-30δ protons in the diamagnetic form are also in reasonable agreement with the predictions of ring current calculations [21]. Resonances *f* and *h* are therefore firmly assigned to Pro-30δ, together with those at −0.3 and −0.9 ppm which we assign to Pro-30γ. Similar arguments lead to the assignment of resonance *g* and the peak at −0.7 ppm to the α protons of Gly-29, and this is confirmed by saturation transfer from *g* to −0.12 ppm with a relayed effect at 3.70 ppm, which were recently assigned to the α-CH_2 of Gly 29 in the reduced form [22]. The X-ray model also shows a Pro-30δ-Gly-29α separation of 2.3 Å, which appears to be an upper limit for the observation of NOEs in the core of this paramagnetic protein. It is also interesting to note

Table 1

Assignments of proton chemical shifts in horse cytochrome *c* at pH 6.8 and 30°C

Assignment	Reduced		Oxidised
Heme			
methyl 8	2.18 [27]	A	34.6 [27,28]
methyl 3	3.86 [27]	B	32.0 [27,28]
methyl 5	3.60 [27]	C	10.1 [27,28]
Thioether 2			
methyl	1.48 [27]	D	−2.3 [27,28]
Propionate 7			
β	n.d.		1.7
β	n.d.		−0.2
α	3.61	*b*	18.6 [11,12]
α	4.16	*e*	11.4 [11,12]
His-18			
β	1.09	*c*	14.2 [11,12]
C2	0.51 [13,14]	*i*	−24.3 Met-80 γ CH [15]
C4	0.14 [13,14]	*a*	23.4 His-18 C2/C4 [11]
Gly-29			
α	−0.12 [22]	*g*	−4.1 Heme meso [15]
α	3.70 [22]		−0.7
Pro-30			
γ	n.d.		−0.9
γ	n.d.		−0.3
δ	1.46	*f*	−2.1 Met-80 β-CH_2 [12]
δ	1.00	*h*	−6.1 Heme meso [15], Met-80 β-CH_2 [12]
Leu-68			
δ-CH_3	0.37 [22]	E	−2.6 [29]
Met-80			
α	3.09 [14,19]		2.8
β	−0.19 [19]	*d*	12.1 His-18 C2/C4 [11]
β	−2.58 [31]		3.0
γ	−3.73 [31]	*k*	−27.6 [30]
ϵ	−3.28 [31]	F	−23.7 [2]

References are given in the appropriate column for resonances which have been assigned previously, with the earlier assignment if it is now revised. The well resolved resonances are labelled as in fig. 1, n.d., not determined

that the well resolved resonances of the highly conserved proline residue present the opportunity to measure pure dipolar shifts close to the heme in other cytochromes.

Our completion of the assignment of the sixteen well resolved resonances of horse ferricytochrome *c* leaves one intriguing problem: we can confirm that resonance *k* arises from one of the γ protons of Met-80, but our attempts to find the other outside the range shown in fig.1 have been unsuccessful. Both γ-CH_2 protons of the axial methionine have been identified in the high field region of spectra of the ferricytochromes *c*-551 from *Pseudomonas stutzerii* and *P. mendocina* [23]. However, an early analysis of electron exchange effects in the proton NMR spectrum of *P. aeruginosa* cytochrome *c*-551 predicted a shift to low field for at least one of the γ-CH_2 protons of the axial methionine [24]. Thus we cannot exclude the possibility that the missing resonance lies underneath the main envelope of signals in horse ferricytochrome *c* but is too broad for us to detect an NOE from resonance *k*. The resonances of the heme meso protons may be similarly obscured.

The present work (summarised in table 1) illustrates the value of using both saturation transfer and NOE measurements for the assignment of proton resonances from the heme cavity of paramagnetic proteins; a similar methodology recently proved very successful in the assignment of heme resonances in horseradish peroxidase [25]. This work provides the only example of the assignment of β-CH_2 protons of an axial methionine or of the C2 proton of an axial histidine in the oxidised form of any native *c*-type cytochrome studied to date. The assignment of the Gly-29 and Pro-30 resonances may also be significant since these residues are conserved in almost all mitochondrial and most bacterial *c*-type cytochromes [26], possibly because of the importance of controlling the orientation of the axial histidine through hydrogen bonding [3]. Several NMR parameters have been compared among a number of class I *c*-type cytochromes in an attempt to determine which aspects of the geometry of the iron coordination are significant with respect to enzymatic activity, redox potential, or electronic structure [3]. The assignments presented here should give a much wider basis to the search for understanding of the structure/function relationship in cytochromes.

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