^{13}C and proton NMR studies of horse cytochrome *c*

Assignment and temperature dependence of methyl resonances

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The ^{13}C and proton chemical shifts of the 55 methyl groups of horse cytochrome *c* have been determined over a range of temperatures both in the diamagnetic ferrocytochrome and in the paramagnetic ferricytochrome. Specific assignments of many proton resonances have been published previously and all of the remaining methyl proton resonances are now specifically assigned. The corresponding ^{13}C assignments follow directly, including those of contact shifted ^{13}C resonances which are reported for the first time.

(Horse) Cytochrome c ^{13}C-NMR ^{1}H-NMR 2D shift correlation Hyperfine shift Temperature dependence Methyl resonance

1. INTRODUCTION

Horse cytochrome *c* has been the subject of intensive NMR study for many years [1–7]. It is a small and freely available globular protein which offers a model for studies of proteins in general and heme proteins in particular. Despite this interest, relatively few specific assignments were available for the aliphatic region of the proton spectrum until recent work extended the assignment to ~90% of the methyl resonances [8,9]. Isotopic substitution allowed the methyl resonances of Met 65 and Met 80 to be assigned in the ^{13}C spectrum of the ferrocytochrome, but only Met 65 was observed in the oxidized protein [10]; none of the contact shifted ^{13}C resonances associated with the heme has been reported previously. A combination of techniques including two-dimensional experiments correlating ^{13}C chemical shifts with those of the attached protons [11,12] allowed several more specific ^{13}C assignments to be made in the reduced form [13].

Dedicated to Professor S.P. Datta

We now describe the application of similar methods to horse ferricytochrome *c* and show how correlation of proton and ^{13}C hyperfine shifts allows the assignment of the proton and ^{13}C resonances of the 55 methyl groups to be completed in both oxidation states.

Studies of the paramagnetic ferricytochrome have 3 major purposes. First, they assist the cross assignment of resonances in the less well-resolved spectra of the ferrocytochrome, and this may provide some insight into the origin of ^{13}C shifts in diamagnetic proteins. The pseudocontact contribution to the hyperfine shifts of specifically assigned resonances may then be used for the elucidation of the three-dimensional structure [14] and to determine which residues participate in redox linked conformational changes [15]. The assignment of ^{13}C resonances is particularly important for this purpose since the temperature dependence of the chemical shifts indicates that they are more sensitive to conformational change than those of the protons [13]. Finally, determining the contact contribution to the hyperfine shift allows estimates to be made of the unpaired elec-

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tron spin density in the porphyrin and axial ligands.

2. EXPERIMENTAL

The basic experimental techniques used were described in [13]. Further experiments were performed using reduced samples of horse cytochrome *c* (Sigma, type VI) from which the acetate was removed by gel filtration in a column of Sephadex G-25. Ferricytochrome was prepared by addition of potassium ferricyanide and purified by adsorption onto a column of Whatman CM-52 equilibrated with 5 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.6. The protein was eluted with a 10–250 mM gradient of NaCl in the same buffer, then dialysed against H_2O and freeze dried. Solutions of 15 mM ferricytochrome were prepared in 90% H_2O/10% D_2O with a trace of ferricyanide and adjusted to pH 6.7. Experiments were performed in the range 27–51°C, the temperature being determined from the chemical shift of the methyl protons of Leu 68 and thioether bridge 2. The main envelope of proton resonances from ~ -2.5 to 12.5 ppm could be pulsed and decoupled in individual experiments at 300 MHz using field strengths corresponding to 90° pulses of 34 μs for polarization transfer [11,16] and of 110 μs for decoupling [17]. The extreme proton shifts of heme methyls 3 and 8 and of Met 80 required that selective decoupling difference experiments be used to determine their ^{13}C shifts. For these, the protons were irradiated continuously with a power of 0.5 W with the frequency on-resonance and $\sim$1.5 kHz off-resonance on alternate transients.

3. RESULTS AND DISCUSSION

Of the 55 methyl resonances of horse cytochrome *c*, 53 were resolved in the spectrum of the diamagnetic protein by experiments reported previously [13]. A form of two-dimensional spectroscopy was used in which the chemical shift of each ^{13}C nucleus is correlated with the shifts of the protons which are bonded to it [11]. It is therefore necessary only that 2 groups should be resolved either in the proton spectrum or in the ^{13}C spectrum for them to be fully resolved in the two-dimensional spectrum. The observations in [13] have been confirmed and one further methyl resonance revealed by the removal of acetate. Examination of peak intensities shows that 2 resonances remain unresolved in both proton and ^{13}C spectra over the range of temperatures studied. The proton shift of the methyl group previously obscured by the acetate resonance is close to that previously assigned to the methyl of the heme thioether bridge 2. This introduces an ambiguity, but the peak assigned previously is much sharper than those of thioether 4, the heme methyls or the newly revealed peak and, unlike these resonances, does not appear to broaden in the presence of a trace of oxidized protein. Furthermore, its temperature-dependent ^{13}C shift is typical of Leu, and it is reassigned to Leu 94 in accordance with the proton shift reported by Moore et al. [9]. The proton shift of the thioether methyl was established by proton autocorrelation experiments [18]. This agrees with the shift of the new peak, which is assigned accordingly. The methyl resonance tentatively assigned to Ile 75 or Ile 81 [13,19] has since been firmly assigned in the proton spectrum to Leu 68 [8,9], and therefore we reassign the ^{13}C shift.

The ^{13}C shifts of the remaining methyl groups of horse ferrocytochrome *c* may be assigned specifically by comparison with the recent proton assignments of Moore et al. [9] with the exception of 5 out of the 6 Ile δCH_3 resonances for which only primary assignments have been given [13]. The situation is reversed for Ile δCH_3 resonances in the proton spectrum of horse ferricytochrome *c*, with only Ile 75 δCH_3 and Leu 32 lacking specific assignments [8]. All 6 Ile δCH_3 resonances of the oxidized protein are readily distinguished by their ^{13}C chemical shifts in shift correlation spectra such as that shown in fig.1, so they may be assigned firmly and the peak with a proton shift of 2.01 ppm at 30°C is assigned to Ile 75 δCH_3 by elimination.

The difference between the proton shift of individual methyl groups assigned in the oxidized and reduced forms of the protein shows a good correlation with the difference of their ^{13}C shifts, as shown in fig.2, indicating that they are dominated by the pseudocontact interaction with the unpaired electron of the low-spin Fe(III). If the Fermi contact interaction contributed significantly, as it does for the heme and its axial ligands, then no such correlation would be expected. Some scatter is anticipated since the local field may

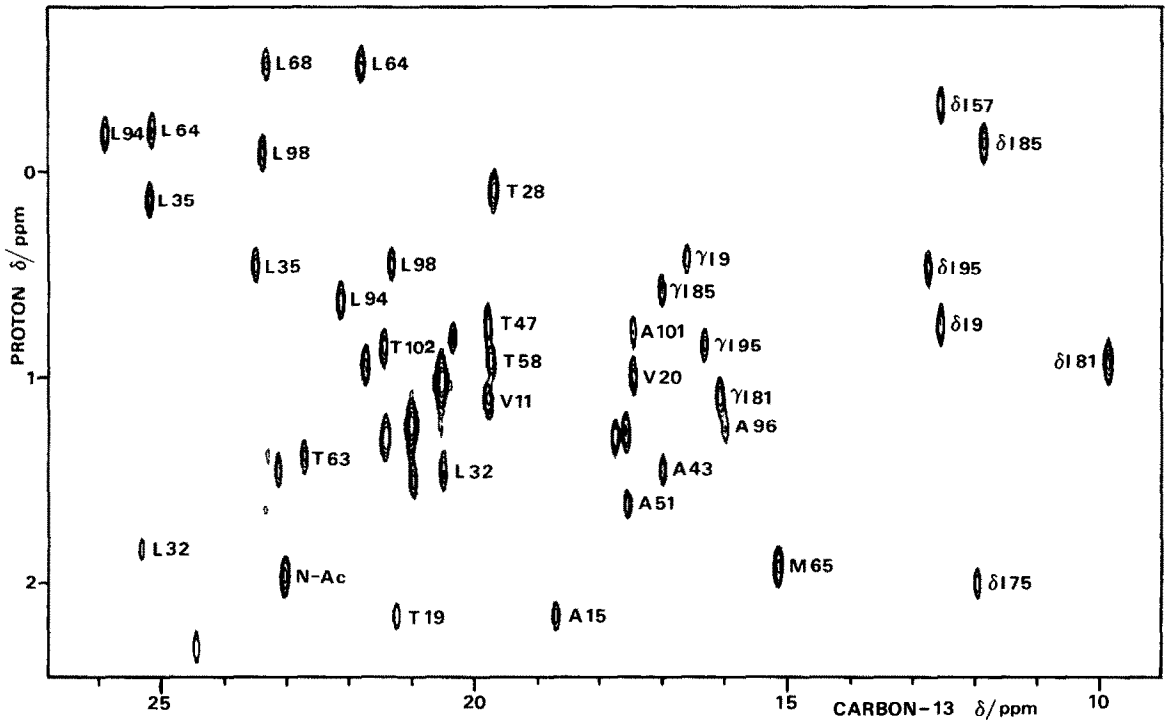

Fig.1. Part of the proton-^{13}C shift correlation spectrum of horse ferricytochrome *c* at 35°C showing the assignment of several methyl resonances. Only the contact shifted methyl resonances fall outside the range of ^{13}C shifts shown; the resonance of Thr 78 and one from Leu 68 are excluded because of large shifts in the proton dimension.

change significantly between the protons and carbon nuclei in some groups and variations in the diamagnetic shifts may be expected in case of redox linked changes in conformation. However, the correlation is good enough to cross assign the Ile δCH_3 resonances of the reduced protein, as shown in table 1. The small shift difference between Ile 9 δCH_3 and Ile 85 δCH_3 in the reduced protein leaves a slight possibility that the assignment should be interchanged; in either case the 4 conserved residues are found to have similar proton shifts in tuna ferrocytochrome *c* [9].

The specific assignment of the ^{13}C and proton resonances of the methyl groups is thereby completed in the reduced protein, and the 2 resonances of Leu 32 are all that remain to complete the assignment of the methyl protons in the oxidized form, with the methyl groups of Met 80 and the heme yet to be assigned in the ^{13}C spectrum. The proton shifts of the 48 methyl groups in the main envelope of the spectrum were determined from shift correlation spectra [11] after the ^{13}C shifts of methyl resonances had been distinguished by polarization transfer experiments [16] over a range of temperatures. Two of these proton shifts have strikingly large temperature dependencies and correlate with ^{13}C shifts in the region characteristic of Leu. Their assignment to Leu 32 can be confirmed by elimination now that the proton shifts of all of the remaining methyl groups have been specifically assigned. Excepting only Met 80, this completes the assignment of both proton and ^{13}C resonances of the methyl groups of the polypeptide chain, and the temperature dependence of the ^{13}C shifts is plotted in fig.3 for comparison with fig.3 of [13]. The structural implications of the differences between these chemical shifts in the oxidized and reduced protein are being analysed in collaboration between this department and the Inorganic Chemistry Laboratory in Oxford.

The methyl group of the axial ligand, Met 80, and the 4 heme methyls all show substantial contact shifts in the proton spectrum of the oxidized protein. The 2 methyls of the thioether bridges

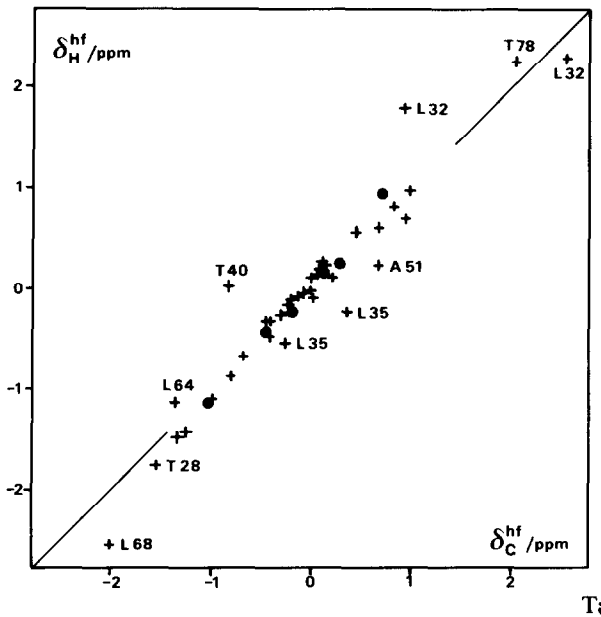

Fig.2. A good correlation is observed between the displacement of the proton and ^{13}C chemical shifts of methyl groups which occurs when the protein is oxidized. (+) Displacements of the 42 polypeptide methyls assigned in both oxidation states at 50°C. (●) Ile δCH_3 resonances assigned in the reduced form by means of this correlation.

may also be affected. The assignments of these resonances and of the corresponding proton shifts in the reduced protein were among the earliest reported [3,4,7], and the ^{13}C shifts of these groups in horse ferrocytochrome *c* were reported recently [13], but no contact shifted ^{13}C resonance has been observed previously. In our own experiments, the resonances from heme methyls 1 and 5 and thioether bridges 2 and 4 were characterized in

Table 1

Assignments of ^{13}C and proton chemical shifts of the methyl groups in horse cytochrome *c* referred to in the text

	Ferrocytochrome *c*				Ferricytochrome *c*			
	^{13}C shifts (ppm)[a]		Proton shifts (ppm)[b]		^{13}C shifts (ppm)[a]		Proton shifts (ppm)[b]	
	30°C	50°C	30°C	50°C	30°C	50°C	30°C	50°C
Leu 32	22.39 [13]	22.38	−0.77 [19]	−0.77	25.43[e]	24.93	1.89[e]	1.51
Leu 32	19.41 [13]	19.39	−0.61 [19]	−0.61	20.52[e]	20.33	1.51[e]	1.18
Leu 68	20.56[c]	20.59	0.35 [9]	0.34	18.20[c]	18.58	−2.61 [8]	−2.21
Leu 94	22.92[c]	23.06	1.54 [9]	1.54	22.10[c]	22.26	0.60 [8]	0.67
δIle 9	12.85[f]	12.71	0.99[f]	1.01	12.60[c]	12.50	0.71 [8]	0.78
δIle 57	12.21 [13]	12.16	−0.63 [19]	−0.50	12.57[c]	12.43	−0.36 [19]	−0.25
δIle 75	11.14[f]	11.20	0.94[f]	0.96	11.98[e]	11.90	2.01[e]	1.91
δIle 81	9.51[f]	9.82	0.70[f]	0.73	9.86[c]	9.94	0.90 [8]	0.89
δIle 85	12.94[f]	12.86	1.10[f]	1.10	11.86[c]	11.85	−0.18 [8]	−0.03
δIle 95	13.24[f]	13.11	0.95[f]	0.96	12.78[c]	12.67	0.44 [8]	0.52
Met 80	14.01 [10]	14.01	−3.30 [2]	−3.30	13.7[d]	13.6[g]	−23.72 [1]	−21.84
Heme 1	13.86 [13]	13.77	3.46 [7]	3.46	−17.4[c]	−16.6	7.11 [7]	7.65
Heme 3	13.74 [13]	13.79	3.84 [4]	3.84	−53.6[d]	−49.4	31.76 [4]	30.10
Heme 5	13.51 [13]	13.45	3.58 [7]	3.58	−24.1[c]	−22.8	10.11 [7]	10.84
Heme 8	11.56 [13]	11.57	2.16 [4]	2.16	−68.0[d]	−62.0	34.57 [4]	32.43
Thioether 2	24.81[c]	24.80	1.46 [7]	1.48	40.8[c]	38.7	−2.28 [7]	−1.75
Thioether 4	21.15 [13]	21.15	2.57 [7]	2.57	84.3[c]	80.8	3.08 [7]	3.08

[a] ± 0.02 or ± 0.05 ppm, dioxane ≡ 67.80 ppm
[b] ± 0.02 ppm, dioxane ≡ 3.73 ppm
[c] By cross-assignment from proton shifts in two-dimensional spectra
[d] By selective decoupling of assigned proton resonances
[e] By elimination of methyl resonances in both ^{13}C and proton spectra
[f] By correlation of ^{13}C and proton shift differences from the oxidized form
[g] T = 37°C

References are given in the appropriate column for resonances which have been assigned previously, otherwise the principal method of assignment is indicated by a–g

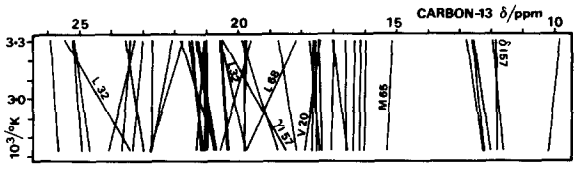

Fig.3. The temperature dependence of the ^{13}C chemical shifts of the methyl groups of horse ferricytochrome *c*, excluding those with contact shifts. Measurements were made in the range 27–51°C and are shown extrapolated to an arbitrary temperature to emphasize the gradients; these are determined by variations in both the diamagnetic and pseudocontact contributions.

one-dimensional ^{13}C spectra then assigned from their proton shifts in shift correlation experiments in a similar manner to the methyl groups of the polypeptide. Polarization transfer from the protons to ^{13}C of heme methyls 3 and 8 was observed with a low field proton irradiation frequency, but the effect is weak because of the short proton T_2. Accordingly, no transfer was detected from the methyl protons of Met 80. The ^{13}C shifts of these groups were assigned specifically using difference spectra with selective proton decoupling. The shift obtained for Met 80 is remarkable, but it should be noted that the samples used showed no trace of the denatured protein reported by Wooten et al. [10]. The contact contribution to the ^{13}C shifts of the methyl groups and other CH_n groups which have been assigned provides information about the delocalization of the unpaired electron which will be discussed in a future article. Here, we note simply that the shifts are in broad agreement with ^{13}C studies of model compounds [20] with allowance made for the asymmetry of the spin density shown by the contact contribution to the proton shifts.

In conclusion, it is clear that the observation of hyperfine shifts is of considerable assistance in the assignment process as well as being of intrinsic interest. The eclectic methodology for ^{13}C and proton NMR studies outlined [13] offers similar advantages but is not restricted to metalloproteins. Horse cytochrome *c* is particularly fortunate in its physical properties, even so, ^{13}C NMR has been the key factor in completing the assignment of the methyl proton resonances.

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