^{13}C-NMR studies of horse ferrocytochrome *c*

Assignment and temperature dependence of methyl resonances

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The ^{13}C and proton chemical shifts of 53 of the 55 methyl resonances of horse ferrocytochrome *c* have been determined by editing natural abundance ^{13}C spectra according to the number of attached protons, observing the temperature dependence of the chemical shifts, and correlating ^{13}C and proton chemical shifts in two-dimensional spectra. Previous assignements of proton shifts allow 16 of the ^{13}C resonances to be assigned firmly.

Horse ferrocytochrome c *^{13}C-NMR Two-dimensional NMR Shift correlation Temperature dependence Methyl resonance*

1. INTRODUCTION

If NMR is to realise its potential for the study of the structure and function of proteins, the first requirement is that unambiguous assignments of resonances should be routinely available. Proton spectra have received most attention despite their limited resolution since information is readily obtained from double-resonance experiments or their two-dimensional analogs [1,2]. ^{13}C has the advantage of a much greater dispersion of chemical shifts, and studies of its homonuclear couplings provide the most direct route to the total assignment of spectra of moderately large organic molecules [3]. However, the low natural abundance of this isotope makes this approach impracticable for studies of proteins, and peaks are still often unresolved. What is required is a collection of spectral properties sufficient to distinguish every group, and this might be made possible by regarding each carbon atom and its attached protons as a single spectroscopic unit. The resolving power of this eclectic approach is most clearly manifest in the heteronuclear two-dimensional shift correlation experiment [4], and its effectiveness has been demonstrated in studies of the ^{13}C-enriched proteins ferredoxin [5,6] and plastocyanin [7], and in the smaller ovomucoids with the isotope in natural abundance [8].

Most of the aromatic proton resonances of horse cytochrome *c* have been firmly assigned [9,10], as well as several of the non-protonated carbons in the aromatic region [11,12]. However, the aliphatic region is far more complex and correspondingly few resonances have been assigned [9,13–15], with just two carbon assignments resulting from isotopic substitution of the methionine methyl groups [16]. Here, we demonstrate the use of editing the natural abundance ^{13}C spectrum according to the number of attached protons, in conjunction with the large temperature dependence of ^{13}C chemical shifts, to resolve 53 of the 55 methyl group resonances in the spectrum of horse ferrocytochrome *c*. Two-dimensional spectra correlating ^{13}C and proton shifts provide an additional parameter to resolve the resonances.

2. EXPERIMENTAL

A 15 mM solution of horse cytochrome *c* (Sigma type VI) was prepared in 90% H_2O/10% D_2O, reduced by addition of solid dithionite and adjusted to pH 6.7. A trace of dioxane was added to

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provide a secondary reference designated 67.80 ppm for ^{13}C and 3.73 ppm for protons. An impurity of approx. 50 mM acetate was identified but did not interfere with the measurements. NMR spectra were obtained using a Bruker AM300 spectrometer operating at 300.13 MHz for protons and 75.47 MHz for carbon. Since the use of proton decoupling causes significant sample heating, the equilibrium temperature established in each experiment was measured by acquiring a proton spectrum within 5 s to determine the shift of the δCH_3 of Ile-57, which has a large and approximately linear temperature dependence in the range studied [14]. Proton decoupling by means of a train of 180° pulses [17] was used to minimise the heating effect for spectra obtained below 50°C. ^{13}C resonances arising from methyl, methylene, and methine groups were separated by magnetisation transfer from protons according to the method of Doddrell et al. [18] at 27, 37 and 45°C, and their temperature dependence was monitored in ordinary proton decoupled spectra in the range 29–61°C. Two-dimensional spectra correlating the chemical shifts of protonated ^{13}C nuclei with the shifts of the attached protons were obtained by the method of Maudsley et al. [4] with the decoupling during the evolution period replaced by a single 180° pulse at its midpoint [19] and the fixed delays set at approx. $1/2\ J_{CH}$ and $1/5\ J_{CH}$ to optimise signals from methyl and methylene groups. The spectral width in the dimension of ^{13}C shifts was restricted to 73.6 ppm, excluding the aromatic and carbonyl resonances, with 4096 points acquired for each free induction decay. The evolution period was incremented in 256 steps of 200 μs to give a spectral width of 16.7 ppm in the second dimension covering the complete proton spectrum. A maximum of 296 transients was time-averaged for each increment in an experiment lasting 32 h. Exponential weighting corresponding to a 5 Hz line broadening was applied in each dimension prior to Fourier transformation, and the data were zero filled to double the number of points in the dimension of proton shifts.

3. RESULTS AND DISCUSSION

Horse cytochrome *c* is expected to show 55 methyl resonances, 49 arising from the methyl groups of the amino acids and 6 from the heme and its thioether bridges to the polypeptide chain [9]. Separating the overlapping methylene and methine resonances by transferring magnetisation from the attached protons [18] allowed 49 methyl resonances to be distinguished, as shown in fig.1. Four more methyl resonances were distinguishable by monitoring the temperature dependence of the chemical shifts, using proton shifts obtained from shift correlation spectra such as that shown in fig.2 to resolve overlapping peaks. This also allowed the proton shift of each methyl group to be determined and compared with assignments made previously, as shown in table 1. The proton shifts of Thr-19 [15] and Thr-47 [13] have also been determined, but the corresponding carbon shifts are ambiguous in this crowded region.

In all cases the shifts were a linear function of temperature within experimental error, in agreement with the observation that ferrocytochrome *c* has no temperature-induced phase transitions before denaturation [20]. The temperature dependences of the carbon shifts are generally large, and they are shown extrapolated to a notional temperature of 200°C in fig.3, for com-

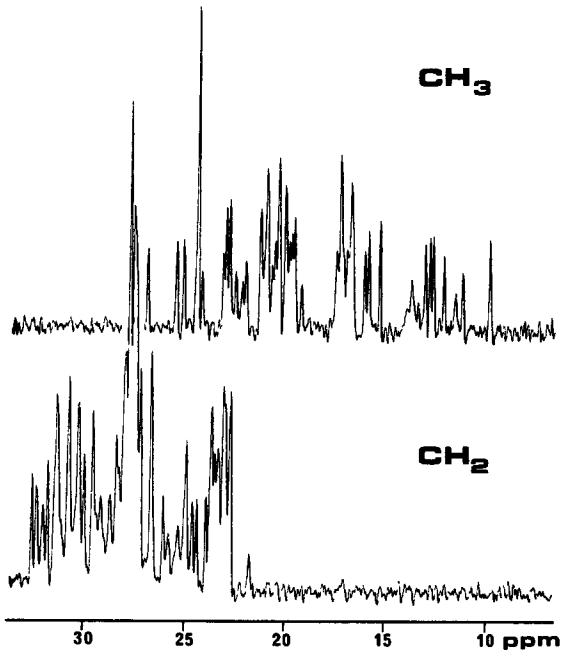

Fig.1. Part of the ^{13}C-NMR spectrum of horse ferrocytochrome *c* at 45°C and pH 6.7 decomposed into signals arising from methyl and methylene groups.

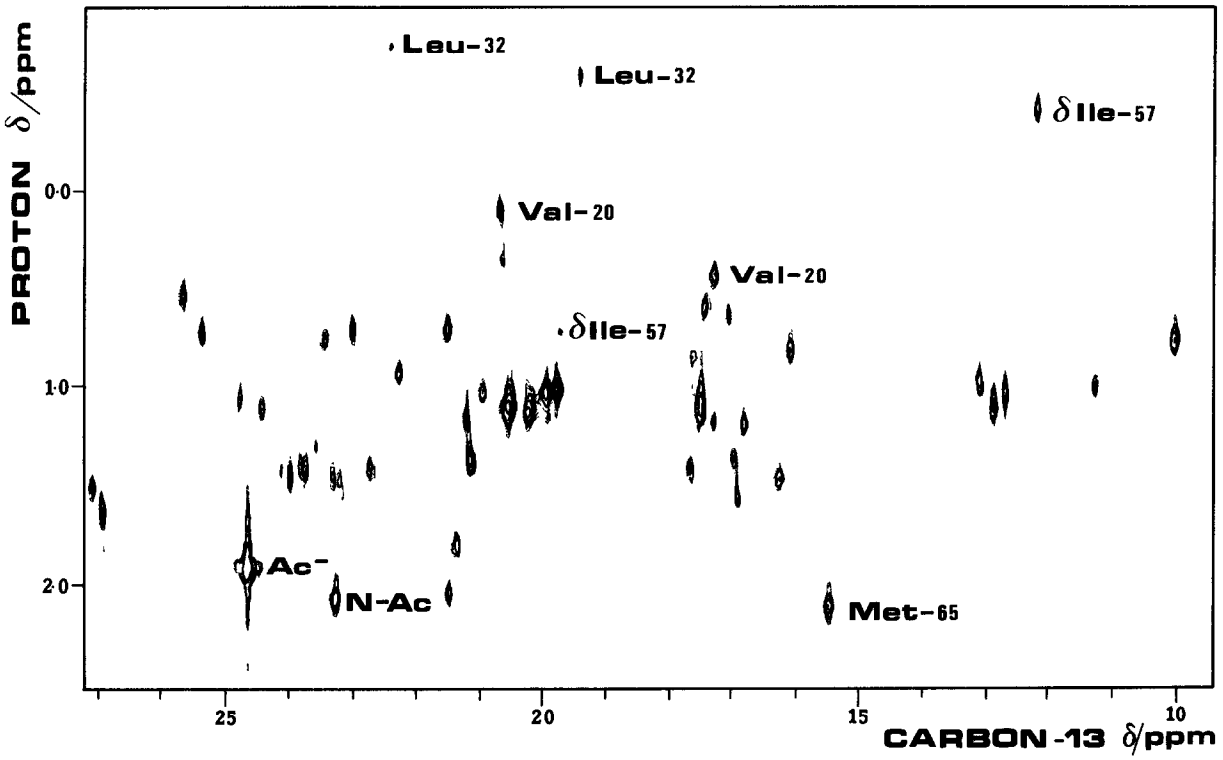

Fig.2. Part of the proton-^{13}C shift correlation spectrum of horse ferrocytochrome *c* at 57°C covering the full range of ^{13}C shifts of methyl groups but excluding a few extreme methyl proton shifts (see table 1).

parison with the shifts observed for isolated amino acids [21]. It is reasonable to suppose that the general trend of chemical shifts will be to move in the direction of the fully solvated values with increasing temperature, though the approximately linear dependence observed in the range 29–61°C cannot persist, and the gradients will be modified by variations in structural integrity. Even so, it is clear that 6 resonances converge in the region expected for the δCH_3 of isoleucine, in agreement with the number of residues in the protein. The shift of the γCH_3 of Ile-57 is the most heavily temperature-dependent, as might be predicted on the basis of its large deviation from the random coil value, but extrapolation indicates the correct primary assignment. On the other hand, the resonances of Met-80 and Leu-32 are virtually independent of temperature despite substantial deviations in their shifts, and this may reflect the stability of the protein conformation in the region of the heme.

Only about 10% of the proton shifts have a significant temperature dependence, and the dependence of proton and ^{13}C shifts is essentially uncorrelated. For example, the δCH_3 of Ile-57 has the most strongly temperature-dependent proton shift, and this has been interpreted in terms of a changing conformation in that region [20]. The proton and ^{13}C shifts of the γCH_3 of Ile-57 also show significant temperature dependence and yet the ^{13}C shift of the δCH_3 is quite stable.

In conclusion, we note that proton and carbon shifts in combination with their temperature dependence are sufficient to characterise 96% of the methyl groups in this protein. Longitudinal relaxation times and pH dependence offer two further parameters which should allow resolution of most CH_n groups in a protein of this size. Once resolved in terms of a complete set of spectral parameters, it should be possible to interpret the diagnostic proton-proton interactions far more clearly than has been possible hitherto, but the

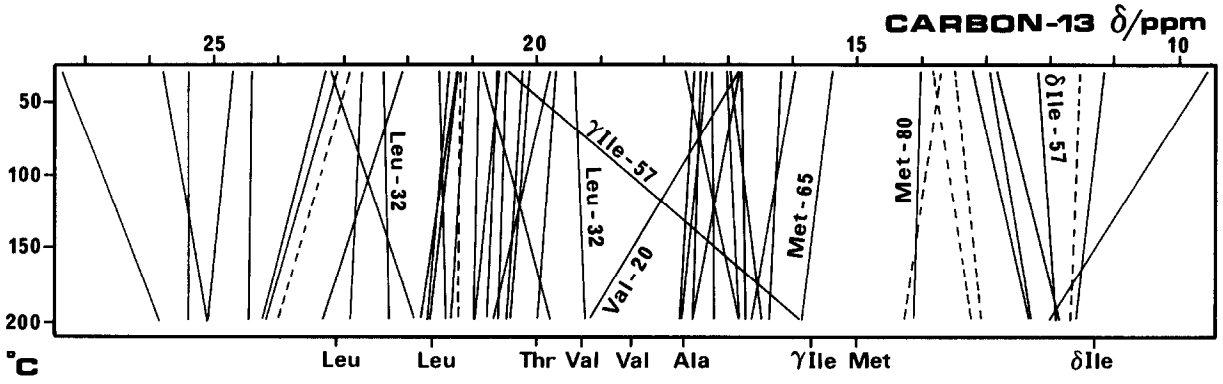

Fig.3. The temperature dependence of the ^{13}C chemical shifts of the methyl resonances of horse ferrocytochrome *c*. Measurements were made in the range 27–61°C and are shown extrapolated for comparison with the shifts of free amino acids. The shifts of the methyl groups of the heme and its thioether bridges are indicated by dashed lines.

Table 1

Assignment of ^{13}C chemical shifts of methyl groups in horse ferrocytochrome *c*

^{13}C shifts (ppm[a])		Proton shifts (ppm[b])	Assignment of proton shifts
29°C	57°C	57°C	
9.56	9.97	0.76	Ile δCH_3
11.15	11.22	0.99	Ile δCH_3
11.54	11.56	2.17	Heme CH_3 8 [22]
12.18	12.14	−0.43	Ile-57 δCH_3 [13]
12.81	12.65	1.02	Ile δCH_3
12.93	12.82	1.09	Ile δCH_3
13.20	13.05	0.96	Ile δCH_3
13.48	13.41	3.57	Heme CH_3 5 [22]
13.72	13.81	3.86	Heme CH_3 3 [22]
13.83	13.73	3.47	Heme CH_3 1 [22]
13.99	14.01	−3.29	Met-80 [23]
15.37	15.45	2.11	Met-65 [13]
16.89	17.27	0.42	Val-20 [15]
19.42	19.39	−0.60	Leu-32 [13]
20.46	19.70	0.71	Ile-57 γCH_3 [14]
20.56	20.62	0.35	?Ile-75/81 γCH_3 [13]
20.81	20.65	0.07	Val-20 [15]
21.10	21.12	1.35	Thr-89 [13]
21.17	21.18	2.59	Thioether 4 [22]
22.39	22.37	−0.76	Leu-32 [13]
23.11	23.29	2.07	*N*-acetyl [13]
22.93	23.11	1.54	Thioether 2 [22]

[a] ± 0.01 ppm, dioxane ≡ 67.80 ppm; [b] ± 0.02 ppm, dioxane ≡ 3.73 ppm

utility of the approach for larger proteins remains questionable.

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