PULSE METHODS FOR THE SIMPLIFICATION OF PROTEIN NMR SPECTRA

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1. Introduction

In the nuclear magnetic resonance (nmr) spectra of large molecules, such as proteins, it is frequently difficult to resolve resonances of individual nuclei because of the large number of overlapping resonances. One general method of spectral simplification which is being increasingly used is difference spectroscopy [1,2]. In this paper we shall discuss the application of another general method, the use of different pulse sequences for the simplification of complex nmr spectra. These pulse sequences have previously been used to measure relaxation times [3,4]. We shall illustrate the application of the pulse sequences to ^{1}H nmr spectra of lysozyme and myoglobin and show that resonances may be observed selectively because of intrinsic differences in relaxation times or in multiplet structure and coupling constants. These methods are generally applicable to different systems and different nuclei.

2. Materials

Lysozyme from hen egg white was obtained from Sigma, and dialysed and lyophilised before use as described previously [5]. Myoglobin from sperm whale was obtained from Koch-Light Laboratories.

The proteins were dissolved in 99.8% D_2O to a concentration of approx. 5 mM, and the pH adjusted using dilute DCl and NaOD.

3. Pulse methods

Spectra were obtained using a 270 MHz Bruker spectrometer with an Oxford Instrument Co. magnet and a Nicolet 1085 computer. The pulses were generated with a Nicolet 293 pulse unit under computer control. The phase shift in the radiofrequency pulse was effected by swichting in delay lines [6].

Four different pulse sequences were examined, all using non-selective 180° (40 μsec) and 90° (20 μsec) pulses. The sequences were A) $180°-\tau-90°$ and B) $(90°-\tau-90°)_n$ which are commonly used to measure the spin lattice relaxation time, T_1 [3,7]: C) $90°-\tau-180°-\tau$, the Carr–Purcell method A and D) $90°-\tau-(180°-2\tau)_n-180°-\tau$, the Carr–Purcell method B (with a 90° phase shift on the 90° pulse). C and D have both been used to measure the spin-spin relaxation time T_2 [4,8]. In C and D we denote the time between the 90° pulse and the start of data acquisition as T; $T = 2\tau$ in C and $2(n + 1)\tau$ in D.

4. Results and discussion

4.1. *Selection on the basis of relaxation times*

If the pulse sequences are to be used to distinguish between resonances on the basis of relaxation times (T_1 and T_2), there must be significant differences between the relaxation times of different resonances. In particular, to be valuable for spectral simplification, there must be differences between those resonances in the same region of the spectrum. Measurements of T_1 and T_2 using pulse sequences A and D respectively have been made in a number of proteins, and will be described separately. These sequences can result in spectral simplification in several ways. The most effective is sequence D. Since the area of resonances with short T_2 values rapidly decays to zero, the resonances with longer values of T_2 can be observed selectively by choosing s suitable value of T. This

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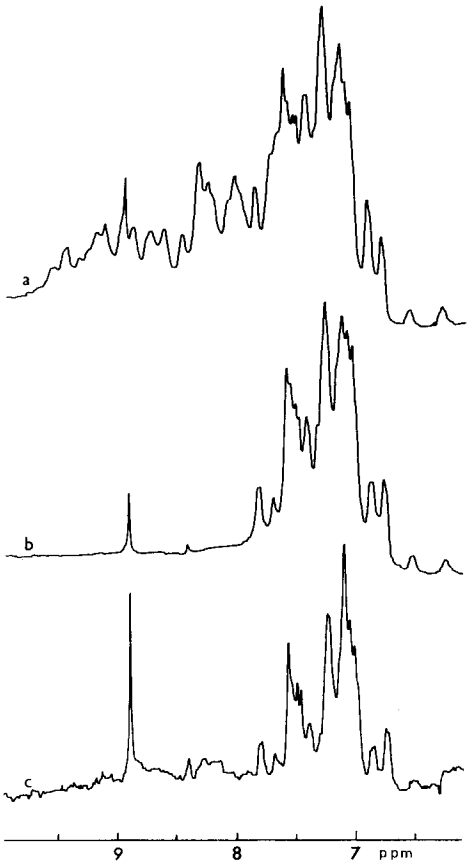

Fig.1. Aromatic region of the ^1H spectrum of lysozyme at 54°C, pH 4; a) normal Fourier transform spectrum of lysozyme in D_2O; b) as a) but after exchanging the NH resonance by heating to 80°C (5); c) spectrum obtained using sequence D with $T = 90$ msec, $\tau = 1$ msec.

method has been found valuable in the following cases.

i) For removal of NH resonances in order to observe separately the aromatic proton resonances, as illustrated for lysozyme in fig.1. In this case T_2 for the peptide NH resonances is approx. 7 msec while those for the aromatic protons are nearly all longer than 30 msec. The difference in T_2 values arises because the ^{14}N nucleus has a spin while the ^{12}C nucleus has not.

ii) The $C_{(2)}$ and $C_{(4)}$ proton resonances of histidines exposed to solvent have particularly long T_2 values. This arises because these protons are relatively far from other protons, especially in a deuterated solvent and surface histidines have a relatively high mobility. (For example, in lysozyme the $C_{(2)}$ histidine proton has a T_2 of about 150 msec at 25°C whereas the other aromatic protons have T_2 values in the 40 msec range.) The observation of histidine resonances in myoglobin is illustrated in fig.2. This spectrum was accumulated using sequence C. This sequence is particularly simple to apply and is within the capabilities of most existing commercial pulsed nmr spectrometers. The possibilities for selection on the basis of relaxation times are by no means limited to the above examples. For example, in lysozyme a peak assigned to the ϵ-CH_2 groups of lysines has a particularly long T_2 value.

iii) The resonances of small molecules in the presence of large molecules are often easy to detect using sequence D because of the difference in T_2 values. In certain cases T_1 sequences are also valuable, particularly sequence B where the overall accumulation time is considerably less than for sequence A. For example,

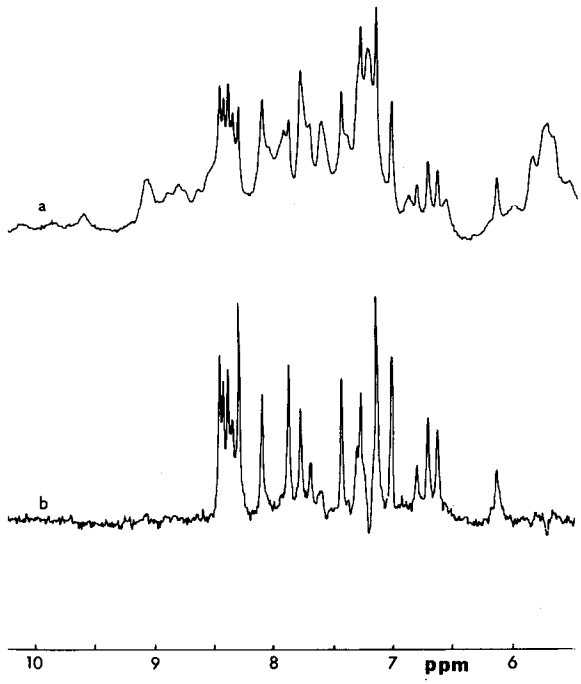

Fig.2. Aromatic region of the ^1H spectrum of myoglobin at 36°C, pH 5.9 a) normal spectrum; b) spectrum obtained using sequence C with $\tau = 30$ msec, $T = 60$ msec.

T_1 sequences have been used in the study of the whole adrenal medulla of rats [9]. In general however, we find T_2 sequences better for selection purposes.

These methods have all been concerned with the observation of the class of resonances with long relaxation times. Resonances with short relaxation times may be observed using difference spectroscopy in conjunction with these pulse sequences.

4.2. *Selection on the basis of multiplet structure*

Unless the value of τ is short in pulse sequence D, the decay of the spectrum as a function of the total time (T) between the 90° pulse and the measurement of magnetisation is modulated by spin coupling effects. This modulation is readily observed using pulse sequence C. Singlet resonances decay exponentially with a time constant T_2, but a doublet of coupling constant J not only decays in this way but also changes phase such that at total time $T = 1/J$, it is 180° out of phase (i.e. inverted) with respect to a singlet. At $T = 2/J$, the doublet is in phase with the singlet again, and so on. In the case of a triplet, the central component decays in the manner of a singlet, but the two outer components are inverted at $T = 1/2J$, and in phase at $1/J$ [4].

We have found that sequence C is of great value for interpreting protein nmr spectra, as information concerning the multiplet structure of resonances is obtained. However, in this paper the use of the sequences to simplify spectra is illustrated. In protein spectra, the aromatic resonances from phenylalanine, tyrosine and the benzenoid part of tryptophan consist, to a first approximation, of doublets and triplets with $J = 8.5 \pm 0.5$ Hz. The resonances from histidine and the five membered ring of tryptophan are singlets. All the resonances also have second order couplings of less than 2 Hz, but since the natural linewidth is of this order, these may be neglected.

Fig.3a shows the aromatic region of lysozyme (with NH protons exchanged) obtained using sequence D with $\tau = 1$ msec, and $T = 120$ msec. Using a τ value as short as this results in a spectrum without J modulation [4]. Fig.3b shows a spectrum obtained using sequence C with $\tau = 60$ msec and $T = 120$ msec. The doublets are now inverted since $T \simeq 1/J$. Singlets and triplets have normal phase. In order to observe doublet resonances separately, these spectra are simply subtracted as fig.3c shows. In order to observe singlets and triplets separately, the spectra are added, fig.3d. For example, the three resonances at 6.28 ppm, 6.49 ppm and 6.82 ppm have been shown to be tryptophan triplets by spin-decoupling [10]. The triplet at 6.82 ppm is normally obscured by the doublet resonances, known to be of tyrosine, which are clearly observed in fig.3c.

The multiplicity selection method is not limited to the aromatic region. Protein methyl group resonances,

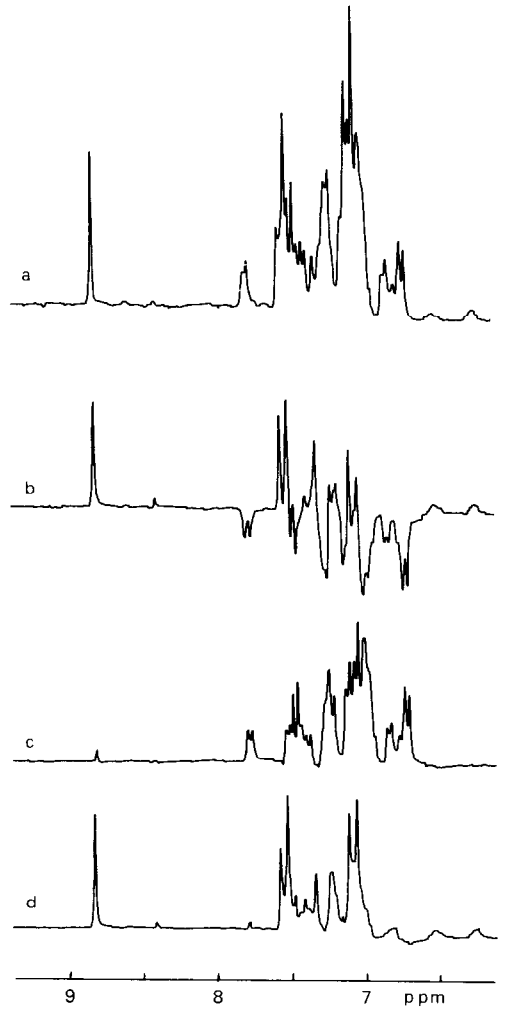

Fig.3. Illustration of multiplet selection. a) aromatic region of lysozyme (54°C, pH 4) obtained using sequence D, T = 120 msec, τ = 1 msec. b) spectrum obtained using sequence C, T = 120 msec, τ = 60 msec. The doublet resonance are inverted. c) spectrum a) minus b) showing doublets only. d) spectrum a) plus b) showing singlets and triplets.

for example, are mainly doublets with J values of 6.5 ± 1 Hz. The methionine methyl resonances are, however, singlets, and one of the isoleucine resonances is a triplet. In particular we have found that methionine resonances can be detected very easily in many proteins.

We have described just one of the ways in which multiplets may be selected using these pulse sequences. Variation of τ values and of T values allows other combinations of spectra to be used.

5. Conclusions

When resonances and their multiplet structure are well resolved, as in lysozyme, selection of a small number of resonances on the basis of relaxation times or multiplet structure can be achieved. This is an important simplification of complex spectra. For larger proteins, when T_1 or T_2 becomes much less than $1/J$ for many resonances, some resonances, particularly of histidine, can be simply and clearly separated from a complex spectrum.

Acknowledgements

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