PULSED NMR METHODS FOR THE OBSERVATION AND ASSIGNMENT OF EXCHANGEABLE HYDROGENS: APPLICATION TO BACITRACIN

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1. Introduction

NMR is an established technique for the examination of the conformation of peptides and proteins in solution. One common structural approach is to relate measured peptide NH-αCH proton coupling constants to the angle between the NH and CH bonds (e.g. [1]). The solvent of most interest is, of course, water but the observation of NH proton resonances in aqueous solution is technically difficult because the resonances of the peptide must be observed in the presence of a very large H_2O resonance. This dynamic range problem has usually meant that the versatile and sensitive pulsed Fourier transform technique cannot be used. Another problem is that in order to assign observed NH resonances to particular amino acids it is usually necessary to irradiate the αCH resonances in order to correlate the NH resonances with αCH and βCH resonances by decoupling. The αCH resonances, however, occur in the region of the H_2O resonance and are therefore obscured and difficult to irradiate [2]. In this paper we show how these problems may be overcome by using pulsed multiple resonance. Three separate irradiation frequencies are applied to the sample, one to observe the spectrum using Fourier transform techniques, one to suppress the H_2O resonance and one to decouple the resonances of the αCH protons from those of the NH and βCH protons.

The application of the pulsed triple resonance method to the cyclic polypeptide bacitracin (mol. wt 1420) will be briefly described. The mechanism of action of this antibiotic has been established (e.g. [3]). The primary structure is known (e.g. [4]) to consist of a seven membered ring of amino acids joined to a 'tail' at a lysine residue. The 'tail' is unusual in that it contains a thiazolene ring (see fig. 1). Little is known about the conformation of this molecule in solution, although Galardy et al. [4] have suggested a compact structure from thin-film dialysis studies. In the same paper one amide proton was observed to exchange more slowly than the others by means of hydrogen/tritium exchange experiments. Some work using ^{13}C NMR has also been described [5].

2. Materials and methods

Bacitracin was obtained from the Sigma Chemical Corporation and freeze dried from water before use to remove volatile impurities. This compound exists as a mixture of closely related species but the dominant component is bacitracin A, especially in freshly made solution. pH measurements were made using a combined glass electrode and a Radiometer 24 pH meter. The con-

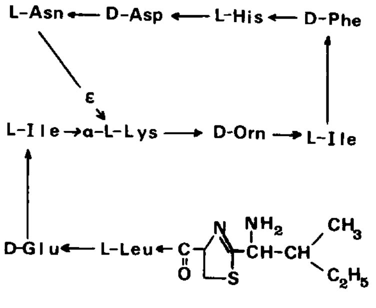

Fig. 1. Structure of bacitracin A.

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centration used was 20 mM. 10% D_2O was added to the H_2O samples to provide the internal field frequency lock. Chemical shift values are quoted in parts per million (ppm) downfield from 2:2-dimethyl-2-sila-pentane-5-sulphonate (sodium salt), employed as an internal standard.

The experiments were performed on a 270 MHz Bruker spectrometer, and a Nicolet 1085 computer with a 294 disc system and a 293 pulse controller. The spectra shown were accumulated using an interpoint time of 166 μsec and a pulse repetition time of approximately 1.2 sec.

Two irradiation frequencies (f_2 and f_3) were applied to the sample in addition to the observation pulse at frequency f_1. The frequency f_2 was tuned to coincide with the H_2O resonant frequency and applied continuously for 0.4 sec, finishing 80 μsec before the f_1 pulse. A second irradiation frequency, f_3, was derived from a Schomandl frequency synthesiser at close to 30 MHz and multiplied by 9. This frequency was applied, using a Bruker time sharing unit, in pulses during the acquisition time [6].

3. Results and discussion

3.1. *Pulsed triple resonance*

The ratio of the concentration of protons in H_2O and bacitracin is 5000 to 1 in these studies. This dynamic range problem is best overcome by avoiding the excitation

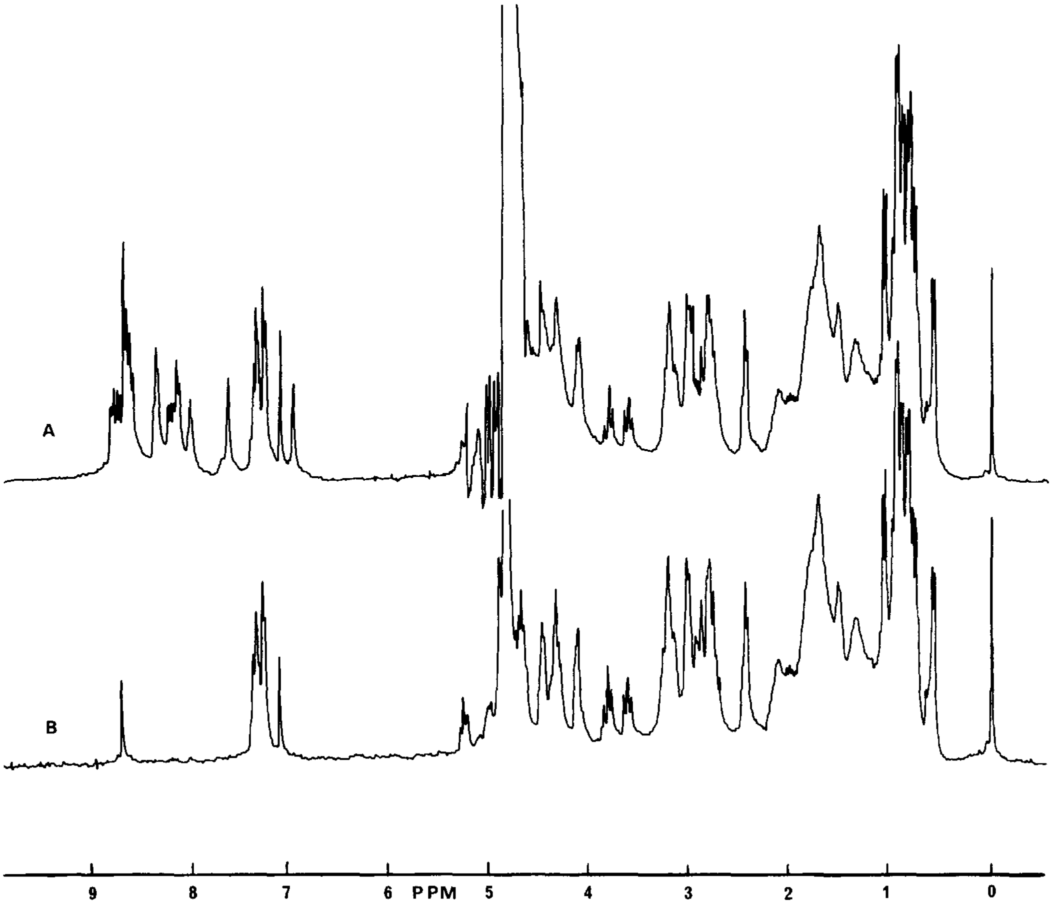

Fig. 2. (a) 270 MHz Fourier transform spectrum of 20 mM bacitracin. pH 3.15, 20°C. in 90% H_2O and 10% D_2O. The H_2O resonance was suppressed using a saturation pulse. (b) spectrum under the same conditions but 99% D_2O.

of the H_2O resonance [7]. This may be done using a continuous wave method, in which only a small region of the spectrum is observed. The sensitivity of this method can be improved using rapid scanning techniques [8] although this method is sometimes difficult to apply to a system with a wide range of linewidths. In addition the aliphatic and aromatic regions of the spectrum cannot be observed simultaneously. Other solvent suppression techniques have been proposed, including different null methods [9,10] and pulsed stochastic resonance [11] but it is very difficult to achieve the required reduction of the H_2O resonance (about 1000-fold) using these methods. The method we have used is to saturate selectively the H_2O resonance by applying a radiofrequency field at the H_2O resonant frequency (denoted f_2). This frequency was applied in a long low-power (and therefore selective) pulse just before the usual high-power (non-selective) observation pulse in a Fourier transform experiment. This method was originally applied to ^{13}C experiments [12]. As Freeman et al. have pointed out [13], a pulse applied in this manner can result in saturation and thus give rise to nuclear Overhauser effects but the spin coupling is unaffected. Therefore NH-αCH spin coupling will be observed in the NH resonance even if the αCH resonance is directly under the H_2O resonance and is thus saturated by the f_2 pulse. By adjusting the magnitude of the f_2 pulse it is normally possible to reduce the H_2O resonance by a factor of around 1000 without significantly affecting peaks more than about 0.5 ppm away (see fig. 2). The sensitivity of the instrument under these conditions is only slightly worse than that obtained using 99.8% D_2O solvent without the application of the f_2 pulse. As can be seen in the figures, transfer of saturation from the H_2O position to the peptide NH resonances [1] is not usually a serious problem, although there probably is some cross saturation of the peaks at 6.9 and 7.6 ppm. (see section 3.2.)

The problem of irradiating peptide αCH resonances in aqueous solution has been discussed by Dadok et al. [2] and it was shown that careful detuning of the amplification network in their spectrometer allowed irradiation to be used. This method is difficult to apply, however, and does not allow Fourier transform experiments to be performed. Perhaps the most common method of circumventing this double irradiation problem is to perform the decoupling in another solvent e.g. dimethyl sulphoxide (DMSO) and then to extrapolate the resonance positions to the aqueous solution situation by gradually changing from 100% DMSO to 100% H_2O. This method is laborious and sometimes it is not easy to follow the resonances when they cross over each other [14]. Moreover, some polypeptides (e.g. bacitracin) yield broad, poorly resolved spectra in DMSO. However, once the solvent has been suppressed as described in the previous paragraph, normal homonuclear decoupling methods can be applied to the sample in aqueous solution. This third frequency, f_3, was applied here using a time-sharing pulse technique [6], which is equivalent to a continuous application of radio frequency power provided the applied f_3 pulses are less than 90° pulses [15]. This type of irradiation readily decouples the NH resonances, as shown in fig. 3.

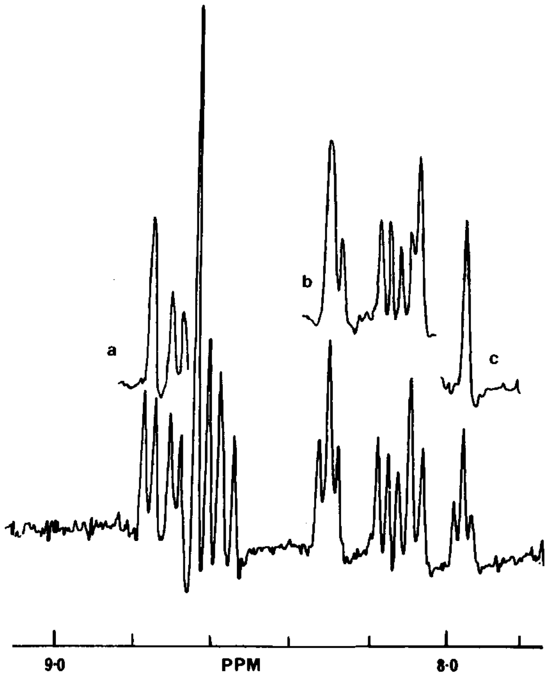

Fig. 3. Decoupling of bacitracin peptide NH proton resonances using pulsed triple resonance. The spectra shown correspond to convolution differences as described in [18]. (a) shows the decoupling of the doublet at 8.73 ppm caused by irradiation at 4.71 ppm. (b) effect of irradiation at 4.20 ppm. (c) effect of irradiation at 3.23 ppm.

3.2. *NH resonances of bacitracin*

Bacitracin contains 10 NH protons of normal peptide bonds and one NH proton from a lysine NH group of an amide linkage. In addition there is an amide ($CONH_2$) group of asparagine, and there are amine groups of the terminal isoleucine and of ornithine. The resonances of protons of the NH_2 groups are not observed under the conditions used here, but those of the amide protons of asparagine (singlets) are observed at 6.9 and 7.6 ppm (see fig. 2). As shown in the figs. 10 doublet resonances and one triplet resonance are also observed. The triplet resonance can be confirmed as being that of the ϵ-NH proton of lysine by irradiating at 3.23 ppm (fig. 3).

The 10 doublet resonances correspond to the normal peptide NH protons. These resonances can be collapsed separately by selective irradiation in the region around the H_2O resonance position where resonances of αCH protons are expected. The assignments shown in fig. 4 follow from these decoupling experiments and from previous work in our laboratories [16] on the assignment of αCH proton resonances. A complete assignment of the NH proton resonances is possible by making use of perturbations by pH, temperature and paramagnetic probes and will be described separately. The decoupling methods described in this paper are of particular importance for bacitracin, as the multiplet structure of the NH resonance is difficult to resolve in DMSO [16].

Having characterized the spectrum of bacitracin in H_2O, studies on the proton solvent exchange rates were performed. Fig. 5 shows the results of one of these experiments. One hour after being dissolved in D_2O under these conditions, no NH proton resonances are observed (fig. 5D). The spectrum of the CH protons is identical to the spectrum in H_2O except where the coupling pattern is altered by the exchange of NH to ND. The exchange of the NH protons can be observed by recording spectra rapidly after dissolution (figs. 5B and C). These studies have shown that the slowest exchange rate is for the ϵ-NH lysine proton which has a half-life of about 15 min. This is probably the slowly exchanging proton observed by Galardy et al. [4].

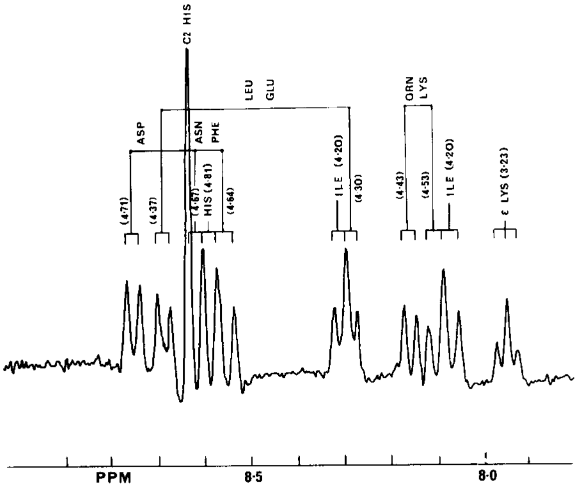

Fig. 4. The positions (in ppm) at which decoupling is achieved for each multiplet is shown in parenthesis (see fig. 2). Some spectral assignments are given. A horizontal line (e.g. between Asp, Asn and Phe) indicates that distinction between these resonances has not been made here.

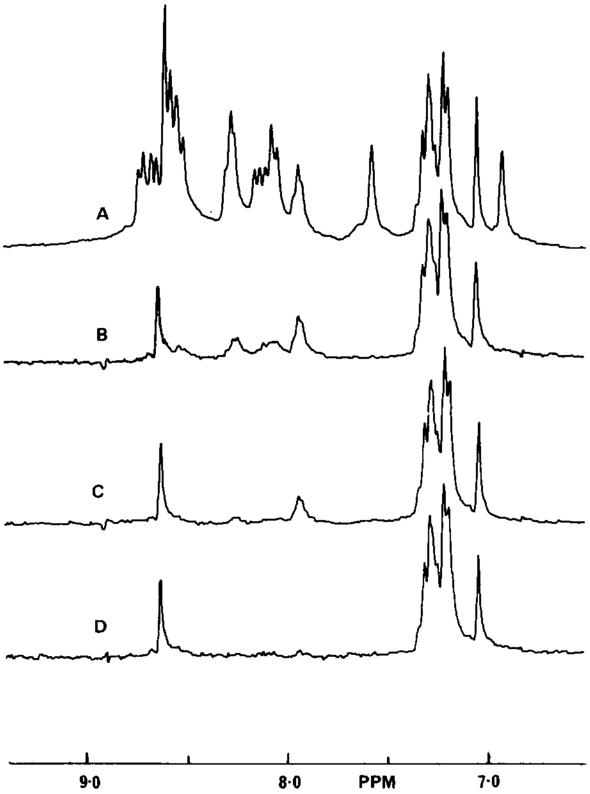

Fig. 5. (a) spectrum of bacitracin in 90% H_2O, 10% D_2O (as in fig. 1a). (b) spectrum recorded 3 min after dissolving bacitracin in 99.8% D_2O. (c) as (b) but after 17 min. (d) as (b) but after 61 min.

4. Conclusions

In this article we have shown that the ability to apply up to three frequencies to the proton spectrum of bacitracin has permitted the assignment of the NH proton resonances. The sensitivity of Fourier transform techniques together with the two pulsed irradiation frequencies makes it possible to carry out a complete decoupling of the NH and βCH resonances from the αCH resonances of bacitracin with comparative ease. This method is generally applicable in the study of peptides, proteins and other molecules. This ability to perform experiments in H_2O rather than the more usual D_2O is particularly important. We have applied the same solvent resonance suppression techniques to lysozyme solutions, thus allowing a study of the exchangeable (NH) hydrogens of this enzyme. It is possible to assign and follow the rates of exchange of back-bone NH protons in proteins. We have also suppressed both the CH_3 and OH peaks when using methanol as solvent by applying both f_2 and f_3 in the long saturation pulse before the f_1 pulse.

Acknowledgements

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