CARBON-13 FOURIER TRANSFORM NUCLEAR MAGNETIC RESONANCE STUDIES OF PEPTIDES

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Summary: The carbon-13 nuclear magnetic resonance (CMR) spectra of several small peptides have been obtained at 25.1 MHz and natural abundance of ^{13}C using the Fourier transform technique with proton noise decoupling. Chemical shift values have been studied as a function of pH. The peptide corresponding to the 1-15 sequence of ribonuclease has been synthesized both with normal and 15% ^{13}C enriched Phe in position 8, and the CMR spectra of these two products are compared.

We wish to report ^{13}C chemical shift data for several peptides, as a basis for studies of protein structure and function. Our approach to the application of CMR to proteins includes the chemical synthesis of peptides which are specifically enriched in ^{13}C at selected residues, and which participate in well-characterized, enzymically active, non-covalent complexes. In the present case, we report the synthesis and CMR spectrum of [^{13}C-Phe-8]-ribonuclease-(1-15).

CMR data have been recorded previously for several classes of compounds[1-3], including the amino acids[4]. These studies have demonstrated the greater simplicity of spectra and improved resolution obtainable by CMR than by proton magnetic resonance (PMR)[4], thus indicating the potential application to peptides and proteins[5]. More recently, the CMR spectra of the cyclic decapeptide antibiotic gramicidin S-A[6] and ribonuclease[7], both at natural abundance and 15.1 MHz, have been reported.

In order to carry out CMR studies, signal enhancement is necessary since the carbon-13 nucleus has a much lower sensitivity than the hydrogen nucleus (1.6% for equal numbers of nuclei at the same magnetic field) and exists in

low natural abundance (1.1%). Techniques used include proton noise decoupling[8,9] and automatic repetitive spectral accumulation. For the latter, the pulse mode-Fourier transform (FT) technique[10] has advantages over conventional time-averaging. In the FT method, a pulse of radiofrequency energy is applied to the sample and the resultant free induction signal is measured

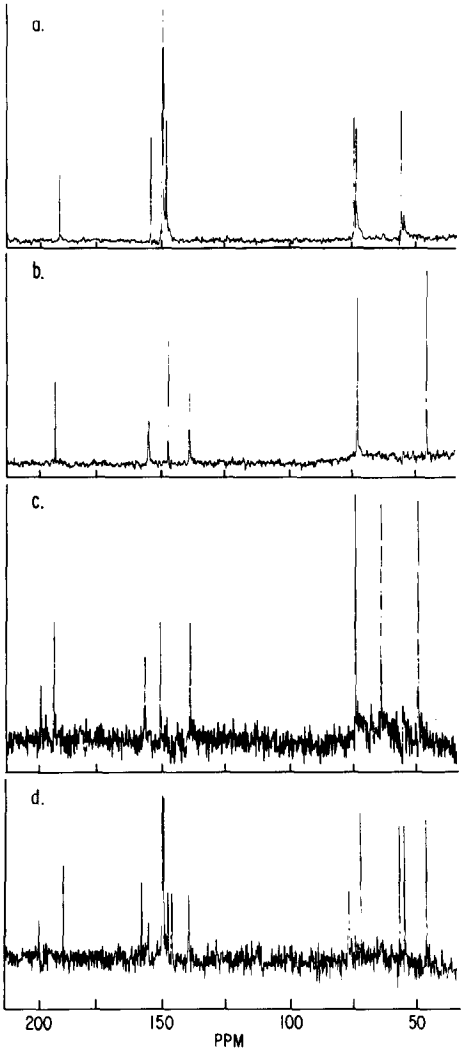

Fig. 1. CMR-FT spectra at 25.1 MHz and natural abundance with proton noise decoupling. a) Phe methyl ester, pH 1.86, satd. soln., 10^4 pulses (50 min.); b) His, pH 1.14, satd. soln., 10^4 pulses; c) His-Gly, pH 3.51, 100 mg/ml, 10^4 pulses; d) His-Phe, pH 3.33, 100 mg/ml, 1.5 x 10^4 pulses. Spectra were recorded with a pulse width of 40 μsecs and an acquisition time of 0.3 sec. Chemical shift values are in ppm downfield from external $^{13}CH_3I$.

as a function of time. The Fourier transformation of this signal gives the slow sweep (CW) high resolution spectrum. The successive addition of responses to repetitive pulses and the use of a computer to perform the calculation make it possible to reduce the time taken to achieve the same signal/noise level by one to two orders of magnitude compared with conventional methods[7,10].

We have used a Varian Associates HA-100D spectrometer operating at 25.1 MHz with a V-4357/FFT-100 system with fast arithmetic, utilizing a Varian 620i digital computer. This was equipped with a V-3512 proton noise decoupler and homonuclear frequency lock. Spinning 8 mm sample tubes were maintained at a probe temperature of 32 ± 2°C with a flow of dry air. The lock signal was provided by 65% ^{13}C-methyl iodide (Merck of Canada) in a capillary.

Several representative spectra of amino acids and small peptides are shown in Fig. 1. Excellent resolution (up to 4 Hz) was routinely obtained. The carbonyl and aromatic quaternary carbon resonances are generally less intense than would be expected. This is due to their relatively long relaxation times (T_1) resulting from the lack of bound hydrogen atoms[4], and differential nuclear Overhauser enhancement[9]. Chemical shift data for several peptides are shown in Fig. 2. Excellent consistency was found with previously reported chemical shift data for the amino acids[4]. The effects of the peptide bond formation were relatively small. The pH dependence and the assignments[4] of the resonances of histidine and phenylalanine methyl ester are shown in Figs. 3 and 4. The changes in chemical shift in Fig. 3 were shown not to be due to differences in concentration by dilution (x4) of Phe methyl ester at both high and low pH. As a result of previous experience with PMR studies of proteins[5], we feel that it is important to make such studies of small peptides[11] at least concurrently with those of proteins.

It is clear from Figs. 3 and 4 that several resonances which would be expected to show the effects of titration are little affected. Thus, the

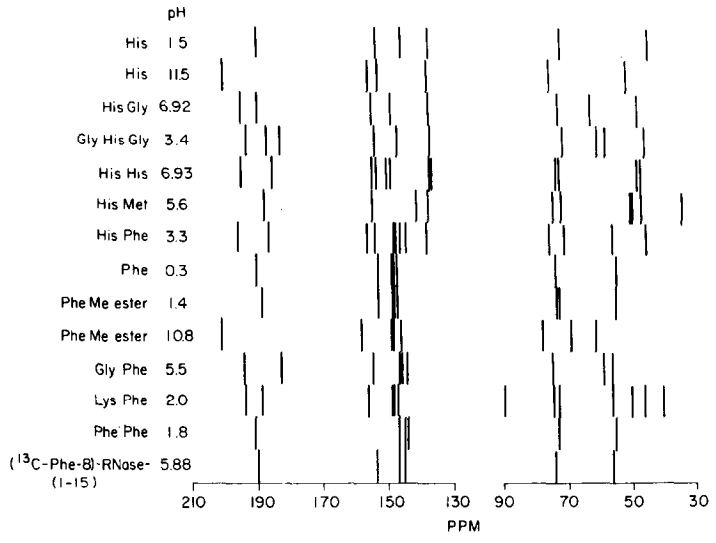

Fig. 2. Representation of CMR-FT spectra at 25.1 MHz of several amino acids and peptides (all in the L-form) obtained at natural abundance, and of [^{13}C-Phe-8]-RNase-(1-15) peptide (line intensities have been normalized). The peptides His-Met and Lys-Phe correspond to the sequences 12-13 and 7-8 of ribonuclease respectively. Assignments of several resonances are given in Figs. 3 and 4. Solutions of amino acids were saturated, solutions of peptides were 100 mg/ml in 0.1 M NaCl. Low pH values were used in order to increase solubility.

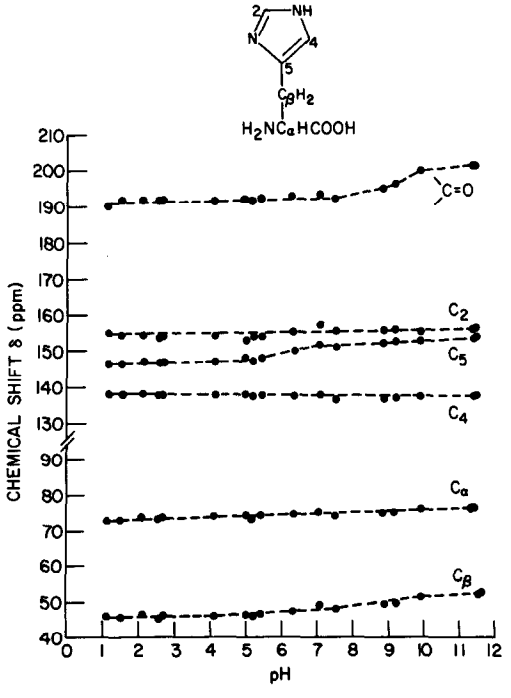

Fig. 3. Titration data for L-histidine. Assignments are based on the correlations deduced in ref. 4, except for C_5 (cf. ref. 7).

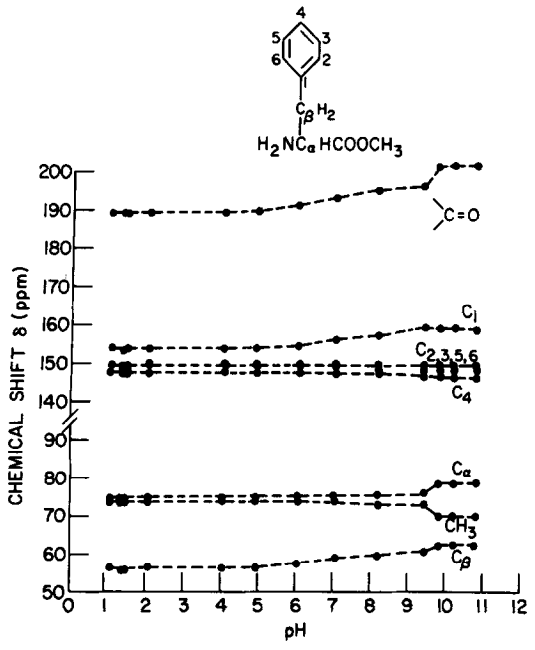

Fig. 4. Titration data for L-phenylalanine methyl ester. The assignments are based on ref. 4, except for C_1 (cf. ref. 7) and the methyl resonance which was assigned by comparison with Phe.

carboxyl carbon resonances shift very little between pH 1 and 4. This phenomenon has been noted previously[4,12] and explained in terms of conservation of electron density at the carbon atom[4,13]. Also, the C-2 and C-4 carbon resonances of the imidazole group of histidine show very little shift[3], although the hydrogen atoms attached to them are well known to be deshielded as a result of π-electron delocalization upon protonation[14,15]. However, somewhat surprisingly in both the case of His and Phe methyl ester it is the quaternary bridge-head carbons which show pH-dependent changes in their chemical shifts, relfecting the titration of the imidazole (pK ≃ 6.5) and amino-terminal groups (pK ≃ 9.5) respectively. Also, the carboxyl and β-carbon atoms show changes in the higher pH range[13]. The independent observation of asymmetric titration curves for the C-2 proton resonances of certain His peptides[16] also is indicative of an amino-imidazole interaction. The precise nature of the electronic effects involved, i.e. whether a through-space or bond effect, is not known at the present time.

A further approach to the improvement of signal observation is the use of ^{13}C isotopic enrichment, for example in the study[4] of 15% ^{13}C enriched amino acids derived from algae grown on enriched ^{13}C-carbon dioxide[17]. Since satisfactory spectra can now be obtained in relatively short time periods at natural abundance using the presently available techniques (See Fig. 1) it is no longer essential to enrich whole molecules to observe their constituent carbon resonances. On the other hand, the selective enrichment of one residue in a macromolecule would enable one to study its resonance in the presence of several other such residues.

We have applied the principle of selective enrichment to peptides prepared synthetically by the Merrifield procedure[18,19] as adapted previously[20,21]. Two parallel syntheses were carried out. One was for the normal sequence of Residues 1 through 15 of ribonuclease (RNase-(1-15)) (Fig. 5), an active derivative of ribonuclease S-peptide[22-24]. A second was for the above peptide with ^{13}C-enriched phenylalanine[17] at position 8 ([^{13}C-Phe-8]RNase-(1-15)). The yields were 350 mg (ca. 75%) and 390 (ca. 95%) respectively. The amino acid composition of the ^{13}C-enriched peptide obtained after acid hydrolysis (6N HCl, 24 hours, 110°C in vacuo) was as follows: Lys 2.14 (2), His 1.01 (1), Arg. 0.96 (1), Asp. 1.06 (1), Ser. 0.92 (1), Glu 2.84 (3), Ala. 3.08 (3), Met. 0.25 (1) and Phe 0.91 (1), where the expected values are given in parentheses. Both partially purified peptides generated 24-28% of the enzymic activity[25] effected with native ribonuclease S-peptide when each was added in equimolar amounts to S-protein. This extent of activity compares favorably with that (7-11%) previously obtained for this peptide prepared by the solid phase procedure and at the same stage of puri-

1 5 10 15
NH_2-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-COOH

Fig. 5. Amino acid sequence of residues 1 through 15 of ribonuclease A.

fication[26]. A comparison of the CMR spectra for these synthetic peptides is shown in Fig. 6. The presence of ^{13}C enriched phenylalanine increases markedly the intensity of the phenylalanine enriched side-chain resonances relative to the background. Spectra of ribonuclease A have also been obtained as well as of its C-peptide (sequence 1-13)[27] and S-peptide (1-20)[22]. Comparison between them and the RNase-(1-15) peptide should enable us to obtain definitive assignments of resonances.

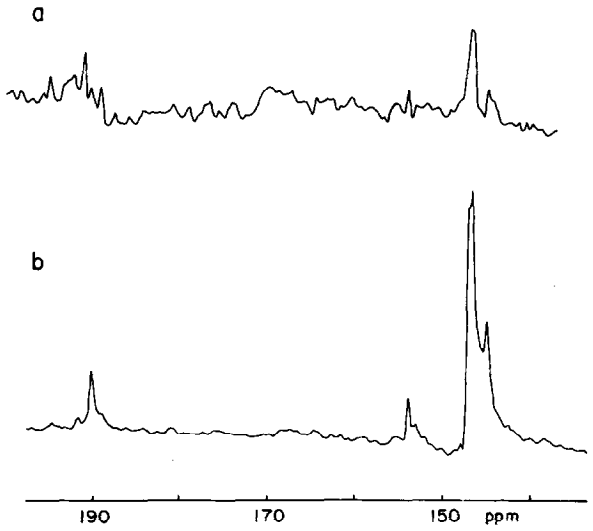

Fig. 6. Downfield region of CMR-FT spectra at 25.1 MHz of a) RNase-(1-15) peptide (see Fig. 5), natural abundance ^{13}C; 9 x 10^4 pulses (4 hr.). pH 6.14. b) [^{13}C-Phe-8]RNase-(1-15) peptide; 1.5 x 10^4 pulses (40 min.) pH 5.88. Concentrations of these peptides were 4.5 x 10^{-2} M, aquisition time was 0.1 sec. The following are the actual chemical shift values compared to those of Phe methyl ester (in parenthesis) at the same pH; C=0, 192.6 (191.6); Aromatic, 156.2 (155.0), 149.0 (149.2), 148.6 (148.8), 147.4 (147.0); Cα, 75.1 (74.7); Cβ, 57.2 (57.4).

Our use of ^{13}C-enriched phenylalanine is designed not only to improve our ability to observe its ^{13}C resonances (Fig. 6) but more as a means to distinguish it from like residues when the peptide forms part of the ribonuclease S complex. In this latter sense, enrichment allows us to use Phe-8 as a "reporter group" without disturbing the structure of the system in any significant way. There is evidence that this Phe residue is important for the formation of active ribonuclease S, perhaps by its

participation in hydrophobic interactions[28,28]. Studies with synthetic peptides such as that described here should provide a means to characterize the formation and properties of enzymic complexes.

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