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Carbon-13 Fourier Transform Nuclear Magnetic Resonance, II. Ribonuclease

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Abstract. High-resolution Fourier transform nuclear magnetic resonance was used to observe the natural abundance ^{13}C spectrum of a 0.02 M solution of bovine pancreatic ribonuclease A. Peaks assignable to only three carbons were easily observed after 10 hr of signal averaging. A number of tentative assignments were made.

As expected, the ^{13}C spectrum was appreciably richer in detail than the corresponding proton spectrum. Natural abundance ^{13}C Fourier transform nmr appears to be a practical tool for the study of biopolymers in solution.

The nuclear magnetic resonance technique can be used in several ways to obtain information about proteins in solution.[1] Nearly all direct observations of protein nmr spectra have involved the proton nuclei, and the resulting structural information has been limited by the relatively small range of chemical shifts. Horsley, Sternlicht, and Cohen[2] have pointed out the possible advantages of carbon-13 nmr. We are aware of only one observation of a ^{13}C spectrum of a protein, a recent report by Lauterbur[3] on lysozyme, involving several days of signal averaging per spectrum on a conventional nmr spectrometer. The problem of low signal-to-noise ratios in carbon-13 nmr studies of dilute biopolymer solutions can be partially alleviated either by ^{13}C labeling or by using the Fourier transform nmr method.[4–7] We have chosen the latter approach.

The experiments were performed on a high-resolution ^{13}C Fourier transform nmr spectrometer consisting mainly of a Varian high-resolution 14.1 kG electromagnet, a "home-built" pulsed nmr apparatus operating at 15.08 MHz, a Fabri-Tek 1074 signal averaging system, and a PDP-8/I computer. The apparatus included an external ^{19}F nmr lock and noise-modulated proton decoupling. The sample tubes had an inside diameter of 11.6 mm. A detailed description of the apparatus will be given elsewhere. The "ordinary" Fourier transform procedure[4] was used. Spin-echo refocusing techniques such as DEFT[6] and SEFT[7] are unnecessary for biopolymer nmr studies, because the natural linewidths are relatively large. A 1.1 M aqueous solution of sucrose gave the spectrum of Fig. 1 after less than 5 min of accumulation time. Many hours of signal averaging would be required to obtain a comparable spectrum by continuous wave nmr.[8]

Bovine pancreatic ribonuclease A has been thoroughly studied by proton nmr. Only the C-2 imidazole hydrogens of the four histidine residues have been

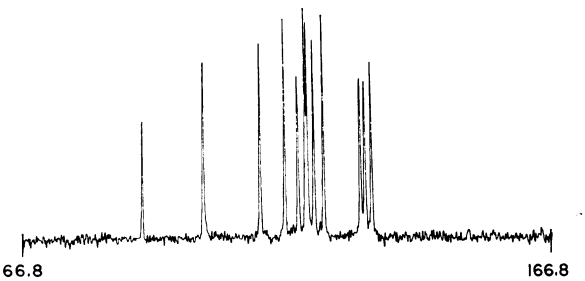

FIG. 1. Proton-decoupled natural abundance carbon-13 nmr spectrum of aqueous 1.1 M sucrose at 38°C, obtained at 15.08 MHz by the Fourier transform method, in 4.6 min of accumulation time (128 scans). Horizontal scale is in parts per million "upfield" from neat carbon disulfide.

unambiguously identified as single proton resonances at the low-field edge of an envelope of many overlapping lines. Furthermore, Jardetzky and co-workers[9] have succeeded in assigning the C-2 peaks to the four histidine residues on a one-to-one basis. Studies on the effects of pH and inhibitors on these resonances have given important information about ribonuclease in solution.

In view of the large range of carbon-13 chemical shifts, it may be possible to resolve more single-atom resonances in the ^{13}C spectra of proteins than in their proton spectra. In this preliminary report we wish to demonstrate the feasibility of observing peaks arising from only one or two carbons in the ^{13}C spectra of proteins of low molecular weight. We also report some tentative assignments in the spectrum of ribonuclease as a first step in the possible use of ^{13}C Fourier transform nmr in protein chemistry.

Bovine pancreatic ribonuclease A contains 575 carbons distributed among 124 amino acid residues.[10] Fig. 2 shows the proton-decoupled ^{13}C spectrum of a

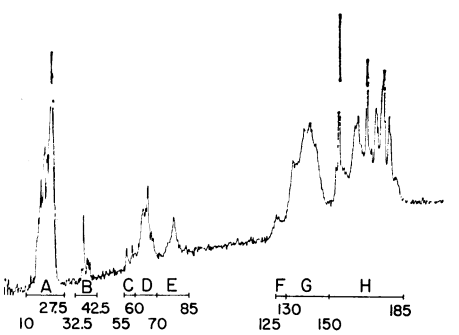

FIG. 2. Proton-decoupled natural abundance carbon-13 nmr spectrum of aqueous 0.02 M ribonuclease A at 38°C and pH 4.02, obtained at 15.08 MHz by the Fourier transform method, in 10 hr of accumulation time (65,000 scans). Horizontal scale is in parts per million "upfield" from neat carbon disulfide.

0.02 M solution of this enzyme (RNase A, lyophilized, phosphate-free, Worthington, Freehold, N.J.) after 65,000 scans obtained in 10 hr (38°C, pH 4.02). Most of the peaks were observable after only 1 hr. The spectral assignments given below are based on the known ^{13}C chemical shifts of the amino acids.[2] However, the experimental values of four amino acids contained in ribonuclease, namely asparagine, cysteine, glutamine, and tyrosine, have not been reported. We have determined the chemical shifts of the first three (see Table 1). For the highly insoluble tyrosine, we used the calculated values reported by Horsley, Sternlicht, and Cohen.[2,11,‡]

Spectral band *A* in Fig. 2 arises from the 151 carbonyl carbons. Region *B*

TABLE 1. *Carbon-13 chemical shifts of some amino acids.**

Amino acid	Chemical shift†				
	Co	C-α	C-β	C-γ	C-δ
Cys (1 M)	10.0	136.5	167.5	...	...
Asn (0.2 M)	17.8	140.9	157.6	19.0	...
	or 19.0	...	...	or 17.8	...
Gln (0.3 M)	15.0	138.2	166.2	161.6	18.6
	or 18.6	...	...	...	or 15.0

* Signal averaging time per spectrum from 10–40 min, depending on concentration; pulse recycle time was 4.35 sec. Listed values refer to pH 7; Asn and Gln chemical shifts did not change appreciably when the pH was lowered to 4.

† In parts per million "upfield" from neat carbon disulfide. The chemical shift of an internal dioxane reference was 126.1 ppm.

can be assigned to a total of 10 carbons: the aromatic C-6 carbons of the six tyrosine residues and the ϵ-carbons of the four arginine residues (notations follow ref. 2). All these carbons are quaternary. The signals in regions *A* and *B* should have a very small Overhauser enhancement.[12]

The two peaks in region *C* can be assigned to the imidazole C-2 carbons of the four histidine residues and the quaternary carbons of the three phenylalanine residues. It is known that the proton chemical shifts of the hydrogens bound to the imidazole C-2 carbons are pH dependent. At a pH of 4, three of the proton peaks nearly coincide. We intend to carry out a systematic study of the pH dependence of the ^{13}C spectrum.

Spectral band *D* arises from 10 quaternary and 27 nonquanternary carbons. The former are the aromatic C-3 carbons of the six tyrosines and the imidazole C-5 carbons of the four histidines. The latter are the 12 aromatic C-4 carbons of the tyrosines and the 15 aromatic (C-4, C-5, C-6) carbons of the three phenylalanines. Band *E* consists of 16 nonquaternary carbon peaks: the imidazole C-4 carbons of the histidines, and the aromatic C-5 carbons of the tyrosines. Band *F* probably contains the 10 β-carbon peaks of the threonine residues. Band *G* contains the β-carbons of serine and all the α-carbons except those of glycine. The remaining carbon peaks are in Band *H*.

Table 2 shows a comparison of the experimental integrated intensities with

TABLE 2. *Relative integrated intensities in the proton-decoupled carbon-13 spectrum of ribonuclease A.*

Spectral band*	Total intensity (%)	
	Experimental	Calculated
A	20.8	15.5
B	1.7	1.0
C	0.9	1.1
D	6.3	6.6
E	2.6	3.3
F + G + H	67.7	72.5

* See Fig. 2.

those calculated on the basis of our assignments. We used an Overhauser enhancement factor of 2 for all the nonquaternary carbons. Grant and coworkers[12] have shown that if the carbon-13 relaxation is dominated by dipolar coupling to one or more protons, then the Overhauser enhancement factor will

be about 3, regardless of the number of hydrogen atoms bound to the carbon. In practice, an enhancement factor of about 2 is more commonly observed in peptide spectra (Gurd, F. R. N., P. J. Lawson, E. Wenkert, and D. W. Cochran, unpublished results). In view of the uncertainty in our estimate of the Overhauser enhancement, the calculated intensities in Table 2 must be considered tentative. The good agreement with the experimental values is encouraging.

It is apparent that natural abundance carbon-13 nmr can be a practical tool in protein chemistry, even at low resonance frequencies. It is likely that spectra taken at much higher frequencies in superconducting solenoids[13] will be even more informative.

‡ ***Note added in proof:*** The resonance of the quaternary aromatic carbon in phenylalanine was not observed by Horsley, Sternlicht, and Cohen (ref. 2). We have found this resonance at 57.3 ppm upfield from carbon disulfide at pH 5.7. The other chemical shifts were in agreement with those in reference 2. We have also obtained the chemical shifts for the highly insoluble amino acid, tyrosine, by using glycyl-L-tyrosine (pH 7.0). The quaternary aromatic carbons were observed at 38.2 and 63.0 ppm. Other tyrosine resonances had chemical shifts in good agreement with the estimated values in reference 2.

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[11] The chemical shifts of one carbon each in His, Phe, and Tyr have not been reported. The imidazole C-5 in His is 60.8 parts per million "upfield" from external carbon disulfide at pH 7.05 (F. R. N. Gurd and J. Morrow, private communication). We estimated the other unknown values by the procedure used in ref. 2. See note added in proof.‡
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