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Spin-Echo and Spin-Lock Natural-Abundance Carbon-13 Fourier Transform NMR of Proteins Using a Sideways-Spinning 20-mm Tube Probe*

In a recent communication (*1*) Lilley and Howarth reported an empirical investigation of the spin-echo determination of short T_2 values in proton-decoupled natural-abundance carbon-13 NMR spectra of ribonuclease A (EC 2.7.7.16). Using a high-sensitivity high-resolution "homebuilt" Fourier transform nuclear magnetic resonance spectrometer equipped with a sideways-spinning 20-mm tube sample probe (*2*), we have investigated the apparent spin–spin and rotating frame spin–lattice relaxation rates of both protonated and nonprotonated carbons in aqueous solutions of the protein hen egg-white lysozyme (EC 3.2.1.17). Our results indicate that, as expected, large differential (T_2 or $T_{1\varrho}$) relaxation rates are observed between the protonated and nonprotonated carbon atom sites. Low resolution and low sensitivity precluded this observation in the earlier study (*1*). Use of the spin-echo Fourier transform (SEFT) or spin-lock Fourier transform (SLFT) technique is shown to be a useful alternative to weak-decoupling convolution-difference schemes (*3–5*) for removing the broad bands of protonated carbon resonances from natural-abundance carbon-13 Fourier transform nuclear magnetic resonance spectra of native proteins.

Carbon-13 spectra were obtained by the Fourier transform method (*6*) at 37.723 MHz using a "homebuilt" spectrometer (*2*), which consists of a Nalorac Incorporated high-resolution 4.0-in. bore superconducting magnet (Nalorac Division, Nicolet Instrument Corporation, Stanwell Industrial Park, Concord, Calif.), a Nicolet NIC-820 computer (Nicolet Instrument Corporation, Madison, Wis.) together with a homebuilt sideways-spinning 20-mm probe and other associated digital and radiofrequency electronics (*2*). The spectrometer was not equipped with any form of field-frequency stabilization. The 90° pulse width for ^{13}C was 22 μsec when using 65 W of power. Time domain data were generally accumulated in quadrature using two 8192-word blocks and were processed using Nicolet software. Conventional two-pulse Carr–Purcell spin-echo, and $T_{1\varrho}$ pulse sequences were used as described elsewhere (*1*, *7*). Data acquisition was begun either at the echo maximum (two-pulse Carr–Purcell spin-echo experiment) or immediately following the ^{13}C spin-lock pulse, to generate the appropriate spin-echo Fourier transform (SEFT) or spin-lock Fourier transform (SLFT) spectra. Full proton decoupling was used in all cases.

In Fig. 1 we show a series of proton-decoupled carbon-13 spin-echo Fourier transform NMR spectra of hen egg-white lysozyme, and in Fig. 2 we present expanded versions of these spectra in the region of the aromatic and arginine C^{ζ} resonances. A full discussion of the types of carbon atom contributing to each region of the spectrum of a

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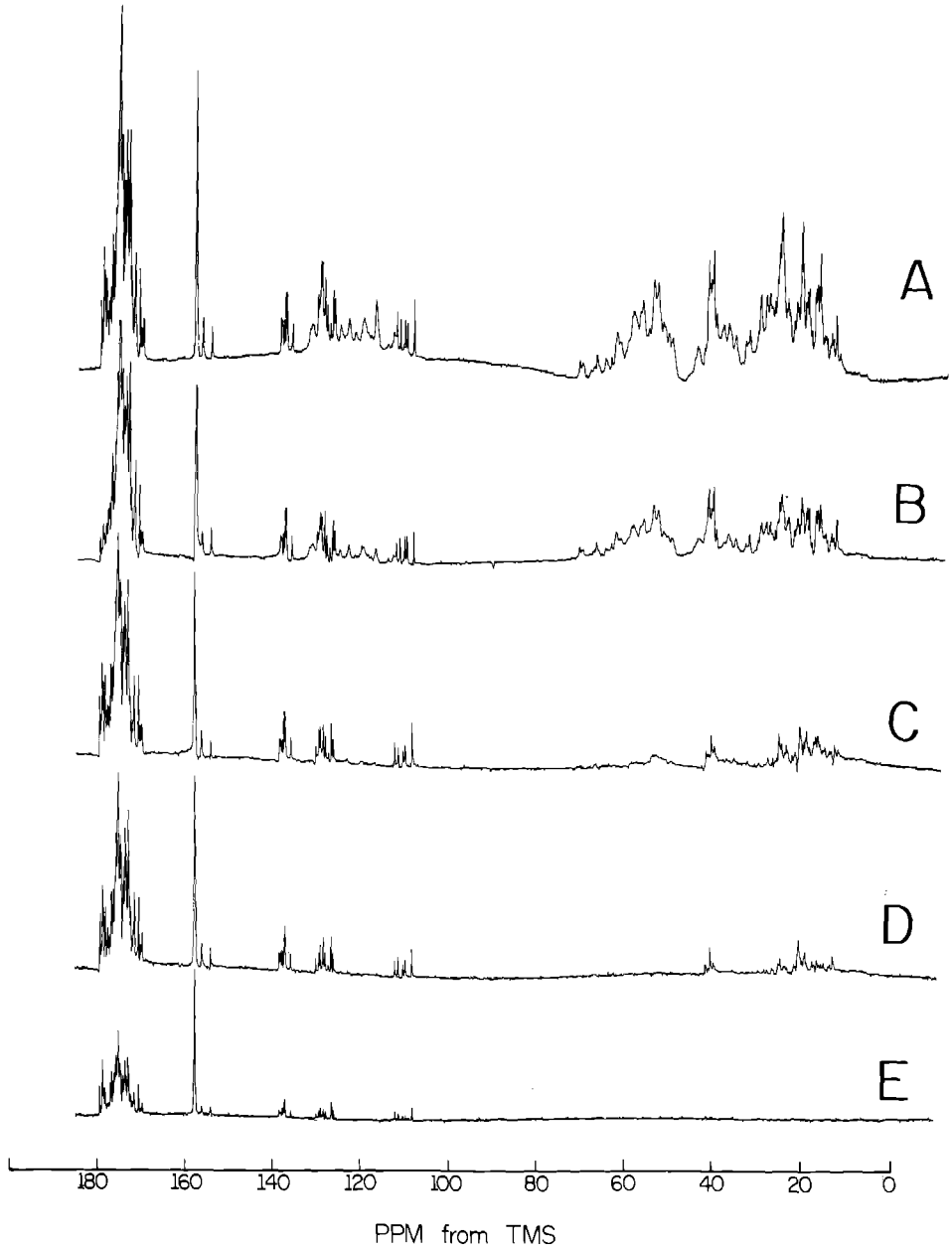


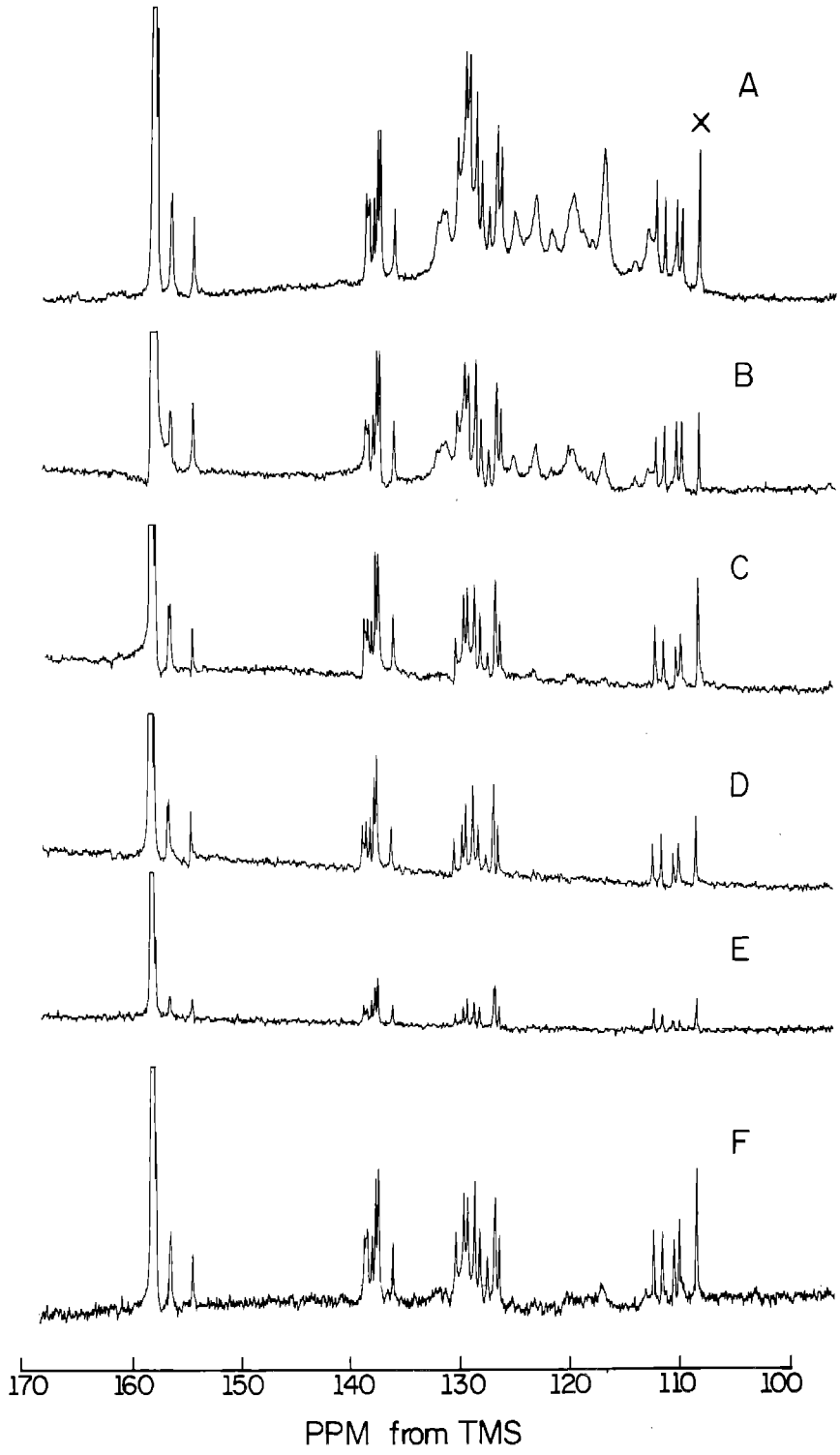
FIG. 1. Proton-decoupled natural-abundance carbon-13 normal and spin-echo Fourier transform NMR spectra of 6.5-ml aqueous solutions of hen egg-white lysozyme (EC 3.2.1.17) obtained using a sideways-spinning 20-mm tube probe. (A) Hen egg-white lysozyme (Sigma Chemical Co., St. Louis, Missouri, Type I, further purified by chromatography on diethylaminoethyl–Sephadex) in H_2O (19 m*M*, pH 2.86, about 37°C, ^{13}C frequency 37.723151 MHz, ^{1}H frequency 150.009428 MHz, pseudorandom-noise phase-modulation, 1800-Hz bandwidth, 5.6-W decoupling power, 6.0-sec recycle time, 22-μsec 90° pulse width, 8547-Hz spectral width, 350-μsec acquisition delay time, 2 × 8192 data points, 12,000 scans, 5000-Hz four-pole Butterworth low-pass filters, 8-bit A/D resolution, 1.5-Hz line broadening). (B)–(E), as (A) but spin-echo spectra obtained using the Carr/Purcell method with the following τ values and delay times: (B) 6 msec, (C) 20 msec, (D) 40 msec, (E) 150 msec.

protein is given elsewhere (*5*). An examination of the peak intensities in Figs. 1A–E clearly shows that all resonances in the range 0 to 80 ppm downfield from tetramethylsilane (TMS) are characterized by relatively short spin–spin relaxation times, while many resolved resonances in the range 100 to 190 ppm downfield from TMS have relatively long spin–spin relaxation times.

All narrow resonances in the downfield region of the spectrum are expected to arise from nonprotonated carbons: carbonyl groups; C^{ζ} of arginine and tyrosine: C^{γ} of phenylalanine, tyrosine, histidine, and tryptophan; plus $C^{\epsilon 2}$ and $C^{\delta 2}$ of tryptophan. In Fig. 2 we present vertical and horizontal expansions of the downfield regions of the spectra of Fig. 1. There are clearly two types of relaxation behavior present in Fig. 2. The narrow peaks in the range 100 to 190 ppm downfield from TMS are characterized by long T_2 relaxation times while the broad bands have relatively short T_2 values. Thus, in Fig. 2C, which corresponds to a τ value of 20 msec, only the narrow resonances remain. Similar differential relaxation rates are observed for rotating frame relaxation experiments. One example is illustrated in Fig. 2F.

Relaxation of protonated carbons in macromolecules is dominated by the carbon–hydrogen dipolar mechanism, at currently available magnetic field strengths (*8*). As a result we may readily calculate (*9*, *10*) a protein rotational correlation time from T_1 and T_2 data, assuming that the CH group under consideration is irrotationally bound. Such a situation is almost certainly true for most backbone α carbons and for many aromatic amino acid sidechains (*11*). Using the data of Fig. 1 together with additional τ values, we estimate that the α-carbon spin–spin relaxation time is 20 ± 4 msec, which indicates a rotational correlation time (τ_R) in the range 8.0 to 13 nsec. Spin–lattice relaxation time measurements on the same sample used in Fig. 1 give a T_1 value of 74 ± 10 msec, corresponding to a τ_R of 5.4 to 9 nsec. We have thus used a value of τ_R of 8 nsec to estimate the spin–spin relaxation rates of the nonprotonated aromatic carbon resonances of Fig. 2. Peak X of Fig. 2A, for example, arises from C_γ of tryptophan residues, in this case Trp-111 C^{γ} and Trp-28 C^{γ} (*12*). Using equations presented elsewhere (*5*, *9*, *10*) we calculate a dipolar contribution of 0.62 Hz to the linewidth for a Trp-C^{γ} interacting with three hydrogen atoms 2.16 Å distant. The contribution to the linewidth from relaxation via chemical shielding anisotropy, assuming an axially symmetric shielding tensor having $|\sigma_{\parallel} - \sigma_{\perp}| = \Delta\sigma = 200$ ppm (*8*, *13*, *14*), is about 0.56 Hz, giving a total theoretical linewidth of 1.18 Hz, or T_2 of about 270 msec. The experimentally determined T_2 values for C^{γ} of Trp obtained from the data of Figs. 1 and 2 is 110 ± 10 msec, a poor agreement with that predicted theoretically. The $T_{1\varrho}$ obtained from our unpublished results is 130 ± 20 msec. However, comparisons of the experimentally determined Trp C^{γ} spin–lattice relaxation time T_1 with that measured experimentally are in good agreement (unpublished results; and see, for example, the results of Ref. (*8*)). We intend to investigate the precise reasons for the discrepancy between the calculated and observed spin–spin relaxation rates. Possible candidates are inadequate decoupler power (H_2), rotating frame resonance effects (*15*), or even perhaps anisotropic rotation of the lysozyme molecule (*16*).

The results presented in Figs. 1 and 2 nevertheless clearly indicate that spin-echo and spin-lock Fourier transform NMR spectra are useful techniques for facilitating the study of non-protonated carbon atom sites in proteins. A similar resolution enhancement, of the histidine $H^{\delta 2}$ and $H^{\epsilon 1}$ *proton* resonances in a myoglobin sample, was reported previously by Campbell and co-workers (*17*) using the Carr–Purcell spin-echo


A
X
B
C
D
E
F
170
160
150
140
130
120
110
100
PPM from TMS

method A: long-range dipolar interactions dominate the relaxation of $H^{\delta 2}$ and $H^{\epsilon 1}$ (especially in deuterated solvents); thus T_2 is long. In addition, further line narrowing may occur because of fast internal rotation of the His residues (*17*). However, because of homonuclear scalar coupling, the method is not universally applicable for resolution enhancement in proton NMR spectroscopy of proteins. For natural-abundance carbon-13 NMR, convolution-difference weak-decoupling techniques, which provide results essentially identical to those shown in Fig. 2, require operator "baseline adjustment" to remove residual broad bands of protonated carbon atom resonances, together with the negative wings of sharp absorption lines (see, for example, Refs. (*4*, *5*)). These operations are unnecessary when using spin-echo or spin-lock pulse sequences.

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ERIC OLDFIELD
ROBERT P. SKARJUNE

School of Chemical Sciences
University of Illinois at Urbana–Champaign
Urbana, Illinois 61801

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FIG. 2. Aromatic and C^{ζ} of arginine region of the proton decoupled natural-abundance carbon-13 normal, spin-echo, and spin-lock Fourier transform NMR spectra of 6.5-ml aqueous solutions of hen egg-white lysozyme. (A)–(E), horizontal and vertical expansions of the downfield regions of the spectra shown in Fig. 1A–E. (F), Spin-lock spectrum obtained under the conditions of Fig. 1A with the addition of a 40-msec spin-lock pulse. Peak intensities are similar to those of (C).