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Studies of Proteins in Solution by Natural-Abundance Carbon-13 Nuclear Magnetic Resonance. Spectral Resolution and Relaxation Behavior at High Magnetic Field Strengths

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Abstract: Natural-abundance ^{13}C nuclear magnetic resonance spectra of hen egg-white lysozyme at 63.4 kG are compared with spectra at 14.2 kG. The increase in resolution when going from the low to the high field is much greater for aliphatic and methine aromatic carbon resonances than for carbonyl and nonprotonated aromatic carbon resonances. This result is consistent with experimental and calculated spin–lattice relaxation times, which demonstrate the dominance of chemical shift anisotropy as a relaxation mechanism for nonprotonated unsaturated carbons of a native protein at magnetic field strengths much above 40 kG. For the aliphatic and methine aromatic carbons, the ^{13}C–^{1}H dipolar relaxation mechanism should be dominant at all magnetic field strengths now available for NMR.

Most ^{13}C nuclear magnetic resonance studies of proteins have been carried out at low magnetic field strengths, such as 14.2[1-3] or 23.5 kG.[4,5] With the increased availability of high-field high-resolution Fourier transform NMR spectrometers,[6,7] it becomes important to evaluate the effect of high magnetic field strengths on the information content of ^{13}C NMR spectra of proteins. It is not necessarily true that resolution and sensitivity will automatically increase with magnetic field strength. It has been suggested[8] that, for some carbons of native biopolymers, *sensitivity* at high magnetic field strengths (such as 50–90 kG) may be adversely affected by long spin–lattice relaxation times (T_1). This suggestion was based on the assumption of purely ^{13}C–^{1}H dipolar relaxation for all carbons. It is now well documented that, in general, this relaxation mechanism is dominant for ^{13}C resonances of proteins at 14.2 kG, even when dealing with nonprotonated carbons.[2] However, there is evidence that at 63.4 kG chemical shift anisotropy (CSA) contributes strongly to the relaxation of nonprotonated unsaturated carbons of organic molecules in solution.[7] If, as expected, CSA is also the dominant relaxation mechanism for some carbons of a protein (at high field), then the T_1 values of these carbons may be significantly shorter than predicted on the basis of the ^{13}C–^{1}H dipolar mechanism. However, the dominance of the CSA relaxation mechanism will adversely affect the use of high magnetic field strengths for improving spectral *resolution* (see below).

In this report we present natural-abundance ^{13}C Fourier transform NMR spectra and spin–lattice relaxation data for

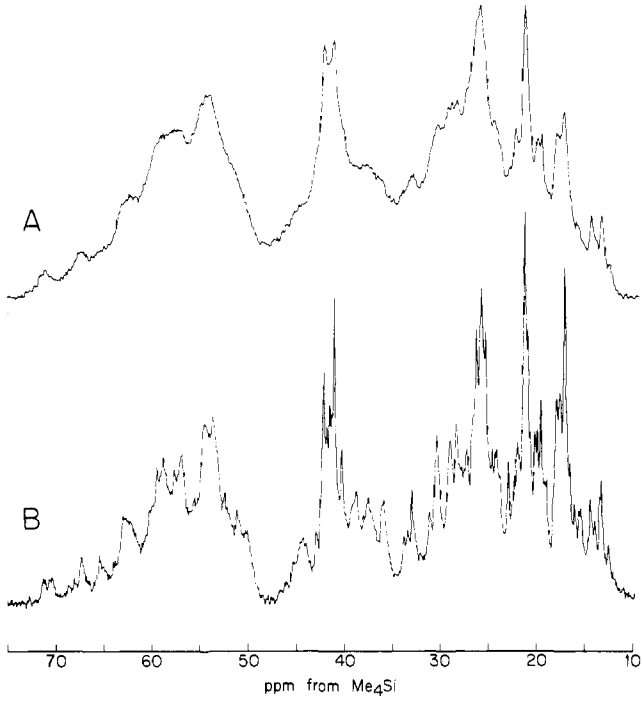

Figure 1. Region of aliphatic carbons in the fully proton-decoupled natural-abundance ^{13}C Fourier transform NMR spectra of hen egg-white lysozyme in H_2O and 0.1 M NaCl: (A) at 14.2 kG (15.2 MHz), using 14.1 mM lysozyme (volume 10.5 ml, 2.1 g protein, pH 4.0, 36 °C), a spectral width of 3788 Hz, 16 384 time-domain addresses, 32 768 accumulations with a recycle time of 2.205 s (20-h total time), and 0.88-Hz digital broadening; (B) at 63.4 kG (67.9 MHz), using 15.4 mM lysozyme (volume 2 ml, 0.44 g protein, pH 3.1, 30 °C), 32 768 accumulations with a recycle time of 3.016 s (27 h total time), and 2.78-Hz digital broadening.

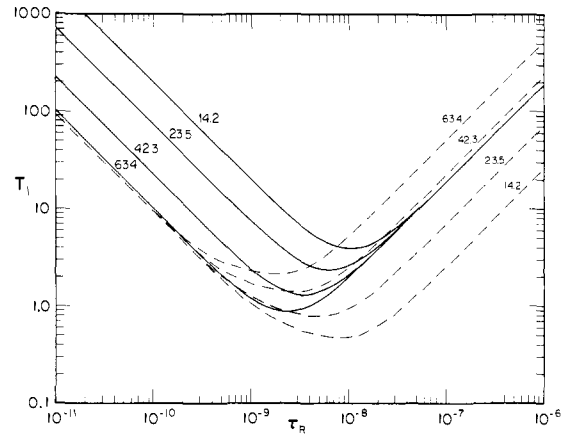

Figure 2. Log–log plots of T_1 vs. τ_R (both in seconds) for a ^{13}C spin relaxing by the chemical shift anisotropy mechanism with $\Delta\sigma$ = 200 ppm (solid lines), and by dipolar interactions with three hydrogens 2.16 Å away (dashed lines), in the case of isotropic rotational reorientation and under conditions of proton-decoupling. Plots are given for four magnetic field strengths, indicated in kilogauss.

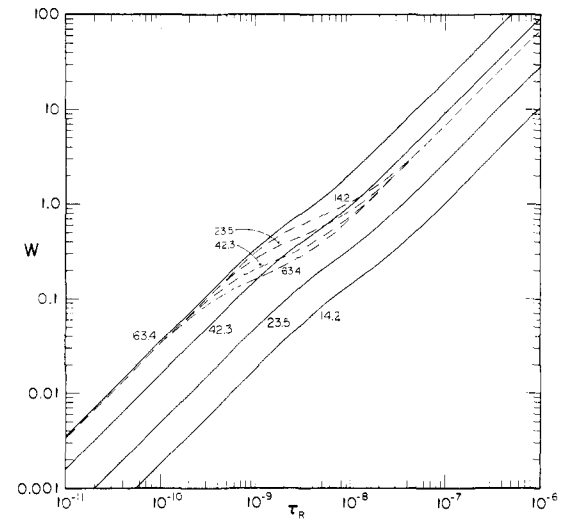

Figure 3. Log–log plots of line width (in Hertz) vs. τ_R (in seconds) for four magnetic field strengths, indicated in kilogauss. The meaning of solid and dashed lines is given in the caption of Figure 2.

hen egg-white lysozyme, obtained at 63.4 kG under conditions of sufficient sensitivity for the observation of single-carbon resonances. We compare the spectral resolution and relaxation behavior at 63.4 kG with previous results obtained at 14.2 kG.[2] We also present theoretical results for the relative contributions of CSA and ^{13}C–^1H dipolar relaxation to $1/T_1$ and to natural line widths (W), at various magnetic field strengths. To our knowledge, only two studies of high-field ^{13}C NMR spectra of native proteins have been published, both on hen egg-white lysozyme at 63.4 kG.[6,9] In one case,[9] the signal-to-noise ratio was not sufficient to permit a detailed comparison with spectra of lysozyme obtained at low fields. In the other case,[6] the sensitivity was sufficient for the detection of single-carbon resonances, but only the carbonyl region was examined, and the relaxation behavior of the resonances was not studied.

Experimental Section

Lysozyme samples were prepared as described previously.[3] Carbon-13 NMR spectra at 15.2 MHz (14.2 kG) were obtained as described previously.[2]

Carbon-13 NMR spectra at 67.9 MHz (63.4 kG) were obtained on a spectrometer consisting of a Bruker high-resolution superconducting magnet, Bruker 10-mm probe, home-built radiofrequency electronics, and a Nicolet 1085 computer. The spectrometer was not equipped with a field-frequency lock. The ^1H irradiation for proton decoupling (at 270 MHz) had a peak field strength of 0.8 G (3.4 kHz) and a random noise modulation bandwidth of about 300 Hz. Separate fully proton-decoupled spectra were recorded for the unsaturated and saturated carbon regions, with the ^1H irradiation centered 6.7 and 2.2 ppm, respectively, downfield from the ^1H resonance of Me_4Si. For noise-modulated off-resonance proton-decoupled spectra of the aromatic carbon region,[3] the ^1H irradiation was centered on Me_4Si. For ^{13}C excitation, 90° pulses were used in all cases. For spectra of the unsaturated carbon region, the width of the 90° pulse was 23 μs (11-kHz peak field strength), and the frequency was set 13.7-kHz downfield from the ^{13}C resonance of Me_4Si. Spectra of the saturated carbon region were recorded after spectrometer improvements, which yielded a 90° pulse of 13 μs (19-kHz peak field strength), with the frequency set 1.0-kHz upfield from Me_4Si. Time-domain data were accumulated in 16 384 addresses, with a spectral width of 14 286 Hz. The method of progressive saturation[10] was used to obtain T_1 values.

Broad bands were removed digitally from some spectra by means of the convolution difference procedure,[11] as described previously.[3] For spectra at 63.4 kG, the values of τ_1, τ_2, and K (defined by Campbell et al.[11]) were 0.115 s, 0.0057 s, and 0.9, respectively. At 14.2 kG, the corresponding values were 0.36 s, 0.033 s, and 0.9.

Results and Discussion

Theoretical Considerations. Theoretical values of $1/T_1^D$ and W^D (dipolar contributions to $1/T_1$ and W) for protonated and nonprotonated carbons, as a function of the rotational correlation time (τ_R), have been published.[2,8] The expressions for $1/T_1^{CSA}$ and W^{CSA} (contributions to $1/T_1$ and W from the CSA relaxation mechanism) for the isotropic rigid rotor model are:[12]

$$1/T_1^{CSA} = (2/15)\gamma^2 H_0^2 (\Delta\sigma)^2 \tau_R (1 + \gamma^2 H_0^2 \tau_R^2)^{-1} \quad (1)$$

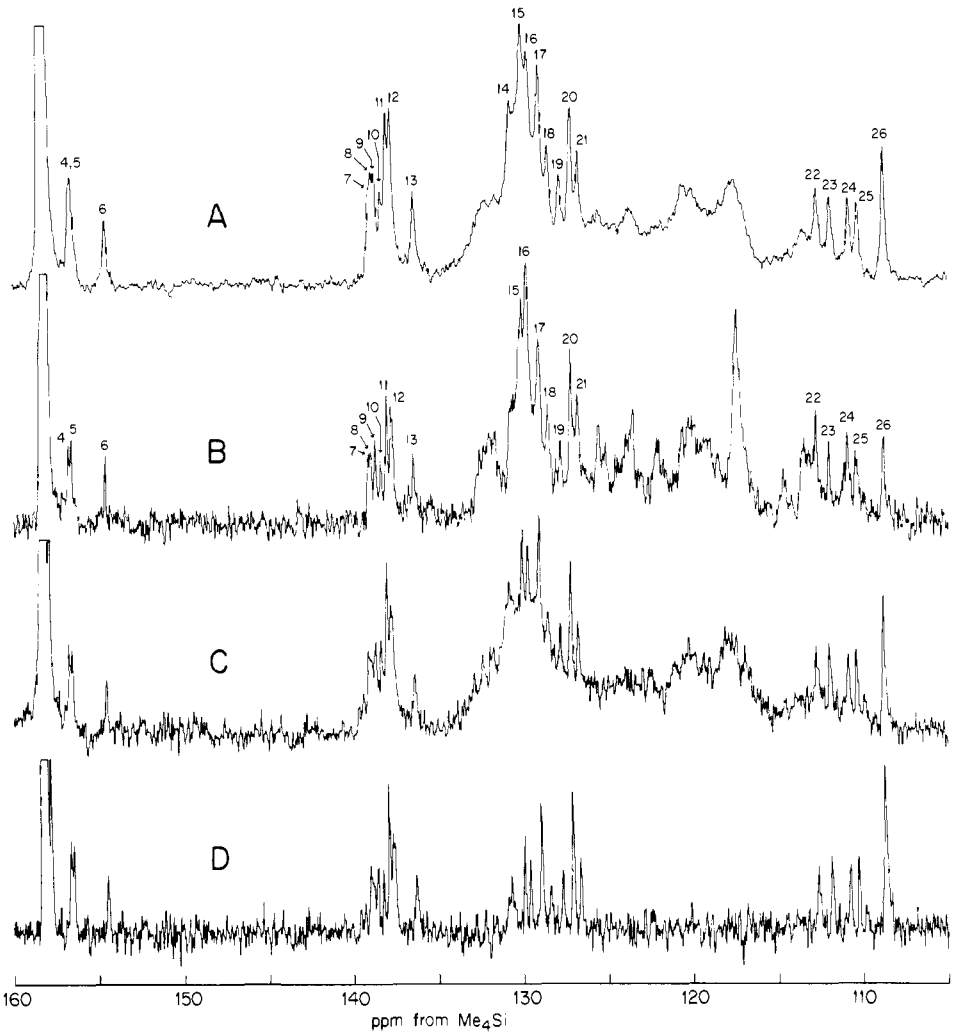

Figure 4. Region of aromatic carbons and C^{ζ} of arginine residues in the natural-abundance ^{13}C Fourier transform NMR spectra of hen egg-white lysozyme in H_2O (0.1 M NaCl, pH 3.1). (A) At 14.2 kG (15.2 MHz), using 10.5 ml of 14.5 mM lysozyme (2.2 g protein) at 42 °C. Spectrum was recorded under conditions of full proton decoupling, with a spectral width of 3788 Hz, 8192 time-domain addresses, 32 768 accumulations, a recycle time of 1.105 s (10 h total time), and 0.88-Hz digital broadening. Resonances of nonprotonated aromatic carbons are numbered as in ref 2 and 3. The truncated band at about 158 ppm arises from C^{ζ} of the 11 arginine residues (peaks 1-3[2,3]). (B) At 63.4 kG (67.9 MHz), using 2 ml of 15.9 mM lysozyme (0.45 g protein) and full proton decoupling. Other spectral conditions were as in Figure 1B. (C) As in B, except that the spectrum was recorded under conditions of noise-modulated off-resonance proton decoupling, with 40 960 accumulations and a recycle time of 5.016 s (57 h total time). (D) Convolution difference spectrum derived from the same time-domain data as in C, with $\tau_1 = 0.115$ s, $\tau_2 = 0.0057$ s, and $K = 0.9$ (τ_1, τ_2, and K are defined in ref 3 and 11).

$$W^{CSA} = (45\pi)^{-1}\gamma^2 H_0^2 (\Delta\sigma)^2 \tau_R [4 + 3(1 + \gamma^2 H_0^2 \tau_R^2)^{-1}] \quad (2)$$

Here γ is the gyromagnetic ratio, H_0 is the magnetic field strength, and $\Delta\sigma$ is given by

$$(2/3)(\Delta\sigma)^2 = (\sigma_{11} - \sigma_{av})^2 + (\sigma_{22} - \sigma_{av})^2 + (\sigma_{33} - \sigma_{av})^2 \quad (3)$$

where the σ_{ii} are the eigenvalues of the shielding tensor, and

$$3\sigma_{av} = \sigma_{11} + \sigma_{22} + \sigma_{33} \quad (4)$$

If the shielding is axially symmetric ($\sigma_{33} = \sigma_{\parallel}$, and $\sigma_{11} = \sigma_{22} = \sigma_{\perp}$), then $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$.

In general, the isotropic rigid rotor model can be used for α carbons,[2,13] backbone carbonyls,[13] and aromatic carbons[2] of a native spherical protein. In the case of the somewhat nonspherical hen egg-white lysozyme,[14] corrections for anisotropic overall rotation are necessary.[15] Furthermore, internal motions must be considered when dealing with the relaxation of some side-chain carbons.[13] However, even when the isotropic rigid rotor model is not applicable, this model will yield reasonable estimates of the *ratios* T_1^{CSA}/T_1^{D} and W^{CSA}/W^{D}, if the effective rotational correlation times for the C–H bond vectors and for the anisotropic chemical shift tensor are similar.

Aliphatic Carbons. With the use of the isotropic rigid rotor model and published values of shielding tensors for aliphatic carbons ($\Delta\sigma \lesssim 20$ ppm),[16-18] we calculate that W^{CSA} and $1/T_1^{CSA}$ should be negligible relative to W^{D} and $1/T_1^{D}$, respectively, even at a magnetic field strength as high as 200 kG. Consequently, the highest available magnetic field strengths should yield the greatest resolution.[8]

Figure 1 shows the aliphatic regions[2] of fully proton-decoupled ^{13}C NMR spectra of hen egg-white lysozyme at 14.2 and 63.4 kG. As expected, the resolution (reciprocal of the line width in parts per million) is considerably greater at 63.4 kG (Figure 1B) than at 14.2 (Figure 1A) and 23.5 kG.[4,5] However, even at 63.4 kG, few, if any, of the aliphatic carbons of hen egg-white lysozyme yield resolved single-carbon resonances.

Aromatic Carbons. We used eq 1 and 2 to calculate T_1^{CSA} and W^{CSA} for an aromatic carbon with $\Delta\sigma = 200$ ppm,[16,17] at various magnetic field strengths. The solid lines of Figures 2 and 3 are the resulting log–log plots for T_1^{CSA} and W^{CSA}, re-

Table I. Calculated and Experimental T_1 Values of Nonprotonated Carbons of Hen Egg-White Lysozyme at 63.4 kG

Carbon	Calcd[a] T_1^D	Calcd[a] T_1	Expt[b]
Carbonyl	7.9	1.9	1.6 ± 0.7[c]
Arg C^ζ	3.0[d]	1.4[d]	1.2 ± 0.3
Tyr C^ζ	6.8	1.8	1.4 ± 0.6
Phe C^γ	5.1	1.7	*e*
Trp $C^{\epsilon 2}$	7.9	1.9	1.9 ± 0.8
His C^γ	4.4[f]	1.6	2.5 ± 1.3[g]
Tyr C^γ	5.1	1.7	1.2 ± 0.5
Trp $C^{\delta 2}$	20.5	2.2	1.8 ± 1.2
Trp C^γ	6.8	1.8	1.7 ± 0.7

[a] Theoretical spin-lattice relaxation times (in seconds) for τ_R = 13 ns.[15] Values of T_1^D were computed from eq 6 of ref 2 by considering $^{13}C-^1H$ dipolar interactions with hydrogens, two bonds removed (using a C-H distance of 2.16 Å), and $^{13}C-^{14}N$ dipolar interactions with directly bonded nitrogens (using a C-N distance of 1.35 Å). Values of T_1 were computed by assuming that $1/T_1 = 1/T_1^D + 1/T_1^{CSA}$. $1/T_1^{CSA}$ was computed from eq 1 of text, with $\Delta\sigma = 2 \times 10^{-4}$. [b] Experimental T_1 value, in seconds, obtained by the method of progressive saturation[10] from spectra recorded with recycle times of 0.716, 1.516, 3.016, and 5.016 s. Sample and instrumental conditions are those of Figure 4D, except that 65 536 accumulations were used for the spectra recorded with recycle times of 0.716, 1.516, and 3.016 s. Assignments of peaks 1-26 of Figure 4 were taken from ref 3 (peaks 1-3 are truncated in Figure 4). Unless otherwise indicated, each T_1 value is an arithmetic average of the measured values of at least two peaks, as follows (peak designations are from Figures 4B and 5D): peaks b-d for carbonyl, peaks 1-3 for Arg C^ζ, peaks 4-6 for Tyr C^ζ, peaks 11 and 13 for Trp $C^{\epsilon 2}$, peaks 14 and 17 for Tyr C^γ, peaks 16 and 18-21 for Trp $C^{\delta 2}$, and peaks 22-26 for Trp C^γ. [c] 1.7 ± 0.7 s for peak a and 1.6 ± 0.7 s for the main carbonyl band centered at about 176 ppm (Figure 5D). [d] The rigid rotor model may not be applicable to some resonances of C^ζ or arginine residues.[2] [e] The resonances of C^γ of phenylalanine residues are peaks 7, 9, and one-half of peak 12 of Figure 4 (see Table I of ref 3). These poorly resolved resonances did not yield usable T_1 values. [f] Computed for the imidazolium form of the histidine. [g] Peak 15 of Figure 4.

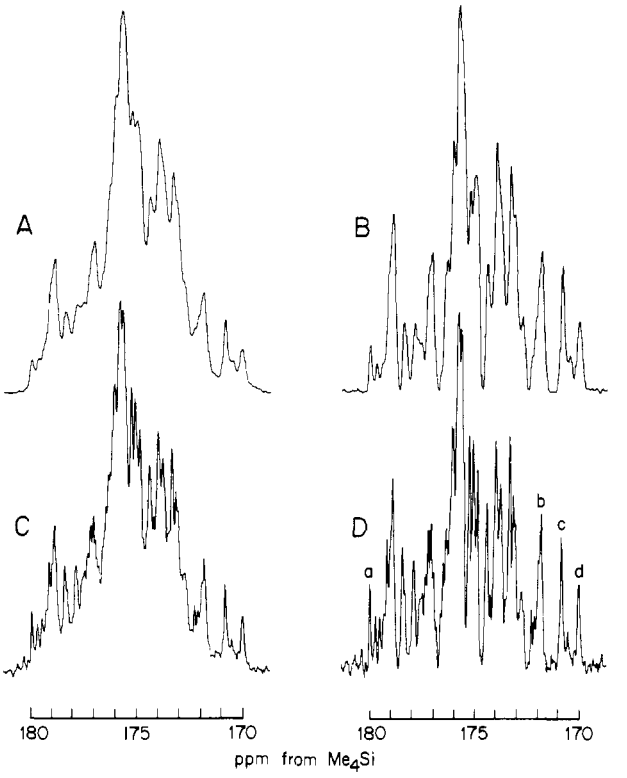

Figure 5. Carbonyl region in fully proton-decoupled natural-abundance ^{13}C Fourier transform NMR spectra of hen egg-white lysozyme in H_2O (0.1 M NaCl, pH 3.1). (A) At 14.2 kG (15.2 MHz). Sample and spectral conditions were those of Figure 4A. (B) Convolution difference spectrum derived from the same time-domain data as in A, with the use of τ_1 = 0.36 s, τ_2 = 0.033 s, and K = 0.9.[3,11] (C) At 63.4 kG (67.9 MHz). Sample and spectral conditions were those of Figure 4B. (D) Convolution difference spectrum derived from the same time-domain data as in C, with the use of τ_1 = 0.115 s, τ_2 = 0.0057 s, and K = 0.9.[3,11]

spectively. The dashed lines of Figures 2 and 3 are the dipolar values T_1^D and W^D, respectively, for a *nonprotonated* carbon interacting with three hydrogens, two bonds removed.[2,19] Figures 2 and 3 indicate that, at magnetic field strengths well above 40 kG, CSA should be the dominant relaxation mechanism for nonprotonated aromatic carbons of a native protein ($\tau_R \gtrsim 10$ ns). An increase in magnetic field strength much above 40 kG will decrease the resolution of the nonprotonated aromatic carbon resonances of a native protein.

The theoretical results for T_1^{CSA} and W^{CSA} in Figures 2 and 3 are equally applicable to nonprotonated and methine aromatic carbons. However, the values of T_1^D (Figure 2) must be divided by a factor of about 20, and the values of W^D (Figure 3) must be multiplied by this factor, before using them for methine aromatic carbons.[2] We estimate that the $^{13}C-^1H$ dipolar mechanism should dominate the relaxation of methine aromatic carbons at magnetic field strengths $\lesssim$150 kG.

A comparison of the aromatic regions in fully proton-decoupled ^{13}C NMR spectra of hen egg-white lysozyme at 14.2 (Figure 4A) and 63.4 kG (Figure 4B) yields an experimental verification of the contrasting behavior of nonprotonated and methine aromatic carbons. In the spectrum at 14.2 kG, all the narrow resonances (peaks 4-26 of Figure 4A) arise from nonprotonated aromatic carbons, and the broad bands arise from methine carbons.[1] We estimate that instrumental broadening (from field inhomogeneity and drift, and from digital processing) is about 1-2 Hz at 14.2 kG and about 4-6 Hz at 63.4 kG. The observed resolution (reciprocal of the line width in parts per million) of the nonprotonated aromatic carbon resonances increases about two-fold when going from 14.2 (Figure 4A) to 63.4 kG (Figure 4B). A comparable increase in resolution is expected when instrumental broadening is negligible (Figure 3). The observed improvement in resolution when going from 14.2 to 63.4 kG (Figures 4A and 4B) is much greater for the methine aromatic carbons than for the nonprotonated aromatic carbons. Inefficient proton decoupling[1-3] is more necessary at 63.4 kG than at 14.2 kG for distinguishing the two types of resonances (compare Figures 4B and 4C). Furthermore, at 63.4 kG, application of the convolution difference procedure[11] for eliminating the resonances of the methine aromatic carbons[3] is not practical if the spectrum of the protein is recorded under conditions of full proton decoupling, but is effective if noise-modulated off-resonance proton decoupling is used (Figure 4D).

Measured T_1 values confirm the dominance of the CSA relaxation mechanism for nonprotonated aromatic carbons of hen egg-white lysozyme at 63.4 kG. The experimental T_1 values are considerably shorter than calculated T_1^D values, but are in reasonable agreement with calculated T_1^{CSA} values (Table I). In contrast, at 14.2 kG there is good agreement between the experimental T_1 values of nonprotonated aromatic carbons of lysozyme and the corresponding calculated values of T_1^D.[2] The good agreement between the experimental and theoretical T_1 values at 63.4 kG (Table I) indicates that $\Delta\sigma \approx 200$ ppm is probably a good choice for *all* aromatic carbons (of amino acid residues) in a protein.

When $T_1 \approx T_1^D$ for all nonprotonated aromatic carbons, then the resonances of $C^{\delta 2}$ and $C^{\epsilon 2}$ of tryptophan residues can

be identified by means of partially-relaxed Fourier transform spectra.[3] This method has been used successfully at 14.2 kG,[3] but it is not applicable when $T_1 \approx T_1^{CSA}$, because in this case all nonprotonated aromatic carbons have similar T_1 values (Table I).

Carbonyl Resonances. Ordinarily, all carbonyl carbons of a protein are nonprotonated. Their values of $\Delta\sigma$ are comparable to those of aromatic carbons,[16,17] and their $^{13}C-^{1}H$ dipolar interactions are comparable in strength to those of nonprotonated aromatic carbons.[2] The analysis presented in the previous section for nonprotonated aromatic carbons should be applicable to backbone carbonyls. Indeed, this expectation is supported by experimental observations: our spectral resolution in the carbonyl region increases about twofold when going from 14.2 (Figures 5A and 5B) to 63.4 kG (Figures 5C and 5D). At 63.4 kG, measured T_1 values of the multiple-carbon peaks a–d (Figure 5D) and of the carbonyl region "as a whole" are similar to measured T_1 values of nonprotonated aromatic carbons (Table I). Peaks b–d probably arise only from backbone carbonyls.[20] Side-chain carbonyls and the C-terminal carbonyl should yield resonances in the downfield half of the carbonyl region.[6,20] The two-carbon peak a has been very tentatively assigned to the C-terminal carboxyl and a side-chain carboxyl of an aspartic acid residue.[6] Note that the rigid rotor model may not be applicable to side-chain and backbone terminal carbonyl resonances.

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