Nuclear Magnetic Resonance Studies of Amino Acids and Proteins. Rotational Correlation Times of Proteins by Deuterium Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: We show that measurement of the spin–lattice (T_1) and spin–spin (T_2) relaxation times (or line widths) of irrotationally bound ^2H nuclei in macromolecules undergoing isotropic rotational motion *outside of the extreme narrowing limit* (i.e., for the case $\omega_0^2\tau_R^2 \gg 1$) permits determination of both the rotational correlation time (τ_R) of the macromolecule *and* the electric quadrupole coupling constant (e^2qQ/h) of the ^2H label. The technique has the advantage over ^{13}C nuclear magnetic resonance (NMR) that no assumptions about bond lengths (which appear to the sixth power in ^{13}C relaxation studies) or relaxation mechanisms need to be made, since relaxation will always be quadrupolar, even for aromatic residues at high field. Asymmetry parameter (η) uncertainties are shown to cause negligible effects on τ_R determinations, and in any case it is shown that both e^2qQ/h and η may readily be determined in separate solid-state experiments. By way of example, we report ^2H NMR results on aqueous lysozyme (EC 3.2.1.17) at 5.2 and 8.5 T (corresponding to ^2H-resonance frequencies of 34 and 55 MHz). Interpretation of the results in terms of the isotropic rigid-rotor model yields e^2qQ/h values of $\approx$170 or $\approx$190 kHz, respectively, for the imidazolium and free-base forms of [ϵ_1-^2H]His-15 lysozyme in solution, in excellent agreement with e^2qQ/h values of $\sim$167 and $\sim$190 kHz obtained for the free amino acids in the solid state. In principle, the method may in suitable cases permit comparison between the dynamic structures of proteins in solution and in the crystalline solid state.

During the past decade there has been considerable interest in using nuclear magnetic resonance (NMR)[1] spectroscopic techniques to determine the rotational correlation times of proteins in solution. Early studies concentrated on the use of carbon-13 NMR, where, for sp^3 carbons having attached hydrogens (protonated carbons), the ^{13}C–^1H dipolar relaxation mechanism is expected to overwhelmingly dominate ^{13}C relaxation (Allerhand et al., 1971a,b). Accurate determination of ^{13}C spin–lattice relaxation times (T_1), spin–spin relaxation times (T_2), and nuclear Overhauser enhancements (NOEs) should thus provide the required correlation times through use of the appropriate theoretical relationships (Oldfield et al., 1975). Unfortunately however, it has been clear for some time that there are differences between correlation times (τ_R) obtained at different field strengths. Explanations involving anisotropic motion (Wilbur et al., 1976), longer, vibrationally distorted C–H bond lengths (Llinas et al., 1977; Dill & Allerhand, 1979), or librational motions (Howarth & Lilly, 1978; Howarth, 1979; Levy et al., 1981a,b) have all been investigated, the results indicating in general that longer bonds and/or librational motions could account for the discrepancies. The situation would be simplified, however, if the extreme (sixth-power) sensitivity of the observed T_1s and T_2s on the C–H bond length could somehow be dispensed with, since r_{CH} is never actually measured but is used essentially as a free parameter in most of the studies reported. In addition, determination of rotational correlation times would also be facilitated if the nature of the relaxation mechanism(s) was known with high accuracy. For aromatic ^{13}C nuclei at ultrahigh field ($\geq$12–14 T), relaxation contributions from both dipolar mechanisms (r_{CH}^6) and from chemical shift anisotropy (CSA) causes become significant. Thus, determination of τ_R (or, in general, any τ_i) will require knowledge not only of r_{CH} values but also of complete chemical shift tensors, i.e., σ_{11}, σ_{22}, and σ_{33} values, and their orientation. Since ^{13}C line widths are difficult to measure accurately (because they are generally small), NOEs for proteins are often small and have a shallow τ_R dependence, and because very few complete ^{13}C chemical shift tensors are available for amino acid side chains (Naito et al., 1981), ^{13}C NMR determinations of rotational correlation times are at best difficult.

In this paper, we try to indicate that ^2H NMR determinations of protein rotational correlation times will not suffer from most of the difficulties outlined above. Instead, (1) relaxation will almost always be quadrupolar, even for aromatic species at very high field (e.g., 14 T), and (2) unknown bond lengths do not appear in the relaxation expressions. The unknown parameter is the electric quadrupole coupling constant (e^2qQ/h). This may be determined from a combined T_1 and T_2 measurement for a species tumbling outside of the extreme narrowing limit ($\omega_0^2\tau_R^2 \gg 1$). It may also be determined (in principle) directly from the spectrum of the protein in the solid state, although, in general, this determination may best be carried out on a suitable solid amino acid.

Librational motion or anisotropic rotation etc. will also affect ^2H NMR measurements in much the same way as in ^{13}C NMR, but the elimination of r_{CH}^6 terms and possible multiple relaxation mechanisms by use of ^2H NMR, for $\omega_0^2\tau_R^2 \gg 1$, is shown to offer considerable promise for new, more accurate τ_R determinations. Our results are thus an extension of earlier

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[1] Abbreviations: NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; NOE, nuclear Overhauser enhancement; e^2qQ/h, electric quadrupole coupling constant; η, electric field gradient tensor asymmetry parameter; τ_R, rotational correlation time; CSA, chemical shift anisotropy; r_{CH}, carbon–hydrogen bond length; NMA, *N*-methyl[*N*-^2H]acetamide; EDTA, ethylenediaminetetraacetic acid.

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^{2}H NMR studies of proteins (Oster et al., 1975a,b; Wooten & Cohen, 1979) in which either only fast motions were observed ($\omega_0^2\tau_R^2 \ll 1$, Oster et al., 1975a,b) or where e^2qQ/h values were not determined (Wooten & Cohen, 1979).

Experimental Procedures

Nuclear Magnetic Resonance Spectroscopy. Deuterium Fourier-transform NMR spectra were obtained on two home-built spectrometers, which operate at 5.2 and 8.5 T, respectively (corresponding to ^{2}H resonance frequencies of 34 and 55 MHz). The high-field spectrometer consists of an 8.5-T, 3.5 in. bore high-resolution superconducting solenoid (Oxford Instruments, Osney Mead, Oxford, U.K.), together with a variety of digital and radio-frequency electronics. We used a Nicolet 1180 computer, 293B pulse programmer, and Model NIC-2090 dual-channel 50-ns transient recorder (Nicolet Instrument Corp., Madison, WI) for experiment control and rapid data acquisition, together with a dual Diablo Model 40 disc system for data storage (Diablo Systems, Inc., Haywood, CA). In order to generate high-power radio-frequency pulses (3 μs 90° pulse widths), we used an Amplifier Research (Souderton, PA) Model 200L amplifier to drive a retuned Henry Radio (Los Angeles, CA) Model 2006 transmitter to a ~1000–1500-W output power level. The deuterium resonance frequency was 55.273 MHz. Deuterium NMR spectra were recorded on this instrument using an 800-μL sample volume and with single-90° or 180°–τ–90° pulse sequences. Low-field spectra were obtained with the instrument described previously (Oldfield et al., 1978) by using Nicolet software package NTCFT. The 90° pulse width on this system was ~3–3.5 μs, more than adequate for the relatively narrow (≈1–2-kHz) line-width spectra observed.

Sample temperature was regulated either by means of a liquid nitrogen boil-off system or by using a heated air flow. The temperatures reported were measured with a calibrated Doric Trendicator (San Diego, CA) with a copper–constantan thermocouple, and the temperatures recorded are the gas-flow temperatures measured next to the sample. Separate experiments indicate that this temperature is accurate to ±1–2 °C over the entire sample volume. All samples had normal optical absorption spectra and enzymatic activity after NMR spectroscopy.

Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory.

Preparation of Deuterated Samples. (*A*) [ϵ_1-^{2}*H*]*His-15 Lysozyme* (*EC 3.2.1.17*). The synthesis of [ϵ_1-^{2}H]His-15 lysozyme (EC 3.2.1.17) was carried out by using basically the procedure of Wooten & Cohen (1979). Lysozyme (Boehringer Mannheim, GmbH) was exchanged at pH 9.0 in 99.7% ^2H_2O (Merck Sharp & Dohme, Montreal, Canada) at 37 °C in a sealed tube under nitrogen for approximately 1 week. The pH of the solution was then lowered to 2.6 by addition of HCl, followed by lyophilization. To ensure complete back-exchange of all amide protons, the lyophilized protein was then taken up in ^{2}H-depleted water (Aldrich Chemical Co., Milwaukee, WI) and subjected to a temperture of 80 °C for 2 min (Campbell et al., 1975). To remove residual D_2O, the sample was then twice lyophilized, again from ^{2}H-depleted water.

(*B*) [*amide*-^{2}*H*]*Lysozyme.* The procedure for perdeuterioamidolysozyme was basically as above except instead of an exhaustive back-exchange final step, the sample was only lyophilized twice from ^{2}H-depleted H_2O, using 1-h room temperature exchanges.

(*C*) [ϵ_1-^{2}*H*]*Histidine Hydrochloride* (*His-HCl*). L-Histidine hydrochloride (Sigma Chemical Co., St. Louis, MO) was exchanged at pH 7.0 in 99.7% ^2H_2O at 80 °C for 2 days (Matthews et al., 1977). The pH of the solution was then lowered to 2.6 and the sample lyophilized. Crystals of pure [ϵ_1-^{2}H]histidine hydrochloride were obtained from the deuterated material basically according to Greenstein & Winitz (1961).

(*D*) [ϵ_1-^{2}*H*]*Histidine* (*His*). Pure crystals of [ϵ_1-^{2}H]histidine hydrochloride, 1.0 g, were dissolved in 12 mL of H_2O by warming. The cooled solution was then treated with 6 mL of 1.0 N LiOH, followed by the addition of absolute ethanol to 80% concentration to precipitate the white crystals of [ϵ_1-^{2}H]histidine.

(*E*) *N-Methyl*[*N*-^{2}*H*]*acetamide* (*NMA*). *N*-Methyl[*N*-^{2}H]acetamide was synthesized by refluxing *N*-methylacetamide (Aldrich) at pH 2.0 in 99.7% ^2H_2O for 24 h, followed by lyophilization.

Isotopic purities of all deuterated species were established with ^{1}H NMR spectroscopy at 220 (Varian HR-220) or 360 MHz (Nicolet NT-360), the ^{2}H labeling being in all cases ~90 ± 5% complete. Purities of histidine, histidine hydrochloride, and *N*-methylacetamide were all checked by elemental analysis and melting-point determinations. Lysozyme concentrations were estimated spectrophotometrically at 280 nm with an extinction coefficient of 38 500 M^{-1} cm^{-1} (Sophianopoulos et al., 1962). Enzymatic activity was determined as the rate of lysis of *Micrococcus lysodeikticus* cells (Shugar, 1952). The buffer used was 0.1 M potassium phosphate, pH 7.0. *M. lysodeikticus* cells (Sigma), 9 mg of dried cells, were suspended in 55 mL of buffer solution. The protein solution was diluted prior to assay to a concentration of 150–500 units/mL, where 1 unit is equal to a decrease in turbidity of 0.001/min at 450 nm, pH 7.0, 25 °C. A high activity of enzyme is defined at ≳12 000 units/mg of protein. The His, His-HCl, and NMA samples were dried over P_4O_{10} prior to NMR spectroscopy.

Theory

As stated in the introduction, the accurate determination of ^{13}C T_1s, T_2s, and nuclear Overhauser enhancements should provide the required rotational correlation times through use of the appropriate theoretical relationships. If one assumes only dipolar relaxation and the applicability of the isotropic rigid-rotor model, then, as is now well-known (Allerhand et al., 1971a,b)

$$\frac{1}{T_1} = \frac{\hbar^2\gamma_C^2\gamma_H^2}{10r_{CH}^6}\chi_H \tag{1}$$

$$\frac{1}{T_2} = \frac{\hbar^2\gamma_C^2\gamma_H^2}{20r_{CH}^6}\left(\chi_H + 4\tau_R + \frac{6\tau_R}{1+\omega_H^2\tau_R^2}\right) \tag{2}$$

and

$$\text{NOE} = 1 + \frac{\gamma_H}{\gamma_C\chi_H}\left[\frac{6\tau_R}{1+(\omega_H+\omega_C)^2\tau_R^2} - \frac{\tau_R}{1+(\omega_H-\omega_C)^2\tau_R^2}\right] \tag{3}$$

where

$$\chi_H = \frac{\tau_R}{1+(\omega_H-\omega_C)^2\tau_R^2} + \frac{3\tau_R}{1+\omega_C^2\tau_R^2} + \frac{6\tau_R}{1+(\omega_H+\omega_C)^2\tau_R^2} \tag{4}$$

where $\hbar$ is Planck's constant, γ_C and γ_H are the gyromagnetic

ratios for ^{13}C and ^{1}H, and ω_C and ω_H are the Larmor frequencies for ^{13}C and ^{1}H. Unfortunately, accurate determinations of nuclear Overhauser enhancements and line widths are quite difficult at natural abundance, and ^{13}C labeling of proteins is also very difficult; consequently, the problem of finding τ_R has been underdetermined. Early studies all invoked r_{CH} values of 1.09 Å, but this value was later seen to generate problems in that τ_R values appeared field dependent (Wilbur et al., 1976; Llinas et al., 1977). This problem has been resolved in a number of ways, including changing r_{CH} from 1.09 to 1.11–1.15 Å, due to vibrational averaging (Diehl & Niederberger, 1973; Szeverenyl et al., 1976; Jackman & Trewella, 1976; Vold et al., 1977; Llinas et al., 1977; Dill & Allerhand, 1979), or by invoking or calculating various rapid librational motions (Howarth, 1979; Levy et al., 1981a,b). Since a number of models may explain the results observed, and there are other difficulties associated with analysis of ^{13}C relaxation data, e.g., the sixth-power dependence of T_1 on r_{CH}, and the observation that in many cases T_1 is only weakly dependent on τ_R due to the location of the T_1 minimum, we have investigated further the utility of ^{2}H NMR as a means of determining protein τ_R values. As noted previously, CSA contributions to relaxation become significant as the field strengths of NMR instruments are increased, but with ^{2}H, such contributions are never likely to be of any consequence, in contrast to the case with ^{13}C NMR.

For isotropic motion, we have (Abragam, 1961)

$$\frac{1}{T_1} = \frac{3\pi^2}{10}\left(\frac{e^2qQ}{h}\right)^2\left(1+\frac{\eta^2}{3}\right)\left[\frac{\tau_R}{1+\omega_0^2\tau_R^2}+\frac{4\tau_R}{1+4\omega_0^2\tau_R^2}\right] \quad (5)$$

and

$$\frac{1}{T_2} = \frac{3\pi^2}{20}\left(\frac{e^2qQ}{h}\right)^2\left(1+\frac{\eta^2}{3}\right)\times \left[3\tau_R+\frac{5\tau_R}{1+\omega_0^2\tau_R^2}+\frac{2\tau_R}{1+4\omega_0^2\tau_R^2}\right] \quad (6)$$

For the case of ^{2}H relaxation, there are three unknowns: the rotational correlation time, τ_R, the asymmetry parameter, η, and the quadrupole coupling constant, e^2qQ/h. Fortunately however, η values in aliphatic species are <0.04 (Wooten et al., 1979) while for aromatic species η values of about 0.05 ± 0.02 are common, so typical uncertainties in η in the range 0–0.05 can only have an ~0.1% effect on T_1 or T_2 and may therefore reasonably be neglected. The maximum effect of an uncertainty in η (η = 1) can in fact only cause a 25% change in T_1 or T_2, and such asymmetry parameters will not occur for C–^{2}H-labeled sites in rigid biopolymers. More importantly, we note that T_1 and T_2 depend only quadratically on e^2qQ/h rather than the sixth-power dependence on bond length (r_{CH}) found for dipolar relaxation, so that small (~5%) errors in e^2qQ/h determinations will be less significant than having similar errors in r_{CH}. In addition, as we indicate below, both e^2qQ/h and η may be determined in separate solid-state experiments, e.g., on crystalline amino acids or on frozen solutions, leading to independent determinations of these parameters.

The considerations discussed above indicate that accurate determination of protein rotational correlation times (τ_R) *and* electric quadrupole coupling constants (e^2qQ/h) should in principle be possible by using experimental T_1 and T_2 (or

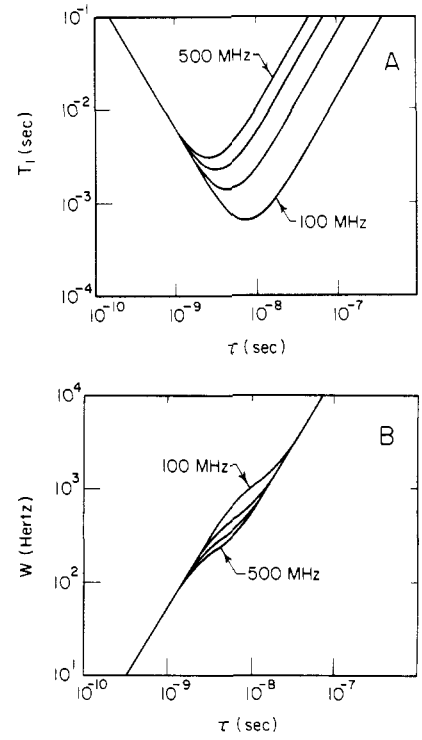

FIGURE 1: Theoretical plots of deuterium quadrupole relaxation behavior. (A) Spin–lattice relaxation times (T_1, s) and (B) line widths (Hz) vs. rotational correlation time (τ_R, s) for the case of a deuterium nucleus undergoing isotropic rotational motion and having a quadrupole coupling constant of 190 kHz and an asymmetry parameter η = 0.09. Values for magnetic field strengths of 2.36, 5.2, 8.5, and 11.7 T are shown, corresponding to ^{1}H resonance frequencies of 100, 220, 360, and 500 MHz or 15.4, 34.1, 55.3, and 77 MHz ^{2}H resonance frequencies. Only the "long" correlation times ($\tau_R \gg \omega_0^{-1}$) give good agreement between the experimental T_1 and line-width values for aqueous lysozyme (Table I).

line-width) results and ignoring asymmetry parameter effects, *but only outside the extreme narrowing limit.* For fast motions such that $\omega_0^2\tau_R^2 \ll 1$, eq 5 and 6 reduce to

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{3}{2}\left(\frac{e^2qQ\pi}{h}\right)^2\tau_R \quad (7)$$

in which case τ_R and e^2qQ/h cannot be determined simply by measurement of T_1 and T_2. This situation in the "extreme narrowing" limit is of course the same as with ^{13}C NMR.

However, in the limit $\omega_0^2\tau_R^2 \gg 1$, we may write expressions for the spin–lattice relaxation time (T_1) and the line width (W) in the following simplified forms:

$$\frac{1}{T_1} = \frac{3}{5}\left(\frac{e^2qQ\pi}{h\omega_0}\right)^2\frac{1}{\tau_R} \quad (8)$$

and

$$W = \frac{1}{\pi T_2} = \frac{9\pi}{20}\left(\frac{e^2qQ}{h}\right)^2\tau_R \quad (9)$$

Here, we find that $T_1 \neq T_2$ and both T_1 and the line width [$=1/(\pi T_2)$] are directly proportional to τ_R, as shown in Figure 1. Note also that due to the strong nature of the quadrupolar interaction, deuterium T_1 and T_2 relaxation in macromolecules is very effective and short T_2s (large line widths) and short T_1s are expected. Broad line widths, however, make possible relatively accurate T_2 measurements, since instrumental broadening factors are much less significant than in, for example, ^{13}C NMR line-width measurements (Oldfield et al., 1975). Since it is relatively easy to ^{2}H label a variety of sites

Table I: Deuterium NMR Spin-Lattice Relaxation Times (T_1), Line Widths, Rotational Correlation Times (τ_R), and Electric Quadrupole Coupling Constants (e^2qQ/h) for Deuterated Lysozymes[a]

protein	T_1 (ms)[c]	line width (Hz)[d]	τ_R (ns)[e]	e^2qQ/h (kHz)[f]
lysozyme, pH 4.2, 6 mM, 24 °C, 5.2 T[b]	3.9	664	14	168[g]
lysozyme, pH 4.2, 13 mM, 19 °C, 5.2 T[b]	4.6	831	17	172[g]
lysozyme, pH 7.2, 13 mM, 40 °C, 5.2 T[b]	4.4	1086	20	187[h]
lysozyme, pH 7.2, 13 mM, 22 °C, 5.2 T[b]	6.2	1693	30	194[h]
lysozyme, pH 4.4, 13 mM, 25 °C, 8.5 T[b]	12.0	830	17	174[g]
lysozyme, pH 4.1, 11 mM, 26 °C, 8.5 T[i]	5.5	950	13	219[j]

[a] Obtained by the Fourier-transform technique at the field indicated. The 90° pulse widths were both ≈3–3.5 μs. [b] Lysozyme was deuterated at H^{ϵ_1} of His-15 as described under Experimental Procedures. Spectra were obtained in ^2H-depleted water. [c] Obtained using a 180°–τ–90° pulse sequence. The accuracy is ±5–10%. From eq 5 and 6 (text). [d] Obtained from partially relaxed spectra in which the HO^2H signal is "nulled". Line width was estimated by means of a spectral simulation, and the error is about ±10%, on the basis of an average of between 2 and 4 PRFT spectra. [e] Correlation time required to fit experimental T_1 and line width results, assuming an asymmetry parameter $\eta = 0$. The values obtained by assuming the solid-state experimental values for e^2qQ/h and η are the same, to two significant figures. The estimated error on τ_R is about ±10–15%. [f] Quadrupole coupling constant required to fit experimental T_1 and line-width data, assuming the asymmetry parameter $\eta = 0$. The values obtained by assuming the solid-state experimental value for the asymmetry parameter are the same, to three significant figures. The estimated errors on e^2qQ/h are ~±5%. [g] The quadrupole coupling constant and asymmetry parameter for the model compound [ϵ_1-^{2}H]histidine hydrochloride were 167 ± 3 kHz and 0.00 ± 0.02, respectively. [h] The quadrupole coupling constant and asymmetry parameter for the model compound [ϵ_1-^{2}H]histidine were 190 ± 3 kHz and 0.09 ± 0.02, respectively. [i] Lysozyme was deuterated primarily at the backbone amide positions. [j] The quadrupole coupling constant and asymmetry parameter for the model compound *N*-methyl[*N*-^{2}H]acetamide were 210 ± 10 kHz and 0.10 ± 0.03, respectively, and for [*amide*-^{2}H]lysozyme 210 ± 10 kHz and 0.2 ± 0.05, respectively.

in proteins by chemical exchange and to thus obtain accurate T_1 and W measurements, eq 8 and 9 indicate that it should be relatively straightforward to solve for both τ_R and e^2qQ/h. In addition, as shown below, "cross-checks" of e^2qQ/h (and η) may be obtained by solid-state NMR methods, although here there is the potential problem of "structural" differences between crystal and solution species.

Results and Discussion

A typical line-width determination for a 13 mM, pH 4.4 sample of [ϵ_1-^{2}H]His-15 hen egg white lysozyme (EC 3.2.1.17) at 25 °C is shown in Figure 2A. In order to facilitate an accurate (10%) line-width estimate, we used a 180–τ–90° pulse sequence, with τ chosen to null the background HO^2H peak. In this case, a τ of 100 ms was used, and the protein line width was determined by means of a spectral simulation, Figure 2A. For this sample, at 55.3 MHz, W was found to be ~830 Hz (Figure 2A and Table I).

Spin–lattice relaxation times (T_1) were also determined with a 180°–τ–90° pulse sequence, and a PRFT data set on [*amide*-^{2}H]lysozyme and two typical recovery curves are shown in Figure 2B,C. The samples were pH 4, 13 mM, 25 °C [ϵ_1-^{2}H]His-15 lysozyme (T_1 = 12.0 ms, Figure 2C) and [*amide*-^{2}H]lysozyme (T_1 = 5.5 ms, Figure 2B,C). Both sets of data were recorded at 55.3 MHz (corresponding to a magnetic field strength of 8.5 T). The accuracy on the T_1 values is estimated to be ±5–10% and is, of course more accurate for the massively ^{2}H-labeled amide species. Numerical T_1 and W results for a variety of lysozyme samples at different pH values, field strengths, and temperatures are given in Table I.

For the neutral imidazole species of lysozyme, we obtained agreement with the experimental (T_1, T_2) results using the following parameters: for a pH 7.2, 13 mM, 40 °C sample, e^2qQ/h = 187 kHz and τ_R = 20 ns; for the same sample at 22 °C, e^2qQ/h = 194 kHz and τ_R = 30 ns. The average value of e^2qQ/h for the free-base (imidazole) form of [ϵ_1-^{2}H]His-15 lysozyme is thus considerably in excess of the ~170-kHz value found for the pH 4.2, imidazolium form, Table I. Support for this large e^2qQ/h value for the free-base form is obtained by examination of the solid-state ^{2}H NMR spectrum (at 8.5 T) of the corresponding free amino acid, [ϵ_1-^{2}H]histidine. We show in Figure 3 the solid-state ^{2}H NMR spectrum of [ϵ_1-^{2}H]histidine, obtained with the quadrupole-echo method (Davis et al., 1976), Figure 3A, together with its spectral simulation. From the simulation, Figure 3B, we find that e^2qQ/h = 190 kHz and η = 0.09 ± 0.02, in excellent agreement with the

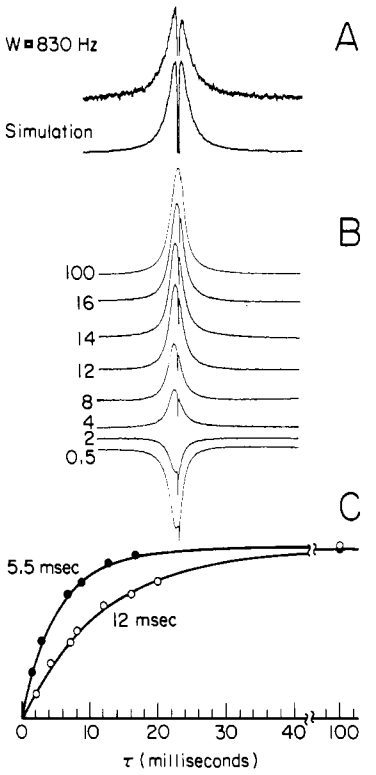

FIGURE 2: Experimental and theoretical line-width and spin–lattice relaxation time results for deuterated lysozymes. (A) 55.3 MHz partially relaxed Fourier-transform NMR spectrum, together with its spectral simulation, of [ϵ_1-^{2}H]His-15-labeled lysozyme (EC 3.2.1.17), 13 mM, pH 4.4, 25 °C, 8000 scans, ~4-μs 90° pulse width. The line width is 830 Hz (after correcting for instrumental broadening). The τ value used to null the HO[^{2}H] resonance was 100 ms. (B) 55.3 MHz partially relaxed Fourier-transform NMR spectra of [*amide*-^{2}H]lysozyme, 11 mM, pH 4.1, 26 °C, 10 000 scans, 3.7-μs 90° pulse width. The number to the left of each PRFT spectrum is τ, in milliseconds. (C) Computer-fitted recovery curves of ln ($A_\infty - A_\tau$) vs. τ (ms) for a 180°–τ–90° sequence for pH 4, 13 mM, 25 °C samples of [ϵ_1-^{2}H]His-15 lysozyme (T_1 = 12 ms) and [*amide*-^{2}H]lysozyme (T_1 = 5.5 ms), both at 55.3 MHz.

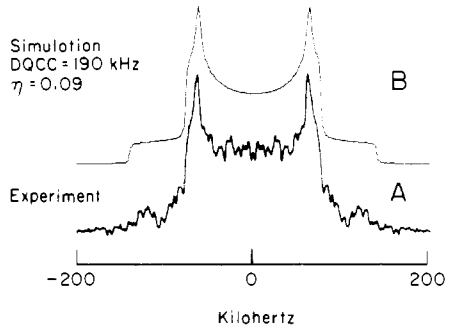

FIGURE 3: (A) Experimental deuterium Fourier-transform NMR spectrum (at 55.3 MHz) of [ϵ_1-^{2}H]histidine, in the solid state at ~25 °C with (B) spectral simulation having e^2qQ/h = 190 kHz, η = 0.09.

187–194-kHz range found in lysozyme in solution, Table I. If we had assumed η = 0.09 for the solution relaxation data, there would be no change in τ_R (Table I) for the reasons discussed above ($\eta^2/3 \rightarrow 0$).

The excellent agreement between the solution and solid-state results imparts some confidence in the use of the method. Even so, as may be seen from the results of Table I, the τ_R and e^2qQ/h values so deduced are still quite susceptible to small errors in T_1 or line-width measurement. For example, in the case of the pH 4.2, 19 °C and pH 7.2, 40 °C samples, the T_1 values are the same, within experimental error, while the line widths differ by only ≈25%. Accurate line-width (and T_1) data are thus, as expected, still critical for determination of accurate τ_R and e^2qQ/h values, and we must emphasize that in general the direct measurement of e^2qQ/h (and η) values in the system of interest, or an apropriate model compound, should be carried out. In addition, determination of T_1 and W for several values of concentration, temperature, operating frequency, etc. should also of course be carried out when the most reliable τ_Rs are required (Dill & Allerhand, 1979). The good agreement between amino acid crystal results and the solution e^2qQ/h values (Table I) nevertheless gives some considerable confidence in the present results, where, notably, we have not had to assume values for C–^{2}H bond lengths. As noted previously, a spread of ~40% in r_{CH}[6] values has been previously reported in the literature.

We should note at this point that similar values for τ_R of lysozyme have been obtained by using only ^{2}H NMR line-widths by other workers (Wooten & Cohen, 1979). These workers choose e^2qQ/h values of 168 kHz (η = 0.04) for the charged imidazolium species, by assuming sp^3 hybridization and using the literature data for CDH_2I (Wooten et al., 1979) and 192 kHz (η assumed ~ 0.04) for the neutral imidazole on the basis of a previous study of [1,3,5-$^2H_3$]benzene (Diehl & Reinhold, 1978). While it is likely that a low value of the deuteron quadrupole coupling constant is consistent with a reduction in the number of delocalized electrons, increasing the electronic contribution to the electric field gradient (Rinnē & Dēpireux, 1974), there seems to be no reason for choosing these numbers over the wide variety of others available (Rinnē & Dēpireux, 1974), the assumption of sp^3 hybridization for the imidazolium species being particularly suspect. Thus, the agreement between the two studies is quite fortuitous. In addition, T_1 and W measurements in our laboratory on samples prepared as described by Wooten & Cohen (1979), yielding similar line widths, have exhibited very short T_1s (Figure 2B and Table I), which are not due to paramagnetic impurities (as determined by similar measurements on samples containing EDTA). The resulting quadrupole coupling constants are ~215 kHz (Table I), consistent with an amide N–^{2}H species (Brevard & Kintzinger, 1978). The correlation times of

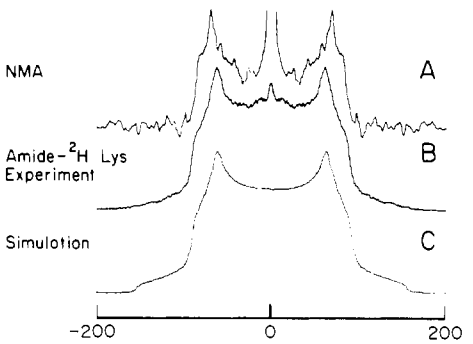

FIGURE 4: 55.3-MHz deuterium quadrupole echo Fourier-transform NMR spectra of (A) N-methyl[N-^{2}H]acetamide and (B) [*amide*-^{2}H]lysozyme, both in the crystalline solid state: 2000 scans, 2.7-μs 90° pulse widths, at ~25 °C for both spectra. (C) Spectral simulation of the lysozyme spectrum having e^2qQ/h = 210 kHz, η = 0.2.

samples prepared in this way are also consistent with those of the back-exchanged samples, Table I. The e^2qQ/h values obtained from the solution ^{2}H NMR results, which we believe arise from [*amide*-^{2}H]lysozyme, are within experimental error the same as those found for the model compound N-methyl-[N-^{2}H]acetamide, Figure 4A, where e^2qQ/h = 210 kHz and η = 0.1. The e^2qQ/h (and η) results are also in good agreement with the solid-state ^{2}H NMR spectrum of [*amide*-^{2}H]lysozyme, Figure 4B, and its spectral simulation, Figure 4C, where e^2qQ/h = 210 kHz and η = 0.2. All of these results are thus consistent with those of Campbell et al. (1975), who clearly show that lysozyme must be heated to ~80 °C to effect rapid amide proton exchange. The results on [*amide*-^{2}H]-lysozyme do support the applicability of the rigid-rotor model for lysozyme, since good agreement is obtained between the uniformly labeled and monosubstituted lysozyme and with solid- and solution-state NMR measurements.

Conclusions

The results presented above suggest that accurate protein rotational correlation times and deuterium electric quadrupole coupling constants may be determined for (irrotationally bound) ^{2}H-labeled sites of macromolecules in solution, outside of the extreme narrowing limit ($\omega_0^2\tau_R^2 \gg 1$), by combined measurment of spin–lattice relaxation times (T_1) and ^{2}H NMR line widths. The quadrupole coupling constants (e^2qQ/h) for [ϵ_1-^{2}H]histidine in the imidazole and imidazolium forms of His-15 lysozyme are in excellent agreement with those obtained using solid-state NMR on the crystalline amino acids. These results support the applicability of the (isotropic) rigid-rotor model for His-15 of lysozyme in solution, consistent with the presence of two hydrogen bonds to His-15 seen in the X-ray crystallographic structure (Blake et al., 1965). In contrast to ^{13}C NMR, the method does not suffer from uncertainties in bond lengths, relaxation mechanisms, or numbers of interacting spins although the method may be limited to proteins having molecular weights of ≤200 000 because of the difficulty in determining very broad lines (≥10–20 kHz).

There are, however, a number of points that can be raised in criticism of such τ_R determinations by ^{2}H NMR spectroscopy, including primarily the applicability of the isotropic rigid-rotor model and the accuracy of the experimental T_1 and T_2 values that are used (in eq 8 and 9) to solve for e^2qQ/h and τ_R. Both criticisms are, however, also applicable to τ_R determinations by ^{13}C (or ^{1}H) NMR spectroscopy. Indeed, it is clearly rather regrettable that, historically, the somewhat aspherical molecule lysozyme (a/b ratio ~2; Blake et al., 1965) has been used in so many correlation time determina-

tions, not only in NMR (Oldfield et al., 1975; Bauer et al., 1975; Wilbur et al., 1976; Dill & Allerhand, 1979; Howarth, 1979; Wooten & Cohen, 1979) but also in fluorescence depolarization (Irwin & Churchich, 1971; Rawitch, 1972) and inelastic light scattering (Dubin et al., 1971; Bauer et al., 1975). The effects of such anisotropic motion are potentially most severe when only a single-site label is used, since an unfortunate C–^{2}H vector orientation can seriously undermine the accuracy of the τ_R determination. That such anisotropic motion is *probably* unimportant in the present case is suggested by the good agreement between τ_Rs determined with the [ϵ_1-^{2}H]His-15 label and the presumably relatively uniformly ^{2}H-labeled amide species, Table I. It is also unfortunate that the more sensitive optical determinations are carried out at much lower concentrations than the NMR measurements, but it is conceivable that with even higher field instrumentation that ^{2}H NMR measurements will be feasible in proteins at a <1 mM concentration level.

Finally, we note that there will always be fast, small-amplitude thermal motions that may have an effect on relaxation. However, by combining molecular dynamic methods (Levy et al., 1981a,b) with ^{2}H NMR T_1 and T_2 determinations on proteins labeled at different sites, it should in the future be possible to obtain even more accurate rotational correlation times of proteins than are currently available, together with in some cases more accurate analyses of side-chain dynamics.

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Registry No. Lysozyme, 9001-63-2; [ϵ_1-^{2}H]His-HCl, 85479-43-2; [ϵ_1-^{2}H]His, 62595-10-2; NMA, 3669-70-3; L-histidine hydrochloride, 645-35-2; *N*-methylacetamide, 79-16-3; deuterium, 7782-39-0.

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