Variability of the ^{15}N Chemical Shift Anisotropy in *Escherichia coli* Ribonuclease H in Solution

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Abstract: The ^{15}N chemical shift anisotropy, $\Delta\sigma$, is reported for 81 well-ordered backbone amide sites in the *Escherichia coli* enzyme ribonuclease H. The values of $\Delta\sigma$ were determined from ^{15}N relaxation rate constants measured at static magnetic field strengths of 11.7, 14.1, and 18.7 T; the data analyzed included both autorelaxation rate constants and ^{1}H−^{15}N dipolar/^{15}N chemical shift anisotropy relaxation interference rate constants. For this data set, the values of $\Delta\sigma$ are approximately Gaussian distributed with a mean of -172 ± 13 ppm. The standard deviation of the site-to-site variation of the chemical shift anisotropy is 5.5 ppm and a 95% confidence limit for this variation is 9.6 ppm. The site-to-site variation in the chemical shift anisotropy is similar to the variation observed in solid state NMR studies of specifically labeled polypeptides. Variability in the value of $\Delta\sigma$ becomes a significant factor in the interpretation of spin relaxation rate constants at 18.7 T, but is less significant at lower field strengths, for the precision of relaxation data presently achievable by solution NMR. Ribonuclease H is the second protein for which an extensive set of $\Delta\sigma$ values is available. Comparison of results for ribonuclease H with results reported for well-ordered sites in ubiquitin (Fushman, D.; Tjandra, N.; Cowburn, D. *J. Am. Chem. Soc.* **1998**, *120*, 10947−10952) reveals differences primarily in the larger number of statistically significant values far from the mean in ubiquitin.

Introduction

A nuclear spin in a molecule is shielded from external magnetic fields by its local electronic environment. The magnitude and orientational dependence of the shielding is described by the nuclear chemical shift tensor.[1] Experimental or theoretical knowledge of chemical shift tensors is required for accurate interpretation of many nuclear magnetic resonance (NMR) experiments designed for investigations of molecular dynamics[2] and structure.[3] Relaxation rate constants for ^{15}N spins have been used extensively to characterize backbone dynamics in proteins.[4] Most commonly, a single value for the amide ^{15}N chemical shift anisotropy (CSA) is assumed for all spins even though the effects of conformation-dependent variations in the CSA have been discussed.[5,6] The range of variability expected for the ^{15}N chemical shift tensors in proteins has not been established definitively. Only relatively small databases of experimentally determined chemical shift tensors exist[7,8] and theoretical calculations, while qualitatively suggestive, have not achieved yet the requisite level of accuracy.[1] In the backbone of a polypeptide chain, the amide ^{15}N chemical shift tensor is influenced by local factors such as nearby dihedral angles, the presence of a hydrogen bond involving the covalently attached proton, and the presence of a hydrogen bond involving the oxygen of the adjacent carbonyl group; and long-range factors such as solvent effects and electrostatics.[1,3] Large variations exist in these structural parameters in proteins, which warrants an assessment of the variability in ^{15}N chemical shift tensors from site to site throughout the polypeptide backbone.

Until recently, the only method for determining ^{15}N chemical shift tensors in biological macromolecules was solid state NMR using samples specifically ^{15}N enriched at the site of interest.[9,10] Investigations of specifically labeled peptides suggest that amide ^{15}N chemical shift anisotropies in polypeptides have a narrow distribution with a standard deviation of 6.3 ppm (as calculated from Table 1 in Lee et al.[8]). Solid state NMR is prohibitively inefficient for application to a large number of ^{15}N sites in a protein due to the difficulties of producing the necessary samples. In a noteworthy advance, Fushman et al.[6] used ^{15}N spin relaxation rate constants measured by high-resolution solution NMR spectroscopy to determine the anisotropy, $\Delta\sigma$, of the ^{15}N chemical shift tensor at 63 backbone amide sites in the protein ubiquitin. This study reported an unexpectedly large spread in $\Delta\sigma$ between −125 ppm and −216 ppm, with a weighted mean value of $\langle\Delta\sigma\rangle = -157$ ppm.

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To further assess the variability of chemical shift tensors in proteins, the current study reports the chemical shift anisotropies for 81 well-ordered backbone amide ^{15}N nuclei in the *Escherichia coli* enzyme ribonuclease H (RNase H). The values of $\Delta\sigma$ are determined from the static magnetic field dependence of ^{15}N spin relaxation rate constants using an approach similar to that of Fushman et al.[6] The values of $\Delta\sigma$ are approximately Gaussian-distributed with a mean value of -172 ± 13 ppm. The standard deviation of the site-to-site variation of the CSA is estimated as 5.5 ppm, and a 95% confidence limit for this variation is 9.6 ppm. Assessment of protein dynamics using ^{15}N spin relaxation data requires accurate values of dipole−dipole and CSA coupling constants. For the level of precision in the ^{15}N spin relaxation rate constants achieved for RNase H, the degree of variability in $\Delta\sigma$ significantly affects the interpretation of spin relaxation data acquired at 18.7 T, but not at the lower fields, 14.1 and 11.7 T.

Materials and Methods

Experimental Procedures. High-resolution solution state measurements of ^{15}N longitudinal and transverse autorelaxation rate constants (R_1 and R_2, respectively) and ^{1}H−^{15}N heteronuclear cross relaxation rate constants (σ_{NH}) were determined at static magnetic field strengths of 11.7, 14.1, and 18.7 T. Longitudinal and transverse ^{15}N CSA/^{15}N−^{1}H dipolar interference relaxation rate constants (η_z and η_{xy}, respectively) were determined at static magnetic field strengths of 11.7 and 14.1 T. The sample was 0.8 mM (99% ^{15}N, 97% ^{2}H) RNase H (100 mM $NaCO_2CD_3$, 1 mM dithiothreitol-d_{10}, 2 mM NaN_3, 90%/10% H_2O/D_2O, pH = 5.5), prepared as described previously.[11] All NMR spectroscopy was performed using Varian Inova NMR spectrometers equipped with triple-resonance three-axis gradient probeheads. Sample temperatures were calibrated to 300 K using a 100% methanol sample. Relaxation rate constants were measured using standard experimental protocols; σ_{NH} was determined from the $\{^1H\}-^{15}N$ steady-state NOE.[11,12] Experimental uncertainties in resonance intensities were estimated from duplicate NMR spectra; uncertainties in fitted R_1, R_2, η_z, and η_{xy} relaxation rate constants were obtained using the jackknife procedure;[13] and uncertainties in σ_{NH} were obtained by error propagation.

Data Analysis. The set of measurements performed for RNase H consists of autorelaxation rates at three magnetic field strengths, and transverse and longitudinal interference relaxation rates at two magnetic field strengths. The data set used by Fushman et al.[6] consisted of autorelaxation rates and transverse cross relaxation rates obtained at three magnetic field strengths, but the longitudinal interference relaxation rate constant was not measured. As a result, the two studies employ different methods of data analysis to determine the ^{15}N CSA. In the analysis of the RNase H data, linear combinations of relaxation rate constants that depend only on the value of the spectral density function, $J(\omega)$, at zero frequency are defined using reduced spectral density mapping assuming that $J(\lambda\omega_H) \propto (\lambda\omega_H)^{-2}$ for $\lambda \approx 1$.[14,15] Assuming that the ^{15}N chemical shift tensor is axially symmetric and overall rotational motion of the protein is isotropic, the autorelaxation rate constants can be expressed as[14,16]

$$\Gamma_{auto} = R_2 - 0.5R_1 - 0.454\sigma_{NH} = (3d^2 + 4c^2)J(0)/6 + R_{ex} \quad (1)$$

and the interference relaxation rate constants as[11]

$$\Gamma_{cross} = (\eta_{xy}/\eta_z - 0.5)(R_1 - 1.249\sigma_{NH}) = (3d^2 + 4c^2)J(0)/6 \quad (2)$$

in which $d = (\mu_0/4\pi)\hbar\gamma_N\gamma_H\langle r_{NH}^{-3}\rangle$, $c = \omega_N\Delta\sigma/3^{1/2}$, μ_0 is the permeability of free space, $\hbar$ is Planck's constant divided by 2π, γ_X is the gyromagnetic ratio for nucleus X, $\langle r_{NH}^{-3}\rangle^{-1/3} = 1.02$ Å is the vibrationally averaged effective N−H bond length, ω_N is the ^{15}N Larmor frequency, $\Delta\sigma$ is the ^{15}N chemical shift anisotropy, and R_{ex} is the contribution to R_2 from chemical exchange. In the following, exchange is assumed to be fast on the NMR chemical shift time scale. Solid-state NMR studies[7,8] have shown that the ^{15}N chemical shift tensors in peptide groups deviate from axial symmetry. In addition, previous studies[11] have shown that rotational diffusion for RNase H is not isotropic. However, as will be discussed below, these deviations lead to small effects on the measured $\Delta\sigma$ values when compared to the experimental uncertainties.

In the case of negligible R_{ex} contributions to R_2, $\Gamma_{auto} = \Gamma_{cross} = \Gamma$, and

$$\Gamma = (2/9)\Delta\sigma^2 J(0)\omega_N^2 + (1/2)d^2 J(0) = m\omega_N^2 + b \quad (3)$$

In eq 3, Γ is linear in ω_N^2 and has slope $m = 2\Delta\sigma^2 J(0)/9$ and intercept $b = (1/2)d^2 J(0)$. The values of m and b are determined by linear least squares optimization, the absolute value of $\Delta\sigma$ is given by

$$|\Delta\sigma| = (3d/2)(m/b)^{1/2} \quad (4)$$

and $\Delta\sigma$ is known to be negative from other measurements.[7,8] If R_{ex} contributes significantly to R_2

$$\Gamma_{cross} = (2/9)\Delta\sigma^2 J(0)\omega_N^2 + (1/2)d^2 J(0) = m_{cross}\omega_N^2 + b$$

$$\Gamma_{auto} = [(2/9)\Delta\sigma^2 J(0) + \Theta]\omega_N^2 + (1/2)d^2 J(0) = m_{auto}\omega_N^2 + b \quad (5)$$

In eq 5, Γ_{cross} is linear in ω_N^2 with slope $m_{cross} = m$. Γ_{auto} also is linear in ω_N^2; however, the slope $m_{auto} = m_{cross} + \Theta$ contains contributions from $R_{ex} = \Theta\omega_N^2$, in which Θ depends on the rate of interconversion between magnetically distinct sites, the difference in populations of the various sites, and the method used to determine R_2.[2] Thus, the presence of a significant R_{ex} contribution to R_2 results in $m_{auto} > m_{cross}$, which can be detected by comparing linear least-squares fits of eq 3 and 5 to the Γ_{auto} and Γ_{cross} data.

The method described here differs from that of Fushman et al.,[6] in which the ratios R_2/η_{xy} are analyzed as a function of magnetic field strength. This ratio is given by[6]

$$\frac{\omega_N R_2}{\eta_{xy}} = \frac{3d^2 + 4c^2}{\epsilon} = (3d^2/\epsilon) + (4\Delta\sigma^2/3\epsilon)\omega_N^2 = m_{ratio}\omega_N^2 + b_{ratio} \quad (6)$$

in which $\epsilon\ \omega_N$ is the relaxation interference coupling constant.[17] In the case of isotropic molecular rotation and an axially symmetric ^{15}N chemical shift tensor oriented with its principal axis at an angle β with respect to the N−H bond vector, ϵ reduces to $-2(3)^{1/2}cd(3\cos^2\beta - 1)$.[17] After determining m_{ratio} and b_{ratio}, from eq 6, $|\Delta\sigma|$ is determined from eq 4 by substituting m_{ratio} and b_{ratio} for m and b, respectively. Clearly the value of $\Delta\sigma$ obtained from eqs 4 and 6 is independent of ϵ. Thus, both eqs 3 and 6, in conjunction with eq 4, determine $\Delta\sigma$ from the field dependence of R_2, and the differences between $\Delta\sigma$ values determined using these two methods are expected to be minor. Which method of analysis should be applied in a given case depends primarily upon which relaxation parameters are available. Very recently, Damberg et al.[18] performed an analysis analogous to eqs 3 and 4 to characterize a ^{13}C chemical shift tensor for the Tyr C$^\delta$ spin from the field dependence of R_1.

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As a practical point, the fractional dependence of R_2 on variation in $\Delta\sigma$ is given by

$$\frac{\delta R_2}{R_2} = \left(\frac{8c^2}{3d^2 + 4c^2}\right)\frac{\delta\Delta\sigma}{\Delta\sigma} \tag{7}$$

For example, if $\Delta\sigma = -172$ ppm and $\delta\Delta\sigma = 5$ ppm (vide infra), then the fractional variation $\delta R_2/R_2$ due to the variation in $\Delta\sigma$ is 1.2% at 500 MHz and 2.3% at 800 MHz; consequently extremely high precision data are required to measure small site-to−site conformational variations in $\Delta\sigma$.

In studies of protein dynamics by ^{15}N NMR spectroscopy, relaxation rate constants are interpreted using either the model-free formalism[19,20] or reduced spectral density mapping.[14,15] In the latter approach, values of $J(0)$, $J(\omega_N)$, and $J(0.87\ \omega_H)$ are calculated as follows:

$$J(0) = 6\Gamma_{auto}/(3d^2 + 4c^2) \tag{8}$$

$$J(\omega_N) = 4(R_1 - 1.249\sigma_{NH})/(3d^2 + 4c^2) \tag{9}$$

$$J(0.87\omega_H) = 4\sigma_{NH}/(5d^2) \tag{10}$$

in which either a single average value of $\Delta\sigma$ is used for all residues, as has been historical practice, or $\Delta\sigma$ values determined by the methods outlined above are used for individual residues. Alternatively, when data are available at multiple static magnetic fields, $J(0)$ and $J(\omega_N)$ can be determined as

$$J(0) = 2b/d^2 \tag{11}$$

$$J(\omega_N) = 4b(R_1 - 1.249\sigma_{NH})/(3d^2\Gamma_{auto}) \tag{12}$$

without the intermediate step of first determining site specific values of $\Delta\sigma$. For residues subject to exchange broadening, Γ_{cross}, rather than Γ_{auto}, must be used in eqs 8 and 12. If individual values of $\Delta\sigma$ are used in eqs 8 and 9 or if eqs 11 and 12 are used, uncertainties in the spectral density values are obtained by Monte Carlo simulation to account for covariance between m and b and the relaxation rate constants.

Results

RNase H has 149 backbone amide groups, however, resonance overlap prevented measurement of the relaxation rates for 26 residues. Thirty sites exhibit extensive internal motion on ps−ns time scales as evidenced by a $\{^1H\}-^{15}N$ steady-state nuclear Overhauser enhancement <0.7 at 11.7 T. For these spins, the approach for determining $\Delta\sigma$ embodied in eqs 1−4 is not applicable because the assumption that $J(\lambda\omega_H) \propto (\lambda\omega_H)^{-2}$ may not be valid. In addition internal motions may render $\Delta\sigma$ a time dependent quantity,[21,22] which complicates determination of the CSA. Furthermore, internal motions complicate assessments of the effects of asymmetry of the CSA tensor and rotational diffusion anisotropy (vide infra). Therefore, these 30 mobile sites were excluded from further analysis. Using eqs 1 and 2, three independent Γ_{auto} measurements and two independent Γ_{cross} measurements were obtained for the remaining 93 amide ^{15}N spins. The mean relative uncertainties were 1.6% for Γ_{auto} and 3.7% for Γ_{cross}. After the initial analysis of the Γ values using eq 3, the sum-squared-residuals were larger than expected statistically, presumably because the uncertainties in individual Γ_{auto} and Γ_{cross} values determined at a single static magnetic field strength do not include any contribution from experiment-to-experiment variability. To avoid systematically underestimating the uncertainties in m and b, the data were analyzed again after scaling the uncertainties in all Γ values by an empirical factor of 1.7. This procedure resulted in a distribution of sum-of-squared residuals for fits of eq 3 that closely matched the quartiles of a χ^2-distribution with three degrees of freedom. After scaling, the mean relative uncertainties were increased to 2.7% for Γ_{auto} and 6.3% for Γ_{cross}. The scaled uncertainties were used for the final analyses.

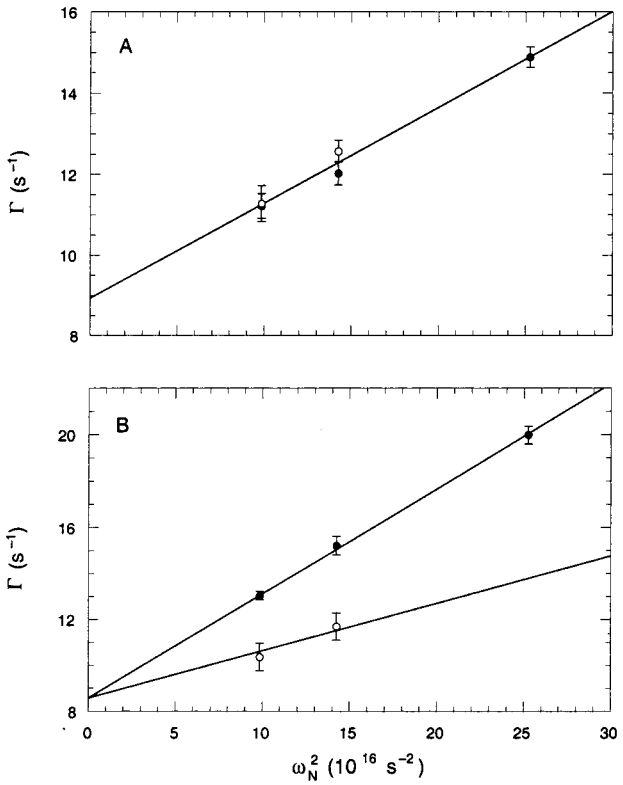

Figure 1. Magnetic field dependence of ^{15}N spin relaxation. Values of Γ_{auto} (●) and Γ_{cross} (○) for (A) Arg 106 and (B) Trp 90 are plotted versus ω_N^2. Solid lines represent linear least-squares fits to (A) eq 3 and (B) eq 5. The two-parameter fit for Arg 106 yields $\Delta\sigma = -173 \pm 12$ ppm.

Incommensurate Γ_{auto} and Γ_{cross} data were identified by comparing two-parameter fits using eq 3 and three-parameter fits of eq 5 to the five Γ data points. In the two-parameter fits, the data were analyzed simultaneously to obtain m and b. In the three-parameter fits, individual slopes, m_{auto} and m_{cross} were determined together with a common intercept b. F-statistical testing at a confidence level $\alpha = 0.05$ was used to identify sites for which eq 5 with $m_{auto} \neq m_{cross}$ provided an improved fit compared with eq 3. Eight sites were excluded because $m_{auto} > m_{cross}$, as predicted if $R_{ex} > 0$. Four other sites, three of which are glycines, were excluded because $m_{auto} < m_{cross}$. The cause of this unexpected behavior is unknown, but appears to affect a restricted set of residues. A plot of Γ versus ω_N^2 for residue Arg 106 is shown in Figure 1a. For comparison, a plot of Γ versus ω_N^2 for Trp 90, one of the eight residues for which $\Gamma_{auto} > \Gamma_{cross}$ that has previously been identified as being subject to conformational exchange broadening,[11,23] is shown in Figure 1b.

The final data set consists of 81 residues: 41 sites from α-helical regions of the protein, 28 sites from β-strands, and 12 sites from loops or termini. A plot of the 162 Γ_{cross} values

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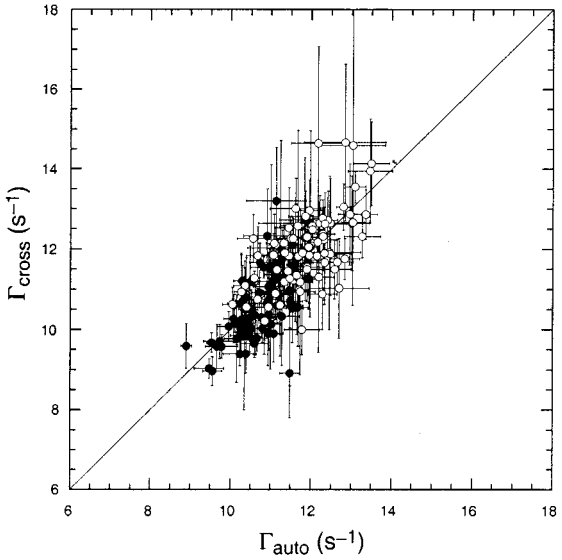

Figure 2. Agreement between Γ_{auto} and Γ_{cross}. Values of Γ_{cross} are plotted versus Γ_{auto} at static magnetic field strengths 11.7 T (●), and 14.1 T (○) for the 81 ^{15}N sites used for CSA determination. The uncertainties displayed are scaled by an empirical factor of 1.7, as described in the text.

versus the corresponding Γ_{auto} values obtained from data recorded at 11.7 and 14.1 T is shown in Figure 2. To measure the degree of similarity between the two data sets, a χ^2 statistic for fitting the data to a line of slope unity and intercept zero is defined as

$$\chi^2 = \sum (x_i - y_i)^2/(dx_i^2 + dy_i^2) \qquad (13)$$

in which dx_i and dy_i are the uncertainties in x and y dimensions, respectively. For Figure 2, $\chi^2 = 165$, and the p value is 0.42, indicating that Γ_{auto} and Γ_{cross} values are statistically indistinguishable. The observed equality between Γ_{auto} and Γ_{cross} confirms previous conclusions,[11] that significant systematic errors in the relaxation data are absent. For the selected 81 ^{15}N spins, Γ values were fit using eq 3, $\Delta\sigma$ was determined from m and b using eq 4, and the uncertainties in $\Delta\sigma$, denoted s, were determined by Monte Carlo simulations to account for covariance between m and b.

The values of the ^{15}N $\Delta\sigma$ for RNase H are shown as a function of amino acid sequence in Figure 3a. The weighted mean is $\langle\Delta\sigma\rangle = -172$ ppm with a weighted uncertainty (not standard error) $\langle s\rangle = 13$ ppm. The median $\Delta\sigma$ and uncertainty values of -173 and 13 ppm agree with the weighted values, indicating that the weighted statistics are not dominated by a small number of outlying data points that deviate significantly from average values. The standard deviation in site-to-site variability in $\Delta\sigma$, denoted Λ, was estimated from the distribution of the standardized variable $Z = (\Delta\sigma - \langle\Delta\sigma\rangle)/s$, as shown in Figure 3b. This distribution is approximately Gaussian with a standard deviation of $\sigma_Z = 1.09$, and using a large-sample test,[24] $\sigma_Z < 1.25$ with 95% confidence. The increase in σ_Z above unity reflects either an underestimation of s, or significant site-to-site residue-specific variation in the chemical shift tensor. Assuming that the experimental uncertainties and the site-to-site variation are independent, $\sigma_Z{}^2 = 1 + (\Lambda/\langle s\rangle)^2$. Consequently, $\Lambda = 5.5$ ppm is obtained for $\sigma_Z = 1.09$, and $\Lambda < 9.6$ ppm with 95% confidence.

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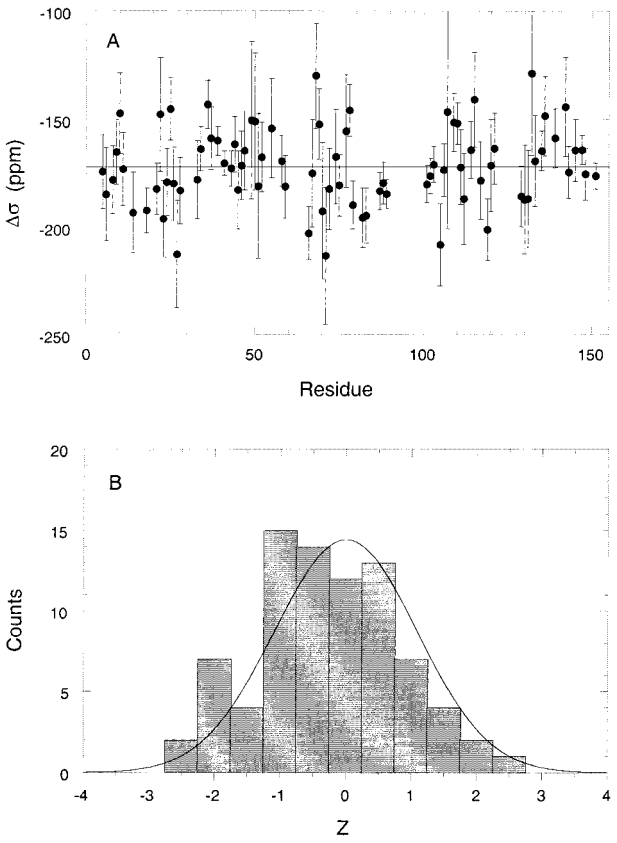

Figure 3. ^{15}N chemical shift anisotropy for RNase H. (A) Values of $\Delta\sigma$ are plotted versus residue number. The horizontal solid line is drawn at the position of the weighted mean value of $\langle\Delta\sigma\rangle = -172$ ppm. Residues in secondary structure elements are β1:4−13, β2:18−27, β3:32−42, α1:43−58, β4:64−69, α2:71−80, α3:81−88, α4:100−112, β5:115−120, α5:127−142. (B) Distribution of the standardized values $Z = (\Delta\sigma - \langle\Delta\sigma\rangle)/s$. The solid line represents a Gaussian distribution with a mean of 0 and a standard deviation of 1.09.

Table 1. χ^2 Values Comparing $J(0)$ Values Calculated from Average versus Individual $\Delta\sigma$ Values[a]

uncertainty in $\langle\Delta\sigma\rangle$	magnetic field strength (T)		
	11.7	14.1	18.7
0 ppm	38.2 ($p > 0.99$)[b]	60.4 ($p = 0.96$)	138.2 ($p < 0.001$)
5.5 ppm	37.7 ($p > 0.99$)	54.7 ($p = 0.99$)	108.8 ($p = 0.022$)
9.6 ppm	31.8 ($p > 0.99$)	46.4 ($p > 0.99$)	79.5 ($p = 0.53$)

[a] χ^2 values are calculated from eq 13 [b] p values are calculated from a χ^2 distribution with 81 degrees of freedom. p values <0.05 reflect >95% confidence that the relationship between data pairs is not described by a line of slope unity and intercept zero.

To assess the effect of site-to-site variability in $\Delta\sigma$ on the interpretation of spin relaxation rates, Figure 4 compares $J(0)$ values obtained using an average $\langle\Delta\sigma\rangle$ of -172 ppm to those obtained using the individual residue-specific $\Delta\sigma$ values. In all cases, $J(0)$ was determined from autorelaxation rates using the reduced spectral density mapping method given in eqs 8−10. To measure the degree of similarity between the sets of $J(0)$ values, χ^2 values were determined using eq 13, and are reported in the first row of Table 1. For magnetic field strengths of 11.7 and 14.1 T, χ^2 values correspond to $p > 0.95$, which suggests that $J(0)$ values calculated using $\langle\Delta\sigma\rangle$ and values calculated using residue-specific $\Delta\sigma$ values are indistinguishable. The χ^2 observed for the data collected at 18.7 T, however, indicates a significant difference between $J(0)$ values derived from the individual $\Delta\sigma$ values and from $\langle\Delta\sigma\rangle$. At currently achievable levels of precision in the relaxation rate constants, this discrep-

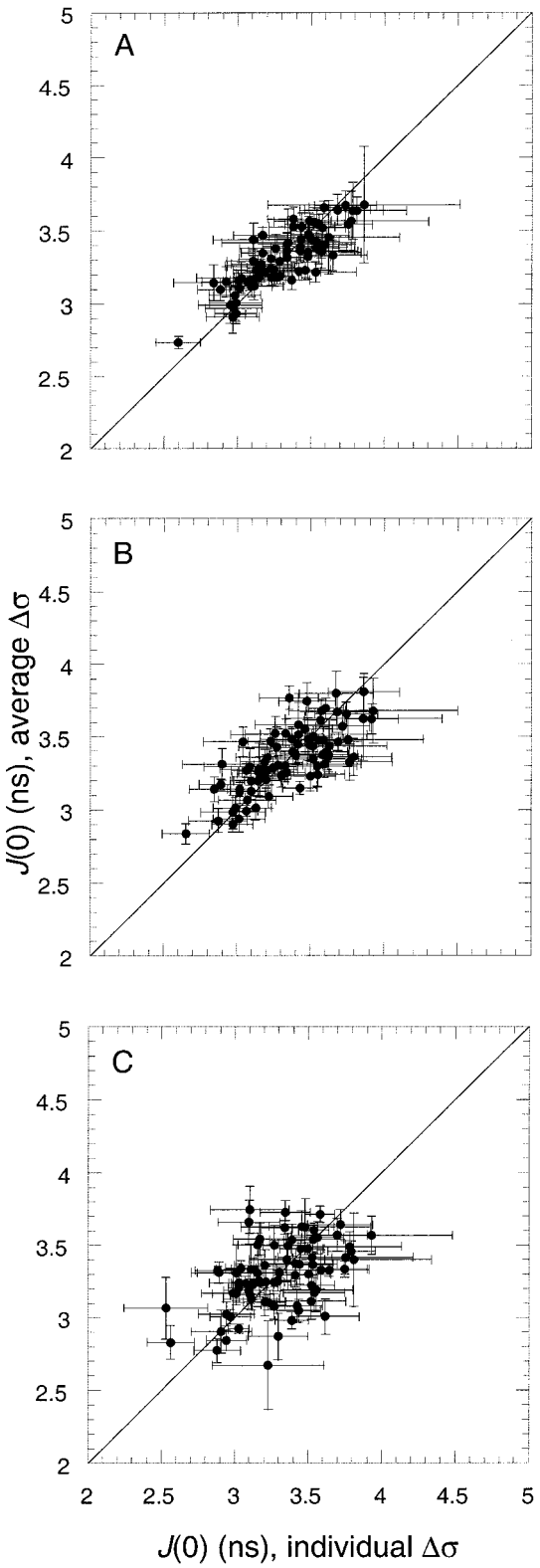

Figure 4. Comparison of $J(0)$ values determined using average versus individual $\Delta\sigma$ values. $J(0)$ is calculated using reduced spectral density mapping approach of eqs 8−10[14,15] and either an average $\Delta\sigma$ of −172 ppm or the individual values of $\Delta\sigma$ presented in Figure 3. χ^2 values for a fit to a line of slope unity and zero intercept are (A) 38.2 ($p >$ 0.99) at 11.7 T, (B) 60.4 ($p = 0.96$) at 14.1 T, and (C) 138.2 ($p <$ 0.001) at 18.7 T.

ancy arises only for the 18.7 T data because the CSA interaction provides a more efficient relaxation mechanism at higher magnetic fields. The variability in $\Delta\sigma$ must therefore be taken in to account when calculating internal dynamics parameters such as $J(0)$ using data acquired at field strengths >14.1 T. One drawback to determining $J(0)$ using individual $\Delta\sigma$ values, however, is that the precision in $J(0)$ suffers from propagating the error in $\Delta\sigma$ if, as in the present case, $s > \Lambda$. Thus, using individual $\Delta\sigma$ values potentially yields more accurate, but lower precision values of $J(0)$, while using $\langle\Delta\sigma\rangle$ for all ^{15}N sites generates higher precision, but biased, values of $J(0)$. An alternative method consists of treating the site-to-site variability, Λ, as a random uncertainty in $\langle\Delta\sigma\rangle$. In doing this, errors of magnitude Λ, rather than s, are propagated into $J(0)$ estimates. In essence, this approach reduces the precision in $J(0)$ only enough to make the systematic bias smaller than the uncertainty in $J(0)$. Table 1 gives χ^2 values comparing $J(0)$ values determined using individual $\Delta\sigma$ values to $J(0)$ values obtained assuming the average $\langle\Delta\sigma\rangle = -172 \pm 5.5$ ppm, and -172 ± 9.6 ppm. χ^2 for data collected at 18.7 T becomes acceptable when an uncertainty in $\langle\Delta\sigma\rangle$ between 5.5 and 9.6 ppm is used, in agreement with the analysis of site-to-site variability in $\Delta\sigma$. An additional assessment of the effect of variability of $\Delta\sigma$ is obtained by comparing values of $J(0)$ calculated at 11.7 and 18.7 T using $\langle\Delta\sigma\rangle = -172$ ppm, as shown in Figure 5. If an uncertainty of zero is assumed for $\langle\Delta\sigma\rangle$, $\chi^2 = 93.9$ ($p = 0.15$) is slightly larger than expected, but assuming an uncertainty for $\langle\Delta\sigma\rangle$ of 5.5 or 9.6 ppm reduces the χ^2 to acceptable values of 54.9 ($p = 0.99$) or 32.5 ($p > 0.99$).

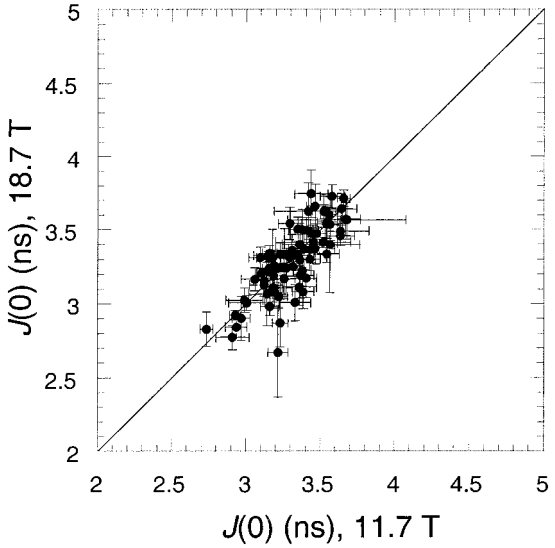

Figure 5. Comparison of $J(0)$ values determined at 18.7 T versus 11.7 T using an average $\langle\Delta\sigma\rangle$ of −172 ppm and no uncertainty. The χ^2 for a fit to a line of slope unity and zero intercept is 93.9 ($p = 0.15$).

Discussion

The values of $\Delta\sigma$ have been determined for 81 backbone ^{15}N sites in RNase H from the magnetic field dependence of spin relaxation rates in the liquid state. The weighted mean $\langle\Delta\sigma\rangle$ is −172 with a weighted uncertainty of $\langle s\rangle = 13$ ppm. Although this number is slightly more negative than the value reported in a similar study,[6] it is consistent with other liquid-state NMR investigations of polypeptide backbone ^{15}N CSA values.[8,25,26] The observed distribution of $\Delta\sigma$ values was partitioned into a component from experimental uncertainty and a component that arises due to site-to-site variation. The estimated standard

(25) Tjandra, N.; Wingfield, P.; Stahl, S.; Bax, A. *J. Biomol. NMR* **1996**, *8*, 273−84.
(26) Ottiger, M.; Tjandra, N.; Bax, A. *J. Am. Chem. Soc.* **1997**, *119*, 9, 9825−9830.

deviation in $\Delta\sigma$ due to site-to-site variability is $\Lambda = 5.5$ ppm, with a 95% confidence limit of 9.6 ppm. As shown by eq 4, the absolute values of $\Delta\sigma$ depend on the value of the N−H bond length used to calculate d. Increasing the bond length by 0.01−0.02 Å[27] reduces the values of $\Delta\sigma$, and the estimates of Λ, by 3−6%. The value of Λ obtained for RNase H is in good agreement with solid-state NMR studies; for example, the standard deviation of $\Delta\sigma$ values compiled in Table 1 of Lee et al.[8] is 6.3 ppm.

The derivations leading to eq 4 assume that the ^{15}N chemical shift tensor has axial symmetry and that RNase H has an isotropic diffusion tensor. For an asymmetric tensor, values of $\Delta\sigma$ are overestimated approximately by a factor $(1 + \eta^2/3)^{1/2}$, in which η is the asymmetry parameter of the tensor.[17] For peptide amide ^{15}N spins, η values typically are ≤ 0.4 with an average value of $\eta = 0.22$;[7,8] consequently, this error is ≤ 5 ppm with an average value of 1.4 ppm. If the angle between the N−H bond vector and the symmetry axis of the CSA tensor is $\beta \neq 0$, then the $J(0)$ terms appearing in eqs 1−3 differ for the dipolar and CSA interactions in a manner that depends on the anisotropy of the molecular rotational diffusion tensor.[28,29] For an axially symmetric rotational diffusion tensor with $0.5 < D_{||}/D_{\perp} < 2$, the values of $J(0)$ for the CSA and dipolar interactions for well-ordered amide moieties can be determined using the local diffusion approach.[30,31] In this case, the apparent local diffusion constant is given by $D_{iso}[1 - \kappa P_2(\cos\theta_i)]$ in which D_{iso} is $^1/_3$ the trace of the diffusion tensor, $\kappa = (D_{||}/D_{\perp} - 1)/(D_{||}/D_{\perp} + 2)$, and θ_i is the angle between the symmetry axis of the tensor for the ith relaxation mechanism and the symmetry axis of the diffusion tensor. Thus, values of $\Delta\sigma$ corrected for noncollinearity of the CSA and dipole symmetry axes are obtained by multiplying values determined using eq 4 by the factor

$$\xi = \left[\frac{1 - \kappa P_2(\cos\theta_{CSA})}{1 - \kappa P_2(\cos\theta_{DD})}\right]^{1/2} \qquad (14)$$

in which θ_{CSA} and θ_{DD} refer to the CSA and dipolar tensor orientations, respectively. Rotational diffusion of RNase H is described by an axially symmetric diffusion tensor, with $D_{||}/D_{\perp} = 1.19$.[11] Values of θ_{DD} were determined using the atomic coordinates for RNase H from the PDB file 1rnh.[32] In determining θ_{CSA}, the symmetry axis of the CSA tensor was assumed to be located in the plane of the peptide group and rotated by $\beta = 17°$ toward the C′ atom.[29] Values of ξ calculated using eq 14 ranged from 0.98 to 1.02, corresponding to corrections in $\Delta\sigma$ ranging from −3.5 to +3.5 ppm. The corrected values of $\Delta\sigma$ yielded $\langle\Delta\sigma\rangle = -172$ ppm and $\Lambda = 6.5$ ppm, which differ negligibly from results obtained using $\xi = 1$. The effects $\eta \neq 0$ and $\beta \neq 0$ are much smaller than experimental uncertainties in the present results; therefore, neglect of these effects does not affect any of the conclusions derived from the $\Delta\sigma$ values for RNase H.

Site-specific values of the ^{15}N $\Delta\sigma$ have been determined for two proteins, ubiquitin and RNase H. Figure 6 shows the ^{15}N CSA values reported for ubiquitin.[6] Sites present in residues that undergo significant internal motion (residues 8−11, 62, 73, and 75)[33] have been removed from this figure to allow direct comparison with the RNase H results. For the remaining 56 sites, the weighted mean and uncertainty are $\langle\Delta\sigma\rangle = -160$ ppm, and $\langle s\rangle = 3$ ppm, respectively. The median values for $\Delta\sigma$ and s are −156 and 10 ppm, respectively. The difference between the weighted and median values of s indicate that a subset of data points have uncertainties that are much smaller than the remainder of the data. In Figure 6a, $\Delta\sigma$ is plotted versus residue number. The absolute range in $\Delta\sigma$ values are similar for RNase H (−129 ppm to −213 ppm) and ubiquitin (−125 ppm to −216 ppm); however, absolute $\Delta\sigma$ values far from the mean invariably have large uncertainties in the RNase H data, but frequently have small uncertainties in the ubiquitin data. As a result of this difference, the distribution of Z values in ubiquitin shown in Figure 6b spans a much larger range (−21 to +14) than that observed for RNase H (−3 to +3), and many of the outliers present in the tails of the distribution plotted in Figure 6b arise from small values of s. If only the central cluster of data points in Figure 6b are analyzed in the manner utilized for RNase H (sites in which $|Z| < 4$), $\langle s\rangle = 4.4$ ppm and $\sigma_Z = 1.52$, which yields $\Lambda = 5.0$ ppm. Thus, except for the nine outliers with $|Z| > 4$ in Figure 6b, the distribution of $\Delta\sigma$ values in ubiquitin and RNase H predict a similar limited site-to-site variation in $\Delta\sigma$

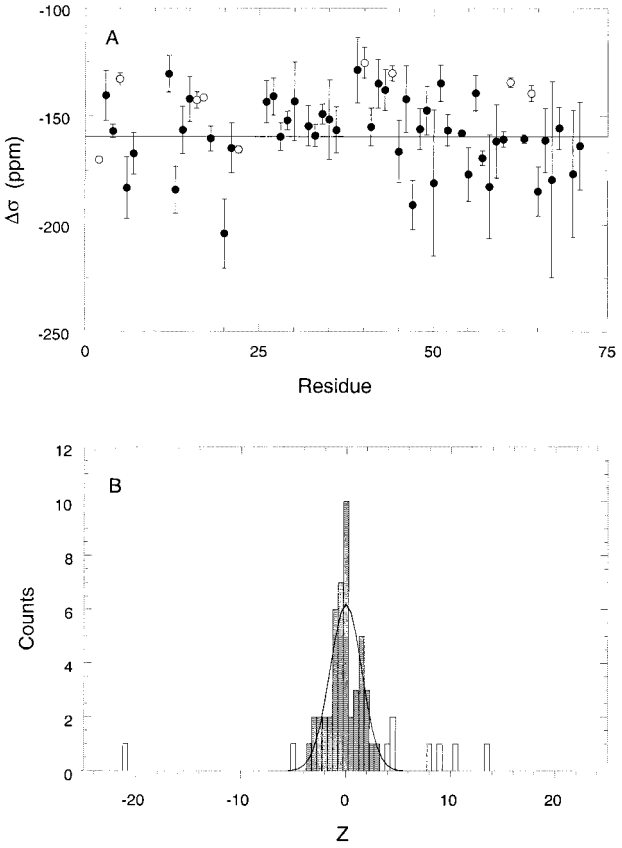

Figure 6. ^{15}N chemical shift anisotropy for ubiquitin. (A) Values of $\Delta\sigma$ for ^{15}N sites not subject to extensive internal motion are taken from Fushman et al.[6] and are plotted versus residue number. The horizontal line is drawn at the position of the weighted mean value of $\langle\Delta\sigma\rangle = -160$ ppm. Residues in secondary structure elements are β1:1−7, β2: 10−17, α1:23−34, β3:40−45, β4:48−50, α2:56−59, β5:64−72. (B) Distribution of the standardized values of Z calculated using the weighted mean of $\Delta\sigma$ values that are within 4 standard deviations of $\langle\Delta\sigma\rangle$ (shaded bars). Open bars with $|Z| > 4$ represent data points plotted as open circles in (A). The solid line represents a Gaussian distribution with a mean of 0 and a standard deviation of 1.52.

(27) Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 12334−12341.
(28) Boyd, J.; Redfield, C. *J. Am. Chem. Soc.* **1998**, *120*, 9692−9693.
(29) Fushman, D.; Cowburn, D. *J. Biomol. NMR* **1999**, *13*, 139−147.
(30) Brüschweiler, R.; Liao, X.; Wright, P. E. *Science* **1995**, *268*, 886−889.
(31) Lee, L. K.; Rance, M.; Chazin, W. J.; Palmer, A. G. *J. Biomol. NMR* **1996**, *9*, 287−298.
(32) Yang, W.; Hendrickson, W. A.; Crouch, R. J.; Satow, Y. *Science* **1990**, *249*, 1398−1405.
(33) Tjandra, N.; Feller, S. E.; Pastor, R. W.; Bax, A. *J. Am. Chem. Soc.* **1995**, *117*, 12562−12566.

for the majority of well-ordered residues in proteins. Additional investigations in other proteins will be necessary to determine whether the absence of extreme values of $\Delta\sigma$ for well-ordered residues of RNase H is exceptional.

The spread in $\Delta\sigma$ observed in the current study has important consequences for the investigation of protein dynamics by ^{15}N spin relaxation. Most notably, at 18.7 T, but not at the lower field strengths, the discrepancy between $J(0)$ values predicted using individual $\Delta\sigma$ values versus those predicted using an average $\Delta\sigma = -172$ ppm is statistically significant. Consequently, the site-to-site variation in $\Delta\sigma$ should be incorporated into the analysis of ^{15}N relaxation rate constants using reduced spectral density mapping or model-free approaches. With the current experimental methods, the site-to-site variability, Λ, is less than typical uncertainties in the individual values of $\Delta\sigma$ (for example, both RNase H and ubiquitin median values of s are greater than 10 ppm). Therefore more precise values of spectral densities or model-free parameters, particularly at fields >14.1 T, are obtained using the mean $\langle\Delta\sigma\rangle$ for all residues plus an uncertainty in $\Delta\sigma$ equal to Λ, rather than using residue specific $\Delta\sigma$ values. As discussed above, for the determination of $J(0)$, this approach yields biased estimates of motional parameters but the estimated uncertainties are correspondingly increased to include the bias. The precision in model-free or reduced spectral density mapping analyses will be higher if residue specific $\Delta\sigma$ values are used only if typical values of s are less than Λ. Thus, further improvements in the methods used to determine $\Delta\sigma$ are necessary, both through future availability of additional magnetic field strengths and additional methodological developments. However, as $\Delta\sigma$ determinations become more precise, deviations from axial symmetry in the chemical shift tensor and noncollinearity of the ^{15}N CSA with the N−H bond vector will be proportionally more important. Methods for determining $\Delta\sigma$ that are less sensitive to these effects will be invaluable in elucidating the structural factors that determine the ^{15}N chemical shift tensor. Advances in solid-state NMR methods for obtaining resonance assignments and chemical shift tensors in fully labeled proteins[34] and in high resolution NMR methods for characterizing chemical shift tensors of weakly oriented systems[26,35] may prove particularly decisive.

(34) Gu, Z.; Opella, S. J. *J. Magn. Reson.* **1999**, *138*, 193−198.
(35) Boyd, J.; Redfield, C. *J. Am. Chem. Soc.* **1999**, *121*, 7441−7442.

Conclusion

Methods similar to those of Fushman et al.[6] have been used to determine the distribution of 81 well-ordered backbone amide ^{15}N CSA values in *E. coli* RNase H. The observed distribution has a weighted mean of −172 ppm, and a weighted standard deviation of 13 ppm. The site-to-site variability in $\Delta\sigma$ is 5.5 ppm, and is less than 9.6 ppm with 95% confidence. Four principal conclusions emerge from the current study: (1) the observed site-to-site variability in $\Delta\sigma$ in RNase H is similar to the range of $\Delta\sigma$ values observed in solid-state NMR studies of peptides; (2) in contrast to the results for ubiquitin, no extreme outlying values of $\Delta\sigma$ are observed for RNase H ($|Z| < 3$) when comparing well-ordered ^{15}N sites; (3) at the currently achievable degree of precision in ^{15}N spin relaxation measurements, the site-to-site variability in $\Delta\sigma$ becomes a significant factor in interpreting such data at fields >14.1 T; and (4) at the currently achievable degree of precision in the determination of $\Delta\sigma$, the site-to-site variability in $\Delta\sigma$ can be treated as a random variation in $\langle\Delta\sigma\rangle$ when analyzing ^{15}N spin relaxation data. The further development of methods for determining chemical shift anisotropies using solution NMR methods is likely to considerably improve both spin relaxation studies of protein dynamics and theoretical understanding of the structural determinants of chemical shift tensors.

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Note Added in Proof: D. Fushman, N. Tjandra, and D. Cowburn (*J. Am. Chem. Soc.* **1999**, *121*, 8577−8582) recently have used an approach similar to eq 1 to re-analyze relaxation data for ubiquitin previously analyzed using eq 6 in reference 6.

Supporting Information Available: One table containing measured values of $\Delta\sigma$ and three tables containing spin relaxation rates at 11.7, 14.1, and 18.7 T for 81 ^{15}N spins in *E. coli* RNase H (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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