An Approach to Direct Determination of Protein Dynamics from ^{15}N NMR Relaxation at Multiple Fields, Independent of Variable ^{15}N Chemical Shift Anisotropy and Chemical Exchange Contributions

David Fushman,† Nico Tjandra,‡ and David Cowburn*,†

Contribution from The Rockefeller University, 1230 York Avenue, New York, New York 10021, and The Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-0380

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Abstract: An approach to protein dynamics analysis from ^{15}N relaxation data is demonstrated, based on multiple-field relaxation data. This provides a direct, residue-specific determination of both the spectral density components, the ^{15}N chemical shift anisotropy (CSA) and the conformational exchange contribution to the ^{15}N line width. Measurements of R_1, R_2, and ^{15}N{^{1}H} NOE are used. The approach is free from any assumption about the values of the CSA or of the conformational exchange. Using this approach, the spectral densities, the values of ^{15}N CSA, and the conformational exchange contribution to the ^{15}N line width are directly determined from the relaxation data for human ubiquitin, collected at 360, 500, and 600 MHz. The spectral densities are analyzed in terms of the order parameter and the correlation time of local motion, using an axially symmetric overall rotational diffusion model. The residue-specific values of ^{15}N CSA and the spectral densities obtained using this approach are in agreement with those derived previously [Fushman, Tjandra, and Cowburn. *J. Am. Chem. Soc.* **1998**, *120*, 10947−10952] from CSA/dipolar cross-correlation analysis. Accurate determination of spectral densities and order parameters from ^{15}N relaxation may be accomplished by analysis of multiple-field data without assumption of constant CSA or zero chemical exchange contributions.

Introduction

Current approaches to obtaining information on protein dynamics from ^{15}N relaxation data are based on either "model-free" approaches[1,2] or the mapping of spectral density functions.[3] These approaches assume a constant value of −160 ppm for the ^{15}N chemical shift anisotropy (CSA), as inferred from solid-state NMR data.[4] Recent analysis of multiple-field ^{15}N relaxation and CSA/dipolar cross-correlation data for human ubiquitin[5] has indicated a spread in ^{15}N CSA values significantly larger (from −125 to −216 ppm, with the mean value of −157 ppm, median of −157 ppm, and quartiles of −142 and −165 ppm (52 values)) than anticipated from previous peptide studies. These site-specific variations in CSA render inaccurate analyses based on the assumption of a uniform single value of CSA. The spectral density functions, $J(0)$, derived using site-specific values of ^{15}N CSA differ from those obtained assuming a uniform CSA value.[5] Here we describe approaches to the direct determination of protein dynamics from ^{15}N relaxation data, R_1, R_2, and ^{15}N-{^{1}H} NOE, at various fields, which require no assumption about ^{15}N CSA or additionally the chemical exchange contribution, R_{ex}. Using these approaches, the spectral densities, the values of ^{15}N CSA, and the conformational exchange contribution to ^{15}N transverse relaxation are directly determined from the relaxation data for human ubiquitin.

* To whom correspondence should be addressed. E-mail: cowburn@rockefeller.edu. Phone: (212) 327-8270. Fax: (212) 327-7566.
† The Rockefeller University.
‡ National Institutes of Health.

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Theory

The spectral density functions $J(\omega)$ characterizing protein dynamics are related to experimentally measured ^{15}N relaxation rates, R_1 and R_2, and steady-state heteronuclear NOE as follows:

$$R_1 - 3(d^2 + c^2)J(\omega_N) + 6.25d^2J(0.87\omega_H) \quad (1)$$

$$2R_2 - R_1 - 4.54d^2J(0.87\omega_H) = 4(d^2 + c^2)J(0) + 2R_{ex} = 4d^2J(0) + 2\omega_N{}^2[\rho_{ex} + 2(CSA/3)^2J(0)] \quad (2)$$

$$(1 - NOE)R_1\frac{\gamma_N}{\gamma_H} = 5d^2J(0.87\omega_H) \quad (3)$$

where $d = -(\mu_o/(4\pi))\gamma_H\gamma_N h/(4\pi r_{HN}{}^3)$, $c = \gamma_N B_o(\sigma_{||} - \sigma_\perp)/3$, r_{HN} is the internuclear ^{15}N−^{1}H distance, $\sigma_{||} - \sigma_\perp$ is the anisotropy of the ^{15}N chemical shift tensor (CSA), γ_H, γ_N and ω_H, ω_N are gyromagnetic ratios and resonance frequencies of the nuclei, h is Planck's constant, and R_{ex} is the conformational exchange contribution, if any, to measured R_2; $\rho_{ex} \equiv R_{ex}/\omega_N{}^2$ is

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independent of the field strength.[6] These equations were obtained by modification of the standard expressions,[7] by the assumption that $J(\omega) \propto \omega^{-2}$ at $\omega \approx \omega_H$; hence, $J(\epsilon\omega_H) = (0.87/\epsilon)^2 J(0.87\omega_H)$.[8] This high-frequency component, $J(0.87\omega_H)$, of the spectral density function can be directly determined from the experimental data, R_1 and NOE, using eq 3, independent of ^{15}N CSA. Accurate derivation of the other two components, $J(0)$ and $J(\omega_N)$, is less straightforward, and requires knowledge of ^{15}N CSA and R_{ex}, which are, in general, not known. Although experimental approaches to measure R_{ex} have been suggested,[9] the absence of information on site-specific CSA poses a significant problem for accurate analysis of protein dynamics from relaxation data measured at a single field. Direct determination of ^{15}N CSA from relaxation data at multiple fields using CSA/dipolar cross-correlation rates, η, can, however, be done,[5] and similar approaches[10] could be developed for this purpose.

The approach suggested here does not require cross-correlation rate measurements, and uses a standard set of relaxation measurements (R_1, R_2, and NOE) at multiple field strengths to derive $J(0)$ without any preexisting knowledge of CSA and/or R_{ex}. Since $J(0)$ is independent of magnetic field, eq 2 provides a method of obtaining $J(0)$ directly from a standard linear-regression fit of the left-hand side of this equation to a linear dependence versus ω_N^2 ($\propto B_0^2$), with a slope m and intercept b: $b + m\omega_N^2$. As a result of such fit, $J(0)$ can be determined as

$$J(0) = b/(4d^2) \tag{4}$$

and for R_{ex} and CSA

$$2R_{ex}/\omega_N^2 + b(\mathrm{CSA}/d)^2/9 = m \tag{5}$$

The spectral density component $J(0)$ derived using this approach is independent of CSA and R_{ex}. Equation 5 contains both ^{15}N CSA and R_{ex}, and, therefore, allows determination of one of these parameters given the value of the other. Simultaneous solution for both values requires a more complex approach shown below, but eq 5 can still be valuable. For example, in those cases when the conformational exchange contribution is negligible, the ^{15}N CSA can be directly determined from eq 5 as $\mathrm{CSA} = 3d(m/b)^{1/2}$ (further referred to as method A).

When Neither R_{ex} nor CSA Is Known. A direct determination of CSA and R_{ex} is possible using the following approach (method B), when neither R_{ex} nor CSA is known a priori. The idea of this approach is to derive ^{15}N CSA directly from experimental R_1 values. Method A, above, makes no assumptions as to the distribution of spectral density values, only that R_{ex} is negligible. If the Lipari−Szabo formalism for the distribution of spectral densities is accepted, then the values of $J(0)$ and $J(\omega_H)$, determined above, can be used for a parametrization of the spectral density function, in terms of the model-free parameters,[1] S^2 and τ_{loc}.[15]

Using these parameters, the spectral density component $J(\omega_N)$ can be interpolated (see Materials and Methods) and then substituted into eq 1 to determine the ^{15}N CSA directly from the measured value of R_1:

$$\mathrm{CSA} = \frac{3d}{\omega_N}\left(\frac{R_1 - 6.25d^2J(0.87\omega_H)}{3d^2J(\omega_N)} - 1\right)^{1/2} \tag{6}$$

This derivation of ^{15}N CSA is independent of any chemical exchange contribution. The value of R_{ex} can then be directly determined from eq 5. This approach provides a direct determination of both CSA and R_{ex}. It depends, however, on the accuracy of the interpolation of $J(\omega_N)$, and, therefore, the use of the Lipari−Szabo formalism for derivation.

Although the interpolation of $J(\omega_N)$ from spectral densities at rather distant frequencies, $\omega = 0$ and ω_H, might appear to be a complex and uncertain procedure, its robustness is demonstrated by our computer simulations (see Materials and Methods). A simpler way is possible to determine CSA from R_1 values using method B, for those residues characterized by restricted motions, typically observed in the protein core. This approach, described below, is a first-order approximation to the full interpolation procedure, and thus can be considered a truncated version of method B. Using the Lipari−Szabo formalism, the contributions to the lower frequency spectral densities from local motions are usually very small. These contributions are the terms containing τ_e (eq 12, Materials and Methods) or τ_{ke} in eq 11. For the core residues of ubiquitin ($S^2 > 0.8$ and $\tau_{loc} < 50$ ps), these contributions are less than 1% and 0.3% to $J(\omega_N)$ and $J(0)$, respectively, and therefore, can be safely neglected for these spectral density components. The same assumption is usually made when the hydrodynamic properties of proteins are determined using the R_2/R_1 ratio.[11−14] Both $J(\omega_N)$ and $J(0)$ then scale as S^2 (eqs 11 and 12, Materials and Methods), and therefore, the following relation holds between the two spectral density components:

$$J(\omega_N) = J(0)/B \tag{7}$$

where B is independent of local dynamics. From eqs 11 and 12

$$B = 1 + (\omega_N\tau_c)^2 \tag{8}$$

for the case of isotropic overall rotation and

(6) For a two-site exchange with a rate Γ and a chemical shift difference (ppm) between the equally populated sites 2δ, R_{ex} can be written in the fast-exchange regime, $\Gamma \gg 2\omega_N\delta$, as $R_{ex} = \omega_N^2\delta^2/(2\Gamma) = \omega_N^2\rho_{ex}$ (ref 7, p 450).

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(15) In the model-free approach[1] the local motions are assumed to be independent of the overall tumbling of the molecule and are characterized by the order parameter S and the correlation time of the local motion, τ_{loc}. The overall rotational diffusion can be characterized using the R_2/R_1 ratio [11−14] which is independent of S^2, τ_{loc}, and ^{15}N CSA to the first order, for those residues belonging to a well-defined protein core. Those amides with extremely large R_2/R_1 ratios should be excluded as possibly influenced by conformational exchange. The number of remaining NH groups available for the analysis is usually sufficient for a statistically robust characterization of the overall rotational diffusion. Given the overall rotational parameters determined independently, a set of the two spectral density components, at $\omega = 0$ and $\omega = \omega_H$, is sufficient for determination of S^2 and τ_{loc} ($0 \le S^2 \le 1$; $0 \le \tau_{loc} \le 1/\omega_H \sim 300$ ps). More complex models using additional parameters[2] could also be used, employing $J(\omega_H)$ values at $\omega_H = 360$ and 500 MHz, in the case when NOE data at these field strengths are available.

$$B = [(3\cos^2\theta - 1)^2\tau_1 + 3(\sin^2 2\theta)\tau_2 + 3(\sin^4\theta)\tau_3]/\left[(3\cos^2\theta - 1)^2\frac{\tau_1}{1+(\omega_N\tau_1)^2} + 3(\sin^2 2\theta)\frac{\tau_2}{1+(\omega_N\tau_2)^2} + 3(\sin^4\theta)\frac{\tau_3}{1+(\omega_N\tau_3)^2}\right] \quad (9)$$

in the case of anisotropic (axially symmetric) overall motion. A similar expression for B can be obtained for the fully anisotropic overall motion. Substitution for $J(\omega_N)$ from eq 7 into eq 6 then gives

$$\mathrm{CSA} = \frac{3d}{\omega_N}\left(\frac{R_1 - 6.25d^2J(0.87\omega_H)}{3d^2J(0)}B - 1\right)^{1/2} \quad (10)$$

Equation 10 provides a method of deriving CSA directly from the longitudinal relaxation rate, using assumptions identical to those used for obtaining overall correlation time, from the values of the spectral density components, $J(0.87\omega_H)$ and $J(0)$, determined as described above. This method is expected to yield accurate values of ^{15}N CSA for those amides which belong to the well-structured regions in a protein. The degree of agreement of this simplified method with the more complete interpolation is then some measure of the soundness of this approach.

Materials and Methods

The set of ^{15}N relaxation data for human ubiquitin taken for this analysis consists of R_1, R_2, and NOE at 600 MHz,[16] and R_1 and R_2 at 500 and 360 MHz.[5] The high-frequency components, $J(0.87\omega_H)$ at 600 MHz, were derived according to eq 3, and the values at other field strengths were obtained as described[5] assuming the scaling properties of $J(0.87\omega_H)$ discussed above.

A self-consistency test for matching the conditions between the data acquired at different fields and spectrometers is shown in Figure 1.

Derivation of S^2 and τ_{loc} from the Spectral Density Components $J(0)$ and $J(\omega_H)$. The following functional form of the spectral density function was used (see e.g. ref 16):

$$J(\omega) = \frac{1}{10}\left\{(3\cos^2\theta - 1)^2\left[S^2\frac{\tau_1}{1+(\omega\tau_1)^2} + (1-S^2)\frac{\tau_{1e}}{1+(\omega\tau_{1e})^2}\right] + 3(\sin^2 2\theta)\left[S^2\frac{\tau_2}{1+(\omega\tau_2)^2} + (1-S^2)\frac{\tau_{2e}}{1+(\omega\tau_{2e})^2}\right] + 3(\sin^4\theta)\left[S^2\frac{\tau_3}{1+(\omega\tau_3)^2} + (1-S^2)\frac{\tau_{3e}}{1+(\omega\tau_{3e})^2}\right]\right\} \quad (11)$$

Here $\tau_1^{-1} = 6D_\perp$, $\tau_2^{-1} = 5D_\perp + D_\parallel$, $\tau_3^{-1} = 2D_\perp + 4D_\parallel$; $D_\parallel$ and $D_\perp$ are principal components of the rotational diffusion tensor assumed here to be axially symmetric, θ is the angle between a given NH vector and the unique principal axis of the tensor, the local motion of the NH vector is characterized by the order parameter S and a correlation time τ_{loc}, and $\tau_{ke}^{-1} \equiv \tau_k^{-1} + \tau_{loc}^{-1}$ (k =1, 2, or 3).

In the case of isotropic rotational diffusion, $D_\parallel = D_\perp = D$, $\tau_1 = \tau_2 = \tau_3 = \tau_c = 1/(6D)$, this equation reduces to the conventional "model-free" form[1]

$$J(\omega) = \frac{2}{5}\left[S^2\frac{\tau_c}{1+(\omega\tau_c)^2} + (1-S^2)\frac{\tau_e}{1+(\omega\tau_e)^2}\right] \quad (12)$$

where $\tau_e^{-1} \equiv \tau_c^{-1} + \tau_{loc}^{-1}$.

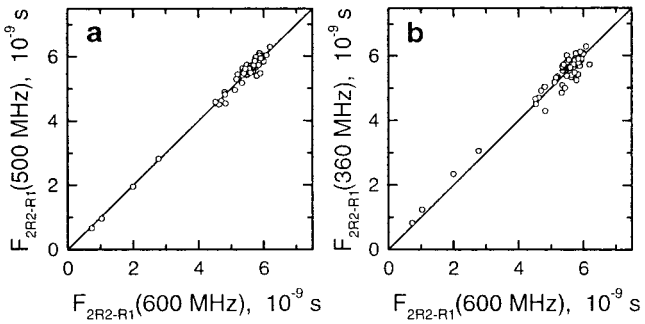

Figure 1. Consistency test for the experimental data, 500 MHz vs 600 MHz (a) and 360 MHz vs 600 MHz (b). To test the consistency of the data from different fields, the following self-consistency test can be used (see also ref 5): the variable $F_{2R2-R1} \equiv [2R_2 - R_1 - 4.54d^2J(0.87\omega_H)]/[d^2 + \omega_N^2(\mathrm{CSA}/3)^2]$ must be field-independent. To achieve this, the $2R_2 - R_1 - 4.54d^2J(0.87\omega_H)$ values at 500 MHz were uniformly scaled by 0.988 (1.24%) and the corresponding data at 360 MHz by 0.966 (3.56%). The scaling factor was determined as the average ratio of the corresponding F_{2R2-R1} values for all residues excluding Ile[23] and Asn[25], subjected to conformational exchange (not shown), and the four C-terminal residues Leu[73]-Gly[76] (the lowest left points) exhibiting a high degree of local flexibility.[16] This small uniform scaling compensates for possible deviations in the experimental conditions, as well as, possibly, for any systematic errors introduced by deriving $J(0.87\omega_H)$ for 500 and 360 MHz from the 600 MHz data. For these plots, the approximation was to take CSA = −157 ppm as an average value for ubiquitin.[5] The spread of the points around the diagonal is caused by site-specific variations in CSA and by experimental errors. The correlation coefficient r is 0.99 and 0.98 for the data shown in (a) and (b), respectively, and 0.98 for 500 vs 360 MHz data. Note that the F_{2R2-R1} values at different fields could also be made consistent by adjusting the average CSA value in the denominator of F_{2R2-R1}. The values of CSA necessary to make the data consistent (−141 and −137 ppm for (a) and (b), respectively, and −132 ppm for 500 vs 360 MHz data) are much lower in absolute value than the average CSA values reported in the previous studies.[4,5,18] The calculations show that these values of the scaling factor might correspond to a temperature decrease of 1.6 °C for the 360 MHz and 0.55 °C for the 500 MHz measurements, compared to 600 MHz.

In method A, the model-free parameters, S^2 and τ_{loc}, were determined by solving numerically a set of three equations (11), for $\omega = 0$, ω_N, and $0.87\omega_H$, corresponding to the experimentally determined values of $J(0)$, $J(\omega_N)$, and $J(0.87\omega_H)$. The solution was based on minimization of the differences between the measured and calculated values of $J(\omega)$ (least-squares method), and was performed using the simplex algorithm.[17] The following expression for the target function was used:

$$E = \left[\frac{J(0)^{\mathrm{exptl}} - J(0)^{\mathrm{calcd}}}{\sigma_0}\right]^2 + \left[\frac{J(0.87\omega_H)^{\mathrm{exptl}} - J(0.87\omega_H)^{\mathrm{calcd}}}{\sigma_H}\right]^2 + \left[\frac{J(\omega_N)^{\mathrm{exptl}} - J(\omega_N)^{\mathrm{calcd}}}{\sigma_N}\right]^2 \quad (13)$$

where superscripts "exptl" and "calcd" refer to measured and calculated (using eq 11 or 12) values of $J(\omega)$, and σ_0, σ_H, and σ_N denote estimated experimental errors in the corresponding spectral densities. The model-free parametrization in method B uses eq 11 twice, corresponding to $J(0)$ and $J(0.87\omega_H)$, with the last term in eq 13 omitted. In this case, both the least-squares approach (above) and a direct analytical solution (assuming $\tau_{loc} \ll \tau_1$, τ_2, and τ_3) were used; both approaches yielded similar results. The same procedures were applied in the case of the isotropic model, using eq 12.

After the model-free parametrization of the spectral density was performed as described here, the $J(\omega_N)$ component was calculated from

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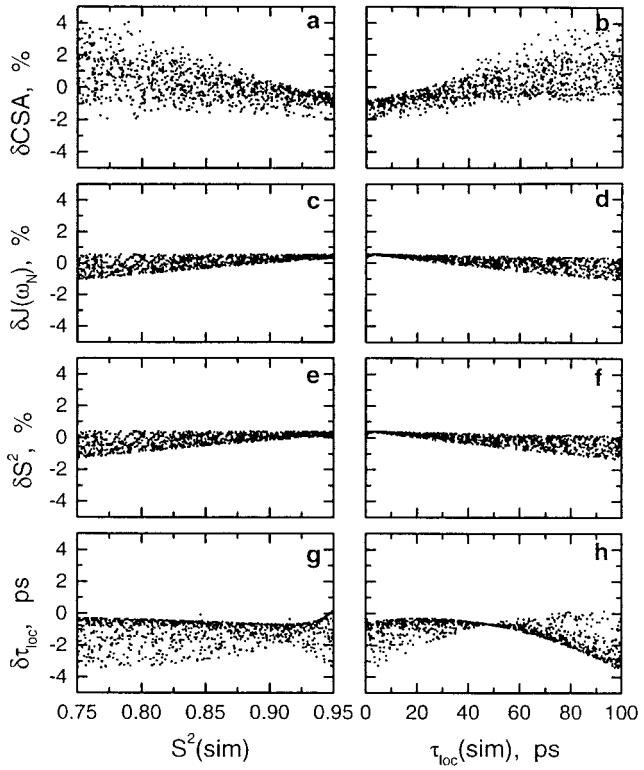

Figure 2. Validation of the precision and reproducibility of method B based on interpolation of $J(\omega_N)$ from $J(0)$ and $J(0.87\omega_H)$. One thousand synthetic relaxation data sets were generated as described in the text and subsequently analyzed using method B. Shown are deviations in the calculated values from the simulated ones for CSA (a, b), $J(\omega_N)$ (c, d), and S^2 (e, f), all in percents, and τ_{loc} (g, h), in picoseconds. Left and right panels represent the deviations as a function of S^2 and τ_{loc}, respectively. These results demonstrate that the expected inaccuracy of method B, based on $J(\omega_N)$ interpolation, is within 1% for $J(\omega_N)$ and S^2 and within 4% in CSA. The R_{ex} values derived in these calculations were below the level of experimental errors in R_2. As expected, the method using a simple model-free approach[1] becomes less accurate for larger amplitudes ($S^2 < 0.75$) and longer correlation times ($\tau_{loc} > 100$ ps) of the NH bond reorientations; in these cases the "extended" model[2] might be necessary for an accurate data parametrization. Since the majority of amides in ubiquitin are characterized by $S^2 > 0.8$ and $\tau_{loc} < 50$ ps, the expected errors in CSA are below 2%, consistent with the observed values.

eq 11 (eq 12 for the isotropic model) for $\omega = \omega_N$, by substituting the derived values of the model-free parameters into the right-hand side of this equation.

All this analysis was performed using a locally produced Matlab program package, JCSA.

Computer Simulation Test of Method B. Method B is based on interpolation of the spectral density component $J(\omega_N)$, given $J(0)$ and $J(\omega_H)$. To verify the accuracy of this method assuming the model-free approach, 1000 synthetic data sets were simulated, each comprising R_1, R_2, and NOE at 600 MHz, and R_1 and R_2 at 500 and 360 MHz, thus mimicking the available experimental data. For this simulation, an isotropic rotation with $\tau_c = 4.1$ ns was assumed; the values of the other relevant parameters were generated randomly, uniformly distributed in the following intervals: −110 to −210 ppm (CSA), 0.75 to 0.95 (S^2), and 0 to 100 ps (τ_{loc}), corresponding to the ranges of these parameters observed in ubiquitin (CSA) or typical for the model-free approach (S^2 and τ_{loc}). The relaxation parameters thus generated were analyzed using the approach of method B, in the same way as the real experimental data, assuming the level of experimental errors as observed in experiment. The results (Figure 2) validate the precision and robustness of the algorithmic implementation of method B based on $J(\omega_N)$ interpolation.

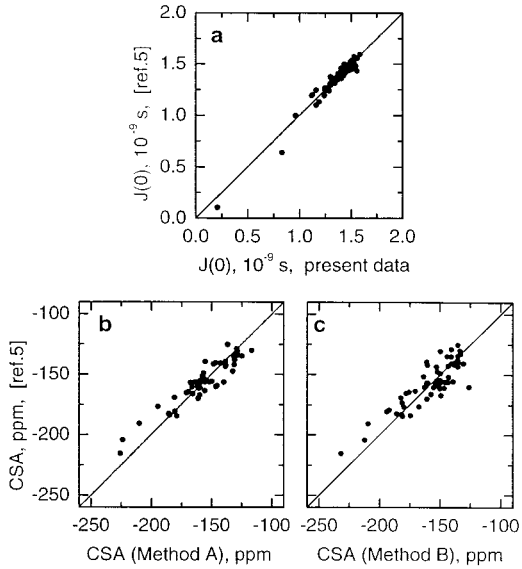

Figure 3. Comparison of the present results with the ones derived from dipolar/CSA cross-correlation:[5] (a) the spectral density function component $J(0)$ and (b, c) the ^{15}N CSA values. The present CSA data shown in (b) were derived using eqs 4 and 5 and assuming $R_{ex} = 0$ (method A), while those in (c) were obtained from eq 6 (method B). The correlation coefficient r between the data is 0.98 (a), 0.94 (b), and 0.85 (c). Those residues with significant R_{ex} were excluded from (c); the correlation coefficient between CSA values obtained using method A and method B is 0.89.

Results and Discussion

The ^{15}N relaxation data for human ubiquitin, comprising the R_1, R_2, and NOE at 600 MHz,[16] and R_1 and R_2 at 500 and 360 MHz,[5] were analyzed as described above.

As outlined previously,[5,18] small variations of conditions between experimental sets may need correction (Figure 1). The experimental values of $2R_2 - R_1 - 4.54d^2J(0.87\omega_H)$ were fit to a linear dependence vs ω_N^2 using the standard linear regression method.[17] No correlation was observed between the slope m and the intercept b of this dependence (correlation coefficient $r = 0.14$). The values of $J(0)$ derived according to eq 4 are in a good agreement with those obtained previously from CSA/dipolar cross-correlation analysis[5] (Figure 3a). In contrast to that previous approach, values of $J(0)$ are now available for Ile[23] and Asn[25] which had been excluded from the previous analysis as influenced by conformational exchange.

Further analysis of these data including extraction of CSA, R_{ex}, and model-free parameters was performed separately for methods A and B as described above.

The four C-terminal residues, Leu[73]-Gly[76], with NOEs below 0.34 were excluded from further analysis as not amenable to the simple model-free treatment.[1]

Method A. The ^{15}N CSA values derived from eq 5 (range −111 to −226 ppm, with the mean value of −157, median of −156, and quartiles of −138 and −167 ppm (61 residues)) are in good agreement with the results of our previous analysis based on the η/R_2 ratio[5] (Figure 3b). These values assume that R_{ex} is zero. Using these values of CSA, the spectral density components $J(\omega_N)$ were determined from the measured values of R_1, according to eq 1. The model-free parameters characterizing local backbone dynamics in ubiquitin, S^2 and τ_{loc} (Figure 4b,c), were then derived from the spectral densities $J(\omega_N)$ and $J(0.87\omega_H)$ at 600 MHz and $J(0)$, assuming an axially symmetric

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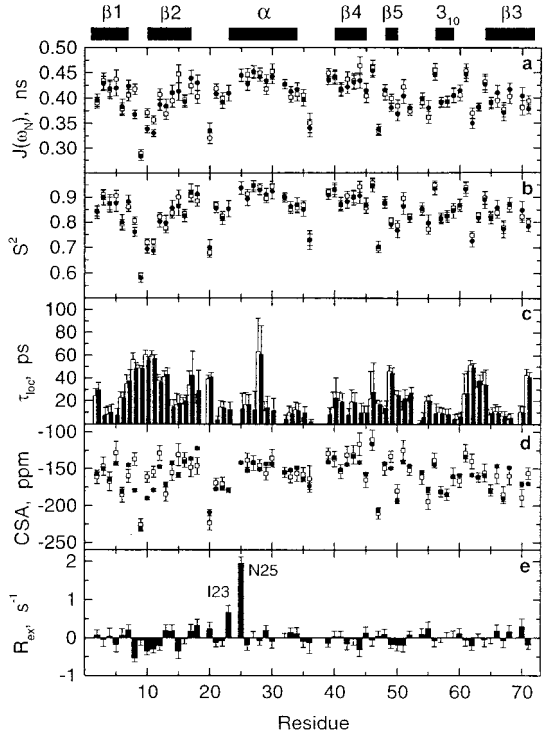

Figure 4. Backbone spectral density component $J(\omega_N)$ (a), the model-free parameters, S^2 (b) and τ_{loc} (c), ^{15}N CSA (d), and the conformational exchange contribution R_{ex} (e) in human ubiquitin, obtained using the approaches proposed here. The elements of secondary structure of human ubiquitin are indicated on the top. Panels a−d provide a comparison of the parameters derived using the method A (open symbols/bars) and method B (solid symbols/bars). The error bars represent standard errors in the parameters. The model-free parametrization of the spectral densities was performed assuming axially symmetric overall rotational diffusion of the protein, as described in Materials and Methods. The overall rotational diffusion tensor was characterized by the following parameters: $\tau_c = 4.12$ ns and $D_\parallel/D_\perp = 1.17$. The orientation of the unique principal axis of the tensor with respect to the protein coordinate frame (1ubq.pdb) was given by the Euler angles $\alpha = 46°$ and $\beta = 40°$. These hydrodynamic characteristics of the protein were derived from the ^{15}N relaxation data in ref 16. Note that the ^{15}N CSA values are now available for Ile[23] and Asn[25] (indicated in e) which had been excluded from the previous analyses as influenced by conformational exchange. Note also much smaller experimental errors (2.6 ppm or 1.6%, on average) in the CSA values obtained from R_1 (method B), as compared to the ones derived from the η/R_2 analysis.[5] Statistically significant positive R_{ex} values are obtained for Ile[23] and Asn[25], in good agreement with the previous studies. The observed small negative values of R_{ex} are not statistically significant, except those for Leu[8], Gly[10], and Lys[11] (see the text).

overall rotational diffusion of the molecule.[20] This model of the overall motion was previously used to yield a fit to the ^{15}N relaxation data in ubiquitin.[16] The derived parameters exhibit little sensitivity to the model of overall rotation. Comparison of this model and an isotropic one is available in the Supporting Information.

Method B. To illustrate this method and to verify our assumptions regarding the conformational exchange contributions in ubiquitin, the model-free parameters, S^2 and τ_{loc} (Figure 4b,c), were derived directly from $J(0)$ and $J(0.87\omega_H)$ (cf. Materials and Methods). These values of the model-free parameters are free from any assumption about site-variable CSA or R_{ex}. The values of $J(\omega_N)$ calculated using S^2 and τ_{loc} agree well with those obtained directly from the experimental values of R_1, method A, for all residues except Leu[8], Gly[10], and Lys[11] (Figure 4a). The ^{15}N CSA derived using eq 6 (range −116 to −231 ppm, mean value −159, median −155, quartiles −176 and −143 ppm (63 residues)) are consistent with those of method A (Figure 4d) and with the results of our previous analysis based on the η/R_2 ratio[5] (Figure 3c), for those residues with negligible chemical exchange contributions.[22]

The truncated version of method B, based on eqs 9 and 10, yielded CSA values in excellent agreement with those derived using the full interpolation. The correlation coefficient between the ^{15}N CSA values derived using these two versions of method B was 0.98, when all residues were included, and 0.99 if only those residues belonging to the well-defined secondary structure were used. This agreement also validates the performed interpolation of $J(\omega_N)$, using $J(0)$ and $J(\omega_H)$. Note that the truncated version of method B is less generally applicable than the full-interpolation approach, since it is based on additional assumptions than the Lipari−Szabo formalism.

For the majority of the amide groups in human ubiquitin (58 out of 63 analyzed), the observed conformational exchange contribution is negligible, within the experimental errors (Figure 4e). Positive values of R_{ex} were observed as statistically significant in Ile[23] and Asn[25]. The conformational exchange contribution was anticipated for these residues from the η/R_2 analysis[23] and from the previous analysis of relaxation data.[16,24] The R_{ex} values determined here for Ile[23] and Asn[25], 0.67 ± 0.18 and 1.95 ± 0.16 s^{-1}, respectively, are in good agreement with those estimated earlier[24] from the relaxation data (0.68 and 2.09 s^{-1}) and from the correlation between R_2/R_1 and residual dipolar coupling (0.8 ± 0.3 and 2.1 ± 0.4 s^{-1}). As outlined above with method A, a comparison between the anisotropic axially symmetric model and an isotropic model was performed. For the isotropic model, statistically significant positive R_{ex} values were also calculated in Glu[18], Asp[52], and Lys[63]; these are likely to be apparent conformational exchange contributions arising from inadequacy of the isotropic rotational diffusion model.[25] With the anisotropic axially symmetric model, three residues,

(19) Fushman, D.; Cowburn, D. *J. Biomol. NMR* **1999**, *13*, 139−147.

(20) We have shown separately[19] that the noncollinearity between the ^{15}N CSA and $^1H-^{15}N$ dipolar interaction can lead to site-specific variations in R_2 and R_1 which are not accounted for in eqs 1 and 2 but might become substantial in the case of significant rotational anisotropy. This effect is not considered here, as the corresponding contributions are estimated[19] to be negligible in the case of the small rotational anisotropy observed in human ubiquitin.

(21) Saupe, A. *Z. Naturforsch.* **1964**, *19a*, 161−171.

(22) A weak correlation can be seen from Figure 4b,d between the ^{15}N CSA and the values of S^2, mostly pronounced in the cases of extreme values of CSA and S^2 and suggesting increased absolute values of CSA for those residues with large amplitudes of apparent local motion. The effect is present even if those amides with the extreme values of the order parameter ($S^2 < 0.77$ and $S^2 > 0.92$) are excluded: the correlation coefficient is $r = 0.62$ for both methods A and B. This effect may be caused by relaxation mechanisms/contributions not accounted for in the standard approach, eqs 1−3. For example, modulation of the strength of the ^{15}N CSA tensor and/or its orientation relative to the dipolar tensor frame, caused by local protein dynamics, or limitations of the selected simple model-free representation of the spectral density by the truncation of the order parameter series[21] may be responsible. This correlation is not intrinsic to the approaches suggested here, since a similar correlation ($r = 0.65$) holds between the CSA values derived from the η/R_2 ratio,[5] which are independent of $J(0)$, and the values of $J(0)$ derived here, which are independent of CSA. Further analysis, including complete measurements at additional fields and in other proteins, and testing various model-free models, is required to solve this issue.

(23) Fushman, D.; Cowburn, D. *J. Am. Chem. Soc.* **1998**, *120*, 7109−10.

(24) de Alba, E.; Baber, J. L.; Tjandra, N. *J. Am. Chem. Soc.* **1999**, *121*, 4282−3.

(25) Fushman, D.; Cowburn, D. Studying protein dynamics with NMR relaxation. In *Structure, Motion, Interaction and Expression of Biological Macromolecules*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: Albany, NY, 1998; pp 63−77.

Leu[8], Gly[10], and Lys[11], exhibit small but significant negative R_{ex} values (confidence level of 99%).[26] These have no apparent physical significance, and presumably are caused by accumulation of experimental errors associated with these positions, although inadequacy of the simple model-free model when applied to Leu[8], Gly[10], and Lys[11] characterized by NOEs below 0.65 cannot be excluded. The values of the order parameter determined from methods A and B are statistically comparable. The linear regression fit of $2R_2 - R_1 - 4.54d^2J(0.87\omega_H)$ vs ω_N^2 for Leu[8] and Lys[11] yielded the goodness-of-fit probability[17] below 1%. This implies that there is a less than 1% probability that these residues' data have the same underlying set of interrelationships as others. Similarly, Leu[8], Gly[10], and Lys[11] are anomalous for analysis with method A, with goodness-of-fit probability below 5% for the model-free fit. These residues provided a poor fit in the previous analysis of cross-relaxation data.[5] On one hand, most proteins appear to have a number of residues whose relaxation data are only poorly fitted by any current model, and so the current results are not exceptional; on the other hand, it remains a challenge to achieve a higher degree of completion of analysis, which would clearly be critical for useful applications of dynamic interpretation, e.g., estimates of local entropy.[27−29]

Despite the essential differences of approach between methods A and B suggested here, their results are in generally good agreement for the analyzed amide groups in ubiquitin. The agreement between the ^{15}N CSA values determined here from the slope in the $2R_2 - R_1$ vs ω_N^2 dependence (method A), from the rates of longitudinal relaxation R_1 (method B), and from those obtained using the η/R_2 values[5] appears to validate the applicability of these methods of ^{15}N CSA determination in solution, and the range of values observed. The η/R_2 and methods A and B are not fully independent because both depend on the values of R_2; however, the CSAs derived arise from η and R_1 data sets independently derived. It remains to be determined whether the ranges of ^{15}N CSA observed in ubiquitin are generally applicable to other proteins.

Previously, others[30] have used the magnetic field dependence of $2R_2 - R_1$ for spectral density mapping and for determination of R_{ex}. In that work, however, a constant value of −160 ppm was assumed for ^{15}N CSA. The values of $J(\omega_N)$ and R_{ex} reported in ref 30 are likely to be affected by the site-specific variations in the residue-specific CSA values.

There is considerable interest in using the simple analysis of spectral density components to describe protein dynamics from relaxation studies. It is well recognized that accurate derivation relies on correct estimates of contributions to relaxation from CSA and chemical exchange contributions. In combination with our previous studies,[5,23] several alternatives are now possible: (1) use of cross-correlation of CSA and dipolar contributions at multiple fields to estimate residue-specific CSA, when R_{ex} is either assumed negligible or directly measured in combination with measurements of R_1, R_2, and NOE; (2) use of method A of this paper, with similar assumptions concerning R_{ex}, and measurements of R_1, R_2, and NOE at multiple fields, or (3) the assumption of the validity of the Lipari−Szabo formalism and direct determination of residue-specific CSA and R_{ex} terms with measurements of R_1, R_2, and NOE at multiple fields. It remains to be seen which of these approaches will provide the most accurate and useful data for protein dynamics and structural investigation. Such studies, involving other proteins, as well as ubiquitin, and focused on comparison of various methods, including the traditional approach which neglects site-specific variations in ^{15}N CSA, and of various spectral density models, are currently in progress.

Conclusion. The proposed approach to protein dynamics analysis based on multiple-field relaxation data provides a direct, residue-specific determination of both the spectral density components, ^{15}N CSA and R_{ex}. This approach is demonstrated here to be applicable to the more rigid residues in ubiquitin. This applicability is significant for accurate analysis of the dynamically simplest components of the protein motion. The approach is free of any assumption about residue-specific CSA or conformational exchange. It depends, however, on the accuracy of the interpolation of $J(\omega_N)$ and hence on the Lipari−Szabo formalism for the spectral density parametrization. Caution should be exercised when this method is applied to more flexible parts of a protein, where direct contributions to spectral densities from complex local motion cannot be neglected. Further development is needed to test whether this model, incorporating physical variables associated with residue-specific CSA and chemical exchange, is more generally informative than models incorporating additional parameters describing model-free motional modes, the extended Lipari−Szabo approach.[2] The approach suggested here does not require cross-correlation measurements, and it provides a direct determination of R_{ex}, in contrast to our previous approach to CSA determination.[5] The value of ^{15}N CSA can then also be used to determine the angle θ between the N−H vector and the unique principal axis of the ^{15}N CSA tensor, from the η/R_2 ratio.[23]

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Supporting Information Available: Three tables containing the results of the analysis, one figure illustrating the agreement between the $J(\omega_N)$ values from the two methods, and two figures with comparison of the results obtained using anisotropic and isotropic models (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) Similar magnitude, negative values for apparent chemical exchange contribution have been previously reported (Figure 6 in ref 10).

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