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Deuterium Spin Probes of Backbone Order in Proteins: A ^2H NMR Relaxation Study of Deuterated Carbon α Sites

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Abstract

^2H spin relaxation NMR experiments to study the dynamics of deuterated backbone α -positions, D^α , are developed. To date, solution-state ^2H relaxation measurements in proteins have been confined to side-chain deuterons - primarily $^{13}\text{CH}_2\text{D}$ or $^{13}\text{CHD}_2$ methyl groups. It is shown that quantification of ^2H relaxation rates at D^α backbone positions and the derivation of associated order parameters of C^α - D^α bond vector motions in small [^{15}N , ^{13}C , ^2H]-labeled proteins is feasible with reasonable accuracy. The utility of the developed methodology is demonstrated on a pair of proteins - ubiquitin (8.5 kDa) at 10°C, 27°C, and 40°C, and a variant of GB1 (6.5 kDa) at 22°C. In both proteins, the D^α -derived parameters of the global rotational diffusion tensor are in good agreement with those obtained from ^{15}N relaxation rates. Semi-quantitative solution state NMR measurements yield an average value of the quadrupolar coupling constant, QCC , for D^α sites in proteins equal to 174 kHz. Using the uniform value of QCC for all D^α sites, we show that C^α - D^α bond vectors are motionally distinct from the backbone amide N-H bond vectors, with ^2H -derived squared order parameters of C^α - D^α bond vector motions, $S^2_{\text{CaD}\alpha}$, on average slightly higher than their N-H amides counterparts, S^2_{NH} . For ubiquitin, the ^2H -derived backbone mobility compares well with that found in a 1- μs molecular dynamics simulation.

Introduction

It is becoming increasingly apparent that protein function is oftentimes predicated upon the character and amplitudes of motions undergone by protein structures - that is molecular dynamics.^{1–4} As a result, the past decade has witnessed an up-surge in the development of both experimental and theoretical approaches for studying motional processes in proteins on a variety of time scales. NMR spectroscopy is a particularly powerful experimental method for studying dynamics in proteins because it provides site-specific information that covers a wide range of motional time scales.^{5–12} The amide ^{15}N nuclei are the most commonly used

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Supporting Information Available: One figure showing D^α $R^Q(D_+)$, $R^Q(D_z)$ relaxation rate profiles along with ^{15}N R_1 , R_2 profiles in GB1 at 22°C as a function of the protein sequence (Figure S1). One figure showing the dependence of $R^Q(D_+)/R^Q(D_z)$ on the angle α formed by C^α - D^α bonds and the principal axis of the diffusion tensor in GB1 at 22°C and ubiquitin at 27°C (Figure S2). One figure showing the correlation plot of the D^α quadrupolar splittings, ν_Q , and $^1\text{D}_{\text{Ca-H}\alpha}$ residual dipolar couplings measured for a subset of 11 peaks in the oriented sample of GB1 (Figure S3). One figure showing $S^2_{\text{CaD}\alpha}$ and S^2_{NH} as a function of sequence for GB1 at 22°C (Figure S4). One figure showing $S^2_{\text{CaD}\alpha}$ and S^2_{NH} for Ubiquitin at 27°C obtained using a common diffusion tensor and the QCC value of 171 kHz (Figure S5). One figure showing the agreement between $S^2_{\text{CaH}\alpha}$ and $S^2_{\text{CaC}\beta}$ values found in the 1- μs MD simulation of ubiquitin at 27°C (Figure S6). One Table listing $S^2_{\text{CaD}\alpha}$ and τ_f values obtained in ubiquitin at 10°C, 27°C and 40°C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

spin probes of backbone motions in proteins, and ^{15}N -based NMR relaxation studies of fast dynamics at backbone ^{15}N - ^1H amide positions of small- to medium-sized proteins are now largely routine.^{5,13}

The popularity of ^{15}N as a nuclear spin probe of motional processes in backbones of proteins stems from the ease and affordability of selective incorporation of ^{15}N into backbone amide positions (without concomitant ^{13}C -labeling of neighboring nuclear sites) as well as high sensitivity of ^{15}N relaxation measurements.^{5,13} The rates of decay of ^{15}N magnetization in amide moieties is expressed as the sum of dipolar (^{15}N - ^1H) and ^{15}N chemical shielding anisotropy (CSA) contributions.^{5,13,14} Quantitative interpretation of conventional ^{15}N NMR relaxation data in terms of microdynamic characteristics of fast (pico- to nanosecond) motions requires, however, prior knowledge of a number of parameters, such as (i) exact ^{15}N - ^1H bond lengths, (ii) amide ^{15}N CSA values, and (iii) contributions to the relaxation rates due to chemical exchange, R_{ex} , that, in principle, should be quantified independently. Although ^{15}N relaxation rates in small- to medium-sized proteins can be usually measured with high accuracy, the uncertainties in any or all of the above parameters leads to ambiguities in the obtained measures of backbone order expressed as generalized order parameters (amplitudes) of N-H bond vector fluctuations, S_{NH} .^{15,16} That is why significant efforts have been dedicated recently to development of NMR methodology that would provide 'exchange-free' and 'CSA-free' amide ^{15}N relaxation data in proteins.^{17,18}

In search of a 'cleaner' spin probe of backbone order in protein molecules, we designed ^2H -based spin relaxation experiments for the studies of dynamics at deuterated backbone α -positions, D^α . Deuterium relaxation is dominated by a strong and local quadrupolar interaction,¹⁹ and the knowledge of only a single parameter, the anisotropy of ^2H quadrupolar tensor or quadrupolar coupling constant (QCC), is needed to quantitatively describe D^α ^2H relaxation data in terms of order parameters of C^α - D^α bond motions, S_{CaDa} . Since the seminal introduction of deuterium relaxation measurements to the field of protein NMR by Kay and co-workers,²⁰ deuterium has been recognized as a particularly favorable probe for the studies of dynamics. Millet *et al.*²¹ and Skrynnikov *et al.*²² have demonstrated the utility of deuterium spin probes of side-chain dynamics by the measurement of five relaxation rates per deuteron in $^{13}\text{CH}_2\text{D}$ methyl groups of a 6.5-kDa protein L. The motional properties of ^{13}CHD methylene positions in N-terminal drk SH3 domain have been studied by ^2H relaxation.²³ Comparisons of ^2H -derived and ^{13}C -derived methyl three-fold axis order parameters (S^2_{axis}) in proteins have been provided.^{24,25} To date, however, ^2H relaxation measurements in proteins have been confined to side-chain deuterons, primarily methyl groups of the $^{13}\text{CH}_2\text{D}$ ^{20,21,26,27} or $^{13}\text{CHD}_2$ ^{27,28} variety. We show that the availability of increasingly sensitive NMR instrumentation allows the quantification of ^2H relaxation rates at D^α positions of protein backbones as well as the associated order parameters of C^α - D^α bond vector motions in small [^{15}N , ^{13}C , ^2H]-labeled protein molecules.

The developed NMR methodology has been applied to a pair of small proteins - an 8.5-kDa ubiquitin at three temperatures and a variant of a 6.5-kDa protein GB1. The parameters of the global molecular reorientation can be reproduced using D^α ^2H R_2/R_1 ratios, with the extracted diffusion tensors in good agreement with those derived from ^{15}N data in both proteins. Relying on a number of previous solid-state NMR measurements and our own semi-quantitative solution state results we propose the use of a uniform QCC value of 174 kHz for D^α sites in proteins. Using this QCC value, it is shown that C^α - D^α bond vectors are motionally distinct from the backbone amide N-H bond vectors. In particular, ^2H -derived S^2_{CaDa} values are similar to, but are on average slightly higher than their ^{15}N -derived S^2_{NH} counterparts. Importantly, D^α rates can sample protein backbone motions that are inaccessible through ^{15}N relaxation measurements. Although for sensitivity reasons the developed methodology is expected to be limited to small protein molecules, a number of advantages of using ^2H relaxation of D^α sites

turn it into a useful complement to the existing array of NMR techniques for quantitative studies of motional order in proteins.

Materials and Methods

NMR Samples

The following isotopically labeled samples of wild-type human ubiquitin and a variant of GB1 protein have been used in this work: (i) [U-¹⁵N]-labeled (both proteins), (ii) [U-¹⁵N, ¹³C, ²H]-labeled (both proteins), and (iii) a mixture of [U-¹⁵N, ¹³C, ²H]-labeled and [U-¹⁵N, ¹³C]-labeled GB1. The deuterated samples of both proteins have been obtained using [U-¹⁵N, ¹³C]-glucose as the main carbon source in the 99.9% D₂O-based *E. coli* media. Since carbon α positions are completely deuterated in proteins obtained using D₂O as the solvent in bacterial medium,^{29, 30} residual protonation of some aliphatic and aromatic sites is of no consequence for the present study. All samples of GB1 have been obtained using a co-expression vector where the sequence of GB1 serves as a removable tag, resulting in the addition of seven residues (-Ser-Ser-Gly-Leu-Val-Pro-Arg) to the C-terminus of the protein.

The [U-¹⁵N, ¹³C, ²H]- and [U-¹⁵N]-labeled NMR samples of human ubiquitin were 3.2 mM and 1.0 mM in protein concentration, respectively, and were dissolved in a 20 mM 90% H₂O/10% D₂O sodium phosphate buffer (pH 6.8) containing 0.03% NaN₃ and a cocktail of protease inhibitors. The [U-¹⁵N, ¹³C, ²H]- and [U-¹⁵N]-labeled samples of GB1 were 5.8 mM and 1.2 mM in protein concentration, respectively, and were dissolved in a 25 mM 90% H₂O/10% D₂O sodium phosphate buffer (pH 6.5) containing 50 mM NaCl, 0.03% NaN₃ and a cocktail of protease inhibitors. A third sample of GB1 contained a mixture of the [U-¹⁵N, ¹³C, ²H]- and [U-¹⁵N, ¹³C]-labeled protein in the approximate ratio of 4.5:1 (final concentrations of [U-¹⁵N, ¹³C, ²H]- and [U-¹⁵N, ¹³C]-GB1 of 4.5 and 1 mM, respectively), and was dissolved in a 25 mM 90% H₂O/10% D₂O sodium phosphate buffer (pH 6.5) as above to enable the measurements of D ^{α} quadrupolar splittings and ¹D_{C α -H α residual dipolar couplings (RDCs) in the same protein solution (see below).}

Experimental NMR pulse sequence details

Figure 1 shows the pulse-schemes that have been designed for the measurements of $R^Q(D_+)$, $R^Q(D_z)$, $R^Q(D_+D_z+D_zD_+)$, $R^Q(3D_z^2-2)$ at D ^{α} sites of deuterated proteins. The scheme derives from a gradient sensitivity enhanced HN(COCA) experiment³¹ and involves additional magnetization transfers to and from D ^{α} nuclei whose relaxation is measured by parametrically varying delay T . Insets A and B correspond to the measurement of $R^Q(D_+)$ and $R^Q(D_z)$, respectively. The part of the scheme that is enclosed in a dashed rectangle should be modified as it is shown in the figure to measure the relaxation of the $D_+D_z+D_zD_+(3D_z^2-2)$ elements in insets C(D). All narrow (wide) rectangular pulses are applied with the flip angles of 90°(180°) along the x -axis unless indicated otherwise. The ¹H(²H; ¹⁵N) carriers are positioned at 4.7(4.5; 119) ppm. The ¹³C carrier is placed at 177 ppm, switched to 57 ppm before the first 90° ¹³C α pulse (after the gradient g5), and switched back to 177 ppm after the last 90° ¹³C α pulse (after the gradient g5). ¹⁵N WALTZ-16³² decoupling during acquisition is achieved using a 1.25 kHz field, while ²H GARP-1³³ decoupling uses a 0.9 kHz field. ²H decoupling is interrupted for the application of gradients g6 and g8 (insets A,B).³⁴ SEDUCE³⁵ ¹³C α decoupling is implemented with 300 μ s seduce-shaped pulses applied at ¹³C α frequency by phase-modulation of the carrier.^{36,37} ¹H WALTZ-16³² decoupling is applied with a 7 kHz field. All ¹H, ²H and ¹⁵N pulses are applied with maximum possible power, while 90° (180°) ¹³C pulses shown with filled rectangles are applied with a field strength of $\Delta/\sqrt{15}(\Delta/\sqrt{3})$ where Δ is the difference in Hz between the ¹³C α and ¹³CO chemical shifts.³⁸ Vertical arrows at the beginning and the end of $2\tau_c$ periods indicate the position of the ¹³CO Bloch-Siegert shift compensation pulses.³⁸ ¹³C α shaped pulses marked with asterisks in the middle

of $2\tau_d$ periods are 350 μ s RE-BURP³⁹ pulses centered at 43 ppm by phase modulation of the carrier and cover the range of all ¹³C aliphatic chemical shifts. The ²H pulses shown with open rectangles in inset C have a flip angle of 45°. The ¹³C α pulses shown with open dashed rectangles (insets A–C) are optional. All the experiments described in this work have been performed with these pulses included. The ¹H pulse shown with an arc (preceding the gradient g3) is implemented as a water-selective 1.5 ms pulse of rectangular shape. Delays are: $\tau_a = 2.7$ ms; $\tau_b = 12.5$ ms; $\tau_c = 4.5$ ms; $\tau_d = 14$ ms; $\delta = 5.4$ ms; $\epsilon = 11$ ms; $\zeta = 7$ ms; $\Delta = 0.4$ ms. The phase-cycle is: $\phi_1 = x, -x$; $\phi_2 = 2(x), 2(-x)$ (insets A, B) and $4(x), 4(-x)$ (insets C, D); $\phi_3 = x$; $\phi_4 = 8(x), 8(-x)$; $\phi_5 = x$; $\phi_6 = 4(x), 4(-x)$ (inset A, C) and $2(0^\circ), 2(45^\circ), 2(90^\circ), 2(135^\circ)$ in inset D; $\phi_7 = 2(x), 2(y), 2(-x), 2(-y)$ (inset A) and $8(x), 8(-x)$ (inset C); $\phi_8 = x, -x, y, -y$ (inset C); rec. = $2(x, -x), 2(-x, x)$ (inset A), $x, 2(-x), x, -x, 2(x), -x$ (inset B), $x, 2(-x), x, -x, 2(x), -x, -x, 2(x), -x, x, 2(-x), x$ (inset C), $x, 2(-x), x, -x, 2(x), -x$ (inset D). Quadrature detection in t_1 is achieved via the Rance-Kay scheme: for each t_1 value a pair of spectra is recorded with ($\phi_5 = x$; g11) and ($\phi_5 = -x$; -g11) and manipulated post-acquisition.^{40,41} The phase ϕ_3 is inverted for each t_1 point.⁴² Durations and strengths of pulsed-field gradients in units of (ms; G/cm) are: g1 = (1; 15); g2 = (0.25; 5); g3 = (1.2; 12); g4 = (0.5; 8); g5 = (0.6; 10); g6 = (0.3; 5); g7 = (0.7; 15); g8 = (0.35; 5); g9 = (0.5; 12); g10 = (0.4; 10); g11 = (1.25; 20); g12 = (0.25; 8); g13 = (0.3; 5); g14 = (0.0625; 20); g15 = (0.0625; -20); g7' = (0.6; 10); g8' = (0.8; 12).

D α and ¹⁵N Relaxation Measurements

All ²H and ¹⁵N spin relaxation experiments were performed on a 600 MHz Bruker Avance III spectrometer equipped with a room temperature triple-resonance z-gradient probe. NMR data sets recorded with the pulse scheme of Figure 1 (D α , [U-¹⁵N, ¹³C, ²H]-labeled samples) comprised [512, 40] complex points in the [¹HN, ¹⁵N] dimensions with corresponding acquisition times of [64 ms, 24 ms]. Typically, a recovery delay of 1.5 s was used along with 128 scans/FID giving rise to net acquisition times of ~4.8 hr./experiment. $R^Q(D_+)$ rates in ubiquitin samples were recorded with parametrically varied delays T (inset A in Figure 1) of (0.02; 0.2; 0.4; 0.6; 0.8; 1.2; 1.6) ms, (0.02; 0.3; 0.6; 0.9; 1.2; 1.6; 2.0) ms, and (0.02; 0.2; 0.4; 0.6; 0.8; 1.2; 1.6) ms at 10°C, 27°C and 40°C, respectively, while $R^Q(D_z)$ rates (Figure 1, inset B) were recorded with parametrically varied delays T of (0.02; 2.0; 4.0; 6.0; 8.0; 10.0; 12.0; 14.0) ms, (0.02; 1.0; 2.0; 3.0; 4.0; 6.0; 8.0; 10.0) ms, and (0.02; 1.0; 2.0; 3.0; 4.0; 5.0; 6.0; 8.0) ms at 10°C, 27°C and 40°C. $R^Q(D_+)$, $R^Q(D_z)$, $R^Q(D_+D_z+D_zD_+)$, $R^Q(3D_z^2-2)$ relaxation rates of α deuterons in GB1 have been measured at 22°C using the delays T of (0.02; 0.2; 0.4; 0.6; 0.8; 1.2; 1.6; 2.0) ms, (0.02; 2.0; 4.0; 6.0; 8.0; 10.0; 13.0) ms, (0.02; 0.2; 0.4; 0.6; 0.8; 1.2; 1.6; 2.0) ms, and (0.02; 1.0; 2.0; 3.0; 4.0; 6.0; 8.0; 10.0) ms, respectively. All NMR spectra were processed using the NMRPipe/NMRDraw suite of programs⁴³ and associated software. Rates were obtained by fitting peak intensities to a single exponential function of the form $I = I_0 \exp(-RT)$, where I is the measured intensity and R is the relaxation rate. Errors in peak intensities have been estimated from duplicate measurements or from the noise-floor level of the spectra, whichever was the highest, and subsequently propagated to the errors in the extracted rates using Monte-Carlo analysis.⁴⁴ The estimated errors in $R^Q(D_+)$ ($R^Q(D_z)$) measurements are on the order of 2.0% (1.5%) on average in ubiquitin.

¹⁵N $R_{1\rho}$, R and ¹H-¹⁵N NOE measurements on [U-¹⁵N]-labeled samples of both proteins have been performed using standard procedures.^{45,46} Typically, NMR data sets comprised [512, 64] complex points in the [¹HN, ¹⁵N] dimensions with corresponding acquisition times of [64 ms, 38 ms]. Recovery delays of 1.0s and 4.3s (including a 2s presaturation period) for ¹⁵N $R_{1\rho}$, R and ¹H-¹⁵N NOE measurements respectively, along with 16 scans/FID gave rise to respective net acquisition times of ~0.7 and 2.5 hr./experiment. The spin-lock field strength of 1.9 kHz was used in ¹⁵N $R_{1\rho}$ measurements. $R_{1\rho}$ data have been subsequently corrected for resonance offset to obtain ¹⁵N R_2 rates.⁵ Error analysis of peak intensities and the extracted relaxation rates closely followed the procedure used for ²H relaxation measurements at D α positions.

The relaxation rates of $N_z C'_z C_z^\alpha$ 3-spin order, $R(N_z C'_z C_z^\alpha)$, have been measured using a pulse-scheme derived from the gradient sensitivity enhanced HN(COCA) experiment³¹ using the same acquisition parameters as for ^2H relaxation measurements. A recovery delay of 1.0 s was used along with 16 scans/FID giving rise to net acquisition times of ~ 30 min./experiment. The relaxation delays T of (0.01; 50; 100; 150; 200; 300; 350; 400; 450; 500; 600) ms have been typically used for both proteins at all temperatures. The extracted $R(N_z C'_z C_z^\alpha)$ rates have been subsequently subtracted from the obtained $D^\alpha R^Q[D]$ rates before analysis (see text).

Data Analysis

The relaxation rates of ^2H transverse and longitudinal magnetization are given by¹⁹

$$R^Q(D_+) = R_2^Q = \frac{\pi^2}{20} \left(\frac{e^2 q Q}{h} \right)^2 [9J(0) + 15J(\omega_D) + 6J(2\omega_D)] \quad (1)$$

$$R^Q(D_z) = R_1^Q = \frac{3\pi^2}{10} \left(\frac{e^2 q Q}{h} \right)^2 [J(\omega_D) + 4J(2\omega_D)], \quad (2)$$

where $(e^2 q Q/h)$ is the quadrupolar coupling constant, QCC , and $J(\omega_D)$ is the spectral density function evaluated at ω_D frequency. A uniform value of $QCC = 174$ kHz has been used for α deuterons in this work (see text). It is usually assumed that the ^2H electric field gradient tensor is axially symmetric with its principal axis parallel to the direction of the deuteron bond, so that only the anisotropy of the tensor, $(e^2 q Q/h) = QCC$, enters into eq 1–2. Indeed, the asymmetry values of the ^2H tensor, η , in aliphatic deuterons are known to be < 0.04 .^{47,48} If the assumption of axial symmetry is dropped, the relaxation rates in eq 1–2 above should be

multiplied by $\left(1 + \frac{\eta^2}{3}\right)$, that would contribute $< 0.06\%$ to $R(D_+)$ and $R(D_z)$. Clearly, such small contributions are well within the errors of the relaxation rate measurements and can be safely neglected.

All ^2H data have been analyzed using the following Lipari-Szabo model-free spectral density function for the axially symmetric molecular tumbling,^{15,16,49}

$$J(\omega) = S_{CaD\alpha}^2 \left(\frac{A_1 \tau_1}{1 + (\omega \tau_1)^2} + \frac{A_2 \tau_2}{1 + (\omega \tau_2)^2} + \frac{A_3 \tau_3}{1 + (\omega \tau_3)^2} \right) + \left(1 - S_{CaD\alpha}^2 \right) \frac{\tau'}{1 + (\omega \tau')^2}, \quad (3)$$

where $S_{CaD\alpha}$ is the generalized order parameter describing the fluctuations of C^α - D^α bond vectors, $A_1 = (3/4)\sin^4(\alpha)$, $A_2 = 3\sin^2(\alpha)\cos^2(\alpha)$, $A_3 = [(3/2)\cos^2(\alpha) - 0.5]^2$, $\tau_1 = (4D_{\parallel} + 2D_{\perp})^{-1}$, $\tau_2 = (D_{\parallel} + 5D_{\perp})^{-1}$, $\tau_3 = (6D_{\perp})^{-1}$, D_{\parallel} and D_{\perp} are the parallel and perpendicular components of the molecular diffusion tensor, α is the angle between the C^α - D^α bond vector (assumed collinear with C^α - H^α bond vector in protein structures) and the unique diffusion axis, and $1/\tau' = 1/\tau_f + 1/\tau_{c,eff}$ with $\tau_{c,eff} = (2D_{\parallel} + 4D_{\perp})^{-1}$ the effective correlation time of overall rotation and τ_f the correlation time of fast local motions. Direction cosines for the C^α - H^α vectors of ubiquitin and GB1 have been obtained from the x-ray structures with the respective PDB accession codes 1ubq⁵⁰ and 2qmt⁵¹.

Diffusion tensors have been estimated by minimization of the error function χ^2 expressed as,

$$\chi^2 = \sum_{i=1}^N \left| \frac{(R_2^{expt}/R_1^{expt})_i - (R_2^{calc}/R_1^{calc})_i}{(\sigma_{R_2/R_1})_i} \right|^2, \quad (4)$$

where the summation extends over all sites included in analysis, R_2^{expt} and R_1^{expt} are the experimentally measured $^2\text{H}/^{15}\text{N}$ rates, R_2^{calc} and R_1^{calc} are the rates calculated using diffusion tensor parameters (eq 3) and the expressions for R_2^Q , R_1^Q (eq 1–2) $^{15}\text{N}R_2$, R_1 rates, and σ_{R_2/R_1} is the uncertainty in the experimental R_2/R_1 ratios. A fit to a fully anisotropic diffusion tensor⁵² was not warranted for either protein because of relatively high errors in extracted $D^\alpha R^Q[D]$ relaxation rates that preclude a statistically significant differentiation between the axially symmetric and fully anisotropic models. The diffusion tensor parameters obtained by minimization of χ^2 (eq 4) are sensitive to the choice of residues whose dynamics is properly described by the first term of eq 3. Therefore, in both proteins only the residues in the secondary structure elements have been used. Likewise, the residues with missing coordinates (C-terminus of GB1), $^1\text{H}-^{15}\text{N}$ NOE < 0.6 and the residues undergoing chemical exchange (^{15}N data only) have been excluded from analysis. Errors in the fitted diffusion parameters (polar angles θ , ϕ describing the orientation of the unique axis of the diffusion tensor with respect to the inertial frame, and $D_{||}$, D_{\perp}) were estimated using 300 Monte-Carlo simulations⁴⁴ with random additions of experimental errors to the measured rates. The standard deviations in the fitted parameters were used as uncertainties in diffusion parameters.

$D^\alpha(^{15}\text{N})$ relaxation rates have been interpreted in terms of motional parameters using their corresponding ^2H -(^{15}N)-derived diffusion tensor characteristics. Motional parameters of D^α sites, $S^2_{CaD\alpha}$ and the corresponding τ_f values, were obtained by fitting the ^2H relaxation data to the corresponding expressions for relaxation rates (eq 1–2) using eq 3 for the spectral density function (see 'Results and Discussion'), whereas the motional parameters of N-H bond vectors, S^2_{NH} and τ_f , were obtained using the program Dynamics^{53,54} that models ^{15}N relaxation data selecting for the appropriate motional model (that can be different from the simplest form of eq 3) based on statistical criteria. ^{15}N relaxation data have been analyzed with standard expressions for ^{15}N relaxation in $^{15}\text{N}-^1\text{H}$ spin pairs. ^{15}N R_2, R_1 and $^1\text{H}-^{15}\text{N}$ NOE data were included in analysis that used the spectral density function of eq 3 where the squared order parameter describing the fluctuations of the backbone amide N-H bond vector, S^2_{NH} , was used instead of $S^2_{CaD\alpha}$ together with the corresponding N-H direction cosines. The N-H bond distance of 1.02 Å and a uniform ^{15}N CSA of -170 ppm were used in all calculations.

D^α quadrupolar splitting and $^1D_{C\alpha-H\alpha}$ measurements in oriented GB1

Analysis of $D^\alpha QCC$ closely followed the work of Mittermaier and Kay where QCC values have been determined for methyl deuterons using the ratios of quadrupolar splittings, ν_Q , and $^{13}\text{C}^{\text{methyl}}$, ^{13}C RDCs measured in an oriented protein.⁵⁵ The same approach can be used for α deuterons provided that $^1D_{C\alpha-H\alpha}$ RDCs are measured in the same sample. To enable the measurements of D^α splittings and $^1D_{C\alpha-H\alpha}$ RDCs in the same protein sample, a mixture of $[\text{U}-^{15}\text{N}, ^{13}\text{C}, ^2\text{H}]$ -labeled and $[\text{U}-^{15}\text{N}, ^{13}\text{C}]$ -labeled GB1 was used (see 'NMR samples' above). The use of the same sample with two different labeling schemes (one having ^2H nuclei and the other ^1H nuclei at α positions) ensures that the alignment characteristics and dynamical properties are exactly the same for the ν_Q and $^1D_{C\alpha-H\alpha}$ measurements. The 'mixed' GB1 sample was aligned in 18 mg/ml pf1 bacteriophage⁵⁶ (quadrupolar D_2O splitting of 24.7 Hz). $^1D_{C\alpha-H\alpha}$ RDCs have been obtained using the HNCO-type experiment of Yang *et al.*⁵⁷ modified to eliminate the resonances of $^{13}\text{C}^\alpha$ nuclei attached to deuterons arising from the (more concentrated) $[\text{U}-^{15}\text{N}, ^{13}\text{C}, ^2\text{H}]$ -labeled protein. This experiment allows for convenient

measurements of $^1D_{C\alpha-H\alpha}$ in H₂O protein solutions by recording the modulation of carbonyl chemical shifts by ($^1J_{C\alpha-H\alpha} + ^1D_{C\alpha-H\alpha}$) couplings (see *ref.*⁵⁷). The quadrupolar splittings ν_Q in α deuterons have been obtained using the scheme of Figure 1 (inset A) with parametrically varied delays T of (0.02; 0.4; 1.0; 1.1; 1.6; 2.0; 2.3; 2.8; 3.1; 3.5; 4.0; 4.3; 4.8; 5.1; 5.5; 6.0; 6.3; 6.8; 7.1; 7.6; 8.0; 8.6) ms. The $^1D_{C\alpha-H\alpha}$ and ν_Q measurements have been performed twice and the obtained $\nu_Q/{}^1D_{C\alpha-H\alpha}$ ratios have been averaged.

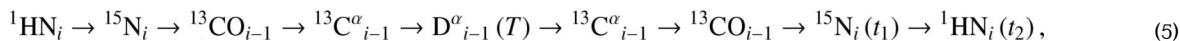
Molecular dynamics simulations

A 1 μ s molecular dynamics (MD) trajectory of ubiquitin was performed as described elsewhere.⁵⁸ Briefly, the MD simulation was performed using the AMBER 9 package⁵⁹ with the AMBER99SB force field,⁶⁰ which was shown previously to accurately reproduce the native state dynamics of ubiquitin.^{59,61–63} The SHAKE algorithm⁶⁴ was employed to constrain all bonds involving hydrogen atoms, and a time step of 2 fs was used. Non-deuterated ubiquitin was embedded in a cubic box with SPC/E water models and long-range electrostatic interactions were handled using the PME method⁶⁵ with an 8 Å cutoff. The starting coordinates were taken from the crystal structure of ubiquitin (PDB entry 1ubq), and the simulation was run for 1000 ns at 300 K under NPT conditions after application of standard minimization and heating protocols. The values of $S^2_{C\alpha H\alpha}$ have been extracted using the iRED method⁶⁶ averaged over time windows of 1 ns and 5 ns, *i.e.* on the order of the experimental global tumbling correlation time of ubiquitin.

Results and Discussion

NMR experiments for the measurement of 2H relaxation rates at D^α sites of proteins

Figure 1 shows the HN(COCA)D experiment used for the measurements of relaxation rates at D^α sites. The pulse scheme is derived from the gradient sensitivity-enhanced HNCOCA experiment³¹ with additional delays at $^{13}C^\alpha$ nuclei to allow the magnetization to evolve to and from D^α positions whose relaxation is measured during time period T (see 'Materials and Methods' for the pulse-scheme details). The transfer of magnetization can be summarized by,



where the transfer from one spin to the next is achieved via one-bond scalar couplings and t_1, t_2 are acquisition times. A series of two-dimensional data sets is recorded as a function of T . The cross-peaks obtained at the frequencies ($\omega_{N_i}; \omega_{HN_i}$) decay with the relaxation rates of α -deuterons belonging to the previous residue, D^α_{i-1} . A region of such a 2D spectrum recorded on ubiquitin at 27°C using the pulse scheme of Figure 1 (inset A, $T = 0$) is shown in Figure 2a. Relaxation rates can be extracted directly by fitting the intensity of each correlation to a single-exponential decay curve (Figures 2b–c). Since in glycines the magnetization is transferred from α carbons to *both* α -deuterons, the relaxation rates of the two (non-equivalent) Gly D^α positions can not be separated. Therefore, glycine residues (*i.e.* the amide correlations of the residues following glycines) have been excluded from analysis in this work. Likewise, D^α relaxation rates of residues preceding prolines can not be quantified using the experiment of Figure 1. The relaxation rates of proline D^α sites can be measured, however, through the amide correlation of the following (non-proline) residue. Although, as shown below, robust measures of backbone dynamics can be obtained from D^α relaxation rates, the D^α relaxation rate measurements using the scheme of Figure 1 are associated with very significant sensitivity losses compared to conventional ^{15}N relaxation experiments. Taking into account different sample concentrations and acquisition times used for each time-point in the $D^\alpha/^{15}N$ rate measurements (see 'Materials and Methods'), we estimate that the scheme of Figure 1 is

approximately 20 times less sensitive than ^{15}N relaxation experiments necessitating the use of more concentrated protein samples and/or cryogenically cooled probes for data collection.

D^α relaxation rates

The R_1 and R_2 relaxation rates of D^α nuclei, $R^Q(D^+)$ and $R^Q(D_z)$, have been measured using the experiments shown in insets A and B of Figure 1, respectively. Both experiments measure the relaxation rates of magnetization terms $N_z C'_z C^\alpha_z [D]$, where $[D]$ is any deuterium spin operator and A_z is a longitudinal spin operator of nucleus A (carbonyl/carbon- α of residue $i-1$ and nitrogen of residue i). As is the case in the previous deuterium relaxation studies,^{20,21,67} it can be shown that to a very good approximation $R(N_z C'_z C^\alpha_z [D]) = R(N_z C'_z C^\alpha_z + R[D])$, and to obtain a 'pure' deuterium relaxation rate it is sufficient to subtract $R(N_z C'_z C^\alpha_z)$ from the rates obtained in the experiments of Figure 1. In practice, however, the rates $R(N_z C'_z C^\alpha_z)$ are very small compared to $R(N_z C'_z C^\alpha_z [D])$. For example, the $R(N_z C'_z C^\alpha_z D_+)$ rates measured in ubiquitin vary between 372 and 1042(321 and 675; 370 and 569) s^{-1} at 10(27;40) $^\circ\text{C}$, whereas $R(N_z C'_z C^\alpha_z D_z)$ rates in ubiquitin at 10(27;40) $^\circ\text{C}$ vary between 62 and 115(90 and 129; 119 and 153) s^{-1} . The rates $R(N_z C'_z C^\alpha_z)$ measured as described in 'Materials and Methods' vary between 2.1 and 2.8(2.1 and 3.8; 1.9 and 4.0) s^{-1} at 10(27;40) $^\circ\text{C}$. Clearly, these rates are within measurement errors of $R(N_z C'_z C^\alpha_z D_+)$. This is, however, not necessarily the case for $R(R(N_z C'_z C^\alpha_z D_z))$ measurements - although the corrections remain small. To avoid introduction of systematic errors to the quantified deuterium relaxation rates, the $R(N_z C'_z C^\alpha_z)$ values have been subtracted on a residue-specific basis from all measured D^α rates in this work. It is important to emphasize that ^2H relaxation of backbone D^α sites is completely dominated by the strong quadrupolar interaction, as is also the case in the previous ^2H relaxation studies of $^{13}\text{CH}_2\text{D}^{20,21,27,68}$ and $^{13}\text{CHD}_2^{27,28}$ methyl groups, ^{13}CHD methylene sites in proteins²³ as well as deuterated sugars and bases of RNA molecules.⁶⁷ For example, calculations using typical sets of motional parameters obtained in this work for proteins tumbling with isotropic correlations times in the range between 2 and 10 ns show that the maximal contribution of the dipolar interaction of D^α deuterons with the directly bonded $^{13}\text{C}^\alpha$ spins to transverse (longitudinal) D^α relaxation rates $D_+(D_z)$ is 1.0(0.25) s^{-1} . Evidently, such small contributions are within the errors of D^α relaxation rate measurements and can be safely ignored.

Figure 3 shows the profiles of experimental $R^Q(D_z)$ and $R^Q(D_+)$ values obtained in ubiquitin at 10 $^\circ\text{C}$ (Figure 3a,d), 27 $^\circ\text{C}$ (Figure 3b,e) and 40 $^\circ\text{C}$ (Figure 3c,f). For comparison, ^{15}N R_1 and R_2 rate profiles are included in the same plots. The average $R^Q(D_+)$ rates measured at 10(27;40) $^\circ\text{C}$ in ubiquitin are 880(597;500) s^{-1} , whereas average $R^Q(D_z)$ rates are 72(107;132) s^{-1} . Higher than average degree of backbone flexibility is manifested in decreased $R^Q(D_+)$ values in (i) the loop between residues Thr⁷ and Lys¹¹ with average $R^Q(D_+)$ of 813(571) s^{-1} at 10(27) $^\circ\text{C}$ (Figures 3d and 3e; the residues in this region could not be reliably quantified at 40 $^\circ\text{C}$); (ii) near the Lys⁴⁸ polymerization site (residues Ala⁴⁶-Lys⁴⁸) at all temperatures with average $R^Q(D_+)$ values of 855(566;467) s^{-1} at 10(27;40) $^\circ\text{C}$, and (iii) the C-terminus starting from Arg⁷² (Figures 3 d-f). These trends are largely mirrored by ^{15}N R_2 rates. Interestingly, $R^Q(D_z)$ profiles show that higher disorder at the C-terminus is accompanied by an increase in $R^Q(D_z)$ rates while ^{15}N R_1 rates decrease for the same residues (Figures 3a-c; some $R^Q(D_z)$ rates could not be quantified at the C-terminus at 40 $^\circ\text{C}$ because of resonance overlap). Notably, the $R^Q(D_z)$ and $R^Q(D^+)$ rates of Gly D^α positions have to be excluded from the plots in Figure 3 resulting in a loss of information as some of Gly residues in ubiquitin (e.g. Gly¹⁰ and Gly⁴⁷) fall in the regions of increased backbone flexibility according to ^{15}N data. It is apparent from Figures 3d-f that chemical exchange on the micro-to-millisecond time-scale - most notably affecting the amide of Asn²⁵ with ^{15}N R_2 rate of 17.2(8.6;5.8) s^{-1} , while the average ^{15}N R_2 rates in ubiquitin are 9.0(6.0;4.6) s^{-1} at 10(27;40) $^\circ\text{C}$ - does not affect the D^α rates to any significant extent. Large exchange contributions to R_2 relaxation rates can significantly complicate ^{15}N relaxation analysis, while D^α relaxation data (with the exception of double-

quantum coherences, D_+^2 , *vide infra*) remain unaffected by contributions from chemical exchange.

Because the deuteron is a spin 1 nucleus, a total of five relaxation rates can be measured at D^α sites. As described by Millet *et al.*²¹ for the case of methyl groups, in addition to 'rank-1' coherences D_+ and D_z , the relaxation rates of 'rank-2' elements ($D_+D_z+D_zD_+$, $3D_z^2-2$ and D_+^2) can be quantified in the same ^2H site. Indeed, one of the principal advantages of ^2H spin relaxation lies in the possibility to assess the self-consistency of the obtained rates prior to analysis in terms of motional parameters. Jacobsen and co-workers⁶⁹ have shown that so long as $J(0) \geq J(\omega_D) \geq J(2\omega_D)$, where $J(\omega_D)$ is the spectral density function evaluated at the ^2H Larmor frequency, ω_D , (see 'Materials and Methods') the following inequalities must hold: $(5/3)R^Q(D_+D_z+D_zD_+) \geq R^Q(D_+) \geq (5/3)R^Q(3D_z^2-2) \geq R^Q(D_z)$, where the relaxation rates of anti-phase ^2H magnetization, ($D_+D_z+D_zD_+$), and the quadrupolar order, ($3D_z^2-2$), are given by,

$$R^Q(D_+D_z+D_zD_+) = \frac{\pi^2}{20} \left(\frac{e^2qQ}{h} \right)^2 [9J(0) + 3J(\omega_D) + 6J(2\omega_D)] \quad (6)$$

$$R^Q(3D_z^2-2) = \frac{3\pi^2}{10} \left(\frac{e^2qQ}{h} \right)^2 [3J(\omega_D)] \quad (7)$$

The pulse-scheme that can be used for $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ measurements at D^α positions of proteins are shown in insets C and D of Figure 1, respectively. In contrast to ^2H relaxation studies of methyl deuterons, however, the $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ measurements at backbone D^α sites suffer from very poor sensitivity because of fast ^2H spin-flips occurring during prolonged $^{13}\text{C}^\alpha_{i-1} \rightarrow D_{i-1}(T) \rightarrow ^{13}\text{C}^\alpha_{i-1}$ magnetization transfer steps (two time periods $\tau_d + \zeta$ in Figure 1, insets C–D). As a result, it was not possible to measure $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ of D^α nuclei in ubiquitin within a reasonable time frame. Nevertheless, we have been able to quantify the $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ rates in a more concentrated sample of a smaller protein GB1. Figure 4 shows that the inequalities $(5/3)R^Q(3D_z^2-2) \geq R^Q(D_z)$ (Figure 4a) and $(5/3)R^Q(D_+D_z+D_zD_+) \geq R^Q(D_+)$ (Figure 4b) are indeed satisfied without a single exception in GB1 at 22°C representing a useful semi-quantitative consistency test of the experimental raw data. The profiles of $R^Q(D_+)$, $R^Q(D_z)$ and ^{15}N R_2, R_1 rates measured in GB1 are compared in Figure S1 of the Supporting Information. Notably, the flexibility of the backbone in the loop between Gly⁹ and Leu¹³ and the C-terminus of GB1 is 'picked-up' by D^α relaxation data with decreased $R^Q(D_+)$ and $R^Q(D_+D_z+D_zD_+)$ rates in these regions (Figure 4b and Figure S1 of the Supporting Information). Unfortunately, the presence of two glycine residues (Gly³⁸ and Gly⁴¹) prevents the quantification of D^α rates in another flexible loop of GB1 extending from Asn³⁷ to Gly⁴¹ as it is apparent from ^{15}N R_1, R_2 rate profiles (Figure S1b).

As described above, fast ^2H spin-flips lead to on average 4-to-5-fold sensitivity losses in the $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ experiments (Figure 1, insets C–D) compared to the $R^Q(D_+)$ measurements (Figure 1, inset A) in GB1 at 22°C. This situation is similar to ^2H relaxation measurements in the sugar and base moieties of RNA reported earlier.⁶⁷ Because of low sensitivity of 'rank-2' relaxation measurements and, as a result, substantial errors in the derived $R^Q(D_+D_z+D_zD_+)$ and $R^Q(3D_z^2-2)$ rates, these data have not been used in further analysis. Mainly for the same reasons, no attempt has been undertaken to measure the relaxation rate of the fifth double-quantum ^2H coherence, D_+^2 , at D^α sites.

D^α relaxation derived diffusion tensor parameters

Prior to obtaining the ²H-derived measures of backbone order it is important to establish the diffusion parameters of the global molecular reorientation. In analogy to the case of ¹⁵N relaxation in protein backbones, ²H R_2/R_1 ratio, $R^Q(D_+)/R^Q(D_z)$, is, to a good approximation, independent of the amplitude and time-scale of rapid internal motions.^{13,52} It therefore serves as a good measure of the rate at which the C^α-D^α vector reorients with global tumbling.⁷⁰ Table 1 compares the parameters of the (axially symmetric) diffusion tensors derived from D^α $R^Q(D_+)/R^Q(D_z)$ and ¹⁵N R_2/R_1 ratios for ubiquitin at three temperatures (see 'Materials and Methods'). Since the diffusion tensor of ubiquitin has a relatively low diffusion anisotropy, we have chosen to test whether the $R^Q(D_+)/R^Q(D_z)$ ratios can reproduce a significantly more anisotropic reorientation on the sample of GB1 modified at the C-terminus (Table 1). Figure S2 of the Supporting Information shows the plots of $R^Q(D_+)/R^Q(D_z)$ as a function of the angle α that each of the C^α-D^α bond vectors subtends with respect to the unique axis of the diffusion tensor, along with the best fits of the data for GB1 (22°C) and ubiquitin (27°C). Although the D^α-derived orientations of the principal axes (polar angles θ and ϕ in the inertial coordinate frame) are less well defined in comparison to ¹⁵N-derived values, a good agreement between the ¹⁵N- and D^α-derived tensors is observed in all cases. The ratios of $\tau_{c,eff} = (2D_{\parallel} + 4D_{\perp})^{-1}$ values obtained at 10, 27 and 40°C in ubiquitin are very close to those expected based on the Stokes-Einstein relationship. Despite the fact that D^α relaxation rates in ubiquitin have been obtained using a 3-fold more concentrated protein sample than ¹⁵N rates, the D^α-derived values of $\tau_{c,eff}$ in ubiquitin are somewhat lower (3.6%, 5.6% and 2.8% at 10°C, 27°C and 40°C, respectively) than their ¹⁵N-derived counterparts at the same temperature (Table 1). Interestingly, in a recent study exchange-free, purely dipolar contributions to ¹⁵N relaxation rates, R_{dd} , in ubiquitin have been derived.¹⁷ These exchange-free rates indicated the presence of non-zero R_{ex} contributions to ¹⁵N $R_{1\rho}$ rates for many residues in ubiquitin.¹⁷ This implies that D^α $R^Q(D_+)/R^Q(D_z)$ ratios that are not affected by chemical exchange to any significant degree might serve as more robust estimates of the global reorientation parameters in small proteins.

The choice and estimation of D^α QCC values

Although the value of D^α QCC is not needed for determination of the diffusion tensor, the knowledge of its accurate value is of paramount importance for interpretation of D^α relaxation rates in terms of motional parameters. Several solid-state NMR studies have reported the values of QCC for aliphatic deuterons attached to sp³-hybridized carbons ranging from 168 ± 2 to 174 ± 2 kHz.⁷¹ Solid-state NMR measurements of Haerberlen and co-workers provided the QCC values of the two α -deuterons in zwitterionic glycine equal to 159.9 and 169.4 kHz.⁷² The authors explained the large difference between the two values by formation of weak C-H...O hydrogen bonds leading to a decrease of QCC in one of the two α -deuterons. Notably, oxygen acceptors are located in an adjacent layer of glycine molecules⁷² - a situation that cannot be encountered in solution. In another low-temperature liquid crystal NMR study, the QCC value obtained for deuterons in a CD₃ methyl group of toluene (165 kHz) has been compared to the QCC of deuterons in cyclohexane-d₁₂ (174 ± 2 kHz). The two values can be brought into exact agreement if the tetrahedral angle θ of the methyl group is assumed to be equal to 111° instead of 109.5°. ⁷³ Interestingly, using solution NMR techniques Mittermaier and Kay determined the QCC value of deuterons in ¹³CH₂D methyl groups of proteins equal to 167 ± 1 kHz assuming $\theta = 109.5^\circ$.⁵⁵ What is effectively determined in this study is the product $P_2(\cos\theta)QCC$, where $P_2(x) = 0.5(3x^2 - 1)$ is the second order Legendre polynomial. If the value of θ is increased by only 1°, ⁷⁴ the methyl QCC increases to 174 kHz. We note here that what is normally determined in single-crystal NMR measurements (unless they are performed at very low temperatures) is the product $\sqrt{S^2}QCC$, where S is the order parameter of local motions. Here, local motions are assumed to be axially symmetric, and S includes the effects of slow motions and (potentially small) contributions from 'rocking' motions of the molecule relative

to the crystal lattice. If S^2 is assumed to be equal to 0.95,^{75,76} then the measurements of Haeberlen and co-workers in α -glycine⁷² yield the value of $QCC = 173.8$ kHz for the non-hydrogen bonded deuteron.

To obtain an independent estimate of QCC of α -deuterons in proteins we followed the approach of Mittermaier and Kay.⁵⁵ Briefly, in an oriented protein, during the period T in the pulse-scheme of Figure 1 (inset A) the ^2H magnetization evolves according to^{55,77}

$$D_y \rightarrow D_y \cos(\pi \nu_Q T) - \{D_x D_z + D_z D_x\} \sin(\pi \nu_Q T), \quad (8)$$

, where the quadrupolar splitting $\nu_Q = \frac{3}{4} QCC \langle 3 \cos^2 \theta - 1 \rangle$, θ is the angle between the principal axis of the electric field gradient tensor and the magnetic field, and the brackets $\langle \rangle$ denote ensemble averaging. The relaxation rates of D_y and $(D_x D_z + D_z D_x)$ coherences are described by eq 1 (the rate of in-phase ^2H magnetization, R_I) and eq 6 (the rate of anti-phase ^2H magnetization, R_A), respectively. Including relaxation during the period T , the evolution of the D_y magnetization (that is detected in the end of the experiment) is described by⁵⁵

$$D_y(T) = \frac{A}{2} \exp\left\{-\frac{(R_I + R_A)}{2} T\right\} \left[(1 - \Delta) \exp\left\{\frac{\Omega}{2} T\right\} + (1 + \Delta) \exp\left\{-\frac{\Omega}{2} T\right\} \right], \quad (9)$$

where $\Omega = \sqrt{(R_I - R_A)^2 - 4\pi^2 \nu_Q^2}$ and $\Delta = (R_I - R_A)/\Omega$. In contrast to the study of methyl groups,⁵⁵ here, the average rate of magnetization decay, $(R_I + R_A)/2$, is 2-to-3 fold higher than the maximal achievable quadrupolar splitting ν_Q , making the analysis of the decay of D_y (eq 9) extremely unreliable except for a (small) subset of residues where ν_Q is close to its maximal value and the zero-crossing of the magnetization decay can be achieved experimentally. In pf1 phage-aligned, highly concentrated sample of GB1 (see 'Materials and Methods'), a small subset of 11 peaks has been chosen where the zero-crossing of the D_y magnetization decay is apparent and where the extraction of a reliable albeit inexact value of ν_Q is still feasible. Although for this subset of residues the condition $(2\pi \nu_Q)^2 \gg (R_I - R_A)^2$ is always satisfied, simulations using synthetic data and average R_I , R_A rates and ν_Q values for GB1 showed that the fitting of the decay function D_y to a simpler 3-parameter expression with a single effective $(R_I + R_A)/2$ rate⁷⁸ would produce errors in the derived ν_Q values of 5 to 8%. We have chosen therefore to fit the decay curves to the full expression in eq 9 above with four variable parameters (R_I , R_A , A and ν_Q). Two examples of such fits for Leu¹² and Asp⁴⁰ from the chosen subset of 11 peaks in GB1 are shown in Figures 5a–b.

The value $^1D_{C\alpha-H\alpha}$ RDC measured as described in 'Materials and Methods' is expressed as, $^1D_{C\alpha-H\alpha} = -(\mu_0/4\pi) (\gamma_C \gamma_H h / 4\pi^2 r_{C\alpha H\alpha}^3) \langle 3 \cos^2 \theta - 1 \rangle$, where γ_i is the gyromagnetic ratio of nucleus i , μ_0 is the vacuum permeability constant, and r_{CH} is the distance between C^α and H^α nuclei. Figures 5c–d show the results of $^1D_{C\alpha-H\alpha}$ measurements for the same pair of residues in GB1 as in Figures 5a and 5b, respectively. As shown by Mittermaier and Kay for methyl groups, the ratio of $\nu_Q / ^1D_{C\alpha-H\alpha}$ taken for the same α position, is independent of magnetic alignment ($\langle 3 \cos^2 \theta - 1 \rangle$) and local motions.⁵⁵ QCC value can then be obtained from the $\nu_Q / ^1D_{C\alpha-H\alpha}$ ratio following the relationship,

$$QCC = \frac{1}{12} \frac{\mu_0 \gamma_C \gamma_H h}{\pi^3 r_{C\alpha H\alpha}^3} \left(\frac{\nu_Q}{^1D_{C\alpha-H\alpha}} \right) \quad (10)$$

Note that unlike in the case of methyl groups,⁵⁴ according to eq 10, $D^\alpha QCC$ does not depend on dihedral angles. The correlation of the 2H quadrupolar splitting ν_Q with the $^1D_{C\alpha-H\alpha}$ dipolar coupling for the subset of 11 peaks in GB1 is shown in Figure S3 of the Supporting Information. Linear regression analysis of this correlation provided a slope of -5.66 ± 0.34 (Pearson $R = 0.979$) when the (statistically insignificant) intercept was fixed at zero. The sign of ν_Q can not be determined using this procedure, and ν_Q values have been assumed to have a sign opposite to that of the measured $^1D_{C\alpha-H\alpha}$. Using eq 10 and assuming a standard r_{CH} distance of 1.095 \AA ^{79–81} we obtain the $D^\alpha QCC$ value of 173.7 kHz. Notably, any effects of local dynamics are cancelled out by taking the ratio of ν_Q and $^1D_{C\alpha-H\alpha}$ allowing one to use the value of r_{CH} distance 'uncorrected' for any type of local motions.⁸¹

The procedure described above for the derivation of QCC values of D^α deuterons is only semi-quantitative due to large uncertainties associated with the derivation of ν_Q . From the variation of individual $\nu_Q/{}^1D_{C\alpha-H\alpha}$ ratios for 11 peaks in GB1 and from propagated errors in (fitted) ν_Q and ${}^1D_{C\alpha-H\alpha}$ values we estimate the uncertainty obtained in $D^\alpha QCC$ measurements on the order of 6–8% (~10–14 kHz). However, *on average* we have been able to obtain the value of $D^\alpha QCC$ that agrees quantitatively with the results of solid-state measurements in α -glycine if dynamics is taken into account (see above). In what follows, we therefore used a uniform value of $D^\alpha QCC$ equal to 174 kHz in all calculations. It is unlikely that the variation in individual $\nu_Q/{}^1D_{C\alpha-H\alpha}$ ratios in GB1 reflects the actual variation in $D^\alpha QCC$ values in a protein rather than the uncertainties of the measurement. Although hydrogen bonding is known to significantly affect quadrupolar couplings of deuterons,⁷⁶ α -deuterons in proteins are unlikely to participate in sufficiently strong hydrogen bonds. Of note, DFT calculations of QCC values of aliphatic deuterons bound to sp^3 -hybridized carbons show that QCC is a highly local parameter whose value is dependent almost exclusively on the choice of the r_{CD} bond length (N.R. Skrynnikov, personal communication).

Comparison of 2H - and ^{15}N -derived backbone order

Since, commonly, only two rates, $R^Q(D_+)$ and $R^Q(D_-)$, are available for the derivation of motional parameters of C^α - D^α vectors in proteins, caution must be exercised in a model-free-type analysis of D^α relaxation data to avoid over-interpretation when the results are compared to ^{15}N -derived dynamics parameters. Therefore, we have chosen to fit the $R^Q(D_+)$ and $R^Q(D_-)$ rates first to the simplest possible motional model that excludes the term describing local motions in the spectral density function of eq 3 ($\tau' = 0$). The majority of the residues in ubiquitin could be fit using such a single-parameter ($S^2_{CaD\alpha}$) spectral density function with 5% probability of exceeding χ^2 .⁸² If the χ^2 of the fit exceeded this level of confidence, the $R^Q(D_+)$ and $R^Q(D_-)$ rates have been fit to the full expression in eq 3 with 2 variable parameters ($S^2_{CaD\alpha}; \tau_f$). The resulting values of $S^2_{CaD\alpha}$ in ubiquitin at 10°C, 27°C and 40°C versus residue numbers are shown with blue rectangles in Figures 6a–c. For convenience of comparison we have included in the plots the values of S^2_{NH} derived from ^{15}N relaxation data (red circles in Figures 6a–c). Table S1 in the 'Supporting Information' lists all the dynamics parameters obtained at D^α positions of ubiquitin at the three temperatures. Figure 7 shows the ribbon structure of ubiquitin where the $H^\alpha(D^\alpha)$ atoms (Figure 7a) and HN atoms (Figure 7b) are color-coded according to the values of $S^2_{CaD\alpha}$ and S^2_{NH} , respectively. Overall, lower $S^2_{CaD\alpha}$ and S^2_{NH} values are observed in the loop region between residues Thr⁷ and Lys¹¹, in the bend near the polyubiquitination site (residues Ala⁴⁶-Lys⁴⁸) and in several C-terminal residues.

C^α - D^α bond vectors are motionally distinct from the N-H bond vectors, with 2H -derived $S^2_{CaD\alpha}$ values on average slightly higher than their S^2_{NH} counterparts in N-H amides in agreement with several earlier molecular dynamics simulations^{83,84} as well as NMR spin relaxation^{85,86} and liquid crystal studies.⁸¹ In particular, the average $S^2_{CaD\alpha}$ values of 0.86 (0.85;0.84) are obtained in ubiquitin at 10(27;40)°C for the full protein and 0.89(0.87;0.85)

when the flexible C-terminal residues are excluded from analysis, while the respective average S^2_{NH} values are 0.82(0.81;0.81) for the full protein and 0.84(0.83;0.82) when the C-terminus is excluded. For those residues where both $S^2_{CaD\alpha}$ and S^2_{NH} could be quantified, $S^2_{CaD\alpha}$ values are higher than S^2_{NH} on average, with their respective average values of 0.86(0.86;0.85) and 0.82(0.81;0.80) at 10(27;40)°C. The difference in order parameters is even more pronounced in GB1. The values of $S^2_{CaD\alpha}$ and S^2_{NH} in GB1 at 22°C are plotted as a function of the protein sequence in Figure S4 of the Supporting Information. The higher rigidity of the C^α-H^α (C^α-D^α) bond vectors in proteins is a consequence of the high correlation of their motions with those of the C^α-C^β bond vector of the same amino acid.⁸⁷ The latter anchors the side-chain to the backbone, and thereby any reorientational motion will need to involve the bulk of the side chain. By contrast, the N-H^N amides are strongly affected by local crank-shaft-type motions of the peptide bond plane,^{84,88} which involve anti-correlated modulations of the associated backbone φ_i and ψ_{i-1} dihedral angles. There are notable exceptions to this general trend, however. For example, in the C-terminal region of ubiquitin following Leu⁷¹, average $S^2_{CaD\alpha}(S^2_{NH})$ of 0.61(0.63) have been obtained at 27°C, whereas in GB1 the average $S^2_{CaD\alpha}(S^2_{NH})$ of the C-terminal residues are 0.22(0.24). Interestingly, the D^α rates of the C-terminal residues in both proteins can not be fit to the simplest ($S^2_{CaD\alpha}$ only) Lipari-Szabo model because the increased $R^Q(D_z)$ rates at the C-terminus (Figure 3a–c) can not be explained without invocation of fast local motions. In principle, direct comparison of $S^2_{CaD\alpha}$ and S^2_{NH} on a per-residue basis can be ambiguous since higher S^2_{NH} values may result from the use of different motional models for the derivation of $S^2_{CaD\alpha}$ and S^2_{NH} as well as significantly different time-scales of N-H and C^α-D^α bond vector fluctuations. Furthermore, since their respective diffusion tensor parameters have been used for the derivation of $S^2_{CaD\alpha}$ and S^2_{NH} , any small differences in these parameters (Table 1) can skew the differences between $S^2_{CaD\alpha}$ and S^2_{NH} ($S^2_{CaD\alpha} - S^2_{NH}$) in favor of somewhat smaller values adding additional uncertainty to direct comparisons of backbone order at the amide and D^α sites (*vide infra*).

Since different motional processes are sampled by D^α and ¹⁵N relaxation rates, it would be of interest to compare the degree of backbone order in the elements of protein secondary structure where C^α-H^α(D^α) and N-H bond vectors are oriented in different directions. For example, ¹⁵N relaxation data do not sample fast local motions that occur around the helical axis, such as a rolling motion of an α -helical shaft,⁹⁰ because in α -helices all N-H bonds lie approximately parallel to the helical axis forming hydrogen bonds with a carbonyl oxygen acceptor of residue $i-4$. In contrast, C^α-H^α(D^α) bond vectors are directed outward making angles of up to $\sim 70^\circ$ with the α -helical axis. Interestingly, in the α -helical segment of ubiquitin (residues Ile²³-Glu³⁴), $S^2_{CaD\alpha}$ are noticeably higher for the residues whose C^α-H^α(D^α) bond vectors are directed towards a short 3_{10} -helical stretch the protein structure (Val²⁶, Ile³⁰ or oriented towards the interior of the protein at the other face of the α -helix (Glu²⁴, Ala²⁸, Gln³¹). Figure 7c shows a schematic representation of the α -helix in ubiquitin viewed from its N-terminal end approximately along the axis of the helix, with $S^2_{CaD\alpha}$ (27°C) shown for each D^α site. In GB1, the residues of the α -helix (Ala²³-Asp³⁶) facing the hydrophobic interior of the protein (Ala²⁶, Phe³⁰, Tyr³³ and Ala³⁴) also tend to have elevated $S^2_{CaD\alpha}$. The resulting alternating patterns of $R^Q(D_+)$ rates (Figures 3d–f and Figures S1 of the Supporting Information) and $S^2_{CaD\alpha}$ values (Figures 6a,b, Figure S4 of the Supporting Information) are apparent in the helical regions of both proteins indicating that the backbone order as probed by ²H spin relaxation at D^α positions is likely to be related to the tightness of hydrophobic packing in protein cores. Specifically, the values of $S^2_{CaD\alpha}$ in the α -helix of ubiquitin (Figure 7c) are weakly but statistically significantly correlated with the buried surface area and the hydrophobic contact potential^{91,92} at C^α positions (Pearson $R > 0.5$). We note that the 'zigzag' patterns of $R^Q(D_+)$ rates and the resulting $S^2_{CaD\alpha}$ values cannot be explained by the anisotropy of the global molecular tumbling, as the unique diffusion axis of ubiquitin subtends an angle of only $\sim 10^\circ$ relative to the α -helical axis.

Molecular dynamics simulations and alternative analyses of D^α ^2H rates

To gain more confidence in the ^2H -derived measures of backbone order, we have performed molecular dynamics simulations for ubiquitin at 27°C (see 'Materials and Methods'). Interestingly, the best agreement between MD-derived $S^2_{\text{CaH}\alpha}$ and experimental $S^2_{\text{CaD}\alpha}$ order parameters is achieved when the D^α QCC value is (slightly) lowered to 171 kHz. A comparison between experimental $S^2_{\text{CaD}\alpha}$ ($QCC = 171$ kHz) and MD-derived $S^2_{\text{CaH}\alpha}$ values obtained using a $1\ \mu\text{s}$ MD trajectory of ubiquitin averaged over 1 ns and 5 ns time windows, is shown in Figure 8. A good agreement is found for the loop region of the N-terminal β -hairpin and the flexible C-terminus. The experimentally observed decrease in the order parameters of loop residues Ile⁴⁴, Ala⁴⁶, Lys⁴⁸ and Glu⁴⁹, whose side-chains point towards the solvent, is well reproduced by the MD simulation. Furthermore, the 'zigzag' pattern displayed by the experimental $S^2_{\text{CaD}\alpha}$ values in the Tyr⁵⁹-Lys⁶³ loop is also clearly observable in the simulation profile (Figure 8). For this region, the order parameters directly reflect the orientation of the associated side-chain, with lower S^2 for inward oriented side-chains and higher S^2 for more solvent exposed ones. Lys⁶³ is a notable exception with a relatively high $S^2_{\text{CaH}\alpha}$ despite its high solvent accessibility. The close relationship between $S^2_{\text{CaD}\alpha}$ and the associated side chain properties is corroborated by the very close agreement between $S^2_{\text{CaD}\alpha}$ and $S^2_{\text{CaC}\beta}$ values found in the MD simulation of ubiquitin (see Supporting Information, Figure S6).

The comparisons of ^{15}N - and ^2H -derived measures of backbone order described here have been performed using their respective (^{15}N -derived or ^2H -derived) overall diffusion tensor parameters (Table 1). Obviously, there is a unique diffusion tensor that characterizes the rotational reorientation of a molecule. Therefore, it would be of interest to ascertain that the relation between $S^2_{\text{CaD}\alpha}$ and S^2_{NH} noted above holds if analysis is performed using a common set of diffusion tensor parameters for both nuclei. We have repeated the analysis as described in the 'Comparison of ^2H - and ^{15}N -derived backbone order' section above for ubiquitin at 27°C using common diffusion tensor parameters for ^{15}N and ^2H data with the averaged values of $\tau_{c,\text{eff}} = (2D_{\parallel} + 4D_{\perp})^{-1} = 4.05$ ns; $D_{\parallel}/D_{\perp} = 1.20$; $\theta = 7^\circ$; $\phi = -12^\circ$, and the D^α QCC value of 171 kHz as obtained from MD simulations. The results of this analysis are presented in Figure S5 of the Supporting Information. The main conclusions obtained in the case when separate diffusion tensors have been used, remain in force. In particular, the average $S^2_{\text{CaD}\alpha}(S^2_{\text{NH}})$ values of 0.87(0.84) are obtained for all residues of ubiquitin, while $S^2_{\text{CaD}\alpha}(S^2_{\text{NH}})$ values of 0.89(0.86) are obtained when the flexible C-terminal and loop regions are excluded from analysis. However, when the same analysis (common diffusion tensor, $QCC = 171$ kHz) is performed for GB1, a number of residues give $S^2_{\text{CaD}\alpha}$ values higher than the theoretical limit of unity. When the D^α QCC value is 'reset' to 174 kHz, the relationship between $S^2_{\text{CaD}\alpha}$ and S^2_{NH} still holds in both proteins although the average $S^2_{\text{CaD}\alpha}$ values become only insignificantly higher than S^2_{NH} in ubiquitin.

Concluding remarks

In summary, we have shown that deuterium relaxation rates measured at deuterated carbon- α positions (D^α) serve as robust measures of backbone order in proteins. D^α relaxation rates are straightforward to interpret in terms of motional parameters ($S^2_{\text{CaD}\alpha}$; τ_f) provided that a uniform quadrupolar coupling constant (QCC) is assumed for all D^α sites in the protein molecule. To the best of our knowledge, this is the first attempt to use ^2H relaxation as a probe of backbone dynamics in proteins. Clearly, the experiments developed for D^α relaxation rate measurements are not sufficiently sensitive for routine applications requiring the use of concentrated [$U\text{-}^2\text{H}$, ^{13}C , ^{15}N]-labeled protein samples. Most of the D^α relaxation measurements described in this work have been performed on a 3.2 mM [$U\text{-}^2\text{H}$, ^{13}C , ^{15}N]-ubiquitin using a room-temperature probe. Obviously, at such high protein concentrations, potential aggregation of protein molecules in NMR samples is of concern. The data on diffusion tensor characteristics summarized in Table 1 show that this is apparently not the case for both

proteins studied in this work. With the increased sensitivity of NMR measurements due to the continuing development of cryogenically-cooled detection devices and the availability of higher magnetic fields, we anticipate that $R^Q(D_+)$, $R^Q(D_z)$ measurements would become feasible on protein samples with regular protein content (0.5–1.0 mM). Moreover, with the *increasing* molecular weight of a protein under study ^2H spin-flipping rates would *decrease* (Figures 3a–c). Therefore, the methods described in this work may be applicable to well-behaved medium-sized proteins (up to ~200 residues), as the sensitivity of the experiment in Figure 1 is primarily determined by the rate of ^2H spin-flips at the step when the magnetization is transferred from $^{13}\text{C}^\alpha$ spins to D^α and back.

For sensitivity reasons, because of the requirement for $[\text{U-}^2\text{H}, ^{13}\text{C}, ^{15}\text{N}]$ labeling, and because less data is available per nuclear probe in the case of D^α measurements at a single spectrometer field ($R^Q(D_+)$, $R^Q(D_z)$ rates versus ^{15}N R_1 , R_2 and $^1\text{H-}^{15}\text{N}$ NOEs) the developed methodology is not meant to substitute for the commonly used ^{15}N amide nuclei as NMR spin probes of backbone dynamics. Rather, it can be envisaged to serve as a useful complement to existing more sensitive techniques. Indeed, it should be kept in mind that the D^α relaxation rates measured in this work sample motions occurring in the protein backbone at positions that are distinct - chemically, structurally and motionally - from those normally sampled by conventional ^{15}N -based techniques. This work represents an attempt to get an alternative view of dynamic processes in protein molecules and to establish the range of applicability and utility of the described NMR methodology. In this context, it is noteworthy that, in principle, the measurements of $^{13}\text{C}^\alpha$ relaxation in proteins^{93,94} would provide the same information about backbone dynamics in a protein molecule as D^α methodology described here. The use of (protonated) carbon- α probes would, however, suffer from the same set of shortcomings and uncertainties as ^{15}N -based methods (the uncertainty in $^{13}\text{C}^\alpha\text{-H}^\alpha$ distances; site-specific ^{13}C CSA variation; contributions to ^{13}C relaxation from dipolar interactions with $^{13}\text{C}^\beta$ and ^{13}CO spins *etc.*). We have chosen to compare the D^α -derived motional parameters with more commonly used ^{15}N spin probes. An NMR study that quantifies $^{13}\text{C}^\alpha$ relaxation rates in selectively $^{13}\text{C}^\alpha$ -enriched protein samples⁹⁵ and compares the measures of backbone order thus obtained with the D^α -derived data reported here, is in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- (1). Fersht, A. Structure and Mechanism in Protein Science. Freeman & Co.; New York: 2002.
- (2). Frauenfelder H, Sligar SG, Wolynes PG. Science 1991;254:1598–1603. [PubMed: 1749933]
- (3). Dobson CM, Karplus M. Curr. Opin. Struct. Biol 1999;9:92–101. [PubMed: 10047588]
- (4). Karplus M, Kuriyan J. Proc. Natl. Acad. Sci. USA 2005;102:6679–6685. [PubMed: 15870208]
- (5). Peng JW, Wagner G. Methods Enzymol 1994;239:563–596. [PubMed: 7830599]
- (6). Palmer AG. Curr. Opin. Struct. Biol 1997;7:732–737.

- (7). Ishima R, Torchia DA. *Nat. Struct. Biol* 2000;7:740–743. [PubMed: 10966641]
- (8). Palmer AG, Kroenke CD, Loria JP. *Methods Enzymol* 2001;339:204–238. [PubMed: 11462813]
- (9). Kay LE. *Nat. Struct. Biol. NMR Suppl* 1998;5:513–516.
- (10). Palmer AG. *Annu. Rev. Biophys. Biomol. Struct* 2001;30:129–155. [PubMed: 11340055]
- (11). Fushman D, Cowburn D. *Methods Enzymol* 2001;339:109–26. [PubMed: 11462809]
- (12). Korzhnev DM, Billeter M, Arseniev AS, Orekhov VY. *Prog. Nucl. Magn. Reson. Spectrosc* 2001;38:197–266.
- (13). Kay LE, Torchia DA, Bax A. *Biochemistry* 1989;28:8972–8979. [PubMed: 2690953]
- (14). Cavanagh, J.; Fairbrother, WJ.; Palmer, AG.; Rance, M.; Skelton, NJ. *Protein NMR Spectroscopy*. Elsevier Academic Press; 2007.
- (15). Lipari G, Szabo A. *J. Am. Chem. Soc* 1982;104:4559–4570.
- (16). Lipari G, Szabo A. *J. Am. Chem. Soc* 1982;104:4546–4559.
- (17). Hansen DF, Yang D, Feng H, Zhou Z, Wiesner S, Bai Y, Kay LE. *J. Am. Chem. Soc* 2007;129:11468–11479. [PubMed: 17722922]
- (18). Xu J, Millet O, Kay LE, Skrynnikov NR. *J. Am. Chem. Soc* 2005;127:3220–3229. [PubMed: 15740163]
- (19). Abragam, A. *Principles of Nuclear Magnetism*. Clarendon Press; Oxford: 1961.
- (20). Muhandiram DR, Yamazaki T, Sykes BD, Kay LE. *J. Am. Chem. Soc* 1995;117:11536–11544.
- (21). Millet O, Muhandiram DR, Skrynnikov NR, Kay LE. *J. Am. Chem. Soc* 2002;124:6439–6448. [PubMed: 12033875]
- (22). Skrynnikov NR, Millet O, Kay LE. *J. Am. Chem. Soc* 2002;124:6449–6460. [PubMed: 12033876]
- (23). Yang D, Mittermaier A, Mok YK, Kay LE. *J. Mol. Biol* 1998;276:939–954. [PubMed: 9566198]
- (24). Ishima R, Petkova AP, Louis JM, Torchia DA. *J. Am. Chem. Soc* 2001;123:6164–6171. [PubMed: 11414851]
- (25). Lee AL, Flynn PF, Wand AJ. *J. Am. Chem. Soc* 1999;121:2891–2902.
- (26). Millet O, Mittermaier A, Baker D, Kay LE. *J. Mol. Biol* 2003;329:551–563. [PubMed: 12767834]
- (27). Tugarinov V, Ollerenshaw JE, Kay LE. *J. Am. Chem. Soc* 2005;127:8214–8225. [PubMed: 15926851]
- (28). Tugarinov V, Kay LE. *J. Am. Chem. Soc* 2006;128:12484–12489. [PubMed: 16984199]
- (29). Rosen MK, Gardner KH, Willis RC, Parris WE, Pawson T, Kay LE. *J. Mol. Biol* 1996;263:627–636. [PubMed: 8947563]
- (30). Gardner KH, Rosen MK, Kay LE. *Biochemistry* 1997;36:1389–1401. [PubMed: 9063887]
- (31). Bax A, Ikura M. *J. Biomol. NMR* 1991;1:99–104. [PubMed: 1668719]
- (32). Shaka AJ, Keeler J, Frenkiel T, Freeman R. *J. Magn. Reson* 1983;52:335–338.
- (33). Shaka AJ, Barker PB, Freeman R. *J. Magn. Reson* 1985;64:547–552.
- (34). Kay LE. *J. Am. Chem. Soc* 1993;115:2055–2057.
- (35). McCoy MA, Mueller L. *J. Magn. Reson* 1992;98:674–679.
- (36). Patt SL. *J. Magn. Reson* 1992;96:94–102.
- (37). Boyd J, Soffe N. *J. Magn. Reson* 1989;85:406–413.
- (38). Kay LE, Ikura M, Tschudin R, Bax A. *J. Magn. Reson* 1990;89:496–514.
- (39). Geen H, Freeman R. *J. Magn. Reson* 1991;93:93–141.
- (40). Kay LE, Keifer P, Saarinen T. *J. Am. Chem. Soc* 1992;114:10663–10665.
- (41). Schleucher J, Sattler M, Griesinger C. *Angew. Chem. Int. Ed. Engl* 1993;32:1489–1491.
- (42). Marion D, Ikura M, Tschudin R, Bax A. *J. Magn. Reson* 1989;85:393.
- (43). Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. *J. Biomol. NMR* 1995;6:277–293. [PubMed: 8520220]
- (44). Kamith U, Shriver JW. *J. Biol. Chem* 1989;264:5586–5592. [PubMed: 2647722]
- (45). Farrow NA, Muhandiram R, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T, Forman-Kay JD, Kay LE. *Biochemistry* 1994;33:5984–6003. [PubMed: 7514039]

- (46). Ferrage F, Piserchio A, Cowburn D, Ghose R. *J. Magn. Reson* 2008;192:302–313. [PubMed: 18417394]
- (47). Wooten JB, Savitsky GB, Jacobus J, Beyerlein AL, Emsley JW. *J. Chem. Phys* 1979;70:438–442.
- (48). Schramm S, Oldfield E. *Biochemistry* 1983;22:2908–2913. [PubMed: 6871171]
- (49). Wöessner DE. *J. Chem. Phys* 1962;37:647–654.
- (50). Vijay-Kumar S, Bugg CE, Cook WJ. *J. Mol. Biol* 1987;194:531–544. [PubMed: 3041007]
- (51). Frericks-Schmidt HL, Sperling LJ, Gao YG, Wylie BJ, Boettcher JM, Wilson SR, Rienstra CM. *J. Phys. Chem. B* 2007;111:14362–14369. [PubMed: 18052145]
- (52). Tjandra N, Feller SE, Pastor RW, Bax A. *J. Am. Chem. Soc* 1995;117:12562–12566.
- (53). Fushman D, Cahill S, Cowburn D. *J. Mol. Biol* 1997;266:173–194. [PubMed: 9054979]
- (54). Hall JB, Fushman D. *J. Am. Chem. Soc* 2006;128:7855–7570. [PubMed: 16771499]
- (55). Mittermaier A, Kay LE. *J. Am. Chem. Soc* 1999;121:10608–10613.
- (56). Hansen MR, Mueller L, Pardi A. *Nat. Struct. Biol* 1998;5:1065–1074. [PubMed: 9846877]
- (57). Yang D, Tolman JR, Goto NK, Kay LE. *J. Biomol. NMR* 1998;12:325–332.
- (58). Showalter SA, Brüschweiler R. *J. Chem. Theory Comput* 2007;3:961–975.
- (59). Case DA, Cheatham TE, Darden T, Gohlke H, Luo R, Merz KM, Onufriev A, Simmerling C, Wang B, Woods RJ. *J. Comp. Chem* 2005;26:1668–1688. [PubMed: 16200636]
- (60). Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C. *Proteins* 2006;65:712–725. [PubMed: 16981200]
- (61). Showalter SA, Brüschweiler R. *J. Am. Chem. Soc* 2007;129:4158–4159. [PubMed: 17367145]
- (62). Showalter SA, Johnson E, Rance M, Brüschweiler R. *J. Am. Chem. Soc* 2007;29:14146–14147. [PubMed: 17973392]
- (63). Maragakis P, Lindorff-Larsen K, Eastwood MP, Dror RO, Klepeis JL, Arkin IT, Jensen MO, Xu HF, Trbovic N, Friesner RA, Palmer AG, Shaw DE. *J. Phys. Chem. B* 2008;112:6155–6158. [PubMed: 18311962]
- (64). Ryckaert JP, Ciccotti G, Berendsen HJC. *J. Comput. Chem* 1977;23:327–341.
- (65). Darden T, York D, Pedersen L. *J. Chem. Phys* 1993;98:10089–10092.
- (66). Prompers JJ, Brüschweiler R. *J. Am. Chem. Soc* 2002;124:4522–4534. [PubMed: 11960483]
- (67). Vallurupalli P, Kay LE. *J. Am. Chem. Soc* 2005;127:6893–6901. [PubMed: 15869313]
- (68). Yang D, Kay LE. *J. Magn. Reson. Series B* 1996;110:213–218.
- (69). Jacobsen JP, Bildsøe HK, Schaumburg K. *J. Magn. Reson* 1976;23:153–164.
- (70). Grzesiek S, Bax A. *J. Am. Chem. Soc* 1994;116:10196–10201.
- (71). Burnett LH, Muller BH. *J. Chem. Phys* 1971;55:5829–5831.
- (72). Muller C, Schajor W, Zimmermann H, Haeberlen U. *J. Magn. Reson* 1984;56:235–346.
- (73). Rowell JC, Phillips WD, Melby LR, Panar M. *J. Chem. Phys* 1965;43:3442–3454.
- (74). Ottiger M, Bax A. *J. Am. Chem. Soc* 1999;121:4690–4695.
- (75). Henry ER, Szabo A. *J. Chem. Phys* 1985;82:4753–4761.
- (76). LiWang AC, Bax A. *J. Magn. Reson* 1997;127:54–64. [PubMed: 9245630]
- (77). Sörensen OW, Eich GW, Levitt MH, Bodenhausen G, Ernst RR. *Prog. Nucl. Magn. Reson. Spectrosc* 1983;16:163–192.
- (78). Kuboniwa H, Grzesiek S, Delaglio F, Bax A. *J. Biomol. NMR* 1994;4:871–878. [PubMed: 7812158]
- (79). Jeffrey, G. *Accurate molecular structures, their determination and importance*. Oxford University Press; Oxford: 1992.
- (80). Demaison J, Wlodarczak G. *Struct. Chem* 1994;5:57–66.
- (81). Ottiger M, Bax A. *J. Am. Chem. Soc* 1998;120:12334–12341.
- (82). Bevington, PR.; Robinson, DK. *Data Reduction and Error Analysis for the Physical Sciences*. WCB/McGraw-Hill; New York: 1992.
- (83). Palmer AG, Case DA. *J. Am. Chem. Soc* 1992;114:9059–9067.
- (84). Fadel AR, Jin DQ, Montelione GT, Levy RM. *J. Biomol. NMR* 1995;6:221–226. [PubMed: 8589611]

- (85). Dellwo MJ, Wand AJ. *J. Am. Chem. Soc* 1989;111:4571–4578.
- (86). LeMaster DM, Kushlan DM. *J. Am. Chem. Soc* 1996;118:9255–9264.
- (87). Brüschweiler R. *J. Chem. Phys* 1995;102:3396–3403.
- (88). Brüschweiler R, Wright PE. *J. Am. Chem. Soc* 1994;116:8426–8427.
- (89). Koradi R, Billeter M, Wüthrich K. *J. Mol. Graphics* 1996;14:51–55.
- (90). Fischer MWF, Zeng L, Majumdar A, Zuiderweg ERP. *Proc. Natl. Acad. Sci. USA* 1998;95:8016–8019. [PubMed: 9653132]
- (91). Lee B, Richards FM. *J. Mol. Biol* 1971;55:379–400. [PubMed: 5551392]
- (92). Kurochkina N, Lee B. *Protein Eng* 1995;8:437–442. [PubMed: 8532664]
- (93). Lee LK, Rance M, Chazin WJ, Palmer AG. *J. Biomol. NMR* 1997;9:287–298. [PubMed: 9204557]
- (94). Idiyatullin D, Nesmelova I, Daragan V, Mayo KH. *Protein Sci* 2003;12:914–922. [PubMed: 12717014]
- (95). Lundström P, Teilum K, Carstensen T, Bezsonova I, Wiesner S, Hansen DF, Religa TL, Akke M, Kay LE. *J. Biomol. NMR* 2007;38:199–212. [PubMed: 17554498]

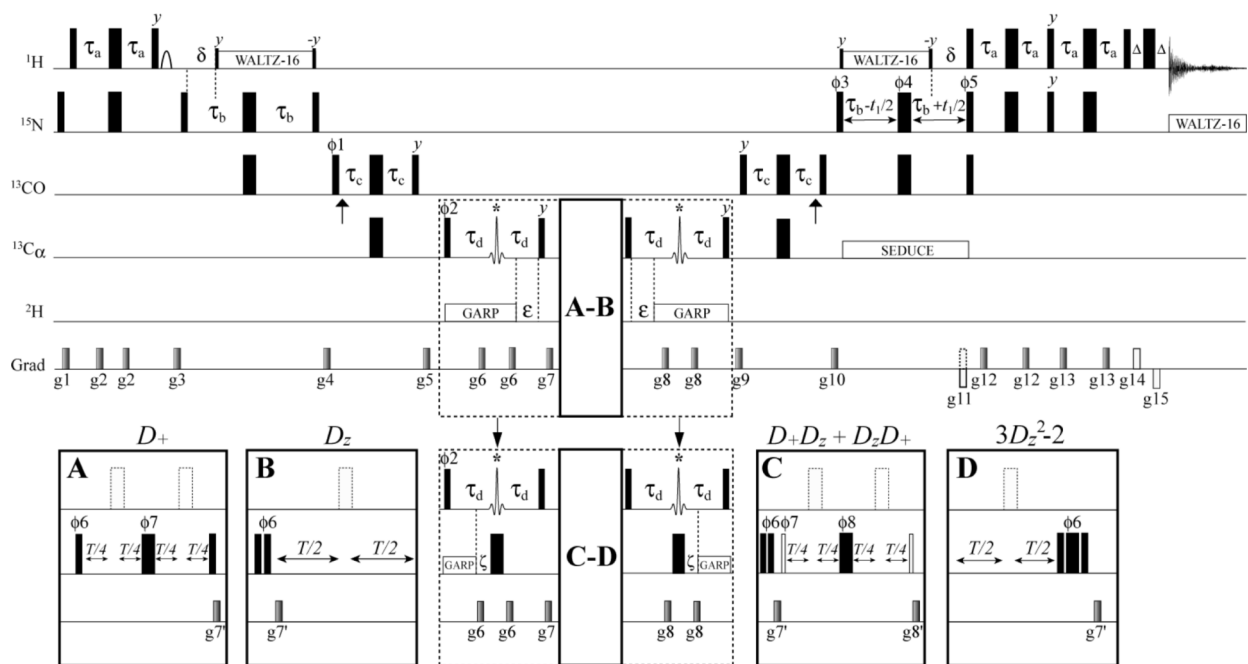


Figure 1. The HN(COCA)D pulse scheme for the measurement of relaxation rates of α -deuterons in proteins. See 'Materials and Methods' for the pulse-sequence details.

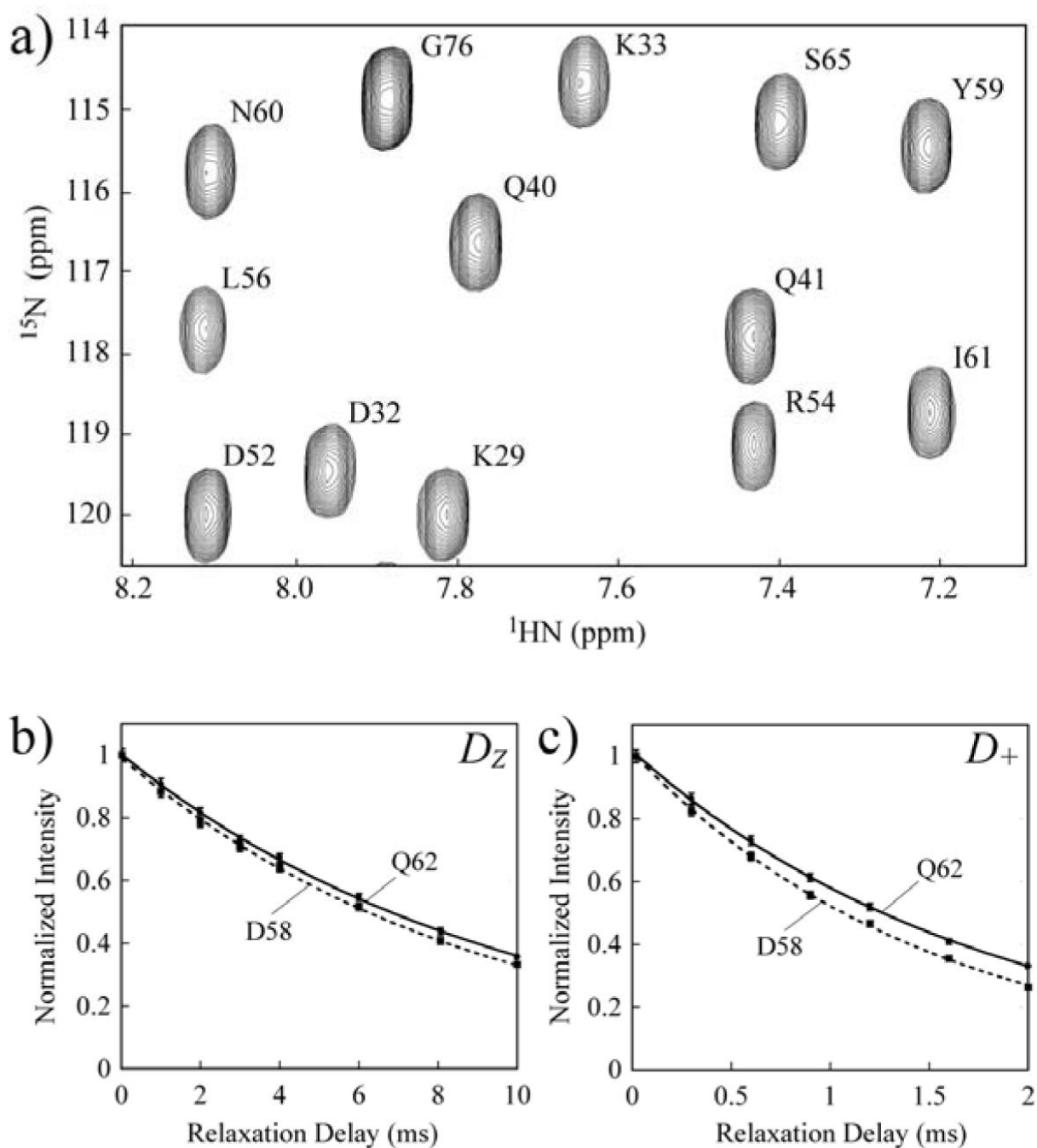


Figure 2.

a) A region of the ${}^1\text{H}$ N- ${}^{15}\text{N}$ correlation map of the $[\text{U-}{}^{15}\text{N}, {}^{13}\text{C}, {}^2\text{H}]$ -labeled 3.2 mM ubiquitin (27°C; 600 MHz) acquired as the first measurement point using the experiment of Figure 1 (inset A, $T = 0$). Assignments of the cross-peaks are shown according to their ${}^1\text{H}$ N- ${}^{15}\text{N}$ frequencies (located at position i if $i-1$ is the position of the decaying D^α nucleus). Typical decay curves of **b)** D_z and **c)** D_+ magnetization are shown for Asp⁵⁸ (dashed curves) and Gln⁶² (solid curves) of ubiquitin.

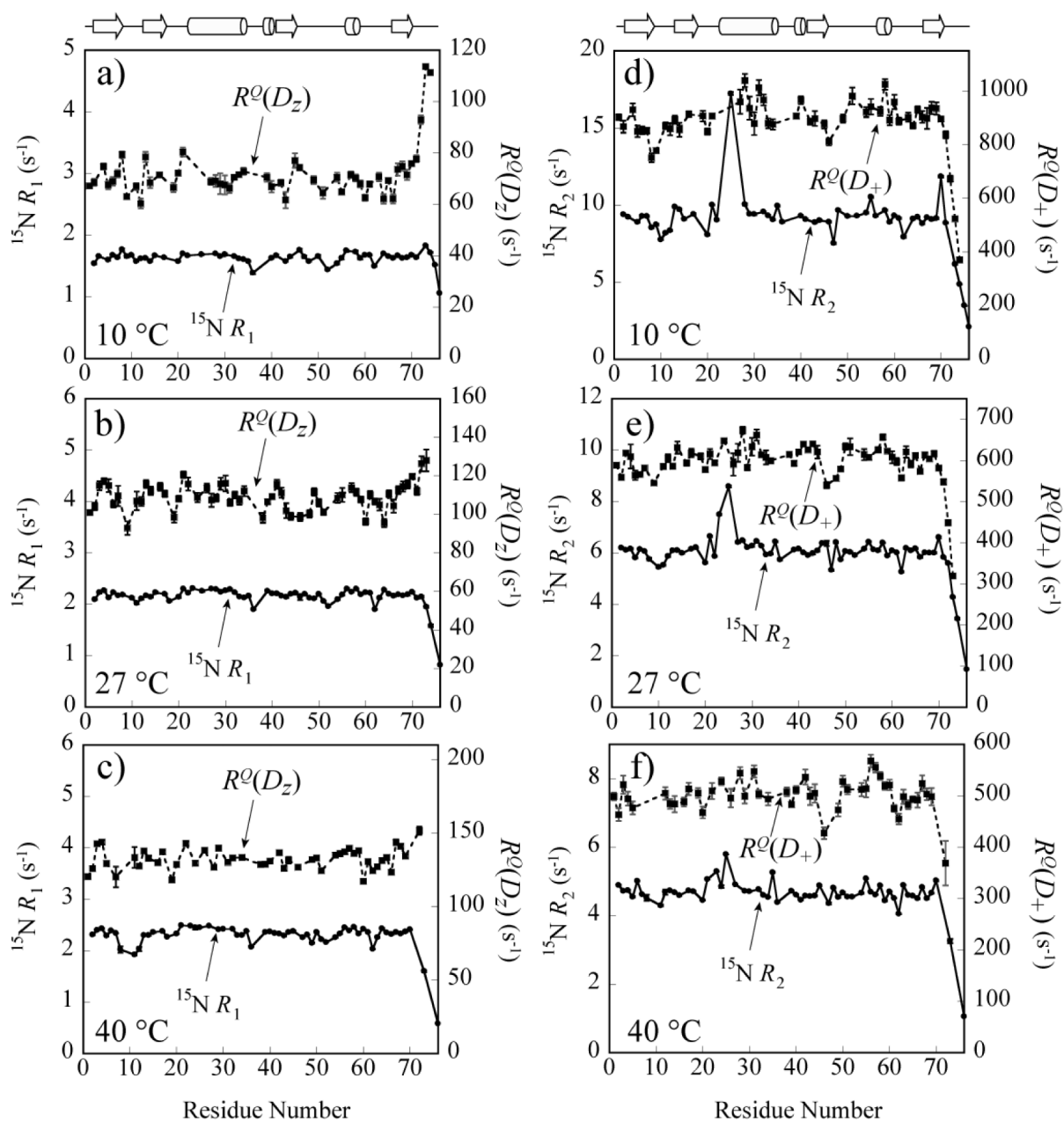


Figure 3.

Plots of D^α (shown with black rectangles and dashed lines) and ^{15}N (black circles and solid lines) relaxation rates in ubiquitin as a function of the protein sequence. $R^Q(D)$ and $^{15}\text{N } R_1$ rates are shown at **a)** 10°C, **b)** 27°C, **c)** 40°C, while $R^Q(D_+)$ and $^{15}\text{N } R_2$ rates are shown at **d)** 10°C, **e)** 27°C, **f)** 40°C. The lines connecting the symbols on the plots are intended solely to guide the eye. Schematic representation of the secondary structure of ubiquitin is shown on top: β -sheets are depicted with arrows, while α -helices are represented with cylinders.

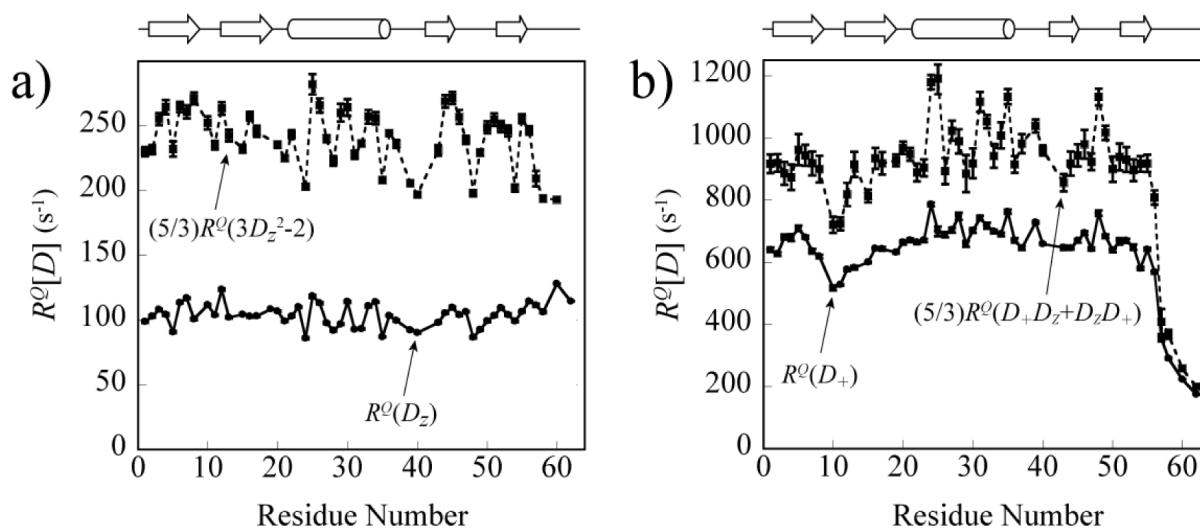


Figure 4. Consistency plots of D^a relaxation rates. **a)** $(5/3)R^Q(3D_z^2-2)$ (shown with black rectangles and dashed lines) and $R^Q(D_z)$ (black circles; solid lines); **b)** $(5/3)R^Q(D_+D_z+D_zD_+)$ (black rectangles; dashed lines) and $R^Q(D_+)$ (black circles; solid lines) rates measured in GB1 at 22° C (600 MHz) are plotted as a function of GB1 sequence. Schematic representation of the secondary structure of GB1 is shown on top.

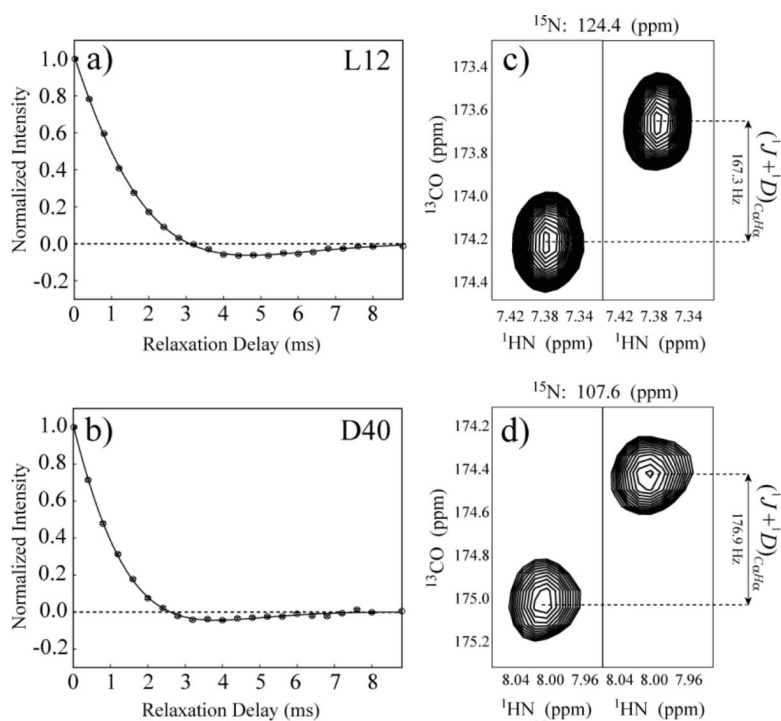


Figure 5.

Examples of the decay curves are shown for **a)** for Leu¹² and **b)** Asp⁴⁰ of GB1(22°C) from the chosen subset of 11 peaks in GB1 that show clear-cut zero-crossing. The decay curves are fit to eq 9. Zero normalized intensity is shown with a dashed line. The $^1\text{H-N}^{13}\text{C}$ correlations obtained in the experiment of Yang et al.⁵⁶ that allows the measurement of $^1D_{\text{C}^{\alpha}\text{-H}^{\alpha}}$ residual dipolar couplings for **c)** for Leu¹² and **d)** Asp⁴⁰. The measured frequency differences ($^1J + ^1D_{\text{C}^{\alpha}\text{-H}^{\alpha}}$) are shown for each pair of peaks.

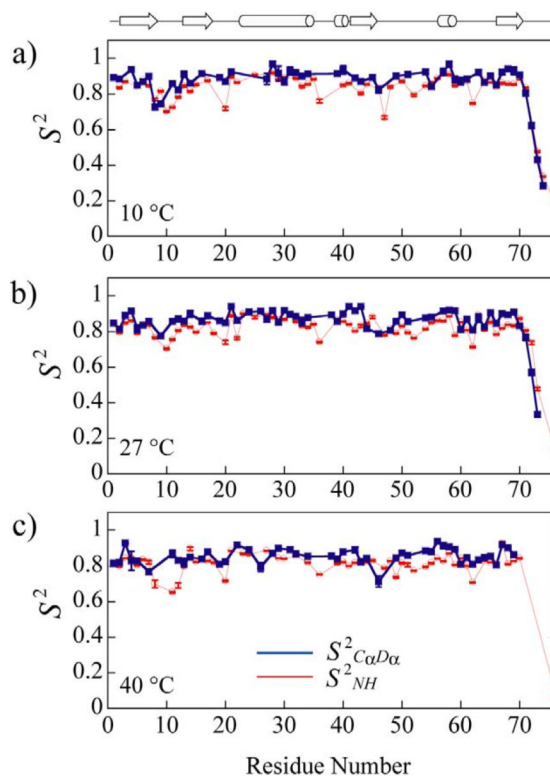


Figure 6. D^{α} -derived $S^2_{C\alpha D\alpha}$ (blue rectangles and lines) and ^{15}N -derived S^2_{NH} (red open circles and lines) in ubiquitin at **a)** 10°C, **b)** 27°C and **c)** 40°C plotted as a function of protein sequence. Schematic representation of the secondary structure of ubiquitin is shown on top: β -sheets are depicted with arrows, while α -helices are represented with cylinders.

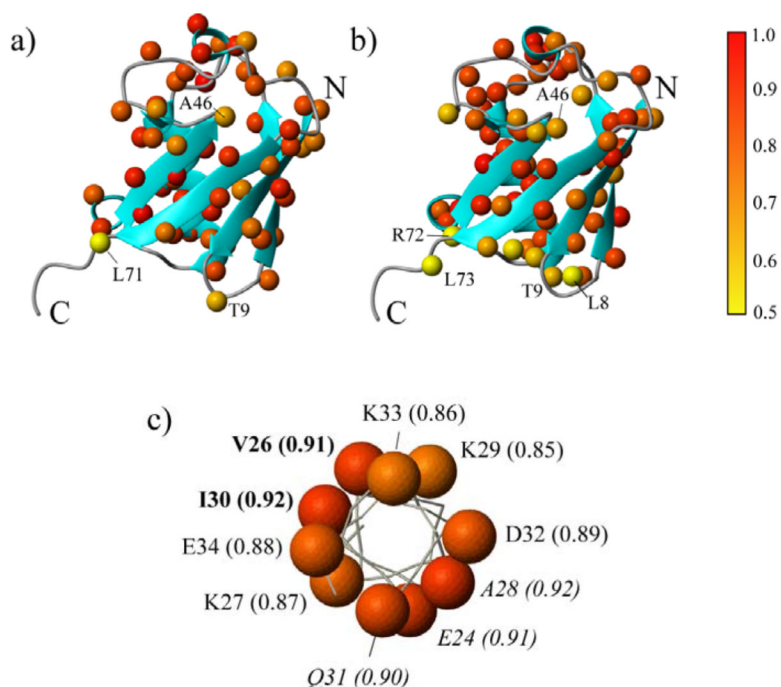


Figure 7. Ribbon diagrams of ubiquitin crystal structures with **a)** D^α and **b)** HN atoms represented as balls color-coded according to the values of $S^2_{C\alpha D\alpha}$ and S^2_{NH} (27°C), respectively. The elements of the secondary structure are shown in blue. The placement of a number of residues discussed in the text is indicated with residue numbers. **c)** Schematic representation of the α -helical stretch of ubiquitin (residues Ile²³-Glu³⁴) viewed from the top along the helical axis. The color-coded balls represent D^α atoms. The values of $S^2_{C\alpha D\alpha}$ are listed along with residue numbers. $S^2_{C\alpha D\alpha}$ values of residues with C^α-D^α vectors directed towards a short 3_{10} -helical stretch (Pro³⁸-Gln⁴⁰) are shown in bold, whereas those directed towards the interior of the protein core on the other side of the α -helix are shown in italics. The color-coding scale is shown to the right of panel (b). The diagrams have been generated using the program Molmol.

89

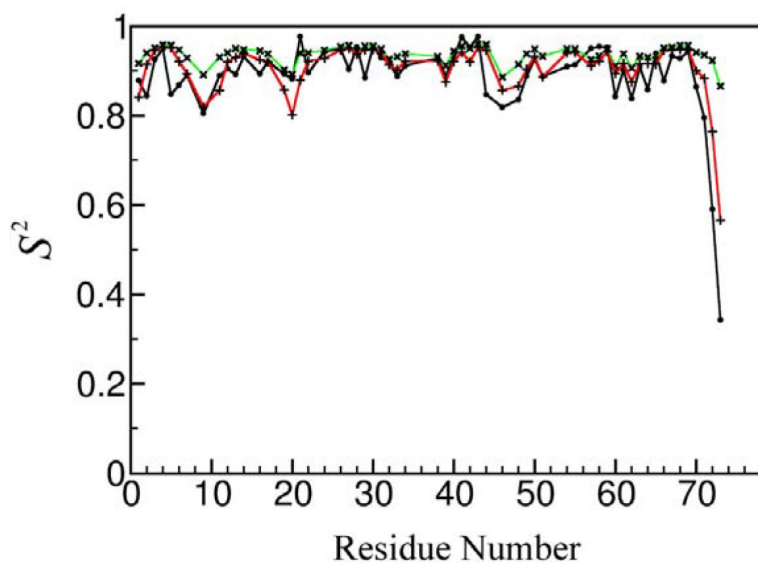


Figure 8. Experimental S^2_{CaDa} order parameters of ubiquitin at 27°C (black line with closed circles) determined using a uniform QCC value of 171 kHz, in comparison with the MD-derived S^2_{CaHa} values obtained from a 1 μ s MD trajectory of ubiquitin averaged over 1 ns (green line with 'x' symbols) and 5 ns (red line with '+' symbols) time windows. See 'Materials and Methods' for the details of MD simulations.

Table 1

Comparison of $D^\alpha R^Q(D_+)/R^Q(D_z)$ - and $^{15}\text{N } R_2/R_1$ -derived Diffusion Tensor Parameters of Ubiquitin (10°C, 27°C, 40°C) and GB1 (22°C).^{a)}

	$\tau_{c,eff}$ ^{b)}	D_{\parallel}/D_{\perp}	θ ^{c)}	φ ^{c)}
Ubiquitin (10 °C)	6.68 ± 0.03 (6.92 ± 0.02)	1.28 ± 0.04 (1.23 ± 0.02)	12 ± 4 (6 ± 2)	-22 ± 12 (-19 ± 5)
Ubiquitin (27 °C)	3.94 ± 0.03 (4.16 ± 0.01)	1.22 ± 0.03 (1.18 ± 0.02)	8 ± 3 (6 ± 2)	-8 ± 12 (-16 ± 4)
Ubiquitin (40 °C)	2.90 ± 0.02 (2.98 ± 0.01)	1.26 ± 0.03 (1.23 ± 0.01)	16 ± 2 (6 ± 1)	-34 ± 14 (-48 ± 8)
GB1 (22 °C)	4.43 ± 0.02 (4.42 ± 0.01)	1.45 ± 0.03 (1.39 ± 0.02)	20 ± 6 (24 ± 1)	-61 ± 26 (-99 ± 8)

^{a)} $^{15}\text{N } R_2/R_1$ -derived diffusion parameters are shown in parentheses and italics below the $D^\alpha R^Q(D_+)/R^Q(D_z)$ -derived values. The orientation of the unique axis of the axially symmetric diffusion tensor is specified in the inertial frame for both proteins. The inertia tensors and direction cosine values for $\text{C}^\alpha\text{-H}^\alpha$ and N-H bond vectors have been calculated using the coordinates of protonated x-ray structures of ubiquitin and GB1 with respective pdb accession codes 1ubq.pdb⁵⁰ and 2qmt.pdb.⁵¹ Seven C-terminal residues of GB1 do not have crystallographic coordinates and have been excluded from the derivation of the diffusion tensor. See 'Materials and Methods' for the details of diffusion tensor determination.

^{b)} $\tau_{c,eff}$ is given in units of nanoseconds (ns).

^{c)} Angles are given in degrees. The angle γ between the D^α and ^{15}N -derived diffusion axes can be estimated using the relationship $\cos\gamma = \cos\theta_1\cos\theta_2 + \sin\theta_1\sin\theta_2\cos\phi_1 - \phi_2$, where $\{\theta_i, \phi_i\}$ are the polar angles that define the mean orientations of the unique axes of the two compared diffusion tensors. These angles are equal to 6(2,10)° for ubiquitin at 10(27,40)°C and 14.5° for GB1 (22°C).