Overall rotational diffusion and internal mobility in domain II of protein G from *Streptococcus* determined from ^{15}N relaxation data

MARCUS L. TILLET¹, MARTIN J. BLACKLEDGE², JEREMY P. DERRICK³,
LU-YUN LIAN^{1,3} AND TIMOTHY J. NORWOOD^{4,5}

¹Biological NMR Centre, Department of Biochemistry, Leicester University, University Road,
Leicester LE1 7RH, United Kingdom

²Institut de Biologie Structurale–Jean-Pierre Ebel C.N.R.S.-C.E.A., 41 Avenue des Martyrs, 38027 Grenoble Cedex, France

³Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology,
P.O. Box 88, Manchester M60 1QD, United Kingdom

⁴Department of Chemistry, Leicester University, University Road, Leicester LE1 7RH, United Kingdom

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Abstract

The backbone dynamics and overall tumbling of protein G have been investigated using ^{15}N relaxation. Comparison of measured R_2/R_1 relaxation rate ratios with known three-dimensional coordinates of the protein show that the rotational diffusion tensor is significantly asymmetric, exhibiting a prolate axial symmetry. Extensive Monte Carlo simulations have been used to estimate the uncertainty due to experimental error in the relaxation rates to be $D_{\parallel}/D_{\perp} = 1.68 \pm 0.08$, while the dispersion in the NMR ensemble leads to a variation of $D_{\parallel}/D_{\perp} = 1.65 \pm 0.03$. Incorporation of this tensorial description into a Lipari–Szabo type analysis of internal motion has allowed us to accurately describe the local dynamics of the molecule. This analysis differs from an earlier study where the overall rotational diffusion was described by a spherical top. In this previous analysis, exchange parameters were fitted to many of the residues in the alpha helix. This was interpreted as reflecting a small motion of the alpha helix with respect to the beta sheet. We propose that the differential relaxation properties of this helix compared to the beta sheet are due to the near-orthogonality of the NH vectors in the two structural motifs with respect to the unique axis of the diffusion tensor. Our analysis shows that when anisotropic rotational diffusion is taken into account NH vectors in these structural motifs appear to be equally rigid. This study underlines the importance of a correct description of the rotational diffusion tensor if internal motion is to be accurately investigated.

Keywords: anisotropic; dynamics; model-free approach; ^{15}N relaxation; protein G; rotational diffusion

Measurement of heteronuclear relaxation rates has been widely used to investigate the backbone dynamics of proteins (Palmer, 1997; Kay, 1998). Commonly, ^{15}N R_1 and R_2 autorelaxation rates and the heteronuclear ^1H – ^{15}N NOE are measured for each accessible residue in the protein. The time autocorrelation function, describing the angular reorientation of the dipole–dipole and ^{15}N CSA interactions, which dominate ^{15}N relaxation, is thus investigated directly. The different relaxation rates measured are sensitive to motion on different timescales. These measurements are normally insufficient to differentiate between distinct motional models due to the small number of experimental measurements and their relative imprecision compared to the complexity of the different analytical descriptions of the motion. This problem was first

identified by Lipari and Szabo, who proposed a model-free approach (Lipari & Szabo, 1982). This describes all internal angular motion, irrespective of its true character, in terms of a generalized order parameter S^2 , which describes the amplitude of the movement, and a characteristic internal correlation time τ_i . For those residues whose motion cannot be satisfactorily characterized using this simple model, further parameters can be introduced. This can be done by using either the extended model-free approach (Clare et al., 1990), which allows internal motion on two different timescales to be described, or by fitting an exchange parameter, which allows the effects of slow motion on the sub-millisecond timescale due to chemical or conformational exchange, to be considered (Luginbuhl et al., 1997). This approach has the advantage of being quantitative, intuitive, and relatively easy to test for statistical significance (Mandel et al., 1995), although it is nonspecific and is only valid in restricted timescales with respect to the overall motion. This simple analysis has gained popularity with the routine

Reprint requests to: Lu-Yun Lian, Biomolecular Sciences, UMIST, P.O. Box 88, Manchester, M60 1QD, United Kingdom; e-mail: yun@le.ac.uk.

⁵Deceased November 13, 1999.

investigation of ^{15}N labeled proteins to become a standard measure in the solution study of proteins using heteronuclear NMR spectroscopy. However, in many cases, this approach is incorrect and/or inadequate to allow accurate and precise interpretation of the ^{15}N relaxation data.

The time autocorrelation function is affected by both internal and overall motions, implying that both contributions require detailed attention if the motion of the interactions and consequently the relaxation are to be adequately described. In the simplest analysis, overall motion is assumed to be isotropic, and this contribution is described by a single exponential. In the case of rotational diffusion anisotropy, the time correlation is described by a multi-exponential function, whose relative weighting depends on the orientation of the different relaxation interactions with respect to the principal components of the diffusion tensor. Not surprisingly, the interpretation of heteronuclear relaxation using the model-free approach assuming isotropic motion can artificially evoke fictive contributions to the internal motion if the rotational diffusion tensor is actually significantly anisotropic (Schurr et al., 1994; Cordier et al., 1998). To interpret the internal motion with confidence, an accurate description of the contribution to the time correlation function due to overall motion is therefore necessary. It has been shown that this tensor can be determined from the relaxation rate ratio R_2/R_1 (Kay et al., 1989; Kordel et al., 1992), which becomes independent of internal motion in the fast motion limit and is highly sensitive to overall diffusion. To reduce the possibility of contributions from internal motion in this analysis, it is important to select residues whose interaction directions are likely to be in the rapid motion limit (i.e., $\tau_i < 30$ ps). This is achieved either by analysis of the actual relaxation rates with respect to the mean values for the whole molecule, or by identifying those residues present in secondary structural elements that are assumed to have similar rigidity. Once the rotational diffusion tensor has been determined using a subset of the available residues, this can be applied to a model-free analysis of the entire molecule.

In this paper we consider the importance of selecting the correct model for the overall motion of the protein for the analysis of relaxation data. We describe the analysis of ^{15}N relaxation data of domain II from *Streptococcus* protein G, a 64 residue immunoglobulin G (IgG)-binding protein, consisting of a four stranded beta sheet and a single alpha helix (Lian et al., 1992). Uncertainty in the analysis of the anisotropic rotational diffusion tensor has been estimated using Monte Carlo sampling methods and compared to the dispersion present in the NMR ensemble. The results of this analysis are compared to an equivalent analysis of the same data assuming isotropic overall tumbling and a previous analysis making this same assumption (Barchi et al., 1994). The model invoked to describe the rotational diffusion is shown to be of fundamental importance for the accurate analysis of internal mobility. The use of rotational diffusion anisotropy to analyze ^{15}N -relaxation data has been previously highlighted (e.g., Tjandra et al., 1995).

Results

Measurement of the ^{15}N relaxation data for 51 of the 64 residues was made. Data were unavailable for 11 residues due to spectral overlap or rapid conformational exchange, and the two remaining residues were proline (3 and 63). The errors for R_1 and R_2 relaxation rates were determined from the data and had an average value of 5.0 and 1.6%, respectively. The error in the nuclear Over-

hauser effect (NOE) measurements was estimated from the noise level and had an average value of 7%. The ^{15}N relaxation data are shown in Figure 1.

The first step in the correct analysis of relaxation data is the proper characterization of the overall motion of the protein, i.e., the rotational diffusion tensor for the protein. The existence of anisotropic rotational diffusion was suggested by the highly different ratios of R_2/R_1 present in the alpha helix when compared to the almost geometrically orthogonal beta sheet regions. This is shown in Figure 2. ^{15}N relaxation data for the 38 residues present in these motifs of secondary structure were incorporated into the fit of the rotational diffusion tensor from the experimental R_2/R_1 ratio. Structure number 6 from the NMR ensemble was used in this analysis (Protein Data Bank (PDB) entry code: 2igg), as this is predicted to be most representative of the solution structure of protein G using the Olderado program (Kelley et al., 1997). The results of the least-squares fitting of the isotropic and anisotropic spectral density functions (given in Equation 3) are shown in Table 1.

It has recently been shown that two orthogonal minima exist in the parametric space available when a truly anisotropic system is approximated by a simpler, axially asymmetric tensor (Blackledge et al., 1998). The relative significance of these minima is indicative

Fig. 1. Experimental R_1 and R_2 relaxation rates and heteronuclear ^1H - ^{15}N NOE measurements for protein G; error bars represent ± 1 standard deviation.

Fig. 2. Plot of the R_2/R_1 ratio for protein G, with the average value of this ratio of the alpha-helix and beta-sheet regions indicated by the solid lines.

of the nature of the diffusion tensor. In the case of the protein G, the oblate minimum has a much higher χ^2_{exp} (65.6) than that of the prolate (19.8), indicating that the latter tensorial model is most relevant of the two. Not surprisingly, the oblate model is not statistically significant, while the prolate model is statistically acceptable as judged by the Monte Carlo derived 95% confidence levels.

To ensure that the prolate model adequately describes the overall diffusion rotation tensor, we have also performed a fit of the diffusion tensor using the fully anisotropic description of the spectral density functions. The fully anisotropic model yields a lower value for the χ^2_{exp} (17.3), which is within the 95% confidence limit defined by the simulated data sets. It is therefore necessary to test the significance of this improvement compared to the simpler model using the so-called *F*-test. We have compared the improvement in the χ^2 due to random statistical fluctuation by fitting the simulated data sets derived from the prolate model with the parameters determined for the fully anisotropic diffusion tensor. This determines the expected improvement in χ^2 achieved when the use of the more complex fit is actually unnecessary. The experimental *F*-value is slightly lower than the threshold for statistical significance of the improved fit assuming 90% confidence limits ($p = 0.11$).

Uncertainties in the parameterization of the all diffusion tensors have been determined using extensive Monte Carlo simulations. The errors cited in Table 1 are taken from the standard deviations of the simulations present in the relevant minima, and the relevant statistical tests were performed using these Monte Carlo simulations.

The prolate rotational diffusion tensors for each of the 27 structures from the NMR ensemble have also been calculated (Lian et al., 1992). The eigenvalues of the average tensor taken over the NMR ensemble has a significantly smaller dispersion than the dispersion estimated from the experimental error of the relaxation rates (Table 1), so that the total uncertainty is largely dominated by the latter. The uncertainty in the directions appears to have similar contributions from the coordinate and relaxation rates ($\sigma = 5^\circ$ in both cases). The average values are close to those fitted using the prolate model to the structure chosen as the most representative solution structure.

Fitting our data using the isotropic rotational diffusion tensor gave a correlation time of 3.69 ± 0.03 ns, using the same set of residues used for determining the anisotropic diffusion tensor, with $\chi^2_{exp} = 103.1$. The model-free analysis, using this correlation time, is shown in Figure 3. Slightly more than half of the residues, 32 out of 51, can be fitted using model 1 (see Materials and methods for a definition of the models used) and a further seven residues can be fitted to model 2 with $\tau_i < 120$ ps. A total of six residues required the inclusion of an exchange parameter and are fitted to models 3 and 4. The C-terminal residue, Glu64, requires the extended form of the model-free approach used in model 5, which allows for internal motion on two timescales. The remaining five residues (Ala29, Phe35, Ala39, Asn40, Asp41) are not adequately fitted by any of the five models.

By using the axially symmetric prolate rotational diffusion tensor for the model-free analysis, shown in Figure 4, the majority of the residues, 43 out of 51, can be fitted to model 1. Four residues required model 2 and one residue is best fit by each of models 3, 4, and 5. The remaining residue (Ala29) is not fit adequately by any of the models.

Discussion

An initial concern when analyzing relaxation data is whether the protein can be considered monomeric. If the fraction of monomer is in the range 0.2 to 0.8, the fitted values of S^2 and τ_i can significantly exceed their true values. Even a small amount of dimer,

Table 1. Axially symmetric and fully anisotropic diffusion parameters from R_2/R_1 relaxation rate ratios for protein G

Tensor ^a	$D_{ }/D_{\perp}$	D_{xx}^b (10^7 s^{-1})	D_{yy}^b (10^7 s^{-1})	D_{zz}^b (10^7 s^{-1})	θ^c (deg)	ϕ (deg)	ψ	χ^2_{exp}	$\omega_{0.05}^d$
NMR ensemble (prolate)	1.65 ± 0.03	3.86 ± 0.03	3.86 ± 0.03	6.37 ± 0.09	-64.9 ± 4.0	-6.0 ± 5.0	—	19.2 ± 3.1	—
Prolate	1.68 ± 0.08	3.83 ± 0.06	3.83 ± 0.06	6.43 ± 0.21	-58.4 ± 4.1	-5.1 ± 4.9	—	19.8	48.1
Oblate	0.71 ± 0.02	3.54 ± 0.12	5.00 ± 0.09	5.00 ± 0.09	36.0 ± 6.1	-3.5 ± 8.7	—	65.7	47.8
Fully anisotropic	—	3.55 ± 0.13	4.09 ± 0.14	6.44 ± 0.23	-43.2 ± 22.9	-4.4 ± 5.2	-57.0 ± 4.3	17.3	33.6

^aIsotropic model gives $D_{iso} = 4.52 \pm 0.03$, 10^7 s^{-1} with $\chi^2_{exp} = 103.1$.

^bThe fitted parameters $D_{||}$ and $D_{\perp}$ are shown here as D_{xx} , D_{yy} , and D_{zz} for direct comparison with the generally asymmetric diffusion tensor. For the prolate approximation $D_{||} = D_{zz}$, $D_{\perp} = D_{xx} = D_{yy}$; for the oblate approximation $D_{||} = D_{xx}$, $D_{\perp} = D_{zz} = D_{yy}$.

^cAngles (θ, ϕ, ψ) describe the orientations of the rotational diffusion tensor in the NMR structure frame. $D_{zz} > D_{yy} > D_{xx}$.

^d $\chi^2_{0.05}$ refers to the ($\alpha = 0.05$) confidence limit for the fit derived from 1,000 MC simulations.

Fig. 3. Plot of fitted model-free parameters for protein G using an isotropic rotational diffusion tensor. **A:** Order parameter, $\bullet$ represents S^2 and $\diamond$ represents S^2 . **B:** Internal correlation time τ_i . **C:** Chemical exchange term R_{ex} . The solid bar and the crosses represent beta-sheet and alpha-helical regions, respectively. All error bars represent ± 1 standard deviation. Solid bar represents beta sheets, and $\times$ represent alpha-helical regions.

Fig. 4. Plot of fitted model-free parameters for protein G using a prolate axially symmetric rotational diffusion tensor. **A:** Order parameter, $\bullet$ represents S^2 and $\diamond$ represents S^2 . **B:** Internal correlation time τ_i . **C:** Chemical exchange term R_{ex} . The solid bar and the crosses represent beta-sheet and alpha-helical regions, respectively. All error bars represent ± 1 standard deviation.

such as when the fraction of monomer is 0.9, can have quite significant effects on the fitted values of S^2 and τ_i (Schurr et al., 1994). The measured translational diffusion coefficient for protein G of $1.35 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ (Tillett et al., 1999) is consistent with a monomer (Krishnan, 1997). This evidence, coupled with the lack of observable broadening of peaks in the ^1H one-dimensional (1D) spectrum with increased concentration, allows confidence in results obtained from the analysis of ^{15}N relaxation data from protein G with the assumption of a monomeric protein. The previous analysis of ^{15}N relaxation data for protein G (Barchi et al., 1994) also assumed a monomeric protein at similar concentrations.

The most appropriate overall correlation time for the protein G, calculated using the vectors present in the secondary structural elements, was in broad agreement with the previous study of the dynamic behavior of this molecule [$3.69 \pm 0.03 \text{ ns}$ in this study of the 64 residue construct at 300 K compared to $3.30 \pm 0.02 \text{ ns}$ for the 56 residue construct at 299 K in Barchi et al. (1994)]. In this study, when using an isotropic model for the rotational diffusion tensor for the model-free analysis of the ^{15}N relaxation data for protein G, it is surprising to note that 11 residues require the inclusion of an exchange parameter, of which five do not fulfill the

95% confidence limits even with the inclusion of this R_{ex} term. Nine of the residues requiring an exchange parameter are in the alpha helical region of the protein. The interaction vectors in the alpha helix are oriented differently to those in the beta sheet. If protein G was a spherical protein and tumbled isotropically in solution, the contribution to the relaxation of each residue due to the overall molecular motion would be identical, regardless of its location in the three-dimensional (3D) structure of the protein. However, because of the anisotropy of the rotational diffusion of protein G due to its shape, the interaction vectors in the alpha helix experience a longer effective correlation time than the interaction vectors in the beta sheet. If an isotropic model for the rotational diffusion is used in this case, two possible consequences can be identified. In the first case, if the longer correlation time is used to fit residues whose interaction vectors are *not oriented* along the long axis of the protein, these residues will be fitted with compensatory slow internal correlation times τ_i . These slow internal correlation times will have no physical reality and are only an artifact of the fitting process. In the second case, if the shorter correlation time is used to fit residues whose interaction vectors are *oriented* along the long axis of the protein these residues will

be fitted with exchange parameters R_{ex} . Again, these exchange parameters also have no physical reality and do not represent a slow conformational process due to exchange between two chemically different sites.

In both the current analysis, using the isotropic model for the rotational diffusion tensor, and the previously published analysis (Barchi et al., 1994), the second case stated above is true. The preponderance of residues in the alpha helix that require the inclusion of an exchange parameter for the data to be fitted was previously interpreted as reflecting a small motion of the alpha helix with respect to the beta sheet. The order parameters S^2 for the residues in the alpha helix have a large dispersion in their values and the value also appears to oscillate with respect to the residue number. This anomaly could be due to the use of an isotropic model for the rotational diffusion tensor, reflecting changes in the orientation interaction vectors along the alpha helix.

In light of these observations, we therefore reanalyzed the overall rotational diffusion of protein G. This can be adequately described by an axially symmetric tensor with an anisotropy of $D_{||}/D_{\perp} = 1.68 \pm 0.08$. The orientation of the unique axis of the tensor is in agreement with inertial considerations, following the axis of the barrel form of the protein. The orientation of the prolate tensor in the molecular frame is illustrated in Figure 5.

This model of the rotational diffusion tensor for the model-free analysis allows 48 of the 51 residues to be fitted without the need for an exchange parameter, and only Ala29, for reasons that are not immediately apparent at this stage, cannot be adequately fitted by any of the models. One of the residues requiring an exchange parameter, Leu9, is present in the first beta strand, but the exchange contribution is only 1.03 s^{-1} and is required because of the deviation in the measured R_2 rate. Many of the residues in loop regions or at the ends of secondary structure elements show increased motion. Residues near the N-terminus, Ala4 and Val5, show a large amount of internal motion with S^2 values of 0.35 ± 0.01 and 0.54 ± 0.01 , respectively, which are much lower than the average for secondary structure elements of protein G. The C-terminal residue, Glu64, also shows a large amount of internal motion though on both the fast and slow timescales, with $S_f^2 = 0.71 \pm 0.04$, $S_s^2 = 0.19 \pm 0.05$, and $\tau_i = 908 \pm 65 \text{ ps}$.

Residues between the first and second beta strands, Leu17 to Glu20, exhibit lower than average order parameters. This corre-

lates well with an increase in the root-mean-square deviation (RMSD) calculated from the average structure in this region of the protein (Lian et al., 1992). The highest RMSD is, however, shifted by one residue toward the N-terminus when compared with the lowest S^2 value (Leu17). This observation was previously reported (Barchi et al., 1994), but can now be substantiated, because the rotational diffusion tensor has been properly described. For Asp45 and Gly46, which are located in the loop region between the alpha helix and the third beta strand, the lower than average order parameter S^2 and the increased internal correlation times are not matched with an increase in the RMSD. This may be due to the fact that relaxation is more sensitive to changes in internal motion than is the RMSD.

The last region of interest is the loop between the third and fourth beta strands, comprising residues Ala53 and Thr54. We have previously suggested that these residues adopt a major and a minor conformation (Lian et al., 1992). A small deviation in the S^2 values for these residues is observed. Although this observation suggests that this region of the protein is not completely rigid, the conformation exchange is on a slow time scale that is not accessible with ^{15}N relaxation measurements.

Finally, it is also possible to detect a lower than average S^2 value for Thr49 (0.77 ± 0.01); this residue is known to deviate by $\sim 60^\circ$ from its expected ψ angle (Lian et al., 1992), implying greater flexibility.

It is clear from the analysis of the rotational diffusion tensor that protein G cannot be approximated by an isotropic model. In the past, this approximation has led to many of the residues in the alpha helix being fitted with an exchange parameter, which were consequently misinterpreted as motion of the slow timescale. By using a prolate axially symmetric rotational diffusion tensor, both this fictitious slow motion and the apparent oscillation in the order parameters are eliminated. This analysis gave more accurate S^2 values and therefore allowed greater confidence in the interpretation of the relaxation data. This study highlights the complementary importance of accurate descriptions of the contribution to the vectorial reorientation functions from global and internal motions. Analysis of relaxation data for protein G demonstrates the importance of using the correct model for the rotational diffusion tensor to avoid incorrect biological conclusions being drawn and erroneous interpretation of the dynamics of a protein.

Fig. 5. An illustration of the prolate axially symmetric diffusion tensor of protein G, with an anisotropy of $D_{||}/D_{\perp} = 1.68 \pm 0.08$. The line represents the orientation of the unique axis, with the positions determined from 1,000 Monte Carlo simulations represented by the symbol +.

Materials and methods

The 64-residue uniformly ^{15}N -labeled protein G domain II was obtained by growing the transformed *Escherichia coli* in $2 \times \text{M9}$ medium containing 1 g/L of ^{15}N ammonium chloride as the sole nitrogen source, with 200 g/mL ampicillin. The protein purification was similar to that described in Lian et al. (1991). A 4 mM solution of protein G was used in 50 mM NaH_2PO_4 buffer with 90% $\text{H}_2\text{O}/10\%$ D_2O at pH 6.2.

The NMR relaxation measurements were performed on a Bruker DMX 500 MHz spectrometer operating at 500.1 MHz for ^1H , using a 5 mm triple resonance ($^{15}\text{N}/^{13}\text{C}/^1\text{H}$) probe equipped with a single-axis actively shielded gradient, the sample temperature was set to 300 K. The pulse sequences used to measure ^{15}N R_1 and R_2 relaxation rates and the heteronuclear ^1H - ^{15}N NOE have been previously described (Farrow et al., 1994). Two-dimensional ^1H - ^{15}N spectra were acquired with 1,024 (^1H) and 128 (^{15}N) complex points averaging 8 (R_1 and R_2) or 64 (^1H - ^{15}N NOE) transients per t_1 increment. ^{15}N R_1 experiments were carried out at 17 mixing times of 10.04, 20.09, 40.18, 70.31, 110.48, 140.62, 180.79, 220.97, 271.19, 331.45, 391.72, 462.02, 542.38, 652.86, 793.48, 1,004.40, and 1,255.50 ms. ^{15}N R_2 experiments were carried out at 17 mixing times of 5.94, 17.83, 29.72, 41.61, 53.50, 65.38, 77.27, 95.10, 112.94, 130.77, 148.60, 172.38, 196.15, 225.87, 261.54, 303.14, and 356.64 ms. The ^1H - ^{15}N NOE was measured from two spectra, with and without ^1H saturation (which comprised of 120° pulses). A recycling delay of 1.5 s was allowed for the ^{15}N R_1 and R_2 experiments and 3 s for the NOE experiments. Data processing and cross-peak intensity measurements were performed using FELIX 97.2 (Biosym/MSI Technologies, San Diego, California). ^{15}N R_1 and R_2 relaxation rates and accompanying errors were calculated from a single exponential fit of the data. The ^1H - ^{15}N NOE error was determined from the noise level.

The measured ^{15}N heteronuclear relaxation parameters R_1 , R_2 , and NOE are functions of the spectral density function $J(\omega)$, defined as the Fourier transformation of the time correlation function:

$$R_1 = d^2 [J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N)] + c^2 J(\omega_N) \quad (1A)$$

$$R_2 = (d^2/2) [4J(0) + J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N) + 6J(\omega_H)] + (c^2/6) [4J(0) + 3J(\omega_N)] \quad (1B)$$

$$\text{NOE} = 1 + d^2 (\gamma_H/\gamma_N) [6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)]/R_1 \quad (1C)$$

where $d^2 = (1/10)(\mu_0/2\pi)^2 \gamma_H^2 \gamma_N^2 \hbar^2 (r_{NH}^{-3})^2$ and $c^2 = (2/15) \omega_N^2 (\sigma_{\parallel} - \sigma_{\perp})^2$, $\hbar$ is Planck's constant divided by 2π , γ_H and γ_N the gyromagnetic ratios of the ^1H and ^{15}N , respectively, ω_H and ω_N their Larmor frequencies, and r_{NH} the internuclear distance (assumed to average to 1.01 Å). $\sigma_{\parallel}$ and $\sigma_{\perp}$ are the parallel and perpendicular components of the axially symmetric ^{15}N chemical shift tensor, which is assumed to be parallel to the dipolar interaction [$\sigma_{\parallel}/\sigma_{\perp}$ is taken to be -170 ppm (Tjandra et al., 1996)].

For protein G, the rotational diffusion tensor was determined by minimization of Equation 2, optimizing the orientation and amplitude of the eigenvalues and vectors of the tensor in the coordinate frame of the NMR structure.

$$\chi^2 = \sum_n [(R_2^{\text{meas}}/R_1^{\text{meas}})^2 - (R_2^{\text{calc}}/R_1^{\text{calc}})^2]/\sigma^2. \quad (2)$$

For anisotropic diffusion, the spectral density function introduced by Woessner (1962) was used:

$$J(\omega) = \sum_j \left\{ \frac{A_j \tau_j}{1 + (\omega \tau_j)^2} \right\} \quad (3)$$

where $A_1 = 3\mathbf{y}^2\mathbf{z}^2$, $A_2 = 3\mathbf{x}^2\mathbf{z}^2$, $A_3 = 3\mathbf{x}^2\mathbf{y}^2$, $A_{4,5} = 0.25\{3(\mathbf{x}^4 + \mathbf{y}^4 + \mathbf{z}^4) - 1\} \pm (1/12)\{\delta_x((3\mathbf{x}^4 + 6\mathbf{y}^2\mathbf{z}^2 - 1) + \delta_y((3\mathbf{y}^4 + 6\mathbf{x}^2\mathbf{z}^2 - 1) + \delta_z((3\mathbf{z}^4 + 6\mathbf{y}^2\mathbf{x}^2 - 1)\}$ and $\tau_{1,2,3} = (4D_{xx} + D_{yy} + D_{zz})^{-1}$, $(D_{xx} + 4D_{yy} + D_{zz})^{-1}$, $(D_{xx} + D_{yy} + 4D_{zz})^{-1}$, $\tau_{4,5} = (6D_{iso}^2 \pm 6(D_{iso}^2 - L^2)^{1/2})$, $D_{iso} = (D_{xx} + D_{yy} + D_{zz})/3$, $L^2 = (D_{xx}D_{yy} + D_{xx}D_{zz} + D_{yy}D_{zz})/3$ and $\delta_m = (D_{mm} - D_{iso})/(D_{iso}^2 - L^2)^{1/2}$. $m = (x, y, z)$ and $(\mathbf{x}, \mathbf{y}, \mathbf{z})$ are the direction cosines of the N-H vector in the principal axis frame of the diffusion tensor. For the general case, this is a six parameter fit (D_{xx} , D_{yy} , D_{zz} , θ , ϕ , and ψ), in the case of the axially symmetry diffusion tensor four parameters are optimized ($D_{\parallel}$, $D_{\perp}$, θ , and ϕ), and for an isotropic diffusion tensor, one single parameter is required (τ_c). For the axially symmetry diffusion tensor, the function can have two minima, corresponding to the prolate and oblate approximations to the real tensor.

The NMR structure of protein G (Lian et al., 1992) (PDB entry code: 2igg) has been elucidated and contains an ensemble of 27 structures, of which Oldorado (Kelley et al., 1997) predict there is only one subfamily with the most representative structure being number 6. Structure number 6 from the ensemble was therefore used in the relaxation data analysis. To investigate the sensitivity of the rotational diffusion parameterization to the dispersion present in the NMR ensemble, we have determined rotational diffusion tensor for each of the 27 structures.

The different models for the rotational diffusion tensor were used to fit the tensor using a selection of residues, for which the internal mobility is assumed to be negligible. In the case of the protein G, we have taken residues present in secondary structural motifs (the alpha helix and the beta sheet), in total 38 of these residues for which relaxation data were measured were used.

The relaxation data were then analyzed and fitted to one of five models using the model-free spectral density function. Assuming isotropic rotation diffusion, the Lipari and Szabo (1982) form is given by Equation 4.

$$J(\omega) = \left\{ S^2 \frac{\tau_c}{1 + (\omega \tau_c)^2} + (1 - S^2) \frac{\tau'}{1 + (\omega \tau')^2} \right\} \quad (4)$$

$$\frac{1}{\tau'} = \frac{1}{\tau_c} + \frac{1}{\tau_i}$$

where S^2 is the generalized overall order parameter and τ_i is the internal correlation time. In models 1 and 4, τ_i are <20 ps, and in Equation 4, therefore, $\tau_i = 0$. Model 2 is given by Equation 4 and contains two parameters S^2 and τ_i . Models 3 and 4 are the same as models 1 and 2, respectively, with the addition of an exchange parameter R_{ex} to R_2 . An extended model-free spectral density function has been proposed (Clare et al., 1990), Equation 5, in which a second internal motion on a slower time scale can be considered.

$$J(\omega) = S_f^2 \left\{ S_s^2 \frac{\tau_c}{1 + (\omega \tau_c)^2} + (1 - S_s^2) \frac{\tau'}{1 + (\omega \tau')^2} \right\}. \quad (5)$$

Slow internal motion is characterized by an order parameter given by S_s^2 and a correlation time τ_i . Fast internal motion is characterized by an order parameter given by S_f^2 ; the correlation time for fast internal motion is assumed to be negligible. Model 5 is taken from Equation 5 and contains three parameters S_f^2 , S_s^2 , and τ_i . We have used the anisotropic diffusion tensor in a hybrid anisotropic diffusion/Lipari–Szabo spectral density function to determine the local mobility in the anisotropically tumbling molecule:

$$J(\omega) \approx S_f^2 \sum_j \frac{A_j \tau_j}{1 + (\omega \tau_j)^2} + (1 - S_s^2) \frac{\tau'}{1 + (\omega \tau')^2} \quad (6)$$

where

$$\frac{1}{\tau'} = 6D_{iso} + \frac{1}{\tau_i}.$$

To avoid overinterpretation of the data, we have developed and applied a rigorous statistical testing procedure, similar to that developed by Modelfree (Mandel et al., 1995), except that an anisotropic diffusion tensor is available to describe the overall tumbling.

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References

- Barchi JJ, Grasberger B, Gronenborn AM, Clore GM. 1994. Investigation of the backbone dynamics of the IgG-binding domain of streptococcal protein-G by heteronuclear 2-dimensional H-1-N-15 nuclear-magnetic-resonance spectroscopy. *Protein Sci* 3:15–21.
- Blackledge M, Cordier F, Dosset P, Marion D. 1998. Precision and uncertainty in the characterization of anisotropic rotational diffusion by N-15 relaxation. *J Am Chem Soc* 120:4538–4539.
- Clore GM, Szabo A, Bax A, Kay LE, Driscoll PC, Gronenborn AM. 1990. Deviations from the simple 2-parameter model-free approach to the interpretation of N-15 nuclear magnetic-relaxation of proteins. *J Am Chem Soc* 112:4989–4991.
- Cordier F, Caffrey M, Brutscher B, Cusanovich MA, Marion D, Blackledge M. 1998. Solution structure, rotational diffusion anisotropy and local backbone dynamics of *Rhodobacter capsulatus* cytochrome *c2*. *J Mol Biol* 281:341–361.
- Farrow NA, Muhandiram R, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T, Formankay JD, Kay LE. 1994. Backbone dynamics of a free and a phosphopeptide-complexed Src homology-2 domain studied by N-15 NMR relaxation. *Biochemistry* 33:5984–6003.
- Kay LE. 1998. Protein dynamics from NMR. *Nat Struct Biol* 5:513–517.
- Kay LE, Torchia DA, Bax A. 1989. Backbone dynamics of proteins as studied by N-15 inverse detected heteronuclear NMR spectroscopy—Application to staphylococcal nuclease. *Biochemistry* 28:8972–8979.
- Kelley LA, Gardner SP, Sutcliffe MJ. 1997. An automated approach for defining core atoms and domains in an ensemble of NMR-derived protein structures. *Protein Eng* 10:737–741.
- Kordel J, Skelton NJ, Akke M, Palmer AG, Chazin WJ. 1992. Backbone dynamics of calcium-loaded calbindin-D(9k) studied by 2 dimensional proton-detected N-15 NMR-spectroscopy. *Biochemistry* 31:4856–4866.
- Krishnan VV. 1997. Determination of oligomeric state of proteins in solution from pulsed-field-gradient self-diffusion coefficient measurements. A comparison of experimental, theoretical, and hard-sphere approximated values. *J Magn Reson* 124:468–473.
- Lian LY, Derrick JP, Sutcliffe MJ, Yang JC, Roberts GCK. 1992. Determination of the solution structures of domain-Ii and domain-Iii of protein-G from streptococcus by H-1 nuclear-magnetic-resonance. *J Mol Biol* 228:1219–1234.
- Lian LY, Yang JC, Derrick JP, Sutcliffe MJ, Roberts GCK, Murphy JP, Goward CR, Atkinson T. 1991. Sequential H-1-NMR assignments and secondary structure of an IgG-binding domain from protein-G. *Biochemistry* 30:5335–5340.
- Lipari G, Szabo A. 1982. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results. *J Am Chem Soc* 104:4559–4570.
- Luginbuhl P, Pervushin KV, Iwai H, Wüthrich K. 1997. Anisotropic molecular rotational diffusion in N-15 spin relaxation studies of protein mobility. *Biochemistry* 36:7305–7312.
- Mandel AM, Akke M, Palmer AG. 1995. Backbone dynamics of *Escherichia coli* ribonuclease hi-correlations with structure and function in an active enzyme. *J Mol Biol* 246:144–163.
- Palmer AG. 1997. Probing molecular motion by NMR. *Curr Opin Struct Biol* 7:732–737.
- Schurr JM, Babcock HP, Fujimoto BS. 1994. A test of the model-free formulas—Effects of anisotropic rotational diffusion and dimerization. *J Magn Reson Ser B* 105:211–224.
- Tillett ML, Horsfield MA, Lian LY, Norwood TJ. 1999. Protein-ligand interactions measured by 15N-filtered diffusion experiments. *J Biomol NMR* 13:223–232.
- Tjandra N, Feller SE, Pastor RW, Bax A. 1995. Rotational diffusion anisotropy of human ubiquitin from N-15 NMR relaxation. *J Am Chem Soc* 117:12565–12566.
- Tjandra N, Szabo A, Bax A. 1996. Protein backbone dynamics and N15 chemical shift anisotropy from quantitative measurement of relaxation interference effects. *J Am Chem Soc* 118:6986–1991.
- Woessner DE. 1962. Nuclear spin relaxation in ellipsoids undergoing rotational Brownian motion. *J Chem Phys* 37:647–654.