Published in final edited form as:

J Biomol NMR. 2016 November ; 66(3): 163–174. doi:10.1007/s10858-016-0064-7.

Quantifying protein dynamics in the ps-ns time regime by NMR relaxation

Griselda Hernández¹ and David M. LeMaster^{1,*}

¹Wadsworth Center, New York State Department of Health and Department of Biomedical Sciences, School of Public Health, University at Albany - SUNY, Empire State Plaza, Albany, New York, 12201

Abstract

Both ¹⁵N chemical shift anisotropy (CSA) and sufficiently rapid exchange linebroadening transitions exhibit relaxation contributions that are proportional to the square of the magnetic field. Deconvoluting these contributions is further complicated by residue-dependent variations in protein amide ¹⁵N CSA values which have proven difficult to accurately measure. Exploiting recently reported improvements for the implementation of T₁ and T_{1ρ} experiments, field strength-dependent studies have been carried out on the B3 domain of protein G (GB3) as well as on the immunophilin FKBP12 and a H87V variant of that protein in which the major conformational exchange linebroadening transition is suppressed. By applying a zero frequency spectral density rescaling analysis to the relaxation data collected at magnetic fields from 500 MHz ¹H to 900 MHz ¹H, differential residue-specific ¹⁵N CSA values have been obtained for GB3 which correlate with those derived from solid state and liquid crystalline NMR measurements to a level similar to the correlation among those previously reported studies. Application of this analysis protocol to FKBP12 demonstrated an efficient quantitation of both weak exchange linebroadening contributions and differential residue-specific ¹⁵N CSA values. Experimental access to such differential residue-specific ¹⁵N CSA values should significantly facilitate more accurate comparisons with molecular dynamics simulations of protein motion that occurs within the timeframe of global molecular tumbling.

Keywords

Chemical shift anisotropy; exchange linebroadening; field strength dependence; GB3; FKBP12

Introduction

In the ongoing efforts to experimentally test and further develop molecular force fields for the modeling of biomacromolecular motion, solution NMR relaxation measurements offer a particularly promising source for extensive dynamics information as monitored at the level of individual nuclei throughout the protein structure. Unfortunately, the full exploitation of this approach has been hindered by various practical considerations. The great majority of

*Correspondence to: David M. LeMaster, Wadsworth Center, New York State Department of Health, Empire State Plaza, Albany, New York, 12201. Telephone: (518)474-6396, Fax: (518)473-2895, david.lemaster@health.ny.gov.

studies reporting the standard T_1 , T_2 relaxation time and heteronuclear NOE experiments have been applied to backbone ^{15}N resonances (Jarymowycz and Stone 2006) along with a smaller number of ^{13}C and ^2H methyl relaxation studies (Igumenova et al. 2006). In recent years much of the effort in this field has focused on the conformational exchange linebroadening effects on T_2 values that arise from motion in the μs -ms time regime. Although of interest in their own right, these motions are generally too slow to be adequately modeled by unbiased full atom molecular dynamics simulations to serve as tests for the robustness of these algorithms.

Beyond these slower chemical/conformational exchange effects, standard T_1 , T_2 and NOE values are sensitive only to dynamical processes that occur within the timeframe of global molecular tumbling (~ 5 -10 ns for small globular proteins) or faster. Under the assumption that the protein does not more slowly switch to well-populated alternate conformations that have distinctly differing dynamical properties, molecular simulations of $\sim \mu\text{s}$ duration can provide the roughly 100-fold sampling needed to properly evaluate the relatively high frequency motions which occur within the timeframe of molecular tumbling (Lu and vandenBout 2006). Such simulations should be sufficient for predicting either the S^2 order parameters obtained via the widely used Lipari-Szabo model-free analysis (Lipari and Szabo 1982) or, more fundamentally, the spectral density values from which the individual NMR experimental values can be directly derived. For the case of well-ordered backbone ^{15}N resonances in particular, the order parameter is overwhelmingly determined by the global tumbling time of the protein τ_m and the zero frequency spectral density value $J(0)$:

$$J(0) = 2/5[S^2\tau_m + (1 - S^2) \cdot \tau] \quad (1)$$

where $\tau^{-1} = \tau_m^{-1} + \tau_e^{-1}$ with τ_e being the effective time constant for internal motion. Analysis of the model-free formalism has indicated its validity for τ_e values less than ~ 0.3 ns (Lipari and Szabo 1982). Note that for non-spherical globular proteins, each ^1H - ^{15}N bond vector has a distinct effective global tumbling time that is characteristic of its orientation with respect to the rotational anisotropy tensor (Woessner 1962). With a typical S^2 value of 0.85 for well-ordered backbone segments, the second term of the equation will generally be less than 1% of the first term.

Within the reduced spectral density approximation for the small $J(\omega_H)$ dependent term (Farrow et al. 1995), a more fundamental representation of the zero frequency spectral density value can be derived directly from the experimental NMR relaxation rate measurements (Phan et al. 1996):

$$(R_2 - R_1/2) = (2\omega_N^2 \Delta\sigma^2 / 9 + d^2/2) \cdot J(0) + 0.6(\gamma_N/\gamma_H)(\text{NOE} - 1)R_1 + R_{\text{ex}} \quad (2)$$

where R_{ex} is the exchange linebroadening factor. The dipolar coupling factor d equals $[\mu_0 h \gamma_H \gamma_N / (8\pi^2)] \langle 1/r_{\text{NH}}^3 \rangle$, ω_N equals $\gamma_N B_0$, and σ equals the anisotropy of the chemical

shift tensor ($\sigma_{\parallel} - \sigma_{\perp}$). As illustrated by the small B3 domain of streptococcal protein G considered below, at 600 MHz the NOE term is only 1% of the total in Eq. 2 for well-ordered residues and is a correspondingly smaller contribution for more slowly tumbling proteins and for higher magnetic fields.

For conformational exchange transitions exhibiting sufficiently slow kinetics, relaxation dispersion experiments can directly determine the magnitude of the R_{ex} term. However, for the common case of conformational linebroadening dynamics which approach the fast exchange limit, an alternate approach is required. Experimental studies for quantifying R_{ex} due to conformational dynamics near the fast exchange limit most commonly have utilized R_2 measurements at multiple magnetic fields so as to exploit the B_0^2 dependence of this effect. Unfortunately, the accuracy of such analyses is degraded by several factors including uncertainty in the chemical shift anisotropy factor σ which also gives rise to corrections that vary with B_0^2 . On the expectation that, for many proteins, exchange linebroadening will affect only a subset of the backbone ^{15}N resonances while the CSA effects are ubiquitous, a number of field-dependent relaxation studies have been conducted in order to quantify the residue-specific CSA contributions. GB3 has been widely used to such studies, in part due to its apparent lack of significant conformational exchange linebroadening for the backbone ^{15}N resonances. While initial studies indicated a quite large variability among the residue-specific ^{15}N CSA values (125 to 216 ppm (Fushman et al. 1998)), subsequent solution NMR studies have reported smaller standard deviations for the distribution of estimated residue-specific CSA values, although still on the order of $\sim 10\%$ (Damberg et al. 2005; Hall and Fushman 2006; Kroenke et al. 1999). However, much of this spread appears to reflect experimental uncertainty (Kroenke et al. 1999).

Solid state NMR techniques provide a more direct means of assessing the chemical shift tensor and its anisotropy. Nevertheless, the challenges of accurate quantitation are indicated by two recent solid state NMR studies on the B1 domain of the protein G (closely homologous to GB3) which yields estimates for the average ^{15}N CSA anisotropy value of 164 ppm (Wylie et al. 2007) and 174 ppm (Hung et al. 2015). Such discrepancies are largely transparent in model-free analyses of solution ^{15}N relaxation measurements at a single magnetic field since the predicted S^2 values scale with the assumed average ^{15}N CSA value and thus yield equivalent statistical fits to the observed relaxation data.

In practice, molecular dynamics analysis of protein NMR relaxation is typically completely insensitive to the accuracy of the assumed average ^{15}N CSA value. For the common case of comparison against experimentally derived S^2 values, only the time averaged correlation of the ^1H - ^{15}N bond vector orientations is typically computed. For the more demanding predictions of the experimental NMR relaxation values themselves, the theoretical coupling factor must be rescaled to reflect the fact that MD simulations standardly utilize rigid models for hydrogen-containing covalent bonds so that the contributions to relaxation arising from high frequency classical/quantum mechanical librations are not adequately predicted (Case 1999). While this rescaling of the theoretical coupling factor to optimally match the experimental data is typically expressed in terms of an altered effective ^1H - ^{15}N bond length, in reality any error in the assumed average ^{15}N CSA value is simultaneously corrected for as well. Consistent with Eq. 2, the ratio of the dipolar and CSA contributions to both R_1 and R_2

is closely approximated by $(2\omega_N - \sigma/3d)^2$. As a result, at 600 MHz ^1H the predicted relaxation effects from a 3% (~ 5 ppm) elevation in the average ^{15}N CSA factor can be accurately compensated for by a 0.004 Å increase in the assumed bond length. With the exception of the still rare occasion in which MD simulations are used for quantitative prediction of NMR relaxation values at multiple magnetic field strengths, the continued uncertainties in the absolute magnitude of the protein ^{15}N CSA values do not pose a substantial practical limitation for molecular dynamics analysis.

On the other hand, such a rescaling protocol can not correct for the deviations which will arise if the ^{15}N CSA values significantly differ at various positions along the protein backbone. Indeed, the solid state NMR studies mentioned above, as well as an NMR study utilizing liquid crystalline aligned samples (Yao et al. 2010), have found that the residue-specific chemical shift anisotropies of GB1/GB3 are conformationally dependent. In particular, the α -helical residues of these proteins exhibit ^{15}N CSA values that are $\sim 6\%$ higher than those in β -sheets. At 600 MHz, a 6% difference in ^{15}N CSA values yields a 3.2% change in the predicted S^2 values. As a result, if equivalent CSA values are applied to the analysis of helical and sheet residues, equally well-ordered helices and sheets would be incorrectly predicted to have relatively more dynamic sheet segments. As an illustration, predicted S^2 values of 0.823 for sheets and 0.850 for the helices based on uniform CSA values would actually correspond to equivalent order parameter dynamics. Molecular dynamics simulations model the time dependency for the loss of structural correlation, that is to say $(1-S^2)$. In this illustration, the improper assumption of a uniform ^{15}N CSA value yields an 18% error in the prediction of the relative dynamics of the helical vs. the sheet segments for 600 MHz NMR data which then increases to a 31% error (0.804 vs. 0.850) for the analogous 900 MHz data.

This evidence for a potentially significant source of incorrect modeling of experimental data on protein conformational dynamics is reinforced by the fact that the automated fragmentation quantum mechanics/molecular mechanics calculations of Tang and Case (Tang and Case 2011) closely reproduced the average differential ^{15}N CSA values for the helical and sheet segments of GB1/GB3 domains as well as finding a somewhat smaller differential for the ^{15}N CSA values of ubiquitin, consistent with previously published experimental data (Loth et al. 2005). Unfortunately, experimental measurements of residue-specific CSA values have not been generally applied to other proteins systems using either the solid state NMR or liquid crystalline solution NMR techniques so that the quantification and correction for these effects in NMR relaxation studies has been impeded.

The present study reconsiders the utility of the magnetic field strength dependence of ^{15}N relaxation in isotropic solution measurements, taking advantage of recently introduced improvements in the standard T_1 , $T_{1\rho}$ (Lakomek et al. 2012) and NOE (Ferrage et al. 2010) experiments and a differential scaling approach that compensates for small differences in the effective $J(0)$ values among datasets. While the GB1/GB3 domains have been particularly favored for the analysis of residue-specific CSA values, in part due to the apparent absence of conformational exchange linebroadening effects on the backbone ^{15}N resonances, the recent report of evidence for small conformational exchange linebroadening for ^{13}C and ^1H sidechain resonances in GB3 (Smith et al. 2015) warrants a reinvestigation of similar effects

for the mainchain ^{15}N resonances. This differential analysis approach is then applied to the immunophilin FKBP12 and a mutational variant which has previously been shown to selectively suppress a prominent conformational exchange linebroadening transition. This protocol is analyzed for its capacity to enhance the quantitation of both residue-specific ^{15}N CSA values and weak exchange linebroadening effects as well as more fully characterizing the spatial distribution of the residues exhibiting those exchange linebroadening effects.

Methods

Sample preparation

The expression and purification of the FKBP12 proteins has been previously described (Mustafi et al. 2013). Gene sequence for the B3 domain of streptococcal protein G (Hall and Fushman 2003) was chemically synthesized (Genscript), with codon optimization for expression in *Escherichia coli*. The gene sequences were cloned into the expression vector pET11a and the plasmids were transformed into the BL21Star(DE3) strain (Invitrogen) for expression. Protein expression and purification of GB3 followed that for FKBP12 (Mustafi et al. 2013), excepting the absence of reducing agent in the buffers, until the final ion exchange chromatography step. For the case of GB3, the eluant from the Sephadex G-50 column was loaded onto a Q-Sepharose column equilibrated to the same Tris buffer and then eluted with a gradient to 0.25 M NaCl. The NMR sample was concentrated via centrifugal ultrafiltration and then equilibrated into a 25 mM NaH_2PO_4 buffer (pH 6.3) containing 0.01% NaN_3 and 6% D_2O by a series of centrifugal concentration steps to a final concentration of 2 mM protein.

NMR Spectroscopy and Analysis

NMR relaxation data were collected on a Bruker Avance II 500 MHz spectrometer, a Bruker Avance III 600 MHz spectrometer, a Bruker Avance II 800 MHz spectrometer and a Bruker Avance II 900 MHz spectrometer at 25°C as calibrated against methanol- d_4 . All spectrometers were equipped with cryo-temperature probes. HSQC-based T_1 , $T_{1\rho}$ and heteronuclear NOE experiments were carried out as described by Lakomek et al. (Lakomek et al. 2012) and Ferrage et al. (Ferrage et al. 2010). Spin lock fields were calibrated by offset-dependent scalar coupling measurements (Palmer et al. 2001). To enhance statistical sampling and minimize ^{15}N offset corrections, the $T_{1\rho}$ experiments were repeated at equally spaced ^{15}N carrier frequencies (three sets at 500, four sets at 600, and five sets at higher fields). In calculating R_2 values for each resonance from the R_1 and $R_{1\rho}$ data, the R_2 determinations for each $R_{1\rho}$ set were then averaged according to the ^{15}N offset using a linear weighting varying from 1.0 on resonance to 0.0 at an offset equal to 60% of the ^{15}N spin lock field strength. Recycle times of 3s, 3s, and 10s were used for the R_1 , $R_{1\rho}$ and heteronuclear NOE experiments, respectively. FELIX software (Felix NMR) was used for NMR data processing.

Results and discussion

Magnetic field strength dependence of ^{15}N relaxation in GB3

The correlation between changes in $(R_2 - R_1/2)$ values and the square of the magnetic field strength has been commonly used as a means of assessing the internal consistency between analogous relaxation datasets collected at different field strengths (Fushman et al. 1999; Morin and Gagne 2009; Phan et al. 1996). In the absence of exchange linebroadening and excepting the generally small $J(\omega_H)$ -dependent term (Eq. 2), the factor $(2\omega_N^2 - \sigma^2/9 + d^2/2)$ provides the proportionality between the $(R_2 - R_1/2)$ value and the spectral density $J(0)$. Thus if the $J(0)$ values for each nucleus were identical in both datasets, the ratio of the $(R_2 - R_1/2)$ values at different magnetic field strengths could be directly predicted from the relative magnitudes of the dipolar coupling factor and the anisotropy of the CSA tensor.

However, even when using the same NMR sample at differing magnetic field strengths, slight differences in sample temperature can substantially alter the solvent viscosity and hence the τ_m value for the protein. As the viscosity of water differs by 2.3% between 24°C and 25°C, a proportional change in the $(R_2 - R_1/2)$ values imposes severe experimental constraints, particularly given the substantial power input required for the CPMG or spin lock sequences used in measuring R_2 or $R_{1\rho}$ which complicates accurate sample temperature calibration and regulation. The challenge of accurately matching the $J(0)$ values for different datasets is obviously further exacerbated when comparisons are made between different protein samples. Taking advantage of the fact that the $J(0)$ values are generally proportional to τ_m (Eq. 1) and that variations in $J(0)$ affect the dipolar and CSA contributions to the $(R_2 - R_1/2)$ values in a proportional manner, a relaxation analysis that is based upon the ratio of the $(R_2 - R_1/2)$ values at different magnetic field strengths enables the corrections for small variations in the $J(0)$ values for different datasets to be incorporated directly.

Recently Bax and colleagues (Lakomek et al. 2012) described systematic improvements in the implementation of the ^{15}N T_1 and $T_{1\rho}$ experiments. The use of amide resonance-selective 180° ^1H IBURP pulses spaced by 40 ms during the relaxation delay of the T_1 experiment averages the effects of the differently relaxing components arising from interference between the ^{15}N - ^1H dipolar and ^{15}N CSA interactions, while minimizing saturation of the water magnetization. While this T_1 experiment can exhibit small systematic errors due to uncontrolled saturation of aliphatic protons when samples lacking background perdeuteration are used (Gairí et al. 2015), this artifact is suppressed by using longer recycle times (3s used herein). The use of a pair of composite ^1H 180° pulses during the relaxation delay of $T_{1\rho}$ experiment achieves a similar effect, while adiabatic half passage pulses encapsulated by z filter pulses (Mulder et al. 1998) achieve precise alignment of the spin lock field. In their original study (Lakomek et al. 2012), a sample of $\text{U-}^2\text{H}, ^{15}\text{N}$ enriched GB3 protein was used to demonstrate the performance of these improved pulse sequences as measured at 600 MHz (14.1 T). An additional potential benefit of utilizing $T_{1\rho}$ experiments for differential analyses similar to the one described herein is that under reasonably broad conditions, the exchange linebroadening contribution to the rotating-frame relaxation rate is proportional to the square of the magnetic field well outside of the fast exchange limit (Baldwin and Kay 2013; Trott and A. G. Palmer 2002).

Extensive magnetic field dependent ^{15}N relaxation measurements have been reported by the laboratories of Fushman (Hall and Fushman 2006) on GB3 and Mayo (Idiyatullin et al. 2003) on GB1 with the former study also including a determination of residue-specific CSA values. The correlation coefficients for the $(R_2-R_1/2)$ values from the 600 MHz datasets of these two studies, relative to the more recent data of Bax and colleagues (Lakomek et al. 2012) are 0.960 and 0.952, respectively (Table 1). The correlation coefficients appreciably decrease when this reference 600 MHz dataset is compared against either the 500 MHz or 800 MHz datasets from the Fushman and Mayo studies. The internal consistency within these field dependent studies is somewhat higher as indicated by the correlation against their own 600 MHz data.

Using the optimized pulse sequences, we have collected ^{15}N relaxation data at 500, 600, 800 and 900 MHz in which $T_{1\rho}$ measurements were conducted at multiple ^{15}N offsets, and then the derived R_2 values were averaged (deposited in the BMRB under accession number 26845). For the datasets at each magnetic field, the correlation coefficients with respect to the 600 MHz dataset of Bax and colleagues were substantially higher than for the earlier field dependent studies. Internal consistency within our own datasets is also higher (Table 1). These measurements indicate that the use of robust relaxation pulse sequences can provide a quite high degree of correlation among $(R_2-R_1/2)$ values obtained over a wide range of magnetic field strengths. Furthermore, the comparatively strong correlation observed between the 600 MHz data of Lakomek et al. (Lakomek et al. 2012) and our own data sets suggest the potential to substantially compensate for the variations in the $J(0)$ values arising from independently prepared samples that somewhat differ in protein concentrations and buffer conditions as well as presumably modestly differing data collection temperatures.

Assessing the relaxation contribution arising from residue-specific ^{15}N CSA values in GB3

A linear scaling between the $(R_2-R_1/2)$ values for two different magnetic fields was carried out based on the ratio of the mean $(R_2-R_1/2)$ values for each dataset. Since a normal distribution for the resultant differences need not apply, the more statistically robust median values were also monitored, but yielded quite similar results. As illustrated by the relaxation datasets for the GB3 protein from 600 MHz and 800 MHz, inclusion of the $0.6(\gamma_{\text{N}}/\gamma_{\text{H}})(\text{NOE}-1)R_1$ term in Eq. 2 alters the derived individual residue values for $(R_2-R_1/2)_{800/600}$ by less than 0.2% of the $(R_2-R_1/2)$ value for all residues except for the highly mobile Gly 41 residue which differs by 0.33%. As these differences are well below the overall precision of this analysis, the NOE term has not been included in the subsequent calculations.

Assuming no exchange linebroadening contributions, the magnetic field dependence of the $(R_2-R_1/2)$ values should arise solely from the ^{15}N CSA anisotropy factors. The signed magnitude of the difference in the scaled $(R_2-R_1/2)$ values for each nucleus should reflect the degree to which the CSA anisotropy σ for that nucleus deviates from the mean (i.e. $\overline{\Delta\sigma}$). Let σ be expressed as $(1+\delta_c)\overline{\Delta\sigma}$, and let D_B represent the average coupling term $(2/9 \cdot \omega_B^2 \cdot \overline{\Delta\sigma}^2 + d^2/2)$ to express the differential scaled $(R_2-R_1/2)$ value for each ^{15}N nucleus:

$$\Delta(R_2 - R_1/2)_{B'/B''} = \left(D_{B'} + 2/9 \cdot (2\delta_c + \delta_c^2) \cdot \omega_{B'}^2 \cdot \overline{\Delta\sigma^2} \right) \cdot J_{B'}(0) - (\tau_{B'}/\tau_{B''}) (D_{B'}/D_{B''}) \cdot \left(D_{B''} + 2/9 \cdot (2\delta_c + \delta_c^2) \cdot \omega_{B''}^2 \cdot \overline{\Delta\sigma^2} \right) \cdot J_{B''}(0) \quad (3)$$

where the ratio of the global molecular correlation times τ for the two data sets is assumed to model the change in $J(0)$ for each nucleus under potentially slightly differing collection conditions.

Utilizing the ratio of the τ values to cancel out the spectral density terms at each field yields:

$$\Delta(R_2 - R_1/2)_{B'/B''} = (2\delta_c + \delta_c^2) \cdot (2/9 \cdot \overline{\Delta\sigma^2}) \cdot (\omega_{B'}^2/D_{B'} - \omega_{B''}^2/D_{B''}) \cdot D_{B'} \cdot J_{B'}(0) \quad (4)$$

The product of the second and third factors in this equation corresponds to the difference at the two magnetic fields in the fraction of the relaxation which arises from the average CSA contribution. Assuming an ^{15}N CSA value of 168 ppm (Anderson and LeMaster 2012) and a ^1H - ^{15}N bond length of 1.020 Å, this difference equals 0.18 (0.44-0.26) for B' at 900 MHz and B'' at 600 MHz, respectively.

The product $D_{B'} \cdot J_{B'}(0)$ corresponds to $(R_2 - R_1/2)_{B'}$ under the assumption that σ equals $\overline{\Delta\sigma}$. Normalizing the scaled differences in the $(R_2 - R_1/2)$ values to the $(R_2 - R_1/2)$ value at the higher field yields:

$$\begin{aligned} \Delta(R_2 - R_1/2)_{B'/B''} / (R_2 - R_1/2)_{B'} \\ = (2\delta_c + \delta_c^2) \cdot (2/9 \cdot \overline{\Delta\sigma^2}) \cdot (\omega_{B'}^2/D_{B'} - \omega_{B''}^2/D_{B''}) \cdot D_{B'} / \left(D_{B'} + 2/9 \cdot (2\delta_c + \delta_c^2) \cdot \omega_{B'}^2 \cdot \overline{\Delta\sigma^2} \right) \end{aligned} \quad (5)$$

This equation can be robustly approximated by the simpler linearized form:

$$\Delta(R_2 - R_1/2)_{B'/B''} / (R_2 - R_1/2)_{B'} \cong 2\delta_c \left(2/9 \cdot \overline{\Delta\sigma^2} \right) \cdot (\omega_{B'}^2/D_{B'} - \omega_{B''}^2/D_{B''}) \quad (6)$$

such that the normalized scaled difference in $(R_2 - R_1/2)$ is given by the product of δ_c and the difference at the two magnetic fields in the fraction of the relaxation which arises from the average CSA contribution. At a δ_c value of 0.10 (~2-fold larger than the standard deviation for the experimentally reported σ values discussed herein), the deviation arising from the linear approximation is 0.005, 4-fold smaller than the experimental uncertainties in the δ_c values estimated for the FKBP12 protein considered below.

Thus, this ratio of experimentally observed relaxation values is predicted to depend solely upon the proportionality factor δ_c , the mean σ value, the effective ^1H - ^{15}N bond length, and elemental constants. The estimate of δ_c is highly insensitive to either the assumed mean σ value or the effective ^1H - ^{15}N bond length. The most robust experimental estimates for the mean value of σ for protein amides have generally yielded results within $\sim 3\%$ of the 168 ppm value assumed in the present calculations. Given the quadratic field dependence of these effects, such a 3% change in $\overline{\Delta\sigma}$ and the resultant 6% change in the CSA relaxation contribution yields a 1.7% change in the predicted δ_c value based on a pairwise analysis of 900 MHz and 600 MHz data. Even for the extreme case of the highly mobile Gly 41 of GB3, which exhibits a strikingly low ^{15}N CSA anisotropy, such a 1.7% shift in the δ_c value would change the predicted σ value by only 0.6 ppm, far less than any currently practical experimental accuracy. Similarly, a large increase in the assumed ^1H - ^{15}N bond length from 1.020 Å to 1.030 Å would decrease the predicted dipolar contribution by 6%, yet likewise would yield a predicted change in the δ_c value of only 1.7%.

Assessing the information content of this $(R_2-R_1/2)$ analysis is significantly complicated by the difficulties that have confronted obtaining robust residue-specific CSA anisotropy values by independent experimental approaches. As noted above, the two solid state NMR studies on GB1 (Hung et al. 2015; Wylie et al. 2007) and the liquid crystalline solution study on GB3 (Yao et al. 2010) differ among themselves with regards to the mean CSA anisotropy value. Furthermore, correlation analysis among the individual residue values from these three studies exhibits only a modest level of internal agreement (Table 2). The correlation between the two solid state NMR studies ($r = 0.75$) is arguably inflated by the outlier effect from the low ^{15}N CSA value for Gly 41. It should be noted that the exceptionally small CSA value for this residue has been proposed to arise from systematic error due to increased mobility at that site (Tang and Case 2011; Wylie et al. 2007). No ^{15}N CSA value was reported for Gly 41 in the liquid crystalline solution study on GB3 as that study specifically excluded loop residues that might exhibit substantial internal mobility (Yao et al. 2010).

The differential analysis for the relaxation datasets at 900 MHz and 600 MHz yielded correlation coefficients with respect to the various solid state NMR and liquid crystalline solution NMR measurements of the residue-specific CSA anisotropies of a quality similar to the correlation values among those previously reported datasets (Table 2). Similar comparisons utilizing the 900 MHz dataset and either the 500 MHz or 800 MHz datasets yielded somewhat lower correlation values. As anticipated from the modest degree of correlation indicated in Table 2, there is considerable dispersion when the various sets of residue-specific ^{15}N CSA anisotropy values are plotted with respect to the protein sequence in terms of the percentage by which each residue value differs from the mean CSA anisotropy value for that dataset (Fig. 1). Given the discussion in the literature referred to above as to whether the markedly lower ^{15}N CSA values reported for Gly 41 in both solid state NMR studies might arise from conformational dynamics effects, it should be noted that the $(R_2-R_1/2)$ -derived δ_c value lies midway between the results from those earlier studies. With respect to the seemingly robust prediction of the anomalously low ^{15}N CSA value for Gly 41 that has been obtained from the $(R_2-R_1/2)$ -based analysis, it would appear relevant

that, in contrast to the other experimental approaches considered here, these solution NMR measurements are not dependent upon the degree of orientational ordering for the nuclei.

Despite the variations among the different sets of residue-specific ^{15}N CSA anisotropy estimates, there are still clearly significant commonalities in the patterns exhibited, most notably the elevated values of δ_c seen for the central portion of the protein sequence corresponding to the single α -helix extending from Asp 22 to Asn 37. Averaging across the helical residues and those of the four β -strands in GB1/GB3 (Gln 2-Asn 8, Lys 13-Lys 19, Val 42-Asp 46, Thr 51-Thr 55) the difference in the mean CSA anisotropy for the two secondary structural states was found to be 7.4% and 4.9% in the earlier (Wylie et al. 2007) and later (Hung et al. 2015) solid state NMR studies, respectively, and 6.3% in the liquid crystalline NMR study (Yao et al. 2010). The δ_c values predicted from the experimental $(R_2-R_1/2)_{900/600}$ data gave a 5.5% differential between the α -helical and β -strand residues. Previous studies to derive residue-specific CSA anisotropy values from magnetic field dependent isotropic solution measurements on GB3 yielded a corresponding secondary structure differential value of -2.1% (Hall and Fushman 2006).

It should be noted that the present analysis ascribes all deviations in the experimental $(R_2-R_1/2)$ values to changes in the δ_c parameter. Given the substantial variation among the various experimental estimates of the residue-specific δ_c values of GB3/GB1 indicated in Fig. 1, it is plausible to expect for some residues that measurement errors may account for a significant proportion of the derived δ_c correction factors. To avoid potentially augmenting rather than decreasing the errors which result from assuming a uniform ^{15}N CSA value, it may prove useful to apply the δ_c -based corrections to the residues in which large deviations in the residue-specific ^{15}N CSA values most clearly appear to dominate the observed $(R_2-R_1/2)$ values. There are seven residues for which the magnitude of the $(R_2-R_1/2)$ -derived δ_c values are greater than 1.5 times the standard deviation for all residues. In each case, the average δ_c value obtained from the two solid state NMR and the liquid crystalline NMR studies predicts the same sign as that of the $(R_2-R_1/2)$ -derived δ_c value. If each of those seven average δ_c values is subtracted from the corresponding $(R_2-R_1/2)$ -derived δ_c values, the resultant 'CSA-corrected' values are, on average, two-fold smaller than the initial $(R_2-R_1/2)$ -derived δ_c values, suggesting that deviations in the residue-specific ^{15}N CSA values do account for the majority of the experimental $(R_2-R_1/2)$ values.

The present analysis indicates that exchange linebroadening contributions to the ^{15}N relaxation of the backbone amides of GB3 are quite small, $\sim 0.15\text{ s}^{-1}$ or less at 900 MHz ^1H for a sample at 298 K. In a recent NMR relaxation dispersion study of the ^1H and ^{13}C methyl resonances in GB3, the dispersion curves for the $^1\text{H}^{\text{N}}$ resonances for three residues - Lys 10, Leu 12 and Lys 13 were also presented (Smith et al. 2015). These authors reported $^1\text{H}^{\text{N}}$ exchange linebroadening contributions of $\sim 10\text{ s}^{-1}$ for Leu 12 and $\sim 5\text{ s}^{-1}$ for Lys 10. Not only are these exchange effects substantially larger than any analogous contributions to their reported sidechain ^1H and ^{13}C methyl resonance measurements, they are at least 50-fold larger than the exchange linebroadening effect for any backbone ^{15}N resonance of GB3 as assessed by the present study. While exchange linebroadening contributions need not be equivalent for the $^1\text{H}^{\text{N}}$ and ^{15}N resonances of a given residue, it seems surprising to observe such dramatic differences in magnitude for both Lys 10 and Leu

12. Since those relaxation dispersion measurements were carried out at 275 K (Smith et al. 2015), a steep temperature dependence for the underlying linebroadening transition might partially account for the far larger exchange effects seen in the $^1\text{H}^{\text{N}}$ measurements. However, earlier ^{15}N $T_{1\rho}$ measurements of GB1 at 278 K provide no evidence for any discernible exchange linebroadening at lower temperatures (Idiyatullin et al. 2003).

Mutational analysis of residue-specific ^{15}N CSA and conformational exchange linebroadening in FKBP12

The immunophilin FKBP12 exhibits extensive exchange linebroadening which arise from at least three distinct conformational transitions (Mustafi et al. 2013). Standard magnetic field strength dependence measurements as well as more direct relaxation dispersion studies (Brath and Akke 2009; Brath et al. 2006; Sapienza et al. 2011) have demonstrated that the rates of these transitions occur near the fast exchange limit. The most spatially extensive exchange linebroadening transition is centered at Gly 89 near the tip of long β_4 - β_5 loop (Mustafi et al. 2014a). This loop plays a central role in the protein-protein interactions involved in the signaling functions for various members of the structurally homologous family of FKBP domain proteins (LeMaster and Hernández 2016). We recently found that introducing the H87V mutation into FKBP 12 selectively suppresses the conformational exchange linebroadening within the β_4 - β_5 loop (Mustafi et al. 2013). The NMR chemical shift pattern and high resolution crystal structure are quite closely preserved in the H87V variant, consistent with this mutation selectively suppressing the transition to the minor linebroadening conformational state seen in the wild type protein (Mustafi et al. 2014a). If this model is true in detail, the joint magnetic field dependent NMR relaxation analysis of the wild type and H87V variant of FKBP12 should facilitate quantifying the residue-specific ^{15}N CSA values as well as the magnitude of the ^{15}N conformational exchange linebroadening.

Determination of the $(R_2-R_1/2)_{900/600}$ values for both the wild type and H87V proteins (Fig. 2A and B, respectively) illustrate the suppression of conformational linebroadening not only in the β_4 - β_5 loop but also in the β_{3b} - α loop which lies ~ 20 Å away on the opposite side of the catalytic cleft. As the previous discussion has indicated, the $J(\omega_{\text{H}})$ -dependent contribution to $R_2-R_1/2$ for this protein, which is two-fold larger than GB3, is well below the precision of this analysis. As a result, the $(R_2-R_1/2)_{900/600}$ values should reflect the combination of exchange linebroadening, residue-specific ^{15}N CSA variations, and uncertainty in the relaxation measurements. Only the exchange linebroadening contributions should be systematically positive in sign and $(R_2-R_1/2)_{900/600}$ values larger than $\sim 0.5 \text{ s}^{-1}$ can straightforwardly be assigned to this effect. Indeed, this set of exchange linebroadened residues was excluded for the calculation of the scaling factors relating the different magnetic field data sets of Fig. 2.

Given the close similarity between the crystal structures of the wild type and H87V variant of FKBP12 (0.25 Å C $^{\alpha}$ rmsd (Mustafi et al. 2013)), a $(R_2-R_1/2)$ analysis comparing these two proteins could be expected to cancel out the conformational contributions to the differences that arise from residue-specific ^{15}N CSA values. Furthermore, if the H87V mutation blocks the exchange linebroadening transition centered within the β_4 - β_5 loop

without significantly altering the conformational dynamics in the ps-ns timeframe, then a $(R_2-R_1/2)$ analysis could also potentially yield a robust measure of comparatively small changes in exchange linebroadening which arise from that mutation.

When a $(R_2-R_1/2)_{900/600}$ analysis is applied to the wild type and the H87V variant of FKBP12, a calculation for all residues in which this differential is less than 0.5 s^{-1} yields a number of residues which have positive differences that are appreciably elevated above the rest (Fig. 3A). Under the assumption of equal relaxation effects for each of the two protein samples arising from the residue-specific ^{15}N CSA and ps-ns dynamics contributions, the observed differential signals should reflect the summation of a systematic positive contribution from conformational exchange linebroadening in the wild type FKBP12 protein and the effectively random variations arising from the individual experimental measurements. To operationally distinguish these two contributions to the $(R_2-R_1/2)_{900/600}$ values, a $+2\sigma$ trim filter was iteratively applied. Since any signal more than 2σ above the mean has only a 2.5% probability of arising from random measurement error, these signals were removed and the statistics for the remaining population were then recalculated to obtain a revised σ value.

Ultimately six residues were putatively identified as exhibiting weak differential exchange linebroadening effects (Fig. 3B). The standard deviation for the remaining fifty residues is 0.126 s^{-1} , which corresponds to 1.2% of the mean $(R_2-R_1/2)_{900}$ values for the two FKBP12 proteins. If random errors primarily contribute, then by propagation of errors analysis the $(R_2-R_1/2)$ calculation should yield a standard deviation which is $\sqrt{2}$ larger than for the $(R_2-R_1/2)$ analysis which, in turn, is consistent with the estimated experimental uncertainties of the R_2 measurements dominating the variance of $(R_2-R_1/2)_{900/600}$ values reported in Fig. 3B.

The largest exchange linebroadening effect identified by the $+2\sigma$ trim filter was observed for Asp 37 which is in the β_{3a} strand juxtaposed to the tip of the β_4 - β_5 loop. The novel observation of a small degree of exchange linebroadening at this position gains significance due to the occurrence of a much larger conformational exchange linebroadening effect that we have recently reported for the corresponding Asp 68 in the structurally homologous FK1 domain of FKBP51 which is absent in the closely homologous FKBP52 domain (LeMaster et al. 2015; Mustafi et al. 2014b). The β_{3a} strand of FKBP51 undergoes a conformational exchange linebroadening transition, centered at an interstrand bifurcated hydrogen bonding interaction, in which the sidechain of Phe 67 (Phe 36 of FKBP12) is flipped out toward the solvent phase from its native position underneath the tip of the β_4 - β_5 loop (Gaali et al. 2015; LeMaster et al. 2015). While the transition within the β_{3a} strand is mechanistically distinct from the exchange linebroadening transition centered within the β_4 - β_5 loop, these two transitions are energetically coupled (LeMaster et al. 2015; Mustafi et al. 2014b).

This transition within the β_{3a} strand has provided the structural basis for the development of the first FKBP51-selective inhibitor (Gaali et al. 2015), potentially addressing a substantial pharmacological challenge for the highly structurally conserved family of the FKBP domain proteins. Since the development of this selective inhibitor was initiated from earlier studies on mutations of the buried Phe 36 of FKBP12 which destabilized the interactions with the

β_4 - β_5 loop (Clackson et al. 1998; Yang et al. 2000), tentative evidence presented here that an analogous transition in the β_{3a} strand may be experimentally observable in wild type FKBP 12 is of some interest. Within FKBP51, the largest linebroadening effects for the β_{3a} transition are observed at Lys 66 and Asp 68. Given the severe spectral overlap of the homologous Lys 35 with Gln 3 of FKBP 12, the exchange linebroadening of Asp 37 is the most readily available direct NMR evidence which might be anticipated for such a transition.

Of the other five residues in FKBP 12 which were identified as exhibiting modest levels of exchange linebroadening, four of these (Trp 59 + Glu 60 and Phe 99 + Val 101) are adjacent to residues which exhibit much larger conformational exchange effects, suggesting that the spatial mapping of the regions directly affected by such conformational transitions may be usefully enhanced. The last of the six residues identified as exhibiting a modest level of exchange linebroadening is Ile 76. The potential significance of observing exchange linebroadening effects at this position arises from the fact that, although the closely homologous FKBP 12.6 undergoes a conformational exchange linebroadening transition within the β_4 - β_5 loop similar to that of FKBP 12, no evidence for dynamical coupling to the distant β_{3b} - α loop is apparent (Chen et al. 2014; Mustafi et al. 2014a). FKBP 12.6 contains a cysteine substitution at residue 76 which is well positioned to potentially help modulate the degree of dynamical coupling that occurs between these two distant loop segments.

Having thus applied a $(R_2-R_1/2)$ analysis to identify each FKBP12 residue which gives rise to a differential exchange linebroadening effect in excess of the estimated experimental uncertainty, the remaining fifty residues were further examined to assess whether they might offer a suitably robust measure of the residue-specific ^{15}N CSA values. Using this subset of residues to conduct a slight ($\sim 0.2\%$) re-optimization of the scaling factors relating the $(R_2-R_1/2)$ values at 600 MHz and 900 MHz, the differential values for both the wild type and H87V variant were obtained (Fig. 4). The normalized $(R_2-R_1/2)$ values were then utilized to derive δ_c values for each protein according to Eq. 6. The correlation coefficient between the δ_c values for these two proteins was 0.824. The mean absolute difference between these two data sets was 0.029, substantially smaller than the standard deviation (0.054) obtained from averaging the individual δ_c estimates from the two protein data sets (Table 3). With the exclusion of significant exchange linebroadening contributions for these fifty residues, the consistency between the datasets from the wild type and H87V variant of FKBP12 appears to indicate a comparatively robust measure of the differential ^{15}N CSA values at these sites. Given the $1/2$ smaller values for differences with respect to the mean, the experimental uncertainty in the derived ^{15}N SCA values can be estimated to be 2% (~ 3.4 ppm).

Concluding remarks

Given the substantial relaxation contribution due to ^{15}N chemical shift anisotropy, it has long been recognized that residue-specific variations in these CSA values might significantly complicate the accurate dynamical interpretation of protein NMR data. To date, solid state (Hung et al. 2015; Wylie et al. 2007) and liquid crystalline solution (Yao et al. 2010) NMR techniques have yielded the most robust measures of the residue-specific ^{15}N CSA values. However, both the experimental demands of these techniques as well as their still modest

level of inter-dataset consistency have hindered the potential integration of such residue-specific variations into the quantitative dynamical interpretation of the complementary molecular dynamics simulation results. Taking advantage of recent improvements in the solution measurement of the requisite transverse and longitudinal relaxation times, a $J(0)$ scaling approach has been applied to proteins for which backbone exchange linebroadening dynamics are either absent or else are approaching the fast exchange limit. This analysis appears capable of providing residue-specific ^{15}N CSA values of a quality comparable to the other available methods, while at the same time enabling the quantitation of exchange linebroadening effects down to near the uncertainty limits of the relaxation measurements. Protein ^{15}N chemical shift anisotropy analysis remains significantly limited in terms of both the accuracy of the available experimental measurements and the restricted set of proteins to which such methods have been effectively applied. Given the potential relevance of these analyses for quantitative MD-based predictions of NMR relaxation data, it is anticipated that the described solution NMR analysis will usefully assist to quantifying the conformational basis for variations in the ^{15}N CSA values and thus facilitate further insights into the dynamical characteristics of proteins.

Acknowledgments

We acknowledge the use of the NMR facility and Applied Genomic Technologies cores at the Wadsworth Center. This work was supported in part by National Institutes of Health [GM 088214]. The NMR data collected at NYSBC was made possible by a grant from NYSTAR and ORIP/NIH facility improvement grant CO6RR015495. The 900 MHz NMR spectrometers were purchased with funds from NIH grant P41GM066354, the Keck Foundation, New York State Assembly, and U.S. Dept. of Defense.

References

- Anderson JS, LeMaster DM. Rotational Velocity Rescaling of Molecular Dynamics Trajectories for Direct Prediction of Protein NMR Relaxation. *Biophys Chem.* 2012; 168-9:28–39.
- Baldwin AJ, Kay LE. An R1 ρ expression for a spin in chemical exchange between two sites with unequal transverse relaxation rates. *J Biomol NMR.* 2013; 55:211–218. [PubMed: 23340732]
- Brath U, Akke M. Differential responses of the backbone and side-chain conformational dynamics in FKBP12 upon binding the transition-state analog FK506: Implications for transition-state stabilization and target protein recognition. *J Mol Biol.* 2009; 387:233–244. [PubMed: 19361439]
- Brath U, Akke M, Yang D, Kay LE, Mulder FAA. Functional dynamics of human FKBP12 revealed by methyl ^{13}C rotating frame relaxation dispersion NMR spectroscopy. *J Am Chem Soc.* 2006; 128:5718–5727. [PubMed: 16637639]
- Case DA. Calculations of NMR dipolar coupling strengths in model peptides. *J Biomol NMR.* 1999; 15:95–102. [PubMed: 10605083]
- Chen H, Mustafi SM, LeMaster DM, Li Z, Héroux A, Li H, Hernández G. Crystal structure and conformational flexibility of the unligated FK506-binding protein FKBP12.6. *Acta Cryst.* 2014; D70:636–646.
- Clackson T, et al. Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. *Proc Natl Acad Sci U S A.* 1998; 95:10437–10442. [PubMed: 9724721]
- Damberg P, Jarvet J, Gräslund A. Limited Variations in ^{15}N CSA Magnitudes and Orientations in Ubiquitin are Revealed by Joint Analysis of Longitudinal and Transverse NMR Relaxation. *J Am Chem Soc.* 2005; 127:1995–2005. [PubMed: 15701036]
- Farrow NA, Zhang O, Forman-Kay JD, Kay LE. Comparison of the backbone dynamics of a folded and an unfolded SH3 domain existing in equilibrium in aqueous buffer. *Biochemistry.* 1995; 34:868–878. [PubMed: 7827045]

- Ferrage F, Reichel A, Battacharya S, Cowburn D, Ghose R. On the measurement of ^{15}N - $\{^1\text{H}\}$ nuclear Overhauser effects. 2. Effects of the saturation scheme and water signal suppression. *J Magn Reson.* 2010; 207:294–303. [PubMed: 20951618]
- Fushman D, Tjandra N, Cowburn D. Direct measurement of ^{15}N chemical shift anisotropy in solution. *J Am Chem Soc.* 1998; 120:10947–10952.
- Fushman D, Tjandra N, Cowburn D. An approach to direct determination of protein dynamics from ^{15}N ^{15}N chemical shift anisotropy and chemical exchange. *J Am Chem Soc.* 1999; 121:8577–8582.
- Gaali S, et al. Selective inhibitors of the FK506-binding protein 51 by induced fit. *Nature Chemical Biology.* 2015; 11:33–37. DOI: 10.1038/nchembio.1699 [PubMed: 25436518]
- Gairi M, Dyachenko A, González MT, Feliz M, Pons M, Giralt E. An optimized method for ^{15}N R_1 relaxation measurements in non-deuterated proteins. *J Biomol NMR.* 2015; 62:209–220. [PubMed: 25947359]
- Hall JB, Fushman D. Characterization of the overall and local dynamics of a protein with intermediate rotational anisotropy: Differentiating between conformational exchange and anisotropic diffusion in the B3 domain of protein. *G J Biomol NMR.* 2003; 27:261–275.
- Hall JB, Fushman D. Variability of the ^{15}N Chemical Shielding Tensors in the B3 Domain of Protein G from ^{15}N Relaxation Measurements at Several Fields. Implications for Backbone Order Parameters *J Am Chem Soc.* 2006; 128:7855–7870. [PubMed: 16771499]
- Hung I, Ge Y, Liu X, Liu M, Li C, Gan Z. Measuring $^{13}\text{C}/^{15}\text{N}$ chemical shift anisotropy in [^{13}C , ^{15}N] uniformly enriched proteins using CSA amplification. *Solid State NMR.* 2015 doi:dx.doi.org/10.1016/ssnmr.2015.09.002.
- Idiyatullin D, Nesmelova I, Daragan V, Mayo KH. Heat capacities and a snapshot of the Energy Landscape in protein GB1 from the Pre-denaturation temperature dependence of backbone NH nanosecond fluctuations. *J Mol Biol.* 2003; 325:149–162. [PubMed: 12473458]
- Igumenova TI, Frederick KK, Wand AJ. Characterization of the fast dynamics of protein amino acid side chains using NMR relaxation in solution. *Chem Rev.* 2006; 106:1672–1699. [PubMed: 16683749]
- Jarymowycz VA, Stone MJ. Fast time scale dynamics of protein backbones: NMR relaxation methods, applications, and functional consequences. *Chem Rev.* 2006; 106:1624–1671. [PubMed: 16683748]
- Kroenke CD, Rance M, Palmer AG I. Variability of the ^{15}N chemical shift anisotropy in *Escherichia coli* Ribonuclease H in solution. *J Am Chem Soc.* 1999; 121:10119–10125.
- Lakomek NA, Ying J, Bax A. Measurement of ^{15}N relaxation rates in perdeuterated proteins by TROSY-based methods. *J Biomol NMR.* 2012; 53:209–221. [PubMed: 22689066]
- LeMaster DM, Hernández G. Conformational Dynamics in FKBP Domains: Relevance to Molecular Signaling and Drug Design. *Curr Molec Pharmacol.* 2016; 9:5–26.
- LeMaster DM, Mustafi SM, Brecher M, Zhang J, Héroux A, Li H, Hernández G. Coupling of conformational transitions in the N-terminal domain of the 51 kDa FK506-binding protein (FKBP51) near its site of interaction with the steroid receptor proteins. *J Biol Chem.* 2015; 290:15746–15757. DOI: 10.1074/jbc.M115.650655 [PubMed: 25953903]
- Lipari G, Szabo A. Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 1. Theory and Range of Validity. *J Am Chem Soc.* 1982; 104:4546–4559.
- Loth K, Pelupessy P, Bodenhausen G. Chemical shift anisotropy tensors of carbonyl, nitrogen, and amide proton nucleic in proteins through cross-correlated relaxation in NMR spectroscopy. *J Am Chem Soc.* 2005; 127:6062–6068. [PubMed: 15839707]
- Lu CY, vandenBout DA. Effect of finite trajectory length on the correlation function analysis of single molecule data. *J Chem Phys.* 2006; 125:124701. [PubMed: 17014194]
- Morin S, Gagne SM. Simple tests for the validation of multiple field spin relaxation data. *J Biomol NMR.* 2009; 45:361–372. [PubMed: 19842046]
- Mulder FAA, deGraaf RA, Kaptein R, Boelens R. An off-resonance rotating frame relaxation experiment for the investigation of macromolecular dynamics using adiabatic rotations. *J Magn Reson.* 1998; 131:351–357. [PubMed: 9571112]

- Mustafi SM, Brecher M, Zhang J, Li H, LeMaster DM, Hernández G. Structural Basis of Conformational Transitions in the Active Site and 80's loop in the FK506-binding Protein FKBP12. *Biochem J.* 2014a; 458:525–536. [PubMed: 24405377]
- Mustafi SM, Chen H, Li H, LeMaster DM, Hernández G. Analyzing the visible conformational substates of the FK506-binding protein FKBP12. *Biochem J.* 2013; 453:371–380. DOI: 10.1042/BJ20130276 [PubMed: 23688288]
- Mustafi SM, LeMaster DM, Hernández G. Differential conformational dynamics in the closely homologous FK506-binding domains of FKBP51 and FKBP52. *Biochem J.* 2014b; 458:525–536. DOI: 10.1042/BJ20140232 [PubMed: 24405377]
- Palmer AG, Kroenke CD, Loria JP. Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules. *Methods Enzymol.* 2001; 339:204–238. [PubMed: 11462813]
- Phan IQH, Boyd J, Campbell ID. Dynamic studies of a fibronectin type I module pair at three frequencies: Anisotropic modeling and direct determination of conformational exchange. *J Biomol NMR.* 1996; 8:369–378. [PubMed: 20859776]
- Sapienza PJ, Mauldin RV, Lee AL. Multi-timescale dynamics study of FKBP 12 along the rapamycin-mTOR binding coordinate. *J Mol Biol.* 2011; 405:378–394. [PubMed: 21073880]
- Smith CA, et al. Population shuffling of protein conformations. *Angew Chem Int Ed.* 2015; 54:207–210.
- Tang S, Case DA. Calculation of chemical shift anisotropy in proteins. *J Biomol NMR.* 2011; 51:303–312. [PubMed: 21866436]
- Trott O, Palmer AG I. $R_{1\rho}$ relaxation outside of the fast-exchange limit. *J Magn Reson.* 2002; 154:157–160. [PubMed: 11820837]
- Woessner DT. Nuclear Spin Relaxation in Ellipsoids Undergoing Rotational Brownian Motion. *J Chem Phys.* 1962; 37:647–654.
- Wylie BJ, Sperling LJ, Frericks HL, Shah GJ, Franks WT, Rienstra CM. Chemical-Shift Anisotropy Measurements of Amide and Carbonyl Resonances in a Microcrystalline Protein with Slow Magic-Angle Spinning NMR Spectroscopy. *J Am Chem Soc.* 2007; 129:5318–5319. [PubMed: 17425317]
- Yang W, et al. Investigating protein-ligand interactions with a mutant FKBP possessing a designed specificity pocket. *J Med Chem.* 2000; 43:1135–1142. [PubMed: 10737745]
- Yao L, Grishaev A, Cornilescu G, Bax A. Site-specific Backbone Amide ^{15}N Chemical Shift Anisotropy Tensors in a Small Protein from Liquid Crystal and Cross-Correlated Relaxation Measurements. *J Am Chem Soc.* 2010; 132:4295–4309. [PubMed: 20199098]

Fig. 1.

Percent deviation from the mean for experimental measurements of residue-specific ^{15}N chemical shift anisotropies of the closely homologous proteins GB1 and GB3. Solid state NMR measurements on GB1 have been reported by Wylie et al. (Wylie et al. 2007) (red $\square$) and Hung et al. (Hung et al. 2015) (blue ∇). Liquid crystalline solution NMR measurements have been reported by Yao et al. (Yao et al. 2010) (green $\circ$). Under the assumption of no exchange linebroadening, the difference between R_2 - $R_1/2$ measurements (O) scaled between two different magnetic field strengths (600 and 900 ^1H) have been used to calculate ^{15}N CSA according to Eq. 6, assuming a mean CSA anisotropy value of 168 ppm (Anderson and LeMaster 2012) and a ^1H - ^{15}N bond length of 1.02 Å.

Fig. 2.

Difference between $R_2 - R_1/2$ measurements on FKBP12 scaled between 600 and 900 1H field strengths. Extensive conformational exchange linebroadening observed in the β_4 - β_5 loop of the wild type protein (A) is strongly suppressed in the H87V variant (B), while the exchange linebroadening in the β_{3b} - α loop is only partially suppressed in the mutational variant and the dynamics within the β_{3a} - β_{3b} loop are largely unaffected by the mutation.

Fig. 3. Difference between $(R_2-R_1/2)_{900/600}$ measurements on the wild type and H87V variant of FKBP12. Analysis of all residues for which the $(R_2-R_1/2)_{900/600}$ values were less than 0.5 s⁻¹ (A). A +2 σ trim filter was iteratively applied to the residues in panel A to identify a subset of residues (Asp 37, Trp 59, Glu 60, Ile 76, Phe 99, Val 101) for which the $(R_2-R_1/2)_{900/600}$ value was greater than twice the value of the standard deviation of the final distribution (B).

Fig. 4.
 $A(R_2 - R_1/2)_{900/600}$ analysis for residues in the wild type (red $\triangle$) and H87V variant (blue ∇) of FKBP12 lacking detectable exchange linebroadening contributions in both proteins.

Table 1
Correlation of (R_2 - $R_1/2$) values for GB1/GB3 with respect to 600 MHz data (Lakomek et al. 2012)^a

	(Hall and Fushman 2006)	Internal correlation	(Idiyatullin et al. 2003)	Internal correlation	This work	Internal correlation
500 MHz	0.941	0.949	0.822	0.864	0.988	0.991
600 MHz	0.960	-	0.952	-	0.987	-
800 MHz	0.805	0.845	0.867	0.919	0.972	0.982
900 MHz					0.971	0.978

^a excluding Gln 2

Table 2
Correlation between experimental ^{15}N CSA anisotropy values for GB1/GB3

	(Yao et al. 2010)	(Hung et al. 2015)	(Hall and Fushman 2006)	$(R_2-R_1/2)_{900/600}$
(Wylie et al. 2007)	0.71(0.81) ^a	0.75(0.60) ^b	0.14	0.69(0.61) ^b
(Yao et al. 2010)		0.34(0.53) ^a	0.05	0.51(0.53) ^a
(Hung et al. 2015)			0.07	0.47(0.34) ^b

^a deleting Val 39

^b deleting Gly 41

Table 3
Differential ^{15}N residue-specific chemical shift anisotropy values for positions in FKBP12 lacking discernible exchange linebroadening contributions

	CSA(%)		CSA(%)		CSA(%)		CSA(%)		CSA(%)
Gln 2	2.7	Thr 21	-4.4	Lys 47	-4.1	Gly 69	-6.4		
Val 4	-2.3	Cys 22	-8.5	Phe 48	0.2	Ala 72	5.1		
Ile 7	2.5	Val 23	-5.3	Met 49	0.6	Lys 73	-2.8		
Ser 8	1.5	Val 24	-3.2	Leu 50	9.4	Leu 74	0.1		
Gly 10	8.0	His 25	-8.9	Gly 51	-0.2	Thr 75	-10.1		
Asp 11	2.5	Tyr 26	7.6	Lys 52	5.0	Ser 77	5.2		
Arg 13	6.5	Thr 27	6.8	Glu 61	-2.9	Tyr 80	2.8		
Thr 14	-2.4	Gly 28	3.6	Gly 62	2.7	Glu 102	-3.7		
Phe 15	-3.3	Met 29	8.1	Val 63	2.7	Leu 103	-4.7		
Lys 17	-4.6	Leu 30	-5.3	Gln 65	-3.7	Lys 105	0.3		
Arg 18	-1.0	Glu 31	-0.4	Met 66	3.0	Leu 106	-11.2		
Gly 19	-8.7	Gly 33	2.4	Val 68	2.8	Glu 107	-8.0		
Gln 20	5.2	Lys 34	9.3						