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COMMUNICATIONS

Protein Backbone ^{15}N Relaxation Rates as a Tool for the Diagnosis of Structure Quality

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In the work reported herein we define a structure validation factor that depends on protein backbone ^{15}N relaxation rates. This is an alternative method to the previously defined quality factors derived from anisotropic chemical shifts or residual dipolar couplings. We have used the structure dependence of ^{15}N relaxation rates of anisotropically tumbling proteins to calculate this structure diagnosis factor and have used it to demonstrate the improvement of protein structures refined with residual dipolar couplings.

Key Words: **NMR; ^{15}N relaxation; protein structure; dipolar couplings; liquid crystal.**

INTRODUCTION

The residual dipolar coupling has proven to be a NMR parameter complementary to the nuclear Overhauser effect (NOE) in the three-dimensional structure determination of biopolymers (*1–4*). The "long-range" nature of the structural information it provides makes it considerably valuable in the structure calculation of multidomain proteins for which sparse interdomain NOEs are observed (*5*). Among other important advantages, the utilization of residual dipolar couplings improves the quality of the determined structure (*3, 6*). The calculation of the recently defined Q factor is a simple way to test this improvement (*7, 8*). The Q factors are derived either from chemical shift anisotropy (*7*) or from residual dipolar couplings (*8*).

In order to observe either of these two parameters it is necessary to have a certain degree of molecular alignment under the magnetic field. This has been successfully achieved using liquid crystals formed by bicelles (*2*), bacteriophages (*9, 10*), or purple membranes (*11*) as the orienting medium. Although the mechanism of molecular alignment in the liquid crystal solutions is still under debate, it has been proposed that, in the case of micelles and neutral bicelles, the alignment force can be of steric origin (*12, 13*). Other types of mechanism cannot be ruled out, since there is evidence of the influence of electrostatic interactions when using charged bicelles (*14*). In the cases where dipolar couplings have been used for protein structure refinement, NMR data suggest that structural modifications due to the presence of the liquid crystal, if any, are minimal (*2*). Evidence for this is the absence of significant chemical shift changes in the presence of liquid crystals, excluding those contributions from the chemical shift anisotropy. Despite this, it is still reasonable to question whether the interactions between the aligned molecules and the liquid crystal media can perturb the structure. These possible structural changes may modify the value of the residual dipolar coupling or chemical shift anisotropy with respect to the ones that would be observed if those interactions were nonexistent. A structure validation factor that does not depend on any parameter measured in the presence of the liquid crystals seems in this sense advantageous.

Recently, it has been demonstrated that the combined use of dipolar couplings and ^{15}N relaxation rates allows the identification of protein backbone conformational exchange (*15*). This has been possible due to the related structure dependence of ^{15}N T_1/T_2 ratios and dipolar couplings in proteins that tumble anisotropically, as well as to the similar orientations of the alignment and the rotational diffusion tensors in neutral bicelles. This is an example that ^{15}N T_1/T_2 ratios can be used as structural probes. Therefore, it is expected that the higher the quality of a structure is, the better the predicted ^{15}N T_1/T_2 ratios will fit the experimental ones. In a recently determined protein structure a better correlation between the calculated and the measured ^{15}N T_1/T_2 ratios for the protein refined with dipolar couplings has been observed (*6*). This is a result of the improvement in structure quality, and it is not related to the characteristics of the diffusion and the alignment tensors. That is, the two tensors do not need to be similar. On the contrary, for the identification of residues undergoing conformational exchange, both tensors must have close orientations.

A structure validation factor that depends only on relaxation data measured in the absence of orienting media would allow

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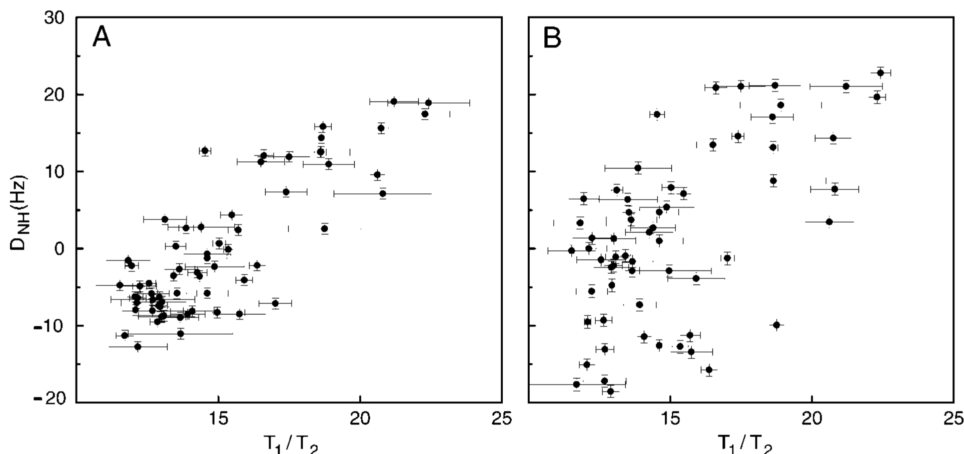

FIG. 1. Amide NH dipolar couplings of GAIP observed in the presence of bicelles (A) and bacteriophages (B) versus backbone ^{15}N T_1/T_2 ratios. Data for residues identified as undergoing conformational exchange are not included. Bicelles were prepared using a DMPC:DHPC molar ratio of 3:1 (GAIP) and 3.5:1 (KH3) present in 3.2% (w/v) and protein concentration of 0.7 mM (GAIP) and 0.6 mM (KH3). The fd bacteriophages liquid crystals were prepared by the method of Hansen *et al.* (*10*). The concentration of bacteriophages and protein was ~15 mg/ml and 0.7 mM, respectively. NH dipolar couplings were measured and calculated using the procedure applied by Ottiger *et al.* (*26*). ^{15}N longitudinal and transverse relaxation rates were measured in the absence of liquid crystals, using previously described methods (*20, 27*) with pulse field gradients to suppress the water signal. $T_{1\rho}$ values instead of T_2 values were measured using a ^{15}N spin-lock field strength of 2.5 KHz. These values were corrected for resonance offset using the equation by Davis *et al.* (*32*). Residues with heteronuclear NOEs lower than 0.65 and residues identified as undergoing conformational exchange are not included in the calculation of Q_{relax}. The orientation and magnitude of the rotational diffusion tensor components are searched by optimizing the agreement between the measured and the calculated T_1/T_2 ratios through Powell minimization (*21*).

us in principle to identify structure perturbations caused by the alignment medium, provided that this factor is calculated for a structure determined with and without dipolar coupling information. This factor will as well indicate whether there is any improvement in the quality of the determined structure when it has been refined with residual dipolar coupling data. According to these ideas we define a new structure diagnosis factor that depends on ^{15}N T_1/T_2 ratios (measured in the absence of orienting media) which is described as

$$Q_{\text{relax}} = \{\sum[(T_1/T_2)_{\text{m}} - (T_1/T_2)_{\text{c}}]^2\}^{1/2} / \{\sum[(T_1/T_2)_{\text{m}} - \langle T_1/T_2\rangle]^2\}^{1/2}, \quad [1]$$

Where m and c denote measured and calculated values, respectively, and the term $\langle T_1/T_2\rangle$ refers to the average of the measured values.

The same criteria used to calculate the overall correlation time of the molecule from ^{15}N relaxation rates apply when calculating Q_{relax}. That is, ^{15}N relaxation rates influenced by conformational exchange or by the presence of large-amplitude internal motions should be excluded from the data set. Measurements of transverse relaxation rates at different magnetic fields (*16*), dipolar and chemical shift anisotropy interference (*17*), ROESY $T_{1\rho}$ measurements of amide protons in perdeuterated proteins (*18*), and off-resonance rotating frame relaxation rates (*19*) provide information on exchange processes on the microsecond to millisecond time scale. Another method uses the T_2 and T_1 deviations from the average values as criteria to identify conformational exchange (*20*). In the work presented here we have used this method according to the equations reported in Barbato *et al.* (*20*), together with the method that takes advantage of the correlation between ^{15}N T_1/T_2 ratios and dipolar couplings of NH bond vectors (*15*) (*vide infra*). Large-amplitude internal motions can easily be identified by low ^{15}N–^{1}H NOEs (e.g., <0.65). In order to properly predict the ^{15}N T_1/T_2 ratios it is necessary to know the orientation of the diffusion tensor. This will be the orientation that minimizes the difference between the measured and the calculated T_1/T_2 ratios (*21*).

RESULTS

We have calculated the Q_{relax} factors for two protein structures that have been refined with and without dipolar couplings of NH, $C_\alpha H_\alpha$, $C_\alpha C'$, and $C'N$ bond vectors. These proteins are the G_α interacting protein (GAIP) (*22*) and the C-terminal domain of hnRNP K (KH3) (*6*). Two orienting media (bicelles and bacteriophages) have been used to obtain dipolar coupling data for GAIP. The orientation of the alignment is different in each medium and two alignment tensors were used for structure calculation purposes. Only the dipolar couplings obtained in the presence of bicelles have a strong correlation with the T_1/T_2 ratios (Fig. 1A), indicating that in this medium the alignment and the diffusion tensors have similar orientations (*15*). The angle between the principal axis of the diffusion tensor and the alignment tensor in the presence of bicelles is 8.7 degrees. This correlation allows the identification of resi-

dues that are subjects to conformational exchange. Residues that deviate more than two times the standard deviation of the plot represented in Fig. 1A are considered involved in conformational exchange. The same result is obtained with the method of Barbato *et al.* (*20*) using one standard deviation as the cutoff. This indicates the reliability of both methods. The similarity between the two tensors is also reflected in the correlation factor of Fig. 1A, which is 0.83. In contrast, the correlation factor of the plot represented in Fig. 1B (dipolar couplings obtained in the presence of bacteriophages) is 0.59. Both correlation factors have been calculated excluding residues identified as undergoing conformational exchange. Thus, the orientation of the alignment and the diffusion tensors differs under the presence of bacteriophages. This has a clear effect in the combined use of T_1/T_2 ratios and dipolar couplings to detect conformational exchange, but has no influence in the assessment of structure quality improvement by Q_{relax} for structures refined with dipolar couplings, since this factor is independent of the relative orientation of both tensors. In the cases where dipolar couplings are obtained in the presence of orienting media that have other than steric-type interactions with the protein, the method that correlates dipolar couplings and T_1/T_2 ratios cannot be employed to detect conformational exchange. Alternative methods described earlier can be used in place of the correlation method. Nevertheless, the comparison between the Q_{relax} factor calculated for the structure determined with and without dipolar couplings will still indicate whether the incorporation of dipolar data is improving the quality of the structure.

The Q_{relax} values for the mentioned proteins are

$$Q_{relax}[\text{GAIP without dipolar couplings}] = 0.70$$
$$Q_{relax}[\text{GAIP with dipolar couplings}] = 0.45$$
$$Q_{relax}[\text{KH3 without dipolar couplings}] = 0.62$$
$$Q_{relax}[\text{KH3 with dipolar couplings}] = 0.45.$$

According to the value of the factor Q_{relax}, the quality of both structures improves when these have been refined with dipolar coupling information. As an additional example we have calculated this factor for the solution and crystal structure of the N-terminal domain of the protein Enzyme I of *Escherichia coli* (*23, 24*).

$$Q_{relax}[\text{Enzyme I, solution structure}] = 0.92$$
$$Q_{relax}[\text{Enzyme I, crystal structure}] = 0.84$$

When the solution structure of the N-terminal domain of the protein Enzyme I is refined using ^{15}N relaxation derived restraints (*25*), the Q_{relax} drops to 0.32, as expected.

To provide a clearer idea of the significance of the Q_{relax} value in terms of structure quality, we have calculated the average deviation of the angles that the NH bond vectors form with the Z axis of the diffusion tensor, with respect to the theoretical values obtained using Eq. [2] for an axially symmetric rotor (*28*):

$$T_1/T_2 \sim T_1/T_2(0) \times [(1 + \epsilon \sin^2\theta)]^{-1}, \qquad [2]$$

where T_1/T_2 (0) is the ratio in the case of isotropic diffusion, $\epsilon = (D_\parallel/D_\perp - 1)$, and θ is the angle between the unique axis of the diffusion tensor and the NH bond vector.

The average angle deviation for the crystal structure of Enzyme I ($Q_{relax} = 0.84$) is 15.6°, for the solution structure ($Q_{relax} = 0.92$) it is 16.0°, and for the structure refined with ^{15}N relaxation data ($Q_{relax} = 0.32$) it is 8.8°.

Any expression that depends on the measured relaxation data is influenced by the distribution of NH bond vector orientation. This is the case of the denominator part of the Q_{relax} factor. A simple way to avoid biases due to a nonuniform NH bond vector distribution is to numerically simulate the complete distribution of T_1/T_2 ratios (*29*) or to use Eq. [2] along with the fitted diffusion tensor to calculate the total spread of the tensor. On the contrary, the presence of nonuniformity in the orientation of NH bond vectors is irrelevant when comparing Q_{relax} values calculated for the same protein at different stages of the refinement process.

DISCUSSION

Q_{relax} is analogous to the previously defined Q factors that are derived from dipolar coupling and chemical shift anisotropy. However, there are some aspects that differentiate them. The main difference is that Q_{relax} is calculated using data obtained in the absence of orienting media, while the other Q factors need data obtained in the presence of liquid crystals. Another difference concerns the errors in relaxation rate measurements which are larger, relative to T_1/T_2 value ranges, than typical errors in dipolar coupling. These errors influence the accuracy with which the tensor is determined and, as mentioned above, the appropriate determination of the diffusion tensor orientation is necessary for the calculation of T_1/T_2 ratios. In addition, a nonuniform distribution of N–H bond vectors will also affect tensor determination. In this case the use of ^{13}C relaxation data from α and carbonyl carbons will homogenize the distribution of the data, therefore helping in the determination of the diffusion tensor (*30*). A similar idea has been previously applied to better define the alignment tensor orientation, using dipolar coupling information derived from different bond vectors (*31*).

The principal utility of Q_{relax} is as a structure diagnosis tool to check structure quality improvement upon the addition of different parameters in structure refinement. Q_{relax} is particularly useful to confirm the absence of structural changes in the presence of orienting media, since it does not depend on any parameter measured in them. Additionally, Q_{relax} may as well

indicate the presence of motions between protein domains that might not be easily detected by relaxation data. In this case, if the protein has been refined with dipolar couplings assuming a unique alignment tensor, a large deviation between the calculated and the measured T_1/T_2 ratios is expected.

CONCLUSION

The structural information inherent to ^{15}N relaxation rates of proteins with anisotropic rotational diffusion has allowed us to propose an alternative factor for protein structure validation (Q_{relax}). It is important to note that the relaxation rates of other heteronuclei can be used analogously. We have demonstrated that Q_{relax} serves to check structure quality improvement upon the addition of residual dipolar couplings in the structure refinement process. Additionally, Q_{relax} can be used to detect structural changes in the presence of orienting media. The combined use of J coupling values or chemical shifts, both providing local structure validation, together with Q_{relax}, which accounts for global structure quality, will offer a comprehensive tool to check the quality of structures determined by NMR.

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