The Use of Residual Dipolar Coupling in Concert with Backbone Relaxation Rates to Identify Conformational Exchange by NMR

Eva de Alba, James L. Baber, and Nico Tjandra*

Laboratory of Biophysical Chemistry, Building 3
National Heart, Lung, and Blood Institute
National Institute of Health
Bethesda, Maryland 20892-0380

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The slight alignment of a macromolecule in the presence of liquid crystal allows the measurement of dipolar couplings which otherwise would be averaged out by fast rotational diffusion of the molecule. Dipolar couplings provide important and unique structural information.[1] Although the mechanism of alignment is still not clear, it has been suggested that the liquid crystal bicelles[2,3] formed by neutral lipids induce alignment through steric interactions with the macromolecules.[4] This type of mechanism can only be dependent on the shape of the macromolecule. Since the molecular shape also governs the rotational diffusion, we suggest that the alignment and the rotational diffusion tensors may have similar orientations. The two tensors would not be comparable in any other way, that is, their magnitudes in general would be uncorrelated. For nonisotropic diffusors T_1/T_2 ratios are related to the orientation of the bond vector with respect to the principal axis system of the diffusion tensor.[5] By examining the expression of the local diffusion constants suggested by Lee *et al.*,[6] it becomes apparent that the dependence of this parameter and the dipolar coupling with respect to bond orientation in their respective tensors is similar. Therefore, should our hypothesis concerning the similarity between the orientations of the alignment and diffusion tensors prove true, a good correlation between T_1/T_2 ratios and dipolar couplings is expected. In the work reported herein a respectable correlation between these two parameters is found. Furthermore, it is also shown that this correlation can be used to identify residues whose backbones undergo conformational exchange. The applicability of this approach is illustrated using experimental data for three proteins, human ubiquitin, C-terminal domain of hnRNP K (KH3), and human G-alpha interacting protein (GAIP).

Heteronuclear relaxation data has been used to reveal internal dynamics,[7−10] and to characterize the anisotropic rotational diffusion parameters of macromolecules.[6,11,12] When the rotational diffusion anisotropy is favorable, the heteronuclear T_1/T_2 ratios can even provide important structural information in the form of bond orientation with respect to the rotational diffusion coordinate frame.[13] ^{15}N T_1/T_2 ratios are typically used to define the global diffusion parameters,[7] since to a good approximation their dependence with rapid internal motions and with the magnitude of the chemical shift anisotropy has been shown to be negligible.[11] Residues that exhibit large-amplitude internal motions on time scale greater than a few hundred picoseconds as well as residues that have contribution to their transverse ^{15}N relaxation rates from conformational exchange must be excluded from the analysis. Residues undergoing large-amplitude internal motions can be identified through the low heteronuclear NOE values. Typically an NOE cut off of 0.65 is used. A proper identification of residues with substantial conformational exchange contribution to the transverse relaxation rate is still a challenge. The consequences of failing to exclude these residues from the analysis are overestimates of the overall correlation time and of the diffusion anisotropy ($D_\parallel/D_\perp$). From the different methods used to identify exchange processes,[12,14−16] one of the simplest reveals residues involved in conformational exchange if their relaxation parameters satisfy the following equation:[17]

$$(\langle T_2\rangle - T_{2,n})/\langle T_2\rangle - (\langle T_1\rangle - T_{1,n})/\langle T_1\rangle > 1.5 \times \mathrm{SD} \quad (1)$$

where $T_{2,n}$ is the T_2 value for residue n, and $\langle T_2\rangle$ is the average T_2 value. SD is the standard deviation of the left-hand side of eq 1. The factor 1.5 is the usual cutoff used.[17] However, as the diffusion anisotropy increases, it is not trivial to determine which cutoff to use. This approach mistakenly identifies as residues with conformational exchange those having unusually large T_1/T_2 ratios as a result of their bonds being nearly parallel to the major diffusion axis.

As mentioned before, if the alignment and diffusion tensors have similar orientations, a good correlation between the dipolar couplings and the T_1/T_2 ratios is expected. Figure 1 shows the results obtained for the above mentioned proteins. The correlation factors for each plot are; 0.67, 0.81, and 0.83, respectively. Residues that deviate from the linear correlation observed contain additional nonstructural features (i.e., not related to the orientation of the NH bond vector) that affect the two quantities differently. One nonstructural contribution to the dipolar coupling is **S**, which is the order parameter of the dipolar interaction vector. Since the measured dipolar couplings are proportional to **S** and only residues with heteronuclear NOE higher than 0.65 are included in Figure 1, it is apparent that within that NOE limit the changes in the dipolar couplings due to the variation in **S** are still within the scatter of the data.[1] This fact may indicate that, when comparing the dipolar couplings of different NH vectors, the variation of their order parameters with respect to the average does not have a substantial effect, at least when S^2 is larger than ca. 0.75. This is not surprising since the S^2 values found in the structured regions of proteins approximately range from 0.75 to 0.95, with 0.85 as the average value. Therefore, the error introduced in the dipolar coupling for not considering its real S value is only around 5%. Conformational exchange is a nonstructural feature that can affect the transverse relaxation rate (R_2) substantially and therefore the T_1/T_2 ratios. As mentioned above, T_1/T_2 ratios and dipolar couplings have a similar dependence on the angle that the NH vector forms with the main axis of the diffusion and the alignment

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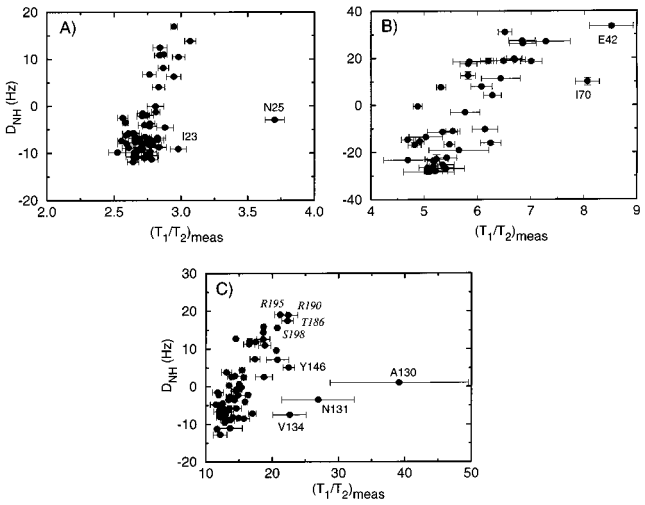

Figure 1. Correlation plots of heteronuclear T_1/T_2 ratios and the measured $^{15}N-^1H^N$ dipolar couplings for (A) human ubiquitin, (B) KH3, and (C) GAIP. Only residues with heteronuclear NOE greater than 0.65 are included. Residues that are subject to conformational exchange are labeled. Only a few residues in GAIP that are mistakenly identified as undergoing conformational exchange using eq 1 are indicated in italic for clarity. Error bars represent reproducibility of the data sets. Correlation factors of the plots shown are; (A) 0.67, (B) 0.81, (C) 0.83. Sample conditions for T_1 and T_2 measurements were: 0.3 mM, 0.6 mM, and 0.8 mM protein concentrations for ubiquitin, KH3, and GAIP, respectively, in H_2O:D_2O (10% by volume). Residual dipolar couplings were obtained on samples of 0.3 mM, 0.6 mM, and 0.7 mM protein concentration with bicelles prepared using a molar DMPC:DHPC ratio of 2.9:1 (ubiquitin), 3.5:1 (KH3), and 3.0:1 (GAIP) present in 5.0% (w/v) (ubiquitin), 3.2% (w/v) (KH3), and 3.2% (w/v) (GAIP). Heteronuclear relaxation data were acquired and processed as described previously.[11] NH dipolar couplings were measured and calculated using the procedure applied by Ottiger et al.[19]

tensor, respectively. Conformational exchange of an NH vector implies that there exists a variation in this angle depending on the conformation adopted by the vector. In this respect, when the exchange process is fast in the time scale of the NMR measurement, both the T_1/T_2 ratio and the residual dipolar coupling represent an average of the different angles adopted by the NH vector, and this averaging affects both parameters equally. Apart from the averaging, the rate of the exchange process affects the transverse relaxation time, and this produces an additional effect on the T_1/T_2 ratios that is not present in the dipolar coupling. For this reason residues involved in conformational exchange are expected to have T_1/T_2 ratios that deviate from the observed correlation.

In human ubiquitin only residues I23 and N25 have been identified previously to exhibit conformational exchange.[11] In KH3, according to the analysis of the $T_{1\rho}/T_2$ ratios and T_2 field dependence[12] (data not shown), only residues H41, E42, and I70 have been identified to undergo conformational exchange. Using eq 1 with a cutoff factor of 1.5 the same result is obtained. Indeed, these are residues with substantially worse correlation between the T_1/T_2 ratio and their dipolar coupling as illustrated in Figures 1A and B. Their T_1/T_2 ratios are much higher than expected due to T_2 values shortened by conformational exchange. Dipolar coupling for residue H41 in KH3 was not obtained due to spectral overlap, therefore this residue was excluded from the correlation plot. In the case of GAIP the correlation plot only reveals residues A130, N131, V134, and Y146 as possible residues with conformational exchange. In contrast, eq 1 using the cutoff factor of 1.5 suggests A130, N131, Y146, H175, L182, Q183, Y185, T186, R190, Y193, R195, and S198 as subject to conformational exchange. It is interesting to note that this procedure failed to identify residue V134. Only after adjusting the cutoff factor to 1.4 can one identify this residue. The additional residues that have been mistakenly identified as residues with conformational exchange are those at the extreme end (large T_1/T_2 ratio and large dipolar coupling) of the correlation plot. This indicates that the dipolar interaction vectors are nearly parallel to the major axis of the molecule. No appropriate value for the cutoff factor of eq 1 will clearly separate residues experiencing conformational exchange from those with bond vectors lying parallel to the long axis of the molecular frame. No other methods apart from the ones mentioned have been used to identify residues undergoing conformational exchange in these three proteins. The agreement between the results obtained by these methods and the one we propose indicates that the later can be used as a simple and reliable method to find residues involved in conformational exchange processes. Furthermore, we demonstrate in the case of GAIP that misleading results obtained using eq 1 can be identified by the presence of a good correlation between T_1/T_2 ratios and dipolar couplings.

A careful inspection of Figure 1A can provide an estimate for the exchange contribution to transverse relaxation rate (R_2^{exch}) values for residues I23 and N25 of human ubiquitin. This rough estimate of the R_2^{exch} can only be made under the assumption that the deviations of the T_1/T_2 ratios from the best fitted line through the data points in Figure 1A are solely due to conformational exchange. The basis for this assumption is that conformational averaging affects T_1/T_2 ratios and dipolar coupling differently only in terms of the influence of the exchange rate on T_2 (*vide supra*). The R_2^{exch} are estimated to be 11.8 ± 4.5 and $25.3 \pm 4.4\%$ of the observed R_2 which correspond to R_2^{exch} of 0.8 ± 0.3 and 2.1 ± 0.4 sec^{-1} for I23 and N25, respectively. The expected R_2 values in the absence of conformational exchange can also be calculated from exhaustive fit of the relaxation data to the high-resolution X-ray structure.[11] The R_2 values are 6.17 sec^{-1} for both I23 and N25. These would translate to R_2^{exch} of 0.68 sec^{-1} and 2.09 sec^{-1} for I23 and N25, respectively. Clearly these R_2 values are in reasonable agreement with the estimates made through the use of the simple correlation plot. Similar calculations can also be carried out for residues subject to conformational exchange in KH3 as well as in GAIP.

The structural information obtained through dipolar coupling depends on the orientation as well as on the magnitude of the alignment tensor. Since the information gained through the use of different alignments is complementary,[18] it would be desirable to have a better understanding of the mechanism of interaction between the macromolecule and the liquid crystal medium, so as to be able to modify the alignment in a controlled manner. The correlation found between the T_1/T_2 ratios and the dipolar couplings indicates that the alignment and the diffusion tensors have similar orientations. This can be explained if the principal force producing the alignment in the neutral bicelles is steric and therefore dependent only on the shape of the molecule. In contrast, the orientation of the alignment can be modified by changing the pH or the overall charge of the protein when using nonneutral bicelles,[18] indicating that interactions other than steric may have an influence in the alignment process. In addition, we demonstrate that the use of dipolar couplings in conjunction with heteronuclear T_1/T_2 ratios is a simple and reliable tool to identify residues exhibiting conformational exchange.

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Supporting Information Available: One figure which shows correlations between KH3 NH dipolar couplings and T_1/T_2 ratios for different rotations of the alignment tensor with respect to the diffusion tensor (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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