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Accurate Measurement of H^N–H^α Residual Dipolar Couplings in Proteins

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A method for accurately measuring H^N–H^α residual dipolar couplings is described. Using this technique, both the sign and magnitude of the coupling can be determined easily. Residual dipolar coupling between $H^N(i)$–$H^\alpha(i)$ and $H^N(i)$–$H^\alpha(i\text{-}1)$ were measured for the FK506 binding protein complexed to FK506. The experimental values were in excellent agreement with predictions based on an X-ray crystal structure of the protein/ligand complex, suggesting that these residual dipolar couplings will provide accurate structural constraints for the refinement of protein structures determined by NMR. © 1999 Academic Press

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Partial alignment of biomolecules in a magnetic field leads to anisotropic averaging of tensorial interactions (*1*). The anisotropic value that is observed depends on the orientation of the tensorial property with respect to the alignment tensor. An example is the dipolar interaction between two nuclei. In this case, the tensor is oriented along the internuclear vector connecting the two atoms. In an isotropic medium, the tumbling of the molecule averages the dipolar tensor so that no net dipolar coupling is observed. However, in a partially oriented sample, the observed dipolar coupling can be nonzero depending on the relative orientation of the internuclear vector and the alignment tensor (*2, 3*).

The large number of NMR observables in a biomolecule that are orientation dependent has fueled the recent development of experiments to measure the residual anisotropic effect (*2–10*). To date, most experiments have focused on the measurement of residual dipolar couplings observed for fixed distance interactions between either two hetero atoms or a proton and a hetero atom. In all of these cases, the elements of the dipolar tensor are known, and only the size and orientation of the alignment tensor need to be determined. Less effort has been placed on quantitatively measuring residual dipolar couplings between protons (*3, 7*). Proton–proton residual dipolar couplings are more difficult to quantitate and interpret because the sign of the coupling is not easily determined and most interproton distances are not fixed.

In this Communication, we describe a method for accurately measuring $H^N(i)$–$H^\alpha(i)$ and $H^N(i)$–$H^\alpha(i\text{-}1)$ dipolar couplings in proteins. The couplings are measured using a nonproton decoupled HNCA experiment to generate an E.COSY effect between H^N and H^α from which an accurate measurement of both the magnitude and the sign of the residual dipolar couplings can be made. Using this method, more than 90% of the possible $H^N(i)$–$H^\alpha(i)$ and 52% of the $H^N(i)$–$H^\alpha(i\text{-}1)$ residual dipolar couplings were obtained for the FK506 binding protein (FKBP) complexed to FK506. The measured residual dipolar couplings are compared to values predicted from an X-ray crystal structure of FKBP/FK506 (PDB access code 1FKF, *11*).

The pulse scheme used in the present study is shown in Fig. 1. The experiment is similar to the Soft HNCA–E.COSY experiment (*12–14*). The current experiment differs in the placement of the carbonyl decoupling pulses in t2 and the use of gradients to reduce artifacts and aid in water suppresion. An interesting feature of the Soft HNCA–E.COSY experiment is the final INEPT transfer. This transfer utilizes a net 360° rotation of the proton magnetization that results in the refocusing of the coupling between the nitrogen and H^N but still retains the terms dependent on H^α coupling in the carbon evolution period. This leads to an E.COSY effect that is resolved by the large C^α–H^α coupling and allows for accurate measurement of the H^N–H^α coupling (*12–14*).

Figure 2 shows a region of the Soft HNCA–E.COSY spectrum, recorded with the pulse scheme shown in Fig. 1. The size of the displacement in the f3 dimension corresponds to the sum of the $J(H^N$–$H^\alpha)$ scalar and residual dipolar coupling. The sign of the coupling can easily be determined by comparing the spectra from the aligned samples (Figs. 2B and 2C) to the sample that was run under normal isotropic conditions (Fig. 2A). For example, the residual dipolar couplings for H^N(L74)–H^α(K73) and H^N(L74)–H^α(L74) have the opposite sign as the scalar *J*-coupling observed in the reference spectrum. In the sample aligned by phage (Fig. 2C), the alignment tensor has a different orientation which results in a positive residual dipolar coupling for H^N(L74)–H^α(K73).

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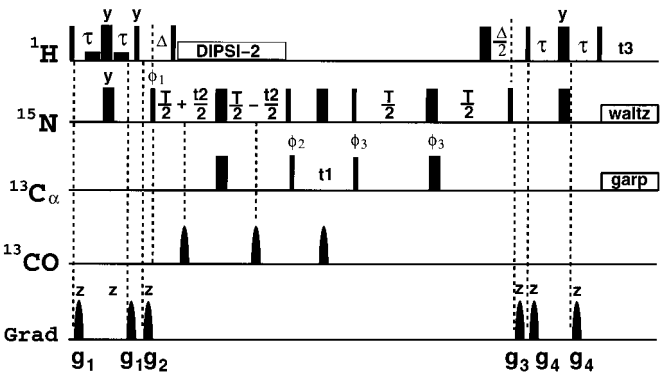

FIG. 1. Pulse scheme of the Soft HNCA–E.COSY experiment (*12–14*). Narrow and wide pulses correspond to 90° and 180° flip angles, respectively. A Watergate sequence (*15*) is used in the initial INEPT step to aid in water suppression. Selectivity between C^{α} and CO pulses were obtained by using a sinc pulse. Pulses without an explicit phase cycle are along the *x* axis. The remaining phases are $\phi_1 = x, -x, \phi_2 = 2(x), 2(-x), \phi_3 = 4(x), 4(-x)$, and RCVR = $x, 2(-x), x, -x, 2(x), -x$. The delays were D = 5.4 ms, T = 27.2 ms, and τ = 2.25 ms. The same gradient strength was used for all of the gradient pulses. The lengths of the gradients g1–g4 are 0.3, 1.0, 1.5, and 0.4 ms, respectively. Data were processed using the program NMRPipe (*16*) and analyzed using the program PIPP (*17*).

In Fig. 3, residual dipolar couplings for FKBP/FK506 partially aligned by the DMPC/DHPC/SDS bicelles are compared to those predicted from an X-ray crystal structure of FKBP/FK506 (*11*). The predicted values were obtained using

$$^{1}D_{HN-HA} = -S\,\frac{\mu_0}{4\pi}\,\frac{\gamma_H\gamma_H h}{4\pi^2 r^3}\times\left[A_a(3\cos^2\theta - 1) + \frac{3}{2}A_r(\sin^2\theta\times\cos 2\phi)\right], \qquad [1]$$

where S is the generalized order parameter for internal motion, γ_H is the proton gyromagnetic ratio, h is Planks constant, r is the internuclear proton distance, A_a and A_r are the axial and rhombic components of the molecular alignment tensor, and θ and ϕ are the cylindrical coordinates of the bond vector in the principal axis system of A (*3*). The order parameter S was assumed to be one in all of the calculations. All distances and angles were derived from the coordinates of the X-ray structure. The values used for A_a, A_r, and the Euler angles relating the molecule fixed frame and the alignment tensor were the same as those obtained from a fit of $N–H^N$ and $C^{\alpha}–H^{\alpha}$ residual dipolar couplings (data not shown).

As shown in Fig. 3, the experimental data for the $H^N(i)–H^{\alpha}(i)$ and $H^N(i)–H^{\alpha}(i\text{-}1)$ dipolar coupling compare favorably to predictions based on the X-ray crystal structure with correlation coefficients of 0.90 and 0.89, respectively. The quality of the fit suggests that even in the case of variable distance interactions residual dipolar couplings will yield valuable constraints for determining protein structures by NMR. It is also interesting to note that the experimental data fit the X-ray structure better than they fit any of the earlier NMR-derived structures of FKBP/ligand complexes (data not shown).

In larger proteins, systematic errors are expected in the measurement of J coupling constants using the Soft HNCA–E.COSY experiment (*18, 19*). The observed splittings in the experiment are biased toward smaller values due to differential relaxation rates of coherences that are generated to observe the E.COSY effect. These errors can be reduced in this experiment by shortening the length of the refocusing delays after the carbon evolution period and by the use of selective H^N pulses to avoid H^{α} transverse relaxation in the final reverse INEPT (*19*).

The constraint derived from the $H^N(i)–H^{\alpha}(i)$ residual dipolar coupling can be used to restrain the backbone angle ϕ while the $H^N(i)–H^{\alpha}(i\text{-}1)$ coupling restrains the backbone angle ψ. The measured values are very sensitive indicators of structure, since they depend on both the orientation and the magnitude of the interproton vector. Small changes in the interproton distance have a dramatic effect on the size of the dipolar coupling constant due to the inverse cube dependence on distance. For example, the difference between a 2 and 2.5 Å internuclear distance is almost a factor of two difference in the measured residual dipolar coupling. In addition, unlike NOEs, the measured residual dipolar couplings are not affected by spin diffusion.

We have demonstrated a method for measuring residual dipolar couplings between $H^N(i)–H^{\alpha}(i)$ and $H^N(i)–H^{\alpha}(i\text{-}1)$ in proteins. The measured values for the FKBP/FK506 complex show an excellent correlation with those predicted from a high-resolution X-ray structure. These results suggest that re-

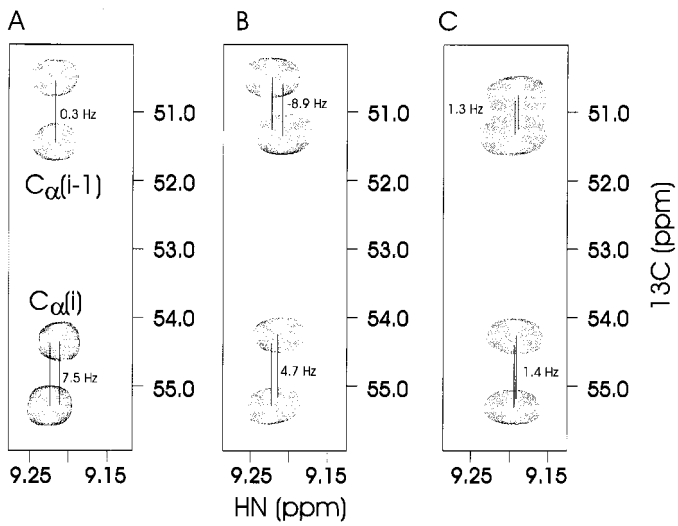

FIG. 2. Small regions of the Soft HNCA–E.COSY spectra showing data for H^N(L74)–H^{α}(L74) and H^N(L74)–H^{α}(K73). All samples contained 1 mM FKBP/FK506 and 50 mM potasium phosphate buffer at pH 6.5. Experiments were performed at 311 K at 600 MHz on a Bruker DRX-600 spectrometer. Soft HNCA–E.COSY spectra acquired using samples containing (A) FKBP/FK506 and phosphate buffer (isotropic conditions), (B) FKBP/FK506, phosphate buffer, and 5% DMPC/DHPC/SDS at a molar ratio (3:1:0.1) (aligned sample), or (C) FKBP/FK506, phosphate buffer, and Pf1 phage (aligned sample). The phage samples were prepared as described in Ref. (*7*).

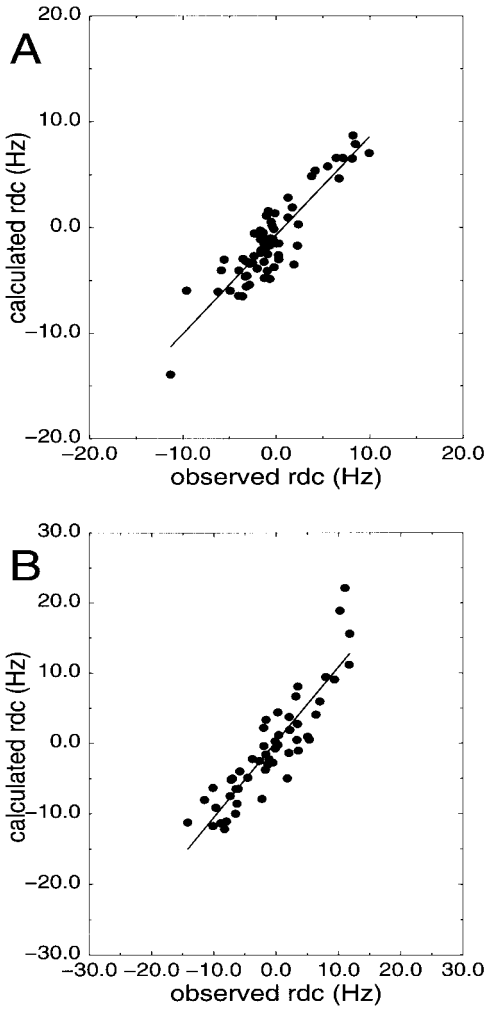

FIG. 3. Correlation of the measured residual dipolar couplings obtained from the difference in the *J* couplings measured under isotropic conditions and when aligned in the presence of DMPC/DHPC/SDS bicelles. (A) $H^N(i)$–$H^\alpha(i)$ and (B) $H^N(i)$–$H^\alpha(i\text{-}1)$ residual dipolar couplings compared to predicted values derived from an X-ray structure of FKBP/FK506 (*11*). The straight line in the figures is from a least-squares fit of the data. The magnitude of the alignment tensor used in the fit are 1.26×10^{-4} and 1.03×10^{-4} for the axial and rhombic components, respectively.

sidual dipolar couplings can provide additional useful structural constraints for determining protein structures by NMR spectroscopy.

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