Measurement of Magnitude and Sign of H,H-Dipolar Couplings in Proteins

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The determination of three-dimensional structures of proteins and nucleic acids by NMR relies on the measurement of a large number of NOE's and homonuclear and heteronuclear couplings.[1] Recently cross correlated relaxation rates[2] and dipolar couplings,[3−5] were also introduced as new parameters for structure elucidation. Residual heteronuclear dipolar couplings proved to be a tool for orientation restraints which provide long-range structural information in proteins and have special impact on the structure determination of multidomain proteins.[6,7] Size and sign of heteronuclear dipolar couplings can be measured from the difference of the splitting observed in partially aligned molecules, by comparison with the scalar couplings measured in isotropic solutions. Provided the amount of alignment is so weak that the absolute value of the dipolar coupling does not exceed the absolute value of the scalar coupling, the dipolar coupling can be directly extracted. This requirement is easily met for directly bound H−C and H−N pairs. For proton−proton dipolar couplings, however, this requirement cannot be met due to the much smaller size of proton−proton *J*-couplings. Therefore, experiments which allow measurement of size and sign of H,H dipolar couplings are of interest.

In this communication, we propose the measurement of H,H dipolar couplings based on the E.COSY principle[8−10] in which the H,H dipolar coupling is related to an ^{15}N−H splitting ($J + D$) whose sign is known. This method provides size and sign information for the H,H dipolar coupling which was only possible so far to measure for interresidual H^N,H^α dipolar couplings in ^{13}C,^{15}N labeled samples.[11] Transfer of coherence between the protons without decoupling the ^{15}N and a planar mixing step before detection as achieved in a J_{HH} sequence[12] (Figure 1) provides an E.COSY type pattern in a ^{15}N−H_1,H_2 moiety. The J_{HH} sequence is built from a ^{15}N edited NOESY spectrum with planar mixing steps before and after the NOESY step. Such pulse sequences have been used for reliable extraction of *J*-coupling values.[13−15] The relevant product operators are given under the pulse sequence in Figure 1. It turns out that in the first planar segment[16−18] the operator transformations, $N_{1y}H_{1\alpha} \rightarrow H_{1z}N_{1\alpha}$ and similarly $N_{1y}H_{1\beta} \rightarrow H_{1z}N_{1\beta}$, are unique apart from other nondetectable coherences. Both transfers have an efficiency of $\sin[\pi(J + D)\Delta]$. However, for the second planar mixing, in addition to the desired transfer (full arrow), two undesired transfers are also possible (broken arrows) if Δ deviates from $(2(J + D))^{-1}$ (Figure 1). This complication will only occur in anisotropic (Bicelle, Phages) media where the values of $J + D$ are no longer uniform. The transfer amplitudes for the desired and undesired transfers are $(\sin[\pi(J_1 + D_1)\Delta](1 + \sin^2[\pi(J_1 + D_1)\Delta]))/2$ and $(\sin[\pi(J_1 + D_1)\Delta](\cos^2[\pi(J_1 + D_1)\Delta]))/2$, respectively, and $(J + D)(N_1,H_1)$ has been abbreviated by $(J_1 + D_1)$. The expected cross-peak pattern of the J_{HH} NOESY experiment is shown in Figure 2. In Figure 2 also sections out of the 2D spectra with displacement vectors are shown. To remove the undesired peak contributions the value of $(J + D)(N_1,H_1)$ needs to be determined. Then the amplitudes of the desired and undesired peak components can be derived. Taking into account that the biggest N,H^N dipolar couplings in our case are about ±25 Hz, the biggest peak contribution is 8%. The undesired peak contributions can be completely removed as previously described.[19] Undesired peak contributions also arise from spin flips during the NOESY mixing

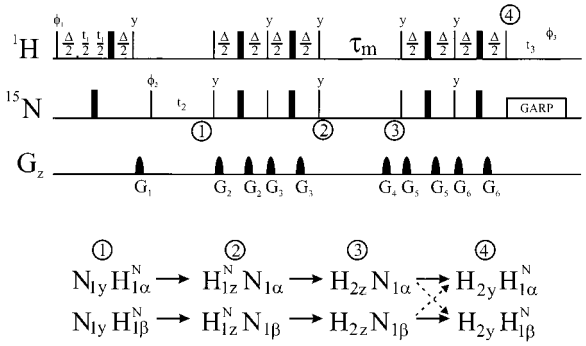

Figure 1. Pulse sequence of the 3D J_{HH}-NOESY. Narrow and thick bars represent 90° and 180° pulses. The default phase for pulses is *x*. Phase cycling: $\phi_1 = 2(x), 2(-x)$, $\phi_2 = x, -x$, $\phi_3 = x, 2(-x), x$. Quadrature detection in both dimensions is obtained by altering ϕ_1 and ϕ_2, respectively, in the States-TPPI manner.[28] Water suppression was done with presaturation during the 2 s relaxation delay. Delay durations: Δ = 5.4 ms and τ_M = 200 ms. Carrier positions: ^{1}H = 4.65 ppm; ^{15}N = 114.5 ppm. Proton pulses are applied using a 20.7 kHz rf field. ^{15}N pulses are at a field of 8.2 kHz and GARP[29] decoupling during acquisition is applied with a 1 kHz field. Gradients (sine bell shaped): G_1 = (1 ms, 30G/cm), G_2 = (1 ms, 8G/cm), G_3 = (1 ms, 10G/cm), G_4 = (1 ms, 50G/cm), G_5 = (1 ms, 9G/cm), G_6 = (1 ms, 11G/cm). 24 scans per t_1 (32 complex points, spectral width 7183.9 Hz) and t_2 (32 complex points, spectral with 1666.6 Hz) experiment were recorded with 1024 complex points in t_3 (spectral width 7183.9 Hz). A repetition delay of 1.3 s was used between scans, giving rise to a total measurement time of 50 h for the 3D experiment. The measurement time for the 2D versions was 15 h. The relevant operator transfers are given around the two planar mixing periods and the NOE transfer step. Details are explained in the text. The pathway of operators is given under the pulse sequence for the crucial points in the sequence.

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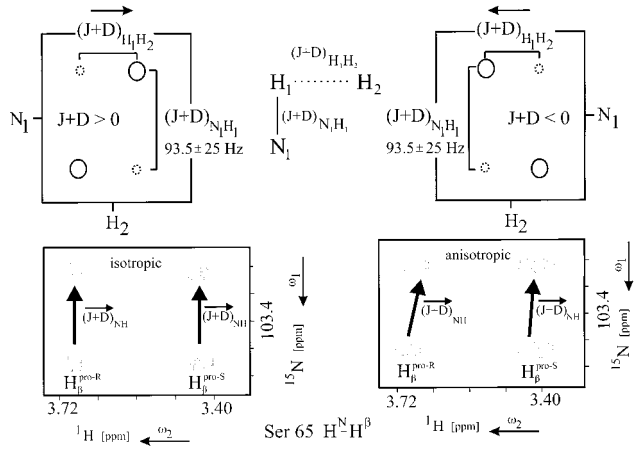

Figure 2. Schematic cross-peak pattern for the J_{HH}−NOESY. Due to the defined sign of $J_1 + D_1$ (93.5 ± 25 Hz provided sufficiently weak alignment is used), the sign of the H,H dipolar coupling can be extracted. The dotted cross-peak contributions arise from the normally weak undesired coherence transfers shown in Figure 1. They cannot be avoided since it is impossible to match Δ to all values of $J + D$. The dotted cross-peak contributions are also modulated by spinflips during the NOESY mixing time as indicated in the text. Also two peaks out of the spectra of Ubiquitin are shown. They display the peaks of Ser 65 H^N−H^β. In isotropic solution the ^4J coupling is zero but in anisotropic solution a through space dipolar coupling can be extracted.

time. Therefore it is important that the ^{15}N T_1 time is longer than the NOESY mixing time. This condition is increasingly more easily accomplished going to larger proteins. The average error on the coupling constants was determined to be ±0.8 Hz based on repeating the experiment.

The experiment was applied to ^{15}N labeled Ubiquitin (VLI Research, Inc., Malvern, PA). The alignment of the sample was achieved with the CHAPSO/DLPC/CTAB system (10:50:1) with 5% total lipid concentration. The reference sample was free of lipids. For both samples we used 3 mg of ^{15}N-labeled Ubiquitin (10 mM phosphate-buffer pH 6.5; H_2O/D_2O 90/10) in a 250 μL Shigemi microcell tube. This system allowed us to measure all experiments at 303 K. For the measurement of Ubiquitin, 2D versions of the J_{HH} sequence were also recorded. The digital resolution was put to 0.22 Hz/point in ω_2. For small proteins, the couplings can be extracted from the 2D versions of the pulse sequence. The quality of the spectra is shown on representative traces in Figure S1. As expected the dipolar couplings show different signs. A total of 67 H,H dipolar coupling could be identified (Supporting Information).

We compared the measured dipolar couplings with the dipolar couplings calculated for structures of Ubiquitin derived from X-ray[20] crystallography and from NMR.[21,22] The alignment tensor was calculated from the $^1D_{NH}$ dipolar coupling constants[23] in the frame of the pdb file using the software *DipoCoup*.[24] The *Q*-values of the H,N^H and the H,H dipolar couplings are listed in Table 1. It is observed that the dipolar H,H couplings fit the calculated NMR structure much better than the X-ray structure. Similar results on carbonyl chemical shifts were observed previously.[21]

Table 1. Comparison of the Different *Q* Values[21]

	X-ray	⟨NMR⟩	NMRopt	Δ*Q*	$\langle D^{exp}/D^{\langle NMR\rangle}\rangle$
$D(N-H^N)$	0.2312	0.2006	0.1991	14%	0.95
$D(H^N-H^X)$	1.0639	0.8324	0.8288	22%	0.70
$D(H^N-H^\alpha)$	0.9232	0.6843	0.6880	25%	0.78

[a] Out of the 67 measured H^N,H^X dipolar couplings as indicated in Table S2 in the Supporting Information 53 are used for the calculation of the H^N,H^X *Q*-factor. 14 dipolar couplings mainly H^N,H^γ and H^N,H^δ were not used because of lack of stereospecific assignment. All 29 measured H^N,H^α couplings are used for the calculation of the H^N,H^α *Q*-factor. The percentages indicate the improvement of the *Q*-factor (Δ*Q*) referring to the X-ray structure versus the NMR structure. ⟨NMR⟩ is the average NMR structure of the 10 best structures and NMRopt is the best structure.[22]

The percentage of decrease of the *Q*-factors for the H,H couplings from the X-ray structure to the NMR structure is even higher compared with that of the N,H^N couplings (Table 1). The larger discrepancies (*Q*-factors) of experimental and structure derived dipolar couplings for the side chain have also been observed for CH dipolar couplings[25] and have been attributed to larger dynamics of the side chains compared to the protein backbone. This is substantiated by our finding that the slope of $D_{HH}^{exp}/D_{HH}^{\langle NMR\rangle} = 0.7$ instead of 1 indicating that $\langle S_{HH}\rangle = 0.7\langle S_{HN}\rangle$. The larger *Q*-factors observed for the H,H dipolar couplings underlines the potential of these couplings to improve the description of protein structures and dynamics.[26]

In conclusion, we have presented a method for measuring the sign and value of H,H dipolar couplings. These dipolar couplings are easily extractable and can be used as long-range distance restraints in biomacromolecules. Due to the knowledge of the sign of the proton−proton dipolar couplings also angular restraints can be evaluated. This method allows one to extract all possible H,H dipolar couplings out of two spectra. In principle, all methods for the measurement of H,H scalar couplings based on E.COSY or DQ/ZQ methodology can be used for sign and size determination of H,H dipolar couplings. The E.COSY method proposed here is among the most sensitive methods even for large proteins. It requires minimal isotopic labeling and can be combined with S^3E[27] elements for further resolution enhancement.

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Supporting Information Available: A listing of the eigenvalues of the alignment tensor and the orientation in the bicelle medium used for the measurement and formula for calculation of the dipolar couplings; all measured H,H and N,H^N dipolar couplings in Ubiquitin, graphs for the correlation of the experimental and the calculated theoretical values, and some representative traces are shown; transferamplidues for the J_{HH} NOESY experiment are discussed (PDF). This material is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

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