Direct Observation of Hydrogen Bonds in Proteins by Interresidue $^{3h}J_{NC'}$ Scalar Couplings

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A number of NMR observations give indirect evidence for the existence of hydrogen bonds in proteins.[1] These parameters include hydrogen-exchange rates,[2,3] isotope shifts,[4,5] isotropic and anisotropic chemical shifts,[6−10] the quadrupolar coupling constant of a deuteron involved in the bond,[11,12] and the sequential one-bond $^1J_{C'N}$ coupling constants.[13,14] Scalar couplings transmitted through the hydrogen bond were first observed in proteins between a backbone amide proton and metal ions coordinated by a cysteine residue.[15,16]

Recently, it has been shown that direct evidence for the existence of hydrogen bonds can be established in nucleic acid base pairs by trans-hydrogen bond scalar couplings to the ^{15}N hydrogen bond acceptor nucleus.[17,18] These scalar couplings seem to correlate with hydrogen bond length and can be used to identify the coupling partner directly. Here we report a similar observation of cross-hydrogen bond ^{15}N−^{13}C′ scalar couplings ($^{3h}J_{NC'}$) in proteins. This coupling establishes an electron-mediated connection between an amide nitrogen and carbonyl carbon nucleus of two residues involved in a N−H···O═C hydrogen bond.

To detect this scalar coupling a long-range, water flip-back[19] HNCO experiment[20,21] (see Supporting Information) was carried out with a modification of the nitrogen to carbonyl dephasing time. In a standard HNCO experiment, this time, 2*T*, for the INEPT transfer between in-phase N^y_i and anti-phase $2N^x_iC'^z_{i-1}$ magnetization is usually set to values slightly shorter than $1/(2*{}^1J_{NC'})$.[21] In proteins, values for $^1J_{NC'}$ range between 13 and 17 Hz with an apparent correlation to the strength of the hydrogen bond.[13] In contrast to the standard HNCO experiment, the long-range $J_{NC'}$ HNCO experiment was carried out with the transfer time 2*T* set to 133 ms $\approx 2/{}^1J_{NC'}$ such that the one-bond transfer from N^y_i to $2N^x_iC'^z_{i-1}$ is approximately refocused. If a second long-range $J_{NiC'j}$ coupling between the nitrogen ^{15}N nucleus of residue *i* and the carbonyl ^{13}C nucleus of residue *j* is active during this transfer, then the intensity of a cross-peak to this nucleus will be proportional to $\sin^2(2\pi J_{NiC'j}T)*\cos^2(2\pi {}^1J_{NiC'i-1}T) \approx \sin^2(2\pi J_{NiC'j}T)*\cos^2(2\pi) = \sin^2(2\pi J_{NiC'j}T)$.

Figure 1 shows the result of such a two-dimensional, long-range $J_{NC'}$ HNCO experiment for a sample of uniformly ^{15}N/^{13}C-enriched human ubiquitin. A total of 31 backbone trans-hydrogen bond $^{15}N_i-{}^{13}C'_j$ correlations could be detected after 12 h of total measuring time. In addition to these previously not observed connectivities, a number of intraresidue two-bond $^{15}N_i-{}^{13}C'_i$ and sequential one-bond $^{15}N_i-{}^{13}C'_{i-1}$ correlations are present. The fact that not all one-bond correlations are completely suppressed after an INEPT ^{15}N to ^{13}C transfer time of 133 ms is a consequence of the variation[13] in the one-bond $J_{NC'}$ coupling constants.

To determine the size of these long-range ^{15}N to ^{13}C′ coupling constants by a quantitative *J*-correlation technique,[15] a second, reference HNCO spectrum was recorded with the same total length 2*T* for the ^{15}N to ^{13}C′ INEPT and reverse INEPT periods, but with the ^{13}C′ 180°-pulses shifted by 16.5 ms relative to the ^{15}N 180°-pulses in both INEPT steps (see Supporting Information). This has the effect of reducing the effective time for the ^{15}N to ^{13}C′ defocusing and refocusing to a value of $2*(T - 16.5$ ms) while keeping the relaxation losses identical to the original experiment. In this reference experiment, the intensities of one-bond $^{15}N_i$ to $^{13}C'_{i-1}$ cross-peaks will be proportional to $\sin^2(2\pi {}^1J_{NiC'i-1}[T - 16.5\text{ms}])*\cos^2(2\pi J_{NiC'j}[T - 16.5\text{ms}])$, if a second $J_{NiC'j}$ scalar coupling is present. Thus, the ratio of the intensities of a $^{15}N_i$ to $^{13}C'_j$ correlation in the long-range experiment relative to the $^{15}N_i$ to $^{13}C'_{i-1}$ correlation in the reference experiment is given by $I_{lr}/I_{ref} = \sin^2(2\pi J_{NiC'j}T)*\cos^2(2\pi {}^1J_{NiC'i-1}T)*\sin^{-2}(2\pi {}^1J_{NiC'i-1}[T - 16.5\text{ms}])*\cos^{-2}(2\pi J_{NiC'j}[T - 16.5\text{ ms}])$. The size of the long-range coupling $J_{NiC'j}$ can be determined from the measured intensity ratio and the known value of $J_{NiC'i-1}$[13] either by a numerical inversion of this implicit equation or from the relation $J_{NiC'j} \approx (I_{lr}/I_{ref})^{1/2}/(2\pi T)$ which is valid for $|2\pi J_{NiC'j}T| \ll 1$ and values of $^1J_{NiC'i-1}$ close to 15 Hz. In the present case, this approximation introduces errors of less than 0.03 Hz for all the derived long-range coupling constants.

Values for the trans-hydrogen bond $^{3h}J_{NiC'j}$ couplings determined by this quantitative-*J* correlation technique range between 0.25 and 0.9 Hz (Supporting Information). The largest couplings are found in β-sheet conformations with an average of 0.65 ± 0.14 Hz ($N = 15$), whereas $^{3h}J_{NiC'j}$-couplings in α-helical structures have a mean value of 0.38 ± 0.12 Hz ($N = 10$). As in the case of the trans-hydrogen bond $^{2h}J_{NN}$ couplings in nucleic acids,[17] the size of the couplings seems to anti-correlate with the hydrogen bond length. For the considered residues, the mean distance between the proton and the oxygen acceptor in the α-helical region of ubiquitin is 2.05 ± 0.09 Å, whereas the mean distance in the β-sheet conformations is 1.91 ± 0.09 Å, as calculated from coordinates of the 1.8 Å resolution crystal structure.[22] The measured intraresidue $^2J_{NiC'i}$ coupling constants range between 0.26 and 1.1 Hz (Supporting Information).

Figure 2 shows a two-dimensional correlation map of all the backbone $^{15}N_i$ to O═$^{13}C_j$ hydrogen bonds detected for ubiquitin

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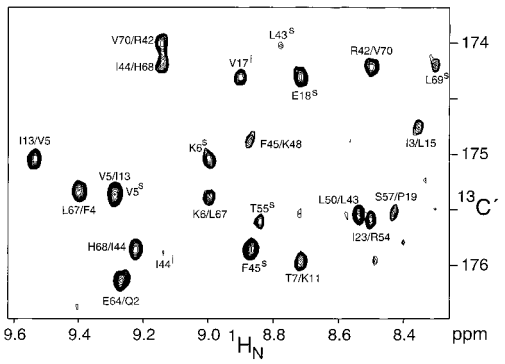

Figure 1. Selected region of the two-dimensional, long-range, quantitative-$J_{NC'}$ H(N)CO spectrum recorded on a 1.6 mM sample of $^{15}N/^{13}C$ labeled ubiquitin, 95% H_2O/5% D_2O, pH 4.6, 45 °C. The data matrix consisted of 65* (t_1) × 512* (t_2) data points (where n* refers to complex points) with acquisition times of 39 ms (t_1) and 53 ms (t_2). Total measuring time was 12 h on a Bruker DMX600 instrument. Compared to a conventional three-dimensional constant-time HNCO experiment, the ^{15}N to $^{13}C'$ defocusing and refocusing times were set to 133 ms, and the chemical shift evolution of the ^{15}N nucleus was not recorded. Cross-peaks marked as Res_i/Res_j are due to $^{3h}J_{NiC'j}$ trans-hydrogen bond scalar couplings between the ^{15}N nucleus of residue i and $^{13}C'$ nucleus of residue j. Residue names marked by the superscript s denote not completely suppressed, sequential, one-bond correlations between the ^{15}N nucleus of residue i and $^{13}C'$ nucleus of residue $i-1$. The superscript i demarks intraresidue two-bond $^{15}N_i-^{13}C'_i$ correlations.

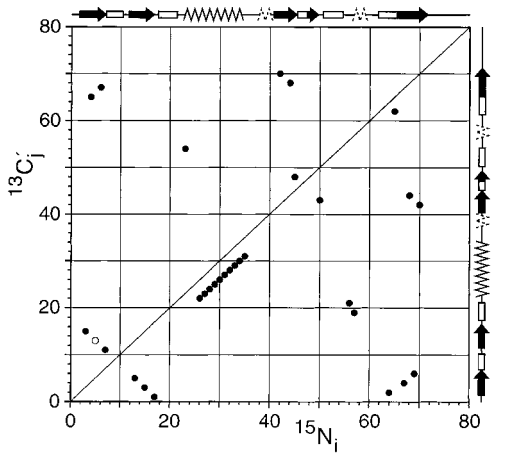

Figure 2. Two-dimensional map representing the $^{3h}J_{NiC'j}$ trans-hydrogen bond correlations in ubiquitin observed in the long-range, quantitative-$J_{NC'}$ HNCO experiment with a N−C′ dephasing time of 133 ms (12 h experimental time). The open circle denotes an ambiguous correlation due to resonance overlap. Secondary structure elements are indicated as filled arrows for β-strands, open boxes for β-turns, zigzag for the α-helix, and dashed zigzags for the 3_{10}-helices.

by the long-range HNCO experiment. Regular α-helical and β-sheet hydrogen bonds comprise 25 of these observations, whereas 6 correlations are detected in more irregular secondary structure elements. Excluding the flexible residue R72, the observed correlations correspond to 83% of all backbone to backbone hydrogen bonds expected from the crystal structure[22] with a proton to carbonyl oxygen distance smaller than 2.2 Å.

It is apparent from Figure 1 that many trans-hydrogen bond $^{15}N_i$ to $O{=}^{13}C_j$ correlations are stronger in the H_N downfield region of the HNCO spectrum. The isotropic[8,23−26] and more recently the anisotropic chemical shift[9,10] of amide protons have been used

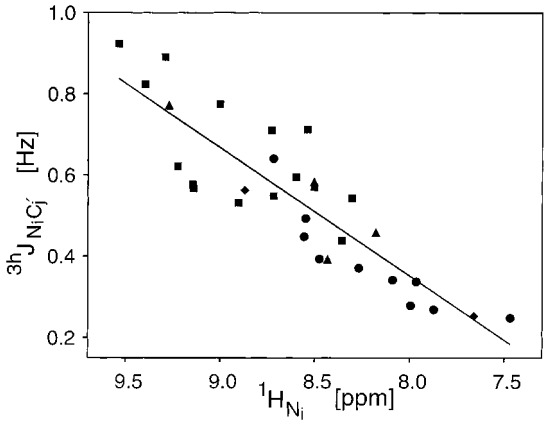

Figure 3. Correlation between $^{3h}J_{NiC'j}$ and the $^1H_{Ni}$ isotropic chemical shift in human ubiquitin. Amide protons are marked according to their secondary structure classification (● = α-helix; ■ = β-sheet; ◆ = turn; ▲ = irregular). The solid line shows a linear fit to the data according to the equation $^{3h}J_{NiC'j} = 0.32$ Hz/ppm * $\delta_{Hi} - 2.18$ Hz.

as an indicator for the strength of the hydrogen bond in proteins. In particular, there is a strong correlation between the hydrogen bond length and the isotropic shift[8,23−26] of the amide proton as well as between the amide proton CSA and the isotropic shift.[9] Short hydrogen bond lengths (R_{HO}) correspond to larger values for the isotropic chemical shift. Figure 3 shows that there is also a strong linear correlation ($r = 0.88$) between the amide proton isotropic chemical shift and the trans-hydrogen bond $^{3h}J_{NiC'j}$ coupling constant. Large coupling constants correlate with a downfield shift of the amide proton, and therefore with short amide proton to carbonyl oxygen distances.

In summary, we have shown that trans-hydrogen bond $^{3h}J_{NiC'j}$ scalar couplings exist in proteins. At present, these small couplings have been observed with reasonable sensitivity for ubiquitin and other proteins of similar size (data not shown). For larger systems, the shorter ^{15}N transverse relaxation times will clearly limit the sensitivity of the proposed long-range HNCO experiment. However, methods which strongly increase the ^{15}N relaxation time, such as the TROSY experiment,[27] can be expected to make the trans-hydrogen bond correlations observable also for medium-sized proteins. Observation of the trans-hydrogen bond correlations establishes valuable and previously inaccessible tertiary structure information. This information is available at the stage of the backbone assignment of a protein.

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Supporting Information Available: Pulse scheme and parameters for a quantitative-$J_{NC'}$ HNCO experiment, list of long-range $J_{NC'}$ coupling constants observed in human ubiquitin. This material is available free of charge via the Internet at http://pubs.acs.org.

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