Identification of the Hydrogen Bonding Network in a Protein by Scalar Couplings

Gabriel Cornilescu, Jin-Shan Hu, and Ad Bax

Laboratory of Chemical Physics National Institute of Diabetes and Digestive and Kidney Diseases National Institutes of Health Bethesda, Maryland 20892-0520

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Hydrogen bonds (H bonds) are of key importance for stabilizing biomolecular structure, and for modulating the substrate binding specificity and reaction rate of virtually any enzymatic reaction. In macromolecules, the presence of H bonds is indicated by the spatial proximity and relative arrangement of the atoms involved, after the structure has been solved by either crystallography or NMR. A variety of NMR spectroscopic parameters has also been used for characterizing these pivotal interactions. These include the effect of such bonds on isotropic chemical shifts and chemical shift anisotropy, and on the quadrupole coupling of H involved in H bonds, on protection of exchange of the labile hydrogen with solvent, and on H/H fractionation.

In a recent remarkable report, Dingley and Grzesiek were the first to demonstrate the presence of surprisingly large hydrogen itself and the H bond accepting 15 N nucleus. These couplings confirm the interaction between the electronic orbitals of the atoms involved, and most importantly they identify unambiguously the pairs of atoms involved in a given H bond.

Although in organic chemistry the possibility of J couplings between atoms separated by less than the sum of the van der Waals radii has long been known, in particular for cases involving C-H, H/H bond donation and accepting 15 N nuclei in a Watson–Crick base pair in double-stranded RNA. This finding was confirmed by Per-vushin et al., and these authors additionally discovered the presence of a smaller (2–4 Hz) J couplings between the imino hydrogen itself and the H bond accepting 15 N nucleus. These couplings confirm the interaction between the electronic orbitals of the atoms involved, and most importantly they identify unambiguously the pairs of atoms involved in a given H bond.

Here, we demonstrate for the protein ubiquitin that small J couplings can be observed across H bonds between the donating 15 N atom and the accepting carboxyl/carboxyl 13 C. The scheme used for detecting this through-H-bond J connectivity (J_{HC}) is essentially the regular CT-HNCO experiment,11 optimized for the detection of these small 15 N–13 C couplings (Figure 1). The magnitude of J_{HC} is then determined through the principle of quantitative analysis of cross-peak intensity.12 In brief, the scheme of Figure 1 is executed three times: (A) with the dephasing time, 27, tuned to 1/(2 J_{NC})-(B) with 27 = 3/(2 J_{NC})-(C) with 27 = 4/(2 J_{NC}). Values of J_{HC} in polypeptides are quite homogeneous (15 ± 1 Hz),13 much larger than the 15 N–13 C which are the focus of this study. The signal intensity observed for a correlation of N and C with coupling J_{NC} is proportionate to \exp(-4 D T_{J}^2) sin^2(\alpha J_{NC} T_{J}) \cos^2(\pi T_{N} \alpha) where the product extends over all other carboxyl/carboxyl carbons coupled to the 15 N of interest with coupling constants J_{NC}, and T_{J} is the 15 N transverse relaxation time. The dephasing times, 27, used in this study have a maximum duration of 133.2 ms, and \cos^2(\pi T_{N} \alpha) \approx 1 for T_{N} \leq 1 Hz. Hence, a good approximation for T_{J} as obtained from the ratio between experiments A and B. To a good approximation, therefore, the magnitude of the small J coupling between N and k can be derived from

$$\sin^2(2 \pi T_{J} \alpha) = \hat{F} \exp(1/(2 J_{NC} T_{J})/\hat{B})$$

where \hat{F} is the intensity of the (weak) correlation between 15 N and k, observed in experiment C, and \hat{B} is the intensity of the

of these intraresidue connectivities falls below the signal-to-noise threshold, indicating $|\gamma_{J_{NC}}| < 0.25$ Hz.

As discussed above, the intensity ratio in the spectra recorded with 133.2 and 100 ms dephasing periods can be used directly to obtain a quantitative measure for the magnitude of the small $J_{NC}$ couplings giving rise to the through-H-bond correlations. Values observed for through-H-bond couplings range from 0.3 Hz for R72H$\rightarrow$Q40C$^\cdot$ to 0.8 Hz for E64H$\rightarrow$Q2C$.^\cdot$ For the $\beta$-sheet H bonds we find an average $\langle J_{NC} \rangle$ value of 0.56 $\pm$ 0.10 Hz. For ubiquitin’s $\alpha$-helix, all but one of its H bonds (K33H$\rightarrow$K29C$^\cdot$) present in the crystal (and NMR) structure give rise to observable cross-peaks. For the missing correlation, $\langle J_{NC} \rangle$ falls below the detection limit of $\sim 0.25$ Hz. Excluding this unobservably small $J_{NC}$ coupling, the average for the $\alpha$-helical $\langle J_{NC} \rangle$ values is 0.37 $\pm$ 0.15 Hz. None of the $3_{10}$ H bonds observed in the crystal and NMR structures, which tend to be longer than $\beta$-sheet H bonds, give rise to observable correlations. In contrast, a number of irregular types of H bonds, not part of helix or sheet, give rise to substantial correlations (e.g., S57H$\rightarrow$P19C$^\cdot$ and I23H$\rightarrow$R54C$^\cdot$). All of these correspond to short (< $2 \text{~Å}$) H$^\cdot$O distances in the X-ray structure.

Detection of the weak $J_{NC}$ connectivities relies on the use of long de- and rephasing intervals. Although for small proteins such as ubiquitin this does not present a significant problem, for larger proteins the long intervals during which $^{15}$N magnetization is relaxing at the transverse relaxation rate result in much lower sensitivity. One way to alleviate this problem is to modify the pulse scheme in such a way that only the most slowly relaxing $^{15}$N doublet component is conserved. As shown by Pervushin et al., transverse relaxation times for the downfield $^{15}$N doublet component remain long in larger, perdeuterated proteins in which the exchangeable hydrogens are protonated.\(^\text{15}\) A pulse scheme suitable for such measurements is presented in the Supporting Information, together with the full 2D H(N)CO spectrum. An additional advantage of this latter method is that H bonds involving side chain NH$_2$ groups are also visible with this method, although none were observed in ubiquitin. Note that such correlations are eliminated with the scheme of Figure 1, where the $^{15}$N$\rightarrow$[H] rephasing delays are optimized for NH and not NH$_2$ groups. For smaller, protonated proteins such as ubiquitin, the scheme of Figure 1 offers about 50% higher sensitivity, however.

Experimental observation of $J$ coupling through H bonds is invaluable in NMR structure determination, where unambiguous identification of the H bond acceptor carbonyl can be particularly difficult. Moreover, the presence of $J_{NC}$ coupling is of conceptual interest as it provides unequivocal evidence for the partial covalent character of even regular, weak H bonds in proteins. Data obtained on ubiquitin indicate that only H bonds for which the H$^\cdot$O distance is less than 2.2 Å give rise to an observable $J_{NC}$ connectivity, and $J_{NC}$ may prove to be a useful parameter for characterizing the strength of H bonds. In this case, $J_{NC}$ will be an ideal parameter for studying such interactions in the active sites of enzymes, where H bond strengths frequently are of key interest.

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Supporting Information Available: One table with the $J_{NC}$ couplings observed in ubiquitin; one pulse sequence for recording 2D H(N)CO spectra on larger, perdeuterated proteins, and one 2D H(N)CO spectrum, recorded with this pulse scheme for ubiquitin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.