NMR Spectroscopy of Proteins

DOI: 10.1002/anie.201209641

N–H Spin–Spin Couplings: Probing Hydrogen Bonds in Proteins**

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Hydrogen bonds are essential for the structure of many biochemical compounds. Protein folding, the formation of amyloid aggregates, enzymatic catalysis, drug–receptor interactions, and many other phenomena are intrinsically connected to hydrogen bonding. NMR chemical shifts,[1–3] anisotropies of the chemical shifts,[1,4,5] and nuclear quadrupole coupling constants[1,6] are influenced by hydrogen bonding, making NMR spectroscopy a key tool for the detection and characterization of hydrogen bonds. The experimental observation of NMR spin–spin coupling constants across hydrogen bonds allows the direct detection of hydrogen bonding in proteins.[9] In addition, detailed insight into the nature of hydrogen bonds can be obtained by theoretical calculations.[10–12]

Hydrogen bonding in proteins involves, in most cases, arrangements of the type C=O···H–N. Six spin–spin coupling constants should be affected by hydrogen bonding, four directly because they are spin–spin coupling constants across the hydrogen bond and two indirectly because they involve atoms participating in hydrogen bonding. However, only the ^{13}C–^{15}N three-bond scalar coupling, $^{3}J_{NC}$, across the hydrogen bond and the one-bond ^{1}H–^{15}N scalar coupling, $^{1}J_{NH}$, can be currently measured in proteins. The magnitude of the $^{1}J_{NH}$ spin–spin coupling constant is dependent upon the electronic structure of the molecule.[11,13,14] Theoretical studies on small model systems have shown that $^{1}J_{NH}$ is negative and becomes more negative upon the formation of a hydrogen-bond.[11,14] Moreover, conformational changes due to ligand binding can induce changes in $^{1}J_{NH}$ spin–spin coupling constants.[16]

Although it is possible to accurately measure $^{1}J_{NH}$ scalar couplings, they have not been employed for the detection and characterization of hydrogen bonds in biomolecules. We attribute this to the fact that changes in $^{1}J_{NH}$ coupling constants due to hydrogen-bond formation are predicted to be small,[11,14] a systematic analysis of $^{1}J_{NH}$ spin–spin coupling constants in disordered proteins, which might serve as reference, is lacking, and the influence of pH and temperature is unknown. To overcome these limitations we have measured a large set of $^{1}J_{NH}$ coupling constants in intrinsically disordered proteins as a function of pH and temperature and compared these to values in folded proteins. Our study demonstrates that the magnitude of $^{1}J_{NH}$ spin–spin coupling constants increases by up to 1.6 Hz upon hydrogen-bond formation. Thus $^{1}J_{NH}$ spin–spin couplings can provide a sensitive tool for the study of hydrogen bonds.

We measured $^{1}J_{NH}$ scalar couplings in the 441 residue protein Tau at a static magnetic field strength of 21.1 T and a temperature of 278 K (see Table S1 in the Supporting Information).[17] Tau belongs to the class of intrinsically disordered proteins and displays only transient secondary structure.[18] A total of 367 $^{1}J_{NH}$ values were obtained for nonoverlapping residues. Experimental values were observed for all non-proline amino acid types except tryptophan, which is not present in the primary sequence of Tau. $^{1}J_{NH}$ values belonging to the same amino acid type were grouped and median $^{1}J_{NH}$ values were calculated (Figure 1 a). Glycine and alanine residues have the most negative $^{1}J_{NH}$ values (−94.2 Hz and −93.4 Hz, respectively). For other amino acids, values ranged from −93.3 Hz (serine) to −93.0 Hz (valine) (Table S2).

To systematically investigate the influence of pH and temperature, we measured $^{1}J_{NH}$ couplings in the intrinsically disordered protein α-synuclein at different temperatures and pH values (pH 5.7: 278 K, 288 K, 298 K; pH 6.0: 278 K); pH 6.5: 278 K, and pH 7.4: 278 K, 288 K; Table S3). As a result of increased amide proton exchange the signal-to-noise ratio decreased and the experimental error in $^{1}J_{NH}$ increased at higher pH and temperature. However, at all tested temperatures and pH values $^{1}J_{NH}$ values were highly correlated (Figure 1 b,c and Figure S1), indicating that $^{1}J_{NH}$ values in α-synuclein are stable against pH and temperature variations. In addition, comparison of $^{1}J_{NH}$ values in the 130 residue Tau fragment K18 observed at 400 and 600 MHz proton frequency (Figure S2) suggests that the contribution of the dynamic frequency shift[7,19,20] is negligible in intrinsically disordered proteins. Accordingly, mean values of $^{1}J_{NH}$ observed in α-synuclein at 700 MHz and in Tau at 900 MHz were identical within experimental error (Figure S3).

The intrinsically disordered proteins α-synuclein and Tau populate transient secondary structures; for Tau ^{428}LADEVSASLA^{437} the contribution of the helical structure can reach 25 %.[17] Figure 1 d,e show secondary $^{1}J_{NH}$ values, $\Delta^{1}J_{NH}$, that is, the difference between experimental $^{1}J_{NH}$ spin–spin coupling constants and the random-coil values from Table S2. For both intrinsically disordered proteins, $\Delta^{1}J_{NH}$ values were below 0.35 Hz, which corresponds to the estimated uncertainty in $\Delta^{1}J_{NH}$ due to uncertainties in the random-coil values (Figure 1 a) and any remaining influence of pH and temperature (Figure 1 b,c and Figure S1). Only K96 in α-synuclein showed systematically negative $\Delta^{1}J_{NH}$ values at different temperatures and pH values (Table S3). The data

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[**] We thank Dr. Jacek Biernat and Dr. Eckhard Mandelkow for providing Tau samples.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201209641.

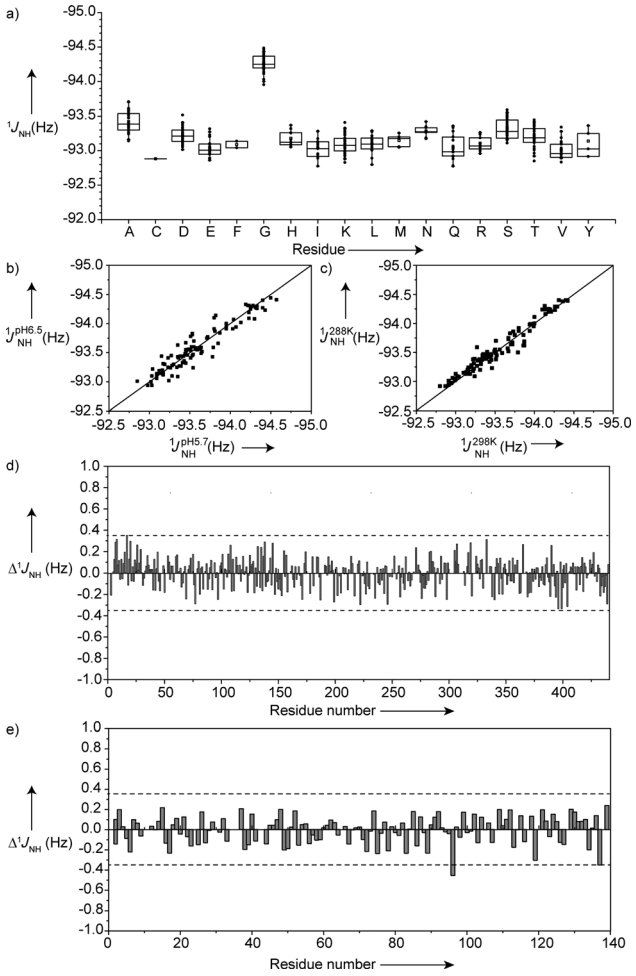

Figure 1. a) $^1J_{NH}$ spin–spin coupling constants observed in the intrinsically disordered protein Tau using $^1J_{NH}$-modulated 2D ^1H–^{15}N heteronuclear single quantum coherence (HSQC) experiments.[7] $^1J_{NH}$ values were grouped according to amino acid type and subjected to a box plot analysis. The center of the box indicates the median value for each residue type, while bottom and top correspond to 25th and 75th percentile, respectively. The mean values are outlined with an empty box (□). b) Correlation between $^1J_{NH}$ values in α-synuclein at pH 6.5 and pH 5.7, 278 K. c) Correlation between $^1J_{NH}$ values in α-synuclein at 288 K and 298 K, pH 5.7. Data in (b) and (c) were measured using IPAP-HSQC, in which long-range couplings are not suppressed.[15] d,e) Deviation of $^1J_{NH}$ spin–spin coupling constants observed in Tau (d) and α-synuclein (e), both at pH 6.0, 278 K, from their amino acid specific random-coil values as a function of residue number. The dashed line indicates the estimated error range of 0.35 Hz for Δ^1J_{NH} taking into account uncertainties in the random-coil values (a) and potential influence of pH and temperature (b,c).

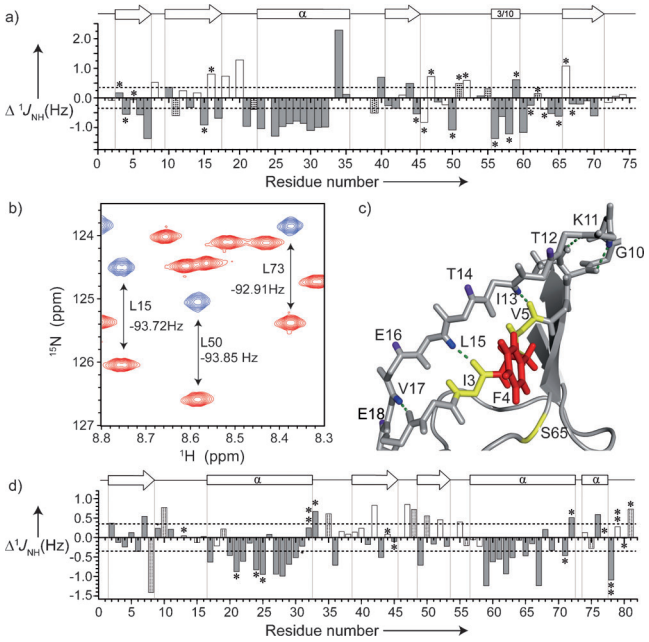

Figure 2. $^1J_{NH}$ spin–spin coupling constants in globular proteins. a) Deviation of $^1J_{NH}$ spin–spin coupling constants observed in ubiquitin from the amino acid specific random-coil values as a function of residue number. Filled and hatched bars mark amide protons involved in hydrogen bonds to the backbone or a side chain, respectively. Stars indicate spatial proximity (< 6 Å) to aromatic rings. Secondary structures identified by DSSP[8] in the crystal structure of ubiquitin (pdb code: 1ubq) are marked above. b) Selected part of a BSD-IPAP-HSQC spectrum of ubiquitin. Doublet components are shown in red and blue. c) Expanded region of the crystal structure of ubiquitin highlighting the hydrogen bonds between β-strands 1 and 2. Hydrogen bonds are in green. Amide protons involved in hydrogen bonds are in blue while those not in hydrogen bonds are in purple. The aromatic ring of Phe4 is shown in red and residues in spatial proximity (< 6 Å) are shown in yellow. d) Deviation of $^1J_{NH}$ couplings observed in DinI from the amino acid specific random-coil values as a function of residue number. Secondary structures identified by DSSP[8] in the 3D structure (pdb code: 1ghh) are marked above.

demonstrate that $^1J_{NH}$ random-coil values are highly stable. Hydrogen bonds potentially present in transient secondary structures of Tau and α-synuclein do not have sufficiently long lifetimes to lead to a detectable change in the $^1J_{NH}$ coupling constant.

Having established a set of robust amino acid specific reference values, we studied the variation of $^1J_{NH}$ coupling constants in folded proteins (Figure 2). Particularly suited for this investigation is the protein ubiquitin as it has been extensively studied by NMR spectroscopy.[5,9,21] Figure 2a shows secondary $^1J_{NH}$ values in ubiquitin as a function of residue number. Both negative and positive Δ^1J_{NH} were found that cover a range from about −1.3 Hz to +2.3 Hz. Ile23–Asp32 within the α-helix of ubiquitin showed a continuous set of negative Δ^1J_{NH} values with an average value of approximately −1.0 Hz (Figure 2a). Positive Δ^1J_{NH} values were predominantly observed in loop regions and at the edges of helices and strands.

According to theoretical studies on small model systems $^1J_{NH}$ is predicted to become more negative upon hydrogen-bond formation.[11,14] Figure 2a demonstrates that amide protons with Δ^1J_{NH} <-0.35 Hz are involved in hydrogen bonds as predicted by the program Whatif.[22] Note that hydrogen bonds to backbone and side chains were taken into account. We also included hydrogen bonds of residues such as Thr22. This residue is not predicted to be hydrogen-bonded on the basis of the crystal structure of ubiquitin (PDB code: 1ubq), but has a high probability for hydrogen-bond formation with its own side chain or the side chain of Asn25 according to the Whatif prediction for the ensemble of NMR

structures of ubiquitin (PDB code: 1d3z). Indeed, the amide proton of Thr22 was previously suggested to be involved in a hydrogen bond because of its small temperature coefficient.[23] The tight connection between hydrogen bonds and negative Δ^1J_{NH} values is further supported by experimental data for the protein DinI: all amide protons of DinI with negative Δ^1J_{NH} values are involved in hydrogen bonds (PDB code: 1ghh; Figure 2d).

There are amide protons such as Ala46 in ubiquitin that have negative Δ^1J_{NH} values but are not predicted to be hydrogen-bonded on the basis of the deposited 3D structure. In addition, the most positive Δ^1J_{NH} value was detected for Glu34, at the C-terminal end of the α-helix of ubiquitin, although its amide proton is hydrogen-bonded to Ile30.[9,21] For Glu34, a large value of the amide proton chemical shift anisotropy and a small value of the deuterium quadrupolar coupling constant in the N–^{2}H···O=C bond was observed, pointing to destabilization of the hydrogen bond.[5,6] Moreover, nearby aromatic groups might influence $^1J_{NH}$ spin–spin coupling constants (Figure 2c).[16,24] However, the $^1J_{NH}$ couplings for the residues at the flexible C-terminus of ubiquitin were very close to their random-coil values (Figure 2a), providing support for the identification of hydrogen bonds on the basis of $^1J_{NH}$ coupling constants.

Theoretical calculation of $^1J_{NH}$ spin–spin coupling constants is currently not accurate enough for detailed comparison with experimental data.[12] However, $^1J_{NH}$ values do not correlate with the dihedral angle ρ (∢H···O=C-N), which can distinguish α-helix from β-sheet, nor with the length of the hydrogen bond (Figure S4). Thus $^1J_{NH}$ coupling constants do not simply report on the presence of secondary structure, but are intimately linked to the presence of hydrogen bonds. Indeed, for residues 14–17 in ubiquitin's β-strand 2, which forms the edge of a twisted β-sheet, an alternating pattern of positive and negative Δ^1J_{NH} values was observed in line with the presence and absence of hydrogen bonds (Figure 2a,c). A similar alternating pattern of Δ^1J_{NH} and hydrogen bonds is present in the edge β-strands 2 and 3 of protein G (Figure S5). The influence of hydrogen bonds on $^1J_{NH}$ coupling constants is further highlighted by the large negative Δ^1J_{NH} values of Leu50 (Figure 2a,b), which is located at the beginning of a short β-turn region and forms a hydrogen bond with Leu43.

Identification of hydrogen bonds on the basis of $^1J_{NH}$ couplings also complements trans-hydrogen bond ^{13}C–^{15}N scalar couplings. $^3J_{NC}$ couplings were observed for most hydrogen bonds in the α-helix and the β-strands of ubiquitin, but not in the 3_{10}-helix.[9,21] The failure to detect there $^3J_{NC}$ couplings stems from an unfavorable geometry of the hydrogen bonds in 3_{10}-helices.[10] Indeed, $^3J_{NC}$ couplings in 3_{10}-helices are only detectable in conformationally rigidified peptides at very high concentrations (ca. 10 mM).[25] In contrast, large negative Δ^1J_{NH} values were observed for residues 56 to 58, consistent with the location of hydrogen bonds in the 3_{10}-helix of ubiquitin (Figure 2a).

In summary, we showed that $^1J_{NH}$ spin–spin coupling constants in proteins are increased by up to 1.6 Hz due to hydrogen-bond formation. Our data suggest that $^1J_{NH}$ couplings can be valuable for identification of transient hydrogen bonds, such as those of Thr22 in ubiquitin, which are difficult to identify by other means. We also expect that our study will provide a basis for investigating the connection between hydrogen bonds and $^1J_{NH}$ couplings by theoretical calculations. We conclude that $^1J_{NH}$ spin–spin coupling constants are a sensitive tool for studying hydrogen bonds in proteins.

Experimental Section

$^1J_{NH}$ coupling constants in hTau40 and α-synuclein (at pH 6.0, 278 K) were measured using two-dimensional $^1J_{NH}$-modulated HSQC experiments[7] recorded on a Bruker Avance 900 MHz spectrometer (hTau40) and on a Bruker Avance 700 MHz spectrometer (α-synuclein). Spectra were recorded in an interleaved manner with 15 different mixing times: 31.9 ms, 31.1 ms, 30.7 ms, 30.3 ms, 29.9 ms, 26.5 ms, 25.5 ms, 25.1 ms, 24.6 ms, 24.3 ms, 21.2 ms, 20.3 ms, 19.8 ms, 19.5 ms, and 19.1 ms. At each time point a two-dimensional HSQC was recorded, with $334(^{15}N)\times512(^1H)$ complex points for hTau40, and $256(^{15}N)\times512(^1H)$ complex points for α-synuclein. $^1J_{NH}$ values were determined by fitting cross-peak intensities as described previously.[7]

$^1J_{NH}$ couplings in hTau40, ubiquitin, and α-synuclein were also measured using broad-band-selective-decoupled IPAP-HSQC (BSD-IPAP-HSQC) experiments,[26] in which long-range couplings such as $^2J_{NH\alpha}$, $^3J_{NH\beta}$, and $^3J_{HNH\alpha}$ were refocused by REBURP pulses centered at $\delta = 2.4$ ppm and covering a bandwidth of $\delta = 2.8$ ppm. The experimental error derived from repeat measurements was 0.05 Hz. $^1J_{NH}$ couplings in K18 were also measured by BSD-IPAP-HSQC experiments on Bruker Avance 600 MHz and 400 MHz spectrometers with z-axis gradient, triple resonance, room-temperature probes. In-phase and anti-phase spectra were recorded interleaved with $256(^{15}N)\times512(^1H)$ complex points. $^1J_{NH}$ couplings in ubiquitin were measured at 400 MHz, 600 MHz, and 900 MHz to correct for the effects of dynamic frequency shifts (DFS) and residual dipolar couplings, which are caused by anisotropic magnetic susceptibility of the protein.[7] BSD-IPAP-HSQC experiments were recorded as interleaved in-phase and anti-phase spectra with $256(^{15}N)\times512(^1H)$ complex points. Data processing was conducted according to Ref. [26]. Additional experimental details have been provided as Supporting Information.

Received: December 2, 2012
Published online: February 10, 2013

Keywords: amides · coupling constants · hydrogen bonds · NMR spectroscopy · proteins

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