Direct NMR Detection of Bifurcated Hydrogen Bonding in the α-Helix N-Caps of Ankyrin Repeat Proteins

Matthew R. Preimesberger, † Ananya Majumdar, † Tural Aksel, ‡ Kevin Sforza, † Thomas Lectka, ‡ Doug Barrick, † and Juliette T. J. Lecomte*†‡

†T. C. Jenkins Department of Biophysics and ‡Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, United States

Abstract: In biomolecules, bifurcated H-bonds typically involve the interaction of two donor protons with the two lone pairs of oxygen. Here, we present direct NMR evidence for a bifurcated H-bonding arrangement involving nitrogen as the acceptor atom. Specifically, the H-bond network comprises the N61 atom of histidine and both the backbone N–H and side-chain Oγ–H of threonine within the conserved TXTH motif of ankyrin repeat (AR) proteins. Identification of the H-bonding partners is achieved via solution NMR H-bond scalar coupling (HBC) and H/D isotope shift experiments. Quantitative determination of 2JHN HBCs supports that Thr N–H–N61 His H-bonds within internal repeats are stronger (~4 Hz) than in the solvent exposed C-terminal AR (~2 Hz). In agreement, pKa values for the buried histidines bridging internal ARs are several units lower than those of the C-terminus. Quantum chemical calculations show that the relevant 2J and 13J couplings are dominated by the Fermi contact interaction. Finally, a Thr-to-Val replacement, which eliminates the Thr Oγ–H–N61 His H-bond and decreases protein stability, results in a 25% increase in 2JHN attributed to optimization of the Val N–H–N61 His H-bond. Overall, the results provide new insights into the H-bonding properties of histidine, a refined structural rationalization for the folding cooperativity of AR proteins, and a challenging benchmark for the calculation of HBCs.

Hydrogen bonds (H-bonds) are essential structural elements in the self-assembly, stability, and remarkable catalytic properties of biomolecules. These low energy interactions participate in processes essential to life either singly or as intricate networks conveying structural and thermodynamic cooperativity. Yet, the presence and configuration of intra-molecular H-bonds, along with their relative strength, are difficult to establish with direct experimental methods. By default, H-bonds are often modeled in crystallographic structures using theoretical idealized geometry, therefore leaving the precise configuration unknown and important instances of strained H-bonds unnoticed.

Ankyrin repeat (AR) proteins have highly cooperative folding–unfolding transitions. Most of the available X-ray models show a conspicuous array of H-bonds extending from repeat to repeat. This array involves conserved TXTH motifs initiating the first α-helix of each repeat (Figure 1). Histidine plays an essential role within and between adjacent TXTH motifs. Specifically, a side-chain/main-chain interaction involving the His Nδ1 and Thr NH caps the N-terminus of the α-helix. In addition, successive ARs pack against each other by using the Nz2H of the TXTH histidine as an H-bond donor to the carbonyl group of the residue preceding the TXTH threonine of the next repeat. We produce the detailed description of the H-bond network necessary to rationalize thermodynamic properties, we are pursuing solution NMR studies of consensus AR proteins. Here, we provide direct evidence for stable bifurcated H-bonds in TXTH helix capping motifs and demonstrate the importance of the Thr hydroxyl group for the stability of the AR fold.

Amide H/D exchange rates and the response of amide 1H chemical shifts to temperature are routinely used to infer the presence of H-bonds in proteins. These approaches are experimentally straightforward, but they do not identify acceptor atoms or report on local structure. In contrast, scalar couplings across H-bonds (HBCs) are challenging to measure, but provide information not otherwise experimentally accessible. In particular, the magnitude of the HBC is exquisitely sensitive to changes in bonding geometry, which makes this

Received: October 20, 2014
Published: January 12, 2015

Figure 1. Ribbon diagram of the first four ARs in E3_19 (PDB: 2BKG) as a model for the NRRC protein discussed in this work (residue numbers correspond to NRRC). Each AR is an ~33-residue helix-turn-helix module followed by an extended β-hairpin loop. The Thr and His of interest are shown in ball-and-stick representation (green, 2nd repeat T44–H47; magenta, 3rd repeat T77–H80; cyan, C-terminal repeat T110–H113). NRRC contains an additional N-terminal TXTH motif (T11–H14).

DOI: 10.1021/jacs.4b11074g
J. Am. Chem. Soc. 2015, 137, 1008–1011
parameter well suited for interrogation of H-bond strain and relaxation.19

The most common H-bond in proteins is of the N–H···O=C type and has 1H JNNC1 and 15N JNNC1 HBCs below 1 Hz.10,13,20,21 The small 1H values and the yet smaller variations caused by different structural contexts often limit the feasibility and utility of these measurements to relatively small proteins. However, the 2H JNN values will o

cap is found in several non-AR protein structures (Table S1). These will offer interesting opportunities for comparative studies. In prior structural work, we assigned the backbone 1H, 15N, and 13C NMR signals of the three-repeat consensus AR protein NRRC, where N refers to the N-terminal AR, R to an internal AR, and C to the C-terminal AR.1 Here, we extend our study to the four-repeat AR protein NRRC, which contains an additional TXXH cap is found in several non-AR protein structures (Table S1). These will offer interesting opportunities for comparative studies. In prior structural work, we assigned the backbone 1H, 15N, and 13C NMR signals of the three-repeat consensus AR protein NRRC, where N refers to the N-terminal AR, R to an internal AR, and C to the C-terminal AR.1 Here, we extend our study to the four-repeat AR protein NRRC, which contains an additional TXXH motif. From 1H−15N LR HMOC spectra (Figure S1B,C) we deduce that each capping histidine adopts the Ne2H tautomer23 in agreement with data published on the naturally occurring AR protein gankyrin.4 Intra- and inter-repeat NOEs (Figure S2A) orient the imidazole rings as in Figure 1.

In NRRC, a soft HNN-COSY experiment detects three Thr(i) N–H···Nδ1 His(i+3) H-bonds through 2H JNNC-mediated cross peaks between the NH of T11 (T44, T77) and Nδ1 of H14 (H47, H80) (Figure S3). In NRRC, there are four detectable signals (Figure 2a, black peaks). A complete NMR connectivity map demonstrating the helix capping Thr N–H···Nδ1 His H-bonds in NRRC is presented in Figure S1.

To investigate how the attributes of Thr N–H···Nδ1 His H-bonds vary from repeat to repeat (e.g., R1 to R2 to C in NRRC), we measured the magnitude of 2H JNN by a quantitative (Q) spin–echo difference method.24,25 Figure 2b shows portions of Q-2H JNN 1-D HSQC spectra collected on NRRC. The NH signals of T44, T77, and T110 undergo 2H JNN modulation with frequencies reporting on each coupling constant. Peak heights were determined as a function of the 2H JNN modulation period (r) and fitted according to the relationship25 I(r) = A cos (ω r t) to extract 2H JNN values. In NRRC (Figure 2c), the internal TPLH H-bonds (T44−H47, T77−H80) have 2H JNN values of ∼3.9−4.0 Hz, whereas the C-terminal TPEH H-bond has an attenuated value (T110−H113, ∼2.1 Hz). Because of H overlap, only an upper bound for the N-terminal T11−H14 HBC was determined (2H JNN < 4.1 Hz, not shown). The varying magnitude of 2H JNN from internal to terminal repeat (R1 = R2 > C) results from differences in H-bond geometry, time-averaged populations, or both. The C-terminal T110−H113 motif has high solvent exposure and no carbonyl acceptor for H113 Nε2-H, which likely destabilizes the N–H···Nδ1 bond relative to those in internal repeats. Similar differences between internal (T44−H47, 2H JNN ∼4.1 Hz) and C-terminal (T77−H80, 2H JNN ∼1.8 Hz) H-bonds are observed in NRRC (Table 1, Figure S4A).

Table 1. Chemical Shifts, 2H JNN Coupling Constants, and 2HΔ1H Isotope Shifts in AR TXXH Motifs (298 K, pH 6.6)

<table>
<thead>
<tr>
<th>Protein and proton</th>
<th>H-bond partners</th>
<th>δ (ppm)</th>
<th>1H JNN (Hz)</th>
<th>2HΔ1H (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRC NH</td>
<td>T44−H47</td>
<td>9.98</td>
<td>4.1 ± 0.2</td>
<td>nd</td>
</tr>
<tr>
<td>NRRC NH</td>
<td>T77−H80</td>
<td>9.18</td>
<td>1.8 ± 0.2</td>
<td>nd</td>
</tr>
<tr>
<td>NRRC NH</td>
<td>T44−H47</td>
<td>9.79</td>
<td>4.0 ± 0.2</td>
<td>−53 ± 5</td>
</tr>
<tr>
<td>NRRC OγH</td>
<td>T44−H47</td>
<td>6.36</td>
<td>−47 ± 5</td>
<td></td>
</tr>
<tr>
<td>NRRC NH</td>
<td>T77−H80</td>
<td>9.58</td>
<td>3.9 ± 0.2</td>
<td>−53 ± 5</td>
</tr>
<tr>
<td>NRRC OγH</td>
<td>T77−H80</td>
<td>6.75</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>NRRC NH</td>
<td>T110−H113</td>
<td>9.13</td>
<td>2.1 ± 0.1</td>
<td>nd</td>
</tr>
<tr>
<td>T44V NH</td>
<td>T44−H47</td>
<td>11.27</td>
<td>5.2 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>T44V NH</td>
<td>T77−H80</td>
<td>9.57</td>
<td>3.4 ± 0.5</td>
<td>−55 ± 5</td>
</tr>
<tr>
<td>T44V OγH</td>
<td>T77−H80</td>
<td>6.70</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

*Data collected at 308 K, pH 7.5. Most N–H···N type H-bonds undergo thermal expansion (longer distance, lower 2H JNN) with increasing temperature.31 Not determined.

The trend in 2H JNN values is mirrored in other physicochemical properties. For example, we measured the apparent pK of each TXXH histidine within NRRC, a protein containing identical repeats at internal (R1) and C-terminal (R2) positions, by following the His Hε1, Hδ2, and Nδ1 resonances as a function of pH (Figure S5). H47, within R1, remains in the neutral state at pH values below ∼3, until the protein begins to undergo global acid unfolding. H14 of the N-terminal AR is buried and shows similar behavior. In contrast, the ionization midpoint of H80 (within R2) is only moderately depressed (apparent pK = 5.7). Protonation of histidine necessarily breaks the N–H···Nδ1 H-bond, and greater pK depression should in part result from stronger H-bonds. Therefore, the data are consistent with the use of the 2H JNN HBC as a proxy for relative H-bond strength. The difference between buried and solvent exposed N–H···Nδ1 H-bonds in AR repeats is analogous to the difference observed between the middle and ends of nucleic acid secondary structures, where fraying leads to smaller 2H JNN values.16

We next sought to detect Thr N–H···Nδ1 His 2H JNN HBCs in NRRC by using a high-sensitivity 1H−15N LR HMOC approach.23 These 2H JNN were not observed, but surprisingly, the experiment yielded J-correlations between T44 (T77) Oγ−H

Figure 2. (a) Overlay of 600 MHz soft HNN-COSY spectra collected on 15N-labeled consensus AR proteins for detection of backbone N–H···Nδ1 histidine H-bonds: NRRC (pH 6.6, 298 K, black) and T44V NRRC (pH 7.5, 308 K, red). Labels identify NH,Nδ1 bonding partners. (b) Downfield region of quantitative 2H JNN-modulation 1-D HSQC spectra collected on NRRC. (c) Intensity-normalized peak heights from (b) plotted as a function of the modulation period, t. Solid lines represent the best fit of the data to a cosine wave (see text).
and H47 (H80) N\(\delta 1\) (Figure 3a,b). LR HSQC modulation experiments (Figure S6) confirmed the buildup of the weak T44 H-bonds in nucleic acids. Correlations are labeled in red. The accompanied by splitting of T44 and T77 N atom at the adjacent H-bond. (f) Proposed origin for \(2^h\)H/D exchange experiment was performed to test the hypothesis that the population of T44 H/D acceptor to T44 (T77) N\(\delta 1\) H-bond. An H/D exchange experiment was performed to test the proposed bifurcated H-bond scheme. When an \(^{15}\)N-labeled NRRC NMR sample was diluted into a 50:50 H\(_2\)O/D\(_2\)O solvent mixture, we observed H/D equilibration of Thr hydroxyl groups, these spectra, a 3-Hz upper limit for bifurcated H-bond acceptor to T44 (T77) N\(\delta 1\) H-bonds in nucleic acids. The observation of \(2^h\)HBCs strongly suggests that H47 (H80) N\(\delta 1\) serves as a bifurcated H-bond acceptor to T44 (T77) N\(\delta 1\) H-bonds in the oxyanion hole of ketosteroid isomerase (Figure S11, Table S2) are consensus protein (Figure S8). Figure 2a shows the HNN-COSY spectrum of the variant (red peaks). H-bond detection in T44V NRRC is consistent with a \(\alpha\)-helix N-cap TXXH motif. The consequences of Thr O\(\gamma\)−H···N\(\delta 1\) His H-bond deletion were explored with the isosteric T44V replacement in NRRC. We reasoned that elimination of the bifurcated N\(\gamma\)−H···N\(\delta 1\) O\(\gamma\)−H interaction would perturb the remaining N\(\gamma\)−H···N\(\delta 1\) His H-bond. Denaturation experiments conducted at pH 8.0 indicate that T44V NRRC is destabilized by \(\sim 2.6\) kcal/mol relative to the consensus protein (Figure S8). Figure 2a shows the HNN-COSY spectrum of the variant (red peaks). H-bond detection in T44V NRRC is difficult because of protein aggregation at concentrations above \(\sim 100\) \(\mu\)M; nevertheless, the weak T77 N\(\gamma\)−H···N\(\delta 1\) H-bond correlation is observable and overlays well with the reference NRRC signal. In contrast, the V44 N\(\gamma\)−H···N\(\delta 1\) H47 cross peak has increased intensity compared to the reference T44 N\(\gamma\)−H···N\(\delta 1\) H47 cross peak. Also remarkable are the large downfield shifts of both V44 amide \(^1\)H (\(\sim 1.5\) ppm) and H47 \(2^h\)N\(\delta 1\) (\(\sim 10\) ppm) (Figures 2a and S9), signifying N\(\gamma\)−H···N\(\delta 1\) H-bond reconfiguration and a decrease in bond length.\(^{14,16,27,29,30}\) Measurement of \(2^h\)J\(_{NN}\) due to the V44-H47 (\(\sim 5.2\) Hz) and T77-H80 (\(\sim 3.4\) Hz) H-bonds provides insight into the repercussions of the T44V replacement relative to the TPLH helix caps in NRRC. In the T44-H47 and T77-H80 bifurcated interactions, the \(2^h\)J\(_{NN}\), \(1^h\)J\(_{NN}\) and \(2^h\)H/D isotope shift data support that the His ring orients its N\(\delta 1\) atom between Thr N\(\gamma\) and O\(\gamma\)\(\delta\), adopting a nonlinear geometry for both H-bonds and leading to relatively small T44-H47 and T77-H80 N\(\gamma\)−H···N\(\delta 1\) \(2^h\)J\(_{NN}\) couplings (\(\sim 4\) Hz). Upon T44V replacement, the observed \(\sim 25\%\) increase in \(2^h\)J\(_{NN}\) (Figure S4B,C) suggests a straightening (and concomitant shortening) of the Val N\(\gamma\)−H···N\(\delta 1\) His H-bond.

To gain insight into the nature of the N\(\gamma\)−H···N\(\delta 1\)−H···O\(\gamma\)−H-bond network, quantum chemical calculations (described in the Supporting Information) were performed using Gaussian 09\(^{31}\) on fragments of 2BKG mimicking an internal repeat. Energy minimization from multiple starting geometries (Figure S10) validated the use of the X-ray coordinates for the calculations. The computed HBCs (Figure S11, Table S2) are \(2^h\)J\(_{NN}\) = \(+3.0\) Hz, \(1^h\)J\(_{HH}\) = \(+2.0\) Hz (N\(\gamma\)−H\(\delta 1\)), and \(1^h\)J\(_{NH}\) = \(+2.2\) Hz (N\(\delta 1\)−H···O\(\gamma\)) all dominated by the Fermi contact (FC) contribution. These values are in reasonable agreement with the experimental numbers. Along with the geometry of the TXXH unit, the HBCs suggest that the His N\(\delta 1\) sp\(^3\) lone pair is the major contributor to both hydrogen bonds. H-bonding may also be augmented by interaction with the His N\(\pi\) system, as in a cation−π interaction.\(^{33}\) The larger \(2^h\)J\(_{NN}\) in T44V NRRC is consistent with a...
repositioning of the histidine ring that improves the orbital overlap and enhances the FC effect (Figure S12, Table S2).

Collectively, the data illuminate a relationship between the magnitude of $\Delta J_{NN}$ scalar couplings and H-bond sharing. The N-H...·N HBCs measured for bifurcated H-bonds in buried consensus ARs ($\sim$4 Hz), 22,25,36 an indication of weaker bonds and nonideal geometry in the former. Stability compensation is likely provided by the bifurcated arrangement. Importantly, our description of the H-bond network clarifies the role of the threonine hydroxyl group and contributes a comparative view of the TXXH motif within individual repeats. Further work will extend to longer AR proteins in order to explore the generality of the bifurcated N–H...N or H–O...H bond and determine the factors controlling its formation.

**ASSOCIATED CONTENT**

1. Supporting Information
Sample preparation; PDB survey results; NMR information; HSQC, LR HMQC, HNN-COSY NRRC spectra; HNN-COSY NRRC spectra; NRRC His Nε2H NOEs, $\Delta J_{NN}$ modulation curves; NRRC His pKα determination, NRRC LR HSQC spectra, $\Delta H$ isotope shifts (NRRC HSQC), NRRC and T44V denaturation curves; T44V NRRC HSQC, LR HMQC, HNN-COSY spectra; computational results. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**
*lecomte_tj@iu.edu*

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Financial support was provided by NSF MCB-0843439 to J.T.J.L., NIH R01-GM-068462 to D.B., T32-GM-008403 for M.R.P., and T32-GM-007231 for K.S. The authors thank George Rose and Christopher Falzone for helpful discussions.

**REFERENCES**

(1) Aksel, T.; Majumdar, A.; Barrick, D. Structure 2011, 19, 349.
(23) Pelton, J. G.; Torchia, D. A.; Meadow, N. D.; Roseman, S. Protein Sci. 1993, 2, 543.