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# Direct Observation of Dipolar Couplings and Hydrogen Bonds across a $\beta$-Hairpin in 8 M Urea

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The detailed, quantitative description of the unfolded state ensemble of proteins is a largely unsolved task. Structural probability distributions for such ensembles are very difficult to determine from the limited amount of existing experimental data. However, a detailed description should help to understand protein folding since a certain bias toward the native state must exist within the unfolded ensemble in order to resolve Levinthal's paradox.[1]

Indeed, elements of residual structure are sometimes detected by NMR under strongly denaturing conditions. Thus nuclear Overhauser enhancements[2] have revealed a native-like hydrophobic cluster in the urea-denatured form of the 434-repressor; relaxation and mutational studies[3] have proven long-range interactions between hydrophobic clusters in unfolded proteins, and chemical shifts or three-bond scalar couplings[4] report on certain conformational preferences on the background of a "random coil" structural distribution.

More recently, residual structure in unfolded states has been detected by residual dipolar couplings (RDCs) in weakly anisotropic solutions.[5] Since large numbers of RDCs are easily measured and since they have a simple geometrical dependence on the length and orientation of the internuclear distance vector,[6] they bear particular promise for a detailed, quantitative characterization of unfolded conformations. Indeed, fair quantitative agreement has been reported between measured $^1D_{NH}$ RDCs in unfolded proteins and predictions according to a structural ensemble that samples the Protein Data Bank coil conformations.[7] Such one-bond RDCs and the statistical coil models are, however, limited to local order and do not offer direct insights into longer-range interactions.

Here we show that long-range information for an unfolded, perdeuterated protein (urea-denatured ubiquitin) can be detected with very high sensitivity from $H^N-H^N$ RDCs. Besides numerous sequential contacts, the RDCs give evidence for the persistence of native-like structure in ubiquitin's first $\beta$-hairpin. This native-like structure is corroborated by chemical shifts, $^3J_{HNHA}$ couplings, relaxation rates, and the direct detection of hydrogen bonds (H-bonds) by $^{h3}J_{NC'}$ couplings.

The long-range $H^N-H^N$ RDCs were obtained by a quantitative $^{15}$N-edited $H^NH^N$ COSY experiment[8] on perdeuterated/amide protonated $^{15}$N-labeled ubiquitin aligned in strained polyacrylamide gel (Figure 1). In total, 66 $H^N_i-H^N_{i+1}$, 53 $H^N_i-H^N_{i+2}$, and 17 $H^N_i-H^N_{i+3}$ RDCs could be detected unambiguously and quantified (Supporting Information). In addition to these sequential RDCs, the experiment also revealed RDCs across the first $\beta$-turn of the native state (T7-G10 and T7-K11, Figures 1 and 3). An additional RDC, V5-I13, which connects the adjacent amino acid pair of the $\beta$-hairpin, may be present but could not be assigned unambiguously due to overlap. ROESY data confirmed the spatial proximity of these natively H-bonded residues (Supporting Information).

The presence of a subpopulation of native-like structure for the $\beta$-hairpin in the urea-denatured form was corroborated by an

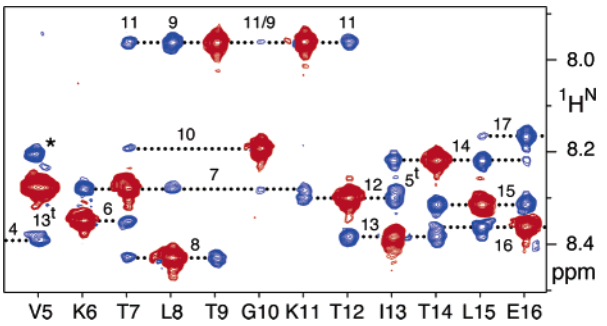


***Figure 1.*** $^1$H/$^{15}$N strips from a 3D quantitative, $^{15}$N-edited $H^NH^N$ COSY for the detection of $H^N-H^N$ RDCs in urea-denatured ubiquitin. Data are extracted for the amide resonances of residues V5 to E16 in the N-terminal hairpin. Cross-peaks are marked with assignment information. In case of ambiguity, information from mirror cross-peaks was used to make assignments unique. Tentative assignments of RDCs between residues V5 and I13 are marked as $13^t$ and $5^t$. An overlapping resonance from the amide of V70 is marked by an asterisk. Sample conditions: 2 mM $^2$H/$^{15}$N ubiquitin aligned in strained polyacrylamide gel [10% w/v, max($|^1D_{NH}|$) = 15 Hz], 8 M urea, pH 2.5, 5% $D_2O$/95% $H_2O$, 25 °C. Data were collected on a Bruker DMX600 MHz spectrometer equipped with a TXI probe. Total experimental time: 48 h.

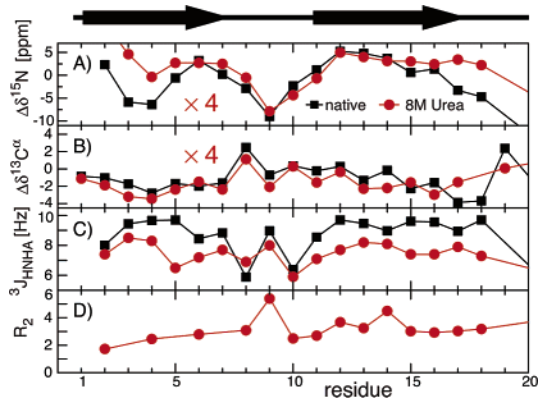


***Figure 2.*** Evidence for native-like backbone conformations of ubiquitin's first $\beta$-hairpin in 8 M urea at pH 2.5 from isotropic NMR parameters. Data for native ubiquitin (water, pH 4.6) are shown in comparison. (A,B) $^{15}$N and $^{13}C^\alpha$ secondary chemical shifts (see Supporting Information for details). The secondary chemical shifts are scaled by a factor 4 for the denatured state. (C) Scalar $^3J_{HNHA}$ couplings. (D) Transverse $^{15}$N relaxation rates.

analysis of $^{13}C^\alpha$ and $^{15}$N secondary chemical shifts, which are strongly dependent on backbone torsion angles (Figure 2). A scaling by a factor of 4 of the secondary shifts in 8 M urea leads to almost exact coincidence with the secondary shifts of the native form in the $\beta$-hairpin region. In the simplest approximation, this suggests that the native hairpin backbone conformation is populated to about 25% in 8 M urea. Likewise, $^3J_{HNHA}$ couplings and $^{15}$N relaxation data[9] point to native-like local structure and slowing of dynamics


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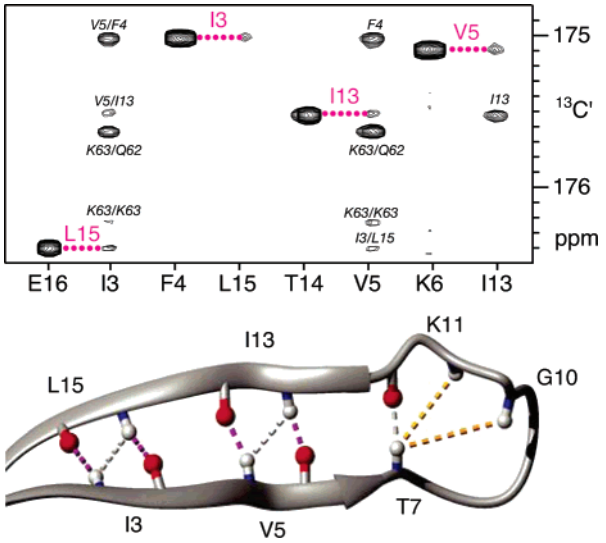


***Figure 3.*** Top: $^{1}$H/$^{15}$N strips from a 3D long-range HNCO−TROSY experiment[13] showing $^{h3}J_{NC'}$ correlations across H-bonds in urea-denatured ubiquitin. Overlapping resonances are marked in italics. Sample conditions: 2 mM $^{2}$H/$^{13}$C/$^{15}$N ubiquitin, 8 M urea pH 2.5, 5% $D_2O$/95% $H_2O$, 25 °C. Data were collected for a total of 72 h on a Bruker DMX800 MHz spectrometer equipped with a TCI cryoprobe. Bottom: Summary of long-range interactions in the first $\beta$-hairpin of urea-denatured ubiquitin. Detected interactions are shown as dashed cylinders for $^{h3}J_{NC'}$ (magenta) and $D_{HNHN}$ (gold). Ambiguous correlations due to spectral overlap are shown in white.

***Table 1.*** Ubiquitin $^{h3}J_{NC'}$ Couplings [Hz] under Various Conditions

| donor N | I3 | L15 | V5 | I13 |
|---|---|---|---|---|
| acceptor C′ | L15 | I3 | I13 | V5 |
| $^{h3}J_{NC'}$ 8 M urea | −0.05 | −0.06 | −0.05 | −0.06 |
| $^{h3}J_{NC'}$ native[13] | −0.45 | −0.62 | overlap | −0.73 |
| $^{h3}J_{NC'}$ A-state[14] | −0.44 | −0.49 | −0.48 | −0.55 |

to the microsecond time scale near the $\beta$-bulge of the N-terminal hairpin (Figure 2).

There is some debate about the relative importance of hydrophobic side chain interactions, amino acid conformational preferences, and H-bonding for the stabilization of model $\beta$-sheet structures. A recent combined NMR and IR study on an isolated $\beta$-hairpin showed that the IR signature of H-bonding was absent, while $^{1}H^{\alpha}$ chemical shifts were consistent with a $\beta$-hairpin structure.[10] The presence and identity of hydrogen-bonded pairs can be monitored directly by H-bond scalar couplings ($^{h3}J_{NC'}$).[11,12] Since the native-state $^{h3}J_{NC'}$ couplings for ubiquitin's first $\beta$-hairpin are close to −0.6 Hz,[13] even an only 25% population of formed H-bonds should be detectable in a long-range HNCO experiment. Indeed, cross signals were obtained between four H-bond donors and acceptors in the N-terminal hairpin (Figure 3, Table 1).

The size of the $^{h3}J_{NC'}$ couplings was quantified from the signal intensities of the long-range HNCO experiment as described.[11] For the four H-bonds in amino acid pairs I3-L15 and V5-I13, the $^{h3}J_{NC'}$ values range between −0.05 and −0.06 Hz with a statistical error of about 0.02 Hz (Table 1). Thus the $^{h3}J_{NC'}$ values are significantly smaller than expected for a 25% population of closed native-state H-bonds. This additional reduction is not caused by the loss of contacts to other secondary structure elements since the $^{h3}J_{NC'}$ values in ubiquitin's A-state, where the $\beta$-hairpin is isolated, are only slightly reduced (about −0.5 Hz) relative to the native state (Table 1). Taken together, this suggests that despite the 25% native backbone torsion angles indicated by the $^{15}$N and $^{13}C^{\alpha}$ chemical shifts, the H-bonds of the hairpin are formed to a lesser degree in urea.

In conclusion, we have shown that a very large number of long-range $H^N$−$H^N$ RDCs can be detected in perdeuterated, denatured proteins. The RDCs reveal the persistence of native-like elements even under strongly denaturing conditions in ubiquitin. Although the RDC detection may emphasize to a certain extent more strongly aligning conformations, the native-like structure elements are independently confirmed by chemical shifts, $^{3}J_{HNHA}$, and $R_2$ values as well as $^{h3}J_{NC'}$-detected H-bonds. This quantitative information should help to improve the current models of the denatured state. Efforts in our groups are directed to this goal.

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**Supporting Information Available:** Details of the chemical shift assignments, full strip plots of $^{15}$N-edited $H^NH^N$ COSY and ROESY, and $D_{HNHN}$ and $^{1}D_{HN}$ values. This material is available free of charge via the Internet at http://pubs.acs.org.

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