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# Heteronuclear three-dimensional NMR spectroscopy of a partially denatured protein: The A-state of human ubiquitin

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## SUMMARY

Human ubiquitin is a 76-residue protein that serves as a protein degradation signal when conjugated to another protein. Ubiquitin has been shown to exist in at least three states: native (N-state), unfolded (U-state), and, when dissolved in 60% methanol:40% water at pH 2.0, partially folded (A-state). If the A-state represents an intermediate in the folding pathway of ubiquitin, comparison of the known structure of the N-state with that of the A-state may lead to an understanding of the folding pathway. Insights into the structural basis for ubiquitin's role in protein degradation may also be obtained. To this end we determined the secondary structure of the A-state using heteronuclear three-dimensional NMR spectroscopy of uniformly $^{15}$N-enriched ubiquitin. Sequence-specific $^{1}$H and $^{15}$N resonance assignments were made for more than 90% of the residues in the A-state. The assignments were made by concerted analysis of three-dimensional $^{1}$H-$^{15}$N NOESY-HMQC and TOCSY-HMQC data sets. Because of $^{1}$H chemical shift degeneracies, the increased resolution provided by the $^{15}$N dimension was critical. Analysis of short- and long-range NOEs indicated that only the first two strands of β-sheet, comprising residues 2–17, remain in the A-state, compared to five strands in the N-state. NOEs indicative of an α-helix, comprising residues 25–33, were also identified. These residues were also helical in the N-state. In the N-state, residues in this helix were in contact with residues from the first two strands of β-sheet. It is likely, therefore, that residues 1–33 comprise a folded domain in the A-state of ubiquitin. On the basis of $^{1}H^{\alpha}$ chemical shifts and weak short-range NOEs, residues 34–76 do not adopt a rigid secondary structure but favor a helical conformation. This observation may be related to the helix-inducing effects of the methanol present. The secondary structure presented here differs from and is more thorough than that determined previously by two-dimensional $^{1}$H methods [Harding et al. (1991) *Biochemistry*, **30**, 3120–3128].

## INTRODUCTION

Ubiquitin is a small, 76-residue protein that is involved in protein degradation. Conjugation of ubiquitin to other proteins in an ATP-dependent fashion serves as a signal for degradation of the

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protein (for a review see Hershko (1988)). The structure of the native protein, termed the N-state, has been determined by both X-ray crystallography (Vijay-Kumar et al., 1987) and by solution NMR spectroscopy (Di Stefano and Wand, 1987; Weber et al., 1987). All three structures reveal an extremely compact protein consisting of five strands of β-sheet, an α-helix, a $3_{10}$ helix, and several reverse turns.

In solution, the protein has been shown to exist in at least two other structural states: unfolded, termed the U-state, at 350 K in 8 M urea or at 298 K in 6 M guanidinium chloride, and partially folded, termed the A-state, at 298 K in 60% methanol:40% water (Harding et al., 1991). If the A-state represents an intermediate in the folding pathway of ubiquitin, comparison of the N-state and the A-state structures may lead to an understanding of the folding pathway, as well as to an understanding of the structural basis for its role in protein degradation.

To this end, the solution conformation of the A-state has been investigated by two-dimensional NMR methods (Harding et al., 1991). In this investigation, it was found that the A-state contained residual secondary structure consisting of the first three strands of the β-sheet and one α-helix. However, because of the tremendous degeneracy of the chemical shifts, complete resonance assignments were not possible on the basis of the two-dimensional $^1H$–$^1H$ spectra alone, and some of the structural information obtained was tenuous. We were able to make nearly complete $^1H$ and $^{15}N$ resonance assignments for the backbone atoms in the A-state, using heteronuclear three-dimensional NMR techniques on uniformly $^{15}N$-enriched ubiquitin. The secondary structure revealed by characteristic NOE patterns differs from and is more thorough than that determined with two-dimensional methods alone.

## MATERIALS AND METHODS

### *Protein enrichment and sample preparation*

Uniformly $^{15}N$-enriched human ubiquitin was purchased from VLI Research (Wayne, PA) and was used without further purification. The N-state sample used for the NMR experiments contained 2 mM ubiquitin in 25 mM [$^2H_4$]acetic acid at pH 4.7. Partially denatured A-state ubiquitin was prepared by lyophilizing the N-state sample, dissolving it in 60% [$^2H_4$]methanol:40% $^1H_2O$, and adjusting the pH to 2.0.

### *NMR spectroscopy*

All NMR spectra were recorded at 300 K on a Bruker AMX-600 spectrometer equipped with a multi-channel interface. Proton chemical shifts were referenced to the $^1H_2O$ signal at 4.76 ppm. Nitrogen chemical shifts were referenced to external $^{15}NH_4Cl$ (2.9 M) in 1 M HCl at 24.93 ppm relative to liquid ammonia. Data were processed on a Silicon Graphics Personal Iris 4D35 workstation, using the software package FELIX from Hare Research, Inc.

Two-dimensional $^1H$–$^{15}N$ HSQC spectra (Bodenhausen and Ruben, 1980) were acquired for both A-state and N-state ubiquitin by using $^1H$ and $^{15}N$ sweep widths of 9 090 Hz and 1 562 Hz, respectively. For each of 256 $t_1$ values, 96 scans were recorded. Quadrature in $t_1$ was accomplished with the method of States et al. (1982). Continuous wave low-power saturation was used during the 700-ms relaxation delay to attenuate the intensity of the $^1H_2O$ resonance. GARP decoupling (Shaka et al., 1985) was used during acquisition to decouple $^{15}N$.

Three-dimensional $^1H$–$^{15}N$ NOESY-HMQC and TOCSY-HMQC spectra of A-state ubiquitin

were recorded with standard pulse sequences (Marion et al., 1989; Zuiderweg and Fesik, 1989). The $\omega_1$, $\omega_2$ and $\omega_3$ sweep widths used were 7353, 1111, and 9090 Hz, respectively. The 18 ppm $^{15}N$ sweep width used was minimized to achieve the highest spectral resolution possible in this dimension. Each three-dimensional experiment was acquired as a series of two-dimensional $^1H$–$^1H$ NOESY data sets with incremented $^{15}N$ evolution periods. Sixteen scans were recorded for each of 256 $t_1$ values and 32 $t_2$ values. The total recording time for each experiment was 48 h. Quadrature in $t_1$ and $t_2$ was accomplished by using TPPI (Marion and Wüthrich, 1983). $^{15}N$ was decoupled during $t_1$ by a 180° pulse in the center of the evolution period, and during $t_3$ by using a GARP sequence (Shaka et al., 1985). Continuous wave low-power saturation was used during the 1.0-s relaxation delay to attenuate the intensity of the $^1H_2O$ resonance. The SCUBA method (Brown et al., 1988) was used to recover intensity of saturated $^1H^\alpha$ resonances resulting from irradiation of the $^1H_2O$ resonance. A mixing time of 150 ms was used for the NOESY experiment. A DIPSI-2 (Shaka et al., 1988) spin-lock time of 32 ms was used for the TOCSY experiment.

## RESULTS

Two-dimensional $^1H$ NMR was sufficient to characterize the N-state of human ubiquitin (Di Stefano and Wand, 1987; Weber et al., 1987), but, because of resonance overlap, it was not sufficient for a thorough study of the A-state (Harding et al., 1991). The tremendous difference in resonance dispersion between the two structures is shown in Fig. 1, which compares the $^1H$–$^{15}N$ HSQC spectra obtained for the N- and A-states of ubiquitin. The resonance dispersion in the spectrum for N-state ubiquitin is typical for a folded protein. In contrast, the dramatically collapsed appearance of the spectrum of A-state ubiquitin indicates that much of the structure present in the N-state is absent in the A-state, causing the chemical shifts to migrate to their random coil values. As discussed below, $^1H$–$^{15}N$ correlations that remain dispersed in the A-state arise from residues that remain structured. It is clear that without the $^{15}N$ dimension, most of the $^1H^N$ resonances of the A-state would be hopelessly overlapped. Even though the resolution afforded by the $^{15}N$ dimension in the A-state is much less than in the N-state, it was sufficient to allow assignment of the resonances.

Sequential resonance assignments for A-state ubiquitin were accomplished by concerted analysis of two data sets: $^1H$–$^{15}N$ NOESY-HMQC and TOCSY-HMQC (Marion et al., 1989). The spectra were analyzed in a three-step process. First, symmetry related pairs of $^1H^N$–$^1H^N$ correlations in the $^1H$–$^{15}N$ NOESY-HMQC spectrum were identified. This type of NOE is useful for identifying sequential residues. For the A-state, a total of 24 such NOEs were identified. Second, $^1H^N$ resonances were classified into a particular type of spin system by using the $^1H$–$^{15}N$ TOCSY-HMQC data set. In most cases, magnetization transfer extended from the $^1H^N$ resonance to the $^1H^\alpha$ and $^1H^\beta$ resonances. Many of the side chains were easily identified owing to the nearness to random coil values of their $^1H$ chemical shifts. Longer side chains, which look quite similar by this criteria, were difficult to distinguish and were usually only assignable in the third step. The final step involved aligning the residues by using standard techniques based on the uniqueness of the primary sequence (Wüthrich, 1986). As illustrative examples, assignment of three stretches of residues will be discussed, corresponding to regions of β-sheet, α-helix, and no regular structure.

Residues 8–16 comprise one of two strands of β-sheet in the N-state that is also present in the A-state. The assignments for this stretch of residues in the A-state are shown in Fig. 2. Each panel

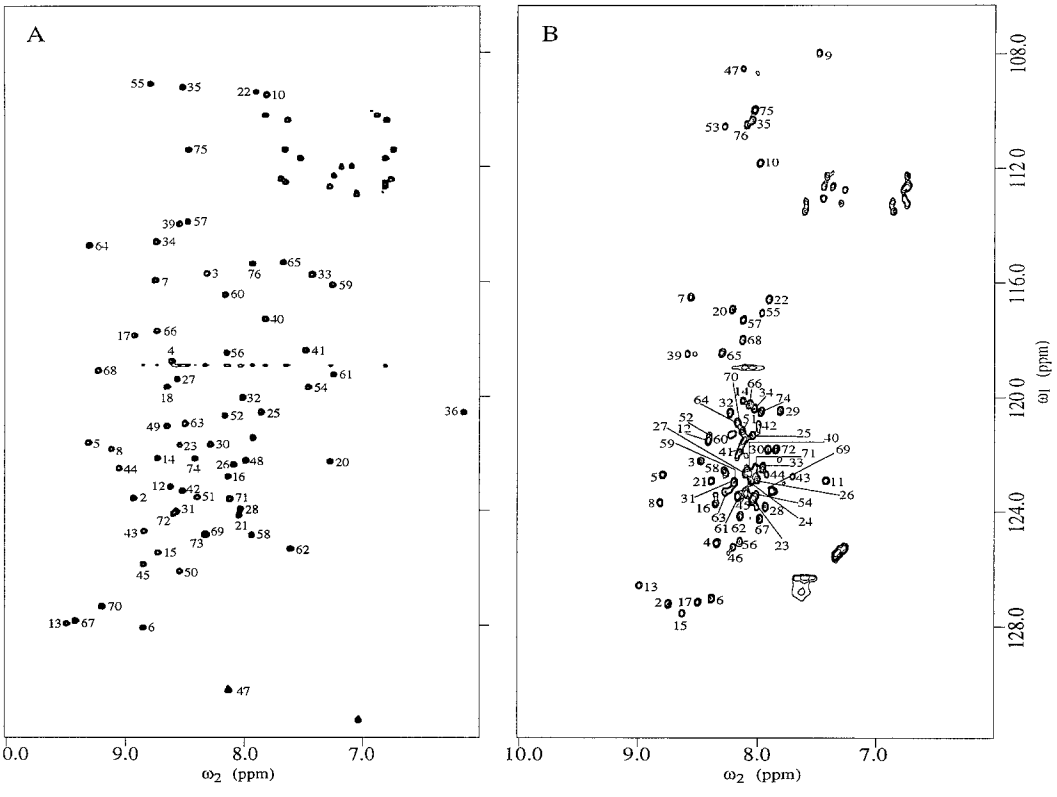


Fig. 1. Region of the 14.2 T $^{1}H$–$^{15}N$ HSQC spectrum of uniformly $^{15}N$-enriched ubiquitin in the N-state (A) and A-state (B). Assigned correlations are labeled according to residue sequence number. Assignments for the N-state were based on those of Di Stefano and Wand (1987) and Schneider et al. (1992). Unlabeled resonances with $^{1}H$ chemical shifts of 7.0 to 8.0 ppm and $^{15}N$ chemical shifts near 113.0 ppm arise from asparagine and glutamine side-chain δ and ε NH groups, respectively. The main-chain correlation for residue 47 in (A) is folded in the $^{15}N$ dimension.

represents a small $\omega_1$, $\omega_3$ slice of the $^{1}H$–$^{15}N$ NOESY-HMQC spectrum corresponding to the $^{1}H^{N}$ group of the residue indicated. Intraresidue NOEs are boxed. Arrows drawn indicate sequential dNα(i,i−1) and dNβ(i,i−1) NOEs used to make the assignments. Horizontal lines identify sequential dNN NOEs. Sequential dNα(i,i−1) correlations were observed for all residues in this stretch. The downfield $^{1}H^{\alpha}$, $^{1}H^{N}$, and $^{15}N$ chemical shifts, as well as the intense sequential dNα(i,i−1) correlations, are typical for residues in β-sheet conformations (Wüthrich, 1986). From the $^{1}H$–$^{15}N$ TOCSY-HMQC spectrum, nearly complete side-chain assignments were made for all residues except for the longer side chains. Sequential $^{1}H^{N}$–$^{1}H^{N}$ correlations observed for residues 8–11 locate a type I turn at this position, similar to that observed in the N-state. The following cross-strand NOEs to main-chain $^{1}H$s of residues 1–7, which serve to orient the two β-sheet strands, are labeled in Fig. 2: K11 $^{1}H^{N}$ to T7 $^{1}H^{N}$, I13 $^{1}H^{N}$ to V5 $^{1}H^{N}$, L15 $^{1}H^{N}$ to I3 $^{1}H^{N}$, and L15 $^{1}H^{N}$ to F4 $^{1}H^{\alpha}$. The orientation of the two strands defined here is identical to that obtained from two-dimensional data (Harding et al., 1991) and suggests a hydrogen-bonding network which is consistent with that defined by the slowly exchanging $^{1}H^{N}$ resonances (Harding et al., 1991). The increased resonance dispersion of the three-dimensional data allowed us to extend what was

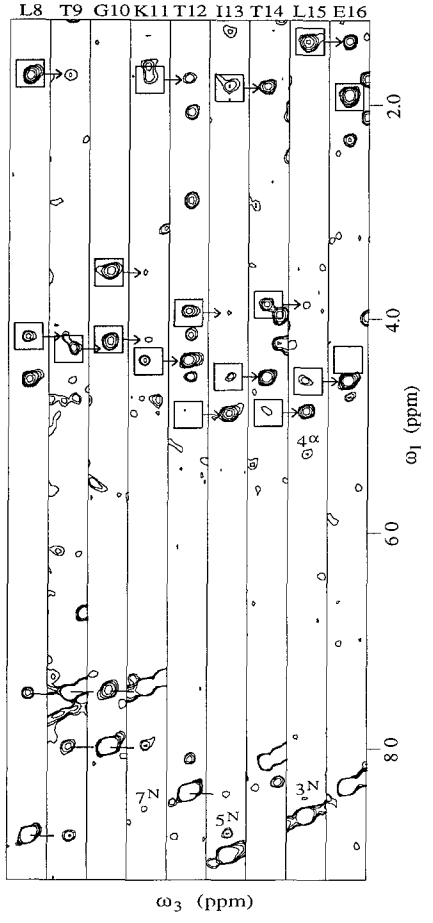


Fig. 2. Selected $\omega_1$, $\omega_3$ slices corresponding to residues 8–16 taken from the 14.2 T three-dimensional $^1H$–$^{15}N$ NOESY-HMQC spectrum of ubiquitin in the A-state. Slices were taken at the $^{15}N$ frequency (listed in Table 1) corresponding to the residue indicated at the top of each panel. Each slice represents 0.19 ppm in $\omega_3$, with the center located at the frequency of the indicated $^1H^N$ resonance (listed in Table 1). Intraresidue dNα and dNβ correlations are boxed. Sequential dN α(i,i−1) and dNβ(i.i−1) correlations are indicated by arrows beginning at the boxed intraresidue correlation in the preceding slice. Horizontal lines identify sequential dNN NOEs. Cross-strand NOEs are labeled according to residue number and atom type. In the T12 and E16 slices, the weak intraresidue dNα correlation was not seen at this level; however, a strong correlation was seen at the corresponding position in the $^1H$–$^{15}N$ TOCSY-HMQC spectrum.

defined previously to include the turn conformation of residues 8–11, and thus to improve the description of the conformation of residues 1–16 in the A-state. Long-range NOEs observed between these two strands of β-sheet were the only ones identified in the A-state.

Residues 25–33 were found to comprise an α-helix in the A-state, similar to what is observed in the N-state. As shown in Fig. 3, unambiguous sequential dNα(i,i−1) and dNβ(i,i−1) correlations were observed for all residues, except for the degenerate $^1H^\beta$ resonances of K29 and I30, in the $^1H$–$^{15}N$ NOESY-HMQC data set. Many of the side-chain resonances of residues in this stretch were assigned from the $^1H$–$^{15}N$ TOCSY-HMQC data set. The α-helical conformation of these residues is indicated by characteristic dNN and dNα(i,i−3) NOEs (Wüthrich, 1986).

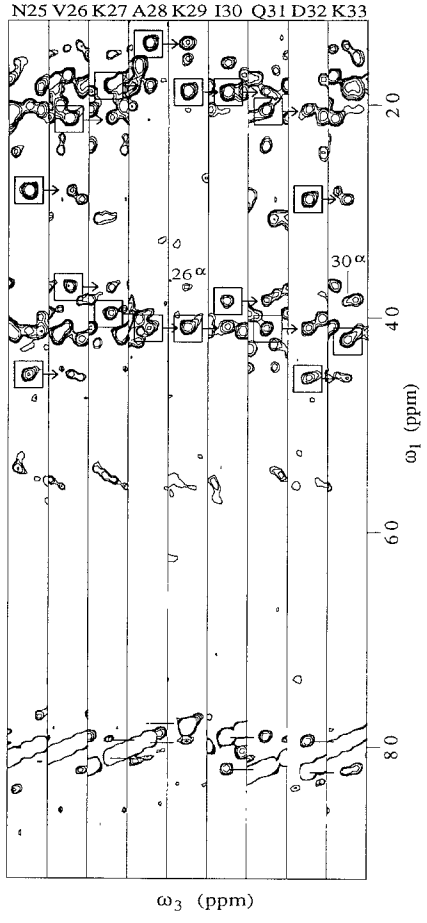


Fig. 3. Selected $\omega_1$, $\omega_3$ slices corresponding to residues 25–33 taken from the 14.2 T three-dimensional $^1H$–$^{15}N$ NOESY-HMQC spectrum of ubiquitin in the A-state. Slices are labeled as described in the legend to Fig. 2. dNα(i,i−3) NOEs are labeled according to residue number and atom type.

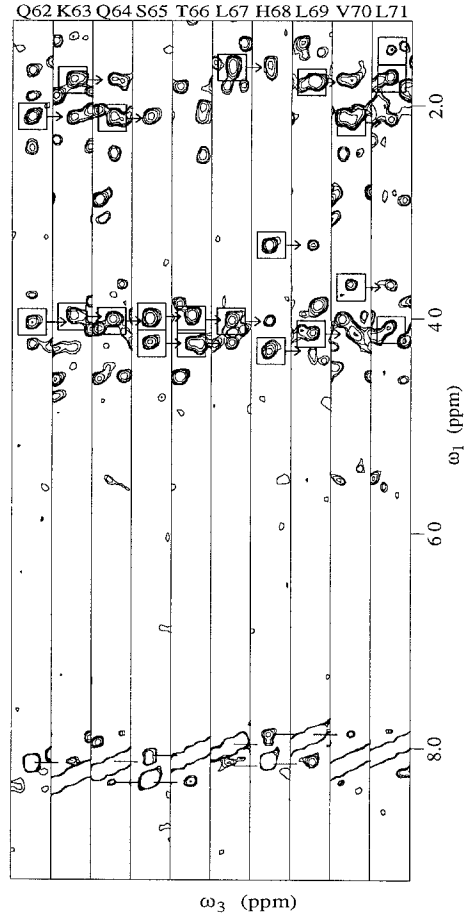


Fig. 4. Selected $\omega_1$, $\omega_3$ slices corresponding to residues 62–71 taken from the 14.2 T three-dimensional $^1H$–$^{15}N$ NOESY-HMQC spectrum of ubiquitin in the A-state. Slices are labeled as described in the legend to Fig. 2.

Sequential dNN NOEs were observed for residues 27–31 and 32–33, but $^1H^N$ degeneracies prohibited identification of dNN NOEs for residues 25–27 and 31–32. Short-range dNα(i,i−3) NOEs involving the $^1H^N$ resonances of A28, A29, and K33 were unambiguously identified. $^1H^\alpha$ degeneracies, however, prohibited unequivocal identification of dNα(i,i−3) NOEs involving the $^1H^N$ resonances of Q31 and D32. No other clusters of dNN and dNα(i,i−3) NOEs were observed for A-state ubiquitin.

An example of assignments for a stretch of residues that does not adopt a regular secondary structure is illustrated for residues 62–71 in Fig. 4. Unambiguous dNα(i,i−1) correlations were identified for each pair of sequential residues in the $^1H$–$^{15}N$ NOESY-HMQC data set. Several residues also had additional sequential dNβ(i,i−1) and dNN correlations. The observation of

sequential dNN NOEs but few other short-range NOEs indicates that this stretch of residues may prefer a helical conformation but is not a rigid α-helix in the A-state.

In total, main-chain assignments were made for all but six non-prolyl residues. Side-chain assignments, in some cases complete, have been made for many of these residues. Previously, 17 of the 76 residues of A-state ubiquitin were assigned (Harding et al., 1991). Our assignments for residues 2–7 and 12–17 agree quite well with those determined from two-dimensional $^1$H data (Harding et al., 1991). Our data do not agree with the assignments for residues 66, 68 and 70 reported by Harding et al. (1991). A summary of the sequential and medium-range NOE correlations identified is shown in Fig. 5. A list of all assigned resonances is presented in Table 1.

## DISCUSSION

The heteronuclear three-dimensional data presented here define three elements of secondary structure in the ubiquitin A-state: two strands of β-sheet and one nine-residue α-helix. The two strands of β-sheet comprise residues 1–7 and 12–17, and are connected by a well-defined reverse turn, identical to the structure observed in the N-state. The α-helix spans residues 25–33. As shown in Fig. 6, the secondary structure present in the A-state is a subset of that present in the N-state. Although the α-helix associates with the two β-sheet strands in the N-state, as shown in Fig. 6A, no definitive NOEs have been assigned that indicate the presence of this association in the A-state. However, it seems likely that these elements form a single, folded domain in the A-state, as shown in Fig. 6B. Further characterization of NOEs between side-chain protons may provide concrete evidence for this hypothesis.

That the β-sheet and α-helix are associated in the A-state is supported by hydrogen exchange characteristics (Pan and Briggs, 1992). Based on hydrogen exchange data, Pan and Briggs (1992) concluded that the A-state of ubiquitin is a molten globule-like structure with native-like but highly dynamic secondary and tertiary structure. However, our results indicate that residues 1–33 adopt a more stable conformation than the remainder of the protein.

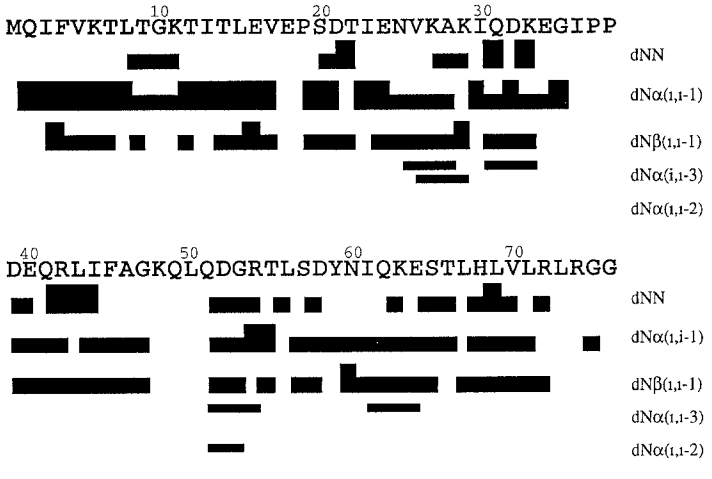


Fig. 5. Summary of sequential resonance assignments of human ubiquitin in the A-state. A bar between two residues indicates that a dNN, dNα(i,i−1), dNβ(i,i−1), dNα(i,i−2), or dNα(i,i−3) NOE was observed between the two residues. Wide bars indicate a strong intensity NOE, while narrow bars indicate a weak or medium intensity NOE.

TABLE 1
CHEMICAL SHIFTS OF ASSIGNED $^{1}H$ AND $^{15}N$ RESONANCES OF A-STATE UBIQUITIN[a]

| Residue | $N^{\alpha}$ | $H^{N}$ | $H^{\alpha}$ | $H^{\beta}$ | Other |
|---|---|---|---|---|---|
| Met[1] | | | 4.09 | | |
| Gln[2] | 127.2 | 8.75 | 4.89 | 1.80 | |
| Ile[3] | 122.2 | 8.48 | 4.35 | 1.72 | $H^{\gamma 1}$ 0.95; $H^{\gamma 2}$ 0.75 |
| Phe[4] | 125.1 | 8.35 | 5.29 | 2.83 | $H^{\delta}$ 7.13 |
| Val[5] | 122.7 | 8.79 | 4.36 | 1.96 | $H^{\gamma}$ 0.77, 1.07 |
| Lys[6] | 127.0 | 8.39 | 5.02 | 1.64, 1.73 | |
| Thr[7] | 116.5 | 8.57 | 4.56 | | $H^{\gamma 2}$ 1.18 |
| Leu[8] | 123.7 | 8.82 | 4.16 | 1.74 | |
| Thr[9] | 108.0 | 7.48 | 4.28 | | $H^{\gamma 2}$ 1.19 |
| Gly[10] | 111.8 | 7.98 | 3.56, 4.21 | | |
| Lys[11] | 122.9 | 7.44 | 4.39 | 1.65, 1.74 | |
| Thr[12] | 121.5 | 8.42 | 4.90 | 3.93 | |
| Ile[13] | 126.6 | 8.99 | 4.54 | 1.82 | $H^{\gamma 2}$ 0.80 |
| Thr[14] | 120.1 | 8.13 | 4.86 | 3.87 | |
| Leu[15] | 127.5 | 8.64 | 4.57 | 1.41 | $H^{\delta}$ 0.92, 0.76 |
| Glu[16] | 123.7 | 8.36 | 4.30 | 1.91 | |
| Val[17] | 127.2 | 8.51 | | 1.83 | $H^{\gamma}$ 0.78 |
| Glu[18] | | | | | |
| Pro[19] | | | 4.38 | 1.97, 2.26 | |
| Ser[20] | 116.9 | 8.22 | 4.32 | 3.85 | |
| Asp[21] | 122.9 | 8.39 | | 2.89 | |
| Thr[22] | 116.6 | 7.91 | 4.21 | | $H^{\gamma 2}$ 1.19 |
| Ile[23] | 123.6 | 8.05 | 3.92 | 1.88 | $H^{\gamma 2}$ 0.88 |
| Glu[24] | 123.0 | 8.07 | 4.16 | 2.02 | |
| Asn[25] | 121.3 | 8.05 | 4.54 | 2.82 | |
| Val[26] | 122.9 | 8.02 | 3.71 | 2.14 | |
| Lys[27] | 122.7 | 8.11 | 3.96 | 1.81 | |
| Ala[28] | 123.8 | 7.94 | 4.10 | 1.42 | |
| Lys[29] | 120.5 | 7.81 | 4.09 | 1.88 | |
| Ile[30] | 121.8 | 7.92 | 3.85 | 1.88 | $H^{\gamma 1}$ 0.84; $H^{\gamma 2}$ 1.13 |
| Gln[31] | 123.0 | 8.20 | 4.10 | 2.05 | |
| Asp[32] | 120.5 | 8.24 | 4.57 | 2.89 | |
| Lys[33] | 122.3 | 7.96 | 4.21 | 1.84 | |
| Glu[34] | 120.4 | 8.03 | 4.28 | 2.03, 2.11 | |
| Gly[35] | 110.3 | 8.05 | 3.88 | | |
| Ile[36] | | | | | |
| Pro[37] | | | | | |
| Pro[38] | | | 4.29 | | |
| Asp[39] | 118.5 | 8.59 | 4.49 | 2.90 | |
| Glu[40] | 122.5 | 8.07 | 4.16 | 2.01 | |
| Gln[41] | 121.8 | 8.15 | 3.97 | 2.05 | |
| Arg[42] | 121.0 | 8.00 | 4.14 | 1.84 | |
| Leu[43] | 122.8 | 7.71 | 4.15 | 1.54, 1.78 | |
| Ile[44] | 122.7 | 7.93 | 3.83 | 1.80 | $H^{\gamma 2}$ 0.75 |
| Phe[45] | 123 4 | 8.10 | 4.37 | 3.06, 3.16 | |
| Ala[46] | 125.2 | 8.21 | 4.16 | 1.45 | |
| Gly[47] | 108.6 | 8.12 | 3.80, 3.88 | | |
| Lys[48] | | | | | |

TABLE 1 (continued)

| Residue | $N^{\alpha}$ | $H^{N}$ | $H^{\alpha}$ | $H^{\beta}$ | Other |
|---|---|---|---|---|---|
| $Gln^{49}$ | | | | | |
| $Leu^{50}$ | | | | | |
| $Gln^{51}$ | 121.2 | 8.12 | 4.17 | 2.07 | |
| $Asp^{52}$ | 121.4 | 8.41 | 4.54 | 2.90 | |
| $Gly^{53}$ | 110.6 | 8.29 | 3.86, 3.95 | | |
| $Arg^{54}$ | 123.5 | 8.05 | 4.22 | 1.87 | |
| $Thr^{55}$ | 117.1 | 7.97 | 4.10 | | $H^{\gamma 2}$ 1.19 |
| $Leu^{56}$ | 125.0 | 8.15 | 4.23 | 1.67 | |
| $Ser^{57}$ | 117.3 | 8.13 | 4.28 | 3.90 | |
| $Asp^{58}$ | 122.6 | 8.29 | 4.49 | 2.84, 2.96 | |
| $Tyr^{59}$ | 123.0 | 8.17 | 4.25 | 3.08 | |
| $Asn^{60}$ | 121.3 | 8.23 | 4.45 | 2.89 | |
| $Ile^{61}$ | 123.5 | 8.16 | 3.78 | 1.93 | |
| $Gln^{62}$ | 124.2 | 8.15 | 4.04 | 2.11 | |
| $Lys^{63}$ | 123.3 | 8.28 | 3.97 | 1.76 | |
| $Glu^{64}$ | 120.9 | 8.18 | 4.00 | 2.11 | |
| $Ser^{65}$ | 118.4 | 8.31 | 4.23 | 3.98 | |
| $Thr^{66}$ | 120.2 | 8.08 | 3.97 | 4.23 | $H^{\gamma 2}$ 1.20 |
| $Leu^{67}$ | 124.3 | 7.99 | 4.02 | 1.64 | |
| $His^{68}$ | 118.0 | 8.13 | 4.32 | 3.32 | |
| $Leu^{69}$ | 123.2 | 7.90 | 4.14 | 1.78 | |
| $Val^{70}$ | 120.9 | 8.14 | 3.69 | 2.15 | $H^{\gamma}$ 1.00 |
| $Leu^{71}$ | 122.5 | 8.03 | 4.09 | 1.48, 1.74 | |
| $Arg^{72}$ | 121.8 | 7.85 | 4.13 | 1.88 | |
| $Leu^{73}$ | | | | | |
| $Arg^{74}$ | 120.5 | 7.97 | 4.26 | 1.78, 1.93 | |
| $Gly^{75}$ | 110.0 | 8.05 | 3.89, 4.00 | | |
| $Gly^{76}$ | 110.5 | 8.09 | 3.92 | | |

[a] Proton chemical shifts are ± 0.02 ppm. Nitrogen chemical shifts are ± 0.1 ppm.

In contrast to the β-sheet structure defined by two-dimensional NMR, the three-dimensional data do not support the presence of a third β-strand adjacent to residues 1–7. The presence of this third strand, residues 65–70, was suggested by observation of four slowly exchanging backbone amide protons and one cross-strand NOE (Harding et al., 1991). The chemical shifts and sequential NOEs defined here for these residues are not characteristic of β-sheet residues (Wüthrich, 1986). In addition, no cross-strand NOEs to residues 1–7 were identified. In contrast, medium and strong sequential dNN NOEs indicate that this stretch of residues prefers a helical conformation in the A-state. The incomplete nature of the resonance assignments made by Harding et al. (1991) led to an incorrect interpretation of the structure in this region, a problem not encountered when using three-dimensional methods.

Although at first glance it may seem trivial to distinguish regions of folded structure from regions of random structure on the basis of chemical shifts alone, this was not the case. Figure 7 shows a plot of the $^{1}H^{\alpha}$ $\Delta\delta$ values (observed minus random coil chemical shifts) for each assigned

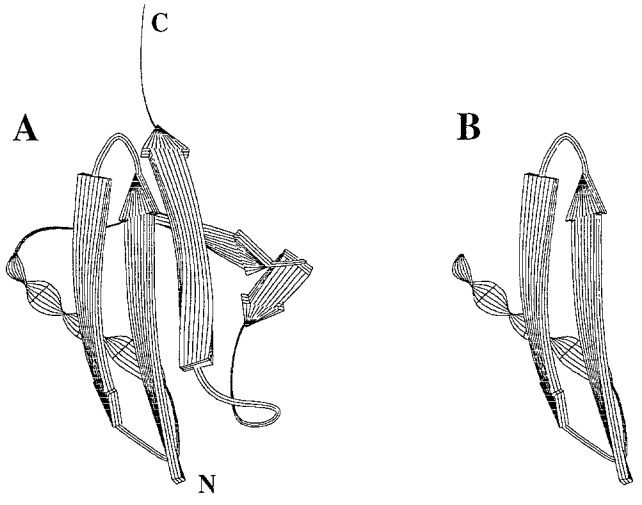


Fig. 6. Ribbon diagram of the secondary structure present in the N-state (A) and in the A-state (B) of human ubiquitin.

residue in the N- and A-states. Distinct positive and negative $\Delta\delta$ values (Wishart et al., 1991) were observed for β-sheet and α-helix residues, respectively, in the N-state. While the β-sheet residues (1–17) in the A-state also displayed an overall tendency for positive $\Delta\delta$ values, most of the remaining residues showed $\Delta\delta$ values in the range of −0.1 to −0.3. This was expected for residues 25–33, since these residues participate in an α-helix, as determined by characteristic sequential and short-range NOEs, but was not expected for residues 34–76.

The $\Delta\delta$ values for residues 34–76 were roughly 0.2 ppm more negative than those observed in the urea-unfolded form of the phage 434 repressor (Neri et al., 1992). One possible explanation for the larger negative $\Delta\delta$ values for residues 34–76 is the helix-inducing properties of the methanol present. Residues 34–76 may exist transiently in helical conformations. This is supported by observation of a number of dNN NOEs dispersed throughout the second part of the A-state. Lack of any short-range dN$\alpha$(i,i−3) NOEs indicates that no rigid α-helices were present. The difference between the $\Delta\delta$ values in the A-state compared to those of the phage 434 repressor may thus result from the difference in the denaturing conditions used.

It is unclear whether the ability of ubiquitin to exist in a partially folded state has any bearing on its physiological role in protein degradation. The attachment of ubiquitin to proteins targeted for destruction occurs through the COOH-terminal glycine residue. This reaction may mimic the formation of the A-state by destabilizing the latter half of the ubiquitin molecule while leaving the residue 1–33 domain intact, and thus may serve as a signal for protein degradation. The reversibility of the N-state to A-state transition would be consistent with the observation that ubiquitin is recycled after protein degradation. Given the resonance assignments presented here, both of these hypotheses are testable by conjugating $^{15}$N-enriched ubiquitin to a substrate protein and determining the resulting ubiquitin structure.

Finally, the results presented here demonstrate the utility of heteronuclear three-dimensional NMR spectroscopy to obtain structural information about incompletely folded proteins. This has been demonstrated previously by the complete assignment of the urea-denatured phage 434

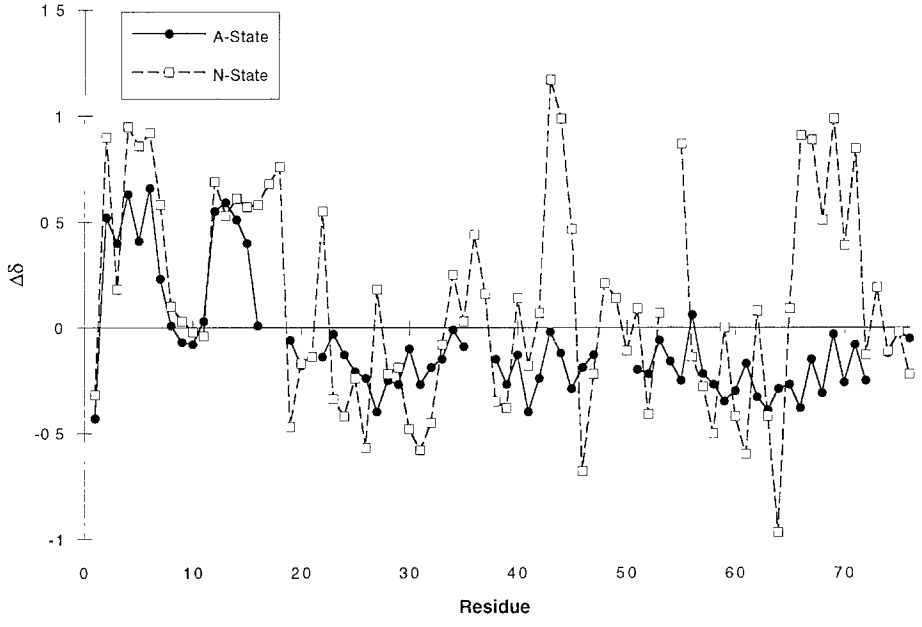


Fig. 7. Comparison of $^{1}H^{\alpha}$ $\Delta\delta$ values for N-state and A-state ubiquitin. Breaks in the plots are located at unassigned resonances. Chemical shifts for N-state ubiquitin were taken from Di Stefano and Wand (1987). Random coil chemical shifts were those of Wishart et al. (1992).

repressor (Neri et al., 1992). In denatured phage 434 repressor, lack of spectral resolution was overcome by delineating resonance assignments from chemical exchange with folded 434 repressor, for which assignments had already been obtained. In contrast, resonance degeneracy in the ubiquitin A-state was overcome by spreading the $^{1}H$–$^{1}H$ correlations into an $^{15}N$ chemical shift dimension and analyzing spectra of the partially denatured protein only. This technique should be widely applicable to other systems.

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