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# Close Identity between Alternatively Folded State $N_2$ of Ubiquitin and the Conformation of the Protein Bound to the Ubiquitin-Activating Enzyme

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Supporting Information

**ABSTRACT:** We present the nuclear Overhauser effect-based structure determination of the Q41N variant of ubiquitin at 2500 bar, where the alternatively folded $N_2$ state is 97% populated. This allows us to characterize the structure of the "pure" $N_2$ state of ubiquitin. The $N_2$ state shows a substantial change in the orientation of strand $\beta_5$ compared to that of the normal folded $N_1$ state, which matches the changes seen upon binding of ubiquitin to ubiquitin-activating enzyme E1. The recognition of E1 by ubiquitin is therefore best explained by conformational selection rather than induced-fit motion.

Ubiquitin (Ub) is a post-translational modifier consisting of 76 amino acid residues and plays crucial roles in many important cell functions, such as the ubiquitin-proteasomal degradation of damaged proteins.[1,2] By solution nuclear magnetic resonance (NMR) investigations, several dynamic ensembles of ubiquitin have been described, in particular DER,[3] EROS,[4] and ERNST.[5] Because the EROS ensemble covers the structural heterogeneity of the protein seen in complex with other proteins in crystals, molecular recognition of substrate proteins by ubiquitin was suggested to occur by conformational selection rather than induced-fit motion.[4] However, the ensemble does not cover the structure of the protein in the complex with ubiquitin-activating enzyme E1, as shown in Figure S1 (Supporting Information).

Our previous high-pressure NMR investigations revealed that ubiquitin exists in a conformational equilibrium among the natively folded $N_1$ state, a totally unfolded U state, and at least two high-energy states: an alternatively folded $N_2$ state and a locally disordered I state.[6−8] We also found that similar high-energy states are conserved in a group of post-translational modifiers having the E1−E2−E3 cascade reaction, specifically NEDD8.[9] These results allowed us to speculate that similar conformational fluctuations among different states in ubiquitin-like modifiers are important for the E1−E2−E3 cascade reaction. However, investigation of the structures of high-energy states of proteins at atomic resolution is difficult, thereby limiting our understanding of high-energy states. So far, we reported the solution structures as well as backbone dynamics of ubiquitin at 3 kbar where the $N_2$ state is 77% populated,[7] and its Q41N variant at 1 bar where the $N_2$ state is 70% populated.[8] The $N_2$ conformers produced by high pressure and by the Q41N mutation were generally similar in both structure and dynamics, but different in detail. The differences likely originate partly from the low detection sensitivity of the capillary pressure-resistant NMR cell and an insufficient population of the $N_2$ state in the sample for those NMR structure determinations.

Here, we present the structure determination of the Q41N variant of ubiquitin at 298 K and 2500 bar, where the $N_2$ state is 97% populated (Supporting Information). To obtain high-quality structural constraints, we use a ceramic pressure-resistant cell (Daedalus Innovations),[10] which has a sensitivity ∼10-fold higher than that of our previous quartz capillary NMR cell,[6−9] on an AVANCE3-800 spectrometer (BrukerBioSpin Co.). Details of the structural determination are given in the Supporting Information. Structural models for the "pure" $N_2$ state were calculated using 1245 distance and 72 torsion angle constraints. Structural constraints and geometrical statistics of the calculated 20 models are listed in Table S1 and Figure S2 (Supporting Information). The geometrical statistics show the quality of the structural models obtained by high-pressure NMR spectroscopy to be almost the same as that of conventional 1 bar structures.[8] The normal folded structure of $N_1$ [wild type (WT) at 1 bar] and the alternatively folded structure of $N_2$ (Q41N at 2500 bar) of ubiquitin are compared in Figure 1. Displacements of C$\alpha$ atoms in the $N_2$ state (Q41N

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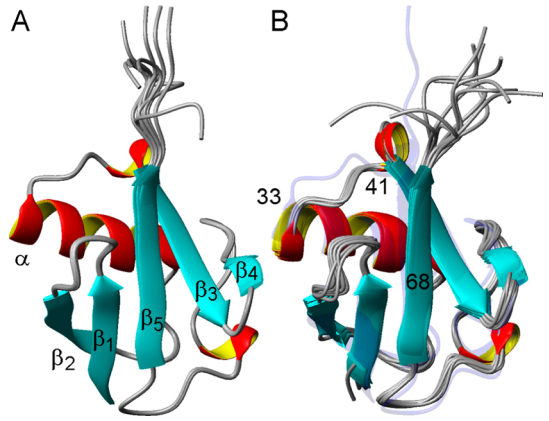


**Figure 1.** Solution structure of WT ubiquitin at 1 bar, which is a model for the basic folded conformation $N_1$ [Protein Data Bank (PDB) entry 1D3Z] (A), and the Q41N variant at 2500 bar, which is a model for the alternatively folded conformation $N_2$ (PDB entry 2RU6) (B). As a comparison, the transparent model of the WT is superposed on Q41N.

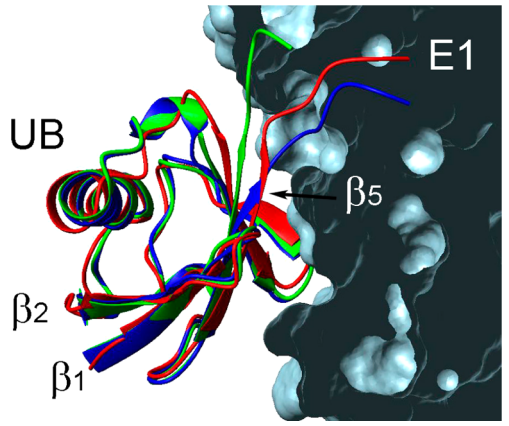


**Figure 2.** Superposition of WT ubiquitin at 1 bar (green, PDB entry 1D3Z) and Q41N at 2500 bar (red, PDB entry 2RU6) on the ubiquitin−E1 complex (blue, PDB entry 3CMM). E1 is presented as a molecular surface (gray).

at 2500 bar) from the corresponding positions in the $N_1$ state (WT at 1 bar) are shown in Figure S3A (Supporting Information). Panels B and C of Figure S3 show torsion angles, $\phi$ and $\psi$, respectively, in the $N_1$ and $N_2$ states. The resultant structure of the pure $N_2$ state reveals a large displacement of the C-terminal $\beta_5$ strand (after residue 68) with some displacements of the $\alpha$-helix and the following loop regions (residues 33−41). These structural changes arise from changes in backbone dihedral angles $\phi$ and $\psi$. For instance, significant changes in $\phi$ and $\psi$ are observed at residues 34−42 and 68−72 (~50°), while those in the other regions are rather small. A displacement of the C-terminal $\beta_5$ strand under high pressure can also explain a decrease in the $^{h3}J(H^N,C')$ hydrogen bond scalar coupling between the two magnetic nuclei, $^1H_N$ of R42 and $^{13}C'$ of V70, which was previously observed in WT ubiquitin under high pressure.[11]

To evaluate the structural change, we back-calculated the chemical shifts from the Q41N structure at 2500 bar by using SHIFTX2 version 1.07.[12] There are highly significant correlations between observed and back-calculated chemical shifts ($R$ values of 0.90 for amide H, 0.92 for amide N, 0.85 for C′, 0.99 for C$\alpha$, 0.92 for H$\alpha$, and 0.99 for C$\beta$), which validate the backbone orientations of the structure determined by high-pressure NMR.

Figure 2 shows a superposition of WT ubiquitin at 1 bar (green), Q41N at 2500 bar (red), and the WT in the complex with the E1 enzyme at 1 bar (blue). The C-terminal region of ubiquitin interacts with E1. It is striking that the conformational change observed at the C-terminal side of the protein matches the change seen on binding of ubiquitin to E1 but is not observed in complexes with other ubiquitin-interacting proteins such as ubiquitin-associated proteins (UBA) and proteins containing the ubiquitin-interacting motif (UIM).[13,14] Interestingly, a similar reorientation of strand $\beta_5$ was also observed in NEDD8, when the protein binds to the NEDD8-activating enzyme E1 (Figure S4 of the Supporting Information).[15] Because the $N_1$−$N_2$ conformational fluctuation is evolutionarily conserved between ubiquitin and NEDD8,[9] it is plausible that recognition of E1 by both ubiquitin and NEDD8 occurs by conformational selection rather than induced-fit motion.

Interestingly, a similar change in the orientation of the C-terminal $\beta_5$ strand (after residue 68) was also observed in ubiquitin variant L69S.[14] According to $^1H$ and $^{15}N$ chemical shift changes in the variant, the $N_2$ state is likely to be highly populated in the protein, at a level similar to that of the present Q41N variant (e.g., large upfield shifts of the $^{15}N$ chemical shift of Val70 and a large downfield shift of the $^1H$ chemical shift of I36). Interestingly, both the glutamine and the leucine residues, corresponding to Q41 and L69 in WT ubiquitin, respectively, are highly conserved in the subgroup of the ubiquitin family that includes both ubiquitin and NEDD8.[16] The $NH_2$ group of Q41 makes a hydrogen bond with the backbone CO group of I36, and L69 makes a hydrophobic interaction with other hydrophobic groups in the core of ubiquitin. These interactions are highly conserved in the subgroup. The fact that these conserved interactions seem to be important for keeping the energy balance between the $N_1$ and $N_2$ states is noteworthy.

Ubiquitin plays crucial roles in many cell functions and seems to use different interaction modes according to the specific protein target. For instance, the same hydrophobic surface of the protein consisting of L8, I44, and V70 is involved in interactions with many Ub-binding proteins, including UIMs and UBAs. In the complex with the E2 enzyme, two canonical recognition surfaces are identified in ubiquitin: one consisting of a $\beta_1$−$\beta_2$ turn and strand $\beta_3$ and the other consisting of the $\alpha$−$\beta_3$ loop.[17,18] It is plausible that each conformation facilitates an interaction with the particular proteins by providing a suitable binding surface for them. This implies that nature has evolved conformational fluctuations of the protein that are structurally and thermodynamically suitable for the different interactions.

Here, we revealed the atomic coordinates of Q41N at 2500 bar, which is a snapshot of the protein fluctuating between $N_1$ and $N_2$ states on the 1−10 $\mu$s time scale.[7,8] The preexistence of $N_2$ in the structural ensemble of ubiquitin is a prerequisite for conformational selection in the binding of the protein to E1. This finding can be general for the ubiquitin-like post-translational modifiers that have a similar functional strategy of the E1−E2−E3 cascade reaction. Unambiguous structure determination of alternative states requires ~100% population of the alternative state. The novel strategy used here, of high pressure combined with a suitably chosen point mutation, is


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simple and generally applicable and has the potential to greatly improve our understanding of alternative states.

## ■ ASSOCIATED CONTENT

### Supporting Information

Materials and methods, Table S1, and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

R.K., S.K., and M.P.W. wrote the manuscript.


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### Notes

The authors declare no competing financial interest.

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