

Characterization of the molten globule conformation of V26A ubiquitin by far-UV circular dichroic spectroscopy and amide hydrogen/deuterium exchange

Soon-Ho Park*

Department of Biochemistry and Molecular Biology, College of Dentistry and Research Institute of Oral Sciences, Kangnung National University, Gangneung, Korea

The molten globular conformation of V26A ubiquitin (valine to alanine mutation at residue 26) was studied by nuclear magnetic resonance spectroscopy in conjunction with amide hydrogen/deuterium exchange. Most of the amide protons that are involved in the native secondary structures were observed to be protected in the molten globule state with the protection factors from 1.2 to 6.7. These protection factors are about 2 to 6 orders of magnitude smaller than those of the native state. These observations indicate that V26A molten globule has native-like backbone structure with marginal stability. The comparison of amide protection factors of V26A ubiquitin molten globule state with those of initial collapsed state of the wild-type ubiquitin suggests that V26A ubiquitin molten globule state is located close to unfolded state in the folding reaction coordinate. It is considered that V26A ubiquitin molten globule is useful model to study early events in protein folding reaction. [BMB reports 2008; 41(1): 35-40]

INTRODUCTION

The mechanism by which a protein acquires the native three-dimensional structure, the protein folding problem, is not clearly understood yet. It was considered that a protein folds through progressively more structured ensemble intermediate states to a unique native structure (1-4). Thus, it is thought that the analysis of conformation of these ensemble intermediate states would provide valuable information to understand protein folding problem. The ensemble folding intermediate states were observed in the early phase of folding reaction explored by the kinetic folding measurements for several proteins, including cytochrome c (5), B1 domain of protein G (6, 7), ubiquitin variants (8-10), α -lactalbumin (11-13), and apomyoglobin

(14, 15). Unfortunately, due to the highly cooperative nature of protein folding process in the condition that favors the native conformation, these intermediate states were transiently populated so that the detailed structural analysis was not feasible. Interestingly, it has been observed that some proteins form a conformation that was neither the native nor unfolded random coil state in a solution that mildly destabilizes the native conformation (4, 16). This conformation was named as the molten globule, since it had a globular shape without tightly packed side-chains. Although well-defined tertiary structures were absent, significant amount of native-like secondary structures were observed in the molten globule state. Furthermore, the molten globule state has been shown to have similar conformational aspects with the transiently populated partially folded state observed in the kinetic folding experiments (11, 17-20). Therefore, it is considered useful to study the conformational properties of the molten globule to expand our understanding of the protein folding problem.

Among the small globular proteins, ubiquitin has been widely used as a model to study the protein folding process (8, 10, 21-25). Wild-type ubiquitin has been shown to form a loosely collapsed conformation in an acidic solution containing 60% (v/v) methanol. This conformational state was named as the alcohol form (26). Although the alcohol form was widely studied to understand ubiquitin folding reaction, there were some doubts whether this conformation was true folding intermediate in aqueous condition (27). Recently, a mutant ubiquitin with valine to alanine mutation at residue position 26 (V26A ubiquitin) was observed to form neither the native nor fully unfolded random coil conformation in solution at pH 2 (25). The far-UV circular dichroic (CD) spectrum indicated that significant amount of secondary structures were present in the acid-denatured V26A ubiquitin, while the near-UV CD spectrum of V26A ubiquitin indicated the absence of tightly packed tertiary structure. Acid-denatured V26A ubiquitin observed to bind to the hydrophobic dye, 8-anilinonaphthalene-1-sulfonic acid (ANS), indicating that the presence of loosely collapsed hydrophobic cluster. The urea-induced unfolding of V26A ubiquitin at acidic solution was observed to be less cooperative than that observed in neutral pH. All these

*Corresponding Author. Tel: 82-33-640-2458; Fax: 82-33-642-6410; E-mail: spark9@kangnung.ac.kr

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observations strongly indicated that V26A ubiquitin formed a molten globule in the solution at pH 2. In this report, (a) the conformations of alcohol form and molten globule form of V26A ubiquitin were compared by using far-UV CD spectroscopy, and (b) the conformational properties of V26A ubiquitin molten globule were studied by using nuclear magnetic resonance (NMR) spectroscopy in conjunction with amide hydrogen/deuterium (H/D) exchange.

RESULTS AND DISCUSSION

Wild-type ubiquitin has been shown to form a partially folded conformation in a solution containing 60% (v/v) methanol at pH 2 (26–28). This conformation was named as the alcohol form of ubiquitin and considered to be an intermediate state that may appear during the ubiquitin folding reaction. Recently, partially folded molten globule conformation was observed for hydrophobic core residue variant ubiquitin (V26A ubiquitin) in the aqueous solution at pH 2 (25). This molten globule form of V26A ubiquitin was also considered to be an intermediate state of ubiquitin folding reaction. To compare the conformational properties of these states and to find their role in the folding process, the polypeptide backbone structures of the alcohol form of wild-type and V26A ubiquitin, and the molten globule form of V26A ubiquitin were measured by far-UV CD spectroscopy. The far-UV CD spectrum of the native V26A ubiquitin in neutral pH (Fig. 1, dashed line) shows two negative bands at 208 and 220 nm with molar ellipticity values of $-6,500$ and $-5,000$ deg cm² dmol⁻¹, re-

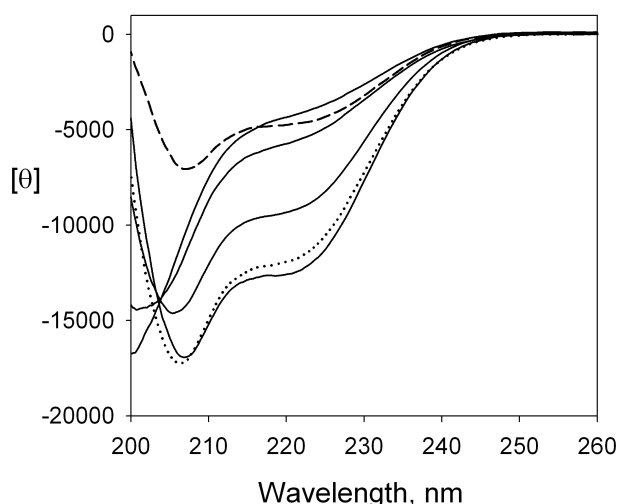


Fig. 1. Far-UV CD spectra. Solid lines represent the far-UV CD spectra of V26A ubiquitin at pH 2 with 0%, 20%, 40%, and 60% methanol, reading downward at 220 nm. Dashed line represents the native state far-UV CD spectrum of V26A ubiquitin at pH 5. Dotted line represents the far-UV CD spectrum of wild type ubiquitin in 60% (v/v) methanol at pH 2. $[\theta]$ denotes the molar ellipticity with unit of deg cm² dmol⁻¹.

spectively, which is consistent with that of the native wild-type ubiquitin (9). The far-UV CD spectrum for the molten globule form of V26A ubiquitin at pH 2 shows strong negative molar ellipticity near 200 nm with a shoulder at 220 nm, features typical to the peptide backbone containing some random coil conformation (29). The observed molar ellipticity value of $-4,400$ deg cm² dmol⁻¹ at 220 nm for V26A ubiquitin molten globule was slightly less negative than that observed for the native state at neutral pH. The far-UV CD spectra of V26A ubiquitin at pH 2 became more negative by the addition of methanol. Furthermore, the negative band at 208 nm became conspicuous so that the far-UV CD spectrum of the alcohol form of V26A ubiquitin in 60% methanol at pH 2 had two negative bands near 208 and 220 nm, which are the spectral features typical to the α -helical conformation (29). The molar ellipticity values at 208 and 220 nm for the alcohol form of V26A ubiquitin in 60% methanol at pH 2 were $-17,000$ and $-13,000$ deg cm² dmol⁻¹, respectively. The far-UV CD spectrum for the alcohol form of V26A ubiquitin appeared to be nearly superimposed to that of the wild-type ubiquitin (dotted line in Fig. 1), indicating that the alcohol form of V26A ubiquitin has the same backbone conformation as that of the wild-type ubiquitin.

It has been known that the molar ellipticity value at 220 nm reflects the content of secondary structures in a polypeptide backbone (30). The more negative molar ellipticity value represents the more secondary structures in the polypeptide backbone. The observed far-UV CD spectrum suggests that there still some secondary structure remains, albeit disrupted as compare to the native state, in the molten globule form of V26A ubiquitin. The content of secondary structures in the molten globule form of V26A ubiquitin appears to be less than those of the native state V26A ubiquitin. Based on the spectral features and molar ellipticity value at 220 nm, the content of secondary structures, especially α -helical conformation, in the alcohol form of V26A ubiquitin appears to be more than those in the native state V26A ubiquitin. This observation suggests that the regions of polypeptide backbone which are not α -helical structure in the native state assume α -helical conformation in the presence of methanol. Therefore, it can be concluded that the alcohol form of V26A ubiquitin contains nonnative α -helical backbone structures. If the alcohol form of V26A ubiquitin is ever occurred during the folding process, the non-native α -helical structures should be unfolded prior to the formation of the native conformation. It is considered that the alcohol form of V26A ubiquitin would be more like off-pathway intermediate that is trapped in a local energy minimum. Furthermore, no such highly α -helical folding intermediate was observed in the folding kinetics experiment for the wild-type ubiquitin measured by stopped-flow CD device (23). All these observations suggest that alcohol form of ubiquitin may not be a proper model to study ubiquitin folding process. By contrast, the molten globule form of V26A ubiquitin appeared to have less secondary structures than the native state.

Although there are less secondary structures in the molten globule state than the native state of V26A ubiquitin, it does not necessarily mean that there are no nonnative secondary structures in the molten globule form of V26A ubiquitin. However, it could be considered that the molten globule form of V26A ubiquitin might have subset of native secondary structures, and would be worth to study the conformation in more detail.

Since far-UV CD spectrum reflects content of secondary structures only and cannot tell the location of the secondary structures in the polypeptide chain, the detailed structure

should be studied using other experimental tools. In order to study the detailed conformational properties of V26A ubiquitin molten globule, the degree of amide proton protection was measured by using two-dimensional NMR spectroscopy in conjunction with amide H/D exchange method.

Fig. 2 shows the peptide fingerprint region of total correlation spectroscopy (TOCSY) spectra of V26A ubiquitin at 1, 21, and 41 min of H/D exchange, respectively. Assigned chemical shifts of peptide finger print region are shown in Fig. 2A. As illustrated, about 30 amide protons exchanged slow enough to be detected. The cross-peak intensities observed in the early stage of H/D exchange were gradually diminished as the time elapsed, indicating that the amide protons were exchanged with solvent deuterons. The time dependent proton occupancies for representative amides are shown in Fig. 3. As shown by the solid lines in Fig. 3, proton occupancies as a function of time were fitted to a single exponential equation and the rates for H/D exchange (k_{ex}) were obtained for observed amides. The protection factors were obtained as described in Materials and Methods (Supplementary Table 1). The protection factors of the amides in V26A ubiquitin molten globule state were observed to be from 1.2 to 6.7 with the average of 3.5. The protection factors of V26A ubiquitin molten globule were smaller than those of the native wild-type ubiquitin by about 2 to 6 orders of magnitude (28), suggesting that the hydrogen bonds in V26A ubiquitin in the molten globule state are significantly destabilized. Fig. 4 illustrates the residues in V26A ubiquitin whose amide protection factor is bigger than 3 in the molten globule state. As illustrated, the protected amides are distributed throughout the region that formed the native secondary structures. Interestingly, the amide protons located in the edge of the β -strand 1, β -strand 2, and α -helix were not pro-

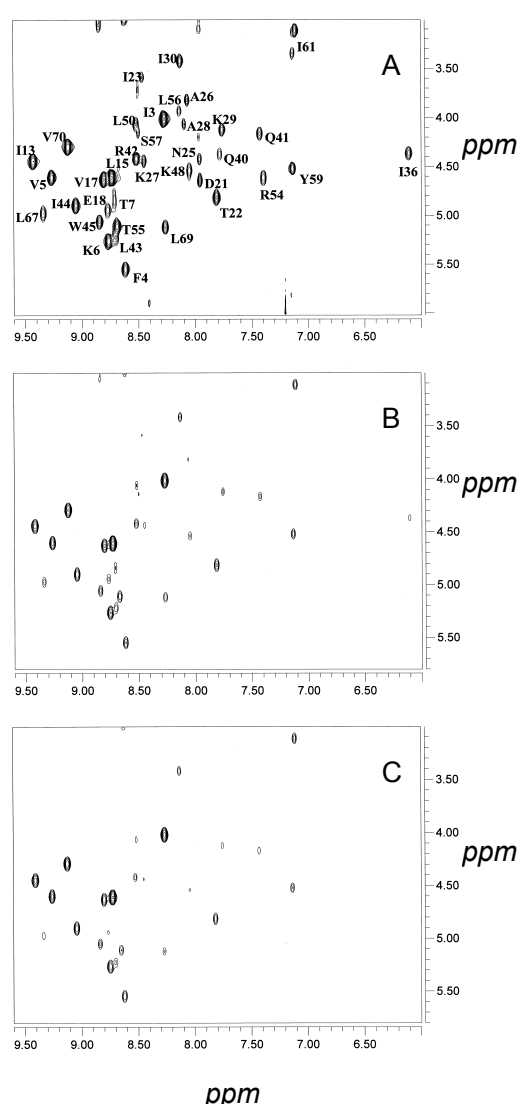


Fig. 2. Peptide finger print region of TOCSY spectra of V26A ubiquitin. Panels A, B, and C illustrate the spectra for V26A ubiquitin after 1, 21, and 41 min of amide H/D exchange, respectively.

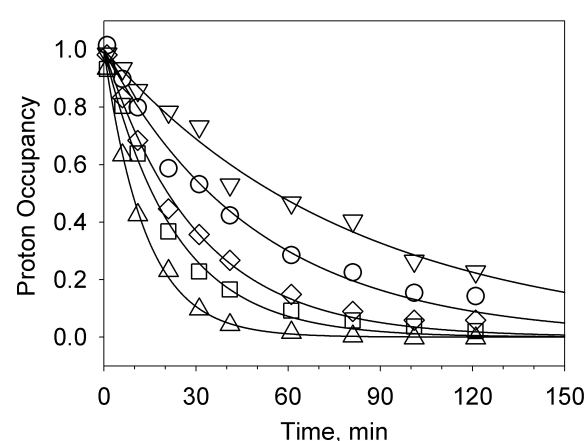


Fig. 3. Time course of proton exchange for representative amides of V26A ubiquitin. Proton occupancies as a function of time for Ile3 (circle), Val17 (square), Ile23 (diamond), Tyr59 (triangle), and Ile61 (inverted triangle) are illustrated. Lines represent the non-linear least squares fit of time vs. proton occupancies with a single exponential equation.

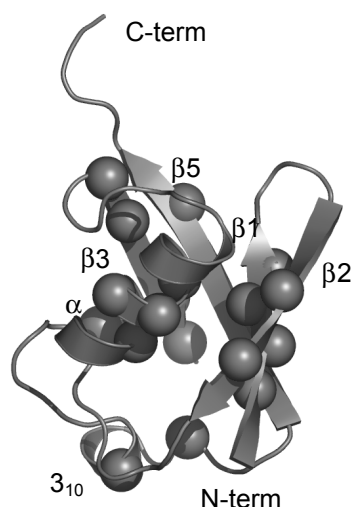


Fig. 4. Amide protection pattern of V26A ubiquitin molten globule. Spheres represent the α -carbon of residues whose amide protection factor is bigger than 3 in the molten globule state. Helix and strands are represented as α and β , respectively. N-term and C-term denote amino-terminus and carboxyl-terminus, respectively.

tected in the molten globule state, indicating that the secondary structures in these regions of the V26A ubiquitin molten globule are frayed. These observations suggest that polypeptide backbone of V26A ubiquitin molten globule is in the native-like topology with marginal stability.

Gladwin and Evans measured the H/D exchange of wild-type ubiquitin occurred in the dead-time of quenched-flow device (23). This method is highly useful to detect the hydrogen bonding pattern in the initial collapsed state in the protein folding reaction. The protection factors observed for most of the amides in the initial collapsed state of wild-type ubiquitin were observed to be below 2, indicating that there were virtually no hydrogen bonded secondary structures in the initial collapsed state of wild-type ubiquitin folding reaction. The fact that the protection factors observed in this study are slightly bigger than those measured by Gladwin and Evans suggests that the molten globule state of V26A ubiquitin would be the state that formed after the initial collapsed state. Went and Jackson studied the structure and energetics of ubiquitin folding transition state ensemble by using folding kinetics measurements in conjunction with protein engineering method (31). In the ubiquitin folding transition state ensemble, the main α -helix was appeared to be fully formed and stabilized by packing against the partially formed first β -turn near amino terminus. If the amino-terminal region of ubiquitin has fully formed hydrogen bonded secondary structures in the folding transition state ensembles, the protection factor for the amide protons in the folding transition state ensemble would expected to be bigger than those observed in the molten globule state measured in this study. Furthermore, the folding kinetics

of V26A ubiquitin from urea-induced unfolded state to the native state and that from the molten globule state to the native state were observed to be same (25). These observations indicated that the molten globule state and unfolded state of V26A ubiquitin were kinetically indistinguishable, and both unfolded state and molten globule state were occurred prior to the major folding transition state in the folding reaction coordinate. The results of amide protection in the dead-time and the conformational properties of folding transition state ensemble suggest that it is highly plausible that the molten globule state of V26A ubiquitin would be a conformational state that could be placed between the fully unfolded random coil state and major folding transition state. It is considered that the molten globule state of V26A ubiquitin would reflect the events occurring in the early stage of folding reaction. It would be worth to study the conformational dynamics of V26A ubiquitin molten globule using ^{13}C and ^{15}N labeled V26A ubiquitin coupled with multi-dimensional NMR for more detailed analysis of early folding events of V26A ubiquitin.

We have characterized conformational properties of the alcohol form and the molten globule form of V26A ubiquitin by using far-UV CD spectroscopy and NMR spectroscopy in conjunction with amide H/D exchange. The alcohol form of V26A ubiquitin appeared to have nonnative α -helical structure, suggesting that this form would be an off-pathway intermediate and may be inappropriate to study folding process. On the other hand, V26A molten globule in an acidic solution appeared to have native-like backbone topology with diminished stability. The amide protection factors suggest that the molten globule state of V26A ubiquitin would be a useful model to study the early events in the ubiquitin folding process.

MATERIALS AND METHODS

Materials

The wild-type and V26A ubiquitins were prepared as described previously (24). Deuterium oxide, sodium deuterioxide, and deuterium chloride were purchased from Sigma Chemical Co. Other chemical reagents were reagent grade or better.

Circular dichroic spectra measurements

Far-UV CD spectra were measured using JASCO J-810 spectropolarimeter as previously described (25). CD signals were reported as mean residue ellipticity, $[\theta]$, having the unit of $\text{deg cm}^2 \text{dmol}^{-1}$.

Hydrogen/deuterium exchange

The H/D exchange reaction was initiated by dissolving 100 mg of lyophilized V26A ubiquitin in 9 ml of deuterium oxide ($^2\text{H}_2\text{O}$) at pH 2 and 25°C . All the pH values reported were direct meter reading uncorrected for the deuterium isotope effect. After desired exchange time (1, 6, 11, 21, 31, 41, 61, 81, 101, and 121 min), 1 ml aliquot was withdrawn and so-

dium deuterioxide was added to bring the final pH of sampled aliquot to 4 so that V26A ubiquitin would return to the native conformation. By this way, the H/D exchange reaction was quenched, since the amide H/D exchange rates for native state ubiquitin were observed to be extremely slow (28). The sampled aliquots were lyophilized and stored in -80°C freezer until use.

NMR measurements and protection factor calculation

The lyophilized aliquots were dissolved in $^2\text{H}_2\text{O}$, pH 5, right before NMR measurements. The protein concentrations for NMR samples were around 2 mM. TOCSY spectra were recorded on a Bruker DMX600 spectrometer located in Korea Basic Science Institute. Spectral width was 7507.51 Hz for both dimensions. A total of 24 transients of 2048 complex data points were recorded for each of 512 t_1 increments with the total acquisition time of ~ 1.3 hr. All the NMR measurements were made at 25°C . The free induction decay was processed using the program Xwin-NMR to obtained TOCSY spectrum. The cross-peak volumes were obtained by peak integration option included in Xwin-NMR. All the cross-peak volumes were scaled to the volume of non-exchangeable proton (Phe4 CH_2) to correct for protein concentration variations. Proton occupancy was obtained by dividing the scaled cross-peak volumes by the scaled cross-peak volume at zero exchange time, obtained by extrapolation. The H/D exchange rates (k_{ex}) were obtained by fitting time vs. proton occupancy to a single exponential equation. The protection factor (PF) was calculated by dividing the exchange rate of unprotected amide proton (k_{int}) by the observed exchange rate (k_{ex}), $\text{PF} = k_{\text{int}}/k_{\text{ex}}$. The proton exchange rate for unprotected amide was calculated by using the measured exchange rate for poly-D, L-alanine as an unstructured peptide model with modification that accommodates local sequence effects (32).

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