Journal of Biochemistry and Molecular Biology, Vol. 37, No. 6, November 2004, pp. 676-683

JOURNAL OF Biochemistry and Molecular Biology

Hydrophobic Core Variant Ubiquitin Forms a Molten Globule Conformation at Acidic pH

Soon-Ho Park*

Department of Dentistry, College of Dentistry, Kangnung National University, Gangneung 210-702, Korea

Received 13 April 2004, Accepted 3 July 2004

The conformational properties of hydrophobic core variant ubiquitin (Val26 to Ala mutation) in an acidic solution were studied. The intrinsic tryptophan fluorescence emission spectrum, far-UV and near-UV circular dichroic spectra, the fluorescence emission spectrum of 8-anilinonaphthalene-1-sulfonic acid in the presence of V26A ubiquitin, and urea-induced unfolding measurements indicate this variant ubiquitin to be in the partially folded molten globule conformation in solution at pH 2. The folding kinetics from molten globule to the native state was nearly identical to those from the unfolded state to the native state. This observation suggests that the equilibrium molten globule state of hydrophobic core variant ubiquitin is an on-pathway folding intermediate.

Keywords: Folding intermediate, Molten globule, Ubiquitin

Introduction

In order to perform their biological function, it is necessary for proteins to fold into their unique three-dimensional structure. It has been well known since the early 1960's that the information required for a protein chain, at least for relatively small globular proteins, to acquire its unique three-dimensional structure is encoded in its amino acid sequence (Anfinsen, 1973). However, the mechanism by which this information is decoded to a three-dimensional structure remains to be clearly understood. It was thought that a protein folds through progressively more structured intermediate states to acquire its unique three-dimensional structure (Kim and Baldwin, 1982; Kim and Baldwin, 1990; Matthews, 1993; Ptitsyn, 1995). Thus, it is considered that the characterization of the conformational properties of a folding intermediate will provide valuable information about a protein folding pathway.

Since the protein folding reaction is a highly cooperative process, partially folded intermediates are seldom present under solvent conditions favoring the native conformation. However, partially folded states are present at equilibrium under mildly denaturing conditions, such as a low solvent pH or low denaturant concentrations. This partially folded state was named the "molten globule", since it had a globular shape with a loosely collapsed hydrophobic core (Ptitsyn, 1995; Kuwajima and Arai, 2000). Far-UV circular dichroic (CD) spectra and hydrogen-deuterium exchange experiments suggested that the molten globule state had some native-like secondary structures. Furthermore, the molten globule state has been shown to have some conformational similarities with the kinetic folding intermediate that was transiently populated in the early phase of the folding reaction (Kuwajima *et al.*, 1985; Arai and Kuwajima, 1996; Heidary *et al.*, 1997; Raschke and Marqusee, 1997; Mizuguchi *et al.*, 1998). This observation strongly suggests the conformational characterization of the molten globule will help in the understanding of the early event in the folding reaction.

Ubiquitin is a small globular protein with 76 amino acids that has been widely used as a model to study the folding process (Briggs and Roder, 1992; Khorasanizadeh *et al.*, 1993; Gladwin and Evans, 1996; Khorasanizadeh *et al.*, 1996). The folding kinetics of tryptophan containing ubiquitin has indicated the presence of a kinetic folding intermediate that was transiently populated in the early phase of the folding reaction (Khorasanizadeh *et al.*, 1996). Thus, the comparison of conformational aspects of kinetic folding intermediate and molten globule of ubiquitin was considered to provide valuable information in the understanding of the early folding event. However, wild type ubiquitin did not form the molten globule in an aqueous environment. Conversely, the hydrophobic core variant (Val26 to Ala, V26A) ubiquitin (Khorasanizadeh *et al.*, 1996) was expected to form the molten globule in aqueous solution. This was based on the analysis that found the formations of the molten globule depend on the ratio between the mean hydrophobicity and mean net charge

*To whom correspondence should be addressed.
Tel: 82-33-640-2458; Fax: +82-33-642-6410
E-mail: spark9@kangnung.ac.kr

(Uversky, 2002). Under mildly denaturing conditions, proteins with a relatively high mean hydrophobicity over mean net charge stay in the native state, while those with a relatively low mean hydrophobicity over mean net charge have a tendency to form the molten globule conformation. Since the valine to alanine mutation is considered to decrease the mean hydrophobicity over mean net charge, V26A ubiquitin may form the molten globule under mildly denaturing conditions. Thus, the possibility of V26A ubiquitin forming the molten globule was explored in this study.

Materials and Methods

Materials The V26A ubiquitin used in this study was generated on WT* ubiquitin, and kindly provided by Heinrich Roder (Khorasanizadeh *et al.*, 1996). The WT* ubiquitin contained a tryptophan instead of a phenylalanine at the residue position 45, which allows the study conformational change using fluorescence spectroscopy (Khorasanizadeh *et al.*, 1993). The three dimensional structure of WT* ubiquitin was nearly identical to wild type ubiquitin, except for local structural rearrangement near the mutation site (Laub *et al.*, 1995). Purification of the WT* and V26A ubiquitin were carried out as previously described by Khorasanizadeh *et al.* (Khorasanizadeh *et al.*, 1993), with additional Sephacryl S-100 column chromatography after the DEAE-Sephacel column chromatography. The purified ubiquitin was more than 98% pure, as adjudged by a densitometric analysis of Coomassie brilliant blue stained sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The mutation at sequence position 26 was checked by amino acid sequencing using a Perkin Elmer Model 491 protein sequencer (Foster City, USA). The ultrapure grade urea was purchased from ICN Biomedical Inc (Aurora, USA). All other chemicals were reagent grade or better.

Spectral measurements The fluorescence emission spectra were measured using a JASCO FP6500 spectrofluorometer (Tokyo, Japan). For tryptophan fluorescence measurements, 3~10 μM ubiquitin was excited at 295 nm and the emission spectrum was recorded between 300 nm and 450 nm. The fluorescence emission spectra of 8-anilinonaphthalene-1-sulfonic acid (ANS) were measured from 400 nm to 600 nm after excitation at 350 nm. The concentration of ANS was ten-fold higher than the concentration of ubiquitin. The slit widths for excitation and emission of the tryptophan and ANS fluorescence emission measurements were 5 nm and 10 nm, respectively. Circular dichroic spectra were measured using a JASCO J-810 spectropolarimeter (Tokyo, Japan). Cells with 1 mm and 1 cm path length were used for far-UV CD measurements and near-UV CD measurements, respectively. The protein concentrations were around 30 and 50 μM for far-UV and near-UV CD measurements, respectively. All the spectral measurements were made at 25°C. The concentration of ubiquitin was determined by absorbance at 280 nm in 6 M guanidinium chloride (GdnCl) solution. In 6 M GdnCl solution, the extinction coefficient of ubiqutin was calculated to be 6970 M^{-1} cm^{-1} (Edelhoch, 1967).

Equilibrium unfolding measurements For pH-induced unfolding measurements, tryptophan emission intensity at 355 nm after excitation at 295 nm was monitored as a function of the solvent pH. Ubiquitin (3~5 μM) was incubated in 25 mM acetate, with the designated pH, for 30 min before spectral measurements. The pH was adjusted by the addition of concentrated HCl. For urea-induced equilibrium unfolding measurements, unfolded ubiquitin stock solution in ~9 M urea and native ubiquitin stock solution in buffer were prepared at the same concentration in 25 mM acetate solution at pH 2 or 5. 3 ml native stock solution was placed in a cell for measuring the far-UV CD signal of the native state at 220 nm. After the far-UV signal measurement, an aliquot of the native solution was removed and an equal volume of unfolded stock solution then added to increase the urea concentration without altering the protein concentration. The mixed solution was stirred for 4~5 min to reach equilibrium before the spectral measurement. The spectral measurements in the increased urea concentration were carried out by repeating the procedure mentioned above until the concentration of urea was close to 9 M. The refractive index of the removed aliquot was measured to determine the urea concentration.

Stopped-flow fluorescence measurements The time course from the acid denatured state or denaturant-induced unfolded state to the native state of V26A ubiquitin was followed by monitoring changes in the fluorescence emission as a function of time. All measurements were made using a BioLogic SFM-4/QS device (Claix, France) in the stopped-flow mode. The fluorescence of tryptophan was excited at 295 nm and the change in fluorescence emission above 324 nm was measured using a filter with a 324 nm cut-off. Unfolded ubiquitin in 6 M urea solution (pH 5) was diluted 11-fold in 25 mM acetate buffer (pH 5) to initiate folding. Acid denatured ubiquitin was diluted 11-fold in 25 mM acetate solution (pH 5.5) to initiate folding. The final pH of the solution was measured as 5, where ubiquitin is in the native state. The refolding of acid denatured ubiquitin was performed in the presence of 0.54 M urea to produce the same final conditions as the refolding from a urea-induced unfolded state.

Results

Acid-induced denaturation of hydrophobic core variant ubiquitin Fig. 1 shows the acid-induced conformational change in ubiquitin monitored by the fluorescence emission intensity at 355 nm after excitation at 295 nm. The WT* ubiquitin showed fairly constant fluorescence emission intensities from pH 5.1 to 1.2. This observation indicates that WT* ubiquitin is in the native conformation, even in a highly acidic solution. However, V26A ubiquitin shows increased fluorescence emission intensities as the pH of solution was lowered from 3.5 to 2.5. It appears that the fluorescence emission reaches a maximum near pH 2. This observation suggests that the native conformation of V26A ubiquitin is disrupted in a solution at pH 2. The fluorescence emission intensities of V26A ubiquitin at varying solvent pH were observed to be lower than the fluorescence emission intensity of the fully unfolded state (dotted line in Fig. 1).

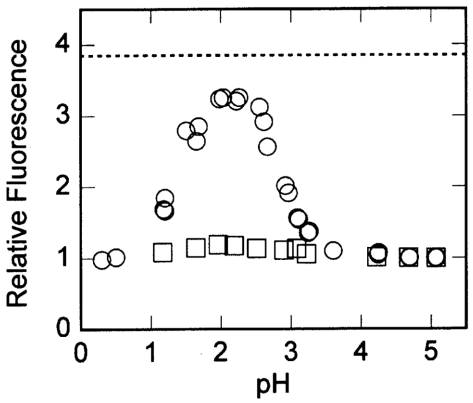

Fig. 1. Effect of pH on the fluorescence emission intensity of ubiquitin. The fluorescence emission intensities at 355 nm in the various solvent pH are illustrated. Squares and circles represent the fluorescence emission intensities as a function of pH for WT* and V26A ubiquitin, respectively.

Interestingly, the fluorescence emission intensities of V26A ubiquitin were observed to decrease as the solvent pH was lowered below 2. The fluorescence emission intensity of V26A ubiquitin becomes the same as that of the native state at around pH 0.5. The decrease in the fluorescence intensities below pH 2 may be due to aggregation of partially denatured ubiquitin, as proteins under moderate denaturing conditions tend to aggregate (Fink, 1998; Dobson, 2004). However, as shown in Fig. 2A, the far-UV CD signal at 220 nm of V26A ubiquitin at pH 2 was constant with protein concentrations up to 1 mM. The constant far-UV CD signal as a function of the protein concentration indicates that the decreased fluorescence intensities at low pH are not likely due to aggregation (Dabora *et al.*, 1996). Native polyacrylamide gel electrophoresis of V26A ubiquitin showed no indication of aggregation (data not shown). Another plausible explanation for the decreased fluorescence intensities at the pH lower than 2 is the formation of a partially folded A-state (Goto *et al.*, 1990; Fink *et al.*, 1994). It has been shown that the acid-unfolded state becomes an A-state upon the addition of neutral salts or further decrease in pH by the addition of HCl. The side-chains of acidic amino acids become protonated at low pH, while those of basic amino acids remain positively charged. Thus, the native conformation of a protein tends to unfold by charge repulsion as the solvent pH is lowered by addition of HCl. The further decrease of pH by addition of HCl, however, eventually increases the concentration of chloride anions, which screen the charge repulsion to drive the acid-denatured protein into a partially folded A-state. As shown in Fig. 2B, the fluorescence intensity change of V26A ubiquitin in pH 2 solution upon the addition of neutral salt, KCl, appears to be superimposed with that observed with a decreased solvent (filled circle). This result strongly suggests that the fluorescence intensity change in V26A ubiquitin at a pH below 2 is due to the conformational change caused by the charge screening effect of chloride anions. The fluorescence emission intensity of V26A ubiquitin at chloride concentrations above 0.2 M is virtually the same as that of the native state. This observation suggests that, unlike other proteins that form an A-state, V26A ubiquitin acquires the native conformation at increased concentrations of neutral salt at pH 2.

The change in the fluorescence emission intensities of V26A ubiquitin at varying solvent pH suggest that V26A ubiquitin, whose native state was destabilized about 3 kcal/mole compared to the wild type protein (Khorasanizadeh *et al.*, 1996), undergoes acid-induced denaturation. Based on the fluorescence intensity, the conformation of acid-denatured V26A ubiquitin at around pH 2 appears to be different from that of the denaturant-induced unfolded state, which has a higher fluorescence emission intensity than that of the acid-denatured state. It appears that some residual structures remain in V26A ubiquitin at around pH 2.

Tryptophan fluorescence spectra of V26A ubiquitin The tryptophan fluorescence emission spectra of V26A ubiquitin

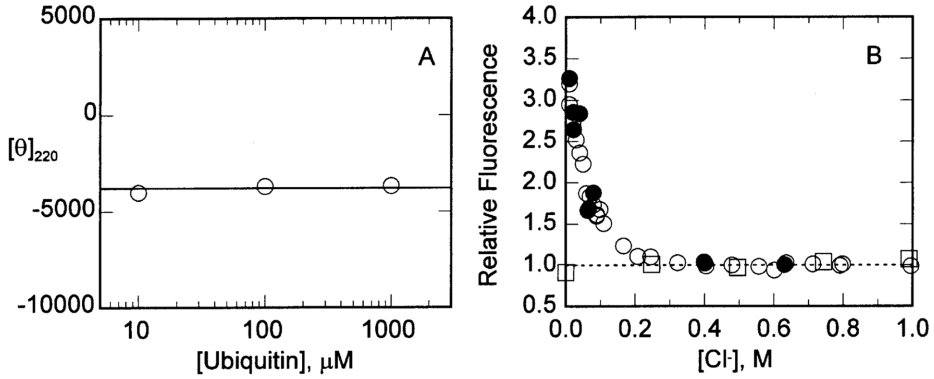

Fig. 2. Effect of V26A ubiquitin concentrations on the far-UV CD signal at 220 nm, and the effect of the neutral salt concentrations on the fluorescence emission intensities of V26A ubiquitin at pH 2. Panel A illustrates the far-UV CD signal at 220 nm as a function of V26A ubiquitin concentration. Line represents the average molar ellipticity value. The unit for molar ellipticity, [θ], is deg cm^2 $dmol^{-1}$. Panel B illustrates the fluorescence emission intensities at 355 nm of WT* ubiquitin (squares) and V26A ubiquitin (circles and filled circles) at pH 2 with various concentrations of chloride ions.

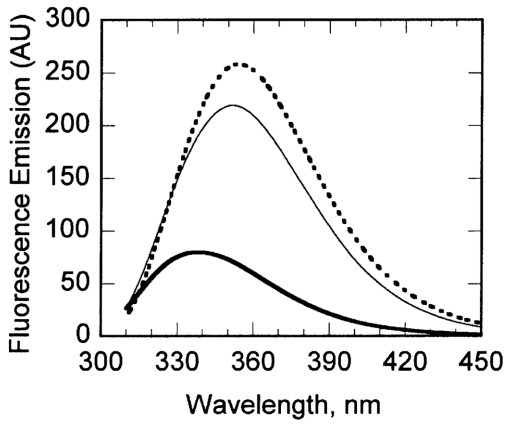

Fig. 3. Intrinsic tryptophan fluorescence emission spectra of V26A ubiquitin. Thick, thin and dotted lines represent the fluorescence emission spectra of V26A ubiquitin at the same protein concentration in pH 5, 2 and 6 M GdnCl (pH 5) solutions, respectively. AU denotes arbitrary units.

are shown in Fig. 3. The native state of V26A ubiquitin has a tryptophan fluorescence emission maximum at 338 nm, indicating that the tryptophan side-chain is partially shielded from the aqueous environment (Stryer, 1968). The tryptophan fluorescence emission spectrum of unfolded V26A ubiquitin in 6 M GdnCl solution showed an increased emission intensity, with an emission maximum at 355 nm, which was consistent with the previously observed tryptophan fluorescence of unfolded WT* ubiquitin (Khorasanizadeh *et al.*, 1993). At pH 2, the tryptophan fluorescence emission of V26A ubiquitin was stronger than that of the native state. The tryptophan fluorescence emission maximum was observed at 350 nm. These observations indicate that the native conformation of V26A ubiquitin is disrupted in pH 2 solution. However, the tryptophan fluorescence of V26A in an acidic solution was observed to be different from that in the unfolded state. The tryptophan fluorescence emission of V26A ubiquitin at pH 2 was weaker than that in the unfolded state. Furthermore, the tryptophan fluorescence emission maximum of V26A ubiquitin at pH 2 solution showed a blue shift by about 5 nm compared to that in the unfolded state. These observations suggest that the V26A ubiquitin has residual tertiary structures in pH 2 solution.

Circular dichroic spectra of V26A ubiquitin Fig. 4A shows the far-UV CD spectra of V26A ubiquitin. V26A ubiquitin in the native state showed a far-UV CD spectrum with negative bands near 220 and 208 nm, and a molar ellipticity value at 220 nm of around −4600 deg cm^2 dmol^{-1}. This was consistent with the previously observed far-UV CD spectra of the wild type ubiquitin (Wilkinson and Mayer, 1986; Cox *et al.*, 1993), indicating that the backbone structure of V26A ubiquitin is not significantly changed upon mutation. The far-UV CD spectrum of V26A ubiquitin in the 6 M GdnCl solution showed the typical far-UV CD spectra of an unfolded polypeptide chain, having a significantly less negative molar ellipticity value at 220 nm (Greenfield and Fasman, 1969). The far-UV CD spectrum of V26A ubiquitin at pH 2 had a strong negative signal near 200 nm, with a shoulder near 220 nm, suggesting that the backbone conformation of V26A ubiquitin at pH 2 is different to that in neutral pH solution. The most conspicuous feature of the far-UV CD spectrum of V26A ubiquitin at pH 2 was that the molar ellipticity value at 220 nm, −4500 deg cm^2 dmol^{-1}, was similar to that of the native state. Since the CD signal at 220 nm reflects the content of secondary structure of a polypeptide chain, this observation suggests that a significant amount of secondary structures remain in V26A ubiquitin under acidic conditions.

Fig. 4B shows the near-UV CD spectra of V26A ubiquitin. The near-UV CD spectrum of the native state showed a strong near-UV CD signal near 270 nm, indicating that the aromatic side-chains are tightly packed in the interior of V26A ubiquitin. The near-UV CD spectrum of V26A ubiquitin in the unfolded state showed no particular signal in this wavelength range, indicating that the aromatic side-chains are rotating freely under this solvent condition. Furthermore, the near-UV CD spectrum of V26A ubiquitin in 6 M GdnCl was

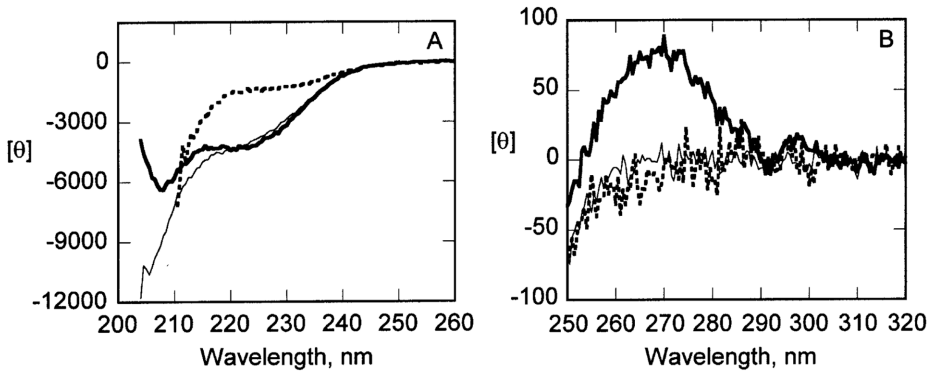

Fig. 4. Circular dichroic spectra of V26A ubiquitin. Panels A and B illustrate the far-UV and near-UV CD spectra, respectively. Thick, thin and dotted lines represent the spectra of V26A ubiquitin in pH 5, 2 and 6 M GdnCl (pH 5) solutions, respectively. The molar ellipticity, [θ], has a unit of deg cm^2 dmol^{-1}.

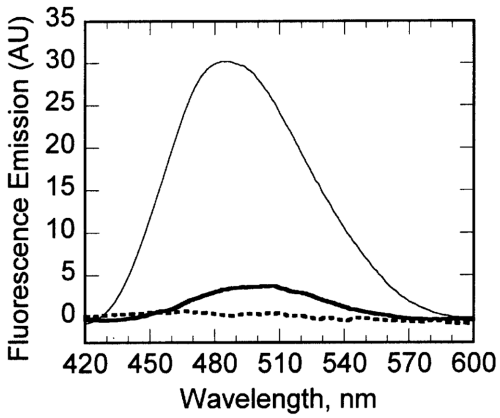

Fig. 5. Fluorescence emission spectra of ANS in the presence of V26A ubiquitin. Thick, thin and dotted lines represent the fluorescence emission spectra of ANS with V26A ubiquitin in pH 5, 2 and 6 M GdnCl (pH 5) solutions, respectively. AU denotes arbitrary units.

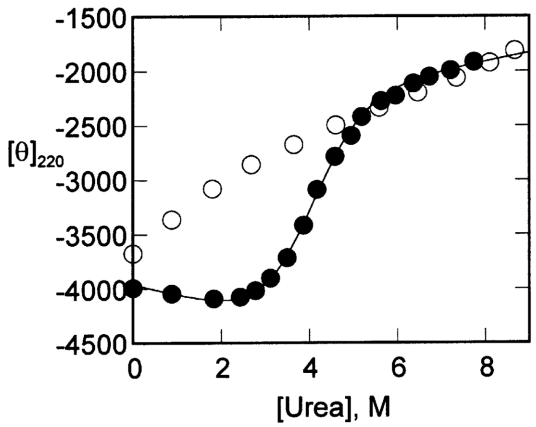

Fig. 6. Urea-induced equilibrium unfolding of V26A ubiquitin. Filled and open circles represent the equilibrium unfolding transitions of V26A ubiquitin at pH 5 and 2, respectively. Line represents the nonlinear least squares fit of the unfolding data at pH 5, based on a two-state transition model.

virtually identical to the near-UV CD spectrum in pH 2 solution. Since the near-UV CD spectrum reflects the tertiary interactions near the residues with aromatic side-chains, little tertiary interactions were considered to present near the aromatic residues of V26A ubiquitin in pH 2 solution.

The far-UV and near-UV CD spectra of V26A ubiquitin in pH 2 solution indicate that V26A ubiquitin has a significant secondary structure, without tightly packed tertiary structures, typical to the molten globule conformation of a polypeptide chain (Ptitsyn, 1992).

ANS binding of hydrophobic core variant ubiquitin at pH 2 ANS fluorescence has been shown to be a particularly useful spectral probe for identification of the molten globule (Azzi, 1974; Goto and Fink, 1989; Semisotnov *et al.*, 1991). The fluorescence emission spectrum of ANS in a hydrophobic environment, such as binding to a loosely folded hydrophobic cluster of molten globule, showed a fluorescence emission with maximum at around 480 nm. As illustrated in Fig. 5, ANS at pH 2 in the presence of V26A ubiquitin shows a fluorescence emission spectrum with the maximum at around 480 nm. This observation indicates that V26A ubiquitin has hydrophobic clusters that allow ANS to bind and fluoresce. By contrast, virtually no ANS fluorescence emission was observed in the presence of native or unfolded V26A ubiquitin. The hydrophobic core of V26A ubiquitin in the native state is considered to be tightly packed, so the hydrophobic clusters are not exposed for ANS to bind. On the other hand, although ANS may bind to hydrophobic groups exposed under highly denaturing conditions, it is considered that the fluorescence emission of ANS bound to unfolded protein under aqueous conditions would be severely quenched by the surrounding water due to the lack of a hydrophobic cluster to shield the ANS from the solvent. The ANS fluorescence in the presence of V26A ubiquitin was consistent with the interpretation that this variant ubiquitin acquires the molten globule conformation in acidic solution.

Equilibrium unfolding of hydrophobic core variant ubiquitin A urea-induced equilibrium unfolding experiment was performed on V26A ubiquitin. Since the conformation of ubiquitin appeared to be influenced by salt (Fig. 2B), urea was used as a denaturant in this study. Fig. 6 illustrates the urea-induced change of the peptide backbone conformation of V26A ubiquitin measured by the far-UV CD signal at 220 nm at pH 2 and 5. The equilibrium unfolding at pH 5 appeared to be a typical cooperative transition curve, with a sigmoidal shape. By contrast, the urea-induced unfolding of V26A ubiquitin at pH 2 showed a gradual increase of the far-UV CD signal at 220 nm, indicating that the transition was less cooperative than that observed at pH 5. Since the molten globule conformation does not have a tightly packed hydrophobic core, its equilibrium unfolding normally shows far less cooperative transition compared to that of the native state (Luo *et al.*, 1997). This observation provides further evidence that V26A ubiquitin forms a partially folded molten globule at pH 2.

Folding kinetics of hydrophobic core variant ubiquitin The role of the partially folded molten globule state of V26A ubiquitin to the overall folding process was studied by measuring the folding kinetics using stopped-flow fluorescence. The fluorescence signal for the molten globule state of V26A ubiquitin observed by the stopped-flow fluorescence device was shown as circles when V26A ubiquitin molten globule was mixed with pH 2 buffer. The reaction progress from the native to molten globule state is shown as an increased fluorescence signal as a function of time (diamonds), while the folding reaction was shown as a decreased fluorescence signal as a function of time (squares and triangles).

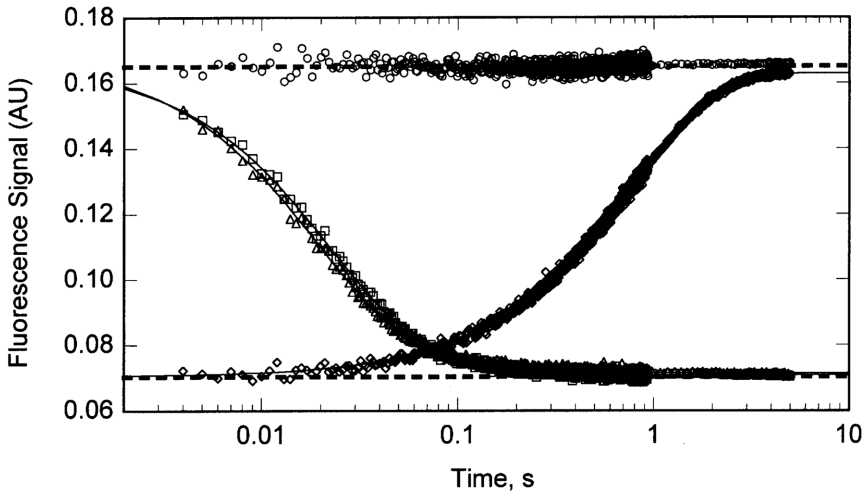

Fig. 7. Kinetic traces for V26A ubiquitin folding/unfolding reactions measured by stopped-flow fluorescence. The observed fluorescence emission above 324 nm is plotted as a function of time in a log scale. Squares represent the refolding kinetic trace of V26A ubiquitin from the molten globule to the native state, which was obtained by mixing V26A ubiquitin in pH 2 solution with a buffer (25 mM acetate, pH 5.5) containing 0.6 M urea. The final pH and urea concentration were 5 and 0.54 M, respectively. Triangles represent the refolding kinetic trace when unfolded V26A ubiquitin in 6 M urea solution (pH 5) was mixed with 25 mM acetate, pH 5. The final pH and urea concentration were 5 and 0.54 M, respectively. Diamonds represent the unfolding kinetic trace when native V26A ubiquitin in 25 mM acetate, pH 5, was mixed with 25 mM acetate, pH 1.9. The final pH was measured as 2. Circles represent the kinetic trace when V26A ubiquitin in acidic solution (pH 2) was mixed with 25 mM acetate, pH 2. Solid lines represent the nonlinear least squares fit of the kinetic traces, with three exponential equations for the refolding and a single exponential equation for the unfolding reactions. The dotted lines represent the fluorescence signal for the native and molten globule state of V26A ubiquitin.

The folding kinetic trace from denaturant-induced unfolded V26A ubiquitin to the native state is illustrated as triangles in Fig. 7. As previously observed, three folding phases were detected (Briggs and Roder, 1992; Khorasanizadeh *et al.*, 1993; Khorasanizadeh *et al.*, 1996). Of these folding phases, the first exponential phase had about 80% of the total amplitude and the two later phases around 20%. The later phases have been considered to be due mainly to the cis/trans isomerization of the peptide bond preceding proline residue (Briggs and Roder, 1992). Thus in the analysis of ubiquitin folding kinetics, only the first exponential phase was considered as a conformational folding process (Khorasanizadeh *et al.*, 1996). The time constant of the major folding phase from the unfolded to the native state was 18 ± 1 ms.

The folding kinetic trace of V26A ubiquitin from the molten globule to the native state is shown as squares in Fig. 7. The folding reactions from unfolded to the native state, and the molten globule to the native state were nearly superimposed on each other. The time constant of the major folding phase from the molten globule to the native state was 21 ± 2 ms. This observation suggests that the acid-induced molten globule and the denaturant-induced unfolded states of V26A ubiquitin are kinetically indistinguishable, although their conformational properties are fairly different judging by the various spectroscopic properties. The fact that the folding rates were nearly identical in the folding reaction initiated from the molten globule state as in the folding reaction initiated from denaturant-induced unfolded state suggests that the partially folded conformation at pH 2 would be an on-pathway folding intermediate. As shown in the proposed reaction coordinate of an on-pathway folding reaction (Fig. 8), the interactions stabilizing the molten globule intermediate state may persist in the transition state, so that the activation energy of the folding reaction from the acid-denatured to the native state would remain nearly the same as that of the folding reaction from unfolded to the native state. If the partially folded conformation at pH 2 is an off-pathway intermediate, the folding reaction initiated from this state should be slower than the folding reaction initiated from the denaturant-induced unfolded state, since this off-pathway intermediate should be unfolded prior to refolding, as shown in the proposed reaction coordinate of the folding reaction with the off-pathway intermediate.

Discussion

The conformation of V26A ubiquitin in acidic solution was studied using various spectroscopic methods. The intrinsic tryptophan fluorescence emission spectrum, far-UV and near-UV CD spectra, fluorescence emission spectrum of ANS in the presence of V26A ubiquitin, and the urea-induced unfolding measurements in acidic solvent strongly indicate that V26A ubiquitin is in the partially folded molten globule conformation in the absence of neutral salt.

The behavior of proteins in acidic solvent has been well

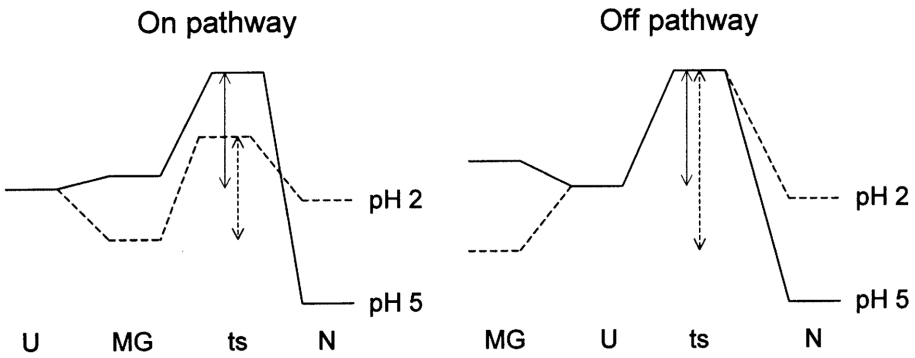

Fig. 8. Proposed reaction coordinates of the V26A ubiquitin folding reaction. U, MG, ts and N represent the unfolded, molten globule, transition and the native states, respectively. Thin lines with double arrows denote the activation energies of the U to N reaction. Broken lines with double arrows denote the activation energies of the MG to N reaction.

documented by Fink *et al.* (1994). The native state of proteins is destabilized by charge-charge repulsion in highly acidic solution, since the side-chains of the basic amino acids are positively charged, while those of the acidic amino acids are neutral. The destabilizing interactions counteract the stabilizing noncovalent interactions, such as hydrophobic interactions, van der Waals interactions and hydrogen bonds. Thus, it was considered that the balance between the destabilizing charge-charge repulsion and stabilizing interactions determined the conformational state of proteins in acidic solution. If the stabilizing interactions are strong enough to overcome the destabilizing charge-charge repulsion, proteins stay in the native state, as with WT* ubiquitin (Fig. 1). If the destabilizing charge-charge repulsions are stronger than the stabilizing interactions, proteins attain an unfolded state, like cytochrome *c* and β-lactamase, etc. (Fink *et al.*, 1994). These proteins tend to acquire a partially folded molten globule conformation in the presence of neutral salt, due to the charge screening effect of the salt. Some proteins directly form a partially folded molten globule conformation in acidic solution without the addition of neutral salt. The charge-charge repulsion by the positively charged groups and the stabilizing interactions are considered to be balanced in the molten globule state, even in the absence of the charge screening effect of a neutral salt.

The hydrophobic core variant ubiquitin in this study appeared to belong to the family of proteins that directly form the molten globule conformation in acidic solution, without the addition of a neutral salt. In the presence of a neutral salt, V26A ubiquitin appeared to attain the native conformation, as shown by the fluorescence emission intensity (Fig. 2B). On the other hand, the WT* ubiquitin appeared to belong to the family of proteins that stay in the native state, even in highly acidic solution. The mutation of valine to alanine, a less hydrophobic amino acid, decreased the stability of the native state of ubiquitin about 3 kcal/mol (Khorasanizadeh *et al.*, 1996). The charge-charge repulsion by the positively charged groups and the stabilizing interactions are considered to be balanced in the molten globule state of V26A ubiquitin in acidic solvent with low concentrations of neural salt, while the stability of the native state of WT* ubiquitin is strong enough to overcome the repulsive interactions of the positively charged groups, even in highly acidic solution.

The formation of a partially folded molten globule in V26A ubiquitin suggests that this mutant ubiquitin would be a useful model to study the conformational properties of the ubiquitin folding intermediate. First, as shown in the folding kinetics measurement, the V26A molten globule appears to be an on-pathway folding intermediate (Fig. 8). It is considered that the interactions that stabilize the ubiquitin molten globule would be the key interactions guiding the unfolded ubiquitin chain toward the native three-dimensional conformation. Second, the molten globule state of V26A ubiquitin is in the monomeric form for a reasonably longer time at millimolar concentrations. Thus, the conformational properties of the V26A molten globule could be explored by high resolution nuclear magnetic resonance spectroscopy. Third, the structure of the folding transition state of a protein was explored by folding kinetics measurement in conjunction with protein engineering (Fersht, 1995). This method can be readily applied to V26A ubiquitin to provide an opportunity to compare the conformational properties of the equilibrium folding intermediate and the folding transition state in a single protein.

Ptitsyn proposed the conformational state of folding intermediate as a pre-molten globule, a molten globule and a dry molten globule state based on the compactness (Ptitsyn, 1995). The dry molten globule state is highly compact with the interior of protein dehydrated. This folding intermediate is close to the native state. The pre-molten globule state is the least compact intermediate state, located close to the unfolded state in the folding process. The molten globule is in the middle of pre- and dry molten globule states in the overall folding process. The protein folding process would be the process that the unfolded polypeptide chain passes through the pre-molten globule, molten globule and dry molten globule states to finally arrive at the native state. The fact that hydrophobic core variant ubiquitin acquired the molten

globule conformation suggests that the molten globule states, with various conformational states, such as pre-molten globule, molten globule or dry molten globule, could be generated on a single protein by the systematic mutation of the hydrophobic core residue with varying degrees of hydrophobicity. It is considered that the detailed structural analysis of molten globules, with different conformational states generated on a single protein, would expand our understanding of the protein folding process.

Acknowledgments Author appreciates to Dr. Heinrich Roder (Fox Chase Cancer Center, Philadelphia, USA) for providing ubiquitin gene. Author also appreciates Dr. Myeong-Hee Yu (Korea Institute of Science and Technology, Korea) for providing stopped-flow device. Author deeply thanks to Dong-Hyung Kim, Yu-Sun Lee, Eun-Kyung Choi, and Mi-Jin Baek for their assistance. The operation of protein sequencer was supported by Shared Research Equipment Assistance Program Grant R23-2002-000-00001-0(2002) from Korea Basic Science Institute. This work was supported by a Korea Science and Engineering Foundation Grant R05-2003-000-12064-0 (2003).

References

Anfinsen, C. B. (1973) Principles that govern the folding of protein chains. *Science* **181**, 223-229.

Arai, M. and Kuwajima, K. (1996) Rapid formation of a molten globule intermediate in refolding of α-lactalbumin. *Fold. Des.* **1**, 275-287.

Azzi, A. (1974) The use of fluorescent probes for the study of membranes. *Methods Enzymol.* **32**, 234-246.

Briggs, M. S. and Roder, H. (1992) Early hydrogen-bonding events in the folding reaction of ubiquitin. *Proc. Natl. Acad. Sci. USA* **89**, 2017-2021.

Cox, J. P. L., Evans, P. A., Packman, L. C., Williams, D. H. and Woolfson, D. N. (1993) Dissecting the structure of a partially folded protein. Circular dichroism and nuclear magnetic resonance studies of peptides from ubiquitin. *J. Mol. Biol.* **234**, 483-492.

Dabora, J. M., Pelton, J. G. and Marqusee, S. (1996) Structure of the acid state of *Escherichia coli* ribonuclease H1. *Biochemistry* **35**, 11951-11958.

Dobson, C. M. (2004) Principles of protein folding, misfolding and aggregation. *Semin. Cell Dev. Biol.* **15**, 3-16.

Edelhoch, H. (1967) Spectroscopic Determination of Tryptophan and Tyrosine in Proteins. *Biochemistry* **6**, 1948-1954.

Fersht, A. R. (1995) Characterizing transition states in protein folding: an essential step in the puzzle. *Curr. Opin. Struct. Biol.* **5**, 79-84.

Fink, A. L. (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold. Des.* **3**, R9-23.

Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T. and Palleros, D. R. (1994) Classification of Acid Denaturation of Proteins: Intermediates and Unfolded States. *Biochemistry* **33**, 12504-12511.

Gladwin, S. T. and Evans, P. A. (1996) Structure of very early protein folding intermediates: new insights through a variant of hydrogen exchange labelling. *Fold. Des.* **1**, 407-417.

Goto, Y., Calciano, L. J. and Fink, A. L. (1990) Acid-induced folding of proteins. *Proc. Natl. Acad. Sci. USA* **87**, 573-577.

Goto, Y. and Fink, A. L. (1989) Conformational states of β-lactamase: molten-globule states at acidic and alkaline pH with high salt. *Biochemistry* **28**, 945-952.

Greenfield, N. and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108-4116.

Heidary, D. K., Gross, L. A., Roy, M. and Jennings, P. A. (1997) Evidence for an obligatory intermediate in the folding of interleukin-1β. *Nat. Struct. Biol.* **4**, 725-731.

Khorasanizadeh, S., Peters, I. D., Butt, T. R. and Roder, H. (1993) Stability and folding of a tryptophan-containing mutant of ubiquitin. *Biochemistry* **32**, 7054-7063.

Khorasanizadeh, S., Peters, I. D. and Roder, H. (1996) Evidence for a three-state model of protein folding from kinetic analysis of ubiquitin variants with altered core residues. *Nat. Struct. Biol.* **3**, 193-205.

Kim, P. S. and Baldwin, R. L. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu. Rev. Biochem.* **51**, 459-489.

Kim, P. S. and Baldwin, R. L. (1990) Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* **59**, 631-660.

Kuwajima, K., Hiraoka, Y., Ikeguchi, M. and Sugai, S. (1985) Comparison of the transient folding intermediates in lysozyme and α-lactalbumin. *Biochemistry* **24**, 874-881.

Laub, P. B., Khorasanizadeh, S. and Roder, H. (1995) Localized solution structure refinement of an F45W variant of ubiquitin using stochastic boundary molecular dynamics and NMR distance restraints. *Protein Sci.* **4**, 973-982.

Luo, Y., Kay, M. S. and Baldwin, R. L. (1997) Cooperativity of folding of the apomyoglobin pH 4 intermediate studied by glycine and proline mutations. *Nat. Struct. Biol.* **4**, 925-930.

Matthews, C. R. (1993) Pathways of protein folding. *Annu. Rev. Biochem.* **62**, 653-683.

Mizuguchi, M., Arai, M., Ke, Y., Nitta, K. and Kuwajima, K. (1998) Equilibrium and kinetics of the folding of equine lysozyme studied by circular dichroism spectroscopy. *J. Mol. Biol.* **283**, 265-277.

Ptitsyn, O. B. (1995) Molten globule and protein folding. *Adv. Protein Chem.* **47**, 83-229.

Raschke, T. M. and Marqusee, S. (1997) The kinetic folding intermediate of ribonuclease H1 resembles the acid molten globule and partially unfolded molecules detected under native conditions. *Nat. Struct. Biol.* **4**, 298-304.

Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F. and Gilmanshin, R. I. (1991) Study of the molten globule intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119-128.

Stryer, L. (1968) Fluorescence Spectroscopy of Proteins. *Science* **162**, 526-533.

Uversky, V. N. (2002) Cracking the folding code. Why do some proteins adopt partially folded conformations, whereas other don't. *FEBS Lett.* **514**, 181-183.

Wilkinson, K. D. and Mayer, A. N. (1986) Alcohol-induced conformational changes of ubiquitin. *Arch. Biochem. Biophys.* **250**, 390-399.