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Urea Induced Inactivation and Unfolding of Arginine Kinase from the Sea Cucumber *Stichopus japonicus*

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Abstract—Urea titration was used to study the inactivation and unfolding equilibrium of arginine kinase (AK) from the sea cucumber *Stichopus japonicus*. Both fluorescence spectral and circular dichroism spectral data indicated that an unfolding intermediate of AK existed in the presence of 1.0 to 2.0 M urea. This was further supported by the results of size exclusion chromatography. The spectral data suggested that this unfolding intermediate shared many structural characteristics with the native form of AK including its secondary structure, tertiary structure, as well as its quaternary structure. Furthermore, according to the residual activity curve, this unfolding intermediate form still retained its catalytic function although its activity was lower than that of native AK. Taken together, the results of our study give direct evidence that an intermediate with partial activity exists in unfolding equilibrium states of AK during titration with urea.

Key words: arginine kinase, conformational change, equilibrium intermediate, urea denaturation

Arginine kinase (AK; ATP:arginine N-phosphotransferase, EC 2.7.3.3) in invertebrates catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP [1]. It plays a central role in cellular metabolism with the temporal buffering of ATP levels in cells with fluctuating energy requirements, such as muscle and nerve cells [2]. The enzyme from the sea cucumber *Stichopus japonicus* is made up of two identical 42 kD polypeptide chains of 370 residues of known sequence [3]. Its three dimensional structure has not yet been determined. Although AK is a phosphagen kinase and plays a physiological role similar to creatine kinase, it has not been investigated as extensively as creatine kinase.

Much research has focused on the evolution of phosphagen kinases and the evolutionary relationship between arginine kinase and creatine kinase [3-8]. Previous equilibrium denaturation studies focused on creatine kinase [9-17] and found several well described folding intermediates. As a dimer enzyme, AK from the sea cucumber *Stichopus japonicus* shows high structural homology to creatine kinase, especially the amino acid sequence. This study focused on the unfolding equilibrium of AK by urea titration. The present research showed that an unfolding intermediate with partial enzyme activity exists in the unfolding equilibrium states of AK. This unfolding intermediate shares many structural properties with native AK and has partial enzyme activity. Our findings suggest that this dimeric intermediate state is the "highly-ordered molten globule state" existing in the unfolding process of AK. This conclusion is important for describing the folding pathway of oligomeric proteins, especially the events of subunit association and later conformational adjustment.

MATERIALS AND METHODS

Arginine kinase was a purification product overexpressed by *Escherichia coli* BL21 (DE3) containing recombinant plasmid pET-21b-AK. The protein was expressed in a soluble and functional form in *Escherichia coli* and purified by Blue Sepharose CL-6B, DEAE-32, and Sephadex G-100 chromatography as previously described [18].

Ultra pure urea, Tris, and 8-anilino-1-naphthalenesulfonic acid (ANS) were from Sigma (USA). All other reagents were local products of analytical grade.

Abbreviations: AK) arginine kinase; CD) circular dichroism; ANS) 8-anilino-1-naphthalene-sulfonic acid; SEC) size exclusion chromatography.

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Protein concentration was determined by the Bradford method [19] with BSA as the standard. The activity assay was an improved version of the previous phosphate determination method [20]. Enzyme (10 μl) was added to 290 μl assay mixture (10 mM arginine, 2 mM ATP, and 3 mM magnesium acetate dissolved in 0.1 M Tris/acetate, pH 8.1) and incubated for 0.5 min at 25°C. The reaction was stopped by the addition of 250 μl of 2.5% TCA, after which the mixture was put in boiling water for 1 min to hydrolyze the phosphoarginine, then immediately cooled on ice for another minute and equilibrated at 25°C for 5 min. Phosphate determination reagent (450 μl 44 mM bismuth nitrate, 1.33 M nitric acid, 31 mM ammonium molybdate, and 0.11% ascorbic acid) was added to the above equilibrated solution to give 1 ml final volume of a color developing system. After 3 min, the absorbance was measured at 700 nm. The activity and concentration were measured with an Ultrospec 4300 pro UV/visible spectrophotometer (Sweden). To get the unfolding equilibrium, all samples were denatured in 20 mM Tris-acetate buffer (pH 8.1), 0.1 mM dithiothreitol, with urea of different concentrations for at least 24 h.

Fluorescence was measured with an F-2500 fluorescence spectrophotometer (Hitachi, Japan) using 1 cm pathlength cuvettes. For intrinsic fluorescence emission spectra, the excitation wavelength was 280 nm; for the ANS fluorescence emission spectra it was 380 nm. The final ANS concentration was 40 μM. CD spectra were recorded on a Jasco 715 spectrophotometer (Japan) with a 2 mm pathlength cell over the wavelength range 200-250 nm.

Size exclusion chromatography (SEC) was carried out with a Superdex 200 column on a Pharmacia FPLC apparatus (Sweden). The stock solutions of AK and other control samples containing urea at different concentration were centrifuged at 15,000 rpm before being loaded on the column equilibrated with the same buffer. All the buffers were passed through a 0.22 μm filter before use. The flow rate was 0.6 ml/min and the effluent was monitored at 280 nm.

RESULTS

Activity changes of AK in the presence of urea at different concentrations. Before activity measurement, AK was incubated with urea of different concentrations for 24 h. The residual activity was plotted versus urea concentration (Fig. 1). It was obvious that the residual activity of AK decreased correspondingly as the urea concentrations increased. When urea concentrations were above 3.5 M, no residual activity could be detected.

Tertiary structural changes of AK induced by urea titration. To detect the tertiary structural changes of AK in the presence of urea with different final concentrations, the intrinsic fluorescence and ANS fluorescence spectra of AK were measured. The original intrinsic fluorescence spectra shown in Fig. 2a revealed that the maximum emission wavelength gradually red-shifted from 332 to 350 nm as urea concentrations increased from 0 to 4 M or even higher. The curve (Fig. 2b) of emission maximum versus urea concentration showed a plateau at urea concentrations between 1.0 and 2.0 M.

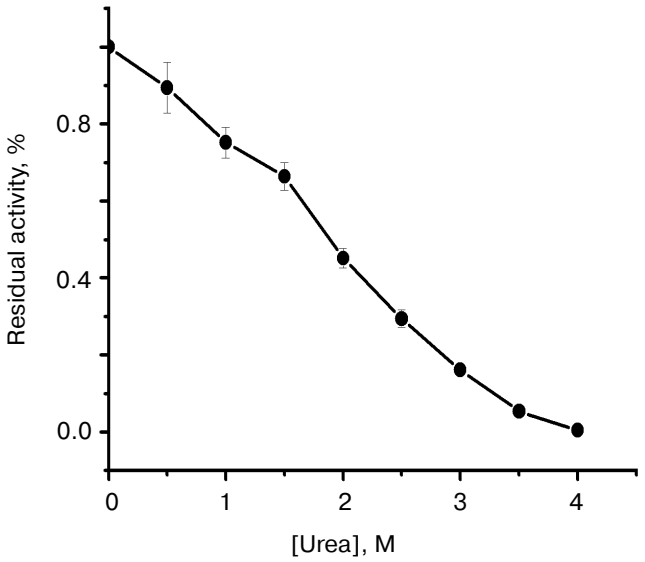

Fig. 1. Residual activity of AK in different concentrations of urea. Arginine kinase was treated with urea for 24 h. The final urea concentrations were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 M. The final AK concentration was 6 μM. The experiments were carried out at 25°C.

Figure 3a shows the original ANS-binding fluorescence spectra of AK in the presence of different urea concentrations, indicating that native AK and its unfolding equilibrium state in 2.0 M urea both showed high fluorescence intensity. The curve of Fig. 3b further demonstrates the changes of ANS-binding fluorescence intensity during urea titration. At urea concentrations below 1 M, the fluorescence intensity slightly decreased as urea concentration increased. However, as urea concentrations increased up to 2 M, the ANS-binding fluorescence intensity increased to a peak value, then gradually came down to a plateau as urea concentrations further increased.

Secondary structural changes of AK induced by urea titration. Secondary structural changes of AK in different urea solutions were studied by far-UV CD spectra shown in Fig. 4a. The figure shows that the secondary structure was significantly destroyed as urea concentration increased. The curve of Fig. 4b shows the changes in the ellipticity at 222 nm of AK in the presence of different urea concentrations. Consistent with that of intrinsic emission maximum, the curve also shows a plateau at urea concentrations

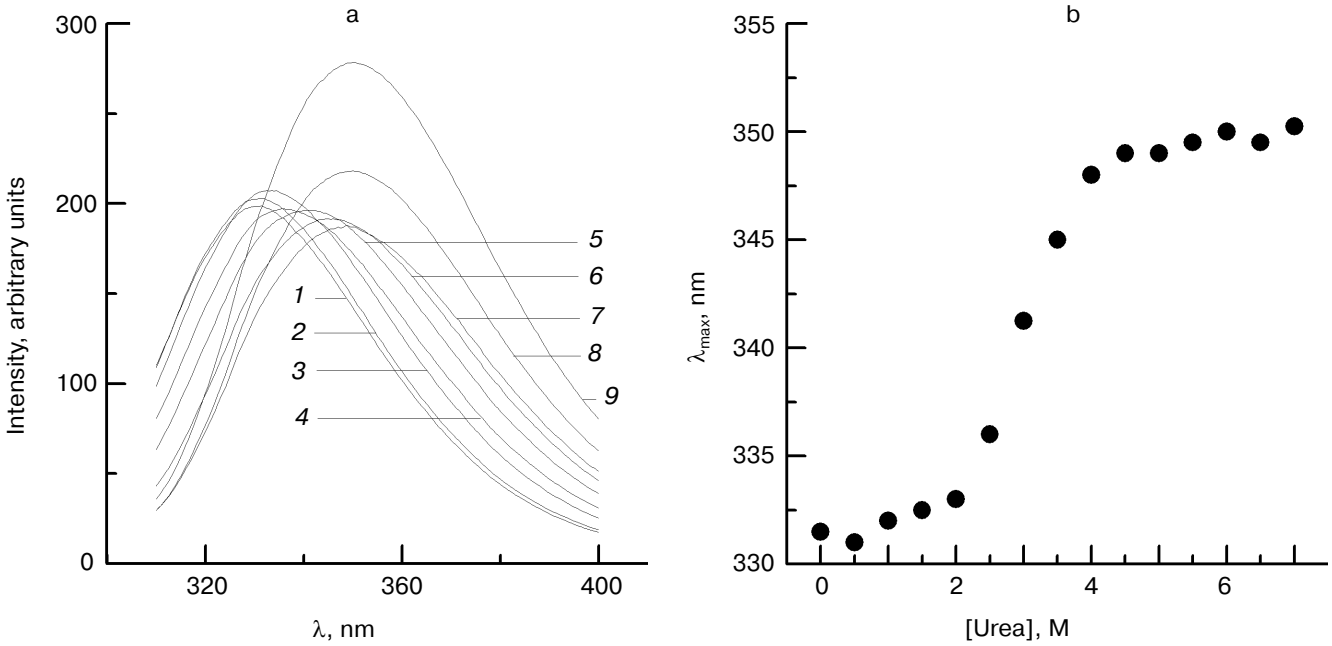

Fig. 2. Effect of urea on AK fluorescence. a) Fluorescence emission spectra of AK at different urea concentrations. Arginine kinase was treated with urea for 24 h. The fluorescence emission spectra were then measured at a final enzyme concentration of 6 μM. The excitation wavelength was 280 nm. The final urea concentrations were 0, 0.5, 2, 2.5, 3, 3.5, 4.5, 6, and 7 M for curves *1-9*, respectively. The experiments were carried out at 25°C. b) Red shift of AK at 280 nm in different concentrations of urea. The points are the experimental values of red shift of AK at maximum intensity in different urea concentrations that were calculated from Fig. 2a.

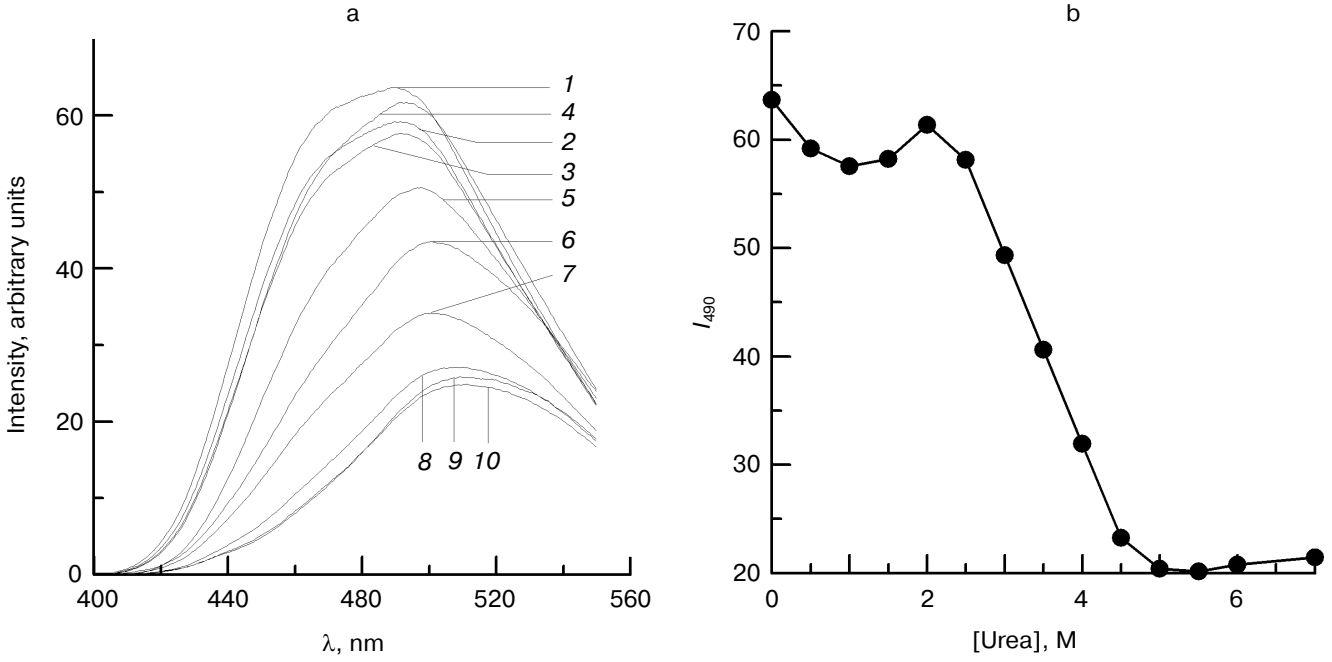

Fig. 3. Effect of urea on AK-ANS binding fluorescence. a) ANS fluorescence emission spectra in the presence of AK at different urea concentrations. Arginine kinase was treated with urea for 24 h. The ANS fluorescence emission spectra were then measured at a final protein concentration of 6 μM. The final ANS concentration was 40 μM. The excitation wavelength was 380 nm. The final urea concentrations were 0, 0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5, and 5 M for curves *1-10*, respectively. The experiments were carried out at 25°C. b) Changes in ANS fluorescence intensity of AK in different concentrations of urea. The points are the experimental values of the fluorescence intensity monitored at 490 nm. The experimental conditions were as for Fig. 3a.

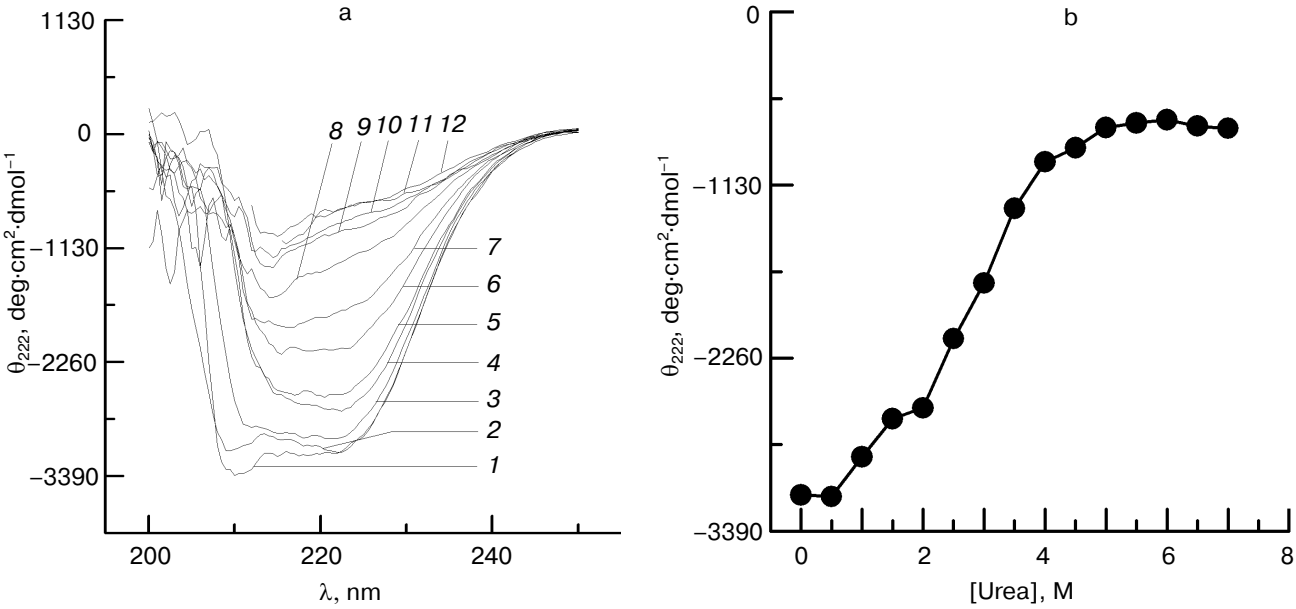

Fig. 4. Effect of urea on AK far-UV CD spectra. a) Far-UV CD spectra of AK in different concentrations of urea. AK was treated with urea for 24 h. The CD spectra were then measured at a final protein concentration of 6 μM. The final urea concentration were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, and 5.5 M for curves *1-12*, respectively. The experiments were carried out at 25°C. b) Changes of ellipticity at 222 nm in the presence of different concentrations of urea. The ellipticity at 222 nm of AK treated with different urea concentrations were calculated from Fig. 4a.

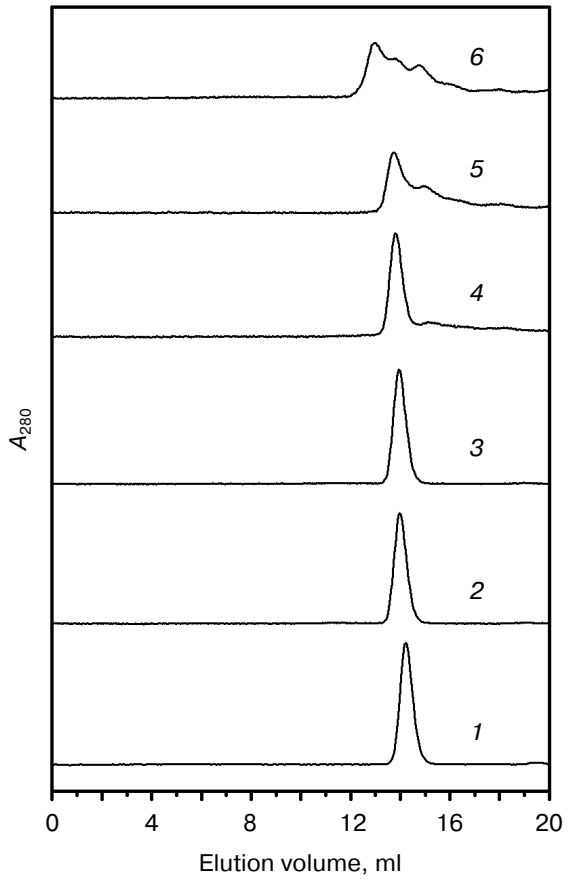

Fig. 5. Elution profiles of AK in the presence of different concentrations of urea. SEC was carried out with a column of Superdex 200 on a Pharmacia FPLC apparatus. Arginine kinase was treated with urea for 24 h. The final concentration of AK was 6 μM. The final urea molar concentrations used were: *1*) 0; *2*) 1; *3*) 2; *4*) 2.5; *5*) 3; *6*) 4. The flow rate was 0.6 ml/min.

between 1 and 2 M. Moreover, at urea concentrations below 1 M, the ellipticity only slightly changed.

Subunit dissociation of AK induced by urea titration. For a two-subunit protein, subunit dissociation is an important event during protein unfolding. Therefore, SEC was used here to detect subunit dissociation of AK during urea-induced unfolding. Results shown in Fig. 5 demonstrate that no dissociation occurs at urea concentrations below 2.5 M. However, as urea concentrations increased further, an elution peak appeared following the peak of dimeric AK. Furthermore, the SEC behavior of AK showed that at least three forms existed in the presence of 4 M urea, including dimer, extensively unfolded monomer, and partly unfolded monomer corresponding to the elution order.

DISCUSSION

By detecting the environmental changes of tryptophan, the intrinsic fluorescence spectra indicated that at urea concentrations between 1 and 2 M, AK exists as a form of unfolding intermediate. Combined with the results of activity measurements, our findings suggest that this unfolding intermediate is a relatively compact dimeric form with partial enzyme activity of 45-75% of that of native AK and shared many properties with native AK. About 76.3-82.5% of the secondary structure still remains in the intermediate. The emission maximum of the intrinsic fluorescence was 331.5 nm for native AK and 333 nm for the intermediate in 2 M urea, this being the same within experimental error. ANS-

binding fluorescence spectra further described this intermediate as having an extensive exposure of hydrophobic surface. Results of SEC indicated that, like the native state of AK, this unfolding intermediate was also in dimeric form.

Previous studies have suggested that in addition to the "molten globule state", two kinds of intermediates called the "pre-molten globule state" and the "highly-ordered molten globule state" may also exist during protein unfolding and refolding [21, 22]. Compared with a typical "molten globule" intermediate, the latter has more structure and is much more similar to the native state. All of our findings in this study indicated that the unfolding intermediate of AK has the characteristics of a "highly-ordered molten globule state". Therefore, we conclude that the urea-trapped intermediate with partial enzyme activity is the "highly-ordered molten globule state" that exists in the unfolding process of AK. This provides direct evidence for a hierarchical mechanism of protein folding [23, 24]. This conclusion is important for describing the folding pathway of oligomeric proteins, especially the events of subunit association and later conformational adjustment.

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