Intermediate studies on refolding of arginine kinase denatured by guanidine hydrochloride

Hong-Min Tang and Hong Yu

Abstract: The refolding course and intermediate of guanidine hydrochloride (GuHCl)-denatured arginine kinase (AK) were studied in terms of enzymatic activity, intrinsic fluorescence, 1-anilino-8-naphthalenesulfonte (ANS) fluorescence, and far-UV circular dichroism (CD). During AK refolding, the fluorescence intensity increased with a significantly blue shift of the emission maximum. The molar ellipticity of CD increased to close to that of native AK, as compared with the fully unfolded AK. In the AK refolding process, 2 refolding intermediates were observed at the concentration ranges of 0.8–1.0 mol/L and 0.3–0.5 mol GuHCl/L. The peak position of the fluorescence emission and the secondary structure of these conformation states remained roughly unchanged. The tryptophan fluorescence intensity increased a little. However, the ANS fluorescence intensity significantly increased, as compared with both the native and the fully unfolded states. The first refolding intermediate at the range of 0.8–1.0 mol GuHCl/L concentration represented a typical "pre-molten globule state structure" with inactivity. The second one, at the range of 0.3–0.5 mol GuHCl/L concentration, shared many structural characteristics of native AK, including its secondary and tertiary structure, and regained its catalytic function, although its activity was lower than that of native AK. The present results suggest that during the refolding of GuHCl-denatured AK there are at least 2 refolding intermediates; as well, the results provide direct evidence for the hierarchical mechanism of protein folding.

Key words: arginine kinase, guanidine-denatured, refolding, intermediate, molten globule state.

Résumé : Le processus et les étapes intermédiaires du repliement de l'arginine kinase (AK) dénaturée par le chlorhydrate de guanidine (GuHCl) ont été étudiés par la mesure de l'activité enzymatique, de la fluorescence intrinsèque, de la fluorescence au 1-anilino-8-naphtalènesulfonate (ANS), et par dichroïsme circulaire (DC) dans l'UV lointain. Durant le repliement de l'AK, l'intensité de fluorescence a augmenté tel que démontré par un déplacement significatif de l'émission maximale vers le bleu. L'ellipticité molaire en DC a augmenté à un niveau similaire à celui de l'AK native, comparativement à celle de l'AK complètement dépliée. Lors du processus de repliement de l'AK, deux intermédiaires ont été observés dans des conditions de concentrations de GuHCl allant de 0.8 à 1.0 mol/L et 0.5 à 0.3 mol/L. La position du pic de l'émission de fluorescence et la structure secondaire de ces états de conformation étaient grosso modo inchangés. L'intensité de fluorescence du tryptophane a augmenté légèrement. Cependant l'intensité de leur fluorescence respective à l'ANS a augmenté de façon significative. Le premier intermédiaire de repliement observé dans des conditions de concentration de GuHCl allant de 0.8 à 1.0 mol/L représentait un état de structure globulaire pré-fusion inactif. Le deuxième intermédiaire trouvé entre 0.5 et 0.3 mol/L de GuHCl partageait plusieurs caractéristiques avec l'AK native, y compris sa structure secondaire, sa structure tertiaire et sa fonction catalytique, quoique cette activité ait été plus faible que celle de l'AK native. Les résultats présents suggèrent que durant le repliement de l'AK dénaturée par le GuHCl, au moins deux intermédiaires sont formés et fournissent une évidence directe d'un mécanisme hiérarchique dans le repliement de la protéine.

Mots clés : Arginine kinase, dénaturation par la guanidine, repliement, intermédiaire, état globulaire pré-fusion.

[Traduit par la Rédaction]

Introduction

Arginine kinase (ATP: L-arginine phosphotransferase EC 2.7.3.3), similar to creatine kinase (ATP: N-creatine phosphotransferase EC 2.7.3.2) in vertebrates, is a phosphagen kinase participating in cell metabolism that catalyzes the reversible transfer of a phosphoryle group from MgATP to arginine, leading to phosphoarginine and MgADP (Newsholme et al. 1978). It plays an important role in cellular energy metabolism in invertebrates (Strong and Ellington 1995). Phosphagen

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Abbreviations: AK, arginine kinase; CK, creatine kinase; GuHCl, guanidine hydrochloride; ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism; DTT, dithiothreitol.

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Fig. 1. The effect of temperature and different final concentrations of refolded arginine kinase (AK) on aggregation during refolding of 3.5 mol/L GuHCl-denatured AK by 60-fold dilution into refolding buffers. From curve 1 to 5, the final concentrations of AK were 6.32, 3.31, 3.27, 7.16, and 6.48 μmol/L, and the temperatures were 4, 10, 20, 20, and 25 °C, respectively.

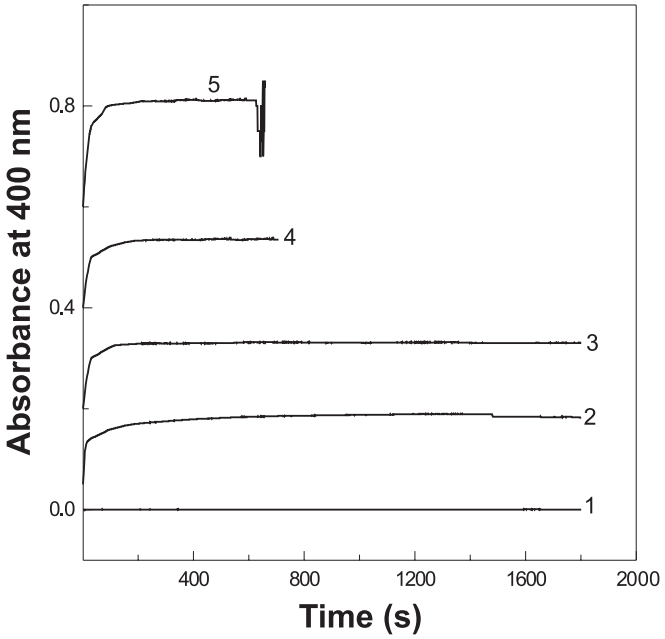
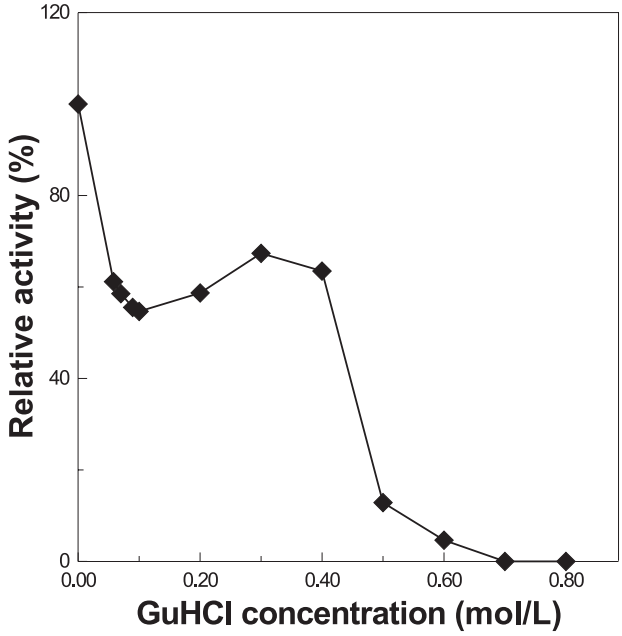


Fig. 2. Relative activity of arginine kinase (AK). The enzyme was denatured at 25 °C in a solution containing 3.5 mol GuHCl/L in 0.1 mol Gly-NaOH/L, 1 mmol dithiothreitol/L buffer, pH 8.6, for 3 h. Denatured enzyme was diluted into standard buffer for 3 h at 4 °C in the presence of different concentrations of GuHCl, and the enzymatic activity (◆) was measured at 25 °C. The final enzyme concentration of AK was 3.12 μmol/L. The native enzymatic activity at the same concentration was taken as 100%. The enzymatic activity was monitored by measuring the absorption at 575 nm.



kinases, such as creatine kinase, arginine kinase (AK), glycocyamine kinase and so on, are a family of highly conserved enzymes. They provide an excellent model system for elucidating how enzymes develop the recognition site for the substrate during evolution. Recently, some papers focusing on the evolutionary relation between AK and creatine kinase were published (Suzuki et al. 1997, 1999).

The study of folding intermediate is a key point in protein folding investigations. Apart from the molten globule state, 2 others, the so-called "highly-ordered molten globule state" and "pre-molten globule state", may also exist as intermediates between the native and the fully unfolded state in general protein folding (Redfield et al. 1994; Ptitsyn 1995). Creatine kinase, as a model protein, has been used to study the problem of protein folding for 40 years. However, there are only a few papers focusing on the folding of shrimp AK (Gross et al. 1995; France and Grossman 1996; Yu and Li 2003; Pan et al. 2004). Studying AK will bring us abundant knowledge about enzyme evolution and provide further information to compare the different folding mechanisms of AK and creatine kinase. Therefore, it is important to investigate the folding of AK in detail.

In the present paper, we studied the refolding course and intermediate of guanidine hydrochloride(GuHCl)-denatured AK. The results show that during the refolding of GuHCl-denatured AK there are 2 refolding intermediates. The first is very similar to that of the pre-molten globule state, and the second has the characteristics of the highly ordered molten globule state with partial activity.

Materials and methods

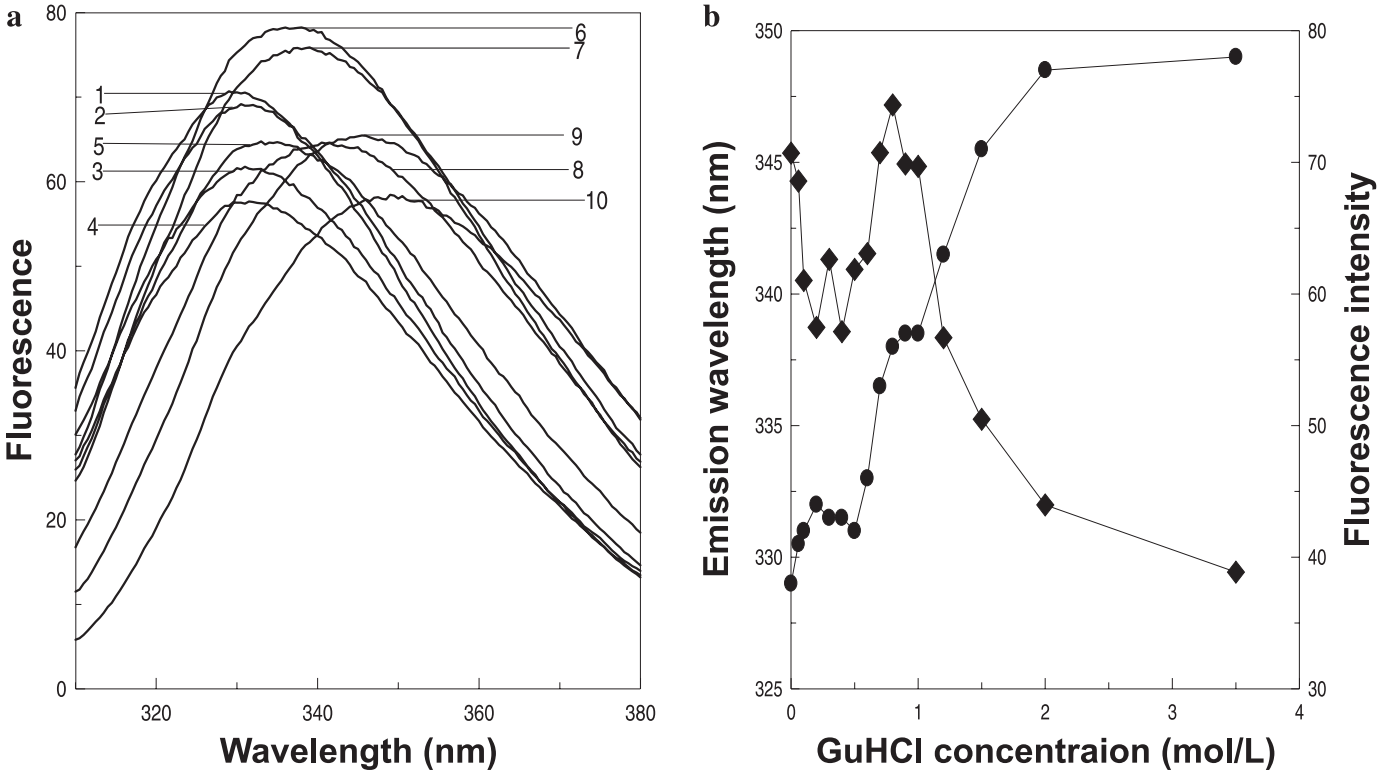
AK was prepared from the tail muscle of shrimp *Fenneropenaeus chinensis*, as described in France et al. (France et al. 1997). The purified enzyme was found to be homogenous, and the molecular mass of 40 kDa was determined by isoelectric focusing and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE) (Laemmli 1970). The enzyme concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient $A^{1\%}_{1\,cm} = 6.7$ (Virden et al. 1965). AK activity was assayed using the method developed by Yu et al. (Yu et al. 2002). The activity of the same concentration of native AK was taken as 100%.

ATP, arginine, glycine, GuHCl, 1-anilino-8-naphthalenesulfonate (ANS), dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were local products of analytical grade.

AK was diluted 60-fold into the standard buffer (0.1 mol Gly-NaOH/L, 1 mmol DTT/L, pH 8.6) at different temperatures. The final AK concentrations were 3.27, 3.31, 6.32, 6.48 and 7.16 μmol/L. Aggregation during the refolding course of 60-fold dilution of GuHCl-denatured AK was measured by recording the absorbance at a wavelength of 400 nm in an Amersham Pharmacia Biotech (Uppsala, Sweden) Ultrospec 4300 pro UV/visible spectrophotometer.

The enzyme was denatured at 25 °C in a solution containing

Fig. 3. Intrinsic fluorescence emission spectra of refolded arginine kinase (AK) in different concentrations of GuHCl. AK was denatured in 3.5 mol GuHCl/L for 3 h at 25 °C. Refolding occurred by diluting the unfolded enzyme into the standard buffer (0.1 mol/L Gly-NaOH/1 mmol dithiothreitol/L, pH 8.6) in the presence of various concentrations of GuHCl. The final enzyme concentration of AK was 3.12 μmol/L. The excitation wavelength was 295 nm. (*a*) Intrinsic fluorescence spectra of refolded AK were measured after dilution for 3 h. The native AK curve was labeled as 1, curves 2 to 10 were measured in different GuHCl concentrations of 0.058, 0.1, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, and 3.5 mol/L, respectively. The reactions all occurred at 4 °C. (*b*) Intrinsic fluorescence intensity changes (◆) and emission maximum blue shift (●). The points were calculated from (*a*).



3.5 mol GuHCl/L in a buffer of 0.1 mol Gly-NaOH/L, 1 mmol DTT/L, pH 8.6, for 3 h. In the refolding studies, the enzyme denatured as described was diluted at 4 °C to a final concentration of 3.12 μmol/L into the standard buffer (0.1 mol Gly-NaOH/L, 1 mmol DTT/L, pH 8.6) containing GuHCl solution of different concentrations for 3 h, and then the enzymatic activity was measured. In addition, after this dilution the circular dichroism (CD) spectra and the intrinsic fluorescence emission spectra were recorded, and then 10 μL of ANS was added to 1 mL of the sample that had been used to measure the intrinsic fluorescence in the dark, so that the final ANS concentrations were 30 μmol/L, and the ANS fluorescence emission spectra were recorded after 30 min.

The intrinsic fluorescence emission spectra were measured with a Hitachi F-2500 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) using 1 cm path-length cuvettes, with an excitation wavelength of 295 nm over a wavelength range of 310–380 nm. For ANS detection of hydrophobic exposure fluorescence emission spectra, the excitation wavelength was 380 nm, and the emission was recorded from 420–580 nm. CD spectra were acquired on a Jasco J-715 spectrophotometer (Jasco (UK) Ltd., Great Dunmow, UK) over a wavelength range of 205–250 nm with a path length of 2 mm. Each spectrum was the result of 5 scans obtained by collecting data at 0.5-nm intervals with an integration time of 0.5 s. The CD intensity at 222 nm was used to calculate the amount of helical second structure.

All measurements were carried out at 4 °C, expect for the activity assay and aggregation.

Results

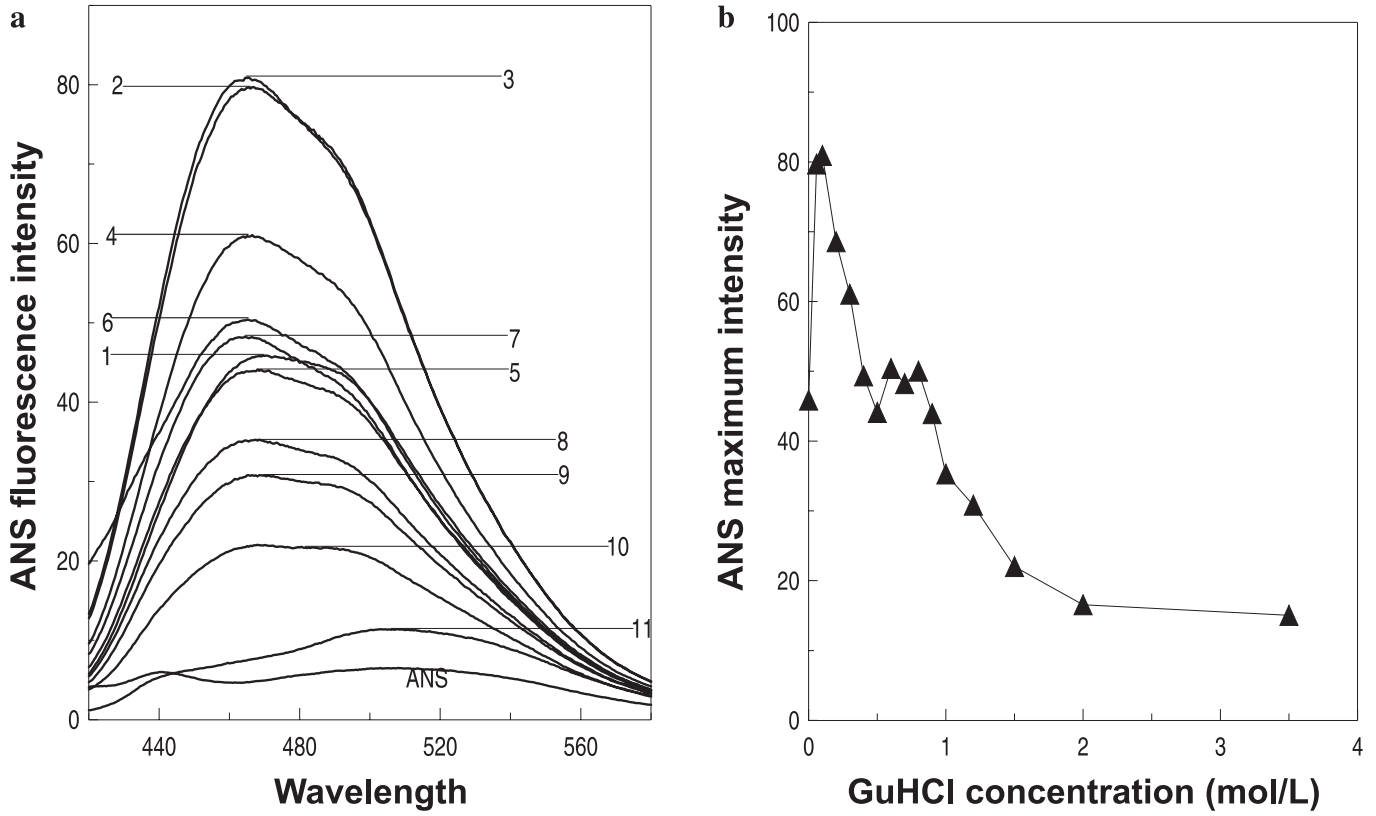
Aggregation of the refolding of diluted AK

AK aggregation depends on protein concentrations and solution temperatures (Yu and Li 2003). The result was similar to the aggregation of CK (Li et al. 2001). After AK had been denatured it was diluted at different temperatures, and aggregation was measured by monitoring the turbidity at 400 nm. Aggregation did not take place at 4 °C if the enzyme concentration was lower than 6.32 μmol/L. The results are shown in Fig. 1.

Changes of the activity of refolding AK at different GuHCl concentrations

In the refolding studies, the denatured enzyme was diluted at 4 °C to a final concentration of 3.12 μmol/L into the standard buffer containing GuHCl solution of different concentrations for 3 h and its enzymatic activity measured. The activity of AK after it had been renatured for 3 h regained more than 50% as compared with the native AK activity

Fig. 4. 1-anilino-8-naphthalenesulfonte (ANS) fluorescence emission spectra of refolding arginine kinase (AK) in different concentrations of GuHCl. The procedures were the same as for Fig. 2. 10 μL of ANS (3 mmol/L) was added to 1 mL of each diluted sample after 3 h, and ANS fluorescence emission spectra of refolded AK were measured after 0.5 h. The excitation wavelength was 380 nm. The final AK and ANS concentrations were 3.12 and 30 μmol/L, respectively. (*a*) The native AK was labeled as 1, curves 2 to 11 were original spectra for AK renatured in GuHCl of different concentrations of 0.058, 0.1, 0.3, 0.5, 0.6, 0.7, 1.0, 1.2, 1.5, and 3.5 mol/L, respectively. The reactions all occurred at 4 °C. (*b*) ANS fluorescence intensity changes. The points were calculated from (*a*).



with no GuHCl. No activity was detected after diluting AK at a GuHCl concentration of more than 0.6 mol/L. The results are shown in Fig. 2.

Changes of the tertiary structure of refolding AK at different GuHCl concentrations

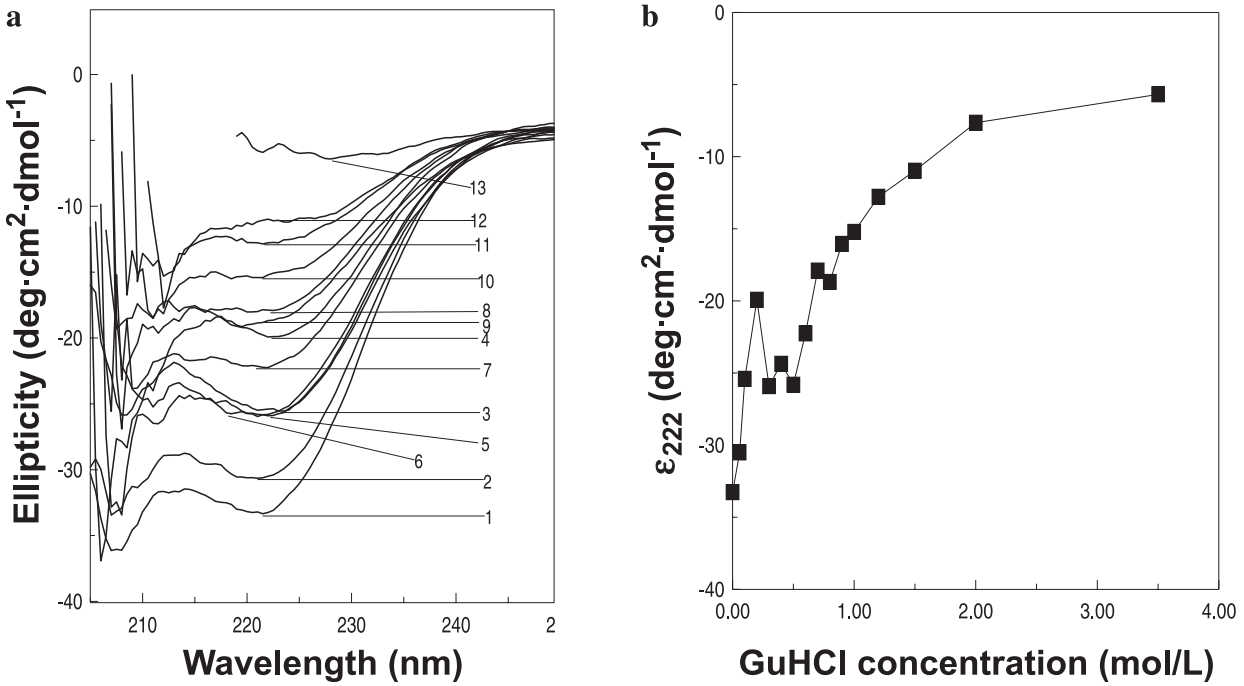
The tertiary structural changes of AK during refolding in different concentrations of GuHCl were studied by analysis of intrinsic fluorescence spectra with the excitation wavelength of 295 nm. Changes in the intrinsic fluorescence emission spectra of AK after it had been renatured for 3 h in GuHCl of different concentrations are shown in Fig. 3*a*. Decreasing the GuHCl concentration caused spectra blue shifting. The fluorescence spectra blue shifted from 349 nm to 329.5 nm when GuHCl concentrations were reduced from 3.5 to 0.058 mol/L. Figure 3*b* shows the plot of the emission maximum blue shifting and the fluorescence intensity increasing versus the GuHCl concentration. As shown by the plot, decreasing GuHCl concentrations caused significant changes in the blue shifting of the emission maximum consistent with the fluorescence intensity increasing. This occurred in 3 stages: at GuHCl concentrations in the higher ranges, decreasing the concentration to 1 mol/L caused an obvious blue shift of the emission maximum (from 349 to 338.5 nm). This also shifted significantly from 338 to 331 nm and from 331.5 to 329 nm at the ranges of 0.8–0.5 mol GuHCl/L and 0.3–0 mol GuHCl/L, respectively. However, at the ranges of 1.0–0.8 and 0.5–0.3 mol GuHCl/L there was little change in the emission maximum.

The tertiary structural changes of AK during refolding in different concentrations of GuHCl were also studied by ANS fluorescence spectra excited at 380 nm. The fluorescence emission of ANS is known to increase when the dye binds to the hydrophobic regions of a protein (Stryer 1965). Here, ANS binding was used as another criterion to identify the refolding intermediate of the protein. The results in Fig. 4*a* show that decreasing the GuHCl concentration caused the fluorescence emission intensity of ANS binding in renatured protein to increase. Figure 4*b* shows the plot of the fluorescence emission intensity of ANS binding versus the GuHCl concentration. The fluorescence emission intensity of ANS binding increased markedly at both ranges of 0.058–0.4 mol/L and 0.6–0.8 mol/L.

Changes of the secondary structure of refolding AK at different GuHCl concentrations

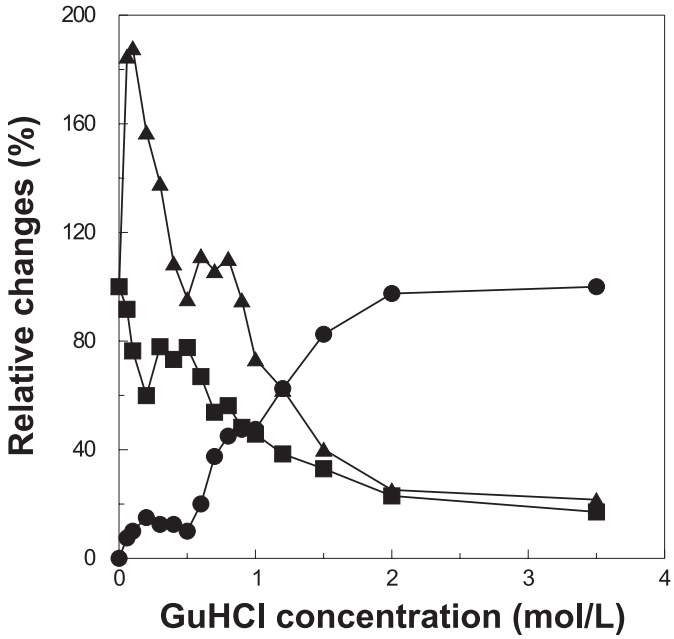
Secondary structural changes of refolding AK were studied by far-UV CD. The far-UV CD intensity is dependent on the amount of α-helical residues in the protein (Gross et al. 1995). The CD intensity at 222 nm was used to calculate the amount of helical secondary structure. The far-UV CD spectra of AK is shown in Fig. 5*a*. The figure shows that the sec-

Fig. 5. Circular dichroism spectra of refolded arginine kinase (AK) in different concentrations of GuHCl. The procedures were the same as for Fig. 2. Circular dichroism spectra of the refolded AK were measured over a wavelength range of 205–250 nm. (*a*) The native AK was labeled as 1, curves 2 to 13 were original spectra for AK renatured in GuHCl of different concentrations of 0.058, 0.1, 0.2, 0.3, 0.5, 0.6, 0.7, 0.8, 1.0, 1.2, 1.5, and 3.5 mol/L. The reactions all occurred at 4 °C. (*b*) Changes in ellipticity at 222 nm. The points were calculated from (*a*).



ondary structure was significantly increased as GuHCl concentration decreased in the refolding course. The plot of the relative ellipticity at 222 nm versus GuHCl concentration (Fig. 5*b*) shows that the secondary structure of refolding AK was changed little at the ranges of 0.8–1.0 and 0.3–0.5 mol GuHCl/L, consistent with that of intrinsic emission maximum.

Fig. 6. Refolding course and intermediates were monitored by changes in the blue-shift of intrinsic fluorescence (●), the fluorescence emission intensity of ANS binding (▲), and ellipticity at 222 nm (■) between 0 and 3.5 mol GuHCl/L as described in the text.



Discussion

It has been generally recognized that protein denaturation is a highly cooperative process, which for small globular protein may be approximated by a 2-state model (Aune and Tanford 1969). The refolding pathway may be the rapid reverse of the unfolding pathway. However, recent results show that many globular proteins exhibit an intermediate conformational state at equilibrium (Gross et al. 1995; France and Grossman 1996; Bai et al. 1999; Tang et al. 2003). Molten globule state, 1 of these states, was characterized by Goto (Goto and Fink 1989). Several studies have suggested that 2 kinds of intermediates, a highly ordered molten globule state and re-molten globule state, may exist during protein folding (Redfield et al. 1994; Ptitsyn 1995) in addition to the molten globule state. Compared with a typical molten globule intermediate, the re-molten globule state is much more similar to the unfolding state, and the highly ordered molten globule state is close to native state.

In the present investigation, the concentration-dependent transitions of fluorescence and the ellipticity at 222 nm of AK are clearly shown in Fig. 6. The results demonstrate that in the process of AK refolding there are 2 forms of refolding intermediates. The corresponding activity measurements showed that the first refolding intermediate at the range of 0.8–1.0 mol GuHCl/L was inactive and accounted for approx-

imately 45%–48% of the secondary structure. The tryptophan fluorescence intensity increased from 69.69 to 74.35, and the fluorescence emission maximum had a slight blue shift in the first refolding intermediate. The fluorescence emission intensity of ANS binding increased from 35.27 to 50.01, indicating the transfer of the ANS molecules from a hydrophilic to hydrophobic environment.

This characterization of the first refolding intermediate is very similar to that of the re-molten globule state However, the second refolding intermediate, in the range of 0.3–0.5 mol GuHCl/L, regained partial activity, as much as 63.5%–67.3% of that of native AK, and reclaimed about 77% of the secondary structure. The tryptophan fluorescence intensity also increased, from 57.14 to 62.63, and the fluorescence emission maximum had a slight blue shift in the second refolding intermediate; ANS binding fluorescence spectra suggested the formation of a hydrophobic core. All of these results indicate that the second refolding intermediate of AK has the characteristics of a highly ordered molten globule state.

These results imply that during the refolding of GuHCl-denatured AK there exist at least 2 refolding intermediates in the experimental conditions. Pan et al. (2004) also suggested that the refolding course of urea-denatured AK contained at least 2 equilibrium refolding intermediates. The present study provides direct evidence for the hierarchical mechanism of protein folding (Baldwin and Rose 1999*a*, 1999*b*) and may be useful for determining the folding pathway of proteins.

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