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Intermediates in the Inactivation and Unfolding of Dimeric Arginine Kinase Induced by GdnHCl

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Equilibrium studies of guanidine hydrochloride (GdnHCl)–induced unfolding of dimeric arginine kinase (AK) from sea cucumber have been performed by monitoring by enzyme activity, intrinsic protein fluorescence, circular dichroism (CD), 1-anilinonaphthalene-8sulfonate (ANS) binding, size-exclusion chromatography and glutaraldehyde cross-linking. The unfolding is a multiphasic process involving at least two dimeric intermediates. The first intermediate, I_1, which exists at 0–0.4 M GdnHCl, is a compact inactive dimer lacking partial global structure, while the second dimeric intermediate, I_2, formed at 0.5–2.0 M GdnHCl, possesses characteristics similar to the globular folding intermediates described in the literature. The whole unfolding process can be described as follows: (1) inactivation and the appearance of the dimeric intermediate I_1; (2) sudden unwinding of I_1 to another dimeric intermediate, I_2; (3) dissociation of dimeric intermediate I_2 to monomers U. The refolding processes initiated by rapid dilution in renaturation buffers indicate that denaturation at low GdnHCl concentrations (below 0.4 M GdnHCl) is reversible and that there seems to be an energy barrier between the two intermediates (0.4–0.5 M GdnHCl), which makes it difficult for AK denatured at high GdnHCl concentrations (above 0.5 M) to reconstitute and regain its catalytic activity completely.

Key words: dimeric arginine kinase, dissociate, equilibrium intermediate, protein folding, refolding.

Arginine kinase (ATP: arginine N-phosphotransferase, EC 2.7.3.3) catalyzes the reversible phosphorylation of arginine (Arg) by ATP, and the formation of a high-energy compound phosphoarginine (PArg). As a member of the phosphogen kinase family, it is mainly distributed in invertebrates, and plays a key role in the interconnection of energy production and utilization, which is analogous to the creatine kinase reaction in vertebrates (*1*, *2*).

AK in *Stichopus japonicus*, one of the dimeric arginine kinases found in echinoderms, is composed of two identical subunits, each with 371 amino acid residues and a molecular weight of about 42,000 (*3*). This dimeric AK has raised interest recently because of its special position in evolution. Sequence analysis indicated that the dimeric arginine kinase was evolutionarily closer to creatine kinase, while its catalytic site was still conserved with that of monomeric arginine kinase. Moreover, the success in obtaining the matrix-bound heterohybrids muscle creatine kinase (M-CK) and muscle arginine kinase (M-AK) also indicated that there was a close relationship between these two enzymes (*4*). Therefore, it has been proposed that *Stichopus japonicus* arginine kinase evolved at least twice during the evolution of phosphogen kinase: first at an early stage of phosphogen kinase evolution (its descendants are molluscan and arthropod arginine kinases), and second from creatine kinase at a later time in metazoan evolution (*5*).

Understanding the folding/unfolding and self-assembly processes of oligomeric proteins remains a major problem. Equilibrium denaturation studies of such proteins provide important information on the relationship of folding and oligomerizatoin processes and on the influence of quaternary structure on protein stability (*6*, *7*). Investigations on denaturant-induced unfolding and refolding of other phosphogen kinases, such as monomeric lobster AK, dimeric rabbit M-CK, octameric chicken mitochondrial creatine kinase (Mi-CK), were undertaken previously and they suggested that the occurrence of equilibrium globular folding intermediates might be a general feature inherent in the common structure type of phosphogen kinase (*8*). Because of this, it is interesting to investigate the transitions between native and denatured states of this specific dimeric AK.

In this paper, we have investigated the dissociation and unfolding of the dimeric AK in GdnHCl using the following methods: (1) enzyme catalytic activity, to indicate the disruption of the active site regions; (2) red shift of wavelength of the maximum fluorescence emission λ_{max}, to monitor global structural changes induced by the denaturant; (3) ellipticity at 220 nm (θ_{220}) in the CD spectrum, to detect secondary structural changes induced by GdnHCl; (4) ANS binding, to detect the appearance of hydrophobic patches in the enzyme molecules during unfolding; (5) size-exclusion chromatography and glutaraldehyde cross-linking, to monitor dissociation steps at different GdnHCl concentrations. Our results indicate that the unfolding of dimeric AK is a multiphasic process, involving at least two dimeric intermediates, one of which

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corresponds to the globular folding intermediate mentioned in the literature (*8*). In addition, there seems to be an energy barrier between these two intermediates, which makes it difficult for denatured AK to reconstitute and regain its catalytic activity completely.

MATERIALS AND METHODS

Materials—AK was prepared from the muscle of sea cucumber *Stichopus japonicus* according to the procedure of Anosike et al. (*9*). The purified enzyme was homogeneous on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. ATP, Arg, ANS and ultra-pure GdnHCl were purchased from Sigma. All the other reagents were local products of analytical grade used without further purification.

Enzyme Concentration and Activity Assay—The enzyme concentration was determined by the Coomassie blue protein dye binding method of Bradford with bovine serum albumin as standard (*10*). The enzyme activity was assayed by phosphate determination method, based on the spectrophotometric determination of an ascorbic acid–reduced blue ternary heteropolyacid composed of bismuth, molybdate and the released phosphate from the acid-labile PArg formed in the forward catalysis reaction (*11*). The absorbance maximum at 700 nm was measured using an analytic spectrophotometer Specord 200 UV VIS (Jena, Germany) and the molar extinction coefficient was 15.97 mmol liter^{-1} cm^{-1} (*12*).

Enzyme Unfolding and Refolding—All unfolding and refolding experiments were performed at 25°C.

The enzyme samples were prepared by mixing a solution of the AK in 20 mM Tris-acetate, 0.1 mM DTT (pH 8.1) buffer with 6 M GdnHCl in the same buffer to the final concentration of 0.12 mg/ml, followed by incubation overnight to ensure equilibration. Longer incubation up to 10 days did not result in further changes in spectral and fast performance liquid chromatography (FPLC) measurements.

The refolding reaction was induced by adding aliquots of AK denatured at desired GdnHCl concentrations to buffers containing varied concentrations of GdnHCl. Each sample was mixed by vortexing and incubated overnight before being evaluated.

Spectral Measurements of Unfolding and Refolding Transitions in GdnHCl—All fluorescence emission spectra were collected on a Hitachi 850 spectrofluorimeter at 25°C. The excitation slits and emission slits were set at 5 nm. Excitation was accomplished at 280 nm, while the emission spectra were collected from 300 nm to 400 nm. The contribution of the buffer was subtracted.

To determine the hydrophobic surface exposure during the GdnHCl denaturation, samples were incubated with a 50-fold molar excess of ANS for 30 min at 25°C in the dark, then fluorescence emission spectra were measured from 400 to 500 nm with excitation at 380 nm.

Far-UV circular dichroism spectra were measured on a Jasco 500C CD spectropolarimeter at 25°C, using a 1 mm pathlength cell over the wavelength range 200–250 nm. Five spectra signals were averaged for each sample.

Size Exclusion Chromatography—Gel filtration was carried out with a Superdex 200HR 10/30 column (fractionation range 10,000–600,000) on a Pharmacia FPLC apparatus at 25°C. All solutions prepared were passed through a filter and degassed. A 100 μl sample was injected into a column pre-equilibrated with 20 mM Tris-acetate, 0.1 mM DTT (pH 8.1) buffer, with or without GdnHCl at desired concentrations ranging from 0–6 M. The flow rate was maintained at 0.5 ml/min and absorbance at 280 nm was monitored. The distribution coefficient K_{av} ($K_{av} = (V_e - V_0) / (V_t - V_0)$, with V_e being the elution volume, V_0 the void volume and V_t the column total volume) was used to monitor Stokes radius changes of the enzyme molecules during the GdnHCl denaturation.

Glutaraldehyde Cross-Linking—Glutaraldehyde was used as the cross-linking reagent to evaluate the dimeric structure of AK. Aliquots (100 μl) of denatured AK at various GdnHCl concentrations were withdrawn and a small volume of glutaraldehyde (25 %, w/v) was added at 2 % (v/v) of the final mixture. After 2 min, cross-linking was quenched by the addition of solid $NaBH_4$ to give a final molar ratio of $NaBH_4$ / glutaraldehyde of 10. Nine hundred microliters of dd H_2O was added to the cross-linking reaction after 15 min to dilute the GdnHCl, which facilitated AK precipitation, accomplished by adding 8 μl of 25% deoxycholic acid solution and 25 μl of 78%TCA (*13*). The samples were centrifuged at 14,000 rpm for 10 min, and the precipitates were washed with distilled water several times, then redissolved in 30 μl of 1.5 M Tris-HCl buffer (pH 8.8) containing 1% (w/v) SDS and 50 mM dithioerythritol. Finally, the samples were boiled for 10 min before SDS-PAGE was performed at room temperature using the tris-glycine buffer system described by Laemmli (*14*).

Quantitative Analysis of Unfolding Curves—Since a simple two-state model failed to fit the fluorescence data, we assumed the existence of dimeric intermediate species (I_1, I_2) in equilibrium with both the native enzyme (N_2) and the completely unfolded monomer (U):

$$N_2 \overset{K_{ni}}{\leftrightarrow} I_1 \rightarrow I_2 \overset{K_{iu}}{\leftrightarrow} 2U$$

Equilibrium constants for the two unfolding transitions (K_{ni} and K_{iu}) could be calculated by the following equation (*15*):

$$K_{ni} = \frac{f_{I1}}{f_{N2}} = \frac{\lambda - \lambda_{N2}}{\lambda_{I1} - \lambda} \text{ and } K_{iu} = 2[P] \times \frac{f_{2u} \times f_{2u}}{f_{I2}},$$

with f_{N2}, f_{I1}, f_{I2} and f_{2u} being the fraction of native AK, intermediate I_1, intermediate I_2 and completely unfolded monomers, respectively; λ, λ_{N2} and λ_{I1} being the wavelength measured at the desired GdnHCl concentrations, at the native state and at the intermediate I_1 state, respectively; and [P] the total protein concentration in monomer units. In addition, K_{ni} and K_{iu} were related to the respective free energy values ΔG_{ni} and ΔG_{iu} (in monomer units) ($\Delta G_i = -RT\ln K_i$), which were assumed to vary linearly with the denaturant concentration [D] (*16*):

$$\Delta G_i = \Delta G_{i,H_2O} - m_i [D] \quad (1)$$

In the multiphasic unfolding process, there were four unknown parameters ($\Delta G_{0,ni}$, $\Delta G_{0,iu}$, m_{ni}, and m_{iu}) whose values could easily be obtained by two preliminary fits: fitting the two-state model to both the first transition $N_2 \leftrightarrow I_1$ and the third transition $I_2 \leftrightarrow 2U$.

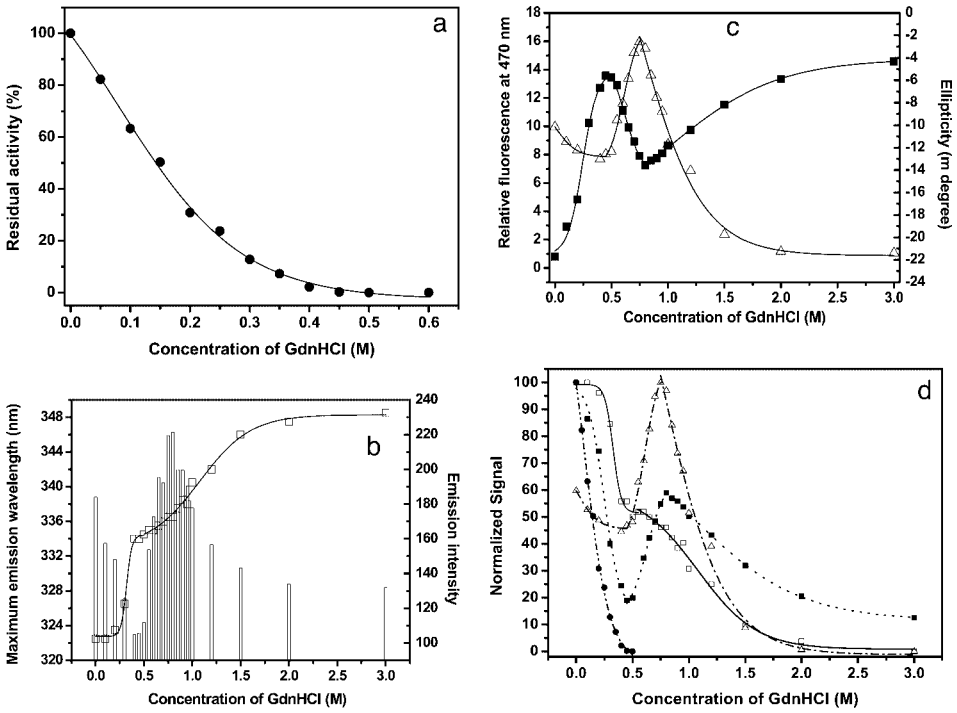


Fig. 1. **Inactivation and unfolding of AK by GdnHCl.** (a) Dependence of the catalytic activity of AK on GdnHCl concentration. (b) Dependence of the wavelength of maximum fluorescence emission (λ_{max}) (open squares) and emission intensity (bars) on GdnHCl concentration. (c) Dependence of the intensity of anilinonaphthalene sulfonic acid (ANS) at 480 nm (λ_{exc} = 380 nm) (open triangles) and ellipticity at 220 nm (solid squares) in circular dichroism on GdnHCl concentration. (d) Comparision of the change of the normalized signal monitored by different methods during the inactivation and unfolding of AK by GdnHCl. Catalytic activity of enzyme (solid circles), fluorescence emission maximum wavelength (open squares), ellipticity at 220 nm in circular dichroism (solid squares) and the intensity of ANS fluorescence (open triangles). The enzyme concentration was 0.12 mg/ml.

RESULTS

Changes in Enzyme Activity, Intrinsic Protein Fluorescence, ANS Binding, CD and Ultraviolet Difference Spectra during GdnHCl Denaturation—Figure 1 shows the transition curves obtained after native AK was incubated in different concentrations of GdnHCl overnight, as determined by catalytic activity, intrinsic fluorescence, ANS binding and circular dichroism.

The catalytic activity of AK as a function of the GdnHCl concentration (Fig. 1a) indicated that increasing GdnHCl concentration caused a marked decrease in AK activity. The enzyme was completely inactivated when the GdnHCl concentration reached 0.45 M, and GdnHCl concentration at this transition midpoint was 0.13 M.

The wavelength of maximum fluorescence emission (λ_{max}) as a function of GdnHCl can give a skewed tracking of the states and was very useful for characterizing the environments of the indole side chains as a function of denaturant concentration (*17*). Figure 1b shows that the unfolding of AK exhibited a strongly biphasic profile when monitored by the red shift of λ_{max}, indicating the equilibrium unfolding process, from the native (N_2) to the fully unfolded (U) state, involved stable intermediates (I), as do those of other guanidine kinases (*8*). The first transition was centered at 0.33 M and resulted in a 13 ± 0.5 nm (321.5 nm to 335.0 nm) red shift of λ_{max} with a concerrent decrease of the fluorescence intensity. In addition, this transition was paralleled by inactivation of the modified enzyme. The second transition centered at 1.15 M and was accompanied by a further large red shift of λ_{max} to 348.5 nm, suggesting a fully exposed indole fluorophore (*18*). Its fluorescence intensity rose sharply after the first transition, reached a maximum at 0.8 M GdnHCl concentration, then decreased gradually.

The effect of increasing GdnHCl concentration on the secondary structure of AK was studied by CD spectra in the far-UV region. Figure 1c shows that the ellipticity at 220 nm decreased sharply with the $N_2 \leftrightarrow I$ transition at low GdnHCl concentrations of 0–0.5 M. The residual secondary structure was 19.8% of the native enzyme at 0.5 M of GdnHCl. As GdnHCl concentration was increased further, the ellipticity began to increase and reached a maximum at 0.8 M GdnHCl, with the secondary structure 59% of the native enzyme, indicating that an intermediate with more secondary structure was induced. Above 0.8 M GdnHCl, the ellipticity again decreased, leveling off to about 12.6% of the native ellipticity at 6 M GdnHCl.

ANS, as a fluorophore, increases its fluorescence quantum yield upon binding hydrophobic regions of proteins. Thus, the changes in ANS fluorescence can be related to the increase in accessible hydrophobic surface upon protein unfolding. Figure 1c shows that the native AK bound ANS without GdnHCl. The addition of low concentrations of GdnHCl (0–0.45 M) caused the ANS fluorescence to decrease slightly, indicating a decrease in the hydrophobic areas in the native AK that could bind ANS. However, between 0.5 and 0.75 M GdnHCl, the ANS fluorescence rose steeply. Further increase of GdnHCl concentration caused a gradual decrease of ANS binding, reflecting the progressive loss of the hydrophobic patches where ANS binds.

Effect of AK Concentration on the Unfolding Transition—Since AK is a homodimer, it is necessary to investigate the concentration dependence of the unfolding process. Figure 2a shows that the inactivation ($N_2 \leftrightarrow I$) was independent of concentration, suggesting that AK did not dissociate during this process. When we used the λ_{max} to monitor the unfolding process at different AK concentrations (Fig. 2b), no obvious changes were observed during the $N_2 \leftrightarrow I$ transition, which was consistent with the concentration independence of inactivation. However, in the

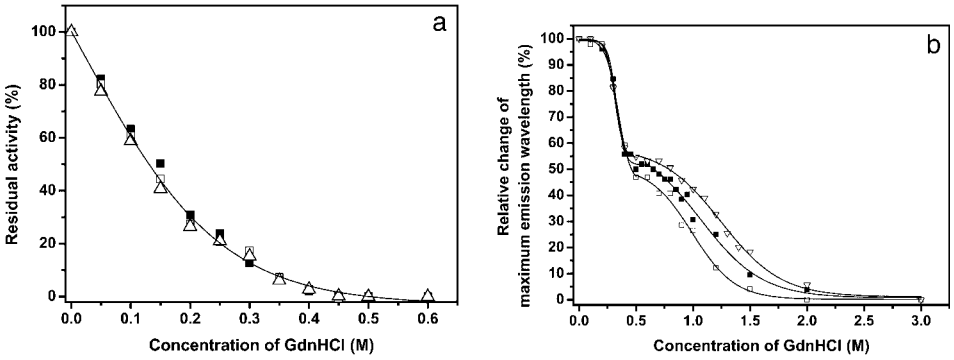


Fig. 2. **Dependence of inactivation and equilibrium unfolding of AK on protein concentration.** (a) Inactivation was monitored at protein concentration of 0.056 mg/ml (open squares), 0.12 mg/ml (solid squares), and 0.20 mg/ml (open triangles). (b) Unfolding was monitored by fluorescence emission with excitation at 280 nm at the same as protein concentrations in (a).

second unfolding transition there was a shift in the position of the midpoint to higher GdnHCl concentration (from 1.02 M to 1.30 M) (Table 2). The results indicated that subunits dissociated during the second unfolding process ($I_2 \leftrightarrow U$). Therefore, we inferred that the unfolding process involved at least one inactive homodimeric intermediate.

Equilibrium Folding Followed by Size-Exclusion Chromatography—Size-exclusion chromatography can be applied to the study of protein unfolding, because it is able to resolve changes in the hydrodynamic properties along the denaturation pathway and to detect the presence of intermediate states that are kinetically stable within the time scale of the chromatographic run (*19*).

A plot of coefficient K_{av} *vs*. GdnHCl concentration (Fig. 3b) was also used to monitor Stokes radius changes of the enzyme molecules during GdnHCl denaturation. Native AK showed a single elution peak (Fig. 3a) with elution volume 12.20 ml. At low GdnHCl concentrations (below 0.1 M GdnHCl), K_{av} increased sharply to about 0.37, while at concentrations between 0.1 and 0.4 M, K_{av} increased gradually, suggesting the appearance of an intermediate (I_1) with a much more compact structure than the native state (N_2). The peak split into two peaks in 0.5 M GdnHCl, and K_{av} also decreased greatly at this GdnHCl concentration due to the dissociation of dimer. Peak 1 (solid circles), still a dimer but with its enzyme molecules significantly loose compared to I_1, was defined as I_2. Peak 2 (open circles) was suggested to be an expanded monomer, smaller than the native dimer and larger than the native compact monomer. With the increase of GdnHCl concentration from 0.5 to 2.0 M, the elution volumes of P_1 and P_2 shifted gradually and continuously became smaller, indicating the gradual swelling of the intermediate I_2 and expanded monomer (indicated by the gradual decrease of K_{av}) (*20*). With further increase of the GdnHCl concentration to 3 M, there was a single peak with an elution volume of 11.38 ml. This peak remained the same when the GdnHCl concentration was increased to 6 M, suggesting that it corresponded to an unfolded monomer (U). The above results together suggested that two different dimeric intermediates, I_1 and I_2, were involved in the unfolding process, with I_1 existing below 0.5 M GdnHCl and I_2 above 0.5 M GdnHCl.

Glutaraldehyde Cross-Linking: Monitoring the Dissociation upon GdnHCl Unfolding—To confirm the results of FPLC, glutaraldehyde was used to cross-link AK in the presence of GdnHCl. Figure 4 shows the SDS-PAGE gel of the cross-linked AK. From 0 to 0.4 M GdnHCl, AK existed as a dimer. A transition from dimer to monomer was investigated in 0.5 M GdnHCl. From 0.5 M to 2.0 M, bands corresponding to a dimer and a monomer could be

Table 1. **Aggregation during reconstitution of arginine kinase denatured at high enzyme concentrations (2.4 mg/ml).**

Denaturant GdnHCl (M)	Concentration of AK in the solution (mg/ml)											
	Concentration of GdnHCl in the dilution buffer (M)											
	0	0.2	0.3	0.4	0.6	0.8	1.0	1.2	1.4	1.5	2.0	3.0
1.5	*↓	0.027↓	0.073↓	0.10↓	0.12	0.12	0.12	0.12	0.12	0.12	–	–
2.0	*↓	0.023↓	0.069↓	0.10↓	0.12	0.12	0.12	0.12	0.12	0.12	0.12	–
3.0	*↓	0.016↓	0.070↓	0.10↓	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12

↓, AK aggregates after dilution; *↓, AK aggregates after dilution. There was not enough AK left in buffer to measure by the Bradford method.

Table 2. **Fluorescence-derived thermodynamic parameters obtained from GdnHCl equilibrium denaturation of AK.**

AK (mg/ml)	$\Delta G_{0,ni}$ (kJ/mol)	$\Delta G_{0,iu}$ (kJ/mol)	m_{ni} (kJ·l/mol^2)	m_{iu} (kJ·l/mol^2)	$D_{50,ni}$ (M)	$D_{50,iu}$ (M)
0.056	18.2 ± 1.6	30.7 ± 2.8	53.6 ± 5.2	20.3 ± 2.7	0.34	1.02
0.12	17.8 ± 2.6	30.2 ± 2.3	56.0 ± 8.4	23.4 ± 2.6	0.33	1.15
0.20	18.5 ± 0.3	31.4 ± 1.6	59.0 ± 0.9	20.1 ± 1.4	0.32	1.30
Average	18.2 ± 1.5	30.8 ± 2.2	56.2 ± 4.8	21.3 ± 2.2	0.33	–

The data were obtained by fitting of the λ_{max} (the maximum emission wavelength, Fig. 2(b)) to a two-state denaturation model (details in "MATERIALS AND METHODS"). $\Delta G_{0,ni}$ and $\Delta G_{0,iu}$ were calculated in monomer units. $D_{50,ni}$ and $D_{50,iu}$ are the calculated GdnHCl concentrations at each transition midpoint.

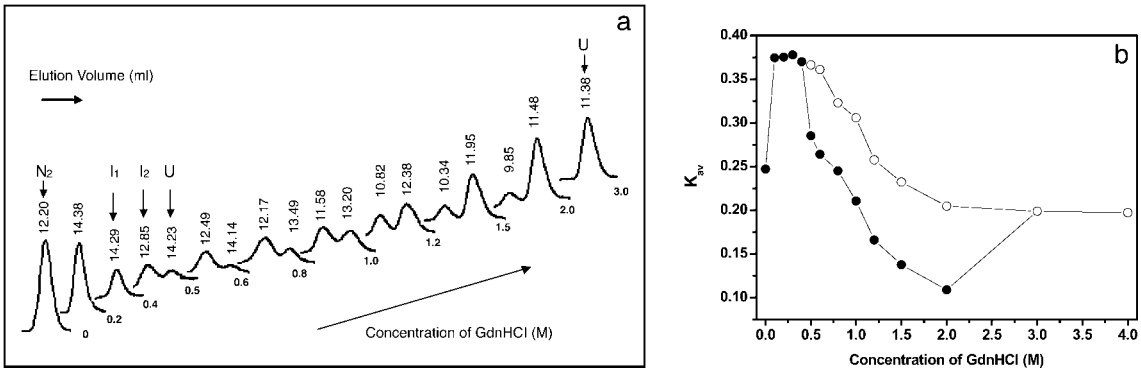


Fig. 3. **(a) Elution profiles in size exclusion chromatography (FPLC) of denatured AK.** Separation was carried out at 25°C. The elution volumes (ml) and the GdnHCl concentrations (M) are indicated in the figure. The enzyme concentration was 0.20 mg/ml. **(b) Effect of GdnHCl on K_{av} ($K_{av} = (V_e - V_0) / (V_t - V_0)$) of the AKs.** Curve (solid circles): dimer; curve (open circles): monomer.

seen clearly, with the former decreasing and latter increasing as the GdnHCl concentration rose. At 3 M GdnHCl, only the monomer band remained.

Reactivation and Reconstitution of AK upon Dilution of GdnHCl—To investigate the reversibility of the unfolding process, AK denatured at various concentrations of GdnHCl overnight was reconstituted in dilution buffers. We first chose the enzyme concentration of 0.25 mg/ml to ensure the soluble equilibrium conditions during denaturation and used fluorescence to monitor the structural changes of AK during the reconstitution process. Figure 5a indicates that AK denatured at below 0.4 M GdnHCl regained more than 80% of its catalytic activity, and its λ_{max} after dilution was around 322.5 nm, the same as for the native enzyme; AK denatured at above 0.5 M could not regain its catalytic activity, and its λ_{max} after dilution remained at around 335.5 nm, which corresponded to the λ_{max} when the enzyme was denatured at 0.5 M GdnHCl; and AK denatured at 0.4–0.5 M seemed to be in transition between the two states mentioned above. Since the refolding pathway of proteins often differs from the unfolding pathway, we tried to monitor the process by gradual dilutions. The results shown in Fig. 5b confirmed that the denaturation of AK at 0–0.4 M GdnHCl was reversible. They also indicated that AK denatured at 0.5–3.0 M GdnHCl refolded well when the dilution buffer contained a GdnHCl concentration of above 0.5 M; while at lower concentrations, the λ_{max} of AK remained at around 335.5 nm, indicating only partial refolding. When we increased the concentration of denatured AK to 0.45 mg/ml, results were similar except that AK began to aggregate partly when the dilution buffer contained a GdnHCl concentration of below 0.5 M. Further increase of the concentration of denatured AK lead to three different conditions as follows (Table 1; conditions when AK was denatured at 2.4 mg/ml): (*1*) at 0–0.4 M GdnHCl concentration, the unfolding processes were also reversible; (*2*) at 0.5–1.0 M, almost all AK molecules aggregated during

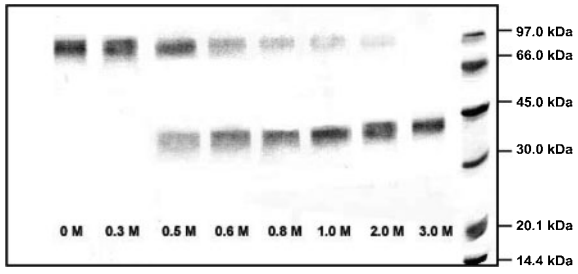


Fig. 4. **SDS-PAGE of AK during denaturation as a function of GdnHCl concentration (after cross-linking with glutaraldehyde).** The GdnHCl concentrations (M) are indicated in the figure.

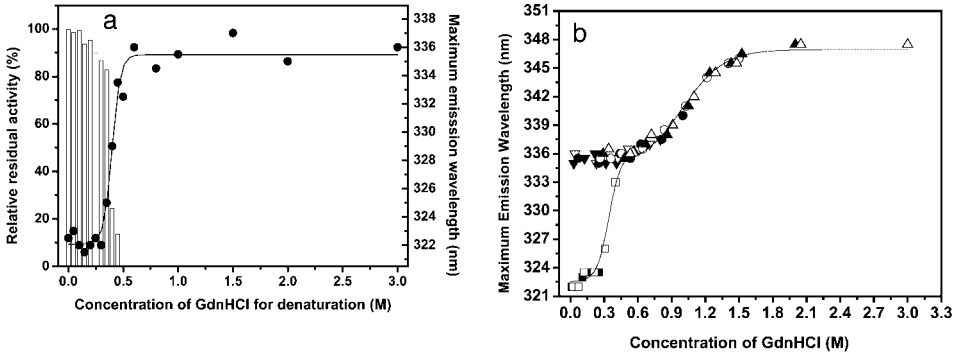


Fig. 5. **Reactivation and refolding of AK denatured in GdnHCl.** (a) Reactivation and refolding of AK *vs.* denaturation concentration of GdnHCl. AK denatured at various concentrations of GdnHCl was diluted 10-fold with dilution buffer and left to stand overnight at 25°C. Bars indicate the percentage of the reactivation and solid circles indicate the emission wavelength of maximum fluorescence after dilution. (b) The wavelength of maximum fluorescence emission as a function of GdnHCl concentration after the gradual reconstitution of AK. AK denatured at various concentrations of GdnHCl (solid squares,0.25 M; open squares, 0.40 M; inverted solid triangles, 0.60 M; inverted open triangles, 0.80 M; solid circles 1.0 M; open circles, 1.5 M; solid triangles, 2.0 M; open triangles, 3.0 M) was gradually reconstituted by 10-fold dilution with dilution buffer. The solid line was obtained from denaturation, in the same way as in Fig. 1b. Reconstitutions was carried out overnight at 25°C. The enzyme concentration in all experiments was 0.25 mg/ml.

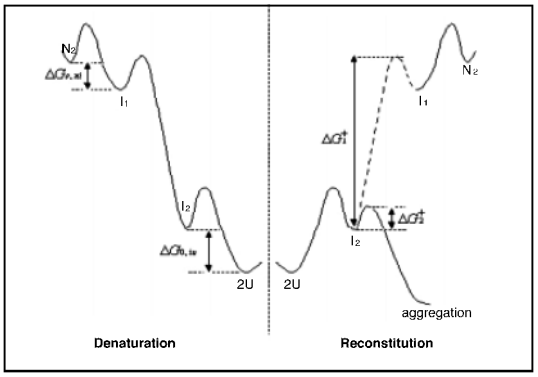


Fig. 6. **Free energy coordinate diagrams consistent with the behavior of AK under denaturation and reconstitution.** The curve on the left shows the denaturation conditions, and the values of $\Delta G_{0,ni}$ and $\Delta G_{0,iu}$ were as calculated in Table 2. The curve on the right shows the reconstitution of denatured AK in dilution buffer. Since ΔG_1^+, separating I_1 and I_2, was insurmountable, while ΔG_2^+, separating I_2 and aggregation proteins, was much smaller and surmountable, partly folded I_2 was prone to aggregate when dilution further. The solid curve indicates that the process is available, and the dashed curve that it is unavailable.

denaturation; (*3*) at 1.5–3.0 M, AK refolded well when the dilution buffer contained a GdnHCl concentration of above 0.5 M, while at lower concentration, the enzyme molecules partly aggregated, and λ_{max} of those soluble in the buffer remained at around 335.5 nm. The above results are all consistent well with the existence of a barrier at 0.4–0.5 M GdnHCl that prevented denatured AK from refolding to its native state.

Quantitative Evaluation of the GdnHCl Unfolding—The above results suggest that the multiphasic GdnHCl unfolding involves two dimeric intermediates (I_1 and I_2) and two reversible steps ($N_2 \leftrightarrow I_1$ and $I_2 \leftrightarrow 2U$). Each of the two steps was analyzed using a two-state equation (details in "MATERIALS AND METHODS"). If this model provides a reasonable thermodynamic description of the denaturation reaction, the values for the conformational stability from experiments performed at different protein concentrations should be the same. Results of fitting measured values to the model are given in Table 2. Analysis of the separate denaturation experiments showed that in the protein concentration range from 0.056–0.20 mg/ml, the ΔG values were almost identical. The free energy change, ($\Delta G_{0,ni}$, and the denaturant m value, m_1, associated with the first reversible step in GdnHCl denaturation, were 18.2 kJ/mol and 56.2 kJ liter/mol^2, respectively. The second reversible step involved subunit dissociation of AK. The free energy change, $\Delta G_{0,iu}$, and the denaturant m value, m_2, were 30.8 kJ/mol and 21.3 kJ liter/mol^2, respectively, also constant over the protein concentration range used.

Table 3. **Thermodynamic parameters obtained from inactivation of AK by GdnHCl.**

AK (mg/ml)	ΔG_i (kJ/mol)	m_i (kJ l/mol^2)	$D_{50,i}$ (M)
0.056	4.5 ± 0.5	31.9 ± 2.0	0.13
0.12	4.4 [1; 2] ± 0.28	30.5 ± 1.4	0.14
0.20	4.3 ± 0.5	32.4 ± 3.0	0.12
Average	4.4 ± 0.43	31.6 ± 2.1	0.13

The data were obtained by fitting of the enzyme activity (Fig. 2a) to a two-state denaturation model (details in "MATERIALS AND METHODS"). $D_{50,i}$ is the calculated GdnHCl concentration at each transition midpoint.

Moreover, in addition to the fluorescence methods, we used enzymatic activity to monitor the denaturation of AK. The same samples were used as in the fluorescence spectroscopic GdnHCl unfolding analysis. Analysis of the inactivation curves led to the results shown in Table 3. The calculated free energy changes ΔG_i and $D_{50,i}$ were 4.4 kJ/mol and 0.13 M, both much smaller than those determined from fluorescence measurement. Thus, it was reasonable to infer that the inactivation took place before the unfolding of the molecule as a whole during the first transition state $N_2 \leftrightarrow I_1$.

DISCUSSION

The results reported in this paper from several independent measurements indicate that the equilibrium unfolding of dimeric AK is a multiphasic process. The progressive addition of GdnHCl leads first to a compact dimeric intermediate I_1, accompanied by the disappearance of the catalytic activity, second to the sudden unwinding of I_1 to I_2, which is still dimeric but looser in structure due to the dissociation of the intermediate dimer to monomers; and third to further dissociation of I_2 to monomers U, accompanied by continuous swelling of both I_2 and U.

Inactivation and the Appearance of the Dimeric Intermediate I1 ($N_2 \leftrightarrow I_1$)—In the native state, AK bound to a significant amount of ANS. It was previously suggested that ANS bound to nucleotide sites on proteins and was displaced by the addition of nucleotides (*21*, *22*). Since ATP is one of the substrates of AK, ANS binding to native enzymes could be due to an interaction with the ATP binding sites on the enzyme. At low GdnHCl concentrations of 0–0.4 M, AK was inactivated quickly. Its tertiary structure monitored by fluorescence and the secondary

Table 4. **Thermodynamic parameters of some proteins in equilibrium denaturation (*31*).**

Protein name	M.W. (kDa)	Denaturant	Model	$\Delta G_{0,ni}$ (kJ/mol)	$\Delta G_{0,iu}$ (kJ/mol)	Reference
Arginine kinase (monomer)	1 × 40	GdnHCl	$N \leftrightarrow I \leftrightarrow U$	15.8	9.2	(*8*)
Arginine kinase (dimer)	2 × 42	GdnHCl	$N_2 \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow 2U$	18.2	30.8	This paper
Creatine kinase	2 × 43	GdnHCl	$N_2 \leftrightarrow 2I \leftrightarrow 2U$	18.7/16.4	10.3/10.9	(*8*) (*30*)
Organophosphorus hydrolase	2 × 6	Urea (GdnHCl)	$N_2 \leftrightarrow I_2 \leftrightarrow 2U$	18.0	150.9	(*24*)
Prostatic acid phosphatase	2 × 0	GdnHCl	$N_2 \leftrightarrow 2I \leftrightarrow 2U$	17.7	14.2	(*31*)
Ascorbate oxidase	2 × 70	GdnHCl	$N_2 \leftrightarrow I_2 \leftrightarrow 2U$	15.1	49.6	(*28*)

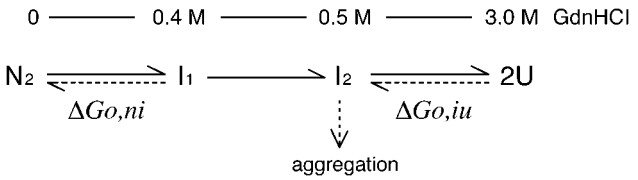
M.W.: molecular weight; $\Delta G_{0,ni}$ and $\Delta G_{0,iu}$: free energy changes (in monomer units) associated with transitions from the native to intermediates and intermediates to the unfolded states, respectively.

structure measured by CD decreased sharply, accompanied by an increase in compactness of the enzyme molecules, which was indicated by the increased elution volume in FPLC and the gradual decrease in the ANS binding. In addition, both the independence of the enzyme activity on its concentration (Fig. 4) and the result of the glutaraldehyde cross-linking showed that dimer dissociation did not appear in this period. All these results suggested that the inactivation preceded the dissociation and the unfolding process involved a dimeric intermediate. We noticed that during this period, the CD of the intermediate in the far-UV (220 nm) was decreased by about 80%, suggesting partial unfolding and disruption of considerable secondary structure. The decrease maybe due to a local loss of structure in an exposed part of the protein (*23*). However, the change in CD from N_2 to I_1 may not reflect just secondary structure content, since acromatic residues also contributed to the CD in this region (*24*). Anyway, it was obvious (Fig. 1d) that lower concentrations of GdnHCl were required to bring about inactivation than were required to produce significant structural changes of the enzyme molecule, indicating that the AK inactivation was due to the distortion of the active site, and the active site was more flexible than the whole enzyme molecule (*25*).

Sudden Unwinding of I_1 to Another Dimeric Intermediate I_2 ($I_1 \rightarrow I_2$)—When GdnHCl concentration rose from 0.4 to 0.5 M, although other spectroscopic measurements did not change greatly during this period, results of FPLC suggested that sudden changes took place in this period: the compact dimeric intermediates I_1 (elution volume: 14.29 ml) unwound themselves to I_2 (elution volume: 12.85 ml) and then began to dissociate into monomers. A comparison of the molecular compactness between I_1 and I_2 indicated that I_2 was more like native AK (elution volume: 12.20 ml) than was I_1.

Dissociation of Dimeric Intermediate I_2 to Monomers U ($I_2 \leftrightarrow 2U$)—At 0.5–3.0 M GdnHCl, I_2 gradually dissociated into monomers. Specific features of the intermediates I_2 can be summarized as follows: (1) the buried hydrophobic residues of the native state are exposed, as indicated by the increase in ANS binding fluorescence; (2) it is prone to aggregate at high enzyme concentration (above 0.45 mg/ml); (3) its tertiary sturcture is further substantially disordered, as indicated by the further red shift of 13 nm of λ_{max}; (3) there is a significant residual amount of secondary structure, 59% of the native AK at most, as indicated by θ_{220}. These features resemble those of the molten globule states already described in the unfolding of both smaller proteins (*26*, *27*) and the "molten dimer" state in larger oligomeric proteins (*28*). Interestingly, we found that CD ellipticity at 220 nm (θ_{220}) increased from 0.5 M to 0.8 M GdnHCl and then decreased again. It is known that GdnHCl at low concentrations could induce refolding from the acid –unfolded state, due to its anion effect (*29*). However, further experiments are required to find an exact explanation for the increase in θ_{220}.

Reactivation and Reconstitution—To investigate the refolding process from the point of view of equilibrium, reconstitution of denatured AK was monitored by gradual dilution in various reconstitution buffers. The critical concentration for the refolding process seems to lie between 0.4 M and 0.5 M GdnHCl, because AK denatured at GdnHCl concentrations below 0.4 M could regain over 80% of its catalytic activity, while that denatured at above 0.5 M reconstituted only to states similar to AK denatured in 0.5 M GdnHCl at low enzyme concentration, and increasing the enzyme concentration did not increase the proportion of folded protein but lead to aggregation instead. Since I_1 and I_2 were the major existing states of AK present in 0.4 M and 0.5 M GdnHCl, respectively, it was reasonable for us to infer that the failure of AK to reconstitute from 0.5 M GdnHCl to 0.4 M GdnHCl may be because the large free energy of activation ΔG_1^+, which separated I_2 and I_1, was insurmountable during dilution. On the contrary, since ΔG_2^+, which separated I_2 and the aggregated proteins, was much smaller and surmountable, AK tended to aggregated in this transition state as its concentration was increased (Fig. 6).



Scheme 1.

As a result of our studies, the following simplified scheme of denaturation and reconstitution pathway of AK was proposed (Scheme 1).

In this scheme, solid arrows indicate the denaturation pathway and dashed arrows the reconstitution. It shows that the whole denaturation process involves two reversible processes and at least two dimeric intermediates (I_1 and I_2). Moreover, that the denatured AK could not refold to its native state by simple dilution may be due to the insurmountable free energy of activation ΔG_1^+, which separates these two intermediates. Intermediate I_1 is an inactive intermediate with a compact dimeric structure, while I_2 possesses some specific characteristics similar to molten globule states.

Comparison of the Energetics of Unfolding in the Phosphagen Kinases and Other Dimeric Proteins—Studies on unfolding of proteins shed light on the role of quaternary interactions in the formation and stabilization of protein subunits. Previous studies on the unfolding of dimeric proteins presented two models: one involving a monomeric intermediate ($N_2 \leftrightarrow 2I \leftrightarrow 2U$), and one involving a dimeric intermediate ($N_2 \leftrightarrow I_2 \leftrightarrow 2U$) (*31*). In energetics, the difference of between the two pathways focuses on the second step: ΔG of the second pathway, which involves the unfolding and dissociation of the dimeric intermediate, is much higher than that in the first pathway. Examples of several dimeric proteins and their thermodynamic parameters are listed in Table 4. The table shows that the ΔG values of the first step in these proteins are comparable regardless of their unfolding pathway. Moreover, since dimeric AK stands at a special position between monomer AK and dimeric CK in the family of phosphagen kinase, the energetics of unfolding was compared among them (Table 4). Although the dimeric AK is closer to CK in evolution, their unfolding pathways differ greatly: one involved dimeric intermediates (this

paper) and the other a monomeric one (*30*). Therefore, ΔG of the intermediates to the completely unfolded monomers in the dimeric AK (30.8 kJ/mol) was much higher than CK (21.8 kJ/mol) (*30*). As to the monomer AK, ΔG (9.2 kJ/mol) (*8*) of its intermediate to the unfolded monomers was also much lower than the dimeric one. Therefore, we conclude that in the dimeric AK, the quaternary structure is very important in its overall stability. That is, the interactions between the dimeric AK's two subunits stabilize the general rather than the local structure.

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