Refolding of urea-denatured adenylate kinase

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The refolding of urea-denatured adenylate kinase (EC 2.7.4.3) has been followed by formation of the secondary structure, change of surface hydrophobicity and recovery of catalytic activity. During refolding of adenylate kinase with a 20–80-fold dilution of 4 M urea-denatured enzyme at 10 °C, the formation of the secondary structure is a fast process with a rate constant of $> 0.16 \text{ s}^{-1}$. Transient enhancement of the 8-anilino-1-naphthalenesulphonate (ANS) fluorescence intensity is followed by a fluorescence decrease to the level equal to the value characteristic of native enzyme. The desorption of ANS binding fluorescence is relatively slow and can be fitted to a first order reaction with a rate constant of 0.004 s^{-1} when the ANS is present in the dilution buffer. The desorption of ANS-binding fluorescence is accelerated in the presence of nucleotide sub-

strates. The rate constants are increased to 0.049, 0.029, 0.028 and 0.029 s^{-1} in the presence of 1 mM AMP, MgATP, ATP and ADP respectively. The refolding rate constant calculated from the initial fluorescence intensity after mixing ANS with protein at different refolding intervals is 0.016 s^{-1} , which is faster than those obtained when ANS is present throughout the refolding process, indicating that the binding of ANS with a partially folded intermediate retards its further refolding to its native structure. The reactivation rate is even faster than the rates of refolding monitored in the absence of substrates, showing that the refolding is accelerated in the presence of the substrates. A possible refolding pathway and the accelerating effect of substrates are discussed.

INTRODUCTION

Many studies have suggested that protein folding proceeds via transient partially ordered intermediates known as the molten globule state, which exhibits native-like secondary structure, compact globularity, large exposed hydrophobic surfaces, and the absence of rigid side-chain packing [1–6]. Stopped-flow CD, absorbance and fluorescence have provided powerful ways to follow the characteristics of rapidly formed intermediates [7–9]. ANS (8-anilino-1-naphthalene sulphonate) fluorescence has also been used to detect the molten globular intermediate since it binds ANS, leading to stronger fluorescence than with the native and completely unfolded proteins [10]. The application of this method to several enzymes has produced useful results [9,11,12]. In the present study adenylate kinase (AK) was chosen for studying the mechanism of protein folding.

AK (EC 2.7.4.3) is a small monomeric enzyme that catalyses the phosphorylation of AMP by ATP to form two ADP molecules [13]. The rabbit muscle cytosolic enzyme contains 194 amino acid residues in a single peptide chain. It has two structural domains responsible for MgATP and AMP binding, which lead to conformational changes of the enzyme [14–17] and large domain movements [18,19]. The substrate binding sites and the mechanism of enzyme catalysis have been well investigated [20–22]. However, the conformation and activity changes during unfolding and refolding of this enzyme and the importance of conformational integrity for its activity have not been thoroughly explored. Previous work in this laboratory has studied the activity and conformational changes of AK during thermal denaturation [23] and guanidine unfolding [24]. The results show that the unfolding of AK by guanidine hydrochloride does not follow an ‘all-or-none’ mechanism but that partially folded intermediates exist on the denaturation pathway. To further understand the folding process of this enzyme, the refolding of urea-denatured AK has been followed by secondary structure formation, change of surface hydrophobicity, and recovery of catalytic activity.

MATERIALS AND METHODS

Materials

ADP, NADP, hexokinase, and glucose-6-phosphate dehydrogenase were Sigma products. Urea was a Nacalai Tesque (Japan) product. Other reagents were local products of analytical grade used without further purification. Urea solutions were always freshly prepared. The enzyme AK was prepared according to Zhang et al. [23].

Activity assay of AK

Native AK was assayed by following the reduction of NADP in a coupled enzyme solution with hexokinase and glucose-6-phosphate [23]. The reaction mixture contained 2.5 mM ADP, 2.1 mM Mg acetate, 6.7 mM glucose, 0.67 mM NADP, 10 units hexokinase, and 5 units glucose-6-phosphate dehydrogenase in 50 mM Tris/HAc buffer (pH 8.1). The activity of AK in different concentrations of urea was measured by the acid-stopped method. The denatured enzyme was first mixed into the substrate solution containing 25 mM MgADP and the same concentration of urea for a certain period of time. Then an equal volume of 1 M trichloroacetic acid was added to quench the reaction. BSA was then added to a final concentration of 10 mg/ml as a carrier protein, and this solution was centrifuged for 10 min at room temperature at 13000 r.p.m. The pH of the supernatant was immediately adjusted to 8. The residual activity of the denatured enzyme was calculated from the amount of ATP in the supernatant, measured in the same coupled assay system as for the native enzyme. The concentration of AK was determined by absorption at 280 nm with a specific absorbance coefficient of $A_{1\text{cm}}^{1\%} = 5.2$.

Conformational changes during unfolding and refolding

A Jasco-720 spectropolarimeter was used for CD measurements in the far-UV region of 200–250 nm. In the time course of

Abbreviations used: AK, adenylate kinase; ANS, 8-anilino-1-naphthalenesulphonate; DTT, DL-dithiothreitol.

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refolding, ellipticity was followed at 222 nm for changes of secondary structure. Changes of absorbance at 287 nm were measured with a Beckman DU-7500 spectrophotometer. The fluorescence intensity of ANS bound to AK was measured with a Perkin-Elmer MPF-66 fluorescence spectrofluorimeter with excitation at 378 nm and emission at 478 nm. The kinetics of ANS fluorescence change during refolding were monitored using a Hitachi F-4500 spectrofluorimeter with the same conditions as in the steady-state measurements. The data was fitted to the eqn. (1):

$$A_t = A_0 \exp(-kt) + A_\infty \quad (1)$$

Equilibrium unfolding and refolding

Equilibrium unfolding of AK was studied by incubating the native enzyme with various concentrations of urea for 24 h in 50 mM Tris/HCl buffer, pH 8.1, containing 1 mM EDTA and 1 mM DL-dithiothreitol (DTT) at 25 °C. The equilibrium refolding was effected by dilution of 4 M urea denatured enzyme with 50 mM Tris/HCl buffer containing 1 mM EDTA, 10 mM DTT and various concentrations of urea and incubating for 24 h at 25 °C.

RESULTS

Equilibrium denaturation of adenylate kinase

The initial and final states of reactivation and refolding were defined by studying the equilibrium unfolding and refolding of AK in different concentrations of urea.

Figure 1 Comparison of activity and conformational changes of AK in different concentrations of urea

Relative changes of enzyme activity, θ_{222} , A_{287} and ANS fluorescence are shown as a function of urea concentration during equilibrium unfolding and refolding. Enzyme concentrations were 20 nM, 10 μ M, 20 μ M and 5 μ M for activity, θ_{222} , A_{287} and ANS fluorescence respectively. For the procedure of equilibrium unfolding and refolding see the Materials and methods section.

Table 1 Thermodynamic denaturation parameters for adenylate kinase

Data were taken from Figure 1.

Method	$\Delta G_{N-U}^{\circ}(\text{kJ})$	$m_{N-U}(\text{kJ} \cdot \text{M}^{-1})$	$C_m \text{ (M)}$
CD (222 nm)	19.6 ± 2.0	16 ± 1.6	2.1 ± 0.2
Absorbance (287 nm)	16.2 ± 1.6	9.3 ± 0.9	2.1 ± 0.2
ANS binding fluorescence (487 nm)	15.6 ± 1.6	7.0 ± 0.7	2.1 ± 0.2

Table 2 Comparison of refolding rate constants measured by different methods

Method	Activity	CD		ANS fluorescence				
		Fast	Slow	1 mM AMP	1 mM MgATP	1 mM ATP	1 mM ADP	
Rate constant (s ⁻¹)	0.025	> 0.16	—	0.004	0.049	0.029	0.028	0.029

Activity

The enzyme activity of AK in the presence of different concentrations of urea obtained from equilibrium unfolding (●) and refolding (○) (see Materials and methods section) are shown in Figure 1. The enzyme is activated in the presence of low concentrations of urea and the activity is 1.5 times the native activity in the presence of 1.0 M urea. The enzyme activity then decreases with increasing urea concentration, with 50 % native activity being retained in 2.1 M urea, and total inactivation in the presence of 3 M urea. The curves derived from unfolding and refolding treatments are superimposable showing that the change of enzyme activity is thermodynamically reversible. Thermodynamic denaturation parameters for AK are shown in Table 1.

Secondary and tertiary structure

Adenylate kinase is an α/β -type protein rich in both α -helix (62.9 %) and β -sheet (12.4 %) [25]. Its CD spectrum typically shows two negative peaks, at 222 and 208 nm [23,26]. Figure 1 (◇ and ◆) shows the decrease in relative magnitude of the ellipticity at 222 nm (θ_{222}) with increasing urea concentration. There is no obvious change of θ_{222} in 1 M urea in which the enzyme showed maximum activity. There was a 50 % change in θ_{222} at a urea concentration of 2.1 M.

Figure 1 (□ and ■) shows the dependence of the relative change of absorbance at 287 nm (A_{287}) on urea concentration. The trends are consistent with the change in θ_{222} . The absorption spectra monitor the structural environment of the aromatic residues in the molecule, and the change of A_{287} is more sensitive to the microenvironmental changes in tyrosine residues. Comparison of the CD and absorption results (Table 1) show that the unfolding of AK during urea denaturation is highly co-operative.

Surface hydrophobicity

The fluorescent ligand ANS was used to probe the surface hydrophobicity of AK. Our previous investigation showed that native AK can bind one ANS molecule with a K_d of about 56 μ M and that the presence of MgATP, ADP or AMP decreases the fluorescence emission intensity, suggesting that the ANS binding site is at or close to the substrate-binding sites [24]. The emission

Figure 2 Kinetics of the reactivation of adenylate kinase at 10 °C

Curve 1: time course of reactivation of 4 M urea-denatured AK upon 100-fold dilution in a buffer of 50 mM Tris/HCl (pH 8.1) containing 1 mM EDTA and 10 mM DTT. Curve 2: time course of reactivation of 4 M urea denatured enzyme in the same buffer as curve 1, but containing 1.04 M urea. Initial concentration of enzyme was 2 μ M; final enzyme concentration was 0.02 μ M.

intensity at 478 nm of ANS bound to AK gradually decreases with increasing urea concentration (Figure 1, ∇) as a result of the decrease of the ANS binding and/or the quantum yield of bound-ANS fluorescence emission. In 1.0 M urea, in which the enzyme showed maximum activity, the fluorescence at 478 nm decreased to 85% of the native state value, suggesting that a subtle conformational change around the enzyme active site might accompany the enzyme activation.

Reactivation kinetics for AK

Denatured AK is almost fully unfolded in 4 M urea, Figure 1. The refolding of AK was initiated with a 20- to 80-fold dilution of 4 M urea-denatured enzyme to 50 mM Tris/HCl buffer, pH 8.1, containing 1 mM EDTA and 10 mM DTT at 10 °C. Aliquots (100 μ l) of refolding enzyme were taken at various refolding time intervals and assayed at 10 °C to measure the restored enzyme activity. In this experiment, the estimated dead time between mixing the solution twice and recording the first data point was 18 s. Figure 2 presents the increase of enzyme activity versus the refolding time. Curve 1 is the time course of reactivation of 4 M urea-denatured AK in the buffer containing 0.04 M urea. The activity is 100% restored in 200 s with a rate constant of 0.025 s⁻¹. Curve 2 shows the time course of reactivation of denatured enzyme in a buffer solution containing 1.04 M urea. The enzyme refolds to the activated state (with an activity 1.4 times greater than the native enzyme) with a rate constant of 0.011 s⁻¹, suggesting that the activated state of AK might appear in its refolding pathway as a transient intermediate or lies off the refolding pathway. The increase of enzyme activity in the presence of low concentrations of urea could be due to changes of minor conformation and/or solvation.

Figure 3 Kinetics of secondary structure formation

Lower curve: time course of ellipticity at 222 nm after 20-fold dilution of 4 M urea denatured AK at 10 °C with buffer containing 50 mM Tris/HCl, 10 mM DTT and 1 mM EDTA at pH 8.1. Upper curve, the time course of 4 M urea-denatured AK after 20-fold dilution with buffer containing 4 M urea. Final enzyme and urea concentrations were 12.5 μ M and 0.2 M respectively.

Kinetics of secondary structure formation

Figure 3 shows the time course of the change of ellipticity at 222 nm (θ_{222}) during refolding of AK. The upper curve is a control showing that θ_{222} for 4 M urea-denatured AK does not vary with time when the enzyme is unfolded and that there is no

Figure 4 Kinetics of ANS fluorescence desorption

Time courses of desorption of ANS fluorescence after addition ANS to the refolding system at times of 0, 12, 40, 91, 121, 188, 320, 478, 725 and 1090 s (curves 1 to 10) after the beginning of the refolding process. The concentration of adenylate kinase, ANS and urea in the refolding system were 5 μ M, 0.2 mM and 0.2 M respectively. For rate constants see Table 3.

Figure 5 Comparison of the kinetics of ANS binding desorption fluorescence (curve 2) and of refolding in the absence of ANS (curve 1) of AK

Data taken from Figure 4 curves 1 to 10. Initial fluorescence intensities at various refolding times after the delayed addition of ANS were obtained by extrapolation of the decay curve to zero time.

Table 3 ANS desorption rate constants

Delay time (s)	0	12	40	91	121	188
Rate constant (s^{-1})	0.004	0.004	0.0035	0.0034	0.0034	0.0028

time-dependent change of θ_{222} upon enzyme dilution. The lower curve shows very rapid formation of secondary structure within a burst phase. The first signal recorded in this experiment is 18 s after a 20-fold dilution of 4 M urea-denatured enzyme at 10 °C in a cuvette with 1 mm light pathlength. Some 95 % of the secondary structure is formed in the dead time, and the θ_{222} signal then gradually decreases to the value of the native state. The rate constant of the secondary structure formation is larger than $0.16 s^{-1}$, indicating that the early step of refolding is the fast formation of an intermediate which contains most of the secondary structure.

Kinetics of ANS binding fluorescence and its desorption

The time courses of ANS binding and its desorption during the refolding of AK are shown in Figure 4. Curve 1, the time course obtained when using the conventional approach in which ANS is in the dilution buffer, shows the very rapid transient enhancement of the ANS fluorescence followed by a slower return to a low level as the protein folds to its native conformation. The desorption-of-ANS fluorescence induced by the decrease of the exposed surface hydrophobicity as the protein folds further can be fitted with a single exponential phase with a rate constant of $0.004 s^{-1}$. Curves 2–10 show the time courses of the ANS desorption fluorescence by adding ANS at different time intervals (from 16 to 1106 s) after the refolding of AK had been allowed to proceed in the absence of ANS. The rate constant calculated from the initial intensity of ANS fluorescence at different time intervals thus reflects the refolding rate of the protein in the absence of ANS. This refolding curve, Figure 5 curve 1, also follows a single exponential function but with a rate constant of $0.016 s^{-1}$, which is faster than the rate measured from desorption

Figure 6 Effect of substrates on the refolding of adenylate kinase

Curve 1: time course of ANS fluorescence desorption during refolding of AK under the same conditions as Figure 4. Curves 2, 3, 4 and 5 were in the presence of 1 mM of MgATP, AMP, ATP and ADP respectively.

of ANS fluorescence (Table 3). This indicates that the binding of ANS on a folded intermediate retards further refolding.

Effect of nucleotide substrates on refolding kinetics

The refolding of AK in the presence of different adenosine nucleotides has also been followed by ANS fluorescence. Figure 6 shows the time course of ANS desorption during refolding when ANS and various substrates are present in the dilution buffer. Curve 1 represents the course of ANS desorption fluorescence during refolding of AK. The time courses of ANS desorption fluorescence during refolding of AK in the presence of MgATP, AMP, ATP and ADP are shown in Figure 6 curves 2 to 5 respectively. The rate constant is increased to 0.049, 0.029, 0.028 and $0.029 s^{-1}$ in the presence of 1 mM AMP, MgATP, ATP and ADP respectively (Table 2), suggesting that the folding of AK is accelerated in the presence of its substrates.

DISCUSSION

The refolding of AK was successfully followed by change of enzyme activity, θ_{222} , A_{287} and ANS fluorescence.

The refolding pathway of adenylate kinase

In protein folding studies, the two-state theory of kinetics of folding is based on identical relative changes in conformation measured by different methods in the folding–unfolding transitions [27]. Experimental studies of protein folding and unfolding have revealed the existence of intermediates in the pathway of folding and unfolding of many proteins, in both kinetic and equilibrium studies [28]. It has been reported that the unfolding of AK by guanidine hydrochloride is a sequential process and that there are at least two intermediate states recognized by size-exclusion chromatography [24]. In the present investigation, both 95 % of the native ellipticity and pronounced ANS binding occur within 18 s at 10 °C, indicating that secondary structure and hydrophobic clusters are both formed to a significant extent in the early folding event. This intermediate is consistent with the so-called ‘molten globule state’, which contains pronounced secondary structure and a much more

exposed hydrophobic core than the native state. The further refolding of the intermediate gradually leads to correct side-chain packing and builds the tertiary structure to the activated state or the native state, depending on the final urea concentration, as indicated by the absorbance at 287 nm, ANS fluorescence and enzyme activity. The further adjustment of the structure leads to its native state. Based on the above results, the activated state could be an on-folding pathway intermediate (eqn. 2):

or an off-folding pathway intermediate (eqns. 3 and 4):

where U is the unfolded enzyme, I is the fast-forming, collapsed intermediate, which contains most of the secondary structure and extensively exposed hydrophobic regions, N' is the activated state and N is the native state. In the on-folding pathway mechanism (eqn. 2), a transient intermediate (N') other than the molten globule state (I) is present on the folding pathway before reaching the native state, suggesting that, during refolding of AK *in vitro*, the recovery of biological activity may not reflect the structure returning to the final native state [29].

The folding kinetics of adenylate kinase are altered by ANS binding to the folding intermediate

In previous investigations, the interpretation of ANS binding has been based on two assumptions: (i) that ANS is bound instantaneously so that any time-dependent effects could be assumed to reflect conformational changes in the protein; and (ii) interaction with ANS would not significantly perturb the folding pathway or its kinetics. A number of investigations have shown that these assumptions are correct since no influence of ANS on the refolding of carbonic anhydrase was observed [30,31]. However, Engelhard and Evans [32] have reviewed the method and suggested that a combination of methods is likely to be necessary to build up a reasonable picture of early folding events. In previous kinetic studies, ANS fluorescence has generally been investigated by the conventional method of simply mixing with ANS in the refolding buffer. In the present study, ANS was added to the refolding system after folding had proceeded for a certain time and the folding process was reconstructed by plotting the initial fluorescence intensity as a function of the folding time. The rate constant of refolding measured by the initial fluorescence intensity using delayed addition of ANS is faster than that measured by ANS desorption fluorescence when ANS is present throughout the entire process. This result provides strong evidence that binding of ANS with folding intermediates retards further refolding to the native state but there is no evidence that it alters the folding pathway. The limitation of further refolding of ANS-bound intermediates could be due to the slow release of the bound ANS from the folding intermediate.

Refolding of AK is accelerated in the presence of substrates

During protein folding *in vitro*, the recovery of biological activity has been assumed to reflect the recovery of the protein structure

to its native state. The folding pathway reported above shows that AK gradually refolds from its unfolded state to its native state; hence the enzyme can only regain its activity after refolding to the N' and/or N state. If so, the reactivation rate of AK should be equal to or slower than the refolding rate measured by CD and ANS fluorescence. However, the above results show that the reactivation rate is faster than the spectroscopically monitored refolding rate and the apparent inconsistency can be explained by the substrate acceleration effect. The refolding of enzyme molecules is generally measured in the absence of substrate, but reactivation must be followed by measuring the rate of the substrate reaction. The refolding rates measured by ANS binding and desorption are faster in the presence of substrates, which is the acceleration effect that substrates have on the refolding of AK. How the substrate competes with ANS binding and accelerates the folding is not clear; however, it seems likely that substrates bind to the transition state preferentially or more strongly than ANS.

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