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Activation of Adenylate Kinase by Denaturants Is Due to the Increasing Conformational Flexibility at Its Active Sites

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The unfolding of adenylate kinase in urea or guanidine hydrochloride solutions was measured by UV absorbance at 287 nm, circular dichroism at 222 nm and 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence. At concentrations less than 1.8 M of urea, the secondary and tertiary structures of AK were not noticeably perturbed. In contrast, the activity of the enzyme underwent significant changes, increasing about 1.6-fold when the urea concentration was increased to 1 M. The enzyme activity then decreased with further increases of the urea concentration. We also observed that the kinetics of ANS binding to AK by fluorescence was biphasic. The fast phase completed within the dead-time of the stopped-flow apparatus used, while the slow phase ended in about 10 minutes. The slow phase fluorescence rate constants increased from 0.0073 s^{-1} in the absence of denaturants to 0.0100 s^{-1} (about 1.4-fold) at 1 M urea and then decreased at higher urea concentrations. Similar results were obtained when guanidine hydrochloride was used as a denaturant. The change of the enzyme activity coincided with that of the rate of ANS binding during denaturation by low concentration of denaturants, suggesting that the activation of AK by denaturants may be due to the increasing conformational flexibility at its active site. © 1997 Academic Press

Key Words: **adenylate kinase; activation; ANS fluorescence; denaturants; conformational flexibility; active sites.**

A series of studies in this and other laboratories that compared the activity with conformational changes of enzymes during unfolding by chemical denaturants have demonstrated that for some enzymes (see refs. 1-3 and references there-in) [1-3] inactivation generally precedes unfolding of the molecules as a whole, leading to the hypothesis that the active sites of these enzymes were situated in limited and more flexible regions and were therefore more easily perturbed than the rest of the molecules. Recently we reported that chicken liver dihydrofolate reductase (DHFR) [4] can be activated by urea or guanidine hydrochloride over a wide range of concentration even though urea and guanidine hydrochloride have been commonly used as protein denaturants. Such an effect suggested that the active site of DHFR was more easily perturbed by denaturants. Based on these lines of evidence, the activated enzyme appears to be more open and flexible at the active site, which may enhance the catalytic potential of the enzyme [1-4]. However, since the activation of DHFR by denaturants was very fast, the kinetics of this process was very difficult to be measured. Direct evidence for enzyme activation due to increased flexibility of the conformation at the active site remains uncovered.

Adenylate Kinase (AK, EC2.7.4.3), the smallest phosphotransferase, catalyzes the transfer of a phosphoryl group following this reaction: MgATP + AMP ⇌ MgADP + ADP. It is abundant in muscle and mitochondria where the metabolic rate is high [5]. Its physicochemical and kinetic properties from several sources have been the subject of a number of investigations (see refs. 5, 6 and references there-in) [5-6]. Moreover, AK is generally considered to be a very flexible enzyme which undergoes large conformational changes upon binding with substrates [7]. Thus it is an ideal system for studying the relationship between the enzymatic activity and the flexibility at its active site. Here we demonstrate that AK can be activated in the presence of denaturants such as urea and guanidine hydrochloride.

MATERIALS AND METHODS

Materials. Glucose-6-phosphate dehydrogenase, hexokinase, NADP, ADP and ANS (8-anilino-1-naphthalenesulfonic acid) were obtained

Abbreviations: AK, adenylate kinase; ANS, 8-anilino-1-naphthalenesulfonic acid.

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from Sigma; urea, Nacalal Tesque, Inc., Japan; Guanidine hydrochloride, Fluka. Other reagents were of analytical grade. The urea solution was always freshly prepared just before being used.

Preparation and activity assay of adenylate kinase. The enzyme was prepared essentially as described in detail in Zhang et al. [8]. The yield was usually about 60 mg pure enzyme per kilogram of rabbit muscle. The final preparation generally yielded AK with specific activity greater than 1600 units/mg that showed only a single peak in SDS electrophoresis, gel filtration and reversed-phase FPLC. One unit is defined as 1 μmole ATP generated from ADP per minute.

The enzymatic activity of native adenylate kinase was quantified by measuring the reduction of NADP in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase [8]. The reaction mixture contained 2.5 mM ADP, 2.1 mM Mg acetate, 6.7 mM glucose, 0.67mM NADP, 10 units of hexokinase and 5 units of glucose-6-phosphate dehydrogenase in 50 mM Tris-HAc buffer (pH8.1). The activity of the enzyme at low denaturant concentrations was measured by using an acid-stopped assay. The perturbed enzyme was mixed into a solution containing 25mM ADP-Mg for a specified period of time, then the reaction was quenched by adding an equal volume of 1M trichloride acetate acid. BSA was added to the reaction system to a final concentration of 10 mg/ml, and the solution was finally centrifuged at 13,000 rpm and the pellet was discarded. The pH of the supernatant was adjusted to 8. The residual activity of the denatured enzyme was calculated according to the amount of ATP in the supernatant and compared with that of the native enzyme [8]. The concentration of AK was determined by the absorption at 280 nm with $A_{1cm}^{1\%}$ =5.2.

Conformational changes in denaturant solution. The global conformational change of adenylate kinase was detected by the extent of Tyr exposure which was indicated by the change of the UV absorption spectrum recorded at 287 nm on a Beckman DU-7500 spectrophotometer. The change of the secondary structures were monitored by measuring the ellipticity at 222 nm on a Jasco-500A or a Jasco-720 spectropolarimeter.

ANS Binding. The time courses of ANS binding to AK were followed by mixing enzyme and ANS in a stopped-flow apparatus (Applied Photophysics) with an exciting wavelength at 378 nm and a filter for emission wavelength cut-off at 410nm. Aliquots of AK at 10 μM was first added in urea at specified concentrations at 25°C for 24 hours, then mixed at a ratio of 1:1 (v/v) with solutions of 400 μM ANS containing the same concentration of urea used for denaturation. Under such conditions, about 80% of the enzyme molecules can form complexes with ANS. The concentration of ANS was determined by using ϵ_{1cm}= 4.95×10^3 $M^{-1}cm^{-1}$ at 350 nm. Data collected at various urea concentrations were fit to the equation using a non-linear regression program

$$A_t = A_s (1 - \exp(-k\,t)) + A_f$$

where A_t is the total fluorescence intensity measured at time t, A_f is the fast phase intensity (dead-time about 15 ms), A_s is the slow phase intensity and k is the rate constant of the slow phase.

RESULTS

Conformational and activity changes of AK in urea and guanidine hydrochloride. The unfolding of AK in urea was monitored by measuring changes in ellipticity at 222 nm and UV absorbance at 287 nm. Curves 1 and 2 in Figure 1 show that these changes as a function of urea concentration. There were no significant changes in the absorbance with up to 1.8 M urea, indicating AK is not unfolded at these low urea concentrations [9]. The absorbance decreased sharply at higher concentrations of urea, indicating unfolding of AK.

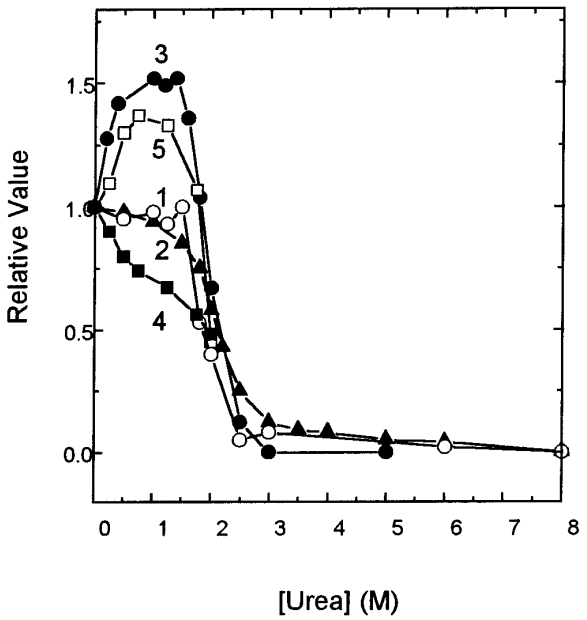

FIG. 1. Comparison of activity with conformational changes of adenylate kinase in different urea solutions. The curves show the change in absorption at 287 nm (○), the change of the CD at 222 nm (▲), the relative activity of the enzyme (●), the relative intensity of the total ANS binding fluorescence (■) and the relative value of the slow phase rate constant of ANS binding fluorescence (□). The enzyme concentrations in 50 mM Tris-HCl, 1 mM EDTA and 1 mM DTT were 25 μM (○), 10 μM (▲), 0.02 μM (●) and 5 μM (■), respectively. The enzyme was incubated with urea at 25°C and pH 8.1 for 24 hours.

The changes of activity of AK with increasing concentrations of urea are shown by curve 3 in Figure 1. Initially the activity increased as the concentration of urea was raised up to 1 M, but decreased subsequently at higher concentration of urea. In 1 M urea, the enzyme activity reached a maximum (1.6-fold enhancement compared to in the absence of urea). The activation of AK was a very fast process that finished within the dead-time of the measurement (data not shown). Similar results were obtained by using guanidine hydrochloride as a denaturant, except that the maximum activation occurred at 0.25 M of guanidine hydrochloride (1.6-fold enhancement compared to in the absence of urea, data not shown).

Effect of urea or guanidine hydrochloride on ANS binding fluorescence. ANS binds to AK at the same pocket of ATP binding site with a dissociation constant, k_d, of 56 ± 5 μM, [10-12]. The binding of ANS to AK enhances the quantum yield of ANS fluorescence as

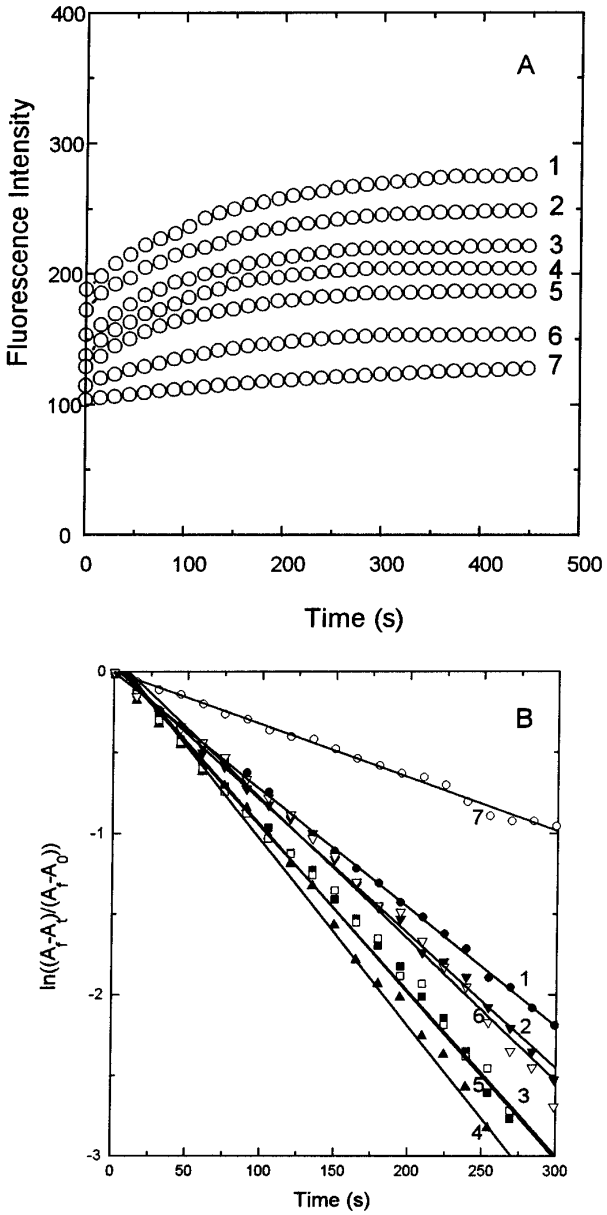

FIG. 2. Fluorescence time course of ANS binding to AK. (A) Curves 1–7 are the time courses of ANS binding to AK in urea concentrations of 0, 0.25, 0.50, 0.75, 1.25, 1.75 and 2.00 M, respectively. Solutions of 10 μM AK were first denatured in different urea concentration at 25°C for 24 h and then mixed 1:1 (v/v) with solutions of 400 μM ANS containing the same concentration of urea used for the denaturation. The other conditions were the same as shown in figure 1. (B) Semi-logrithmic plots of the slow phase fluorescence vs. time in different urea concentrations. Curves 1–7 correspond to urea concentrations of 0, 0.25, 0.50, 0.75, 1.25, 1.75, and 2.00 M, respectively.

well as shifts the emission peak to a shorter wavelength [12]. Figure 2A shows the time courses of ANS fluorescence upon binding to AK at different urea concentrations. The binding of ANS to AK was a biphasic process both in the presence and absence of urea. The fast phase completed within the dead-time of the stopped-flow apparatus used, while the slow phase ended in about 10 minutes. The binding fluorescence decreases markedly with the increasing of the urea concentration (Curve 4 of Fig. 1). Control experiments demonstrated that urea had no obvious effect on the fluorescence of free ANS (not shown), indicating that the decrease of the ANS fluorescence in the presence of urea could not be attributed to the interactions between urea and ANS. The binding rate constant of the slow phase fluorescence was determined by curve-fitting as described above (Figure 2B). The rate constants obtained from those plots are listed in Table 1. The rate constants increased from 0.0073 s^{-1} in the absence of urea to 0.0100 s^{-1} (about 1.4-fold) at 1 M urea, then decreased at higher urea concentrations. Curve 5 of Figure 1 shows the relative change of the rate constant of the slow phase as a function of the urea concentration. In the guanidine hydrochloride denaturation process, similar results were obtained (Table 1).

DISCUSSION

Conformation at the active site of AK is more flexible than the molecule as a whole. For concentrations up to 1.8 M urea or 0.25 M guanidine hydrochloride, the secondary and tertiary structures of AK are not noticeably perturbed as demonstrated by our CD and UV measurements. In contrast, the activity of the enzyme underwent significant changes, increasing about 1.6-fold when the urea concentration was increased to 1 M or guanidine hydrochloride to 0.25 M. The enzyme activity then decreased with further increases of either the urea or guanidine hydrochloride concentration. Moreover, the total fluorescence intensity of the enzyme-bound ANS is significantly decreased, implying that the conformation at the active site of the enzyme

TABLE 1

Slow Phase Rate Constants of ANS Binding to AK in Different Concentrations of Urea and Guanidine Hydrochloride

Urea (M)	Rate constant (s^{-1})	GudHCl (M)	Rate constant (s^{-1})
0	0.0073	0	0.0073
0.25	0.0080	0.05	0.0079
0.50	0.0095	0.10	0.0092
0.75	0.0100	0.15	0.0105
1.25	0.0097	0.20	0.0109
1.75	0.0078	0.25	0.0107
2.00	0.0033	0.35	0.0106
—	—	0.40	0.0083
—	—	0.60	0.0059

is perturbed. This observation is consistent with our previous reports on a number of enzymes whose active sites are situated in a limited region and are more flexible than the molecules as a whole [1-3].

Activation of AK by denaturants is due to increasing conformational flexibility at its active sites. ANS is widely used as a hydrophobic probe of protein structures. The fluorescence intensity of ANS binding to protein is dependent on the microenvironment of the binding site and the solvent environment [13]. AK has one ANS binding site on the same pocket of ATP binding site [10-12]. The fast phase fluorescence should result from rapid binding of the enzyme with the dye, whereas the slow phase fluorescence may arise from the subsequent conformational adjustment of the ANS-AK complex or the conversion of multiple conformers of AK (see discussion below). In both cases, the slow phase rate constant represents the conformational flexibility at the ANS binding site, i.e. the active site of AK. Increases in the slow phase rate constant indicate that the conformation at the binding site is more flexible. Our observation that changes in the enzyme activity and the rate constant of the slow phase fluorescence coincided with those of the urea/guanidine hydrochloride concentration reveals an intrinsic characteristic of AK activation by denaturants: activation by denaturants due to increased conformational flexibility at its active sites.

Based on the comparison of AK crystal structures representing the enzyme in different complexed forms, apo-form (from pig muscle), enzyme-AMP binary complex (from beef heart mitochondrial matrix), and enzyme-AP_5A complex (from *E. coli*), Schulz and co-workers suggested that AK should undergo large structural changes upon binding substrates [7, 14-16]. These conformational changes can take place in two phases. In the first phase binding of AMP only involves the displacement of the small α-helical domain (residues 30-59 in *E. coli* AK), whereas the second phase occurs upon additional binding of substrate ATP that mainly involves the displacement of the LID domain (residues 122-159 in *E. coli* AK). ANS binds to AK at the same pocket of ATP, so the binding of ANS to AK may also induce the domain movement of AK. It is conceivable that the binding of ANS to AK results in fast phase fluorescence changes, then the enzyme-ANS complex undergoes subsequent conformational adjustment, which causes the slow phase fluorescence changes. The increasing flexibility derived from the denaturants facilitated conformational change, resulting in the increased enzymatic activity and rate of the slow phase fluorescence. Another possible explanation is that apo-AK may adopt multiple conformations in solution. Two crystal forms, A and B, of adenylate kinase from pig muscle have been reported [10, 17], which are interconvertable dependent on the pH of the mother liquid. The experiments of ANS or ATP soaking with AK revealed that ANS- or ATP-binding crystal could only be obtained in the case of crystal form B. Conner & Russell [18] interpreted the activation of renewed AK by DTT as the existence of active and inactive conformers of AK in solution that slowly interconvert. Sinev et al.[19] measured the time-resolved dynamics of nonradiative excitation energy transfer of mutant *E. coli* AK, in which the solvent-accessible residues valine 169 and alanine 55 were replaced by tryptophan (the donor of excitation energy) and cysteine labeled with either 5- or 4-acetamidosalicylic acid (the acceptor), respectively. The experimental results confirmed the stepwise manner of the domain closure of the enzyme upon binding substrates and revealed the presence of multiple conformation of *E. coli* AK in solution. Perhaps one conformer (denoted N_1) could bind to ANS while the other (denoted N_2) could not. N_1 and N_2 equilibrate with each other in the absence of ANS. On addition of ANS to AK solution, the fast phase fluorescence change results in the binding of ANS to the equilibrium concentration of N_1 in the absence of ANS. Then N_2 interconverts to N_1 which binds to ANS causing the slow phase fluorescence change. The low concentration of denaturants made the AK conformers more flexible which facilitated the interconversion, resulting in the activation of AK and the increasing rate constant of the slow phase fluorescence.

Although the detailed mechanism for the slow phase of ANS binding fluorescence and the activation of AK is still unclear, our results suggest that the activation of AK by low concentrations of denaturants is due to the increased flexibility of the conformation at the enzyme active site. Our observations provides strong evidence that the flexibility of the enzyme at its active sites is favorable for the full exploitation of its catalytic power [2-4].

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