Evidence for at Least Two Native Forms of Rabbit Muscle Adenylate Kinase in Equilibrium in Aqueous Solution*

(Received for publication, December 2, 1997, and in revised form, January 12, 1998)

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The time course of 8-anilino-1-naphthalenesulfonic acid (ANS) binding to adenylate kinase (AK) is a biphasic process. The burst phase ends in the dead-time of the stopped-flow apparatus (about 15 ms), whereas the slow phase completes in about 10 min. A Job's plot tests of the binding stoichiometry demonstrates that there is one ANS binding site on AK, but only about 70% of the enzyme can rapidly bind with ANS, indicating that the conformation of native AK molecules is not homogeneous. Further kinetic analysis shows that the effects of ANS and substrates concentration on the burst and slow phase fluorescence building agree well with the multiple native forms mechanism. One form (denoted N₁) binds with ANS, whereas the other (denoted N₂) does not. ANS binding to N₁ results in a burst phase fluorescence increase, followed by the interconversion of N₂ to N₁, to give the slow phase ANS binding. Under urea denaturation conditions, N₂ is easily perturbed by urea and unfolds completely at low denaturant concentrations, whereas N₁ is relatively resistant to denaturation and unfolds at higher denaturant concentrations. The existence of multiple native forms in solution may shed some light on the interpretation of the enzyme catalytic mechanism.

It has been accepted that a globular protein in its native state adopts a single, well defined conformation. However, this concept has been challenged by several reports that some proteins may exist in more than one distinct folded form in equilibrium. Evidence for distinguishing multiple native forms of staphylococcal nuclease has come from electrophoretic and NMR studies (1–6). For calbindin D₉k, evidence of multiple forms came not only from NMR studies, but also from x-ray crystal structure (7, 8).

Adenylate kinase (AK; EC 2.7.4.3) catalyzes the phosphoryl transfer reaction: MgATP + AMP ⇌ MgADP + ADP (9, 10). The enzyme contains two distinct nucleotide binding sites: the MgATP site, which binds MgATP and MgADP, and the AMP site, which is specific for AMP and uncomplexed ADP. The substrate-induced conformation changes in AK have been the subject of a number of investigations (11–22). Based on the comparison of AK crystal structures representing the enzyme in different ligand forms, apo-form (from pig muscle), enzyme-AMP binary complex (from beef heart mitochondrial matrix), and enzyme-AP₅A complex (from *Escherichia coli*), Schulz and co-workers (15, 18) suggested that AK undergoes large structural changes upon substrates binding. These conformational changes can be subdivided into two steps; the first change, corresponding to binding to AMP, only involves the displacement of the small α-helical domain (residues 30–59 in *E. coli* AK), whereas the second change, occurring with additional binding of substrate ATP, mainly involves the displacement of the LID domain (residues 122–159 in *E. coli* AK). However, it is not clear whether the enzyme achieves its catalytic conformation only upon substrate binding or whether the above conformation is an intrinsic property (15). In other words, is there more than one distinct folded native form of AK in the absence of the substrate in solution?

Two crystalline forms of adenylate kinase from pig muscle have been reported, which can be interconverted into each other depending on the pH of the medium. The conformational change from form A to B is believed to be an intrinsic property of the enzyme (22, 23). Russell et al. (14) reported that the higher apparent *M*ₘ values of rabbit muscle adenylate kinase determined from gel filtration data obtained in the presence of DTT, higher pH, and higher substrate concentration were due to the conformational changes due to alterations of the intramolecular charge distribution induced by pH, DTT, and the substrates. Sinev et al. (21) performed measurements of time-resolved dynamic radiation-less 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), whereas the cysteine was 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 conformations of *E. coli* AK in solution. However, despite numerous studies using ³H NMR on AK with different ligands, AMP, ADP, ATP, and AP₅A (13, 24–27), the information on the solution structures is limited due to the relatively large size of the enzyme.

In a previous study from this laboratory (28), it was found that the time course of 8-anilino-1-naphthalenesulfonic acid (ANS) binding to AK was a biphasic process. The burst phase ended in the dead-time of the stopped-flow apparatus (about 15 ms), whereas the slow phase completed in about 10 min. The results were interpreted to indicate either conformational changes during AK binding with ANS or the existence of multiple forms of the enzyme. In the present study, a kinetic approach was used to explore the mechanisms of the fluorescence building. The results clearly reveal the existence of at least two native forms of AK in equilibrium in solution. *This work was supported in part by Projects 39625008 and 39670157 from the National Natural Science Foundation of China and the Pandeng Project of the China Commission for Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: AK, adenylate kinase; ANS, 8-anilino-1-naphthalenesulfonic acid; DTT, dithiothreitol.
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EXPERIMENTAL PROCEDURES

Reagents
Glucose-6-phosphate dehydrogenase, hexokinase, NADP, ADP, and ANS were Sigma products; urea was from Nacalai Tesque Inc. (Japan); and other reagents were local products of analytical grade. Urea solution was always freshly prepared.

Preparation and Activity Assay of Adenylate Kinase
The enzyme was prepared essentially according to Zhang et al. (29). The yield was usually about 60 mg of pure enzyme/kg of rabbit muscle. The final preparation usually had a specific activity greater than 1600 units/mg and showed only a single peak in SDS electrophoresis, gel filtration, and reversed-phase FPLC. One unit is defined as 1 μmol of ATP generated from ADP/min.

The activity assay was made by following the reduction of NADP in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture contained 2.5 mM ADP, 2.1 mM Mg acetate, 6.7 mM NADP, 20 units hexokinase, and 10 units glucose-6-phosphate dehydrogenase in 50 mM Tris/HCl buffer (pH 8.1) The concentration of AK was determined by the absorption at 280 nm with a DU-7500 UV spectrophotometer for steady state and with a Carry 219 spectrophotometer for the time course of unfolding. The data were obtained after subtracting the baseline under the same conditions.

The time course of ANS binding to AK fluorescence was measured using an Applied Photophysics Spectro Workstation (Archemedes). Apparent observed first-order rate constants (kobs) were obtained by nonlinear least-squares fits of the data to Equation 1.

\[ A_t = A_t(1 - \exp(-k_{obs}t)) + A_i \]  

(A) is the total fluorescence intensity measured at time t, A is the amplitude of the burst phase reaction, A is the amplitude of the slow phase reaction, and kobs is the observed rate constant of the slow phase reaction.

Kinetics
Two possible mechanisms can be proposed to interpret the biphasic fluorescence increase of ANS binding to AK. 1) The rapid formation of AKANS complex is followed by a slow conformational transformation to result in the slow phase reaction (this is designated as a conformational transformation mechanism). 2) At least two native forms of AK exist in solution, of which one binds rapidly with ANS and the other does not (designated as a multiple forms mechanism). These two mechanisms can be distinguished by kinetic analysis.

Conformational Transformation Mechanism—The conformational transformation mechanism can be expressed by Reaction 1.

\[ \text{AK} + \text{ANS} \rightarrow \text{[AK\cdotANS]} \rightarrow \text{[AK\cdotANS]} \rightarrow \text{AK\cdotANS} \]  

REACTION 1

AK and ANS first rapidly complex to form an intermediate ([AK·ANS]), resulting in the burst phase fluorescence. This intermediate then undergoes a conformational adjustment to give the slow phase fluorescence increase. If this mechanism is correct, the fraction of burst and slow phases should remain constant with varying ANS concentration.

Multiple Forms Mechanism—Consider that there are two native forms of AK in equilibrium in solution, as shown in Reaction 2. One form (denoted N1) binds with ANS, whereas the other (denoted N2) does not. ANS binding to N1 makes the burst phase fluorescence, as shown in Reaction 3. N2 is then converted to N1 and binds with ANS to give the slow phase fluorescence increase, as shown in Reaction 4.

\[ N_1 \rightarrow N_1 \rightarrow N_1 \cdot ANS \]  

REACTION 2

\[ K_1 = \frac{[N_1]_b}{[N_1]_0} \]  

(Eq. 2)

and

\[ [N_2]_b = [N_2]_0 \]  

(Eq. 3)

\[ N_1 + \text{ANS} \rightarrow N_1 \cdot \text{ANS} \]  

REACTION 3

\[ k_3 \]  

REACTIO 4

\[ N_1 + \text{ATP} \rightarrow N_1 \cdot \text{ATP} \]  

REACTION 5

Here, [N1]0, [N2]0, and [N]0 are the equilibrated concentration of forms N1, N2, and total enzyme, respectively. The kinetics of ANS binding to AK are based on the following assumptions: 1) Steady-state conditions are instantaneously reached between N1, ANS, and N1·ANS and also between N2, ATP, and N2·ATP, and the interconversion of N2 to N1 is the rate-limiting step. 2) Only N1 binds with ANS and ATP. The first assumption is justified because the enzyme binding with substrates or substrate analogs is usually a very fast reaction. The second assumption seems also to be justified because experiments of soaking of crystals with ANS or ATP revealed that crystalline form B of AK binds with ANS or ATP (12). Under the above conditions, we obtain the following equations.

\[ K_2 = \frac{[N_1]_0 [\text{ANS}]}{[N_1]_0 \cdot \text{ANS}} \]  

(Eq. 4)

\[ K_3 = \frac{[N_2]_0 [\text{ATP}]}{[N_2]_0 \cdot \text{ATP}} \]  

(Eq. 5)

\[ [N_1]_b = [N_1]_0 + [N_2]_0 + [N_1]_0 \cdot \text{ANS} + [N_1]_0 \cdot \text{ATP} \]  

(Eq. 6)

\[ \frac{d[N_1]}{dt} = k_3 \times [N_1]_0 + k_1 \times [N_1] \]  

(Eq. 7)

Substituting Equations 4–6 into Equation 7 gives Equation 8.

\[ \frac{d[N_1]}{dt} = k_3 \times [N_1]_0 + \frac{k_1}{1 + \frac{[\text{ANS}]}{K_2} + \frac{[\text{ATP}]}{K_3}} \times \frac{[N_1]_0 [\text{ANS}]}{[N_1]_0 \cdot \text{ANS}} \]  

(Eq. 8)

\[ \frac{d[N_1]}{dt} = \frac{A - B[N_1]}{A - B[N_1]} - k_1 \times [N_1]_0 - k_3 \times [N_1]_0 \]  

(Eq. 9)

where

\[ A = \frac{k_1 \times [N_1]_0}{1 + \frac{[\text{ANS}]}{K_2} + \frac{[\text{ATP}]}{K_3}} \]  

(Eq. 10)
Integrating Equation 9, under the boundary conditions that at \( t = 0 \), \([N_2] = [N_2]_0\), and that at \( t = t \), \([N_2] = [N_2]_1\), gives Equations 12 and 13.

\[
[N_1 \cdot \text{ANS}] = \frac{[N_1]_0 - [N_1] - [N_2] - [N_1 \cdot \text{ATP}]}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} + \frac{[N_2]_0 - \frac{[N_1]_0}{[\text{ANS}]} A}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} (1 - e^{-kt}) \tag{Eq. 13}
\]

The observed slow phase fluorescence building rate constant \( k_{obs} \) is shown below.

\[
k_{obs} = B = k_{-1} + \frac{k_1}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} \tag{Eq. 14}
\]

Substitute \( A \) and \( B \) into Equation 15 to give Equation 16.

\[
[N_1 \cdot \text{ANS}] = \frac{[N_1]_0 - \frac{[N_1]_0}{[\text{ANS}]} A}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} + \frac{[N_2]_0 - \frac{[N_1]_0}{[\text{ANS}]} A}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} (1 - e^{-kt}) \tag{Eq. 16}
\]

Here \( k_s = k_{-1} h_{1} \). The amplitude of the total fluorescence \( (F_t) \) is shown in Equation 17.

\[
F_t = C \times [N_1 \cdot \text{ANS}] = C \times \frac{[N_1]_0 \times [\text{ANS}]}{K_2} \times \frac{1}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} \tag{Eq. 17}
\]

At \( t = 0 \),

\[
[N_1 \cdot \text{ANS}] = \frac{[N_1]_0}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} \tag{Eq. 18}
\]

The amplitude of the burst phase fluorescence \( (F_b) \) is given by Equation 19.

\[
F_b = \frac{[\text{ANS}]}{1 + \left( \frac{[\text{ATP}]}{K_3} \right) \left( \frac{[\text{ANS}]}{K_2} \right)} (1 - e^{-kt}) \tag{Eq. 19}
\]
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the quantum yield of ANS fluorescence as well as shift the emission peak to lower wavelengths. Fig. 1 shows the time course of fluorescence increase by ANS binding to AK at pH 8.1 in the presence of DTT. The fluorescence increase is obviously a biphasic process. The burst phase completes within the dead-time of mixing in the stopped-flow apparatus, and the slow phase ends in about 10 min.

Stoichiometry of ANS Binding to AK—To check the stoichiometry of ANS binding, the fluorescence increase for both the burst and the total increase were treated by the method of Job (31). The results are shown in Fig. 2. The maximum intensity of the total fluorescence increase is at a 1:1 molar ratio of ANS to AK, indicating one ANS binding site on AK. However the burst fluorescence intensity reaches a maximum at the molar ratio 0.4:0.6 of ANS to AK, indicating only about 70% of the enzyme can rapidly bind with ANS. The above results suggest that the conformation of AK molecules in solution is not homogeneous.

Effects of ANS Concentration on ANS Binding to AK—The effect of ANS concentration on the kinetics of ANS binding to AK was explored by measuring the burst and total fluorescence increase of a series of solutions containing increasing concentrations of ANS. Fig. 3A shows the amplitudes of the burst phase (Fs) and total (Ft) fluorescence increase as functions of the ANS concentration at pH 8.1. The inset in Fig. 3A shows the fractions of the burst (Fs/Ft) and slow phases (Fs/Ft) as functions of ANS concentration, showing that Fs/Ft decreases and Fs/Ft increases with increasing ANS concentration and that both gradually approach a constant value at higher concentrations of ANS. Similar results were obtained at pH 6.0 and pH 9.0 (data not shown).

According to Equations 26 and 27, when [ANS] \( \rightarrow 0 \), then \( F_F/F_B \rightarrow 1 \) and \( F_F/F_T \rightarrow 0 \); when [ANS] \( \rightarrow \infty \), then \( F_F/F_B \rightarrow K_B/(1 + K_B) \), \( F_F/F_T \rightarrow 1/(1 + K_B) \), and \( F_F/F_B = K_B \). The equilibrium values of \( F_F/F_B \) is 0.47, equivalent to a \( K_B \) value of 2.1. The solid lines in the inset plot of Fig. 3A are from calculated values using Equations 26 and 27 with \( K_B = 37 \) mM. These values agree well with the experimental results.

Fig. 3B shows the double-reciprocal plot of \( 1/F_s/F_T \) and \( 1/F_s/1/[ANS] \); both give straight lines with the same slope. The kinetic parameters obtained by fitting the data with Equations 24 and 25 are listed in Table I.

Table I: Kinetics parameters of AK binding to ANS

| \( K_1 \) | \( K_2 \) | \( K_3 \) | \( K_4 \) | \( K_5 \) |
|---------|---------|---------|---------|---------|
| 6.0     | 0.84    | 0.79    | 0.0167  | 0.0132  | 0.27    | 50     |
| 8.1     | 0.34    | 0.31    | 0.0091  | 0.006   | 0.51    | 37     |
| 9.0     | 0.35    | 0.31    | 0.0050  | 0.0027  | 0.85    | 70     |

Fig. 4 shows that the observed first-order rate constant (\( k_{obs} \)) of the slow phase fluorescence change decreases with increasing ANS concentration. The interconversion rate constants \( k_1 \) and \( k_{-1} \) can be obtained by fitting the date with Equation 20. According to Equation 20, when [ANS] \( \rightarrow 0 \), \( k_{obs} \rightarrow k_{-1} + k_1 \), and when [ANS] \( \rightarrow \infty \), \( k_{obs} \rightarrow k_{-1} \). From the data in Fig. 4, \( k_{-1} \) can be estimated to a little less than 0.006. Equation 20 has three unknowns: \( k_1 \), \( k_{-1} \), and \( K_B \). A nonlinear fit can be avoided by rewriting Equation 20 as shown in Equation 28.
When \(k_{-1}\) is chosen properly, a plot of \(1/(k_{\text{obs}} - k_{-1})\) versus ANS concentration yields a straight line. When \(k_{-1}\) is chosen too high, the curve bends upward, and, when chosen too low, downward. As can be seen from the inset in Fig. 4, a good fit is obtained using \(k_{-1} = 0.0055 \text{ s}^{-1}\), which yields \(k_1 = 0.013 \text{ s}^{-1}\) and \(K_2 = 55 \text{ mM}\).

\[
\frac{1}{k_{\text{obs}} - k_{-1}} = \frac{1}{k_1} \left(1 + \frac{[\text{ANS}]}{K_2}\right) \quad \text{(Eq. 28)}
\]

When \(k_{-1}\) is chosen properly, a plot of \(1/(k_{\text{obs}} - k_{-1})\) versus ANS concentration yields a straight line. When \(k_{-1}\) is chosen too high, the curve bends upward, and, when chosen too low, downward. As can be seen from the inset in Fig. 4, a good fit is obtained using \(k_{-1} = 0.0055 \text{ s}^{-1}\), which yields \(k_1 = 0.013 \text{ s}^{-1}\) and \(K_2 = 55 \text{ mM}\).

**Effect of AK Concentration on ANS Binding to AK**—Fig. 5 shows the effect of AK concentration on the amplitudes of the burst and slow phase fluorescence. Both increase linearly with increasing concentration of AK. This result indicates that there is no dimer or aggregation formation in this enzyme concentration range.

**Substrate Effects on the Kinetics of ANS Binding to AK**—The overall effects of substrates on the kinetics of ANS binding to AK were also investigated. Specified concentrations of substrates were first added to ANS solution, then mixed 1:1 with AK solution by stopped-flow. Fig. 6 shows that the substrates and their analogue, AMP, ADP, ATP, and AP₅A, inhibit both the burst (Fig. 6A) and slow phase (Fig. 6B) fluorescence building. The inhibition efficiency is in the order: AMP < ADP < ATP < AP₅A. Further experiments were carried out to study the effects of ATP and MgATP concentration on the kinetics of ANS binding to AK. Fig. 7 shows \(1/F_f\) (A) and \(1/F_t\) (B) versus \(1/[\text{ANS}]\) for various ATP concentrations. Fig. 8 shows \(1/F_f\) (A) and \(1/F_t\) (B) versus \(1/[\text{ANS}]\) for various MgATP concentrations. The results clearly show that ATP and MgATP act as competitive inhibitors for ANS binding to AK.

**Effect of pH on ANS Binding to AK**—The dependence of the burst and slow phase fluorescence building on pH (Fig. 9) illustrates that the fraction of the slow phase increases but that of the burst phase decreases with increasing pH in the pH range from 6 to 9 in the presence of DTT or \(\beta\)-mercaptoethanol. At pH 8.1 in the absence of either DTT or \(\beta\)-mercaptoethanol, the AK binding ANS fluorescence shows the same kinetics behavior as in the presence of DTT or \(\beta\)-mercaptoethanol except that the amplitude of the slow phase reaction increases to 0.43. Similar results were also obtained at other pH values (data not shown).

Denaturation of Adenylate Kinase in Urea—Urea unfolding of AK was studied by measuring the changes of CD at \(\theta_{222}\) nm and of the UV absorbance at 287 nm (Fig. 10). Both measurements changed little up to 1.8 M urea and changed sharply between 1.8 and 2.5 M urea. The concentration of urea required to produce changes of 50% in either method was about 2.0 M.

The unfolding kinetics were followed using the time courses of ellipticity at 222 nm and absorbance at 287 nm. Fig. 11 shows the kinetics of unfolding, with the early time history recorded in the inset plot. The unfolding of the tertiary and
secondary structures of the enzyme are both biphasic processes. The amplitude of slow phase increased with increasing urea concentration, whereas that of the burst phase was nearly constant in the range of 2.3–3.4 M urea (Fig. 12). The unfolding rate constant of the slow phase increased from 0.005 s\(^{-1}\) in 2.3 M urea to 0.02 s\(^{-1}\) in 3.4 M urea, whereas the change of the burst phase rate constant was too fast to determine (data not shown).

Unfolding Kinetics of the AK\(_z\) ANS Complex—The unfolding of the AK\(_z\) ANS complex was studied by first incubating 0.2 mg/ml AK with 200 mM ANS for 1 h at 25 °C. This equilibrated solution was then mixed 1:1 with a 5.6 M urea solution containing 200 \(\mu\)M ANS. The time course of unfolding, monitored by ellipticity at 222 nm (Fig. 13, line a), shows a monophasic process with a rate constant of 0.016 s\(^{-1}\). A control experiment showed that there was no influence of ANS on the CD spectra of AK (data not shown). For comparison, the unfolding of AK in the absence of ANS, monitored by ellipticity at 222 nm (Fig. 13, line b) is clearly a biphasic process.

DISCUSSION

The above studies provide evidence to suggest rabbit muscle adenylate kinase exists in solution in at least two folded forms in equilibrium. One form (denoted N\(_1\)) rapidly binds with ANS, whereas the other (denoted N\(_2\)) does not. N\(_1\) binding with ANS causes the burst phase fluorescence increase. N\(_2\) is slowly converted to N\(_1\) and then binds with ANS, resulting in the slow phase of fluorescence increase.

**FIG. 7.** Effect of ATP concentration on the kinetics of ANS binding to AK. A, \(1/F_f\) versus 1/[ANS] with varying ATP. B, \(1/F_t\) versus 1/[ANS] with varying ATP. The experimental conditions were the same as in Fig. 6. The concentrations of ATP are: 1, 0.0 mM; 2, 4 mM; 3, 8 mM; 4, 12 mM; 5, 16 mM; 6, 20 mM.

**FIG. 8.** Effect of MgATP concentration on the kinetics of ANS binding to AK. A, \(1/F_f\) versus 1/[ANS] with varying MgATP. B, \(1/F_t\) versus 1/[ANS] with varying MgATP. The experimental conditions were the same as in Fig. 6. The concentrations of MgATP are: 1, 0.0 mM; 2, 4 mM; 3, 8 mM; 4, 12 mM; 5, 16 mM; 6, 20 mM.

ANS is widely used as a probe of hydrophobic region in protein structures. The fluorescence intensity of ANS bound to a protein is dependent on the microenvironment of the binding site (32). Experiments wherein AK crystals were soaked with ANS or ATP revealed that ANS or ATP binding occurred only with AK of crystalline form B. ANS occupies the pocket formed between the \(\beta\)-sheet, loop 16–22, helix 23–30, and the C-terminal helix. This pocket was originally assigned to the adenosine moiety of AMP site (12), but has recently been recognized as the ATP site (33, 34). Compared with the results obtained in the present study, it appears highly suggestive that N\(_1\) relates to the crystalline form B, and N\(_2\) to crystalline form A.

AK undergoes a domain movement when it binds with substrates or their analogues. ANS binding to AK should also induce a conformational change of the enzyme. If the slow phase fluorescence comes from this domain movement, the fraction of the burst and slow phases should keep constant with varying ANS concentration (see “Experimental Procedures”). Our observation is that the fraction of the burst phase decreases and that of the slow phase increases with increasing ANS concentration, and this is incompatible with the conformational transformation mechanism.

The present experimental results demonstrate that the fraction of N\(_1\) decreases and that of N\(_2\) increases at higher pH,
consistent with the study of crystal structures. N$_1$ and N$_2$ equilibrate with each other in solution. The fractions of N$_1$ and N$_2$ depend on the solution pH. High and low pH values favor N$_2$ and N$_1$ respectively consistent with the observation of the prevalence of crystalline forms A and B at different pH values.

The report by Russell et al. (14) that AK adopts a more open conformation at higher pH in the presence of 1 mM DTT seems to contradict our observations and crystal studies. This contradiction may arise from the different definitions of the "open" and "closed" forms. The open and closed forms determined by apparent $M_r$ refer to the whole size of the enzyme, whereas the open and closed forms discussed here and in the crystal structure studies refer to the hydrophobic pocket of the enzyme. According to the data provided by Sachsenheimer and Schulz (11), the crystal size of form A, which has a closed hydrophobic pocket, is slightly bigger than that of the form B, which has an open hydrophobic pocket.

Under urea denaturation, the unfolding amplitude of the burst phase is independent of the urea concentration in the range of 2.3–3.5 M, whereas the amplitude of the slow phase increases at higher urea concentrations, suggesting the existence of at least two native forms of AK in solution. The biphasic process of unfolding cannot be interpreted as the formation of an unfolding intermediate, because the unfolding amplitude of the burst would not then remain constant. Perhaps one form of AK is easily perturbed by urea and unfolds completely at lower concentrations of urea, so that the unfolding amplitude of the burst phase is independent of the denaturant concentration. The other form is then relatively resistant to urea denaturation and its unfolding fraction increases at higher denaturant con-
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The urea denaturation kinetics of the AK-ANS complex also confirms the suggestion of at least two native forms of AK in equilibrium in solution. At equilibrium with 0.2 mM ANS, about 65% of the AK molecules are in the form of N1-ANS complex, 25% in the form of N2, and 10% are in form of N2. N2 unfold fast, whereas the unfolding of N2 is relatively too small to observe, so the observed unfolding curve is single phase.

From the above discussion, the mechanisms of ANS binding and urea denaturation of AK can be written as shown below, where U represents the denatured state of AK.

\[
\begin{align*}
\text{burst & can bind to ANS and ATP} \\
N_1 & \xrightarrow[k_1]{k_{-1}} N_2 \\
\text{slow & can't bind to ANS and ATP} \\
N_2 & \xrightarrow[k_3]{k_{-3}} N_1 \\
U & \\
\end{align*}
\]

From unfolding amplitudes, we obtain Equation 29.

\[
K'_1 = \frac{[N_2]}{[N_1]} = \frac{\text{[burst unfolding phase]}}{\text{[slow unfolding phase]}} = \frac{0.3}{0.7} = 0.43
\]

\[
\text{(Eq 29)}
\]

From ANS binding amplitude, we obtain Equation 30.

\[
K'_1 = \frac{[N_2]}{[N_1]} = \frac{\text{[slow binding phase]}}{\text{[burst binding phase]}} = \frac{0.32}{0.68} = 0.47
\]

\[
\text{(Eq 30)}
\]

Here \(N_1\) denotes the form which unfolds slowly and can bind with the ANS burst, and \(N_2\) denotes the form which unfolds burst and cannot bind with ANS. The equilibrium constants calculated from both experiments agree very well and the interconversion free energy \(\Delta G^0\) from \(N_1\) to \(N_2\) is calculated according to Equation 31.

\[
\Delta G^0 = -RT \ln K'_1 = 1.87 \text{ kJ/mol}
\]

\[
\text{(Eq 31)}
\]

A previous study reported that the slow phase fluorescence building can be catalyzed by peptidyl prolyl cis/trans isomerases (28). The calculated free energy \(\Delta G^0\) from \(N_1\) to \(N_2\) is close to that of proline isomerization, giving further evidence that the interconversion of the two forms involves the cis/trans peptidyl prolyl isomerization of proline residue.

The multiple forms of AK co-existent in solution may shed some light on the interpretation of the catalytic mechanism of the enzyme. One form of AK may fit the substrates MgATP and AMP as well as fit the inhibitor AP5A, so that AP5A acts as a competitive inhibitor for the forward reaction, whereas the other form can fit the substrates MgADP and ADP, but not fit the inhibitor AP5A so that AP5A acts as a noncompetitive inhibitor for the reverse reaction.

In summary, the evidence presented here suggests that at least two native forms of AK co-existent in solution. One form unfolds slowly and binds rapidly with ANS, whereas the other form unfolds quickly and does not bind with ANS. In the absence of substrates, these two folded forms equilibrate with each other in solution. Changes of the solution pH or the presence of substrates shifts the equilibrium to fit the new conditions. Furthermore, interconversion of these two forms may involve the cis/trans peptidyl prolyl isomerization.

Acknowledgment—We express our sincere thanks to Professor C. L. Tsou of the Institute of Biophysics, Academia Sinica, for many helpful suggestions.

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J. Biol. Chem. 1998, 273:7448-7456.
doi: 10.1074/jbc.273.13.7448

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