Both native conformers of rabbit muscle adenylate kinase are active

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Abstract There are two forms of rabbit muscle adenylate kinase (AK) with different 8-anilino-1-naphthalenesulfonic acid (ANS) binding properties in equilibrium solution. One form (about 70%, denoted N_1) binds rapidly with ANS, whereas the other (about 30%, denoted N_2) does not. Furthermore, native forms of AK should adopt different conformations for binding with substrates and products, which should be pre-existing for performing its catalytic function. The present experiments demonstrate both forms of AK distinguished by ANS probe are active. The activity of N_2 is about 0.8 fold higher than N_1 and shows higher susceptibility to proteolysis by trypsin. This means that the native state of AK might be an ensemble of kinetically attainable conformers and the energy landscapes of AK folding should be rugged with more than one local minimum.

Key words: Adenylate kinase; Multiple native conformer; Proteolysis susceptibility; Proline isomerization

1. Introduction

Based on the refolding experiments of RNase A, Anfinsen proposed the 'thermodynamic hypothesis', that in its normal physiological milieu the three-dimensional structure of a native protein has the lowest Gibbs free energy in the whole system [1]. In contrast, Levinthal pointed out that it is impossible for an unfolded protein to find the native state by random searching through the entire space of possible conformations. This led him to postulate that a protein must follow a specific pathway which guides it to the native state, and therefore folding must be under kinetic control [2].

The thermodynamic hypothesis was challenged by the accumulated experimental evidence that some proteins may exist in more than one distinct folded form in solution. Evidence for distinguishing multiple native forms of staphylococcal nuclease comes from electrophoretic and NMR results [3–8] and of calbindin D_{9K} from both NMR and X-ray crystal structure studies [9,10].

On the other hand, the 'Levinthal paradox' implies that the energy landscape of folding was a golf-course-like landscape with the native conformation in the lowest energy state and the others in equal energy states [11]. Since the interactions that stabilize folded proteins are individually weak and are able to stabilize folded conformations, possibly due to their high number, the energy landscapes of folding should be rugged with more than one local minimum. The golf-course-like landscape is not a good model for protein folding.

Previous reports from this laboratory indicated two different conformational forms of rabbit muscle adenylate kinase (AK) with different 8-anilino-1-naphthalenesulfonic acid (ANS) binding properties and two native forms for binding products and binding substrates [12,13]. If the native forms involved in the catalytic cycle are different from those distinguished by ANS probe, AK might exist in more native forms in equilibrium. In the present work, we demonstrate that both conformations distinguished by the ANS probe of AK are active, implying the native state of AK might be an equilibrium ensemble of kinetically attainable conformations. This gives a good example of the rugged energy landscape.

2. Materials and methods

2.1. Reagents

ADP (adenosine diphosphate), AMP (adenosine monophosphate), ATP (adenosine triphosphate), NADH, NADP, hexokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, ANS, BPTI (bovine pancreas trypsin inhibitor) and trypsin (type XIII, tosylphenylalanylchloromethane (TPCK)-treated) were all Sigma products. All other reagents were local products of analytical grade. Tris–HCl buffer was always freshly prepared just before use.

2.2. Methods

2.2.1. Preparation and activity assay of adenylate kinase. The enzyme was prepared essentially according to Zhang et al. [16]. The final preparation had a specific activity greater than 1800 units per mg which showed only a single peak in SDS electrophoresis, gel filtration and reversed-phase FPLC. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol ATP generated per minute in backward reaction or the formation of 1 μmol ADP (MgADP) generated per minute in forward reaction.

The backward activity of AK (MgADP+ADP → MgATP+AMP) was assayed by following the reduction of NADP at 340 nm in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture contained 3.0 mM ADP, 2.1 mM Mg acetate, 6.7 mM glucose, 0.67 mM NADP, 15 units of hexokinase, 8 units of glucose-6-phosphate dehydrogenase, 7.6 μM bovine serum albumin (BSA) and 10 mM β-mercaptoethanol in 50 mM Tris–HCl buffer, pH 8.1.

Measurement of the velocity of the forward reaction (MgATP+AMP → MgADP+ADP) was performed by monitoring the oxidation of NADH at 340 nm coupling with pyruvate kinase and lactate dehydrogenase. The final assay mixture was: 50 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol, 75 mM KCl, 4 mM phosphoenolpyruvate, 0.2 mM NADH, 15 units ml^{-1} pyruvate kinase, 20 units ml^{-1} lactate dehydrogenase, 7.6 μM BSA and 2 mM Mg^{2+}. UV-absorbance at 340 nm was measured either with a UV-2501 or with a UV-1601 spectrophotometer (Shimadzu Corp., Japan).

2.2.2. Digestion of AK by trypsin. Digestion of AK with trypsin was performed in 50 mM Tris–HCl buffer, pH 8.1, 1 mM EDTA and 10 mM β-mercaptoethanol at 20°C. The complete digestion reaction system contained 22 μM AK and 0.05 μM trypsin with a molar ratio of about 440:1. To stop the proteolysis, 1 μM BPTI as a competitive

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Abbreviations: AK, rabbit muscle adenylate kinase; ANS, 8-anilino-1-naphthalenesulfonic acid; BPTI, bovine pancreatic trypsin inhibitor; PPIase, peptidyl prolyl *cis*/*trans*-isomerase

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irreversible inhibitor of trypsin was used at a molar ratio of about 20:1 (in the same buffer).

2.2.3. The time course of ANS binding to AK fluorescence. The time course of ANS binding to AK fluorescence was measured using a Shimadzu RF-5301PC spectrofluorophotometer. The excitation and the emission wavelengths were separately at 378 nm and 478 nm with the slit widths of 3/3 nm. The final concentration of ANS was 0.2 mM in 50 mM Tris–HCl buffer, pH 8.1, 1 mM EDTA and 10 mM β-mercaptoethanol. All the data were obtained after subtracting the baseline under the same conditions.

3. Results

3.1. Both N_1 and N_2 conformations are active

There are two forms of AK with different ANS binding properties co-existent in solution. One form (about 70%, denoted N_1) binds rapidly with ANS, whereas the other (about 30%, denoted N_2) does not but can convert to N_1 and then binds to ANS [13]. To assay the activities of these two forms, AK and ANS were mixed with final concentrations of 0.02 mM AK and 0.2 mM ANS in 50 mM Tris–HCl, 1 mM EDTA, and 10 mM β-mercaptoethanol, at 25°C, pH 8.1. At various time intervals this AK and ANS mixture solution was 50 fold diluted into an assay system to measure the observed activity. Fig. 1 shows the results. Both observed activities of forward and backward reactions are decreasing first with

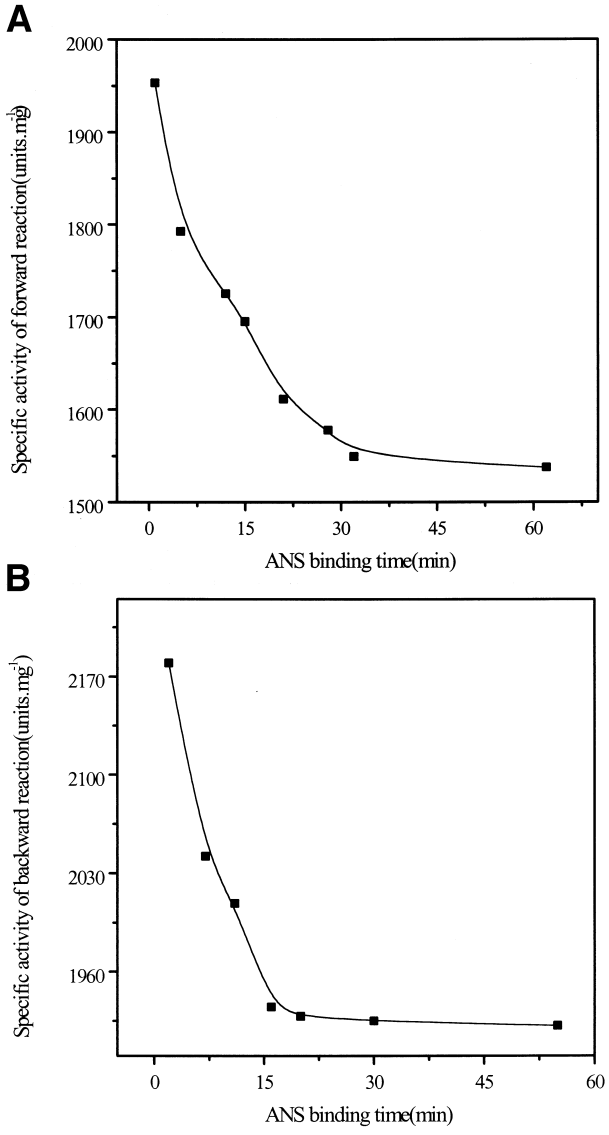

Fig. 1. The time course of observed activity change during AK binding with ANS. Preparation and activity assaying of adenylate kinase are described in Section 2. AK and ANS were mixed with final concentrations of 0.02 mM AK and 0.2 mM ANS in 50 mM Tris–HCl, 1 mM EDTA, and 10 mM β-mercaptoethanol, at 25°C, pH 8.1. At various intervals this AK and ANS mixture solution was 50 fold diluted into the assay system to measure the observed activity. A: Activity change of forward reaction. B: Activity change of backward reaction.

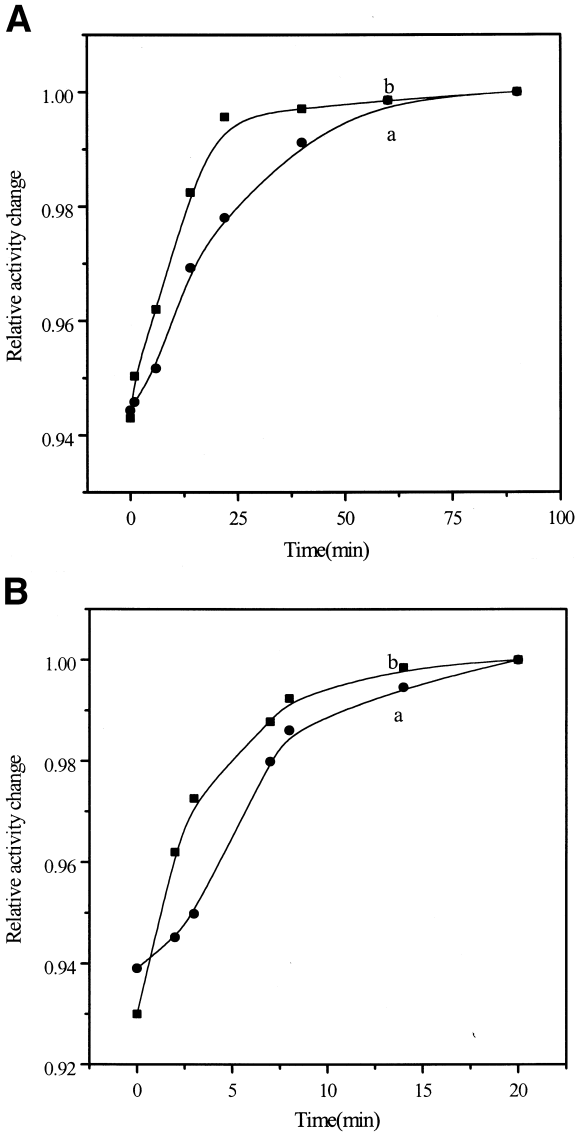

Fig. 2. The time course of observed activity recovery of AK and ANS complex after removing ANS. AK and ANS were incubated with final concentration of 0.02 mM AK and 0.2 mM ANS in 50 mM Tris–HCl, 1 mM EDTA, 0.02 mM BSA, and 10 mM β-mercaptoethanol, at 25°C, pH 8.1 for about 30 min. The equilibrated solution with nearly all AK in N_1ANS complex form then was 1:50 diluted in the same buffer to remove ANS. The observed activity was measured at various intervals after dilution. A: Activity change of forward reaction, line a: in the absence of prolyl *cis*/*trans*-isomerase, line b: in the presence of prolyl *cis*/*trans*-isomerase (trigger factor). B: Activity change of backward reaction, line a: in the absence of prolyl *cis*/*trans*-isomerase, line b: in the presence of prolyl *cis*/*trans*-isomerase (trigger factor).

ANS binding time and then remain constant. This means that both N_1 and N_2 are active but the activity of N_2 is higher than that of N_1. It can be roughly calculated that the activities of N_1 are 1900 for the forward reaction and 1500 for the backward reaction, whereas the activities of N_2 are 2700 for the forward reaction and 2800 for the backward reaction.

3.2. The conversion of N_1 to N_2

The conversion of N_1 to N_2 was explored by first incubating AK and ANS with a final concentration of 0.02 mM AK and 0.2 mM ANS in 50 mM Tris–HCl, 1 mM EDTA, 0.02 mM BSA, and 10 mM β-mercaptoethanol, at 25°C, pH 8.1 for about 30 min. The equilibrated solution with nearly all AK in N_1ANS complex form then was diluted by 1:50 in the same buffer to remove ANS. After dilution the time courses of activity change for forward and backward reactions are shown in Fig. 2. It appears that by dilution removing ANS N_1 converts to N_2. Both forms equilibrate at a ratio of 0.7:0.3.

Previous reports from this laboratory show that prolyl *cis*/*trans*-isomerase accelerates the conversion of N_2 to N_1 [14]. The conversion rate of N_1 to N_2 is also accelerated in the presence of prolyl *cis*/*trans*-isomerase (Fig. 2), providing further evidence that the interconversion of N_1 and N_2 might involve proline isomerization.

The conversion of N_1 to N_2 was also explored by trypsin digestion (Fig. 3). Both forms N_1 and N_2 show different susceptibilities to trypsin. During trypsin digestion the ratio of fast phase ANS binding fluorescence increases, while that of the slow phase decreases, implying that N_2 is easily digested by trypsin. It should be pointed out that the changes of fast and slow phases ratios are not due to the existence of some fragments which could bind fast with ANS. Control experiments show: (1) During digestion, the enzyme activity and UV_{287} absorption decrease simultaneously, while the total ANS binding fluorescence decreases slightly faster than enzyme activity and UV_{287} absorption, implying that the partly digested enzyme shows higher susceptibility to proteolysis by trypsin than the whole molecule (data not shown); (2) The completely digested enzyme gives no ANS binding fluorescence. This is also evidenced by addition of competitive irreversible inhibitor BPTI to stop digestion. After terminating digestion, the fractions of fast and slow phase fluorescence recovered to 0.7 and 0.3, respectively (Fig. 4).

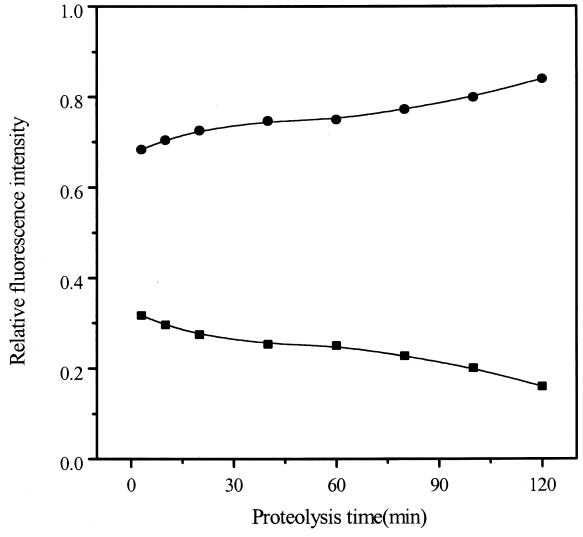

Fig. 3. The effect of trypsin proteolysis on ANS binding to AK. 22 μM AK was mixed with 0.05 μM trypsin in 50 mM Tris–HCl (containing 1 mM EDTA, 10 mM β-mercaptoethanol, pH 8.1) at 20°C. At different intervals the proteolysis was terminated with 1.0 μM BPTI, then the ANS and AK binding fluorescence were measured at 20°C. Circles: the fraction of fast phase fluorescence; squares: the fraction of slow phase fluorescence.

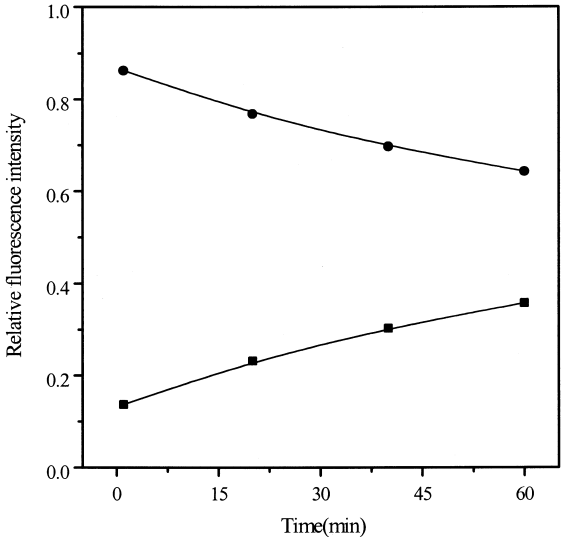

Fig. 4. The changes of ANS binding fluorescence of partly digested AK versus incubation time after the proteolysis was terminated. 55 μM AK was digested by 0.065 μM trypsin for 120 min at 20°C (about 30–40% intact AK molecules remaining), the proteolysis was stopped with 1.55 μM BPTI in 50 mM Tris–HCl buffer, pH 8.1, 1 mM EDTA, 10 mM β-mercaptoethanol plus 0.5 mg ml^{-1} BSA. Then at various intervals, ANS binding fluorescence was measured. Circles: the fraction of fast phase fluorescence; squares: the fraction of slow phase fluorescence.

4. Discussion

The above experiments demonstrate that both forms of AK with different ANS binding properties are active. When N_1 bind with ANS, N_2 would convert to N_1 and removing ANS, N_1 would convert to N_2. The interconversion of N_1 and N_2 might involve proline isomerization. In the absence of substrates the ratio of N_1:N_2 is 0.7:0.3. Furthermore, native forms of AK should adopt different conformations for binding with substrates and products. These conformations of AK should be pre-existing for performing its catalytic function [13]. This means that the native state of AK should be an ensemble of kinetically attainable conformers and the energy landscapes of AK folding should be rugged with more than one local minimum.

Typical values of the difference in free energies of native and unfolded states for small natural proteins are −20 to −40 kJ mol^{-1} [15]. Since the possible compact conformations of a given sequence are enormous with subtle energy difference, if there were no kinetic barriers, folded protein should be a thermodynamic equilibrium ensemble of these conformations. A rugged energy landscape is more reasonable than a golf-course-like energy landscape for describing protein folding. On the other hand, protein is also the product of evolution. These sequences with many attainable conformations in which

only few fractions are bio-active should be rejected by evolution. Perhaps, our view about the protein native state should be changed from the lowest Gibbs energy state to an equilibrium ensemble of kinetically attainable conformations.

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