FEBS 19099 FEBS Letters 413 (1997) 429–432

Domain movement in rabbit muscle adenylate kinase might involve proline isomerization

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Received 25 April 1997; revised version received 15 June 1997

Abstract The fluorescence probe, 8-anilino-1-naphthalenesulfonic acid (ANS), was used to monitor the induced-fit conformational movement in rabbit muscle adenylate kinase. In 50 mM Tris-HCl buffer (pH 8.1), the time course of ANS binding to rabbit muscle adenylate kinase is a biphasic process. The fast phase completes within the dead-time of the stopped-flow equipment used (about 15 ms), while the slow phase ends in about 10 minutes. In the presence of 2.0 μM peptidyl prolyl *cis*/*trans*-isomerase, the rate constant of the slow phase reaction is accelerated about 2.4-fold, suggesting that the domain movement during ANS binding to rabbit muscle adenylate kinase may involve proline isomerization. The activation energy of the slow phase was determined to be 74.6 kJ/mol, which is comparable to the activation energy of proline *cis*/*trans*-isomerization (about 80 kJ/mol).

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Key words: Rabbit muscle adenylate kinase; Proline isomerization; 8-Anilino-1-naphthalenesulfonic acid; Domain movement

1. Introduction

Adenylate kinase (AK, EC 2.7.4.3) catalyzes the phosphoryl transfer reaction: MgATP+AMP7⇌MgADP+ADP [1,2]. 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 [3,4]. Previous studies have suggested that adenylate kinase undergoes substrate-induced conformational changes to shield the substrate from water by surrounding it with catalytic residues, and to prevent the escape of kinetic intermediates [5–7]. X-ray analysis of enzyme crystals revealed that the AMP-binding domain undergoes a movement of 8 Å upon AMP binding and that the ATP-binding domain moves up to 30 Å upon binding with ATP [7,8]. This was strongly supported by investigations of different ligand forms of AK in solution using several methods [9–16]. However, these and other published investigations have paid little attention to whether these substrate-induced conformational changes involve proline isomerization.

A well-known property of proline residue is its unique ability to form *cis*-peptide bonds and to undergo *cis*/*trans*-isomerization. About 7% of all prolyl peptide bonds are *cis* in native proteins of known three-dimensional structure [17,18]. The equilibrium between *cis* and *trans*-conformation of an Xaa-Pro peptide bond induces conformational heterogeneity and appears to be widespread in proteins and of importance for the biological function of a protein [19].

The prolyl isomerization can be catalyzed by peptidyl prolyl *cis*/*trans*-isomerases (PPIase, EC 5.2.1.8), which were discovered in porcine kidney by Fischer and his co-workers in 1984 [20]. PPIases not only efficiently catalyze prolyl peptide bond isomerizations in oligopeptides, but also accelerate slow, proline-isomerization-limited steps in the folding of numerous proteins [21].

Two crystal forms, A and B, of adenylate kinase from porcine muscle have been reported [3], which are interconvertable depending 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. ANS occupies the pocket formed between the β-sheet, loop 16–22, helix 23–30 and the C-terminal helix. This pocket was originally assigned to the adenosine moiety of AMP site[3], which turned out recently as ATP site [4,22]. ANS binds to AK with a dissociation constant, k_d, of 56 ± 5 mM [10–12,23]. The binding of ANS to AK enhances the quantum yield of ANS fluorescence as well as shifts the emission peak to a shorter wavelength [23]. A previous study in this laboratory found that the time course of the fluorescence of ANS binding to AK was a biphasic process. The fast phase fluorescence completes within the dead-time of the stopped-flow equipment used, whereas the slow phase reaction completes in about 10 minutes, which was interpreted to result from the conformational changes during AK binding to ANS. Substrates, AMP, ADP and ATP decrease both the fast and slow phase fluorescence. The inhibition efficiency is in the order of AMP < ADP < ATP. ATP was found to bind competitively with AK respect to ANS, but AMP not. ANS has also been found to be an inhibitor of AK for both the fast and slow steps [24].

Investigation of the fluorescence property of ANS binding to AK should develop the understanding of the induced-fit conformational changes. The present communication reports the effects of PPIase on the slow phase reaction of ANS binding to AK. The results suggest that the domain movement of AK during binding with ANS may involves the proline isomerization.

2. Materials and methods

2.1. Materials

Peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was purchased from Peptide Institute Inc. in Japan. Chymotrypsin (Type VII: from Bovine Pancreas, TLCK treated) was a product of Sigma. Glucose-6-phosphate dehydrogenase, hexoskinase, NADP and ANS were Sigma products. Urea was product of NACALA TESQUE, INC. (Japan).

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Abbreviations: AK, rabbit muscle adenylate kinase; ANS, 8-anilino-1-naphthalenesulfonic acid; PPIase, peptidyl prolyl *cis*/*trans*-isomerases; CK, creatine kinase

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PII S0014-5793(97)00951-4

Glucose, Mg acetate and Tris-HCl were local products of analytical grade. Tris-HCl buffer was always freshly prepared.

2.2. Methods

The porcine 17-kDa PPIase was purified according to the method described by Kofron et al. [25]. The enzyme migrates as a single band in reducing SDS-PAGE. The prolyl isomerase activity was measured using the chromogenic peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide in a coupled assay with chymotrypsin as reported by Takahashi et al. [26]. The assay buffer (855 μl of 35 mM HEPES containing 5 mM 2-mercaptoethanol, pH 7.8 at 25°C) was equilibrated in the spectrometer cell (UV-250) before adding the peptide substrate (stored in DMSO, final concentration 42 μM) in the same buffer. Samples were equilibrated for 1 min, and then PPIase (10 μl, 0.1–0.2 μg) was incubated with the substrate for 30 s. The reaction was then started by adding 20 μl 0.76 mM of chymotrypsin (final concentration 15.2 μM). The control measurement was performed under the same conditions in the absence of PPIase. The formation of *p*-nitroaniline was followed at 360 nm. The *trans*-isomer (approximately 90% of equilibrium population) of the substrate was consumed by chymotrypsin hydrolysis during the mixing time as indicated by sudden absorbance change. Further change represented the rate of *cis/trans*-isomerization of the peptide substrate. The enzyme concentration was determined by using the molar extinction coefficient $\varepsilon_{280} = 8730\ M^{-1}\ cm^{-1}$ calculated on the basis of aromatic amino acid content [27].

Rabbit muscle adenylate kinase was prepared essentially according to Zhang et al. [28]. The yield was usually about 60 mg of pure enzyme per kg rabbit skeletal muscle. The final preparation usually had a specific activity greater than 1600 units/mg and showed only one 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/minute ATP generated from ADP.

The activity was assayed 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 absorption at 280 nm with $\varepsilon^{0.1\%}_{1cm} = 0.52$.

The kinetics of ANS binding were measured using a SPF-17 stopped-flow system with a syringe ratio of 1:1 (Applied Photophysics Ltd., UK). One syringe contained a specified concentration of ANS in 50 mM Tris/HCl containing 1 mM EDTA at pH 8.1, 25°C while the other syringe contained appropriate protein samples. The concentration of adenylate kinase was 4.8 μM for all experiments unless otherwise specified. Creatine kinase (CK), prepared as described [29,30], was used in the ANS-binding experiment with the same concentration as AK as a comparison. The final ANS concentration was 200 μM. The ANS concentration was determined using the extinction coefficient $\varepsilon = 4.85 \times 10^3\ M^{-1}\ cm^{-1}$ in aqueous solution. The samples were excited at 378 nm and the emission above 410 nm was detected using a wavelength cutoff filter. The dead-time in the experiments was about 15 ms. In all measurements, the baseline was calibrated with ANS solution.

Digitized kinetic traces were analyzed using the software provided with the Applied Photophysics Kinetic Spectrometer Workstation (Archimedes). Apparent first-order rate constants (k) were obtained by non-linear least-squares fits of the data to:

$$A_t = A_s(1-\exp(-kt)) + A_f$$

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

3. Results

3.1. Effect of PPIase on ANS binding to AK

The binding kinetics of ANS to AK were measured in the presence of 0 to 2.0 μM PPIase. Both in the absence and presence of PPIase the time courses of ANS binding to AK are biphasic process. The fast kinetic phase was unaffected by PPIase but the slow binding interaction was accelerated by PPIase (Fig. 1A). The time constant (τ) of the slow phase was reduced from τ = 480 s in the absence of PPIase to τ = 200 s in the presence of 2.0 mM PPIase, equivalent to a 2.4-fold acceleration. In the control experiment, the time course of 2.0 mM PPIase mixed with 200 mM ANS showed no evidence of ANS binding to PPIase inducing fluorescence changes under our experimental conditions. The dependence of the apparent rate constants on the concentration of PPIase is shown in Fig. 1B.

3.2. Heat-inactivated PPIase or non-specific proteins have no catalysis effect on the kinetics of ANS binding to AK

To clarify whether the catalysis of AK to ANS binding kinetics arises from the biological function of PPIase or

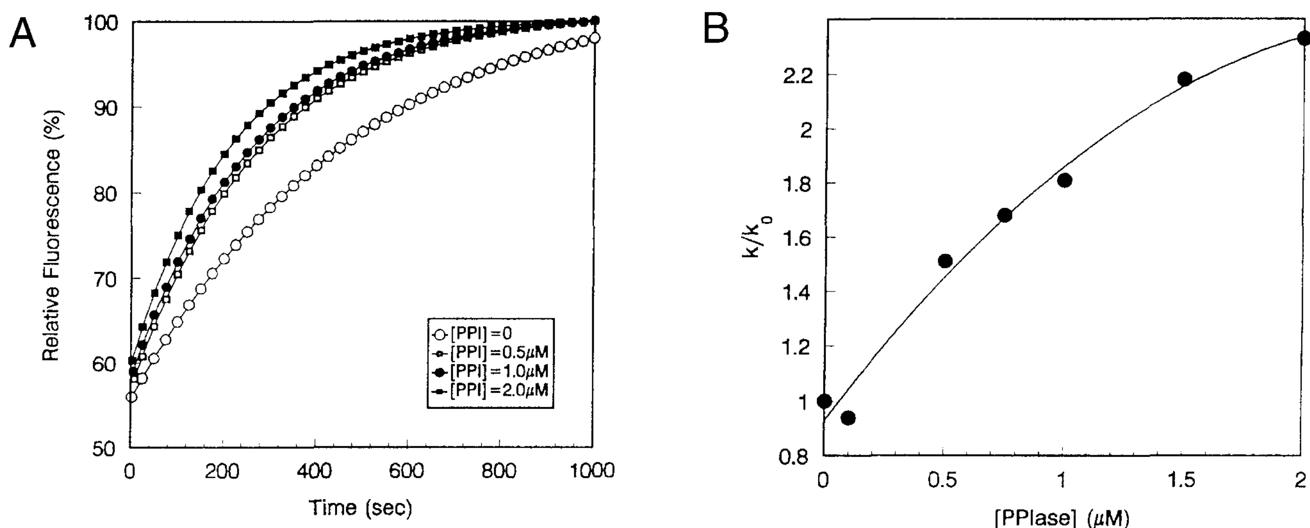

Fig. 1. (A) Catalysis of ANS binding kinetics monitored by fluorescence changes in the presence of increasing concentrations of PPIase from porcine kidney. The increase in ANS fluorescence excited at 378 nm and the emission above 410 nm is shown as a function of time in 50 mM Tris-HCl, pH 8.1, at 25°C in the presence of the indicated concentrations of PPIase. The total fluorescence change during the measurement was defined as 100%. (B) The apparent rate constants of ANS fluorescence changes for various PPIase concentrations. The data is presented as the ratio of the observed rate constants in the presence, k, and in the absence, k_0, of PPIase.

from the protein effect, the binding kinetics of ANS binding to AK were measured in the presence of heat-inactivated PPIase. The isomerase activity of PPIase was suppressed irreversibly by heat inactivation (30 min at 80°C) [31]. No rate enhancement was observed in the presence of 2.0 μM heat-inactivated PPIase.

Creatine kinase (CK) was used to test the unspecified protein effect, because the fluorescence kinetics of ANS binding to CK showed only one fast phase. The fluorescence signal nearly overlaid the base line during the entire course of the measurement for as long as 1000 s (data not shown). No increase in the rate of the slow-binding phase of ANS-AK could be detected in the presence of 0.6 μM CK, thus ruling out any unspecified protein effect. These results are shown in Fig. 2 for clarity.

3.3. Activation energy of ANS binding to AK

The above experiments demonstrated that proline isomerization may be involved in the conformational changes during ANS binding to AK. The activation energy of proline *cis/trans*-isomerization is about 80 kJ/mol [21]. For comparison, the activation energy of the conformational changes during ANS binding to AK was determined by measuring the temperature dependence of the slow phase rate constant of the fluorescence changes during ANS binding to AK at pH 8.1. Fig. 3 shows the Arrhenius plot of the rate constant of the slow phase of ANS binding to AK versus temperature. The apparent activation energy for this reaction was found to be 74.6 kJ/mol.

4. Discussion

ANS is widely used as a hydrophobic probe of protein structures. The fluorescence intensity of ANS binding to a protein is dependent on the microenvironment of the binding site and the solvent environment [32]. AK has one ANS-binding site around the adenosine moiety of ATP-binding site [3,4,22], so that the property of binding ANS to AK can represent, at least to some extent, that of binding ATP to

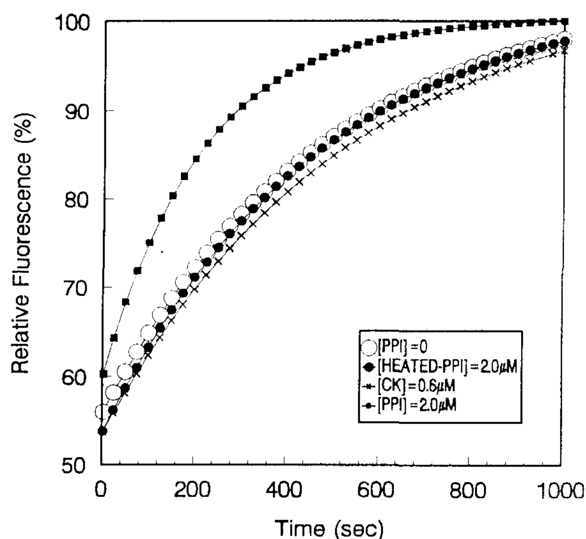

Fig. 2. Effect of inactivated PPI or non-specific protein, creatine kinase (CK), on ANS-binding kinetics monitored by fluorescence changes. Kinetic measurements were carried out as described in Fig. 1.

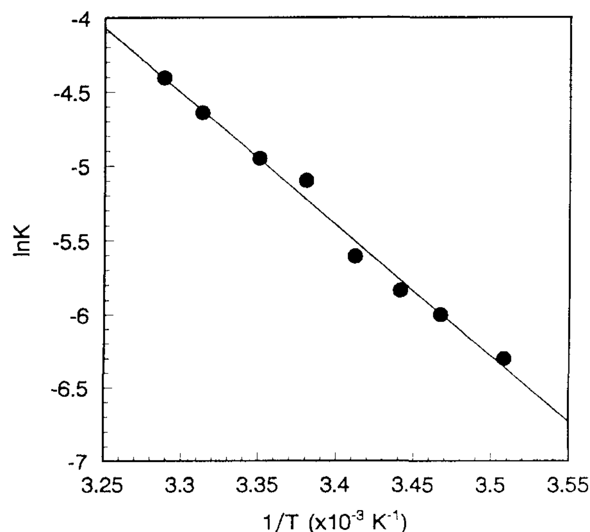

Fig. 3. The Arrhenius plot of the rate constants of the ANS binding to AK as a function of temperature. Kinetic measurements were carried out as described in Fig. 1.

AK. The fast phase fluorescence should come from the rapid combination of the enzyme with the dye, whereas the slow phase fluorescence may arise from the conformational changes during ANS binding to AK. Our observation that PPI can catalyze the slow phase ANS-binding fluorescence suggests that the induced-fit domain movement in AK might involve proline isomerization. The activation energy of the slow phase, which was determined from the temperature dependence of the binding kinetics of ANS to AK to be 74.6 kJ/mol, is similar to that of proline isomerization [21] which supports our suggestion that a proline isomerization is possibly involved in this process.

Sequencing and X-ray crystallography results show that there are 6 proline residues in rabbit muscle adenylate kinase [33]. Three of the proline residues are conserved: one *cis*-form (Pro^{96}) is in the active center while the other two *trans*-form (Pro^{17} and Pro^{159}) are located at loop 16–22 and alpha-helix 145–165, respectively. The active center is located in a hydrophobic pocket deep in a cleft between two domains. Another hydrophobic pocket behind loop 16–22 and helix 23–30 has been assigned to the binding site of ATP and ANS [3,4,22].

Although it is difficult to distinguish which proline residue is involved in the isomerization equilibrium, the following evidence suggests that proline 17 might be a likely candidate even though it is in *trans*-form in the native structure of AK.

1. The existence of Gly^{16} preceding Pro^{17} suggests that the Gly-Pro sequence could undergo *cis/trans*-isomerization. Analysis of the protein sequence data bank MIPSX36 for the fractions of X-Pro bonds in the total pool of established sequences [34] clearly demonstrates that *cis*-peptide bond occur with the highest frequency when X is Tyr, Leu, Ser, Gly, and Phe. Moreover, as reported earlier the sequence of loop 16–22 is Gly-Pro-Gly-Ser-Gly-Lys-Gly, i.e. every second position has a glycine residue, which allows for a large range of dihedral angles at their Cα atoms. The glycine-rich sequence has now been recognized as a fingerprint sequence for nucleotide-binding sites. Consequently, the conformation

of loop 16–22 is so flexible that it could be in one conformation with Pro[17] in *trans*-form or with Pro[17] in *cis*-form.

2. Tagaya et al. [35] characterized two mutants of chicken adenylate kinase in which Pro[17] was replaced by Gly or Val. Their results suggest that Pro[17] plays an important role for the binding of substrates, but not for catalytic efficiency, although it does not directly interact with substrates. It is conceivable that the rigid turn at Pro[17] in the loop structure is important for the binding of the substrate nucleotides since the replacement of Pro[17] by Val would perturb the turn and replacement by Gly would make the turn loose.
3. X-ray diffraction analysis of porcine AK at 4.7 Å resolution indicated the existence of three crystalline forms depending on the pH of the mother liquor [3]. Form A exists in pH 6.9–8.0 while form B exists in pH 5.7–6.0. An experiment with ANS soaking with AK revealed that the ANS-binding crystal could only be obtained with crystalline form B [3]. The Gly-rich loop moves about 6 Å during the transition between two crystalline forms. Therefore, form B was more 'open' in structure than form A. The *trans*-peptide bond between Gly[16] and Pro[17] in crystal form A was deduced from the refined structure of porcine AK at 2.1 Å resolution [33]. However, the structure of form B at high resolution is unavailable because of the labile crystal quality [33].

The present study provides new information on induced-fit movement in adenylate kinase. The proline isomerization involving in induced-fit movement is of importance for understanding the biological function of adenylate kinase.

Acknowledgements: We express our sincere thanks to Professor C.L. Tsou of the Institute of Biophysics, Academia Sinica, for many helpful suggestions. This work was supported in part by the Pandeng Project of the China Commission for Science and Technology, and projects 39625008 and 39670157 from the National Natural Science Foundation of China.

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