Native states of adenylate kinase are two active sub-ensembles

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Abstract There are two kinds of conformational forms of adenylate kinase (AK) in equilibrium in solution with different ANS-binding properties. Furthermore, the nature of AP_5A inhibition suggests also that the native forms of AK for binding with different substrates pre-exist in the absence of substrates. In the present study, a kinetics approach was used to explore the native forms distinguished by ANS-binding properties and by the nature of AP_5A inhibition. The results revealed that the native forms distinguished by ANS probe are two conformational sub-ensembles. Both sub-ensembles are active and consist of a series of forms, which pre-exist in solution and can bind with different substrates. The K_m values of N_1 for AMP, ADP and MgATP are larger than that of N_2, indicating that the N_2 sub-ensemble is more specific for binding substrates. This is consistent with the previous observation that the activity of N_2 is about 1.8-fold of that of N_1.

Key words: Adenylate kinase; AP_5A; Multiple native conformers; Ensemble; Active energy; Kinetic control; Thermodynamic control; Protein folding

1. Introduction

It has become clear that some proteins may exist in more than one distinct folded form in solution. Evidence for distinguishing multiple native forms of staphylococcal nuclease come from electrophoretic and NMR results [1–6], and for calbindin D_{9K} from both NMR and X-ray crystal structure studies [7,8].

Adenylate kinase (AK, EC 2.7.4.3) catalyzes the phosphoryl transfer reaction: MgATP+AMP⇌MgADP+ADP [9–12]. Previous reports from this laboratory indicated two different conformational forms of rabbit muscle AK in equilibrium in solution with different 8-anilino-1-naphthalenesulfonic acid (ANS)-binding properties [13,14]. One form (denoted N_1) binds with ANS, whereas the other (denoted N_2) does not. ANS binding to N_1 results in a burst phase fluorescence increase, followed by the interconversion of N_2 to N_1, to give the slow phase ANS binding. Furthermore, the nature of AP_5A changes qualitatively from competitive inhibition with respect to either substrate in the forward reaction (MgATP or AMP) to a mixed non-competitive in the backward reaction with either substrate (MgADP or ADP) [15,16], suggesting that the native forms of the enzyme binding with different substrates pre-exist in the absence of substrates [17].

It is not clear whether the conformations distinguished by ANS probe are consistent with those distinguished by AP_5A inhibition. The rate of enzyme conformational interconversion is on the order of 10^2 s^{-1} in the catalytic cycle [17], which is much higher than that of determined by ANS fluorescence probe [13] (on the order of 10^{-2} s^{-1}). This can perhaps be explained by the fact that substrates decrease whereas ANS increases the activation energy of enzyme conformational interconversion. Another possibility is that the native forms involved in catalytic cycle are different from those distinguished by ANS probe. The native states of AK are two sub-ensembles. One sub-ensemble binds with ANS, whereas the other does not. Both sub-ensembles consist of a series of forms, which pre-exist in solution and can bind with different substrates.

In the present study, a kinetic approach was used to explore the competitive reaction of substrates and ANS binding to the enzyme. The competitive equations derived from the suggested model of two conformational ensembles agree well with the experimental observations. This result provides evidence that the native states of AK are two sub-ensembles of forms.

2. Materials and methods

2.1. Reagents

2.1.1. Preparation and activity assay of AK. The enzyme was prepared from rabbit muscle essentially according to Zhang et al. [18]. The final preparation had a specific activity greater than 1800 units/mg, which showed only a single peak in SDS electrophoresis, gel filtration and reversed phase fast protein liquid chromatography. One unit of activity 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 methods of activity assay were described previously [13,16,18].

2.2. Methods

The time course of ANS binding to AK fluorescence was measured by SPF-17 stopped-flow system with a syringe ratio of 1:1 (Applied Photophysics). The samples were excited at 378 nm, and the emission above 410 nm was detected using a wavelength cut-off filter. The dead-time in these experiments was about 15 ms. In all measurements, the baseline was calibrated with ANS solution.

2.3. Kinetics

Two possible models can be proposed to interpret the discrepancy of the conformational interconversion rates determined by ANS probe

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

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and found in catalytic cycle. (1) There are two sub-ensemble native forms of AK existing in solution, of which one binds rapidly with ANS and the other does not. Both sub-ensembles consist of a series of forms and can bind different substrates (the two sub-ensemble model). (2) There are two native forms of AK existing in solution, of which one binds with substrates AMP, MgATP, as well as ANS and AP_5A, whereas the other binds with ADP and MgADP but not with ANS and AP_5A. Substrates decrease whereas ANS increases the activation energy of enzyme conformational interconversion (the two forms model). Those two models can be distinguished by competitive kinetics of substrates and ANS binding to the enzyme.

2.3.1. Two sub-ensemble model. The two sub-ensemble model can be expressed by Reactions 1–4:

$$N_1 \overset{k_1/k_{-1}}{\leftrightarrow} N_2 \quad \text{(Reaction 1)}$$

$$N_1 + ANS \leftrightarrow N_1{\cdot}ANS \quad \text{(Reaction 2)}$$

$$N_1 + S \leftrightarrow N_1{\cdot}S \quad \text{(Reaction 3)}$$

$$N_2 + S \leftrightarrow N_2{\cdot}S \quad \text{(Reaction 4)}$$

and:

$$K_1 = \frac{[N_1]}{[N_2]} \quad (1)$$

$$K_2 = \frac{[N_1][ANS]}{[N_1{\cdot}ANS]} \quad (2)$$

$$K_3 = \frac{[N_1][S]}{[N_1{\cdot}S]} \quad (3)$$

$$K_4 = \frac{[N_2][S]}{[N_2{\cdot}S]} \quad (4)$$

$$[N_1] + [N_1{\cdot}S] + [N_1{\cdot}ANS] + [N_2] + [N_2{\cdot}S] = [N_0] \quad (5)$$

Here S is the substrate (AMP, ADP or MgATP), N_1 and N_2 are the two conformational sub-ensembles, respectively. N_1 binds with ANS, whereas N_2 does not. Both sub-ensembles consist of a series of forms and can bind different substrates. The conformational interconversion rates in one sub-ensemble are very much faster than that of between two sub-ensembles, so that the forms in one ensemble act as a single form in the time scale of N_1 and N_2 interconversion. $[N_1]$, $[N_2]$, $[N_1{\cdot}S]$, $[N_2{\cdot}S]$, $[N_1{\cdot}ANS]$ and $[N]_0$ are the concentration of forms N_1, N_2, binary complexes $N_1{\cdot}S$, $N_2{\cdot}S$, $N_1{\cdot}ANS$ and total enzyme, respectively. Since the binding Reactions 2, 3 and 4 are very fast, steady-state conditions are instantaneously reached for Reactions 2, 3 and 4 and the interconversion of N_2 to N_1 is the rate-limiting step. So we obtained:

$$-\frac{d([N_1] + [N_1{\cdot}S] + [N_1{\cdot}ANS])}{dt} = k_1[N_1] - k_{-1}[N_2] \quad (6)$$

Substituting Eqs. 1–5 into Eq. 6 gives Eq. 7.

$$-\frac{d[N_1{\cdot}ANS]}{dt} = A[N_1{\cdot}ANS] - B \quad (7)$$

Where

$$A \equiv \frac{k_1}{1 + [ANS]/K_2 + [S]/K_3} + \frac{k_{-1}}{1 + [S]/K_4} \quad (8)$$

$$B \equiv \frac{k_{-1}[N_0][ANS]/K_2}{(1 + [S]/K_4)(1 + [ANS]/K_2 + [S]/K_3)} \quad (9)$$

Integrating Eq. 7, t from 0 to t.

$$[N_1{\cdot}ANS] = [N_1{\cdot}ANS]_0 e^{-At} + \frac{B}{A}(1 - e^{-At}) \quad (10)$$

Here the subscripts '0' denotes the concentration at $t = 0$. At $t = 0$, Reactions 2, 3 and 4 instantaneously reach equilibrium:

$$[N_1]_0 + [N_1{\cdot}S]_0 + [N_1{\cdot}ANS]_0 = [N_1]^{eq} + [N_1{\cdot}ANS]^{eq} \quad (11)$$

Here, the superscript 'eq' denotes the pre-equilibrated concentration of AK and ANS solution. Substituting Eqs. 2 and 3 into Eq. 11 gives Eq. 12:

$$\frac{[N_1{\cdot}ANS]^{eq}}{[N_1{\cdot}ANS]_0} = 1 + \frac{1/K_3}{1 + [ANS]/K_2}[S] \quad (12)$$

Substituting K_2 and the concentration of ANS into Eq. 12, K_3 can be determined from the slope of plot $[N_1{\cdot}ANS]^{eq}/[N_1{\cdot}ANS]_0$ vs. the concentration of substrate.

At $t = \infty$:

$$[N{\cdot}ANS]_\infty = \frac{B}{A} \quad (13)$$

Substituting Eqs. 2, 8 and 9 into Eq. 13 gives Eq. 14:

$$\frac{[N_1{\cdot}ANS]^{eq}}{[N_1{\cdot}ANS]_\infty} = 1 + \frac{1/(K_1K_4) + 1/K_3}{1 + 1/K_1 + [ANS]/K_2}[S] \quad (14)$$

Substituting K_1, K_2, K_3 and the concentration of ANS into Eq. 14, K_4 can be determined from the slope of plot $[N_1{\cdot}ANS]^{eq}/[N_1{\cdot}ANS]_\infty$ vs. the concentration of substrate.

At any time t, the concentration of $N_1{\cdot}ANS$ can be calculated from:

$$[N_1{\cdot}ANS] = [N_1{\cdot}ANS]_0 e^{-At} + [N_1{\cdot}ANS]_\infty (1 - e^{-At}) \quad (15)$$

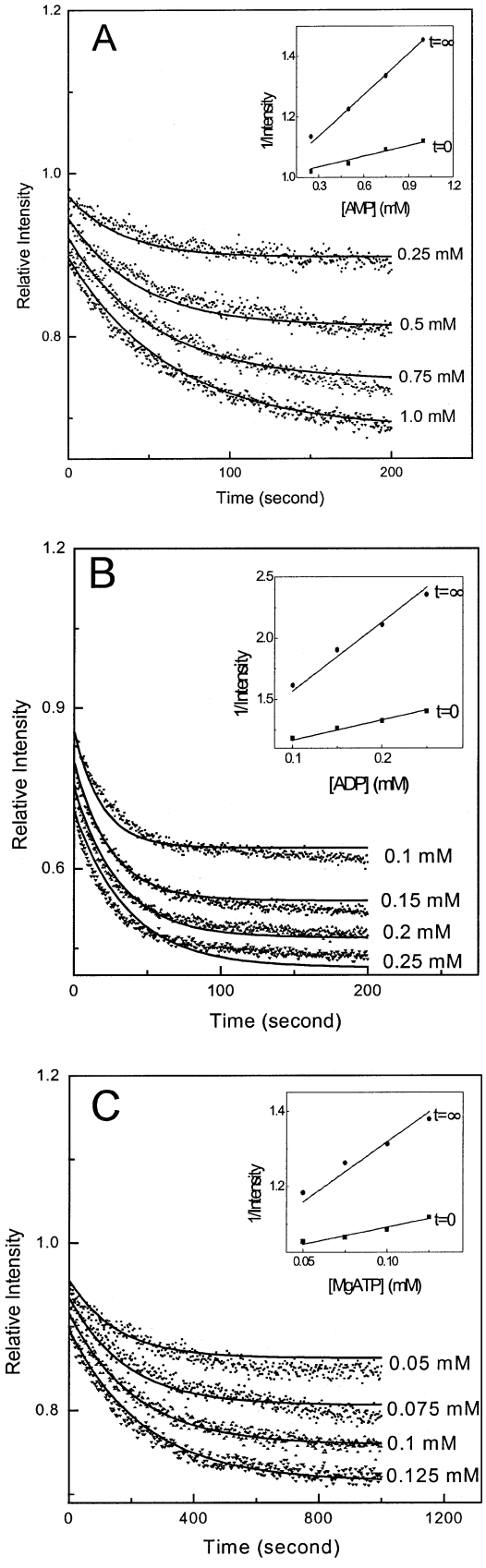
According to Eq. 15, initiating the competitive reaction by adding substrate to the enzyme and ANS pre-equilibrated solution, two phases of fluorescence decreasing, a burst and a slow, could be observed. While initiating the competitive reaction by adding ANS to the enzyme and substrate pre-equilibrated solution, two phases of fluorescence increasing, a burst and a slow, could be observed.

2.3.2. Two forms model. According to the two forms model, one form (N_1) binds with substrates AMP, MgATP, as well as ANS and AP_5A, whereas the other (N_2) binds with ADP and MgADP but does not with ANS and AP_5A. In the enzyme and substrate AMP or MgATP pre-equilibrated solution nearly all the enzyme should be in the N_1 form. Initiating the competitive reaction by adding ANS to this pre-equilibrated solution, only the burst phase fluorescence increasing could be observed, while in the enzyme and substrate ADP pre-equilibrated solution, nearly all the enzyme should be in the N_2 form. Initiating the competitive reaction by adding ANS to this pre-

Table 1
Kinetics parameters determined by ANS binding to AK fluorescence

Substrate	ANS competitive kinetics		Enzyme kinetics[a]
	K_m (mM) N_1 ensemble	K_m (mM) N_2 ensemble	
AMP	1.039 ± 0.016	0.547 ± 0.009	0.12 ± 0.02
ADP	0.072 ± 0.003	0.046 ± 0.002	0.028 ± 0.002
MgATP	0.130 ± 0.002	0.081 ± 0.001	0.06 ± 0.01

Here K_m of N_1 ensemble is K_3 and K_m of N_2 ensemble is K_4.
[a]The K_m determined by enzyme kinetics was reported by Sheng et al. [12].



equilibrated solution, only the slow phase fluorescence increasing could be observed.

3. Result

3.1. Initiating the competitive reaction by adding different concentrations of substrate to the fixed concentration of enzyme and ANS pre-equilibrated solution

Freshly prepared AK usually had a specific activity of 2000 ± 100 units/mg. Then AK was diluted to 20 μM and mixed with 0.4 mM ANS with a rate of 1:1. The mixture with a final concentration of 10 μM AK and 0.2 mM ANS was incubated for 15 min in 50 mM Tris–HCl, 1 mM EDTA and 1 mM β-mercaptoethanol, pH 8.1, 25°C. The competitive reaction was initiated by adding various concentrations of substrate to this pre-equilibrated AK and ANS solution. One syringe contains a fixed concentration of ANS and AK, whereas the other syringe contains different concentrations of AMP, ADP or MgATP with the same concentration of ANS, respectively. The final concentration of the reaction system was about 5 μM AK, 0.2 mM ANS and different concentrations of substrate. As Fig. 1A–C show, there was a burst phase decreasing of ANS binding to AK fluorescence completed in the dead-time of the SPF-17 stopped-flow system. Then ANS binding to AK fluorescence decreased slowly with prolonging of reaction time, and at last to a limit. The higher concentration of substrate was, the smaller the limit was. The values of K_3 for different substrates were determined from the slope of plots $[N_1{\cdot}ANS]^{eq}/[N_1{\cdot}ANS]_0$ vs. the concentration of substrate (Fig. 1A–C, inset), using the values of $K_1 = 0.46$ and $K_2 = 0.027$ mM [13]. The values of K_4 for different substrates were determined from the slope of plots $[N_1{\cdot}ANS]^{eq}/[N_1{\cdot}ANS]_\infty$ vs. the concentration of substrate (Fig. 1A–C, inset) using the values of $K_1 = 0.46$, $K_2 = 0.027$ mM [13] and the values of K_3 obtained from the above calculation. All the obtained kinetic parameters are listed in Table 1. The values of conformational interconversion rate between the two sub-ensembles N_1 and N_2 in the presence of different substrates were obtained by non-linear least-squares fits of the experimental data using Eq. 15. Solid lines in Fig. 1A–C are the fitting results, agreeing well with the experimental data. The obtained values of k_1 and k_{-1} in the presence of different substrates are listed in Table 2. It shows that the rate of conformational interconversion between the two sub-ensembles N_1 and N_2 is indeed accelerated in the presence of substrates, but is still much lower than that of conformational interconversion in catalytic cycle. We also added ATP and MgAMP to the enzyme and ANS pre-equilibrated solution, and got experiment curves similar to the first three substrates. Since ATP and MgAMP are not in fact the substrates of AK, those data are not shown here.

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Fig. 1. Initiating the competitive reaction by adding different concentration of substrate (A: AMP; B: ADP; and C: MgATP) to the fixed concentration of enzyme and ANS pre-equilibrated solution. The solid lines are the fitting lines using Eq. 15 with $K_1 = 0.46$ and the value of K_3 and K_4 derived from the inset plot. The inset plot shows the reciprocal of relative fluorescence intensity versus the concentration of substrates (A: AMP; B: ADP; C: MgATP) at $t = 0$ and $t = \infty$, and the solid lines are the fitting lines. AK concentration is about 5 μM; buffer solution is 50 mM Tris–HCl, containing 1 mM EDTA and 1 mM β-mercaptoethanol, pH 8.1, at 25°C.

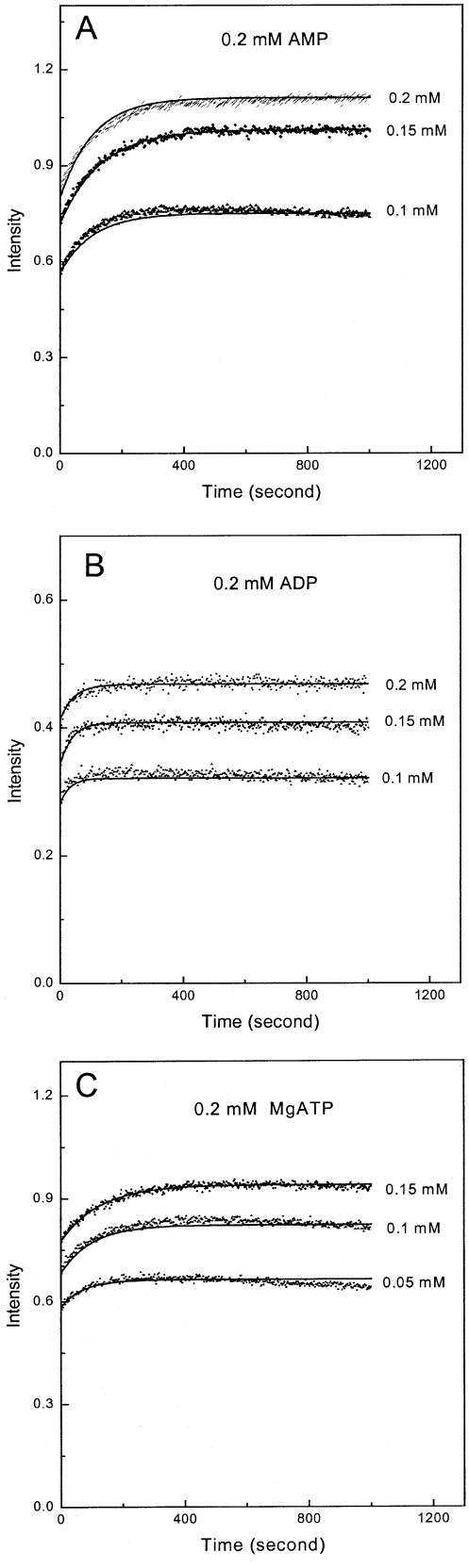

Fig. 2. Initiating the competitive reaction by adding different concentrations of ANS to the fixed concentration of enzyme and substrate (A: AMP; B: ADP; and C: MgATP) pre-equilibrated solution. The solid lines are calculated using Eq. 15 with $K_1 = 0.46$, K_3 and K_4 shown in Table 1. AK concentration is about 5 μM; buffer solution in 50 mM Tris–HCl, containing 1 mM EDTA and 1 mM β-mercaptoethanol, pH 8.1, at 25°C.

Table 2
Rate constants of conformational interconversion between two ensembles

Rate constants	In the presence of substrates			In the absence of substrate[a]
	AMP	ADP	MgATP	
k_1	0.09468	0.3472	0.02287	0.013
k_{-1}	0.04363	0.16	0.01054	0.0055

k_1 and k_{-1} are the rate constants of interconversion between N_1 and N_2 (Reaction 1). Here $K_1 = k_1/k_{-1}$.
[a]The rate constants in the absence of substrate was reported by Zhang et al. [13].

3.2. Initiating the competitive reaction by adding different concentrations of ANS to the fixed concentration of enzyme and substrate pre-equilibrated solution

AK was prepared with the same procedure as above. Instead of ANS, AK was mixed with 0.4 mM AMP, MgAMP, ADP, ATP or MgATP, respectively. Then the pre-equilibrated AK and substrate solution was mixed with a solution containing the same concentration of substrate and different concentrations of ANS monitored by the SPF-17 stopped-flow system. As Fig. 2A–C show, there was a burst phase fluorescence increasing completed in the dead-time of the SPF-17 stopped-flow system. Then ANS binding to AK fluorescence increased slowly with prolonging of reaction time, and at last to a limit. The solid lines in Fig. 2A–C were calculated by Eq. 15, using the parameters derived by the first experiment data, agreeing well with the experimental results.

4. Discussion

4.1. The energy landscapes of AK folding should be rugged with more than one local minimum

The above experimental observed kinetic patterns of ANS and substrates binding to AK competitive reactions agree well with the prediction of the two sub-ensemble forms model, providing evidence that the energy landscapes of AK folding should be rugged with at least two local minimums. The folded native forms of AK are two sub-ensembles with different ANS-binding properties. Both conformational ensembles of AK, which consist of a series of forms binding with different substrates, are active [19]. The fact that AP_5A acts as a competitive inhibitor for the forward reaction and a mixed non-competitive inhibitor for the backward reaction evidenced that the forms of AK for binding with different substrates pre-exist.

4.2. Forms in N_2 sub-ensemble are more specific for substrate binding

Previous reports from this laboratory indicated that both sub-ensembles of AK distinguished by ANS probe are active. The activity of N_2 is about 1.8-fold of that of N_1 and shows higher susceptibility to proteolysis by trypsin [19]. As Table 1 shows, the K_m values of AMP, ADP and MgATP of N_1 sub-ensemble are larger than those of in N_2 sub-ensemble, indicating that the N_2 sub-ensemble is more specific for binding substrates. The fact that the forms in N_1 sub-ensemble can bind with ANS and that forms in N_2 sub-ensemble does not, is also evidence that N_2 is more specific for binding substrates than N_1.

The K_m values of AMP, ADP and MgATP determined by

the present experiment are different from those determined by enzyme kinetics [13]. The difference arose from two reasons. (1) The K_m values determined by enzyme kinetics are the average values of the two ensembles. (2) AK contains two distinct nucleotide-binding sites: one is specific for AMP (or ADP), whereas the other less specific one is for MgATP (or MgADP). By means of the enzyme kinetics, we changed the concentration of MgATP (or MgADP) that was added to AMP (or ADP)-saturated AK solution to determine the K_m of MgATP (or MgADP), since AMP (or ADP) could bind not only to AMP sites but also to MgATP sites. The K_m of MgATP (or MgADP) determined by the enzyme kinetics method should be lower than the actual value. While in the present experiment, the K_m of MgATP was determined in the absence of AMP, and this value should be more close to the actual value. On the contrary, the K_m of AMP determined in the present experiment should be higher than the actual value, since some AMP molecules would bind to MgATP sites, while the K_m of AMP determined by the enzyme kinetics method should be closer to the actual value. Both methods, the enzyme kinetics and the competitive kinetics, are complemental for correctly determining the K_m values of AK.

4.3. Substrates decrease the active energy of conformational interconversion between two ensembles

The rate constants of conformational interconversion between the two sub-ensembles determined in the presence of substrates are much faster than that determined in the absence of substrates (see Table 2), indicating that the substrates decrease the conformational interconversion active energy between N_1 and N_2. The decreasing efficiency is in the order ADP > AMP > MgATP.

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