An Iso-random Bi Bi Mechanism for Adenylate Kinase*

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An iso-random Bi Bi mechanism has been proposed for adenylate kinase. In this mechanism, one of the enzyme forms can bind the substrates MgATP and AMP, whereas the other form can bind the products MgADP and ADP. In a catalytic cycle, the conformational changes of the free enzyme and the ternary complexes are the rate-limiting steps. The AP5A inhibition equations derived from this mechanism show theoretically that AP5A acts as a competitive inhibitor for the forward reaction and a mixed noncompetitive inhibitor for the backward reaction.

Adenylate kinase (AK)1 (EC 2.7.4.3) catalyzes the reaction MgATP + AMP ⇔ MgADP + ADP, which is essential for cell survival (1–8). This small kinase has also been considered as a “model kinase” in the study of the structure-function relationship of kinases (9). Although the catalytic kinetics of AK has been studied extensively (10–14), a full understanding mechanism is still lacking. The basic kinetic pattern is random Bi Bi (11), but whether the chemical step or the physical step(s) is rate-limiting is still controversial. Furthermore, it was reported in an early work of Kuby et al. (12) that the nature of the AP5A inhibition changes qualitatively from competitive inhibition with respect to either substrate in the forward reaction (MgATP or AMP) to noncompetitive (mixed noncompetitive) in the backward reaction with either substrate (MgADP or ADP). There is still no convincing explanation for the inhibition nature of AP5A.

In a series of previous studies in this laboratory, it was found that there are multiple native forms of AK in equilibrium in solution (15–18), which may help improve our understanding of the catalytic mechanism of AK. In this study, the catalysis and inhibition kinetics of AK was re-examined. An iso-random Bi Bi catalytic mechanism was proposed. The AP5A inhibition equations derived from this mechanism show that AP5A acts as a competitive inhibitor for the forward reaction and a mixed noncompetitive inhibitor for the backward reaction, agreeing well with experimental results.

EXPERIMENTAL PROCEDURES

Reagents—Pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, phosphoenolpyruvate, NADP, NADH, AMP, ADP, ATP, and AP5A were Sigma products, and other reagents were local products of analytical grade.

Preparation and Activity Assay of Adenylate Kinase—The enzyme was prepared essentially according to Zhang et al. (19) The yield was usually about 60 mg of pure enzyme per kilogram of rabbit muscle. The final preparation usually had a specific activity greater than 1,600 units/mg and showed only a single peak in SDS electrophoresis, gel filtration, and reversed-phase fast protein liquid chromatography. One unit is defined as 1 μmol of ADP (MgADP) generated per minute in forward reaction and 1 μmol of ATP generated per minute in backward reaction.

Methods—UV absorbance at 340 nm was measured with either Cary 219 (Varian) or U-3000 (Hitachi, Japan) spectrophotometer.

The rate of the backward reaction (MgADP + ADP → MgATP + AMP) was measured by following the reduction of NADP at 340 nm in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase. The final assay mixture was: 50 mM Tris-HCl, pH 8.1, 2 mM β-mercaptoethanol, 6.7 mM glucose, 0.67 mM NADP, 7.6 μM bovine serum albumin, 10 units/ml hexokinase, 10 units/ml glucose-6-phosphate dehydrogenase, varying and calculated concentrations of MgAC2 and ADP.

Measurements 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, 2 mM β-mercaptoethanol, 75 mM KCl, 4 mM phosphoenolpyruvate, 1.0 mM of MgAc2 as free Mg2+, 0.2 mM NADH, 10 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, 7.6 μM bovine serum albumin, plus varying and calculated amounts of MgAc2, ATP, and AMP.

Iso-random Bi Bi Mechanism—The catalysis and inhibition mechanism of adenylate kinase is suggested as shown Scheme 1. Using the combined equilibrium and steady-state treatment developed by Cha (20) and described by Huang (21), Scheme 1 can be reduced to Scheme 2 and Eq. 1.

Here $E_i$ and $E_2$ denote two native forms of enzyme, one form ($E_i$) can bind with AMP ($M$) and MgATP ($T$), and the other one with ADP ($D_i$) and MgADP ($D_j$). Both interconversions of the ternary complexes and free enzymes are rate-limiting steps. This mechanism is, in principle, a random Bi Bi type, similar to that proposed by an earlier study (11), but with the major modification that two native forms of enzyme are introduced. This mechanism is based on the fact that AK undergoes large domain movements upon substrate binding (22). After aligning 17 known structures of nucleoside monophosphate kinases, Vonrhein et al. (23) proposed a common fold for this class of enzymes.
(23) gave movies to demonstrate the catalytic cycle of AK, which showed that the conformation of ternary complex of enzyme binding with AMP and ATP is different from that of enzyme binding with two ADP. That is to say, AK undergoes at least two steps of conformational changes. One step is the change of a ternary complex with bound substrates to one with bound products during the reaction, and the other is the change of the free enzyme in different forms. These conformational changes should be the rate-limiting steps in the catalytic cycle of AK, whereas the binding of substrates to and the release of products from the enzyme are quickly equilibrated. AP₅A (I) can only bind to the other less specific one is MgATP (25), so the reaction system is rapidly removed by the coupled enzymes, so that the concentration of ternary complex DᵢEᵢDᵢ approaches zero.

In the absence of AP₅A, we have Eq. 2. 

\[ v = \frac{k_4 [M][T]}{k_{-1} + \left( \frac{[M]}{K_M} \right) \frac{[T]}{K_T} + \frac{[M][T]}{K_M^2 K_T} + k_1 + k_2 \frac{[M][T]}{K_M K_T}} \] (Eq. 2)

Here a factor of two is introduced, because the forward reaction produces two ADP molecules.

In the presence of AP₅A, we have Eq. 3. 

\[ \frac{v}{E_0} = \frac{2 \cdot k \cdot J \cdot [M][T]}{k_{-1} \left( \frac{[M]}{K_M} \right) \frac{[T]}{K_T} + \frac{[M][T]}{K_M^2 K_T} + k_1 + k_2 \frac{[M][T]}{K_M K_T}} \] (Eq. 3)

Eq. 3 shows that AP₅A acts as a competitive inhibitor for the forward reaction.

In the backward reaction, one binding site is specific for AMP, whereas the other less specific one is MgADP (25), so the reaction system consists of free enzymes Eᵢ and binary complexes DᵢEᵢDᵢ, and EᵢT and ternary complexes MEᵢT and MEᵢM. The products, Dᵢ and Dᵢ, are rapidly removed by the coupled enzymes, so that the concentration of ternary complex DᵢEᵢDᵢ approaches zero.

In the absence of AP₅A, we have Eq. 4. 

\[ \frac{v}{E_0} = \frac{k \cdot J \cdot [D][D]}{K_D K_{D_2}} \] (Eq. 4)

1. The full set of data in Figs. 1 and 2 was fitted to Eqs. 2 and 4 to calculate kinetic parameters by a computer program. The best fit kinetic parameters are summarized in Table I and were used to draw the solid lines in Figs. 1 and 2, which are in good agreement with the experimental results.

\[ \text{AP₅A Inhibition Patterns—It has been reported by Kuby et al. (12) that AP₅A acts as a competitive inhibitor for the forward reaction and a noncompetitive inhibitor (the origin figures show a mixed noncompetitive) for the backward reaction, here the AP₅A inhibition patterns were re-examined.} \]

Because the affinities of AMP to MgATP site as well as ADP to MgADP site are relatively smaller, Eqs. 10 and 12 can be treated as linear. Fig. 3, A and B, shows the AP₅A inhibition patterns in the forward reaction with varied AMP (Fig. 3A) and MgATP (Fig. 3B) concentrations, respectively, indicating that AP₅A acts as a competitive inhibitor for the forward reaction.
Fig. 1. Catalytic pattern of adenylate kinase at pH 7.4 in the forward reaction. The free magnesium ion concentration was kept constant at 1 mM. A, AMP as variable substrate at various fixed MgATP concentrations as indicated; B, MgATP as variable substrate at various fixed AMP concentrations as indicated. In all experiments, the final concentration of adenylate kinase was 2.3 nM unless otherwise specified.

DISCUSSION

There are two nucleotide-binding sites of AK, one for the magnesium complexes and the other for uncomplexed nucleotides. X-ray and NMR studies suggested that the latter sub-

Fig. 2. Catalytic pattern of adenylate kinase at pH 8.1 in the backward reaction. A, magnesium ion as variable substrate at various fixed ADP concentrations as indicated; B, ADP as variable substrate at various fixed magnesium ion concentrations as indicated.

TABLE I

| Kinetic parameters of adenylate kinase |
|----------------------------------------|
| The Michaelis constants $K_m$ equal to the dissociation constants, i.e. $K_m$(AMP) = $K_M$, $K_m$(MgATP) = $K_T$, $K_m$(ADP) = $K_D$, $K_m$(MgADP) = $K_M$, $K_m$(AP5A) = $K_D$, and $K_m$(AMP) = $K_M$. |
| Constants                  | Value (mM)       |
|---------------------------|------------------|
| $K_m$(AMP)                | 3.3 ± 0.3        |
| $K_m$(MgATP)              | 0.06 ± 0.01      |
| $K_m$(ADP)                | 0.028 ± 0.002    |
| $K_m$(MgADP)              | 0.91 ± 0.1       |
| $K_m$(AP5A)               | 0.033 ± 0.003    |
| $k_1$                     | 14,000 ± 2,000 μmol·min⁻¹·mg⁻¹ |
| $k_2$                     | 8,000 ± 800 μmol·min⁻¹·mg⁻¹ |
| $k_{-1}$                  | 710 ± 70 μmol·min⁻¹·mg⁻¹ |
| $k_{-2}$                  | 960 ± 100 μmol·min⁻¹·mg⁻¹ |
strate site is more specific, whereas the former can bind uncomplexed substrates to some extent (24). The above experiments demonstrate that AMP or ADP shows substrate inhibition in the forward or the backward reaction, respectively. This result, which was also observed in bakers’ yeast AK by Russell and co-workers (25), is in agreement with the x-ray and NMR findings.

The equilibrium concentrations of $E_1$ and $E_2$ in the absence of substrates should be different from those at steady state, so a lag (or burst) phase should occur in the beginning of the catalytic reaction. However, because the activity of AK was assayed with a coupled enzyme system, the initial process is too complicated to follow.

![Graphs](image)

**FIG. 3.** Inhibition pattern of AP$_5$A in the forward reaction. A, the MgATP concentration was kept constant at 0.05 mM, and AMP as variable substrate at various fixed AP$_5$A concentrations as indicated; B, the AMP concentration was kept at 0.1 mM, and MgATP as variable substrate at various fixed AP$_5$A concentrations as indicated.

**FIG. 4.** Inhibition pattern of AP$_5$A in the backward reaction. A, the MgADP concentration was kept at 0.1 mM, and ADP as variable substrate at various fixed AP$_5$A concentrations as indicated; B, the ADP concentration was kept at 0.03 mM, and ADP as variable substrate at various fixed AP$_5$A concentrations as indicated.

The fact that AP$_5$A acts as a competitive inhibitor for the forward reaction and a mixed noncompetitive inhibitor for the backward reaction cannot be explained by the normal random Bi Bi mechanism. However, these inhibition patterns are consistent with an iso-random Bi Bi mechanism in which the substrates can bind to one isoform while the products bind to another isoform of the enzyme. This strongly supports the suggestion that two native forms of AK might be involved in the catalytic reactions.

The rate of enzyme conformational changes is on the order of $10^2$ s$^{-1}$ in the catalytic reactions, which is much higher than that determined by ANS fluorescence probe (about $10^{-2}$ s$^{-1}$, see Ref. 15). This perhaps can be explained that substrates decrease whereas ANS increases the activation energy of en-
zyme conformational changes. It was indeed observed that ANS inhibits the folding of AK, whereas AMP, ADP, and ATP can accelerate the folding (16). Another possibility is that the two native forms involved in the catalytic cycle are different from those distinguished by ANS probe, and AK might exist in more native forms in equilibrium. The current experimental results provide no information to distinguish the above possibilities.

It has been reported 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 electrophoresis and NMR studies (27–32). For calbindin D_{9K}, the evidence of multiple forms has come from not only NMR studies but also from x-ray crystal structures (33, 34). These results shed light on the understanding of the protein folding problem but give little information on the biological role of the multiple native forms. Is the multiple native forms necessary for protein to perform its biological functions or only the results of protein folding? Our results suggest that the multiple native forms are necessary for AK to perform its catalytic function. AK, a small nucleoside monophosphate kinase, undergoes large domain movements during catalysis (23), because it has to shield its active center from water to avoid ATP hydrolysis. Because the folded forms of AK must be flexible, multiple native forms with small free energy difference can exist in equilibrium in the absence of substrates.

In summary, two rate-limiting steps are involved in the catalytic cycle of AK. Only one form (E_{1}) of AK can bind with AP_{2}A, so AP_{2}A acts as a competitive inhibitor for the forward reaction and a noncompetitive inhibitor for the backward reaction.

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