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Short communication

The kinetic study of arginine kinase from the sea cucumber *Stichopus japonicus* with 5,5′-dithiobis-(2-nitrobenzoic acid)

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Abstract

The *Stichopus japonicus* arginine kinase (AK) is a significant dimeric enzyme. Its modification and inactivation course with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and the reactivation course of DTNB-modified AK by dithiothreitol were investigated on the basis of the kinetic theory of the substrate reaction during the modification of enzyme activity. The results show that the modification is a biphasic course while the inactivation is monophasic, with one essential reactive cysteine per subunit. The Cys^{274} (numbering from the *Stichopus* sequence) is exposed to DTNB and is near the ATP binding site. The modified AK can be reactivated by an excess concentration of dithiothreitol in a monophasic kinetic course. The presence of ATP or the transition-state analog markedly slows the apparent reactivation rate constant. The analog components, arginine-ADP-Mg^{2+} can induce conformational changes of the modified enzyme, but adding NO_3^- cannot induce further changes that occur with the native enzyme. The reactive cysteines' location and its role in the catalysis of AK are discussed. The results suggest that the cysteine may be located in the hinge area of the two domains of AK. The reactive cysteine of AK, which was proposed to be Cys^{274}, may play an important role not in the binding of the transition-state analog but in the conformational changes caused by the transition-state analog.

Keywords: Dimeric arginine kinase; Dithiothreitol; Modification; Reactive cysteine; Reactivation kinetic

1. Introduction

Arginine kinase (AK; ATP; arginine *N*-phospho-transferase, EC 2.7.3.3) in invertebrates catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP [1]. As a member of the phosphagen kinase family, it is mainly distributed in invertebrates, and plays a key role in the interconnection of energy production and utilization, which is analogous to the creatine kinase (CK) reaction in vertebrates [2,3]. Most AKs are monomers with a 40 kDa subunit; however, some phosphagen kinases are dimeric or octameric as in the case of mitochondrial CK [4].

The important roles proposed for various amino acid residues in either binding of substrates or in the catalytic activity of the enzyme have been investigated by techniques such as chemical modification and NMR spectroscopy. Cysteine [5,6], histidine [7], lysine [8], and tryptophan [9] all are essential for activity of the enzyme. The interconversion of substrates on the surface of the enzyme is not the rate-determining step in the overall reaction with NMR [10]. Moreover, it was confirmed that the transition-state analog (TSA) was detected during the catalytic process of AK with the addition of NO_3^- to the dead-end complex, AK-MgADP-Arg, and that the transition-state analog complex (TSAC), AK-MgADP-Arg-NO_3^-, was formed step by step with the gradual conformational change [11,12].

Recently, a crystal structure of the monomeric AK from *Limulus* was determined, suggesting that AKs with two intra-domains have a unique substrate binding system [13]. ATP or ADP is accommodated in the C-domain, and arginine or arginine phosphate mainly contacts the N-domain.

Abbreviations: AK, arginine kinase; Arg, arginine; CK, creatine kinase; TSA, transition-state analog; ANS, 1-anilinonaphtalene-8-sulfonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 2-mercapto-5-nitrobenzoic acid; DTT, dithiothreitol; CD, circular dichroism; FPLC, fast performance liquid chromatogram

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The catalytic center is located in the C-domain, where the reversible transfer of the phosphate is achieved. The dead-end or the transition-state analog (TSA) complex with Mg^{2+}ADP, nitrate (mimicking a planar phosphoryl in transition), and arginine associates with the enzyme. Substantial conformational changes are induced in different parts of the enzyme as intimate interactions are formed with both analog components. Although induced fit occurs in a number of phosphoryl transfer enzymes, the conformational changes in phosphagen kinases appear to be more complicated than in prior examples [14–16]. The large and small domains undergo a hinged 13° rotation, and several loops become ordered and adopt different positions in the presence of substrate [17,18].

AK in sea cucumber *Stichopus japonicus*, one of the dimeric AKs found in echinoderms, is composed of two identical subunits, each with 370 amino acid residues and a molecular weight of about 42 kDa [19,20]. A comparison of the sequence with those of other enzymes belonging to the phosphagen kinase family indicates that the entire amino acid sequence of *Stichopus* AK is apparently much more similar to vertebrate CKs than to most other AKs. But the GS region is of the AK type: five amino acid deletions in the flexible loop region that might help to accommodate larger guanidine substrates in the active site [20]. Therefore, it has been proposed that *Stichopus* AK with a two domain structure evolved at least twice during the evolution of phosphagen kinase: first at an early stage of phophogen kinase evolution (its descendants are molluscan and arthropod AKs), and secondly from CK later in metazoan evolution, when the sequence of the GS region might have been replaced by the AK type via exon shuffling [20]. So *Stichopus* AK was very important in the evolution of phosphagen kinase. Four residues in the GS region, Ser^{63}, Gly^{64}, Val^{65}, Tyr^{68} (numbering from the *Limulus polyphemus* sequence), are highly conserved in almost all of the AKs known so far and are associated with substrate binding [21]. However, none of these four conserved residues are present in *Stichopus* AK, which implies that the enzyme may have another completely different substrate-binding system.

We investigated the important roles of various amino acid residues in either binding of substrates or in the catalytic activity of the enzyme by chemical modification and site mutagenesis, and gained significant information about the dimeric AK. Trp^{218} [22], Cys^{274} [23,24], Arg^{283} [24], and His^{287} [24] are essential for activity of the enzyme. Extensive chemical modification studies were carried out with different reagents [5,25–27]. The modification of dimeric AK with *o*-phthalaldhyde revealed one essential cysteine, and it was proposed that the Cys^{274} (numbering from *Stichopus* AK sequence) of dimeric AK acts the same as the Cys^{271} (numbering from *Limulus*) of *Limulus* AK [13,23]. There are several hypotheses [11,12,28–30] that the cysteine may not be catalytically important, but may be involved in synergism between the binding of the two substrates or in a hinge movement required for the enzyme to become active. The functions and the roles of cysteines in dimeric AK from sea cucumber *S. japonicus* have not been determined.

Although chemical modifications of the reactive thiol group of CK and AK were extensively studied [5,6,25–27,31–33] and the kinetic course of reactivation of CK was reported [34], the modifications of thiol groups and the kinetic course of reactivation have never been reported so far. In this study, the modification of thiol groups with DTNB was investigated. The reactivation course of AK-TNB was analyzed using the substrate kinetic described by Tsou [30] to demonstrate the function of reactive cysteine. In addition, the roles of the cysteine in the catalysis and conformational change upon substrate binding were also examined.

2. Materials and methods

AK of sea cucumber *S. japonicus* was prepared as described by Guo [19]. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate. The enzyme concentration was determined by the coomassie blue protein dye binding method of Bradford with bovine serum albumin as standard [36]. The activity of AK was assayed using direct continuous pH-spectrophotometric assay [35].

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), ATP, ADP, Arginine (Arg), 1-anilinonaphtalene-8-sulfonate (ANS) were Sigma products. Dithiothreitol (DTT) was from Progma. All other reagents were local products of analytical grade.

Modification of the enzyme was carried out in 20 mM Tris-HAc buffer, pH 8.1, with 100 M excess of DTNB. Excess reagent was removed by gel filtration through a Sephadex G-200 column, and AK-TNB was obtained.

All absorption spectra were measured by an analytic spectrophotometer Specord 200 UV–vis (Jena, Germany). The AK-TNB was compared with the native enzyme at 30 °C. All fluorescence emission spectra were collected on a Hitachi 850 spectrofluorimeter, and gel filtration was carried out with a Superdex 200HR 10/30 column on a Pharmacia FPLC apparatus [22].

The intrinsic fluorescence emission spectra were recorded after AK and AK-TNB were added to the analog components, Mg^{2+} and ADP, for 0.5 h. $NaNO_3$ was added to the reaction mixture above for another 0.5 h. An excitation wavelength of 295 nm was used to determine the AK tryptophan fluorescence intensity. In order to determine the hydrophobic surface exposure of the enzyme in the dead-end complex and TSAC, samples were incubated with a 50-fold molar excess of ANS for 0.5 h in the dark, followed by measurement of fluorescence emission spectra from 400 to 600 nm with excitation at 380 nm.

2.1. Kinetic analysis during modification course

The absorbance was measured at 412 nm and the number of modified thiol groups was determined according to Ellman [37].

The time course of the absorbance change at 412 nm and inactivation of enzyme activity were analyzed according to Eq. (1). Initial estimates of the rate constants and amplitudes were obtained from semilogarithmic plots as described earlier [34]

$$(\Delta A_\infty - \Delta A_t) = A_1\, e^{-k_1 t} + A_2\, e^{-k_2 t} \quad (1)$$

where ΔA_t is the corrected absorbance increase at time t, ΔA_∞ the corrected absorbance increase when all the accessible thiol groups have reacted with excess DTNB, k_1 and k_2 are the pseudo-first-order rate constants and A_1 and A_2 are amplitudes (expressed as absorbance increase so that $A_1 + A_2 = \Delta A_\infty$) of the fast and slow phases, respectively.

2.2. Kinetic analysis of the reactivation

The reactivation course of AK-TNB was studied by the kinetic method of the substrate reaction in the presence of an inactivator or an activator as previously described by Tsou [38]. In this method, 10 μl of 1.3 μM AK-TNB was incubated with a reaction mixture containing various concentrations of substrate. The modified enzyme showed no activity. Different volumes of DTT solution were then added to start reactivation. The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. The reaction was carried out at a constant temperature of 30 °C.

The time course of the substrate reaction in the presence of different DTT concentrations showed that at each activator concentration, the rate increased from zero with time elapsing until a straight line was approached. This can be written as

$$[P]_t = \frac{V(t + (e^{-a[Y]t} - 1))}{(A[Y])} \quad (2)$$

where $[P]_t$ is the concentration of the product formed at time t, V the maximum recovered activity of AK, [Y] the concentration of the activator, DTT and A is the apparent forward rate constant of reactivation.

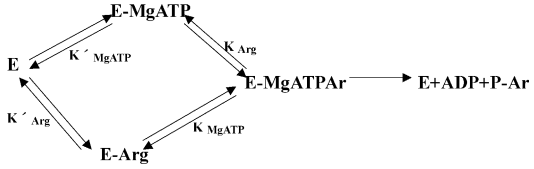
Rearranging Eq. (2) gives

$$\ln([P]_t - [P]_{calc}) = \ln\left(\frac{V}{A[Y]}\right) - A[Y]_t \quad (3)$$

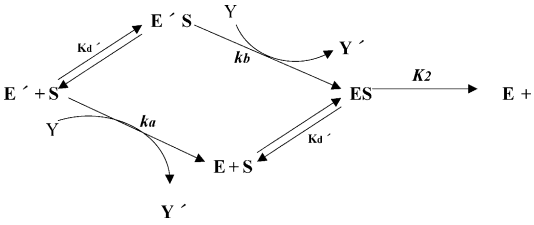
The product concentration, $[P]_{calc}$, to be expected from the straight line is given by the following equation:

$$[P]_{calc} = V\left(\frac{t-1}{(A[Y])}\right) \quad (4)$$

Since the binding sequence of the two substrates to AK, as shown in Scheme 1, is random and the binding rapidly reaches equilibrium [39,40], the reaction kinetics in the present study can be simplified when the concentration of one of the substrates is fixed. Scheme 2 depicts the irreversible reactivation of AK-TNB where E and E′ are the native and AK-TNB, respectively. S is the substrate, MgATP or arginine; P is the product; k_a, k_b, and k_2 are the corresponding micro-rate



Scheme 1.



Scheme 2.

constants. K_d and K_d' are the dissociation constants of the enzyme substrate complex. The apparent rate constant of reactivation, A, is given by

$$A = \frac{(K_d' k_a + [S]k_b)}{(K_d' + [S])} \quad (5)$$

A plot of A versus $[S]/(K_d' + [S])$ gives a straight line whose intercept can be used to calculate k_a and k_b.

3. Results

3.1. The modification course of the AK with DTNB

The modification of native dimeric AK by OPTA modifies its reactive cysteine and causes the loss of activity [23]. After modification of the reactive cysteine by DTNB, the enzyme loses its activity entirely. However, the slight decrease of ellipticity, fluorescence intensity, and the close elution volume showed that the conformation of AK-TNB did not have any detectable variation from the native enzyme (data not shown here).

AK is a dimeric enzyme composed of identical subunits with 10 cysteine residues [20]. The number of modified thiol groups was obtained using the method described by Ellman [37]. There were about six cysteine residues of AK that were modified by DTNB, and four others were inaccessible (Table 1), as calculated from Fig. 1. The time course of absorbance change at 412 nm and inactivation of the enzyme

Table 1
Data of the modification process of AK with DTNB

Number of thiol groups	
Accessible	6.1 ± 0.1
Inaccessible	3.9 ± 0.1
Rate constant ($\times 10^{-4}$ s^{-1})	
Slow phase (k_1)	1.6 ± 0.1
Quick phase (k_2)	23 ± 1
Inactivation (k_3)	93 ± 1

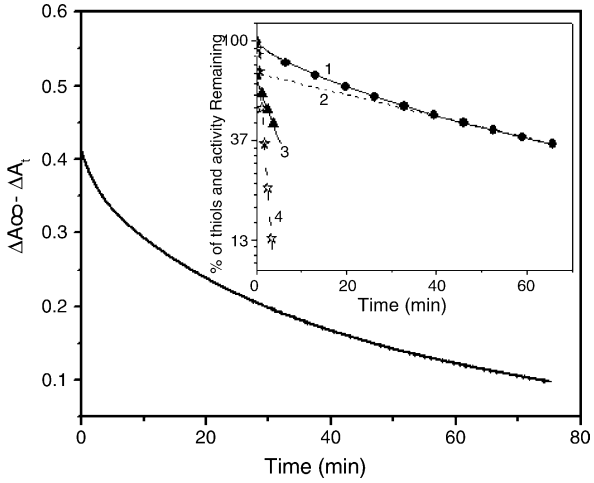


Fig. 1. The kinetic course of absorbance change at 412 nm and inactivation of the enzyme activity during modification of dimeric AK in the presence of DTNB. The reaction mixture contained 5 μM AK and 0.5 mM DTNB in 20 mM Tris-HAc buffer, pH 8.1. The reaction was followed at 412 nm and plotted with a semilogarithmic inset plot of the pseudo-first-order reaction. The temperature was 25 °C. (●) Experimental data, (▲) points obtained by subtracting contribution of the slow phase from curve (- - -), (☆) the remaining activity (%) during the modification of AK with DTNB.

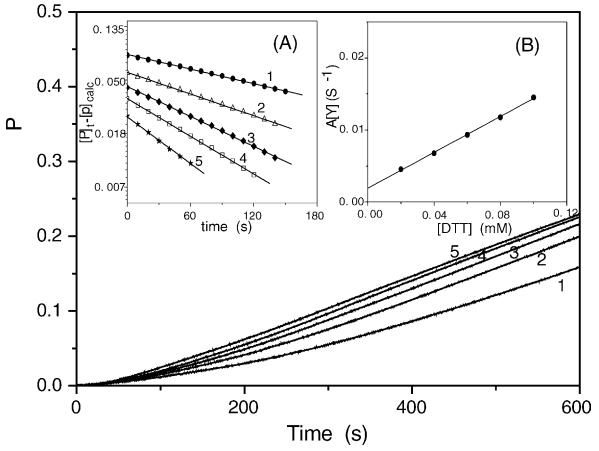


Fig. 2. Reactivation kinetic course of substrate reaction of AK-TNB at different DTT concentrations. The reaction mixture contained 5.7 mM arginine, 4.8 mM ATP, 6.6 mM magnesium acetate, complex acid–base indicator (consisting of 0.15% thymol blue and 0.025% cresol red), and 6.5 nM AK-TNB. Concentrations of DTT were 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 mM, respectively, for curves 1–5. The production of protons was followed by the absorbance change at 575 nm at 30 °C, here P represents the product. (Inset plot A) semilogarithmic plots of lines 1–5 according to Eq. (2), (inset plot B) plot of A[Y] against [DTT]. The data points for A[Y] were calculated from the inset plot A.

activity were recorded (Fig. 1), and analyzed according to Eq. (1).

Fig. 1 shows that there is almost a 'burst' of absorbance increase (initial 3 min), followed by a slower reaction (completed in 25 min) and a very slow absorbance increase, which shows a linear correlation with time and is not completed even after 70 min. The modification course and an inset of a semilogarithmic plot showed a biphasic reaction, which can be considered as the pseudo-first-order reaction. The rate constant for the slow phase, k_1, was obtained from experimental data with a linear fit as shown in the inset plot of Fig. 1. The rate constant for the quick phase, k_2, was obtained by subtracting the contribution from the slow phase, k_2 is much larger than k_1 (Table 1). Two cysteine residues calculated from the data shown in the inset of Fig. 1 were essential for the quick phase, and the other four for the slow phase, as indicated by line 2.

Moreover, the kinetics of inactivation with DTNB as followed by the substrate reaction in the presence of the modifiers is monophasic (data not shown here). The inactivation rate constant in the presence of DTNB is close to the rate constant for the quick phase during the modification with the same concentration of inhibitor.

3.2. Kinetic course of the substrate reaction of AK-TNB in the presence of DTT

The modified enzyme, AK-TNB, was purified by gel filtration through a Sephadex G-200 column. In the presence of reductive reagent, such as DTT, the CK-TNB can be reactivated [41,42]. Similarly, the AK-TNB can be reactivated by DTT.

Fig. 2 shows the course of the substrate reaction of AK-TNB at various DTT concentrations in the presence of fixed arginine, ATP, and Mg^{2+} concentrations. The general shapes of the curves agree with those predicted by Eq. (2). The reactivation rates of the AK-TNB increase with increasing DTT concentrations. The straight line portions of the curves represent the recovered activities of the reactivated enzyme. From Eq. (3), a plot of $\ln([P]_t - [P]_{calc})$ versus t gives a series of straight lines at different concentrations of DTT with slopes of $-A$[Y], as shown in inset A. The apparent forward rate constant A can be obtained from the slopes of these straight lines. Inset A of A[Y] versus [DTT] gives a straight line, which nearly intersects the ordinate at zero, as shown in inset B. The value of A does not change with various DTT concentrations, indicating that the reduction of the AK-TNB by DTT is an irreversible second-order reaction, but not a complex formation type of modification.

3.3. Effect of MgATP on the reactivation of AK-TNB

Fig. 3 shows the reactivation course of AK-TNB at different ATP concentrations in the presence of a fixed DTT concentration. A plot of $\ln([P]_t - [P]_{calc})$ versus t gives a series of straight lines at different concentrations of the substrate (not shown here). The apparent forward rate constant A decreases with increasing concentration of [MgATP], as shown in inset A. The plot of A versus [MgATP]/([MgATP] + K_d') reveals a straight line (inset plot B) with K_d' being determined to be 2.1 mM by a least squares fitting.

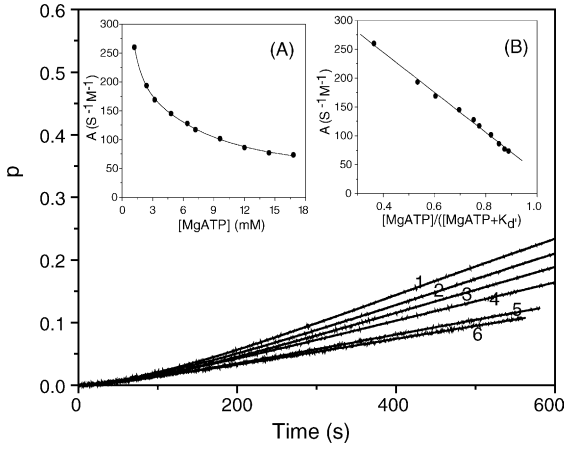


Fig. 3. Course of substrate reaction at different MgATP concentrations. The conditions were the same as for Fig. 2 except that the DTT concentration was 0.08 mM and the concentrations of ATP were 7.2, 6.4, 4.8, 3.2, 2.4 and 1.6 mM, respectively, for curves 1–6. Inset plot A is a plot of A against [MgATP]. Inset plot B is a plot of A against [MgATP]/([MgATP] + K_d').

3.4. Effect of MgADP and transition-state analog on the reactivation of AK-TNB

The reactivation courses of AK-TNB in various concentrations of MgADP and $NaNO_3$ were observed. The presence of MgADP remarkably decreased the activity of the reactivated enzyme. This indicated that the ADP is a competitive inhibitor of the forward reaction. While there was a slight decrease of the apparent rate constant of the reactivation with the increase of MgADP concentration (Fig. 4), a significant decrease of the apparent reactivation rate constant was observed when the reaction mixture contained the transition-state analog, MgADP-Arg-NO_3 (Fig. 5), accompanied by the decrease of the activity of the reduced enzyme.

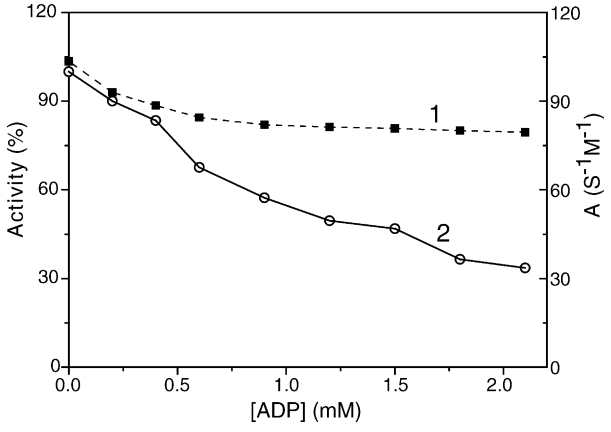


Fig. 4. Reactivation of AK-TNB in different concentrations of MgADP. The concentration of DTT was 0.08 mM. Other concentrations were the same as for Fig. 2. Curve 1 (■) is the apparent rate constant A of reduced enzyme, while curve 2 (◯) is the relative activity.

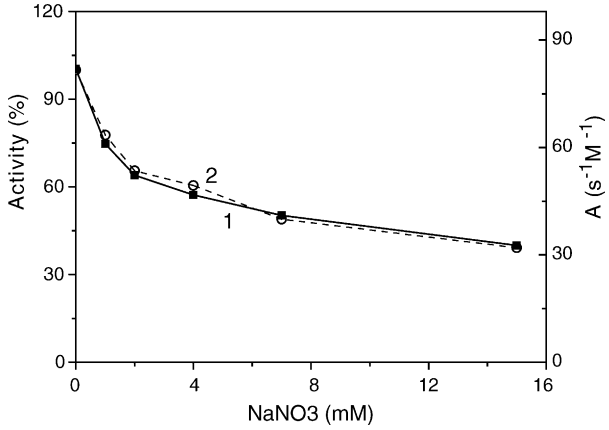


Fig. 5. Reactivation of AK-TNB in the presence of MgADP and different concentrations of $NaNO_3$. The MgADP fixed concentration was 0.9 mM. Other conditions were the same as for Fig. 4. Curve 1 (■) is the apparent rate constant A, while curve 2 (◯) is the relative activity of the reactivated enzyme.

3.5. Substrate analog components or transition-state analog induced conformational changes of AK-TNB

The changes of protein tertiary structure and the hydrophobic surface exposure can be investigated with intrinsic fluorescence and ANS fluorescence. Figs. 6 and 7 compare the intrinsic fluorescence and ANS fluorescence spectra for the native enzyme and AK-TNB in the presence of either the substrate analog components or the transition-state analog.

Fig. 6 and its inset plot clearly show that analog components to the native enzyme, Arg-ADP-Mg^{2+}, can induce a certain change of the intensity in the intrinsic fluorescence and ANS spectra. Furthermore, additional NO_3^- could

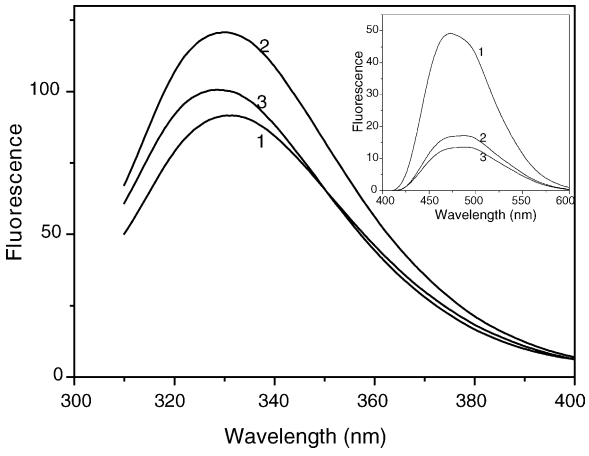


Fig. 6. Intrinsic and ANS fluorescence spectra for native dimeric AK with the substrate analog and the transition-state analog. Final concentration was 14 μM enzyme in 20 mM Tris-HAc buffer, pH 8.1, 5.7 mM arginine, 0.9 mM ADP and 6.6 mM magnesium acetate. $NaNO_3$ (0.5 mM) was also added in addition to the substrate analog components for the measurement of the intrinsic and ANS fluorescence of the complex with the transition-state analog. The inset shows the ANS fluorescence spectra of native AK. Curve 1 is the apo-enzyme, curve 2 is the dead-end complex and curve 3 is the complex with the transition-state analog.

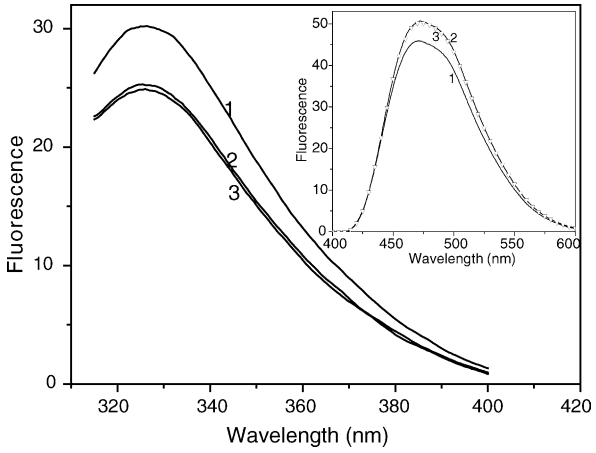


Fig. 7. Intrinsic and ANS fluorescence spectra for AK-TNB with the substrate analog components and the transition-state analog. The conditions were the same as for Fig. 6. The inset shows the ANS fluorescence spectra AK-TNB. Curve 1 is the apo-enzyme, curve 2 is the dead-end complex, curve 3 is the complex with the transition-state analog.

induce further conformational change of the native enzyme. From Fig. 7 and its inset plot, Arg-ADP-Mg^{2+} also can induce the conformational change of the AK-TNB, while additional NO_3^- could not induce further changes in its conformation.

4. Discussion

Unlike the monomeric 40 kDa AK from molluscs and arthropods, the *S. japonicus* AK is a dimeric AK, like the vertebrate creatine kinase. It has been suggested that AKs such as *S. japonicus* evolved at least twice during the evolution of phosphagen kinase: first at an early stage of phosphagen kinase evolution and secondly from CK later in metazoan evolution [20]. Because of its special position, it played a significant role during evolution processes.

The modification reaction with DTNB is biphasic. The DTNB molecules are accessible to about six cysteine residues out of the 10 in *Stichopus* AK, among them two for quick phase and the other four for slow phase. The reactive rate of thiol groups in the quick phase is the same, and faster than that of thiol groups in the slow phase. It is clear that two of the reactive thiol groups react faster than the other four. On the other hand, the inactivation course is monophasic. The inactivation rate constant ($9.3 \times 10^{-3}\ s^{-1}$) is close to the rate constant ($2.3 \times 10^{-3}\ s^{-1}$) of the quick phase in the modification process with the same concentration of DTNB. Further, the two reactive cysteine residues in the dimeric enzyme for the quick phase, one per subunit, may correspond to the catalysis mechanism.

Recently, we found that the mutant AKC274A lost its activity [24] and in the present study, the number of the accessible thiol groups to DTNB in mutant AKC274A [24] is four, while that in the native enzyme is six. This explicitly suggests that Cys^{274} is exposed to DTNB and reacts with DTNB faster than other accessible cysteine residues with the loss of enzyme activity.

Kinetic analysis of the course of reactivation of AK-TNB shows that the modified AK can also bind MgATP, and the binding of MgATP can decrease the apparent rate constant of reactivation, but does not fully stop the reactivation. The dissociation constant of 2.1 mM for the modified AK–ATP complex is much larger than 0.5 mM for the native AK–ATP complex. These results suggest that the reactive cysteine is not located in the ATP binding site, but is very close to it. This means that the binding of ATP can somewhat influence the accessibility of the added TNB group, and the TNB group can enable ATP to more easily dissociate from the enzyme. This conclusion is consistent with the recently reported crystal structure of AK from horseshoe crab [13], in which the reactive cysteine residue is near the γ-phosphate. Moreover, it has also been reported that the binding of ATP-Mg^{2+} affected the modification of AK by iodoacetamide [5]. Combined with the present analysis, it can be concluded that Cys^{274} is exposed to DTNB, which is the reactive cysteine relevant to activity. These results strongly suggest that the Cys^{274} of the dimeric AK is near the ATP binding site.

In the presence of the substrate analog arginine-ADP-Mg^{2+} or the transition-state analog MgADP-arginine-NO_3^-, the enzyme undergoes a conformational change as indicated by absorbance changes in the UV region [43]. The intrinsic and ANS fluorescence spectra are conventional means to use for demonstrating the conformational change [22,44]. The analog components, arginine-ADP-Mg^{2+}, can induce a certain change of the intensities in the intrinsic fluorescence and ANS spectra (Fig. 6). The change of intensities also occurred in AK-TNB (Fig. 7). This implies that MgADP can bind with the AK-TNB. Comparing the changes of the reactivation rate constants between MgATP (Fig. 3) and MgADP (Fig. 4), the apparent reactivation rate constant of AK-TNB does not markedly decrease with the concentration of ADP. This result suggests that the effect of MgADP on the accessibility of the TNB group is slight. The transition-state analog is believed to mimic the binding of the substrate at the transition-state during catalysis involving possible conformational changes of the enzyme molecule [11,12]. The present results suggest that the AK-TNB can bind with the transition-state analog, which decreases the accessibility of the added TNB group in the reaction mixture, which markedly decreases the apparent reactivation rate constant (Fig. 5), although the adding of NO_3^- did not induce further intensity changes of AK-TNB (Fig. 7). The reactive cysteine might be involved in the conformational changes, which are believed to be essential for the coordinated binding of substrates and enzyme catalysis. The effect of modifying the reactive cysteine is mainly to prevent the transition-state analog inducing conformational changes, but not the binding of the transition-state analog. Similar results were contained in reports showing that a mutant of an amino acid of an iron protein of nitrogenase from *Azotobacter vinelandii* had normal substrate binding ability, but the substrate-induced long distance conformational change of

the hinged motion of its two subunits disappeared [4,7]. The results suggest that the reactive cysteine of dimeric AK may play an important role not in the binding to the transition-state analog but in the conformational changes caused by the transition-state analog, similar to the functions of creatine kinase [45]. It is known that large and small domains in native AK undergo a hinged 13° rotation, which can lead to the conformational changes in the presence of substrate [17,18]. Thus, we infer that the reactive cysteine of dimeric AK is located in the hinge area of the two domains of the enzyme subunit.

Recently, researchers proposed an interesting suggestion that the reactive cysteine helps enhance the catalytic rate instead of mediating a substrate-induced conformational change [46], though this contradicts prevailing speculations [11,12,18,28–30]. The potential roles of a conserved active site cysteine in phosphagen kinase have been being investigated and debated for more than 40 years. Because the roles of reactive cysteine determined by the different types of phosphagen kinase and the species may be complicated and multiple, to completely clarify these issues still needs further studies.

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