Biochemistry (Moscow), Vol. 69, No. 12, 2004, pp. 1336-1343. Translated from Biokhimiya, Vol. 69, No. 12, 2004, pp. 1639-1648.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-047, June 27, 2004.

Evidence for Proximal Cysteine and Lysine Residues at or near the Active Site of Arginine Kinase of *Stichopus japonicus*

Qin Guo, BaoYu Chen, and XiCheng Wang*

Department of Biological Science and Biotechnology, School of Life Science and Engineering, Tsinghua University, Beijing 100084 China; fax: 8610-62784977; E-mail: wangxic@mail.tsinghua.edu.cn

Received February 26, 2004
Revision received April 21, 2004

Abstract—Inactivation of arginine kinase (AK) of *Stichopus japonicus* by *o*-phthalaldehyde (OPTA) was investigated. The modified enzyme showed an absorption peak at 337 nm and a fluorescent emission peak at 410 nm, which are characteristic of an isoindole derivative formed by OPTA binding to a thiol and an amine group in proximity within the enzyme. Loss of enzymatic activity was concomitant with an increase in fluorescence intensity at 410 nm. Stoichiometry studies by Tsou's method showed that among the cysteine residues available for OPTA modification in the enzyme, only one was essential for the enzyme activity. This cysteine residue is located in a highly hydrophobic environment, presumably near ATP and ADP binding region. This conclusion was verified by 5,5′-dithiobis(2-nitrobenzoic acid) modification. In addition, these results were supported by means of electrophoresis and ultraviolet, fluorescence, circular dichroism spectroscopy and fast performance liquid chromatography. Sequence comparison suggested that this essential cysteine residue maybe the conservative Cys274.

Key words: arginine kinase, cysteine, OPTA, Tsou's method

Arginine kinase (AK) (ATP:arginine N-phosphotransferase, EC 2.7.3.3) catalyzes the reversible phosphorylation of arginine by ATP and yields the high-energy compound phosphoarginine (PArg). As a member of the phosphagen kinase family, it is mainly distributed in invertebrates, and it plays a key role in the interconnection of energy, like creatine kinase (CK) in vertebrates [1, 2].

Stichopus japonicus AK, one of the dimeric AKs found in echinoderms, has raised interest recently because of its special position in evolution. Sequence analysis indicated that the dimeric AK is evolutionarily closer to CK, while its catalytic site still resembles that of monomeric AK [3]. Since extensive studies have been carried out on the catalytic mechanisms of both CK and monomeric AK [4-7], investigations on the dimeric AK are necessary to elucidate its evolutional role from a viewpoint of enzymatic mechanism.

The focus of the present study is to probe the involvement of cysteine and lysine residues in the catalytic center of AK. For this purpose, we used *o*-phthalaldehyde (OPTA), a homobifunctional cross-linking reagent that reacts with proximal cysteine thiol and lysine ε-amine groups that are about 3 Å apart to form a characteristically fluorescent isoindole derivative [8]. This unique property has been extensively utilized to probe the role of cysteine and lysine residues in the active centers of many enzymes [9-13].

The present investigation reveals that a cysteine residue is located in close proximity to a lysine residue at or near the ATP/ADP binding region of AK.

MATERIALS AND METHODS

Materials. AK was prepared from the muscle of sea cucumber *Stichopus japonicus* according to the procedure of Anosike et al. [14]. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis (PAGE) in the presence and absence of sodium dodecyl sulfate (SDS). ATP, Arg, OPTA, 5,5′-dithiobis(2-nitrobenzoic

Abbreviations: FPLC) fast performance liquid chromatography; AK) arginine kinase; CD) circular dichroism; CK) creatine kinase; DTNB) 5,5′-dithiobis(2-nitrobenzoic acid); OPTA) *o*-phthalaldehyde; PArg) phosphoarginine.

* To whom correspondence should be addressed.

0006-2979/04/6912-1336 ©2004 MAIK "Nauka/Interperiodica"

acid) (DTNB), PArg, and urea (ultra-pure) were purchased from Sigma (USA). All other reagents were local products of analytical grade and were used without further purification.

Enzyme concentration and activity assay. Enzyme concentration was determined by the Coomassie blue protein dye binding method of Bradford with bovine serum albumin as the standard [15]. Enzyme activity was assayed by a phosphate determination method, based on spectrophotometric determination of an ascorbic acid-reduced blue ternary heteropolyacid composed of bismuth, molybdate, and the phosphate released from acid-labile PArg formed in the forward reaction [16]. The absorbance at 700 nm was measured in a Specord 200 UV VIS spectrophotometer (Jena, Germany) using the molar extinction coefficient of 15.97 $mmol \cdot liter^{-1} \cdot cm^{-1}$ [17].

OPTA modification. OPTA was dissolved freshly in 1% distilled methanol. The modification was carried out by incubating AK in 20 mM sodium borate buffer, pH 8.1, with varying concentrations of OPTA at 25°C. At different time intervals, 5-μl aliquots were taken from the reaction mixture and mixed with an equal volume of quenching solution containing 40 mM cysteine, 10 mM β-mercaptoethanol, and 20 mM sodium borate, pH 8.1. This terminated further reaction of OPTA with the enzyme [10, 18]. Residual activities were then measured as described above.

DTNB modification. The enzyme was modified by DTNB by incubating the AK with DTNB at desired concentration at 25°C. At different time intervals, 10-μl aliquots were withdrawn and the residual enzyme activities were measured.

Double inhibition studies. AK was first incubated with DTNB. The DTNB-modified AK was loaded on a Sephadex G-200 column to remove the excess DTNB and the released TNB^- ion. Then, the purified DTNB-AK was modified by OPTA. In another set of experiments, the modification reagents were added to native AK in the reverse order. Control experiments in the absence of modification agents or in the presence of only one agent were run concurrently.

Substrate protection. For substrate protection, proper concentrations of substrates or analogs were incubated with the enzyme for 6 min prior to the addition of OPTA.

Spectroscopy. After modification by OPTA and subsequent quenching by 40 mM cysteine and 10 mM β-mercaptoethanol, the reaction mixture was dialyzed against 20 mM sodium borate buffer, pH 8.1, overnight before spectroscopy measurements and fast performance liquid chromatography (FPLC). Ultraviolet spectra from 200 to 410 nm were measured on a Specord 200 UV VIS spectrophotometer. Fluorescence measurements were performed on a Hitachi 850 spectrofluorimeter. Fluorescence emission spectra were recorded from 350 to 480 nm with an excitation wavelength of 337 nm, and fluorescence excitation spectra were recorded from 300 to 370 nm with an emission wavelength of 410 nm. Circular dichroism (CD) spectra were recorded on a Jasco 500C spectropolarimeter using a cell of 1 mm pathlength. The resulting spectra were averages of five successive scans spanning from 190 to 260 nm.

Size exclusion chromatography. Gel filtration was carried out with a Superdex 200HR 10/30 column (fractionation range 10-600 kD) on a Pharmacia (Sweden) FPLC apparatus at 25°C. AK (5.81 μM) was incubated with 2.5 mM OPTA for 10 sec, 20 sec, and 6 min and then dialyzed against 20 mM sodium borate buffer, pH 8.1, overnight after stopping the reaction with cysteine. Samples were then freeze dried and redissolved in 100 μl of distilled water before being applied to the Superdex 200HR column which was preequilibrated with 20 mM sodium borate buffer, pH 8.1. The flow rate was maintained at 0.5 ml/min, and absorbance at 280 and 337 nm was monitored.

Stoichiometric measurements. For OPTA modification, stoichiometric measurements were carried out by recording the ultraviolet absorbance changes at 337 nm at 25°C. The formed isoindole derivative has an absorption coefficient value of 7.66 $mM^{-1} \cdot cm^{-1}$ [19]. This value was taken for stoichiometric calculations where the molecular weight of the dimeric AK was taken as 86 kD [3]. The value of absorption obtained as a result of isoindole derivative formation was corrected for both the absorption of 0.5 mM OPTA and the unmodified enzyme. Tsou's method was used to calculate the number of Cys-Lys pairs essential for AK activity. Assuming that the reaction rates were neither the same nor differed greatly between the essential and unessential Cys-Lys pairs during modification, an equation could be obtained by Tsou's method [20] as follows:

$$nx = pa^{1/i} + (n-p)a^{\alpha/i}, \quad (1)$$

with α being the residual activity of AK, i being the number of Cys-Lys pairs essential for enzyme activity; n the total number of Cys-Lys pairs in AK ($n = 10$ here), x the average fraction of unmodified Cys-Lys pairs; p the number of Cys-Lys pairs with fast reaction rates, and a the ratio of the first-order rate constants for the slowly and rapidly reacting Cys-Lys pairs. Equation (1) can also be expressed in the following form:

$$\log\left(n\frac{x}{\alpha^{1/i}} - p\right) = \log(n-p) + \left(\frac{a-1}{i}\right)\log\alpha. \quad (2)$$

Variables i and p were conferred every possible numerical value and then logα versus $\log(nx/\alpha^{1/i} - p)$ was plotted. If a linear relationship between logα and $\log(nx/\alpha^{1/i} - p)$ was observed, this meant that the assumed numerical values of i and p were reasonable.

As to DTNB modification, all methods for stoichiometric measurements were the same except that absorbance changes were recorded at 412 nm and the absorption coefficient value was 13.6 $mM^{-1} \cdot cm^{-1}$ [20].

Activity staining for AK. The activity staining for AK was conducted according to the methods used for CK except that PArg was used instead of phosphocreatine [21].

Glutaraldehyde cross-linking. Glutaraldehyde was used as a cross-linking reagent according to a protocol described previously [22].

Electrophoresis. SDS-PAGE, native-PAGE, and PAGE in 8 M urea were carried out as previously described [23].

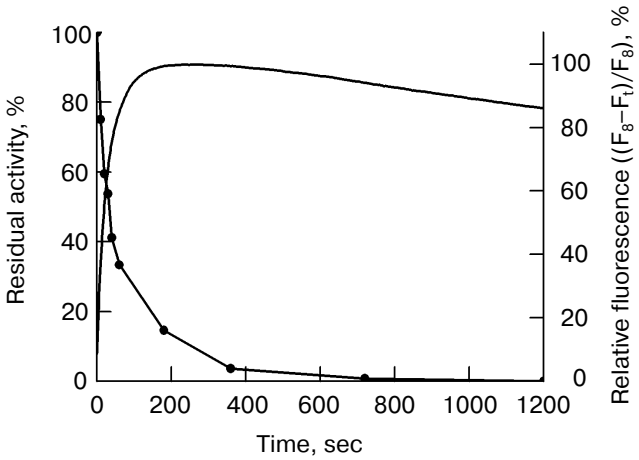

Fig. 1. Time course of the loss of arginine kinase activity and the increase in fluorescence intensity during OPTA modification. The concentration of the enzyme was 3.64 μM and that of OPTA was 0.5 mM. The excitation and emission wavelengths were 337 and 410 nm, respectively.

RESULTS

Inactivation of AK by OPTA. At a concentration of 0.5 mM, OPTA inactivated 3.64 μM AK rapidly (Fig. 1). The inactivation was concomitant with the enhancement of fluorescence intensity. The enzyme lost 86% of its activity after 200 sec of OPTA incubation, when the intensity of fluorescence reached a maximum. Extending incubation time to 20 min completely depleted the enzyme activity, while the fluorescence intensity began to decrease after 200 sec of OPTA incubation. This decrease may result from bleaching of fluorescence due to continuous scanning.

Spectral analysis of OPTA-modified AK. The absorption spectrum of AK modified by OPTA exhibited a peak at 337 nm (Fig. 2a), which was not present in the absorption spectrum of unmodified enzyme. The fluorescence emission spectrum (λ_{ex} at 337 nm) was characterized by a maximum at 410 nm, while the excitation spectrum (λ_{em} at 410 nm) showed an excitation maximum at 337 nm (Fig. 2b). All the spectral results suggested the formation of isoindole derivatives during modification, formed by OPTA cross-linking of proximal thiol and ε-amine groups of cysteine and lysine in the enzyme molecule [10, 12, 13, 24-34]. OPTA does not form a fluorescent isoindole derivative with amine groups alone [35]. The polarity of the AK active site was probed by studying the microenvi-

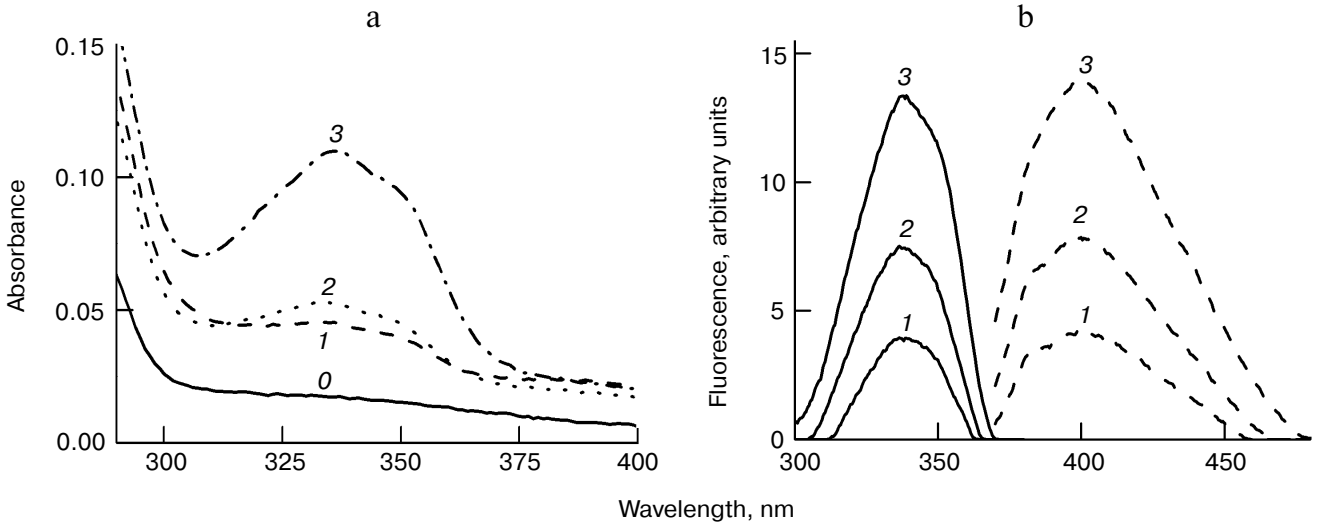

Fig. 2. Ultraviolet (a) and fluorescence (b) spectra of OPTA modified arginine kinase. Samples *1*, *2*, and *3* correspond to the 5.81-μM AK incubated with 2.5 mM OPTA for 10 sec, 20 sec, and 6 min, respectively, and then dialyzed against 20 mM sodium borate buffer, pH 8.1, overnight after stopping the reaction with cysteine. Sample *0* corresponds to native AK without OPTA modification. In panel (b), the solid curves are the excitation spectra with emission wavelength 410 nm and the dashed curves are the emission spectra with excitation wavelength 337 nm.

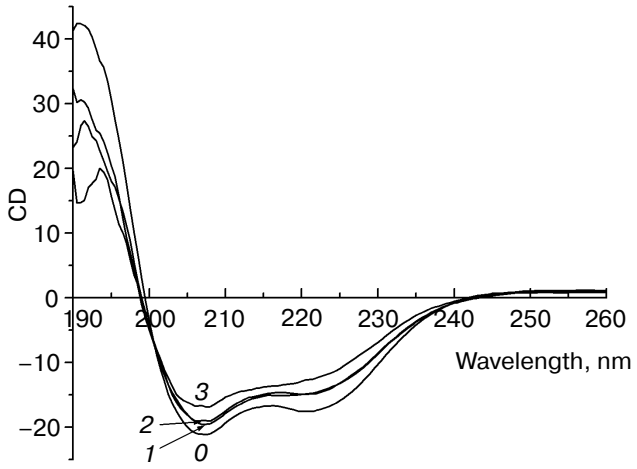

Fig. 3. CD spectra of native and OPTA modified arginine kinase. Samples *0-3* were the same as in Fig. 2 legend.

ronment of the isoindole ring bound to its active site. Polar solvents could cause bathochromic shifts in fluorescence of the isoindole derivative. In *n*-hexane, a completely hydrophobic environment, the fluorescence emission maximum wavelength was 407 nm and in water, a polar environment, it was 452 nm when the excitation wavelength was fixed at 337 nm [24]. In our experiment, the λ_{em} of 410 nm suggested that the fluorescent isoindole derivative formed was located in a highly hydrophobic environment.

CD spectra (Fig. 3) revealed a small decrease of the α-helix and β-sheet content of AK as the OPTA modification preceded, suggesting that although conformational unfolding took place after modification, such an effect was confined to a limited region, and the enzyme molecules retained most of their secondary structure after modification.

Kinetic analysis of inactivation of AK by OPTA. Incubation of AK with different concentrations of OPTA ranging from 0-2.5 mM resulted in a time- and concentration-dependent loss of enzyme activity as shown in Fig. 4. The reaction could be characterized as a two-phase pseudo first order kinetic reaction. The rate constants of the fast and slow phases in the inactivation were defined as k' and k'', respectively. Subtracting the contribution of the slow phase from the fast phase gave the real fast phase rate constant k_1 of the inactivation. According to the Hollenberg' equation [36]:

$$k_1 = k_n \cdot [\mathrm{I}]^n$$

or

$$\log k_1 = \log k_n + n \cdot \log [\mathrm{I}] ,$$

with k_1 being the first order rate constant of the inactivation, k_n the *n*th-order rate constant, [I] the concentration of OPTA, and *n* the order of the reaction. In our experiments, the constant k_1 was linearly related to the concentrations of OPTA. The slope of the plot yielded a second-order rate constant k_2 of 6 $M^{-1}\cdot sec^{-1}$ [24, 32]. Analysis of the order of the inactivation by the double logarithmic plot (Fig. 4) gives a slope of 0.99, indicating that one molecule OPTA binds to one molecule of AK.

Stoichiometric analysis of the number of Cys-Lys pairs essential for AK activity. Stoichiometry of OPTA reaction with AK was monitored by the absorbance enhancement at 337 nm (extinction coefficient 7.66 $mM^{-1}\cdot cm^{-1}$ [19]). Measuring the residual activity, the number of Cys-Lys pairs essential for AK activity could be determined by Tsou's method [20].

According to the change of absorbance at 337 nm in three independent experiments, in which the molar ratio of AK and OPTA increased to about 1 : 50, 2.45 μM AK modified by 0.125 mM OPTA formed 20.3 ± 0.4 μM fluorescent isoindole derivative (data not shown), indicating that 8.30 isoindole derivatives formed per enzyme molecule, which suggested that about eight Cys-Lys pairs in an AK dimer could be modified by OPTA under these conditions.

Analysis by Tsou's method [20] (Fig. 5a) showed that when $i = p = 1$, a linear relationship between logα and $\log(nx/\alpha^{1/i} - p)$ (details in "Materials and Methods") could be obtained, and $a = 0.78$. The results indicated that among the Cys-Lys pairs modified by OPTA per

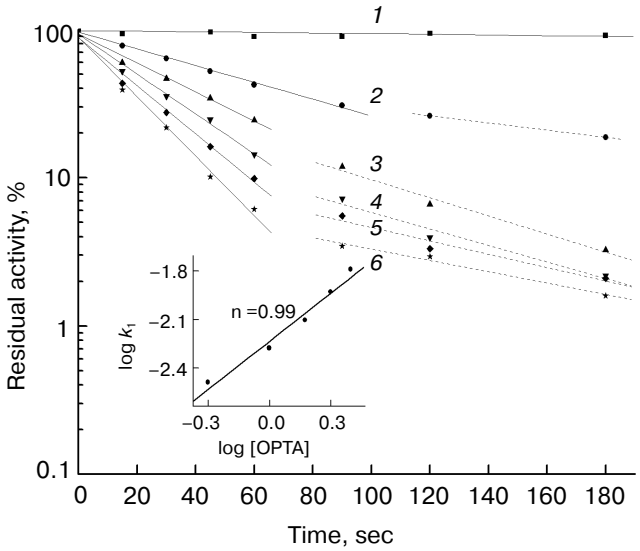

Fig. 4. Inactivation kinetics of OPTA modified arginine kinase. The solid curve is the fast phase of the reaction and the dotted curve is the slow phase. The enzyme concentration was 3.64 μM and the concentrations of OPTA were 0 (*1*), 0.5 (*2*), 1 (*3*), 1.5 (*4*), 2 (*5*), and 2.5 (*6*) mM. The insert shows a secondary plot of pseudo first order rate constant as a function of log OPTA concentration. The slope was 0.99.

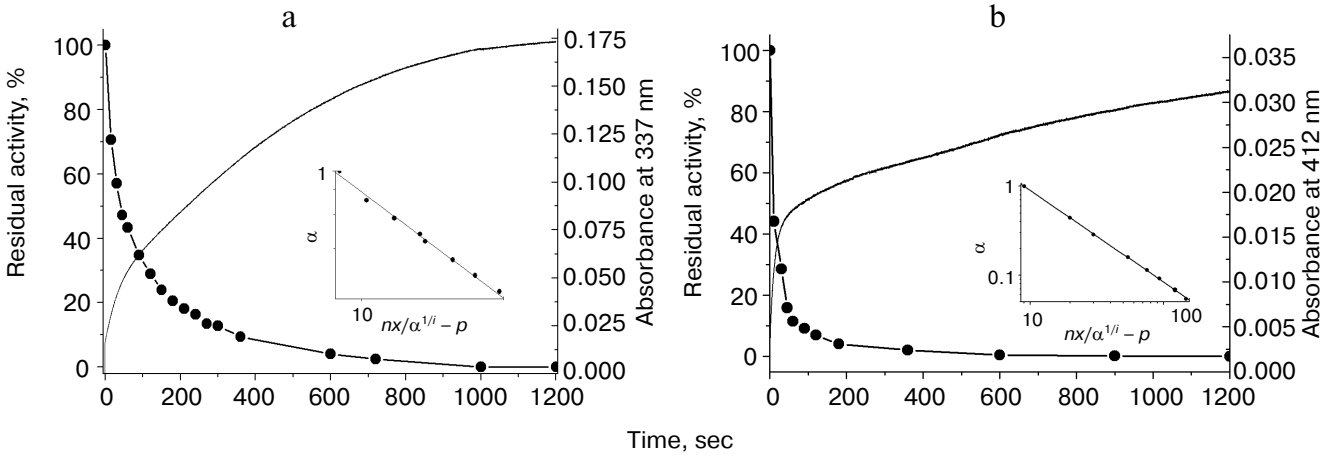

Fig. 5. Stoichiometric analysis of the OPTA and DTNB modification of AK. a) Time course of the loss of AK activity and increase in absorbance at 337 nm following treatment with OPTA. The concentration of the enzyme was 3.96 μM and that of OPTA was 0.5 mM. The insert illustrates Tsou's method to determine the number of the Cys-Lys pairs essential for the catalytic activity of AK. b) Time course of the loss of AK activity and increase in absorbance at 412 nm following treatment with DTNB. The concentration of the enzyme was 1.96 μM and that of DTNB was 6 μM. The insert illustrates Tsou's method to determine the number of the Cys residues essential for AK activity.

enzyme molecule, only one was essential for enzyme activity, even if the other could also be modified at a rate of 0.78 times of that for the essential one.

To verify the involvement of cysteine residues in isoindole formation, DTNB, a reagent specific for cysteine, was used. It is shown in Fig. 6 that DTNB-modified AK could also be modified by OPTA (monitored by the absorbance at 337 nm), with releasing of TNB^- ions (monitored by absorbance at 412 nm). In another set of experiments, when DTNB was added to the OPTA-modified AK, no increase was observed in A_{412}. Moreover, two sets of control experiments were performed wherein AK was modified only by OPTA or DTNB, respectively. Results in Fig. 6 showed that both the values of A_{337} and A_{412} were close to those in the DTNB-modified AK. Since the absorption coefficient values of OPTA and DTNB modification were 7.66 and 13.6 $mM^{-1}\cdot cm^{-1}$, respectively, it was calculated that under these conditions, about eight Cys residues were modified by OPTA, while about four modified by DTNB. It was thus inferred that OPTA could modify more Cys residues than DTNB and most important of all, it could competitively modify almost all the Cys residues that were previously modified by DTNB. The data on DTNB modification were analyzed by Tsou's method (Fig. 5b), which indicated that among the cysteine residues available for DTNB modification, only one was essential for the enzyme activity. Therefore, all the results mentioned above made it reasonable for us to conclude that Cys residues participated in the isoindole formation by OPTA modification, and only one Cys residue was essential for AK activity.

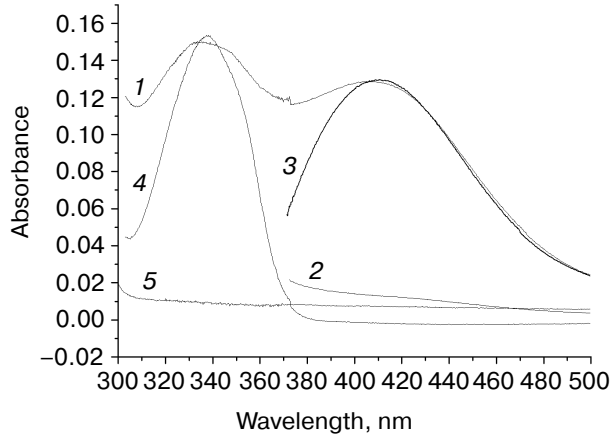

Fig. 6. Ultraviolet spectra of doubly modified AK. Curves: *1*) DTNB-modified AK was incubated with OPTA; *2*) OPTA-modified AK was incubated with DTNB; *3*) native AK was incubated with DTNB; *4*) native AK was incubated with OPTA; *5*) native AK. The enzyme concentration was 2.45 μM and the concentration of the modifying agents (OPTA and DTNB) was 0.125 mM. (Ultraviolet regions of curves *2* and *3* are not shown because excess DTNB interfered greatly in this region.)

Protecting effect of substrates and other reagents on AK inactivation by OPTA. The effects of substrates and their analogs on AK inactivation by OPTA are shown in table. ATP and ADP, whether with or without Mg^{2+}, could protect AK against OPTA. This suggested that the Cys-Lys pair whose modification by OPTA results in the loss of AK activity may be located in or near the active site where the ATP, ADP, ATP-Mg^{2+}, or ADP-Mg^{2+} bind.

Physical properties of OPTA-modified AK. Modification of AK by OPTA led to changes of its physi-

cal properties, such as electrophoretic characteristics and the molecule size monitored by FPLC.

Native PAGE showed that the longer the enzyme was modified, the faster the enzyme migrated and the relative migration ratio paralleled the fraction of the residual activity, which was validated by electrophoresis in 8 M urea, in which the effect of molecular shape was excluded. All the above suggested that the increased migration ratio was determined by the number of the modified Cys-Lys pairs, due to the changes of charge intensity. In addition, all types of electrophoresis of the modified enzyme, regardless of its modification extent, showed bands in the stacking gel, indicating polymers formed during OPTA modification.

Effect of potential protecting reagents on the inactivation of arginine kinase by OPTA*

Agent	Residual activity, %
Control	3
Arg (10 mM)	3.8
Arg (20 mM)	4.0
PArg (10 mM)	0.1
PArg (20 mM)	1.0
Metal ions:	
Mg^{2+} (20 mM)	1.0
Ca^{2+} (20 mM)	0.5
Mn^{2+} (20 mM)	0.4
Nucleotides (no metal):	
AMP (10 mM)	1.9
AMP (20 mM)	2.1
ADP (10 mM)	18.4
ADP (20 mM)	32.8
ATP (10 mM)	20.6
ATP (20 mM)	38
Mg–nucleotide complex:	
AMP (5 mM) + Mg^{2+} (10 mM)	2.0
AMP (10 mM) + Mg^{2+} (20 mM)	2.3
ADP (5 mM) + Mg^{2+} (10 mM)	10.6
ADP (10 mM) + Mg^{2+} (20 mM)	15.3
ATP (5 mM) + Mg^{2+} (10 mM)	22.5
ATP (10 mM) + Mg^{2+} (20 mM)	35.2

* Protection studies were carried out by incubating the reagents shown in the table with 3.64 μM AK for 6 min followed by another 10 min incubation with 0.5 mM OPTA. The reaction was quenched by 20 mM cysteine and 5 mM β-mercaptoethanol. Appropriate controls in each case without OPTA were carried out concurrently. AK activity that remained in the reaction mixtures was subsequently determined.

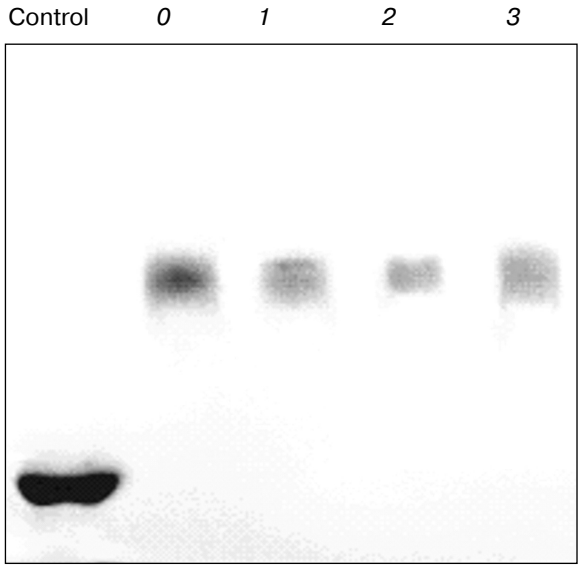

Fig. 7. SDS-PAGE analysis of cross-linking by glutaraldehyde. Samples *0-3* were the same as in Fig. 2 except for some later treatments (details in "Materials and Methods"). The control sample was native AK without cross-linking by glutaraldehyde.

Use of FPLC helped to understand the effect of OPTA modification on AK molecules. First, elution peaks were observed in the void volume, verifying the conclusion that the modifications by OPTA lead to polymer formation. Second, OPTA modification made the native AK split into two peaks, with one remaining active and the other not. Since SDS-PAGE of the glutaraldehyde cross-linking experiment (Fig. 7) indicated that modification by OPTA did not lead to dissociation of this dimeric enzyme and both peaks in FPLC had absorbance at 337 nm, it was inferred that AK eluting at either peak had been modified and the modification led to two different dimeric spatial conformations: one active and one inactive. As modification proceeded, the active peak disappeared and became a completely inactive peak, suggesting the conformation changes induced by the modification of the active essential Cys-Lys pair determined the overall modified enzyme conformation, which could mask the changes induced by the modification of the nonessential Cys-Lys pairs.

DISCUSSION

The results of our experiments showed that different modification positions of AK led to different spatial conformations: modification of the essential Cys-Lys pair made the enzyme conformation loose while modification

Fig. 8. Alignment of the amino acid sequences of dimeric *S. japonicus* AK with monomeric *Limulus* AK. Data were obtained from the PDB and processed by ClustalX1.8 software. The symbol ∿ stands for α-helix; → stands for the β-sheet; ⬭ stands for the position of the two conservative flexible loops; letters in italic and bold stand for the GS region; △ stands for the position of cysteine in sea cucumber AK, and the rectangle stands for the position of the conservative cysteine.

of the other pairs made it compact. In addition, as the modification of the essential Cys-Lys pair had a determinant effect on the enzyme conformation, the compactness degree of AK molecules in the final modification state was similar to that with only the active essential Cys-Lys modified. In previous studies, comparison of the open substrate-free structure of CK and the closed substrate-bound structure of AK suggested the crux of the catalysis of the two-substrate phosphagen kinases is the relative rotation of the N-terminal and C-terminal domains, together with the subsiding of two conservative flexible loops towards the substrates binding region. These rearrangements acted to exclude water molecules and provide a hydrophobic environment to minimize wasteful ATP hydrolysis [37]. In our experiments, conformation changes of AK molecules whose active Cys-Lys pair was modified by OPTA validated the conclusion that conformation changes at or near the substrate-binding region, which was located in a highly hydrophobic environment, related closely to the overall enzyme conformation. In addition, it could also be inferred from our results that the active sites of AK have a certain flexibility and independence, because although the modification of the nonessential Cys-Lys pairs led to an obvious conformational change, the enzyme still retained its activity.

AK of sea cucumber is a dimeric enzyme of identical subunits [38]. However, the experimental data reported above indicated that among the Cys-Lys pairs modified by OPTA in AK, only one Cys-Lys pair is essential for activity. That means modification of the active essential Cys-Lys pair in one subunit would lead to a conformation change that inactivates the whole enzyme. The high reactivity of the initial OPTA binding to AK may be due to the cooperativity of the AK inactivation. Such cooperativity was already found in the modification and catalysis of many other oligomeric enzymes, such as CK, indicating

that the reciprocity and information exchange between the subunits play an important part in the function of oligomeric enzymes [39-41].

Previous comparison of the amino acid sequences of dimeric *S. japonicus* AK and monomeric *Limulus* AK (Fig. 8) [6] showed, that all five cysteines are located in the C-terminal domain, including the four nonconservative (Cys137, Cys284, Cys303, and Cys343) and the one conservative (Cys274), which corresponds to Cys271 in the *Limulus* sequence. Zhou et al. suggested that Cys271 plays an important part by interacting with the guanidine group of Arg in the monomeric AK [6]. Therefore, we inferred that the active essential cysteine, which was detected by both OPTA and DTNB modification in our experiments, maybe the Cys274. However, the exact identification requires further investigations.

This work was supported by the Laboratory of Protein Structure and Function in the Department of Biotechnology and Biological Science, Tsinghua University, China.

REFERENCES

1. Watts, D. C. (1971) in *Evolution of Phosphagen Kinases in Biochemical Evolution and the Origin of Life* (Schoffeniels, E., ed.) North-Holland, Amsterdam, pp. 150-173.
2. Watts, D. C. (1975) *Symp. Zool. Soc. Lond.*, **36**, 105-127.
3. Suzuki, T., Kamidochi, M., Inoue, N., Kawamichi, H., Yazawa, Y., Furukohri, T., and Ellington, W. R. (1999) *Biochem. J.*, **340**, Pt. 3, 671-675.
4. Sheikh, S., Mukunda, K., and Katiyar, S. S. (1993) *Biochim. Biophys. Acta*, **1203**, 276-281.
5. Yilmaz, S., and Ozer, I. (1990) *Arch. Biochem. Biophys.*, **279**, 32-36.
6. Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W. R., and Chapman, M. S. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 8449-8454.
7. Yousef, M. S., Clark, S. A., Pruett, P. K., Somasundaram, T., Ellington, W. R., and Chapman, M. S. (2003) *Protein Sci.*, **12**, 103-111.
8. Benson, J. R., and Hare, P. E. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 619-622.
9. Huynh, Q. K. (1990) *J. Biol. Chem.*, **265**, 6700-6704.
10. Puri, R. N., and Roskoski, R., Jr. (1988) *Analyt. Biochem.*, **173**, 26-32.
11. George, S. P., Ahmad, A., and Rao, M. B. (2001) *Biochem. Biophys. Res. Commun.*, **282**, 48-54.
12. Pandey, A., Sheikh, S., and Katiyar, S. (1996) *Biochim. Biophys. Acta*, **1293**, 122-128.
13. Chen, C. Y., Emig, F. A., Schramm, V. L., and Ash, D. E. (1991) *J. Biol. Chem.*, **266**, 16645-16652.
14. Anosike, E. O., Moreland, B. H., and Watts, D. C. (1975) *Biochem. J.*, **145**, 535-543.
15. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
16. Xiang, L. B. Q. (1996) *Beijing Agric. Sci.*, **14**, 39-40.
17. Chen, B. Y., Guo, Q., Guo, Z., and Wang, X. C. (2003) *Tsinghua Sci. Technol.*, **8**, 422-427.
18. Zhou, H. M., Zhang, X. H., Yin, Y., and Tsou, C. L. (1993) *Biochem. J.*, **291**, Pt. 1, 103-107.
19. Simons, S. S., Jr., Thompson, E. B., and Johnson, D. F. (1979) *Biochemistry*, **18**, 4915-4922.
20. Zhou, H. M., and Wang, H. R. (1998) *Chemical Modification of Proteins* [in Chinese], Tsinghua University Press, Beijing.
21. Font, B., Vial, C., Goldschmidt, D., Eichenberger, D., and Gautheron, D. C. (1981) *Arch. Biochem. Biophys.*, **212**, 195-203.
22. Zheng, Y. B., Wang, Z., Chen, B. Y., and Wang, X. C. (2003) *Int. J. Biol. Macromol.*, **32**, 191-197.
23. Creighton, T. E. (1997) *Protein Structure: A Practical Approach*, Oxford University Press, London.
24. George, S. P., and Rao, M. B. (2001) *Eur. J. Biochem.*, **268**, 2881-2888.
25. Ahn, J. Y., Choi, S., and Cho, S. W. (1999) *Biochimie*, **81**, 1123-1129.
26. Reddy, Y. V., and Rao, D. N. (1998) *J. Biol. Chem.*, **273**, 23866-23876.
27. Stoops, J. K., Henry, S. J., and Wakil, S. J. (1983) *J. Biol. Chem.*, **258**, 12482-12486.
28. Kobayashi, M., Miura, M., and Ichishima, E. (1992) *Biochem. Biophys. Res. Commun.*, **183**, 321-326.
29. Bhagwat, A. S., Blokesch, A., Irrgang, K. D., Salnikow, J., and Vater, J. (1993) *Arch. Biochem. Biophys.*, **304**, 38-44.
30. Khan, Y. M., Wictome, M., East, J. M., and Lee, A. G. (1996) *Biochem. J.*, **317**, Pt. 2, 433-437.
31. Rider, M. H., and Hue, L. (1989) *Biochem. J.*, **262**, 97-102.
32. Puri, R. N., Bhatnagar, D., Glass, D. B., and Roskoski, R., Jr. (1985) *Biochemistry*, **24**, 6508-6514.
33. Yilmaz, S., and Ozer, I. (1990) *Arch. Biochem. Biophys.*, **279**, 32-36.
34. Sheikh, S., Mukunda, K., and Katiyar, S. S. (1993) *Biochim. Biophys. Acta*, **1203**, 276-281.
35. Palczewski, K., Hargrave, P. A., and Kochman, M. (1983) *Eur. J. Biochem.*, **137**, 429-435.
36. Hollenberg, P. F., Flashner, M., and Coon, M. J. (1971) *J. Biol. Chem.*, **246**, 946-953.
37. Suzuki, T., Yamamoto, Y., and Umekawa, M. (2000) *Biochem. J.*, **351**, Pt. 3, 579-585.
38. Guo, S. Y., Guo, Z., Guo, Q., Chen, B. Y., and Wang, X. C. (2003) *Protein Exp. Purif.*, **29**, 230-234.
39. Wang, X. C., Zhou, H. M., Wang, Z. X., and Tsou, C. L. (1990) *Biochim. Biophys. Acta*, **1039**, 313-317.
40. Reddy, S. R., and Watts, D. C. (1994) *Comp. Biochem. Physiol. Biochem. Mol. Biol.*, **108**, 73-78.
41. Cox, J. M., Davis, C. A., Chan, C., Jourden, M. J., Jorjorian, A. D., Brym, M. J., Snider, M. J., Borders, C. L., Jr., and Edmiston, P. L. (2003) *Biochemistry*, **42**, 1863-1871.