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Two Fused Proteins Combining *Stichopus japonicus* Arginine Kinase and Rabbit Muscle Creatine Kinase

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Abstract—Two fused proteins of dimeric arginine kinase (AK) from sea cucumber and dimeric creatine kinase (CK) from rabbit muscle, named AK-CK and CK-AK, were obtained through the expression of fused AK and CK genes. Both AK-CK and CK-AK had about 50% AK activity and about 2-fold K_m values for arginine of native AK, as well as about 50% CK activity and about 2-fold K_m values for creatine of native CK. This indicated that both AK and CK moieties are fully active in the two fused proteins. The structures of AK, CK, AK-CK, and CK-AK were compared by collecting data of far-UV circular dichroism, intrinsic fluorescence, 1-anilinonaphthalene-8-sulfonate binding fluorescence, and size-exclusion chromatography. The results indicated that dimeric AK and CK differed in the maximum emission wavelength, the exposure extent of hydrophobic surfaces, and molecular size, though they have a close evolutionary relationship. The structure and thermodynamic stability of AK, CK, AK-CK, and CK-AK were compared by guanidine hydrochloride (GdnHCl) titration. Dimeric AK was more dependent on the cooperation of two subunits than CK according to the analysis of residual AK or CK activity with GdnHCl concentration increase. Additionally, AK and CK had different denaturation curves induced by GdnHCl, but almost the same thermodynamic stability. The two fused proteins, AK-CK and CK-AK, had similar secondary structure, tertiary structure, molecular size, structure, and thermodynamic stability, which indicated that the expression order of AK and CK genes might have little effect on the characteristics of the fused proteins and might further verify the close relationship of dimeric AK and CK.

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Creatine kinase (CK) (ATP:creatine N-phosphotransferase, EC 2.7.3.2) is an important enzyme for energy metabolism in vertebrate animals, transferring high-energy phosphate from ATP to creatine as the following reversible reaction [1, 2]:

MgATP + creatine ↔ MgADP + phosphocreatine.

Considering its stable characteristics and special catalytic mechanism, CK has been extensively used as a model for research on protein folding [3-6] and as a paradigm for understanding the mechanisms of bimolecular reactions [7-10]. The cytoplasmic isozymes of CK occur in three tissue-specific and dimeric forms: CK-MM (muscle type) and CK-BB (brain type), as well as CK-MB (heart type), which can be regarded as the hybrid of the other two forms [11-13]. We have succeeded in the hybridization of CK-MM and CK-BB by urea denaturation or freeze drying method, and verified that the subunit of CK may be the minimal functional unit [13, 14]. CK used in the experiments is from rabbit muscle and composed of two identical subunits, each of which has 385 amino acids and a molecular weight of about 43 kD [15].

Arginine kinase (AK) (ATP:arginine N-phosphotransferase, EC 2.7.3.3) in invertebrates catalyzes the reversible phosphorylation of arginine by MgATP to form a high-energy compound phosphoarginine (Parg) and MgADP [16-18]:

MgATP + arginine ↔ MgADP + phosphoarginine.

Abbreviations: AK) arginine kinase; ANS) 1-anilinonaphthalene-8-sulfonate; CD) circular dichroism; CK) creatine kinase; DTT) dithiothreitol; FPLC) fast protein liquid chromatography; GdnHCl) guanidine hydrochloride; IPTG) isopropyl-β-D-thiogalactopyranoside; PCR) polymerase chain reaction; SEC) size-exclusion chromatography.

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Although the function of AK is analogous to that of CK in vertebrates, it has not been investigated as extensively as CK. While most CKs are dimers, AK exists as a monomer except in some annelids, echinoderms, and sipunculids [19]. AK from sea cucumber *Stichopus japonicus* is composed of two identical subunits, each of which has 371 amino acids and a molecular weight of about 42 kD [20]. Dimeric AK was evolutionarily related to dimeric CK by sequence comparison [21]. Besides, the success in obtaining heterohybrids of muscle creatine kinase (M-CK) and muscle arginine kinase (M-AK) could more or less convince us that there was a close evolutionary relationship between these enzymes [22]. However, there was almost no AK activity left in such heterohybrids, so it was impossible to infer the minimal subunit of AK.

In this work, we succeeded in obtaining two fused proteins of dimeric AK and CK by the expression of fused AK and CK genes. The two fused proteins were named AK-CK and CK-AK, respectively. The activity assay indicated that both of them had AK and CK activity. And through measuring the circular dichroism (CD), intrinsic fluorescence, 1-anilinonaphthalene-8-sulfonate (ANS) binding fluorescence, and size-exclusive chromatography (SEC) of the two native enzymes and the two fused proteins, we investigated the difference between dimeric AK and CK. In addition, the structure and thermodynamic stability of the four proteins were compared by guanidine hydrochloride (GdnHCl) induced denaturation.

MATERIALS AND METHODS

Materials. *Escherichia coli* expression vector pET-21b and the strain BL21 (DE3) were purchased from Novagen (USA). All the enzymes used for plasmid construction were purchased from Takara (Japan), and the Ni^{2+}-nitrilotriacetic acid column used for protein purification was from Qiagen (Germany). ATP, arginine, creatine, dithiothreitol (DTT), GdnHCl, isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, acrylamide, Tris, and ANS were purchased from Sigma (USA), but other reagents were local products of analytical grade.

Coexpression of AK and CK. The mutations were introduced on a template of wild type PET-21b/*Stichopus*-AK by PCR-based mutagenesis. The primers used for the construction of fused protein AK-CK were: 5′-CACACCGAGAGTGAGGGCGGTGTC-TACGATATCTCAAAC-3′ (upstream), and 5′-GGAG-CTCGAGCTTCTGGGCGGGGATCATGTC-3′ (downstream).

And the primers for CK-AK were: 5′-GGAATTC-CATATGATGCCGTTCGGCAACAAC-3′ (upstream), and 5′-GGAGCTCGAGCTTGGGGACAAGGTCAT-CGAT-3′ (downstream). After the PCR product and plasmid PET-21b were treated with two restriction enzymes, they were ligated by T4 DNA ligase at 4°C for 24 h. Then the genes of the two heterohybrids were transformed into *E. coli* DH5α cells. The fidelity of the mutation was confirmed by sequencing the entire gene.

Expression and purification of AK, CK, AK-CK, and CK-AK. Plasmids encoding AK, CK, AK-CK, and CK-AK were transformed into the *E. coli* BL21 (DE3) strain, and the transformed cells were incubated at 37°C to A_{600} of 0.6-0.8. Then 0.2 mM IPTG was added to induce the protein expression for about 24 h at 20°C. All the proteins were purified *via* Ni^{2+}-nitrilotriacetic acid column according to the manufacturer's protocol. Further purification was performed with a Superdex 200HR 10/30 column on a Pharmacia (Sweden) FPLC apparatus at 4°C. Each enzyme exhibited a single band on SDS-PAGE. Protein concentrations were determined by the Bradford method with bovine serum albumin as the standard [23].

Enzyme activity assay and K_m determination. The AK activity of AK, AK-CK, and CK-AK was assayed by an improved version of the phosphate determination method [24], while the CK activity of CK, AK-CK, and CK-AK was measured by the pH-stat method [25]. The kinetic parameters K_m values were estimated by Lineweaver–Burk plot and fitted by the least-squares method using Microsoft Excel.

Equilibrium denaturation. All samples were incubated with different concentrations of GdnHCl in 20 mM Tris-acetate buffer with 0.1 mM DTT (pH 8.1) for 24 h, and all the experiments were performed at 25°C. The final concentrations of the proteins were 0.2 mg/ml.

CD profiles. Far-UV CD profiles were collected on a Jasco 500C (Jasco, Japan) CD spectropolarimeter at 25°C, using a 2 mm pathlength cell over the wavelength range of 200-250 nm. Four spectra signals were averaged for each sample.

Fluorescence measurement. All fluorescence emission spectra were determined on a Hitachi F-2500 (Hitachi, Japan) fluorescence spectrophotometer at 25°C, and the excitation slits and emission slits were both set at 5 nm. Excitation was accomplished at 280 nm, and all the emission spectra were collected from 300 to 400 nm. ANS binding fluorescence was performed after incubating AK and its mutants with 100-fold molar excess of ANS (160 μM) for 30 min in the dark. Then we collected the spectra from 400-600 nm with the excitation wavelength set at 380 nm.

Quantitative studies by equilibrium denaturation. To obtain profiles suitable for quantitative calculation, the intensity-weighted average emission wavelength ($\langle\lambda\rangle$) was plotted versus GdnHCl concentration [26, 27]. After the plot using $\langle\lambda\rangle$ versus GdnHCl concentration, we found that the two-state model fitted the fluorescence data, a native dimer (N_2) and a completely unfolded monomer (U) [28]. So the equilibrium constants K_{nu} could be calculated. The success in obtaining the value of K_{nu} makes the calculation of free energy ($\Delta G = -RT\ln K$)

possible. According to previous studies, there is a linear relationship between the Gibbs free energy ΔG and denaturant concentration [D] during the transition from any A state to the unfolding B state [27, 29]. Obviously, the ΔG_0 value by the extrapolation to zero denaturant concentration (intercept) reflects the conformation stability of the enzyme:

$$\Delta G_{ab} = \Delta G_{0,ab} - m_{ab}[D].$$

Size-exclusion chromatography (SEC). SEC was performed with a Superdex 200HR 10/30 column (fractionation range 10-600 kD) on a Pharmacia FPLC apparatus at 4°C. All solutions used in this experiment were passed through a filter and degassed. A 100-μl sample was analyzed.

RESULTS AND DISCUSSION

Activity assay and kinetic parameters. The specific activity of AK-CK and CK-AK are shown in Table 1. Consistent with our previous studies that verified the subunit of CK was the minimal functional unit, the two fused proteins, AK-CK and CK-AK, had 58.76 and 60.96% CK activity compared with native CK. And the AK activity of AK-CK and CK-AK were 50.56 and 53.36% of native AK, respectively. All these results indicated that both AK and CK moieties are fully active in the two fused proteins.

Table 1. Specific activity of AK-CK and CK-AK

Enzyme	AK activity	CK activity
AK-CK	50.56 ± 0.26	53.36 ± 1.15
CK-AK	58.76 ± 4.28	60.96 ± 2.94
AK	100	0
CK	0	100

Compared with the K_m value for arginine of native AK, 0.52 mM, the K_m values of AK-CK and CK-AK increased about twofold, 1.04 and 0.96 mM, respectively (Table 2). Analogously, the K_m values for creatine (6.44 mM for native CK) increased for AK-CK and CK-AK also about twofold (13.62 and 13.35 mM, respectively).

Structure comparison of AK, CK, AK-CK, and CK-AK. A comparison of the secondary structure of the four proteins is shown in Fig. 1. AK and CK shared a similar shape of CD profiles, and the CD profiles of AK-CK and CK-AK almost overlapped each other.

To detect the difference in the tertiary structures of the four proteins, we collected the intrinsic fluorescence profiles from 300 to 400 nm, with the excitation wavelength set at 280 nm (Fig. 2). The maximum emission wavelength of AK was 320.5 nm, while that of CK was 330.5 nm. The two fused proteins, AK-CK and CK-AK, had a maximum emission wavelength of 328.5 nm, unlike both AK and CK.

ANS can bind to the hydrophobic regions of a protein, so it can be used as an indicator of the changes in the hydrophobic surface of a protein. Figure 3 indicates that of the four proteins, AK had the least exposed hydrophobic surface under the native state. The exposure extent of the hydrophobic surface of AK-CK and CK-AK was almost the same, larger than that of AK.

The separation of different proteins depends on their own molecular size in SEC. Dimeric AK and CK had a little difference in elution volume, 10.8 and 11.2 ml, respectively (Fig. 4). However, the SEC data indicated that AK-CK and CK-AK might exist as tremendous multiple complexes, for their elution volumes were both about 7.5 ml, which was equal to the void volume of the column.

Equilibrium unfolding of AK, CK, AK-CK, and CK-AK induced by GdnHCl. Figure 5 shows the transition curves obtained after the four proteins were treated in different concentrations of GdnHCl overnight. The residual CK activity of CK, AK-CK, and CK-AK was plotted versus GdnHCl (Fig. 5a). It was clear that the CK activity of these three proteins decreased almost to the same extent with GdnHCl concentration increase. But Fig. 5b indicates that the rate at which AK activity decreased in AK-

Table 2. Kinetic analysis of initial and used AK and CK

Enzyme	CK activity		AK activity	
	K_m^{Cr}	K_m^{ATP}	K_m^{Arg}	K_m^{ATP}
AK	n.d.	n.d.	0.52 ± 0.02	1.01 ± 0.01
CK	6.4 ± 0.9	0.40 ± 0.03	—	—
AK-CK	14 ± 1	0.86 ± 0.16	1.0 ± 0.2	1.5 ± 0.1
CK-AK	13 ± 1	0.73 ± 0.06	1.0 ± 0.2	1.6 ± 0.2

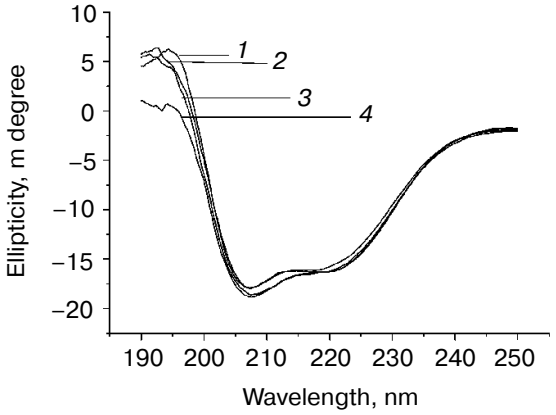

Fig. 1. CD profiles of AK, CK, CK-AK, and AK-CK (curves *1-4*, respectively).

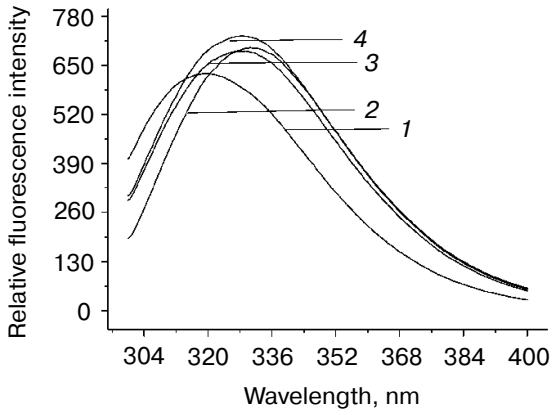

Fig. 2. Intrinsic fluorescence profiles of AK, CK, AK-CK, and CK-AK (curves *1-4*, respectively).

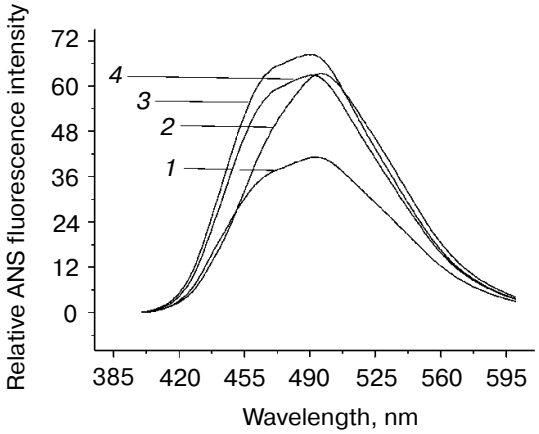

Fig. 3. ANS profiles of AK, CK, AK-CK, and CK-AK (curves *1-4*, respectively).

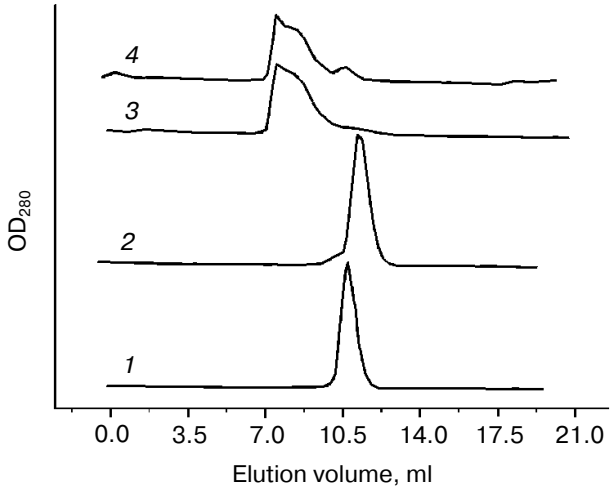

Fig. 4. SEC profiles of AK, CK, AK-CK, and CK-AK (lines *1-4*, respectively).

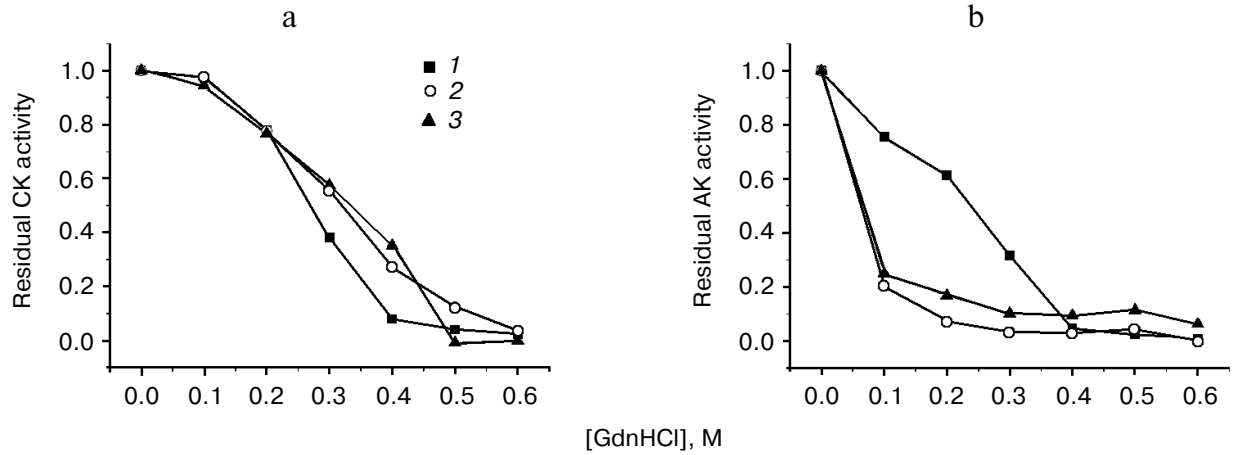

Fig. 5. Effect of GdnHCl on CK (a) and AK (b) activities. Curves: *1*) CK (a) or AK (b); *2* and *3*) AK-CK and CK-AK, respectively.

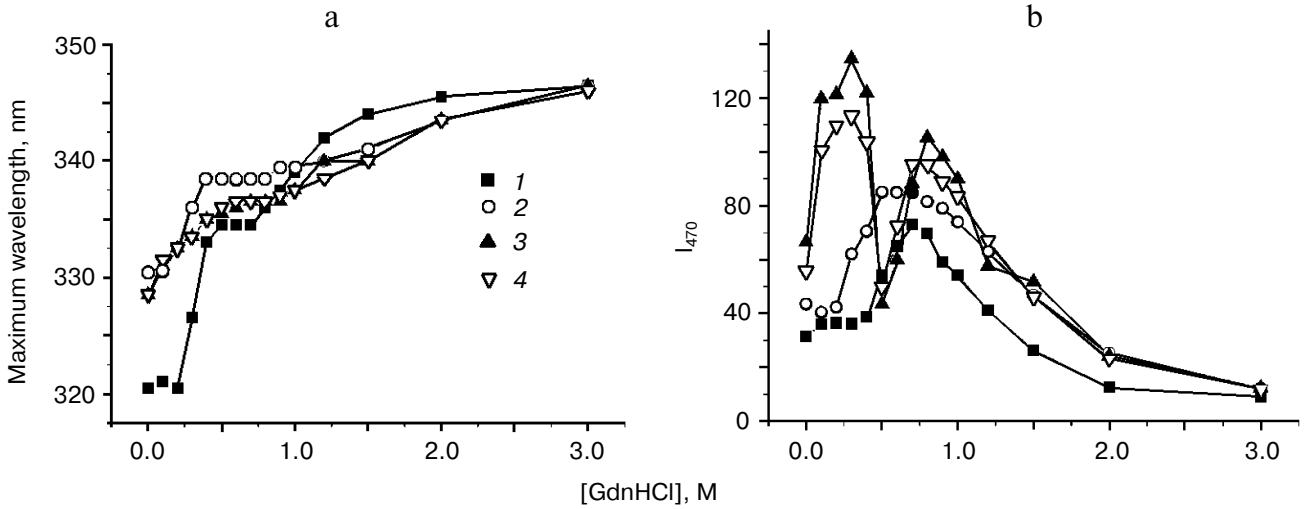

Fig. 6. Effect of GdnHCl on the position of the intrinsic fluorescence maximum (a) and ANS fluorescence (b) of AK, CK, AK-CK, and CK-AK (lines *1-4*, respectively). The proteins were treated with different concentrations of GdnHCl for 24 h at 25°C. b) Final ANS concentration was 160 μM. The points are the experimental values of fluorescence intensity monitored at 470 nm.

CK and CK-AK as the GdnHCl concentration increased was faster than that of AK; only 0.1 M GdnHCl would lead to about 80% loss of AK activity in the two fused proteins.

The effect of increasing denaturant concentration on the tertiary structure of the four proteins was studied by intrinsic fluorescence spectra. Figure 6a shows that the maximum emission wavelength of AK and CK both exhibited biphasic profiles during unfolding. The first transition of AK resulted in a 14-nm (320.5 to 334.5 nm) red shift of the maximum emission wavelength, and the second transition was accompanied by a further red shift of the maximum emission wavelength to 346.5 nm, while the two transitions of CK were both 8 nm, from 330.5 to 338.5 nm and from 338.5 to 346.5 nm, respectively. All these results are consistent with previous studies [4, 30]. However, the similar unfolding process of AK-CK and CK-AK was quite different from that of AK or CK; the maximum emission wavelength red-shifted from 328.5 to 346.5 nm.

The changes in hydrophobic surfaces of the four proteins during GdnHCl denaturation could be indicated through the ANS binding fluorescence (Fig. 6b). The relative ANS intensity at 470 nm of AK changed a little below 0.4 M GdnHCl, then the ANS intensity began to increase and reached a peak at 0.7 M GdnHCl. The ANS fluorescence profile of CK was different from that of AK; its acceleration began at 0.2 M GdnHCl and reached a plateau at 0.5-0.7 M GdnHCl. Interestingly, the ANS fluorescence profiles of AK-CK and CK-AK both had two peaks; one was at 0.3 M GdnHCl and the other was at 0.8 M GdnHCl.

The Gibbs free energy of AK, CK, AK-CK, and CK-AK was evaluated through GdnHCl titration (28.71, 27.83, 28.89, and 27.22 kJ/mol, respectively). It is noted that all four proteins have similar thermodynamic stability.

Although dimeric AK and CK are both phosphagen kinases and have an evolutionary relationship [15], there is much difference between them. For one thing, the interactions between two subunits might be different. The residual AK or CK activity of the two fused proteins with denaturant concentration increase (Fig. 5) might indicate that the resistance to the disturbance of the environment of AK is more dependent on the cooperation of its two subunits than that of CK, for only 0.1 M GdnHCl decreased the AK activity of AK-CK and CK-AK by about 80% (Fig. 5b) while the CK activity of the two fused proteins decreased by not more than 10% at the same concentration of denaturant (Fig. 5a). For another thing, though AK and CK share a similar secondary structure (Fig. 1) and thermodynamic stability, they could be distinguished from each other by the measure of intrinsic fluorescence (Fig. 2), ANS fluorescence (Fig. 3), and SEC (Fig. 4), as well as from the changes of the maximum emission wavelength and ANS intensity induced by GdnHCl titration.

Thus, although the gene expression order of AK-CK and CK-AK was different, the two fused proteins were almost the same in specific AK or CK activity, secondary structure, tertiary structure, and molecular size, as well as thermodynamic stability. All these results suggest that the gene expression order of the AK gene and CK gene might have little influence on the structure and characteristics of their fused expression products. This indicated that AK and CK might share the same protein folding mechanism and might provide further proof that dimeric AK and CK have a close evolutionary relationship.

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