Kinetic properties and structural characteristics of an unusual two-domain arginine kinase of the clam *Corbicula japonica*

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Abstract **Arginine kinase (AK) from the clam *Corbicula japonica* is a unique enzyme in that it has an unusual two-domain structure with molecular mass of 80 kDa. It lacks two functionally important amino acid residues, Asp-62 and Arg-193, which are conserved in other 40 kDa AKs and are assumed to be key residues for stabilizing the substrate-bound structure. K_m^{arg} and V_{max} values for the recombinant two-domain AK were determined. These values were close to those of usual 40 kDa AKs, although *Corbicula* AK lacks the functionally important Asp-62 and Arg-193. Domain 2 of *Corbicula* AK was separated from the two-domain enzyme and was expressed in *Escherichia coli*. Domain 2 still exhibited activity. However, kinetic parameters for domain 2 appeared to be slightly, but significantly, different from those of two-domain AK. Thus, it is likely that the formation of the contiguous dimer alters the kinetic properties of its constituent domains significantly. Comparison of K_d^{arg} and K_m^{arg} for two-domain AK and its domain 2 showed that the affinity of the enzyme for arginine is greater in the presence of substrate ATP than in its absence. Presumably this difference is correlated with the large structural differences in the enzyme in the presence or absence of substrate, namely *open* and *closed* structures. We expressed three mutants of *Corbicula* AK domain 2 (His-60 to Gly or Arg, Asp-197 to Gly), and determined their K_m^{arg} and V_{max} values. The affinity for the substrate arginine in mutant enzymes was reduced considerably, accompanied by a decrease in V_{max}. These results suggest that His-60 and Asp-197 affect the substrate binding system, and are consistent with the hypothesis that a hydrogen bond is formed between His-60 and Asp-197 in *Corbicula* AK as a substitute for the Asp-62 and Arg-193 bond in normal AKs.**

Key words: Arginine kinase; Creatine kinase; Mutagenesis; *Corbicula*

1. Introduction

Phosphagen kinases are enzymes that catalyze the reversible transfer of the high-energy phosphoryl group of ATP to naturally occurring guanidine compounds such as creatine, glycocyamine, taurocyamine, lombricine, and arginine to produce a phosphorylated high-energy guanidine called a phosphagen. Members of this enzyme family (creatine kinase (CK), arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK), and lombricine kinase (LK)) play key roles in the coupling of energy production and utilization in animals [1–4]. The homologous amino acid sequences of phosphagen kinases suggest that they have evolved from a common ancestor [5,6], but the evolutionary processes are not fully understood. At present, it is clear that two strains (CK and AK strains) diverged from their ancestral gene before metazoan evolution, and based on phylogenetic analyses, GK, LK, and presumably TK radiated from the CK strain [7,8]. The presence of CK dates back at least to the evolution of the phylum Porifera [9], and its dimeric subunit structure is also present in GK, TK, and LK. It is well known that in mammals there are three types of CK isozymes: muscle type, brain type and mitochondrial type [10]. Recent sequence determination of mitochondrial type CK from a marine worm indicates that its origin is rather ancient [11].

AK is most widely distributed among invertebrates [1–3]. Most AKs are monomers with a 40 kDa subunit, however other phosphagen kinases, CK, GK and LK, are dimeric or octameric as in the case of mitochondrial CK [10]. Interestingly, AK appears to have evolved at least twice during the evolution of phosphagen kinases: at an early stage of phosphagen kinase evolution before metazoan evolution (its descendants are monomeric 40 kDa AKs) and from CK at a later time in metazoan evolution (dimeric AKs in Echinodermata) [7,12].

An unusual 80 kDa AK was first isolated from the primitive sea anemone *Anthopleura*, and was shown to have a two-domain structure [13]. Moreover, Soga and Yazawa [14] isolated an unusual 86 kDa AK with a cDNA-derived 724 amino acid sequence and a two-domain structure [15] from the adductor muscle of the marine clam *Pseudocardium sachalinensis*. Some other clams also contain unusual two-domain AKs; we recently reported the cDNA-derived amino acid sequences of *Solen strictus* and *Corbicula japonica* AKs [16]. Thus, two-domain AKs have been observed in two diverse invertebrate groups, a sea anemone and a mollusc, and it is clear that gene duplication and subsequent fusion has occurred frequently, and likely independently, during the course of the evolution of AKs.

Here we have cloned and expressed the 80 kDa two-domain AK from the clam *Corbicula* and have determined several kinetic parameters to characterize the unusual AK. We also show that although *Corbicula* two-domain AK lacks the functionally important hydrogen bond between Asp-62 and Arg-193 [17], the His-60 and Asp-197 bond in *Corbicula* AK might serve as a substitute for the former hydrogen bond [16].

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Throughout this paper, the sequence numbering of *Limulus* AK [18] is used.

2. Materials and methods

The open reading frames of *Corbicula* two-domain AK (2172 bp, 723 amino acid residues) and *Corbicula* AK domain 2 (1080 bp, 359 residues) were cloned into the *Bam*HI/*Pst*I site of pMAL-c2 (termed *Corbicula* two-domain AK/wt and *Corbicula* AK domain 2/wt, respectively). We tried unsuccessfully to obtain the recombinant enzyme for *Corbicula* AK domain 1. The maltose binding protein–*Corbicula* AK fusion protein was expressed in *Escherichia coli* TB1 cell by induction with 1 mM IPTG at 25°C for 24 h. The soluble protein was extracted with B-PER reagent (Pierce) and the fusion protein was purified by affinity chromatography using amylose resin (NEB). Purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were placed on ice until use, and enzymatic activity was determined within 12 h.

Polymerase chain reaction (PCR)-based mutagenesis was done as described previously [12,17]. The mutations (His-60 (CAT) to Gly (GGT), His-60 (CAT) to Arg (CGT), Asp-197 (GAT) to Gly (GGT)) were introduced in the template of *Corbicula* domain 2/wt using the following primers (introduced mutations are underlined): His-60 to Gly: GGTTTAGGCAGCAAAGTAGGCAT and AATACATCCTGAGTTGATAC, His-60 to Arg: CGTCTAGGCAGCAAAGTAGG CAT and AATACATCCTGAGTTGATAC, and Asp-197 to Gly: GTGCCGGTGGTTACAAGTACT and CGCGCAACACGTCGTCATCG. *KOD*$^{+}$ DNA polymerase (Toyobo) was used as the amplifying enzyme. PCR products were digested with *Dpn*I and the target 7000 bp DNA fragment was recovered by EasyTrap Ver.2 (Takara). After blunting and kination, the DNA was self-ligated, and the mutated protein was expressed as described above. cDNA of the insert was completely sequenced to confirm that only the intended mutations were present.

Enzyme activity was assayed spectrophotometrically at 25°C [19]. Protein concentration was estimated from the absorbance at 280 nm (0.77 AU at 280 nm in a 1 cm cuvette corresponds to 1 mg protein/ml). To estimate K_m for arginine (K_m^{arg}, mM) and V_{max} (μmol Pi/min/mg of protein), either a Lineweaver–Burk plot was made and fitted by the least-square method using Microsoft Excel, or data were fitted directly to the equation of Michaelis–Menten using the software Hyper.exe (ver.1.0, distributed by J.S. Easterby (1992)). Since the kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [20], the reaction velocity (v) is given by the following equation:

$$v = \frac{V_{max}}{\frac{K_m^{ATP} K_d^{arg}}{[arginine][ATP]} + \frac{K_m^{ATP}}{[ATP]} + \frac{K_m^{arg}}{[arginine]} + 1} \quad (1)$$

Here, K_d^{arg} is the dissociation constant of arginine in the absence of ATP, and is obtained graphically (see Section 3) or by fitting data directly to Eq. 1 according to the method of Cleland [21], using the software written by Dr. R. Viola (Enzyme Kinetics Programs, ver.2.0).

3. Results and discussion

All of the recombinant enzymes used in this study (*Corbicula* two-domain AK, *Corbicula* AK domain 2, and three mutants of *Corbicula* AK domain 2 (His-60 to Gly, His-60 to Arg, and Asp-197 to Gly) were expressed as soluble proteins, successfully purified by affinity chromatography, and confirmed to be highly purified by SDS–PAGE. Kinetic parameters, K_m^{arg}, K_d^{arg}, K_m^{ATP}, K_d^{ATP}, k_{cat} (catalytic rate constant) and V_{max} of the recombinant wild-type enzymes and mutants were obtained for the forward reaction (phosphagen formation) and are listed in Table 1.

AK from the clam *Corbicula japonica* is a unique enzyme in that it has an unusual two-domain structure with molecular mass of 80 kDa. It lacks two functionally important amino acid residues, Asp-62 and Arg-193, which are conserved in other 40 kDa AKs from Mollusca and Arthropoda and are assumed to be key residues for stabilizing the substrate-bound structure [12,17]. Each of the two domains shows about 60% amino acid sequence identity, and still has a strong homology (~50%) with the typical 40 kDa AK from *Limulus* [18]. Despite the unusual features of *Corbicula* AK, the enzyme isolated directly from adductor muscle has an activity comparable to those of molluscan 40 kDa AKs (data not shown).

K_m^{arg} and V_{max} values for recombinant two-domain AK were determined to be 0.42 ± 0.14 mM and 110 ± 15.4 μmol Pi/min/mg of protein, respectively. Although two-domain AK lacks the functionally important Asp-62 and Arg-193, the K_m^{arg} value is close to those of usual 40 kDa AKs. Furthermore, the V_{max} shows the highest value among molluscan AKs (see Table 1). The similarity of kinetic parameters of recombinant *Corbicula* two-domain AK with usual 40 kDa AKs results suggests that there is some mechanism to compensate for the lack of Asp-62 and Arg-193 in *Corbicula* AK since the enzyme activity of the mutated protein (Asp-62 to Gly in *Nautilus* and *Stichopus* AKs) was reduced markedly [17,12].

On the other hand, although domain 2 is separated from the two-domain enzyme, it still exhibits activity (K_m^{arg} = 0.26 ± 0.086 mM, and V_{max} = 67.0 ± 6.86 μmol Pi/min/mg of protein). These parameters appear to be slightly, but significantly, different from those of two-domain AK. Comparison of the K_m^{arg} values suggests that the substrate arginine has much more affinity (1.6-fold) to domain 2 than two-domain AK. In addition, the V_{max} of two-domain AK is 1.5-fold higher than that of domain 2. Thus, it is likely that the formation of the contiguous dimer alters the kinetic properties of its constituent domains significantly.

Table 1
Kinetic parameters of *Corbicula* two-domain AK, domain 2 and mutated domain 2

Species	K_m^{arg} (mM)	K_d^{arg} (mM)	K_m^{ATP} (mM)	K_d^{ATP} (mM)	k_{cat} (s^{-1})	V_{max} (μmol Pi/min/mg of protein)
Corbicula two-domain	0.42 ± 0.14	2.49 ± 0.11	0.46 ± 0.05	2.75 ± 0.27	73.3 ± 10.3	110.0 ± 15.4
Corbicula domain 2	0.26 ± 0.086 (100)	0.67 ± 0.08	0.97 ± 0.13	2.45 ± 0.05	44.7 ± 4.57	67.0 ± 6.86 (100)
His-60 to Gly	3.60 ± 0.97 (1390)	ND	ND	ND	8.93 ± 2.14	13.4 ± 3.21 (20)
His-60 to Arg	1.02 ± 0.19 (390)	ND	ND	ND	18.1 ± 1.61	27.1 ± 2.42 (40)
Asp-197 to Gly	0.52 ± 0.12 (200)	ND	ND	ND	9.53 ± 0.41	14.3 ± 0.61 (21)
Scapharca[a]	1.49 ± 0.09				52.2 ± 1.63	78.3 ± 2.45
Crassostrea[a]	0.38 ± 0.03				48.3 ± 2.71	72.4 ± 4.07
Octopus[a]	1.26 ± 0.19				11.9 ± 1.09	17.8 ± 1.64

Data for *Scapharca*, *Crassostrea* and *Octopus* AKs are shown for comparison. All of the enzymes in this table are recombinant enzymes expressed as fusion protein with maltose binding protein. All parameters were obtained at 25°C under the same conditions. Values in parentheses are the relative value (%) to its wild-type. Averages and standard errors of at least three runs are shown. ND, not determined.
[a]Unpublished data by Suzuki, Takeuchi, Fujimoto, Ryotani and Uda.

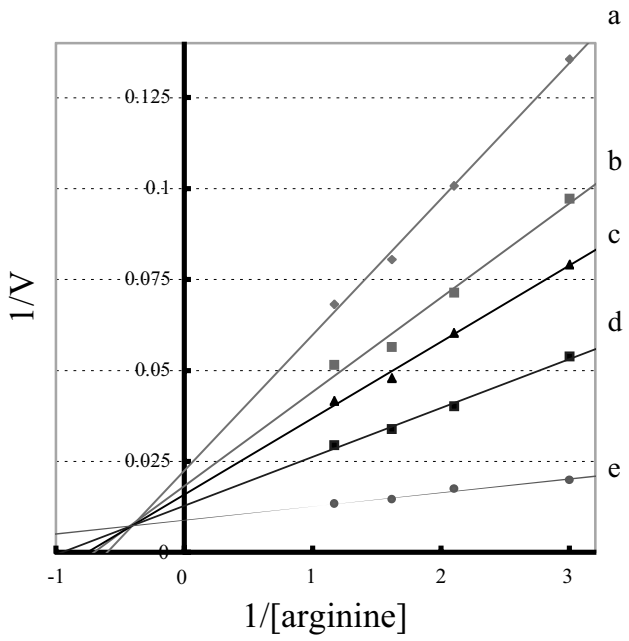

Fig. 1. Lineweaver–Burk plots ($1/v$ vs. 1/[arginine]) of *Corbicula* two-domain AK reaction with various concentrations of ATP (mM): 0.33 (a), 0.47 (b), 0.67 (c), 0.95 (d), and 4.76 (e).

We calculated parameter K_d^{arg}, the dissociation constant of substrate arginine in the absence of substrate ATP, to more clearly demonstrate the functional difference between two-domain AK and domain 2. The slope (p) and an intercept (q) from the Lineweaver–Burk plot with arginine (Fig. 1) were substituted into Eq. 1 to give the following equation for K_d^{arg}:

$$K_d^{arg} = \frac{pV_{max} - K_m^{arg}}{qV_{max} - 1} \quad (2)$$

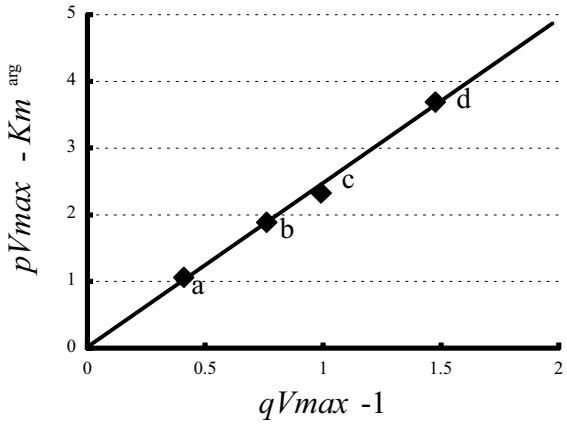

Fig. 2. Graphical method of estimating K_d^{arg}; the slope of the straight line of the plot of $(pV_{max} - K_m^{arg})$ vs. $(qV_{max} - 1)$ is K_d^{arg}. Here p and q are the slope and $1/v$ axis intercept of each graph in Fig. 1a–d.

K_d^{arg} can also be depicted graphically as the slope of the plot of $(pV_{max} - K_m^{arg})$ vs. $(qV_{max} - 1)$ (Fig. 2). In the case of AK reactions where K_m^{arg} and K_m^{ATP} are very close (both usually less than 1.5 mM), this graphical method appears to be more reliable than the direct curve fitting to Eq. 1. Kinetic parameters on ATP binding, K_m^{ATP} and K_d^{ATP}, were also determined for *Corbicula* two-domain AK and its domain 2 using data in Fig. 1 by the graphical method (see Table 1).

The K_d^{arg} values for two-domain AK and domain 2 were determined to be 2.49 ± 0.11 and 0.67 ± 0.08 mM, respectively. For two-domain AK, the affinity for the substrate arginine in the absence of ATP decreased 3.7-fold more than domain 2, as in K_m^{arg}. On the other hand, K_d^{ATP} values for two-domain AK and domain 2 were very similar (2.75 ± 0.27 and 2.45 ± 0.05 mM, respectively) (Table 1).

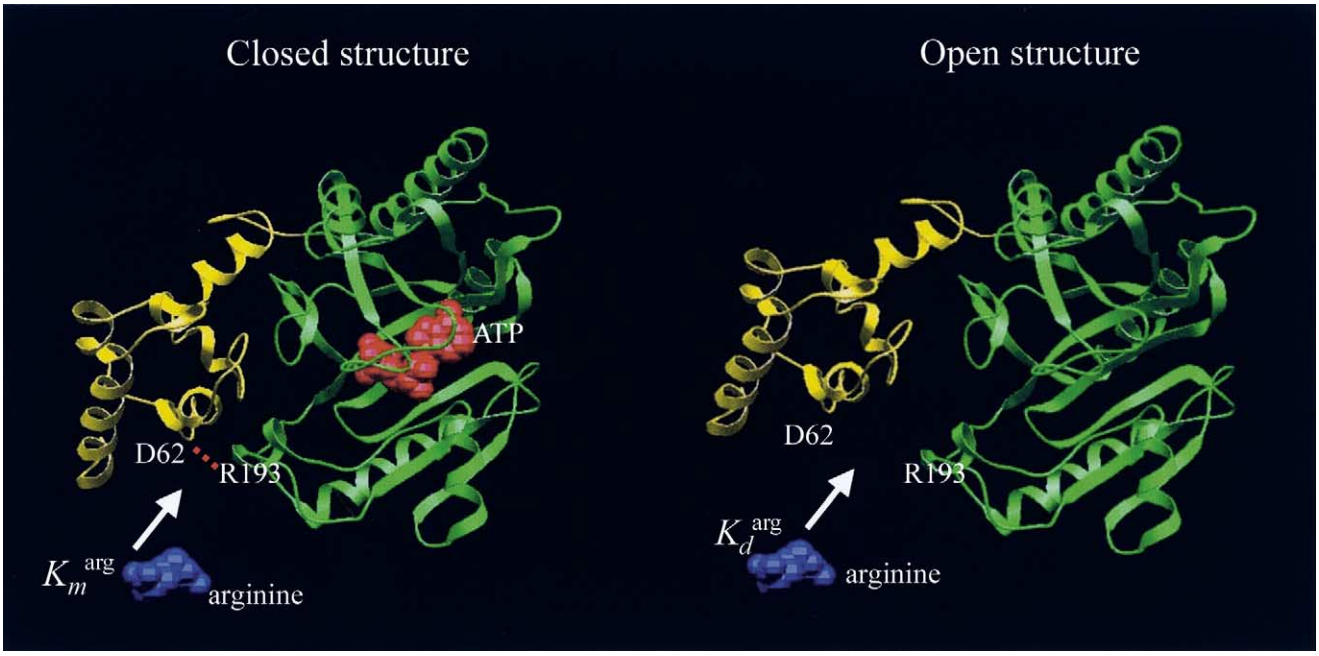

Fig. 3. Schematic presentation of the *open* and *closed* structures of AK and the kinetic parameters K_m^{arg} and K_d^{arg}. In this model, the hydrogen bond between Asp-62 and Arg-193 stabilizes the substrate-bound closed structure. This figure was made by the SwissPdbViewer using *Limulus* AK [26] as a template (PDB Id: 1BG0). The N-terminal small domain (N-domain) is colored yellow, and the C-terminal large domain (C-domain) is green. The substrate arginine and ATP are shown by blue and red balls, respectively.

The comparison of K_d^{arg} and K_m^{arg} (or K_d^{ATP} and K_m^{ATP}) is of great importance. In two-domain AK, K_d^{arg} is six-fold larger than K_m^{arg} indicating that the affinity of the enzyme for arginine is greater in the presence of substrate ATP than in its absence. Presumably this difference is correlated with the large structural differences in the enzyme in the presence or absence of substrate [22–24], namely *open* and *closed* structures (Fig. 3). The ratio of K_d^{arg}/K_m^{arg} (or K_d^{ATP}/K_m^{ATP}) ($\sim$2.5) for domain 2 is lower than that ($\sim$6.0) for two-domain AK, suggesting that the structural change of the separated domain 2 is smaller.

Transition state analog complex structural analysis of *Limulus* AK indicates that it consists of two domains: a N-terminal small α-helical segment consisting of residues 1–111 and a C-terminal segment possessing an eight-stranded antiparallel β-sheet flanked by seven α-helices consisting of residues 112–357 [25]. ATP or ADP is accommodated in the C-segment, while arginine or arginine phosphate mainly makes contact with the N-segment. The catalytic center where the reversible transfer of phosphate occurs is located in the C-segment.

Substantial conformational changes take place upon substrate binding in CK [22]. Recent crystal structures of CK [25] and AK [26] provide very compelling insights into the nature of these structural changes. The structural changes in the N-terminal small segment and flexible loop during catalysis have recently been modeled for CK by Forstner et al. [23] and for AK by Zhou et al. [24]. The enzyme assumes an *open* structure in the absence of substrate and has a *closed* structure when bound to substrate, as schematically shown in Fig. 3.

Using the above model, we propose that in AK, the hydrogen bond between Asp-62 located in the N-terminal small segment and Arg-193 in the C-terminal large segment is the key to stabilizing the substrate-bound, *closed* structure [17,12]. It should be noted that Asp-62 and Arg-193 are *not* associated with substrate binding, but are conserved strictly in all usual 40 kDa AKs. Namely, upon substrate binding, the flexible loop bearing Asp-62 would move nearer to the active center, and the hydrogen bond between Asp-62 and Arg-193 is formed to link the N-terminal and C-terminal segments of AK. The mutants of *Nautilus* and *Stichopus* AKs (Asp-62 to Gly or Arg-193 to Gly) are destabilized in the *closed* state and/or perhaps the unique topology of the catalytic pocket is disrupted, leading to very weak enzyme activity [17,12] (V_{max} less than 7% of the wild-type, unpublished results).

While *Corbicula* two-domain AK lacks both Asp-62 and Arg-193 (replaced by Gly and Asp, respectively [16]) and thus the hydrogen bond, enzyme activity of the recombinant *Corbicula* AK is comparable to those of other molluscan 40 kDa AKs with Asp-62 and Arg-193 (see Table 1). We proposed in a previous paper that in clam two-domain AKs, the hydrogen bond between His-60 located in the N-terminal segment and Asp-197 in the C-terminal segment serves as a substitute for the Asp-62 and Arg-193 bond [16].

We expressed three mutants of *Corbicula* AK domain 2 (His-60 to Gly or Arg, Asp-197 to Gly) in *E. coli*, and determined their K_m^{Arg} and V_{max} values (Table 1). The K_m^{arg} values for the His-60 to Gly, His-60 to Arg, and Asp-197 to Gly mutants were 3.60 ± 0.97, 1.02 ± 0.19, and 0.52 ± 0.12 mM, respectively, and V_{max} were 13.4 ± 3.21, 27.1 ± 2.42, and 14.3 ± 0.61 μmol Pi/min/mg of protein, respectively. When these values were compared with wild-type domain 2, K_m^{arg} was increased 2–14-fold and V_{max} reduced 20–40% compared to wild-type. This means that the affinity of the substrate arginine in mutant enzymes was reduced considerably, accompanied by a decrease in V_{max}. These results of mutant domain 2 enzyme activities suggest that His-60 and Asp-197 affect the substrate (arginine) binding system, and are consistent with the hypothesis that a hydrogen bond is formed between His-60 and Asp-197 in *Corbicula* AK as a substitute for the Asp-62 and Arg-193 bond in normal AKs.

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