CMLS, Cell. Mol. Life Sci. 61 (2004) 110–117
1420-682X/04/010110-08
DOI 10.1007/s00018-003-3384-1

CMLS Cellular and Molecular Life Sciences

Research Article

Unique evolution of Bivalvia arginine kinases

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Received 14 October 2003; accepted 1 November 2003

Abstract. The clams *Pseudocardium*, *Solen*, *Corbicula* and *Ensis* possess a unique form of arginine kinase (AK) with a molecular mass of 80 kDa and an unusual two-domain structure, a result of gene duplication and subsequent fusion. These AKs also lack two functionally important amino acid residues, Asp^{62} and Arg^{193}, which are strictly conserved in other 40-kDa AKs and are assumed to be key residues for stabilizing the substrate-bound structure. However, these AKs show higher enzyme activity. The cDNA-derived amino acid sequences of 40-kDa AKs from the blood clam *Scapharca broughtonii* and the oyster *Crassostrea gigas* were determined. While Asp^{62} and Arg^{193} are conserved in *Scapharca* AK, these two key residues are replaced by Asn and Lys, respectively, in *Crassostrea* AK. The native enzyme from *Crassostrea* and both of the recombinant enzymes show an enzyme activity similar to that of two-domain clam AKs and at least twofold higher than that of other molluskan AKs. Although the replacement of Asp^{62} or Arg^{193} by Gly in normal AK causes a considerable decrease in V_{max} (6–15% of wild-type enzyme) and a two- to threefold increase in K_m for arginine, the same replacement in *Scapharca* AK had no pronounced effect on enzyme activity. Together with the observation that bivalve AKs are phylogenetically distinct from other molluskan AKs, these results suggest that bivalve AKs have undergone a unique molecular evolution; the characteristic stabilizing function of residues 62 and 193 has been lost and, consequently, the enzyme shows higher activity than normal.

Key words. Arginine kinase; kinetic property; two-domain enzyme; *Scapharca*; *Crassostrea.*

Members of the phosphagen kinase enzyme family play a key role in the coordination of energy production and utilization in animals [1–4]. These enzymes catalyze the reversible transfer of high-energy phosphoryl groups of ATP to naturally occurring guanidine compounds such as creatine, glycocyamine, taurocyamine, lombricine and arginine. The phosphorylated high-energy guanidine is referred to as a phosphagen.

$$Mg^{2+}ATP + \text{natural guanidine} \overset{\text{phosphagen kinase}}{\Leftrightarrow} Mg^{2+}ADP + \text{phosphagen}$$

In vertebrates, phosphocreatine is the only phosphagen, and the corresponding phosphagen kinase is creatine kinase (CK). By contrast, invertebrates have phosphocreatine and at least six unique phosphagens: phosphoarginine, phosphoglycocyamine, phosphotaurocyamine, phospholombricine, phosphohypotaurocyamine and phosphoopheline. The kinases corresponding to each of the first four phosphogens have been identified and characterized [1–4]: arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK) and lombricine kinase (LK). The high extent of sequence identity among phosphagen kinases suggests that they have evolved from a common ancestor [5, 6], but the evolutionary processes are not fully understood. At present, that two strains (CK and AK) diverged from their ancestral gene before

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metazoan evolution in dear, and based on phylogenetic analyses, GK, LK and presumably TK radiated from the CK strain [7, 8].

AKs are the most widely distributed among invertebrates and are probably closely related to the ancestral phosphagen kinase [3, 9–11]. Most of the AKs are 40-kDa monomers, while the other phosphagen kinases, CK, GK and LK, are dimeric, or octameric in the case of the mitochondrial CK isoform [12]. Interestingly, AK appears to have evolved at least twice during the evolution of phosphagen kinases: first at an early stage of phosphagen kinase evolution, and second, from CK at a later time in metazoan evolution [8].

An unusual 80-kDa AK was first isolated from the primitive sea anemone *Anthopleura*, and shown to have a two-domain structure (a contiguous dimer), a result of gene duplication and subsequent fusion [11]. In 1996, Soga and Yazawa [13] discovered that the marine clam *Pseudocardium sachalinensis* has an unusual 86-kDa AK in the adductor muscle based on SDS-PAGE analysis. The yield of the arginine kinase was as high as 5% of the soluble proteins in the muscle, and the enzymatic properties were comparable to those of the usual 40-kDa AKs. In addition, *Pseudocardium* does not appear to coexpress a 40-kDa AK. We determined that *Pseudocardium* AK has a two-domain structure based on the 724 residues of cDNA-derived amino acid sequence [14]. 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 [15], and Ellington's group reported the same type of two-domain AK from *Ensis* [16]. Thus, two-domain AKs have been observed in two diverse invertebrate groups, a sea anemone and a mollusk, clearly indicating that gene duplication and subsequent fusion has occurred frequently, and likely independently, during the course of evolution of AKs. The clam two-domain AKs also lack the pair of two functionally important amino acid residues, Asp^{62} and Arg^{193} [8, 17, 18], in both domains of a contiguous dimer, which are strictly conserved in other 40-kDa AKs and are assumed to be key residues for stabilizing the substrate-bound structure by forming a hydrogen bond. However, they display higher enzyme activity. In *Nautilus* and *Stichopus* AKs, the D62G and R193G mutants have markedly reduced AK activity [17, 18].

In this study, we determined the cDNA-derived amino acid sequences of the 40-kDa AKs from the blood clam *Scapharca broughtonii* and the oyster *Crassostrea gigas*. We show that Asp^{62} and Arg^{193} are conserved in *Scapharca* AK, but are replaced by Asn^{62} and Lys^{193} in *Crassostrea* AK. In addition, we show by a site-directed mutagenesis study that the role of Asp^{62} and Arg^{193} is apparently less important in *Scapharca* AK, compared with normal AKs. These results lead us to conclude that bivalve AKs are the result of a unique molecular evolution. Throughout this paper, the sequence numbering of *Limulus* AK [19] is used.

Materials and methods

Isolation of AKs from *C. gigas* and *S. broughtonii*

All procedures were carried out at 4–8 °C. The adductor muscles (130 g) of *Crassostrea* or *Scapharca* were homogenized with 200 ml of 10 mM Tris-acetate buffer (pH 8.1) containing 0.1 mM dithiothreitol (DTT). The tissue extract was fractionated with 50–80% saturated ammonium sulfate. The precipitate was dissolved in a small volume of the same buffer, and applied to a buffer-equilibrated Sephacryl S-200H/R column (3 × 75 cm). The fraction possessing AK activity was pooled and applied to a DEAE-Sepharose CL-6B column (1.5 × 15 cm) equilibrated with 10 mM Tris-acetate buffer (pH 8.1) containing 0.1 mM DTT. The column was washed with the same buffer, and then eluted along a linear gradient of 0–200 mM NaCl. The purified AK was stored on ice until analysis.

SDS-PAGE was carried out in a 15 % acrylamide gel containing 0.087% *N*,*N*'-methylenebisacryl-amide, 0.375 M Tris/HCl (pH 8.8) and 0.1% SDS. The sample was incubated in 0.75% SDS at 100 °C for 5 min in the presence of 2-mercaptoethanol before electrophoresis.

Partial amino acid sequence determination of *Scapharca* AK

The isolated protein was prepared for amino acid sequencing by pyridylethylation and digestion with lysyl endopeptidase at an enzyme/substrate ratio of 1:100 in 0.1 M Tris-HCl (pH 8.0) at 37 °C for 6 h. The digested products were isolated by reverse-phase chromatography on a Cosmosil $5C_{18}$-300 (Nacalai Tesq), 4.6 × 150 mm column equilibrated with 0.1% TFA. Products were eluted on a linear gradient of 0–90% acetonitrile in 0.1% TFA over 90 min at a flow rate of 1 ml/min. The amino acid sequences of peptides were determined by an automated protein sequencer (Applied BioSystems 476A).

Enzyme assay and determination of K_m and K_d for the substrate arginine

Enzyme activity was measured with an NADH-linked assay at 25 °C [20, 21] and determined for the forward reaction (phosphagen systhesis). The reaction mixture (total 1.05 ml) contained 0.65 ml of 100 mM Tris/HCl (pH 8), 0.05 ml of 750 mM KCl, 0.05 ml of 250 mM Mg acetate, 0.05 ml of 25 mM phosphoenolpyrvate made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 5 mM NADH made up in Tris/HCl (pH 8), 0.05 ml of lactate deydrogenase/pyruvate kinase mixture made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of an appropriate concentration of ATP made up in 100 mM imidazole/HCl

(pH 7) and 0.05 ml of an appropriate concentration of arginine made up in 100 mM imidazole/HCl (pH 7).

The initial velocity values for all wild-type recombinant enzymes and the selected three mutants were obtained by varying the concentration of one substrate (arginine) versus five fixed concentrations of the second substrate (ATP), resulting in a 7 × 5 matrix. For other mutants, only the K_ms for arginine were measured under 5 mM ATP. 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 per milligram protein), a Lineweaver-Burk plot was made and fitted by the least-square method using Microsoft Excel. Since the kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [22], the reaction velocity (v) is given by the following equation.

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

Here, K_d^{arg} is the dissociation constant of arginine in the absence of ATP, and is obtained graphically [23] or by fitting data directly to equation 1 according to the method of Cleland [24], using the software written by Dr R. Viola (Enzyme kinetics Programs, ver.2.0).

cDNA sequence determination of AKs from *Crassostrea* and *Scapharca*

mRNA was prepared from the adductor muscle of *Crassostrea* or *Scapharca* with the QuickPrep Micro mRNA Purification Kit (Pharmacia). The single-stranded cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Pharmacia) using the oligo-dT adaptor as a primer. The 3′ half of the cDNA was first amplified for 30 cycles, each consisting of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 2 min at 72 °C for primer extension by polymerase chain reaction (PCR). Extra *Taq* DNA polymerase (Takara) was used as the amplifying enzyme. The primers used were the oligo-dT adaptor and a 512-fold 'universal' redundant oligomer (Phos.con.), 5′GT(ACGT)TGG(AG)T(ACGT)AA(TC)-GA(AG)GA(AG)GA(TC)CA, designed for amplification of phosphagen kinases [6]. The amplified products were cloned into the pCR2.1 vector and sequenced, or sequenced directly with the dye terminator cycle sequencing FS ready reaction kit using a Model 373-18 DNA sequencer (Applied BioSystems). The universal redundant oligomer (Phos.con.) was used as a sequence primer.

The 5′ half of the cDNA was amplified as follows. The poly(G)$^+$ tail was added to the 3′ end of the single-stranded cDNA with a terminal deoxynucleotidyl transferase. Then the 5′ half of the cDNA was amplified, using the oligo-dC (20mer) and the specific primer designed from the 3′-half sequence.

Expression of recombinant enzymes of *Crassostrea* and *Scapharca* AKs

The open reading frames of *Crassostrea* AK (350 amino acid residues) and *Scapharca* AK (348 residues) were cloned into the *Eco*RI/*Pst*I site of pMAL-c2. The maltose-binding protein-AK fusion protein was expressed in *E. coli* TB1 cells 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 SDS-PAGE. The proteins were placed on ice until use, and enzymatic activity was determined within 12 h.

Mutagenesis of *Scapharca* and *Crassostrea* AKs

PCR-based mutagenesis was done as described previously [17, 18]. The mutations (D62G, D62E, D62N, D71G, D71E, R193G, R193K, and a double mutation: D62N/R193K) were introduced into the wild-type template of *Scapharca* AK. 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.

Results and discussion

AKs from the adductor muscle of the oyster *Crassostrea* and the blood clam *Scapharca* were purified to homogeneity using ammonium sulfate fractionation, Sephacryl S-200 gel filtration and DEAE-Sepharose chromatography. SDS-PAGE in the presence of a reducing agent showed that the isolated AKs were purified, and the molecular mass of the subunit was about 40 kDa, and not 80 kDa as observed for the other clams *Pseudocardium*, *Solen*, *Corbicula* and *Ensis* [14–16].

The native enzyme isolated from *Crassostrea* gave a K_m^{arg} value of 0.33 mM for arginine and a V_{max} value of 84.2 μmol Pi/min per milligram protein for the forward reaction (table 1). The enzymatic properties for the native AK isolated from *Scapharca* were not obtained because of its protein instability, presumably derived from the contamination of a protease.

The 3′ and 5′ halves of the cDNAs of *Crassostrea* and *Scapharca* AKs were amplified separately by PCR and the 1228-bp and 1340-bp nucleotide sequences, respectively, were determined. The open reading frame for *Crassostrea* AK is 1053 nucleotides in length and

Table 1. Comparison of kinetic parameters of *Scapharca* AK, its mutant, and *Crassostrea* AK with those of other molluskan AKs (*Corbicula* two-domain, *Nautilus* and *Octopus*) and an echinoderm AK (*Stichopus*).

AK enzymes	K_m^{arg} (mM)	K_d^{arg} (mM)	K_m^{ATP} (mM)	K_d^{ATP} (mM)	V_{max} µmol Pi/min per milligram protein	k_{cat} (s^{-1})
Scapharca WT (recombinant)	1.44 ± 0.28	2.57 ± 0.29	0.65 ± 0.15	1.16 ± 0.25	108.1 ± 11.18	72.1 ± 7.45
D62G	1.33 ± 0.10	ND	ND	ND	56.91 ± 1.78	37.9 ± 1.19
D62E	1.26 ± 0.009	ND	ND	ND	75.47 ± 0.29	50.3 ± 0.19
D62N	2.52 ± 0.09	2.22 ± 0.016	1.32 ± 0.14	1.16 ± 0.084	77.07 ± 1.89	51.4 ± 1.26
D71E	1.49 ± 0.05	ND	ND	ND	80.76 ± 0.99	53.8 ± 0.66
D71G	1.77 ± 0.05	ND	ND	ND	0.52 ± 0.01	0.35 ± 0.007
R193G	2.13 ± 0.021	ND	ND	ND	52.0 ± 0.39	34.7 ± 0.26
R193K	2.44 ± 0.08	6.52 ± 0.39	0.62 ± 0.12	1.66 ± 0.17	57.04 ± 1.43	38.0 ± 0.95
D62N/R193K (double mutation)	1.08 ± 0.006	2.53 ± 0.05	0.73 ± 0.16	1.71 ± 0.056	84.08 ± 1.11	56.1 ± 0.74
Crassostrea						
WT (native)	0.33 (av. of two runs)	ND	ND	ND	84.2 (av. of two runs)	56.1
WT (recombinant)	0.35 ± 0.01	0.82 ± 0.37	0.97 ± 0.25	2.26 ± 0.59	71.3 ± 3.08	47.5 ± 2.05
Corbicula two-domain [23]	0.42 ± 0.14	2.49 ± 0.11	0.46 ± 0.05	2.75 ± 0.27	110.0 ± 15.4	73.3 ± 10.3
Nautilus WT (recombinant) [17; unpublished results]	0.670 ± 0.113	2.26 ± 0.0687	1.40 ± 0.108	4.72 ± 0.364	2.57 ± 0.171	1.71 ± 0.11
D62G	1.135 ± 0.330	ND	ND	ND	0.162 ± 0.025	0.108 ± 0.017
R193G	2.214 ± 0.463	ND	ND	ND	0.384 ± 0.027	0.256 ± 0.018
Octopus WT (recombinant) [unpulished results]	0.948 ± 0.033	3.776 ± 0.049	0.745 ± 0.121	2.58 ± 0.39	44.12 ± 1.08	29.4 ± 0.72
D62G	3.955 ± 0.258	ND	ND	ND	5.46 ± 0.066	3.64 ± 0.044
R193G	5.344 ± 0.168	ND	ND	ND	2.92 ± 0.191	1.95 ± 0.13
Stichopus WT (recombinant) [18; unpublished results]	0.413 ± 0.0593	1.249 ± 0.231	0.814 ± 0.032	2.46 ± 0.097	38.5 ± 1.894	25.7 ± 1.26
D62G	0.965 ± 0.125	ND	ND	ND	1.37 ± 0.03	0.91 ± 0.02
R193G	1.460 ± 0.463	ND	ND	ND	0.665 ± 0.132	0.44 ± 0.09

All of the recombinant enzymes in this table were expressed as a fusion protein with maltose-binding protein. All parameters were obtained at 25 °C under the same conditions. Averages and SEs of at least three runs are shown. ND, not dertermined.

encodes a protein of 350 amino acids, while the reading frame for *Scapharca* is 1047 nucleotides and encodes 348 amino acids. The reliability of the cDNA-derived amino acid sequence of *Scapharca* AK was supported by chemical sequencing of the internal lysyl endopeptidase peptide (28 amino acid residues). The nucleotide and amino acid sequences have been submitted to DDBJ (accession number AB118649 for *Scapharca* AK; accession number AB118650 for *Crassostrea* AK).

All of the recombinant enzymes 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}, V_{max} and k_{cat} of the recombinant wild-type enzymes and the selected three mutants obtained for the forward reaction (phosphagen formation) are listed in table 1.

All bivalve AKs sequenced so far from the clams *Pseudocardium*, *Solen*, *Corbicula* and *Ensis* are unique in that they consist of a contiguous dimer with a molecular mass of 80 kDa. They lack the pair of two functionally important amino acid residues, Asp^{62} and Arg^{193}, in both domains of a contiguous dimer, which are conserved in all other AKs from Cnidaria, Mollusca, Arthropoda and Echinodermata, and are assumed to be key residues for stabilizing the substrate-bound structure by forming a hydrogen bond [8, 17, 18]. A preliminary site-directed

mutagenesis study using *Nautilus* and *Stichopus* AKs indicated that the mutants of D62G or R193G have markedly reduced AK activity [17, 18]. However, in *Solen*, *Corbicula*, *Pseudocardium* and *Ensis* AKs, the Asp^{62} is replaced by Gly, except in *Pseudocardium* domain 2, and Arg^{193} is replaced by Ala, Ser or Asp, thus eliminating the hydrogen bond. Despite this unusual feature, the recombinant enzymes from *Corbicula* [23] and *Ensis* [16] have a higher activity (V_{max}) than those of molluskan 40-kDa AKs from *Nautilus* and *Octopus* and an echinoderm AK from *Stichopus* (table 1). Furthermore, Compaan and Ellington [16] suggest that the first domain of the *Ensis* contiguous dimer might be inactive.

The clams *Pseudocardium, Solen, Corbicula* and *Ensis* belong to the order Veneroida among the bivalves. To determine whether these unusual features are common in all clam AKs, we determined the cDNA-derived amino acid sequences of AKs from the oyster *C. gigas* (order Ostreoida) and the blood clam *S. broughtonii* (order Arcoida). The sequences show that *Scapharca* AK retains the two functional key residues, Asp^{62} and Arg^{193}, while these residues are replaced by Asn^{62} and Lys^{193}, respectively, in *Crassostrea* AK. A hydrogen bond between Asn^{62} and Lys^{193} like that of Asp^{62} and Arg^{193} in *Limulus* AK [25] appears possible. The crystal structure of the transition state analog complex of *Limulus* AK shows that four amino acid residues (Ser^{63}, Gly^{64}, Val^{65} and Tyr^{68}) form hydrogen bonds with the amino and carboxylate groups of the substrate arginine [25]. These residues are well conserved in *Crassostrea* AK, but not in *Scapharca* AK: in an unusual replacement, Gly^{64} and Val^{65} are replaced by His and Cys, respectively, and this might be reflected in a lower affinity for the substrate arginine (K_m^{arg} = 1.44 mM) compared to other AKs (see table 1).

K_m^{arg} and V_{max} values for recombinant wild-type AK enzymes of *Scapharca* (1.44 ± 0.28 mM and 108.1 ± 11.18 µmol Pi/min per milligram protein, respectively) and *Crassostrea* (0.35 ± 0.01 and 71.3 ± 3.08, respectively; table 1) are comparable to kinetic values for two-domain recombinant AK enzymes (K_m^{arg}, 0.42 ± 0.14; V_{max}, 110.0 ± 15.4) [23] of the clam *Corbicula* (table 1), which lacks the functionally important Asp^{62} and Arg^{193}. Thus, we conclude that all bivalve AKs examined so far share a common enzymatic property characterized by an at least twofold higher V_{max} (70–110 µmol Pi/min per milligram of protein) than that (3–45) of other molluskan (*Nautilus* and *Octopus*) or *Stichopus* AKs (see table 1), regardless of the presence or absence of the two key residues, Asp^{62} and Arg^{193}.

For *Scapharca* AK, the K_d^{arg} (2.57 mM), or apparent dissociation constant for arginine in the absence of another substrate ATP, was 1.8-fold greater than the K_m^{arg} (1.44 mM) (table 1), indicating a synergism. This is common for most wild-type AKs, such as those from *Corbicula* [23], *Nautilus*, *Stichopus* [unpublished results], *Octopus* [unpublished data] and *Limulus* [16]. However, the magnitude of the ratio K_d^{arg}/K_m^{arg} (1.8) in *Scapharca* AK appears to be smaller than those (3.4–5.9) of molluskan AKs from *Corbicula, Octopus* and *Nautilus* (see table 1). In *Crassostrea* AK, the K_d^{arg}/K_m^{arg} ratio (2.3) is closer to that of *Scapharca* AK.

Based on these results, we assume that the role of Asp^{62} and Arg^{193} has become less important during the evolution of bivalve AKs compared to other AKs. To evaluate this hypothesis, we replaced Asp^{62} and Arg^{193} with Gly in *Scapharca* AK. In the cases of *Nautilus*, *Octopus* and *Stichopus* AKs (normal AKs), D62G or R193G mutations caused a remarkable decrease in V_{max} (4–15% of the wild type), accompanied by a two- to threefold increase in K_m^{arg} (table 1). However, the same mutations in *Scapharca* AK produced no pronounced effect on the kinetic properties: K_m^{arg} is comparable to the wild type and the V_{max} was kept at 50% of wild type (see table 1). Thus, in wild-type recombinant *Scapharca* AK, the role of residues Asp^{62} and Arg^{193} seems to be less important, similar to clam two-domain AK from *Corbicula* [23].

The pattern described above was further reinforced by the following *Scapharca* AK mutants: D62E, D62N (converting residue 62 to *Crassostrea* type), R193K (converting residue 193 to *Crassostrea* type) or a double mutation of D62N/R193K (table 1). All four of the mutants showed the following similar kinetic properties as the wild type: K_m^{arg} (1.08–2.52 mM), K_d^{arg} (2.22–6.52 mM) and V_{max} (53–78 µmol Pi/min per milligram protein) (table 1). Thus, we conclude that replacement of residues 62, 193, or both does not significantly affect the kinetic properties of *Scapharca* AK.

Asp^{71} is conserved not only in all AKs (see fig. 1), but also other phosphagen kinases, CK, GK and LK. We introduced the Asp^{71} to Glu or Gly mutation in *Scapharca* AK. The mutant D71E produced no significant effect on the kinetic properties, but the D71G mutant caused a remarkable decrease in V_{max} (4.8% of the wild type) while K_m^{arg} remained comparable to the wild type (table 1). Thus, a negatively charged group is apparently required at position 71. The residue is located near the end of the N-terminal flexible loop in crystal structures of AK and CK [25–27] and the side chain forms hydrogen bonds with Thr^{44}, Glu^{73} and Ser^{74} in the substrate-unbound structure in *Limulus* AK (accession number: 1M80), but with Thr^{44} and Ser^{74} in the transition state analog complex (accession number: 1M15).

The amino acid sequences of AKs of *Crassostrea* and *Scapharca* were aligned with those of the clam *Corbicula* (two-domain AK), cephalopods *Nautilus* and *Octopus* and the arthropods *Limulus* and *Drosophila* (AK and AK-related sequence designated as AK2) in figure 1 in a ClustalW alignment. The sequences of *Crassostrea* and *Scapharca* AKs show 50–60% identity with other molluskan and *Limulus* AKs.

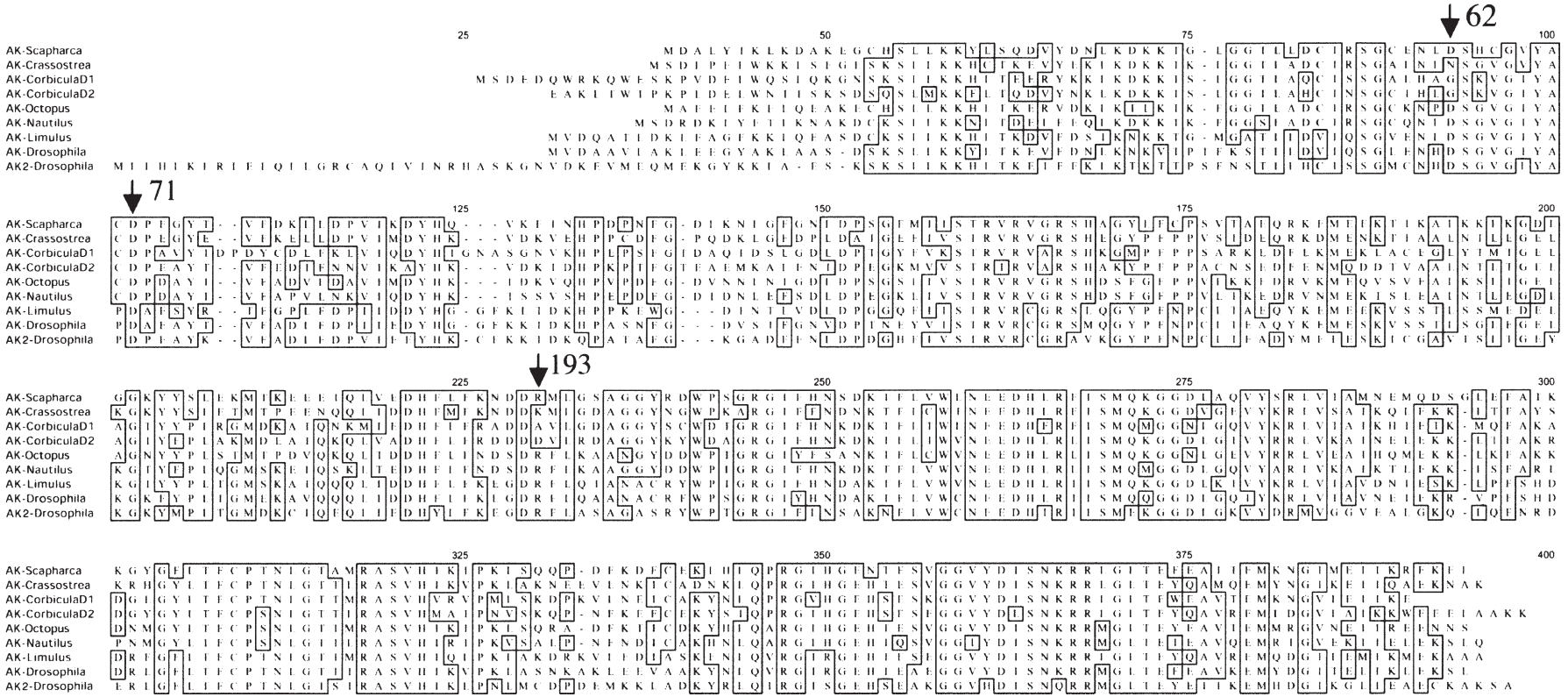

Figure 1. Alignment of AK amino acid sequences of *Crassostrea* and *Scapharca* with those of *Corbicula* (two-domain AK), *Nautilus* and *Octopus*, *Limulus* and *Drosophila* (AK sequence, and an AK-related sequence designated AK2-*Drosophila*). Residues 62, 71 and 193 (mutated positions) are indicated by an arrow. The alignment was made with ClustalW (http://www.ddbj.nig.ac.jp/Welcome-j.html). D1, domain 1 of two-domain AK; D2, domain 2. The residues conserved in at least five sequences are boxed.

We constructed a phylogenetic tree of all known AK and AK-related amino acid sequences from the phyla Cnidaria, Arthropoda and Mollusca by the neighbor-joining method [28] (fig. 2). The aim of the tree construction is to make clear the phylogenetic position of bivalvia AKs in molluskan enzymes.

The molluskan AK sequences, used in figure 2, represent four major classes Polyplacophora, Gastropoda, Cephalopoda and Bivalvia, and the topology of the molluskan AK cluster in figure 2 clearly shows that the Bivalvia AK group diverged first, followed by the radiation of the Polyplacophora, Gastropoda and Cephalopoda AKs. A similar tree topology for molluskan AKs was also obtained with the maximum-likelihood method [29].

Here it should be noted that the sequences for bivalve AKs are placed in a unique position. As mentioned above, the importance of Asp^{62} and Arg^{193} has diminished in bivalve AKs, and in turn, they have acquired a higher enzyme activity (V_{max} at least two to threefold larger than other molluskan enzymes) without significant alternation of K_m^{arg}. Finally, these results suggest that bivalve AKs have undergone a unique molecular evolution: gene duplication and subsequent fusion yielding a two-domain enzyme in some clams, loss of the functional role of Asp^{62} and Arg^{193} leading to amino acid replacement of the two conserved residues (Asp^{62} to Gly or Asn, and Arg^{193} to Ala, Ser, Asp or Lys), and acquisition of a higher enzyme activity.

In vertebrate CK systems, four isoforms of CK polypeptides are known: two cytoplasmic forms and two mitochondrial forms [4, 5]. Mitochondrial CK isoforms, targeted to the mitochondrial intermembrane space, play a key role in the energy transfer system from mitochondria to the cytoplasmic matrix [12]. Therefore, an analog to vertebrate mitochondrial CK is likely also present in AK-possessing invertebrate organisms. There have been extensive studies to isolate a mitochondrial AK isoform at the protein or gene level [30–34]. However, although AK activity is evident in some invertebrate mitochondria, no mitochondrial AK sequence has been determined. The phylogenetic tree in figure 2, constructed from all the 40-kDa AK (and AK-related) sequences, does not suggest clearly the presence of isoforms of AK. However, the AK2 sequence from *Drosophila melanogaster* might be a

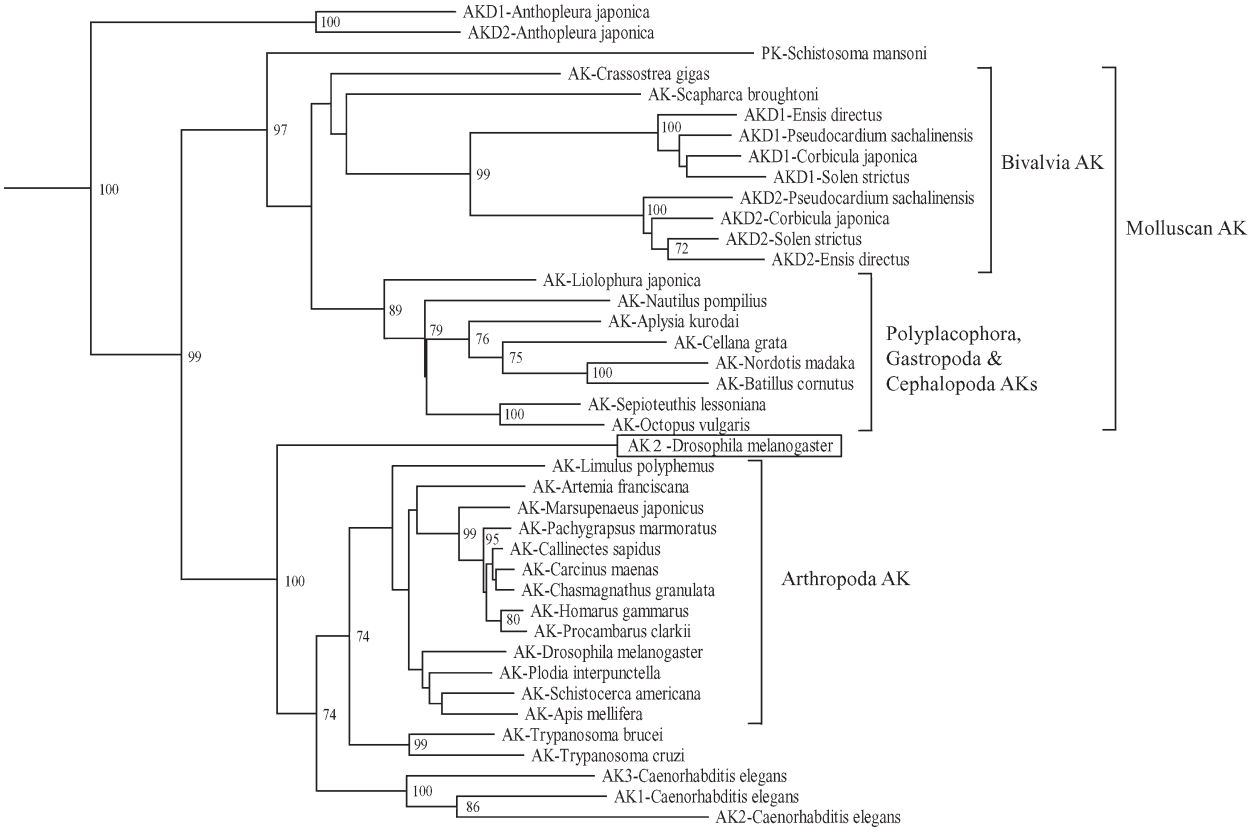

Figure 2. Phylogenetic tree of amino acid sequences of AKs. This tree was made with the neighbor-joining (NJ) method [28]. We made an NJ tree including all 40 sequences of AKs so far known and selected 65 sequences of CKs, GKs, LKs and a *Stichopus* AK. The latter 65 sequences were used as an out group. The portion of the AK cluster was extracted from a complete tree, and is shown in the figure. D1, domain 1 of two-domain AK; D2, domain 2. Bootstrap values over 70% are shown at the branching point. The *Drosophila* AK2 sequence (accession number: AE003553-38) is boxed.

candidate for a mitochondrial AK isoform. It appears to have a putative signal peptide (see the long N-terminal extension of the sequence shown in fig. 1.) In addition, the *Drosophila* AK2 sequence is placed outside the other arthropod, *Trypanosoma* and *Caenorhabditis* AK sequences (see fig. 2).

Acknowledgements. We thank S. Susuki and Y. Ikuta for experiments at an early stage of this work.

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