Two-domain arginine kinase from the deep-sea clam *Calyptogena kaikoi* - Evidence for two active domains.

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Abbreviations used: AK, arginine kinase; CK, creatine kinase; 2D[WT], two-domain AK (wild-type); D1[WT], domain 1 of the two-domain AK (wild-type); D2[WT], domain 2 of the two-domain AK (wild-type); 2D[Y68A in D1], two-domain AK with Y68A mutation in domain 1; 2D[Y68A in D2], two-domain AK with Y68A mutation in domain 2; 2D[Y68A in D1 & D2], two-domain AK with Y68A mutation in domain 1 and 2; D2[Y68A], domain 2 with Y68A mutation.

Abstract

The cDNA and deduced amino acid sequences for arginine kinase (AK) from the deep-sea clam *Calyptogena kaikoi* have been determined revealing an unusual two-domain (2D) structure with molecular mass of 80 kDa, twice that of normal AK. The amino acid sequences of both domains contain most of the residues thought to be required for substrate binding found in the horseshoe crab *Limulus* AK, a well studied system for which several X-ray crystal structures exist. However, two highly conserved residues, D62 and R193, that form a salt bridge thereby stabilizing the substrate bound structure have been replaced by G and N in domain 1, and G and P in domain 2, respectively. The present effort probes whether both domains of *Calyptogena* AK are catalytically competent. Recombinant constructs of the wild-type enzyme, of both single domains, and of selected mutants of the *Calyptogena* AK have been expressed as fusion proteins with the maltose-binding protein. The wild-type two-domain enzyme (2D[WT]) had high AK activity (k_{cat} = 23 s^{-1}, average value of the two domains), and the single domain 2 (D2[WT]) showed 1.5-times higher activity (k_{cat} = 38 s^{-1}) than the wild-type 2D[WT]. Interestingly, the single domain 1 (D1[WT]) showed only a very low activity (k_{cat} ~ 0.016 s^{-1}). Introduction of a Y68A mutation in both domains virtually abolished catalytic activity. On the other hand, significant residual activity was observed (k_{cat} = 2.8 s^{-1}), when the Y68A mutation was introduced into domain 2 of the two-domain enzyme. A similar mutation in domain 1 of the two-domain enzyme reduced activity to a much lower extent (k_{cat} = 11.1 s^{-1}) . Although the domains of this “contiguous” dimeric AK each have catalytic capabilities, the presence of domain 2 strongly influences the stability and activity of domain 1.

Introduction

Arginine kinase (AK) is one of the phosphagen kinases (guanidino kinases) which catalyzes the reversible transfer of the phosphoryl group of ATP to the naturally occurring guanidino compound, arginine. The phosphorylated high-energy guanidine is referred to as a phosphagen. Members of this enzyme family play a key role in animals as ATP buffering systems in cells that display high and variable rates of ATP turnover [Kenyon and Reed, 1983; Wyss et al., 1992; Ellington, 2001]. AK is most widely distributed among organisms; its activity has been observed in arthropods, molluscs, nematoda, cnidarians, poriferae (the most ancient multi-cellular organisms), protozoans (ciliates, *Trypanosoma* and choanoflagellates) and bacteria [Watts and Bannister, 1970; Noguchi et al., 2001; Pereira et al., 2000; Uda et al., 2006; Conejo et al., 2008; Andrews et al., 2008], indicating an ancient origin of AK. Most AKs are monomers with a relative molecular mass of approximately 40 kDa [Morrison, 1973].

From the standpoint of evolutionary history, phosphagen kinases can be divided into two superclusters. The first cluster (CK: creatine kinase cluster) A CK (creatine kinase) cluster including CK, glycocyamine kinase, taurocyamine kinase and lombricine kinase. In addition, deuterostome AKs and a unique AK from the polychaete *Sabellastarte* are part of this supercluster and appear to have evolved from an ancestral mitochondrial CK-like gene [Ellington and Suzuki, 2006; Tanaka et al., 2007]. The second super cluster consists of typical AKs distributed widely in animals and protozoans, and of a hypotaurocyamine kinase of sipunculids [Uda et al., 2006; Uda et al., 2005]. In contrast to the relatively conserved exon/intron organization in the genes of the CK cluster [Suzuki et al., 2004; Tanaka et al., 2007], those of the AK cluster seem to be highly divergent, suggesting that a frequent loss or gain of introns has occurred

during the course of AK evolution [Uda et al., 2006]. It should be noted that during the course of AK evolution, unusual 80 kDa AKs evolved independently four times - in protozoa, cnidarians, plathyhelminths and heterodont clams. These enzymes have a two-domain structure, and are believed to result from gene duplication and subsequent fusion. In effect, these AKs are “contiguous” dimers.

An 80 kDa AK enzyme was first isolated from the primitive sea anemone *Anthopleura japonicus*, and the cDNA-derived amino acid sequence clearly showed that it has a two-domain structure (“contiguous” dimer) [Suzuki et al., 1997]. Subsequently, the same type of two-domain AKs was isolated in the heterodont clams *Pseudocardium* [Suzuki et al., 1998], *Corbicula* and *Solen* [Suzuki et al., 2002] as well as *Ensis* [Compaan and Ellington, 2003].

With respect to the enzymatic properties of the clam two-domain AKs, there is some controversy as to whether both domains have catalytic ability or not. From the kinetic measurements of recombinant *Corbicula* two-domain AK and its separated domain 2, Suzuki et al. (2003) assumed that both domains have a distinct AK activity. This was supported by the observation that most of the residues responsible for substrate binding have been conserved in both domains. However, the failure to obtain a soluble domain 1 enzyme made this proposal uncertain. Compaan and Ellington (2003) expressed NusA-His tagged domain 1 and 2 of the two-domain *Ensis* AK, refolded its domain 1, and showed that the domain 1 has no AK activity. Thus, they concluded that only the domain 2 functions as AK. However, in the *Ensis* enzyme, too, most of the substrate-binding residues have been conserved in both domains, like in *Corbicula* AK.

In the present study, we have determined the cDNA sequence of the two-domain AK from the deep-sea heterodont clam *Calyptogena kaikoi*, and cloned it

into the plasmid pMAL. In order to clarify the catalytic ability of the two domains, we prepared the two-domain enzyme (wild-type), its separated domains 1 and 2, and mutated enzymes (Y68A) in either one or both domains 1 or 2, and determined their kinetic parameters. Our results suggest that both domains of *Calyptogena* AK are catalytically competent, although domain 2 strongly influences catalysis in domain 1.

Materials and Methods

cDNA amplification, sequence determination and cloning of Calyptogena kaikoi AK

The single stranded cDNA, which had been synthesized in our previous work (the mRNA origin: adductor muscle of *C. kaikoi*) [Suzuki et al., 2000b], was used as a template for PCR. The 3'-half of the cDNA of *C. kaikoi* AK was amplified using the oligo-dT primer and a 256-fold universal primer (5'-GTNTGGGTNAAYGARGARGAYCA) designed from the highly conserved sequences of phosphagen kinases [Suzuki and Furukohri, 1994]. Ex *Taq* DNA polymerase (Takara, Kyoto, Japan) was used as amplifying enzyme. PCR amplification was performed for 30 cycles, each consisting of 30 sec at 94^{o}C for denaturation, 30 sec at 60^{o}C for annealing and 2 min at 72^{o}C for primer extension. The amplified products (~900 bp) were purified by agarose gel electrophoresis and subcloned into the pGEM-T Easy Vector (Promega, WI, USA). Nucleotide sequence was determined with an ABI PRISM 3100-Avant DNA sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

A poly $(G)^{+}$ tail was added to the 3' end of the single-stranded cDNA with terminal deoxynucleotidyl transferase (Promega, WI, USA). The 5'-half of the *C. kaikoi* AK cDNA was then amplified using the oligo-dC primer (5'-GAATTC$_{18}$-3') and a

specific primer (5'-TGTACACCTCGCCAAGGTC-3' or 5'-ACAGCTTCCCATTCAGTGAGACC-3') designed from the sequence of the 3' region. The amplified products (~1800 bp) were subcloned and sequenced.

The open reading frames of the *C. kaikoi* two-domain AK (named *Calyptogena* 2D[WT]) and its single domains 1 and 2 (named *Calyptogena* D1[WT] and D2[WT], respectively) were amplified using specific primers, and cloned into the *Bam* HI/*Sal* I site of pMAL-c2 (New England Biolabs, MA, USA).

Site-directed mutagenesis of Calyptogena kaikoi and Corbicula japonica AKs

The pMAL-c2 plasmids with inserts of *Calyptogena* 2D[WT], D1[WT], D2[WT], and of the bivalve *Corbicula japonica* two-domain AK and its separated domains (*Corbicula* 2D[WT], D1[WT] and D2[WT]) which had been constructed previously [Suzuki et al., 2003], were used as templates of the mutagenesis. The *Corbicula* two-domain AK construct was used for comparative purposes.

Tyrosine 68 in *Limulus* AK plays a major role in arginine binding in the catalytic pocket (Zhou et al., 1998). To probe aspects of catalysis in the “contiguous” dimers, the Y68 equivalent residues in both *Calyptogena* and *Corbicula* AKs were mutated to alanine. PCR based mutagenesis was performed as described previously [Suzuki et al., 2000a]. The mutations (2D[Y68A in D1], 2D[Y68A in D2], 2D[Y68A in D1 & D2]) and D2[Y68A] were introduced into the templates using the following primers: for *Calyptogena* Y68A in domain 1, TGCCTGCGACCCGGAAGTTTACACGGAC and GCAATACCCACCTTGCTCCCTTCGTGAAG; for *Calyptogena* Y68A in domain 2, TGCTTGCGATCCTGAAGCATACACGGTG and

GCGATTCCCACTTTACTACCGAGATGG; for *Corbicula* Y68A in domain 1, TGCCTGTGACCCCGCTGTGTACACTGAC and GCAATACCAACCTTGCTGCCCGCGTGC; for *Corbicula* Y68A in domain 2, TGCCTGTGATCCCGAAGCTTACACAGTA and GCTATGCCTACTTTGCTGCCTAGATGAATAC; mutated positions underlined). *KOD*$^{+}$ DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products (8000 bp or 9000 bp) were purified by agarose gel electrophoresis. After blunting and kination, the DNA was self-ligated. The cDNA insert was completely sequenced to confirm that only the intended mutations were introduced.

Protein expression

The recombinant enzymes were expressed as MBP-enzyme fusion proteins in *E. coli* TB-1 cells by induction with 0.1 mM IPTG at 18°C for 36 hr. The cells were resuspended in 2 ml of MBP column buffer (50 mM Tris, 0.2 M NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol), sonicated, and the soluble protein was extracted. The MBP-tagged enzymes were purified by affinity chromatography using amylose resin (New England Biolabs, MA, USA). The purity of the recombinant enzyme was verified by SDS-PAGE.

Enzyme assays

Enzyme activity was measured with an NADH-linked assay at 25 °C and determined for the forward reaction (phosphagen synthesis) [Morrison, 1973; Ellington, 1989]. The reaction mixture (total 1.0 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

phosphoenolpyruvate 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 pyruvate kinase/lactate dehydrogenase mixture made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 100 mM ATP made up in 100 mM imidazole/HCl (pH 7) and 0.05 ml of recombinant enzyme. The reaction was started by adding 0.05 ml of an appropriate concentration of arginine made up in 100 mM Tris–HCl (pH 8). The initial velocity values were obtained by varying the concentration of arginine, to estimate the K_m for arginine (K_m^{arg}) and k_{cat} in the presence of 4.76 mM ATP. The kinetic parameters were determined by our previous method [Fujimoto et al., 2005]. Protein concentration was estimated from the absorbance at 280 nm. The extinction coefficient at 280 nm in $M^{-1}cm^{-1}$ (or mg/ml) was obtained using ProtParam (available from the URL http:/ca.expasy.org/tools/protparam.html). The protein concentration for the AK enzyme moiety was obtained by excluding the portion of the MBP tag.

Phylogenetic analysis

Forty-five amino acid sequences of AKs and a *Homo* muscle type CK (outgroup) were aligned using the ClustalW program available on the DDBJ homepage (http://www.ddbj.nig.ac.jp/). The default setting was used for the alignment except that the PAM model was used to construct the distance matrix. Phylogenetic trees were generated using two different approaches - the maximum likelihood method (ML) in the Phylip package v3.65 [Felsenstein, 1989] and the neighbor joining (NJ) method on the DDBJ homepage (http://www.ddbj.nig.ac.jp/).

Results

The full-length *Calyptogena kaikoi* AK cDNA was successfully amplified by RT-PCR. The cDNA comprises 2681 bp with 62 bp of 5' untranslated region (UTR), 2181 bp of ORF coding for a 726-amino acid residue protein, and 438 bp of 3' UTR. The translated protein has a calculated molecular mass of 81,999 Da and an estimated pI of 6.74. The sequence is available through the DDBJ database (Accession No. AB186413). The cDNA-derived amino acid sequence of *Calyptogena kaikoi* AK displayed a typical two-domain structure, domain 1 consisting of 365 amino acids with a calculated mass of 41,119 Da, and domain 2 consisiting of 361 amino acids with a mass of 40,848 Da (Fig. 1). Figure 1 shows that the *Calyptogena* AK displays considerable sequence identity to molluscan AKs as well as to the AK from the horseshoe crab *Limulus*.

Figure 2 is a schematic representation of the recombinant enzyme constructs (*Calyptogena* and *Corbicula*) investigated in this study. In addition to the wild-type enzymes (2D[WT], D1[WT] and D2[WT]), Y68A mutated enzymes were prepared. All the recombinant enzymes, except for *Corbicula* AK domain 1, were successfully obtained as a soluble protein fused to maltose binding protein (MBP), and purified to near homogeneity by affinity chromatography. *Corbicula* domain 1 was not obtained as a soluble enzyme even when expression conditions were varied [Suzuki et al., 2003].

The kinetic parameters for the recombinant wild-type and mutated enzymes were obtained for the forward reaction and are listed in Table 1. The K_m^{arg} values for *Calyptogena* 2D[WT], D2[WT], 2D[Y68A in D1] and 2D[Y68A in D2] were determined to be 0.72 -1.0 mM, which are comparable to those of typical AKs. The K_m^{arg} values for D1[WT], D2[Y68A] and 2D[Y68A in D1 & D2] were not determined due to their very low activities. The k_{cat} value for D2[WT] (37.7 s^{-1}) was much higher

than the corresponding value for 2D[WT] (22.9 s^{-1}). When the Y68A mutation was introduced in domain 1 (2D[Y68A in D1]) or domain 2 (2D[Y68A in D2]), the k_{cat} values were decreased to 11.1 s^{-1} (~50 % of 2D[WT]) or 2.8 s^{-1} (~12% of 2D[WT]), respectively, indicating that these mutants still retained significant AK activities.

The K_m^{arg} values for *Corbicula* 2D[WT], D2[WT], 2D[Y68A in D1] and 2D[Y68A in D2] were determined to be 0.17 - 0.38 mM, reflecting a higher binding capacity for arginine than *Calyptogena* AK (Table 1). The binding constants for D2[Y68A] and 2D[Y68A in D1 & D2] (K_m^{arg} = 2.1 - 3.6 mM) were 10-times lower than those of the wild-type enzymes, indicating that Y68 is indeed involved in the binding of the substrate arginine. Unlike *Calyptogena* AK, the k_{cat} values for D2[WT] and 2D[WT] were almost identical (87 s^{-1}). When the Y68A mutation was introduced in domain 1 (2D[Y68A in D1]) or domain 2 (2D[Y68A in D2]), the k_{cat} values decreased to 42 or 27 s^{-1} (less than 50 % that of the 2D[WT]), respectively, but these mutants still maintained significant AK activities, like *Calyptogena*. Interestingly, the catalytic efficiency (k_{cat}/K_m^{arg}) for the single domain 2 (D2[WT]) was two-times higher than that for the two-domain enzyme (2D[WT]), in both *Calyptogena* and *Corbicula* AKs (Table 1).

The above kinetic results with respect to the *Calyptogena* AK can be summarized as follows: (a) when expressed alone, domain 1 displayed minimal activity, (2) when expressed alone, domain 2 had significantly higher activity and catalytic efficiency that the two-domain wild-type AK, (c) when domain 1 was inactivated using the Y68A mutation, activity was ~50% of the wild-type enzyme and (d) when domain 2 was inactivated using the Y68A mutation, activity was retained at ~12% of the wild-type level. The kinetic behavior of the *Corbicula* constructs showed similarities to the *Calyptogena* AK, particularly in the retention of significant activity in the 2D[Y68A

in D2] mutant as well as in the enhanced catalytic efficiency in the domain 2 when expressed alone.

Discussion

cDNA sequence determination of Calyptogena kaikoi AK and characteristics of its amino acid sequence

Calyptogena kaikoi is a deep-sea clam living in the cold-seep area at a depth of 3761 m of Nankai Trough, Japan. The AK cDNA amplified in this work originates from the adductor muscle of *Calyptogena kaikoi* [Suzuki et al., 2000b]. So far, the cDNA sequences of bivalve AKs are known for pteriomorphian (*Scapharca*, *Crassostrea*, *Pecten* and *Argopecten*) and heterodont (*Pseudocardium*, *Corbicula*, *Solen* and *Ensis*). The pteriomorphian enzymes are of the typical 40 kDa AK, but they have some unique features in amino acid sequence [Takeuchi et al., 2004]. In contrast, all the known heterodont AKs have an unusual two-domain structure. The homologous exon/intron organization of each domain of the *Pseudocardium* AK gene and the presence of "bridge-intron" indicate that the two-domain AK resulted from a gene duplication and a subsequent fusion event [Suzuki and Yamamoto, 2000c]. In this study, it was shown that *Calyptogena kaikoi*, which belongs to the heterodonts but has a habitat is quite different from other heterodonts, also has a two-domain AK consisting of 726 amino acid residues (domain 1, 365 residues; domain 2, 361 residues).

We have aligned the amino acid sequences of the two domains of *Calyptogena kaikoi* AK in Figure 1 with those of other heterodont two-domain AKs and the *Limulus* AK. The sequence of *Calyptogena* domain 1 showed 59% amino acid identity with that of *Calyptogena* domain 2. However, each domain shows a much higher degree of identity (over 80%) with the corresponding domain of other heterodont AKs. The

crystal structure of the substrate-bound, closed state of *Limulus* AK revealed a variety of residues interacting with the substrates, arginine and ADP, as well as the key residues involved in stabilization towards large conformational changes upon substrate binding [Zhou et al., 1998; Yousef et al., 2003]. Comparison of the amino acid sequence of *Calyptogena* AK with that of *Limulus* AK indicated that most of the residues interacting with the substrates are also conserved in both domains of *Calyptogena* AK (residues marked by asterisks in Fig. 1). However, D62 and R193 in the *Limulus* sequence, which are conserved in most AK sequences [Suzuki et al., 2000a] and are supposed to play a key role in stabilizing the substrate-bound structure by forming an ion pair, have been replaced by G and N in domain 1 and G and P in domain 2, respectively, in *Calyptogena* AK (Fig. 1). This is a notable feature common to all heterodont two-domain AKs- the residue at position 62 (typically D) being occupied by G or D in domain 1, and the residue at position 193 (typically R) by N, A, S, P or D as shown in Fig. 1.

In a previous communication, we reported that residues 62 and 193 play the key role in regulating the synergism of substrate binding, and that the replacement of residue 193 substantially increases the K_m^{arg} [Fujimoto et al., 2005]. In spite of the unique replacement of these residues in the heterodont two-domain AKs, these AKs show a binding capacity for the substrate arginine comparable to that of typical, 40 kDa AKs with the D62 and R193 residues. Considering that the two-domain AK lacks the ion pair formed by D62 and R193, we suggest that there exists an alternative mechanism for stabilizing the substrate-bound structure in the heterodont two-domain AKs.

Phylogenetic relationships with other arginine kinases

A phylogenetic tree was constructed from the amino acid sequences of 44 AKs from Porifera, Cnidaria, Arthropoda and Mollusca by NJ method. A portion of the molluscan AK cluster is shown in Figure 3. A similar topology was obtained with the ML method (data not shown). The molluscan AK cluster, including four major classes Polyplacophora, Bivalvia, Gastropoda and Cephalopoda, clearly indicates that the heterodont AKs with two-domain structure diverged first. If the AK genes of heterodonts and pteriomorphians in Figure 3 are orthologous, the evolution of Bivalvia appears to be paraphyletic, consistent with recent phylogenetic analyses of 18S or LSU/SSU rRNA sequence [Winnepenninckx et al., 1996; Passamaneck et al., 2004]. The heterodont AK cluster suggests that gene duplication and fusion occurred at the immediate ancestor of *Solen*, *Corbicula*, *Ensis*, *Pseudocardium* and *Calyptogena*. We estimated in the previous paper that the two domains of the heterodont AKs diverged 280 million years before present (MyrBP) [Suzuki et al., 2002], assuming the Mollusca and Arthropoda diverged about 550 MyrBP.

Are both of the two domains of two-domain AK active? Kinetic analyses of wild-type and mutant enzymes using Calyptogena kaikoi and Corbicula japonica AKs

It is somewhat controversial whether each of the two domains of heterodont two-domain AK has catalytic ability or not. Suzuki et al. (2002) assumed that both domains will function as AK, mainly based on the observation that most of the substrate-interacting residues are conserved as in Fig. 1. On the other hand, using *Ensis* two-domain AK Compaan and Ellington (2003) concluded that only domain 2 has catalytic activity because the refolded NusA-His tagged domain 1 showed no activity. In the case of the present effort, both separated domains 1 of *Corbicula* and *Ensis* AKs

were insoluble as recombinant proteins rendering it impossible to elucidate the nature of domain 1.

To make clear the catalytic ability in domain 1 of the two-domain AKs, we expressed *Calyptogena* two-domain AK (2D[WT]) and its individual domains 1 (D1[WT]) and 2 (D2[WT]) as fusion proteins with MBP. All the three recombinant enzymes are, fortunately, soluble, could be easily purified and displayed AK activity. As shown in Table 1, the 2D[WT] and D2[WT] showed high AK activity (k_{cat} = 23-38 s^{-1}) with a K_m^{arg} value of 0.7-0.9 mM, but the D1[WT] showed minimal AK activity (k_{cat} ~ 0.016 s^{-1}), a value less than 0.05% that of D2[WT]. Here we considered the possibility that the remarkably weak, but not zero, activity in the isolated D1[WT] might be due to some conformational destabilization caused by the absence of some portions of the domain 2 polypeptide.

To explore the relative contributions of domains 1 and 2 to catalytic activity, Y68 mutations were engineered into the *Calyptogena* two-domain AK. This residue forms a hydrogen-bond with the amino group of the substrate arginine in a crystal structure of the transition state analog complex of *Limulus* AK [Zhou et al., 1998]. The Y68 residue is conserved in all AKs, without exception, including two-domain clam AKs, and we have previously shown that Y68S or Y68R mutants in *Nautilus* AK reduced its enzyme activity dramatically [Suzuki et al., 2000a; Uda and Suzuki, 2004]. We prepared four mutants, 2D[Y68A in D1], 2D[Y68A in D2], 2D[Y68A in D1 & D2] and D2[Y68A] for *Calyptogena* AK, and the same four mutants for *Corbicula* AK, for comparison. The results of kinetic measurements for these mutants are listed in Table 1. Figure 4 shows the impact of Y68 mutations in *Calyptogena* and *Corbicula* AKs in terms of k_{cat} value.

Kinetic analysis of domain 2 mutations of *Calyptogena* AK showed that k_{cat} values of 2D[Y68A in D2] and D2[Y68A] were reduced to 2.81 (12.3% that of the 2D[WT]) and 0.031 s^{-1} (less than 0.1% that of the D2[WT]), respectively (Table 1 and Fig. 4). The reduced but significant residual activity (12.3%) of 2D[Y68A in D2] suggests that domain 1 has AK catalytic ability. However, it appears that the activity in domain 1 is lower than that in domain 2. On the other hand, in the case of a Y68A mutation in domain 1, the k_{cat} value of 2D[Y68A in D1] was reduced to 11.1 s^{-1} (48.5% that of the 2D[WT]) (Table 1 and Fig. 4). It should be noted that the mutant with Y68A in both domains 1 and 2 (2D[Y68A in D1 & D2]) gave a very low k_{cat} value (0.04 s^{-1}, 1.8% that of 2D[WT]).

The sum of the k_{cat} values (11.1 + 2.81) of the mutants 2D[Y68A in D1] and 2D[Y68A in D2] corresponds only to 60% of that of the 2D[WT] suggesting that the mutation affects not only the target position but also the overall three-dimensional structure of the enzyme, or that some domain-domain interaction has been generated when taking a two-domain structure. Similar subunit-subunit interaction has been reported for dimeric CK [Hornemann et al., 2000]. In addition, Hoffman et al. (2008) produced seven different constructs of a “contiguous” trimeric flagellar CK in which all combinations of D1, D2 and D3 domains were inactivated using a reactive C to S mutation. They showed that the domains are non-equivalent in terms of activity and that the extent of activity depends upon which combination of domain(s) is inactivated. The present results, as summarized, reveal that domain 1 in the *Calyptogena* two-domain AK is catalytically competent, but its ability is not equivalent to that of domain 2. It is possible that domain 1 when expressed may be structurally unstable. An intriguing alternative possibility is that the presence of domain 2 may be required for full activity

by facilitating the formation of the closed state required for catalysis in domain 1.

To further validate the above observations, we undertook the same experiment using *Corbicula* AK. Like *Calyptogena* AK, the k_{cat} values of 2D[Y68A in D2] and D2[Y68A] were reduced to 27.6 (31.6% that of the 2D[WT]) and 2.79 s^{-1} (3.2% that of the D2[WT]), respectively (Table 1 and Fig. 4). The residual 31.6% activity in 2D[Y68A in D2] indicates that domain 1 retains sufficient AK activity. On the other hand, the k_{cat} value of 2D[Y68A in D1] was 42.1 s^{-1} (48.2% that of the 2D[WT]). The sum of the k_{cat} values (42.1 + 27.6) of the mutants 2D[Y68A in D1] and 2D[Y68A in D2] corresponds to 80% of that of 2D[WT], similar to the case of *Calyptogena* AK. The mutant with Y68A in both domains 1 and 2 (2D[Y68A in D1 & D2]) gave a low k_{cat} value of 4.0 s^{-1} (4.6% that of the 2D[WT]). These results are generally consistent with the kinetic data for the *Calyptogena* two-domain AK and further support the lack of equivalency of the two domains in spite of the presence of the requisite residues for catalysis.

Very recently, we have examined the properties of the “contiguous” dimeric AK from the sea anemone *Anthopleula japonicus* [Tada et al., 2008]. *Anthopleula* two-domain and *Calyptogena* two-domain AKs are enzymes which evolved independently in quite different lineages [Uda et al., 2006]. Tada et al. (2008) were able to express the two-domain wild-type enzyme as well as individual domains 1 and 2. Both individual domains were shown to be active but the two domains displayed a much higher catalytic efficiency indicating interaction of domains important in determining the properties of the two-domain AK as a whole. The results from the studies of *Calyptogena* and *Corbicula* two-domain AKs reported in this communication, coupled with kinetic analysis of similar enzymes, are consistent with the view that

multiple catalytic sites in multi-domain phosphagen kinases are indeed active, and the overall kinetic character of the multi-domain enzyme may be altered and influenced significantly by the presence of adjacent domains.

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References

Andrews, L.D., Graham, J., Snider, M.J., Fraga, D., Characterization of a novel bacterial arginine kinase from *Desulfotalea psychrophila*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. (in press)

Cleland, W.W., 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. 63, 103–138.

Compaan, D.M., Ellington, W.R., 2003. Functional consequences of a gene duplication and fusion event in an arginine kinase. J Exp Biol 206, 1545-1556.

Conejo, M., Bertin, M., Pomponi, S., Ellington, W., 2008. The early evolution of the phosphagen kinases--insights from choanoflagellate and poriferan arginine kinases. J Mol Evol 66, 11-20.

Edmiston, P.L., Schavolt, K.L., Kersteen, E.A., Moore, N.R., Borders, C.L., 2001. Creatine kinase: a role for arginine-95 in creatine binding and active site organization. Biochim Biophys Acta 1546, 291-298.

Ellington, W.R., 1989. Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens. J Exp Biol 143, 177-194.

Ellington, W.R., 2001. Evolution and physiological roles of phosphagen systems. Annu Rev Physiol 63, 289-325.

Ellington, W.R., Suzuki, T., 2006. Evolution and divergence of creatine kinase genes. In: Vial, C. (Ed.) Molecular Anatomy and Physiology of Proteins: Creatine Kinase, Nova Science, New York, pp. 1-27

Felsenstein, J., 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5, 161-166.

Fujimoto, N., Tanaka, K., Suzuki, T., 2005. Amino acid residues 62 and 193 play the key role in regulating the synergism of substrate binding in oyster arginine kinase. FEBS Lett 579, 1688-1692.

Hoffman, G., Davulcu, O., Sona, S., Ellington, W., 2008. Contributions to catalysis and potential interactions of the three catalytic domains in a contiguous trimeric creatine kinase. FEBS J 275, 646-654.

Hornemann, T., Rutishauser, D., Wallimann, T., 2000. Why is creatine kinase a dimer? Evidence for cooperativity between the two subunits. Biochim Biophys Acta 1480, 365-373.

Kenyon, G.L., Reed, G.H., 1983. Creatine kinase: structure-activity relationships. Adv Enzymol Relat Areas Mol Biol 54, 367-426.

Morrison, J.F., 1973. Arginine kinase and other invertebrate guanidino kinases. In: Boyer, P.C. (Ed.) The Enzymes., Academic Press, New York, pp. 457-486

Noguchi, M., Sawada, T., Akazawa, T., 2001. ATP-regenerating system in the cilia of Paramecium caudatum. J Exp Biol 204, 1063-1071.

Passamaneck, Y., Schander, C., Halanych, K., 2004. Investigation of molluscan phylogeny using large-subunit and small-subunit nuclear rRNA sequences. Mol Phylogenet Evol 32, 25-38.

Pereira, C., Alonso, G., Paveto, M., Iribarren, A., Cabanas, M., Torres, H., Flawi・ M., 2000. *Trypanosoma cruzi* arginine kinase characterization and cloning. A novel energetic pathway in protozoan parasites. J Biol Chem 275, 1495-1501.

Suzuki, T., Fukuta, H., Nagato, H., Umekawa, M., 2000a. Arginine kinase from *Nautilus pompilius*, a living fossil. Site-directed mutagenesis studies on the role of amino acid residues in the Guanidino specificity region. J Biol Chem 275, 23884-23890.

Suzuki, T., Furukohri, T., 1994. Evolution of phosphagen kinase. Primary structure of glycocyamine kinase and arginine kinase from invertebrates. J Mol Biol 237, 353-357.

Suzuki, T., Kawamichi, H., Ohtsuki, R., Iwai, M., Fujikura, K., 2000b. Isolation and cDNA-derived amino acid sequences of hemoglobin and myoglobin from the deep-sea clam *Calyptogena kaikoi*. Biochim Biophys Acta 1478, 152-158.

Suzuki, T., Kawasaki, Y., Furukohri, T., 1997. Evolution of phosphagen kinase. Isolation, characterization and cDNA-derived amino acid sequence of two-domain arginine kinase from the sea anemone *Anthopleura japonicus*. Biochem J 328 , 301-306.

Suzuki, T., Kawasaki, Y., Unemi, Y., Nishimura, Y., Soga, T., Kamidochi, M., Yazawa, Y., Furukohri, T., 1998. Gene duplication and fusion have occurred frequently in the evolution of phosphagen kinases--a two-domain arginine kinase from the clam

*Pseudocardium sachalinensi*s. Biochim Biophys Acta 1388, 253-259.

Suzuki, T., Mizuta, C., Uda, K., Ishida, K., Mizuta, K., Sona, S., Compaan, D., Ellington, W., 2004. Evolution and divergence of the genes for cytoplasmic, mitochondrial, and flagellar creatine kinases. J Mol Evol 59, 218-226.

Suzuki, T., Sugimura, N., Taniguchi, T., Unemi, Y., Murata, T., Hayashida, M., Yokouchi, K., Uda, K., Furukohri, T., 2002. Two-domain arginine kinases from the clams *Solen strictus* and *Corbicula japonica*: exceptional amino acid replacement of the functionally important D(62) by G. Int J Biochem Cell Biol 34, 1221-1229.

Suzuki, T., Tomoyuki, T., Uda, K., 2003. Kinetic properties and structural characteristics of an unusual two-domain arginine kinase of the clam *Corbicula japonica*. FEBS Lett 533, 95-98.

Suzuki, T., Yamamoto, Y., 2000c. Gene structure of two-domain arginine kinases from *Anthopleura japonicus* and *Pseudocardium sachalinensis*. Comp Biochem Physiol B Biochem Mol Biol 127, 513-518.

Tada, H., Nishimura, Y., Suzuki, T., 2008. Cooperativity in the two-domain arginine kinase from the sea anemone *Anthopleura japonicus*. Int J Biol Macromol 42, 46-51.

Takeuchi, M., Mizuta, C., Uda, K., Fujimoto, N., Okamoto, M., Suzuki, T., 2004. Unique evolution of Bivalvia arginine kinases. Cell Mol Life Sci 61, 110-117.

Tanaka, K., Uda, K., Shimada, M., Takahashi, K., Gamou, S., Ellington, W., Suzuki, T., 2007. Evolution of the cytoplasmic and mitochondrial phosphagen kinases unique to annelid groups. J Mol Evol 65, 616-625.

Uda, K., Fujimoto, N., Akiyama, Y., Mizuta, K., Tanaka, K., Ellington, W.R., Suzuki, T., 2006. Evolution of the arginine kinase gene family. 1, 209-218.

Uda, K., Iwai, A., Suzuki, T., 2005. Hypotaurocyamine kinase evolved from a gene for arginine kinase. FEBS Lett 579, 6756-6762.

Uda, K., Suzuki, T., 2004. Role of amino acid residues on the GS region of *Stichopus* arginine kinase and *Danio* creatine kinase. Protein J 23, 53-64.

Watts, D., Bannister, L., 1970. Location of arginine kinase in the cilia of *Tetrahymena pyriformis*. Nature 226, 450-451.

Winnepenninckx, B., Backeljau, T., De Wachter, R., 1996. Investigation of molluscan phylogeny on the basis of 18S rRNA sequences. Mol Biol Evol 13, 1306-1317.

Wyss, M., Smeitink, J., Wevers, R., Wallimann, T., 1992. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim Biophys Acta 1102, 119-166.

Yousef, M., Clark, S., Pruett, P., Somasundaram, T., Ellington, W., Chapman, M., 2003. Induced fit in guanidino kinases--comparison of substrate-free and transition state analog structures of arginine kinase. Protein Sci 12, 103-111.

Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W., Chapman, M., 1998. Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. Proc Natl Acad Sci U S A 95, 8449-8454.

Figure legends

Figure 1: Alignment of the amino acid sequence of *Calyptogena* two-domain AK with those of other heterodont AKs and *Limulus* AK. The substrate arginine binding residues are indicated by (#), and substrate ATP binding residues by (*) in *Limulus* AK crystal structure [Zhou et al., 1998]. Highly conserved residues, D62 and R193 according to the number of the *Limulus* sequence, are indicated by $. Guanidino substrate specific residues are marked by % [Edmiston et al., 2001].

Figure 2: Schematic representation of the construction of wild-type and its mutants of *Calyptogena* and *Corbicula* AKs and these mutants.

Figure 3: Neighbor-joining tree for the amino acid sequences of molluscan AKs. Bootstrap value (1000 replications) is shown at the branching point.

Figure 4: Comparison of k_{cat} values for wild-type and mutants of *Calyptogena* and *Corbicula* AKs. Values represent means +/- 1 SD.

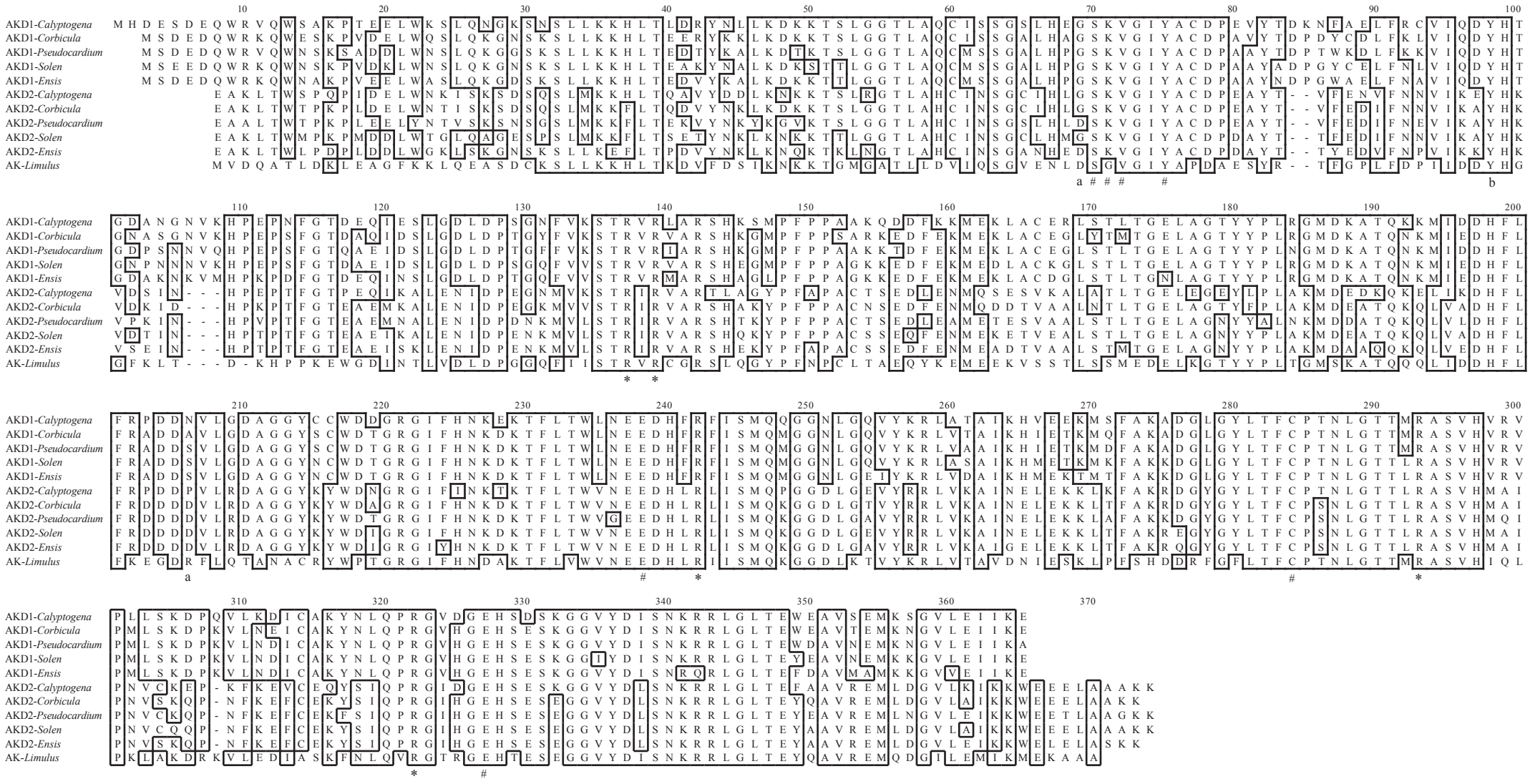

AKD1-Calyptogena
AKD1-Corbicula
AKD1-Pseudocardium
AKD1-Solen
AKD1-Ensis
AKD2-Calyptogena
AKD2-Corbicula
AKD2-Pseudocardium
AKD2-Solen
AKD2-Ensis
AK-Limulus

Fig. 1

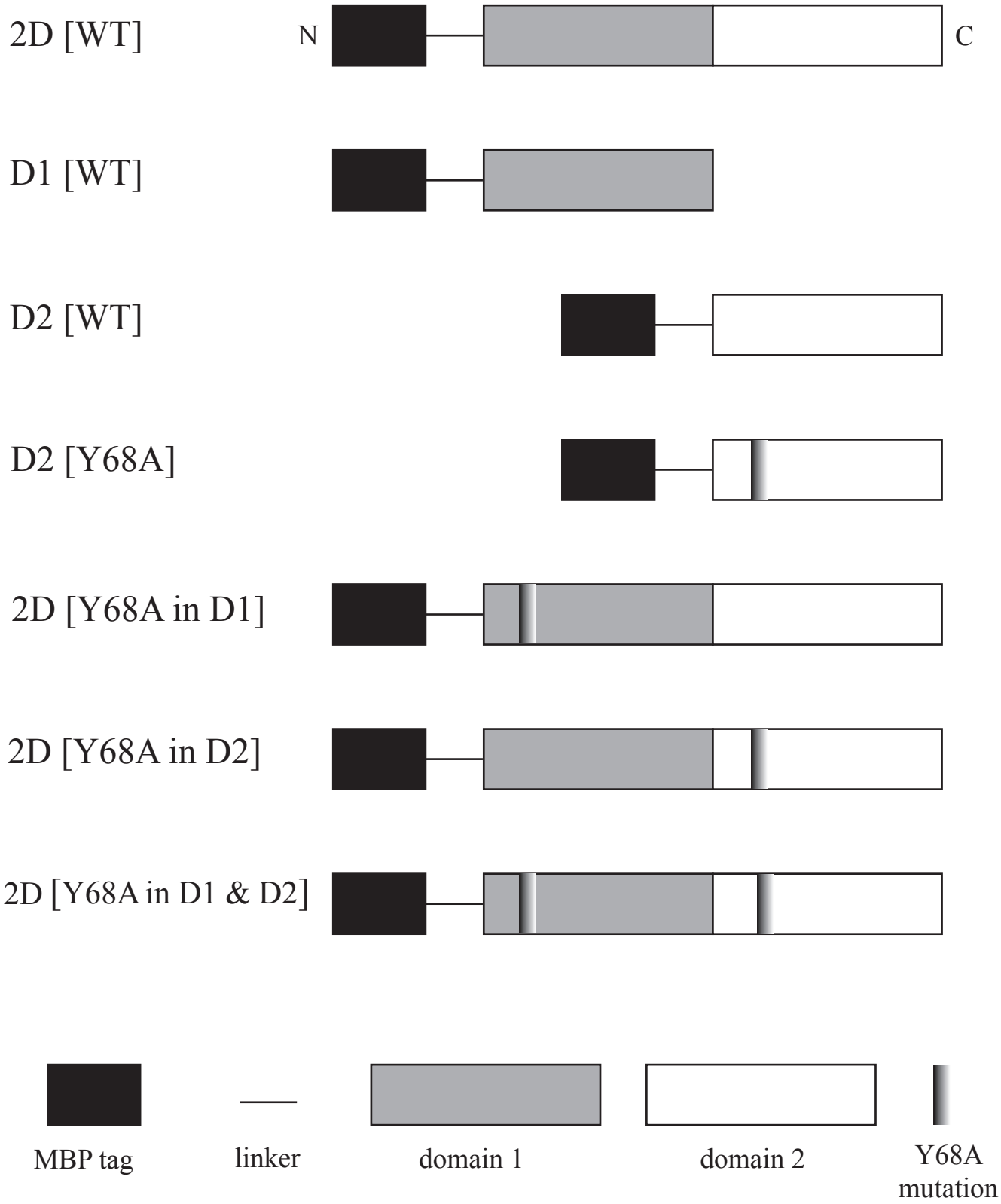
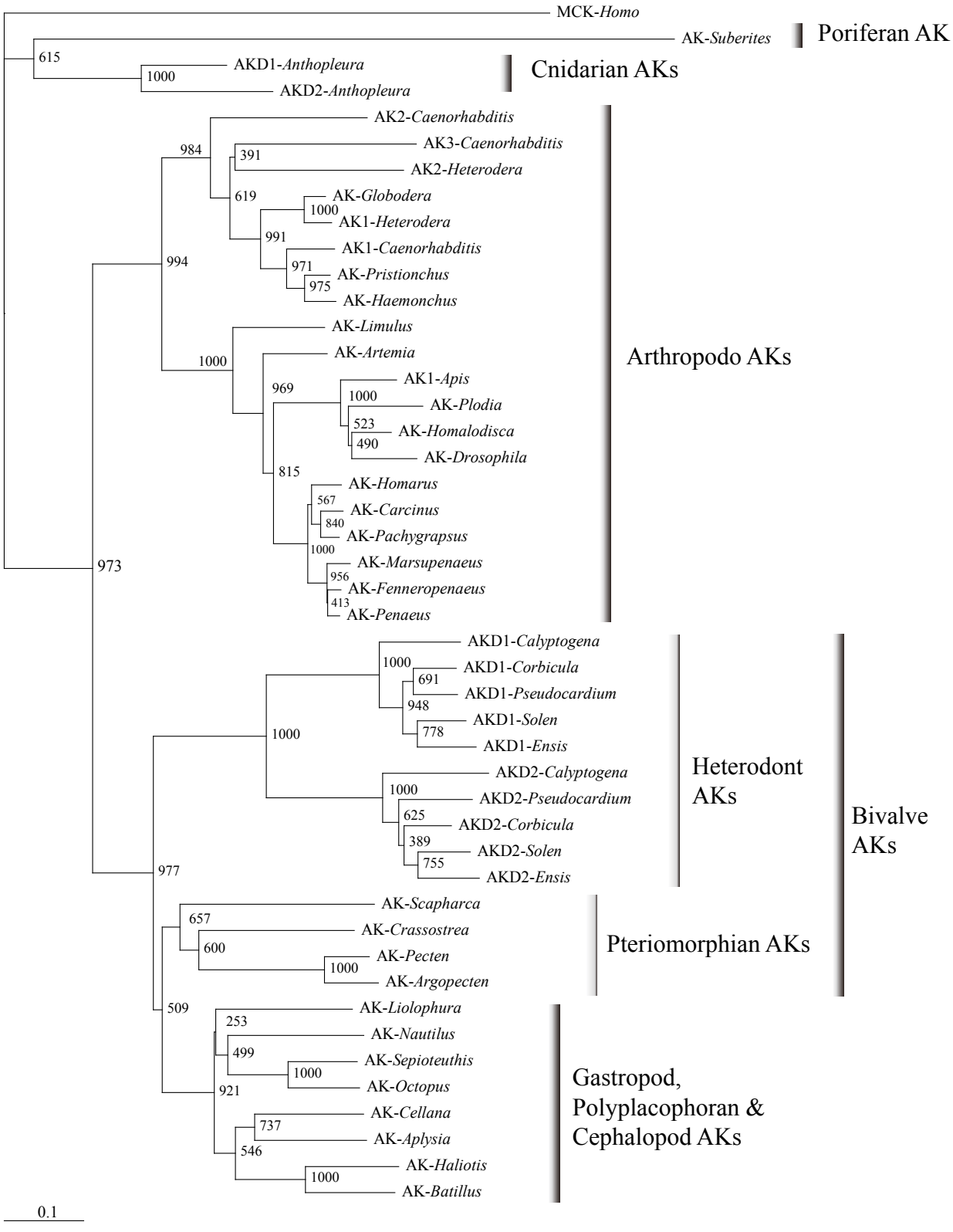

2D [WT]
N
C
D1 [WT]
D2 [WT]
D2 [Y68A]
2D [Y68A in D1]
2D [Y68A in D2]
2D [Y68A in D1 & D2]
MBP tag
linker
domain 1
domain 2
Y68A mutation

Fig. 2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AK-*Suberites*
Poriferan AK
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1000
AKD1-*Anthopleura*
AKD2-*Anthopleura*
Cnidarian AKs
AK2-*Caenorhabditis*
AK3-*Caenorhabditis*
AK2-*Heterodera*
984
391
619
AK-*Globodera*
1000
AK1-*Heterodera*
991
AK1-*Caenorhabditis*
994
971
AK-*Pristionchus*
975
AK-*Haemonchus*
AK-*Limulus*
AK-*Artemia*
1000
AK1-*Apis*
969
1000
AK-*Plodia*
523
AK-*Homalodisca*
490
AK-*Drosophila*
815
AK-*Homarus*
567
AK-*Carcinus*
840
AK-*Pachygrapsus*
1000
973
AK-*Marsupenaeus*
956
AK-*Fenneropenaeus*
413
AK-*Penaeus*
Arthropodo AKs
AKD1-*Calyptogena*
1000
AKD1-*Corbicula*
691
AKD1-*Pseudocardium*
948
AKD1-*Solen*
1000
778
AKD1-*Ensis*
AKD2-*Calyptogena*
1000
AKD2-*Pseudocardium*
625
AKD2-*Corbicula*
389
AKD2-*Solen*
977
755
AKD2-*Ensis*
Heterodont AKs
Bivalve AKs
AK-*Scapharca*
657
AK-*Crassostrea*
600
AK-*Pecten*
1000
AK-*Argopecten*
Pteriomorphian AKs
509
AK-*Liolophura*
253
AK-*Nautilus*
499
AK-*Sepioteuthis*
1000
AK-*Octopus*
921
AK-*Cellana*
737
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AK-*Haliotis*
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AK-*Batillus*
Gastropod, Polyplacophoran & Cephalopod AKs
0.1

Fig. 3

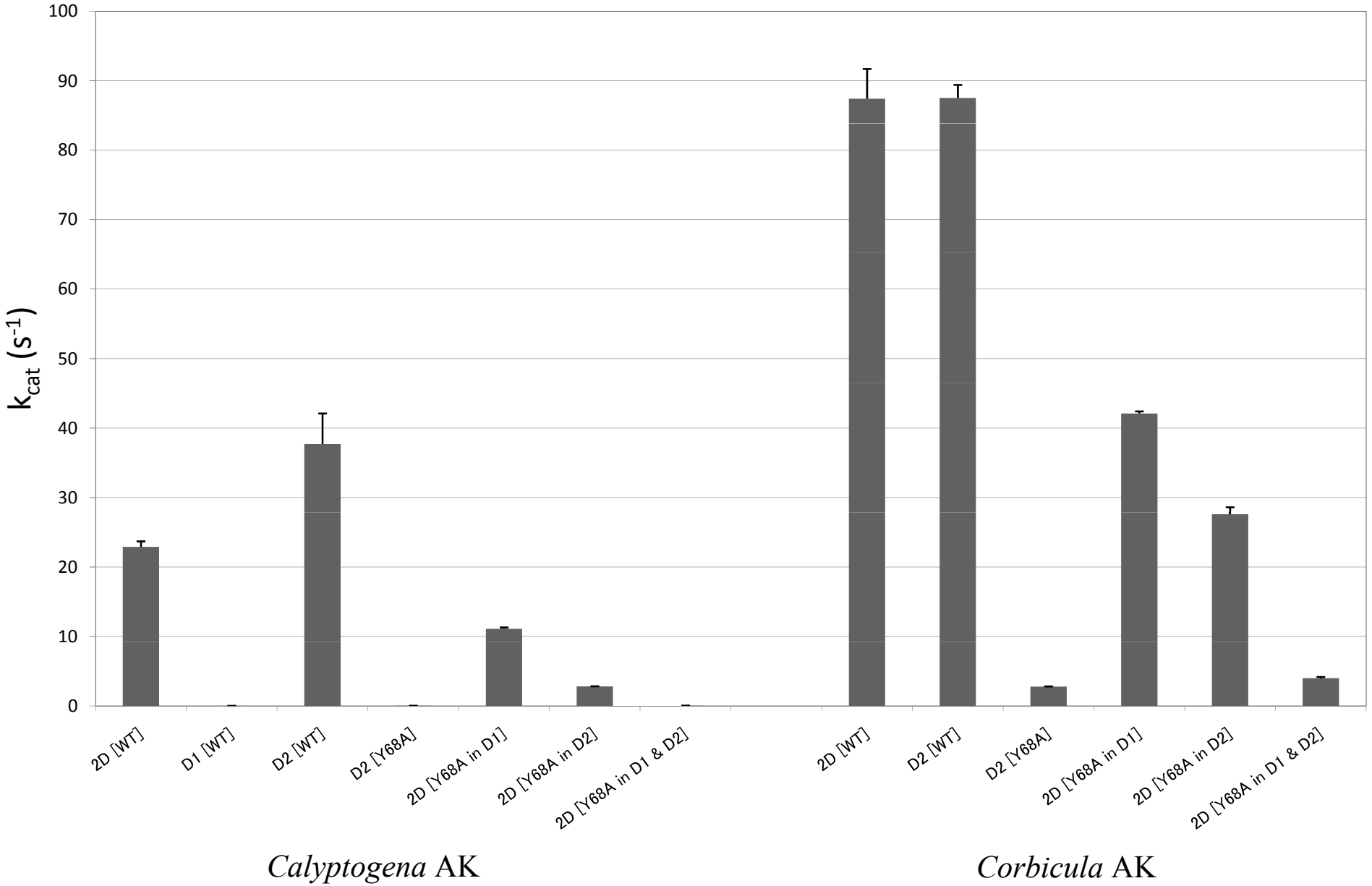
100
90
80
70
60
50
40
30
20
10
0
k_{cat} (s^{-1})
2D [WT]
D1 [WT]
D2 [WT]
D2 [Y68A]
2D [Y68A in D1]
2D [Y68A in D2]
2D [Y68A in D1 & D2]
2D [WT]
D2 [WT]
D2 [Y68A]
2D [Y68A in D1]
2D [Y68A in D2]
2D [Y68A in D1 & D2]
Calyptogena AK
Corbicula AK

Table 1 Kinetic parameters for the forward reaction of the wild-type and mutants of *Corbicula japonica* AK and *Calyptogena kaikoi* AK.

		K_m^{Arg} (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
Calyptogena	2D [WT]	0.912 ± 0.031	22.9 ± 0.8	25.2 ± 0.6
	D1 [WT]	-	0.0161 ± 0.0065*	-
	D2 [WT]	0.724 ± 0.030	37.7 ± 4.4	52.5 ± 8.6
	D2 [Y68A]	-	0.0308 ± 0.0064*	-
	2D [Y68A in D1]	0.828 ± 0.029	11.1 ± 0.2	13.4 ± 0.5
	2D [Y68A in D2]	1.06 ± 0.08	2.81 ± 0.02	2.66 ± 0.21
	2D [Y68A in D1 & D2]	-	0.0401 ± 0.0124*	-
Corbicula	2D [WT]	0.341 ± 0.026	87.4 ± 4.3	257 ± 10
	D1 [WT] insoluble	-	-	-
	D2 [WT]	0.167 ± 0.007	87.5 ± 1.9	526 ± 28
	D2 [Y68A]	2.10 ± 0.21	2.79 ± 0.02	1.34 ± 0.13
	2D [Y68A in D1]	0.283 ± 0.011	42.1 ± 0.3	149 ± 6
	2D [Y68A in D2]	0.379 ± 0.026	27.6 ± 1.0	72.9 ± 2.4
	2D [Y68A in D1 & D2]	3.61 ± 0.19	4.00 ± 0.17	1.11 ± 0.01

* These v values were obtained in the presence of 9.5 mM arginine..

The k_{cat} of the two-domain enzyme are the average values calculated for one domain.