Evolution of phosphagen kinase¹

Isolation, characterization and cDNA-derived amino acid sequence of two-domain arginine kinase from the sea anemone *Anthopleura japonicus*

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Arginine kinase (AK) was isolated from the body wall muscle of the primitive sea anemone *Anthopleura japonicus* by Ultrogel AcA34 gel filtration, DEAE-32 chromatography and elution on a Cosmogel-SP column. The denatured molecular mass as determined with SDS/PAGE was 80 kDa, twice that of the usual AK subunit, indicating that this AK has an unusual two-domain structure. The native form was eluted on a Superose 12 column with the same retention time as that of rabbit homodimeric creatine kinase, indicating that *Anthopleura* AK is a monomer of 80 kDa. The isolated enzyme gave a specific activity of 100–120 μmol of P_i /min per mg of protein in the pH range

7.9–9.1 for the forward reaction. The enzyme is fully activated by Ca^{2+} , as it is with Mg^{2+} . The cDNA-derived amino acid sequence of 715 residues of *Anthopleura* AK was determined. The validity of the sequence was supported by chemical sequencing of internal tryptic peptides. A bridge intron of 686 bp, which separates the two domains of *Anthopleura* AK, is present between the second and third nucleotide in the codon of Ala-364. This is the first two-domain AK to be sequenced. *Anthopleura* AK shows 48–54 % amino acid sequence identity with known invertebrate AKs, and also shows a lower, but significant, similarity (39–46 %) to marine worm glycocyamine kinase and rabbit creatine kinase.

INTRODUCTION

Phosphagen kinases are the enzymes that catalyse the reversible transfer of the high-energy phosphoryl group of ATP to naturally occurring guanidino compounds such as creatine, glycocyamine, taurocyamine, lombricine and arginine, and have a key role in the interconnection of energy production and utilization in animals [1–3]. The phosphorylated high-energy guanidine is referred to as phosphagen:

In vertebrates the only phosphagen is phosphocreatine, and the corresponding phosphagen kinase is creatine kinase (CK). Three types of isoform, M-CK, B-CK and Mi-CK, are present in muscle, brain and mitochondria respectively [4]. In invertebrates, at least six unique phosphagens (phosphoarginine, phosphoglycocyamine, phosphotaurocyamine, phospholombricine, phosphohypotaurocyamine and phospho-opheline) are present in addition to phosphocreatine, and the corresponding kinases for the first four, arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase and lombricine kinase, have also been identified [1,2]. Like vertebrate CKs, several isoforms have been reported in arthropod AKs [5,6]. The homologous amino acid sequences of vertebrate CKs, sea urchin CK, marine worm GK, and lobster, shrimp and *Limulus* AKs suggest that they have evolved from a common origin [2,7,8]. Thus phosphagen kinases provide an excellent model system for elucidating how enzymes developed the recognition site for substrate during evolution. One of the major interests in phosphagen kinase evolution is in determining which enzyme is the ancestral type. Although AK is most widely distributed among invertebrates, this does not necessarily mean that AK is the ancestral form of all phosphagen kinases. This problem can be most effectively be resolved with the analyses of phosphagen kinases from lower invertebrates. Here we report the isolation, characterization and cDNA-derived

amino acid sequence of an unusual two-domain AK from a primitive invertebrate, *Anthopleura japonicus* (sea anemone).

MATERIALS AND METHODS

Isolation of *Anthopleura* AK

All procedures were performed at 4–8 °C. Body wall muscle (50 g) of the sea anemone *Anthopleura japonicus* was homogenized with 250 ml of 10 mM Tris/acetate buffer, pH 8.1, containing 3 $\mu\text{g}/\text{ml}$ PMSF. The tissue extract was fractionated with 50–70 % satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in a minimum volume of the same buffer and applied to an Ultrogel AcA 43 column (3 cm $\times$ 90 cm) equilibrated with the same buffer. The fraction possessing AK activity was pooled and applied to a DEAE-cellulose column (1.5 cm $\times$ 10 cm) equilibrated with 10 mM Tris/acetate buffer, pH 8.1, containing 0.2 mM dithiothreitol. The column was washed with the same buffer and then eluted with a linear gradient of 0–150 mM NaCl in 10 mM Tris/acetate buffer. Because the AK activity was found in the unabsorbed fraction, it was then applied to a Cosmogel-SP column (7.5 cm $\times$ 75 mm; Nakalai Tesque) and eluted with a linear gradient of 0–450 mM NaCl in 10 mM phosphate buffer, pH 7.2, at a flow rate of 1 ml/min. The purified AK was stored on ice or at –80 °C until use.

Enzyme assay

Measurement of enzymic activity for the forward reaction was done as follows. The reaction mixture (1.35 ml) contained 12.5 mM L-arginine, 1 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 40 mM Tris/acetate (usually pH 8.1) and purified enzyme. After equilibration at 30 °C for 5 min, the reaction was started by the addition of 0.15 ml of 10 mM ATP at pH 7.2. The reaction was stopped by the addition of 0.5 ml of 0.4 M perchloroacetic acid and stored

Abbreviations used: AK, arginine kinase; CK, creatine kinase; GK, glycocyamine kinase.

¹ This is no. 4 in the series. No. 1 is [8], no. 2 is [31] and no. 3 is [19].

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB008014.

Figure 1 Ultrogel AcA43 gel filtration of *Anthopleura* muscle extracts

The column (3 cm × 90 cm) was equilibrated and eluted with 10 mM Tris/acetate buffer, pH 8.1. The fraction size was 6 ml per tube.

on ice for 10 min. The reaction tube was then placed in a boiling-water bath for 1 min, and cooled immediately on ice for 5 min. The released inorganic phosphate from phosphoarginine was determined with the method of Nakamura [9]. Protein concentration was estimated from the absorbance at 280 nm (an A_{280} of 0.77 in a 1 cm cuvette corresponds to 1 mg/ml protein) [10].

Protein analysis

The molecular mass of native AK was estimated on a gel-filtration column of Superose 12 (1 cm × 30 cm; Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.2. The column was eluted with the same buffer at a flow rate of 0.4 ml/min and calibrated with rabbit CK (80 kDa), *Battilus* indoleamine dioxygenase-like myoglobin (40 kDa) and sperm-whale myoglobin (18 kDa).

The isolated protein was pyridylethylated and digested with trypsin at an enzyme-to-substrate ratio of 1:100 in 0.1 M NH_4HCO_3 at 37 °C for 2 h. The digested products were isolated by reverse-phase chromatography. The column (Cosmosil 5C₁₈-300; 4.6 mm × 150 mm) was equilibrated with 0.1 % trifluoroacetic acid and eluted with a linear gradient of 0–90 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid over 120 min at a flow rate of 1 ml/min. Peptides for sequence analysis were purified further by rechromatography on the same column with a linear gradient of acetonitrile in 10 mM ammonium acetate [11]. The amino acid sequences of the whole AK and tryptic peptides were determined by an automated protein sequencer (Applied BioSystems 476A). SDS/PAGE was performed in 15 % (w/v) polyacrylamide gel containing 0.087 % *N,N'*-methylenebisacrylamide, 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 or absence of 2-mercaptoethanol before electrophoresis.

cDNA and genomic analyses of *Anthopleura* AK

Total RNA was prepared from the body wall muscle of *Anthopleura japonicus* by the method of Chomczynski and Sacchi [12]; poly(A)⁺ RNA was purified with a FirstTrack mRNA Isolation Kit (Invitrogen). The single-stranded cDNA was synthesized with avian reverse transcriptase by using oligo-dT adaptor, 5'-GGATTCGAATTCCCCGGGT₁₇-3' as a primer.

Figure 2 Further purification of *Anthopleura* AK

Upper panel: Cosmogel-SP chromatography of *Anthopleura* AK. The column (7.5 mm × 75 mm; Nakalai Tesque) was eluted with a linear gradient of 0–450 mM NaCl in 10 mM phosphate buffer, pH 7.2, at a flow rate of 1 ml/min. Lower panel: SDS/PAGE of purified *Anthopleura* AK. The peaks labelled 1 to 4 in the upper panel were subjected to electrophoresis in the presence of a reducing agent. The standard marker proteins (indicated at the left) were: a, phosphorylase b (94 kDa); b, BSA (67 kDa); c, rabbit CK (42 kDa); d, carbonic anhydrase (30 kDa); e, trypsin inhibitor (20 kDa); f, sperm-whale myoglobin (17 kDa).

The 3' half of the cDNA of *Anthopleura* AK was first amplified for 30 cycles, each consisting of 1 min at 94 °C for denaturation, 1.5 min at 55 °C for annealing and 2 min at 72 °C for primer extension, by PCR [13]. Extra *Taq* DNA polymerase (Takara) was used as enzyme. The primers used were the oligo(dT) adaptor and a 512-fold 'universal' redundant oligomer, GT(ACGT)TGG(AG)T(ACGT)AA(TC)GA(AG)GA(AG)GA(TC)CA, designed for amplification of phosphagen kinases [8]. This universal oligomer is based on the conserved amino acid sequence of Val-Trp-(Val/Ile)-Asn-Glu-Glu-Asp-His [8]. The 600 bp products were subcloned in the *Sma*I site of pUC18 and three independent clones were sequenced with a PRISM dye terminator cycle sequencing kit by using a Model 373-18 DNA sequencer (Applied BioSystems). The 5' half of the cDNA was amplified as follows. The single-stranded cDNA was newly synthesized with the non-redundant primer Anth.R1 (5'-CCTGTTCCAAGGT-TGGTGGGACA-3') and the poly(A)⁺ tail was added to the 3'

end with a terminal deoxynucleotidyl transferase. Then the 5' half of the cDNA was amplified by the method described above, with the oligo-dT adaptor and the non-redundant primer Anth.R2 (5'-TCGCTGCCTTTCTCCATGCTGAT-3'). The 850 bp products were subcloned in the *Sma*I site of pUC18 and three independent clones were sequenced. The whole length of cDNA of *Anthopleura* AK was amplified with Anth.FW (5'-GGACTATCAACACATCTCTGC-3'), designed from the sequence of 5' clones, and the oligo(dT) adaptor by PCR and subcloned in the *Sma*I site of pUC18; three clones were sequenced. The genomic DNA was purified from a single specimen of *Anthopleura* with a conventional phenol/chloroform method. Genomic DNA (1 µg) and two non-redundant primers (1 pmol each), Anth.F1 (5'-GAAAACATCCTAACCAAGTA-3') and Anth.R3 (5'-TTCAGGGAGTCAAACAGTTC-3') were used to amplify a DNA fragment containing a bridge intron by PCR. The PCR products were isolated by 0.7% agarose-gel electrophoresis, and sequenced by direct sequencing with a PRISM dye terminator cycle sequencing kit (FS).

RESULTS

Figure 1 shows the elution profile on Ultrogel AcA43 chromatography of crude aqueous extracts of *Anthopleura* muscle. Fractions 35–45, possessing AK activity (maximum 12 µmol of

Figure 3 Effect of pH on the activity of *Anthopleura* AK

The pH dependence of *Penaeus* AK is also shown for comparison [10].

Figure 4 Effects of Mg^{2+} and Ca^{2+} on the activity of *Anthopleura* AK

The reaction mixture contained 1 mM ATP.

GACCCCTTAAGAAAGGTAACGCTTACTTTTCTACGGCGGTGGATTGAGGAAGGTTCTGGTGACAAACAGCTT	75
<u>Anth.FW</u>	
GGACTATCAACACATCTCTGCAAAATCAAGGTACCGGTGACAGTAAAGAAACAATGGCAGATCCAGAGACA	150
M A D P E T	
GCTGCGAAATTCAAATCAAGAATGCATTCCTGATCCTCTCAAGCAGCCCAATGCAACCCCAAGTCTTGTC	225
A A K F K S K N A F P D P L N D P K N P K S L V	
AAGAAGTATTTGACTCCCAAGTGTTCGAGTCCCTCAAGAACAGAAACAAGCTTGGTATTTACTCTGTGGGAT	300
K K Y L T P K V F E S L K N K K T K L G I T L W D	
TGCATCAATTCAAGGGTTGTCAATCTTGATTCTGGAGTCGGTGTATATGACAGTGATGAAGAGTCATACACATT	375
C I N S G V V N L D S G V G V Y A G D E E S Y T L	
TTTGGTCCCTTGTTCGATGCCATCATCGAAGATTACCAAGTCCCTCAAACTTCACTGGACACCACTCAGAT	450
F G F L F D A I I E D Y H S P Y K L A T G H N S D	
ATGAACCCAGCACATGTCAAGGCCAGACCTGGACCCAGCCAGCATGATATCCGATCAACAGAAATTCGCGTG	525
M N P A H V K A P D L D P A N R Y I R S T R I R V	
GCTCGAAGCTCAAGGGTATGGCTTGCACAGCGCTTACCAAGGCCATCGCTTGGAAATAGAAAGAGGTG	600
A R S L K G Y G L A P G V T K A H R L E I E K K V	
GTTGGAGTACGACATCTTGTGCTGCTGCTGGCAGCAATATTAACCACTGTCTGGTATGGACGAGAAC	675
V G V L T S L T G D L A G K Y Y P L S G M D E K T	
AGGCAACAGCTGTAGATGATCAATTTCTTGTTCAGAAAGGTGACCGCTTCTGGAGAGCTGCTGATTAACAG	750
R Q Q L V D D D H F L F K K G D R F L E A A G I N K	
<u>PK consensus</u>	
GAGTGCCCTGAAGGAAGGAATCTACCATCAACATGACAAGACCTTCCTCGTGGCTGAACGAGAGATCAT	825
E W P E G R G I Y H N N D K T F L V W L N E E D H	
<u>Anth.R2</u>	
CTCGATATCAAGTGAAGAAAGCAGCATTTGGTTCAGTGTTCAGCAGACTTTCCCGTGTCTCAACGAG	900
L R I I S M E K G S D I G S V F S R L C R A V N E	
ATTGATAAAGTGTGGGCTCCAGCATCAAGAACATGTTACCTGACATCTGTCCTCAACAGCTTGGTACA	975
I D K K L G F Q H T K K H G Y L T C G P S N L G T	
<u>Anth.F1</u>	
GGGATCGCTCAAGTGTCCATGTCAAGATCCACATGCTAAGAACACCCCTGACTTTGAAACATCTTAACCA	1050
G M R A S V H V K I P H A K E H P D F E N I L T K	
TACCACATCCAGGCCGTGGCATCCATGGCAGCATTCGAGTCACTGGGAGGATGCTGGTGTCTATGACATT	1125
Y H I Q A R G I H G E H S E T G G V T C D I	
AGTAAACCGTAGAGCTTTGGTCTTTCTGAGGTTCACTGTGTACAAGATACATGATGCTCAAGGCCCTGATG	1200
S N R R R L G L S E V Q C V Q D M Y D G V K A L M	
<u>Bridge Intron</u>	
GAGTTGGAAGGAAGCAATTCGGAAGAACGTTTCAGTGTTCCTGAAGTCTCTCAAGAACCCGAGGTCAATCC	1275
E L E K E A I A K K R S V F P E V L K N P E V K S	
<u>Anth.R3</u>	
CTGCTACGAAGTACCTCAGCGCTGAACGTTTGAAGTCTGAAAGACAGAAACAGCAGCAAGGAATCTCCCTG	1350
L L R K Y L T F E L F D S L K D K K T A K G I S L	
YDCGACTGCATCACTCAGCGCTTGAAGAACGTCCTCTGAGTGTATGCGGTGATGAAGAAATGCTCTAC	1425
T D C I N S G V E N L D S S C G V Y A G D E E C Y	
ACACTCTTTGACCATTGTTTGACAAGATCGTTGAGGATTACTCTCCCATCAAACTTGCCAAATAACATACC	1500
T L F A P L F D K I V E D Y H S P Y K L A N K H T	
TCTGATGACCCAGAGAGGTTGACGCGCAATTTGGACCCGAGGAGCACTATATCAGGTCTACGAGATC	1575
S D M N F E K V D A P N L D P E G T Y I R S T R I	
CGTGTGCTGTAACGTGAAGGATATGCTCTGACTCCAGGACTCACAGGAATGAAGATTGGATATTCAGCGGT	1650
R V A R N V K G Y A L T P G L T R N E R L D I E R	
AAGTGGTTGGGTGCTTCTCTTTGACCGGTGACTTGGCAGGCCAGTACTACCGCTGACAGGAATGGATGAA	1725
K V V G V L S S L T G D L A G Q Y Y P L T G M D E	
GCAACAGCGCAAGAAATGGTCAACGACCACTTCTTGTTCAGAAAGGCGCTTCTTGGAAAGCACTGGAGTA	1800
A T R Q K L V N D H F L F K K G D R F L E A A G V	
<u>PK consensus</u>	
AACAACTCTGGCTGAAGGAAGGAGGATCTTCCACAAACAGCAACCTTCCTTGTCTGGATTAACAGAGAA	1875
N K L W P E G R G I F H N N D K T F L V W I N E	
<u>Anth.R2</u>	
GATCAGCTGCGTATCATCAGCATGGAGAAAGCAGCATATTGGATCACTGCTCGGAGGCTTGGCTGCTGTC	1950
D Q L R I I S M E K G S D I G S V F G R L C R A V	
AACGAGATTGATAAACAACCTGGGTTTCCAAACACCGGATGCTCATGGTGACCTGTCTGCTGCTCCACCACTT	2025
N E I D K Q L G F Q H T D A H G Y L S G C P T N L	
<u>Anth.R1</u>	
GGAAACGAATGAGAGCAAGTGTTCAGTCAAGATTCCTCAAGCAAGTGCCACCTGATTCCAGAAATCTGTG	2100
G T G M R A S V H V K I P K A S A H P D F Q K I C	
GACGAGTTCACATCCAAAGCCGTGGTATCCATGCTGATGCTGCTACCGGAGAGATGCTGGTGTATTT	2175
D E F H I Q A R G I H G E H S V S T G E D A G V F	
GACATCAGTAACCTCGACGCTTGTGTCTGCTGAAGTACAATGTGACAAGATGTATGATGAGTCAAGAA	2250
D I S N R R R L G L S E V Q C V Q D M Y N G V K K	
CTGCTGGAAGTGAAGATCAACCAATAAGAAACAGTCTCTGACTATACCAAAACGTTGCCATATCAAGTTAA	2325
L L E I E K S T K	
AAAAAGACGAGAAATTTGTTCTGCTTGTGTTTACCGAGTGTGTTGATAGATGTGTAGTGTGTTTGTGTTGTT	2400
TTGTTATTTTGAAGGATGAAGTACTGCTGTTTAAACCAAGCAATTAATAA _n	2462

Figure 5 Nucleotide and derived amino acid sequences of the cDNA of *Anthopleura* AK

The polyadenylation signal (AATAAA) is boxed. Arrows indicate primers used for amplification. Amino acid sequences determined chemically are underlined.

P_i /min per mg of protein), were pooled and applied to a DEAE-cellulose column. AK activity was found in the unadsorbed fraction, and this step gave an approx. 10-fold purification (70–100 µmol of P_i /min per mg of protein). The *Anthopleura* AK was finally purified on a Cosmogel-SP column (Figure 2, upper panel) and the specific activity was increased to 100–120 µmol of P_i /min per mg of protein. SDS/PAGE in the presence of a reducing agent showed that the isolated AK was highly purified, and the molecular mass of the subunit was approx. 80 kDa

Figure 6 For legend see opposite page

(Figure 2, lower panel), twice that (40 kDa) of the usual phosphagen kinase. *Anthopleura* AK was eluted on a Superose 12 column with the same retention time as rabbit CK (results not shown). Thus the molecular mass of the native form was estimated to be approx. 80 kDa. No N-terminal amino acid of the whole protein was detected by protein sequencing, indicating that the N-terminus is blocked.

Figure 3 shows the effect of pH on the enzymic activity for the forward reaction of *Anthopleura* AK. The enzyme gave a maximum activity (120 μmol of P_i /min per mg of protein) over a wide pH range (7.9–9.1).

Figure 4 shows the effects of Ca^{2+} and Mg^{2+} on the activity of *Anthopleura* AK in the presence of 1 mM ATP. Mg^{2+} ion activated the enzyme at concentrations greater than 1 mM, indicating that the MgATP^{2-} complex is required for enzymic activity, but the maximum effect was at 1.5 mM Mg^{2+} . Our enzyme assay usually contained equimolar concentrations of ATP and Mg^{2+} ; thus the ATP was not all present as MgATP^{2-} . The enzyme was also activated fully by Ca^{2+} .

The 3' half of the cDNA of *Anthopleura* AK was amplified by PCR with the phosphagen consensus primer and oligo(dT) adaptor. Although two intrinsic binding sites for the phosphagen consensus primer are present in the cDNA sequence (Figure 5), the second (nt 1858–1880) was utilized, giving a product of 600 bp. Two new oligomers, Anth.R1 and Anth.R2, were designed for amplification of the 5' half. Anth.R2 had two intrinsic binding sites (see Figure 5). Nevertheless the 5' half amplification with Anth.R2 and the oligo(dT) adaptor, by using newly synthesized cDNA with Anth.R1 as a primer, gave a product of 850 bp, indicating that the first binding site (nt 834–857) was used. Finally, the full-length cDNA of *Anthopleura* AK was amplified with the primers Anth.FW and oligo(dT) adaptor, subcloned in pUC18 and sequenced.

The complete nucleotide sequence of 2462 bp of the cDNA of *Anthopleura* AK, of which the first 75 nt were derived from sequencing of 5' clones and the remaining sequence from the Anth.FW–oligo(dT) clone, is shown in Figure 5. The sequence contains 132 and 182 bp of 5' and 3' untranslated sequences respectively. A polyadenylation signal, AATAAA (boxed in Figure 5), is present 20 bp upstream of the 3' terminus. The open reading frame is 2148 nt long and encodes a protein containing 715 residues. The molecular mass was calculated to be 79933 Da. The cDNA-derived amino acid sequence of *Anthopleura* AK was completely consistent with the chemical sequencing of tryptic peptides (underlined in the amino acid sequence of Figure 5). This is the first two-domain AK to be sequenced.

A bridge intron that separates the two domains was amplified with genomic DNA as template, and the sequence of 686 bp, which begins with GT and ends with AG, was determined by direct sequencing of PCR products (results not shown).

DISCUSSION

In the present study, AK from the sea anemone *Anthopleura japonicus*, a member of one of the most primitive invertebrate groups, has been isolated by gel filtration and ion-exchange

chromatography. The isolated enzyme is highly specific for arginine and could not use creatine, glycoylamine or taurocyamine as substrate. The molecular masses by SDS/PAGE and Superose 12 indicate that *Anthopleura* AK is a monomeric enzyme of 80 kDa. Because the subunit mass of all AKs so far examined has been 40 kDa, the unusual 80 kDa unit of *Anthopleura* AK suggests a two-domain structure. This was confirmed later by cDNA sequencing of *Anthopleura* AK.

Baker [14] partly purified AK from the sea anemone *Condylactis aurantiaca*. This enzyme differs markedly from *Anthopleura* AK in that *Condylactis* AK was found to be a tetramer of 40 kDa subunits. We have not found any AK with a 40 kDa subunit in the sea anemone *Anthopleura*.

Multi-domain CKs are known for a sea urchin, *Strongylocentrotus purpuratus* [15], and the trematode *Schistosoma mansoni* [16]. The sea urchin CK has a three-domain structure, each of the domains showing 66–70% amino acid sequence identity. The trematode CK has an incomplete two-domain structure, of which the second domain lacks the C-terminal 50 residues. The presence of two-domain phosphagen kinases in the primitive invertebrates *Schistosoma* and *Anthopleura* suggests that gene duplication events occurred frequently during the evolution of phosphagen kinase. Such events are expected to act as a driving force in generating various phosphagen kinases including GK, taurocyamine kinase and lombricine kinase.

The specific activity (100–120 μmol of P_i /min per mg of protein) for the purified *Anthopleura* AK was rather low compared with that of the shrimp *Penaeus* AK (Figure 3). We do not know the reason for this but the phosphagen kinases from invertebrates are structurally unstable in general, except arthropod AKs [2]. Thus a fraction of the total AK protein in the assay could have been in the inactive state.

The pH optimum for the forward reaction of *Anthopleura* AK occurs over a wide pH range, 7.9–9.1 (Figure 3), but the overall pH profile is very similar to that of shrimp AK. The pH optima of various phosphagen kinases are reported to be in the pH range 8.4–9.1 [2].

Mg^{2+} ions are required for the phosphagen kinase activity to generate the MgATP^{2-} complex. With most phosphagen kinases, Ca^{2+} also partly activates them. For earthworm lombricine kinase and shrimp AK, an appropriate concentration of Ca^{2+} gave approx. 30% activation in the absence of Mg^{2+} ions [10,17]. In this respect it should be noted that *Anthopleura* AK is fully activated by Ca^{2+} , as it is by Mg^{2+} (Figure 4).

We have amplified the bridge intron that separates the two domains of 686 bp in the gene of *Anthopleura* two-domain AK. The intron was located between the second and third nucleotides in the codon of Ala-364. Thus the two domains of *Anthopleura* AK can be separated between Ala-364 and Lys-365.

The cDNA-derived amino acid sequences of domains 1 and 2 of *Anthopleura* AK are aligned in Figure 6 (upper panel) with those of lobster AK [18], shrimp AK [8,19], *Limulus* AK [20], *Battillus* AK [21], abalone AK [8], chiton AK [21], *Schistosoma* CK [16], marine worm GK [8] and rabbit CK [22], by using the algorithm of Feng and Doolittle [23].

In the alignment (Figure 6, upper panel), there are 63 amino acid residues (indicated by asterisks) conserved in all of the

Figure 6 Comparison of amino-acid sequences of AK, GK and CK, and their phylogenetic relationship

Upper panel: alignment of the amino acid sequence of *Anthopleura* AK with those of rabbit CK, marine worm GK and invertebrate AKs. This alignment was obtained with the algorithm of Feng and Doolittle [23]. Abbreviations: s.aneD1, sea anemone domain 1; s.aneD2, sea anemone domain 2; *Schist.*, *Schistosoma*; m.worm, marine worm. Symbols: *, invariant residue; #, candidate residue for acid/base catalysis; @, residue involved in co-ordinating Mg^{2+} to ATP. Lower panel: a phylogenetic tree constructed from the 11 sequences of phosphagen kinases aligned in the upper panel. The numbers at each branching point show bootstrap values (200 replications).

phosphagen kinases. These residues include the cysteine residue (position 288) that is located in the centre of the active site [3,24], tryptophan residues (positions 216 and 233) involved in the binding site of ATP [25], and a histidine residue (position 99) that is the most likely candidate for a general acid/base catalyst that deprotonates the guanidium group [26,27]. These residues are marked with # in Figure 6 (upper panel). A recent crystal structure of chicken cardiac mitochondrial CK has established the involvement of these residues in its function [28], together with Glu-236, Glu-237 and Asp-238 (marked with @ in Figure 6, upper panel), which are strictly conserved through all phosphagen kinases and are involved in co-ordinating Mg^{2+} to ATP.

Domains 1 and 2 of *Anthopleura* AK show a high identity (77%), and the level of similarity between *Anthopleura* AK and other AKs is still high (48–53%). These sequence homologies would be enough to conclude that CK, GK and AK are derived from a common origin.

A phylogenetic tree was constructed from the sequence alignment in Figure 6 (upper panel) with the neighbour-joining method in the PHYLIP package, version 3.5c [29]. As shown in Figure 6 (lower panel), the AK sequences are clearly separate from the GK and CK sequences. The AK cluster consists of three subclusters, those of molluscan AKs, arthropod AKs and sea anemone AKs. The domain 1 sequence of *Schistosoma* CK was placed near the molluscan AK subcluster. The enzymic activity of *Schistosoma* CK is reported to be extremely weak but reproducible [16], but the sequence of *Schistosoma* CK is apparently homologous with AKs rather than CK (see Figure 6). The subcluster containing two sequences of *Anthopleura* AK is outside the molluscan and arthropod AKs, consistent with the traditional phylogeny.

The evolutionary origin of phosphagen kinases is of primary concern. In this study we sequenced an unusual two-domain AK from a primitive invertebrate. This is the first observation of a two-domain structure for any AK. Of the phosphagen kinases, AK is the most widely distributed in animals: it is found even in protozoans such as *Tetrahymena* [30]. Furthermore the substrate of AK, arginine, is obtained without evolving any special enzyme, whereas the biosynthesis of the remaining guanidine substrates (creatine, glycocyamine, taurocyamine and lombricine) needs a specific enzyme [7]. These results have been used to support the hypothesis that AK is closer to an ancestral phosphagen kinase. Our phylogenetic analysis (Figure 6, lower panel) is fully supportive of this proposition.

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