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# EXPRESSION OF ARGININE KINASE ENZYMATIC ACTIVITY AND mRNA IN GILLS OF THE EURYHALINE CRABS *CARCINUS MAENAS* AND *CALLINECTES SAPIDUS*

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## Summary

**Phosphagen kinases catalyze the reversible dephosphorylation of guanidino phosphagens such as phosphocreatine and phosphoarginine, contributing to the restoration of adenosine triphosphate concentrations in cells experiencing high and variable demands on their reserves of high-energy phosphates. The major invertebrate phosphagen kinase, arginine kinase, is expressed in the gills of two species of euryhaline crabs, the blue crab *Callinectes sapidus* and the shore crab *Carcinus maenas*, in which energy-requiring functions include monovalent ion transport, acid–base balance, nitrogen excretion and gas exchange. The enzymatic activity of arginine kinase approximately doubles in the ion-transporting gills of *C. sapidus*, a strong osmoregulator, when the crabs are transferred from high to low salinity, but does not change in *C. maenas*, a more modest osmoregulator. Amplification and sequencing of arginine kinase cDNA from both species, accomplished by reverse transcription of gill mRNA and the polymerase chain reaction, revealed an open reading frame coding for a 357-amino-acid protein. The predicted amino acid sequences showed a minimum of 75 % identity with arginine kinase sequences of other arthropods. Ten of the 11 amino acid residues believed to participate in arginine binding are completely conserved among the arthropod sequences analyzed. An estimation of arginine kinase mRNA abundance indicated that acclimation salinity has no effect on arginine kinase gene transcription. Thus, the observed enhancement of enzyme activity in *C. sapidus* probably results from altered translation rates or direct activation of pre-existing enzyme protein.**

Key words: phosphagen kinase, arginine kinase, osmoregulation, gene expression, gill, crab, *Callinectes sapidus*, *Carcinus maenas*.

## Introduction

Phosphagen kinases belong to a class of enzymes that catalyze the reversible transfer of a phosphoryl group from a guanidino phosphagen to adenosine diphosphate (ADP), generating a molecule of adenosine triphosphate (ATP). At least seven functionally distinct but phylogenetically related phosphagen kinases occur in the animal kingdom: arginine kinase, creatine kinase, glycocyamine kinase, hypotaurocyamine kinase, lombricine kinase, opheline kinase and taurocyamine kinase (Suzuki et al., 1997). Creatine kinase is the only guanidino kinase found in vertebrates, but it is also present in some invertebrates such as sponges, polychaetes, phoronids and echiuroids (Urich, 1990). Arginine kinase is the most widely distributed phosphagen kinase, being found throughout the invertebrates and in hemichordates and tunicates as well, and is usually considered to be most closely related to the ancestral phosphagen kinases (Suzuki et al., 1997).

The phosphagen kinases have been studied primarily in muscle tissues, where they function in buffering the ATP supply during periods of high energy demand by regenerating depleted ATP supplies (Wallimann et al., 1992). In some tissues, phosphagen kinases may also function as an energy shuttle between mitochondria and the cytosol, a process facilitated by the presence of separate isoforms in each compartment (Bessman and Carpenter, 1985). Because the phosphagens are typically present in higher concentrations than ADP and ATP, and because they are thought to diffuse more rapidly, the shuttle system increases the capacity of high-energy phosphate transport between mitochondria and cytosol. While the buffer function relies only on a cytoplasmic phosphagen kinase, the shuttle system depends on both mitochondrial and cytosolic forms.

Tissues requiring a large continuous delivery of ATP may utilize the shuttle process, while tissues that go through short bursts of energy demand may use mainly the buffering function of the phosphagen kinases. This suggestion is supported by the observation that the proportion of mitochondrial phosphagen kinase activity in vertebrate cardiac muscle (up to 35 % of total activity) is much higher than that in fast-twitch skeletal muscle (0.5–2 %) (Wyss et al., 1995).

Tissues other than muscle that experience a high energy demand may also benefit from an ATP-buffering or energy-shuttle system. Among such tissues are the gills of euryhaline crustaceans, which achieve high rates of osmoregulatory ion uptake from reduced-salinity environments, driven by the hydrolysis of ATP. Active influx of $Na^+$ from the medium across the gill epithelium is energized in part by the $Na^+$ pump ($Na^+/K^+$-ATPase) situated in the basolateral membrane of specialized ion-transporting cells (Towle and Kays, 1986; Towle, 1990; Lucu, 1993). In addition, a V-type $H^+$-ATPase is present in the gills of some crab species, where it may generate an electrochemical gradient that facilitates ion uptake, particularly in freshwater-acclimated animals (Onken and Putzenlechner, 1995; Weihrauch and Towle, 1997; Wieczorek et al., 1999). These demands for phosphate bond energy suggest that the euryhaline crab gill may benefit from an ATP-buffering or energy-shuttle system that would restore the ATP supply depleted by active ion transport in fluctuating environmental salinities.

Evidence of a high-energy phosphate reserve in the gills of the blue crab *Callinectes sapidus* was obtained following treatment of isolated gills with cyanide and iodoacetate (Burnett and Towle, 1990). In spite of the presence of these metabolic inhibitors, ATP content remained surprisingly high and the rate of $Na^+$ influx was maintained at 20–50 % of control levels after 90 min of exposure. If phosphoarginine serves as a high-energy phosphate reserve in euryhaline crab gills, we would expect to find substantial activity levels of the cognate phosphagen kinase, namely arginine kinase.

In this study, we demonstrate that arginine kinase is indeed expressed in crustacean gills, at the level of both protein and mRNA. Although the enzyme activity levels in gills are much lower than those measured in heart and claw muscle, both anterior (respiratory) and posterior (ion-transporting) gills exhibit substantial levels of arginine kinase activity. An insignificant contribution of mitochondrial arginine kinase to the total activity in posterior gills suggests that arginine kinase is serving mainly as an ATP buffer rather than as part of a high-energy phosphate shuttle system. Interestingly, branchial arginine kinase activity is significantly enhanced by exposure to low salinity in *C. sapidus*, a strong osmoregulator (Mantel and Farmer, 1983), but not in *Carcinus maenas*, a more modest osmoregulator (Siebers et al., 1982).

Amplification and sequencing of arginine kinase cDNA from the gills of *C. sapidus* and the shore crab *C. maenas* revealed close sequence homologies with other arthropod arginine kinases and, indeed, with phosphagen kinases in general. Analysis of mRNA expression indicated that the accumulation of arginine kinase gene transcripts is not measurably responsive to acclimation salinity in either of the two species examined.

## Materials and methods

### *Animals*

Shore crabs (*Carcinus maenas*) were obtained from the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, USA, or were collected from intertidal regions adjacent to Mount Desert Island Biological Laboratory, Salsbury Cove, Maine, USA. Blue crabs (*Callinectes sapidus*) were obtained from the Waukegan (Illinois, USA) Oriental Food Store or from Gulf Specimen Marine Laboratory, Panacea, Florida, USA. Crabs were kept at 15 °C (*C. maenas*) or 20 °C (*C. sapidus*) in recirculating biologically filtered Instant Ocean sea water and were fed cleaned squid twice weekly.

### *Enzyme and protein assays*

Gills and other tissues were removed from crabs following anesthesia on ice and homogenized immediately or were stored at −80 °C for later use. Homogenization was accomplished in 250 mmol $l^{-1}$ sucrose, 6 mmol $l^{-1}$ disodium EDTA, 10 mmol $l^{-1}$ Tris, pH 7.4 (50 mg tissue $ml^{-1}$ homogenizing solution) using an Omni International TH rotor-stator homogenizer. The homogenate was filtered through two single layers of cheesecloth and diluted 20 times in homogenizing solution prior to the assay. In some experiments, the mitochondrial fraction was removed from the homogenate by centrifugation at 10 000 ***g*** for 30 min. All samples were stored on ice for immediate assay of enzyme activity.

The arginine kinase assay was a modification of an enzyme-linked creatine kinase assay employing phosphoarginine as substrate, with glucose, NADP, hexokinase and glucose-6-phosphate dehydrogenase in the assay buffer (Sigma Chemical Co.) (Nealon and Henderson, 1976). The appearance of NADPH following addition of substrate was monitored in a Hitachi U2000 ultraviolet-visible spectrophotometer at 340 nm and 25 °C. The assay was standardized with known amounts of ATP.

Total protein was determined in each sample using a Coomassie Brilliant Blue assay (Bradford, 1976). Arginine kinase activity was expressed on the basis of fresh tissue mass as nmol ATP $min^{-1}$ 50 $\mu g^{-1}$ tissue. Specific activity was calculated as nmol ATP $min^{-1}$ $\mu g^{-1}$ protein. Each individual homogenate or tissue fraction was assayed in triplicate, and the results were averaged. A mean of arginine kinase activities in 3–6 tissue samples is presented for each treatment. Values are presented as means ± S.E.M. Statistical analyses were performed by analysis of variance and Newman–Keuls multiple-comparison tests ($P<0.05$).

### *Preparation of RNA and cDNA*

Gills were removed following anesthesia on ice and were immediately homogenized in guanidinium isothiocyanate for the isolation of total RNA under RNAase-free conditions (Chomczynski and Sacchi, 1987) using materials supplied by Promega Corporation. RNA concentrations were quantified by measuring absorbance at 260 nm. Single-stranded cDNA was reverse-transcribed from poly(A)-containing mRNA in the total RNA samples using SuperScript II RNAse $H^-$ reverse transcriptase (Gibco-BRL) and an oligo(dT) primer.

Table 1. *Oligonucleotide primers employed to amplify arginine kinase cDNA from* Carcinus maenas *and* Callinectes sapidus

| Primer | Nucleotide sequence (5′→3′) | Location in final cDNA |
|---|---|---|
| Degenerate sense primers | | |
| 1F | GGN GTN GGN ATH TAY GCN CC | 247–266 |
| 2F | YAA RAC NTT YYT NGT NTG GTG | 702–722 |
| Specific sense primers | | |
| AUAP | GGC CAC GCG TCG ACT AGT AC | 61–80 |
| GSPF | TCC AGT CCG GTG TTG AGA | 218–235 |
| F10 | TTT CCT CCA CCC TGT CCA ACC T | 512–533 |
| F20 | TCA TCG ACG ACC ACT TCC TCT T | 599–620 |
| F51 | CGC TGA GTC TAA GAA GGG ATT | 7–27 |
| Degenerate anti-sense primers | | |
| 1R | TGR TCY TCR TTR CAC CA | 718–734 |
| 2R | YTG RAA YTC NGT NAR NCC CAT | 1048–1068 |
| ENDR2 | GCN TGG AAT GTT GNC GGG GNC T | 1303–1324 |
| Specific anti-sense primers | | |
| GSPR | CTG CTT GAA ACC CTT GTG | 325–342 |
| R10 | TCA ACA CCG GAC TGG ATC ACA T | 212–233 |
| R31 | GAT ACC GTC CTG CAT CTC CTT | 1075–1095 |
| ENDR1 | CCG GCT CGC CCT CAC CCA TAG A | 1147–1168 |

Location in the final arginine kinase cDNA sequence from *C. maenas* is presented.
H, A/C/T; N, A/C/G/T; R, A/G; Y, C/T.

### *Amplification of arginine kinase cDNA*

The initial amplification of arginine kinase cDNA was achieved serendipitously in the course of other molecular work on gill ion transport in *C. maenas*. In an application of the rapid amplification of cDNA ends (RACE) technique, the abridged universal anchor primer (AUAP) provided as a reverse primer with the 3′ RACE kit from Gibco-BRL served effectively as a forward primer for amplification of arginine kinase cDNA by the polymerase chain reaction (PCR) (Kotlyar et al., 1997). A BLAST search of GenBank (Altschul et al., 1997) indicated that the resulting PCR product was similar to arginine kinase sequences already in the database. We eventually found that the AUAP oligonucleotide matches the corresponding arginine kinase sequence from *C. maenas* at 14 of 20 bases near the 5′ terminus (Table 1). The sequence of the initial PCR product was used as the basis for designing specific oligonucleotide primers employed with degenerate primers to obtain the complete sequence of arginine kinase cDNA from *C. maenas*.

Degenerate primers were based on conserved regions in the published amino acid sequence of arginine kinases from one chelicerate, the horseshoe crab *Limulus polyphemus* (Strong and Ellington, 1995), and two crustaceans, the shrimp *Penaeus japonicus* (Furukohri et al., 1994) and the European lobster *Homarus vulgaris* (Dumas and Camonis, 1993). These degenerate primers and other non-degenerate primers were designed with the assistance of Primer Premier software (Premier Biosoft International) and were synthesized by Operon Technologies (Table 1).

Polymerase chain reactions were initiated by the hot start method using *Taq* DNA polymerase (Boehringer-Mannheim or Sigma Chemical Co.) and 30 cycles at 92 °C (60 s), 45 °C (60 s) and 72 °C (120 s) followed by a final incubation at 72 °C for 5 min in an MJ Research thermocycler. PCR products were analyzed by gel electrophoresis in 1 % agarose and 1× TAE buffer (40 mmol $l^{-1}$ Tris, 20 mmol $l^{-1}$ acetic acid, 2 mmol $l^{-1}$ EDTA, pH 7.6). DNA bands were visualized by staining with ethidium bromide and photographing over an ultraviolet light source.

The 5′ and 3′ ends of the arginine kinase cDNA from *C. maenas* were amplified using the RACE systems from Gibco-BRL. Amplification of the 5′ end was facilitated with reverse primers GSPR and R10, and the 3′ end was amplified using forward primers GSPF, F10 and F20 (Table 1). cDNA representing the complete open reading frame of *C. sapidus* arginine kinase was amplified using primers F51, R31, ENDR1 and ENDR2 (Table 1).

### *cDNA sequencing and analysis*

PCR products were excised from the agarose gels and purified using a Qiagen gel extraction kit. Eluted DNA was analyzed for purity and concentration by agarose gel electrophoresis. Purified DNA fragments were sequenced by the dideoxynucleotide method (Sanger et al., 1977) either manually using $^{35}$S-labeled dATP and 5 % polyacrylamide gels or by automated sequencing with fluorescently labeled nucleotides. Sequencing primers were selected to give appropriate overlaps with each template. Nucleotide sequence was read from both template strands with at least three independent sequencing reactions for each segment. The resulting automated traces, produced at the Marine DNA Sequencing Center at Mount Desert Island Biological Laboratory, were edited with Chromas software and assembled with the SeqMan component of DNASTAR. Multiple alignments were produced with the CLUSTALW component of ANTHEPROT software (http://www.ibcp.fr/ANTHEPROT/) (Geourjon and Deleage, 1995).

### *mRNA expression analysis*

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was accomplished by incorporating biotinylated dUTP in the PCR reaction mixture and visualizing the resulting products in the logarithmic phase of amplification using streptavidin-linked alkaline phosphatase and a chemiluminescent substrate (Phototope system, New England Laboratories) (Towle et al., 1997). Reverse transcription of each tissue RNA sample was initiated with exactly 2.0 µg of total RNA. One-tenth of the resulting cDNA was used to initiate amplification by PCR, and proportional samples were employed throughout the remaining procedure to enable final comparisons of image intensity.

## Results

The enzymatic activity of arginine kinase, expressed on the basis of wet tissue mass, was rather low in gill homogenates from *C. maenas*, particularly compared with heart and claw muscle (Fig. 1). Claw muscle, for example, showed more than 18 times the fresh-mass-based activity of gills. When the mitochondrial fraction was removed from homogenates by centrifugation at 10 000 ***g*** for 30 min, no significant changes in enzyme activity were noted in any of the tissues (Fig. 1). Calculation of specific activities revealed a relationship between the tissues similar to that observed with fresh mass activities, with heart and claw muscle exhibiting the highest values (data not shown).

A comparison of arginine kinase activities in gills of *C. maenas* and *C. sapidus* showed that *C. sapidus* gills contain 3–4 times more arginine kinase activity than *C. maenas* gills, measured on the basis of fresh tissue mass (Fig. 2). In high salinity (35 ‰), posterior gills, in which the majority of the ion-transporting epithelial cells reside, contained arginine kinase activities similar to those of the anterior gills, which are specialized for gas exchange (Taylor and Taylor, 1992). Acclimation salinity had no effect on arginine kinase activities in gills of *C. maenas*, but exposure of *C. sapidus* to low salinity (5 ‰) led to a doubling of arginine kinase activity in the posterior gills (Fig. 2).

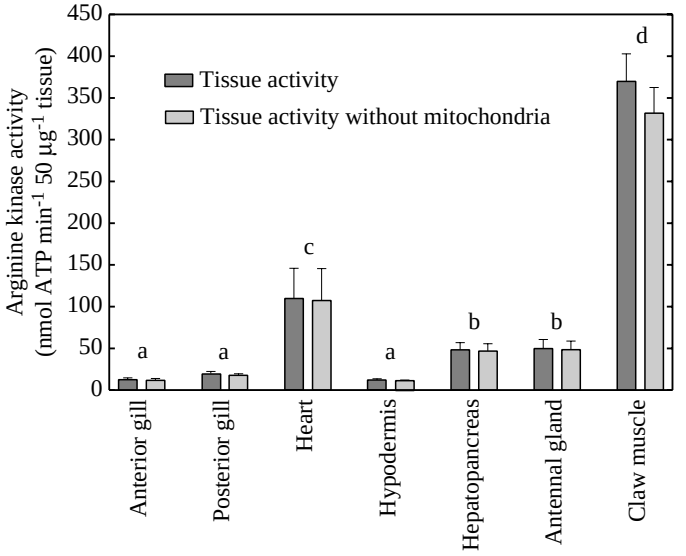


Fig. 1. Arginine kinase activity in selected tissues of the shore crab *Carcinus maenas* measured using an enzyme-linked assay method with phosphoarginine as substrate (Nealon and Henderson, 1976). Total arginine kinase activity is expressed on the basis of fresh tissue mass (dark-colored columns). Tissue homogenates were centrifuged at 10 000 ***g*** for 30 min to pellet the mitochondrial fraction; the resulting arginine kinase activities are depicted in the light-colored columns. Values are means ± S.E.M. of triplicate assays on each of three different tissue preparations. Analysis of variance across all tissue types revealed no statistical differences between total arginine kinase activities and activities without mitochondrial fractions ($P$>0.05). Activity comparisons among the tissue types were performed using an unplanned multiple-comparisons test (Student–Newman–Keuls) that showed significant differences among four groups of tissues (a–d; $P$<0.05). Columns marked with the same letter are not significantly different.

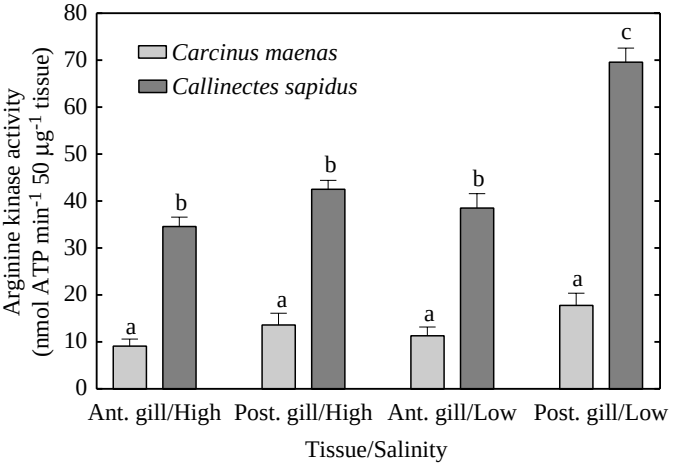


Fig. 2. Arginine kinase activities of anterior (Ant.) and posterior (Post.) gills of shore crabs *Carcinus maenas* and blue crabs *Callinectes sapidus* acclimated to high salinity (35 ‰) or low salinity (10 ‰ for *C. maenas* and 5 ‰ for *C. sapidus*). Values are means ± S.E.M. of triplicate measurements on each of five (*C. maenas*) or six (*C. sapidus*) separate preparations. A multiple-comparisons test (Student–Newman–Keuls) revealed significant differences between species (a *versus* b and c) and a significant effect of acclimation salinity in posterior gills of *C. sapidus* (b *versus* c) ($P$<0.05).

To determine whether the observed increase in arginine kinase activity in the gills of low-salinity-acclimated *C. sapidus* was the result of enzyme activation or enhancement of gene transcription, we amplified and sequenced arginine kinase cDNA from both *C. maenas* and *C. sapidus*. Degenerate and specific oligonucleotide primers (Table 1) were used to amplify arginine kinase cDNA segments. The resulting PCR products were separated on agarose electrophoresis gels (Fig. 3) and then extracted to serve as templates for sequencing.

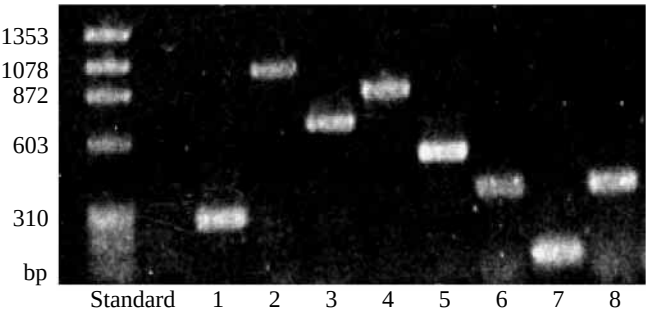


Fig. 3. Agarose gel electrophoresis of polymerase chain reaction products amplified from cDNA prepared from posterior gills of the shore crab *Carcinus maenas*. Primer combinations, designed to amplify arginine kinase cDNA, were as follows: lane 1, AUAP/GSPR; lane 2, AUAP/2R; lane 3, AUAP/1R; lane 4, GSPF/2R; lane 5, GSPF/1R; lane 6, 1F/2R; lane 7, GSPF/GSPR; lane 8, 2F/2R (see Table 1). Base pair (bp) size standards are shown in the left-hand lane.

The putative arginine kinase cDNA from *C. maenas* gill consists of a 1475-nucleotide sequence (GenBank Accession Number AF167313) with an open reading frame coding for a 357-amino-acid protein (Fig. 4). The molecular mass of the predicted protein was calculated to be 40 235 Da. Using the 1475-nucleotide cDNA as the query sequence, a gapped

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   1  ACT CGC CGC TGA GTC TAA GAA GGG ATT GGA CGC CAA GGG TCT TCA CAG      48
  49  AAC ACA ACA ATG GCC GAC GCT GCA ACT ATT ACC AAG CTG GAG GAG GGC      96
   1              M   A   D   A   A   T   I   T   K   L   E   E   G      13

  97  TTC AAG AAG CTG GAG GCT GCC ACT GAC TGC AAG TCT CTC CTG AAG AAG     144
  14   F   K   K   L   E   A   A   T   D   C   K   S   L   L   K   K      29

 145  TAC CTC ACC AAG AGT GTG TTC GAC CAG CTC AAG GCC AAG AAG ACT AGC     192
  30   Y   L   T   K   S   V   F   D   Q   L   K   A   K   K   T   S      45

 193  CTG GGC GCC ACC CTC CTC GAT GTG ATC CAG TCC GGT GTT GAG AAC CTG     240
  46   L   G   A   T   L   L   D   V   I   Q   S   G   V   E   N   L      61

 241  GAC TCC GGC GTT GGT GTG TAT GCC CCT GAC GCC GAG GCC TAC ACT CTC     288
  62   D   S   G   V   G   V   Y   A   P   D   A   E   A   Y   T   L      77

 289  TTC TCC CCA CTG TTC GAC CCC ATC ATC GAG GAC TAC CAC AAG GGT TTC     336
  78   F   S   P   L   F   D   P   I   I   E   D   Y   H   K   G   F      93

 337  AAG CAG ACT GAC AAG CAC CCT AAC AAG GAC TTC GGC GAT GTC AAC CAG     384
  94   K   Q   T   D   K   H   P   N   K   D   F   G   D   V   N   Q     109

 385  TTC GTC AAC GTG GAC CCC GAT GGC AAA TTC GTG ATC TCT ACT CGC GTG     432
 110   F   V   N   V   D   P   D   G   K   F   V   I   S   T   R   V     125

 433  CGT TGC GGT CGC TCC ATG GAG GGC TAC CCC TTC AAC CCC TGT CTC ACC     480
 126   R   C   G   R   S   M   E   G   Y   P   F   N   P   C   L   T     141

 481  GAG GCT CAG TAC AAG GAG ATG GAA TCA AAG GTT TCC TCC ACC CTG TCC     528
 142   E   A   Q   Y   K   E   M   E   S   K   V   S   S   T   L   S     157

 529  AAC CTC GAA GGC GAG CTC AAG GGT ACC TAC CAT GCT CTT ACT GGC ATG     576
 158   N   L   E   G   E   L   K   G   T   Y   H   A   L   T   G   M     173

 577  ACC AAG GAT GTC CAG CAG AAG CTC ATC GAC GAC CAC TTC CTC TTC AAG     624
 174   T   K   D   V   Q   Q   K   L   I   D   D   H   F   L   F   K     189

 625  GAG GGT GAC CGT TTC CTG CAG GCC GCC AAT GCT TGC CGC TAC TGG CCC     672
 190   E   G   D   R   F   L   Q   A   A   N   A   C   R   Y   W   P     205

 673  ACC GGC CGT GGC ATC TAC CAC AAC GAC AAC AAG ACC TTC CTT GTC TGG     720
 206   T   G   R   G   I   Y   H   N   D   N   K   T   F   L   V   W     221

 721  TGT AAC GAA GAG GAT CAC CTC CGC ATC ATC TCC ATG CAG ATG GGT GGT     768
 222   C   N   E   E   D   H   L   R   I   I   S   M   Q   M   G   G     237

 769  GAC CTG GGC CAG GTT TAC CGC CGC CTT GTC ACT GCT GTC AAC GAT ATT     816
 238   D   L   G   Q   V   Y   R   R   L   V   T   A   V   N   D   I     253

 817  GAG AAG CGT GTT CCC TTC TCC CAC CAC GAC CGC CTG GGC TTC CTC ACC     864
 254   E   K   R   V   P   F   S   H   H   D   R   L   G   F   L   T     269

 865  TTC TGC CCC ACC AAC CTT GGC ACC ACT GTA CGT GCC TCC GTC CAC ATC     912
 270   F   C   P   T   N   L   G   T   T   V   R   A   S   V   H   I     285

 913  AAG CTC CCC AAG CTC GCC GCC AAC CGT GAC AAG CTC GAG GAA GTC GCT     960
 286   K   L   P   K   L   A   A   N   R   D   K   L   E   E   V   A     301

 961  GGC AAG TAC AGT CTC CAG GTT CGC GGC ACC CGC GGC GAG CAC ACC GAG    1008
 302   G   K   Y   S   L   Q   V   R   G   T   R   G   E   H   T   E     317

1009  GCT GAG GGT GGT GTT TAC GAT ATC TCC AAC AAG CGC CGC ATG GGT CTT    1056
 318   A   E   G   G   V   Y   D   I   S   N   K   R   R   M   G   L     333

1057  ACT GAA TTC CAG GCT GTT AAG GAG ATG CAG GAC GGT ATC CTT GAG CTC    1104
 334   T   E   F   Q   A   V   K   E   M   Q   D   G   I   L   E   L     349

1105  ATC AAG ATT GAG AAG GAG ATG CAG TAA AGT TCG GCT CTT CCC TCT ATG    1152
 350   I   K   I   E   K   E   M   Q                                     357

1153  GGT GAG GGC GAG CCG GTC TCT GCT GTA GGA AGA GGG CGC CCC ACC CTG    1200
1201  GAC CCG GGG CCC AGG AGA GTG GCG CTG GAT TAG GCG CTG GAT TAG GCA    1248
1249  GTG ATC AGC CCG GCA ACA AAC CAA ATA ACC TAG CTA GCT GAT GAA GCC    1296
1297  AAC CTT AGA CCC CGC CAA CAT TCC ACG CAG CCG GGG CTT GTA GCC GGG    1344
1345  CCG GGC GTG GTG GCC TTG TAC ATA CCA TCT ATC GTA AAG ACG CGC CCA    1392
1393  GCC CTC GCT ACA TCA CTT TCA ACT GTT ATC ACA TTA ACA TTA TAA CAG    1440
1441  TAA ACC TCT AAA AAT CCT CAA AAA AAA AAA AAA AA                     1475
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Fig. 4. Complete cDNA sequence of arginine kinase from the gills of the shore crab *Carcinus maenas* determined by direct sequencing of polymerase chain reaction products. Partial sequences were edited using Chromas software and assembled using the SeqMan component of DNASTAR. Two in-frame stop codons (red) are indicated in the 5′ untranslated region upstream from the probable start codon, which is framed by a consensus sequence **A**CA*ATG***G** (Kozak, 1991) (GenBank Accession Number AF167313). A polyadenylation signal cannot be stringently assigned; however, an AT-rich region occurs approximately 20 nucleotides upstream from the start of the poly(A) site (underlined).

BLAST search (Altschul et al., 1997) of the GenBank database on 15 February 2000 revealed 22 related sequences with bit scores greater than 300, all identified as arginine kinases from a variety of invertebrate phyla.

Amplification and sequencing of arginine kinase cDNA from *C. sapidus* gill produced a partial cDNA sequence of 1256 nucleotides (GenBank Accession Number AF233355) that contained a complete open reading frame coding for a 357-amino-acid protein (Fig. 5). The calculated molecular mass of the predicted arginine kinase protein from *C. sapidus* is 40 313 Da.

The arginine kinase cDNA sequences from both crab species

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   1  CGC CAG GGG TCT TCA CAG AAC ACA ATG GCT GAC GCT GCT ACC ATT GCC    48
   1                              M   A   D   A   A   T   I   A      8

  49  AAG CTG GAG GAG GGC TTC AAG AAG CTG GAG GCT GCC ACT GAC TGC AAG    96
   9   K   L   E   E   G   F   K   K   L   E   A   A   T   D   C   K    24

  97  TCT CTC CTC AAG AAG TAC CTC ACC AAG TCT GTG TTC GAC CAG CTC AAG   144
  25   S   L   L   K   K   Y   L   T   K   S   V   F   D   Q   L   K    40

 145  GAC AAG AAG ACA AGC CTG GGC GCC ACC CTC CTC GAT GTG ATC CAG TCT   192
  41   D   K   K   T   S   L   G   A   T   L   L   D   V   I   Q   S    56

 193  GGT GTT GAG AAC CTG GAC TCT GGC GTT GGT GTG TAC GCC CCT GAC GCC   240
  57   G   V   E   N   L   D   S   G   V   G   V   Y   A   P   D   A    72

 241  GAG GCC TAC ACC CTC TTC GCC CCT CTG TTC GAC CCC ATC ATC GAG GAC   288
  73   E   A   Y   T   L   F   A   P   L   F   D   P   I   I   E   D    88

 289  TAC CAC AAG GGT TTC AAG CAG ACT GAC AAG CAC CCT AAC AAG GAC TTC   336
  89   Y   H   K   G   F   K   Q   T   D   K   H   P   N   K   D   F   104

 337  GGC GAT GTC AAC CAG TTC GTC AAC GTG GAC CCC GAT GGC AAG TTC GTG   384
 105   G   D   V   N   Q   F   V   N   V   D   P   D   G   K   F   V   120

 385  ATC TCC ACT CGC GTG CGT TGC GGT CGC TCC ATG GAG GGC TAC CCC TTC   432
 121   I   S   T   R   V   R   C   G   R   S   M   E   G   Y   P   F   136

 433  AAC CCC TGT CTC ACC GAG GCC CAG TAC AAG GAG ATG GAG TCC AAG GTT   480
 137   N   P   C   L   T   E   A   Q   Y   K   E   M   E   S   K   V   152

 481  TCC TCC ACC CTG TCC AAC CTC GAG GGC GAG CTC AAG GGC ACT TAC TTC   528
 153   S   S   T   L   S   N   L   E   G   E   L   K   G   T   Y   F   168

 529  CCT CTC ACC GGC ATG ACC AAG GAA GTG CAG CAA AAG CTG ATC GAC GAC   576
 169   P   L   T   G   M   T   K   E   V   Q   Q   K   L   I   D   D   184

 577  CAC TTC CTC TTC AAG GAG GGT GAC CGC TTC CTG CAG GCC GCT AAT GCC   624
 185   H   F   L   F   K   E   G   D   R   F   L   Q   A   A   N   A   200

 625  TGC CGC TAC TGG CCC ACT GGC CGT GGC ATC TAC CAC AAC GAC AAC AAG   672
 201   C   R   Y   W   P   T   G   R   G   I   Y   H   N   D   N   K   216

 673  ACC TTC CTG GTG TGG TGC AAC GAA GAG GAT CAC CTC CGC ATC ATC TCC   720
 217   T   F   L   V   W   C   N   E   E   D   H   L   R   I   I   S   232

 721  ATG CAG ATG GGT GGT GAC CTG GGC CAG GTC TAC CGC CGC CTT GTC AGC   768
 233   M   Q   M   G   G   D   L   G   Q   V   Y   R   R   L   V   S   248

 769  GCT GTT AAC GAG ATT GAG AAG CGC GTG CCC TTC TCC CAC CAT GAC CGC   816
 249   A   V   N   E   I   E   K   R   V   P   F   S   H   H   D   R   264

 817  CTG GGC TTC CTC ACC TTC TGC CCC ACC AAC CTT GGC ACC ACC GTG CGT   864
 265   L   G   F   L   T   F   C   P   T   N   L   G   T   T   V   R   280

 865  GCC TCC GTC CAC ATC AAG CTC CCC AAG CTG GCC GCC AAC CGC GAA AAG   912
 281   A   S   V   H   I   K   L   P   K   L   A   A   N   R   E   K   296

 913  CTT GAG GAA GTC GCC GGC AAG TAC AGC CTC CAG GTT CGC GGC ACC CGC   960
 297   L   E   E   V   A   G   K   Y   S   L   Q   V   R   G   T   R   312

 961  GGC GAG CAC ACC GAG GCT GAG GGC GGC GTG TAC GAC ATC TCC AAC AAG  1008
 313   G   E   H   T   E   A   E   G   G   V   Y   D   I   S   N   K   328

1009  CGC CGC ATG GGT CTC ACT GAG TAC CAG GCT GTC AAG GAG ATG CAG GAC  1056
 329   R   R   M   G   L   T   E   Y   Q   A   V   K   E   M   Q   D   344

1057  GGT ATC CTT GAG CTC ATC AAG ATT GAG AAG GAA ATG CAG TAA AGT TCG  1104
 345   G   I   L   E   L   I   K   I   E   K   E   M   Q              357

1105  GCT CCT CCC TCT ATG GGT GAG GGC GAG CCG GTC TCT GCT GTA GGA AGA  1152
1153  GGG TGC CCC ACC CTG GAC CCG GGG CCC AGG AGA GTG GCG CTG GAT TTG  1200
1201  GCA TTT GTC AGC CCG GCA ACA AAC CAT CTT TAA CCT AGC GAG CTG ATG  1248
1249  AAG CAG CT                                                     1256
```

Fig. 5. Partial cDNA sequence of arginine kinase from the gills of the blue crab *Callinectes sapidus* determined by direct sequencing of polymerase chain reaction products generated with oligonucleotide primers F51, ENDR1 and ENDR2 (see Table 1). A complete open reading frame coding for a 357-amino-acid polypeptide is included within the cDNA sequence (GenBank Accession Number AF233355). A Kozak consensus sequence (**A**CA*ATG***G**) frames the probable start codon, as in the *Carcinus maenas* sequence.

contain a consensus sequence around the likely start codon (**A**CA*ATG***G**) (Kozak, 1991) (Figs 4, 5). Although the most frequently found polyadenylation signals (AATAAA or ATTAAA) do not appear near the 3′ terminus, an AT-rich region occurs approximately 20 nucleotides upstream from the beginning of the poly(A) site (Fig. 4). A single nucleotide substitution within this region would give one of the common signal hexamers, consistent with the variations noted in polyadenylation signals in a variety of non-mammalian species (Graber et al., 1999).

The predicted amino acid sequences of the two brachyuran arginine kinases reported here were aligned with previously published arthropod arginine kinases: European lobster *Homarus gammarus* (Dumas and Camonis, 1993), shrimp *Penaeus japonicus* (Furukohri et al., 1994), honeybee *Apis mellifera* (Kucharski and Maleszka, 1998) and horseshoe crab *Limulus polyphemus* (Strong and Ellington, 1995) (Fig. 6). The arginine kinase amino acid sequences of the six selected arthropod species are highly conserved, showing at least 75 % amino acid identity when compared pairwise with each other. The amino acid sequences of *C. maenas* and *C. sapidus* arginine kinases are 96 % identical. An unrooted phylogenetic tree generated from the multiple alignment clustered the brachyuran crabs within a larger cluster that included all crustacean species (Fig. 7).

Estimation of arginine kinase mRNA abundance by quantitative RT-PCR indicated that our method is sufficiently sensitive to detect a twofold difference in template availability (Fig. 8A), confirming an earlier study on $Na^+/H^+$ exchanger mRNA in gills of *C. maenas* (Towle et al., 1997). We were unable to detect any substantial differences in arginine kinase mRNA abundance between anterior and posterior gills of *C. maenas* and *C. sapidus* (Fig. 8B). Moreover, acclimation salinity appeared to have no measurable effect on accumulation of arginine kinase mRNA in either species. Thus, the increase in enzymatic activity noted in the gills of *C. sapidus* acclimated to reduced salinity is not likely to be the result of an enhanced abundance of arginine kinase gene transcript but rather may be produced by altered translation rate or, more likely, enzyme activation.

## Discussion

Although arginine kinase activities in gills are much lower than those in claw muscle and heart (Fig. 1), the gills of osmoregulating crabs do contain measurable levels of arginine

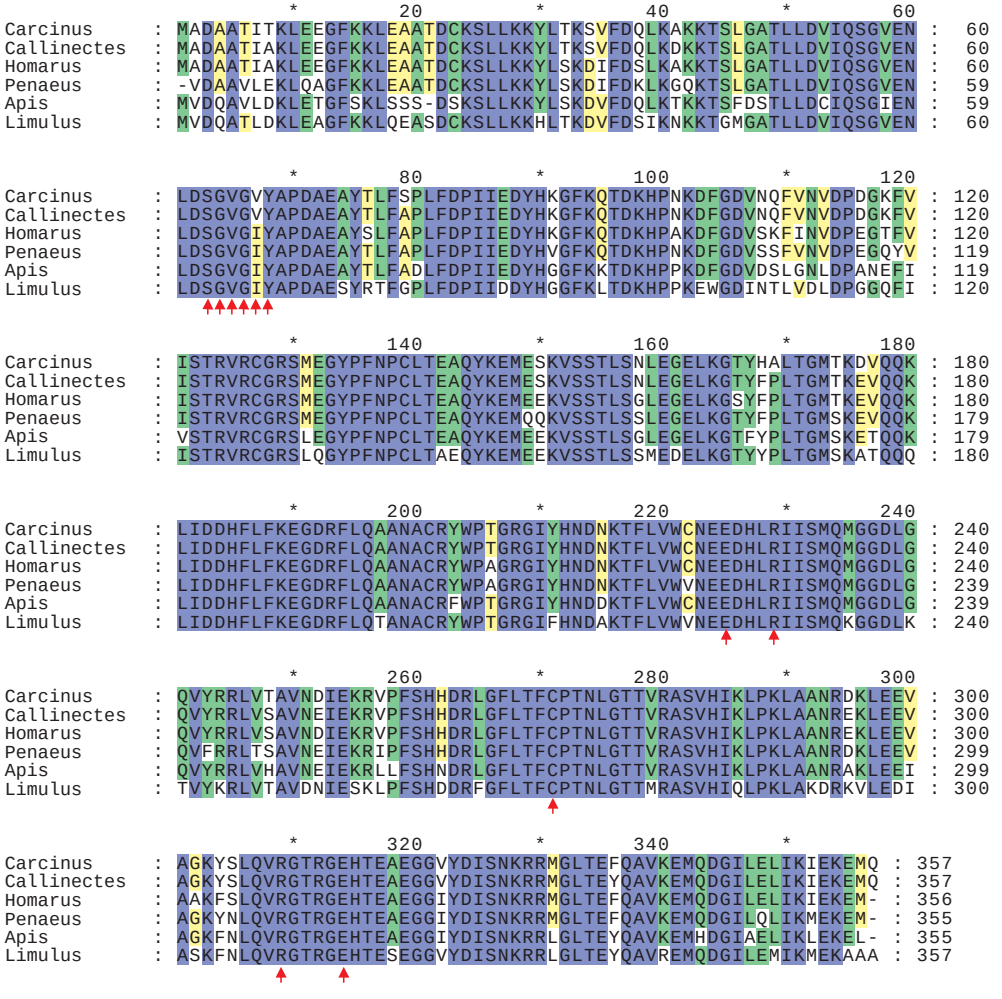


Fig. 6. Multiple alignment of *Carcinus maenas* and *Callinectes sapidus* arginine kinase amino acid sequences with four arthropod sequences from the literature (accession numbers in parentheses following each citation): European lobster *Homarus gammarus* (Dumas and Camonis, 1993) (P14208), shrimp *Penaeus japonicus* (Furukohri et al., 1994) (P51545), honeybee *Apis mellifera* (Kucharski and Maleszka, 1998) (AAC39040) and horseshoe crab *Limulus polyphemus* (Strong and Ellington, 1995) (P51541). Alignment was produced using CLUSTALW (http://www.ibcp.fr/antheprot.html) and GeneDoc (http://www.concentric.net/Ketchup/genedoc.shtml). The blue background signifies complete homology, green signifies homology in five of the six species, and yellow signifies homology in four of the six species. Residues shown to be involved with arginine binding by the arginine kinase of *L. polyphemus* (Zhou et al., 1998) are indicated by red arrows.

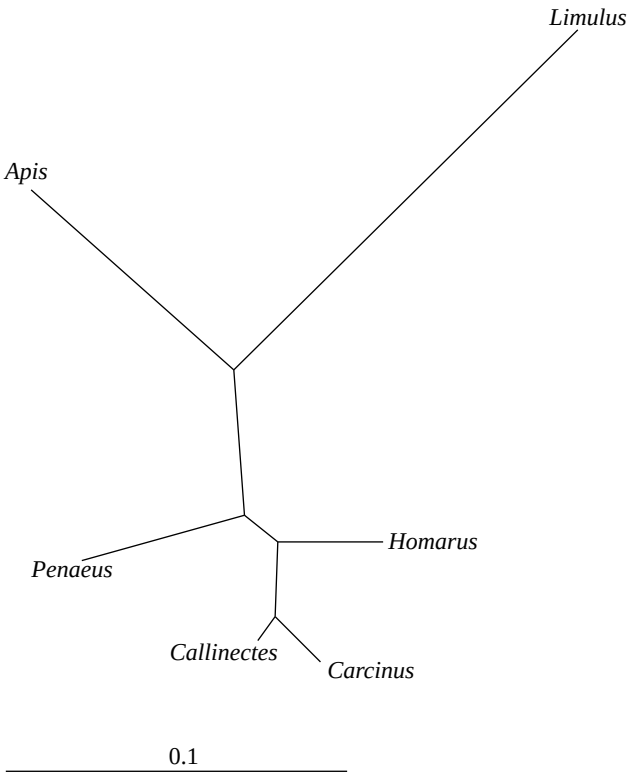


Fig. 7. Unrooted phylogenetic tree depicting distance relationships of six arthropod arginine kinase sequences, produced using CLUSTALW and TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The scale represents 10 % (0.1) amino acid divergence.

kinase activity, suggesting that phosphoarginine plays some role in the metabolic support of gill function. None of the tissues examined appeared to contain a significant contribution of mitochondrial arginine kinase activity. A mitochondrial form of arginine kinase is rare among invertebrate species, although it is found in the heart of the horseshoe crab *Limulus polyphemus* (Doumen and Ellington, 1990) and the crayfish *Procambarus clarkii* (Ellington and Hines, 1991) and in the midgut of the tobacco hornworm *Manduca sexta* (Chamberlin, 1997). The possibility of a mitochondrial arginine kinase in the flight muscle of the fruit fly *Drosophila melanogaster* is controversial (Wyss et al., 1995). Of particular relevance to the present study is the immunolocalization of arginine kinase in the mitochondria of heart muscle in the blue crab *C. sapidus* (Pineda and Ellington, 1998), supporting an earlier indication of a mitochondrial arginine kinase in the hepatopancreas of the same species (Chen and Lehninger, 1973). The apparent abundance of cytosolic arginine kinase in our assays may have masked any significant mitochondrial contribution.

The apparent lack of mitochondrial arginine kinase in gills suggests that the arginine kinase/phosphoarginine system is serving primarily as an ATP buffer in the cytosol. Because the gill is a multifunctional organ, participating in acid–base balance, nitrogen excretion and ion transport as well as gas exchange (Taylor and Taylor, 1992), it is difficult to partition the most likely destinations of high-energy phosphate reserves. One indication of a possible role of arginine kinase in ion transport is gained from our observation of increased enzymatic activity in ion-transporting posterior gills of blue crabs (*C. sapidus*) transferred from high salinity (35 ‰) to low salinity (5 ‰) (Fig. 2). Moreover, the strongly osmoregulating blue crab showed substantially higher branchial arginine kinase activity overall compared with the more modestly osmoregulating shore crab.

ATP buffering by phosphoarginine and arginine kinase is likely to be temporal as well as spatial in the sense that it may help to meet rapidly rising requirements for ATP as a consequence of tidal salinity reductions or rainfall runoff. The comparatively low $K_{eq}$ for the arginine kinase reaction (13 % of the $K_{eq}$ for creatine kinase) and the relative stability of phosphoarginine are thought to increase the efficiency of phosphoarginine as an energy buffer when the ATP/ADP ratio is low, as might be expected under conditions of osmotic stress (Ellington, 1989). *In vivo* measurements of mobile phosphorus metabolites in the leg muscle of *C. maenas* using $^{31}$P-NMR

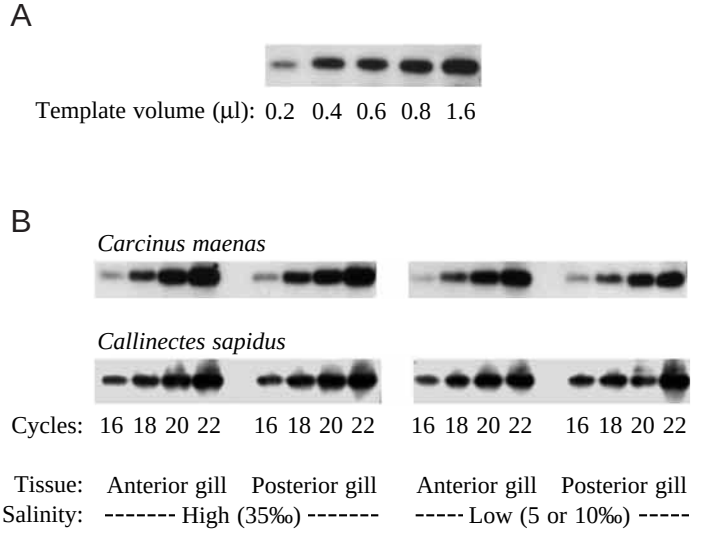


Fig. 8. Expression analysis of arginine kinase mRNA abundance using quantitative reverse transcription polymerase chain reaction. (A) Relationship between template availability (μl of cDNA template per 50 μl reaction volume) and product abundance after 18 cycles of amplification. (B) Apparent arginine kinase mRNA abundance in total RNA extracted from anterior and posterior gills of shore crab *Carcinus maenas* and blue crab *Callinectes sapidus* acclimated to high salinity (35 ‰) or low salinity (10 ‰ for *C. maenas* and 5 ‰ for *C. sapidus*). Amplification was initiated with 2 μl of cDNA template (the product from reverse-transcribing 200 ng of total RNA) per 100 μl reaction volume, and samples were taken after the number of cycles indicated.

revealed concentrations of 78.7 mmol $l^{-1}$ for phosphoarginine, 9.0 mmol $l^{-1}$ for ATP and 2.6 mmol $l^{-1}$ for inorganic phosphate (Briggs et al., 1985). The abundant phosphoarginine would serve as a very effective energy reserve delivery system, a function that may also exist in ion-transporting cells of the gill.

The arginine kinase/phosphoarginine system may also serve to provide locally high concentrations of ATP associated with specific ATP-utilizing pathways, including $Na^+/K^+$-ATPase in the basolateral membrane of branchial ion-transporting cells (Towle and Kays, 1986) and the V-type $H^+$-ATPase that may reside either in intracellular vesicles or on the apical membrane (Onken and Putzenlechner, 1995; Weihrauch and Towle, 1997). An intriguing role for phosphoarginine itself has been described in squid giant axons, in which phosphoarginine (in the absence of ATP) is apparently capable of stimulating $Na^+/Ca^{2+}$ exchange directly (Dipolo and Beauge, 1995). Whether it supplies such a function in crustacean gill is unknown.

Prior to 1993, the only phosphagen kinase described at the molecular level was creatine kinase. Since then, a large number of invertebrate phosphagen kinase sequences have been determined, the first being arginine kinase from the lobster *Homarus vulgaris* (Dumas and Camonis, 1993). Phylogenetic analysis of 18 phosphagen kinase sequences suggests a bifurcated tree, with arginine kinases in one cluster and the other phosphagen kinases in a second cluster (Suzuki et al., 1997). An interesting variant to this scheme is the dimeric arginine kinase of the sea cucumber *Stichopus japonicus*, which appears to be more closely related to vertebrate creatine kinases than to the other invertebrate arginine kinases (Suzuki et al., 1999). The arginine kinases from *C. maenas* and *C. sapidus* reported here clearly fit within the major cluster of invertebrate arginine kinases.

Phosphagen kinase sequences are highly conserved throughout evolution, showing little more than 50 % difference between even distantly related species. An X-ray crystallographic analysis of the structure of arginine kinase from *L. polyphemus* offers at least a partial explanation for the observed sequence conservation (Zhou et al., 1998). Of the 11 amino acid residues known to be involved with binding arginine, including part of the previously predicted guanidino specificity region (Suzuki et al., 1997), 10 residues are completely conserved within the six arthropod species for which arginine kinase sequences have been published (Fig. 6). One of these residues, the reactive cysteine at position 271, is part of the signature pattern C-P-(S/T)-N-(I/L)-G-T that is characteristic of all guanidino kinases (Bairoch, 1991).

The apparently constitutive levels of arginine kinase mRNA observed in anterior and posterior gills suggest that phosphoarginine is not serving a specialized role as an energy reserve exclusively for ion transport, since the anterior gills are generally believed to function mainly in gas exchange while the posterior gills are thought to contain most of the ion-transporting capability (Taylor and Taylor, 1992). However, the stronger osmoregulator (*C. sapidus*) does exhibit higher levels of arginine kinase enzymatic activity than the more modest osmoregulator (*C. maenas*), and the activity doubles in posterior gills when *C. sapidus* is exposed to low salinity (Fig. 2). The enzymatic response is similar to the changes in $Na^+/K^+$-ATPase activity noted in the posterior gills of *C. maenas* and *C. sapidus* upon acclimation to low salinity (Siebers et al., 1982; Towle et al., 1976). The consequent increased demand on ATP reserves may be met by increased arginine kinase activity observed in *C. sapidus* but not *C. maenas*, perhaps offering a partial explanation for the higher osmoregulatory capacity of *C. sapidus*. The mechanism by which arginine kinase activity is enhanced in *C. sapidus* seems not to depend on up-regulation of arginine kinase gene transcription. Whether the enhancement in activity results from changes in mRNA translation or from direct activation of pre-existing enzyme remains to be investigated.

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