

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

SIMON KOTLYAR*, DIRK WEIHRAUCH, RYAN S. PAULSEN AND DAVID W. TOWLE‡

Department of Biology, Lake Forest College, Lake Forest, IL 60045, USA and Mount Desert Island Biological Laboratory, Salsbury Cove, ME 04672, USA

*Present address: Program in International Medicine, Ben Gurion University, Beer-Sheva 84105, Israel

‡Author for correspondence (e-mail: towle@lfc.edu)

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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, glycoamine 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; Wiczorek 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 000g 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 µg⁻¹ tissue. Specific activity was calculated as nmol ATP min⁻¹ µg⁻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 ³⁵S-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

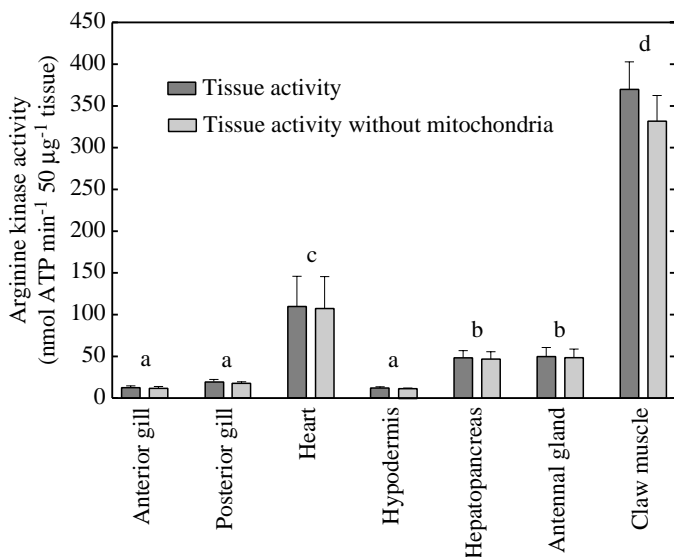


Fig. 1. Arginine kinase activity in selected tissues of the shore crab *Carcinus maenas* measured using an enzyme-linked assay method with phosphoarginine as substrate (Nealon and Henderson, 1976). Total arginine kinase activity is expressed on the basis of fresh tissue mass (dark-colored columns). Tissue 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 \pm 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.

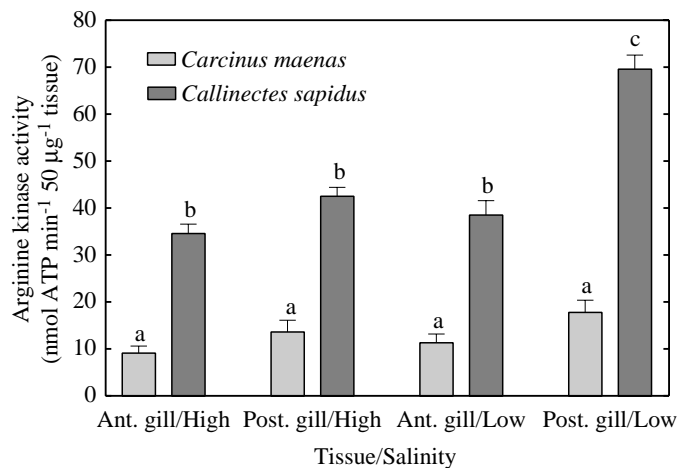


Fig. 2. Arginine kinase activities of anterior (Ant.) and posterior (Post.) gills of shore crabs *Carcinus maenas* and blue crabs *Callinectes sapidus* acclimated to high salinity (35‰) or low salinity (10‰ for *C. maenas* and 5‰ for *C. sapidus*). Values are means \pm S.E.M. of triplicate measurements 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) ($P < 0.05$).

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).

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.

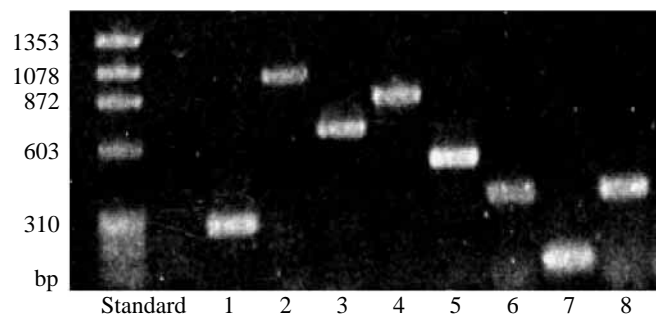


Fig. 3. Agarose gel electrophoresis of polymerase chain reaction products amplified from cDNA prepared from posterior gills of the shore crab *Carcinus maenas*. Primer combinations, designed to amplify arginine kinase cDNA, were as follows: lane 1, AUAP/GSPR; lane 2, AUAP/2R; lane 3, AUAP/1R; lane 4, 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 40235 Da. Using the 1475-nucleotide cDNA as the query sequence, a gapped

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	AAA	AA				1475

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 ACAATGG (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

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																	24
97	TCT	CTC	CTC	AAG	AAG	TAC	CTC	ACC	AAG	TCT	GTG	TTC	GAC	CAG	CTC	AAG	144
25																	40
145	GAC	AAG	AAG	ACA	AGC	CTG	GGC	GCC	ACC	CTC	CTC	GAT	GTG	ATC	CAG	TCT	192
41																	56
193	GGT	GTT	GAG	AAC	CTG	GAC	TCT	GGC	GTT	GGT	GTG	TAC	GCC	CCT	GAC	GCC	240
57																	72
241	GAG	GCC	TAC	ACC	CTC	TTC	GCC	CCT	CTG	TTC	GAC	CCC	ATC	ATC	GAG	GAC	288
73																	88
289	TAC	CAC	AAG	GGT	TTC	AAG	CAG	ACT	GAC	AAG	CAC	CCT	AAC	AAG	GAC	TTC	336
89																	104
337	GGC	GAT	GTC	AAC	CAG	TTC	GTC	AAC	GTG	GAC	CCC	GAT	GGC	AAG	TTC	GTG	384
105																	120
385	ATC	TCC	ACT	CGC	GTG	CGT	TGC	GGT	CGC	TCC	ATG	GAG	GGC	TAC	CCC	TTC	432
121																	136
433	AAC	CCC	TGT	CTC	ACC	GAG	GCC	CAG	TAC	AAG	GAG	ATG	GAG	TCC	AAG	GTT	480
137																	152
481	TCC	TCC	ACC	CTG	TCC	AAC	CTC	GAG	GGC	GAG	CTC	AAG	GGC	ACT	TAC	TTC	528
153																	168
529	CCT	CTC	ACC	GGC	ATG	ACC	AAG	GAA	GTG	CAG	CAA	AAG	CTG	ATC	GAC	GAC	576
169																	184
577	CAC	TTC	CTC	TTC	AAG	GAG	GGT	GAC	CGC	TTC	CTG	CAG	GCC	GCT	AAT	GCC	624
185																	200
625	TGC	CGC	TAC	TGG	CCC	ACT	GGC	CGT	GGC	ATC	TAC	CAC	AAC	GAC	AAC	AAG	672
201																	216
673	ACC	TTC	CTG	GTG	TGG	TGC	AAC	GAA	GAG	GAT	CAC	CTC	CGC	ATC	ATC	TCC	720
217																	232
721	ATG	CAG	ATG	GGT	GGT	GAC	CTG	GGC	CAG	GTC	TAC	CGC	CGC	CTT	GTC	AGC	768
233																	248
769	GCT	GTT	AAC	GAG	ATT	GAG	AAG	CGC	GTG	CCC	TTC	TCC	CAC	CAT	GAC	CGC	816
249																	264
817	CTG	GGC	TTC	CTC	ACC	TTC	TGC	CCC	ACC	AAC	CTT	GGC	ACC	ACC	GTG	CGT	864
265																	280
865	GCC	TCC	GTC	CAC	ATC	AAG	CTC	CCC	AAG	CTG	GCC	GCC	AAC	CGC	GAA	AAG	912
281																	296
913	CTT	GAG	GAA	GTC	GCC	GGC	AAG	TAC	AGC	CTC	CAG	GTT	CGC	GGC	ACC	CGC	960
297																	312
961	GGC	GAG	CAC	ACC	GAG	GCT	GAG	GGC	GGC	GTG	TAC	GAC	ATC	TCC	AAC	AAG	1008
313																	328
1009	CGC	CGC	ATG	GGT	CTC	ACT	GAG	TAC	CAG	GCT	GTC	AAG	GAG	ATG	CAG	GAC	1056
329																	344
1057	GGT	ATC	CTT	GAG	CTC	ATC	AAG	ATT	GAG	AAG	GAA	ATG	CAG	TAA	AGT	TCG	1104
345																	357
1105	GCT	CCT	CCC	TCT	ATG	GGT	GAG	GGC	GAG	CCG	GTC	TCT	GCT	GTA	GGA	AGA	1152
1153																	1200
1201	GCA	TTT	GTC	AGC	CCG	GCA	ACA	AAC	CAT	CTT	TAA	CCT	AGC	GAG	CTG	ATG	1248
1249																	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 (ACAATGG) frames the probable start codon, as in the *Carcinus maenas* sequence.

contain a consensus sequence around the likely start codon (ACAATGG) (Kozak, 1991) (Figs 4, 5). Although the most frequently found polyadenylation signals (AATAAA or ATTTAAA) 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

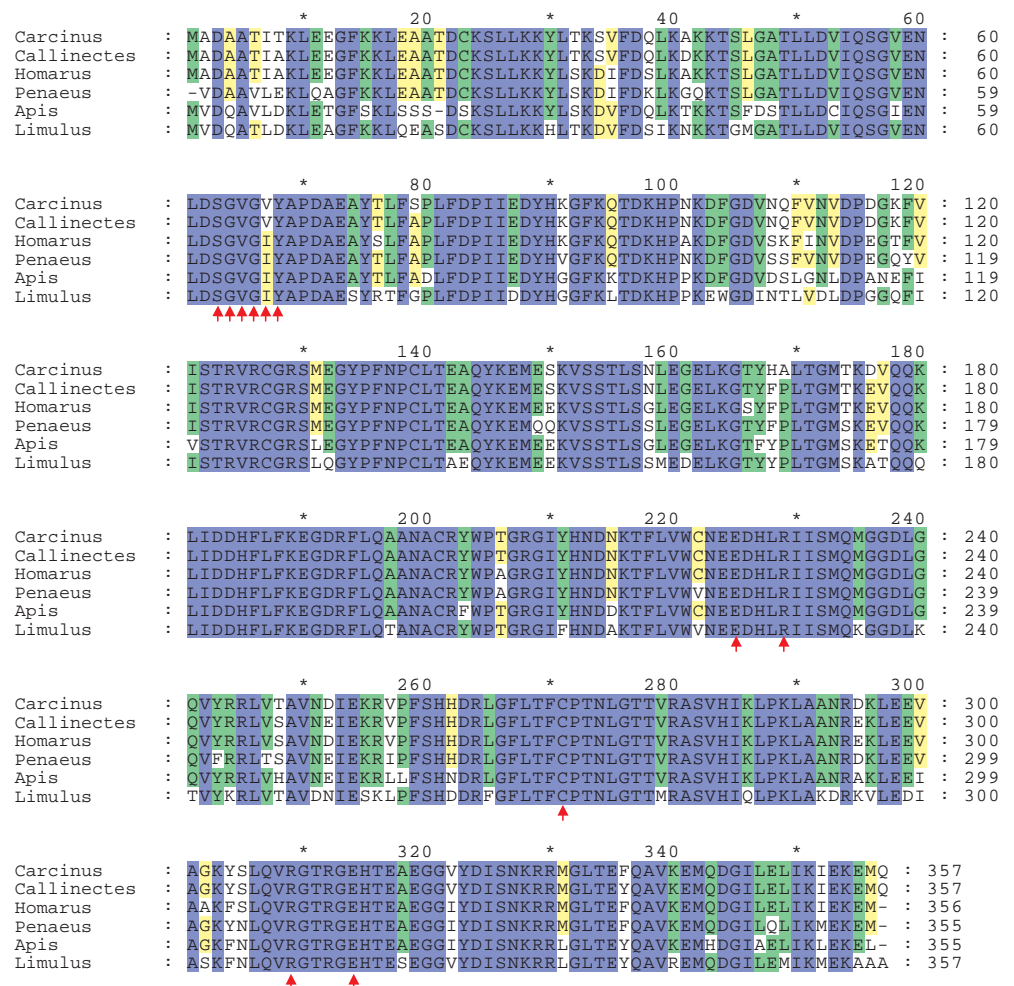
brachyuran crabs within a larger cluster that included all crustacean species (Fig. 7).

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.



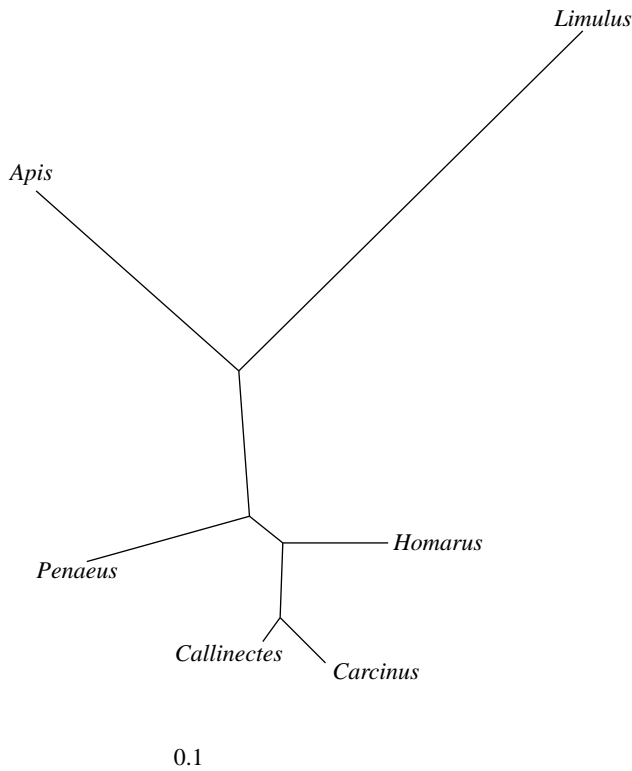


Fig. 7. Unrooted phylogenetic tree depicting distance relationships of six arthropod arginine kinase sequences, produced using CLUSTALW and TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). The scale represents 10% (0.1) amino acid divergence.

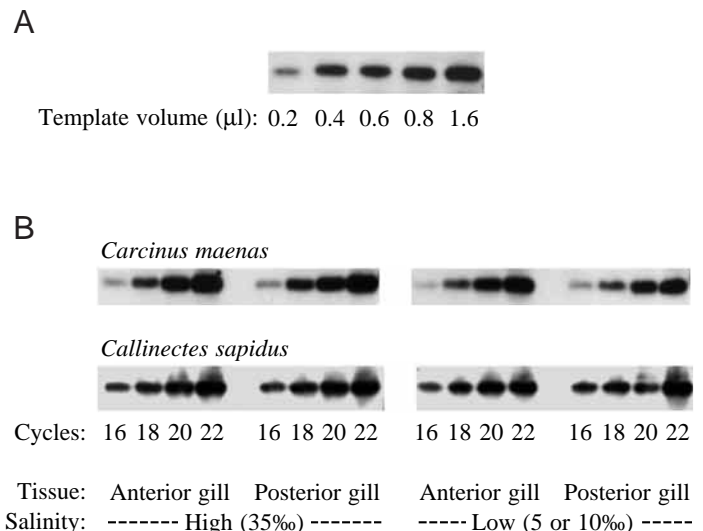
kinase activity, suggesting that phosphoarginine plays some role in the metabolic support of gill function. None of the tissues examined appeared to contain a significant contribution of mitochondrial arginine kinase activity. A mitochondrial form of arginine kinase is rare among invertebrate species, 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 $50\mu\text{l}$ 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 $2\mu\text{l}$ of cDNA template (the product from reverse-transcribing 200 ng of total RNA) per $100\mu\text{l}$ 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 $\text{Na}^+/\text{Ca}^{2+}$ 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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