Physical, kinetic, and immunological studies of monomeric (Periplaneta americana) and dimeric (Isostychopus badonotus) arginine kinases

Brianne Wright-Weber

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Physical, Kinetic, And Immunological Studies Of Monomeric (Periplaneta Americana) And Dimeric (Isostychopus Badonotus) Arginine Kinases

by

Brianne Wright-Weber

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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July 13, 2007

Keywords: phosphotransferase, enzyme characterization, purification, quaternary structure, echinoderm

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Dedications

I would like to dedicate this to my family, friends, and most importantly my husband. Without the support from all of my family and friends I would never have made it through these last 5 years. To my husband, I would like to say thank you for all of your time, patience, and support. You have always believed in me, even when I had doubts in myself. You have been my best friend and I can’t imagine my life without you.
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Next, I would like to thank Dr. Ashli Brown and Dr. Brenda Held who both graduated from Dr. Grossman’s lab before me. The two of you became part of my family during graduate school and I don’t know what it would have been like if I had not had you here with me. I will always remember the fun times we shared together and I will miss you both very much.

I would also like to say thank you to all of my fellow graduate students (especially the Ming Lab Students) who helped and supported me along the way. I don’t think that I will ever find a more intelligent, kind, and fun group of friends and colleagues.

Thanks to my committee members: Dr. Ming, Dr. Livingston, Dr. Harmon, and Dr. Bisht for serving on my committee and guiding me through this process.

Lastly, and most importantly, I would like to thank my family for their continuous love and support. I don’t think anyone else has a family that can compare to you. You have always been there for me and I know you always will be. Thank you.
Note to Reader

Note to Reader: The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the USF library in Tampa, Florida.
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<th>Description</th>
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<tbody>
<tr>
<td>ACA</td>
<td>Polyacrylamide and Agarose Chromotography</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’ Diphosphate</td>
</tr>
<tr>
<td>AK</td>
<td>Arginine Kinase</td>
</tr>
<tr>
<td>Arg~p</td>
<td>Phosphoarginine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ Triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CD</td>
<td>Circular Dichroism</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CK</td>
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<td>CM</td>
<td>Carboxy Methylchloride</td>
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<td>Diethylaminoethyl</td>
</tr>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl-Formamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNTP</td>
<td>Deoxyribonucleotide Triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
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x
ES₁S₂: Ternary Complex
GK: Glycocyamine Kinase
GS: Guanidino Specificity Region
HCl: Hydrochloric Acid
Kₙᵃᵣ: Binary Complex Dissociation Constant
KCl: Potassium Chloride
kD: Kilo Daltons
Kᵢ: Inhibitor Dissociation Constant
Kₙᵣᵢᵣ: Ternary Complex Dissociation Constant
LB: Luria Broth
LK: Lombricine Kinase
MgADP: Magnesium Adenosine 5’ Diphosphate
MgATP: Magnesium Adenosine 5’ Triphosphate
NaCl: Sodium Chloride
NaCH₃CO₂: Sodium Acetate
NAD: Nicotinamide Adenine Dinucleotide
NADP⁺: Nicotinamide Adenine Dinucleotide Phosphate (oxidized)
NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NaI: Sodium Iodide
NaN₃: Sodium Azide
NaNO₂: Sodium Nitrite
NaNO₃: Sodium Nitrate
<table>
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<td>NaOH</td>
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</tr>
<tr>
<td>NaSCN</td>
<td>Sodium Thiocyanate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl-Sulfonyl Fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) Aminomethane</td>
</tr>
<tr>
<td>TSAC</td>
<td>Transition State Analog Complex</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Initial Velocity</td>
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Physical, Kinetic, and Immunological Studies of Monomeric (*Periplaneta americana*) and Dimeric (*Isostychopus badonotus*) Arginine Kinases

Brianne Wright-Weber

ABSTRACT

Arginine kinase catalyzes the reversible phosphorylation of arginine using ATP. This phosphotransferase is found throughout invertebrate species; whereas a homologous enzyme, creatine kinase, is found in both vertebrate and invertebrate species. Arginine kinases are found as monomers of 40 kDa or 80 kDa and dimers of 80 kDa while creatine kinases are found as dimers of 80 kDa, monomers of 150 kDa, or octamers of 320 kDa. The significance or advantage of the dimeric state or various quaternary structures is still not understood for this family of enzymes. Here, arginine kinase from *Isostychopus badonotus* muscle was purified to homogeneity and analyzed for physical, kinetic, and immunological characteristics. The results indicate that arginine kinase from the sea cucumber, *I. badonotus*, is a dimer with a molecular weight of 87 kDa that displays physical and kinetic characteristics similar to other arginine kinases regardless of their weight or subunit composition. However, immunological cross-reactivity using *I. badonotus* polyclonal antibodies shows that dimeric arginine kinase from the sea cucumber can react with dimeric arginine and creatine kinases but not with monomeric arginine or creatine kinases. Comparable results are seen with polyclonal antibodies raised against purified monomeric arginine kinase from the American cockroach,
*Periplaneta americana.* Monomeric arginine kinase from the cockroach reacted with monomeric arginine kinases but not with dimeric arginine or creatine kinases or monomeric creatine kinases. Arginine kinase from the sea cucumber and the cockroach is substantially inhibited by the anion nitrate which mimics the transferable phosphoryl group in the assumed rapid equilibrium, random addition reaction. Here, nitrate has been shown to inhibit both the initial velocity and percent of product formed from arginine kinase in *I. badonotus* and *P. americana.* Difference spectra for each enzyme in the presence of varying components of the transition state analog suggest that nitrate has an effect on the enzyme itself and inhibits through a mechanism beyond that of stabilization of the dead-end complex. Further characterization of the dimeric state in these enzymes on a structural level included the elucidation of the protein sequence from the American cockroach and a comparison with dimeric arginine and creatine kinases.
Chapter 1. Introduction
Literature Review

Physiological Role, Distribution, and Quaternary Structure

Phosphagen kinases are a highly conserved family of enzymes that catalyze the reversible phosphorylation of guanidino compounds using ATP (Figure 1). These phosphagens include: lombricine phosphate, glycocyamine phosphate, taurocyamine phosphate, hypotaurocyamine phosphate, creatine phosphate, and arginine phosphate, with each phosphagen having its own corresponding phosphagen kinase (Figure 2).

Phosphagen kinases are most widely known as ATP buffering systems that maintain ATP homeostasis during high or fluctuating energy demands in cells that experience rapid, large bursts of energy like neurons and skeletal muscle (Kladis et al., 1997; Wyss & Kaddurah-Daouk, 2000). This balance between the production and consumption of ATP is essential due to the fact that ATP is required for metabolic reactions and cellular functions in both single and multi-cellular organisms.

The most accepted role of the phosphagen kinases is as a “temporal energy buffer” which stabilizes the cellular ATP/ADP ratios (Meyer et al., 1984). The buffering capacity of phosphagen kinases minimizes the changes in $\Delta G_{\text{ATP}}$ values when there is a shift in equilibrium between ATP hydrolysis and ATP synthesis. Phosphagen kinases have the ability to synthesize large pools of high energy phosphates under standard
Figure 1. Reaction Catalyzed by Phosphagen Kinases:

Phosphagen Kinase

Quanidine Acceptor + MgATP $\rightleftharpoons$ Phosphagen + MgADP + H$^+$
Figure 2. Structure of Phosphagens:

The structures of phosphoglycocyamine (GP), phosphocreatine (CP), phosphoarginine (AP), phospholombricine (LP), phosphohypotaurocyamine (HTP), and phosphotaurocyamine (TP) are shown. Figure from Ellington, 2001.
conditions that can then be used to replenish ATP during high metabolic activity when
ATP concentrations are low (Wallimann et al., 1992). The net effect of buffering ATP
and ADP levels by the phosphagen kinases can be seen in the following reactions:

Phosphagen kinase: Phosphagen + MgADP + H\(^+\) → guanidine acceptor + MgATP
ATPase: MgATP → MgADP + Pi + H\(^+\)
Net: phosphagen → guanidine acceptor + Pi

Phosphagen kinases are also said to have a “proton buffering” capacity by
consuming protons when regenerating ATP. They are able to trap inorganic phosphate
(Pi) during the net phosphagen hydrolysis reaction (above) and use it to be an effective
H\(^+\) buffer. If protons released by cellular ATPases were not buffered by inorganic
phosphate they would acidify the cytosol (Wallimann et al., 1992). In 1981, Griffiths
determined that inorganic phosphate concentrations in resting muscle were low and
suggested that this might be a factor in suppressing glycogenolysis during resting
conditions. Since inorganic phosphate is released during net phosphagen hydrolysis it is
also believed that kinases can support short bursts of cellular activity until pathways such
as oxidative phosphorylation, glycolysis, and glycogenolysis are turned on (Pereira et al.,
2003).

The most controversial function that has been reported for phosphagen kinases is
that of a “spatial energy buffer” (Meyer et al., 1984). The energy transport, or
phosphocreatine shuttle theory, suggests that a large part of cellular high energy
phosphate is transported as phosphocreatine rather than ATP (Bessman & Geiger, 1981;
Bessman & Carpenter, 1985). Due to CK being localized in mitochondria (Jacobs et al.,
1964), at the ATP source, as well as in skeletal and cardiac muscle (cytosol), at the ATP sink, it is thought that phosphocreatine is used to transfer the high energy phosphate because of its larger diffusion coefficient compared to ATP and ADP (Hubley et al., 1995). This would allow $\Delta G_{\text{ATP}}$ values to remain high at sites of ATP turnover.

Creatine and arginine kinases are the most extensively studied phosphagen kinase enzymes. Creatine kinase is the only phosphagen kinase found in vertebrate species while arginine kinase is found as the most prevalent phosphagen in invertebrate species (Watts, 1973; Morrison, 1973). However, creatine kinase, lombricine kinase, glycocyamine kinase, taurocyamine kinase, and hypotaurocyamine kinase are also found in invertebrate species (Watts, 1971). Tissue specific expression of phosphagens can occur in certain organisms but interestingly, a large number of organisms contain creatine kinase in their spermatozoa regardless of which phosphagen system they possess in somatic tissue or eggs (Robin, 1964; Thoai & Robin, 1969; Robin 1974; Ellington & Kinsey, 1998).

The significance of particular phosphagen kinases found in specific phylogenetic groups is currently unknown. The advantages ascribed to organisms containing phosphocreatine are said to be: (a) a much higher apparent equilibrium constant (Lawson & Veech, 1979), that allows the CK system to maintain high $\Delta G_{\text{ATP}}$ values over a broader range of free energy change (Ellington, 2001); (b) the intrinsic diffusivity of phosphocreatine which is higher then other phosphagens; and (c) creatine is metabolically inert and cannot be used by other enzymatic reactions. However, not all organisms that contain phosphocreatine are able to synthesize creatine and must rely on
acquiring it from their diet (Van-Pilsum et al., 1972). The advantage for organisms that contain phosphoarginine, or one of the other phosphagens as their substrate, is said to be due to their physico-chemical properties (Ellington, 2001). These phosphagens are more acid stable than phosphocreatine and might be beneficial to organisms with low intracellular pH (yeast) or those that thrive in more acidic environments (Sauer & Schlattner, 2004). Organisms containing arginine might have a disadvantage in the fact that arginine is an essential amino acid in many metabolic pathways.

The quaternary structure, like the distribution of the phosphagen kinases, can vary. Arginine kinases are typically found as monomers consisting of a single polypeptide chain with a molecular weight of 40 kDa or dimers consisting of two similar (or identical) polypeptide chains with a molecular weight of 80 kDa. In general, monomeric arginine kinases are found in arthropods such as the shrimp, honey bee, and cockroach (France et al., 1997; Kucharski & Maleszka, 1998; Brown et al., 2004) while dimeric arginine kinases are found in echinoderms such as the sea cucumber and sea urchin (Seals & Grossman, 1988; Wright-Weber et al., 2006). Exceptions to the general trend do occur with a tetrameric arginine kinase of 150 kDa existing in some marine worms (Robin et al., 1969) and more recently in bivalves which have been found to contain large two-domain monomers of 80 kDa (Suzuki et al., 1998; Suzuki & Yamamoto, 2000; Suzuki et al., 2002).

The quaternary structure of creatine kinases is not as varied as that of arginine kinases. Creatine kinases are typically classified as being dimers with very few exceptions existing. However, a special feature of the creatine kinase system is the
occurrence of multiple isozymes localized in different compartments. Vertebrate creatine kinases located in the cytoplasm, such as CK-MM (muscle), CK-BB (brain), and CK-MB (heart) exist as dimers of 80 kDa (Eppenberger et al., 1967) while CK localized in the mitochondria are large octamers of 320 kDa (Wyss et al., 1990). On the other hand, invertebrate creatine kinases like that found in the sperm of the sea urchin, *S. purpuratus*, were determined to be large monomers with a molecular weight of 150 kDa (Tombes & Shapiro, 1985; Tombes et al., 1987).

**Physical Characterization**

Both creatine kinase and arginine kinase were discovered in the late 1920’s in vertebrate and crayfish muscle, respectively. One of the first successful creatine kinase purifications was performed by Kuby et al. (1954) by isolating CK from rabbit muscle with the use of ethanol fractionation. The first arginine kinase to be purified in high yield was described by Ennor et al. (1956) from crayfish muscle. The studies performed by Kuby et al. and Ennor et al. not only established purification protocols for arginine and creatine kinase but also outlined assay procedures which allowed for detailed examinations of other phosphagen kinases to be achieved.

Today, arginine kinase and creatine kinase have been purified from numerous species such as the lobster (Pradel et al., 1964), tarantula (Blethen & Kaplan, 1968), sea cucumber (Anosike et al., 1975), sea urchin (Ratto & Christen, 1988), shark (Gray et al., 1986), and even a sponge (Ellington, 2000). Typically purifications utilize basic purification techniques such as homogenization, ammonium sulfate fractionation, gel
chromatography, ion exchange chromatography, and ultrafiltration, in a multi-step protocol to isolate the enzyme of interest. There are now several assays available to monitor AK and CK activity in both the forward and reverse directions of the reversible reaction. The reverse reaction (synthesis of ATP) is routinely measured using an enzyme-coupled spectrophotometric assay described by Rosalki (1967) while the forward reaction (synthesis of ADP) is measured directly using a pH stat assay (Grossman, 1983) or an enzyme-coupled spectrophotometric assay (Fujimoto et al., 2005).

The ability to readily purify and monitor activity of an enzyme is essential for studying the physical characteristics needed to understand enzyme structure and function. As stated earlier, there are a variety of molecular weights associated with the phosphagen kinases although they are generally found as monomers of 40 kDa or dimers of 80 kDa. The first physical characterizations identified then are usually their native molecular weight, determined through gel chromatography, and their subunit molecular weight, determined through SDS-PAGE (Laemmli, 1970).

Another physical characteristic examined for the phosphagen kinases is the thermal stability of the enzyme. Arginine and creatine kinase thermal stabilities vary regardless of their prospective substrate, molecular weight, or quaternary structure (Table 1). This is determined by heating the enzyme at varying temperatures and recording the temperature at which 50% of the enzyme activity remains after 10 minutes. Monkey CK-MM has one of the highest thermal stabilities by retaining 50% of its activity after heating for 10 minutes at 61°C (Grossman & Mollo, 1979). Monkey CK-BB, monomeric cockroach AK, and monomeric butterfly AK all have relatively high thermal stabilities
by retaining 50% activity at 50 °C, 50 °C, and 48 °C respectively (Grossman & Mollo, 1979; Brown et al., 2004; Wright-Weber et al., 2006). Dimeric arginine kinase from the sea cucumber, *C. arenicola*, retains 50% activity at 38 °C while dimeric arginine kinases from sea urchin eggs, *S. purpuratus* and *P. lividus*, retain 50% activity at only 26 °C and 20 °C, respectively (Seals & Grossman, 1988; Wright-Weber et al., 2006; Ratto & Christen, 1988). The difference in thermal stabilities between the last three dimeric enzymes, all of which are found in echinoderms, suggests that thermal stability might be affected by the environment in which the enzyme is found. The sea cucumber *C. arenicola* resides in warm waters while the sea urchins *S. purpuratus* and *P. lividus* both reside in cooler waters. This could suggest that nature selects an enzyme structure or composition to advance that is suited to the environment in which it will be found.

A common characteristic examined for the phosphagen kinases is the isoelectric point (pI) of each native enzyme. An isoelectric point is the pH at which a protein carries no net electric charge. Factors that can affect the isoelectric point are amino acid composition of each enzyme as well as the charged amino acid side chains that are exposed to each other and the solvent in the tertiary structure. The isoelectric point of an enzyme can be useful in distinguishing between different isozymes contained in a single organism, like the three isoforms of AK in the honey bee (Kucharski & Maleszka, 1998) or the three isoforms of CK in vertebrates (Eppenberger et al., 1967). A general trend among the phosphagen kinases is that the isoelectric points of monomeric arginine kinases tend to be slightly more acidic than dimeric arginine or creatine kinases (Table 2). This is shown to hold true for monomeric AK for the cockroach, *P. americana*,
whose pI is 5.8 (Wright-Weber et al., 2006) and from the squid, *S. oulaniensis*, that has a pI of 5.3 (Storey, 1977). However, exceptions due exist with CK-BB from the monkey, *C. verus*, displaying an isoelectric point of only 4.8 compared to a pI of 6.9 for CK-MM from the same species (Grossman & Mollo, 1979). Although isozymes of CK are 80% homologous (Muhlebach et al., 1994), it is likely that the isoelectric points are different due to additional basic amino acids in the MM form or differences in conformations between the two isozymes.

pH optima of phosphagen kinases are often generated to determine the optimal pH at which to run kinetic measurements. Enzymes are sensitive to pH changes and are typically active in the narrow range of pH 5 to 9. The pH of a reaction is important in regulating enzymes which catalyze physiological reversible reactions because it can have an effect on the pKa’s of amino acids that make up the active site of the enzyme (Hochacka & Somero, 1973). Arginine kinases display pH optima in the reverse direction (synthesis of ATP) from ~6.6 to 7.2 and in the forward direction (synthesis of ADP) from ~8.4 to 9.1 (Morrison, 1973). Similarly, creatine kinases display pH optima in the reverse direction from ~6.5 to 7.0 while in the forward direction a broader range is seen from ~7.5 to 9.5 (Watts, 1973). The comparable ranges in pH optima for both the forward and reverse directions of arginine and creatine kinases suggest the catalytic mechanism is conserved throughout the phosphagen kinases. However, it should be noted that the optima for the creatine and arginine kinases reported here are just effects of pH on the initial rates of the reaction and not detailed pH profiles for the reaction.
Table 1. Thermal Stability of Creatine and Arginine Kinases:

The temperature shown for each species is the approximate temperature at which 50% activity remains after heating for 10 minutes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature °C</th>
<th>Reference</th>
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<td><em>C. verus</em></td>
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<td>Sea Cucumber AK</td>
<td>38</td>
<td>Seals &amp; Grossman, 1988</td>
</tr>
<tr>
<td><em>C. arenicola</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea Urchin Egg AK</td>
<td>26</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td><em>S. purpuratus</em></td>
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<td>Sea Urchin Egg AK</td>
<td>20</td>
<td>Ratto &amp; Christen, 1988</td>
</tr>
<tr>
<td><em>P. lividus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cockroach AK</td>
<td>50</td>
<td>Brown et al., 2004</td>
</tr>
<tr>
<td><em>P. americana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butterfly AK</td>
<td>48</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td><em>V. cardui</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shrimp AK</td>
<td>37</td>
<td>France et al., 1997</td>
</tr>
<tr>
<td><em>P. azteces</em></td>
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Table 2. Isoelectric Points (pI) of Creatine and Arginine Kinas:

<table>
<thead>
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<th>Species</th>
<th>pI</th>
<th>Reference</th>
</tr>
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<tr>
<td>Monkey CK-MM</td>
<td>6.9</td>
<td>Grossman &amp; Mollo, 1979</td>
</tr>
<tr>
<td><em>C. verus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey CK-BB</td>
<td>4.8</td>
<td>Grossman &amp; Mollo, 1979</td>
</tr>
<tr>
<td><em>C. verus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea Cucumber AK</td>
<td>7.8</td>
<td>Seals &amp; Grossman, 1988</td>
</tr>
<tr>
<td><em>C. arenicola</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea Urchin Egg AK</td>
<td>6.7</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td><em>S. purpuratus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cockroach AK</td>
<td>5.8</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td><em>P. americana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squid AK</td>
<td>5.3</td>
<td>Storey, 1977</td>
</tr>
<tr>
<td><em>S. oulaniensis</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Although not as extensively studied as the previous characteristics discussed two
techniques, circular dichroism (CD) and hybridization, which examine the structural
features of proteins, are worth mentioning. Circular dichroism is a technique which
investigates the secondary structure of proteins through the unequal absorption of right
and left circularly polarized light caused by the asymmetry of proteins during optical
activity (Creighton, 1984). The CD spectra of proteins can determine the percentage of
both alpha helical and beta sheets contained in a protein and can be used to examine if
these secondary structures are intact when proteins are exposed to heat or denaturants.
Rabbit CK shows an alpha helical content of 17.8%, 21%, and 24% for MM, BB, and
MB isozymes, respectively (Grossman & Sellers, 1998). Dimeric arginine kinase from
the sea cucumber, \textit{S. japonicus}, has an alpha helical content of 31% (Guo et al., 2003)
while monomeric arginine kinases from the cockroach, \textit{P. americana}, and the shrimp, \textit{P.
aztecu}s, show alpha helical contents of 12% and 16%, respectively (Wright-Weber et al.,
2006; France & Grossman, 1996). These results indicate that monomeric arginine
kinases may have a lower alpha helical content then either of the other dimeric
phosphagen kinases.

To compare the conserved structural features possessed by the phosphagen
kinases required for subunit association, catalytic activity, and formation of quaternary
structure a technique known as hybridization can be utilized. It has been well established
that CK isozymes from the same tissue in two different species (ie. chicken CK-MM and
rabbit CK-MM) are more closely related than intraspecies isozymes and can form
functionally efficient dimers (Eppenbeger et al., 1967; Grossman & Mollo, 1979). The
ability of dimeric AK and CK to form functional heterodimers was also shown in 1988 by Seals and Grossman from arginine kinase in sea cucumber muscle and creatine kinase from rabbit brain. However, a hybrid containing monomeric arginine kinase and dimeric arginine kinase can not be found in the literature suggesting that monomers do not contain the conserved structural features required for subunit association.

**Immunological Properties**

To date, the immunological properties of several phosphagen kinases from both vertebrate and invertebrate species have been examined. Immunogenic reactivity between different enzymes contained in a protein family can be used to establish similarities in either their structure or their sequence. Reactivity is tested by using polyclonal antibodies (heterologous antibodies made against several epitopes) or monoclonal antibodies (antibodies made against one specific epitope). In 1970, Viala et al. determined that there was no cross-reactivity between antibodies made against creatine kinase and monomeric arginine kinase or between antibodies made against monomeric arginine kinase and creatine kinase. The authors concluded that polyclonal antibodies used to test cross-reactivity were most likely made from surface epitopes, amino acids close to each other in the folded protein but separated in linear sequence, and not from sites in the active site of the protein. They suggested that using denatured proteins to make antibodies might help establish cross-reaction of the phosphagens by exposing epitopes not available on the outside surface of the natured protein. Robin et al. (1976) then supported the theory of Viala and coworkers by creating polyclonal
antibodies to denatured monomeric lobster muscle AK and rabbit muscle CK by oxidizing the proteins with performic acid. Cross-reactivity was seen between denatured arginine kinase antibodies and CK as well as denatured creatine kinase antibodies and AK.

An interesting study on the cross-reactivity between isozymes of CK was performed by Grossman and Mollo in 1979. Polyclonal antibodies made from monkey CK-MM reacted with monkey CK-MM, human CK-MM, and monkey CK-MB but not with monkey CK-BB. Although the creatine kinase isozymes contain high sequence homology with each other the authors speculated that CK-MM and CK-BB might have enough differences between their amino acid sequences to create conformational changes which make them antigenically distinct. More recently the antigenicity of monomeric shrimp arginine kinase was tested with monomeric AK from arthropods and mollusks as well as vertebrate CK and dimeric AK from echinoderms (Brown et al., 2004). All organisms in the classes known to contain monomeric AK reacted with shrimp antibodies while organisms that contained dimeric AK or CK did not cross-react. Currently, there is no evidence for the generation of dimeric arginine kinase antibodies and their ability to react with monomeric arginine kinases or dimeric creatine kinases in the literature. A study involving dimeric arginine kinase would be interesting because this enzyme contains the same substrate as AK monomers but shares a similar structure to dimers of creatine kinase.
Mechanism and Kinetic Analysis

Initial kinetic investigations of CK were performed in the 1950’s using rabbit muscle (Kuby et al., 1954). These initial studies showed that the enzyme operated through a bisubstrate reaction and had a strong dependence on the Mg$^{2+}$ concentration (which was interpreted as meaning that MgADP and MgATP were the active substrates). Further characterization showed that the over-all equation was reversible and that at pH 8.0, or above, the enzyme followed a rapid equilibrium, random addition mechanism (Morrison & James, 1965; Morrison & Cleland, 1966; Morrison & White, 1967). This mechanism (Figure 3) is classified by having no order to the binding of substrates and states that all steps are in rapid equilibrium except those involved with the conversion of the central ternary complex (Segal, 1975). At pH 7.0 or below, Schimerlik and Cleland (1973) determined that the situation was more complex and in the forward direction obeyed an ordered equilibrium mechanism while in the reverse direction the reaction remained random.

Kinetic investigations of AK were first performed in the late 1950’s and 1960’s on extracts from the tail of the crayfish (Morrison et al., 1957; Griffiths et al., 1957; Smith & Morrison, 1969). Initially it was suggested that the enzyme obeyed a ping-pong mechanism by proceeding through a phosphorylated enzyme intermediate (Uhr et al., 1966). However, this suggestion was re-evaluated and further studies proved that both monomeric and dimeric arginine kinases obeyed a rapid equilibrium, random addition mechanism like the homologous creatine kinase enzyme (Virden et al., 1965; O’Sullivan et al., 1969; Blethen, 1972; Anosike et al., 1972). Also like CK, the presence of a
divalent metal ion was necessary for the arginine kinase reaction (Watts, 1973). Through the use of the paramagnetic properties of the manganous ion and monomeric arginine kinase from two shrimp species, O’Sullivan et al. (1969) determined that the metal binds with the nucleotide before the complex binds to the active site. The cation is needed in concentrations at least 1 mM excess over the concentration of the ATP and only manganese or calcium can be substituted for it in the reaction (Blethen, 1972). Several cationic metals such as Cu$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$, were ruled out as replacements of Mg$^{2+}$ because they reacted with a thiol group that was essential for activity (Morrison, 1973).

The dissociation (or binding) constants for numerous CK and AK species have been obtained for both the forward (synthesis of ADP) and the reverse (synthesis of ATP) directions (Tables 3A and B and 4A and B). These binding constants are determined through initial velocity studies where one of the substrates in the bisubstrate reaction is held constant and the other is varied at several fixed concentrations. This is done for both substrates so that the dissociation constants, $K_{bi}$, for the binary complex [ES] and $K_{ter}$ for the ternary complex [ES$_1$S$_2$] can be determined. The dissociation constants, $K_{bi}$ and $K_{ter}$, refer to the intrinsic $K_M$ and the apparent $K_M$, respectively where $K_{bi}$ would be the effect one substrate had on the enzyme and $K_{ter}$ would be the effect that both substrates had on the enzyme. The $K_M$ value determines how well a substrate binds the enzyme so a lower $K_M$ suggests that a substrate binds very tightly and can indicate greater substrate specificity or catalytic efficiency (Segal, 1975).
Figure 3. Rapid Equilibrium Random Addition Mechanism:

Phosphagen kinases are said to obey a rapid equilibrium random, addition mechanism in which either the nucleotide or the guanidine substrate can bind first.
For CK-MM and CK-BB isozymes in chicken, rabbit, and human the $K_{bi}$ for ATP vary from 0.99 to 3.26 mM and the $K_{ter}$ vary from 0.33 to 0.89 mM in the forward direction (Table 3A) (Morrison & James, 1965; Hornemann et al., 2000; Chen et al., 2000). Dissociation constants for the substrate creatine vary from 6.0 to 45.6 mM for the binary complex and 4.63 to 9.5 mM for the ternary complex (Table 3A). Table 4A shows the dissociation constants for human and rabbit CK-MM and CK-BB in the reverse direction. The $K_{bi}$ for ADP ranges from 0.02 to 0.17 mM while the $K_{ter}$ values are between 0.03 and 0.05 mM (Morrison & James, 1965; Wang et al., 2001; Wang et al., 2006). The binary complex dissociation constants for phosphocreatine range from 0.22 to 8.6 mM and the ternary complex constants are in a narrower range of 0.51 to 2.9 mM. The wide variety in $K_M$ values here and for the other phosphagen kinases can usually be accounted for by the types of assays chosen to run the experiments (enzyme-coupled, direct pH stat, or inorganic phosphate determinations), the range of substrate concentrations used, or the use of recombinant enzyme samples verses native enzyme samples.

Tables 3B and 4B show the dissociation constants from representative monomeric and dimeric arginine kinases in both the forward and reverse directions. A trend can be seen in the forward direction with most arginine kinases (except the American cockroach) exhibiting $K_{bi}$ and $K_{ter}$ values right around 1.0 mM (Seals & Grossman, 1988; Uda & Suzuki, 2004; Brown & Grossman, 2004; Tanaka et al., 2007). The American cockroach displays lower dissociation constants which could indicate that muscle contraction in the cockroach is faster, or at least greater than, the other organisms displayed and suggests
that the AK in the cockroach plays a greater role than it does in the other organisms. The $K_{bi}$ and $K_{ter}$ values for the reverse direction are more varied in the arginine kinases than they are in the forward direction. The $K_{bi}$ for ADP ranges from 0.06 to 0.20 mM for the sea urchin, cockroach, and squid while the $K_{ter}$ ranges from 0.06 to 0.09 mM (Storey, 1977; Brown & Grossman, 2004; Held et al., 2007). The $K_{bi}$ for phosphoarginine ranges from 1.0 to 3.5 mM and the $K_{ter}$ varies from 0.94 to 1.31 mM. One interesting point to note is the difference in $K_M$ values for creatine and arginine in their respective kinases. As a general trend the binary and ternary dissociation constants for arginine are much lower than the dissociation constants for creatine. This might suggest that arginine is in greater demand in species that utilize it as a substrate. If less substrate was available, it would be important for the enzyme to hold onto the substrate when it became available.
Tables 3A and B. Kinetic Constants [mM] for CK and AK Enzymes in the Forward Direction:

$K_{bi}$ and $K_{ter}$ refer to the dissociation constants from the binary complex [ES] and the ternary complex [ES$_1$S$_2$] respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_{bi}$ ATP</th>
<th>$K_{ter}$ ATP</th>
<th>$K_{bi}$ Arg</th>
<th>$K_{ter}$ Arg</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit CK-MM</td>
<td>1.2</td>
<td>0.48</td>
<td>15.6</td>
<td>6.1</td>
<td>Morrison &amp; James, 1965</td>
</tr>
<tr>
<td>Chicken CK-BB</td>
<td>3.26</td>
<td>0.33</td>
<td>45.6</td>
<td>4.63</td>
<td>Hornemann et al., 2000</td>
</tr>
<tr>
<td>Human CK-MM</td>
<td>1.2</td>
<td>0.89</td>
<td>14.6</td>
<td>9.5</td>
<td>Chen et al., 2000</td>
</tr>
<tr>
<td>Human CK-BB</td>
<td>0.99</td>
<td>0.81</td>
<td>6.0</td>
<td>4.9</td>
<td>Chen et al., 2000</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_{bi}$ ATP</th>
<th>$K_{ter}$ ATP</th>
<th>$K_{bi}$ Arg</th>
<th>$K_{ter}$ Arg</th>
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</tr>
</thead>
<tbody>
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<td>1.31</td>
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<td>1.7</td>
<td>Seals &amp; Grossman, 1988</td>
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<tr>
<td>Sea Cucumber AK</td>
<td>0.81</td>
<td>2.46</td>
<td>0.41</td>
<td>1.25</td>
<td>Uda &amp; Suzuki, 2004</td>
</tr>
<tr>
<td>Cockroach AK</td>
<td>0.17</td>
<td>0.14</td>
<td>0.45</td>
<td>0.49</td>
<td>Brown &amp; Grossman, 2004</td>
</tr>
<tr>
<td>Beetle AK</td>
<td>0.92</td>
<td>0.95</td>
<td>0.99</td>
<td>1.01</td>
<td>Tanaka et al., 2007</td>
</tr>
</tbody>
</table>
Tables 4A and B. Kinetic Constants [mM] of CK and AK Enzymes in the Reverse Direction:

$K_{bi}$ and $K_{ter}$ refer to the dissociation constants from the binary complex [ES] and the ternary complex [ES$_1$S$_2$] respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_{bi}$ ADP</th>
<th>$K_{ter}$ ADP</th>
<th>$K_{bi}$ CrP</th>
<th>$K_{ter}$ CrP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit CK-MM</td>
<td>0.17</td>
<td>0.05</td>
<td>8.6</td>
<td>2.9</td>
<td>Morrison &amp; James, 1965</td>
</tr>
<tr>
<td>Human CK-MM</td>
<td>0.07</td>
<td>0.03</td>
<td>3.7</td>
<td>1.33</td>
<td>Wang et al., 2001</td>
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<td>Human CK-BB</td>
<td>0.02</td>
<td>0.04</td>
<td>0.22</td>
<td>0.51</td>
<td>Wang et al., 2006</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Species</th>
<th>$K_{bi}$ ADP</th>
<th>$K_{ter}$ ADP</th>
<th>$K_{bi}$ ArgP</th>
<th>$K_{ter}$ ArgP</th>
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<td>Sea Urchin AK</td>
<td>0.06</td>
<td>0.06</td>
<td>1.30</td>
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<td>Held et al., 2007</td>
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<tr>
<td>Cockroach AK</td>
<td>0.12</td>
<td>0.09</td>
<td>1.00</td>
<td>0.94</td>
<td>Brown &amp; Grossman, 2004</td>
</tr>
<tr>
<td>Squid AK</td>
<td>0.20</td>
<td>3.5</td>
<td></td>
<td></td>
<td>Storey, 1977</td>
</tr>
</tbody>
</table>
In a bisubstrate reaction the ratio of the binding constants, \( K_{bi}/K_{ter} \), for a particular substrate is known as the degree of substrate binding cooperativity or “synergism” (Segal, 1975). Synergism occurs when the binding of one substrate influences the binding of the second substrate. According to Segal (1975) synergism can be both positive and negative with the binding of the first substrate enhancing the binding of the second substrate or where the binding of the first substrate decreases the binding of the second substrate. Positive and negative synergism is determined when the ratio of \( K_{bi}/K_{ter} \) deviates from one. For the creatine kinases, synergism seems to be a common characteristic displayed, although the degree of cooperativity can be considerably variable (Watts, 1973; Jacobs & Kuby, 1970; Chegwidden & Watts, 1975). On the other hand, the appearance of synergism for the arginine kinases is more variable with monomeric AK from the beetle and cockroach showing no degree of synergism while lobster AK displays a significant degree of synergism (Tanaka et al., 2007; Brown & Grossman, 2004; Virden et al., 1965). However, two dimeric arginine kinases from sea cucumbers, \( H. forskali \) and \( C. arenicola \), both exhibited synergism in the forward direction (Anosike et al., 1975; Seals & Grossman, 1988). In 2000, Hornemann et al. suggested that this type of cooperativity was associated with subunit-subunit interaction which would give significance to the dimeric state of these enzymes. Once again it is important to note that multiple types of reaction conditions and the use of recombinant enzymes can cause the values of synergism to vary (Morrison & James, 1965; Novak et al., 2004).
An interesting question investigated in enzyme kinetics is whether or not substrate analogs can be used in the enzyme reaction for catalysis. Studies utilizing substrate analogs can give important information into the way in which a substrate binds into the active site of the enzyme and how that binding influences product formation. Creatine kinases are fairly absolute when it comes to the specificity of their substrate, presumably due to the narrow slot that creatine fits into during binding (Watts, 1973; McLeish and Kenyon, 2005). Unlike creatine kinases, arginine kinases appear to not be absolutely specific for their substrate L-arginine. Enzymes from the muscles of *S. pavonina*, *L. polyphemus*, and *P. americana* can catalyze reactions with related substrate analogs such as homoarginine, canavanine, and D-arginine with some of the analogs being potent inhibitors to the preferred substrate L-arginine (Robin et al., 1971, Blethen, 1972; Brown & Grossman, 2004).

Early kinetic investigations determined that sulfate and phosphate anions inhibited the activity of CK (Nihei et al., 1961; Kumudavalli et al., 1970). These anions affected the initial velocities and the shape of the progress curves by mimicking the transferable phosphoryl group which stabilizes the dead-end complex and forms a highly stable transition state analog (Milner-White & Watts, 1971). The use of kinetic, NMR, spectroscopic, and X-ray techniques confirmed that the dead-end complex consists of the enzyme, a substrate, and a product (CK-creatine-MgADP) and that planar anions can stabilize the dead-end complex by forming a transition state analog consisting of the enzyme, a substrate, a product, and an anion (CK-creatine-MgADP-nitrate) (Reed & Cohn, 1972; Reed & Leyh, 1980; Lahiri et al., 2002). Milner-White and Watts (1971)
suggested that catalysis of CK proceeded by an in-line transfer reaction through a $S_N2$ type mechanism and that the tetrahedral phosphate binding site, for the transferable phosphoryl group of the substrate, changes conformation into a trigonal bipyrimidal site ($sp^3 d$ hybrid) in the transition state. Similarly, the formation of a catalytic dead-end complex and a transition state analog stabilized by planar anions in both monomeric and dimeric arginine kinases has been confirmed through the use of kinetic and NMR studies (Buttlaire & Cohn, 1974; Anosike et al., 1975; Brown et al., 2004).

Milner-White and Watts (1971) showed that small anions had varying effects on creatine kinase. Acetate was shown to increase activity in CK while planar anions, some halides, and tetrahedral anions such as: $\text{HCO}_3^-$, $\text{HCO}_2^-$, $\text{NO}_3^-$, $\text{NO}_2^-$, $\text{Cl}^-$, $\text{Br}^-$, $\text{F}^-$, $\text{SO}_4^{2-}$, $\text{HPO}_4^{2-}$, $\text{ClO}_4^-$, and $\text{BF}_4^-$ could have inhibitory effects. Anosike and Watts (1975) performed similar studies with monomeric, $H. vulgarus$, and dimeric, $H. forskali$, arginine kinases and found that both enzymes were activated, like CK, by the acetate anion. Unlike creatine kinase these enzymes were activated by the chloride anion. However, monomeric arginine kinases from the lobster, $H. americanus$, and the cockroach, $P. americana$, were found to be inhibited by the chloride anion (Buttlaire & Cohn, 1974; Brown et al., 2004). All arginine kinases studied thus far seem to be inhibited, to varying degrees, by the nitrate anion (Anosike and Watts, 1975; Buttlaire & Cohn, 1974; Brown et al., 2004). In 2004, Brown et al. showed that the nitrate anion was the most effective inhibitor of cockroach AK activity in terms of both the initial velocity and the percent of product formed. This led the authors to suggest that nitrate might be
inhibiting AK from the cockroach through a mechanism beyond that of just stabilization of the dead-end complex.

The first studies utilizing difference spectra for the phosphagen kinases were performed on monomeric arginine kinase and dimeric creatine kinase (Roustan et al., 1970; Roustan et al., 1968). This technique looks at small conformational changes in the environment (binding site) of a protein and can offer information regarding the structure of the active site. Roustan et al. (1970) was able to demonstrate what phosphate groups, \( \gamma \) and \( \beta \), from the nucleotides ATP and ADP, respectively, were able to participate in nucleotide binding by looking at spectral changes from the four substrates in the reaction (phosphoarginine, arginine, MgATP and MgADP).

Previous establishment of certain monovalent anions that could stabilize the dead-end complex of phosphagen kinases initiated studies on their effects towards the difference spectra of phosphagen kinases (Focant & Watts, 1973; Anosike & Watts, 1975; Lum et al., 1978). CK-BB from the ox and CK-MM from the rabbit both show pronounced minima around 260 nm in the presence of the complete transition state analog (creatine-MgADP-nitrate). However, the addition of the nitrate anion has a more pronounced affect on the rabbit muscle enzyme (Focant & Watts, 1973). Grossman and Garcia-Rubio (1987) determined that monkey CK-BB displayed significant differences only in the presence of the complete transition state analog mixture. Similarly, results seen with monomeric arginine kinase from the lobster showed that the enzyme was affected with nitrate in the presence of the transition state analog but not with nitrate alone (Anosike & Watts, 1975). The nitrate anion is known for having a profound affect
on tyrosine residues and it has been suggested that a tyrosine located in the active site of phosphagen kinases might cause the difference spectra and inhibition results seen with nitrate (Anosike & Watts, 1975). Interestingly, difference spectra in the presence of monovalent anions besides nitrate can not be found in the literature and results for the spectral changes of dimeric arginine kinases and nitrate have not been performed.

**Amino Acid Sequence, Structure, and Theories of Evolution**

Four independent genes have been found to encode the individual isozymes of CK and the mature gene products range from about 40 kDa to 44 kDa (Muhlebach et al., 1994). The two cytosolic isozymes exhibit 80% sequence homology toward each other with the mitochondrial isozymes displaying a similar homology when compared. Alternatively, the cytosolic and mitochondrial isozymes show only 60 to 65% sequence identity when compared to each other (Muhlebach et al., 1994). It was determined that there are six regions of extensive homology in the CK isozymes flanked by seven more variable regions. Two of these regions are highly conserved sequences (a negatively charged NEED-box and a region surrounding a cysteine residue), which have been found across all phosphagen kinases and are thought to play a role in the catalytic mechanism (Eder et al., 2000; Cantwell et al., 2001).

The first arginine kinases were sequenced in 1994 by Suzuki and Furukohri from monomeric sources. Since then, numerous monomeric and dimeric arginine kinases have been sequenced and when compared are found to contain about 40% sequence homology (Suzuki et al., 1999). Interestingly, when dimeric arginine kinase from *S. japonicus* was
compared to creatine kinase sequences, it was found to be more homologous (58-62%) with the vertebrate CK sequences then it was with other monomeric arginine kinases (33-45%) (Suzuki et al., 1999). Similarly, Suzuki et al. (2000) determined that the intron and exon make-up (6 and 7 respectively) as well as the splice junctions of dimeric AK from *S. japonicus* were conserved in the same positions as they appeared in human CK-MM. Sequence alignments by Suzuki and Furukohri also displayed a highly conserved block of 15 amino acids in monomeric AK, dimeric AK, and dimeric CK. The authors suggested that these residues might be conserved residues because they included amino acid candidates that could be involved with ATP binding and general acid/base catalysis. In 2001, a representative CK sequence from rabbit muscle and a representative AK sequence from the horseshoe crab were used to analyze sequence homology between 29 creatine kinase and 16 arginine kinase amino acid sequences found in GenBank (Edmiston et al., 2001). The authors found that 53% of the CK residues were either fully or highly conserved between all creatine kinases analyzed while only 31% of the AK residues were fully or highly conserved in all arginine kinases examined (Figure 4). The sequence homology comparisons also show that 76 residues were fully or highly conserved in all of the CK and AK sequences examined suggesting that those residues may have similar roles in all guanidino kinases.
Figure 4. Sequence Homology of Creatine and Arginine Kinases:

The sequence homology of 29 creatine kinases and 16 arginine kinases were compared to the representative sequences of dimeric rabbit muscle CK (top row) and monomeric horseshoe crab AK (bottom row). Colored residues indicate positions that are either fully conserved (caps) or highly conserved (>90%, lower case) conserved. Blue: conserved in either CK or AK but not both; Red: conserved in both CK and AK; and Green: conserved in CK and conserved as a different residue in AK. A list of all CK and AK sequences used can be found in Edmiston et al., 2001.
While the above comparisons clearly demonstrate that arginine and creatine kinases are part of a highly conserved enzyme family, the phosphagen kinases do have some distinct differences. Suzuki et al. (1997) noticed through sequence alignments of the phosphagen kinases that each kinase might have unique guanidino specificity (GS) regions used for guanidine recognition. The GS region is characterized by amino acid deletions or insertions that seem to correspond to the size of the guanidine substrate utilized. Glycocyamine kinase has no amino acid deletions, CK has a one amino acid deletion, and AK and LK have a 5 amino acid deletion, presumably due to their relatively large guanidine substrates (Figure 5). The sequence of dimeric arginine kinase from the sea cucumber, *S. japonicus*, highlights the substrate specificity of each kinase by sharing the same deletions and insertions as those for creatine kinases except in the GS region (Suzuki et al., 1999). To further characterize the specificity of the GS region, Uda and Suzuki (2004) introduced mutations around the GS region of *S. japonicus* AK. The authors found that an insertion or deletion mutation near the GS region caused the $V_{max}$ of the mutant enzyme to be significantly decreased compared to the wild-type enzyme. The loss of activity was presumed to be due to the structure around the substrate binding site being altered and led the authors to suggest that the length of the GS region was critical for recognition of the guanidine substrate. Further kinetic analysis suggested that amino acids Phe 63 and Leu 65 are associated with arginine binding because replacement of either amino acid to Gly caused a dramatic increase in the $K_{M}^{arg}$. 
Figure 5. Alignment of Dimeric AK against CK Enzymes:

A comparison of the amino acids in dimeric arginine kinase and creatine kinase.

Red: guanidino specificity region; Green: amino acids in nucleotide binding; Blue: amino acids in arginine binding; Pink: amino acids in creatine binding. Figure from Held, 2007.

Sequences used:
- Danio CK: gi|18858427|
- Sea Urchin Sperm: gi|125302|
- Sea Cucumber: gi|4586462|
- Cnidarian: gi|110083395|

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Although the phosphagen kinases display specificity in their guanidino regions, arginine and creatine kinase do exhibit very similar crystal structures (Eder et al., 1999; Fritz-Wolf et al., 1996; Lahiri et al., 2002). The first reported crystal structure of creatine kinase occurred in 1996 by Fritz-Wolf et al. using sarcomeric chicken cardiac (Miβ-CK) in the presence and absence of NaATP. Four identical independent monomers were shown to consist of a small N-terminal domain (residues 1 to 112) and a larger C-terminal domain (residues 113 to 380) with the ATP binding site located in a cleft between the two domains (Figure 6). Two loops, residues 60 to 65 and 316 to 326, were found to be highly flexible and the previously observed six regions of conserved sequence homology (Muhlebach et al., 1994) were found to form the core structure covering the active site of the enzyme.

The crystal structure helped identify residues in the active site of the enzyme which are important for substrate binding or catalysis. Previous studies pointed to a histidine acting as a general acid/base catalyst near the binding of ATP and that an ionized carboxylic acid group was required for the binding of both creatine and phosphocreatine (Rosevear et al., 1981; Cook et al., 1981). The X-ray structure showed that no histidines resided within 12 Å of the γ-phosphate ATP and that two glutamic acid residues, Glu 226 and Glu 227, as well as an aspartic acid, Asp 228, were located in the active site. The phosphate group of ATP was found to interact with four arginine residues and that two more are located within 5 Å of the γ-phosphate (Figure 5). The specificity for creatine was determined in a later structure of CK-MM and appears to be located in a pocket formed by Ile 69 and Val 325 while the substrate is held in place by
Figure 6. Ribbon Diagram of Sarcomeric Chicken Creatine Kinases Monomer:

The two flexible loops are highlighted and the ATP binding site is identified by the two phosphates in the cleft between the N and C terminal domains. Figure from McLeish & Kenyon, 2005. Coordinate used from PDB 1CRK (Fritz-Wolf et al., 1996).
the amino acids Glu 232 and Cys 283 (Lahiri et al., 2002). A tryptophan, Trp 223, was found in the active site and supported early theories on its importance for catalysis (Vasak, et al. 1979; Messmer & Kagi, 1985; Gross et al., 1994). A cysteine residue, Cys 278, found to be located in the active site and positioned near acidic residues and the γ-phosphate of ATP is thought to be involved in creatine binding. This finding supported numerous studies over the years which implicated a cysteine residue in the active site of creatine kinase (Mahowald et al., 1962; Wyss et al., 1993; Buechter et al., 1992).

The first crystal structure of arginine kinase from the horseshoe crab *Limulus* was reported in 1998 by Zhou et al. bound to its transition state analog complex. Like the structure for creatine kinase, AK also displayed a small N-terminal domain and a larger C-terminal domain with the catalytic site in between the two (Figure 7). Two loops, residues 63-68 and 309-318, were found in *Limulus* AK, but unlike creatine kinase, which contained two disordered loops, the loops in *Limulus* AK are found in a substantially different conformation. The structure also indicated seven amino acids (Ser 63, Gly 64, Val 65, Tyr 68, Glu 225, Cys 271, and Glu 314) were involved with arginine binding and that 5 arginine amino acids were involved with ADP binding. The amino acids involved in nucleotide binding for AK are identical to the results from the CK structure (Fritz-Wolf et al., 1996). This similarity highlights the importance of arginine amino acids in binding phosphate moieties in the phosphagen kinases (Edmiston et al., 2001). It has been suggested that arginine residues function by pulling electrons towards the oxygens attached to the phosphate in order to prepare it for a nucleophilic attack (Zhou et al., 1998). The residues Ser 63, Gly 64, Val 65, and Tyr 68 appear in the
guanidine specificity region of Limulus. These residues were not found to be amino acids conserved in dimeric *S. japonicus* AK (Uda & Suzuki, 2004) but a comparison of several one and two-domain arginine kinase sequence compositions showed that these amino acids were highly conserved throughout the arginine kinases (Uda et al., 2006).

A comparison of AK and CK sequences and structures shows distinct similarities in their conserved amino acid residues. A superimposed figure of AK and CK crystal structures further demonstrates this similarity by showing that the enzymes display a three dimensional structure that is nearly identical (Figure 8). Clearly, there is no doubt that the structure of the phosphagen kinases is closely linked to their function. However, the original crystal structures of Miβ-CK and AK showed discrepancies in a few of their arrangements (ie. loop structures). It was suggested that discrepancies between crystal structures could be attributed to what the enzyme was bound too when crystallized. Miβ-CK crystals were not bound to both substrates while AK crystals were bound to the complete transition state analog complex (TSAC). These two distinct enzyme states where referred to as “open” (enzyme not bound to substrate) and “closed” (enzyme bound to substrate) conformations. It was evident that the loops moved upon substrate binding which allowed a substantial conformational change to take place (Forstner et al., 1998; Yousef et al., 2003). A later crystal structure, of *Torpedo* CK bound to MgADP and the TSAC, allowed a direct comparison and conformation of the open and closed conformations in a single enzyme of the phosphagen kinase family (Lahiri et al., 2002).
Figure 7. Ribbon Diagram of Horseshoe Crab Arginine Kinase:

The N-terminal is shown in yellow and the C-terminal is shown in green. The transition state analog complex ligands are shown in ball-and-stick and lie left to right. MgADP: light blue; Nitrate and Arginine: red; Two Loops: red (63-68) and blue (309-318). Figure from Zhou et al., 1998.
Figure 8. Arginine Kinase Structure Superimposed on Creatine Kinase Structure:

The structure of arginine kinase is from PDB 1RL9 (Azzi et al., 2004) and the structure of creatine kinase is from PDB 1VRP (Lahiri et al., 2002).
Crystal structures of AK and CK bound to substrate allowed for comparisons of the two closed structures to be made. Uda and Suzuki (2004) linked Asp 62, in the GS region, and Arg 193, in the C-terminal domain of *Limulus* AK with amino acids His 66 and Asp 326, respectively, in *Torpedo* CK. They suggested that these amino acids were significant in stabilization of the closed structure through hydrogen bonding. Similarly, these structures led to a different understanding of the catalytic mechanism in which substrate alignment appeared to more important than other mechanisms. It is suggested that substrate binding induces large loop conformational changes which allow the loops to interact with substrates, and possibly play a role in substrate specificity (Suzuki et al., 1997; Cantwell et al., 2001). However, Azzi et al. (2004) demonstrated that deletions or mutations to loop structures had no apparent effect on the enzyme suggesting their role in substrate specificity was not as important as previously implied. Currently, it is viewed that loop movements are likely involved in rearranging critical elements of the active site and closing off the active site to exterior solvent during the reaction (Zhou et al., 2000; Azzi et al., 2004).

For over 30 years the evolutionary relationships between the phosphagen kinases have been questioned and continually revised. Initial studies deduced that arginine kinase was the most primitive phosphagen kinase due to the following facts: (a) it was found in invertebrate species; (b) it was widely distributed; (c) it was typically found as a monomer; (d) the homologous enzyme creatine kinase was the only phosphagen kinase present in vertebrate species; and (e) it used the amino acid arginine as a substrate rather than a secondarily derived guanidine compound (Moreland & Watts, 1967; Van Thoi,
1968; Watts, 1971). This basic theory remained unchanged but was modified by Anosike et al. (1975) who suggested that arginine kinase had actually evolved twice through the course of evolution. Their suggestion was based on the finding of cooperativity in monomeric and dimeric organisms (honey bee and sea cucumber) that were considered to be more advanced species. Organisms such as the crab and annelid which evolved earlier on the evolutionary tree were not found to contain cooperativity. The authors pointed out that cooperativity was always seen in mammalian creatine kinases (more advanced organisms) but since the honey bee and sea cucumber were sufficiently separated from mammals on the evolutionary tree; they thought cooperativity, like dimerization, had occurred more than once during phosphagen evolution.

Although it had been known for quite some time that echinoderms contained arginine kinase in their eggs and creatine kinase in their sperm (Moreland et al., 1967) Ratto et al. (1989) studied a variety of echinoderms to see which phosphagen they contained in other tissues. They determined that both enzymes were distributed throughout the echinoderm family and suggested that since there would be no need for a single organism to contain two functionally similar enzyme systems that the echinoderms were currently transitioning from one system to another. Echinoderms branched off from other deutrostromians soon after the protostomian and deuterostomian split which led Ratto et al. (1989) to hypothesize that the present day phosphagen kinases diverged from one metazoan ancestor or that a common ancestor before the split contained both creatine and arginine kinase activities.
As more advanced techniques became available, the amino acid sequences for a series of phosphagen kinases were deduced and evaluated. The first dimeric arginine kinase, *S. japonicus*, was sequenced by Suzuki et al. (1999), aligned with several creatine kinases, glycocyamine kinases, arginine kinases, and lombricine kinases known at the time, and found to share the most sequence identity with the creatine kinases. A phylogenetic tree was constructed (with the maximum-likelihood method) which separated the sequences into 2 major clusters. One cluster contained all of the protosome arginine kinases while the other cluster contained all creatine kinases, glycocyamine kinases, lombricine kinases, and dimeric AK from *S. japonicus*. Suzuki et al. (1999) also suggested that AK evolved twice and that members of the phosphagen kinase family are derived from two genes, one for AK and one for CK, which arose from the duplication of an ancestral gene. They concluded that dimeric arginine kinases evolved from creatine kinases later in metazoan evolution while arginine kinases from mollusks and arthropods evolved at an earlier stage of evolution.

A more recent discovery by Ellington (2000) reported that CK was present in the most primitive of all metazoans species. Previously, sponges were known to contain arginine kinase activity and most were thought to be monomeric in structure (Robin & Guillou, 1980). Recently, creatine kinase from the sponge *Tethya auranita* was shown by Ellington to be a dimer; which implied that CK was expressed earlier in evolution than was previously thought, along with the occurrence of quaternary structure. Ellington (2001) suggested that AK and CK evolved early in the course of metazoan evolution and that they are on two distinct evolutionary trajectories. Ellington also stated that the CK
gene most likely underwent multiple gene duplications resulting in the other phosphagen kinases (GK and LK) as well as the multiple isozymes characterized in CK such as flagellar, mitochondrial, MM, and BB creatine kinases.

Figure 9 shows the relationships between protozoans, invertebrates, and vertebrates along with the phosphagen kinases that appear in each class on a phylogenetic tree (Held, 2007). The phylogenetic tree shows protozoan monomeric arginine kinase from *T. cruzi* to be the most basal phosphagen kinase and implies that monomeric AK is the primordial phosphagen. It should be noted however, that this arginine kinase has been shown by Pereira et al. (2000) to cluster with arthropod AK and the authors suggest that parasitic *T. cruzi* acquired AK from arthropod hosts through horizontal gene transfer. If the protozoan results are set aside then sponges would be left as the basal organism on the phylogenetic tree. Since sponges have been confirmed to contain both AK and CK it can be assumed that a primitive organism prior to sponges must have contained both AK and CK. These observations indicate that the traditional characterizations of phosphagen kinase evolution (a primitive monomeric AK to an advanced dimeric CK) are inconsistent with present day data and should no longer be supported.
Figure 9. Phylogenetic Tree for the Relationship of Vertebrates and Invertebrates:
A phylogenetic tree representing the accepted relationships of vertebrates to invertebrates and protozoans was created. The phosphagen kinases which appear in each class were mapped onto the phylogenetic tree. Figure from Held, 2007.
Significance

Since the 1960’s it has been known that cockroaches have been a factor in allergenic disease by producing potent allergens that are an important cause of asthma (Bennett & Spink, 1968). Sensitized individuals living in urban, inner-city settings are particularly susceptible to cockroach allergen exposure and increased asthma morbidity (Zhou et al., 1998; Yun et al., 2001). The America cockroach, *Periplaneta americana*, is recognized as one of the most common domiciliary cockroach species along with the German cockroach, *Blattella germanica*. Initially there was interest in studying arginine kinase from the American cockroach not because the roach was an organism that produced allergens towards humans but because of its role as a domestic pest (Brown et al., 2004). Arginine kinase from the cockroach was viewed as a potential target to control cockroach populations because this enzyme’s catalytic activity is essential for the motility of the roach. This view of population control was previously shown for AK from the parasite causing Chagas disease (Pereira et al., 2000) and led Brown and Grossman (2004) to look for ways to inhibit AK activity through substrate and transition state analogs.

It was not until 1995 when Crespo et al. identified that arginine kinase from the shrimp was one of the enzymes responsible for allergic reactions in individuals that scientist started looking at AK as a primary etiological agent. In 2001 through molecular characterization it was discovered that arginine kinase from the moth, *P. interpunctella*, caused a high prevalence of IgE reactivity and demonstrated that this allergen has IgE cross-reactive homologs in several invertebrate species including the American
cockroach (Binder et al., 2001). Since that time, seven *P. americana* produced allergens (including one from arginine kinase) have been identified, characterized, and officially recognized by the World Health Organization/International Union of Immunological Societies showing the significance of studying arginine kinase from the roach (Gore & Schal, 2007; Sookrung et al., 2006).

Studies pertaining to the isozymes of CK are important since these enzymes are key regulators of vertebrate energy metabolism, as pointed out when discussing the physiological role of phosphagen kinases (Wallimann et al., 1992; Schlattner et al., 2002). More recently, research suggests that CK may be involved in human conditions such as neuromuscular and neurodegenerative disease, where it is being recognized as an important metabolic regulator (Wallimann et al., 1998). In 2000 it was shown that CK-BB is over-expressed in malignant tumors and can apply cell protective functions in neurodegenerative and age related diseases (Wyss & Kaddurah-Daouk, 2000; Wyss & Schulze, 2002). Similarly, a number of phenotypes support the importance of the CK system in tissues that contain it. In transgenic mice where one or both CK isozymes were knocked out in muscle cells a number of biochemical, morphological, and metabolic adjustments were seen (Steeghs et al., 1998) with the high energy demanding processes being the most impaired by the loss of the phosphagen system (de Groof et al., 2002).

Finally, the comparison of dimeric arginine kinase to creatine kinase and monomeric arginine kinase is interesting in light of the evolutionary questions regarding the phosphagen family. Dimeric arginine kinase shares a substrate with the monomeric arginine kinases but it been shown to be more homologous to the dimeric creatine kinases.
in terms of sequence and structure (Suzuki et al., 1999; Suzuki et al., 2000). Current molecular techniques have made it possible to re-evaluate the evolutionary views regarding the phosphagen kinase family and suggest that the traditional characterizations of phosphagen evolution might no longer hold true (Ellington, 2001). Dimeric arginine kinases can be used to compare phosphagen kinase dimers and monomers towards a better understanding for the significance of the dimeric state in these enzymes and can provide information on why some organisms retained the dimeric state while others did not.

Although the phosphagen kinases have been studied for over 40 years, many questions about this enzyme family remain unanswered. New discoveries of their significance are continually being found and questions regarding the structures, functions, and even regulation of the phosphagen kinases still need to be addressed. The significance of the various subunit compositions found scattered throughout vertebrate and invertebrate species, as pointed out earlier, is one such question that has yet to be elucidated. Clearly further examination of the phosphagen kinases is warranted, especially given the key roles they play in energy metabolism.
Chapter 2. Materials and Methods
Part I. Standard Assays and Procedures

Protein Determination

Determination of protein concentrations were performed using the dye binding method described by Bradford (1976) with bovine serum albumin as the calibration standard. Protein concentrations were also measured using the Micro Protein Determination kit from Sigma Chemical Company (St. Louis, MO). Bovine serum albumin was used as the protein standard for calibration and absorbance was read at 725 nm.

Enzyme Assays

Arginine kinase activity was routinely determined for the reverse direction, production of L-arginine and ATP (Figure 10), using the enzyme-coupled assay (Figure 11) as described by Rosalki (1967) for the creatine kinases. The final mixture (0.5 mL) contained 10 mM arginine phosphate, 1.125 mM ADP, 0.4 mM NADP, 0.5 U/mL hexokinase, 1 U/mL glucose-6-phosphate dehydrogenase, 10 mM dithiothreitol, 3 mM magnesium acetate, and 20 mM glucose in 0.1 mM Tris/HCL buffer, pH 8.0. After addition of enzyme samples, the increase in absorbance was measured at 340 nm on a Beckman Coulter DU 640 Spectrophotometer (Fullerton, CA).
An enzyme-linked colorimetric assay (Florini, 1989) was used to measure AK activity in the reverse direction (Figure 12). The assay mixture contained 10 mM arginine phosphate, 1 mM ADP, 10 mM magnesium acetate, 20 mM glucose, 0.4 mM thiolated NAD, 10 mM DTT, 0.5 U/mL hexokinase, and 1 U/mL glucose-6-phosphate dehydrogenase. The production of the reduced pyridine nucleotide of a thiolated NAD produces a yellow color which is measured at 405 nm.

Arginine kinase activity in the forward direction (Figure 10), synthesis of L-arginine phosphate and ADP, was routinely measured by a direct pH stat assay (Figure 13), using a Radiometer Analytical Autoburette ABU 901 (Chicago, IL). The final saturating assay mixture (2 mL) contained 2.0 mM ATP, 5 mM L-arginine, 3.0 mM magnesium acetate, and 2.0 mM dithiothreitol adjusted to pH 8.0. Dry nitrogen continuously purged the reaction vessel and the temperature was held constant at 25°C with a circulating water bath. A 0.01 M solution of NaOH (which had been calibrated against a standard solution of HCl) was used as the titrant. Prior to assay, the enzyme was dialyzed against 2 mM Tris/HCl pH 8.0 buffer containing 1 mM EDTA and 2 mM DTT.

An enzyme-coupled spectrophotometric assay (Fujimoto et al., 2005) was used to measure AK activity in the forward direction. The assay mixture (1 mL) contained 37.5 mM KCl, 12.5 mM magnesium acetate, 1.25 mM phosphoenolpyruvate, 40 mM L-arginine, and 5 mM ATP in 0.1 M imidazole/HCl pH 7.0, 0.25 mM NADH in Tris/HCl pH 8.0, 0.1 mL pyruvate kinase/lactate dehydrogenase mixture, and 0.55 mL of 0.1 M
Tris/HCl pH 8.0. A decrease in absorbance was read at 340 nm after the addition of 0.050 mL of arginine kinase enzyme.

**Arginine Kinase Reactions**

**Reverse Reaction**

\[ \text{MgADP} + \text{L-Arginine Phosphate} \rightleftharpoons \text{MgATP} + \text{L-Arginine} \]

**Forward Reaction**

\[ \text{MgATP} + \text{L-Arginine} \rightleftharpoons \text{MgADP} + \text{L-Arginine Phosphate} + \text{H}^+ \]

Figure 10. Arginine Kinase Reactions:
Figure 11. Enzyme-Coupled Assay for the Reverse Reaction:

Enzyme-Coupled Spectrophotometric Assay

\[ \text{L-Arginine Phosphate} + \text{ADP} \underset{\text{AK}}{\rightleftharpoons} \text{L-Arginine} + \text{ATP} \]

\[ \text{ATP} + \text{D-Glucose} \underset{\text{HK}}{\rightleftharpoons} \text{ADP} + \text{D-Glucose-6-Phosphate} \]

\[ \text{D-Glucose-6-Phosphate} + \text{NADP}^{+} \underset{\text{G6PDH}}{\rightleftharpoons} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^{+} \]

An increase in absorbance at 340 nm due to the formation of NADPH + H⁺ is directly proportional to arginine kinase activity (in the reverse direction).
Figure 12. Enzyme-Linked Colorimetric Thio-NAD Assay:

\[
\begin{align*}
\text{AK} & \quad \text{Arginine Phosphate} + \text{ADP} \rightarrow \text{Arginine} + \text{ATP} \\
\text{HK} & \quad \text{ATP} + \text{D-Glucose} \rightarrow \text{ADP} + \text{D-Glucose-6-Phosphate} \\
\text{G6PDH} & \quad \text{D-Glucose-6-Phosphate} + \text{Thio-NAD}^+ \rightarrow 6\text{-Phosphogluconate} + \text{Thio-NADH} + H^+
\end{align*}
\]

An increase in absorbance at 405 nm due to the formation of Thio-NADH + H⁺ is directly proportional to arginine kinase activity (in the reverse direction).
Figure 13. Direct pH Stat Assay for the Forward Reaction:

\[ \text{L-Arginine} + \text{ATP} \xrightarrow{\text{AK}} \text{L-Arginine Phosphate} + \text{ADP} + \text{H}^+ \]

This assay measures arginine kinase activity (in the forward direction) directly by the production of H⁺.
**SDS-PAGE Electrophoresis**

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS) was performed as described by Laemmli (1970). Slabs were comprised of a 12.5% separating gel and a 4% stacking gel. Prior to electrophoresis, protein samples were denatured by addition of an equal volume of loading buffer containing 0.5 M Tris/HCl pH 6.8, 10% SDS, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% bromophenol blue followed by boiling for 5 minutes. Electrophoresis running buffer contained 25 mM Tris/HCL pH 8.3, 192 mM glycine, and 0.1% SDS. A constant voltage (150 volts) was maintained for electrophoresis until the dye front approached the bottom of the gel. Prestained molecular weight markers (Bio-Rad, Richmond, CA) were electrophoresed along with protein samples. The gels were stained with a 4% Coomassie blue, 40% methanol, and 10% glacial acetic acid solution for 5 min followed by extensive destaining with a 1:100 glacial acetic/water solution with 10% methanol. The destaining solution was changed multiple times over a period of several hours.

**Native Gel Electrophoresis**

Native gels were prepared and electrophoresed as described for SDS-PAGE electrophoresis with the omission of SDS in all steps. Gels were stained for catalytic activity with an assay reagent (Figure 14) containing 30 mM arginine phosphate (or creatine phosphate), 2 mM ADP, 20 mM glucose, 10 mM magnesium acetate, 2 mM NADP, 2 U/mL hexokinase, 1.6 U/mL glucose-6-phosphate dehydrogenase, 22 μM phenazine methosulfate, and 0.36 mM tetranitroblue in a total volume of 25 mL of 1 M
Tris/acetate, pH 7.5. After development, the gels were destained in a 70% methanol-5% acetic acid solution for 30 minutes. Standards obtained from Sigma (CK 3 in 1) were electrophoresed along with samples to determine electrophoretic mobility. Arginine kinase activity is indicated by a purple precipitate (production of tetranitroblue tetrazolium formazan).

![Catalytic Activity Stain](image)

**Catalytic Activity Stain**

\[
\begin{align*}
\text{AK} & : \quad \text{L-Arginine Phosphate} + \text{ADP} \rightarrow \text{L-Arginine} + \text{ATP} \\
\text{HK} & : \quad \text{ATP} + \text{Glucose} \rightarrow \text{Glucose-6-Phosphate} + \text{ADP} \\
\text{G6PDH} & : \quad \text{Glucose-6-Phosphate} + \text{NADP}^+ \rightarrow \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+ \\
\text{NADPH} + \text{H}^+ + \text{Phenazine Methosulfate} & \rightarrow \text{NADP}^+ + \text{Phenazine Methosulfate} \\
\text{Phenazine Methosulfate(reduced)} + \text{Tetranitroblue Tetrazolium} & \rightarrow \text{Phenazine Methosulfate(oxidized)} + \text{Tetranitroblue Tetrazolium Formazan (TNBT)} \\
& \quad \text{(Purple precipitate)}
\end{align*}
\]

The production of TNBT formazan produces a purple precipitate that is directly proportional to AK activity.

Figure 14. Catalytic Activity Stain for Native Gel Electrophoresis:
Part II. Purification and Characterization of Sea Cucumber Arginine Kinase

Purification

The purification of arginine kinase from the Giant Sea Cucumber, *Isostychopus badonotus* (Gulf Specimen Marine Laboratory, Inc., Panacea, FL), is a modification of the method developed by Seals and Grossman (1988) for the purification of AK from the sea cucumber *Caudina arenicola*. A total of 30-50 g of frozen sea cucumber muscle was homogenized in 50 mM Tris/acetate, 10 mM 2-mercaptoethanol, 1 mM EDTA, 25 uM phenylmethylsulfonylfluoride, 50 uM NaN₃, protease inhibitor cocktail tablets (1 tablet per 0.1 L) from Roche (Indianapolis, IN), pH 8.0 for 1 min in a Waring blender. The homogenate was centrifuged at 14,500 x g for 25 min at 4°C and filtered through glass wool. The supernatant was centrifuged at 81,000 x g for 1 hr at 4°C and the pellet discarded. Following ultracentrifugation, the supernatant that resulted was made 50% saturated with ammonium sulfate, stirred on ice for 10 min, and then centrifuged at 4,000 x g for 20 minutes. The supernatant obtained was made 82% saturated with ammonium sulfate and the precipitate was collected by centrifugation at 4,000 x g for 20 min. The 50-82% ammonium sulfate precipitation yielded the majority of AK activity in the pellet. The pellet was solubilized in 50 mM Tris/acetate pH 8.0, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 25 uM PMSF to a total volume of 7.5 mL and the solution applied to an ACA 34 (Ciphergen, Freemont, CA) gel filtration column (3.8 cm x 76 cm) and eluted with the same buffer. Fractions containing 16 mL were collected and assayed for activity. Active fractions were combined and dialyzed extensively against 0.02 M potassium phosphate buffer containing 10 mM 2-mercaptoethanol and 1 mM EDTA at
pH 7.5. The fractions were applied to a column (4 cm x 12.7 cm) of Hydroxyapatite (Bio-Rad, Richmond, CA) and eluted with a potassium phosphate gradient of 0.02 M to 0.5 M, pH 7.5. Fractions containing 10 mL were collected and assayed for activity. The most active fractions were combined and concentrated using an Amicon ultra filtration cell (Millipore Corp., Bedford, MA) containing a YM-30 membrane. Figure 15 summarizes the protocol for the purification.

Molecular Weight and Subunit Composition

The molecular weight of native arginine kinase from the sea cucumber was determined using gel filtration chromatography. A purified sample (0.25 mL) of AK (0.2 mg/mL) was placed on a Sephadex G-100 column (1.9 cm x 53.3 cm) and eluted with 50 mM Tris/HCl pH 8.0, 10 mM 2-mercaptoethanol, 1 mM EDTA, 5 μM NaN₃ in 1 mL fractions. The column was calibrated with 1 mg/mL of purified creatine kinase (MM) and monomeric arginine kinase (shrimp). Activities were determined using the enzyme-coupled spectrophotometric assay for the reverse direction.

The subunit molecular weight of denatured AK was determined using calibrated SDS-PAGE. Myosin (210 kDa), beta-galactosidase (131 kDa), bovine serum albumin (87 kDa), carbonic anhydrase (42.2 kDa), soybean trypsin inhibitor (32.3 kDa), lysozyme (18.8 kDa), and aprotinin (8.2 kDa) were used as molecular weight standards. A sample of 11 μg of AK was applied to the gel.
Figure 15. Flow Chart for the Purification of Sea Cucumber Arginine Kinase:

1. Homogenize 30-50 g Sea Cucumber
   - 50mM Tris-acetate pH 8.0, 10mM 2-mercaptoethanol, 1mM EDTA, 25μM PMSF
   - Centrifuge 14,500 x g for 25 min

   - Pellet: Discard
   - Supernatant: Centrifuge 81,000 x g for 1 hr

2. Supernatant
   - Pellet: Discard
   - Supernatant
     - Ammonium Sulfate 0-50%
     - Stir on ice 10 min
     - Centrifuge 4,000 x g for 20 min

   - Supernatant
     - Ammonium Sulfate 50-82%
     - Stir on ice 10 min
     - Centrifuge 4,000 x g for 20 min

3. Gel Chromatography
   - ACA-34 column

4. Collect Active Fractions
   - Combine
   - Dialyze

5. Adsorption Chromatography
   - Hydroxyapatite column

6. Collect Active Fractions
   - Combine
   - Concentrate
**Thermal Inactivation**

Partially purified samples were incubated at varying temperatures for 1-15 min, rapidly cooled on ice, and tested for arginine kinase activity in the reverse direction using the enzyme-coupled spectrophotometric assay.

**pH Optimum**

The pH optimum for the reverse reaction was determined using the enzyme-linked colorimetric thio-NAD assay (Figure 12) described above. Sea cucumber AK (12 μg) was incubated for 15 minutes in the colorimetric assay mixture and change in absorbance recorded. The buffer systems included 0.1 M acetic acid/acetate for pH values between 4.5 and 6.0, 0.1 M sodium phosphate buffer for pH values between 6.5 and 7.5, and 0.1 M Tris/HCl for pH values between 8.0 and 10.0. The pH optimum in the forward direction was measured using the pH stat assay as described, with the pH being directly varied and controlled by fixing the “stat” pH.

**pH Stability**

The pH stability of arginine kinase was determined using 0.1 M sodium phosphate, 1 mM EDTA, and 2 mM DTT buffer over a pH range of 6.4-8.25. Arginine kinase (0.2 mL) was incubated in each buffer (1 mL) and stored over a period of three weeks. The activity of each enzyme sample was tested using the standard enzyme-coupled spectrophotometric assay in the reverse direction at varying days throughout the three week time span.
**Isoelectric Focusing**

Isoelectric focusing was performed using a Rotofor Preparative IEF Cell (Bio-Rad, Richmond, CA). A 50 mL sample containing 5 units (1 unit = 1 µmol NADH + H⁺ produced/min) of AK and 1% ampholytes (pH 3-10) were electrofocused for 6 h at 6°C. Two mL fractions were collected and the pH of each fraction was measured with a Radiometer IE 7.5 mm x 103 mm electrode. The activity of each fraction was determined by using the enzyme-coupled spectrophotometric assay for the reverse direction.

**Absorption Spectrum**

An absorption spectrum (250 to 600 nm) was determined for a sample of sea cucumber arginine kinase (0.22 mg/mL) in 0.05 M sodium phosphate buffer, pH 8.0.

**Circular Dichroism**

Circular dichroic spectra in the far ultraviolet (190 nm to 240 nm) were performed on a purified protein sample of AK. The protein concentration was 0.07 mg/mL in 2 mM sodium phosphate, 1 mM 2-mercaptoethanol, pH 8.0 buffer. An Aviv Model 215 spectropolarimeter (Rheometric Scientific, Inc., Lakewood, NJ) and a cuvette with a 1 mm cell pathlength were used to record spectral measurements. Alpha helical content was obtained using the equation $f_n = \theta_{222} + 2340/30,300$ according to Chen et al. (1972).
Electrophoretic mobility

The electrophoretic mobility of native sea cucumber AK was determined using the native gel electrophoresis procedure previously described. A sample of purified sea cucumber AK (0.05 mL) and purified cockroach AK, *Periplaneta americana*, (0.05 mL) were applied to the gel along with a standard containing three isozymes of CK with known relative electrophoretic mobilities.

Reactivation

A purified sample of AK (0.1 mL) was denatured by incubation with 8 M urea (0.1 mL) in 0.1 M Tris/HCl, 2 mM DTT, pH 8.0 for 15 minutes. The final concentration of urea during denaturation was 4 M. The sample was then renatured for 30 min directly in the spectrophotometric assay for the forward direction (previously described). The final concentration of urea during renaturation was 0.2 M. A sample of AK (0.1 mL) and buffer (0.1 mL) without urea was assayed parallel to the above sample for comparison.

Hybridization of AK subunits

Purified samples of sea cucumber (13 µg) and sea urchin egg (36 µg), *Strongylocentrotus purpuratus* (Marinus Scientific, Garden Grove, CA), were mixed to a total volume of 1 mL and then diluted with 6 M guanidine hydrochloride in 0.05 M Tris/HCl, 2 mM DTT, 1 mM EDTA, 50 µg NaN₃, pH 8.0 buffer to a final concentration of 3 M guanidine hydrochloride. After incubation for 30 min at 4 °C the sample was
renatured using extensive dialysis against the above buffer without guanidine hydrochloride. Samples of (a) native sea cucumber AK, (b) native sea urchin egg AK, (c) mixed native sea cucumber and sea urchin egg AK, (d) mixed denatured/renatured sea cucumber and sea urchin egg AK, (e) denatured/renatured sea urchin egg AK, and (f) denatured/renatured sea cucumber AK were then subjected to non-denaturing electrophoresis at 4°C. Gel slabs containing a 10% separating gel and a 4% stacking gel were prepared, developed (4 h at 4°C), and destained as described under the Native Gel Electrophoresis section.
Part III. Polyclonal Antibody Production and Western Blotting of Sea Cucumber AK

Preparation of Polyclonal Antibodies

Polyclonal antibodies against sea cucumber AK were developed by ProSci, Inc. (Poway, CA) from a 1 mg sample of purified protein. Polyclonal antibody fractions from serum were purified and concentrated by 33% ammonium sulfate precipitation. The serum was made 33% saturated with ammonium sulfate, stirred on ice for 10 min, and then centrifuged at 4,000 x g for 20 minutes. The precipitate was then solubilized and dialyzed extensively against 0.05 M sodium phosphate pH 7.5 buffer containing 0.9% NaCl (PBS). After dialysis the solubilized precipitate was aliquoted and stored at -80°C.

Preparation of Extracts for Western Blot Analysis

High speed supernatant extracts were prepared from whole organisms listed below by homogenizing 0.25 g of tissue in 1 mL of 0.05 M Tris/HCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50 µM NaN₃, and 25 µM PMSF, at pH 8.0. Homogenization was followed by high speed centrifugation at 22,000 x g for 10 minutes at 4°C. Phosphagen kinase activity of the supernatants was determined with arginine phosphate and creatine phosphate as substrate using the enzyme-coupled spectrophotometric assay. Prior to Western blot analysis, samples were diluted and catalytic rates matched to 0.50 absorbance units per minute. Negative controls tested for western blotting experiments included pre-bleed anti-sera, hemoglobin, alcohol dehydrogenase, aldolase, lysozyme, and ribonuclease A.
Local species were collected in the Tampa bay area or purchased from Gulf Specimen Marine Laboratory Inc. (Panacea, FL). Species included *Gryllus rubens* (Cricket), *Periplaneta americana* (Cockroach), *Tenebrio molitor* (Mealworm), *Veronicella floridan* (Slug), *Thyonella gemmata* (Striped Sea Cucumber), *Penaus aztecus* (Shrimp), *Paracheirodon innesi* (Tetra), *Xiphophorus maculates* (Platy), *Plecia nearctica* (Love Bug), *Anolis sagrei* (Lizard), *Xenopus laevis* (Frog), *Harmonia axyridis* (Lady Bug), *Pomacea bridgesii* (Snail), *Sabella melanostigma* (Feather Duster), *Chione cancellata* (Clam), *Clypeaster subdepressus* (Sea Biscuit), *Echinaster spinulosus* (Starfish) *Ophiothrix angulata* (Brittle star) and *Isostichopus badonotus* (Giant Sea Cucumber). Pure CK-MM and CK-BB were purchased from CalBiochem (San Diego, CA). Live sea urchins (*Strongylocentrotus purpuratus*) and sponges (*Tethya aurnatium*) were obtained from Marinus Scientific (Garden Grove, CA). Butterflies (*Vanessa cardui*) were purchased from Greathouse Butterflies, Inc. (Gainsville, FL).

**Western Blotting**

Western blotting was performed with polyacrylamide gels (12.5% running, 4% stacking) containing 10% SDS. Five microliters of each sample was loaded on the gel and subjected to electrophoresis. Gels were preincubated in a 0.05 M Tris/HCl, 0.19 M glycine, 20% methanol, pH 8.3 buffer for 10 min, placed in an electroblotting cell, and transferred to an Immuno-Blot PVDF membrane (Bio-Rad, Richmond, CA) by electrophoresis for 45 minutes. Detection of AK was determined by the method of Dunbar (1994). After electrophoresis, unbound sites were blocked in a 5% (w/v) non-fat
dry milk/Tris buffered saline solution of 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4 for 40 min on a constant motion rotator. The membrane was rinsed 2-3 times with a 0.1% (w/v) non-fat dry milk/Tris buffered saline solution. Incubation with primary antibody (1:2,000) diluted in 1% non-fat dry milk/Tris buffered saline containing 0.2% (v/v) Tween 20 was done for 45 min followed by rinsing in 0.1% non-fat dry milk/Tris buffered solution as before. The membrane was incubated in a dilution of 1:20,000 of secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase) in 1% non-fat dry milk/Tris buffered solution and rinsed for a final time. Development of the membrane utilized an alkaline phosphatase reaction solution containing 50 mL of 0.2 M Tris/HCl pH 9.5 buffer with 5 mg nitroblue tetrazolium, 3.7 mg 5-bromo-4-chloro-3-indoyl phosphate p-toluidine in DMF, and 1.2 mM magnesium chloride in Tris buffered saline. A positive cross reaction was indicated by a purple precipitate.
Part IV. Kinetic Analysis of Sea Cucumber Arginine Kinase

**Kinetic Analysis**

Kinetic analysis of arginine kinase was measured for the reverse reaction using an enzyme-coupled spectrophotometric assay. The final mixture (0.5 mL) contained varying amounts of L-arginine phosphate (0.5 mM – 3.0 mM), ADP (0.125 mM – 3.0 mM), magnesium acetate (0.3375 mM – 8.0 mM), 20 mM glucose, 2 mM DTT, 0.4 mM NADP, 1 U/mL glucose-6-phosphate dehydrogenase, and 1 U/mL hexokinase in 0.1 M Gly-Gly buffer pH 6.75. The magnesium acetate concentration was held 2.7 times above the ADP concentration to account for the association between the magnesium ion and ADP (4,150 M⁻¹) (Morrison et al., 1961).

Kinetic analysis for the forward reaction was determined using the pH stat procedure previously described except with varying amounts of L-arginine (0.16 mM – 2 mM), ATP (0.0625 mM – 0.75 mM), and magnesium acetate (1.0625 mM – 3 mM), held 1 mM in excess of the ATP concentration.

Data for the binary and ternary complexes in both catalytic directions are represented using Lineweaver-Burk plots. Resulting y-intercepts for each substrate in the binary complex were replotted to obtain a secondary plot (ternary complex) for each substrate as described by Florini and Vestling (1957). To calculate the kinetic constants and error for each reaction, data were fit to non-linear least squares equations (Equations 1, 2, 3, and 4) (Marangoni, 2003) using SigmaPlot 8.0 where A and B represent individual substrates in the bi-substrate reaction. Each reported rate is an average of at least three separate determinations.
Binary Complex Reactions

\[
v = \frac{V_{\text{max}}^A[A]}{K_{A_{\text{hi}}} + [A]} \quad [1] \quad v = \frac{V_{\text{max}}^B[B]}{K_{B_{\text{hi}}} + [B]} \quad [2]
\]

Ternary Complex Reactions

\[
V_{\text{max}}^A = \frac{V_{\text{max}}[A]}{K_{BA_{\text{ter}}} + [A]} \quad [3] \quad V_{\text{max}}^B = \frac{V_{\text{max}}[B]}{K_{AB_{\text{ter}}} + [B]} \quad [4]
\]

Effect of Monovalent Anions

Phosphagen kinases have been shown to be inhibited by monovalent anions that mimic the transferable phosphoryl group in the reaction. These anions stabilize the catalytic dead-end complex by forming a highly stable transition state analog (AK-arginine-MgADP-anion). A test for stabilization of the catalytic dead-end complex by monovalent anions involved the pH stat assay previously described with saturating substrate conditions. The assay was supplemented with 50 mM of the following anion salts: NaCl, NaI, NaSCN, NaNO₂, NaNO₃, and NaCH₃CO₂. Reaction rate progress curves for AK in the presence of monovalent anions were recorded over 25 minutes in order to examine both the initial velocity and the percent of product formed at the completion of the time course.
**Nitrate Inhibition**

The monovalent anion that showed the most profound effect on both the initial velocity and percent of product formed was nitrate. The reaction rate progress curve of nitrate exhibited a lack of curvature in the time course (which is a measure of the formation of the dead-end complex and transition state analog). It is believed that the binding of the NO\(^{3-}\) anion may be more complex than simply dead-end stabilization alone, so an investigation into the effect of nitrate concentration on initial rates of dimeric AK was performed. A series of reaction rate progress curves for AK were run with varying amounts of the anion (3.125 mM – 50 mM) using the pH stat assay described under conditions of substrate saturation.

**Substrate Specificity**

Arginine kinases react primarily with L-arginine but have been known to utilize other amino acids as substrates. An investigation into the substrate specificity of AK from the sea cucumber was performed using the pH stat assay at saturating substrate conditions substituting the L-arginine with D-arginine, creatine, L-histidine, L-canavanine, L-ornithine, glycocyamine, and aminoguanidine. Time courses were initiated with the addition of 0.1 mL of enzyme.

**Difference Spectroscopy**

To investigate possible conformational changes due to nitrate as a stabilizer of the dead-end complex of AK difference spectra were performed. Purified sea cucumber
arginine kinase (0.08 mg/mL) in 0.1 M Tris/acetate, 1 mM EDTA, 2 mM DTT buffer, pH 8.0 and transition state analog components were scanned from 200-600 nm in a 1 cm tandem cuvette at 25°C. The final mixture (0.9 mL) contained 0.2 mM ADP, 1.2 mM magnesium acetate, 5 mM L-arginine, and 50 mM nitrate. All solutions were made in the above buffer and the pH adjusted to 8.0 before use. The left chamber contained 0.9 mL of enzyme and the right chamber contained 0.9 mL of varying combinations of the above components. The recorded spectrum was stored in the instrument’s memory. The chambers were then mixed and allowed to sit for 1 min before being scanned again. The difference spectrum obtained before mixing of the cuvette was subtracted from the difference spectrum obtained after mixing the cuvette on a Beckman Coulter DU 640 Spectrophotometer.
Cockroach Purification

Arginine kinase from *Periplaneta americana* (American cockroach) was purified by the method described by Brown et al. (2004). Six to seven grams of frozen cockroach bodies are minced and centrifuged at 4,500 x g at 4°C. The purification then utilizes a heat step (50°C for 1 min) followed by a CM-cellulose suspension (volume ratio 1:1) to separate extraneous proteins. The resulting supernatant is applied to a size exclusion column (Sephadex G-100, 4 cm x 70 cm) followed by an ion exchange column (Sephadex DEAE-50, 2.5 cm x 14.5 cm) in which arginine kinase is eluted in one step with a buffer containing 0.3 M NaCl.

Polyclonal Antibody Production

Polyclonal antibodies against purified cockroach arginine kinase (1.2 mg) were developed by ProSci, Inc. (Poway, CA). Polyclonal antibody fractions from serum were purified in the same manner as the sea cucumber (concentrated by 33% ammonium sulfate precipitation). The precipitate was solubilized and dialyzed extensively against 0.05 M sodium phosphate, pH 7.5 containing 0.9% NaCl. After dialysis the solubilized precipitate was aliquoted and stored at -80°C.
Western Blotting

Western blotting using roach polyclonal antibodies was performed using polyacrylamide gels (12.5% running, 4% stacking) containing 10% SDS. Five microliters of each sample was loaded on the gel and electrophoresed. Gels were preincubated in a 0.05 M Tris/HCl, 0.19 M glycine, 20% methanol, pH 8.3 buffer for 10 min, placed in an electroblotting cell, and transferred to an Immuno-Blot PVDF membrane (Bio-Rad, Richmond, CA). Detection of AK was determined by the method of Dunbar (1994). After electrophoresis, unbound sites were blocked in a 4% (w/v) non-fat dry milk/Tris buffered saline solution of 0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4 for 40 min with constant rotation. The membrane was rinsed 2-3 times with a 0.1% (w/v) non-fat dry milk/Tris buffered saline solution. Incubation with primary antibody (1:250) diluted in 1% non-fat dry milk/Tris buffered saline containing 0.2% (v/v) Tween 20 was done for 45 min followed by rinsing in 0.1% non-fat dry milk/Tris buffered solution as previously described. The membrane was incubated in a dilution of 1:5,000 of secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase) in 1% non-fat dry milk/Tris buffered solution and rinsed for a final time. Membrane development was performed using an alkaline phosphatase reaction solution containing 50 mL of 0.2 M Tris/HCl pH 9.5 buffer with 5 mg nitroblue tetrazolium, 3.7 mg 5-bromo-4-chloro-3-indoyl phosphate p-toluidine in DMF, and 1.2 mM magnesium chloride in Tris buffered saline. A positive cross reaction was indicated by a purple precipitate.

Species used for Western blot analysis included *Periplaneta americana* (Cockroach), *Penaus aztecus* (Shrimp), *Thyonella gemmata* (Striped Sea Cucumber),
*Xiphophorus maculates* (Platy), *Chione cancellata* (Clam), and *Strongylocentrotus purpuratus* (Sea Urchin sperm). High speed supernatant extracts were homogenized and centrifuged as described previously in the sea cucumber polyclonal antibody Western blotting section. Phosphagen kinase activity for each sample was determined using arginine phosphate and creatine phosphate as substrate in the enzyme-coupled spectrophotometric assay and catalytic rates were matched to 0.50 absorbance units per minute prior to Western blotting analysis.

**Difference Spectroscopy**

Difference spectra were performed in order to further investigate possible conformational changes due to nitrate and borate as stabilizers of the dead-end complex of arginine kinase. Purified cockroach arginine kinase (0.275 mg/mL) in 0.1 M Tris/acetate, 1 mM EDTA, 2 mM DTT buffer, pH 8.0 and transition state analog components were scanned from 200-600 nm in a 1 cm tandem cuvette at 25°C. The final mixture (0.9 mL) contained 0.2 mM ADP, 1.2 mM magnesium acetate, 5 mM L-arginine, and 50 mM nitrate or borate. All solutions were made in the above buffer and the pH adjusted to 8.0 before use. The left chamber contained 0.9 mL of enzyme and the right chamber contained 0.9 mL of varying combinations of the above components. The recorded spectrum was stored in the instrument’s memory. The chambers were then mixed and allowed to sit for 1 min before being scanned again. The difference spectrum obtained before mixing of the cuvette was subtracted from the difference spectrum obtained after mixing the cuvette on a Beckman Coulter DU 640 Spectrophotometer.
Roach Rearing and Maintenance

Cockroaches were collected and identified as the American cockroach. Five specimens were added to a 5 gallon bucket. The bucket was covered by a screen secured with an o-ring. Vaseline was placed on the inside edge of the bucket to prevent the roaches from crawling on the screen. Wood chips were placed in the bucket as well as 2 small Petri dishes to hold water. The cockroaches were fed twice a week with 20 mL of a concentrated sucrose solution and once a week with a grain mix. The bucket was kept in a dark, damp area. Over several months, a colony of approximately 100 roaches was established and was maintained in the above conditions.

Isolation of Total RNA

Isolation of total RNA from the American cockroach was performed using the RNA-Bee kit from Tel-Test, Inc. (Friendswood, TX). Frozen cockroach tissue (50 mg) was homogenized in 1 mL of RNA-Bee solution using a glass-glass homogenizer. Chloroform (0.2 mL) was added to the suspension followed by shaking for 30 seconds. The suspension was placed on ice for 5 min and centrifuged at 12,000 x g for 15 min at 4°C. The RNA containing aqueous phase was transferred to a clean microcentrifuge tube, mixed with 0.5 mL isopropanol, stored at room temperature for 10 min, and centrifuged at 12,000 x g for 5 min at 4°C. After centrifugation, the RNA precipitate formed at the bottom of the tube so the supernatant was removed. The RNA pellet was washed with
75% ethanol, vortexed, and centrifuged at 7,500 x g for 5 min at 4°C. The supernatant was removed and the RNA pellet was briefly air dried before being dissolved in 0.1 mL of diethylaminoethyl (DEPC) water followed by incubation at 55°C for 10 minutes.

Reverse Transcription

In order to synthesize double stranded DNA from single stranded RNA, a reverse transcription reaction was performed. Template RNA (1.5-2.0 µg) was incubated with random primers or oligo dT primers at 70°C for 5 min then transferred to ice. M-MLV 5x reaction buffer (Fisher, Pittsburg, PA), a complete set of deoxyribonucleotide triphosphates (DNTP’s), RNase Inhibitor solution, MLV-RT Enzyme (Fisher, Pittsburg, PA), and sterile H2O were added to the RNA and primer solution to a total volume of 50 µL and incubated at 37°C for 1 hr.

DNA Gel Electrophoresis

DNA agarose gels were prepared using a 1.5% agarose solution in 1X TAE (50 mM Tris/acetate, 1 mM EDTA, pH 8.0 buffer) that was heated until all of the agarose was dissolved. Ethidium bromide (10 mg/mL) was added to the agarose before it was allowed to solidify. The gel was run in 1X TAE buffer and a constant voltage (125 volts) was maintained for electrophoresis until the dye front reached the bottom of the gel. Samples contained 8 µL DNA, 2 µL 1X TAE buffer, and 2 µL loading dye (Promega, Madison, WI). DNA ladders (100 bp) from Promega (Madison, WI) were electrophoresed parallel to the samples.
DNA Fragment and PCR Product Purification

In order to purify DNA fragments from agarose gels, a S.N.A.P. Gel Purification Kit from Invitrogen was utilized (Carlsbad, CA). DNA from the gel was excised using a razor blade rinsed in autoclaved water and transferred to a sterile microcentrifuge tube. Sodium iodide (6.6 M) was added to the microcentrifuge tube, vortexed, and incubated at 42-50°C until the agarose was completely melted. The tube was then placed at room temperature and Binding buffer (7 M guanidinium/HCl) was added and mixed with the solution. The mixture was added to a S.N.A.P. purification column and centrifuged at 3,000 x g for 30 sec at 25°C. The liquid that passed through the column was poured back into the column and centrifuged again at 3,000 x g for 30 sec. This step was repeated again to allow all DNA to bind to the column. After the third centrifugation the liquid that passed through the column was discarded and 0.4 mL of 1X Final Wash (400 mM NaCl diluted with 100% ethanol) was added to the column. The column was centrifuged at 3,000 x g for 30 sec at 25°C. The previous step was repeated and the liquid that passed through the column was discarded. The column was centrifuged at 10,000 x g for 2 min at 25°C to dry the column resin and then transferred to a new microcentrifuge tube. Sterile water (0.04 mL) was added to the column and allowed to incubate for 1 min at room temperature to absorb into the column. The column was centrifuged at 10,000 x g for 1 min to elute the DNA. The DNA was stored at -20°C until use.

In order to purify PCR products, we used the Montage PCR kit from Millipore (Billerica, MA). Distilled water (0.3 mL) was placed into the sample reservoir of the Montage PCR centrifugal filter unit. The PCR reaction (0.1 mL) was added to the
reservoir and centrifuged at 1,000 x g for 15 min at 25°C. The filtrate was discarded and 0.02 mL of distilled water was placed in the reservoir. The reservoir was inverted, placed into a clean vial, and centrifuged at 1,000 x g for 2 min at 25°C. The PCR product was stored at -20°C until use.

Degenerate Primers

A central region of cDNA from the American cockroach was amplified using degenerate primers. Degenerate primers were designed from highly conserved amino acid sections using 27 AK amino acid sequences. The following sequences were used: gi|1078951|, gi|1085649|, gi|1346366|, gi|13647103|, gi|13647113|, gi|15886861|, gi|16518985|, gi|1708615|, gi|2543067|, gi|2543072|, gi|2543073|, gi|2543074|, gi|2543075|, gi|2543078|, gi|3183056|, gi|3183057|, gi|3183060|, gi|3183062|, gi|3831705|, gi|4586462|, gi|5726565|, gi|585342|, gi|7243761|, gi|7768860|, gi|7770085|, gi|7770087|, and gi|8099051|. The amino acid sequences were entered in the Block Maker program at the Blocks WWW Server (http://blocks.fhcrc.org/blocks/). The results from the Block Maker program were then entered into the CODEHOP program on the Blocks WWW Server (http://blocks.fhcrc.org/blocks/). Four primer sequences were then selected from three separate blocks (C, D, and E). These primers (listed below) made it possible to obtain the entire sequence between blocks C and E.

C Primer: 5’- GCA GCA GCT GAT CGA YCA YTT YYT – 3’

E Primer: 5’- CAT GGT GGT CCC CSG GTT NSW NGG RCA - 3’
Insert Sequence PCR

PCR amplification was performed using a Mastercycler Gradient Thermal Cycler from Eppendorf (Westbury, NY). Each reaction contained 10 µL MasterMix (Eppendorf, Westbury, NY), 1 µM Block C forward primer, 1 µM Block E reverse primer, 1 µL cDNA, and molecular biology grade water to 25 µL. The reactions were run at 55, 57, and 62°C using the protocol for touchdown PCR. A product of ~ 450 bp was identified by DNA gel electrophoresis.

Insert Sequence Cloning and Transformation

The insert sequence was cloned and transformed using a TOPO TA Cloning kit from Invitrogen (Carlsbad, CA). The cloning reaction (6 µL) contained 1 µL salt solution, 2 µL of PCR product, 2 µL water, and 1 µL TOPO vector. The reaction was gently mixed, incubated for 5 min at room temperature, and stored on ice. The cloning reaction (2 µL) was then transformed into One Shot TOP10 Cells by pipetting it into a vial of OneShot Chemically Competent E. coli and allowing it to incubate on ice for 30 min. The cells were then heat-shocked for 30 sec at 42°C and immediately transferred to ice. S.O.C. medium (0.25 mL) was added and the reaction was shaken horizontally (200 rpm) for 1 hr at 37°C. The transformation reaction (10-50 µL) was spread onto LB plates containing ampicillin (50 µg/µL) and incubated overnight at 37°C. Colonies were selected and cultured overnight in 3 mL LB/ampicillin broth with shaking (300 rpm).
Isolation of Insert Plasmid DNA

Isolation of plasmid DNA was performed at room temperature using the Eppendorf (Westbury, NY) Perfectprep Plasmid Mini kit. Bacterial culture (1.5 mL) was transferred to a sterile tube and centrifuged at 12,000 x g for 20 sec to pellet cells. The supernatant was decanted and 0.1 mL of Solution 1 was added to the cell pellet. The pellet was resuspended by vortexing and 0.1 mL of Solution 2 was added to the resuspension to lyse the bacteria. Solution 3 (0.1 mL) was added and immediately mixed by inversion. The suspension was centrifuged at 12,000 x g for 30 sec and the supernatant transferred to a spin column. DNA Binding Matrix (0.45 mL) was added to the spin column, mixed with the suspension, and centrifuged at 12,000 x g for 30 sec. The filtrate was decanted and 0.4 mL of Diluted Purification Solution was added to the spin column followed by centrifugation at 12,000 x g for 60 sec. The spin column was spun once more to remove any excess Purification Solution and the filtrate decanted. Sterile water (50 µL) was placed into the spin column to elute plasmid DNA.

Restriction Digest

A restriction digest reaction was performed on the purified plasmid insert DNA. The reaction contained 1.0 µL buffer H, 0.1 µL BSA, 0.2 µL EcoR 1 (Promega, Madison, WI), 4.7 µL water, and 6 µL DNA to a total volume of 10 µL. The reaction was incubated at 37 °C for 4 hr and analyzed using DNA gel electrophoresis as previously described. The insert was sequenced by the Molecular Biology Core facility at the
Moffitt Cancer Research Center using TOPO TA vector specific sequencing primers (T3 or T7).

**Design of 5’ and 3’ Primers**

In order to obtain full-length 5’ and 3’ ends from cockroach cDNA, the GeneRacer Kit from Invitrogen (Carlsbad, CA) was initially utilized in conjunction with gene-specific primers created from the insert sequence for arginine kinase from the American cockroach. Race Ready cDNA was prepared to use in the GeneRacer Kit by treating total RNA (1-5 µg) with calf intestinal phosphatase (CIP) to dephosphorylate non-mRNA or truncated mRNA. After the dephosphorylation reaction incubated at 50°C for 1 hr, RNA was precipitated using DEPC water, phenol:chloroform, mussel glycogen (10 mg/mL), sodium acetate (3 M), and 95% ethanol followed by vortexing and freezing on dry ice for 10 minutes. RNA was then pelleted by centrifugation and rinsed with 70% ethanol for use in the next steps. Dephosphorylated RNA was incubated with 10X TAP buffer, RNaseOut (40 U/µL) and TAP (0.5 U/ µL) for 1 hr at 37°C in order to remove the 5’ cap structure from full-length mRNA. RNA was precipitated as described above and incubated at 65°C for 5 min to relax RNA secondary structure. A ligation reaction was performed using 10X Ligase buffer, 10 mM ATP, RNaseOut (40 U/µL), and T4 RNA ligase (5 U/µL) followed by incubation at 37°C for 1 hour. RNA was precipitated and reverse transcribed into cDNA with the use of GeneRacer oligo dT primer that contains the priming sites for the GeneRacer primers and Random Primers (100 ng). A cloned AMV RT reaction was performed by adding the desired primer and dNTP mix (25 mM)
to the ligated RNA followed by incubation at 65°C for 5 min and centrifugation. The following reagents were added to the above mixture: 5X RT buffer, Cloned AMV RT (15 U/μL), sterile water, and RNaseOut (40 U/μL) followed by incubating at 45°C for 1 hr, incubating at 85°C for 15 min to inactivate the RT enzyme, and centrifugation before the cDNA was used for amplification.

The following GeneRacer 5’ and 3’ primers (listed below) were used for initial amplification towards each end of AK gene. The inability to obtain a full sequence from a PCR between the GeneRacer 5’ primer and a gene-specific primer or the GeneRacer 3’ primer and a gene-specific primer lead to the design and synthesis (Integrated DNA Technologies, Inc., Coralville, IA) of new gene-specific primers throughout the sequence. Small fragments starting from the insert sequence out toward both the 5’ and 3’ end of the gene were amplified, sequenced, and used to create the new gene-specific primers (listed in the Results chapter). This “primer walking” approach was used to obtain the entire sequence of AK from *P. americana*, including the 5’ and 3’ ends.

GeneRacer 5’: 5’- CGA CTG GAG CAC GAG GAC ACT GA- 3’
GeneRacer 5’ nested: 5’- GGA CAC TGA CAT GGA CTG AAG GAG TA- 3’
GeneRacer 3’: 5’- GCT GTC AAC GAT ACG CTA CGT AAC G- 3’
GeneRacer 3’ nested: 5’- CGC TAC GTA ACG GCA TGA CAG TG- 3’
5' and 3' PCR and Sequencing

Each sequence segment between the 5’ end and 3’ end primers being used was amplified using PCR. Each reaction contained 10 µL MasterMix (Eppendorf, Westbury, NY), 1 µM appropriate forward primer, 1 µM appropriate reverse primer, 1 µL cDNA, and molecular biology grade water to 25 µL. The reactions were run at 57°C using the protocol for touchdown PCR. Products were identified by DNA gel electrophoresis, S.N.A.P. gel purified or Montage Kit purified, and sent to the Molecular Biology Core facility at the Moffitt Cancer Research Center for sequencing directly from a PCR product using the appropriate gene-specific primers created or GeneRacer 5’ and 3’ primers from Invitrogen (Carlsbad, CA).

Sequence Alignment

Sequences from several arginine and creatine kinases were obtained from the National Center for Biotechnology Information (NCBI) database. The sequences chosen are representative phosphagen kinase sequences from both invertebrate and vertebrate species and include: *Danio rerio* gi|18858427|, *Stichopus japonicus* gi|4586462|, *Apis mellifera* gi|58585146|, *Strongylocentrotus purpuratus* gi|125302|, *Tethya aurantia* gi|45643637|, *Suberites domuncula* gi|66862284|, *Trypanosoma cruzi* gi|3831705|, *Anthopleura japonica* gi|2554612|, *Dendronephytha gigantean* gi|110083397|, *Heterodera glycines* gi|31247902|, *Haliotis madaka* gi|439719|, *Calyptogena kaikoi* gi|71834059|, and *Crassostrea gigas* gi|44885729|. These sequences and the deduced sequence of arginine kinase from the American cockroach were aligned with ClustalX.
(Thompson et al., 1997) and MEGA (Kumar et al., 2004). Neighbor joining trees were constructed using Mega to assess the sequence homologies between arginine and creatine kinases. Boot strap values were determined by collapsing the tree to 50% or greater.
Chapter 3. Results
Part I. Purification and Characterization

Purification

The isolation of arginine kinase from the Giant Sea Cucumber, *Isostychopus badonotus*, involves a modification of the procedure used previously to purify the sea cucumber *Caudina arenicola* (Seals & Grossman, 1988). The purification utilizes ultracentrifugation and ammonium sulfate precipitations followed by gel filtration chromatography (Figure 16). After separation based on molecular weight the enzyme is further purified with the use of adsorption chromatography by application to a Hydroxyapatite column and eluted with a buffer gradient (Figure 17). Table 5 summarizes the stepwise results of the isolation from a total of 30-50 g frozen sea cucumber muscle. The purification results in a 57-fold enrichment, a 21% yield, and 8.8 mg of enzyme which is shown to be pure using SDS-PAGE electrophoresis (Figure 18).

Molecular Weight and Subunit Composition

The molecular weight of the native protein was determined by gel filtration chromatography using a calibrated Sephadex G-100 column. The column was calibrated with purified dimeric creatine kinase (MM) and purified monomeric arginine kinase (shrimp) exhibiting relative molecular weights of 80,000 daltons and 40,000 daltons,
respectively. Native arginine kinase from the sea cucumber eluted from the column in fractions corresponding to that of creatine kinase (Figure 19).

The subunit molecular weight of the denatured protein was determined using calibrated SDS-PAGE. The migrations during electrophoresis were obtained and the results plotted in relation to the log of the molecular weights of several calibration standards (Figure 20). Analysis of the purified denatured sample exhibited a single protein staining band at a molecular mass corresponding to 43,700 daltons. The results of the native and denatured molecular weight determinations show that arginine kinase from *Isostychopus badonotus* is a dimer with a molecular mass of approximately 87,000 daltons.

**Thermal Inactivation**

Arginine kinase from the sea cucumber is fairly thermal stable when compared to other phosphagen kinases. Arginine kinase activity was tested over a temperature range of 25°C to 55°C for 1, 5, 10, and 15 minutes (Figure 21). Approximately 50% of the activity was lost when arginine kinase was heated at 46°C for 10 minutes. Complete or near complete inactivation was seen at 5, 10, and 15 min with a sharp decline of activity at 50°C.

**pH Optimum**

The pH at which AK activity is optimal in both the forward (synthesis of L-arginine phosphate and ADP) and the reverse (synthesis of L-arginine and ATP) direction
was determined (Figure 22). The dependence of activity on pH for the forward direction exhibits a maximum at pH 8.0 while the dependence of activity on pH for the reverse direction exhibits a maximum at pH 6.0. Rate determinations used in kinetic measurements were performed at the pH optima in both directions.
Figure 16. ACA 34 Column Elution Profile:

The solubilized pellet from the 50-82% ammonium sulfate precipitation was placed on an ACA 34 gel filtration column (3.8 cm x 76 cm). Fractions containing 16 mL were collected after elution with 50 mM Tris/acetate buffer, pH 8.0. AK activity was measured using the enzyme-coupled spectrophotometric assay in the reverse direction. Protein concentrations were determined using the Bradford assay (1976). Activity (▼). Protein Concentration (●).
Figure 17. Hydroxyapatite Column Elution Profile:

Active fractions from the ACA 34 column were dialyzed against 0.02 M potassium phosphate buffer, pH 7.5 and applied to a column of Hydroxyapatite (4 cm x 12.7 cm). AK was eluted with a potassium phosphate gradient of 0.02 M to 0.5 M, pH 7.5 and 10 mL fractions were collected. AK activity was measured using the enzyme-coupled spectrophotometric assay in the reverse direction. Protein concentrations were determined using the Bradford assay (1976). Activity (■). Protein Concentration (▼). Ionic Conductivity (●).
Table 5. Purification of Arginine Kinase from the Sea Cucumber:

Activity was determined using the enzyme-coupled spectrophotometric assay in the reverse direction and protein concentrations were determined using the Bradford assay (1976).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total Volume [mL]</th>
<th>Total Protein [mg]</th>
<th>Total Activity [µmole NADPH + H⁺/min]</th>
<th>Specific Activity [Total activity/mg]</th>
<th>Fold Enrichment</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td>255</td>
<td>2379.9</td>
<td>68.4</td>
<td>0.03</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>High Speed Centrifugation</td>
<td>215</td>
<td>1257.6</td>
<td>65.3</td>
<td>0.05</td>
<td>1.8</td>
<td>95.4</td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitation</td>
<td>7.5</td>
<td>65.4</td>
<td>9.9</td>
<td>0.15</td>
<td>5.3</td>
<td>14.6</td>
</tr>
<tr>
<td>Gel Filtration (ACA 34)</td>
<td>84</td>
<td>31.7</td>
<td>21.2</td>
<td>0.66</td>
<td>23.2</td>
<td>30.9</td>
</tr>
<tr>
<td>Adsorption Chromatography (Hydroxyapatite)</td>
<td>55</td>
<td>8.8</td>
<td>14.5</td>
<td>1.66</td>
<td>57.6</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure 18. SDS-PAGE Results from the Sea Cucumber Purification Protocol:

Samples along each step of the purification procedure were analyzed by SDS-PAGE.

Lane 1: Kalediscope molecular weight standards.  Lane 2: Low speed centrifugation.
Lane 3: Ultra centrifugation.  Lane 4: Ammonia Sulfate precipitation pellet.  Lane 5:
ACA-34 combined active fractions.  Lane 6: Concentrated ACA active fractions.
Lane 7: Concentrated Hydroxyapatite active fractions.
Figure 19. Molecular Weight Determination of Native Sea Cucumber AK:

A purified sample of AK (0.2 mg/mL) was placed on a Sephadex G-100 column (1.9 cm x 53.3 cm) and eluted with 50 mM Tris/HCL pH 8.0, 10 mM 2-mercaptoethanol, 1 mM EDTA, 5 µM NaN₃ in 1 mL fractions. The column was calibrated with 1 mg/mL purified creatine kinase (MM) and monomeric arginine kinase (Shrimp). Activities were determined using the enzyme-coupled spectrophotometric assay in the reverse direction. Monomeric arginine kinase (●). Sea Cucumber arginine kinase (■). Dimeric creatine kinase (▲).
Figure 20. Calibration Curve for the Subunit Molecular Weight of AK:

The migrations during electrophoresis were obtained from SDS-PAGE electrophoresis (Laemmli, 1970) and the results plotted in relation to the log of the molecular weights of standard proteins.
Figure 21. Thermal Inactivation:

Partially purified enzyme samples were incubated at the indicated temperatures, rapidly cooled on ice, and tested for arginine kinase activity using the enzyme-coupled spectrophotometric assay in the reverse direction. 1 min (●). 5 min (▼). 10 min (■). 15 min (♣).
Figure 22. pH Optimum for AK Activity in the Forward and Reverse Directions:

Activity in the forward direction (●) was determined using the pH stat assay with pH being directly varied and controlled by fixing the “stat” pH. Activity in the reverse direction (○) was determined using the colorimetric thio-NAD assay. Sea cucumber AK (12 µg) was added to assay reagents prepared with buffers over the following ranges: 0.1 M acetic/acetate (pH 4.5-6.0), 0.1 M sodium phosphate buffer (pH 6.5-7.5), and 0.1 M Tris/HCL (pH 8.0-10.0).
**pH Stability**

The pH stability of sea cucumber AK was tested over a period of three weeks in 0.1 M sodium phosphate, 1 mM EDTA, 2 mM DTT buffer of varying pH (6.4 to 8.25). For the duration of the experiment the enzyme was most active at the higher pH values and less active at the lower pH values (Figure 23). Over-all, arginine kinase from the sea cucumber proves to be relatively stable by maintaining approximately 25% of the initial activity at most pH values for the entire 26 days.

**Isoelectric Focusing**

The isoelectric point (measured over a pH range of 3-10) of arginine kinase from the sea cucumber was determined to be 6.0 (Figure 24) although the difference between the activities isolated at pl 6.0 and pl 6.5 for sea cucumber AK is slight. Two mL fractions were collected and assayed for AK activity using the enzyme-coupled spectrophotometric assay in the reverse direction. The pH of each fraction was measured with a Radiometer IE 7.5 mm x 103 mm electrode.

**Absorption Spectrum**

Purified AK displayed an absorption spectrum of a protein that contains no absorbing prosthetic groups or cofactors when scanned from 250 nm to 600 nm (Figure 25).
Figure 23. pH Stability of Arginine Kinase from the Sea Cucumber:

The pH stability of AK (0.2 mL) over time was tested using the enzyme-coupled spectrophotometric assay in the reverse direction for the increments of time stated above. The enzyme was in 0.1 M sodium phosphate, 1 mM EDTA, 2 mM DTT buffer (pH range 6.4-8.25). pH 6.4 (—). pH 6.7 (—). pH 6.88 (—). pH 7.0 (—). pH 7.3 (—). pH 7.5 (—). pH 7.7 (—). pH 7.9 (—). pH 8.25 (—).
Figure 24. Isoelectric Focusing of Sea Cucumber AK:

A 50 mL sample containing 5 units (1 unit = 1 µmole NADH + H⁺ produced/min) of AK and 1% ampholytes (pH 3-10) was focused for 6 hours at 6°C. The activity of each fraction was measured using the enzyme-coupled spectrophotometric assay in the reverse direction and the pH was determined with a Radiometer IE 7.5 mm x 103 mm electrode. Activity (●). pH (○).
Figure 25. Absorption Spectrum of Sea Cucumber AK:

A purified sample of AK (0.22 mg/mL) in 0.05 M sodium phosphate buffer, pH 8.0 was scanned from 250 nm to 600 nm.
Figure 26. Circular Dichroism Spectrum of Sea Cucumber AK:

A purified sample of arginine kinase (0.07 mg/mL) in 2 mM sodium phosphate, 1 mM 2-mercaptoethanol, pH 8.0 buffer was scanned from 190 nm to 240 nm. An Aviv Model 215 spectropolarimeter and a cuvette with a 1 mm cell pathlength were used to record spectral measurements.
**Circular Dichroism**

The circular dichroic spectrum for a purified sample of sea cucumber arginine kinase displays characteristic minima at 222 nm and 208 nm (Figure 26). Using calculations derived from Chen et al. (1972) the alpha helical content for the sea cucumber was determined to be 9%. Although characteristic minima are apparent for sea cucumber arginine kinase, the circular dichroic spectrum is anomalous compared to other CD spectra in that it does not level off near zero towards the end of the spectrum. In order to account for this anomaly, the CD spectrum for sea cucumber AK was repeated multiple times with the appropriate corrections and the results were always consistent.

**Electrophoretic Mobility**

Native gel electrophoresis was performed to determine the relative electrophoretic mobility of sea cucumber arginine kinase (Figure 27). A purified sample of sea cucumber AK was run parallel to purified monomeric cockroach AK (*P. americana*) and a standard containing the three isozymes of dimeric CK (of known pI). Sea cucumber arginine kinase migrated with CK-MM (pI 7) while monomeric cockroach arginine kinase migrated with CK-BB (pI 4). This result suggests that the pI, the conformation, or a combination of both for sea cucumber AK is more similar to CK-MM than it is to the other isozymes of CK or monomeric AK.
**Reactivation**

The reactivation of denatured arginine kinase in 8 M urea was measured continuously by allowing the denatured protein sample to renature in the forward spectrophotometric assay reagent after being denatured for 15 minutes. No arginine kinase activity was detected while the reaction was monitored over a period of 30 minutes. This result suggests that the experimental conditions lead to irreversible denaturation of the enzyme. Irreversible denaturation of an enzyme can be caused by exposure of the denaturant being too lengthy, the concentration of the denaturant being too high, or from possible aggregation out-competing renaturation during refolding. In order to fully characterize denaturation of sea cucumber arginine kinase, reactivation should be done for a series of urea concentrations.

**Hybridization of AK Subunits**

Purified arginine kinase from sea cucumber muscle and purified dimeric arginine kinase from sea urchin eggs (*Stongylocentrotus purpuratus*) were mixed and denatured in 6 M guanidine hydrochloride. After incubation and renaturation samples were subjected to native gel electrophoresis (Figure 28). Experiments with the mixed denatured/renatured enzymes resulted in a functional hybrid between a homodimer from the sea cucumber muscle and a homodimer from the sea urchin. The electrophoretic mobility of the hybrid is different from that of mixed native and non-mixed denatured/renatured sea cucumber AK and sea urchin AK mobilities. The migration pattern for the hybrid lies in between that of denatured/renatured sea cucumber and sea
urchin enzymes. This study suggests that dimeric phosphagen kinases possess conserved structural features required for subunit association, catalytic activity, and formation of appropriate quaternary structure.
Figure 27. Electrophoretic Mobility of Sea Cucumber and Cockroach AK:

Samples of monomeric arginine kinase (10 µL) from the cockroach (*Periplaneta americana*) and dimeric arginine kinase (20 µL) from the sea cucumber were electrophoresed under native conditions along with a standard (20 µL) containing the 3 isozymes of CK (of known pI). After electrophoresis the gel was stained for catalytic activity using the colorimetric assay described in the Native Gel Electrophoresis section of the Methods chapter. Lane 1: Isozymes of CK. Lane 2: Cockroach AK. Lane 3: Sea Cucumber AK.
Figure 28. Hybridization of Sea Cucumber and Sea Urchin AK:

Purified sea cucumber AK (13 µg) and sea urchin AK (36 µg) were denatured in guanidine hydrochloride (final concentration 3 M) for 30 minutes. After renaturation through extensive dialysis, samples were subjected to native gel electrophoresis. The gel was stained for catalytic activity using the colorimetric assay described in the Native Gel Electrophoresis section of the Methods chapter. All above steps were performed at 4°C.

Lane 1: Native Sea Cucumber AK. Lane 2: Native Sea Urchin Egg AK. Lane 3: Mixed Native Sea Cucumber and Sea Urchin Egg AK. Lane 4: Mixed Denatured/Renatured Sea Cucumber AK and Sea Urchin Egg AK. Lane 5: Denatured/Renatured Sea Urchin Egg AK. Lane 6: Denatured/Renatured Sea Cucumber AK.
Part II. Polyclonal Antibody Production and Western Blotting of Sea Cucumber AK

Preparation of Polyclonal Antibodies

Polyclonal antibodies against purified dimeric arginine kinase from the sea cucumber were developed by ProSci, Inc. (Poway, CA). Antibody fractions were purified and concentrated through ammonium sulfate precipitation. The precipitate was dialyzed against PBS, aliquoted, and frozen at -80°C. Polyclonal antibodies raised against sea cucumber AK displayed cross-reactivity with *Isostichopus badonotus* enzyme when samples were subjected to SDS-PAGE followed by immunoblotting (Figure 29).

Western Blotting

Antigenic reactivity towards 23 different types of phosphagen kinases (with varying molecular weights and subunit associations) was determined using polyclonal antibodies raised against dimeric arginine kinase from the sea cucumber (Table 6). All of the species known to contain dimeric AK reacted with antibodies against sea cucumber AK while each of the species tested in which AK is known to be monomeric, displayed no cross-reactivity (Figure 30). A positive reaction was seen with monomeric AK from the clam, but it is a two domain monomer with a molecular weight of 80 kDa. Strong positive reactions were obtained with two purified homodimeric isozymes of CK (*Homo sapien* MM and BB) as well as extracts from several species known to contain CK (Figure 31). Sea cucumber antibodies did not cross-react with the large, multidomain monomer of CK found in sea urchin sperm or the tetrameric AK from the sabellid worm.
Figure 29. Western Blotting Results of Sea Cucumber Arginine Kinase:

A purified sample of sea cucumber arginine kinase (5 µL) was subjected to SDS-PAGE and transferred to a nylon derivatized membrane for immunoblotting. The purified and concentrated polyclonal antibody raised against Giant Sea Cucumber AK was used to verify cross-reactivity on the Western blot. The primary antibody dilution was 1:2,000 and the secondary antibody dilution was 1:20,000. Detection of AK was determined by the method of Dunbar (1994). Lane 1: Kalediscope molecular weight markers. Lane 2: Giant Sea Cucumber AK.
Table 6. Catalytic Activity and Immunological Cross-Reactivity of Phosphagen Kinases with Dimeric Arginine Kinase Polyclonal Antibody:

Activity was measured with the enzyme-coupled spectrophotometric assay in the reverse direction using arginine phosphate and creatine phosphate as substrates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzymatic Activity</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AK</td>
<td>CK</td>
</tr>
<tr>
<td><strong>Monomeric</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gryllus sp. (Cricket)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Periplaneta americana (Cockroach)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tenebrio molitor (Mealworm)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Penaeus aztecs (Shrimp)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Plecia nearctica (Love Bug)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Harmonia axyridis (Lady Bug)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chione cancellata (Clam)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Echinaster spinulosus (Starfish)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ophiophorus angulata (Brittle star)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Dimeric</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyonella gemmata (Striped Sea Cucumber)</td>
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<td>-</td>
</tr>
<tr>
<td>Strongylocentrotus p. (Sea Urchin Egg)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clypeaster subdepressus (Sea Biscuit)</td>
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<td>-</td>
</tr>
<tr>
<td>Tethya aurantia (Sponge)</td>
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<td>+</td>
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<tr>
<td>Paracheirodon innesi (Fish)</td>
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</tr>
<tr>
<td>Xiphophorus maculates (Fish)</td>
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<td>Anolis sagrei (Lizard)</td>
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<tr>
<td>Xenopus laevis (Frog)</td>
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<td>+</td>
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<tr>
<td>Homo sapien (CK-BB human)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Homo sapien (CK-MM human)</td>
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<td>+</td>
</tr>
<tr>
<td><strong>Tetrameric</strong></td>
<td></td>
<td></td>
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<tr>
<td>Sabella melanostigma (Feather duster)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Monomeric and Trimeric</strong></td>
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<td></td>
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<tr>
<td>Strongylocentrotus p. (Sea Urchin Sperm)</td>
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<td>+</td>
</tr>
<tr>
<td><strong>Undetermined</strong></td>
<td></td>
<td></td>
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<tr>
<td>Veronicella floridan (Slug)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pomacea bridgesii (Snail)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Two domain monomer with molecular weight of 80 kDa (Suzuki et al., 2002).
*b Monomer with molecular weight of 150 kDa (Ratto et al., 1989).
Figure 30. Western Blotting Results of Monomeric and Dimeric Arginine Kinase:

Extracts were prepared by homogenizing 0.25 g of tissue in 1 mL of 0.05 M Tris/HCL, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50 µM NaN₃, and 25 µM PMSF, pH 8.0. Extracts were centrifuged at 22,000 x g for 10 min and tested for phosphagen kinase activity using the enzyme-coupled spectrophotometric assay in the reverse direction. Prior to Western blot analysis, samples were diluted and catalytic rates matched to 0.50 absorbance units per minute. Samples (5 µL) were subjected to SDS-PAGE and transferred to a nylon derivatized membrane for immunoblotting. The polyclonal antibody raised against the Giant Sea Cucumber was used to determine cross-reactivity on the Western blot (primary antibody dilution = 1:2,000 and secondary antibody dilution = 1:20,000). Detection of AK/CK was determined by the method of Dunbar (1994). Lane 1: Slug AK. Lane 2: Shrimp AK. Lane 3: Stripped Sea Cucumber AK. Lane 4: Giant Sea Cucumber AK. Lane 5: Kalediscope molecular weight markers.
Figure 31. Western Blotting Results of Dimeric Creatine Kinase:

Extracts were prepared by homogenizing 0.25 g of tissue in 1 mL of 0.05 M Tris/HCL, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50 µM NaN₃, and 25 µM PMSF, pH 8.0. Extracts were centrifuged at 22,000 x g for 10 min and tested for phosphagen kinase activity using the enzyme-coupled spectrophotometric assay in the reverse direction. Prior to Western blot analysis, samples were diluted and catalytic rates matched to 0.50 absorbance units per minute. Samples (5 µL) were subjected to SDS-PAGE and transferred to a nylon derivatized membrane for immunoblotting. The polyclonal antibody raised against the Giant Sea Cucumber was used to determine cross-reactivity on the Western blot (primary antibody dilution = 1:2,000 and secondary antibody dilution = 1:20,000). Detection of AK/CK was determined by the method of Dunbar (1994). Lane 1: Kalediscope molecular weight markers. Lane 2: Giant Sea Cucumber AK. Lane 3: Fish (*Xiphophorus*) CK. Lane 4: Fish (*Paracheirodon*) CK. Lane 5: Lizard CK. Lane 6: Frog CK.
Part III. Kinetic Analysis of Sea Cucumber Arginine Kinase

**Kinetic Analysis**

Arginine kinases, and other phosphagen kinases, are believed to obey a rapid equilibrium, random addition mechanism (Smith & Morrison, 1969) which is shown in Figure 3. A kinetic analysis was performed on sea cucumber arginine kinase for both the reverse and the forward reactions. Results of initial velocity studies for the sea cucumber in the reverse direction, where L-arginine phosphate was varied at several fixed concentrations of MgADP and where MgADP was varied at several fixed concentrations of L-arginine phosphate, are shown as double-reciprocal plots in figures 32 and 33. The binary complex \([ES]\) dissociation constants \((K_{bi})\) for L-arginine phosphate and MgADP are 1.00 mM and 0.50 mM, respectively. The resulting y-intercepts from the primary plots were replotted as a secondary plot (Figure 34) to obtain the dissociation constants \((K_{ter})\) for the ternary complex \([ES_1S_2]\) as described by Florini and Vestling (1957). The \(K_{ter}\) for L-arginine phosphate is 1.00 mM while the \(K_{ter}\) for MgADP is 0.33 mM.

Results of initial velocity studies in the forward direction, where L-arginine was varied at several fixed concentrations of MgATP and where MgATP was varied at several fixed concentrations of L-arginine, are shown as double-reciprocal plots in figures 35 and 36. The \(K_{bi}\) for L-arginine and MgATP are 0.36 mM and 0.034 mM, respectively. The \(K_{ter}\) values obtained from replotting the y-intercepts in the secondary plot (Figure 37) are 0.37 mM (L-arginine) and 0.07 mM (MgATP).
A comparison of all dissociation constants for the substrates of sea cucumber AK is shown in Table 7. The ratio of binding constants, \( K_{bi}/K_{ter} \), for the same substrate in the binary and ternary complexes represents the degree of substrate binding cooperativity or “synergism”. Synergism is a measure of how the binding of the first substrate influences the binding of the second substrate (whether through enhancing or decreasing the binding). The ratios of \( K_{bi}/K_{ter} \) for sea cucumber AK in the reverse direction are 1.00 and 1.51 for L-arginine phosphate and MgADP, respectively. The ratios of \( K_{bi}/K_{ter} \) for AK in the forward direction are 0.97 and 0.49 for L-arginine and MgATP, respectively. The fact that there is no significant difference, within experimental error, in the dissociation constants between the binary and ternary complexes indicates that there is limited, if any, cooperativity or synergism for the binding of the substrates in sea cucumber AK.
Figure 32. Effect of Phosphoarginine on Initial Velocity:

L-arginine phosphate was varied at several fixed concentrations of MgADP. The magnesium acetate concentration was held 2.7 times above the ADP concentration to account for magnesium ADP binding (4,150 M\(^{-1}\)) (Morrison et al., 1961). 3.0 mM MgADP (○). 1.0 mM MgADP (●). 0.50 mM MgADP (△). 0.25 mM MgADP (▼). 0.20 mM MgADP (□). 0.125 mM MgADP (■).
Figure 33. Effect of ADP on Initial Velocity:

MgADP was varied at several fixed concentrations of L-arginine phosphate. The magnesium acetate concentration was held 2.7 times above the ADP concentration to account for magnesium ADP binding ($4,150 \text{ M}^{-1}$) (Morrison et al., 1961). 3.0 mM Arg~P (○). 2.0 mM Arg~P (●). 1.0 mM Arg~P (Δ). 0.75 mM Arg~P (▼). 0.5 mM Arg~P (□).
Figure 34. Secondary Plot for the Reverse Reaction:

The secondary plot was obtained by replotting y-intercept data from Figures 32 and 33 as described by Florini and Vestling (1957). Arg~P mM (○). MgADP mM (●).
Figure 35. Effect of L-Arginine on Initial Velocity:

L-arginine was varied at several fixed concentrations of MgATP. The magnesium acetate was held 1 mM in excess of the ATP concentration. 0.75 mM MgATP (●). 0.5 mM MgATP (○). 0.25 mM MgATP (▼). 0.125 mM MgATP (∆). 0.0625 mM MgATP (■).
Figure 36. Effect of ATP on Initial Velocity:

MgATP was varied at several fixed concentrations of L-arginine. The magnesium acetate was held 1 mM in excess of the ATP concentration. 2.0 mM L-Arg (●). 1.0 mM L-Arg (○). 0.50 mM L-Arg (▼). 0.25 mM L-Arg (△). 0.16 mM L-Arg (■).
Figure 37. Secondary Plot for the Forward Direction:

The secondary plot was obtained by replotting y-intercept data from Figures 35 and 36 as described by Florini and Vestling (1957). MgATP mM (○). L-Arg mM (●).
Table 7. The Dissociation Constants of Arginine Kinase from the Sea Cucumber:

Dissociation constants are expressed as mM. $K_{bi}$ refers to the dissociation constants from the binary complex [ES] and $K_{ter}$ refers to the dissociation constants from the ternary complex [ES$_1$S$_2$] for a given reaction. $K_{bi}/K_{ter}$ represents the degree of substrate binding cooperativity or “synergism” that the first substrate has on the binding of the second substrate. Dissociation constants and error were calculated by fitting data to non-linear least squares equations (Marangoni, 2003) using SigmaPlot 8.0. Equations and additional information are in Materials and Methods.

<table>
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<th>Reverse Reaction</th>
<th>$K_{bi}$</th>
<th>$K_{ter}$</th>
<th>$K_{bi}/K_{ter}$</th>
<th>$K_{bi}$</th>
<th>$K_{ter}$</th>
<th>$K_{bi}/K_{ter}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg~P</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.08</td>
<td>1.00</td>
<td>0.50 ± 0.04</td>
<td>0.33 ± 0.03</td>
<td>1.51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Forward Reaction</th>
<th>$K_{bi}$</th>
<th>$K_{ter}$</th>
<th>$K_{bi}/K_{ter}$</th>
<th>$K_{bi}$</th>
<th>$K_{ter}$</th>
<th>$K_{bi}/K_{ter}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg</td>
<td>0.36 ± 0.06</td>
<td>0.37 ± 0.04</td>
<td>0.97</td>
<td>0.034 ± 0.008</td>
<td>0.07 ± 0.01</td>
<td>0.49</td>
</tr>
</tbody>
</table>
Effects of Monovalent Anions

Phosphagen kinases have been shown to be inhibited by monovalent anions that mimic the transferable phosphoryl group in the rapid equilibrium, random addition reaction. These anions stabilize the catalytic dead-end complex consisting of the enzyme, a substrate, and a product (AK-arginine-MgADP) by forming a highly stable transition state analog of enzyme, a substrate, a product, and anion (AK-arginine-MgADP-nitrate). The stabilization of monovalent anions on dimeric arginine kinase from the sea cucumber was determined in a series of reaction rate progress curves with the assay reagent containing: NaCl, NaI, NaSCN, NaNO₂, NaNO₃, or NaCH₃CO₂ (Figure 38). The curvature in the time course is due to the formation of the dead-end complex (Milner-White & Watts, 1971) and the more pronounced curvature in the presence of certain monovalent anions is attributed to additional stabilization of the dead-end complex and formation of a transition state analog. The reaction rate progress curves in the presence of monovalent anions were used to look at both the initial velocity (Table 8) and the percent of product formed at the completion of each time course. The acetate anion had a slight activation effect (5.5%) on the initial velocity of sea cucumber AK, while the chloride, iodide, and thiocyanate anions all showed slight inhibition (9%, 13%, and 16% respectively) towards the initial velocity of arginine kinase. In terms of both initial velocity and percent of product formed at the completion of the time course, the nitrate anion was the most effective towards sea cucumber AK with an inhibition of 88% on the initial velocity.
Figure 38. Effect of Monovalent Anions on Sea Cucumber AK:

Progress curves for sea cucumber AK were monitored using the pH stat assay in the forward direction under saturating substrate conditions supplemented with 50 mM of anions salts. The assay was initiated by the addition of 0.05 mL of enzyme. No anion supplemented (——). NaCH₃CO₂ (—). NaCl (—). NaI (—). NaSCN (——). NaNO₂ (——). NaNO₃ (——).
Table 8. Effect of Monovalent Anions on the Initial Velocity of Sea Cucumber AK:

The initial velocity of sea cucumber AK under saturating substrate conditions was compared to the initial velocity of sea cucumber AK in the presence of several monovalent anions. The activation or inhibition of sea cucumber AK was determined by dividing the initial velocity for the anion in question by the initial velocity under saturating conditions followed by multiplying by 100. The value obtained was then subtracted from 100 to determine the percent inhibition.

<table>
<thead>
<tr>
<th>Ion</th>
<th>% Inhibition (-) or Activation (+) of Initial Velocity ($V_o$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCH$_3$CO$_2$</td>
<td>+ 5.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>- 9</td>
</tr>
<tr>
<td>NaI</td>
<td>- 13</td>
</tr>
<tr>
<td>NaSCN</td>
<td>- 16</td>
</tr>
<tr>
<td>NaNO$_2$</td>
<td>- 76</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>- 88</td>
</tr>
</tbody>
</table>
Nitrate Inhibition

Due to the substantial inhibition by the nitrate anion (50 mM) on sea cucumber arginine kinase, an investigation into the effect of nitrate concentration on the initial rates of AK was performed. A series of reaction rate progress curves (Figure 39), in the presence of varying concentrations of nitrate, show an inhibition in the initial velocity (Figures 40 and 41) before significant production of ADP. The absence of curvature in the time courses, which is a measure of the formation of the dead-end complex and transition state analog, suggest the effect of nitrate on arginine kinase is more complex than simple stabilization of the dead-end complex.

Substrate Specificity

Unlike some of the other phosphagen kinases, arginine kinase does not seem to be absolutely specific for L-arginine. The substrate specificity of AK from the sea cucumber was evaluated from reaction rate progress curves using D-arginine, creatine, L-histidine, L-canavanine, L-ornithine, glycocyamine, and aminoguanidine instead of L-arginine as substrate. Arginine kinase from the sea cucumber was able to utilize L-canavanine as a substrate. L-canavanine exhibited 15% of the initial velocity when compared to L-arginine as substrate (Table 9). Arginine kinase from the sea cucumber was not able to utilize D-arginine, creatine, L-histidine, L-ornithine, glycocyamine, or aminoguanidine as substrates.
Figure 39. Effect of Nitrate Concentration on Sea Cucumber AK:
Progress curves for sea cucumber AK were monitored using the pH stat assay in the forward direction under saturating substrate conditions supplemented with varying amounts (3.125 mM – 50 mM) of sodium nitrate. The assay was initiated by the addition of 0.05 mL of enzyme. No anion supplemented (—). 3.125 mM NaNO₃ (——). 6.25 mM NaNO₃ (—). 15 mM NaNO₃ (——). 25 mM NaNO₃ (——). 50 mM NaNO₃ (——).
Figure 40. Initial Velocity of Arginine Kinase at Various Concentrations of NaNO₃:

The initial velocity of arginine kinase from the sea cucumber was determined for varying concentrations of sodium nitrate (3.125 mM – 50 mM). Tangents to the progress curves were used to determine the initial velocity.
Figure 41. Percent Inhibition of Sodium Nitrate on Sea Cucumber AK:

The initial velocity of AK under saturating substrate conditions was compared to the initial velocity of AK in the presence of varying amounts (3.125 mM – 50 mM) of sodium nitrate. The inhibition of sea cucumber AK was determined by dividing the initial velocity for the concentration in question by the initial velocity under saturating conditions followed by multiplying by 100. The value obtained was then subtracted from 100 to determine the percent inhibition.
Table 9. Substrate Specificity of Sea Cucumber Arginine Kinase:

To determine the substrate specificity of AK from the sea cucumber the pH stat assay in the forward direction was used. The assay was run using saturating substrate conditions substituting L-arginine (5 mM) with D-arginine, creatine, L-histidine, L-canavanine, L-ornithine, glycocyamine, and aminoguanidine. Time course were initiated with the addition of 0.1 mL of enzyme and the initial velocity of each substituted substrate was compared to that of L-arginine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Initial Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>100</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>-</td>
</tr>
<tr>
<td>L-Canavanine</td>
<td>15</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>-</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>-</td>
</tr>
<tr>
<td>Creatine</td>
<td>-</td>
</tr>
<tr>
<td>Glycocyamine</td>
<td>-</td>
</tr>
<tr>
<td>Aminoguanidine biocarbonate salt</td>
<td>-</td>
</tr>
</tbody>
</table>
**Difference Spectroscopy**

The monovalent anion with the most profound effect on sea cucumber arginine kinase was nitrate. Both the initial velocity and percent of product formation of arginine kinase were inhibited by nitrate suggesting a mechanism other than stabilization of the dead-end complex alone. In order to further investigate this inhibition, difference spectra were recorded in the presence of different components of the transition state analog (Figure 42). The addition of nitrate appears to cause a slight, broad minimum from 270 nm to 300 nm to the spectra containing it. The most notable spectrum is the one containing all of the components of the transition state analog in which a more pronounced minimum from 250 nm to 310 nm is found. Another notable comparison is between the spectrum containing enzyme and L-arginine and the spectrum containing enzyme and nitrate. These spectra are markedly different and may indicate that nitrate has an effect on the enzyme itself as well as in the presence of the transition state analog components. To further comment on the inhibition of nitrate on sea cucumber AK a full kinetic inhibition profile should be performed.
Figure 42. Difference Spectra for Sea Cucumber AK in the Presence of Nitrate:

Purified sea cucumber arginine kinase (0.08 mg/mL) in 0.1 M Tris/acetate, 1 mM EDTA, 2 mM DTT buffer, pH 8.0 along with transition state analog components were scanned from 200-600 nm in a 1 cm tandem cuvette. The final assay mixture (0.9 mL) contained 0.2 mM ADP, 1.2 mM magnesium acetate, 5 mM L-arginine, and 50 mM nitrate.

Additional information in Materials and Methods. Enzyme and L-arginine (——). Enzyme and nitrate (——). Enzyme, L-arginine, and nitrate (——). Enzyme, L-arginine, magnesium, and ADP (——). Enzyme, nitrate, L-arginine, magnesium, and ADP (——).
Part IV. Characterization of Cockroach Arginine Kinase

**Purification**

Arginine kinase from the American cockroach, *Periplaneta americana*, was purified by the method described by Brown et al. (2004). An analysis of the stepwise results of the isolation from the cockroach and a single protein staining band at a molecular mass corresponding to 43,000 daltons from SDS-PAGE indicates that the purification was successful and homogeneous.

**Polyclonal Antibody Production**

Polyclonal antibodies against purified arginine kinase from the cockroach, *Periplaneta americana*, were developed by ProSci, Inc. (Poway, CA). Antibody fractions were purified and concentrated through ammonium sulfate precipitation. The precipitate was dialyzed against PBS, aliquoted, and frozen at -80°C. Polyclonal antibodies raised against cockroach AK displayed cross-reactivity with *Periplaneta americana* enzyme when samples were subjected to SDS-PAGE followed by immunoblotting (Figure 43).

**Western Blotting**

The antigenic reactivity towards 5 representative types of phosphagen kinases (of varying molecular weights and subunit associations) was determined using polyclonal antibodies raised against monomeric arginine kinase from the cockroach. A positive
reaction was seen with shrimp arginine kinase, which is known to contain monomeric AK. Roach antibodies did not cross-react with dimeric arginine kinase from the sea cucumber, dimeric creatine kinase from the fish, monomeric (two domain) arginine kinase from the clam, or monomeric creatine kinase (multidomain) from sea urchin sperm (Figure 44).

**Difference Spectroscopy**

Arginine kinase from the cockroach has been shown to be affected the most by two monovalent anions, nitrate and borate (Brown and Grossman, 2004). In terms of both the initial velocity and percent of product formed the nitrate anion is the most effective inhibitor of AK activity. In the presence of 50 mM nitrate, the initial velocity of AK is decreased by 92% while 89% of activity is lost in 10 min (Brown et al., 2004). On the other hand, the borate anion exhibits the best ability to stabilize the dead-end complex by decreasing the initial velocity of AK by 30% while only inhibiting product formation by 51% (Brown and Grossman, 2004). These results indicate that nitrate and borate must be acting upon AK through two different mechanisms.

Additional examination of the difference between nitrate and borate on arginine kinase from the cockroach was performed from measurements of difference spectra in the presence of different components of the transition state analog. The addition of nitrate (in any of the samples that contain it) appears to cause a slight, broad minimum from 270 nm to 300 nm and small maximum around 320 nm (Figure 45). Very little difference was exhibited by the exposure of the enzyme to nitrate, nitrate and L-arginine, or even
The complete transition state analog (enzyme, nitrate, arginine, MgADP). In fact, the biggest impact seems to occur in the spectrum containing enzyme and nitrate when compared to the spectrum of just enzyme and L-arginine alone. This result indicates that the effect that nitrate has on the system occurs whether or not it is in the presence of transition state analog components. Over-all, difference spectra containing nitrate as a component of the transition state analog are unremarkable, further supporting the theory that nitrate works to inhibit AK through a mechanism beyond that of stabilizing the dead-end complex.

The addition of borate (Figure 46) causes a slight decrease around 280-290 nm when compared to the spectrum of just enzyme and L-arginine alone. This decrease is even more pronounced in the spectrum containing enzyme, arginine, and borate and might account for the decrease (30%) in initial velocity of AK in the presence of borate seen in previous studies. The most notable spectrum is that of the complete transition state analog (enzyme, borate, arginine, MgADP) where a strong, sharp minimum is exhibited at 260 nm. This result is a clear indication that borate is in fact interacting with the other components of the transition state analog and further supports the conclusion that the mechanism in which borate inhibits is through stabilization of the dead-end complex and formation of a transition state analog.
Figure 43. Western Blotting Results of Cockroach Arginine Kinase:

A purified sample of cockroach arginine kinase (5 µL) was subjected to SDS-PAGE and transferred to a nylon derivatized membrane for immunoblotting. The purified and concentrated polyclonal antibody raised against American cockroach AK was used to verify cross-reactivity on the Western blot. The primary antibody dilution was 1:250 and the secondary antibody dilution was 1:5,000. Detection of AK was determined by the method of Dunbar (1994). Lane 1: Kalediscope molecular weight markers. Lane 2: American cockroach AK.
Figure 44. Western Blotting Results of Phosphagen Kinases:

Extracts were prepared by homogenizing 0.25 g of tissue in 1 mL of 0.05 M Tris/HCL, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50 µM NaN₃, and 25 µM PMSF, pH 8.0. Extracts were centrifuged at 22,000 x g for 10 min and tested for phosphagen kinase activity using the enzyme-coupled spectrophotometric assay in the reverse direction. Prior to Western blot analysis, samples were diluted and catalytic rates matched to 0.50 absorbance units per minute. Samples (5 µL) were subjected to SDS-PAGE and transferred to a nylon derivatized membrane for immunoblotting. The polyclonal antibody raised against the American cockroach was used to determine cross-reactivity on the Western blot (primary antibody dilution = 1:250 and secondary antibody dilution = 1:5,000). Detection of AK/CK was determined by the method of Dunbar (1994).

Lane 1: Kalediscope molecular weight markers. Lane 2: American cockroach AK. Lane 3: Shrimp AK. Lane 4: Sea Cucumber AK. Lane 5: Fish CK. Lane 6: Clam AK. Lane 7: Sea Urchin Sperm CK.
Figure 45. Difference Spectra for Cockroach AK in the Presence of Nitrate:

Purified cockroach arginine kinase (0.275 mg/mL) in 0.1 M Tris/acetate, 1 mM EDTA, 2 mM DTT buffer, pH 8.0 along with transition state analog components were scanned from 200-600 nm in a 1 cm tandem cuvette. The final assay mixture (0.9 mL) contained 0.2 mM ADP, 1.2 mM magnesium acetate, 5 mM L-arginine, and 50 mM nitrate.

Additional information in Materials and Methods. Enzyme and L-arginine (—).
Enzyme and nitrate (—). Enzyme, L-arginine, and nitrate (—). Enzyme, L-arginine, magnesium, and ADP (—). Enzyme, nitrate, L-arginine, magnesium, and ADP (—).
Figure 46. Difference Spectra for Cockroach AK in the Presence of Borate:

Purified cockroach arginine kinase (0.275 mg/mL) in 0.1 M Tris/acetate, 1 mM EDTA, 2 mM DTT buffer, pH 8.0 along with transition state analog components were scanned from 200-600 nm in a 1 cm tandem cuvette. The final assay mixture (0.9 mL) contained 0.2 mM ADP, 1.2 mM magnesium acetate, 5 mM L-arginine, and 50 mM borate.

Additional information in Materials and Methods. Enzyme and L-arginine (—). Enzyme and borate (—). Enzyme, L-arginine, and borate (—). Enzyme, L-arginine, magnesium, and ADP (—). Enzyme, borate, L-arginine, magnesium, and ADP (—).
Roach Rearing and Maintenance

In order to obtain a group of American cockroaches with similar genetic make-up a colony was established and maintained. Cockroaches were bred until a colony of approximately 100 roaches was established. The entire colony was then frozen, with the use of liquid nitrogen, and stored at -80°C. These cockroaches were then used in all molecular biology experiments performed using the cockroach, Periplaneta americana.

Insert Sequence

The insert sequence of American cockroach arginine kinase was deduced using PCR amplification. Degenerate primers (Block C forward primer and Block E reverse primer) were used at 55, 57, and 62°C in the protocol for touchdown PCR. A PCR product of ~ 450 bp was identified by DNA gel electrophoresis, cloned and transformed using a TOPO TA Cloning kit, and the plasmid DNA was isolated using a Perfectprep Plasmid Mini kit. After a restriction digest and analysis by DNA gel electrophoresis, the insert was sequenced (see below) by the Molecular Biology Core Facility at the Moffitt Cancer Research Center. Sequence blast analysis using NCBI showed the insert sequence to be homologous with several species of arginine kinase.
5’ -
GCAGCTGATCGACGATCATTTCCTCTTCAAGGAGGGCGATCGCTTCTTGCA GG
CTGCCAACGCATGCCGCTTCTGGCCCACTGGACGAGGCATCTACCACAACGA
CGCCAAGACGATGCCTCTCCTGGTGCAATGAGGAGGATCACTTGCGAATCAT C
TCTATGCAGATGGGCGGCGACCTGGGACAGGTGTACCGCCGTCTGGTGACGG
CTGTGAATGACATCGAGAAGCGCATCCCCTTCTCGCACGACGACCCTCTGGG
CTTCCCTCACTTCTGTCCCTCCAACCGGGGACCACCATG
- 3’

5’ and 3’ Primers

The insert sequence previously shown was used to create gene-specific primers to obtain the entire 5’ to 3’ sequence of the arginine kinase gene from the cockroach. Primers made from the insert sequence included: For 1, For 2, Rev 1, and Rev 2. For 1 and Rev 2 primers were made to amplify DNA towards the 3’ and 5’ ends, respectively, and For 2 and Rev 1 primers were made as nested primers to the first set.

For 1: 5’- ACG TTC CTG GTC TGG TGC AAT G -3’
For 2: 5’- GCT GTG AAT GAC ATC GAG AAG C -3’
Rev 1: 5’- GCC TCT CGA TGT CAT TCA CAG C -3’
Rev 2: 5’- CAT TGC ACC AGA CCA GGA ACG T -3’

Due to the inability to obtain a full sequence from the primers listed above and the GeneRacer 5’ and 3’ primers, the design and synthesis of new gene-specific primers was necessary. Small fragments of DNA starting from the gene-specific primers synthesized
from the insert sequence out toward both the 5’ and 3’ ends were amplified using Race Ready cDNA. These short fragments were sequenced and used to create new gene-specific primers (listed below) throughout the sequence in a “primer walking” style approach. These additional primers were used to gain both the 5’ and 3’ ends and the entire sequence of arginine kinase from the American cockroach.

F1: 5’- AAG GAG ATG GAG GAC AAG GTG -3’
F2: 5’- ACC GGC ATG ACC AAG GAG GTC -3’
R1: 5’- GAC CTC CTT GGT CAT GCC GGT -3’
R2: 5’- CAC CTT GTC CTC CAT CTC CTT -3’
DONE: 5’- GAC AAA CTG GAG GAG GTT GC -3’
EREV: 5’- GCC CCA CCA ACC TGG GCA CCA CCG TA -3’
PW1F: 5’- TCA CGG ATA CGA GAA TTC ATA ACC -3’
PW2F: 5’- GCC ACT ATT GAT TTC AAG AAC GGA -3’
PW1R: 5’- GGT TAT GAA TTC TCG TAT CCG TGA -3’
PW2R: 5’- AGA CTG GAT TAC TAC AAG AAG TG -3’

5’ and 3’ PCR and Sequencing

The sequence of arginine kinase from the American cockroach was deduced using PCR and sequenced by the Molecular Biology Core Facility at Moffitt Cancer Center. The entire sequence contains 1,071 base pairs which correlate to 357 amino acids. Blast results using NCBI, confirmed that the sequence isolated was homologous to AK from a
variety of species. The nucleotide sequence was translated using the ExPASy Translate Tool (http://us.expasy.org/tools/dna.html).

Nucleotide Sequence

5’-
ATGGTGAGCAGCCGACTTCTGGAGAAAGCTGGAGGCGCGCTTCGCAAAATGGCGGCTC
CTCCGACAGCAAGTCCCTGCTCAAAGAAATCTCGACACAAGGAAGGTGTTGCAGAAT
CTCAAGACAAAGAAGACTCTTTCTATTGGACTACACTCTTCTTGATGTAATCCAGTG
GTCTCGAGAACACGACTCCGGCGTTGGACATCAGCTCCAGATGCTGGAGCTTAT
GCCGTGTTCTGCTGACCTGTGGCCACCCATCTATTGAGCTACCACATGGGTGCTGCAA
GAAGACGGACAAGCACCCTCCCAAGGTACGGGGATGGGTGACAGCGCAGCAGGCAAT
CTGGACCCCTGCTGGCGAGTACATCATCTCCACACGAGTGAGGTGGCGGTCCCAT
GCAGGGCTACCCCTTTAACCCTGCTTGAAGCCCAGTACAAGAGAGTGGAG
GACAGGTGCTCCAGCACAGCTGTCCGGCCTGGAGGCTGAGCTGAAGGCGAGCTCT
ACCCCTCACCAGATGACCAAGGAGGCTCCAGCAGAAGCTCATGATGACCGACTTC
CTCTCAAGGAGGCGATCGCTTCTTGCAGGGCTCCACAGCAGTGGCGCTTGGCC
CAGTGACGAGGCATCTACAACACGAGCACCAGCTCTCCTGCTGGTGCAATG
AGGAGGATCATTGCGAATCATCTCTATGCAATGGGCGGCGACCTGGGGACAGGT
GTACCAGCCTGTGGAGCGGTGTAAGACTGAGAGCAGCGCTCAGAGCAGCATCCCTTCTCGC
ACGAGCAGCCGTCTGGGCTCTCACCCTTTCTGCCCCACCCACCTGGGCAACCCGGTA
CGTGCGTCTGTCACATCAAGTGGCCAAGGCTGGCTGCGGACAAGGCCAGGGCTTG
AGGAGGTGCTGGCAAGTACAACTGTCAGGTCCGTGCGACCCTGTCGGCAGACAC
AGAGGCTGAGGCGGTGCTACAGCAGCTCCAACAAGCGCGGCATGGGCGCTGACA
GAGTACGAGCGCCGTCAGGAGATGAACGACGCCATC GCCGAGCTGATCAAGCTGG
AGAGCTCGCTCTAA

-3’
Protein Sequence

MVDAAVLEKLEAGFAKLAASDSKSLKKYLTKEVF
DNLKTKKTPSFGGSTLLLVDVIQSGLENHDSGVGITYAPDA
EAYAVFADLFDPIEDYHGFGKKTDDKHPPKDWGDVD
TLGNLDPAGEYIIISTRVRCGRSMQGYPFNPCLTEAQ
YKEMEDKVSSTLSGLEGELKGQFYPLTGMTKEVQ
QKLIIDHFLFKEGDRFLQAAANACRFWPTGRIYHND
AKTFLVWCNEEDHLRIISMQMGGDLGQVYRRLVT
AVNDIEKRIPFSDDRDLGFNTFCPTHTLGGTVRASVHI
KVPKLAAADKAKLREEVAGKNLQVRGTRGEHTEAEG
GVYDISNKRRMGLTEYDAVKEMNDGIAELIKLES

Sequence Alignment

A neighbor joining tree based on sequence homologies was constructed by aligning several representative sequences of phosphagen kinases from vertebrate and invertebrate species, including the arginine kinase sequence deduced for *P. americana* (Figure 47). The sequence from the American cockroach clusters with other arthropod arginine kinases in comparison to the dimeric arginine kinases which cluster with creatine kinases from both invertebrate and vertebrate species. AK from the sponge, *Porifera*, clusters alone while CK from the sponge clusters with the other creatine kinases.
Figure 47. Phylogenetic Tree of a Variety of Arginine and Creatine Kinases from Both Vertebrates and Invertebrates:

A neighbor joining tree displaying the sequence alignments of arginine and creatine kinases was created. Sequences were obtained from NCBI. Boot strap values were determined by collapsing the tree to 50% or greater. Information regarding alignment programs utilized and accession numbers for species can be found in the Material and Methods section.
Chapter 4. Discussion
Part I. Purification and Characterization

In order to understand the direct relationship between the structure and function of an enzyme, many physical characteristics must first be evaluated. The individual properties of an enzyme can be determined by following a purification protocol designed to obtain both high yield and purity of the protein. A purification protocol should be specific for a given enzyme and can be designed by adapting other purification procedures from enzymes in the same family as the one being studied. Obtaining pure enzyme will enable the determination of characteristics unique to that protein and will allow a comparison between it and enzymes in the same family in regards to their molecular weights, quaternary structures, and structural evolution.

In the present study, arginine kinase has been purified to homogeneity from the sea cucumber, *Isostichopus badonotus*, to examine the physical characteristics of this enzyme. The purification procedure involved ammonium sulfate fractionation, size exclusion chromatography, and adsorption chromatography to yield 8.8 mg of pure enzyme from 50 g of sea cucumber muscle. The purity of the enzyme was tested using SDS-PAGE, which resulted in a single protein staining band at 43.7 kDa. To determine the native molecular weight of the enzyme, and to evaluate the quaternary structure of arginine kinase from *I. badonotus*, calibrated gel chromatography was utilized. The
native molecular weight of arginine kinase from the sea cucumber was shown to be approximately 80 kDa which shows, along with results from the calibrated SDS-PAGE, that AK from the sea cucumber is a dimer made up of two identical subunits.

Table 10 compares sea cucumber arginine kinase to AK and CK from various species. An examination of sea cucumber AK and arginine kinase from other echinoderms indicates that these enzymes are all dimers with a molecular weight around 80 kDa (Seals & Grossman, 1988; Wright-Weber et al., 2006). The quaternary structure of arginine kinase found in echinoderms is similar to the quaternary structure found in most creatine kinases from vertebrates, such as the monkey C. verus (Grossman & Mollo, 1979). However, the majority of other arginine kinases, like that of the roach P. americana, are found as monomers with a molecular weight of 40 kDa (Brown et al., 2004). The importance of determining the molecular weight and subunit compositions within an enzyme family, such as the phosphagen kinase family, is to note variations and observe trends between species. Arginine kinases show a more variable range in subunit compositions with the occurrence of monomers, dimers, two-domain monomers, and tetramers (Suzuki et al., 2002; Robin et al., 1969). Creatine kinases are less variable with exceptions being found only in sperm (large monomers) and mitochondria (octamers) (Tombes & Shapiro, 1985; Wyss et al., 1990). A comparison of the distribution in molecular weight and subunit composition of the phosphagen kinases could suggest that although AK from I. badonotus utilizes a different substrate than vertebrate species, it might be evolutionarily closer to these species based on its molecular weight and quaternary structure.
Table 10. Comparison of the Molecular Weight and Subunit Composition of Known Phosphagen Kinases:

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular Weight</th>
<th>Subunit Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cockroach AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. americana</em></td>
<td>43 kDa</td>
<td>1</td>
<td>Brown et al., 2004</td>
</tr>
<tr>
<td>Shrimp AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aztecus</em></td>
<td>40 kDa</td>
<td>1</td>
<td>France et al., 1997</td>
</tr>
<tr>
<td>Hornworm AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. sexta</em></td>
<td>40 kDa</td>
<td>1</td>
<td>Rosenthal et al., 1977</td>
</tr>
<tr>
<td>Sea Cucumber AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>I. badonotus</em></td>
<td>87 kDa</td>
<td>2</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td>Sea Urchin Egg AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. purpuratus</em></td>
<td>81 kDa</td>
<td>2</td>
<td>Wright-Weber et al., 2006</td>
</tr>
<tr>
<td>Sea Cucumber AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. arenicola</em></td>
<td>80 kDa</td>
<td>2</td>
<td>Seals &amp; Grossman, 1988</td>
</tr>
<tr>
<td>Clam AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. strictus</em></td>
<td>80 kDa</td>
<td>1</td>
<td>Suzuki et al., 2002</td>
</tr>
<tr>
<td>Sabellid AK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pavonina</em></td>
<td>150 kDa</td>
<td>4</td>
<td>Robin et al., 1969</td>
</tr>
<tr>
<td>Monkey CK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. verus</em></td>
<td>82 kDa</td>
<td>2</td>
<td>Grossman &amp; Mollo, 1979</td>
</tr>
<tr>
<td>Shark CK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. cirratum</em></td>
<td>85 kDa</td>
<td>2</td>
<td>Gray et al., 1979</td>
</tr>
<tr>
<td>Sea Urchin Sperm CK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. lividus</em></td>
<td>150 kDa</td>
<td>1</td>
<td>Tombes &amp; Shapiro, 1985</td>
</tr>
<tr>
<td>Mitochondrial CK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>320 kDa</td>
<td>8</td>
<td>Wyss et al., 1990</td>
</tr>
</tbody>
</table>
Arginine kinase from the sea cucumber is fairly thermal stable when compared to other phosphagen kinases (Table 1). *Isostichopus badonotus* displays 50% activity when heated for 10 minutes at 46 °C followed by a sharp decline of activity near 50 °C for any of the times examined. This sharp decline of activity at 50 °C is not seen in other thermal stable phosphagen kinases, like AK from the American cockroach, which still show 50% activity at this temperature (Brown et al., 2004). A comparison of thermal stabilities from various phosphagen kinases (Table 1) suggests that this physical characteristic varies regardless of the substrate, molecular weight, or quaternary structure of this enzyme. Sea cucumber AK does, however, have a higher thermal stability then some of the other echinoderms listed in the table. This points to the conclusion that thermal stabilities might have more to do with the environment in which the enzyme is found. The thermal stability from *I. badonotus* is more suited for the environment (warmer waters) this species inhabits, while the lower thermal stabilities seen in AK from *S. purpuratus* and *P. lividus* are more suited for the environment (cooler waters) they inhabit (Wright-Weber et al., 2006; Ratto & Christen, 1988).

Phosphagen kinases show comparable ranges in pH optima for the forward (synthesis of ADP) and reverse (synthesis of ATP) directions of the reversible enzymatic reaction. The forward pH optima are usually broader, from 7.5-9.5, while the reverse pH optima are typically more narrow, from 6.5-7.0 (Morrison, 1973; Watts, 1973). The sea cucumber is no exception and displays a pH optimum in the forward direction at 8.0 and a pH optimum in the reverse direction at 6.0. This suggests that the catalytic mechanism is conserved throughout the phosphagen kinase family since the active sites of these
enzymes have similar sensitivities to changes in pH. There are numerous structural and
catalytic properties of phosphagen kinases that can account for the difference in pH
optima for the forward and reverse direction such as ionizable side chains of amino acids
that make up the active site or the affect of pH on the various substrates involved (ie.
ADP and ATP). However, the pH optima ranges make sense when the other components
of the reversible catalyzed reactions are examined. The forward reaction would occur in
a more basic environment because it is producing MgADP and H⁺ while the reverse
reaction would need to occur in a more acidic environment because it utilizes MgADP
and H⁺ to produce MgATP. The differences in optimal pH are exploited by the muscle in
a unique physiological adaptation that produces a sharp drop in pH upon muscle
contraction (ATP utilization). The drop in pH shifts the enzyme towards the optimum in
the reverse direction thereby facilitating the production of more ATP from MgADP and

The isoelectric point (pI) of an enzyme is a physical characteristic that can help
determine the overall amino acid composition of the enzyme, the types of charged amino
acid side chains exposed to each other and a solvent, as well as distinguish between
different isozymes contained in a single organism. The isoelectric point for *I. badonotus*
was found to be 6.0 which is comparable to other dimeric enzymes in the phosphagen
kinase family (Table 2). Monomeric arginine kinases tend to have more acidic isoelectric
points than dimeric arginine kinases or creatine kinases (Storey, 1977; Grossman &
Mollo, 1979; Wright-Weber et al., 2006). However, looking at Table 2 it can be seen that
the isozymes of CK have a range of isoelectric points from 6.9 in CK-MM to 4.8 in CK-
BB (Grossman & Mollo, 1979). Although both of these enzymes are dimeric the
difference in isoelectric points is presumably due to the MM form containing additional
basic amino acids which would account for the higher pI.

The differences in their isoelectric points, tertiary structures, and shapes are what
allow the three isozymes of CK to be distinguished from each other under native gel
electrophoresis conditions (omission of SDS). This type of experiment can be used to
compare the migration of phosphagen kinases and can highlight the differences between
them for future techniques. Arginine kinase from the sea cucumber was shown to
migrate parallel to CK-MM (pI 7) under native conditions while dimeric arginine kinase
from sea urchin eggs (pI 6.7) and monomeric arginine kinase from the cockroach (pI 5.8)
were shown to migrate parallel to CK-BB (pI 4) (Wright-Weber et al., 2006). Although
the isoelectric points from the sea urchin, *S. purpuratus*, and the cockroach, *P.
americana*, have a difference of almost one, and are much larger than the pI of CK-BB,
there must be something in their structural conformations that allow them to migrate
parallel to each other on a gel.

The differences between the isoelectric points and native gel migration between
dimeric AK from the sea cucumber and dimeric AK from sea urchin eggs were exploited
to allow for a comparison of conserved structural features required for subunit
association, catalytic activity, and formation of quaternary structure possessed by the
dimeric phosphagen kinases (Wright-Weber et al., 2006). This was accomplished
through a technique known as hybridization, where the enzymes were combined,
denatured in the presence of guanidine hydrochloride, renatured, and subjected to native
gel electrophoresis (Figure 28). The presence of a functional heterodimer was seen with a migration pattern in between that of sea cucumber (migrates as though more basic) and sea urchin egg (migrates as though more acidic) AK (Wright-Weber et al., 2006). It should also be noted that neither of these two enzymes were able to form a functional hybrid with monomeric arginine kinase indicating that monomers do not contain the conserved structural features required for subunit association.

This result is consistent with other reports showing CK isozymes from two different species forming functional dimers (Eppenbeger et al., 1967; Grossman & Mollo, 1979) as well as dimeric AK and CK forming functional heterodimers (Seals & Grossman, 1988). More recently, two fused proteins consisting of dimeric AK from sea cucumber muscle and dimeric CK from rabbit muscle were made through the expression of fused AK and CK genes by Zhang et al. (2006). The fused proteins (AK-CK and CK-AK) both displayed approximately 50% AK and CK activity with 2-fold increases in $K_m$ values for their prospective guanidine substrates showing that both moieties are active in the two fused proteins. The proteins (regardless of the gene expression order) had similar secondary, tertiary, and quaternary structure, molecular size, and thermodynamic stability. The authors suggested that AK and CK might share the same protein folding mechanism and that the results provide further proof that dimeric AK and CK have a close evolutionary relationship.

Circular dichroism is used to investigate the secondary structure of proteins in the far UV region (below 250 nm). Circular dichroic spectra measure the absorption of right and left circularly polarized light seen from the asymmetry of proteins during optical
activity (Creighton, 1984). The extent of alpha helical, beta sheet, and random coil structures in a protein can be determined by looking at characteristic minima and maxima that each structure displays at various wavelengths. The alpha helical content of arginine kinase from the sea cucumber was evaluated using circular dichroism and found to contain 9% alpha helices using calculations from the characteristic minima at 222 nm (Chen et al., 1972). This percentage of alpha helices is low when compared to other dimeric proteins in the phosphagen kinase family (Grossman & Sellars, 1998; Guo et al., 2003). The 9% alpha helical content in the sea cucumber is more comparable to the monomeric arginine kinases that typically display percentages between 10-15% (Brown et al., 2004; France & Grossman, 1996). It should be noted that although the characteristic minima at 222 nm and 208 nm were present in the CD spectrum of AK from *I. badonotus*, the spectrum is anomalous compared to others based on the fact that there is no leveling off of the absorption near zero towards the end of the spectrum. Although several attempts were made to account for this anomaly, it could explain why the alpha helical percentage is low in the sea cucumber and suggest that this number might not be indicative of other dimeric phosphagen kinases.
Part II. Immunological Analysis

Immunological reactivity can be used to establish similarities in the structure and sequence of different enzymes. A cross-reaction is seen when epitopes from the enzyme in question recognize antibodies made to a specific protein. Antibodies from a native protein are typically made from epitopes on the surface of the folded protein. A positive reaction with antibodies from a native protein would suggest that the enzyme in question has a similar surface structure to the protein in which the antibodies were made. Similarly, the amino acids contained in those surface epitopes are found close together in the folded protein but not necessarily in the linear sequence and are most likely not found in a central region, such as the active site, of the protein. Although the immunological properties of several phosphagen kinases have already been examined, the literature is currently lacking a report of antibodies made against dimeric arginine kinases. The substrate for monomeric AK and dimeric AK is the same while dimeric AK and CK share the same quaternary structure. A study utilizing dimeric arginine kinase antibodies would be interesting as a comparison between the immunological reactions displayed by monomeric arginine kinases and dimeric creatine kinases with these antibodies.

In the present study arginine kinase from the sea cucumber was used to create polyclonal antibodies and compared to 23 different types of phosphagen kinases of various molecular weights and quaternary structures (Table 6). No positive cross-reaction was seen with any of the proteins that are known to contain monomeric arginine kinase except for AK from the clam, *C. cancellata*, which is known to be a large two-
domain monomer of 80 kDa (Suzuki et al., 2002). This result could suggest that although the clam contains monomeric AK, its two-domain composition is arranged in a manner that might display similar epitopes to dimeric AK from the sea cucumber. No reaction was detected for arginine kinase from *S. melanostigma* which is known to be a tetramer of 150 kDa (Robin et al, 1969). Positive cross-reactions were seen with all species known to contain dimeric creatine kinase, two of which were purified forms of CK-MM and CK-BB isozymes. However, no cross-reaction could be detected from large monomeric creatine kinases from the starfish, *E. spinulosus*, the brittle star, *O. angulata*, or sea urchin sperm, *S. purpuratus* (Ratto et al., 1989; Tombes & Shapiro, 1985).

The most interesting result was seen with the positive cross-reaction from the sponge, *T. aurantia*, which is now known to contain dimeric creatine kinase. It had previously been thought that sponges, which are among the oldest organisms available, contained arginine kinase. It was assumed that AK from the sponge had a monomeric subunit composition because monomeric AK was believed to be the most primitive phosphagen kinase (Watts, 1971; Watts, 1975). In 2000, Ellington reported purifying a phosphagen kinase from a sponge and found that it contained creatine kinase activity and had a dimeric subunit composition. This finding revealed that the appearance of creatine kinase occurred earlier in evolution then was previously thought, as well as the occurrence of quaternary structure. The results here support this finding and demonstrate that the more “modern” dimeric AK from the sea cucumber, *I. badonotus*, contains conserved features to one of the most basal organisms on the evolutionary tree (Figure 9), most likely associated with their shared quaternary structure.
The present results using antibodies made against dimeric arginine kinase from *I. badonotus* support the numerous immunological studies performed for the phosphagen kinases. In 1970, Viala et al. determined that antibodies made from dimeric creatine kinase did not cross-react with monomeric arginine kinase enzymes and that antibodies made from monomeric arginine kinase did not cross-react with dimeric creatine kinase enzymes. Although all of the phosphagen kinases share high sequence homologies to one another, there is enough difference in their amino acid sequences to create conformational changes that make them antigenically distinct. A lack of conserved structural or surface regions caused by amino acid differences can even be seen in the isozymes of CK. Antibodies from CK-MM can react with MM isoymes from other species but they can not cross-react with the BB isozyme from the same species in which the antibody was made (Grossman & Mollo, 1979).

To further compare the differences between the immunological cross-reactivity between phosphagen kinases the present study made polyclonal antibodies against purified monomeric arginine kinase from the cockroach, *P. americana*. The cross-reactivity for several representative types of phosphagen kinases with various molecular weights and quaternary structures can be seen in Table 11, along with a comparison of the results found with antibodies made against *I. badonotus*. As previously demonstrated (Viala et al., 1970; Brown et al., 2004) antibodies made from monomeric AK from the cockroach reacted with monomeric AK but not dimeric AK or CK. Data previously not reported shows that large two-domain monomeric AK and large monomeric CK also display no cross-reaction with antibodies made from monomeric AK from the cockroach.
The result with the large two-domain AK monomer is consistent with the previous statement that this enzyme has a composition or structural arrangement that is more similar to dimeric enzymes than it is to monomeric enzymes.

The comprehensive information regarding the immunological properties of the phosphagen kinases suggests that the dimeric enzymes are more antigenically similar to each other than they are toward monomeric enzymes. The substrate specificity of the enzyme does not seem to play an important role in antigenicity and monomeric phosphagen kinases do not seem to contain similar conserved features necessary for cross-reactivity to occur with dimeric or large monomeric phosphagen kinases. The explanation for this could be that the various phosphagen kinases have evolved through divergent evolution. Although they have a common evolutionary origin they have evolved over time through gene duplication or fusion and some phosphagen kinase genes have diverged enough to create slight differences in their immunological properties.
Table 11. Comparison of Immunological Cross-Reactivity of Phosphagen Kinases with Monomeric Arginine Kinase and Dimeric Arginine Kinase Polyclonal Antibodies:

The antigenic cross-reactivity towards 5 representative types of phosphagen kinases with varying molecular weights and quaternary structures are compared using cockroach and sea cucumber polyclonal antibodies.

<table>
<thead>
<tr>
<th>Representative Phosphagen Kinase</th>
<th>Cross-Reactivity with Roach Antibody</th>
<th>Cross-Reactivity with Sea Cucumber Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomeric AK</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Dimeric AK</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Dimeric CK</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Monomeric AK 2 Domain</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Monomeric CK Multidomain</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Part III. Kinetic Analysis

A kinetic analysis is performed to characterize the mechanism an enzyme follows, the enzymes maximal velocity and product formation, or to determine dissociation constants for the substrates involved in the reaction. Phosphagen kinases catalyze a reversible bisubstrate reaction so dissociation constants can be determined for the binary complex [ES] (the effect one substrate has on the enzyme) and the ternary complex [ES₁S₂] (the effect both substrates have on the enzyme) (Kuby et al., 1954). These constants are referred to as $K_{bi}$ and $K_{ter}$ for the binary and ternary complexes respectively.

In the reverse reaction, arginine kinase from the sea cucumber has a $K_{bi}$ and $K_{ter}$ of 1.00 mM for phosphoarginine and dissociation constants of 0.5 mM ($K_{bi}$) and 0.33 mM ($K_{ter}$) for MgADP. For the forward reaction, sea cucumber arginine kinase has a $K_{bi}$ and $K_{ter}$ of approximately 0.36 mM for arginine and dissociation constants of 0.034 mM ($K_{bi}$) and 0.07 mM ($K_{ter}$) for MgATP (Held et al., 2007).

The analysis of initial velocity kinetics for sea cucumber arginine kinase is represented through Lineweaver-Burk plots. The plots indicate that AK from *I. badonotus* follows a rapid equilibrium, random addition mechanism by displaying lines that intersect left of the ordinate and above the abscissa (Watts, 1973). These results are consistent with reports found for almost all creatine and arginine kinases examined (Morrison & James, 1965; Morrison & Cleland, 1966; Virden et al., 1965; O’Sullivan et al., 1969). This mechanism is characterized as having no order to the binding of
substrates and assumes all steps are in rapid equilibrium except for those involved in the conversion of the ternary complex (Arg-E-MgATP to PArg-E-MgADP) (Segal, 1975).

A comparison of the dissociation constants from AK in the sea cucumber, cockroach, and rabbit can be seen in Table 12. These numbers are indicative of the general trend seen in dissociation constants for the phosphagen kinases. Nucleotide values for both the forward and reverse reactions are typically comparable (0.034 – 1.2 mM: ATP, 0.05 – 0.5 mM: ADP) for a majority of phosphagen kinases (Morrison & James, 1965; Brown & Grossman, 2004; Held et al., 2007). However, dissociation constants for the guanidino substrates tend to vary. Creatine kinases generally have higher values for the substrate creatine and lower values for the phosphocreatine (Morrison & James, 1965; Hornemann et al., 2000; Chen et al., 2000). On the other hand, phosphoarginine for arginine kinases all have $K_{bi}$ and $K_{ter}$ values around 1.00 mM while the dissociation constants for arginine are more variable (Seals & Grossman, 1988; Uda & Suzuki, 2004; Storey 1977). An exception to this trend can be seen Table 12 with AK from the cockroach displaying much lower dissociation constants for phosphoarginine then AK from the sea cucumber (Brown & Grossman, 2004; Held et al., 2007). The lower dissociation constants for the cockroach indicate that the substrates bind more tightly to the roach enzyme then they do to the sea cucumber enzyme. These numbers might suggest that AK from the cockroach has a catalytic efficiency that is greater than arginine kinase from the sea cucumber.

The ratio of dissociation constants $K_{bi}/K_{ter}$ for a given substrate is a characteristic known as substrate binding cooperativity or “synergism” (Segal, 1975). This ratio
reflects how the binding of one substrate affects the binding of the second substrate and is seen when the ratio deviates from one. Arginine kinase for the sea cucumber has a ratio of approximately 1.00 for both arginine and phosphoarginine but has ratios of 0.5 and 1.5 for MgADP and MgATP respectively (Held et al., 2007). Although the ratios for the nucleotides deviate slightly from one for the sea cucumber, it would still be concluded that no substrate cooperativity is displayed. These numbers are within experimental error and are not as high as those typically displayed by the other phosphagen kinases. As shown in Table 12 creatine kinases, to varying degrees, all display synergistic substrate binding while the results are more varied for the arginine kinases (Watts, 1973; Chegwidden & Watts, 1975; Morrison & James, 1973; Brown & Grossman, 2004, Tanaka et al., 2007). It was once thought (Hornemann et al., 2000) that synergism was associated with subunit-subunit interactions and that it ascribed significance to the dimeric state. However, results like those from *I. badonotus* show that not all dimeric phosphagen kinases exhibit synergism (Held et al., 2007).
Table 12. Comparison of Monomeric AK, Dimeric AK, and Dimeric CK Dissociation Constants [mM]:

<table>
<thead>
<tr>
<th></th>
<th>$K_{bi}^{guanidine}$</th>
<th>$K_{ter}^{guanidine}$</th>
<th>$K_{bi}^{MgATP}$</th>
<th>$K_{ter}^{MgATP}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Reaction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cockroach</td>
<td>1.00</td>
<td>0.94</td>
<td>0.12</td>
<td>0.09</td>
<td>Brown &amp; Grossman, 2004</td>
</tr>
<tr>
<td>Sea Cucumber</td>
<td>0.36</td>
<td>0.37</td>
<td>0.034</td>
<td>0.07</td>
<td>Held et al., 2007</td>
</tr>
<tr>
<td>Rabbit</td>
<td>15.6</td>
<td>6.1</td>
<td>1.2</td>
<td>0.48</td>
<td>Morrison &amp; James, 1965</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>$K_{bi}^{guanidine}$</th>
<th>$K_{ter}^{guanidine}$</th>
<th>$K_{bi}^{MgADP}$</th>
<th>$K_{ter}^{MgADP}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reverse Reaction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cockroach</td>
<td>0.45</td>
<td>0.49</td>
<td>0.17</td>
<td>0.14</td>
<td>Brown &amp; Grossman, 2004</td>
</tr>
<tr>
<td>Sea Cucumber</td>
<td>1.00</td>
<td>1.00</td>
<td>0.50</td>
<td>0.33</td>
<td>Held et al., 2007</td>
</tr>
<tr>
<td>Rabbit</td>
<td>8.6</td>
<td>2.9</td>
<td>0.17</td>
<td>0.05</td>
<td>Morrison &amp; James, 1965</td>
</tr>
</tbody>
</table>


Several substrates were tested with arginine kinase from the sea cucumber to examine their effect on initial velocity and product formation. The substrates D-arginine, L-histidine, L-canavanine, L-ornithine, creatine, glycocyamine, and aminoguanidine were chosen because they are natural substrates of the other phosphagen kinases or known substrate analogs of other arginine kinases (Figure 48). Arginine kinase from *I. badonotus* only exhibited activity with its known substrate, L-arginine, and with the nonprotein amino acid L-canavanine (15% initial velocity) (Wright-Weber et al., 2006). This result is consistent with AK enzymes being classified as one of the phosphagen kinase enzymes with a narrow substrate specificity (Morrison, 1973).

Similarly, L-canavanine has been demonstrated as a substrate analog for arginine kinase from organisms such as the cockroach, sea urchin, and the tobacco hornworm (Brown & Grossman, 2004; Wright-Weber et al., 2006; Rosenthal et al, 1977). This is not surprising given the structural similarities between L-canavanine and L-arginine compared to the other substrate analogs tested (Figure 48). L-canavanine and D-arginine have been shown to act as competitive inhibitors of AK from several insects when in the presence of L-arginine (Alonso et al., 2001; Blethen, 1972; Morrison, 1973; Brown & Grossman, 2004). The D-isomers of several amino acids are also known to be toxic to many insects due to their inability to be oxidized. Although D-arginine was not able to be utilized as a substrate by the sea cucumber, the reports of nonprotein amino acids (which are non-toxic to humans) as inhibitors of AK is interesting considering their potential as effective tools in insect population control.
Figure 48. Comparison of the Substrates used in Substrate Specificity Determination:
The rapid equilibrium, random addition mechanism said to be obeyed by the phosphagen kinases is capable of forming two dead-end complexes consisting of the enzyme, a substrate, and a product (Milner-White & Watts, 1971). Anions have the potential to stabilize this dead-end complex and form a highly stable transition state analog consisting of the enzyme, a substrate, a product, and an anion (Buttlaire & Cohn, 1974). To examine the stabilization of the dead-end complex in dimeric AK from the sea cucumber (data currently absent in the literature) several monovalent anions were tested in the forward reaction (synthesis of ADP) through a series of progress curves (Figure 39). Initial velocity and product formation of an enzyme can both be measured in this manner. The curvature in the time course signals formation of the dead-end complex and ADP production while a decrease in the curvature signifies inhibition through the transition state analog (Watts, 1973).

Of the monovalent anions investigated for sea cucumber AK, only the acetate ion showed an increase in initial velocity measurements of 5.5%. The acetate anion did not affect the curvature of the time course or the amount of product formation suggesting that it does not stabilize the formation of the dead-end complex. The rest of the anions caused a decrease in the initial velocity of sea cucumber AK with nitrite and nitrate having the greatest inhibition effects with a decrease in $V_o$ of 76% and 88% respectively. Nitrates potent inhibitory affect is seen for many phosphagen kinases although it shows the greatest impact for arginine kinases while nitrite affects creatine kinases the most (Milner-White & Watts, 1971, Morrison, 1973; Buttlaire & Cohn, 1974). It is thought
that these anions cause inhibition by mimicking the transferable phosphoryl group in the phosphagen kinase catalyzed reaction.

As can be seen from Figures 39, 40, and 41 nitrate affects the initial velocity, time course curvature, and product formation of arginine kinase from *I. badonotus*. This inhibition was displayed with the cockroach, *P. americana*, and led the authors to suggest that nitrate inhibits AK by a more complex mechanism than just stabilization of the dead-end complex (Brown & Grossman, 2004). Brown and Grossman also found that borate, not tested for *I. badonotus*, was the best inhibitor of the dead-end complex by decreasing initial velocity by only 30% and product formation by 51%. Further kinetic analysis to examine the mechanisms of inhibition by nitrate and borate on cockroach AK revealed that nitrate was a non-competitive inhibitor of L-arginine (\(K_i = 8.0 \text{ mM}\)) and that borate was a competitive inhibitor of L-arginine (\(K_i = 5.5 \text{ mM}\)). The kinetic results confirm the distinct difference seen in the progress curves for nitrate and borate with cockroach and sea cucumber AK. A non-competitive inhibitor binds to enzyme sites that participate in both substrate binding and catalysis where as a competitive inhibitor only binds to enzyme sites that participate in substrate binding (Segal, 1975). Clearly the inhibition by nitrate is more complex than what was once proposed.

Due to the differing affects of nitrate and borate on arginine kinases the present study used difference spectroscopy to further characterize the inhibition by these monovalent anions on AK from both the sea cucumber, *I. badonotus*, and the cockroach, *P. americana*. Difference spectroscopy is a technique that looks at small conformational changes in the environment of a protein (from 200-600 nm). Proteins and nucleic acids
absorb strongly below 300 nm and conformational changes can typically be seen between 280-300 nm. The amino acids tryptophan and tyrosine both have strong absorptions at ~280 nm and phosphagen kinases are known for containing a tyrosine residue in their active sites (Anosike & Watts, 1975). Examining arginine kinases in the presence of various components of the transition state analog allows changes in the active site of the enzyme upon binding of various substrates to be monitored.

Spectra recorded for the proteins before mixing with various components were subtracted from spectra recorded after mixing with various components to see the difference caused in each spectrum. Difference spectra for cockroach AK and sea cucumber AK in the presence of nitrate are similar (Figures 42 and 45). Addition of nitrate, to any of the spectra containing it, causes a slight broadening of the minimum from 270-300 nm. The spectrum with the biggest impact for both sea cucumber and cockroach AK contains just nitrate and enzyme which suggests that the affect nitrate has on the system occurs whether other components of the transition state analog are present or not. This is not consistent with results seen in CK from the rabbit (Grossman & Garcia-Rubio, 1987) or monomeric AK from the lobster (Anosike & Watts, 1975) however, it is consistent with reports that nitrate alters the structure of CK (Madelian & Warren, 1977) and that it binds to AK alone (Raimbault et al., 1996).

Difference spectra for arginine kinase from the cockroach in the presence of borate are the first reported in the literature. Addition of borate causes a slight decrease around 280-290 nm in all spectra that contain it (Figure 46). The spectrum containing enzyme, arginine, and borate has a more pronounced minimum. This might account for
the 30% decrease seen in the $V_o$ of AK from the cockroach in the presence of borate (Brown & Grossman, 2004). The spectrum containing the complete transition state analog (enzyme, arginine, borate, and MgADP) displays a strong, sharp minimum at 260 nm and is a clear indication that borate interacts with other components of the TSA. The transition state analog spectrum for borate is strikingly different than the transition state analog spectrum for nitrate. These results support the theory by Brown and Grossman (2004) that nitrate inhibits arginine kinases through a mechanism beyond that of stabilizing the dead-end complex while borate inhibits arginine kinases through stabilization of the dead-end complex and formation of a transition state analog.
The entire 5’ to 3’ nucleotide sequence for arginine kinase from the American cockroach, *Periplaneta americana*, was deduced. The sequence was determined by utilizing several molecular biology techniques such as: isolation of total RNA from the cockroach, reverse transcription of single stranded RNA to obtain double stranded cDNA, generation of degenerate and gene-specific primers, touchdown PCR, and sequencing from the Molecular Biology Core Facility at the Moffitt Cancer Research Center. The entire sequence from start codon to stop codon is 1,071 base pairs which correlates to a total of 357 amino acids. Sequence blast analysis using NCBI shows that the sequence from the American cockroach is homologous to several other species which are known to contain monomeric arginine kinases.

The protein sequence for arginine kinase from the cockroach, *P. americana*, contains all of the amino acids which are thought to be conserved in arginine kinases for nucleotide and guanidine binding. The first crystal structure of AK bound to its transition state analog was reported for the horseshoe crab *Limulus* in 1998 by Zhou et al. This structure indicated that the amino acids involved with arginine binding in AK were Ser 63, Gly 64, Val 65, Tyr 68, Glu 225, Cys 271, and Glu 314 which can all be found in the sequence for the American cockroach (Figure 49). The *Limulus* structure also highlighted amino acids Arg 124, Arg 126, Arg 229, Arg 280, and Arg 309 as being important for nucleotide binding and again, these residues are found in the protein sequence of the American cockroach (Figure 49).
In 1997 Suzuki et al. compared sequence alignments of several phosphagen kinases and concluded that each kinase has a unique guanidino specificity (GS) region used for guanidine recognition. This unique region is characterized by amino acid insertions or deletions that correspond to the size of the guanidine substrate used by the enzyme. Arginine kinases are known to contain a 5 amino acid deletion due to the large size of their guanidine substrate. Figure 49 shows that arginine kinase from *P. americana* contains a 5 amino acid deletion between amino acids 62 and 63, when compared to other phosphagen kinases (Uda & Suzuki, 2004). This deletion corresponds to the fact that *P. americana* utilizes arginine as its natural substrate. Figure 49 also highlights two other features common to all of the phosphagen kinases. Arginine kinase from the cockroach contains the highly conserved negatively charged NEED-box that was first identified in creatine kinases and is thought to play a role in the catalytic mechanism (Eder et al., 2000; Cantwell et al., 2001). Similarly, the sequence contains amino acids (underlined in Figure 49) that make up the C terminal and N terminal flexible loops found in phosphagen kinases. These loops are thought to rearrange critical elements of the active site as well as close off the active site to exterior solvent during catalysis (Zhou et al., 2000; Azzi et al., 2004).
**Figure 49. Nucleotide Sequence for Arginine Kinase from the American Cockroach and a Comparison of Several Conserved Amino Acids and Structures:**

The protein sequence of arginine kinase from the American cockroach was determined as described in the Materials and Methods section. Green Highlights: Conserved amino acids involved in nucleotide binding. Blue Highlights: Conserved amino acids involved in arginine binding. Yellow Highlights: Indicate the 5 amino acid deletion for the Guanidine Specificity (GS) region. Underlining: Amino acids that make up the N (N-A) and C (G-V) terminal flexible loops.
A comparison of arginine and creatine kinase crystal structures (Figure 8) clearly demonstrates the similarity between these two enzymes by showing that their three-dimensional structure is nearly identical. Despite the similarities in their structures, monomeric arginine kinases share only a ~60% sequence homology to dimeric creatine and arginine kinases (Suzuki et al., 1999). This difference in sequence identity must, in part, account for the differences in quaternary structure between the monomeric and dimeric phosphagen kinases. In 2003, Cox et al. identified residues from the crystal structure of rabbit muscle CK (from both subunits) that might be involved in dimerization. Residues Glu 18, Asp 53, Gln 57, Asp 61, Ser 146, Arg 147, Arg 150, Arg 151, and Asp 209 are thought to form a bridge of polar interactions between the two subunits while Arg 147, Arg 151, and Asp 209 each make three salt bridges or hydrogen bonding interactions within the dimer interface (Cox et al., 2003). Monomeric arginine kinase from the cockroach, *P. americana*, does not contain any of these conserved amino acids thought to be involved in dimerization.

As stated previously, synergism or substrate binding cooperativity was thought to be associated with subunit-subunit interactions thereby giving significance to the dimeric state (Hornemann et al., 2000). In 2005 Fujimoto et al. suggested that cooperativity in the phosphagen kinases might be linked to certain amino acids. The amino acids Asp 62 and Arg 193 (numbering from *Limulus* AK) are highly conserved throughout arginine kinases. Site-directed mutagenesis by Fujimoto et al. (2005) led them to implicate these two residues in playing key roles in mediating synergism in substrate binding. Kinetic results from the cockroach, *P. americana*, show that no synergism in substrate binding
occurs for any substrate in the reversible reaction (Brown & Grossman, 2004). Results shown here from the protein sequence of the American cockroach reveal that residues Asp 62 and Arg 193 are conserved in *P. americana* and suggest that synergistic substrate binding may not necessarily accompany the presence of these amino acids (Held et al., 2007). Similar results were seen recently from monomeric arginine kinase from the beetle, *C. cephalotes*, which was also found to contain residues Asp 62 and Arg 193 while not exhibiting synergistic substrate binding (Tanaka et al., 2007).

The traditional view of phosphagen kinase evolution is currently being questioned. It was previously thought that monomeric arginine kinase was the most primitive phosphagen kinase, which evolved into the more advanced dimeric arginine kinases and creatine kinases (Moreland & Watts, 1967; Watts, 1971; Anosike et al. 1975). By looking at the phylogenetic tree in Figure 9 the relationships between protozoans, invertebrates, and vertebrates along with the phosphagen kinases that appear in each class become clear (Held, 2007). Monomeric arginine kinase from the cockroach would be placed on the insect branch of the tree which is most closely related to other monomeric arginine and phosphagen kinases. Dimeric AK from the sea cucumber would be placed on the branch containing other echinoderms which display both dimeric AK and CK enzymes. It is important to note that the phylogenetic tree places echinoderms as being more closely related to vertebrates than to any of the other invertebrates. Protozoan monomeric AK seems to be the most basal phosphagen kinase but it is thought that protozoans acquired AK from arthropod hosts through horizontal gene transfer (Pereira et al., 2000). If this is so then sponges, which contain both AK and CK
activities, would be the most basal organism on the phylogenetic tree and would imply that a primitive organism prior to the sponges must have contained both AK and CK activities.

A phylogenetic tree like the one in Figure 47, which is based off of the sequence alignments of various phosphagen kinases, can be used to examine the homology between sequences. The monomeric AK protein sequence determined for *P. americana* clusters with all of the other arthropod arginine kinases. Dimeric arginine kinases for the echinoderms cluster with creatine kinases from the echinoderms, vertebrates, and invertebrates. The tree confirms reports from Pereira et al. (2000) by showing that monomeric arginine kinase from the protozoan, *T. cruzi*, clusters with arthropod arginine kinases and suggests it did acquire the phosphagen through horizontal gene transfer. Interestingly, arginine kinase from the sponge, *Porifera*, clusters alone as the most basal organism on the phylogenetic tree while CK from the sponge clusters with the other dimeric creatine kinases.
Phosphagen kinases catalyze the reversible phosphorylation of guanidino compounds using ATP. The enzymes that make up this family are key regulators of energy metabolism by acting as ATP buffering systems through maintaining ATP homeostasis during increased or fluctuating energy demands. There are still many interesting questions regarding the structures, functions, and regulation of the phosphagen kinases to be answered. Arginine kinase is a member of the phosphagen kinase family and can occur as a monomeric or dimeric protein. This study takes a look at monomeric arginine kinase, *Periplaneta americana*, from the cockroach and dimeric arginine kinase, *Isostychopus badonotus*, from the sea cucumber to compare and contrast physical, kinetic, immunological, and molecular characteristics of these enzymes with each other and with other members of the phosphagen kinase family.

Arginine kinase from the sea cucumber *I. badonotus* was purified to homogeneity and found to be a dimer of ~80 kDa. In contrast, arginine kinase from the cockroach *P. americana* was purified previously by Brown et al. (2004) and found to be a monomer of ~40 kDa. While the enzymes utilize the same substrate to catalyze the reversible reaction, they do not share the same quaternary structure. Dimeric arginine kinases, like the vertebrate creatine kinases, are made up of two nearly identical polypeptide chains each containing one active site for enzyme catalysis. An unanswered question of the phosphagen kinases is why some of these enzymes have quaternary structure and what is the functional significance, if any, to their dimeric state?
Although the phosphagen kinases are highly similar in primary and tertiary structures, the distinct quaternary structures exhibited by some of the phosphagen kinases are unclear given that there is no evidence of active site communication between the subunits (Bickerstaff & Price, 1978; Morrison & James, 1965). Many studies have attempted to answer the question regarding the significance of the dimeric state by looking at the enzymatic ability of individual subunits in a dimer. Cox et al. (2003) mutated several amino acids at the dimer interface and concluded that dimerization was not a prerequisite for activity but loss of structure and stability upon formation of a monomer did occur. More recently, Awama et al. (2007) opposed this view by stating that activity in CK was dependent on its dimeric state and that it was required for the proper stabilization of the monomers. Clearly the debate regarding the significance of quaternary structure in the phosphagen kinases is still ongoing in the literature and worth examining further.

Many of the monomeric arginine kinases are contained in arthropods and several of these invertebrate proteins have proven to be significant allergens in humans (Crespo et al., 1995; Binder et al., 2001; Gore & Schal, 2007). Arginine kinase from \textit{P. americana} has been identified as one of these allergens and is known for eliciting an allergic response in humans, causing asthma especially to sensitized individuals living in urban settings (Bennet & Spink, 1968). The antigenicity of AK from \textit{I. badonotus} and \textit{P. americana} was investigated by obtaining polyclonal antibodies made against each enzyme. Dimeric arginine kinase from the sea cucumber reacted with other dimeric arginine kinases, dimeric creatine kinases, and one large two-domain monomer.
Monomeric arginine kinase from the cockroach only showed cross-reactivity with other monomeric arginine kinases. Although the substrate specificity of the two enzymes is the same, their difference in quaternary structure seems to account for the variation in their antigenicity towards each other and other phosphagen kinases.

Kinetic comparisons of the two arginine kinases show that they both follow the same rapid equilibrium, random addition mechanism, have a narrow substrate specificity, and have low dissociation constants for the substrate arginine (Brown et al., 2004; Brown & Grossman, 2004). These results are consistent with reports from other arginine kinases but differ when compared to creatine kinases (Morrison & James, 1965). Creatine kinases tend to have a much higher dissociation constant for their guanidino substrate creatine. This shows that although sea cucumber AK shares the same quaternary structure as CK, the specificity of its substrate has the greatest impact over its kinetic characteristics. Arginine kinases from *I. badonotus* and *P. americana* are both absent of substrate binding cooperativity. This result also contrasts the creatine kinases which all exhibit synergism to varying degrees. The display of substrate cooperativity had been suggested to be associated with subunit-subunit interactions, which would ascribe significance to the dimeric state (Hornemann et al., 2000), however, results such as the data reported here do not support these conclusions.

The initial velocities of monomeric and dimeric arginine kinase from the cockroach and the sea cucumber are affected by monovalent anions in a similar manner. The acetate anion has an activation affect while nitrate displays a profound inhibitory affect on both enzymes. Findings of inhibition by monovalent anions are seen across the
phosphagen kinase family but arginine kinases are more affected by nitrate while creatine kinases are more affected by nitrite (Milner-White & Watts, 1971, Morrison, 1973; Buttlaire & Cohn, 1974). A kinetic analysis performed by Brown & Grossman (2004) determined that nitrate was a non-competitive inhibitor of cockroach AK while another monovalent anion, borate, was a competitive inhibitor of the enzyme. These results along with progress curves of initial velocity and product formation in the presence of these anions led the authors to conclude that nitrate inhibited AK through a mechanism that was more complex than just stabilization of the dead-end complex while borate inhibited through dead-end complex stabilization and formation of a transition state analog.

Difference spectra of P. americana and I. badonotus in the presence of nitrate and borate were performed and compared. Nitrate had an affect on each enzyme without the presence of other components of the transition state analog, while borate had the greatest impact in the presence of the transition state analog. These results support the theory by Brown & Grossman (2004) which suggest borate is the best transition state analog inhibitor of AK. Insects are considered to be pests to homes, as well as crops, and arginine kinase is ideal as a potential target for insect population control because it is essential for the organism’s motility. Boric acid has been used as an inexpensive and low risk chemical to control insect infestations, particularly for the cockroach (Zurek et al., 2003). Additional results, reported in the present study, on the affects nitrate and borate have on arginine kinase suggest a mixture containing both of these anions might be an effective treatment for population control to be examined.
Dimeric arginine kinases have been shown to be more homologous in sequence and structure to creatine kinases than to monomeric arginine kinases (Suzuki et al., 1999; Suzuki et al., 2000). Determination of the amino acid sequence for arginine kinase from cockroach, *P. americana*, reveals that despite their differences in quaternary structure, dimeric and monomeric arginine kinases exhibit several of the same amino acids, presumably due to their substrate specificity. Arginine kinase from the cockroach has amino acids conserved in a majority of known sequences for nucleotide and arginine binding as well as a 5 amino acid deletion region unique to all arginine kinases (Zhou et al., 2000; Uda & Suzuki, 2004). On the other hand, the sequence from the cockroach contains amino acids conserved throughout all of the phosphagen kinases which are important to their catalytic mechanism, highlighting the close evolutionary relationship between this family of enzymes (Eder et al., 2000; Zhou et al., 2000; Cantwell et al., 2001).

The purpose of the present study was to compare and contrast monomeric arginine kinases and dimeric creatine kinases with results found for dimeric arginine kinase from *I. badonotus* in order to investigate the significance of the dimeric state. While the advantage of quaternary structure seen in some arginine and creatine kinases remains unclear, several characterizations not previously seen in the literature for dimeric arginine kinases are reported herein. Similarly, further characterization of arginine kinase from the American cockroach *P. americana* has been performed including the elucidation of the entire 5’ to 3’ amino acid sequence of the protein.
List of References


Blethen, S. L. (1972). Kinetic properties of the arginine kinase isoenzyme of *Limulus polyphemus*. *Archives in Biochemistry and Biophysiology* 149, 244-251.


distribution of phosphagens in primitive-type spermatozoa. *Biological Bulletin*
195, 264-272.


enzymology of creatine kinases. Isolation and characterization from chicken and
rabbit tissues. *Journal of Biological Chemistry* 242, 204-209.

allow monitoring at 405 nm. *Analytical Biochemistry* 182, 399-404.

Florini, J. R., & Vestling, C. S. (1957). Graphical determination of the dissociation
constants for two-substrate enzyme systems. *Biochimica et Biophysica Acta* 25,
575-578.

from Ox smooth muscle. Anion effects compared with pyruvate kinase.


and hydrodynamic properties of arginine kinase from Gulf shrimp (*Penaeus aztecs*). *Archives of Biochemistry and Biophysics* 345, 73-78.


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