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The Active Site Cysteine of Arginine Kinase: Structural and Functional Analysis of Partially Active Mutants

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THE FLORIDA STATE UNIVERSITY
COLLEGE OF ARTS AND SCIENCES

THE ACTIVE SITE CYSTEINE OF ARGININE KINASE: STRUCTURAL AND
FUNCTIONAL ANALYSIS OF PARTIALLY ACTIVE MUTANTS

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ABSTRACT

Arginine kinase buffers cellular ATP levels by catalyzing reversible phosphoryl transfer between ATP and arginine. A conserved cysteine has long been thought important in catalysis. Here, cysteine 271 of horseshoe crab arginine kinase has been mutated to serine, alanine, asparagine, or aspartate. Catalytic turnover rates were 0.02-1.0% of wild type, but the activity of uncharged mutations could be partially rescued with chloride. Steady state binding constants were slightly increased, more so for phospho-L-arginine than ADP. Substrate binding synergy observed in many phosphagen kinases was reduced or eliminated in mutant enzymes. The crystallographic structure of the alanine mutant at 2.3Å resolution, determined as a transition state analog complex with arginine, nitrate, and MgADP, was nearly identical to wild-type. Enzyme-substrate interactions are maintained as in wild-type, and substrates remain at least roughly aligned for in-line phosphoryl transfer. Homology models with serine, asparagine, or aspartate replacing the active site cysteine similarly show only minor structural changes. Most striking, however, is the presence in the C271A mutant crystallographic structure of a chloride ion within 3.5Å of the non-reactive N₇ substrate nitrogen, approximating the position of the sulfur in the wild-type's cysteine. Together the results contradict prevailing speculation that the cysteine mediates a substrate-induced conformational change, confirm that it is the thiolate form that is relevant to catalysis, and suggest that one of its roles is to help enhance the catalytic rate through electrostatic stabilization of the transition state.

INTRODUCTION

The Catalysis of Phosphagen Kinases

Arginine kinase is a member of the phosphagen kinases family of enzymes, which catalyze the reversible transfer of the terminal phosphoryl group of ATP to a phosphagen substrate. A variety of phosphagens and phosphagen kinases are used in nature to buffer cellular ATP concentration in cells with fluctuating energy needs. Phosphagen kinases supplement metabolic processes in these cells to maintain optimal energy of hydrolysis of ATP. Phosphagen substrates vary significantly in size as well as electrostatic properties but all share a terminal guanidinium group to which the γ -phosphoryl of ATP is transferred (Ellington 2001). The reaction catalyzed by arginine kinase is:

The phosphagen kinase family displays at least 40% sequence homology between the most distant members (Suzuki et al. 1999), and most have subunits with a molecular mass near 40kDa. A variety of multimeric forms of phosphagen kinases have been observed in nature including monomeric and dimeric forms of arginine kinase, dimeric and octameric forms of creatine kinase, and dimeric forms of lombrocine kinase. *Limulus polyphemus* arginine kinase, the focus of the current study, functions as a monomer.

Phosphagen kinases have been the focus of many structural and biochemical investigations over the last 40 years. Classical enzymology and spectroscopic techniques have provided a vast foundation for understanding phosphagen kinases. However, important details of catalysis have remained elusive despite numerous kinetic studies and several crystal structures. Phosphagen kinases catalyze a direct, in-line phosphoryl transfer between ADP or ATP and one of several guanidinyll group containing substrates (Hansen and Knowles 1981). Substrates bind and products release sequentially in

random order with rapid equilibrium of enzyme:substrate and enzyme:product complexes (Blethen 1972). NMR studies on both arginine kinase and creatine kinase give evidence that conformational changes required by these enzymes are rate limiting (Rao et al. 1976).

Figure 1. The arginine kinase reaction scheme. Kinetic dissociation constants $K_{\text{substrate}}$, and the rate limiting step (k_{cat}) occurs at the chemical step of phosphoryl transfer.

Crystal structures of both arginine kinase (Zhou et al. 1998; Yousef et al. 2003) and creatine kinase (Fritz-Wolf et al. 1996; Lahiri et al 2002) in open and closed conformations have shown extensive conformational changes accompanying substrate binding in both enzymes. Analysis of conformational changes in arginine kinase between the native substrate free structure and the transition state form, identified three dynamic domains which move relative to a fourth domain upon formation of the transition state analog complex (Yousef et al. 2003).

Two extreme views of the reaction mechanism have been proposed for the in-line phosphoryl transfer catalyzed by phosphagen kinases. By a dissociative mechanism, bond breakage at the leaving group would precede bond formation by the incoming nucleophile, resulting in a fully dissociated meta-phosphate transition state (Knowles 1980). The dissociative mechanism might occur with retention of the stereochemistry of the reactant molecules. Alternatively, an associative mechanism might occur if bond formation from the incoming nucleophile preceded bond dissociation at the leaving group. The transition state would then involve a pentavalent phosphoryl with partial bonds to both the incoming nucleophile and the leaving group. The associative mechanism proceeds via complete inversion of configuration of the transferred phosphoryl (Cullis 1987). Experimental evidence is unclear, but suggests that the

mechanism is likely to be a hybrid between the two extremes with bond dissociation and bond breakage occurring near-simultaneously (Knowles 1980; Lahiri et al. 2003a).

Recently a crystal structure of β -phosphoglucomutase was determined in which the enzyme appeared to stabilize a high energy oxyphosphorane intermediate, similar to the proposed transition state of the phosphagen kinase reaction (Lahiri et al. 2003b). β -phosphoglucomutase catalyzes a reversible phosphoryl transfer between the C(6)O and C(1)O positions of hexose phosphates starting and ending with a phosphorylated aspartate side chain on the enzyme. The chemical mechanism of phosphoryl transfer is thought to occur via the same in-line geometry as the reaction catalyzed by phosphagen kinases. The structure determination of β -phosphoglucomutase revealed a penta-coordinate phosphoryl that was equally separated from the leaving and attacking groups. The P-O distance to the leaving group and nucleophile were shorter than expected in a fully dissociated transition state, but were longer than a fully associative transition state (Knowles 2003). Whether or not the structure represents an actual transition state, or a stabilized reaction intermediate has not been firmly established, but the structure does provide the first crystallographic information about the chemical reaction mechanism of phosphoryl transfer.

Depending on the associative or dissociative character of the reaction mechanism, the transferred phosphoryl is arranged in either trigonal bipyramidal, or planar geometry with three oxygen atoms separated by 120° at the transition state. The transition state analog components used for structural studies of arginine kinase include two natural substrates, arginine and magnesium bound ADP, along with the non-covalently bound analog nitrate, similar in size and shape to the transferred phosphoryl at the transition state.

A variety of chemical modification and mutagenesis studies have given insight into the mechanism of phosphagen kinase catalysis by disrupting optimal enzyme function in known ways, demonstrating the importance of conserved glutamate and cysteine residues, among others, in phosphagen kinases (Price and Hunter 1976; Maggio and Kenzyon 1977; Furter et al 1993; Strong and Ellington 1996; Hornemann et al. 2000). In 1996 the first phosphagen kinase structure was published of octameric Mi-CK

in an "open" or substrate free conformation (Fritz-Wolf et al. 1996). Solution scattering, fluorescence studies, and crystallographic structures of arginine kinase and creatine kinase have shown that Mg^{2+} is required in complex with nucleotide to induce conformational changes. Additional, smaller conformational changes can result from binding the phosphagen substrate, although the structural effect of binding the phosphagen substrate alone is unknown (Lahiri et al. 2002). Crystallographic structures of phosphagen kinases in complex with MgADP along with nitrate and phosphagen substrate to form a transition state analog complex, are considered "closed" forms of phosphagen kinases. The crystallographic transition state analog structure at 1.86Å determined by Genfa Zhou in 1998 (Zhou et al 1998) was the first phosphagen kinase structure in the "closed" form. This structure includes the enzyme and two substrates

bound in the active site along with nitrate. Bound precisely between the two substrates for reactions in opposite directions, nitrate is a nearly perfect structural analog of the transferred phosphoryl group at the transition state of the reaction. (Figure 2).

Figure 2 Part of the active site of the wild type arginine kinase transition state analog complex showing substrates and selected active site residues that interact with the guanidinium of substrate arginine.

The transition state analog structure of arginine kinase was the first to demonstrate the precision of substrate alignment achieved by phosphagen kinases. Substrate molecules and nitrate are held in position only through non-covalent interactions between substrates and the enzyme. Angles between groups that would be reacting in the true transition state are very close to ideal, within 4° of optimal angles (Zhou et al. 1998). The “closed” transition state analog structure of arginine kinase provides a better tool for understanding catalysis than structures of the native enzyme or single substrate complexes because achieving the transition state requires ideal enzyme: substrate interactions. Analysis of the transition state analog structure allows visualization of exactly which enzyme: substrate and substrate: substrate interactions occur at the highest energy point of the reaction where the enzyme is thought to bind substrates most effectively.

Chemical Modifications of the active site cysteine

Many chemical modification studies have been performed on creatine kinase, and fewer on other phosphagen kinases including lombricine kinase and arginine kinase (Watts and Rabin 1962; Virden et al. 1965; Watts and Moreland 1970). These studies were the first to identify a conserved cysteine residue involved in catalysis of several different phosphagen kinases. The homologous residue in *Limulus* AK is C271 and is located in the active site (Zhou et al 1998). Chemical modifications that involved addition of small non-polar groups to the active site cysteine resulted in an enzyme with some residual activity (Maggio and Kenyon 1977). Rabbit muscle CK was modified by addition of a -S-CH₃ group by methanethiolsulfonate to the thiol of C283 (Maggio and Kenyon 1977). S-CH₃ modified enzyme was about 20% active and exhibited K_M similar to native CK. Although formation of the ternary complex was not significantly affected by modification, characteristic substrate binding synergy was lost. Native CK exhibited substrate binding synergy with K_M lower than K_{ia}, but modified CK showed a decrease in K_{ia} with little change in K_M and a reduction of catalytic activity by approximately 80% (Maggio and Kenyon 1977). The observed loss of substrate binding synergy was hypothesized to involve blockage of a required conformational change by this chemical

modification. The presumed conformational change in the native enzyme would move important functional groups of the enzyme to improve binding of the second substrate and increase catalysis.

The study by Maggio et. al. was particularly interesting because the enzyme retained enough activity for kinetic analysis, although, it was difficult to rule out the possibility of incomplete binding of the modifying agent (Maggio and Kenyon 1977), (Furter et al. 1993). More current studies focus on chemical modifications that could potentially occur in vivo. Glutathione is found in oxidized and reduced forms in cells, and the ratio of oxidized/reduced or GSSH to GSH can change when cells are exposed to conditions of oxidative stress (Reddy et al. 2000). It has been shown that GSH can reversibly form a covalent disulfide bond with the thiol of the active site cysteine of creatine kinase, and that modification is reversible with removal of the modifying group accomplished by varying the ratio of GSH to GSSH (Reddy et al. 2000). Covalent attachment of glutathione to the active site cysteine resulted in complete loss of measurable enzyme activity. Since this inactivation is reversible and accomplished by varying the concentration of common cellular compounds, it was hypothesized that it could occur in vivo as a protective measure for a cell to avoid irreversible creatine kinase inactivation (Reddy et al. 2000).

The active site cysteine of creatine kinase is also a target of the fungal toxin gliotoxin, found in many infectious fungi (Hurne et al. 2000). Incubation of dimeric rabbit muscle creatine kinase with gliotoxin resulted in formation of a disulfide that inactivated CK (Hurne et al. 2000). Recent evidence suggests there may be significant physiological roles for the active site cysteine in regulating activity (Hurne et al. 2000), (Reddy et al. 2000). Structural studies of mutations of this residue in arginine kinase will extend current understanding of phosphagen kinase catalysis by demonstrating the extent of conformational changes resulting from substitution of this residue.

While chemical modification studies gave significant evidence that a single cysteine residue per subunit was important to phosphagen kinase activity, these studies were plagued by an inability to quantify completeness of the modification reactions

(Furter et al 1993; Hornemann et al 2000), and led to use of the more thorough and specific technique of site directed mutagenesis when the technology became available.

Mutagenesis of the active site cysteine of creatine kinase

The residual partial activity remaining after some chemical modifications could be interpreted either as evidence that the active site cysteine was not essential to catalysis, or that chemical modification was incomplete. Such ambiguities motivated studies using site directed mutagenesis as the technology became available. Cysteine 278 of octameric chicken cardiac mitochondrial creatine kinase (Mi-CK) was replaced by serine, alanine, glycine, aspartate, and asparagine (Furter et al. 1993). Kinetic measurements of wild type and mutants revealed that some residual activity remained in mutant enzymes. Among the mutants, C278G was the most active. At low pH this mutant showed about 50% of optimal wild type turnover. Addition of chloride specifically activated creatine kinase mutants with uncharged side chains, while slightly inhibiting the wild type and C278D turnover (Furter et al. 1993).

Many phosphagen kinases exhibit a form of cooperative behavior known as substrate binding synergy. Substrate binding synergy involves changes in the affinity of a bimolecular enzyme for one particular substrate, dependent on whether or not the other substrate is already bound by the enzyme. Substrate binding synergy is described by the coefficient $\alpha = K_M / K_{ia}$. When $\alpha < 1$ prior binding of a substrate improves binding of the second substrate. When $\alpha = 1$, binding of one or the other substrate does not affect binding of the second substrate, and $\alpha > 1$ suggests that prior binding of one substrate actually decreases the affinity of the enzyme for the second substrate. Favorable substrate binding synergy was found for wild type Mi-CK, with K_{ia} higher than K_M ($\alpha < 1$). Mutants C278G and C278S showed that binding of the first substrate was slightly improved by mutation; K_{ia} values were actually lower for mutants than wild type. K_M is an apparent kinetic dissociation constant for dissociation of a substrate from the active ternary complex. K_M was increased relative to K_{ia} in both C278G, and C278S, leading to the loss of substrate binding synergy. Thus in Mi-CK mutants, binding of the first

substrate had a slightly unfavorable effect on binding the second substrate (α was greater than 1) (Furter et al. 1993).

Kinetic analysis revealed that the active site cysteine of Mi-CK is likely to function in a thiolate form since addition of chloride partially rescued mutants that did not have a negatively charged side chain in this position. After detailed kinetic analysis of creatine kinase mutants indicated that this residue is not essential for creatine kinase catalysis, but that it is essential for synergistic substrate binding. The authors, aware of substrate induced conformational changes in phosphagen kinases, speculated that the active site cysteine must be required for a conformational change.

Recently, the active site cysteine of cytosolic chicken creatine kinase was replaced with serine (Hornemann et al. 2000). In the cytosol, creatine kinase functions as a dimer, and multiple creatine kinase genes are expressed in different tissue leading to formation of distinct homodimers and heterodimers. Individual creatine kinase subunits are identified as M or B, descriptive of the tissue type from which they were originally isolated (M = muscle, B = brain). Subunits can combine to form homo or heterodimers. In order to better understand intersubunit cooperativity, creatine kinase was recombinantly expressed as BB, MM, or MB. To evaluate the role of the active site cysteine in this intersubunit, or heterotropic cooperativity, the active site cysteine was replaced by serine in each isoform and dimers were purified in which one subunit contained an active site cysteine and the other did not. Dimers in which both subunits had active site cysteines replaced by serine showed about 2.5% of wild type activity, similar to the level of residual activity remaining after mutation of the corresponding residue in Mi-CK. However, heterodimers formed from one native and one mutant subunit yielded only 30-45% of wild type activity, not slightly more than 50% as expected from a dimer composed of one completely active and one partially active subunit. Heterodimers also displayed complex changes in substrate binding synergy. Mutagenesis of chicken cytosolic creatine kinase demonstrated that dimeric CK exhibits both intrasubunit cooperativity (substrate binding synergy) as well as intersubunit cooperativity. Arginine kinase is stable as a monomer in solution and therefore is not capable of producing intersubunit cooperativity making AK an ideal model system for

studying the details of (intrasubunit) substrate binding synergy in the phosphagen kinase family.

pK of the active site cysteine of creatine kinase

Spectroscopic and enzyme kinetic experiments comparing native and cysteine mutant creatine kinase, have been used to determine the pK of the active site cysteine. Enzyme kinetic measurements over a range of pH values for the wild type enzyme and creatine kinase with serine substituting the active site cysteine revealed the loss of a kinetic pK at pH 5.4. The pK of the active site cysteine was also determined by measuring absorbance at 240nm as a function of pH in the wild type and the mutant with serine replacing the active site cysteine. Thiol (S-H) groups absorb strongly at this wavelength, whereas thiolate absorbance is minimal. By measuring absorbance at 240nm over a range of pH values the authors measured the loss of an inflection point at a pH of 5.6 +/- 0.1 in a graph of A_{240} vs pH for C282S relative to the wild type.

Figure 3. The two structural domains of arginine kinase as seen in the transition state analog complex.

Calculated pK values for the active site cysteine of arginine kinase based on the transition state analog complex compare well with those measured for creatine kinase (Eliza Ruben personal communication). However, pK calculations based on crystal structures of arginine kinase suggest the reduction in pK is primarily a result of binding the phosphagen substrate as the pK calculated for the active site

cysteine drops from 7.5, using the substrate free crystal structure, to 6.1 in the transition state structure.

The transition state structure of arginine kinase showed that arginine kinase was composed of two structural domains similar to those previously observed in a substrate free structure of creatine kinase (Zhou et al. 1998). Residues 1-95 in arginine kinase constitute the small domain, while residues 105-357 form the large structural domain. (Figure 3)

The structure of wild type arginine kinase offered the first chance to clearly visualize a fully formed active site of a phosphagen kinase. The view pictured in figure 2 shows two glutamate residues in position to act as acid/base catalysts and cysteine 271 positioned near the non-reactive nitrogen of the guanidinium group (Zhou et al. 1998). Although C271 is not in position to abstract a proton from the reactive nitrogen, the side chain of C271 does make contact with the guanidinium of the substrate arginine at the non-reactive nitrogen. In this location, it could still act to remove a proton from the guanidinium, or stabilize a particular electrostatic configuration of the transition state. Thus, from the transition state wild-type structure a catalytic role of the active site cysteine cannot be ruled out. The structure of AK-TSAC is consistent with the hypothesis that C271 is a thiolate in the active site of arginine kinase as postulated for creatine kinase (Zhou et al 1998). The proximity of sulfur of C271 to the partial positive charge of the substrate guanidinium, and the hydroxyl of T273, should stabilize a negatively charged thiolate and support an unusually low pK for this group.

In order to better understand the role of the active site cysteine residue in arginine kinase catalysis, the enzyme has been perturbed by site directed mutagenesis and examined by steady state enzyme kinetics, crystallographic structure, and optimized homology models. Steady state enzyme kinetics determined details of the effect of mutating the active site cysteine on arginine kinase catalysis, and the crystallographic structure of an active site cysteine mutant in the transition state conformation added conclusive evidence about the necessity of cysteine in mediating induced fit conformational changes.

METHODS

The present study was performed to provide information on the role of an active site cysteine of arginine kinase. In order to evaluate how the cysteine is involved in arginine kinase catalysis, this residue has been substituted by a variety of residues using site directed mutagenesis. The effects of four of these substitutions has been evaluated by steady state enzyme kinetics in order to measure changes in the overall rate of reaction catalyzed by each mutant enzyme, and to determine the effect on substrate binding relative to the wild type enzyme. Similar studies have been performed on the homologous enzyme creatine kinase in which an equivalent cysteine was substituted or chemically modified by covalent attachment of additional chemical groups to the sulfur of the active site cysteine. One major goal of the current study is to contribute experimental and theoretical structural information in order to better understand changes in the function of these mutant enzymes.

Primer synthesis and mutagenesis.

Oligonucleotide primers were designed to incorporate the desired mutations, and used to produce mutagenic DNA with the QuikChangeTM mutagenesis kit (Stratagene, Inc.). Single amino acid substitutions were accomplished by 1-3 nucleotide replacements for each amino acid substitution. Mutant plasmid DNA was transfected into commercially produced BL21-DE3 *E. coli* competent cells (Stratagene, Inc.).

After transfection cells were plated on agar plates with ampicillin and colonies were grown by incubation at 37°C overnight. A single colony was selected and a 5ml bacterial cultures was incubated at 37°C for 5 hours in Luria broth (LB) media with 1mM ampicillin. After 5 hours, 700 µl of cultures were removed and added to 300 µl of sterile

glycerol and mixed. 30% glycerol stocks were then frozen at -80 °C and used for subsequent protein expression. This process was repeated for each mutant plasmid.

Expression and Purification

After mutagenic primers were synthesized and used to incorporate specific mutations to the arginine kinase gene sequence, each mutant enzyme had to be recombinantly expressed in *E. coli* and purified in a soluble, active form. Mutants C271S, C271A, C271N, C271D, C271G, C271T, C271M and C271Y were expressed in BL21-DE3 *E. coli* and purified from insoluble inclusion bodies using published methods (Zhou et al., 1997). Each side chain substitution had its own merits. The serine substitution represented the most conservative mutation possible, with sulfur of cysteine replaced by oxygen of similar size and chemical properties. The alanine mutant was included to test the effect of simply deleting sulfur from the side chain. The aspartate substitution was performed to replace cysteine with a side chain which was negatively charged at neutral pH, and due to the increased size of aspartate, asparagine was substituted to calibrate the effect of the increased size of aspartate. The glycine mutant was expressed predominately for comparison with a creatine kinase C278G mutant which gave nearly 50% of wild type activity at reduced pH (Furter et al 1993). The threonine substitution added additional bulk and hydrophobic character at this position. The methionine substitution was similar to the effect of methyl-thiosulfonate modifications on creatine kinase which resulted in residual catalysis, and the tyrosine substitution was produced by an accident of molecular biology where the mutagenic primer for threonine was mistakenly replaced by the codon for tyrosine. All expression was performed in LB media with 1mM ampicillin (LB:amp) to select for *E. coli* that contained the pET vector which expresses beta-lactamase to confer resistance to ampicillin. 50ml cultures of sterile LB:amp media were inoculated with a sterile loop and incubated at 37 °C overnight. Overnight cultures were added to 950ml of sterile LB:amp and incubated at 37 °C until the absorbance, measured at 600nm, was 0.4 - 0.6 indicating exponential growth. Isopropyl thiogalactoside (IPTG) was then added, at a final concentration of 1mM, to induce expression of arginine kinase. Induction continued at 37 °C for 5 hours before cells were centrifuged at 6, 000 g for 20 minutes. Cell pellets were resuspended in

about 40ml of 50mM Tris with 1mM DTT and 1mM EDTA at pH 8. Cells were lysed at high pressure using either a microfluidizer to provide high pressure nitrogen, or a French pressure cell. Insoluble cellular debris including cell walls, membranes, and insoluble inclusion bodies were isolated from soluble cellular components by centrifugation at 10,000 g for 30 minutes, supernatant was discarded. Inclusion bodies were then resuspended in 40ml of washing solution consisting of 1M urea, 2 % triton X-100, 50mM Tris, pH 8. The purpose of the washing solution was to separate soluble protein and nucleic acids from the insoluble arginine kinase in inclusion bodies. Inclusion bodies were resuspended in washing buffer followed by centrifugation at 10,000 g for 25 minutes. This process was repeated a total of three times with the washing buffer and a forth “rinse” step was performed using 10mM Tris, 10mM KCl, 1mM EDTA and 1mM DTT at pH 8, in place of the washing buffer. Insoluble protein was then solubilized by addition of 10ml of buffer containing 8M urea, 10mM Tris, 1mM DTT, 10mM KCl at pH 8. This 8M urea buffer completely solubilized protein from inclusion bodies, but not cell walls or cell membranes. The denatured solution was incubated at room temperature for 1 hour, then centrifuged at 10, 000 g for 1 hour. The supernatant after centrifugation was filtered through a 0.2 μ m filter, and insoluble debris was discarded.

At this stage, arginine kinase and contaminating protein were in a denatured, or unfolded form. Purification of active site cysteine mutants of arginine kinase continued with a series of chromatographic steps that were controlled by either an AKTA FPLC at medium pressure, or a Gradifrac at low pressure. First, a S300 size exclusion step was performed on protein in the unfolded state in a solution with 6M urea, 10mM Tris, 10mM KCl, 1mM DTT pH 8. Fractions were chosen that contained arginine kinase as determined by SDS-PAGE.

Following an initial gel filtration step, arginine kinase was refolded by removal of urea from the protein solution. Refolding was accomplished by either sequential dialysis in buffers with reducing urea concentration, or dilution of the protein directly into buffer with 10mM Tris, 10mM KCl, 1 mM EDTA, and 10mM DTT pH 8. The sequential dialysis method (Zhou et al 1997) began by diluting pooled fractions containing arginine kinase following the S300 column, in a buffer with 6M urea. The final protein

concentration was 0.2 mg/ml in buffer containing 6M urea, 10mM Tris, 10mM KCl, 1mM DTT, and 1mM EDTA.

Throughout the refolding and purification concentration was measured by UV absorbance as follows. In non denaturing conditions the extinction coefficient 0.76 ml/mg(cm^{-1}) was used (Zhou et al 1997). In denatured solutions an extinction coefficient of 0.6 ml/mg(cm^{-1}) as calculated from the amino acid sequence (Zhou et al 1997). The dilute protein solution was added to dialysis tubes and the solution was placed in a 4 liter beaker with a magnetic stir bar. Dialysis began with approximately 500-1000ml of protein solution against 3 liters of buffer with 4M urea and 0.25% v/v β -mercaptoethanol replacing DTT. All dialysis steps were performed at 4 °C for at least 4 hours with gentle stirring by a magnetic stir bar. After dialysis against buffer with 4M urea, dialysis tubes were transferred to buffer containing 2M urea. This sequential dialysis process was repeated with solutions containing 0.5M urea, and finally two dialysis steps against buffer with no urea. The resulting solution was filtered through a 0.2 μ m filter and concentrated to a volume of 10-13 ml.

An alternative to the previously published protocol for refolding arginine kinase was employed for wild type and C271A arginine kinase. This procedure involved rapid dilution of a small volume of denatured arginine kinase. Fractions containing arginine kinase in 6M urea buffer were diluted into a large volume of 10mM Tris, 10mM DTT, 1mM EDTA, and 10mM KCl. Dilution was performed such that the final protein concentration was less than 0.1 mg/ml, and the final urea concentration was less than 500mM. Additional DTT was added during refolding to ensure complete reduction of cysteine residues. In the dilution method of refolding, protein was added to buffer which was equilibrated to room temperature, but upon addition of protein, the dilute protein solution was incubated over night at 4 °C with gentle stirring by a magnetic stir bar. After incubating overnight, the solution containing refolded arginine kinase was filtered through a 0.2 μ m filter and concentrated to a volume of 10-13ml. Urea remaining in solution after refolding by dilution was separated from arginine kinase during the subsequent gel filtration chromatographic step.

Concentrated arginine kinase was filtered again through a 0.2 μ m filter then loaded on an S100 column, for additional separation by size exclusion. Fractions

containing arginine kinase were dialyzed against buffer with no added salt (10mM Tris, 1 mM DTT, 1mM EDTA pH 8) and then subjected to a final chromatographic purification using a DEAE anion exchange column with a KCl gradient to elute. Arginine kinase bound the DEAE column equilibrated with 10mM Tris, 1mM DTT, and 1mM EDTA at pH between 8 and 8.2. The protein was eluted using an elution buffer which was identical to the loading buffer with the exception of 1M NaCl. The gradient used to elute arginine kinase increased salt concentration from 0 to 100mM over 4 column volumes. Arginine kinase eluted from the DEAE column at approximately 70mM NaCl. Following the gradient, salt concentration was increased directly to 1M in order to wash the column. Following the DEAE column, arginine kinase samples were exceptionally pure as judged by SDS-PAGE.

Enzyme kinetics

Steady state enzyme kinetics were performed to evaluate the effect of mutation on steady state parameters

Active site cysteine mutant enzyme kinetics were performed in the “reverse” reaction direction of ATP formation. A procedure for the coupled assay was originally published in 1967 (Rosalki 1967). Protocols listed herein were adapted from those used in the laboratory of W. Ross Ellington and are presented with additional annotation to educate a reader who is unfamiliar with, but interested in performing enzyme kinetic investigations on phosphagen kinases. For final estimates of enzyme kinetic parameters, the data processing procedure has been updated from the prior double-reciprocal method using linear regression, to a more modern process using non-linear least squares methods to more accurately fit measured rates to the rate equation describing a rapid equilibrium bi bi enzyme mechanism (equation 1).

Monitoring Enzymatic activity

A convenient coupled assay involves monitoring change in absorbance associated with reduction of NADP⁺ to NADPH by glucose-6-phosphate dehydrogenase at 340 nm (Rosalki 1967) All enzyme kinetic measurements on wild type and active site cysteine

mutants were performed in the reverse reaction direction. The reaction catalyzed by arginine kinase produced ATP which was used as a substrate for hexokinase along with added glucose. The hexokinase reaction was in turn coupled to the glucose-6-phosphate dehydrogenase reaction which produced NADPH.

In the reverse reaction direction:

Preparation of Reagents

Several components of the coupled enzyme assay degrade with time and most stock solutions were prepared as needed for experiments performed in a single day. Described below are stock solutions used for enzyme kinetic measurements of wild type arginine kinase, followed by protocols for standardizing phosphoarginine concentration (Table 2), and testing for contaminating enzyme activity. The assay buffer used for enzyme kinetics was composed of 100 mM imidazole + 1mM DTT (pH 7 at room temperature), 1 liter stock solutions were made and used to dissolve or dilute reagents following in this list. The pH was adjusted by adding concentrated hydrochloric acid or glacial acetic acid. For assays in 60mM chloride, 6M hydrochloric acid was added volumetrically until the pH = 7. HCl added approximately 30mM chloride to the assay buffer, additional chloride was added in the form of solid KCl to a final concentration of 60mM for half of the trials of the wild type and mutant arginine kinase. For assays performed in 1mM chloride, the pH was adjusted with glacial acetic acid.

Table 1 Stock solutions used for enzyme kinetic analysis. All reagents were dissolved or diluted in assay buffer composed of 100mM Imidazole and 1mM DTT at pH 7.

60 mM Magnesium Acetate. 10ml stock solutions of 60mM magnesium acetate were prepared, enough for 200 reactions.
250 mM Magnesium Acetate. 10ml stock solutions.
200 mM D-glucose in assay buffer, 2.5 ml for 50 assays
50 mM NADP in assay buffer, 2.5 ml for 50 assays
10 mM phospho-L-arginine in assay buffer. 10 ml is enough for 50 assays. Enzymatic measurement of actual concentration is described below.
Coupling enzymes. 945 EU of hexokinase+ 250 µg glucose-6-phosphate dehydrogenase + assay buffer until the final volume is 2.5 ml.
Arginine kinase. 2,5 ml was prepared for 50 assays. For reactions measuring wild type activity a concentration of 0.2 mg/ml was used, and for less active mutants a concentration between 3-5 mg/ml was used. In either case, 50µl of enzyme solution was added to each reaction.
1.5 mM ADP in assay buffer. 30ml was prepared for 50 assays. The actual concentration of ADP was verified by measuring absorbance at 260nm. The millimolar extinction coefficient for ADP used was 15.4 mMcm ⁻¹ .

Table 2 Reagents added to a 3.5ml plastic cuvette for enzymatic phosphoarginine standardization.

Reagent	Volume
1.5 mM ADP	2.75 ml
200 mM D- glucose	0.05 ml
50 mM NADP	0.05 ml
Coupling enzymes	0.05 ml
250 mM Magnesium acetate	0.1 ml
Phosphoarginine stock (~10mM for wild type arginine kinase)	0.02 ml

Cuvettes containing the above reagents were mixed by inversion, and placed in the spectrophotometer. A_{340} was monitored until the baseline stabilized, then 5-15 μg of highly pure wild type arginine kinase was added. A_{340} was recorded until no further change was observed, about 3-6 minutes. The observed increase in absorbance at 340nm comes from NADPH reduced by glucose-6-phosphate dehydrogenase in the enzyme linked assay. NADPH and ATP are produced in a 1:1 stoichiometric ratio. The amount of NADPH produced was determined using the change in absorbance observed, and the millimolar extinction coefficient for NADPH at 340nm. The total change in absorbance is proportional to the amount of phospho-arginine available for reaction. A vast excess of ADP encouraged complete conversion of phosphoarginine, resulting in a maximum level of NADPH produced in the reaction.

Once substrate concentrations were standardized, samples were tested for contaminating enzymes. Some arginine kinase preparations have shown evidence of contamination with adenylate kinase (aka myokinase) which interferes with the coupled assay by converting $\text{ADP} + \text{ADP} \rightarrow \text{ATP} + \text{AMP} + \text{H}^+$. A simple test was performed to quantify myokinase activity in samples prior to measuring reaction rate. All components of the coupled enzyme assay were added to a standard 3.5 ml cuvette, with the exception of phosphoarginine. Change in absorbance at 340nm was monitored for 5 minutes at 25°C. Any increase in absorbance was attributed to contaminating enzyme activity since a required substrate was unavailable for the reaction catalyzed by arginine kinase. No myokinase activity was observed in wild type or cysteine mutant enzymes purified from inclusion bodies. Below is a description of the reagents used for testing myokinase activity in an arginine kinase sample.

Myokinase test: The following reagents were added to a 3.5 ml cuvette and mixed by inversion (Table 3). After the baseline stabilized, 0.05ml of arginine kinase was added and A_{340} was monitored for 5 minutes. Any activity that is measured by this test can be subtracted from activity measurements, but no myokinase activity was observed in the current study.

Table 3 Conditions used to test for contaminating myokinase activity.

Stock solution	Volume
Assay buffer	0.618ml
~1.5 mM ADP	1.6 ml
60mM Magnesium acetate	0.082 ml
200mM D- glucose	0.05 ml
50 mM NADP	0.05 ml
coupling enzyme dilution	0.5 ml
	NO PHOSPHOARGININE

Kinetic evaluation of activity

Prior enzyme kinetic evaluations have shown that the arginine kinase reaction mechanism involves sequential substrate binding in random order (Blethen 1972). Catalytic rate was monitored using the coupled assay described above, by measuring rate of product formation over six different combinations of each substrate. Velocity measurements were fitted to the Michaelis-Menton equation in a form appropriate for a random order bimolecular enzyme (equation 1). V_{max} along with K_{ia} and α for each substrate were determined by non-linear curve fitting performed in the program SigmaPlot. Each enzyme kinetic experiment was performed in triplicate with values averaged, and error estimates were determined as the standard deviation between three independent evaluations. K_M was determined from $\alpha * K_{ia} = K_M$.

Equation 1: Reaction equation for a rapid equilibrium, random order sequential substrate binding, bimolecular -bimolecular reaction.

$$v = \frac{V_{max} [\text{phosphoarginine}][\text{ADP}]}{\alpha (K_{ia(\text{phosphoarginine})} K_{ia(\text{ADP})} + K_{ia(\text{ADP})} [\text{phosphoarginine}] + K_{ia(\text{phosphoarginine})} [\text{ADP}] + [\text{phosphoarginine}][\text{ADP}])}$$

K_{ia} and K_M are apparent dissociation constants deduced from reaction rate measurements under variable substrate concentrations. K_M is the Michaelis constant which represents the concentration of a substrate which produces half maximal velocity, assuming that the second substrate is available at saturating levels. K_{ia} is an apparent

dissociation constant for a substrate assuming that the second substrate is extrapolated to zero concentration. In ideal cases, these kinetically measured dissociation constants are exactly the inverse of substrate binding constants. The overall scheme of the arginine kinase reaction is shown in Figure 1. Double-headed arrows indicate that each step in the reaction is reversible. Kinetic dissociation constants are shown above or next to the corresponding reaction. The enzyme turnover number, k_{cat} , is noted above the arrow designating the rate limiting step of the overall reaction. An increase in apparent dissociation constants, K_{ia} or K_M , corresponds to a decrease in affinity between enzyme and substrate in the complex specified by a particular constant. In the case of the wild type enzyme, prior determination of apparent dissociation constants by steady state enzyme kinetics have compared well with direct measurements determined by equilibrium binding experiments (Azzi et al. 2004).

Data collection

The change in absorbance at 340 nm was measured as a function of time for all combinations of 6 concentrations of each substrate for a total of 36 reactions. Reagents were prepared independently for each set of 36 reactions. The protocol below shows volumes of stock solutions used to prepare 36 individual reaction conditions containing all possible combinations of 6 concentrations of phosphoarginine and ADP. Each row represents a single reaction and the total volume for each reaction is 2.5ml.

Table 4 Reagents added for a 6X6 matrix of enzyme kinetic measurements.

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
2.157	0.043	0.05	0.05	0.05	0.05	0.05
2.132	0.043	0.05	0.05	0.05	0.075	0.05
2.082	0.043	0.05	0.05	0.05	0.125	0.05
2.007	0.043	0.05	0.05	0.05	0.2	0.05
1.907	0.043	0.05	0.05	0.05	0.3	0.05
1.757	0.043	0.05	0.05	0.05	0.45	0.05

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
2.104	0.044	0.05	0.05	0.05	0.05	0.1
2.079	0.044	0.05	0.05	0.05	0.075	0.1
2.029	0.044	0.05	0.05	0.05	0.125	0.1
1.954	0.044	0.05	0.05	0.05	0.2	0.1
1.854	0.044	0.05	0.05	0.05	0.3	0.1
1.704	0.044	0.05	0.05	0.05	0.45	0.1

Table 4 continued.

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
2.003	0.047	0.05	0.05	0.05	0.05	0.2
1.978	0.047	0.05	0.05	0.05	0.075	0.2
1.928	0.047	0.05	0.05	0.05	0.125	0.2
1.853	0.047	0.05	0.05	0.05	0.2	0.2
1.753	0.047	0.05	0.05	0.05	0.3	0.2
1.603	0.047	0.05	0.05	0.05	0.45	0.2

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
1.798	0.052	0.05	0.05	0.05	0.05	0.4
1.773	0.052	0.05	0.05	0.05	0.075	0.4
1.723	0.052	0.05	0.05	0.05	0.125	0.4
1.648	0.052	0.05	0.05	0.05	0.2	0.4
1.548	0.052	0.05	0.05	0.05	0.3	0.4
1.398	0.052	0.05	0.05	0.05	0.45	0.4

Table 4 continued.

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
1.388	0.062	0.05	0.05	0.05	0.05	0.8
1.363	0.062	0.05	0.05	0.05	0.075	0.8
1.313	0.062	0.05	0.05	0.05	0.125	0.8
1.238	0.062	0.05	0.05	0.05	0.2	0.8
1.138	0.062	0.05	0.05	0.05	0.3	0.8
0.988	0.062	0.05	0.05	0.05	0.45	0.8

Assay buffer (ml)	60 mM Magnesium Acetate (ml)	200mM Glucose (ml)	50 mM NADP (ml)	Coupling enzyme dilution (ml)	Phospho- arginine (ml)	ADP (ml)
0.568	0.082	0.05	0.05	0.05	0.05	1.6
0.543	0.082	0.05	0.05	0.05	0.075	1.6
0.493	0.082	0.05	0.05	0.05	0.125	1.6
0.418	0.082	0.05	0.05	0.05	0.2	1.6
0.318	0.082	0.05	0.05	0.05	0.3	1.6
0.168	0.082	0.05	0.05	0.05	0.45	1.6

Reagents were added to standard 3.5ml cuvettes, mixed by inversion, and reactions were performed in groups of six phosphoarginine concentrations with a fixed ADP concentration. Six cuvettes were placed in the spectrophotometer and equilibrated to 25°C for 5 minutes prior to addition of enzyme samples to start reactions. The spectrophotometer was blanked and 50ul of arginine kinase stock solution was added to start six reactions simultaneously. Change in absorbance was recorded for 5 minutes for wild type arginine kinase and 15 minutes for mutant arginine kinase.

Data processing

Initial estimates of steady state enzyme kinetic parameters were determined using double reciprocal, Lineweaver-Burke plots, followed by secondary plots and linear regression analysis to determine estimates of $V_{\max}$, K_M and K_{ia} (for each substrate). Secondary plots graphed $K_M/V_{\max}$ (slope from each line of the double reciprocal plot), and the inverse of $V_{\max}$ (Y-intercept from the double reciprocal plot) values versus the inverse of the second substrate concentration. Parameters were estimated following a single 6X6 set of assays to determine if changes needed to be made to substrate concentrations to cover a range of 0.5-5 K_M for each substrate.

Many commercially available software packages are available for fitting experimental data to an equation and determining a series of parameters by refining a least-squares fit of the data to a user defined equation. Raw rate measurements were converted from units of (A_{340}/min) to specific activity units of ($\mu\text{moles}/\text{min}\cdot\text{mg}$) prior to fitting to the rate equation. Unit conversion involved using the Beer-Lambert law and the molar extinction coefficient of NADPH at 340nm, the pathlength of the spectrophotometer used, and measured values of the change in absorbance of 340nm light. This converted the rate in units of A_{340}/min into $\mu\text{moles}/\text{min}$. Dividing by the amount of arginine kinase added to a single reaction, in milligrams, converted overall enzyme activity to specific activity.

Rate measurements were converted to units of specific activity and entered into SigmaPlot. Steady state parameters from the rate equation for a rapid equilibrium bimolecular enzyme mechanism (equation 1, see introduction), K_{ia} for each substrate, a , and $V_{\max}$ were fitted to the observed data from initial estimates based on previously determined values from the wild type enzyme. K_M for each substrate was then calculated from $K_M = aK_{ia}$. Output from SigmaPlot included the values of parameters determined from experimental data, and the standard error of the parameter. Steady state parameters were determined in triplicate for each enzyme sample, or assay condition. Triplicate values were then averaged to get a final value for a given parameter. Standard deviation was then calculated as the standard deviation between three independent determinations for each parameter. The above procedure is the standard method, found in the

phosphagen kinase literature, of determining enzyme kinetic parameters for this family of enzymes. In the final processing specific activity was converted to a turnover number, k_{cat} , in the units of events per second using Avagadro's number. Converting to k_{cat} allowed easier and more direct comparison with literature values from other phosphagen kinases and is a more commonly used unit of describing catalytic rate.

An alternative data processing strategy resulted in higher, but likely more accurate error estimates, with statistically insignificant changes in steady state parameters. Data from all independent evaluations for a given sample were not averaged, instead parameters were determined by fitting initial estimates against all 108 rate measurements at once using the rate equation. The error determined this way reflected not just the reproducibility of individual measurements, but also some of the systematic errors and inconsistencies between the measured data and the rate equation for a model reaction mechanism.

Crystallization

C271A was concentrated to 27mg/ml and dialyzed against components of the transition state analog complex (Zhou et al., 1998). Crystals were obtained using the hanging drop vapor diffusion method. Reservoir solutions contained 100mM $MgCl_2$ and 100mM HEPES in 18% or 20% polyethylene glycol molecular weight 6000. Hanging drops were formed by addition of 4 μ l of reservoir solution to 4 μ l of protein solution containing 27mg/ml C271A arginine kinase, 100mM $MgCl_2$, 100mM HEPES, 50mM nitrate, 4mM ADP, 20mM arginine, and 1mM DTT. All solutions used for crystallization were at pH 7.5, and 4°C. Crystals appeared between 2-4 weeks, and grew to 0.1-1 mm in two dimensions and approximately 0.05 mm in the third dimension.

Crystallization trials were also performed on C271S, N, and D. All crystallization trials were all performed in the presence of transition state analog components, MgADP, nitrate, and arginine, and protein concentration between 20-25 mg/ml. Initial crystallization trials with each mutant were performed in conditions established for wild type transition state analog complex arginine kinase (Zhou, 1998). Trials continued by varying substrate, PEG, and salt concentrations. Crystal screens 1 and 2 from Hampton Research were performed with C271S, N, and D. These commercial crystal screen kits

contain about 50 different conditions and were designed to provide a sparse matrix of conditions which varied precipitant and buffer concentration, as well as ionic strength and pH (Jankarik and Kim 1991; Cudney et al 1994). Crystal screens 1 and 2 from Hampton Research supply a sparse matrix of conditions based on conditions most likely to crystallize a variety of proteins. Crystal screen 1 was produced first and was the result of exhaustive analysis of conditions that had produced protein crystals. Crystal screen 2 is an extension of the original crystal screen 1 which includes novel precipitating agents and covers a wider range of pH values (Cudney et al 1994). Two conditions from crystal screen 1 resulted in small crystals in trials with C271S, and C271D but the crystals may not have been protein (see results). These conditions (number 13, 38 from Crystal screen 1) were reproduced with variable precipitant concentrations. The conditions numbered 13, and 38 included:

Condition #13:

0.2 M Sodium Citrate

0.1 M Tris

30% PEG 400

pH = 8.5

Condition #38

0.1 M Hepes

1.4M Sodium Citrate

pH =7.5

Precipitant concentrations were varied based on the above conditions over a range of 20-35% PEG400 for conditions near crystal screen 1 #13, and sodium citrate between 0.6-2.0 M for conditions near crystal screen 1 #38.

Structure Determination of C271A. Crystallographic structural studies were performed on active site cysteine mutants in an attempt to rationalize changes in enzyme kinetic parameters resulting from mutation. Crystallization trials were conducted with each

mutant enzyme in the presence of transition state analog components with hopes of determining mutant enzyme structure in a transition state form. Only crystallization trials with C271A were successful in producing measurable diffraction. Crystals of C271A were frozen in liquid nitrogen, after brief soaking in mother liquor containing 30% glycerol. Crystallographic data for the initial structure determination were collected at 100K using a Rigaku Raxis and an imaging plate as described previously for wild-type (Zhou et al. 1997). This data set was replaced during refinement with a slightly improved diffraction data set collected at the Advance Photon Source (at Argonne National Laboratory) from two crystals. Due to the crystals' small size, radiation damage was limiting. The rotating anode data set was collected from a single crystal. 180 frames of diffraction data were processed using the HKL suite of programs (Otwinowski and Minor 2001) then the later frames were culled to strike an appropriate compromise between completeness and quality. The wild type arginine kinase transition state structure was used as a probe for cross rotation and translation searches using the CNS software package. The correct solution at 7.4 sigma was three times greater than the next highest peak. The solution showed the enzyme in virtually the same position and orientation as in the wild-type form 2 crystals. The program "O" was used for manual adjustment of the model to fit the electron density, which was alternated with automated refinement using CNS (Kleywegt and Brünger 1996; Brünger et al. 1998). Automated water picking was performed with CNS and confirmed by visual inspection of $2F_o - F_c$ electron density maps calculated without solvent or substrates present in the phasing model. For the model submitted to the database, refinement was completed with conjugate gradient refinement of atomic positions and restrained individual B-factors. An alternative model was used just to check the nature of bound solvent and ions. The B-factors of these atoms were reset to their average, and then their occupancies were refined to estimate the number of electrons in each bound ion/molecule to help in their identification. The deduced atom types were used in the final refinement, but otherwise the alternative occupancy-refined model did not contribute to the final model (see results, Identification of chloride).

Homology Modeling. In an effort to rationalize the changes observed in enzyme kinetic parameters of additional active site cysteine substitutions, homology models were built

and optimized for C271S, C271D, and C271N arginine kinase. Homology modeling of arginine kinase active site cysteine mutants provided relatively tractable examples of homology modeling because closely related structures could be used as starting points, and experimental structures of mutants (Pruett et al. 2003) were available to evaluate the computational procedures. The starting point for homology modeling was the 1.2 Å resolution transition state analog structure refined to R^{cryst} of 12% (Yousef et al. 2002b). Initial models were constructed using the builder module of InsightII or DeepView/Swiss-PdbViewer (Guex and Peitsch 1997). The side chain of residue 271 was changed to S, N, or D and modeled as the standard rotamer with least steric conflicts. Energy minimization was performed using the AMBER94 force field as a part of the Sander module of the AMBER6 software package (Case et al. 1999). Simulations included the entire protein (residues 2-357), substrates arginine and ADP, magnesium ion, nitrate mimicking the transition state γ -phosphoryl, and 501 crystallographically observed water molecules. Simulations also included a shell of 3774 explicitly simulated water molecules (that had not been observed crystallographically) forming a truncated octahedron surrounding the protein and crystallographically observed solvent atoms by at least 5 Å. Protons and the explicit solvent shell were added using the Leap module of AMBER6 (Case et al. 1999).

Energy minimizations were performed with a truncated octahedral periodic box, with a harmonic restraint of 1 kcal/mol·Å on solute atoms. Minimizations proceeded until the second derivative of the energy function decreased below 0.05 kcal/mol·Å, over about 5000 cycles. Then each model was subjected to simulated annealing followed by slow cooling. The annealing schedule increased the temperature from 1K to 800K over the first 1000 cycles (1ps). The temperature was maintained at 800K from 5-10ps, then the system was slowly cooled from 800K to 0K over the next 5 to 15ps. During simulated annealing, atoms beyond a 15 Å radius of residue 271 were restrained to their starting locations with a harmonic restraint of 50kcal/mol, while most atoms within the 15 Å radius were restrained with a weak 0.2kcal/mol·Å force constant. Details of the simulated annealing protocol were determined empirically in order to minimize the difference between control models and mutant crystal structures. The strong restraints far from the active site allowed conformational searches using aggressive annealing

strategies without disrupting the overall structure. Weak restraints close to a site of perturbation are commonly applied, as here, to suppress gratuitous changes. Active site residues with atoms that were moved in any experimental mutant structure, and those closest to Cys₂₇₁, were free to move without any harmonic restraints. Unrestrained residues included 270-272, 224-226, and 313-316, from three of the four active site loops that contact the guanidinium of substrate arginine.

The ability of the protocols to predict structures was tested with control simulations, the results of which could be compared to known experimental structures. These were increasingly ambitious, but started with the wild-type structure, to verify that the procedures did not cause the structure to diverge from the experimental one. The root mean square deviation (rmsd) for the unrestrained atoms near Cys₂₇₁ was 0.68 Å, elevated due to apparent flexibility in the side chains of Glu₃₁₄ and His₃₁₅. Prediction of the C271A structure from the wild-type was a realistic test for the prediction of other Cys₂₇₁ conservative mutations. The simulated model successfully predicted the largest changes, modest movements of the substrate arginine and movement of His₃₁₅. The rmsd for the unrestrained atoms close to residue 271 was 0.43 Å (compared to the experimental structure). Prediction of the E314D structure (Pruett et al. 2003) was a test of the method's ability to predict larger perturbations – about 1 Å in the backbone of Asp₃₁₄ and His₃₁₅, larger in the side chains. Predicted models replicated the backbone shift within 0.5 Å, and correctly identified a 180° rotation of His₃₁₅ about χ_2 , leading to an interaction with the carboxylate of the substrate arginine. The predicted and experimental conformations of the mutated side chain at residue 314 show different positions of C_B, but rotations about χ_1 and χ_2 torsion angles result in a single carboxylate oxygen interacting with substrate arginine - as observed crystallographically. Thus, even in challenging cases, where there is some discrepancy in atomic positions, the procedures correctly predict the principal atomic interactions.

RESULTS

Mutagenesis, expression, and purification

Success of mutagenesis was confirmed by verifying the mutant DNA sequence. Expression of active site cysteine mutants resulted in similar yields as wild type arginine kinase with approximately 100-200mg of protein resulting from a 1 L bacterial culture (Zhou et al., 1997). Purification of active site cysteine mutants did show specific characteristics that were dependent on the specific mutation. Wild type and C271A arginine kinase were successfully refolded from insoluble inclusion bodies by both the sequential dialysis method, and the rapid dilution method described in Methods. Other mutants in this study did not produce measurable enzymatic activity, or a size exclusion profile characteristic of properly folded arginine kinase, following refolding by the dilution method. C271G was inactive, and in spite of testing many refolding protocols, consistently gave an S100 size exclusion chromatographic profile characteristic of

misfolded arginine kinase. C271T yielded low levels of activity, and an S100 profile indicating both folded and misfolded enzyme similar to wild type arginine kinase. C271M and C271Y yielded no measurable activity. Full kinetic analysis was continued with the C271A, C271S, C271N and C271D

mutants that could be folded normally, while mutants C271G, C271T, C271M and

C271Y were not pursued further. Mutants and wild type gave indistinguishable chromatographic profiles from the S300 column performed in denaturing 6M urea buffer. (Figure 4)

Figure 5 S100 profiles following refolding of mutants and wild type varied, showing evidence of greater or lesser proportion of faster eluting peak (characteristic of larger molecular radius of misfolded or potentially aggregated arginine kinase) relative to the active arginine kinase peak. The two peaks eluted at approximately 90 and 140 ml using a 26mm X 60cm Sephacryl S100 column.

Refolding of C271A was performed by sequential dialysis or dilution of samples in 6M urea into buffer with no urea. This method was much quicker than sequential dialysis, but resulted in lower yields of active enzyme. S100 chromatographic profiles following sequential dialysis of C271A were identical to the profile for the wild type enzyme shown in Figure 5. Refolding by dilution precipitated a larger fraction of the sample during refolding than the sequential dialysis method resulting in reduced yield. However, the S100 profile showed a higher proportion of properly folded enzyme relative to misfolded arginine kinase. (Figure 6)

Figure 6 S100 profile for C271A following refolding by dilution.

C271G was never properly refolded after initial isolation from insoluble inclusion bodies. Attempts to properly refold C271G included extending the time between steps of sequential dialysis as well as additional steps of sequential dialysis, addition of amino

acids to the refolding buffer, and addition of transition state analog components to the refolding buffer. (Figure 7)

Figure 7. Chromatographic profile for C271G. There is no peak at 140ml suggesting that the overall shape of the enzyme is significantly changed relative to the native wild type form of the enzyme.

For mutants which did refold, following the S100 size exclusion step, fractions which showed corresponding to properly folded arginine kinase were pooled and dialyzed against buffer with no added

salt and loaded onto a DEAE anion exchange column. The DEAE column used a KCl gradient (see Methods, expression and purification) to elute arginine kinase and functioned as a final purification step, a sample result, achieved with wild type arginine kinase is shown below. (figure 8)

Figure 8 DEAE profile for wild type arginine kinase. The blue line shows UV absorbance, the brown line is conductivity, and the yellow line is % of the high salt buffer. Wild type and mutants gave indistinguishable profiles.

Enzyme Kinetics

Increase in absorbance at 340nm as a function of time was recorded for the wild type and C271S, C271A, C271N, and C271D arginine kinase mutants. Enzyme velocity was measured in groups of six reactions, and rates were recorded as A_{340}/min .

Representative data sets are shown below for measurements performed on wild type and C271N arginine kinase (Tables 5 and 6). These data sets show the two extremes of enzyme turnover measured in the current study. Enzyme and substrate concentrations used and measured rates are presented in tables below. Double reciprocal plots (Figures 9 and 11) showing the inverse of rate vs. the inverse of substrate concentration were used to construct secondary plots (Figures 10 and 12). The secondary plots consisted of plotting V_{max} / K_M measured for variable phosphoarginine vs. the inverse concentration of ADP. The secondary plots were used for estimating steady state parameters. From the double reciprocal plots, intersection of lines from different “fixed” substrate concentrations show that the enzyme functions by a direct transfer of the reactive group. Parallel lines would have suggested a “ping-pong” mechanism including a covalent enzyme intermediate rather than a direct transfer of the reactive group. The point at which lines intersect also gives information about the enzyme mechanism. Intersection of lines to the left of the Y-axis, and above the X-axis demonstrated that the wild type enzyme functions by a random order substrate binding mechanism that occurred with favorable substrate binding synergy (Figure 9).

Table 5 Raw rate measurements for wild type arginine kinase in 60mM chloride.

ADP (mM)	0.05	0.1	0.2	0.4	0.8	1.6
phosphoarginine (mM)	rate A_{340}/min	rate A_{340}/min	rate A_{340}/min	rate A_{340}/min	rate A_{340}/min	rate (A_{340}/min)
0.078	0.0199	0.044	0.062	0.0809	0.1019	0.1183
0.117	0.0298	0.0575	0.0826	0.1202	0.1456	0.1673
0.194	0.0482	0.0839	0.1255	0.176	0.2114	0.2474
0.311	0.0659	0.1225	0.175	0.2391	0.2837	0.3266
0.469	0.0858	0.1556	0.2244	0.3061	0.3618	0.4112
0.700	0.1096	0.1958	0.275	0.3739	0.4236	0.4815

Figure 9. Double reciprocal plots of wild type arginine kinase activity as a function of substrate concentration. The intersection of lines above the X-axis and left of the Y-axis indicates random order substrate binding in the bimolecular reaction.

Data from the primary double reciprocal plots was then replotted by first tabulating slope and Y-axis intercept values from the primary plots. Slope and y-intercept values were then plotted vs. the inverse of the substrate concentration not used in the primary double reciprocal plots above. (Figure 10)

Figure 10. Secondary plots used to determine K_{ia} , K_m , and V_{max} . Regression equations in the form of $y = \text{slope}(x) + y\text{-int}$, were calculated using linear regression analysis of the 6 data points shown.

Double reciprocal plots constructed from C271N kinetic activity measured in 1mM chloride demonstrate some important differences between this mutant and wild type activity. Values of inverse rate are much higher demonstrating the reduction in activity observed for C271N relative to wild type arginine kinase. The point at which lines intercept one another is below the X-axis demonstrating unfavorable substrate binding synergy, or a > 1 .

Table 6 Raw rate measurements of C271N activity in 1mM chloride.

ADP (mM)	0.04	0.08	0.15	0.31	0.62	1.23
phosphoarginine (mM)	rate	rate	Rate	rate	rate	rate
	A_{340}/min	A_{340}/min	A_{340}/min	A_{340}/min	A_{340}/min	A_{340}/min
0.72	0.0006	0.0008	0.0012	0.0015	0.0021	0.0022
1.07	0.0006	0.001	0.0014	0.0019	0.0024	0.0026
1.79	0.0008	0.0012	0.0017	0.0024	0.0028	0.0031
2.86	0.0009	0.0014	0.0021	0.0027	0.0034	0.0038
4.30	0.001	0.0015	0.0023	0.0031	0.0037	0.0043
6.44	0.001	0.0016	0.0025	0.0034	0.0042	0.0047

Figure 11. Double reciprocal plots resulting from activity measured from C271N in 1mM chloride. Comparison with the graphs shown above from wild type arginine kinase activity shows that activity is much lower in C271N, and a is greater than 1 for C271N since lines intersect below the x-axis.

Figure 12 Secondary plots of C271N activity measured in 1mM chloride.

Kinetic evaluation of Cys271 mutants. All Cys271 mutants displayed dramatically reduced k_{cat} (turnover) values ranging from 0.02-1.0% of wild type (Table 7). Increased chloride concentration from 1 mM to 60 mM elicited 20- and 4-fold increases in the k_{cat} of C271A and C271S mutants, respectively (Table 7). In fact, the highest activity observed among mutants was C271A in 60mM chloride. The C271D mutant showed approximately 30-fold higher activity than the isosteric C271N (Table 7). C271D was slightly less active than C271A, but its activity ($k_{cat} = 0.8\%$ wild type) was independent of chloride concentration. In the absence of a negatively charged side chain at residue 271, or chloride in the assay mixture, serine, alanine, and asparagine mutants displayed only 0.02%-0.06% of wild type activity.

Steady state kinetic measurements of were made for the wild type and mutants in solutions containing 1mM or 60mM chloride, measuring rate in the reverse direction of the reaction forming ATP. Kinetic constants for the wild type arginine kinase were consistent with prior studies (Pruett et al. 2003). All kinetic plots for wild type arginine kinase and mutants were consistent with the random order bi-bi mechanism (Figure 12). Here, the Cleland notation is used with K_{ia} denoting an “initial” (binary) kinetic dissociation constant between a single substrate and free enzyme (denoted by other

authors sometimes as K_s or K_d). K_M is the (ternary) kinetic dissociation constant for the Michaelis complex with both substrates. The synergy coefficient α is the ratio of K_M/K_{ia} . A value of $\alpha < 1$ indicates that the binding of the second substrate is enhanced by the presence of the first (Segel 1975).

The pattern of change in phosphoarginine and ADP binding constants in Cys271 mutants is complex with significant increases being observed in K_{ia} and K_M for C271D, and chloride dependent changes in K_{ia} and K_M for C271A and C271S mutants (Table 7). C271A showed a significant increase in K_{ia} and K_M for phosphoarginine upon addition of 60mM chloride to the reaction mixture, while C271S showed an opposite effect as K_{ia} and K_M for phosphoarginine decreased from significantly elevated levels in 1mM chloride to near wild type values in 60mM chloride. Comparison of C271D steady state constants with those determined for isosteric C271N showed that K_{ia} and K_M for phosphoarginine were increased in C271D, while C271N values were much closer to wild type.

Table 7 Enzyme kinetic parameters determined for C271S,A,N,D in conditions containing (A) 1mM chloride and (B) 60mM chloride. Parameters were determined by averaging 3 independent evaluations, and error is given as the standard deviation between the three evaluations.

(A) 1mM chloride	Wild type	C271S	C271A	C271N	C271D
K_{ia} (phosphoarginine)	1.7 +/- 0.2	7.4 +/- 2.0	1.5 +/- 0.1	0.9 +/- 0.1	6.5 +/- 1.7
K_M (phosphoarginine)	0.45 +/- 0.06	7.6 +/- 0.4	1.6 +/- 0.1	1.2 +/- 0.2	4.4 +/- 1.3
K_{ia} (ADP)	0.16 +/- 0.01	0.2 +/- 0.1	0.26 +/- 0.05	0.12 +/- 0.04	0.14 +/- 0.02
K_M (ADP)	0.04 +/- 0.01	0.2 +/- 0.2	0.30 +/- 0.09	0.17 +/- 0.01	0.10 +/- 0.04
alpha	0.27 +/- 0.04	1.0 +/- 0.2	1.1 +/- 0.2	1.4 +/- 0.4	0.8 +/- 0.5
k_{cat}	104 +/- 24	0.07 +/- 0.02	0.08 +/- 0.03	0.022 +/- 0.002	1.2 +/- 0.4
% Wild type k_{cat}	72%	0.05%	0.06%	0.02%	0.82%

(B) 60mM chloride	Wild type	C271S	C271A	C271N	C271D
K_{ia} (phosphoarginine)	0.8 +/- 0.2	0.9 +/- 0.1	11.5 +/- 2.2	0.75 +/- 0.25	6.5 +/- 0.1
K_M (phosphoarginine)	0.30 +/- 0.09	0.8 +/- 0.3	5.4 +/- 1.9	1.30 +/- 0.08	7.5 +/- 3.6
K_{ia} (ADP)	0.10 +/- 0.05	0.18 +/- 0.03	0.37 +/- 0.05	0.19 +/- 0.05	0.19 +/- 0.06
K_M (ADP)	0.04 +/- 0.02	0.17 +/- 0.07	0.17 +/- 0.04	0.31 +/- 0.03	0.21 +/- 0.04
alpha	0.40 +/- 0.01	0.95 +/- 0.2	0.5 +/- 0.15	1.7 +/- 0.5	1.2 +/- 0.6
k_{cat}	145 +/- 14	0.3 +/- 0.2	1.5 +/- 0.4	0.042 +/- 0.003	1.2 +/- 0.2
% Wild type k_{cat}	100%	0.22%	1.02%	0.03%	0.83%

Several overall trends in the kinetic parameters are obvious. The kinetic dissociation constants of phosphoarginine are more greatly affected by mutation at Cys₂₇₁ than those of the nucleotide. This result suggested that the enzyme affinity for phospharginine was decreased more than the affinity for ADP was decreased, and was expected due to the proximity of Cys₂₇₁ to the guanidino substrate. For the nucleotide substrate, it is the K_M that is affected more than K_{ia} , consistent with an indirect interaction between Cys₂₇₁ and the nucleotide substrate mediated by binding of the other (phosphagen) substrate. With the exception of C271S, the effects of Cys₂₇₁ mutation and added chloride upon kinetic measures of phosphoarginine binding can be explained in terms of electrostatic repulsion between phosphoarginine and the variable side chain of residue 271, or a chloride ion. In the thiolate form of cysteine (wild-type) or the carboxylate in C271D, a negatively charged side chain may repel phosphoarginine which has an overall negative charge. In C271A, where extra space is available, a chloride

replacing the thiolate sulfur at high concentration, may also impair phosphoarginine binding.

All mutants exhibited less substrate synergy than wild type arginine kinase, with α values closer to unity. Lost synergy resulted from larger changes in K_M than K_{ia} for mutants relative to wild type. In contrast to significantly unfavorable synergy observed in active site cysteine mutants of Mi_b -CK, only the C271N mutant displayed any unfavorable substrate synergy with α near 2. However, in other ways the kinetic effects of cysteine mutations in arginine kinase are similar to those observed for similar mutations in Mi_b -CK (Furter et al. 1993), giving no evidence that the function of these homologous residues is fundamentally different.

Crystallization

Extensive efforts to crystallize active site cysteine mutants resulted in diffraction quality crystals only for the C271A mutant. Single crystals of C271A arginine kinase were obtained in the transition state analog form using conditions that were similar to wild type arginine kinase with the exception of increased buffer and $MgCl_2$ concentrations. Crystals of C271A appeared after 4 days and single crystals grew up to about 0.25 x 0.25 x 0.10 mm in 2-4 weeks. Crystallization attempts with C271S, C271N, and C271D resulted in crystal formation for each mutant, but no crystals showed measurable diffraction implying high disorder. Crystals of C271N were obtained in conditions very similar to wild type arginine kinase, but these crystals were small, two dimensional and gave no measurable diffraction. In trials with C271S, and C271D, crystals appeared under conditions similar to crystal screen 1 conditions 13, and 38(see Methods), but these crystals showed x-ray diffraction patterns characteristic of a salt of unknown composition with a few very intense diffraction spots, but none of the close spacings characteristic of diffraction from a macromolecular crystal.

Structure determination of C271A-transition state analog complex

Crystals of C271A were briefly soaked in mother liquor plus 30% glycerol and flash frozen in liquid nitrogen. Diffraction data was collected at 100K on a Rigaku Raxis with rotating anode x-ray generator, using an image plate to record diffraction. Data collected at Argonne National Laboratory at the Advanced Photon Source was collected with a Mar CCD detector. Crystals of C271A were nearly isomorphous to the wild type crystal form 2 (Zhou, 1997), and the structure was solved by molecular replacement using CNS to perform rotation and translation searches. The final model includes residues 2-357, one ADP molecule, one nitrate ion, one magnesium ion, one chloride ion and 313 water molecules. Model statistics include $R^{\text{free}} = 23.1\%$ and $R = 20.6\%$. Cross-validated Luzzatti analysis gives an estimated coordinate error of $0.34\text{\AA}$ (Table 8).

Arginine Kinase	C271A
Crystal Form	P2 ₁ 2 ₁ 2 ₁
a, Å	64.9
b, Å	71.3
c, Å	80
Temperature, K	100
Resolution, Å	2.3 (2.38-2.30)
Completeness	93.2% (92.2%)
Redundancy ^a	3.3
$R_{\text{merge}}^{\text{b}}$	7.5% (19.9%)
$R_{\text{cryst}}^{\text{c}}$	20.9% (32.8%)
$R^{\text{free d}} (46)$	23.6% (33.5%)
Cross-validated Luzzati error (46)	0.34 (10-2.3Å)

Table 8. Crystallographic and Refinement Statistics. a Average number of observations of each reflection; b $R_{\text{merge}} = S |I_h - \langle I_h \rangle| / S |I_h|$, where $\langle I_h \rangle$ is the average intensity of symmetry equivalent observations; c $R_{\text{cryst}} = S |F_o - F_c| / S |F_o|$; the conventional crystallographic refinement R-factor. d R^{free} is the cross-validated R-factor, calculated the same way, except with a random set (3%) of reflections omitted from the structure determination and refinement.

Identification of chloride in the active site.

After placement of 313 water molecules and individual B-factor refinement, the average B-factor for solvent atoms was $52\text{\AA}^2$, but one water molecule had an exceptionally low B-factor of $7\text{\AA}^2$. The exceptionally low B-factor suggested that this “water” molecule was either very tightly coordinated, restricting motion, or that this density was actually occupied by a heavier atom in the crystal. B-factors for all solvent atoms were set equal to the average for solvent atoms and the occupancy values of these atoms were refined. The water molecule of interest refined to an occupancy value of 3, clearly above the occupancy of all other solvent water molecules, and suggested that scattering from this position was more intense than would be expected

from a species with the 10 electrons of water. The electrostatic potential was calculated for this “water” site by numerically solving the linear Poisson-Boltzmann equation using the program Delphi (Rocchia et al. 2001). The site coincided with a positive peak in the electrostatic potential. Based on the high occupancy, positive electrostatic potential, and the presence of 100mM MgCl_2 in crystallization setups, this water molecule was replaced by a chloride ion in the final model.

Position of chloride. Chloride ions act as hydrogen bond acceptors, and are usually coordinated by three or four ligands in trigonal or pyramidal configurations, and like water, there is not great directional specificity to the coordination (Jeffrey and Saenger 1991). In C271A four polar ligands are observed to coordinate chloride: the $\text{N}_{\gamma 1}$ of the substrate arginine, a water molecule, the peptide nitrogen of, and the hydroxyl of threonine 273. Among the ligands, the presumptive hydrogen bonding interactions between chloride and protein atoms are similar to those seen in small organic molecules (Steiner et al. 1998), and in atomic resolution protein crystal structures (Ridder et al. 1999; Backstrom et al. 2002). The coordination geometry is close to that expected for a water molecule except that 3 of the 4 coordinating ligands are found at distances slightly longer than expected for water (Such subtle distinctions would not be significant on their own, but combined with the electrostatic potential and high occupancy suggest the presence of chloride over water). One methyl group and one methylene group also contact chloride at distances between 3.45Å and 3.6Å. (Figure 13)

Comparison between transition state structures of C271A and wild type arginine

kinase. The overall fold of C271A is nearly identical to the wild type AK transition state structure, with a rmsd of 0.36Å over all 356 C α atoms. Comparison between wild-type and C271A shows 5 stretches of 4 or more residues with statistically significant differences between the two models. In the amino terminal domain, residues 2-9, 34-38, and 89-92 are displaced approximately 0.5Å from wild type positions. These changes are minor, distant from the active site, and therefore not likely to be important to catalysis. The largest changes are in the carboxy terminal domain between residues 291 and 300. Residues 294-300 are α -helical as they are in the wild-type transition state structure, but not in the substrate-free structure. However, in C271A the helix is extended by 3 amino acids making it longer than the helix observed in the wild type model by about 1 turn. Extension of this helix in C271A results in deviation of backbone atoms between residues 292-296 of greater than 2Å. Movement of these residues results in new interactions for Arg₂₉₄ - a hydrogen bond to the backbone oxygen of Thr₃₁₆, and, through a water molecule, to His₃₁₅. Glu₃₁₄ and His₃₁₅ interact with the substrate arginine, the side chain of His₃₁₅ moving 0.8Å to form a hydrogen bond with the substrate arginine N ϵ . Neighboring loop residues 315-321 have backbone displacements of approximately 0.5Å (Figure 14)

Figure 14. Stereo image showing the largest conformational change between C271A (red) and wild type arginine kinase (blue) occurs between residues 292-296. This conformational change can be traced to a new hydrogen bond in the mutant active site that is not seen in wild type. These conformational changes in no way resemble the substrate free form of the enzyme.

With the kinetic binding constants suggesting weaker binding, movement of loops away from substrate arginine might have been expected, but, in fact, the loop comes closer to substrate arginine. Other flexible active site loops are unchanged relative to the wild-type transition state structure. Overall, the locations of the greatest changes are similar to the E314D mutant (Pruett et al. 2003) and do not suggest that mutation of the active site cysteine could inhibit substrate induced conformational changes.

Active Site. The positions of ADP and nitrate in the C271A structure do not differ from wild type significantly (atom positions deviate less than the cross-validated Luzatti estimates of coordinate error). The changes in the substrate arginine are slightly greater. The guanidino N₇ atoms move 0.4-0.5Å toward the space vacated by the sulfur in C271A. This shift of the guanidinium of arginine relative to nitrate and MgADP

results in modest perturbation of substrate alignment in the transition state analog complex. Although the guanidinium displacement is nominally greater than the average coordinate error (0.34 Å), the refined C271A structure fits the 2.3 Å $2F_o - F_c$ electron density map only marginally better than wild-type, giving little support for the significance of this structural difference. The difference map, calculated with $F_o(\text{wild-type}) - F_o(\text{C271A})$ coefficients should be most sensitive to conformational differences yet gives no indication of changes in guanidinium atom positions.

The highest positive peak in the $F_o(\text{wild-type}) - F_o(\text{C271A})$ electron density map corresponds to the loss of Cys₂₇₁'s sulfur, and the largest negative peak corresponds to the insertion of the neighboring chloride in C271A. In summary, the diffraction data show clearly that the C271A mutation has introduced a new chloride-binding site within 2 Å of the sulfur that is now missing, but the impact upon the rest of the active site is minimal, barely detectable at 2.3 Å resolution.

C271S,N,D homology models. Homology models of C271S, C271N, and C271D were constructed to help rationalize the kinetic effects of Cys₂₇₁ substitutions in mutants that were not amenable to crystallization. C271S was modeled without and with an active site chloride, initially positioned as in the C271A crystal structure, but allowed to move unrestrained during energy minimization and simulated annealing.

In homology models, the side chains of E314 and H315 showed a variety of conformations of nearly equal energy, suggesting a variety of conformations are possible in this loop (Figure 15). There are several experimental indications that this region is intrinsically less stable than other parts of the active site. The loop holding residues equivalent to 314-321 of arginine kinase was completely disordered in all phosphagen kinase structures determined in the absence of bound substrates, but is clearly observed in transition state analog complexes of both AK and CK as well as a binary complex of creatine kinase with MgADP (Zhou et al. 1998; Lahiri et al. 2002). In arginine kinase, the loop structure is modestly affected by mutation at residue 225, another active site residue with which it makes no direct interaction (Pruett et al. 2003). Thus, it appears that variability between members of a molecular dynamics ensemble (each member

started with a different random trajectory) is at least a qualitative indicator of the stability of active site residues. (Figure 15)

Figure 15. Stereo image of a control experiment for the simulated annealing protocol. The wild type model was subjected to simulated annealing with 10 different random number seeds used to apply different initial trajectories. The resulting models show flexibility in the loop holding residues 314 and 315.

Side chain conformational variability was also seen at the serine in the C271S mutant. In the initial model, several rotamers could be built without steric overlap, so the rotamer homologous to the native cysteine was chosen. Simulated annealing of a chloride-free model with different initial random trajectories resulted in three side chain positions with nearly equal energy. The slightly reduced size and charge neutrality of the serine appear to give greater freedom to the serine than the native cysteine. One conformation was similar to the wild type cysteine, but with the γ -hydroxyl moved slightly closer to the hydroxyl of T273 and the substrate guanidinium. Two other rotamers offer alternative hydrogen bonds for the hydroxyl, either with a carboxylate oxygen of Glu₂₂₅ and the guanidinium of substrate arginine, or away from the Glu₂₂₅ carboxylate and toward a simulated water molecule. In simulations with a chloride ion, the ion remained within 1 Å of its position in the experimental C271A structure, between 3.5 and 4 Å from the positively charged guanidinium of substrate arginine. Simulations of C271S with chloride resulted in different side chain orientations than simulations without chloride. Three distinct side chain conformations were found with interactions of

its γ -hydroxyl to both the chloride (3 Å) and the non-reactive N_η of substrate arginine (3-3.1 Å). A 4th rotamer was found within 2.7 Å of a carboxylate oxygen of Glu₂₂₅, nearly 4 Å from the N_η of substrate arginine, and 5 Å from the chloride ion. (figure 16)

Figure 16. Stereo image of C271S models after simulated annealing. Models shown in green contained a single chloride ion during the simulated annealing procedure in the position observed in the C271A crystal structure.

By [MSC1]contrast, there was less ensemble variation in either the side chain positions of residue 271 of C271D or C271N mutants. In C271D, one carboxylate oxygen interacted with the non-reactive N_η of substrate arginine and a bound water molecule, the other carboxylate oxygen formed hydrogen bonding interactions with hydroxyl oxygen and backbone nitrogen of Thr₂₇₃, as well as the backbone nitrogen of Pro₂₇₂, all at distances between 2.6-3.0 Å. The carboxylate of Glu₂₂₅ was also displaced from its wild type position in one of the C271D ensemble models, but mostly it remained in its wild-type conformation. In the C271N models the side chain amide oxygen makes the closest interactions with the guanidinium (3.2-3.4 Å) from the non-reactive N_η , but forms a more optimal hydrogen bonding interaction with the hydroxyl of Thr₂₇₃ at 2.8 Å. The amide nitrogen of the substituted asparagine hydrogen bonds to the backbone carbonyl of Ile₆₇ and a water molecule. The simulations show that all of the conservative mutations expressed experimentally can be accommodated without major perturbations to the active site.

DISCUSSION

Characterization of active site cysteine mutants of arginine kinase recasts the cysteine as a direct participant in phosphagen kinase catalysis. Attempts to quantify details of phosphagen kinase catalysis should therefore consider this residue an active participant in catalysis, with no essential role in substrate induced conformational changes. More specifically, the transition state structure of C271A suggested that earlier ideas of how cysteine was involved in catalysis were incorrect. Enzyme kinetic characterization of cysteine mutants demonstrated a similar level of residual activity as was observed from mutants of the active site glutamates, previously thought to be more directly involved in catalysis. Enzyme kinetic results from cysteine and glutamate mutants demonstrate that several residues contribute on a similar level to the observed catalysis of phosphagen kinases, and there may be no single amino acid that is absolutely essential for catalysis.

Enzyme kinetic analyses of arginine kinase and creatine kinase show many similarities. They catalyze chemically identical reactions on similar millisecond time scales (Kuby et al. 1954; Blethen 1972). The enzymes have similar substrate-binding affinities with K_M on the order of 1mM for phosphoarginine or phosphocreatine and 0.1mM for MgADP (Furter et al. 1993; Pruett et al. 2003). Here, it is found that mutations of the active site cysteine yielded similar results to those for Mi_b -CK (Furter et al. 1993) and human muscle CK (Wang et al. 2001) in that catalysis was markedly reduced in conditions near ideal for the wild type enzyme, and kinetic dissociation constants were slightly increased for the phosphagen substrate indicating weaker binding. The most active arginine kinase cysteine mutants were slightly less active than creatine kinase. No direct comparison was possible to the 10%-active Mi_b -CK C278G mutant due to mis-folding of the arginine kinase C271G mutant. However the arginine kinase mutant C271A has activity (1%) that is nearly as high as this and the HMCK C282S mutant (4%) (Wang et al. 2001). Enzyme kinetic analysis also supports prior biochemical studies of

the homologue, creatine kinase, which indicated a reduced pK of the active site cysteine in creatine kinase.

Taken together, the kinetic result of mutating the active site cysteine in homologous family members present a strong argument that the cysteine is not absolutely essential for catalysis, even though in some family members, conservative mutations have led to complete loss of activity (Lin et al. 1994), perhaps because there is ample opportunity for collateral damage to the active site. The overall similarity in the kinetics of cysteine mutants also make a strong case that while differences may exist, the cysteine acts in a fundamentally similar role in each of these homologous enzymes. That said, the availability of a high resolution mutant structure (C271A) for arginine kinase now eliminates the popularly held belief that the active site cysteine is essential for proper execution of the large substrate-induced conformational changes and that it is not directly involved with catalysis (Furter et al. 1993). The C271A structure is in the transition state form, and apart from very local changes, is in a wild-type configuration. Clearly, some other rationalization of the importance of the cysteine is needed.

Past considerations of whether the active site cysteine was catalytically essential or key to conformational changes were based on a binary categorization of mutants as active or inactive. In fact, partial activity is critical to understanding the role of the cysteine. Several chemical modifications of the active site cysteine of phosphagen kinases have eliminated catalytic activity in different family members, but as with the proposed catalytic base, Glu₂₂₅ (Pruett et al. 2003), mutation of Cys₂₇₁ can lead to partial loss of activity. Since conservative mutations led to an enzyme with residual activity, the native amino acid is not absolutely required for activity. The ability to produce mutant enzymes with partial activity allowed for detailed kinetic analysis to determine how the native properties of the enzyme were changed by mutation. The case is made below, that there are several contributors to the rate enhancement of these enzymes, with several active site amino acids playing roles in the catalytic chemistry or the alignment of substrates. The leading role of the cysteine may be catalytic, even if it is only responsible for part of the catalytic effect. The near identity of the structure of a partially active mutant to wild-type eliminates a leading role in protein conformation, and similarities to

kinetic results of other active site mutants implicates the cysteine as one of several residues contributing to catalysis.

The kinetics and structure reported here give strong and mutually consistent support to the proposal that the cysteine functions as a thiolate ion (Watts 1973; Furter et al. 1993; Wang et al. 2001). As observed previously with creatine kinase (Furter et al. 1993), chloride can partially rescue the activity of Cys₂₇₁ mutants. The C271A structure indicates that the chloride binds close to the position of the negatively charged sulfur in wild-type, substituting in part for its role. The identification of chloride was on the basis of the size peak in the electron density, coordination, and electrostatic potential of the site. A more definitive indication would have come through anomalous diffraction, but the crystals had been used before the need for such an experiment was apparent, and crystallization was not readily accomplished.

The rescue effect is greatest for the C271A mutation where the chloride gets closest to the native sulfur position. Simulation of the C271S structure shows that, consistent with the chloride-dependence of its kinetics, a chloride can be accommodated in the active site, but would require movement of the serine side chain from the position of cysteine in the wild type structure. Simulation of the C271N structure predicts that a chloride would not be bound in the immediate vicinity of the guanidinium, consistent with the kinetics showing little effect of chloride – less than observed in Mi_bCK (Furter et al. 1993). As might be expected, chloride provides no further help to the C271D mutant that is already negatively charged. The predicted C271D structure indicates that the wild-type thiolate-guanidinium interaction is replaced by a carboxylate-guanidinium salt bridge (2.7Å), although the carboxylate oxygen position differs slightly from that of the sulfur.

The C271A mutant shows that the effect of the chloride is mediated through catalysis. In fact, substrate binding is actually less favorable with higher K_M and K_{ia} for phosphoarginine as chloride concentration is increased. The earlier studies with Mi_bCK found unfavorable impact of chloride upon phosphocreatine binding in most cysteine mutants (Furter et al. 1993). Here, it is seen only for the alanine mutation that allows chloride binding close to the native thiolate position, and not for the asparagine and

aspartate mutants. This, and the higher dissociation constants for C271D, can be rationalized in terms of electrostatic repulsion between phosphoarginine and a negatively charged active site. Direct electrostatic effects can not explain the behavior of C271S. One of the several rotamers predicted by homology modeling moves the hydroxyl away from the guanidino nitrogen to hydrogen bond to Glu₂₂₅, perturbing the pK of Glu₂₂₅ upwards, and likely diminishing the interaction between Glu₂₂₅ and substrate arginine.

The loss of synergistic substrate-binding upon mutation or chemical modification of creatine kinase had been cited as supporting the previously proposed role of the cysteine in a substrate-induced conformational change (Maggio and Kenyon 1977; Furter et al. 1993). Here, with arginine kinase, the effects upon synergy are less pronounced. In both the prior work, and that reported here, there are substantial errors in measuring kinetic dissociation constants for mutants with low activity. Detailed interpretation of the synergy of low-activity mutants can probably not be justified, and the crystallographic demonstration of limited impact on the conformational change in the C271A mutant is the stronger evidence. Furthermore, synergistic effects need not be tied to the large conformational changes that are substrate-induced (Fritz-Wolf et al. 1996; Lahiri et al. 2002; Yousef et al. 2002a). Electrostatic effects are clearly important in understanding the role of the cysteine.

Substrate binding synergy observed in C271A was dependent on added chloride as the value of α was near 1 in conditions containing 1mM chloride, and 0.5 when assay conditions included 60mM chloride. The crystal structure of C271A demonstrated that chloride is bound in the active site in position to form an ion pair with the non reactive nitrogen of substrate arginine. The position of chloride in the C271A active site suggests that disruption of synergistic substrate binding is due to local electrostatic changes in the active site and that the same changes in the local environment are the likely cause of increased turnover in C271A upon addition of chloride. After characterization of cysteine mutants by enzyme kinetics and crystallography, the role of cysteine in mediating substrate synergy remains unclear, but synergy may result from nothing more than modulation of the electrostatic interactions directly between the two charged substrates.

Future Outlook

Continued attempts to understand phosphagen kinase substrate binding synergy would benefit from improvements in experimental measurements of such processes. Nuclear magnetic resonance (NMR) spectroscopy can be an accurate method for measuring substrate binding by arginine kinase. TROSY experiments using ^{15}N labeled enzyme have been performed over a range of single substrate concentrations with a fixed arginine kinase concentration (Davulcu, unpublished). Spectra were recorded at each substrate concentration and are in the process of being assigned to individual residues in the arginine kinase sequence. The main goal of these experiments is to measure the dynamics of arginine kinase residues undergoing changes during the transition between substrate free and transition state forms. A by-product of this analysis will be dissociation constants, K_d , for arginine, phosphoarginine, MgADP, and MgATP, and the transition state analog complex (arginine, MgADP, nitrate). Analogous dissociation constants were determined for creatine kinase by monitoring changes in intrinsic fluorescence as a function of substrate concentration to determine binding constants for individual substrates and the transition state analog complex with creatine, nitrate, and MgADP (Borders, 2002). This method verified earlier enzyme kinetic studies on rabbit muscle creatine kinase that showed substrate binding synergy in the native enzyme (Maggio and Kenyon 1977), and provided experimental evidence that creatine kinase has a higher affinity for the transition state analog complex than the sum of binding affinities of the individual components (Borders, 2002).

The homology models do not provide a clear explanation for subtle differences observed in enzyme kinetic experiments. The models do suggest that the side chain substitutions studied by enzyme kinetics can each be accommodated in the active site with only minor changes in atom positions beyond the substituted amino acid. C271S homology models suggest that a small reduction in size relative to the wild type cysteine can allow a wide variety of side chain positions, and some of these may interfere chemically with the nearby E225 side chain. Homology models that include aspartate or asparagine side chains at position 271 are likely to be forced into a single conformation by steric constraints of the active site, but the extra bulk of these side chains need not

cause collateral changes or close contacts. In the case of the aspartate side chain, a favorable ionic interaction with the guanidinium of substrate arginine may result in a closer interaction with the substrate arginine than in C271N. The interactions with substrate arginine observed in C271D, and C271N homology models are consistent with differences in catalytic rate between these two mutants, but offer no explanation for reduced binding interactions expected from comparison of K_M values.

It has been noted previously that the reported pK of creatine kinase's active site cysteine is perturbed down from 8.5 to one of the kinetic pKs at 5.4 (Wang et al. 2001). Titration of the 240nm absorption, characteristic of thiol groups, was used to show that the pK of the cysteine was low (pK 5.6) even in the absence of substrates (Wang et al. 2001). While there are other precedents of active site cysteines with low pK, in the phosphagen kinase substrate-free form, there is no basic residue with which the cysteine could form an ion pair, the usual mechanism for pK perturbation (Polgar and Halasz 1973; Lo Bello et al. 1993; Gladysheva et al. 1996). The cysteine's pK is perturbed by one unit by the hydrogen bond with Thr₂₇₃, (Zhou et al. 1998) or a homologous serine in creatine kinase (Wang et al. 2001), but the means with which a larger pK perturbation is made remains a mystery. Here, application of the Poisson-Boltzmann equation (Yang et al. 1993) to the refined transition state analog arginine kinase structure (Yousef et al. 2002b) gives a calculated pK of 6.1 (Eliza Ruben, personal communication). This excellent agreement with the kinetic pK (Cook et al. 1981; Wang et al. 2001) demonstrates that the electrostatic environment in the substrate-bound state is sufficient to account for the low pK. By contrast, similar calculation using the substrate-free structure (Yousef et al. 2003) yields a slightly perturbed Cys₂₇₁ pK of 7.5. Comparisons with experimental pKs of many other proteins has demonstrated that the accuracy of such calculations is usually better than 1 pK unit (Antosiewicz et al. 1994). Here there is a caveat that the calculations omit an active site loop that has not been ordered in any of the substrate-free structures (Yousef et al. 2003), but the loop is remote from Cys₂₇₁, and unlikely to have great impact. The current calculation makes intuitive sense, suggesting that the low pK thiolate form is stabilized with the formation of an ion pair as the guanidino substrate binds. Thus, substrate-binding, and the rationalization of kinetic

dissociation constants is complicated by the likely loss of the thiolate proton as the substrate binds.

Experimental determination the pK of the active site cysteine would strengthen the current understanding of the function of this residue. A series of NMR titration experiments could accurately establish the pK of the active site cysteine in the free enzyme and in complex with arginine, phospho-arginine, or the transition state analog (arginine, nitrate, and MgADP). NMR titrations could provide accurate estimates of the protonation state of the active site cysteine, but requires significant resources and spectroscopic skills. Alternatively, titrations of the wild type and C271A mutants could be monitored by absorbance at 240nm as was done with creatine kinase (Wang, 2001). Thiol groups absorb strongly at this wavelength, whereas thiolate ions do not. Graphing absorbance vs. pH for wild type and C271A should show the loss of an inflection point in the mutant curve. The pH value of this inflection point corresponds to the pK of the mutated cysteine. Since calculated pK values of the active site cysteine of arginine kinase suggest a dependence of this pK on the presence of substrates, titration experiments could also be performed in the presence of arginine or phospho-arginine to determine whether binding these substrates differentially influences the pK of the active site cysteine. However, the high absorbance at 240nm of ADP and ATP limits this technique to pK determinations of C271 in the substrate free arginine kinase, AK:arginine, or AK:phosphoarginine complexes.

The coupled enzyme assay could be used to determine a kinetic pK of the active site cysteine by measuring catalysis as a function of pH for the wild type and C271A arginine kinase. Measuring rate over 36 combinations of reactant concentrations at each pH value for the wild type and C271A arginine kinase would result in K_M and V_{max} determinations over a range of pH values. Determining K_M in addition to measuring rate at each pH would help distinguish effects pH dependent effects on the catalytic rate from changes in substrate binding over a range of pH values. This method would establish the protonation state of the active site cysteine which results in optimal catalysis. The combination of pK values determined spectroscopically and kinetically would more thoroughly describe the protonation states of the active site cysteine over the entire catalytic process.

Questions about cysteine's protonation state in the substrate-free form relate to matters of substrate-binding. However, the larger impact of the Cys₂₇₁ mutations is upon catalytic rate. Many arguments now implicate the thiolate form in catalysis: (1) kinetic pKs determined from homologous creatine kinase mutants (Wang et al. 2001); (2) of mutations at the active site cysteine in several homologous enzymes, negatively charged aspartate substitutions are among those partially active (Furter et al. 1993)(and this work); (3) for mutational substitution of small amino acids for the cysteine, chloride is able to partially rescue activity in several of these enzymes (Furter et al. 1993)(and this work); (4) in arginine kinase, the chloride has been shown to bind to mutants at a site close to the native location of the negatively-charged sulfur, and to form analogous interactions with the substrate arginine (this work); (5) electrostatic calculations using the atomic structure indicate that when substrates are bound, Cys₂₇₁ of arginine kinase has a pK of 6.1 and is predominantly deprotonated (this work).

How the cysteine contributes to catalytic enhancement without being absolutely essential is open to speculation. A reversible acid/base role for the cysteine could be responsible for effects of this residue on catalysis. A cysteine thiolate acting as a base could abstract a proton from the non reactive nitrogen of substrate arginine in the forward reaction (producing phosphoarginine). This would enhance the nucleophilicity of the reactive nitrogen, and encourage N-P bond formation by causing the double bond of the guanidinium to be focused away from the reactive nitrogen. In the reverse reaction direction (ADP production), cysteine would have an acidic thiol side chain that donates a proton to the non reactive nitrogen of phosphoarginine. Protonating the non reactive nitrogen would shift the double bond of the guanidinium toward the reactive nitrogen, contributing to N-P bond cleavage. Such a role could involve formal proton transfer from guanidinium to cysteine, and later back again, or the effect might fall short of formal transfer, requiring only that the proton be drawn towards the thiolate.

Another possibility is that the cysteine functions in both directions with a negatively charged thiolate. The negative charge and proton affinity (basic character) of the thiolate would draw positive charge to the unreactive N_η, perturbing the resonance of the guanidinium. The effect would be to increase the double-bonded nature between the

C_ζ and the unreactive N_η , while decreasing it between the C_ζ and the reactive N_γ . For the forward reaction (phosphoarginine-forming), this would enhance the nucleophilicity of the reactive N_η whose lone pair attacks the γ -phosphorus of ATP. For the reverse reaction (ATP-forming), minimization of any double-bonding character of the reactive N_η would enhance the leaving-group properties of the arginine, and assist the phosphoryl transfer to ADP. Such details are of course unknown at this time, or indeed whether another role for the active site cysteine would be equally consistent with available data.

The potential role of a conserved active site cysteine has been debated for over 40 years. The wealth of data on the kinetic effects of chemical modification and mutagenesis upon a variety of homologous phosphagen kinases has often been confusing with apparently conflicting results sometimes. Now with a variety of mutations made to the active sites of several related enzymes, it has become clear that mutation may have unanticipated consequences, and that the loss of activity at a particular site, does not preclude partial activity being recovered for a homologous mutation later. Thus, residues such as this cysteine, once considered “essential” might not be absolutely required for catalysis. With this realization came the search for an alternative non-catalytic role, thought for a decade to be mediation of substrate-induced conformational changes (Furter et al. 1993). Here, with the addition of structure, such a role is ruled out, implicating the cysteine in catalysis again. However, these and other studies of active site residues (Pruett et al. 2003) indicate that the enzyme embodies several strategies simultaneously for enhancing the catalytic rate, and that there are several residues that contribute to the overall rate enhancement, none of which are absolutely required for the reaction to proceed at measurable rate.

APPENDIX A

Homology modeling

Homology modeling was performed to explain enzyme kinetic results from mutants that could not be crystallized. Although experimental structure would have been preferred, two factors made modeling active site cysteine substitutions especially tractable. First, a high quality crystal structure of the wild type enzyme was available as a template for homology modeling. Second, three single point mutants of active site residues had been crystallized, and crystal structures of these variants suggested that modification of active site residues was likely to have a very localized effect on structure. Homology models were produced and optimized using readily available software and modest computational resources. The following appendix is provided as a detailed supplement to the methods section of chapter 1.

AMBER introduction

The software package AMBER6 was used for energy minimization and simulated annealing of homology models. AMBER version 6 is composed of 60 programs that work together to set up a system, perform molecular mechanics calculations, and analyze the results of these calculations. In the molecular mechanics simulations on arginine kinase, the “system” included Cartesian coordinates for all protein and substrate atoms, crystallographically observed solvent water molecules, and artificially modeled solvent surrounding the protein by 5Å. In addition to atomic coordinates, energy programs in AMBER require the topology of the system, force field parameters for each atom and bond, and input commands describing details of the calculations to be performed. Topology includes connectivity, atom names, atom types, residue names, and charges assigned to each atom. Force field parameters include definitions of all the bond lengths

and angles, dihedrals, and atom types in the system. Topology, and force field parameters for common amino acids, nucleotides, and water molecules are provided in a database with the program package.

Getting the starting model

Cysteine mutant homology models were based on the wild type transition state analog complex refined against 1.2Å diffraction data (Yousef et al. 2002b). The wild type model was modified to incorporate the desired amino acid change at residue 271 using SwissPdbViewer/DeepView (Guex and Peitsch 1997). Residues were modeled in standard rotamer positions chosen to minimize close atom contacts (C271N, D), or maximize homology with the native cysteine side chain (C271S).

Parameterization of the AK transition state analog complex

The *Sander* module requires Cartesian coordinates for every atom in the system, topology of the atoms, force field parameters for each atom and bond, and an input file describing calculations to be performed. Coordinates were obtained from the starting model in protein data bank format. The default force field parameters of amber94 were used for most protein atoms and were applied using the program *Leap*. In the wild type arginine kinase model, the active site cysteine was modeled as CYX instead of the default CYS to account for biochemical evidence that the cysteine is a negatively charged thiolate.

Topology files and force field modifications for ADP were obtained by personal communication with Heather Carlson and Kristen Meagher of the University of Michigan. These parameters were derived from a combination of experimentally measured bond lengths and angles and partial charges determined from theoretical quantum calculations (Meagher et al. 2003).

Running LEaP

The AMBER package includes a program for preparing a new system for simulation. *Leap* was used to generate topology files from atomic coordinate files using information from amber residue libraries, force field parameter libraries, and additional

topology and force field parameters supplied for ADP. Topology information for substrate arginine was generated based on an amino terminal arginine residue with an additional oxygen atom added to form the carboxylate group of a free amino acid.

Leap was also used to add explicit hydrogen atoms and solvent molecules. Solvent was added in a truncated octahedron surrounding protein atoms by at least 5 Å using the WATBOX216 solvent model. Sodium ions were added to the system in place of modeled solvent to neutralize the overall charge without affecting protein atoms near the active site.

Energy minimization

Energy minimization was performed with *Sander* after preparing coordinate and topology files with *Leap*. The purpose of energy minimization was to alleviate any close atom contacts present in the starting structure or resulting from addition of explicit protons, solvent molecules or mutation in the enzyme. Energy minimizations were performed with a positional restraint of 1 kcal/mol on all protein and substrate atoms and no restraint on modeled solvent. These variable restraints were imposed because of the greater confidence in position of protein atoms observed crystallographically relative to artificially modeled solvent atoms added around the exterior of the protein. Models were minimized until the second derivative of the energy function decreased below 0.05 kcal/mol*Å. Below is a sample command line input used to start energy minimization with *Sander*. A protocol for performing energy minimization is provided at the end of the chapter under the heading Input files.

```
% sander -O -i all.in -o all.out -p prmtop.test -c restart.test -r restart.test1 -ref  
restart.test &
```

The command line parameters are described below:

- O overwrite existing files
- i *input file*
- o *log file* (to be written)
- p *topology file*
- c *coordinate file* in amber format
- r *output coordinate file* in amber format
- ref *reference coordinates* for harmonic restraints

& run calculation in background
all.in

Energy minimization resulted in small changes in atom positions in all models. Below are root mean squared deviations in atom positions for each model before and after minimization. Values are given for all protein and substrate atoms, and an average value for side chain atoms of residue 271.

Table 8 Root mean square deviation in atom positions following energy minimization

Model	Overall rms (Å)	average rms of side chain atoms of residue 271 (Å)
wild type (cys thiolate)	0.16	0.19
C271S no chloride	0.17	0.36
C271S with chloride	0.17	0.67
C271D	0.16	0.60
C271N	0.11	0.33

Simulated Annealing

Simulated annealing was performed with *Sander* in an attempt to find a more global optimum in homology model atom positions that need not be a minor modification of the starting model. Simulated annealing simulations were short duration molecular dynamics simulations during which the system was heated to allow atoms to move, and then slowly cooled. As the temperature was reduced, atom positions were adjusted in 1 fs time steps to minimize potential energy, allowing models to overcome energy barriers associated with conformational changes. Simulated annealing of active site cysteine mutants of arginine kinase was performed with variable positional restraints based on the distance between solute atoms and the active site cysteine. Positional restraints allowed a higher temperature of simulated annealing required to sample conformational space of unrestrained atoms, within a computationally tractable simulation time. In the absence of positional restraints the simulated annealing protocol resulted in significant unfolding of the protein. Since the goal of the homology models was to predict the position of active

site atoms assuming the majority of the structure was unperturbed, residues located greater than 15Å from the active site cysteine were heavily restrained with a 50kcal/molÅ harmonic restraint. The 50kcal/molÅ harmonic restraint functioned to constrain these atoms to positions very close to their starting positions, retaining the overall shape of the enzyme while allowing atoms to move closer to the active site area. Atoms outside the active site, but within a 15Å radius of residue 271 were weakly restrained with a 0.2 kcal/molÅ harmonic restraint, allowing some movement of these atoms. Substrates and residues contacting the guanidinium of substrate arginine were unrestrained during simulated annealing. Unrestrained residues included 224, 225, 226, 270, 271, 272, 313, 314, and 315. Coordinates following energy minimization served as starting coordinates, and reference coordinates for positional restraints. Below is an example of the command line input supplied to start a simulated annealing calculation. A sample input file is provided at the end of the chapter under the heading Input files.

```
% sander -O -i sal.in -o sal.out -p prmtop.thiolate -c restart.thiolate -r restart.sal.thiolate
-ref restart.thiolate &
```

```
-O overwrite existing files
-i calls input file named sal.in
-o writes log file called sal.out
-p topology file
-c coordinate file output from energy minimization
-r coordinate output file after simulated annealing
-ref reference coordinate file for harmonic restraints
& run calculation in background
```

Simulated annealing with positional restraints allowed atoms in the active site to move by adding “heat energy” to system. The system was then cooled slowly to 0 K in order to reach a minimum energy level. Simulated annealing allowed the system to sample a greater amount of conformational space than a simple energy minimization, and allowed control models to overcome small energy barriers to adopt a conformation closer crystallographic structures. Preliminary simulated annealing runs were cooled to a more realistic final temperature of 300K, near room temperature, but analysis of potential energy calculated from the resulting models sometimes showed higher potential energy than that that calculated from the model prior to simulated annealing. Although the target

of simulated annealing was to most closely replicate crystallographic results, cooling to 0K ensured that the energy level also dropped following the simulated annealing protocol.

The protocol given in the input file sa1.in was determined empirically to reduce the rms deviation in atom positions between simulated and crystallographic coordinates.

Figure 17 Superimposed models of A) wild type arginine kinase (blue) and E314D (green) determined crystallographically and B) homology models of E314D following simulated annealing.

Optimization of the simulated annealing procedure began with the negative control of causing no damage to the wild type crystallographic structure with protons and artificial solvent added. Next, a homology model of C271A was simulated with and without a chloride ion placed near the position of sulfur of C271 in the wild type structure. A more difficult test involved optimizing homology models of E314D. The crystal structure of this mutant showed that the position of substrate arginine was unaffected in the transition state analog complex, but the loop holding D314 was translated greater than 1Å toward substrate arginine to maintain an interaction with two guanidinium nitrogens (N_7 and N_6). Homology models of E314D based on the wild type structure showed qualitatively similar differences as observed in the crystal structure. Backbone atoms of D314 and H315 move greater than 1Å toward substrate arginine, and the arginine molecule was stationary. Other active site side chains contacting the guanidinium were unperturbed in the homology models and crystal structure of D314 arginine kinase (Figure 17). In some models, a large amount of variation was observed in the output of closely related

simulated annealing simulations. This led to changing the random number seed used by *Sander* to assign initial velocities to atoms at the start of simulated annealing.

Variation of the random number seed sometimes resulted in very different side chain conformations for some active site side chains. Control tests, as well as simulations of mutants of unknown structure show conformational variability in predictable locations. Thus, the variability is not only a measure of the uncertainty of the computational predictions, but a reflection of real disorder in the molecule. More specifically, simulated annealing of the wild type model with different random number seeds resulted in a variety of atom positions for Glu314 and His315 (figure 7). Residues in this loop are completely disordered in substrate free models of both creatine kinase and arginine kinase, and it is reasonable to assume that this loop region may be more flexible than other active site loops. Homology models of mutants do not show significant changes in atom positions other than for these two residues with the exception of the C271S homology models. In the C271S models, the serine side chain of residue 271 is found in a variety of positions. This result is also reasonable, as several side chain rotamers could be modeled in the initial model without causing steric conflict. A variety of hydrogen bonding partners are also available for the serine side chain allowing several different conformations of similar energy.

Analyzing the results

Coordinate files that were output from *Sander* in amber format were converted to protein data bank format using the program *ambpdb*. The command below was used to convert the amber coordinate file, *restart.thiolate*, to the pdb style *thiolate.pdb*.

```
% ambpdb -p prmtop.thiolate <restart.thiolate> thiolate.pdb
```

Protein data bank, or pdb format coordinate files can be visualized by a variety of commonly available software, and analysis of output files should include visual inspection of the models. Statistical differences such as rms deviation in atom positions, can be analyzed using the amber program *Carnal*. *Carnal* is capable of performing a variety of comparisons between pairs of coordinate files. The input file supplied at the

end of the chapter, specifies a reference coordinate file, and an coordinate file resulting from simulated annealing. The script tells *Carnal* to compare atomic positions and list differences in a table with rms deviation of individual atom positions. The GROUP input variable describes which atoms in the coordinate file should be compared. The included example specifies that only atoms of 4 active site residues should be compared for the table, and TABLE command specifies that values in the table should be listed by individual atoms. A variety of other output options are available, as documented in the AMBER6 manual.

Input files

all.in

Minimization with Cartesian restraints

```
&cntrl
  imin=1, maxcyc=10000, {minimization, max of 10000cycles}
  igb=0, ntb=1, cut=8.0, {const. pressure, nonbonded interactions calculate within 8Å}
  ntp=5, DRMS = 0.05    {stop when rms of energy term < 0.05 kcal/mol}
  ntr=1, {harmonic restraint of atoms below, no restraint for unspecified atoms}
&end
Allow all atoms to move:
1          { 1kcal/molÅ harmonic restraint on atom positions}
RES 1 862  { apply restraint to all protein, substrate, and crystallographic water atoms)
END
END
```

sa1.in

15 ps simulated annealing run with restraints

```
&cntrl
nstlim=15000, ntt=1, scee=1.2, ntp=50, pencut=0.1, ipnlty=1,    {max 15000 cycles}
vlimit=20, ntb=1, ntr=1, nmropt=1, tempi=1, heat= 1E-06, ig=702375,{ig=random seed}
&end
{commands below specify temperature and pressure controls during simulation}
&wt type='TEMP0', istep1=0,istep2=1000,value1=10., value2=800., &end
&wt type='TEMP0', istep1=1001, istep2=5000, value1=800., value2=800., &end
&wt type='TEMP0', istep1=5001, istep2=15000, value1=0., value2=0.0, &end
&wt type='TAUTP', istep1=0,istep2=3000,value1=0.2, value2=0.2, &end
&wt type='TAUTP', istep1=3001,istep2=11000,value1=4.0, value2=2.0, &end
&wt type='TAUTP', istep1=11001,istep2=13000,value1=1.0, value2=1.0, &end
&wt type='TAUTP', istep1=13001,istep2=14000,value1=0.5, value2=0.5, &end
&wt type='TAUTP', istep1=14001,istep2=15000,value1=0.05, value2=0.05, &end
```

```

&wt type='END' &end
&rst iat=0,
&end
Group 1 are in the active site, most interesting movement, no restraint:
0.0          {Groups 1,2,3,4 represent different harmonic restraint}
RES 223 226
RES 269 271
RES 312 314
RES 358 358
END
Group 2 are within 15A of active site, less restrained: {harmonic restraint=0.2kcal/mol}
0.2
RES 23 29
RES 43
RES 45
RES 47 92
RES 98
RES 123
RES 125 131
RES 133 139
RES 144
RES 147
RES 187 188
RES 191 200
RES 207
RES 220
RES 222 222
RES 227 229
RES 258 268
RES 272 280
RES 304
RES 306 308
RES 323
RES 325 332
RES 334
RES 337
RES 359 362
RES 367 370
RES 373 374
RES 378
RES 380 381
RES 386
RES 391 392
RES 394 395
RES 397
RES 399

```


RES 402
RES 404
RES 408
RES 412
RES 417 418
RES 420
RES 423
RES 430
RES 432
RES 435
RES 437
RES 447
RES 451
RES 453
RES 457
RES 459
RES 466
RES 471
RES 476
RES 478
RES 489 491
RES 503
RES 510
RES 512
RES 517
RES 526
RES 542 543
RES 571
RES 575
RES 577
RES 579 585
RES 588 590
RES 592 593
RES 605
RES 608
RES 623
RES 626
RES 646
RES 649
RES 658 661
RES 664
RES 669
RES 679
RES 682
RES 699
RES 709

RES 732
RES 737
RES 744
RES 762
RES 768
RES 772
RES 783
RES 803
RES 845
RES 862
END

Group 3 is just substrate arginine, holding should improve fit to exp:

1.0

RES 357 357

END

Group 3 protein and xtal tip outside the 15A sphere, need to restrain more:

50

RES 1 22

RES 30 42

RES 44

RES 46

RES 93 97

RES 99 122

RES 124

RES 132

RES 140 143

RES 145 146

RES 148 186

RES 189 190

RES 201 206

RES 208 219

RES 221

RES 230 257

RES 281 289

RES 297 303

RES 305

RES 309 311

RES 315 322

RES 324

RES 333

RES 335 336

RES 338 356

RES 362 366

RES 371 372

RES 375 377

RES 379

RES 382 385
RES 387 390
RES 393
RES 396
RES 398
RES 400 401
RES 403
RES 405 407
RES 409 411
RES 413 416
RES 419
RES 421 422
RES 424 429
RES 431
RES 433 434
RES 436
RES 438 446
RES 448 450
RES 452
RES 454 456
RES 458
RES 460 465
RES 467 470
RES 472 475
RES 477
RES 479 488
RES 492 502
RES 504 509
RES 511
RES 513 516
RES 518 525
RES 527 541
RES 544 570
RES 572 574
RES 576
RES 578
RES 586 587
RES 591
RES 594 604
RES 606 607
RES 609 622
RES 624 625
RES 627 645
RES 647 648
RES 650 657
RES 662 663

RES 665 668
RES 670 678
RES 680 681
RES 683 698
RES 700 708
RES 710 731
RES 733 736
RES 738 743
RES 745 761
RES 763 767
RES 769 771
RES 773 782
RES 784 802
RES 804 844
RES 846 861
END
END

carnal1.in

FILES_IN
PARM p1 prmtop.thiolate;
STREAM s1 p1 restart.thiolate800nocl;
STATIC ref_set inpcrd.thiolate;
FILES_OUT
TABLE tab1 rms.test2;
DECLARE
GROUP g1 (RES 224, 270, 313, 314);
RMS fit1 FIT g1 s1 ref_set;
OUTPUT
TABLE tab1 fit1%atoms;
END

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BIOGRAPHICAL SKETCH

Relevant Skills:

Recombinant protein expression, protein purification, liquid chromatography; native and denaturing gel electrophoresis; UV/Vis spectroscopy; protein crystallization; single crystal x-ray diffraction data collection, solution and powder x-ray scattering data collection (synchrotron and home source); crystallographic data processing; molecular replacement and crystallographic refinement using: automated refinement, electron density map calculation, and model building; steady state enzyme kinetic assay and data processing; protein homology model building and optimization.

Education:

Ph.D. Biochemistry. Florida State University, Tallahassee, Fl. **2004**

Advisor: Michael Chapman.

Dissertation title: "The active site cysteine of arginine kinase, structure and function of partially active enzyme mutants."

B.S. Biochemistry. University of Virginia, Charlottesville, Va. **1997**

Experience:

Research Assistant- Florida State University, Tallahassee, Fl. **1998 to present**

- Conducted all aspects of a crystallographic and steady state enzyme kinetic analysis of partially active enzyme mutants, including mutagenic primer design, protein expression and purification, steady state kinetic analysis, crystallization, diffraction data collection and processing, structure determination and refinement, and mutant structure analysis.
- Mentored new graduate students in techniques of protein purification, crystallization, crystallographic structure determination, and steady state enzyme kinetic analysis.
- Experienced with Unix, Linux, and Windows operating systems and many software applications.

Teaching Assistant- Florida State University, Tallahassee, FL **1997- 1998**
2002-2003

- Developed leadership, and communication skills teaching recitation sections of 15-30 students.
- Assisted in test and quiz preparation.
- Responsible for organizing and leading study sessions with larger groups of 100-200 students

Presentations:

- "Probing catalysis of a bimolecular phosphagen transferase; Arginine Kinase structure/function." James Gattis, Pamela Pruett, Arezki Azzi, Shawn Clark, Jeff Bush, Mohammad Yousef, Felcy Fabiola, W. Ross Ellington, and Michael Chapman. 2001. American Crystallographic Association annual meeting. Los Angeles, Ca. Poster presentation.
- "Orbital steering and entropy: A bimolecular phosphoryl transferase transition state analog at 1.86Å resolution." James Gattis, Genfa Zhou, Pamela S. Pruett, T. Somasundaram, W. Ross Ellington, and Michael S. Chapman. 1998. Buffalo, NY. American Crystallographic Association Annual Meeting. Lecture.

Publications:

- James L. Gattis, W. Ross Ellington, and Michael S. Chapman. "The active site cysteine of arginine kinase; structural and functional characterization of partially active mutants." 2004. Biochemistry. Accepted pending minor revisions.
- Pamela S. Pruett, Arezki Azzi, Shawn A. Clark, Mohammad Yousef, James L. Gattis, T. Somasundaram, W. Ross Ellington, and Michael S. Chapman. "The putative catalytic bases have, at most, an accessory role in the mechanism of arginine kinase." 2003. Journal of Biological Chemistry. Jul 18; 278(29) 26952-7.
- Mohammad Yousef, Felcy Fabiola, James L. Gattis, T. Somasundaram, and Michael S. Chapman. "Refinement of the arginine kinase transition-state analogue complex at 1.2Å resolution: mechanistic insights." Acta Crystallographica D Biological Crystallography. 2002. Dec; 58(Pt 12): 2009-2017.

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- Hoffmann merit award, Florida State University, 1997.
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