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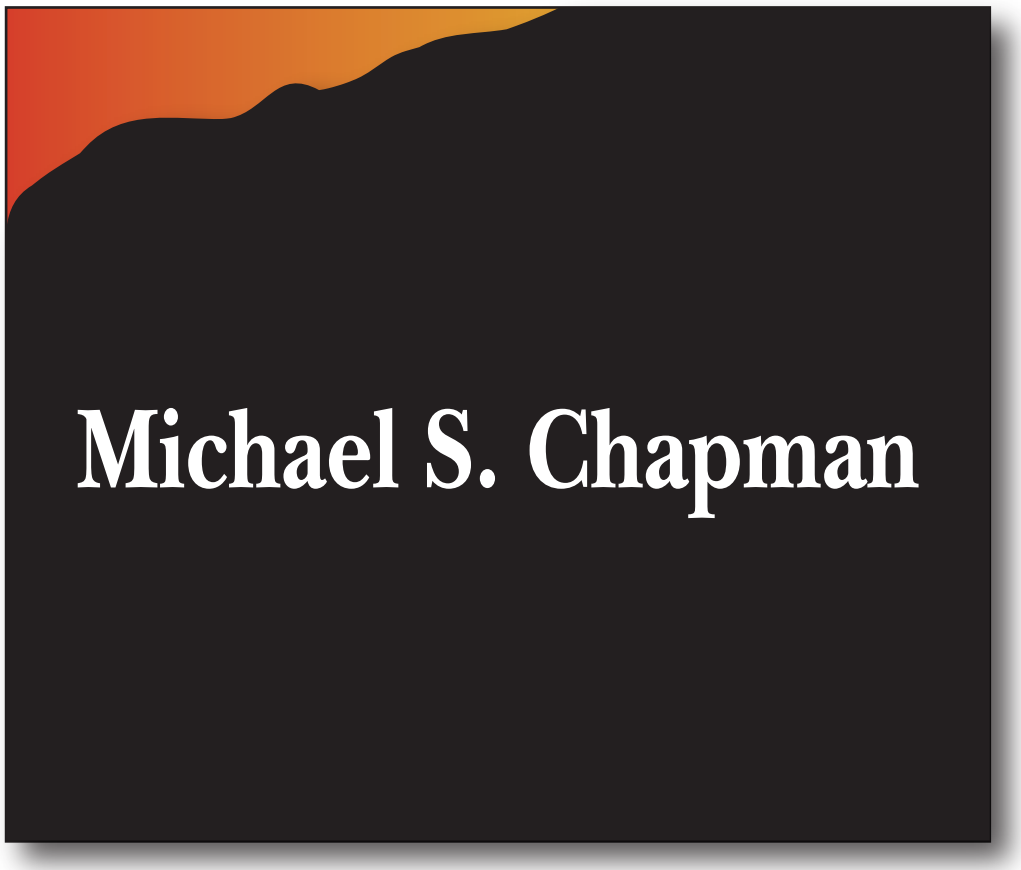
In: "Creatine Kinase": Volume in *Molecular Anatomy and Physiology of Proteins*
Editor: Christian Vial
ISBN: 1-59454-715-7
2006



In: Creatine Kinase
Editor: Christian Vial, pp. 69-94
ISBN 1-59454-715-7

Chapter 3

THE STRUCTURAL ENZYMOLOGY OF ARGININE KINASE AND ITS IMPLICATIONS FOR CREATINE KINASE

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ABSTRACT

The monomeric nature of arginine kinase has facilitated high resolution structural studies. These, and the mutagenic and kinetic analyses guided by structure, have led to re-evaluation of the phosphagen kinase mechanisms of catalysis and specificity. There is not a single mechanism, but several rate-enhancing effects. Base catalysis is likely by a glutamate rather than a histidine, suggesting that proton abstraction is subsequent to N-P bond formation. The "essential" cysteine does not mediate a large confirmation of change, but also appears to be a catalytic accessory. Specificity appears not to be mediated universally by some of the simple lock-and-key mechanism previously proposed. It appears the precise substrate alignment may be a part of the mechanisms of both specificity and catalysis, and that this alignment is a dynamic process coordinated with induced-fit conformational changes.

INTRODUCTION

In a volume about creatine kinase, why is there a chapter about arginine kinase? Arginine kinase offers advantages over creatine kinase in terms of the biochemical and biophysical characterizations possible with a monomeric enzyme, compared to the larger dimers and octamers of creatine kinase (Ellington, this volume). There is often also value in comparing the structures and properties of homologues in determining which are conserved invariants

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and potentially functionally significant features. Combined, these motives have ensured that this arthropod homologue of creatine kinase has been extensively studied [1-3], sometimes leading to insights that would not have been apparent from studies of creatine kinase alone.

Arginine kinase catalyzes the reversible reaction:

$$\text{MgATP} + \text{arginine} \leftrightarrow \text{MgADP} + \text{phospho-L-arginine} + H^{+}.$$

This is analogous to the creatine kinase reaction:

$$\text{MgATP} + \text{creatine} \leftrightarrow \text{MgADP} + \text{phosphocreatine} + H^{+}.$$

Both reactions involve the transfer of a phosphate group to and from a nitrogen atom within a guanidinium group (Figure 1). Away from the reactive guanidinium, the substrates differ in size and charge, creatine with its negatively charged carboxylate, arginine with its zwitterionic amino acid. It is arginine's N_{η} that becomes phosphorylated while N_{ε} is protonated. Creatine differs in that the nitrogen corresponding to the N_{ε} is methylated (Figure 1). In both cases the role of the reaction is in cellular energy homeostasis [4, 5], buffering the concentrations of ATP through reversible transfer of the terminal γ-phosphate to and from the energy storage phosphagen molecule. Creatine kinase is widespread throughout the animal kingdom, including vertebrates, while arginine kinase is present in many invertebrates and lower chordates [2, 5, 6].

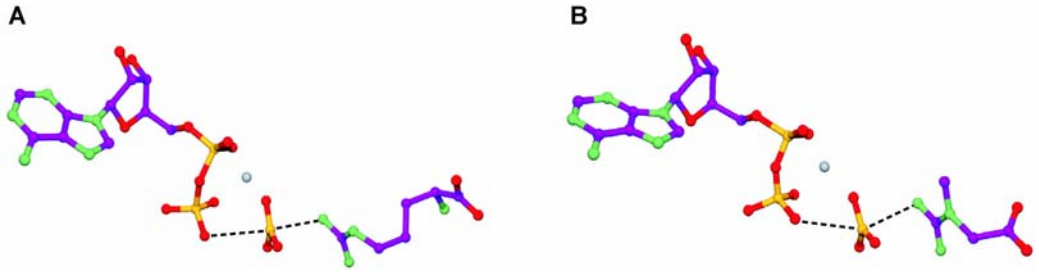

Figure 1: The chemical reactions of arginine kinase (A) and creatine kinase (B) compared, illustrated with the presumptive transition state structures. The atomic coordinates were taken from the crystal structures of the enzyme-bound transition state analog complexes for arginine kinase [12, 32] and creatine kinase [11]. The dotted lines show the bonds, one of which is breaking, the other forming as the phosphoryl group is transferred between the nucleotide (left) and guanidinium (right) of arginine or creatine. The phosphate in substrate and product is tetrahedral, but is thought to pass through a trigonal bipyrimidal phosphorane transition state with inversion of configuration.

On what authority might one presume to extrapolate from the properties of arginine kinase to creatine kinase? *Firstly*, there is the sequence homology. Even the evolutionary divergence of these enzymes is thought to have been ~750 million ago (Ellington, this volume), they share ~ 40 % sequence identity [7-10]. *Secondly*, there is the structural homology. Although the enzymes differ in quaternary structure, each is built around ~ 42 kDa subunits that share the same domain structure (Figure 2). The N-terminal domain of ~100 residues is α-helical with an N-terminal ~30-residue extension in mitochondrial creatine kinases that is associated with targeting. The C-terminal region consists of an 8 stranded anti-

parallel β-sheet flanked by 7 α-helices. Substrates bind in a pocket mostly in the larger C-terminal domain, but bridging to the N-terminal domain. As discussed later, homology extends to the details of the active site. A recent transition state creatine kinase structure [11] is superimposable, nearly exactly, upon the corresponding arginine kinase structure [12, 13]. A *third* reason to expect similar mechanism is the similarity in steady state kinetics, isotopic exchange and product inhibition. These indicate rapid equilibrium, random, bimolecular-bimolecular reactions, although there is a pH-dependent ordering of the reaction in creatine kinase [2, 14, 15]. This final detail sounds a cautionary note, that while we might expect the enzymes to work in predominantly the same way, there may be some subtle differences.

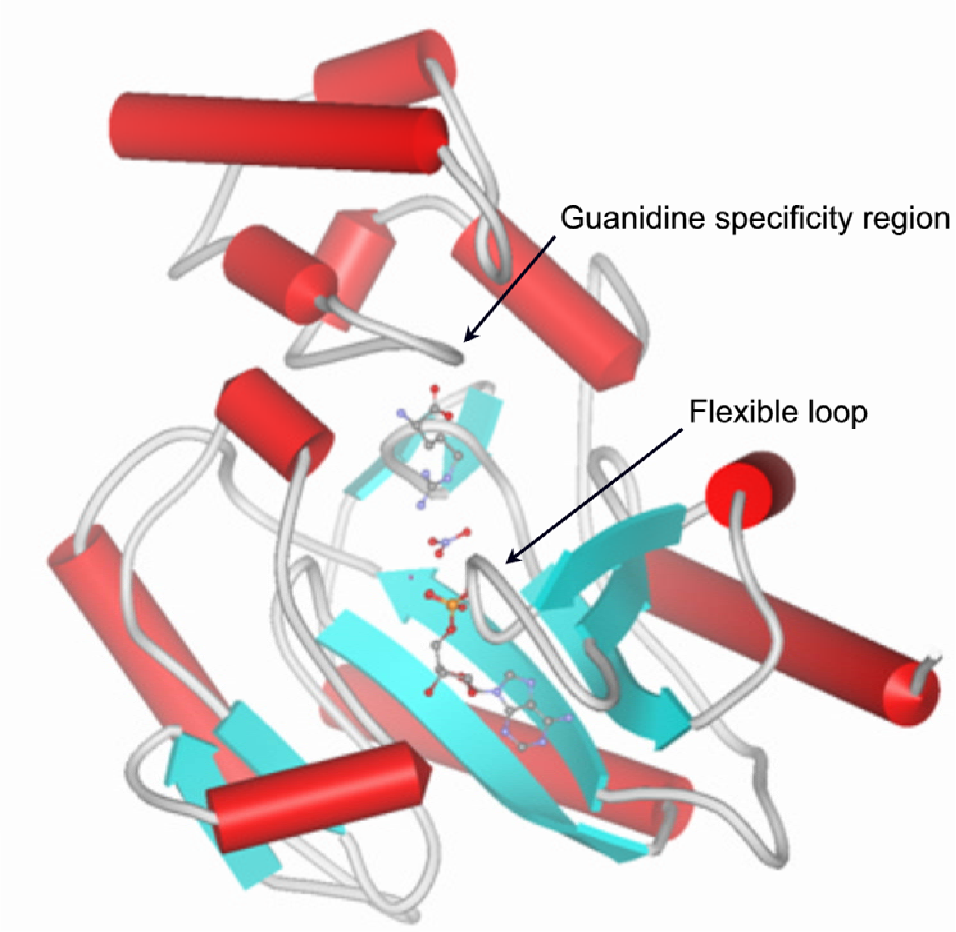

Figure 2: The backbone structure of arginine kinase [12, 13]. Helices are shown as red cylinders, the strands of sheets as blue ribbons. The N-terminal domain comprises the top 1/3 of the structure, and is connected to the C-terminal domain by a flexible loop running down the back. Transition state analog components are shown in ball-and-stick, bridging between the two domains.

This review will focus mostly on advances of the last decade stemming from studies of the structure, specificity and conformational changes of arginine kinase, and their implications for creatine kinase, but we start with some history.

HISTORY OF ARGININE KINASE AS A MODEL; MAGNETIC RESONANCE STUDIES

There has now been a 70 year history of investigating the biochemistry of arginine kinase as one of the models of the phosphagen kinase family. Early reviews cover the classical kinetics, specificity, evidence for conformational change, and identification of active site residues by chemical modification [2]. Subsequent reviews reflect the increasing emphasis on creatine kinase [6, 16], much of it relevant to arginine kinase, and the latter [16] summarizing the understanding of phosphagen kinase enzymology prior to the atomic structures in the mid-1990's.

Use of arginine kinase as a model continued, especially with magnetic resonance studies. These had started in the laboratories of Nathan Kaplan and Mildred Cohn [17, 18]. EPR established that the magnesium salt of ATP was the form required [17]. ^{31}P NMR measurements led to binding constants for the substrates, equilibrium constants for the reaction, and rate constants for the chemical steps (following substrate binding) of the forward and reverse reactions (192 and 154 s^{-1} respectively) [18]. With improved kinetic measurements we now know these to be commensurate with overall turnover rates. Comparative ^{31}P NMR spectroscopy of binary (nucleotide) and transition state analog complexes (see below) provided the first conformational parameters for the nucleotide [19]. Further constraints were added through ^{1}H NMR with paramagnetic ions substituting for the Mg^{++} of the ATP salt [20], and through transferred NOE spectroscopy measurements on nucleotide and transition state analog complexes [21]. Comparison with parallel studies on creatine kinase [22, 23] established that detailed catalytic geometry was homologous in the two enzymes. High consistency between the NMR-derived nucleotide conformational parameters and the subsequent crystallographic transition state analog structure would later establish the relevance of the solid state structure to the solution state reactive form that had been analyzed in the NMR of Rao and colleagues [12]. The recent culmination of Rao's spectroscopy is the tracking of a 1 Å change in the ion – γ-phosphorus distance during the actual reaction with a cobalt salt of ATP [24], offering perhaps the first experimental constraint on a future understanding of the dynamics of the enzyme reaction.

Thus, use of arginine kinase as a model system continued through the 1980's, but its use for biophysical studies expanded with the first cloning, and sequencing of arginine kinase genes in the early 1990's. These were the first phosphagen kinases other than creatine kinase to be sequenced, and the studies were directed mostly towards comparative and evolutionary questions [8, 10, 25, 26]. Cloning also opened the door to mutagenic characterization and over-expression [27, 28]. This led to the availability of large quantities of homogenous samples, facilitating the structural enzymology that has cast new light upon the mechanism of action for phosphagen kinases.

CATALYTIC RESIDUES WITHIN THE ACTIVE SITE

The publication of the (octameric) chicken mitochondrial creatine kinase (Mi_bCK) marked several milestones. Although this family of enzymes were arguably the focus of the largest number of classical enzymological investigations [29], none had succumbed to

structure determination in 25 years of efforts. Now it became possible to interpret the extensive biochemical literature with a structural perspective. The backbone fold was revealed (see above), and by comparing an ADP complex with a substrate-free structure, the active site was located [30]. This structure facilitated later higher resolution structure determinations of arginine and creatine kinases, because experimental phases were no longer required, they could be calculated from a known homologous structure by molecular replacement [31].

The following discussion is based on a transition state analog structure of arginine kinase [12], now refined to atomic resolution (1.2 Å) [32]. The essential features of the active site are conserved in creatine kinase, as established recently by the *Torpedo* transition state structure at 2.1 Å resolution [11]. In both cases, the transition state is thought to involve a pentacovalent (γ-)phosphorane [33, 34] that is partially covalently bonded to both the β-phosphate of the nucleotide and the guanidinyl nitrogen of creatine or arginine (Figure 1). The analog is a highly inhibitory dead-end complex [35, 36] in which the γ-phosphorane is mimicked by a trigonal planar nitrate. The nitrate has no covalent bonding to the other analog components – ADP and arginine (or creatine), but the nitrate mimics the phosphorus and equatorial oxygens. Evidence that the analog complex has similar configuration to the transition state comes circumstantially from its strong inhibition of the reaction, and directly from NMR characterization of the nucleotide structure [19]. Thus the enzyme-analog interactions that are crystallographically observable in the stable complex are likely a close approximation to the reality of the transition state.

Nucleotide Subsite

The most striking feature of the active site is the concentration of arginines counteracting the negatively charged nucleotide phosphates, interacting through salt bridges. Five arginines (1 24, 126, 229, 280 and 309) surround the three phosphates with two nitrogens of each arginine forming salt bridges with one or usually two different phosphate oxygens (Figure 3). All of these arginines are absolutely conserved among all phosphagen kinases, except Arg_{280} which is a leucine or cysteine in the arginine kinases of *Drosophila* and sea hare respectively [unpublished – genbank accession No. P48610 & 37]. All of the phosphate oxygens participate in these arginine interactions except the $O_{1\alpha}$ and the $O_{1\gamma}$, which with $O_{1\beta}$ form the nucleotide ligands to the magnesium ion.

The magnesium ion is in an octahedral coordination with three phosphate oxygen ligands and three waters, all at near-ideal distances between 2.01 and 2.10 Å. At first sight, it may seem strange that there are not direct protein interactions with the Mg^{++}, but each of the water ligands is hydrogen-bonded to a neighboring glutamate, conserved Glu_{224} and Glu_{225}, or Glu_{314} (which is not conserved in creatine kinases). Thus, albeit indirectly, the Mg^{++} bridges between the negatively charged regions of enzyme and substrates.

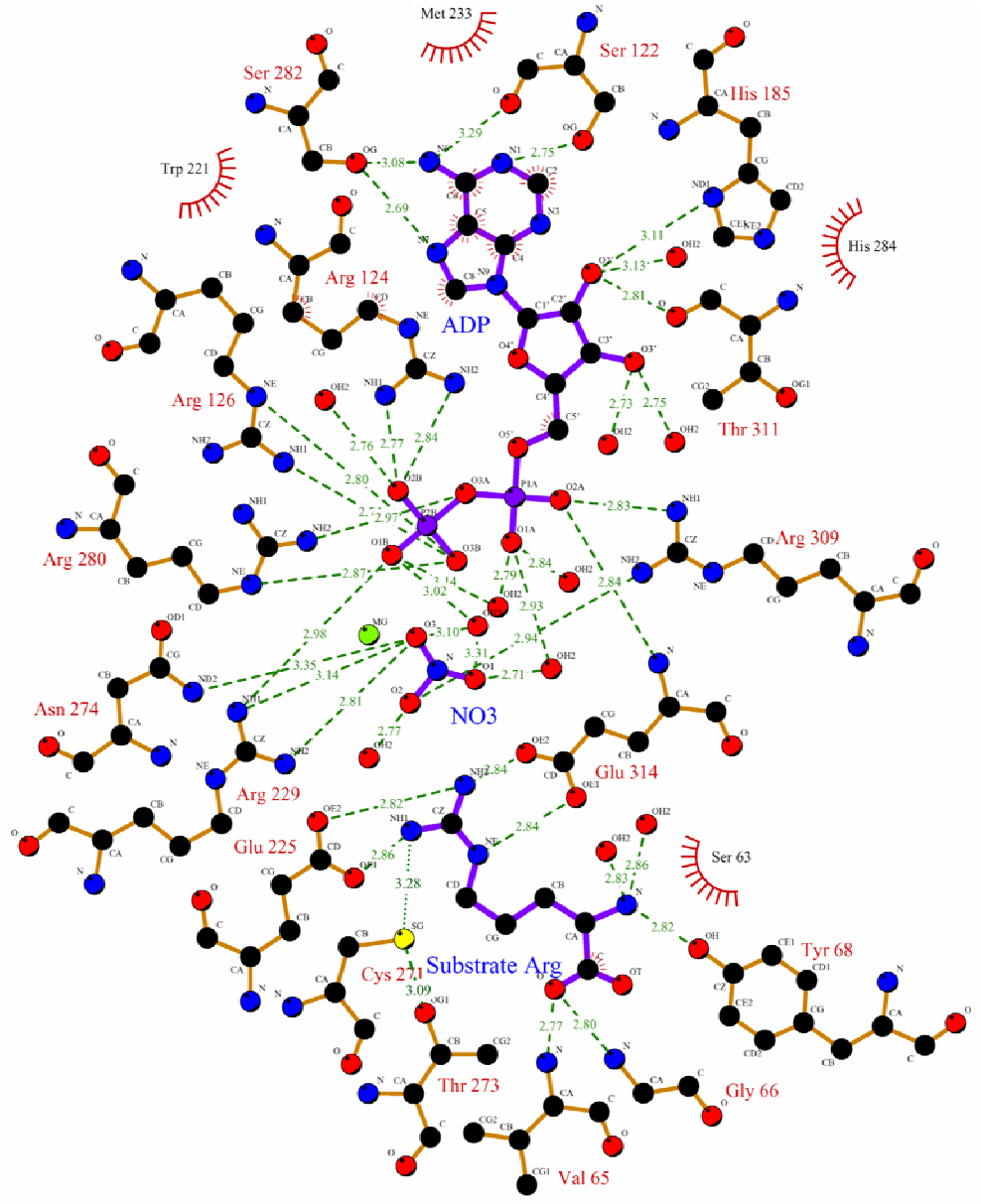

Figure 3: Active site interactions in arginine kinase, drawn schematically with LigPlot [81]. Substrates are shown with purple covalent bonds, active site amino acids with brown bonds. Atoms are colored according to chemical type. Green dashed lines and distances (Å) denote interactions whose geometries are consistent with substrate-active site hydrogen bonding (most protein-protein interactions are not marked). A green dotted line shows an electrostatic interaction between the thiolate of Cys_{271} and the substrate arginine. Water molecules are shown as (isolated) red (oxygen) spheres with an "OH2" label. Hydrophobic contacts are denoted by radial lines showing the contact surfaces of the enzyme residue and substrate atoms. In this schematic figure, atoms in 3D-space have been projected onto a flat plane, then moved to restore appropriate bonding stereochemistry, and to resolve conflicts between overlapping atoms or annotation. It is not intended to convey spatial juxtaposition (which is heavily distorted), but only the hydrogen bonding and salt bridge interactions between enzyme and substrates.

Taken together, the five arginines and magnesium ion counterbalance the negative charges of the nucleotide phosphates. They provide a web of salt-bridge interactions that will have some directional specificity, helping to localize the phosphates. This is particularly true of the $O_{3\beta}$ oxygen that is involved in the formation / breakage of the phosphodiester linkage to the γ-phosphate in ATP. The $O_{3\beta}$ oxygen has three salt-bridge interactions with Arg_{126} and Arg_{280}. Not only does this help localize the reactive oxygen atom, but it influences the relative protonation states of the phosphate oxygens. $O_{2\beta}$ has fewer (two) salt-bridge interactions, and Poisson-Boltzmann calculations [32] give a pK_a indicative that it is mostly in the double-bonded state. ^{31}P NMR measurements show a β-phosphate pK_a elevated from 6 to 7.5 [19], indicating, like the Poisson-Boltzmann calculations, that the remaining ($O_{1\beta}$ and $O_{3\beta}$) oxygens would be significantly protonated in the transition state, sharing about 1.3 protons between the two sites. $O_{1\beta}$ appears to have less electrostatic stabilization of a negative charge. It has a single arginine salt-bridge plus an ionic Mg^{++} interaction, but also two negative charges in close (2.8 Å) proximity ($O_{1\alpha}$ and nitrate (γ-phosphate) O_1). Poisson-Boltzmann calculations indicate pK_as shifted up for $O_{1\beta}$ and down for $O_{3\beta}$. Thus the active site environment is ensuring that in the bound ADP, $O_{3\beta}$ is less protonated than $O_{1\beta}$ and ready for nucleophilic attack upon the γ-phosphorus. It also ensures that there is a proton localized between the $O_{1\beta}$ and the nitrate (γ-phosphate) ready for a concerted transfer from the β- to the γ-phosphate upon making the new phosphodiester bond when ATP is formed [32]. Concerted proton transfers are thought to occur in some other phosphoryl transfer reactions [38, 39].

The γ-phosphate, represented by the nitrate in the transition state analogue structure, is likewise surrounded by salt-bridge and electrostatic interactions. It is sandwiched between conserved Arg_{229} and Arg_{309}, which form salt-bridges to the O_3 and O_2 respectively. The primary interaction of O_1 is with the Mg^{++} ion. All three γ-phosphate oxygen atoms have additional hydrogen-bonded water ligands, again creating a network that constrains the substrate atom positions. The salt-bridge and counter-charge interactions likely draw negative charge away from the phosphorus, enhancing its susceptibility to nucleophilic attack [33].

Phosphagen Subsite

Greatest interest has focused on the guanidino sub-site to which we now move. In the forward reaction, the reactive atoms are the γ-phosphorus and a guanidino η-nitrogen. Interactions with the γ-phosphorus are indirect, as described above, through the phosphate oxygens. By contrast, there is the potential for direct catalytic interactions with the guanidino N_η. Indeed, it was long thought that a catalytic base would figure prominently in proton abstraction.

Candidate Catalytic Bases

An enigma remained following the Mi_bCK structure. Enzyme kinetics and their pH-dependence had implicated a histidine in the proton abstraction from the guanidinium in an early rate-limiting step of the mechanism [15]. The Mi_bCK structure (in the open form) showed no histidine in an appropriate location, but several regions of the active site were disordered. This suggested that a histidine might move into position upon closure of the

active site during the substrate-induced conformational changes [30]. The arginine kinase transition state analog structure revealed the closed form of the active site [12]. Although there was a histidine in the nucleotide binding site, there was still no histidine close to the site of the chemical reaction.

What other residues were in positions to be candidate catalytic bases? We turn again to the arginine kinase transition state analog structure [12]. Through proximity to the γ-phosphoryl-mimicking nitrate, the two chemically equivalent N_η nitrogen atoms of the substrate arginine can be distinguished into "reactive" and "non-reactive" which have been designated arbitrarily $N_{\eta 2}$ and $N_{\eta 1}$ respectively in the atomic structure. In contact with the reactive nitrogen are the carboxylate oxygens of Glu_{225} and Glu_{314}. In sequence alignments [25, 26], Glu_{225} is conserved throughout the family. Glu_{314} was aligned to a valine in creatine kinases, but in a region of poor homology that could accommodate re-alignment to pair Glu_{314} with an adjacent aspartate [12]. This proposal prompted kinetic analysis of site-directed mutants of the aspartate in creatine kinase [40, 41] which appeared consistent with a role in substrate alignment or specificity, but not chemical catalysis. It also prompted analysis of arginine kinase mutants, including a multi-site mutant containing the E314V and other neighboring substitutions, which retained 83% of wild-type activity [42]. By this time, the transition state structure of the *Torpedo* creatine kinase had been determined [11], showing that it was Val_{325} (not its neighbor Asp_{326}) that superimposed upon Glu_{314} of arginine kinase. Together these studies showed that the proposed sequence re-alignment had been incorrect. Glu_{314} was not conserved, and some mutations at this site had little impact. It could be concluded that Glu_{314} did not play a key role.

The transition state arginine kinase structure showed also that Glu_{225} interacted with both the reactive and non-reactive N_η of the substrate [12, 13]. The *Torpedo* creatine kinase transition state structure shows the corresponding Glu_{232} making an analogous bidentate salt bridge with creatine [11]. Visualization of this interaction prompted site-directed mutagenesis in both creatine kinase and arginine kinase [40-42]. In different creatine kinases, conservative mutations to aspartate or glutamine resulted in attenuation of wild-type activity by factors ranging from 500-fold to 90,000-fold respectively [40, 41], leading to the view that this was a critical catalytic residue. Activity in arginine kinase mutants was also attenuated, but to lesser degree. The aspartate and glutamine conservative mutations led to 300 to 400-fold attenuations, but even the non-conservative alanine mutation had detectable activity (3,000-fold attenuated) [42]. This indicates that Glu_{225} may be important, but not absolutely critical, and that the catalytic effect of the glutamate may be one of several that each enhance rate by a couple of orders of magnitude.

The One-time "Essential" Cysteine

For forty years, the role of a completely conserved cysteine (Cys_{271} in arginine kinase) has been debated. Early chemical modification studies with iodoacetamide identified a single essential cysteine per subunit in both arginine and creatine kinases [43, 44]. Creatine kinase, modified by the addition of a smaller ($–SCH_3$) retains significant activity, implying that the cysteine is not essential [45], or that the enzyme was incompletely modified [46]. Mutation in different creatine kinases has led to a variety of results: complete loss of activity in the human brain isoform [HBCK, 47], reduction of activity in the chicken brain [CBCK, 48]]), chicken cardiac mitochondrial (Mi_b-CK) [49] and human muscle (HMCK) [50] isoforms. However

~ ½-wild-type activity is retained at low pH in the C278G mutant of Mi_b-CK [49]. Several of the mutants had altered synergy between the binding of the two substrates, and the consensus emerged that the cysteine was not essential catalytically, but might help mediate substrate-induced conformational changes [45, 49]. Cited in support of a similar role in arginine kinase, were the reactivation kinetics of an enzyme reversibly modified at the cysteine, and the effect of the modification on substrate-induced changes to the UV and fluorescence spectra [51].

A C271A mutant arginine kinase structure in a transition state analog complex is at odds with the purported role of the cysteine in mediating conformational change. The C271A and wild-type structures are nearly identical [52]. All of the major conformational changes between substrate-free and bound forms seen in wild-type [53] have apparently been successfully completed, in spite of the absence of the essential cysteine. The transition state structure showed the cysteine sulfur in contact with the guanidino substrate [12], explaining, through steric overlap, the loss of activity resulting from bulky additions at this site through chemical modification or mutation [43, 44, 48-50]. How can the effects of more conservative modifications or mutations be rationalized? In both arginine and creatine kinases, the loss of activity under different conditions ranges from large to very modest [45, 49, 50, 52]. The contact of the cysteine is to the non-reactive substrate N_η (Figure 3) [32]. Thus, it is possible that modest local changes can be tolerated with loss in activity, depending on the extent of active site perturbation. As discussed later, there is increasing evidence that the cysteine may play an accessory catalytic role, not strictly essential, but enhancing rate through stabilizing a transition state, to an extent that varies with conservative mutation.

The similarity of wild-type and C271A structures [52] also impacts our interpretation of the effects of cysteine mutation upon the substrate-binding synergy that is seen kinetically [49, 50, 52]. Previously the impact of one substrate upon the binding of the other was assumed to be allosteric, mediated by a protein conformational change in which the cysteine was involved [49]. As the cysteine is not mediating a conformational change, it is more likely that synergy involves direct steric or electrostatic interactions between the two neighboring charged substrates that are affected by the proximity of the cysteine, which is likely present in thiolate form [50, 52].

General Lessons from Mutagenesis of the Active Site

Clear from the discussions above, the structures have helped to focus the efforts of several laboratories on a small number of residues. We now have the luxury of comparing the effects at the corresponding sites of many different types of residue substitution in different backgrounds. There is surprising variability in the activities of homologous mutations, even when all are conservative. No trends have emerged that might indicate differences in the reaction mechanisms, rather, we are encountering the vagaries of the methodology. There are many ways that activity can be attenuated, often in unanticipated and collateral effects, perhaps involving perturbation of structure or the electrostatic environment. The details appear to depend upon the background sequence of neighboring residues. Several residues where substitutions were first seen causing massive changes in activity, are now seen as less critical. It suggests that before a residue is deemed critical, that it should be mutated to several other types of residues, and in several of the homologous enzymes. Even this may not be enough. We have an example were one non-conservative mutation was tolerated, but only if a number of neighboring residues were also mutated [42]. An exhaustive search through combinations of multi-site mutations is not generally practical, so there will always remain a

doubt that a way of tolerating a substitution at a particular site has been overlooked, and that a residue is less critical than it appears.

A second lesson from the parallel studies, is that it is no longer sufficient to categorize mutant enzymes as either active, or inactive, and amino acids to be either critical or non-catalytic. It is always tempting to seek a simplified "text-book" mechanism in which there is a dominant way that the enzyme increases rate, such as by a side-chain providing a critical acid-base catalyst. For phosphagen kinases, it appears that several residues have significant, but not dominant impact, and that rate is likely enhanced in several ways that combine to achieve the overall catalytic effect.

SUBSTRATE SPECIFICITY

Further discussion of the catalytic mechanism will follow a review of specificity which also leads to mechanistic insights.

Arginine and creatine kinases are each highly specific for their substrates. Other members of the family a somewhat less specific, e.g. lombricine kinases [54]. Studies with arginine kinase have had implications for the three leading nonexclusive hypotheses proposed to explain the high specificity of arginine and creatine kinases:

The Guanidine Specificity (GS) Region

This region is centered at *Limulus* arginine kinase residues 61 through 65 in the N-terminal domain, a region that is poorly conserved between different phosphagen kinases (Table 1, Figure 2). Here, Suzuki noticed that the length of sequence insertions in lombricine, glycocyamine and creatine kinases are rank-correlated to the size of the guanidino substrate, suggesting a role in specificity [55]. The argument has recently been strengthened by the sequencing of the *Stichopus* arginine kinase sequence [56]. In most respects, this sequence is more similar to creatine kinase than to other arginine kinases, yet it still lacks a creatine-kinase-like insertion at this point. The original prediction was made following the first creatine kinase structure determination [30], but this was in the absence of a creatine substrate, and so the proposal came from sequence alignment alone. However, the substrate-bound structure of arginine kinase [12] showed neighboring (Ser_{63} through Tyr_{68}) residues forming multiple close contacts and hydrogen bonds with the amino acid end of the substrate arginine (Figure 3). This is not the chemically reactive end of the phosphagen, but is the part that differs between the various substrates of the enzyme family. It was very plausible that this loop mediated specificity through a Fisher lock-and-key mechanism [57], in which a long loop physically excluded larger substrates, endowing specificity for the shorter phosphagen substrates.

Table 1: Aligned sequences in two regions proposed to be important in substrate specificity.

	57 68	304 320
GK-sandworm	GVDNPGNKFYGKKTGCVF	RLQGKRGTGGESSLAED
CK-pacific torpedo ray	GVDNPGHP*FIMTVGCVA	RLQ*KRGTGGVDTAAVG
CKB-chicken	GVDNPGHP*FIMTVGIVA	RLQ*KRGTGGVDTAAVG
CKB-Human	GVDNPGHP*YIMTVGCVA	RLQ*KRGTGAVDTAAVG
AK-Horseshoe crab	GVENLDS*****GVGIYA	NLQ*VRGTRGEHTESEG
AK-lobster	GVENLDS*****GVGIYA	SLQ*VRGTRGEHTEAEG
AK-*Drosophila*	GLENLDS*****GVGIYA	NLQ*VANPR*EHTEAEG
LK-earth worm	SVDNTG*****RIIGLVA	HLQ*KRGTGGEHTEAVD

Numbering is according to horseshoe crab arginine kinase. The alignment is a modification of that of Azzi et al. [58]] which was, based upon alignment of the horseshoe crab and pacific ray atomic structures [11, 12] and differs from some others. Asterisks represent imposed alignment gaps

A number of single site mutations in the guanidine specificity region increase real and apparent (kinetic) binding constants, as well as reduce turnover rate [56, 58], apparently supporting the proposed role of the region. However, a chimeric construct indicates that the loop may not be as dominant a specificity determinant or act in the way previously supposed [58]. In this construct, four residues from creatine kinase were inserted into an arginine kinase background. The multi-site mutation clearly affects kinetic measures of phosphoarginine binding (K_M is increased 80-fold) without greatly affecting nucleotide binding (K_M increased two-fold). However, a lock-and-key mediation of specificity would predict that the long loop of creatine kinase would completely exclude arginine phosphate from the active site, yet there is 16% of wild-type turnover.

How could these results for the chimeric construct be reconciled with the mutagenesis of the *Stichopus* enzyme that had greater impact [56]? Firstly, it should be noted that in the work of Azzi *et al.* high turnover was obtained only after several iterations in which the activity was increased in modest steps with mutations at several additional sites. It appears that activity may be acutely sensitive to perturbations at a number of locations close to the active site, more often perhaps through "collateral damage" than through ways that specifically address particular enzyme functions. Had the work of Azzi *et al.* stopped with the first round of low activity mutants, one might have concluded, like Uda and Suzuki [56], that the GS region was a dominant specificity determinant. This is another lesson that mutational loss of activity needs to be interpreted cautiously.

Secondly, Azzi *et al.* [58] speculated that further optimization of the construct might yield more wild-type-like activity. However, it could also be that 16% is the highest activity attainable. Then the partial activity in both *Limulus* and *Stichopus* enzymes could be interpreted as implicating the GS region as one specificity determinant, just not a dominant one. In either case, the third conclusion from the high activity chimeric construct is that substrate is clearly binding and that any influence of the GS region upon specificity is not mediated by a lock-and-key mechanism. Other possibilities could include a role in required

induced-fit conformational changes [57], or substrate alignment – if indeed the region has a role in specificity.

The GS region falls within the N-terminal domain that undergoes an 18º rotation between substrate-free and transition state forms [53]. Regions 223 – 226 and 262 – 275, containing respectively the putative catalytic residues Glu_{225} and Cys_{271} (see above) move with the N-terminal domain as a *quasi*-rigid group [53]. This is consistent with the possibility that domain reorientation might be required to configure catalytic residues, and that the GS region might trigger it in a substrate-specific way. Formation of a salt bridge between Asp_{62} and Arg_{193} appears to stabilize the closed form of the enzyme. Mutational disruption of the interaction in the *Stichopus* and *Nautilus* arginine kinases result in K_M(Arginine) values increased 2 – 4-fold, and activities reduced to 2 – 16% of wild-type [56]. A corresponding D62G mutation within *Limulus* resulted in a 6-fold increase in K_M(Phosphoarginine), but it retained 23% of wild-type activity [58]. (These residues are not conserved in creatine kinases, but the interaction might be replaced by an analogous His_{66}-Asp_{326} interaction [11]). Uda and Suzuki [56] conclude that the salt bridge is crucial, but when *Limulus* is also considered, the results are not unequivocal. If domain motions are involved in specificity, then a number of individual interactions might be important, and site-directed mutagenesis alone might never be fully conclusive. In fact, the structure of the mis-matched creatine – arginine kinase complex (below [58]) indicates that specificity is not mediated through gross conformational changes, as the protein structure is essentially identical to the closed form of the cognate (native) transition state analog complex.

That the GS region may not be the dominant specificity determinant is also suggested by the recent transition state analog complex structure of *Torpedo* creatine kinase [11]. Extrapolation from the arginine kinase complex [12] might have predicted intimate interactions between the GS region and the carboxylate group of creatine, perhaps modified to complement the negative charge of the substrate. Instead, one finds only one direct enzyme-substrate interaction, between the backbone nitrogen of Val_{72} and creatine's carboxylate. The other interactions with the carboxylate are all water-mediated hydrogen bonds – there are no potentially substrate-specific salt-bridges.

Creatine Methyl Specificity Pocket

In the transition state analog complex structure of *Torpedo* creatine kinase, Lahiri *et al.* [11] noted another possible specificity determinant - the methyl group that is unique among phosphagens to creatine fits into a pocket formed by Ile_{69} and Val_{325}. This pocket is not pre-formed, but the residues come together from flexible loops, as indicated by comparing the nucleotide-bound and transition state structures [11]. Ile_{69} and Val_{325} are conserved among, but unique to creatine kinases. Ile_{69} is one of the 4 residues inserted (relative to arginine kinases) in the GS region. Val_{325} occupies the position of Glu_{314} in *Limulus* arginine kinase. This change complements differences between the substrates. Glu_{314} forms a salt bridge with arginine's N_ε. In creatine, the corresponding nitrogen lacks the proton and is methylated instead. The smaller, hydrophobic Val_{326} is more appropriately matched in size and non-polarity perhaps to discriminate between creatine and other phosphagen substrates [34, 36].

The methyl specificity pocket hypothesis has been tested through three mutational studies. Among glycine or alanine mutations to five sites in the GS region of *Danio* creatine

kinase, I69G had second highest K_M(creatine) and second lowest V_{max} [56]. The changes are modest (6-fold on K_M; 1.5% wild-type activity), but this might be expected of an isoleucine to glycine mutation that would not sterically block creatine binding. In a second study, the V325A mutation in human muscle creatine kinase led to a 6-fold increase in the activity with a glycocyamine substrate, and a 100-fold decrease in the specificity for creatine relative to glycocyamine [59]. This is consistent with the methyl specificity pocket hypothesis, because glycocyamine lacks the methyl group, but is otherwise identical to creatine.

The third study involved creation of a chimeric construct in which the entire flexible loop in arginine kinase was converted to its creatine kinase sequence-equivalent [58]. This involved five substitutions R312G, E314V, H315D, H317V and E319V, with E314V corresponding to the Val_{326} site in *Torpedo* creatine kinase. An interesting side point, is that this multi-site mutant was much more active than either of the single site "conservative" mutants E314D or E314Q [42], highlighting again the context dependence of mutational effects and the challenge of interpreting decreased activity or binding. The main point, however, was that the multi-site mutant carried 64% of the wild-type activity, and the relative binding of arginine *versus* creatine was unchanged. Interpreting together the arginine kinase chimeric construct and the creatine kinase V325E mutant, it seems likely that the methyl pocket is a specificity determinant in creatine kinase, but is not important in arginine kinase, and presumably the other phosphagen kinases because their substrates that also lack the guanidinium methyl group.

Summarizing to this point, the "GS region" and "creatine methyl specificity pocket" hypotheses initially looked promising, but neither appear to be dominant determinants of specificity throughout the family. To this point, nothing fully accounts for all of the high specificity seen in many of the phosphagen kinases.

Substrate-specific Substrate Alignment

In an attempt to cast light upon this enigma, the atomic structure of a mis-matched complex has been determined – arginine kinase with creatine and MgADP bound [58]. Crystallographic observation that creatine is able to bind in the arginine kinase active site has the immediate qualitative implication that this specificity is not mediated by lock-and-key exclusion. Differences between the cognate arginine complex and the mismatched creatine complex are subtle, with a root mean square C_α difference of 0.5 Å, implying that creatine is just as capable as arginine of inducing most, if not all of the substrate-induced conformational changes. All of the differences from the cognate arginine kinase complex [32], and from the cognate *Torpedo* cognate creatine-creatine kinase complex [11] are small, but potentially the most significant are in the guanidinium group of the substrate. The methyl group (that is unique to creatine) would overlap with arginine kinase's Glu_{314} without the 38º rigid rotation of the guanidinium that is observed in the mis-matched complex. This change stands out, because the substrates in the cognate complexes of creatine and arginine kinases superimpose nearly exactly [11, 32]. The result is that the reactive $N_{\eta 2}$ is withdrawn 0.32 Å from its position in cognate complexes. This impacts the alignment of substrates. The complex was crystallized in the presence of creatine and ATP (no nitrate), but in the structure, the ATP has apparently been hydrolyzed, so the location of the γ-phosphate must be estimated by

superimposing the nitrate-containing transition state analog complex on the mismatched complex. This indicates that the angle of approach at the reactive η-nitrogen has been perturbed by 12º in the non-cognate complex.

A second difference in the mis-matched complex is elevation of the substrate thermal "B" parameters. Thermal factors are proportional to the mean square displacement of atoms about their mean position. In the cognate arginine kinase complex, the average B-factor for substrate arginine atoms (12.6 Å²) is slightly lower than that of the average protein atom (15.6 Å²). Small molecule substrates have fewer covalent constraints upon atomic positions than residues within a polypeptide chain. Thus, low B-factors indicate enzyme-substrate interactions that hold substrate atoms in relatively fixed positions. In the mis-matched complex, the creatine atoms have an average B-factor that is nearly three times higher, indicating that the enzyme fails to constrain the position of this substrate analog. Taken together, it looks as if in the mis-matched complex, the enzyme is less able to precisely pre-align the substrates and hold them fixed in this configuration. Both effects could lead to a loss of reactivity.

Failure to hold substrates in a favorable pre-alignment could be the denominator common to mutational studies of the GS region and the large domain flexible loop. Such an explanation should be regarded as hypothetical at the moment. The structural work provides a qualitative rationale for one mis-matched complex, but the extrapolation of its results to the interpretation of mutational data, while plausible, has not yet been tested.

IMPLICATIONS FOR THE CATALYTIC MECHANISM

Substrate Alignment May be One of Several Catalytic Effects

The residues that appear to have greatest direct impact upon catalysis (Glu_{225} and Cys_{271}) each offer enhancements of two orders of magnitude, perhaps less, falling at least two orders of magnitude short of the overall catalytic effect. The estimated catalytic impacts are upper limits – mutants characterized in the future may show activity more like wild-type. Additional points should be noted. Glu_{225} and Cys_{271} could be exerting their effects in any one or a combination of ways, including general acid-base catalysis (with or short of formal proton transfer), strain favoring the transition state over the Michaelis complex (sterically through hydrogen-bonding, or through electrostatic polarization), or through their effects in constraining an optimal precise pre-alignment of substrates. Precise alignment could account for part the impact of Glu_{225} and Cys_{271}, as well as some of the hitherto unexplained catalytic effect. There are several reasons for suspecting that this is true:

1. A role for pre-alignment is also indicated by the structure of the unreactive mis-matched complex discussed in the previous section [58].
2. In the structures of the partially active mutants E314D and E225Q, subtle mis-orientations of the substrates are among the few changes seen [42].
3. Perturbation of precise pre-alignment could offer a superficial rationalization for the wide variation seen in the activities of analogous mutations introduced into different enzyme homologues [40-42, 49, 50, 56, 58, 60, 61].

Note that a central role for substrate alignment is emerging through exclusion of prior hypotheses. There are certainly other active site residues whose effects have not yet been studied. The argument for substrate pre-alignment remains largely circumstantial without strong direct proof.

Several Catalytic Effects

The case was made above that substrate pre-alignment might have an important role. Here, it is emphasized that it is not the only catalytic effect. Text book enzyme mechanism schematics generally emphasize the importance of one or two active site residues in, for example, acid-base catalysis. This would be an over-simplification for phosphagen kinases in which several modest catalytic effects appear to be combined.

Cys_{271} Electrostatic and Acid-base Effects

The partial attenuation of activity in some mutations at Cys_{271} indicate that the cysteine is a contributor to (just) part of the catalytic effect without being absolutely essential [52]. While its interaction with the guanidium could help to fix the substrate in a preferred orientation, at least some of the catalytic effect can be attributed to electrostatics, as indicated by the partial rescue of non-negatively charged mutants (e.g. C271A) with chloride ions [52]. There are several other lines of evidence indicating that the cysteine is active in thiolate form: (1) Kinetic pK_as determined from creatine kinases mutated at the "essential" cysteine [50]; (2) Poisson-Boltzmann electrostatic calculations for the arginine kinase transition state analog structure that show the pK_a of Cys_{271} perturbed down by > 2 pH units to 6.1 [52]; (3) Finding of a chloride ion at the site of the missing sulfur in a structure of the C271A arginine kinase mutant, the presence of which presents a clear rationale for the chloride rescue of selected mutants [52].

How could the catalytic effect of Cys_{271} be mediated through electrostatics? Although the cysteine is a neighbor of the non-reactive guanidinium nitrogen (Figure 3), the presence of the negatively charged thiolate form could draw positive charge to the non-reactive $N_{\eta 1}$ away from the reactive $N_{\eta 2}$. This would perturb the resonance of the guanidinium, and enhancing the nucleophilicity of the reactive $N_{\eta 2}$ for the forward reaction in its lone-pair attack upon the γ-phosphorus of ATP [52]. For the reverse reaction, the same electrostatic perturbation of the guanidinium would accentuate the leaving-group properties of the arginine substrate. Taken to an extreme, the electrostatic perturbation might go as far as formal abstraction of a proton by the cysteine from the non-reactive N_{η}, though there is certainly no need for this in achieving some catalytic enhancement [52]. Taken with the lack of impact upon conformational change (the C271A transition state structure discussed earlier), it seems likely that through direct electrostatic interactions, Cys_{271} modestly enhances catalysis as well as impacts substrate binding synergy.

Glu_{225} Positioning, Acid-base and Strain Effects

Glu_{225}, the other residue implicated as a catalytic accessory, could act in three ways. Firstly, it has salt-bridge interactions with both N_{η} of the substrate arginine, and helps to precisely position the guanidinium group. There are subtle changes in substrate alignment in

the E225Q structure, such as perturbation of the attack angle at the reactive $N_{\eta 2}$ by 9º [42]. It is not yet known whether such subtle perturbations can fully account for the observed 3,000-fold attenuation of activity [42], but there are other ways that Glu_{225} could enhance activity. The salt bridge hydrogen bonds from Glu_{225} and Glu_{314} are out of plane, distorting the geometry of the reactive nitrogen towards an sp^3 configuration that would result presumably from the addition of the new N—P bond [12], although such distortions in hydrogen positions are thought not to be of sufficient energy to have great impact upon catalysis. The greatest contribution to catalysis might be Glu_{225}'s potential general acid-base catalysis [12, 42]. It had long been thought that proton abstraction from the reactive nitrogen would be an early rate-limiting step [15]. However, if proton abstraction preceded N—P bond formation, then catalysis by a high-pK group would be expected for efficient isoergonic protein transfer. Glutamate has a low pK, ideal for proton abstraction only if the reactive $N_{\eta 2}$ has changed from basic to acidic with the prior formation of the new N—P bond [12]. This would require a transient tetracovalent configuration of the nitrogen, supported by the out-of-plane Glu_{225}/Glu_{314} salt-bridges that appear to be stabilizing such a configuration (above).

Concerted Proton Transfer

As ATP product is formed in the reverse reaction, the γ-phosphate must gain a proton. The precision of the 1.2 Å resolution transition state arginine kinase structure is sufficient to indicate that it comes from the β-phosphate. The van der Waals separation of two oxygen atoms is 3.2 Å, but the observed distance is 2.8 Å ± 0.06 Å (error estimated from a cross-validated Luzzati plot) [32]. The closer separation is only possible with a hydrogen bond between the two oxygen atoms. Molecular mechanics calculations were consistent with the structure only if the $O_{1\beta}$ of the ADP of the transition state analog were uncharged – double-bonded or protonated [32], while Poisson-Boltzmann calculations indicate that it is predominantly protonated at pH 7.5 due to the proximity (~2.8 Å) of charged α- and γ-phosphate oxygens in the bound configuration of the nucleotide. Transfer of the proton in the reverse reaction would push electrons from $O_{1\beta}$ to P_β, reducing the double-bonded character of the $O_{3\beta}$—P_β bond, enhancing the nucleophilicity of $O_{3\beta}$ (Figure 4). Nucleophilic attack upon P_γ would release electrons from the $O_{1\gamma}$ for attack upon the $H_{1\beta}$, completing a cycle. This all indicates a concerted proton transfer as proposed for some other kinases [38, 39], with the proton and phosphoryl transfer reactions enhancing each other.

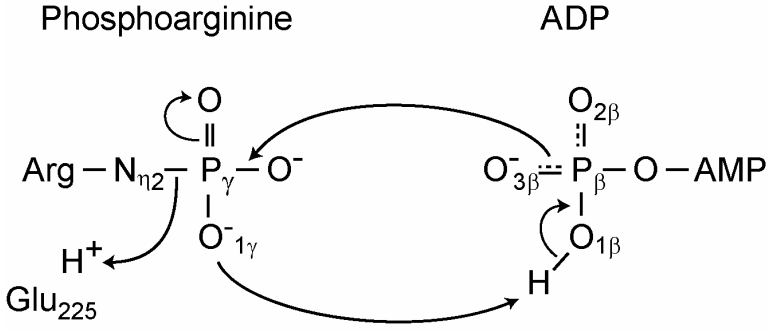

Figure 4: Proposed concerted proton transfer, shown here schematically in the reverse reaction (adapted from [32]).

A Tentative Catalytic Mechanism

It is helpful to bring together the potential rate enhancement effects into a plausible catalytic mechanism. The discussion above has detailed why a mechanism would, in detail, be almost completely different from prior prevalent proposals for creatine kinase involving initial proton abstraction by a catalytic histidine [15]. However, it is still thought that the mechanisms within enzyme family would be fundamentally the same. The mechanism proposed is consistent with the preponderance of structural and kinetic data from arginine and creatine kinases, but not with all. It should be regarded as very tentative, with several ambiguities, but nevertheless useful in helping to frame the future tests that might establish the mechanism on a firmer footing.

The proposed mechanism is shown for the forward reaction in Figure 5. Rate-enhancing effects can start upon substrate-binding with preparation for nucleophilic attack. The reactive groups are held in close to ideal alignment, Glu_{225}, Cys_{271} and (in some phosphagen kinases) Glu_{314} positioning the guanidinium, while Arg_{229} and Arg_{309} help position the γ-phosphate. Many of these residues may have other roles. Cys_{271} likely acts by polarizing the guanidinium to give the reactive $N_{\eta 2}$ more lone-pair character, thereby enhancing its nucleophilicity. This is an indirect effect, exerted through the non-reactive $N_{\eta 2}$. Simple electrostatics are certainly in play as indicated by the partial rescue of activity in cysteine mutants, through addition of chloride bound close to the sulfur position [49, 52]. It is also possible that full or partial proton abstraction could contribute with the Cys_{271} thiolate acting as a general base or as a hydrogen bond acceptor, although it is noted that the angle of the sulfur's approach is far from that of an ideal hydrogen bond acceptor.

The susceptibility of the P_γ to nucleophilic attack will be increased by enhancement of its electrophilicity [33]. This could be accomplished by the salt-bridges and positive counter-charges surrounding the γ-phosphate (Arg_{229}, Arg_{309}, Mg^{++}) which would draw electron density towards the oxygens, which would also stabilize the putative pentacovalent phosphorane [34] of the transition state (Figure 1). Formation of the new N—P bond would also leave the $N_{\eta 2}$ at least transiently in a tetracovalent configuration, favored, but perhaps only marginally, by the salt bridge interactions of Glu_{225} and Glu_{314} that are out of plane and straining away from the starting trigonal-planar $-N_{\eta 2}H_2$ configuration. This now-acidic nitrogen would tend to rehybridize with loss of a proton. Glu_{225}, already salt-bridged to the $N_{\eta 2}$, would appear to be the most natural proton acceptor. It is not yet known what part of Glu_{225}'s impact upon rate (~400-fold as judged from the E225Q mutation [42]) could be attributed to general base catalysis of this proton abstraction. It is also remotely possible that Cys_{271} could abstract a proton, then one be transferred from $N_{\eta 2}$ to $N_{\eta 1}$. Another unknown is when the proton abstraction happens. The lack of a high-pK catalytic base indicates that the active site has been optimized for isoergonic transfer from an acidic $N_{\eta 2}$, and that proton abstraction would likely follow or be simultaneous with N—P bond formation [12].

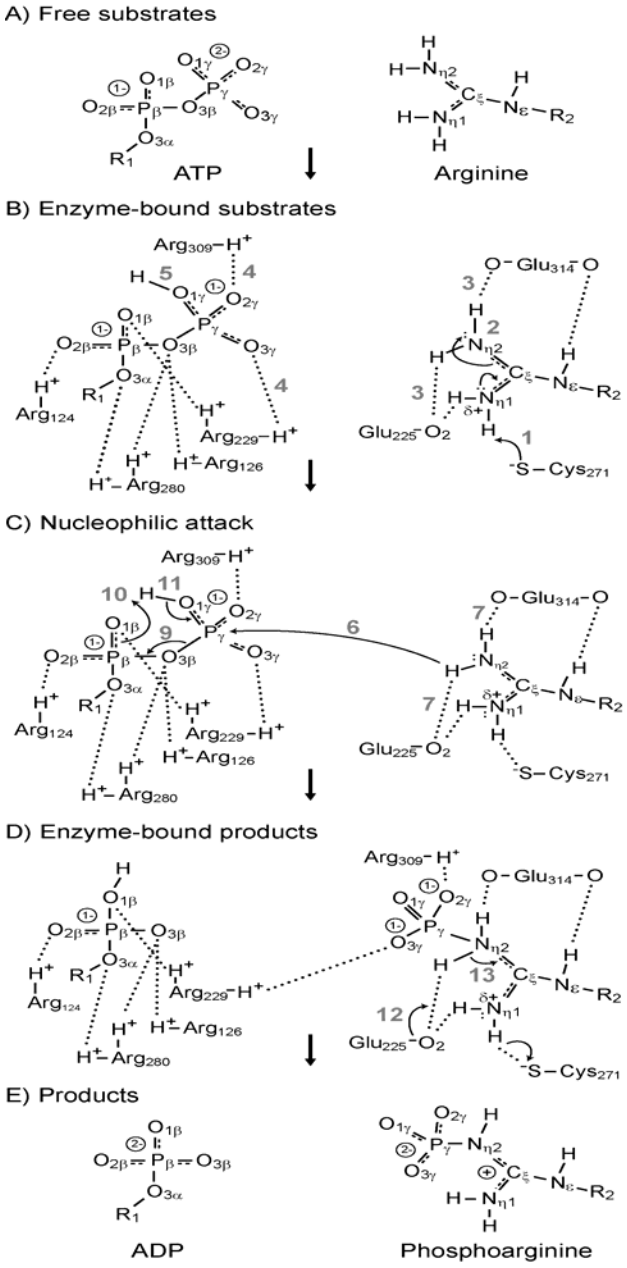

Figure 5: A tentative catalytic mechanism for the arginine kinase forward reaction. The schematic is consistent with the prevailing data available at this time, but it has not been tested experimentally, or through quantum calculations, so should be regarded as a proposal. Substrates for the forward reaction are shown in panel A in one of several possible tautomeric forms. Salt bridges are shown in dotted lines, as determined for the transition state analog structure [12, 32], and are therefore a slight extrapolation to the Michaelis and product complexes shown in panels (B) through (E). Opportunities to enhance the rate are annotated 1 through 13, and begin with substrate binding (panel B). (1) The thiolate form of Cys_{271} likely pushes electrons towards the reactive nitrogen in either of two ways: (a) perhaps by (partially) abstracting a proton from $N_{\eta 1}$ through formal proton transfer or through hydrogen bonding; and probably (b) electrostatically, by pushing electron charge from the $N_{\eta 1}$ and favoring a polarization of the guanidinium in which $N_{\eta 1}$ and C_{ζ}. are predominantly double-bonded; (2) The

consequence would be to reduce the double-bonding between C_ζ and $N_{\eta 2}$, allowing the development of more lone pair character on the reactive $N_{\eta 2}$. (3) Salt bridges (dotted lines) from Glu_{225} and Glu_{314} may help to pre-align the guanidinium ready for nucleophilic attack. (4) Salt-bridges from Arg_{229} and Arg_{309} similarly may help to align the γ-phosphate, draw negative charge away from the P_γ electrophile, and (5) favor protonation of $O_{1\gamma}$ over other sites. (6) Formation of the new N—P bond is initiated by lone pair attack from the $N_{\eta 2}$ upon the P_γ.. (7) Addition of a new bond would result in a tetracovalent configuration of the $N_{\eta 2}$, made more favorable by the out-of-plane salt bridges of Glu_{225} and Glu_{314}. (8) It also results in a pentacovalent transition state phosphorus, stabilized by the electron-withdrawing effects of the salt-bridges to $O_{3\gamma}$ and $O_{2\gamma}$.. (9) This would break down upon cleavage of the P_γ—$O_{3\beta}$ bond, with migration of the electrons to the $O_{3\beta}$ favored by the salt bridges from Arg_{126} and Arg_{229}. (10) This in turn likely prompts a migration of electrons from the P_β—$O_{1\beta}$ bond to the proton that is transferred concertedly. (11) The resulting migration of electrons to the $O_{1\gamma}$—P_γ bond would increase its double-bonded character, further facilitating cleavage of the P_γ—$O_{3\beta}$ bond. (12) Following formation of the new N—P bond, the tetrahedral acidic $N_{\eta 2}$ would be expected to rehybridize and loose a proton. The most likely proton acceptor would appear to be Glu_{225}. (13) Electron migration from the abstracted proton would restore $N_{\eta 2}$ to a trigonal configuration and the guanidinium back to its resonant form.

The pentacovalent phorphorane of the transition state would also be unstable. Cleavage of the P_γ—$O_{3\beta}$ would be facilitated bond by the salt bridges from Arg_{126} and Arg_{229} which would favor migration of the electrons to the $O_{3\beta}$. The proposed concerted proton transfer (above) would apply, but now in the direction of the forward reaction. The increased double-bonded character of P_β—$O_{3\beta}$ will decrease the double bonding in P_β—$O_{1\beta}$ with electron migration towards the $O_{1\gamma}$ proton that appears to be transferred to the $O_{1\beta}$ concertedly with phosphoryl transfer. Loss of the proton would increase the double-bonded character of the $O_{1\gamma}$—P_γ bond which, in turn, would further facilitate cleavage of the P_γ—$O_{3\beta}$ bond.

In summary, there are many points in this proposed mechanism at which the enzyme might enhance the rate. Quantitatively, the relative impact of these potential effects remains to be established.

SUBSTRATE-INDUCED CONFORMATIONAL CHANGE

Members of the phosphagen kinase family have long been known to undergo large substrate-induced conformational changes [62, 63]. Prior to the atomic structures, these were best documented through radius of gyration measurements by x-ray solution scattering for both arginine and creatine kinases [3, 64, 65]. The radii differed according to the quaternary structure that was different for the two enzymes, but these studies established that the subunit conformational changes were likely fundamentally similar. Changes in both cases were greater upon addition of MgATP than for the addition of arginine or creatine. Completion of the transition state analog complex from the binary nucleotide complex led to minor additional changes. The magnesium salt of ATP was required to elicit the change, free nucleotide having no effect.

By 1998, there were in hand atomic structures of the open form of creatine kinase [30] and the closed transition state analog complex form of arginine kinase [12]. It then became possible to model the closed form of creatine kinase by homology to arginine kinase [66], or the open form of arginine kinase by homology to creatine kinase [67]. While most of the differences were likely due to the substrate-induced changes, these extrapolations glossed

over the possible effects of dissimilarity between creatine and arginine kinases of quaternary structure and primary sequences. These were considered necessary evils while crystals of closed form of creatine kinase and open form of arginine kinase remained elusive. With the *Torpedo* creatine kinase structure has come the possibility of comparing transition state and MgADP forms with the same sequence [11], and of comparing this closed form with open structures of creatine kinase that have the same quaternary structure and more similar sequences [66, 68, 69]. The most recent arginine kinase structure, in substrate-free form, now allows detailed comparison of open and closed forms with exactly the same sequence, finally removing this uncertainty and superseding the earlier homology models [53].

An approximate view of the conformational changes can be sketched as a ~18º rotation of the N-terminal domain (residues 1 – 99) relative to the C-terminal domain (residues 100 – 357). The latter contains most of the active site, and a flexible loop (residues 309 – 320) that refolds itself over the active site following substrate-binding [67]. However, closer comparison of the paired substrate-free and transition state arginine kinase structures reveals that the details are more complicated [53].

Transformations that map individual amino acids from one conformation to the other, cluster into four groups that correspond each to *quasi*-rigid-group rotations [53]. One can be arbitrarily fixed for reference, and we choose the one that corresponds to most of the C-terminal region (residues 98 – 352), excepting several regions that themselves move predominantly as independent rigid groups, known as “dynamic domains”. These differ from conventional domains where the term usually applies to a distinct globular unit containing contiguous elements of sequence. By contrast, “dynamic domains” consist of residues that move together and are therefore often close in three-dimensional space, but need not be contiguous in sequence [70]. Within the C-terminal region, there are two dynamic domains in addition to the fixed domain. Dynamic domain 2 comprises residues 169 – 219, except for 7 loop residues (198 – 204) (

Figure). Domain 2 contains one of the residues (conserved His_{185}) that moves furthest, closing the gap with conserved His_{284} of the fixed domain, and sandwiching the base of the nucleotide between. In the closed configuration, a new salt bridge is formed between Arg_{192} of this dynamic domain and Asp_{62} of the N-terminal region, offering a tantalizing suggestion of how nucleotide binding might be communicated to the N-terminal domain. However, the salt bridge is not conserved in creatine kinases, and mutation of either residue to disrupt the salt bridge leads to different levels of attenuation in the arginine kinases from different organisms, leading to differing views of its importance [56, 58].

Dynamic domain 3 contains two distinct regions of the sequence (112 – 123 and 286 – 328) that come together in the folded subunit. Rotation of this group appears to be associated with bringing flexible loop 309 – 320 into position over the active site. Changes to this loop represent the largest of the local conformational changes that are overlaid upon the rigid domain rotations. In substrate-free structures of both arginine and creatine kinases, this loop has either indistinct or no electron density, characteristic of a highly flexible or disordered loop [30, 53, 66, 68, 69, 71, 72]. Its structure becomes well defined only in association with substrates [11, 53]. The loop appears to be functionally important, not only in helping to close off the active site [67], but also in dynamically forming critical parts of the active site. In arginine kinase, Glu_{314} forms salt bridges to the reactive $N_{\eta 2}$ and the N_{ε} of the substrate [12]. In creatine kinase, the Val_{325} at this position forms part of the creatine methyl specificity pocket, while Asp_{326} forms a salt bridge with His_{66} of the guanidino specificity region of the

N-terminal domain (see above) [11]. Residues 311 and 314 (arginine kinase numbering) form conserved backbone hydrogen bonds with a ribose hydroxyl and the α-phosphate respectively (Figure 3), while the side chain of Arg_{309} forms conserved salt bridges with the α and γ-phosphates. With all of these nucleotide interactions, it is hardly surprising that the loop configuration changes upon binding.

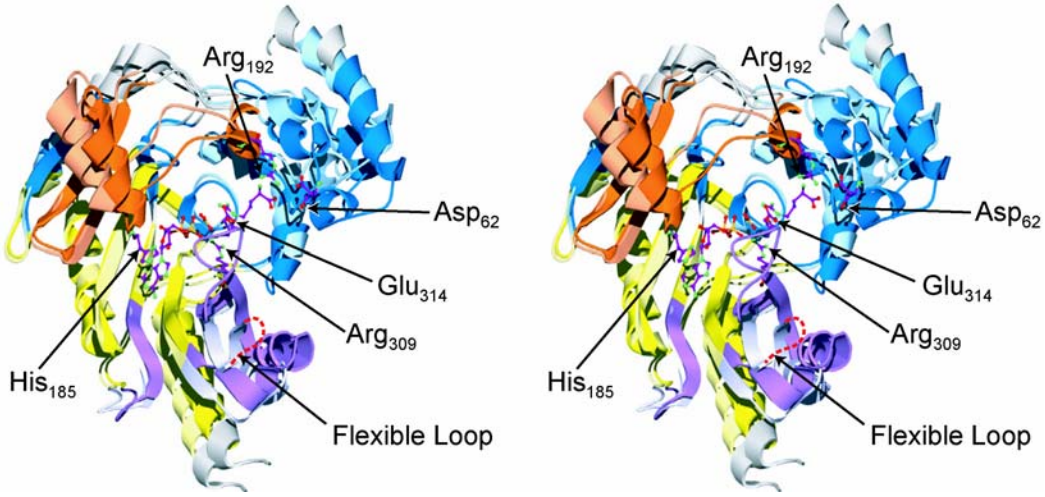

Figure 6: Conformational changes in arginine kinase. Stereo images show ribbon representations of the open substrate-free (feint hues) and closed transition state forms (darker hues), color coded according to dynamic domains [53]: Blue (#1) – residues 7 – 97, 129 – 136, 147 – 155, 164 – 168, 223 – 226 and 262 – 275; Orange (#2) – residues 169 – 197 and 205 – 219; Purple (#3) – residues 112 – 123 and 286 – 328; Yellow (fixed) – residues 98 – 111, 124 – 127, 156 – 163, 220 – 222, 227 – 261, 276 – 285 and 329 – 352. Substrates are shown in ball-and-stick, as are the side chains of several residues to highlight particular aspects of the conformational changes. These include His_{185} of dynamic domain 2 that undergoes a large motion towards the nucleotide; Arg_{192} and Asp_{62} that form a salt bridge; Glu_{314} and Arg_{309} that are part of the disordered flexible loop (unseen in the substrate-free form) that folds into a more ordered configuration on top of the substrates.

Dynamic domain 1 contains the entire N-terminal (globular) domain including the guanidino specificity region, and also including four segments from the C-terminal (globular) domain. Two of these segments contain catalytically important conserved residues that interact with the phosphagen substrate. Glu_{225} that has been discussed in regard to substrate positioning and base catalysis (above) falls within a conserved NEEDH sequence that moves with the N-terminal domain [53]. The "essential" Cys_{271} that has figured prominently in discussions of catalysis (above) also moves with the N-terminal domain, linked in part by a hydrogen bond between conserved neighbor Pro_{272} and Tyr_{72} that is retained through the conformational changes [53].

The conformational changes likely have purposes similar to those suggested for other induced-fit kinases. Closure of the dynamic domains and flexible loops around the substrates restricts solvent access, diminishing the chance of wasteful ATP hydrolysis, as does configuration of essential active site residues only after substrates have been bound [73, 74]. Although motions between rigid groups can in principle be of either shear or rotational type [75, 76], all of the motions here are hinged rotations. This is consistent with analysis of the atomic resolution (1.2 Å) refinement of the transition state analog complex [32]. At such high

resolution it is possible to analyze the anisotropic components of the small harmonic fluctuations in atomic positions that reflect dynamic motions or static disorder. Atoms whose anisotropic displacement parameters are correlated can be grouped into *quasi*-rigid groups [77]. Such analysis for arginine kinase bore close, but not exact semblance to the dynamic domains defined by comparison of open and closed structures. The rigid-body components of the motions could account for most of the variation in displacement parameters by residue number, and decomposition of the rigid motions into translation, libration and screw tensors [78] showed that the motions were almost entirely librational rotations about axes that corresponded closely with the axes of dynamic domain hinge rotations between open and closed forms. The close correspondence of harmonic fluctuations in the transition state structure with the much larger, presumably non-harmonic changes between conformational states, suggests strongly that the enzyme has intrinsically "soft" modes of inter-domain rotational flexibility that are extended greatly when substrate-induced transitions occur.

Loop flexibility has also been measured for creatine kinase in solution using hydrogen/deuterium exchange followed by quenching, proteolysis and measurement of the deuteration distribution by mass spectrometry [79]. Out of peptide fragments that covered almost all of the rabbit muscle creatine kinase sequence, those that contained the guanidino specificity region, and the C-terminal flexible loop had the largest proportion of fast exchange backbone amides. Details are covered elsewhere in this volume, but these studies indicate that the regions identified crystallographically as the most flexible are also those most mobile in solution.

Dynamics in both creatine and arginine kinases are likely to remain of continued interest, because it has become clear that the alignment of substrates and configuration of active site residues that are important to rate enhancement and specificity are achieved only after substrate-induced conformational changes. The turnover rates of both arginine and creatine kinases are commensurate with the rates of other enzymes limited by large conformational changes. Thus it is likely that an understanding of the enzyme dynamics will be key to understanding how the enzyme works.

CONCLUSION

Although the catalytic mechanisms are fundamentally similar, idiosyncratic differences between corresponding mutations engineered in different phosphagen kinases have shown the value of pursing parallel mechanistic investigations in at least two of the homologs. In recent years, progress on a new understanding of the catalytic mechanism and in how specificity is achieved has been led by studies with arginine kinase, inspired by and corroborated by studies of creatine kinase. Progress has been easier, in part, due to the greater tractability of sample preparation, biochemical and biophysical characterization with a monomeric enzyme. Creatine kinase will be the prime focus of studies relevant to mammalian physiology, quaternary structure or cellular targeting, but the technical tractability of arginine kinase will ensure that it will continued to be probed as a mechanistic model for the phosphagen kinase family.

NOTE ADDED IN PROOF

Quantum mechanical calculations are suggesting further ways that the reaction would be catalytically enhanced. Natural bond orbital analysis indicates that the phosphate protonation state affects and electronic transition from a lone pair orbital of a phosphate oxygen to an anti-bonding orbital in phosphoguanidines. This presents an additional opportunity for the enzyme to enhance cleavage in the reverse reaction [80].

ACKNOWLEDGEMENTS

The author gratefully acknowledges the contributions of many co-workers and colleagues who have contributed to the material reviewed here: Arezki Azzi, Donald "Jeff" Bush, Shawn Clark, Omar Davulcu, W. Ross Ellington, James Gattis, Pam Pruett, Eliza Ruben, Thayumanasamy Somasundaram, and Genfa Zhou. Nancy Myer and James Gattis and are thanked for help with the illustrations. This work was supported in part by NIH grant GM 1R01-55837.

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