The Putative Catalytic Bases Have, at Most, an Accessory Role in the Mechanism of Arginine Kinase*

Arginine kinase is a member of the phosphagen kinase family that includes creatine kinase and likely shares a common reaction mechanism in catalyzing the buffering of cellular ATP energy levels. Abstraction of a proton from the substrate guanidinium by a catalytic base has long been thought to be an early mechanistic step. The structure of arginine kinase as a transition state analog complex (Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W. R., and Chapman, M. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8449–8454) showed that Glu-225 and Glu-314 were the only potential catalytic residues contacting the phosphorylated nitrogen. In the present study, these residues were changed to Asp, Gln, and Val or Ala in several single and multisite mutant enzymes. These mutations had little impact on the substrate binding constants. The effect upon activity varied with reductions in $k_{cat}$ between 3000-fold and less than 2-fold. The retention of significant activity in some mutants contrasts with published studies of homologues and suggests that acid-base catalysis by these residues may enhance the rate but is not absolutely essential.

Crystal structures of mutant enzymes E314D at 1.9 Å and E225Q at 2.8 Å resolution showed that the precise alignment of substrates is subtly distorted. Thus, pre-ordering of substrates might be just as important as acid-base catalysis. Pre-ordering and alignment of substrates likely contribute more to the catalysis of bimolecular reactions than the better characterized unimolecular reactions, but there is little consensus on its importance relative to acid-base chemistry, strain, and other catalytic effects (4–6). One challenge was a lack of high resolution structures of multisubstrate enzyme complexes in which the alignment of two substrates could be viewed without perturbation or constraint, an obstacle overcome first with the structure of arginine kinase (7).

Several phosphagen kinase structures have become available recently, including creatine kinases from chicken muscle mitochondria (8), rabbit muscle (9), chicken brain (10), human mitochondria (ubiquitous) (11), and arginine kinase from horseshoe crab (12), all structures determined in the open, inactive configuration. The structure of arginine kinase was also determined as a transition state complex (TSAC educate Mg-ADP, nitrate, arginine) (7) in a closed conformation with ordered active site loops. This structure prompted re-examination of the catalytic mechanism here and elsewhere (see below).

The recent structure of creatine kinase from Torpedo californica revealed one subunit in a binary Mg-ADP complex, the other as TSAC, confirming that the transition state structures of creatine and arginine kinases were very similar (13). Prior to these structures, biochemical kinetics, chemical modification, and site-directed mutagenesis led to a proposed mechanism of in-line phosphoryl transfer involving abstraction of a guanidinyl proton as an early step, catalyzed, it was long thought, by a histidine (14). The arginine kinase TSA structure, in fact, showed only two residues, Glu-225 and Glu-314, in contact with the phosphorylated guanidinium nitrogen (Fig. 1). If the guanidines of the glutamates were matched to the reactive arginine for efficient isoergonic proton transfer, then it implied that proton abstraction would not be initial but subsequent to or coincident with addition of the new N—P bond when the nitrogen would have lower pK (7). Consistent with this, the glutamates were positioned for interactions favoring a tetrahedral reactive nitrogen. The TSAC structure, now refined to 1.2 Å resolution (15), suggested another potential role of the glutamates. The reactants were all aligned precisely within 3° of optimal for in-line transfer. This raised interesting questions.

Phosphagens and their corresponding phosphagen kinases play a central role in energy homeostasis in cells with highly variable rates of ATP turnover (1, 2). Phosphagen kinases are a conserved family of phosphofructokinase enzymes that includes creatine, arginine, glycoamylase, laminin, tauocytamine, and hypotaurine. All are thought to share a common mechanism by which one of the guanidinyl nitrogens becomes phosphorylated. Arginine kinase is widespread in invertebrates and may be the primordial enzyme because of its widely available substrate, monomeric structure, and presence in protozoa (2). The reversible reaction catalyzed by arginine kinase is: phosphoarginine + Mg-ADP ⇔ arginine + Mg-ATP.

Phosphagen kinases are among the most intensively studied by classic enzymology (3) and constitute paradigms for the fundamentals of catalysis of multisubstrate reactions. Pre-ordering of substrates likely contribute more to the catalysis of bimolecular reactions than the better characterized unimolecular reactions, but there is little consensus on its importance relative to acid-base chemistry, strain, and other catalytic effects (4–6). One challenge was a lack of high resolution structures of multisubstrate enzyme complexes in which the alignment of two substrates could be viewed without perturbation or constraint, an obstacle overcome first with the structure of arginine kinase (7).

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about the relative roles of acid-base chemistry and substrate pre-ordering in the catalysis of this multisubstrate reaction and the roles in each played by these two glutamates.

Glu-225 is highly conserved, and mutagenesis soon suggested that the corresponding residues in creatine kinase were critical for (acid-base) catalysis: Glu-227 and Glu-232, respectively, in the human mitochondrial and muscle enzymes (16, 17). In contrast, the role of arginine kinase in substrate pre-ordering is less well defined, with a number of mutations in the enzyme in different species and in heterologous expression systems that suggest a different interpretation of catalysis and kinase structure (13), it could also be aligned with a neighboring aspartate, conserving a carboxylate throughout the family (7). Mutagenesis of Asp-326 in human muscle creatine kinase indicated an essential role, perhaps in substrate binding (17). Here we report mutagenesis and structural studies that suggest a different interpretation of catalysis and the roles of these amino acids.

EXPERIMENTAL PROCEDURES

Materials—All were obtained from Sigma, Fisher Scientific, and Roche Applied Science unless otherwise noted.

Original Clone—Two variants of an arginine kinase expression system were used, both derived from a pET 22b (Novagen) plasmid clone (19) that contains a selectable ampicillin resistance gene. Escherichia coli strain BL21(DE3)/pLyS8 was transformed for expression of protein, whereas strain DH5α was used to produce plasmid DNA for mutagenesis. The first expression system was the same as used in the original structure determination (7, 20) and is termed AKorig. Sequencing, prompted by the structure, revealed PCR-generated nucleotide mis-incorporations leading to four amino acid changes, E103Q, D112G, G116A, and K351R. These were reverted back to the native sequence using the QuikChange mutagenesis kit (Stratagene) consecutively, in the order G112D, Q103E, A116G, R351K, using plasmid from the prior round of mutagenesis as the template for the next round. Sequences were confirmed using an ABI model 3100 DNA sequencer. The final native clone has been designated as reverted wild type AKrev.

Mutagenesis was carried out using both AKorig and AKrev templates and the QuikChange kit. Sequences of constructs were confirmed using an ABI model 3100 DNA sequencer. The final native clone has been designated as reverted wild type AKrev.

Expression and Protein Purification from AKorig—Small cultures of E. coli containing the plasmid were grown overnight at 37 °C in Luria broth with 100 μg/ml ampicillin. One-liter cultures were inoculated with 20 ml of this overnight culture, grown to mid log phase (optical density 660 nm = 0.5), and protein expression was induced with a final concentration of 1 μM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested at 5–6 h postinduction and lysed in a French pressure cell. Protein was expressed as inclusion bodies, unfolded in 8 M urea, and refolded by sequential dialysis against decreasing concentrations of urea. Enzyme from this system was purified by previously reported chromatographic methods (20).

Expression and Protein Purification from Wild Type AKrev—The same procedures were used for E. coli culture and induction. However, protein was mostly in the soluble fraction after lysis. The properly folded and active protein was put directly onto a DEAE anion exchange column after dialysis against running buffer (10 mM Tris, pH 8, 0.1 M KC1, 1 mM dithiothreitol, 1 mM EDTA, 0.02% NaN3) and eluted with a 0–200 mM KC1 gradient. This was followed by size exclusion chromatography on a S100 Sephacryl column. Chromatography was carried out using an APTA FPLC (Amersham Biosciences) system.

Concentration—Protein was concentrated for crystallization using Centricron-10 microconcentrators or an Amicon cell with 10-kDa molecular mass cutoff YM-10 membranes. Protein concentration was determined spectrophotometrically at A280, using an extinction coefficient of 0.76 mmol−1 cm−1. For enzyme dialyzed against TSA components, the concentration was determined microscopically and by gel electrophoresis.

Enzyme Kinetics—Phosphoryl transfer was assayed in the reverse direction by measurement of the increase in absorbance at 340 nm due to production of NADPH resulting from linkage of ATP production to the hexokinase and glucose-6-phosphate dehydrogenase reactions (21). Note, however, that the substrate was creatine kinase, the native binding constants for arginine and Mg-ATP for the forward reaction are similar to those for the reverse direction (phosphoarginine and Mg-ADP) (22, 23). Kinetics assays were set up in a 6 × 6 matrix of ADP versus phosphoarginine concentrations, with phosphoarginine between 0.2 and 3.2 mM and ADP between 0.03 and 0.06 mM. Concentrations of ADP in assay stocks were verified by UV absorbance, whereas phosphoarginine concentrations were determined by a spectrophotometric enzymatic assay using highly purified arginine kinase. Assays were conducted in triplicate at 25 °C using a Varian Cary 3E UV-VIS spectrophotometer. Data were analyzed using SigmaPlot (SPSS, Inc.).

Crystallization—AKrev protein at 10–20 mg/ml was dialyzed against transition state analog components (7) and crystalized by vapor diffusion at 4 °C. Crystals of E314D measuring ~1.0 × 0.3 × 0.3 mm2 were obtained through both macro-seeding (24) and de novo set-ups. For seeding, a 10-μl drop comprised of 5 μl of 20 mM/ml protein plus 5 μl of 26% PEG6000, 0.025 mM HEPES, pH 7.5, 0.05 mM MgCl2 was aliquoted onto a coverslip and then seeded with a piece of an AKrev crystal (grown using the same conditions). The drop was then equilibrated for vapor diffusion against 26% PEG6000. For de novo crystallization of E314D, the drop contained 5 μl of 20 mg/ml protein plus 5 μl of 32% PEG6000, 0.025 mM HEPES, pH 7.5, and 0.05 mM MgCl2, for a drop concentration of 16% PEG6000. It was equilibrated by vapor diffusion against 28% PEG6000. The same conditions were used for de novo crystallization of E225Q, except that drops were made from 2 μl of ~24 mg/ml protein solution plus 2 μl of 26% PEG6000. De novo crystals of E225Q were not of sufficient quality but were used for microseeding (24) identical conditions.

Data Collection—Diffraction data were collected with an R-Axis II image plate detector (Rigaku/Molecular Structure Corporation) and a rotating anode x-ray source at our in-house facility. E314D/TSA and E225Q/TSA data were collected at 100 K using 25% PEG6000 + 20% glycerol as the cryoprotectant.

Structure Determination—The Denzo/Scalepack/HKL suite (25) was used for data processing. Rotation and translation searches were carried out with GLRF (26), using TSAC arginine kinase as a model. Manual rebuilding using the program "O" (27) was alternated with atomic refinement using the CNS package (28).

RESULTS

Expression and Crystalization—Conversion of the original arginine kinase construct (AKrev) to the wild type construct (AKorig) unexpectedly increased the solubility of the expressed enzyme. AKrev found predominantly in inclusion bodies, had to be unfolded and refolded before purification (20), resulting in final yields of 10–30 mg/liter of E. coli culture. The conversion of AKrev to AKorig (actually the change G112D) resulted in more enzyme in the soluble fraction. Each 1-liter culture of E. coli provided 100–200 mg of protein, yielding 80–100 mg after purification. The recombinant enzymes from AKorig and AKrev as well as that isolated from horseshoe crab muscle, had indistinguishable kinetic parameters (data not shown). However, high quality crystals have only been obtained for AKrev. Extensive efforts to crystallize the four conservative single-site mutant proteins led to diffraction grade crystals for two, E225Q and E314D.

Kinetics—Kinetic values from the 6 × 6 matrix assay yielded Km, Vmax, and kcat values for the wild type mutants (Table I). Here, Km is defined as the ternary steady-state constant (a measure of binding the second substrate in this random-order rapid equilibrium reaction (22)), whereas Kmax denoted as KA by some authors, is the “initial” binary steady-state constant. Kinetic plots for the wild type mutants were consistent with the random order bi-bi mechanism (data not shown). Individual mutations to Asp and Glu at positions E225 and E314 and the double mutation E225Q/E314Q had little impact on the Km, Kmax, and kcat values for ADP and phosphoarginine yet reduced the catalytic activity 60–500-fold (Table I). Substrate binding synergy, one substrate facilitating the binding of the second and characterized by a = Km/KS < 1, is similar to that of chicken cardiac mitochondrial and rabbit muscle creatine kinases (29, 30). There is a partial loss of synergy with mutation at Glu-225, but the change is not always experimentally significant and involves different effects upon Km and Kmax for different mutants. Overall, catalytic activity is affected much more than substrate binding.
### Table I

**Kinetic analysis of the wild type and seven mutants in the reverse direction (phosphoarginine → ADP)**

<table>
<thead>
<tr>
<th>Arginine kinase</th>
<th>Wild type (15)</th>
<th>E314D</th>
<th>E225Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_r$ (ADP) mm</td>
<td>0.29 ± 0.09</td>
<td>0.16 ± 0.03</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (ADP) mm</td>
<td>0.16 ± 0.06</td>
<td>0.12 ± 0.02</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.36 ± 0.02</td>
<td>0.14 ± 0.04</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.14 ± 0.04</td>
<td>1.3 ± 0.5</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.48 ± 0.25</td>
<td>0.88 ± 0.25</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.48 ± 0.11</td>
<td>0.77 ± 0.19</td>
<td>ND*</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.48 ± 0.24</td>
<td>0.49 ± 0.26</td>
<td>0.039 ± 0.039</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.48 ± 0.11</td>
<td>0.49 ± 0.26</td>
<td>0.039 ± 0.039</td>
</tr>
<tr>
<td>$K_r$ (Arginium) mm</td>
<td>0.48 ± 0.24</td>
<td>0.49 ± 0.26</td>
<td>0.039 ± 0.039</td>
</tr>
</tbody>
</table>

*The percentage rate shown for E225A is based on specific activity, which was too low for measurement of bimolecular steady state kinetic parameters.

### Table II

**Crystalllographic and refinement statistics**

<table>
<thead>
<tr>
<th>Arginine kinase</th>
<th>Wild type (15)</th>
<th>E314D</th>
<th>E225Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal form</td>
<td>P2$_1$2$_1$2$_1$</td>
<td>P2$_1$2$_1$2$_1$</td>
<td>P2$_1$2$_1$2$_1$</td>
</tr>
<tr>
<td>a, Å</td>
<td>65.4</td>
<td>65.4</td>
<td>65.5</td>
</tr>
<tr>
<td>b, Å</td>
<td>70.3</td>
<td>71.1</td>
<td>71.3</td>
</tr>
<tr>
<td>c, Å</td>
<td>80.1</td>
<td>80.5</td>
<td>80.1</td>
</tr>
<tr>
<td>Temperature, K</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>6-1.2</td>
<td>10-1.9</td>
<td>10-2.8</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.2</td>
<td>98</td>
<td>97.4</td>
</tr>
<tr>
<td>Redundancy*</td>
<td>7.8</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>0.06</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>4.8</td>
<td>6.6</td>
<td>12.8</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>10.8</td>
<td>18.1</td>
<td>17.2</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>12.3</td>
<td>23.5</td>
<td>24.6</td>
</tr>
<tr>
<td>Cross-validated Luzzati</td>
<td>0.04</td>
<td>0.27</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*Average number of observations of each reflection

### The most surprising result came from a multisite mutant that included the non-conservative mutation E314V. This chimeric construct, containing several substitutions of creatine kinase residues into arginine kinase, was part of an ongoing study of substrate specificity. It became relevant to this study with the unexpected finding of only 2-fold loss of activity despite a non-conservative change at one of the putative catalytic bases. The non-conservative mutation at the other glutamate, E225A, showed 3,000-fold reduction in activity relative to wild type, precluding full kinetic analysis but confirming that this residue also is not absolutely essential for catalysis.

### E314D/TSA Structure

The crystal of E314D was nearly isomorphous with wild type (Table II), and its structure was solved by molecular replacement using the CNS package (28). Cross-rotation and translation searches using the wild type transition state model (7) yielded a unique solution, rotated 1.9° from the wild type with slightly different crystal packing. The final model includes an ADP molecule, a nitrate, one Mg$^{2+}$, a substrate arginine, and 395 water molecules. It has $R/R_{free}$ of 0.18/0.24 and only 0.6% of disallowed residues.

Overall, the E314D mutant structure is similar to wild type, with an overall root mean square difference between the Cα atoms of the two structures of 0.39 Å. Localized differences near the loop containing 314 include (Fig. 1C): (i) movement of the side chain carboxylate of E314D by 0.7 Å so that separate $O_{41}$ and $O_{42}$ are replaced by a single bridging interaction of $O_{42}$ to both the $N_{35}$ and $N_{42}$ (Fig. 1D); (ii) movement of the loop near 314 by up to 0.9 Å (Fig. 1C); (iii) small changes in the alignment of the substrate analogs (Fig. 1E and Table III), due to movements of the ADP phosphates, Mg$^{2+}$, NO$_3^-$, and arginine by up to 0.25 Å (detailed below); and (iv) a movement of His-315 away from the active site, 1.5 Å at the Cγ atom (Fig. 1C). The movement of His-315 and the 180° flipping of its $\chi_2$ side chain torsion angle establishes a possible 2.6-Å salt bridge interaction with the terminal carboxyl group of the substrate arginine that is not present in wild type (Fig. 1D). It also disrupts a water-mediated interaction to the backbone carbonyl of residue 62, on the other flexible loop, reducing the distance from 5.9 to 3.7 Å, too short for an intervening water but too long for a direct hydrogen bond. However, this has little impact on the backbone structure near residue 62.

The changes in substrate alignment arise mostly from a translation of the nitrate by −0.25 Å away from Arg-309 and a
small tilting of the nitrate plane so that it is not so orthogonal to the direction of in-line transfer. Although the mutated Asp-314 is a contact residue 3.7 Å from the nitrate O1, there is not a specific interaction that causes the move, but the mutation would change the local electrostatic environment. The move of the nitrate is small but clear from the electron density. The ADP/H9252-phosphate and substrate arginine guanidinium trans-rate roughly parallel but by a smaller amount (0.2 Å). The result is little change in the directions of nucleophilic attack from the ADP O3 or the guanidinium N2 but distortions in the angle of approach to the nitrate plane (that is mimicking the transferring phosphoryl) of up to 9°.

Excluding the loop containing the E314D, the protein is essentially unchanged and the root mean square difference is nearly halved to 0.22 Å. Conserved arginines 124, 126, 229, 280, and 309 maintain unchanged interactions with substrate phosphate groups with, at most subtle, change to accommodate the slightly altered phosphate positions. The “essential” Cys-271 and its interactions are unperturbed.

E225Q/TSA Structure—The crystal was also nearly isomorphous with wild type (Table II), and its structure was similarly solved by molecular replacement. The final model contained an ADP, a nitrate, an Mg2+-ion, a substrate arginine, and 126 water molecules. It has R/Rfree values of 0.17/0.25, and only 0.6% of φ, ψ angles are “generously allowed” (none disallowed) (31) (Table II).

Overall, the changes of E225Q compared with wild type are more modest than for E314D. The overall root mean square difference in Ca atoms between E225Q and wild type is 0.27 Å. At the site of mutation, the backbone shift at residue 225 is small, <0.2 Å. The largest changes are once again in the side chain of His-315, with a shift at Cy of 0.5 Å (Fig. 1D), smaller than in E314D and not enough to disrupt the water-mediated interaction with the carbonyl of residue 62. The closer interac-
tion of His-315 with the substrate carboxylate (Fig. 1D) appears to have little impact upon binding (Table I), presumably because it is offset by the loss of similar ionic interactions between the guanidinium and Glu-225 as it is changed to Gln. Although there are other changes in hydrogen bonding, the interactions of the reactive N$_{\text{ae}}$ with residues 225 and 314 that favor transition toward a tetrahedral nitrogen appear to be unaffected (Fig. 1D). As in E314D, the linearity of phosphoryl transfer is perturbed (Fig. 1D). As in E314D, the linearity of phosphoryl transfer is perturbed (Fig. 1D). As in E314D, the linearity of phosphoryl transfer is perturbed (Fig. 1D).

The transition state structure of arginine kinase (7) had implicated Glu-225 and/or Gln-314 as likely catalytic bases in the prevailing reaction mechanism (14). They have been mutated to aspartate to change the juxtaposition of the proton acceptor, to glutamine to block formal proton transfer while likely retaining hydrogen bonding, and to valine or alanine to completely disrupt the interaction.

Near wild type activity (83% $k_{\text{cat}}$) for a multisite mutant protein containing an E314V substitution shows that Glu-314 is not a critical catalytic base. There is now no reason to pair it with aspartates in sequence alignments (7). In fact, the recent TSA structure of $T$. californica creatine kinase aligns Val-325 with arginine kinase Glu-314, whereas Asp-326 is more remote from the creatine nitrogens (13). Val-325 interacts with the CH$_2$ that is unique to creatine. It remains to be seen whether the drastic effects of mutating the aspartate in creatine kinase (17) reflect differences between creatine and arginine kinases or whether more innocuous mutations remain to be discovered as for arginine kinase Glu-314.

The 300- to 3000-fold attenuation of activity for the E225Q/D/A mutations is similar to the E314D/Q mutations at a non-essential site and less than expected for a base in the primary means of catalysis. Comparing to corresponding creatine kinase mutants, they are similar to a 500-fold attenuation in the human muscle E232D mutant (17) but differ from the 90,000- fold attenuation in the mitochondrial E227Q mutant (16) and greater attenuations with non-conservative mutations (16, 17). Retention of significant activity in the arginine kinase E225A mutant suggests that Glu-225, like Glu-314, is not absolutely essential. Arginine kinase might be unique with possible redundancy offered by the juxtaposition of a second glutamate (Glu-314). This possibility is eliminated by the double mutant E225Q/E314Q having activity similar to the E225Q single mutant (Table I). Thus, the conserved Glu-225 has a more modest role in a multifaceted catalytic mechanism than implied from earlier creatine kinase mutants (16).

The similar kinetic effects of mutations at Glu-225 and Glu-314 raise the possibility that they impact the reaction in similar ways, not necessarily involving formal proton transfer. Conservative mutations at Glu-314 or Gln-225 reduce activity somewhat without greatly affecting substrate binding (Table I). In the E225Q structure, a hydrogen bond is preserved between the substrate and Gln-225 that could contribute to catalysis, short of formal proton transfer, by lowering the pK of the reactive nitrogen, perhaps explaining the modest additional loss of activity without the hydrogen bond in the E225A mutant. Significant activity in E225A indicates that Glu-225 acts in a supplemental, not an essential, capacity. Similar kinetics in the arginine kinase E225D and human muscle creatine kinase E232D mutants (17) support a common role, but the disparity with mutations in other homologs (16, 17) indicates a susceptibility to collateral damage. Thus, the difference in E225A and E225Q kinetics could be attributed to the absence of pK perturbation, collateral damage, or perturbation of substrate alignment. The non-additivity of the kinetic effects of E225Q and E314Q in a double mutant suggests that their effects are mediated through a common means, mostly likely substrate alignment, having ruled out pK effects for Glu-314 (see above).

Conformational changes are larger in E314D than E225D and mostly involve distortions to the loop containing 314 as it maintains an interaction with the substrate arginine despite a shorter side chain. There is a greater loss of activity in E225Q despite smaller overall changes to the active site, indicating that the two may not be strongly correlated. Changes to the atoms involved in the phosphoryl transfer may be more critical: up to 0.25 Å for E314D and 0.34 Å for E225Q. These changes are commensurate with the estimated errors of the structures.
(0.27 and 0.38 Å, respectively), and only slightly increase distortion of the linear transfer (from 173° in wild type to 170° in E225Q and 168° in E314D, Table III). The angles of nucleophilic attack at the guanidinium $N_H$ and ADP $O_{5\beta}$ remain close to perfect in E314D but are distorted 5 and 10°, respectively, in E225Q. Obvious from the E225Q electron density is a change in the tilt of the nitrate as the oxygens are moved 0.5 to 0.9 Å, leading to a 22° distortion of the angle at which lone pairs of the guanidinium or ADP would attack the phosphate in each direction of the reaction. Thus, precise substrate alignment may be an important role for Glu-225.

Catalytic rate could also be enhanced by restricting substrate motion (entropy). Crystalllographic correction of the reaction. Thus, precise substrate alignment may restrict active site motion, but they were similar in wild type leading to a 22° distortion of the nitrate as the oxygens are moved 0.5 to 0.9 Å, leading to a 22° distortion of the angle at which lone pairs of the guanidinium or ADP would attack the phosphate in each direction of the reaction. Thus, precise substrate alignment may be an important role for Glu-225.

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The Putative Catalytic Bases Have, at Most, an Accessory Role in the Mechanism of Arginine Kinase

Pamela S. Pruett, Arezki Azzi, Shawn A. Clark, Mohammad S. Yousef, James L. Gattis, Thayumanasamy Somasundaram, W. Ross Ellington and Michael S. Chapman

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