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Crystal structures of arginine kinase in complex with ADP, nitrate, and various phosphagen analogs

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Abstract

Arginine kinase catalyzes the reversible transfer of a phosphoryl group between ATP and L-arginine and is a monomeric homolog of the human enzyme creatine kinase. Arginine and creatine kinases belong to the phosphagen kinase family of enzymes, which consists of eight known members, each of which is specific for its own phosphagen. Here, the source of phosphagen specificity in arginine kinase is investigated through the use of phosphagen analogs. Crystal structures have been determined for *Limulus polyphemus* arginine kinase with one of four arginine analogs bound in a transition state analog complex: L-ornithine, L-citrulline, imino-L-ornithine, and D-arginine. In all complexes, the enzyme achieves a closed conformation very similar to that of the cognate transition state analog complex, but differences are observed in the configurations of bound ligands. Arginine kinase exhibits no detectable activity towards ornithine, citrulline, or imino-L-ornithine, and only trace activity towards D-arginine. The crystal structures presented here demonstrate that phosphagen specificity is derived neither from a lock-and-key mechanism nor a modulation of induced-fit conformational changes, but potentially from subtle distortions in bound substrate configurations.

Keywords

Arginine kinase; Phosphagen kinase; Substrate analog; Substrate specificity; X-ray crystallography

Introduction

Arginine kinase, a homolog of the human enzyme creatine kinase, is a member of the phosphagen kinase family of enzymes which buffer cellular ATP levels in cells with high and/or fluctuating energy demands. To this end, these enzymes catalyze reversible phosphoryl transfer between ATP and their respective phosphagens, substrates with guanidinium groups to and from which the phosphoryl group is transferred. Through these

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enzyme-catalyzed reactions, cells are capable of maintaining a constant ATP/ADP ratio, thus retaining a high free energy of ATP hydrolysis which can be used to fuel a number of cellular processes [1].

Phosphagen substrates vary by organism, but all have a guanidino group that is reversibly phosphorylated. Crystal structures for several homologous phosphagen kinases have been determined, including arginine kinase (AK), creatine kinase (CK), glycyamine kinase (GK), and lombricine kinase (LK) in substrate-free “open” forms [2,3,4,5,6,7]. AK, CK and GK structures have also been determined as transition state analog (TSA) complexes, composed of the phosphagen, MgADP, and nitrate [2,4,8,9,10]. In these closed-conformation complexes, the planar nitrate group mimics the phosphoryl group being transferred. Comparison of open- and closed-form structures reveals a conserved set of conformational changes on substrate binding that may mediate substrate alignment, position catalytically important residues, and/or exclude bulk solvent from the enzyme’s active site, potentially preventing the wasteful hydrolysis of ATP [8,11,12,13]. NMR has demonstrated that a subset of the conformational changes observed crystallographically occur intrinsically in substrate-free arginine kinase and that they are likely turnover-limiting [14,15,16].

The structure of the arginine kinase TSA complex, solved at a resolution of 1.2 Å, provides a precise and unperturbed view of substrate alignment [9]. Substrates were aligned within 4 degrees of optimal for in-line phosphoryl transfer [9,10]. Two glutamates, E225 and E314, were shown to interact with the substrate guanidinium. Mutation of these glutamates reduces activity to 0.5% – 1.7% of wild type (wt) respectively, but does not eliminate activity. Structures of E225Q and E314D mutants as TSA complexes showed subtle distortions in the precise alignment of substrates [17]. The TSA complex structure of another active site mutant, C271A, exhibits 15° distortions of the phosphagen and nucleotide attack angles, although loss of catalysis has been thought to be an electrostatic/stereoelectronic effect of losing a thiolate [18]. The structure of the non-cognate complex of wtAK with ADP and creatine, a phosphagen for which AK has no detectable activity, suggests that the lack of activity may result from AK’s inability to position precisely this smaller phosphagen [19]. Taken together, a number of observations can be interpreted to indicate that substrate alignment plays an important role in rate enhancement and/or substrate specificity.

The role of substrate alignment is further investigated here through structural studies of AK with a set of phosphagen analogs. Crystal structures are determined for arginine kinase from the Atlantic horseshoe crab *Limulus polyphemus* with one of four phosphagen analogs bound in a TSA-like complex with nitrate and MgADP. No enzymatic activity is observed for three of the analogs: L-ornithine, imino-L-ornithine, and L-citrulline; and trace activity is detected for D-arginine. The enzyme structures are very similar to the native TSA complex, but significant distortions are observed in substrate configurations [9,10]. The crystal structures show the non-cognate substrate analogs bound at high occupancy, i.e. they are not excluded in a lock-and-key fashion, so that it appears that activity and substrate specificity are mediated through appropriate substrate alignment.

Materials and Methods

Protein expression, purification, and crystallization

Limulus polyphemus arginine kinase was expressed and purified as previously described [20]. Initial crystals were grown at room temperature by hanging drop vapor diffusion. Purified arginine kinase at 30 mg/ml was mixed with substrate analog and crystallant solutions in 4:4:1 ratio. The crystallant solution contained 26% (w/v) PEG 6000, 50 mM HEPES, and 100 mM MgCl₂ at a pH of 8.0. The substrate analog solution contained 25 mM MgCl₂, 20 mM K-ADP, 250 mM NaNO₃, 25 mM NaN₃, 5 mM DTT, and phosphagen

analog (100 mM L-citrulline, L-ornithine, or iminoethyl-L-ornithine or 250 mM D-arginine) at pH 7.5. Diffraction quality crystals were obtained by microseeding initial crystals into new drops prepared identically.

Crystallographic data collection and structure determination

For all analogs, crystals were transferred into a *cryo*-protectant solution containing 20% glycerol and analog solution described above at a working concentration. Diffraction was collected in-house using a Rigaku R-axis II rotating anode x-ray generator and a MarCCD detector. Data were processed using the HKL software package [21]. To avoid bias in cross-validation, test sets were chosen to match the Miller indices of the high resolution arginine kinase TSA complex [9,10].

The L-ornithine and L-citrulline crystals were isomorphous the AK TSA crystal, with unit cell lengths differing by < 1% [9,10]. Initial phases for the iminoethyl-L-ornithine and D-arginine complexes were obtained by molecular replacement using the AutoMR module of Phenix [22,23]. For all initial phasing models, the phosphagen and nitrate were omitted to avoid potential bias. Refinement proceeded by alternating between optimization in Phenix and manual model rebuilding in Coot [24]. Phenix refinement incorporated backbone dihedral angle restraints derived from the high resolution TSA structure [9,10]. During the final stage of refinement in Phenix, B-factors were optimized by combination of a TLS model and individual B-factors. Five TLS groups were used, corresponding to the five “dynamic domains” involved in closure of arginine kinase around bound substrates [5]. To differentiate between rotameric states of imino-L-ornithine about the C_δ - N_ε bond, we turned to quantum mechanical energy calculations of each of the *cis* and *trans* isomers, which indicated that the *trans* isomer was favored by nearly 2 kcal/mol. Thus, the *trans* isomer of imino-L-ornithine was used in model refinement.

Superposition of structural models

Protein conformational changes were compared between the four phosphagen analogs presented here, the TSA structure, and the structures of other phosphagen kinases using the LSQKAB program in the CCP4 suite [25,26]. Initially, structures were aligned based on the “fixed” domain, composed of residues 103–128, 156–166, 216–220, 233–257, 277–290, and 330–357 [5]. These pre-aligned models form the basis for further comparisons. Previously determined multiple sequence alignments were utilized when aligning homologous enzymes [1].

Enzyme activity assays

Arginine kinase activity for the phosphagen analogs was measured using a standard, coupled enzyme procedure in the forward direction (synthesis of phosphorylated phosphagen/analog) [27,28]. The final reaction mixture included 100 mM Tris-acetate (pH 8), 6 mM magnesium acetate, 133 mM potassium chloride, 1.2 mM phosphoenolpyruvate, 0.5 mM NADH, 6.5 EU each of pyruvate kinase and lactate dehydrogenase, 5 mM ATP, and 100 mM phosphagen analog. Purified arginine kinase was added to initiate the reaction, which was allowed to proceed for 15 minutes. Conversion of NADH to NAD⁺ was measured through via absorbance at 340 nm.

Results and Discussion

Crystallographic structure determination

Crystal structures were successfully determined for four complexes of arginine kinase bound each of the phosphagen analogs L-citrulline, L-ornithine, iminoethyl-L-ornithine, and D-arginine. Crystallographic and refinement statistics are shown in Table I. The unit cells of all

phosphagen analog complexes except D-arginine are very similar to that of the transition state analog (TSA) complex of arginine kinase [9,10]. In fact, molecular replacement was only necessary for the iminoethyl-L-ornithine and D-arginine complexes. The availability of a high resolution (1.2 Å) related structure was a distinct advantage, supporting higher quality structures than usual at modest resolution, particularly for the D-arginine complex which yielded R/R_{free} of 18.6/22.7, even though diffraction was limited to 3.0 Å resolution. The refined models have been deposited into the Protein Data Bank (Table I).

All crystals were grown in the presence of magnesium, ADP, nitrate, and phosphagen analog. Clear electron density can be observed in the enzyme active site for all of these components in the L-ornithine, imino-L-ornithine, and D-arginine maps (Figure 1). No density for an active site nitrate ion was present in the L-citrulline map; instead, three water molecules are located near where the nitrate oxygens would be.

Overall, the structures of the phosphagen analog complexes are very similar to TSA structure of arginine kinase [9,10]. After superposition of the fixed domains onto the cognate TSA complex, the largest RMS differences are observed in the L-ornithine complex, with 0.47 Å for C_α atoms and 0.62 Å for all atoms (Table II). Compared to a maximum likelihood estimated mean coordinate error of 0.30 Å, the overall difference between non-cognate cognate TSA complexes is quite modest. However, the complexes of all phosphagen analogs reveal local deviations from the cognate TSA complex, as described below (Figure 2).

Subdomain motions associated with phosphagen analog binding

The conformational changes of arginine kinase associated with substrate binding have been investigated, both crystallographically and with NMR. They approximate quasi rigid-body motions of 5 dynamic domains, clusters of non-contiguous residues whose motions can be described by common rotational and/or translational operations [5]. Arginine kinase is an intrinsically flexible enzyme across a wide range of timescales. In the absence of substrates, micro- to millisecond motions have been observed in the N-terminal domain and a loop spanning residues 182–209; pico- to nanosecond motions are present in another loop, residues 308–322 [15]. All of these dynamic regions contain residues that interact directly with the substrates [7,10].

Subdomain orientations in the phosphagen analog structures are very similar to those of the cognate TSA, implying similar conformational changes on substrate binding. Rotations of subdomains 1, 2 and 3 are within one degree of those observed in the TSA complex. The largest difference is with subdomain 5, which is over-rotated by 4 – 6 degrees in all of the phosphagen analog models presented here, relative to the TSA complex. Subdomain 5, which borders the C-terminal domain specificity loop (residues R309-G320), is also the site of most prominent differences between the various homologous phosphagen kinases. Substrate-associated rotations range from 6.1° in *Apostichopus japonicus* arginine kinase, to 14.0° in rabbit and *Torpedo californica* creatine kinases, to 27.3° in *Limulus polyphemus* arginine kinase complexed with ADP and non-cognate substrate, creatine [8,19,29,30]. Refinement of anisotropic atomic displacement parameters, possible with the atomic resolution (1.2 Å) data of the *Limulus polyphemus* arginine kinase TSA structure, showed that the principle axes of subdomain 5 motion were correlated with the differences between homologs [9]. Taken together, these observations suggest that the structures of subdomain 5 in the various homologs are being sampled from orientations in which there is significant intrinsic flexibility.

Although the C-terminal domain specificity loop is flanked each side by the over-rotated subdomain 5, the loop configuration in the phosphagen analog complexes is very similar to

that of the cognate TSA structure. The loop, comprising residues R309-G320, interacts extensively with the substrates and is important for catalysis [9,10,17,19]. In spite of differences in the phosphagen analogs themselves, all-atom RMS differences for the loop, relative to the cognate TSA complex, are less than 0.53 Å. The subtlety of differences in the loop indicates that it is not its extensive substrate interactions that are dictating subdomain 5 orientations, which differ between the phosphagen analogs and the cognate substrate. It appears that there is intrinsic flexibility in the orientation of subdomain 5, and that the selection for each crystal structure between configurations of presumably near equal energy depends on subtle factors that are not obvious.

Active site configuration

The positions of active site residues are very similar to those in the TSA structure, with all-atom RMS deviations of 0.22 – 0.32 Å for residues that interact with substrates [9,10]. Given that the estimated overall coordinate error of the phosphagen analog models is 0.21 – 0.33 Å, most of the differences in active site coordinates are not significant (Table II). Nevertheless, there are meaningful differences in the configurations of specific active site residues, the largest occurring at H315. These differences are discussed below in the context of each of the phosphagen analogs.

Among the phosphagen analogs presented here, L-citrulline binds in a manner that is the least similar to substrate arginine in the TSA complex, with its R-group bent nearly 180 degrees away from ADP. Active site residues, however, remain in positions very similar to the TSA, with an all atom RMS of 0.22 Å. At first glance, the chemical structure of citrulline looks similar to arginine, with an oxygen replacing one of the guanidinium nitrogens, but, unlike arginine, citrulline's R-group is non-titratable and is not charged. As a consequence, there are not the favorable salt bridge interactions with E225 and C271 (thiolate) that help stabilize the cognate L-arginine configuration [9,10,18]. With rotation, primarily about the C_γ - C_δ of citrulline, a water molecule of the L-arginine TSA complex is displaced, but two new water molecules are introduced where the guanidinium would have been.

Nitrate was not observed in the citrulline complex, even though it was present in the crystallization solutions. Three solvent molecules take its place. Thus, interactions with the enzyme alone are not sufficient for nitrate binding, suggesting that the presence of positively charged L-arginine substrate contributes to the binding of the γ-phosphate. This shows that there is an inter-substrate component to substrate binding synergy which has traditionally been considered mediated by induced protein conformational change [31]. Furthermore, the observation of the closed protein configuration in the citrulline complex shows that interactions of the substrate guanidinium are not a primary driver of the conformational changes.

The enzyme structure of the arginine kinase TSA-like complex with ornithine is the least similar to the cognate TSA complex, with an all atom RMS difference of 0.62 Å and an active site difference of 0.34 Å. The largest difference is at H315, with shifts of 1.3 Å in the imidazole ring, reducing the distance between N_ε2 of H315 and the carboxylate of ornithine from 3.6 Å in the cognate complex to 3.0 Å. Other hydrogen bonding interactions between the carboxylate of ornithine and the substrate specificity loop are maintained within 0.2 Å of corresponding interactions in the cognate TSA complex [9,10].

In L-ornithine, the guanidinium group is replaced by a smaller primary amine group, resulting in a more extended R-group configuration than L-arginine as the N_ε moves 1.1 Å into the space vacated by arginine's guanidinium. Nevertheless, at a distance of 4 Å from E225/E314, ornithine's N_ε is unable to maintain the near optimal hydrogen bonding of the

cognate L-arginine. This might afford greater freedom to the R-group configuration of ornithine. Ornithine's lack of a guanidinium group also changes the position of the nitrate ion. The nitrate is translated ~ 1.5 Å towards ornithine and rotated $\sim 30^\circ$. It appears that with the loss of the guanidinium, there is greater freedom in positioning of the nitrate with a trade-off in active site interactions that may not greatly affect the total interaction energy.

Imino-L-ornithine binds in a fashion that is very similar to substrate arginine in the cognate TSA structure, with an all-atom RMS of 0.47 Å for the whole protein and 0.13 Å for the active site. This is not surprising, as the only chemical difference between the components of these two models is the identity of a single atom: one of the guanidino nitrogen atoms in substrate arginine is substituted by a methyl group in imino-L-ornithine. As a result, the lengths of all important hydrogen bonds between ligands (imino-L-ornithine, nitrate, or ADP) and active site residues are within 0.2 Å of their counterparts in the cognate TSA complex [9,10,17,18]. A subtle rotation about χ_1 can be observed in E314, possibly due to the lack of hydrogen bond donating character of the methyl group in imino-L-ornithine, leading to slightly more flexibility in this side chain. These differences, however, are all within the estimated coordinate error of 0.27 Å for the imino-L-ornithine complex.

The D-arginine complex is unique from the others, marked by deviations from the cognate TSA at the α -amino group. The inversion of chirality about the C_α affects the phosphagen structure, but has little impact on the active site configuration, with an all atom RMS difference of 0.24 Å compared to the cognate TSA [9,10]. The carboxylate of D-arginine is rotated away from the N-domain substrate specificity loop slightly, but brought closer to the backbone amines of V65 and G64. As in the ornithine complex, the other carboxylate oxygen has a more favorable interaction with H315 N₂ (3.0 *cf.* 3.2 Å), but this is due to rotation of the D-arginine carboxylate, rather than movement of the H315 side chain. There is a smaller shift in the α -amino group, but the hydrogen bond with the hydroxyl of Y68 remains unchanged.

Crystal structures of *Limulus* arginine kinase bound by four phosphagen analogs in transition state-like complexes have been compared to the cognate TSA complex. The overall enzyme structures are very similar. The most prominent difference is that subdomain 5 rotates 4 – 6 degrees more on binding the four analogs than the cognate substrate. However, on retrospective examination of TSA complexes with a variety of homologs, wide variation is found in the orientation of subdomain 5, and there is nothing unique about the enzyme interactions of the phosphagen analogs to indicate that the observed orientation is anything more than casual selection from configurations of near equal energy.

In contrast to the near identical protein structures for the four complexes, the phosphagen analogs exhibit significant variation in bound configurations. Taken together, it can be seen that specificity is not mediated by lock-and-key substrate-binding, nor by modulation of induced-fit conformational changes known to occur for the phosphagen kinase family of enzymes. Although specificity cannot be fully explained at this point, it is clear that it results partly from stringent requirements for precise substrate alignment that are not satisfied when substrate analogs are subtly mis-aligned. Furthermore, the citrulline complex, which lacks a bound nitrate ion, highlights an inter-substrate component of substrate binding synergy that was previously thought to arise solely from protein conformational changes.

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Abbreviations

AK	Arginine kinase
CK	Creatine kinase
GK	Glycocyamine kinase
LK	Lombricine kinase
TSA	Transition state analog
wt	Wild type

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- X-ray structures of arginine kinase bound to four phosphagen analogs are presented.
- The structures are of the transition state analog complex, with ADP and nitrate.
- The protein conformation with analogs bound is similar to the cognate complex.
- Analog configurations, however, differ from that of bound L-arginine.
- Substrate binding synergy may have an inter-substrate component.

Figure 1. Electron density, contoured at 1.5σ , for ligands in the phosphagen analog models. The underlying protein model is shown with transparency.

Figure 2.

Active site interactions with phosphagen analogs. Red lines depict selected side chains on the backbones (pink ribbon) of the phosphagen analog complexes; blue lines show the side chains for cognate TSA complex. Opaque sticks show the substrate analogs in the non-cognate complexes; transparent sticks show the ligands in the cognate TSA complex.

Table I

Crystallographic and refinement statistics

	L-citrulline	D-arginine	L-ornithine	Iminoethyl-L-ornithine
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å) ¹	<i>a</i> = 65.4 <i>b</i> = 70.3 <i>c</i> = 80.3	<i>a</i> = 64.1 <i>b</i> = 65.4 <i>c</i> = 85.9	<i>a</i> = 65.4 <i>b</i> = 70.3 <i>c</i> = 80.3	<i>a</i> = 65.2 <i>b</i> = 71.1 <i>c</i> = 80.0
Contents of asymmetric unit (no. of non-H atoms)	Protein: 2867 Ligands: 40 Waters: 258	Protein: 2850 Ligands: 44 Waters: 85	Protein: 2861 Ligands: 41 Waters: 243	Protein: 2859 Ligands: 44 Waters: 183
Resolution range (Å) ²	17.6 – 2.09 (2.25 – 2.09)	35.9 – 2.96 (3.73 – 2.96)	29.6 – 2.16 (2.37 – 2.16)	32.5 – 2.45 (2.80 – 2.45)
Unique reflections	20717	7468	19862	13808
Completeness (%)	92.1 (81.0)	93.9 (91.0)	96.7 (91.0)	97.2 (95.0)
R _{sym} (%)	8.40 (27.5)	4.90 (16.1)	7.80 (30.2)	5.60 (21.4)
I/σ	21.1 (2.2)	14.3 (6.8)	19.3 (6.4)	16.6 (9.3)
R _{work} /R _{free} (%)	17.5/21.1 (21.9/25.3)	19.4/23.1 (22.1/26.7)	19.3/22.9 (24.7/31.8)	17.8/21.0 (22.3/27.5)
RMS deviation from ideal				
Bond length	0.006	0.003	0.006	0.007
Bond angles	0.975	0.758	0.892	0.833
Mean B-value (Å ²)	Protein: 37.7 Ligands: 24.2 Solvent: 40.4	Protein: 22.8 Ligands: 18.4 Solvent: 12.7	Protein: 40.8 Ligands: 24.3 Solvent: 41.7	Protein: 33.9 Ligands: 18.6 Solvent: 34.6
Maximum likelihood estimated coordinate error (Å)	0.21	0.33	0.30	0.27
PDB ID	4GVY	4GVZ	4GW2	4GW0

¹The citrulline and ornithine crystals were assumed to be fully isomorphous to that of the arginine kinase transition state analog.

²Values in parenthesis refer to the outermost resolution shell.

Table II

Root mean square deviations between phosphagen analog and TSA structures

Complex	RMS deviation from TSA structure (Å)			Coordinate Error (Å; Table I)
	C _α	All-atom	Active site All-atom	
L-Citrulline	0.45	0.57	0.22	0.21
L-Ornithine	0.47	0.62	0.32	0.30
D-Arginine	0.45	0.56	0.25	0.33
Imino-L-ornithine	0.35	0.47	0.13	0.27

RMS deviations were calculated after alignment of the fixed domain, subdomain 4. Active site residues are those that directly interact with substrates: 64–66, 68, 124, 126, 225, 229, 271, 273, 280, 309, and 314–315.