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Crystallization and X-ray diffraction studies of arginine kinase from the white Pacific shrimp *Litopenaeus vannamei*

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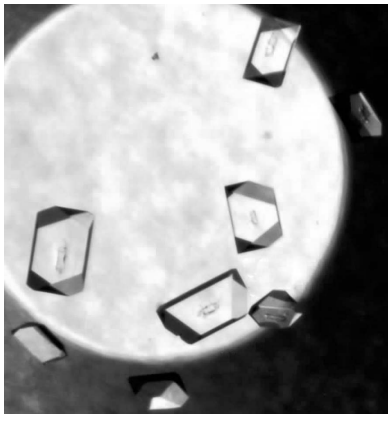


Crystals of an unligated monomeric arginine kinase from the Pacific whiteleg shrimp *Litopenaeus vannamei* (*Lv*AK) were successfully obtained using the microbatch method. Crystallization conditions and preliminary X-ray diffraction analysis to 1.25 Å resolution are reported. Data were collected at 100 K on NSLS beamline X6A. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 56.5$, $b = 70.2$, $c = 81.7$ Å. One monomer per asymmetric unit was found, with a Matthews coefficient (V_M) of 2.05 Å^3 Da^{-1} and 40% solvent content. Initial phases were determined by molecular replacement using a homology model of *Lv*AK as the search model. Refinement was performed with *PHENIX*, with final R_{work} and R_{free} values of 0.15 and 0.19, respectively. Biological analysis of the structure is currently in progress.

1. Introduction

The phosphagen kinase (PK) family, also known as the guanidine kinase group, is comprised of enzymes that play an essential role in regulating the cellular ATP pool *via* high-energy phosphagen storage (Ellington, 2001). These enzymes catalyze the reversible transfer of the γ-phosphate group of ATP to a guanidine substrate (creatine, arginine or lombricine). Despite structural variation of the phosphate acceptor (also known as the phosphagen), PKs are well conserved, with approximately 40% amino-acid sequence identity between them (Dumas & Camonis, 1993). Arginine kinase (AK) and creatine kinase (CK) are the most representative enzymes of the PK family; AK is found in invertebrates, while its homologue CK is found in vertebrates (Babbitt *et al.*, 1986). AK (EC 2.7.3.3) is present as a monomer of 40 kDa, except in the sea cucumber (*Stichopus japonicas*) and other equinoderms, where it is found as a homodimer of 80 kDa (Guo *et al.*, 2003). Despite these quaternary-structural differences, all AKs use arginine as a substrate,

$$\text{ATP-Mg} + \text{arginine} \longleftrightarrow \text{ADP-Mg} + \text{arginine-PO}_4 + \text{H}^+.$$

In recent decades, several AKs have been described by biochemical, kinetic and structural studies (Ellington, 2001). Crystal structures of AK and other PKs from several sources are available: octameric human ubiquitous mitochondrial CK (Fritz-Wolf *et al.*, 1996), dimeric CK from human brain and muscle, *Torpedo californica*, chicken brain and bovine retinal (Bong *et al.*, 2008; Lahiri *et al.*, 2002; Tisi *et al.*, 2001), dimeric AK from sea cucumber (Wu *et al.*, 2010) and monomeric AK from *Trypanosoma cruzi* (Fernandez *et al.*, 2007) and the horseshoe crab *Limulus polyphemus* (Zhou *et al.*, 1998). Monomeric AK is comprised of two domains: a small N-terminal domain (~100 residues), which has a mainly α orthogonal bundle fold consisting of an irregular array of six short α-helices, and a larger C-terminal domain (~250 residues). The C-terminal domain has an $\alpha\beta$ two-layer sandwich fold consisting of seven α-helices with a central core of eight-stranded antiparallel β-sheets (Zhou *et al.*, 1998). A detailed analysis of the crystal structure of the ternary dead-end complex of horseshoe crab AK revealed a large conformational change upon substrate binding and closure of both domains (Yousef *et al.*, 2002). The substrate-free enzyme is in the 'open form' and changes to the 'closed form' upon substrate binding. This conformational change requires a 15° rotation between the N-terminal domain and the C-terminal domain. In the 'closed form' a nitrate ion mimics the

trigonal γ-phosphoryl geometry that occurs during phosphate transfer between arginine and ADP-Mg to form the ternary dead-end complex (Zhou *et al.*, 1998). In addition to the domain rotations mentioned above, large conformation changes occur in the loop comprising residues 309–320. This loop serves as a 'lid' that protrudes into the active site when it is in the 'closed form' to provide an extra 'wall' for active-site stabilization, specifically through interaction between residues Arg309, Thr311 and Glu314 and both the ATP-Mg and arginine moieties. In contrast, in the 'open form' this loop is fully disorganized and is not visible in electron-density maps.

AK has been isolated from several invertebrate species and is a well conserved enzyme with approximately 70% amino-acid sequence identity. The arginine kinase from the Pacific white shrimp *Litopenaeus vannamei* (*Lv*AK; GenBank ABI98020.1) was purified from shrimp muscle (yield of ∼2 mg AK per gram of tissue) for biochemical and structural studies. The amino-acid sequence was deduced by cDNA sequencing and was confirmed by mass spectrometry of tryptic peptides from the purified protein (García-Orozco *et al.*, 2007). Proteomic expression analysis indicates that *Lv*AK is up-regulated in shrimps infected with yellow head virus (YHV; Rattanarojpong *et al.*, 2007). Biophysical and structural studies will help to understand the role of *Lv*AK in energy metabolism and its possible relation to the antiviral response mechanism to YHV in the white shrimp.

In this paper, we report the crystallization and preliminary structural analysis of arginine kinase from the marine crustacean decapod shrimp *L. vannamei* in the absence of substrate or ligands.

2. Materials and methods

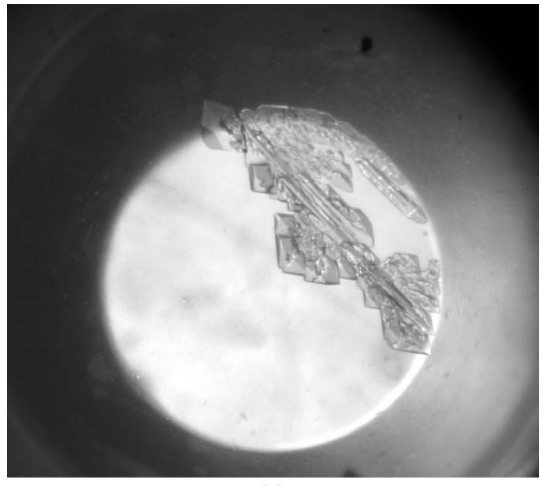
2.1. *Lv*AK purification and crystallization

*Lv*AK was purified from shrimp tail muscle as reported previously (García-Orozco *et al.*, 2007). Briefly, 5 g white shrimp tail muscle was homogenized in 50 ml 100 m*M* Tris–HCl pH 8.0, 10 m*M* β-mercaptoethanol, 1 m*M* EDTA, 25 µ*M* PMSF, incubated overnight at 277 K with constant stirring and clarified by centrifugation. The supernatant was precipitated using 70 and 90% saturated ammonium sulfate and centrifuged at 30 000*g* for 20 min at 277 K. The pellet was redissolved in 10 m*M* Tris–HCl pH 8.0, 10 m*M* β-mercaptoethanol, 0.1 m*M* EDTA. The homogenate was spun down (30 000*g*, 30 min, 277 K) and the supernatant was loaded onto a Q-Sepharose column (GE Healthcare). The enzyme was eluted using a linear gradient from 0 to 1 *M* NaCl. The purity was verified by the presence of a single protein band (∼40 kDa) on silver-stained SDS–PAGE. The protein was stored at 277 K until use. The protein concentration was determined by the bicinchoninic acid method (Pierce; Smith *et al.*, 1985) using bovine serum albumin as a standard. The *Lv*AK activity was measured using a direct colorimetric assay in the forward direction following the synthesis of arginine phosphate (Yu *et al.*, 2003).

*Lv*AK was extensively dialyzed against 10 m*M* Tris–HCl pH 8.0, 1 m*M* DTT and concentrated with a 10 kDa ultrafiltration membrane (Amicon, Millipore) to a final protein concentration of 20 mg ml^{-1}. This protein was immediately used in crystallization trials with Crystal Screen (Hampton Research) using the microbatch method (Greiner plates, Hampton Research). The plates were prepared as follows: 1 µl concentrated *Lv*AK solution was mixed with 1 µl of each crystallization solution and 10 µl paraffin oil in each well and incubated at 290 K. After 3 d, a large cluster of *Lv*AK crystals appeared in a condition consisting of 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.5, 30%(*w*/*v*) PEG 8000 (solution No. 28 of Crystal Screen). 3 d later, a small individual *Lv*AK crystal appeared bound to the well plate under the same condition (Fig. 1*a*). These crystals were small; the conditions were thus refined in a single matrix using the condition described above, varying the pH from 5.8 to 7.4 in increments of 0.2 pH units. Single *Lv*AK crystals appeared at pH values ranging from 6.6 to 7.4 and grew over 3 d until they reached dimensions of 0.44 × 0.23 × 0.14 mm (Fig. 1*b*). A cat whisker was used to carefully separate a crystal from the well. Before cryocooling, the crystal was transferred to a cryoprotectant solution obtained by replacing the water and PEG 8000 in the mother liquor with 30%(*v*/*v*) PEG 400. A single crystal grown at pH 7.4 was soaked in the cryoprotectant solution for 5 min, loop-mounted and flash-cooled at 100 K using a dry nitrogen steam.

2.2. X-ray data collection and crystallographic analysis

Data collection from the *Lv*AK crystals was performed on beamline X6A of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, New York, USA using an ADSC Quantum 270 detector. X-ray diffraction data were collected from a single crystal at 0.9795 Å (12 672 eV). The crystal-to-detector distance was kept at 110 mm, with an oscillation range per image of 0.5°, collecting a data set with a total oscillation range of 180° and exposing each frame for 10 s. For data collection, crystals were soaked in a cryoprotectant solution as described above, loop-mounted and



(*a*)

(*b*)

Figure 1
*Lv*AK crystals were obtained by the microbatch method using Greiner plates. (*a*) A large cluster of crystals appeared in a condition consisting of 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.5, 30%(*w*/*v*) PEG 8000 within 3 d at 290 K. (*b*) *Lv*AK crystals obtained after refinement of the initial condition to pH 7.4 with dimensions of 0.44 × 0.23 × 0.14 mm. These crystals were used for X-ray data collection.

Table 1
Crystallographic data and data-collection statistics for *Lv*AK.

Values in parentheses are for the highest resolution bin.

X-ray source	NSLS X6A
Wavelength (Å)	0.9795
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 56.5, b = 70.2, c = 81.7
Resolution range (Å)	28.10–1.25 (1.28–1.25)
Total reflections	654679 (47535)
Unique reflections	91314 (6709)
R_{merge}†	0.054 (0.545)
R_{meas}‡	0.058 (0.588)
Completeness (%)	100 (100)
$\langle I/\sigma(I)\rangle$	22.2 (3.9)
Multiplicity	7.1 (7.0)
R_{work}	0.1584
R_{free} (5% of reflections)	0.1968
Mean B value (Å^2)	16.0
B value from Wilson plot (Å^2)	16.4
PDB code	4am1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements. ‡ R_{meas} is a redundancy-independent version of R_{merge}: $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

flash-cooled in a dry nitrogen stream at 100 K. Diffraction images were integrated using *XDS*, scaling was performed with *XSCALE* and mtz conversion was performed with *XDSCONV*, all from the *XDS* suite (Kabsch, 2010).

The *Lv*AK crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 56.5, b = 70.2, c = 81.7 Å. A total of 654 679 reflections were integrated to a resolution of 1.25 Å and were merged to obtain 91 314 unique reflections with an overall R_{merge} and R_{meas} of 0.05 and a completeness of 100%. Matthews coefficient calculations indicated that there was one molecule per asymmetric unit (V_M = 2.05 Å^3 Da^{-1}, 40% solvent content; Matthews, 1968). Data-collection statistics are listed in Table 1.

Initial phases for *Lv*AK were determined by molecular replacement in *Phaser* (McCoy *et al.*, 2007) using a homology model of the *Lv*AK amino-acid sequence (GenBank ABI98020.1) based on the three-dimensional structure of arginine kinase from *L. polyphemus* (PDB entry 3m10; Niu *et al.*, 2011). The best solution from *Phaser* was obtained after the search model was divided into two domains comprising 91 residues from the N-terminus and 255 residues from the C-terminus and had a log-likelihood gain of −1.844, an RFZ of 10.5 and a TFZ of 15.9. After the first electron-density map from the solution obtained in *Phaser* was visually inspected, most of the main-chain atoms were properly fitted; however, several residues were adjusted and/or constructed by hand in several cycles of model rebuilding and phase calculation (this process was made less difficult owing to the high resolution available). The original R_{work} and R_{free} values calculated after molecular replacement were 0.48 and 0.49, respectively. Refinement was performed using the programs *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010), giving final R_{work} and R_{free} values of 0.15 and 0.19, respectively. The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entry 4am1. Biological analysis of the crystallographic structure is in progress.

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