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Crystallization and preliminary crystallographic studies of arginine kinase from the ciliate *Paramecium tetraurelia*

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ABSTRACT: The arginine kinase from the ciliate *Paramecium tetraurelia* catalyzes the reversible transfer of phosphoryl groups from adenosine triphosphate to arginine, generating adenosine diphosphate and arginine phosphate. The ciliate *Paramecium tetraurelia* has four arginine kinases (*Pt*AK1 to 4). *Pt*AK3 showed typical substrate inhibition toward arginine, and the enzymatic activity markedly decreased when the arginine concentration increased. To elucidate the substrate inhibition mechanism of *Pt*AK3, we performed a crystallographic study of *Pt*AK3. Here we report the crystallization and preliminary X-ray diffraction analysis of *Pt*AK3. The diffraction data were collected and processed with a 2.6 Å resolution based on the *Pt*AK3 crystals. The preliminary crystallographic analysis revealed that the *Pt*AK3 crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 77.5, b = 162.5, c = 68.8 Å. They contained two molecules per asymmetric unit.

1. Introduction

Phosphagen kinases (PKs) are enzymes that catalyze the reversible transfer of the γ-phosphoryl group of adenosine triphosphate (ATP) to guanidine substrates, generating adenosine diphosphate (ADP) and phosphorylated guanidine compounds referred to as phosphagens (Morrison & James, 1965). These enzymes play a key role in maintaining a constant ATP concentration, especially in cells that consume a significant amount of ATP energy (Ellinton, 2001, Ellington & Suzuki 2006). Arginine kinase (AK), a member of the phosphagen kinase family, is widely distributed in invertebrates (Uda *et al*., 2006), including unicellular organisms such as bacteria (Andrews *et al*., 2008; Suzuki *et al*., 2013) and protozoan ciliates (Uda *et al*., 2006; Magida & Fraga, 2007). Substrate inhibition is the phenomenon where enzymes are inhibited by their own substrate. This occurs approximately for 20% of all enzymes (Chaplin & Bucke, 1990) and often has important biological functions (Kuehl, 1994; Reed *et al.*, 2010).

The ciliate *Paramecium tetraurelia* contains four types of arginine kinase (*Pt*AK1 to *Pt*AK4). In a previous study (Yano *et al*., 2017), we showed that PtAK3 is characterized by typical arginine substrate inhibition and that enzymatic activity markedly decreased with a concentration of 1 mM arginine substrate (Yano *et al.*, 2017; Yano & Suzuki, 2018). Substrate inhibition in wild-type PKs was first observed with *Pt*AK3. In these same studies—by using three models—the substrate inhibition kinetics of wild-type and mutant *Pt*AK3 were also analyzed to understand the various occurring mechanisms. The results suggested that after primary arginine substrate binding, the binding of another arginine at the secondarily induced inhibitory site is accelerated to form the two substrate-enzyme complex, causing substrate inhibition (Yano & Suzuki, 2018). In the current study, we conducted a crystallographic investigation to elucidate the reaction mechanism of *Pt*AK3 at the atomic level.

2. Materials and Methods

2.1. Crystallization

Recombinant *Pt*AK3 was purified according to the protocol described previously (Yano *et al.*, 2017). The resulting recombinant *Pt*AK3 was dialyzed in 10 mM Tris-HCl (pH 8.0). For crystallization, the purified *Pt*AK3 solution was concentrated to 18 mg/ml by the ultrafiltration method using an Amicon Ultra-4 concentrator (Merck, USA). The purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels (Laemmli, 1970). A preliminary search for crystallization conditions was performed using the sitting-drop method in 24-well Cryschem plates (Hampton Research, USA). Initial crystallization screening conditions were constructed using several commercially available kits:

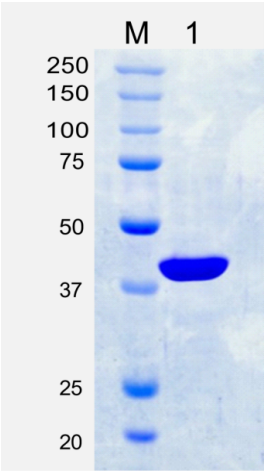

Figure 1.
A 12.5% SDS-PAGE of purified *Pt*AK3. The gel was stained with Coomassie Brilliant Blue. Lane M, molecular-mass markers (labelled in kDa); Lane 1, purified *Pt*AK3.

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Crystal Screen and Crystal Screen 2 (both from Hampton Research, USA). Typically, a 1.0 µl droplet consisting of 18 mg/ml *Pt*AK3 in 10 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol was mixed with an equal volume of reservoir solution. This droplet was allowed to equilibrate against 500 µl of reservoir solution. All crystallizations were carried out at 285 K.

2.2. X-ray data collection

Data collection was performed with the beamline BL26B1 of the SPring-8 synchrotron-radiation source (Hyogo, Japan). The crystals were soaked in a cryoprotectant solution and then mounted in a cryoloop. The crystal was immediately flash-cooled in a stream of nitrogen gas at 100 K. Diffraction data sets were collected using 0.5° oscillations with a crystal-to-detector distance of 200 mm. The diffraction data were processed and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

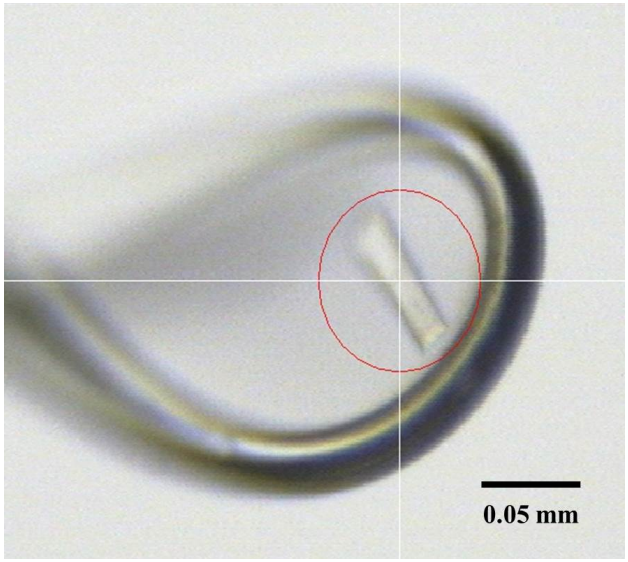

Figure 2.
Crystal of *Pt*AK3 prepared by sitting-drop vapour diffusion.

3. Results and Discussion

Purified *Pt*AK3–with a calculated molecular weight of 44.6 kDa–migrated as a single band on the matrix gel using SDS–PAGE. The analysis showed a homogeneous preparation with a molecular weight of 45 kDa (Fig. 1). A preliminary search for crystallization conditions was performed using Hampton Crystal Screens. Microcrystals of *Pt*AK3 were obtained as clusters from several PEG-based reagents. The crystals from the cluster exhibited poor diffraction quality and were too small for diffraction experiments. The use of a seeding technique in an attempt to improve the crystals proved unsuccessful. Therefore, we tried to further refine the crystallization conditions. Consequently, the crystallization conditions were optimized by changing the PEG concentration and the pH. As a result, suitable *Pt*AK3 crystals with dimensions of 0.02 × 0.02 × 0.2 mm appeared at 285 K within a week. The best crystallization condition for X-ray diffraction measurement was obtained with a reservoir solution consisting of 20% (w/v) PEG4000 and 0.1 M Hepes-NaOH (pH 7.0). The crystallization condition is summarized in Table 1.

Table 1.
Crystallization conditions

Method	Sitting-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	285
Protein concentration (mg/ml)	10
Buffer composition of protein solution	10 mM Tris-HCl (pH 8.0), 1.0 mM DTT
Composition of reservoir solution	0.1 M Hepes-NaOH (pH 7.0), 20% (w/v) PEG4000
Volume and ratio of drop	2 µl, 1:1 (protein solution: reservoir solution)
Volume of reservoir (µl)	900

Table 2.
Crystal parameters and data-collection statistics for *Pt*AK3.

Values in parentheses are for the outer shell.

X-ray source	SPring-8 (BL26B1)
Wavelength (Å)	0.90
Temperature (K)	100
Detector	Eiger 4M CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	0.5
Total rotation range (°)	211.5
Exposure time per image (s)	20
Space group	$P2_12_12_1$
Unit-cell parameters	
a, b, c (Å)	77.5, 162.5, 68.8
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.29
Resolution range (Å)	50 - 2.60
(High-resolution shell)	(2.64- 2.60)
Total No. of reflections	150025
No. of unique reflections	24813
Completeness (%)	88.4 (74.1)
Multiplicity	6.0 (3.6)
Mean $I/\sigma(I)$	4.4 (1.7)
R_{merge}[§] (%)	18.6 (56.3)
Overall B factor from Wilson plot (Å^2)	24.8

[§] $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observed intensity of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity over symmetry-equivalent measurements.

X-ray diffraction experiments were performed under a stream of nitrogen gas at 100 K. A crystal was mounted in a nylon loop, soaked rapidly in the 15% (v/v) glycerol, 20% (w/v) PEG4000, 0.1 M Hepes-NaOH (pH 7.0) and then immediately flash-cooled. The wavelength was fixed at 0.9 Å. A total of 423 frames were recorded with an oscillation angle of 0.5°, an exposure time of 20 s per frame and a crystal–to–detector distance of 200 mm. Diffraction data were processed and scaled using the HKL-2000 program package (Otwinowski & Minor, 1997). Analysis of the symmetry and the systematic absences in the recorded diffraction pattern indicated that the crystals belonged to the cubic space group $P2_12_12_1$, with unit-cell parameters a = 77.5, b = 162.5, c = 68.8 Å. Assuming the presence of two *Pt*AK3 molecules (2 × 45 kDa) in the asymmetric unit, the V_M value is calculated to be 3.1 Å3Da-1, with an estimated solvent content of 49.4 %; these values are

within the range commonly observed for protein crystals (Matthews, 1968). Calculation of the Matthews coefficient suggested that two monomers exist in the asymmetric unit. A total of 150025 observed reflections were merged into 24813 unique reflections in the 50.0-2.6 Å resolution range. Data-collection and processing statistics are summarized in Table 2. Structural analysis of the *Pt*AK3 is now in progress. We anticipate that our structural study of the *Pt*AK3 will contribute to elucidation of the substrate inhibition mechanism of the *Pt*AK3.

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