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## Expression, purification, crystallization and preliminary X-ray analysis of *Plasmodium falciparum* GTP:AMP phosphotransferase

Adenylate kinases (AKs) are phosphotransferase enzymes that catalyze the interconversion of adenine nucleotides, thereby playing an important role in energy metabolism. In *Plasmodium falciparum*, three AK isoforms, namely *PfAK1*, *PfAK2* and GTP:AMP phosphotransferase (*PfGAK*), have been identified. While *PfAK1* and *PfAK2* catalyse the conversion of ATP and AMP to two molecules of ADP, *PfGAK* exhibits a substrate preference for GTP and AMP and does not accept ATP as a substrate. *PfGAK* was cloned and expressed in *Escherichia coli* and purified using two-step chromatography. Brown hexagonal crystals of *PfGAK* were obtained and a preliminary diffraction analysis was performed. X-ray diffraction data for a single *PfGAK* crystal were processed to 2.9 Å resolution in space group  $P3_121$  or  $P3_221$ , with unit-cell parameters  $a = b = 123.49$ ,  $c = 180.82$  Å,  $\alpha = \beta = 90$ ,  $\gamma = 120^\circ$ .

### 1. Introduction

Malaria is a parasitic infectious disease that is widespread in tropical and subtropical regions and affects 350–500 million people a year (Wells & Poll, 2010; Hay *et al.*, 2004). Amongst the five *Plasmodium* species that account for human infections (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*), *P. falciparum* is the most virulent (World Health Organization, 2009; Sinclair *et al.*, 2009). With the increasing drug resistance of the parasite and no vaccine in sight, new chemotherapeutic approaches are required (Petersen *et al.*, 2011).

Adenylate kinases (AKs) are cell energy regulators that play important roles in complex phosphotransfer networks that control intracellular ATP-production processes (Dzeja *et al.*, 2002; Dzeja & Terzic, 2003). In many organisms these enzymes participate in a variety of physiological functions, such as muscle contraction and cell motility (Hancock *et al.*, 2006; Cao *et al.*, 2006). The malaria parasite *Plasmodium* has a high rate of ATP turnover, as merozoite invasion involves high-performance ATP-dependent actomyosin motors and the mature parasite has to supply the host erythrocytes with ATP (Webster & Whaun, 1981). These processes require AKs, making them attractive antimalarial targets.

AKs belong to the nucleotide monophosphate (NMP) kinase superfamily. They exhibit a typical  $\alpha/\beta$  protein fold consisting of a CORE ATP-binding domain with a central five-stranded  $\beta$ -sheet surrounded by  $\alpha$ -helices, a smaller NMP-binding domain composed of three  $\alpha$ -helices and a LID domain of flexible residues covering the phosphate-donor site (Yan & Tsai, 1999). There are two forms of AKs, the short form and the long form, which differ by a 20–30 amino-acid-residue insertion in the LID domain (Müller & Schulz, 1992). Mammalian AKs are short AKs, while those from bacteria, yeast and mitochondria are long AKs. Different oligomeric forms are observed for AKs: eubacterial AKs are monomeric, while archaeobacterial AKs exist in a trimeric form (Vonnrhein *et al.*, 1998). In addition, dimeric forms of AK have also been reported (Perrier *et al.*, 1998).

Three isoforms of AK have been identified in *P. falciparum*, all of which belong to the long form. Of these isoforms of *P. falciparum* AK, *PfAK1* and *PfAK2* specifically bind AMP and favour ATP over other nucleotide triphosphates, whereas GTP:AMP phosphotrans-



ferase (*PfGAK*) exhibits a substrate preference for GTP and AMP (Ulschmid *et al.*, 2004). *PfGAK* corresponds to mammalian AK3 and shares 25% sequence identity with human AK3. While human AK3 accepts both GTP and ATP as phosphate donors, *PfGAK* specifically utilizes only GTP for phosphate transfer (Ulschmid *et al.*, 2004). In addition, it has been shown that the nucleotide analogue GP<sub>5</sub>A strongly inhibits *PfGAK* activity (Ulschmid *et al.*, 2004); this makes GP<sub>5</sub>A an attractive lead compound to assist in the design of drugs that are specific against this enzyme. Here, we report the expression, purification and crystallization of *PfGAK* and a preliminary X-ray diffraction analysis.

## 2. Methods and materials

### 2.1. Expression and purification

The coding region for *PfGAK* (Gene ID PFD0755c) was amplified using PCR from genomic DNA of *P. falciparum* strain 3D7 and was inserted into the bacterial expression vector pET28b (Novagen) via *NdeI* and *XhoI* restriction sites. The resultant plasmid encodes *PfGAK* with an N-terminal 6×His tag with the sequence MGS-HHHHSSGLVPAGSH-. The *PfGAK* plasmid was transformed into *Escherichia coli* BL21 (DE3) Rosetta 2 cells (Novagen). The transformed cells were grown in 2×TY medium containing 30 µg ml<sup>-1</sup> kanamycin and 30 µg ml<sup>-1</sup> chloramphenicol at 310 K with shaking at 225 rev min<sup>-1</sup> until the OD<sub>600</sub> of the cells reached ~0.8. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were incubated for a further 16–18 h at 289 K.

The cells were harvested, resuspended in lysis buffer (25 mM Tris-HCl pH 8.5, 50 mM NaCl, 20 mM imidazole, 0.1 mM PMSF) and lysed by sonication. After centrifugation at 12 000g for 45 min at 277 K, the cleared lysate was loaded onto a 5 ml HisTrap column (GE Healthcare) pre-equilibrated with lysis buffer. Recombinant *PfGAK* was eluted with a linear gradient of 20–250 mM imidazole in lysis buffer. Fractions containing *PfGAK* were pooled and concentrated. The concentrated *PfGAK* protein was loaded onto a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated with 25 mM Tris-HCl pH 8.5, 40 mM KCl, 2 mM DTT, from which it eluted as a dimer. In each purification step, fractions containing the protein were analyzed by 12% SDS-PAGE and appropriate fractions were pooled. After size-exclusion chromatography, fractions containing *PfGAK* were pooled and concentrated using Amicon

Ultra centrifugal concentrators (10 kDa cutoff, Millipore). The concentration of *PfGAK* was determined spectrophotometrically at 280 nm with a NanoVue Plus (GE Healthcare) using its theoretical extinction coefficient of 12 170.

### 2.2. Crystallization

Screening of crystallization conditions was performed by a Mosquito liquid handler (TTP LabTech) at 291 K using the sitting-drop vapour-diffusion method, mixing 200 nl *PfGAK* at 12 mg ml<sup>-1</sup> with an equal volume of crystallization solution. Of the 576 conditions screened, initial hits were observed in Index (Hampton Research) condition No. 4 (2.0 M ammonium sulfate, 0.1 M bis-Tris-HCl pH 6.5) and Protein Complex Suite (Qiagen) condition No. 76 (2.0 M ammonium sulfate, 0.1 M Tris-HCl pH 8.0). After optimization, improved crystals of *PfGAK* were grown in 1.9–2.1 M ammonium sulfate, 0.1 M Tris-HCl pH 8.0 by mixing 2 µl *PfGAK* solution with 2 µl well solution.

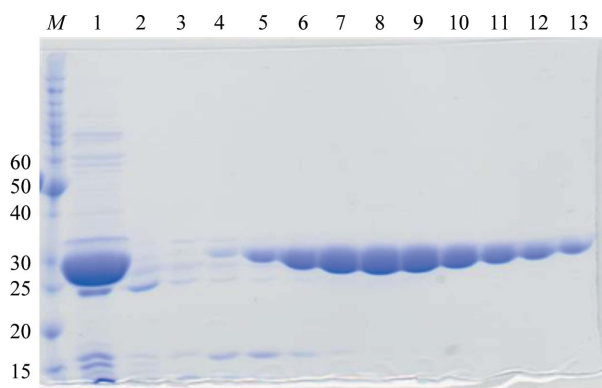
### 2.3. Data collection and processing

*PfGAK* crystals were soaked in a cryobuffer consisting of 4 M ammonium sulfate, 0.1 M Tris-HCl pH 8.0 for about 5 min and flash-cooled by immersion in liquid nitrogen. Data collection was carried out on beamline 13B1, National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. Data collection from a single *PfGAK* crystal was performed with an exposure of 20 s per frame (1° oscillation) and at a wavelength of 1 Å. A total of 90 frames were collected using an ADSC Quantum 315 CCD detector. Diffraction intensities were integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997).

## 3. Results and discussion

The DNA segment encoding *PfGAK* was cloned into the pET28b expression vector, resulting in the addition of an N-terminal 6×His tag. Recombinant *PfGAK* was expressed in *E. coli* BL21 (DE3) Rosetta 2 cells (Novagen) and purified (Fig. 1). Crystals of *PfGAK* appeared after one week of incubation in the initial crystallization screen at 291 K. After optimization, single hexagonal crystals of *PfGAK* which were brown in colour grew to maximum dimensions of 0.3 × 0.1 × 0.1 mm over a period of 3–5 d (Fig. 2).

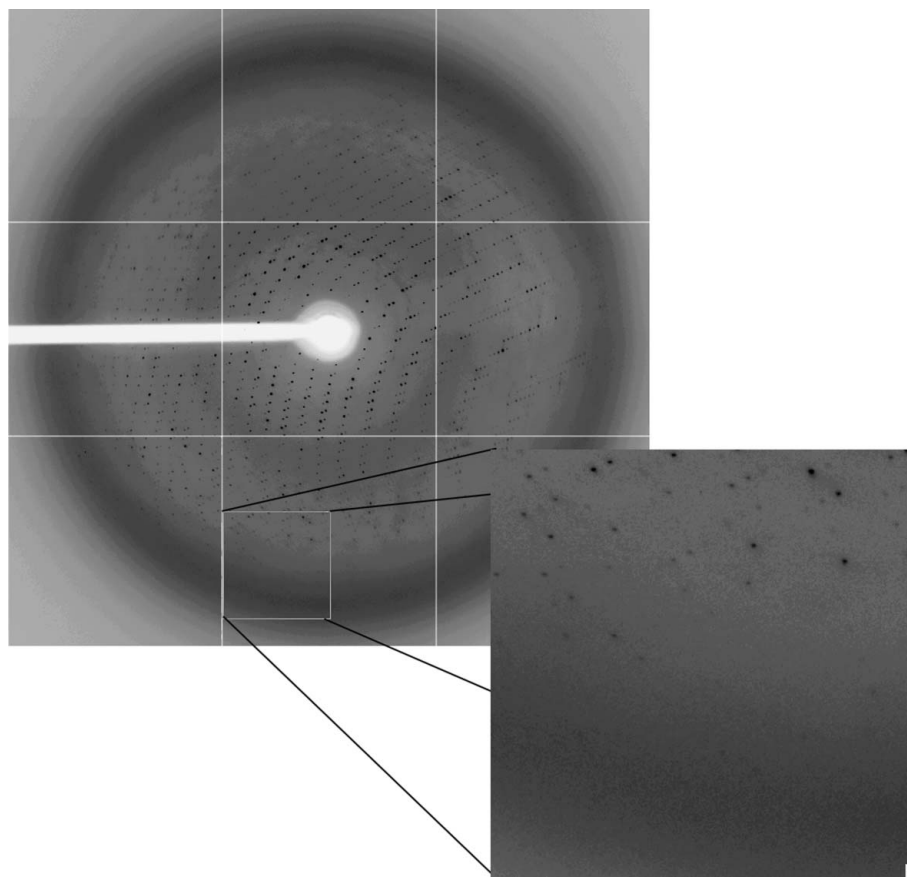
X-ray diffraction and data collection of *PfGAK* crystals were performed on beamline 13B1 at NSRRC, Taiwan. A complete data set was collected from a single crystal to 2.9 Å resolution (Fig. 3) and was processed in space group *P*321 without a screw component.



**Figure 1**  
12% SDS-PAGE analysis of *PfGAK* purified by size-exclusion chromatography. Lane M, molecular-weight marker (labelled in kDa); lane 1, semi-purified *PfGAK* before size-exclusion chromatography; lanes 5–13, fractions of purified *PfGAK* following size-exclusion chromatography.



**Figure 2**  
Crystal of *PfGAK*. Crystals typically grew to maximum dimensions of 0.3 × 0.1 × 0.1 mm.



**Figure 3** Diffraction image of the *PfGAK* crystal collected at NSRRC, Taiwan. The resolution limit at the edge is 2.9 Å. The insert shows an enlarged view of the diffraction at the edge of the detector.

**Table 1** Crystallographic data-collection statistics for *PfGAK*.

Values in parentheses are for the highest resolution shell.

X-ray source	13B1, NSRRC
Wavelength (Å)	1.00
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å, °)	$a = b = 123.49$ , $c = 180.82$ , $\alpha = \beta = 90$ , $\gamma = 120$
Resolution (Å)	30.0–2.90 (3.00–2.90)
Observed reflections	170651
Unique reflections	34852
Multiplicity	4.9 (4.1)
Completeness (%)	97.0 (82.5)
$R_{\text{merge}}^{\dagger}$ (%)	9.8 (71.7)
Average $I/\sigma(I)$	19.1 (1.5)

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

Systematic absences were then used to determine the exact space group as either  $P3_121$  or  $P3_221$ , with unit-cell parameters  $a = b = 123.49$ ,  $c = 180.82$  Å,  $\alpha = \beta = 90$ ,  $\gamma = 120^\circ$ . Despite *PfGAK* being a dimer in solution, the asymmetric unit was estimated to contain four to seven copies of *PfGAK*, with the crystal volume per unit molecular weight,  $V_M$ , calculated to be between 2.10 and 3.68 Å<sup>3</sup> Da<sup>-1</sup>, corresponding to a solvent content of 41.6–66.6% (Matthews, 1968). Attempts to detect the self-rotation function of *PfGAK* in the asymmetric unit using *POLARFN* and *MOLREP* from the *CCP4* suite (Winn *et al.*, 2011) were inconclusive. As *PfGAK* exists as a dimer in solution, we predict that the asymmetric unit of the crystal should contain four to six copies of *PfGAK*, corresponding to two to three dimers. The data-collection statistics are summarized in Table 1.

The solution of the structure of *PfGAK* is currently being actively pursued by the use of both heavy-atom derivatization and selenomethionine incorporation of the protein for MIR and MAD/SAD phasing, respectively, as molecular replacement using homologous proteins such as the AKs from *E. coli* (PDB entry 1ake; 31% homology; Müller & Schulz, 1992), *Saccharomyces cerevisiae* (PDB entry 3aky; 33% homology; Spurgin *et al.*, 1995) and *Bacillus subtilis* (PDB entry 2osb; 32% homology; R. Counago, C. J. Wilson, J. Myers, G. Wu, Y. Shamoo & P. Wittung-Stafshede, unpublished work) was unsuccessful.

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