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

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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 *Pf*AK1, *Pf*AK2 and GTP:AMP phosphotransferase (*Pf*GAK), have been identified. While *Pf*AK1 and *Pf*AK2 catalyse the conversion of ATP and AMP to two molecules of ADP, *Pf*GAK exhibits a substrate preference for GTP and AMP and does not accept ATP as a substrate. *Pf*GAK was cloned and expressed in *Escherichia coli* and purified using two-step chromatography. Brown hexagonal crystals of *Pf*GAK were obtained and a preliminary diffraction analysis was performed. X-ray diffraction data for a single *Pf*GAK 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, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

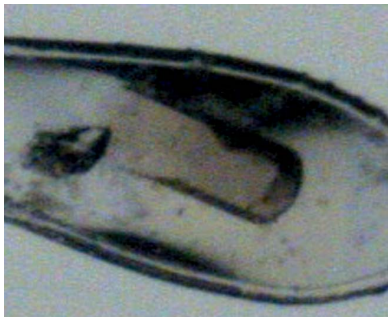
1. Introduction

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 α/β protein fold consisting of a CORE ATP-binding domain with a central five-stranded β-sheet surrounded by α-helices, a smaller NMP-binding domain composed of three α-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 archaebacterial AKs exist in a trimeric form (Vonrhein *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, *Pf*AK1 and *Pf*AK2 specifically bind AMP and favour ATP over other nucleotide triphosphates, whereas GTP:AMP phosphotrans-



ferase (*Pf*GAK) exhibits a substrate preference for GTP and AMP (Ulschmid *et al.*, 2004). *Pf*GAK corresponds to mammalian AK3 and shares 25% sequence identity with human AK3. While human AK3 accepts both GTP and ATP as phosphate donors, *Pf*GAK specifically utilizes only GTP for phosphate transfer (Ulschmid *et al.*, 2004). In addition, it has been shown that the nucleotide analogue GP_5A strongly inhibits *Pf*GAK activity (Ulschmid *et al.*, 2004); this makes GP_5A 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 *Pf*GAK and a preliminary X-ray diffraction analysis.

2. Methods and materials

2.1. Expression and purification

The coding region for *Pf*GAK (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* *Nde*I and *Xho*I restriction sites. The resultant plasmid encodes *Pf*GAK with an N-terminal 6×His tag with the sequence MGSH-HHHHSSGLVPAGSH–. The *Pf*GAK 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^{-1} kanamycin and 30 µg ml^{-1} chloramphenicol at 310 K with shaking at 225 rev min^{-1} until the OD_{600} of the cells reached ∼0.8. Protein expression was induced with 0.5 m*M* 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 m*M* Tris–HCl pH 8.5, 50 m*M* NaCl, 20 m*M* imidazole, 0.1 m*M* PMSF) and lysed by sonication. After centrifugation at 12 000*g* 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 *Pf*GAK was eluted with a linear gradient of 20–250 m*M* imidazole in lysis buffer. Fractions containing *Pf*GAK were pooled and concentrated. The concentrated *Pf*GAK protein was loaded onto a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated with 25 m*M* Tris–HCl pH 8.5, 40 m*M* KCl, 2 m*M* 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 *Pf*GAK were pooled and concentrated using Amicon Ultra centrifugal concentrators (10 kDa cutoff, Millipore). The concentration of *Pf*GAK was determined spectrophotometrically at 280 nm with a NanoVue Plus (GE Healthcare) using its theoretical extinction coefficient of 12 170.

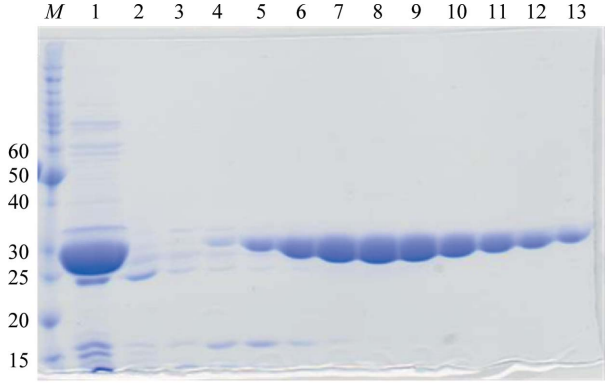


Figure 1
12% SDS–PAGE analysis of *Pf*GAK purified by size-exclusion chromatography. Lane *M*, molecular-weight marker (labelled in kDa); lane 1, semi-purified *Pf*GAK before size-exclusion chromatography; lanes 5–13, fractions of purified *Pf*GAK following size-exclusion chromatography.

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 *Pf*GAK at 12 mg ml^{-1} 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 *Pf*GAK were grown in 1.9–2.1 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.0 by mixing 2 µl *Pf*GAK solution with 2 µl well solution.

2.3. Data collection and processing

*Pf*GAK 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 *Pf*GAK 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 *Pf*GAK was cloned into the pET28b expression vector, resulting in the addition of an N-terminal 6×His tag. Recombinant *Pf*GAK was expressed in *E. coli* BL21 (DE3) Rosetta 2 cells (Novagen) and purified (Fig. 1). Crystals of *Pf*GAK appeared after one week of incubation in the initial crystallization screen at 291 K. After optimization, single hexagonal crystals of *Pf*GAK 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 *Pf*GAK 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.

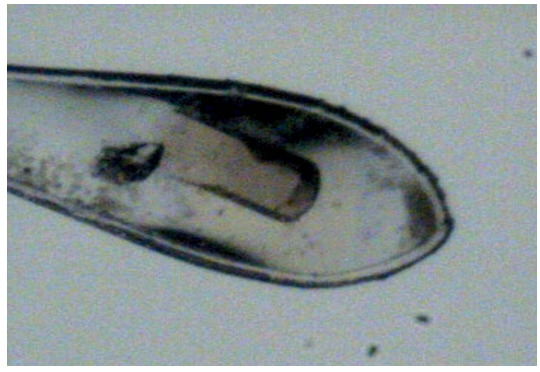


Figure 2
Crystal of *Pf*GAK. Crystals typically grew to maximum dimensions of 0.3 × 0.1 × 0.1 mm.

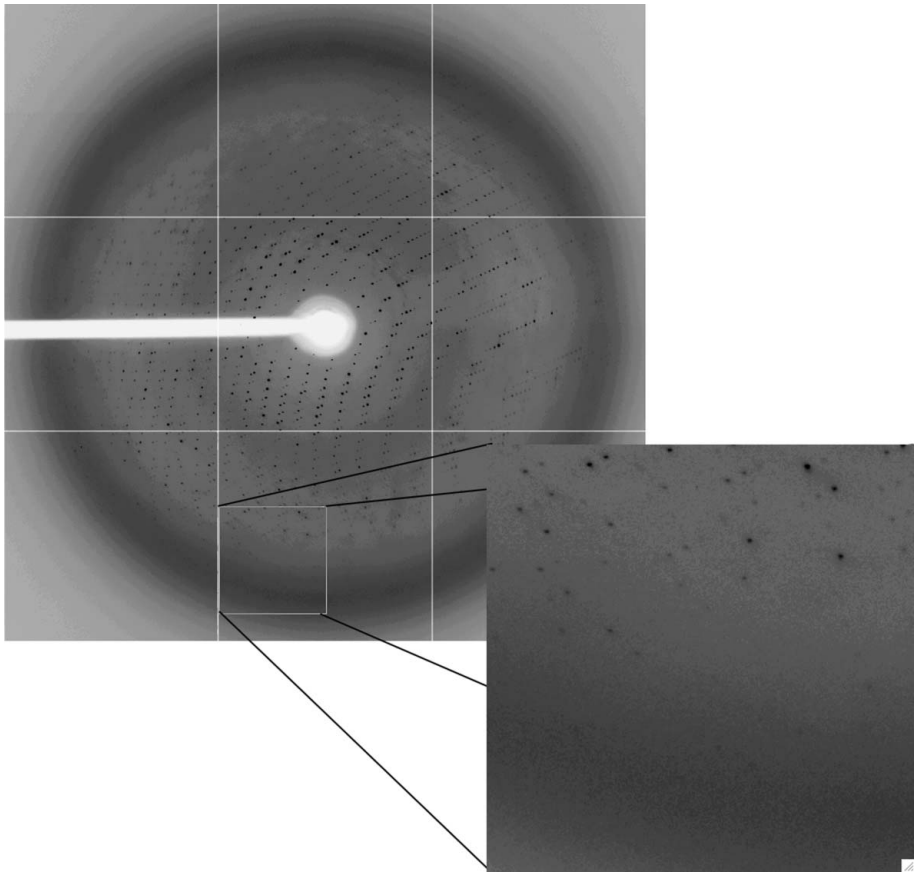


Figure 3
Diffraction image of the *Pf*GAK crystal collected at NSRRC, Taiwan. The resolution limit at the edge is 2.9 Å. The insert shows a enlarged view of the diffraction at the edge of the detector.

Table 1
Crystallographic data-collection statistics for *Pf*GAK.

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)
$R_{\rm merge}$† (%)	9.8 (71.7)
Average $I/\sigma(I)$	19.1 (1.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

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 *Pf*GAK being a dimer in solution, the asymmetric unit was estimated to contain four to seven copies of *Pf*GAK, with the crystal volume per unit molecular weight, $V_{\rm M}$, calculated to be between 2.10 and 3.68 Å^3 Da^{-1}, corresponding to a solvent content of 41.6–66.6% (Matthews, 1968). Attempts to detect the self-rotation function of *Pf*GAK in the asymmetric unit using *POLARRFN* and *MOLREP* from the *CCP*4 suite (Winn *et al.*, 2011) were inconclusive. As *Pf*GAK 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 *Pf*GAK 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; Spuergin *et al.*, 1995) and *Bacillus subtilus* (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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