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Purification, crystallization and preliminary X-ray analysis of the aspartate aminotransferase of *Plasmodium falciparum*

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Aspartate aminotransferases (EC 2.6.1.1) catalyse the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate in a reversible manner. Thus, the aspartate aminotransferase of *Plasmodium falciparum* (*Pf*AspAT) plays a central role in the transamination of amino acids. Recent findings suggest that *Pf*AspAT may also play a pivotal role in energy metabolism and the *de novo* biosynthesis of pyrimidines. While therapeutics based upon the inhibition of other proteins in these pathways are already used in the treatment of malaria, the advent of multidrug-resistant strains has limited their efficacy. The presence of *Pf*AspAT in these pathways may offer additional opportunities for the development of novel therapeutics. In order to gain a deeper understanding of the function and role of *Pf*AspAT, it has been expressed and purified to homogeneity. The successful crystallization of *Pf*AspAT, the collection of a 2.8 Å diffraction data set and initial attempts to solve the structure using molecular replacement are reported.

1. Introduction

Malaria remains one of the most prevalent parasitic diseases worldwide, infecting a quarter of a billion people annually and killing almost one million people a year over the last decade (Trigg & Kondrachine, 1998; World Health Organization, 2008). Protozoan parasites of the genus *Plasmodium* are the causative agents of malaria and are transmitted to humans by female *Anopheles* mosquitoes. Of the various species, *P. falciparum* is responsible for the most lethal and severe form of human malaria (Guerin *et al.*, 2002).

No effective vaccine is currently available for malaria and infections have mostly been treated through the use of antimalarial drugs such as chloroquine (a quinine derivative), antifolates or artemisinin derivatives. Prophylactic drugs are taken by visitors to malaria-endemic regions and must be taken continuously to interfere with the development of the parasite. The drugs in current use belong to a limited collection of chemical structures that act on a small number of partially characterized biochemical targets. The use of closely related compounds has promoted the spread of multidrug-resistant parasites, particularly of *P. falciparum* (Wongsrichanalai *et al.*, 2002; Arora *et al.*, 2008; Dondorp *et al.*, 2009; Lim *et al.*, 2009; Noedl *et al.*, 2009; Olliaro, 2001; Sutanto *et al.*, 2009), severely limiting their efficacy. Thus, there is a pressing need for more basic research into the proteins of *P. falciparum* in order to identify areas of parasite vulnerability and to be able to generate new therapeutics that can uniquely target the malarial parasite.

Aspartate aminotransferases (AspATs; EC 2.6.1.1) typically catalyze the reversible reaction of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate and have been classified into aminotransferase subgroup I (Mehta *et al.*, 1993), which has been further subdivided into subgroups Ia and Ib (Okamoto *et al.*, 1996). Analysis of the homologue from *P. falciparum* (*Pf*AspAT) placed the enzyme into the Ia subfamily, but the malarial enzyme was the most divergent of the Ia subfamily members (24–31% identity with all other Ia family members). The plasmodial AspATs (*P. falciparum*, *P. vivax* and *P. berghei*) also exhibit the mutation of an invariant glycine 197 that

is present in all other AspAT members (Berger *et al.*, 2001; Mehta *et al.*, 1993).

Although methionine is an essential component to sustain life, many organisms lack the ability to synthesize this amino acid, necessitating its uptake from the environment, in which it is present in scarce quantities. Methionine is required in the biosynthesis of cysteine, phospholipids and polyamines, which are synthesized in large amounts in rapidly growing cells immediately prior to DNA replication. Interference with polyamine biosynthesis or function can completely halt cellular growth. Indeed, compounds that interfere with polyamine biosynthesis have been used successfully against the parasitic protozoa causing African sleeping sickness (Marton & Pegg, 1995) and malaria (Klenke *et al.*, 2003; Das Gupta *et al.*, 2005; Muller *et al.*, 2008). In order to alleviate the scarcity of methionine, a pathway exists in mammals and bacteria that regenerates methionine from methylthioadenosine (Heilbronn *et al.*, 1999) and it has been postulated that interference with other enzymes that are involved in the regeneration of methionine could lead to cell death in *P. falciparum* (Sufrin *et al.*, 1995).

Berger and coworkers demonstrated that *Pf*AspAT (PlasmoDB PFB0200c) is able to efficiently use aspartate and tyrosine as substrates to catalyze the transamination of α-ketomethiobutyrate (KTMB) to regenerate methionine. However, other researchers have identified branched-chain aminotransferases as being responsible for the amino acid–KMTB aminotransfer reaction in *Homo sapiens* (Davoodi *et al.*, 1998). Thus, humans and the malarial parasite potentially use different enzymes and different substrates in the regeneration of methionine. While doubts have recently been cast upon methionine regeneration in *P. falciparum* (Ting *et al.*, 2005), suggesting that the transamination activity of *Pf*AspAT may be involved in other pathways, the essential nature of *Pf*AspAT has been demonstrated by the finding that the aminooxy compound canaline can inhibit its activity in parasite homogenates and has antimalarial effects *in vitro* (Berger, 2000; Berger *et al.*, 1998; Heilbronn *et al.*, 1999).

Thus, AspAT represents a novel and potentially highly selective target for therapeutics against *P. falciparum*. Elucidation of the structure of *Pf*AspAT will aid in the design of drugs to selectively inhibit the plasmodial AspAT and will provide further information about the role and function of *Pf*AspAT within the malarial parasite. In this communication, we report the purification, crystallization and preliminary X-ray diffraction of *P. falciparum* AspAT.

2. Methods

2.1. Cloning and expression of pASK-IBA3-*Pf*AspAT

The open reading frame encoding *Pf*AspAT was amplified by PCR from *P. falciparum* 3D7 genomic DNA using sequence-specific antisense (5′-GCGCGCGGTCTCAGCGCTTTAATGATGATGATGATGATGGCCCTGAAAATAAAGATTCTCTATTTGACTTAGCGAAAGACAAATTTTGTCGG-3′) and sense (5′-GCGCGCGGTCTCCAATGGATAAGTTATTAAGCAGCTTAG-3′) oligonucleotides. The PCR for the construct was performed using *Pfu* polymerase (Promega, USA) and the following PCR program: denaturation for 5 min at 368 K followed by 30 cycles of 50 s at 368 K, 90 s at 315 K and 2 min at 345 K. The generated PCR product was digested with *Bsa*I and cloned into the *Escherichia coli* expression plasmid pASK-IBA3 previously digested with the same enzyme, resulting in the expression construct *Pf*AspAT-IBA3. The antisense oligonucleotide encodes a TEV protease site (ENLYFQ) immediately following the native C-terminus followed by a single Gly and a 6×His tag that allows purification of the recombinant fusion protein using Ni–NTA agarose (Qiagen). The final construct consists of 418 amino acids and has a molecular weight of 48 680 Da. The nucleotide sequence was verified by automated nucleotide sequencing (MWG, Germany). Nucleotide and amino-acid analyses were performed with the *Gene Runner* software (Hastings Software Inc.). Protein expression was performed using *E. coli* strain BL21 (DE3) and was induced with 200 ng ml^{-1} anhydrotetracycline at an OD_{600} of 0.5 and a temperature of 310 K. Cell cultures were harvested by centrifugation 4 h post-induction, resuspended in 50 m*M* sodium phosphate buffer pH 7.5 containing 300 m*M* NaCl and 10 m*M* imidazole (buffer *L*) and frozen at 253 K prior to purification. The final yield of purified protein was approximately 1.3 mg per litre of culture.

2.2. Purification of *Pf*AspAT

Following cell lysis in buffer *L* and clarification by centrifugation, the soluble fraction of the lysate was applied onto an Ni–NTA column previously incubated in buffer *A* (100 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl) with an additional 20 m*M* imidazole (buffer *W*). The bound sample was washed extensively in buffer *W* and eluted in buffer *A* with an additional 300 m*M* imidazole (buffer *E*). Following overnight dialysis against buffer *A*, the sample was applied onto a MonoQ HR 5/5 anion-exchange column (GE Healthcare) previously equilibrated in buffer *A*. The protein was eluted using a linear gradient from 0.15 to 1.0 *M* NaCl and eluted as a single peak between 0.35 and 0.38 *M* NaCl. The peak fractions were pooled, concentrated to 1 ml and injected onto a Superdex-75 16/60 size-exclusion column previously equilibrated in buffer *A*. *Pf*AspAT eluted as a highly homogeneous single peak as determined using static light scattering (Nettleship *et al.*, 2008). The purity of the sample was also assessed after ion-exchange and size-exclusion chromatography on SDS–PAGE (Laemmli, 1970; Fig. 1) and concentrations were determined using an absorption coefficient calculated from the primary sequence [$\varepsilon_{o\,(280\,nm)}$ = 0.852 mg^{-1} ml].

In order to improve protein stability and homogeneity, the cofactor pyridoxal 5′-phosphate (PLP) was added to the purified protein at a concentration of 2.5 m*M* to ensure full cofactor binding. The sample was then concentrated to 6 mg ml^{-1} in buffer *A* prior to initial crystallization screening. No removal of the affinity tag was performed.

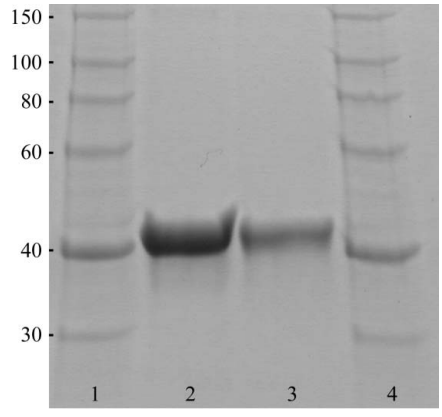

Figure 1
An SDS–PAGE gel indicating the protein quality during the final stages of purification. Lanes 1 and 4, protein markers (Roti-Mark 10-150; Roth, Karlsruhe, Germany; labelled in kDa); lane 2, pooled fraction of *Pf*AspAT following anion-exchange chromatography; lane 3, *Pf*AspAT after subsequent size-exclusion chromatography and immediately prior to crystallization.

3. Results and discussion

3.1. Crystallization of *Pf*AspAT

Once purified, the protein was screened for crystallization using the EMBL Hamburg high-throughput robotic crystallization facility (Mueller-Dieckmann, 2006) and the presence of crystals was confirmed using a protein-specific fluorescent dye (Groves *et al.*, 2007). The preliminary crystallization conditions were (i) 1.4 *M* trisodium citrate, 0.1 *M* HEPES pH 7.5 and (ii) 2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5.

While the initial crystallization conditions resulted in highly disordered clusters of crystals, subsequent grid screening (Weber, 1990; McPherson, 1990) based upon the above conditions resulted in a single crystal (with a minor satellite) that was suitable for diffraction data collection (Fig. 2). The optimized crystal used in diffraction data collection was grown from 1.5 µl protein solution at a concentration of 5.4 mg ml^{-1} in buffer *A* mixed with an equal volume of reservoir solution (2.0 *M* ammonium sulfate, 0.1 *M* bis-tris–HCl pH 7.0) at 293 K.

3.2. Diffraction experiment and data processing

While only a single diffraction-quality protein crystal was obtained, cryoprotection conditions could be established using the disordered plate clusters, which exhibited reasonably strong disordered diffraction to better than 5 Å resolution. Crystals were transferred into a stabilizing solution [2 *M* ammonium sulfate, 25%(*w*/*v*) glycerol, 0.1 *M* bis-tris–HCl pH 7.0] based upon an initial estimate of cryoconditions from Garman & Mitchell (1996) and were subsequently flash-cooled in a nitrogen-gas stream at 100 K. Initial frames were collected on beamline ID23-2 at ESRF to allow crystal characterization and space-group assignment using the software *MOSFLM* (Leslie, 2006) and *BEST* (Bourenkov & Popov, 2006; Popov & Bourenkov, 2003). Data were integrated with *XDS* (Kabsch, 1988) and scaled using *SCALA* (Evans, 2006). The crystals of *Pf*AspAT belonged to space group *H*32, with unit-cell parameters $a = b = 101.0$, $c = 240.7$ Å. An examination of the expected solvent content strongly suggested the asymmetric unit to consist of one molecule, with a Matthews coefficient (V_M) of 2.49 Å^3 Da^{-1} and a solvent content of 50.8% (Kantardjieff & Rupp, 2003; Matthews, 1968). In order to make efficient use of the small beam size and reduce the effect of radiation damage, four independent data sets were collected from different parts of the crystal (Fig. 2) to 2.8 Å resolution as a total of 110 non-overlapping 1° oscillation images. A total of 28 1° oscillations were collected for the first three data sets, with the fourth data set being composed of 26 1° oscillations. A summary of the data-collection parameters and integration statistics of all four data sets is given in Table 1.

Table 1
Results of data collection from a single *Pf*AspAT crystal on beamline ID23-2, ESRF.

Values in parentheses are for the highest resolution bin.

Data-collection parameters	
Wavelength (Å)	0.8726
Temperature (K)	100
Oscillation range (°)	1
Crystal-to-detector distance (mm)	304.82
Data-integration statistics	
Space group	*H*32
Unit-cell parameters (Å, °)	$a = b = 101.0$, $c = 240.7$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution limits (Å)	42.6–2.8 (3.0–2.8)
Total No. of reflections	62404 (9899)
No. of unique reflections	22737 (3580)
Multiplicity	2.8 (2.8)
Completeness (%)	97.2 (96.9)
R_{merge}†	0.081 (0.487)
Mean $I/\sigma(I)$	11.4 (1.4)
V_M‡ (Å^3 Da^{-1})	2.49
Solvent content‡ (%)	50.8

† R_{merge} is defined as $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity from multiple observations. ‡ For one molecule in the asymmetric unit.

Molecular replacement using *BALBES* (Long *et al.*, 2008), with PDB entry 2q7w (Liu *et al.*, 2007; Berman *et al.*, 2000) as a search model, resulted in an initial model with R and R_{free} factors of 0.344 and 0.416, respectively. The search model possesses an overall sequence identity of 31%. Model building using *Coot* (Emsley & Cowtan, 2004) is currently under way and the structure and analysis will be described in a subsequent manuscript.

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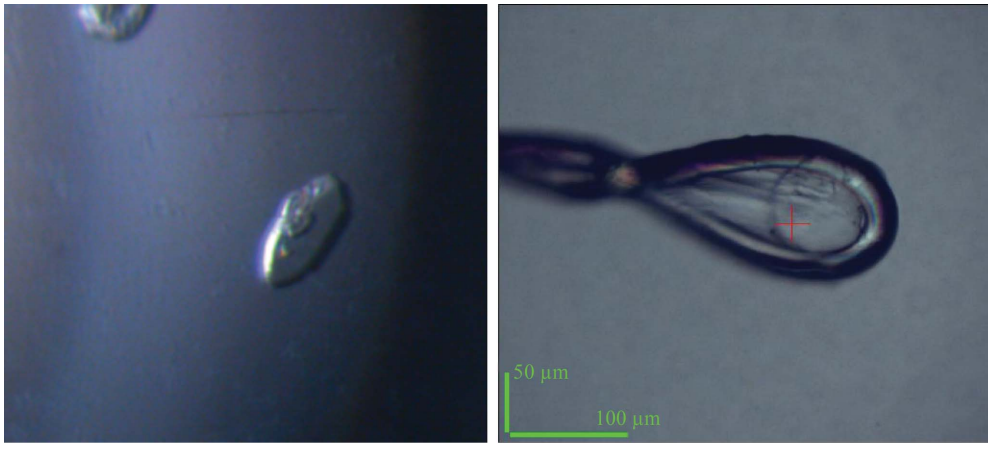

(*a*) (*b*)

Figure 2
Crystallization of and data collection from a *Pf*AspAT crystal. Optimization of the crystallization conditions resulted in a single *Pf*AspAT crystal with a maximum dimension of ∼80 µm (*a*). Multiple (four) data sets were collected from this crystal using a beam size of 20 × 20 µm on beamline ID23-2 at the ESRF (*b*), with care being taken to avoid the satellite crystals (highlighted with an arrow). Diffraction maxima were recorded to a maximum resolution of 2.8 Å.

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