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Expression, purification, crystallization and preliminary X-ray analysis of the sexual stage-specific protein Pfg27 from *Plasmodium falciparum*

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The differentiation and development of sexual stages in *Plasmodium falciparum* is a complex process which involves the expression of several sexual stage-specific proteins. Pfg27 is one of the most crucial proteins and is expressed abundantly at the onset of gametocytogenesis. An expression and purification system for Pfg27 has been established that yields $\sim$5 mg l^{-1} of purified protein in a soluble form. This protein has been crystallized by the hanging-drop vapour-diffusion method using PEG 8000 as a precipitant. The original crystal size was improved significantly by the addition of glucose to the reservoir solution. Pfg27 crystals belong to the space group $C222_1$, with unit-cell parameters a = 58.9, b = 113.2, c = 91.6 Å. Native diffraction data were collected under cryogenic conditions and phase resolution by a selenomethionine-aided multiple-wavelength anomalous dispersion technique is in progress. The Pfg27 structure will provide a framework for functional and biochemical studies aimed at understanding gametocyte development in *P. falciparum*.

1. Introduction

Malaria continues to exact a heavy toll on human life each year in the world. Various chemotherapeutic interventions and vector-control campaigns have only been partly successful. The species of *Plasmodium* that infect humans and cause malaria are *P. falciparum*, the most deadly of the *Plasmodium* species, *P. vivax*, *P. ovale* and *P. malariae* (Newton & White, 1999; Enserink, 2000). The parasite life cycle begins when sporozoites are injected into the blood during a mosquito's blood meal. These reach the liver parenchyma within 1 h, where they develop as intracellular hepatic schizonts. After 7–10 d, the latter rupture and release several thousands merozoites which invade the red blood cells. Here, the parasites multiply asexually and a smaller population differentiates into sexual forms (male and female gametocytes). The male and female gametocytes are ingested by mosquitoes during a subsequent blood meal and initiate a sexual reproduction cycle within the mosquito (Alano & Carter, 1990).

Sexual differentiation within the human host is an essential step in the transmission of *Plasmodium* parasites. During parasite development, the decision for sexual differentiation takes place before the burst of the schizont; the gametocytes then undergo several cellular transformations (Alano *et al.*, 1991). The production of *P. falciparum* sexual stage-specific protein (Pfg27) is one of the first events in this process (Carter *et al.*, 1989; Kumar, 1997). It is a soluble protein expressed 30–40 h after red blood cell invasion by the merozoites, when the parasite is not yet morphologically recognizable as a gametocyte (Carter *et al.*, 1989; Alano *et al.*, 1995). The high level and timely expression of Pfg27 makes it an important protein in the cell specialization.

The gene for Pfg27 (TrEMBL accession code Q27336) is located in a subtelomeric region of chromosome 13 and the promoter upstream region of this gametocyte-specific gene has been reported to show polymorphism in different parasite lines. This mainly arises from the structure of a repetitive DNA region located at about 500 bp from the Pfg27 coding sequence (Sallicandro *et al.*, 2000). The Pfg27 gene has been cloned and shown to be transcriptionally regulated in the blood stages (Alano *et al.*, 1996; Pologe, 1994). The Pfg27 gene locus was successfully disrupted using homologous recombination and these parasites failed to develop into gametocytes (Lobo *et al.*, 1999). Morphological studies also revealed massive vacuolation of developing parasites, followed by disintegration of sexually committed parasites in culture (Lobo *et al.*, 1999). These studies demonstrated a critical role for Pfg27 during sexual differentiation and development. However, the structural and biochemical features of Pfg27 that underlie its crucial role are still unknown. Towards this end, we report the overexpression, purification, crystallization and preliminary X-ray diffraction analysis of Pfg27. A detailed structure–function analysis of Pfg27 will help

to elucidate its exact biological role and may provide a framework for the development of transmission-blocking antimalarials.

2. Experimental

2.1. Cloning of Pfg27 from pRSET to pMAL-p2 expression vector

An effort to express recombinant Pfg27-His_6 fusion protein was made by taking advantage of a prokaryotic expression vector, pRSET-C (Invitrogen). The coding sequence of Pfg27 was PCR amplified using gene-specific primers. The antisense primer lacked the final stop codon and the PCR product was cloned into the *Pvu*II site of pRSET-C. Subsequent sequence analysis as well as expression upon induction confirmed protein production, but in an insoluble form. In order to obtain large quantities of Pfg27, its gene from the pRSET-C plasmid was excized by restriction digestion with *Pst*I and *Hin*dIII. The insert thus obtained was cloned into pMAL-p2 (NEB) plasmid previously digested with the same enzymes. Sequence at the cloning junction was confirmed by DNA-sequence analysis of the clone Pfg27-pMAL-p2.

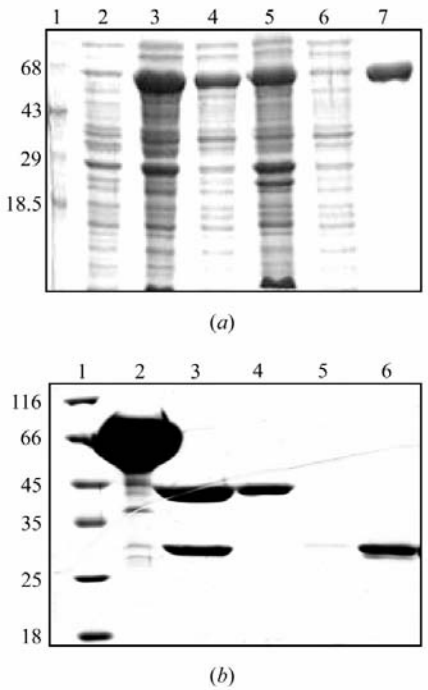

Figure 1
Protein expression and purification analysis of Pfg27 by SDS–PAGE. (*a*) Lane 1, protein standards; lane 2, uninduced cells; lane 3, induced cells; lane 4, induced cell supernatant; lane 5, induced cell pellet; lane 6, amylose column flowthrough; lane 7, eluted MBP-Pfg27 fusion protein. (*b*) Lane 1, protein standards; lane 2, MBP-Pfg27 fusion protein; lane 3, fusion protein after factor Xa cleavage; lane 4, heparin column flowthrough; lane 5 and lane 6, eluted Pfg27 fractions.

2.2. Overexpression and purification of Pfg27

Pfg27-pMAL-p2 was introduced into *Escherichia coli* BL21 (B834 DE3) cells by heat-shock transformation and the transformants were grown in Luria–Bertani (LB) broth in the presence of carbenicillin (50 µg ml^{-1}) and 0.2% glucose. For protein production, a bacterial culture was grown at 310 K and then induced with 0.4 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) at OD $\simeq$ 0.6. The temperature of the incubator was lowered from 310 to 303 K and the culture grown for another 4 h. It was then spun down in an SS34 rotor (Sorvall) at 6000 rev min^{-1} and the pellet was suspended in lysis buffer (20 m*M* Tris pH 7.5, 200 m*M* NaCl, 1 m*M* EDTA and 1 m*M* PMSF) and subjected to sonication. The cell lysate was centrifuged at 15 000 rev min^{-1} for 30 min in a SS34 rotor (Sorvall) and the cleared lysate loaded onto an amylose resin (NEB) column pre-equilibrated in lysis buffer. After washing the column with 12 volumes of lysis buffer, the MBP-Pfg27 fusion protein was eluted with 10 m*M* maltose and the eluted fractions were checked on SDS–PAGE and pooled (Fig. 1*a*). Purified fusion protein at $\sim$1 mg ml^{-1} was cleaved with factor Xa in lysis buffer containing 10 m*M* $CaCl_2$ at 293 K for 12 h and the reaction was stopped with 1 m*M* PMSF. The cleavage mixture was loaded on a heparin Sepharose column equilibrated in 50 m*M* Tris pH 7.5, 50 m*M* NaCl (buffer *A*). The column was washed with 10 column volumes of buffer *A* and Pfg27 protein was eluted with a gradient of NaCl from 50 m*M* to 2 *M* in buffer *A* (Fig. 1*b*). The heparin-eluted fractions were concentrated to $\sim$4 mg ml^{-1} and stored at 203 K. Using this protocol, crystallography-grade protein could be obtained readily.

3. Results and discussion

3.1. Crystallization of Pfg27

Highly pure and concentrated Pfg27 ($\sim$4 mg ml^{-1}) was used in crystallization trials based on the sparse-matrix method (Jancarik & Kim, 1991). Both Crystal Screen I and II reagents (Hampton Research) were used in the trials. All experiments were performed using the hanging-drop vapour-diffusion method in 24-well tissue-culture plates at room temperature. In each trial, 1 µl of protein and 1 µl of reservoir solution were mixed in wells that contained 500 µl of the screening solution. Thin plate-like crystals ($\sim$10 $\times$ 75 $\times$ 75 µm) grew over two weeks (similar to those shown in Fig. 2) in 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate and 30%(*w*/*v*) PEG 8000. Several rounds of optimization were carried out, including changing variables such as temperature, pH, protein concentration, various alcohols and the addition of glucose. The most significant improvement in crystal size was obtained by using glucose as an additive to the reservoir solution. Best crystals were obtained in 0.1 *M* MES pH 6.5, 0.2–0.3 *M* glucose and 11–16%(*w*/*v*) PEG 8000. Addition of glucose to the reservoir solution enhanced the size of the crystals, reduced the number of nucleation sites and gave mostly single crystals.

3.2. X-ray analysis of Pfg27 crystals

A freshly grown Pfg27 crystal was transferred into its reservoir solution (supplemented with 22% glycerol), scooped in a home-made nylon loop and flash-cooled to 100 K in a nitrogen stream (Oxford Cryosystems) at the Protein Crystallography beamline 5.2R of Elettra, Trieste, Italy. A minimum bathing time of 1 min in the cryosolution was required to obtain the best diffraction both in terms of resolution limit as well as crystal mosaicity. A Pfg27 crystal

Table 1
Native diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.0
Space group	$C222_1$
Unit-cell parameters (Å)	a = 58.9, b = 113.2, c = 91.6
Resolution (Å)	25–2.1
Total observations	72581
Unique reflections	18053
Completeness (%)	99 (99)
$\langle I/\sigma(I)\rangle$	37.3 (3.4)
R_{sym}† (%)	3.9 (28.2)

† $R_{sym} = \sum|I_{obs} - I_{avg}/\sum I_{obs}$, where the summation is over all reflections.

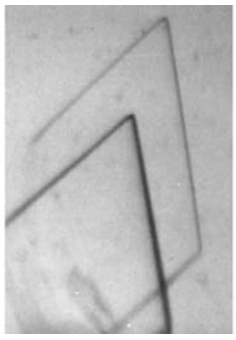

Figure 2
Typical crystals of the Pfg27 protein from *P. falciparum*. The dimensions of these crystals are approximately 20 $\times$ 150 $\times$ 150 µm.

of dimensions ~60 × 200 × 200 µm was used for data collection on the X-ray beamline using a MAR CCD detector. The crystal-to-detector distance was set to 150 mm, the exposure time per frame to 20 s and the oscillation range to 1° per frame. A total of 103 frames were collected with these parameters. Data processing indicated that Pfg27 crystals belong to the orthorhombic space group $C222_1$ (Table 1). The data were processed, reduced and scaled with *DENZO*/*SCALEPACK* programs (Otwinowski & Minor, 1997). Under the stated conditions, Pfg27 crystals diffracted to 2.1 Å and relevant data statistics are given in Table 1. Based on the unit-cell parameters and a molecular weight of 27 kDa, it is likely that there is one molecule of Pfg27 per asymmetric unit. This gives a solvent content of 45% and a V_M of 2.9 Å^3 Da^{-1} (Matthews, 1968).

We are currently producing selenomethionine-substituted Pfg27 using standard protocols (there are four methionines in the 217 amino-acid residue sequence) and we expect to solve the structure using established MAD techniques (Hendrickson *et al.*, 1990). The three-dimensional structure of Pfg27 will provide a platform for functional analysis. A combined structure–function thrust will elucidate the biological role of Pfg27 in controlling the sexual development of *P. falciparum*.

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