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Crystallization and preliminary X-ray analysis of the *Plasmodium vivax* sexual stage 25 kDa protein Pvs25, a transmission-blocking vaccine candidate for malaria

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The *Plasmodium vivax* sexual stage 25 kDa protein Pvs25 is expressed on the surface of the ookinete form of the parasite. Monoclonal antibodies directed against Pvs25 block the development of *P. vivax* oocysts in the mosquito host. Thus, Pvs25 is a potential vaccine candidate for eliciting transmission-blocking immunity in individuals living in malaria-epidemic regions. Pvs25 which was expressed and purified for clinical trials was crystallized using polyethylene glycol as the precipitating agent and diffracts X-rays to 2.3 Å. The orthorhombic Pvs25 crystal form belongs to space group $P2_12_12_1$, with unit-cell parameters a = 42.6, b = 59.8, c = 66.8 Å and one molecule in the asymmetric unit. Reductively methylated Pvs25 crystallized in two forms: an orthorhombic $P2_12_12_1$ form with unit-cell parameters a = 43.4, b = 62.9, c = 66.9 Å and one molecule in the asymmetric unit and a monoclinic $P2_1$ form with unit-cell parameters a = 53.5, b = 43.3, c = 65.3 Å, β = 104.0° which was predicted to have one or two molecules in the asymmetric unit. Several native and heavy-atom data sets have been collected from Pvs25 and methylated Pvs25 crystals for use in MAD or MIR techniques.

1. Introduction

Malaria is a disease caused by infection with protozoan parasites of the genus *Plasmodium* and transmitted by *Anopheles* mosquitoes. The global burden of malaria is estimated to be 500 million clinical cases each year, of which 70–80 million cases are *P. vivax* malaria. *P. vivax* exhibits significant mortality, although lower than that caused by *P. falciparum* malaria, and is responsible for the most recurrent form of malaria, causing high morbidity for millions of people in tropical and subtropical countries outside sub-Saharan Africa (Mendis *et al.*, 2001). *Plasmodium* has a complicated life cycle and vaccines are being developed that would interfere with each developmental stage of the parasite (Good *et al.*, 1998; Hoffman *et al.*, 1998; Kaslow, 1997). The development of vaccines targeting the antigens expressed on the surface of the sexual stage parasite (gametocyte, gamete, zygote, ookinete and oocyst) is considered to be a promising strategy for malaria control (Tsuboi *et al.*, 1997, 2003; Carter, 2001). Such vaccines induce antibodies in the vertebrate host, which inhibit parasite development in the mosquito midgut and consequently prevent the transmission of the parasite to another host.

Pvs25, a leading transmission-blocking vaccine candidate, is expressed on the surface of the ookinete form of *P. vivax* and has been shown to confer transmission-blocking immunity. Mice immunized intraperitoneally with yeast-produced Pvs25 adsorbed to alum (aluminium hydroxide) developed strong antibody responses. The antisera completely inhibited the development of oocysts in mosquitoes when the antisera were ingested with the *P. vivax* parasite (Hisaeda *et al.*, 2000).

In *P. vivax* and most *Plasmodium* species there are two closely related proteins, Pvs25 and Pvs28. The genes of Pvs25 and Pvs28 have been cloned and have about 40% sequence identity (Tsuboi *et al.*, 1998). Both of these proteins are important for the development of malaria parasites in mosquitoes (Tomas *et al.*, 2001) and the three-dimensional structure of one member of the family will assist in rational vaccine design for this important group of molecules. Pvs25 shares the typical family characteristics of the orthologous proteins of other *Plasmodium* parasites, consisting of a signal sequence at the N-terminus, four tandem epidermal growth factor-like domains and a C-terminal glycosyl-phosphatidyl-inositol (GPI) moiety anchoring the protein to the parasite surface (Kaslow *et al.*, 1988; Gozar *et al.*, 1998).

In this report, we describe the crystallization and preliminary X-ray crystallographic studies of Pvs25. The detailed structure–function analysis of Pvs25 will help to elucidate its

biological role and may aid in the development and the improvement of transmission-blocking anti-malaria vaccines.

2. Methods

2.1. Expression and purification

The expression and purification was as described previously (Hisaeda *et al.*, 2000; Miles *et al.*, 2002). Briefly, the gene encoding Ala23–Leu195 of Pvs25 (*P. vivax* Salvador I strain) was inserted into the yeast episomal plasmid YEpRPEU-3 and transformed into *Saccharomyces cerevisiae* VK1 cells. An expression-positive colony was grown in a fermenter and recombinant protein was produced by ethanol induction of the ADH2 promoter. Pvs25 was purified using nickel-affinity, hydrophobic interaction and size-exclusion chromatographies and was characterized by N-terminal sequencing and mass spectrometry. Purified Pvs25 contains 186 amino-acid residues: five N-terminal residues from the yeast α-factor secretion signal (Glu-Ala-Glu-Ala-Ser-), 173 residues of Pvs25 and eight residues from the purification affinity tag, -Gly-Pro-His_6.

2.2. Crystallization of Pvs25

The purified Pvs25 protein was screened for crystallization at 293 K using the hanging-drop vapor-diffusion technique by mixing the protein solution (1 µl, 5–7 mg ml^{-1}) in 20 m*M* Tris–HCl pH 7.4 with equal volumes of the solutions from the Hampton Crystal Screen I kit (Hampton Research). Initial conditions were optimized by systematically varying the pH, buffer concentration and precipitant and the precipitant concentration. The final crystallization condition was 30–40%(*w*/*v*) polyethylene glycol (PEG) 1500, 100 m*M* sodium 2-(*N*-morpholino)ethanesulfonic acid (NaMES) pH 6.0. Crystals appeared after 2–3 d and grew to typical dimensions of 0.3 × 0.2 × 0.1 mm (Fig. 1), but most had flaws when examined under the microscope.

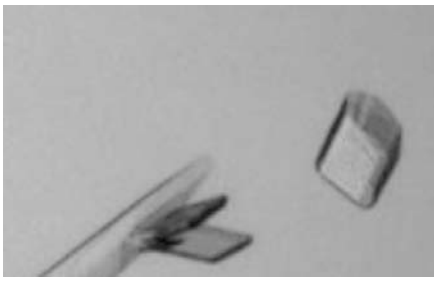


Figure 1
Orthorhombic crystals of unmethylated Pvs25. This crystal size is typical and is about 0.3 × 0.2 × 0.1 mm.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the last resolution shell.

	Pvs25		Methylated Pvs25	
Data set	Native *A*	K_2PtCl_4	Native *B*	Native *C*
Resolution (Å)	20–2.30 (2.38–2.30)	20–2.79 (2.89–2.79)	20–2.80 (2.90–2.80)	36–3.00 (3.11–3.00)
X-ray source	Cu *Kα*	X9B	Cu *Kα*	Cu *Kα*
Wavelength (Å)	1.5418	1.0675	1.5418	1.5418
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_1$
Unit-cell parameters (Å, °)	*a* = 42.6, *b* = 59.8, *c* = 66.8	*a* = 42.9, *b* = 59.4, *c* = 67.1	*a* = 43.4, *b* = 62.9, *c* = 66.9	*a* = 53.5, *b* = 43.3, *c* = 65.3, β = 104.0
Observations	94479	34459	38647	15889
Unique reflections	7839 (740)	7712 (745)	4816 (475)	5680 (569)
Completeness (%)	97.5 (95.0)	93.5 (91.0)	99.5 (100)	94.6 (97.3)
R_{merge}† (%)	7.0 (30.7)	7.1 (24.4)	7.8 (21.4)	9.6 (30.7)
$\langle I\rangle/\langle\langle I\rangle\rangle$	34.4 (9.1)	16.3 (6.9)	25.4 (11.2)	11.0 (4.2)

† $R_{merge} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i |I_{h,i}|$, where $I_{h,i}$ is the ith intensity measurement of reflection h and $\langle I_h\rangle$ is the average intensity of that reflection.

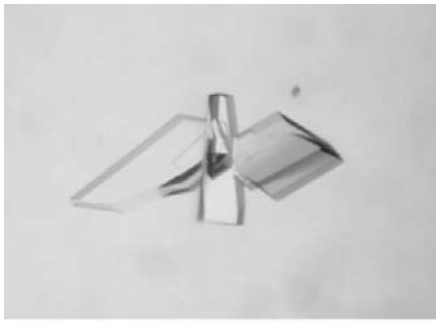
The crystals frequently started growth as 'forks', but would appear more plate-like upon further growth. These crystals were usually multiple and diffracted X-rays anisotropically, as the diffraction spots were streaky and elongated in one direction. Despite extensive trials that sampled a large variety of crystallization conditions with Pvs25, little improvement in diffraction quality was obtained. Data statistics from two of the best crystals are given in Table 1.

2.3. Reductive alkylation of Pvs25

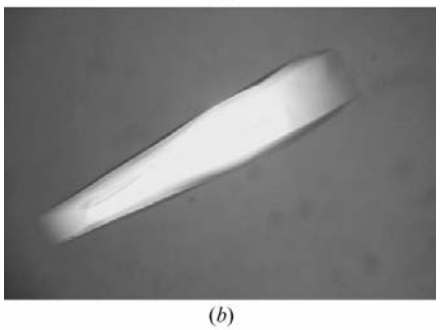
To improve the Pvs25 crystals, Pvs25 was subjected to reductive methylation of the lysine residues as described by Rayment (1997). Briefly, the protein solution (1 ml at 6.4 mg ml^{-1}) was dialyzed against 200 m*M* phosphate buffer pH 7.4 at 277 K. The dialyzed protein solution was brought to 20 m*M* in borane–dimethylamine complex (Sigma–Aldrich), followed immediately by the addition of formaldehyde (Electron Microscopy Sciences) to 88 m*M* and gently mixing at 277 K for 2 h. These additions and the 2 h incubation were repeated. Additional borane–dimethylamine complex was then added (to 10 m*M*) and the reaction mixture was incubated overnight at 277 K. Glycine (100 m*M*) was added to quench the reaction and the solution was extensively dialyzed against 20 m*M* Tris–HCl pH 7.4 for crystallization experiments. Mass spectrometry of the methylated protein revealed a molecular weight that was consistent with two methyl groups having been added to each of the 18 lysines and the N-terminus of Pvs25.

2.4. Crystallization of methylated Pvs25

Methylated Pvs25 was crystallized at room temperature by the hanging-drop vapor-diffusion method by mixing the protein solution (1 µl, 5–7 mg ml^{-1}) with either 30–40%(*w*/*v*) PEG 1500, 100 m*M* NaMES pH 6.0 or 30%(*w*/*v*) PEG 4000, 200 m*M* ammonium acetate, 100 m*M* sodium acetate pH 4.6. The orthorhombic crystal form appeared in both conditions (Fig. 2*a*), while the monoclinic crystal form appeared only in the pH 6.0 condition (Fig. 2*b*). Crystals of methylated protein did not exhibit fork-like growth, but grew uniformly as plates and bars.



(*a*)



(*b*)

Figure 2
Crystals of methylated Pvs25. (*a*) This orthorhombic crystal is typical in size and is approximately 0.4 × 0.3 × 0.1 mm. (*b*) This monoclinic crystal is one of the largest that appeared and is about 0.6 × 0.2 × 0.15 mm.

3. Results and discussion

Both unmethylated and methylated Pvs25 crystals were flash-frozen either in the Cryostream or in liquid nitrogen for data collection. Since all the crystals grew in solutions containing 30–36%(*w*/*v*) PEG, X-ray data were collected without further cryoprotection. Data collection at room temperature was not pursued. Native diffraction data were collected in-house at 100 K using an R-AXIS IV^{++} image-plate detector fitted to a Rigaku rotating-anode generator producing Cu $K\alpha$ radiation. All diffraction images were processed using *DENZO* and the integrated intensities were scaled and analyzed using *SCALEPACK* from the *HKL*2000 package (Otwinowski & Minor, 1997). Crystallographic parameters and data-collection statistics are given in Table 1.

A crystal of unmodified Pvs25 was soaked with K_2PtCl_4 by transferring it to a 2 m*M* K_2PtCl_4, 40%(*w*/*v*) PEG 1500, 100 m*M* NaMES pH 6.0 solution in a sitting drop overnight and yielded the K_2PtCl_4 data set in Table 1, in which the F^+ and F^- measurements were scaled separately. The anomalous signal-to-noise ratio $[\langle|F^+ - F^-|\rangle/\sigma(F^+ - F^-)\rangle]$ from analysis in *XPREP* (Bruker AXS, Wisconsin, USA) was 1.67 in the 4.2–4.0 Å resolution range. Three Pt sites were obtained from *SHELXD* (Schneider & Sheldrick, 2002) and are being used in phasing calculations.

For unmethylated Pvs25, the $P2_12_12_1$ crystal form contains one molecule in the asymmetric unit based on the unit-cell parameters and the molecular weight of Pvs25 (20.6 kDa). This predicts a solvent content of 40% and a V_M value of 2.1 Å^3 Da^{-1}, which is within the range usually observed in protein crystals (Matthews, 1968). For methylated Pvs25, the $P2_12_12_1$ crystal form contains one molecule per asymmetric unit, corresponding to a solvent content of 44% and a V_M value of 2.2 Å^3 Da^{-1}. The $P2_1$ crystal form contains one or two methylated Pvs25 molecules per asymmetric unit, corresponding to a solvent content of 65% and a V_M value of 3.6 Å^3 Da^{-1} or a solvent content of 31% and a V_M value of 1.8 Å^3 Da^{-1}, respectively.

Methylation of Pvs25 allowed us to find orthorhombic and monoclinic single crystals that diffract isotropically, produce diffraction spot shapes that are symmetrical and are more suitable for generating phasing-quality X-ray data. We were not able to grow such crystals from unmethylated protein, although the unmethylated protein crystals diffracted to higher resolution. We are collecting several additional heavy-atom derivative data sets from methylated crystals and expect to solve the structure by MIR and/or MAD techniques. The three-dimensional structure of Pvs25 will help in the understanding of its function and may play a key role in the further development of transmission-blocking vaccines.

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