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Crystallization and preliminary X-ray analysis of the aspartic protease plasmepsin 4 from the malarial parasite *Plasmodium malariae*

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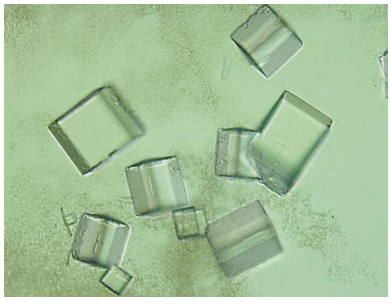


Plasmepsin 4 from the malarial parasite *Plasmodium malariae* (PmPM4) is a member of the plasmepsins (*Plasmodium* pepsins), a subfamily of the pepsin-like aspartic proteases whose ortholog in the malarial parasite *P. falciparum* is involved in hemoglobin digestion in its digestive vacuole. Crystals of PmPM4 in complex with the small-molecule inhibitor AG1776 have been grown from a precipitant of 15% PEG 4000 and 200 m*M* ammonium sulfate in 100 m*M* sodium acetate pH 4.5. X-ray diffraction data were collected on a Rigaku rotating-anode generator from a single crystal under cryoconditions, with a maximal useful diffraction pattern to 3.3 Å resolution. The crystals are shown to be orthorhombic and have been assigned to space group $P2_12_12$, with unit-cell parameters a = 95.88, b = 112.58, c = 90.40 Å and a scaling R_{sym} of 0.104 for 14 334 unique reflections. Packing consideration and self-rotation function results indicate that there are two molecules per asymmetric unit. It is expected that in the near future the structure of PmPM4 will be obtained using molecular-replacement methods, obtaining phases from previously determined plasmepsin structures. Elucidation of the structure of PmPM4 in complex with inhibitors may be paramount to producing new antimalarial therapeutic agents.

1. Introduction

Protozoan parasites of the genus *Plasmodium* are the etiologic agents of malaria, a devastating human disease that afflicts up to 500 million people a year and kills an estimated two million (World Health Organization, 1995). Four *Plasmodium* species infect humans: *P. falciparum* (Pf), *P. vivax* (Pv), *P. ovale* (Po) and *P. malariae* (Pm). During infection with *P. falciparum*, the parasite invades erythrocytes and degrades up to 75% of the hemoglobin content in a specialized digestive vacuole through a semi-ordered sequence of proteolytic events involving aspartic, cysteine and metallopeptidases (Francis *et al.*, 1997; Murata & Goldberg, 2003; Rosenthal *et al.*, 2002; Klemba *et al.*, 2004). Genes encoding ten different plasmepsin enzymes have been identified in the *P. falciparum* genome and four of them [plasmepsin 1 (PfPM1), plasmepsin 2 (PfPM2), plasmepsin 4 (PfPM4) and histidine aspartic protease (HAP)] are active in the digestive vacuole (Banerjee *et al.*, 2002). Previous studies have concluded that the genomes of the non-falciparum species *P. berghei*, *P. knowlesi*, *P. yoelii*, *P. malariae*, *P. vivax* and *P. ovale* all encode a plasmepsin homologous to those in the digestive vacuole of *P. falciparum* (Humphreys *et al.*, 1999; Carlton *et al.*, 2002; Dame, 1996; Bernstein *et al.*, 2003; Dame *et al.*, 2003). Furthermore, these plasmepsins are orthologs of PfPM4 and these other species lack genes encoding PM1, PM2 and HAP (Dame *et al.*, 2003). Therefore, plasmepsin 4 could be a common target for therapeutic compounds against all malarial parasites.

So far, the structural knowledge of this family of enzymes has been limited to three plasmepsins: PfPM2 (329 amino acids; PDB codes 1sme, 1lee, 1lf2; Silva *et al.*, 1996; Asojo *et al.*, 2002), PfPM4 (329 amino acids; PDB code 1ls5; Asojo *et al.*, unpublished work) and PvPM4 (327 amino acids; PDB code 1qs8; Bernstein *et al.*, 2003). These studies have shown that plasmepsins have the characteristic bilobal shape (N- and C-terminal domains) of the pepsin family of aspartic proteases and that the active site is located in a deep cleft between the two lobes (Fig. 1*a*).

Here, we report for the first time the crystallization and preliminary crystallographic analysis of PmPM4 (328 amino acids).

2. Methods

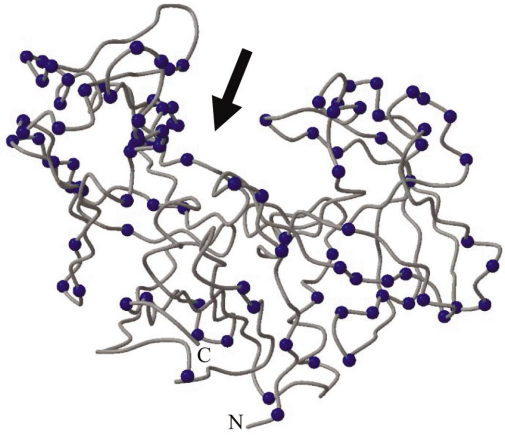
2.1. Expression and purification

Recombinant PmPM4 was cloned as described by Dame *et al.* (2003). The enzyme was expressed using the pET3a expression vector from Novagen and transformed into the *Escherichia coli* expression cell line BL21(DE3)pLysS from Stratagene. Protein expression, inclusion-body isolation, protein refolding and purification were carried out as described by Westling *et al.* (1997) with the following modification. After refolding and anion-exchange chromatography, the enzyme was further purified by size-exclusion chromatography using a Superdex 75 16/60 column from Amersham Pharmacia. The enzyme was then activated in 0.1 *M* sodium formate buffer pH 4.4 at 310 K for 5 min. The activated PmPM4 was then diluted into 20 m*M* Tris pH 8.0, purified using a Resource Q column from Amersham and eluted off the column using a gradient of 0–1 *M* NaCl in 20 m*M* Tris pH 8.0 buffer.

2.2. Crystallization

PmPM4 was concentrated to 8.5 mg ml^{-1} (0.2 m*M*) using a 30 kDa Centricon (Amicon) and preincubated with the small molecule AG1776 (formerly known as JE2147), a peptidomimetic inhibitor of HIV protease (Yoshimura *et al.*, 1999), in a twofold to fivefold molar excess (0.4–1.0 m*M*) for 120 min at 277 K in 100 m*M* sodium acetate pH 4.5 prior to crystallization. Initial crystallization trials were conducted using the hanging-drop vapour-diffusion method (McPherson, 1982), screening the precipitants 10–40% polyethyleneglycol molecular weight 4000 (PEG 4000), 0.5–1.0 *M* ammonium sulfate and combinations of both in 100 m*M* sodium acetate pH 4.5. Crystal drops were prepared by mixing 2 µl of enzyme solution with 2 µl of precipitant solution and equilibrated by vapor diffusion against 1 ml precipitant solution at 298 K.

Based on the results of the crystallization screening, useful X-ray diffraction-quality crystals of PmPM4 were obtained by mixing 10 µl of enzyme (pre-incubated with the inhibitor AG1776) with 4 µl of precipitant solution consisting of 15% PEG 4000 and 0.2 *M* ammonium sulfate.



(*a*)

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PfPM2   1-SSNDNIELVDFQNIM FYGDAEVGDNQQPFT FILDTGSANLWVPSV- 45
PmPM4   1-SENDSIELDDVANLM FYGEGQIGTNKQPFM FIFDTGSANLWVPSV- 45

PfPM2  46-KCTTAGCLTKHLYDS SKSRTYEKDGTKVEM NYVSGTVSGFFSKDL- 90
PmPM4  46-NCDSIGCSTKHLYDA SASKSYEKDGTKVEI SYGSGTVRGYFSKDV- 90

PfPM2  91-VTVGNLSLPYKFIEV IDTNGFEPTYTASTF DGILGLGWKDLSIGS-135
PmPM4  91-ISLGDLSLPYKFIEV TDADDLEPIYSGSEF DGILGLGWKDLSIGS-135

PfPM2 136-VDPIVVELKNQNKIE NALFTFYLPVHDKHT GFLTIGGIEERFYEG-180
PmPM4 136-IDPVVVELKKQNKID NALFTFYLPVHDKHV GYLTIGGIESDFYEG-180

PfPM2 181-PLTYEKLNHDLYWQI TLDAHVGNIMLEKAN CIVDSGTSAITVPTD-225
PmPM4 181-PLTYEKLNHDLYWQI DLDIHFGKYVMQKAN AVVDSGTSTITAPTS-225

PfPM2 226-FLNKMLQNLDVIKVP FLPFYVTLCNNSKLP TFEFTSENGKYTLEP-270
PmPM4 226-FLNKFFRDMNVIKVP FLPLYVTTCDNDDLP TLEFHSRNNKYTLEP-270

PfPM2 271-EYYLQHIEDVGPGLC MLNIIGLDFPVPTFI LGDPFMRKYFTVFDY-315
PmPM4 271-EFYMDPLSDIDPALC MLYILPVDIDDNTFI LGDPFMRKYFTVFDY-315

PfPM2 316-DNHSVGIALAKKNL-329
PmPM4 316-EKESVGFAVA-KNL-328
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(*b*)

Figure 1
(*a*) Superimposed onto the crystal structure of PfPM2 (C^{α} grey coil) are the amino-acid positions that differ between PfPM2 and PmPM4 (blue spheres). The arrow indicates the active-site cleft between the N- and C-terminal domains. This figure was made using *BOBSCRIPT* (Esnouf, 1997, 1999; Kraulis, 1991). (*b*) Pairwise sequence alignment of PfPM2 and PmPM4. Amino acids that differ between PmPM4 and PfPM2 are highlighted in blue.

2.3. Data collection

Data were collected using an R-AXIS IV^{++} image-plate system with Osmic mirrors and a Rigaku HU-H3R CU rotating-anode generator operating at 50 kV and 100 mA. A 0.3 mm collimator was used with a crystal-to-detector distance of 150 mm and the 2θ angle fixed at 0°. A single crystal was briefly dipped into a cryoprotectant (30% glycerol mixed with precipitant solution) and placed on a thin nylon crystal-mounting loop (Hampton Research) and flash-frozen (Oxford Cryosystems). All diffraction data frames were collected using a 0.5° oscillation angle with an exposure time of 600 s per frame at 105 K. The data set was indexed using *DENZO* and scaled and reduced with *SCALEPACK* software (Otwinowski & Minor, 1997).

2.4. Sequence alignment and model building

A pairwise sequence alignment of PmPM4 and PfPM2 was performed using *CLUSTALW* (Thompson *et al.*, 1994). Based on the previously determined three-dimensional crystal structure of the PfPM2 (PDB code 1lf2; Asojo *et al.*, 2002) and the *CLUSTALW* sequence alignment, a model of PmPM4 was built using *SWISS-MODEL* (Peitsch, 1995, 1996).

2.5. Rotation and translation search

Self-rotation function calculations (Rossmann & Blow, 1962) were computed using the program *GLRF* (Tong & Rossmann, 1990). Cross-rotation and translation-function calculations were performed with the software *MOLREP* (Vagin & Teplyakov, 1997) using the

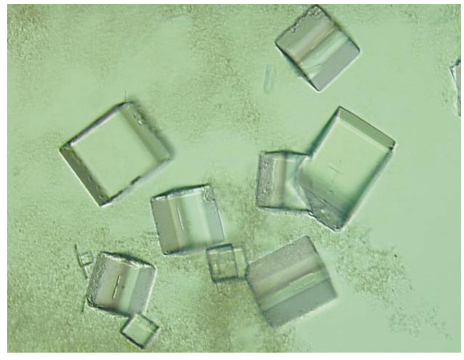

Figure 2
Optical photograph of crystals of PmPM4. The bar indicates 0.2 mm. The photograph was taken with a Bio-Rad 1024 ES Confocal Microscope with an Olympus IX 70 transmission.

PmPM4 model truncated to a poly-L-alanine chain in order to avoid phase bias.

3. Results and discussion

3.1. Crystallization

Crystals suitable for X-ray diffraction studies appeared around three months after equilibration against the precipitant solution. The crystal habit was regular rectangular parallelepipeds with approximate dimensions of 0.2 × 0.2 × 0.2 mm (Fig. 2). Interestingly, the quality of the diffraction of these crystals apparently improved with time. Diffraction data obtained from 'freshly' formed crystals provided usable data to only ∼6 Å resolution, whereas the X-ray diffraction data reported in this paper were obtained from a crystal tray that had been set up six months prior to data collection and the crystal selected had sat in the crystal tray for three months. Hence, in light of this crystal-aging phenomenon of improving diffraction quality with time, fully grown crystals are currently being left undisturbed in crystal trays so that we can observe whether this trend continues. However, we are also exploring variations on our current crystallization conditions and incubating PmPM4 with a variety of inhibitors in an attempt to improve the resolution of diffraction.

We also expect that these PmPM4 crystals are in complex with the inhibitor AG1776, as using comparable crystallization conditions for PmPM4 alone only produced very irregular crystalline entities.

3.2. Data collection

A total of 180° of data were collected (360 images) from a single crystal and a total of 60 347 reflections were measured to a maximum useful resolution of 3.3 Å. The data were initially processed in the Laue crystal system *P*222, with unit-cell parameters a = 95.88, b = 112.58, c = 90.40 Å. The data were merged and reduced to a set of 14 334 independent reflections (93.9% complete, 84.6% in the outer resolution shell), resulting in an $R_{\rm sym}$ of 0.104 (0.259 in the outer resolution shell; Table 1).

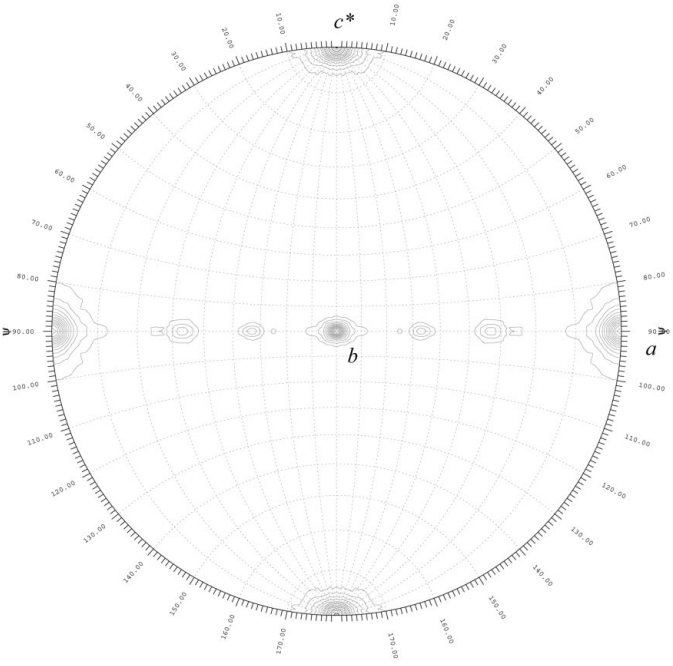

Figure 3
Self-rotation function stereographic projection of the $P2_12_12$ reduced data set for κ = 180°, calculated for data between 10 and 4 Å resolution using the *GLRF* program (Tong & Rossmann, 1990).

Table 1
Summary of data statistics.

Values in parentheses are for the outer resolution shell.

Temperature (K)	105
Resolution range (Å)	25–3.3 (3.42–3.30)
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 95.88, b = 112.58, c = 90.40
X-ray source	Rigaku Cu rotating-anode generator
Wavelength (Å)	1.5418
Solvent content (%)	62.6
Crystal dimensions (mm)	0.2 × 0.2 × 0.2
$V_{\rm M}$ (Å^3 Da^{-1})	3.3
Total No. reflections	60347 (5317)
No. unique reflections	14334 (1263)
Mosaicity	0.71
$R_{\rm sym}$†	0.104 (0.259)
Completeness (%)	93.9 (84.6)
Average $I/\sigma(I)$	14.4 (3.8)

† $R_{\rm sym}$ is defined as $\sum|I-\langle I\rangle|/\sum I$, where I is the intensity of an individual reflection and $\langle I\rangle$ is the average intensity for this reflection; the summation is over all intensities.

On inspection of the intensities of the h00, 0k0 and 00l reflections, the existence of two twofold screw axes along the a and b axes could be inferred, but not along the c axis, therefore implying that the space group was $P2_12_12$. From the unit-cell volume and the molecular weight of PmPM4, a $V_{\rm M}$ value (Matthews, 1968) of 3.3 Å^3 Da^{-1} was calculated for two molecules per asymmetric unit (with a solvent fraction of 62.6% assuming a partial specific volume of 0.74 cm^3 g^{-1}) using *CNS* v.1.1 (Brünger *et al.*, 1998). This observation indicates that the PmPM4 may have crystallized as a dimer. Interestingly, this has also been the case for both the crystal structures of PfPM2 (Silva *et al.*, 1996) and PvPM4 (Bernstein *et al.*, 2003), where each of the two molecules in the crystallographic asymmetric unit exhibits conformational differences in the C-terminal domains. The PmPM4 crystals are highly solvated, so this may explain why the current diffraction is limited to 3.3 Å resolution and that crystals seem to improve in diffraction quality with time, which might be a consequence of crystal dehydration in aging drops. Table 1 gives a full summary of the data-collection statistics.

3.3. Sequence alignment and model building

A pairwise sequence alignment between PmPM4 and PfPM2 showed that 99 out of 328 residues differ, giving an overall sequence identity of 70% (Fig. 1*b*). The r.m.s. difference between the crystal structure of PfPM2 and the PmPM4 model (generated using *SWISS-MODEL*; Peitsch, 1995, 1996) was 0.09 Å over 328 C$^\alpha$ atoms (Fig. 1*b*). The modeling studies would suggest that a poly-L-alanine model of PmPM4 (the side-chain conformations might be unreliable and were therefore removed in order to reduce model phase bias) would be suitable for molecular-replacement methods of phasing the data (Rossmann, 1990).

3.4. Rotation and translation search

A self-rotation function calculation for κ = 180° was performed using data between 10 and 4 Å resolution. This clearly showed four peaks (with peak heights approximately half that of the crystallographic twofolds; Fig. 3). These peaks were perpendicular to the c axis in the bc plane, with φ values of 34, 58, 124 and 148°, respectively. This confirmed the existence of one non-crystallographic symmetry dimer of PmPM4 in the crystallographic asymmetric unit, which had been previously inferred from the calculated $V_{\rm M}$ values (Table 1).

A cross-rotation function using the poly-L-alanine PmPM4 model, assuming the Laue group $P222$, was calculated to determine the PmPM4 relative orientations in the unit cell. This resulted in two clear solutions, A ($\alpha = 58.42$, $\beta = 73.47$, $\gamma = 69.96^\circ$) and B ($\alpha = 172.36$, $\beta = 77.10$, $\gamma = 71.05^\circ$), with peak heights of 9.06σ and 8.04σ above the mean, respectively.

Translation functions using the rotation-function results were performed assuming all four possible translational space groups of the Laue group $P222$. The space group $P2_12_12$ (as expected from inspection of the data) yielded the best solutions, with PmPM4 rotation solution A having a position of $T_x = 0.652$, $T_y = 0.659$, $T_z = 0.224$ (fractional coordinates) with an R factor of 0.473 and a correlation coefficient of 0.212 and rotation solution B having a position of $T_x = 0.282$, $T_y = 0.062$, $T_z = 0.256$ (fractional coordinates) with an R factor of 0.487 and a correlation coefficient of 0.187. The combined R factor for the two PmPM4 monomers in the crystallographic asymmetric unit was 0.432 with a correlation coefficient of 0.365.

This work represents the preliminary steps towards obtaining the first crystal structure of PmPM4. It is expected that the structure of PmPM4 in complex with the small-molecule inhibitor AG1776 will be obtained relatively quickly and that this will allow direct structure–function comparisons between the active sites of PmPM4 with PfPM2, PfPM4 and PvPM4. This will also provide insight into the differences in catalytic efficiency and drug inhibition between these plasmepsins.

We are currently 'homing in' on our current crystallization conditions and hope to grow higher resolution diffracting crystals soon, which will be required for the rational drug design of therapeutic compounds targeted to the malarial parasite. We are also planning to collect data using synchrotron radiation, which may facilitate obtaining higher resolution data.

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