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Expression, purification and crystallization of the *Plasmodium falciparum* enoyl reductase

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New hope has been gained in the control of the malaria parasite *Plasmodium falciparum* (pf) with the discovery that the parasite contains a prokaryotic type II fatty-acid synthase (FAS). Since enzymes of this type are absent in humans, they are potential targets for the development of new drugs. The enoyl reductase enzyme (ENR) belonging to this pathway is of particular interest because it has been shown to be inhibited by submicromolar concentrations of the antimicrobial agent triclosan. Here, the development of an efficient overexpression system for pfENR as a fusion protein with maltose-binding protein, its simple one-step purification and cleavage from its fusion protein and crystallization under new conditions with bound NAD^+ cofactor and triclosan are reported. The crystals belong to the space group $P2_1$, with approximate unit-cell parameters $a = 88.2$, $b = 82.4$, $c = 94.8$ Å, $\beta = 90.77^\circ$, and contain a tetramer in the asymmetric unit. Cryocooled crystals (100 K) diffracted to beyond 2.2 Å resolution at the Daresbury Synchrotron Radiation Source.

1. Introduction

The malaria parasite, *P. falciparum*, is responsible for over 500 million cases of malaria per year, killing more than 2 million people annually (Breman, 2001). Recently, a new area for drug development has come to light with the discovery of a prokaryotic type II fatty-acid synthase (FAS) in this parasite (Waller *et al.*, 1998; Waters *et al.*, 2002). Type II FAS systems, in which the enzymes of this pathway are found on separate polypeptides, are common in bacteria and plants (Magnuson *et al.*, 1993). In contrast, in the type I FAS systems found in metazoans the domains that carry out the equivalent catalytic steps are quite different in amino-acid sequence and reside on one or two much larger multifunctional proteins (Smith, 1994). Thus, the enzymes of type II FAS systems are prime targets for the discovery of drugs with potentially low toxicity in man. Previous studies have shown that enoyl reductase (ENR) is a key enzyme in type II systems and is the target of several classes of antimicrobial compounds, including the diazaborines and triclosan (Baldock *et al.*, 1996; Levy *et al.*, 1999, 2001). More recently, it has been shown that triclosan also inhibits pfENR with a K_i of 0.4 nM (Kapoor *et al.*, 2001) and inhibits the growth of *P. falciparum* in culture with an IC_{50} of 0.7 μM (Surolia & Surolia, 2001).

The sequence of the pfENR shows significant sequence similarity to enoyl reductases from other species with, for example, 47% identity to the enzyme from *Brassica napus*. However, the pfENR sequence differs from all other ENR sequences determined to date in that it contains a 43 amino-acid insert in a loop which in the ENRs from other species is known to undergo substantial conformation change on inhibitor (and presumably substrate) binding (Levy *et al.*, 1999, 2001; Qiu *et al.*, 1999). The structure of this loop is therefore of particular interest in the exploitation of pfENR for drug discovery owing to its possible interactions with substrate, cofactor or inhibitors. Recently, the structure of pfENR has been solved to 2.4 Å (Perozzo *et al.*, 2002), but unfortunately no density could be seen for the 43 amino-acid insert owing to disorder in this part of the structure, thus leaving an important piece of the structure undetermined. In order to address this problem, we have developed a new expression system for this enzyme. We report the successful expression and purification of pfENR to give crystals of a new form which diffract to high resolution.

2. Materials and methods

2.1. Expression and purification of pfENR using the pMALc2x vector

The coding sequence of *P. falciparum* ENR was amplified from gDNA of the 3D7 strain of *P. falciparum* using PfuTurbo polymerase (Stratagene). The primers (forward) 5′-GGTGGT**GAATTC**TCAAACATAAACAAAATTAAAGAAG-3′ and (reverse) 5′-GGTGGT-

GTCGACTTATTCATTTTCATTGCGATATATATC-3′ were used to amplify nucleotides encoding amino acids 85–432 and to introduce a proximal *Eco*RI and distal *Sal*I site (bold) in the PCR product. Nucleotides encoding the amino-terminal 84 residues of pfENR were excluded because this region is principally composed of a signal peptide and an organellar transit peptide. The resulting amplicon was digested with *Eco*RI and *Sal*I and ligated into the pMALc2x vector (New England Biolabs). The resulting pSTP6 was transformed into BL21 Star(DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid isolated from BL21-CodonPlus(DE3) cells (Stratagene) and used for the expression of MBP-ENR fusion protein. Cells were grown in LB medium at 310 K to an optical density at 600 nm of 0.8 and then induced by addition of IPTG to a final concentration of 0.4 m*M*. The culture was maintained in shaker flasks at 293 K for 12 h and then harvested by centrifugation.

Cells were resuspended in lysis buffer [20 m*M* sodium/potassium phosphate pH 7.5, 1 mg ml^{-1} lysozyme (Sigma), 2.5 µg ml^{-1} DNAse I (Sigma), 200 m*M* NaCl] and sonicated. Cell lysate was clarified by centrifugation and applied to a 10 ml amylose column (New England Biolabs) for affinity purification (Fig. 1*a*, lane 2). Purified MBP-ENR fusion protein was digested with factor Xa (New England Biolabs) at a ratio of 1 mg factor Xa per 500 mg of fusion protein in the presence of 1 m*M* calcium chloride at 277 K (Fig. 1*a*, lane 3). The reaction mixture was desalted with a HiPrep 26/10 desalting column (Pharmacia) and applied to an SP Sepharose cation-exchange column (Pharmacia). Column fractions containing pure pfENR protein were pooled for further analysis.

2.2. Expression and purification of pfENR using the pMALcHT vector

A second construct of pfENR also containing residues 85–432 was designed for *in vivo* cleavage by the TEV (tobacco etch virus) protease. The amplicon described above was ligated into a modified version of the pMALc2x vector (pMALcHT) in which the linker region was altered to contain nucleotides encoding a TEV (tobacco etch virus) protease cleavage site followed by a six-histidine tag (Fig. 1*b*). The resulting ligation product, pSTP7, was transformed into BL21 Star(DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid from BL21-CodonPlus(DE3) cells (Stratagene) and plasmid (pKM586) encoding the TEV protease (Kapust & Waugh, 2000). Cells were grown, harvested and lysed as above. Cell lysate was clarified by centrifugation and applied to a 5 ml HiTrap Chelating HP column (Pharmacia). Column fractions containing cut pfENR were desalted with a HiPrep 26/10 desalting column (Pharmacia) and loaded onto a 5 ml HiTrap SP Fast Flow column (Pharmacia). Pure pfENR (Fig. 1*a*, lane 4) was then concentrated to 12 mg ml^{-1} for crystallization trials.

2.3. Crystallization of pfENR and data collection.

Crystals of pfENR were grown using the hanging-drop vapour-diffusion technique by mixing 2.5 µl of the protein solution (12 mg ml^{-1} pfENR in 20 m*M* sodium/potassium phosphate pH 8.0, 150 m*M* NaCl, 5 µ*M* NAD^+ and 6 µ*M* triclosan) with 2.5 µl of the reservoir solution at 290 K. Initial screening of crystallization conditions was conducted using Crystal Screen 1, Crystal Screen 2 and PEG/Ion Screen (Hampton Research), of which PEG/Ion screen solution 11 [20%(*w*/*v*) PEG 3350 and 200 m*M* KI] produced the best-quality crystals. This aforementioned screen was optimized, changing both the pH and precipitant concentrations, to achieve an optimal reservoir solution composed of 19.5%(*w*/*v*) PEG 3350 and 230 m*M* KI. This condition produced good-quality crystals which took four to five weeks to reach dimensions of 0.15 × 0.10 × 0.10 mm. X-ray analysis at the Daresbury Synchrotron Radiation Source (SRS) of crystals frozen at 100 K using 20% glycerol as a cryoprotectant showed that they diffracted to beyond 2.2 Å. Rotation images were collected with 1° oscillation width and 1 min exposure times on an ADSC Quantum 4 detector at station 14.1. These crystallization conditions were very different to those previously reported by Perozzo *et al.* (2002), which were based on ammonium sulfate. They also give crystals whose diffraction properties appear to be superior, giving a lower $R_{\rm merge}$ (0.095 compared with 0.123) at a higher resolution of 2.2 *versus* 2.4 Å.

3. Results and discussion

We were able to efficiently overexpress pfENR as a fusion protein with the maltose-binding protein (MBP) using the pMALc2x expression vector. The resulting MBP-ENR fusion protein was readily purified through affinity chromatography (Fig. 1*a*, lane 2). However, endoproteolytic digestion of pure

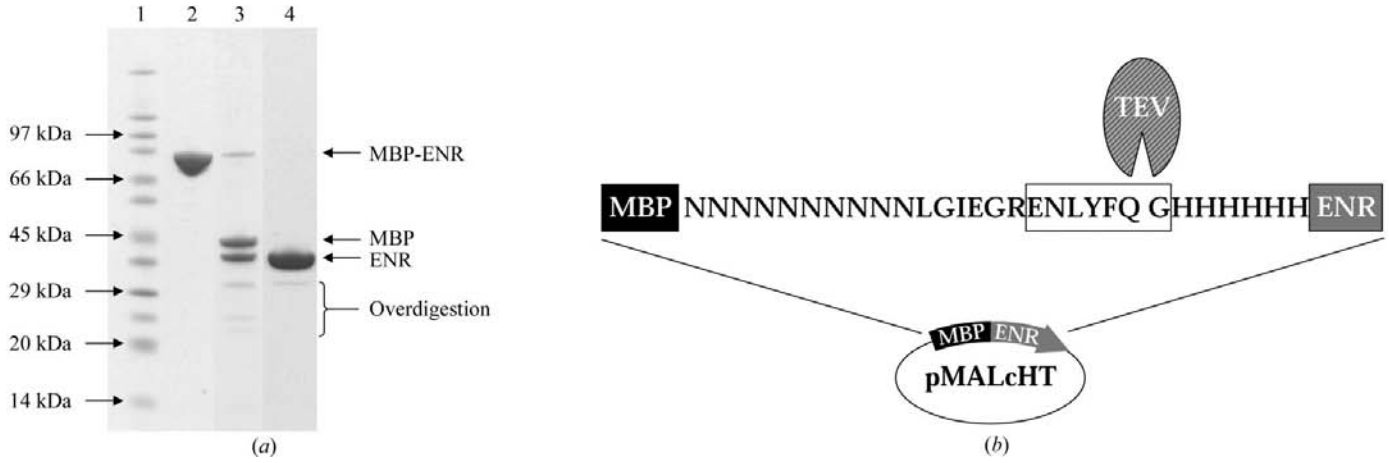

Figure 1
(*a*) SDS–PAGE of pfENR purification. SigmaMarker wide-range molecular-weight markers are shown in lane 1. Pure MBP-ENR fusion protein produced by the pMALc2x vector (lane 2) and factor Xa-digested fusion protein (lane 3) are shown. Lane 4 shows pfENR produced by *in vivo* cleavage using the pMALcHT vector. (*b*) Schematic representation of the pMALcHT vector. Amino acids in the linker region between the maltose-binding protein (MBP) and pfENR (ENR) are shown using single-letter abbreviations. The seven-residue TEV protease recognition site is boxed and a gap indicates the protease cleavage site.

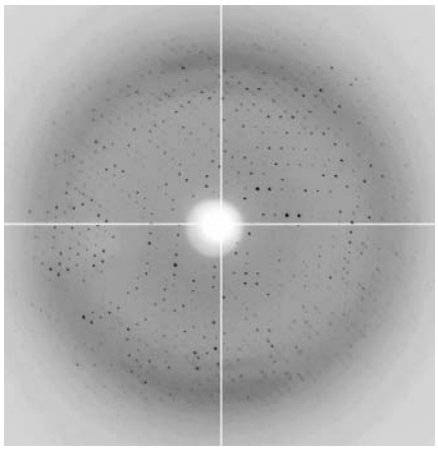

Figure 2
A representative 1° oscillation image of data collected from a crystal of pfENR complexed with NAD^+ and triclosan on a Quantum Q4 CCD detector on station 14.1 at the SRS Daresbury Laboratory. The resolution at the edge of the image corresponds to a resolution of 2.2 Å.

MBP-ENR with factor Xa protease yielded overdigestion products under conditions that did not completely digest all of the MBP-ENR fusion protein (Fig. 1*a*, lane 3). Better results were obtained using *in vivo* cleavage of the fusion protein by the tobacco etch virus (TEV) protease. The linker region of the pMALc2x vector was modified to contain a TEV protease cut site followed by a six-histidine tag, generating the pMALcHT expression vector (Fig. 1*b*). This vector was cotransformed into *Escherichia coli* along with a plasmid encoding the TEV protease (pKM586) and a plasmid encoding three rare tRNAs (pRIL). A long low-temperature induction period during the expression of the MBP-ENR fusion protein also served to expose the fusion protein to digestion by constitutively expressed TEV protease. The resulting pfENR digestion product could then be purified *via* a histidine tag exposed by cleavage. Since pfENR is a multimer (see below), uncut MBP-ENR will tend to copurify with cut pfENR. We were able to remove trace amounts of uncut MBP-ENR by using a subtractive amylose column.

The data collected at the Daresbury Synchrotron Radiation Source (SRS) were processed and scaled using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997). Analysis of the diffraction data using the autoindexing routine in the program *DENZO* showed that the crystals belong to the primitive monoclinic system,

Table 1
Data collection and processing for pfENR crystals.

Values in parentheses refer to data in the highest resolution shell.

Space group	$P2_1$
Wavelength used (Å)	0.9600
Resolution range (Å)	0–2.18 (2.26–2.18)
Unique reflections	77321 (7521)
Multiplicity	3.1 (3.0)
Completeness (%)	97.2 (94.7)
$I/\sigma(I) > 3$ (%)	72.6 (49.6)
$R_{\rm merge}$† (%)	0.095 (0.395)

† $R_{\rm merge} = \sum_{hkl} |I_i - I_m| / \sum_{hkl} I_i$, where I_i and I_m are the observed intensity and mean intensity of related reflections, respectively.

with unit-cell parameters $a = 88.18$, $b = 82.37$, $c = 94.82$ Å, $\alpha = \gamma = 90$, $\beta = 90.77°$. Reflections were observed along the $0k0$ axis (b^*) only where they satisfied the condition $k = 2n$, indicating that the crystals belonged to space group $P2_1$. Significant reflections were observed to the edge of the image-plate detector (Fig. 2) and a good-quality data set was collected to 2.2 Å. Data-collection and processing statistics can be found in Table 1. Gel-filtration studies indicate that pfENR is a tetramer in solution like the enzymes from *E. coli* and *B. napus* (Baldock *et al.*, 1998; Rafferty *et al.*, 1995); consideration of the possible values of $V_{\rm M}$ suggest that the asymmetric unit contains a complete tetramer with a $V_{\rm M}$ of 2.2 $Å^3$ Da^{-1}, which is within the range observed for protein crystals (Matthews, 1974).

A preliminary attempt to solve the structure using molecular replacement with the *AMoRe* program (Navaza, 1994) as implemented within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) has been carried out using the coordinates of *B. napus* ENR (PDB code 1d70) as a search model. A promising solution was obtained and ultimately it is hoped that refinement of the structure to high resolution may provide insights into the details of inhibitor binding and allow us to see any important interactions with the additional loop present in the pfENR.

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