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Expression, crystallization and preliminary X-ray analysis of the phosphoribosylglycinamide formyltransferase from *Streptococcus mutans*

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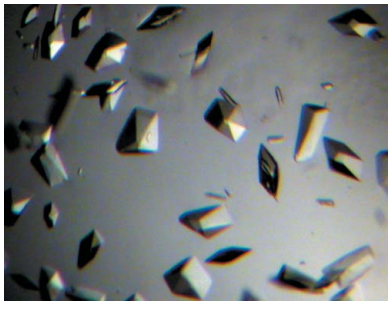
Phosphoribosylglycinamide formyltransferase (PurN) from *Streptococcus mutans* was recombinantly expressed in *Escherichia coli*. An effective purification protocol was established. The purified protein, which had a purity of >95%, was identified by SDS–PAGE and MALDI–TOF MS. The protein was crystallized using the vapour-diffusion method in hanging-drop mode with PEG 3350 as the primary precipitant. X-ray diffraction data were collected to 2.1 Å resolution. Preliminary X-ray analysis indicated that the crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 52.25$, $b = 63.29$, $c = 131.81$ Å.

1. Introduction

Streptococcus mutans is frequently isolated from human dental plaque; it has been implicated as a primary cause of dental caries (Loesche, 1986; Hamada & Slade, 1980) and as a primary pathogen in infective endocarditis (Ullman *et al.*, 1988). It is an opportunistic pathogen that is commonly found as a member of the resident flora of individuals without caries and only expresses its pathogenicity under specific environmental conditions. Apart from the ecological advantages that are provided to *S. mutans* from changes in its genome, it also adapts and responds to environmental stress in order to survive. Generally, adaptive and stress-response mechanisms such as tolerance of acids, starvation, oxygen, fluoride and the expression of urease can be regarded as having evolved to assist *S. mutans* to survive the stresses that are common to its habitats (Borden, 2000).

Phosphoribosylglycinamide formyltransferase is a key folate-dependent enzyme in the *de novo* purine-biosynthesis pathway (Zhang *et al.*, 2002; Welin *et al.*, 2010). Nucleotide bases are essential for all living organisms. The biosynthesis of nucleotides is a crucial process for the production of the building blocks for RNA and DNA, as well as to provide nucleotides for additional cellular processes in which they serve as energy carriers or signalling molecules (as secondary messengers or neurotransmitters; Aimi *et al.*, 1990; Welin *et al.*, 2010; Zhang *et al.*, 2002).

Nucleotides can be obtained by two different routes: synthesis *via* the *de novo* pathway or salvage from the breakdown products of nucleic acids. The function of most normal cells (except liver and T cells) relies on the salvage of purines, whereas tumour cells cannot salvage purines efficiently and depend on *de novo* synthesis (McGuire, 2003; Zhang *et al.*, 2009). The *de novo* purine-biosynthesis pathway consists of ten enzymatic reactions (Gooljarsingh *et al.*, 2001; Hartman & Buchanan, 1959; Welin *et al.*, 2010), two of which involve folate-dependent enzymes: phosphoribosylglycinamide formyltransferase (PurN) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (PurH). PurN catalyses the third step of the purine-biosynthesis pathway and transfers the formyl group from N^{10}-formyltetrahydrofolate (fTHF) to glycinamide ribonucleotide (GAR) to produce formyl-glycinamide ribonucleotide (fGAR) and tetrahydrofolate (Almassy *et al.*, 1992; Zhang *et al.*, 2009).



Exploiting the requirement of tumour cells for *de novo* purine biosynthesis, enzymes of nucleotide metabolism constitute potential antiproliferative drug targets for the treatment of, for example, cancer and autoimmune diseases (Welin *et al.*, 2010; Zhang *et al.*, 2002; Aimi *et al.*, 1990). PurN has been developed clinically as an anticancer drug target, with 5,10-dideazatetrahydrofolate (DDATHF) being the first antifolate inhibitor of purine synthesis to reach clinical trials (Boger *et al.*, 1997). Furthermore, enzymes of nucleotide metabolism in pathogens also provide interesting targets for the development of antibacterial (Welin *et al.*, 2010; Samant *et al.*, 2008) and antiviral agents (McGuire, 2003) on the basis of the differences between the bacterial or viral and human enzymes involved in purine metabolism.

Structures are available of three bacterial PurN enzymes, those from *Escherichia coli* (PDB entries 1cde and 1grc; Almassy *et al.*, 1992; Chen *et al.*, 1992), *Aquifex aeolicus* (PDB code 2ywr; W. Kanagawa, S. Baba, S. Kuramitsu, S. Yokoyama, G. Kawai & G. Sampei, unpublished work) and *Mycobacterium tuberculosis* (PDB entries 3dcj and 3da8; Zhang *et al.*, 2009), and one eukaryotic enzyme, the PurN domain of a trifunctional three-domain human enzyme (PDB entry 1zlx; Zhang *et al.*, 2002).

The reported crystal structures of *E. coli* and human PurN contain numerous examples of ligand binding, including the substrate GAR, cofactor analogues and cofactor-based inhibitors, as well as structures with multisubstrate adduct inhibitors (Zhang *et al.*, 2002, 2009; Almassy *et al.*, 1992). This wealth of information has played a major part in the structure-based drug discovery of antifolate compounds for cancer chemotherapy and provides an important knowledge base for the design of potential antibacterial agents once the similarities and differences between the bacterial and human enzymes have been mapped. Given the slow growth and persistence of *S. mutans*, compounds that target purine biosynthesis are attractive drug-development options. *S. mutans* PurN protein shares only 34, 36, 36 and 38% sequence identity at the protein level with the human, *A. aeolicus*, *M. tuberculosis* and *E. coli* PurN proteins, translating into potentially significant structural differences that can be exploited for selective drug discovery.

To date, the detailed structural characteristics of phosphoribosylglycinamide formyltransferase from *S. mutans* remain unknown. Its three-dimensional structure will help us to further elucidate its biological function in infection by *S. mutans*.

In this study, we report the expression, crystallization and preliminary crystallographic analysis of phosphoribosylglycinamide formyltransferase from *S. mutans*.

2. Materials and methods

2.1. Materials

Enzymes for recombinant DNA technology such as *Pfu* polymerase, T4p DNA ligase, *Bam*HI and *Xho*I were purchased from New England Biolabs. PCR amplification kits (including PCR buffer and dNTP Mix) were also obtained from New England Biolabs. The DNA Quick Purify/Recover Kit and Plasmid Mini Kit were products of Omiga Co.

S. mutans strain UA159 was a gift from Professor Lihong Guo of Peking University.

2.2. PCR amplification

The primers spanning the coding sequence of PurN were designed according to the published nucleotide sequence of *S. mutans* strain UA159 (GenBank Accession No. AE014133). In order to facilitate the subsequent cloning, *Bam*HI and *Xho*I restriction endonuclease sites were attached to the 5′-termini of the upstream and downstream primers, respectively: forward, 5′-CGCGGATCCATGGCTCAAA-AAATTGCTG-3′; reverse, 5′-CCGCTCGAGTTATCTTCTCTCG-ATCCC-3′. The polymerase chain reaction was carried out using the genomic DNA of UA159 as a template. The PCR product was separated on an agarose gel containing 1% agarose and was purified using the DNA Quick Purify/Recover Kit.

2.3. Cloning, expression and purification

The purified PCR product was digested with *Bam*HI and *Xho*I overnight and cloned into the expression vector pET-28a (Novagen) treated with the same enzymes. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3).

Protein expression was induced by addition of IPTG (0.1 m*M* final concentration) when the OD_{600} of the culture reached approximately 0.7. The cultures were allowed to grow for a further 12 h at 291 K. The cells were then harvested by centrifugation at 5000 rev min^{-1} for 10 min at 277 K. The bacterial cells were resuspended in lysis buffer (25 m*M* Tris pH 8.0, 500 m*M* NaCl, 5 m*M* imidazole, 1 m*M* DTT and 1 m*M* PMSF) and homogenized by sonication. The cell lysate was centrifuged at 20 000*g* for 45 min at 277 K to remove the cell debris completely.

The following purification steps were performed at 289 K. The clear supernatant was applied onto a self-packaged Ni–NTA affinity column (3 ml Ni–NTA His·Bind resin; Qiagen) and the contaminant proteins were washed away with wash buffer (lysis buffer plus 10 m*M* imidazole). The protein was eluted with a linear gradient of imidazole from 20 to 500 m*M* in lysis buffer. The fractions containing the target protein were pooled, concentrated and desalted using buffer *A* (25 m*M* Tris–HCl pH 8.5, 40 m*M* NaCl, 2 m*M* DTT). The desalted protein sample was further purified using a Resource Q column (Pharmacia) and a Superdex 75 column (Pharmacia).

The purified protein was analyzed by SDS–PAGE and MALDI–TOF MS according to the method described previously (Zhang *et al.*, 2010). The fractions containing the target protein were pooled and concentrated to 22 mg ml^{-1}. The protein concentration was measured using the Bradford method (Bradford, 1976).

2.4. Crystallization

The protein sample was centrifuged at 20 000*g* for 45 min to clarify the solution before starting the crystal screening trials. Initial screening was performed at 291 K in 48-well plates by the sitting-drop

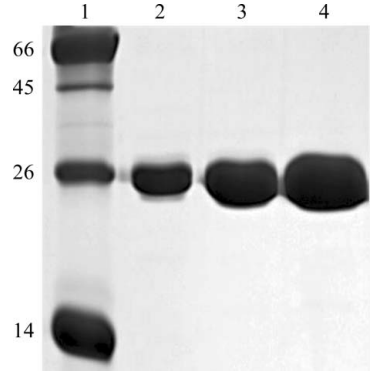


Figure 1
SDS–PAGE of purified PurN. Lane 1, molecular-weight markers (kDa); lanes 2–4, purified PurN used for crystallization.

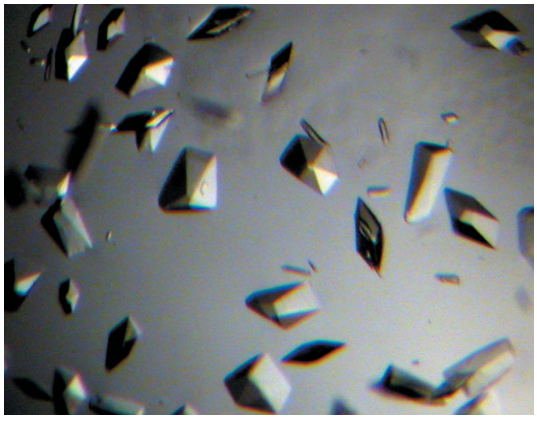


Figure 2
Crystals of PurN.

vapour-diffusion method using sparse-matrix screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, Crystal Screen Lite and Natrix) and was followed by a series of refinements of the conditions by variation of the precipitant, the pH and the protein concentration and use of additives. Generally, droplets consisting of 1 µl protein solution and an equal volume of reservoir solution were equilibrated against 200 µl reservoir solution.

2.5. Data collection and X-ray analysis

X-ray diffraction data sets were collected from the crystals on BL17U of Shanghai Synchrotron Radiation Facility (SSRF) at a wavelength of 0.9793 Å on a MAR CCD 245 image-plate detector.

The crystal was immersed in a cryoprotectant solution (reservoir solution supplemented with 15% glycerol) for 5–10 s, picked up in a loop and then flash-cooled in a nitrogen-gas stream at 100 K. A data set consisting of 220 frames was collected. The exposure time per frame was 0.8 s, the crystal-to-detector distance was 250 mm and the oscillation range per frame was 1°. All intensity data were indexed, integrated and scaled using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997).

Table 1
Data-collection and processing statistics.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 52.25, b = 63.29, c = 131.81
Wavelength (Å)	0.9793
Resolution (Å)	50–2.1
No. of observed reflections	223964
No. of unique reflections	25769
Completeness (%)	98.4 (86.1)
$\langle I/\sigma(I)\rangle$	21.7 (3.0)
$R_{\rm merge}$ (%)	11.1 (36.4)

3. Results and discussion

A 555 bp DNA fragment was obtained by PCR amplification and cloned into pET-28a. The bacteria harbouring the recombinant plasmid were identified by PCR and plasmid digestion and confirmed by DNA sequencing.

PurN fused with an additional 34 amino acids including a 6×His tag (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS) at the N-terminus was solubly expressed in *E. coli* with high yield. After purification, the protein was >95% pure on SDS–PAGE stained with Coomassie Brilliant Blue (Fig. 1). MALDI–TOF MS of trypsin-digested purified protein provided convincing evidence that this protein is the PurN protein from *S. mutans*.

Small crystals appeared after about 5 d from one condition of the Crystal Screen kit (Hampton Research) consisting of 20% PEG 3350, 0.2 *M* lithium chloride. The condition was further optimized by variation of the precipitant, the buffer pH and the protein concentration and larger crystals (Fig. 2) were obtained at 291 K using the vapour-diffusion method in hanging-drop mode by mixing 1 µl protein with 1 µl reservoir solution (0.2 *M* lithium chloride, 0.1 *M* sodium cacodylate trihydrate pH 6.6, 14% PEG 3350) and equilibrating against 200 µl reservoir solution. The larger crystals were reproducible and were suitable for X-ray diffraction.

An X-ray diffraction data set was collected from a single crystal to 2.1 Å resolution (Fig. 3). The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 52.25, b = 63.29, c = 131.81 Å (see Table 1). It is estimated that there are two molecules per asymmetric unit, giving a Matthews coefficient $V_{\rm M}$ of 2.48 Å^3 Da^{-1} and a solvent content of 50.3%. A self-rotation function showed a peak in the

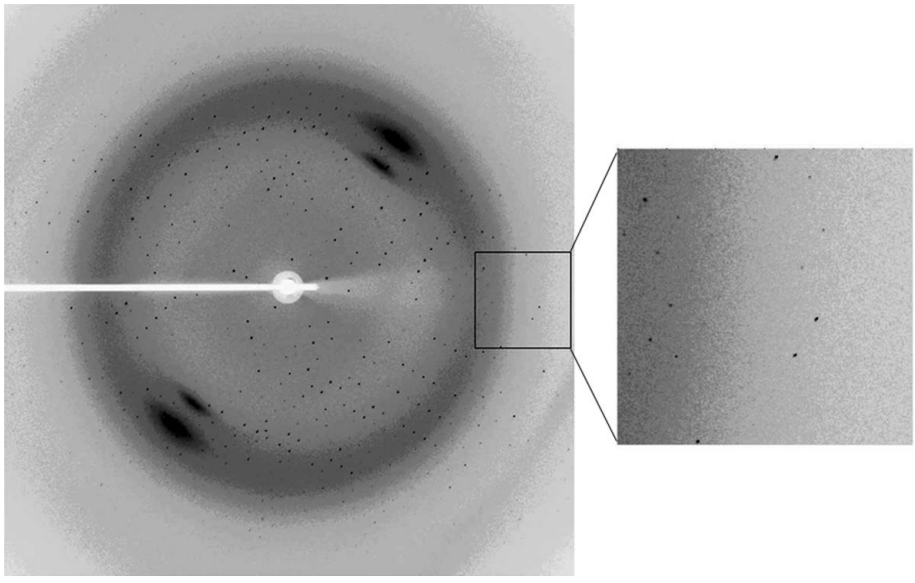


Figure 3
X-ray diffraction pattern from a crystal of PurN. The crystal diffracted to 2.1 Å resolution.

$\kappa = 180^\circ$ section, which confirmed that there are two molecules per asymmetric unit.

Although we have collected several full sets of diffraction data from crystals of PurN expressed in the native form, unfortunately we have so far been unable to determine the structure of PurN by the molecular-replacement method. *S. mutans* PurN shares 34 and 38% sequence identity with human and *E. coli* PurN, respectively. We tried human PurN (PDB entry 1zlx) and *E. coli* PurN (PDB entry 1grc) as models in the molecular-replacement method to solve the structure of *S. mutans* PurN, but we could not obtain a satisfactory result. The multiple-wavelength anomalous dispersion (MAD) or single-wavelength anomalous dispersion (SAD) methods will be used to solve the structure; the preparation and crystallization of selenomethionine-derivatized PurN is now in progress.

The three-dimensional structure of *S. mutans* PurN will facilitate a good understanding of its specific functions and may also be useful in the rational design of anti-*S. mutans* drugs.

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