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Purification, crystallization and preliminary X-ray analysis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase of *Streptococcus pneumoniae*

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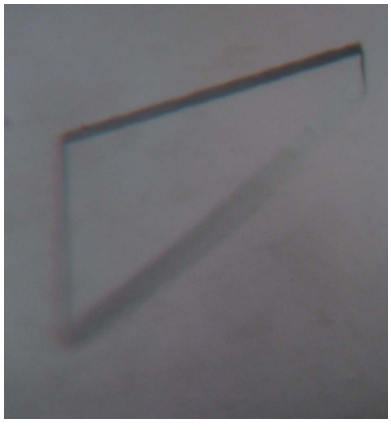

Class II 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductases are potential targets for novel antibiotic development. In order to obtain a precise structural model for use in virtual screening and inhibitor design, HMG-CoA reductase of *Streptococcus pneumoniae* was cloned, overexpressed and purified to homogeneity using Ni–NTA affinity chromatography. Crystals were obtained using the hanging-drop vapour-diffusion method. A complete data set was collected from a single frozen crystal on a home X-ray source. The crystal diffracted to 2.3 Å resolution and belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 773.4836, b = 90.3055, c = 160.5592 Å, $\alpha = \beta = \gamma = 90^\circ$. Assuming the presence of two molecules in the asymmetric unit, the solvent content was estimated to be 54.1% (V_M = 2.68 Å^3 Da^{-1}).

1. Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductases catalyze the reductive deacylation of (*S*)-HMG-CoA to (*R*)-mevalonate (Rodwell *et al.*, 2000). By targeting this rate-controlling enzyme of the mevalonate pathway, HMG-CoA reductase inhibitors such as statins are widely used as cardiovascular disease drugs to lower serum cholesterol (Corsini *et al.*, 1995; Hebert *et al.*, 1997; Eisenberg, 1998).

Two classes of HMG-CoA reductases have been distinguished in eukaryotes, archaea and bacteria. Class I enzymes are present in eukaryotes and most archaea, while class II enzymes exist in eubacteria and some archaea (Bochar *et al.*, 1999). The crystal structures of the *Homo sapiens* class I enzyme and the *Pseudomonas mevalonii* class II enzyme show that the two classes of enzymes share a common catalytic mechanism, but that the number of conserved residues and the location of the active-site lysine are different (Istvan, 2001; Istvan & Deisenhofer, 2001; Lawrence *et al.*, 1995; Tabernero *et al.*, 1999, 2003). The two classes of enzymes have a significant difference in their sensitivity to inhibition by statins. The K_i values of the class II enzymes are over four orders of magnitude higher than those of the class I reductases (Hedl & Rodwell, 2004).

The survival of many Gram-positive pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* depends on a functional mevalonate pathway and class II HMG-CoA reductase is vital for these pathogens (Hedl *et al.*, 2004). The significant divergence between the human and bacterial HMG-CoA reductases allows the development of selective inhibitors of the bacterial enzymes; thus, the class II enzymes could be an efficient target of novel antibiotics.

Our groups are currently working on virtual compound screening and structure-based inhibitor design aimed at the class II reductases from *S. pneumoniae*. Although a high-resolution structure of the *S. pneumoniae* class II reductase was resolved by the pharmaceutical company PanTherix Ltd in Glasgow, Scotland by X-ray crystallography in 2003, to the best of our knowledge no results were made publicly available apart from a meeting abstract (Gourley *et al.*, 2003). Moreover, our preliminary results indicated that the homology structure model based on the *P. mevalonii* enzyme crystal structure was not satisfactory despite its 42% sequence identity. This report describes our attempts to obtain protein crystals and determine the structure of class II HMG-CoA reductase from *S. pneumoniae*.

2. Methods

2.1. Cloning, expression and purification

The 1275 bp open reading frame encoding HMG-CoA reductase was PCR-amplified from *S. pneumoniae* strain R6 genomic DNA (NCBI reference NP_359162) template using high-fidelity DNA polymerase (Novagen) and primer pairs CCATGAAGATAAGTTGGAATGGA and GCTTATTCGTCTGAGTTTTTATG. The PCR-amplified fragment was cloned into pET28a(+) vector (Invitrogen). The final plasmid construct encodes HMG-CoA with 15 additional amino acids (MRGSHHHHHHGMASH–), including a hexahistidine tag, at the N-terminus. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells. The transformed cells were inoculated into LB broth medium and allowed to grow at 310 K until the OD at 600 nm reached 0.8. Protein expression was induced for 12 h by the addition of 0.1 m*M* isopropyl *β*-D-1-thio-galactopyranoside at 289 K. Cultured cells were harvested by centrifugation at 3000*g* for 30 min at 277 K. The cell pellet was resuspended in binding buffer (20 m*M* Tris pH 8.0, 500 m*M* NaCl and 20 m*M* imidazole) and disrupted using a French press at 277 K. The crude lysate was centrifuged at 25 000*g* for 30 min at 277 K. The supernatant was loaded onto an Ni^{2+}-chelating HisTrap FF column (GE Healthcare, USA) which had been pre-equilibrated with binding buffer. The column was washed with binding buffer followed by washing buffer (20 m*M* Tris pH 8.0, 50 m*M* NaCl and 50 m*M* imidazole). The protein was eluted with elution buffer (20 m*M* Tris pH 8.0, 400 m*M* NaCl and 250 m*M* imidazole). The purified protein was concentrated to 10 mg ml^{-1} using a spin filter in buffer consisting of 5 m*M* Tris pH 8.0 and 400 m*M* NaCl, 5% glycerol prior to initial crystallization screening. No removal of the affinity tag was performed. The purity of the protein was examined by 10% SDS–PAGE and determined to be >99%.

2.2. Crystallization

Initial crystallization experiments on native protein were carried out at 297 K in 24-well plates using the hanging-drop vapour-diffusion crystallization method. Crystallization drops consisted of 2 µl 10 mg ml^{-1} protein solution and 2 µl crystallization cocktail. Crystallization screening was initially carried out using Crystal Screen and Crystal Screen 2 from Hampton Research. Initial crystals were observed using condition No. 39 of Crystal Screen, which consists of 2.0 *M* ammonium sulfate, 2% PEG 400, 0.1 *M* HEPES pH 7.5. This condition was optimized by variation of the precipitant concentration and the pH. The optimum condition consisted of 2.0 *M* ammonium sulfate, 6% PEG 400, 0.1 *M* MES pH 6.5. High-quality plate-shaped crystals were obtained (Fig. 1) for diffraction data collection at 277 K.

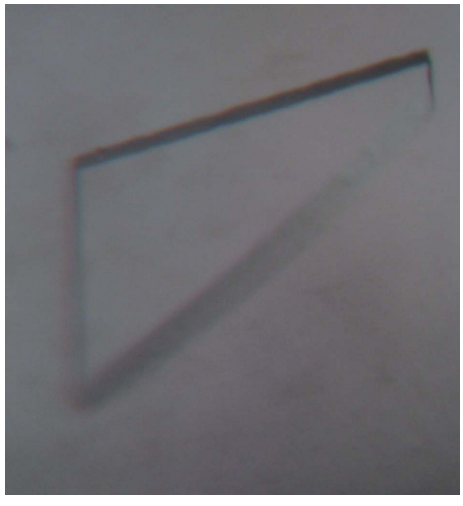

Figure 1
A plate-shaped crystal of HMG-CoA reductase with dimensions of 0.10 × 0.30 × 0.05 mm.

2.3. X-ray diffraction data collection

For X-ray diffraction experiments, a crystal was picked up from the crystallization drop using a nylon loop and flash-frozen in a dry nitrogen-gas stream at 100 K. A complete X-ray diffraction data set was collected at a wavelength of 1.5418 Å on our in-house Oxford Diffraction Xcalibur Nova diffractometer operating at 50 kV and 1 mA. The exposure time and crystal oscillation angles were set to 150 s and 0.5°, respectively. The crystal-to-detector distance was maintained at 120 mm. The crystal diffracted X-rays to 2.3 Å resolution. A total of 404 images were recorded using a 165 mm Onyx CCD detector. Data sets were processed and scaled using *CrysAlis*^{Pro} (v.1.171.33.42; Oxford Diffraction) and programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A typical diffraction image of the HMG-CoA reductase crystal is shown in Fig. 2.

3. Results and discussion

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase of *S. pneumoniae* R6 (NCBI reference NP_359162.1) was cloned in the pET28a(+) vector (Invitrogen) with an N-terminal hexahistidine tag and the protein was purified using Ni–NTA affinity column chromatography. Crystals of HMG-CoA reductase (0.10 × 0.30 × 0.05 mm) that were suitable for structural studies were obtained in 2.0 *M* ammonium sulfate, 6% PEG 400, 0.1 *M* MES pH 6.5 after 2 d

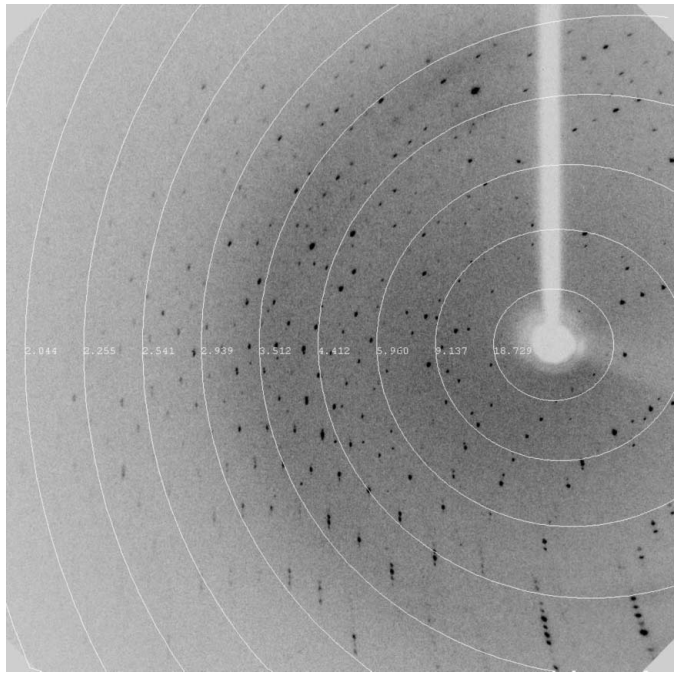

Figure 2
A typical diffraction pattern of an HMG-CoA reductase crystal. The exposure time was 150 s, the crystal-to-detector distance was 120.0 mm and the oscillation range per frame was 0.5°. The diffraction image was collected on a 165 mm Onyx CCD detector.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Crystallization conditions	2.0 *M* ammonium sulfate, 6% PEG 400, 0.1 *M* MES pH 6.5
Wavelength (Å)	1.5418
Temperature (K)	100
Resolution range (Å)	24.72–2.30 (2.42–2.30)
Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 73.4836, b = 90.3055, c = 160.5592, $\alpha = \beta = \gamma = 90$
Observed reflections	88608 (10136)
Unique reflections	24163 (3489)
Data completeness (%)	99.9 (100)
Multiplicity	3.7 (2.9)
$\langle I/\sigma(I)\rangle$	8.8 (3.4)
$R_{\rm merge}$ (%)	0.115 (0.291)
Matthews coefficient (Å^3 Da^{-1})	2.68
Solvent content (%)	54.1

using the hanging-drop vapour-diffusion crystallization method and diffracted X-rays to 2.3 Å resolution on a home X-ray source. The X-ray diffraction data collected from a single crystal were processed using *CrysAlis*^{Pro} (Oxford Diffraction) and scaled using *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The crystal belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 73.48, b = 90.31, c = 160.56 Å, $\alpha = \beta = \gamma = 90^\circ$. The data-collection and processing statistics are summarized in Table 1. The Matthews coefficient (Matthews, 1968) of 2.68 Å^3 Da^{-1} and the solvent content of 54.1% strongly suggested the asymmetric unit to consist of one molecule. The HMG-CoA reductase structure was solved by molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010). The sequences of the *P. mevalonii* and *S. pneumoniae* HMG-CoA reductases have 42% identity, so the monomer of the *P. mevalonii* HMG-CoA reductase crystal structure (PDB code 1qax; Tabernero *et al.*, 1999) was used to create the search model. In 1qax, the two monomers in the dimer are related by noncrystallographic symmetry; the C-terminal region (377–428) of the first monomer (chain *A*) is not visible in the second monomer (chain *B*). A significant molecular-replacement solution was found with a contrast score of 18.07 by using 1qax chain *B* as the search model (chain *A* gave a contrast score of 3.85). After several rounds of model building and refinement with *Coot* (Emsley & Cowtan, 2004) and *REFMAC*5 (Murshudov *et al.*, 1997), the model converged with reasonable R and $R_{\rm free}$ factors of close to 0.23 and 0.26, respectively. Our efforts are now aimed at collecting a high-quality data set at a synchrotron station.

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