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

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 *C*22₁, 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% ($V_M = 2.68$ Å³ 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; Taberner *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 CCATGAAGATAAGTT-GGAATGGA 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 mM isopropyl β -D-1-thiogalactopyranoside at 289 K. Cultured cells were harvested by centrifugation at 3000g for 30 min at 277 K. The cell pellet was resuspended in binding buffer (20 mM Tris pH 8.0, 500 mM NaCl and 20 mM imidazole) and disrupted using a French press at 277 K. The crude lysate was centrifuged at 25 000g for 30 min at 277 K. The supernatant was loaded onto an Ni²⁺-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 mM Tris pH 8.0, 50 mM NaCl and 50 mM imidazole). The protein was eluted with elution buffer (20 mM Tris pH 8.0, 400 mM NaCl and 250 mM imidazole). The purified protein was concentrated to 10 mg ml⁻¹ using a spin filter in buffer consisting of 5 mM Tris pH 8.0 and 400 mM 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 μ l 10 mg ml⁻¹ protein solution and 2 μ l crystallization cocktail. Crystallization screening was initially carried out using Crystal Screen and Crystal Screen 2 from Hampton Research. Initial crystals were

Figure 1

A plate-shaped crystal of HMG-CoA reductase with dimensions of 0.10 $\times$ 0.30 $\times$ 0.05 mm.

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.

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 CCP4 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 $\times$ 0.30 $\times$ 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_{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 *CCP4* suite (Collaborative Computational Project, Number 4, 1994). The crystal belonged to the orthorhombic space group C222₁, 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 Å³ 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 *REFMAC5* (Murshudov *et al.*, 1997), the model converged with

reasonable R and R_{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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