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Crystallization and preliminary X-ray study of a (2*R*,3*R*)-2,3-butanediol dehydrogenase from *Bacillus coagulans* 2-6

(2*R*,3*R*)-2,3-Butanediol dehydrogenase (*R,R*-BDH) from *Bacillus coagulans* 2-6 is a zinc-dependent medium-chain alcohol dehydrogenase. Recombinant *R,R*-BDH with a His₆ tag at the C-terminus was expressed in *Escherichia coli* BL21 (DE3) cells and purified by Ni²⁺-chelating affinity and size-exclusion chromatography. Crystals were grown by the hanging-drop vapour-diffusion method at 289 K. The crystallization condition consisted of 8% (v/v) Tacsimate pH 4.6, 18% (w/v) polyethylene glycol 3350. The crystal diffracted to 2.8 Å resolution in the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 88.35, *b* = 128.73, *c* = 131.03 Å.

1. Introduction

2,3-Butanediol (BD) is known as a by-product of sugar metabolism in microorganisms and is widely used in the food production, cosmetics, fuel, aeronautic, astronautic and pharmaceutical fields (Celińska & Grajek, 2009; Syu, 2001). 2,3-Butanediol dehydrogenases (BDHs), which catalyze the NAD⁺-dependent redox reaction between acetoin (AC) and BD, can be classified into at least three types based on their stereospecificity for their substrates and products: (2*S*,3*S*)-BDH (*S,S*-BDH) catalyzes the conversion of (2*S*,3*S*)-BD to (3*S*)-AC, *R,R*-BDH catalyzes the conversion of (2*R*,3*R*)-BD to (3*R*)-AC and *meso*-BDH catalyzes the conversion of *meso*-BD to (3*R*)-AC (Ui *et al.*, 1984). All isomers of BD and AC are major sources of chiral groups in drugs and high-value pharmaceuticals (Xiao *et al.*, 2010; Yamada-Onodera *et al.*, 2002; Yu *et al.*, 2011). The crystal structures of *meso*-BDH from *Klebsiella pneumoniae* IAM 1063 (PDB entry 1geg, Otagiri *et al.*, 2001) and *S,S*-BDH from *Brevibacterium saccharolyticum* C-1012 (PDB entry 3a28, Otagiri *et al.*, 2010) have been reported previously. These two enzymes share 50% sequence homology and belong to the short-chain dehydrogenase/reductase (SDR) family (Ui *et al.*, 1998). Structural comparison revealed that these two enzymes are similar in overall structure and cofactor-binding mode, implying that they may have similar catalytic mechanisms. However, the difference in orientation of the C3 chiral C atom in the catalytic pocket between *meso*-BDH and *S,S*-BDH may allow them to recognize different diastereomers. This leads to the possible conclusion that the topological difference at the substrate-binding site is responsible for its stereospecificity.

R,R-BDH from *Bacillus coagulans* 2-6 belongs to the medium-chain alcohol dehydrogenase family (Persson *et al.*, 2008). Its sequence is 43% homologous to those of the *R,R*-BDHs from *Bacillus subtilis* and *Paenibacillus polymyxa* ATCC 12321, but is less similar to *meso*-BDH from *K. pneumoniae* IAM 1063 and *S,S*-BDH from *B. saccharolyticum* C-1012. There is no structure report to date on any *R,R*-BDH. Here, we report the crystallization and preliminary X-ray diffraction data analysis of *R,R*-BDH from *B. coagulans* 2-6. Its three-dimensional structure potentially will elucidate its role in substrate recognition and its structural relationship to *meso*-BDH and *S,S*-BDH.

Table 1

Diffraction data statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.5418
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 88.35$, $b = 128.73$, $c = 131.03$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	50.00–2.79 (2.84–2.79)
Total No. of reflections	272873 (10427)
No. of unique reflections	37899 (1862)
Average multiplicity	7.2 (5.6)
Completeness (%)	99.8 (98.5)
$R_{\text{merge}}^{\dagger}$ (%)	6.3 (26.1)
Average $I/\sigma(I)$	27.7 (4.0)
V_M (Å ³ Da ^{−1})	2.42
Solvent content (%)	49.19

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of all symmetry-related reflections.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding *R,R*-BDH was amplified from *B. coagulans* 2-6 genomic DNA (GenBank accession No. NC_015634) using the primers 5'-TGC**ATATG**AAAGCAGCAGTATGGCACGG-3' and 5'-GG**CTCGAG**TTTCTCCCCGCTCAATTAAACC-3' containing *Nde*I and *Xho*I restriction sites (bold sequences), respectively. The amplified PCR products were digested with *Nde*I and *Xho*I and subsequently inserted into pET-22b(+) (Novagen, USA). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen, USA). The transformants were grown at 310 K in Luria–Bertani (LB) medium containing 100 µg ml^{−1} ampicillin until the OD₆₀₀ reached 0.6–0.8 and induced overnight with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

The cells cultured overnight were harvested by centrifugation at 8600g for 30 min and resuspended in 25 ml lysis buffer [20 mM HEPES pH 7.2, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 4 mM β-mercaptoethanol (BME)]. After the cells had been homogenized, the cell debris was removed by centrifugation at 48 400g for

30 min. The supernatant was filtered and loaded onto a Ni²⁺-chelating affinity chromatography column equilibrated with 40 ml lysis buffer. The column was further rinsed with 100 ml wash buffer [20 mM HEPES pH 7.2, 300 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, 4 mM BME] to remove unbound proteins. The tightly bound *R,R*-BDH was eluted with 10 ml elution buffer [20 mM HEPES pH 7.2, 300 mM NaCl, 10% (v/v) glycerol, 50 mM imidazole, 4 mM BME]. The *R,R*-BDH protein was further purified by size-exclusion chromatography with a Superdex 200 10/300 GL column (GE Healthcare, USA). Before crystallization, the *R,R*-BDH protein was dialyzed against 20 mM HEPES buffer pH 7.2 with 10% (v/v) glycerol and 4 mM BME and concentrated to 20 mg ml^{−1} by centrifugation at 277 K using an Amicon Ultra centrifugal filter device (30 kDa molecular-weight cutoff, Millipore). The purity of the protein was analyzed by 12% SDS–PAGE and the protein concentration was determined with a NanoDrop device (Thermo Scientific, USA).

2.2. Crystallization

Initial crystallization screening was performed at 289 K using the hanging-drop vapour-diffusion method with Hampton Research Crystal Screen, Crystal Screen 2, PEG/Ion and Index kits. The protein concentrations used were 8 and 10 mg ml^{−1}. Drops consisting of 1 µl protein sample plus 1 µl crystallization solution were used in the screening. After the initial crystals were found, the crystallization condition was further optimized to produce the crystals for X-ray data collection.

2.3. X-ray data collection and processing

For X-ray diffraction experiments, the crystals were quick-soaked in reservoir solution containing 20% (v/v) glycerol as a cryoprotectant for 15 to 20 s and then flash-cooled in liquid nitrogen (Teng, 1990; Parkin & Hope, 1998). The preliminary X-ray data collection was performed using an in-house X-ray system (Rigaku MicroMax-007 HF desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA) at a wavelength of 1.5418 Å. 720 diffraction images were collected with 0.5° oscillation per image. The data were processed using *HKL-2000* (Otwinowski & Minor, 1997). The data quality was assessed by using *SFHECK* (Vaguine *et al.*, 1999) and the solvent content was calculated with *MATTHEWS_COEF* in the *CCP4* package (Winn *et al.*, 2011).

Figure 1

12% SDS–PAGE analysis of *R,R*-BDH. Lane *M*, molecular-mass standards (labelled in kDa); lane 1, purified *R,R*-BDH (40 kDa).

Figure 2

Typical crystals of *R,R*-BDH.

Figure 3
Typical X-ray diffraction pattern from a *R,R*-BDH crystal.

3. Results

The 1050 bp DNA fragment encoding *R,R*-BDH was amplified and cloned into the expression vector pET-22b(+) to construct the plasmid pET-*R,R*-BDH. Recombinant *R,R*-BDH with a His₆ tag was expressed in a soluble form and purified to electrophoretic homogeneity through Ni²⁺-chelating affinity chromatography and size-exclusion chromatography. After several trials, the expression level of *R,R*-BDH reached a high yield of 50 mg protein per litre of culture. The molecular weight of recombinant *R,R*-BDH was estimated to be about 40 kDa by SDS-PAGE analysis (Fig. 1), coinciding with the calculated molecular weight of 38.5 kDa.

Crystals of *R,R*-BDH protein crystallized readily. Crystals with different shapes were found in several initial crystallization screening conditions after 24–48 h: (i) 0.2 M sodium formate, 20% (w/v) polyethylene glycol 3350; (ii) 0.2 M ammonium phosphate monobasic, 20% (w/v) polyethylene glycol 3350; (iii) 8% (v/v) Tacsimate pH 5.0, 20% (w/v) polyethylene glycol 3350; (iv) 2% (v/v) Tacsimate pH 6.0, 0.1 M bis-tris pH 6.5, 20% (w/v) polyethylene glycol 3350; and (v) 0.2 M sodium bromide, 20% (w/v) polyethylene glycol 3350. The crystals grown in the condition 8% (v/v) Tacsimate pH 5.0, 20% (w/v) polyethylene glycol 3350 diffracted to a reasonable resolution (8 Å). A series of optimizations were performed around this condition by adjusting the pH and the concentration of the precipitant polyethylene glycol 3350. Crystals suitable for X-ray data collection were grown to about 0.1 × 0.12 × 1 mm in size (Fig. 2) in a solution consisting of 8% (v/v) Tacsimate pH 4.6, 18% (w/v) polyethylene glycol 3350.

Crystallographic data statistics are summarized in Table 1. The crystal diffracted to 2.8 Å resolution (Fig. 3) in the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 88.35, *b* = 128.73, *c* = 131.03 Å. This data set has 99.8% overall completeness and 98.5% completeness in the highest resolution shell. The overall *R*_{merge} is 6.3%. The crystal has a solvent content of 49.19%. The Matthews coefficient is 2.42 Å³ Da⁻¹, indicating the presence of four protein molecules in the asymmetric unit (Matthews, 1968).

Human sorbitol dehydrogenase (PDB entry 1pl7; Pauly *et al.*, 2003) has 31% sequence similarity to *R,R*-BDH from *B. coagulans* 2-6. The molecular-replacement (MR) method using *AMoRe* (Navaza, 1994) as implemented in the *CCP4* package (Winn *et al.*, 2011) was

employed to determine the initial phases using the structure of PDB entry 1pl7 as a search model. After rotational and translational search, one of the MR solutions gave very promising *R*_{work} and *R*_{free} values of 21.24 and 28.42%, respectively. Further model building and refinement of the structure are in progress. Determination of the *R,R*-BDH structure will provide insights into the biochemical properties of this novel enzyme from *B. coagulans* 2-6 and illustrate the substrate-recognition mechanism.

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