Crystallization and preliminary X-ray diffraction analysis of domain-chimeric L-(2S,3S)-butanediol dehydrogenase

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A domain-chimeric L-2,3-butanediol dehydrogenase (chimera L-BDH), which was designed to possess both the *S*-configuration specificity of L-BDH and the stability of *meso*-BDH, was constructed by exchanging the respective domains of these two BDHs. However, chimera L-BDH possessed a lower enzymatic function than expected based on the two original enzymes. To elucidate the causes of the decreased stability and substrate specificity, crystallization of the protein was performed. Chimera L-BDH was purified to homogeneity *via* ammonium sulfate fractionation and three column-chromatography steps, and was crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to space group *C*222₁, diffracted synchrotron radiation to 1.58 Å resolution and were most likely to contain two molecules in the asymmetric unit.

1. Introduction

2,3-Butanediol (BD) and acetoin (AC, the precursor of BD) are typical metabolic products of oxidative bacteria. 2,3-Butanediol dehydrogenase (BDH) catalyzes an oxidation–reduction reaction between BD and AC in an NAD-dependent manner. There are three types of BD stereoisomers (*D*-BD, L-BD and *meso*-BD), and there has been considerable interest in BDHs with respect to the isomeric production of BD and AC for possible application of these compounds as chiral moieties in fine chemicals, including drugs and liquid crystals (Ui *et al.*, 2004). *meso*-BDH from *Klebsiella pneumoniae* and L-BDH from *Brevibacterium saccharolyticum* belong to the short-chain dehydrogenase/reductase (SDR) family. Although they share a highly similar structure, their substrate specificities and stabilities differ from each other. Focusing on the structural similarities, a domain-chimeric L-2,3-butanediol dehydrogenase (chimera L-BDH) was obtained by exchanging the domains of the two BDHs in order to potentially combine their beneficial properties (Shimegi *et al.*, 2011). Chimera L-BDH consists of the ‘basic’ domain from *meso*-BDH, which probably contributes to stability, and the ‘leaf-like’ domains of L-BDH, which provide the *S*-configuration substrate specificity towards L-BD. As expected, the chimera L-BDH exhibited a higher stability than wild-type L-BDH and had an activity towards L-BD comparable to that of the wild-type enzyme. However, chimera L-BDH also had relaxed stereoisomeric recognition and significant activity towards other related compounds. These results show not only the potential of the domain-exchange method in enzyme engineering, but also unexpected effects. To understand the reason why chimera L-BDH has this relaxed stereoisomeric recognition, its three-dimensional structure is required. In addition, the structure could provide hints for preferred amino-acid modifications in the enzyme. This study reports the crystallization and preliminary X-ray diffraction analysis of chimera L-BDH.

2. Materials and methods

2.1. Protein expression and purification

The chimera L-BDH gene constructed previously in pUC119 (Shimegi *et al.*, 2011) was amplified using PCR, digested using *Nde*I/*Bam*HI and ligated into the pET-11a expression vector (Novagen,

Germany) using Ligation high Ver.2 (Toyobo, Japan). The reconstructed vector was transformed into *Escherichia coli* BL21(DE3) competent cells. The cells were grown in LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin at 303 K. Protein expression was induced at an OD_{600} of 0.5 by adding isopropyl β -D-1-thiogalactopyranoside to a final concentration of 1.0 mM. After an overnight culture, the cells were harvested by centrifugation (5000g, 10 min, 277 K) and suspended in a buffer consisting of 50 mM Tris-HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride. The cell suspension was sonicated at 277 K and separated *via* centrifugation (38 900g, 30 min, 277 K). The supernatant was fractionated using 35 and 55% saturated ammonium sulfate. The 35–55% precipitate was dissolved in 10 mM Tris-HCl pH 7.5 (buffer A). The protein was applied onto a 2×5 ml HiTrap Phenyl HP column (GE Healthcare Life Science, UK) equilibrated with buffer A supplemented with 1 M ammonium sulfate and eluted using a linear gradient of 1.0–0 M ammonium sulfate. The fractions containing BD activity were diluted with buffer A to decrease the electrical conductivity and applied onto a 5 ml HiTrap Q HP column (GE Healthcare Life Science) also equilibrated with buffer A. Elution was performed with a linear gradient of 0–0.5 M sodium chloride. The pooled fractions were further purified using a Superdex 200 pg column (GE Healthcare Life Science) equilibrated with a buffer consisting of 10 mM Tris-HCl pH 7.5, 250 mM NaCl. The purified protein was concentrated to 30–50 mg ml^{-1} using an Amicon Ultra-15 50K centrifugal filter (Millipore, USA) for crystallization.

The concentration of protein was determined by the Bradford assay (Bradford, 1976) after each purification step.

2.2. Crystallization and diffraction data collection

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method at 293 K. 2 μl protein solution consisting of 10 mg ml^{-1} protein and 1 mg ml^{-1} NAD^+ was mixed with an equal volume of reservoir solution and equilibrated against 500 μl reservoir solution. Since *meso*-BDH and L-BDH were crystallized using PEG 6000 and PEG 4000 as precipitants, respectively (Otagiri, Kurisu, Ui, Tagusagawa *et al.*, 2001; Otagiri, Kurisu, Ui, Ohkuma *et al.*, 2001), the initial screening for chimera L-BDH

crystallization was performed with high-molecular-weight polyethylene glycols at pH values ranging from 6.0 to 8.0. The reservoir solutions contained glycerol as a cryoprotectant and β -mercaptoethanol (ME), which is an inhibitor of L-BDH from *B. saccharolyticum* (Takusagawa *et al.*, 2001). X-ray diffraction data were collected using an ADSC Quantum 210r CCD detector on beamline NE-3A of the Photon Factory-Advanced Ring, Tsukuba, Japan. The data sets were indexed, integrated and scaled with the HKL-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Chimera L-BDH protein expressed in *E. coli* was purified to homogeneity *via* ammonium sulfate fractionation, hydrophobic interaction chromatography, anion-exchange chromatography and gel-filtration chromatography. During Phenyl Sepharose chromatography, the protein eluted at approximately 220–20 mM ammonium sulfate, with the peak being at around 70 mM. The protein yield was 9.4 mg from a 200 ml LB medium culture. SDS-PAGE analysis with Coomassie Brilliant Blue staining identified the purified protein as a single band around 26 kDa, which matches the calculated molecular mass of the chimera L-BDH monomer (Fig. 1). The purity of the protein was sufficiently high for crystallization. According to gel-filtration chromatography (column calibrated with protein standards), the elution volume of the chimera L-BDH corresponded to a molecular weight of approximately 103 kDa. Therefore, the biological unit of the chimera L-BDH was confirmed to be a tetramer, as for native BDHs.

In the initial crystallization screening, clusters of needle-shaped crystals appeared under several sets of conditions using PEG 6000. The conditions were optimized in order to produce larger single crystals, and rod-shaped crystals were obtained when a reservoir solution consisting of 17% (w/v) PEG 6000, 15% (w/v) glycerol and 1% ME in 100 mM MES buffer pH 6.4 was used. Diffraction-quality crystals appeared within one week at 293 K and grew to maximum dimensions of $350 \times 50 \times 20$ μm (Fig. 2). *meso*-BDH was also crystallized using PEG 6000 as a precipitant. However, for *meso*-BDH, magnesium acetate and sodium chloride were required as additives. In addition, the optimum pH for crystallization of *meso*-BDH was found to be 7.2, which is higher than that for chimera L-BDH (6.4). This suggests that the precipitant used in crystallization of the native protein is also effective for that of the domain-chimeric protein,

Figure 1
SDS-PAGE analysis. The black box around the single band at approximately 26 kDa identifies the purified protein. The chimera L-BDH protein was separated using 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane A, 16 μl cell culture; lane B, cell-free extract; lane C, ammonium sulfate fractionation; lane D, HiTrap Phenyl HP; lane E, HiTrap Q HP; lane F, Superdex 200 pg; lane M, molecular-weight marker (labelled in kDa). 10 μg protein was applied to each lane except for lane A.

Figure 2
Crystals of the chimera L-BDH protein.

Figure 3

Diffraction image of a chimera L-BDH crystal collected to 1.58 Å resolution at a wavelength of 1.0 Å.

although the optimized procedures, including additives, might differ from one protein to the next. A diffraction data set (data set 1) was collected to 1.58 Å resolution at a wavelength of 1.0 Å (Fig. 3), and another data set (data set 2) was collected at a wavelength of 1.8 Å from the same crystal in order to determine the positions of S atoms *via* the single-wavelength anomalous dispersion (SAD) method (Dauter *et al.*, 1999; Table 1). Data analysis shows that the crystal belonged to the centred orthorhombic space group $C222_1$, with unit-cell parameters $a = 81.2$, $b = 134.6$, $c = 87.8$ Å, which is a different space group from the crystals of *meso*-BDH and L-BDH (Otagiri *et al.*, 2010). The asymmetric unit was expected to contain two monomers of chimera L-BDH, with a Matthews coefficient of $2.25 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 45.4% (Matthews, 1968). This suggests that a biological tetramer is formed in the crystal lattice by two dimers with crystallographic twofold symmetry, similar to other tetramic SDR-family enzymes (Hadfield *et al.*, 2004; Yasutake *et al.*, 2007). Structure determination *via* the molecular-replacement method using *meso*-BDH (PDB entry 1geg; Otagiri, Kurisu, Ui, Takusagawa *et al.*, 2001) or L-BDH (PDB entry 3a28; Otagiri *et al.*, 2010) as search models is currently in progress.

A high-resolution crystal structure determination and subsequent analysis of the substrate-binding site of chimera L-BDH is expected to

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell. Data set 2 was only used for the determination of the positions of S atoms *via* the SAD method.

	Data set 1	Data set 2
Beamline	NE-3A, Photon Factory-Advanced Ring, Japan	
Space group	$C222_1$	$C222_1$
Unit-cell parameters		
a (Å)	81.2	81.3
b (Å)	134.6	134.8
c (Å)	87.8	87.9
Wavelength (Å)	1.0000	1.8000
Resolution range (Å)	50–1.58 (1.61–1.58)	50–2.57 (2.61–2.57)
Observed reflections	584841	223527
Unique reflections	65845	15793
Oscillation width (°)	0.5	1.0
Total rotation range (°)	180	360
Multiplicity	8.9 (6.4)	14.3 (12.5)
Completeness (%)	99.4 (98.5)	99.1 (96.7)
$R_{\text{merge}}^{\dagger}$ (%)	6.0 (31.1)	5.1 (11.6)
$\langle I/\sigma(I) \rangle$	27.9 (6.4)	42.7 (20.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl) \rangle$ is the mean value for that reflection.

reveal a relaxed substrate specificity which could be caused by the domain-chimeric assembly. Determination of S-atom positions by SAD analysis (Dauter *et al.*, 1999), in particular for ME, will elucidate the binding mode of ME at the active site, which could provide a guide for inspection of the binding of various substrates in ongoing and future work.

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