Cloning, expression, purification, crystallization and X-ray crystallographic analysis of the (S)-3-hydroxybutyryl-CoA dehydrogenase PaaH1 from *Ralstonia eutropha* H16

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The (S)-3-hydroxybutyryl-CoA dehydrogenase PaaH1 from *Ralstonia eutropha* (RePaaH1) is an enzyme used in the biosynthesis of *n*-butanol from acetyl-CoA by the reduction of acetoacetyl-CoA to (S)-3-hydroxybutyryl-CoA. The RePaaH1 protein was crystallized using the hanging-drop vapour-diffusion method in the presence of 1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.0, 0.2 M sodium chloride at 295 K. X-ray diffraction data were collected to a maximum resolution of 2.6 Å on a synchrotron beamline. The crystal belonged to space group $P3_221$, with unit-cell parameters $a = b = 135.4$, $c = 97.2$ Å. With three molecules per asymmetric unit, the crystal volume per unit protein weight (V_M) is $2.68 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 54.1%. The structure was solved by the single-wavelength anomalous dispersion method and refinement of the structure is in progress.

1. Introduction

Current concerns about fossil fuel insufficiency and increasing oil prices have led to the use of alternative energies such as solar, wind, geothermal and water power. However, these energy sources cannot be easily or efficiently utilized in transportation systems (Connor & Liao, 2009; Connor & Atsumi, 2010). To overcome fuel shortages, biofuels such as *n*-butanol may be employed as an alternative motor energy in the future. If *n*-butanol is to be considered as a potential next-generation biofuel source, its biosynthetic efficiency must be improved, and there have been multiple attempts to do this (Schiel-Bengelsdorf *et al.*, 2013; Jang *et al.*, 2012; Lütke-Eversloh & Bahl, 2011). For example, many engineering efforts ranging from genetic modifications to microbial culture optimization have aimed to increase *n*-butanol production during acetone–butanol–ethanol (ABE) fermentation (Mitchell, 1998; Inui *et al.*, 2008). However, the main limitation of ABE fermentation is the toxic effect of *n*-butanol on microbes, which leads to a low production rate of *n*-butanol (Zheng *et al.*, 2009). Therefore, to solve the toxicity problem as well as to increase the final concentration of butanol, attempts have been made to search for highly butanol-tolerant microbial strains or to develop real-time toxicity-excluding techniques such as extraction, gas stripping, adsorption and pervaporation (Mariano *et al.*, 2012; Li *et al.*, 2011).

Recently, alternative methods to enhance the *n*-butanol yield have been reported; these involve the use of metabolically engineered hosts such as *Escherichia coli*, *Pseudomonas putida* and *Bacillus subtilis* in the *n*-butanol biosynthetic pathway (Tran *et al.*, 2014; Nikel & de Lorenzo, 2014; Choi *et al.*, 2014). For example, about fivefold higher titres of *n*-butanol are obtained in *E. coli* by a chimeric pathway using heterologous enzymes from three different species (Steen *et al.*, 2008; Nicolaou *et al.*, 2010).

While many enzymes are involved in *n*-butanol biosynthetic pathways in clostridial species, (S)-3-hydroxybutyryl-CoA dehydrogenase (HBD) catalyzes the second step in the biosynthesis of *n*-butanol from acetyl-CoA by the reduction of acetoacetyl-CoA to (S)-3-hydroxybutyryl-CoA (Jones & Woods, 1986). It has been

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reported that the (*S*)-3-hydroxybutyryl-CoA dehydrogenase PaaH1 from *Ralstonia eutropha* (RePaaH1) is a homologue of clostridial HBD, and the enzyme can be utilized in *n*-butanol biosynthesis in *E. coli* (Machado *et al.*, 2012). The crystal structure of (*R*)-3-hydroxybutyryl-CoA dehydrogenase from *R. eutropha* (RePhaB) has been reported (Kim *et al.*, 2014). Although RePaaH1 and RePhaB utilize the same acetoacetyl-CoA substrate, there is no similarity in amino-acid sequence, oligomeric state or length of polypeptide between these two proteins. RePaaH1 and RePhaB produce (*S*)- and (*R*)-isomers of 3-hydroxybutyryl-CoA, respectively, which can be further processed to *n*-butanol and used in polyhydroxyalkanoate (PHA) biosynthesis (Machado *et al.*, 2012). Based on this report, we cloned the RePaaH1 coding gene and purified the recombinant RePaaH1 protein. Diffraction-quality crystals of RePaaH1 were obtained by the hanging-drop vapour-diffusion method. Diffraction data were collected to 2.6 Å resolution, and the structure was determined by the single-wavelength anomalous dispersion (SAD) method. Here, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the RePaaH1 protein. The crystal structure of RePaaH1 will provide valuable structural information for the development of a highly efficient enzyme that can be utilized to obtain an increased *n*-butanol production rate.

2. Expression and purification of recombinant RePaaH1

The forward and reverse primers were designed as 5'-GCGCG-CATATGAGCATCAGGACAGTGGGCATCG-3' and 5'-GCGCG-CTCGAGCTTGCTATAGACGTACACGCCG-3' to introduce *Nde*I and *Xho*I restriction sites, respectively. The RePaaH1 coding gene was amplified from *R. eutropha* H16 chromosome by polymerase chain reaction (PCR). The PCR product was then subcloned into pPET-30a (Invitrogen) with a 6×His tag at the C-terminus. The resulting expression vector pPET-30a:RePaaH1 was transformed into *E. coli* B834 strain and the cells were grown overnight in LB medium containing 100 µM ampicillin at 310 K. At an OD₆₀₀ of 0.6, RePaaH1 protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h at 291 K, the cells were harvested by centrifugation at 4000g for 15 min at 277 K. The cell pellet was resuspended in buffer A (40 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13 500g for 25 min and the lysate was applied onto a Ni-NTA agarose column (Qiagen). After washing

Figure 1

SDS-PAGE of purification of recombinant RePaaH1 protein. Lane 1 contains molecular-weight markers (labelled in kDa). Lanes 2–12 show the purification procedure of RePaaH1 using Ni-NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, pellet fraction and supernatant after centrifugation of whole cell extract, respectively; lane 5, flow-through from Ni-NTA column; lanes 6–10, wash with 2, 5, 10, 15 and 20 mM imidazole, respectively; lanes 11 and 12, elution with 150 and 300 mM imidazole, respectively.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost shell.

	SeMet peak	RePaaH1
Beamline	7A (SBI), PAL	7A (SBI), PAL
Wavelength (Å)	0.97855	1.000
Temperature (K)	100	100
Oscillation (°)	1.0	1.0
Total rotation range	357	100
Space group	<i>P</i> ₃ ₂ ₁	<i>P</i> ₃ ₂ ₁
Unit-cell parameters		
<i>a</i> = <i>b</i> (Å)	136.4	135.4
<i>c</i> (Å)	97.5	97.2
α = β (°)	90.00	90.00
γ (°)	120.00	120.00
Resolution (Å)	50–2.42 (2.47–2.42)	50.0–2.60 (2.69–2.60)
Total reflections	1007767	122476
Unique reflections	47313	30900
Completeness (%)	99.7 (99.6)	96.8 (95.4)
<i>R</i> _{merge} [†] (%)	12.0 (44.5)	8.7 (37.4)
$\langle I/\sigma(I) \rangle$	56.6 (10.2)	17.0 (2.6)
Multiplicity	21.3 (20.4)	4.0 (3.0)

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections.

with buffer A containing 20 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, the trace amount of contaminants was removed by size-exclusion chromatography using a Superdex 200 prep-grade column (320 ml, GE Healthcare) equilibrated with buffer A containing 1 mM dithiothreitol (DTT). The protein eluted at a molecular weight of ~60 kDa, indicating that the RePaaH1 protein, with a molecular weight of 30 kDa, forms a dimeric structure. All purification experiments were performed at 277 K. The purity of the final protein was assessed by SDS-PAGE (Fig. 1). The purified protein was concentrated to 30 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT. The selenomethionine (SeMet) derivative of the RePaaH1 protein was expressed in M9 minimal medium (Maniatis *et al.*, 1982) supplemented with 25 mg l⁻¹ SeMet and was purified in the same manner as the wild type.

3. Crystallization

Crystallization of the purified RePaaH1 protein was initially performed with commercially available sparse-matrix screens,

Figure 2

Trigonal crystals of RePaaH1. The crystals with the best quality were crystallized using 1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.0, 0.2 M sodium chloride and reached maximum dimensions of 0.5 × 0.3 × 0.2 mm.

including Wizard I and II from Emerald Bio, Index from Hampton Research, and Structure Screen 1 and 2 from Molecular Dimensions, using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 2.0 μ l protein solution (20 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT) with 2.0 μ l reservoir solution and then equilibrating against 0.5 ml reservoir solution. *RePaaH1* crystals were observed from three crystallization screening conditions: (i) 2 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.5, 0.2 M sodium chloride, (ii) 10% PEG 8K, 0.1 M imidazole pH 8.0, 0.2 M calcium acetate and (iii) 25% PEG 3350, 0.1 M bis-tris pH 5.5, 0.2 M lithium sulfate. After several rounds of crystal improvement, suitable crystals for diffraction experiments were obtained using

1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.0, 0.2 M sodium chloride and reached their maximum dimensions of $0.5 \times 0.3 \times 0.2$ mm within 7 d (Fig. 2). The crystallization of the SeMet-derived protein was carried out using the same procedures as described for the native protein.

The crystals were transferred to a cryoprotectant solution consisting of 1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.0, 0.2 M sodium chloride, 30% glycerol, fished out with a loop larger than the crystals and flash-cooled by immersion in liquid nitrogen at 100 K. Data were collected to a resolution of 2.6 Å on beamline 7A at the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using a Quantum 270 CCD detector (ADSC, USA) (Fig. 3). All data were indexed, integrated and scaled together using the *HKL-2000* software package (Otwinowski & Minor, 1997). The crystals of *RePaaH1* belonged to space group *P*3₂21, with unit-cell parameters $a = b = 135.4$, $c = 97.2$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. With three molecules per asymmetric unit, the crystal volume per unit protein weight (V_M) is $2.68 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 54.1% (Matthews, 1968). Two molecules form a dimer and the third molecule forms another dimer *via* the twofold crystallographic axis.

SAD data were collected from a SeMet-derivatized crystal on beamline 7A of PAL at a wavelength of 0.97855 Å. 36 Se atoms of the expected 39 in the asymmetric unit were identified using *SOLVE* (Terwilliger & Berendzen, 1999) at 2.42 Å resolution. The electron density was improved by density modification using *RESOLVE* (Terwilliger, 2000), resulting in 54% of the residues being automatically built (Fig. 4). The data statistics are summarized in Table 1. After the crystallographic model building and refinement of the SeMet-derivative structure, we performed molecular replacement using the SeMet-derivative structure as a search model for the refinement of the native structure. The refinement of the native structure to 2.6 Å resolution is in progress.

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