CrossMark

Acta Crystallographica Section F
Structural Biology Communications
ISSN 2053-230X

Cloning, expression, purification, crystallization and X-ray crystallographic analysis of (*S*)-3-hydroxybutyryl-CoA dehydrogenase from *Clostridium butyricum*

Eun-Jung Kim and Kyung-Jin Kim*

School of Life Sciences, KNU Creative BioResearch Group (BK21 Plus Program), Kyungpook National University, Daehak-ro 80, Daegu 702-701, Republic of Korea

Correspondence e-mail: kkim@knu.ac.kr

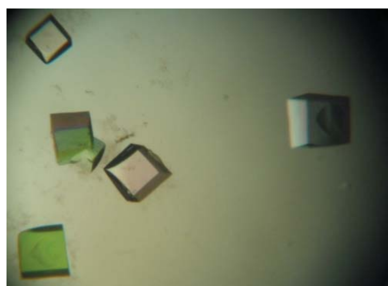
Received 10 February 2014
Accepted 25 February 2014

(*S*)-3-Hydroxybutyryl-CoA dehydrogenase from *Clostridium butyricum* (*Cb*HBD) is an enzyme that catalyzes the second step in the biosynthesis of *n*-butanol from acetyl-CoA by the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. The *Cb*HBD protein was crystallized using the hanging-drop vapour-diffusion method in the presence of 2 *M* ammonium sulfate, 0.1 *M* CAPS pH 10.5, 0.2 *M* lithium sulfate at 295 K. X-ray diffraction data were collected to a maximum resolution of 2.3 Å on a synchrotron beamline. The crystal belonged to space group *R*3, with unit-cell parameters $a = b = 148.5$, $c = 201.6$ Å. With four molecules per asymmetric unit, the crystal volume per unit protein weight (V_M) is 3.52 $Å^3$ Da^{-1}, which corresponds to a solvent content of approximately 65.04%. The structure was solved by the molecular-replacement method and refinement of the structure is in progress.

1. Introduction

n-Butanol is one of the most promising biofuel sources, and the widely known anaerobic bacterial strain *Clostridium acetobutylicum* efficiently produces *n*-butanol through a carbohydrate catabolic pathway (Mitchell, 1998; Inui *et al.*, 2008). Compared with bioethanol, *n*-butanol has several advantages as a biofuel source such as high energy content, lower corrosiveness, lower water solubility and ease of blending with motor-vehicle fuels (Dürre, 2007, 2008; Lee, Park *et al.*, 2008). The *n*-butanol synthetic pathway consists of six tightly regulated steps catalyzed by independent proteins (Jones & Woods, 1986). Despite intensive studies to improve *n*-butanol production, *n*-butanol titres in *Clostridium* strains are usually less than 20 g l^{-1} (Mitchell, 1998), which prohibits their utilization in industrialized processes. Recently, the production of *n*-butanol using industrial hosts such as *Escherichia coli*, *Pseudomonas putida* and *Bacillus subtilis* has been attempted because their genetic and physiological characteristics are well defined and there are various genetic tools available to support their modification (Inui *et al.*, 2008). However, the final titre of *n*-butanol was even worse than that of *Clostridium* strains, and did not exceed 1 g l^{-1} (Inui *et al.*, 2008; Dürre, 2007, 2008; Lee, Park *et al.*, 2008).

Engineering non-solventogenic microbes to produce a large amount of *n*-butanol has also been performed for the following reasons. Firstly, *n*-butanol may be toxic in bacterial cells and inhibits the growth of *E. coli* (Jones & Woods, 1986; Ezeji *et al.*, 2010). Secondly, *n*-butanol synthesis disrupts the balance of energy carriers such as $NADH/NAD^+$, which in turn results in a decrease in *n*-butanol production (Atsumi *et al.*, 2008; Felnagle *et al.*, 2012; Shen & Liao, 2008). Thirdly, the activities of the heterologous enzymes for *n*-butanol synthesis are host-cell specific, so each enzyme of the pathway should be optimized depending on the heterologous host. For example, about a fivefold higher titre of *n*-butanol is obtained in *E. coli* by a chimeric pathway using proteins from three different species (Steen *et al.*, 2008; Nicolaou *et al.*, 2010). These indicate that understanding the detailed enzymatic reactions and regulatory mechanisms of the key enzymes involved in the *n*-butanol biosynthetic pathway is inevitable for increased *n*-butanol production (Lee, Chou *et al.*, 2008; Felnagle *et al.*, 2012).



© 2014 International Union of Crystallography
All rights reserved

(S)-3-Hydroxybutyryl-CoA dehydrogenase from *C. butyricum* (*Cb*HBD) is an enzyme that catalyzes the second step in the biosynthesis of *n*-butanol from acetyl-CoA by reducing acetoacetyl-CoA to 3-hydroxybutyryl-CoA (Fig. 1; Jones & Woods, 1986). A *BLAST* search using the PDB revealed that *Cb*HBD has 43% amino-acid sequence homology to human heart short-chain L-3-hydroxy-acyl-CoA dehydrogenase (HuHAD), an enzyme that catalyzes the oxidation of the hydroxyl group of L-3-hydroxyacyl-CoA to a keto group in the β-oxidation pathway (Barycki *et al.*, 1999). As a step towards elucidating the structural and substrate-binding properties of *Cb*HBD, we cloned the *Cb*HBD coding gene and purified the recombinant *Cb*HBD protein. Crystals of *Cb*HBD with diffraction quality were obtained by the hanging-drop method. The crystal diffracted well and data were collected to a resolution of 2.3 Å; the structure was determined by the molecular-replacement method. Here, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the *Cb*HBD protein.

2. Expression and purification of the recombinant *Cb*HBD

The forward and reverse primers were designed as 5′-GCGCG-**CATATG**AAAAAAGTATTTGTACTTGGTGCAG-3′ and 5′-GCGCG**CTCGAG**TTTAGAATAATCGTAGAATCCTTTTC-3′ to introduce *Nde*I and *Xho*I cleavage sites, respectively. The *Cb*HBD coding gene (Met1–Lys282, molecular mass 30.6 kDa) was amplified by polymerase chain reaction (PCR) using *C. butyricum* chromosomal DNA as a template. The PCR product was then subcloned into pET-30a (Invitrogen) with a 6×His tag at the C-terminus. The resulting expression vector pET30a:*Cb*HBD was transformed into an *Escherichia coli* B834 strain, which was grown in 1 l LB medium containing kanamycin (50 mg ml^{-1}) at 310 K. At an OD_{600} of 0.8, *Cb*HBD protein expression was induced by adding 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h at 291 K, the cells were harvested by centrifugation at 4000*g* for 20 min at 277 K. The cell pellet was resuspended in buffer *A* (40 m*M* Tris–HCl pH 8.0, 5 m*M* β-mercaptoethanol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13 500*g* for 30 min and the lysate was applied onto an Ni–NTA agarose column (Qiagen). After washing with buffer *A* containing 20 m*M* imidazole, the bound proteins were eluted with 300 m*M* imidazole in buffer *A* (Fig. 2). 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 m*M* dithiothreitol (DTT). The protein eluted at a molecular mass of ~60 kDa, indicating that the *Cb*HBD protein with 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. The purified protein was concentrated to 30 mg ml^{-1} in 40 m*M* Tris–HCl pH 8.0, 1 m*M* DTT.

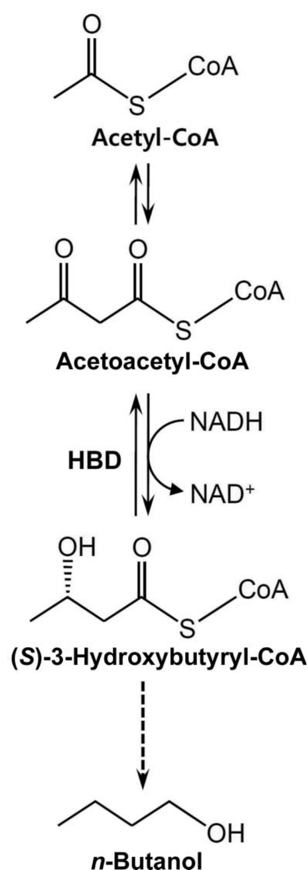


Figure 1
Enzymatic reaction of *Cb*HBD. *Cb*HBD is an enzyme involved in *n*-butanol biosynthesis by converting acetoacetyl-CoA to 3-hydroxybutyryl-CoA.

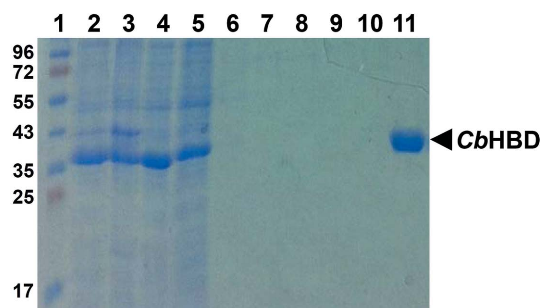


Figure 2
SDS–PAGE of purification of recombinant *Cb*HBD protein. Lane 1 shows molecular-weight markers (labelled in kDa). Lanes 2–11 show the purification procedure of *Cb*HBD using Ni–NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, pellet fraction and supernatant after centrifugation of the whole cell extract, respectively; lane 5, flowthrough from Ni–NTA column; lanes 6–10, wash with 0, 2.5, 5, 7.5 and 10 m*M* imidazole, respectively; lane 11, elution with 300 m*M* imidazole. Eluted *Cb*HBD protein with a 6×His tag at the C-terminus is indicated on the right side of the gel.

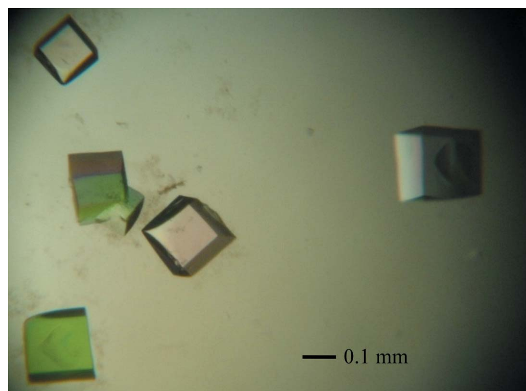


Figure 3
Trigonal crystals of *Cb*HBD. Crystals of the best quality were crystallized at 2 *M* ammonium sulfate, 0.1 *M* CAPS pH 10.5, 0.2 *M* lithium sulfate.

3. Crystallization

Crystallization of the purified *Cb*HBD protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald Bio using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 1.5 µl protein solution (20 mg ml^{-1} in 40 m*M* Tris–HCl pH 8.0, 1 m*M* DTT) with 1.5 µl reservoir solution and then equilibrating this drop against 0.5 ml reservoir solution. *Cb*HBD crystals were observed from several crystallization screening conditions. After several steps of improvement using the hanging-drop vapour-diffusion method, crystals of the best quality appeared in 7 d and reached maximum dimensions of 0.2 × 0.2 × 0.2 mm using 2 *M* ammonium sulfate, 0.1 *M* CAPS pH 10.5, 0.2 *M* lithium sulfate as the reservoir solution (Fig. 3).

4. X-ray analysis

The crystals were transferred to cryoprotectant solution consisting of 0.2 *M* lithium sulfate, 0.1 *M* CAPS pH 10.5, 2 *M* ammonium sulfate, 30%(*v*/*v*) glycerol, fished out with a loop larger than the crystals and flash-coooled using a liquid-nitrogen cryostream at 100 K. The data were collected to a resolution of 2.3 Å on the 7A beamline (MXII) at the Pohang Accelerator Laboratory (PAL; Pohang, Republic of Korea) using a Quantum 270 CCD detector (ADSC, USA) (Fig. 4). The data were then indexed, integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The crystals belonged to space group *R*3, with unit-cell parameters $a = b = 148.5$, $c = 201.6$ Å. With four molecules of *Cb*HBD per asymmetric unit, the crystal volume per unit of protein mass was 3.52 Å^3 Da^{-1} (Matthews, 1968), which corresponds to a solvent content of approximately 65.04%.

We attempted the molecular-replacement method of phase determination and a solution was found using human L-3-hydroxyacyl-CoA dehydrogenase (HuHAD; PDB entry 1f0y; Barycki *et al.*, 2000) as a search model. *Cb*HBD has an amino-acid sequence identity of 43% to HuHAD (Barycki *et al.*, 2000). *MOLREP* (Vagin & Teplyakov, 2010) located four polypeptide-model molecules in the asymmetric unit. The resulting solution had a correlation coefficient and *R* factor of 0.505 and 57.6%, respectively. After rigid-body refinement using *REFMAC*5 (Murshudov *et al.*, 2011) from the *CCP*4 suite (Winn *et al.*, 2011) in the resolution range 50–2.3 Å, the *R* factor and $R_{\rm free}$ were 54.4 and 55.7%, respectively, with an overall correlation coefficient of 0.662. The initial electron-density map, which was of good quality with backbones well defined by electron density, allowed us to build a three-dimensional model of *Cb*HBD. After initial model building of 100 *Cb*HBD amino-acid residues and restrained refinement, the *R* factor and $R_{\rm free}$ were 33.5 and 35.6%, respectively, with an overall correlation coefficient of 0.812. Crystallographic model building and refinement of the structure to 2.3 Å resolution are in progress. The data statistics are summarized in Table 1.

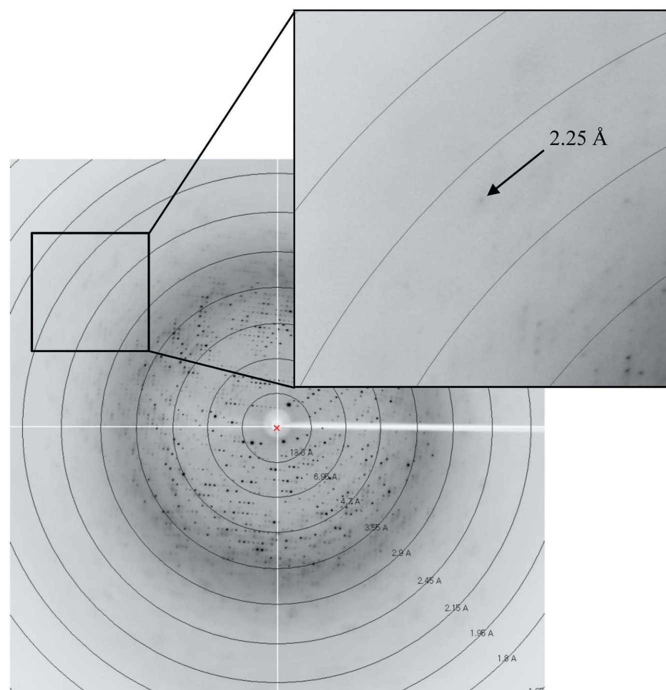


Figure 4
Diffraction pattern of the *Cb*HBD crystal. The crystal diffracted to a maximum resolution of 2.3 Å.

Table 1
Data-collection statistics of *Cb*HBD.

Values in parentheses are for the highest resolution shell.

Beamline	7A, PAL
Wavelength (Å)	1.0
Temperature (K)	100
Oscillation (°)	1.0
Mosaicity (°)	0.81
Total rotation range (°)	180
Space group	*R*3
Unit-cell parameters (Å, °)	$a = b = 148.5$, $c = 201.6$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Resolution (Å)	50.0–2.30 (2.38–2.30)
Total reflections	243475
Unique reflections	65804
Completeness (%)	93.5 (89.6)
$R_{\rm merge}$† (%)	6.6 (29.6)
$\langle I/\sigma(I)\rangle$	29.9 (2.9)
Multiplicity	3.7 (3.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, 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.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (NRF-2009-C1AAA001-2009-0093483) and by the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the MEST (ABC- 2012-053895).

References

Atsumi, S., Hanai, T. & Liao, J. C. (2008). *Nature (London)*, **451**, 86–89.
Barycki, J. J., O'Brien, L. K., Bratt, J. M., Zhang, R. G., Sanishvili, R., Strauss, A. W. & Banaszak, L. J. (1999). *Biochemistry*, **38**, 5786–5798.
Barycki, J. J., O'Brien, L. K., Strauss, A. W. & Banaszak, L. J. (2000). *J. Biol. Chem.* **275**, 27186–27196.
Dürre, P. (2007). *Biotechnol. J.* **2**, 1525–1534.
Dürre, P. (2008). *Ann. N. Y. Acad. Sci.* **1125**, 353–362.
Ezeji, T., Milne, C., Price, N. D. & Blaschek, H. P. (2010). *Appl. Microbiol. Biotechnol.* **85**, 1697–1712.
Felnagle, E. A., Chaubey, A., Noey, E. L., Houk, K. N. & Liao, J. C. (2012). *Nature Chem. Biol.* **8**, 518–526.
Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N. & Yukawa, H. (2008). *Appl. Microbiol. Biotechnol.* **77**, 1305–1316.
Jones, D. T. & Woods, D. R. (1986). *Microbiol. Rev.* **50**, 484–524.
Lee, S. K., Chou, H., Ham, T. S., Lee, T. S. & Keasling, J. D. (2008). *Curr. Opin. Biotechnol.* **19**, 556–563.
Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J. & Jung, K. S. (2008). *Biotechnol. Bioeng.* **101**, 209–228.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Mitchell, W. J. (1998). *Adv. Microb. Physiol.* **39**, 31–130.
Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* D**67**, 355–367.
Nicolaou, S. A., Gaida, S. M. & Papoutsakis, E. T. (2010). *Metab. Eng.* **12**, 307–331.
Otwinowski, Z. & Minor, W. (1997). *Method Enzymol*, **276**, 307–326.
Shen, C. R. & Liao, J. C. (2008). *Metab. Eng.* **10**, 312–320.
Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., Ouellet, M. & Keasling, J. D. (2008). *Microb. Cell Fact.* **7**, 36.
Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* D**66**, 22–25.
Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.