

Yoshitaka Nakajima, Kiyoshi Ito,
Emi Ichihara, Kyohei Ogawa,
Takashi Egawa, Yue Xu and
Tadashi Yoshimoto*

Biotechnology Department, Graduate School of
Biomedical Sciences, Nagasaki University,
1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Correspondence e-mail:
yosimoto@net.nagasaki-u.ac.jp

Received 23 August 2004
Accepted 1 October 2004
Online 9 October 2004

Crystallization and preliminary X-ray characterization of D-3-hydroxybutyrate dehydrogenase from *Pseudomonas fragi*

A recombinant form of D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) from *Pseudomonas fragi* has been crystallized by the hanging-drop method using PEG 3000 as a precipitating agent. The crystals belong to the orthorhombic group $P2_12_12$, with unit-cell parameters $a = 64.3$, $b = 99.0$, $c = 110.2$ Å. The crystals are most likely to contain two tetrameric subunits in the asymmetric unit, with a V_M value of 3.29 Å³ Da⁻¹. Diffraction data were collected to a 2.0 Å resolution using synchrotron radiation at the BL6A station of the Photon Factory.

1. Introduction

D-3-Hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.30) is an enzyme that reversibly oxidizes 3-hydroxybutyrate to acetoacetate using NAD as coenzyme. In mammals, the acetyl-CoA that is formed during oxidation of fatty acids is metabolized by two pathways. One is oxidation *via* the citric acid cycle, while the other pathway leads to acetoacetate and D-3-hydroxybutyrate, which, together with acetone, are collectively called ketone bodies. The free acetoacetate so produced is reversibly reduced by HBDH, a mitochondrial enzyme, to D-3-hydroxybutyrate.

One of the severe complications of diabetes mellitus is diabetic ketoacidosis (DKA). DKA is caused by an increase of ketone bodies and often leads to an unconscious condition. In diabetes mellitus patients, particularly type 1, glucose cannot be absorbed and utilized by cells owing to insulin deficiency and fatty-acid metabolism is elevated to produce acetyl-CoA. This acetyl-CoA, however, cannot enter the citric acid cycle because of the loss of oxaloacetate as an intermediate in the cycle. Excess acetyl-CoA is converted to ketone bodies. Therefore, monitoring of ketone bodies in blood and urine is important for the treatment of patients with diabetes mellitus.

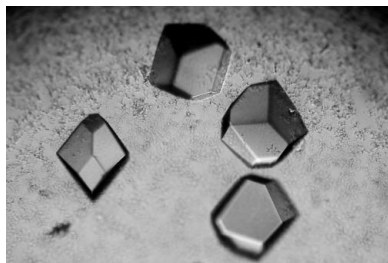
HBDH was described in respiratory particles from pig heart by Green *et al.* (1937) and has also been demonstrated to be present in liver mitochondria (Lehninger *et al.*, 1960). HBDH has been purified from human heart (Marks *et al.*, 1992), bovine heart and rat liver (Churchill *et al.*, 1992). The enzyme has a specific requirement for phosphatidylcholine for activity (Marks *et al.*, 1992). The enzyme has also been found in many microorganisms (Bergmeyer *et al.*, 1967). However, only the enzyme from *Rhodobacter* sp. has been cloned (Kruger *et al.*, 1999). In contrast to mammalian HBDHs, bacterial enzymes showed no requirement for phospholipids for activity. These microbial enzymes have been used for the diagnostic analysis of ketone bodies for diabetes mellitus.

We found the HBDH in *Pseudomonas fragi*, cloned the enzyme gene and expressed the enzyme in *Escherichia coli*. This is the first report of the crystallization of HBDH and its preliminary analysis by X-ray crystallography.

2. Experimental

2.1. Expression and purification

An overexpression plasmid was prepared by inserting the structural gene amplified by PCR into a pKK223-3 prokaryotic expression vector (Pharmacia). Molecular cloning of the D-3-hydroxybutyrate



dehydrogenase gene from *P. fragi* will be described elsewhere (manuscript in preparation). The enzyme gene was amplified with *P. fragi* chromosomal DNA as a template by PCR using oligonucleotide primers designed from homologous regions of several bacterial genomic sequences assumed to encode HBDH and cloned in a pGEM-T-easy vector (Promega). Upstream and downstream parts of the gene were amplified by an *in vitro* cloning kit (TaKaRa shuzo). They were combined using a unique *Nco*I site to produce pHBDHfull1. The DNA fragment of the structural gene for D-3-hydroxybutyrate dehydrogenase containing a unique *Eco*RI recognition site was amplified by PCR using the upstream primer HBDHATG15 (5'-CCCGAATTCATGCTCAAAGGAAAAGT-CGC-3'), the downstream primer HIII-T7 (5'-GCCCAAGCTTTG-TAATACGACTCACTATAG-3') and the plasmid pGEM-T-easy containing the entire gene for D-3-hydroxybutyrate dehydrogenase as a template. The resultant 0.99 kbp fragment was digested with *Eco*RI and *Hind*III and ligated into similarly digested pKK223-3 to give a plasmid pHBDH11. The nucleotide-sequence data of *P. fragi* HBDH appears in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number AB183516.

E. coli DH5 α transformed with the pHBDH11 plasmid was aerobically cultured in 20 l N-broth containing 50 $\mu\text{g ml}^{-1}$ ampicillin at 310 K for 12 h using a New Brunswick jar fermenter. The cells were suspended in 20 mM Tris-HCl buffer pH 7.5 and were disrupted with glass beads in a Dyno-Mill. The cell lysate was centrifuged at 15 000g for 60 min in order to remove cell debris. 2% protamine sulfate was added to the supernatant in a dropwise fashion to a final concentration of 17 mg per gram of wet cells in order to remove chromosomes and viscous materials. After centrifugation, the supernatant was saturated with 45% ammonium sulfate and applied onto a column of HW65C (Tosoh) equilibrated with 20 mM Tris-HCl buffer pH 7.5 containing 45% saturated ammonium sulfate. Enzymes were eluted using a linear gradient of ammonium sulfate concentration from 45 to 0% saturation. The enzyme was then purified using a Toyopearl SuperQ-650C column equilibrated with the same buffer using a linear gradient from 0 to 500 mM sodium chloride. The purified enzyme was homogeneous on SDS-PAGE. The purified enzyme was concentrated to 15 mg ml $^{-1}$ using a Centricon 10 (Millipore) and stored at 193 K.

2.2. Crystallization

HBDH crystals were grown by the hanging-drop vapour-diffusion method at 293 K. Initial screening was performed using sparse-matrix screens (Jancarik & Kim, 1991) based on the commercially available Crystal Screens I and II (Hampton Research Inc.) and Wizard Screens I and II (Emerald Biostructures). Several crystal forms were obtained and one of the more promising crystallization conditions was optimized. A 2 μl droplet of 10.8 mg ml $^{-1}$ protein solution mixed with the same amount of reservoir solution was equilibrated against 100 μl reservoir solution [12%(w/v) PEG 3000, 100 mM sodium cacodylate buffer pH 6.5 and 200 mM MgCl $_2$] to give HBDH crystals.

2.3. Data collection

Data collection was performed at 100 K using a wavelength of 1.00 Å from the synchrotron-radiation source at the Photon Factory BL6A station (Tsukuba, Japan) with an ADSC Quantum 4R CCD detector system. For data collection under cryogenic conditions, crystals were soaked for a few seconds in a prepared solution containing 30%(v/v) glycerol, 12%(w/v) PEG 3000, 100 mM sodium cacodylate buffer pH 6.5 and 200 mM MgCl $_2$. Crystals were mounted in a nylon loop and flash-cooled in a liquid-nitrogen stream at 100 K.

Table 1

Crystal data and intensity statistics.

Values in parentheses are for the last resolution shell.

Space group	$P2_12_12$
Unit-cell parameters (Å)	$a = 64.3, b = 99.0, c = 110.2$
Temperature (K)	100
Wavelength (Å)	1.00
Resolution range (Å)	30.0–2.00 (2.11–2.00)
No. observed reflections	193929 (26311)
No. of unique reflections	47802 (6880)
Completeness (%)	99.1 (99.2)
R_{sym} (%)	7.8 (30.6)
Mean $I/\sigma(I)$	4.1 (3.8)

The data were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The open reading frame of pHBDH11 consisted of 780 bp coding for 260 amino-acid residues. The isoelectric point was calculated to be 6.72. The purified HBDH showed a single band of 27 kDa on SDS-PAGE, which is in good agreement with the calculated value of 26 684 Da. A gel-filtration experiment gave a molecular weight of 120 kDa, suggesting that the HBDH exists as a tetramer.

Crystals appeared within 3 d of incubation and grew to maximum dimensions of 0.4 \times 0.4 \times 0.2 mm (Fig. 1). From diffraction data collection, the space group was determined to be orthorhombic $P2_12_12$. Assuming two tetrameric subunits in the asymmetric unit, the Matthews coefficient V_M was calculated to be 3.29 Å 3 Da $^{-1}$, indicating a solvent content of approximately 63% in the unit cell. These values are within the range typical for protein crystals (Matthews, 1968).

A native data set with 47 802 unique reflections was collected, giving a data-set completeness of 99.1% in the resolution range 30.0–2.00 Å, with an R_{sym} of 7.8% (Table 1). These data indicate the good quality of the crystals for X-ray structural analysis. The crystals showed no significant decay upon exposure.

HBDH belongs to the short-chain dehydrogenase/reductase (SDR) family and shows sequence homology to several SDRs of known structure. We have attempted to carry out molecular replacement with *AMoRe* (Navaza, 1994) using monomeric, dimeric

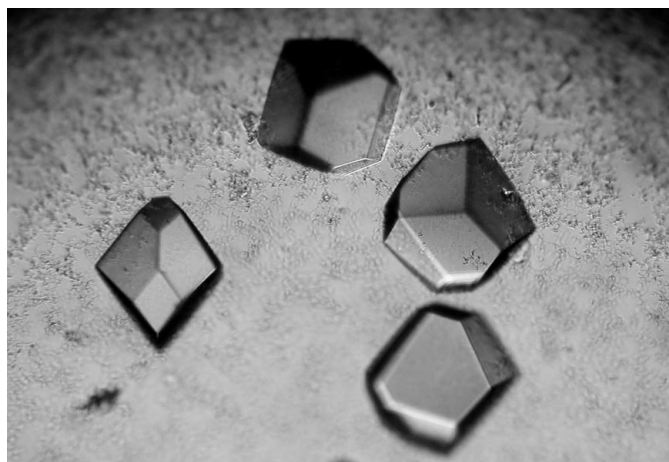


Figure 1

Crystals of D-3-hydroxybutyrate dehydrogenase as grown by the hanging-drop method. The average dimensions of these crystals were 0.2 \times 0.2 \times 0.1 mm.

or partial models of $3\alpha,20\beta$ -hydroxysteroid dehydrogenase (PDB code 2hsd) from *Streptomyces hydrogenans*, tropinone reductase-I (PDB code 1ae1) from *Datura stramonium*, sorbitol dehydrogenase (PDB code 1k2w) from *Rhodobacter sphaeroides*, $3\beta/17\beta$ -hydroxysteroid dehydrogenase (PDB code 1hxx) from *Comamonas testosteroni* and 7α -hydroxysteroid dehydrogenase (PDB code 1ahh) from *E. coli* as search models. These enzymes showed 30.8, 27.3, 26.9, 24.5 and 22.3% sequence identity to *P. fragi* HBDH, respectively. However, no solutions of either molecular orientation or molecular packing were discovered.

HBDH possesses three methionine residues and 1878 non-H atoms per subunit. The Bijvoet diffraction ratio is expected to be 3.2% (Hendrickson & Ogata, 1997), suggesting that a selenomethionyl derivative of HBDH should be produced and should have a sufficient anomalous signal for the multiple anomalous dispersion (MAD) method. We have recently succeeded in obtaining a selenomethionyl derivative of HBDH and plan to solve the crystal structure using the MAD method.

References

- Bergmeyer, H. U., Gawehn, K., Klotzsch, H., Krebs, H. A. & Williamson, D. H. (1967). *Biochem. J.* **102**, 423–431.
- Churchill, P., Hempel, J., Romovacek, H., Zhang, W.-W., Brennan, M. & Churchill, S. (1992). *Biochemistry*, **31**, 3793–3799.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst. D* **50**, 760–763.
- Green, D. E., Dewan, J. G. & Leloir, L. F. (1937). *Biochem. J.* **31**, 934.
- Hendrickson, W. A. & Ogata, C. M. (1997). *Methods Enzymol.* **276**, 494–523.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Kruger, K., Lang, G., Weidner, T. & Engel, A. M. (1999). *Appl. Microbiol. Biotechnol.* **52**, 666–669.
- Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960). *J. Biol. Chem.* **235**, 2450–2455.
- Leslie, A. G. W. (1992). *Jnt CCP4/ESF-EAMCB Newsl. Protein Crystallogr.* **26**.
- Marks, A. R., McIntyre, J. O., Duncan, T. M., Erdjument-Bromage, H., Tempst, P. & Fleischer, S. (1992). *J. Biol. Chem.* **267**, 15459–15463.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Navaza, J. (1994). *Acta Cryst. A* **50**, 157–163.