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Crystallization and preliminary X-ray crystallographic studies of NADP-dependent 3-hydroxyisobutyrate dehydrogenase from *Thermus thermophilus* HB8

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3-Hydroxyisobutyrate, a central metabolite in the valine catabolic pathway, is reversibly oxidized to methylmalonate semialdehyde by a specific NADP-dependent dehydrogenase (HIBADH). HIBADH from *Thermus thermophilus* HB8 has been overexpressed in *Escherichia coli* and crystallized by the microbatch method using lithium chloride as a precipitant at 296 K. X-ray diffraction data have been collected to 1.80 Å resolution at 100 K using synchrotron radiation. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 85.878, b = 106.367, c = 168.639 Å. A homotetramer of HIBADH is likely to be present in the asymmetric unit, giving a V_M of 3.0 Å^3 Da^{-1} and a solvent content of 59.3%.

1. Introduction

3-Hydroxyisobutyrate (HIBA), a central metabolite in the valine catabolic pathway, is reversibly oxidized to methylmalonate semialdehyde by a specific NADP-dependent dehydrogenase (HIBADH or 3-hydroxy-2-methyl-propanoate:NADP$^+$ oxidoreductase; EC 1.1.1.31) present in mitochondria (Robinson & Coon, 1957). HIBADH is essential for valine catabolism and is widely distributed in yeasts, bacteria and mammalian tissues (Bannerjee *et al.*, 1979; Hasegawa, 1981). Unlike other intermediates of valine catabolism which are acyl-CoA derivatives, HIBA diffuses across the mitochondrial and plasma membranes. Because of this, HIBA has been suggested to be the most important valine catabolite, being a better substrate for hepatic gluconeogenesis than α-ketoisovalerate (Letto *et al.*, 1986). The pathway of catabolism of valine is as shown below.

Valine → 2-oxoisovalerate → isobutyryl-CoA
→ methylacrylyl-CoA
→ 3-hydroxyisobutyryl-CoA
→ 3-hydroxyisobutyrate
→ methylmalonate semialdehyde
→ propionyl-CoA.

The catabolism of valine is unique among the three branched-chain amino acids (leucine, isoleucine and valine) because of an essential step in which a CoA ester is hydrolyzed to give a carboxylic acid. Whether a tissue catabolyzes or releases 3-hydroxyisobutyrate is dependent on the level of HIBADH. When valine catabolism is initiated in muscle, some of the carbon is released as 2-oxoisovalerate and some is metabolized through the branched-chain 2-oxoacid dehydrogenase and subsequent enzymes of valine catabolism.

The control of HIBADH is of interest because elevated levels of HIBA in serum and urine have been reported in hydroxyisobutyric aciduria, brain disorders and methymalonic acidaemia (Congdon *et al.*, 1981; Landaas, 1975; Ko *et al.*, 1991). If the level of HIBA in an organism is changed from its equilibrium, the process of valine catabolism is disturbed, leading to fatality of the organism. HIBADH is sensitive to NADPH inhibition, which may be a factor in the short-term physiological control of valine catabolism (Rougraff *et al.*, 1989). Enzyme inhibition by NADPH may regulate the complete oxidation of valine beyond the formation of 3-hydroxyisobutyrate. This enzyme does not require a metal ion for catalysis and has a number of features in common with the short-chain alcohol dehydrogenases (Hawes *et al.*, 1995; Chowdhury *et al.*, 1996).

The structure of HIBADH may provide an understanding of how to overcome disturbance to the process of valine catabolism, determine the catalytic mechanism and accurately model the interactions of the enzyme with substrate and inhibitor. However, no three-dimensional structure of HIBADH has been reported to date. As a first step towards the structure, we report here the expression, crystallization and preliminary X-ray crystallographic study of the HIBADH homologue from *Thermus thermophilus* HB8.

2. Experimental

2.1. Protein expression and purification

The gene for HIBADH was amplified by the polymerase chain reaction (PCR) using the genomic DNA of the *T. thermophilus* HB8

strain as a template. The PCR product was ligated with pT7blue (Novagen). The plasmid was digested with *Nde*I and *Bgl*II and the fragment was inserted into the expression vector pET-11a linearized with *Nde*I and *Bam*HI. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) cells and was grown at 310 K in Luria–Bertani medium containing 509 µg ml^{-1} ampicillin for 20 h. The cells were harvested by centrifugation at 6500 rev min^{-1} for 5 min at 277 K. The cell pellet was suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* sodium chloride and 5 m*M* 2-mercaptoethanol and was then homogenized by ultrasonication. The supernatant was heated at 343 K for 12 min and the cell debris and denaturated protein were then removed by centrifugation (14 000 rev min^{-1}, 30 min); the supernatant solution was used as the crude extract for purification. The crude extract was desalted with a HiPrep 26/10 desalting column and applied onto a Super Q Toyopearl 650M column equilibrated with 20 m*M* Tris–HCl pH 8.0. The fraction containing the protein was eluted with a linear gradient of 0–0.3 *M* sodium chloride. Protein was then dialyzed against 20 m*M* Tris–HCl pH 8.0 and subjected to a Resource Q column (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0. Fractions containing protein were again eluted with a linear gradient of 0–0.3 *M* sodium chloride. The protein was desalted with a HiPrep 26/10 desalting column with 10 m*M* sodium phosphate pH 7.0 and applied onto a Bio-Scale CHT20-I column (Bio-Rad) equilibrated with 10 m*M* sodium phosphate pH 7.0. Protein was again eluted with a linear gradient of 10–150 m*M* sodium phosphate. The protein was desalted with a HiPrep 26/10 desalting column with 20 m*M* Tris–HCl pH 8.0 containing 0.05 *M* sodium chloride and applied onto a Mono Q column (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 0.05 *M* sodium chloride. The fraction containing protein was eluted with a linear gradient of 0–0.5 *M* sodium chloride. The fraction containing protein was cooled, concentrated by ultrafiltration (Vivaspin, 10 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex 75 pg column (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 0.05 *M* sodium chloride. The homogeneity and identity of the protein were assessed by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis. Finally, the purified enzyme was concentrated to 30.3 mg ml^{-1} by ultrafiltration and was stored at 203 K.

2.2. Dynamic light-scattering studies

A dynamic light-scattering experiment was performed on a DynaPro MS/X instrument from Protein Solutions (Lakewood, New Jersey). The measurements were made at 291 K on the purified protein at 0.4–0.9 mg ml^{-1} in buffer solution containing 20 m*M* Tris–HCl and 200 m*M* sodium chloride.

2.3. Crystallization and X-ray data collection

Crystallization was performed by a microbatch method using NUNC HLA plates. Each crystallization drop was prepared by mixing 1.0 µl of the precipitant solution (27.5% PEG 4000, 0.1 *M* Tris–HCl, 1 *M* $LiCl_2$ pH 8.1) and 1.0 µl of the protein solution at 32.3 mg ml^{-1}. The crystallization drop was then overlaid with a 1:1 mixture of silicon and paraffin oils, allowing slow evaporation of water in the drop, and was stored at 296 K. Initial crystallization conditions were screened using the TERA (automatic crystallization) system (Sugahara & Miyano, 2002) from 144 conditions.

A crystal of HIBADH from *T. thermophilus* was flash-frozen using a solution comprising 27.5% PEG 4000, 0.1 *M* Tris–HCl, 1 *M* lithium chloride and 30%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data were collected at 100 K on a Rigaku R-AXIS V image-plate detector at beamline BL26B1, SPring8, Japan. The crystal was rotated through a total of 130°, with a 0.5° oscillation range per frame. The wavelength of the synchrotron radiation was 0.80 Å. Data were processed and scaled using the programs *HKL*2000 and *SCALEPACK* (Otwinowski & Minor, 1997).

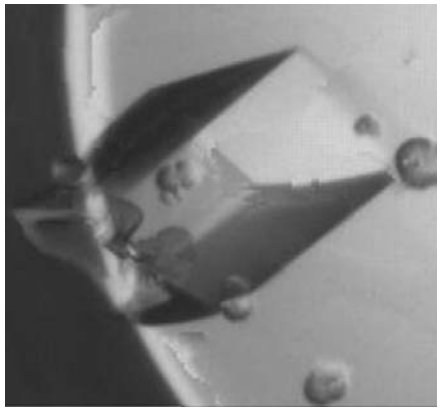

Figure 1
Crystal of 3-hydroxyisobutyrate dehydrogenase from *T. thermophilus* HB8. Its approximate dimensions are 0.1 × 0.1 × 0.1 mm.

Table 1
Statistics of diffraction data-collection and reduction.

Values in parentheses are for the highest resolution shell.

X-ray wavelength (Å)	0.80
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	85.878
b (Å)	106.367
c (Å)	168.639
No. of molecules in AU	4
V_M (Å^3 Da^{-1})	3.0
Resolution limits (Å)	30–1.80 (1.86–1.80)
Total/unique reflections	890536/139317
Data completeness (%)	97.1 (91.2)
R_{merge}† (%)	6.8 (45.0)
Average $I/\sigma(I)$	13 (4.9)

† $R_{merge} = \sum_h \sum_j |\langle I(h)\rangle - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the intensity of the *j*th observed reflection and $\langle I(h)\rangle$ is the mean intensity of reflection *h*.

3. Results

Well diffracting crystals of HIBADH were obtained using a precipitant solution consisting of 27.5% PEG 4000, 0.1 *M* Tris–HCl pH 8.1 and 1 *M* lithium chloride. The crystals grew to approximate dimensions of 0.1 × 0.1 × 0.1 mm within 2 d (Fig. 1). A set of X-ray diffraction data were collected to 1.80 Å resolution at 100 K using synchrotron radiation. A total of 890 536 measured reflections were merged into 139 317 unique reflections with an R_{merge} of 6.8%. The merged data set was 97% complete. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters *a* = 85.878, *b* = 106.367, *c* = 168.639 Å. Data-collection statistics are summarized in Table 1. The presence of a tetramer in each asymmetric unit gives a crystal volume per protein mass (V_M) of 3.0 Å^3 Da^{-1} and a solvent content of 59.3% (Matthews, 1968). Dynamic light-scattering experiments also showed the presence of a tetramer in solution, supporting the biological significance of the formation of a tetramer. The structure determination of the *T. thermophilus* HIBADH is now under way using the multiple anomalous dispersion method (Hendrickson *et al.*, 1990).

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