Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

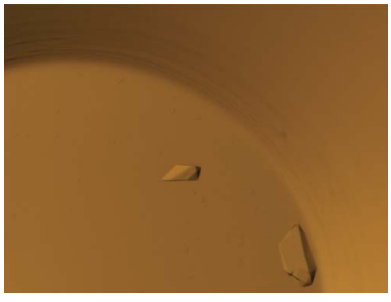
Crystallization, preliminary X-ray diffraction and structure analysis of *Thermotoga maritima* mannitol dehydrogenase

Jennifer Puttick,[a] Claire Vieille,[b] Seung H. Song,[b] Michel N. Fodje,[c] Pawel Grochulski[c] and Louis T. J. Delbaere[a]*

[a]Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada, [b]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824-1319, USA, and [c]Canadian Macromolecular Facility, Canadian Light Source Inc., University of Saskatchewan, Saskatoon, Saskatchewan S7N 0X4, Canada

Correspondence e-mail: louis.delbaere@usask.ca

Received 16 January 2007
Accepted 19 March 2007



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Diffraction data have been collected from a crystal of *Thermotoga maritima* mannitol dehydrogenase at the Canadian Light Source. The crystal diffracted to 3.3 Å resolution and belongs to space group $P2_12_12_1$, with unit-cell parameters a = 83.43, b = 120.61, c = 145.76 Å. The structure is likely to be solved by molecular replacement.

1. Introduction

Industrial polyols are sugar alcohols that are used in the pharmaceutical, chemical and food-ingredient industries. Mannitol is used as a low-caloric and low-cariogenic sweetener (in particular in diabetic foodstuffs), as a pharmaceutical formulating agent (*e.g.* as a diuretic in the manufacture of intravenous fluids and tablets, in dental hygiene products and as a low-reactivity drug filler; Le & Mulderrig, 2001) and as a specialty chemical in other types of industries (Soetaert *et al.*, 1995; Le & Mulderrig, 2001). *SpecChemOnline* estimates the global market for mannitol to be about 22 million Euros (Challener, 2004). Currently, 50 000 tons per year of mannitol are produced by the hydrogenation of 50% fructose/50% glucose syrup at high pressures and temperatures using a Raney nickel catalyst (Kulbe *et al.*, 1987; Soetaert *et al.*, 1999). The fructose/glucose syrup is converted to a 30% mannitol/70% sorbitol mixture, from which mannitol is purified by low-temperature crystallization (Soetaert *et al.*, 1999). Developing new simplified biological processes for mannitol production could lower production costs, increase the chemical yield and lower the downstream processing costs. Biological synthesis routes are now being developed (Kaup *et al.*, 2005; Silveira & Jonas, 2002; Soetaert *et al.*, 1999) to lower production costs.

Mannitol is produced enzymatically from fructose by mannitol dehydrogenase (MtDH) using NAD(P)H as the cofactor (Jörnvall *et al.*, 1984, 1987; Schneider & Giffhorn, 1989; Slatner *et al.*, 1999). Many MtDH-encoding genes have been cloned and sequenced; their enzymes have been purified and characterized (Aarnikunnas *et al.*, 2002; Brünker *et al.*, 1997; Sasaki *et al.*, 2005; Schafer *et al.*, 1997; Schneider & Giffhorn, 1989; Stoop & Mooibroek, 1998). These enzymes belong to three different dehydrogenase/reductase families. Firstly, some fungal MtDHs belong to the short-chain dehydrogenase/reductase family (Hörer *et al.*, 2001; Suvarna *et al.*, 2000). These fungal MtDHs do not require metals for catalysis and contain a conserved catalytic triad (Hörer *et al.*, 2001; Persson *et al.*, 1991). Secondly, the medium-chain dehydrogenase/reductase family contains some bacterial MtDHs (Aarnikunnas *et al.*, 2002). These enzymes are often zinc-dependent enzymes (Nordling *et al.*, 2002; Sasaki *et al.*, 2005). Thirdly, other bacterial MtDHs belong to a polyol-specific long-chain dehydrogenase group (Schneider *et al.*, 1993). These long-chain MtDHs are often monomeric; they are characterized by the catalytic consensus motif Lys-Xaa(4/5)-Asn-Xaa(2)-His and do not require metals for catalysis (Kavanagh *et al.*, 2002*a*,*b*; Klimacek & Nidetzky, 2002). The X-ray structures of *Agaricus bisporus* MtDH (a short-chain dehydrogenase; Hörer *et al.*, 2001) and of *Pseudomonas fluorescens* MtDH (a long-chain dehydrogenase; Kavanagh *et al.*, 2002*a*,*b*) are known. None of the medium-chain MtDHs have known X-ray structures. However, all

biological routes being currently developed for mannitol production use medium-chain MtDHs (Kaup *et al.*, 2005; Soetaert *et al.*, 1999). Thus, it is important to understand the structure–function relationships in this group of enzymes, particularly if enzyme engineering is required for process optimization. For this reason, we cloned and characterized the medium-chain MtDH from the hyperthermophilic bacterium *Thermotoga maritima* (Song *et al.*, in preparation). *T. maritima* MtDH (TmMtDH, GenBank No. TM0298) shares 32% identity and 55% similarity with the mesophilic medium-chain *Leuconostoc mesenteroides* and *Lactobacillus reuteri* MtDHs. TmMtDH is optimally active at temperatures above 363 K. It reduces fructose optimally at pH 6.0, while it oxidizes mannitol optimally at pH 8.3. This NADH-dependent enzyme also shows activity with NADPH, but with a threefold lower affinity for this cofactor (Song *et al.*, in preparation); NADH is also a more economically viable cofactor for industrial use than NADPH. Here, we describe the crystallization conditions, characterization and initial structural analysis of TmMtDH crystals.

2. Experimental results

2.1. TmMtDH expression and purification

The *T. maritima mtdh* gene was cloned into the *Nde*I and *Xho*I sites of pET24a(+) (Novagen, Madison, WI, USA) to yield pTmMtDH (Song *et al.*, in preparation). From this construct, TmMtDH is expressed with a C-terminal His tag. To produce TmMtDH, *Escherichia coli* BL21(DE3) cells (Novagen) containing pTmMtDH were grown in 1 l super broth (24 g yeast extract, 12 g tryptone, 13 ml glycerol, 1 m*M* $MgSO_4$, 15.3 g K_2HPO_4 and 1.7 g KH_2PO_4 per litre) containing 50 µg ml^{-1} kanamycin at 310 K. TmMtDH expression was induced at an OD_{600} of 1.4 by adding 0.6 m*M* isopropyl thiogalactoside and growth continued for a further 16 h. After centrifugation (4000*g*, 10 min), the cell pellet was resuspended (3 ml buffer per gram of wet pellet) in 50 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.0 containing 5 m*M* *β*-mercaptoethanol and protease inhibitors (Complete Mini, Roche Diagnostics, Indianapolis, IN, USA). Cells were disrupted by two passes through a French pressure cell using a pressure drop of 96 MPa. After centrifugation (25 000*g*, 30 min), the supernatant was heat treated at 358 K for 20 min and then centrifuged again (20 000*g*, 20 min). The heat-treated extract was then loaded onto a 20 ml nickel–nitrilotriacetic acid agarose (Qiagen, Valencia, CA, USA) column. TmMtDH purification from this column followed the Gibco BRL procedure for Protein Expression System, pRoEX-1 vector (catalogue No. 10197-010, Gaithersburg, MD, USA), washing with 20 volumes of buffer *A*, five volumes of buffer *B* and five volumes buffer of *A* before eluting with 5–10 volumes of buffer *C*. The purified protein was dialyzed against 50 m*M* MOPS pH 7.0 containing 5 m*M* *β*-mercaptoethanol and stored frozen at 203 K. Protein purity was assessed on an SDS–PAGE gel after staining with Coomassie blue. Protein concentrations were quantified using the Biorad Protein Dye (Biorad, Hercules, CA, USA) with using bovine serum albumin as the standard.

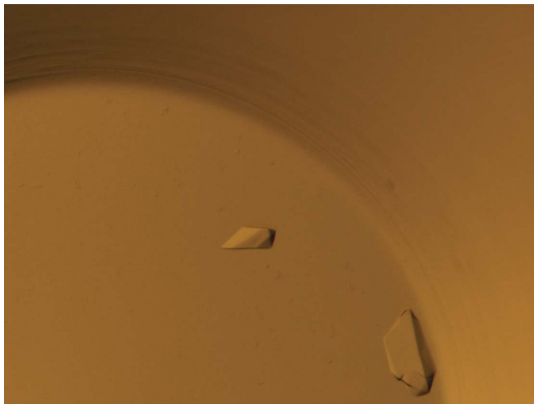

Figure 1
A TmMtDH crystal. The largest dimension is approximately 0.1 mm.

Table 1
Data-collection statistics for a crystal of TmMtDH.

Values in parentheses are for the highest resolution shell.

Temperature (K)	150
Beamline	08ID-1, Canadian Light Source
Detector	MAR 225 CCD
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 83.43, b = 120.61, c = 145.76
Matthews coefficient (Å^3 Da^{-1})	2.64
Solvent content (%)	53
Unit-cell volume (Å^3)	1470000
Molecular weight (Da)	34870 [317 amino-acid residues]
No. of molecules in ASU	4
No. of measured reflections	314666
Total No. of unique reflections collected	47727
Resolution range (Å)	7.0–3.3 (3.8–3.3)
Completeness (%)	99 (92.4)
Redundancy	7.20 (6.59)
$R_{\rm merge}$†	0.024 (0.027)
$\langle I/\sigma(I)\rangle$	8.46 (6.55)

† $R_{\rm merge} = |I - \langle I\rangle|/\sum I$, where $\langle I\rangle$ is the average intensity over symmetry-related reflections and I is the measured intensity.

2.2. TmMtDH crystallization

10 mg ml^{-1} TmMtDH protein in 50 m*M* Tris pH 8.5 buffer solution was screened at room temperature against the MPD and Classics screening kits from Nextal Biotechnologies (Montreal, Canada) using the microbatch-under-oil method. Drops were formed by mixing an equal volume of TmMtDH protein solution and MPD screening kit solution and were covered with 100% mineral oil. The protein crystallized in two Nextal Classics conditions, but the largest and most defined crystals were found in the MPD screening kit solution G4 (30% MPD, 0.1 *M* Na HEPES pH 7.5). The C-terminal His tag was retained on the protein construct prior to crystallization. The MPD-grown crystals were approximately 0.1 × 0.05 × 0.05 mm in size and were rectangular in shape (Fig. 1). Crystals were flash-cooled without additional cryoprotectant because of the high concentration of MPD in the precipitant solution.

2.3. TmMtDH diffraction and data collection

Diffraction of the TmMtDH crystals took place at the 08ID-1 protein crystallography beamline at the Canadian Light Source (Saskatoon, Saskatchewan, Canada). The data collected from the TmMtDH crystals are summarized in Table 1. Intensity data were indexed, integrated and scaled using *XDS* (Kabsch, 1993).

3. Structure solution

A molecular-replacement solution for the diffraction data set from the TmMtDH crystal was found using the *3D-PSSM* search engine (Kelly *et al.*, 2000). The sequence of ketose reductase (sorbitol dehydrogenase) from silverleaf whitefly (PDB code 1e3j) is 24% identical to that of TmMtDH. This identity level is low, so the structure of NADP(+)-dependent *Bacillus stearothermophilus*

alcohol dehydrogenase (PDB code 1rjw), which has 23% sequence identity with TmMtDH, may also be used to aid in calculating phases using the program *Phaser* (Storoni *et al.*, 2004).

LTJD is a Tier 1 Canada Research Chair in Structural Biochemistry. This research was funded by a CIHR Operating Grant and an NSERC Discovery Grant to LTJD and by a USDA grant (No. 2005-35504-16239) to CV. The research described in this paper was performed at the Canadian Light Source, which is supported by NSERC, NRC, CIHR and the University of Saskatchewan.

References

Aarnikunnas, J., Ronnholm, K. & Palva, A. (2002). *Appl. Microbiol. Biotechnol.* **59**, 665–671.
Brünker, P., Altenbuchner, J., Kulbe, K. D. & Mattes, R. (1997). *Biochim. Biophys. Acta*, **1351**, 157–167.
Challener, C. (2004). *Spec. Chem.* **26**, 24–25.
Hörer, S., Stoop, J., Mooibroek, H., Baumann, U. & Sassoon, J. (2001). *J. Biol. Chem.* **276**, 27555–27561.
Jörnvall, H., Persson, B. & Jeffery, J. (1987). *Eur. J. Biochem.* **167**, 195–201.
Jörnvall, H., von Bahr-Lindstrom, H. & Jeffery, J. (1984). *Eur. J. Biochem.* **140**, 17–23.
Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
Kaup, B., Bringer-Meyer, S. & Sahm, H. (2005). *Appl. Microbiol. Biotechnol.* **69**, 397–403.
Kavanagh, K. L., Klimacek, M., Nidetzky, B. & Wilson, D. K. (2002*a*). *J. Biol. Chem.* **277**, 43433–43442.
Kavanagh, K. L., Klimacek, M., Nidetzky, B. & Wilson, D. K. (2002*b*). *Biochemistry*, **41**, 8785–8795.
Kelly, L. A., MacCallum, R. M. & Sternberg, M. J. E. (2000). *J. Mol. Biol.* **299**, 501–522.
Klimacek, M. & Nidetzky, B. (2002). *Biochem. J.* **367**, 13–18.
Kulbe, K. D., Schwab, U. & Gudernatsch, W. (1987). *Ann. NY Acad. Sci.* **506**, 552–568.
Le, A. S. & Mulderrig, K. B. (2001). *Alternative Sweeteners*, 3rd ed., edited by L. O'Brien-Nabors, pp. 317–334. New York: Marcel Dekker.
Nordling, E., Jörnvall, H. & Persson, B. (2002). *Eur. J. Biochem.* **269**, 4267–4276.
Persson, B., Krook, M. & Jörnvall, H. (1991). *Eur. J. Biochem.* **200**, 537–543.
Sasaki, Y., Laivenieks, M. & Zeikus, J. G. (2005). *Appl. Microbiol. Biotechnol.* **68**, 36–41.
Schafer, A., Stein, M. A., Schneider, K. H. & Giffhorn, F. (1997). *Appl. Microbiol. Biotechnol.* **48**, 47–52.
Schneider, K. H. & Giffhorn, F. (1989). *Eur. J. Biochem.* **184**, 15–19.
Schneider, K. H., Giffhorn, F. & Kaplan, S. (1993). *J. Gen. Microbiol.* **139**, 2475–2484.
Silveira, M. & Jonas, R. (2002). *Appl. Microbiol. Biotechnol.* **59**, 400–408.
Slatner, M., Nidetzky, B. & Kulbe, K. D. (1999). *Biochemistry*, **38**, 10489–10498.
Soetaert, W., Buchholz, K. & Vandamme, E. J. (1995). *Agro Food Industry Hi-Tech*, **6**, 41–44.
Soetaert, W., Vanhooren, P. & Vandamme, E. (1999). *Biotechnology*, **10**, 261–275.
Stoop, J. M. & Mooibroek, H. (1998). *Appl. Environ. Microbiol.* **64**, 4689–4696.
Storoni, L. C., McCoy, A. J. & Read, R. J. (2004). *Acta Cryst.* D**60**, 432–438.
Suvarna, K., Bartiss, A. & Wong, B. (2000). *Microbiology*, **146**, 2705–2713.