Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic analysis of mannitol dehydrogenase (MtDH) from the common mushroom *Agaricus bisporus*

Judyth Sassoon,[a]*† Stefan Hörer,[a]† Johan Stoop,[b] Hans Mooibroek[c] and Ulrich Baumann[a]

[a]Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, 3012 Berne, Switzerland, [b]Nutrition and Health, DuPont, Wilmington, DE 19880, USA, and [c]Wageningen University and Research Center, Agrotechnological Research Institute (ATO BV), Department of Bioconversion, NL-6700 AA Wageningen, The Netherlands

† These authors contributed equally to this paper.

Correspondence e-mail: sassoon@izb.unibe.ch

Received 4 January 2001
Accepted 5 February 2001

Mannitol dehydrogenase (MtDH) is a key enzyme controlling the reductive synthesis of mannitol from fructose in the common mushroom *Agaricus bisporus*. A better understanding of the control of mannitol metabolism can be obtained by studying the structure of this enzyme. Here, the purification and crystallization of recombinant MtDH are reported. Crystals generally belonged to the space group $C2$, with unit-cell parameters $a = 227$, $b = 125$, $c = 133$ Å, $\beta = 118^\circ$, and diffracted to at least 1.8 Å resolution, although a tantalum derivative belonged to the space group $P2_1$ and diffracted to the lower resolution of 2.9 Å.

1. Introduction

Mannitol, an acyclic six-carbon polyol, is one of the most abundant sugar alcohols occurring in nature (Jennings, 1984). In the button mushroom *A. bisporus*, mannitol is synthesized from fructose by the enzyme mannitol dehydrogenase (MtDH; E.C. 1.1.1.138). MtDH is a mannitol:fructose 2-oxidoreductase that uses NADPH as its cofactor and has a strict specificity for fructose and mannitol (Ruffner *et al.*, 1978). MtDH was initially purified from *A. bisporus* fruit bodies and biochemically characterized by Ruffner *et al.* (1978) and Morton *et al.* (1985). More recently, MtDH was purified to homogeneity and *mtdh* cDNA was cloned and expressed in *Pichia pastoris* (Stoop & Mooibroek, 1998).

Mannitol metabolism appears to be of great physiological importance in *A. bisporus* as it is the main storage carbon, contributing up to 20% of the mycelium dry weight and up to 50% of the fruit-body dry weight (Rast, 1965). In spite of this, its physiological role is not completely elucidated, although several possible functions have been proposed (Jennings, 1984). According to Hammond & Nichols (1975), it may serve as the main respiratory source during post-harvest development and fruit-body senescence. The direct production of NADPH during mannitol oxidation and its capacity to be shuttled into the mitochondrion for conversion to ATP indicates that mannitol is a very efficient energy source. Evidence for this was observed in celery suspension cultures, where the conversion of mannitol to cell dry weight was 27% more efficient than the conversion of sucrose (Stoop *et al.*, 1995). Mannitol synthesis and metabolism can also play a role in growth regulation (Dütsch & Rast, 1972) by providing and storing reducing power produced during synthesis as NADP. This can then become available for the oxidative reactions of the pentose phosphate shunt, which are controlled by NADP/NADPH ratios.

As an osmoregulatory compound, mannitol might be critical for absorbing water from the surroundings to enhance sporophore development by supporting turgor pressure within the tissues (Holtz, 1971; Jennings, 1984). The metabolic conversion of the disaccharide trehalose, the other major soluble carbohydrate in the sporophore, to mannitol is accompanied by a doubling in osmotic potential. The presence of soluble carbohydrate could also suggest an osmoregulatory function for mannitol (Hammond & Nichols, 1976).

Since mannitol is such an important yet functionally elusive compound for *A. bisporus*, it is clear that the more information we have regarding the regulation of its synthesis the better we will understand its function(s). A better understanding of mannitol dehydrogenase, the critical enzyme in mannitol synthesis, may hold clues to regulation and a crucial part of such a study is the understanding of the enzyme structure. Thus, a crystallographic study of the mannitol dehydrogenase enzyme was undertaken. The protein has been overexpressed and purified from *Escherichia coli* and the crystallization conditions and preliminary characterization are described here.

2. Materials and methods

2.1. Cloning and protein expression

The *mtdh* gene of *A. bisporus* (Stoop & Mooibroek, 1998) was subcloned by PCR methods into the *E. coli* expression vector pET28 (Novagen) using the *Nde*I and the *Xho*I sites, resulting in the plasmid pET28-MtDH.

This coded for a hexahistidine tag and thrombin cleavage site on the N-terminus of the 262 residues of MtDH. Sequencing of four independent clones revealed a conflict with the published sequence at residue 89, the published alanine being replaced by proline. Plasmid pET28-MtDH was used to transform *E. coli* BL21(DE3) and was selected on Luria–Bertani (LB) agar plates containing 50 µg ml^{-1} kanamycin. Bacteria cultivated at 310 K in LB broth were induced for expression of MtDH with 0.5 m*M* ispropyl-β-D-thiogalactopyranoside at an OD_{600} of 0.6 and cell growth continued for 2 h. Cells were harvested by centrifugation (3000*g*) at 277 K, resuspended in 20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole and stored at 253 K. Cells were thawed on ice, sonicated at 277 K and the debris pelleted by centrifugation at 277 K (40 000*g*) for 25 min. The supernatant was applied to a 10 ml metal-chelate affinity column (Ni-NTA, Qiagen) and the column was washed with 20 m*M* imidazole and 80 m*M* imidazole until the baseline absorption at 280 nm stabilized. MtDH was eluted with 250 m*M* imidazole in the same buffer and 5 m*M* β-mercaptoethanol was added to the column fractions immediately after elution. Column fractions were checked for purity and quantity by SDS–PAGE. 0.2 U of thrombin protease (Pharmacia) (where U is the amount of enzyme that cleaves $\geq$ 90% of 100 µg of a GST fusion protein when incubated in 1× PBS at 295 K for 16 h) per milligram of MtDH was added to the pooled protein fractions and dialyzed in 20 m*M* Tris–HCl pH 7.5 at 293 K for 16 h. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 m*M* to inhibit thrombin protease. The features of a typical preparation of MtDH were the following: (i) purity was >98% as checked by SDS–PAGE; (ii) the yield of purified protein was around 30 mg l^{-1} of growth medium; (iii) electrospray mass-spectroscopy analysis gave an MW of 28 324.13 $\pm$ 1.08 Da for one subunit. This confirmed the presence of an A89P mutation at the DNA level in our expression clone, a one-base G265C exchange, and explains the slight difference in mass between our protein and the published theoretical MW of 28 352.07 Da (Stoop & Mooibroek, 1998); (iv) dynamic light-scattering analysis showed a monomodal size distribution with an apparent molecular weight of 112 kDa, which corresponds to the mass of the tetramer. This quaternary structure was also confirmed by gel filtration. Prior to crystallization, the protein was concentrated and dialyzed by ultrafiltration (Amicon, Centriprep). The final concentration of the protein was determined by UV-absorbance spectroscopy using a calculated extinction coefficient ε = 26 120 l mol^{-1} cm^{-1} at 280 nm (Gill & von Hippel, 1989).

2.2. Crystallization

Initial crystallization experiments were based on the sparse-matrix sampling method (Jancarik & Kim, 1991) using Crystal Screens I and II and the Natrix Screen (Hampton Research). Trials were duplicated at 277 and 293 K. Crystalline precipitates were obtained under various conditions: 6, 9, 40 and 41 from Screen I, and condition 25 from the Natrix Screen. These conditions were refined and the best crystals were obtained within a few days using the vapor-diffusion method with a sitting drop consisting of 4 µl MtDH (10 mg ml^{-1}) and 4 µl 90 m*M* Tris–HCl pH 7.5, 18% PEG 4K, 9% 2-propanol equilibrated against 100 µl of 90 m*M* Tris–HCl pH 7.5, 18% PEG 4K, 9% 2-propanol at 293 K. Typical crystals were needle-shaped, as shown in Fig. 1.

2.3. Data collection and analysis

Diffraction data were collected with an R-AXIS IV image-plate detector mounted on a Rigaku RU-300 rotating-anode equipped with Yale mirrors (Cu $K\alpha$, 50 kV, 100 mA) operating at 0.3 × 0.3 mm focus. Data collection was performed at 110 K; cryocooling required the crystal to be immersed in cryoprotectant (crystallization buffer containing increasing concentrations of glycerol: 5, 10, 15, 20% for several seconds) prior to mounting and freezing. The crystal-to-detector distance was 250 mm with oscillations of 0.5°. Autoindexing and processing of the data was performed with the program *MOSFLM* (Leslie, 1996); the intensities were scaled and truncated to amplitudes with the programs *SCALA* (Evans, 1997) and *TRUNCATE* from the *CCP*4 suite of crystallographic programs (Collaborative Computational Project, Number 4, 1994). Data-collection statistics are given in Table 1.

As mentioned above, MtDH forms a homotetramer in solution. A Matthews coefficient V_M (Matthews, 1968) of 2.43 Å^3 Da^{-1} is calculated assuming three homotetramers in the asymmetric unit, with a solvent volume of about 47.5%. The self-rotation function was calculated with the program *GLRF* (Tong & Rossmann, 1990) and was consistent with 222 symmetry (Fig. 2). There are numerous structures of the SDR superfamily known which could provide models for an attempt to determine the structure by the method of molecular replacement. However, the proteins of the SDR superfamily for which three-dimensional structures are available share less than 30% sequence identity with MtDH and so far no model has given a convincing solution. For that reason, a selenomethionine derivative was produced by the method of methionine-synthesis pathway inhibition (Van Duyne *et al.*, 1993). The crystals were grown under the same condition as the wild-type protein except for the

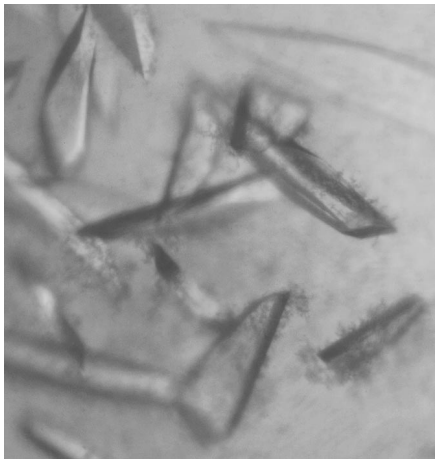

Figure 1
Needle-shaped crystals of recombinant mannitol dehydrogenase, with typical dimensions of 1.5 × 0.2 × 0.1 mm.

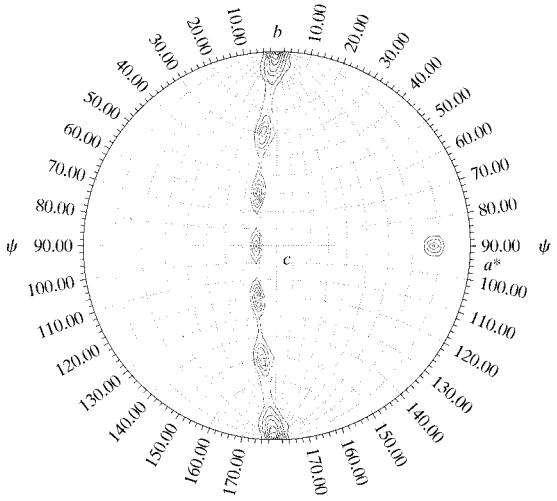

Figure 2
The self-rotation function, κ = 180° section, calculated using all data in the range 4–20 Å with a radius of integration in the Patterson function of 20 Å.

addition of 10 m*M* dithiothreitol to all buffers. These crystals were very fragile and only diffracted to a resolution of 2.9 Å. So far it has not been possible to collect a MAD data set, but the selenomethionine derivative may be useful to confirm solutions of the molecular replacement. A search for heavy-atom derivatives has also been initiated and various mercury and platinum compounds have so far been investigated. When soaked with 1 m*M* Ta_6Br_{14}, a transformation of the space group from *C*2 to $P2_1$ (Table 1) was observed. Judging from the unit-cell size, two tetramers per asymmetric unit are expected (V_M = 2.27 Å^3 Da^{-1}, solvent content 43.7%). Attempts to determine the structure in this crystal form by further heavy-atom derivatives and more extensive molecular-replacement calculations are also under way.

We gratefully acknowledge Professor Robert Huber for the generous gift of Ta_6Br_{14}. This work was supported by SNF grant No. 31-52398.97. The MtDH cloning was supported by an EU Human Capital and Mobility fellowship granted to JMHS (BIO4-CT96-5002).

References

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dütsch, G. A. & Rast, D. (1972). *Phytochemistry*, **11**, 2677–2681.
Evans, P. R. (1997). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **33**, 22–24.
Gill, S. C. & von Hippel, P. H. (1989). *Anal. Biochem.* **182**, 319–326.
Hammond, J. B. W. & Nichols, R. (1975). *J. Sci. Food. Agric.* **26**, 835–842.
Hammond, J. B. W. & Nichols, R. (1976). *J. Gen. Microbiol.* **93**, 309–320.
Holtz, R. B. (1971). *J. Agric. Food. Chem.* **19**, 1272–1273.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jennings, D. H. (1984). *Adv. Microb. Physiol.* **25**, 149–193.
Leslie, A. G. W. (1996). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **32**, 7–8.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Morton, N., Dickerson, A. G. & Hammond, J. B. W. (1985). *J. Gen. Microbiol.* **131**, 2885–2890.
Rast, D. (1965). *Planta*, **64**, 81–93.
Ruffner, H. P., Rast, D., Tobler, H. & Karesch, H. (1978). *Phytochemistry*, **17**, 865–868.
Stoop, J. M., Williamson, J. D., Conkling, M. A. & Pharr, D. M. (1995). *Plant Physiol.* **108**, 1219–1225.
Stoop, J. M. H. & Mooibroek, H. (1998). *Appl. Env. Microbiol.* **64**, 4689–4696.
Tong, L. & Rossmann, M. G. (1990). *Acta Cryst.* A**46**, 783–792.
Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.