The Crystallographic Structure of the Mannitol 2-Dehydrogenase NADP⁺ Binary Complex from *Agaricus bisporus*

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Mannitol, an acyclic six-carbon polyol, is one of the most abundant sugar alcohols occurring in nature. In the button mushroom, *Agaricus bisporus*, it is synthesized from fructose by the enzyme mannitol 2-dehydrogenase (MtDH; EC 1.1.1.138) using NADPH as a cofactor. Mannitol serves as the main storage carbon (up to 50% of the fruit body dry weight) and plays a critical role in growth, fruit body development, osmoregulation, and salt tolerance. Furthermore, mannitol dehydrogenases are being evaluated for commercial mannitol production as alternatives to the less efficient chemical reduction of fructose. Given the importance of mannitol metabolism and mannitol dehydrogenases, MtDH was cloned into the pET28 expression system and overexpressed in *Escherichia coli*. Kinetic and physicochemical properties of the recombinant enzyme are indistinguishable from the natural enzyme. The crystal structure of its binary complex with NADP⁺ was solved at 1.5-Å resolution and refined to an R value of 19.3%. It shows MtDH to be a tetramer and a member of the short chain dehydrogenase/reductase family of enzymes. The catalytic residues forming the so-called catalytic triad can be assigned to Ser⁴⁴⁹, Tyr⁴⁶⁹, and Lys¹⁷³.

Mannitol is the most abundant sugar alcohol in nature, occurring in bacteria, algae, lichens, fungi, and many vascular plants (1, 2). In *Agaricus bisporus*, it is the main storage carbon and can contribute up to 20% of the mycelium dry weight and up to 50% of the fruit body dry weight (3). Mannitol has been reported to accumulate in response to environmental stresses such as salt stress (2, 4, 5). In fungi, its function as an osmo-regulatory compound might also be critical in providing an influx of water from the environment to support growth and fruit body development (2, 6). Other physiological roles of mannitol are also likely including service as the main and most efficient respiratory source during postharvest development and fruit body senescence (7). The advantage of mannitol metabolism over sucrose may be explained by the fact that NADPH is produced during mannitol oxidation and can be converted to ATP, resulting in a more efficient system than in organisms that metabolize sugars.

Furthermore, mannitol metabolism may be involved in the recycling of NADP⁺ and NADPH. NADP⁺ produced during the conversion of fructose to mannitol becomes available for the oxidative reactions of the pentose phosphate shunt, which in turn release NADPH. The interaction between these two biochemical pathways may result in a fine regulation of the cellular NADP⁺/NADPH ratio and suggests a role for mannitol metabolism in growth regulation (8). The production of transgenic mushrooms with altered characteristics is possible, because transformation procedures have been established (9–11). The lack of detailed information at the molecular level regarding genes and proteins involved in central metabolic processes (such as mannitol metabolism) has restricted the breeding of *A. bisporus* by genetic methods.

Apart from its agrotechnological importance, mannitol dehydrogenases are being evaluated as an alternative way to synthesize mannitol from fructose. The ability to produce large quantities of pure and active enzyme using microbial expression systems together with the more efficient enzymatic mannitol production process (compared with the chemical reduction of fructose) may enable commercial production of pure mannitol for use in the food industry.

Given the importance of mannitol metabolism in fungi, the MtDH gene of *A. bisporus* has been cloned and characterized (4). In addition, the knowledge of the three-dimensional structure reported in this work makes it easier and more rational to produce mutated variants of mannitol dehydrogenase.

**EXPERIMENTAL PROCEDURES**

Crystallographic Structure of the MtDH-NADP⁺ Binary Complex—Expression of the *A. bisporus* MtDH gene within the *Escherichia coli* host BL21 (DE3), purification of the resulting soluble protein, and production of monoclonal crystals (space group C2) have been reported previously (12). Crystals of the binary complex (MtDH and NADP⁺) were obtained under similar conditions using the sitting drop vapor diffusion method. NADPH was added to the protein solution to a final concentration of 1 mM prior to crystallization. The drop was created by mixing 5 μl of the protein solution (10–20 mg/ml and 1 mM NADPH) with 5 μl of well solution (90 mM Tris-HCl, pH 7.5, 18% PEG 4K, and 9% isopropanol at 20 °C).

X-ray Diffraction Data Collection—X-ray diffraction data were collected at 1.5-Å resolution at 120 K using a MAR345 image plate detector at beamline BM1A at the European Synchrotron Radiation Facility in Grenoble (wavelength = 0.800 Å). The detector was placed 280 mm from the crystal. Each image was exposed in dose mode for ~5 min and consisted of a 0.5° rotation of the crystal. The data from one crystal (1.5 × 0.2 × 0.1 mm) were processed using MOSFLM (13) and SCALA (14, 15). Data collection statistics are listed in Table I.

Initial Phase Determination and Model Refinement—Molecular replacement was done with the CNS software package (16). Initial mo-

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1 The abbreviations used are: MtDH, mannitol 2-dehydrogenase; SDR, short-chain dehydrogenase/reductase; TLS, translation, liberation, screw rotation.
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Table I

X-ray diffraction data statistics

| Space group | C2 |
|-------------|----|
| Lattice constants | a = 227.253 Å, b = 124.848 Å, c = 132.685 Å, α = 90°, β = 118.55°, γ = 90° |
| Completeness | 98.4% |
| Symmetry | 97.0% |
| Rsym (based on I) | 6.3% |
| Redundancy | 2.7 |
| 〈I/σI〉 | 11.8 |

Table II

TLS refinement

Comparison of average B factors before and after TLS refinement.

| Tetramer | Average B factors | Number of crystal contacts |
|----------|------------------|--------------------------|
| Before TLS refinement | After TLS refinement |
| 1 (ABCD) | 27.1 | 13.3 | 38 |
| 2 (EFGH) | 16.0 | 13.3 | 50 |
| 3 (IJKL) | 16.6 | 13.3 | 49 |

Table III

Model refinement statistics

| R factor = ∑|Fobserved - Fcalculated|/∑|Fobserved| × 100; Free R factor = R value of portion of data omitted from model refinement; F, amplitude. |
|-------------|------------------|----------------|------------------|------------------|
| R factor | 19.3% |
| Free R factor | 20.9% |
| Test set size | 1.0% |
| Bond length | 0.007 Å |
| Bond angles | 1.36° |

| Atom type | Number of non-hydrogen atoms | Average temperature factor |
|-----------|-----------------------------|---------------------------|
| Protein | 23412 | 13.3 |
| NADP | 576 | 14.0 |
| Nickel ions | 6 | 31.6 |
| Water molecules | 3191 | 51.7 |
| Total | 27185 | 15.5 |

* Root mean square deviation from ideal stereochemistry.
RESULTS

Overall Tertiary and Quaternary Structure of MtDH—MtDH is a member of the SDR family of enzymes, also known as the tyrosine-dependent oxidoreductase family (reviewed in Ref. 26). The characteristic of this family is the extension of the typical Rossmann fold consisting of two $\beta$-$\alpha$-$\beta$ motifs $\beta$A-$\alpha$B-$\beta$B-$\alpha$C-$\beta$C and $\beta$D-$\alpha$E-$\beta$E-$\alpha$F-$\beta$F (27) (reviewed in Ref. 28) by a seventh $\beta$-strand with a left-handed cross-over connection be-

FIG. 1. Stereo drawing of the tertiary and quaternary structure of MtDH. $a$, ribbon diagram of a single subunit of MtDH. red, $\alpha$-helices; green, $\beta$-strands. The bound NADP$^+$ is shown in Corey-Pauling-Koltun (CPK) representation. $b$, the homotetramer of MtDH. Each monomeric subunit is shown in a different color (cyan, red, green, and magenta). The C termini of subunits 1 and 3 (cyan and green) coordinate one nickel ion as well as the C termini of subunits 2 and 4. This view is along the axis that intersects the two nickel ions such that only one ion is visible. The figure was prepared with MOLSCRIPT (52) in combination with RASTER-3D (53).

FIG. 2. View of the NADP$^+$ binding pocket of MtDH. Those residues that contribute to the binding of the cofactor by hydrogen bonds and eight water molecules involved in the hydrogen bonding network are shown. Putative hydrogen bonds are shown as dotted lines. The figure was prepared with DINO (www.bioz.unibas.ch/~xray/dino) and RASTER-3D (53).

FIG. 3. Mannitol binding to MtDH. Mannitol, the NADP, and the residues of MtDH involved in binding of the substrate are shown. Putative hydrogen bonds and distances are shown. In the crystal structure, a water molecule can be found within 0.88 Å distance to the $O_2$ oxygen, which must be expelled upon mannitol binding. Hydrogen bonds are shown as dashed lines, and the distance between the $O_2$ of mannitol and the C4 position of the nicotinamide ring is indicated with a dotted line. WAT, water molecule. The figure was prepared with MOLSCRIPT (52) and RASTER-3D (53).
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between strands F and G. Secondary structure elements were assigned according to the convention described by Ghosh et al. (29). The loop between \( \beta F \) and \( \alpha G \) contains two small helices (\( \alpha FG1 \), residues 204–209 and \( \alpha FG2 \), residues 211–220) and one \( \beta \)-strand (\( \beta FG1 \), residues 227–229), which form one side of the active site cavity. Within the SDR family this loop is responsible for the substrate specificity among the different enzymes (30–35). The other side of the active site cavity is lined by the insertion loop between \( \beta E \) and \( \alpha F \), which in the case of MtDH contains a small antiparallel \( \beta \)-sheet, \( \beta EF1 \) (residues 157–159) and \( \beta EF2 \) (residues 162–164) (Fig. 1A).

Size exclusion chromatography and dynamic light scattering showed that MtDH is a tetramer in solution. Our kinetic data showed cooperativity in neither the oxidative nor the reductive reaction of MtDH. The crystal structure revealed that the asymmetric unit contains three tetramers. Each tetramer possesses internal 222 symmetry. The interaction surface is 1460 Å² between monomers 1 (cyan) and 2 (red), 660 Å² between monomers 1 and 3 (green), and 1740 Å² between monomers 1 and 4 (magenta) (color coding according to Fig. 1B; calculations were done with the CNS software package (16, 36)). The total surface area buried in each monomer therefore is 3850 Å² or -32%.

The C termini of monomer 1s and 3 and the C termini of monomers 2 and 4 coordinate one metal ion each. The absence of anomalous scattering for this metal ion using Cu-K\( \alpha \) radiation (1.54 Å) combined with the presence of anomalous scattering peaks using synchrotron radiation (0.80 Å) give good evidence that this metal ion indeed is nickel, which is probably a purification artifact. Also, the trigonal prismatic coordination sphere of each metal ion (two C termini from two individual subunits of one tetramer and four water molecules) is not evidence that this metal ion indeed is nickel, which is probably a purification artifact. Also, the trigonal prismatic coordination sphere of each metal ion (two C termini from two individual subunits of one tetramer and four water molecules) is not uncommon for nickel under conditions at which steric hindrance prevents octahedral coordination.

**Location and Conformation of the NADP⁺ Substrate**—The NADP⁺ binding site is located at the C-terminal end of the \( \beta \)-sheet forming the Rossmann fold (Fig. 1A). The substrate binding crevice forms an oval-shaped cavity with dimensions of \( \sim 20 \times 12 \times 7 \) Å with the cofactor at the bottom of the cavity. Both riboses of the NADP⁺ have \( \gamma \-E \) (C\sub{2}-endo) puckering, the nicotinamide ring is in the \( \text{syn} \) conformation, and the adenine in the \text{anti} conformation (nomenclature according to International Union of Pure and Applied Chemistry-International Union of Biochemistry nomenclature for polynucleotides (37)). This conformation classifies MtDH as B-stereospecific and is typical for SDR enzymes (38). The cofactor is bound to the enzyme by an extensive network of hydrogen bonds, which also involves seven water molecules (Fig. 2). The loops at the C-terminal end of \( \beta \)-strands A, B, and C are responsible for the binding of the adenosine moiety. In the first loop, Asn\sub{69} and Arg\sub{21} form hydrogen bonds to the phosphate group of the ribose and the O\sub{2} of the ribose, respectively. The main-chain amide groups of Arg\sub{43}, Ser\sub{44}, and Ala\sub{45} also form hydrogen bonds to the phosphate group. In addition, the side chains of Arg\sub{43} and Ser\sub{44} coordinate two water molecules together with the phosphate group. The Asn\sub{69} hydrogen bonds to the adenosyl amino group, whereas the main chain amide of Val\sub{70} interacts with the N1 ring nitrogen. Gln\sub{206} and Ile\sub{23} form direct hydrogen bonds to the pyrophosphate moiety, whereas Gly\sub{18}, Arg\sub{21}, Gly\sub{24}, Asn\sub{96}, Gly\sub{98}, and Thr\sub{204} form hydrogen bonds mediated by water molecules.

The nicotinamide moiety also shows several residues in hydrogen bonding distance. The active site Lys\sub{173} forms a bifurcate hydrogen bond to the O\sub{2} and O\sub{3} of the ribose; the active site tyrosine also forms a hydrogen bond to the O\sub{2}. The amide group of the nicotinamide is kept in place by a contact to Val\sub{202} and Thr\sub{204} and an additional water molecule.

**Location of the Mannitol Substrate and Mechanism of Catalysis**—All attempts to obtain a structure of MtDH with various combinations of NADP, NADPH, mannitol, and fructose to date have failed. For that reason mannitol was modeled into the active site cavity with the program AUTODOCK. The cluster with the lowest energy contained 38 of 100 docked structures. This orientation was assumed to be the best orientation possible for mannitol. In our model, the substrate can form seven hydrogen bonds to the protein. The active site serine (Ser\sub{151}) is in hydrogen-bonding distance to O\sub{1} and O\sub{2}; Ser\sub{151} also interacts with O\sub{3}. Further contacts are possible between O\sub{2} and the amide group of the nicotinamide and between O\sub{2} and Gln\sub{166}. This orientation places the C\sub{2} of a distance of 3.4 Å to the C4 of the nicotinamide, to which the hydrogen is transferred during catalysis (Fig. 3). Structural analysis of other members of the SDR family has shown that three residues are crucial for catalysis, forming the so-called catalytic triad (30, 31, 33, 39–45). The SDR family catalytic triad of MtDH is Ser\sub{149}, Tyr\sub{169}, Lys\sub{173}. The role of the lysine is to orient the nicotinamide moiety of the NADP by forming a bifurcate hydrogen bond to the O\sub{2} and O\sub{3} of the ribose (46). The proposed reaction mechanism suggests Tyr\sub{169} to be deprotonated. This is facilitated by the positively charged environment provided by Lys\sub{173} and the NADP⁺ itself. During catalysis, the tyrosine directly interacts with the substrate hydroxyl group. In the structure presented here, the putative position of the substrate is occupied by a water molecule, which is hydrogen-bonded to both of the active site residues participating in catalysis, Ser\sub{149} and Tyr\sub{169}, and to Ser\sub{151}. This water molecule lies within a distance of 0.88 Å to the proposed position of the O\sub{2} of the mannitol obtained by modeling. A schematic drawing of the reaction mechanism is shown in Fig. 4.

**Enzymatic Activity of Recombinant MtDH**—The enzymatic parameters of recombinant MtDH. The \( K_m \) values of the native enzyme are 34 mM for mannitol and 190 mM for fructose as determined by Ruffner et al. (47).

| Reaction       | \( K_m \) (mM) | \( k_{cat} \) (s⁻¹) | \( k_{cat}/K_m \) (s⁻¹ M⁻¹) | pH  |
|---------------|---------------|-------------------|----------------------------|-----|
| Mannitol oxidation | 29.3 ± 1.1    | 2.20 ± 0.08 × 10³ | 7.4 × 10⁴                   | 9.0 |
| Fructose reduction | 58.7 ± 4.8    | 7.31 ± 0.17 × 10³ | 1.3 × 10⁴                   | 9.0 |
parameters of MtDH in steady-state kinetics have been determined for recombinant MtDH (Table IV). The results are comparable with the data determined for the native enzyme isolated from *A. bisporus* sporocarps (47).

**DISCUSSION**

**Structure Determination**—Three steps proved to be crucial for fast structure determination and refinement in the case of MtDH. First, different members of the SDR family, for which the three-dimensional structure was available, were used as probes in the molecular replacement calculations. Of those, only one (mouse lung carbonyl reductase; Protein Data Bank entry 1cyd) gave interpretable solutions for the cross-rotation and translation function. In addition, the probe had to be modified. On the basis of a sequence alignment and modeling with the program SWISSMODEL (48), all nonconserved residues were changed to alanine (except for the glycines, which were kept), and differing loops were deleted. Second, automatic model building with ARP/wARP made fast model building possible, keeping in mind that the asymmetric unit of the crystal consists of three tetramers that are not similar enough to apply real-space averaging. Third, describing the difference between the three tetramers as independent rigid body movements and the separate treatment of this behavior with the three parameters $T$, $L$, and $S$ for each individual subunit led to remaining $B$ factors that were very similar between the subunits and allowed for subsequent application of noncrystallographic symmetry restraints. This procedure dropped the $R$ factor and $R_{free}$ by about 3%. The movement of the three tetramers can be described with a set of three nonintersecting screw axes for each monomer.

![Figure 5](image-url)  
**FIG. 5.** Rigid body movement of the three different tetramers in the asymmetric unit cell. The three tetramers forming the asymmetric unit of the crystal and the three nonintersecting screw axes for each monomer are shown. The length of the axes is proportional to the amplitude of the movement. The axes were calculated with the program TLSANL (23).

| Table V | Putative hydrogen bonds between mannitol and MtDH. |
|----|----|----|----|
| Substrate | Mannitol | Sorbitol | Arabinol |
| O1 | Ser<sup>149</sup> | Ser<sup>149</sup>, Ser<sup>151</sup>, Tyr<sup>69</sup> | Ser<sup>149</sup>, Ser<sup>151</sup>, Tyr<sup>69</sup> |
| O2 | Ser<sup>149</sup>, Ser<sup>151</sup>, Tyr<sup>69</sup> | NADP amide group | NADP amide group |
| O3 | NADP amide group | NADP amide group |
| O4 | Gln<sup>166</sup> | Ser<sup>100</sup> |
| O5 | Ser<sup>100</sup> |

![Figure 6](image-url)  
**FIG. 6.** Sequence alignment of SDR family members with respect to cofactor specificity. Sequences of proteins with known three-dimensional structure and less than 1.9 Å root mean square deviation compared with MtDH were aligned based on the three-dimensional structure. Amino acids are colored as follows: dark gray, glycine; light gray, apolar; black background, negatively charged; gray background, positively charged. The consensus sequence is based on a minimum plurality of four. *, MtDH is listed here for comparison.
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each subunit (Fig. 5). A comparison with Table II clearly shows that tetramer 1 (monomers A, B, C, and D), having the highest B factors, also undergoes the largest movements in the crystal. An analysis of the crystal contacts formed by the three tetramers is in accordance to the hypothesis of Hery et al. (24), who theorized that crystal contacts can restrict the movement of whole molecules. The number of contacts is 38 for tetramer 1 compared with 50 and 49 for tetramers 2 and 3, respectively. The treatment of the data as rigid body movement of whole monomers significantly improved the quality of the electron density in tetramer 1, but it is still worse than for the two other tetramers.

Enzymatic Activity and Mechanism of MtDH—The kinetic constants of recombinant MtDH are comparable with those of the native enzyme. The very low affinity for the substrates has been reported for mannitol dehydrogenases from other organisms (47). MtDH can also to some extent use sorbitol or arabinol as a substrate. This is also demonstrated by our modeling studies. Mannitol can form seven hydrogen bonds to MtDH (see Table V and Fig. 3), whereas sorbitol and arabinol are only able to form six hydrogen bonds. The high $K_m$ values may explain why we have not succeeded until now to co-crystallize the binary complex of MtDH and NADP$^+$ with either mannitol or fructose. The high $K_m$ value for fructose is probably because of the fact that it is actually the open and not the hemiketal form of the sugar that undergoes the reaction. The equilibrium between the open and ring forms leaves very little substrate in the crystal. Therefore the distance between the catalytic tyrosine and the lysine can be correlated to the pH dependence of the different enzymes in the SDR family (49).

Cofactor Specificity—An interesting possibility is to change the cofactor specificity for the cofactor NADP to the much cheaper NAD. This arginine is in accordance to the hypothesis of Hery et al. (24), who theorized that crystal contacts can restrict the movement of whole molecules. The number of contacts is 38 for tetramer 1 compared with 50 and 49 for tetramers 2 and 3, respectively. The treatment of the data as rigid body movement of whole monomers significantly improved the quality of the electron density in tetramer 1, but it is still worse than for the two other tetramers.

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