Long-chain mannitol dehydrogenases are secondary alcohol dehydrogenases that are of wide interest because of their involvement in metabolism and potential applications in agriculture, medicine, and industry. They differ from other alcohol and polyol dehydrogenases because they do not contain a conserved tyrosine and are not dependent on Zn$^{2+}$ or other metal cofactors. The structures of the long-chain mannitol 2-dehydrogenase (54 kDa) from Pseudomonas fluorescens in a binary complex with NAD$^+$ and ternary complex with NAD$^+$ and D-mannitol have been determined to resolutions of 1.7 and 1.8 Å and R-factors of 0.171 and 0.176, respectively. These results show an N-terminal domain that includes a typical Rossmann fold. The C-terminal domain is primarily α-helical and mediates mannitol binding. The electron lone pair of Lys-295 is steered by hydrogen-bonding interactions with the amide oxygen of Asn-300 and the main-chain carbonyl oxygen of Val-229 to act as the general base. Asn-191 and Asn-300 are involved in a web of hydrogen bonding, which precisely orients the mannitol O2 proton for abstraction. These residues also aid in stabilizing a negative charge in the intermediate state and in preventing the formation of nonproductive complexes with the substrate. The catalytic lysine may be returned to its unprotonated state using a rectifying proton tunnel driven by Glu-292 oscillating among different environments. Despite low sequence homology, the closest structural neighbors are glycerol-3-phosphate dehydrogenase, N-(1-β-carboxyethyl)-L-norvaline dehydrogenase, UDP-glucose dehydrogenase, and 6-phosphoglucuronate dehydrogenase, indicating a possible evolutionary relationship among these enzymes.

Mannitol, a six-carbon non-cyclic polyol, is the most abundant sugar alcohol found in nature. Produced in plants, fungi, protozoa, and bacteria as a storage compound for carbon and reducing equivalents, it also functions in response to oxidative stress and as an osmoregulator (1–5). D-Mannitol is used extensively in the food and pharmaceutical industry because of its favorable bulking properties and the fact that it does not cause tooth decay and is safe for diabetics. The traditional method of industrially producing mannitol involves the reduction of fructose using a metal catalyst and hydrogen gas, resulting in nearly equal amounts of D-sorbitol and D-mannitol, which must then be separated.

In general, mannitol dehydrogenases (MDH)$^{1}$ catalyze the NAD(P)$^+$-dependent reversible oxidation of D-mannitol or D-mannitol-1-phosphate to the corresponding ketose, D-fructose, or D-fructose 6-phosphate. These secondary alcohol dehydrogenases are specific for the C2i(R) configuration of polyhydroxylated compounds and are of interest because of their potential applications in chiral synthesis. More recently, mannitol dehydrogenases have been identified in plants that catalyze the oxidation of D-mannitol to D-mannose, an aldose (6). MDHs have been characterized from plants and fungi that are members of the medium-chain zine-containing dehydrogenase/reductase family (7, 8). Other MDH from fungi are members of the short-chain dehydrogenase/reductase (SDR) family (3, 9). Often, bacterial MDHs do not share significant similarity with either of these families (10) but instead belong to a family of long-chain MDH that so far includes 54 recognized members. These members are classified by the Protein Families Data Base as family 01232 (11). More recent work increased this number to 66.$^{2}$ Sequence identity with other long-chain dehydrogenases is low, typically around 10%.

Most members of the prokaryotic long-chain MDH family have been identified by primary sequence alone (11), and a limited number of these proteins have had their specificity characterized and activity quantitated. The proteins that have been studied are monomeric long-chain dehydrogenases of a molecular mass of ~54 kDa (10, 13, 14). This family so far includes mannitol 2-dehydrogenases, mannitol-1-phosphate 5-dehydrogenases, D-mannionate dehydrogenases, sorbitol dehydrogenases, L-sorbose reductase, fructuronate reductase, and other.

$^{1}$ The abbreviations used are: MDH, mannitol dehydrogenase; 6PGDH, 6-phosphoglucuronate dehydrogenase; MAD, multiwavelength anomalous dispersion; CENDH, N-(1-β-carboxyethyl)-L-norvaline dehydrogenase; G3PDH, glycerol-3-phosphate dehydrogenase; NAD$^+$, nicotinamide adenine dinucleotide (oxidized); pNPDH, P. fluorescens mannitol 2-dehydrogenase/SBR, short-chain dehydrogenase/reductase; UDPGPDH, UDP-glucose dehydrogenase; SSRL, Stanford Synchrotron Radiation Laboratory.

$^{2}$ Klimacek, M., Kavanagh, K., Wilson, D., and Nidetzky, B., in press.
onate oxidoreductases, and D-arabinitol dehydrogenases. In addition to their significance as an alcohol dehydrogenase that employs a novel mechanism, interest in long-chain MDHs originates from several potential uses: (i) transgenic expression of bacterial MDHs in plants has been tested to improve salt tolerance and resistance to oxidative stress in agricultural crops (15), (ii) quantitative analysis of mannitol concentration in serum and urine via a simple and sensitive enzymatic assay has potential clinical use (16), and (iii) enzymatic production of D-mannitol from fructose would reduce downstream purification (17).

An inductible mannitol 2-dehydrogenase belonging to the long-chain MDH family was isolated from Pseudomonas fluorescens DSM50106 (pMDH, EC 1.1.1.167) (18). It catalyzes the reversible oxidation of D-mannitol to D-fructose, D-arabinitol to D-xylulose, and D-sorbitol to L-sorbose by transferring the C2 hydride to the pro-S position on the nicotinamide without the use of metal cations. It is specific for the C2(4) configuration of polyols with a minimum of five carbons, and no activity is measurable with mannitol 1-phosphate, fructose 6-phosphate, or 5,6-dIDEOXY-D-fructose.\(^3\) How specificity for polyol substrates is achieved is not well understood. Although able to use both NADH and NADPH as cofactor, the activity with NADH is greater. Other alcohol dehydrogenases use metal ions (medium-chain dehydrogenase/reductases and some long-chain dehydrogenases) or a conserved tyrosine (SDRs) for catalysis. Neither is present in long-chain MDHs, so presumably, these alcohol dehydrogenases use a novel catalytic mechanism. Biochemical data implicate an enzyme side-chain with a pKa of 9.34 (13). This study was undertaken to gain an understanding of how specificity is achieved for substrate and cofactor as well as elucidate the mechanism for catalysis in the structurally uncharacterized family of long-chain MDHs.

MATERIALS AND METHODS

Expression and Purification—Recombinant wild-type MDH from P. fluorescens DSM 50106 was expressed in Escherichia coli and purified as previously described (13). The selenomethionine-substituted protein was expressed in the presence of 60 mg/liter selenomethionine accompanied by amino acids inhibiting de novo synthesis of methionine (19). Purification was carried out using a protocol similar to the previously described wild-type preparation (13). A final gel filtration step was included to obtain highly purified protein. Gel filtration was carried out on an Aktaexplorer 100 system (Amersham Biosciences) using 140-mL Superdex 75 prep-grade material packed into a 1.670-cm column. Approximately 15 mg of protein in 50 mM Tris, pH 7.2, were applied to the column equilibrated with 50 mM Tris, 200 mM NaCl. The protein was eluted at a flow rate of 0.75 mL/min. Fractions containing enzyme activity were pooled and concentrated to ~11 mg/mL. The purified protein migrated as a single band in SDS-PAGE and non-denaturing anionic PAGE. Semiquantitative densitometric analysis of the Coomassie Blue-stained gels suggested that the purity of pMDH was 99% or better. The selenium-substituted protein retains full wild-type activity.

Crystallization—Wild-type pMDH was concentrated to 14 mg/mL, and the buffer changed to 10 mM Tris, 25 mM NaCl, pH 7.5. Hanging drop vapor diffusion experiments were conducted at 277 and 293 K using both apoprotein and protein solution containing 5 mM NADH. Initial crystals of a binary complex with NADH took five months to appear at 293 K over a well containing 30% (v/v) polyethylene glycol 4000, 200 mM ammonium acetate, 100 mM sodium citrate, pH 5.6. A single crystal was flash-cooled in a buffer containing 75% (v/v) well solution and 25% (v/v) ethylene glycol. Diffraction intensities to 1.7 Å were collected at Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-1 and processed using the program Denzo (20). This indicated that the spacegroup was \(P2_12_12_1\) with unit cell dimensions of \(a = 107.0 \text{ Å}, b = 104.5 \text{ Å}, \text{ and } c = 101.5 \text{ Å} \). Systematic extinctions indicated that the spacegroup was \(P2_1\), \(2_1\). The calculation of the Matthews' constant \((V_M) = 2.6 \text{ Å}^3/\text{dAton} \) implied two protein molecules/asymmetric unit. Molecular replacement using the holo-MDH structure stripped of water molecules as a search model was implemented using the program EMPeror (23). An initial solution was found for two molecules using data between 30 and 4 Å, which yielded a correlation coefficient of 0.603 and an initial \(R_{cryst} = 0.41 \) in this resolution range.

In both cases before refinement commenced, 5% of the data was flagged for calculation of \(R_{free} \). Alternating rounds of manual fitting and crystallographic refinement using the programs O and CNS (24) resulted in the final structures of which statistics appear in Table II. Ordered water molecules were picked in CNS and manually checked for appropriate hydrogen bonding. Despite the inclusion of 1 mM NAD\(^+\) in the mannitol soak, the density of the NAD\(^+\) indicated occupancy of <1. Because it is not possible to accurately refine occupancy and temperature factors at this resolution, values between 0.5 and 1.0 were tested and the occupancy for the NAD\(^+\) in the ternary model was set to 0.8, a value that gave both reasonable temperature factors and minimized features in difference maps.

RESULTS AND DISCUSSION

Structure Determination and Model Quality—The structure of the binary complex of pMDH with NADH was initially determined to 2.3 Å resolution by MAD using selenomethionine-substituted protein (Table I). A data set to 1.7 Å was subsequently collected and used for refinement (Table II). Crystals of a ternary complex of pMDH with NADH and mannitol were obtained by including 10 mM D-mannitol in the protein solution and soaking the crystals for 12 h in a solution containing 300 mM mannitol. Diffraction intensities to 1.8 Å resolution were collected on a ternary crystal (Tables I and II).

In both cases, the model for MDH includes residues 1–492. The final C-terminal residue was disordered and not fit. The holo model includes 1 pMDH monomer, 1 NADH molecule, and

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\(^3\) B. Nidetzky, unpublished results.
and helices α10 and α15 in domain 2. In addition, helix α13 contributes a conserved arginine, Arg-361, which is engaged in interdomain salt bridges to Asp-230 on β11 and Glu-259 located on the loop at the C terminus of β12.

The active site is at the interface of the two domains with the majority of residues that bind NADH contributed by the N-terminal domain and residues that bind mannitol coming primarily from the C-terminal domain. Arg-373 is the only residue from the C-terminal domain that interacts directly with the bound NAD⁺. Important secondary structural elements that contribute residues to the active site are α1 and loop regions at the C termini of β3, β7, and β10 from domain 1 and α10 from domain 2. A conserved lysine located on α10, Lys-295, is poised to act as the general acid/base in the reaction.

**Domain Structure**—Residues 1–285 form the NAD⁺-binding domain, domain 1. Helix α1 is in the center of domain 1 and is surrounded by the six-strand parallel β-sheet of the dinucleotide binding motif, a three-strand β-sheet formed by the extension of the final strand in the Rossmann fold with two antiparallel strands, and a four-strand mixed β-sheet. Although the relative order of the strands in the parallel sheet are the same as a Rossmann fold (CBADEF), there are insertions within it that suggest that the additional elements are not merely appended (Fig. 2). The three-strand sheet is formed wholly from residues inserted between the last strand in the dinucleotide binding fold (strand F) and the C-terminal domain, whereas the four-sheet strand is formed from residues at the N terminus, an insertion between strands B and C and the C-terminal insertion. A small solvent-accessible β-hairpin is located at the C terminus of β7. Asn-191 and Asp-230 are the only residues from domain 1 that directly bind mannitol, whereas Glu-133 has a water-mediated interaction.

Eleven α-helices and a small β-hairpin make up the C-terminal domain, which can be further divided into two subdomains. Residues 289–375 compose helices α10–α14, forming domain 2A, an antiparallel three-helix bundle with two short connecting helices. Domain 2B, residues 378–493, consists of α15–α20 and the β-hairpin. Helices α15, α16, and α19 form a three-helix bundle of different structural arrangement with α15 and α16 antiparallel and α16 and α19 parallel. Helix α15 has a pronounced 40° bend because of two proline residues, Pro-383 and Pro-388, in the middle of the helix. At least one proline is found in this region in all long-chain MDH sequences examined, suggesting that the bend in α15 is a common feature for the family. Helix α15 lies antiparallel to α10 and contributes several contacts to domain 1 including three water-mediated hydrogen bonds and one salt link between Lys-384 and Asp-140. Also located on α15 is Lys-381, which hydrogen bonds to the terminal hydroxyl of mannitol away from the point of oxidation.

Residues 289–313 compose α10, the first helix in the C-terminal domain that lies near the cleft between domains 1 and 2 and provides several interdomain contacts. It also contributes three residues important in substrate binding, Asn-300, His-303, and the apparent catalytic acid/base Lys-295. Arg-361 located on α13 is involved in salt links across
the domain interface to Glu-259 and Asp-230, and the loop following α13 provides hydrogen bonds and van der Waals contacts to residues Leu-88, Asp-90, and Met-258 in domain 1. Arg-373 on α14 donates hydrogen bonds to hydroxyls on carbons 3 and 4 of the substrate and is the only residue from the C-terminal domain within 4 Å of the NAD⁺. It is located parallel but slightly offset by 3.15–3.89 Å abutting the amide on the nicotinamide, contributing weak stacking or van der Waals interactions. Although there is a lack of extensive interaction between the NAD⁺ and domain 2, a closer inspection shows that the loops from domain 1 that recognize the NAD⁺ provide 40% of the interdomain contacts. Therefore, a conformational change upon binding NAD⁺ is possible and is predicted from the kinetic mechanism (13).

**NAD⁺ Binding and Specificity**—The NAD⁺ is bound with the adenine anti and the nicotinamide syn between the two domains. The sugar pucker of the adenosine ribose is C₂-endo, and the nicotinamide ribose is C₃-endo. There is an intramolecular hydrogen bond between the nicotinamide N7 and pyrophosphate oxygen NO1. Using the method of Lee and Richards (26) with a probe of radius 1.4 Å, 80% of the accessible surface of NAD⁺ is buried in the complex with pfMDH (26). Cofactor contacts are mainly from domain 1 and primarily from the canonical dinucleotide binding motif. As is commonly found in enzymes with a Rossmann fold, the pyrophosphate moiety is situated at the N terminus of α1. The glycine-rich turn in the sequence HIGVGGFHR precedes α1. HXGXGXXR is the conserved fingerprint motif for the long chain MDH family with the exception of altronate dehydrogenases that have a glutamine at position 31. A hydrophobic Ile, Leu, or Phe follows this residue. The Ne of invariant Arg-39 orients the amide of Gly-36 to interact with the pyrophosphate by hydrogen bonding to the carbonyl oxygen of Gly-35. Phe-37 stacks against the A side of the nicotinamide, making the B side accessible to substrate and promoting transfer of the 4-pro-S hydride. A Phe or Ile is present at this position for all members of the long-chain MDH family and probably stacks against the pyridine ring. Thr-233, located on the loop following 611, has main-chain hydrogen bonds to the cofactor amide oxygen and nitrogen (Fig. 3).

The adenine packs against Ile-131 and is shielded from solvent by Arg-66. Isomerization of the loop containing residues 65–69 is required for cofactor binding and release and could explain why the release of NADH is the rate-limiting step in the direction of oxidation of mannitol (13). Loops at the C termini of strands B, D, and E contribute residues that hydrogen bond with the ribose hydroxyls. Although significant activ-
ity with NADPH is still observed, pfMDH exhibits a strong preference for NADH over NADPH (13). Asp-69, which hydrogen bonds to both adenosine ribose hydroxyls, almost certainly contributes toward specificity for NAD$^+$ over NADP$^+$ by discouraging phosphate binding. However, it is located on a mobile loop, allowing Asp-69 to have a different conformation with NADP$^+$ bound. Arg-66 on the same loop could make favorable contacts with a phosphate moiety and partially compensate for the negative Asp-69 interaction. Other members of this family showing a preference for NADP$^+$ have a loop containing an aspartate and several lysines that could occupy this position. However, the arginine is less well conserved.

The single residue found in a disallowed region of the Ramachandran plot, Thr-132, is located between Ile-131 (mentioned above) and Glu-133, which binds a nicotinamide ribose hydroxyl. The strained conformation observed in Thr-132 is probably dictated by the required arrangement of this loop.

**Mannitol Binding and Specificity**—Mannitol binds 16–23 Å deep in the active site cleft with C2 (or C5) above the nicotinamide C4 (Fig. 4A). Each half of D-mannitol is equivalent to the other because of the fact that the chirality of C5(R) is the same as C2(R) and C4(R) is the same as C3(R). Consequently, the numbering choice for mannitol is arbitrary. This becomes important when considering alternate substrates such as D-sorbitol, D-mannonate, or mannitol 1-phosphate. The mannitol is bound in an extended conformation with a pseudo-2-fold axis of rotation between C3 and C4 (Fig. 4B). Ten direct polar interactions are made between the protein and the polyl as well as one additional water-mediated interaction (Fig. 4C). Three of the direct and the water-mediated interactions are with residues from the N-terminal domain; the remaining seven are with residues from the C-terminal domain. The substrate C2 is located within hydride transfer distance 2.9 Å from the nicotinamide C4.

Of the seven residues whose side chains are involved in specific interactions with mannitol, Asp-230 and Lys-295 (discussed below) are invariant throughout the long-chain MDH family. Asp-230 hydrogen bonds to the C1 hydroxyl, preventing a phosphate or carboxylate from binding at that position. It is also involved in an important interdomain salt-link with Arg-381. Aan-300, His-303, and Lys-381 are all conserved or have conservative replacements. Asn-191 is replaced by leucine in the altronate dehydrogenases but is conserved in all other cases. The most variable residue is Arg-373, which may be replaced by Gln, Ser, or Asp. It is impractical at this time to make predictions regarding whether substitutions in the active site are involved in specificity for alternate substrates such as mannitol 1-phosphate, arabinitol, mannonate, or altronate, because few proteins of the long-chain MDH family have been enzymatically characterized.

pfMDH is completely inactive with phosphorylated substrates D-mannitol 1-phosphate and D-fructose 6-phosphate. In the reaction catalyzed by mannitol-1-phosphate 5-dehydrogenase, a phosphate moiety replaces the hydroxyl distal to catalysis on the substrate. Although Lys-381, which interacts with O6, could potentially bind a phosphate at that position in pfMDH, there is no steric room for this additional group. The lack of steric volume near O1 and the presence of Asp-230 prohibit binding of a phosphate or carboxylate moiety directly adjacent to the catalytic site. D-Arabinitol and D-sorbitol are alternate polyl substrates of pfMDH with $K_m = 14$ and 460 mM, respectively. The specificity constant of pfMDH for D-arabinitol ($k_{cat}/K_m = 3.4 \text{ mM}^{-1} \text{s}^{-1}$) is 5-fold lower than that for D-mannitol ($K_m = 1.2 \text{ mM}$, $k_{cat}/K_m = 18 \text{ mM}^{-1} \text{s}^{-1}$) (13). Arabinitol, a five-carbon polyol, could potentially make 10 of the 11 hydrogen bonds that mannitol makes. In addition, mannitol can fit into the active site with either C2 or C5 in position to be reduced, whereas D-arabinitol can only bind productively in one orientation. Both of these factors would increase the affinity of the enzyme for D-mannitol relative to D-arabinitol. The 600-fold reduced catalytic efficiency with D-sorbitol ($k_{cat}/K_m = 0.03 \text{ mM}^{-1} \text{s}^{-1}$) is less easily understood. D-Mannitol and D-sorbitol differ only in that the configuration of C2 is S in D-sorbitol. For D-sorbitol, it is C5(R) that is oxidized to produce L-sorbose, such that the C2 is distal to the active site. Modeling sorbitol into the active site in a conformation equivalent to that of mannitol revealed no major steric clashes. The C2 hydroxyl of sorbitol, which is equivalent to O5 of mannitol, could no longer hydrogen bond to Asn-300 but would be located between His-303 and Arg-373 in a suboptimal orientation for hydrogen bonding. In the binary complex of pfMDH with NAD$^+$, a water molecule is found at a position equivalent to O5 of mannitol and makes hydrogen bonds to His-303 and Asn-300. Presumably, sorbitol would displace this water because C2 would be too close. This would leave the hydrogen bonds to His-303 and Asn-300 unfilled, a thermodynamically unfavorable situation. In fact, a loss of binding energy of $-3.5 \text{ kcal/mol}$ for sorbitol relative to mannitol is observed (13) and may be attributed to the loss of one or two hydrogen bonds. More importantly, the perturbation of the precise hydrogen-bonding network surrounding the Asn-300 side chain could disrupt catalysis (see below).

**Catalytic Mechanism**—In converting mannitol to fructose, the hydroxyl at C2 is oxidized. This is done by the sequential abstraction of a proton from the sugar O2, the transfer of this proton to bulk solvent followed by the transfer of a hydride from the sugar C2 to the nicotinamide C4. The three hydrogen bond O2 makes to the protein identify residues involved in the catalytic mechanism. Lys-295 is positioned to act as the proton acceptor. The observed inflection point in catalytic efficiency at pH 9.3, therefore, is probably the result of the titration of this side chain (13). Hydrogens on the amide nitrogens of Asn-191 and Asn-300 would stabilize the partial negative charge on the
O2 in the transition state (Fig. 5). In functioning as hydrogen bond donors to the two lone pairs of the substrate oxygen, the asparagines also direct the O2 proton toward Lys-295. Carbonyles of Val-229 and Asn-300 accept hydrogen bonds from Lys-295 and direct the lone pair of electrons on the lysine to accept a hydrogen bond from the substrate O2. The side-chain conformation of Asn-300 is therefore critical for catalysis because it functions in orienting both enzyme and substrate groups. The Asn-300 side chain is additionally oriented by a hydrogen-bonding interaction with the mannitol O5. Polyols must have five or six carbons to be substrates of pfMDH (13). The fact that no activity has been detected with four-carbon polyols is presumably because this interaction with the substrate O5 is necessary. A lack of measurable activity with 5,6-dideoxy-D-fructose3 supports this hypothesis.

The proposed mechanism for mannitol oxidation requires Lys-295 to be unprotonated. Substrate inhibition patterns suggest that isomerization of the pfMDH-NAD+ complex is required before it can productively bind mannitol (13). Based upon the hydrogen-bonding network surrounding Lys-295, it would seem probable that dissociation of the proton from this lysine producing the active form of the enzyme is this necessary step. Because the mannitol O2 accepts two hydrogen bonds from protein side chains, its proton will be directed toward the Nε of Lys-295. If the lysine is protonated, a productive complex will not form easily because there would be unfavorable contacts between the O2 proton and the Lys-295 proton.

In the binary complex, there is a well ordered water molecule 3.15 Å from Nε of Lys-295 on the opposite side from the active site. This water is held in place by interactions with the main-
chain oxygen of Val-229 and the side chain of Glu-292. The side chain of Glu-292 is buried in a pocket lined with hydrophobic side chains from residues such as Met-228 and Ile-296, probably elevating its $pK_a$. The same glutamate in the ternary complex is found with the carboxylate oxygen rotated 6.5 Å out of the pocket and making contact with the bulk solvent where it

Fig. 4. A, surface rendering of pfMDH with α-carbons portrayed as a blue ribbon and NAD$^+$ (red) and α-mannitol (green) represented by Van der Waals radii. The view of image on the left is down into the active site, and the image on the right is rotated 90° about the y axis. GRASP was used to produce this figure (12). B, representative electron density for α-mannitol taken from ternary molecule A. A $F_o - F_c$ map calculated without the substrate is contoured at 4σ with the refined final mannitol molecule superimposed. C, schematic of mannitol bound to molecule A with hydrogen bonds drawn as dotted lines.
FIG. 5. A, overlay of the binary and ternary complexes of pfMDH showing substrates NAD and mannitol with Lys-295 and mechanistically important interactions. The binary complex is gray, and in the ternary complex, residues from the N-terminal domain are colored red, those from the C-terminal domain are colored green, and waters are colored gold. Selected hydrogen bonds are drawn for each complex, and an arrow shows the rotation of the side chain of Glu-292 toward bulk solvent (see “Results and Discussion”). B, a proposed high pH reaction mechanism based upon the structure of the enzyme.
presumably has a lower pKₐ (Fig. 5). Strand F (containing residues 222–230) is shifted 0.56 Å toward the active site in the ternary complex, presumably because of interactions between substrate O1 and residues 230 and 231. The water molecule is shifted away from the lysine to a distance of 3.76 Å. A solvent-lined channel is also observed in the ternary complex. These conformational differences and the well ordered water molecules observed in the binary and ternary complexes suggest two possible mechanisms by which the proton could be shuttled to bulk solvent.

In the first mechanism, Glu-292 could function as the proton shuttle. In its solvent-accessible conformation, it is located within 4.6 Å of Glu-293 and has a water-mediated interaction with this residue. Repelled by this negative charge, Glu-292 swings down toward the active site. After returning to the hydrophobic pocket, it neutralizes the charge by abstracting a proton from the water molecule. This water then abstracts a proton from Lys-295. The binding of the mannitol substrate brings strand F (residues 222–230) closer to the active site. This includes the carbonyl oxygen of Val-229, which is now within 2.34 Å of the water. The water moves back to a new position 1.15 Å away, hydrogen bonded to the main-chain nitrogen of Val-229, and is now 3.76 Å from Lys-295. The new water position is 1.46 Å from Glu-292, and Glu-292 swings out and loses the proton to the bulk solvent. Lys-295 abstracts a proton from the mannitol, which is then kinetically observed to be transferred to bulk solvent before hydride transfer occurs (27). The hydride is transferred, and products are released. Strand F readjusts to the position observed in the binary complex once NAD⁺ is bound, the water moves back in close to Lys-295, Glu-292 swings down, and the cycle is repeated.

The second possible mechanism utilizes Glu-292 in a slightly different manner. In the binary complex, Glu-292 polarizes the water molecule, which abstracts a proton from Lys-295. The binding of polyol substrate brings residues 222–230 closer, causing the protonated water to move away from Lys-295. The new water position is 1.46 Å of Glu-292, and Glu-292 is ejected from the pocket. Glu-292 moves to its solvent-accessible conformation, opening a channel by which the proton is shuttled to bulk solvent along a chain of three water molecules. Lys-295 picks up a proton from substrate and shuttles it again to the solvent. Hydride transfer occurs, products are released, and the strand containing residues 222–230 moves back out. This allows the water to again move close to Lys-295 and Glu-292 to swing back in. In this mechanism, Glu-292 acts as a gate opening and closing the solvent channel. Although further studies are necessary to elucidate the exact mechanism of proton transfer to bulk solvent, a direct loss of the proton from the lysine to the solvent is very unlikely because of the sequenced nature of the active site and the kinetically observed deprotonation of the lysine prior to hydride transfer (27).

These proposed mechanisms cannot apply to the whole family, because Glu-292 is conserved only among a subgroup of the long-chain MDHs. This subgroup of 15 members includes mannitol 2-dehydrogenases, D-arabinitol 4-dehydrogenases, and mannionate dehydrogenases. The altronate dehydrogenase sequences have a lysine or arginine at this position, whereas the mannitol-1-phosphate 5-dehydrogenases have an isoleucine or valine. These other groups necessarily would use a different mechanism to return the lysine to the unprotonated state.

**Structural Neighbors—**A search for structural neighbors was conducted using the program Dali (28). As expected, many proteins showed similarity to the NAD⁺-binding domain. Absent was the SDR family member tetrameric NAD⁺-dependent mannitol 2-dehydrogenase from *Agaricus bisporus* (3). A number were identified that also had similarity in the C-terminal domain. The top three, 6-phosphogluconate dehydrogenase (29) (6PGDH), UDP-glucose dehydrogenase (30) (UDPGDH), and N-(1-d-carboxylethyl)-L-norvaline dehydrogenase (31) (CENDH), were chosen for closer inspection. Overall root mean square deviations were 3.8–4.2 Å with 7–10% sequence identity. All of them catalyze more complex reactions: NAD⁺-dependent oxidative decarboxylation in the case of 6PGDH, 2-fold NAD⁺-dependent oxidation for UDPGDH, and NADH-dependent reductive condensation between an α-keto acid and an amino acid for CENDH. When the N- and C-terminal domains were searched separately, a similarity in both was also identified with glycerol-3-phosphate dehydrogenase (32) (G3PDH). Because the C-terminal domain of G3PDH was rotated ~50° compared with pMDH, this relationship was initially missed. The domains in G3PDH are proposed to come closer upon substrate binding, explaining the difference in domain arrangement when compared with pMDH. G3PDH catalyzes the NADH-dependent interconversion of dihydroxyacetone phosphate and 1-glycerol 3-phosphate, a reaction similar to that catalyzed by pMDH. A superposition of pMDH with these structural neighbors is shown in Fig. 6.

Each of these enzymes has an eight-strand β-sheet in the N-terminal domain that consists of the canonical six parallel strands with an additional two antiparallel strands. UDPGDH and 6PGDH also have a conserved Lys-X₅-Asn on the central α-helix homologous to Lys-295 and Asn-300 in pMDH. The role of the lysine as the general base in these reactions has been proposed. Although CENDH and G3PDH do not contain a comparable lysine, they do share a homologous asparagine. The conservation of secondary structural elements as well as residues at equivalent positions that are contributed to the active
sites implies that these enzymes are related by divergent evolution.

Conclusion—The structure presented here is the first crystal structure of an enzyme from the long-chain mannitol dehydrogenase family. It reveals that pfMDH is a monomer composed of two domains with the catalytic site 20 Å deep in a cleft between the two domains. The N-terminal nucleotide-binding domain includes a Rossmann fold that is extended by two antiparallel β-strands. Helix α1 has the HXGXGXXR fingerprint motif located at its N terminus. The complexes with NAD⁺ and mannitol allow the identification of residues involved in substrate and cofactor binding. Conservation of many of these residues suggests that other long-chain MDH family members recognize their substrates similarly. Unlike other alcohol and polyol dehydrogenases that contain a catalytic tyrosine or metal ion, Lys-295 is poised to act as the general base in the reaction. Asn-191 and Asn-300 are positioned to direct the O2 proton toward Lys-295 and stabilize a negative charge on the substrate oxygen in the transition state. Asn-300 is involved in a precise hydrogen-bonding network with Lys-295 and substrate O2 and O5 that appears to be essential for efficient catalysis. An unusual proton tunnel/shuttle utilizing the mobile Glu-292 side chain could be involved in returning the lysine to its uncharged state in preparation for the next catalytic cycle. Even though pMDH is a monomer, the closest structural neighbors are dimeric with the C-terminal domain involved in dimerization as well as substrate binding. Conserved secondary structural elements and conservation of residues contributed to the active site suggest a common ancestor for this heterogeneous group.

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