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SUBSTRATE STEREOSPECIFICITY OF THE NAD-DEPENDENT MANNITOL DEHYDROGENASE FROM CELERY

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Key Word Index—*Apium graveolens*; Umbelliferae; celery; D-mannitol dehydrogenase; 1-oxidoreductase; substrate specificity; polyol; aldose.

Abstract—The NAD-dependent mannitol dehydrogenase (MTD) of celery catalyses the interconversion of D-mannitol and D-mannose. This 1-oxidoreductase is uniquely different from all NAD-dependent polyol dehydrogenases described to date, which are 2-oxidoreductases. The stereospecificity of mannitol dehydrogenase was tested in the oxidative direction in the presence of polyol and NAD cofactor and in the reductive direction in the presence of aldose and NADH. The enzyme would be expected to show the same stereospecificity in either direction. The stereospecificity in the reductive direction was tested by attempted reduction of all eight D- and L-pentoses and 15 of the 16 D- and L-hexoses. Stereospecificity in the oxidative direction was tested with the four pentitols and four of the hexitols. Mannitol dehydrogenase showed a marked preference for aldopentose and aldohexose substrates with the same absolute configuration at C-2 as that of D-mannose. Reduction of L-idose by mannitol dehydrogenase was the only exception to the stated stereochemical preference among 23 aldoses and eight alditols tested. The sugar D-threose that occurs rarely in nature is a competitive inhibitor (K_i = 18 mM) of mannitol oxidation. The physiologically important hexitols, galactitol and glucitol, are oxidized by MTD to aldoses that are not metabolized by higher plants. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Acyclic polyols (alditols) are widely distributed in nature and occur in bacteria, algae, fungi and higher plants [1, 2]. These polyols have important functions relating to growth, carbon storage, recycling of reductant and efficient carbon fixation [3]. Mannitol, a six-carbon non-cyclic sugar alcohol, also plays an important role in stress tolerance of fungi, lichens and higher plants because of its function as a compatible solute and free radical scavenger [3, 4]. Most of the polyol oxidizing enzymes described to date are pyridine nucleotide linked, requiring either NAD or NADP as a co-substrate. Some exceptions include the glucitol oxidase in apple [5] and the mannitol oxidase in the land snail [6] which use oxygen as the electron acceptor. Until recently all NAD(P) dependent polyol dehydrogenases reported were 2-oxidoreductases catalysing the conversion of an acyclic polyol to its corresponding ketose sugar [1, 2] including a report of an uncharacterized mannitol dehydrogenase in the parasitic vascular plant *Orabanche ramosa* [7]. Recently, a novel NAD-dependent mannitol dehydrogenase (MTD) that oxidizes D-mannitol to D-mannose was isolated from sink tissues of celery (*Apium graveolens* L. var. *dulce* [Mill.] Pers.) and celeriac (*Apium graveolens* L. var. *rapaceum*) [8]. This dehydrogenase is uniquely different from all previously reported alditol reductases in that it is a 1-oxidoreductase. MTD was purified to homogeneity from celery suspension cultures [9]. MTD provides the first step by which mannitol is committed to central metabolism and plays a critical role in regulating the mannitol concentration in the plant. In celery, mannitol is a major phloem translocated photoassimilate which is utilized specifically in sink tissues [8].

Although many NAD(P)-dependent polyol dehydrogenases have been isolated and characterized, there have been few studies of substrate specificity, including both oxidation of polyols and reduction of the corresponding sugars. The NAD-dependent ribitol dehydrogenase (EC1.1.1.56) from *Enterobacter agglomerans* [10], the L-glucitol dehydrogenase from *Pseudomonas* [11] and the NAD-dependent D-arabinitol dehydrogenase from *Pichia stipitis* [12] can use various polyols as substrates. The detailed stereochemical requirements for polyol dehydrogenases have been determined in a

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few cases. Bertrand [13] described the stereospecific oxidation of polyols by an NAD-dependent mannitol dehydrogenase (NAD-MtDH) from *Acetobacter xylinum*, and subsequent observations led to the confirmation of Bertrand's rule as expanded by Richtmyer et al. [14]. The Bertrand–Hudson rule in modern terminology predicts that the 2*R*-hydroxyl of an alditol having *erythro* relative stereochemistry at C-2 and C-3 will be oxidized to a ketone by *A. xylinum* and *A. suboxydans* (Fig. 1A, NAD-MtDH). For example, glucitol is oxidized at C-5 (C-2 sorbitol numbering) to L-sorbose. Richtmyer *et al.* [14] showed that the unpredicted oxidation of L-fucitol to 1-deoxy-D-xylo-3-hexulose conformed to the Bertrand rule with the recognition that the enzyme accepts the methyl group of the CH_3CHOH terminus of fucitol as a near steric equivalent of one of the hydrogen atoms of the more usual CH_2OH terminus.

The NAD-dependent D-glucitol (sorbitol) dehydrogenase of rat liver (NAD-SDH, E.C. 1.1.1.15) does not follow the Bertrand–Hudson rule [15]. This enzyme, also referred to as L-iditol:NAD 2-oxidoreductase or ketose reductase, catalyses oxidation of the 2*S*-hydroxyl of alditols having an *erythro* relationship between the chiral centres at C-2 and C-4 (Fig. 1B, NAD-SDH). Chirality at intervening C-3 and more distal chiral centres is not critical to substrate recognition by this enzyme.

An NAD-dependent galactitol dehydrogenase was partially purified from a *Pseudomonas* sp. by Shaw [16] and shown to oxidize the 2*S* hydroxyl of pentitols and hexitols having *threo* relative stereochemistry at C-2 and C-3 (Fig. 1C, NAD-GaDH). Evidence was also presented for the presence of a separate -iditol dehydrogenase specific for 2*R-threo* configuration at C-2 and C-3.

A recently isolated L-glucitol dehydrogenase from *Pseudomonas* sp. had very high specificity for oxidation of L-glucitol to sorbose, but oxidized other 2*S*-pentitols and hexitols less rapidly [11]. The enzyme oxidized 2*S-threo*-alditols more slowly than 2*S-erythro*-alditols and had no activity on tetritols. A ribitol dehydrogenase isolated from *E. agglomerans* oxidizes all 2*S*-alditols to 2-ketoses regardless of the relative configuration at C-3 [10].

The MTD from celery is the first NAD-dependent enzyme known to oxidize alditols at C-1 to aldoses. In the present study we report the substrate specificity of this NAD-dependent 1-oxidoreductase.

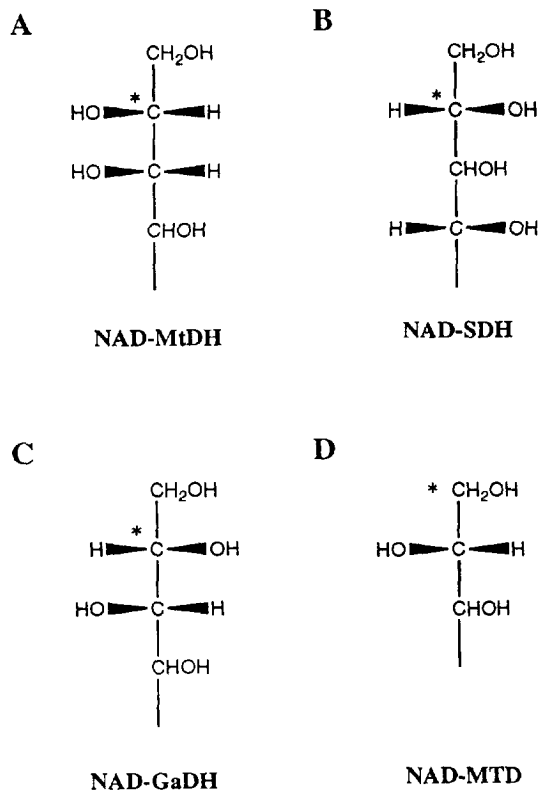

Fig. 1. Substrate stereospecificity of NAD-dependent polyol dehydrogenases: (A) NAD-mannitol dehydrogenase (NAD-MtDH) from *Acetobacter suboxydans*; (B) NAD-glucitol (sorbitol) dehydrogenase (NAD-SDH) from rat liver; (C) NAD-galactitol dehydrogenase (NAD-GaDH) from a *Pseudomonas* sp.; and (D) NAD-mannitol dehydrogenase (NAD-MTD) from *Apium graveolens*. Site of oxidation is indicated by (*). See text for details.

RESULTS

Substrate specificity

In order to establish a complete picture of the substrate specificity of MTD, all commercially available aldose sugars that can be reduced to the respective hexitols, pentitols and tetritols were tested as substrates for MTD. Both D- and L-pairs of polyols and aldose sugars were assayed when commercially available. Initial rates of hexitol- and pentitol oxidation and aldose reduction are shown in Fig. 2. Highest activity was observed in the polyol oxidation reaction with D-mannitol as the substrate (100% is 22 nkat mg^{-1} MTD) followed by the pentitols D-arabinitol (37%), *i*-ribitol (36%) and the hexitols *i*-galactitol (26%), D-glucitol (22%). No activity was observed with L-arabinitol, and less than 10% activity was observed with *i*-xylitol and L-iditol. The other hexitols D-iditol, D-altritol (D-talitol), *i*-allitol, L-glucitol, L-altritol and L-mannitol are not commercially available and were not tested. The tetritols *i*-erythritol and D-threitol did show some activity, 16 and 25%, respectively, relative to mannitol (100%).

However, because it was unknown how much D-threitol was present in the impure D-erythritol preparation (and vice versa) these data were not considered for determinations of stereospecificity of MTD. In Fig. 2, the aldose sugars are positioned on the basis of the configuration of the secondary alcohol in close proximity to the carbonyl of the carbon that will be reduced by MTD. Interestingly, one can immediately observe that

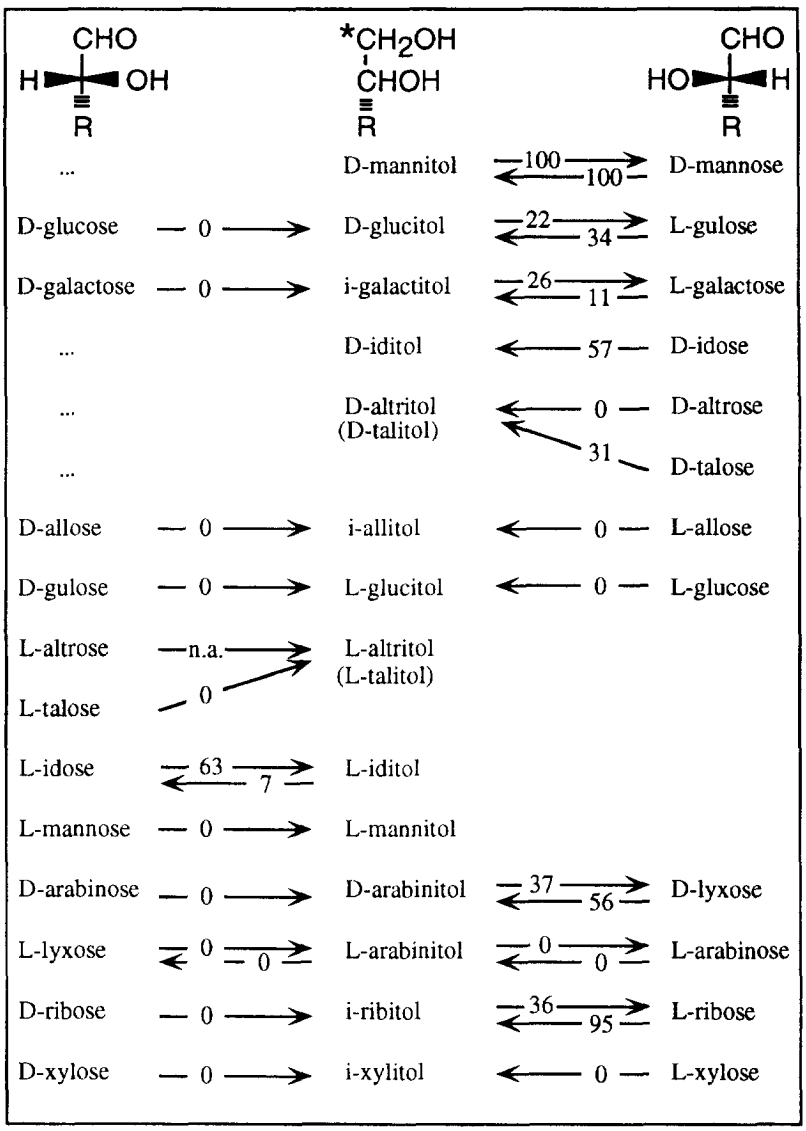

Fig. 2. Initial oxidation rates of polyols and reduction rates of aldose sugars catalysed by the NAD-dependent mannitol dehydrogenase (MTD) from celery. Rates are relative to D-mannitol (oxidation) and D-mannose (reduction). R = $(CHOH)_n$-CH_2OH, where $n = 2$ or 3; n.a. = not available.

aldoses which are chemically reducible to alditols having 2*S*-chirality are not reduced by MTD with the exception of D-idose. Seven of 11 aldoses which are chemically reducible to alditols having 2*R*-chirality are reduced by MTD. The highest activity in the aldose reduction reaction was obtained with D-mannose as the substrate (100% is 13 nkat mg^{-1} MTD), followed by L-ribose (95%), D-idose (57%), D-lyxose (56%), L-gulose (34%), D-talose (31%) and L-galactose (11%). Because the purity of the commercially available tetrose sugars D-threose and D-erythrose ranged between 50 and 75% they were not included in the evaluation of the stereochemical recognition by MTD.

Other substrates such as 6-deoxy-D-galactose (D-fucose), 6-deoxy-L-galactose (L-fucose), 6-deoxy-L-mannose (L-rhamnose) and D-mannose-6-P were not reduced by MTD (data not shown). The ketose sugars such as D-sorbose, D-tagatose, L-sorbose, and D-fructose were not reduced by MTD (data not shown) as expected for a 1-oxidoreductase.

Verification of identities of D- *and* L-*idose*

Because of the unusual observation that both enantiomers of idose were reduced by MTD, the D- and L-idoses were examined more closely to verify their identities. The samples of D- and L-idose co-migrated with each other and were resolved from all other hexoses and pentoses except glucose upon electrophoresis in borate buffer. In sodium arsenite buffer D- and L-idose also co-migrated and were well separated from glucose. Specific rotations for the D-idose sample

were: $[\alpha]^{22}$ +19.7 (589 nm), +23.5 (546 nm) and +38.7 (435 nm). Specific rotations for L-idose were: $[\alpha]^{22}$ −15.6 (589 nm), −18.0 (546 nm) and −22.5 (435 nm). The reported specific rotation of syrupy D-idose is $[\alpha]$ +15.8 (589 nm) and for syrupy L-idose, $[\alpha]$ −17.4 (589 nm) [17].

D-*Threose as a competitive inhibitor of mannitol oxidation*

During the survey of aldose substrates for MTD, we observed that D-threose, which occurs infrequently in nature [2], had a 17-fold higher activity than D-mannose in the reduction direction. D-Erythrose showed only 50% of the activity of D-mannose. Because the D-erythrose preparation contained an unknown amount of D-threose, it is likely that the rate observed with D-erythrose was due to the contamination with D-threose. The high rate observed with D-threose led us to examine its possible effect on mannitol oxidation. Mannitol oxidation was measured at pH 7.5 and not at pH 9.5, because the D-threose preparation exhibited increasing absorbance at 340 nm in the absence of MTD or NADH at pH 9.5. The source of this absorption is unknown, but it did not occur at pH 7.5. D-threose acts as a competitive inhibitor for mannitol oxidation with a K_i of 18 mM (Fig. 3). D-Mannose also acted as a competitive inhibitor, but the K_i (200 mM) was much higher than that of D-threose.

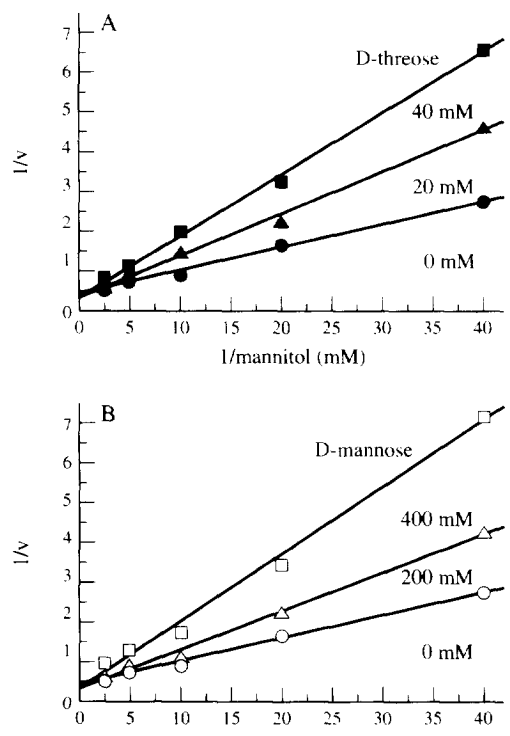

Fig. 3. Inhibition of mannitol dehydrogenase (MTD) by D-threose or D-mannose. Lineweaver–Burk plots in the presence of (A) 0 mM (●), 20 mM (▲) or 40 mM (■) D-threose or (B) 0 mM (○), 200 mM (△) or 400 mM (□) D-mannose. Inhibition by both compounds is competitive with respect to mannitol. K_i for D-threose is 18 mM, and K_i for D-mannose is 200 mM.

DISCUSSION

The identification of a novel NAD-dependent MTD that catalyses the conversion of D-mannitol to the aldose D-mannose [8] and the subsequent purification of MTD to homogeneity [9] allowed us to conduct a thorough analysis of substrate stereospecificty of MTD. While all previously reported pyridine nucleotide-dependent alditol dehydrogenases interconvert alditols and 2-ketoses [10–13, 15, 17], MTD is unique in interconverting alditols and aldoses. MTD shows many similarities with other alditol dehydrogenases such as optimal alditol oxidation rates at high pH, low affinity towards its alditol substrates and reaction velocity highly affected by NADH/NAD ratios [8].

Substrate specificity of MTD in the reductive direction was assessed using 15 of the 16 aldohexoses and all eight of the aldopentoses. Study of substrate specificity of the oxidation of alditols by 2-dehydrogenases in earlier work required product isolation and identification, because two different ketoses are potentially generatable from an alditol. Study of 2-alditol dehydrogenases in the reductive direction still requires product identification because a new chiral centre is created, and one of two alditols are potential products in each reduction. However, our study of the action of MTD on aldoses in the reductive direction leads to only one possible alditol, removing the need for product identification in every case. The enzyme substrate stereospecificity was also probed in the oxidative direction with all commercially available alditols (4/10 hexitols and 4/4 pentitols). All substrate activities detected in the oxidative direction were consistent with those determined in the reductive direction. The substrate stereospecificity analysis for MTD (Fig. 2) indicates that a minimal requirement for an alditol to serve as a substrate for MTD is the presence of 2*R* chirality at the carbon adjacent to the primary carbon undergoing the oxidation (Fig. 1D).

Chirality at centres remote from C-2 plays no very significant role in determining the suitability of alditols as substrates for MTD. The physiological product of the enzyme in celery, D-mannose, has the highest reduction rate, followed very closely by L-ribose. D-Mannitol has 2*R-erythro* stereochemistry at C-2 and C-3, and *i*-ribitol has the same stereochemistry at one end. Several other aldoses giving 2*R-erythro*-alditols are reduced at appreciable rates (D-lyxose, L-gulose and D-talose), but so are two aldoses (L-idose and D-galactose) yielding 2*R*-threo-alditols. Therefore, chirality at C-3 plays no obvious modifying role in determining substrate suitability. The requirement of 2*R* chirality in the alditol is, however, only a minimal requirement. The following aldoses did not serve as substrates for mannitol dehydrogenase even though they have the appropriate chirality at C-2: D-altrose, L-allose, L-glu-

cose and L-xylose. There is no obvious pattern of chirality at more remote centres, which would explain why seven aldoses yielding 2*R*-alditols are substrates for the enzyme while four others are not.

The enzyme MTD fails to reduce the commercially available 6-deoxyhexoses D-fucose (6-deoxy-D-galactose), L-fucose or L-rhamnose (6-deoxy-L-mannose). This is perhaps to be expected since the enzyme reduces L-galactose only slowly and does not reduce D-galactose or L-mannose at all. If the 6-deoxyaldoses are viewed as pentoses with a methyl group replacing one of the hydrogens at C-5, as in the Hudson–Richtmeyer modification of the Bertrand rule [14], then D-fucose can be viewed as a modified L-arabinose, L-fucose as a modified D-arabinose, and L-rhamnose as a modified L-lyxose. However, none of these three pentoses are reduced by MTD. Hence, it remains to be determined whether a terminal carbinol is required by MTD or whether a methyl group could replace a hydrogen atom on the terminal carbinol.

The C-2 chirality of the seven aldose substrates of MTD all yield 2*R*-alditols on reduction. Ten other aldoses which are chemically reducible to 2*S*-alditols did not serve as substrates. However, L-idose was reduced to L-iditol (2*S*) at 63% of the initial rate of D-mannose, while its enantiomer D-idose was reduced to D-iditol (2*R*) at 57% of the rate of D-mannose. In view of the unexpected finding that both idose enantiomers were reduced by MTD at comparable rates, we felt it desirable to confirm the identity of these two substrates. The relative stereochemical identity of the two idoses was confirmed by borate electrophoresis capable of resolving all eight aldoses, and the enantiomeric relationship between them was confirmed by their specific optical rotations of opposite signs in agreement with published rotation data. Shaw [16] observed anomalous oxidation of a few alditols which did not fit a much larger pattern of consistent substrate stereospecificity requirements for a bacterial galactitol dehydrogenase. The activity responsible for oxidation of the non-conforming alditols disappeared with time and could be reduced by fractional ammonium sulphate precipitation, leading to the conclusion that the anomalous oxidations were due to the presence of a second less-stable alditol dehydrogenase with different stereospecificity. In our case the MTD has been purified to a high state of purity, and it is doubtful that the anomalous reduction of L-idose could be due to the presence of a second alditol dehydrogenase. The MTD used here was purified to apparent homogeneity based upon mobility as a single band on native and SDS-PAGE, a signal consistent with a single 40 kD protein when subjected to matrix-assisted laser desorption ionization mass spectrometry, and a tryptic peptide digest that yielded three independent fragments (38 total amino acids) giving an amino acid sequence that matched the predicted sequence from a cDNA encoding MTD [9, 18].

The 2*R*-tetritol, D-threitol, is also a substrate for mannitol dehydrogenase as expected. The initial oxidation rate is only 25% that of D-mannitol. However, the reduction rate of D-threose by mannitol dehydrogenase is 17 times that of D-mannose, the physiological product of this enzyme. The seemingly anomalously high activity of the enzyme acting on D-threose relative to D-mannose, but only low activity in the reverse direction, is largely due to the inability of a C-4 aldose to form a pyranose, although threose does form a mixture of 90% furanose and 10% open chain form in water [19]. At physiological pH, all of the other pentoses and hexoses studied are present in water as pyranoses (with a small percentage of furanose, dependent on the stereochemistry of the sugar). The equilibrium between pyranose and open-chain aldose lies very much in favour of the pyranose (>99.9%, >2 kcal mol^{-1} more stable), but the actual substrate for the enzyme is necessarily the free carbonyl, open-chain aldose. The necessity for opening the chain normally adds *ca.* 2 kcal mol^{-1} to the free energy of activation in the reductive direction. However, this step is not necessary for D-threose, lowering the free energy of activation with corresponding acceleration of the reduction rate. Actually, a difference in free energy of activation of 1.67 kcal mol^{-1} is adequate to account for the observed 17-fold greater rate of reduction of D-threose than D-mannose. Since cyclization of the aldose product occurs *after* the enzymic step in the reverse, oxidative direction, cyclization of the product does not alter the free energy of activation or the rate of the enzymatic reaction in the oxidative direction, and the difference in rate of oxidation of D-threitol and D-mannitol is much less than the difference in rate of reduction of D-threose and D-mannose. Commercial D-erythrose, known to contain some epimerized D-threose, does consume NAD at 50% of the initial rate of D-mannitol in the presence of MTD, but much of this activity is likely due to the very reactive threose impurity. The possible utility of D-threose as an inhibitor of mannitol oxidation may be important in the light of recent evidence that mannitol is involved in the tolerance of plants to salt stress [3, 20].

Competitive inhibition of MTD by D-threose, if introduced into these plants, might result in a higher steady state level of mannitol in the plant. Such inhibition might serve as an alternative to silencing or antisense technologies in trying to down regulate the MTD activity in vascular plants in order to enhance mannitol accumulation.

Finally, it is interesting to observe that MTD can oxidize D-glucitol (sorbitol) and *i*-galactitol at appreciable rates relative to D-mannitol. These are the three most abundant acyclic hexitols in vascular plants, although they seldom, if ever, occur within the same species. For instance, celery contains D-mannitol, but not other acyclic hexitols, and many members of the Rosaceae contain D-sorbitol, but not D-galactitol or D-mannitol [2]. The oxidation of D-glucitol and *i*-galactitol by MTD yields L-gulose and L-galactose, respectively, and neither of these aldoses normally occur in vascular plants. The production of uncatabolizable L-

gulose as a product of the oxidation of D-glucitol by MTD is consistent with the observation that celery suspension cells do not grow significantly on this hexitol despite the fact that they contain MTD that will oxidize the hexitol [3].

EXPERIMENTAL

Plant material and chemicals. Celery cell cultures were grown on D-mannitol as sole carbon source as described in ref. [21]. MTD was purified to homogeneity from celery suspension cultures as described in ref. [9]. Only this highly purified MTD was utilized in this study. Purified MTD was stable for several months when stored at −80°. All chemicals at highest purity were obtained from Sigma, except D-erythritol and D-talose, which were obtained from Aldrich. Commercially available hexitols and pentitols ranged in purity from 97 to 100%. Purity of tetritols and triols were significantly lower. The hexitols *i*-allitol, D-altritol, D-iditol, L-altritol, L-mannitol and L-glucitol are not commercially available and thus were not included in this study.

MTD activity assay. MTD activity was assayed in both polyol oxidation and aldose reduction directions. The polyol oxidation assay mixt. contained 100 mM Bis–Tris propane (pH 9.5), 4 mM NAD, 150 mM polyol and was initiated with 1.6 μg purified MTD. The sugar reduction assay mixt. contained 100 mM MES (pH 6.5), 0.1 mM NADH and 200 mM sugar and was initiated with 1.6 μg purified MTD. Reduction (oxidation) of NAD (NADH) was observed spectrophotometrically at 340 nm. Enzyme activity is expressed in μmol sec^{-1} (katals).

Inhibition studies. Kinetic constants for inhibition of mannitol oxidation by D-mannose and D-idose were determined at room temp. in 100 mM MOPS (pH 7.5), with varying mannitol and inhibitor concs. All reactions were initiated with 1.6 μg MTD in a total of 1 ml and the rate of NAD oxidation was observed spectrophotometrically at 340 nm.

Verification of identities of commercial D- *and* L-idose. Differentiation of idose from the other 7 hexoses and the 4 pentoses was performed by high voltage electrophoresis (40 V cm^{-1}) in a 1% borax buffer (pH 9.0) and 0.1% Na arsenite buffer (pH 9.6) [22]. Reducing sugars were detected with aniline phosphate reagent. Optical rotations were measured in a Rudolph Autopol III spectropolarimeter at 22° in a 1 dm cell at a conc. of 10 mg ml^{-1}. Measurements were made at 589, 546 and 435 nm.

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