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Substrate and Phospholipid Specificity of the Purified Mannitol Permease of Escherichia coli

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D-Mannitol is transported and phosphorylated by a specific enzyme II of the phosphotransferase system of Escherichia coli. This protein was purified previously in detergent solution and has been partially characterized. As one approach in understanding the structure and mechanism of this enzyme/permease, we have tested a number of sugar alcohols and their derivatives as substrates and/or inhibitors of this protein. Our results show that the mannitol permease is highly, but not absolutely, specific for D-mannitol. Compounds accepted by the enzyme include those with substitutions in the C-2 (=C-5) position of the carbon backbone of the natural substrate as well as D-mannonic acid, one heptitol and one pentitol. All of these compounds were both inhibitors and substrates for the mannitol permease except for D-mannoheptitol, which was an inhibitor but was not phosphorylated by the enzyme. No compound examined, however, exhibited an affinity for the enzyme as high as that for its natural substrate. We have also investigated the phospholipid requirements of the mannitol permease using phospholipids purified from E coli. The purified protein was significantly activated by phosphatidylethanolamine, but little activation was observed with phosphatidylglycerol or cardiolipin. These observations partially delineate requirements for interaction of sugar alcohols and phospholipids with the mannitol permease. They suggest approaches for the design of specific active site probes for the protein, and strategies for stabilizing the enzyme's activity in vitro.

Key words: phosphotransferase system, sugar transport, mannitol permease, substrate specificity, transport inhibitors, phospholipid requirements, integral membrane proteins

We have been studying the structure and mechanism of the integral membrane permease responsible for the concomitant transport and phosphorylation of the hexitol D-mannitol in Escherichia coli [for a recent review, cf 1]. This protein, the D-mannitol-specific enzyme II (hereafter called the mannitol permease) of the bacterial

Abbreviations used: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PEP, phosphoenolpyruvate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

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phosphotransferase system (PTS), has been purified in detergent solution and partially characterized [2,3], and has been functionally reconstituted into proteoliposomes [4]. Its primary amino acid sequence has been deduced recently from the base sequence of the *mtlA* gene [5]. Recent studies also have suggested that the mannitol permease (M_r = 60,000) spans the phospholipid bilayer of the E coli inner membrane asymmetrically, with a significant proportion of its mass exposed to the cytoplasmic compartment [5,6].

The phosphotransfer reactions catalyzed by the mannitol PTS are as follows:

$$\text{phosphoenolpyruvate} + \text{HPr} \underset{\text{(cytoplasm)}}{\overset{\text{enzyme I}}{\longleftrightarrow}} \text{phospho-HPr} + \text{pyruvate} \quad (1)$$

$$\text{phospho-HPr} + \text{D-mannitol}_{(\text{out})} \xrightarrow[\text{(membrane)}]{\text{mannitol permease}} \text{HPr} + \text{mannitol-1-P}_{(\text{in})} \quad (2)$$

Enzyme I and HPr are general phosphotransfer proteins for all sugars transported by the PTS in E coli, and each is covalently phosphorylated on a histidine residue as an intermediate step in this series of reactions [7]. Sugar-specific membrane permeases (enzymes II) are then responsible for concomitant transport and phosphorylation of their respective substrates. At least seven such permeases have been identified in E coli, showing specificities for hexoses and hexitols, all of the D-configuration [8]. Furthermore, there are several independent lines of evidence that suggest that at least some of these PTS permeases are themselves covalently phosphorylated as obligatory intermediates in the transport/phosphorylation reaction [9–14].

Elucidation of the mechanism of transport carried out by the PTS will rely on a number of kinetic, physicochemical, and genetic approaches. The best-characterized PTS permease, that specific for D-mannitol, appears for several reasons to be the subject of choice for these investigations [1]. In this report, we present the results of experiments designed to delineate the requirements for interaction of sugar alcohols and phospholipids with this protein. They also suggest several logical approaches for the design and use of probes specific for the active site of the mannitol permease.

METHODS

Hexoses, hexitols, pentitols, D-mannosamine, D-mannoheptitol (perseitol), 1,6-dibromo-1,6-dideoxy-D-mannitol, 2-deoxy-D-glucose, D,L-threitol, L-threitol, and erythritol all were purchased from Sigma Chemical Co. D-Mannono-1,4-lactone was from P-L Biochemicals, and was converted to the acid by titrating a 0.5 M solution to pH 9.1 with 5 M NaOH, followed by acidification to pH 8.0 with 1 M HCl [15]. D-Mannitol, D-glucitol, D-mannose, D-mannosamine, 2-deoxy-D-glucose, D-galactose, D-ribose, and glycerol, all labeled with carbon 14, were products of New England Nuclear. D-Arabinose and D-xylitol, also so-labeled, were products of ICN and Amersham, respectively. Some sugar alcohols were prepared by $NaBH_4$ reduction of their corresponding aldoses. Unlabeled sugar alcohols were synthesized using a sugar concentration of 0.1 M as described [3]. Labeled sugar alcohols were prepared by the same procedure except that 1 mM solutions of the corresponding labeled sugar (5 mCi/mmol) were used and the $NaBH_4$ concentration was lowered accordingly. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL), all

purified from E coli B, were obtained from Supelco, Inc. The mannitol permease was purified from E coli in the nonionic detergent Lubrol PX as described [2]. Unless otherwise stated, phosphoenolpyruvate (PEP)-dependent phosphorylation of labeled compounds was tested at 37°C at final substrate concentrations of 0.1 mM in the presence of a rate-limiting amount of purified mannitol permease, an excess of a soluble fraction from Salmonella typhimurium strain LJ144 (as a source of enzyme I and HPr), potential inhibitors (if present) and 10 mM PEP at pH 8.0 as described [6].

The effects of inhibitors of the mannitol permease on glycerol uptake in S typhimurium, strain SB1476 *(ptsI17)* were tested as follows: Cultures (150 ml) were grown at 37°C to a density of 20 Klett units on nutrient broth, and D-mannitol was added to a final concentration of 0.5% to induce the mannitol permease. After 90 min of induction, cells were harvested and washed with 2 × 100 ml of medium 63 (0.1 M potassium phosphate, 15 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, pH 7.0) at 4°C. The final cell pellet was taken up in 20 ml of medium 63 and kept on ice until used. Glycerol uptake was measured at a final concentration of 0.1 mM labeled glycerol (5 mCi/mmol) by preincubating separately 1.8 ml of cell suspension and 0.6 ml of labeled glycerol (containing inhibitor, if present) at 37°C for 2 min followed by rapid mixing of the two solutions. Samples (0.5 ml) were removed at 1, 3, 5, and 10 min, each was added to 3 ml of 0.1 M LiCl at 4°C to quench uptake, and these suspensions were filtered through nitrocellulose filters (0.45 μm, Millipore) followed by 3 × 1-ml washes with ice-cold 0.1 M LiCl. Filters were air-dried and counted in 5 ml of standard toluene/Triton X-100 scintillation fluid. Uptake was found to be a linear function of time up to 10 min under these conditions.

RESULTS

Recent experiments have established that, of the six D-stereoisomers of D-mannitol, only D-glucitol is an inhibitor of the PEP-dependent phosphorylation of D-mannitol by the mannitol permease [3]. Since D-glucitol differs in configuration from D-mannitol about carbon-2, it was suggested that the mannitol binding site could accept alterations at this position (=carbon-5 of the symmetrical molecule D-mannitol), but not easily at carbons 3 and 4 [3]. In Table I, we show that two other compounds differing in structure from D-mannitol at carbon-2, 2-deoxy-D-mannitol and 2-amino-2-deoxy-D-mannitol, are also inhibitors of the mannitol permease. These compounds were tested at a concentration of 2 mM, 20-fold higher than the concentration of the substrate. Higher concentrations could not be used, because we found that above 2 mM, reaction products from the $NaBH_4$ reduction reaction used to synthesize these compounds began to inhibit activity (not shown). These results confirm that the enzyme is not absolutely specific with regard to substitutions or altered stereochemistry at carbon-2(5) of the substrate. In order to determine if other compounds that are structurally related to D-mannitol could compete with this compound for the active site, we carried out the inhibition experiments shown in Figure 1. Of the three D-pentitols, only D-arabitol significantly inhibited the mannitol permease. D,L-Threitol, L-threitol, erythritol, and glycerol did not inhibit, even at high concentrations. Of the six and seven carbon derivatives tested, D-mannonic acid and D-mannoheptitol were inhibitory, but 1,6-dibromo-1,6-dideoxy-D-mannitol was not (Fig. 1).

To determine if any of the compounds shown to be inhibitory in Table I and

TABLE I. Inhibition of PEP-Dependent Mannitol Phosphorylation Catalyzed by Purified Mannitol Permease by Unlabeled Mannitol Analogs*

Inhibitor (2 mM)	Relative activity (%)
None	100
D-mannitol	9
D-glucitol	73
2-deoxy-D-mannitol	74
2-amino-2-deoxy-D-mannitol	63

*Each mannitol derivative was synthesized from its corresponding hexose by $NaBH_4$ reduction. As a control, D-mannitol was reduced by this procedure from D-mannose (second entry). No inhibition was observed with any of the unreduced hexoses. The labeled D-mannitol concentration in the assays was 0.1 mM and the specific activity of the mannitol permease in these and the following experiments was 1 μmol mannitol-1-P formed per minute per milligram protein. Each value is the average of three independent experiments.

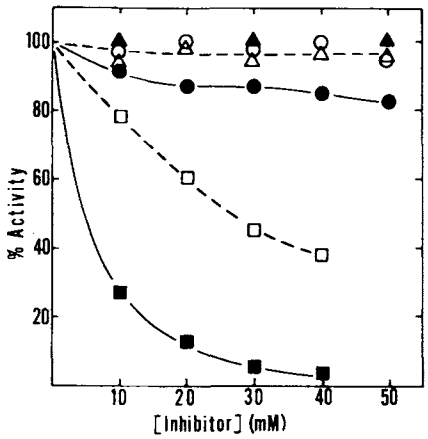

Fig. 1. Inhibition of the mannitol permease by polyols. PEP-dependent phosphorylation of D-mannitol (0.1 mM) by the purified mannitol permease was measured at varying concentrations of potential inhibitors. No significant inhibition was found with D-xylitol (○) or D-ribitol (△), and no inhibition at all was detected under these conditions with D,L-threitol, L-threitol, erythritol, glycerol, or 1,6-dibromo-1,6-dideoxy-D-mannitol (all symbolized by ▲). Significant inhibition was observed with D-arabitol (●), D-mannonic acid (□), and D-mannoheptitol (■).

Figure 1 were also substrates of the purified mannitol permease, we tested each, labeled with carbon 14, in PEP-dependent phosphorylation assays. The results, summarized in Table II, show that D-glucitol, 2-deoxy-D-mannitol, 2-amino-2-deoxy-D-mannitol, and D-arabitol were also substrates for this reaction. (The first two of these compounds had previously been suggested by others to be substrates of the mannitol permease, but purified enzyme was not used in these studies [16, 17].) D-Ribitol and D-xylitol, shown not to be inhibitors in Figure 1, were also not substrates, nor was D-galactitol which was previously reported to be noninhibitory [3]. We have further shown that compounds found to be substrates in the PEP-dependent reaction in Table II are likewise substrates in mannitol-1-phosphate-dependent transphosphor-

TABLE II. Substrate Specificity of the Mannitol Permease*

Labeled compound	Specific activity (at 0.1 mM substrate)	Km[a] (μM)
D-mannitol	1.0	10
2-amino-2-deoxy-D-mannitol	0.50	200
2-deoxy-D-mannitol	0.32	500
D-glucitol	0.37	n.d.[b]
D-arabitol	0.11	>500
D-galactitol	0.0	—
D-ribitol	0.0	—
D-xylitol	0.0	—

*All compounds were prepared from their corresponding labeled aldoses by $NaBH_4$ reduction except for D-mannitol, D-glucitol, and D-xylitol, which were purchased from sources listed in Methods. Specific activity is given in micromoles phosphorylated product formed per minute per milligram protein. No activity was observed with any of the unreduced aldoses.

[a]K_m's were determined at substrate concentrations varied between 5 μM and 2 mM from plots of [substrate]$^{-1}$ against (velocity)$^{-1}$.

[b]Not determined.

ylation, a reaction also catalyzed by the mannitol permease [1], (data not shown).

Two inhibitors, D-mannonic acid and D-mannoheptitol, could not be tested as substrates in this manner because of their unavailability in a radioactive form. Instead, we carried out the the experiments shown in Table III. Preincubation of mannitol permease with D-mannonic acid, soluble PTS proteins, and rate-limiting amounts of PEP followed by addition of labeled mannitol led to greater inhibition of mannitol-1-phosphate formation than when the inhibitor was added at the same time as the radiolabeled substrate. This indicated that preincubation depleted the concentration of PEP, and hence that D-mannonic acid was phosphorylated. In contrast, preincubation with D-mannoheptitol, which is also an inhibitor (Fig. 1), did not cause more inhibition under the same conditions than was observed when inhibitor and substrate were added together (Table III). Since this compound was tested in this experiment at a concentration giving approximately 50% inhibition (without preincubation), these results suggest that D-mannoheptitol is an inhibitor, but not a substrate, under these conditions.

To obtain further evidence for this, we tested the effects of these compounds on glycerol uptake in a S typhimurium strain containing a "leaky" enzyme I mutation *(ptsI17)*. In such strains, glycerol uptake has been shown to be hypersensitive to inhibition by PTS substrates [18]. For such inhibition to occur, a compouind must be taken up and phosphorylated by a PTS permease [cf 8]. The results presented in Table IV show that D-mannitol (0.1 mM) and D-mannonic acid (25 mM) strongly inhibited glycerol uptake in this strain to nearly the same extent, while little, if any, inhibition was observed in the presence of D-mannoheptitol (5 mM). Concentrations of D-mannonic acid and D-mannoheptitol were used in these experiments that gave 50% inhibition of the mannitol permease at 0.1 mM mannitol (cf Fig. 1). Thus, if either inhibitor were a substrate, it should inhibit glycerol uptake at these concentrations to approximately the same extent as 0.1 mM mannitol. Therefore, by this criterion as well, D-mannonic acid is clearly a substrate, while D-mannoheptitol is not.

TABLE III. Effects of Inhibitor Preincubation on D-Mannitol Phosphorylation by the Mannitol Permease*

Inhibitor	No preincubation	Preincubation
None	100	100
D-mannonic acid (10 mM)	88	52
D-mannoheptitol (5 mM)	39	43

*PEP-dependent phosphorylation was measured as described in Methods except that the PEP concentration was 0.1 mM, which was found to be rate-limiting (not shown). Preincubation was for 1 hr at 37°C in the presence of all assay constituents except labeled D-mannitol. Assays were started by addition of the substrate and were for 45 min at 37°C. The specific activity of the mannitol permease under these conditions without inhibitor was 0.51 μmol mannitol-1-P formed per min per mg protein, with or without preincubation. All values are expressed as relative percent activity of control samples without inhibitor.

TABLE IV. Effects of Mannitol Permease Substrates and Inhibitors on Glycerol Uptake in S typhimurium *(ptsI17)*

Compound added	Relative uptake rate[a]
None	1.00
D-mannitol (0.1 mM)	0.06
D-mannonic acid (25 mM)	0.09
D-mannoheptitol (5 mM)	0.95

[a]The uninhibited rate (1.00) corresponded to 5 nmol glycerol taken up per min per ml assay mixture (of the composition described in Methods) at 37°C.

Finally, we asked whether the ionic state of the one charged substrate that has a pK_a over the pH range in which the mannitol permease is active [3], 2-amino-2-deoxy-D-mannitol, in any way affected its ability to be phosphorylated by the enzyme. As shown in Table V, however, the phosphorylation of both this compound and of D-mannitol showed similar pH-activity responses over the range pH 7 to pH 9.5, indicating that at least the protonated form of the former compound can serve as a substrate for this reaction.

Recent experiments have established that the mannitol permease, purified in the presence of the nonionic detergent Lubrol PX, is activated by phospholipids in the PEP-dependent phosphorylation reaction [3]. However, phospholipids from various heterologous sources were used in these studies. In order to determine the specificity of phospholipid interaction with the mannitol permease in a homologous system, we carried out the experiments shown in Figure 2. Purified E coli phospholipids were assessed for their abilities to stimulate PEP-dependent mannitol phosphorylation catalyzed by the mannitol permease. The most stimulatory compound in these studies was PE, the most abundant phospholipid in E coli [19]. PG and CL from E coli had little effect on the activity of the mannitol permease, even at high concentrations (Fig. 2).

TABLE V. pH-Dependence of PEP-dependent Phosphorylation of D-Mannitol and 2-Amino-2-deoxy-D-mannitol by the Mannitol Permease[a]

pH	D-Mannitol	2-Amino-2-deoxy-D-mannitol
7.0	0.47	0.21
8.0	1.0	0.60
9.0	0.14	0.04
9.5	0.09	0.01

[a]PEP-dependent phosphorylation was measured as described in Methods except that the buffers used were potassium phosphate (pH 7), Tris-HCl (pH 8 and 9), and glycine-HCl (pH 9.5), all at 0.1 M. The 2-amino-derivative was prepared from labeled mannosamine as described in Methods. The specific activities listed are in the same units as in Table II.

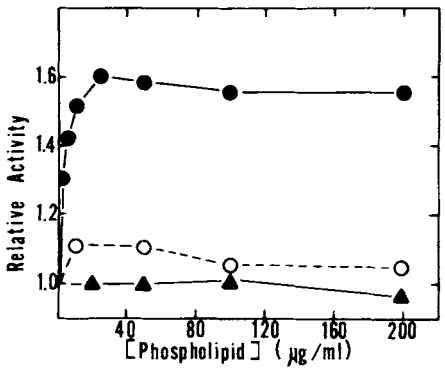

Fig. 2. Activation of the mannitol permease by E coli phospholipids. PEP-dependent activities were determined as described in Methods in the presence of the indicated concentrations of sonicated phospholipids, prepared as described in [3]: (●), phosphatidylethanolamine; (○), phosphatidylglycerol; (▲), cardiolipin. Relative activity without phospholipid is set at a value of 1.0.

DISCUSSION

Fischer projection structures of compounds we have shown to be substrates and/or inhibitors of the mannitol permease are given in Figure 3. The compounds are aligned in the manner in which they presumably interact with the binding/active site of the enzyme. Several conclusions can be drawn about this site, and the mechanism of phosphorylation of these compounds, from our results. First, as has been suggested [3], alterations in structure about carbon-2(5) are tolerated by the enzyme, although all of these compounds interact much less well with the mannitol permease than the natural substrate. This is shown by the fact that a 20-fold molar excess of compounds II–IV over substrate inhibits the enzyme less than 50% (Table I), that phosphorylation rates of these compounds as substrates are less than for the same concentration of D-mannitol, and that the apparent K_m's for analogs substituted at the 2-position are

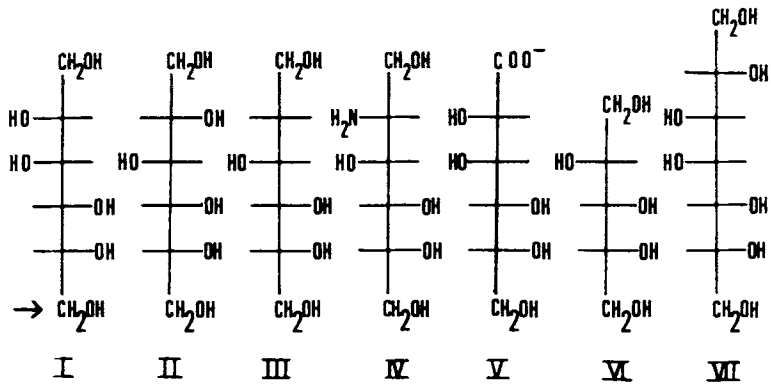

Fig. 3. Substrates and/or inhibitors of the E coli mannitol permease. Compounds I–VI are both substrates and inhibitors, while compound VII is an inhibitor, but not a substrate. The arrow shows the predicted position of phosphorylation in I–VI. I, D-mannitol; II, D-glucitol; III, 2-deoxy-D-mannitol; IV, 2-amino-2-deoxy-D-mannitol; V, D-mannonic acid; VI, D-arabitol; VII, D-mannoheptitol (perseitol).

considerably higher than that for the natural substrate (Table II). The order of efficiency of these compounds as substrates/inhibitors deduced from the data in these tables (2-amino-2-deoxy-D-mannitol > 2-deoxy-D-mannitol ~ D-glucitol) suggests that an interaction of the hydroxyl (or amino) group at carbon-2 of the enzyme, presumably a hydrogen bond, is important, but not absolutely essential. Whether the substituent at carbon-2 is positively charged or not does not seem to influence this interaction greatly as evidenced by the pH-dependence of 2-amino-2-deoxy-D-mannitol phosphorylation. In contrast, although D-mannonic acid is a substrate and inhibitor of the enzyme, 50% inhibition is seen when this compound is present in 250-fold molar excess over the natural substrate. D-Mannoheptitol, which is at least as bulky as the acid derivative, is nonetheless a much better inhibitor of the mannitol permease (Fig. 1). Thus, a negative charge at the 1-position inhibits interaction, suggesting that the binding site itself contains at least one negatively charged group, presumably a carboxylate anion.

A second series of conclusions from these studies concerns size constraints on the active site. While a 5-carbon polyol, D-arabitol, can interact with the enzyme, shorter polyols do not. On the other hand, although a 7-carbon polyol, D-mannoheptitol, is a good inhibitor and thus must bind to the active site, it is not phosphorylated. This suggests that the binding of this larger compound induces strain in it, in the active site, or both, such that the phosphorylation reaction cannot proceed. Consistent with this suggestion is that the even larger compound, 1,6-dibromo-1, 6-dideoxy-D-mannitol, does not interact with the enzyme at all, although the absence of hydroxyl groups both positions 1 and 6 in this compound may also explain this result.

An examination of the compounds tested that act as substrates for the mannitol permease (I–VI in Fig. 3) suggests that it is the 6-position in compound V, and the 5-position in compound VI, that are phosphorylated by the enzyme. The only way in which a terminal hydroxyl can occupy the same relative position in these two compounds and still satisfy the stereochemical requirements of the hydroxyls at positions 2–5 (relative to the D-mannitol molecule) is the alignment shown in Figure

3. These terminal hydroxyls are the one in the 6-position of compound V and the 5-hydroxyl group of arabitol (VI). We are currently attempting to confirm this conclusion by direct structural analyses of these products. However, this conclusion is not surprising since it is the 6-position that is phosphorylated by all the other aldohexose and hexitol-specific PTS permeases in E coli [8]. It is still possible, of course, that compounds II–IV could be phosphorylated at either the 1- or 6-position if the stereochemical requirements at the 5-position are similar to those at position 2. Further experiments will be required to resolve this question.

An examination of the phospholipid specificity of the purified mannitol permease using E coli phospholipids revealed that PE was a good activator, but PG and CL were not (Fig. 2). In an earlier report [3], PG (from egg) was found to stimulate mannitol permease activity more effectively than PE (from E coli). Thus, not only the polar head group, but also the fatty acid composition of a particular phospholipid must influence its degree of favorable interaction with the mannitol permease. In the case of E coli phospholipids, however, the neutral compound PE appears to interact better than the negatively charged lipids PG and CL. Possibly, this reflects the fact that the mannitol permease itself is a slightly acidic protein [2], although this does not explain activation of the mannitol permease by egg PG. We are currently investigating the roles that fatty acyl groups play in phospholipid interactions with the mannitol permease using synthetic phospholipids of defined fatty acid compositions. Furthermore, it is not yet known whether the purified protein still contains bound phospholipid. If so, then identification of any such lipids would also help define the phospholipid specificity of the mannitol permease. Although conclusions regarding the specificity of phospholipid interactions with the protein must still be considered preliminary, the results reported here strongly suggest that the mannitol permease interacts most favorably with PE in E coli.

Besides partially delineating the structural constraints of the hexitol site of the mannitol permease and its phospholipid requirements, our results also suggest further experiments that will be useful in the study of the mechanism and structure of this transmembrane permease. For example, a rapid and highly efficient method of purifying this protein relative to the published procedures [2,3] would be very useful. The specificity studies reported here suggest logical ways in which to couple mannitol derivatives to solid supports for potential affinity chromatography without destroying recognition of the coupled compound by the enzyme. Fluorescent and/or reactive derivatives of D-mannitol might also be useful in probing the structure of the active site, and our results also point to reasonable strategies for the synthesis of such compounds. Furthermore, the discovery of an apparent "dead end" inhibitor of the mannitol permease, D-mannoheptitol, should be useful in kinetic studies of the transport and phosphorylation mechanisms of this enzyme. Such studies become particularly important, and are potentially very informative, because of recent predictions concerning the topography of this protein in the membrane based on its sensitivity to various modification agents [6] and its amino acid sequence [5].

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