Binding of the Substrate Analogue Perseitol to Phosphorylated and Unphosphorylated Enzyme II^{mtl} of the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*†

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ABSTRACT: Enzyme II^{mtl} catalyzes the concomitant transport and phosphorylation of the hexitol mannitol. Here we demonstrate that the heptitol perseitol is not phosphorylated and not transported by the enzyme. However, the enzyme binds perseitol with an affinity comparable to the affinity for mannitol. Apparent affinities of the phosphorylated enzyme for perseitol were inferred from the inhibition by perseitol of mannitol phosphorylation and uptake. Apparent affinities of the unphosphorylated enzyme follow from the inhibition of mannitol binding to the enzyme. Mechanistic interpretations of the apparent inhibition constants are discussed, and it is concluded that phosphorylation of the cytoplasmic domain of enzyme II^{mtl} has little effect on the affinity of the membrane-bound domain of the enzyme for perseitol.

Enzyme II^{mtl} [1] belongs to a class of transport proteins that couple the translocation of sugar substrates over the bacterial cytoplasmic membrane to the phosphorylation of the sugar. The phosphoryl group originates from the high-energy metabolite phosphoenolpyruvate and is transferred to the incoming sugar via a number of proteins, the last one being the transporter itself. The phosphoryl group donor that phosphorylates the mannitol-specific enzyme II^{mtl} is a small protein, termed HPr [for reviews see Postma and Lengeler (1986), Meadow et al. (1990), and Lolkema and Robillard (1992)]. Therefore, the reaction catalyzed by enzyme II^{mtl} is described by

$$\text{mannitol}_{out} + \text{P-HPr} \xrightarrow{\text{II}^{mtl}} \text{mannitol-P}_{in} + \text{HPr} \qquad (1)$$

Enzyme II^{mtl} is a 68-kDa protein with a well-defined domain structure. It consists of two hydrophilic domains that protrude into the cytoplasm and one hydrophobic transmembrane domain (Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,b). Each of the two cytoplasmic domains contains a phosphoryl group binding site that is transiently phosphorylated during turnover of the enzyme (Pas & Robillard, 1988). The membrane-bound domain contains the mannitol binding site (Lolkema et al., 1990) and is responsible for the transport activity. We have proposed a model for the mechanism of enzyme II^{mtl} that closely relates its function to its domain structure (Lolkema et al., 1991a). In the model, the vectorial phosphorylation of mannitol (reaction 1) would proceed, mechanistically, in two separate steps. In one step, mannitol bound to the transmembrane domain is translocated from the periplasmic side of the membrane to the cytoplasmic side. In the second step, the phosphoryl group bound to the cytoplasmic domain is transferred to mannitol. Mannitol bound to the cytoplasmic-facing binding site would be in contact with the cytoplasmic volume. Consequently, mannitol would be able to access this site from the cytoplasm and become phosphorylated, resulting in phosphorylation without transport. Therefore, enzyme II^{mtl} solubilized in detergent may be expected to catalyze, mechanistically, both vectorial and nonvectorial phosphorylation according to the following scheme:

$$\begin{array}{l} \text{Mtl} + \text{EP}_{out} \rightleftharpoons \\ \text{Mtl}\bullet\text{EP}_{out} \rightleftharpoons \text{Mtl}\bullet\text{EP}_{in} \rightleftharpoons \text{Mtl—P}\bullet\text{E}_{in} \rightleftharpoons \text{E}_{in} + \text{Mtl—P} \\ \qquad\qquad \rightleftharpoons \\ \qquad\qquad \text{Mtl} + \text{EP}_{in} \end{array} \qquad (2)$$

in which EP and E represent the phosphorylated states and unphosphorylated states of the enzyme, respectively, and the subscripts denote the periplasmic (out) and cytoplasmic (in) orientations of the mannitol binding site. Simulation of the kinetic behavior of a kinetic scheme based upon this model showed that the model could explain much of the complex phosphorylation kinetics of enzyme II^{mtl} solubilized in detergent or embedded in the membrane of vesicles with an inside-out (ISO) orientation (Lolkema, 1993; Lolkema et al., 1993a).

An important feature of the model is the regulation of the translocation activity of the membrane-bound domain by the state of phosphorylation of the cytoplasmic domain. In the unphosphorylated state the energy barrier between the two states of the enzyme with the binding site facing the periplasm and the cytoplasm would be high; the translocator would be almost inactive (Lolkema et al., 1990, 1992). Phosphorylation of the cytoplasmic domain would lower the activation energy considerably and, thereby, activate the translocator. Evidence for a functional interaction between the two domains followed from studies in which the effects of chemical or mutational modifications of the cytoplasmic domain upon the translocator domain were measured (Lolkema et al., 1991b; 1993b).

In this report, we investigate the effect of phosphorylating the cytoplasmic domain on the substrate affinity of the translocator domain. Four states of the binding site on enzyme

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[1] Abbreviations: ISO, inside-out; RSO, right-side-out; DTT, dithiothreitol; decylPEG, decyl poly(ethylene glycol) 300; mtl, mannitol; ptl, perseitol; pts, phosphotransferase system.

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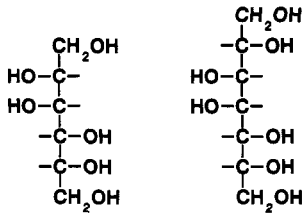
Chart I: Fischer Projections of Mannitol (Left) and Perseitol



II^{mtl} may be discriminated; the states with the two opposite orientations in both the phosphorylated and unphosphorylated enzyme. Measurement of the binding affinity of the site for mannitol in the phosphorylated state is not possible because of the mannitol phosphorylation activity of the enzyme. Therefore, we have measured the binding affinity of the four states of the binding site for the heptitol perseitol (Chart I), a substrate analogue that is not phosphorylated by enzyme II^{mtl} (Jacobson et al., 1983) but for which unphosphorylated enzyme II^{mtl} has a comparable high affinity as observed for mannitol.

EXPERIMENTAL PROCEDURES

Materials

[1-^{3}H(N)]-D-Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. [1-^{14}C]-D-Mannitol (2.2 Gbq/mmol) and [^{14}C]phosphoenolpyruvate (2.2 GBq/mmol) were from Amersham. Decyl poly(ethylene glycol) 300 (decylPEG) was synthesized by B. Kwant in our laboratory. Perseitol was purchased from Pfanstiehl Laboratories Inc., Waukegan, IL.

Methods

Growth Conditions. Escherichia coli strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing either 0.5% mannitol or 0.5% glucose as the carbon source. Cells were grown in 5-L flasks filled with 2 L of medium for membrane preparations or in 500-mL flasks, filled with 100 mL of medium for the uptake studies. The cultures were aerated by continuous shaking. Cells were harvested at an OD_{650} of 1.0.

Membrane vesicles were prepared essentially as described (Reenstra et al., 1980). The vesicles were washed once with 25 mM Tris, pH 7.5, 1 mM DTT, and 1 mM NaN_3. Aliquots of 50 μL containing approximately 1 mg/mL membrane protein (Bradford, 1976) were stored in liquid nitrogen. Samples were thawed rapidly before use.

Enzyme Purifications. The *E. coli* phosphotransferase enzyme, E_I, necessary for the phosphorylation of HPr, and Hpr, were purified as described (Robillard et al., 1979; van Dijk et al., 1990). Enzyme II^{mtl} was purified as described (Roossien et al., 1984) with modifications (Robillard & Blaauw, 1987; Lolkema et al., 1993c).

Uptake Measurements. Cells grown on glucose or mannitol were washed once with a buffer containing 50 mM KP_i, pH 7.5, and subsequently resuspended at an OD_{650} of about 10 in the same buffer. These suspensions were stored on ice until use. Aliquots of 100 μL at the specified OD_{650} were incubated at 30 °C for at least 10 min, after which [^{3}H]mannitol was added to the suspension to the desired concentration. The uptake reaction was stopped at time intervals of 10 s by addition of 2 mL of ice-cold buffer containing 1 mM $HgCl_2$, after which the cells were separated from the medium by rapid filtration using GF/F microfiber filters (Whatman International Ltd., Maidston, England). The filters were washed twice with 2 mL of quench buffer, after which the retained radioactivity was determined in a liquid scintillation counter.

Phosphorylation Assays. All experiments were performed at 30 °C. The activity of enzyme II^{mtl} was measured by following the formation of [^{3}H]mannitol-P or [^{14}C]mannitol-P in time in a total volume of 100 μL. The buffer contained 25 mM Tris, pH 7.5, 5 mM DTT, and 5 mM $MgCl_2$. Four samples of 20 μL each were withdrawn at consecutive times and analyzed for labeled mannitol-P as described (Robillard & Blaauw, 1987). A fifth sample of 10 μL was used to relate the labeled mannitol concentration to the total radioactivity in the sample.

Membranes were solubilized by suspending ISO membranes in a buffer containing 25 mM Tris, pH 7.5, 5 mM DTT, and 0.25% decylPEG. Subsequently, these solubilized membranes were diluted into the assay mixture that contained 0.25% decylPEG as well.

Pyruvate Burst Assays. The conversion of [^{14}C]phosphoenolpyruvate into [^{14}C]pyruvate was measured as described (Brouwer et al., 1980).

Binding assays were performed with the flow dialysis technique as described (Lolkema et al., 1990). The flow dialysis cell was thermostated at 30 °C.

Evaluation of the Data

Competitive Uptake and Phosphorylation. The binding characteristics of perseitol to phosphorylated enzyme II^{mtl} were deduced from the inhibition by perseitol of in vivo mannitol uptake and in vitro mannitol phosphorylation. The inhibition was treated as purely competitive, relating the rate (v) as follows to the mannitol concentration ([mtl]) and the perseitol concentration ([ptl]):

$$v = V_{max}\frac{[mtl]}{[mtl] + K_M^{mtl}\left(1 + \frac{[ptl]}{K_I^{ptl}}\right)} \quad (3)$$

in which K_M and K_I are the kinetic affinity constant for mannitol and the inhibition constant for perseitol, respectively. Equation 3 may be used to derive eq 4, in which v_0 is the rate in the absence of perseitol:

$$\frac{v_0}{v} = 1 + \frac{K_M^{mtl}}{K_I^{ptl}(K_M^{mtl} + [mtl])}[ptl] \quad (4)$$

Plots of v_0/v as a function of the perseitol concentration at constant mannitol concentrations show a set of lines that intersect at the positive y-axis ($v_0/v = 1$). The kinetic parameters follow from a secondary plot relating the negative x-axis intercept of each line to the mannitol concentration:

$$-[ptl]_{v_0/v = 0} = K_I^{ptl} + \frac{K_I^{ptl}}{K_M^{mtl}}[mtl] \quad (5)$$

Competitive Binding. The binding characteristics of the binding of perseitol to enzyme II^{mtl} in the unphosphorylated state were deduced from competition for the binding sites between mannitol and perseitol. The equation describing the concentration of bound mannitol ($[mtl]_b$) as a function of the free mannitol concentration ($[mtl]_f$) and the total perseitol concentration ($[ptl]_t$) is as follows:

$$[mtl]_b = \frac{-b + (b^2 - 4ac)^{1/2}}{2a} \quad (6)$$

with

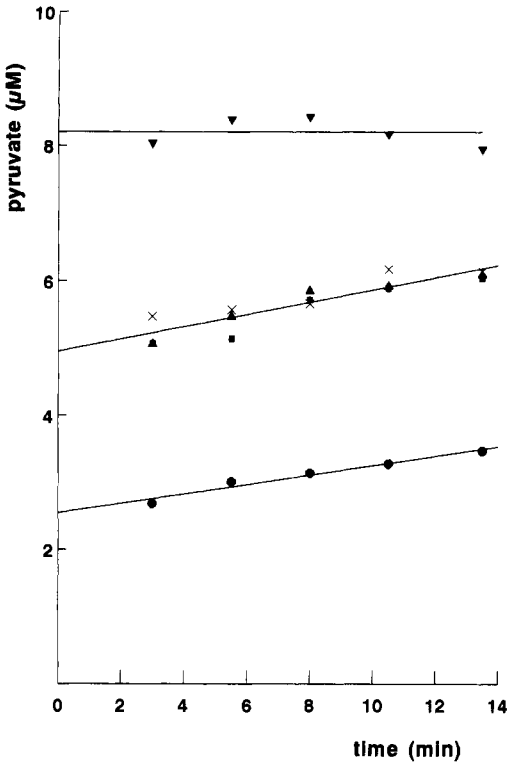


FIGURE 1: Formation of [^{14}C]pyruvate by addition of mannitol and perseitol to the components of the mannitol-specific phosphotransferase system. The assay mixtures contained EI and HPr (●), EI, HPr, and II^{mtl} (■), EI, HPr, II^{mtl}, and 50 μM mannitol (▼), EI, HPr, II^{mtl}, and 50 μM perseitol (×), and EI, HPr, II^{mtl}, and 500 μM perseitol (▲). The concentrations of the pts components were 0.2 μM EI, 0.6 μM HPr, and 1.1 μM purified enzyme II^{mtl}.

$$a = K_D^{mtl}(K_D^{mtl} + [mtl]_f)$$

$$b = K_D^{mtl}[mtl]_f(K_D^{ptl} - \epsilon + [ptl]_t) + [Mtl]_f^2 K_D^{ptl}$$

$$c = -[mtl]_f^2 K_D^{ptl}\epsilon$$

in which K_D^{mtl} and K_D^{ptl} are the dissociation constants for mannitol and perseitol, respectively, and ϵ is the concentration of binding sites. The data were fitted by a nonlinear fitting procedure.

RESULTS

Perseitol Is Not Phosphorylated by Enzyme II^{mtl}. In this study we aim to measure the binding affinity of phosphorylated enzyme II^{mtl} for the substrate analogue perseitol. In order to do so, it should be absolutely clear that perseitol is not phosphorylated by enzyme II^{mtl}. Jacobson et al. (1983) suggested that this might be the case since preincubation of the pts components with perseitol and rate-limiting concentrations of phosphoenolpyruvate did not result in depletion of the phosphoenolpyruvate pool. The much more sensitive experiment shown in Figure 1 confirms their conclusion by showing that no pyruvate is formed under similar conditions but with stoichiometric amounts of the enzyme. Mixing of 8.2 μM [^{14}C]phosphoenolpyruvate with the general pts components EI and HPr results in a "burst" of [^{14}C]pyruvate followed by a slow increase of the [^{14}C]pyruvate concentration (Figure 1, ●). The burst is due, in part, to a fraction of hydrolyzed [^{14}C]phosphoenolpyruvate already present in the stock solution and, in part, to the rapid incorporation of phosphoryl groups in EI and HPr. The slow increase is due to the continuous hydrolysis of P-EI and P-HPr and rephosphorylation (Brouwer et al., 1980). Including 1.1 μM enzyme II^{mtl} in the assay mixture results in a 2.2 μM increase of the [^{14}C]pyruvate burst due to the incorporation of two phosphoryl groups per molecule of enzyme II^{mtl} (■). Addition of mannitol in excess of phosphoenolpyruvate converts all [^{14}C]phosphoenolpyruvate instantaneously into [^{14}C]pyruvate, indicative of rapid mannitol phosphorylation (▼). However, if perseitol is added at concentrations of 50 or 500 μM, no additional burst shows up (× and ▲, respectively). A single turnover of the enzyme II^{mtl} pool in 14 min would have resulted in an increase of the [^{14}C]pyruvate concentration of 1.1 μM on top of the [^{14}C]pyruvate formed from hydrolysis of the phosphorylated enzymes. Since this does not happen, the turnover number in the perseitol phosphorylation reaction is $\ll 0.1$ min^{-1}, which is at least 4 orders of magnitude smaller than the turnover number in the mannitol phosphorylation reaction (about 1000 min^{-1}, Lolkema et al., 1993a). It seems fair to conclude that perseitol cannot be phosphorylated by enzyme II^{mtl}.

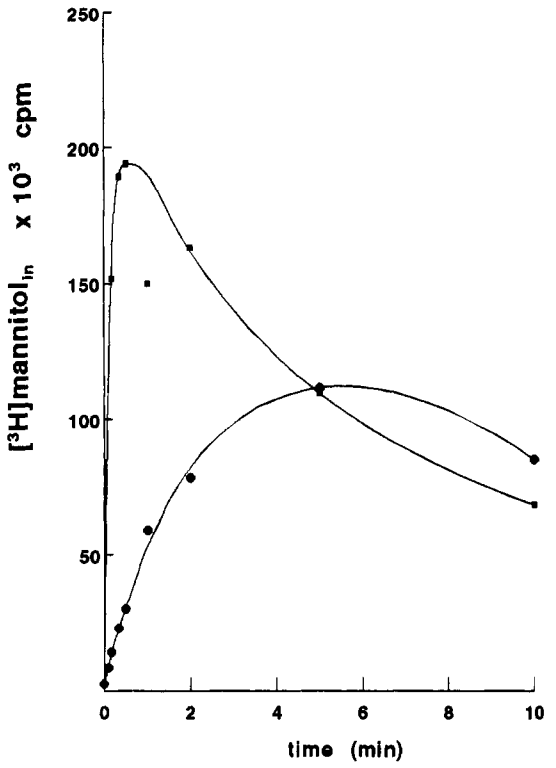


FIGURE 2: Time course of mannitol uptake by cells grown on mannitol (■) and glucose (●). Cells were resuspended at an OD_{650} of 0.5. The [^{3}H]mannitol concentration was 0.2 μM, which was equivalent to 400 kcpm in the sample.

Inhibition of Mannitol Uptake. E. coli strain ML308-225 grown on mannitol takes up and metabolizes mannitol at such a high rate that no initial rates could be estimated from the time course of uptake (Figure 2, ■). To overcome these problems, the cells were grown in the presence of glucose, which results in much lower levels of expression of both enzyme II^{mtl} and mannitol-P dehydrogenase [see also Lolkema et al. (1990)]. Uptake of mannitol in these cells is linear for at least the first 30 s (Figure 2, ●).

Inhibition of mannitol uptake by perseitol was measured by adding mixtures of [^{3}H]mannitol and perseitol to suspensions of glucose-grown cells. The resulting data were analyzed as described under Methods and are shown in Figure 3. It follows from the inset that the affinity constant for mannitol uptake, K_M^{mtl}, equals 2.1 μM and that the inhibition constant for perseitol, K_I^{ptl}, equals 10.9 μM.

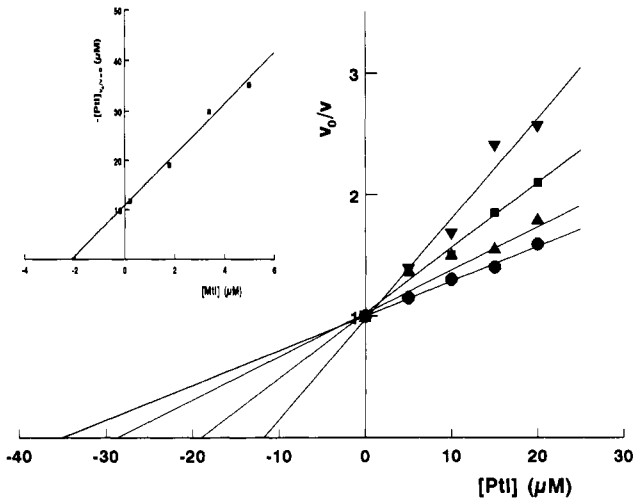


FIGURE 3: Analysis of the inhibition of mannitol uptake in whole cells by perseitol. Glucose-grown cells were resuspended to an OD_{650} of 1.0. The [^{3}H]mannitol concentrations were 0.2 μM (▼), 1.8 μM (■), 3.4 μM (▲), and 5.0 μM (●). The data were analyzed according to eqs 4 (main plot) and 5 (inset) in the Experimental Procedures section. The uptake rate at 5 μM mannitol in the absence of perseitol was 0.25 μM/min.

The effect of preincubation of cells with perseitol was investigated by incubating cells for 1 h at 30 °C in the absence and presence of 10 μM perseitol. The uptake activity of both samples was measured by adding 0.2 μM [^{3}H]mannitol to the suspensions with and without 10 μM perseitol, respectively. No difference in uptake rate could be detected (data not shown).

Inhibition of Phosphorylation. The kinetics of mannitol phosphorylation at saturating concentrations of P-HPr is biphasic with respect to the mannitol concentration when catalyzed by enzyme II^{mtl} in both solubilized and intact cytoplasmic membranes. The high-affinity regime was characterized by affinity constants for mannitol of 2.4 μM and 9.3 μM with the solubilized and intact membranes, respectively. The phenomenological affinity constant in the low-affinity regime was about 60 μM. Up to concentrations of about 10 μM mannitol, only the high-affinity phase is manifest (Lolkema et al., 1993a).

Figure 4 shows the inhibition of mannitol phosphorylation catalyzed by cytoplasmic membranes in the high-affinity regime in the presence (■) and absence (●) of 0.25% decylPEG over an extended range of perseitol concentrations. The inhibition with the intact membranes was much stronger than observed with the solubilized membranes. Moreover, whereas competitive inhibition, as described by eq 4, was observed with the solubilized membranes, the inhibition with the intact membranes resulted in a nonlinear relationship when treated identically. A small fraction of the rate (<10%) was much more difficult to inhibit by perseitol. Since this was not observed with the membranes after solubilization, we believe that this relates to inhomogeneity of the membrane preparation.

The inhibition by perseitol of mannitol phosphorylation catalyzed by the solubilized membranes was further analyzed with the same range of [^{3}H]mannitol and perseitol concentrations used in the uptake experiments described above (Figure 3). The data showed a good fit to eqs 4 and 5. The affinity constant for mannitol, K_M^{mtl} = 2.2 μM, was in good agreement with earlier reports (Lolkema et al., 1993a), and the inhibition constant for perseitol, K_I^{ptl}, was 3.9 μM (Table I). A similar analysis with the intact membranes was

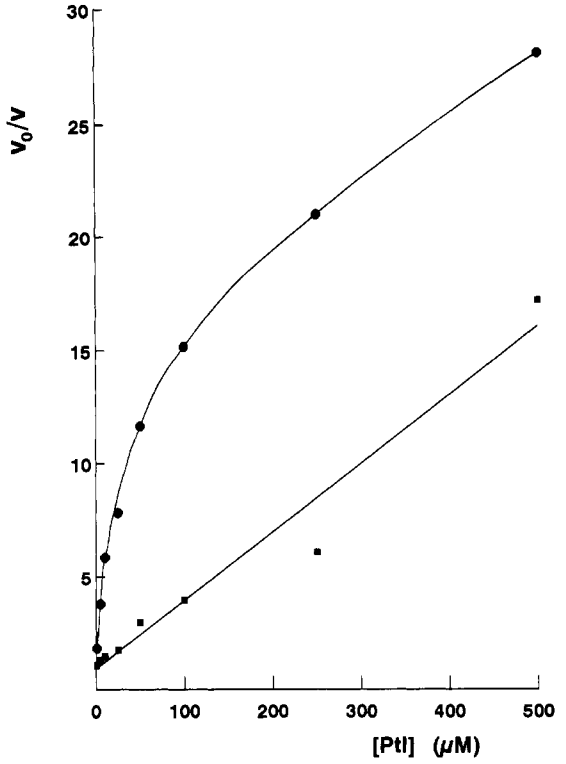


FIGURE 4: Inhibition by perseitol and mannitol phosphorylation catalyzed by intact (●) and solubilized (■) cytoplasmic membranes. The assay mixture contained 5 μM [^{3}H]mannitol, 0.2 μM EI, and 6 μM HPr. The membrane protein concentrations were 33 ng/mL (●) and 3.3 ng/mL (■). The data were plotted according to eq 3. The phosphorylation rates were 36.2 and 71.0 nmol/(min·mg) in the absence and presence of decylPEG, respectively.

Table I: Kinetic Parameters from Competition Studies between Mannitol and Perseitol in Mannitol Phosphorylation and Uptake Activities of Enzyme II^{mtl}[a]

enzyme II^{mtl} activity	K_M^{mtl} (μM)	K_I^{ptl} (μM)
uptake	2.1	10.9
phosphorylation (solubilized)	2.2	3.9
phosphorylation (ISO membranes)	9.5	0.7

[a] The assay conditions were as described in the legends of Figures 3 and 4.

performed in the lower perseitol concentration range of Figure 4. The perseitol concentrations were chosen such that the inhibition was never larger than a factor of 3, which provided a linear relationship when the data were plotted according to eq 3. The secondary plot indicated a affinity constant for mannitol, K_M^{mtl}, of 9.5 μM and an inhibition constant for perseitol, K_I^{ptl}, of 0.7 μM (Table I).

Inhibition of Binding. Binding of perseitol to unphosphorylated enzyme II^{mtl} was measured by mixing stoichiometric amounts of [^{3}H]mannitol, perseitol, and enzyme. Subsequently, the fractions of free and bound [^{3}H]mannitol were measured by flow dialysis after equilibration. Unlike with the inhibition studies of uptake and phosphorylation, the bound perseitol concentration in these binding studies is not necessarily a negligible fraction of the total concentration. This leads to the rather complicated equation (eq 6) in the Methods section that relates the measurable parameters $[mtl]_b$ and $[mtl]_f$ to the total perseitol concentration ([ptl]) in the case of competition for a single binding site on the enzyme.

The competition between mannitol and perseitol was analyzed for three different conditions of the enzyme: (i) embedded in the membrane of ISO vesicles, (ii) embedded in

Table II: Dissociation Constants for Mannitol and Perseitol Binding to Enzyme II^{mtl} at 30 °C[a]

membranes	K_D^{mtl} (nM)	K_D^{ptl} (nM)	ϵ (nM)	error (%)
ISO, intact	144	813	344	1.8
ISO, solubilized	212	1264	357	4.6
RSO, intact, prep 1	300	1436	260	10
RSO, intact, prep 2	290	1282	178	1.2

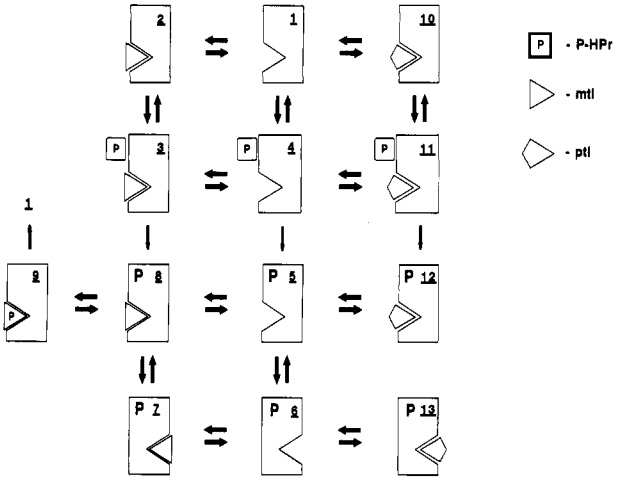
[a] Bound [^{3}H]mannitol was measured at total concentrations of 100, 200, 400, and 600 nM [^{3}H]mannitol in the presence and absence of 1 μM perseitol. ISO membranes were solubilized with 0.5% decylPEG. The binding to the RSO membranes was measured with two independent membrane preparations (1 and 2). The membrane concentration were 0.5 mg/mL (ISO), 0.4 mg/mL (RSO, preparation 1), and 0.14 mg/mL (RSO, preparation 2). The data were fitted to eq 6 by nonlinear regression. The last column gives the mean deviation of the data points to the calculated line.

the membrane of RSO vesicles, and (iii) solubilized in detergent. The flow dialysis cell was thermostated at 30 °C to allow a comparison with the affinities for perseitol inferred from the inhibition of transport and phosphorylation activities. The results were summarized in Table II. At 30 °C, the affinity of enzyme II^{mtl} for mannitol was somewhat lower than at room temperature (Lolkema et al., 1990). The total number of sites found with the intact ISO membranes was identical to the total number of sites observed after solubilization of the membranes, indicating equilibration of the binding site over the membrane, which is also observed at room temperature (Lolkema et al., 1990) but not at 4 °C (Lolkema et al., 1992). The apparent affinity constant of the binding site was a factor of 2 higher when measured with RSO membrane vesicles as compared to ISO membrane vesicles. The affinities for perseitol were a factor of 5–6 lower than observed for mannitol. The higher affinity for mannitol of the ISO vesicles relative to the solubilized enzyme was also observed for perseitol.

DISCUSSION

In this paper we aim to investigate whether the effect of phosphorylation of the cytoplasmic domain on the translocator domain of enzyme II^{mtl} is confined to a lowering of the activation energy for translocation or whether the affinities for mannitol are affected as well. This is relevant for understanding the nature of the interaction between the two domains and is of particular interest for the analysis of the kinetics of the enzyme. To address the question, the binding affinities to both the phosphorylated and unphosphorylated states of the enzyme have to be measured. Obviously, this is not possible for mannitol, but it is for perseitol, a substrate that cannot be phosphorylated by enzyme II^{mtl} (Figure 1). The measured parameters for perseitol are true binding affinities, not perturbed by turnover of the enzyme. Unfortunately, perseitol is not available in a radioactive form. Therefore, the affinities have to be inferred from competition experiments with mannitol. The inhibition constants for perseitol in the binding experiments relate directly to the binding of perseitol to unphosphorylated enzyme II^{mtl}. The inhibition constants in the mannitol uptake and phosphorylation activities follow from the analysis by back-extrapolation to zero mannitol concentration (eq 5). This provides not only that the parameters are mannitol concentration-independent but also that the affinity constants characterize the binding of perseitol to the phosphorylated enzyme. For both states of the enzyme, the mannitol concentration-independent binding affinities for perseitol may, and probably will, be functions of

Scheme I: Kinetic Scheme for Enzyme II^{mtl}-Catalyzed Mannitol Phosphorylation in the Presence of Perseitol[a]



[a] The left side of each state represents the cytoplasmic side of the membrane. Capital P indicates the phosphorylated state of enzyme II^{mtl}, Hpr, and mannitol. The phosphoryl group transfers from Hpr to enzyme II^{mtl} and from enzyme II^{mtl} to mannitol are assumed to be irreversible (initial rates). States 1–4 indicate the random binding of mannitol and P-Hpr to the unphosphorylated enzyme. The states showing binding of P-HPr to the enzyme are not relevant for the present study but were included for completeness. The isomerization of the binding site on the unphosphorylated enzyme is too slow to play a role in the kinetics. States 5–8 makes the transport cycle of the enzyme. Perseitol is neither transported nor phosphorylated by enzyme II^{mtl} (see text). The scheme is largely based on previous studies [see Lolkema (1993)].

true dissociation constants and intrinsic transitions of the enzyme. These will be discussed next in the context of a kinetic scheme that we recently have proposed and tested for enzyme II^{mtl} (Lolkema, 1993).

States 1–9 in Scheme I make a kinetic scheme for monomeric enzyme II^{mtl} that was shown to explain the main characteristics of the mannitol phosphorylation kinetics catalyzed by enzyme II^{mtl} in a noncompartmentalized system (Lolkema et al., 1993a; Lolkema, 1993). The two kinetic phases with respect to the mannitol concentration that show up under this condition for the enzyme at saturating concentrations of P-HPr reflect two different pathways through the scheme. These two pathways come about by virtue of the high and low accessibility of the mannitol binding site when facing the periplasm and cytoplasm, respectively. The binding affinities of the binding site in the two orientations are high and not very different. In the high-affinity kinetic phase, the major pathway is from state 1 via states 4–7 to "productive" state 8 where the phosphoryl group is transferred to mannitol to give state 9. Mannitol phosphorylation via this pathway reflects vectorial phosphorylation since it involves the binding of mannitol to the periplasmically-oriented binding site (6 → 7) and the translocation step (7 → 8). In the low-affinity phase, the low accessibility of the cytoplasmically-oriented binding site is overcome by high concentrations of mannitol. This results in direct binding of mannitol to either state 1 or state 5 to form "productive" state 8 via states 2 and 3 or directly. This pathway reflects cytoplasmic mannitol phosphorylation and is faster because the rate-determining translocation steps are short-circuited. Both kinetic phases show up in an "open system" because the mannitol binding site faces the same mannitol concentration, irrespective of its orientation. The "open system" may refer to the enzyme solubilized in detergent or to the enzyme in open membrane fragments. The latter is believed to be the condition of a fraction of enzyme II^{mtl} in an inside-out vesicle preparation. This fraction would be solely responsible for the

high-affinity phase seen in the kinetics of the inside-out membranes (Lolkema et al., 1993a).

States 10–13 in Scheme I represent those states that are involved in perseitol binding. The inhibition of mannitol phosphorylation by perseitol was investigated in the high-affinity kinetic phase for mannitol. Since perseitol is not phosphorylated by enzyme II^{mtl}, the dynamics of the perseitol binding equilibria are irrelevant for the steady-state distribution. Therefore, the mannitol concentration-independent inhibition constant determined from the competition experiments will be determined by binding to both sides of the phosphorylated enzyme. The states involved are states 5, 6, 12, and 13 in the following scheme:

$$\text{Ptl}\cdot\text{EP}_{out} \overset{K^{ptl}_{D,out}}{\rightleftharpoons} \text{EP}_{out} \overset{K_t}{\rightleftharpoons} \text{EP}_{in} \overset{K^{ptl}_{D,in}}{\rightleftharpoons} \text{Ptl}\cdot\text{EP}_{in} \qquad (7)$$

The apparent binding constant ($K_D{}^{ptl}$) is the average of the two binding constants weighted by the translocation equilibrium:

$$K_D{}^{ptl} = \frac{K^{ptl}_{D,out} K^{ptl}_{D,in} (1 + K_t)}{K^{ptl}_{D,in} + K^{ptl}_{D,out} K_t} \qquad (8)$$

Mannitol uptake follows the same kinetic pathway within the model described by Scheme I as mannitol phosphorylation in the high-affinity phase (see above). However, the inhibition by perseitol is different since, in the uptake experiment, it can only bind to the periplasmically-facing binding site. The inhibition involves states 5, 6, and 13 according to the following scheme:

$$\text{Ptl}\cdot\text{EP}_{out} \overset{K^{ptl}_{D,out}}{\rightleftharpoons} \text{EP}_{out} \overset{K_t}{\rightleftharpoons} \text{EP}_{in} \qquad (9)$$

The apparent affinity is lower than the true affinity at the periplasmic site:

$$K_D{}^{ptl} = K^{ptl}_{D,out}(1 + K_t) \qquad (10)$$

The translocation of the binding site on the unphosphorylated enzyme was shown to be very slow and irrelevant under turnover conditions of the enzyme (Lolkema et al., 1990). However, in equilibrium binding experiments the two orientations of the binding sites equilibrate and the binding of perseitol to unphosphorylated enzyme II^{mtl} is described by equations similar to eqs 7 and 8 with the important difference that all parameters apply to the unphosphorylated state of the enzyme.

It follows from the analysis above that the inhibition of the phosphorylation activity and the competitive binding experiments provide a direct comparison between the affinity of the phosphorylated and the unphosphorylated enzyme for perseitol, respectively. In the solubilized state, the affinity of the phosphorylated enzyme is a factor of 3 lower than that observed for the unphosphorylated enzyme (3.9 μM and 1.3 μM, respectively; Tables I and II). With the membrane-bound enzyme the difference is even smaller, 0.7 μM in the phosphorylated state and 0.8 μM and about 1.3 μM in the unphosphorylated state for the ISO and RSO membrane vesicles, respectively. The affinity for perseitol to both states of the enzyme is higher in the intact membranes than observed in the solubilized state. The same is observed for the binding affinity for mannitol to the unphosphorylated enzyme, but it is the opposite for the kinetic affinity for mannitol in the phosphorylation activity. The differences in binding affinity may reflect changes in the true binding constants or in the translocation equilibria (eq 8). In conclusion, phosphorylation of enzyme II^{mtl} induces no important changes in the affinity for perseitol. Apparently, the structure of the binding site is not affected significantly by phosphorylation of the enzyme per se. Provided that the results obtained with perseitol may be extrapolated to mannitol, it may be concluded that the effects of phosphorylation of the cytoplasmic domain are confined to activation of the translocation step. This suggests that the structure of the binding site is independent of the translocation mechanism and could mean that the structure is not very different in the two orientations of the binding site.

The inhibition constant for perseitol from the uptake experiments provides the upper limit of the true binding constant for perseitol at the periplasmic side of the membrane: $K^{ptl}_{D,out} = 10.9\ \mu M$ when $K_t \ll 1$ (eq 10). Together with the analysis of the inhibition of the mannitol phosphorylation activity, an upper limit is set to the affinity constant for perseitol binding to the cytoplasmically-oriented binding site: $K^{ptl}_{D,in} = 0.75\ \mu M$ when $K_t \gg 1$ (combine eqs 8 and 10). A value for K_t of 14.6 would result in a symmetrical carrier ($K^{ptl}_{D,out} = K^{ptl}_{D,in} = 0.7\ \mu M$).

Analysis of eqs 8 and 10 shows that the apparent binding affinity for perseitol should be lower in the upake experiments than in the phosphorylation experiments, which is in line with the results (Table I). Consequently, equilibration of perseitol over the cytoplasmic membrane before the addition of [^{3}H]-mannitol to the cells in the uptake experiments will results in an increased inhibition. Our experiments showed no increased inhibition after preincubation of the cells with perseitol for 1 h. Therefore, we conclude that perseitol is not transported by enzyme II^{mtl} (see also Scheme I). Apparently, the binding site on enzyme II^{mtl} has a high affinity for perseitol irrespective of its orientation, but the larger perseitol molecule as compared to the mannitol molecule prevents both translocation and phosphorylation (Chart I). It is tempting to conclude that the cytoplasmically-oriented binding site on the translocator domain binds the C_1–C_5 part of mannitol, thereby positioning the C_6 part correctly relative to the phosphoryl group bound to the cytoplasmic domain. The C_7 part of perseitol would stick out too far from the binding site, preventing both phosphoryl group transfer and closing of the binding pocket [see also Lolkema et al. (1992)].

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