Radding, W., Corfield, P. W. R., Levinson, L. S., Hashim, G. A., & Low, B. W. (1988) *FEBS Lett. 231*, 212–216.
Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871–1875.
Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, J. L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261–3266.
Rayner, D. M., Krajcarski, T., & Szabo, A. G. (1977) *Can. J. Chem. 56*, 1238–1245.
Saito, Y., Tachibana, H., & Hayashi, H. (1981) *Photochem. Photobiol. 33*, 289–295.
Shi, Q., Colson, K. L., Lentz, T. L., Armitage, I. M., & Hawrot, E. (1988) *Biophys. J. 53*, 94a.
Steinberg, I. Z. (1968) *J. Chem. Phys. 48*, 2411–2415.
Stryer, L. (1966) *J. Am. Chem. Soc 88*, 5708–5712.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Szabo, A., Lynn, K. R., Krajcarski, D. T., & Rayner, D. M. (1978) *FEBS Lett. 94*, 249–252.
Tao, T., Lamkin, M., & Lehrer, S. (1983) *Biochemistry 22*, 3059–3066.
Teale, F. W. J., & Weber, G. (1957) *Biochem. J. 65*, 476–482.
Weber, G., & Young, L. B. (1964) *J. Biol. Chem 239*, 1424–1430.
Weber, G., Borris, D. P., De Robertis, E., Barrantes, F. J., La Torre, J. L., & De Carlin, M. C. L. (1971) *Mol. Pharmacol. 7*, 530–537.
Weinberg, R. B. (1988) *Biochemistry 27*, 1515–1521.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.
Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790–8794.
Wilson, P. T., Hawrot, E. & Lentz, T. L. (1988) *Mol. Pharmacol. 34*, 643–650.

The Membrane-Bound Domain of the Phosphotransferase Enzyme II^{mtl} of *Escherichia coli* Constitutes a Mannitol Translocating Unit†

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ABSTRACT: The orientation of the mannitol binding site on the *Escherichia coli* phosphotransferase enzyme II^{mtl} in the unphosphorylated state has been investigated by measuring mannitol binding to cytoplasmic membrane vesicles with a right-side-out and inside-out orientation. Enzyme II^{mtl} is shown to catalyze facilitated diffusion of mannitol at a low rate. At equilibrium, bound mannitol is situated at the periplasmic side of the membrane. The apparent binding constant is 40 nM for the intact membranes. Solubilization of the membranes in detergent decreases the affinity by about a factor of 2. Inside-out membrane vesicles, treated with trypsin to remove the C-terminal cytoplasmic domain of enzyme II^{mtl}, showed identical activities. These experiments indicate that the translocation of mannitol is catalyzed by the membrane-bound N-terminal half of enzyme II^{mtl} which is a structurally stable domain.

The mannitol-specific transport protein enzyme II^{mtl} [for reviews, see Postma and Lengeler (1985) and Robillard and Lolkema (1988)][1] catalyzes mannitol transport and phosphorylation via the following reaction in which P-enolpyruvate serves as the initial phosphoryl group donor.

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

Phosphorylated enzyme II^{mtl} is an intermediate in this reaction. Enzyme II^{mtl} is a 68-kDa polypeptide chain for which the primary sequence has been deduced from the base sequence of the cloned mtlA gene (Lee & Saier, 1983). Hydropathy analysis divides the protein into a hydrophobic N-terminal half and a hydrophilic C-terminal half. This is in agreement with topographical studies that demonstrate that half of the enzyme is globular and situated at the cytoplasmic side of the membrane whereas the other half is integrated in the membrane (Stephan & Jacobson, 1986). The latter is assumed to be responsible for the translocation of the solute. The hydrophilic C-terminal half consists of two separate domains that have been subcloned from the mtlA gene and shown to be functional in complementation assays (van Weeghel et al., 1990; White & Jacobson, 1990). Each of these two domains contains a phosphorylation site. His554 accepts the phosphoryl group from P-Hpr and passes it on, via an intramolecular transfer, to Cys384 in the second domain. Phosphorylated Cys384 is the donor for the incoming sugar (Pas & Robillard, 1988).

A high-affinity mannitol binding site on the unphosphorylated enzyme has been demonstrated with purified enzyme II^{mtl} by ultrafiltration (Pas et al., 1988). Data have been presented showing that the site is localized in the N-terminal half of the protein (Grisafi et al., 1989). The present study focuses on the orientation of the binding site on enzyme II^{mtl} with respect to the two sides of the membrane. The binding of mannitol was measured to membrane vesicles with a right-side out (RSO) or an inside-out (ISO) orientation.

Experimental Procedures

Materials

D-[1-^{3}H(N)]Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. Decylpoly(ethylene glycol) 300 (decylPEG) was synthesized by B. Kwant in our labora-

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[1] Abbreviations: ISO, inside-out; RSO, right-side-out; DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol.

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tory. Lysozyme (15 000 units/mg) was from Merck and trypsin TPCK from Worthington. The *Escherichia coli* phosphotransferase enzymes E_I and Hpr were purified as described (Robillard et al., 1979; Dooijewaard et al., 1979).

Methods

Growth Conditions. Escherichia coli strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing, unless otherwise stated, 0.5% mannitol as the carbon source. Cells were harvested at an OD_{650} of 1.0.

ISO membrane vesicles were prepared essentially as described (Reenstra et al., 1980). The buffer used throughout the whole procedure was 25 mM Tris, pH 7.6, 1 mM DTT, and 1 mM NaN_3. The vesicles were washed once with the same buffer. Aliquots of 200 μL containing 1–4 mg/mL membrane protein (Lowry et al., 1951) were stored in liquid nitrogen.

RSO membrane vesicles were prepared by the osmotic lysis procedure (Kaback, 1971) except that 50 μg/mL lysozyme was used to make the spheroplasts. In the final steps, the phosphate buffer was replaced with a 25 mM Tris, pH 7.6, buffer. The vesicle preparation was purified on a sucrose gradient to remove any contaminating cells or spheroplasts. A vesicle suspension containing approximately 1 mg/mL membrane protein in 25 mM Tris, pH 7.6, 1 mM DTT, and 10 mM EDTA was layered on top of 20 mL of a 50% (w/v) sucrose solution in the same buffer and centrifuged for 45 min at 50000*g* in a Sorvall SS34 rotor. The membranes at the interface were collected and washed twice with 200 mL of the same buffer without EDTA. The purification is absolutely essential to prevent the metabolism of mannitol in the binding experiments. The membranes were stored in liquid nitrogen.

Activity Measurements. The residual enzyme II^{mtl} activity of trypsin-treated membranes was assayed by measuring the rate of exchange between 1 mM mannitol-P and 0.4 μM mannitol. The exchange rate and P-enolpyruvate-dependent mannitol phosphorylation rate were measured as described (Robillard & Blaauw, 1987).

Flow Dialysis Experiments. The flow dialysis system consists of two compartments separated by a dialysis membrane. Buffer flows continuously through the lower compartment, a coil-shaped channel. Membranes equilibrated with radioactively labeled substrate are loaded in the upper compartment, which is stirred with a magnetic stirring bar. A small amount of substrate diffuses continuously into the dialyzate in the lower compartment. The amount of radioactivity there is proportional to the free substrate concentration in the upper compartment. The response time of the system indicates the time required for a change in the mannitol concentration in the sample compartment to equilibrate with the dialyzate.

All experiments were performed in 25 mM Tris, pH 7.6, 5 mM DTT, and 5 mM $MgSO_4$ at room temperature. The upper compartment was loaded with 400 μL of membrane suspension. No addition changed the volume by more than 3%. A flow rate through the lower compartment of 0.4 mL/min and a sampling time of 1 min resulted in a half-time of the response of 31 s. The half-time was 14 s with a flow rate of 0.6 mL/min and a sampling time of 12 s. An aliquot from each sample was transferred to a scintillation vial, and 2.5 mL of scintillation emulsifier was added.

A change in the free concentration in the upper compartment was analyzed by treating the response of the system as an exponential. This approach neglects the initial slow phase in the response, which is due to sampling of the dialyzate. Changes in the free concentration were characterized by half-times that were taken as the difference between the $t_{1/2}$ of the displacement experiment and the system response.

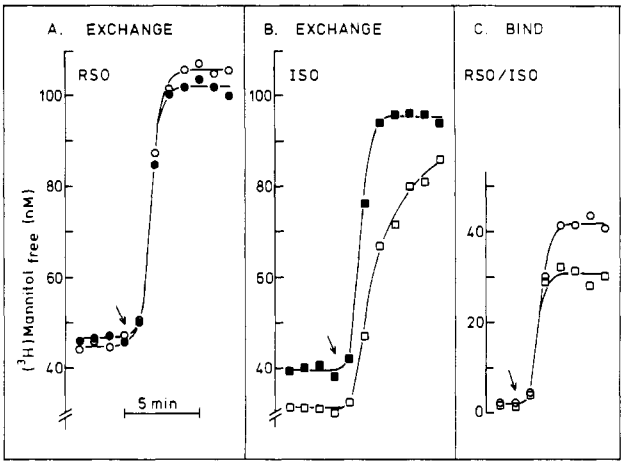


FIGURE 1: Flow dialysis plots of mannitol binding to RSO and ISO membrane vesicles. (A and B) RSO membrane vesicles (A) or ISO membrane vesicles (B) were incubated for 10 min with 100 nM [^{3}H]mannitol in the presence (●, ■) or absence (○, □) of 0.5% decylPEG and loaded in the flow dialysis cell. At the indicated time (arrow), 100 μM unlabeled mannitol was added. (C) The upper compartment was loaded with RSO (○) or ISO (□) membranes. At the indicated time, the suspensions were made 100 nM in [^{3}H]-mannitol. Membrane protein concentrations in all experiments were 0.14 and 0.24 mg/mL for RSO and ISO membranes, respectively. Response time of the system $t_{1/2}$ = 31 s.

RESULTS

Mannitol Binding Measured with Flow Dialysis. The flow dialysis technique allows a continuous measurement of the concentration of free substrate in equilibrium with the enzyme–substrate complex (Colowick & Womack, 1969). When cytoplasmic membranes derived from cells induced for the mannitol transport system were solubilized with the detergent decylPEG and subsequently equilibrated in the upper compartment with 100 nM [^{3}H]mannitol, the radioactivity in the dialyzate in the lower compartment was equivalent to approximately 40 nM [^{3}H]mannitol [Figure 1A (●) and Figure 1B (■)]. Therefore, about 60 nM mannitol was bound to a component solubilized from the membranes, presumably enzyme II^{mtl}. The solubilization procedure made it irrevelant whether the membranes were RSO (Figure 1A) or ISO (Figure 1B) vesicles.

The concentration of free label increased rapidly up to 100 nM upon the addition of 100 μM unlabeled mannitol. Excess unlabeled mannitol displaced essentially all labeled mannitol at the binding sites. The rate at which the free label concentration increased was identical with the response of the system to a stepwise change of the label concentration in the upper compartment. Therefore, with solubilized membranes, the exchange of bound and free mannitol was much faster than the response time of the system.

The accessibility of the binding site on enzyme II^{mtl} was investigated by measuring mannitol binding to intact RSO and ISO membranes. Comparison of the open and closed symbols in Figure 1A,B shows that, after equilibration of the intact membranes with 100 nM [^{3}H]mannitol, the levels of free mannitol were comparable with those found with the solubilized membranes; the amount of mannitol bound to intact versus solubilized membranes was more or less the same irrespective of the orientation. The potentially excluded volume inside the membranes is of no importance in this measurement since the internal volume of the membrane suspensions at these protein concentrations is well below 1% [for the larger RSO membranes, see, for instance, Beneski et al. (1982)] while the

Table I: Binding of Mannitol to Intact ISO Membranes Derived from Cells Grown on Different Carbon Sources[a]

C source	$[mannitol]_{free}$ (nM)	$[mannitol]_{bound}$ (pmol/mg)	enzyme II^{mtl} act. (nmol min^{-1} mg^{-1})
mannitol	50.8	164	998
glucose	55.8	2.6	21
glycerol	49.4	8.4	46

[a] Cells were grown on medium 63 with 0.5% of the indicated carbon sources. The binding was measured after equilibration of 0.3, 3.5, and 1.9 mg/mL membrane protein for the mannitol-, glucose-, and glycerol-induced cells, respectively. The total concentration of [^{3}H]mannitol was 100 nM for the first experiment or 65 nM for the latter two. The second and third column, give the equilibrium concentrations of free and bound mannitol, respectively. Enzyme II^{mtl} activity was measured as the rate of mannitol phosphorylation in the presence of 0.6 mM mannitol, 5 mM P-enolpyruvate, 3.6 μM HPr, and 0.1 μM enzyme I. The buffer contained 25 mM Tris, pH 7.6, 5 mM DTT, 5 mM $MgSO_4$, and 0.5% decylPEG.

amount of substrate bound is approximately 60%.

The data in Table I show that the observed binding is due to the presence of enzyme II^{mtl}. Membranes isolated from cells grown on glucose or glycerol bind only a fraction of the mannitol that is bound by membranes isolated from cells grown on mannitol (Table I). The low levels of binding correlate well with the low rates of mannitol phosphorylation catalyzed by these membranes, indicating that the binding is to enzyme II^{mtl} that is constitutively expressed by the cells. Therefore, all binding can be accounted for by binding to enzyme II^{mtl}; aspecific binding is negligible.

Scatchard Analysis of the Binding. Scatchard plots of the binding to solubilized ISO and RSO membranes in the presence of decylPEG revealed similar binding constants as expected (Figure 2, closed symbols). The K_d values of 97 and 72 nM, respectively, are close to the value of 100 nM that has been reported for the binding of mannitol to purified enzyme II^{mtl} in decylPEG (Pas et al., 1988). The affinity of enzyme II^{mtl} for mannitol in intact membranes was found to be consistently higher. Typical K_D's were 35 and 47 nM for ISO and RSO membranes, respectively (Figure 2, open symbols). Grisafi et al. (1989) have reported a K_D of 1 μM for mannitol binding to intact membranes. However, this number is based upon an incorrect analysis of a nonlinear Scatchard plot (Dahlquist, 1978). Subtraction of the low-affinity contribution from the high-affinity part of the curve, in their data, will indicate a much lower value for the latter.

The maximal number of sites increased some 20% upon solubilization of both RSO and ISO membranes for some unknown reason. Nevertheless, the results show that essentially all sites are accessible both in RSO and in ISO membranes. This, together with the similar K_D's for the two preparations, strongly indicates that at equilibrium the complex between mannitol and enzyme II^{mtl} is the same with ISO and RSO vesicles.

The maximal number of sites in the ISO and RSO preparations in Figure 2 were 550 and 400 pmol/mg of membrane protein, respectively. This corresponds to 7.6% and 5.5% of the membrane protein being enzyme II^{mtl}, assuming one binding site per dimer (Pas et al., 1988). This unusually high level of expression of enzyme II^{mtl} was confirmed when the mannitol phosphorylation activity of membranes of this strain of *E. coli* (ML 308-225) was compared to the same activity of a known concentration of purified enzyme II^{mtl}. The total number of sites differed considerably between different batches of one type of membranes, indicating different levels of expression of enzyme II^{mtl} in the original cells. This explains why the specific binding to the RSO membranes in Figure 1A is 1.5 times higher than to the ISO membranes in Figure 1B,

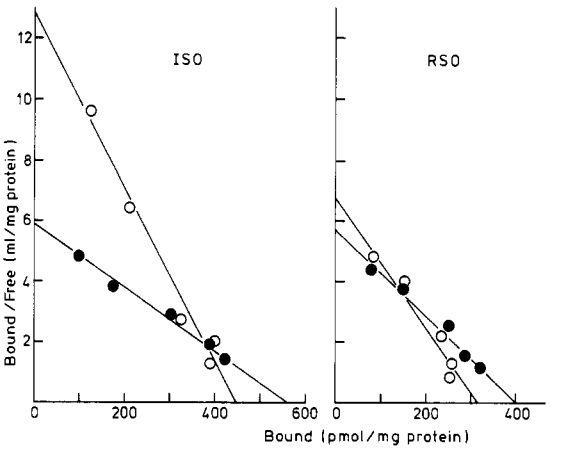


FIGURE 2: Scatchard analysis of the binding of mannitol to intact (○) and solubilized (●) membrane vesicles. ISO and RSO membrane vesicles were incubated with 50, 100, 200, 300, and 400 nM [^{3}H]-mannitol. Free mannitol and bound mannitol were measured in the presence (●) and absence (○) of 0.5% decylPEG. Membrane protein concentration was 0.32 and 0.4 mg/mL for ISO and RSO membranes, respectively. The affinity constants derived from the plots are 35 nM (ISO, intact), 96 nM (ISO, solubilized), 47 nM (RSO, intact), and 72 nM (RSO, solubilized).

whereas the reverse holds in Figure 2 where other batches of membranes were used.

All titrations in Figure 2 show single populations of binding sites. A possible second site with a lower affinity (Pas et al., 1988) would not show up, however, since the binding was only titrated up to total concentrations of 500 nM. The emphasis in this report is on the high-affinity binding site.

Kinetics of Exchange and Binding with Intact Membranes. An identical rate of increase of free label was observed upon addition of excess unlabeled mannitol either to intact RSO vesicles or to solubilized membranes equilibrated with 100 nM [^{3}H]mannitol [compare (○) and (●), Figure 1A]. This indicates a rapid exchange between bound and free mannitol. However, with intact ISO vesicles, the increase was much slower than the response time of the system [Figure 1B (□)]. Exchange of free mannitol with mannitol bound to ISO membranes was much slower than with mannitol bound to RSO vesicles or solubilized membranes. Apparently, mannitol bound to intact ISO membranes is located at the periplasmic side of the membrane (the inner face of the ISO vesicle, the outer face of the RSO vesicle).

The slow exchange can be explained in one of two ways. First, passive diffusion of mannitol across the membrane is the slow process; the binding site on enzyme II^{mtl} is fixed at the periplasmic side. Second, translocation of the loaded binding site followed by dissociation is the slow process; enzyme II^{mtl} catalyzes facilitated diffusion. Discrimination between the two possibilities can be made by analyzing the kinetics of the binding event itself (Figure 1C). In the fixed-site option, [^{3}H]mannitol added to intact ISO membranes would first have to diffuse passively across the membrane before it could bind to the binding sites. This diffusion would be slower than the response time of the system (see above). Consequently, when free label was added at the arrow in Figure 1C, a slow binding would result in a free label concentration that would be transiently much higher than the equilibrium concentration seen in experiment 1B (□). The fixed-site option predicts an overshoot in the free label concentration upon addition of [^{3}H]mannitol to intact ISO membranes. Figure 1C (□) shows, however, that the free concentration immediately rises to the equilibrium concentration, indicating that the binding event itself is much faster than the response time of the system. This

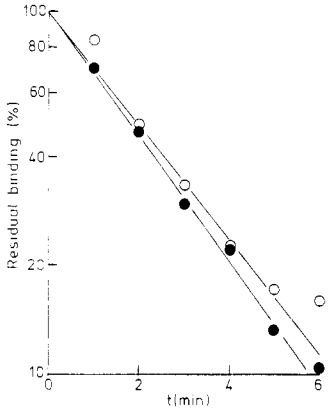


FIGURE 3: Rate of exchange of mannitol bound to ISO membranes with excess unlabeled mannitol, before (●) and after (○) trypsin treatment. Residual binding after addition of 100 μM unlabeled mannitol to the upper compartment is plotted on an exponential scale. Total [^{3}H]mannitol concentration was 100 nM. The slopes of the lines are 0.4 min^{-1} (●) and 0.37 min^{-1} (○), which after correction for the response time of the system ($t_{1/2}$ = 31 s) results in $t_{1/2}$ values for the exchange process of 73 s for the untreated membranes and 82 s for the trypsin-treated membranes.

is not consistent with the fixed-site option. This leaves the second option that the loaded binding site on enzyme II^{mtl} translocates between the two faces of the membrane. The initial event in the binding is binding to the unloaded site at the external face of the membranes. Since the binding to ISO as well as RSO membranes is fast (Figure 1C), we must conclude that the unloaded binding site isomerizes rapidly between the two faces of the membrane. Once mannitol is bound, the loaded site is situated preferentially at the periplasmic side of the membrane (inside the ISO vesicle). For exchange of bound label with unlabeled mannitol [Figure 1B (□)], the loaded site must translocate to the exterior of the ISO vesicle and release the label. The overall rate constant for these steps is different from the overall rate constant for the same steps in the opposite direction [Figure 1C (□)]. This difference in rate constants in two directions is typical for enzyme-catalyzed translocation and inconsistent with passive diffusion.

The time course of the exchange of [^{3}H]mannitol bound to ISO membranes with excess unlabeled mannitol is analyzed in Figure 3 (●). The decay of bound [^{3}H]mannitol followed a single exponential, indicating that, at equilibrium, all bound mannitol is situated at the periplasmic side of the membrane. It should be noted, however, that rapid initial decays up to about 10% would be difficult to detect because of limitations in the rate of sampling of the dialyzate in the lower compartment.

We have attributed the slow exchange rate in Figure 1B (□) to translocation of the loaded carrier to the exterior followed by dissociation. However, one could postulate that the rate of exchange is determined instead by exchange of unlabeled mannitol with a pool of free [^{3}H]mannitol diluted with unlabeled mannitol. Assume, for instance, that unlabeled mannitol entered the vesicle and exchanged with bound [^{3}H]mannitol, leading to an internal pool of [^{3}H]mannitol of lower specific activity. Many turnovers of the enzyme, depending on the dilution ratio, would be necessary to reach equilibrium of the internal [^{3}H]mannitol of low specific activity and the external unlabeled mannitol. This could be the "slow process" in Figure 1B (□). For this to be correct, the observed rate of exchange should be dependent on the mannitol concentrations used since that will determine the dilution factor of the [^{3}H]mannitol. This was examined by preequilibrating

Table II: Rate of Exchange of [^{3}H]Mannitol Bound to Intact ISO Membranes with Different Concentrations of Free [^{1}H]Mannitol[a]

[^{3}H]mannitol concn (μM)	[^{1}H]mannitol concn (μM)	$t_{1/2}$ (s)
1.0	10	58
0.2	10	64
0.2	100	69
0.2	1000	61

[a] ISO membranes were equilibrated with the [^{3}H]mannitol concentrations given in the first column. [^{1}H]Mannitol was added at the concentrations in the second column, and the decrease of binding was followed with time. The response was analyzed as described under Methods. System response $t_{1/2}$ = 14 s. Membrane protein concentration in the experiment with 1.0 μM [^{1}H]mannitol was 1.5 mg/mL; for the other experiments, it was 0.44 mg/mL.

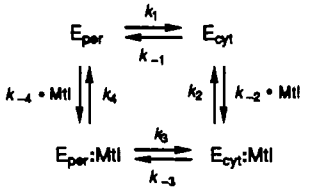
the membranes with two different [^{3}H]mannitol concentrations and varying the unlabeled mannitol concentrations 1000-fold. The data in Table II show that, within experimental error, the rate of exchange was independent of the bound [^{3}H]mannitol concentration or the concentration of excess unlabeled mannitol. We consistently observe a decrease of the binding according to a single exponential and to a level that could be predicted solely based upon the ratio of labeled and unlabeled mannitol. Therefore, the exchange process demonstrates single turnover of the bound mannitol pool.

Binding to Trypsin-Treated ISO Membranes. The cytoplasmic domain of enzyme II^{mtl} embedded in the membrane of an ISO vesicle can be removed by trypsinolysis (Stephan & Jacobson, 1986). Addition of 50 μg/mL trypsin to ISO membranes equilibrated with 100 nM [^{3}H]mannitol had no effect on the level of binding (not shown). Control experiments showed that this treatment instantaneously abolished over 90% of the mannitol phosphorylation activity. Addition of excess unlabeled mannitol released bound mannitol at a rate comparable to that observed with the untreated ISO membranes [Figure 3, (○) versus (●)]. These experiments emphasize that the membrane-bound part of enzyme II^{mtl} is a structurally stable domain that catalyzes mannitol transport even in the absence of the cytoplasmic domains.

Discussion

Facilitated diffusion catalyzed by enzyme II of the bacterial phosphotransferase system has been reported before. The function was inferred from the efflux of accumulated pts sugars from cells (Solomon et al., 1983; Reizer et al., 1983; Reizer & Saier, 1983) or from steady-state phosphorylation by ISO membranes (Lolkema & Robillard, 1985). Facilitated diffusion in these studies was from the cytoplasmic side of the membrane to the periplasmic side and under phosphorylating conditions. The present study demonstrates that the *E. coli* enzyme II^{mtl} catalyzes facilitated diffusion of mannitol across the cytoplasmic membrane in the absence of P-HPr. It was shown that all of the binding sites on enzyme II^{mtl} were accessible both in RSO and in ISO membranes. The different rates of binding and expelling of mannitol to and from ISO membranes are consistent with the translocation of mannitol by enzyme II^{mtl}. The facilitated diffusion property of the unphosphorylated mannitol carrier has no relevance for mannitol catabolism for the very simple reason that *E. coli* does not possess a kinase or dehydrogenase to utilize mannitol. In addition, the carrier would be kinetically incompetent for two reasons: (i) the rate of transport into the cell is very slow; (ii) due to the high affinity of the carrier for mannitol, the transporter would become trapped in the loaded form at submicromolar internal concentrations of mannitol. The diffusion of mannitol into the cell may play a role in induction

Scheme I



of the genes for mannitol metabolism.

Mechanism of Translocation. The present results are consistent with a single translocation site model as depicted in Scheme I. The insensitivity of the rate of exchange to very high concentrations of unlabeled mannitol indicates no involvement of a second site with lower affinity in the translocation mechanism (Table II). In models where mannitol moves within the protein from a binding site at one side of the membrane to a second site at the opposite side of the membrane, high concentrations of unlabeled mannitol would occupy the second site and inhibit the exchange rate. The slow exchange between mannitol bound to ISO membranes and excess unlabeled mannitol demonstrates the transition between E_{per}:Mtl and E_{cyt}:Mtl, followed by dissociation of mannitol at the cytoplasmic side (E_{cyt}:Mtl → E_{cyt}). The single-exponential time course of the displacement (Figure 3) indicates that, at equilibrium, all bound mannitol is situated at the periplasmic side (state E_{per}:Mtl). Association/dissociation at this side is demonstrated by the exchange of mannitol bound to RSO membranes with excess unlabeled mannitol (E_{per}:Mtl ↔ E_{per}, Figure 1A). This equilibrium is fast relative to the response time of the system. Binding to ISO membranes involves, among other steps, the translocation of the loaded carrier from the cytoplasmic to the periplasmic side of the membrane (E_{cyt}:Mtl → E_{per}:Mtl). The equilibrium distribution between E_{cyt}:Mtl and E_{per}:Mtl indicates that this transition is much faster than the reverse transition ($k_3 \gg k_{-3}$). The steps involved in the binding events (Figure 1C) depend on the equilibrium distribution of the unloaded carrier over E_{cyt} and E_{per}. Nevertheless, the rapid binding kinetics to both RSO and ISO membranes at room temperature demonstrate that the binding site isomerizes rapidly between the two states.

Mechanistic Relevance. The overall catalytic activity of enzyme II^{mtl} is the coupled transport and phosphorylation of mannitol. However, in terms of catalytic mechanism, the transport and kinase activities are separable functions that the enzyme somehow manages to couple. The cytoplasmic domain contains the kinase activity. The hydrophobic membrane-bound half of the protein constitutes the machinery to transport mannitol. The structural and catalytic integrity of this domain is supported by the trypsinolysis results. The present data show that the transport of mannitol from periplasm to cytoplasm catalyzed by the N-terminal domain in the absence of P-HPr is slow. Transport coupled to phosphorylation is about 3 orders of magnitude faster as estimated from the turnover number for steady-state phosphorylation of mannitol catalyzed by purified enzyme II^{mtl}. Therefore, phosphorylation of the cytoplasmic domain of enzyme II^{mtl} speeds up the transport process catalyzed by the membrane-bound domain. The measured rate constant for the displacement of mannitol bound to intact ISO membranes is a combination of the rate constants for the translocation of the loaded binding site (k_3 and k_{-3}) and the dissociation constant at the cytoplasmic side of the membrane (k_2). Phosphorylation of the enzyme may affect one or both of these steps. In the case of an increased translocation rate, the mechanism of coupling is at the level of the transport step itself. In the case that the dissociation of mannitol is the slow step, the coupling is at the level of phoshorylation of mannitol, the slow dissociation of mannitol being replaced by the fast dissociation of mannitol-P. At present, experiments are in progress to determine the relative contribution of these two steps in the slow exchange process. This will allow us to determine the mechanism of coupling between the kinase and transport activity of enzyme II^{mtl}.

Registry No. Mtl, 69-65-8; phosphotransferase enzyme II^{mtl}, 37278-09-4.

References

Beneski, D. A., Misko, T. P., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14656–14575.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Dahlquist, F. (1978) *Methods Enzymol. 48*, 270–299.

Dooijewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry 18*, 2990–2996.

Grisafi, R. L., Scholle, A., Sugayama, J., Briggs, L., Jacobson, G. R., & Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719–2727.

Kaback, H. R. (1971) *Methods Enzymol. 22*, 99–120.

Lee, C. A., & Saier, M. H. (1983) *J. Biol. Chem. 258*, 10761–10767.

Lolkema, J. S., & Robillard, G. T. (1985) *Eur. J. Biochem. 147*, 69–75.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Pas, H. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5835–5839.

Pas, H. H., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5520–5525.

Postma, P. W., & Lengeler, J. W. (1985) *Microbiol. Rev. 49*, 232–269.

Reenstra, W. W., Patel, L., Rottenberg, H., & Kaback, H. R. (1980) *Biochemistry 19*, 1–9.

Reizer, J., & Saier, M. H. (1983) *J. Bacteriol. 156*, 236–242.

Reizer, J., Novotny, M. J., Panos, C., & Saier, M. H. (1983) *J. Bacteriol. 156*, 345–361.

Robillard, G. T., & Blaauw, M. (1987) *Biochemistry 26*, 5796–5803.

Robillard, G. T., & Lolkema, J. S. (1988) *Biochim. Biophys. Acta 947*, 493–519.

Robillard, G. T., Dooijewaard, G., & Lolkema, J. S. (1979) *Biochemistry 18*, 2984–2989.

Saier, M. H., Jr., Simoni, R. D., & Roseman, S. (1976) *J. Biol. Chem. 251*, 6584–6597.

Solomon, E., Miyai, K., & Lin, E. C. C. (1973) *J. Bacteriol. 114*, 723–728.

Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 8230–8234.

van Weeghel, R. P., Meyer, G. H., Keck, W. H., & Robillard, G. T. (1990) *Biochemistry* (submitted for publication).

White, D. W., & Jacobson, G. R. (1990) *J. Bacteriol. 172*, 1509–1515.