Mechanistic coupling of transport and phosphorylation activity by enzyme IIm" of the Escherichia coli phosphoenolpyruvate-dependent phos-photransferase system?

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Mechanistic Coupling of Transport and Phosphorylation Activity by Enzyme II^{mtl} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System†

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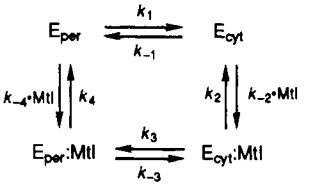
ABSTRACT: Mannitol bound to enzyme II^{mtl} could be trapped specifically by rapid phosphorylation with P-HPr. The assay was used to demonstrate transport of mannitol across the cytoplasmic membrane with and without phosphorylation of mannitol. The latter was 2–3 orders of magnitude slower. The fraction of bound mannitol molecules that was actually phosphorylated, the efficiency of the trap, was less than 50%. The efficiency was not very different for enzyme II^{mtl} embedded in the membrane of vesicles with an inside-out orientation or solubilized in detergent. Subsequently, it is argued that the fraction of the bound mannitol molecules that was not phosphorylated dissociated into the cytoplasmic space. A model for the catalytic mechanism of enzyme II^{mtl} is proposed on the basis of interpretations of the present experiments. The main features of the model are the following: (i) mechanistically, the coupling between transport and phosphorylation is less than 50%; (ii) in the physiological steady state of mannitol transport and metabolism, the coupling is 100%; (iii) phosphorylated enzyme II^{mtl} catalyzes facilitated diffusion at a high rate; (iv) the state of phosphorylation of the cytoplasmic domain modulates the activity of the translocator domain; (v) the enzyme catalyzes phosphorylation of free cytoplasmic mannitol at least as fast as it catalyzes transport plus phosphorylation of free periplasmic mannitol.

The majority of hexoses and hexitols are transported into bacterial cells via a P-enolpyruvate-dependent phosphotransferase system and appear as sugar phosphates in the cytoplasm. The transport proteins, enzymes II, catalyze both transport and phosphorylation [for reviews, see Postma and Lengeler (1985) and Robillard and Lolkema (1988)]. The flow of the phosphoryl group from P-enolpyruvate to the sugar is mediated through sequential phosphorylation and dephosphorylation of a number of proteins, the last one being enzyme II. The mannitol-specific transport protein enzyme II^{mtl}[1] receives the phosphoryl group from P-HPr, which itself is phosphorylated by P-enolpyruvate in a reaction catalyzed by enzyme I. Therefore, the overall reaction catalyzed by enzyme II^{mtl} is

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

Enzyme II^{mtl} is a 68-kDa polypeptide; half of the protein is membrane-bound; the other half protrudes into the cytoplasm (Lee & Saier, 1983; Stephan & Jacobson, 1986). The latter consists of two domains, each one carrying a phosphoryl group binding site (Saier et al., 1988; Pas & Robillard, 1988; Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a). The most C-terminal domain is phosphorylated by P-HPr, after which the phosphoryl group is transferred to the second domain. Mannitol accepts the phosphoryl group directly from this second cytoplasmic domain. Intuitively, this indicates that even though in the overall reaction (eq 1) transport and phosphorylation are coupled events, mechanistically the two activities may very well be separate steps; i.e., mannitol is first transported to an internal site on the membrane-bound part of the enzyme from where it is phosphorylated by the cytoplasmic domain. Recently, we have presented data that support this notion (Lolkema et al., 1990). The membrane-bound half of enzyme II^{mtl} was shown to be a structurally stable domain that appeared to catalyze transport of mannitol across the membrane in the absence of phosphorylation (Scheme I). The state where mannitol is bound to the cytoplasmic facing binding site (E_{cyt}:Mtl) is likely to be the site where mannitol gets phosphorylated under phosphorylating conditions. The very low rate of translocation of the binding site in the absence of phosphorylation suggests that this facilitated diffusion is a manifestation of part of the catalytic cycle of the enzyme, rather than a metabolically relevant process. The translocator slips to some extent. Clearly, coupling of phosphorylation to transport leads to a significant activation of the translocator. The coupling mechanism would involve interaction between the cytoplasmic domain and the membrane-bound domain.

Scheme I



The translocator could be described by a four-state model (Scheme I). E_{cyt} and E_{per} denote those conformations of the enzyme in which a single binding site is exposed to the cytoplasmic and periplasmic side of the membrane, respectively.

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[1] Abbreviations: ISO, inside out; DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol; pts, phosphotransferase system.

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At equilibrium, mannitol bound to enzyme II^{mtl} embedded in the membrane of a vesicle with an inside-out (ISO) orientation is situated at the inner face of the membrane (state E_{per}:Mtl). In the present paper, the coupling of phosphorylation to transport is studied by taking this situation as a starting point and following the fate of bound mannitol upon phosphorylation of enzyme II^{mtl}. The effects of phosphorylation will be shown by trapping specifically those mannitol molecules that are bound to enzyme II^{mtl}.

Materials and Methods

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 laboratory. The *Escherichia coli* phosphotransferase enzymes E_I and HPr were purified as described (Robillard et al., 1979; van Dijk et al., 1990).

Escherichia coli strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing 0.5% mannitol as the carbon source. Cells were grown in 1-L serum bottles and aerated by blowing in sterile air near the bottom of the bottle. Cells were harvested at an OD_{650} of 1.0.

ISO membrane vesicles were prepared essentially as described (Reenstra et al., 1980). Instead of phosphate buffer, the buffer used throughout the whole procedure was 25 mM Tris, pH 7.6, 1 mM DTT, and 1 mM NaN_3. In addition, a batch of vesicles was prepared in 25 mM KP_i, pH 7.6, 1 mM $MgCl_2$, and 1 mM NaN_3 for some control experiments. 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. Samples were thawed rapidly before use. Each sample was used only 1 time.

Activity Measurements. Binding of [^{3}H]mannitol to enzyme II^{mtl} was measured with flow dialysis as described (Lolkema et al., 1990). P-Enolpyruvate-dependent mannitol phosphorylation catalyzed by enzyme II^{mtl} was measured as described (Robillard & Blaauw, 1986).

Mannitol-P Burst Experiments. ISO membrane vesicles in 25 mM Tris, pH 7.6, 5 mM DTT, and 5 mM $MgCl_2$ were incubated with the appropriate concentration of [^{3}H]mannitol in a total volume of 75 μL for 10 min and in duplicate (see also Scheme II). To one of the tubes (the experiment) was added 5 μL of water: to the other (the control) was added 5 μL of 200 mM unlabeled mannitol. Both samples were incubated for 10 min at 30 °C or for 20 min at 20 °C. A volume of 20 μL containing 2.5 μM P-HPr and 50 mM unlabeled mannitol is then added to the experiment sample, and an equal volume of 2.5 μM P-HPr solution without unlabeled mannitol is added to the control sample. Both samples are then assayed for [^{3}H]mannitol-P at intervals of 1 min as described (Robillard & Blaauw, 1986). The mannitol-P burst was measured as a function of the P-HPr concentration and shown to be maximal with 0.5 μM.

The two P-HPr solutions were prepared by incubating 2.5 μM HPr, 110 nM E_I, and 5 mM P-enolpyruvate in 25 mM Tris, pH 7.6, 5 mM DTT, and 5 mM $MgCl_2$. One of them in addition contained 50 mM unlabeled mannitol. The mixtures were incubated for 10 min, irrespective of the assay temperature.

Results

Trapping of Bound Mannitol by Rapid Phosphorylation. Mannitol bound specifically to enzyme II^{mtl} could be trapped as mannitol-P by the procedure outlined in Scheme II. ISO membranes derived from cells induced for the mannitol transport system were equilibrated with a low concentration

Scheme II

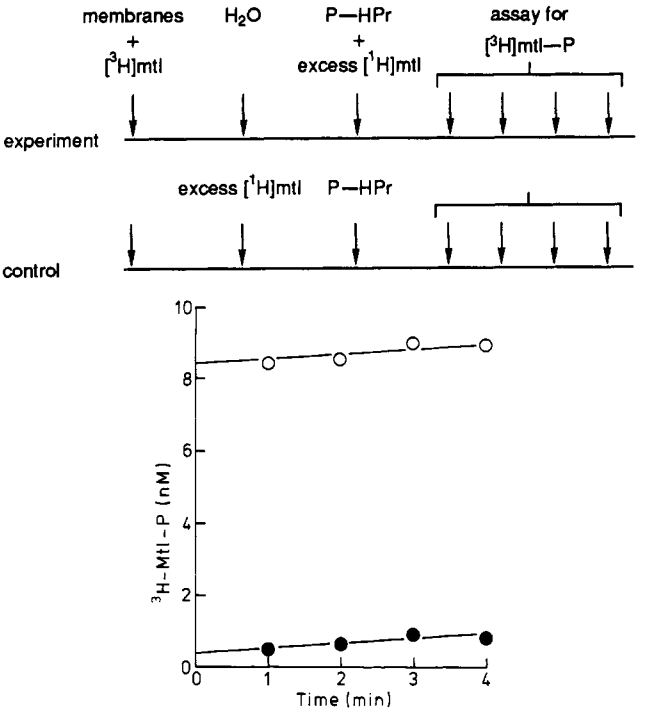


FIGURE 1: Trapping of bound mannitol by rapid phosphorylation. ISO membranes (80 μg/mL) were incubated with 100 nM [^{3}H]mannitol. The experiment was initiated by adding 0.5 μM P-HPr and 10 mM unlabeled mannitol (○). In the control experiment (●), 10 mM unlabeled mannitol is added 10 min before 0.5 μM P-HPr. For details, see Materials and Methods.

of labeled mannitol. Rapid phosphorylation of bound mannitol followed upon the addition of the phosphoryl group donor P-HPr. A large excess of unlabeled mannitol was added together with the phosphoryl group donor to prevent binding and subsequent phosphorylation of free [^{3}H]mannitol. In a control experiment, bound [^{3}H]mannitol was allowed to exchange with excess unlabeled mannitol before P-HPr was added. This led to undetectable levels of labeled mannitol bound to enzyme II^{mtl}, but the steady-state rate of phosphorylation was the same. Bound mannitol showed up as an offset between the two lines (Figure 1).

Coupling of Phosphorylation to Transport. Mannitol bound to enzyme II^{mtl} in membrane vesicles with an inside-out (ISO) orientation has been shown to be situated at the inner, periplasmic face of the membrane (state E_{per}:Mtl; Lolkema et al., 1990). The phosphorylation trap converts this pool of bound mannitol to mannitol-P which ends up at the cytoplasmic side of the membrane. The mannitol-P burst shows that transfer from the periplasmic binding site to the cytoplasmic volume is very rapid. It is too fast to be estimated from these single-turnover measurements, but the process correlates with the steady-state rate of vectorial phosphorylation at saturating mannitol concentrations, where the periplasmic facing binding site is continuously saturated with substrate. Recently, this maximal rate was estimated from P-enolpyruvate-driven mannitol uptake into proteoliposomes reconstituted with purified enzyme II^{mtl}. The turnover number was 243 min^{-1} at 30 °C (Elferink et al., 1990).

The rate of transfer of bound mannitol to the cytoplasmic space uncoupled from phosphorylation may be measured by the phosphorylation trap by varying the time between the addition of unlabeled mannitol and P-HPr (see the control experiment in Scheme II). The experiment described in Figure 1 was repeated with increasing intervals between the addition

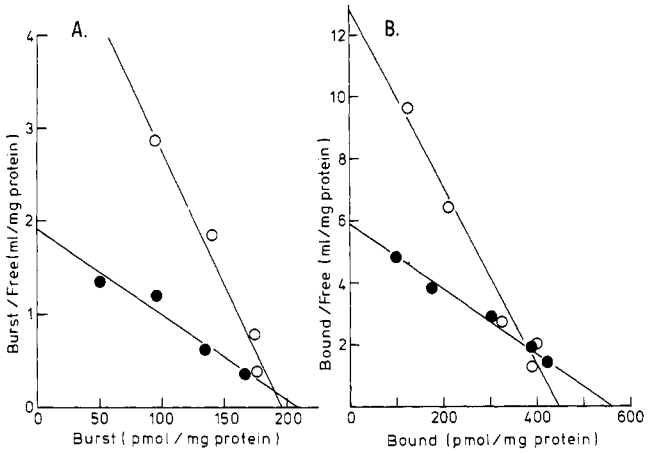


FIGURE 2: Scatchard analysis of the mannitol-P burst (A) and mannitol binding (B). (A) ISO membranes (80 μg/mL) were incubated at 20 °C with 50, 100, 150, and 250 nM [^{3}H]mannitol in the presence (●) and absence (○) of 0.25% decylPEG. The burst of mannitol-P was measured as described. The free mannitol concentrations were computed from the binding parameters determined from the experiments in (B). The affinity constants calculated from the figure are 109 (●) and 35 nM (○). (B) [^{3}H]Mannitol binding was measured to the same membrane preparation with flow dialysis. The data were reproduced from Lolkema et al. (1990). The slopes indicate affinity constants of 95 (●) and 35 nM (○).

of [^{1}H]mannitol and P-HPr, which resulted in a decreasing burst of mannitol-P. In these experiments, the phosphorylation trap is simply used to measure the fraction of the [^{3}H]mannitol molecules that did not yet exchange with the unlabeled mannitol. A logarithmic plot of the residual burst versus time resulted in rate constants of 0.51 min^{-1} at 20 °C and 1.44 min^{-1} at 30 °C. These data show that bound mannitol (E_{per}:Mtl) exchanged slowly with excess unlabeled mannitol added to the cytoplasmic volume before enzyme II^{mtl} was phosphorylated. The exchange measures the rate of transfer of mannitol bound at the inner face of the vesicle (state E_{per}:Mtl) to the cytoplasmic space (state E_{cyt}). The same process was measured by a direct binding assay using flow dialysis (Lolkema et al., 1990). The release of [^{3}H]mannitol bound to ISO membranes was followed in time after the addition of a large excess of unlabeled mannitol. When the same membrane preparation was used as in the above experiment at a temperature of 20 °C, the rate constant for the exchange was 0.57 min^{-1}, in fair agreement with the 0.51 min^{-1} obtained from the mannitol-P burst experiments.

In conclusion, transport of bound mannitol across the membrane is accelerated by at least 2 orders of magnitude when coupled to phosphorylation.

Titration of the Binding Sites. The mannitol-P burst was measured after equilibration of membranes with different concentrations of [^{3}H]mannitol. A Scatchard analysis of the data is presented in Figure 2A. At room temperature, the affinity constants in the presence and absence of the detergent decylPEG were 109 and 35 nM, respectively. In parallel, the binding of mannitol to the same membrane preparation was measured directly with the flow dialysis technique. It was demonstrated before that all binding measured in this way could be accounted for by binding to enzyme II^{mtl} (Lolkema et al., 1990). The Scatchard analysis of the binding data revealed similar K_D's in the presence and absence of the detergent decylPEG, 97 and 35 nM, respectively (Figure 2B). The maximal mannitol-P burst (Figure 2A) together with the maximal number of binding sites (Figure 2B) gives the efficiency of the trap, the fraction of bound molecules that is actually phosphorylated. The efficiency was 44% and 37% for the intact and solubilized membranes, respectively.

Table I: Affinity of Enzyme II^{mtl} for Mannitol As Measured with Mannitol-P Burst and Efficiency of the Mannitol Phosphorylation Trap Measured under Different Conditions[a]

temperature (°C)	decylPEG	K_d (nM)	efficiency (%)
20	-	35	44
	+	109	37
30	-	52	42
	+	277	32

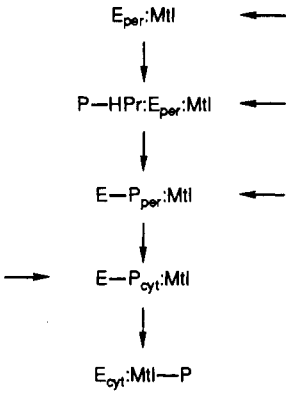
[a] The experiments were performed as described in the legend of Figure 2. The efficiency is defined as the ratio of the burst at infinite mannitol concentration to the total number of binding sites.

Scheme III

(A) E:*Mtl + P—HPr ⟶ E + HPr + *Mtl—P

E:*Mtl + Mtl ⟶ E:Mtl + *Mtl

(B)



We have repeated the titration of the binding sites using the phosphorylation burst at 30 °C which has been the assay temperature for most kinetic measurements in the past. The K_d of the solubilized enzyme for mannitol was raised considerably to 277 nM, whereas the K_d of the enzyme in intact membranes was elevated only slightly to 52 nM. In both cases, the efficiency of the trap was somewhat lower, 32 and 42%, respectively (Table I).

To exclude possible effects of increased leakiness due to the preparation of the vesicles in Tris buffers, we have repeated the titration of the binding sites and the mannitol-1-P burst with ISO membranes prepared in phosphate buffer. The efficiency of the trap measured in 25 mM KP_i, pH 7.6, 5 mM $MgCl_2$, and 5 mM DTT was 46% at room temperature.

Efficiency of the Phosphorylation Trap. Not all [^{3}H]-mannitol molecules bound to enzyme II^{mtl} will be trapped since a fraction exchanges with unlabeled mannitol, added together with P-HPr, before it gets phosphorylated. The efficiency is determined by the rate of phosphorylation relative to the rate of exchange (Scheme IIIA). The latter depends solely on the dissociation rate of bound mannitol, since unlabeled mannitol is added in excess. Scheme IIIB indicates the steps that lead to the phosphorylation of labeled mannitol bound to enzyme II^{mtl}: (i) association of P-HPr to enzyme II^{mtl}; (ii) transfer of the phosphoryl group to the cytoplasmic domain of enzyme II^{mtl}; (iii) isomerization of the loaded binding site; and (iv) transfer of the phosphoryl group to mannitol. Only the very first step in this sequence can be controlled experimentally. Even though the concentration of P-HPr in the assays was chosen such that higher concentrations did not result in a higher burst, more than 50% of the bound mannitol pool is released unphosphorylated (Table I). Exchange with unlabeled mannitol may take place at the periplasmic side (right arrows Scheme IIIB) or at the cytoplasmic side (left arrow) of the membrane (E_{per}:Mtl and E_{cyt}:Mtl, respectively). With intact

Table II: Mannitol-P Burst When Quenched with Different Concentrations of Unlabeled Mannitol[a]

excess mannitol concn (μM)	mannitol-P burst (nM)	
	preparation 1	preparation 2
10	4.2	3.3
100	3.3	3.3
1000	3.6	3.2
10000	3.5	3.1

[a] Membranes were incubated with 50 nM [^{3}H]mannitol at 20 °C. P-HPr was added together with unlabeled mannitol at the concentrations in the first column. Membrane protein concentrations were 67.5 and 45 μg/mL for membrane preparations 1 and 2, respectively. The steady-state rates of phosphorylation with 10 μM unlabeled mannitol that followed the burst were 3.2 and 3.5 nM/min for preparations 1 and 2, respectively. The efficiency of the trap with preparation 1 was 42%; with preparation 2, it was somewhat lower, 31%.

ISO membranes, exchange at the periplasmic side would require competitive rates of phosphorylation and influx of unlabeled mannitol, followed by exchange with labeled mannitol at the binding sites. The latter process cannot be excluded since (i) routinely the excess of unlabeled mannitol is chosen very high (factor of 10^5) to reduce the steady-state rate of phosphorylation, (ii) facilitated diffusion at a considerable rate catalyzed by enzymes II^{mtl} under phosphorylating conditions has been reported (Solomon et al., 1973; Reizer et al., 1983; Reizer & Saier, 1983; Lolkema & Robillard, 1985), and (iii) exchange of free and bound mannitol at the periplasmic site is fast (Lolkema et al., 1990). Nevertheless, we will argue that exchange at the periplasmic side of the membrane is not responsible for the low efficiency of the phosphorylation trap by showing that, even if this happened, it would not result in a reduced burst in the case of intact ISO membranes.

Conditions in the experiment shown in Table II were such that much lower concentrations of unlabeled mannitol were sufficient to quench the mannitol-P burst. The burst did not change significantly when quenched with concentrations of unlabeled mannitol from 10 μM to 10 mM. Assuming, for the sake of argument, that equilibration of unlabeled mannitol over internal and external phases was instantaneous and that the internal volume was 4 μL/mg of membrane protein, it can be calculated that 10 μM mannitol amounts to 40 pmol of internal mannitol per milligram of protein. This small amount of mannitol is the substrate in the normal vectorial phosphorylation reaction catalyzed by enzyme II^{mtl} (eq 1). Consequently, it is pumped out of the vesicle at a high rate. The rate of phosphorylation of mannitol catalyzed by the membranes solubilized in detergent, at 10 μM mannitol and 0.5 μM HPr, was measured to be 660 pmol s^{-1}·(mg of protein)$^{-1}$. As a result, it takes a fraction of a second to pump the 40 pmol of free mannitol out of the vesicles. Under steady-state phosphorylation conditions, the internal concentration of mannitol is extremely low. This very fact was experimentally demonstrated with enzyme II^{fru} of *Rhodopseudomonas sphaeroides* (Lolkema & Robillard, 1985). Therefore, even if the exchange at the internal face of the ISO vesicle happened, all labeled mannitol which was initially bound at the periplasmic side would still show up in the burst. Finally, our data show that such an exchange does not occur since (i) the burst is not significantly lower when quenched with 10 mM mannitol (Table II) and (ii) the efficiency of the trap is very similar with ISO vesicles versus membranes solubilized in detergent (Table I).

In conclusion, more than half of the mannitol molecules bound to enzyme II^{mtl} are lost in the phosphorylation trap. It can be argued that this is due to two competing processes with comparable rates, the dissociation from the cytoplasmic facing binding site and the phosphoryl group transfer to mannitol at this binding site.

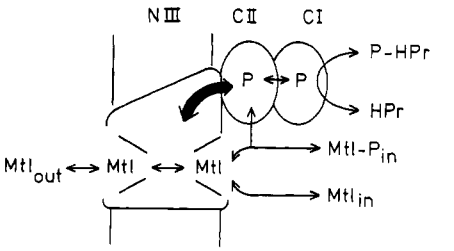


FIGURE 3: Schematic representation of the catalytic mechanism of enzyme II^{mtl} in relation to its domain structure. CI, CII, and NIII indicate the three domains of the enzyme. Capital P's in the cytoplasmic domains represent phosphoryl group binding sites. The interaction between CII and the translocator NIII is indicated by the wide arrow. For further explanation, see the text.

Discussion

Domain Structure and Activity. Evidence is accumulating that enzyme II^{mtl} consists of three domains: a membrane-bound domain, which is about half of the molecule, and two smaller cytoplasmic domains. Partial activities that together make up the overall activity of enzyme II^{mtl} may be recognized in these domains (Saier et al., 1988; Pas & Robillard, 1988; Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,b; Lolkema et al., 1990). On the basis of the present results and other experimental data, we propose a model of enzyme II^{mtl} which relates the kinetic mechanism and the domain structure (Figure 3). The N-terminal domain constitutes a mannitol translocator. Cytoplasmic domain CI accepts the phosphoryl group from P-HPr and transfers it to cytoplasmic domain CII. The latter takes care of the mannitol kinase activity together with the internally oriented binding site on the translocator. The kinetic features of enzyme II^{mtl} in this model will be discussed below in relation to the present results.

Coupled and Uncoupled Transport. The trapping of bound mannitol by rapid phosphorylation with P-HPr provides a direct demonstration of the effect of coupling of phosphorylation to the transport of mannitol. Mannitol bound to enzyme II^{mtl} embedded in the membrane of ISO vesicles is situated at the periplasmic side of the membrane. Slow transfer to the cytoplasmic space, catalyzed by the translocator, was demonstrated by addition of excess unlabeled mannitol at the cytoplasmic side of the membrane. The process is accelerated 2–3 orders of magnitude when coupled to phosphorylation of mannitol as evidenced by the burst of phosphorylated mannitol molecules upon addition of P-HPr.

Facilitated Diffusion. The efficiency of the mechanism of trapping of bound mannitol depends on the rate of phosphoryl group transfer to the bound sugar relative to the rate of dissociation of the bound sugar. Over 50% exchanges with unlabeled mannitol and, consequently, does not show up in the burst (Table I). Even though the evidence is indirect, it is very likely that the dissociation takes place, after translocation, into the cytoplasmic volume. If so, these single-turnover measurements indicate that the rate of dissociation of mannitol into the cytoplasm

$$E\text{–}P_{cyt}\text{:Mtl} \rightarrow E\text{–}P_{cyt} + Mtl_{in}$$

is at least as fast as the phosphoryl group transfer to mannitol followed by dissociation of mannitol-P

$$E\text{–}P_{cyt}\text{:Mtl} \rightarrow E_{cyt} + Mtl\text{–}P_{in}$$

(see also Figure 3). In the sequence presented in Scheme IIIB, the steps involved in transport of mannitol from periplasm to

cytoplasm with or without phosphorylation of mannitol are identical up to state E–P_{cyt}:Mtl. Consequently, phosphorylated enzyme II^{mtl} catalyzes facilitated diffusion into the cell at a rate comparable to the rate of mannitol transport coupled to phosphorylation.

In the model, enzyme II^{mtl} catalyzes two modes of facilitated diffusion: a low-activity mode in the unphosphorylated state (E) and a high-activity mode in the phosphorylated state (E–P). The state of phosphorylation of the cytoplasmic domain is signaled to the translocator and modulates the activation energy of the transport process. The modulation requires direct interaction between the two domains as indicated by the wide arrow in Figure 3. In the following paper, we will provide more direct evidence for this domain/domain interaction (Lolkema et al., 1991).

Kinase Activity. According to the model, roughly one out of every two mannitol molecules that arrive at state E–P_{cyt}:Mtl after transport from the periplasm is actually phosphorylated to mannitol-P under the experimental conditions used in this study. This implies that the coupling between transport and phosphorylation of mannitol is less than 50%. On the other hand, since the dissociation of unphosphorylated mannitol in the cytoplasm is a reversible process, free mannitol in the cytoplasm should be able to bind to the binding site on the translocator and be phosphorylated. In the model, the kinase within enzyme II^{mtl} uses the inwardly directed binding site on the translocator as substrate binding site. The steps leading to state E–P_{cyt}:Mtl would be irrelevant to the phosphorylation reaction. State E–P_{cyt}:Mtl follows upon translocation of the loaded site from the periplasmic side of the membrane (E–P_{per}:Mtl → E–P_{cyt}:Mtl) or upon direct binding of mannitol in the cytoplasm (E–P_{cyt} → E–P_{cyt}:Mtl). Mechanistically, there would be no obligatory coupling between transport and phosphorylation. In the model, mannitol phosphorylation from the cytoplasm should be at least as fast as transport plus phosphorylation from the periplasm, since the transport step is omitted in the former reaction.

The interpretation of the results provides a mechanistic explanation for efflux of unphosphorylated pts sugars from cells (Gachelin, 1970; Solomon et al., 1973; Reizer et al., 1983; Reizer & Saier, 1983) as well as enzyme II catalyzed phosphorylation of pts sugars in the cytoplasmic space (Thomson & Chassy, 1985; Delobbe et al., 1976).

Mannitol Transport. Assuming a high phosphorylation potential inside the cell, the model proposed in Figure 3 predicts the following events upon addition of mannitol to the outside of the cell. Initially, only half of the molecules that are transported into the cell become phosphorylated. The other half is released as free mannitol inside the cell. External and internal free mannitol will rapidly equilibrate. Once this physiological steady state is reached, the substrate in the phosphorylation reaction may come either from the cytoplasm or from the periplasm. As a result, the phenomenological coupling between transport and phosphorylation under these conditions reaches 100%. How does enzyme II^{mtl} do the job? Basically it is a facilitated-diffusion enzyme with a built-in sugar trap.

The most surprising conclusion of our studies is that the coupling between phosphorylation and transport is not at the level of the phosphorylation of the sugar but at the level of the transport of the sugar. The translocator is activated by phosphorylation of the enzyme.

Steady-state kinetics of fructose phosphorylation catalyzed by the fructose-specific enzyme II^{frc} in *Rhodopseudomonas sphaeroides* have led to a model for the mechanism of enzymes II that at certain points agrees with the present findings but at other points has to be modified and extended (Robillard & Lolkema, 1988). In the model based on those measurements, the accessibility of a single high-affinity binding site from a given side of the membrane depended on the state of phosphorylation of the carrier. In the unphosphorylated state, the binding site faced the cytoplasm; in the phosphorylated state, it faced the periplasm. Transport was achieved by a continuous phosphorylation of enzyme II by P–HPr, followed by dephosphorylation by the sugar. In addition, facilitated diffusion catalyzed only by the phosphorylated enzyme was part of the model.

Clearly, single-turnover measurements as presented in this paper and in Lolkema et al., (1990) add much more detail to the mechanism. The present data support the single translocating binding site of the model and confirm the facilitated diffusion catalyzed by the phosphorylated enzyme. The isomerization of the binding site on the unphosphorylated enzyme most likely did not show up in steady-state phosphorylation kinetics since it was too slow. Even though there is an apparent coupling between the orientation of the binding site and the state of phosphorylation of the enzyme under turnover conditons, it is not an essential step in the mechanism. In the present interpretation, the coupling is not obligatory.

The present data indicate that enzyme II^{mtl} should be able to phosphorylate mannitol in the cytoplasm directly, a process that was not foreseen in the model based upon the *Rhodopseudomonas sphaeroides* data. Analysis of the steady-state rate of phosphorylation of mannitol by the detergent-solubilized enzyme II^{mtl} as a function of mannitol concentration shows biphasic kinetics, in accord with phosphorylation through the periplasmic and cytoplasmic facing binding sites (unpublished results). The same analysis with enzyme II^{fru} showed monophasic kinetics (Lolkema & Robillard, 1985). A complete analysis of the steady-state kinetics of phosphorylation of mannitol catalyzed by enzyme II^{mtl} and their relation to the present results will be published elsewhere.

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Interaction between the Cytoplasmic and Membrane-Bound Domains of Enzyme II^{mtl} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System†

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ABSTRACT: Sulfhydryl reagents affected the binding properties of the translocator domain, NIII, of enzyme II^{mtl} in two ways: (i) the affinity for mannitol was reduced, and (ii) the exchange rate of bound and free mannitol was increased. The effect on the affinity was very much reduced after solubilization of enzyme II^{mtl} in the detergent decylPEG. The effects were caused exclusively by reaction of the sulfhydryl reagents with the cysteine residue at position 384 in the primary sequence. Interaction between two domains is involved, since Cys384 is located in the cytoplasmic domain, CII. When Cys384 was mutated to serine, the enzyme exhibited the same binding properties as the chemically modified enzyme. The data support our proposal that phosphorylation of enzyme II^{mtl} drastically reduces the activation energy for the translocation step through interaction between domains CII and NIII [Lolkema J. S., ten Hoeve-Duurkens, R. H., Swaving Dijkstra, D., & Robillard, G. T. (1991) *Biochemistry* (preceding paper in this issue)]. Functional interaction between the translocator domain, NIII, and domain CI was investigated by phosphorylation of His554, located in domain CI, in the C384S mutant. No effect on the binding properties was observed. In addition, the binding properties were insensitive to the presence of the soluble phosphotransferase components enzyme I and HPr.

Enzyme II^{mtl} [1] of the bacterial P-enolpyruvate-dependent phosphotransferase system is responsible for the transport of mannitol into the cell. It combines the transport activity with the phosphorylation of mannitol at the expense of P-enolpyruvate. Mannitol appears as mannitol-P in the cytoplasm [for recent reviews, see Postma and Lengeler (1985) and Robillard and Lolkema (1988)]. The primary phosphoryl group donor is P-HPr that itself is phosphorylated by P-enolpyruvate in a reaction catalyzed by enzyme I. Phosphorylated enzyme II^{mtl} is a catalytic intermediate in the phosphoryl group transfer from P-HPr to mannitol.

The primary sequence of enzyme II^{mtl} from *Escherichia coli* was deduced from the base sequence of the cloned MtlA gene (Lee & Saier, 1983). Studies in which fragments of the gene were subcloned or the gene was partially digested from the C-terminal end suggest that the enzyme consists of three domains (Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991b,c). These have been called CI, CII, and NIII from the C-terminal to the N-terminal of the protein, respectively. About half of the protein constitutes the N-terminal domain NIII. It is very hydrophobic and presumably completely membrane-bound. NIII and the complementary C-terminal half of the protein, termed CIII, were expressed separately in *E. coli*. Mannitol phosphorylation was restored after bringing the two together. CIII was shown to protrude into the cytoplasm (Stephan & Jacobson, 1986). It has been isolated as a soluble protein which consists of the two domains CI and CII. CI, CII, and CIII have been expressed separately in *E. coli* and shown to be functional proteins.

Enzyme II^{mtl} is phosphorylated at two distinct sites during turnover, a histidine residue at position 554 in the primary sequence and a cysteine residue at position 384, both located in the cytoplasmic domains (Pas & Robillard, 1988b). His554 in domain CI accepts the phosphoryl group from P-HPr after which transfer takes place to Cys384 in domain CII. From

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[1] Abbreviations: ISO, inside out; RSO, right side out; DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol; pts, phosphotransferase system; NEM, *N*-ethylmaleimide; diamide, 1,1'-azobis(*N*,*N*-dimethylformamide).

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