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Functional Reconstitution of the Purified Phosphoenolpyruvate-Dependent Mannitol-Specific Transport System of *Escherichia coli* in Phospholipid Vesicles: Coupling between Transport and Phosphorylation

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Purified mannitol-specific enzyme II (EII) from *Escherichia coli* was reconstituted into phospholipid vesicles with the aid of a detergent-dialysis procedure followed by a freeze-thaw sonication step. The orientation of EII in the proteoliposomes was random. The cytoplasmic moiety of the inverted EII could be removed with trypsin without effecting the integrity of the liposomal membrane. This enabled us to study the two different EII orientations independently. The population of inverted EII molecules was monitored by measuring active extrusion of mannitol after the addition of phosphoenolpyruvate, EI, and histidine-containing phosphocarrier protein (HPr) at the outside of the vesicles. The population of correctly oriented EII molecules was monitored by measuring active uptake of mannitol with internal phosphoenolpyruvate, EI, and HPr. A low rate of facilitated diffusion of mannitol via the unphosphorylated carrier could be measured. On the other hand, a high phosphorylation activity without translocation was observed at the outside of the liposomes. The kinetics of the phosphoenolpyruvate-dependent transport reaction and the nonvectorial phosphorylation reaction were compared. Transport of mannitol into the liposomes via the correctly oriented EII molecules occured with a high affinity (K_m, lower than 10 μM) and with a relatively low V_{max}. Phosphorylation at the outside of the liposomes catalyzed by the inverted EII molecules occurred with a low affinity (K_m of about 66 μM), while the maximal velocity was about 10 times faster than the transport reaction. The latter observation is kinetic proof for the lack of strict coupling between transport and phosphorylation in these enzymes.

Enzyme II mannitol (EII^{Mtl}) of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) in *Escherichia coli* is an integral membrane protein, responsible for the coupled transport and phosphorylation of its substrate, mannitol. The transport reaction occurs at the expense of PEP. The phosphoryl group is transferred from PEP via two cytoplasmic proteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr), to the actual transport protein, EII, according to the scheme in Fig. 1.

Some systems, such as the glucose PTS, contain another cytoplasmic protein, EIII, which transfers the phosphoryl group from HPr to glucose-specific EII. The nucleotide sequences of various EIIs and EIIIs have been compared. On basis of these analyses, it was proposed that EII species which operate without a separate EIII possess an EIII-like domain at their C termini (19). A review of the structure and sugar transport mechanism of the different EIIs (17) and a general review of the PTS (14) have appeared previously.

EII^{Mtl} was the first EII to be purified to homogeneity (5) and sequenced (7) and, consequently, has been the most extensively studied. The enzyme consists of a membrane-bound domain and a cytoplasmic domain of about equal size (30 kDa) (4, 20). The cytoplasmic part forms a globular protein, which can be removed from the membrane by trypsinolysis (20). The remaining membrane-bound domain is still able to bind mannitol (3). Purified EII^{Mtl} possesses one high-affinity and one low-affinity binding site per dimer (13). In agreement with the proposed EIII function coupled to EII, EII^{Mtl} contains two phosphoryl-binding sites (12). The phosphoryl group coming from HPr is first donated to histidine 554 and then transferred to cysteine 384. The enzyme is active only in the reduced form and can be irreversibly inactivated with *N*-ethylmaleimide (NEM).

The catalytic activity of EII^{Mtl} in the solubilized form is usually measured by monitoring the phosphorylation of mannitol. The actual transport activity of the purified enzyme can be studied only if the enzyme is first reconstituted in liposomes. The major advantage of reconstitution is that no interference from other transport systems can occur. Reconstitution of EII^{Mtl} has been reported previously (8); however, in the preparation of Leonard and Saier, only the transphosphorylation reaction, mannitol transport, and phosphorylation at the expense of internal mannitol 1-phosphate could be observed (8).

(Some of the results reported here have been presented at the FEMS Symposium on Group Translocation in Bacterial Membrane Transport at the Pasteur Institute, Paris, France, 7 to 9 September 1988 [18].)

MATERIALS AND METHODS

D-[1-^{14}C]mannitol (59 mCi/mmol) and [^{14}C]PEP (38.6 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, Buckinghamshire, United Kingdom). D-[^{3}H]mannitol (19.1 Ci/mmol) was from NEN. *E. coli* L-α-phosphatidylethanolamine (type IX) was obtained from Sigma. The phospholipid preparation was further purified by acetone-ether extraction, suspended in 50 mM potassium phosphate (pH 7.5)–2 mM dithiothreitol (DTT), and stored in

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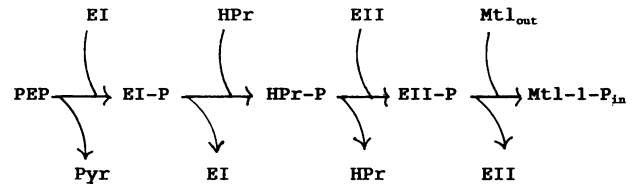

FIG. 1. Transfer of phosphoryl group from PEP to mannitol via EI, HPr, and EII.

liquid nitrogen. EII^{Mtl} was purified from *E. coli* ML 308/225 as described elsewhere (15). EI and HPr were purified from *E. coli* as described previously (1, 16). Decyl-polyethylene glycol 300 (decyl-PEG) was synthesized by B. Kwant in our laboratory. Sodium deoxycholate was recrystallized twice from acetone-H_2O. Trypsin tolylsulfonyl phenylalanyl chloromethyl ketone was obtained from Worthington. Trypsin inhibitor soybean type I-S was from Sigma.

Reconstitution of EII^{Mtl}. The procedure to reconstitute EII^{Mtl} was essentially as described by Driessen et al. (2). Phosphatidylethanolamine was suspended in 0.24% deoxycholate by sonication and mixed with purified EII suspended in decyl-PEG at a protein/lipid ratio of 1 to 925 (wt/wt). The preparation was dialyzed once for 24 h at 4°C against 20 mM Trischloride (pH 7.5)–2 mM DTT in the presence of 0.35% deoxycholate and twice for 9 and 16 h at 4°C against 50 mM potassium phosphate (pH 7.5)–1 mM DTT in the absence of deoxycholate. The preparation was then stored in liquid nitrogen. Before use, a small sample of the liposomes was thawed and sonicated for 8 s with a probe-type sonicator (Vibra cell; Sonics & Materials Inc, Danbury, Conn.). It was possible to entrap [^{14}C]mannitol or a mixture of PEP, EI, and HPr inside the liposomes by adding these components before sonication. The lipid concentration in the final preparation was 32.5 mg/ml, and the EII concentration was 0.58 μM. The internal volume was calculated from the fraction of ^{14}C-mannitol or ^{14}C-PEP entrapped inside the liposomes during sonication. Sonication resulted in equal concentrations of the radioactive compound on both sides of the membrane. To determine the internal volume, we measured the total radioactivity per unit of volume, filtered off the liposomes, and measured the total radioactivity of the liposome fraction. The latter value divided by the former gave the fractional internal volume. The internal volume of the concentrated liposomes was 8%, which corresponds to 2.5 μl/mg of lipid.

Phosphorylation of mannitol. The phosphorylation activity of reconstituted EII^{Mtl} was measured in 50 mM potassium phosphate (pH 7.5) at 30°C by measuring the amount of [^{14}C]Mtl-P formed. The procedure was performed essentially as described elsewhere (15). The reaction was started by the addition of [^{14}C]^{Mtl} to the liposome suspension in the presence of PEP, EI, and HPr. At distinct time intervals, samples (20 to 50 μl) were taken and loaded on a column containing a 1-ml bed volume of Dowex AG 1-X2 resin. The nonphosphorylated sugar was removed by washing three times with 2 ml of H_2O. Subsequently, the phosphorylated sugar was eluted with two 2-ml volumes of 0.2 M HCl directly into scintillator vials. Then 2 ml of Packard liquid scintillator TM 299 was added, and the radioactivity was counted. When the total amount of mannitol phosphate after transport was measured, the samples were first diluted 80 times into 0.5% lubrol PX to completely dissolve the liposomes.

Vectorial transport of mannitol. Efflux of mannitol out of the liposomes was measured by using liposomes to which 10 or 100 μM [^{3}H]mannitol (790 and 79 mCi/mmol, respectively) or 1 mM [^{14}C]mannitol (4 to 12 mCi/mmol) was added before sonication. Efflux was measured after a 10-fold dilution of the concentrated liposome suspension in 50 mM potassium phosphate (pH 7.5). At indicated time intervals, samples (50 to 100 μl) were diluted in 2 ml of 50 mM potassium phosphate (pH 7.5), rapidly filtered through cellulose nitrate membrane filters with a pore size of 0.2 μm (BA83; Schleicher & Schuell), and washed once with another 2 ml of buffer. The filters were put into scintillation vials with 4 ml of Packard liquid scintillator TM 299 and counted.

Active extrusion of mannitol was measured similarly after the addition of PEP, EI, and HPr at the outside.

Active uptake of mannitol into the liposomes was measured by using liposomes preloaded with a mixture of PEP (10 mM), EI (0.5 μM), and HPr (6 μM) during sonication. ^{14}C-mannitol (7 μM, 59 mCi/mmol) was added at the outside of the liposomes, and the accumulation of mannitol was measured after sampling and filtration as described above. Specific conditions for each experiment can be found in the figure legends.

Polyacrylamide-sodium dodecyl sulfate slab gel electrophoresis was performed according to the method of Laemmli (6). The gel contained 12.5% acrylamide. After electrophoresis, the gel was stained with silver according to the method of Wray et al. (22).

Electron microscopy. For freeze fracturing, concentrated proteoliposomes in 10% glycerol were rapidly frozen in liquid Freon 12. Freeze-etch replicas were prepared with a Balzer BA 360 freeze-etching unit (Balzer, Liechtenstein).

RESULTS

Preparation of proteoliposomes. Purified *E. coli* EII^{Mtl} was reconstituted into liposomes as described in Materials and Methods. For complete removal of decyl-PEG, it was necessary to include deoxycholate in the first dialysis buffer. The total EII-dependent phosphorylation activity of the proteoliposomes, in the presence of 1% decyl-PEG, was 85% of the activity of the original EII preparation, indicating that 15% of the activity was lost during the entire procedure of liposome preparation.

Figure 2A shows an electron micrograph of a negatively stained liposome preparation. The size distribution varied between 200 and 500 nm. Figure 2B shows a freeze-fracture electron micrograph of the same preparation. EII^{Mtl} is visible as randomly distributed small particles.

Orientation of EII in proteoliposomes. EII^{Mtl} consists of a membrane-bound domain and a cytoplasmic domain. Trypsin inactivates EII in inverted vesicles but not in spheroplasts because it cleaves off the cytoplasmic domain (4, 20). We have treated proteoliposomes with trypsin in the presence or absence of detergent and stopped the reaction with trypsin inhibitor after 10, 20, or 30 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the treated liposomes illustrated the results (Fig. 3). Untreated proteoliposomes are shown (lane 1) with a 60-kDa band representing intact EII^{Mtl}. When liposomes were treated with trypsin in the absence of detergent, the intensity of the 60-kDa band decreased and a 34-kDa band with an intensity approximately equal to that of the remaining 60-kDa band appeared (lanes 2 to 4). The 34-kDa band represents the membrane-bound portion of the inverted EII population. The cytoplasmic portion is further degraded and cannot be

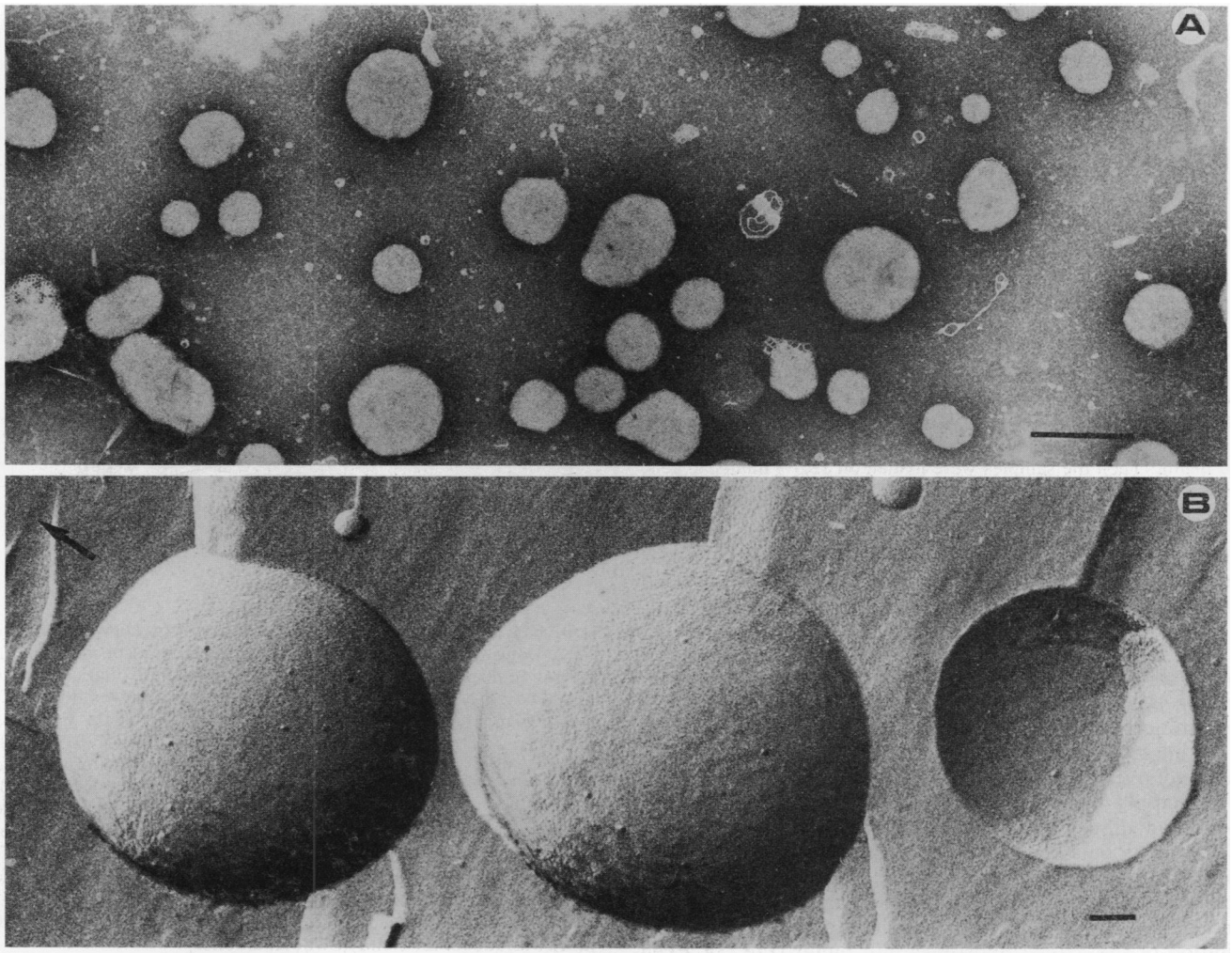

FIG. 2. (A) Electron micrograph of a negatively stained liposome preparation. Bar = 500 nm. (B) Freeze-fracture replica of a similar preparation. The arrow indicates the direction of shadowing. Bar = 50 nm.

seen on these gels (20). Trypsin treatment in the presence of detergent resulted in the complete loss of the 60-kDa band. (lanes 5 to 7). Since trypsin treatment of the intact liposomes degraded approximately half of the EII, it would appear that there is a random orientation of EII. This was substantiated by measuring the phosphorylation activity in the presence of 1% decyl-PEG, 6 μM HPr, 0.5 μM EI, 5 mM PEP, and 1 mM mannitol before and after trypsin treatment of the intact liposomes. The activity after trypsin treatment was 14 nmol · min^{-1} · mg of $lipid^{-1}$, which was 50% of the original activity, confirming that trypsin degrades half of the EII molecules in intact liposomes, consistent with an equal number of EII molecules facing in each direction.

Active extrusion of mannitol. When mannitol is trapped inside the liposomes, the EIIs with inverted orientations should be able to catalyze the active extrusion at the expense of externally added PEP, EI, and HPr (see scheme B in Fig. 8a). Trypsin should inhibit this process by cleaving off the outwardly oriented cytoplasmic domains. The effect of trypsin on the active extrusion of mannitol was measured in two different preparations (Fig. 4A). In the first preparation, trypsin was present during the first 32 min of the experiment to trypsinolyse the cytoplasmic part of the inverted EIIs. Before the addition of a mixture of EI, HPr, and PEP, trypsin inhibitor was added to prevent the inactivation of the soluble PTS components. In the second preparation, trypsin inhibitor was added before trypsin to prevent the digestion of the inverted EIIs. Both preparations showed only a slow efflux of mannitol in the first 35 min. However, a rapid extrusion of mannitol was observed in the preparation with intact EII after addition of a mixture of PEP, EI, and HPr at the outside of the liposomes at the time indicated by the arrow in Fig. 4A. Removal of the cytoplasmic domain with trypsin did not influence the slow efflux in the first 35 min but completely blocked the PEP-dependent extrusion of mannitol. NEM, which inactivates EII^{Mtl}, also inhibited the PEP-dependent transport of mannitol, although not completely, while the slow efflux of mannitol in the presence of EI and HPr was unchanged (Fig. 4B). Upon the addition of PEP at 24 min, active extrusion of mannitol in the untreated preparation was observed, while extrusion via the NEM-treated liposomes was significantly inhibited. A control sample was taken from both preparations before the addition of PEP to measure the phosphorylation activity separately. The control experiment showed that the phosphorylation activity after NEM treatment was 8% of the untreated vesicles, explaining why some PEP-dependent efflux still occurred with the NEM-treated preparation. In contrast, the phos-

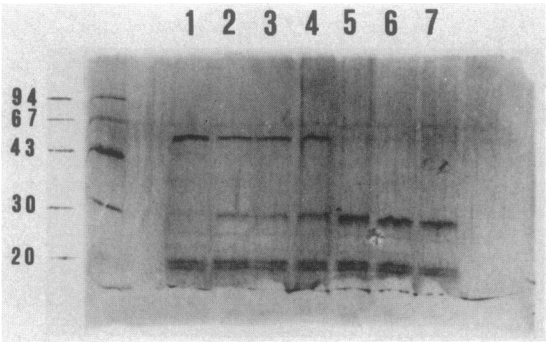

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of liposomes after treatment with trypsin and decyl-PEG. Liposomes were diluted fourfold in 50 mM potassium phosphate (pH 7.5) and incubated with trypsin (5 μg/ml) in the absence and in the presence of 1% decyl-PEG. After 10, 20, and 30 min, a sample was taken and trypsin inhibitor was added to a final concentration of 0.5 mg/ml. Then the samples were loaded on 12.5% mini-slab gels. Lane 1, Intact proteoliposomes; lanes 2, 3, and 4, proteoliposomes incubated with trypsin for 10, 20, and 30 min, respectively; lanes 5, 6, and 7, proteoliposomes incubated with trypsin and decyl-PEG for 10, 20, and 30 min, respectively. The two lower bands present in all samples represent trypsin and trypsin inhibitor. Markers in the left lane are indicated in kilodaltons.

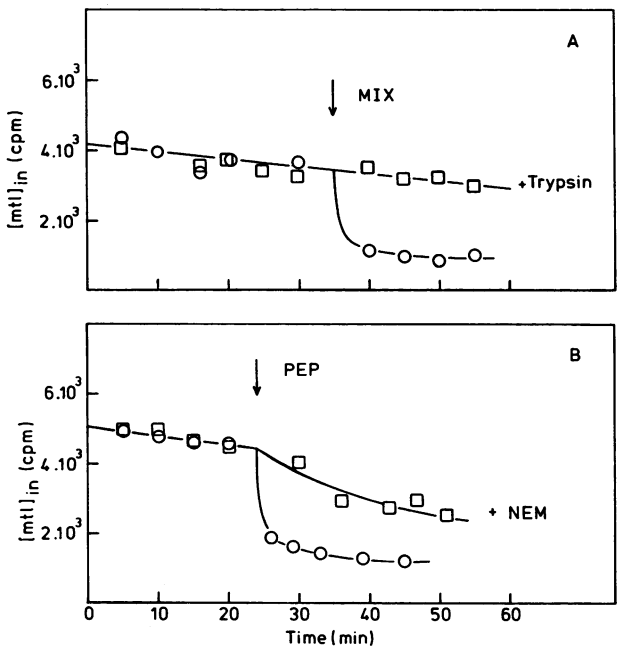

FIG. 4. Active extrusion of mannitol. (A) Proteoliposomes were loaded during sonication with 1 mM [^{14}C]mannitol (4 mCi/mmol). Efflux of mannitol was measured after a 10-fold dilution of the liposome suspension containing 1 mM internal and external ^{14}C-mannitol in 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, 5 mM NaF, and trypsin (0.5 μg/ml) (□) or trypsin inhibitor (0.5 mg/ml) plus trypsin (0.5 μg/ml) (○). After 32 min, trypsin inhibitor was also added to the first preparation. At 35 min, a mixture of PEP (10 mM), EI (0.5 μM), and HPr (6 μM) was added. (B) Proteoliposomes were loaded as for panel A and incubated with 4 mM NEM for 1 min at 30°C. The reaction was terminated by the addition of 10 mM DTT. Efflux of mannitol was measured after a 10-fold dilution of the liposomes in 50 mM potassium phosphate (pH 7.5)–5 mM $MgSO_4$–5 mM NaF–5 mM DTT–0.5 μM EI–6 μM HPr. After 24 min, PEP (10 mM) was added. □, NEM; ○, no additions.

phorylation activity at the outside of the liposomes after trypsin treatment was about 0.5 to 1% that of the original preparation.

Facilitated diffusion of mannitol. The slow movement of mannitol over the phospholipid membrane in the absence of PTS activity could be caused by passive diffusion or by facilitated diffusion via the unphosphorylated carrier. To discriminate between these two possibilities, we measured the efflux rate at different mannitol concentrations. Liposomes were loaded during sonication with 10 μM, 100 μM, or 1 mM mannitol, and efflux was measured after a 10-fold dilution of the loaded liposomes into buffer (Fig. 5). Even though the concentration gradient was the same in all three cases, efflux was faster and equilibrium was reached more quickly at the lower mannitol concentrations. This is the expected behavior for an enzyme-catalyzed efflux. Low internal substrate concentrations are exhausted more rapidly than high internal substrate concentrations. The involvement of EII^{Mtl} in this efflux process was confirmed by examining the same process by using glucose, a nonspecific sugar, in place of mannitol. In this case, efflux and the approach to equilibrium followed the same time course for the two substrate concentrations measured, indicating that glucose exited the liposome by passive diffusion. Finally, when liposomes without EII^{Mtl} were loaded with 1 mM or 100 μM mannitol and diluted 10-fold, efflux and the approach to equilibrium also followed the same time course expected for passive diffusion.

After equilibration of mannitol was achieved, about 30% of the initial label was still inside or associated with the liposomes, instead of the 10% expected on the basis of a 1:10 dilution. This could be caused by the presence of small liposomes which did not contain EII.

Active uptake of mannitol. Figure 6 reports the ability of the proteoliposomes to accumulate mannitol via the correctly oriented EII molecules. Liposomes, containing enclosed PEP, EI, and HPr, were diluted 10-fold, and [^{14}C]mannitol was added to the medium. No mannitol was accumulated in the absence of internal PEP, EI, and HPr. At a substrate concentration of 7 μM, an accumulation ratio of 50 was measured, representing an uptake of 22% of the total amount of mannitol present. The factor determining the steady-state level of accumulation was the phosphorylation activity at the outside of the liposomes, since PEP, EI, and HPr were still present, although at levels a factor of 10 lower than levels inside, after dilution. The EII population with outwardly facing cytoplasmic domains was able to deplete the mannitol pool by converting it to mannitol phosphate before it could be transported. This was shown by measuring the phosphorylation and transport activity simultaneously. When the steady-state level of accumulation was reached, all of the mannitol at the outside was present as mannitol 1-phosphate. Elimination of external phosphorylation activity was accomplished by dialyzing away most of the external PEP and adding pyruvate kinase and ADP to the incubation mixture during the transport experiment in order to convert the remaining external PEP to ATP. This procedure resulted in 90% of the mannitol being taken up inside the liposomes (Fig. 6).

Since the external mannitol concentration at the start of the transport experiment was 7 μM and the internal volume of the liposome preparation after inclusion of PEP, EI, and HPr and subsequent 1-to-10 dilution was 0.53%, the uptake of 90% of the label indicates that the internal mannitol concentration was 1.1 mM.

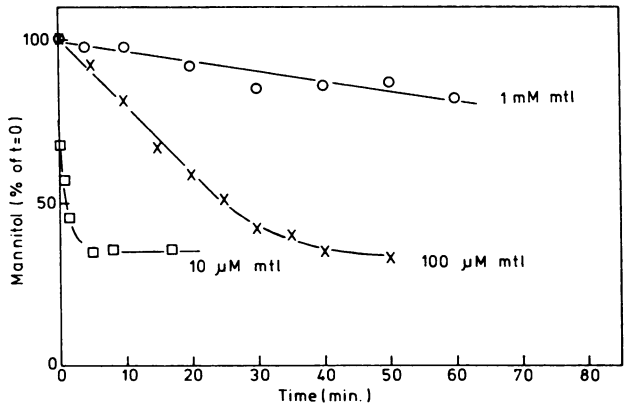

FIG. 5. Facilitated diffusion via the unphosphorylated carrier. Proteoliposomes were loaded during sonication with 1 mM ^{14}C-^{Mtl} or 100 or 10 μM ^{3}H-^{Mtl}. Efflux of mannitol was measured after a 10-fold dilution of the liposome suspension in 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, and 5 mM DTT.

The mannitol, once taken up into the liposomes as mannitol 1-phosphate, did not come out again. When, after a steady-state level of accumulation had been reached, a 100-fold excess of unlabeled mannitol was added at the outside of the liposomes, no efflux of label out of the liposomes could be observed. This indicates that exchange or transphosphorylation did not occur at a measurable rate.

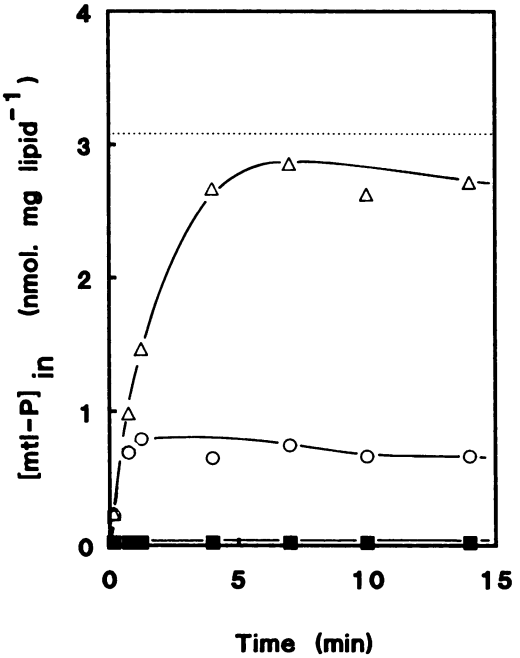

FIG. 6. Active uptake of mannitol. Proteoliposomes were loaded during sonication with a mixture of PEP (10 mM), EI (0.5 μM), HPr (6 μM), DTT (1 mM), and $MgCl_2$ (5 mM). Uptake of mannitol was measured after a 10-fold dilution of the liposomes in potassium phosphate (pH 7.5) and preincubation for 5 min at 30°C. [^{14}C] mannitol (59 mCi/mmol) was added at the outside to a concentration of 7 μM. ○, Liposomes without any further additions; △, dialyzed liposomes after incubation with pyruvate kinase (100 μg/ml) and 2 mM ADP for 10 min at 30°C; ■, liposomes without inclusion of PEP, EI, and HPr. The dotted line represents the total amount of mannitol present in the reaction mixture.

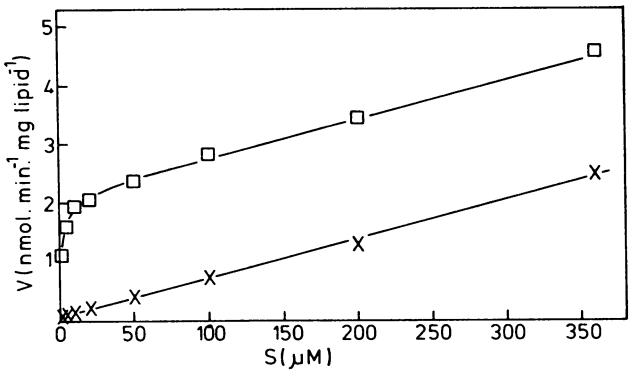

FIG. 7. Kinetics of vectorial transport into the liposomes. Liposomes were loaded during sonication with PEP (10 mM), EI (0.5 μM), HPr (100 μM), DTT (4 mM), and $MgCl_2$ (4 mM). Then the liposomes were incubated with trypsin (20 μg/ml) for 10 min at 30°C. The rate of mannitol transport into the liposomes was determined from the amount of accumulated label during the first 10 s in triplicate. The incubation medium contained 50 mM potassium phosphate (pH 7.5) and the liposomes diluted 10-fold with respect to the original preparation. [^{14}C]Mtl (59 mCi/mmol) was added in concentrations between 2 and 360 μM. The sample volume was 100 μl. □, Rate of transport plotted as a function of the mannitol concentration; ×, influx of [^{14}C]mannitol measured under the same experimental conditions in unloaded liposomes.

Kinetics of transport and phosphorylation. In the previous sections, we have demonstrated the vectorial transport processes into and out of the liposome (see schemes C and B, Fig. 8a) and we have been confronted with nonvectorial phosphorylation, the formation of external Mtl-P when PEP, HPr, EI, and Mtl were present outside the liposome, catalyzed by inverted EII^{Mtl} molecules (see scheme D in Fig. 8a). The purpose of the kinetic measurements is to compare the rates of transport with the rates of nonvectorial phosphorylation. Initial rates of transport were measured by using liposomes loaded with PEP (10 mM), EI (0.5 μM), and HPr (100 μM). The HPr concentration was increased in these experiments to minimize rate limitation from HPr at high mannitol concentrations. Trypsin treatment was used to inactivate inversely oriented EII. The initial uptake rates were the same as after removing external PEP by dialysis. The uptake was measured in triplicate during the first 10 s after the addition of mannitol. The resulting data were biphasic (Fig. 7). There was a rapid uptake, which tended to saturate at low substrate concentrations, followed by a linear increase with increasing mannitol concentrations. The control experiment to measure binding and passive and facilitated diffusion was executed in exactly the same way with liposomes lacking EI, HPr, and PEP. The data (Fig. 7) were monophasic and showed no saturation at any mannitol concentration used. Subtraction of the control values from the transport values revealed a normal Michaelis Menten saturation curve, with a V_{max} of 2.1 nmol · min^{-1} · mg of lipid^{-1} calculated by a nonlinear fitting program. The correlation coefficient was 0.989. Because of the low apparent K_m for mannitol, it was impossible to maintain initial rate conditions in the transport experiments at mannitol concentrations below the K_m long enough to obtain accurate rate data. Consequently, accurate K_m values could not be extracted from the transport data. It is obvious from Fig. 7, however, that the K_m is below 10 μm.

The kinetics of the phosphorylation activity were measured between 1 μM and 1 mM mannitol at the same PEP,

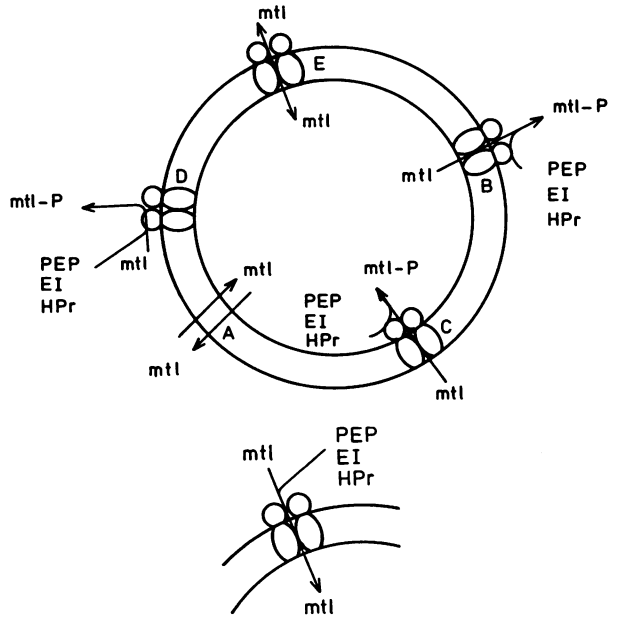

FIG. 8. (a) Schematic representation of the reactions observed to occur in the proteoliposomes. (A) Efflux (passive diffusion) of mannitol via the phospholipid membrane; (B) active extrusion via the inverted carriers; (C) active uptake via the carriers with a correct orientation; (D) external phosphorylation; (E) facilititated diffusion via the unphosporylated carrier. (b) Representation of a possible facilitated diffusion pathway mediated by phosphorylated EII^{Mtl}.

EI, and HPr concentrations used for the transport kinetics. The measurements were done on a similar liposome preparation which was not treated with trypsin, and the data were analyzed with the nonlinear fitting program. The kinetics of phosphorylation were best fit with a single K_m of 66 μM and V_{max} of 21.7 nmol · min^{-1} · mg of $lipid^{-1}$, with a correlation coefficient of 0.998. This comparison indicates that nonvectorial phosphorylation occurs at a rate 10 times faster than that of vectorial transport and phosphorylation.

DISCUSSION

The reactions which were observed to occur in the proteoliposomes are represented in Fig. 8a. Although both passive and facilitated diffusion of mannitol are very slow across the proteoliposome membrane, high phosphorylation activity was observed when PEP, EI, HPr, and mannitol were added at the outside of the liposomes, and mannitol phosphate appeared exclusively at the outside of the liposomes. A similar observation was reported earlier by Lolkema and Robillard (9) for fructose phosphorylation mediated by the fructose PTS in inside-out membrane vesicles of *Rhodobacter sphaeroides*. In that system, however, the data indicated that fructose first had to enter the vesicle before it could be phosphorylated (Fig. 8b). The kinetic analysis of both the vectorial transport reaction and the phosphorylation reaction indicates that this is not the case for the mannitol transport system. Transport of mannitol from the outside to the inside of the liposomes occurs with a high affinity (K_m, <10 μM) and a low V_{max} (2 nmol · min^{-1} · mg of $lipid^{-1}$), while the phosphorylation reaction at the outside has a low affinity (K_m, 66 μM) and a high V_{max} (21.7 nmol · min^{-1} · mg of $lipid^{-1}$).

A direct comparison of the two reaction rates is, however, allowed only when the same number of carriers is involved in both reactions. The distribution of EII^{Mtl} over the liposomal membrane was shown to be random. But for all correctly oriented EII molecules to participate in the transport reaction, there must also be a random distribution of PEP, EI, and HPr. The internal volume of the liposomes was calculated on the basis of the amount of internal $[^{14}C]^{Mtl}$ after loading of the liposomes with 1 mM mannitol. This value correlated well with the amount of [^{14}C]PEP which could be entrapped inside the liposomes. In order to ascertain whether EI and HPr were distributed comparably to PEP over the entire liposome population, we measured the final accumulation level after transport of an excess of mannitol in trypsinolysed liposomes. The internal PEP concentration in the liposomes was 10 mM; therefore, the maximal internal mannitol concentration would be 10 mM, in case of a random distribution of PEP, EI, and HPr. At an external mannitol concentration of 1 mM, the internal mannitol concentration at the steady-state level of accumulation was 7 mM. We conclude therefore that EI, HPr, and PEP were comparably distributed over the EII-containing liposomes.

Since the phosphorylation reaction is 10 times faster than the vectorial transport reaction, the phosphorylation reaction as we observed at the outside of the liposomes cannot be coupled to a vectorial process and must be described by scheme D of Fig. 8a.

The vectorial transport reaction is characterized by a high affinity and a low V_{max}, while the phosphorylation in the absence of detergent has a low affinity and a high V_{max}. Since a high affinity was observed when EII was accessible for mannitol at its periplasmic side only and a low affinity was observed when EII was accessible for mannitol at its cytoplasmic side only, we would expect to observe two affinities in permeabilized liposomes, in which EII^{Mtl} is accessible for its substrate on both sides. Preliminary kinetics of phosphorylation in detergent clearly showed biphasic behavior which could be fit with two K_m and V_{max} values. It appears therefore that there is a correlation between the observed affinities and the two orientations of EII. In this respect, it is interesting to note that two mannitol-binding sites, one high-affinity and one low-affinity binding site per dimer, have been detected on purified EII^{Mtl} in detergent (13). These two binding sites may also be correlated with the two sides of EII.

Our experimental results indicate that transport and phosphorylation are not strictly coupled. In the in vivo transport process, mannitol first binds to the periplasmic side of EII and is then transported to the cytoplasmic side (17). Here it is phosphorylated by EII and released. When mannitol was available at the cytoplasmic side of the enzyme, however, it could be phosphorylated after binding on this side. For the in vivo situation, this means that EII^{Mtl} is able to phosphorylate free mannitol inside the cell. The same conclusion has been drawn for EII mannose (21) and for EII glucose in *E. coli* cells (11). That phosphorylation can occur independently of transport is also indicated by the recent isolation of a transport-negative mutant of EII^{Mtl} which retains phosphorylation activity (10).

The calculated V_{max} for vectorial transport of 2.1 nmol of mannitol · min^{-1} · mg of $lipid^{-1}$ corresponds with a specific EII activity of 243 nmol/nmol of correctly oriented EII per min. By contrast, facilitated diffusion was about 0.3 to 2.5 nmol · nmol of EII^{-1} · min^{-1} (both orientations) for the experimental conditions in Fig. 5, i.e., 1 mM to 10 μm mannitol and a concentration gradient of 10. The comparison

indicates that facilitated diffusion by nonphosphorylated EII does not play a significant role in vectorial transport of mannitol into the cell. As has been suggested earlier, facilitated diffusion could be important for induction and also for detoxification if high internal mannitol concentrations occur.

The highest turnover number was found when mannitol was phosphorylated at the cytoplasmic side of the carrier: 2,513 nmol of mannitol · nmol of inverted EII^{-1} · min^{-1}. After solubilization in decyl-PEG, mannitol was phosphorylated with a maximal velocity of 2,788 nmol · nmol of EII^{-1} · min^{-}, which is in the same order as the phosphorylation at the cytoplasmic side of the membrane. The large difference between the maximum rate of transport and that of phosphorylation is unequivocal evidence that phosphorylation can occur without transport.

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