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Active Transport of Maltose in Membrane Vesicles Obtained from *Escherichia coli* Cells Producing Tethered Maltose-Binding Protein

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Attempts to reconstitute periplasmic binding protein-dependent transport activity in membrane vesicles have often resulted in systems with poor and rather inconsistent activity, possibly because of the need to add a large excess of purified binding protein to the vesicles. We circumvented this difficulty by using a mutant which produces a precursor maltose-binding protein that is translocated across the cytoplasmic membrane but is not cleaved by the signal peptidase (J. D. Fikes and P. J. Bassford, Jr., J. Bacteriol. 169:2352–2359, 1987). The protein remains tethered to the cytoplasmic membrane, presumably through the hydrophobic signal sequence, and we show here that the spheroplasts and membrane vesicles prepared from this mutant catalyze active maltose transport without the addition of purified maltose-binding protein. In vesicles, the transport requires electron donors, such as ascorbate and phenazine methosulfate or D-lactate. However, inhibition by dicyclohexylcarbodiimide and stimulation of transport by the inculsion of ADP or ATP in the intravesicular space suggest that ATP (or compounds derived from it) is involved in the energization of the transport. The transport activity of intact cells can be recovered without much inactivation in the vesicles, and their high activity and ease of preparation will be useful in studies of the mechanism of the binding protein-dependent transport process.

Many active transport systems in *Escherichia coli* belong to the periplasmic binding protein-dependent or osmotic-shock-sensitive class (1). In contrast to the shock-resistant or proton motive force-dependent system, each of which is composed of a single protein that functions as a proton symporter, the shock-sensitive systems are generally quite complex, each system usually composed of at least four protein components (1). The classical study of Berger and Heppel (3) suggested that the shock-sensitive systems are probably energized by ATP. Since that report, however, there has been much controversy on the identity of the primary energy source for these processes (7, 13, 27, 28).

In the study of the proton motive force-dependent transport systems, the use of membrane vesicles, introduced by Kaback (16), has been instrumental in establishing the true nature of the energy source. Hunt and Hong (14) made a pioneering contribution in showing that active membrane vesicles can be prepared for investigation of shock-sensitive transport systems, and subsequently some success has been achieved in a few other laboratories in reconstituting binding protein-dependent transport in membrane vesicles (29; H. Nikaido, unpublished data). However, the need to add a very large amount of purified periplasmic binding protein to vesicles not only made the method difficult but also created additional problems because the added binding proteins usually contributed a large number of tightly bound, unlabeled ligand molecules and possibly other undesirable contaminants. Thus, a recent review (1) points out that in all of the published reports the vesicles transported at rates less than 1% of that of whole cells.

More recently, two of us have characterized an unusual mutation in the signal sequence-coding region of the *malE* gene, which codes for the maltose-binding protein (MBP). This mutation, *malE24-1*, changes the alanine residue at the −3 position of the signal sequence to aspartic acid and was originally recognized by its negative effect on the secretion of a precursor MBP (pMBP) species that already harbored two other alterations in its signal sequence (9). When separated from these other mutations, pMBP encoded by the *malE24-1* allele was shown to be translocated efficiently across the cytoplasmic membrane. However, the maturation of pMBP24-1 was prevented by the alteration in the signal peptide recognition sequence, and the unprocessed pMBP remained tethered to the cytoplasmic membrane, presumably with its signal sequence serving as a membrane anchor (10). We found that these mutant cells could transport maltose quite actively (10). This study was initiated with the expectation that *malE24-1*-carrying cells would produce spheroplasts and vesicles with tethered, functional MBP molecules, allowing us to reconstitute active maltose transport without adding back large amounts of free MBP.

MATERIALS AND METHODS

Bacterial strains. Plasmid pJF13, containing the *malE24-1* allele under *lacUV5* promoter control, was described previously (10). This *malE* allele was genetically recombined into the *E. coli* chromosome as follows. Plasmid pJF13 was transformed into strain BAR1000, a derivative of strain MC4100 (5) that harbors a large, in-frame, internal *malE* deletion designated *malEΔ323* (9). This strain was lysogenized with phage λapΔ*malB13* (*c*I857 *S*7) (22), onto which the *malEΔ323* deletion had been recombined as previously described (2), and a heat-induced lysate was prepared. Transient recombination between homologous *malE* sequences present on both the phage and plasmid resulted in a small population of phage in which the *malEΔ323* allele had been replaced by the *malE24-1* allele. Such phages were selected for by infecting strain BAR1000 and plating it on maltose minimal agar at 30°C. Only cells lysogenized with λapΔ*malB13 malE24-1* were able to utilize maltose as the sole carbon source in the absence of isopropyl β-D-thiogalactopyranoside. A Mal$^+$ λc^r colony was purified, and the

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strain in which the *malE24-1* allele was recombined into the chromosome was isolated by selecting for Mal$^+$ λ-cured survivors at 42°C. The resulting strain, designated CC142, had the *malE24-1* allele in the chromosome under the normal *malEp* promoter control.

For transport studies, it was desirable to avoid the metabolism of transported maltose by the enzyme amylomaltase, the product of the *malQ* gene. A Tn*10* insertion closely linked to *malQ* was moved by P1 transduction from donor strain JGC127 (K-12 F$^-$ *argG metB his leu thyA malA dnaM zhg*::Tn*10 xyl mtl gal lacY rpsL tonA tsx*) (11) into strain MH70 [K-12 F$^-$ *thi araD139 ΔlacU169 rpsL1 relA flbB malQ*(Am)] (12). Although the donor contained a *malA* mutation, this was probably a *malT* mutation, since the strain showed negligible levels of both amylomaltase and maltodextrin phosphorylase activities. In contrast to the donor, the *malQ*(Am) mutation in the recipient, MH70, made the strain somewhat maltose sensitive, and it produced colonies with many white papillae on maltose-McConkey plates (31). One of the tetracycline-resistant transductants exhibiting this colony morphology characteristic of *malQ* mutants and undetectable amylomaltase activity but full maltodextrin phosphorylase activity was saved as strain HN606. The *malQ*(Am) mutation in strain HN606 was then transferred by P1 cotransduction with *zhg*::Tn*10* into CC142. The final construct, HN596 [K-12 F$^-$ *ΔlacU169 araD139 rpsL150 thi flbB5301 deo-7 ptsF25 relA malE24-1 malQ*(Am) *zhg*::Tn*10*], was used in most of the experiments. As a control with the wild-type *malE* gene, we used HN598 [K-12 F$^-$ *ΔlacU169 araD139 rpsL150 thi flbB5301 deo-7 ptsF25 relA malQ*(Am) *zhg*::Tn*10*), which was constructed by transducing the *malQ*(Am) and *zhg*::Tn*10* alleles from HN606 into ECB526, which is BAR1091 (10) whose *malEΔ312* allele was replaced by the wild-type *malE*$^+$ allele. HN599 was a *lamB* derivative of HN596 selected by plating on λvir. The absence of the LamB protein in this strain was confirmed by analyzing the outer-membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Growth and induction. Cells were grown in mineral medium M63 (23) containing 0.4% glycerol and 0.1 μg of thiamine per ml at 37°C with aeration by shaking. Maltose (0.2%) was added as the inducer during the exponential phase of growth, and the cells were harvested usually after 2 h of induction.

Spheroplasts. Cells were converted to spheroplasts by the procedure of Witholt et al. (38). Cells were recovered by centrifugation usually from a 200-ml culture, washed once in 0.2 M Tris chloride buffer (pH 8.0) and resuspended in the same buffer. An equal volume of 1 M sucrose–0.2 M Tris chloride buffer (pH 8.0) was added, followed by lysozyme and EDTA to final concentrations of 60 μg/ml and 0.5 mM, respectively. The suspension was diluted rapidly with an equal volume of distilled water, and after a few minutes $MgCl_2$ was added to 3 mM and pancreatic DNase was added to 20 μg/ml. Examinations under a phase-contrast microscope showed that more than 98% of the cells were converted to spheroplasts, which were collected by centrifugation at 4,000 × *g* for 15 min.

Vesicles. Right-side-out vesicles were prepared by a modification of the osmotic-lysis procedure of Kaback (17). However, because the spheroplasts prepared by the Witholt procedure are very stable, there was very little lysis when they were diluted into 100 mM phosphate buffer as specified (17). Furthermore, in some cases we wanted to make vesicles containing added small molecules and to minimize the enzymatic degradation of these compounds. The procedure finally adopted was as follows. Cells (usually from a 200-ml culture at a density of about 0.25 mg [dry weight]/ml) were converted to spheroplasts as described above, except that 1 mM EDTA was used. Spheroplasts, recovered by centrifugation, were suspended in less than 1 ml of 0.05 mM KPO_4 buffer (pH 6.6)–0.25 M sucrose–5 mM $MgCl_2$–DNase (1 mg/ml), and the suspension was added to 40 ml of 8 mM KPO_4 buffer (pH 6.6) which was being stirred in an ice-water bath and which sometimes contained additional components as described below. After 5 min, EDTA was added to a final concentration of 2 mM, and after 5 min of further stirring, $MgCl_2$ was added to 3 mM. After 5 min, unlysed spheroplasts were removed by centrifugation at 5,000 × *g* for 10 min, the supernatant was removed to a clean centrifuge tube, and vesicles were collected by centrifugation at 30,000 × *g* for 15 min. The pellet was suspended in a small volume (usually 0.1 ml or less) of 20 mM KPO_4–3 mM $MgCl_2$ and used immediately (unwashed vesicles) or suspended in 10 to 20 ml of the same buffer at 0°C and centrifuged again at 30,000 × *g* for 15 min before use (washed vesicles). The low osmotic activity of the lysis buffer was necessary to obtain efficient lysis of the spheroplasts, and all procedures after the osmotic lysis were performed at 0 to 4°C and as rapidly as possible to avoid the degradation of added compounds.

Transport assays. Cells in the exponential phase of growth were centrifuged, washed twice with M63, and suspended in M63 at a density of 0.1 to 0.3 mg (dry weight)/ml. The assay was initiated by adding [^{14}C]maltose (specific activity, 20 mCi/mmol; Amersham Corp.) to a final concentration of 10 μM, and 0.1-ml portions of the incubation mixture were filtered every 30 s through a Millipore HA filter, which was washed with 5 ml of 0.1 M LiCl, and radioactivity retained on the filter was determined in a liquid scintillation counter.

Spheroplasts, recovered by centrifugation, were suspended in M63, since spheroplasts prepared by the Witholt procedure are not lysed, even without osmotic protection, as long as Mg^{2+} is present (38). Transport assays were then performed as described above.

Vesicle suspensions were diluted in 20 mM KPO_4 (pH 6.6)–3 mM $MgCl_2$ (at room temperature) to a final concentration of 0.5 to 1.5 mg of protein per ml, and electron donors (10 mM D-lactate or 10 mM ascorbate–0.1 mM phenazine methosulfate [PMS]) were added as indicated. The substrate, either [^{14}C]maltose (specific activity, 125 mCi/mmol) or [^{14}C]proline (specific activity, 285 mCi/mmol; Amersham), was added to a final concentration of 10 μM, and a 25-μl sample was removed every 30 s and filtered through a Millipore HA filter which was washed with 5 ml of 0.05 M LiCl, dried, and counted in a liquid scintillation counter.

Enzyme assay. Amylomaltase and maltodextrin phosphorylase were assayed essentially as described previously (32, 37), by using sonicated extracts of cells.

Enzyme-linked immunosorbent assay. The amounts of tethered pMBP and MBP present in HN596 and HN598 were determined by using serial dilutions of purified MBP as standards in an enzyme-linked immunosorbent assay (6) and comparing these with dilutions of crude extracts from HN596 and HN598. Purified MBP or crude protein extract was bound to polystyrene plates, specific rabbit antiserum to MBP was reacted with the antigen, goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad Laboratories) was reacted with the rabbit antiserum, and *o*-phenylenediamine was mixed with hydrogen peroxide and reacted with the peroxidase. The reaction was stopped after 10 min by addition of sulfuric acid, and the A_{490} was read in a Bio-Tek microplate reader.

Continuous labeling and purification of pMBP and MBP. HN596 and HN598 were grown in 20 ml of M63 as described earlier and induced for three generations with 0.2% maltose in the presence of 250 μCi of [^{3}H]leucine (158 mCi/mmol). HN598 was harvested, washed with 50 mM Tris chloride buffer (pH 7.3), and subjected to osmotic shock to release the periplasmic components (25). HN596 was washed and disrupted by sonication, and its cytoplasmic membrane proteins were extracted twice with 1% Triton X-100 in the presence of 5 mM $MgCl_2$ (30). The Triton X-100-insoluble material was removed by centrifugation at 20,000 × *g* for 20 min. MBP and pMBP were purified from the shock fluid from HN598 and the Triton X-100-soluble fraction from HN596, respectively, by affinity chromatography on a cross-linked amylose column (8). The MBP species were eluted with 10 mM maltose, and all of the fractions were analyzed by polyacrylamide gel electrophoresis (20) and liquid scintillation counting.

Determination of cellular dry weight and protein. Cell mass was obtained from the optical density of the suspension by using a calibration curve, and protein was determined by the Lowry method (18) with bovine serum albumin as the standard.

Determination of vesicle volume. The vesicle volume was determined as the dextran-impermeable but glycerol-permeable volume by equilibrating vesicle preparations for 1 h at 0°C in 20 mM potassium phosphate buffer–3 mM $MgCl_2$ (pH 6.6) containing 1 mM [^{14}C]glycerol (New England Nuclear Corp.; diluted to 0.5 mCi/mmol with nonradioactive glycerol) and 10 mg of [^{3}H]dextran (Amersham; diluted to 0.25 mCi/g with nonradioactive dextran) per ml. After centrifugation at 70,000 × *g* for 5 min, the ^{14}C and ^{3}H contents of both the supernatant and the resuspended pellet were determined. The controls and calculations were essentially as specified by Stock et al. (34).

RESULTS

Amount of pMBP produced by mutant cells. Pulse-labeling of cells containing the *malE24-1* allele on plasmids showed that substantial amounts of pMBP are synthesized and exported rather efficiently into the periplasm (10). However, the strain used in this work, HN596, contained the *malE24-1* allele on the chromosome, and we were interested in knowing the steady-state levels of pMBP and its processed product. Continuous labeling of HN596 and HN598, a strain which contains wild-type MBP, with [^{3}H]leucine and purification of MBP from these cells allowed direct comparison of the amounts of MBP present. Bands of molecular weights corresponding to those of the MBP in HN598 and the pMBP in HN596 and of almost equal intensity were present on stained polyacrylamide gels (Fig. 1). Similar amounts of radioactivity were present in the purified proteins from both strains, confirming that equal amounts were produced. It should be noted that the protein from HN596 contained no MBP; thus, pMBP is not processed detectably in this strain.

These results were supported by experiments using an enzyme-linked immunosorbent assay with antisera to MBP (data not shown). Again, similar amounts of pMBP and MBP per milligram of cell protein were present in both strains.

Transport in intact cells. Intact cells of HN596, which produce tethered pMBP, transported maltose efficiently. The apparent K_m of the transport was 0.9 μM and was similar to that found in HN598, the wild-type strain, 1.0 μM. The V_{max} of transport, 3.2 nmol/mg of protein per min, was identical to that in HN598.

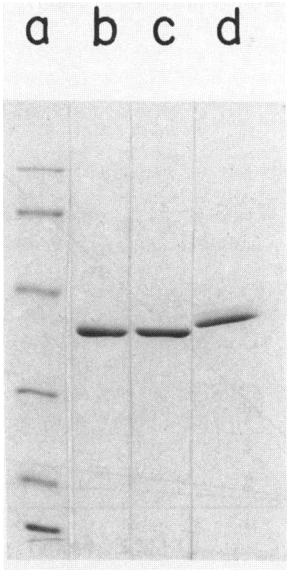

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins with maltose-binding capacity. Lanes: A, molecular weight standards (from the top) phosphorylase *b* (M_r, 97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400); B, MBP (2 μg); C and D, proteins with the maltose-binding capacity purified from HN598 and HN596, respectively. Cell extracts (7.4 mg of protein) were applied to a column of cross-linked amylose, which was then eluted with 10 mM maltose as described in Materials and Methods. One-tenth of the total protein eluted by maltose was applied to each lane. The starting material for purification was the supernatant obtained after osmotic shock for HN598, whereas for HN596, the extract obtained by Triton X-100 solubilization of the sonically disrupted cells was used (see Materials and Methods). Lanes that are not relevant were removed before photography.

These V_{max} values were rather low for transport systems catalyzing the uptake of major carbon sources. A partial explanation is that the cells, after 2 h of growth in the presence of maltose, are not yet fully induced. When CC142 and ECB526, the *malQ*$^+$-carrying parent strains of HN596 and HN598, were fully induced by growth in minimal media containing maltose as the sole carbon source, the V_{max} values obtained were 12 and 28 nmol/mg of protein per min, respectively, much higher than the values described above. Even under these conditions, the strain with tethered pMBP transported maltose at relatively high rates. We also noted that although induction of the maltose regulon inhibited the growth of strains containing the *malE24-1* allele on plasmids (10), a strain containing a single copy of this allele on its chromosome, CC142, was able to grow in maltose-minimal medium.

Transport in spheroplasts. Spheroplasts of HN596 transported maltose at a significant rate (Fig. 2). In contrast, spheroplasts of HN598, a strain producing normal, free MBP could not transport maltose at all, presumably because most of the MBP was lost during the formation of spheroplasts.

The transport rate observed with HN596 spheroplasts was about 20% of that of intact cells and was too rapid for a process carried out by the remaining intact cells in the spheroplast population. Two lines of evidence further confirmed that spheroplasts, rather than intact cells, were transporting maltose. (i) The spheroplasts from HN596 lost the

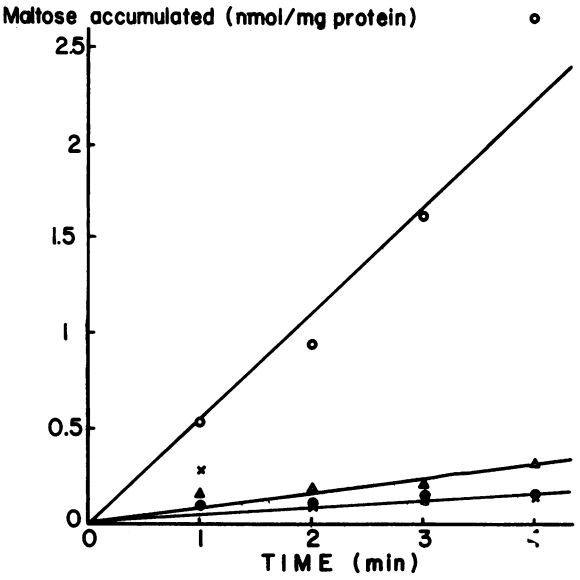

FIG. 2. Accumulation of maltose by spheroplasts. Spheroplasts from HN596 (a strain with the tethered pMBP) and HN598 (a strain with the wild-type MBP free in the periplasm) were made as described in Materials and Methods but in the presence of 4 mM EDTA. The spheroplasts were collected by centrifugation at 5,000 × *g* for 10 min and washed with 0.2 M Tris chloride buffer (pH 8.0)–0.25 M sucrose–5 mM $MgCl_2$–DNase (1 mg/ml) and resuspended in the same mixture. Spheroplasts were incubated at room temperature in the presence of 10 μM [^{14}C]maltose as described in the text. Symbols: ○, HN596 spheroplasts in M63; ●, HN596 spheroplasts diluted in water; △, HN598 spheroplasts in M63; ×, HN598 spheroplasts diluted in water.

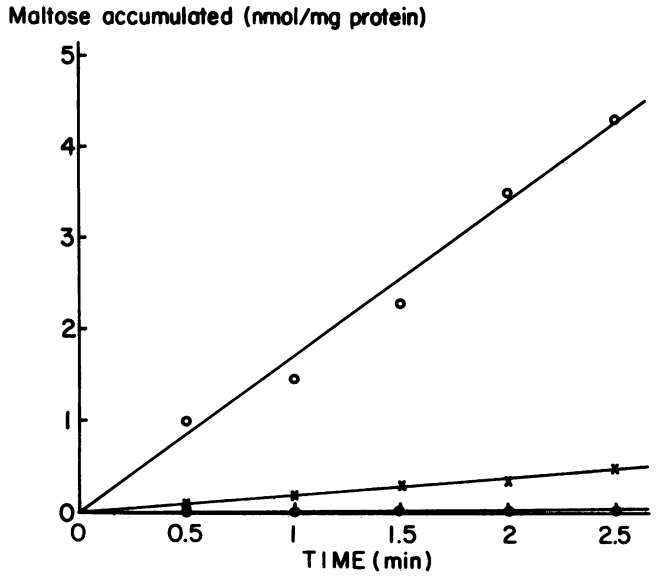

FIG. 3. Accumulation of maltose by membrane vesicles. Unwashed membrane vesicles from HN596 and HN598 were incubated at room temperature in the presence of 10 μM [^{14}C]maltose as described in Materials and Methods. Symbols: ○, HN596 vesicles in the presence of 10 mM ascorbate–0.1 mM PMS; ×, HN596 vesicles in the presence of 10 mM D-lactate; ●, HN596 vesicles in the absence of an exogenous electron donor; ▲, HN598 vesicles in the presence or the absence of electron donors. In the presence of ascorbate-PMS, the maltose accumulation achieved by HN598 vesicles was less than 1% of that attained by HN596 vesicles. In contrast, HN598 vesicles accumulated [^{14}C]proline as rapidly as did HN596 vesicles under these conditions (data not shown).

ability to transport when they were first diluted in water (Fig. 2), undoubtedly a treatment leading to the release of most cytoplasmic constituents. (ii) The K_m for maltose transport in intact cells of HN599, a *lamB* mutant of HN596, was much elevated (370 μM) in comparison with that of HN596 (0.9 μM), because in these cells the diffusion of maltose across the outer membrane became the rate-limiting process owing to the absence of the maltose-specific LamB channel (19, 35). In contrast, the spheroplasts of HN599 showed a low K_m (16 μM) similar to that found with the spheroplasts of HN596 (12 μM), indicating that the entity that transports maltose here is not surrounded by a functional outer membrane.

Transport in vesicles. Rapid transport of maltose was observed in vesicles from HN596, with the tethered pMBP; in contrast, vesicles from HN598, producing the wild-type MBP, showed no transport in the absence of added MBP under the same conditions (Fig. 3).

The transport with HN596 vesicles was dependent on electron donors, such as ascorbate-PMS or D-lactate. In the experiment shown in Fig. 3, ascorbate-PMS showed a much stronger stimulation of transport, but in others D-lactate was as effective or even slightly more effective. Usually, the transport rate decreased to less than 5% in the absence of these donors (Fig. 3).

The energization of transport by the electron donors appeared to involve the generation of a proton motive force, since the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone or valinomycin-nigericin completely abolished the transport (Fig. 4).

Maltose concentration in vesicles. The volume of vesicles, determined as described in Materials and Methods, was 6 μl/mg of protein. The volume of intact cells is 2.4 μl/mg (dry weight) (34) or 4.4 μl/mg of protein, since 55% of the dry weight of *E. coli* is protein (24). Thus, if we assume that 30% of the protein belongs to the envelope (see below) and the vesicles are formed with no loss of volume, we would expect the vesicle volume of 14.4 μl/mg of protein. The observed value shows that some but not extensive fragmentation of membranes occurs during the preparation of vesicles.

The vesicles accumulated up to 5 or 6 nmol of maltose per mg of protein in a few minutes (Fig. 3 to 5). Thus, the intravesicular concentration of maltose was close to 1 mM. In comparison with the maltose concentration in the incubation medium, 10 μM, this represented a 100-fold concentration inside the vesicles.

When the vesicles recovered by filtration were extracted with boiling water and the content was examined by thin-layer chromatography on cellulose, all of the radioactivity was associated with the spot with the mobility of maltose (data not shown). There was no evidence for hydrolysis or modification of the substrate within the time frame of our transport experiments.

Evidence for involvement of ATP. Although the observation that the transport in vesicles was energized by electron donors appeared superficially very similar to that on the proton motive force-dependent transport systems, we found some evidence that ATP (or compounds that can be readily generated from ATP) is involved in maltose transport.

(i) Addition of *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of F_0F_1 ATPase, decreased maltose transport drastically (Fig. 5) without affecting proline transport (data not shown). Proline is apparently transported by a sodium

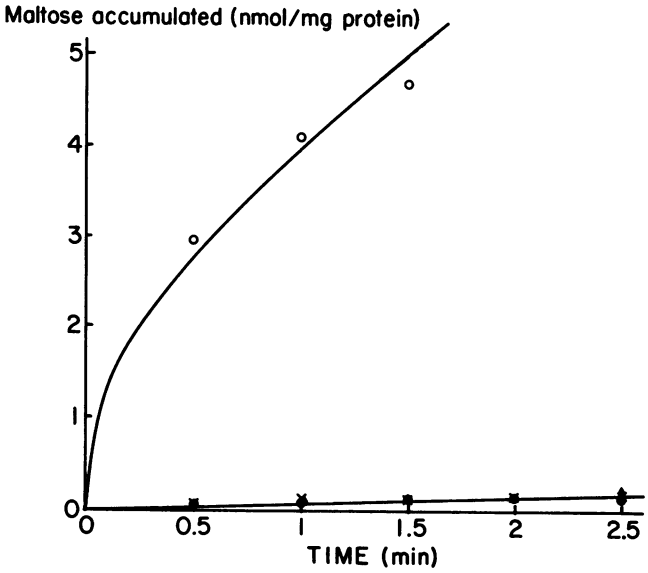

FIG. 4. Inhibition of maltose accumulation by *m*-chlorophenylhydrazone and valinomycin-nigericin. Washed membrane vesicles from HN596 were incubated in the presence of 10 μM [^{14}C]maltose and 10 mM ascorbate–0.1 mM PMS (○). Maltose accumulation was abolished when the incubation mixture also contained 1 μM *m*-chlorophenylhydrazone (×) or a combination of 5 μM valinomycin and 1 μM nigericin (▲). There was negligible transport also when ascorbate-PMS was omitted from the incubation mixture (●).

symport system (4). However, because enough sodium is present in our system (e.g., from sodium lactate) and because generation of a sodium gradient depends on proton motive force, the system behaves as though it were driven by the proton motive force alone (see reference 4).

(ii) Preparing vesicles by performing the lysis in the presence of ATP often enhanced maltose uptake. The extent of stimulation was variable, and only two- to threefold stimulation was commonly seen when the spheroplasts were prepared from mid-exponential-phase cells, presumably with high intracellular levels of ATP and ADP. When starved cells or cells about to enter the stationary phase were used, the stimulation was sometimes higher, and one example is shown in Fig. 6. In a similar experiment, lysis in the presence of ADP had the same effect as that in the presence of ATP, and it seems probable that electron transport from the donors created a proton motive force, which in turn allowed F_0F_1 ATPase to convert ADP to ATP, finally causing energization of the transport process.

Comparison of vesicle activity with the transport activity of whole cells. The vesicles of HN596 transported maltose very actively, usually at a rate of 2 to 3 nmol/mg of protein per min (Fig. 3 to 5). When made by lysis in the presence of ATP, the activity was often even higher, reaching almost 6 nmol/mg of protein per min in the experiment of Fig. 6. These rates are equal to or two to three times higher than the rates observed in intact cells (see Transport in intact cells, above). Indeed, when the transport rates per milligram of protein in intact cells and in vesicles of strain HN596 were compared in a single experiment, the latter was about twofold higher than the former (data not shown). These data can be compared with the specific activity expected if the transport capacity was not inactivated during vesicle preparation. For this calculation, we have to know the fraction of cellular proteins present in the envelope fraction. Osborn et al. (26) found that the protein/phospholipid weight ratio in

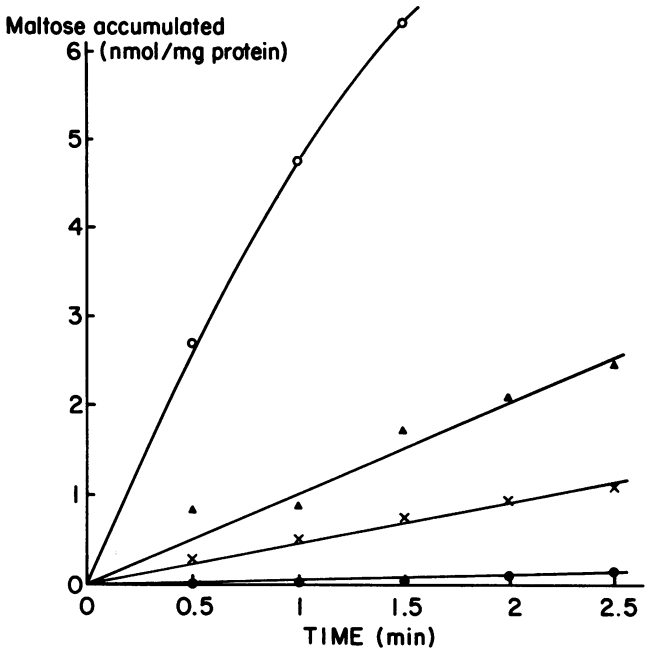

FIG. 5. Inhibition of maltose accumulation by DCCD. Washed vesicles from HN596 were incubated with 10 μM [^{14}C]maltose and 10 mM D-lactate (○). In two tubes, DCCD at 50 (●) and 100 (×) μM was added to vesicles 2 min before the assay was begun by addition of D-lactate and [^{14}C]maltose. Vesicles were also assayed without D-lactate addition as a control (△). Under these conditions, DCCD showed negligible inhibition of proline accumulation by vesicles from HN596 (data not shown).

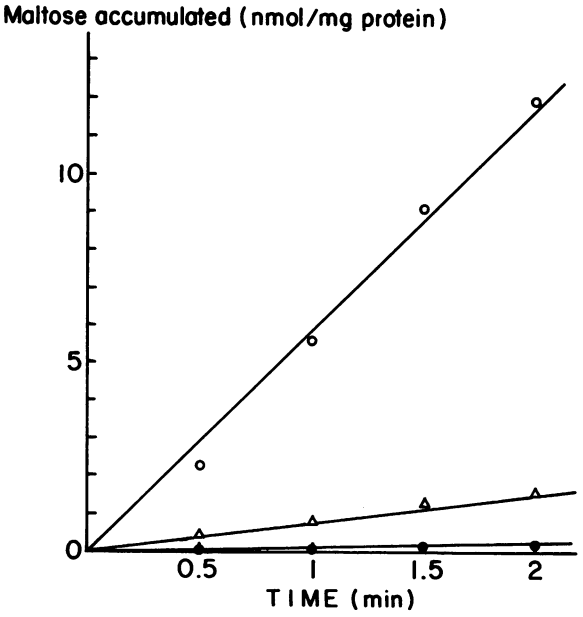

FIG. 6. Vesicles prepared by lysis in the presence of ATP. Spheroplasts were prepared from HN596 cells about to enter the stationary phase (cell density, 0.49 mg [dry weight]/ml) and were lysed in 8 mM KPO_4 buffer (see Materials and Methods) containing 0 or 0.5 mM ATP. Unwashed membrane vesicles were assayed for maltose transport in the presence of 10 mM D-lactate. Symbols: ○, vesicles made in the presence of ATP; △, vesicles made in the absence of ATP; ●, both types of vesicles assayed in the absence of D-lactate.

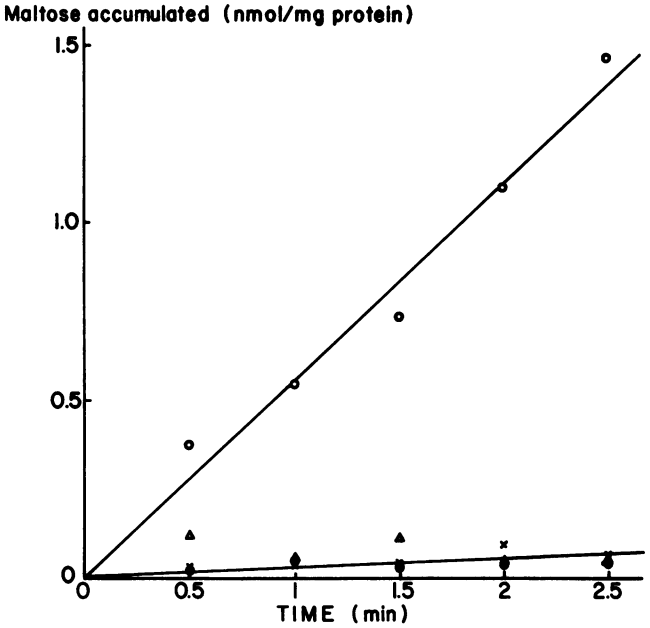

FIG. 7. Accumulation of maltose by membrane vesicles with exogenous MBP. Unwashed membrane vesicles from HN598 were incubated in the presence or absence of purified MBP. Vesicles were incubated at room temperature in the presence of 10 μM [^{14}C]maltose as described in Materials and Methods. Symbols: ○, vesicles in the presence of 0.1 mM MBP and 10 mM ascorbate–0.1 mM PMS; ●, vesicles in the presence of 0.1 mM MBP but without exogenous electron donors; △ and ×, vesicles in the absence of MBP and in the presence or absence, respectively, of exogenous electron donors.

the envelope was 3.1. Reportedly, 9% and 55% of the cellular dry weight, respectively, correspond to phospholipids and proteins (24). Combining these two series of numbers, one comes to expect about 50% of the cellular protein to be in the envelope. On the other hand, experimental recovery of proteins in the envelope fraction never exceeded 18% of the total cellular protein (26). Thus, the envelope fraction contains 18 to 50% of the cellular proteins, and we can expect that the specific transport activity per milligram of protein of the vesicles should be two to five times higher than that of intact cells. The values obtained were close to these expected values, and the transport capacity seems to have been recovered in the vesicles without much inactivation.

Reconstitution of maltose transport in wild-type vesicles. We attempted to see whether maltose transport can be reconstituted at such a high efficiency with vesicles from HN598, a strain producing a normal MBP. Since we suspected that a major reason for past failures in reconstitution was contamination of the binding protein preparation by nonradioactive ligand molecules, we performed extensive dialysis of the MBP based on the theoretical predictions of Silhavy et al. (33). Activity was obtained (Fig. 7), although the level was much lower than with the vesicles of strain HN596. It seems possible that during the protracted dialysis of the MBP some protein molecules were denatured or damaged, and the use of membranes with tethered binding proteins allows us to circumvent such a difficulty. An alternative possibility is that many of our vesicles were still surrounded by the outer membrane, which limits the access of an exogenously added MBP (see Discussion), although it is transparent for smaller maltose molecules (see Transport in spheroplasts, above).

DISCUSSION

The mutation *malE24-1*, which changes the alanine residue at the −3 position of the signal sequence of the MBP into aspartic acid, inhibits cleavage of the signal peptide nearly completely (10). This result is not unexpected, because charged amino acid residues have not been found at this position in an extensive survey of natural signal peptide sequences (36). However, it was considered somewhat surprising that pMBP24-1 was exported to the periplasm, was folded correctly as judged from the protease resistance of the pMBP in spheroplasts (10), and functioned efficiently in maltose transport as shown in this study.

Intact cells of HN596, containing the tethered pMBP, transported maltose at a rate similar to the rate observed in the strain containing the wild-type MBP. Osmotic shock releases only trace amounts of MBP from *malE24-1* cells compared with isogenic cells carrying *malE*$^+$ (10). On the other hand, it is known that the rate of maltose transport decreases to one-half when the MBP concentration reaches 9% of the wild-type level (21). Thus, with intact-cell experiments we cannot rigorously rule out the possibility that the uptake of maltose in the strain carrying *malE24-1* was catalyzed by the small amounts of pMBP and MBP released into the periplasm, although such a possibility seemed unlikely.

With spheroplasts and vesicles, however, there is no such ambiguity. Even if a small fraction of pMBP or MBP were released during incubation, many of the released molecules would escape into the buffer solution with its large volume. We know that spheroplasts and vesicles from wild-type cells were totally inactive in the absence of high concentrations of exogenously added MBP, and thus it is extremely unlikely that any pMBP or MBP released into the medium from the HN596 vesicles would have contributed to the transport process. Thus, there is no question in our minds that maltose transport into vesicles and spheroplasts of HN596 occurs via tethered pMBP molecules. It was quite unexpected that these tethered proteins were so active in transport, especially because binding of the ligand and interaction with the membrane-associated components of the transport system, involving the MalF and MalG proteins, are expected to involve major conformational changes of the MBP.

We used the Witholt procedure (38) to prepare spheroplasts. The ease, reproducibility, and efficiency of this procedure are excellent, and possibly the small extent of membrane damage produced during this process also contributed to the high transport activity of our vesicles. However, this method produces spheroplasts that are apparently surrounded by a seemingly intact outer membrane (38), and thus there was some concern about whether exogenously added molecules are able to reach the cytoplasmic membrane. It is thus important to stress that the outer membrane in the spheroplasts did not constitute a permeation barrier for maltose (see Results). However, we cannot rule out the possibility that the poor transport activity of the wild-type vesicles in the presence of the MBP (Fig. 7) was due to the restricted access of the MBP to the cytoplasmic membrane.

Although reconstitution of binding protein-dependent transport processes in vesicles has been achieved by Hunt and Hong (14, 15), the use of vesicles has not contributed much toward our understanding of the molecular mechanism of these processes. One of the major reasons for this lack of

success has been the very poor recovery of activity (1). When the activity is so low, it becomes impossible to study the stoichiometry between ATP hydrolysis and transport, for example, because the background rate of ATP hydrolysis is hundreds of times higher than that expected from the transport process. In our system, we obtained a nearly quantitative recovery of transport activity in vesicles, and this system will be very useful for studying many features of the transport process, including energy coupling.

Although the decisive results must await the use of vesicles made from mutants lacking F_0F_1 ATPase, a study currently in progress, the results with *unc*$^+$ vesicles already suggest strongly the involvement of ATP in the transport process, because maltose accumulation was inhibited strongly by DCCD and because the inclusion of ADP or ATP in the intravesicular space had a strong stimulatory effect, at least in some experiments.

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