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Overexpression of Mal61p in *Saccharomyces cerevisiae* and Characterization of Maltose Transport in Artificial Membranes

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For maltose uptake in *Saccharomyces cerevisiae*, multiple kinetic forms of transport as well as inhibition of transport by high concentrations of maltose at the *trans* side of the plasma membrane have been described. Most of these studies were hampered by a lack of genetically well-defined mutants and/or the lack of an artificial membrane system to study translocation catalysis in vitro. A genetically well-defined *S. cerevisiae* strain lacking the various *MAL* loci was constructed by gene disruption. Expression of the maltose transport protein (Mal61p) was studied by using various plasmid vectors that differed in copy number and/or type of promoter. The expression levels were quantitated by immunoblotting with antibodies generated against the N-terminal half of Mal61p. The levels of expression as well as the initial uptake rates were increased 20-fold compared with those in a yeast strain carrying only one chromosomal *MAL* locus. Similar results were obtained when the transport activities were compared in hybrid membranes of the corresponding strains. To generate a proton motive force, isolated membranes were fused with liposomes containing cytochrome *c* oxidase as a proton pump. Fusion was achieved by a cycle of freeze-thawing, after which the hybrid membranes were passed through a filter with a defined pore size to obtain unilamellar membrane vesicles. Proton motive force-driven maltose uptake, maltose efflux down the concentration gradient, and equilibrium exchange of maltose in the hybrid membranes vesicles have been analyzed. The data indicate that maltose transport by the maltose transporter is kinetically monophasic and fully reversible under all conditions tested.

Yeasts such as *Saccharomyces cerevisiae* are capable of converting maltose, the primary sugar in brewing and baking, to CO_2 and ethanol. Utilization of maltose requires two gene products, i.e., maltose transport protein (encoded by *MALX1*) (*X* stands for one of the five unlinked *MAL* loci), and maltase (encoded by *MALX2*). An activator of transcription of the *MALX1* and *MALX2* genes is encoded by *MALX3*. The three genes are clustered in the *MAL* locus, which is repeated at different chromosomal locations (*MAL* 1 to 4 and *MAL* 6) (33). The different *MAL* loci show extensive sequence and functional similarities (8). In the presence of maltose and the absence of other sugars, the expression of maltase and the transport protein is increased to levels that are 600-fold higher than those in glucose-grown cells (33). The Mal61 protein is homologous to other (sugar) transport proteins found in *S. cerevisiae* but also to transporters in prokaryotes and higher eukaryotes (31). Maltose transport has been described as a symport reaction in which one proton is taken up per molecule of maltose (42, 51). Maltose transport follows biphasic kinetics with apparent affinity constants (K_m^{app}) of 4 and 70 mM (6, 10). The low-affinity transport activity may be caused by nonspecific binding of maltose to cell wall and/or plasma membrane components (3) or, alternatively, by passive diffusion of the solute across the plasma membrane at the high concentrations of sugars used (19). In most studies, however, it is not clear whether Mal61p, other MalX1 proteins, or more than one transport system contribute to maltose uptake.

Studies of solute-proton symport in whole cells of *S. cerevisiae* have revealed unusual features of the energetics of uptake. It has been suggested that the proton symport systems in *S. cerevisiae* facilitate unidirectional transport and catalyze only proton motive force (Δp)-driven uptake, and no efflux, upon dissipation of the electrochemical H^+ gradient or removal of the external substrate (16, 26). Moreover, it has been suggested that the transport systems require a Δp for activity even under conditions of downhill influx (16, 35). Unidirectional arginine-proton symport has been observed not only in intact cells but also in isolated plasma membrane vesicles (40).

Because of the complexity of processes in cells, it is often difficult to draw unequivocal conclusions regarding the mechanisms of energy coupling to solute transport or the regulation of transport. Isolated plasma membrane vesicles, in which the effects of cellular metabolism are eliminated, have been used to study facilitated diffusion of sugars (18, 37, 40) and uptake of leucine, arginine, galactose, and maltose in symport with protons (38, 39, 50, 51). However, such isolated plasma membrane vesicles are not well sealed, and a large fraction is composed of membrane sheets (43). These membranes are leaky for protons and other ions, which limits their use in studies of energy transduction. To improve the tightness of the membranes and to introduce an efficient proton pump in the membranes, the plasma membrane vesicles can be fused with liposomes containing cytochrome *c* oxidase (COVs) (14).

In this paper, we report the overexpression of Mal61p in *S. cerevisiae* lacking other *MAL* loci and an improved procedure for the preparation of hybrid membrane vesicles. Studies with these hybrid membrane vesicles indicate that the maltose transport system is fully reversible and capable of catalyzing influx as well as efflux transport.

MATERIALS AND METHODS

Strains and growth conditions. *S. cerevisiae* 6001B (*MAT***a** *SUC1 MAL11 MAL12 MAL13 ura3-52 leu2-3,112*) and 6001BΔ11 (*MAT***a** *SUC1 MAL12*

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*MAL13 mal11*Δ::*URA3 leu2-3,112*) (8) were gifts from C. Michels (Queens College New York, N.Y.). The strains were grown in batch culture on YP medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone [pH 6.6]) supplemented with 1% glucose or 1% maltose or on minimal medium containing, per liter, 0.2 g of $MgSO_4$, 3.0 g of NaH_2PO_4, 0.7 g of K_2HPO_4, 2.5 g of $(NH_4)_2SO_4$ (pH 6.0), 1 ml of Vishniac solution, 1 ml of vitamin solution (51), different sugars, and when appropriate, 1 ml of uracil (40 mg/ml), leucine (125 mg/ml), or adenine (20 mg/ml). For growth in chemostat cultures, the cells were grown in the minimal medium except that K_2HPO_4 was omitted and NaH_2PO_4 was replaced by 1 g of KH_2PO_4. Chemostat cultures were run with a dilution rate of 0.1 h^{-1} at 30°C and pH 5.0 (kept constant by titration with 1 M KOH).

DNA manipulations. For gene manipulation in *Escherichia coli*, strains JM101 (32) and DH5α (22) were used. DNA manipulations were performed as described previously (41). Yeast cells were transformed according to the protocol of Gietz et al. (20). *S. cerevisiae* 6001BΔ11Δ12 was constructed by disruption of the *MAL12* gene by using a truncated *MAL62* gene and strain 6001BΔ11 as the parent. A 64-bp *Pst*I-*Cla*I fragment (positions 573 and 637) of *MAL62* was removed from pY6 (34). The DNA was made blunt ended with T4 DNA polymerase and ligated, yielding pYM10. Subsequently, pYM10 was digested with *Bgl*II (positions 146 and 1561), and the 1,351-bp fragment containing the 5′- and 3′-truncated *MAL62* gene was ligated into the *Bam*HI site of the integrative plasmid YDp-L (4), yielding pYM20. pYM20 was linearized by digestion with *Sau*I (position 415 of *MAL62*). *S. cerevisiae* 6001BΔ11 was transformed with the opened plasmid, and disruption of the chromosomal *MAL12* gene was allowed to occur by homologous recombination at the *Sau*I site. The integration event was assayed by leucine prototrophy. The two hybrid *MALX2* genes were allowed to recombine again after the integration event, thereby deleting one of the hybrid *MALX2* genes and plasmid YDp-L. This event was followed by leucine auxotrophy. The resulting strain, 6001BΔ11Δ12, contains a hybrid *Mal12-Mal62* gene on the chromosome containing a *Pst*I-*Cla*I deletion. Disruption of the *Mal12* gene was confirmed by loss of *p*-nitrophenyl-α-D-glucoside-hydrolyzing activity. The 5,900-bp *Bam*HI-*Sac*I fragment of pY6, containing the entire *MAL61* gene, was ligated into pBluescript SK^- (Stratagene, La Jolla, Calif.). *MAL61* was isolated from the resulting plasmid by digestion with *Hin*dIII and subsequently ligated into YEp13 (5), containing the *ADC1* promoter and terminator located on a *Bam*HI fragment and separated by a unique *Hin*dIII site (11).

Preparation of cell extracts. The cell extract used for immunoblotting was obtained as described by Volland et al. (52). The cell extract used for the maltase assay and purification of the antibodies was prepared by vortexing 0.1M potassium phosphate (pH 6.8)-washed cells for 4 min in the presence of glass beads (diameter, 0.23 to 0.33 mm). Whole cells and debris were removed by centrifugation. Protein concentrations were determined in the presence of 0.5% (wt/vol) sodium dodecyl sulfate (SDS) by using a modified Lowry assay (15).

Immunoblot analysis. Proteins were separated by electrophoresis in SDS–10% polyacrylamide and transferred to polyvinylidene difluoride (Millipore) sheets by semidry electroblotting (28). Antibodies raised against a Mal61p-LacZ fusion protein (29) were a generous gift from M. Herweijer (Gist Brocades, Delft, The Netherlands). The antibodies were purified by incubation with cell extracts of *S. cerevisiae* 6001BΔ11Δ12, and serum dilutions of 1:15,000 were used. Primary antibodies were detected with a chemiluminescence detection system by using the CSPD chemiluminescent substrate as described by the manufacturer (TROPIX, Inc., Bedford, Mass.).

Maltase assay. Maltase activity was measured by monitoring the hydrolysis of *p*-nitrophenyl-2-D-glucoside at 400 nm. The reaction mixture consisted of 1 mM *p*-nitrophenyl-α-D-glucoside in 100 mM K-phosphate (pH 6.8) at 30°C. The reaction was started by the addition of cell extract containing 0.2 mg of protein in a total volume of 3 ml. Enzyme activity is expressed as micromoles of substrate converted · $minute^{-1}$ · mg of $protein^{-1}$.

Plasma membrane isolation. Plasma membranes were isolated as follows. Cells were washed three times with ice-cold demineralized water and once with buffer A (0.1 M glycine, 0.3 M KCl, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0). Cells (21 g [wet weight]) were suspended in 21 ml of buffer A and homogenized with 49 g of glass beads (diameter, 0.23 to 0.33 mm) by using a Braun MSK homogenizer. The cells were homogenized for 2 min (10 s on, 10 s off) with cooling by liquid carbon dioxide expansion. The supernatant was collected, and cells and debris were removed by low-speed centrifugation (10 min, 2,100 × *g*). The supernatant was filtered through a glass fiber filter (Sigma; retention, >3.3 μm), and crude plasma membranes were collected by high-speed centrifugation (30 min, 27,000 × *g*). The plasma membrane pellet was washed and suspended in 21 ml of buffer A, after which the pH of the mixture was titrated to 4.9 with 0.1 M HCl. At this pH, the mitochondria aggregate and can be removed by centrifugation (10 min, 2,100 × *g*). The supernatant was adjusted to pH 7.0 with 0.1 M KOH, incubated for 5 min, acidified to pH 5.0, and centrifuged at 7,700 × *g* for 3 min. The supernatant was adjusted to pH 7.0, and plasma membranes were collected by high-speed centrifugation (45 min, 27,000 × *g*), washed with buffer B (10 mM Tris, 1 mM EDTA, pH 7.0), resuspended to approximately 5 mg of protein per ml, and stored in liquid nitrogen until used. The purity of the plasma membranes was assayed by measuring the activity of the azide-sensitive mitochondrial and vanadate-sensitive plasma membrane ATPases (21). The plasma membranes usually contained less than 10% of mitochondria. The protein concentration was measured in the presence of 0.5% (wt/vol) SDS by using modified Lowry assay (15).

TABLE 1. Maltase and maltose transport activities in *S. cerevisiae*[a]

Yeast strain	Maltase activity (μmol · min^{-1} · mg of $protein^{-1}$) with the following carbon source:		Maltose transport activity (nmol · min^{-1} · mg of $protein^{-1}$) with the following carbon source:	
	1% galactose–1% maltose	1% maltose	1% glucose	1% maltose
6001B	0.23	37.6	No uptake	0.26
6001BΔ11Δ12	<0.010	No growth	No uptake	No growth
6001BΔ11Δ12/YEpY18	ND[b]	No growth	4.46	No growth

[a] The strains were grown in batch culture on minimal medium plus the sugars indicated. Uptake in intact cells was assayed as described in Materials and Methods and was started by the addition of 40 μM [^{14}C]maltose.

[b] ND, not determined.

Membrane fusion. Cytochrome *c* oxidase was isolated from beef heart according to the procedure of Soulimane and Buse (44), using Na-deoxycholate to solubilize the mitochondria and NH_4SO_4 precipitation to collect the enzyme. Cytochrome *c* oxidase was reconstituted in liposomes composed of acetone-ether-washed *E. coli* lipid and egg yolk phosphatidylcholine in a ratio of 3:1, and using 0.225 nmol of heme *a* per mg of lipid (12). COVs (10 mg of lipid) were mixed with purified plasma membranes (1 mg of protein), rapidly frozen in liquid N_2, and thawed slowly at room temperature (14). The resulting multilamellar hybrid membranes were sized and converted into unilamellar vesicles with a small-volume extrusion apparatus (Avestin Inc., Ottawa, Canada) (30), using polycarbonate filters with pore sizes of 400 and 200 nm (Avestin). Alternatively, the multilamellar hybrid membranes were sonicated for 5 to 10 s at 4°C with a probe sonicator (MSE Scientific Instruments, West Sussex, United Kingdom). Fusion was quantitated by monitoring the decrease of R_{18} (octadecyl rhodamine β-chloride) fluorescence quenching of *S. cerevisiae* membranes labeled with R_{18} (4 mol% phospholipid phosphorous) as described previously (13, 23). Labeled membranes were fused with COVs, and the R_{18} fluorescence (excitation and emission wavelengths of 560 and 590 nm, respectively) was determined prior to and after the addition of 1% (vol/vol) Triton X-100. The intensity with the labeled plasma membranes was taken as the zero value.

Transport studies. Cells were harvested at an optical density at 600 nm of 1, washed twice in buffer C (100 mM K-citrate PO_4, pH 5.5), and concentrated 60-fold. Cells were diluted 10 times in buffer C (to approximately 1 mg of protein per ml) at 30°C, and the uptake reaction was started with the addition of [U-^{14}C]maltose (Amersham; 582 Ci/mol). At given time intervals, samples of 15 μl were taken, diluted into 2 ml of ice-cold 0.1 M LiCl, and filtered immediately on 0.45-μm-pore-size cellulose-nitrate filters (Schleicher & Schuell). The filters were washed once with 2 ml of 0.1 M LiCl and transferred to scintillation vials. The amount of radioactivity was determined with a liquid scintillation counter (Packard-Tri-Carb 460 CD; Packard Instruments). The amount of [U-^{14}C]maltose absorbed to the filters was estimated in separate experiments and subtracted from the total amount taken up.

Hybrid membranes were diluted to a final concentration of approximately 1 mg of protein per ml in buffer C containing 5 mM $MgSO_4$ and the electron donor system K-ascorbate (30 mM)–*N,N,N,N*,-tetramethyl-*p*-phenylenediamine (TMPD) (150 μM)–horse heart cytochrome *c* (7.5 μM). After 1 min of incubation, [^{14}C]maltose was added and samples were processed as described above.

For exchange and efflux experiments, purified plasma membranes were fused with liposomes (*E. coli* lipid/phosphatidylcholine ratio, 3:1) without cytochrome *c* oxidase. Hybrid membranes were concentrated by high-speed centrifugation (30 min, 50,000 × *g*), resuspended to approximately 20 mg/ml in buffer C with various concentrations of [^{14}C]maltose, and allowed to equilibrate overnight in the presence of 1 μM valinomycin plus 1 μM nigericin. The exchange reaction was started by diluting the membrane suspension 1,000-fold with buffer C containing equal concentrations of nonradioactive maltose and 1 μM valinomycin plus 1 μM nigericin. The efflux reaction was started by diluting the membranes 1,000-fold with buffer C containing no maltose. Samples of 1 ml were withdrawn, filtered immediately, and processed as described above. The results of the exchange and efflux experiments were analyzed after subtracting the radioactivity retained on the filter after 60 min of incubation from the amount of radioactivity at given times.

RESULTS

Expression of the maltose transport protein. To obtain a genetically well-defined *S. cerevisiae* strain lacking the genes required for maltose uptake and hydrolysis, *MAL12* of strain 6001BΔ11 (containing a truncated *MAL11* gene) was disrupted by using an integrative plasmid containing a truncated *MAL62*

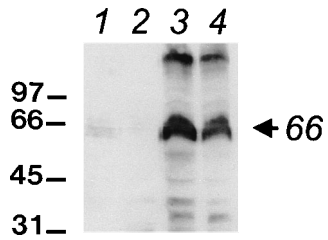

FIG. 1. Immunoblot visualizing the expression of the maltose transport protein in different strains grown in batch culture. Lane 1, strain 6001B grown on 1% maltose; lane 2, strain 6001BΔ11 grown on 1% glucose; lane 3, strain 6001BΔ11, containing YEpY18, grown on 1% glucose; lane 4, strain 6001BΔ11Δ12, containing YEpY18, grown on 1% glucose. Each lane contains 50 μg of protein. Numbers on the left and right are molecular masses in kilodaltons.

gene. The resulting strain, 6001BΔ11Δ12, was unable to grow on maltose, and maltose-hydrolyzing activity was virtually absent when the cells were grown on 1% glucose or on a mixture of 1% maltose and 1% galactose (Table 1). Strains 6001B Δ11 and 6001BΔ11Δ12 were used as hosts to monitor the expression of the maltose carrier from plasmid YEpY18. YEp Y18 contains the *MAL61* gene (encoding a maltose transport protein) under control of the *ADC1* promoter. YEpY18 is able to complement *S. cerevisiae* 6001BΔ11 on minimal medium containing maltose as the sole carbon and energy source (data not shown). The maltose transport activity observed in strain 6001BΔ11Δ12/YEpY18 grown on 1% glucose was about 20-fold higher than that of strain 6001B grown on 1% maltose (Table 1). Expression of the maltose transport protein, visualized by Western blotting (immunoblotting) (Fig. 1), correlated well with the transport activities in the different strains.

Preparation of hybrid membranes. Isolated plasma membranes of *S. cerevisiae* 6001B, grown in a maltose-limited chemostat, were fused with COVs. The hybrid vesicles were extruded through filters with different pore sizes in order to obtain unilamellar hybrid membrane vesicles with a rather well-defined diameter. Alternatively, the membrane preparations were sonicated after freeze-thawing, which results in unilamellar hybrid membrane vesicles with more heterogenous sizes (17). The fusion efficiency was quantitated with the R_{18} fusion assay (23) and was higher than 90% for the different membrane preparations (data not shown). Figure 2A shows that the rate of maltose uptake and accumulation levels in hybrid membrane vesicles prepared by extrusion were at least

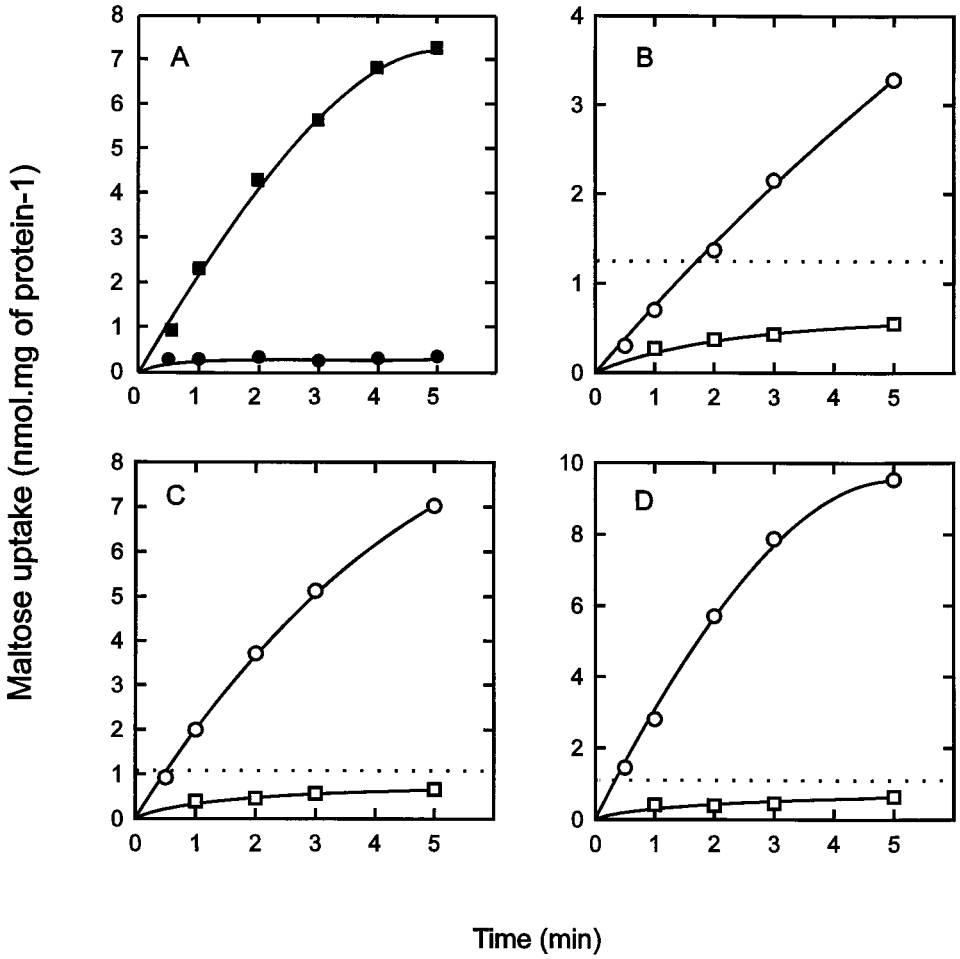

FIG. 2. (A) Uptake of maltose in hybrid membranes isolated from strain 6001B grown in a maltose-limited chemostat. Following freezing and thawing at room temperature, the hybrid membranes were prepared by sonication with a probe-type sonicator (●) or by subsequent extrusion through 400- and 200-nm-pore-size filters (■). (B, C, and D) The multilamellar membranes were sized by extrusion through polycarbonate filters with defined pore sizes of 400 (B), 200 (C), or 100 nm (D). The dotted line represents the equilibration level. □, uptake without electron donor; ○, uptake in the presence of K-ascorbate, cytochrome *c*, and TMPD. Uptake experiments were performed in 100 mM K-citrate, pH 5.5. The uptake experiment was started by the addition of 40 μM [^{14}C]maltose.

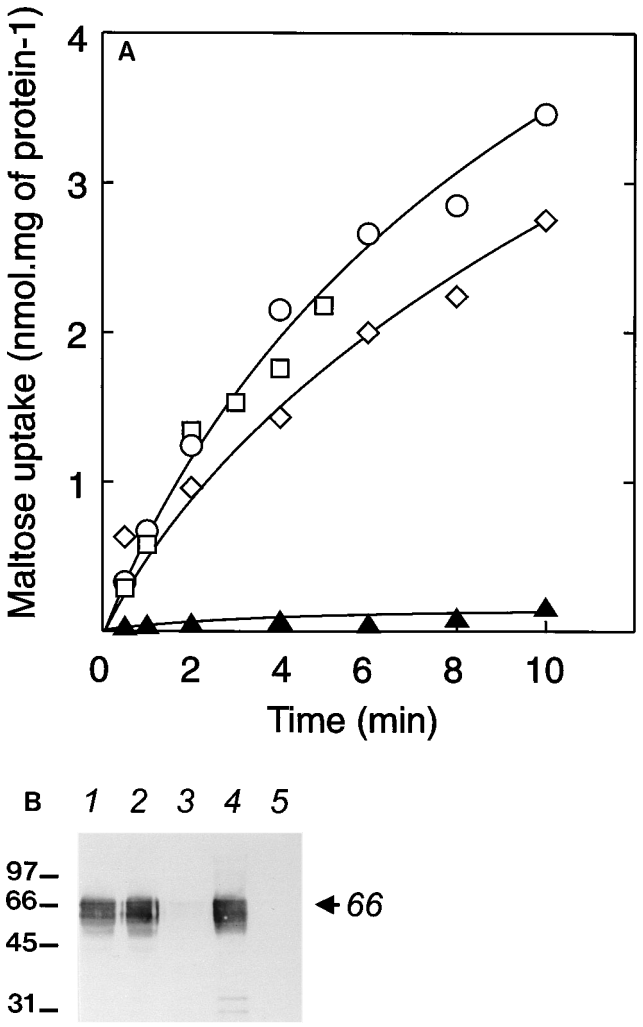

FIG. 3. Activity of Mal11p or Mal61p (A) and immunoblot quantifying the expression of the maltose transport proteins (B) in membranes prepared from different strains. ○ and lane 1, strain 6001BΔ11Δ12, containing YEpY18, grown in batch culture on 1% glucose; ◇ and lane 2, strain 6001BΔ11Δ12, containing YEpY18, grown in a glucose-limited chemostat; ▲ and lane 3, strain 6001B, grown in batch culture on 1% maltose; □ and lane 4, strain 6001B grown in a maltose-limited chemostat; lane 5, hybrid membranes prepared from strain 6001BΔ11Δ12 grown in a glucose-limited chemostat. For panel B, each lane contains 50 μg of protein and numbers on the left and right are molecular masses in kilodaltons. Uptake was assayed in the presence of K-ascorbate, cytochrome *c*, and TMPD as described in the legend to Fig. 2.

10-fold higher than those in sonicated membrane vesicles. Moreover, we observed that the reproducibility of the results was much higher with the extrusion method than when sonication was employed. To optimize the extrusion procedure further, polycarbonate filters with different pore sizes were compared (Fig. 2B, C, and D). Smaller pore sizes of the filters resulted in increased specific uptake activities. However, extrusion through 100-nm-pore-size filters also resulted in loss of material, most probably by retention of membranes on the filter. The conditions found to be optimal were passage of the membranes first through a 400-nm-pore-size filter and then through a 200-nm-pore-size filter.

Maltose transport in hybrid membrane vesicles. Plasma membrane vesicles were prepared from *S. cerevisiae* 6001B and 6001BΔ11Δ12/YEpY18 grown in batch or in chemostat cultures and in the presence of maltose or glucose. These membranes were fused with COVs, and the transport activities were compared. The initial rate of maltose uptake in plasma membrane vesicles from strain 6001B prepared from maltose-grown cells increased from 0.12 to 4.02 nmol · min^{-1} · mg of $protein^{-1}$ when maltose-limited chemostat cultures instead of batch cultivation were used (Fig. 3A). Importantly, the maltose uptake in hybrid membranes obtained from strain 6001BΔ11Δ12/YEpY18 grown on 1% glucose in a batch culture was comparable to that of 6001B grown in a maltose-limited chemostat and even somewhat higher than of strain 6001BΔ11Δ12/YEpY18 grown in a glucose-limited chemostat. The expression of the different maltose transport proteins, as visualized by Western blotting (Fig. 3B), correlates well with the transport activities of the different hybrid membranes.

Energetics and kinetics of maltose transport. The use of isolated plasma membrane vesicles fused with proteoliposomes allows a detailed analysis of the energetics and kinetics of transport. As shown before (51), uptake of maltose is dependent on both the ΔpH and the ΔΨ (Fig. 4A). Maltose uptake in the hybrid membranes, driven by the Δp, displayed Michaelis-Menten kinetics with a K_m^{app} of 4.6 ± 0.3 mM and a V_{max} of 52 ± 2 nmol/min · mg of protein (data not shown); these values are similar to those reported for intact cells (49). The maltose concentration gradients ($[maltose]_{in}/[maltose]_{out}$) were approximately 20 after 5 min of uptake and at a substrate concentration of 40 μM (using a specific internal volume of 10 μl/mg of protein); the $[maltose]_{in}/[maltose]_{out}$ was about 10 at a substrate concentration of 5 mM. In both cases, however, the maltose concentration gradients were not yet in equilibrium with the Δp (Fig. 4). At low (40 μM) and high (5 mM) maltose concentrations, dissipation of the Δp by valinomycin plus nigericin resulted in the efflux of accumulated maltose. Upon the addition of a 100-fold excess of nonradiolabeled maltose at the outside of the hybrid membrane vesicles, a rapid exit (exchange) of [^{14}C]maltose was observed at the high substrate concentration (Fig. 4B). At the low substrate concentration the exit of radiolabeled maltose was not complete (Fig. 4A). The rates of efflux down a concentration gradient and of equilibrium exchange were studied in more detail after equilibrating the hybrid membranes passively with increasing concentrations of [^{14}C]maltose. Exchange was faster than efflux at all substrate concentrations tested (Fig. 5). The exchange and efflux data could be fitted with the Michaelis-Menten equation and yielded K_m^{app} values of about 9 ± 1.6 and 46 ± 10.5 mM for efflux and exchange, respectively. In membrane vesicles prepared from strain 6001BΔ11Δ12 grown in a glucose-limited chemostat, no significant efflux or exchange could be observed (Fig. 5).

DISCUSSION

Expression of Mal61p in *S. cerevisiae* 6001BΔ11Δ12 from a multicopy plasmid resulted in a maltose transport activity 20-fold (for whole cells) to 30-fold (for isolated membranes) higher than that of the parental strain 6001B. The immunoblots indicate that the increased activity is due to enhanced expression. The expression of the maltose transport protein in 6001B cells grown in a maltose-limited chemostat is similar to the expression of the maltose transporter from YEpY18 grown on 1% glucose in batch culture (Fig. 3B). Growth of strain 6001BΔ11Δ12/YEpY18 in a glucose-limited chemostat, which induces the *ADC1* promoter (1), did not result in a further increase in maltose transport activity or protein expression. This level of expression might be close to the maximum possible in the plasma membrane of *S. cerevisiae*. Limitations may stem from proteolysis of the transport protein (catabolite in-

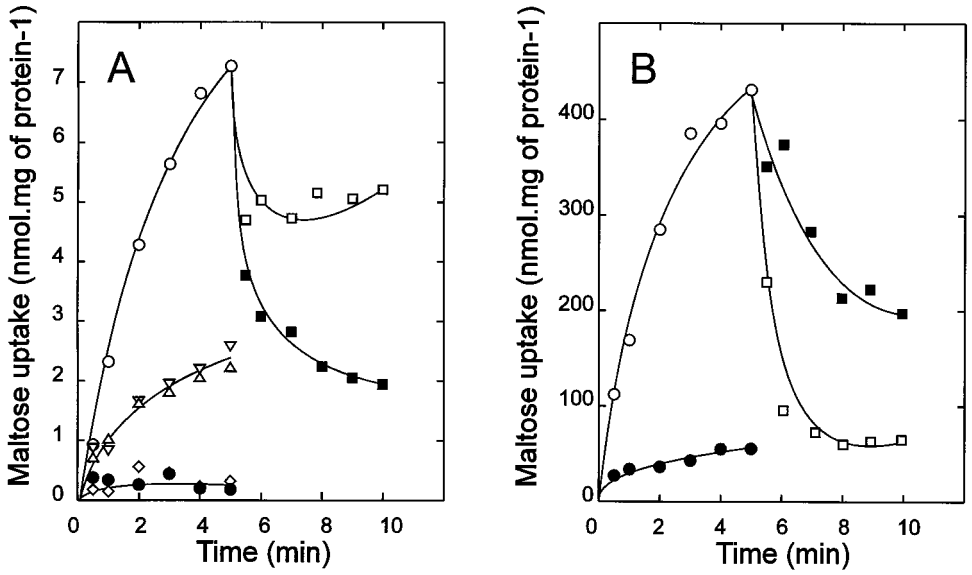

FIG. 4. Uptake of [^{14}C]maltose in hybrid membranes, isolated from strain 6001B grown in a maltose-limited chemostat, at initial substrate concentrations of 40 μM (A) and 5 mM (B). Uptake of [^{14}C]maltose was assayed in the presence of K-ascorbate, cytochrome *c*, and TMPD without further additions (○) or with 0.1 μM nigericin (△), 0.1 μM valinomycin (▽), or 0.1 μM valinomycin plus 0.1 μM nigericin (◇). After 5 min of accumulation of maltose, a 100-fold excess (4 or 500 mM) of unlabeled maltose (□) or 0.1 μM valinomycin plus 0.1 μM nigericin (■) was added. ●, uptake in the absence of an electron donor.

activation) (6, 10, 24, 29) or a limited capacity of the secretory pathway to deliver proteins to the plasma membrane. Limitations in expression have also been observed for other transport proteins in *S. cerevisiae*, such as the uracil and cytosine transporter (25), the arginine transporter (38), and the lysine transporter (46).

A rapid and irreversible inactivation of proteins is frequently observed in *S. cerevisiae* when the nitrogen source in the medium is exhausted, glucose or other readily fermentable substrates are added, protein synthesis is inhibited, or stress conditions are imposed (6, 10, 24, 52). The presence of immunogenic low-molecular-weight polypeptides in isolated plasma membranes suggests proteolytic breakdown of the maltose transport protein during plasma membrane isolation (Fig. 3B). It has been shown before that the antibodies can react with three or more bands in the 66-kDa region (29). These bands may reflect different degradation products and/or phosphorylation states of the protein (47). Our current research is aimed at isolating Mal61p mutants and *S. cerevisiae* strains in which this heterogeneity in the maltose transport protein(s) is absent.

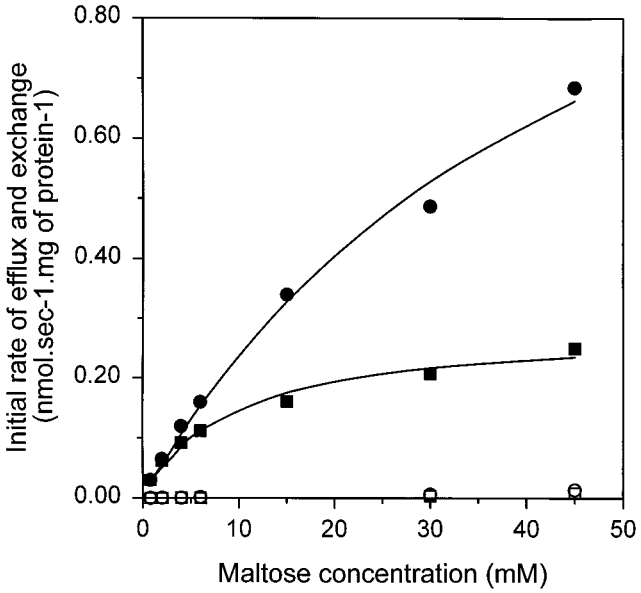

FIG. 5. Efflux and exchange in hybrid membranes prepared from chemostat-grown cells of strain 6001B grown under maltose limitation. ●, equilibrium exchange; ■, maltose efflux down the concentration gradient. The lines are based on a fit of the data according to the Michaelis-Menten equation. The exit of maltose in strain 6001BΔ11Δ12, grown under glucose limitation, in the presence (○) and absence (□) of an equal external concentration of unlabeled maltose is also shown. Experiments were performed in 100 mM K-citrate buffer (pH 5.5) in the presence of 1 μM valinomycin plus 1 μM nigericin.

Some reports on the reconstitution of Δp-driven transport systems of *S. cerevisiae* have appeared (7, 38, 39, 51). A glucose transport system from *Arabidopsis thaliana* was expressed in *S. cerevisiae*, and transport was studied in isolated plasma membranes (45). Recently, Pho84p, a inorganic phosphate transporter from *S. cerevisiae*, has been purified and functionally reconstituted into liposomes (2). To generate a Δp in these plasma membranes or reconstituted systems, it is often essential to incorporate powerful Δp-generating systems into the membranes. This can be done by fusing the plasma membranes with COVs by a freeze-thaw procedure. This results in multilamellar membrane structures which, in this form, are not suited for transport studies. The conversion of multilamellar structures into unilamellar membranes can be realized by sonication (14). The isolated plasma membranes of *S. cerevisiae*, however, are very sensitive to sonication, and this method does not give very reproducible results. In this paper we show that better results can be obtained by extruding the multilamellar membranes through polycarbonate filters with a defined pore size (30). This method has the additional advantage that membranes with a more uniform size compared with that of sonicated membranes are obtained (17).

In several secondary transport systems the Δp or its components are part of the driving force for solute accumulation (36).

Usually, this reaction is reversible and accumulated solutes will leak out down the concentration gradient when the Δp is dissipated. Transport studies with intact yeast cells have frequently indicated that secondary transport systems in *S. cerevisiae* are not reversible but rather operate unidirectionally. This apparent unidirectionality can be caused by several factors: (i) the internal pH could convert the transported solute into a species which is not recognized by the transport system; (ii) the transported solute could be chemically modified following translocation across the plasma membrane; (iii) the transported solute could be sequestered into another compartment so that it is no longer accessible to the transport system; or (iv) the kinetic parameters for entry and exit transport could differ. It has also been suggested that accumulated solutes inhibit the action of the transporter by a process termed *trans* inhibition (27). Accumulated solutes bind to the transport protein on the *trans* side of the membrane, thereby inhibiting the turnover of the enzyme (26). The unidirectionality of maltose transport was thought to be caused by such *trans* inhibition (16, 26). The experiments whose results are shown in Fig. 4A do not confirm this suggestion. The partial release of [^{14}C]maltose at a low substrate concentration upon the addition of a 100-fold excess of unlabeled maltose (Fig. 4A) can be explained by the low affinity of Mal61p for maltose. At a 100-fold excess of unlabeled maltose, the total maltose concentration is similar to the K_m^{app} of the uptake reaction. Under these conditions, uptake of the remaining radiolabeled maltose from the outside will compensate for the loss of radiolabeled maltose from the vesicle lumen. The efflux and exchange experiments described in this paper show unequivocally that high concentrations of the solute on the *trans* side of the membrane do not inhibit the activity of the transporter. Furthermore, it is evident that Mal61p catalyzes downhill transport in the absence of a Δp, which contrasts with other suggestions (16, 35). It has also been reported that arginine transport in hybrid membranes is unidirectional (38). However, at low concentrations of arginine, normal efflux of the substrate is observed after the driving force is collapsed, indicating that also in this case the symport process is fully reversible (48). The insignificant exit of maltose (even at high substrate concentrations) in the hybrid membranes prepared from strain 6001BΔ11Δ12 indicates that diffusion does not play a significant role in translocation of maltose across the membrane.

In conclusion, the method for the preparation of hybrid membranes presented here is a major step forward in the development of a model system for the study of transport processes in yeast plasma membranes. Studies of transport in these hybrid membranes allow detailed investigations of the mechanism of transport. This model membrane system is essential for the characterization of transport mutants that differ in kinetic properties and for the analysis of the effects of posttranslational modifications.

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