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Mechanism of glucose and maltose transport in plasma-membrane vesicles from the yeast *Candida utilis*

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Transport of glucose and maltose was studied in plasma-membrane vesicles from *Candida utilis*. The yeast was grown on a mixture of glucose and maltose in aerobic carbon-limited continuous cultures which enabled transport to be studied for both sugars with the same vesicles. Vesicles were prepared by fusion of isolated plasma membranes with proteoliposomes containing bovine heart cytochrome *c* oxidase as a proton-motive-force-generating system. Addition of reduced cytochrome *c* generated a proton-motive force, consisting of a membrane potential, negative inside, and a pH gradient, alkaline inside. Energization led to accumulation of glucose and maltose in these vesicles, reaching accumulation ratios of about 40–50. Accumulation also occurred in the presence of valinomycin or nigericin, but was prevented by a combination of the two ionophores or by uncoupler, showing that glucose and maltose transport are dependent on the proton-motive force. Comparison of sugar accumulation with quantitative data on the proton-motive force indicated a 1:1 H^+/sugar stoichiometry for both transport systems. Efflux of accumulated glucose was observed on dissipation of the proton-motive force. Exchange and counterflow experiments confirmed the reversible character of the H^+–glucose symporter. In contrast, uncoupler or a mixture of valinomycin plus nigericin induced only a slow efflux of accumulated maltose. Moreover under counterflow conditions, the expected transient accumulation was small. Thus the H^+–maltose symporter has some characteristics of a carrier that is not readily reversible. It is concluded that in *C. utilis* the transport systems for glucose and maltose are both driven by the proton-motive force, but the mechanisms are different.

INTRODUCTION

In yeasts, sugar transport can proceed by at least two different mechanisms. Facilitated diffusion, a widespread mechanism among yeasts [1], is a carrier-mediated transport where net uptake of a sugar ceases when the intracellular concentration reaches that of the external medium. In addition, many yeasts are capable of transporting sugars against a concentration gradient [1,2]. In most of these cases, coupling of sugar uptake and proton influx has indicated involvement of proton–sugar symport mechanisms.

Sugar-transport studies in yeasts have long relied on the use of intact cells. A major problem of intact-cell studies is interference of intracellular metabolism with transport, which may obscure transport parameters. Furthermore it is virtually impossible to selectively manipulate the driving force for active transport, thereby complicating quantitative studies on energy-coupled transport. Finally, transport studies are hindered by intracellular compartments of yeast cells. These problems can be overcome by using membrane vesicles. Isolated yeast plasma membranes, however, do not form well-sealed vesicles, even though they can be used to measure counterflow [3]. Sealing is improved by fusion of plasma-membrane vesicles with liposomes [4], a technique that has been used to study sugar transport in *Saccharomyces cerevisiae* [5,6]. An additional advantage of membrane fusion, which has had great impact on studies of secondary active transport in bacteria [7], is that a stable proton-motive-force (PMF)-generating system can be co-reconstituted.

Fusion of yeast plasma membranes with proteoliposomes, containing bovine heart cytochrome *c* oxidase as a PMF generator, was first used in studies on ion and leucine transport in *S. cerevisiae* [8,9]. Postma [10], studying glucose and arginine transport in cytochrome *c* oxidase-containing plasma-membrane vesicles of *Candida utilis*, found only low solute accumulation ratios and, moreover, a poor reproducibility. The methodology for measuring transport in vesicles, prepared by fusion of isolated plasma membranes and proteoliposomes containing cytochrome *c* oxidase, was improved by van Leeuwen et al. [11]. The improved technique was used for the characterization of galactose and lactose transport in *Kluyveromyces marxianus* [11,12] and also enabled quantitative kinetic studies on maltose transport in *S. cerevisiae* [13]. The potential of the membrane-fusion technique for sugar-transport studies in yeasts was supported in a recent study [14], which confirmed uphill maltose transport in plasma membranes of *S. cerevisiae*.

C. utilis ('fodder yeast') is a popular organism for physiological studies on sugar metabolism in yeasts, and it stands as a model for the group of Crabtree-negative yeasts that can exhibit active glucose transport [15]. So far its sugar-uptake systems have only been investigated in intact cells. Studies on uptake of radio-labelled glucose by *C. utilis* cells revealed the involvement of at least two kinetically distinct and differentially regulated components [16,17]. Since the kinetic constants of glucose-dependent alkalinization of weakly buffered cell suspensions were identical with those of the high-affinity component ($K_m \approx 20$ μM glucose), it was concluded that this component was probably a proton–glucose symporter. This differs from *S. cerevisiae*, which appears to transport glucose via facilitated diffusion [1,2]. Maltose uptake by *C. utilis* has been reported to involve a system with a K_m of about 0.4 mM [18]. Attempts in our laboratories to study the

Abbreviations used: PMF, proton-motive force; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; TPP^+, tetraphenylphosphonium; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; R18, octadecyl-rhodamine B.

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mechanism of maltose uptake in this yeast were hampered by binding of labelled disaccharide to the cells. Furthermore, unlike the situation in *S. cerevisiae*, maltose-dependent alkalinization of weakly buffered *C. utilis* cell suspensions is sluggish and poorly reproducible (M. A. H. Luttik and R. A. Weusthuis, unpublished work).

The aim of the present work was to characterize and compare the mechanisms of glucose and maltose transport in *C. utilis* membrane vesicles, prepared by fusion of plasma membranes with proteoliposomes containing cytochrome *c* oxidase. In order to have the maltose and glucose carriers simultaneously present in the same membranes, cells expressing both uptake systems were obtained from carbon-limited chemostat cultures grown on a mixture of the two sugars.

EXPERIMENTAL

Growth conditions

C. utilis CBS 621 was grown in continuous culture in a 1-litre laboratory fermenter (Applikon) at 30 °C and a stirrer speed of 1000 rev./min in a medium described previously [13]. The medium was supplied with a mixture of glucose and maltose, each at a final concentration of 5 g/l, at a dilution rate of 0.1 h^{-1}. The pH was maintained at 5.0 by addition of 2 M KOH, and air was flushed at a rate of 1 l/min. This resulted in a dissolved oxygen tension higher than 50 % of air saturation. These growth conditions resulted in a cell density of 5 g dry weight/l.

Isolation of plasma membranes

Plasma membranes were isolated essentially as described previously [13]. Briefly, *C. utilis* cells were washed in buffer A (0.3 M KCl, 0.1 M glycine, pH 7.0), and then 15 g were resuspended in 15 ml of buffer A, and PMSF was added (0.1 mM). After homogenization with 35 g of glass beads (0.25–0.32 mm) for 75 s in a Braun cell homogenizer MSK, the suspension was separated from the glass beads by filtration on a 25D2 glass filter, and centrifuged for 10 min at 2100 ***g*** in a Sorvall SS34 rotor. The supernatant was filtered through a Sartorius glass-fibre filter (13400-47-S), and centrifuged for 20 min at 6200 ***g*** in a Sorvall SS34 rotor. The supernatant was again centrifuged for 20 min at 6200 ***g*** and the two pellets were combined and washed once with buffer A. The pellet was resuspended in a large volume (50–100 ml) of buffer A, frozen in liquid nitrogen and defrosted at room temperature. After centrifugation (20 min, 6200 ***g***) the pellet was resuspended in 15 ml of buffer A containing 0.1 mM PMSF and titrated to pH 4.9 with 50 mM HCl in buffer A. After centrifugation for 10 min at 2100 ***g***, the supernatant was brought to pH 7.0, and the pellet was resuspended, acidified once more and centrifuged for 10 min at 2100 ***g***. The supernatant was brought to pH 7.0, combined with the first supernatant and frozen in liquid nitrogen. After thawing, it was centrifuged for 20 min at 100000 ***g*** in a Kontron TFT 70 rotor. The pellet was washed once with 50 mM potassium phosphate/1 mM $MgCl_2$, pH 6.2, and was finally resuspended in this buffer at a concentration of about 3–5 mg of protein/ml and stored in small aliquots in liquid nitrogen.

Protein was determined by the method of Lowry et al. [19] with BSA as standard. The purity of the plasma membranes was checked by measuring the ATPase activity. Briefly, plasma membranes were incubated in 25 mM Tris/maleate, containing 50 mM KCl and 5 mM $MgCl_2$, at pH 5, 6, 7, 8 and 9. ATP was added to a concentration of 4 mM, and after 45 min incubation at 30 °C the amount of P_i liberated was measured as described by Tijssen et al. [20].

Preparation of liposomes with reconstituted cytochrome *c* oxidase

Cytochrome *c* oxidase was isolated as described by Yu et al. [21], and activity was measured by the method of Yonetani [22]. Commercially obtained phospholipids were extracted with acetone/ether [23]. The lipids were dissolved in chloroform and stored at −20 °C under nitrogen gas. Cytochrome *c* oxidase-containing proteoliposomes were routinely prepared in 50 mM potassium phosphate, pH 6.2, by the method of van Leeuwen et al. [11], using *Escherichia coli* lipids (containing 50 % phosphatidylethanolamine). In some cases, the buffer system was 50 mM sodium phosphate or 50 mM Tris/phosphate, pH 6.2.

Fusion of cytochrome *c* oxidase-containing liposomes with yeast plasma membranes

Freeze–thaw–sonication technique

Cytochrome *c* oxidase-containing liposomes (5 mg of phospholipid) and plasma membranes (0.25 mg of protein, unless stated otherwise) were defrosted and mixed in a volume of 300 μl in an Eppendorf reaction vessel. The buffer contained, unless indicated otherwise, 50 mM potassium phosphate and 1 mM $MgCl_2$ (final concentrations) at the indicated pH. The mixture was frozen in liquid N_2, thawed at room temperature and sonicated twice for 10 s in an ultra K42 sonic bath sonifier.

Freeze–thaw–extrusion technique

The procedure was identical with the freeze–thaw–sonication technique, except that the sonication was replaced by extrusion essentially as described by MacDonald et al. [24]. Briefly, the freeze–thawed liposome preparation was passed 9 or 11 times through a polycarbonate membrane (pore size 200 or 400 nm) in a LiposoFast extruder (Avestin Inc.).

The hybrid cytochrome oxidase-containing liposome–plasma membrane vesicles were used immediately. The internal volume was determined with L-[^{14}C]glucose or [^{14}C]lactose [11].

Measurement of $\Delta\psi$ and ΔpH

The membrane potential was determined from the distribution of tetraphenylphosphonium (TPP^+), using a TPP^+-selective electrode [25]. Vesicles (0.021 mg of protein) were suspended in 1.5 ml of 50 mM potassium phosphate/1 mM $MgCl_2$ at the indicated pH, and 2 μM TPP^+ was added. Energization was started with a solution containing 100 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 10 μM cytochrome *c* and 20 mM ascorbate (adjusted to the indicated pH with KOH). $\Delta\psi$ was calculated assuming symmetrical binding [26,27].

ΔpH was determined after measurement of the intravesicular pH using the technique of Clement and Gould [28]. The buffer system and electron donors were the same as for the $\Delta\psi$ determination.

Fusion assay

Fusion of plasma membranes with liposomes was measured using the fluorescence-dequenching method of Hoekstra et al. [29]. Briefly, octadecylrhodamine B (R18; 6 μg) was added to 600 μl of plasma membranes (1 mg of protein) in 50 mM potassium phosphate/1 mM $MgCl_2$ with continuous vortexing. After 20 min of incubation at room temperature in the dark, unbound R18 was removed on a Sephadex G-75 spin column. The membranes were washed twice and resuspended in 50 mM potassium phosphate/1 mM $MgCl_2$, pH 5.7. Hybrid plasma-membrane–liposome vesicles were prepared as described above.

R18 fluorescence was measured in a Perkin–Elmer luminescence spectrometer LS50B with excitation at 560 nm and emission at 590 nm. Fluorescence in the presence of 0.5 % Triton X-100 was taken as 100 % dequenching.

Measurement of sugar transport

Uptake of sugar in vesicles was determined as described previously [11] with some minor modifications. Vesicles were diluted in the same buffer as used for the preparation of the vesicles, with a final volume of 300 μl and a vesicle concentration of about 50–100 μg of protein/300 μl. Energization was performed by adding a solution containing 10 μM cytochrome *c*, 100 μM TMPD and 20 mM ascorbate (adjusted to the pH of the buffer). Transport was started by adding ^{14}C-labelled sugar, usually 5 μl from the radioactive stock, giving an external concentration of 5.3 μM maltose or 11 μM glucose. Samples of 15 μl were withdrawn, diluted in 2 ml of ice-cold 0.1 M LiCl, filtered on a cellulose nitrate filter, after which the filter was washed once with 2 ml of LiCl. Radioactivity on the filter was measured by liquid-scintillation counting. For kinetic analysis of uptake, 20 μl vesicles were placed in 70 μl of buffer containing cytochrome *c*, TMPD and ascorbate. After 2 min at 30 °C 10 μl of ^{14}C-sugar of the appropriate specific radioactivity was added. Transport was measured after 30 s of incubation as described previously. Binding of radioactivity to vesicles and the filter was determined by filtering and washing samples where the ^{14}C-labelled sugar was added to 2 ml of LiCl containing the appropriate amount of vesicles plus cytochrome *c*/TMPD/ascorbate. Kinetic constants are given as the mean ± S.D. for three independent measurements.

Equilibrium exchange

Vesicles (300 μl) were prepared by mixing 5 mg of liposomes, not containing cytochrome *c* oxidase, with 0.25 mg of plasma-membrane protein in the presence of 2 mM glucose or 10 mM maltose. The buffer was 50 mM potassium phosphate/1 mM $MgCl_2$, pH 5.7. After freeze–thaw–sonication the vesicles were incubated at 30 °C, 1 μM carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP) was added and exchange was started by adding 5 μl of ^{14}C-sugar. A zero-*trans* influx control was performed by preparing vesicles in the absence of sugar, and initiating transport by adding 5 μl of ^{14}C-sugar plus unlabelled sugar giving a final concentration of 2 mM glucose or 10 mM maltose. Transport was assayed as described before.

Counterflow

Vesicles (300 μl), not containing cytochrome *c* oxidase, were prepared (1:20 protein/lipid ratio) in the presence of 2 mM glucose plus 11 μM [^{14}C]glucose or 10 mM maltose plus 5.3 μM [^{14}C]maltose. The buffer was 50 mM potassium phosphate/1 mM $MgCl_2$, pH 5.7. After freeze–thaw–sonication, vesicles were centrifuged and resuspended in 50 μl of the same buffer, containing the same concentrations of unlabelled and labelled sugar. Subsequently 20 μl concentrated vesicles were diluted in 600 μl of buffer, containing only labelled sugar (11 μM [^{14}C]glucose or 5.3 μM [^{14}C]maltose), and 1 μM FCCP was added immediately. Samples of 40 μl were taken, and transport was measured as described above.

Chromatography of vesicle extracts

Intravesicular sugars were analysed by TLC [30]. Briefly, 300 μl vesicles were energized with cytochrome *c*/TMPD/ascorbate after which ^{14}C-sugar was added. At 10 and 20 min after addition of the sugar, 120 μl of this mixture was diluted in 2 ml of ice-cold 0.1 M LiCl, filtered and washed once with 2 ml LiCl. The filters were extracted with 100 μl of 80 % ethanol for 30 min with continuous agitation. After separation of the ethanolic extract from the filter, the volume of this extract was reduced to ± 20 μl under a current of nitrogen gas. A 5 μl aliquot was loaded on a cellulose F TLC plate. Elution was performed in formic acid/butanone/*t*-butanol/water (15:30:40:15, by vol.). After the chromatogram had been dried, autoradiography was performed, and quantitative data were obtained by cutting the chromatogram into 2 mm bands and measuring radioactivity by liquid-scintillation counting.

Materials

D-[U-^{14}C]Glucose (10.8 GBq/mmol), [U-^{14}C]maltose (23 GBq/mmol), L-[1-^{14}C]glucose (2.04 GBq/mmol) and [D-*glucose*-1-^{14}C]lactose (2.1 GBq/mmol) were obtained from Amersham International. L-α-Phosphatidylethanolamine (type IX from *E. coli*, P6398), nigericin, cytochrome *c* and PMSF were obtained from Sigma. Tetraphenylphosphonium bromide, octyl β-D-glucopyranoside, FCCP, nonactin and monensin were from Fluka. TMPD was obtained from BDH, L-ascorbic acid from J. T. Baker, R18 from Molecular Probes, pyranine from Eastman and Scintillator 299 from Packard Instruments Co. Cellulose nitrate filters with pore size 0.45 μm were purchased from Schleicher & Schuell, and cellulose F TLC plastic sheets from Merck, Darmstadt, Germany.

RESULTS

Characteristics of *C. utilis* plasma-membrane vesicles

In order to estimate the purity of isolated plasma membranes, ATPase activity was measured. It was found that the membrane preparation contained an ATPase with maximal activity at pH 6.5–7.0, as reported for other *Candida* species [31,32]. At pH 6, 0.1 μM orthovanadate inhibited activity by more than 90 %, whereas inhibition with 5 mM azide was less than 10 %. Since at pH 8.5 also azide did not inhibit ATPase activity, it is concluded that the isolated plasma-membrane preparations were not significantly contaminated with mitochondrial membranes [32].

Fusion of isolated plasma membranes with proteoliposomes containing bovine heart cytochrome *c* oxidase was achieved through freeze–thaw–sonication as described previously [11]. To determine the efficiency of fusion, R18 fluorescence was measured. It was found that fusion of proteoliposomes with plasma membranes containing R18 resulted in R18 fluorescence dequenching. The fusion efficiency was calculated to be higher than 95 %.

Transport in vesicles

Addition of cytochrome *c*, TMPD and ascorbate to the vesicles generated a PMF, negative and alkaline inside. Under energized conditions, both glucose and maltose were accumulated by the vesicles (Figure 1). On the basis of an internal volume of 20 μl/mg of protein, determined as described by van Leeuwen et al. [11], it can be calculated that accumulation ratios of 40–50 were reached. Although plasma-membrane vesicles should be devoid of cytoplasmic enzymes, it could be expected that a small fraction of these enzymes co-purified with the plasma membranes. This might lead to modification of the sugar, thus perturbing accumulation studies. To test whether intravesicular sugar was

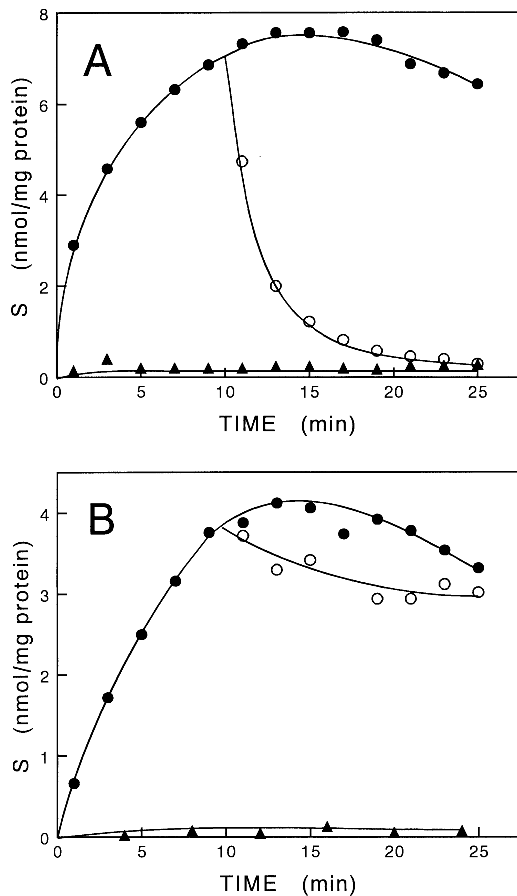

Figure 1 Effect of uncoupler on glucose and maltose transport in energized vesicles

Vesicles were incubated in 50 mM potassium phosphate/1 mM $MgCl_2$ at pH 5.7. The 10 μM cytochrome *c*/100 μM TMPD/20 mM ascorbate solution was added 1 min before the sugar, followed at $t = 0$ by 11 μM [^{14}C]glucose (**A**) or 5.3 μM [^{14}C]maltose (**B**). 10 μM FCCP was added at $t = -1$ min (▲) or at $t = 10$ min (○). No FCCP was added to the control (●). Transport was measured as described in the Experimental section, and is given as the intravesicular concentration of sugar (S).

modified, vesicle extracts were analysed by TLC. It was found that glucose was not modified in a 20 min uptake experiment. In the case of maltose transport, a slight increase in radioactive material co-migrating with the glucose standard was observed, i.e. from 0.3 % in the radioactive stock solution to 1.6 % after 20 min of transport. However, even after 20 min of uptake, 98 % of the intravesicular radioactivity co-migrated with maltose, indicating that the vesicles did not contain a significant amount of maltase.

It has been reported that sonication can be deleterious to the activity of the maltose-transport system from *S. cerevisiae* [14]. To test whether this was the case for maltose transport in *C. utilis*, transport was compared in vesicles prepared by freeze–thaw–sonication and by freeze–thaw–extrusion. For extrusion, vesicles were passed 11 times through a 400 nm filter, or alternatively, first 9 times through a 400 nm followed by 9 times through a 200 nm filter. No differences in influx velocity or accumulation were observed between the extrusion and sonication method (results not shown). Therefore the freeze–thaw–sonication method was used throughout this paper to analyse transport in vesicles from *C. utilis*.

The 40–50-fold intravesicular accumulation of sugar shown in Figure 1 was obtained with vesicles prepared by fusion of membranes with liposomes in a ratio of 0.25 mg of protein/5 mg of phospholipid. In order to study the dependence of the accumulation ratio on the protein/phospholipid ratio, vesicles were prepared with 5 mg of phospholipid and various amounts of plasma membranes. For glucose transport lower accumulation ratios than observed in Figure 1 were obtained when less than 100 μg of membrane protein/5 mg of phospholipid was used, whereas for maltose uptake this occurred with less than 175 μg of membrane protein per 5 mg of phospholipid. Since the data indicated that maximal accumulation ratios were achieved at a 1:20 protein/phospholipid ratio, all further experiments were performed at this plasma-membrane/phospholipid ratio.

Transport kinetics

Kinetic analysis of glucose and maltose transport revealed that uptake was linear with time for at least 30 s. Therefore 30 s uptake experiments were used to study influx kinetics. Monophasic uptake kinetics were observed for maltose transport, measured in a concentration range from 50 μM to 5 mM. The apparent K_m was 0.37 ± 0.17 mM with a V_{max} of 26 ± 8 nmol/min per mg of protein. For glucose transport, biphasic kinetics were measured in the concentration range from 13 μM to 20 mM. Computer curve fitting of the data [33] yielded for the first component an apparent K_m of 78 ± 9 μM with a V_{max} of 28 ± 10 nmol/min per mg of protein. Although the kinetic constants of the second component could not be estimated accurately, the data indicated a $K_m > 20$ mM. Whether this system represents a low-affinity transporter or reflects passive diffusion was not investigated. In order to measure the activity of the high-affinity glucose carrier without interference from the low-affinity transport system, further experiments were performed at low external glucose concentrations (11 μM). It was estimated from the kinetic constants that, at this concentration, less than 5 % of transport activity was accounted for by the low-affinity system.

To determine whether glucose could be transported by the maltose carrier, and vice versa, inhibition kinetics were studied. Influx of [^{14}C]glucose (11 μM initial concentration) was not inhibited by maltose even at a concentration of 2 mM (five times the zero-*trans* K_m). Maltose influx, measured at an initial external concentration of 5.3 μM, was slightly inhibited by glucose. At the highest glucose concentration tested, i.e. 0.5 mM (six-times the zero-*trans* influx K_m of the high-affinity system), inhibition was only 13 %, showing that high-affinity glucose transport does not proceed via the maltose carrier.

Effect of ionophores

To study the influence of the PMF on glucose and maltose transport, the effect of various ionophores on transport was studied. As shown in Figure 1 the uncoupler FCCP completely prevented accumulation. In the presence of FCCP, or a mixture of valinomycin plus nigericin, uptake did not exceed the passive diffusion equilibrium. In the presence of either valinomycin or nigericin, accumulation still took place (Figure 2), although to a lower extent (especially in the case of maltose transport). These data show that the glucose- and maltose-uptake systems are dependent on the PMF.

Addition of FCCP or a mixture of valinomycin and nigericin induced rapid and complete efflux of accumulated glucose (Figures 1 and 2). Dissipation of the PMF with uncoupler or valinomycin plus nigericin resulted in only a slow efflux of maltose. Addition of excess non-radioactive maltose to vesicles

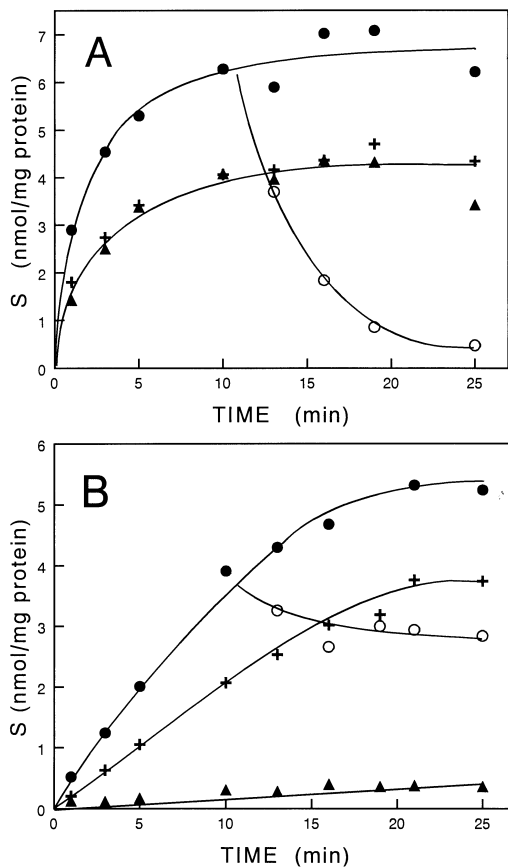

Figure 2 Effect of nigericin and valinomycin on glucose and maltose transport in vesicles

Glucose (**A**) and maltose (**B**) transport were measured under the conditions described for Figure 1. ●, Control; ▲, 100 nM valinomycin added at $t = -1$ min; +, 37 nM nigericin added at $t = -1$ min; ○, 100 nM valinomycin + 37 nM nigericin added at $t = 10$ min.

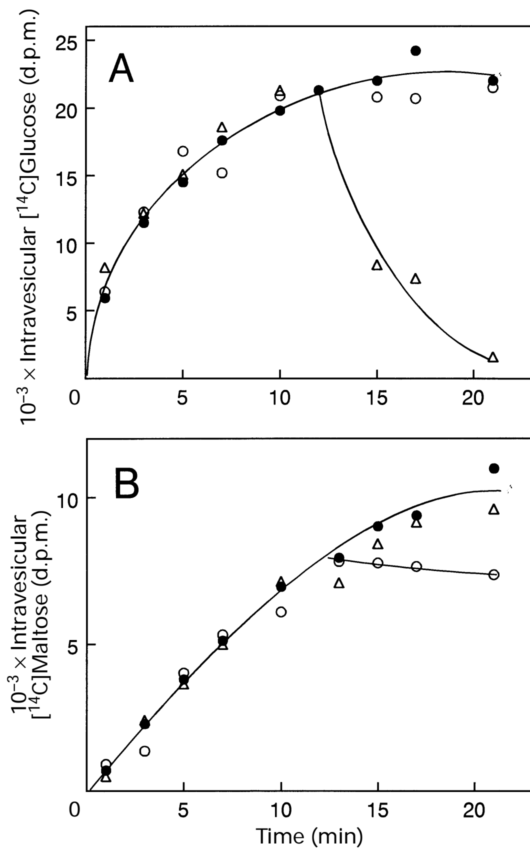

Figure 3 Effect of excess sugar on accumulation of ^{14}C-sugar in vesicles

Vesicles incubated in 50 mM potassium phosphate/1 mM $MgCl_2$ at pH 5.7 were energized at $t = -1$ min by adding 10 μM cytochrome *c*, 100 μM TMPD and 20 mM ascorbate. At $t = 0$, 11 μM [^{14}C]glucose (**A**) or 5.3 μM [^{14}C]maltose (**B**) was added, and transport was measured as described before. At $t = 12$ min, 1 mM non-radioactive glucose (△) or 5 mM non-radioactive maltose (○) was added. ●, Control without extra addition.

that had accumulated [^{14}C]maltose only caused a slow decrease in intravesicular radioactivity (Figure 3B), whereas under similar conditions unlabelled glucose induced a rapid efflux of accumulated [^{14}C]glucose (Figure 3A). Thus the data indicate that glucose transport is readily reversible, whereas maltose transport seems to be poorly reversible.

The poor reversibility of maltose transport could be due to inhibition of carrier activity by high concentrations of maltose, at either the *cis* or the *trans* side. To evaluate this possibility, the effect of FCCP on maltose efflux was tested with external maltose concentrations of 0.3–30 μM. On energization, the intravesicular sugar concentrations reached levels ranging from 17 μM (= 0.34 nmol/mg of protein) at an external maltose concentration of 0.3 μM to 1.8 mM (= 36 nmol/mg of protein) at the highest external maltose concentration used (30 μM). At all maltose concentrations studied FCCP caused only very slow efflux. This shows that maltose transport was poorly reversible even at very low external and internal sugar concentrations. Another way to measure *trans*-inhibition is to determine substrate influx in vesicles preloaded with non-radiolabelled substrate, essentially as described for amino acid transport in intact yeast cells [34–36]. It would be expected that, if the maltose transporter is sensitive to *trans*-inhibition, high intravesicular concentrations of unlabelled maltose would inhibit influx of [^{14}C]maltose. Therefore vesicles were preincubated with 20 μM unlabelled maltose plus cytochrome *c*/TMPD/ascorbate. At various preincubation times [^{14}C]maltose influx was measured. After preincubation of vesicles with maltose, maltose accumulation occurred (Table 1), reaching, after 10 min, an internal maltose concentration of

Table 1 Effect of the incubation time of vesicles with maltose on sugar accumulation and sugar influx

Vesicles were incubated in 50 mM potassium phosphate/1 mM $MgCl_2$ at pH 5.7. At $t = -1$ min, cytochrome *c*/TMPD/ascorbate was added, followed at $t = 0$ by the addition of 20 μM maltose. Samples of 90 μl were taken at the times indicated, mixed with 1 μM [^{14}C]maltose and the intravesicular radioactivity was measured 45 s after mixing [^{14}C]maltose with the vesicles. The intravesicular maltose concentration (S_{in}) was measured by adding at $t = 0$, 19 μM maltose + 1 μM [^{14}C]maltose, and measuring the intravesicular ^{14}C at the times indicated.

Time (min)	S_{in} (nmol/mg of protein)	[^{14}C]Maltose influx (d.p.m./min)
0	0	607
5	2.4	773
7.5	3.3	572
10	4.0	527

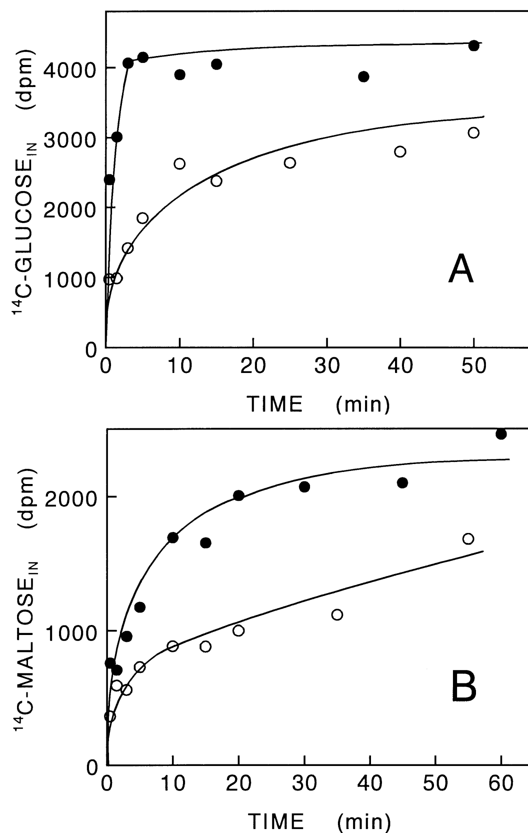

Figure 4 Uptake of ^{14}C-labelled sugar under equilibrium exchange and zero-*trans* conditions in non-energized vesicles

Vesicles, not containing cytochrome *c* oxidase, were preloaded with 2 mM glucose (**A**) or 10 mM maltose (**B**), and subsequently, at $t = 0$, 11 μM [^{14}C]glucose or 5.3 μM [^{14}C]maltose was added, after which transport was measured as described before (●). Uptake under zero-*trans* conditions (○) was measured in the same way, except that the vesicles were not preloaded with unlabelled sugar.

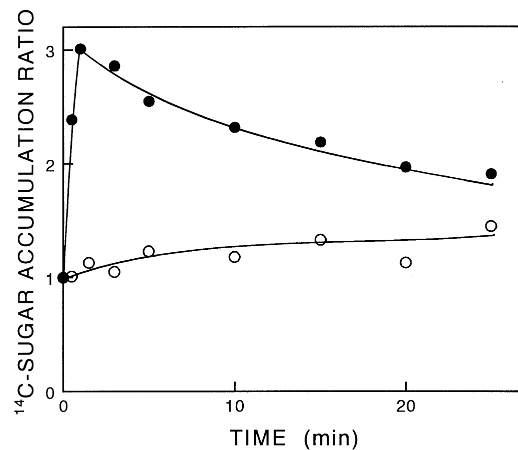

Figure 5 Uptake of ^{14}C-labelled sugar under counterflow conditions in non-energized vesicles

The conditions are given in the Experimental section. ●, Glucose transport; ○, maltose transport.

about 4 nmol/mg of protein. The [^{14}C]maltose influx was not very sensitive to the internal maltose since, even at the highest intravesicular maltose concentration, [^{14}C]maltose influx was only slightly lower than the control. The small decrease in maltose influx can be related to a change in PMF, since during a 10 min incubation the PMF decreased slightly (about 10–15 mV).

To study whether the pH had an influence on the rate of maltose efflux, the transport was measured in experiments with vesicles prepared at pH 5.7, 6.1 and 6.5. At all pH values, maltose accumulation was observed on energization, even though at pH 6.5 accumulation was reduced to about 40 % of the level measured in buffer of pH 5.7. Under none of the conditions, however, did FCCP induce a rapid efflux of accumulated maltose (results not shown).

It has been reported that, depending on the conditions, K^+ may either stimulate or inhibit maltose transport in *S. cerevisiae* [37]. This indicates that this cation can have a complex effect on the activity of the transporter. Thus it might be hypothesized that the low efflux of maltose in *C. utilis* membrane vesicles could be due to K^+ in the medium. To test this, the effect of uncoupler was studied in media where 50 mM potassium phosphate was replaced by 50 mM sodium phosphate or 50 mM Tris/phosphate. On energization, intravesicular accumulation of maltose was observed with all media. However, the presence of univalent cations other than K^+ did not alter the uncoupler effect, as FCCP induced identical low maltose efflux in the three media used.

Exchange and counterflow

To study further the reversibility of glucose and maltose transport in *C. utilis* vesicles, equilibrium exchange and counterflow were measured under de-energized conditions. Under equilibrium exchange conditions, [^{14}C]glucose uptake was considerably faster than zero-*trans* [^{14}C]glucose uptake (Figure 4A), indicating that transport proceeds through a reversible carrier with the return of the unloaded carrier as the rate-limiting step [38]. Also [^{14}C]maltose influx was higher under exchange conditions than during zero-*trans* influx (Figure 4B), although the effect was less prominent than for glucose uptake.

One of the best ways to demonstrate the action of a mobile carrier mechanism is to measure counterflow, i.e. transient uphill transport of a substrate driven by the downhill flow in the opposite direction of a structurally similar compound [39]. Counterflow data, presented in Figure 5, revealed transient internal accumulation of [^{14}C]glucose when an outwardly directed concentration gradient of non-radioactive glucose was imposed. This contrasted with the data for maltose transport, for which [^{14}C]maltose accumulation was much lower.

Proton/sugar stoichiometry

An important parameter in PMF-driven transport is the proton/sugar stoichiometry. This can be determined by assuming that in the steady state an equilibrium is obtained between the sugar accumulation ratio and the PMF. The apparent proton/sugar stoichiometry (n_{app}) can then be estimated from the equation [40]:

$$n_{app} = \Delta\mu_S/(\mathrm{PMF} \times F)$$

Table 2 Relationship between PMF and sugar accumulation in vesicles

ΔpH and $\Delta\psi$ were measured as described in the Experimental section at the time that sugar accumulation was maximal. The value for sugar accumulation is that of maximal accumulation. All measurements were performed at pH 5.7.

Sugar	Ionophore	$\Delta\psi$ (mV)	ΔpH (mV)	PMF (mV)	$\Delta\mu_S/F$ (mV)	n_{app}
Glucose	–	−55	−60	−115	96	0.83
Glucose	Nigericin	−82	0	−82	74	0.90
Glucose	Valinomycin	0	−74	−74	75	1.01
Maltose	–	−55	−60	−115	103	0.90
Maltose	Nigericin	−80	0	−80	95	1.19
Maltose	Valinomycin	0	−67	−67	41	0.61

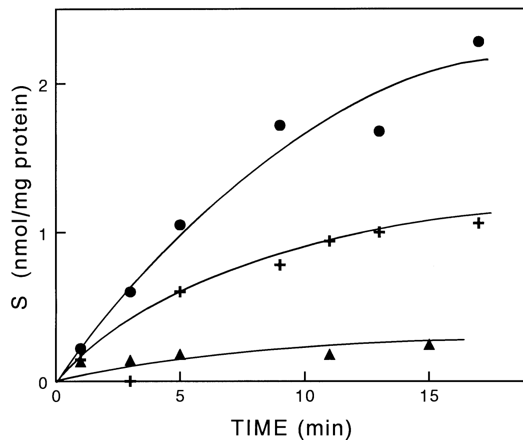

Figure 6 Effect of monensin and nonactin on maltose transport in vesicles in Na^+-containing buffer

Vesicles were prepared and incubated in a buffer containing 50 mM sodium phosphate and 1 mM $MgCl_2$ at pH 5.7. After the addition of 10 μM cytochrome *c*, 100 μM TMPD and 20 mM ascorbate at $t = -1$ min, transport was initiated by the addition of 5.3 μM [^{14}C]maltose. ●, Control; ▲, 10 μM nonactin added at $t = -1$ min; +50 nM monensin added at $t = -1$ min.

where $\Delta\mu_s$ is the chemical sugar gradient, and F is the Faraday constant. For glucose transport, the apparent proton–sugar stoichiometry was close to 1, both under fully energized conditions and under conditions where $\Delta\psi$ or ΔpH were dissipated with valinomycin or nigericin (Table 2). Under fully energized conditions, maltose transport displayed an n_{app} of 0.9. In the presence of nigericin the apparent stoichiometry slightly increased whereas in the presence of valinomycin it decreased. These data suggest that maltose transport is not very sensitive to ΔpH, and that, under the conditions used, $\Delta\psi$ is the main driving force. This could indicate that the coupling ion in maltose transport might not be H^+, but another univalent cation. Owing to the composition of the buffer routinely used, K^+ would be a plausible candidate. However, maltose accumulation was also observed in buffers devoid of K^+ (sodium phosphate or Tris/phosphate), indicating that K^+ is not a cosubstrate for the maltose permease.

A low accumulation of maltose when valinomycin is present does not necessarily reflect a $\Delta\psi$ effect. It might be due to an artifact such as aspecific inhibition of carrier activity by valinomycin or inhibition of maltose flux by high intravesicular K^+ concentrations. Therefore the influence of ionophores on maltose transport was studied in a buffer containing 50 mM sodium phosphate instead of potassium phosphate. In Na^+ buffer $\Delta\psi$ can be dissipated with nonactin and ΔpH with monensin [41,42]. Figure 6 shows that the effects of monensin and nonactin were similar to those achieved with nigericin and valinomycin respectively in K^+ medium. In both cases, a $\Delta\psi$-dissipating ionophore strongly reduced maltose accumulation. Therefore these data indicate that $\Delta\psi$ has a stronger influence on maltose influx than ΔpH.

DISCUSSION

The results presented in this paper show that glucose and maltose can be accumulated to high levels in *C. utilis* plasma-membrane vesicles equipped with bovine heart cytochrome *c* oxidase as a PMF-generating system. Experiments where vesicles were prepared with various amounts of plasma membranes at a fixed quantity of liposomal phospholipid showed that, at a membrane protein/phospholipid ratio of 1:20 or higher, maltose and glucose accumulations were maximal. With less membrane protein per vesicle lower accumulations were observed. Obviously vesicles that do not contain a carrier cannot accumulate sugar, whereas vesicles containing at least one transport system will be able to accumulate sugar. As long as a vesicle contains at least one carrier, the accumulation ratio will be relatively independent of the number of carriers present and will give maximal accumulation. Thus the data indicate that, at a 1:20 ratio of protein/phospholipid, all vesicles participated in uphill transport, whereas at lower ratios part of the vesicle population was not capable of accumulating sugars. Therefore we conclude that at a 1:20 ratio each vesicle contained at least one glucose and one maltose transporter. In previous studies on reconstitution of sugar carriers from *S. cerevisiae* and *K. marxianus* it was found that part of the vesicles was not participating in active transport [11,13]. This suggests that the amount of carrier protein is higher in *C. utilis* than in the other two yeasts.

The results illustrate the advantages of sugar-limited chemostat cultivation for studies on sugar transport in yeasts: the low residual sugar concentrations in the cultures lead to a strong induction of high-affinity uptake systems [2,16], thus obviating the need for genetic overexpression of carrier proteins. Furthermore the alleviation of carbon catabolite repression in such cultures led to the simultaneous utilization of sugar mixtures (glucose and maltose) [2,43], thus enabling the isolation of membrane vesicles that contain different sugar transporters from cells grown under a single defined set of growth conditions.

The high accumulation of sugars in the vesicles and the fact that the kinetic parameters of initial influx in vesicles were close to those observed with intact cells (see refs. [16–18]) suggest that the vesicle system closely mimics the situation *in vivo*. Recently it was reported that maltose transport in *S. cerevisiae* was very sensitive to sonication and that vesicle preparation by freeze–thaw–sonication would not give reproducible transport results [14]. Better results would be obtained using the extrusion technique as described by MacDonald et al. [24]. We, however, could not find any significant improvement in maltose transport in *C. utilis* if freeze–thaw–extrusion was used instead of freeze–thaw–sonication. Even in studies on maltose transport by *S. cerevisiae* vesicles, we did not find any improvement in uptake when the extrusion technique was used. We conclude that, for the preparation of plasma-membrane vesicles, freeze–thaw–extrusion and freeze–thaw–sonication can both yield vesicles with high sugar-transport capacities.

Maltose solutions often contain considerable amounts of

glucose. Since high-affinity glucose transport has, in intact cells, a K_m value about 20 times lower than that for maltose uptake, contaminating glucose might contribute significantly to transport parameters measured with maltose. It might even be hypothesized that the weak alkalinization of the extracellular medium observed after addition of maltose to *C. utilis* cells (results not shown) is due to glucose–proton symport. Similarly it could not *a priori* be excluded that, after addition of labelled maltose, accumulation of ^{14}C-labelled material in vesicles was due to uptake of labelled glucose. Chromatographic analysis of vesicle extracts, however, unequivocally established that maltose was taken up in the disaccharide form, and thus maltose uptake is an uphill process. It also followed from this analysis that maltase activity in the vesicle preparations was low, and did not lead to significant hydrolysis of intravesicular maltose.

Role of the PMF

Glucose accumulated in vesicles in response to the generation of a PMF (inside alkaline and negative), but also under conditions where only a $\Delta\psi$ or ΔpH was present (presence of nigericin and valinomycin respectively), showing that glucose transport is PMF-dependent. The proton/glucose stoichiometry of 1 (Table 2) is identical with that found in intact cells [17]. Dissipation of the PMF induced a rapid efflux of accumulated glucose, indicating that transport is fully reversible. Equilibrium exchange and counterflow experiments showed enhancement of the uptake of [^{14}C]glucose and a characteristic transient accumulation of radiolabelled glucose respectively. This clearly demonstrates the presence of a reversible proton–glucose symporter in *C. utilis*.

As observed with glucose, maltose also accumulated in the vesicles in response to the generation of a PMF. Also when nigericin was present, dissipating ΔpH, high accumulation was found. This contrasts with the situation where valinomycin was present ($\Delta\psi = 0$) leading to a decreased accumulation level, thereby resulting in a low apparent proton/maltose stoichiometry. This can be explained in three ways: (a) maltose accumulation is mainly $\Delta\psi$ driven with ΔpH having negligible influence, (b) the H^+/maltose stoichiometry is variable, or (c) the H^+/maltose stoichiometry measured in the presence of valinomycin is underestimated. Explanation (a) implies that maltose transport is not coupled to H^+ uptake but rather to that of another univalent cation. Maltose accumulation was independent of the univalent cation composition of the medium, and was even observed in a medium with $Tris^+$ as the main univalent cation. This makes it unlikely that a cation other than H^+ is the coupling ion, and thus it should be expected that the PMF and not just $\Delta\psi$ is the driving force. Explanation (b) implies that the H^+/maltose stoichiometry in the presence of valinomycin is truly different from that measured in the presence of nigericin. Variable H^+/substrate stoichiometries have been suggested for some bacterial secondary active transport systems [44,45], where they were dependent on the external pH. However, it seems highly unlikely that, at a fixed pH, $\Delta\psi$ contributes in a more efficient way to the energization of maltose transport than ΔpH. Situation (c) gives the best explanation for the low stoichiometry when $\Delta\psi$ was dissipated. Underestimation of the stoichiometry is achieved when maltose influx is low, thereby preventing sugar accumulation from entering the thermodynamic equilibrium with the PMF. Moreover, passive leakage from the vesicles will decrease the accumulation ratio even more. Indeed it was observed that the maltose-uptake velocity was low when valinomycin was present, suggesting reduced transport activity. In vesicles from *Streptococcus cremoris*, a similar underestimation of accumulation ratios was observed for serine and alanine transport [46], which was explained by a negative effect of high internal pH on transport activity. Thus dissipation of the ΔpH with nigericin gave an increase in serine and alanine influx. However, we did not observe a similar effect of nigericin on maltose influx (results not shown). This makes an effect of the internal pH less likely. Alternatively it was considered that K^+ might be inhibitory, as has been observed for maltose transport in *S. cerevisiae* [37]. However, maltose influx was also low in Na^+-containing medium when $\Delta\psi$ was dissipated with nonactin. Thus it is concluded that maltose transport is slow when $\Delta\psi$ is dissipated, and that this leads to an underestimation of the proton/maltose stoichiometry. It has been reported for other transport systems in yeast that initial rates of transport can be much more sensitive to membrane energization than the final accumulation ratios [47]. Our conclusion that influx of maltose is highly sensitive to $\Delta\psi$ extends this view.

Reversibility of maltose transport

A striking characteristic of maltose transport in *C. utilis* membrane vesicles was the virtual absence of efflux of accumulated sugar when the PMF was dissipated. It has been reported for a number of transport systems in yeast that substrate efflux induced by uncoupler for example was slow [35,48,49]. A number of explanations have been given for this apparent irreversibility, namely compartmentalization, *trans*-inhibition, inactivation of the carrier by low internal pH or low PMF and unidirectionality of the transporter [47,50–53]. Obviously, this study, in which membrane vesicles were used, excludes the possibility that the lack of maltose efflux is caused by compartmentalization. *trans*-Inhibition has been found for a number of amino acid-transport systems in yeast, where high internal amino acid concentrations inhibit the activity of the transporter, probably by binding to a specific regulatory site at the internal face of the membrane. However, it is unlikely for two reasons that *trans*-inhibition is the explanation for uncoupler not causing efflux of maltose. First, an effect of *trans*-inhibition is only to be expected at high internal maltose concentrations. The uncoupler-induced efflux was, however, also extremely slow with very low maltose concentrations (external maltose at 0.3 μM and internal maltose at 17 μM). Secondly, experiments in which vesicles were preloaded with unlabelled maltose showed that this did not have a significant effect on maltose influx (Table 1). This insensitivity of maltose carrier activity to high internal maltose concentrations strongly argues against *trans*-inhibition.

Sugar transport in *Chlorella vulgaris* is extremely sensitive to the internal pH [51,52]. At internal pH values lower than 6.0, sugar transport (both influx and efflux) is completely inhibited. This is not the case for the maltose transporter of *C. utilis*, since influx was high when vesicles were energized at pH 5.7 and ΔpH was dissipated by nigericin. Still, this did not rule out the possibility that the slow efflux observed after de-energization of the vesicles is due to a low internal pH. Experiments performed at pH values up to 6.5 showed that, at high pH values also, uncoupler did not induce any significant outflow of accumulated maltose, showing that it is not a low internal pH that prevents maltose from leaving the vesicles.

It has been reported that even downhill transport of substrates in yeast requires a proton gradient [47,50], suggesting that, in the absence of a PMF, carrier activity would be low. Since our data indicated that initial uptake of maltose was highly sensitive to $\Delta\psi$, an attractive hypothesis is that activity of the maltose permease, in whatever direction, is low in the absence of a membrane potential. This would explain why maltose efflux was low when the PMF was dissipated with uncoupler, or a mixture

of valinomycin and nigericin, and why exchange and counterflow were low in de-energized vesicles. It, however, does not explain why maltose does not induce counterflow under energized conditions (Figure 3B), indicating that the dependence of the carrier on membrane potential cannot (fully) explain the apparent irreversibility of the maltose permease.

For arginine transport in reconstituted plasma-membrane vesicles from *S. cerevisiae*, it was also found that uncoupler did not induce efflux of accumulated arginine [53], leading to the conclusion that the arginine transporter is unidirectional. This view has been challenged [14] by claiming that, at low arginine concentrations, uncoupler could induce efflux. Thus arginine transport might be sensitive to *trans*-inhibition. Our data show that maltose transport in *C. utilis* plasma-membrane vesicles is not sensitive to substrate or *trans*-inhibition. Still maltose efflux is slow when the PMF is dissipated. Maltose does, however, show the phenomenon of equilibrium exchange, indicating that the carrier catalyses the reversible translocation of maltose. The fact that some counterflow was observed for maltose (Figure 5) is in agreement with this view. We therefore conclude that the maltose permease of *C. utilis* is a reversible carrier which acts in an asymmetrical way. Thus the kinetics of inwardly moving maltose are different from those of outwardly moving maltose, leading to slow efflux compared with influx.

In summary, this paper shows that, within one membrane preparation, two distinct uphill sugar-transport systems can be found which are kinetically different. This shows that, in *C. utilis*, the PMF-driven sugar-transport systems are mechanistically diverse, and suggests that the corresponding transport proteins also have some characteristic differences. Future work should focus on characterizing these proteins, a study that may help to resolve the molecular background of the asymmetry in the maltose transporter.

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