The Effects of pH on Proton Sugar Symport Activity of the Lactose Permease Purified from Escherichia coli*

Malcolm G. P. Page†, Jürg P. Rosenbusch, and Ichiro Yamamoto‡

From the European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, D-6900 Heidelberg, Federal Republic of Germany, and the Biozentrum der Universität Basel, Abteilung Mikrobiologie, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

The lactose permease, which catalyzes galactoside-proton symport into Escherichia coli, has been purified and reconstituted in active form into artificial lipid vesicles. The roles of many detergents and phospholipids in solubilization and stabilization of the activity of the permease have been examined with a view to its eventual crystallization. Initial rates of uptake into reconstituted proteoliposomes determined by rapid mixing techniques proved that the activity of the permease can be comparable to that observed in the intact cell, while the best values for uptake rates obtained with conventional techniques were comparable to those reported for vesicles. The activity of the purified protein has been monitored over time periods of hours to weeks. It is shown that, under the best current conditions, the permease retains full activity for 1 to 2 weeks. Although this is still marginal for its crystallization, future improvements can now be assayed by rather stringent criteria.

The mechanism of galactoside transport into reconstituted proteoliposome has been investigated by examining the effects of pH on influx into the vesicles. It is shown that the observed effects are entirely consistent with the predictions of a simple model of proton symport. The apparent increase in rate of uptake that is observed in the presence of a pH gradient is not so much due to an acceleration by a component of the protonmotive force as to the relaxation of inhibition by a product (internal protons) of the symport reaction.

The lactose permease of Escherichia coli (1) is one of the most extensively characterized proton-coupled active transport systems (2, 3). One-to-one coupling of galactoside and proton influx has been demonstrated under a wide variety of conditions in intact cells (4, 5) and in membrane vesicles (6). The pH dependence of galactoside binding to the permease in the intact cell has been shown to be consistent with the formation of a 1:1:1 complex between proton, galactoside, and permease (7, 8), but the stoichiometry of proton transport and the pH dependence of the kinetics of uptake have not yet been investigated in such detail in membrane vesicles (3, 9). In order to investigate the structure of the permease and the molecular mechanism of the symport process in more detail, it is necessary to purify, to characterize, and to reconstitute the permease in a well defined system. Although activity has been reconstituted, a stable, highly active preparation that would be suitable for high resolution structural studies has not yet been obtained. Newman and colleagues (10–12) have purified the protein in the presence of octyl glucoside and recovered activity after reconstituting liposomes by removing the detergent through dialution. The galactoside-flux reactions catalyzed by these preparations have been investigated in some detail and shown to be similar to those of the permease in native membrane vesicles (3). Wright and colleagues (13, 14) have also solubilized the permease using dodecyl maltoside and reconstituted transport activity after removal of the detergent. This preparation has been physically well characterized, and some of the galactoside-flux reactions have been described. While we were examining various kinds of detergents for solubilization, we found that the permease activity was stabilized in the presence of deoxycholate and could also be reconstituted by removal of detergent by dialysis. Here we describe the details of purification and reconstitution of the permease, and the kinetic characterization of the reconstituted system using rapid mixing techniques to measure initial rates of transport into vesicles. The dependence of uptake on external and internal pH is similar to that already determined for the intact cell (7, 8) and is entirely consistent with the role of the proton as a substrate in galactoside symport. We also demonstrate galactoside-induced proton movements, both uptake from the external medium and release within the vesicle and show that these are compatible with a stoichiometry of 1 for the symport reaction.

MATERIALS AND METHODS

Protein Purification—E. coli T206 (15) was grown in 100-liter batches in minimal medium M9 supplemented with glyceral (0.5% by volume) and casein hydrolysate (0.2% w/v). The cells were harvested by centrifugation, frozen rapidly, and stored at −20°C. The frozen cell paste (50–100 g) was thawed in 400 ml of 10 mM Tris, 25 mM MgSO4, 1 mM sodium EDTA, 1 mM dithiothreitol, pH 7.8, containing 10 μg/ml bovine pancreatic DNase and cells broken by passage through a French pressure cell (16). The cytoplasmic membranes were collected by centrifugation in an IEC A170 rotor for 90 min at 40,000 rpm at 4°C and then washed by resuspension in the following sequence of buffers: (i) 10 mM Tris, 10 mM sodium EDTA, 1 mM dithiothreitol, adjusted to pH 8.5 with HCl; (ii) 20 mM Tris, 3 mM KCl, 1 mM dithiothreitol adjusted to pH 8.5 with acetic acid; (iii) two washes with 1 mM Tris, 1 mM sodium EDTA, 1 mM dithiothreitol, 10% glycerol, pH 8.5; (iv) 10 mM KPO4, 50 mM lactose, 1 mM dithiothreitol, pH 5.8. The final pellet was resuspended in 50 ml of 10 mM KPO4, 50 mM lactose, 1 mM dithiothreitol at pH 5.8 and an equal volume of freshly prepared solution of 2.5% β-octyl glucoside in 10 mM KPO4 at pH 5.8 was mixed with the membrane suspension by
driving the two solutions simultaneously through a T-shaped plastic connecting piece. The mixture was stirred for 20 min and then centrifuged as before.

The supernatant was applied to a 100-ml (bed volume) column of DEAE-cellulose (Whatman DE52) that had been pre-equilibrated with 10 mM KPi, 1 mM dithiothreitol, 1 M octylglucoside at pH 5.8, and the column was washed with 200 ml of this buffer after application of the sample. The flow-through fractions having absorbance at 280 nm greater than 0.1 were collected, pooled, and concentrated as rapidly as possible (within 2 h) to approximately 30% of the initial volume by ultrafiltration using an Amicon Corp. PM-30 filter. The concentrate was applied to a 5-ml (bed volume) column of CM-cellulose, pre-equilibrated with the same buffer as above. After application of the sample, the column was washed with elution buffer at pH 7.2 until absorbance at 280 nm returned to baseline and then the elution buffer was changed for one comprising 50 mM potassium borate, 0.1 M KCl, 0.1% sodium deoxycholate, 1 mM dithiothreitol, 1.5% β-octyl glucoside, pH 9.

Variations of this procedure that have been used are as follows. (i) The use of cytoplasmic membranes prepared by osmotic lysis and sucrose density gradient centrifugation (17) reduces the amount of contaminants in the DEAE-fractions but is not suitable for large scale preparations or for use of frozen cells. (ii) Replacing the sequential washing by an extraction with 50 mM potassium phosphate buffer, 50 mM lactose, 4% sodium cholate, 1 mM dithiothreitol at pH 7.8 removes many peripheral membrane proteins but also extracts to 30% of the lactose permease; it also necessitates the addition of at least 0.1 mg/ml of phosphatidylethanolamine to the solubilization buffer (11). (iii) Solubilization of the permease from washed or cholate-extracted membranes in 10 mM Tris, 10 mM sodium EDTA, 1 mM dithiothreitol, 1% Lubrol-PX, 10% glycerol, pH 7.8. The permease is weakly bound to the DEAE-cellulose column and can be eluted between 0.05 and 0.07 M KCl in the same buffer. More contaminants result in the DEAE-fraction, but these are removed by the CM-cellulose step. These variations make no detectable difference to the final specific activity of the protein.

Reconstitution—The fractions containing the 280 nm absorbance peak that eluted with cholate buffers in the CM-cellulose chromatography above were pooled, and the protein concentration was measured. One of the phospholipid mixtures described in Table IV, dissolved in 0.1 M KPi, 10 mM dithiothreitol, 10 mM sodium EDTA, 10% sodium cholate, 1% sodium deoxycholate, pH 9.0, was added to give a protein-to-lipid ratio of between 1:150 and 1:200 (by weight). Reconstitution was achieved by dialysis against 1 liter of 50 mM KPi, buffer, 0.1 M KCl, 0.5 mM MgSO4, 0.1 mM dithiothreitol, pH 7.8. and either immediately or rapidly frozen in liquid N2 and stored at -80°C.

Proton Uptake—Protoproteoliposomes were reconstituted in DPPE/DOPE/DPFG/DOPG mixture, H and Table IV) and then given two cycles of thawing, rapid freezing, and sonication in medium comprising 50 mM potassium phosphate, 0.27 M KSO4, 1 mM pyranine (24), 0.2 mM valinomycin, pH 7.5, or, for measurement of external pH changes (proton uptake), 50 mM potassium phosphate, 0.25 M KSO4, 0.2 mM valinomycin, pH 7.5. To measure proton release, the vesicles (0.02 mg of protein) were diluted 100-fold into 3 ml of medium comprising 50 mM potassium phosphate, 0.25 M KSO4, 0.2 mM valinomycin, pH 7.5, 100 mM valinomycin, 100 mM nigericin and 50 μM 4-methylumbellifor- one, 0.2 mM valinomycin at pH 7.5. The change in fluorescence was followed (excitation 340 nm, emission 470 nm) and then lactose, dissolved in dilution buffer, was added to give a final concentration of 5 mM. To measure proton uptake, the vesicles were dilute into medium comprising 5 mM potassium phosphate, 0.25 M KSO4, and 50 μM 4-methylumbellifer- one, 0.2 mM valinomycin at pH 7.5. The change in fluorescence was followed (excitation 340 nm, emission 470 nm) and then lactose, dissolved in dilution buffer, was added to give a final concentration of 5 mM. Fluorescence changes were calibrated by suspending vesicles in media of known pH between 7.0 and 8.0 that included 10 mM carbonyl cyanide m-chlorophenylhydrazone as protonophore. In an identical series of experiments, the uptake of [3H]Lactose (shown by solid circles) was measured at 5-s intervals by withdrawing 100-μl aliquots from the reaction mixture and treating as for galactose uptake. All solutions were degassed and flushed with N2 before use; the reactions were performed in stirred, sealed cuvettes at 37°C.

Galactoside Uptake—Protoproteoliposomes, reconstituted as described, were given two cycles of rapid freezing, thawing, and sonication in the uptake medium described in Table I. Concentrated vesicles (0.02-0.05 mg of protein in 0.2 ml of uptake medium) were then diluted into 500 volumes of dilution medium, and samples were taken at intervals between 2 and 600 s after mixing. Each sample was quenched with an equal volume of 10 mM HgCl2 in dilution medium, filtered through Millipore filters (0.22-μm pore size), and washed three times with 1 ml of dilution medium containing 1 mM HgCl2. Radioactivity was measured by liquid scintillation counting. To measure pH dependent, phosphate buffers were used in the range of pH 6.8-2.2 and borate buffers in the range of pH 7.8-9.5. The magnitude of ion gradients established in the vesicles were estimated from the accumulation of [3H]methyltritylphosphonium iodide (19, 20). The internal volume of the vesicles was estimated as the lactose-diffusible space (21).

The apparent initial rate of uptake, determined in conventional assays, appeared to be considerably less than those obtained with intact cells (27), although no truly linear phase was observed. Rather, the uptake followed a curve deviating from linearity, even in the first 100 ms, and

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Kinetics of Lactose Transport in Reconstituted Vesicles

TABLE I
Composition of galactoside uptake media

| Assay          | pH | KPi | NaPi | K2SO4 | Na2SO4 | Valinomycin |
|----------------|----|-----|------|-------|--------|-------------|
| Influx         |    |     |      |       |        |             |
| Uptake medium  | 7.5| 0.05| 0    | 0.25  | 0      | 0          |
| Dilution       | 7.5| 0   | 0.05 | 0     | 0.25   | 0.2         |
| Efflux         |    |     |      |       |        |             |
| Uptake medium  | 7.5| 0.05| 0    | 0.25  | 0      | 0.2         |
| Dilution       | 7.5| 0   | 0.05 | 0     | 0.25   | 0           |
| Exchange       |    |     |      |       |        |             |
| Uptake         | 7.5| 0.05| 0    | 0.25  | 0      | 0.2         |
| Dilution       | 7.5| 0   | 0.05 | 0     | 0.25   | 0           |
| Counterflux    |    |     |      |       |        |             |
| Uptake         | 7.5| 0.05| 0    | 0.25  | 0      | 0.2         |
| Dilution       | 7.5| 0   | 0.05 | 0     | 0.25   | 0.5         |

*To measure pH dependence, phosphate buffers were used in the range of pH 5–8.2 and borate buffers in the range pH 7.8–9.5. No difference was observed between the rates obtained in phosphate and borate buffers at the same pH.

For experiments with melibiose, 6-[3H]melibiose was used as the label at concentrations 0.01–2 mM for influx, 2 mM for efflux and exchange, and 0.05 mM for counterflux. Unlabeled melibiose was used at 2 mM concentration.

TABLE II
Kinetic parameters of lactose and melibiose uptake via the purified permease

Permease was reconstituted in E. coli PE + PG and transport assayed at 20 °C. Binding sites were determined by equilibrium binding of thiodigalactoside to the proteoliposomes. The $K_a$ and $K_i$ are the Michaelis constants for proton and galactoside, respectively, and $K_a$ is the apparent dissociation constant for the substrate proton (7, 18). These constants and $K_{ic}$ are obtained from pH dependence of sugar uptake shown in Fig. 3. The rates of efflux, counterflux, and exchange are obtained in the same way in the appropriate experiments. In the intact cell, with lactose as substrate, they have the values $K_a = 7 \times 10^{-7} M$, $K_{ic} = 5 \times 10^{-7} M$, $K_i = 2.5 \times 10^{-8} M$. The rate of uptake decreased quite rapidly thereafter. The rate of uptake of lactose increased when the pH of the internal of the vesicles was raised above that of the external medium. This was due to an increase in maximum velocity ($V$) and a decrease in the apparent affinity constant for lactose ($K_a$) (Fig. 3). In addition, a decrease in $V$ was observed between pH 5.5 and 7.0 when either external or internal pH was lowered. The combination of these effects, attributed to protonation at two types of site (8), gives rise to the complicated pH profiles shown in Fig. 3 and described by Equation 1 in the legend to that figure. In Fig. 3A, where the pH is low and the first type of site is fully protonated, it is the second type of site that largely contributes to the variation in $V$, whereas in Fig. 3B, where the pH is high and the second type of site is deprotonated, the variation in both $V$ and apparent $K_a$ is mostly due to changes in protonation of the first type of site (pK, 6.3). In Fig. 3C, the complete pH profile is shown for $V$, which describes the behavior of the ternary complex and hence shows inhibition at low pH values due to protonation at the second type of site. Fig. 3D shows the complete pH profile for the specificity constant $V/K_a$, which

![Graphs and images](image-url)

**FIG. 1.** Phospholipid specificity of binding and transport of galactosides in the reconstituted system. Purified lactose permease was reconstituted as described in the text using the following phospholipid mixtures: partially purified E. coli phospholipids (B), 90% DMPE plus 10% (by weight) DMPG (A), DMPG alone (C), DMPG (Δ), and DMPS (○). Shown are: binding of $[6,6'$-3H]thiodigalactoside to the vesicles at 4 °C in the presence of 10 mM NaI; $r$ represents the ratio of bound and free thiodigalactoside (A); isotope exchange with 10 mM lactose in the external medium and vesicles loaded with 10 mM $[3H]$lactose at 37 °C (B); influx with 1 mM external $[14C]$lactate at 37 °C (C); counterflux with 0.2 mM external $[14C]$lactate and vesicles loaded with 10 mM lactose at 37 °C (D); efflux at 15 °C from vesicles loaded with 10 mM $[3H]$lactose in the presence of 10 mM NaI.

M. G. P. Page, unpublished results.
describes the behavior of the permease at infinitely low lactose concentrations (i.e., the unloaded permease). Here, there is inhibition at low pH values due to protonation at the second type of site and inhibition setting in at higher pH values due to protonation at the first type of site on the internal face of the membrane.

**Stoichiometry of Uptake**—The vesicles could be loaded with the pH-sensitive dye pyranine (18), which allowed the proton uptake during lactose influx to be followed (Fig. 4A). Comparison of the initial rate of proton uptake (i.e., release within the vesicle) with the rate of lactose uptake in the same experiment gave values between 0.50 and 1.1 (mean ± 0.22) for the stoichiometry of proton:lactose symport. Similar experiments using the pH-sensitive dye 4-methylumbelliferone to follow changes in external pH (Fig. 4B) gave a mean value of 0.98 ± 0.31 for the proton:lactose stoichiometry.

**DISCUSSION**

The activity of the permease can be monitored at several levels of stringency: retention of binding, retention of exchange activity (including counterflux), and, ultimately, retention of coupled sugar-proton symport (influx and efflux). The substrate dependence of the influx reaction, catalyzed by the purified preparation, was found to be characterized by affinity constants very similar to those reported for uptake into intact cells (7, 18). The maximum velocity obtained in using conventional sampling techniques was found to be considerably less than that reported for the intact cell, but there are a number of problems associated with the accurate determination of rates of uptake into vesicles. Many of these are the result of the small internal volume of the vesicles relative to that of the intact cells. In particular, the small volume may result in a rapid build-up of internal substrate and, consequently, inhibition of further influx, even within the first seconds of uptake. Practically, this effect can be minimized by closely controlling two factors: (i) obtaining vesicles with reproducibly large diameters and (ii) determining the initial rate from points taken as early as possible in the time course of uptake experiments (28). In this respect, it is noteworthy that the maximum velocities obtained using conventional assays (with reaction times of 10 s or more) are at least 5-fold lower than the rate of uptake into intact cells (29, 30) and nearly 15-fold lower than rates reported for uptake into well energized cells (31, 32); the results obtained are comparable to the rate of lactose uptake into reconstituted vesicles (standardized by thiodigalactoside binding) reported by Wright and Overath (14) and to that described by Kaback (3) for a vesicle preparation standardized by photoaffinity labeling. However, using rapid mixing and quenched flow to obtain samples between 25 and 100 ms gives estimates of the initial rate that can be nearly 10-fold higher than those obtained in the
Fig. 3. Effect of internal pH on the initial rate of lactose uptake. The data from experiments in A and B are presented as the Eadie-Hofstee plot where the initial rates of uptake (v) are given in moles of lactose influx per mol of thiodigalactoside binding site per s. Lactose concentration was varied between 0.1 and 5 mM. Proteoliposomes were reconstituted with E. coli PE + PG or with the synthetic mixture H (Table SIV) in the uptake medium described for galactoside uptake assays, except that pH was varied as follows. In A, transport into vesicles having the following internal pH values were examined: pH 5.5 (○), pH 6.0 (□), pH 6.5 (A), pH 7.0 (O) at external pH 5.5. In some experiments, 1 mM fluorescein 6-carboxylic acid was included in the loading medium in order to assay the internal pH. The pH measured in this way was within 0.1 pH unit of the expected values (results not shown). The reactions occurred in dilution medium (as described for the uptake assay) at a pH adjusted to 5.5. In B, transport into vesicles having internal pH 7.0 (O), 7.5 (□), and 8.0 (△) was examined at pH 7.0, and, in some experiments, 1 mM pyranine was included to assay the internal pH. C, replott of the apparent maximum velocity (intercepts on the velocity axis in A and B) as a function of internal and external pH from several experiments such as shown in A and B. The external medium was pH 5.5 (○), 6.0 (□), 6.5 (A), 7.0 (□), or 8.0 (△). The broken lines are extrapolated to internal pH 8.0. D, replott of the intercepts on the velocity/substrate concentration axis as a function of internal and external pH from several experiments such as shown in A and B. The external medium was pH 5.5 (○), 6.0 (□), 6.5 (A), 7.0 (□), or 8.0 (△). The dependence of the initial rate of uptake on galactoside concentration and pH is described by the following equation (see Ref. 15 for further discussion):

\[ v = \frac{V[H]^n[S]}{K_n(K_o + [H]^o + K_o[S])} \]

where \( K_o \) is the dissociation constant for the proton-permease complex and \( K_n \) is the dissociation constant of proton from the ternary complex. \( K_n \), \( K_{II} \), and \( K_{III} \) are inhibitor constants, \([H]^o \) and \([H] \) are the external and internal values of proton concentration, respectively. At an external pH of 5.5, the effect of internal pH on V is described by an inhibition constant \( K_o \) of 4.5 \times 10^{-7} M (pKo = 6.5), while the effect on \( V/K_m \) is described by an inhibition constant \( K_{II} \) of 6 \times 10^{-16} M (pKi = 9.2). At an external pH of 7.0, the effect of internal pH on V is described by an inhibition constant of 4 \times 10^{-7} M (pKII = 6.3) and the effect on \( V/K_m \) is described by an inhibition constant of 1.2 \times 10^{-9} M (pKIII = 8.9). At an internal pH of 8.0, the effect of external pH on V and \( V/K_m \) is described by an inhibition constant of 3 \times 10^{-11} M (pKi = 6.5). The effects of the ionization described by \( K_o \) (Table II) are not detectable in the range of pH used for these experiments.

Fig. 4. Time course of lactose-H+ symport into reconstituted proteoliposomes. Lactose uptake was followed by radioactivity measurements (solid circles), while pH was monitored by fluorometry (recorder traces). The fluorescence corresponding to 20 ng of H+ is indicated in both panels by the bar. In A, proton release (indicated by the change in fluorescence of pyranine trapped in the vesicles) is compared to uptake of lactose. In B, proton uptake (indicated by the change in fluorescence of 4-methylumbelliferone in the external medium) is compared to the uptake of lactose.

conventional assays and hence comparable to the rate of uptake into the intact cell. This suggests that product inhibition is indeed contributing quite considerably to rate limitation in the time range of the conventional assay. Of course, using rapid mixing has its own problems, one of which is that the vesicles might be deformed by high pressures developed during flow and mixing, resulting in loss of internal substrate (for example, up to 20% of the trapped pyranine is released during the reaction). Such a loss will lead to an underestimation of uptake, so that the rates obtained still have to be regarded as lower estimates of the real initial rate. Questions as to how much variation of the lateral pressure in the membrane affects the activity of individual molecules remain, as do those of the orientation of the permease molecules in the vesicles and whether this affects the observed uptake activity. The orientation of permease in these preparations has not been assessed; Seckler and Wright (33) have suggested that the permease is randomly oriented in their preparations while Carrasco et al. (34) have claimed that the molecules have native orientation but that the C terminus may be dislocated in some cases. Whatever the orientation, we measure kinetic constants for influx that are similar to those determined in the intact cell where all the permease molecules may be assumed to be uniquely oriented. Simply, this suggests either that the permease is kinetically symmetrical or that we only detect those molecules with normal orientation with respect to the driving force.

The lactose/proton stoichiometry of uptake has been measured by comparing the initial rate of lactose uptake to the rate of either proton uptake from the medium or proton release within the vesicle, using fluorescent, pH-indicating dyes. The ratio of fluxes was found to be approximately 1, in agreement with previous findings with intact cells using pH-complex. \( K_o \), \( K_{II} \), and \( K_{III} \) are inhibitor constants, \([H]^o \) and \([H] \) are the external and internal values of proton concentration, respectively.
sensitive electrodes to measure external pH (4, 5, 19). Previous estimates of the stoichiometry of transport by reconstituted vesicle systems have been made by comparing the steady state level of galactoside accumulation to the nominal value of the proton motive force and have yielded stoichiometries of up to 0.7 proton/galactoside (14). Galactoside-induced proton uptake by a reconstituted system was also demonstrated by Foster et al. (12), but the stoichiometry was not reported.

The reconstituted system lends itself to the facile manipulation of the intravesicular compartment. Thus, the effects of internal pH on transport activity could be investigated in a more readily controlled manner than possible in the intact cell and without the complication of the outer membrane and periplasm of the intact cell. The regulation of activity by proton concentration on either side of the membrane can be interpreted in terms of two types of proton binding sites exposed on each face of the membrane. The first type of site, which appears to represent binding of the symported proton, is involved in substrate binding, for protonation of this site on the external face (apparent pK, 8.9-9.2) increases the affinity of the permease for galactoside. Binding of the proton to this type of site exposed to the internal face of the membrane decreases the apparent affinity of the permease for galactoside. Binding of the proton to this type of site exposed to the internal face of the membrane decreases the apparent affinity of the permease for external galactoside through competitive (product) inhibition. The second type of site, which has a much lower affinity for protons (pK, 6.3), appears to affect only the maximum velocity of the permease, and protonation of this site causes a marked decrement in turnover rate between pH values of 7.5 and 5.5 on either side of the membrane. These findings appear relevant to the understanding of the mechanism of the symport reaction. Firstly, they confirm the prediction of steady state kinetic analysis that the internal protons, as a product of the reaction, should compete with influx and confirm the pH dependence described for the intact cell (7, 8). Secondly, it has previously been reported that the ΔpH component of the proton motive force alters the kinetic properties of the carrier in a manner that is dependent on the square of the magnitude of ΔpH (35). The possibility of manipulating the pH on both sides of the membrane afforded by the reconstituted system reveals that this effect is due to rate-limiting product inhibition by internal protons. In conclusion, it appears from this kinetic characterization that the purified and reconstituted permease can have essentially the same activity as it does in the native environment in the intact cell, provided that the limitations imposed by the nature (see Miniprint Section) and the size of the vesicles are taken into consideration.

It is clear that the nature of the phospholipid environment in which the permease is reconstituted greatly affects its activity, and the specificity for PE-rich lipid mixtures has already been reported (10, 36). For reasons unknown to us, we were unable to obtain full activity when PS was the major lipid species (unlike Chen and Wilson (36), who reported that PS was as effective as PE). From our results, it appeared that equilibrium binding was not greatly affected by the phospholipid environment, but reactions involving net turnover of the permease were dependent on both the nature of the phospholipid head group and the fluidity of the lipid core. Thus, it appears that the permease can be incorporated in its native state into a variety of lipid bilayers, but either is not able to turn over so rapidly in some environments as it does in others or is not as tightly coupled in some membranes as it is in others. Comparison of the initial rates of lactose uptake with those of the lactose-induced proton uptake suggests that coupling is as tight in all the reconstituted systems as it is in the native membranes and that the low velocities (and corresponding low accumulation ratios) are due to smaller intrin-}

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The effects of phopholipid on activity after reconstitution

The protein was found to have little phospholipid associated with it after (1-2)ml of protein was found in preparations made without exogenous phospholipid with extensive washing of the column with deoxycholate or deoxycholate-detergent as agents. However, the preparations did not yield high levels of activity after reconstitution either by dialysis or by removal of detergent using absorption to polyethylene glycols. Preparations made with phospholipid during all steps contained 10-20 mol phosphorus per mole of protein and were substantially more active than those made using only detergent. Thus, the absence of phospholipid in the reconstituted material appears to be a property of the vesicles. The phospholipid contained in the vesicles appeared to have the same composition as the exogenous lipid: for example, if PC or PE were used, then no PE was found when the lipids were analyzed by two dimensional thin layer chromatography. There did not seem to be any specificity for incorporation of the active protein into membranes with a particular phospholipid composition, an equilibrium binding of bidimensional reconstituted liposomes was not greatly affected by the nature of the phospholipid used (Table IV in the manuscript section). In contrast, the protein did show a marked sensitivity to the head group of the phospholipid in its transport reactions. Phospholipid mixtures that contained up to 60% PE gave high activity in all assays (Table VI) but more than this gave problems with obtaining good vesicles; probably due to the tendency of PE to form bimolecular layers. Phospholipid mixtures that were rich in PC or PE gave low activity in assays that involved migration of labeled solute across the membrane. Active forms, efflux or, to a certain extent, counterflow (Table IV). The initial rate of uptake during counterflow, which is essentially an exchange reaction, was less affected than either influx or efflux and equilibrium exchange was the least dependent of all assays on phospholipid composition. DNS supported binding but gave low activity in the flux reactions while DOPH gave better transport activity. These differences could not be readily ascribed to differences in the size of vesicles, or in the magnitude of imposed driving force, or in the rates of passive flux of the solute (Table IV).
Kinetics of Lactose Transport in Reconstituted Vesicles

Purification of lactose permease

Protein was determined by a modification of the method of Lowry et al. (42, 43). Labelling with N-14C-ethylmaleimide was performed according to the procedure (42) and thiodigalactoside binding was measured by flow dialysis in separate experiments. The figures in parentheses indicate the final recovery as a percentage of the original activity.

Table II

| Protein          | NEM label | Thiodigalactoside binding |
|------------------|-----------|--------------------------|
| (mg)             | (nmol)    | (nmol)                   |
| Cytoplasmic membrane | 100       | 781                       |
| Cholate supernatant | 100       | 35                        |
| pellet           | 100       | 162                       |
| Octylglucoside eluate | 100       | 229                       |
| pellet           | 100       | 40                        |
| DEAE flow through fractions | 100       | 167                       |
| CM-cellulose eluate | 100       | 167                       |
| Reconstituted protein liposomes | 100     | 5                         |

* N.D. = indicates that no activity could be detected.

Table III

The stability of lactose permease in detergent solutions

Protein in solution (A, B) was determined by centrifuging the suspension of protein, containing some lactose permease, protein an hour for 10 min at 10,000 g and measuring the radioactivity in supernatant and pellet. A equals 100% of protein measured in solution, C = 100-20% of protein measured in solution. Binding of 4,4'-thiodigalactoside (AB) was measured in the supernatant or after reconstitution: C = 100-40% of binding activity retained after reconstitution (for in solution for 4.8 x 71). D = 40-20% of binding activity retained after reconstitution (for in solution for 4.8 x 71). E = 20-10% of binding activity retained after reconstitution (for in solution for 4.8 x 71).

Table IV

Sulphidolysis of the lactose permease with various detergents

Detegent | pH | Addition of detergent | Recovery of binding (%) | After reconstitution (%) |
|----------|----|-----------------------|-------------------------|--------------------------|
| Dodecyl-MAAX | 7.4 | n.d. | 63 | 60 |
| Dodecyl-MAAX | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |
| Dodecylglucoside | 7.4 | n.d. | 63 | 60 |

* Detergents were used at 2.5% (w/v) and the sulphidolysis step was performed in the presence of 2.5% (w/v) detergent, pH 7.4.

* N.D. = not detected

* Octol NEM is a fraction obtained by distillation (81).

* D = indicates that less that 24% of original activity was recovered.
## Table 2

Characteristics of lactose transport in reconstituted systems: selection of phospholipid mixture E as optimum.

| Parameter                             | A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
|---------------------------------------|----|----|----|----|----|----|----|----|----|----|----|
| Binding (dissolved lactose)            |    |    |    |    |    |    |    |    |    |    |    |
| att Neu protein                       | 0.91 | 0.92 | 0.87 | 0.80 | 0.84 | 0.84 | 0.83 | 0.87 | 0.83 | 0.86 | 0.85 |
| Kd (mM)                               | 62 | 36 | 27 | 44 | 45 | 43 | 43 | 42 | 43 | 43 | 43 |
| Rate (lactose)                        |    |    |    |    |    |    |    |    |    |    |    |
| Km (mM)                               | 10 | 5 | 2 | 2 | 4 | 0.7 | 0.2 | 0.4 | 0.5 | 0.5 | 0.5 |
| Kd (mM)                               | 0.5 | 0.6 | 0.9 | 1.0 | 0.8 | 0.7 | 1.5 | 3.5 | 3.6 | 3.9 | 0.5 |
| Km (mM)                               | 3.1 | 3.6 | 3.7 | 3.8 | 3.9 | 3.8 | 3.8 | 3.9 | 3.9 | 3.9 | 3.9 |
| Efflux (lactose)                      |    |    |    |    |    |    |    |    |    |    |    |
| Km (Mn, k)                            | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Efflux (lactose)                      |    |    |    |    |    |    |    |    |    |    |    |
| Substrate (mM)                        | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| % at equilibrium                      |    |    |    |    |    |    |    |    |    |    |    |
| Substrate (mM)                        | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

Phospholipid mixture:
- A: DPPC
- B: DPPC + DSPC (50:50)
- C: DOPS + DMPC (50:50)
- D: DOPS + DMPC + cholesterol (10:10:10)
- E: DOPS + DMPC + cholesterol (20:10:10:10)
- F: DOPS + DMPC + cholesterol (30:10:10:10)

The values in the table represent the binding and efflux rates of lactose at various concentrations. The data were obtained by flow dialysis or equilibrium dialysis and the results analyzed by nonlinear regression (Fig. 1). Lactose flux measurements were made as described in Table 1 and protein flow as described in Fig. 4. Kd, in the apparent dissociation constants, is the concentration of ligand at which the rate of dissociation equals the rate of association. In the absence of lactose, the dissociation constant for lactose was determined according to methods described in refs. (18) and (19).