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Transport by Reconstituted Lactose Carrier from Parental and Mutant Strains of *Escherichia coli*

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Summary. The lactose transport carrier from parental (X71/F'W3747) and mutant cells (54/F'5441) was reconstituted into proteoliposomes. Transport by the counterflow assay showed slightly greater activity in proteoliposomes prepared from extracts of the mutant membranes compared with that for the parental cell. The mutant carrier showed a threefold lower K_m but similar V_{max} compared to the parent. On the other hand proteoliposomes from the mutant showed a defect in protonmotive force-driven accumulation, compared with the parent. With a pH gradient (inside alkaline) plus a membrane potential (inside negative) the parental proteoliposomes accumulated lactose 25-fold over the medium concentration while the mutant proteoliposomes accumulated sixfold. In a series of experiments proteoliposomes were exposed to proteolytic enzymes. Chrymotrypsin treatment resulted in 30% inhibition of counterflow activity for the reconstituted carrier from both parent and mutant. Papain produced 84% inhibition of transport by the reconstituted parental carrier but only 41% of that of the mutant. Trypsin and carboxypeptidase Y treatment had no effect on counterflow activity of either parent or mutant. Exposure of purified lactose carrier in proteoliposomes to carboxypeptidase Y resulted in the release of alanine and valine, the two C-terminal amino acids predicted from the DNA sequence.

Key Words reconstitution · lactose transport · membrane potential · pH gradient · proteolytic enzymes

Introduction

The study of membrane transport of small molecules in the living cell is complicated by such factors as metabolism of the substrate and interaction of the carrier molecule with other proteins in the membrane or cytoplasm. A major simplification can be achieved by the extraction of the carrier protein from the biological membrane and reconstitution of transport activity in phospholipid vesicles. Such proteoliposomes are free of cytoplasmic components and thus do not metabolize added substrates. In addition many membrane components are not extracted and most of those that are solubilized do not enter the phospholipid vesicles. This allows the physical separation of the carrier from many of the components of the membrane *in vivo*.

We have used the reconstitution technique to study lactose transport in a mutant with several interesting and unusual properties. The mutant was isolated (Wilson, Kusch & Kashket, 1970) by growth on glycerol in the presence of the toxic galactoside, *o*-nitrophenyl-thio-β-galactoside. Cells possessing a normal lactose transport carrier (lac Y positive) accumulate this galactoside (which inhibits growth probably due to the drain on metabolite energy) and mutants in the lac Y gene outgrow the parental cells (Müller-Hill, Crapo & Gilbert, 1968). One such mutant, X71-54, showed a reduced capacity to accumulate methyl-thio-β-galactoside (Wilson et al., 1970; Wilson & Kusch, 1972) while showing elevated entry rate of another substrate, *o*-nitrophenyl-β-galactoside (Wilson, 1978). An accumulation defect for lactose was inferred from the failure of lactose to induce the lactose operon, although growth on lactose was quite normal where full induction was achieved or if the cell were constitutive (Kusch & Wilson, 1973). These data are consistent with a defect in energy coupling. Indeed, a reduced proton entry with galactosides was demonstrated for the mutant (West & Wilson, 1973).

In the present study the accumulation of the natural substrate lactose was investigated in both counterflow assays and protonmotive force-driven uptake. This study was made possible by the reconstitution technique. The mutant showed a severe defect in protonmotive force-driven lactose accumulation in spite of an elevated counterflow and an increased affinity for the substrate. In addition, the reconstituted mutant carrier showed an altered pH dependence for counterflow and a reduced sensitivity to papain compared with the parent. A defect in the coupling of ion gradients to sugar accumulation is consistent with the data.

Materials and Methods

Bacterial Strains

E. coli strains used in most of these studies were: X71/F'W3747 (lac $i^-z^+y^+$ Pro C^- $Strep^R$ $tryp^-$ B_1^-/F' lac $i^+z^+y^+$ Pro C^+) and 54/F'5441 (lac $i^-z^+y^{un}$ Pro C^- $Strep^R$ $tryp^-$ B_1^-/F' lac $i^-z^+y^{un}$ Pro C^+). The gene which codes for the "energy-uncoupled" transport protein is designated as y^{un}. The isolation of the mutant and some of its properties are given elsewhere (Wilson et al., 1970; Wilson & Kusch, 1972; Kusch & Wilson, 1973). *E. coli* T206 (lac $i^+z^-y^-$/F' lac $i^qz^{u188}y^+$/lac Δi Δz y^+) which carries the lac y gene on a DNA plasmid (Teather et al., 1978), was kindly provided by Dr. Peter Overath at Max Planck-Institut für Biologie, Tübingen, West Germany.

Growth of Bacteria

The cells were grown to midlog-phase in minimal medium 63 (Cohen & Rickenberg, 1956) with 0.4% glycerol as the carbon source plus supplements. The supplement for X71/F'W3747 and 54/F'5441 was 20 μg/ml tryptophan, 0.5 μg/ml thiamine and 200 μg/ml streptomycin. The supplement for T206 was 50 μg/ml methionine, 50 μg/ml threonine, 10 μg/ml thiamine, 100 μg/ml streptomycin and 10 μg/ml tetracycline (Teather et al., 1978). When tetracycline was used, the culture was grown in the dark.

Stock cells in midlog phase were diluted into minimal medium 63 containing glycerol and supplement as described above plus 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were grown to early stationary phase and then harvested for preparation of membrane vesicles.

Preparation of Membrane Vesicles

The cells were harvested, washed once with medium 63 and resuspended in a buffer to 5 ml per gram wet weight of cells. The temperature was 4°C in all steps except where otherwise indicated. The buffer consisted of 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol (DTT), 20 mM lactose, 5 mM magnesium sulfate and 1 mM phenylmethylsulfonylfluoride. DNAase (10 μg/ml) was added to the suspension and the cells were disrupted by passing through an Aminco French pressure cell at 20,000 psi. The unbroken cells were removed by centrifugation at 11,700 × *g* for 10 min. The supernatant was centrifuged at 145,000 × *g* for 1 hr. The pelleted membrane vesicles were washed once with a buffer consisting of 50 mM potassium phosphate, pH 7.5, 1 mM DTT, 20 mM lactose and 1 mM phenylmethylsulfonylfluoride and then centrifuged at 145,000 × *g* for 1 hr. The washed membrane vesicles were resuspended in 50 mM potassium phosphate, pH 7.5, 0.5 mM DTT and 10 mM lactose. For storage, the membrane vesicles were divided into small aliquots, frozen in liquid N_2 and stored at −80°C.

Reconstitution of Lactose Transport

The reconstitution method was that of Newman and Wilson (1980) with the modifications indicated. The membrane vesicles were extracted at 0°C with a buffer containing 100 mM potassium phosphate, 25 mM 4-morpholineethanesulfonic acid (MES), pH 6, 1.3 mM DTT, 3.9 mg/ml *E. coli* lipid and 1.3% octylglucoside. The protein concentration of the suspension was approximately 1 mg/ml. The suspension was incubated on ice for 10 min and then centrifuged at 180,000 × *g* for 1 hr at 4°C. Reconstitution was carried out by adding 650 μl of the supernatant to 165 μl of bath-sonicated *E. coli* liposomes plus 15 μl of 15% octylglucoside. The final concentration of octylglucoside was 1.29%. In order to decrease the concentration of the detergent the suspension was diluted into 25 ml of buffer (at 22°C) to form proteoliposomes. The buffer contained 100 mM potassium phosphate, 25 mM MES, pH 6, and 1 mM DTT with or without 20 mM lactose. In some experiments the dilution buffer was pH 7.5 in order to prepare proteoliposomes with an internal medium of that pH. The proteoliposomes were collected by centrifugation at 145,000 × *g* for 1 hr at 4°C.

Counterflow Assay

The proteoliposome pellet was resuspended in 75 μl of a buffer containing 100 mM potassium phosphate, 25 mM MES, pH 6, 20 mM lactose and 1 mM DTT. Twelve microliters of proteoliposomes were added to 0.6 ml of assay buffer at 22°C. The assay buffer contained 100 mM potassium phosphate, 25 mM MES, pH 6, and 0.5 μCi/ml [^{14}C]-lactose (0.01 mM). The final concentration of lactose in the reaction mixture was 0.41 mM. Samples (100 μl) were removed at various time intervals and placed onto the center of a 0.22 μm Millipore filter (type GSTF) without a chimney. After filtration the proteoliposomes were washed with 5 ml cold buffer. The composition of the wash buffer was the same as the incubation medium without lactose.

Ion Gradient-Driven Transport Assay

The proteoliposome pellets were resuspended in 100 mM potassium phosphate, 25 mM MES and 1 mM DTT. The pH of the buffer was varied depending on the experiments (*see* the text). Valinomycin was added to the resuspended proteoliposomes to give a concentration of 19 μM. These proteoliposomes (12 μl) were added to 1.2 ml of assay buffer (at 22°C) containing 100 mM potassium or sodium phosphate, 25 mM MES (for pH *see* Figure legends) and 1 μCi/ml [^{14}C]-lactose (0.2 mM). Samples (220 μl) were removed at various time intervals, filtered and washed as described above.

Kinetic Measurements

Six microliters of proteoliposomes (preloaded with 20 mM lactose) were diluted into 1.2 ml of counterflow assay medium (at 22°C) containing various lactose concentrations. The final concentration of the assay medium contained 100 mM potassium phosphate, 25 μM MES, pH 6, and 0.5 μCi/ml [^{14}C]-lactose (0.1 to 1.1 mM). The protein concentration was approximately 5 μg/ml. The reaction mixture was incubated at 22°C. Samples (0.55 ml) were removed at various time intervals (20 to 60 sec) and placed into 5 ml of cold buffer in the chimney above the Millipore filter and then filtered. The chimney was removed and the proteoliposomes were washed with 5 ml cold buffer.

Purification of the Lactose Carrier

The method was that of Newman et al. (1981) with the modifications indicated. Approximately 100 mg of T206 membrane vesi-

cles plus [^{3}H]-*p*-nitrophenyl-α-galactoside (α*p*NPG)-labeled membranes were resuspended in 15 ml of buffer containing 50 mM potassium phosphate, pH 7.5, 0.5 mM DTT and 10 mM lactose and then 15 ml of 10 M urea were added dropwise to the suspension. The temperature was 4°C in all steps except where otherwise indicated. The mixture was incubated on ice for 10 min and centrifuged at 145,000 × *g* for 1 hr. The pellet was resuspended in 19 ml of 50 mM potassium phosphate, pH 7.5, and 8 ml of sodium cholate (20%) was added dropwise. The final concentration of sodium cholate was 6%. The suspension was incubated on ice for 20 min and centrifuged at 145,000 × *g* for 1 hr. The pellet was washed once with 25 ml of 10 mM potassium phosphate, pH 6, and then resuspended in 10 ml of 12.5 mM potassium phosphate, pH 6. To the suspension were added 125 μl of 100 mM DTT, 450 μl of 20% lactose, 900 μl of 50 mg/ml *E. coli* lipid and 1040 μl of 15% octylglucoside. Thus the suspension contained 10 mM potassium phosphate, 1 mM DTT, 20 mM lactose, 3.6 mg/ml *E. coli* lipid and 1.25% octylglucoside. This suspension was incubated on ice for 10 min and then centrifuged at 145,000 × *g* for 1 hr.

A 10 ml column of DEAE-Sepharose-CL-6B was prepared as described by Newman et al. (1981). The octylglucoside extract (5 ml) was loaded onto the column and fractions (1.5 ml) were collected. When radioactive α-*p*NPG counts appeared in the eluate, the subsequent 1.5 ml fractions were collected into 0.5 ml of sonicated *E. coli* lipid plus 46 μl of 15% octylglucoside, with periodic mixing with a Pasteur pipette. Each of these fractions was diluted into 75 ml buffer containing 50 mM potassium phosphate, pH 7.5, 20 mM lactose and 1 mM DTT (at 22°C). The reconstituted proteoliposomes were collected by centrifugation at 145,000 × *g* for 1 hr.

Carboxypeptidase Y Digestion of Reconstituted Proteoliposomes Containing Purified Lactose Carrier

In experiment A of Table 4 the reconstituted proteoliposomes containing the purified lactose carrier were resuspended in 3 ml of 10 mM sodium acetate, pH 5. The suspension was centrifuged in a Beckman 50 Ti rotor at 180,000 × *g* for 1 hr at 4°C. The pellet was washed with the same buffer and collected by centrifugation. The supernatant was removed and kept frozen (−20°C) for subsequent amino acid analysis ("control"). The pellet was resuspended in 1 ml of 10 mM sodium acetate, pH 5. Pepstatin A (1 μl of 2 mM in 20% dimethylsulfoxide) was added and then incubated at 22°C for 2 min (Lee & Riordan, 1978). Carboxypeptidase Y (10 μl of 500 μg/ml) and norleucine (40 μl of 50 μM) were added to the suspension. Thus, the final concentration of the reaction mixture contained 270 μg/ml (5.8 nmol/ml) of purified lactose carrier, 5 μg/ml of carboxypeptidase Y, 2 nmol/ml of pepstatin A and 2 nmol/ml of norleucine (a "standard" for the subsequent amino acid analysis). The suspension was incubated at 37°C for 18 hr and then centrifuged in a Beckman 50 Ti rotor at 180,000 × *g* for 1 hr at 4°C. The supernatant was removed and frozen for subsequent amino acid analysis.

The "control" supernatant from washed proteoliposomes and the "experimental" supernatant from carboxypeptidase Y digested proteoliposomes were each diluted into 9 ml of cool acetone and kept at −20°C for 1 hr to precipitate protein. The samples were centrifuged at 3500 × *g* for 10 min. The supernatants were evaporated under N_2 gas to a small volume and then lyophilized. The amino acids were resuspended in 400 μl of 0.15 N lithium hydroxide, 0.15 N citric acid 1% thiodiglycol and 0.1% phenol. A sample (100 μl) was analyzed in a Beckman 121 MB Amino Acid analyzer.

In experiment B proteoliposomes with purified lactose carrier were incubated in 120 μl of a solution containing 5 μg/ml carboxypeptidase, 2 nmol/ml of pepstatin A, 100 mM potassium phosphate, 25 mM MES (pH 6), 20 mM lactose and 1 mM DTT. After on incubation of 16 hr at 22°C the proteoliposomes were sedimented in a Beckman Airfuge for 30 min at 86,000 × *g*. The 120-μl supernatant was added to 80 μl of citric acid buffer (given above) and 100 μl removed for amino acid analysis. The control was a 120-μl wash with the above buffer prior to exposure to carboxypeptidase Y. The experimental values were corrected for low level of amino acids found in the control sample.

Exposure of Proteoliposomes to Proteolytic Enzymes

The proteoliposomes were preloaded with 100 mM potassium phosphate, 25 mM MES, pH 6, 20 mM lactose and 1 mM DTT. Proteoliposomes (12 μl) were mixed with 12 μl of proteolytic enzyme or 12 μl of water (as a control). Papain, chymotrypsin, TPCK-trypsin or carboxypeptidase Y was dissolved with water and the concentration was 100 μg per ml. Thus, the final concentration of the digestion mixture contained 50 mM potassium phosphate, 12.5 mM MES, pH 6, 10 mM lactose and 0.5 mM DTT. The ratio of membrane proteins to proteolytic enzyme was approximately 10 : 1. The mixture was then incubated at 37°C for 1.5 hr. Following the digestion transport was assayed. The counterflow assay buffer (0.6 ml) was added to start the reaction. The final concentration of the lactose in the reaction mixture was 0.41 mM. The sampling, filtering and washing were the same as described in the "Counterflow Assay" section.

Measurement of Internal Water Space of the *E. coli* Liposomes

Liposomes (without added protein) were prepared in a manner to trap radioactive sugars in the internal water space. *E. coli* lipid plus octylglucoside were mixed in the absence of membrane vesicles and centrifuged at 4°C. The supernatant was mixed with sonicated lipid plus octylglucoside as described in the Reconstitution section of Materials and Methods. This lipid-detergent suspension (50 μl) was added to 1.65 ml buffer consisting of 100 mM potassium phosphate, 25 mM MES, pH 7.5, 1 mM DTT, radioactive sugars and nonradioactive sugars. The radioactive sugars were: (A) 0.9 μCi/ml [^{14}C]-lactose (0.9 mM); (B) 3.6 μCi/ml [^{3}H]-raffinose (0.9 mM); (C) 0.9 μCi/ml [^{14}C]-lactose (0.9 mM) plus 3.6 μCi/ml [^{3}H]-raffinose (0.9 mM). The liposomes were centrifuged at 180,000 × *g* for 1 hr. The pellets were resuspended with 1.5 ml buffer containing 100 mM potassium phosphate, 25 mM MES, pH 7.5 and 1 mM DTT. The resuspended liposomes (1 ml) were filtered onto the center of a 0.22 μm Millipore filter (type GSTF) without a chimney. The filter was washed with 10 ml cold buffer containing 100 mM potassium phosphate plus 25 mM MES, pH 6. As a control non-preloaded liposomes were exposed to the radioactive sugar and centrifuged. Samples were filtered and washed as above. This "blank" was subtracted from the experimental values.

Protein Determination

Protein was determined by a modification of the methods of Schaffner and Weissmann (1973) and Newman et al. (1981). The

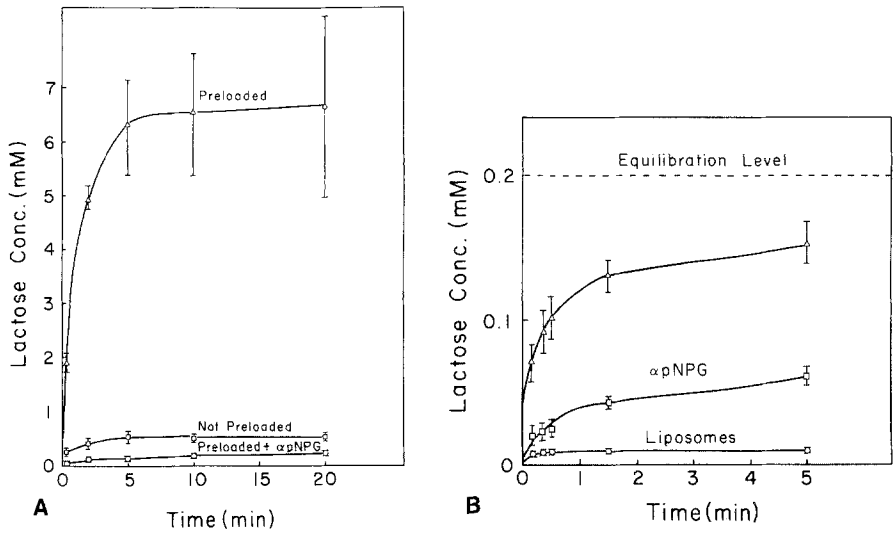

Fig. 1. Lactose uptake in lactose-preloaded and non-preloaded proteoliposomes. *A*. The lactose-preloaded (20 mM) or non-preloaded proteoliposomes were prepared from the extract of *E. coli* X71/F'W3747 membrane vesicles. The final external concentration of [^{14}C]-lactose was 0.41 mM with or without 10 mM α-*p*NPG (pH 6). The protein concentration in the three experiments varied from 15 to 25 μg/ml. △–△: lactose preloaded proteoliposomes; □–□: non-preloaded proteoliposomes; ○–○: lactose-preloaded proteoliposomes with 10 mM α-*p*NPG in the assay medium. Data given represent the mean values of three experiments. The error bar represents the standard error. *B*. Proteoliposomes or liposomes were preloaded with 100 mM potassium phosphate plus 25 mM MES, pH 7.5. Valinomycin was added to the concentrated suspension of proteoliposomes and liposomes to give a concentration of 19 μM. Proteoliposomes or liposomes were diluted 100-fold into an assay medium containing 100 mM potassium phosphate, 25 mM MES, pH 7.5, [^{14}C]-lactose (0.2 mM) with or without 10 mM α-*p*NPG. The protein concentrations in the three different epxeriments varied from 8 to 13 μg/ml (no protein in the liposomes). △–△ and □–□: proteoliposomes; ○–○: liposomes. The data given represent the mean values of three experiments (with standard error)

final concentrations of sodium dodecal sulfate and trichloroacetic acid were increased to 1% and 20%, respectively.

Phospholipid Determination

Phospholipid was determined by the method of Hallen (1980). Bath sonicated *E. coli* lipid was used as standard to determine *E. coli* phospholipid.

Chemicals

Preparation of Acetone/Ether-Washed E. coli Lipid

The crude chloroform/methanol-extracted *E. coli* lipid was purchased from Avanti Biochemicals. The method for preparation of acetone/ether-washed *E. coli* lipid was the same as described by Newman and Wilson (1980).

[^{14}C]-Lactose was obtained from Amersham and was purified by descending paper chromatography in *l*-propanol : H_2O (3 : 1) before use. [^{3}H]-Raffinose was from New England Nuclear.

Octylglucoside was obtained from Calbiochem. DEAE-Sepharose CL-CB was from Sigma. Papain, α-chymotrypsin and TPCK-trypsin were from Worthington. Carboxypeptidase Y was from Pierce.

Results

Counterflow

There are two general methods for assaying membrane transport of lactose in proteoliposomes: ion gradient-driven uptake and facilitated diffusion. Counterflow is one measure of facilitated diffusion and does not depend on ion gradients as a driving force for accumulation. In order to determine whether the uncoupled mutant has a normal sugar recognition site, the properties of the reconstituted lactose carrier from the mutant and parental strains were first compared by the counterflow technique.

In this type of assay system proteoliposomes preloaded with lactose were exposed to a low external concentration of [^{14}C]-lactose. Radioactive molecules enter via the lactose carrier and accumulate within the proteoliposomes due to competitive inhibition of exit by the high concentration of internal nonradioactive sugar. The accumulation is only temporary, however, since the exit of the preloaded sugar via the carrier leads to a progressive fall in its concentration and reduction of its inhibition of exit of radioactive sugar ultimately leading to equilibra-

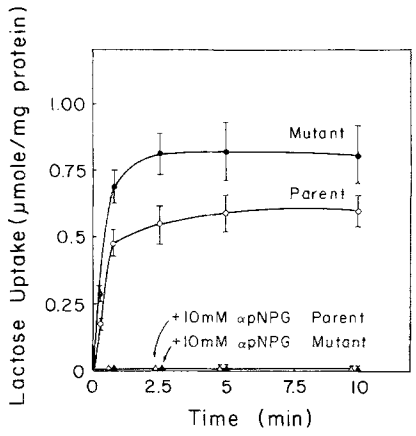

Fig. 2. Counterflow in proteoliposomes from parent and mutant. Lactose-preloaded proteoliposomes were prepared from the extract of *E. coli* X71/F'W3747 or 54/F'5441 membrane vesicles. The assay mixture (pH 6) was the same as described in Fig. 1. ○–○ and △–△: parent; and ●–● and ▲–▲: mutant. Data given represent the mean values of three experiments (with standard error)

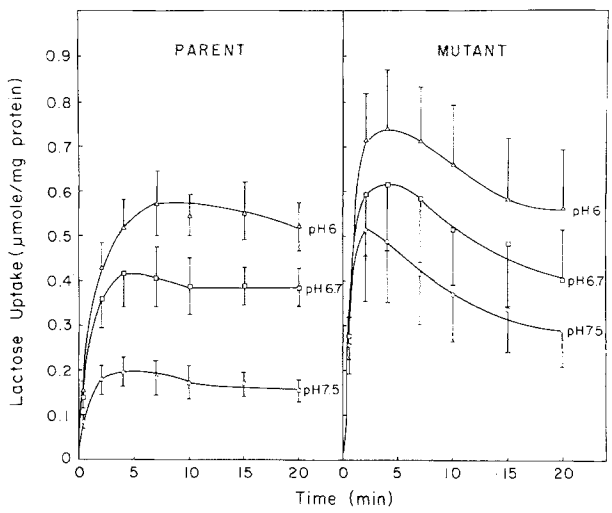

Fig. 3. Effect of pH on the counterflow activity of the proteoliposomes from the parent and mutant. Proteoliposomes were preloaded with 100 mM potassium phosphate, 25 mM MES, pH 6, 6.7 or 7.5, 20 mM lactose and 1 mM DTT. Proteoliposomes were added to the counterflow assay medium which contained the same buffer (without lactose) used for preloading. The final external concentration of [^{14}C]-lactose was 0.41 mM. The protein concentrations in the four experiments varied from 15 to 25 μg/ml. The washing buffer was the same as the incubation buffer, without lactose. △–△: pH 6; □–□: pH 6.7, and ○–○: pH 7.5. The mean values of four experiments are given (with standard error)

tion of radioactive and nonradioactive sugars across the membrane. In the experiments presented here only the uptake portion of the counterflow curve was studied.

The mean values of three counterflow experiments are indicated in Fig. 1*A*. Proteoliposomes preloaded with 20 mM lactose were diluted 50-fold into [^{14}C]-lactose (final concentration 0.41 mM). In this experiment rapid sugar uptake was observed during the first few minutes, reaching an internal concentration of [^{14}C]-lactose of about 7 mM at 20 min. Proteoliposomes not preloaded with sugar showed uptake of [^{14}C]-lactose to a concentration of 0.5 mM. This represents approximate equilibration between external and internal concentrations of the labeled disaccharide. The presence of the competitive inhibitor *p*-nitrophenyl-α-galactoside (α-*p*NPG) reduced the [^{14}C]-lactose uptake in the preloaded proteoliposomes to low levels (0.25 mM).

Assigning concentrations to the values obtained in the experiment of Fig. 1 was based on the determination of internal water volume. Liposomes were prepared in the presence of two different radioactive sugars; they were centrifuged, washed, filtered and counted. The exit rate of these sugars were extremely slow, less than 10% in 1 hr at 25°C (D. Wilson, *unpublished data*). With [^{14}C]-lactose the internal volume was 1.28 μl water/mg lipid (mean of three experiments) and with [^{3}H]-raffinose the value was 1.23 μl water/mg lipid (mean of three experiments).

If each proteoliposome contains at least one transport protein molecule one would expect that internal and external concentrations of lactose would equilibrate in the absence of competitors or ion gradients. Figure 1*B* shows the mean value of three experiments in which proteoliposomes were exposed to [^{14}C]-lactose in the absence of an ion gradient. At 5 min the concentration inside was 75% of equilibration, indicating that at least 75% of the liposomes contained a lactose carrier molecule. In some experiments the concentration in the internal water space equilibrated with that of the external medium (Fig. 1*A*). The competitive inhibitor *p*-nitrophenyl-α-galactoside gave approximately 70% inhibition of uptake. Liposomes prepared in the absence of protein appeared to be almost impermeable to lactose (Fig. 1*B*).

The transport by proteoliposomes prepared with the mutant transport protein was compared with that of the parental cell (Fig. 2). The mean values of three experiments at pH 6 showed that the mutant carrier gave a faster initial rate of entry and sustained somewhat higher levels of counterflow than that of the parental cell. The competitor α-*p*NPG inhibited both equally.

The effect of pH on counterflow activity by proteoliposomes containing mutant and parental carrier was studied (Fig. 3). Accumulation of [^{14}C]-lactose was greater at pH 6 than at pH 7.5, with intermediate values for pH 6.7. This agrees with the

Table 1. K_m and V_{max} values for lactose counterflow in reconstituted proteoliposomes containing the lactose carrier from parent and mutant at pH 6[a]

Strain	K_m (mM)	V_{max} (μmol/mg protein/min)
Parent	0.87 ± 0.06	1.4 ± 0.13
Mutant	0.29 ± 0.02	1.2 ± 0.1

[a] The data represent the mean values from seven experiments (±SE).

findings of Kaczorowski and Kaback (1979) and of Garcia et al. (1983) that the peak of the counterflow curve decreases with increasing pH. At pH 6 and 6.7 the mutant carrier showed significantly higher lactose accumulation than the parental carrier. There was a much larger difference at pH 7.5; the mutant carrier showed almost three times as much lactose uptake as that of the parental cell. Valinomycin, *p*-chlorocarbonylcyanide phenylhydrazine or a combination of valinomycin and nigericin had no effect on the counterflow activity (*data not shown*).

The kinetics for counterflow for both parent and mutant carriers are summarized in Table 1. The mutant carrier showed threefold lower K_m for lactose than the parent. The maximum transport rate was approximately the same for the two strains.

Effect of Ion Gradients

It is well documented that both a pH gradient and a membrane potential drive the active accumulation of lactose against its concentration gradient. In order to determine whether this uncoupled mutant is defective in the proton-lactose coupling mechanism, the effect of pH gradient and membrane potential on transport were studied separately and together.

To produce a pH gradient, proteoliposomes with an internal pH of 7.5 were diluted into buffer at pH 6. With the K^+ concentration of 100 mM on both sides of the membrane the presence of valinomycin prevented the generation of a membrane potential (H^+ entry was compensated by K^+ exit). In the presence of a pH gradient proteoliposomes with mutant carrier accumulated lactose to a level approximately twofold greater than that observed in the absence of a pH gradient (Fig. 4), while similar experiments with the parental transport carrier gave about fivefold accumulation in the presence of a pH gradient. Thus, the mutant carrier shows a defect in ΔpH-driven sugar uptake.

A membrane potential was generated across the

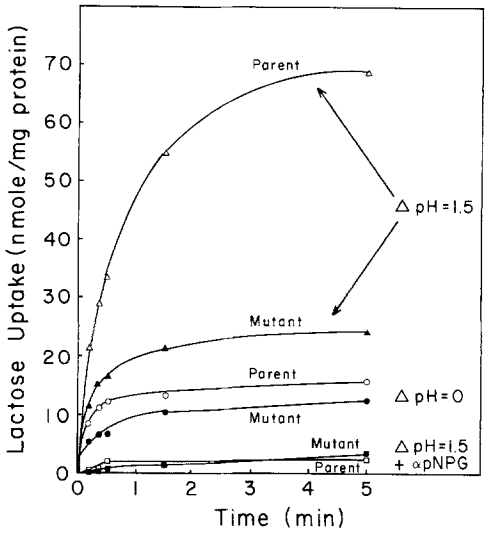

Fig. 4. pH gradient-driven lactose uptake in proteoliposomes from parent and mutant. Proteoliposomes were preloaded with 100 mM potassium phosphate plus 25 mM MES, pH 7.5. The external medium contained 100 mM potassium phosphate, 25 mM MES, pH 7.5 or 6, 0.2 mM lactose with or without 10 mM α-*p*NPG. The concentrations of valinomycin and protein were the same as described in Fig. 1*B*. Open symbols represent parent and closed symbols represent mutant. △–△, ▲–▲: pH gradient (inside pH 7.5, outside pH 6); ○–○ and ●–●: no pH gradient (inside and outside pH 7.5); □–□ and ■–■: pH gradient, plus 10 mM α-pNPG. The data given represent one experiment. Two additional experiments with the parent and one with the mutant gave similar results to that in the Figure. The counts obtained with liposomes (no protein) were subtracted from values obtained with proteoliposomes.

membrane of the proteoliposomes by preloading with 100 mM K^+ and diluting to give a final external concentration of 1 mM K^+ in the presence of valinomycin. Potassium ion exits from the proteoliposomes via the valinomycin down a concentration gradient of approximately 100-fold and generates a membrane potential, inside negative. Such a K^+ diffusion potential was generated at pH 7.5 or 6 (Fig. 5). In these experiments the pH inside and outside the proteoliposome was the same. The lactose accumulation was greater at pH 7.5 than at pH 6. There is a severe defect in ΔΨ-driven lactose accumulation in the mutant at either pH value. The defect is somewhat greater at pH 6.

Additional experiments were performed with both a pH gradient (pH 7.5 inside and pH 6 outside) and a membrane potential (K^+ inside 100 mM and K^+ outside 1 mM with valinomycin). Proteoliposomes with mutant carrier accumulated about sixfold over the equilibration value while those with parental carrier showed 25-fold accumulation (Fig. 6).

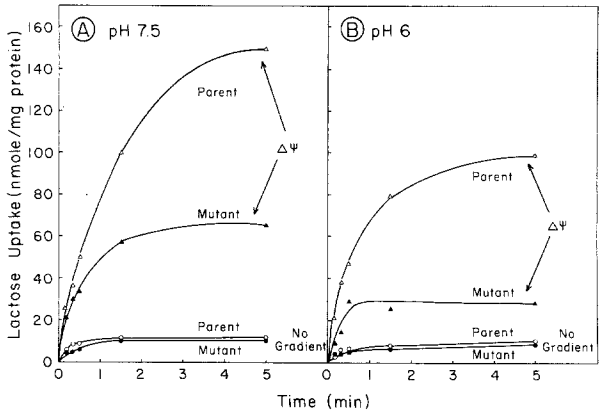

Fig. 5. Membrane potential-driven lactose uptake in proteoliposomes from parent and mutant. Proteoliposomes were preloaded with 100 mM potassium phosphate plus 25 mM MES, pH 7.5 (*A*) or pH 6 (*B*). The external medium contained 100 mM potassium phosphate or sodium phosphate, 25 mM MES, pH 7.5 (*A*) or 6 (*B*) and 0.2 mM lactose. The concentration of valinomycin and protein were the same as described in Fig. 1*B*. The open symbols represent the parent and the closed symbols represent the mutant. △–△ and ▲–▲: membrane potential (K^+ inside, Na^+ outside); ○–○ and ●–●: no membrane potential (K^+ inside and outside). The data represent one experiment. Two additional experiments gave similar results. The counts obtained with liposomes (no protein) were subtracted from values obtained with proteoliposomes

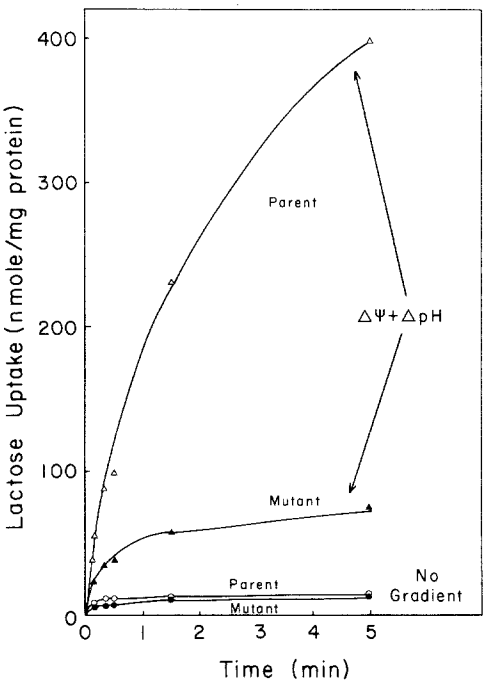

Fig. 6. pH gradient and membrane potential-driven lactose uptake in proteoliposomes from parent and mutant. Proteoliposomes were preloaded with 100 mM potassium phosphate plus 25 mM MES, pH 7.5. The external medium contained 100 mM potassium phosphate or sodium phosphate plus 25 mM MES, pH 7.5 or 6 and 0.2 mM lactose. The concentrations of valinomycin and protein were the same as described in Fig. 1*B*. The open symbols represent the parent and the closed symbols represent the mutant. △–△ and ▲–▲: pH gradient and membrane potential (inside pH 7.5 and K^+; outside pH 6 and Na^+); ○–○ and ●–●: no pH gradient or membrane potential (inside pH 7.5 and K^+; outside 7.5 and K^+). Data represent one experiment. Two additional experiments gave similar results. The counts obtained with liposomes (no protein) were subtracted from values obtained with proteoliposomes

Proteolytic Enzymes

The three-dimensional structure of the lactose carrier in the lipid bilayer may play an important role in the transport mechanism. To gain some information about the orientation of the lactose carrier, the effect of proteolytic enzymes was tested on counterflow by reconstituted proteoliposomes with carriers derived from parental and mutant strains. Trypsin treatment at a concentration of 50 μg/ml for 90 min had extremely little effect on transport (Table 2) in spite of the large number of lysine and arginine residues in the molecule (Büchel, Gronenborn & Müller-Hill, 1980). Chymotrypsin treatment resulted in approximately 30% inhibition of counterflow in both parent and mutant carrier. Papain was of interest because it produced 84% inhibition of counterflow by the reconstituted parental carrier but only 41% of that for the mutant. Carboxypeptidase Y produced no significant effect on transport in either the parent or mutant.

Since carboxypeptidase Y had no effect on transport it was of interest to determine how many amino acids were removed by this treatment. Experiments were carried out with carboxypeptidase Y digestion of reconstituted lactose carrier purified from the plasmid strain T206. The lactose carrier was purified by the method described by Newman et al. (1981). The carrier was reconstituted into proteoliposomes, which were washed carefully and then exposed to carboxypeptidase Y at a concentration of 5 μg/ml. After an 18-hr incubation at 37°C the free amino acids were separated from the proteoliposomes by centrifugation, lyophilized, resuspended and analyzed on an amino acid analyzer. Table 3 shows the results of two preliminary experiments. In both experiments the major amino acids found were the C-terminal two amino acids of the lactose carrier alanine and valine. In the first experiment 1.4 nmol of lactose carrier yielded 0.63 nmol of alanine suggesting that about one-half of protein molecule were accessible to the enzyme. Glutamic acid showed the next highest concentration which was detected at a level of only about one-half that of

the first two amino acids. It is known that carboxypeptidase is far less active on negatively charged amino acids than on neutral ones (Hayashi, Bai & Hata, 1975).

In experiment B transport was carried out before and after carboxypeptidase Y digestion and lactose counterflow found to be the same. Thus, the removal of two or three amino acids from the C-terminal end of the lactose carrier under these conditions has no effect on biological activity.

Discussion

With lactose as substrate, the entry of disaccharide on the carrier from the mutant strain was faster than that of the parental strain. This reflected a greater affinity of the mutant carrier than the parental carrier for lactose. In spite of the greater affinity (and normal V_{max}) of the mutant carrier in the counterflow assay, ion gradient-driven transport was defective. Reduced transport activity (compared with the parent) was observed for the reconstituted carrier from the mutant for the pH gradient (ΔpH), membrane potential ($\Delta\Psi$) or the sum of the two (Figs. 4, 5, and 6). In addition the reconstituted mutant carrier showed an altered pH dependence for counterflow.

It is remarkable that trypsin had no effect on the counterflow activity in spite of 12 lysine and 12 arginine residues in the lactose carrier. This indicates that either the lysine and arginine residues are not accessible to the trypsin or splitting occurs in positions not required for the facilitated transport activity. The fact that the lactose carrier of membrane vesicles is more sensitive to proteolytic enzymes (Goldkorn, Rimon & Kaback, 1983) than the carrier in proteoliposomes of the present study is unexpected and requires further study. Papain has a distinctly greater effect on the parent than the mutant. This suggests that the molecular arrangement of the mutant carrier in the lipid bilayer is different from that of the parent.

Our interpretation of these data is that the lactose carrier from the mutant possesses a sugar recognition site that is intact but a H^+-recognition site which is defective. Consistent with this view was the finding of West and Wilson (1973) that proton transport associated with lactose entry was significantly reduced in this mutant.

It is of interest that the defect in the Y gene in this mutant (mapped by Hobson, Gho & Müller-Hill, 1977) was found to be located in the DNA that codes for a region close to the C-terminal end of the peptide. This map location plus the transport defect of the mutant makes it possible that an amino acid or amino acids near the C-terminal region of the peptide are involved in the determination of the proton recognition site.

An additional point of interest in this work was the release of alanine and valine from the purified lactose carrier treated with carboxypeptidase Y. These two amino acids correspond to the two C-terminal amino acids inferred from the DNA sequence (Büchel et al., 1980). This indicates that there is no post translational processing at the C-terminal end of the molecule.

Table 2. Inhibition by proteolytic enzymes of counterflow activity of proteoliposomes containing the lactose carrier from parent and mutant[a]

Strain	Inhibition (%)			
	Papain	Chymotrypsin	Trypsin	Carboxypeptidase Y
Parent	84	35	10	2
Mutant	41	30	16	8

[a] Lactose uptake was measured in proteoliposomes treated with different proteolytic enzymes. Inhibition of the 3-min uptake is given. The % inhibition at 15 sec was similar (*data not shown*). The data represent the mean values from four experiments.

Table 3. Carboxypeptidase Y digestion of lactose carrier

C-terminal sequence of lactose carrier	Expt. no.	Ala	Val	Glu	Asn	Val	Gln	Arg	Arg	Leu	Leu
pmol found	A[a]	630	820	280	270	—	180	190	—	760	—
	B[b]	371	382	141	50	—	97	47	—	201	—

[a] All other amino acids were less than 300 pmol. *See* Materials and Methods for the procedure.

[b] All other amino acids were less than 100 pmol with the exception of threonine which was 145.

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