Solubilization and Reconstitution of the Lactose Transport System from Escherichia coli*

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The lactose transport system from Escherichia coli was solubilized with octylglycoside and reconstituted into liposomes by an octylglycoside dilution procedure. The reconstituted proteoliposomes exhibited lactose counterflow and membrane potential-driven lactose transport.

The lactose transport system of Escherichia coli is responsible for the active transport of β-galactosides into the cell (1). In 1963, Mitchell (2) postulated that this system functions as a proton-substrate co-transport system which is coupled to the metabolism of the cell via the transmembrane electrochemical proton gradient. Studies with intact cells and cytoplasmic vesicles have provided strong support for this concept (see Ref. 3 for review). In addition, the kinetics (4-6) and substrate specificity (7) of the transport system have been extensively studied. The lacγ gene, which codes for the lactose transport protein, has been cloned on a bacterial plasmid and the nucleotide sequence of the gene has been determined (8). The transport protein has been purified in an inactive form (9).

Despite the fact that the lactose carrier represents one of the most extensively characterized active transport systems, little is known about its subunit structure, the molecular mechanism of active transport, or the mechanism by which the lactose carrier is regulated by the phosphotransferase system (10). Reconstitution of the carrier would provide an assay for the purification of the protein(s) responsible for β-galactoside transport. Furthermore, reconstitution of a purified transport system would greatly facilitate the determination of the molecular mechanisms of carrier function and regulation. Solubilization and reconstitution of lactose transport into transport-negative membrane vesicles have been reported (11). In addition, Padan et al. (12) have reported that lactose transport activity is lost after extraction of membrane vesicles with sodium cholate, and that transport activity in these vesicles is restored upon addition of exogenous phospholipid followed by detergent removal. However, previous attempts to solubilize and reconstitute the lactose transport system into liposomes have been unsuccessful.

Several reports on the reconstitution of bacterial cation-substrate co-transport systems have appeared (13-15), one of which deals with the reconstitution of proline transport from E. coli (13).

This communication describes the solubilization of the lactose transport system and its reconstitution into liposomes prepared from E. coli phospholipid. The reconstituted proteoliposomes exhibited lactose counterflow and membrane potential-driven lactose transport.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Two strains of E. coli were used: ML308-225 (lac i z y') (17) and ML2 (lac i z y') (18).

Materials—Diithiothreitol, lactose, deoxyribonuclease 1, phenylmethylsulfonyl fluoride, 1,10-phenanthroline monochloride, and thiogalactoside were obtained from Sigma. Octylglycoside (stored at -20°C as a 15% stock in 50 mM potassium phosphate, pH 7.5), gramicidin D, CCCP, and valinomycin were obtained from Calbiochem. Omni-Solv aceton and ethanol-stabilized chloroform (CX1082) were obtained from Matheson, Coleman, and Bell. Anhydrated ether and sucrose were from Fisher. N-Ethylmaleimide was from Schwarz/Mann. Asolectin was from Associated Concentrates (Woodside, NY) and was stored (Vortex-dispersed) at 100 mg/ml in 5 mM 2-mercaptoethanol under N2 gas at -80°C. 2-Mercaptoethanol was from Eastman. Cruude chloroform/methanol-extracted E. coli lipid was purchased by special order from Avanti Biochemicals (Birmingham, AL). The crude extract was stored under argon or N2 gas at -80°C in chloroform/methan (91). Radioactive [14C]lactose was obtained from Amersham and was purified by descending paper chromatography in 1-propanol/H2O (3:1) before use. Membrane filters were type GSTF (02500) 0.22 μm from Millipore, and the bath sonicator was 80 watts, 80 kHz, generator model G80-80-1, tank model T80-80-1-RS from Laboratory Supplies Co., Inc. (Hicksville, NY).

Preparation of Acetone/Ether-washed E. coli Lipid—Chloroform/methanol-extracted E. coli lipid was acetone/ether-washed by a modification of the method of Kagawa and Racker (19). Cruude lipid extract, 50 ml of chloroform/methanol (9:1), containing 1 g of lipid, was evaporated to 5 ml under a stream of N2 gas. The material was suspended in 100 ml of N2-bubbled anhdydrous solvent containing 2 mM 2-mercaptoethanol. The suspension was placed in a light-protected 250-ml flask under N2 gas and stirred at low speed (on top of a styrofoam block) on a magnetic stirrer for 12 h at room temperature. The extract was filtered through Whatman No. 1 paper on a Buchner funnel with suction, and the insoluble material was scraped off the filter paper and immediately resuspended (with stirring) in 100 ml of anhydrous ether containing 2 mM 2-mercaptoethanol. This suspension was centrifuged in a glass bottle at 2500 × g (4000 rpm) for 15 min. The supernatant was carefully decanted and placed in a small flask and then evaporated to 10 ml under a stream of N2 gas. The solution was transferred to a preweighed test tube, and the remaining ether was evaporated as above. A small amount of chloroform was added to the tube, and the lipid was dispersed as a film on the bottom and sides of the tube by rotating the tube under a stream of N2 gas. The lipid was lyophilized for 3 h to remove remaining solvent. The tube containing the lipid was weighed, and the lipid was suspended in 2 mM 2-mercaptoethanol at 50 mg/ml. The lipid was vortex-dispersed while under N2 gas, and the suspension was stored in 1-ml aliquots under N2 gas at -80°C. One-half of the starting material was recovered after acetone/ether washing.

Preparation of Liposomes for Reconstitution—Liposomes were prepared by bath sonication. 0.25 to 1.0 ml of acetone/ether-washed E. coli lipid (50 mg/ml), 50 mM potassium phosphate, pH 7.5, 20 mM lactose, 2 mM 2-mercaptoethanol in a Pyrex test tube (15 × 125 mm) under N2 gas for 15 min. The sonicator bath contained 0.02% Triton X-100.
Proteoliposomes into 0.45 ml of 50 mM potassium phosphate, 2 mM theoliposomes was assayed by diluting 7 µl lactose was eliminated from all reconstitution steps and the proteoliposomes (5 mg of lipid) plus 8.3 µl of 15% octylglucoside (final concentration, approximately 1.25%). The mixture was blended on a Vortex mixer, and then homogenized before the addition of 20 µg/ml of DNase and 0.5 mM phenylmethylsulfonyl fluoride. In some initial experiments, 0.5 mM phenanthroline (phenylmethylsulfonyl fluoride and phenanthroline are both protease inhibitors) was also added, but the presence or absence of this inhibitor did not affect the reconstitution.

Cells were disrupted by passage through an Ammuno French pressure cell (model 4-3388) at 19,000 psi. Total pressure and collected in a tube in an ice bath. Unbroken cells were removed by centrifugation at 11,700 x g (10,000 rpm) for 10 min. The supernatant was carefully removed and the centrifugation was repeated. The resulting supernatant was removed and the vesicles were sedimented by ultracentrifugation at 140,000 x g (45,000 rpm) for 2 h. The pellet was resuspended in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 20 mM lactose, 0.5 mM phenylmethylsulfonyl fluoride, and the centrifugation was repeated. The vesicles were resuspended in the same buffer and concentrated to 30 to 70 mg/ml. Vesicles were divided into 50-µl aliquots, frozen in liquid N2, and stored at ~80°C.

Reconstitution of Lactose Transport—Lactose transport was reconstituted by the octylglucoside dilution procedure of Racker et al. (22). Two hundred ninety microliters of 50 mM potassium phosphate, pH 7.5, 5 µl of 100% octylglucoside, 17 µl of 280 mM lactose, 7.2 µl of high pressure vesicles (0.5 mg of protein) and 40 µl of acetone/ether-washed E. coli lipid (from 50 mg/ml of stock) were added to a small test tube in an ice bath, and the tube was blended on a Vortex mixer. Octylglucoside (53 µl of a 15% solution in 50 mM potassium phosphate, pH 7.5) was added (final concentration, 1.25%) and the tube was blended on a Vortex mixer. The suspension was incubated at 4°C for 10 min, blended on a Vortex mixer again, and then incubated at 175,000 x g (45,000 rpm) (4°C) for 1 h. The supernatant (containing 0.2 to 0.3 mg of solubilized membrane protein) was carefully removed with a Pasteur pipette and mixed with 190 µl of bath sonicated liposomes (6 mg of lipid plus 8.3 µl of 15% octylglucoside (final concentration, approximately 1.25%). The mixture was blended on a Vortex mixer and then incubated at 4°C for 10 min. The suspension was then pipetted directly into 15 ml of 50 mM potassium phosphate, pH 7.5, 20 mM lactose, 1 mM dithiothreitol at room temperature, and the tube was blended on a Vortex mixer generator. The resulting proteoliposomes were sedimented by ultracentrifugation in a TY 42.1 rotor at 85,000 x g (35,000 rpm) for 1.5 h. The supernatant was decanted and the tube was wiped with a cotton-tipped applicator. The pellet was resuspended by stirring the pellet with a glass rod and squiring the suspension up and down three times with a 50-µl Hamilton syringe. For membrane potential-driven lactose transport, lactose was eliminated from all reconstitution steps and the proteoliposomes were resuspended in 50 µl of 50 mM sodium phosphate, pH 7.5, 1 mM dithiothreitol. Reconstituted proteoliposomes were stored at +4°C. Membrane potential-driven uptake was assayed immediately. Counterflow was assayed within 12 h.

Transport Assays—Reconstituted proteoliposomes were assayed for counterflow by diluting 9 µl (2.3 µg of protein) of lactose-loaded proteoliposomes into 0.45 ml of 50 mM potassium phosphate, 2 mM MgSO4, containing 0.9 µCi of [14C]lactose (counterflow assay buffer). The final lactose concentration was 0.43 mM. Transport was carried out at room temperature. The tube was blended on a Vortex mixer and, at various times, 0.1-ml samples were removed and dried down on the center of a 0.22-µm Millipore filter (type GSWF) using 25 inches of mercury vacuum suction. The filter was washed with 5 ml of ice-cold 50 mM potassium phosphate and counted in 4 ml of Bray's scintillation fluid at +4°C efficiency of 80%. A blank value, obtained by filtering 0.1 µl of assay buffer without proteoliposomes, was subtracted from all points.

Membrane potential-driven lactose transport in reconstituted proteoliposomes was assayed by diluting 7 µl of potassium phosphate-loaded proteoliposomes (0.4 µg of protein) into 0.7 ml of 50 mM sodium phosphate, pH 7.5, containing 1.4 µCi of [14C]lactose. The final lactose concentration was 0.23 mM. Transport was carried out at room temperature. The tube was blended on a Vortex mixer, and 0.1-ml samples were removed at 15-, 30-, and 45-s time points, filtered as above, washed with 5 ml of ice-cold 50 mM sodium phosphate, and counted. Thiodigalactoside (a competitive inhibitor of lactose transport) also exhibited very low transport activity. Ethylmaleimide-treated lactose-preloaded proteoliposomes (one-half of the accumulated lactose was retained after preloading) was added to give a final concentration of 14 µM, and the tube was blended on a Vortex mixer. At various times, 0.1-ml samples were removed, filtered, washed with 5 ml of ice-cold 50 mM sodium phosphate, and counted as above.

Density Gradient Centrifugation—Linear gradients were formed in Beckman ultracentrifuge tubes (No. 305500, cellulose nitrate) on top of a 0.2-ml 42% or 60% (w/w) sucrose cushion. The gradients were formed from equal volumes (2.1 ml each) of a low density solution of 700 mM glycerol, 50 mM Tris-HCl, pH 7.4, 20 mM lactose, 5 mM 2-mercaptoethanol and a high density solution of 700 mM sucrose, 50 mM Tris-HCl, pH 7.4, 20 mM lactose, 5 mM 2-mercaptoethanol.

Reconstituted proteoliposomes (lactose-preloaded), approximately 1.5 mg of lipid phosphate, 20 µg of protein in 20 µl, were suspended in 0.5 ml of low density solution and layered on one gradient (42% sucrose cushion). High pressure French press vesicles, approximately 0.6 mg of lipid phosphate, 1 mg of protein in 14.5 µl, were suspended in 0.5 ml of low density solution and layered on top of a second, similar gradient (60% sucrose cushion) The gradients were centrifuged for 2.5 h at 58,000 rpm in an SW 65 rotor (4°C).

Results and Discussion

When E. coli membrane vesicles were treated with 1.25% octylglucoside in the presence of exogenous acetone/ether-washed E. coli lipid, as described under "Experimental Procedures," 40 to 60% of the membrane protein was solubilized. This step was followed by a high speed centrifugation to remove all unextracted membrane material. The supernatant was then exposed to liposomes and diluted 30-fold into detergent-free buffer. In the present experiments, 15 to 20% of the solubilized protein was recovered in the reconstituted proteoliposomes centrifuged following the diution step. This represents 7.5 to 10% of the original protein in the French press vesicle material.

Liposomes reconstituted with solubilized membrane protein from E. coli ML308-225 exhibited lactose entrance counterflow (Fig. 1) when preloaded with nonradioactive lactose. The counterflow phenomenon is observed when radioactive substrate is transported into a cell or vesicle and accumulates because its exit is competitively inhibited by a high internal concentration of unlabeled substrate. The subsequent rate of loss of the accumulated radioactive substrate has been shown to be dependent on the number of carriers per cell (25). The time course for counterflow in the reconstituted proteoliposomes (one-half of the accumulated lactose was retained after 70 min) and the lipid to protein ratio used for reconstitution (approximately 20:1) are consistent with the hypothesis that each active proteoliposome contains only one or a few active lactose carriers.

Proteoliposomes which were not preloaded with lactose exhibited very low transport activity (Fig. 1, inset). Lactose-preloaded proteoliposomes assayed for counterflow in the presence of thiodigalactoside (a competitive inhibitor of lactose transport) also exhibited very low transport activity. N-Ethylmaleimide-treated lactose-preloaded proteoliposomes exhibited virtually no transport activity. The same result was obtained with proteoliposomes prepared with solubilized membrane protein from uninduced E. coli ML3 (lac y') French press vesicles.

Table I shows that the addition of exogenous E. coli lipid
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Fig. 1 (left). Time course of lactose counterflow in reconstituted proteoliposomes. Reconstitution was carried out and counterflow was assayed as described under "Experimental Procedures." O—O, 24 μl of lactose-preloaded proteoliposomes, prepared from an extract of ML308-225 French press vesicles, were diluted into 1.2 ml of counterflow assay buffer. Inset, data obtained under similar conditions as above (8 μl of proteoliposomes diluted into 0.45 ml of counterflow assay buffer). Lactose uptake is expressed as nanomoles/mg of protein. ▲—▲, the proteoliposomes were not preloaded with lactose; □—□, lactose-preloaded proteoliposomes were assayed in the presence of 10 mM thiodigalactoside; △—△, lactose-preloaded proteoliposomes were treated with 4 mM N-ethylmaleimide for 15 min at room temperature prior to dilution; ○—○, lactose-preloaded proteoliposomes were prepared from an extract of ML3 (lac y') French press vesicles.

Fig. 2 (center). Membrane potential-driven lactose transport in reconstituted proteoliposomes. Reconstitution was carried out and membrane potential-driven transport was assayed as described under "Experimental Procedures." O—O, proteoliposomes were diluted into 50 mM sodium phosphate, pH 7.5, containing 20 μM CCCP, and valinomycin (14 μM) was added at 60 s; ▲—▲, proteoliposomes were diluted into 50 mM sodium phosphate, pH 7.5, and valinomycin (14 μM) was added at 60 s; □—□, proteoliposomes were treated with 4 mM N-ethylmaleimide for 10 min at room temperature prior to dilution into 50 mM sodium phosphate, pH 7.5, and valinomycin (14 μM) was added at 60 s.

Fig. 3 (right). Isopycnic density gradient centrifugation of reconstituted proteoliposomes and French press vesicles. Gradients were prepared and assayed as described under "Experimental Procedures." Thirteen 0.37-ml fractions were collected from each gradient. A 25-μl aliquot from each of the French press vesicle gradient fractions was assayed for phosphate. In this gradient, the lipid phosphate peak was found in the first fraction (indicated by the arrow). Aliquots of 10 μl from each of the proteoliposome gradient fractions were assayed for phosphate (○—○). The phosphate peak observed at the top of the gradient corresponds to potassium phosphate buffer placed on the gradient with the proteoliposomes. Each fraction was diluted into 15 ml of 50 mM potassium phosphate, pH 7.5, 20 mM lactose, 1 mM dithiothreitol. Proteoliposomes were then sedimented by ultracentrifugation of the suspension at 85,000 × g (35,000 rpm) for 1.5 h. Fraction 7 was the only fraction which yielded a pellet upon centrifugation. This pellet was resuspended in 15 μl of the above buffer, and a 6-μl aliquot was assayed for counterflow at a 2-min time point (○—○). No lactose uptake was observed when a second 6-μl aliquot was assayed in the presence of 10 mM thiodigalactoside.

Table I

| Experiment | Transport | Uninhibited | Thiodigalactoside | nmol lactose/mg protein/15 min |
|------------|-----------|-------------|------------------|-----------------------------|
| 1. Complete system | 525 | 9 |
| 2. No exogenous lipid at solubilization step | 10 | 4 |
| 3. Asolectin | 81 | 3 |

at the membrane solubilization step was required for lactose transport reconstitution. In addition, when asolectin was substituted for acetone/ether-washed E. coli lipid in the reconstitution, only 15% of the E. coli lipid reconstitution activity was recovered.

Energy-depleted whole cells and French press vesicles accumulate lactose against a concentration gradient when a membrane potential, negative inside, is generated across the cytoplasmic or vesicle membrane (26). This can be accomplished by diluting potassium-containing cells or vesicles into low potassium medium and adding valinomycin, thus giving rise to a potassium diffusion potential. Membrane potential-driven lactose accumulation was demonstrated in the reconstituted proteoliposomes (Fig. 2). When potassium phosphate-loaded proteoliposomes were resuspended and diluted in sodium phosphate plus radioactive lactose, uptake of [14C]lactose was observed (approximately 10 nmol of lactose/mg of protein at 60 s). The addition of valinomycin (arrow, Fig. 2) resulted in a transient 5-fold accumulation of radioactive lactose. When a similar experiment was carried out in the presence of the proton conductor CCCP, the addition of valinomycin did not give rise to transient lactose accumulation. The CCCP would be expected to block membrane potential-driven lactose accumulation, since this ionophore collapses the protonmotive force generated by the membrane potential. Dilution of potassium phosphate-loaded proteoliposomes into sodium phosphate and addition of gramicidin instead of valinomycin also resulted in no lactose accumulation. When potassium phosphate-loaded proteoliposomes were diluted into potassium phosphate instead of sodium phosphate, the addition of valinomycin resulted in no lactose accumulation, since no potassium diffusion potential was produced. In the preceding three experiments, lactose presumably equilibrated between the inside and outside of the proteoliposomes containing active transport protein. This view is confirmed by the experiment (Fig. 2) in which proteoliposomes were pretreated with N-ethylmaleimide, which inactivated the carrier and almost completely prevented entry of sugar into the proteoliposomes.
The possibility was considered that the lactose transport activity was not solubilized, but that intact French press vesicles were retained during the reconstitution procedure and were responsible for the final transport activity. However, no activity was found when lactose-loaded high pressure French press vesicles were assayed for counterflow under the same conditions as the proteoliposome counterflow assay (data not shown). Lancaster and Hinkle (26) have shown that French press vesicles pass through 0.22-μm Millipore filters, unless the vesicles are aggregated with (poly)lysine prior to filtration. The absence of activity (by the counterflow assay) in the high pressure French press vesicle preparation used for reconstitution argues against the possibility that the reconstitution transport activity is due to contaminating French press vesicles.

A second possibility was that hybrid vesicles might be formed due to the introduction of exogenous lipid into detergent-disrupted French press vesicles or that hybrid vesicles were formed from large fragments of native membrane and exogenous lipid. This possibility was examined by density gradient centrifugation of the reconstituted vesicles and native French press vesicles in a manner similar to that utilized by Papazian et al. (27). Native membrane material, containing approximately equal amounts of protein and lipid, should sediment to a much denser region of the gradient than liposomes or proteoliposomes containing only a few protein molecules per vesicle. Two similar gradients were prepared. Onto the surface of one was placed reconstituted vesicles; the other gradient received high pressure French press vesicles. After centrifugation, 13 fractions were collected from each gradient. As shown in Fig. 3 (arrow), the peak of lipid phosphate in the French press vesicle gradient was found in the first fraction, which included the 60% sucrose cushion. This position in the gradient corresponds to a density of between 1.09 and 1.29 g/ml. Each fraction from the proteoliposome gradient was assayed for phosphate (closed circles) and then diluted and centrifuged at high speed to pellet any intact proteoliposomes. Only Fraction 7 contained a pellet following this procedure. This pellet was found to contain lactose transport activity (open circle). The position of lactose transport activity on the gradient closely parallels the position of the only lipid phosphate peak (artificial vesicles) and corresponds to a density of 1.046 g/ml, which is close to the theoretical value for pure phospholipid vesicles (1.03 g/ml). This experiment provides strong evidence that the lactose transport system has been solubilized and transferred into an artificial vesicle or proteoliposome.

A preliminary kinetic analysis of initial rates of lactose counterflow in the reconstituted proteoliposomes (data not shown) has yielded maximum velocities of 0.5 to 1.0 pmol of lactose/min/mg of protein. These values are 5 to 10 times higher than the maximum velocity (100 nmol/min/mg of protein) published for lactose counterflow in membrane vesicles (28). Considering that only 7.5 to 10% of the original French press vesicle protein was recovered in the reconstituted proteoliposomes, these results suggest that a partial purification of the lactose transport system may have been achieved.

One of the interesting features of these experiments is the finding that the addition of acetone/ether-washed E. coli lipid at the solubilization step and the use of this material in the detergent dilution procedure significantly improves the activity of the reconstituted proteoliposomes. A requirement for exogenous phospholipid at the time of solubilization has also been demonstrated by Maron et al. (29) for the reconstitution of a catecholamine transporter from bovine chromaffin granules.

The present reconstitution method should be useful as an assay for the purification of an active lactose transport system and make possible the study of several interesting aspects of the molecular mechanism of lactose carrier function.

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