Solubilization and Reconstitution of the Melibiose Carrier from a Plasmid-carrying Strain of Escherichia coli

Tomofusa Tsuchiya, Kathleen Ottina, Yoshinori Moriyama, Michael J. Newman, and T. Hastings Wilson

From the 1Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Okayama 700, Japan and the 2Department of Physiology and Biophysics and the 3Program on Cell and Developmental Biology, Harvard Medical School, Boston, Massachusetts 02115

A strain of Escherichia coli was constructed containing a plasmid from the Clarke-Carbon collection that showed high levels of melibiose transport activity. Membranes from the plasmid-containing strain were extracted with octyl-$\beta$-D-glucopyranoside and melibiose transport was reconstituted in liposomes. The proteoliposomes exhibited counterflow activity, as well as membrane potential and sodium gradient-driven melibiose accumulation.

The melibiose transport system of Escherichia coli is unusual among cation-substrate cotransport carriers in its recognition of three different cations (H$, Na$, and Li$)$ and that the cation specificity depends upon the particular sugar transported (1). While considerable kinetic data has been obtained with whole cells (1-8), little kinetic data has been obtained with isolated systems. For example, the cation specificity of glucose transport in E. coli is not known. The isolation of strains containing plasmids carrying the melibiose genes was carried out as follows: each of the coli in E. plasmid-containing cells from the Clarke-Carbon collection (11) was mated with RAII (mel A $B'$) on minimal plates containing 0.2% melibiose and streptomycin. Clones which appeared on the plates after 48 h were purified and characterized.

Preparation of Acteone/Ether-washed E. coli Phospholipid—Acteone/ether-washed E. coli phospholipid was prepared as described (10).

Preparation of Membranes—Cells (RAII/pLC25-33) (3 liters) were grown to late logarithmic phase in medium 63 (12) (without iron) containing 1% tryptone (Difco) plus 10 mM melibiose and 37 °C. After harvesting, the cells were washed once in medium 63. The cells were suspended at a density of about 1 ga wet weight/5 ml in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 5 mM MgSO$_4$, and 20 mg/ml of DNase. The cells were disrupted by a single passage through an Amino French pressure cell (model 4-3398) at 19,000 p.s.i. and collected in a tube in an ice bath. Centrifugation was carried out at 12,000 $x$ g for 10 min to remove intact cells. The supernatant fluid was then centrifuged at 140,000 $x$ g for 45 min. The pellet was resuspended in 2 ml of 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol, using a 2-ml syringe with a No. 21 needle and the centrifugation was repeated. The pellet was then resuspended at a concentration of about 10 mg of membrane protein/ml in equal volumes of the same buffer (potassium phosphate and dithiothreitol) and 50% glycerol. Membranes were divided into 60-$\mu$al aliquots, frozen in liquid N$_2$, and stored at $-80^\circ$C.

Solubilization and Reconstitution—The technique of Newman and Wilson (10) was followed which is based on the octylglucoside$^1$ dilution procedure developed by Racker et al. (13). In a typical experiment 290 $\mu$l of 40 mM potassium phosphate, 10 mM sodium phosphate, pH 7.5, or 290 $\mu$l of 50 mM potassium phosphate, pH 7.5, were added to a small test tube in an ice bath. To this were added 50 $\mu$l of French press vesicles (10 mg/ml), 5 $\mu$l of dithiothreitol (100 mM), 30 $\mu$l of washed E. coli lipid (50 mg/ml), and 7 $\mu$l of melibiose (1 M). Melibiose was added at this step when sugar preloading was not desired. After blending on a Vortex mixer for 5 s, 34.7 $\mu$l of 15% octylglucoside ($w/v$) in 50 mM potassium phosphate, pH 7.5, were added and the tube was blended again. The suspension was incubated in the ice bath for 10 min and then centrifuged at 140,000 $x$ g for 1 h. The supernatant fluid was carefully removed and 220 $\mu$l were mixed with 66 $\mu$l of bath-solubilized liposomes containing 20 mM melibiose. Melibiose was omitted from this step when sugar preloading was not desired. The bath-solubilized liposomes were prepared as previously described (10), except that lactose was omitted. Octylglucoside (15% in 50 mM potassium phosphate, pH 7.5) was added to bring the final octylglucoside concentration to 1.25%. The suspension was blended on a Vortex mixer and then incubated at 4 °C for 15 min. The suspension was then pipetted into 10 ml of the appropriate phosphate buffer, pH 7.5.

1The trivial name used is: octylglucoside, octyl-$\beta$-D-glucopyranoside.
containing sodium or potassium according to the experiment. The resulting proteoliposomes were centrifuged in a type 42.1 (Beckman) rotor at 100,000 \( \times \) g for 90 min.

Counterflow Assay—The proteoliposome pellet was resuspended with a glass rod after the addition of 50 \( \mu \)l of the appropriate buffer (usually 50 mM potassium phosphate, pH 7.5, or 40 mM potassium phosphate plus 10 mM sodium phosphate, pH 7.5) containing 1 mM dithiothreitol. Resuspension was carried out in an ice bath to prevent leakage of the preloaded substances. Sixteen \( \mu \)l of reconstituted proteoliposomes were added to a solution containing 800 \( \mu \)l of the appropriate phosphate buffer, pH 7.5, plus 40 \( \mu \)M of \( ['H] \) melibiose (20 \( \mu Ci/\mu l \)). Samples (100 \( \mu l \)) were removed at intervals and filtered onto the center of a 0.22 \( \mu l \) Millipore filter (type GSTF). The filter was then washed with 5 ml of ice-cold buffer (of the same ionic composition as the incubation medium) and counted as described (10) at a \(^{3}H\) efficiency of 32%.

Membrane Potential Assay—The proteoliposome pellet was resuspended with a glass rod after the addition of 50 \( \mu \)l of 50 mM potassium phosphate, pH 7.5, plus 1 mM dithiothreitol. Sixteen \( \mu \)l of reconstituted proteoliposomes were added to a solution containing 800 \( \mu \)l of the appropriate phosphate buffer, pH 7.5, plus 40 \( \mu \)M of \( ['H] \) melibiose (80 \( \mu Ci/\mu l \)). Valinomycin (2.5 \( \mu M \)) and 10 \( \mu M \) p-nitrophenyl-\( \alpha \)-D-galactopyranoside were included where indicated in the legend to Fig. 3. Samples (100 \( \mu l \)) were removed at intervals, filtered, washed, and counted as above.

Protein Determination—Protein was assayed by the method of Schaffner and Weissmann (16), which was modified as described (17).

### RESULTS AND DISCUSSION

#### Isolation of Plasmid-containing Strains—The first experiments involved the construction of plasmid-containing strains which produced elevated levels of the melibiose transport protein. All of the Clarke-Carbon (11) plasmid-containing strains were mated to RA11 (mel A' B') and three melibiose-positive clones were obtained. Two of the plasmids (pLC 25-33 and pLC 17-38) contained both mel A' and mel B' genes while one (pLC 16-37) possessed only the mel A' gene (Table I). The presence of the mel B gene in two of the plasmids was verified by high levels of transport in strains RA11/pLC 25-33 (Fig. 1) and RA11/pLC 17-38 (data not shown). The kinetic parameters for thiomethylgalactoside transport in JA200/pLC 25-33 were compared with another strain (JA200/pLC 32-4) taken at random from the Clarke-Carbon collection. The plasmid in the latter strain did not contain the melibiose genes although the chromosome possessed the normal mel A' B' genes. Induced cells of both strains gave a \( K_m \) value of 0.2 mM for thiomethylgalactoside transport in the presence of 10 mM NaCl. The \( V_{max} \) for JA200/pLC 32-4 was 20 nmol/min/mg of cell protein while that for JA200/pLC 25-33 was 160 nmol/min/mg of cell protein. This suggests a high level of expression of the mel B gene product in the cell containing the pLC 25-33 plasmid.

An elevated level of transport activity was also observed with RA11 carrying the plasmid pLC 25-33. Fig. 1 shows that the presence of the plasmid elevates the initial rate (15 s time point) of transport approximately 7-fold compared with RA11 alone. RE16 (mel A' B') carrying the plasmid also showed high levels of transport.

Solubilization and Reconstitution—The transport protein was extracted from membranes of RA11/pLC 25-33 with octylglucoside in the presence of added E. coli phospholipid and reconstituted according to the procedure of Newman and Wilson (10). After removal of the residual membranes by centrifugation, an aliquot of the extract was incubated with bath-sensitized liposomes made from E. coli lipid and then diluted into detergent-free medium. When this type of suspension is diluted in this manner, liposomes form and membrane proteins become incorporated in the phospholipid bilayer (13). The proteoliposomes formed during the dilution procedure were centrifuged and then resuspended in a small volume. Aliquots were taken for \( ['H] \) melibiose uptake experiments.

Fig. 2 shows \( ['H] \) melibiose counterflow by proteoliposomes prepared under various conditions. When proteoliposomes preloaded with 30 \( \mu M \) melibiose and 10 mM Na' were diluted into medium containing 10 mM Na' and \( ['H] \) melibiose (50 \( \mu M \)), the radioactive sugar accumulated in the proteoliposomes reaching a peak level at 20 min and then declining. The presence of the competitive inhibitor p-nitrophenyl-\( \alpha \)-galactopyranoside completely blocked melibiose entry. When proteoliposomes were prepared without preloading with 20 mM melibiose, a low level of uptake was observed. Based on previous experience with the counterflow phenomenon (14) it is presumed that proteoliposomes containing transport protein molecules but not preloaded with melibiose take up external \( ['H] \) melibiose until internal and external concentrations of the sugar equilibrate. When proteoliposomes preloaded with 20
Reconstitution of the Melibiose Carrier

Fig. 2. Melibiose counterflow by reconstituted proteoliposomes. Reconstitution was carried out as described under "Experimental Procedures." The internal buffer composition of the proteoliposomes was altered by diluting the octylglucoside extract into media containing the desired components. The proteoliposomes were centrifuged and resuspended in 50 μl of melibiose-free media with an ionic composition similar to that in which they had been prepared. The experiment was carried out as follows: ○ - ○, proteoliposomes containing 40 mM potassium phosphate, 10 mM sodium phosphate, and 20 mM melibiose were diluted into 40 mM potassium phosphate, 10 mM sodium phosphate, and 50 μM [3H]melibiose; △ - △, proteoliposomes containing 50 mM potassium phosphate and 20 mM melibiose were diluted into 50 mM potassium phosphate and 50 μM [3H]melibiose; ■ - ■, proteoliposomes containing 40 mM potassium phosphate and 10 mM sodium phosphate were diluted into 40 mM potassium phosphate, 10 mM sodium phosphate, and 50 μM [3H]melibiose; ○ - ○, proteoliposomes containing 40 mM potassium phosphate, 10 mM sodium phosphate, and 20 mM melibiose were diluted into 40 mM potassium phosphate, 10 mM sodium phosphate, and 50 μM [3H]melibiose. In all cases the pH was 7.3.

mm melibiose in the absence of Na+ were diluted into Na+-free buffer containing [3H]melibiose, a small transient accumulation of radioactivity was observed. The intravesicular concentration of radioactive sugar then fell to a level corresponding to that observed with proteoliposomes not preloaded with melibiose. The peak accumulation at 1 min was 4 times higher than the nonmelibiose-preloaded curve, but was much lower than the transport observed in the presence of Na+. This observation is expected for the melibiose transport system which is the only sugar carrier in E. coli known to be Na+-dependent. The Na+ concentration (as a contaminant) in the buffer used in this experiment was determined by atomic absorption spectrophotometry and found to be approximately 50 μM. This concentration is far below the K_m of Na+ (0.3 mM) for melibiose transport. Since melibiose-H+ cotransport is known to occur in whole cells (3), presumably the counterflow observed with the reconstituted system in the absence of added Na+ is due to this phenomenon.

Preliminary experiments were carried out to determine the kinetic parameters of the counterflow transport. Proteoliposomes were preloaded with 20 mM melibiose and 10 mM Na+, and diluted into 10 mM Na+. The external concentration of [3H]melibiose that produced one half-maximal transport (K_m) was 0.4 mM, which is similar to the K_m for melibiose accumulation (0.2 mM) in intact cells (6). The V_max was 110 nmol/min/mg of protein.

In another series of experiments attempts were made to drive melibiose accumulation with ion gradients. When proteoliposomes were preloaded with potassium phosphate and diluted into a solution containing sodium phosphate plus

Fig. 3. Effect of membrane potential and Na+ gradient on melibiose transport by reconstituted proteoliposomes. Reconstitution was carried out as described under "Experimental Procedures." In each case proteoliposomes containing 50 mM potassium phosphate were diluted 50-fold into media containing 50 mM [3H]melibiose plus the following: ○ - ○, 50 mM sodium phosphate and 2.5 μM valinomycin; △ - △, 50 mM tetraethylammonium phosphate and 2.5 μM valinomycin; ○ - ○, 50 mM sodium phosphate; x - x, 50 mM potassium phosphate and 2.5 μM valinomycin; ○ - ○, 50 mM sodium phosphate, 2.5 μM valinomycin, and 10 mM p-nitrophyenol-α-D-galactopyranoside. In all cases the pH was 7.5.

valinomycin, a membrane potential (inside negative) and an inwardly directed Na+ gradient were established. Under these conditions a marked accumulation of [3H]melibiose was observed with a peak at 5 min (Fig. 3). In a second experiment only a ΔΨ (inside negative) was imposed by diluting K+ containing proteoliposomes into tetraethylammonium phosphate plus valinomycin. A transient accumulation was again observed, although the initial rate of uptake was less than that of the previous experiment. Since Na+ was not added in this experiment, it is assumed that H+-melibiose cotransport was taking place. In the third experiment an inwardly directed chemical gradient for Na+ (in the absence of a membrane potential) was established by diluting proteoliposomes into sodium phosphate in the absence of valinomycin. Accumulation of radioactive sugar was again observed. Following this accumulation a fall in vesicular sugar concentration was observed. Very little transport was observed when ΔΨ and Na+ gradient transport was carried out in the presence of p-nitrophenyl-α-D-galactopyranoside.

The reconstitution was carried out with a high lipid to protein ratio and it is therefore likely that there is only one or very few transport carriers per proteoliposome (10, 18). This fact plus the ease of altering the composition of fluids on the two sides of the membrane make this preparation useful for the study of various aspects of cation-sugar cotransport. Furthermore, the reconstitution technique may be used as an assay for the melibiose carrier during purification after solubilization. Purification is essential for further studies of the subunit composition and other biochemical properties of the carrier.

REFERENCES


Reconstitution of the Melibiose Carrier

Solubilization and reconstitution of the melibiose carrier from a plasmid-carrying strain of Escherichia coli.
T Tsuchiya, K Ottina, Y Moriyama, M J Newman and T H Wilson


Access the most updated version of this article at [http://www.jbc.org/content/257/9/5125](http://www.jbc.org/content/257/9/5125)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/257/9/5125.full.html#ref-list-1](http://www.jbc.org/content/257/9/5125.full.html#ref-list-1)