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RECONSTITUTION OF THE GalP GALACTOSE TRANSPORT ACTIVITY OF *ESCHERICHIA COLI* INTO LIPOSOMES MADE FROM SOYBEAN PHOSPHOLIPIDS

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Galactose transport activity from *Escherichia coli* was solubilized with octyl glucoside, and reconstituted into liposomes made from soybean or *E. coli* lipid. Galactose counterflow in the proteoliposomes was inhibited by glucose, talose, 2-deoxygalactose and 6-deoxygalactose, confirming that it was due to GalP and not one of the other *E. coli* galactose transport systems.

Introduction

Galactose is transported into *Escherichia coli* by at least seven routes [1]. Only two of these, designated GalP and MglP [2], are induced by galactose or 6-deoxy-D-galactose [3], and they differ in several respects. The GalP system is energized by a proton symport mechanism, while MglP is probably energized by a phosphorylated compound [4,5]; 2-deoxygalactose and talose are substrates for GalP and not for MglP [6,7], whereas methyl β-D-galactoside is a substrate for MglP and not GalP [3,4]; the GalP system probably contains one protein component of apparent M_r about 37000 [8], but the MglP system probably contains three components [9,10] one of which is a periplasmic binding protein [9,11,12]; the gene(s) for the GalP system map at 62.5 min [13], whereas the genes for the MglP system map at 45 min [2], remote from each other and the galactose operon at 17 min [2]. Thus, galactose transport into *E. coli* is much more complex than the relatively well-characterized lactose transport system LacY [14–18]. This report shows that the GalP function of the galactose transport systems can be solubilized and reconstituted into liposomes.

Newman and Wilson [17] succeeded in solubilizing LacY activity in octyl glucoside and reconstituting it into liposomes made from *E. coli* lipid (see also Refs. 18 and 19). Critical features of the procedure appeared to be inclusion of *E. coli* lipid in the solubilization step, and the employment of a narrow range of octyl glucoside concentration around 1.25%. Subsequently, the melibiose carrier of *E. coli* was reconstituted by the same procedure [20], which appears to be applicable to a number of *E. coli* transport systems [21].

Experimental procedures

Organism. *E. coli* strain JM1424 (*galP*$^+$ *mglP his val ileu*) was grown on 20 mM glycerol and salts [4] with the appropriate amino acids and 1 mM D-fucose, conditions which induce high GalP activity about 3-times that of wild-type organisms [8]. *E. coli* strain ML308-225 (*laci, lacz*) was grown in glycerol and salts with 0.3% nutrient broth (Oxoid, London, U.K.).

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Abbreviations: GalP and MglP, galactose transport pathways in *E. coli* designated according to Ref. 2.

Materials. Crude *E. coli* phospholipid was prepared [22] and purified by the procedure of Newman and Wilson [17]. Asolectin was purchased from Associated Concentrates (Woodside, NY, U.S.A.), purified [23], and stored as a 50 mg/ml solution at −80°C. Galactose (glucose-free grade), 6-deoxygalactose, 2-deoxygalactose, glucose, arabinose, methyl β-D-galactoside, methyl β-D-thiogalactoside, dithiothreitol and *N*-ethylmaleimide were from Sigma (Poole, Dorset, U.K.). Octyl β-D-glucopyranoside from Calbiochem (St. Louis, MO, U.S.A.) was dissolved at 13.6% in 50 mM potassium phosphate, pH 7.5, and stored in 0.2-ml samples at −20°C. Unlabelled talose was from Koch-Light (Colnbrook, Bucks., U.K.) and [1-^{14}C]talose was from CEA (Gif-sur-Yvette, France); it was adjusted to a specific activity of 1 Ci/mol (37 GBq/mol) in a 2.5 mM stock solution. [1-^{3}H]Galactose from The Radiochemical Centre (Amersham, Bucks., U.K.) was adjusted to 2 Ci/mmol (74 GBq/mmol) in a 50 μM solution.

Preparation of subcellular membrane vesicles. Spheroplasts [24] of strain JM1424 or ML308-225 were made into vesicles by the method of Kaback [25] and stored at −80°C. The GalP activity of the vesicles was checked by measuring the transport of [^{14}C]talose (40 μM) energized by ascorbate + tetramethylphenylenediamine.

Preparation of liposomes. A Branson sonifier 200 fitted with a microprobe was used instead of a bath-type sonicator [17,20]. The smallest practicable volume was about 0.15 ml, and a typical procedure was as follows. 140 μl of 50 mg/ml lipid (either purified asolectin or *E. coli* lipid), 7 μl M potassium phosphate, pH 7.5, 3.5 μl M galactose and 25 μl 50 mM potassium phosphate, pH 7.5, in a small test tube (0.8 cm i.d. × 4.5 cm) at 0°C were sonicated for 3 min using 50% duty cycle pulses at a power setting of 2. After a 1 min pause the pulsed sonication was repeated for 3 min, which was usually sufficient to clarify the solution, although a further 3 min were sometimes necessary with *E. coli* lipid. A stream of argon was directed over the mixture throughout.

Solubilization and reconstitution of galactose transport activity. The method was very similar to that described for the lactose and melibiose transport systems [17,20], although for some experiments it was practicable to reduce the volumes of the components to the following. Membrane vesicles (0.25 mg protein in 34 μl), 15 μl 50 mg/ml lipid, 112.5 μl 50 mM potassium phosphate (pH 7.5), 3.7 μl 1 M galactose were thoroughly mixed, and 16.5 μl of 13.6% octyl glucoside were added on a vortex mixer. After 15 min the mixture was sedimented at 100000 × *g* for 1 h. Liposomes (about 1.3 mg lipid in 33 μl) were treated with 3.25 μl of 13.6% octyl glucoside and then mixed with 110 μl of the supernatant removed from the sedimentation above, taking care not to disturb the pellet. After 15 min the mixture was diluted with 5 ml of 50 mM potassium phosphate (pH 7.5), 20 mM galactose, 1 mM dithiothreitol, and then centrifuged at 100000 × *g* for 1 h. The centrifuge tube was carefully drained and residual fluid removed from the walls of the tube with tissue. The pellet of proteoliposomes was resuspended in 25 μl of 50 mM potassium phosphate (pH 7.5), 20 mM galactose, 1 mM dithiothreitol using a small glass rod and two passes into a 50 μl microsyringe. All steps were carried out at 0–4°C. The diameters of the proteoliposomes were 20–70 nm when viewed by negative staining in the electron microscope.

Transport assay. The method followed that of Newman and Wilson [17], which was based on the entrance counterflow technique described by Wong and Wilson [26]. The volumes given below were convenient for assays with two to three timed samples, and the procedure was scaled up correspondingly for experiments in which more samples were required. At zero time proteoliposomes (0.6–1.5 μg protein in 5 μl) were diluted into a mixture of 0.235 ml potassium phosphate (pH 7.5), 1 mM dithiothreitol with 0.01 ml [^{3}H]galactose (1 μCi); at 15 s a 0.1 ml sample was filtered (Millipore GSW filters, pore size 0.22 μm, prewashed in the medium below) and washed with about 2 × 2 ml ice-cold 50 mM potassium phosphate, 1 mM dithiothreitol. A second sample was taken at 2 min. The filtration plus washing procedure took about 45 s. The radioactivity appearing in the proteoliposomes retained on the filter was determined by liquid scintillation counting in Bray's fluid (New England Nuclear, Boston, U.S.A.). The assays were carried out at 15°C unless indicated otherwise.

Protein assay. The method of Schaffner and Weissmann [27] was used.

Results and Discussion

Fig. 1 shows the accumulation of labelled galactose in proteoliposomes formed from an asolectin/octyl glucoside extract of fucose-induced JM1424 vesicles reconstituted with asolectin liposomes. The rapid accumulation was followed by slow efflux of label as expected of the entrance counterflow type of kinetics for transport proteins [26]. At 15°C the peak of accumulation occurred at 5–6 min (Fig. 1), and at 25°C it was at 1–2 min (Fig. 1 inset). The time course was very similar when *E. coli* lipid was used instead of asolectin (not shown), and was faster than the 15–20 min peak found at 25°C with reconstituted Lac permease derived from the constitutive strain ML308-225 [17]. This may reflect a difference in the number/activity of the transport proteins in the two strains [26], or a greater stability of the galactose transport protein during the extraction/reconstitution procedure. The assays were routinely performed at 15°C to obtain a more accurate measurement of the initial rate. If the protein was omitted from the reconstitution procedure the uptake of label was reduced by 85–90%.

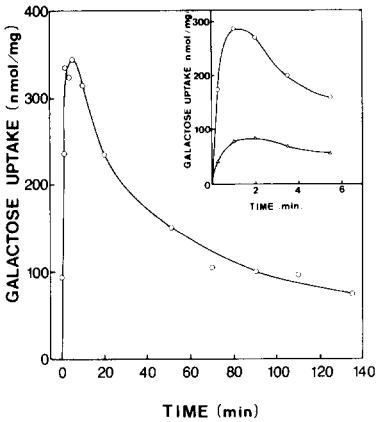

Fig. 1. Galactose counterflow in proteoliposomes made from asolectin. Subcellular vesicles from *E. coli* JM1424 were treated with 0.4% asolectin and 1.25% octyl glucoside, and the solubilized extract was reconstituted with liposomes made from asolectin as described in Experimental procedures except that all amounts were increased 6-fold. The proteoliposomes in 50 mM potassium phosphate (pH 7.5), 20 mM galactose, 1 mM dithiothreitol (25 μl containing 5.9 μg protein) were diluted into 1.34 ml 50 mM potassium phosphate containing 2 nmol [^{3}H]galactose at 15°C; at the indicated times 0.1-ml samples were filtered and washed. Inset, a separate batch of proteoliposomes was prepared in the same way but on half the scale; two samples (10 μl containing 2 μg protein) were each mixed with 2.5 μl of 50 mM potassium phosphate (pH 7.5), 2 mM galactose, 1 mM dithiothreitol, one of which included 20 mM *N*-ethylmaleimide (△) (final concentration 4 mM), at 25°C for 20 min. Galactose counterflow was then measured on 10-μl samples of the treated (△) or untreated (○) proteoliposomes diluted into 0.2 ml 50 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol containing 1 nmol of [^{3}H]galactose at 25°C.

Preincubation of the proteoliposomes with 3–4 mM *N*-ethylmaleimide at 25°C for 20–30 min before the assay reduced the activity by 70–75% (see Fig. 1 inset). This was consistent with the transport activity being due to a protein containing a reactive -SH group, which is the case for the GalP system [8]. The failure to achieve a higher degree of inhibition was partly due to the nonprotein-mediated galactose uptake (above), but may also reflect protection of the protein against *N*-ethylmaleimide by the 20 mM galactose [8].

Since galactose can be a substrate for several different *E. coli* transport systems, as described above, it was important to determine which had been reconstituted. GalP has a substrate specificity unique amongst the known *E. coli* sugar transport systems [3,4,6,7], so the specificity of the reconstituted galactose transport was determined by measuring the efficacy of various sugars or derivatives as inhibitors of the entrance of labelled galactose into the proteoliposomes. Fig. 2 shows one example where glucose, talose and 6-deoxygalactose inhibited labelled galactose uptake, whereas arabinose and methyl β-D-galactoside, which are not substrates of GalP [3,4,6,7], did not. The same type of experiment was performed with just two time points, 15 s and 2 min, on many batches of proteoliposomes made from either asolectin or *E. coli* lipid. The averaged descending order of inhibitor effectiveness was glucose, galactose, talose, 2-deoxygalactose, 6-deoxygalactose, while methyl β-D-thiogalactoside, methyl β-D-galactoside and arabinose were ineffective. This selectivity was sufficient to identify GalP activity unambiguously [3,6,7]. The identification was substantiated by the observation that labelled talose exhibited entrance counterflow kinetics using proteoliposomes preloaded with 20 mM unlabelled talose instead of galactose (data not shown); talose is a substrate for GalP and not for other *E. coli* sugar transport systems [7].

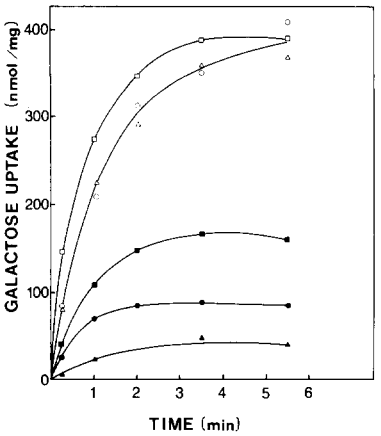

Fig. 2. Inhibition of galactose counterflow by sugars. Protein from subcellular vesicles of *E. coli* JM1424 was solubilized in *E. coli* lipid and octyl glucoside and reconstituted with asolectin liposomes as described in Experimental procedures, but all amounts were increased by four times. Entrance counterflow of galactose was measured as described for the inset to Fig. 1, except that the temperature was 15°C and the indicated sugars (20 mM) were present from the dilution step (zero time). □, methyl β-D-galactoside; ○, no additions; △, L-arabinose; ■, D-fucose; ●, D-talose; ▲, D-glucose.

TABLE I

EFFECT OF LIPID ON RECONSTITUTION OF GALACTOSE TRANSPORT ACTIVITY

Three samples of membrane vesicles were extracted with octyl glucoside as described in Experimental procedures, except that either *E. coli* lipid, asolectin, or an equivalent volume of water was present as indicated. After centrifugation 60 μl of each extract were reconstituted with 36.25 μl of liposomes made from *E. coli* lipid or asolectin and pretreated with 1.25% octyl glucoside. The six samples were diluted, centrifuged, and the pellets resuspended in an identical manner. Galactose transport into 5 μl of each type of proteoliposome was measured in duplicate as described in Experimental procedures.

Lipid for solubilization	Lipid for reconstitution	Protein recovered (μg per 5 μl proteo-liposomes)	Transport activity (nmol/mg per 2 min)
None	*E. coli*	1.28	6.2
None	asolectin	0.80	27.2
E. coli	*E. coli*	0.65	232.3
E. coli	asolectin	1.68	133.5
Asolectin	*E. coli*	1.55	95.5
Asolectin	asolectin	1.06	230.1

In early experiments *E. coli* lipid was used for both the extraction of GalP activity and the manufacture of liposomes, as recommended for the reconstitution of the lactose carrier, LacY [17]. However, it is more convenient to use the commercially available soybean phospholipid, asolectin, so a comparison of the two types of lipid was made. The results of one experiment are shown in Table I. The presence of either lipid was essential during the extraction of GalP activity with octyl glucoside; this was also the case for the extraction of LacY activity [17]. The specific activity was similar when either *E. coli* lipid or asolectin was used at both extraction and reconstitution stages. However, if one lipid was used for extraction and the other for making liposomes, there was a reduction in specific activity associated with increased recovery of protein (Table I). Thus, asolectin was a satisfactory substitute for *E. coli* lipid provided it was used for both extraction and liposome formation. The experiment of Table I was repeated using samples of the same lipid solutions but an octyl glucoside extract of membrane vesicles from *E. coli* ML308-225, which is constitutive for the LacY carrier. LacY activity was reduced by 70–100% when asolectin was used instead of *E. coli* lipid in the octyl glucoside extraction step, confirming the report of Newman and Wilson [17]. Thus, the different lipid effects seem to reflect some inherent difference between GalP and LacY rather than, for example, a difference in the purity or composition of the asolectin samples. *E. coli* lipid differs from asolectin considerably, both in the proportions of different phospholipids [28,29] and in their fatty acid substituents [30,31]; for example, *E. coli* lipid contains phosphatidylglycerol whereas asolectin contains phosphatidylcholine. An explanation for the different lipid requirements of GalP and LacY will need further experiments using purified lipids singly or in mixtures.

The necessity for both lipid and octyl glucoside during extraction showed that activity was not due to carry-over of subcellular vesicles into the proteoliposome preparation.

The concentration of octyl glucoside used for the solubilization can be critical for successful reconstitution [19]. In the case of the galactose transport activity described here a very narrow

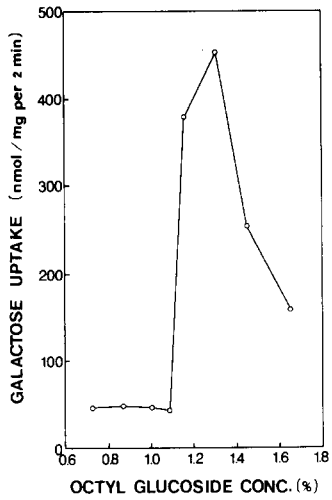

Fig. 3. Dependence of reconstituted activity on the concentration of octyl glucoside used in the solubilization step. Samples of subcellular vesicles from *E. coli* JM1424 were extracted with 0.4% asolectin as described in Experimental procedures except that the octyl glucoside concentration was varied from 0% to 1.65%. The subsequent reconstitution procedure with asolectin liposomes was not changed. Entrance counterflow was measured on 5 μl of each containing 0.23–1.50 μg protein.

range of 1.1–1.3% was optimal for preservation of activity (Fig. 3); this range was very similar to that necessary to reconstitute the *E. coli* lactose and melibiose transport activities [17,20]. For all three systems a concentration of 1.25% was routinely employed.

The GalP transport system operates by a sugar-H^+ symport (or sugar-OH^- antiport) mechanism [4], and, consistently, galactose transport into the proteoliposomes was driven by valinomycin-promoted K^+ efflux (data not shown). However, we could achieve only about 1.5-fold accumulation of galactose using liposomes made from either *E. coli* lipid or asolectin, as opposed to the 4–5-fold accumulation of lactose under the same experimental conditions [17]. The reasons for this relative inefficiency are being investigated.

The reconstitution assay described here should be the basis for purifying undenatured GalP protein(s). Purification is important because kinetic and mechanistic studies of GalP activity must be free from contamination by the other *E. coli* galactose transport systems mentioned in the Introduction. Identification of GalP during isolation will also be facilitated by labelling with *N*-ethylmaleimide and by the knowledge that the apparent monomer M_r of GalP on sodium dodecyl sulphate-polyacrylamide gel electrophoresis is about 37000 [8].

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