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Permeability Properties of *Escherichia coli* Outer Membrane Containing, Pore-Forming Proteins: Comparison Between Lambda Receptor Protein and Porin for Saccharide Permeation

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Outer membrane permeability conferred by λ receptor protein and porins to maltose-maltodextrins and other oligosaccharides was studied in vitro with reconstituted vesicle membranes and in vivo with mutant strains lacking either one of these proteins. The vesicle membranes reconstituted from phospholipids, lipopolysaccharide, and purified λ receptor allowed rapid diffusion of maltose and maltose-maltodextrins of up to six glucose residues, but the membranes acted essentially as a molecular sieve for sucrose, raffinose, stachyose, and inulins of molecular weights 800, 920, and 1,380. The vesicle membranes containing porins allowed rapid diffusion of maltose but not of maltose-maltodextrins larger than maltose. The apparent transport K_m values for maltose-maltodextrins of up to six glucose residues from the strain carrying *lamB*$^+$ *ompB* (λ receptor$^+$, porin$^-$) were similar (about 5×10^{-6} M), whereas the transport K_m values for maltose- and maltotriose of the strain carrying *lamB ompB*$^+$ (λ receptor$^-$, porin$^+$) alleles appeared to be 300 and about $20{,}000 \times 10^{-6}$ M. These results suggest that λ receptor protein forms permeability pores that facilitate the diffusion of maltose-maltodextrins and function as a molecular sieve for other saccharides.

Small hydrophilic molecules cross the outer membrane of gram-negative bacteria by passive diffusion through water-filled permeability pores (6, 8, 9). Our recent studies revealed that the channels or pores in the outer membranes of *Escherichia coli* and *Salmonella typhimurium* function as molecular sieves for uncharged molecules of up to about 600 daltons (8, 9, 11). These pores are formed by the abundant outer membrane proteins, "porins," of about 35,000 daltons (8, 9).

Besides these general permeability pores, the outer membrane of *E. coli* harbors several specific transport proteins facilitating diffusion across the outer membrane of Fe^{3+}-chelator complexes, maltose-maltodextrins, nucleosides, and vitamin B_{12} (see reference 2 for a review). The receptor protein for phage λ is the product of the *lamB* gene located near the *malG-malF-malE* and *malK-lamB* clusters of the maltose operon under the positive control of *malT* (5, 17). The λ receptor protein was shown to increase the outer membrane permeability to Glc_2 and Glc_3 (13, 14) to a certain extent, to lactose and glucose (16), and to ions (1). (Maltose oligosaccharides are expressed as polymers of glucose such as Glc_n.) More recently, it was shown that wild-type *E. coli* K-12 can grow on maltodextrins of up to Glc_7 (17). We have reported (10) that purified λ receptor protein forms permeability pores in reconstituted vesicle membranes that allow diffusion of low-molecular-weight saccharides and certain amino acids. Raffinose (molecular weight, 504) and some amino acids are partially diffusible, but stachyose (molecular weight, 666) is not. Thus, we suggested that pores made of λ receptor protein act as a molecular sieve for saccharides and hold an unknown exclusion mechanism(s) for certain solutes (10).

It is not known, however, whether the λ receptor allows diffusion of maltose-maltodextrins of high molecular weights and, if so, what is the difference between the diffusion of maltose-maltodextrins and that of other substances. Another question concerns the basic difference in diffusion of solutes between the λ receptor and porin pores. In this paper, we show that λ receptor protein forms pores that facilitate diffusion of maltose-maltodextrins across the outer membrane and function as a molecular sieve for other solutes. The pores made of porin allow diffusion of only Glc_2 among the maltose-maltodextrins tested so far.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 substrain T19 (*tsx supE ompB*) and *E. coli* B

were described elsewhere (9, 10). *E. coli* K-12 substrain G6 (13) was grown in M9 medium containing 0.1% Casamino Acids and 0.4% maltose, and exponential-phase cells were used for purification of amylomaltase. *E. coli* K-12 substrain TNE005, a spontaneous mutant resistant to phage λ, was derived from substrain T5 (*tsx supE*). The envelope fraction of the mutant (maltose induced) lacked λ receptor protein as determined by stained gel after electrophoresis in sodium dodecyl sulfate. Mutation at the regulatory gene of the maltose operon is unlikely, since the mutant produced a normal level of the product of the *malQ* gene, amylomaltase, and transported Glc_2 but not maltose-maltodextrins larger than Glc_2 (Table 1) (maltose may be diffused across the outer membrane through the pores made of porin, see below).

Purification of λ receptor protein and porin. λ receptor protein and porin were purified from *E. coli* K-12 substrain T19 and *E. coli* B, respectively, by a procedure described earlier (10). The purified proteins were quantified on the basis of their absorption coefficients at 278 nm (3, 12).

Enzymatic synthesis of ^{3}H-labeled maltose-maltodextrins. The 0.5-ml reaction mixture contained 2 μmol of maltose, 25 μmol of Tris-hydrochloride buffer (pH 6.8), 1 mCi of D-[6-^{3}H(*N*)]glucose (0.092 μmol) (New England Nuclear Corp.), and 0.055 U of amylomaltase, partially purified by ammonium sulfate precipitation by the method of Weismeyer and Cohn (18). The mixture was incubated at 37°C for 180 min, and the reaction was stopped by heating the tube at 100°C for 3 min. The volume of the centrifuged supernatant fluid was reduced to about 30 μl under an N_2 gas stream at 65°C, and the sample was applied as a line onto 5-cm-wide Whatman 3 MM paper (Whatman Ltd.). The paper was developed by descending chromatography with a solvent containing *n*-butanol–pyridine–water (6:4:3, vol/vol) at 25°C for 34 h. Radioactive spots, located with a Dünnschicht Scanner II (Berthold Laboratories) (data not shown), corresponding to authentic maltose-maltodextrins were eluted with distilled water. The purity of radioactive oligosaccharides was 97 to 99% as determined by analytical paper chromatography with two solvent systems (ethylacetate-pyridine-water [12:5:4, vol/vol] and isopropanol-pyridine-water [12:4:4, vol/vol]) and by gel filtration with Bio-Gel P-2 (minus 400 mesh, Bio-Rad Laboratories).

Permeability assay. Three permeability assay methods were used. (i) The transport assay using whole cells was done essentially as described by Szmelcman et al. (14). Cells grown in M9 medium supplemented with 0.1% Casamino Acids, 10 μg of thiamine per ml, and 0.4% maltose were harvested at the exponential phase and washed three times with M9 medium containing 50 μg of chloramphenicol per ml. Cells were resuspended in M9-chloramphenicol medium, and the absorbance at 600 nm was adjusted to 0.6. The suspension was incubated at 25°C for 5 min in the presence of 1 mM glycerol immediately before the transport experiment. At time zero, 0.5 ml of the cell suspension (3×10^8 cells) was mixed with 0.05 ml of radioactive sugar, and the mixture was incubated at 25°C for 30 s. The mixture (0.5 ml) was withdrawn, filtered through a membrane filter (0.45-μm pore size, 2.5-cm diameter; Millipore Corp.), and washed with 5 ml of M9-chloramphenicol medium. The transport assay was completed within 30 s since other workers (13) have reported that the contribution of maltose metabolism to apparent permeability rate is negligible under these conditions. (ii) Permeability rate was also determined by using reconstituted vesicle membranes. The permeability rate of maltose-maltodextrins was determined in vitro by our recently developed technique (15). Vesicle membranes were reconstituted from 2 μmol of phospholipids, 150 nmol of Rc-type lipopolysaccharide, and 4.17 or 0.208 nmol of outer membrane protein (sodium dodecyl sulfate was reduced by dialysis as reported previously [8]) by the procedure described earlier (7). The dried materials were suspended in 100 μl of solution containing about 14 U of amyloglucosidase (dialyzed overnight against a large excess of a solution containing 0.05 M acetate buffer [pH 5.0], 0.05 M NaCl, and 3 mM NaN_3) by sonic oscillation for 1.0 min. The tube containing vesicles was kept at 40°C for 30 min. Vesicles were separated from free amyloglucosidase with a Sepharose 4B column (0.9 by 50 cm, Pharmacia Fine Chemicals, Inc.) equilibrated with a solution containing 0.05 M acetate buffer (pH 5.0), 0.05 M NaCl, and 3 mM NaN_3. The reaction mixture (total, 0.4 ml) contained 20 μmol of acetate buffer (pH 5.0), 20 μmol of NaCl, 4 μmol of $MgCl_2$, and maltose-maltodextrins. The concentrations of Glc_2, Glc_3, Glc_4, Glc_5, Glc_6, and Glc_7 were 20×10^{-4}, 16×10^{-4}, 14×10^{-4}, 10×10^{4}, 9×10^{-4}, and 9×10^{-4} M, respectively (twice the K_m of amyloglucosidase). The reaction was started by tipping the vesicle suspension into the mixture and was stopped by heating in a tube at 100°C for 2 min. After the mixture cooled, 100 μl of 0.3 M Tris base, 15.6 μl of 1 N HCl, and 4 μl of 10% Triton X-100 were added sequentially (final pH of the mixture, 7.6). The glucose released was determined spectrophotometrically by a coupled reaction of hexokinase and glucose 6-phosphate dehydrogenase (4). The total enzyme activity was determined after the membranes were dissolved in the presence of 0.1% Triton X-100, and V_{max} of the enzyme was calculated. Triton X-100 at this concentration did not detectably influence the initial activity of the enzymes used in this study. Extravesicular amyloglucosidase was determined by using membranes made of phospholipids and lipopolysaccharide, and the activity (8.6% of the total enzyme activity) was subtracted from that of the experimentals. Permeability parameters were calculated by the procedure of Zimmermann and Rosselet (19). Enzyme activity was expressed as nanomoles of substrate hydrolyzed per minute per nanomole of LPS, nanomoles of substrate hydrolyzed per minute per 13.3 nmol of phospholipid, or nanomoles of substrate hydrolyzed per minute per 2.8×10^{-2} or 1.4×10^{-3} nmol of pore-forming protein. (iii) The assay for permeability of radioactive solutes through reconstituted vesicle membranes was described previously (7, 8).

Other methods. Extraction and quantitation of lipopolysaccharide and phospholipids were described previously (7), except that strain HN202 (LT2M1) was used.

Chemicals. Nonradioactive maltose-maltodextrins were purchased from the following sources: Glc_2,

Merck & Co., Inc.; Glc_3, Sigma Chemical Co.; Glc_5, Wako Pure Chemicals; and Glc_4, Glc_6, and Glc_7, Seisin Pharmaceutical Co. Chemicals used were the best grade commercially available.

Enzyme. Amyloglucosidase from *Aspergillus niger* was purchased from Boehringer Mannheim GmbH. Michaelis constants (K_m) of the enzyme for Glc_2, Glc_3, Glc_4, Glc_5, Glc_6, and Glc_7 were 10×10^{-4}, 8.3×10^{-4}, 7×10^{-4}, 5.2×10^{-4}, 4.4×10^{-4}, and 4.5×10^{-4} M, respectively, as determined by glucose liberation in 0.05 M acetate buffer (pH 5.0).

RESULTS

Kinetic constants for maltose-maltodextrin transport in mutant strains carrying *lamB*$^+$ *ompB* (λ receptor$^+$, porin$^-$) or *lamB ompB*$^+$ (λ receptor$^-$, porin$^+$). The apparent transport K_m values of Glc_2 through Glc_6 for strain T19 induced with maltose (λ receptor$^+$, porin$^-$) were 3×10^{-6} to 7×10^{-6} M (Table 1). Although one does not know which step of the maltose transport chain is rate limiting, the apparent K_m values for maltose-maltodextrin diffusion (Glc_2 through Glc_6) in this strain were only slightly different. The reason for the deviation of the apparent K_m values for Glc_2 and Glc_3 diffusion from reported values (14) is unknown at present. We confirmed our results by repeated experiments. The diffusion K_m values of Glc_2 and Glc_3 for strain TNE005 (lacking λ receptor protein [porin$^+$]) were 3×10^{-4} and $\approx 200 \times 10^{-4}$ M, respectively (Table 1). Diffusion of Glc_4 through Glc_6 in this strain was practically undetectable under the present assay conditions. The apparent transport K_m for Glc_2 in the strain carrying *lamB ompB* (λ receptor$^-$, porin$^-$) was about 10^{-3} M (T. Nakae, unpublished data). It became evident that λ receptor protein allowed an efficient influx of maltose-maltodextrins of at least up to Glc_6. This result does not, however, exclude the possibility that efficient transport of maltose-maltodextrins by the λ receptor$^+$ strain was a result of the cooperative work of λ receptor protein and other cellular component(s) such as the maltose-binding protein. Such possibility was suggested previously (17).

Determination of maltose-maltodextrin diffusion rates through reconstituted vesicle membranes. The diffusion rate of maltose-maltodextrins was determined by using vesicles containing amyloglucosidase in the intravesicular space (Fig. 1). The theoretical basis for the present assay method is based upon Fick's law: $V = PA\ (C_o - C_i) = C_i \times [V_{max}/(K_m + C_i)]$, where V is the velocity of substrate diffusion, P is the permeability coefficient, A is the surface area, and C_o and C_i are substrate concentrations at the outside and the inside, respectively, of the vesicles. Since A was identical for all experiments (1 nmol of lipopolysaccharide and 13.3 nmol of phospholipids), we could calculate a

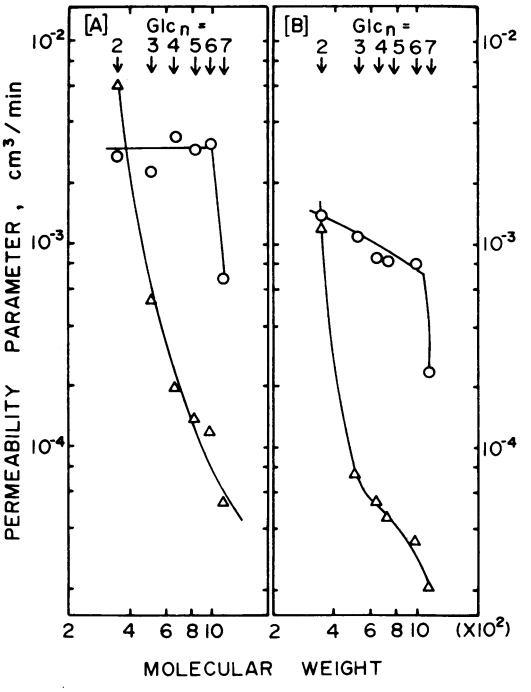

FIG. 1. *Permeability parameters of reconstituted vesicle membranes containing λ receptor protein or porin protein to maltose-maltodextrins. (A) Double logarithmic plot of permeability parameter versus molecular weight of maltose-maltodextrin. Vesicles contained 1 nmol of lipopolysaccharide, 13.3 nmol of phospholipids, and 2.8 × 10⁻² nmol of λ receptor proteins or porins per assay. (B) Vesicles contained 1 nmol of lipopolysachcaride, 13.3 nmol of phospholipids, and 1.4 × 10⁻³ nmol of λ receptor proteins or porins. Symbols: ○, λ receptor, △, porin.*

TABLE 1. *Maltose-maltodextrin transport kinetics by lamB$^+$ ompB and lamB ompB$^+$ strains*

Strain	Glc_2		Glc_3		Glc_4		Glc_5		Glc_6	
	K_m[a]	V_{max}[b]	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
T19 *lamB*$^+$ *ompB*	6.6	1.4	5.8	2	3.5	1	4.4	1.1	5.3	0.7
TNE005 *lamB ompB*$^+$	300	7	≈20,000	≈40	—[c]	—	—	—	—	—

[a] K_m, 10^{-6} M.
[b] V_{max}, 10^{-9} mol/min per 3×10^8 cells.
[c] —, Not determined even at 10 mM substrate.

permeability parameter (*PA*) from the rates of substrate hydrolysis by the intact and detergent-solubilized vesicles. (For theoretical considerations of the present assay method, refer to references 15 and 19.)

The vesicles containing λ receptor protein had permeability parameters for maltose-maltodextrins that were within a range of 2.3×10^{-3} to 3.4×10^{-3} cm^3/min for Glc_2 through Glc_6 and 6.8×10^{-4} cm^3/min for Glc_7 (2.8×10^{-2} nmol of λ receptor protein per assay) (Fig. 1A). If the total pore area of the λ receptor protein is very high, it is possible that the membranes do not function as a rate-limiting step of the entire process. The permeability parameters from the experiment with 1.4×10^{-3} nmol of λ receptor protein were somewhat lower (Fig. 1B). However, the permeability parameters of the membranes for Glc_2 through Glc_6 appeared similar (0.83×10^{-3} to 1.4×10^{-3} cm^3/min) for all of the substrates tested. These results suggest that the pores made of λ receptor protein did not act as a simple molecular sieve, although the substrate concentrations used for the in vitro experiment were much higher than the diffusion K_m of maltose-maltodextrins as determined for the strain harboring λ receptor protein (Table 1). The permeability parameters for Glc_2 in the membrane containing the λ receptor and porin appeared similar, despite the fact that the transport K_m for Glc_2 in strains T19 (porin$^-$) and TNE005 (λ receptor$^-$) showed dramatic differences. This obvious discrepancy may be due to the relatively high concentration of substrate used for the in vitro rate assay. (We could not lower the substrate concentration because of the relatively high K_m value for maltose-maltodextrins of amyloglucosidase.)

We determined the diffusion of maltose-maltodextrins through membrane vesicles containing porins. The permeability parameters for these saccharides of the porin-containing membranes showed that the membranes acted as a molecular sieve (Fig. 1). The results presented in Fig. 1 demonstrate that maltose-maltodextrins larger than maltose were practically impermeable through the porin-containing membranes. The results were consistent with the in vivo transport experiment (Table 1).

Determination of oligosaccharide efflux from preloaded vesicle membranes. The exit of maltose-maltodextrins from preloaded vesicle membranes containing λ receptor protein or porin was determined by the procedure described earlier (8). Glc_2 through Glc_6 were released from the membrane containing λ receptors rapidly, and the extents of release of Glc_2 through Glc_6 were similar (Fig. 2). In contrast, the porin-containing membranes functioned as a molecular

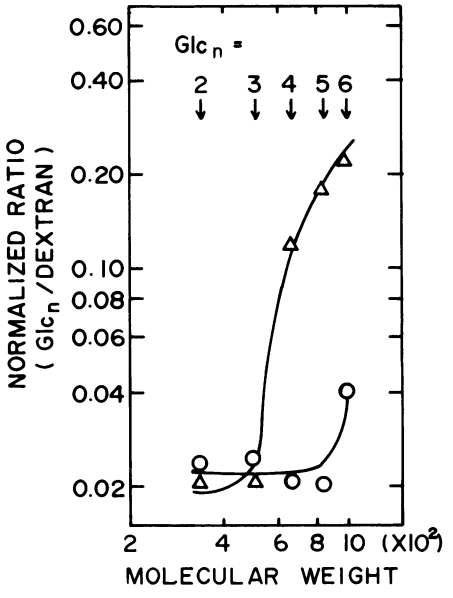

FIG. 2. *Exit of ^{3}H-labeled maltose-maltodextrins from reconstituted vesicle membranes. Vesicles were reconstituted from 0.5 μmol of phospholipids, 0.075 μmol of lipopolysaccharide, and 100 μg of λ receptor proteins or 100 μg of porins (sodium dodecyl sulfate was reduced by dialysis) in the presence of ^{3}H-labeled maltose-maltodextrin and [^{14}C]dextran as described earlier (8, 10). The figure shows the logarithm of the normalized ratio of oligosaccharides and dextran as previously described (10). The permeability assay was done at 25°C 10 min after dilution of vesicles with a 100-fold volume of medium. Symbols: ○, λ receptor, △, porin.*

sieve for these homologous oligosaccharides (Fig. 2). The vesicle membranes containing the λ receptor protein acted essentially as a molecular sieve for sucrose, lactose (molecular weight, 342), raffinose (molecular weight, 504), and stachyose (molecular weight, 666) as reported previously (10) and for fractionated inulins of average molecular weights 800, 920, and 1,380 (data not shown). It must be noted that raffinose and stachyose showed K_{av} values similar to those of Glc_4 and Glc_6, respectively, as determined by gel permeation chromatography with a Bio-Gel P-2 column (Nakae, unpublished data). In addition, the λ receptor pores excluded fractionated inulins of molecular weights 800 and 920 (similar to those of Glc_5 and Glc_6, respectively).

DISCUSSION

The ability of λ receptor protein to promote diffusion of maltose-maltodextrins was determined in vivo by using strains carrying the *lamB*$^+$ and *lamB* alleles and in vitro by using reconstituted vesicle membranes. Maltose-mal-

todextrins of up to Glc_6 were readily diffusible through membranes containing λ receptors, whereas only maltose was diffusible through membranes containing porin. The following question was raised: How do λ receptor proteins allow preferential passage of maltose-maltodextrins? We have reported that λ receptor proteins act as a molecular sieve for sucrose, lactose, raffinose, stachyose, and inulins (10, 15, present paper). Boehler-Kohler et al. suggested that the surface area of the λ receptor pore is 1.77 times larger than that of a pore made of porin, on the basis of the magnitude of single-pore conductance in black-lipid membrane (1). If this is true, can we explain the high permeability of maltose-maltodextrins through the λ receptor pores? The results of our gel permeation chromatography revealed that the molecular sizes of hydrated Glc_4 to raffinose or Glc_6 to stachyose were very close (Nakae, unpublished data). However, the diffusion of Glc_4 and Glc_6 through the λ receptor membranes appeared very great, whereas the diffusion of raffinose or stachyose across the λ receptor membranes was much less. Furthermore, diffusion of maltose-maltodextrins through porin-containing membranes depended on molecular size (Fig. 1 and 2) (15). Thus, we concluded that λ receptor protein facilitated the diffusion of maltose-maltodextrins in such a manner that the protein formed pores allowing the diffusion of maltose-maltodextrins or that the protein loosely interacted with maltose-maltodextrins. The λ receptor protein functioned, on the other hand, as a molecular sieve for other saccharides such as sucrose, raffinose, stachyose and inulins. The molecular basis of the solute selectivity by the λ receptor protein is as yet unsolved.

Recently, it was suggested that periplasmic maltose-binding proteins play an important role(s) in the selective diffusion of maltose-maltodextrins via λ receptor pores (17). The results presented in the present paper revealed that λ receptor protein formed pores that facilitated diffusion of maltose-maltodextrins in vesicles reconstituted from purified λ receptor. Although the substrate concentrations used for the rate assay in vitro were much higher than the transport K_m values in vivo, the concentrations of ^3H-labeled maltose-maltodextrins used for the exit study were lower than the transport K_m values in vivo. Therefore, it is likely that the pores made of λ receptor protein carry solute selectivity. In addition, it is still possible that the λ receptor protein and maltose-binding protein interact to facilitate diffusion of maltose-maltodextrins, as suggested previously (17). We believe that it is feasible to test such a possibility by our in vitro assay techniques.

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