Outer Membranes of Gram-Negative Bacteria

XV. Transmembrane Diffusion Rates in Lipoprotein-Deficient Mutants of Escherichia coli

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Received for publication 1 August 1977

Permeability of the outer membrane to 6-aminopenicillanic acid was unaltered in an lpo mutant, lacking the Braun lipoprotein, a result suggesting that the lipoproteins by themselves form no or few diffusion pores.

The Braun lipoprotein of Escherichia coli and Salmonella typhimurium is a protein that exists in the largest number of copies in these cells (1). Most of the polypeptide chain in this protein is organized as α-helices (1), and Braun and Bosch (2), who determined the amino acid sequence of this protein, noticed and emphasized the presence of the entire hydrophobic "side" as well as a totally hydrophilic "side" on the helix. On the basis of these results, Inouye (8) proposed that several of these protein molecules might aggregate together, with the hydrophilic faces toward the center, and thus create "diffusion pores" that penetrate through the outer membrane.

This hypothesis can now be critically tested, as mutants (lpo) of E. coli K-12 totally lacking the lipoprotein have been isolated (7). We measured the permeability of the outer membrane of these strains as follows. First, the R-factor R1 was transferred by conjugation from YC 219 (E. coli K-12 lac gal mtl xyl ara/R1, a gift of M. Yoshikawa) to stationary-phase cells (F- phenocopies) of man- lpo+ pps+ and man+ lpo pps+ transductants of HfrC man pps, described in Table 2 of reference 7. The presence and absence of Braun lipoprotein in these strains was confirmed by slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9). These strains will be called Lpo+(R1) and Lpo-(R1), respectively. The outer membrane permeability was then estimated by following the hydrolysis of β-lactams by suspensions of intact cells of these strains according to the modification (11) of the method of Zimmermann and Rosselet (15).

This assay for permeability required that the R-factor-coded β-lactamase remained totally in the periplasmic space. However, the lpo mutants are known to leak a significant fraction of the periplasmic enzymes into the media (7), and it was first necessary to minimize this leakage. The observation that the lpo mutant is sensitive to ethylenediaminetetraacetic acid (7) and our previous experience that Mg2+ could prevent the leakage of β-lactamases in S. typhimurium suggested the addition of Mg2+ to the growth medium and the suspension buffer. Thus, the Lpo-(R1) cells were grown at 37°C in L broth containing 5 mM MgSO4 and at room temperature, centrifuged, washed, and suspended in 10 mM sodium phosphate-5 mM MgCl2 (pH 7.0). Assay of β-lactamase activity on the supernatants obtained by centrifugation of these suspensions at about 10 min after their preparation showed that only 1 to 3.5% of the total β-lactamase has leaked into the medium. Although the extent of leakage was greater than the value of 0.2 to 1% found in the Lpo+(R1) strain, it was small enough to make the diffusion assay usable. The amount of the leaked enzyme remained constant when the suspension of Lpo-(R1) cells was kept at room temperature for up to 40 min. Thus, under our conditions, the leakage apparently is not a continuous process and presumably takes place only during the centrifugation and/or suspension process.

When the diffusion rates of a β-lactam antibiotic, cephaloridine (a gift from L. F. Ellis, Eli Lilly & Co.), were measured by determining the rate of its hydrolysis by intact cells as described previously (11), we found that the intact cells of Lpo+(R1) showed about 65% of the total enzymatic activity at the external cephaloridine concentration of 0.8 mM, whereas the Lpo-(R1) cells showed almost full activity, i.e., 98% of the total activity. These results made us suspect that the presence of cephaloridine might lead to the destruction of the permeability barrier. To test this possibility, Lpo-(R1) cells were incubated in a reaction mixture containing 10 mM sodium phosphate (pH 7.0), 5 mM MgCl2, and 0.8 mM cephaloridine at 30°C for 2 min. The
mixture was immediately centrifuged for 1 min in an Eppendorf microcentrifuge, and a portion of the supernatant was incubated for a further 10 min at 30°C before the reaction was stopped and the hydrolysis product was determined. We found that 9.7 nmol of cephaloridine was hydrolyzed in the reaction mixture that originally contained 25 µg (dry weight) of cells of Lpo+(R1). Since this amount of cells hydrolyzed 3.4 nmol of cephaloridine in 2 min, 9.7 - 3.4 = 6.3 nmol of cephaloridine was hydrolyzed by the cell-free supernatant during the second incubation of 10 min. A comparison with the β-lactamase activity of the sonic extract shows that about 40% of the total β-lactamase activity is already released into the medium after 2 min of incubation with cephaloridine. Thus, the Lpo−(R1) cells rapidly lost the barrier properties of the cell wall upon exposure to 0.8 mM cephaloridine. This was rather surprising, especially in view of the fact that the lpo mutants and lpo+ parent strains had similar minimum inhibitory concentrations for various β-lactam antibiotics (Y. Nishimura and Y. Hirota, manuscript in preparation). Possibly the damaging effect observed in the present study may be related to the very high concentration of cephaloridine used.

In view of the destructive effect of cephaloridine, we sought a compound that is readily hydrolyzed by β-lactamases yet is essentially inactive as an antibiotic and is unlikely to perturb the integrity of the cell wall barrier. One such compound is 6-aminopenicillanic acid. This compound (obtained from Sigma Chemical Co.), at various concentrations, was incubated with a sonic extract of Lpo+(R1) cells, an experiment that showed its rapid enzymatic hydrolysis with a Kₘ of 170 µM. Since it is known that 6-aminopenicillanic acid has less than 1% of the antibacterial activity of benzylpenicillin (12), we hoped that the incubation of Lpo−(R1) cells in its presence would not lead to the release of the β-lactamase into the medium. Indeed, experiments similar to that described in the preceding section revealed that after 10 min of incubation of Lpo−(R1) cells with 170 µM 6-aminopenicillanic acid, 3.3% of the total β-lactamase activity was found in the supernatant. Since with this cell suspension 3.3% of the total activity was in the supernatant even without treatment with 6-aminopenicillanic acid, clearly this compound does not produce any increase in the leakage of the β-lactamase.

The rates of hydrolysis of 6-aminopenicillanic acid were then determined with Lpo−(R1) and Lpo+(R1) cells and extracts (Table 1). Clearly, the absence of the murein lipoprotein in the Lpo−(R1) strain does not produce any decrease in the diffusion rate for 6-aminopenicillanic acid.

The outer membrane allows the diffusion of any hydrophilic molecule with a molecular weight lower than 500 to 600 (3). In addition to this nonspecific pathway, it also has specific diffusion mechanisms for the diffusion of maltose and maltodextrins (13), nucleosides (5), vitamin B₁₂ (4), and iron siderophore complexes (6, 14). β-Lactams apparently diffuse through the outer membrane by the nonspecific pathway, as mutants known to be deficient in β-lactam diffusion are also deficient in the diffusion of amino acids and carbohydrates (10). Thus, the diffusion rate of a β-lactam, 6-aminopenicillanic acid, serves as a good indicator of the operation of the nonspecific diffusion pathway, and very similar diffusion rates in Lpo−(R1) and Lpo+(R1) strains clearly do not support the hypothesis (8) that lipoproteins by themselves form nonspecific pores. If lipoprotein pores exist, they must be in such small numbers that they cannot make significant contributions to the hydrophilic permeability of the outer membrane. These conclusions are consistent with the previous observations that isolated lipoprotein could not produce pores in the in vitro reconstitution assay (9) and that the lpo mutants grew rapidly in various media and were permeable to α-nitrophenyl-β-D-galactoside (7).

The permeability of the outer membrane to hydrophobic substances might be altered in lpo mutants, and this remains to be examined. However, the hydrophobic molecules generally appear to penetrate through the outer membrane by dissolving into the hydrocarbon interior regions of the membrane and not through the pores (10).

This study was supported by Public Health Service research grant AI-09644 from the National Institute of Allergy and Infectious Diseases and grant BC-20 from the American Cancer Society to Hiroshi Nikaido and by research grants.

### Table 1. Hydrolysis of 6-aminopenicillanic acid by Lpo+(R1) Lpo−(R1) Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of hydrolysis (nmol/s per mg [dry wt])</th>
<th>Permeability coefficient, β⁺ (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V₀,corr</td>
<td>Vₚ₀,corr</td>
</tr>
<tr>
<td>Lpo+(R1)</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>Lpo−(R1)</td>
<td>0.35</td>
<td>0.32</td>
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</table>

*a Calculated as described in reference 11.

*b Correction was made for the activity of "leaked-out" enzyme, which corresponded to 0.8 and 1.4% of the total β-lactamase in the suspensions of Lpo+(R1) and Lpo−(R1), respectively, in this particular experiment.
944070, 048300, and 058087 from the Ministry of Education, Japan, to Yukinori Hirota.

LITERATURE CITED