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Effect of lipid fluidity upon the activity and structure of the 39 kDa porin from *Enterobacter cloacae* 908S

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The 39 kDa porin from *Enterobacter cloacae* 908S was isolated in a lipopolysaccharide-free form using the non-ionic detergent, octylpentaoxyethylene, and reconstituted into vesicles of dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC), respectively. Porin activity, measured by the rate of hydrolysis of the lipid-impermeant β-lactam cephazoline by entrapped lactamase, could be demonstrated for porin-DMPC but not for porin-DOPC vesicles, and for the former was significantly lower in the gel than in the liquid-crystalline phase. The fluorescence changes are thought to arise from lipid phase-induced structural/dynamic changes of the porin structure.

Membrane; Membrane protein; Reconstitution; Fluorescence

1. INTRODUCTION

Diffusion of many small substances through the outer cell membrane of gram-negative bacteria occurs via a group of channel-forming proteins known as the porins [1]. Several of these proteins have been well-characterized biochemically [2–5]. The *ompF* gene product (36 kDa) from *Escherichia coli* has been crystallized in 2D sheets and shown to exist as trimer in its functional form [6]. The physical mechanism of pore formation and the effect of the lipid environment remain unclear at present. Schindler and Rosenbusch [7,8] were able to incorporate the ompF porin from *E. coli* K12 into planar bilayers and show that channel formation is a dynamic process and that the pores exist in two discrete functional states (open and closed) [7]. In these studies the addition of LPS to bilayers of unsaturated phospholipids was necessary for stable channel formation. Indeed, it has been claimed that the presence of LPS is obligatory for the observation of porin activity [1]. To our knowledge, however, saturated phospholipids have not been used for reconstitution until now.

Here, we have isolated the LPS-free form of the 39 kDa porin from *Enterobacter cloacae* 908S and reconstituted it with saturated (DMPC) and unsaturated (DOPC) phospholipids, respectively. We show that with DMPC, but not with DOPC, stable channels can be formed in the absence of LPS and that porin activity is modulated by the lipid phase. Furthermore, the lipid-induced effects upon porin activity can be correlated directly with changes in the protein structure/dynamics.

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride; NaP, sodium phosphate buffer; O-POE, octylpentaoxyethylene; PAGE, polyacrylamide gel electrophoresis; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; LPS, lipopolysaccharide; T_c, phase transition temperature

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2. MATERIALS AND METHODS

DMPC and O-POE (Bachem, Switzerland), nitrocefin (Hoffmann-La Roche) and cephazoline (Lilly) were used without further purification. All other reagents were analytical grade (Fluka). Partially purified β-lactamase from *Klebsiella oxytoca* was a gift from Dr R. Then. Cells of *E. cloacae* were grown as described [9].

The 39 kDa porin was isolated essentially according to [6]. 28 g *E. cloacae* 908S cells were washed with 10 mM NaP buffer (pH 7.0) and the pellet resuspended in 150 ml of 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.2 mM DTT. After addition of 5 mg DNase and RNase, respectively, and 2 mg PMSF, the cells were broken by three passages through a French pressure cell at 1100 atm. Residual cells were removed by low-speed centrifugation at 3000 × *g* and 4°C for 20 min. The supernatant was centrifuged further for 1 h at 29000 × *g* and 4°C. The crude outer membranes were then extracted repeatedly with 50 mM NaP (pH 7.0), 0.1 M NaCl, 0.1 mM DTT, 3 mM NaN_3, 3% (v/v) O-POE at 37°C for 1 h to yield the porin supernatant fraction, as monitored by SDS-PAGE, after centrifugation of the detergent extract at 30000 × *g* for 30 min at room temperature. The crude porin-containing supernatants were pooled, concentrated by ultrafiltration (Amicon PM10) and then applied to a column of DEAE-cellulose equilibrated in 10 mM NaP (pH 7.0), 3 mM NaN_3, 0.1 mM DTT, 0.6% (v/v) O-POE. Elution was then performed batchwise using the above buffer containing (i) 0.1 M NaCl, (ii) 0.15 M NaCl, and finally (iii) 0.5 M NaCl to obtain the 39 kDa porin. The purity of the preparation was tested by SDS-PAGE [11] using Coomassie blue R250 and silver stains (Bio-Rad), respectively. The porin was stored in the final elution buffer at 4°C. Determination of LPS content was performed as in [12].

Reconstitution of the 39 kDa porin into lipid vesicles was performed as described by Dorset et al. [6]. 5 mg DMPC in ethanolic solution was dried under high vacuum for 12 h, then resuspended in 0.5 ml of 20 mM NaP (pH 7.0), 0.1 M NaCl, and adjusted to 1% (v/v) in O-POE (final volume 1 ml). 0.1 mg porin in 0.6 ml was diluted with 0.3 ml of the above buffer containing 2% (v/v) O-POE and 250 U β-lactamase and then added to the O-POE-solubilized DMPC and allowed to equilibrate at room temperature for 1 h. The reconstitution mixture was then dialyzed extensively against detergent-free buffer at 37°C [6]. After dialysis non-entrapped β-lactamase was removed by repeated (4 ×) centrifugation at 100000 × *g* and the final pellet resuspended in 1 ml detergent-free buffer. The residual β-lactamase activity bound to the external surface of the vesicle was measured with control reconstituted vesicles containing porin but where β-lactamase was added only after reconstitution had been performed. Incorporation of porin into vesicles was quantitative yielding a final *L/P* (w/w) ratio of 50. Control porin-free vesicles were prepared in the same way. β-Lactamase activity was measured by the change in absorption at 482 and 262 nm due to hydrolysis using the substrates nitrocefin and cephazoline, respectively.

Fluorescence measurements were performed using an SLM 4800 fluorimeter fitted with Hamamatsu R928P photomultipliers operating in the ratio mode. Excitation and emission were measured with Glan-Thompson calcite polarizers set to the 'magic angle' [13] and monochromator slit widths were 2 and 4 nm, respectively. The cell holder was always thermostatted and continually flushed with nitrogen during the measurements. All buffers (including that used for the resuspension of the final pellet) were filtered before use. No correction for the inner filter effect was performed since this was generally small.

3. RESULTS

The 39 kDa porin was purified by the method of Dorset et al. [6] and yielded a single protein (>98% pure) as monitored by Coomassie blue and silver staining of 12.5% SDS gels. The preparation contained less than 0.2 mol LPS/mol porin. Reconstitution of the 39 kDa porin with DMPC at a lipid concentration of 1 mg/ml or less led to a homogeneous translucent vesicle suspension quite suitable for absorption or fluorescence spectroscopy.

Three methods of measuring porin activity have been widely used: (i) the swelling assay of Luckey and Nikaido [14]; (ii) conductance in single bilayer films [7]; (iii) enzyme encapsulation [15]. We chose

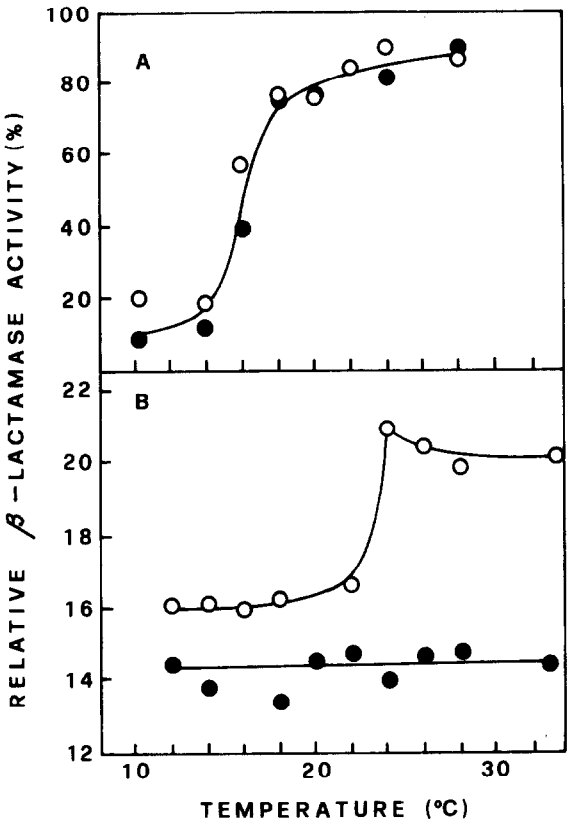

Fig.1. Determination of the effect of the lipid phase upon porin activity. β-Lactamase was entrapped in porin-containing (○) and porin-free (●) DMPC as described in section 2. β-Lactamase activity ratios were determined by adding (A) the lipid-permeant substrate, nitrocefin (0.1 mM) or (B) the lipid-impermeant substrate, cephazoline (0.1 mM) to 20 μg lipid in 20 mM NaP (pH 7.0), 0.1 M NaCl, 3 mM NaN_3, and measuring the linear rate of hydrolysis at 484 and 262 nm, respectively. At each temperature, the total β-lactamase activity was determined by adding an identical vesicle-containing sample made to 1% in O-POE to a separate assay cuvette and measuring the linear rate of β-lactam hydrolysis as above. Porin activity was determined as (β-lactamase activity before O-POE addition) × 100/(total β-lactamase activity). In these experiments the *L/P* (w/w) ratio was 50.

the last method as it avoids some of the problems associated with the swelling assay: light scattering due to vesicle swelling and non-specific clumping of vesicle aggregates is of particular importance here; low concentrations of vesicles may be used for the determination of activity; correction factors due to back-diffusion rates are not necessary.

The main problem with the β-lactamase entrapment method lies in the removal of excess enzyme bound to the outer surface of the vesicles. This was accomplished almost quantitatively by including a resuspension step at the p*I* of the protein (pH 6.5) during the final wash procedures. Thus the residual β-lactamase activity bound to the external surface of the vesicles was only 2% of the total activity (external + entrapped) remaining after the isoelectric resuspension step. A further problem is that the pathway of β-lactam penetration through porin-containing membranes has not been particularly well-defined. Fig.1 shows the temperature dependence for the permeability of DMPC vesicles to nitrocefin, a hydrophobic β-lactam that

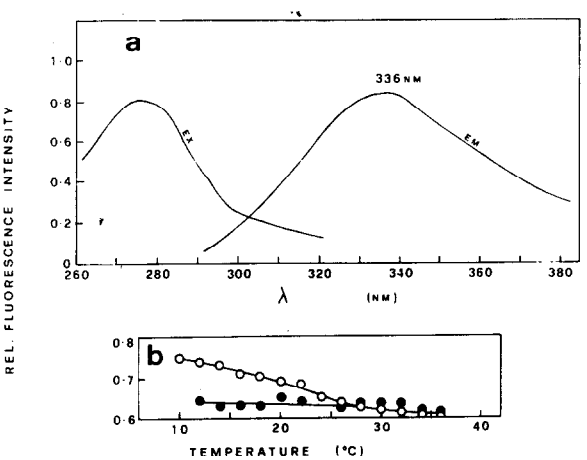

Fig.2. (a) Fluorescence emission and excitation spectra for the 39 kDa porin reconstituted into vesicles of DMPC. (Upper) Excitation (EX.) and emission (EM.) spectra of the intrinsic Tyr/Trp fluorescence. Excitation and emission were measured at 336 and 274.6 nm, respectively. In all cases, slit widths of the monochromators for excitation and emission were set to 2 and 4 nm, respectively. All preparations used for fluorescence studies had an *L/P* (w/w) ratio of 10. (b) Changes of relative fluorescence intensity for the porin-containing vesicles of DMPC (○) and DOPC (●), respectively; intrinsic Tyr/Trp fluorescence of the same samples (○, porin-DMPC; ●, porin-DOPC), respectively. Variations in the temperature were made in successive intervals of 2–5°C and all fluorescence changes were completely reversible. Temperature equilibration was performed in the absence of exciting light and in the event, no photooxidation of Tyr or Trp was observed.

penetrates the lipid bilayer, and cephazoline, which can only traverse the hydrocarbon layer via the porin channel. For the nitrocefin-accessible β-lactamase activity, the temperature vs activity profiles for both porin-reconstituted and porin-free vesicles were identical and showed a sharp decrease (approx. 60%) below the T_c (19°C) of DMPC. This is consistent with the well-known decrease of permeability for phospholipid vesicles in the gel phase. The cephazoline-accessible activity, in contrast, shows only a 4% decrease for porin-containing DMPC vesicles and no abrupt change for the control vesicles. Porin incorporated into DOPC vesicle produced no changes in β-lactam permeability above that of pure DOPC.

The temperature vs fluorescence intensity profiles for the intrinsic fluorescence of porin reconstituted into DMPC showed an abrupt rise of fluorescence as the temperature was raised from 0 to 40°C. However, the influence of the phase transition upon the intrinsic fluorescence did not exactly parallel that of the increase of porin activity observed, the fluorescence increase being less steep. In contrast, the temperature vs fluorescence intensity profile for porin-DOPC vesicles (T_c = −22°C) showed no change over the same temperature range, suggesting that the effects above are due to the lipid environment rather than an intrinsic conformational change of the protein (fig.2a) or large temperature-dependent changes in the lifetimes of Tyr or Trp residues, respectively.

4. DISCUSSION

This study shows for the first time that the channel-forming activity of a membrane porin is influenced by the physical state of the membrane and that these functional changes correlate with a structural/dynamic transition of the protein at the polar interface. The mechanism of channel formation by the outer membrane porins, and the nature of possible lipid-protein interactions involved are poorly understood at present. Schindler and Rosenbusch [7,8] reconstituted 36 kDa porin from *E. coli* into planar lipid bilayers and were able to demonstrate using conductance measurements that pore formation is a dynamic process involving the generation of short-lived channels within the membrane. These data suggest that the porins exist in two rapidly interconverting states (open and closed) and is at variance with the concept of a static pore structure implicit in the previous literature.

Our measurements of the variation of pore activity with temperature in vesicles of DMPC are consistent with the dynamic pore concept above. Thus the transition from a highly 'fluid' liquid-crystalline phase to the much more tightly packed gel phase below 23°C has a corresponding damping effect upon the observable porin activity (fig.1). Nevertheless, in the gel phase porin activity remains finite and measurable, suggesting that the lipid environment is only indirectly involved in modulating porin-mediated diffusion. An interesting result is that the LPS-free porin preparation described here shows significant activity in vesicles of DMPC but not those of DOPC. The latter is consistent with the results of Schindler and Rosenbusch [8] who observed stable channel formation in DOPC only in the presence of added LPS. The obligatory requirement of LPS for the demonstration of porin activity is well described [1] but to our knowledge these studies have been performed exclusively with vesicles formed from unsaturated phospholipids. It has been shown recently [16] that the hydrocarbon chains of the lipid A moiety of LPS are highly ordered at physiological temperatures and it is thus tempting to suggest that the fatty acyl chains of DMPC (which still possess a preponderance of *trans* conformers even at 10–20°C above the T_c [10]) are mimicking the hydrocarbon environment provided by the lipid A moiety.

Lipid-protein interactions were investigated by measuring the intrinsic fluorescence of the 39 kDa porin from *E. cloacae* 908S. It has been shown [16] that the 36 kDa porin from *E. coli* K12 contains 1–2 aqueous phase-accessible tyrosine residues at the polar interface, whereas most of the tryptophan is buried within the hydrocarbon layer. The correspondence between the lipid phase transition and the discontinuous change in porin activity as well as the intrinsic fluorescence of the aromatic residues suggest that the lipid phase plays a role in determining the active conformational state of the protein.

The lipid phase-mediated changes in intrinsic fluorescence probably arise from one or both of two possible mechanisms: (i) the protein ex-

periences a conformational change in the gel phase thereby decreasing radiationless energy loss or quenching by oxygen. A change of environmental polarity is excluded, however, as no lipid phase-dependent shifts of the excitation/emission maxima were observed for any of the fluorescence spectra examined; (ii) dynamic energy transfer [17,18]. In the gel phase a certain fraction of the Tyr/Trp residues becomes immobilized and fluorescence intensity is lost due to internal quenching. As the temperature is raised the aromatic residues become less immobilized, internal quenching is decreased and the observed fluorescence intensity increases. It is interesting that the fluorescence intensity rises continuously whereas the DMPC gel-to-liquid crystal phase transition is quite abrupt. This probably means that the two structural processes are not tightly coupled and/or that several chromophoric populations may be involved. No increase in fluorescence intensity over the same temperature range is shown for porin reconstituted into DOPC, indicating that this effect is indeed linked to the lipid phase transition.

We believe that model (ii) represents the most attractive explanation of the fluorescence data and is in accord with recent studies of other membrane proteins [19–21]. It has been demonstrated for many membrane proteins that the physical state of the bilayer has a significant effect upon functional activity (see [21]) although the structural basis for these observations remains rather speculative. It is thus tempting to suggest that the correlation shown here between rotational diffusion of amino acid residues at the polar interface and porin activity is causal and may be of more general significance.

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REFERENCES

[1] Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
[2] Rosenbusch, J.P. (1974) J. Biol. Chem. 249, 8019–8029.
[3] Nakae, T. (1976) J. Biol. Chem. 251, 2176–2178.
[4] Neuhaus, J.-M. (1982) Ann. Microbiol. 133A, 27–32.
[5] Nikaido, H. and Rosenberg, E.V. (1983) J. Bacteriol. 153, 241–252.
[6] Dorset, D.L., Engel, A., Haener, M., Massalski, A. and Rosenbusch, J.P. (1983) J. Mol. Biol. 165, 701–710.
[7] Schindler, H.-G. and Rosenbusch, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 3751–3755.
[8] Schindler, H.-G. and Rosenbusch, J.P. (1983) Proc. Natl. Acad. Sci. USA 78, 2302–2306.
[9] Then, R.L. and Angehrn, P. (1982) Antimicrob. Agents Chemother. 21, 711–717.
[10] Seelig, J. and Seelig, A. (1974) Biochemistry 13, 4839–4845.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Karkhanis, Y.D., Zeltner, J.Y., Jackson, J.J. and Carlo, D.J. (1978) Anal. Biochem. 85, 595–601.
[13] Cantor, C.R. and Schimmel, P.R. (1980) Biophysical Chemistry, vol.II, pp.349–408, Freeman, San Francisco.
[14] Luckey, M. and Nikaido, H. (1980) Proc. Natl. Acad. Sci. USA 77, 167–171.
[15] Tokunaga, M., Tokunaga, H. and Nakae, T. (1979) FEBS Lett. 106, 85–88.
[16] Schindler, M. and Rosenbusch, J.P. (1982) J. Cell Biol. 92, 742–746.
[17] Eisinger, J. (1976) Q. Rev. Biophys. 9, 21–33.
[18] Esfahani, M. and Devlin, T.M. (1982) J. Biol. Chem. 257, 9919–9921.
[19] Gomez-Fernandez, J.C., Baena, M.D., Tencel, J.A., Villalain, J. and Vidal, C.J. (1985) J. Biol. Chem. 260, 7168–7170.
[20] Kenir, M.A., Gutowsky, H.S. and Oldfield, E. (1984) Nature 307, 383–385.
[21] Cronan, J.E. jr and Gelman, E.P. (1975) Bacteriol. Rev. 39, 232–256.