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Influence of the lipid matrix on incorporation and function of LPS-free porin from *Paracoccus denitrificans*

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Abstract

We have studied the role of lipopolysaccharide (LPS) for the insertion of LPS-free porin from *Paracoccus denitrificans* into planar lipid bilayers and its function therein. For this, we reconstituted the porin into different asymmetric planar lipid bilayers with or without LPS and into symmetric phospholipid bilayers. LPS-free porin added to the various bilayer systems was found to induce a step-wise increase in membrane conductance with different incorporation rates, depending on the presence of LPS in the bilayer leaflet opposite to porin addition. The incorporation rate into asymmetric LPS/phospholipid membranes from the phospholipid side was more than 10-fold higher than that observed for pure phospholipid membranes. The porin formed general diffusion pores without any salt specificity. The mean single-channel conductance did not depend on the presence of LPS and was about 4.2 nS for a subphase containing 1 M KCl in all systems tested. At certain applied transmembrane voltages, which depended on membrane composition and were ≥ 100 mV for the LPS/phospholipid system, single-channel closing in three steps was observed. Differences in the voltage dependence of porin-channel closing could be correlated with the surface charge of the bilayer. From the voltage-dependent gating behaviour proof for an oriented incorporation of the porin molecules, depending on the side of porin addition, and evidence for their orientation could be derived. Measurements at temperatures above and below the $\beta \leftrightarrow \alpha$ phase transition temperature of LPS gave evidence for the influence of membrane rigidity on the gating behaviour.

Key words: Channel gating; Lipopolysaccharide; Outer membrane; Planar membrane; Porin

1. Introduction

The cell envelope of Gram-negative bacteria is composed of the cytoplasmic membrane and the peptidoglycan layer – these are common also to Gram-positive bacteria – and an additional barrier, the outer membrane, which is strictly asymmetric with respect to its lipid composition. Whereas the inner leaflet of this membrane contains only phospholipids (PL), the outer leaflet is built up mainly from lipopolysaccharides (LPS) which consist of an oligo- or polysaccharide moiety and a covalently linked lipid component, termed lipid A, anchoring the LPS in the outer membrane [1]. The outer membrane represents a molecular sieve allowing hydrophilic substrates to permeate through particular pores formed by special outer membrane proteins (Omps), the porins [2]. The porins have a molecular mass between 30 and 50 kDa and normally form trimers in the outer membrane [3,4].

Although porins have been studied intensively for many years (for review see, e.g., Refs. [5–8]) there still exist controversial aspects to some fundamental questions. To these belong, among others, the influence of LPS on functional properties, the biological significance of channel gating, the amplitude of a gating voltage, and the orientation of porin pores in the membrane.

The role of LPS for the biosynthesis and structure, on the one hand, and for incorporation and function of porins, on the other hand, has been subject of various

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investigations (for review see Ref. [6]). Rocque et al. [9] did not succeed in completely removing the LPS from porins isolated from *Escherichia coli* JF733 without denaturing the protein. In accordance with these results, Buehler et al. [10] found that LPS-free porins OmpF, OmpC, and OmpB had no pore-forming activity in LPS-free planar membranes prepared by the method of Schindler [11] but could be activated in the presence of LPS, free lipid A, and lipid A part structures. The minimal chemical structure required for porin activity was found to be the lipid A precursor corresponding to the phosphate-free lipid X, i.e., a glucosamine carrying two hydroxy-myristic acid residues. In contrast to these results, Parr et al. [12] could show that isolated LPS-free OmpF from *E. coli* and protein P from *Pseudomonas aeruginosa* exhibit in phospholipid bilayer membranes the same average conductances, ion selectivities, and incorporation rates as their conventionally purified counterparts. Hoenger et al. [13] deduced from electron microscopic studies on *E. coli* OmpF that the protein forms three distinct channels in the outer leaflet of the outer membrane, containing one LPS-molecule in their center, merging to one channel on the periplasmic side. Weiss et al. [14–16], however, applying X-ray crystallographic methods found the single outlet of the porin pore from *Rhodobacter capsulatus* on the extracellular and the three distinct channels on the periplasmic side, but without sufficient space for the incorporation of an LPS-molecule in their center.

Planar bilayers are a powerful tool to study functional properties of pore-forming proteins via the measurement of conductance changes. In the literature, however, contradictory results on the degree of voltage sensitivity of bacterial porins are reported even for the same porin but investigated in different systems. This holds in particular for OmpF [17,18] and OmpC [10,19] of *E. coli*. Reasons for these discrepancies might be found in the different membrane systems used for reconstitution, salt concentrations, and pH. Thus, Lakey and Pattus [17] compared the voltage dependence of OmpF from *E. coli* reconstituted into symmetric lipid membranes prepared by the Mueller-Rudin, Montal-Mueller, and Schindler techniques. Independent of the chosen method, channel closing of the incorporated pores was observed but at different voltages. Furthermore, for Schindler-type membranes, the pores had to be activated by an initially applied external potential (see also Ref. [20]) but were then not dependent on the sign of the voltage. In patch-clamp investigations, however, an asymmetric closing behaviour of *E. coli* porins has been observed [21–23]. An asymmetry originating from the orientation of a porin pore has very recently been described for the first time by Brunen and Engelhardt [24] for the anion-selective porin Omp34 from *Acidovorax delafieldii* reconstituted into a symmetric phospholipid bilayer.

With the aim of reconstituting the lipid matrix of the outer membrane with respect to its asymmetry – the inner leaflet composed of phospholipids and the outer of LPS – and, at the same time being able to vary the single components and experimental parameters in a controlled way, we have incorporated LPS-free porin isolated from *P. denitrificans*, into different symmetric and asymmetric phospholipid and LPS/PL planar bilayers. For the preparation of the various bilayer systems we have applied the Montal-Mueller technique [25]. This way, we could investigate the influence of lipid asymmetry, especially when originating from the presence of LPS, and of the resulting intrinsic potential differences on porin properties. We have tried to find answers to the following questions: (i) is LPS essential for the incorporation of porin into the membrane, (ii) is the pore of the porin from *P. denitrificans* formed by a trimer or a dimer, (iii) is the presence of LPS essential for porin function, in particular for the gating behaviour, and for the geometry of the pore, and (iv) is there evidence for a directed incorporation of the porin channels and if so, can statements be derived on its direction?

2. Materials and methods

Lipids and other chemicals

Deep rough mutant LPS of *Salmonella minnesota* strain R595 was used in experiments with asymmetric LPS/PL-membranes. Its chemical structure is depicted in Fig. 1. LPS was extracted by the phenol/chloroform/petroleum ether method [26], purified and

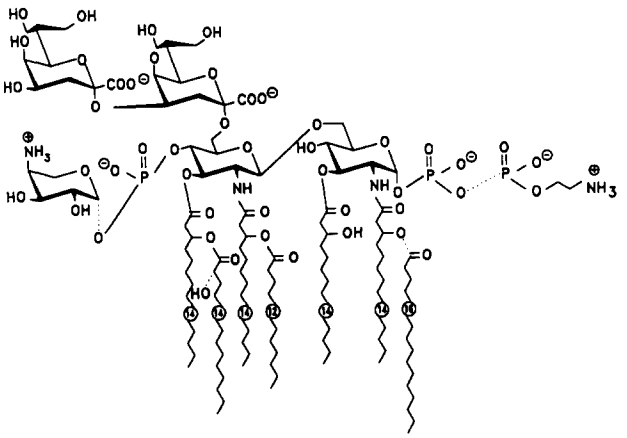

Fig. 1. Chemical structure of the lipopolysaccharide from *Salmonella minnesota* deep rough mutant strain R595. The dotted lines indicate nonstoichiometric substitution.

lyophilized. Phosphatidylcholine (PC) from egg yolk (type XI-E), phosphatidylethanolamine (PE) from bovine brain (type I), phosphatidylglycerol (PG) from egg yolk lecithin (sodium salt) and diphosphatidylglycerol (DPG) from bovine heart (sodium salt) were purchased from Sigma (Deisenhofen, Germany) and used without further purification. Phospholipids (2.5 mg/ml) were dissolved in chloroform at room temperature, LPS (2.5 mg/ml) in chloroform/methanol (10:1 v/v) by heating the mixture to 80°C for a few minutes. The nonapeptide of polymyxin B (PMBN) was obtained from Boehringer (Mannheim, Germany).

LPS-free porin from Paracoccus denitrificans

P. denitrificans ATCC 13543 was grown aerobically in a medium containing 0.4% w/v Bacto-Pepton (Difco, Detroit, MI, USA), 0.2% w/v yeast extract (Difco), 1% w/v K_2HPO_4, and 1% w/v glucose, as described [27]. Cells were broken in a French press. The cell envelope was pelleted at $160000 \times g$ and washed with 0.02 M Tris-HCl (pH 8)/0.01 M $MgCl_2$. The inner membrane and some outer membrane proteins were solubilized with 0.02 M Tris-HCl (pH 8) buffer, containing 1 mM EDTA and 2% w/v SDS, for 30 min at 35°C. Protein content was determined according to the method of Lowry et al. [28]. The protein concentration was adjusted to 2 mg/ml. After centrifugation, the pellet contained primarily porin bound to peptidoglycan. The porin was solubilized with 0.02 M Tris-HCl (pH 8), 0.5 M NaCl, 1 mM EDTA, and 2% w/v lauryldimethylamine oxide (LDAO) for 30 min at 35°C. LPS was assayed by specific silver staining of SDS-gels [29]. Further purification was done by anion exchange chromatography (Q-Sepharose, Pharmacia, Freiburg, Germany). The column was equilibrated with 0.02 M Tris-HCl (pH 8), 1% w/v LDAO. Porin was eluted with a linear 0–1 M NaCl gradient using the same buffer as above. Subjection of the LDAO extract to anion exchange chromatography led to the elution of two bands in the NaCl gradient at 0.3 and 0.7 M, respectively. In SDS gel electrophoresis the first diffuse band showed only an LPS band running at the top. The second narrow band contained the porin. Single bands in SDS-PAGE were seen at apparent molecular masses of 81 kDa and 33 kDa without heating and after heating up to 100°C, respectively. The porin fraction contained no detectable amount of LPS, as shown by electrophoresis. In contrast, porin fractions before anion exchange chromatography were LPS contaminated. According to the detection limit of the silver staining method for R-type LPS given by Parr et al. [12], less than 1/50 mol LPS per mol of porin was present. For porin storage in 0.02 M Tris-HCl (pH 7.5), 0.3 M LiCl, 3 mM NaN_3, and 1% w/v octyl glucoside, LDAO was replaced by octyl glucoside via anion exchange chromatography.

Preparation of planar bilayers and electrical measurement

The planar bilayers were prepared according to the Montal-Mueller technique [25]. The apparatus for membrane formation consisted of two teflon compartments of 1.5 ml volume each, which were separated by a teflon septum (12.5 μm thick) with a small aperture (typically 110 μm diameter). Prior to membrane formation, the septum was pretreated with a hexane/hexadecane mixture (20:1, v/v). Membranes were finally formed by apposing the two identically or differently composed lipid monolayers, each spread on aqueous subphases, over the aperture. For the reconstitution of the lipid matrix of the outer membrane we used the LPS of the deep rough mutant of *Salmonella minnesota* R595 instead of the homologous LPS of *P. denitrificans* for the following reasons: to guarantee sufficient membrane stability, we are restricted with our system to the use of rough mutant LPS, which contains only two additional sugar units in the core region. To our knowledge, a deep rough mutant strain of *P. denitrificans* is not available. Lipid A is, however, known to be the most conservative part of LPS and therefore the use of the nonhomologous Re-LPS should not influence the system significantly. The inner leaflet of the outer membrane was reconstituted from a lipid mixture composed of PE, PG and DPG in a molar ratio of 81:17:2 (PL-membrane). This particular mixture corresponds to that determined for other Gram-negative bacteria, e.g., *Salmonella typhimurium* [30] (see Discussion). For electrical measurements, planar membranes were voltage-clamped via a pair of Ag/AgCl-electrodes (type IVM E255, Zak, Simbach, Germany) which were connected to the headstage of a BLM 120 bilayer membrane amplifier (Biologic, Claix, France) with a feedback resistor of 1 GΩ. For the conductance measurements, a voltage was applied to one electrode (*cis* compartment), while the other (*trans* compartment) was grounded. The potential could be varied in 1 mV steps by a built-in power supply or continuously via an external control voltage. The latter was connected to the membrane amplifier from an analog output card PCI 20093-W (Intelligent Instrumentation, Leinfelden-Echterdingen, Germany) plugged into an AT-compatible microcomputer system. Membrane current and holding voltage were stored on a DAT-tape recorder (DTR 1200, Biologic). The stored signals were sent to the microcomputer system filtered by a 4-pole low-pass Bessel filter (Ithaco Scientific Instruments, Ithaca, NY, USA), the corner of which was adjusted to 10 Hz (−3 dB) and digitized at a sampling rate of 10 Hz with a PCI 20089W-1 analog input board (Intelligent Instrumentation). Current was positive when cation flux was directed towards the grounded compartment.

For incorporation, different concentrations of porin

from *P. denitrificans* were diluted in an aqueous solution containing 150 μg/ml Triton X-100. In most cases porin was added to the grounded side of the membrane system (*trans* compartment) and the subphase was stirred for 30 s prior to measurement.

Membrane potentials

Lipid asymmetry may provoke a potential difference between the two surfaces of the bilayer membrane, the so-called intrinsic membrane potential. An asymmetric potential profile has been observed in membranes with an asymmetric distribution of the surface charge densities on the opposing monolayers [31], in membranes with a symmetric distribution of the charge densities but with an asymmetry with respect to the headgroup conformations of the two leaflets [32], and for symmetric membranes with asymmetry of the subphases [33]. An extreme asymmetry in charge densities as well as in headgroup conformations occurs for the asymmetric LPS/PL-bilayer. At neutral pH, of the phospholipids only the phosphatidylglycerol molecule carries one negative charge, whereas each Re-LPS molecule carries four negative charges. The resulting surface charge densities are -0.58 A s m^{-2} (-3.63 e_0/nm^2) for LPS and -0.05 A s m^{-2} (-0.31 e_0/nm^2) for the PL-mixture, respectively, since the molecular cross section of a Re-LPS molecule is 1.10 nm^2 [34] as compared to 0.55 nm^2 [35] of a diacyl phospholipid. It must be pointed out that for the calculation of the negative charges any possible neutralizing effect of Mg^{2+} has not been considered. This effect is difficult to assess under the experimental conditions where a LPS-monolayer faces a 10 mM $MgCl_2$ solution. The surface potential Φ_s of a lipid layer can be estimated from the Gouy-Chapman equation [36]

$$\Phi_s = \frac{2 \cdot k \cdot T}{e_0} \cdot \text{a sinh} \frac{1.36 \cdot \sigma}{\sqrt{C}} \quad (1)$$

where σ is the surface charge density in electronic charges per nm^2 and C the ionic strength of the membrane bathing solution. From this, the following values could be calculated for the subphase containing 0.1 M KCl and 0.01 M $MgCl_2$ at 37°C used for the measurement of current–voltage (I–U) characteristics (Table 1). Further contributions to the intrinsic membrane potential are due to the dipole moments within the headgroup region, the carbonyl groups of the hydrocarbon chains causing a dipole potential, and from the interfacially bound water (for review on membrane electrostatics see Ref. [37]). The resulting intrinsic membrane potential profiles (Fig. 2) of different membrane types could be determined previously [38] from the shape of I–U curves of the carrier-ion complex valinomycin-K^+ using a fit procedure described by Schoch et al. [33]. Superimposed with the intrinsic membrane potential, an applied transmembrane voltage can decrease or increase the effective potential difference between the two leaflets and may thus change the electrical conditions influencing porin incorporation and function. These complex interrelations ought to be considered when the porin function is interpreted in terms of an effective membrane potential.

Table 1
Surface charge densities σ and resulting Gouy-Chapman potentials Φ_s of membrane surfaces composed of various phospholipids and of Re-LPS for a subphase containing 0.1 M KCl and 0.01 M $MgCl_2$ at a temperature of 37°C

Lipid:	PC	PL	PG	LPS
σ (e_0/nm^2)	0.00	−0.31	−1.82	−3.63
Φ_S (mV)	0	−55	−142	−178

PL represents the lipid mixture of PE, PG and DPG in a molar ratio of 81:17:2.

3. Results

Incorporation of the porin into different bilayer systems

For the experiments described in this section, all planar bilayer membrane systems were prepared on subphases containing 0.1 M KCl and 0.01 M $MgCl_2$ and at a temperature of 37°C. Addition of 5 ng/ml porin to the PL-side of asymmetric LPS/PL-membranes led to a rapid increase of membrane current which decelerated typically after some minutes (Fig. 3). Similar current responses could be obtained with asymmetric PG/PL- or symmetric PL/PL-membranes only when the amount of porin – added to either side of the bilayer – was increased. While the incorporation rates

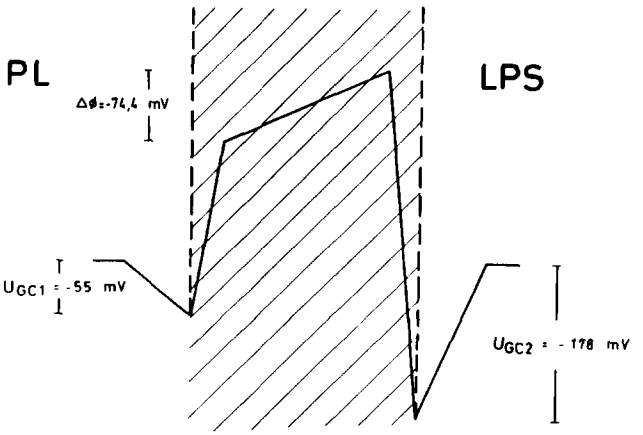

Fig. 2. Potential profile across an asymmetric lipopolysaccharide/phospholipid membrane as derived from measurements with the valinomycin-K^+ complex [38]. U_{GC}, Gouy-Chapman potentials at the membrane surfaces; $\Delta\Phi$, potential difference in the inner membrane region; PL, lipid mixture of PE, PG, and DPG in a molar ratio of 81:17:2.

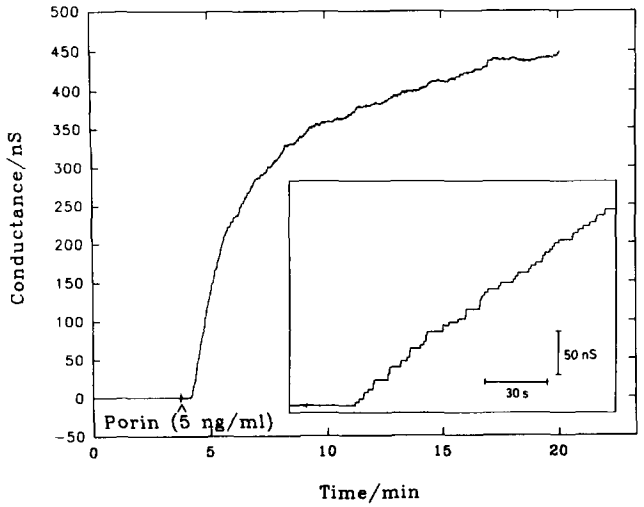

Fig. 3. Macroscopic increase in membrane current after addition of LPS-free porin (5 ng/ml) from *Paracoccus denitrificans* to the PL-side of an asymmetric LPS/PL-membrane (composition of the PL-mixture see Fig. 2). Transmembrane potential + 10 mV (LPS-side positive); subphase: 0.1 M KCl (unbuffered), $T = 37°C$. (Inset) Step-wise membrane current increase after addition of 0.1 ng/ml porin. Subphase 1 M KCl; other conditions unchanged.

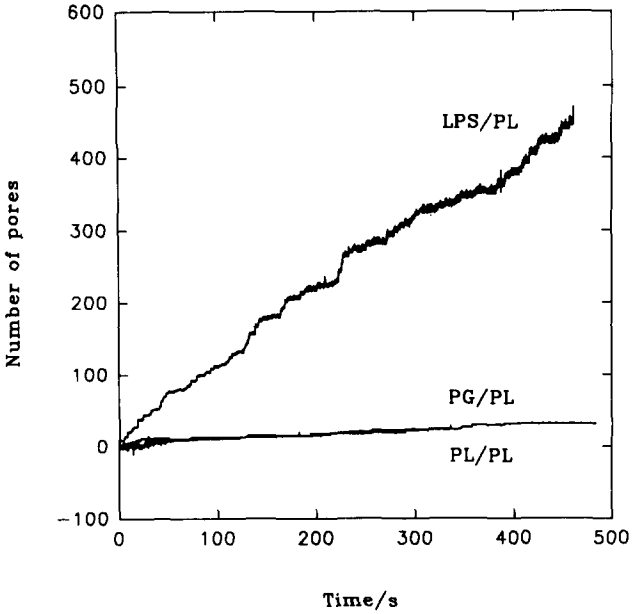

Fig. 4. Typical incorporation rates for LPS-free porin (5 ng/ml) into planar bilayers of different composition. Upper trace, asymmetric LPS/PL*-membrane; lower traces, phospholipid membranes (PG/PL* and PL/PL*) (* marks the side of porin addition; composition of the PL-mixture see Fig. 2). The derived incorporation rates were 55 pores/min and 4 pores/min, respectively. Transmembrane potential + 10 mV (opposite side to porin addition positive); subphase 0.1 M KCl, 0.01 M $MgCl_2$ (unbuffered); $T = 37°C$.

into the same bilayer system varied by up to a factor of five, in particular when they were low, the mean incorporation rate of porin added to the PL-side of the LPS/PL-membrane was found to be more than a factor of ten higher than for the membrane systems without any LPS. Fig. 4 shows a comparison of typical incorporation rates of the porin into the LPS/PL-bilayer and into the PG/PL- and PL/PL-systems, respectively. In all cases, the incorporation of porin was independent of an applied external membrane potential between −80 mV and + 80 mV. Out of this range no measurements were possible due to channel closing. Control experiments with pure detergent solution did not induce any effect. After addition of the same amount of porin to the LPS-side of the membrane system, no increase of membrane current could be detected over a comparable period of time (up to 1 h).

At lower porin concentrations and low clamp voltages (10–20 mV), step-wise current increase could be observed. From these current traces, single-channel conductance was derived. The histograms of the amplitudes of conductance steps are shown in Fig. 5. No significant differences in the single-channel conductance for porin incorporated into the various bilayer systems were observed. The pore diameter could be estimated as 1.5 nm using the relation [39]

$$\Lambda = \frac{\pi \cdot \sigma \cdot r^2}{l} \qquad (2)$$

between Λ (single-channel conductance), σ (specific

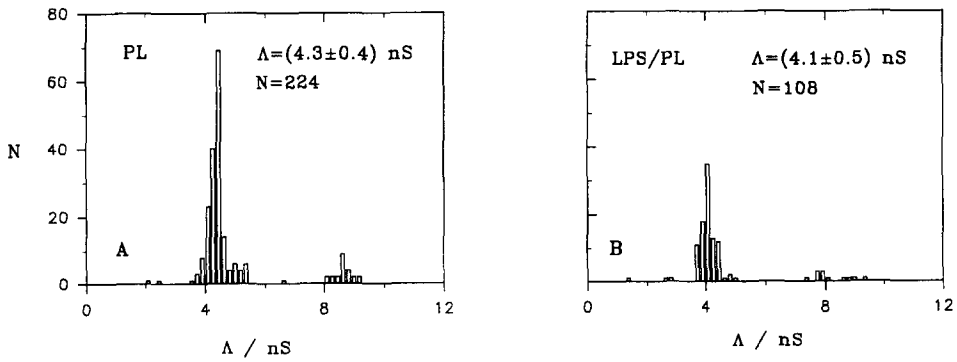

Fig. 5. Histograms of the amplitudes of conductance steps of membrane systems and derived single-channel conductance after addition of 10 pg/ml porin, other conditions as in Fig. 3. (A) Symmetric PL/PL-bilayer; (B) asymmetric LPS/PL-bilayer. Composition of the PL-mixture see Fig. 2.

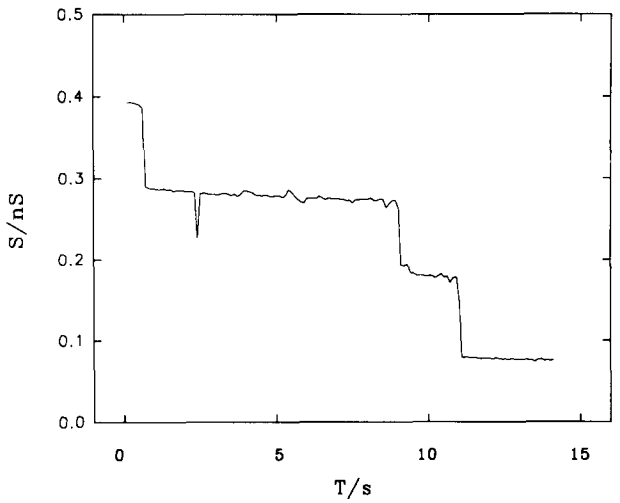

Fig. 6. Voltage-induced closing of a single porin pore in an asymmetric LPS/PL-membrane at an applied voltage $\lesssim -100$ mV on the LPS-side. Experimental conditions as in Fig. 3.

conductance of the subphase) and $l = 6$ nm (length of the pore) with a membrane thickness of 6 nm and assuming that the length of the pore corresponds to the membrane thickness.

Concentration dependence and selectivity

Single-channel conductivity was tested with symmetric lipid bilayers composed of PE, PC, and cholesterol in a molar ratio 81:17:2 for various subphases containing different salts in different concentrations at 25°C (cholesterol was used for reasons of membrane stability in those cases in which the subphase did not contain Mg^{2+}). The results are summarized in Table 2. The temperature of 25°C was chosen for reasons of better comparability with literature data. As will be shown later, Λ and σ had a similar temperature dependence, hence the resulting Λ/σ values were temperature-independent. The ratio Λ/σ did not vary significantly and the average pore conductance was a linear function of the specific conductance of the subphase. The pore did not show any salt specificity. In experiments with a 10-fold concentration gradient between the two compartments no significant zero-current potential was measured.

Table 2
Mean single-channel conductance of porin isolated from *P. denitrificans* for different subphases

Subphase	Λ (nS)	σ (mS·cm^{-1})	Λ/σ (10^{-8} cm)
0.05 M KCl	0.16	6.6	2.43
0.1 M KCl	0.31	12.6	2.46
0.2 M KCl	0.72	24.4	2.95
0.5 M KCl	1.60	58.2	2.75
1.0 M KCl	3.29	112.6	2.92
1.5 M KCl	4.96	164.1	3.02
2.0 M KCl	6.31	211.0	2.99
1.0 M NaCl	2.34	85.3	2.74
0.5 M $MgCl_2$	1.87	62.0	3.02

The porins were reconstituted into symmetric membranes composed of PE, PC, and cholesterol in a molar ratio 81:17:2 using the different unbuffered subphases at 25°C and a clamp voltage of 10 mV. Each value represents the average of at least 50 single-channel events.

Voltage-dependent activity

Porin channels showed voltage-dependent activity when reconstituted into either symmetric or asymmetric bilayer membranes. For all membrane systems tested, at sufficient-high clamp voltages single-channel closing in three discrete steps could be observed. This is shown in Fig. 6 for a porin channel reconstituted

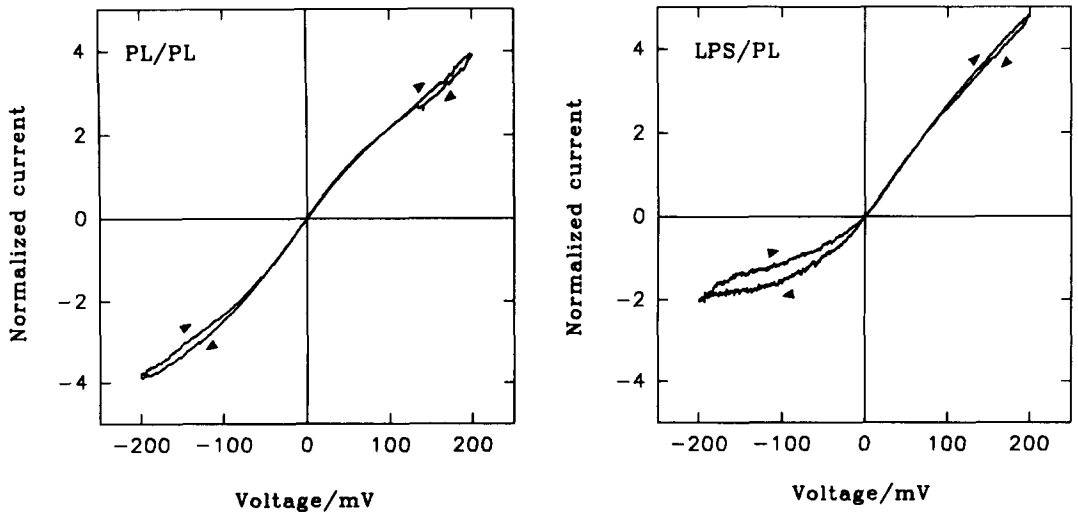

Fig. 7. *I–U* curves of porin-doped symmetric PL/PL*- and asymmetric LPS/PL*-membranes (* marks the side of porin addition). Current is normalized to the value at 40 mV (i.e., when all channels are open). Subphase: 0.1 M KCl, 0.01 M $MgCl_2$ (unbuffered), $T = 37$°C; PL, lipid mixture of PE, PG, and DPG in a molar ratio of 81:17:2.

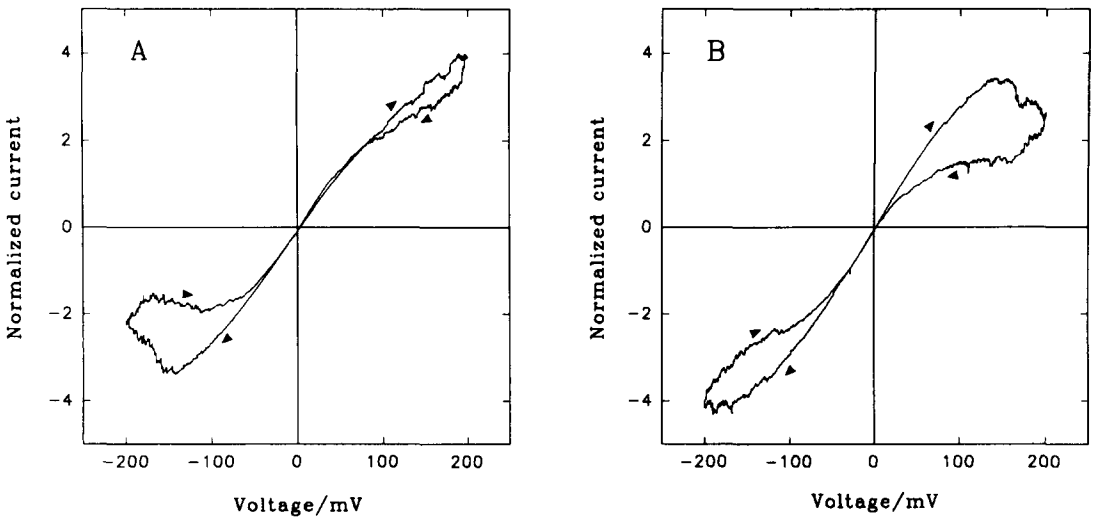

Fig. 8. *I–U* curves of porin-doped PG/PL-membranes in dependence on the side of porin addition (5 ng/ml). Porin added to: the PL-side (A), the PG-side (B). Experimental conditions as in Fig. 7.

into an asymmetric LPS/PL-membrane. Within our detection limits, all closing steps had the same amplitude and were reversible. To compare the amplitude of the voltage necessary for channel gating, we have measured *I–U* curves for the three membrane systems LPS/PL, PG/PL and PL/PL. A low amount of porin (0.05–0.1 ng/ml) was added to the PL-side and a voltage ramp was driven (4 mV/s) when up to three pores were incorporated. The gating voltages differed for the various bilayer systems. They depended on the asymmetry in the charge densities of the two membrane leaflets. For symmetric PL/PL-membranes (charge density $-0.31\ e_0/nm^2$ on both sides), the necessary clamp voltages were $\gtrsim +160$ mV and $\lesssim -160$ mV. For the asymmetric LPS/PL-bilayer system (charge density on the LPS-side $-3.63\ e_0/nm^2$), an asymmetry in the closing voltage $\gtrsim +170$ mV and $\lesssim -100$ mV, respectively, was observed. In all cases, the PL-side was on ground potential.

To study the effect of membrane charge density on the voltage-dependent activity of the porin channel, we also have measured *I–U* curves when the porin incorporation after addition of a higher porin concentration (5 ng/ml) had decelerated or even stopped. The recorded traces (Figs. 7 and 8) exhibited a hysteretic behaviour in each case. Going from zero voltage in either direction to approximately +100 mV or −100 mV, for all membrane systems and independent of the side of porin addition the measured membrane current changed proportional to the applied voltage. Above this range, an underproportional increase, a steady state, or a decrease in membrane current was observed

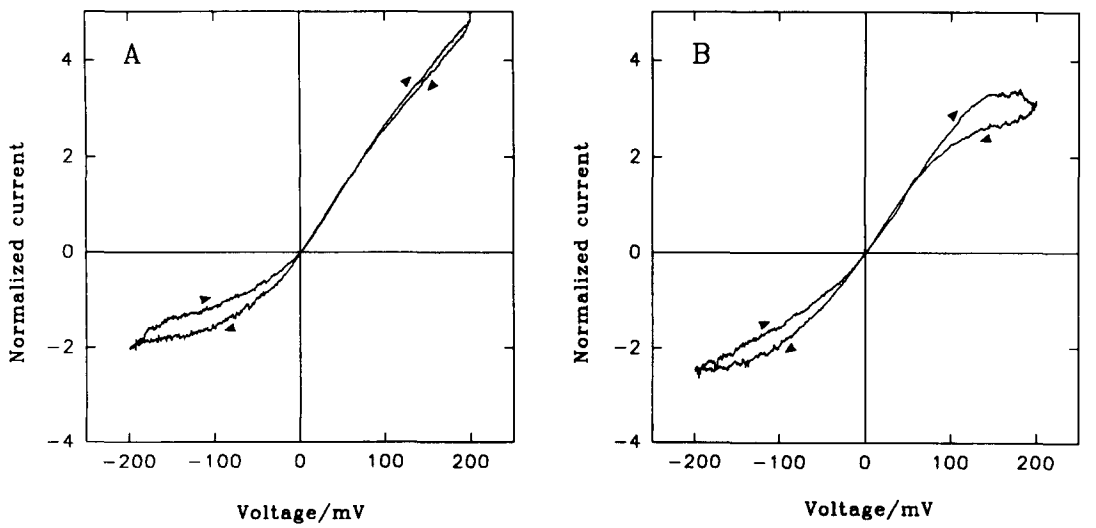

Fig. 9. *I–U* curves of porin-doped asymmetric LPS/PL-membranes before (A) and after (B) addition of 5 μM PMBN to the LPS-side. Experimental conditions as in Fig. 7.

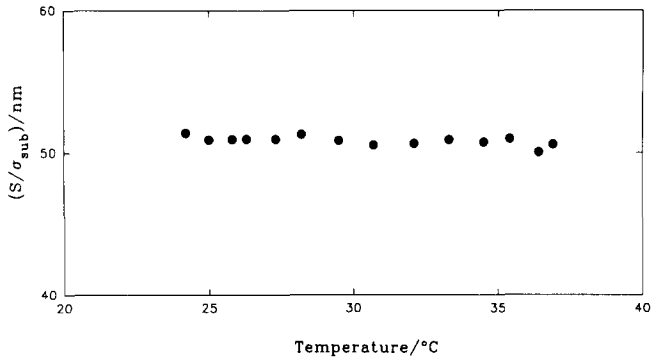

Fig. 10. Conductance S of a porin-doped LPS/PL-membrane normalized to the specific conductance σ of the subphase in dependence on temperature. Subphase: 0.1 M KCl, 0.01 M $MgCl_2$ (unbuffered). PL, lipid mixture of PE, PG, and DPG in a molar ratio of 81:17:2.

with increasing voltage, dependent on the membrane composition. At voltages $\lesssim -100$ mV, corresponding phenomena were observed but in opposite direction. The asymmetry in the shape of the recorded I–U curves, i.e., the voltage at which channel closing started and the area of the hysteresis slopes, again depended on the asymmetry in the charge densities of the membrane leaflets and, in the case of asymmetric PG/PL-membranes, on the side of porin addition (porin did not incorporate into LPS/PL-bilayers when added to the LPS-side).

From the addition of a polycationic substance to a negatively charged membrane, it may be expected that the effective negative surface charge is reduced and this way further information on the influence of the charge density on porin function may be obtained from changes in the respective I–U curves. To this end, we have added the nonapeptide of the polycationic antibiotic polymyxin B, PMBN – which itself does not induce conductance changes [40] – to the LPS-side of LPS/PL-membranes. As can be seen from Fig. 9, the addition of PMBN led, in this case, to a more symmetric gating behaviour.

Influence of LPS phase state on porin activity

Porin incorporation into LPS/PL-membranes (from the PL-side) was even found at 24°C, i.e., below the temperature of the gel to liquid-crystalline phase transition of the LPS fatty acid chains [34]. The open-channel conductivity measured at an applied voltage of 10 mV increased with increasing temperature in the same way as the subphase conductivity (Fig. 10), whereas voltage-dependent gating changed in the case of the LPS/PL-membrane (Fig. 11). The shape of I–U curves for PL/PG-membranes, which exhibit no phase transition in the range from 24°C to 37°C, was not affected by temperature (not shown).

4. Discussion

The aim of this study was to elucidate the role of the LPS-leaflet for the incorporation of porin channels into and their function in the outer membrane of Gram-negative bacteria. To this end, we have reconstituted the LPS-free porin from *P. denitrificans* into various lipid bilayer systems, including an asymmetric lipid matrix of the outer membrane common to a variety of Gram-negative species, determined the incorporation rate, and studied the closing behaviour dependent on the different intrinsic and effective transmembrane potentials. Our results show that LPS-free porin from *P. denitrificans* builds water-filled channels in artificial bilayer membranes (Figs. 3 and 5)

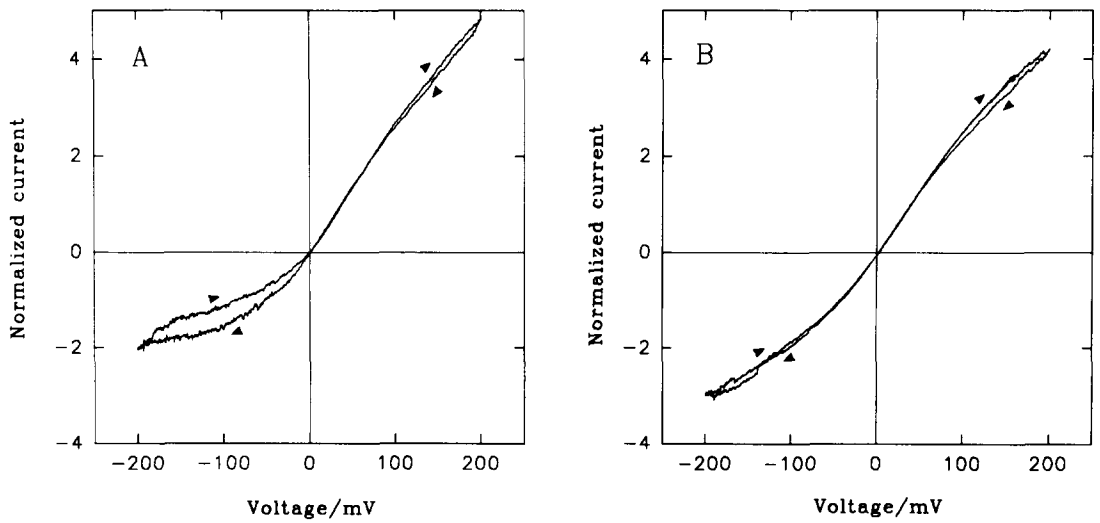

Fig. 11. I–U curves for a porin-doped LPS/PL-membrane (as indicated in Fig. 2) above (A) and below (B) the temperature T_c of the $\beta \leftrightarrow \alpha$ phase transition of the acyl chains. Experimental conditions as in Fig. 7.

as it has been described for other porins from Gram-negative bacteria (for review see, e.g., Refs. [5–8]).

From the dependence of the single-channel conductance (Table 2) on the specific conductance of the subphase, the vanishing zero-current potential in the presence of a salt gradient, and the ohmic behaviour of the *I–U* curves in the non-closing voltage range (for example see Fig. 7), the ion mobility in the pore may be deduced to be the same as in the subphase. Assuming a cylindrical cross section and a length of 6 nm with Eq. (2) we arrived at an effective channel diameter of the porin trimer of 1.5 nm, a value in good agreement to that determined by Zalman and Nikaido [27] with the liposome swelling method. The BLM-studies of Woitzik et al. [41] on the porin of the closely related *R. capsulatus* [42] also led to the same pore size. For this porin, the crystal structure could be determined at 0.18 nm resolution [14–16]. The crystallographic data suggest that the three entries are located at the periplasmic side and have a diameter of 0.7 nm to 0.9 nm, each. According to this, the effective diameter can be calculated to 1.2–1.6 nm assuming one water-filled channel. With this value the BLM-data would be confirmed.

In agreement with investigations on other porins [43], we have not seen any activity of protein monomers. Moreover, contradictory to the experimental evidence published by Zalman and Nikaido [27] that porin from *P. denitrificans* builds dimeric pores, single channel closing in three steps (Fig. 6) is typical for the trimeric porin complex [4,44] and is, again, in accordance with the crystallographic data.

In contrast to observations on porin of *R. capsulatus* [41], we could not obtain evidence for a selectivity for cations. Furthermore, since the investigated porin was proven to be LPS-free (see Materials and methods), we conclude that LPS is not required for porin activity. This result is in accordance with that of Parr et al. [12] on reconstituted LPS-free porin from *E. coli* OmpF and *Pseudomonas aeruginosa* protein P, but in contrast to that of Buehler et al. [10].

LPS does, however, influence other parameters such as the incorporation rate and the gating behaviour. The incorporation rates into LPS-free phospholipid bilayer systems are more than a factor of ten lower than into asymmetric membranes with an LPS-leaflet on the opposite side (Fig. 4). This observation is a strong indication for specific interactions between the porin molecules with the LPS-leaflet and is in correspondence to the model of Weiss et al. [15,16], who propose that the very polar zone on the outer surface of the β-barrel with its numerous negatively charged residues is directed towards the extracellular medium. The carboxylate groups of the porin are, according to these authors, likely to participate in the strong and tight network of divalent cation and carboxylate groups within the layer of LPS-core units in such a way that the interface between these core units and porin would become as tight as the LPS-layer itself.

The differences in the incorporation rates for the different membrane systems cannot be accounted for by differences in the surface charge densities (Table 1) between the two membrane leaflets of each system, because no differences in the incorporation rates into PG/PL- and PL/PL-bilayers, respectively, were observed. This implies the existence of specific forces dragging the porin molecules into the bilayer when they reach the LPS-leaflet. The complete prevention of porin incorporation into the reconstituted lipid matrix of the outer membrane from the LPS-side may be explained in several ways. Benz reported on lipid-dependent incorporation rates for *S. typhimurium*, *E. coli* and *Ps. aeruginosa* porins into black lipid membranes [5]. From his results it could be deduced that porins incorporate into membranes formed by lipids with smaller headgroups at a higher rate. Other reasons might be the rigidity of the LPS-leaflet or the steric hindrance caused by the sugar moiety of the LPS. The prevention of incorporation from the LPS-side has no implication for the biological system, because there the porins are always offered from the periplasmic, i.e., the PL-side of the outer membrane. These findings are in accordance with the observation that an external potential applied to the bilayer system and differently charged phospholipids do not influence the incorporation.

The deceleration of porin incorporation following the linear phase (Fig. 3), typical for all bilayer systems, is not yet understood. Since the conductance steps indicating porin insertion are always directed upwards, the decreasing incorporation rate can, however, not be explained by steady-state processes, in which porin incorporation and release are in equilibrium.

A further aim of our investigations was to elucidate the mechanism responsible for voltage-dependent gating of the porin channel, particularly the influence of LPS. In the literature, gating effects have been discussed controversially. Various groups have examined the voltage-dependent closing of porin channels, especially that of *E. coli* OmpC and OmpF proteins, utilizing different techniques. These included patch-clamp studies both with giant *E. coli* cells or spheroplasts [23], or purified porins OmpF and OmpC reconstituted via outer membrane vesicles into liposomes enlarged to giant proteoliposomes [21,22] as well as electrical measurements on porin channels reconstituted into planar bilayers by different techniques [17]. For most of these systems, gating effects have been reported at voltages $\gtrsim 100$ mV. Sen et al. [45] tried to clarify the biological relevance of the voltage-dependent behaviour of porins by measuring the permeability of porin channels in intact cells at different Donnan potentials. They con-

cluded that voltage gating is not found in vivo but should rather be interpreted as artifact of the reconstitution method used. Very recently, Brunen and Engelhardt [24] have published values of gating voltages of as low as −10 mV for the anion-specific porin Omp34 isolated from *A. delafieldii* and reconstituted into symmetric phospholipid bilayer matrices. Furthermore, they found the gating behaviour to be asymmetric. This they took as evidence for unidirectional orientation of the porin.

With our experimental setup we were able to study gating effects with respect to bilayer asymmetry, in particular with respect to the resulting charge distribution and the arising potential gradient. We have observed voltage-dependent gating for porin channels in symmetric as well as in asymmetric bilayers (Fig. 7); however, this occurred at different voltages in the differently composed systems. Taking into account the surface charges and Gouy-Chapman potentials derived therefrom, a correlation between the heights of the surface potential on the one hand and the shape of the recorded *I–U* curves on the other can be stated. With increasing difference in the Gouy-Chapman potential between both bilayer leaflets from 0 mV in the case of the symmetric PL/PL-membrane over −87 mV for the PG/PL-membrane (PG-side negative) to −123 mV on the LPS-side of the LPS/PL-membrane, the asymmetry in the shape of the *I–U* curves increases. The addition of the polycationic antibiotic derivative PMBN to the LPS-side of the asymmetric LPS/PL-bilayer system diminished the asymmetry of the *I/U* curve (Fig. 9). From this we concluded that the sum of the applied and the intrinsic potentials at the surface, fitted by the Gouy-Chapman potential, may be a determinant factor for channel closing. The influence of the Gouy-Chapman potential agrees well with the observed PMBN-effect in that the four positive charges of each PMBN-molecule may neutralize the negative surface charge of the LPS-leaflet. Our results for an applied negative external potential are in agreement with the model proposed by Brunen and Engelhardt [24] which is based on findings by Weiss et al. [16] from crystallographic data. Brunen and Engelhardt propose that gating occurs due to conformational changes in the negatively charged loops within the so-called 'eyelet' [46]. This eyelet defines the size of the porin channel. It is lined by negatively charged groups of two loops and by positively charged groups in the channel wall. A negative potential difference would provoke a force on the negatively charged loops in each monomer driving them towards the fixed positive charges which obviously leads to a switch-like channel gating process. The observed channel closing in three steps (Fig. 6) is further experimental proof that the gating mechanism must be located in the region of the three separated channels.

In contrast, the interpretation of the results with an applied positive potential is difficult in terms of the above model. Under these conditions, the negatively charged loops should be driven away from the fixed positive charges. Furthermore, simple addition of the applied external potential and the intrinsic Gouy-Chapman potential to an effective potential does not acount for the gating effects observed in the different membrane systems. At present, we have no conclusive explanation for this. It may be speculated that the cations in the subphase are driven towards the membrane surface leading to a neutralization of the negatively charged membrane surface with the consequence that the surface potential decreases very rapidly.

From our experiments with symmetric PL/PL-membranes (Fig. 7) it can be derived that an applied potential difference of at least 160 mV is required for channel closing, independent of the direction of the potential gradient. This fact could be due to a random orientation of the porin molecules in the lipid bilayer or a symmetric gating behaviour of the porin channel itself. From changes in the asymmetry of the *I–U* curves of PG/PL-bilayer systems, however, which depended on the side of porin addition (Fig. 8), we obtained unequivocal proof for a directed insertion of the porin molecules into the bilayer. Porins oriented randomly or in dependence on the surface charges would have to follow the same gating processes independent of the side of addition. From the differences in the gating voltage at an applied negative potential and under the assumption that the gating model discussed above is valid, it may be proposed that the porin is inserted with the single opening in front, i.e., in the case of the LPS/PL-system - and with that in the outer membrane – the porin channel would be oriented with the three openings directed to the periplasmic space. This proposal is in accordance with the orientation deduced by comparison of topological data of PhoE porin from *E. coli* by Tomassen [47] with the structure of porin from *R. capsulatus* [48].

The measured gating voltages for porins incorporated in an LPS/PL-bilayer imply, that in the natural system gating would occur, if the bacterial cell managed to depress the potential at the LPS-surface by approximately −100 mV. This value is about 10-fold higher than that found by Brunen and Engelhardt [24] for Omp34. Because the applied salt concentrations were nearly identical in the two systems, this discrepancy can only be refered to functional differences of the two porins. Independent of these discrepancies in the amplitudes of the required gating voltages, the physiological implications of a negative potential at the extracellular side of the membrane has to be discussed. In this context, corresponding results which we obtained when investigating the effect of polymyxin B (PMB) on the reconstituted outer membrane [40] might

merit attention. Also in this system we found that the effect of PMB could be increased when the potential at the LPS-surface was negative with respect to the PL-side. At present, we are not able to offer a fully satisfying explanation for these observations. However, it might be interesting to discuss a possible contribution of the Bayer's junctions [49] for the adjustment of the potential of the outer membrane. Thus, in a similar way as proposed for the role of the contact sites between the inner and the outer membrane of mitochondria which should transduce the potential of the inner membrane to the outer membrane by capacitive coupling [50], the Bayer's junctions may function as electrical transducers.

LPS from *S. minnesota* R595 undergoes a gel ↔ liquid ($\beta \leftrightarrow \alpha$) phase transition of the hydrocarbon chains at about $T_c = 32°C$ [34]. When the LPS/PL-bilayer – with the incorporated pores – was cooled down below T_c, we could observe this phase transition reflected in porin gating behaviour. The typical hysteretic effects in the recorded I–U curves, caused by a delayed reopening of the porin channels with respect to channel closing, was lost in the asymmetric LPS/PL-membranes at temperatures below approximately 30°C (Fig. 11) but reappeared when the system was reheated to $T > T_c$. By relating the membrane conductance to the specific conductance of the subphase for the different temperatures, the size of the open channel was found unchanged within this temperature range (Fig. 10). In contrast, in membranes built up from natural phospholipids not exhibiting a $\beta \leftrightarrow \alpha$ phase transition in this temperature range, no changes in the shape of the I–U curves were seen (data not shown). This is in accordance with the concept that the gating behaviour is influenced by a phase transition within the membrane lipids but not by one within the porin channel itself. It must, however, be considered that the fluidity within the outer membrane does not change in a step-like manner between the two leaflets, the outer leaflet being the more rigid one, but rather increases gradually towards the periplasm (Eberstein, W., diploma thesis, University of Kiel, Germany). Thus, at $T = 24°C$ the I–U curve for a porin-doped LPS/PL-membrane is still nonlinear and asymmetric with a lower conductance at high voltages. The high negative voltages seem still to change the channel properties either by narrowing the pore diameter or by fully closing single pores, but with a shorter lifetime for the closed state with respect to the lifetime at higher temperatures.

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6. References

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