Electrostatic Potential Barrier in Asymmetric Planar Lipopolysaccharide/ Phospholipid Bilayers Probed with the Valinomycin-K^+ Complex

Ulrich Seydel, Wolfram Eberstein, Guido Schröder, and Klaus Brandenburg
Forschungsinstitut Borstel, Parkallee 10, D-W-2061 Borstel, Bundesrepublik Deutschland

Z. Naturforsch. **47c,** 757–761 (1992); received March 9/June 29, 1992

Lipopolysaccharide, Planar Asymmetric Membrane, Membrane Potential Barrier, Valinomycin

Using the carrier-ion complex valinomycin-K^+, current/voltage *(I/U)* characteristics were registered for planar asymmetric lipid bilayers composed on one side of a phospholipid mixture and on the other side of rough mutant lipopolysaccharide. This system resembles the lipid matrix of the outer membrane of Gram-negative bacteria. The evaluation of the current/voltage curves yielded a highly asymmetric electrical potential barrier. The total potential difference between the phospholipid and the lipopolysaccharide was −85 mV, a result which cannot be explained by contributions of Gouy-Chapman potentials alone. The possible contribution of dipole potentials and influences of headgroup effects are discussed. It is shown that the asymmetry of the *I/U*-characteristic results from the differences of the surface charge densities of the two monolayers but not from those of the states of order of their hydrocarbon chains.

Introduction

Electrostatic phenomena in membranes play a crucial role in many processes ranging from binding of charged species, over insertion and orientation of integral membrane proteins to membrane transport [1]. The electrostatic properties of lipid bilayers are determined by the surface density of charges and their distribution within the headgroups of the particular lipid molecule along the membrane normal as well as over the two leaflets.

Electrostatic properties can be probed with carrier ion complexes *via* the measurement of current/voltage characteristics (*I/U*-characteristics). For a symmetric lipid bilayer (under symmetric solution conditions) symmetric *I/U*-characteristics are obtained from which a symmetric trapezoidal potential barrier for the carrier-ion complex can be derived.

I/U-curves of membranes with an asymmetric lipid distribution may be asymmetric. This can be refered to an asymmetric potential barrier. Asymmetric potential barriers have been observed in membranes with an asymmetric distribution of the surface charge density [2], in membranes with a symmetric distribution of the charge density but with an asymmetry with respect to the headgroup conformation of the two leaflets [3] as well as for symmetric membranes with solution asymmetry [4].

An extreme asymmetry both in the charge density and the headgroup conformation is exhibited in the lipid matrix of the outer membrane of Gram-negative bacteria. The inner leaflet of this membrane is composed of a phospholipid mixture (PL) of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in a molar ratio of 81 : 17 : 2 [5]. The outer leaflet is composed exclusively of lipopolysaccharide (LPS). Under physiological conditions, of the phospholipids only phosphatidylglycerol molecules carry one negative charge each, whereas each LPS molecule carries at least 4 negative charges. Since the LPS molecule requires approx. twice the surface area (1.10 nm^2, [6]) as compared to diacyl phospholipids with the same length of the hydrocarbon chains (0.55 nm^2, [7]), the resulting specific surface charge densities are −0.61 As/m^2 (−3.81 e_0/nm^2) and −0.05 As/m^2 (−0.31 e_0/nm^2), respectively.

Furthermore, LPS differs considerably from phospholipids in its chemical structure and its conformation. It is composed of an oligo- or polysaccharide moiety, which is covalently linked to a lipid component termed lipid A which anchors the LPS molecule in the membrane [8]. Lipid A con-

Abbreviations: LPS, lipopolysaccharide; PL, phospholipid mixture; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; Kdo, 2-keto-3-deoxyoctonate.

Reprint requests to Prof. Dr. U. Seydel.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/92/0900–0757 $ 01.30/0

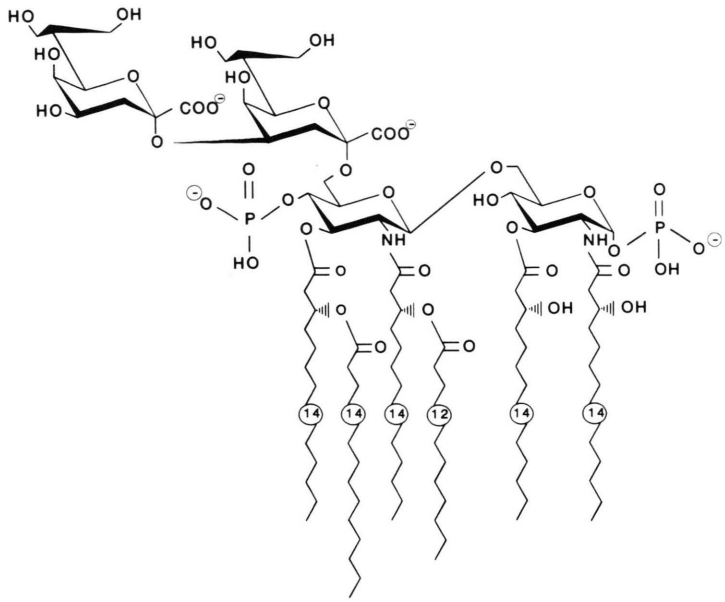

Fig. 1. Chemical structure of deep rough mutant lipopolysaccharide Re from *Escherichia coli* strain F 515.

sists of a β-D-glucosaminyl-(1→6)-α-D-glucosamine disaccharide, phosphorylated in positions 1 and 4′, which carries in ester and amide linkage up to seven hydroxylated and non-hydroxylated saturated fatty acid residues [9]. The length and composition of the sugar moiety depends on the kind of the bacterial mutant. In the present investigation, mainly deep rough mutant LPS Re was used consisting of the lipid A moiety and two additional 2-keto-3-deoxyoctonate (Kdo) monosaccharides.

From the unusual architecture of the outer membrane, electrostatic characteristics deviating from those known for various phospholipid membrane systems should be expected. To obtain information on the role of LPS for the electrical properties of the outer membrane, we have measured I/U-characteristics for asymmetric LPS/PL bilayers composed of deep rough mutant LPS Re doped with the K^+-carrier valinomycin. The evaluation of the asymmetric I/U-characteristic gave evidence for an extremely high dipole potential of the LPS leaflet.

Materials and Methods

Preparation of membranes and electrical measurements

Asymmetric planar bilayers were prepared essentially according to the Montal-Mueller method as has been previously described in detail [10]. Briefly, asymmetric bilayers were formed by apposing monolayers prepared on two aqueous subphases from chloroform solutions of LPS and PL, respectively, at a small aperture punched into a thin teflon foil (12.5 μm thickness). Clear solutions of LPS Re in chloroform/methanol (9:1 by volume) were obtained by heating the suspension to 80 °C for 5 min. Diameters of the aperture were typically 200 μm and the teflon foil was pretreated with a 20:1 (vol/vol) hexane/hexadecane solution. The phospholipid mixture PL consisted of bacterial phosphatidylethanolamine (PE) from *E. coli,* phosphatidylglycerol (PG) from egg yolk lecithin and cardiolipin from bovine heart in a molar ratio of 81:17:2. These lipids as well as phosphatidylcholine (PC) from bovine brain and dipalmitoyl phosphatidylcholine (DPPC) from egg yolk PC were purchased from Sigma (Deisenhofen, F.R.G.). Deep rough mutant LPS Re from *E. coli* strain F 515 (chemical structure see Fig. 1) was extracted by the phenol/chloroform/petrol ether method [11], purified and lyophilized according to standard procedures [12]. For the evaluation of the I/U-curves only such experiments were used, in which the first attempt to form a stable membrane was successful. This way, a possible lipid exchange between the two compartments of the test chamber was excluded. The membrane area was typically 2×10^{-2} mm^2.

As aqueous subphase Hepes buffer was used containing 100 mM KCl and 10 mM $MgCl_2$, but no Ca^{2+}. The pH was adjusted to 7. $MgCl_2$ served for a stabilization of the LPS leaflet. The valinomycin concentration was 2×10^{-7} M.

The electrical measurements were performed using a voltage clamp set-up connected to the membrane *via* a pair of Ag/AgCl-electrodes. To avoid a hysteresis in the I/U-curves, the clamp voltage was changed at a sweep rate of 4 mV s^{-1}. For the evaluation, both the clamp voltage and the current signal were digitized and stored with a microcomputer system.

The voltage is defined as positive when the current flows from the PL side across the membrane to the LPS side. Measurements were conducted at 37 °C, *i.e.* in the liquid crystalline state of the hydrocarbon chains of as well PL as of LPS [6].

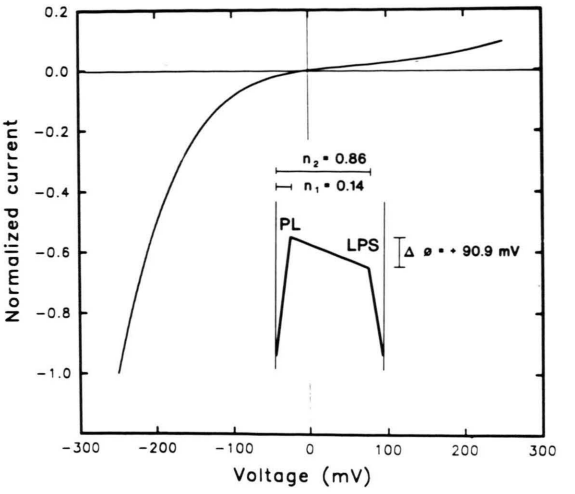

Fig. 2. Calculated current/voltage curve for the asymmetric planar phospholipid/lipopolysaccharide membrane according to Eqn. (1) on the basis of the Gouy-Chapman potentials. Insert: the corresponding potential profile.

Theory

The evaluation of the I/U-curves was done according to a protocol by Schoch *et al.* [4]. In the approximation given in this reference, the current I is given as function of the voltage U applied with the voltage clamp by

$$I = K \cdot \frac{(\Delta\Phi + (n_2 - n_1) \cdot U)}{n_2 - n_1} \cdot \frac{e^{\frac{1}{k \cdot T} \cdot U} - 1}{e^{\frac{1}{k \cdot T} \cdot (\Delta\Phi + n_2 \cdot U)} - e^{\frac{1}{k \cdot T} \cdot n_1 \cdot U}}, \quad (1)$$

where K is a constant for each individual membrane (depending, among others, on the absolute area and the thickness). The meaning of the parameters n_1, n_2 and $\Delta\Phi$, which describe the shape of the trapezoidal energy barrier, is illustrated in Fig. 2 (for details see [3, 4]). T and k have their usual meanings. The parameters n_1, n_2 and $\Delta\Phi$ were determined from the experimental curves by computer fitting to Eqn. (1).

For the interpretation of the experimental curves it is useful to calculate the surface potentials Φ_S on the LPS and PL side, respectively, which can be estimated from the Gouy equation [13]

$$\Phi_S = \frac{2 \cdot k \cdot T}{e_0} \cdot asinh \frac{1.36 \cdot \sigma}{\sqrt{C}}, \quad (2)$$

where σ is the surface charge density in electronic charges per nm^2 and C the ionic strength of the membrane bathing solution. For the LPS side ($\sigma = -3.81\ e_0/nm^2$) a value of -53.2 mV is derived resulting from the contributions of the monovalent K^+ (0.1 M) and divalent Mg^2 (0.01 M). Fig. 2 shows in the insert for comparison with the experimental results the asymmetric barrier profile when exclusively the asymmetry of the surface potentials is taken into account, and the corresponding I/U-curve obtained from Eqn. (1) with $n_1 = 0.14$, $n_2 = 0.86$ [4]. The value of $\Delta\Phi_S$ was set to 90.9 mV on the basis of the following consideration: the left corner of the potential barrier is lowered by a potential $n_1 \cdot \Delta\varrho_S$ ($\Delta\varrho_S$ = (179.4−53.2) mV, surface potential difference) and the right corner by a potential $n_2 \cdot \Delta\varrho_S = (1 - n_1) \cdot \Delta\varrho_S$, resulting in a potential difference between the two corners of $(1 - 2 \cdot n_2) \cdot \Delta\varrho_S$.

Results and Discussion

In Fig. 3, the I/U-curve for the asymmetric LPS/PL bilayer doped with valinomycin is plotted. Apparently, the course of the measured I/U-curve shows an inverse behaviour to that of Fig. 2 which was calculated exclusively on the basis of the estimated Gouy-Chapman potentials.

From the fit of the experimental data to Eqn. (1), an actual potential difference between the left and right side of the potential barrier, respectively, of $\Delta\Phi = -85$ mV was obtained, and the parame-

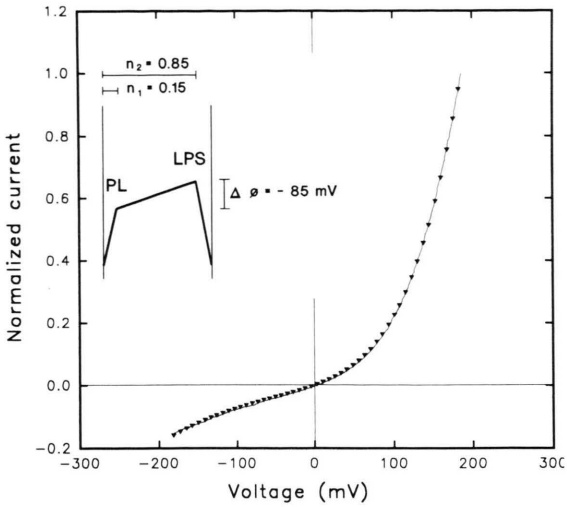

Fig. 3. Measured current/voltage curve for the asymmetric planar phospholipid/lipopolysaccharide membrane doped with valinomycin. Insert: actual potential profile calculated from a fit of the experimental data to Eqn. (1).

ters n_1 and n_2 were determined to $n_1 = 0.15$ and $n_2 = 0.85$. With these values of n_1 and n_2, a best fit of the measured data was achieved, however, the influence of these parameters on the quality of the fit was relatively weak. This implies that the potential borders are not well defined. From the conformation of the LPS molecule and its orientation in the membrane, it is intelligible that the border between the hydrophilic and hydrophobic moieties extends over a relatively wide range, due to the inclination of about 45° of the disaccharide backbone of the lipid A part with respect to the membrane normal [14].

The comparison of the actual barrier shape (insert of Fig. 3) with that of the estimated (Fig. 2) shows, that the left corner of the former is lowered by a potential of approx. −176 mV. For this discrepancy, the following explanations are proposed:

1. This potential may result from contributions of electrical dipole moments, which themselves are provoked by the particular charge distribution of the headgroup of the LPS Re exhibiting drastical differences as compared to phospholipids. The electrical charge is distributed over a wide region along the membrane normal as well as laterally. The extension (length) of the headgroup of LPS Re was calculated with energy minimization procedures to approximately 0.9 nm [15].

2. It has been shown that many physicochemical parameters of LPS Re (*e.g.* phase transition temperature, enthalpy and the three-dimensional supramolecular structure) undergo considerable changes up to water concentrations of approx. 60% [16, 17]. This may be explained by binding of a large number of water molecules in the headgroup area of LPS. A fraction of these bound water molecules may be oriented and, thus, contribute to a resulting electrical dipole moment.

The LPS leaflet of the reconstituted outer membrane has a higher state of order of the acyl chains at the measuring temperature of 37 °C than the phospholipid leaflet [18]. To rule out the possibility that the observed asymmetry in the *I/U*-curves results from this asymmetry in the state of order, we have performed a number of experiments with phospholipid membranes having an asymmetry in the state of order but not in the charge density and *vice versa*. We found that membranes from PC on one side and from 1:1 M PC/DPPC on the other side, the latter having a considerably higher state of order at 37 °C, gave rise to symmetric *I/U*-characteristics, whereas only phospholipid bilayers with an asymmetry in the charge density but a symmetry in the state of order (*e.g.* PC/PG) led to asymmetric *I/U*-curves. Also the lowering of pH on one side of a symmetric PE/PE bilayer led to an asymmetry in the *a priori,* under symmetric pH conditions, symmetric *I/U*-curves.

To study the influence of the chemical structure of LPS on the dipole potential, we have measured *I/U*-curves for membranes composed on one side of phospholipids and on the other side of LPS from Rd_1^- or Rd_1^+ of *Salmonella minnesota* strains R 7 and Rz, respectively. LPS Rd_1^- possesses one further Kdo and two heptoses as compared to LPS Re, and for LPS Rd_1^+, the heptose units carry, in addition, one phosphate group each. Thus, both LPS Rd_1 have a significantly longer sugar chain and LPS Rd_1^+ moreover twice as many phosphate groups as LPS Re. The actual potential difference measured for the PL/LPS Rd_1^- was −40 to −50 mV, *i.e.* approximately only half of that of the PL/LPS Re system, and that for the PL/LPS Rd_1^+ bilayer was again reduced by a factor of two. For the PL/LPS Rd_1^- system, part of this effect can be explained by a reduction of the Gouy-Chapman

potential due to the additional negative charge at the third Kdo molecule. However, this does not explain the difference to the total potential of PL/LPS Re satisfactorily. It seems rather that the electrical dipole moment in the headgroup of the LPS Rd_1^- is reduced by the larger sugar portion. The further significant reduction of the potential for the PL/LPS Rd_1^+ system can hardly be explained by assuming that the dipole potential results exclusively from the headgroup of the LPS. It seems likely that the incorporation of cations and alignment of water molecules contribute to its value. Furthermore, changes of the headgroup conformation by the addition of further sugar units cannot be safely predicted. Therefore, it is not known whether the additional heptose units contribute to an elongation of the headgroup and how the negative charges might influence the latter. Models based on energy minimization concepts [15] cannot unrestrictedly be applied to the "real" structure because they were performed with single molecules *in vacuo, i.e.* by neglecting the influence of water and cations.

From a biological point of view, two parameters of the potential energy barrier are of importance, the potential gradient inside the membrane affecting the activity of intrinsic proteins and the potential between the bulk subphases influencing membrane fusion and the interaction of the membrane with soluble molecules [4]. For the function of porins and other outer membrane proteins, the potential gradient inside the bilayer plays only a minor role whereas the surface potential (Gouy-Chapman) is of major importance. However, within a small transition range also the inner potential might be crucial for the orientation of proteins within the membrane. Our results clearly indicate that the potential gradient inside the membrane cannot be estimated from the "surface potential" as the exclusive parameter, and, moreover, they show that particular headgroup conformations may even reverse the direction of the field.

[1] G. Cevc, Biochim. Biophys. Acta **1031,** 311–382 (1990).
[2] J. E. Hall and R. Latorre, Biophys. J. **15,** 99–103 (1976).
[3] R. Latorre and J. E. Hall, Nature **264,** 361–363 (1976).
[4] P. Schoch, D. F. Sargent, and R. Schwyzer, J. Membr. Biol. **46,** 71–89 (1979).
[5] M. J. Osborn, J. E. Gander, E. Parisi, and J. Carson, J. Biol. Chem. **247,** 3962–3972 (1972).
[6] K. Brandenburg and U. Seydel, Biochim. Biophys. Acta **775,** 225–238 (1984).
[7] M. C. Phillips and D. Chapman, Biochim. Biophys. Acta **163,** 301–313 (1968).
[8] O. Lüderitz, M. A. Freudenberg, C. Galanos, V. Lehmann, E. Th. Rietschel, and D. H. Shaw, Curr. Top. Membr. Transp. **17,** 79–151 (1982).
[9] E. Th. Rietschel, L. Brade, U. Schade, U. Seydel, U. Zähringer, K. Brandenburg, I. Helander, O. Holst, S. Kondo, H. M. Kuhn, B. Lindner, E. Röhrscheidt, R. Russa, H. Labischinski, D. Naumann, and H. Brade, in: Endotoxin (H. Friedman, T. W. Klein, M. Nakano, and A. Novotny, eds.), Plenum Press, New York 1990; Adv. Exp. Med. Biol. **256,** 81–99 (1990).
[10] U. Seydel, G. Schröder, and K. Brandenburg, J. Membr. Biol. **109,** 95–103 (1989).
[11] C. Galanos, O. Lüderitz, and O. Westphal, Eur. J. Biochem. **9,** 245–249 (1969).
[12] H. Brade, C. Galanos, and O. Lüderitz, Eur. J. Biochem. **131,** 195–200 (1983).
[13] S. McLaughlin, Annu. Rev. Biophys. Biophys. Chem. **18,** 113–136 (1989).
[14] H. Labischinski, G. Barnickel, H. Bradaczek, D. Naumann, E. Th. Rietschel, and P. Giesbrecht, J. Bacteriol. **162,** 9–20 (1985).
[15] D. Naumann, C. Schultz, A. Sabisch, M. Kastowsky, and H. Labischinski, J. Mol. Struct. **214,** 213–246 (1989).
[16] K. Brandenburg and U. Seydel, Eur. J. Biochem. **191,** 229–236 (1990).
[17] K. Brandenburg, M. H. J. Koch, and U. Seydel, J. Struct. Biol. **108,** 93–106 (1992).
[18] K. Brandenburg and U. Seydel, Biochim. Biophys. Acta **1069,** 1–4 (1991).