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Understanding the function of bacterial outer membrane channels by reconstitution into black lipid membranes

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Abstract

Structural and functional information is obtained by the reconstitution of membrane channel forming proteins into black lipid membranes. Due to this outstanding sensitivity only little material is needed and single molecule detection can be easily achieved. An overview on different types of detection will be given.

Keywords: Channel; Lipid membrane; Porin; Conductance; Noise; Gating

1. Introduction

Biological systems are exceedingly complex and it is often necessary to reduce the number of parameters in order to gain an understanding of their basic mechanism. Planar lipid bilayers offer an enormous advantage — with a tiny amount of material a first hint on, for example, the size of the channel or functional information such as ion selectivity or ligand translocation can be achieved. This review covers previously introduced applications in the field of bacterial outer membrane research and summarizes the current understanding.

Bacterial outer membrane channels can serve as model systems for other channel forming proteins. Although, they are structurally different from eukaryotic channels (except probably for mitochondrial outer membrane channels) we assume that the underlying transport mechanisms will have much in common. Moreover, many of these channels play an important role in the

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permeation of antibiotics through the outer membrane and are, therefore, important molecules in our understanding of acquired resistance as well as vulnerability towards antibiotics. Understanding antibiotic permeation may enable us to engineer and modify existing antibiotics for improved permeation through blocking of essential channel proteins.

Bacterial outer membrane channels as a model system will attract more interest in basic science as it allows quantitative studies. Most of these proteins can be overproduced to give high yields (tens of milligrams per liter range) and are extremely stable (4 M GuaHCl, 70°C in 2% SDS). This allows the application of a wide range of physical methods to study structure and function. The structures of only six different classes of membrane proteins have been solved showing the difficulties encountered when crystallizing these proteins and limiting the available tools for structure/function research. Since recently the high resolution structure of several bacterial membrane proteins has been elucidated, this creates the basis for an elaborate and rational structure/function analysis.

1.1. The bacterial cell envelope

Gram-negative bacteria like *Escherichia coli* are surrounded by two membranes (Fig. 1). The inner lipid bilayer contains 74% phosphatidylethanolamine, 3% cardiolipin and 19% phosphatildylglycerol-lipid [1]. In contrast, the outer membrane consists of an asymmetric bilayer with a phosphatidylethanolamine-enriched inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. The LPS headgroups protects the cell from damaging agents such as bile salts, lipases, proteases and from recognition or adsorption. Both cell walls are separated by the periplasmic space containing a hydrophilic polymer layer, the peptidoglycan, which provides additional stability. Cells have to continuously exchange substrates across their cell walls. Uptake of nutrients or secretion of proteins through this barrier is accomplished by several pore-forming proteins [2]. Most abundant are the general diffusion pores, which allow the diffusion of hydrophilic molecules with molecular weights up to approximately 300 Da. They have no specificity and exhibit a weak selectivity towards cations or anions. In *E. coli*, two major cation-selective porins, OmpC and OmpF, are synthesized under standard laboratory conditions [3], while the anion-selective porin PhoE is produced under phosphate limitation [4]. However, at very low substrate concentrations this type of channel will not suffice to provide the cell with vital nutrients. The flux through the outer membrane is given by $\phi = (c_o - c_i)\ A\ P$, with $(c_o - c_i)$ the concentration difference between the outside and the inside, A represents the area and P the permeability. When $(c_o - c_i)$ becomes very small, the cell has to increase the permeability to survive. This can be achieved by increasing the number of channels but is limited within a small range. Alternatively, the channel size could be increased but this will be deleterious because harmful agents will also enter the cell. Therefore, another type of channel has evolved, the solute-specific porins [2]. Here, diffusion transport is facilitated by increasing the local substrate concentration due to the presence of a binding site in the channel. For example, maltoporin (also called LamB, the receptor for bacteriophage λ) of *E. coli* shows specificity towards maltooligosaccharides [5–7]. Others show specificity for sucrose (sucrose porin or ScrY) [8,9] or nucleosides (Tsx) [10]. Although it has been shown that RafY displays specificity for raffinose [11], recent studies demonstrated that this channel does not contain a binding site for raffinose [12]. Another category of transporter proteins are the TonB-dependent receptors [13], such as FhuA and FepA, which bind particular iron-chelators. Also the export of proteins is mediated by several channel-forming proteins in the outer membrane (for reviews see [14,15]). Besides the transporters, other proteins are present in the outer membrane like OmpA [16] for which the function still remains elusive, enzymes like the phospholipase PldA [17] and the protease OmpT [18].

1.2. Structure of outer membrane channels

Recently, the three-dimensional structures of

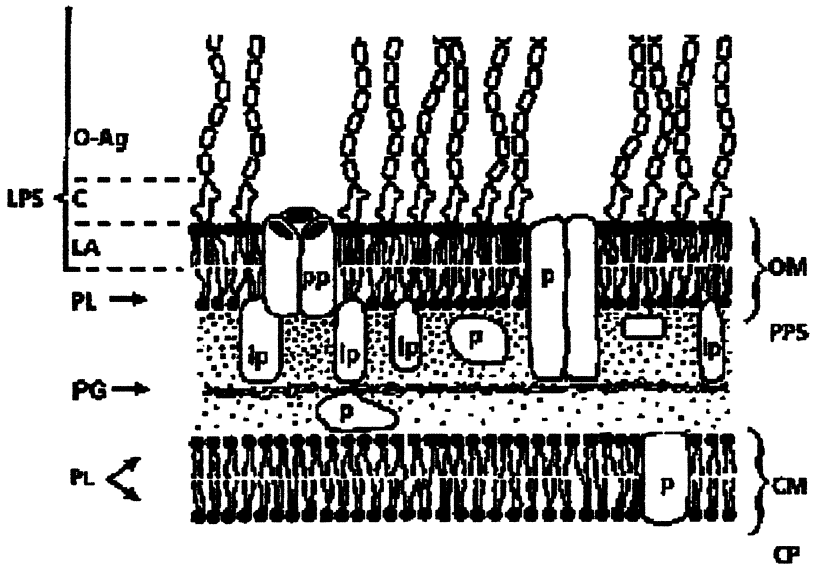

Fig. 1. Scheme of the cell wall of Gram-negative bacteria. LPS: lipopolysaccharide; PL: phospholipid; PG: peptidoglycan; OM: outer membrane; CM: cell membrane; PPS: periplasmic space; CP: cytoplasm; lp: lipoprotein; p: protein; pp: porin.

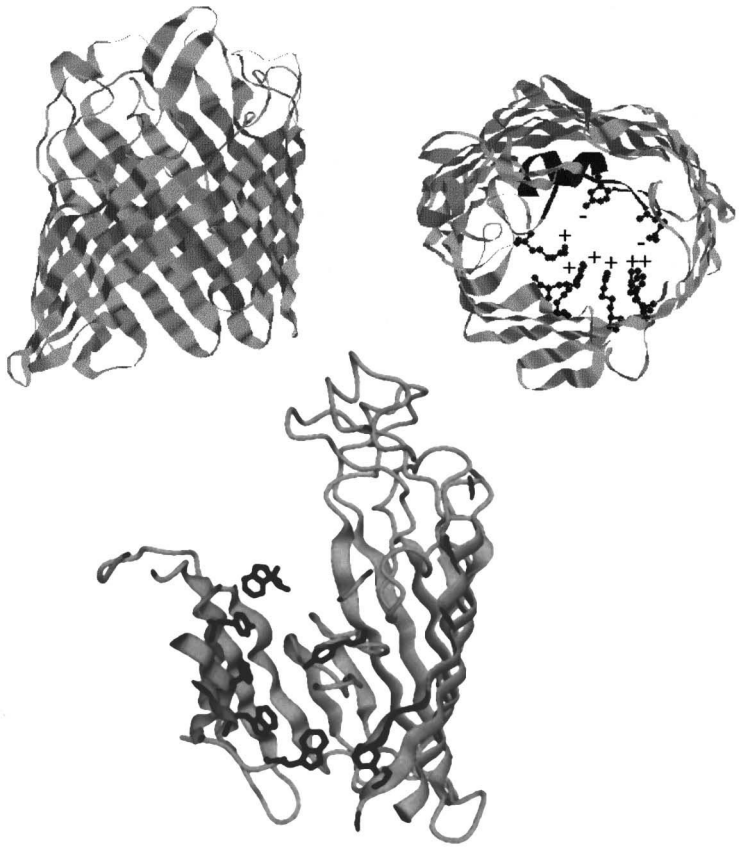

Fig. 2. (a) Structure of OmpF from the side. (b) Structure of OmpF from top with the charged residues highlighted.(c) Structure of maltoporin with the aromatic residues.

the general diffusion channels of *Rhodobacter capsulatus* [19], *Rhodopseudomonas blastica* [20] and *Paracoccus denitrificans* [21], porins OmpF and PhoE of *E. coli* [22] and OmpK36 of *Klebsiella pneumoniae* [23] have been elucidated. Although they are porins from evolutionary distant organisms their three-dimensional structures still consist of a similar channel-forming motif. All these porins form three water-filled channels per trimer, the functional unit of the proteins in the outer membrane. In each monomer, 16 β-strands span the outer membrane and form a barrel with short turns at the periplasmic side and large loops at the outside of the cell (Fig. 2a). Unlike the other loops, the third loop, L3, is not exposed at the cell surface but folds into the barrel, forming a constriction zone at half the height of the channel. Therefore, this loop contributes significantly to permeability properties, such as the exclusion limit, of the pore. At the constriction zone, a strong transverse electrostatic field exists that is caused by acidic residues in loop L3 and a cluster of basic residues in the barrel wall opposite to the internal loop [24] (Fig. 2b). Furthermore, analysis of the OmpF and PhoE channels suggests the existence of numerous electrostatic interactions between L3 and the barrel wall [22]. Therefore, L3 might have a stabilizing function in porin integrity.

The three-dimensional structure of specific porins, which have been elucidated recently, show a similar trimeric β-barrel motif. Although they display only a moderate sequence homology, the structures can be superimposed. The monomer of LamB of *E. coli* [25], LamB of *Salmonella typhimurium* [26] and ScrY of *Salmonella typhimurium* [27] consist of an 18-stranded β-barrel with short turns at the periplasmic side and large irregular loops at the outside of the cell (Fig. 2c). By analogy with the general diffusion channels, the third loop, L3, folds inside the β-barrel thereby forming a constriction at the middle of the channel. Based on the structure of sugar-soaked crystals of LamB, a specific sugar translocation pathway, consisting of an aromatic amino acid 'greasy slide' aligned by polar track residues, has been postulated (Fig. 2c) [25,28]. Sugar residues are in van der Waals contact with the greasy slide via the hydrophobic face of their sugar ring while hydrogen bonds exist between the sugar hydroxyl-groups and the polar track residues. Constant breaking and remaking of these hydrogen bonds has been supposed to ensure movement of the substrate through the channel.

2. Black lipid membranes

2.1. Reconstitution into planar lipid membranes

Giant planar lipid membranes are readily formed by applying one of the classical techniques [29–34] and using a setup of two cells separated by a Teflon septum as schematically shown in Fig. 3. Teflon is inert, easy to handle, available in thin films and thus often used as cuvette material and as septum, separating both compartments. However, Teflon is lipophobic and requires to be pretreated by spreading, for example, a tiny amount (1 μl) of a 1% (v/v) lipid in chloroform solution. After allowing 20 min for drying, the chambers are filled with buffer. The so-called solvent containing or Muller–Rudin bilayers were made from an approximate 1% (v/v) lipid in decane solution [30,33]. This membrane-forming solution is finally smeared with a Teflon loop across the hole of a typical size between 100 and 500 μm radius. The blackening of the film can be followed with a simple microscope.

Later this technique was modified [31,32,34]. For example, we used two half cells separated by a thin Teflon foil (approx. 25 μm thick, Goodfellow, GB) containing a small (approx. 50–100 μm)

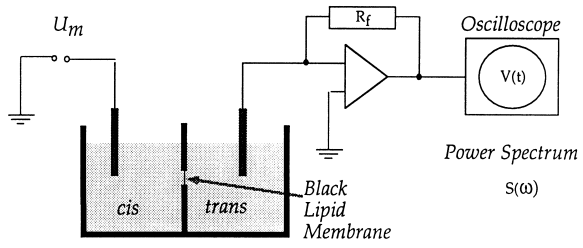

Fig. 3. Schema of a set up for electrical recording with planar lipid membranes. See text for discussion.

hole. Such circular-shaped holes can easily be punched in by a high frequency high voltage spark (Electrotechnic Products, Chicago, USA). The surface of the holes are pre-painted with a 1% (v/v) hexadecane/hexane solution. After the 20 min necessary for drying, the chambers are filled with buffer. According to the suggestion of Montal and Muller, the lipid is dissolved in a volatile organic solvent such as hexane/chloroform and spread on the surface of the buffer. After evaporation (typically 20 min) the buffer level is lowered and raised and by crossing the hole in the septum one of the monolayers is made. Although this method is often named solvent-free some of the organic solvent is still present. A great advantage of this specific technique is the possibility to form asymmetric membranes [35].

These techniques have been successfully used to study membrane active substances (see for example [33]). Unlike liposomes one has direct access to both sides of the membrane and this allows to modify each side individually. Although both techniques exist for a long time, a fundamental comparison on the physical properties is still lacking. The main difference seems to be the lateral stress. Solvent-containing membranes appear to be more flexible than solvent-free ones.

Reconstitution of membrane proteins into planar lipid membranes is the crucial part of this technique. A great variety of protocols were established, ranging from direct mixing with the membrane forming solution, addition into the aqueous phases to insertion into proteoliposomes and subsequent fusion with the lipid bilayer (see [32] for a discussion). To reconstitute porins we add minor amounts (<1 μl) from a diluted solution detergent-containing protein solution. The protein is kept in a detergent solution above the CMC (approx. 1%). After addition to the chamber, the detergent is diluted below the CMC, which causes the detergent to leave the protein. The hydrophobic surface of the protein becomes water-exposed causing aggregation or for a few protein molecules this results in insertion into the membrane. The insertion is then readily seen by a jump in the membrane conductance.

2.2. Orientation of reconstituted proteins

A relevant question related to membrane channels which has been constantly overlooked or underestimated is their orientation in the membrane. The reconstitution of highly oriented channels into artificial lipid bilayers is obviously necessary to characterize the individual asymmetry of the channels or the effect of point mutations on transport. The use of phages, which employ the channel as their receptor, has been proven successful [36]. Often the interaction induces a change in the conductance state of the channel providing an easy probe to determine orientation. Recently, unidirectional insertion of the siderophore receptor FhuA of *E. coli* was observed [37]. FhuA does not form a classical porin, since energy transduction is required to open the channel. However, phage T5, which is unique by circumventing the energy requirement, can convert the receptor into a permanently open channel. This uncoupling has been exploited for a functional in vitro assay that allows determination of channel orientation. Also in the case of LamB, controlled blocking of the porins was attempted by adding phage lambda [36]. Here, sugar transport through channels recognized by the phage was blocked. This yielded an easy way to determine orientation of LamB insertion and to study directional sugar transport. However, phages or toxins, specifically interacting with the channel, are not always available. Therefore, often an intrinsic asymmetrical behavior in conductance or voltage gating [38,39] may give an insight into the orientation of the channels in the planar lipid membrane. Previously, unidirectional insertion has been observed for mitochondrial outer membrane channel VDAC [40], PhoE of *E. coli* [41] and OprD2 of *Pseudomonas aeruginosa* [42].

2.3. Conductance measurements

2.3.1. Channel size

The great advantage of planar lipid membranes is the possibility to record easily the conductance. A planar lipid bilayer represents a perfect insulator, which allows the detection of single channels. Reconstitution of a water-filled channel into a

planar lipid membrane will provoke an immediate jump in conductance (Fig. 4). We used typically Ag/AgCl commercially available or home-made electrodes. One chamber is connected to the ground and called *cis* side (Fig. 3). The other electrode is connected to an amplifier (for example BLM120, Biologic, France). The output is usually recorded on paper, tape and/or on an oscilloscope. Analyzing a larger number of such jumps allows to make conclusions on the single channel conductance. If the molecule creates a well-defined channel one can expect a clear distribution around multiples of a unit conductance. In this case it is tempting to convert the unit conductance into a pore size. Neglecting all kind of interactions with the wall, the resistance of a cylindrical tube representing the channel contains two components, the resistance of the channel itself and the region in front and behind the channel, the so-called access resistance. The conductance is given by

$$G = \kappa[\pi a^2/(d + \pi a/2)] \quad (1)$$

with κ as the bulk conductance, a the pore radius and d the membrane thickness [43]. The bulk conductance can be readily measured with a standard conductometer or can be found in standard tables by summing up the contribution from each individual ions $\kappa = \Sigma\Lambda_i c_i$ with Λ_i as the equivalent conductance of the individual ion and c_i its bulk concentration. Although Eq. (1) looks convincing and is often used to estimate a pore size it often does not reflect the underlying physics. The first argument is quickly understood: the channel wall consists mostly of charged or polar residues, which definitely interact with ions creating the current. A typical distance is the so-called Debye screening length which is the distance over which ions feel each other in an electrolyte solution and is given by

$$\lambda_D = \sqrt{(\varepsilon_w \varepsilon_0 kT/2n_0 e^2)} \quad (2)$$

Here $\varepsilon_w = 80$ is the relative dielectric constant of water, $\varepsilon_0 = 8.85 \times 10^{-12}$ (C^2 m^{-2} N^{-1}) the dielectric permittivity of the vacuum, n_0 the number of ions per volume and the elementary charge $e = 1.6 \times 10^{-19}$ [C] [33]. Moreover, any charge on the surface of a channel will have its counter ion next to it, thus increasing the local ion density and pore conductance. This will apparently increase the pore size. Furthermore, a charge approaching the area of low dielectric constant immediately feels its image charge as the field lines are repelled from the protein surface. This leads to apparently smaller pore sizes. Another effect, usually not accounted for, are polarization forces. This could be tested by ions of different sizes and thus different polarisabilities. Indeed, while in some cases conductance measurements correlate well with the (expected) channel size, very often it shows a breakdown of the theory. Introduction of bulky residues expected to narrow the channel have a neutral effect on conductance while isosteric replacements show a dramatic decrease [44,45]. Moreover, large deletions in the constricting loop L3 of OmpF of *E. coli* [46] and porin of *R. blastica* [47] resulted in a decreased conductance. Mostly the rationale between measured conductance values and channel size is of alien nature with authors desperately trying to explain the ambiguous results. Although many theories have been adopted to explain channel conductance, none of them is to date able to provide satisfying predictions.

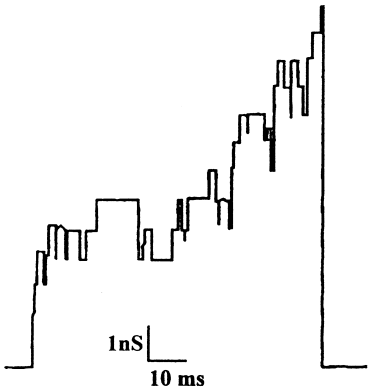

Fig. 4. Typical conductance increase during insertion of outer membrane protein PhoE from *E. coli.* Channels were inserted by applying a positive potential of 120 mV in electrolyte buffer containing 1 M NaCl, 1 mM $CaCl_2$, 5 mM Hepes pH 7.4.

Another technique to achieve information on the pore size is particle exclusion measurements. Efflux measurement with radioactive-labeled hydrophilic markers of a specific size yield information on the pore size [48]. A sufficient number of low molecular weight polyethylene glycoles are commercially available with sizes that are convenient for determining the pore diameter of ion channels [49]. The substrate of interest or the polymer of a specific size is added to the outside under isosmotic conditions. If the substrate or polymer is able to permeate the channel the osmotic stress inside the vesicle is increased and the liposome start to swell. The change in size can be recorded by light scattering [49,50]. A similar effect can also be recorded by conductance measurement [51–53]. The conductance depends on the added percentage of added non-electrolyte polymer. If the channel conductance varies as the bulk conductance, the polymer is supposed to enter the channel. In contrast, if the channel conductance is independent of the bulk concentration of the polymer, the polymer is supposed to be excluded [51]. Again the predicted channel size depends as much on the measured size of the molecules in the bulk being representative of the size inside the channel, which means neglecting conformational changes, hydration and other interaction. More recently the ion current fluctuation has been analyzed in the presence of differently sized polymers [52]. This allows additional conclusions on possible interactions of the polymer with the channel wall [53].

2.3.2. Channel-exposed ionizable groups

The analysis of the ion current fluctuation can also be used to obtain structural information inasmuch as charged residues are exposed to the channel wall [54–56]. Even at high ionic strength the actual number of ions inside the channel is rather low. This can be readily used to probe the pK value of residues exposed to the channel lumen. At a pH below the pK the ion conductance has a channel specific value and in addition some ion current fluctuation. Raising the pH towards the pK creates sudden additional charges, which give rise to an additional ion current fluctuation.

2.3.3. Selectivity

Experimentally the ion-selectivity of reconstituted porins is easily tested by determining the ratio between cation over anion permeability (P_c/P_a) and gives an idea about the overall electrostatic potential within the channel. The lipid membrane separates two aqueous compartments containing ions with different concentrations. On each side of the membrane the number of positive charges equals that of the negative ones. If both ion solutions are connected via a channel the difference in the chemical potential due to a possible concentration gradient causes the ions to flow. Under the specific case that one of the ions is hindered in the flow with respect to the other one, more ions of one charge accumulate and the net charge difference causes a transmembrane voltage ΔV that can be recorded via a voltmeter. This so called zero-current potential is a measure of the channel selectivity. The transmembrane voltage difference is given, for example, an NaCl and KCl solution by the Goldman–Hodgkin–Katz equation [43].

$$\Delta V = \frac{RT}{F} \ln \frac{P_{\mathrm{Na}}[\mathrm{Na}]_{ex} + P_{\mathrm{K}}[\mathrm{K}]_{ex} + P_{\mathrm{Cl}}[\mathrm{Cl}]_{in}}{P_{\mathrm{Na}}[\mathrm{Na}]_{in} + P_{\mathrm{K}}[\mathrm{K}]_{in} + P_{\mathrm{Cl}}[\mathrm{Cl}]_{ex}} \quad (3)$$

Here, R is the molar gas constant, F the Faraday constant, T the temperature, P_x the respective permeability and $[X]_x$ the ion concentration in the respective compartment. Again this equation is derived for the ideal case where all interactions with the channel wall are lumped in the permeability P. As pointed out above, ions do feel an image force, they do see the charged residues and they feel the interaction with the wall by polarization forces.

Some of the porins are a passive filter for charges and passage depends on the sign and size of the substrate. For example, it has been shown previously by chemical modification [57,58] and site-directed mutagenesis [59,60] that lysine and arginine residues contribute to the selectivity filter in the anion-selective porins PhoE of *E. coli* and Omp34 porin of *Acidovorax delafieldii*. Moreover, additional charges present in the channel and the channel mouth of PhoE are important for ion-selectivity [60,61]. Thus, it seems that the total

charge constellation contributes to the creation of a specific electrical field, giving each porin its unique properties. This is consistent with other studies on chemically-modified porin from *E. coli* B [62] and succinylation of porin from *R. capsulatus* [44], the introduction of negatively charged groups rendered the channels more cation-selective. The introduction of a negatively charged residue or the replacement of basic residues at the channel constriction of OmpF of *E. coli* significantly increased its cation-selectivity [63,64] while substitution of acidic side-chains showed a decrease [64]. Conversely, substitutions of basic or acidic residues in the anion-selective channel PhoE of *E. coli* resulted in a decreased and increased anion-selectivity, respectively [59]. Replacement of positively charged residues in the anion-selective porin of *P. denitrificans* even resulted in ion-selectivity reversal [45].

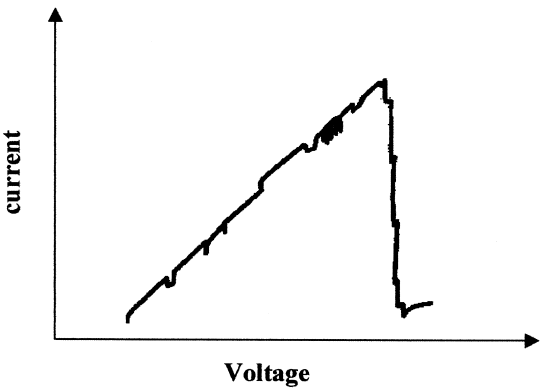

Fig. 5. Voltage dependence of inserted OmpF. An increase in the transmembrane voltage give rise to a larger current. Above a threshold voltage the channels suddenly close. See text for details.

2.3.4. Voltage gating

One of the most intriguing phenomena in channel studies is the voltage gating behavior. The underlying mechanism is not yet understood. Purified porins reconstituted into lipid bilayers can be closed at a certain potential (Fig. 5). The significance of this voltage gating is still not clear since the potentials needed to close the pores are larger than the naturally occurring Donnan potential across the outer membrane [65]. However, porin Omp34 of *A. delafieldii* was shown to close at potentials as low as 10 mV [39]. Furthermore, pore-gating can be mediated by additional means. Naturally occurring components in the periplasm, such as membrane-derived oligosaccharides (MDOs) [66], and outer membrane-associated compounds such as polyamines [67,68] are able to close the pores. Furthermore, the *E. coli* pores have been shown to be pressure-sensitive [69].

It is clear from previous reports that charges present in porin channels are involved in voltage gating. The separation of residues of opposite charges at the constriction zone and in the barrel wall are responsible for the existence of a strong electrical field in the porins [24]. Replacement of the positively charged arginine residues in the constriction zone of OmpF [64] and OmpC [70] resulted in an increased voltage sensitivity. However, substitution of positively charged residues in PhoE [59] and chemical modification of arginine and lysine residues in the anion-selective porin of *A. delafieldii* decreased its voltage-sensitivity [58]. Replacement of negatively charged residues in PhoE had a reverse effect, i.e. increased voltage sensitivity, whereas mutations of negatively charged residues in OmpF resulted in an increased threshold potential [59]. Thus, the importance of charged residues in the constriction zone as sensors for voltage gating was confirmed [59,71].

The mechanism of voltage-gating is still not unraveled. Movement of the complete L3 and subsequent blocking of the channel as a possible gating mechanism as demonstrated in molecular dynamics simulations [72] seems to be very unlikely since this loop has many interactions with the barrel wall. Indeed, tethering the tip of loop L3 of OmpF [46,73] and PhoE [74] to the barrel wall still allowed complete closing of the channel. Previously, molecular dynamics perturbation studies [75,76] suggested that a part of loop L3 from *R. capsulatus* porin, adjacent to two glycine residues, is flexible. This part of the loop may well correspond to the *E. coli* porin L3 region just behind the conserved PEFGG sequence. Although, L3 of OmpF was tethered to the barrel wall at several places [73], this potential flexible

region was overlooked leaving the possibility of movement open. On the other hand, mutant PhoE pore proteins containing mutations in the PEFGG sequence showed reduced permeability, suggesting a smaller channel size [38]. The altered channel properties of this mutant protein may be explained by a change in the flexibility of loop L3. Possibly, the fast flickering observed in planar lipid bilayer [77,78] and patch-clamp experiments [79] may represent small conformational changes in loop L3. Recently, it has been proposed that gating frequency is determined by the hydrogen-bond network between L3 and the wall [80].

An alternative mechanism of voltage gating may be the screening–unscreening of charges [75,81]. At the constriction zone, the water shell of entering molecules has to be stripped and/or the water molecules interacting with the peptide structure in the channel have to be removed to allow the passage of the entering molecule. The removal of the hydration shell, probably aided by the strong transversal electrical field, followed by the redistribution of water molecules (or counter ions) in the channel may result in the unscreening of the charges in the channel and additionally increase the strength of the electrical field [76,81]. This may induce re-orientation of side-chains in the channel, resulting in the closing of the pore.

2.4. Thermodynamic constants

2.4.1. Ligand affinity constants

The previous part describes the effect of the porin structure on the ion conductance and numerous studies are dealing with these properties. However, the biological relevant function of most of the channels is substrate translocation. Again, the modulation of the ion flux across reconstituted porins can be used as a probe for substrate flux [82–88]. Recently this technique has been successfully applied for the characterization of solute transport through maltoporin of *S. typhimurium* [87], Tsx, RafY and the product of the cryptic gene *bglH* of *E. coli* [88] and sucrose porin of *Klebsiella pneumoniae* [88]. A well studied example is sugar transport through maltoporin of *E. coli*. Since it has been shown that the binding of maltooligosaccharide to maltoporin blocks ion translocation in a concentration-dependent manner, this can be used to monitor the binding constants of a sugar [82–84]. The sugar binding site is located in the constriction part of the channel [25] and, therefore, an open maltoporin channel P will bind one sugar molecule M to form the closed sugar-porin complex ($M + P = MP$). Since bound ligands prevent ion flux, measuring conductance for various sugar concentrations allows the sugar-binding constant $K = [MP]/[M][P]$ to be evaluated [84]. The measured conductance in the absence of sugar G_{max} is proportional to the total number of channels $M + MP$ and, therefore, to the number of binding sites. In the presence of a given sugar concentration $[M]$ the conductance $G[M]$ is reduced and proportional to the open channels P. Since the channel is either open or closed the following equation can be used:

$$\frac{G_{max} - G[M]}{G_{max}} = \frac{K[M]}{1 + K[M]} \quad (4)$$

As shown in Fig. 6 measuring the conductance as a function of sugar concentration readily gives the binding constant. This model assumes that each bound sugar will block a single channel and thus a second sugar penetrating the channel region will not be detected. At low sugar concentration, single sugar blocking was observed. Titration to higher sugar concentrations increased the probability of two sugar molecules to be present in a single channel and an apparent lower binding constant was observed. Sometimes such data are fitted by an inverse diagram, the so-called Lineweaver Burk plot. However, such a plot requires accurate data at high sugar concentrations beyond the validity of the model.

2.4.2. Kinetic constants

In the absence of sugar the ion current through open channels is fluctuating around an average value. The presence of sugar in the channel results in additional current fluctuations. These additional fluctuations reflect the on and off rates of sugar binding. General fluctuation theory on the

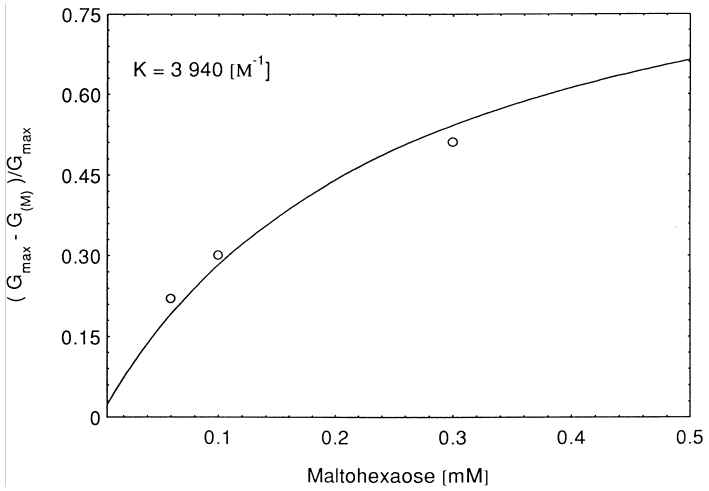

Fig. 6. Binding constant revealed from the conductance measurement according to Eq. (4). Approximately 60 maltoporin trimers were reconstituted into a planar lipid bilayer and different concentration of maltohexaose were added. Here tryptophane residue 358 was replaced by an alanine.

equilibrium of a pseudo-first order reaction predicts an exponentially decaying autocorrelation [85,89] and, thus, a Lorentz power spectrum (Fig. 7a). This model can be applied to maltooligosaccharide binding to a defined number of sites [85]. Assuming a two-state channel (open and closed corresponding to unbound and bound), the fluctuations around the average ionic current value yield the Lorentzian power spectrum (Fig. 7a) [34,85,86,89] which is given by:

$$S(\omega) = \frac{S_0}{1 + \left(\frac{\omega}{\omega_c}\right)^2}, \quad (5)$$

where S_0 is the plateau value at zero frequency and ω_c the corner frequency at which the plateau value decreased to half of its value.

$$S_0 = (8N i^2/k_{off}^2) \quad K[M]/(1 + K[M])^3 \quad (6)$$

with N as the number of inserted channels, i the current through a single channel and $[M]$ the sugar concentration, k_{off} the dissociation constant and K the sugar binding constant described by k_{on}/k_{off}. The second parameter determining the Lorentzian is the so-called corner frequency ω_c, at which the amplitude decayed to $\frac{1}{2}$ S_0 (Fig. 7b). Fluctuation theory shows that this frequency is related to the inverse of the decay time and to the on and off rates of the sugar binding [34,85–87,90]:

$$\omega_c = 1/\tau = k_{on}[M] + k_{off} = k_{off}(K[M] + 1) \quad (7)$$

This equation is valid for a single channel or an ensemble of channels facing the same sugar concentration. In the cases of a single channel or unidirectionally oriented channels one could adopt the above equation and titrate the sugar on one side only. This will give information on the accessibility from each side of the channel and thus an idea about the symmetry of the energy barriers [36].

In previous studies it was assumed that the channel is symmetric with an affinity site in the middle. In this case the on and off rates measured are directly related to the sugar flux from the side with the concentration $[M]$ to the sugar free side [85]:

$$\Phi = \frac{k_{on}[M]_{ex}}{1 + K[M]_{ex}} \quad (8)$$

In the case of an asymmetrical channel such as maltoporin, the situation is more complex and the

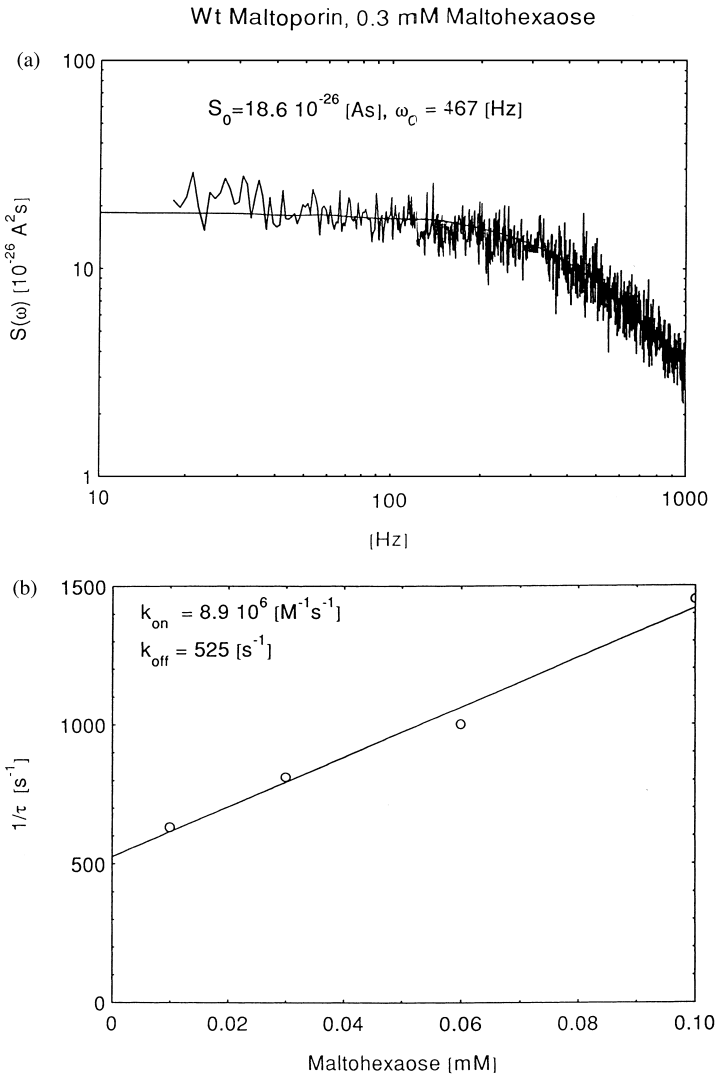

Fig. 7. Power density spectra of the membrane of the W358A maltoporin mutant in the presence of 0.3 mM maltohexaose. The curve was fitted to a Lorentzian according to Eq. (5). (b) On- and off-rates revealed from the power spectra at different concentrations according to Eq. (7).

on and off rate measured with randomly inserted channels will not allow conclusions on the transport.

Several reports demonstrate the usefulness of this technique. Previously it was shown that single-stranded RNA can block specifically alphatoxin. Correlating the number of blocking events with PCR of the subphase on the opposed aqueous phase revealed a nice correlation with ion current fluctuation giving absolute transport rates [91]. Analyzing the ion current fluctuation can also reveal inasmuch the ligand is penetrating via a Brownian motion or via an affinity site. Recently ATP translocation through mitrochondrial ion channels, VDAC, was quantified this way [92].

3. Outlook

For a long time it has been temping to use the

large variety of natural specific channels to construct biosensors. The main difficulty in the commercial application of these biosensors is the inherent instability of these free standing films [93]. This can be overcome by using a solid support. However, often the interaction of the lipid membrane with the support will dominate and a larger length scale defect will occur, visible in a higher background conductance. To overcome this problem we currently work on artificial non-phospholipid membranes, showing a dramatic increase in stability.

Furthermore, porins can provide a unique tool to study single molecule interactions. Since the structure at atomic resolution is available, the porins become an exciting object to understand the physical origin of the substrate specificity and voltage gating at molecular level. Considering the enormous amount of experimental data already gathered from experiments with these membrane channels a definite and good correlation between the theory and experiment is in reach. Moreover, although conductance behavior is interesting from a fundamental point of view, nature has created most of these channels for substrate translocation. Refined conductance measurements can provide quantitative data to understand these processes. Recently we have shown that based on the structure, specific affinity sites can be engineered into such porins as demonstrated by an analysis of ion current fluctuation.

Many outer membrane transporters/receptors require energy transduction such as the proton motive force over the inner membrane for transport of solutes [94–96]. Therefore, a completely new system must be developed which mimics the double membrane system of the bacteria. The ultimate goal will be a double bilayer with receptor molecules in one membrane and an energy-transducing complex in the other membrane. This would allow us to study energy coupled transport and may be used as a sensor-system. We plan to deposit a lipid bilayer onto hollow polyelectrolyte shells. This lipid layer isolates the interior and mimics the internal cell wall. This lipid membrane has to be coated by a separate polymer providing a specific spacing to a second lipid layer. Such an artificial double lipid bilayer can serve as a model for a bacterial cell wall with a potential use for the screening of new antibiotics.

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