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SOLUTE UPTAKE THROUGH GENERAL PORINS

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1. ABSTRACT

General diffusion porins are among the few membrane proteins that have been thoroughly investigated by many techniques, including X-ray crystallography, AFM microscopy, computer modeling, electrophysiology and biochemistry. This had led to a good understanding of the process of solute transport *per se*. However, other aspects of porin function remain enigmatic, such as the molecular basis and physiological relevance of many regulatory processes. After summarizing the most salient structural features, the review provides a description of the techniques used for the functional study of porins. The process of solute transport is presented on the basis of structure-function relationship and modeling studies. Three aspects of regulation are discussed: voltage-dependence, pH sensitivity and modulation by polycations and polyanions. The review ends with a perspective on future porin research, to be targeted at a molecular understanding of the regulatory processes, the deciphering of the physiological context in which these processes take place, and rational drug design.

2. INTRODUCTION

General diffusion porins were the first pore-forming proteins to be identified in bacteria. They remain one of the best characterized channels in prokaryotes from a structural/functional standpoint and in terms of their role in the physiology of the microorganism. In addition, their ubiquitous presence among Gram-negative bacteria and their extremely large abundance in the outer membrane make them an easily tractable protein to investigate. General diffusion porins are distinguished from the specific and the ligand-gated porins by their poor substrate selectivity and their high probability of being in the open conformation in the absence of any specific substrates. Examples of such proteins are OmpF, OmpC and PhoE from *Escherichia coli*. For more examples, the reader should consult a useful organization of transport proteins provided by Milton Saier at the Transport Protein Database. (http://tcdb.ucsd.edu/tcdb/tcfamilybrowse.php?tcname=1.B.1#protein).

3. STRUCTURAL FEATURES

The crystal structure of the porin from *Rhodobacter capsulatus* was the first one published (1), soon to be followed by those of the OmpF and PhoE porins of *Escherichia coli* (2). To date, they are 6 published structures for general diffusion porins in various organisms (Table 1). All these proteins, along with other pore formers

Table 1. General diffusion porins with solved crystal structures at high resolution. Low-resolution X-ray structures, models, AFM and electron microscopy images are not listed

Protein	Bacterium	Ref.
Porin	*Rhodobacter capsulatus*	1
Porin	*Rhodopseudomonas blastica*	4
OmpF	*Escherichia coli*	2
PhoE	*Escherichia coli*	2
OmpK36	*Klebsiella pneumoniae*	5
Omp32	*Comamonas acidovorans*	6

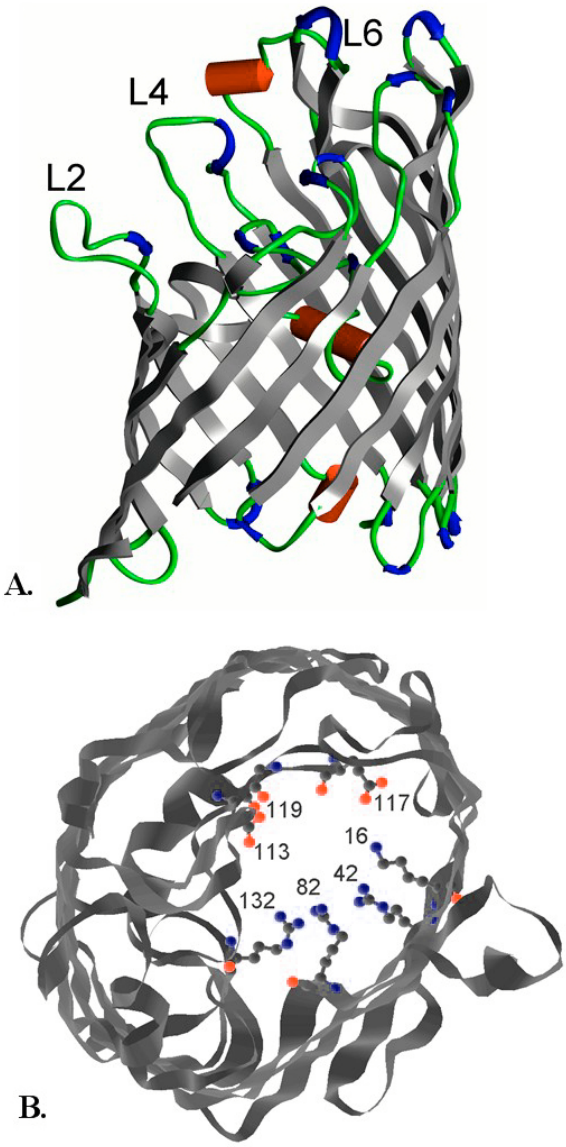

Figure 1. Three-dimensional structure of OmpF porin. **A.** side view of a monomer; beta-strands are shown as a gray ribbon, and alpha helices as red cylinders; extracellular loops L2, L4 and L6 are labeled. **B.** cross section of a monomer, viewed from the extracellular side. Labeled residues are: K16, R42, R82, D113, E117, G119 and R132, and include charged residues projecting into the pore lumen at the level of the constriction zone.

of the outer membrane of Gram-negative bacteria, share the beta-barrel motif, which appears to be a hallmark structure for outer membrane transporters (3).

The quaternary structure of porins is trimeric. Each subunit is a barrel of 16 antiparallel beta-strands connected by long loops on the extracellular side and short loops on the periplasmic side (Figure 1). The monomers make extensive polypeptide chain contacts with each other over the height of the membrane-embedded domains. This large contact surface, along with loop L2 which extends sideways and makes ionic contacts with barrel residues of an adjacent subunit, provide for the unusually high stability of the trimeric assembly. An important structural feature is the presence of a hydrophilic pore within each subunit (and not at the interface of the three subunits, as typically the case for channels based on alpha-helical transmembrane segments). An early report (7) of an image reconstruction of OmpF 2-D crystals examined by electron microscopy suggested that the three pores merged into a single one on the periplasmic side of the protein. However, this structure was clearly shown to be incorrect in later publications of high-resolution structures (see Table 1 for references).

Size exclusion for permeating solutes is provided by the inwardly folded L3 loop, another conserved structural feature among general diffusion porins. Instead of projecting outwards like the other loops, the L3 loop bends inside the pore lumen and constricts the beta-barrel at approximately mid-height (Figure 1). This architecture has the effect of creating a constricted region called the constriction zone or eyelet, within the hollow, hydrophilic cylinder. The eyelet is decorated with a constellation of charges from conserved residues that play an important role in ionic movement and in ionic selectivity (see below). Negatively charged residues are typically found on the L3 loop itself (residues D113 and E117 on OmpF in Figure 1B), and positive charges often form a cluster on the opposite barrel wall (residues K16, R42, R82 and R132 on OmpF in Figure 1B).

4. PORE PROPERTIES

4. 1. Methodologies

Over the years, several techniques have been used to investigate the pore properties of porins. A comparison of the obtained results requires an understanding of the methodologies involved. Each technique offers a different set of advantages and thus the methods are essentially complementary. A short description of each technique and some typical results are presented below.

4.1.1 Liposome swelling assay

Prior to the development of this technique, solute flux through porins had been studied with some limitations using radiolabelled isotopes (8) or hydrolytic enzymes trapped in the liposomes (9). The introduction of the liposome swelling assay allowed a quantitative assessment of the relative rates of permeation of many different solutes. Purified porins are reconstituted in the bilayer of artificial liposomes made in a solution of a large permeant

solute, such as dextran or stachyose (10). After filtration to remove large aggregates, the liposomes are diluted in an isotonic solution of test solutes. As the test solute diffuses through the porins, the resulting osmotic imbalance leads to water influx and swelling of the liposomes. The rate of swelling can be easily determined from the rate of turbidity change and is proportional to the permeability of the test solute. A typical experiment yields the relative permeability of several solutes with respect to a common standard. The technique is suited for determining the permeability of uncharged compounds, but suffers shortcomings in the determination of the permeability of electrolytes, since flux of charged compounds will generate diffusion potentials which themselves will affect the flux rate.

4.1.2. Antibiotic flux in live cells

The technique was essentially developed and optimized by Hiroshi Nikaido and colleagues, after an initial study by Zimmerman and Rosselet (11). The main advantage of this technique is that it can be performed on live cells. Thus the overall permeability of the outer membrane can be investigated in various conditions. The method is based on the idea that the periplasmic beta-lactamase provides an enzymatic sink for the degradation of antibiotics that require general-diffusion porins for permeation. As the rate of degradation of beta-lactam antibiotics by the lactamase is much faster than the rate of permeation, the flux of the antibiotic across porins is the rate-limiting step to the overall disappearance of the antibiotic. A simple measurement of the rate of absorbance decrease at 260 nm (absorbance peak for beta-lactam antibiotics) provides a numerical value for the flux of the antibiotic through the membrane. An excellent description of the technique and the associated calculations can be found in previous publications (12, 13). Several antibiotics were tested (12). Cephaloridine has been the substrate of choice because it combines a measurable permeation rate with a fast enzymatic degradation rate. In addition, it is zwitterionic, and its flux is not affected by charges or ionic strength effects. Importantly, Nikaido and colleagues have shown that cephaloridine flux is absolutely dependent on the presence of general-diffusion porins (14). Thus the measurement of degradation of this antibiotic is a faithful representation of the porin-mediated permeability of the outer membrane. The extent of flux depends of the type and number of open porins present in the membrane. The technique is thus useful in the investigation of not only regulation of porin expression, but also regulation of porin activity (see below "Modulation").

4.1.3 Planar lipid bilayer electrophysiology

Electrophysiology has been a technique of choice for the functional study of porin flux properties. Planar lipid bilayers (also known as "black lipid membranes" or "BLM's") containing reconstituted porins were the first electrophysiological tools to be used for this purpose. They continue to be widely used for the initial study of pore-forming activity of putative porin-like proteins, and for the description of overall pore properties. Detailed description of channel kinetics is usually performed with the patch-clamp technique (see below), although improvements in noise reduction and resolution have recently allowed BLM's to be used for the study of solute transport through OmpF (15).

The BLM technique calls for the formation of a lipid bilayer over an aperture located in a septum separating two chambers. Each chamber is filled with a buffered ionic solution and contains an electrode which is used to measure current due to the flow of ions across the bilayer. The electrodes are also used to maintain ("clamp") the transmembrane potential to a fixed value, independently of the ionic movement. The membrane bilayer can be made in a variety of ways, such as by "painting" the aperture with lipids dissolved in an organic solvent (like n-decane) and subsequent evaporation of the solvent ("painted bilayer" technique) (16), or by slowing rising the buffer level in each half-chamber after a lipid monolayer was formed at the buffer/air interface ("raised-bilayer" technique). The monolayers themselves can be made either from an organic solvent-based mixture of phospholipids (17) or by self assembly from solvent-free lipid vesicles (18). The formation of the bilayer will change the optical properties of the membrane, such that the aperture will appear to turn black, hence the name of the technique. In the absence of added channel proteins, the conductance, i.e. the ease of flow of current across the bilayer, is very low.

Purified, detergent solubilized channel proteins are added to the so-called *cis* side, and spontaneously insert in the bilayer over time. Sometimes, membrane vesicles are also used as a source of protein (18, 19). Applying transmembrane voltages can increase the efficiency of insertion. As channels insert in the membrane, ions now find a hydrophilic passageway to cross the membrane, and hence an increase in electrical current can be detected across the membrane. Discrete individual insertion events of porin channels are typically witnessed as sudden jumps in the conductance, as seen in Figure 2A. Since porins retain their trimeric structure upon detergent solubilization, each conductance step is taken to originate from a single trimer. The size of these individual conductance increments follows a Gaussian distribution. The fit to this distribution yields an average conductance, which can be used as an electrophysiological fingerprint for a given channel.

Once insertion is complete, the current waveform is investigated at various potentials, and yields traces such as the one shown in Figure 2B. Here, channel closures are represented as upward deflections. This trace shows sequential closures of several pores, where each large current jump (~ 140 pA, marked by asterisks) presumably represents the closure of a single monomer in response to the applied potential. Note that frequent channel fluctuations also occur during the time periods in between closures. These fluctuations are usually not the focus of attention of BLM studies, and are typically ignored (in many cases, these fluctuations are not even detected because of high filtering and low sampling rate of the data). For sake of comparison with patch clamp data, it is important to realize that many fluctuations of fairly small amplitudes do exist, as indicated on the expanded trace (bottom trace of panel B).

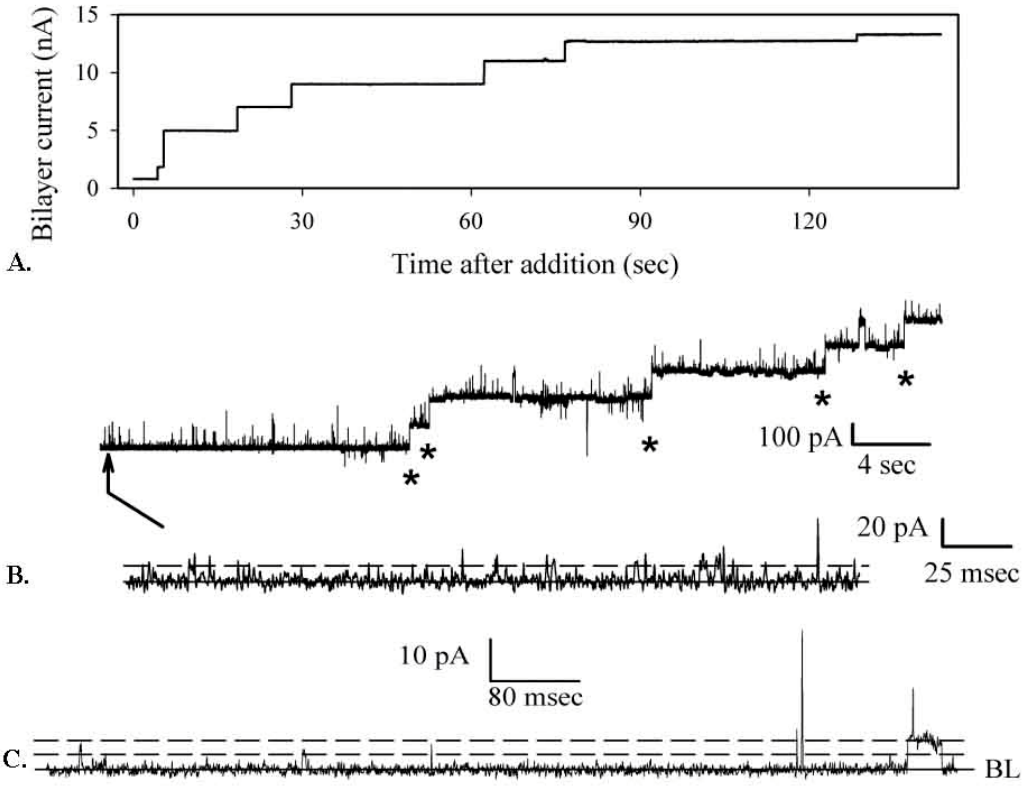

Figure 2. Typical electrophysiology data obtained with purified OmpF. **A.** step-wise increase in current due to sequential insertion events of trimers in BLM; protein was added to the solution on the *cis* side of the bilayer at a final concentration of 100 ng/ml; the membrane potential is – 40 mV (ground is on the *cis* side); solutions contain 1 M KCl, 10 mM Hepes, pH 7.4. **B.** Current trace obtained from a BLM after insertion of porin channels is complete; the membrane potential is –110 mV; the large upward deflections correspond to channel closures in response to the membrane potential and have a conductance of ~ 1320 pS; the expanded part of the trace shows smaller closing transitions of ~ 120 pS. The solutions are the same as in A. **C)** Patch clamp trace obtained at - 100 mV pipette voltage, showing spontaneous closing transitions (upward deflections) from the all-open level (baseline, "BL") of ~ 40 pS (levels marked by dashed lines); bath and pipette solutions are 150 mM KCl, 0.1 mM K-EDTA, 10 microM $CaCl_2$, 5 mM Hepes, pH 7.2; note the different current and time axes for the three traces.

4.1.4. Patch-clamp electrophysiology

The patch clamp technique (20) was initially introduced to the study of ion channels because it allows the investigation of single channels in their native environment, even if they belong to small cells. When first applied to investigating porins, it revealed small size events and channels with fast kinetics of opening and closing, which had not been documented with planar lipid bilayers (21, 22). Initially, the patch clamp technique, which samples the activity of channels in a much smaller patch of membrane (~ 6 μm^2) than in BLM's (10,000 μm^2), provided lower noise and higher time resolution than in black lipid membranes. However, recent advances in BLM technology now allows for fine details of porin behavior to be obtained as well (15, 23).

In the patch-clamp technique, a glass pipette of ~ 1-2 micrometer tip diameter and containing an ionic solution is fitted over a single electrode. With the use of micromanipulators and phase-contrast microscopy, the pipette is placed at the surface of a cell or lipid vesicle. A membrane patch, harboring one or a few channels, is drawn inside the tip, and a tight mechanical and electrical seal is formed between the lipid bilayer and the inner glass surface of the micropipette. The resistance of this seal is very high (in the order of 10^9 ohms), such that currents of very small amplitude (such as those due to ion flow through single ion channel, ~ 10^{-12} A) passing through the ion channels can be detected under a clamped transmembrane voltage. Since the membrane already contains channels, individual insertion events are not observed. Instead, one sees transient flickering in the current trace that represent spontaneous transitions of channels between ion-conducting (open) and non-conducting (closed) states. The technique was applied successfully to live bacterial cells and spheroplasts (24, 25), as well as reconstituted liposomes containing inner or outer membrane fragments (21, 22).

Figure 2C shows a typical patch clamp recording of porin activity. The preferred current level, which we call "baseline" (labeled "BL") represents the total current flowing through all the open porin monomers of the patch. Channel closures are seen as upward deflections from this baseline (in OmpC, frequent downward deflections,

corresponding to openings of additional channels are also seen). The closures can involve two or more channels acting in cooperation, hence the various sizes of the transitions, as shown by the dashed lines (more on this below). Typically the openings of additional channels are extremely transient and appear as downward spikes. The closures, on the other hand, are often more prolonged and appear as square-top deflections. The fact that the kinetics of the openings and closing transitions from a common baseline level are so different indicates that porins can display kinetically distinct states. Presumably they represent different stable closed and open conformations of the proteins that can be visited independently.

4. 2. Ion flow and selectivity

4.2.1. Early studies

Channel size and selectivity are two main parameters that distinguish channels from each other. For porins, the former has been expressed in many different ways depending on the technique used. Channel size can be reported in Angstroms in crystallographic data, in terms of conductance (in picoSiemens, pS) in electrophysiology, or in relative or absolute permeabilities in flux studies. A direct comparison between the different values is somewhat difficult, and care has to be taken in the interpretation because of the limitations of each method of determination.

The initial characterization of the pore properties of OmpF and OmpC by radioactive tracer flux in liposome established the cutoff of 600 Daltons (8). A thorough comparative study of the relative permeability of sugars in the range of 100 to 400 Daltons further established the dependence of the diffusion rates on the solute size (10). It also provided evidence for the OmpC pore being ~ 10% narrower than the OmpF pore. Non-electrophysiological techniques also documented the restricted entry of hydrophobic compounds and ionic preferences (12). In liposome swelling assays, the diffusion rate of negatively charged sugars through OmpF was shown to be much reduced relative to their uncharged counterparts of similar molecular weight (10). This resistance to the flow of anions in OmpF was also observed in the flux of charged antibiotics in liposomes (10) and intact cells (12). On the other hand, the diffusion of anionic compounds through PhoE was accelerated relative to that of their uncharged counterparts (10, 12).

4.2.2. The conductance problem

Nowadays, the characterization of new porins typically relies on the observation that the protein of interest forms ion-conducting units in electrophysiological experiments. When reading the literature, a major point of confusion remains in the value of the conductance of porin channels. Discrepancies over this value likely arise from several factors outlined below.

The first complication stems from the use of the term “single channel” in the literature, as there is uncertainty whether authors refer to single “channel” as a single monomer or as a single protein trimer (i.e. three ion-conducting monomers). The very first study by Benz and colleagues of “matrix protein” (i.e. OmpF) used the size of the current jumps during incorporation of open channels to measure the so-called single channel conductance (26). An average conductance of ~ 1.9 nS and 1.2 nS were found in 1 M KCl or 1M NaCl, respectively. Similar studies performed with OmpC and PhoE yielded values of 1.5 and 1.8 nS, respectively (in 1 M KCl) (27). At the time, the subunit structure was unknown, and it is not surprising that the authors assigned these values to the single channel conductance. They correctly pointed out that these events arise from the incorporation of trimers into the membrane, but there was no reason to believe that trimers actually contained three pores (for example tetramers of eukaryotic K+ channels contain only one pore). On hindsight, it is now clear that the values reported in these papers must represent the trimeric conductances.

Other laboratories have used the conductance steps that occur either at steady-state after insertion is complete, or during voltage-dependent sequential closure of channels at high voltages. Although this is not always clearly stated, it is commonly thought that these transitions correspond to individual monomers. The monomeric conductances for OmpF obtained in such way are in the order of ~ 1.4 nS in 1 M KCl (28) or ~ 0.8 nS in 1 M NaCl (29, 30). Surprisingly these values for the monomer are in the same order of magnitude that those proposed for the trimer by Benz and coworkers (27) (see above). The reasons for this discrepancy are not clear. Conductance values are highly dependent on membrane composition, and porins might exhibit multiple levels of conductance in different conditions (28). In addition, pH is known to affect the distribution of conductances, with a high proportion of smaller conductance events appearing at more acidic pHs (31). Thus, it is possible that the smaller relative monomeric conductance reported by Benz and colleagues stems from the fact that their studies are done at pH 6.0 rather than 7.4.

Finally, the traces obtained with the patch clamp technique provide yet another range of values for the putative single channel conductance. Figure 2 shows that the fast spontaneous fluctuations between open and closed states exist in both BLM and patch clamp traces, but as stated above, these have usually been ignored in papers reporting on BLM work. On the other hand, we and others (32) have focused our attention on these fast transitions that typically exhibit current amplitudes of multiple values. These closing transitions are too infrequent and transient to yield sizeable peaks in amplitude histograms. Therefore, the current amplitudes of individual transitions are typically measured at each voltage, and the conductance is obtained from the slope of the so-called current-voltage relationship, as done for eukaryotic ion channels (33). As the trace exhibits multiple current levels, the amplitudes of individual events tend to cluster around values that are integer multiples of the smallest observed value. Surprisingly, for wildtype OmpC and OmpF, there is no favored amplitude level (as might be expected to represent the trimer conductance). Thus, the question arises at what might be the single monomer conductance in such traces. There is unfortunately no-clear cut answer. Therefore, we made the working hypothesis that the transitions of the

smallest amplitude represent a single monomer (rather than a sub-conductance state), and the largest transitions are those of many monomers opening and closing cooperatively. Cooperativity is a well-documented phenomenon in porins (22, 28, 32, 34), although the molecular mechanism is unclear. The idea that adjacent monomers strongly interact with each other during opening and closing was substantiated by our observation that the large transitions are abolished in an OmpC single-point mutant where the salt bridge that links the latching loop L2 to an adjacent monomer has been disrupted (35). Instead, the kinetic pattern of this porin mutant shows a regular succession of staircase events of identical amplitudes, as expected from a patch that contains independent identical channels.

By assigning the smaller current amplitude to a monomer, we have documented single monomer conductances in the range of 30-60 pS for OmpC, OmpF and PhoE in 150 mM KCl (35, 36, 37). Even when taking into account the differences in salt concentrations, these values are much smaller than previously reported (for example, 1.45 nS in 1 M KCl (28)). Similar values of 50-70 pS in 100 mM KCl (along with transitions of 200 pS) were also obtained by an independent group in a patch clamp study of PhoE (38) and of mixtures of OmpF and OmpC (32). On the other hand, the patch clamp work of Molle and colleagues (39) reports an OmpF conductance of 275 pS in 0.2 M KCl, a value close to the 300-400 pS value derived for OmpF monomers from recent modeling studies in the same ionic conditions (40). A trimeric conductance of 810 pS was also found for OmpF in 100 mM KCl (Winterhalter & Yamato, personal communication).

We need to point out that we cannot rule out that the small conductances measured in patch clamp may represent so-called sub-conductance levels or substates. Substates are extremely frequent in other channels, but are generally characterized by the fact that they are always accompanied by the full-conductance state, which is the dominant conductance level observed in any given condition. Our traces reveal some large conductance levels, but these are usually not dominant, except in the case of some OmpC mutants (35). The fact that the 30-60 pS conductance might be a substate, though, is strongly suggested by a recent study of the block of the OmpF pore by ampicillin, a hydrophilic beta-lactam antibiotic known to use OmpF to gain entry in the cell (15). Here, by studying bilayers that have a single reconstituted protein, the authors were able to demonstrate well-defined current transitions that are likely to represent individual blocking events of one or two monomers. The interruptions in current due to these blocking events would thus directly provide a measurement of the monomeric conductance, which was found to be ~ 1.4 nS in 1 M KCl.

If indeed the 30-60 pS conductance is a substate, an intriguing observation remains that transitions whose conductances are integer multiples of this value are commonly seen in patch-clamp traces. These multiples might represent a series of discreet steps in the gating process, or the cooperative gating of monomers to a single substate. Distinguishing between these – and perhaps other – possibilities will require a better understanding of the molecular mechanism that underlies the spontaneous gating activity.

Finally, one needs to be aware that the conductance is dependent on both ionic concentrations and on the nature of the salt used, as shown in the early work by Benz and colleagues (26, 27). These authors reported that in many cases (but not all), the channel conductance remains proportional to the specific conductance of the bulk solution for the given ion (41). In other words, they interpreted that the differences in conductances seen for different ions can be attributed solely to the intrinsic differences in conductivities of these ions in aqueous solutions. This finding led to the notion that porins are water-filled pores, where ion movement proceeds as in solution, and has contributed to the notion of the poor selectivity of the channel and lack of interactions between ions and porin. As shown below, this notion has to be revised. In fact, deviations in the conductance ratios for different alkali from the bulk conductivity ratios have been shown experimentally (Winterhalter & Yamato, personal communication). The non-linear dependence of the conductance with salt concentration has also been demonstrated by Winterhalter's group. The assumption that the pore is filled with a solution of the same conductivity as the bulk and that the channel is a perfect cylinder of 6 nm in length has led to the estimation of pore diameters. Although the values have turned out to be fairly close to the pore size in the crystal, this approach has been severely criticized based on the knowledge that ions do interact with the pore walls and that the channel architecture is much more complicated than a cylinder (42, 43). This practice has the unfortunate effect to oversimplify the mechanism of solute transport, and has contributed to the erroneous view that porins are simply inert and passive molecular sieves.

In conclusion, one needs to apply caution when comparing values given in the literature. It is not sufficient to simply normalize the values to each other since the linear dependence initially documented (26) has not been reproduced by others in experiments (39) (Winterhalter & Yamato, personal communication), and in modeling (40, 44). There is also evidence that selectivity and kinetics are affected by ionic strength, lending support to the idea of ion-protein interactions (28, 32, 40, 44, 45) (Winterhalter & Yamato, personal communication). Importantly, the ability of monomers to act independently may be affected by salt concentrations (28, 45). Thus, depending on the ionic conditions used, what appears as a current step originating from a monomer may be erroneously assigned. It is clear that a side-by side comparison of patch-clamp and BLM data on the same protein preparation and in the same ionic solutions is needed to shed some light on the discrepancies in conductance.

4.2.3. Selectivity

The measurement of selectivity has been much more straightforward and not biased by the considerations described above. There is general agreement that in the salt conditions at which electrophysiological measurements

have been made 1) OmpF is a fairly non-selective pore with a ratio of potassium to chloride permeability (P_K/P_{Cl}) of ~ 4 (29, 41); 2) OmpC is more cation selective (P_K/P_{Cl} = 7-30 (41, 46, 47); and 3) PhoE is anion-selective (P_K/P_{Cl} ~ 0.4 (41, 48)). There was some debate regarding the existence of a specific polyphosphate binding site inside the PhoE pore (49, 50). The confusion might have arisen from the similarity in the BLM traces showing time- and concentration-dependent inhibition of currents when either polyphosphates are added to PhoE (49) or maltodextrins are added to maltoporin, a well-known specific porin (51). However, the issue appears to be essentially semantic, as all investigators agree that an inhibition of flux of either antibiotics or other ions occur when polyvalent anions are added. In fact this inhibition reflects the prolonged occupancy of the pore by the larger polyphosphate ions, relative to the other ions that give rise to the measured current. There is also agreement that the charge constellation of the PhoE pore plays an important role in the attraction of negatively charged compounds through the pore (49, 50, 52).

Porins are traditionally thought of as very non-selective pores, as clearly indicated in all the electrophysiological experiments done to date. Recent calculations by Im and Roux indicate that the selectivity of OmpF is highly dependent on ionic concentration. They calculated that at low ionic concentrations (below 100 mM), OmpF becomes quite cation-selective, with a ratio of potassium flux to chloride flux through OmpF reaching a value of ~ 1100 (40). The molecular basis for this dependence is presented below. Assuming that this ratio of flux is indicative of selectivity, this would indicate a selectivity comparable to that of eukaryotic potassium channels. Electrophysiological experiments are typically done in hundreds of mM of ions, a condition necessary for both bilayer stability and detectable currents. Recently, Winterhalter and colleagues were able perform BLM experiments in concentrations as low as 10 mM salt. They experimentally corroborated the calculations of Im & Roux by reporting that the ionic selectivity of OmpF was highly dependent on salt concentrations. The permeability ratios are not as high as the flux ratios calculated by Im & Roux, but the P_K/P_{Cl} did increase ~ 5 fold when the ionic gradient is 0.01 M / 0.1 M as opposed to 0.1 M / 1 M (personal communication). Low ionic strength milieus are common for bacteria when outside animal hosts. If substantiated by further experiments, these observations call for a serious re-assessment of the permeability properties of the outer membrane in different external conditions.

4.2.4. Mechanistic considerations

Insights into the molecular mechanism of permeation and selectivity of general diffusion channels have been gained in the past 5 years from the study of mutants and from modeling work. A summary of these accounts is presented below.

4.2.4.1. Mutant work

Even before the publication of the OmpF and PhoE structures, Benson's group provided evidence for the involvement of specific residues in OmpC and OmpF that control the pore properties of these proteins. They cleverly designed a screen for mutants with enlarged pores, and were able to identify 3 residues for OmpC and 4 residues for OmpF, that participate in pore size (53, 54). This discovery was immediately followed by electrophysiological studies of these mutants, which confirmed the increased conductance, and also documented that other pore properties (such a selectivity and voltage dependence) had been equally modified (55, 56). When the crystal structures were published, it was satisfying to note that the residues uncovered by Benson and colleagues indeed decorated the walls of the OmpF pore, precisely at its narrowest part (the constriction zone), where size exclusion and selectivity are primarily achieved.

Since then, the constriction zone has been a primary target for site-directed mutagenesis. In particular the charged residues of the inwardly folded L3 loop and the cluster of positive charges in the barrel wall opposite to L3 have been investigated (see Table 2 and figure 1B for a representation of the OmpF constriction zone with labeled residues).

Effects on conductance have been variable depending on the type of porin. Typically, single-point mutations of OmpF pore residues have negligible effects (<10 %) (29, 57). Exceptions are charge removal at E117 that decreases conductance by 25% (57), and charge substitution at R132 or mutation in G119 that leads to decreased conductance but increased permeation to sugars (39) and alteration in antibiotic flux and susceptibilities (39, 58). Mutations at multiple sites effectively decrease single channel conductance, but enhance permeation of disaccharides (57). For OmpC, the mutations isolated by Misra & Benson at R37 and R74 increased single channel conductance (55, 56), while charge removal at D105 decreased conductance (46). Similar mutations in the equivalent residues of PhoE (R37 and R75) led to opposite effects, i.e. a decrease in conductance (30). Taken together, these results suggest that the channel conductance is governed much more by charge consideration than by steric hindrance. This effect is more pronounced for the smaller OmpC and PhoE pores, where even single point mutations have strong effects. These observations are good evidence that the charge constellation of porins in the constriction zone increases permeability by increasing local ionic concentrations. They are also a good reminder of the flaw in deriving channel size from single-channel conductance.

On the other hand, as can be expected, effects on selectivity are drastic: removal of positive charges at K16 and the arginine sites of OmpF leads to an increased in cation selectivity, while removal of the negative charge at D113 or E117 decreases cation-selectivity (29, 39, 57). Similar findings were found for OmpC (46). These results suggest that selectivity towards cations is largely determined in OmpF and OmpC by the combined attractive and repulsive effects of charged residues of the constriction zone. In PhoE, some loss of selectivity was detected at K18, but the most drastic effect was found at the K125 residue (52). The substitution of glutamic acid for lysine at this position converted PhoE into a cation-selective pore. In

Table 2. Some of the constriction zone residues that are conserved in *E. coli* OmpF, OmpC and PhoE

OmpF	OmpC	PhoE
K16	K16	K18
R42	R37	R37
R82	R74	R75
D113	D105	D106
E117	E109	E110
G119	G110	G112
R132	R124	K125

addition, the binding of polyphosphate, as measured by the inhibition of the channel conductance (open channel block), was also specifically annihilated in this mutant, suggesting that K125 is intimately involved in the interaction with phosphate bearing compounds that require PhoE for transport.

Mutations at the constriction zone revealed two other interesting aspects of porin function: 1) the influence of the pore charge constellation on voltage-dependence, and 2) the existence of a spontaneous form of channel gating (i.e. channel oscillation between closed and open states) which is influenced by the charges of the constriction zone and interactions of L3 with the barrel. The former will be presented in the section on "Voltage-dependence" below. Spontaneous channel gating is clearly observed in patch clamp traces (Figure 2C), even in the absence of a transmembrane potential, when ion current is driven by an ion gradient (hence the term "spontaneous"). We can envision this form of gating as originating from rapid fluctuations between conformations that support different current amplitude. Whether the two conformations are truly closed and open, or whether they represent distinct open states with different conductance values remains to be determined (we have proposed that they do represent closed and open forms). It is clear that the interactions that the L3 loop makes with the adjacent barrel wall and with the opposite barrel wall control this form of gating. We have found that mutations that disrupt a salt bridge connecting the root of L3 to the barrel, or mutations that disrupt the putative H-bond network that exists between the tip of L3 and the adjacent barrel, have the profound effect of increasing the frequency of the spontaneous oscillations (35). Thus it appears that the L3 loop can somewhat "breathe" across the pore, and that these motions might lead to states with various conductances. A recent molecular dynamics simulation of OmpF has confirmed the dynamic fluctuation of the very tip of L3 (59), and suggested that water molecules that creep in between the L3 tip and the barrel wall might lead to this flexibility. Other simulation studies have suggested that the segment between residues 105 and 112 on the L3 loop of OmpF might fluctuate between an α-helix and a 1-3 helix (60). The possible relationship between these short-range motions of L3 and the spontaneous gating is not to be confused with the large-scale movement of L3 that seems not to be involved in the voltage-dependent inactivation (see below).

4.2.4.2. Modeling studies

Porins have provided an ideal system to model properties in ion channels, and several computational papers have appeared over the years (40, 44, 57, 59-63). Different algorithms have been used by various groups, and it is not the purpose of this review to compare these approaches. An excellent account is given in the recent publication of Im and Roux (59). Here we will summarize how the computational studies, together with the wealth of information obtained from electrophysiology on wildtype and mutant channels, are providing a picture of the path of ions along the porin channels, and are giving mechanistic insights into this process.

A study published by Schirmer and colleagues soon after the publication of the crystal structure revealed that the charge constellation at the eyelet (i.e. the negatively charged residues of L3 and the cluster of positive charges in the opposite barrel wall, see Figure 1B) establishes a strong intrinsic electric field, that is perpendicular to the pore axis (64). This field increases channel conductance by attracting charges into the pore, and also has the effect of orienting water molecules perpendicularly to the channel axis (59, 60). OmpF is highly asymmetric on each side of the constriction zone. On the periplasmic side, the so-called internal vestibule is wide, and offers a steeply increasing solvent accessible area as one proceeds from the eyelet to the membrane surface. The external vestibule is more constricted and offers a smaller volume for water molecules and ions (Figure 3A). Amazingly, the average preferred paths for chloride and potassium ions are well separated over the entire length of the channel (44, 59). As one follows the ion density maps provided in a recent molecular dynamics simulation of OmpF (59), charge separation is already clearly evident in the extracellular vestibule, and very crisp at the constriction zone due to the transversal electrostatic field (Figure 3B). The trajectories are more blended in the extracellular vestibule, although chloride tends to stay near the barrel walls, while potassium ions mostly cluster at the outer rim. In addition, the ions trajectories down the pore have a left-handed screw-like shape, resulting in a 180-degree rotation of the positions of the ions at the periplasmic side relative to the extracellular side.

Thus, a potassium ion enters the extracellular side near the interface between monomers, but then follows a twisted path that brings it close to the D113 residue of L3, and eventually exits near the outer rim of the intracellular vestibule. Chloride ions enter the pore near the L4 loop, right above the root of the L3 loop, then move in close proximity to the arginine cluster, opposite to L3, to finally exit along the barrel wall.

However at any given time and any given site, there is always more K+ in the pore than Cl-, in accordance with the cation preference of OmpF. In fact, it appears that the only way Cl- can pass through the constriction zone is because of its strong pairing with K+ which has the effect to screen some of the negative electrostatic potential which would repulse Cl- ions. It is very interesting to note that at low ionic strengths (10 mM KCl), where this ionic screening is reduced, OmpF becomes very cation-selective and the number of Cl- ions in the pore becomes almost negligible (the potassium current becomes several hundred times greater than the Cl flux). A substantial increase in

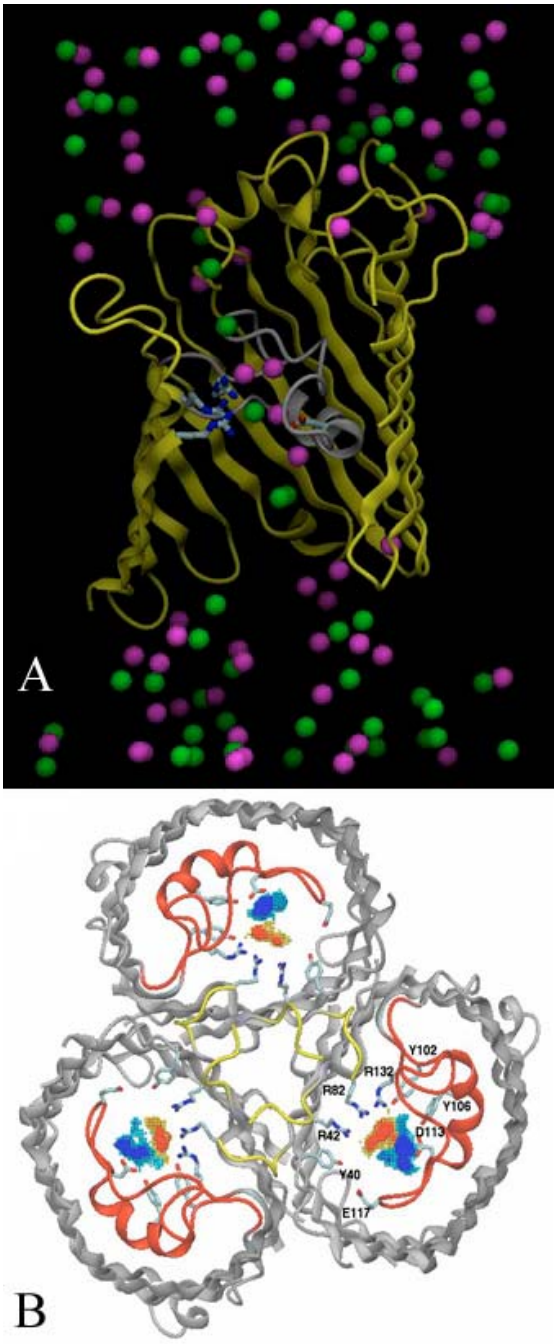

Figure 3. Molecular modeling of ion flow through OmpF pores. **A.** side view of an OmpF monomer showing the L3 loop in gray, K+ ions in magenta and Cl- ions in green; some of the barrel wall has been removed from the front view to allow a better visualization of the channel interior. **B.** cross section of an OmpF trimer, showing ion density maps in a 4 Å slab at the level of the constriction zone; the K+ density is represented in colors ranging from cyan (low density) to blue (high density); the Cl- density is shown in colors ranging from yellow (low density) to red (high density); the segregation of the two paths taken by K+ and Cl- ions is clearly visible (reproduced from ref. 59, with permission).

cation selectivity is already noticeable at physiological concentrations of ~ 0.1.M KCl (40).

Finally, computational studies have established that the diffusion of ions is reduced by about half inside the pore relative to bulk. Permeating ions do trade their solvation with water molecules for interactions with protein residues as they move down the pore. Thus the single channel conductance in various concentrations of KCl does not follow bulk conductivity, but shows a strong deviation from linearity. In addition, the conductance takes slightly different values depending on the direction of ion flow (39). This asymmetry in the conductance is not due to the asymmetric shape of the channel, but to the distribution of charges over the whole length of the ion-conducting pore (40).

Images of ion trajectories can be found at the following web sites:
http://www.biozentrum.unibas.ch/personal/schirmer/
http://thallium.med.cornell.edu/RouxLab/index.html

4.2.4.3. A scenario

In summary, we can envision the following scenario for solute transport across OmpF or OmpC. First only hydrophilic and relatively small solutes will be admitted into the pore. Steric hindrance will be initially more pronounced for solutes entering the cell, than for those exiting the periplasm, although all will meet the highly restrictive barrier of the eyelet at mid-height of the channel. Ionic interactions between the solutes and the channel walls will make crucial contributions in determining rate of flux, and thus ionic strength and pH will have strong influences in the efficiency of transport. Positively charged compounds will be accelerated into the pore, due to the mostly negative electrostatic potential of the channel. As long as they can be accompanied with counterions, anions will be able to pass through, in a path that is somewhat segregated from those of cations. At low ionic strength, only cations will permeate, as the strong negative electrostatic intrinsic potential will repulse anions. As they transit through the pore, small charged species will follow a screw-shaped path across the pore. On the other hand, zwitterionic species will adopt a position that maximizes their electrostatic interactions with the highly asymmetrically charged eyelet. If large enough, they will temporarily block ionic current, as recently shown in an elegant study on beta-lactam antibiotic penetration in OmpF (15). Occasionally, slight fluctuations in L3 loop positions will reduce the pore size, or more likely will distort the shape of the intrinsic electrostatic potential at the eyelet. These conformational changes will lead to changes in the rates of ion flux and abrupt fluctuations in the pore conductance, leading to a spontaneous form of channel gating. Eventually, a very large number of ions – in the order of 1 billion per second – will make their way across the outer membrane.

5. MODULATION OF PORIN ACTIVITY

Porins are traditionally viewed as permanently open pores. Undergraduate textbooks present the outer

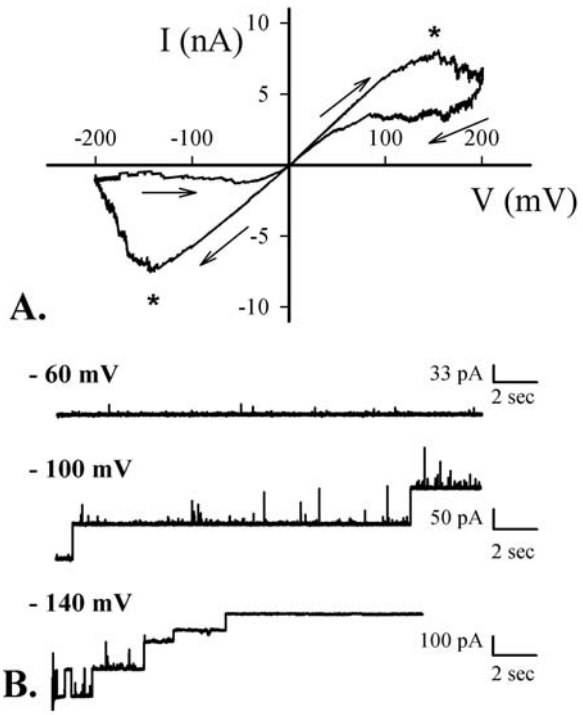

Figure 4. Typical illustrations of porin voltage dependence, **A.** in BLM studies: the voltage-dependence is seen as hysteresis loops in the + and - voltage ranges; this type of data is obtained by plotting on the same graph the current observed during a continuous seesaw voltage ramp going from 0 → 200 mV → 0 mV → - 200 mV → 0 mV (see arrows) at a rate of 0.8 mV/sec; voltage dependent closure is seen as the deviation of the current trace from linearity at the points marked by asterisks; solutions are 1 M KCl, 10 mM Hepes, pH 7.4. **B.** in patch clamp traces; the voltage dependence is seen as a more rapid and more complete sequential closure of channels (upward deflections) upon voltage application; the voltage is switched from 0 mV to the indicated voltage at the beginning of the trace; note that the current remained unchanged for at least 20 sec at the lowest voltage shown (-60 mV). Bath and pipette solutions are 150 mM KCl, 0.1 mM K-EDTA, 10 microM $CaCl_2$, 5 mM Hepes, pH 7.2.

membrane as a dashed line to emphasize the constant and extremely porous nature of this outer layer. The crystallographic data presents a beautiful structure with three widely open pores. But the studies of porin function have challenged this view by providing evidence for the existence of closed states in porins. The AFM study of Muller and Engel also reveals closed porin conformation (65).

5. 1. Voltage dependence

A salient but much debated form of modulation is the voltage-dependent closure of porins. It has been reproducibly observed by numerous laboratories using either BLM's or patch clamp electrophysiology, and a variety of protein sources, from outer membrane fractions to purified channels. It is clear that it is not an artifact originating from the handling the biological samples or the method of study. Its physiological significance, as well as its molecular mechanism, remains to be established.

The voltage sensitivity of porins was first demonstrated by Schindler and Rosenbusch (19) who observed step-wise decrease in the current flowing through porin-doped bilayer when the transmembrane potential had reached a critical threshold voltage (Vc). A common way of documenting voltage dependence in BLM's is to apply successive voltage ramps, that slowly change the voltage to larger values (either positive or negative), and then bring the voltage down again. As the voltage is brought down, channel re-opening shows a distinct and characteristic hysteresis (Figure 4A). In this type of experiment, voltage-dependence occurs in both the positive and negative regimes. Typical values for Vc are ~ 150-170 mV for OmpF (29, 66), ~ 200 mV for OmpC (55) and ~ 135 mV for PhoE (30). Voltage sensitivity was not documented in the work of Benz and colleagues (26, 27). A possible explanation is that differences in purification and reconstitution protocols have been shown to affect voltage dependence (67). If one maintains a constant voltage instead of using ramps, the membrane current drops in steps that have been taken to represent single monomer closures (see Figure 2B above, in the section "Planar Lipid Bilayer Electrophysiology"). The phenomenon is clearly reversible, as excursions to either voltages of opposite polarity or removal of the voltage stimulus restore the original level of macroscopic current.

Patch clamp studies have confirmed the voltage-dependence, but in a lower range of transmembrane potentials (typically less than 100 mV) (22). Here, voltage-dependence is manifested as an increase in the frequency and duration of long closures, and a more rapid onset of sequential closures (Figure 4B). As opposed to their behavior in BLM's, porins closed by high voltages are reluctant to re-open, and the channels appear to have entered a functional state that is more akin to the voltage-inactivated states of some eukaryotic channels. In addition, an asymmetric and opposite voltage dependence was demonstrated for OmpF and PhoE (37), which is usually not observed in BLM's (for example, Figure 4 shows hysteresis loops at both negative and positive voltages, and not just one voltage regime).

The reasons for these discrepancies may reside in experimental conditions, such as ionic strength and the nature of the biological material. As the patch clamp work was performed on outer membrane fractions and not on purified proteins, it is likely that the channels have remained surrounded by more lipopolysaccharides (LPS), which not only allowed for the oriented reconstitution of the channels, but also may influence voltage sensing per se.

Interestingly, the finding that OmpF and PhoE have an opposite voltage-dependence has provided an explanation for the observation that mutations at conserved residues had opposite effects on the voltage dependence (Figure 1B): charge removal of negatively charged residues of L3 renders OmpF less voltage-sensitive, but PhoE more voltage-sensitive (30). Mutations of the positively charged

amino acids cluster of the barrel wall also conferred an increased voltage-sensitivity to OmpF (30) or OmpC (56, 69).

The molecular mechanism for voltage dependent closure (or inactivation) is not known. The L3 loop seems to be a prime candidate for an inactivation gate. A few modeling studies have supported a motion of the L3 loop that could lead to pore closure (61, 62). But they have been criticized because the simulations were done without explicit solvent. Other computational studies involving hydrated states have not found evidence for large motions of L3 (59, 60). Elegant experiments where the L3 loop was tethered in place by engineered disulfide bonds to various parts of the inner barrel have also refuted this hypothesis (68, 70, 71). The focus has now turned to the extracellular loops. Atomic force microscopy has suggested a possible conformational change of the OmpF extracellular domain upon high voltage (65). Recent work on chemical or mutational modifications of lysine residues in the extracellular loops of the Hib porin of *Haemophilus influenzae* also support the involvement of surface-located loops in voltage gating (72, 73). Thus one might envisage that the extracellular loops may form a lid whose movement would control some forms of gating (3).

If the extracellular loops contribute to the voltage-induced conformational change, an important issue remains how to link voltage-dependent modifications in these domains with the voltage sensing that seems to involve the constriction zone. It is not unusual to find that different domains of ion channels perform different functions, and to postulate long-range conformational changes that functionally link these domains. For example, the voltage sensor of the Shaker K+ channels (6 transmembrane segment family) is located along the 4^{th} transmembrane α-helix, and appears to undergo an outward motion upon voltage stimulus (74). It has been recently suggested that the "gate", the protein region that actually controls the conformational changes between closed and open state, is located at the cytoplasmic side of the 6^{th} helix (75). The nature of the coupling between the 4^{th} helix and the gate remains elusive. For porins, one could envisage that a small motion of L3 (which, by the way, also contains a short but very conserved α-helix) in response to potential changes might have far reaching effects on other parts of the protein. Possible candidates to play the role of linkers might be the so-called latching loop L2 that interacts at the root of L3, and the barrel itself, as L3 does not make any direct contact with other extracellular loops.

Perhaps a more likely – but still very speculative – scenario is that the constriction zone *per se* does not play a sensing role, but rather serves as a receptor site for an "inactivating particle" which would be drawn into the open pore upon a specific voltage change. Since it is possible that this inactivating particle (for example an extracellular loop) is charged, it might form ionic interactions with residues of the constriction zone. Thus any modification in the charge constellation of the constriction zone might influence the ability of this inactivation particle to interact with the pore lumen, and thus affect voltage sensitivity, as seen experimentally in site-directed mutants. This type of speculative mechanism would be akin to the "ball and chain" model for inactivation of K+ and Na+ channels, where a peptide segment of the protein swings in the internal mouth of the channel to induce channel block and inactivation (33). Experimental tests of this hypothesis would require site-directed mutagenesis in the external loops, which is currently underway in our laboratory.

Is there any functional relevance to the voltage dependence? Before answering this question, one first needs to address whether potentials across the outer membrane can be created and sustained *in vivo*. Here it is important to consider that biological membranes can support different types of potentials. One is most familiar with the diffusion potential, which is created when a membrane separates two milieus of different ionic concentrations and contains ion specific open pores ("leaks") that allow the diffusion of ions down their concentration gradients. A good example is provided with the K+ selective ionophore valinomycin, which creates a K+ equilibrium potential when added to liposomes in presence of a potassium gradient. Maintaining the potential at the equilibrium value requires the presence of very specific ion channels, otherwise the potential is collapsed by the short-circuiting flux of counterions. The documented low ion specificity of porins precludes the existence of such a potential *in vivo*. However, as shown in modeling studies, if the selectivity of OmpF attains a high level at low ionic strength such that anion flux is essentially nil (40), then a sustained equilibrium diffusion potential might be created.

Another type of potential is the Donnan potential which results from the presence of fixed, impermeant charges on one side of a membrane. Because of the preservation of bulk neutrality, the asymmetric distribution of these impermeant charges forces an asymmetric distribution of counterions, and therefore the creation of a sustained gradient of these counterions and the generation of a diffusion potential, called "Donnan potential" in this special case. Nikaido and coworkers have demonstrated that Donnan potentials are created when cells are grown in low osmolarity medium and have synthesized periplasmic membrane-derived oligosaccharides (MDO's) (76). MDO's constitute a family of negatively charged sugar polymers that are too large to penetrate through the porins. Being trapped in the periplasm, they constitute a pool of fixed negative charges that create a Donnan potential across the outer membrane (potential more negative on the periplasmic side than on the outside). By manipulating the external salt concentration, Nikaido and colleagues were able to generate Donnan potentials of various magnitudes and to show that porin-mediated antibiotic fluxes were not affected by these potentials (76). These results are not surprising in light of the recent discovery that the asymmetric voltage-dependence of OmpF does not lead to OmpF closures at potentials that are negative on the periplasmic side, as generated in this study (37).

Finally, surface potentials are generally present on each side of biological membranes due to charges on lipids. These surface potentials are short-range, as one

moves away from the membrane into the bulk, and are highly sensitive to ionic strength. It is clear that the outer membrane must display very different surface potentials on each side, because of the highly asymmetric nature of the bilayer, and because the charge density is much greater for LPS than for phospholipids (- 0.53 As/m2 for LPS and -0.05 As/m2 for phospholipids (77)). For example, a surface potential of up to ~ -200 mV could be attained at the outer leaflet surface at an ionic strength of 65 mM (77). It is conceivable that changes in surface potentials triggered by changes in medium ionic strength or lipid composition might trigger conformational changes in porin, in particular in the surface-exposed loops which are still within the field of this strong surface potential.

5. 2. pH dependence

The effect of pH on porin function has been examined in numerous studies. It is clear that acidic pH has an inhibitory effect, but the exact nature of this inhibition remains confusing. A decrease in conductance has been suggested (78, 79). McGroarty and colleagues have proposed that porins exist under at least two open-channel configurations with a small and large conductance, and that acidic pH tips the equilibrium towards the small conductance configuration (31). This effect would be somewhat reminiscent of the effect of ionic strength on channel conductance (see above) (28). Acidic pH also shifts the voltage-dependent curve towards lower voltages (i.e. makes the porins more voltage sensitive) (31, 79). The nature of these two open conformations is unclear. It is possible that acidic pH decreases cooperativity and that the small conductance conformation favored at low pH represents a smaller number of pores acting in concert. This interpretation would be consistent with patch clamp data that lacked to detect any effect of acidic pH on conductance (80).

Acidic pH exerts somewhat distinct effects on OmpF or OmpC activity in patch clamp experiments. An increase in the frequency of closures at pH 5.6 relative to pH 7.2 is evident with OmpF (Baslé & Delcour, unpublished), but not with OmpC (80). On the other hand, the macroscopic current flowing through many open OmpC pores is decreased by ~ 20%. Since we have not observed an effect of pH on conductance, our interpretation has been that ~20% of the OmpC pores have shut down. Regardless of the actual mode of action, it is clear that the inhibitory effect of pH on porins can be drastic enough to cause a diminished overall outer membrane permeability. This was indeed demonstrated with antibiotic flux assays done on whole cells (81).

The molecular mechanism of the pH dependence is not yet elucidated. The initial assessment that His-21 of OmpC and OmpF played a role (82) was refuted in another study (66). Muller and Engel reported that a conformational change in the extracellular domain occurs upon acidification of the external milieu (65). More details on this physiologically important mode of regulation are still waiting.

5. 3. Polyamine and polyanion inhibition

OmpC and OmpF are inhibited by the polyamines spermine, spermidine and cadaverine in a concentration- and voltage-dependent manner (36, 83, 84). Similarly, PhoE is inhibited by polyanions, such as ATP (85). However, the nature of both types of inhibition appears different. The inhibition of PhoE by ATP is manifested by a greatly increased open channel noise, in the form of frequent and very transient closing transitions. The kinetic pattern is voltage and concentration dependent, and reminiscent of the flickering of open channel current observed when eukaryotic channels are inhibited by so-called open channel blockers. It appears that ATP, with a molecular weight of 507, blocks ionic flow as it attempts to permeate through the channel. A similar type of inhibition by ATP has been observed with the mitochondrial porin VDAC (voltage-dependent anion channel) (86).

The kinetic effects of polyamines are more complex. Polyamines suppress channel openings and increase the frequency and duration of channel closures. In short, they stabilize closed states, and thus shift the thermodynamic equilibria between open and closed conformations in favor of the closed ones (36, 83). This mechanism, which is distinct from the pure open channel block exerted by polyamines on K+ channels of the heart for example (87), is reminiscent of the allosteric effect exerted by philanthotoxin on nicotinic acetylcholine receptors (88). It seems that multiple sites of interaction are involved, as in eukaryotic channels, since some effects, but not all, are suppressed in some porin mutants (89, 90).

It is clear that polyamines have at least one binding site inside the pore, but the exact nature of this site remains speculative. Attempts to co-crystallize spermine with OmpF have been unsuccessful (Rosenbusch, personal communication). By analogy with polyamine binding proteins of the E. coli Pot system (91), we have made the assumption that the charged groups of the polyamine molecules interact with negatively charged residues in the pore. We had previous evidence that the lumen exposed D113 residue on L3 conferred sensitivity to spermine (90). By postulating that D113 could anchor spermine at one end of the molecule, we searched for pore-exposed residues that could make ionic or H-bonding interactions with spermine, precisely at the amine group sites. Inspection of the OmpF crystal structure revealed two residues, D121 (also on L3) and Y294 (in the barrel wall), that fit this description. We showed that modulation by spermine is lost when any of the three amino acids D113, D121 and Y294 is mutated to alanine (89). These observations support a model whereby spermine would enter the pore in a head-on conformation and effectively bridge the periplasmic and extracellular branches of the L3 loop and connect them to the adjacent barrel wall (89). This saddling over the L3 loop would bring about the allosteric modulation leading to stabilization of closed states and porin inhibition. The involvement of D113 and D121 has been substantiated in a recent computer simulation of spermine docking in the OmpF pore (92). This work also proposed that E62, E117 and D126 might provide anchors to spermine. In some cases, the polyamine might adopt a perpendicular position relative to the pore axis, and partially block the channel lumen (92).

The inhibition of OmpF and OmpC by polyamines has been shown to be voltage dependent (36). Specifically, membrane potentials that are negative on the periplasmic side with respect to the outside enhance inhibition by polyamines applied to the outside, and *vice versa*. Since the sign of the voltage that enhances inhibition depends on the side of application of the drug, it is clear that this voltage dependence is not intrinsic. In other words, it is not the case – as in some eukaryotic channels – that the binding site of polyamine is only revealed upon voltage-induced conformational changes. Rather, the voltage dependence stems from the fact that the positively charged polyamine is electrophoresed to its site of action inside the pore. The net result is that porins close at much smaller voltages in the presence of polyamines that in the absence of these compounds. This synergistic modulation of porins by polyamines and voltage may play important roles *in vivo*, but this remains to be demonstrated.

Could polyamines play the role of gating particles to confer voltage dependence to porins? The voltage-dependence of inward rectifier K+ channels of the heart is due to the channel block that occurs when endogenous cytoplasmic polyamines, such as spermidine or spermine, are attracted inside the pore at depolarizing voltages (i.e. membrane potential more negative on the outside than the inside) (93). In fact, the voltage dependence of these channels is lost when the patch of membrane is isolated from the intracellular milieu. It is unlikely that the same mechanism applies to porins, since the voltage-dependence is still observed with purified proteins.

Whether the mechanisms of polyamine-induced closure and voltage-induced closure are identical or even share some common paths is completely unknown at this point. It is intriguing that the L3 loop residue D113 plays an essential role for both phenomena. One could imagine that if the mechanism of voltage-dependent inactivation did involve pore plugging as described above, this phenomenon might be mimicked by polyamines, since we know that the polyamine site of action is inside the pore. However, polyamines are small enough to permeate the channel, and although they appear to induce some open-channel block, it is much more likely that their mechanism of action is mainly allosteric, rather than purely steric.

The physiological role of polyamine modulation is currently investigated. The best-studied example is the decrease in outer membrane permeability engendered by cadaverine excreted at low pH. Acidic pH activates the cytoplasmic membrane bound environmental sensor CadC protein to bind the *cadBA* promoter, and to induce the *cadBA* operon (94). The operon encodes a lysine decarboxylase (*cadA*) and a lysine-cadaverine antiporter (*cadB*). Increased levels of CadA and CadB lead to the acid-induced synthesis of cadaverine from lysine and the subsequent excretion of CO_2 and of the polyamine, the latter moving through the lysine-cadaverine antiporter. This process requires the consumption of one proton, and may simply represent a means of de-acidifying the cytoplasm, with the release of cadaverine as a waste product. However, we showed that the secretion of cadaverine produces a decrease in outer membrane permeability because cadaverine closes a number of porins, most likely during its passage through the periplasm and through the pores (95). Cadaverine-resistant porin mutants are more sensitive to low pH than their wild-type parents, and thus, cadaverine inhibition of porin may play a role in the adaptive response to acidic conditions (96). This is the first case of porin modulation shown to have direct physiological consequences *in vivo*. Modulating porin function provides a rapid mechanism for limiting outer membrane permeability in stressful conditions. In emergency situations, this process can provide some relief to cells while a more permanent – but also more slowly developing - change in outer membrane permeability is introduced by down-regulating porin expression.

6. PERSPECTIVE

Extensive biochemical and structural investigations over the past 25 years have produced a good understanding of the process of solute passage through general diffusion porins. Emphasis has now shifted towards the functional modulation of porin activity, as evidence has accumulated that general diffusion porins can be inactivated or inhibited by various physico-chemical parameters. Much remains to be done in terms of deciphering the molecular basis for these forms of regulation. The challenge of relating the dynamics of the proteins with their function will likely proceed with the combination of approaches that has been so successful in describing permeation through porins. The use of mutants and biophysical techniques, such as electrophysiology, spectroscopy and molecular imaging, can be powerful in obtaining molecular information on the processes of voltage- or pH-dependent modulation and other forms of inhibition.

Another goal is to find how and where these regulatory processes take place within a physiological context. Here, much can be learned by expanding the research arena to other Gram-negative bacteria. Porins have been described in numerous bacterial species, and little is known about the modulatory aspects of these proteins. It is likely that modulation of porins play a role in the adaptation of bacteria to external environments. The analysis of porins in species that inhabit a variety of milieus, such as for example pathogens, will allow us to identify situations where porin modulation and/or function might play such a role. For example, recent experiments suggest that *Vibrio cholerae* strains expressing different porin types have different abilities in producing virulence factors and colonizing the intestine (97). These properties might be related to the distinct sensitivities of the two porin types to bile components (98, 99), or to the distinct pore properties of these proteins, in particular selectivity (100). Thus, recent discoveries in the biophysics of porins, namely the existence of various forms of modulation, have opened new avenues of research geared towards investigating the impact of regulation of outer membrane permeability in such processes as pathogenicity and environmental adaptation.

Finally, the combination of a better understanding of porin-mediated transport and the application of computational methods is setting up the stage for rational drug design, not only with respect to potential inhibitors of porin function, but also concerning drugs that use porins for access into the cell. For example, recent modeling and molecular dynamic calculations have provided a picture for the docking of spermine (92) or the beta-lactam antibiotic ampicillin (15) inside OmpF. Together with the electrophysiological analysis of antibiotic permeation, these types of approaches are likely to allow the development of methodologies for screening new antibiotics. The need for such new therapies is underscored by reports that antibiotic resistance can arise from mutations that lead to modification in porin function, and not solely from decreased porin expression (101, 102).

7. ACKNOWLEDGEMENTS

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