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Asymmetry of orientation and voltage gating of the *Acidovorax delafieldii* porin Omp34 in lipid bilayers

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The functional properties of the major outer-membrane protein of *Acidovorax delafieldii*, the anion-selective porin Omp34, were investigated in artificial membranes. Detergent-solubilized porin incorporates into the membrane in a unidirectional orientation solely determined by protein features. This enabled us to characterize the vectorial properties of the porin channels. Omp34 is electrostatically asymmetric regarding both the ion conductivity of a single trimer and the macroscopic ion conductance of multiple porin molecules. Voltage-dependent closing occured at negative potentials; 50% of the channels were already closed at −10 mV (switching voltage). Our experimental results suggest that protein charges situated on flexible parts inside the channel are involved in the gating mechanism. A simple model is proposed illustrating the mechanism of voltage-dependent opening and closing of the porin channels. This model explains the functional characteristics of Omp34 and the dependence of the switching voltage on the electrolyte concentration in particular. Further factors influencing voltage gating include the buffer concentration as well as the technique used for membrane formation. Altogether these factors may explain the relatively high voltages needed to obtain voltage gating with other porins.

In recent years a wealth of new information on the structure and function of porins of bacterial outer membranes has been accumulated. A major achievement was the determination of porin structures at atomic resolution, in particular of the *Rhodobacter capsulatus* porin (Weiss et al., 1991 a) and porins from *Escherichia coli* (Jap et al., 1991; Cowan et al., 1992). These structures provided for the first time an insight into the channel morphology and the charge distribution inside the channel.

Many functional investigations confirmed the initial finding of Schindler and Rosenbusch (1978) that porin channels open and close in a voltage-dependent manner. Interestingly the voltages required for channel closing were different even for identical porins in the various studies. This applies to OmpF (Lakey and Pattus, 1989; Morgan et al., 1990) and OmpC (Lakey et al., 1991; Bühler, 1991) of *E. coli* as well as to protein I of *Neisseria gonorrhoeae* (Young et al., 1983; Mauro et al., 1988). A revealing comparison of the effects resulting from the different techniques of membrane formation was given by Lakey and Pattus (1989), but the prerequisites for channel closing at low potentials have not been investigated systematically so far. It is shown here for the porin Omp34 of *Acidovorax delafieldii* that functional experiments together with the structural information available provides further insight into the mechanisms allowing closing and opening of porin channels.

Omp34 of *A. delafieldii* is a strongly anion-selective porin, and recently its biochemical and some of its functional characteristics have been described (Brunen et al., 1991). The bacterium was isolated from soil (Stanier et al., 1966) and has been shown to use a wealth of organic acids as carbon sources (Willems et al., 1990). The bacterium belongs to the β-subdivision of the Proteobacteria and is closely related to *Comamonas acidovorans* which also possesses an anion-selective porin (Engelhardt et al., 1990). The primary structure and a folding model of the latter were published recently (Gerbl-Rieger et al., 1991; Gerbl-Rieger et al., 1992).

The experiments discussed below and the conclusions drawn as to the voltage-induced closing became possible because of the fact that the porin molecules incorporate unidirectionally into the membrane. This process which is determined by protein features only and which was already predicted by Morgan et al. (1990) for *E. coli* porin is proved here experimentally to be correct and has been used to investigate the vectorial properties of the porin Omp34 in more detail.

MATERIALS AND METHODS

Bacterial strains and chemicals

A. delafieldii (previously *Pseudomonas delafieldii* type strain DSM 64, identical with ATCC 17505) was obtained from the German Collection of Microorganisms. Most of the chemicals were purchased from Merck, Triton X-100 from Roth, soy-bean-lipid type IV, *n*-hexane and hexadecane from Sigma. Diphytanoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Biochemicals Inc., *n*-decane and Genapol from Fluka and octyl-polyoxyethylene from Bachem. The porin was purified as described in detail elsewhere (Brunen et al., 1991).

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Conductance measurements

Asymmetric incorporation of porin (Figs 1, 2) was investigated using the Mueller-Rudin technique of membrane formation (also called black-lipid membrane technique, Benz and Bauer, 1988). With this technique a drop of 1% diphytanoyl-*sn*-glycerol-3-phosphocholine in 87% *n*-decane/15% butanol is painted over a circular hole of 0.1 mm^2 bored in the partition wall of the *Teflon* chamber.

All the other conductance measurements were performed with membranes made according to Montal and Mueller (1972). With this technique a hole of 0.05 mm^2 is punched in a thin *Teflon* septum which is placed between the two halves of the *Teflon* cell. Each compartment of the *Teflon* cell contains approximately 1.2 ml. 5 µl of 2% lipid in *n*-hexane is spread on the surface of the aqueous phase in each compartment. After the solvent has evaporated the level in the compartments is raised over the hole where the lipid monolayers form a bilayer membrane across the aperture. This process is facilitated by prior incubation of the aperture with a solution of 2% *n*-hexadecane in *n*-hexane. Similar to black-lipid membranes Montal-Mueller-type membranes form by a thinning process (Niles et al., 1988). The current across the membrane was measured by calomel or Ag/AgCl electrodes, amplified 10^9-fold and recorded by a strip chart recorder. Triangular waves were generated with a Tektronix FG 504 40-MHz function generator. 0.5–3 ng porin solubilized in 1–2 µl 1% Genapol was added to the side of the membrane where the voltage was applied (referred to as *cis* side) unless otherwise stated. The number of incorporated porins was kept constant in the long-lasting Montal-Mueller experiments by exchanging the aqueous phase against a 20-fold excess of buffer on the side containing the porin before the experiments were started. The number of incorporated porin molecules was determined from the stepwise increase of membrane conductivity at 20 mV. 1.3 nS correspond to the conductivity of one Omp34 trimer. Current to voltage curves were recorded by applying a 10^{-3} Hz triangular wave of 30, 100 or 200 mV amplitude usually starting at 0 mV and moving first to negative then to positive voltages.

RESULTS

Unidirectional incorporation

The mode of incorporation of the porin Omp34 was studied using membranes formed by the Mueller-Rudin (or black-lipid membrane) technique. Porin was added to the aqueous phase solubilized by 1% Genapol. It was, therefore, present as a protein/detergent complex. An interesting observation was made when porin was once added to the *cis* side (Fig. 1 A) and once to the *trans* side (Fig. 1 B) of the membrane. After the incorporation of four porin molecules each, a current-to-voltage curve was recorded (Fig. 1 A, B). It is striking that the current-to-voltage characteristic is not linear. The ion current through the porin pore is higher at positive voltages than at the respective negative voltages (Fig. 1 A). It is also notable that there is more noise at positive potential, probably due to rapid switching of several porin channels. If porin is added to the *trans* side, the effects observed are just the opposite (Fig. 1 B). This clearly shows that incorporation of Omp34 into the membrane is a directional process and that Omp34 is an asymmetric molecule regarding its channel conductivity.

Fig. 2 illustrates the conductivity calculated for a single porin trimer incorporated from the different sides under the influence of opposite electric fields. The channel conductivity varies from 1.1 nS at low voltages to 2.2 nS for one porin molecule at either highly negative or highly positive voltages. The curves in Fig. 2 A, B, where porin was added from the *cis* side once at + 20 mV (Fig. 2 A) and once at −20 mV (Fig. 2 B), and the curves in Fig. 2 C, D, where porin was added from the *trans* side at the respective voltages, show that it was not the direction of the applied electric field that determines the orientation of porin in the membrane but the side of porin addition. Since the membrane was composed of identical leaflets of neutral lipid solely the properties of the porin determine the orientation.

Asymmetric voltage-dependent closing

The experiments made with Montal-Mueller bilayers show that ion conductivity is not only asymmetric on the level of single trimers but also on the macroscopic level (Fig. 3). Fig. 3 shows a current trace of *cis*-incorporated porin trimers at −20 mV and + 20 mV, respectively. The number of porin channels closing due to the applied voltage is different in positive and negative electric fields, if porin is incorporated from one side only. Substeps of 0.4 nS and 0.8 nS, which is 33% or 67% of the most frequently observed conductivity step, were also found. Two observations are remarkable. Firstly, at −20 mV under steady state conditions most of the Omp34 channels are closed, while at + 20 mV all of them are open most of the time. Secondly, if the negative electric field is removed, porin channels stay closed for at least some seconds but they open immediately, if an opposite potential of + 20 mV is applied.

Fig. 4 shows that most of the 370 Omp34 trimers, incorporated from the *cis* side at 100 mM KCl and pH 8.3, close at very low negative potentials, while apparently all of them stay open at positive ones.

The conductivity calculated from Fig. 4 for discrete potential values was plotted against the voltage applied (Fig. 4, inset). Conductivity-to-voltage-dependence follows a sigmoidal curve with two voltage-independent conductivity levels between −30 and −15 mV and between + 10 and + 30 mV and a voltage-dependent region in between. The negative part of the graph in Fig. 4 increasing from −30 mV to 0 mV was analyzed on the basis of a two-state model according to Schein et al. (1976). The ratio of open to closed channels (N_o/N_c) at a given membrane potential V is expressed by

$$N_o/N_c = e^{-nq(V-V_0)/kT}$$

where n is the equivalent number of charges which move through the gradient of the transmembrane potential V. The elementary charge is expressed by q, k is the Boltzmann constant and T the temperature. At $V = V_0$ 50% of the channels are closed and the conductivity is reduced to 50% of the maximum level. V_0 is calculated to be −10 mV and n is 3.4 charges for the *A. delafieldii* porin Omp34.

Factors influencing voltage-dependent closing

In order to characterize the factors which are responsible for the sensitivity against low negative voltages, the experimental conditions listed in the legend of Fig. 4 were successively altered. The voltage-sensitive behaviour remained unchanged if the neutral lipid diphytanoyl-*sn*-glycerol-3-phosphocholine was replaced by the soy-bean-lipid mixture or if the cationic buffer Tris/HCl was exchanged by the anionic

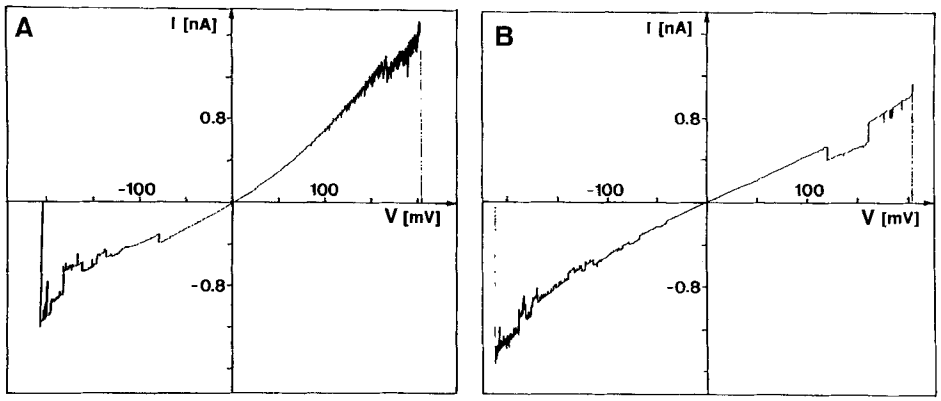

Fig. 1. Asymmetry in voltage dependence of the porin Omp34 from *A. delafieldii*. Four porin molecules were incorporated into a lipid bilayer formed by the Mueller-Rudin technique once from the *cis* compartment (A) and once from the *trans* compartment (B) under the influence of −20 mV on the *cis* side. The experiments were performed in 1 M KCl at a 10^{-3} Hz voltage ramp from ± 200 mV to 0 mV. The membrane was made from 1% diphytanoyl-*sn*-glycerol-3-phosphocholine in *n*-decane/butanol.

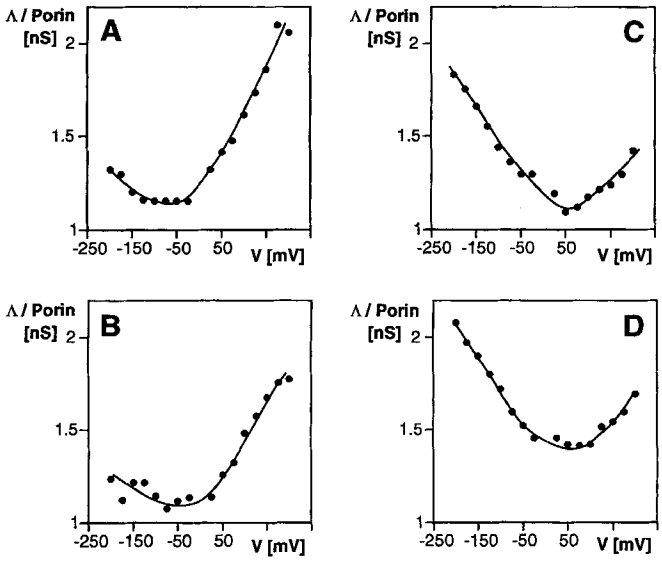

Fig. 2. Conductivity of Omp34 depending on the applied voltage calculated for a single porin molecule. Data were collected from experiments like those presented in Fig. 1 with membranes formed by the Mueller-Rudin technique and containing less then 10 porin molecules each. In A, B porin was added to the *cis* compartment and inserted into the membrane under the influence of + 20 mV (A) and −20 mV (B). In C, D porin was added to the *trans* compartment and inserted into the membrane with an applied voltage of + 20 mV (C) and −20 mV (D). Other conditions were 1 M KCl and 10^{-2} Hz (A, D) or 10^{-3} Hz (B, C). The membrane was made from 1% diphytanoyl-*sn*-glycerol-3-phosphocholine in *n*-decane/butanol.

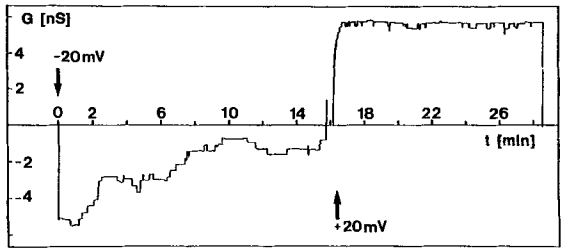

Fig. 3. Voltage-dependent opening and closing of *A. delafieldii* Omp34. After the incorporation of 15−17 porin molecules (from the *cis* side) into a Montal-Mueller type bilayer voltages of −20 mV and + 20 mV were applied for several minutes. Membranes were made from soy-bean lipid in *n*-hexane in a buffer of 100 mM KCl, 10 mM Tris/HCl pH 8.3.

sodium bicarbonate buffer. Using the Mueller-Rudin technique for membrane formation and 87% *n*-decane/15% butanol as a solvent had the effect that voltages more negative than −100 mV were required to obtain porin closing. The same effect occured if the KCl concentration was increased from 100 mM to 800 mM (data not shown). Reducing the buffer concentration to 0.05 mM also removed the effect of porin closing at voltages smaller than −30 mV.

Information about the mechanism of channel closing was obtained by systematically changing the pH value from pH 3 to pH 11. The experiment was performed in two parts only differing in the amount of porin molecules incorporated in the membrane and in the choice of the buffer system (Fig. 5).

When the pH was decreased from 8.3 to 6 and finally to 3.2 in the first experiment (Fig. 5 A−C) the four porin molecules incorporated in the membrane successively lost their sensitivity against negative potentials. At pH 3.2 stable closed states could no longer be observed, but the noise was reproducibly increased (Fig. 5 A). Raising the pH from 8.3 via 9.5 to 11.3 (Fig. 5 D−F) in the second experiment, where seven porins were incorporated, shows the same effects as described above for acidic pH although the noise was somewhat lower at extremely basic pH values.

Another pH-dependent effect is that the ion conductivity was increased about 1.5-times [from 0.45 nS/molecule (Fig. 5 B) to 0.68 nS/molecule (Fig. 5 A) at + 20 mV each] when changing the pH from 6.0 to 3.2. The opposite effect occured at pH 11.4 where only 50% of the total conductivity at pH 8.3 was maintained (Fig. 5 D; 0.39 nS/porin molecule, and Fig. 5 F; 0.2 nS/porin molecule at + 20 mV each). It could be demonstrated by frequently occurring closing events that the effect is due to a real decrease of the single-channel conductivity and does not reflect the inactivation of channels (Fig. 5 E, F). The pH effects were fully reversible.

DISCUSSION

Asymmetric properties of the porin Omp34

A major result of the investigations presented here is the finding that the porin Omp34 from *A. delafieldii* possesses

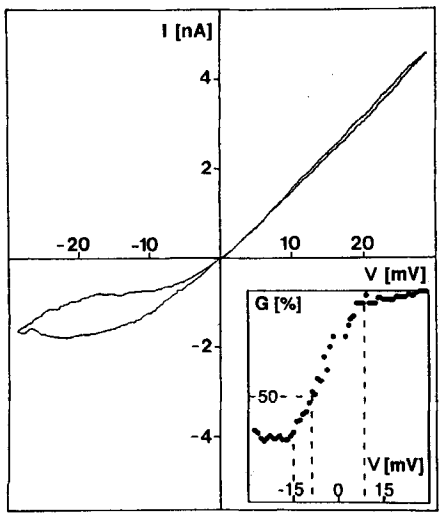

Fig. 4. Current-to-voltage characteristic and conductance-to-voltage characteristic (inset) of a Montal-Mueller type bilayer doped with approximately 370 porin molecules. Porin was added to the *cis* compartment, the membrane was made of 2% diphytanoyl-*sn*-glycerol-3-phosphocholine in *n*-hexane in 100 mM KCl and 10 mM Tris/HCl pH 8.3. A continuous triangular voltage ramp of 10^{-3} Hz and ± 29 mV was applied starting at 0 mV and going first to positive and then to negative potentials. The inset shows the percentage of conductance (100% at + 29 mV) depending on the applied voltage. Vertical dashed lines indicate three important values (from left to right). Lines 1 and 3 mark the voltage range where the ratio of open to closed pores highly depends on the applied voltage (−15 mV to + 10 mV). Line 2 marks the apparent switching voltage, i. e. the voltage where 50% of the channels are closed, V_0 = −10 mV. Conductivity values of the negative part of the curve were multiplied by a factor of 1.04. This is to correct for the fact that single-channel conductivity at −15 mV is approximately 1.04 times smaller compared to the single channel conductivity at + 15 mV (as is shown in Fig. 2 A, B).

vectorial attributes. We observed three types of asymmetric properties. Firstly, an unidirectional incorporation of the porin into the membrane, secondly different single-channel conductivities in positive and negative electric fields and thirdly, the voltage-dependent closing of the channels only functioning in electric fields of a certain polarity.

We can conclude from our experiments that mainly features of the porin itself, or of the protein/detergent complex, are responsible for the directional insertion into the membrane. Neither the lipid chosen nor the application of electric fields had a detectable effect. The directional incorporation of the porin provided the basis to detect and to investigate vectorial properties of its function. Ensuring unidirectional orientation in the lipid bilayer, thus, may have been a problem in studies with other porins where asymmetric features were not observed.

The three-dimensional structure described recently (Weiss et al., 1991 a) showed that the channel of the *Rhodobacter capsulatus* porin is structurally and electrostatically asymmetric. The authors therefore concluded that the diffusion rates for ions should be different in the two directions. This is exactly what can be measured with the anion-selective porin of *A. delafieldii*. Depending on the voltages applied we obtained smaller single-channel conductivities in negative electric fields than in positive ones. This property may be explained by an asymmetric distribution of charges inside the channel which is illustrated in our model below (Fig. 6).

The phenomenon of voltage-dependent closing is also an asymmetric feature of the *A. delafieldii* porin. Closing of the channel is only possible if the potential is negative on the membrane side where the porin inserted. Above + 10 mV and below −15 mV the porin-doped membrane is in an equilibrium and the ratio of open to closed pores does not change. Since closing events did not occur at positive voltages all porin molecules are supposed to be oriented in the same way. The residual conductivity below −15 mV indicates that voltage sensitivity may be different among the incorporated molecules. This could be due to the formation of porin aggregates within the membrane as discussed by Schindler and Rosenbusch (1978). In between these two states the amount of porin channels being open or closed depends on the voltage applied.

Lakey and Pattus (1989) as well as Morgan et al. (1990) have also detected asymmetry in channel gating while with PhoE of *E. coli* and protein I of *N. gonorrhoeae* perfect symmetry of channel closing was observed (Dargent et al., 1986; Young et al., 1983).

Voltage-dependent gating of Omp34

There are several possibilities how a channel gate could work (Hille, 1984; Manella et al., 1992). Our results together with the structural information available enables us to propose a model for Omp34 which may also apply to other bacterial porins exhibiting voltage gating.

The model assumes, firstly, that the gate consists of two flexible parts (or a flexible and a rigid part) which are oppositely charged and predicts, secondly, that the flexible parts undergo conformational changes if an appropriate electric field is applied such that they move together and close the channel.

In the porin of *R. capsulatus* a channel-size-defining structure, the so called eyelet, does exist which fulfills the assumptions of our model. The eyelet is lined by positively charged groups at the channel wall and by negatively charged groups being part of two loops (Weiss et al., 1991 b). A very similar arrangement has been shown to exist in the porins OmpF and PhoE of *E. coli* although they seem to be different regarding details like the tip of the channel-size-defining loop which is conserved amongst them and 12 other porin molecules but not in the *R. capsulatus* porin (Cowan et al., 1992; Weiss et al., 1991 b; Schiltz et al., 1991; Jeanteur et al., 1991). A loop structure corresponding to the large loop of *R. capsulatus* has also been proposed for Omp32 of *C. acidovorans*, which is closely related to *A. delafieldii* (Gerbl-Rieger et al., 1992). The loop lining the eyelet may possibly undergo conformational changes and serve as the flexible part of the channel gate (Cowan et al., 1992).

At moderate pH and in the absence of any electric field the two charged counterparts have a certain distance from each other according to their electrostatic environment. If an appropriate electric field is applied, the charges experience a force and move closer together. In consequence the channel size decreases (Fig. 6 C). This has indeed been observed, minima of single-channel conductance (about 1.2 nS in 1 M KCl) were found between −50 and −100 mV (Fig. 2). At a certain distance the Coulomb force between the opposite charges drags both protein parts together. The conformational change results in a metastable closed state of the channel. This is confirmed by the fact that even some seconds after

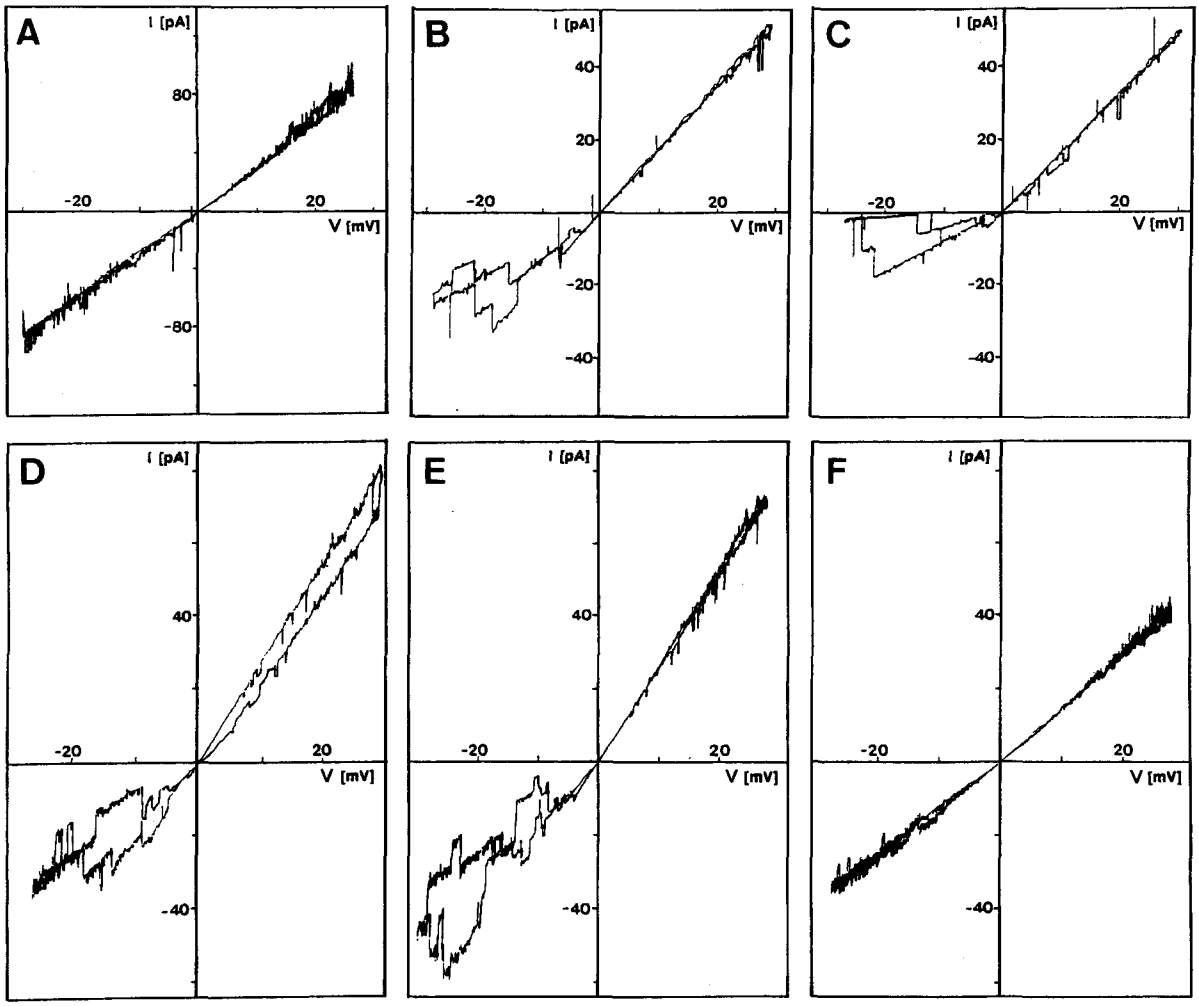

Fig. 5. Current-to-voltage characteristics of *A. delafieldii* Omp34 at various pH values. Two independent experiments with Montal-Mueller type bilayers were made. In the first experiment [1.3 mM $NaHCO_3$, 100 mM KCl, with four porin molecules in (B, C), and five porin molecules in curve (A)] the pH was decreased from pH 8.3 (C) to pH 5.9 (B) and pH 3.2 (A) by the addition of 5 µl and 25 µl 100 mM citric acid. In the second experiment (10 mM Tris, 100 mM KCl, with seven porin molecules) the pH was raised from pH 8.3 (D) to pH 9.5 (E) and pH 11.4 (F) by the addition of 25 µl and 12.5 µl 0.25 M KOH. Porin was added to the *cis* compartment, the membranes were made from 2% diphytanoyl-*sn*-glycerol-3-phosphocholine in *n*-hexane and the voltage was applied as a continuous triangular wave of 10^{-3} Hz and + 27 mV.

the electric field has been removed Omp34 tends to stay closed.

If an electric field of opposite polarity is applied (Fig. 6 D) the channel should become larger which again is the case with Omp34. Channel conductance now continuously increases from about 1.4 nS at + 50 mV to about 2 nS at + 200 mV (Fig. 2). Closing events are not possible.

If one of the charges is removed, closed states should not occur anymore because the electrostatic attraction between both parts of the channel gate is lost. This was simulated by reducing the pH to 3.2 (Fig. 6 A, B) or increasing it to pH 11.4 (Fig. 6 E, F) where at least some of the negative or positive charges are neutralized. The results obtained (Fig. 5) fit nicely into the proposed model.

It should be noted, that some of the effects observed cannot be explained by the interaction of antagonistic charges alone. By comparing Fig. 5 A, F with Fig. 5 C, D it is obvious that ion conductivity is 1.5-times higher at pH 3.2 and two-times lower at pH 11.4, than at moderate pH values. Similar observations were made by Benz et al. (1979) and Bühler et al. (1991). It is quite unlikely that, due to the electric charge distribution, the channel cross section was increased 1.5 times or halved. A phenomenon described previously (Brunen et al., 1991) may account for this effect. Omp34 bears surface charges near the pore mouth which increase the ion concentration in its vicinity. A 20% increase of the net surface charges at pH 3.2 and a 30% decrease at pH 11.4, may account for the change of conductivity (calculated according to Brunen et al., 1991). Since at pH 3.2 negative charges are titrated we can conclude now that the surface charges must be provided mainly by the amino acids arginine and lysine.

Dani (1986) theoretically investigated the influence of surface charges situated in the vestibule of ion channels and showed that these contribute to the asymmetry of conductivity as a function of the voltage. Probably, this must be considered with the porin Omp34.

The switching voltage

The low switching voltage found for the *A. delafieldii* porin is remarkable but it is not unique. Mauro et al. (1988)

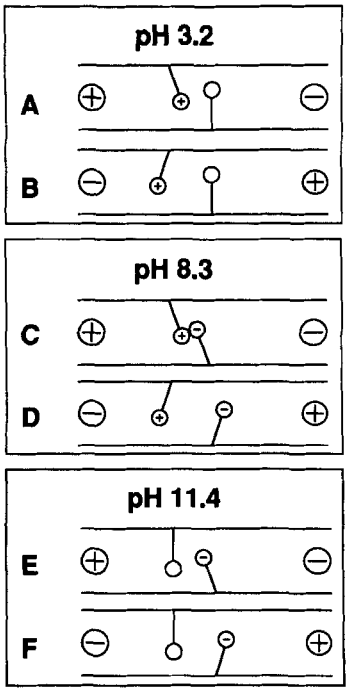

Fig. 6. Model of a channel gate. Two assumptions are made as a result of the experiments: the gate is composed of two flexible protein domains inside the channel; these two protein domains are oppositely charged. For further discussion see text.

as well as Morgan et al. (1990) and Delcour et al. (1989) showed that the porins from *N. gonorrhoeae* and *E. coli*, respectively, do close at voltages below 50 mV. It is striking that in all these investigations, ion concentrations as low as 150 mM to 250 mM (and 100 mM in our study) were used. The critical voltage of gating was clearly higher (90–150 mV) in studies where the salt concentrations approached 1 M (Dargent et al., 1986; Lakey and Pattus, 1989; Bühler et al., 1991; Lakey et al., 1991). The application of 0.8 M KCl indeed prevented channel closing at −30 mV with Omp34. This coincidence is not accidental but can be explained by the fact that charges are involved in the mechanism of channel gating. Shielding these charges by high ion concentrations decreases the force of the electric field exerted on the charged residues. To compensate for the salt effect, higher potentials are needed to induce channel closing. Interestingly, in our experiments channel closing is supported much more in Montal-Mueller or vesicle-derived bilayers than in Mueller-Rudin type (black-lipid membrane type) bilayers which is in agreement with the findings of Lakey and Pattus (1989) for OmpF from *E. coli*. The difference between the two types of lipid bilayers is not clear, but the distribution of solvent remaining in the lipid bilayer may be of importance (Lakey and Pattus, 1989).

Why a low buffer strength also prevents the porin molecules from closing their channels remains to be investigated. One possible explanation could be a binding site which regulates the voltage sensitivity. At present, however, we do not have any evidence for a strong binding of ions, neither from the analysis of single channel data (Brunen et al., 1991) nor from selectivity measurements (Brunen, M. and Engelhardt, H., unpublished results).

Probably the most interesting and important question in the field of porin research remains controversial. Do porins open and close in the living cell? Sen et al. (1988) found that increasing the Donnan potential up to 100 mV did not change the rate of cephaloridine permeation through the outer membrane pores in intact cells. Although our experiments were performed with artifical systems and although it remains to be established that the orientation of the porin in artifical lipid bilayers and in the outer membrane is the same, our results clarify some aspects: it has been shown that the switching voltage (−10 mV) is in the range of the Donnan potential across the outer membrane, i. e. −30 mV (inside negative; Stock et al., 1977) and can be easily modulated by the environmental conditions.

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