Nonlinear and Asymmetric Open Channel Characteristics of an Ion-Selective Porin in Planar Membranes

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ABSTRACT The open channel characteristics of the bacterial porin Omp32 from *Comamonas acidovorans* were investigated by means of conductance measurements in planar lipid bilayers of the Montal-Mueller type. Particularly at low salt conditions (≤30 mM KCl) Omp32 exhibited some unusual asymmetric and nonlinear functional properties. Current-voltage relationship measurements showed that conductance depends on the orientation of porin molecules and is a nonlinear function of the applied membrane potential. Conductance also depends on the salt concentration in a manner not common to porins and the salt concentration modulates the nonlinearity of conductance-voltage relationships. Omp32 is strongly anion-selective. The nonlinear and asymmetric conductance of the open channel is a new observation in porins.

INTRODUCTION

Porins form large water-filled channels in the outer membrane and other diffusion barriers of the bacterial cell wall. Most solutes pass through the pores of porins on their way from the external environment to the periplasmic space of the cell or vice versa. According to the nature of these compounds, i.e., charged, polar or nonpolar, there are specialized porins. We distinguish between nonspecific but ion-selective general diffusion porins and those specific for such molecules as oligomeric sugars or nucleosides (Benz and Bauer, 1988; Jap and Walian, 1996). Knowledge of the structure and function of porins increased substantially when the first atomic models became available (Weiss et al., 1991; Cowan et al., 1992; Kreusch et al., 1994; Schirmer et al., 1995). Common features are the β-barrel structure defining the wall of the channel and a large loop folding back into the channel interior and contributing to functional properties. The structures of native and mutated porins as well as electrophysiological measurements have contributed to our present understanding of porin function. Major conclusions are that a certain arrangement of aromatic amino acids is required for sugar-specific porins (Schirmer et al., 1995) and that charged amino acid residues are particularly important for the function of nonspecific porins.

The channel interior of general diffusion porins is usually hydrophilic and contains charged amino acids whose absolute amount, net charge, and distribution along the pore and around the vestibule contribute to several functional aspects. First, voltage sensing and subtle conformational rearrangements during voltage gating are mediated by charged amino acids (van Gelder et al., 1997; Phale et al., 1997; Brunen and Engelhardt, 1993, 1995). Second, organic compounds modulating the voltage-dependent closure of pores interact with charged amino acid residues in the channel (Liu et al., 1997). Third, ion selectivity is caused by charges located in the constriction zone of the pore (Bauer et al., 1989; Cowan et al., 1992) and by a surface potential at the pore vestibule (Karshikoff et al., 1994; Jap and Walian, 1996). Fourth, diffusion of polar molecules is presumably facilitated by a transverse electric field within the channel (Weiss et al., 1991; Karshikoff et al., 1994). Finally, conductance at very low salt concentrations is enhanced by protein surface charges apparently attracting counter ions from the bulk solution (Brunen et al., 1991; Butz et al., 1993; Trias and Benz, 1993).

Bacterial porins are structurally asymmetric (Cowan et al., 1992; Kreusch et al., 1994), and functional properties depend on fixed protein charges and therefore should also exhibit asymmetry, as discussed by Welte et al. (1997). Asymmetry in function, however, can be detected only if the porin molecules are inserted unidirectionally into the membrane. This limitation has been a problem with electrophysiological experiments performed with planar lipid bilayers but could be overcome in some cases (Lakey and Pattus, 1989; Morgan et al., 1990; Brunen and Engelhardt, 1993; Wiese et al., 1994) or by means of patch clamp measurements (Berrier et al., 1992, 1997). Clear evidence has been accumulated that voltage-induced closing depends on the orientation of the porin relative to the electric field (Berrier et al., 1992; Brunen and Engelhardt, 1993, 1995; Wiese et al., 1994; Mathes and Engelhardt, 1998). Whether this holds true for other functional properties was investigated in the present study which is focused on the open channel characteristics of the bacterial porin Omp32 from *Comamonas acidovorans*. Omp32 inserts unidirectionally into the membrane and enabled us to investigate its asymmetric functional properties. We show here that Omp32 is strongly ion-selective and that the open channels exhibit asymmetric and nonlinear conductance characteristics that are functions of the membrane potential and the salt concentration.

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MATERIALS AND METHODS

Porin preparation

Comamonas acidovorans was originally obtained from the Deutsche Sammlung von Mikroorganismen (type strain DSM39, Braunschweig, Germany). For porin preparations we used strain JL0 which was derived from the type strain by selective cultivation of fast growing cells (Baldermann et al., 1998). Strain JL0 is a spontaneous mutant deficient in the expression of the regular surface protein (Paul et al., 1992) and the synthesis of long lipopolysaccharide side chains (Krezmar, 1991). Cells were grown in complex medium in a 100-liter pilot fermenter as described earlier (Chalcroft et al., 1986). Preparation of the porin Omp32 was performed as outlined in detail elsewhere (Zeth et al., 1998). The purified porin was either dissolved in 10 mM sodium phosphate buffer solution, pH 7.5, 2% *N*-octyl-polyoxyethylene (octyl-POE; Bachem, Heidelberg, Germany), plus 3 mM NaN_3 at 1 mg/ml protein concentration or in 10 mM sodium phosphate buffer solution, pH 8, plus 0.5% octyl-POE at 15 mg/ml. In order to remove possibly associated lipopolysaccharide (LPS) Omp32 was denatured in 6 M guanidinium HCl, 100 mM Tris-HCl at 100°C for 20 min and refolded in 2% *N, N*-dimethyl-*N*-dodecylamine oxide (LDAO), 100 mM Tris-HCl, pH 8.0, at room temperature for several hours.

Most of the chemicals were obtained from Sigma (Deisenhofen, Germany). Hexadecane (HPLC grade), hexane, and Genapol X-080 were obtained from Fluka (Deisenhofen, Germany).

Planar lipid bilayer techniques

Conductance measurements were performed with planar lipid membranes according to the technique of Montal and Mueller (1972). Two Teflon chambers, each containing 1–1.5 ml aqueous solution, are separated by a Teflon septum of 6 or 12 μm width with central holes 110 or 180 μm in diameter, respectively. The holes were either punched with special tools (Schindler, 1989) or burned by means of sparks generated by a system similar to an automobile ignition. We used Ag-AgCl electrodes 1 mm in diameter electrolytically coated in HCl or KCl with currents of 1–2 mA. If the electrodes were coated in KCl the concentration was the same as used in the experiments later. For selectivity measurements electrodes were placed in Eppendorf caps or pipette tips and connected to the chambers with Teflon tubing filled with 2% agarose, 100 mM KCl, plus 10 mM Tris-HCl, pH 8.3. Contents of the two chambers were stirred by small magnetic stirrers when necessary. KCl was used throughout as an electrolyte. Salt solutions at concentrations $\geq$100 mM were supplemented with 10 mM Tris-HCl, pH 8.3. Salt gradients for selectivity measurements were generated by using prepared KCl solutions of the required concentrations for the two chambers. Here Tris-HCl was omitted.

For membrane formation the Teflon septum was pretreated with approximately 1 μl of 0.3–2% hexadecane in hexane. 4.5 μl of the lipid diphytanoyl phosphatidylcholine (DphPC) from Avanti Polar Lipids (Alabaster, AL) dissolved in hexane (20 mg/ml) was added to each side of the chamber. Membranes were formed after approximately 1.5 min when the lateral lipid pressure had approached its final value. Membrane formation was controlled optically by means of a binocular and electrically by application of a triangular voltage while monitoring the membrane capacity and resistance. The porin was added to one side of the membrane in amounts from 1 ng to 4 μg with Genapol from 0.3 to 140 μM (critical micelle concentration [CMC] of Genapol 50–130 μM) and octyl-POE from 0 to 0.2 mM (CMC $\approx$7 mM) depending on the KCl concentration and membrane area. The solution for the refolded porin contained 30 μM Genapol and 15 μM LDAO (CMC 1–2 mM). The experiments were performed at room temperature.

Data acquisition and processing

Our setup for data acquisition consisted of an EPC7 patch clamp amplifier (List-electronic, Darmstadt, Germany), a Tektronix 2211 digital storage oscilloscope (Tektronix, Heerenveen, The Netherlands), a PC486 equipped with an AT-MIO-16F-5 multi-IO data acquisition board from National Instruments (Munich, Germany) with 12-bit AD and DA channels and 8 DIO channels, and a strip chart recorder for analogue documentation. We developed programs for data acquisition and processing using the graphical programming language LabVIEW 3.0.1 and 4.0.1 from National Instruments running on personal computers, separately used for data acquisition and analysis. Data were stored in the binary data format of LabVIEW adapted to our needs for convenient data processing. Data sampling was carried out on-line with 0.25–12 kHz (0 and 12 kHz being the limits of the system). The analogue data were filtered with 3 kHz and various additional filters between 1 and 1.6 kHz. For digital filtering we applied lowpass and bandstop Bessel filters and for data reduction we used different combinations of averaging and median filters. Care was taken that aliasing effects did not affect the data analysis. The gain-dependent offset of our patch clamp amplifier was corrected by means of software-based compensation. It was less than 0.1 pA in the sensitive gain ranges most often used. This enabled us to perform selectivity measurements with as few as five porin molecules at a precision of $\pm$ 1 mV, although better results were obtained with more porins.

Analogue voltages were applied to the membrane via the electrodes using the analogue out channels of the data acquisition board. Our software allows us to design any voltage profile desired. For slow and fast triangular voltages we optimized the scaling factors of the EPC7 hardware such that a maximum of the 12-bit bandwidth of the analogue out channel of the data acquisition board was always used. In order to avoid membrane charging currents for each 1-bit voltage step a 160-Hz filter was installed between the board and the stimulus input of the amplifier. The EPC7 stimulus pathway was corrected by means of a software-based compensation which was calibrated weekly. The accuracy is approximately 0.1% of the applied membrane potential. The settings of the EPC7 such as the gain and stimulus scaling were read via an additional electronic device and the digital IO channels and were stored together with each data point for subsequent data analysis. The program for data display plots up to 40,000 data points simultaneously such as voltage-time, current-time, conductance-time, current-voltage, and conductance-voltage curves. From these curves all required data can be extracted and stored in arbitrary formats for further processing such as filtering, curve fitting, or histogram calculations. Histograms of conductance steps were calculated by semi-automatically evaluating the size of current steps from porin insertions and from closing and opening events.

Voltage application and current measurements were performed via the same electrode. In this report the corresponding side of the membrane is called the "voltage side" and the other the "ground side". Porin was added to the ground-side chamber throughout.

RESULTS

Open channel conductance as a function of voltage

The porin Omp32 from *C. acidovorans* shows a clearly asymmetric behavior in current-voltage diagrams (Fig. 1) when the protein was added to one side of the planar lipid membrane which in our experiments was the ground side of the membrane. Functional asymmetry was highly reproducible, indicating that the structurally asymmetric molecules inserted into the membrane in a certain and constant orientation. However, we cannot yet determine the absolute orientation of the molecules.

Experiments performed in KCl solutions below a concentration of 100 mM, i.e., 3, 10, or 30 mM, showed not only asymmetric but also nonlinear current-voltage curves (Figs. 1–3). *Nonlinearity* is expressed by two features: first, the sudden drop and increase of the current, referred to as

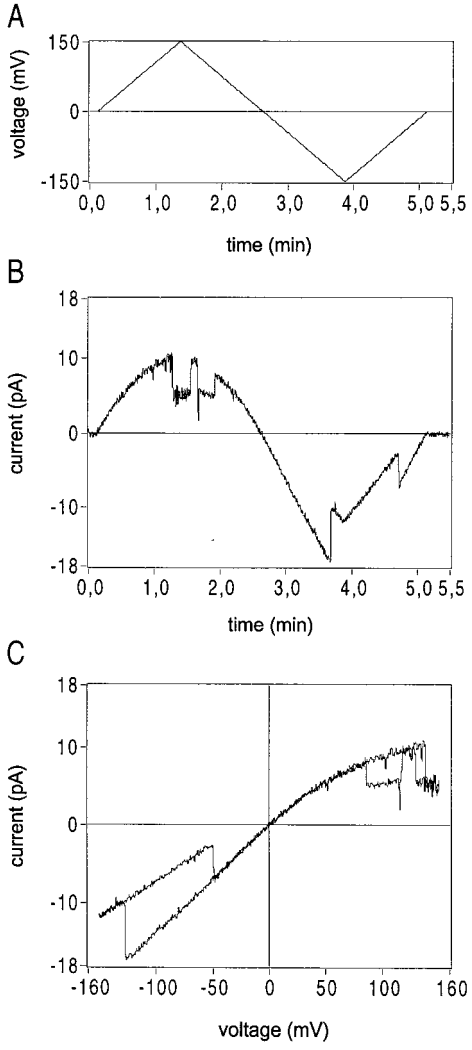

FIGURE 1 Current-voltage characteristic of the *Comamonas acidovorans* porin Omp32 determined at slow triangular voltage in 10 mM KCl. (*A*) Stimulus voltage with a duration of 5 min and an amplitude of 150 mV. (*B*) Current response of three porin molecules. (*C*) Current-voltage curve. Note the asymmetry and nonlinearity of the curves in *B* and *C*.

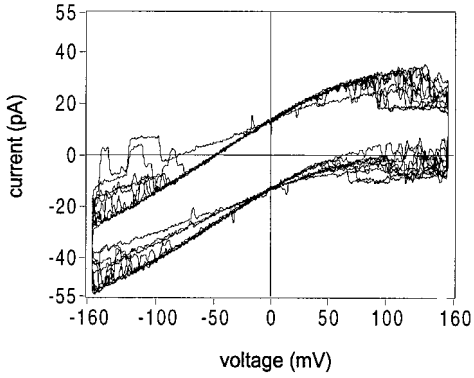

FIGURE 2 Current-voltage characteristics of Omp32 determined at a fast triangular voltage in 10 mM KCl with five porin molecules. The stimulus voltage had a period of 6.25 s and an amplitude of 155 mV, 10 periods were recorded, and the curves superimposed. Due to the membrane charging current the curves are split into two branches with an offset of ±13 pA (membrane capacity 130 pF). The data were recorded at 10 kHz, filtered to reduce noise, and displayed after data reduction.

voltage-dependent closing and opening of porin channels, and second, the nonlinear current-voltage relationship of the open channels. *Asymmetry* is characterized by the probability of channel closing and the minimum modulus of voltage where channels switch to or remain in the closed state, which is usually smaller with positive voltages at the voltage side of the membrane, and by the nonlinear current-voltage response which is consistently more pronounced with positive voltages and results in higher conductance values at corresponding negative voltages (Figs. 1–4). A decreased conductance in response to different voltages may be due to an increased number of closed channels. In order to distinguish between this and other causes of nonlinearity, we usually inserted only a small number of porin molecules. This enabled us to identify closing and opening events of single channels or trimeric porin molecules and to detect the subtle and continuous decrease of current which did not originate from channel closing. Careful analysis of a number of current-voltage curves revealed that about 95% of the molecules had the same orientation in our experiments. In rare cases we found more symmetric current-voltage curves which could be exactly simulated by assuming that one or more porin molecules had opposite orientations and these curves were excluded from further analysis.

In Fig. 1, the characteristic current-voltage curve determined with a slow triangular voltage ($3.3 \cdot 10^{-3}$ Hz) in 10 mM KCl is shown. The current changes originating from permanent channel closure are much higher than the continuous decrease of current in response to changing voltages, particularly in the positive part of the curve. Thus, long lasting channel closures can be excluded as a mechanism for this kind of nonlinearity. The same observation holds true for porins measured with triangular voltages (amplitudes 100–250 mV) being faster by a factor of 50 (0.16 Hz; Fig. 2) to 160 (0.56 Hz; not shown). Even then, closing and opening of porins could be observed but were distinguishable from the curve bending. Because of membrane capacity the curve is split into a rising and a falling voltage branch and the nonlinear response of the porin current is the same in both branches (Fig. 2).

In order to avoid time effects as much as possible and to measure porins which all begin in an open state, we applied constant voltages for about 2–5 min with increasing amplitudes and alternating signs (Fig. 3). Changing the field orientation or applying 0 V caused the closed pores to open immediately. Only the maximum currents at the earliest point of each curve were determined and plotted in Fig. 3 *C*. The first values were taken 20 ms after the voltage had been applied. During this period the membrane charging current had vanished. The advantage of this kind of analysis is that slow processes can be excluded to alter the conductance. The current-voltage characteristic is again the same as that

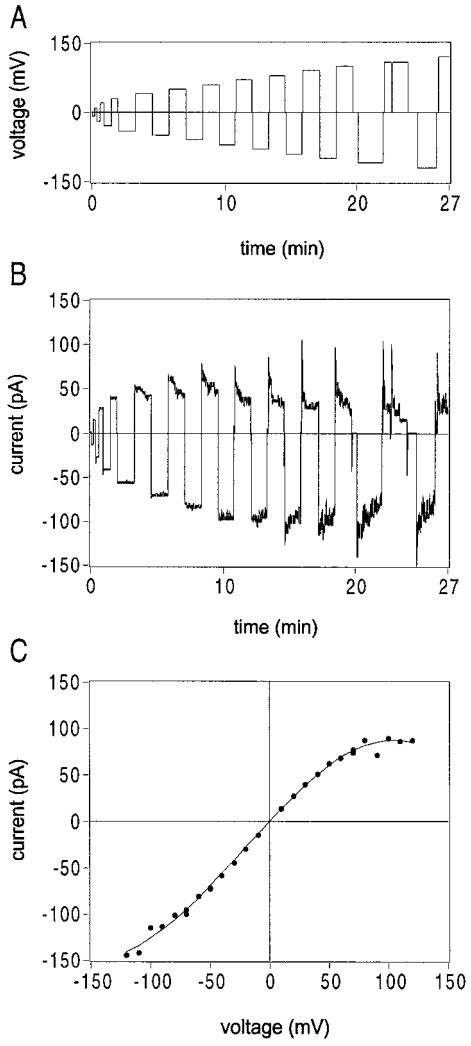

FIGURE 3 Current-voltage characteristic of Omp32 obtained from initial maximum currents of open channels in 10 mM KCl. (*A*) Sequence of constant stimulus voltages applied to a single membrane. (*B*) Current response of ≈ 20 porin molecules. Switching off or changing the sign of the voltage caused the porin channels to open immediately. (*C*) Plot of initial maximum current values 20 ms after the voltage was applied to the membrane, data extracted from (*B*). The curve fitted to the data illustrates the asymmetric and nonlinear current-voltage relationship. The current values at −100 mV and +90 mV deviate significantly from the fitted curve, probably indicating that here one porin closed within the first 20 ms. Data were recorded at 2.5 kHz.

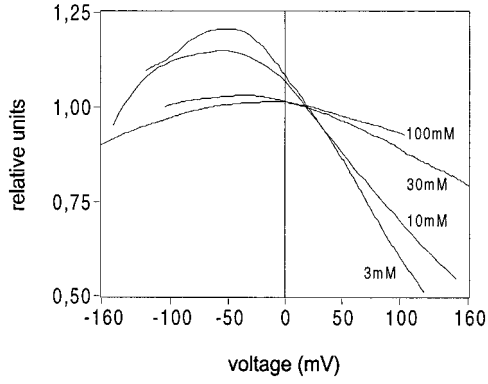

FIGURE 4 Conductance-voltage curves of open porin channels determined in 3, 10, 30, and 100 mM KCl. The curves contain data from various current-voltage measurements as illustrated in Figs. 1 and 2. Data from the curve shown in Fig. 3 are not included. Curve sections free of closing events are normalized with respect to the corresponding conductance values at 20 mV listed in Table 1 and superimposed to obtain the continuous conductance-voltage characteristics shown. In addition the 100 mM KCl sample contained 10 mM Tris-HCl, pH 8.3. The relative root mean square error is approximately proportional to the average values and amounts to ± 16% at −150 mV and ± 20% at 150 mV for the 10-mM curve. The relative errors for the other curves are similar.

obtained in previous experiments performed on different time scales and it shows the nonlinear as well as asymmetric effects.

Voltage-dependent conductance of Omp32 was determined at various KCl concentrations. To this end, data were collected from current-voltage curves like those illustrated in Figs. 1 and 2 which did not contain closing events (i.e., after exclusion of curve sections which exhibited channel closing). By combining a number of different curve sections the continuous characteristics shown in Fig. 4 were obtained. Data from the curve illustrated in Fig. 3 were not included. The results show the relative conductance as a function of voltage, normalized for the corresponding values at +20 mV (Table 1). The nonlinearity of conductance clearly depends on the salt concentration, while measurements at 300 mM KCl and higher did not reveal any significant effect (except for channel closing, which we disregarded here), lower concentrations led to an increase of nonlinearity. The maximum effect was observed with 3 mM KCl between -50 and 150 mV, where the conductance raised by 100%. The relative maximum values were obtained between -20 and -50 mV for each curve (Fig. 4).

In order to exclude a number of possible artifacts from our measurements we changed the side of porin application, used different amounts of porin, examined the concentration effects of different detergents upon addition to the solution, and replaced the membrane with a combination of resistors and capacitors. Neither these experimental parameters nor electronics were responsible for the nonlinear effects observed. In a further experiment we treated Omp32 with guanidine hydrochloride and refolded the protein in presence of lauryl dimethylamine oxide to remove any residual

TABLE 1 Conductance of the *C. acidovorans* porin Omp32 in various salt concentrations

KCl* (mM)	Conductance† (pS)
3	29 ± 6‡
10	56 ± 9
30	89 ± 20
100	183 ± 29
300	278 ± 70
1000	510 ± 20

*Plus 10 mM Tris-HCl, pH 8.3, for [KCl] ≥ 100 mM.
†Normalized to +20 mV for [KCl] < 100 mM.
‡Averages are ±SD from Gaussians fitted to the data.

LPS bound to the porin. The refolded protein showed the same asymmetric and nonlinear current-voltage curves and the same conductance value.

Open channel conductance as a function of salt concentration

One of the functional characteristics often used to compare porins from different sources is the typical conductance value determined at a specific salt concentration. Usually current steps of insertion events are analyzed to calculate an average conductance value for the porin trimer. However, for the reasons discussed above this presents a problem in case of Omp32 with KCl concentrations <100 mM. We have collected conductance values from insertion, closing, and opening events from several experiments performed with 100 mM KCl, 10 mM Tris, pH 8.3, at variable voltage conditions (Fig. 5). On the histogram a clear peak at 0.18 nS and smaller peaks at multiples (2 to 5) of 0.18 nS are evident due to conductance steps of one trimeric porin and corresponding multimeric porin complexes. In addition, there are also events smaller than the conductance unit which can be attributed to single channel closing and opening of the trimeric porin (rather than to the occurrence and insertion of monomeric porin molecules due to the remarkable stability of Omp32 and porins in general). To ensure that the conductance distribution was not blurred by different step sizes for insertion, opening, and closing events, the individual distributions were separated and compared. No significant difference was detectable and therefore the data were combined. Corresponding histograms of the size of conductance steps obtained from experiments performed in low salt conditions did not allow such fine peak resolution as was obtained at or above 100 mM KCl (Fig. 5). To obtain a more significant distribution, the conductance data was corrected by means of the curves shown in Fig. 4. After correction, the resulting conductance values revealed single and multiple porin conductance steps (Fig. 5). However, correction of conductance values determined in 100 mM KCl did not significantly improve the histogram presented in Fig. 5. The expected effect is indeed negligible since the maximum correction is not higher than ≈5% (Fig. 4).

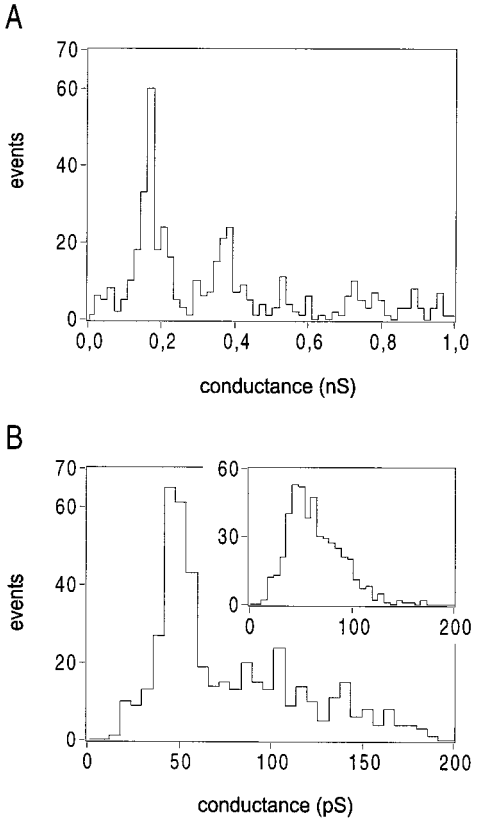

FIGURE 5 Histogram of conductance steps of Omp32 derived from insertion, closing, and opening events. (*A*) Conductance steps determined in 100 mM KCl plus 10 mM Tris-HCl, pH8.3, at voltages between −120 and 120 mV. The most prominent peak corresponds to the conductance of a single trimeric porin molecule at an average value of 0.18 nS. (*B*) Distribution of conductance steps determined in 10 mM KCl at voltages between −200 and 200 mV. Conductance values were corrected and normalized to a voltage of +20 mV using the curve shown in Fig. 4. The average conductance of a single porin molecule is 56 pS. The *inset* illustrates the blurred distribution of conductance values before correction.

In Fig. 6 the average conductance values of single Omp32 trimers are displayed as a function of the KCl concentration. It is striking that the slope of the approximately linear function in the double logarithmic plot is much flatter than that of the *Rhodobacter* capsulatus porin shown for comparison (Przybylski et al., 1996). This effect is attributed to the existence of surface charges at the pore entrance as shown for Omp34 and other channel proteins (Brunen et al., 1991; Nelson and McQuarrie, 1975).

Selectivity

Charges inside the channel as well as charges at the channel entrance create and contribute to the ion selectivity of the

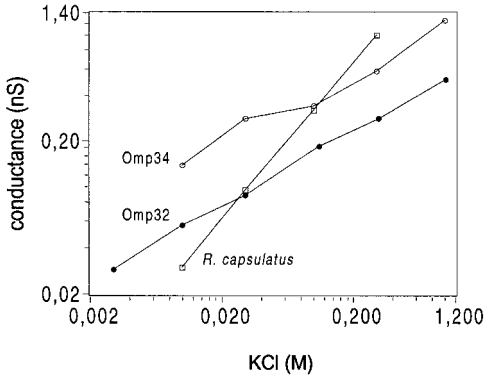

FIGURE 6 Conductance-KCl concentration relationship of porin Omp32. The conductance data at concentrations <100 mM KCl are normalized to +20 mV, experiments with concentrations ≥100 mM KCl contained 10 mM Tris-HCl, pH 8.3. Data of the porins Omp34 from the related bacterium *A. delafieldii* (Brunen et al., 1991; with permission) and of the *R. capsulatus* porin (Przybylski et al., 1996; with permission) are displayed for comparison.

porin pore (Bauer et al., 1989; Karshikoff et al., 1994). The apparent ion selectivity of Omp32 was determined by measuring the membrane potential developed by a salt gradient across the porin pores at zero-current conditions. The potentials obtained for different KCl gradients were consistently positive (in case of our experimental setup, high salt concentration at the voltageside), denoting that the porin Omp32 is anion-selective, i.e., it exhibits a higher permeability for Cl^- than K^+. The selectivity coefficients (P_a/P_c, i.e., permeability for anions over permeability for cations) were calculated by means of the Goldman equation. A gradient of 3–9 mM KCl resulted in a final potential of 24.4 mV and a corresponding selectivity value P_a/P_c of $\approx$ 17. The membrane potential was independent of porin orientation. The potentials developed when porin was added to the low or high concentration side of the membrane were 24.4 $\pm$ 1.3 mV or 24.4 $\pm$ 0.8 mV, respectively (averages of five determinations each).

DISCUSSION

Orientation

Monitoring functional asymmetry requires the unidirectional insertion of porin molecules into the planar lipid membrane. Omp32 behaved asymmetrically with respect to voltage-dependent closing (Fig. 4; A. Mathes and H. Engelhardt, manuscript in preparation) and the shape of current-voltage curves could be judged reliably at low salt conditions only if a few molecules were present in the membrane. Both the method of insertion and the reason for unidirectional incorporation into the membrane are unknown but the latter may be due to the asymmetric structure of porin (Weiss et al., 1991; Cowan et al., 1992) and in particular, the expected asymmetric charge distribution of Omp32. In principle LPS, the negatively charged lipid of the bacterial outer membrane, could contribute to asymmetry. Although we cannot absolutely exclude the possibility that residual LPS was bound to the porin molecules, it is unlikely that LPS was the sole reason for the functional asymmetry and charge effects observed. Omp32, denatured by guanidine hydrochloride and refolded in vitro, behaved like the untreated protein and exhibited the same conductance and asymmetric conductance-voltage characteristics. Previous investigations performed with the closely related porin Omp34 from *Acidovorax delafieldii* are also in accordance with the supposition that LPS is not the source for the charge effects in vitro (Brunen and Engelhardt, 1995).

Conductance

The major and in some aspects new observation of the function of porins is that Omp32 exhibits nonlinear voltage dependence, unusual salt concentration dependence, strongly anion selectivity, and asymmetric conductance characteristics of open channels. The general salt dependence of conductance observed with Omp32 is uncommon, but not unique to porins (Trias and Benz, 1993; Butz et al., 1993; Brunen et al., 1991). If channels were only hollow cylinders and did not exhibit strong selective interactions with ions, ions would diffuse through pores in a manner similar and proportional to ions in the bulk phase. Thus, we would expect to obtain conductance-salt concentration curves parallel to the bulk characteristics in plots such as Fig. 6. This is the case for a number of porins such as OmpF and PhoE (Benz, 1985) and the porins of *R. capsulatus* (Przybylski et al., 1996), *Paracoccus denitrificans* (Saxena et al., 1997) and others, although this does not mean that the ions and the channel interior do not interact (Wohnsland and Benz, 1997). However, the conductance-concentration dependence of Omp32 as well as of Omp34 (Brunen et al., 1991) is quite different. The behavior of Omp32 can be tentatively explained by the effect of surface charges attracting ions at low salt concentrations. Incomplete charge shielding enhances and facilitates the diffusion of ions and increases the conductance. This was shown means of a simple model for Omp34 (Brunen et al., 1991) and can be deduced from more sophisticated theories developed for channels in general (Dani, 1986; Chen et al., 1997). The *Rhodopseudomonas blastica* porin is similar to Omp32 in this respect (Butz et al., 1993) and it possesses an accumulation of negatively charged amino acids at both channel ends (Kreusch et al., 1994).

Interestingly, preliminary information from crystallization and trials to determine the atomic structure by molecular replacement approaches suggest that Omp32 differs from OmpF and the other nonspecific porins whose atomic structures have been determined to date (Zeth et al., 1998). Although its atomic structure is not yet available, a tentative folding model of Omp32 derived from primary structure alignments, secondary structure measurements, proteolysis studies (Gerbl-Rieger et al., 1992), and comparisons with other 16-β-stranded porins provided preliminary information on the location of β-strands and the distribution of charged amino acids. Based on this model we do not expect a significant bulk net charge either in the periplasmic β-turns or in the exoplasmic loops. However, the model indicates an accumulation and surplus of positive charges inside the porin pore but off the channel center. This arrangement would make up an asymmetric potential profile and could create an effective ion filter.

Charged amino acid residues, when distributed asymmetrically along the channel, should have additional orientation-dependent effects on ion flux as theoretical considerations suggest (Chen and Eisenberg, 1993a,b). This was demonstrated for Omp32. The most remarkable effects were observed with 3 mM KCl, for practical reasons, the lowest applied salt concentration. At low concentrations screening (shielding) of protein charges is less effective and charge-dependent properties should therefore be more pronounced. Accordingly, the effects vanished at concentrations higher than 100 mM, where shielding is much more effective as illustrated by, e.g., the Debye length (3 mM KCl: $l_D \approx 6$ nm, 300 mM KCl: $l_D \approx 0.6$ nm). In order not to create

unpredictable effects (Chen et al., 1997), we added neither divalent ions nor large buffer substances to KCl solutions below 100 mM. The conductance of open channels turned out to be strongly voltage-dependent, but the continuous decrease of conductance is not due to permanent channel closure. Nonlinear current-voltage relationships can be explained by the existence of asymmetric potential profiles along the channels that are modulated by the salt conditions and external electric fields and do not need any conformational changes to occur (Chen and Eisenberg, 1993a,b). The decrease of conductance at high voltages in particular can also be explained by limited diffusion of ions from the solution into the channels (Läuger, 1976). In agreement with theoretical expectations we found that the conductance-voltage curve itself is nonlinear and the conductance decreases again beyond −50 mV; however, other phenomena must also be taken into consideration. Although long lasting closure of channels can be excluded as an explanation for nonlinearity, we cannot rule out that conformational or other changes that may appear at high voltages contribute to a continuous decrease of conductance. Among these changes are high frequency fluctuations of the channel (>1 kHz) and cooperative effects (conformational or electrostatic) by clustering of porin trimers (Schindler and Rosenbusch, 1981). The voltage-dependent decrease of conductance and its modulation by salt are uncommon for open porin channels. Measurements with a single porin molecule in the membrane patch would allow study of whether and to what extent clustering of porin trimers modulates the open channel properties. Interestingly, other studies show that the open channel conductance of porins increases with higher voltages (Berrier et al., 1997; Brunen and Engelhardt, 1993), resulting in a conductance-voltage characteristic apparently inverse to that of Omp32. These experiments, however, were performed in salt conditions different from ours (0.1 M KCl plus divalent cations and large buffer substances or 1 M KCl, respectively). Therefore the conductance alterations may be due to different but unknown effects. These experiments illustrate that the open channel characteristics of porins are more complex than previously assumed.

Selectivity

The asymmetric distribution of charges and a surface potential are expected to contribute to the ion selectivity of porin channels (Dani, 1986; Hoyles et al., 1996; Chen et al., 1997). Due to the strength of asymmetric conductance effects, presumably originating from a particular location of charged amino acids, it is not surprising that Omp32 exhibits a selectivity for anions which is much more distinct than that of PhoE from *Escherichia coli* (Benz et al., 1989). The strong anion selectivity is consistent with the selectivity of the related porin Omp34 (Brunen et al., 1991; Brunen and Engelhardt, 1995) and is in agreement with the view that an accumulation of positively charged amino acids make up a putative selectivity filter inside the channel.

CONCLUSION

The anion-selective porin Omp32 from *C. acidovorans* exhibits some unusual orientation-, voltage-, and salt concentration-dependent functional properties of the open channels which are very likely due to the effects of protein charges inside or close to the channel. The strength of these effects and the ability to determine the ion conductance with a fixed unidirectional orientation of the porin molecules suggest that Omp32 is an attractive model system for nonspecific but ion-selective porins. The application of appropriate models describing the ion flux through structurally and electrostatically asymmetric open channels could contribute to our understanding of functional properties of porins like Omp32. Corresponding studies should help to distinguish the features related to electrodiffusion phenomena from those caused by other conformational changes. Of course, a deeper insight into functional and structural properties requires detailed knowledge of the molecular structure. To this end the *C. acidovorans* porin has recently been crystallized (Zeth et al., 1998). Once the x-ray structure is determined the kinetic data can be interpreted in terms of the atomic model.

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