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Identification of Two Porins in *Pelobacter venetianus* Fermenting High-Molecular-Mass Polyethylene Glycols

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Porins were purified from cells of the anaerobic gram-negative bacterium *Pelobacter venetianus* grown with 20-kDa polyethylene glycol. After treatment of the cell envelope fraction with sodium dodecyl sulfate-containing solutions, the murein contained only two major peptidoglycan-associated proteins of 14 and 23 kDa. Both proteins were released from the peptidoglycan by the detergent Triton X-100. Genapol X-80 released only the 23-kDa protein. This protein was purified by chromatography on a hydroxyapatite column. It did not form sodium dodecyl sulfate-resistant oligomers. Reconstituted in lipid bilayer membranes, the 23-kDa protein formed cation-selective channels with a single-channel conductance of 230 pS in 1 M KCl. The channel is not a general-diffusion pore, since its conductance depends only moderately on the salt concentration. The channel conducted ammonium much better than potassium or rubidium ions, suggesting that it is probably involved in ammonium uptake. The outer membrane of *P. venetianus* contains a further, non-murein-associated pore with an unknown molecular mass. It is also cationically selective and has a single-channel conductance of 1.6 nS in 1 M KCl, which suggests that its effective diameter is similar to that of porins from enteric bacteria.

The cell envelope of gram-negative bacteria consists of three different layers, the inner membrane, the murein sacculus, and the outer membrane (2, 18). Pore-forming proteins (porins [15]) allow passage of small hydrophilic solutes across the outer membrane (3, 18). Many porins have been studied in recent years. Most of them form peptidoglycan-associated general-diffusion pores in the outer membrane and preferentially sort hydrophilic solutes by their molecular masses (3). Other porins are induced under special growth conditions (2). Many porins form sodium dodecyl sulfate (SDS)-stable, heat-modifiable oligomers with average molecular masses of the monomer between 30 and 50 kDa. Most porins have been isolated from enteric bacteria. Nothing is known about porins of obligately anaerobic gram-negative bacteria.

Pelobacter venetianus is an anaerobic, gram-negative bacterium originally isolated from marine anoxic sediment (20) and fermentatively degrades high-molecular-mass polyethylene glycols (PEG). Fermentation of PEG 20000 by *P. venetianus* (20) proceeds inside the cytoplasm through a vitamin B_{12}-dependent hydroxy group shift from the terminal to the subterminal C atom, yielding an unstable half-acetal structure which releases acetaldehyde residues (12, 21). Thus, polymeric PEG cross both the outer and inner membranes intact before depolymerization. We therefore chose this bacterium to check for unusual porins in the outer membrane.

MATERIALS AND METHODS

Bacterial strains, medium, and culture conditions. *P. venetianus* Gra PEG1, DSM 2394, was cultivated in saltwater mineral medium at 28°C as described previously (20). Mass cultivation was done in 10-liter glass jars with 0.1% PEG 20000 or 10 mM diethylene glycol as the substrate. Cells were harvested in the late log phase by centrifugation (3 to 4 g of wet cells was obtained from 10 liters of medium), washed once with 20 mM Tris-HCl buffer, pH 7.2, and stored frozen at −20°C as wet cell paste. Control experiments with fresh, nonfrozen cell material gave no indication of significant porin destruction by freezing.

Isolation and purification of porin. The peptidoglycan-associated proteins were obtained basically as described in reference 17. They were suspended in a solution containing 2% Triton X-100, 10 mM Tris-HCl, and 10 mM EDTA, pH 8.0. After centrifugation (1 h, 100,000 × *g*), the supernatant contained predominantly a 23-kDa protein, together with some other proteins. Similar results were obtained when Triton X-100 was replaced by Genapol X-80 (1). The whole-protein isolation procedure was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

For further purification, the supernatant (containing about 1 mg of protein) was applied to a hydroxyapatite column (Bio-Rad) with a volume of about 1.5 ml. The column was equilibrated and eluted with a solution containing 2% Genapol X-80, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0. Fractions of 1 ml were collected. The purity of the protein was checked with SDS–15% polyacrylamide gels. The protein was concentrated and the amount of detergent was decreased as described in reference 25.

Lipid bilayer experiments. The methods used for black lipid bilayer experiments have been described previously (5). Membranes were formed from a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, Ala.) in *n*-decane. Zero-current membrane potentials were measured with a Keithley 610 C electrometer 5 to 10 min after a 10-fold salt gradient was established across the membranes (6).

RESULTS

Isolation of the porin. The cell envelope fraction of *P. venetianus* was treated with 2% SDS to remove lipids and SDS-soluble proteins and obtain the peptidoglycan-associated proteins which contained a large amount of pore-forming activity. The associated proteins could be released

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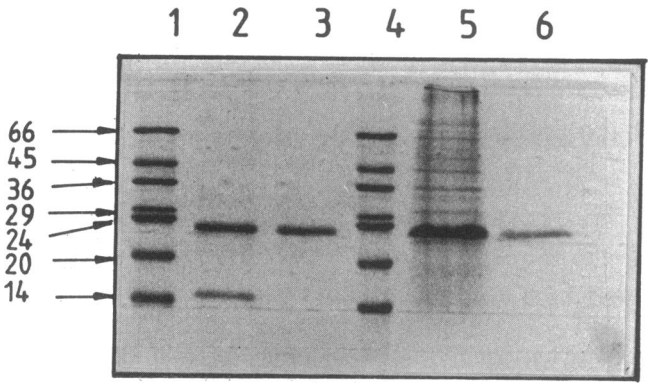

FIG. 1. SDS-PAGE of different purification steps of the 23-kDa porin of *P. venetianus*. Lanes: 1, molecular mass markers (in kilodaltons); 2, supernatant of the wash of the peptidoglycan-associated proteins with Triton X-100-containing solution; 3, eluate of the hydroxyapatite column loaded with the Triton X-100 wash; 4, molecular mass markers; 5, supernatant of the wash of the peptidoglycan-associated proteins with Genapol X-80-containing solution; 6, eluate of the hydroxyapatite column loaded with the Genapol X-80 wash. Proteins were solubilized by being dissolved in sample buffer containing 2% SDS at 100°C for 10 min. Aliquots containing about 10 μg of protein (lanes 2, 3, and 6) or 30 μg of protein (lane 5) were applied to an SDS–15% polyacrylamide gel. The porin migrated with an apparent molecular mass of 23 kDa on the gel system.

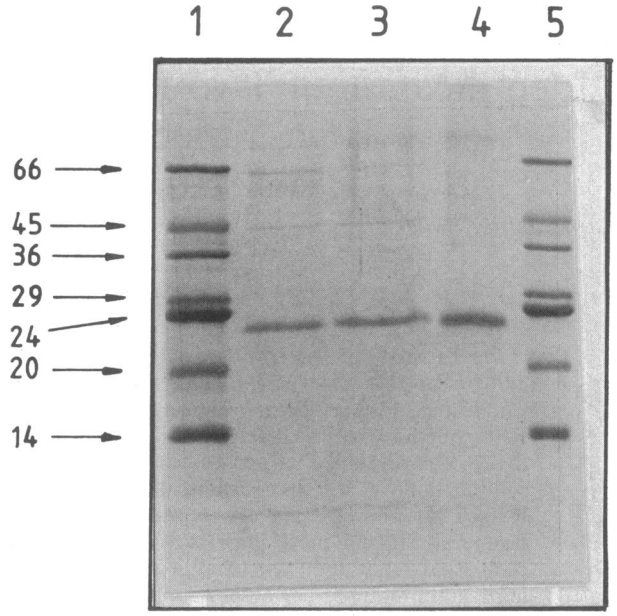

FIG. 2. SDS-PAGE of the supernatant of the wash of the peptidoglycan-associated proteins with Genapol X-80-containing solution containing mainly the 23-kDa porin of *P. venetianus*. Lanes: 1, molecular mass markers (in kilodaltons); 2, protein solubilized in sample buffer containing 2% SDS at 100°C for 10 min; 3, protein solubilized in sample buffer containing 2% SDS at 25°C; 4, protein solubilized in sample buffer containing 2% SDS but without mercaptoethanol at 25°C; lane 5, molecular mass markers. Aliquots containing about 10 μg of protein were applied to an SDS–15% polyacrylamide gel.

from the peptidoglycan by using either Triton X-100 or Genapol X-80. After treatment with Triton X-100, the released protein contained, besides a number of minor bands (Fig. 1), two major proteins, one with a molecular mass of 14 kDa, and one of 23 kDa. Genapol X-80 released only the 23-kDa protein. Use of the outer membranes instead of the cell envelope yielded identical results (data not shown).

During further purification on a hydroxyapatite column, we noticed that the 23-kDa protein did not bind to the column and eluted at high purity, right after the voided volume. The purity of the protein was checked by SDS–15% PAGE (Fig. 1). The apparent molecular mass of the protein was independent of the solubilization temperature, indicating that it did not form SDS-resistant oligomers, as most of the known porins do (3). Also mercaptoethanol had no effect on protein mobility (Fig. 2), which means that the protein did not contain inter- or intrachain disulfide bridges. The porin was identified by testing the different fractions of the column in lipid bilayer membranes (see below). Only the fractions which contained the 23-kDa protein showed pore-forming activity.

Elution of the 23-kDa protein from SDS-PAGE gel. To test whether the 23-kDa protein showed pore-forming activity as a monomer, we performed the following experiment. The pure 23-kDa protein was loaded onto a preparative SDS-PAGE gel, and electrophoresis was performed. The band corresponding to a molecular mass of 22 to 25 kDa was cut out of the gel and eluted overnight with Genapol X-80 buffer. The eluted protein had approximately the same pore-forming activity as it had before being loaded onto the SDS-PAGE gel.

Single-channel analysis. The supernatants obtained after treatment of the murein-associated proteins with the different detergents were tested in the lipid bilayer system. Addition of small amounts of the supernatants (final protein concentration, 50 to 100 ng/ml) to the aqueous solution in contact with a lipid bilayer membrane caused a large increase of the specific membrane conductance (by more than 2 to 3 orders of magnitude). Similar effects were observed with fractions of the hydroxyapatite column which eluted just after the voided volume of the column (fractions 2 to 4). Only these fractions contained the 23-kDa protein. Single-channel experiments were also performed to check the porin fractions for single channels. Figure 3 shows a single-channel recording measured with a lipid bilayer membrane in the presence of 10 ng of Genapol supernatant per ml. The channels had a single-channel conductance of 230 pS in 1 M KCl (Fig. 4). Occasionally, we also observed a transient channel with a larger single-channel conductance of 1.6 nS in 1 M KCl, which comprised about 1 to 3% of all of the channels obtained with the purified protein. This means that it probably represents a contaminant porin. The larger channel was more frequently detected in the supernatants of the SDS wash of the cell envelopes or in the protein obtained by Genapol X-80 treatment (Fig. 4). Neither one of these fractions nor total outer membranes contained heat-modifiable proteins.

Conductance of the two pores was measured in various salt solutions by adding either the 23-kDa porin or the SDS supernatant to the aqueous phase. For the large pore, the single-channel conductance in 0.1 M LiCl was lower than that observed in 0.1 M potassium acetate (Table 1). On the other hand, the single-channel conductance in potassium acetate was only a little lower than or equal to that in KCl. Both results indicated that the two pores are selective for cations. The single-channel conductance of both channels was not a linear function of the bulk aqueous conductance

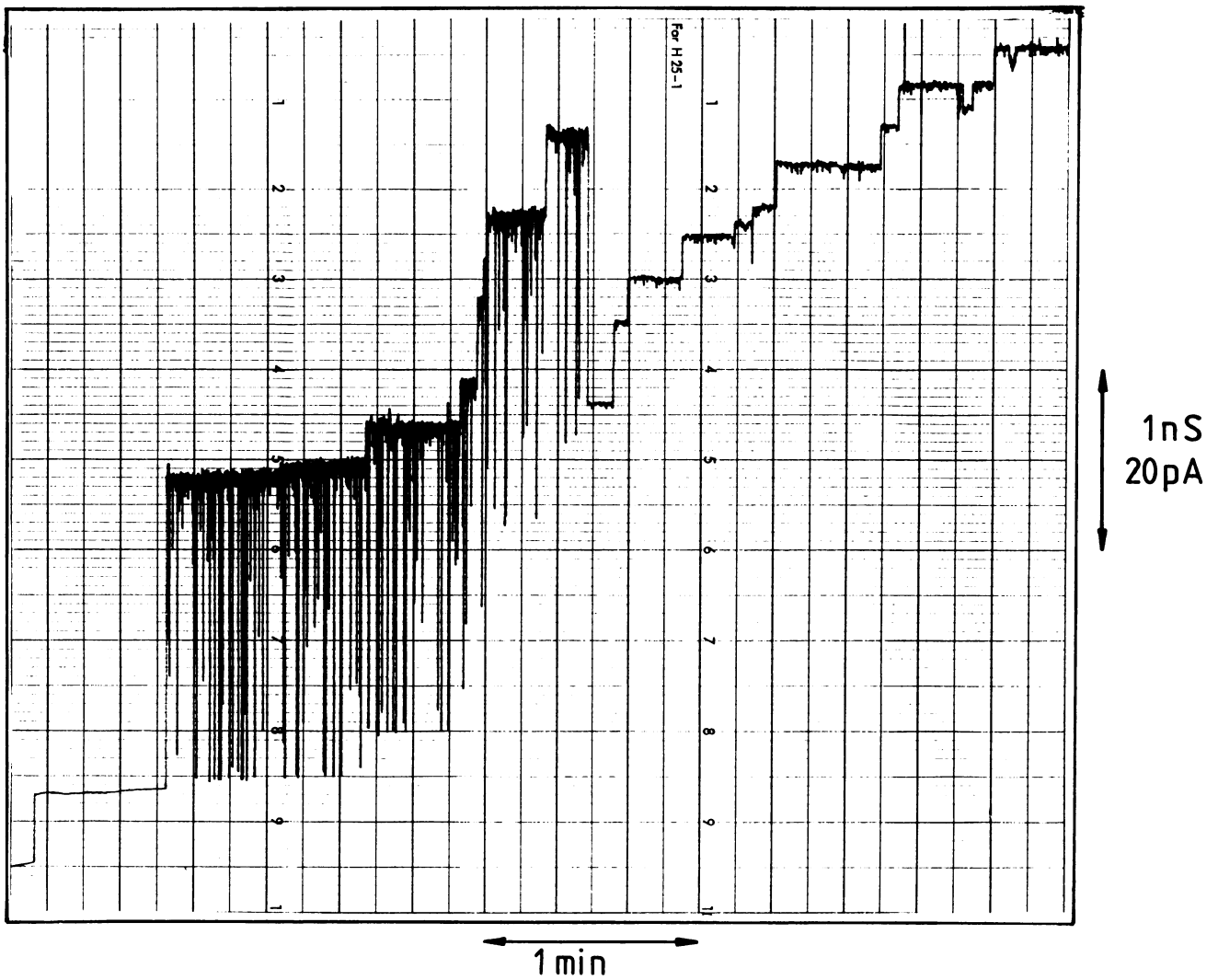

FIG. 3. Single-channel recording of a diphytanoyl phosphatidylcholine membrane in the presence of the supernatant of the Genapol X-80 wash (10 ng of protein per ml) of the peptidoglycan-associated proteins of *P. venetianus*. The aqueous phase contained 1 M KCl. The applied voltage was 20 mV. Temperature, 25°C.

(Fig. 5). The single-channel conductance of the small channel showed unusual concentration dependence.

Effect of PEG on pore conductance and activity. Since *P. venetianus* can degrade PEG up to a molecular mass of 20 kDa, we tested the effect of PEG on the permeability of the isolated and purified 23-kDa porin and the SDS supernatant of the cell envelope fraction containing the large pore. The single-channel conductance of both protein fractions in 1 M KCl did not change at all when the concentration of PEG 20000 was increased to 0.1% (the concentration in growth medium). We also checked the effect of PEG on macroscopic conductance, since this is, under certain conditions, a better indicator of the interaction between the channel and the substrate (8). However, in this case we also observed no effect of PEG on membrane conductance. Only in the presence of diethylene glycol and PEG 200 did we observe a small (less than 10%) decrease in membrane conductance, which may indicate a small viscosity change in the interior of the pore.

Selectivity measurements. The selectivity of the 23-kDa porin was studied by measuring the membrane potential under zero-current conditions in the presence of salt gradients. After incorporation of 100 to 1,000 pores into the membrane, the salt concentration on one side of the membrane was raised from 10 to 100 mM. The zero-current potential was measured, and the permeability ratio, p_{cation}/p_{anion}, was calculated with the Goldman-Hodgkin-Katz equation (6). The results are summarized in Table 2. For all four salts used in these experiments, the more dilute side was always positive, which indicated preferential movement of cations through the pore.

DISCUSSION

We identified two different pores in the envelope fraction of the anaerobic, gram-negative bacterium *P. venetianus* grown on high-molecular-mass PEG. One of them has an unusually small molecular mass of only 23 kDa. The smallest porin known is that of *Paracoccus denitrificans*, with an apparent molecular mass of 30 kDa (27). The solubilization

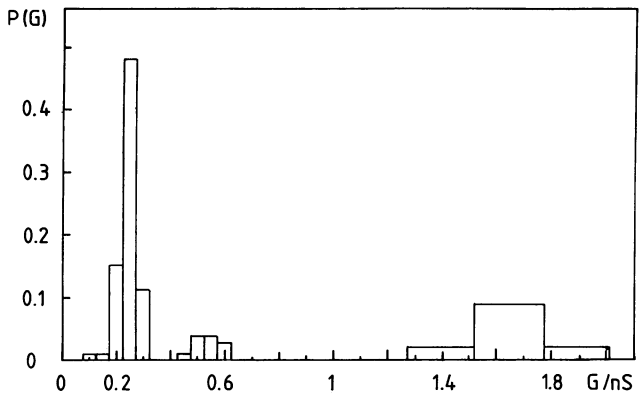

FIG. 4. Histogram of 162 conductance steps observed with diphytanoyl phosphatidylcholine membranes in the presence of the supernatant of the Genapol X-80 wash of the murein-associated proteins of *P. venetianus*. The mean of the left-hand maximum was 230 pS for 130 single events, and that of the right-hand maximum was 1.6 nS for 25 single events. The aqueous phase contained 1 M KCl. Temperature, 25°C; zero-current membrane potential, 20 mV; P, probability; G, conductance.

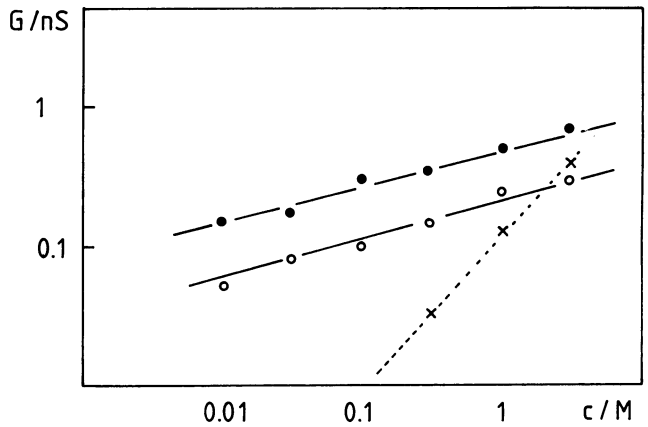

FIG. 5. Single-channel conductance of the 23-kDa porin of *P. venetianus* as a function of the NH_4Cl (●) and the KCl (○) concentration in the aqueous phase. ×, single-channel conductance for 3, 1, and 0.3 M NH_4Cl corrected for the effect of 1.5 negatively charged groups located at the channel mouth and assuming a channel diameter of 0.8 nm. G, conductance; c, concentration; M, molar.

temperature had no influence on the apparent molecular mass of the 23-kDa porin. This means that it is not heat modifiable, as most porins of gram-negative bacteria are (2, 18), except for Tsx of *Escherichia coli* (14) and OprF (protein F) of *Pseudomonas aeruginosa* (26).

Impurities were not responsible for the pore-forming activity of the 23-kDa protein. This was confirmed by eluting the corresponding protein from preparative SDS-PAGE gel, as done for OmpF trimers (19). The eluted protein had the same activity and formed the same channels as the purified 23-kDa porin. This indicates that the 23-kDa porin is one of the few porins known which are active as monomers (3). Only OprF (protein F) of *P. aeruginosa* (26) and the porin of *Rhodobacter sphaeroides* (23) have been found to be active in this form, although the latter is probably a trimer that dissociates on SDS-PAGE (10). Tsx of *E. coli*, which runs on SDS-PAGE gels as a monomer, is inactive after SDS treatment (14). There exists a good possibility that the 23-kDa porin of *P. venetianus* is organized in the outer membrane as a monomer, although possibility of formation of trimers from monomers cannot be excluded completely. The existence of monomers in the outer membrane would be something special, since it is now clear that PhoE of *E. coli* (13) and the porin of *R. capsulatus* (24) both contain three channels in a trimer. In most porin channels, ions move in a fashion similar to the way they move through the aqueous phase (2). This is not the case for the 23-kDa porin, since the single-channel conductance in KCl was similar to that in potassium acetate. Furthermore, the zero-current membrane potential measurements suggest that the permeability of potassium was at least 22 times higher than that for chloride. Since the high-conductance channels may also contribute to the selectivity of the membranes, it is very likely that the 23-kDa porin is exclusively selective for cations. This suggests that the 23-kDa porin represents a channel for cations similar to that which OprP (protein P) of *P. aeruginosa* represents for anions (4). However, the single-channel conductance did not saturate with increasing salt concentration but increased less than fivefold between 10 mM and 3 M salt. This suggests that the channel does not contain a binding site for cations and that the cation selectivity is caused by negative point charges located at the pore mouth. These charges create a potential which attracts cations and repels anions. As a consequence, the single-channel conductance is much larger than expected on the basis of the dimensions of the channel.

A quantitative description of the effect of the point charges on single-channel conductance may be given by the theory of

TABLE 1. Average single-channel conductance of the 23-kDa porin isolated from *P. venetianus* for different 0.1 M salts[a]

Salt	Conductance (pS)	
	Low-conductance pore	High-conductance pore
LiCl	15	270
NaCl	15	245
KCl	100	400
NH_4Cl	300	400
RbCl	125	430
CsCl	150	500
KCH_3COO	100	350

[a] The membranes were formed of diphytanoyl phosphatidylcholine in *n*-decane. The aqueous solutions were unbuffered and had a pH about 6. The applied voltage was 20 mV, and the temperature was 25°C. The average single-channel conductance was calculated from at least 100 single events.

TABLE 2. Zero-current membrane potentials, V_m, of membranes from diphytanoyl phosphatidylcholine–*n*-decane in the presence of the porin of *P. venetianus* measured for a 10-fold gradient of different salts[a]

Salt	V_m (mV)	p_{cation}/p_{anion}
KCl	50	22
NH_4Cl	51	31
LiCl	46	14
KCH_3COO (pH 7)	55	58

[a] V_m is defined as the difference between the potential on the dilute side (10 mM) and the potential at the concentrated side (100 mM). The pH of the aqeous salt solutions was around 6 unless otherwise indicated; temperature, 25°C. The permeability ratio (p_{cation}/p_{anion}) was calculated with the Goldman-Hodgkin-Katz equation (6) on the basis of at least three individual experiments.

Nelson and McQuarrie (16) by assuming that 1.5 negative charges are located at the mouth of the channels and that the channel diameter is 0.8 nm. By introducing the correction for the increased ion concentration (9) into the single-channel conductance, it can be shown (Fig. 5) that the corrected single-channel conductance is a linear function of the bulk aqueous conductivity. This suggests that no positively charged groups are in the vicinity of the negative charges; otherwise, we would not have observed the Gouy-Chapman effects. In fact, the general-diffusion channel of *R. capsulatus* outer membrane contains, according to X-ray diffraction analysis, both negatively and positively charged amino acids inside the channel (24), while the excess of negative charges is responsible for its cation selectivity (11). This channel does not show Gouy-Chapman effects (11). The existence of charged groups at the mouth of a channel represents another way to increase the permeability of a membrane channel for ions.

The large pore found in the SDS and Genapol X-80 supernatants was not a general-diffusion pore either, since the single-channel conductance was also in this case not a linear function of the conductance of the aqueous phase. On the other hand, the influence of the surface charges located at the mouth is rather small at high ionic strength. This means that the single-channel conductance of the large pore is approximately similar to those of *E. coli* general-diffusion pores, which have an effective diameter of about 1.1 nm (7). Such a diameter would allow permeation of hydrophilic solutes up to a molecular weight of about 600 through the pores. The diameter of the 23-kDa porin may be around 0.7 to 0.8 nm, since its single-channel conductance is similar to that of LamB (8) and since diethylene glycol and PEG 200 can obviously enter the channel, thus increasing the internal viscosity and decreasing the pore conductance.

The porins detected and described in this communication do not represent means suited for transport of high-molecular-mass PEG (20 kDa; hydrodynamic coil diameter, up to 15 nm; 22) across the outer cell membrane of *P. venetianus*. However, all existing biochemical evidence indicates that the monomer, as well as the polymer, is degraded only inside the cytoplasm, after crossing both the outer and cytoplasmic membranes.

Necessary transport of PEG into bacterial cells could be provided through unwinding of the PEG coil and entrance of the PEG chain through one of the porins detected. The observed lack of ion transport inhibition by PEG in this study and the high degradation rate which is independent of the molecular mass of PEG degraded both do not speak for this assumption. Transport could also be provided by special proteins which were overlooked in this study or by direct diffusion across the outer membrane. Experiments are in progress in our laboratory to examine these hypotheses.

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