Ion Selectivity Reversal and Induction of Voltage-Gating by Site-Directed Mutations in the *Paracoccus denitrificans* Porin†

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ABSTRACT: The porin from *Paracoccus denitrificans*, a slightly anion specific outer membrane pore protein, was expressed in *Escherichia coli*, isolated from inclusion bodies, and refolded in the presence of urea and detergents. The purified recombinant protein was reconstituted into black lipid bilayer membranes and showed no difference in its functional properties in comparison to the native porin isolated from *P. denitrificans* membranes. To investigate the molecular basis of its ion selectivity and voltage-gating, a series of site-directed mutants was constructed, comprising acidic residues located on the third extracellular loop (L3), which forms the constriction zone of the channel, and basic residues along the opposing barrel wall. Measurements using zero-current membrane potentials indicated that the selectivity changed drastically from a slight anion to a distinct cation selectivity with the exchange of residues R29 and R31 by glutamate, whereas replacements on the L3 loop went largely unaffected. However, when assaying the voltage-dependent closure of channels, only mutations located on the L3 loop showed an effect, in contrast to the voltage-independent recombinant and native *Paracoccus* porin.

Porins are water-filled, channel-forming proteins which span the outer membrane of Gram-negative bacteria, mitochondria, and chloroplasts. They belong to the best-characterized ion channels at the structural and biochemical level (*1*). The nonselective porins permit free diffusion of molecules smaller than 600 Da through the membrane, whereas selective porins have binding sites specific for one class of solutes (*2*, *3*). Bacterial porins form stable homotrimers, where each contains one channel. The main structural motif of these membrane proteins is 16−18 stranded antiparallel β-barrel cylinders, with connections only between neighboring strands (*4*, *5*).

In the general diffusion porins of *Rhodobacter capsulatus* 37b4, *Rhodopseudomonas blastica*, and *Paracoccus denitrificans*, all members of the α-3 subgroup of proteobacteria (*6*), a long loop (L3) connecting strands β5 and β6 runs into the barrel inside and constricts the nonspecific pore (*4*, *7*).

The amino acid residues coating this eyelet show an asymmetric charge distribution. In nearly all other nonspecific porin structures determined to date, the net charge along the inner wall of the channel is negative, while that from the residues of the opposing interior loop is positive (*8*), yielding slightly cation-specific pores. The positive and negative clusters create a strong transverse electrostatic field in the eyelet region, which mainly determines the permeability and the ion selectivity of the pore. The eyelet region of the *P. denitrificans* porin (as shown in Figure 1) is unique in its charge distribution, carrying acidic residues on the third loop that are opposed by basic residues along the barrel wall, resulting in a slight anion selectivity of this porin.

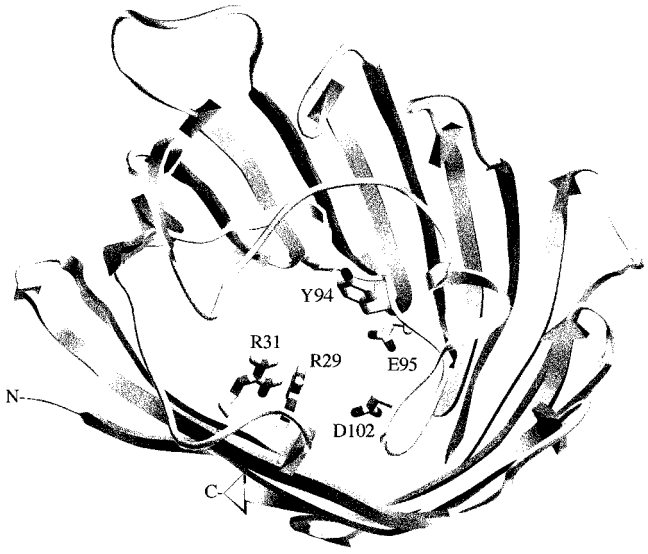

FIGURE 1: Monomeric pore structure of the *P. denitrificans* porin, viewed along the pore axis from the intracellular face. Mutated residues in the restriction zone are highlighted by ball-and-stick representation and numbered, and the N- and C-termini labeled. The figure was created by PovRay based on the coordinates of Hirsch et al. (1997).

Porins have been studied in detail in the black lipid membrane system (*9*, *10*). By increasing the membrane potential, pores tend to close at a certain threshold value. This phenomenon known as voltage-gating was reported by Dargent et al. (*11*) and Lakey (*12*). The physiological significance of this observation is still a matter of debate, since the overall

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potential difference across the outer membrane does not reach values sufficient for closing the pores (*13*). However, it has recently been described that conditions such as pH (*14*), pressure (*15*), and the presence of membrane-derived oligosaccharides (MDO; (*16*)) might initiate voltage-gating effects under physiological outer membrane potentials. Moreover, voltage-gating effects appear to be highly sensitve to the applied experimental conditions (*17*−*19*).

In this paper, we examine the molecular basis of *Paracoccus* porin ion selectivity by mutating selected amino acids in the eyelet region. Zero-current membrane potential measurements were carried out to analyze the selectivity of the mutant porins. In addition, voltage-gating measurements of the porin mutations were performed to study whether replacements of amino acids influence the voltage-independent gating pattern of the *Paracoccus* wild-type porin in lipid bilayer membranes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Wild-type *P. denitrificans* derivative strain Pd1222 (*20*) was grown aerobically at 32 °C with succinate as the major carbon source (*21*). *Escherichia coli* DH5α (Bethesda Research Laboratories) and *E. coli* BL21 DE3 (pLysE) (Novagen) were grown at 37 °C in LB medium. Antibiotics were used, where appropriate, at concentrations of 50 μg/mL for ampicillin, 30 μg/mL for chloramphenicol, and 10 μg/mL for tetracycline.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to the "Altered Sites" system (Promega). The *Nco*I-digested 2.7 kb DNA fragment, encoding the *P. denitificans* porin, *por*G (*22*), was ligated into the *Nco*I site of pALTER-Ex1 (Promega).

Cloning of Paracoccus Porin into an E. coli Expression Vector. The modified *por*G gene described above was ligated into pJC40 (*23*), previously digested with *Nco*I and *Bam*HI. The emerging plasmid pJCPor was transformed into *E. coli* BL21 DE3 (pLysE) (Novagen), transformants grown to an OD_{578nm} of 0.5 and induced by the addition of IPTG[1] to a final concentration of 0.4 mM. The levels of recombinant *Paracoccus* porin expressed at various times after induction were monitored by SDS−PAGE of whole cell lysates followed by staining with Coomassie Brilliant Blue.

Expression and Purification of Recombinant Paracoccus Porins. Wild-type and mutated *Paracoccus* porins expressed in *E. coli* were purified basically according to Schmid et al. (*24*), however, with several minor modifications. *E. coli* BL21 DE3 (pLysE) containing the respective vector was grown in LB medium to an OD_{578nm} of 0.5. Expression was induced by adding IPTG to a final concentration of 0.4 mM, and the culture was grown for an additional 4−5 h before harvesting (Sorvall, GS-3, 10800*g*, 20 min). The pellet was resuspended in TEN buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 mM NaCl), frozen overnight, and then thawed at room temperature. After incubation with benzonase (Merck) for 1 h, cells were broken by sonification (Branson Sonifier 250), the inclusion bodies (IBs) were sedimented by centrifugation (SS34, 4300*g*, 1 h), and the resulting pellet was resuspended in Triton TEN buffer (2% (w/v) Triton X-100 in TEN buffer) and incubated at 37 °C overnight with shaking. The IBs were centrifuged (SS34, 4300*g*, 20 min), followed by resuspension of the pellet in TEN buffer (2 h shaking at 37 °C) and a further centrifugation (SS34, 4300*g*, 20 min).

The IB pellet was solubilized by resuspending in freshly prepared urea buffer (8 M urea in TEN buffer), incubated for 2 h at 37 °C, and centrifuged (SS34, 48000*g*, 20 min). Equal volumes of urea-dissolved IBs—the supernatant after centrifugation—and LDAO-10 buffer (10% (w/v) *N*,*N*-dimethyldodecyl-amine-*N*-oxide (LDAO) in TEN buffer) were mixed and loaded onto a Q-Sepharose fast flow column (Pharmacia) equilibrated in LDAO-0.2 buffer (0.2% (w/v) LDAO; 50 mM Tris-HCl, pH 8.0; 100 mM NaCl). After washing with LDAO-0.2 buffer, a gradient of 0.1−1 M NaCl in LDAO-0.2 buffer was applied and recombinant porin eluted as a single peak at 0.7 M NaCl. Peak fractions containing *Paracoccus* porin were pooled and analyzed by SDS−PAGE.

Membrane Experiments. The methods used for black lipid bilayer experiments have been described previously (*9*). The experiments were performed at 25 °C. All salts were obtained from Merck, Darmstadt, FRG (analytical grade). The pH of the unbuffered salt solutions was adjusted to pH 6 by the addition of the corresponding acid or base. The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall. The membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. Bilayer formation was indicated when the membrane turned optically black to reflected light. The porin was added from the protein stock solution of 1 mg/mL either immediately before membrane formation or after the membrane had turned black. The single-channel conductance of the pores was measured after application of a fixed membrane potential with a pair of Ag/AgCl electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The current through the membrane was boosted by a current amplifier, monitored on a storage oscilloscope, and recorded on a strip chart or tape recorder.

Zero-current membrane potentials were measured as previously described (*25*). The membranes were formed in a 10 mM KCl solution containing a defined protein concentration so that the membrane conductance increased about 100−1000-fold within 10−20 min after membrane formation. At this time, the instrumentation was switched to the measurements of the zero-current potentials, and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value within 2−5 min.

RESULTS

Overexpression of Recombinant Paracoccus Porin in E. coli. Initial attempts to express the *Paracoccus* porin in *E. coli* were without success, possibly because its toxicity to the host, an observation made for several recombinant porins in *E. coli* (*26*, *27*). Supposing that the toxicity is indirectly mediated by its signal peptide which allows transport of the

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IB, inclusion body; IPTG, isopropyl thiogalactoside; KAc, potassium acetate; LDAO, lauryldimethylamine oxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion-selective channel.

porin to the membrane, this sequence was deleted using site-directed mutagenesis, and the truncated *por*G gene was cloned into an expression vector, pJCPor, now coding for the entire mature *Paracoccus* porin (see Experimental Procedures). After transformation into *E. coli* BL21 DE3 (pLysE), expression under the control of an inducible T7 promoter resulted in the accumulation of a 33-kDa inclusion body protein, with no apparent toxicity to the host. Control experiments (not shown) revealed that this protein band was absent when the *E. coli* cells contained only the expression plasmid pJC40 or when no expression was induced in cells. The amount of recombinant *Paracoccus* porin produced after induction was estimated to be about 10% of the total cellular protein.

Purification and Refolding of Recombinant Paracoccus Porins. Fermentation of *E. coli* BL21 DE3 (pLysE) harboring plasmid pJCPor and its point mutations produced recombinant porins in inclusion bodies. The insoluble IBs were readily separated from the majority of *E. coli* proteins by centrifugation of the whole cell lysate. After washing with Triton, the protein was solubilized in 8 M urea. Refolding of recombinant *Paracoccus* porin into its native conformation was achieved by employing a detergent-assisted refolding procedure (*28, 29*). Subsequent purification using an ion exchange chromatography separated folded trimeric from unfolded monomeric porin protein, because the latter passed through the column without binding. The purified refolded recombinant *Paracoccus* porins were free of contaminating *E. coli* proteins as judged by SDS−PAGE (not shown), and both native and recombinant *Paracoccus* porin migrated identically on gels (but distinct from the *E. coli* porins). Typical yields were around 15 mg of refolded protein per liter of bacterial culture.

Comparison of Native and Recombinant Paracoccus Porin. To ascertain that the expression, refolding, and reconstitution of recombinant porin had no influence on the channel features, the functional properties of the heterologously expressed porin were recorded and compared to those of the conventionally expressed and isolated protein. Using black lipid bilayer experiments, single-channel conductance was measured for both the native and the recombinant porin. Single-channel conductivity patterns of both proteins showed no significant difference (2.6 nS in 1 M KCl). The results of these experiments are summarized in Table 1, which shows the average single-channel conductance, *G*, as a function of different salt solutions. Likewise, no difference was seen in the ion selectivity of both porins when 10-fold gradients of KCl, LiCl, and potassium acetate were applied. Differences in channel-forming activities of recombinantly expressed and wild-type porin fall within the normal variability of bilayer reconstitution experiments. Furthermore, recombinant and wild-type porin exhibited no voltage-dependent closure up to 250 mV (see Figure 2). Thus, we can safely conclude that the recombinant porin behaves indistinguishably from native porin isolated from *Paracoccus* membranes.

In addition, these selectivity measurements reveal that two *P. denitrificans* strains show different ion selectivities. Strain Pd1222 (a wild-type derivative, (*20*)) was used for the molecular cloning (*22*), while a wild-type, nominally ATCC 13543 (obtained from Hiroshi Nikaido), was employed for the functional characterization of the *P. denitrificans* porin,

Table 1: Average Single-Channel Conductance, *G*, and Permeability Ratios, *R*, of the *P. denitrificans* Porin Species Probed in Three Salt Solutions

	single-channel conductance *G* (nS)			ion selectivity (*R*)[a]		
porin species	1 M KCl	1 M LiCl	1 M KAc	KCl	LiCl	KAc
wild-type						
isolated from Pd1222	2.6	2.0	1.0	0.35	0.17	0.22
expressed in *E. coli*	2.6	2.3	1.2	0.30	0.11	0.21
E95Q	2.6	1.3	1.0	0.33	0.14	0.34
D102N	2.5	2.5	1.0	0.28	0.15	0.18
E95Q/D102N	2.5	2.3	1.0	0.34	0.13	0.33
D102W	2.5	2.3	0.8	0.26	0.15	0.30
Y94G	3.5	2.5	1.3	0.23	0.12	0.34
R29T	2.0	1.3	0.8	0.74	0.23	1.40
R31T	1.5	2.0	1.3	0.70	0.28	1.40
R29/31T	3.7	1.3	1.6	2.74	0.91	3.90
R29/31E	3.7	1.3	1.6	14.0	7.0	16.0

[a] The permeability ratio ($R = P_{cation}/P_{anion}$) was calculated by the Goldman−Hodgkin−Katz equation (Benz et al., refs 9 and 32) from at least three individual experiments measured for a 10-fold gradient of different salts.

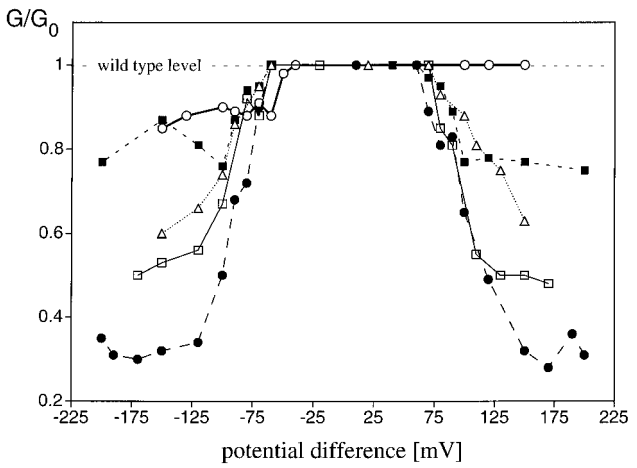

FIGURE 2: Voltage dependence of the *P. denitrificans* porin species reconstituted in black lipid bilayer membranes in the presence of 1 M KCl. The ratios of the conductance *G* (at a given voltage) and G_0 at 20 mV are shown as a function of the applied transmembrane potential for five mutants: E95Q (□), D102N (■), E95Q/D102N (△), D102W (○), and Y94G (●). For clarity, only selected data are displayed in the range +60 to −60 mV.

showing a selectivity ratio of 1.4 in KCl (*22*). A discrepancy in the first amino acid for the porins of both *Paracoccus* strains had already been noted in this previous study, questioning the identity of both strains (*22*), a deviation now further extended to selectivity differences found for both porins. The porin gene of the independently obtained authentic ATCC 13543 strain was sequenced (not shown), indicating that the porin genes of both strains (ATCC 13543 and Pd1222) are identical. The phenomenon of two nominally identical strains having differences in the amino acid sequence was also reported for the porin of *R. capsulatus* by Trieschmann et al. (*30*).

By applying standard site-directed mutagenesis techniques, selected acidic residues (D102N, D102W, E95Q, E95Q/D102N, Y94G) located on the L3 loop which constricts the channel (see Figure 1) and basic residues along the barrel wall (R29T, R31T, R29/31T, R29/31E) opposing this loop were mutated, to examine the molecular basis of porin

selectivity and voltage-gating. The mutant porins were expressed and isolated as described above and investigated by single-channel conductance experiments and zero-current membrane measurements. Furthermore, we also monitored the voltage-gating dependence of the mutants in comparison to the wild-type porin.

Single-Channel Conductance of Paracoccus Porin Mutants. Single-channel experiments revealed that all recombinant mutated porins reconstituted into bilayers as efficiently as wild-type porin. The addition of small amounts of mutant *Paracoccus* porin (10 ng/mL) to lipid bilayer membranes allowed the resolution of stepwise conductance increases. The mean values of the single-channel conductance were calculated from the channel distributions and the results are summarized in Table 1. In four out of the five proteins mutated in residues on the L3 loop (D102N, D102W, E95Q, E95Q/D102N), the differences in channel-forming activities of mutant and wild-type porin fall within the normal variability of bilayer reconstitution experiments (see Table 1). The conductance value of the Y94G mutant was found to be significantly higher than that of the native porin in KCl.

Zero-Current Membrane Potential Measurements. Zero-current membrane potential measurements were carried out to study the selectivity of the recombinant mutant porins in comparison to wild-type *Paracoccus* porin. These measurements were performed by applying 10-fold gradients of KCl, LiCl, and potassium acetate across the membranes containing at least 100 porin pores. The net charge inside the channel determines whether the pore prefers the passage of anions or cations. The mutant porins D102N, D102W, E95Q, E95Q/D102N, and Y94G show no significant changes in their selectivity as compared to the wild-type porin. For all salts, the potential of these mutants was found to be always negative on the more dilute side of the membrane, indicating a slightly preferential movement of anions through the pores. The potentials were quantitated using the Goldman−Hodgkin−Katz equation (*25*). The resulting ratios R (the cation permeability, P_{cation}, divided by the anion permeability, P_{anion}) of the mutant porins were about 0.3, similar to those of the wild-type (see Table 1).

However, significant differences were observed when the two arginine residues (R29 and R31), located along the barrel wall at the channel restriction, were mutated. A replacement by threonine causes a reduction of the anion selectivity by more than twofold for each of the mutants. In the double mutant R29/31T the consequence was even more pronounced, and the anion selectivity was reversed to cationic selectivity. The most drastic effect was obtained when both arginines were exchanged for glutamate (R29/31E), yielding a strong cation selectivity. The resulting ratio R is increased by a factor of nearly 50 over the wild-type in this double mutant.

Voltage-Dependent Closure of Paracoccus Porin Mutants. To study whether replacements of amino acids by site-directed mutagenesis influenced the voltage-independent gating pattern of the *Paracoccus* porin in lipid bilayer membranes, multichannel experiments of the mutant porins were performed and compared to the wild-type. Under such conditions, the decrease of the membrane current following a voltage step can be described by a single exponential decay. Surprisingly, only the mutant porins D102N, D102W, E95Q, E95Q/D102N, and Y94G, in which the mutated residues are located on the third extracellular loop, displayed genuine voltage-dependent closure, whereas the replacements of the basic residues along the barrel wall (R29T, R31T, R29/31T, and R29/31E) resulted in the typical voltage-independent wild-type pattern (see Figure 2).

DISCUSSION

A set of site-directed mutants was devised to study the molecular basis of porin ion selectivity and voltage-gating in the *P. denitrificans* porin. The 3-D structure of this bacterial outer membrane protein is known (*31*), and its gene has been cloned and sequenced (*22*). For the homologous expression of mutant protein, a *Paracoccus* strain deleted in its wild-type copy of the porin gene should prove useful. However, present evidence (Saxena, unpublished) suggests that the *por*G gene codes for the only porin species in *P. denitrificans*, and repeated attempts to construct a porin-deficient strain were indeed unsuccessful. In an alternative approach, we attempted the heterologous production of the *P. denitrificans* porin in *E. coli*.

Heterologous Expression and Refolding of the Porin. Since the heterologous expression of the porin gene may cause lysis in the host cell, as has been reported for several recombinant porins in *E. coli* (*26*, *27*), the region coding for the signal peptide of the *por*G gene was deleted, so that the protein could not reach its target membrane and was prevented from exerting toxic effects to the host cell. This strategy was reported by Qui et al. (*28*) and Pullen et al. (*29*), whereby large amounts of porins were accumulated in IBs. An additional advantage of this expression strategy is the easy purification of recombinant protein by eliminating the majority of *E. coli* contaminating proteins in the supernatant after centrifugation. The truncated *Paracoccus* porin gene, lacking its native signal sequence coding region, was cloned into an inducible T7 expression vector system. Control experiments revealed that the porin from *P. denitrificans* was expressed efficiently in *E. coli* cells and deposited in inclusion bodies without impairing host cell viability. The aggregated porin could be purified according to the procedure of Schmid et al. (*24*), devised for the *R. blastica* porin. This protein, which shows 39% identity to the *P. denitrificans* porin, was successfully refolded from inclusion bodies when expressed in *E. coli* and yielded identical crystal structures for the recombinant and the porin isolated from its native host.

After washing the inclusion bodies containing the aggregated *Paracoccus* porin with a Triton buffer, urea was used to solubilize the recombinant porin. Once soluble, attempts to refold the inclusion body protein by diluting out the denaturant resulted in monomeric misfolded porin, lacking a channel function in black lipid bilayer membranes (data not shown). Refolding of recombinant *Paracoccus* porin into its native conformation was achieved by employing a detergent-assisted refolding procedure as described by Qi et al. (*28*). Subsequent purification using ion exchange chromatography yielded correctly refolded *Paracoccus* porin of high purity.

To test whether the expression and refolding procedure had any influence on the functional properties of the *Paracoccus* porin in the black lipid bilayer system, single-

channel conductance, ion selectivity, and voltage-gating measurements were performed. In all of these biophysical properties, the recombinant *Paracoccus* porin was indistinguishable from the porin isolated from its native host, so that the heterologously expressed porin proved to represent a model suitable for structural and functional studies.

The functional characteristics of porin channels are determined largely by the structure of its constriction site. Analysis of the *Paracoccus* porin 3-D structure revealed that the pore diameter of the β-barrel is reduced to 13 × 11 Å (*31*). In this eyelet region, five arginyl residues (Arg-9, -29, -31, -51, -292) form a cationic cluster on the barrel wall that is opposed by four carboxylate groups (Asp-84, -102, -118, Glu-95) on the opposite side of the constriction on loop L3. For this mutagenesis study, we chose two positions in the arginyl cluster along the barrel wall for their central location in the lumen, and two acidic amino acids in the L3 loop region (see Figure 1). Arg-29 and Arg-31 were both mutated to threonine and glutamate both as single and double mutations, whereas Glu-95 was exchanged for glutamine and Asp-102 for asparagine and tryptophan. To further assess pore size limitations, Tyr-94 pointing toward the channel vestibule, was replaced by glycine. The recombinant porin mutants were investigated by single-channel conductance, ion selectivity, and voltage-gating measurements and were compared to the native porin expressed either in *P. denitrificans* or *E. coli*.

Single-Channel Conductance. Single-channel conductance experiments were performed with three different salt solutions to obtain information on the selectivity of the mutant porins in comparison to the native protein. Potassium and chloride ions have about the same mobility in the aqueous phase, whereas the mobilities of Li^+ and Ac^- are considerably smaller (*32*). In nearly all mutations on the L3-loop (D102N, D102W, E95Q, E95Q/D102N), the single-channel conductance is similar to that observed in wild-type channels. Even with the simultaneous removal of two negative charges on the L3 loop, an influence on the conductance values of the *Paracoccus* porin is only marginal at best (see Table 1). We therefore conclude that these residues do not contribute to the transport properties of the *Paracoccus* porin, as also confirmed by zero-current membrane potential measurements (see Table 1). Only the substitution of Tyr-94 by glycine on the L3 loop displays an increased single-channel conductance (nearly 40%) in KCl, and to some extent also for the two other salt solutions. These findings are easily explained by the fact that a bulky amino acid side chain (Tyr-94) in the constriction zone is exchanged for a small one, increasing the effective pore diameter. However, the replacement of Asp-102 by the bulky amino acid tryptophan does not show a decreased single-channel conductance (see Table 1).

Changes in conductance values are also observed for both arginine replacements R29 and R31. As these arginines have a similiar position in the channel lumen, replacements of the cationic side chains by the uncharged threonine would be expected to cause identical effects. This is actually not the case; both mutants display a reduced conductance in comparison to the wild-type, but to a different degree. The general reduction of single-channel conductance may be explained in two ways: (i) The L3-loop could, due to its disordering caused by the loss of an ionic interaction, obstruct the pore. (ii) The introduction of threonine could initiate new H-bonds, resulting in a reduced pore size. In a computer simulation of the R31T mutation, at least one of the possible rotamer position shows the amide oxygen of Gln-53 in hydrogen bond distance to the hydroxyl group of Thr-31.

The most significant changes in single-channel conductance are displayed by the double mutants R29/31T and R29/31E. The removal of two positive charges at the eyelet diminishes the attractive force for mobile anions in favor of cations transported through the pore, yielding single-channel conductance values which indeed follow the aqueous ion mobility coefficient for cations in contrast to the anion-determined wild-type: the conductances of both double mutants are reduced in LiCl and increased in KAc relative to the wild-type. Surprisingly, the single-channel conductance values are the same for both double mutants in all salt solutions tested, although one mutant (R29/31E) contains two additional negative charges in the constriction zone. This observation deviates strikingly from expectation, because the different pore properties of both double mutants are confirmed by the selectivity measurements (see Table 1).

Selectivity. Most of the selectivity patterns do not differ significantly from those of the wild-type porin. As demonstrated by the single-channel conductance experiments, apparently only the Arg-29 and Arg-31 substitutions had an effect on the selectivity. Besides, the replacement of the uncharged Tyr-94 by the uncharged glycine on the L3 loop also shows no alteration in the selectivity of the pore.

Substituting each one of the arginine residues along the barrel wall by a threonine (R29T, R31T) results in proteins with the same anion selectivity, but both lower than the wild-type. This is in accordance with their similar positions in the channel lumen. When two positive charges in the constriction zone (R29/31T) are deleted simultaneously, attractive forces for mobile anions are further reduced, resulting in a weakly cation-specific porin. The exchange of two positively charged residues R29/31 by glutamates increases the cation selectivity of the channel by an additional factor of nearly 6 over the mutant R29/31T. We conclude that the selectivity of the weakly anion-specific *Paracoccus* porin is mainly determined by the two positively charged arginine residues at position 29 and 31 along the barrel wall opposing the L3 loop.

Voltage Dependence. The effect of voltage-gating of porin channels has been a controversial issue. Reconstituted porins were conventionally found in an open state in planar lipid bilayers; the phenomenon of voltage-dependent closing was explained by a reconstitution artifact, since Sen et al. (*13*) showed that the Donnan potential (up to about −100 mV inside the periplasmic space in dilute media), which exists across the outer membrane, is not sufficient to close OmpF porin channels in intact *E. coli* cells. Furthermore, the rapid equilibration of ions through a large number of porin channels argues against the presence of a significant potential caused by an ion gradient across the outer membrane (*13*, *33*). However, studies of Delcour et al. (*34*−*36*) and Berrier et al. (*37*, *38*) using patch clamp analysis have shown that porins may behave as voltage-dependent channels under physiological outer membrane potentials. Samartzidou and Delcour (*39*) propose that this effect can only be monitored under suitable reconstitution conditions.

We analyzed our mutants for voltage-gating; in contrast to the voltage-independent wild-type and the mutants, in

which the mutated residues are located along the barrel wall, the mutants with replacements on the L3 loop induce genuine voltage-dependent closure of the channels at potentials of about ±70 mV (see Figure 2). The replacements of a negative charge by a neutral amino acid on the L3 loop result in an increased voltage sensitivity, which has also been reported by van Gelder et al. (*40*) for the anionic-specific PhoE.

Unlike the wild-type and the other mutant porins examined, the D102W mutant shows an asymmetric voltage-gating behavior in the planar lipid bilayer system. The effect of closing channels was found only at negative polarity of potential and requires further examination. Since not all mutations in the L3 loop (such as Y94G) disturb the strong electrostatic field in the eyelet region, the reason for the induced voltage-gating is not understood currently.

The eyelet region is probably responsible for the voltage dependence, because the structure of bacterial porin channels itself is stabilized by many hydrogen bonds, requiring considerable energy to break them down (*1*, *4*). Because of the small cross-section at the eyelet constriction, a potential across the membrane will essentially lie across the constriction zone (*41*). Imposed transmembrane potentials could therefore trigger the movement of residues of the L3 loop along the electrostatic field. In contrast to the stable bacterial porins, mitochondrial voltage-dependent anion channels (VDAC), which do not occur as close-packed trimers, undergo reversible, low-voltage-induced partial closures. A possible gating mechanism suggested for VDACs, in which the movement of the N-terminal α-helix out of the lumen wall triggers larger scale structural changes (*42*), is rather unlikely because the removal of the N-terminal region has no effect on channel gating (*43*). Thus, it may be possible that also in this case an external loop between two β-strands is responsible for channel gating (*44*). Two possibilities for the voltage-driven closure of bacterial porin channels were dicussed: (i) a large conformational change of the L3 loop could close the pore eyelet, or (ii) subtle local electrostatic changes in the eyelet region could alter the channel conductivity. A large conformational change of the L3 loop for voltage-gating was also proposed on the basis of molecular dynamic simulation studies by Soares et al. (*45*). Various groups have examined the voltage-dependent closing of porin channels, especially that of *E. coli* OmpF, OmpC, and PhoE. Phale et al. (*46*) demonstrate that tip movement of L3 is not involved in gating. Furthermore, the constructed OmpF mutants by Bainbridge et al. (*47*), in which the L3 loop is fixed by disulfide bonds, show unimpaired voltage-gating. The results of both studies rule out widespread or localized L3 movement, suggesting that the gating effect is exerted solely by the side chains (*40*, *48*). Bainbridge et al. (*49*) conclude that voltage-gating is a general property of the β-barrel pore structure rather than of any particular substructure. The fact that in our present study mutations in the L3 loop of the *P. denitrificans* porin alter the voltage-independent pattern of wild-type clearly contradicts this conclusion, supporting the importance of amino acid residues located on the L3 loop for voltage-gating. Our data are also in contrast to the mutagenesis studies on *R. blastica* porin by Schmid et al. (*50*) which show that distinct changes in the voltage-gating pattern are only observed in porin proteins carrying multiple mutations.

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