Site-Directed Mutagenesis of Tyrosine 118 within the Central Constriction Site of the LamB (Maltoporin) Channel of *Escherichia coli*. I. Effect on Ion Transport

Frank Orlik, Christian Andersen, and Roland Benz
Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

ABSTRACT The three-dimensional structure of the malto-oligosaccharide-specific LamB-channel of *Escherichia coli* (also called maltoporin) is known from x-ray crystallography. The central constriction of the channel formed by the external loop 3 is controlled by a tyrosine residue (Y118). Y118 was replaced by site-directed mutagenesis by ten other amino acids (alanine, isoleucine, asparagine, serine, cysteine, aspartic acid, arginine, histidine, phenylalanine, and tryptophane) including neutral ones, negatively and positively charged amino acids to study the effect of their size, hydrophobicity, and charge on ion transport through LamB. The mutant proteins were purified to homogeneity. They were reconstituted into lipid bilayer membranes and single-channel conductance and ion selectivity were measured to get insight into the mechanism of ion transport through LamB. The mutation of Y118 to any other nonaromatic amino acid led to a substantial increase of the single-channel conductance by more than a factor of six at maximum. The highest effect was observed for Y118D. Additionally, a nonlinear relationship between the salt concentration in the aqueous phase and the channel conductance was observed for this mutant, indicating strong discrete charge effects on ion conductance. For all other mutants, with the exception of Y118R, linear relationships were found between single-channel conductance and bulk aqueous concentration. The individual hydrophobicity indices of the amino acids introduced inside the central constriction of the LamB channel had a somewhat smaller effect on the single-channel conductance as compared with the effect of their size and charge.

INTRODUCTION

The outer membrane of Gram-negative bacteria acts as a molecular sieve, which has a defined exclusion limit for the permeation of hydrophilic solutes (see Nikaido, 1992 and Benz, 1994 for reviews). These molecular sieving properties are due to a major class of proteins called porins that form trimeric channels in the outer membrane. Many porins have an only small or no specificity for solutes and sort mainly according to their molecular mass. They act as general diffusion pathway for the rapid uptake of nutrients across the outer membrane. Other proteins such as LamB (maltoporin) of *Escherichia coli* (Luckey and Nikaido, 1980; Benz et al., 1986) and ScrY of enteric bacteria (Schmid et al., 1991; Schülein et al., 1991) form carbohydrate-specific pores and contain binding sites for these solutes. The expression of these specific pores is induced if the cells are grown on special growth conditions. LamB is part of the maltose uptake system (the mal-system) in *E. coli* and other *Enterobacteriaceae* (Szmelcman and Hofnung, 1975; Palva, 1978). Mutants lacking this protein are impaired in maltose uptake when the concentration of maltose is below 0.1 mM (Szmelcman and Hofnung, 1975). This suggested a high specificity of LamB for carbohydrates (von Meyenburg and Nikaido, 1977), which has been revealed by swelling experiments using reconstituted liposomes (Luckey and Nikaido, 1980) and by lipid bilayer experiments (Benz et al., 1986, 1987).

The LamB proteins from *E. coli* and *Salmonella typhimurium* have been crystallized, and their three-dimensional structures are known from x-ray crystallography (Schirmer et al., 1995; Meyer et al., 1997). The individual channel within a LamB-trimer is formed by 18 antiparallel β-strands, which form a cylinder with a diameter of ~2.5 nm. The diameter of the channel is reduced by the external loop 3 folding into the channel lumen to form a central constriction with a size of ~0.5 × 0.3 nm (see Fig. 1). Carbohydrate transport through the channel is mediated by van der Waals interaction of the malto-oligosaccharides with six aromatic residues lining up the channel interior (the greasy slide, Y6, Y41, W74, W358, W420, and F227) and by hydrogen bonds between the hydroxy groups of the carbohydrates and amino acid residues within the constriction zone such as R8 (Schirmer et al., 1995; Dutzler et al., 1996; Jordy et al., 1996).

It is known that the characteristics of membrane channels, such as LamB or α-hemolysin, are influenced strongly by amino acids localized within the constriction zone (Walker et al., 1994; Jordy et al., 1996). In maltoporin, the bulky aromatic side chain of tyrosine 118 (Y118), which controls the central part of the constriction (see Fig. 1), is of particular importance for the transport properties. In this study, we investigated the effect of Y118 on ion transport in detail. This amino acid was replaced by a variety of amino acids with different aromatic and nonaromatic amino acids of different size and charge. Charged amino acids within the

Submitted July 24, 2001 and accepted for publication January 24, 2002.

Address reprint requests to Roland Benz, Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany. Tel.: +49-931-8884501; Fax: +49-931-8884509; E-mail: roland.benz@mail.uni-wuerzburg.de.

Dr. Andersen's present address is Department of Pathology, Cambridge University, Tennis Court Road, Cambridge CB2 1QP, UK.

0006-3495/02/05/2466/10 $2.00

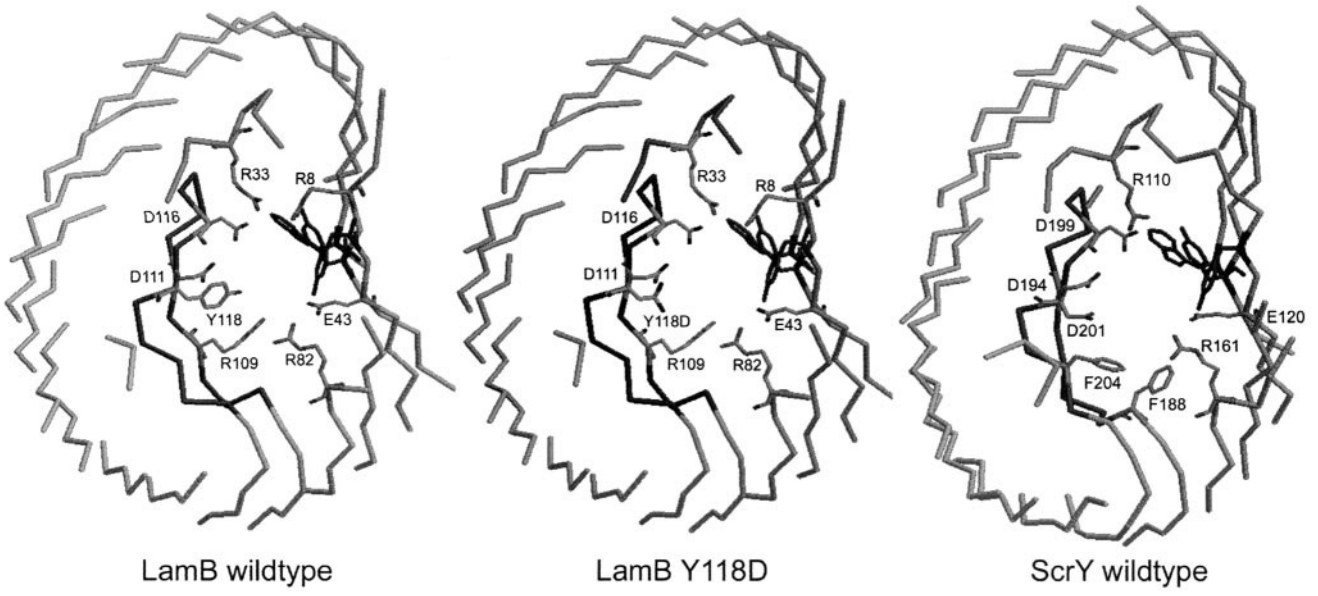

FIGURE 1 Cross sections of the *E. coli* LamB monomer (maltoporin), the LamB Y118D mutant, and the ScrY monomer (sucroseporin). The panel shows loop 3 (*dark gray*) and the amino acid residues (denoted with their numbers from the mature N-terminal end) that are relevant for passage of carbohydrates and ions through the central constriction. The strands of the β-barrel cylinders of the porins are given in light gray. The coordinates of maltoporin were taken from the crystallographic data of Schirmer et al. (1995) and those of sucroseporin from Forst et al. (1998). The cross section of the LamB Y118D mutant monomer was created by using the cross section of LamB and the replacement of the amino acid Y118 by aspartic acid using the program Hyperchem.

central constriction exhibit interesting effects on ion transport. The results suggest indeed that Y118 plays an important role in the permeability of LamB of *E. coli* for ions.

MATERIALS AND METHODS

Materials

Diphytanoyl phosphatidylcholine (DiphPC) was obtained from Avanti Polar Lipids (Alabaster, AL). All salts were analytical grade (Merck, Darmstadt, Germany). Ultrapure water was obtained by passing deionized water through a Milli-Q equipment (Millipore, Bedford, MA). The QuikChange site-directed mutagenesis kit and the XL1-Blue supercompetent cells were bought from Stratagene (Amsterdam, The Netherlands).

Plasmids and DNA manipulations

Plasmids for LamB mutants Y118C (pAM2420), Y118N (pAM2421), Y118F (pAM2422), Y118H (pAM2423), Y118S (pAM2424), and Y118I (pAM2425) were kind gifts of Dr Tom Ferenci, University of Sidney, Australia (Ferenci and Lee, 1982; Clune et al., 1984). The mutants Y118D, Y118R, Y118A, and Y118W were constructed according to standard genetic manipulations using the QuikChange site-directed mutagenesis kit (Stratagene).

Plasmid pAM117-encoding wild-type LamB (Heine et al., 1988) was used as a template for in vitro site-directed mutagenesis. For each mutant, two synthetic oligonucleotide primers were designed (purchased from Carl Roth, Karlsruhe, Germany) each complementary to opposite strands of the plasmid and containing the desired mutation. The oligonucleotide primers were extended during temperature cycling by using *Pfu-turbo* DNA polymerase. Incorporation of the primers generates a mutated plasmid containing staggered nicks. After temperature cycling, the product was treated with *DpnI*. The *DpnI* endonuclease (target sequence: 5′-Gm^{6}ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template. This led to the selection of the mutation-containing synthesized DNA. The nicked vector DNA was then transformed into XL1-Blue supercompetent cells, where the nicks in the mutated plasmid were repaired. All mutated plasmids were controlled by DNA sequencing.

Growth of bacteria and purification of LamB mutants

The LamB mutant Y118F was purified as has previously been described (Jordy et al., 1996). The plasmids that contained the genes of the other LamB mutants were transformed via electroporation into competent cells of the strain KS26 (which lacks most of the outer membrane porins: LamB$^-$, OmpF$^-$, OmpC$^-$, and TolC$^-$) (Schülein et al., 1995). The strains harboring the plasmids were grown in DYT medium (1% (w/v) yeast extract, 1.6% (w/v) Trypton, 0.5% (w/v) NaCl) at 37°C. Although the LamB gene in this plasmid has no controllable promoter, the expression of the LamB gene is sufficient and is not lethal for the cells (Heine et al., 1988). To study the functional integrity of the LamB mutants in vivo, we performed growth experiments of the mutant strain in an artificial medium that contained maltopentaose as sole carbon source. The growth of all mutant strains was similar to the wild-type LamB strain and considerably higher than the KS26 strain that grew very slowly on the artificial medium.

The cells containing the LamB mutants were harvested at an optical density of 1.0 and passed three times through a French pressure cell at 900 psi, and unbroken cells were removed by centrifugation. The cell envelopes were pelleted in an ultracentrifuge (Omega XL90, Beckman Instruments GmbH, München, Germany) at 48,000 rpm for 60 min. Further isolation procedure has been described in detail elsewhere (Jordy et al., 1996). Briefly, the LamB mutants were isolated by extraction of cell envelopes with sodium dodecyl sulfate (SDS) at 30°C and release of LamB mutants

from the protein-peptidoglycan complex by treatment with 0.4 M NaCl solution. The supernatant of a subsequent centrifugation step was applied to a starch column (starch coupled to Sepharose 6B as described by Ferenci and Lee (1982). The column was washed first with a buffer containing 0.1 M $NaHCO_3$, 1% Triton X-100, pH 8.6 (buffer 1) and then with the same buffer supplemented with 1% SDS (buffer 2) to remove unspecifically bound proteins. The column was eluted with buffer 2 containing, in addition, 20% maltose to remove the bound mutant protein from the column. Some of the mutants did not bind sufficiently tight to the starch column to allow the use of the whole elution protocol. In these cases, the column was first washed with buffer 2 and then eluted with buffer 2 supplemented with 20% maltose. Some of the fractions contained pure mutant LamB proteins in their trimeric form as judged by SDS-PAGE using a solubilization temperature of 30°C.

Lipid bilayer experiments

Single channel conductance measurements

Black lipid bilayer membranes were formed as described previously (Benz et al., 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of ~0.3 mm^2. Membranes were formed painting a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane across the holes. The aqueous salt solutions were used unbuffered and had a pH of 6. The LamB mutants were exclusively added in the conductance experiments to only one side of the membrane from concentrated stock solutions. The side to which the mutants were added had no effect on the channel properties irrespective of the sign of the voltage. The temperature was kept at 20°C throughout. The membrane current was measured with a pair of Ag/AgCl electrodes switched in series with a voltage source and a current amplifier (a current-to-voltage converter home-made using a Burr Brown operational amplifier). The amplified signal was monitored with an oscilloscope and recorded with a strip chart recorder. For all conditions used here, ~100 conductance steps were recorded and averaged to give the single-channel conductance value. Its standard deviation was generally below 10% of its mean value.

Selectivity measurements

For the zero-current membrane potentials, the membranes were formed in a 100 mM KCl solution. Protein was added to both sides of the membrane, and the increase of the membrane conductance due to insertion of pores was observed with the electrometer. When a conductance of at least 0.5 nS was reached, corresponding to, at minimum, 100 inserted channels, the instrumentation was switched to the measurement of the zero-current potential, and a KCl gradient was established by adding 3 M KCl solution to one side of the membrane. The zero-current membrane voltage reached its final value after 2–5 min and was analyzed using the Goldman–Hodgkin–Katz equation (Benz et al., 1979).

RESULTS

Replacement of Y118 by neutral amino acids

In a first set of experiments, we investigated the effect of the replacement of Y118 by neutral amino acids on the single-channel conductance of the corresponding mutants: Y118A, Y118I, Y118S, Y118N, Y118W, and Y118C. The data were compared to wild-type LamB (Benz et al., 1986) and Y118F, which had been studied previously (Jordy et al., 1996). All mutants formed well-defined channels in lipid bilayer membranes, indicating no gross pertubation of the channel structure induced by the mutations. Examples for single-channel recordings are shown in Fig. 2 for the mutants Y118N, Y118I, Y118A, and Y118W. The conductance of all insertion events was analyzed in histograms (see Fig. 3) showing a homogeneous size for all LamB mutants. Table 1 shows a summary of the single-channel conductance of the seven mutant channels in different KCl concentrations. The data indicate that the single-channel conductance was, in all cases, an approximately linear function of the bulk aqueous KCl concentration.

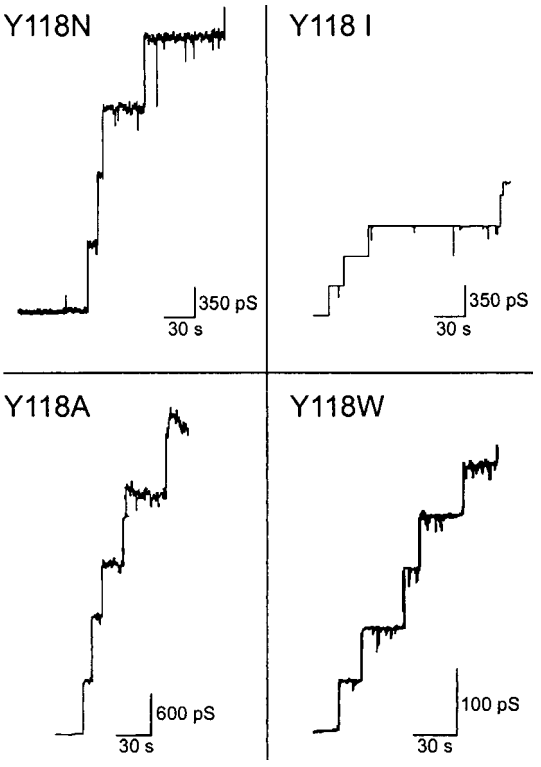

FIGURE 2 Single-channel recordings of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of four different maltoporin mutants Y118N, Y118I, Y118A, and Y118W. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml LamB mutants. The applied membrane potential was 20 mV. $T = 20$°C. Note that current noise of the single-channel recording of wild-type LamB is similar to that of the mutants, indicating that the mutation did not induce a major change of the channel structure.

The highest effect of the mutation was observed for the replacement of Y118 by alanine and cysteine. In these cases the single-channel conductance increased about a factor of six. Somewhat smaller effects were obtained for the mutation Y118N and Y118S, where the enlargement was only about a factor of five. The single-channel conductance increased only two-fold for the isoleucine mutant Y118I. This much smaller effect on ion conductance may be caused in part by the bulky isoleucine side chain and possibly also by the hydrophobicity of isoleucine as compared to that of the other amino acids. The replacement of Y118 by phenylal-

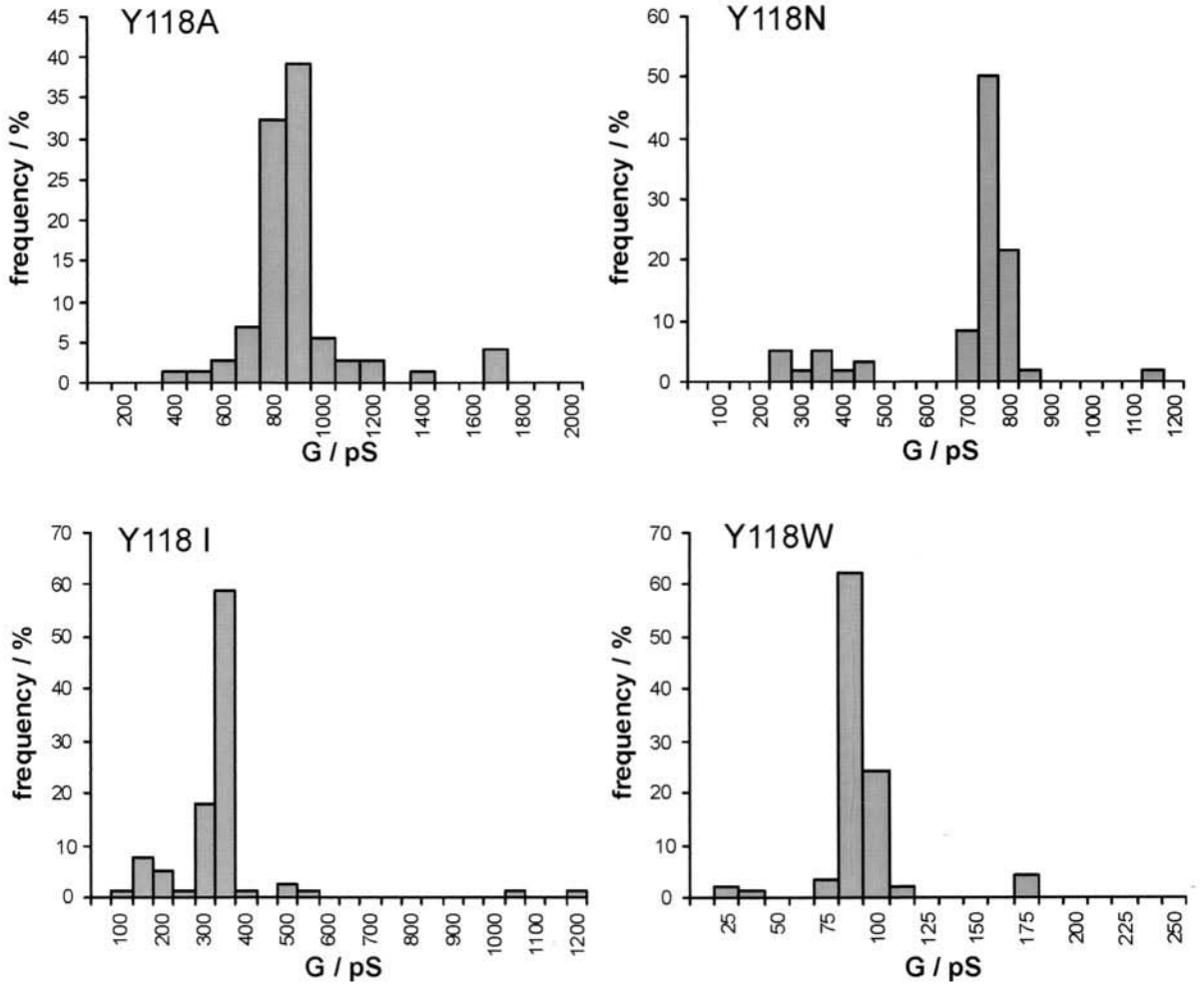

FIGURE 3 Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/*n*-decane in the presence of 10 ng/ml of LamB mutants Y118A, Y118N, Y118I, and Y118W. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV. $T = 20°C$. The average single-channel conductance was 850 pS for 74 single-channel events (Y118A), 750 pS for 72 events (Y118N), 350 pS for 78 events (Y118I), and 90 pS for 80 events (Y118W).

anine and tryptophane had the smallest influence on ion transport through LamB. In these cases, the single-channel conductance decreased a little as compared to wild type. This means that the bulky side chains of these amino acids similarly affect ion transport as in wild type (see Fig. 1). Selectivity measurements with Y118A suggested that the ion selectivity was not changed by substituting Y118 by neutral amino acids. With a KCl gradient across the membrane, the potential of the more diluted side of the membrane became positive, indicating that the permeability ratio

TABLE 1 Single-channel conductance of the Y118 mutants, where tyrosine 118 was replaced by seven neutral amino acids, in KCl-solutions of different concentration, *c***

Mutant	Wild type	Y118A	Y118I	Y118S	Y118C	Y118N	Y118F	Y118W
(c/M)					(G/pS)			
3	590	2500						
1	155	850	350	710	850	750	105	90
0.3	49	300						
0.1	17	150	40	80	95	80	12	10

*The membranes were formed of diphytanoyl phosphatidylcholine dissolved in *n*-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance was calculated from at least 100 single events. *c*, The concentration of aqueous salt solution. The single-channel conductance of wild-type LamB (Benz et al., 1986) and the Y118F mutant (Jordy et al., 1996) are given for comparison.

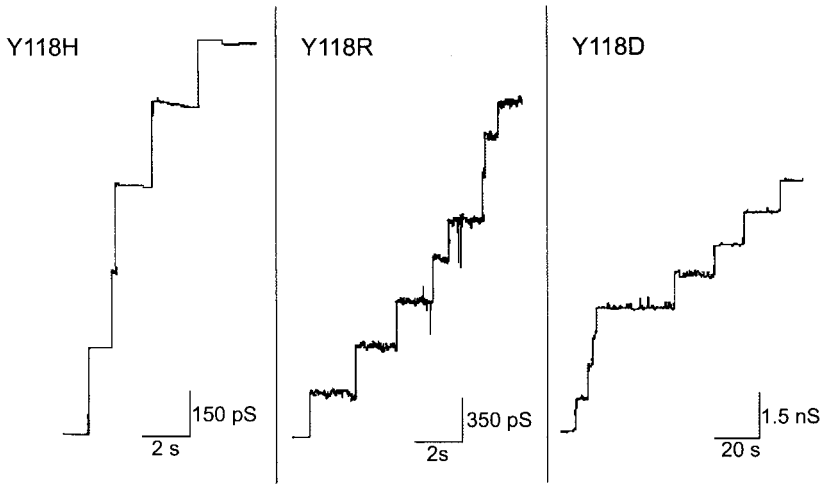

FIGURE 4 Single-channel recordings of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of the maltoporin mutants Y118H, Y118R, and Y118D. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml LamB mutants. The applied membrane potential was 20 mV. $T = 20°C$. Note that current noise of the single-channel recordings of the mutants is small, indicating that the mutation did not induce a major change of the channel structure.

P_K/P_{Cl} was higher than unity. However, it appeared to be reduced as compared to wild type (Y118, $P_K/P_{Cl} = 5.5$; Y118A, $P_K/P_{Cl} = 4.4$).

Effect of charged amino acids on ion transport

The central tyrosine residue Y118 was also substituted by the charged amino acids aspartate (Y118D), arginine (Y118R), and histidine (Y118H). Measurements of the single-channel conductance showed well-defined channels, which do not increase the current noise, indicating a proper folding of the mutant protein. Figure 4 shows single-channel recordings in 1 M KCl of all three mutants. The highest single-channel conductance was observed for Y118D (1050 pS in 1 M KCl), followed by Y118R and Y118H (350 and 250 pS, respectively). Again, the single events were fairly homogeneous as indicated by the histograms of Fig. 5. Single-channel experiments with the Y118H mutant at different pH (5, 6, and 9) suggested that the size of the lateral chain had a much stronger effect on the single-channel conductance than on the charge. Only at pH 5 did we observe some increase of conductance, probably caused by titration of negatively charged amino acids and increasing positive charge of the histidine. The change of pH between 5 and 9 had only a minor influence on the conductance of LamB wild type. We also performed single-channel experiments with salts other than KCl to obtain some information on the selectivity of the mutant channels. The results are summarized in Table 2. For Y118D, the replacement of chloride by the less mobile acetate had only a little, if any, influence on the conductance. The influence of the cations on the single-channel conductance in different 1-M salt solution was more substantial, and the single channel conductance decreased to 400 pS with Li^+ (see Table 2), which suggests that the Y118D channels are highly cation selective. The opposite behavior is found for the mutant Y118R. Exchanging the cation in the salt solution had only little effect on the single-channel conductance, whereas exchanging chloride by the less mobile acetate, the conductivity drops fourfold.

Table 2 also shows the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase for Y118D and Y118R. Interestingly, for both mutants, the relationship between conductance and KCl-concentration was not linear as observed for LamB wild type and the neutral substitutions of Y118. Instead, the slope of the conductance versus concentration curves on a double-logarithmic scale was ~0.5 for Y118D, which indicated the influence of point net charges localized in or near the channels (see also Discussion and Fig. 6). This effect of point charges on the single-channel conductance is explained in more detail in the discussion section.

Selectivity of the Y118D and Y118R channels

Zero-current membrane-potential measurements allow the calculation of the overall permeability ratio P_{cat} (cation) divided by P_{an} (anion) in multichannel experiments. Diphytanoyl phosphatidylcholine/*n*-decane membranes were formed in 100 mM salt solution, and concentrated LamB mutants Y118D and Y118R were added to the aqueous phase when the membranes were in the black state. After increase of membrane conductance, salt gradients were established by addition of small amounts of concentrated salt solution to one side of the membrane, and the zero-current membrane potentials were measured. In both cases, the more diluted side of the membrane became positive, which indicated preferential movement of cations through the channels. The zero-current membrane potentials for KCl were between 10 mV (Y118R) and 17 mV (Y118D) for a three-fold gradient. Analysis of the zero-current membrane potentials using the Goldman–Hodgkin–Katz equation suggested that anions could also have a certain permeability through the Y118D mutant channels because the ratio of the

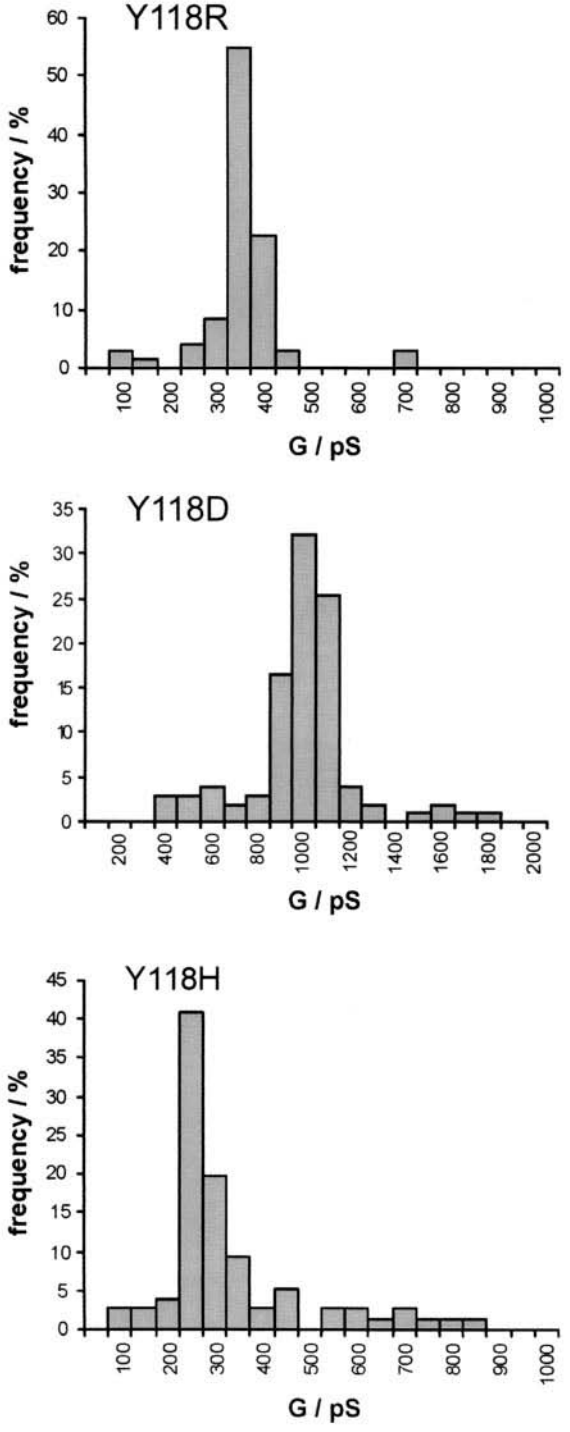

FIGURE 5 Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/*n*-decane in the presence of 10 ng/ml of LamB mutants Y118R, Y118D, and Y118H. The aqueous phase contained 1M KCl. The applied membrane potential was 20 mV. $T = 20°C$. The average single-channel conductance was 1050 pS for 103 single-channel events (Y118D), 350 pS for 71 events (Y118R), and 250 pS for 78 events (Y118H).

permeabilities was 4.2. This result is in some contradiction to the single-channel data, which suggest that anions have a much smaller permeability than cations through the mutant channel, if they have any (see Discussion). Similarly, the ratio P_{cat}/P_{an} was found for the Y118R channel to be 1.9, which does also not make sense when the single-channel conductance data are considered, and which suggest anion selectivity for Y118R. Charge effects are presumably also in this case responsible for the obvious contradiction (see Discussion).

DISCUSSION

The replacement of Y118 increases ion flux through LamB

All mutations of Y118 resulted in increased ion flux through LamB, except for the mutants Y118F (which was previously studied (Jordy et al., 1996)) and Y118W, in which decreased single-channel conductance was observed. This result suggested that the primary effect of the tyrosine within the LamB channel is the restriction of its size. This is demonstrated in Fig. 1, which shows the constriction site, when Y118 is replaced by aspartic acid. The diameter increases considerably, resulting, in turn, in an increased single-channel conductance and probably also in an increased permeability for neutral solutes. This is valid for most of the amino acids introduced in replacement of Y118 with the exception of phenylalanine (Y118F) and tryptophane (Y118W). Highest conductance was observed for Y118D, but some part of the increase was caused by the negative charge, which is obvious, when the single-channel conductance of the asparagine mutant is considered (see also below). It is noteworthy that the sucrose-specific ScrY channel of enteric bacteria (sucroseporin) represents a natural mutant of LamB (Schmid et al., 1991), which has, besides a carbohydrate binding site, also a general diffusion property (Schülein et al., 1991). It has a high single-channel conductance of ~700 pS in 1 M KCl, similar to those of some of the LamB mutants described here. There is an aspartate in position 201 of ScrY (corresponding to Y118 in LamB). This is the reason for the increased size of ScrY wild type as compared to LamB wild type (see Fig. 1).

High conductance increase was also obtained for the alanine mutant despite its high hydrophobicity index, according to Kyte and Doolittle (1982) (the hydrophobicity parameter for the amino acid alanine is +1.8). In contrast, when the much lower single-channel conductance of the isoleucine mutant (350 pS in 1 M KCl) is compared with that of Y118N (750 pS; the hydrophobicity parameters of the two amino acids are +4.5 and −3.5, respectively), which also has a bulky side chain, then, clearly, the hydrophobicity index also plays an important role for ion transport. The difference between alanine and isoleucine has presumably to do with the size of the latter, which means that it intrudes inside the central constriction, interacting with the hydration shell of the cations. This means that it

TABLE 2 Single-channel conductance of the LamB mutant channels Y118D, Y118R, and Y118H in different salt solutions*

Mutant					
Salt	c/M	Wild type	Y118D	Y118R	Y118H
			(G/pS)		
KCl	3	590	3000	1100	
	2		2500	600	
	1	155	1050	350	250
	0.5			150	
	0.3	49	650	75	
	0.1	17	300	17	40
	0.03		150		
	0.01		70		
KCl, pH 5	1	150	—	—	450
KCl, pH 9	1	180	—	—	220
LiCl	1	40	400	340	
Kacetate	1	135	1000	88	
NH_4Cl	1	83	1100	330	

*The membranes were formed of diphytanoyl phosphatidylcholine dissolved in *n*-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance was calculated from at least 100 single events. *c*, The concentration of the aqueous salt solution. The single-channel conductance of wild-type LamB (Benz et al., 1986) is given for comparison.

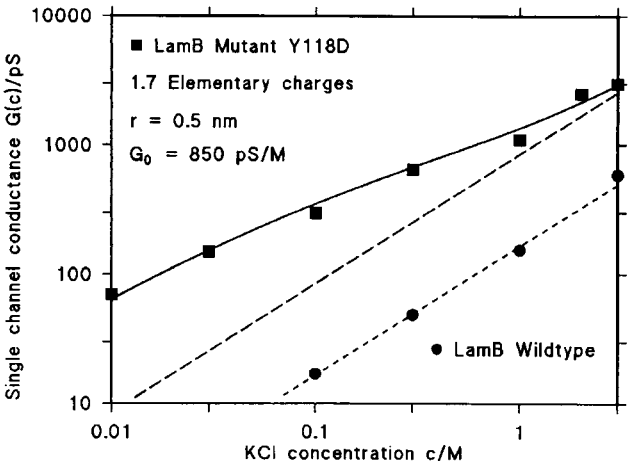

FIGURE 6 Single-channel conductance of Y118D as a function of the KCl concentration in the aqueous phase (*squares*). The solid line represents the fit of the single-channel conductance data with $G(c) = G_0 \cdot c_0^+$ (a combination of Eqs. 1–3 and 5), assuming the presence of negative point charges (1.7 negative charges; $q = -2.72 \times 10^{-19}$ As) within the channel, and assuming a characteristic size (diameter) of 1 nm. *c*, concentration of the KCl-solution in M (molar); *G*, average single-channel conductance in pS (pico Siemens, 10^{-12} S); G_0, single-channel conductance in the absence of point negative charges given in pS/M. The long-dashed line shows the single-channel conductance of the Y118D channel in the absence of point charges and corresponds to a linear function between channel conductance and bulk aqueous concentration (Eq. 5); $G(c) = G_0 \cdot c$). The conductance of wild-type LamB is also a linear function of the bulk aqueous concentration and is given (*short-dashed line*) for comparison (Benz et al., 1987).

may increase the energy barrier for ion movement within the channel, leading to a decreased single-channel conductance. For the other two mutants (Y118S and Y118C) investigated here, the hydrophobicity and the size of the amino acids are more close to one another. Accordingly, they have an approximately similar single-channel conductance under the same conditions.

Charge effect of the Y118D mutant on ion transport

The data shown in Table 2 demonstrate that the single-channel conductance of the Y118D mutant is not a linear function of the bulk aqueous concentration. Instead, a slope of ~0.5–0.6 was observed on a double-logarithmic scale for the conductance versus concentration curve (see Fig. 6). This result indicates that charge effects influence the properties of the LamB mutant channel Y118D in contrast to wild-type LamB, where a linear dependence has been observed (Benz et al., 1987; see also Fig. 6). This means that the charge effects are definitely caused by the aspartate localized in the center of the channel. Its negative charge results in a substantial ionic strength-dependent potential inside the channel, which attracts cations and repels anions. Accordingly, it influences both single-channel conductance and zero-current membrane potential. In particular, the single-channel conductance is larger than expected from the dimensions of the channel. A quantitative description of the effect of point charges on the single-channel conductance may be given by the following considerations. The first one is based on the Debeye–Hückel theory describing the effect of point charges in an aqueous environment. The second treatment was proposed by Nelson and McQuarrie (1975) and first used by Menestrina and Antolini (1981) to discuss the single-channel conductance as a function of the salt concentration in the bulk aqueous phase when the channel contains a discrete charge. In principle, Nelson and McQuarrie (1975) describe the effect of a point charge on the surface of a membrane and did not consider charges attached to a channel. However, this does not represent a serious restriction of its use, and we assume here that the point charge is localized in the mutant Y118D channel.

In case of a negative point charge, q, in an aqueous environment, a potential ϕ is created that is dependent on the distance, r, from the point charge,

$$\Phi = \frac{q \cdot e^{r/l_D}}{4\pi \cdot \varepsilon_0 \cdot \varepsilon \cdot r}. \quad (1)$$

ε_0 (= 8.85×10^{-12} F/m) and ε (= 80) are the absolute dielectric constant of vacuum and the relative constant of water, respectively, and l_D is the so-called Debeye length that controls the decay of the potential (and of the accumulated positively charged ions) in the aqueous phase,

$$l_D^2 = \frac{\varepsilon \cdot \varepsilon_0 \cdot R \cdot T}{2F^2 \cdot c}, \quad (2)$$

where c is the bulk aqueous salt concentration, and R, T, and F ($RT/F = 25.2$ mV at 20°C) have the usual meaning. The potential ϕ created by a negative point charge on the surface of a membrane is twice that of Eq. 1, caused by the generation of an image force on the opposite side of the membrane (Nelson and McQuarrie, 1975). The concentration of the monovalent cations near the point charge increases because of the negative potential. Their concentration is, in both Debeye–Hückel and Nelson–McQuarie cases, dependent on the potential ϕ and given by

$$c_0^+ = c \cdot e^{-\phi \cdot F/R \cdot T}. \quad (3)$$

Similarly, the anion concentration, c_0^-, near the point charge decreases according to

$$c_0^- = c \cdot e^{-\phi \cdot F/R \cdot T}. \quad (4)$$

In the following, we assume that the negative point charge is attached to the channel. In such a case, its conductance is limited by the accumulated positively charged ions and not by their bulk aqueous concentration. A linear dependence of the single-channel conductance on the salt concentration has been found previously for LamB despite its cation selectivity (Benz et al., 1987) and was also found here for the replacement of Y118 by neutral amino acids. In such a case, the single-channel conductance as a function of the ion concentration is given by the linear function,

$$G(c) = G_0 \cdot c, \quad (5)$$

where G_0 is the slope of the conductance–concentration curve. Eq. 3 can be introduced into Eq. 5, and we can try to fit the nonlinear concentration dependence of the single-channel conductance of Y118D given in Table 2. A best fit was obtained using Eqs. 1, 2, and 5 by assuming that 1.7 negatively charged groups ($q = -2.72 \times 10^{-19}$ As) are located at the pore mouth and that its characteristic size (diameter) is ~1 nm. The results of this fit are shown in Fig. 6 for the function $G(c) = G_0 \cdot c_0^+$. The solid line represents the fit of the single-channel conductance versus concentration by using the Debeye–Hückel theory and the parameters mentioned above, together with a specific single-channel conductance, $G_0 = 850$ pS/M. The data also demonstrate that the influence of the surface charges is rather small at high ionic strength, c, i.e., small l_D (see Eq. 2). The broken line corresponds to the single-channel conductance of the Y118D channel without point net charges, i.e., it shows Eq. 5 also assuming $G_0 = 850$ pS/M, and c is given by the bulk aqueous concentration. This leads to a linear relationship between the cation concentration in the aqueous phase and single-channel conductance.

It has to be noted that the number of negative charges involved in the accumulation of cations at the channel mouth has to be considered as tentative. This is because the dielectric constant of their environment is not known. When the dielectric constant is low, then the Nelson and McQuarrie formalism has to be applied, and the q in Eq. 1 has to be replaced by $2 \cdot q$. In the case of a high dielectric constant (that is, when the charge is localized in an aqueous environment), the above-used theory is valid (Debeye–Hückel theory). Because the channel interior has a dielectric constant that is probably more close to that of water, the real charge is probably more close to q than to $2q$, which means that the basic principles of Debeye–Hückel or Nelson and McQuarrie (1975) can both be used with sufficient accuracy. In contrast, clearly the exact number of charges is tentative, whereas the estimated channel radius is probably more precise, as has also been demonstrated elsewhere (Trias and Benz, 1993).

The negative potential created by the negative point charge in the center of the channel has important implications on its ion-transport properties. At a concentration, c, of 100 mM KCl or NaCl, the potential is approximately −100 mV in the channel interior calculated from Eqs. 1 and 2 and assuming $q = -2.72 \times 10^{-19}$ As and $r = 0.5$ nm. This means that the concentration of monovalent cations is increased there to 2 M (bulk concentration 100 mM) calculated from Eq. 3, whereas the concentration of monovalent anions is decreased to ~2 mM (bulk concentration 100 mM; calculated according to Eq. 4). This means that, under these conditions, the LamB mutant channel conducts cations (according to $G(c) = G_0 \cdot c_0^+$) approximately 1000-fold better than anions (according to $G(c) = G_0 \cdot c_0^-$) of the same aqueous mobility without being really selective for cations caused by a binding site for them. This effect of charges near the channel mouth has been predicted by Dani (1986) and Jordan (1987) for ion channels in general and has been experimentally verified in a Ca^{2+}-activated K^+-channel by chemical modification of surface carboxylate groups (MacKinnon et al., 1989). Strategically placed charges near the channel can lower energy barriers inside the channel and accumulate ions to guide them through the channel. Similarly, they may lead to a heavy-metal ion-promoted block of the channels (Walker et al., 1994). It is noteworthy that we observed similar phenomena for the channels formed in lipid-bilayer membranes by some of the so-called RTX-toxins (*R*epeats in *T*o*X*in) produced by certain enteric bacteria (Benz et al., 1994; Maier et al., 1996) and for porins of the cell wall of gram-positive bacteria belonging to the mycolata (Trias and Benz, 1993; Riess et al., 1998).

pH-Dependence of the single-channel conductance of the Y118H mutant

pH had a certain effect on the single-channel conductance of the Y118H mutant. It increased from 220 pS at pH 9 to 250 pS at pH 6 and 450 pS at pH 5. This result suggests that the bulky histidine side chain restricts ion transport at high pH when it is uncharged. At low pH, it seems possible that its protonation leads to an increase of anion transport. However, when it is assumed that the single-channel conduc-

tance of the mutant is 220 pS when the histidine is uncharged (at pH 9) and that the increase of single-channel conductance at pH 6 (250 pS) and pH 5 (450 pS) is exclusively created by the charge of the histidine, we end up with a pK of ~4.5 and an additional pH-dependent single-channel conductance of ~800 pS (besides that of 220 pS) using the Hendersen–Hasselbalch equation. This appears to be not very realistic, which means that we cannot assume that pH influences only the charge of histidine and that there is no cross-talk between the different charged amino acids within the LamB-mutant channel, i.e., the increase of single-channel conductance from pH 6 to pH 5 is probably caused by protonation of histidine and of negatively charged amino acid(s).

Effect of Y118D and Y118R on ion selectivity of LamB

The measurements of ion selectivity lead to some contradictory results concerning the ion selectivity. In principle, we would expect that the Y118D channel is exclusively cation selective because the wild-type channel is already cation selective, caused by an excess of negatively charged groups, although the single-channel conductance is a linear function of the bulk aqueous concentration (Benz et al., 1986). The addition of a further negative charge in a strategic position within the channel should increase the selectivity to 1000-fold according to the estimation shown above. However, when we consider the results of the zero current membrane potential measurements, the selectivity seems to decrease as compared to wild type. The negative charge inside the channel obviously leads to a change of the bulk aqueous cation concentrations on both sides of the channel near the constriction site. As a consequence, only part of the full bulk aqueous gradient drops across the channel caused by the action of D118 on cation concentration near the constriction. This means that the zero-current membrane potential decreases, as we observed. The Y118R channel appears to be anion selective when the single-channel data is considered. Again the zero-current membrane potential measurements suggest the contrary for the concentration range between 0.1 and 0.5 M. This represents an apparent contradiction. It is presumably caused by the excess of negatively charged groups within the LamB wild-type channel, which is highly cation selective (Benz et al., 1987). The positively charged arginine in its center controls the central constriction of the channel and sorts anions according to their aqueous mobility, but it does not seem to be able to change the overall cation selectivity of LamB. The low single-channel conductance of the Y118R channel in 1 M potassium acetate (88 pS) could be because acetate partially blocks the channel. This seems possible because three positively charged Arg residues Y118R, R109, and R82 are located near each other and could form a putative binding site for anions.

The authors would like to thank Tom Ferenci for providing the mutants Y118C, Y118N, Y118F, Y118H, Y118S, and Y118I and Eric Schmid for his help in the early stages of this work.

This work was supported by the Deutsche Forschungsgemeinschaft (Be 865/10), and the Fonds der Chemischen Industrie.

REFERENCES

Benz, R. 1994. Solute uptake through bacterial outer membranes. *In* Bacterial Cell Wall. R. Hackenbek and J.-M. Ghuysen, editors. Elsevier, Amsterdam. 397–423.

Benz, R., K. Janko, W. Boos, and P. Läuger. 1978. Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli. Biochim. Biophys. Acta.* 511:305–319.

Benz, R., K. Janko, and P. Läuger. 1979. Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli. Biochim. Biophys. Acta.* 551:238–247.

Benz, R., E. Maier, D. Ladant, A. Ullmann, and P. Sebo. 1994. Adenylate cyclase toxin (CyaA) of *Bordetella pertussis*. Evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli. J. Biol. Chem.* 269:27231–27239.

Benz, R., A. Schmid, T. Nakae, and G. H. Vos-Scheperkeuter. 1986. Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *J. Bacteriol.* 165:978–986.

Benz, R., A. Schmid, and G. H. Vos-Scheperkeuter. 1987. Mechanism of sugar transport through the sugar-specific LamB channel of *Escherichia coli* outer membrane. *J. Membrane Biol.* 100:12–29.

Clune, A., K. S. Lee, and T. Ferenci. 1984. Affinity engineering of maltoporin: variants with enhanced affinity for particular ligands. *Biochem. Biophys. Res. Commun.* 121:34–40.

Dani, J. A. 1986. Ion-channel entrances influence permeation. *Biophys. J.* 49:607–618.

Dutzler, R., Y. F. Wang, P. J. Rizkallah, J. P. Rosenbusch, and T. Schirmer. 1996. Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. *Structure.* 4:127–134.

Ferenci, T., and K. S. Lee. 1982. Directed evolution of the lambda receptor of *Escherichia coli* through affinity chromatographic selection. *J. Mol. Biol.* 160:431–444.

Forst, D., W. Welte, T. Wacker, and K. Diederichs. 1998. Structure of the sucrose-specific porin ScrY from *Salmonella typhimurium* and its complex with sucrose. *Nat. Struct. Biol.* 5:37–46.

Heine, H. G., G. Francis, K. S. Lee, and T. Ferenci. 1988. Genetic analysis of sequences in maltoporin that contribute binding domains and pore structure. *J. Bacteriol.* 170:1730–1738.

Jordan, P. C. 1987. How pore mouth charge distributions alter the permeability of transmembrane ionic channels. *Biophys. J.* 51:297–311.

Jordy, M., C. Andersen, K. Schülein, T. Ferenci, and R. Benz. 1996. Rate constants of sugar transport through two LamB mutants of *Escherichia coli*: comparison to wild-type maltoporin and to LamB of *Salmonella typhimurium. J. Mol. Biol.* 259:666–678.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Luckey, M., and H. Nikaido. 1980. Diffusion of solutes through channels produced by phage lambda receptor protein of *Escherichia coli*: inhibition of glucose transport by higher oligosaccharides of maltose series. *Biochem. Biophys. Res. Commun.* 93:166–171.

MacKinnon, R., R. Latorre, and C. Miller. 1989. Role of surface electrostatics in the operation of a high-conductance Ca^{++}-activated K^{+} channel. *Biochemistry.* 28:8092–8099.

Maier, E., N. Reinhard, R. Benz, and J. Frey. 1996. Formation of small ion-permeable channels by the RTX-toxins ApxI, ApxII and ApxIII of *Actinobacillus pleuropneumoniae. Infect. Immun.* 64:4415–4423.

Menestrina, R., and R. Antolini. 1981. Ion transport through hemocyanin channels in oxidized cholesterol membranes. *Biochim. Biophys. Acta.* 643:616–625.

Meyer, J. E. W., M. Hofnung, and G. E. Schulz. 1997. Structure of maltoporin from *Salmonella typhimurium* ligated with a nitrophenylmaltotrioside. *J. Mol. Biol.* 266:761–775.

Nelson, A. P., and D. A. McQuarrie. 1975. The effect of discrete charges on the electrical properties of a membrane. *J. Theor. Biol.* 55:13–27.

Nikaido, H. 1992. Porins and specific channels of bacterial outer membranes. *Mol. Microbiol.* 6:435–442.

Palva, E. T. 1978. Major outer membrane protein in *Salmonella typhimurium* induced by maltose. *J. Bacteriol.* 136:286–294.

Riess, F. G., T. Lichtinger, R. Cseh, A. F. Yassin, K. P. Schaal, and R. Benz. 1998. The cell wall porin of *Nocardia farcinica*: biochemical identification of the channel-forming protein and biophysical characterization of the channel properties. *Mol. Microbiol.* 29:139–150.

Schirmer, T., T. A. Keller, Y.-F. Wang, and J. P. Rosenbusch. 1995. Structural basis for sugar translocation maltoporin channels at 3.1 Å resolution. *Science.* 267:512–514.

Schmid, K., R. Ebner, K. Jahreis, J. W. Lengeler, and F. Titgemeyer. 1991. A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. *Mol. Microbiol.* 5:941–950.

Schülein, K., C. Andersen, and R. Benz. 1995. The deletion of 70 N-terminal amino acids of the sugar-specific sucrose-porin ScrY causes its functional similarity to LamB in vivo and in vitro. *Mol. Microbiol.* 17:757–767.

Schülein, K., K. Schmid, and R. Benz. 1991. The sugar specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substrate-specific porins. *Mol. Microbiol.* 5:2233–2241.

Szmelcman, S., and M. Hofnung. 1975. Maltose transport in *Escherichia coli* K-12: involvement of the bacteriophage lambda receptor. *J. Bacteriol.* 124:112–118.

Trias, J., and R. Benz. 1993. Characterization of the channel formed by the mycobacterial porin in lipid bilayer membranes. Demonstration of voltage gating and of negative point charges at the channel mouth. *J. Biol. Chem.* 268:6234–6240.

von Meyenburg, K., and H. Nikaido. 1977. Outer membrane of gram-negative bacteria. XVII. Specificity of transport process catalyzed by the receptor protein in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 78:1100–1107.

Walker, B., J. Kasianowicz, M. Krishnasastry, and H. Bayley. 1994. A pore-forming protein with a metal-actuated switch. *Protein Eng.* 7:655–662.