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Site-directed Mutagenesis within the Central Constriction Site of ScrY (Sucroseporin): Effect on Ion Transport and Comparison of Maltooligosaccharide Binding to LamB of *Escherichia coli*

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Abstract. The 3-D structures of the maltooligosaccharide-specific LamB-channel of *Escherichia coli* (also called maltoporin) and sucrose-specific ScrY (sucroseporin) are known from X-ray crystallography. The central constriction of the channels formed by the external loop 3 is controlled by a number of different amino acids. The most prominent one of these, N192, D201 and F204, were replaced by site-directed mutagenesis into those of LamB, which, according to the 3-D model of both channels are localized at similar places. The ScrY single mutants ScrYN192R, ScrYD201Y and ScrYF204D and the ScrY triple mutant ScrY3113 (N192R + D201Y + F204D) were created together with the triple mutant ScrY3213, which lacks also amino acids 1 to 61 from the N-terminal end. The mutant proteins were purified to homogeneity and were reconstituted into lipid bilayer membranes. In these experiments, the single-channel conductance of the mutants in different salt solutions and the stability constants for binding of different maltooligosaccharides to the mutant channels was measured using titration experiments with carbohydrates. The carbohydrate-induced block of the channel function could also be used for the study of current noise through the different mutant ScrY-channels. The analysis of the power density spectra allowed the evaluation of the on- and off-rate constants (k_1 and k_{-1}) of carbohydrate-binding to the binding site inside the channels. The results suggest that both on- and off-rate constants were affected by the mutations. Most of them showed a substantial effect on carbohydrate binding kinetics. Nevertheless, single-channel conductance and carbohydrate binding of ScrY3113 mutant were still different from that of LamB, suggesting that not only the amino acids of the central constriction but also the general architecture of both channels have a substantial influence on channel properties.

Key words: Noise analysis — ScrY — Lipid bilayer membrane — Sucrose transport — Carbohydrate binding — LamB

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Introduction

The cell envelope of gram-negative bacteria consists of different layers. The inner or cytoplasmatic membrane contains the respiration chain, proteins for the transport of nutrients and proteins involved in the synthesis of phospholipids, peptidoglycan and lipopolysaccharides (Beveridge, 1981; Nikaido & Vaara, 1985). The periplasmic space between the membranes is an aqueous compartment isoosmolar to the cytoplasm (Benz, 1988). It contains the peptidoglycan and a large number of different proteins. The outer membrane is composed of protein, lipid and lipopolysaccharide (Nikaido & Vaara, 1985). It contains only a few major proteins. At least one of the constitutive outer membrane proteins, called porin, forms a general diffusion pore with a defined exclusion limit for hydrophilic solutes within the outer membrane (Nikaido & Vaara, 1985; Hancock, 1987; Benz & Bauer, 1988). In addition to the constitutive porins, the outer membrane may contain porins that are induced under special growth conditions (Szmelcman et al., 1976; Tommassen & Lugtenberg, 1980; Hancock, Poole & Benz, 1982; Bauer et al., 1985). They often form solute-specific channels and

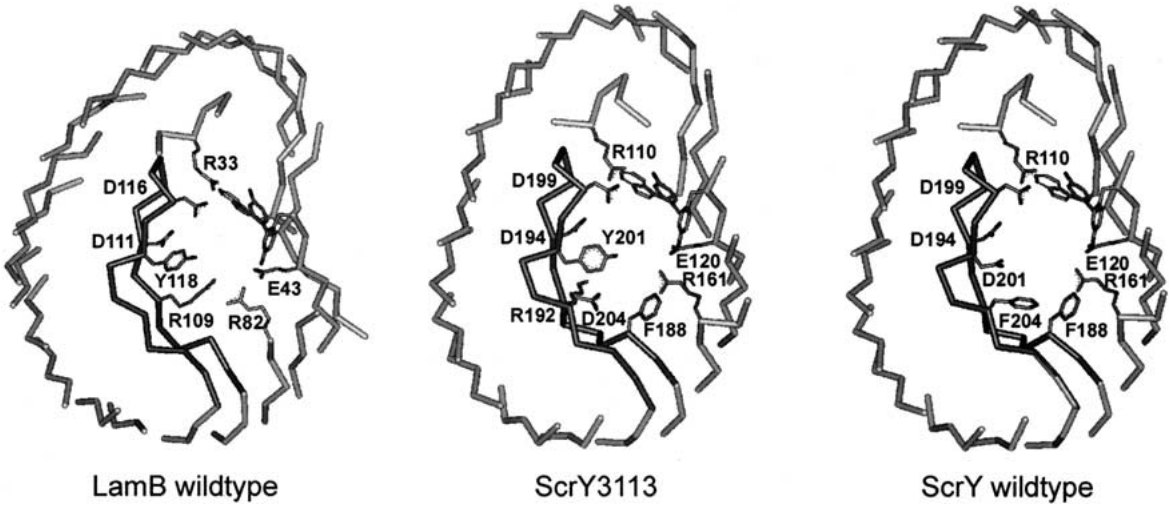

Fig. 1. Cross sections of the *E. coli* LamB monomer (maltoporin), the ScrY monomer (sucroseporin) and the ScrY3113 mutant. The panel shows loop 3 (dark gray) and the amino-acid residues (denoted with their numbers from the mature *N*-terminal end) that are relevant for passage of carbohydrates and ions through the central constriction. The strands of the β-barrel cylinder of the porins are given in light gray. The coordinates of maltoporin were taken (with permission) from the crystallographic data of Schirmer et al. (1995) and that of sucroseporin from Forst et al., (1998). The cross section of the ScrY3113 mutant monomer was created by using the cross section of ScrY and the replacement of the amino acids N192R, D201Y and F204D by using the program Hyperchem®.

contain binding sites for neutral substrates such as carbohydrates (Ferenci et al., 1980; Benz et al., 1986), nucleosides (Maier et al., 1988) and charged solutes such as phosphate (Hancock et al., 1982; Benz & Hancock, 1987). Many of these specific porins are part of uptake and degradation systems, such as the mal system of *Escherichia coli* (Schwartz, 1987) or the single-copy plasmid pUR400 of enteric bacteria (Schmid, Schupfner & Schmitt, 1982). These systems confer to the bacteria the possibility of growth on maltose and maltooligosaccharides and on sucrose as carbon source (Szmelcman et al., 1976; Schmid et al., 1988).

Among the proteins encoded on the maltose operon or on the pUR400 plasmid there are also specific outer-membrane channels that are specific for carbohydrates. LamB of *E. coli* and other enteric bacteria is essential for the uptake of maltooligosaccharides across the outer membrane (Szmelcman et al., 1976), but it has a much lower efficiency for the uptake of sucrose (Schmid et al., 1988). ScrY of pUR400 represents a sucrose-specific channel, but it is also able to replace LamB with respect to maltooligosaccharide uptake. The primary structure of both carbohydrate-specific porins is known from cloning and sequencing and shares high homology at the amino-acid level (Clement & Hofnung, 1981; Schmid et al., 1991). LamB and ScrY have furthermore been crystallized and their 3-D structure is known from X-ray analysis (Schirmer et al., 1995; Forst et al., 1998). The 3-D structure of both carbohydrate-specific porins is highly homologous. In particular, the so-called 'greasy slide', a number of aromatic amino acids (Y6, Y41, W74, F229, W358, and W420 in the sequence of LamB or Y78, Y118, W151, F435 and W482 in that of ScrY) that may interact with the carbohydrates, is present in LamB and ScrY (Dutzler et al., 1996; Forst et al., 1998). However, the central constriction of both channels exhibits some remarkable structural differences (*see* Fig. 1) that are presumably responsible for their different features concerning ion and carbohydrate transport (Schmid et al., 1988; Schülein, Schmid & Benz, 1991). In particular, the cross section of ScrY appears to be wider, although both channels bind carbohydrates, which results also in a much higher single-channel conductance of this channel (Benz et al., 1987; Schülein et al., 1991), although both channels bind carbohydrates with high stability constants (Benz et al., 1987; Schülein et al., 1991).

In this study we mutated different amino acids within the central constriction of ScrY by site-directed mutagenesis into amino acids that are localized at the corresponding positions of LamB to investigate if it is possible to mimick in this way ion and carbohydrate transport in LamB. In particular, we created the mutants ScrYN192R, ScrYD201Y and ScrYF204D (*see* Fig. 1). Furthermore, we investigated also the properties of the triple mutants ScrY3113 (N192R + D201Y + F204D) and ScrY3213 (ScrY Δ1-61 + D201Y + F204D + N192R), which have all been studied in in vivo experiments in a previous study (Ulmke et al., 1999). The mutants were expressed in the porin-deficient *E. coli* KS26 mutant, purified to homogeneity and reconstituted into lipid bilayer membranes. The

mutants were characterized concerning single-channel conductance and carbohydrate binding using titration experiments. Carbohydrate binding kinetics was evaluated using the current-noise approach, which has been applied previously for the study of carbohydrate transport through LamB and ScrY (Nekolla, Andersen & Benz, 1994; Andersen, Jordy & Benz, 1995; Andersen et al., 1998). The results suggest that despite a change of the amino acids in the constriction side ScrY represents a wider channel than LamB and that the amino acids within the constriction side have a major influence on the kinetics of carbohydrate transport.

Materials and Methods

Construction of the ScrY Mutants

ScrY mutants were obtained by site-specific mutagenesis of the wild type allele *scrY* cloned in pPS112 (Schmid et al., 1991). In that plasmid, transcription of *scrY* is under the control of the two promoters *scrYp* and P_{tac}, which are regulated by the Scr repressor (*scrR*) and the Lac repressor ($LacI^Q$) respectively. Additionally, pPSO112 codes for an ampicillin resistance and a spectinomycin resistance allowing efficient selection for plasmid maintenance.

For the amino-acid exchanges D201Y (pPSO117), F204D (pPSO118) and N192R (pPSO119) in loop 3 of the sucrose porin the corresponding subfragment of *scrY* was cloned into pAlter-1, mutagenized using the in vitro mutagenesis system Altered Site™ II (Promega), and transferred back into pPSO112. The triple mutants pUSL3113 and pUSL3213 coding for the three exchanges D201Y, F204D and N192R were constructed in a similar way. The latter differs from pUSL3113 by a partial deletion of the 71-amino-acid long N-terminal extension of ScrY (Schmid et al., 1991; Hardesty et al., 1991; Schülein, Andersen & Benz, 1995). The deletion removes the amino-acid residues 1 to 61 of the mature protein, including the putative coiled-coil structure, which may be involved in carbohydrate binding (Dumas et al., 2000). The construction of this and the other plasmids used in this study was also described earlier in more detail (Schmid et al., 1991; Ulmke et al., 1999).

Isolation and Purification of the ScrY Mutant Proteins

The plasmids pPSO117 (ScrYD201Y), pPSO118 (ScrYF204D), pPSO119 (ScrYN192R), pUSL3113 (ScrY3113; ScrY-D201Y + F204D + N192R) and pUSL3213 (ScrY3213, ScrY Δ1-61 + D201Y + F204D + N192R) were transformed into *E. coli* KS26 TolC$^-$ strain (Schülein et al., 1995). The bacteria containing the different plasmids of an overnight preculture in LB-medium supplemented with tetracycline, spectinomycin and ampicillin were diluted 1:1000 into 200 ml of the same medium. After 3 hr of growth at 37°C, the expression of the mutant proteins was induced by the addition of 10^{-3} M IPTG and 0.2% fructose. After further growth for about 2 hr, the cells were harvested by centrifugation (4,500 × g, 10 min) and washed once with 50 mM Tris-HCl (pH 7.1). The bacteria were resuspended in 5 ml of 50 mM Tris-HCl (pH 7.1) and passed three times through a French pressure cell at 900 psi. Unbroken cells were removed by centrifugation at 4,500 × g for 10 min. The supernatant was centrifuged for 1 hr at 200,000 × g (Beckman Omega 90XL ultracentrifuge, rotor 70.1 Ti). The pellet contained the cell envelopes. It was resuspended in 2 ml of a solution containing 0.1%Triton X-100 and 10 mM Tris-Cl (pH 7.7). After centrifugation (200,000 × g, 30 min, 4°C) this procedure was repeated. The resulting pellet was resuspended in 2 ml of 0.2% lauryldimethylamine (LDAO), 10 mM Tris-HCl (pH 7.7). The supernatant of the subsequent centrifugation (200,000 × g, 30 min, 4°C) contained the ScrYmutant proteins in large amounts and some minor other proteins. The final purification of the ScrY mutants was achieved by Fast Performance Liquid Chromatography (FPLC). 1 ml supernatant of the resuspension with 0.2% LDAO was applied to a Hitrap-Q column. The column was washed with 5 ml of buffer containing 0.4% LDAO, 10 mM Tris-HCl (pH 7.7) and eluted with a linear NaCl gradient between 0 and 1 M NaCl. The mutants eluted from the column at a NaCl concentration of about 0.3 M. After precipitation with ethanol, only one band with an apparent molecular mass of about 52 kDa could be recognized on SDS-PAGE for the mutants ScrYN192R, ScrYD201Y, ScrYF204D and the triple mutant ScrY3113. The triple-mutant gel ScrY3213, which contains also a major deletion in the 71-amino-acid long N-terminal part of the protein, had an apparent molecular mass of 48 kDa (*data not shown*). Wild-type ScrY was isolated and purified as has been described previously (Schülein et al., 1991).

Lipid Bilayer Experiments and Noise Analysis

The ScrY mutants were reconstituted in artificial membranes. The method has been described in detail previously (Benz et al., 1978). The instrumentation consists of a Teflon chamber divided in two compartments. The electrolyte-filled compartments are connected by a small circular hole (diameter 0.5 mm). The membranes were formed by painting a solution of 1% (weight/volume) diphytanoyl phosphatidylcholine in *n*-decane (Avanti Polar Lipids, Alabaster, AL) onto the hole. After the membrane turned to black, a small aliquot of a stock solution of the ScrY mutants was added to the aqueous salt solution in the compartments. The membrane current was measured by a pair of calomel electrodes switched in series with a battery-operated voltage source and a current amplifier (Keithley 427 with a four-pole filter). Feedback resistors between 0.01 and 10 GΩ were used in the experiments. The reconstitution of ScrY and ScrY Δ3-72 in the membranes resulted in an increase of the membrane current. The amplified signal was monitored by a strip-chart recorder and simultaneously analyzed with a digital signal analyzer (Ono Sokki CF 210), which performed fast Fourier transformation of the current noise. The time resolution of the instrumentation was approximately 10 kHz (current amplifier and Ono Sokki signal analyzer), which was limited in the noise experiments by the bandwidth of the current amplifier and a low pass filter (0.3 msec). The spectra were composed of 400 points and they were averaged either 128 or 256 times. They were transferred to an IBM-compatible personal computer (PC) for further analyses. Alternatively, the output of the current amplifier was connected to an AD-converting card of the PC. The digitized data were analyzed with a home-made fast Fourier-transformation program, which yielded identical results compared to the commercial digital signal analyzer.

Analysis of Titration Experiments and Derivation of Rate Constants from the Frequency-Dependence of the Spectral Density

For the analysis of the current noise we used a simple one-site, two-barrier model (Läuger, 1973; Benz et al., 1987; Benz & Hancock, 1987) with a central binding site inside the channel. The binding of the carbohydrates (aqueous concentration *c*) to the central binding

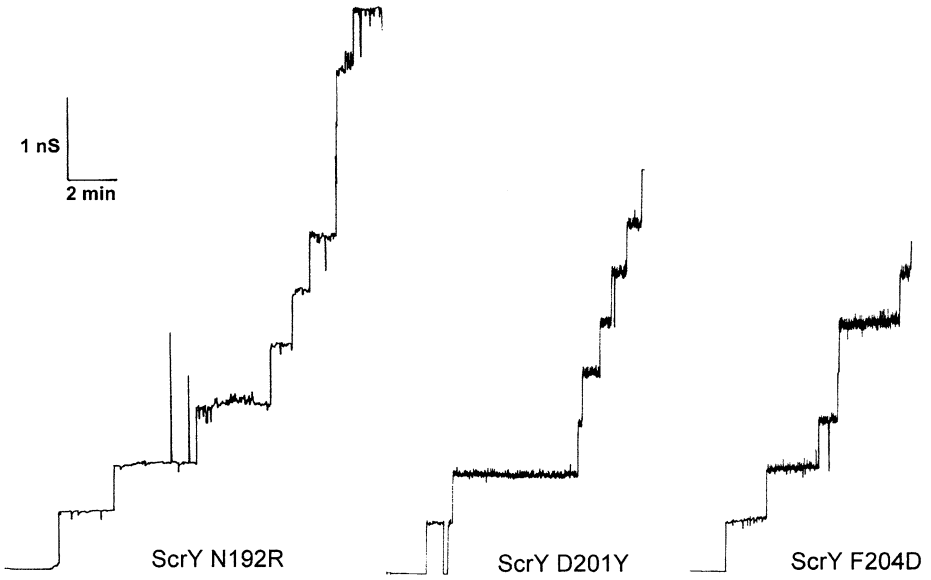

Fig. 2. Single-channel recordings of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 3 different sucroseporin mutants N192R, D201Y and F204D. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml ScrY mutants. The applied membrane potential was 20 mV; $T = 20°C$. Note that current noise of the single-channel recording of wild type ScrY is similar to that of the mutants, indicating that the mutation did not induce a major change of the channel structure.

site inside the channel is described by a first order chemical reaction (on-rate constant k_1 and off-rate constant k_{-1}). The stability constant of the binding of a carbohydrate to the channel is $K = k_1/k_{-1}$. Furthermore, it is assumed that the ScrY-channel is a single-file channel (Benz et al., 1986). This means that ScrY is open when no carbohydrate is bound, and closed when it is occupied. The conductance, $G(c)$ $(= I(c)/V_m$ with V_m membrane voltage), of a ScrY-containing membrane in the presence of carbohydrate (concentration c), which binds with the stability constant, K, is given by the following equation (Benz et al., 1987):

$$\frac{G_{max} - G(c)}{G_{max}} = \frac{I_0 - I(c)}{I_0} = \frac{K \cdot c}{1 + K \cdot c} \tag{1}$$

G_{max} is the membrane conductance before the start of the carbohydrate addition to the aqueous phase, I_0 is the initial current and $I(c)$ is the current at the concentration c. This means that the titration curves can be analyzed using Lineweaver-Burke plots as has been shown in previous publications (Benz et al., 1986; 1987). The half-saturation constant, K_S is given by the inverse stability constant $1/K$.

The measurements of current noise presented here are based on small perturbations of the number of closed channels due to microscopic variations involved in the chemical reaction between carbohydrate and binding site, which can be monitored by current fluctuations. Its reaction rate $1/\tau$ is given by (Verveen & DeFelice, 1974; DeFelice, 1981):

$$\frac{1}{\tau} = 2\pi \cdot f_c = k_1 \cdot c + k_{-1}, \tag{2}$$

where f_c is the corner frequency of the power density spectrum, $S(f)$, given by a "Lorentzian" function. Lorentzian spectra correspond to the noise expected for a random switch with different on and off probabilities, which are coupled by a chemical reaction (Verveen & DeFelice, 1974; Conti & Wanke, 1975; DeFelice, 1981):

$$S(f) = S_0/(1 + (f/f_c)^2) \tag{3}$$

S_0 is the plateau value of the power density spectrum at small frequencies. It is given by (Verveen & De Felice, 1974):

$$S_0 = 4N \cdot i^2 \cdot p \cdot (1 - p) \cdot \tau, \tag{4}$$

where N is the total number of channels (blocked and unblocked) within the membrane, i is the current through one single open channel and p is the probability that the channel is occupied by a sugar (i.e., closed).

Results

Single-Channel Experiments with ScrY-Mutants

In a first set of investigations we studied the effect of the mutations on the single-channel conductance of the ScrY single-mutants ScrYN192R, ScrYD201Y, ScrYF204D and the two triple-mutants ScrY3113 and ScrY3213. The data were compared to wild-type ScrY, which has been studied previously (Schülein et al., 1991). 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 ScrYN192R, ScrYD201Y and ScrYF204D. The conductance steps of the reconstitution experiments with the mutants were analyzed in histograms (Fig. 3). They suggested a homogeneous size for all ScrY mutants. Table 1 shows a summary of the single-channel conductances of the five mutant channels in different KCl concentrations. The results that have previously been

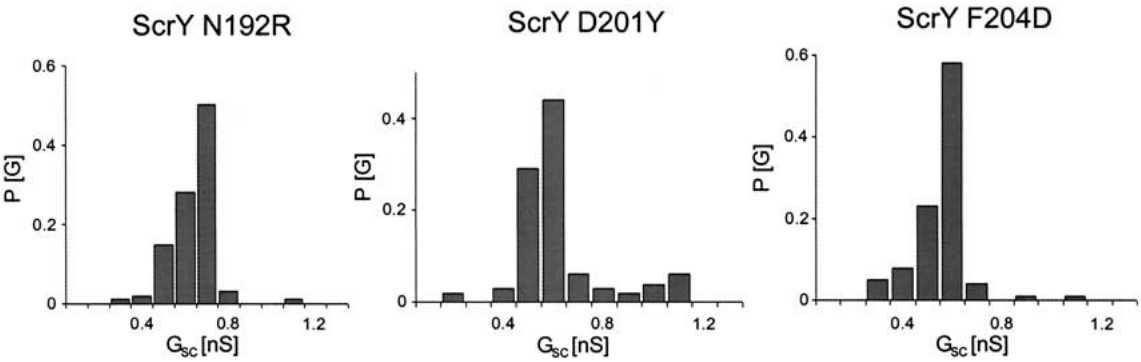

Fig. 3. Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/*n*-decane in the presence of 50 ng/ml of ScrY-mutants N192R, D201Y and F204D. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T = 20°C. The average single-channel conductance was 630 pS for 100 single-channel events (N192R), 580 pS for 97 events (D201Y), and 540 pS for 102 events (F204D).

Table 1. Single-channel conductance of the ScrY mutant channels ScrYN192R, ScrYD201Y and ScrYF204D and the ScrY triple mutants ScrY3113 (N192R + D201Y + F204D) and ScrY3213 (ScrYΔ1-61 + D201Y + F204D + N192R) in different salt solutions

Salt	Concentration (M)	*G* (pS)							
		ScrY*	Δ3-72*	N192R	D201Y	F204D	3113	3213	LamB*
KCl	3	5,000 (2,500)	2,000	2,100	2,250	2,100	900	900	590
	1	1,400 (700)	700	630	580	540	420	380	155
	0.3	460 (230)	200	180	120	120	90	85	49
	0.1	250 (130)	125	62	35	34	40	35	17
	0.03	95 (45)	35	20	–	13	–	–	–
LiCl	1	250	300	340	300	280	250	170	40
KAc (pH7)	1	1,200 (600)	600	400	310	310	250	175	135

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 *G* was calculated from at least 100 single events.

*The single-channel conductances of wild-type ScrY (Schülein et al., 1991) of ScrY Δ3-72 (Schülein et al., 1995) and wildtype LamB (Benz et al., 1986) are given for comparison. The single-channel conductance of ScrY wild-type presumably reflects the reconstitution of two trimers at the same time, which means that it is half of the value of Schülein et al. (1991), and is given in parentheses, with the exception being the conductance in LiCl.

reported for wildtype ScrY (Schülein et al., 1991), the Δ3-72 mutant (Schülein et al., 1995) and wildtype LamB (Benz et al., 1986) are given for comparison. It is noteworthy that with the exception of LiCl, previously a high percentage of simultaneous insertion of dimeric trimers into the bilayers was observed. More recent ScrY samples using other detergents resulted in the reconstitution of single trimers. The data indicate that the single-channel conductance was in most cases affected by the mutation and decreased slightly for the single mutants. A somewhat larger effect was observed for the triple mutants but their single-channel conductance was still more than two times higher than that of wildtype LamB, which means the central constriction of ScrY is larger than that of LamB despite the exchange of the amino acids N192R, D201Y and F204D, which should resemble the central constriction of LamB. In all cases the single-channel conductance was almost a linear function of the bulk aqueous conductance, which suggested that the mutations did not lead to the creation of point charge effects within the ScrY channel.

Selectivity of the ScrY Mutant Channels

Zero-current membrane potential measurements allow the calculation of the overall permeability ratio P_{cation} divided by P_{anion} in multichannel experiments. Diphytanoyl phosphatidylcholine/*n*-decane mem-

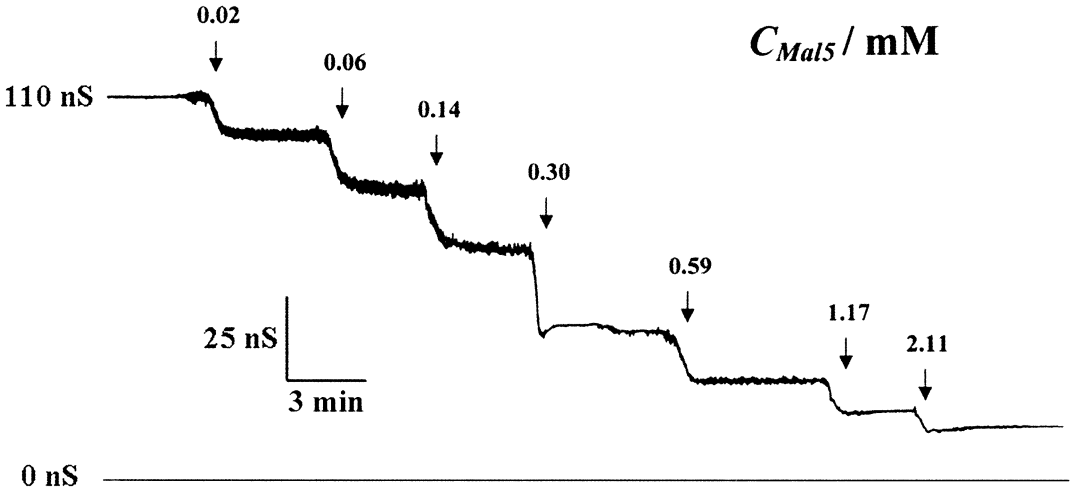

Fig. 4. Titration of ScrYF204D mutant-induced membrane conductance with maltopentaose. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 500 ng/ml protein, 1 M KCl, and maltopentaose at the concentrations shown at the top of the figure. The temperature was 20°C and the applied voltage was 20 mV.

branes were formed in 100 mM salt solution and small amounts of the concentrated ScrY-mutants ScrYN192R, ScrYD201Y, ScrYF204D and the two triple mutants ScrY3113 and ScrY3213 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 all cases studied here, the more diluted side of the membrane became positive when the experiments were performed in KCl, which indicated preferential movement of cations through the mutant channels. However, the more diluted side became negative when the experiments were performed in LiCl, which suggested that the ion selectivity was not an absolute one but changed with the aqueous mobility of the ions. On the other hand, it clearly indicated that the point mutations N192R, D201Y and F204D led to a decrease of the ion selectivity of ScrY.

The analysis of the zero-current membrane potentials was performed using the Goldman-Hodgkin-Katz equation. The results are summarized in Table 2. The point mutations N192R, D201Y and F204D led, indeed, to a strong decrease of the permeability ratio for all three salts employed in this study. This result is in qualitative agreement with the mutations, except for ScrYF204D, which influence the charge near the constriction site. It furthermore also agrees with the single-channel conductance data shown in Table 1, which also suggested that anions could have a higher permeability through the mutant channels, because the single-channel conductance for LiCl and KAc was approximately similar, whereas it is highly different for ScrY wildtype and the Δ3-72 mutant (*see* Table 1). The selectivity change of the single mutations is also reflected in that of the triple mutants ScrY3113 and ScrY3213, which are only a little more selective for potassium over chloride and are anion selective for LiCl. Again we observed a remarkable difference of the ion transport properties between these two mutants and wild-type LamB.

Table 2. Permeability ratio, P_{cation}/P_{anion} of the ScrY-mutants as calculated from the zero-current membrane potentials using the Goldman-Hodgkin-Katz equation (Benz et al., 1979)

Mutant	P_{cation}/P_{anion}		
	KCl	LiCl	KAc
ScrY[a]	8.6	5.8	16
Δ3-72[b]	15	16	18
N192R	1.9	0.60	2.3
D201Y	1.6	0.60	2.4
F204D	1.7	0.32	2.0
3113	1.6	0.57	1.8
3213	1.5	0.48	1.7
LamB[c]	28	12	50

[a] Taken from Schülein et al. (1991).
[b] From Schülein et al. (1995).
[c] From Benz et al. 1986.

Evaluation of the Stability Constants for Carbohydrate Binding with the Different ScrY Mutants

The stability constants for carbohydrate binding to ScrY-mutants were calculated from titration experiments. An example for this type of measurement is given in Fig. 4. ScrY mutant F204D was added while stirring from a concentrated stock solution to the aqueous phase (concentration about 500 ng/ml)

Table 3. Stability constants of maltooligosaccharide binding to different ScrY mutant channels as derived from titration experiments similar to that shown in Fig. 4 by using Lineweaver-Burke-plots (Fig. 5)

	K(M^{-1})				
Mutant	Sucrose	Maltose	Maltotriose	Maltopentaose	Maltoheptaose
ScrY[a]	20	150	550	3,300	4,800
Δ3-72[b]	20	160	910	4,800	5,000
N192R	60	470	1,600	8,200	16,000
D201Y	52	230	1,800	5,200	13,000
F204D	52	220	1,400	5,300	11,000
3113	50	650	23,000	130,000	250,000
3213	20	480	19,000	120,000	150,000
LamB[c]	67	110	2,800	14,000	17,000

The data represent means of at least three individual titration experiments. The standard deviation was typically less than 10% of the mean value. The results of previous titration on experiments with ScrY, ScrY Δ3-72 and LamB are given for comparison.
[a] Taken with permission from Schülein et al. (1991).
[b] From Schülein et al. (1995).
[c] From Benz et al. (1986).

bathing a black lipid bilayer membrane. The corresponding current increase was monitored on a strip-chart recorder. After about 30 min most of the conductance increase was over and the titration experiments were started by the addition of concentrated solution of maltopentaose to both sides of the membrane. This led to a decrease of membrane conductance in a dose-dependent manner as is shown in Fig. 4. At a carbohydrate concentration of 2.1 mM the current through the membrane decreased to almost zero, which means that the ScrYF204D mutant channels were blocked for ions, caused by the binding of maltopentaose to the binding site. The stability constant K for the binding of maltopentaose to the binding site inside the ScrYF204D mutant was evaluated using Lineweaver-Burke plots of the data of Fig. 4, using Eq. (1) (*see* Fig. 5). The stability constant for maltopentaose binding to F204D was 6,300 1/M (half saturation constant 160 μM). This has to be compared with a stability constant of 3,300 1/M (halfsaturation constant 300 μM) for maltopentaose binding to wild-type ScrY (Schülein et al., 1991).

It is noteworthy that the single mutations N192R, D201Y and F204D led to some increase of the stability constant for maltooligosaccharide binding as compared to wild-type ScrY and the Δ3-72 mutant, which have previously been studied (Schülein et al., 1991; 1995). An even higher increase was observed for the two triple-mutants ScrY3113 and ScrY3213, and the stability constant for carbohydrate binding increased between factors of about two (maltose) and about 30 to 50 (maltoheptaose) as compared to wildtype ScrY (Schülein et al., 1991). The results for the stability constant of titration experiments with all ScrY mutants are listed in Table 3 together with the stability constants derived earlier for titration experiments with wild-type ScrY and the Δ3-72 mutant (Schülein et al., 1991; 1995). The results

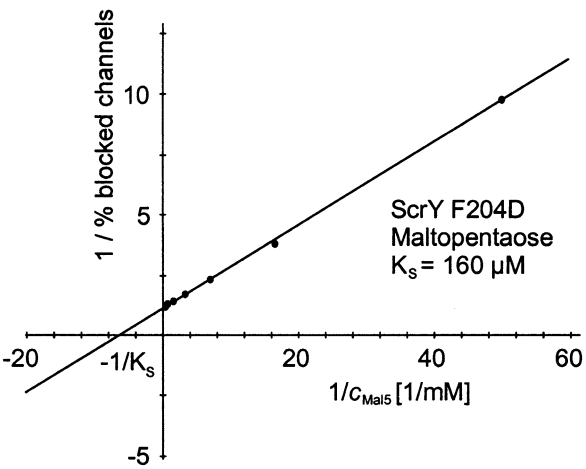

Fig. 5. Lineweaver-Burke plot of the inhibition of the ScrYF204D mutant-induced membrane conductance by maltopentaose. The data taken of Fig. 4 were analyzed using Eqn. (1). For further explanations *see* text. The straight line corresponds to a stability constant K, for maltopentaose binding to the mutant ScrYF204D of 6,300 l/M (K_S = 160 μM).

suggest that the affinity of maltooligosaccharide binding increased for all mutants tested in this study.

Measurement of Current Noise with the ScrY Mutants

Parallel to the titration measurements, the frequency-dependence of the spectral density spectra was measured using fast Fourier transformation of the current noise created by the plugging of the channels by the binding of the carbohydrates. The on- and off-kinetics of this process can be described by a chemical reaction (Nekolla et al., 1994; Andersen et al., 1998). For the measurement of current noise, absolute sta-

tionary conditions are needed, which means that the number of channels in a membrane should be stationary. To ascertain this, the time between formation of the membrane and the start of the measurements had to be considerably longer (about 2 hours) as compared to the titration experiments (about 30 min). After that time, the reference spectrum was taken first. The current noise of the open ScrY-mutant channels exhibited $1/f$ noise in the frequency range between 1 and 50 Hz, as has been described earlier (Wohnsland & Benz, 1997; Andersen et al., 1998). An example for the power spectrum of open-channel noise is given in Fig. 6*A* for the measurement of about 210 ScrYF204D mutant channels without maltopentaose (trace *1*, 0 μM). At small frequencies up to about 100 Hz the spectral density was dependent on $1/f$, which is typical for open bacterial porin channels (Wohnsland & Benz, 1997). The reference spectrum was subtracted from each spectrum taken after the successive addition of carbohydrates in increasing concentration, which led to a considerable increase of the spectral density of the current noise, as Fig. 6*A* clearly indicates.

Figure 6*A*, trace *2*, shows a spectrum taken after addition of maltopentaose (trace *2*; c = 60 μM; the reference spectrum of curve *1* was subtracted) to the membrane containing about 210 ScrYF204D mutant channels. The power-density spectrum of the ScrY-mutant channels could be fitted to two Lorentzian functions after the addition of carbohydrates (*see* Fig. 6*A*, trace *2*). This result agrees well with earlier investigations of ScrY (sucroseporin) wildtype, where also two Lorentzians have been observed (Andersen et al., 1998) in contrast to LamB (maltoporin), where the power density spectra contain only one single Lorentzian (Nekolla et al., 1994; Andersen et al., 1995). The fast Lorentzian could be fitted to Eq. (3) with sufficient accuracy, as has previously been described in full detail (Andersen et al., 1998). It was caused by the addition of carbohydrates, because it was not observed without them in control experiments (*see* Fig. 6*A*), but the reason for this is not clear, as has been discussed previously (Andersen et al., 1998). We tried also to fit the data by the sum of a Lorentzian function and $1/f$-function, but we received the best fit of the experimental data when we fitted them to the sum of two Lorentzian functions. This means that only the corner frequency of the fast process (f_c > 100 Hz) was dependent on the carbohydrate concentration and increased to high frequencies with increasing concentration. It is assumed that this part of the spectra reflects the kinetics of carbohydrate-binding (Andersen et al., 1998), which means that it is analogous to those obtained previously for LamB (Nekolla et al., 1994; Andersen et al., 1995). It may be possible to fit the data with a variable exponential factor, but this does not make much sense because it introduces an additional parameter without any physical meaning. The best fit of the data of Fig. 6*A* and similar experiments was always obtained when we fitted the whole spectra with the sum of two Lorentzian functions (*see* Fig. 6*A*, traces *2* and *4*). In further experiments, the concentration of maltopentaose was increased in defined steps. At other concentrations, the power density spectra could also be fitted to two Lorentzians (*data not shown*).

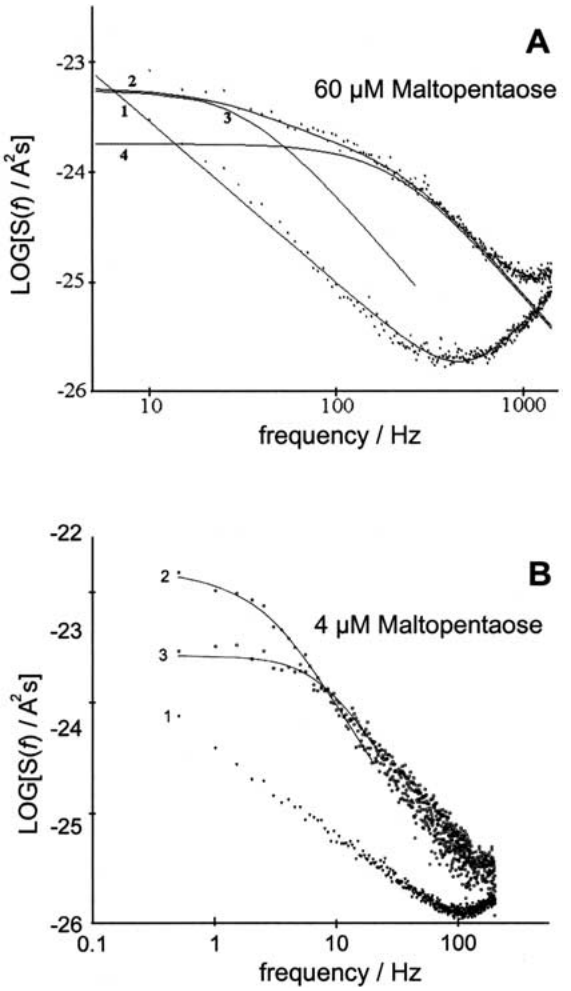

Fig. 6. (*A*) Power density spectrum of maltopentaose-induced current noise of about 210 ScrYF204D mutant channels. Trace *1* shows the control (1 M KCl). Trace *2*: the aqueous phase contained 60 μM maltopentaose and the power density spectrum of trace *1* was subtracted. Trace *3* and *4*: single Lorentzian functions (trace 3: f_c = 38 Hz (τ = 4.2 msec), $S_0 = 4.4 \times 10^{-24}$ A² sec; trace *4*: f_c = 224 Hz (τ = 0.71 msec), $S_0 = 1.9 \times 10^{-24}$ A² sec). (*B*) Power density spectra of maltopentaose-induced current noise of about 240 Scry3113 mutant channels. Trace *1* shows the control (1 M KCl). Trace *2:* the aqueous phase contained 4 μM maltopentaose and the power density spectrum of trace *1* was subtracted (f_c = 2.36 Hz (τ = 67.5 msec); $S_0 = 32.0 \times 10^{-24}$ A² sec). Trace *3*: the aqueous phase contained 30 μM maltopentaose and the power density spectrum of trace *1* was subtracted (f_c = 8.65 Hz (τ = 18.4 msec); $S_0 = 4.9 \times 10^{-24}$ A² sec); T = 20°C; V_m = 20 mV.

The interesting result of the noise measurements with the ScrY3113 and the ScrY3213 mutants was that the power density spectra of the current noise could be fitted to single Lorentzians without any exception. One example for this is shown in Fig. 6*B*, which shows an experiment with the ScrY mutant 3113. Figure 6*B*, trace *1*, shows the control without maltopentaose. This spectrum was subtracted from the spectra when maltopentaose was added in increasing concentration. Trace *2* shows a spectrum taken after addition of maltopentaose ($c = 4$ μM; the reference spectrum of curve *1* was subtracted) to the membrane containing about 240 ScrY3113 channels. It could be fitted to a single Lorentzian function (*see* Fig. 6*B*, trace *2*). A spectrum taken a few minutes later did not show any systematic variation compared to the spectrum taken before. In further experiments, the concentration of maltopentaose was increased in defined steps. At another concentration of maltopentaose (c = 30 μM) the power density spectrum corresponded to that of trace *3* in Fig. 6*B*, which could also be fitted to a single Lorentzian.

The corner frequencies, f_c, of the fast Lorentzians are dependent on the on- and off-rate constant, k_1 and k_{-1}, for carbohydrate-binding to the binding site inside the mutant ScrY channels according to Eq. (2). This means that the corner frequencies, f_c, should increase with increasing carbohydrate concentration. This was the case for all noise measurements including the experiments shown in Fig. 6. The reaction rate $1/\tau$ was plotted as a function of the carbohydrate concentration in the aqueous phase. Fig. 7 shows the fit of the corner frequencies, f_c , of the experiments shown in Fig. 6*A* and 6*B* and of other maltopentaose concentrations (*data not shown*) to Eq. (2). The rate constants for the binding of maltopentaose to the ScrYF204D mutant channels were $k_1 = 3.8 \times 10^6$ 1/(M · sec) and k_{-1} = 923 sec^{-1}, respectively. This corresponds to a stability constant, K, for the binding of maltopentaose to the binding site inside the ScrYF204D mutant channel of 4,100 1/M. Similarly, the rate constants for the binding of maltopentaose to ScrY113 could be evaluated from a similar plot (Fig. 7*B*) (k_1 = 1.5×10^{-6} 1/(M · sec), k_{-1} = 11 sec^{-1} and K = 133,000 1/M). We repeated these experiments several times and obtained similar results for both the rate constants of maltopentaose binding to the mutant channels and also for the stability constants for maltopentaose binding.

Table 4 shows the results of current-noise measurement performed with the different ScrY mutants and maltotriose. The results for wildtype ScrY and the Δ3-72 mutant are given for comparison (Andersen et al., 1998). It is noteworthy that the on- and off-rate constants of maltotriose binding, k_1 and k_{-1}, were fairly independent of the experimental conditions, including the number of reconstituted channels.

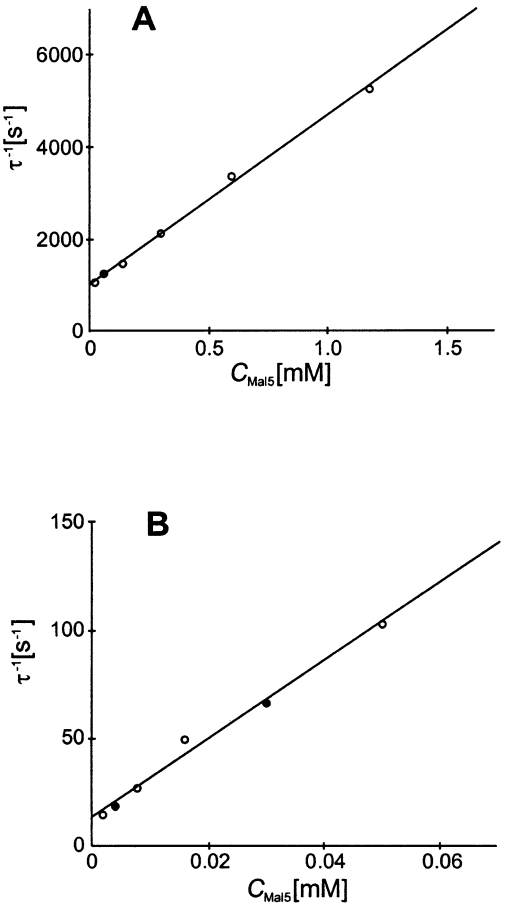

Fig. 7. (*A*) Dependence of the inverse reaction rate $2\pi f_c = 1/\tau$ of the maltopentaose-induced current noise of the ScrY mutant F204D on the maltopentaose concentration in the aqueous phase. The data were derived from the fit of the power density spectra with Lorentzians similar to those given in Fig. 6*A* (*filled circle*, 60 μM) and for other maltopentaose concentrations (*open circles*). The aqueous phase contained 1 M KCl and about 500 ng/ml ScrYF204D. The straight line corresponds to $k_1 = 3.8 \times 10^6$ M^{-1} sec^{-1} and to k_{-1} = 923 sec^{-1}. The applied membrane potential was 20 mV; T = 20°C. (*B*) The panel shows the inverse reaction rates taken from the experiment with ScrY3113 and maltopentaose shown in Fig. 6*B*. The aqueous phase contained 1 M KCl and about 500 ng/ml ScrY3113. The straight line corresponds to k_{-1} = 1.5×10^6 M^{-1} sec^{-1} and to $k_{-1} = 11$ sec^{-1}. The applied membrane potential was 20 mV; T = 20°C.

Similarly, the stability constants, $K = k_1/k_{-1}$ for maltotriose binding agreed within less of a factor of two with one another and with the stability constants derived from the titration experiments described above for the different ScrY mutants (*see* Table 3). Interestingly, we observed some influence of the N192R, D201Y and F204D mutation on the kinetics of maltotriose binding (Table 4). In particular, the on- and off-rate constants increased somewhat as compared to wild-type ScrY. The effect on the maltotriose binding kinetics was more substantial for the two ScrY triple mutants. In particular, the off-rate constants decreased for both mutants by a factor

Table 4. Parameters of maltotriose-induced transport noise in different ScrY-mutants

Mutant	k_1 (10^6 M^{-1} sec^{-1})	k_{-1} (sec^{-1})	K (10^3 M^{-1})
ScrY[a]	1.5	2,100	0.72
Δ3-72[a]	5.5	6,100	0.84
N192R	5.1	3,900	1.3
D201Y	5.1	4,600	1.1
F204D	3.9	4,000	0.98
3113	0.45	18	25
LamB[b]	8.4	1,950	4.3

The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and about 500 ng/ml ScrY mutants, k_1 and k_{-1} were derived from a fit of the corner frequencies as a function of the maltotriose concentration (compare Eq. (2)). K is the stability constant for maltotriose binding derived from the ratio k_1/k_{-1}. The data represent the mean of at least three individual experiments. The standard deviation of the mean value was always smaller than 20% of the mean. The parameters for [a]wildtype ScrY and the Δ3-72 mutant were taken from Andersen et al. (1998) and for [b]wildtype LamB from Andersen et al. (1995), and are given for comparison.

Table 5. Parameters of maltopentaose-induced transport noise in different ScrY-mutants

Mutant	k_1 (10^6 M^{-1} sec^{-1})	k_{-1} (sec^{-1})	K (10^3 M^{-1})
ScrY[a]	2.7	1,900	1.5
Δ3-72[a]	5.9	4,000	1.5
N192R	3.7	480	7.7
D201Y	3.7	680	5.4
F204D	3.7	770	4.8
3113	2.0	15	140
3213	1.9	11	170
LamB[b]	5.3	420	13

The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and about 500 ng/ml ScrY mutants, k_1 and k_{-1} were derived from a fit of the corner frequencies as a function of the maltopentaose concentration (*compare* Eq. (2)). K is the stability constant for maltopentaose binding derived from the ratio k_1/k_{-1}. The data represent the mean of at least three individual experiments. The standard deviation of the mean value was always smaller than 20% of the mean. The parameters for [a]wildtype ScrY and Δ3-72 were taken (with permission) from Andersen et al. (1998) and those for [b]LamB from Andersen et al. (1995). These data are given for comparison.

Table 6. Parameters of maltoheptaose-induced transport noise in different ScrY-mutants

Mutant	k_1 (10^6 M^{-1} sec^{-1})	k_{-1} (sec^{-1})	K (10^3 M^{-1})
ScrY[a]	1.6	840	1.9
Δ3-72[a]	1.6	910	1.8
N192R	3.1	210	15
D201Y	3.7	210	18
F204D	2.8	270	11
3113	5.6	21	260
3213	1.5	15	110
LamB[b]	5.6	180	31

The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and about 500 ng/ml ScrY mutants. k_1 and k_{-1} were derived from a fit of the corner frequencies as a function of the maltoheptaose concentration (compare Eq. (2)). K is the stability constant for maltoheptaose binding derived from the ratio k_1/k_{-1}. The data represent the mean of at least three individual experiments. The standard deviation of the mean value was always smaller than 20%. The parameters for maltoheptaose transport through [a]wildtype ScrY and Δ3-72 (Andersen et al., 1998) and [b]LamB (Andersen et al., 1995) are given for comparison (reproduced with permission).

of about 100, whereas the on-rate decreased only by a factor of about three, which is the reason for the increase of the stability constant.

The carbohydrate-induced current noise of the different ScrY mutants was also investigated for longer-chain maltooligosaccharides, such as maltopentaose and maltoheptaose. Table 5 shows the results of maltopentaose binding kinetics to the different ScrY mutants. Again, the mutations created a substantial change. Whereas the on-rate constants appeared to be only little affected, the off-rate constants strongly decreased for the single mutants N192R, D201Y and F204D by a factor of about three to four. An even stronger decrease of k_{-1} was observed for the two triple mutants 3113 and 3213, where it decreased by a factor of more than 100, similar to the situation described above for maltotriose. A somewhat similar effect on carbohydrate binding was observed for maltoheptaose (*see* Table 6). The on-rates showed only an increase on average by a factor of about two for all ScrY mutants investigated here. The off-rate k_{-1} was again influenced much more. For the single mutants N192R, D201Y and F204D, it decreased by a factor of about four, whereas its decrease by about a factor of 40 in the case of the triple mutants was again much higher. It is noteworthy, that the triple mutants ScrY3113 and ScrY3213 still had binding kinetics completely different from LamB wild-type of *E. coli,* although the design of the single and the triple mutants was such that they should transfer ScrY into LamB (Ulmke et al., 1999).

Discussion

ScrY is thought to function in the outer membrane of enteric bacteria as a channel for sucrose, because experiments have demonstrated that sucrose uptake through LamB is limited (Schmid et al., 1991). Reconstitution experiments using ScrY have, indeed, suggested that ScrY has a high permeability for sucrose, in contrast to LamB of *E. coli*. (Hardesty et al., 1991; Andersen et al., 1995; Andersen et al., 1998). The ScrY mutants studied here and previously

(Ulmke et. al, 1999) were designed in such a way that amino acids within the central constriction, which are in the same position within the 3-D structures of both channels were exchanged in ScrY by the corresponding amino acids of LamB in single mutations (*see* Fig. 1). In addition, triple mutations were designed, in which all three amino acids were exchanged together with a deletion of part of the N-terminal extension (ScrY3113 and ScrY3213). The mutations were performed to investigate if it is possible to transfer ScrY in this simple way into LamB. The in vivo experiments have already demonstrated that the mutant channels designed in this way have a similar function as wild-type LamB or wild-type ScrY, which means that they function as outer-membrane channels for carbohydrates. On the other hand, the in vivo experiments suggested already some specificity change of the mutants (Ulmke et al., 1999). However, in vivo experiments are presumably not an exact measure for the effect of the mutation on ion transport that could be one aspect of membrane channels. Furthermore, they are not able to give precise data for the stability constant of substrate binding to a transport system, which represents another aspect of ScrY and LamB as part of carbohydrate uptake systems in enteric bacteria.

The Mutations Change Ion Flux through ScrY

All mutations of ScrY localized within the constriction forming loop L3 studied here resulted in a decrease of ion flux through ScrY as compared to wild type and also to the Δ3-72 mutant, which have been studied earlier (*see* Table 1). The lowest effect was observed for the N192R mutant, because arginine and asparagine are approximately of the same size and hydrophobicity index. However, zero-current membrane potential measurements demonstrate that this mutation has a major influence on the permeability ratio P_c/P_a and the mutant ScrYN192R became even anion selective in LiCl, where the highly mobile chloride is combined with the less mobile lithium ion. This is presumably caused by the replacement of a neutral amino acid by a positively charged one, which also led to a decrease of the single-channel conductance, because the ScrY wild-type channel is highly cation selective (Schülein et al., 1991). The decrease of the single-channel conductance of the mutant D201Y is also easy to understand on the basis of the introduction of the bulky tyrosine group in the center of the constriction site instead of the negatively charged aspartic acid. This leads also to a change of the ionic selectivity as compared to wild-type ScrY.

The influence of the F204D mutation on single-channel conductance and ion selectivity is somewhat difficult to understand. In principle, an increase of single-channel conductance and a higher cation selectivity would be expected for such a mutation, because the bulky phenylalanine is replaced by a smaller negatively charged group. However, we observed the opposite. Thus, we have to assume that the mutation changes the structure of the central constriction of the ScrY channel to a certain extent, which may decrease the ion permeability. One possibility is that D201 and the newly introduced D204 form some sort of ion bridge to R161 (*see* Fig. 1), which tends to decrease the cross section of the central constriction and thus the ion transport. Indeed, the distance between D204 and R161 is a range that makes this possible. In this respect it is interesting to note that the introduction of a negatively charged amino acid within loop L3 of the central constriction site of OmpF of *Enterobacter aerogenes* also tends to decrease the channel permeability, although it is also not expected in this case (De et al., 2001). The single-channel conductance of the triple mutants ScrY3113 and ScrY3213 is much smaller than that of wild-type ScrY and the Δ3-72 mutant. This is understandable when the central constriction of both mutant channels is considered (*see* Fig. 1). Nevertheless, it is by far not so small as expected when we compare the cross sections of ScrY with LamB and the triple mutant ScrY3113. The mutation of the amino acids in position 192, 201 and 204 of ScrY by the corresponding amino acids of LamB did not transform the ScrY channel in LamB insofar as ion transport is considered, although the mutations have a major influence on ion transport through ScrY. This means presumably that ScrY is not simply a LamB mutant, as it is suggested by the high homology of the primary sequences and also by the high similarity of their 3-D structures (Schirmer et al., 1995; Forst et al., 1998).

The Mutations of N192R, D201Y and F204D have a Major Effect on Maltooligosaccharide Binding Affinity of ScrY

In this study we demonstrated that the single mutations N192R, D201Y and F204D lead to an increased stability constant for binding of the different carbohydrates to the mutants as compared to ScrY wild-type (similar to LamB wild-type; *see* Table 3). This means that the single mutations lead to a substantial change of the binding affinity of the maltooligosaccharides. A much stronger effect of their binding is observed when all three amino acids N192R, D201Y and F204D are exchanged in ScrY by the corresponding amino acids of LamB. Doing this, the carbohydrate binding affinity of the mutants ScrY3113 and also ScrY3213 increased even further, and the stability constant is about a factor of ten higher than that for carbohydrate binding to LamB for long-

chain maltooligosaccharides. This is an interesting result because the ScrY3113 and the ScrY3213 mutants were designed in such a way that they should have the properties of LamB. It is noteworthy that the N-terminal extension of ScrY had only a small influence if any on the carbohydrate binding. This result is in agreement with a previous study of carbohydrate binding to ScrY and the ScrY Δ3-72 mutant (Andersen et al., 1998), but contradicts the results of a recent study where the addition of the N-terminal extension of ScrY to LamB changes the binding affinity of LamB for carbohydrates (Dumas et al., 2000).

Effect of the Mutations on Maltooligosaccharide Binding Kinetics as Derived from the Analysis of Carbohydrate-Induced Current Noise

Besides ion transport properties of the ScrY mutants and their affinity for carbohydrate binding we also studied the effect of the different mutations on the kinetics of maltooligosaccharide binding using the analysis of the current noise. The current noise induced by the plugging of the ScrY mutant channels had two different aspects. The open channel showed $1/f$ noise up to frequencies of about 100 Hz. A major part of this noise represents the characteristics of the open ScrY channels (Wohnsland & Benz, 1997; Andersen et al., 1998). The rest of the $1/f$ noise may be caused by slow closing and opening kinetics of the ScrY-mutant channels (Andersen et al., 1998). Here we tried to avoid this type of current noise and the current recordings were only analyzed for current noise when the membrane current was absolutely stationary.

When carbohydrates were added to the aqueous phase, the block of the channel for ion movement resulted in a drastic change of the power density spectra of the current noise. They showed Lorentzian type of noise in the presence of the carbohydrates, because the plugging of the channel is controlled by a chemical reaction between carbohydrate from the aqueous phase and the binding site (Nekolla et al., 1994; Andersen et al., 1995). Similarly as in previous studies, we also used here a one-site, two-barrier model for the analysis of carbohydrate binding to the different LamB-mutant channels (Nekolla et al., 1994; Andersen et al., 1998). This model provides a good explanation for the experimental data derived here from experiments with the ScrY-mutants. This means that the one-site, two-barrier model allowed the evaluation of the on- and off-rate constants for the binding of maltotriose, maltopentaose and maltoheptaose to the central binding site inside the ScrY mutant channels. For other carbohydrates it was impossible to derive the rate constants from the noise measurements, because the corner frequency could not be obtained from the current noise, i.e., the binding kinetics was too fast or the spectral density was too small We furthermore assumed that the binding of maltooligosaccharides was symmetrical with respect to the sidedness of the ScrY channel, i.e., the on- and off-rate constants were the same from both sides. This assumption has recently been questioned for carbohydrate transport through LamB (Van Gelder et al., 2000). However, in this study we did not find any indication for channel asymmetry concerning maltose and maltooligosaccharide binding to the ScrY-mutants.

The analysis of the current noise indicates that both the on-rates and the off-rates of carbohydrate binding are influenced by the mutation of ScrY (*see* Tables 4 to 6). The degree of change varied somewhat for the three maltooligosaccharides. For maltotriose binding, k_1 varied about 10-fold between the smallest on-rate (ScrY3113, $k_1 = 4.5\times10^5$ $1/(M\cdot sec)$ to the highest one (N192R, $k_1 = 5.1\times10^6$ $1/(M\cdot sec)$). Similarly, the smallest off-rate was measured for the ScrY3113 mutant ($k_{-1} = 18$ sec^{-1}), whereas all the other mutants had off-rates above about 2000 sec^{-1}. The mutations had a more substantial influence on maltopentaose binding kinetics. In particular, the off-rate showed a dramatic decrease for the single mutations N192R, D201Y and F204D and even more on the triple mutants ScrY3113 and ScrY3213, whereas the on-rate was approximately similar for all mutants and also for ScrY and LamB wild-type. Again, the binding kinetics of maltopentaose to the triple mutants did not resemble that of LamB, which suggests that the replacement of the three amino acids N192R, D201Y and F204D was not able to confer ScrY in LamB. The same result was also found for maltoheptaose binding kinetics. The on- and off-rate constants of binding of this carbohydrate to the 5 different ScrY mutants was considerably different from that of ScrY wildtype, but also from LamB wildtype. This means presumably that the replacement of the three amino acids N192R, D201Y and F204D alone or together is not sufficient to transfer ScrY into LamB, although also other important elements, such as the aromatic amino acids of the greasy slide, are also preserved in ScrY (Schirmer et al., 1995; Dutzler et al., 1996; Forst et al., 1998). The reason for this is not obvious, as the comparison of the structures of the central constriction of ScrY, Scry3113 and LamB clearly indicates (*see* Fig. 1). Probably, the central constriction of ScrY is considerably wider than that of LamB, which could mean that the carbohydrates have a somewhat different position inside the ScrY channel as compared to LamB. Then N192R and F204D could form additional hydrogen bonds with the carbohydrates, which increases the stability constant of their binding.

Effect of the Mutations on the Carbohydrate Transport in vivo

The effect of the mutation on carbohydrate transport in vivo has already been studied (Ulmke et al., 1999). These experiments suggest that sucrose, maltose and maltooligosaccharides can pass through the mutant channels, which means that the mutant channels are functional. The in vivo experiments suggest also that the mutations induce a selectivity change of ScrY. However, the in vivo experiments do not allow to evaluate the stability constants of carbohydrate binding and the binding kinetics. The rate constants evaluated here allow us to calculate the flux of carbohydrates through the channel using the one-site, two-barrier model. For this we assume a symmetrical channel because we did not find here and in an earlier investigation (Andersen et al., 1998) much evidence for an asymmetry of carbohydrate binding to ScrY. The net flux of sugar molecules, Φ, through the channel under stationary conditions as the result of a concentration gradient $c''-c'$ across the membrane is given by the net movement of sugar across one barrier of the two identical potential energy barriers (Benz et al.,1987):

$$\Phi = k_1 \cdot c''/(1 + K') - k_{-1} \cdot K'/(1 + K') \quad (5)$$

K' is under symmetrical conditions given by:

$$K' = K \cdot (c' + c'')/2 \quad (6)$$

In equation (5) the rate constants k_1 and k_{-1} are multiplied by the probabilities that the binding site is free or occupied, respectively. Eqn. (5) has in the case $c'' = c, c' = 0$, when carbohydrates are only present on one side of the channel, the following form:

$$\Phi = k_1 \cdot c/(2 + K \cdot c) \quad (7)$$

The latter form may be used to calculate the flux of a given carbohydrate through one of the three channels in a ScrY trimer under the assumption of a symmetrical channel. Eqn. (7) suggests that the maximum permeability of the channel for a carbohydrate with an on-rate k_1 for its binding to the binding site is $k_1/2$, which is obtained for wild-type ScrY at very small carbohydrate concentration ($c \leq$ 100 μM). The flux strongly saturates at high carbohydrate concentration, since the half-saturation constant for carbohydrate flux is $K_S = 1/K$. The maximum turnover number of the channel (similar to the maximum turnover number of an enzyme that is saturated by its substrate) is reached at high carbohydrate concentration on one side of the membrane and is given by k_{-1}. This means that the flux through ScrY is limited at high maltooligosaccharide concentration. It is noteworthy, however, that this is not a serious restriction, since the concentration of substrates is normally small under physiological conditions. For the effective scavenging of nutrients at very small concentrations it seems to be more important to have a high permeability (i.e., a high k_1), which is indeed given for the transport of maltooligosaccharides through the wild-type ScrY channel (Andersen et al., 1998).

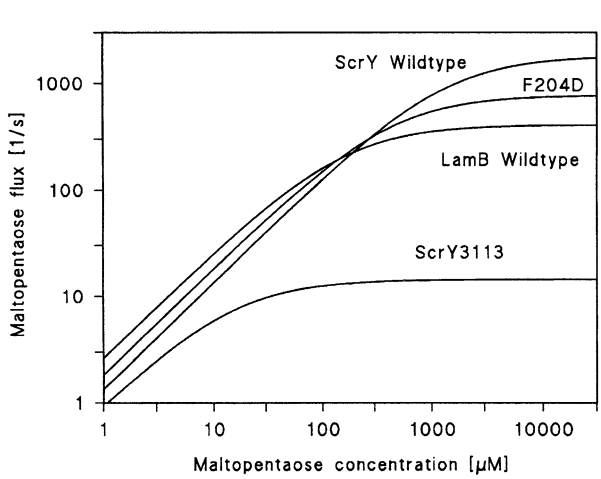

Fig. 8. Flux of maltopentaose through one single wild-type ScrY, ScrYF204D, ScrY3113 and LamB wildtype channel (i.e., through the monomers of these trimeric channels) as a function of the maltopentaose concentration on one side of the channel. The concentration on the other side was set to zero. The flux was calculated using Eqn. (7) and the rate constants are given in Table 5.

Our data allow a comparison of the flux of maltopentaose through wild-type ScrY and some of its mutant channels and through LamB under the same conditions. Fig. 8 shows the maximum flux of maltopentaose through a single ScrY wild-type, ScrYF204D, ScrY3113 and LamB wild-type channel (i.e., through the monomers of these trimeric channels) calculated on the basis of Eqn. (7), under the assumption that the concentration of the maltopentaose on one side (i.e., the periplasmic side) is zero. The curves were calculated using the rate constants given in Table 5. The carbohydrate-specific porins have their maximum permeability in the linear range of the Fig. 8 (left-hand side of the Fig.). This means that all carbohydrate-specific porins have similar permeabilities. The differences in the maximum turnover for the different porins is more substantial. ScrY wildtype had the highest turnover number of 1,900 sec^{-1} as compared to F204D (680 sec^{-1}), LamB wildtype (480 sec^{-1}) and it was lowest for the ScrY3113 mutant (15 sec^{-1}). The comparison of the different fluxes again demonstrates the advantage of a binding site for the maximum scavenging of substrates and the role of the binding site. The expression of LamB in enteric bacteria is always induced with the expression of the periplasmic maltose-binding protein MBP or MalE. MalE is present in the periplasmic space in a concentration in the range of millimolar and confers this space into a sink for

carbohydrates, although it does not modulate LamB channel function (Schwartz, 1987; Brass et al., 1985). This property is an essential part of carbohydrate uptake across the outer membrane, because a carbohydrate bound from the cell surface to the binding site inside the ScrY channel has still two possibilities with equal probabilities for further movement, because the channel is symmetric with respect to its transport properties. It can move back to the cell surface or further on to the periplasmic space. In the latter case it is bound to MBP with a half-saturation constant of considerably less than 10 μM, which means that the carbohydrate is trapped within the periplasmic space, and as long as inner membrane transport functions, it is unlikely that the carbohydrate can be lost through the outer membrane. The N-terminal extension of ScrY and its mutants has probably a similar function in carbohydrate binding and may also confer the periplasmic space in a sink (Dumas et al., 2000). However, similarly as MBP, it has no direct influence on carbohydrate transport in the case of ScrY (Andersen et al., 1998 and this study).

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