Chemical Modification of the Bacterial Porin OmpF: Gain of Selectivity by Volume Reduction

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ABSTRACT OmpF is an essentially nonselective porin isolated from the outer membrane of *Escherichia coli*. Here we report on the manipulation of the ion selectivity of OmpF by chemical modification with MTS reagents (MTSET, MTSEA, and MTSES) and the (rather bulky) tripeptide glutathione, all cysteine specific. When recorded in a gradient of 0.1//1 M $CaCl_2$ or 0.1//1 M NaCl, pH 7.4 solutions, measured reversal potentials of the most cation-selective modified mutants were (virtually) identical to the Nernst potential of Ca^{2+} or Na^{+}. Compared to this full cation selectivity, the anion-selective modified mutants performed somewhat less but nevertheless showed high anion selectivity. We conclude that a low permanent charge in combination with a narrow pore can render the same selectivity as a highly charged but wider pore. These results favor the view that both the electrostatic potential arising form the fixed charge in the pore and the space available at the selectivity filter contribute to the charge selection (i.e., cation versus anion selectivity) of a biological ion channel.

INTRODUCTION

Highly selective nanopores are believed to hold an enormous potential for future nanoscaled devices (1,2), and it is this promise that motivates the study of pores with predictable and controllable permeation characteristics. For design purposes two approaches prevail, typified by Bayley and Jayasinghe (1) as de novo design and redesign, respectively. Examples that follow the first approach include, among others, the synthesis of rigid-rod-β-barrels (3) or peptide ion channels (4), the fabrication of nanopores in synthetic films (5), and the deposition of protein/glutaraldehyde layers on a porous alumina template (6). The alternative approach takes a natural nanopore (or ion channel) as a starting point, keeps its scaffold more or less intact, and engineers (redesigns) its selectivity. Typical examples of this methodology are the selectivity studies on alamethicin (7,8). As fully realized by Bayley and Jayasinghe (1), the distinction is somehow artificial and may at some point become rather fuzzy. A hard to classify example in this respect is the chemical modification of the biological α-hemolysin by dextrin-based adapters (9).

Here we followed a similar hybrid line of research. An advantage of biological over synthetic pores is that molecular structures found in nature already have the proper dimensions. This study describes the engineering of the outer membrane protein F from *E. coli* (OmpF) into a highly selective ion channel. The detailed electrophysiological characterization of OmpF started as early as 1978, notably by the work of Benz (10,11) and Rosenbusch (12) and coworkers. At the narrowest part of the channel lumen, OmpF is ~1-nm wide, but the diameter of most channels is even smaller. The three-dimensional structure of OmpF is known at high resolution (13), and this feature renders an additional advantage because it allows high precision protein design and engineering at the subnanometer scale. Approximately halfway through the channel lumen of wild-type (WT) OmpF, three positively charged arginines (R42, R82, and R132) face a negatively charged aspartate (D113) and glutamate (E117). Together these amino acids make up the selectivity filter of the channel (14,15), and the permeation properties of OmpF arise to a large extent from the charge accumulated here, though other residues contribute as well (16). Substitutions of those residues that line the constriction zone change the permeation properties of OmpF and other porins (17–20).

In this study we hypothesized that WT OmpF is essentially nonselective because of a combination of a low net permanent charge of its selectivity filter and a wide cross section. To support this view we aimed to further improve the ion selectivity of OmpF by increasing the permanent charge in the pore as well as by reducing its volume. The extent to which the filter volume can be manipulated by amino acid substitutions alone is limited by the space occupied by these amino acid residues. For this reason we combined site-directed mutagenesis with chemical modification by relatively bulky cysteine-specific reagents. After the introduction of one or two cysteines in the cysteine-free WT OmpF protein, the mutants were chemically modified by the negatively charged ($-1e$, at pH 7.4) glutathione (GLT) or the more commonly used methanethiosulfonate-based (or MTS) reagents. To the best of our knowledge, the tripeptide GLT (γ-glutamate-cysteine-glycine) has not yet been used for this purpose. Its biochemical nature and its size—it occupies more than three times the volume taken by MTSES (Fig. 1)—make GLT a suitable tool to investigate the exclusive effect of volume reduction on ion selectivity.

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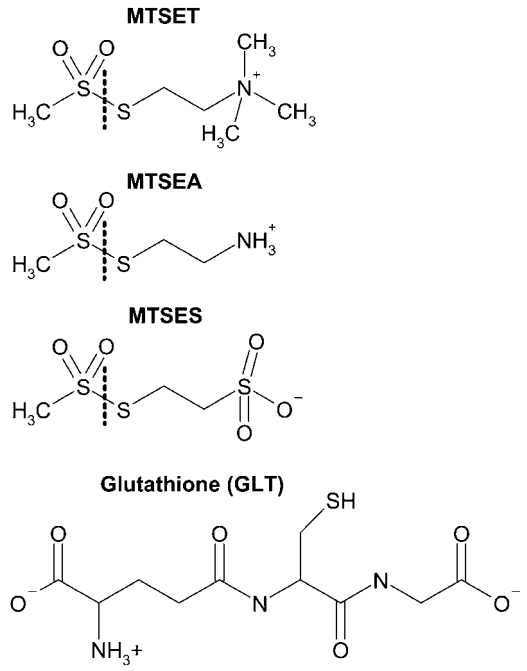

FIGURE 1 Structures of the MTS compounds and the tripeptide GLT (GLT = γ-glutamate-cysteine-glycine) used in this study. The parts on the left-hand side of the dotted lines indicate the methyl-thiosulfonate moiety of the molecules that splits off after covalently binding to the cysteine of the OmpF protein (see also Table 2).

MATERIALS AND METHODS

Mutagenesis of OmpF

The procedures for site-directed mutagenesis, isolation, and purification of OmpF have been described in detail previously (20). In short, (mutated) OmpF monomer was purified from inclusion bodies in large amounts. Subsequent in vitro refolding led to a mixture of mono-, di-, and trimeric protein, from which the latter was isolated with a yield of 5–10 mg per liter cell culture.

The six residues in the constriction zone of WT OmpF considered here are K16, R42, R82, R132, D113, and E117. All residues are assumed either fully ionized or fully protonated (21). Apart from the single and double R42C, R82C, R42C/R82C, D113C, E117C, and D113C/E117C mutants, the triple and quadruple mutants used in this study are listed in Table 1. Volume changes of the constriction zone after mutation and/or modification are expressed relative to the space occupied by the six formal (unmodified) residues in WT and based on the data in Table 2. In this study it has been assumed that all mutated, and for that matter chemically modified, proteins retain their global structural integrity. Experimental evidence that justifies this assumption comes from a crystallographic analysis of Lou et al. (22) that shows the local effects of not only point mutations (of R42, R82, and R132) but also of a mutant deleted of six residues (Δ109–114). In addition, all proteins characterized in this study formed trimers and were functional in the sense that they inserted into bilayers successfully, were able to facilitate ion transport (selectively), and retained their gating ability.

Chemical modification

The procedure followed for modification with GLT can be found in Humphries et al. (23). Cysteine mutant OmpF (5–10 μg/ml) was incubated overnight with 100 mM 1,1′-azobis(*N*,*N*-dimethylformamide) (diamide) and 125 mM GLT (both from Sigma, Zwijndrecht, The Netherlands), in a 200 mM NaP_i buffer at pH 7.5 containing 0.3% *n*-octyl-poly-oxyethylene detergent (OPOE, Alexis Biochemicals, San Diego, CA).

TABLE 1 Triple and quadruple OmpF mutants used in this study

Strain	Mutations
AAA	R42A R82A R132A
ACA	R42A R82C R132A
LACA	K16L R42A R82C R132A
EAE	R42E R82A R132E
LEAE	K16L R42E R82A R132E
LECE	K16L R42E R82C R132E

The MTS-based labels [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET), 2-aminoethylmethanethiosulfonate (MTSEA), and (2-sulfonatoethyl)methanethiosulfonate (MTSES) were purchased from Anatrace (Maumee, OH). To improve the modification efficiency by keeping the cysteine reduced, the protein was pretreated with 1 mM 1,4-dithiothreitol (DTT) for 1 h (24). Chemical modification was achieved after overnight incubation of 5–10 μg/ml mutant OmpF with 50 mM MTS reagents in a 100-mM NaP_i buffer, pH 7.5, containing 0.3% OPOE.

The electrophysiological characterization was performed without any further purification of the chemically modified protein.

Electrophysiology

The entire procedure, including pulse protocol and data analysis, can be found in Miedema et al. (20). Briefly, planar lipid bilayer (PLB) experiments were performed using a chamber and Delrin cuvet (Warner Inst., Hamden, CT). By means of 3 M KCl/2% agar salt bridges, the *cis* compartment was connected to the headstage of the Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and the *trans* compartment was connected to ground. The PLB was painted across a 250-μm diameter aperture and was composed of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in an 8:2 ratio (w/w) and dissolved in *n*-decane. While stirring, ~0.2 μl of a 1–10 μg/ml OmpF stock solution (in 0.1 M KCl buffer supplemented with 1% OPOE) was added to the *trans* side. Potential differences (V) are defined as $V = V_{cis} - V_{trans}$. A positive (outward) current is defined as a flux of positive charge from *cis* to *trans*. Conductance (g) is defined as the slope conductance of the fully opened trimer protein at the reversal potential (E_{rev}). Values of E_{rev} and g were derived from current traces in response to a voltage ramp from −100 to 100 mV in ~2 s. Data were sampled at 5 kHz and filtered at 1 kHz.

TABLE 2 Occupied space by amino acids and MTS compounds in nm^3

Amino acid	Volume in nm^3
Alanine	0.886
Cysteine	0.109
Arginine	0.173
Aspartate	0.111
Glutamate	0.138
Lysine	0.169
Leucine	0.167
Glycine	0.060
Glutathione	0.307
MTS compounds*	
MTSET	0.123 (0.173)
MTSES	0.090 (0.140)
MTSEA	0.063 (0.113)

*Total volumes (in parentheses) taken from Kaplan et al. (26). Actual volumes after covalently binding to cysteines based on the assumption that the leaving group occupies 0.050 nm^3 (see also Fig. 1).

Chemicals, including PE and PC, were purchased from Sigma. Recording buffers contained either 0.1 or 1 M KCl, NaCl, or $CaCl_2$ and 20 mM HEPES, adjusted to pH 7.4 with *n*-methyl-d-glucamine. Before use, all solutions were passed through a 0.2 μm nylon filter. Gradients are represented *cis*/*trans*; for instance, a 0.1//1 M KCl gradient indicates 0.1 M KCl in the *cis* compartment and 1 M KCl in the *trans* compartment. Reversal potentials measured in 1//0.1 M gradients of $CaCl_2$, NaCl, or KCl have all been corrected for liquid junction potentials (LJP) of 19, 11, and 1 mV, respectively. LJPs were measured independently as well as calculated by using Axon's pClamp9 software. Selectivity is expressed as the deviation of E_{rev} from the Nernst potential of the relevant ion species.

RESULTS

Chemical modification of OmpF

The trimeric nature of OmpF is a key characteristic in the context of chemical modification. Here, successfully chemically modified OmpF is defined as trimer protein with all three monomers modified. Following the protocols outlined above, the modification of cysteine-containing mutants of OmpF with either the MTS reagents or GLT was straightforward and rendered no particular problems. The two cysteine mutants R82C and D113C/E117C were the exceptions to the rule. With these two mutants we observed incomplete modification (i.e., not all three monomers modified), a feature that was translated in an E_{rev} that varied with the number of open modified and open unmodified monomers. The efficiency of the modification reaction could be improved by a second or even third addition of the modification reagents, meanwhile maintaining the buffering capacity of the reaction mixture. Eventually, after optimizing the conditions, complete modification could also be achieved with these two mutant proteins, except for the reaction of R82C with GLT. In only three out of 21 attempts did we observe complete modification of R82C by GLT. Curiously, the chemical modification of the double mutant R42C/R82C with either MTSES or GLT had far fewer problems. Examples of current traces from a partly and completely modified R82C-MTSES are shown in Fig. 2. Note that in contrast to Fig. 2 *A*, Fig. 2 *B* shows an E_{rev} that is independent of the number of open monomers, implying that all three cysteines were modified by MTSES.

To determine the cysteine specificity of the MTS reagents, MTSET was added to the cysteine-free WT OmpF. This procedure resulted in an E_{rev} of 21.5 $\pm$ 0.5 mV and a conductance of 1476 $\pm$ 13 pS in 1//0.1 M $CaCl_2$ (see Table 3), not significantly different from the values recorded on WT in the absence of MTSET (20.9 $\pm$ 1.9 mV and 1518 $\pm$ 72 pS).

The reversibility of the chemical modification by the MTS reagents and GLT was tested on a number of mutants: R82C-MTSET, ACA-GLT, R42C/R82C-GLT, D113C-MTSEA, D113C-GLT, and LECE-MTSES. Of these, the modified D113C mutant was the only protein that proved to be sensitive to DTT and of which the modification could be completely reversed by overnight incubation in 100–200 mM DTT, pH 7.5. The modification of the other four mutants tested could not be reversed. An example obtained with D113C-GLT is shown in Fig. 3. Unmodified D113C is characterized by an E_{rev} of 25.9 $\pm$ 0.6 mV and a conductance of 1443 $\pm$ 65 pS (Table 3). With GLT covalently attached, E_{rev} shifts almost 20 mV in the negative direction to 6.1 $\pm$ 2.2 mV, whereas the conductance drastically drops to 159 $\pm$ 32 pS. Exposure of D113C-GLT to DTT results in an E_{rev} and conductance of 26.7 $\pm$ 1.0 mV (10) and 1424 $\pm$ 87 pS (7), respectively, very similar to the values obtained with unmodified D113C. The fast flickering and spiky current behavior at more positive potentials was observed in all mutants that were modified with GLT (compare, e.g., with the trace of LECE-GLT in Fig. 4 *A*).

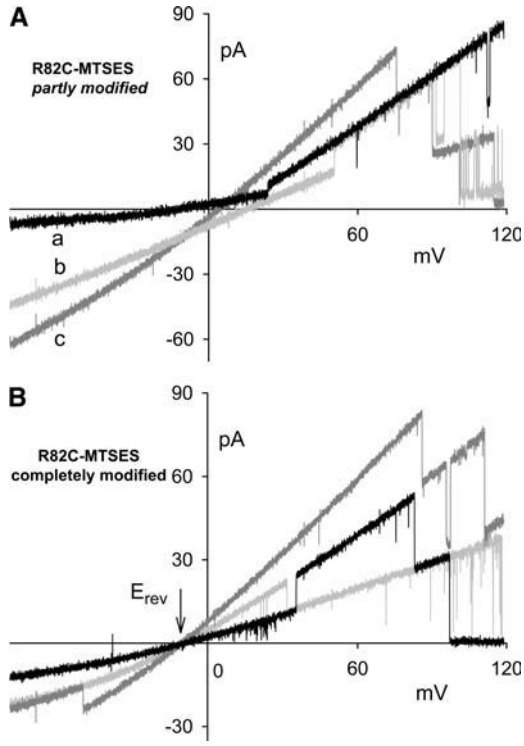

FIGURE 2 Effect of chemical modification of the trimeric OmpF protein on E_{rev}. Examples of current recordings of the partly (*A*) and completely (*B*) MTSES-modified R82C mutant. (*A*) Incomplete modification is evident from the variation in E_{rev}, i.e., E_{rev} depends on the individual contribution of each open modified/unmodified monomer. Traces represent the opening of a single modified (though not fully open) monomer (*a*), two unmodified monomers (*b*), and a combination of two unmodified and one modified monomer (*c*). All three recordings were derived from a single OmpF trimer. (*B*) Optimization of the protocol resulted in the modification of all three monomers as evident from the observation that E_{rev} became independent of the number of open monomers. Recordings in *A* and *B* were performed in gradients of 1//0.1 M $CaCl_2$, pH 7.4.

Cation selectivity

Table 3 lists all the (modified) mutants characterized in 1//0.1 M $CaCl_2$, pH 7.4 solutions and the net charge introduced

TABLE 3 Net charge introduced (ΔCharge, relative to WT), reversal potential (E_{rev}), slope conductance at E_{rev} (g), and estimated volume change at the constriction zone (ΔVol, relative to WT) of (chemically modified) OmpF porin in 1//0.1 M $CaCl_2$, pH 7.4 with $E_{Ca} = -21$ mV and $E_{Cl} = 54$ mV

No.		ΔCharge	E_{rev} in mV	g in pS	ΔVol* in nm^3
1	D113R/E117R	$+4e$	41.3 ± 1.2 (4)	1072 ± 79 (4)	−0.162
2	D113C/E117C- MTSET	$+4e$	42.2 ± 0.9 (12)	644 ± 85 (12)	−0.214
3	D113C/E117C	$+2e$	29.1 ± 1.1 (11)	1056 ± 90 (8)	0.033
4	D113C/E117C-MTSES	$0e$	−6.7 ± 1.0 (5)	140 ± 7 (5)	−0.148
5	D113C-MTSEA	$+2e$	38.2 ± 0.8 (7)	1087 ± 66 (8)	−0.060
6	D113C-MTSET	$+2e$	41.5 ± 1.6 (4)	907 ± 24 (4)	−0.120
7	D113C	$+1e$	25.9 ± 0.6 (7)	1443 ± 65 (8)	0.003
8	E117C	$+1e$	21.6 ± 1.9 (5)	1317 ± 82 (5)	0.030
9	D113C-MTSES	$0e$	14.7 ± 0.4 (5)	682 ± 20 (5)	−0.087
10	D113C-GLT	$0e$	6.1 ± 2.2 (11)	159 ± 32 (11)	−0.304
11	WT	$0e$	20.9 ± 1.9 (20)	1518 ± 72 (12)	0
12	R82C-MTSET	$0e$	11.8 ± 2.0 (9)	1100 ± 93 (9)	−0.058
13	R82C	$-1e$	17 ± 0.1 (6)	1540 ± 40 (6)	0.065
14	R82C-MTSES	$-2e$	−11.3 ± 1.2 (5)	738 ± 39 (5)	−0.025
15	R82C-GLT†	$-2e$	−18.7 ± 2.0 (3)	417 ± 33 (3)	−0.242
16	AAA	$-3e$	−0.6 ± 2.3 (11)	1150 ± 62 (7)	0.254
17	ACA-MTSEA	$-2e$	1.9 ± 1.6 (9)	1137 ± 23 (7)	0.112
18	ACA-MTSET	$-2e$	−4.0 ± 2.5 (7)	874 ± 35 (7)	0.172
19	ACA	$-3e$	−4.5 ± 1.4 (7)	1029 ± 35 (5)	0.235
20	ACA-MTSES	$-4e$	−17.7 ± 0.7 (12)	678 ± 37 (13)	0.145
21	ACA-GLT	$-4e$	−21.4 ± 1.8 (9)	451 ± 16 (4)	−0.073
22	R42C/R82C	$-2e$	5.8 ± 1.2 (6)	1123 ± 129 (7)	0.13
23	R42C/R82C-MTSES	$-4e$	−17.6 ± 1.4 (10)	654 ± 38 (9)	0.04
24	R42C/R82C-GLT	$-4e$	−21.0 ± 1.4 (5)	249 ± 39 (5)	−0.177
25	LACA-MTSET	$-3e$	−6.1 ± 0.7 (2)	835 ± 25 (2)	0.113
26	LACA	$-4e$	−9.4 ± 1.7 (8)	900 ± 46 (5)	0.236
27	LACA-MTSES	$-5e$	−19.7 ± 0.8 (8)	647 ± 20 (6)	0.146
28	EAE	$-5e$	−10.5 ± 0.9 (7)	840 ± 35 (4)	0.155
29	LEAE	$-6e$	−13.9 ± 0.9 (7)	826 ± 41 (5)	0.157
30	LECE-MTSEA	$-5e$	−10.6 ± 0.8 (4)	757 ± 40 (5)	0.074
31	LECE-MTSET	$-5e$	−14.7 ± 0.7 (7)	616 ± 8 (4)	0.014
32	LECE	$-6e$	−12.3 ± 1.5 (11)	840 ± 38 (6)	0.137
33	LECE-MTSES	$-7e$	−21.0 ± 1.2 (17)	527 ± 27 (12)	0.047
34	LECE-GLT	$-7e$	−21.6 ± 1.8 (8)	246 ± 22 (5)	−0.170

*The volume changes (ΔVol) are relative to the space occupied by the six formal residues in the constriction zone of WT OmpF: K16, R42, R82, R132, D113, and E117. A volume change <0, indicates a smaller filter volume than that of WT.
†In only three out of 21 measurements was R82C completely modified by GLT (see text).

(Δcharge) in the constriction zone after the amino acid substitution(s) and subsequent modification compared to WT (i.e., Δcharge of WT = 0). Apart from E_{rev}, Table 3 contains the slope conductance (g) measured at E_{rev}. The conductance data are included because the changes of this parameter after treatment with the MTS reagents or GLT provide additional evidence that the cysteine mutants of the protein were actually modified. The last column of Table 3 gives the estimated change in volume (ΔVol) at the constriction zone, based on the data in Table 2, where a negative change indicates a smaller volume than WT. Note that in contrast to the other compounds used, modification by GLT always resulted in volume reduction, irrespective of the mutant used.

Four out of the 34 (modified) proteins of Table 3 showed a complete Ca^{2+} over Cl^- selectivity (i.e., $E_{rev} \sim E_{Ca} = -21$ mV): ACA-GLT, R42C/R82C-GLT, LECE-MTSES, and LECE-GLT. In addition, R82C-GLT and LACA-MTSES showed a slightly lower but still high Ca^{2+} over Cl^- selectivity as well, reflected in E_{rev}s of −18.7 and −19.7 mV. The high selectivity of these six modified porins is even more remarkable when considering the high ionic strength of the recording solutions (0.1 and 1 M $CaCl_2$), which promotes effective charge screening. Examples of current traces of (modified) LECE are shown in Fig. 4 *A*. Note the marked difference in conductance due to a filter volume that after the reaction with GLT is more than 0.2 nm^3 smaller than after modification with MTSES (see Table 3). Of these six OmpF variants with the highest cation over anion selectivity, only LACA-MTSES and LECE-MTSES possessed a slightly increased filter volume of 0.146 and 0.047 nm^3, respectively. Apparently, the effect of a wider pore on the selectivity is counterbalanced by the large change in permanent charge in the filter of $-5e$ and $-7e$, respectively. The most drastic effects of chemical modification were observed with the R82C mutant. Whereas the substitution of R82 by a cysteine shifted E_{rev} by a mere 4 mV, reaction with MTSES or GLT

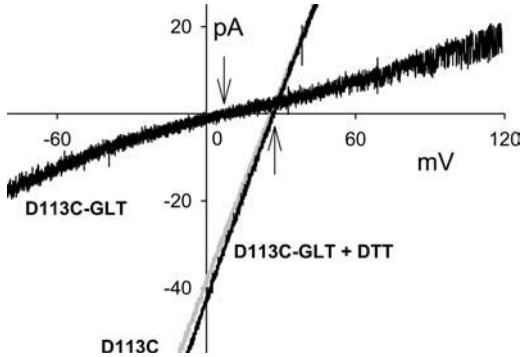

FIGURE 3 The modification of the D113C mutant with GLT can be reversed by DTT. Recordings were performed on three different mutant proteins. The current axis has been enlarged to show that both the conductance and reversal potential changed upon GLT binding. Arrows indicate E_{rev} of D113C-GLT of, on average, 6.1 mV and E_{rev} of ~26 mV of D113C (in *gray*) and D113C-GLT + DTT.

caused E_{rev} to shift another 28 mV (MTSES) or 36 mV (GLT) in the direction of E_{Ca}.

Table 4 summarizes the results of recordings in 1//0.1 M NaCl and KCl, pH 7.4 solutions of R42C/R82C-GLT and MTSES-LECE, two of the modified proteins with the highest Ca^{2+} over Cl^- selectivity. As was the case with $CaCl_2$, both proteins showed nearly full cation selectivity when analyzed in NaCl solutions (note that although the average values of E_{rev} differ from E_{Na} of −49 mV by 1–2 mV, these differences are statistically not or hardly significant). In contrast, when measured in KCl solutions, the E_{rev} of ~−45 mV was still 8 mV removed from E_K (= −53 mV), implying that under these conditions the protein still permits measurable quantities of Cl^- to permeate. These two chemically modified proteins thus show a higher cation over anion selectivity in NaCl than in KCl. Recordings of R42C/R82C-GLT in NaCl and KCl are shown in Fig. 5.

When tested for their ability to discriminate between Na^+ and K^+ under bi-ionic conditions (with 0.1 M KCl in *cis* and 0.1 NaCl in *trans*), all three proteins tested, including WT, demonstrated a very similar selectivity, reflected in a measured E_{rev} of −11.4 to −9.9 mV.

Anion selectivity

Table 5 summarizes the data of two chemically modified mutants at the other end of the selectivity spectrum, i.e., those that showed the highest anion selectivity (in Table 3 those with the most positive E_{rev}s). In contrast to the aforementioned extreme cation selectivity, none of the proteins tested demonstrated full anion selectivity. As for the three most selective, D113C-MTSET, D113C/E117C-MTSET, and D113R/E117R (with no fewer than five arginines in its constriction zone), E_{rev} in 1//0.1 $CaCl_2$ of ~42 mV remained 12 mV displaced from E_{Cl} of 54 mV. Examples of current traces obtained with (modified) D113C in $CaCl_2$ are shown

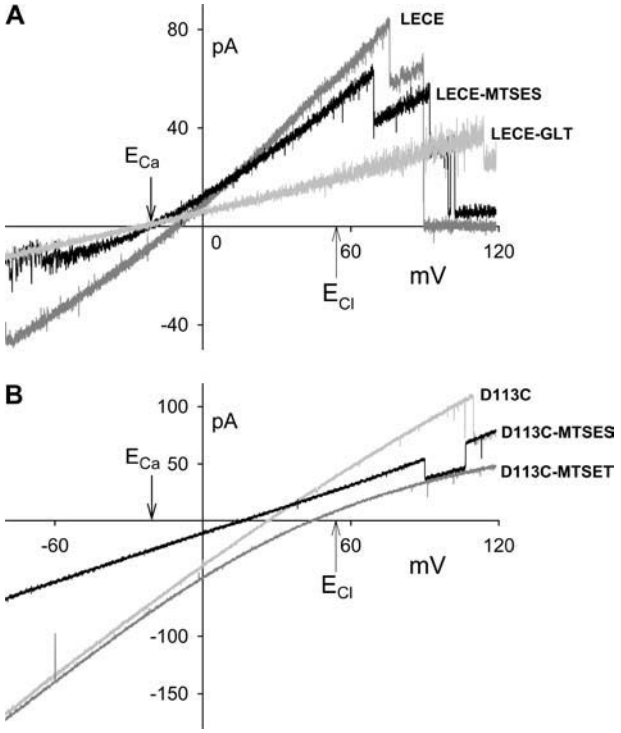

FIGURE 4 Effect of different reagents on the current profile of the LECE and D113C mutant. (*A*) Current traces of the unmodified, MTSES-modified, and GLT-modified LECE mutant. Note the shift of E_{rev} in the direction of E_{Ca} of −21 mV after modification. Also note the substantial reduction in conductance after binding of the bulky GLT. (*B*) The effect of modification on the current profile of the D113C mutant. The introduction of the negatively charged MTSES resulted in a shift of E_{rev} in the direction of E_{Ca} of −21 mV. After reaction with the positively charged MTSET, E_{rev} moved in the opposite direction, i.e., toward E_{Cl} of 54 mV. Recordings in *A* and *B* were performed in gradients of 1//0.1 M $CaCl_2$, pH 7.4.

in Fig. 4 *B*. Remarkably, the effect of the MTSET compound on the conductance vanishes at more negative potentials.

A similar behavior as seen in $CaCl_2$ was observed when these two proteins were tested in 1//0.1 M NaCl and KCl solutions. When recorded in NaCl, the highest anion selectivity was achieved with D113C/E117C-MTSET, showing a 6 mV difference between E_{rev} and E_{Cl}. As observed with the cation-selective proteins, the anion selectivity in KCl was lower than in NaCl. Fig. 5 shows traces from D113C/E117C-MTSET, recorded in either 1//0.1 M NaCl or KCl.

Chemical modification and selectivity

We also addressed the question whether the modification of mutant protein restores WT selectivity, given the final net permanent charge at the constriction zone after the reaction is the same as in WT. The answer to this question is in the negative. Table 6 compares the permeation properties in 1//0.1 $CaCl_2$ of WT and three modified D113C porins. Although all four proteins are equally charged (Δcharge = 0),

TABLE 4 Cation selectivity of WT, R42C/R82C-GLT, and LECE-MTSES in gradients of 1//0.1 M NaCl (E_{Na} = −49 mV) and KCl (E_K = −53 mV)

	WT	R42C/R82C-GLT	LECE-MTSES
ΔCharge	0	−4*e*	−7*e*
ΔVol (nm^3)	0	−0.177	0.047
in 1//0.1 NaCL			
E_{rev}	−16.2 ± 2.2 (9)	−47.0 ± 1.5 (6)	−48.0 ± 1.3 (7)
E_{rev}-E_{Na}	32.8	2.0	1.0
g at E_{rev}	1171 ± 38 (9)	730 ± 37 (5)	1073 ± 49 (4)
in 1//0.1 KCL			
E_{rev}	−20.9 ± 0.5 (8)	−45.6 ± 0.7 (6)	−44.3 ± 1.1 (9)
E_{rev}-E_K	32.1	7.4	8.7
g at E_{rev}	1919 ± 22 (8)	1258 ± 82 (6)	1758 ± 136 (5)

TABLE 5 Anion selectivity of WT, D113C-MTSET, and D113C/E117C-MTSET in 1//0.1 M NaCl (E_{Cl} = 49 mV) and KCl (E_{Cl} = 53 mV)

	WT	D113C-MTSET	D113/E117C-MTSET
ΔCharge	0	2*e*	4*e*
ΔVol (nm^3)	0	−0.120	−0.214
in 1//0.1 NaCL			
E_{rev}	−16.2 ± 2.2 (9)	37.0 ± 1.0 (10)	42.9 ± 1.3 (8)
E_{Cl}-E_{rev}	65.2	12	6.1
g at E_{rev}	1171 ± 38 (9)	539 ± 50 (5)	340 ± 21 (4)
in 1//0.1 KCL			
E_{rev}	−20.9 ± 0.5 (8)	32.0 ± 1.4 (8)	41.4 ± 0.9 (5)
E_{Cl}-E_{rev}	73.9	21	11.6
g at E_{rev}	1919 ± 22 (8)	566 ± 28 (8)	425 ± 20 (7)

the three modified mutants are all more cation selective than WT, reflected in an E_{rev} that is shifted toward E_{Ca} of −21 mV. The measured E_{rev} varies by 28 mV, from 21 mV for WT to −7 mV for D113C/E117C-MTSES. These results point to the fact that charge is not the sole determinant of the ion selectivity of OmpF but that other parameters also contribute to this property. Fig. 6 *A* plots the measured E_{rev} in 1//0.1 $CaCl_2$ in relation to Δcharge of WT and unmodified OmpF mutants (*solid circles*), OmpF modified by MTS reagents (*open circles*), and GLT-modified OmpF (*solid triangles*). The dotted line through the data of unmodified protein is drawn by eye and indicates the effect of Δcharge on E_{rev}. As expected, the more negative the Δcharge, the more cation-selective the protein. But this figure also shows that proteins that carry the same net permanent charge may differ significantly in their selectivity, especially those modified by the bulky GLT. The effect of change of volume on selectivity is highlighted in Fig. 6 *B*, which shows E_{rev} (in 1// 0.1 $CaCl_2$) as a function of the change in pore volume at a constant Δcharge of −4*e*, −2*e*, 0*e*, or 2*e*. The solid lines through the data points present linear fits and only serve to emphasize the trend observed: the larger the volume reduction, the more cation selective (for Δcharge = −4*e*, −2*e*, and 0*e*) or anion selective the channel (for Δcharge = 2*e*).

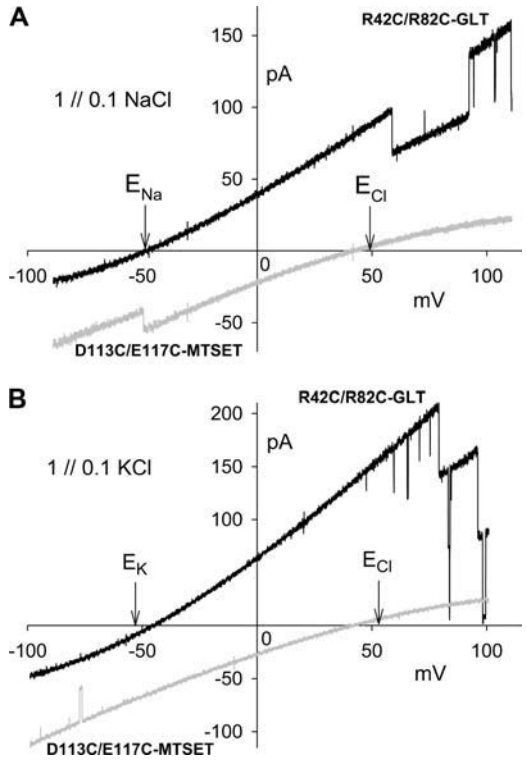

FIGURE 5 High cation or anion selectivity of the two chemically modified double mutants R42C/R82C-GLT and D113C/E117C-MTSET. (*A*) Recordings of both mutants in 0.1//1 M NaCl, pH 7.4 with E_{Na} of −49 mV and E_{Cl} of 49 mV indicated. (*B*) Recordings of both mutants in 0.1//1 M KCl, pH 7.4 with E_K of −53 mV and E_{Cl} of 53 mV indicated.

DISCUSSION

Cysteine-specific modification of OmpF

Scanning (or substituted) cysteine accessibility mutagenesis, or SCAM, has been widely used both to elucidate structural details of ion channel proteins (25–29) as well as in studies that aimed to manipulate the ion selectivity of the channel (30–32). Although OmpF has been chemically modified previously (33–35), we are aware of only two studies that report on the modification of cysteines in the constriction zone of a bacterial porin with sulfhydryl reagents. These studies were conducted on OmpC (24) and OmpF (36) but were focused on protein stability and voltage sensitivity rather than ion selectivity. Here we show the usefulness of

TABLE 6 Permeation properties of WT and modified D113C mutant protein in 1//0.1 M $CaCl_2$ with E_{Ca} of −21 mV

	ΔCharge	E_{rev} in mV	*g* in pS	ΔVol in nm^3
WT	0	20.9	1518	0
D113C-MTSES	0	14.7	682	−0.087
D113C/E117C-MTSES	0	−6.7	140	−0.148
D113C-GLT	0	6.6	159	−0.304

The net permanent charge of the three chemically modified mutant proteins is the same as that of WT (i.e., ΔCharge = 0). The data are ranked according to the estimated change in pore volume (ΔVol).

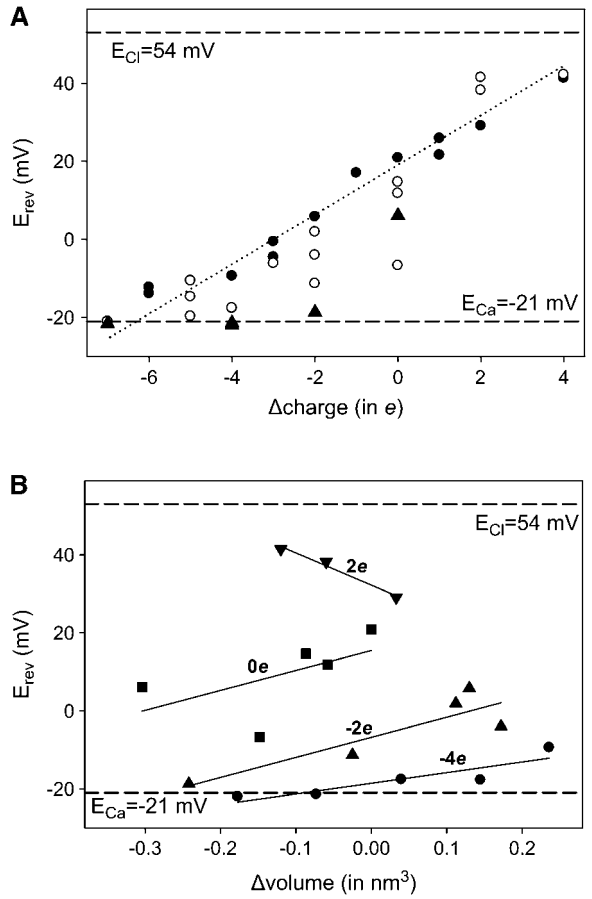

FIGURE 6 Ion selectivity of OmpF in relation to the change in permanent charge and pore volume. (*A*) E_{rev} in 1//0.1 M $CaCl_2$ in relation to Δcharge of WT and OmpF mutants (●), cysteine mutants modified with MTS reagents (○), and GLT-modified cysteine mutants (▲). The dotted line is drawn by eye and shows the trend of the effect of Δcharge on the selectivity (E_{rev}) of WT and mutant but chemically unmodified OmpF. (*B*) E_{rev} in 1//0.1 M $CaCl_2$ as a function of the change in pore volume at a constant Δcharge of $-4e$, $-2e$, $0e$, and $2e$. Equilibrium potentials of Ca^{2+} (-21 mV) and Cl^- (54 mV) are indicated.

these MTS reagents and GLT in manipulating the selectivity of single and double cysteine mutants of OmpF. In combination with the lack of effects on WT OmpF, the reversibility of the chemical modification of the D113C mutant by DTT proves the cysteine specificity of the compounds used.

Obviously, the manipulation of ion channel selectivity by chemical modification is an option only if the particular compound can reach the constriction zone. MTS reagents are cylindrically shaped molecules with a length of ~1.0–1.2 nm (26). The widest part of the MTSEA and MTSES molecules is the sulfonyl group with a 0.48 nm diameter; for MTSET the widest part is the trimethylammonium headgroup with a diameter of 0.58 nm (28). The crystal structure of WT OmpF indicates a minimum cross-sectional area of ~0.2–0.3 nm^2. Molecular dynamics studies led Im and Roux (37) to conclude that the pore undergoes small fluctuations, resulting effectively in a significantly narrower pore. Although the volume before modification of some of the cysteine mutants was substantially increased (e.g., ACA and LACA, Table 3), the volume of others remained fairly constant (e.g., D113C and D113C/E117C). Whatever the precise dimensions, our data are in support of a pore wide enough to give access to the compounds used, even in the case of the bulky GLT. In contrast to the successful chemical modification of the double mutant R42C/R82C with GLT, all efforts to modify R82C by GLT were far less effective. This observation suggests that the flanking R42 contributes somehow to the lack of reactivity of R82C.

We can only speculate on why the D113C mutant was the only protein of the variants tested for which modification could be completely reversed by DTT. Even though DTT fits into a cylinder of just 0.4 nm in diameter (28), the possibility that after modification the pore has become too narrow for DTT to enter cannot be excluded. Lack of or partial reversibility by DTT of cysteine-modified residues has been reported previously (27).

Lin and Chen (29) reported that the accessibility of the ClC-O channel to the charged MTS reagents is dependent on the electrostatic potential inside the pore. Such sensitivity was not observed with the highly negatively charged porins studied here. For instance, the modification of LECE (Δcharge = $-6e$) with the negatively charged MTSES was in no way harder to achieve than using the positively charged MTSET or MTSEA.

Ion selectivity of OmpF

Our previous work on OmpF (20) was motivated primarily by theoretical considerations regarding the mechanism by which calcium channels achieve their high Ca^{2+} over Na^+ selectivity, notably in the context of the charge space competition or CSC model developed by Nonner and co-workers (38,39). Here we focused on the cation versus anion selectivity instead and therefore omitted a discussion of the results in terms of CSC theory.

This study reemphasizes the key role charge plays in the mechanism of charge selection by ion channel proteins. The results show a significant improvement of the selectivity achieved so far. For instance, whereas the LEAE mutant (Table 1) characterized previously showed full Ca^{2+} over Cl^- selectivity in 0.1//0.01 M $CaCl_2$ solutions (20), the LECE mutant modified with MTSES shows a similar selectivity but at much higher ionic strength, i.e., in 1//0.1 M $CaCl_2$. A similar gain in selectivity was achieved when studied in gradients of 1//0.1 M NaCl, pH 7.4. For comparison, single mutations of K16, R42, R82, R132, or D113, as reported by Saint et al. (19) and Bredin et al. (40), resulted in a P_{Na}/P_{Cl} of 14 at most (for K16D). The quadruple mutant LEAE showed a strongly increased selectivity and a P_{Na}/P_{Cl} of 35 (20). Here we applied cysteine chemistry and improved the cation selectivity even further, reflected in an E_{rev} of LECE-MTSES virtually identical to E_{Na} (implying a P_{Na}/P_{Cl} that approaches ∞).

With E_{rev}s of 6–15 mV in 1//0.1 M $CaCl_2$, the D113C mutant modified with either MTSES or GLT is essentially nonselective. The fact that the presence of MTSES or GLT in the pore does not per se result in high selectivity argues against the view that the full cation selectivity obtained with some other mutants modified with MTSES or GLT was actually due to some kind of specific interaction between these reagents and the permeating ion species.

Despite the clear difference in cation selectivity between WT and the two modified mutants R42C/R82C-GLT and LECE-MTSES in NaCl and KCl (Table 4), any difference in E_{rev} between the three porins totally disappeared when measured under bi-ionic conditions (0.1 M KCl//0.1 M NaCl). Moreover, whereas the cation selectivity of the three porins was consistently higher in NaCl than in KCl, E_{rev} of ~ -10.5 mV indicates a slightly higher selectivity for K^+ than for Na^+.

Like OmpF, the anion-selective Omp32 possesses three arginines at its constriction zone but lacks the acidic, negatively charged residues that face this positively charged cluster in OmpF. It has been suggested that this is one of the reasons Omp32 is anion selective (41,42). As shown in Table 3, the neutralization of D113 or both D113 and E117 does indeed shift E_{rev} by 5 and 8 mV, respectively, into the direction of E_{Cl}. The subsequent modification of D113C and D113C/E117C by MTSET results in a further substantial improvement of the anion over cation selectivity by shifting E_{rev} another 13–16 mV toward E_{Cl}. Measured in 1//0.1 M NaCl, E_{rev} of 43 mV is however still 6 mV displaced from E_{Cl} of 49 mV (Table 5). Compared to the observed high cation selectivity of some of the modified porins, there is room left for a further improvement of the anion selectivity of OmpF.

Roles of D113 and E117

Based on a computational study, Danelon et al. (43) hypothesized a crucial role for D113 in the cation selectivity of OmpF. Our results provide experimental evidence for the role a negatively charged residue at the 113 position plays in the cation selectivity of OmpF. Whereas the neutralization of D113 by a cysteine shifts E_{rev} 5 mV away from E_{Ca}, the reintroduction of a negative charge by the modification of D113C with either MTSES or GLT shifts E_{rev} 11 and 20 mV, respectively, back toward E_{Ca}. The data on D113C/E117C and D113C/E117C-MTSES show that the selectivity further improves after the additional neutralization and modification of E117, implying that both D113 and E117 play a role in the cation selectivity of OmpF.

A different picture emerges when considering the effect of both residues on the anion selectivity. E_{rev} of both D113C-MTSET and D113C/E117C-MTSET was 42 mV. Apparently, the additional modification of E117C with the positively charged MTSET does not translate into a further gain in the anion selectivity. This finding is also in agreement with the observation that in contrast to E_{rev} of D113C, E_{rev} of E117C is not significantly different from E_{rev} of WT (Table 3). Of the two residues, D113 clearly dominates the permeation properties. This point is further reinforced by the profound effects of MTSES and MTSET modification of D113C on the current profile as shown in Fig. 4 *B*, with, in the case of MTSET, the marked asymmetry of the effect on the conductance. In addition, the drastic effect on the conductance after modifying D113C with GLT and D113C/E117C with MTSES also points in this direction. The conductance left after the modification of both mutants (140–159 pS) is only $\sim$10% of WT conductance (1518 pS). That in the case of D113C/E117C-MTSES this drop in conductance is not primarily caused by steric hindrance is indicated by the significantly larger g of 644 pS after the modification of D113C/E117C by the slightly more bulky MTSET.

Pore geometry and selectivity

Fig. 6 *B* summarizes the key conclusion of this study: both permanent charge and pore volume contribute to the charge selectivity of the OmpF channel. When comparing equally charged porins, the chemically modified ones were generally speaking more selective, especially those modified by GLT. For example, three out of four proteins with the highest Ca^{2+} over Cl^- selectivity ($E_{rev} \approx E_{Ca} = -21$ mV) contain the bulky GLT (Table 3). Furthermore, whereas Δcharge of LECE-GLT is $-7e$, Δcharge of both ACA-GLT and R42C/R82C-GLT are just $-4e$, yet their E_{rev} is essentially the same. The conclusion that volume contributes to OmpF selectivity is supported by the finding that modification of the D113C mutant did not restore WT characteristics, even though WT and modified mutants are equally charged (Table 6). Partial restoration of selectivity by MTSET-like compounds has been reported previously (30,32).

Of all porins studied, Omp32 is the most (anion) selective and at the same time the porin with the smallest cross section (41). This observation already hints in the direction that structural elements play a role in ion selectivity. The fact that we did not achieve full anion selectivity yet may be due to a pore that is still too wide.

Although it is questionable whether or to what extent the concepts of the electrical double layer (EDL) and the Debye length can be applied at (sub)nanoscaled dimensions, we nevertheless interpreted our results in terms of these parameters. As pointed out by Gu et al. (9), the diameter at the narrowest part of the channel defines to a large extent its selectivity properties. High charge selection occurs as soon as the pore radius is narrowed down to the thickness of the EDL and coions are simply excluded. This has been elegantly demonstrated in synthetic nanopores (5,44,45). The same physical principles may hold for OmpF and for that matter any narrow pore with charged walls. Geometrical aspects are perhaps even more crucial in the selection of two ion species of the same valence, e.g., K^+ and Na^+. In pores as narrow as

biological Na- and K-channels, effects other than the electrostatic potential start to dominate the mechanism of ion selectivity, e.g., free energy differences of dehydration and solvation for the two ion species (46,47). If for OmpF a narrow pore is the key to high anion selectivity and a high Na^+ over K^+ selectivity, it remains to be seen by how much the performance of engineered OmpF porins can be improved. Obviously, at some point volume reduction by chemical modification encounters its limit, since the larger the compounds, the less accessible the pore interior for these reagents.

CONCLUSIONS

At the qualitative level, the results presented here can be interpreted in terms of charge screening, the electric double layer, and the Debye length. High charge selection requires, apart from the presence of permanent charge, a relatively narrow pore with a radius that does not exceed the thickness of the EDL. Such geometry ensures that the permanent charge is not completely screened and coions are effectively repelled. Significantly wider channels are less selective because the composition of the electrolyte solution located beyond the Debye distance starts to dominate (shunting) the permeation properties of the entire pore. Applied to OmpF, the introduction of extra permanent charge and volume reduction resulted in a selectivity that covered almost the entire spectrum from fully cationic to highly anionic.

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