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Role of lysines in ion selectivity of bacterial outer membrane porins

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The ε-amino groups of available lysine residues of the OmpC, OmpF and PhoE porin proteins of *Escherichia coli* and of the protein P porin of *Pseudomonas aeruginosa*, were modified by the bulky reagent trinitrobenzenesulphonic acid. Approximately 78% of the lysines of the anion-selective protein P and PhoE porins were modified whereas only 40–50% of the lysines of the cation selective OmpF and OmpC porins were altered. After modification, the three *E. coli* porins had very similar high selectivities for cations over anions, in contrast to the native porins which varied 86-fold in ion selectivity. Despite the large size of the trinitrophenyl group attached to modified lysines (i.e., a disc of approx. 0.86 nm diameter × 0.36 nm high) relative to the reported size of the constrictions of the *E. coli* porins (1.0–1.2 nm diameter), only the anion-selective PhoE porin was substantially blocked after trinitrophenylation. The protein P porin channel was relatively unaffected by trinitrophenylation, in contrast to previous data showing dramatic effects of acetylation of lysines on protein P conductance and selectivity. This favoured a model in which the critical lysines involved in anion binding by protein P were present in a constriction of the channel that was too small for trinitrobenzenesulphonic acid to enter. Overall, the data suggest that both the number and relative position of charged lysines are major determinants of ion selectivity.

Introduction

The outer membranes of Gram-negative bacteria, like *Escherichia coli* and *Pseudomonas aeruginosa*, contain a class of proteins, termed porins, which form transmembrane, water-filled channels [1,2]. Porin proteins have similar physical properties in that they are generally present in high copy number, usually form sodium dodecyl sulphate (SDS) -resistant trimers with monomer molecular weights of 30000–50000, are often peptidoglycan and lipopolysaccharide associated and, where studied, have a very high β-sheet content [1,2]. In addition, all porins examined to date could be relatively easily purified and their pore-forming functions reconstituted in model bilayer systems [1,2].

Despite these similarities, the channels of porins have a wide range of properties. The channel sizes reconstituted vary from the small 0.6 nm channels (conductance in 1 M KCl of 0.29 nS) formed by *P. aeruginosa* protein P [1,3] through to the large 2.0 nm channels (conductance of 5.6 nS) reconstituted by *P. aeruginosa* protein F [4]. In addition, the ion selectivity of porin channels can vary from anion specific through to strongly cation selective, although most channels have relatively weak ion selectivity [6]. Evidence has been presented that suggests that the basis of ion selectivity is fixed charges on ionized amino acid side chains at the mouth of or within the porin channel [1,3,6–8]. Nevertheless, although one study has identified the position within the primary amino acid sequence of key lysines that are apparently

present within the *E. coli* OmpF porin channel [9], there are few data which describe the positioning of the charged amino acid side chains relative to the diffusion-rate-limiting (i.e., the most constricted) portion of the channel. The one exception is the phosphate-starvation-induced, outer membrane protein P of *Pseudomonas aeruginosa* for which it has been suggested that the phosphate and anion binding site of this anion-specific channel is present at the most constricted portion of the channel [17]. In order to study this in more detail we have utilized the reagent trinitrobenzene sulphonic acid under conditions where all of the ε-amino groups of available lysine residues (as well as the amino terminal α-amino group of the protein) would be expected to become trinitrophenylated. Because the trinitrophenyl group is bulky (i.e., model building with CPK Precision Models, Ealing Co., Watford, U.K. demonstrates that the trinitrophenyl group is shaped like a disc with a diameter of 0.86 nm and a height of 0.36 nm), relative to the estimated diameters of the constrictions (0.6–1.2 nm [5]) in the four porin channels examined here, we predicted that if lysine amino groups were present near the constriction, the trinitrophenylated porins should exhibit a substantial reduction in single channel conductance due to the presence of this bulky group. The results of this paper suggest that the relative positioning of charged amino acids in the channel is a critical factor in determining the degree and nature of the ion selectivity of the channel.

Materials and Methods

Porin purifications. The OmpF porin from *E. coli* B, and the OmpC and PhoE porins from *E. coli* K-12 were purified by Gordon Crockford, exactly as described previously [5]. *P. aeruginosa* protein P was purified as described [3]. We were unable to detect contaminating proteins in these porin preparations by SDS-polyacrylamide gel electrophoresis. The porins ran on SDS-polyacrylamide gels as trimers providing they were not heated to higher than 60°C in solubilization, reduction mix [10] prior to electrophoresis. Before trinitrophenylation, the porin preparations were freed from amine-containing buffers by overnight dialysis against 1000 vol. deionized water. The protein content was then estimated by the technique of Sandermann and Strominger [11].

Trinitrophenylation of porins. The assay used was a slight modification of the procedure of Fields [12], to decrease the amounts of protein used in the assay. Briefly, an 0.25 ml sample containing 50–250 μg of porin or, as controls, different concentrations of bovine serum albumin or lysozyme, was added to 0.25 ml of 0.1 M $Na_2B_4O_7$ in 0.1 M NaOH (pH 9.5). The reaction was started by the addition of 10 μl of freshly prepared 1.1 M trinitrobenzenesulphonate (Sigma Ltd., St. Louis, MO). After incubation at 23°C for 5 min, the reaction was stopped by the addition of 1 ml of freshly prepared 1.9 mM Na_2SO_3 in 99 mM NaH_2PO_4. The number of modified lysines was estimated from the known molar extinction coefficient (19200 $M^{-1} \cdot cm^{-1}$ for trinitrophenylated ε-amino groups) of the sulphite complex at 420 nm [12,13]. The trinitrophenylated proteins were freed from Na_2SO_3 and other ions by exhaustive dialysis against 0.1% SDS, and the content of modified lysines retested at 345 nm (using the known molar extinction coefficient of $1.45 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$).

Black lipid bilayer methods. The methods used for the characterization of the pore-forming ability of the native and modified porins have been previously described in detail [3,4]. The lipid used to form the membrane in the Teflon chamber was 1.5% oxidized cholesterol in *n*-decane. The experiments were done at room temperature, 24°C. Electrical measurements were made by immersing the Ag-AgCl electrodes into the aqueous solutions in compartments on either side of a Teflon divider which was perforated by a small 0.1–2 mm^2 hole over which a membrane had been painted. Bilayer formation was recognized by the membrane turning optically black when viewed by incident light. Current fluctuations were amplified 10^9- or 10^{10}-fold using a Keithley (Cleveland, OH) 427 preamplifier and monitored by a Tektronix (Beaverton, OR) 5115 storage oscilloscope (plug in amplifier 5A22). Observations were recorded on a strip chart recorder for further analysis. Zero-current potential measurements were performed exactly as described previously [3], using a Keithley 610 C electrometer.

Results

Trinitrophenylation of porin proteins

The number of amino groups that were modified by trinitrobenzene sulphonic acid treatment of the four porin proteins used here, is given in Table I. Approx. 40–50% of the total lysines of the cation-selective [5] *E. coli* OmpC and OmpF porins were trinitrophenylated. The number of lysine ε-amino groups of the OmpF protein that were modifiable (Table I) was in good agreement with numbers obtained in previous studies of this porin [8,9,14]. The anion-selective *E. coli* PhoE and *P. aeruginosa* protein P porins showed a higher level (approx. 78%) of modified lysines, than the above cation-selective channels, a result consistent with our previous observations that lysine ε-amino groups are important determinants of the selectivity of these channels [3,7,10].

All trinitrophenylated porins were examined by SDS-polyacrylamide gel electrophoresis after solubilization at 23 and 100°C. After solubilization at 23°C, all of the proteins, whether trinitrophenylated or not, ran with a mobility typical of the trimer form [2,3,10], whereas after boiling, they ran with mobilities typical of the monomer form (data not shown). As noted before for chemically modified porins [3,10], slight shifts in apparent molecular weight (i.e., a 1–2% change in relative mobility), and in the intensity of Coomassie blue staining on SDS-polyacrylamide gel electrophoretograms, were observed. Nevertheless, the retention of the trimeric configuration of all porins suggested that upon modification these proteins suffered no major structural perturbations.

TABLE I

ESTIMATED NUMBER OF AMINO GROUPS ACCESSIBLE TO TRINITROBENZENESULPHONIC ACID (TNBS) MODIFICATION

Means ± S.D. of four or more individual determinations are given. For OmpC, OmpF and PhoE total lysines' data were derived from the primary amino acid sequence [15]. For protein P, the number of lysines was estimated from amino acid analyses after timed hydrolyses of protein P (S. Kielland and R.E.W. Hancock, unpublished results).

Protein	Amino groups modified by TNBS (mol/mol protein)	Total lysines
OmpC	6.8 ± 1.3	15
OmpF	9.0 ± 1.9	18
PhoE	17.9 ± 2.3	23
P	14.8 ± 4.0	19

Single-channel experiments

When the trinitrophenylated porins were added in small quantities (5 ng/ml) to the aqueous salt solutions bathing lipid bilayer membranes, conductance increased in a series of discrete steps, a result previously observed for the corresponding

TABLE II

SINGLE-CHANNEL CONDUCTANCES OF TRINITROBENZENESULPHONIC ACID (TNBS) -MODIFIED PORINS IN DIFFERENT SALT SOLUTIONS

Average single-channel conductances were estimated as the mean of 100–500 conductance increments seen after the addition of 5 ng/ml of porin to the given salt solutions bathing a lipid bilayer membrane. The comparative data for the unmodified, native porins were taken from Ref. 5.

Porin	Average-single-channel conductance (nS)					
	1 M KCl		1 M K^+ Acetate$^-$		1 M LiCl	
	TNBS-modified	(native)	TNBS-modified	(native)	TNBS-modified	(native)
OmpC	1.5	(1.5)	0.75	(0.96)	0.57	(0.68)
OmpF	2.0	(2.1)	1.2	(1.3)	0.61	(0.73)
PhoE	1.0	(1.8)	0.46	(0.62)	0.47	(1.2)
P	0.29	(0.28)	0.020	(0.033)	0.26	(0.27)

native porins [3,5,7]. The conductance increments were, by analogy with previously lipid bilayer experiments, caused by the incorporation of single channels into the membrane. The magnitudes of the conductance increases were not uniform but were distributed around a mean, as described for the native porins (e.g. Ref. 7). A large number of (100–500) events were recorded and the average single-channel conductances of the trinitrophenylated porins in three different salt solutions were calculated and compared to data obtained previously for the native porins (Table II). For the cation-selective OmpF and OmpC channels, trinitrophenylation of the porin had little effect (i.e., a 0–22% reduction) on the single-channel conductance. For the anion-specific protein P channel, only the average single-channel conductance in 1 M potassium acetate was reduced 40% upon trinitrophenylation. The most substantial alterations were observed for the trinitrophenylated PhoE porin. Compared to the native porin, the single channel conductance was reduced 44, 26 and 61% for 1 M KCl, 1 M potassium acetate and 1 M LiCl, respectively.

Ion selectivity

The ion selectivity of the trinitrophenylated porins was examined by measurement of the zero-current potential formed in response to a KCl concentration gradient across membranes into which 100–1000 trinitrophenylated porin molecules were inserted. The permeability ratios of K^+ to Cl^- were obtained by fitting the data to the Goldman-Hodgkin-Katz equation (Table III). The results demonstrated that despite the 86-fold range in ion selectivities of the *E. coli* OmpC, OmpF and PhoE porins, the selectivity of the trinitrophenylated porins differed by less than 30%, with the modified OmpF and PhoE porins becoming as strongly cation-selective as the native or modified OmpC porins. For protein P trinitrophenylation had a much lesser effect on channel selectivity, and the modified porin remained strongly anion selective.

TABLE III

PERMEABILITY RATIOS OF TRINITROBENZENESULPHONIC ACID (TNBS) -MODIFIED PORINS AS CALCULATED BY THE GOLDMAN-HODGKIN-KATZ EQUATION

The data for native OmpC and native PhoE were taken from Ref. 5. All other data were obtained in this study as the mean ± S.D. of at least five independent determinations.

Porin	Permeability of K^+/permeability of Cl^-	
	native	TNBS-modified
OmpF	5.9 ±0.6	29 ± 9
OmpC	(26)	21 ± 6
PhoE	(0.30)	21 ±11
P	0.02 ± 0.02	0.05 ± 0.03

Discussion

The objective of the studies reported here was to determine the location of lysine ε-amino groups relative to the most constricted (channel conductance-determining) part of the channels of four bacterial porins. To do this we covalently modified the ε-amino groups of available lysines with trinitrobenzene sulphonic acid. The modified lysines carried a covalently bound trinitrophenyl group that is bulky (0.86 nm diameter × 0.36 nm high) compared to the predicted diameters of the channels used (0.6–1.2 nm [5], assuming that the channel is a symmetrical cylinder (which it almost certainly is not)). Thus, if the trinitrophenyl group was present at the most constricted part of the channel, the available area for ion movement through the channel should have been severely decreased. As demonstrated in Table II, none of the trinitrophenylated porins had severely decreased single-channel conductances relative to the native porins. Indeed, of the *E. coli* porins, only PhoE showed a substantial decrease in conductance. The effect was larger when a salt with a bulky cation (Li^+ which is heavily hydrated in solution) was used, than when a salt with a bulky anion (acetate) was employed. This is what might be predicted given that the trinitrophenylated PhoE channel had become cation selective (Table III). Nevertheless, these data differ from our previous results in which available lysines of the PhoE porin were acetylated [7]. In that study, acetylation of the lysines of PhoE did not alter the conductance in the presence of 1 M KCl; in contrast, a 44% decrease in conductance in 1 M KCl was observed for trinitrophenylated porin

channels (Table II). In both cases, however, the anion-selective PhoE channel became substantially cation selective upon acetylation [7] or trinitrophenylation (Table III). The simplest explanation for these data is that the trinitrophenyl group caused partial blockage of the PhoE channel. In contrast, it did not substantially affect the single channel conductance of OmpF or OmpC porin channels. Thus, it can be concluded that the native anion-selective PhoE channel has positively charged lysine residues closer to the rate-limiting, constricted region of the channel, than the cation-selective OmpF and OmpC channels.

Interestingly, after trinitrophenylation, the ion selectivity of the three *E. coli* porin channels was very similar. The data of Nakae and collaborators [6,8] suggest that cation selectivity of *E. coli* porins is due to the negatively charged side chains of aspartate and glutamate residues. With this in mind it is interesting that 24 of the 41–43 aspartate and glutamate residues of the three *E. coli* porins (i.e. OmpC, OmpF and PhoE) occupy exactly analogous positions in the primary structure of all three porins [15], whereas a further 13 (total 87%) are conserved in two out of the three porin species [15]. The data described here showing similar ion selectivities of the trinitrophenylated porins, suggest that the positions of negatively charged residues within the OmpF, OmpC and PhoE porins are quite similar. In contrast, the relative placement of lysine ε-amino groups may vary substantially and may well be the major determinant of the differential ion selectivity for these channels. It should be stressed that of the positively charged amino acids, only lysines would have been modified by the treatment described.

The data for protein P suggested little change in conductance (except possibly for the large anion, acetate) or ion selectivity upon trinitrophenylation. This contrasts to other chemical modification experiments in which the ε-amino groups of lysines were modified by smaller reagents [3,17] causing up to 10-fold decrease in conductance and a 30-fold decrease in anion selectivity. This is consistent with our current model [17] suggesting that the lysines that determine the anion specificity of protein P, reside within a channel constriction of 0.6 nm diameter. Thus, the bulky trinitrobenzene sulphonic acid molecule should be sterically excluded from this restriction. In conclusion, it is not only the number, but also the position of the charged amino acid side chains that determines ion selectivity.

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