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Role of the constriction loop in the gating of outer membrane porin PhoE of *Escherichia coli*

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Abstract Porins form voltage-gated channels in the bacterial outer membrane. These proteins are composed of three identical subunits, each forming a 16-stranded β-barrel. In this study, the role in voltage gating of a loop that forms a constriction within the pore was studied. The channel characteristics of mutant PhoE porins, in which the tip of the constriction loop was connected to the barrel wall, were determined. Whereas the properties of several mutant channels were changed, all of these channels could still be closed at high potential, showing that a gross movement of the constriction loop within the channel is not implicated in voltage gating.

Key words: PhoE; Porin; Outer membrane protein; Voltage gating; Constriction loop; *Escherichia coli*

1. Introduction

The outer membrane of *Escherichia coli* is an asymmetrical bilayer containing phospholipids and lipopolysaccharides in the inner and outer leaflet, respectively. This barrier protects the cell from damaging agents such as bile salts, toxins and antibiotics. Several outer membrane proteins are implicated in providing the cell with its nutrients. The major porins of *E. coli*, OmpC, OmpF and PhoE, allow the passive diffusion of small hydrophilic molecules with molecular weights of up to 600 Da. Whereas the OmpF and OmpC pores are cation-selective, PhoE pores are anion-selective [1]. The three-dimensional structures of PhoE and OmpF have been determined [2]. Each monomer of these trimeric proteins is folded as a β-barrel with 16 antiparallel β-strands. The strands are connected by short turns at the periplasmic side and long loops at the surface-exposed side of the membrane. The third loop (L3) is folded into the barrel, thereby forming a constriction at half the height of the membrane. The presence of clusters of negative and positive charges, located on L3 and opposite L3 on the barrel wall, respectively, creates a strong transverse electrostatic field in the constriction zone [2–4], which determines to a large extent the permeability and the ion selectivity of the pores [5–7]. Reconstitution of porins into planar lipid bilayers revealed that the pores could be closed by application of a potential above a certain threshold value [8,9]. The physiological relevance of this phenomenon, known as 'voltage gating', is unclear, since the Donnan potential that exists across the outer membrane was shown to be insufficient to close OmpF pores [10]. Possibly, voltage-dependent closing protects the cells after missorting of porin molecules to the cytoplasmic membrane. In this study, we investigated whether a gross movement of L3 into the channel lumen is implicated in voltage gating. Previously, we have described a series of PhoE mutants with artificial disulfide bonds between residues in the tip of loop L3 and residues in the barrel wall (Fig. 1) [11]. Since, in these mutants, the tip of L3 is tethered to the barrel wall, a gross movement of the loop is prevented. The pore properties of these mutants were characterized in the present study.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The *E. coli* K-12 strain CE1265 [12] is deleted for the *phoE* gene and does not produce the related OmpF and OmpC proteins as a result of an *ompR* mutation. The presence of a *phoR* mutation in this strain results in constitutive expression of the *pho* regulon. Bacteria were grown at 37°C under aeration in L-broth [13]. Plasmid pJP29 is derived from the cloning vector pACYC184 and carries the *phoE* gene [14]. Plasmids pEE1, pEE2, pEE3, pEE4, pEE5, pEE6, and pEE7 are derivatives of pJP29 and carry mutations in the *phoE* gene, resulting in the substitutions K18C, E110C, F111C, D302C, K18C/E110C, E110C/D302C and F111C/D302C, respectively, in the PhoE protein [11].

2.2. Porin isolation and reconstitution into planar bilayers

(Mutant) PhoE trimers were isolated from strain CE1265 as described [15], except that β-mercaptoethanol was omitted from the buffers to avoid reduction of the disulfide bonds. Lipid bilayer experiments were performed to measure the single channel conductance and critical closing potential of the various PhoE forms as described [7]. Briefly, planar lipid bilayers were formed across a pierced (∅ 150 μm) Teflon membrane, pretreated with a solution of *n*-hexadecane in *n*-hexane (1:40 v/v). Bilayer formation and conductance measurements were performed as described [16,17]. Purified porins were added to the aqueous subphase and their insertion into the bilayer was monitored by measuring the membrane current. All experiments were performed at room temperature. The sign of the membrane potential refers to the *cis*-side of the membrane. Measurements of channel conductivities and of critical closing potential were done in 1 M NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4. The critical closing potential was determined by applying a potential ramp across the bilayer from 0 to ca. 250 mV over 100 s.

2.3. Uptake of β-lactam antibiotics in vivo

The rates of permeation of β-lactam antibiotics through the outer membrane of CE1265 cells expressing the various PhoE mutants proteins were determined as described [18]. The cells carried plasmid pBR322 to obtain a high level of β-lactamase in the periplasm.

3. Results

3.1. Pore formation of PhoE mutants in vivo

The residues E110 and F111 in the tip of loop L3 of PhoE are located in close proximity to residues K18 and D302 in the barrel wall (Fig. 1). Hence, we expected that the replacement of these residues by cysteines, two at a time, could lead to the

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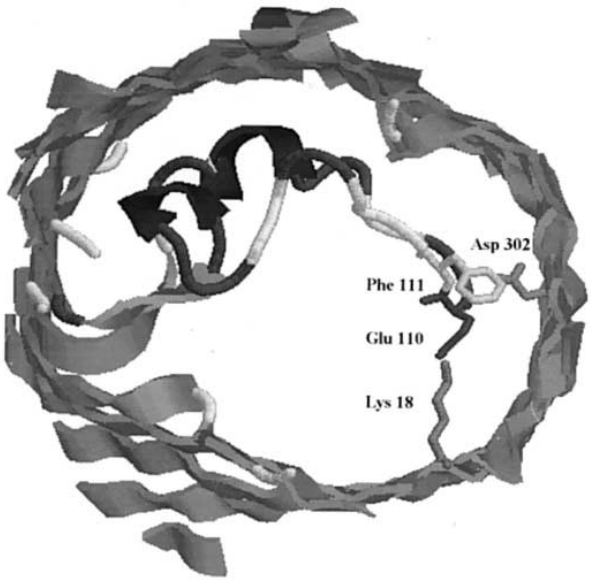

Fig. 1. Rasmol [22] presentation of Swiss model structure [23] of wild-type PhoE. Indicated are residues K18 and D302 in the barrel wall, and E110 and F111 at the tip of loop L3. These residues were replaced, two at a time, by cysteines to create intramolecular disulfide bonds. All loops except L3 are omitted for clarity and the molecule is viewed from the surface-exposed side.

formation of disulfide bonds. The construction of the PhoE double mutant proteins K18C/E110C, E110C/D302C and F111C/D302C and the corresponding single mutants was described previously [11]. All mutant proteins were well expressed and correctly assembled into the outer membrane. The disulfide bonds in the double cysteine mutants were formed, although with varying efficiency [11]. In the K18C/E110C mutant, the efficiency was near 100%, whereas it was approximately 50% for the two other double mutants.

To study whether the mutant proteins form functional pores in vivo, the rates of uptake of the β-lactam antibiotics cephaloridine and cefsulodin by intact cells were determined (Table 1). The uptake of both antibiotics was not or only marginally changed as compared to the wild type in cells expressing the mutant PhoE proteins D302C and F111C/D302C. In the case of the F111C mutant protein, the uptake of both antibiotics was decreased, suggesting that the substitution decreased the channel size. The uptake of the neutrally charged cephaloridine was increased but that of the negatively charged cefsulodin was decreased in cells expressing the mutant protein K18C, suggesting that the anion selectivity of the pores is reduced by this mutation. This result is consistent with the previously proposed role of K18 in the anion selectivity [5]. The cells expressing the E110C mutant protein or the E110C/D302C double mutant protein showed an enormously increased uptake of both cephaloridine and cefsulodin. Interestingly, this effect of the E110C substitution was compensated for to a large extent by the K18C mutation in the K18C/E110C double mutant (Table 1).

3.2. Pore formation in vitro

To study the channel characteristics in further detail, the mutant PhoE proteins were reconstituted into planar lipid bilayers. Wild-type PhoE pores showed a single channel conductance of 0.63 nS and a symmetrical behavior with respect to the polarity of the potential (Table 2). Most of the mutant PhoE pores showed a similar conductance or a conductance that was slightly increased at one polarity of the potential only. For the E110C and E110C/D302C mutants, these results seem to contradict the increased uptake of antibiotics measured in vivo (Table 1; see further Section 4). The pores of mutant F111C showed a drastically decreased conductance (Table 2), in agreement with the reduced uptake of antibiotics. Interestingly, there appeared to be two different pore species, with conductances of 0.37 nS and 0.18 nS, respectively. The same was observed for the F111C/D302C double mutant. In this case, the single channel conductance could not exactly be determined at positive polarity of the potential, because of a noisy signal (Table 2).

3.3. Voltage gating

If the voltage-dependent closing of the PhoE pores is caused by sweeping of the entire loop L3 into the channel, this would be prohibited in the double cysteine mutants in which this loop is tethered to the barrel wall. The critical closing potential of the pores was determined by applying voltage ramps across the bilayers (Table 2). For the wild-type PhoE pores, the critical closing potential was 135 mV under our experimental conditions. The pores formed by the K18C/E110C double mutant showed a similar voltage sensitivity as the wild-type pores (Table 2). Since the disulfide bond in this mutant is formed with nearly 100% efficiency, it can be concluded that the gating behavior is not changed when the tip of loop L3 is tethered to the barrel wall. As expected, incubation of these mutant porins in 10 mM dithiothreitol (DTT) to reduce the disulfides did not change the gating behavior (Table 2). A decreased voltage sensitivity was observed for the K18C mutant. In this case, the critical voltage was asymmetrical and dependent on the polarity of the potential. The mutant pores E110C, E110C/D302C and F111C/D302C showed an increased voltage sensitivity (Table 2). Despite these different characteristics, all mutant pores still showed voltage gating.

4. Discussion

Loop L3 of PhoE folds into the pore and forms a constric-

Table 1
Uptake of β-lactam antibiotics by CE1265 cells expressing mutant PhoE porins

Antibiotic	MW	Charge	Rate of uptake (%)							
			wild type	K18C	E110C	F111C	D302C	K18C/E110C	E110C/D302C	F111C/D302C
Cephaloridine	415	+-	100	167±1*	618±19*	49±12	98±1	216±4	624±2	82±4
Cefsulodin	550	+--	100	66±1	322±3	50±4	119±11	106±3	388±16	94±3

The rate of uptake was calculated in nmol substrate/min per 10^8 cells. The value obtained for the cells expressing wild-type PhoE was set at 100% and those for the mutants are expressed in % relative to the wild type. Experiments were performed at least three times independently and the standard deviations are given. Asterisks indicate data from [7].

Table 2
Electrical properties of wild-type and mutant porin channels in planar lipid bilayers

PhoE form	Conductance (nS)		Critical voltage (mV)	
	+	−	+	−
Wild type	0.63 ± 0.06 (170)	0.62 ± 0.03 (170)	135 ± 8 (26)	133 ± 10 (21)
K18C	0.61 ± 0.05 (35)*	0.74 ± 0.05 (33)	158 ± 8 (7)*	206 ± 14 (7)
E110C	0.60 ± 0.05 (61)*	0.76 ± 0.05 (44)	88 ± 12 (10)*	93 ± 1 (10)
F111C	0.37 ± 0.03 (38)	0.35 ± 0.02 (35)	121 ± 8 (10)	134 ± 9 (10)
	0.18 ± 0.02 (10)	0.17 ± 0.03 (17)		
D302C	0.61 ± 0.06 (49)	0.68 ± 0.05 (35)	103 ± 7 (5)	131 ± 10 (5)
K18C/E110C	0.55 ± 0.04 (39)	0.79 ± 0.03 (49)	145 ± 5 (4)	130 ± 11 (3)
K18C/E110C+DTT	0.63 ± 0.08 (101)	0.74 ± 0.12 (83)	114 ± 6 (10)	130 ± 13 (10)
E110C/D302C	0.65 ± 0.09 (34)	0.63 ± 0.08 (31)	96 ± 9 (10)	88 ± 5 (10)
F111C/D302C	~0.1–0.2	0.44 ± 0.02 (84)	112 ± 14 (8)	92 ± 9 (8)
		0.26 ± 0.03 (46)		

Single channel conductances and critical closing potentials were measured after reconstitution of the porins in planar lipid bilayers. The conductances are single channel conductances and correspond to approximately one third of the conductance observed for one inserted trimeric porin molecule. Measurements were performed at positive (+) and negative (−) polarity of the potential. Values in parentheses are the number of observations. Asterisks indicate data from [7].

tion that determines pore characteristics such as channel size and ion selectivity. The goal of this study was to determine whether this loop is also involved in voltage gating, by sweeping into the channel lumen and thus blocking the pore when a high transmembrane potential is applied. Such a mechanism was recently proposed on the basis of molecular dynamic simulation studies [19]. For this purpose, we employed a previously constructed set of PhoE mutants in which the tip of loop L3 is tethered to the barrel wall by disulfide bonds. In the K18C/E110C mutant, the disulfide bond was formed with nearly 100% efficiency. Still, these mutant pores closed at a high transmembrane potential. Hence, we conclude that pore closings are not mediated by a gross movement of L3 within the channel, but by more subtle rearrangements, involving only part of L3 [20] or the side chains of the charged residues within the constriction zone [7]. Although the disulfide bonds in the other two double mutants, E110C/D302C and F111C/D302C, were formed with only approximately 50% efficiency, the results obtained with these mutants support our conclusion, since all the pores in the bilayers, and not only 50% of them, closed at transmembrane potentials above the critical closing potential. In this respect, it should be noticed that in each experiment typically 5–20 trimers were incorporated in the bilayers, and the experiments with these mutants were repeated 8–10 times (Table 2).

A number of additional properties of the mutant pores are worth noting. The residues E110 and F111 of PhoE are part of a PEFGG sequence, which is highly conserved in a superfamily of bacterial porins [21]. Previously, we have shown that the deletion or the substitution of the two glycines in this sequence reduces the apparent pore size, both in vivo in antibiotic uptake experiments and in vitro in single channel conductance measurements [15]. Now it appears that the substitution of the phenylalanine in this sequence by a cysteine has a similar effect. In the single channel measurements, two types of pores were detected, suggesting some structural heterogeneity in these mutant proteins. Such a structural heterogeneity was previously also observed in an OmpF mutant [6]. Replacement of the glutamate at position 110 by a cysteine resulted in a drastic increase in the uptake of the β-lactam antibiotics (Table 1 and [7]), suggesting a drastically increased pore size. Such an increased pore size was not observed in the single channel measurements. Such apparent discrepancies between the in vivo and in vitro assays have been noted before ([7] and references therein). The increased uptake of antibiotics measured in vivo is not caused by a general leakiness of the outer membrane, caused by insertion of the mutant porin. In similar assays, the uptake of ampicillin (MW 371; one negative charge) was only marginally increased (1.4-fold) in the mutant, whereas that of nitrocefin (MW 520; two positive charges, three negative charges) was increased up to 400-fold (unpublished observations). Furthermore, whereas the sensitivity of the cells to some antibiotics, such as nitrocefin, was increased in filter disk assays, the sensitivity to other antibiotics (e.g. tetracycline, rifampicin) was not changed (data not shown). These observations strongly argue against an increased leakiness of the outer membrane of cells expressing the E110C mutant protein. Apparently, the substitution increases the permeability of the mutant pores for certain compounds, such as certain β-lactam antibiotics used in the in vivo assays, but not for others, such as the ions, measured in the planar lipid bilayer assays. Therefore, each of these assays gives only an apparent pore size, specific for the compound for which the permeability is measured. As argued before [7], the permeability is determined not only by the size of the penetrating molecule, but also by its charges, which have to be oriented within the transverse electric field in the constriction zone.

How could the E110C substitution exert its drastic effect on permeability? Importantly, the effect of this substitution was largely reversed by the K18C substitution in the K18C/E110C double mutant. K18 and E110 are in very close proximity (Fig. 1) and, thus, the removal of the negatively charged glutamate could accentuate the positive charge of K18. This could certainly influence the orientation of the penetrating molecules within the transverse electric field in the constriction zone. For an alternative explanation, it should be noted that E110 is probably hydrogen bonded to D302 in the barrel wall [4]. Thus, the substitution of E110 might increase the flexibility of (the tip of) loop L3, which might increase the permeability for large, charged molecules [15]. By introducing the K18C mutation in the E110C mutant, a disulfide bond is formed [11] which again tethers the tip of L3 to the barrel wall and, thus, restores the original rigidity at the tip of L3.

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