Altered voltage sensitivity of mutant OmpC porin channels

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Received 22 December 1995

Abstract Single OmpC porin channels have been reconstituted in planar bilayer membranes. Wild-type OmpC forms trimers which are largely insensitive to voltages below 250 mV.A single-point mutation of the ompC gene has been prepared resulting in replacement of Trp56 by Cys in the eyelet region of the channel wall in a highly conserved segment of the polypeptide. The monomeric channels of which the trimer is composed have smaller conductivity in 1 M NaCl (400 ± 20 pS, mean and S.E.M., $n = 30$) and increased voltage sensitivity by comparison with the wild-type under similar conditions, whereas other (Dex) mutants form larger channels and display different behaviour. Further, by treatment in SDS solutions at different temperatures, the W56C mutant has been shown to be less stable than either the wild-type or the Dex mutants.

Key words: Porin; Outer membrane protein; Oligonucleotide-directed mutagenesis; Thermal stability; *Escherischia coli*

1. Introduction

Gram-negative bacteria are protected from natural surfactants, toxic substances and changes in osmotic conditions by an outer membrane. *Escherichia coli* as an enteric bacterium, has evolved to resist a number of these challenges particular to the gut. This property is largely determined by the small water-filled channels which characterise the porins, through which small hydrophilic molecules can diffuse. Porins have been observed in many other Gram-negative bacteria as well as the Gram-positive mycobacteria and certain eukaryotic mitochondria and chloroplasts.

The major porins of *E. coli* are the general diffusion porins OmpF and OmpC as well as others which assist the uptake of specific molecules. PhoE, phosphoporin, is weakly anion selective and LamB, maltoporin, allows the uptake of maltodextrins.

These porins have been studied in detail in planar bilayers [1–6], showing each has a characteristic channel conductance and voltage-gating behaviour. Most porins have been observed to open and close in a controlled manner as a function of potential difference (pd). The underlying mechanism is little understood at present but studies of single porin channels in planar bilayer membranes offer the opportunity of functional studies at the molecular level. Several porins from other Gram-negative bacteria have recently been characterised and some have been found to exhibit voltage-gating behaviour similar to that of the *E. coli* porins. Some recent reviews cover porin structure and function in detail [7–9].

In 1988, Misra and Benson [10,11] produced several spontaneous mutations of the *ompC* porin gene by growing a strain expressing solely OmpC, on media with maltodextrins as the only carbon source. In the absence of the LamB porin, cells do not grow well on maltodextrins larger than maltotriose; larger ones are excluded by OmpF and OmpC on account of their size. The mutants obtained, known as OmpC(Dex) mutants show increased channel size and greater voltage sensitivity than the wild-type OmpC which is largely insensitive to membrane pd values below 250 mV, when incorporated into planar bilayers [1].

Further, structural studies of the *E. coli* OmpF porin [12] and the porin of *Rhodobacter capsulatus* 37b4 [13,14] have shown that the porins consist of three monomers making up the trimer. Each monomer is a single channel whose lumen is constricted in the centre by a polypeptide loop (loop 3) projecting from the extracellular surface into the channel. This region of constriction has become known as the eyelet region. Loop 3, and the barrel wall facing the loop, are thought to play a major role in the voltage-gating mechanism.

In this report, the preparation by oligonucleotide directed mutagenesis of a mutation located in the barrel wall opposite loop 3 and the single-channel properties of the mutant porin are described. The object was to substitute Trp-56 with Cys, in a region of the structure which is highly conserved throughout the enteric porins [15]. The results of the oligonucleotide-directed mutagenesis, isolation of the new mutant porin and characterisation of the channel in planar lipid bilayers are discussed in relation to the characteristics of the OmpC(Dex) mutants.

2. Materials and methods

2.1. Materials

The *ompC* gene on plasmid pRAM1006 was kindly donated by S. Benson (University of Maryland, MD). *E. coli* strain oc4261(*ompC*, *lamB*) was also from S. Benson. Molecular procedures were carried out throughout in the *E. coli* strain XL1-blue [11]. Soybean phosphatidylcholine type II-S used in bilayer experiments, was obtained from Sigma. The plasmids pUC18 and pBR322 were obtained from Pharmacia.

2.2. Oligonucleotide-directed mutagenesis

A 1.35 kb *Sal*I-*Eco*RI fragment from pRAM1006 containing approximately 800 bp of the 5′ end of the *ompC* gene was cloned into pUC18, to generate pNB2. Oligonucleotide-directed mutagenesis was performed on linearised pNB2. This was carried out by the 3 primer overlap extension method ('megaprimer' mutagenesis) described by Sarkar and Sommer [17] which utilises the polymerase chain reaction (PCR). The PCR was modified to include a mutagenic primer in order to create a single-point mutation. The method was performed exactly as described previously [17,18]. The final product was re-cloned into pUC18 and its nucleotide sequence determined by the chain termination method. Sequencing reactions were analysed on an ALF Sequencer (Pharmacia) according to the manufacturer's directions. The wild-type and resulting mutant DNA and amino acid sequences are shown in Fig. 1. Following sequencing, the *ompC* gene was reconstructed by cloning

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SSDI 0014-5793(95)01535-3

and the whole gene was transferred to the low copy number plasmid pBR322, forming the plasmid pMUT2. The plasmid pMUT2 was then transformed into *E. coli* strain oc4261 for expression of the mutant OmpC porin. The production of OmpF was repressed in the cells by the addition of 10% sucrose to the medium.

2.3. Porin isolation, purification and electrophoresis

Porin trimers of OmpC mutant (W56C) were isolated by the method of Nikaido (1983) [19], except that the porin trimers were not purified by gel filtration but were dialysed to remove low molecular weight contaminants. In the thermal stability experiments porin trimers were heated for 5 min at 55°C, 65°C, 75°C and 100 °C. Samples were loaded onto a 7.5% stacking/10% separating gel. SDS-PAGE was carried out using a modification of the method of Laemmli [20]. Monomers and trimers were detected by staining with silver nitrate, Fig. 2, using a kit Plusone (Pharmacia).

2.4. Reconstitution into planar bilayers

Formation of planar bilayers was as described previously [1,6] with the following exceptions. The solution used to coat the hole in the teflon septum across which the bilayer was formed, was 1% hexadecane in *n*-pentane and bilayers were formed using Schindler's technique [21]. The buffer used throughout was 10 mM $CaCl_2$, 10 mM HEPES (pH 7.4), containing 1 M NaCl unless otherwise stated. OmpC W56C porin trimers were added to the *cis* side of the teflon septum at a small enough concentration so as to introduce only single channels into the membrane. Porin was diluted in 1% SDS for addition to bilayers to avoid aggregation of porin trimers.

2.5. Electrical measurements

Membranes were tested for stability with pd values of up to 300 mV and, after mixing, porins were inserted into the membrane by applying pd values of ±200 mV. A shift in membrane current under voltage clamp showed the insertion of one or more porin trimers into the membrane. Membrane currents under voltage clamp were measured using an amplifier type HAMK2TC (Montgomery) and Ag/AgCl electrodes. A CED 1401 interface (Cambridge Electronic Design) together with an IBM-PC, was used in conjunction with patch clamp software (supplied by J. Dempster, University of Strathclyde, UK).

3. Results

3.1. Thermal stability

Fig. 2 shows an SDS-PAGE gel which includes samples treated for 5 min in Laemmli buffer [20] at 55°C, 65°C, 75°C and 100°C. At 55°C and 65°C, the OmpC W56C porin was stable and only trimers were seen be present. At 75°C, the porin was of intermediate thermal stability, with most of the trimers having dissociated to monomers. However, at 100°C, the porin was unstable as a trimer having completely broken down into monomers. By contrast, the wild-type OmpC and (not shown) OmpF showed similar thermal stability to one another under these conditions, but were markedly more stable than the mutant so that at 75°C, most of the porin remained in the trimer form.

GGCCAGTGTGAATATCA
Mutagenic Primer H44

CAGCTGACCGGTTACGGCCAGTGGGAATATCAGATCCAGGGCAACAGCGCT
Wild Type *ompC* DNA Sequence

GlnLeuThrGlyTyrGlyGlnTrpGluTyrGlnIleGlnGlyAsnSerAla
Corresponding Amino Acid Sequence

CAGCTGACCGGTTACGGCCAGTGTGAATATCAGATCCAGGGCAACAGCGCT
Mutated *ompC* DNA Sequence

GlnLeuThrGlyTyrGlyGlnCysGluTyrGlnIleGlnGlyAsnSerAla
Corresponding Amino Acid Sequence

Fig. 1. Wild-type OmpC and Mutant OmpC W56C DNA and amino acid sequences before and after mutagenesis. The bases and amino acid shown in bold are the changed sequence.

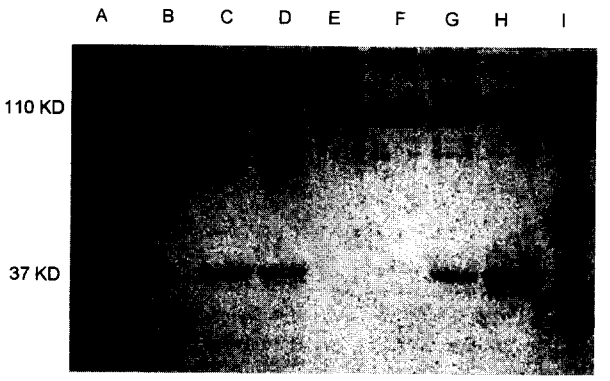

Fig. 2. Comparison of the thermal stability between mutant and wild-type OmpC porin channels. Tracks A–D represent the mutant OmpC (W56C) porin thermal stability by treatment in Laemmli sample buffer [15] for 5 min at 55°C, 65°C, 75°C and 100°C, respectively. Tracks E–H, show wild-type OmpC porin (R105) thermal stability at, 55°C, 65°C, 75°C and 100°C, respectively. Track I shows the markers (Sigma). The polyacrylamide gel electrophoresis was carried out using a modification of the Laemmli method [20]. The gel consisted of 7.5% stacking and 10% separating gels and was stained with silver nitrate.

3.2. Single-channel conductance

The *E. coli* OmpC mutant porin W56C was found to incorporate routinely into planar bilayers forming trimeric channels, comprising three equal-sized monomers. The conductance states of the trimer observed are shown in the histogram, Fig. 3. These give a single-channel conductance of 400 ± 20 pS, mean and S.E.M., $n = 30$, in the 1 M NaCl buffer solution used with −140 mV applied pd

3.3. Symmetry of behaviour

The kinetics of channel behaviour of the mutant OmpC W56C were found to be completely symmetrical with regards closure in response to pd values of opposite polarity and magnitude greater than 150 mV. However, at lower pd values, asymmetrical behaviour was observed, with closures more probable when −ve pd was applied to the *cis* side than when +ve pd was applied. This is especially so at lower pH and is illustrated in Fig. 4.

Engel et al. [22] concludes that in assembly into vesicles the porin channels are orientated smooth side out. This finding is confirmed in the study of Berrier et al. [23] which describes the unidirectional orientation in liposomes on the basis of patch clamping techniques. In the experiments described here, porin is inserted from the *cis* side and should be smooth side out so that the *cis* side in our experiments corresponds to the cytoplasmic side in vivo. Further, the results suggest that a small hyperpolarisation is more likely to close the channel than a small depolarisation.

The question of symmetry of behaviour with respect to pd has been addressed by a number of authors. Voltage symmetry been observed for OmpF, wild-type OmpC [24] and *R. capsulatus* 37b4 [25] and asymmetry of behaviour has been described for the *Acidovorax delafieldii* porin [26] but in none of these studies have low pd values been employed. As far as the high applied pd values in the present study are concerned, the observations suggest that as for *R. capsulatus* 37b4 porin [25], the controller of voltage-dependent opening and closing is situated inside the channel away from either exposed end. If the control-

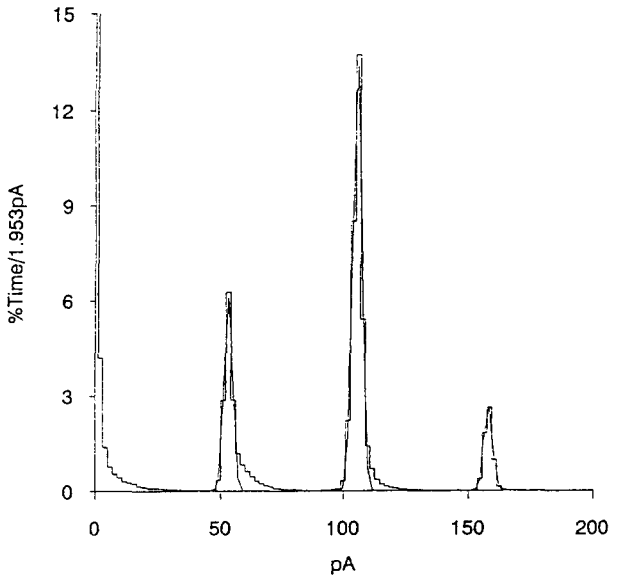

Fig. 3. All points histogram of the conductance values for the trimer associated with OmpC mutant W56C. This histogram is based on channel-gating behaviour in the BLM observed over a period of 120 s, on application of −140 mV to the *cis* compartment of the cell. The size of each monomer was calculated to be 400 ± 20pS (mean and S.E.M., $n = 30$).

ler were towards either end then asymmetrical patterns of voltage-dependent closure in response to high pd values of opposite polarity might be expected. This has not been observed here. At low applied pd values, asymmetrical gating behaviour was observed, which suggests that the mechanism involved here is distinct from the voltage-dependent closure observed at higher pd. This is consistent with a three-state mechanism previously described [27].

3.4. Voltage dependence

The mutant OmpC W56C porin channel appeared to show increased voltage sensitivity when compared with other porins, including the wild-type OmpC porin. The OmpC W56C porin was found to show an increased probability of closure at low pd values (±70 mV to 150 mV) and to open and close (or gate) between substates at pd values as low as +8 mV (Fig. 5). These characteristics are in contrast to those of the wild-type OmpC porin which remains open at low pd and can only be induced to close on application of pd values up to approximately ±300 mV.

Table 1
Comparison of the characteristics of the wild-type OmpC porin and the OmpC(Dex) mutants with the mutant OmpC W56C porin

Mutation	G (pS)	ΔG (pS)	Voltage sensitivity
Wild-type	512	0	none
R37 to H*	633	+119	increased (+)
R37 to C*	690	+178	increased (+)
R74 to S*	626	+114	increased (+)
R74 to G*	643	+131	increased (+)
R74 to C*	623	+121	increased (+)
D105 to C*	614	+102	increased (+)
W56 to C	320	−192	increased (++)

Data for the mutants marked (*) were from [1]. Voltage sensitivity (+) indicates channels closed at ±200 mV, (++) indicated that channels closed at ±70 mV.

4. Discussion

The size, or conductance, of the channel was reduced from approximately 500pS (wild-type OmpC monomer conductance) to 400 pS (mutant OmpC W56C monomer conductance). This is in contrast to the behaviour of the Dex mutant channels which all showed an increased channel size (approximately 600 pS) compared with the wild-type [1], to allow uptake of the maltodextrins necessary for their survival. The voltage dependence has also changed; the channels closing at much lower pd values than for the wild-type OmpC porin, which is largely insensitive to voltages below 250 mV, but can be induced to close at 300 mV. That is, the amino acid substitution from Trp to Cys has increased the voltage sensitivity of the OmpC porin. These observations and comparisons are listed in Table 1.

The residue that was changed in the mutagenesis, Trp-56, was chosen on the basis of its position in the barrel wall near the 3-fold trimer axis, the aromatic ring protruding towards the axis, opposite loop 3, part of the structure believed to be involved in channel-gating. The mutation does not alter the charge distribution inside the pore; both Trp and Cys are neutral. However, the OmpC(Dex) mutants all replace positively

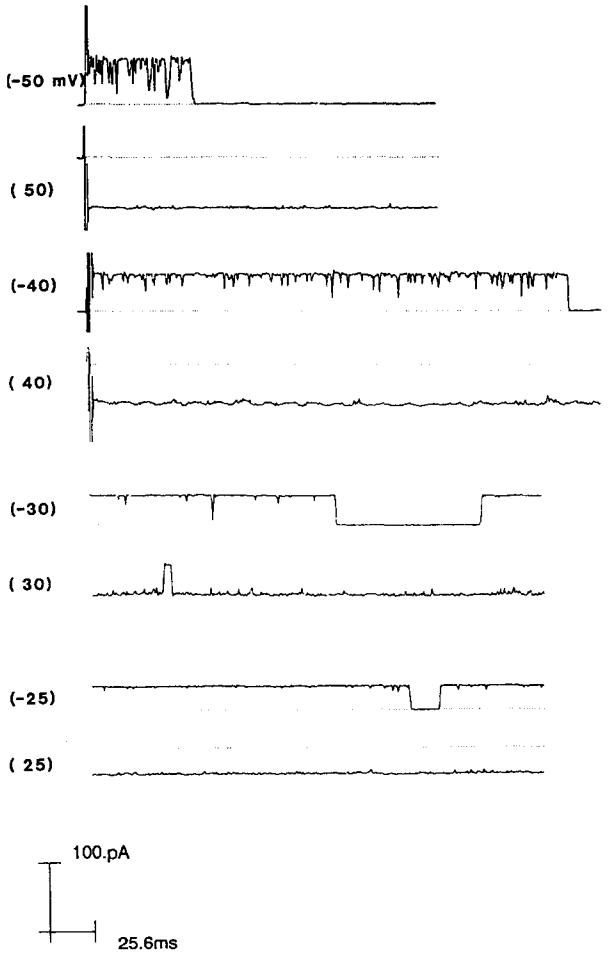

Fig. 4. Typical current traces of the OmpC W56C porin showing the increased voltage sensitivity of the porin and the marked asymmetry exhibited with respect to polarity at low pd. Pd values of 50, 40, 30 and 25 mV were applied. Polarities indicated refer to the *cis* side. The electrolyte contained 1 M NaCl, 10 mM $CaCl_2$, 10 mM Hepes at pH 6.5.

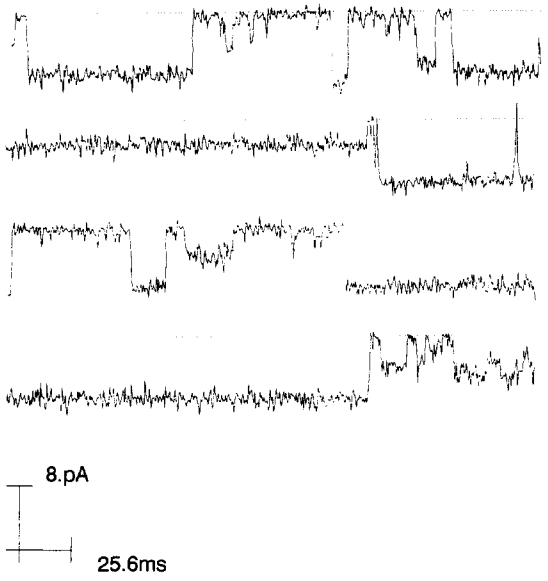

Fig. 5. Typical single-channel record showing current fluctuations and gating in the presence of the mutant OmpC porin (W56C) incorporated in the BLM at +8 mV applied to the *cis* compartment of the cell. The electrolyte contained 1 M NaCl, 10 mM $CaCl_2$, 10 mM Hepes at pH7.4

charged amino acids with neutral ones. Therefore, it seems possible that charged amino acids may be important in keeping the wild-type OmpC porin relatively insensitive to pd values, as when they are removed, voltage sensitivity is increased. However, the mutation Trp to Cys alters the voltage sensitivity to a greater extent than this, inducing closure at as low as ±70 mV. The mutation W56C seems likely to have resulted in a conformational change which has markedly altered the free energy of the system of opposing charges thought to be involved in gating. This is supported by the change in channel size. It would, therefore, seem, from the results presented here, that this region does indeed play a major role in the voltage-gating mechanism and that so sensitive is this region that only one single amino acid change need be involved to make an important change in voltage dependence.

The size of the side chain may also be a consideration. In changing a Trp residue to Cys one, the bulk of the side chain has been decreased, which would be expected to increase the size of the channel lumen. However, the opposite occurs; the size is dramatically decreased, indicating that the bulk of the side chain is not important in this case. Further, the residue Trp-56 is completely conserved throughout the *E. coli* porins [15].

Cowan et al. [12] have suggested that a major contribution to trimer stability is hydrophobic. The monomer monomer interaction has been weakened by the substitution of Trp-56 with Cys-56. The reduction in mutant porin thermal stability reported here is also consistant with this. The implication is, therefore, that a conformational change of the channel has occurred resulting from this single-point mutation which may be a key one in understanding the control of voltage-gating and filtration properties. Future work will characterise the mutant OmpC W56C in greater detail, to elucidate the role of this residue in the altered voltage sensitivity produced.

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