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Characterisation of the porin of *Rhodobacter capsulatus 37b4* in planar lipid bilayers

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Abstract

The outer membrane of Gram-negative bacteria contains aqueous channels, porins, which aid the diffusion of small hydrophilic molecules across it. *Escherischia coli*, as enteric bacteria, are able to survive a hostile environment of proteases, surfactants, and drastic changes of osmotic pressure. *Rhodobacter capsulatus* is not an enteric bacterium and as such has not evolved to resist the same challenges. Porins, which have molecular weight of approximately 35 kDa, form trimeric channels with a solute exclusion limit of about 600 Da. Most of them open and close in a controlled manner as a function of p.d. This function is little understood at present. The functional properties of single trimers of the major porin of *Rhodobacter capsulatus 37b4* have been investigated in planar artificial bilayers. On application of a suitable p.d. the observed trimer closes in approximately three equal steps. The behaviour is completely symmetrical as regards closure in response to p.d.'s of opposite polarity and is strongly cation selective.

Key words: Porin; Outer membrane protein; *Rhodobacter capsulatus*

1. Introduction

The outer membrane of Gram-negative bacteria protects the cell from various natural surfactants, toxic enzymes, and changing osmotic conditions, as well as distinguishing between toxic and essential components of the external medium. *Escherischia coli*, as an enteric bacterium has had to evolve to resist a number of challenges particular to the gut, whereas *Rhodobacter capsulatus*, a non-enteric bacterium, has not encountered the same conditions. These properties are largely determined by small water-filled channels formed by channel-forming proteins, known as porins, through which small hydrophilic molecules can diffuse. Porins have also recently been observed in the Gram-positive Mycobacteria, as well as many other Gram-negative bacteria.

The major porins of *E. coli* are OmpF, OmpC, PhoE (phosphoporin), and LamB (maltoporin), which have molecular weights of approximately 35,000 Da, form trimeric channels held together by lipopolysaccharides with solute exclusion limits of about 600 Da. But they differ in that OmpF and OmpC are weakly cation selective whereas PhoE is weakly anion selective.

These porins have been studied in detail in planar bilayers, showing that 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 (p.d.). The underlying mechanism involved 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. Porins from other Gram-negative bacteria, such as *Rhodopseudomonas blastica, Acidovorax delafieldii, Pasteurella multocida* and *Comomonas acidovorans* have been recently characterised and some have been reported to exhibit voltage gating similar to that of the *E. coli* porins.

The porins all show similar secondary structures, that is, they consist predominantly of β-sheet. OmpF and PhoE of *E. coli* have been studied by X-ray diffraction of detergent/protein crystals at 2.4 Å resolution [1], and the *R. capsulatus 37b4* porin at a resolution of 1.8 Å [2,3]. These studies have revealed the structure of porin to consist of a 16-stranded β-barrel with a polypeptide loop projecting from the extracellular surface to the channel lumen (known as Loop 3, between β-sheets 5 and 6) which partially constricts the channel at its centre, in a region known as the **eyelet region** [4]. Although the *R. capsulatus* porin has low sequence homology with the *E. coli* porins, they have very similar 3-dimensional folds and sequence differences between them provide useful tools for interpretation of differences in gating behaviour and ultimately to the mechanisms involved.

Several recent reviews cover porin structure and function in detail [5–7]. The functional properties of single trimers of the major porin of *Rhodobacter capsulatus 37b4*, first purified by Nestel et al. [8], reconstituted in planar artificial bilayers form the subject of this report. Similarities and differences between the *R. capsulatus 37b4* porin and other porins is discussed in relation to the recently published atomic resolution structures for both the *E. coli* and *R. capsulatus* porins and to earlier measurements of channel activity [9].

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2. Materials and methods

2.1. Materials

The *Rhodobacter capsulatus* strain 37b4 was obtained from A. McEwen, UEA Norwich. Detergents used in the porin preparation were Triton X-100 (Sigma), *N,N*-dimethyldodecylamine-*N*-oxide (Sigma) and octylpolyoxyethylene (Bachem, Switzerland). Soybean phosphatidyl choline type II-S used in bilayer experiments was obtained from Sigma.

2.2. Porin isolation and purification

Porin was isolated from *Rhodobacter capsulatus 37b4* essentially as described by Schilz et al. [10] with several modifications. Cells were grown for two days at 30°C in RCV medium [11] and centrifuged to form the cell pellet (10,000 rpm, 4°C, 20 min, GSA rotor). Cells were washed by homogenizing and centrifuging in 10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0 (10,000 rpm, 4°C, 20 min, GSA rotor). Whole cells were then resuspended in 10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0 containing a small amount of DNase I, and broken using a French press at 10,000 psi. The resulting suspension was centrifuged (15,000 rpm, 4°C, 20 min, Beckman SS34 rotor) to remove whole unbroken cells. The supernatant containing cell fragments was then centrifuged to produce the cell envelope pellet (40,000 rpm, 4°C, 2 h, 70 Ti rotor). This was washed by homogenizing and centrifuging in 10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0 twice until the supernatant was clear (27,000 rpm, 4°C, 30 min, 70Ti rotor).

Pellets were resuspended in 10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0, 2% Triton X-100, stirred for 30 min at 40°C then centrifuged (32,000 rpm, 4°C, 30 min, 70 Ti rotor). This was repeated, stirring for 30 min at 38°C and centrifuging as before. Pellets were washed by homogenizing and centrifuging four times in 10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0 (32,000 rpm, 4°C, 30 min, 70 Ti rotor). Pellets were resuspended in 2% *N,N*-dimethyldodecylamine-*N*-oxide, 0.5 M NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.01% mercaptoethanol, 20 mM Tris-HCl pH 8.0 and stirred at 37°C for 70 min, then centrifuged (32,000 rpm, 4°C, 1 h, 70 Ti rotor). The supernatant was diluted in the same volume of 0.08% *N,N*-dimethyldodecylamine-*N*-oxide, 5 mM NaN_3, 20 mM Tris-HCl pH 7.2 and concentrated down to half volume by vacuum centrifugation. This step was repeated once. The protein solution was then applied to a standardised Sephacryl S200HR column (1 × 60 cm) with a flow rate of 33 ml/h. Fractions were collected every two minutes after elution of the void volume. The porin-containing fractions were stored at 4°C. The column running buffer was 20 mM Tris-HCl pH 7.2, 3 mM NaN_3, 0.3 M LiCl, 0.6% octylpolyoxyethylene. The elution profile of the porin-containing solution showed the porin peak which, on comparison with molecular weight standards, was found to be approximately 96,000 Da, which compared favourably with the molecular weight determined by amino acid sequencing [10].

2.3. SDS-PAGE

SDS-PAGE was carried out on small amounts of the eluted fractions within the porin peak. Samples were loaded onto 4% stacking/20% resolving gels. Trimeric porin sized bands of approximately 96,000 Da molecular weight were detected after staining with Coomassie blue (see Fig. 1). For thermal stability experiments, porin trimers were heated for 5 min at 37°C, 56°C, 75°C or 100°C before loading onto the gel.

2.4. Reconstitution into planar bilayers

Formation of planar bilayers was as described previously [12,13] 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 [14]. The buffer used throughout was 10 mM $CaCl_2$, 10 mM HEPES pH 7.2 containing either 1 M NaCl, 1 M KCl or 1 M potassium-D-gluconate. In experiments where the pH was lowered this was achieved using small amounts of 200 mM citric acid on the *cis* side after incorporation of a porin trimer. *R. capsulatus 37b4* porin was added to the *cis* side of the teflon septum, at approximately 3×10^{-4} μg/ml final concentration, so as to introduce only single channels into the membrane, diluted in column running buffer (as above).

2.5. Electrical measurements

Membranes were tested for stability with applied p.d.'s of up to 250 mV and, after mixing, porins were inserted into the membrane by applying p.d.'s 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). The shortest sampling interval used was 0.2 ms enabling fast events to be monitored.

3. Results

3.1. Incorporation into planar bilayers

The major porin of *R. capsulatus 37b4* was found to incorporate routinely into planar lipid bilayers forming trimeric channels. On incorporation of single trimers of the porin it was immediately observed that these channels open at low or zero p.d. and close in three equal steps (Fig. 2) when a high p.d., such as ±200 mV, is applied (described in Fig. 2 legend). That is, the opening and closing of the porin trimers was voltage dependent. In a number of experiments, several different conductance states were observed for each trimer incorporated (Fig. 3). Monomer states were observed each with a conductance one third of that of a whole trimer. Mini channels were also observed with a conductance of one half that of a monomer. These states have also been observed for the *E. coli* porins OmpC and OmpF in our laboratory. Further, at p.d.'s of approximately 150 mV, fast transitions between various states were observed, much faster than the usual opening and closing behaviour (Fig. 4), as reported previously [15]. These fast transitions will be discussed elsewhere (Lea, Bishop and Lakey, in preparation).

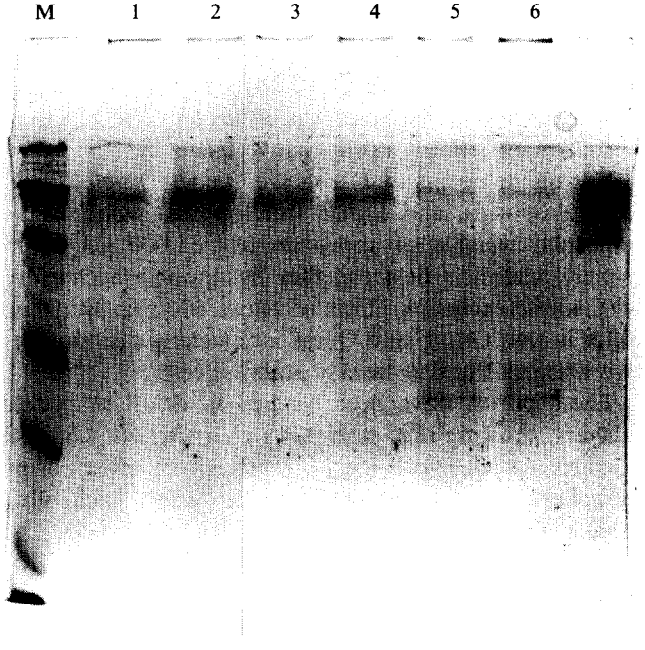

Fig. 1. SDS-PAGE of *Rhodobacter capsulatus 37b4* porin trimers before and after heat treatment. M, marker; 1 and 2, trimers before heat treatment; 3, 37°C; 4, 56°C; 5, 75°C; and 6, 100°C.

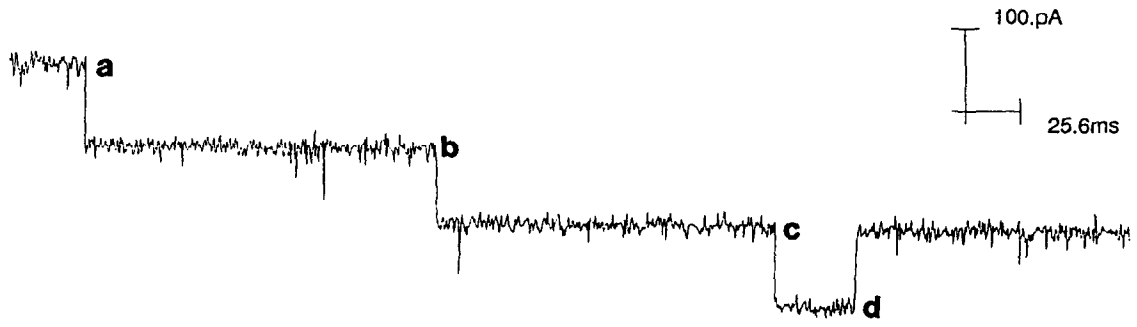

Fig. 2. Closing of a *Rhodobacter capsulatus 37b4* porin trimer on application of −200mV, in three equal steps (a, ~300 pA; b, ~200 pA; c, ~100 pA; d, ~0 pA), each approximately 500 pS. Buffer, 1 M KCl.

3.2. Symmetry of behaviour

In response to p.d.'s of opposite polarity, conductance behaviour of the *R. capsulatus 37b4* porin trimers was found to be completely symmetrical as regards closure. In every experiment we find that the same trimer produces a different pattern of responces when positive or negative p.d.'s are applied. But we have no evidence to suggest the proposition that the channels are functionally asymmetric. This characteristic has also been observed for the *E. coli* porins OmpF and OmpC [16], but contrary to the assymmetry of behaviour described for the *Acidovorax delafieldii* porin [17].

3.3. Selectivity

Conductance of single porin trimers was measured in various electrolytes, the results of which are shown in Table 1. These values show that the porin is strongly selective not only for cations over anions but also, due to the higher conductance value in NaCl compared to that in KCl, selective between cations. The single channel conductivities in KCl and K-d-gluconate are barely distinguishable. Assuming that gluconate is impermeable due to its large size, this means that the channels are cation selective. Further, the ratio of single channel conductivities of Na^+ and K^+ will be 665/468 = 1.42. The selectivity of the channel will be even higher than this because the ratio of conductivity of Na^+ and K^+ respectively, in aqueous solution, is always less than one.

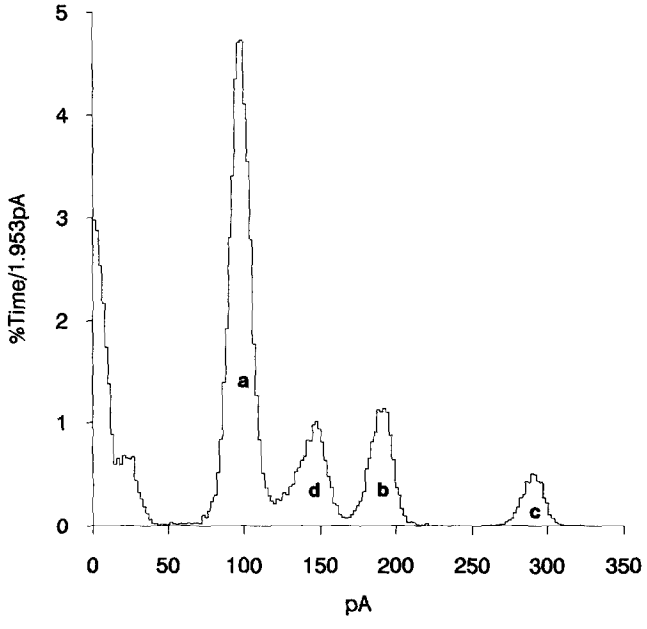

Fig. 3. All points histogram showing the type of conductance states seen in all the buffer solutions used: three equal monomer states (a, ~100 pA; b, ~200 pA; c, ~300 pA), each step approximately 500 pS, making up one trimer and minichannels (d) approximately half the size of a single monomer channel (~250 pS). The buffer used here was 1 M potassium-d-gluconate. The p.d. was −200 mV on the *cis* side.

3.4. Voltage dependence

In addition to porin trimers opening at low p.d. and closing at high p.d., when a range of positive and negative p.d.'s is applied, trimers appeared to spend a decreasing amount of time in the open states as p.d. was increased from ±50 mV to ±200 mV. That is, the open times of trimers were voltage dependent. The average time before a single channel closing event occured after application of a range of p.d.'s was also measured and found to decrease exponentially with increasing pd (Fig. 5a). Further, monomer conductance increased linearly with increasing p.d. (Fig. 5b).

3.5. Effect of a pH change

Lowering pH to approximately 3.2 with a small amount of 200 mM citric acid on the *cis* side resulted in a noisier conductance trace when negative p.d. was applied to the *cis* side whereas at positive p.d. on the *cis* side

Table 1
Conductance measurements, measured in picoSiemens (pS), for the *R. capsulatus 37b4* porin channels in different electrolytes

Buffer	Conductance, *G* (pS) mean ± S.E.
1 M NaCl	665 ± 4 (n = 70)
1 M KCl	468 ± 7 (n = 31)
1 M K-d-gluconate	492 ± 10 (n = 81)

The values quoted are for one third of a trimer.

it remained that same as before the pH change. Whether or not the observed noise is due to fast openings and closings is not clear. If it is, then the events are beyond the 0.2 ms resolution of the system. Trimers were also observed to close down at lower p.d.'s than before the pH change, at as low as ±100 mV. Gating of conductance was observed at lower p.d.'s of about ±50 mV, although the negative p.d. trace was much noisier than the positive p.d. one.

4. Discussion

4.1. Selectivity of the porin trimers

The *R. capsulatus 37b4* porin was found to be highly cation selective. If the charges on Loop 3 of the *R. capsulatus 37b4* porin and OmpF are compared, then it is obvious that the net charge on Loop 3 of *R. capsulatus 37b4* porin is much more negative than that on Loop 3 of OmpF (see Table 3). Bauer et al. [18] have found that only one single amino acid is necessary for controlling the selectivity of the PhoE porin. That is, PhoE, which is anion selective, has a lysine (K131, positively charged) whereas OmpF, which is weakly cation selective, has a glycine (G131, not charged). Mutagenesis of the K125 in PhoE to glutamic acid reverses the ion selectivity to a cation selectivity comparable to that of OmpF, whereas substitution of other lysines above and below the eyelet region has little effect on selectivity. This shows the importance of charged residues in the eyelet region in determining selectivity. In the same position as the K125 responsible for PhoE selectivity, that is, in the eyelet region, *R. capsulatus 37b4* has a number of negatively charged residues D108, D109, E114 and E115 which probably account for the cation selectivity observed. If *R. capsulatus 37b4* is compared to OmpF with respect to overall charged amino acids (see Table 2), then it is clear that *R. capsulatus 37b4* is much more negatively charged than OmpF, which probably accounts for the observed differences in cation selectivity. According to the calculaions of Weiss et al. [4], there is a large area of negative electrostatic potential at the mouth of the barrel, probably attracting cations from the environment and contributing to the cation selectivity of the porin; this is obviously what is observed in these results.

Table 2
Comparison of overall charges between *R. capsulatus* porin and OmpF porin of *E. coli*

	Positive	Negative	Net charge	Ca^{2+}	Net charge after Ca^{2+}
R. capsulatus	18	51	−33	2	−29
OmpF	30	41	−11	0	−11

Positive charges include histidine of which there is only one in both porins.

Table 3
Comparison of charges on Loop 3 of *R. capsulatus 37b4* porin and OmpF of *E. coli*

	Positive	Negative	Net charge	Ca^{2+}	Net charge after Ca^{2+}
R. capsulatus	2	12	−10	2	−6
OmpF	0	6	−6	0	−6

4.2. Symmetry of channel conductance

The *R. capsulatus 37b4* porin channel was found to be completely symmetrical with regards closure in response to p.d.'s of opposite polarity, i.e. patterns of closure in response to opposite p.d. were similar. This symmetry of voltage dependent behaviour points to the suggestion that the controller of voltage dependent opening and closing is not situated on one end or other but is positioned deep inside the channel away from either exposed end. If the controller were towards either end then symmetrical patterns of closure in response to p.d.'s of opposite polarity would not be expected.

It has been suggested that this controller is in fact the polypeptide loop projecting into the channel pore and constricting the channel at its centre, loop 3 [1], and that the control may occur by a conformational change in loop 3 exposing buried charged residues in the barrel wall opposite loop 3 (see Table 4), although it has been found that the loop 3 crystal structure is fairly rigid [2].

Symmetry of conductance would also suggest that this particular channel in its trimer state, is made up of three

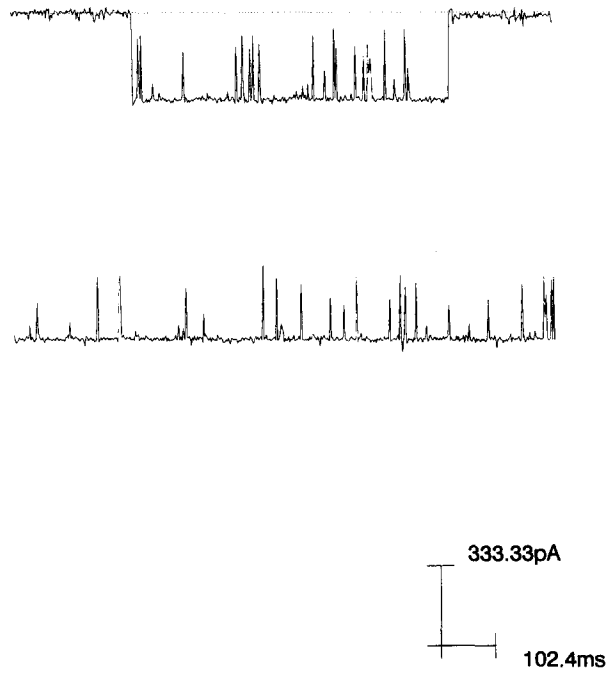

Fig. 4. 'Fast flickering' of conductance of a single *R. capsulatus 37b4* porin trimer. The buffer used here was 1 M NaCl, and the p.d. was +200 mV on the *cis* side.

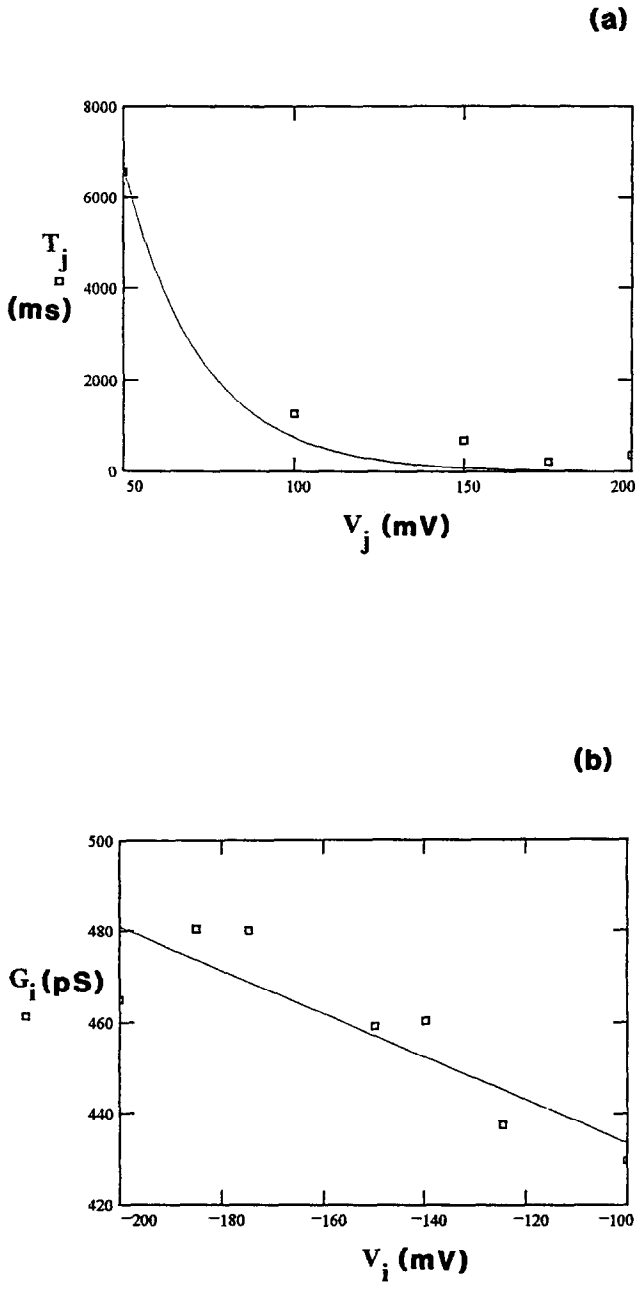

Fig. 5. (a) Average time before a closing event occurs (T_j, ms) as a function of p.d. (V_j, mV), solid line is the exponential curve fitted to the points (boxes). (b) Single channel monomer conductance (G_i, pS) as a function of applied p.d. (V_i, mV), solid line is the regression line fitted to the points (boxes). Data was taken from experiments where 1 M KCl was the buffer.

continuous channels throught the pore, as seen from X-ray structures [1].

4.3. Effects of a pH change

The fact that a lowering of pH in the vicinity of the porin, produces different responses to applied p.d. suggests that charged amino acids must be involved in the voltage gating mechanism. As trimers were observed to close completely at a lower p.d. than without the pH change (±100 mV) and to gate at an even lower p.d. (±50 mV) it would be correct to assume that the channel had become more voltage sensitive, producing reactions at a lower threshold than before.

When pH is lowered, negative charges are titrated so the only charged amino acids present in these conditions are positive *arg* and *lys* residues. Therefore the loss of high amounts of negative amino acids must produce the increased voltage sensitivity. Todt and McGroarty [19], studied effects of pH on bacterial porin function, particularly OmpF, OmpC and PhoE, and observed at least two open channel configurations of low and high conductance; with the small channels detected at low pH and larger channels detected at high pH. The 'mini' channels observed for *R. capsulatus 37b4* could possibly be the small channels detected by Todt et al. [19]. In a second paper [20] they suggest that the one histidine residue in each of these porins is involved in the pH induced switch in channel size. *R. capsulatus 37b4* also has one histidine residue (see Table 2 for overall charges in the porin).

4.4. Voltage dependence

The average time before the first closing event of a single porin trimer after applying p.d., shows an exponential decrease with increasing p.d. (Fig. 5a). Further, average single channel monomer conductance, was found to increase linearly with p.d., showing a dependence of conductance on applied voltage, shown in Fig. 5b, together with the fitted regression line. The type of voltage gating of porins observed here has been reported in *Neisseria gonorrhoeae* [21] and voltage dependence has been reported for *Pasteurella multocida* porin [22] and for OmpF & OmpC by [23] as well as in our laboratory.

4.5. Thermal stability

R. capsulatus 37b4 porin was found to be stable as a trimer at 37°C and 56°C, but breaks down to mainly monomers at 75°C and 100°C. This behaviour was similar to that reported for OmpC wild type porin and spontaneous mutants [12]. On comparison, the same pattern appeared, that is, porin was present in the monomer state at higher temperatures in a higher proportion than trimers. Wild type OmpC was highly thermal stable with only small amounts of trimer remaining at 100°C after

Table 4
Comparison of charges inside the barrel wall of the porin, opposite Loop 3, in both *R. capsulatus 37b4* porin and OmpF porin

	Positive	Negative	Net charge	Ca^{2+}	Net charge after Ca^{2+}
R. capsulatus	6	1	+5	0	+5
OmpF	4	1	+3	0	+3

Figures on Tables 2–4 were calculated from topology models of OmpF and *R. capsulatus 37b4* porin monomers ([1] and [10], respectively).

5 min. By comparison *R. capsulatus 37b4* porin is of intermediate thermal stability.

In a previous study, Woitzik et al. [9] reported some preliminary measurements of *R. capsulatus 37b4* porin channels in bilayers. The p.d.'s used in their study did not exceed 20 mV and no voltage dependent behaviour or channel closure was observed. This could be due to the different porin isolation procedure used. From this characterisation of the *R. capsulatus 37b4* porin it seems that not only is there a common structure between all the porins i.e. similar folding of polypeptide strands, but many of the functional characteristics are also very similar, certainly between *R. capsulatus 37b4* porin and the *E. coli* porins. Future studies will reveal the mechanism of porin function in greater detail, using *R. capsulatus* as a model for comparison with other porin data.

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