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Biophysics of gating phenomena in voltage-dependent OmpC mutant porin channels (R74C and R37C) of *Escherichia coli* outer membranes

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Abstract The mechanism by which the membrane potential closes and opens voltage-dependent β-barrel membrane channels is not fully understood. OmpC porins form trimeric water-filled channels when incorporated into artificial bilayers, each monomer having a conductance of approximately 510 pS in 1 M KCl. These channels are relatively insensitive to membrane potential difference (pd) and close only when the pd exceeds ± 250 mV. Another well-known trimer, OmpF, has a monomer conductance of approximately 780 pS in 1 M NaCl, is more sensitive to pd, and can be closed reversibly when a pd of more than ± 150 mV is applied to the channel-containing membranes. With the aid of the 3D atomic structure of these channels determined by X-ray crystallography, and using site-directed mutagenesis, specific amino acids can be substituted in desired locations in the channel lumen. In this study we have used mutants 37C and 74C and attached fluorescence probes to them to monitor polarity changes in the channel lumen during gating. From the observed changes in polarity, we conclude that conformational changes occur in the channel which interrupt the electrolyte conducting pathway.

Keywords Porin · Channels · Gating · Bilayer · Biophysics

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Introduction

No definite mechanism has been reported so far to explain the voltage-dependent gating processes in bacterial porin channels. The amino acids lining the inside of the channel barrel wall play a crucial role in defining the selectivity of the channel to ions and different hydrophilic and hydrophobic molecules. Channel closure may be the result of the formation of a hydrophobic microenvironment imposed by an applied potential difference (pd). This blockage could be formed through a change in the conformation of the amino acids lining the channel, resulting in the exposure of hydrophobic amino acids and burying hydrophilic ones. This hypothesis has been addressed in this study by examining the hydrophobicity of the inside of the channel lumen at two different positions during gating and in the open and closed states of the channels. Although porins form stable water-filled channels, it may be that their conformation is not completely rigid. For example a "slippery" path is formed by hydrophobic amino acids to facilitate the passage of the dextrins in LamB porins (Klebba and Newton 1998). The resistance to the passage of antibiotics could be another consequence of these characteristics. Formation of a possible transit path for colicins is another indication of the flexibility in the eyelet and also in the channel extracellular mouth region.

The experiments on the OmpC mutant (W56C), with replacement of a tryptophan residue located in the trimeric core of the channel by cysteine, showed a smaller conductance size (30%), an increased voltage sensitivity (40%) and lower heat stability (Bishop et al. 1996). This is another indication of the possible involvement of this region in the gating processes in response to an applied pd. The positively charged amino acids in the so-called "eyelet region" are located on the barrel wall, pointing toward the channel lumen and constricting the channel. Channel selectivity has been studied (Benz 1984) and involvement of four residues (arginines) in the channel

selectivity has been reported (Cowan et al. 1992). Furthermore, it has been shown that gating and closure of the channel in response to the applied pd is not caused by the movement of the entire L3 loop located in the lumen of the channel (Phale et al. 1997; Bainbridge et al. 1998). The effects of introduction of bulky charged molecules in this region have been studied in OmpC channels and the resulting channel conductance and voltage sensitivity have been investigated (Gokce et al. 1997). This group reported a smaller conductance and increased voltage sensitivity in the mutant A37C, caused by labelling cysteine 37 with the fluorescence probe Thiolyte. On the other hand, changes in the bilayer rigidity have shown some effects on the gating and conductance size of the channel (Buehler et al. 1991; Mobasheri and Lea 1998), although where the membrane imposes its effect on the channel is not yet known.

A variety of fluorescence probes has been used to investigate membrane fusion, lipid dynamics, intracellular lipid transfer, bilayer to hexagonal phase transitions in liposomes, protein-lipid interaction and the organization of lipids and proteins in membranes. The fluorescence probe NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole) has been used in a lipid-labelled form (Chattopadhyay and London 1988; Chattopadhyay 1990) to investigate the membrane properties, protein structure and conformational changes (Wakabayashi et al. 1990; Carlier et al. 1994). Thiolyte is another fluorescence probe that has been used to determine the protein-bound thiol component in plasma membranes (Mansoor et al. 1992) and also to study protein structure-function relationships (Gokce et al. 1997). A more selective fluorescence probe, IAEDANS, has been used mainly in fluorescence resonance energy transfer studies to detect structural changes during protein-protein interaction processes (Steer and Merrill 1994). This probe, which is sensitive to the polarity of its surrounding microenvironments, selectively binds to the specific amino acids (in this case to the thiol group) exposed to the aqueous medium. As this probe fluoresces only when it is bound to thiol groups, its binding to the channel can be monitored efficiently. Furthermore, the emission wavelength of the probe varies as a result of changes in the medium hydrophobicity. This makes it a powerful tool to investigate the polarity changes that might occur during the gating process. The fluidity of membranes can be investigated by monitoring the lateral diffusion of membrane lipids labelled with fluorescence probes (Ladha et al. 1996). Fluorescence recovery after photobleaching (FRAP) is a technique by which the lateral diffusion of the unbleached lipids into a membrane spot already bleached by a laser beam is measured. This system, normally used to investigate bilayer characteristics, possesses all the facilities needed to analyze the activity of the fluorescence probe-labelled channel proteins.

A number of different groups has drawn attention to the importance of the amino acids situated in the protein-membrane interface (Gonzalezmanas et al. 1992; Doring et al. 1997; Pattnaik et al. 1997). Others (Liu and Delcour 1998) have suggested that hydrogen bonds between these residues and the adjacent ones located on the L3 loop (D315A, D118Q and R92Q) play a role in spontaneous gating of the channel through disruption of the hydrogen bonds. They have also suggested that the transition between the open and closed states is not driven by the membrane pd, but by thermal oscillations between functionally distinct conformations.

Material and methods

The mutants R37C and R74C resulted from maltoliposaccharide-based selection of *Escherichia coli* porins (Lakey et al. 1991). The fluorescent probe Thiolyte (monobromobimane) was obtained from Calbiochem, NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole) and IAEDANS (1,5-IAEDANS [5-({[(2-iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid] were from Molecular Probes; Sephadex G-25M prepacked PD-10 chromatography columns were from Pharmacia Biotech; dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) were from Sigma; and acetonitrile from BDH. The absorbance of the protein and labelled channels was measured using a Philips UV/Vis spectrometer, and the fluorescence analysis was carried out using a Shimadzu spectrofluorimeter RF-5000.

Bilayer studies

Single channel measurements of labeled and unlabelled channels were carried out as previously described (Bishop et al. 1996).

Flourescent labelling procedures

The purified OmpC mutant channels (R37C and R74C) were labelled with Thiolyte, NBD-Cl and IAEDANS. The protocol used for labelling was slightly different for each probe; thus they are explained separately.

Thiolyte labelling of OmpC cysteine mutants

The fluorescence probe was kept at 50 nM in acetonitrile as a stock solution, at 4 °C in the dark. Once diluted into an aqueous solution, the probe had a half-life of about 4 h at room temperature.

The protein sample was reduced in the presence of an excess amount of DTT at room temperature for 1 h. The mixture was then passed through a G-25 column, already equilibrated with buffer (5 mM $NaHCO_3$, 1% SDS, 0.3 M NaCl, pH 7). The fractions containing maximum concentration of protein (85–95%) were selected according to their absorbance. Excess Thiolyte was then added to the selected vial and left shaking at room temperature overnight. The excess unbound label was removed from the reaction solution by passing it through a G-25 column. The eluates were collected and their absorbance at 280 and 380 nm corresponded to the unlabelled and Thiolyte-labelled proteins, respectively. All manipulations with the probe were carried out in the dark.

Since, for monobromobimane, ϵ_{280} is approximately equal to ϵ_{380}, the labelling stoichiometry (S) was calculated from the two absorbance (A) values at 280 and 380 nm wavelength as $S=(A_{380}/\epsilon_{380})/\{(A_{280}-A_{380})/\epsilon_{280}\}$. The scatter contribution was eliminated by subtraction of appropriate blanks.

NBD-Cl labelling of OmpC mutants

The fluorescence probe NBD-Cl stock solution was 10 mM in 96% ethanol and 0.1 M DTT. The labelling reaction was carried out in 0.1 M phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4) at pH 6. The buffer was boiled and cooled down under nitrogen to reach a 99% nitrogen equilibration. The protein was dialyzed using dialysis tubing with a cut-off of 3 kDa, at room temperature, for 30 min against 0.1 M phosphate buffer to remove SDS from the protein stock solution. Removal of SDS was necessary to avoid sedimentation of SDS when the pH of the solution in the bilayer or fluorescence studies had to be changed.

The cysteine residues of the dialyzed protein were reduced during an incubation process in the presence of 420 μM DTT (10-fold excess over protein) for 1 h at room temperature under nitrogen. The mixture was then dialyzed for 45 min to remove DTT.

The labelling was started by incubating the reduced protein solution in the presence of a 20-fold excess of NBD-Cl at room temperature for 2 h with continuous stirring in the dark. The excess of unbound fluorescent label was removed by further dialysis in 0.1 M phosphate at room temperature in the dark for 1 h.

The protein concentration was measured by means of its absorbance at 280 nm and the stoichiometry of the labelling was calculated using the extinction coefficient of the protein, ϵ_{280}(OmpC) = 59,880 M^{-1} cm^{-1}, and probe, ϵ_{425}(NBD-Cl) = 13,000 M^{-1} cm^{-1} at pH 5–8 (9.8×10^3 M^{-1} cm^{-1} at 336 nm, 2.6×10^4 M^{-1} cm^{-1} at 480 nm).

The labelled protein was excited at 425 nm with a slit width of 5 nm using a Shimadzu RF 5000 spectrofluorimeter and the fluorescence emission was scanned over a range of wavelengths from 400 to 600 nm with an emission slit width of 3–8 nm. The wavelength of the emission maximum was found to be 520 nm.

IAEDANS labelling of OmpC mutants

The cysteine residues were first reduced through incubation in a solution containing 25 mM DTT, 50 mM Tris HCl (pH 8.0), 6 mM β-mercaptoethanol, 2 mM sodium acetate and 5 mM EDTA at 37 °C for 4 h. The buffer was then changed for 50 mM Tris-HCl (pH 8.0), which had already been degassed and equilibrated with 99% nitrogen. The purified protein was passed through a pre-packed chromatography column filled with Sephadex G-25. The elute was added to N_2-purged reaction vessels containing a 5-fold molar excess of IAEDANS which had been dissolved in the same buffer used for the protein. The vessels were sealed, protected from light and stirred at room temperature for 2 h. The mixture was then passed through the same column as before, and fractions of about 400 μL were collected in separate Ependorf vials. The absorbance of each fraction at 280 nm was used to measure the total protein concentration in the vial. The stoichiometry of labelling was determined by UV absorption spectroscopy using the following extinction coefficient: ϵ_{336}(IAEDANS) = 5700 M^{-1} cm^{-1}. The degree of labelling of the protein by IAEDANS was estimated using the following equation: (A_{336}/ϵ_{336})(MW of protein/mg protein mL^{-1}) = moles of dye/moles of protein, in which A_{336} was the absorbance value of the dye at the absorption maximum wavelength and ϵ_{336} was the molar extinction coefficient of the dye at the absorption maximum wavelength.

Fluorescence measurements

The fluorescence of the labelled channels was measured in three different states: in aqueous media, incorporated in liposomes and in artificial planar bilayers.

Fluorescence of labelled protein in aqueous media

Fluorescence measurements were carried out at right angles to the incident light using a Shimadzu RF 5000 spectrofluorimeter equipped with a stirrer in the sample compartment. The probes were excited at their particular excitation wavelength, and the resulting fluorescence emission was measured at a range of wavelengths between 300 and 600 nm. The excitation slit width was set at 3–5 mm and the emission slit width at 3–8 mm, depending on the intensity and the broadness of the fluorescence bandwidth. The temperature of the reaction solution was thermostatted at 20 °C. The pH of the sample was changed, based on the experimental requirements. The exact pH value was measured by a pH meter whose probe had already been installed in the cuvette.

Fluorescence of labelled channels incorporated into liposomes

In this part of the experiment, the labelled proteins were incorporated into single shelled liposomes made from soybean lecithin (Sigma) type II-S. Incorporation was carried out by stirring liposomes in the presence of labelled protein in the dark for 2 h at room temperature.

Fluorescence emission of labelled channels in planar bilayers

The laser-based fluorescence fluorimetery of the labelled protein in bilayers was carried out using a spot photobleaching FRAP set-up (Clark et al. 1990; Ladha et al. 1994, 1996). The apparatus was constructed using components from a Nikon Optiphot microscope mounted on its side with a horizontal optical axis. The binocular eyepiece and nosepiece of the microscope were mounted on a special Nikon bracket, bolted to an optical rail (Newport, Microcontrole) on the laser table (Photon Control, Cambridge). The Montal-Mueller planar lipid bilayer chamber was mounted on a separate bracket attached to a micrometer-controlled transition stage that allowed focusing, and was bolted to the optical rail. The chamber was located inside a metal box acting as a Faraday cage in order to minimize the interference from noise sources during the bilayer studies.

Results

The protein concentration before and after labelling was measured using the absorbance at 280 nm, and only fractions with the highest protein concentration (with a yield of 70–95%) were selected for the labelling experiment. Following the labelling, the protein concentration and the stoichiometry of the reaction were measured, showing a calculated value of about 1.03. At every stage of the labelling procedure the yield of protein, as well as the labelling characteristics, was monitored.

OmpC mutant (R37C)

Wild-type OmpC (Fig. 1) does not have any cysteine residue; thus the presence of a newly introduced cysteine could be checked by the fluorescence of labels that selectively targeted the cysteine residue. The product of this covalently bound complex was excited and fluorescence emission occurred only when the fluorescent probe was bound to the thiol group of the cysteine residue.

The yield of protein labelled with NBD-Cl and IAEDANS, and the efficiency of labelling, was estimated by their absorbance at 280 nm and 336 nm; both were normally high.

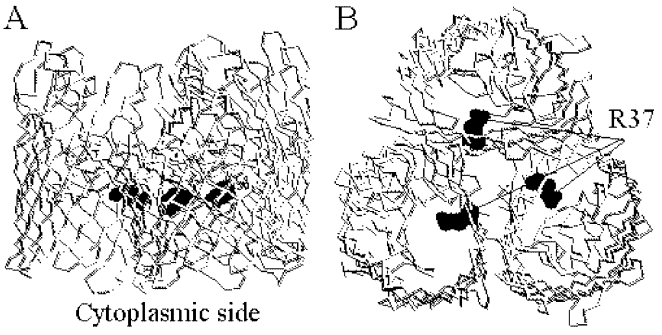

Fig. 1A, B. Location of R37 in the theoretical homology-based model of *S. typhi* OmpC obtained from the Brookhaven Protein Data Bank (code 1IIV) and is represented using "RasMol". Arginine 37 is shown in spacefill mode in black in side view (**A**) and cytoplasmic view (**B**) of the trimeric OmpC channel

Following fluorescence labelling of the protein, two different approaches were used in investigation of conformational changes during gating. Firstly, the channel activity in the artificial bilayer was studied. Secondly, the fluorescence emission of the labelled protein both in aqueous solution and incorporated into liposomes was monitored at different pH values.

Channel activity of OmpC mutant R37C

OmpC mutant R37C porin channels labelled with NBD-Cl and IEADANS were incorporated into artificial bilayers and more than 100 recordings were made of channel activities, focusing on the channel conductance size at the main states and substates and their variations with respect to the applied pd. The voltage at which the gating was initiated in the channel and the voltage required to close the channel were also monitored. Fast flickering, cooperative openings and closure of the channel, and incorporation characteristics were other phenomena that were monitored in this part of the study.

NBD-Cl labelled channels were incorporated easily and stayed in the membrane with no signs of rejection. There were occasions when two or three trimers were incorporated into the membrane at once. The channel showed a monomer conductance of 430 ± 20 pS (Fig. 2a). Furthermore, a variety of substates was identified. Channel gating started at 20 mV and the applied pd values as low as 50 mV could close the channel. Simultaneous closure of two or more monomers was another characteristic of these channels (Fig. 2b). As shown, the channel displayed two different behaviour patterns: one at pH 7.4 with a very steady current level and no frequent transition between different states (Fig. 2a); and the second one at pH 3.0 with frequent gating, fast flickering and fast transitions between the main monomeric states as well as substates (Fig. 2b).

The incorporation of OmpC mutant R37C, labelled with IAEDANS, into artificial bilayers was similar to the wild-type and took place within a maximum period

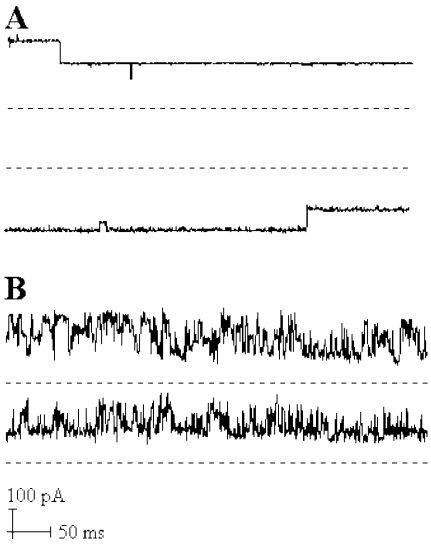

Fig. 2A, B. Current traces of OmpC mutant (R37C) labelled with NBD-Cl at ± 100 mV applied to the *cis* side. Channel activities have been recorded at pH 7.4 (**A**) and pH 3 (**B**). The *dashed line* represents zero pA level. The buffer used was 1 M KCl, 10 mM Hepes, 10 mM $CaCl_2$

of 2 h; no rejection or loss from the membrane was observed. The channel produced a range of different conductances, from which the most frequent monomeric conductance of the channel was identified (440 ± 30 pS). The channel sensitivity to the applied pd was slightly lower than R37C-NBD channels and a minimum of 100 mV was required to close the channel. However, channel gating started at about the same pd, 20 mV, as for the R37C-NBD channels (Fig. 3). Fast flickering was frequently observed for these channels, and simultaneous closure of more than one monomer occurred on a number of occasions.

The kinetics of the channel gating, as well as conductance size of the channel, were analyzed at different pH values. It was clear that acid pH values increased the voltage sensitivity of the channels so that gating in R37C-IEADANS channels initiated at pd values as low as 20 mV. Fast flickering was also monitored at pH 2 when –50 mV was applied to the cis side (Fig. 4). Furthermore, as a result of the pH effects, the channel mean open time decreased by about 30% and most of the monomers spent longer in the closed state.

Fluorescence studies of OmpC mutant R37C

Fluorimetry of the sample containing R37C-NBD with $OD > 0.890$ was carried out using a Shimadzu spectrofluorimeter. Excitation of the protein at 440–470 nm produced a single peak at about 520 nm. The maximum intensity of this peak was observed when the labelled protein was excited at 450 nm and 470 nm (Fig. 5). Both excitation and emission slit widths were 4 nm. The effect of changes in pH on both spectra was similar and did not cause a significant shift in the

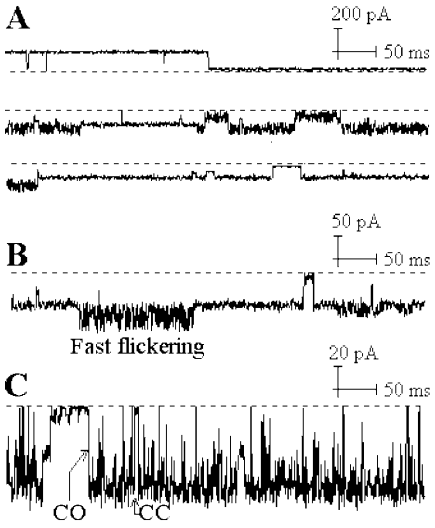

Fig. 3. Current traces of IAEDANS-labelled OmpC mutant (R37C) at 150 mV (**A**) and 100 mV (**B**, **C**). The *dashed line* represents the zero pA level where the channels close. Two cases of cooperative closure (*CC*) and cooperative openings (*CO*) of the channel are shown. The buffer was 1 M KCl, 10 mM $CaCl_2$, 10 mM Hepes, pH 7.2

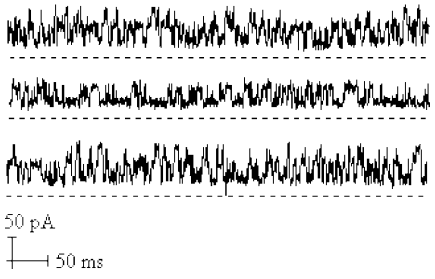

Fig. 4. Current traces of OmpC mutant (R37C) labelled with IAEDANS at pH 2. The channel activities have been recorded at –50 mV. The *dashed line* represents zero pA level. The buffer used was 1 M KCl, 10 mM Hepes, 10 mM $CaCl_2$

emission wavelength. However, the intensities of the fluorescence emissions were different at acid and alkaline pH values.

The wavelength for the fluorescence emission maximum of R37C-IAEDANS was 490 nm with an excitation at 336 nm. Changes in pH had no effect on the wavelength of the emission maximum (see Fig. 6), but the intensity of the fluorescence emission was maximal at pH 7.

OmpC mutant R74C

In this mutant, R74 is located in the trimer core area, facing the channel lumen (see Fig. 7). The purified protein was labelled with NBD-Cl, IAEDANS and Thiolyte fluorescence probes. The corresponding labelled proteins will be called R74C-NBD, R74C-IAEDANS and R74C-Thiolyte, respectively. The yield of labelled protein (80–95%) and labelling stoichiometry

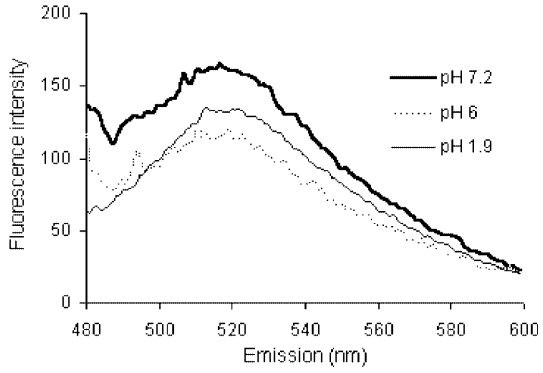

Fig. 5. Fluorescence emission of OmpC mutant (R37C) labelled with NBD-Cl in aqueous solution at different pH values. The labelled protein was excited at 450 nm at different pH values and the corresponding fluorescence emission is shown at 520 nm. The intensity is in arbitrary units

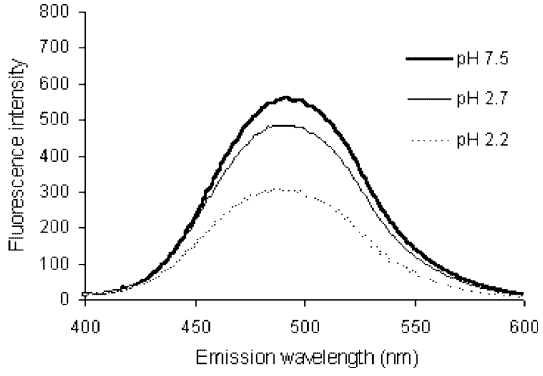

Fig. 6. Fluorescence emission of OmpC mutant (R37C) labelled with IAEDANS in aqueous solution at different pH values. The labelled protein excited at 336 nm. The intensity is in arbitrary units

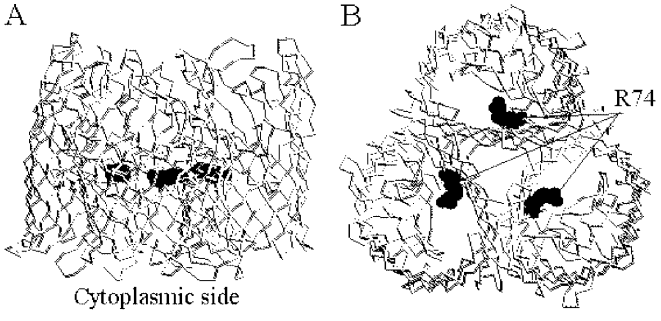

Fig. 7A, B. Location of R74 in the theoretical homology-based model of *S. typhi* OmpC obtained from the Brookhaven Protein Data Bank (code 1IIV) represented using "RasMol". Arginine 74 is shown in spacefill mode in *black* in side view (**A**) and cytoplasmic view (**B**) of the trimeric OmpC channel

(1–1.3) was measured by spectrophotometery. The purified and labelled proteins were incorporated into artificial bilayers.

The channel fluorescence probes were excited at their defined wavelength and the resulting fluorescence emission wavelengths and intensities were measured.

Channel activity of OmpC mutant R74C

OmpC (R74C) was incorporated into soybean artificial bilayers in a similar way to that of wild-type. Incorporation was as easy as wild-type and no ejection or loss from the bilayer was observed. The unlabelled channel produced a conductance of 620 ± 25 pS in 1 M KCl buffer. The gating of the channel began at 60–70 mV, irrespective of the pd polarity, and complete closure took place at pd values higher than ± 100 mV. The channel showed more current fluctuations when a positive pd was applied to the *cis* side, while the current traces at negative pd values tended to be stable most of the time. Fast flickering in this channel was observed mostly at 140–150 mV.

Incorporation of R74C-Thiolyte channels into the bilayer was similar to wild-type and unlabelled mutant. The channel produced a conductance of 450 ± 25 pS, together with substates with conductance of one third to half the monomeric values. Gating at pd values as low as 30 mV indicated higher voltage sensitivity for this channel. The minimum voltage needed to close the channel was ± 50 mV. Some fast flickering at 140–150 mV was also identified in these channels. Another characteristic of this channel was simultaneous cooperative closure of several monomers (Fig. 8).

Labelled OmpC mutant R74C-NBD also incorporated into the bilayer easily and remained in the membrane for the course of the experiment, with no signs of rejection or loss from the bilayer. The channel gave a monomeric conductance value of 470 ± 20 pS at a range of different pd values. The channel gating started at ± 50 mV and complete closure took place at a minimum of ± 50 mV pd. Fast flickering was also identified at 50 mV, during which gating of a fraction of a monomer, whole monomer, and sometimes several monomers all together, took place simultaneously (Fig. 9a,b).

Incorporation of R74C-IEADANS channels into bilayers was more difficult than others and took longer. The monomeric conductance of this channel was 320 ± 20 pS. The channel showed less sensitivity to the applied pd, so that gating was initiated at 80 mV and complete closure took place at voltages higher than 100 mV. The channel showed similar response to opposite polarity (Fig. 9). The I/V curve of these channels showed an ohmic relationship.

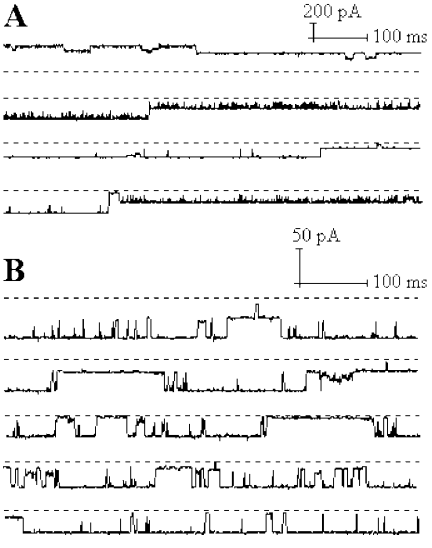

Fig. 8A, B. Current traces of OmpC mutant (R74C) labelled with Thiolyte at pH 7.4. The channel activities have been recorded at 140 mV (**A**) and 50 mV (**B**) applied to the *cis* side. The *dashed line* represents zero pA level. The buffer used was 1 M KCl, 10 mM Hepes, 10 mM $CaCl_2$

Fluorescence studies of OmpC mutant R74C

The fluorescence emission of unlabelled and labelled OmpC (R74C) protein channels was measured using a Shimadzu spectrofluorimeter. The concentration and labelling stoichiometry of the three labelled mutant porin channels, R74C-Thiolyte, R74C-NBD and R74C-IAEDANS, were calculated from UV absorption measurements. The fluorescence emission of these porins was measured in aqueous solutions of different pH values when excited at their corresponding excitation wavelengths. Further, the fluorescence emission of the channels was measured following their incorporation into single-shelled liposomes. The analysis of the effects of the pH changes on the fluorescence emission of these incorporated labelled proteins was the final stage of this part of the study.

When excited at 380 nm, R74C-Thiolyte produced a fluorescence emission peak at about 470 nm. Excitation of the sample at 280 nm also produced a small peak at 470 nm, which was subtracted from the peak produced in response to excitation at 380 nm (Fig. 10). Maximum fluorescence emission of free Thiolyte was found to be at 480 nm and that of the protein-bound probes at about 470 nm.

The fluorescence emission spectra of the protein incorporated into liposomes showed an emission shoulder

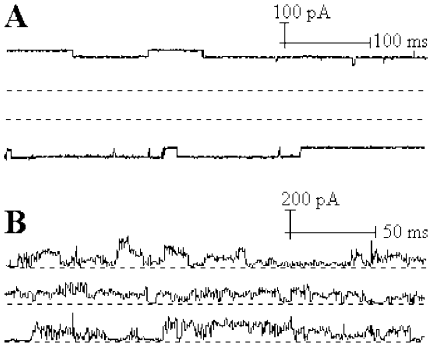

Fig. 9A, B. Current traces of OmpC mutant (R74C) labelled with NBD-Cl at pH 7.4. The channel activities were recorded with 100 mV applied to the *cis* side (**A**). Channel activity of the mutant channel labelled with IAEDANS (**B**) was recorded with 80 mV applied to the *cis* side. The *dashed line* represents zero pA level. The buffer used was 1 M KCl, 10 mM Hepes, 10 mM $CaCl_2$

at about 470 nm, at both excitation wavelengths of 280 nm and 380 nm. The difference between the emission intensities at these two wavelengths was significant, indicating a major emission in response to excitation at 380 nm (Fig. 11).

The emission was measured using a range of excitation wavelengths, 200–600 nm, at pH 2–7. There were three major emission peaks at 390, 470 and 520 nm, produced by labelled proteins. Exciting the unlabelled protein produced no emission peaks. The R74C-NBD fluorescence emission was analyzed at different pH values and showed that the intensity of the emission in all three probes was maximal at acid pH. However, the intensity was found to be time dependent and declined to a lower level which was nevertheless higher than that at neutral pH. This took place over a period of 14–18 min. A similar response was observed at alkaline pH, where the fluorescence emission also increased initially but declined to a lower level in 14 min. In spite of all changes in the intensity of fluorescence emission, the emission wavelength of all three peaks remained constant (Fig. 12). After incorporation into single-shelled liposomes, the emission maximum intensity was found to be at 518–520 nm, similar to that in aqueous solution.

The final stage of this part of the study was fluorescence analysis of the R74C-IAEDANS porin in aqueous solution and in liposomes. The labelled channels were excited at 336 nm and their emission fluorescence was monitored over a range of different wavelengths (200–600 nm), showing an emission maximum at 490 nm. The intensity of the fluorescence emission showed some variation at different pH values. The maximum intensity of the emission was found at neutral pH. The wavelength of the emission maxima was found to be independent of pH (Fig. 13). When incorporated into single-shelled liposomes, the wavelengths of the emission maxima remained unchanged.

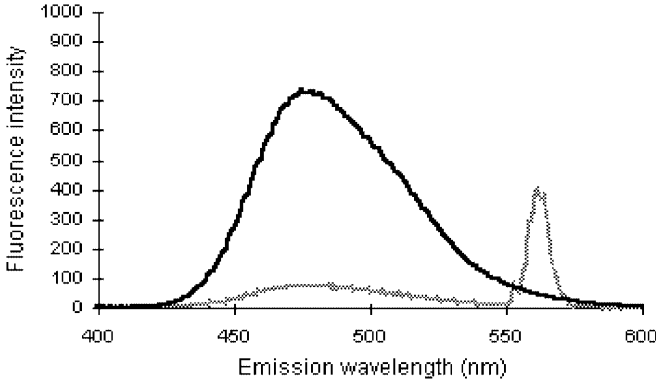

Fig. 10. Fluorescence emission of OmpC mutant (R74C) labelled with Thiolyte. The labelled protein was excited at two wavelengths, 280 nm (*dotted line*) and 380 nm (*solid line*). The resulting emission has produced two peaks at about 470 nm. The intensity is in arbitrary units

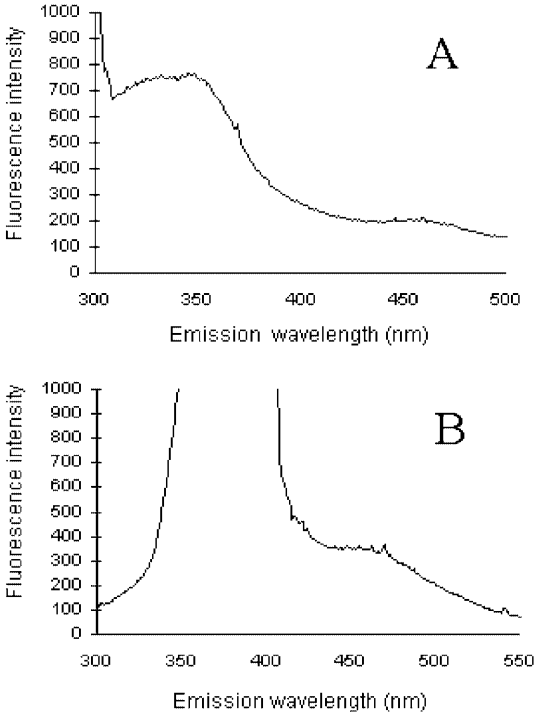

Fig. 11A, B. Fluorescence emission of OmpC mutant (R74C) labelled with Thiolyte incorporated into liposomes. The labelled protein was excited at two wavelengths, 280 nm (**A**) and 380 nm (**B**). The resulted emission produced a peak at about 470 nm. The intensity is in arbitrary units

Fluorescence studies of OmpC mutant channels within artificial bilayers

This study involved measurements of fluorescence from probes attached to the inside of the channel lumen, and at the extracellular mouth of the channel. The labelled protein was incorporated into Montal-Mueller bilayers,

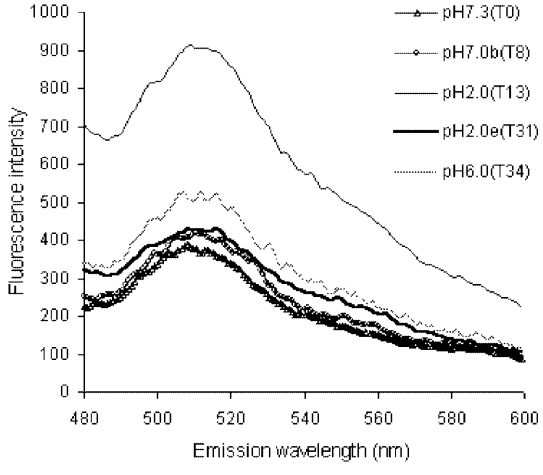

Fig. 12. Fluorescence emission of OmpC mutant (R74C) labelled with NBD-Cl in aqueous solution at different pH values and incubation times. The labelled protein was excited at 336 nm. The incubation times are shown in minutes *in parentheses*. The intensity is in arbitrary units

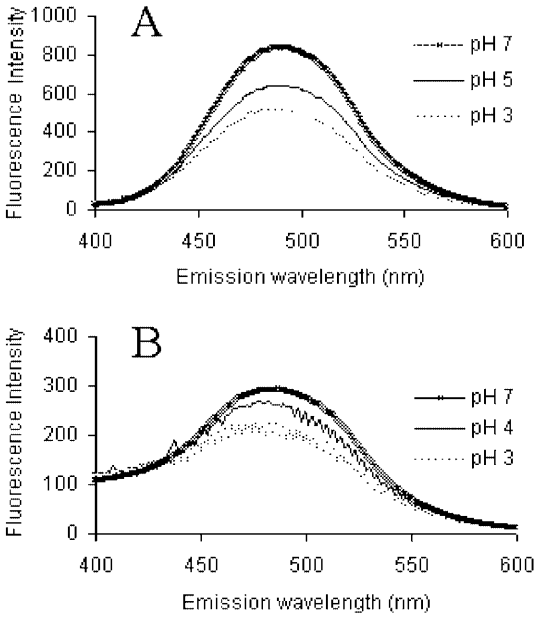

Fig. 13. Fluorescence emission of OmpC mutant (R74C) labelled with IAEDANS in aqueous solution (**A**) and incorporated into liposomes (**B**) at different pH values. The labelled protein was excited at 336 nm. The intensity is in arbitrary units

and its fluorescence emission was measured. The laser based bilayer system, normally used for FRAP experiments (Ladha et al. 1996), was used for this purpose. Although the sensitivity of the system was very high, a specific strategy had to be applied to have a number of trimers incorporated into the bilayer without compromising the stability of the bilayer. In routine bilayer experiments the presence of more than 10 trimers in the bilayer resulted in a large conductance, making it very difficult and sometimes impossible to close them by an applied pd without breaking the bilayer. Consequently, the strategy adopted was to incorporate many trimers into the bilayer at 200 mV, thus keeping them in a closed state before measurement of the fluorescence.

IAEDANS-labelled channels were loaded into the chamber and the background fluorescence was measured. The incorporation of channels into the bilayer was monitored by measurement of the membrane current in the usual way. The fluorescence of the background was high, masking the measurement of the fluorescence of the channels incorporated into the bilayer. Consequently, an attempt was made to focus only on the membrane to monitor the fluorescence emission of the channels. However, the background fluorescence proved to be too high to see significant differences in the fluorescence emission due to the membrane alone. The pd was lowered, with the hope of a distinguishable change in the fluorescence emission of the membrane channels. However, no reliable result was obtained from this experiment. The strategy is promising, however, and will be attempted again in the future, concentrating on the incorporation of a larger number of channels to improve the signal-to-noise ratio and using a more appropriate laser excitation wavelength. The ideal wavelength in the visible range was not available for technical reasons.

Discussion

Two OmpC mutant porins, R37C and R74C, each with one cysteine located in the middle and upper regions of the channel mouth, respectively, were labelled with Thiolyte, NBD-Cl and IAEDANS. Only Thiolyte gave a fluorescence signal in an aqueous phase before attachment to the porins. The fluorescence labelling of these proteins was carried out and the absorbance of the probe attached to the protein was checked at each stage to confirm labelling efficiency.

Bilayer studies show the effects of labelling on the conductance size of the channel as well as its voltage sensitivity. There was an increase in the conductance of the OmpC channel from 512 ± 66 pS in wild-type to 639 ± 94 pS in R37C that is in good agreement with that reported by Lakey et al. (1991). Thiolyte-labelled porin gave different conductance values at opposite polarity of the applied pd: 552 ± 119 pS at +ve pd; 354 ± 70 pS at –ve pd (Gokce et al. 1997). In this study the channel gave smaller conductance values when labelled with NBD-Cl and IAEDANS, irrespective of polarity. Substates of about one-third to one-fourth of the monomeric size were observed for both porin channels, mainly at acid pH. The incorporation of the channels into bilayers was relatively easy and no sign of the rejection or loss from the membrane was observed. The sensitivity of the channels to the applied pd was found to be 50–80% higher than the unlabelled channels. Both of the labelled channels responded to a pd as low as 50 mV and started gating at even lower pd values. The voltage required to close the channel was higher for the channel labelled with IAEDANS than with NBD-Cl, closing at pd values higher than ± 100 mV and ± 50 mV, respectively. Fast flickering at 100–150 mV in both of these channels implied the flexibility of the labelled channels. Analysis of the channel activity at acid pH showed an increase in the voltage sensitivity for both of the above labelled channels, so that both gating voltage and closing voltage were decreased dramatically, and the channel spent more time in the closed state for each value of the applied pd used (Table 1).

Incorporation of the OmpC mutant R74C channels into the bilayer was similar to the wild-type channels with no ejection or loss from the membrane. The conductance of the unlabelled channels was 620 ± 25 pS. The channel began gating when 60–70 mV was applied to the *cis* side. Complete closure of the channel occurred when a minimum of ± 100 mV was applied to the bilayer. Although, in general, the channel showed a symmetrical response to the applied pd, in terms of conductance size, more transitions between different small substates were observed when a positive pd was applied to the *cis* side. The I/V curve of the channel

showed a linear (ohmic) relationship. The conductance of the channels labelled with Thiolyte was 450 ± 25 pS together with substates, one-third to one-half the monomeric conductance size. The channel voltage sensitivity had increased as a result of labelling so that the gating began at 30 mV and closure of the channel occurred at a pd as low as ± 50 mV. The channels labelled with NBD-Cl also showed smaller conductance (470 ± 20 pS) in comparison with the unlabelled channels. The voltage required to close the channel was 50 mV and voltage-dependent gating started at lower pd values. The smallest conductance size was identified in channels that had been labelled with IAEDANS (conductance of 320 ± 20 pS). However, voltage sensitivity did not follow the same pattern as conductance, and showed lower voltage sensitivity by comparison with the other labelled channels. This channel began gating at ± 80 mV and closed when a minimum of ± 100 mV was applied to the bilayer (see Table 2).

The NBD-Cl labelled R37C channels were excited at 450 nm and 470 nm, producing an emission fluorescence at 520 nm. The wavelength of the emission maximum was found to be independent of pH, while the fluorescence intensity did depend on the pH and was maximal at acid pH. Similar results were obtained for the IAEDANS labelled porins.

The porins labelled with Thiolyte were excited at 280 and 380 nm and produced a fluorescence emission at about 470 nm. The major fluorescence emission was produced when the channel was excited at 380 nm. In response to excitation at 280 nm a minor fluorescence was recorded whose intensity was subtracted from the major one, in order to obtain the actual fluorescence emission. The fluorescence emission wavelength showed no significant changes when the channels were incorporated into the single-shelled liposomes. Excitation of the R74C porins labelled with NBD-Cl gave an emission maximum at 520 nm.

When the pH of the medium was changed, the fluorescence intensity was maximal at acid and minimal at neutral and alkaline pH. On the other hand, the fluorescence intensity was time dependent. The intensity produced at acid pH showed a decline over time to a level still above the level produced at neutral pH. The wavelength of the fluorescence emission of the channel incorporated into liposomes was similar to that in aqueous medium, but with a much lower intensity. These porins were also labelled with IAEDANS, and were excited at 336 nm. The channel fluorescence excitation wavelength was maximal at 490 nm with the maximum intensity of the fluorescence emission at neutral pH.

Attention has been drawn by a number of different groups (Gonzalezmanas et al. 1992; Doring et al. 1997; Pattnaik et al. 1997) to the importance of the amino acids situated in the protein-membrane interface. Others (Liu and Delcour 1998) have suggested that hydrogen bonds between these residues and the adjacent ones located on the L3 loop (D315A, D118Q and R92Q) play a role in spontaneous gating of the channel, and increasing the frequency of channel gating. They have also suggested that the transition between the open and closed states is not driven by membrane pd, but due to thermal oscillations between functionally distinct conformations.

The location of the thiol binding site in the OmpC mutants (R37C and R74C), at which the cysteine residues point into the lumen of the pore, are deep within the pore and are unlikely to form intermolecular disulfide bridges. These cysteine residues have been used for the covalent attachment of the fluorescent probes Thiolyte, NBD-Cl and IAEDANS. Investigation of the possible effects of these bulky molecules in the lumen of the channel on the channel conductance size and gating behaviour was the first task that was carried out by experiments at the single-channel level.

The fluorescence probes used were chosen because apart from thiolyte they fluoresce only when bound to cysteine residues. The emission wavelength of these probes depends on the polarity of their microenvironment. This enabled us to monitor potential conformational change(s) close to the probes in the channel lumen. This study will be continued until changes in the hydrophobicity of the entire conducting path of the channel during channel closure is monitored. The closed state probability of the channels reduced as the pH increased. The channel sensitivity to applied pd at acid pH (pH 4–6) greatly increased and resulted in spontaneous gating and increased the probability of closure at pd values as low as 20 mV. The wavelength of the emission maxima was essentially the same whether the labelled porin was in aqueous solution or incorporated in a liposome membrane, indicating that its microenvironment was unchanged by incorporation. Since the single channel experiments at low pH suggested that the

Table 1. Channel activity in wild-type OmpC and its R37C mutant, labelled with three fluorescence probes: Thiolyte, NBD-CL and IAEDANS

Channel	*G* (pS)	Gating pd (mV)	Closure pd (mV)
WT	512 ± 66	80	> 250
R37C	639 ± 94	50	150
R37C-Thiolyte[a]	552 ± 119 (+ ve pd) 354 ± 70 (–ve pd)	50	50
R37C-NBD-Cl	430 ± 20	20	50
R37C-IAEDANS	440 ± 30	20	100

[a] Data taken from Gokce et al. (1997)

Table 2. Channel activity in wild-type OmpC and its R74C mutant labelled with three fluorescence probes: Thiolyte, NBD-Cl and IAEDANS

Channel	*G* (pS)	Gating pd (mV)	Closure pd (mV)
WT	512 ± 66	80	> 250
R74C	620 ± 25	70	100
R74C-Thiolyte	450 ± 25	30	50
R74C-NBD-Cl	470 ± 20	50	50
R74C-IAEDANS	320 ± 20	80	> 100

channel tended towards a closed state, the implication of the fluorescence experiments has to be that this part of the channel is not directly involved in gating, i.e. this part of the channel remains water filled.

The positions of the probe were important for the scanning of the hydrophobicity changes in the eyelet area. The fluorescence spectrum of R74C-NBD was found to be rather different from the others, showing some fluorescence emission (albeit at a different wavelengths) in aqueous media. The fluorescence emission at different wavelengths is not due to labelling of other residues, i.e. lysines as reported by others (Detmers et al. 1981). These authors have reported that NBD attached to a cysteine residue can migrate to a neighboring lysine residue (if there is one). Consequently, although the object was to label the cysteine residues selectively at $pH < 7$, lysine residues, especially nearby ones, may be labelled but emit at different wavelengths from the cysteine-bound probe (Houk et al. 1983). The emission wavelength of the cysteine-bound probes is 520 nm compared with 470 nm for the lysine-bound one; thus the cysteine residue to which the probe is bound can be distinguished with confidence. In general, IAEDANS showed better labelling selectivity, higher binding affinity and sharper and more reproducible fluorescence response than other probes used in this study. The results using IAEDANS attached to residues 37 and 74 (equivalent to 42 and 82, respectively, in OmpF) show that the hydrophobicity of the intralumenal conducting path at least in these regions is not changed during the voltage-dependent gating processes.

The main problem in the experiments that were carried out using IAEDANS-labelled OmpC mutant channels in the planar bilayer system was the background fluorescence. This problem and the difficulty in working with the laser source might be overcome by choosing a probe which fluoresces at visible wavelengths. The characteristics of buffer, lipid, fluorescence probe and the source of the exciting light are amongst the most important factors to be considered. Meanwhile, the preliminary results and the set-up capability are convincing and indicate the applicability of the idea and the feasibility of the protocol.

Changes in the configuration and conformation of the whole trimer might expose more hydrophobic residues to the lumen of the channel, producing a sort of hydrophobic dam in the aqueous ionic conducting pathway. The resulting lack of electrolyte in this area would interrupt the conduction and appear as a channel "closed state".

However, it appears that the mechanism by which ions pass through the channel is different from that by which large non-electrolytes like sugars pass through the channel. There is an apparent paradox in which liposome-swelling experiments demonstrate the existence of large channels through which large sugars pass, but single-channel experiments demonstrate the existence of a much smaller water-filled channel. Must there be a different pathway for sugars? It has been suggested that there is a "slippery pathway" for sugars formed by hydrophobic residues in the channel lumen (Klebba and Newton 1998). This question has yet to be resolved and in this context it has to be remembered that there is a relationship between sugars and ionic channel size. The OmpC Dex mutants (increased porin channel size) are produced by growing *E. coli* with large sugars as the only source of carbon, but in this case the single-channel size is increased. The same state of affairs (two separate mechanisms) seems to be the case for *Brucella abortus* Omp2a porin (Mobasheri et al. 1997).

The results of an unpublished study (H. Mobasheri) in terms of fast flickering and higher voltage sensitivity in the channels incorporated into more fluid membranes are consistent with the published reports. It might be inferred that the tighter or more strongly the surrounding amino acids on the barrel wall interact with the membrane core, the less will be their movement and thus less interaction with their counterparts on the L3 loop. This might explain some of the observed differences in voltage sensitivity, transition between different conductance states and fast flickering in the different mutants and derivatives.

Furthermore, the structure of the channel does not seem to be as rigid as expected, even though the β-sheet structure provides a high degree of stability in the protein. Acid pH is one of the major factors that affects the conformation of the channel, producing different gating patterns. The presence of a fluorescence probe in the right place where hydrophobicity is changed will give a better picture of the gating mechanism and show the amino acids involved during this process. This will form the basis of future studies.

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References

Bainbridge G, Mobasheri H, Armstrong GA, Lea EJA, Lakey JH (1998) Voltage-gating of *Escherichia coli* porin. A cysteine-scanning mutagenesis study of loop 3. J Mol Biol 275:171–176

Benz R (1984) Structure and selectivity of porin channels. Curr Top Membr Transp 21:199–219

Bishop ND, Lea EJA, Mobasheri H, Spiro S (1996) Altered voltage sensitivity of mutant OmpC porin channels. FEBS Lett 379:295–298

Buehler LK, Kusumoto S, Zhang H, Rosenbusch JP (1991) Plasticity of *Escherichia coli* porin channels – dependence of their conductance on strain and lipid environment. J Biol Chem 266:24446–24450

Carlier MF, Didry D, Erk I, Lepault J, Pantaloni D (1994) Myosin subfragment-1-induced polymerization of G-actin – formation of partially decorated filaments at high actin-S(1) ratios. J Biol Chem 269:3829–3837

Chattopadhyay A (1990) Chemistry and biology of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labelled lipids: fluorescent probes of biological and model membranes. Chem Phys Lipids 53:1–15

Chattopadhyay A, London E (1988) Spectroscopic and ionisation properties of *N*-(7-nitroben-2-oxa-1,3-diazol-4-yl)-labelled lipids in model membranes. Biochim Biophys Acta 938:24–34

Clark DC, Dann R, Mackie AR, Mingins J, Pinder AC, Purdy PW, Russell EJ, Smith LJ, Wilson DR (1990) Surface diffusion in SDS stabilized thin liquid films. J Colloid Interface Sci 138:195–206

Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, Jansonius JN, Rosenbusch JP (1992) Crystal structures explain functional properties of two *Escherichia coli* porins. Nature 358:727–733

Detmers P, Weber A, Elzinga M, Stephens RE (1981) 7-Chloro-4-nitrobenzeno-2-oxa-1,3-diazole actin as a probe for actin polymerization. J Biol Chem 256:99–105

Doring K, Beck W, Konermann L, Jahnig F (1997) The use of a long-lifetime component of tryptophan to detect slow orientational fluctuations of proteins. Biophys J 72:326–334

Gokce I, Bainbridge G, Lakey JH (1997) Stabilising and destabilising modifications of cysteines in the *E. coli* outer membrane porin protein OmpC. FEBS Lett 411:201–205

Gonzalezmanas JM, Lakey JH, Pattus F (1992) Brominated phospholipids as a tool for monitoring the membrane insertion of colicin A. Biochemistry 31:7294–7300

Houk TW, Ovnic M, Karipides S (1983) pH and polymerization dependence of the site of labelling of actin by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. J Biol Chem 258:5419–5423

Klebba PE, Newton SMC (1998) Mechanisms of solute transport through outer membrane porins: burning down the house. Curr Opin Microbiol 1:238–247

Ladha S, Mackie AR, Clark DC (1994) Cheek cell membrane fluidity measured by fluorescence recovery after photobleaching and steady state anisotropy. J Membr Biol 142:223–229

Ladha S, Mackie AR, Harvey LJ, Clark DC, Lea EJA, Brullemans M, Duclohier H (1996) Lateral diffusion in planar lipid bilayers – a fluorescence recovery after photobleaching. Investigation of its modulation by lipid composition, cholesterol, or alamethicin content and divalent-cations. Biophys J 71:1364–1373

Lakey JH, Lea EJA, Pattus F (1991) OmpC mutants which allow growth on maltodextrins show increased channel size and greater voltage sensitivity. FEBS Lett 278:31–34

Liu NZ, Delcour AH (1998) The spontaneous gating activity of OmpC porin is affected by mutations of a putative hydrogen bond network or of a salt bridge between the L3 loop and the barrel. Protein Eng 11:797–802

Mansoor MA, Svardal AM, Schneede J, Ueland PM (1992) Dynamic relation between reduced, oxidized, and protein-bound homocysteine and other thiol components in plasma during methionine loading in healthy-men. Clin Chem 28:1316–1321

Mobasheri H, Lea EJA (1998) Gating and size of OmpF porin channels in bilayers: effect of changes in membrane composition. Biophys J 74:A30

Mobasheri H, Fight TA, Marquis H, Lea EJA, Lakey JH (1997) Brucella Omp2a and Omp2b porins; single channel measurements and topology prediction. FEMS Microbiol Lett 155:23–30

Pattnaik BR, Ghosh S, Rajeswari MR (1997) Selective excitation of tryptophans in OmpF: a fluorescence emission study. Biochem Mol Biol Int 42:173–181

Phale PS, Schirmer T, Prilipov A, Lou KL, Hardmeyer A, Rosenbusch JP (1997) Voltage gating of *Escherichia coli* porin channels: role of the constriction loop. Proc Natl Acad Sci USA 94:6741–6745

Steer BA, Merrill AR (1994) Colicin E1 insertion-competent state: detection of structural changes using fluorescence resonance energy transfer Biochemistry 33:1108–1115

Wakabayashi S, Ogurusu T, Shigekawa M (1990) Participation of H^+ in the Ca^{2+}-induced conformational transition of 4-nitro-2,1,3-benzoxadiazole-labelled sarcoplasmic-reticulum ATPase. Biochemistry 29:10613–10620