Stabilising and destabilising modifications of cysteines in the *E. coli* outer membrane porin protein OmpC

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Abstract Three sulfhydryl labels were used to modify two mutated sites, R37C and R74C in the eyelet of the outer membrane porin OmpC. Modification of R37C with the neutral groups Aldrithiol and bimane increases thermal stability but the negatively charged iodoacetate causes a decrease in thermal stability. The effects of substitution at R74C were less significant. Bimane labelling increases the voltage sensitivity and decreases the single channel conductance at R37C asymmetrically with smaller channels being recorded at *cis* negative voltages. Negatively charged acetate does not affect the voltage gating.

Key words: Voltage gating; Porin; Cysteine labeling; Outer membrane; Planar bilayer; Thermal stability

1. Introduction

The porins of the *E. coli* outer membrane form large water filled channels which are permeable to salts and larger aqueous solutes. They can be divided into the non-selective (OmpC, OmpF, PhoE, etc.) [1] and selective porins (Tsx [2], maltoporin or LamB [3] and Scry of *Salmonella* [4] and *Klebsiella*). High resolution structures of members from both groups have been solved by X-ray crystallography [5,6] whilst the overall trimeric β-barrel fold is strongly predicted for a diverse range of Gram-negative bacterial porins [7–9]. All porins could form much larger pores but the large cross section of the β-barrel is occluded by a polypeptide loop which folds back into the pore and reduces the diameter in the so-called eyelet region. This narrow channel is approximately half way through the membrane and therefore has significant effects on selectivity and the voltage gating which these channels exhibit in vitro. The loop 3 of the *Rhodobacter capsulatus* porin has been modelled as a mobile gate in molecular dynamics simulations [10] whereas in maltoporin it participates in the selective uptake of sugar molecules [6]. The OmpF/PhoE eyelet is unusual also in that basic residues face backbone carbonyls and acidic groups to create a strong transverse electric field within the plane of the membrane. This is thought to have peculiar electrostatic effects both on protein structure and ion permeability [11]. OmpC porin has a smaller single channel conductance, higher ion selectivity, and requires higher transmembrane potential differences (>200 mV) for gating compared to OmpF (130 mV) or PhoE (110 mV) [12,13]. It also responds to hydrostatic pressure [14]. The structure of OmpC is not known but it shares 64% and 62% residue identity with OmpF and PhoE respectively and the level of identity rises further when only the beta barrel core and loop 3 region are considered. Hence their backbone atoms should superimpose with an RMS of <2.0 Å [15]. It was shown that mutation of basic residues facing loop 3 across the eyelet of OmpC [16] caused decreased trimer stability, increased channel conductance and greater voltage sensitivity [12,26] and this has recently been confirmed in OmpF [17–19]. In this paper we create a homology model of OmpC and modify two cysteine mutants with sulfhydryl reagents to show the effects of negative and neutral charge alterations in the eyelet region.

2. Materials and methods

PEG (polyethylene glycol MW: 6000), iodoacetic acid, DTT (dithiothreitol) were from Sigma (Poole, UK). Thiolyte (monobromobimane) reagent was from Calbiochem. Aldrithiol (2,2′- dipyridyl disulfide, 2,2′-dithiodipyridine) was from Aldrich. SDS (sodium dodecyl sulfate) was from BDH.

2.1. Preparations of porins and labelling of OmpC mutant proteins with thiol reagents

Porins were purified as previously described employing pre-extractions from the membrane pellet in SDS followed by porin extractions in SDS and 0.5 M NaCl [20,21]. They were concentrated using PEG to extract water from protein in 3.5 kDa cut-off dialysis tubing and then dialysed for 18 h at room temperature against 5 mM $NaHCO_3$ and 0.3 M NaCl containing 1% SDS, pH 7. A stock solution of each reagent was prepared (Aldrithiol and iodoacetic acid in water and bimane in acetonitrile) and used to provide a 10 M excess over cysteine concentration. Before labelling, mutant proteins (R74C and R37C) were incubated for 1 h at 37°C in the presence of 5 mM DTT. Excess DTT was then removed from protein samples by spin columns (Biospin 6-chromatography column (BioRad Laboratories). Labelling reactions were performed overnight at room temperature in 5 mM $NaHCO_3$, 0.3 M NaCl and 1% SDS, followed by removal of the remaining reagent by a spin column. For bimane the stoichiometry of labelling was determined by UV-absorption spectroscopy using the following extinction coefficients, ε_{280} OmpC=59 880 M^{-1} cm^{-1} and ε_{380} monobromobimane = 4000 M^{-1} cm^{-1}. The monobromobimane ε_{280} (3950 M^{-1} cm^{-1}) was calculated from the absorbance spectrum of a bimane-glutathione adduct. Since for monobromobimane $\varepsilon_{280} \approx \varepsilon_{380}$, the labelling stoichiometry (S) was calculated from the two absorbance (A) values at 280 and 380 nm wavelength as:

$$S = \frac{\left(\frac{A_{380}}{\varepsilon_{380}}\right)}{\left(\frac{A_{280}-A_{380}}{\varepsilon_{280}}\right)}$$

2.2. Acid and base denaturation

The pH stabilities of proteins were determined by incubating unbuffered proteins in either 0.2 M Na_2HPO_4-citric acid buffer, pH 3.0 to pH 5 or 0.2 M diethanolamine-0.1 N HCl, pH 8 to pH 10 (at 0.1 pH steps) for 1 h, the presence of monomers was then examined by SDS-PAGE using Coomassie staining.

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2.3. Temperature denaturation

Temperature denaturation studies were performed by heating the labelled, unlabelled and pH treated proteins in 1% SDS, 0.2 M NaCl, 5 mM $NaHCO_3$ for 30 min at different temperatures in a water bath (Grant Instruments). After this treatment, protein samples were cooled to room temperature before loading onto the SDS-PAGE.

2.4. Circular dichroism (CD) measurements

Circular dichroism (CD) measurements were made using a Jobin-Yvon CD6 instrument linked to an IBM computer for the recording of spectra. Quartz cuvettes of 1 cm path-length (Hellma 105.201-QS) and 0.2 mm path-length circular cuvette (121.000-QS) were used. Far and near UV spectra measurements were carried out for both native and 95°C states of OmpC protein. Protein CD spectra were obtained by subtraction of a buffer baseline. Prior to spectroscopic measurements all solutions were filtered and protein samples were centrifuged for 30 min at $10\,000 \times g$.

2.5. Fluorescence measurements

Fluorescence measurements of bimane-labelled proteins were recorded at 25°C using an SLM 8100 spectrofluorometer operating in the ratio mode with spectral bandwidths of 4 nm for both excitation and emission. The excitation wavelength was 380 nm. The inner filter effect was minimised by using 0.5 cm path length cuvettes and the Raman scatter contribution was eliminated by subtraction of appropriate blanks.

2.6. Planar lipid bilayers

Schindler type bilayers [22] were formed of soy bean lecithin (Sigma Type IIa) by apposition of two vesicle spread monolayers on a 0.1 mm diameter hole in a Teflon septum pre-treated with hexadecane. Porins were added as SDS solubilised samples such that the final SDS concentration never exceeded 0.002%. The buffer was 1 M NaCl, 10 mM $CaCl_2$ and 50 mM Tris-acetate pH 7.4. Data was collected via a PCM-2 A/D VCR Adapter (Medical Systems, Greenvale, NY) connected to a PAL-VHS video recorder.

2.7. Structural modelling

The structure of OmpC was modelled using the Modeller [23] facility in Quanta (MSI, Cambridge, UK). Both PhoE (1PHO) and OmpF (2OMF) [5] structures were used as templates for the published alignment [7]. Default parameters were used to produce one structure per modelling run.

3. Results

3.1. Labelling reactions

Bimane-labelled proteins were analysed by SDS-PAGE, UV absorption and fluorescence spectroscopy. Samples of protein were heated at 95°C for 10 min and separated on 10% SDS-PAGE. When viewed under ultraviolet light the gels showed intense fluorescence in a band with the same mobility as monomeric OmpC. The stoichiometry of the reaction was also monitored by ultraviolet scan, the stoichiometry was calculated as 1.03. The fluorescence spectra were recorded and revealed that, whereas free bimane has a fluorescence emission maximum of 480 nm, both R37C and R74C labelled proteins exhibited blue shifted emissions at 470 nm (Fig. 1). The stoichiometry of the reaction of Aldrithiol with R37C could not be determined by ultraviolet-absorption spectroscopy since

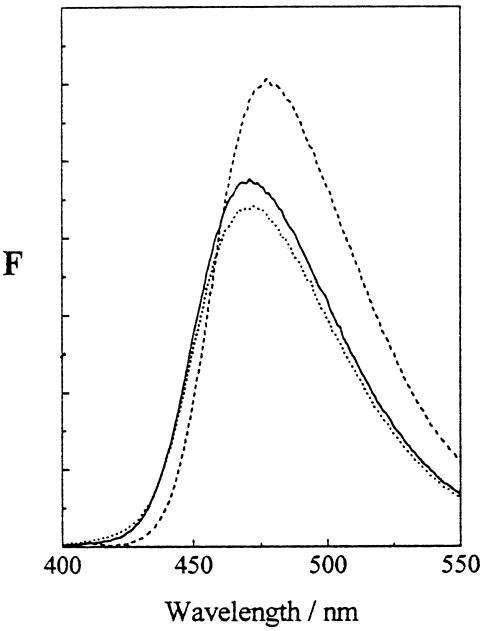

Fig. 1. Fluorescence spectra of monobromobimane-DTT adduct (mM in solution, – – –), bimane-labelled R74C mutant protein (· · ·) and bimane-labelled R37C mutant protein (—). All samples were 10 μg/ml in 1% SDS, 0.2 M NaCl, 5 mM $NaHCO_3$. Excitation wavelength was 380 nm. Temperature = 25°C.

Table 1
Denaturation was measured as the lowest temperature or highest pH at which the sample becomes mostly monomeric on SDS-PAGE (see text)

(A) Thermal denaturation

Protein	Unlabelled	Bimane	Aldrithiol	IAA
Wild type	75°C	n/a	n/a	n/a
R37C	65	70	70	60
R74C	70	70	nd	70

(B) Acid monomerisation

Protein	OmpF	OmpC	R37C	R74C
pH	4.0–4.1	3.5–3.7	3.9–4.0	3.9–4.0

(C) Single channel properties

	WT	R37C Unlabelled	R37C Bimane (+) pd	R37C Bimane (−) pd	R37C IAA
Single channel conductance (pS) ± S.D.	512 ± 66, $n = 191$ (V = 200 mV)	639 ± 94, $n = 232$ (V = 100 mV)	552 ± 119, $n = 214$ (V = +100 mV)	354 ± 70, $n = 145$ (V = −100 mV)	586 ± 56, $n = 249$ (V = 100 mV)
Closing voltage (mV)	> 200 mV	> 100 mV	> 50 mV	> 50 mV	> 100 mV

Single channel conductances were measured as in Ref. [12] using channels that closed due to the applied voltage. Only in the bimane sample did the two polarities have different conductances. The closing voltage is that required to close at least 2/3 of the channels within 2 minutes. (See text). V = transmembrane voltage at which single channel conductances were measured.

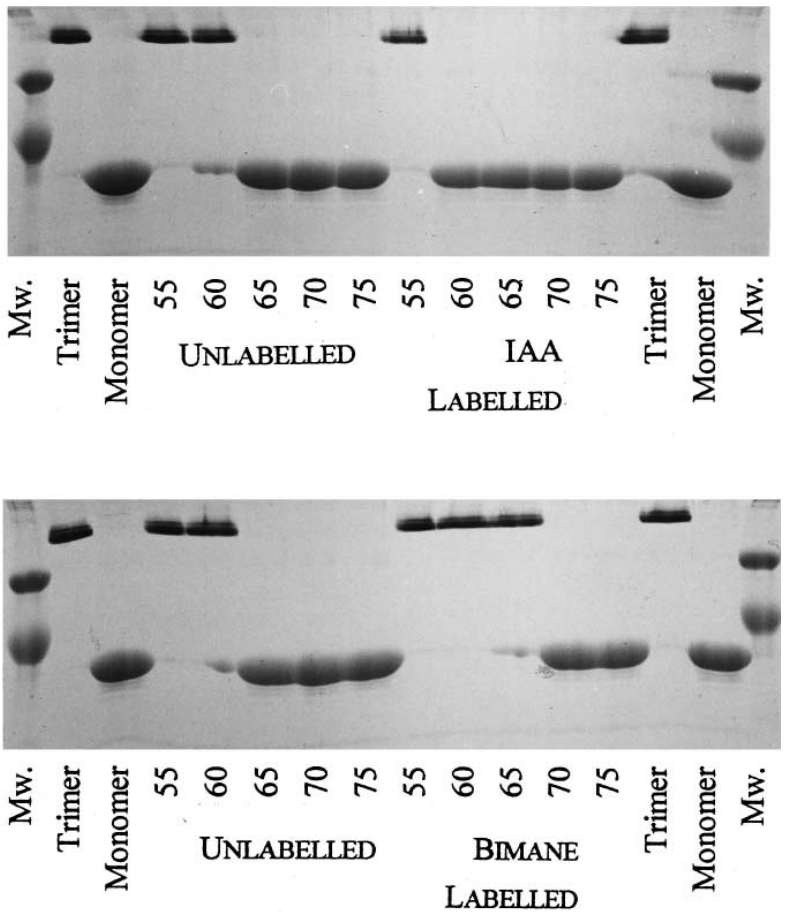

Fig. 2. 14% SDS-PAGE showing the thermal stability of labelled and unlabelled R37C mutant protein. Upper gel: lanes 1 and 16 show MW markers 66 kDa and 45 kDa. Lanes 2 and 14 show trimeric wild-type OmpC protein. Lanes 3 and 15 show monomeric wild-type OmpC protein (incubated at 95°C). Lanes 4 (55°C), 5 (60°C), 6 (65°C), 7 (70°C) and 8 (75°C) show unlabelled R37C mutant protein. Lanes 9 (55°C), 10 (60°C), 11 (65°C), 12 (70°C), 13 (75°C) show iodoacetamide labelled R37C mutant protein. Lower gel: lanes arranged as above except that lanes 9 (55°C) to 13 (75°C) show bimane-labelled R37C mutant protein. All samples were incubated for 30 min at the indicated temperature.

contaminating unreacted Aldrithiol could not be entirely removed.

3.2. Acid and temperature denaturation

All the porins tested retained their native trimeric structure in 8 M urea pH 7.0 and thus another measure of stability was used. High temperature and pH have been used to denature the quaternary structure of porins and the change from trimeric to monomeric protein can easily be visualised on SDS-PAGE as a sharp cooperative transition. The effects of temperature on the secondary and tertiary structure of OmpC was verified by circular dichroism. The steady state spectra of samples previously heated to 90°C show that the results of denaturation were permanent and is similar to that seen for OmpF [24]. We have thus used monomerisation as a measure of each protein's stability at extremes of pH and temperature.

The temperature stability was examined by heat denaturation with the appearance of monomers used to determine the trimer stability of R74C, R37C mutants and WT (wild type) proteins (Table 1(A) and Fig. 2). These studies were performed by heating the protein samples in 1% SDS, 0.3 M NaCl, and 0.5 mM $NaHCO_3$ and reducing sample buffer for 30 min in a water bath, then cooled to room temperature before loading onto SDS-PAGE. Bimane- and Aldrithiol-labelled R37C mutant proteins showed increased heat stability while iodoacetic acid-labelled R37C mutant protein was less heat stable. The heat stability of bimane-labelled R74C mutant protein was the same as the unlabelled protein. The pH values at which acidic denaturation of trimers occurs are shown in Table 1(B). No alkaline denaturation was observed in the range pH 10–11. Schindler and Rosenbusch have studied the stability of OmpF as a function of pH, and they have used SDS-PAGE mobility [25]. They found two acidic transitions at pH 3.5 and pH 1.6 which they attribute to the protonation of a number of carboxylate groups. Our results have shown that this transition occurs for the OmpC porin at approximately the same pH values.

3.3. Single channel measurements

Since R37C was most sensitive to modification, it was further studied at the single channel level. This mutant closed at voltages above 100 mV and its single channel was measured as 640 ± 94 pS which compares to 690 ± 51 pS in our previous study [12]. The conductance was reduced in iodoacetate-labelled samples but the sensitivity to voltage was unchanged. The bimane-labelled samples showed complete channel closure at 50 mV (Table 1(C)) but unusually showed polarity dependent conductance values. This means that although the

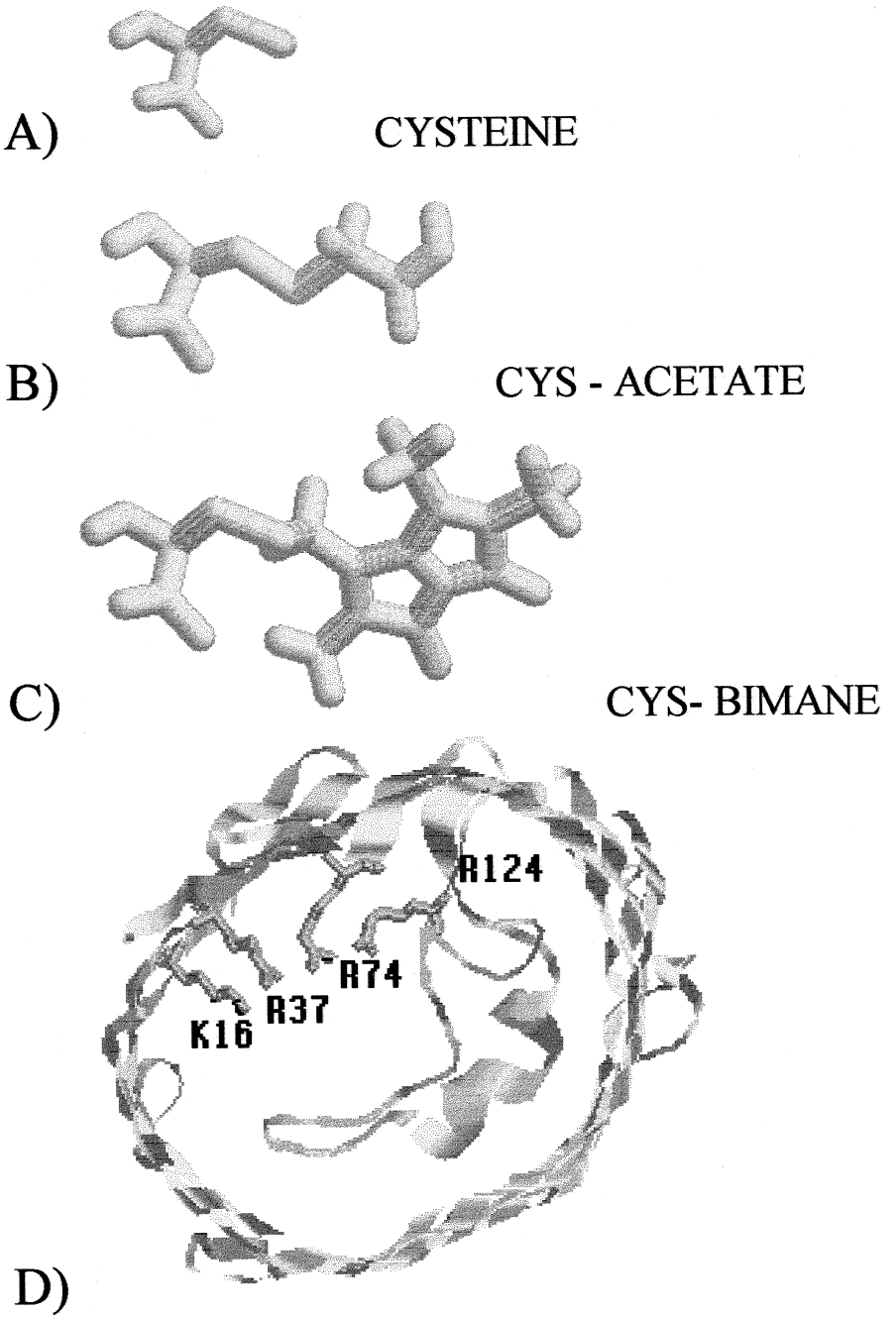

Fig. 3. The relative sizes of (A) cysteine, (B) cys-acetate and (C) cys-bimane side chains. (D) A section through the membrane spanning core of the homology model of OmpC (based upon the structures of OmpF and PhoE). The mutated residues R37 and R74 are shown (along with the adjacent L16 and R124) protruding into the pore lumen bounded on the other face by the loop 3. Figure generated using RASMOL [27].

single channel conductance is close to that of IAA-labelled proteins at positive applied p.d., in the reverse polarity they exhibit very much smaller channels than even the wild type protein.

3.4. Modelling

As expected from the sequence alignment the differences between OmpC and the OmpF/PhoE X-ray models were confined to the loop regions especially loops 4, 6, 8. The amino acid sequence conservation in the barrel core and the loop 3 region are reflected in a clear superposition of most side chains in this region.

4. Discussion

Due to their sites deep within the pore, the cysteines in R37C and R74C are unlikely to form intermolecular disulfide bridges and should react with thiol-reactive reagents. Nevertheless it was impossible to label them without prior reduction in 10 mM DTT. Following this treatment it was clear, from fluorescence and absorption measurements, that bimane-labelled the mutant pore cysteines at near to 100% efficiency. The altered fluorescence emission wavelength and the lack of reactivity of the wild type indicate that the bimane is situated in a buried environment and that this must be due to reaction

with the cysteine. Both R37 and R74 are exposed to the pore lumen and therefore this reactivity was expected.

The instability of R37C and R74C observed in our earlier work [12] was the result of loss of the arginine's bulk, charge or both. We used three probes to test this hypothesis which add mass or negative charge to the thiol site. Although we could not measure the stoichiometry of the Aldrithiol labelling it did increase the stability of R37C by 5°C indicating that the entire sample was labelled. This clearly also true of bimane for which we can measure stoichiometry and the labelling environment for fluorescence emission. Hence the simple reintroduction of side chain bulk restores some of the OmpC stability. Hence some mutant instability is due to the creation of a hole in the packed side chains of the eyelet. The insertion of a negative charge into the centre of this basic region (Lys 16, R37 and R74; Fig. 3) using IAA further reduces the protein's stability and thus local electrostatics contribute significantly to the trimer stability. In contrast the R74C mutant is far less sensitive to the effects of both bimane and IAA. This may indicate that its effect on the pore character is more peripheral than its situation suggests (Fig. 3).

In the previous study it appeared that the loss of trimer stability and increased voltage gating sensitivity were associated. By labelling the pore cysteine residues we have been able to show that although it is possible to regain stability through reaction with bimane this does not increase the closing voltage. On the contrary, bimane labelling decreased the closing voltage whilst the iodoacetate labelling decreased trimer stability and left the voltage sensitivity unaltered. It should be remembered however that all the channels retain a lower trimer stability and a lower closing voltage than the wild type protein. Thus the bulk and charge of the arginine residue both contribute to its high closing voltage (>200 mV) and trimer stability.

The asymmetry of the channel conductance in the bimane-labelled sample was unexpected. Such asymmetry has been seen in some point mutations of OmpF [19] and even though the 3D structure is available the cause of this effect is not clear. Compared to arginine and cys-acetate the bimane molecule is asymmetric and possibly this enables it to adopt a different conformation at each polarity. Since bimane is not charged the potential sensitive element must be elsewhere but the level of sensitivity may be affected by the final stable state of the bimane.

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