Topographic labelling of pore-forming proteins from the outer membrane of *Escherichia coli*

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The topography of three pore-forming proteins from the outer membrane of *Escherichia coli* has been explored by using two labelling techniques. Firstly, the distribution of nucleophilic residues has been investigated by selective chemical modification using arylglyoxals (for arginine residues), isothiocyanates (for lysine residues), carbodi-imides (for carboxy residues) and diazonium salts. Secondly, the membrane-embedded domains have been investigated by labelling with photoactivatable phospholipid analogues and a reagent that partitions into the membrane. Few nucleophilic groups are found to be freely accessible to pore-impermeant probes reacting in the aqueous medium. More groups are accessible to small, pore-permeant probes, suggesting that several groups of each sort are contained within the pore. In addition, there appear to be a number of arginine, lysine, carboxy and many tyrosine residues that are rather inaccessible and that react only with small, hydrophobic probes, if at all. Amongst these more deeply buried residues there are four arginine residues and an as-yet-undetermined number of carboxy residues that appear to be essential to the structural integrity of the oligomeric molecule.

INTRODUCTION

The outer membranes of *Escherichia coli* and other Gram-negative bacteria contain a number of proteins that facilitate the diffusion of small, hydrophilic molecules across the membrane. These proteins have several common properties, as follows. They are rich in acidic and amide residues and poor in aliphatic amino acids. Their secondary structure is mostly β -pleated sheet (Neuhaus, 1982; Kleffel *et al.*, 1985). They form trimers (Palva & Randall, 1978; Nakae *et al.*, 1979) that are stable in SDS solutions at elevated temperatures (Rosenbusch, 1974), in high concentrations of guanidinium chloride and over a considerable range of pH (Schindler & Rosenbusch, 1984). Further, they all form relatively non-specific, voltage-gated pores in planar lipid bilayers (Schindler & Rosenbusch, 1978; Benz *et al.*, 1978; Neuhaus *et al.*, 1983) with an exclusion size for oligosaccharides of about 600 Da (Nikaido & Nakae, 1979). The pores can be seen to be stain-filled channels that run through the trimeric protein in electron micrographs of reconstituted two-dimensional arrays (Steven *et al.*, 1977; Neuhaus *et al.*, 1983; Dorset *et al.*, 1983; Engel *et al.*, 1985). So far four such proteins have been identified from *E. coli*; these are the products of the *ompC*, *ompF*, *phoE* and *lamB* structural genes. The OmpC, OmpF (or porin) and PhoE proteins (or phosphoporin) are very similar in structure (Overbeeke *et al.*, 1980; Inokuchi *et al.*, 1982; Mizuno *et al.*, 1983), whereas the LamB protein (malto-porin or phage lambda receptor) appears to be less closely related to the other three proteins (Clement & Hofnung, 1981; Overbeeke *et al.*, 1983; Mizuno *et al.*, 1983).

The large percentage of hydrophilic amino acids and the absence of long segments of hydrophobic residues (Chen *et al.*, 1979, 1982; Overbeeke *et al.*, 1983; Mizuno *et al.*, 1983) make these proteins an unusual group among the known membrane proteins. It has been suggested that many of the charged and polar residues are contained within the pore or that they are involved in inter- or intra-molecular ion-pairs and hydrogen bonds and thus contribute to the high stability of the trimer (Rosenbusch *et al.*, 1982; Tokunaga *et al.*, 1981; Benz *et al.*, 1984; Schindler & Rosenbusch, 1984; Paul & Rosenbusch, 1985).

One way to investigate the location of the nucleophilic groups is to study their chemical reactivity, since buried residues are often associated with low reactivity, especially towards large or polar molecules. Schindler & Rosenbusch (1982) have investigated the susceptibility of the amino groups of porin to chemical modification by a variety of reagents. They found that large reagents modified only a few groups but that smaller molecules modified many more, and therefore they suggested that two-thirds of these residues are contained within the pore. No effect on oligomeric structure was detected, suggesting that the residues that had been modified were not important to the structural integrity of the trimer. Modification of carboxy groups with carbodi-imides does affect the structure of the porin trimer (Tokunaga *et al.*, 1981), and although their reactivity was not investigated, the groups involved were also proposed to be located within the pore.

In the present study we investigated the reactivity of the common nucleophilic groups in these proteins with reagents of different size and polarity in order to assess

Abbreviations used: TID, 3-trifluoromethyl-3-(*m*-[¹²⁵I]iodophenyl)diazirine; octyl-POE, octyloligo-oxyethylene; DTT, dithiothreitol; BBPG, 2-[(4-benzoyl)benzoyl]-1-palmitoyl-*sn*-glycero-3-phosphoglycerol; PPG, (1,3,6-trisulphopyrenyl)-3-phenylglyoxalsulphonate; PSG, 2,7-disulpho-4-[-2-sulpho-4-(4-sulphophenylazo)phenylazonaphthyl]-3-phenylglyoxalsulphonate; DCCD, dicyclohexylcarbodi-imide; EDCD, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; CMCD, 1-cyclohexyl-3-(2-morpholin-4-ylethyl)carbodi-imide.

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their surface exposure and their contribution to the conformational stability of the trimers. In particular we studied the modification of arginine residues, which has not been examined before, and show that the effect that modification of these residues has on the conformation of the proteins is similar to that produced by modification of carboxy residues. This is attributed to an important role for residues of these two types in maintaining the native conformation of the trimer. Labelling with photoactivatable, membrane-localized reagents demonstrates that the porins have a considerable hydrophobic surface, consistent with their detergent binding, their solubility in organic solvents (Wirz & Rosenbusch, 1984) and the native, membrane-embedded state.

MATERIALS AND METHODS

Protein purification

Maltoporin was extracted from *E. coli* strain POP1021 and purified in the manner described by Neuhaus (1982). Porin was extracted from *E. coli* strain BE and purified in the manner described by Garavito & Rosenbusch (1985). Phosphorin was extracted from *E. coli* strain CE1197 and purified in a manner analogous to that used for OmpF protein. The protein-detergent complexes purified in this way comprise trimers of the porins in a monodisperse state (Garavito & Rosenbusch, 1985) at values of pH between 1.6 and 12.4 (Schindler & Rosenbusch, 1984). The amount of detergent bound is 0.66 g/g of protein (Grabo, 1982).

Labelling with electrophilic reagents

The reagents used, their specificity and relevant physical properties are given in Table 1.

Proteins were labelled in a reaction mixture comprising 0.1–0.5 mg of protein, 10 μ l of polydisperse octyloligo-oxyethylene (octyl-POE; Rosenbusch *et al.*, 1982) and the appropriate amount of reagent/ml of buffer. For labelling with arylglyoxals, aryl isothiocyanates, succinic anhydride and the diazonium salts the buffer was usually 0.1 M-NaHCO₃ adjusted to pH 8.5 with 10 M-NaOH. For labelling of carboxy residues the buffer was 0.1 M-KH₂PO₄ containing the amine (methylamine, 4-aminobenzenesulphonate or 4-aminofluorescein) at pH 4.5 (adjusted with concentrated HCl). Radioactive reagents were used at a specific radioactivity of 1–1.5 Ci/mol.

Samples taken for measurement of radioactivity were mixed with 10 vol. of ice-cold 10% (w/v) trichloroacetic acid. The precipitate was collected either by filtration (Millipore type GSWP 0.22 μ m-pore-size filters were used for small precipitates, whereas Whatman GF/F glass-fibre filters were used for larger amounts) or by centrifugation for 5 min in an Eppendorf centrifuge. The precipitate was washed three times with ice-cold trichloroacetic acid, suspended in 1 ml of water plus 10 ml of Triton X-100/toluene scintillant (Rotiszint 22; Roth, Karlsruhe, Germany) and radioactivity measured by liquid-scintillation counting. Samples taken for spectrophotometry were precipitated as described above and the washed precipitates dissolved in 1 ml of 0.1 M-NaOH. The absorption spectrum between 280 and 600 nm was

Table 1. The electrophilic reagents and some of their properties

Partition coefficients were determined between equal volumes of the appropriate assay buffer (see under 'Labelling with electrophilic reagents') and decane, starting with the normal concentration of labelling agent (see Table 2) in the aqueous layer. The value quoted is the ratio (concentration in the decane layer)/(concentration in the aqueous layer). If the partition coefficient was found to be less than 0.01 in the preliminary experiment, the determination was repeated in a mixture containing buffer and decane in a ratio of 1:100 (v/v). 'Specificity' results are taken from Means & Feeney (1971).

Reagent	M_r	Partition coefficient	Specificity
Phenylglyoxal	134	0.08	Guanidinyll, slow with amino and thiol
Sulphophenylglyoxal	214	< 0.001	As phenylglyoxal
PPG	754	< 0.001	As phenylglyoxal
PSG	844	< 0.001	As phenylglyoxal
DCCD	206	35	Carboxy, thiol, tyrosine, weak with amino and aliphatic hydroxy
EDCD	155	0.12	As DCCD
CMCD	252	0.01	As DCCD
Methylamine	31	< 0.001	Used as a nucleophile for condensation with carboxy groups in the presence of the carbodi-imides
4-Aminobenzenesulphonic acid	173	< 0.001	As methylamine
4-Aminofluorescein	347	< 0.001	As methylamine
4-Diazonium benzenesulphonate	185	< 0.001	Amino, thiol, tyrosine, histidine, arginine; most rapid with tyrosine and histidine groups
7-Diazonium 4-trifluoromethylcoumarin	243	0.001	
4-Diazonium fluorescein	359	< 0.001	
Succinic anhydride	100	1.2	Only forms a stable product with amino groups
Phenyl isothiocyanate	135	20	Amino, thiol and tyrosine groups
4-Sulphophenyl isothiocyanate	215	< 0.001	As phenyl isothiocyanate
Eosin isothiocyanate	705	< 0.001	As phenyl isothiocyanate

recorded by using a Beckman Acta-MV1 spectrophotometer. Samples were prepared for SDS/polyacrylamide-gel electrophoresis by quenching the reaction with 2 vol. of sample buffer [62.5 mM-Tris/2% SDS/10% (w/v) glycerol/0.5% dithiothreitol/0.01% Bromophenol Blue, adjusted to pH 6.8 with conc. HCl] containing 50 mM-arginine (for quenching reaction with arylglyoxals), 2 M-sodium acetate (for quenching reaction with carbodiimides), 30 mM-lysine (for quenching reaction with arylisothiocyanates or succine anhydride) or 30 mM-histidine (for quenching reaction with diazonium salts).

Labelling with photoactivatable reagents

Protein was reconstituted into liposomes from an initial mixture containing protein (0.1–0.5 mg), dimyristoyl phosphatidylcholine (10–50 mg) and 0.01–0.05 mg of the photoactivatable phospholipid analogue (as indicated) in 25 ml of 1% octyl-POE in 0.1 M-potassium phosphate buffer, pH 7.0. Detergent was removed by dialysis for 48 h at room temperature in the dark, with four changes of buffer (0.1 M-potassium phosphate). Liposomes, in which protein orientation is presumably random, were collected by centrifugation and resuspended in a medium containing 0.1 M-potassium phosphate, 5 mM-reduced glutathione, 2 mM-aminobenzenesulphonate and 5 mM-sodium EDTA, pH 7.0, to give a protein concentration of 0.1 mg/ml. For experiments involving 3-trifluoromethyl-3-(*m*-[¹²⁵I]iodophenyl)diazirine (TID, Fig. 1e), 1.5 nmol of the reagent was added in ethanolic solution and the suspension incubated for 30 min in the dark. The suspension was cooled to 0 °C and spread on a glass tray to give a depth of 2–3 mm. The glass tray rested on a metal block maintained at –4 °C in an ice/salt bath. The trays were irradiated with a low-pressure mercury lamp (360 nm peak) located 1–2 cm above the suspension. Samples were collected by centrifugation, washed with 5 mM-sodium EDTA and dissolved in 5 mM-potassium phosphate (pH 7.0)/1% octyl-POE/1 mM-DTT. The clear solution was passed down a 1 ml column of Whatman DE-52 ion-exchange cellulose contained in a 1 ml disposable plastic syringe. Protein was eluted with 0.1 M-potassium phosphate (pH 7.0)/1% octyl-POE, dialysed against water and freeze-dried before assay and subsequent cleavage with CNBr.

Preparation of reagents

The phenylglyoxalsulphonate and sulphonate esters (Figs. 1a–d) were prepared by selenious acid oxidation of the corresponding acetyl derivatives by using aq. 70% (v/v) ethanol as solvent (Riley & Gray, 1943). Diazonium salts were prepared from the corresponding amines by using the method described by Dilley *et al.* (1972) for diazonium benzenesulphonate, except that the final pH of the stock solution was pH 6.0 and the final concentration was 50 mM (based on starting amine concentration). Dipalmitoyl *N*-(4-azido-2-nitrophenyl)phosphatidylethanolamine (Fig. 1f) was prepared by the method of Radhakrishnan *et al.* (1981). 2-[(4-Benzoyl)benzoyl]-1-palmitoyl-*sn*-glycero-3-phosphoglycerol (BBPG, Fig. 1g) was prepared in the following manner. Carbonyldiimidazole (15 mmol) and 4-benzoylbenzoic acid (5 mmol) were dissolved in 2 ml of dry *NN'*-dimethylformamide. After 15 min, 1-palmitoyl-*sn*-glycero-3-phosphocholine (1 mmol), dissolved in 10 ml of dry toluene, was added. The mixture was stirred in the dark for 48 h and then the solvent was removed by evaporation under reduced

Fig. 1. Structures of some of the reagents used for modifications

(a) Phenylglyoxal; (b) 4-sulphophenylglyoxal; (c) PPG; (d) PSG; (e) TID; (f) dipalmitoyl *N*-(4-azido-2-nitrophenyl) phosphatidylethanolamine; (g) BBPG.

pressure. The solids were washed briefly with cold acetone and then dissolved in chloroform/ethanol (1:10, v/v) and applied to a column of Sephadex LH-20 pre-equilibrated with ethanol. Fractions were monitored for u.v. absorbance and by t.l.c. on silica-gel plates (HP-TLC Kieselgel 60) with chloroform/methanol/water (6:3:1 by vol.) as eluent. Only the first peak contained material positive for phosphate and with a u.v. absorption spectrum like that of benzoylbenzoic acid. The fractions containing this peak were evaporated to dryness and the material converted into phosphatidylglycerol by transphosphatidyltransfer with phospholipase D in the presence of [^3H]glycerol (1.5 Ci/mol) by the method of Comfurius & Zwaal (1977). The reaction mixture was chromatographed on CM-cellulose as described by Comfurius & Zwaal (1977); 70% of the original material was recovered as phosphatidylglycerol and 20% of the original material was recovered as phosphatidylcholine. Purity was assessed by two-dimensional t.l.c. on silica-gel plates using chloroform/methanol/water (10:5:1, by vol.) and chloroform/acetone/methanol/acetic acid (5:2:1:1, by vol.).

CNBr cleavage

Cleavage of the proteins was performed in a manner similar to that described by Chen *et al.* (1982). Acid-precipitated protein was dissolved in a small amount of 80% (v/v) formic acid and added to 2.5 vol. of trifluoroacetic acid. CNBr, dissolved in 70% (v/v) trifluoroacetic acid, was added to give a 100 molar excess over methionine residues and the reaction mixture incubated in the dark at 4 °C for 20 h.

RESULTS

Modification of arginine residues

Porin has 12 arginine residues (Rosenbusch, 1974), all of which could be modified with phenylglyoxal (Fig. 2a; Table 2). However, it was apparent that the rates of modification of the individual residues were not identical and that there was a group of four residues per monomer that were modified more slowly than the other eight residues (Fig. 2b; Table 2). The rate of modification of the latter residues showed a pH-dependence similar to that reported for free arginine by Cheung & Fonda (1979), whereas the rate of modification of the four slowly reacting residues showed much less pH-dependence (Fig. 3).

Modification was accompanied by several changes in the electrophoretic mobility of the protein (Fig. 4). The initial product appeared to be an intermediate that migrated slightly more slowly than the trimer. All the protein eventually accumulated as the monomer. Time courses for the appearance of these species during the modification of porin are shown in Fig. 5(a). Although the dissociation into monomers was apparently dependent on modification occurring, it was not concomitant with the reaction. Thus, at pH 8.5, essentially complete modification could be achieved without causing significant amounts of dissociation. The rate of dissociation increased as the pH was increased until, at pH 11.7, the rate of dissociation was coincident with that of modification. 4-Sulphophenylglyoxal did not modify all the residues in the native protein (Table 2) and no changes in mobility in SDS/polyacrylamide-gel electrophoreses were observed. Modification with the larger glyoxal

analogues (PPG and PSG) had similar effects to modification with 4-sulphophenylglyoxal, except that even fewer residues were modified (Table 2).

The results of modification of phosphoporin were very similar to those obtained with the porin (Table 2). The larger maltoporin has more residues, all of which are accessible to phenylglyoxal. Again, the time course of modification is biphasic, there is a group of four slow-reacting residues (Table 2) and the accessibility to large reagents is decreased.

Modification of carboxy residues

The modification of carboxy residues involves two stages: the first is attack by the carbodi-imide and the second is condensation with an amine added during preincubation (Carraway & Koshland, 1972). The reaction can therefore be controlled by selecting the nature both of the carbodi-imide and of the added amine (see Table 1). Independently of the nature of the added amine, the initial attack by carbodi-imides produced a decrease in the electrophoretic mobility of the porin trimer that was very similar to the initial change produced by phenylglyoxal modification (Fig. 4). This effect on mobility occurred most rapidly in the presence of DCCD, less rapidly with EDCD and was slowest in the presence of CMCD. In each case the intermediate with decreased mobility began to dissociate (Fig. 5b) and the eventual products were the monomeric protein and variable amounts of highly aggregated material. Aggregation was most pronounced with DCCD in the absence of an added amine and dissociation was most rapid with CMCD and with aminofluorescein as the added amine.

The initial attack on porin by EDCD in the presence of 1 M-[^{14}C]methylamine resulted in the incorporation of 30 mol of the amine/mol of monomer (Table 2). Further incorporation occurred only after dissociation of the trimers. The initial attack on porin by EDCD in the presence of 0.25 M-4-aminobenzenesulphonate resulted in the incorporation of 4 mol of the amine per mol of monomer before an appreciable change in electrophoretic mobility of the trimer occurred, and then slow incorporation of a further 2 mol of the amine/mol of monomer during the conversion into the slow-moving species (Fig. 6a). The initial rate of modification increased between pH 6 and pH 4.5 (Fig. 3) as expected for the modification of carboxy groups (Khorana, 1953).

The initial attack on porin by EDCD in the presence of 50 mM-aminofluorescein resulted in the rapid incorporation of 1 mol of fluorescein/mol of monomer (Fig. 6a). This was complete before the changes in electrophoretic mobility of the trimer occurred. A further 2–3 mol of fluorescein were incorporated during the conversion into the slow-migrating species (Fig. 6a). The corresponding results obtained with phosphoporin and maltoporin are also given in Table 2.

Modification of tyrosine residues

Reaction of porin with either 4-diazonium benzenesulphonate, 7-diazonium 4-trifluoromethylcoumarin or 4-diazonium fluorescein resulted in the formation of approx. 2 mol of azotyrosine/mol of monomer at 20 °C and pH 7.0 (Table 2). No modification of histidine residues was detected, judged by the absorption spectrum. At higher pH more tyrosine residues were modified at a higher rate (Fig. 6b), but incorporation was concomitant with denaturation. At 0 °C, approx. 1 mol

Fig. 2. Time course of chemical modification with phenylglyoxal

(a) Modification of arginine residues with phenylglyoxal at pH 8.5. Concentrations of the reagent were: ○, 3 mM; ●, 30 mM. (b) Semilogarithmic plot of the data presented in (a) showing the resolution of the total modification into two groups of residues that react at different rates. Closed circles represent the reaction with 3 mM-phenylglyoxal and open circles represent the reaction with 30 mM-phenylglyoxal. Closed triangles represent the reaction occurring during the fast phase of modification with 3 mM-phenylglyoxal and open triangles represent the reaction occurring during the fast phase of modification with 30 mM-phenylglyoxal. The reaction during the slow phase of modification is shown by the broken lines.

of azotyrosine/mol of monomer was formed, irrespective of pH. Similar results were obtained with phosphoporin and maltoporin proteins (Table 2).

Modification of amino groups

The end point of the reaction of porin with succinic anhydride varied slightly with pH, as it increased from approx. 11 residues modified/monomer at pH 8.5 to approx. 14 residues modified/monomer at pH 10.5 (Table 2). The rate of reaction showed considerably more variation with pH (Fig. 3). Prior modification with phenyl isothiocyanate completely blocked subsequent reaction with succinic anhydride, whereas reaction with 4-sulphophenyl isothiocyanate blocked the reaction of some eight residues and eosin isothiocyanate blocked the reaction of two residues (Table 1).

Labelling with photoactivated probes

All of the porins could be labelled by the three membrane-restricted probes shown in Figs. 1(e)–(g); time courses for labelling of porin are shown in Fig. 7(a). The levels of incorporation of the labels into the phosphoporin and maltoporin proteins were very similar to those obtained with porin. There was no detectable effect of labelling on the oligomeric state of the proteins, judged by their electrophoretic mobilities. Labelling with TID had no effect on the incorporation of aminofluorescein in the presence of CMCD, on the reaction with eosin isothiocyanate, with diazofluorescein or with succinic anhydride. It did, however, result in a reduced efficiency of CNBr cleavage, suggesting that, among others, methionine residues had been labelled. Labelling with *N*-(nitrophenylazido)phosphatidylethanolamine decreased the incorporation of succinic anhydride by 1–2 mol/mol of monomer when the former was used at ten times the

normal concentration and in the absence of glutathione, suggesting that at least one amino group is accessible from the head-group region of the bilayer. This reaction with nucleophilic groups is expected for the electrophilic nitrene generated from the nitrophenylazido group (Schrock & Schuster, 1984). Labelling with BBPG had no effect on the incorporation of any reagent or on the CNBr reaction. Phosphoporin and maltoporin are expected to give a number of CNBr fragments (8 and 12 respectively). As is shown for phosphoporin (Figs. 7b and 7c), label was distributed throughout the CNBr peptides.

DISCUSSION

Two approaches to the topographic labelling of three pore-forming proteins have been used. The first has been to use electrophilic reagents that have well-defined specificities to examine the reactivity of the nucleophilic groups in the protein. The second approach has been to use photoactivated reagents that are directed against the membrane-embedded domains of the protein of less-well-defined specificity.

The electrophilic reagents used include small molecules with appreciable solubility in polar organic solvents (for example, phenylglyoxal, DCCD, phenyl isothiocyanate and succinic anhydride) and which may therefore reach groups buried in the membrane or hydrophobic pockets within the protein. Then there are appreciably more polar molecules (such as 4-sulphophenylglyoxal, 4-diazonium benzenesulphonate and 4-sulphophenyl isothiocyanate), which are essentially insoluble in the organic solvents but are small enough to react not only with portions of the protein exposed to the aqueous bulk medium but also within the channels. Finally, there are relatively large reagents, such as the phenylglyoxalsulphonate esters,

Table 2. Extent of modification of pore-forming proteins

Modifications were performed as described in the text, at the reagent concentrations and pH listed below. The values quoted in the Table are taken from the plateau in time courses such as that shown in Fig. 1 and represent the mean of three to nine determinations. The incorporation of phenylglyoxal and its analogues is calculated by assuming that 2 mol of the glyoxal analogue react with 1 mol of arginine (Takahasi, 1968).

Target residue	Reagent	No. of residues									
		Pore-forming protein ...				No. of target residues			Maximum value obtained with denatured protein		
		OmpF*	PhoE†	LamB‡		OmpF	PhoE	LamB	OmpF	PhoE	LamB
Arginine	Phenylglyoxal	12	12	17		12.3	13.0	16.7	11.6	12.1	16.5
	30 mM, pH 8.5								8.2	7.8	12.5
	During fast phase								4.1	4.5	4.1
	After 30 mM-sulphophenylglyoxal										
Carboxy	Sulphophenylglyoxal, pH 8.5					11.4		17.5	8.0		14.1
	30 mM								8.5		
	300 mM								6.9		8.7
	PPG (Fig. 1c), 100 mM, pH 8.5					11.9		16.3	5.1		6.1
Tyrosine	PSG (Fig. 1d), 100 mM, pH 8.5					12.2		16.9			
	EDCD (80 mM)	45	46	55		41.2	47.3	52.3	30.3	27.1	45.6
	Plus 1 M-methylamine, pH 4.5					40.6	45.1	53.1	5.5	5.9	6.0
	Plus 250 mM-aminobenzenesulphonate					42.1	46.3	53.0	3.6	2.9	3.7
Amino	Plus 50 mM-aminofluorescein										
	4-Diazonium benzenesulphonate (10 mM), pH 8.5	25	22	22		24.0	22.9	23.0	1.2	1.4	1.5
	0 °C								1.7	1.9	1.8
	20 °C								1.8		2.1
Amino	7-Diazonium 4-trifluoromethylcoumarin (10 mM) pH 8.5, 0 °C										
	4-Diazonium fluorescein (10 mM), pH 8.5, 0 °C								0.9	1.0	1.1
	Succinic anhydride (50 mM) pH 8.5	19	24	21		19.1	23.8	21.4	11	12	14
	pH 9.5								13		
Amino	pH 10.5								14	15	17
	pH 8.5, after 1 mM-phenyl isothiocyanate								0		

* Rosenbusch (1974).

† Overbeeke *et al.* (1985)

‡ Clement & Hofnung (1981)

Fig. 3. The pH-dependence of the rate of modification of porin

The rate constants for modification were calculated from semilogarithmic plots of the fractional incorporation $[(P - P')/P]$ against reaction time. The plots for phenylglyoxal and EDCD were biphasic, and the method of Ray & Koshland (1961) was used to obtain rate constants for the two phases of reaction (see Fig. 2b). Closed triangles represent the rate of modification with 30 mM-phenylglyoxal (fast phase). Open triangles represent the rate of modification with 30 mM-phenylglyoxal (slow phase). Open circles represent the rate of modification with diazonium benzenesulphonate. Open squares represent the rate of modification with EDCD and 4-aminobenzenesulphonate (fast phase).

diazonium fluorescein and eosin isothiocyanate, which are expected to label only the external regions of the protein because they are larger than the exclusion limit of the pore, which is about 600 Da for penetration of uncharged oligosaccharides (Nikaido & Nakae, 1979).

The essentially stoichiometric modification of arginine residues by phenylglyoxal indicates that all residues can react, but the biphasic nature of the reaction shows that four are much less reactive than others and may therefore be less accessible. The number of residues that are inaccessible to sulphophenylglyoxal and that react slowly with phenylglyoxal is the same (four residues per monomer for each protein), and it is modification of these presumably 'buried' groups that brings about the changes in electrophoretic mobility of the trimers. The difference between the extent of reaction with 4-sulphophenylglyoxal and the larger probes indicates that, of the eight rapidly reacting residues, there are five to seven that occur in positions that are completely exposed to the bulk aqueous phase and that there are a few (three to one) residues that are less accessible (Table 2).

It is clear that only a small number of carboxy residues is freely available to all reagents and that not many more residues are available to small, but charged, reagents (Table 2). Many more groups can only be modified by using small, uncharged reagents such as DCCD or EDCD plus methylamine and hence appear to be less accessible. This suggests that there are three or four residues located at the surface of the protein that is exposed to the aqueous

phase and only one or two exposed within the channel. Another 40 or more residues [including many of those reported by Tokunaga *et al.* (1981) to be modified by EDCD plus glycylamide] appear to be located in the interior of the protein. It is probably the modification of such 'buried' residues that brings about the change in mobility of the trimer on SDS/polyacrylamide-gel electrophoresis.

Modification of tyrosine residues appears completely opposite to the extensive modification of arginine and amino groups, since very few tyrosine residues can be readily modified in the native state, with further modification occurring only with denaturation. This, in part, must reflect the different localization of the functional groups; in soluble proteins at least, arginine, lysine and carboxy residues are most commonly found in surface-exposed positions (Rashin & Honig, 1984), whereas tyrosine residues are more often buried within the hydrophobic core.

Few nucleophilic groups of the types discussed here were expected to be found exposed to the lipid bilayer or embedded in the protein interior. Groups of this sort that are to be found in these regions may be expected to be interacting internally with other groups in the molecule and thus deeply involved in maintaining the tertiary or quaternary structure of the protein. We find that there is a group of four arginine residues and, particularly striking, a group of nearly 40 carboxy residues in all three of the pore-forming proteins which appear to fall into this class. Indeed, modification of these groups occurs consistently slowly, can only be obtained with small hydrophobic molecules and greatly affects the stability of the trimer. Further, several amino and carboxy groups, as well as most of the tyrosine residues, cannot be modified at all in the native protein, although they do react when the protein is in a denatured state. *A priori*, nucleophilic groups may be expected to be found in those parts of the protein that line the pore (Schindler & Rosenbusch, 1982, 1984). The reactivity of these groups will depend on their position in the pore, and their modification may not necessarily have a significant effect on structure, although it may affect function (Tokunaga *et al.*, 1981). It appears noteworthy that of the 25 carboxy residues that were reported to be modified by small reagents, and hence presumed to line the pores (Tokunaga *et al.*, 1981), only five were actually found to react with ionized nucleophiles (Table 2). Selectivity for the size of the chemical reagent relative to the exclusion limit of the pore (600 Da) can be expected, but partitioning into a non-polar phase also appears critical. As shown in Table 2, clear correlations between size and partition coefficient of the reagent may be noted for the number of arginine, amino and carboxy groups that can be modified.

The second approach to topographic labelling has been to use reactive species generated *in situ* by photolysis of stable precursors after their incorporation into the lipid bilayer. TID (Fig. 1e) is a small molecule that partitions into hydrophobic parts of the lipid bilayer or of the protein (Brunner & Semenza, 1981; Krebs *et al.*, 1984). The carbene generated by photolysis (Brunner *et al.*, 1980) shows some selectivity for nucleophilic groups, but labels aliphatic groups quite efficiently as well and should therefore be a good probe for potential lipid-binding sites (Hoppe *et al.*, 1984; Hoppe & Sebald, 1984). The reagent BBPG (Fig. 1g) is expected to be more selective for

Fig. 4. Qualitative effects of modification on electrophoretic behaviour

Samples from a time course such as that shown in Fig. 1 were quenched with Tris/HCl/arginine buffer (the Materials and methods section) and prepared for SDS/polyacrylamide-gel electrophoresis without heat-denaturation. SDS/polyacrylamide-gel electrophoresis was performed by the method quoted in Rosenbusch (1974). Lane 2, native OmpF protein [R_{DF} 0.34]; lanes 3–6, porin treated with 50 mM-phenylglyoxal for 3 h at pH 8.5 (lane 3, R_{DF} 0.30), pH 9.6 (lane 4, R_{DF} 0.30), pH 10.5 (lane 5, R_{DF} 0.30) and pH 11.5 (lane 6, R_{DF} 0.57); lane 7, porin treated with 200 mM-sulphophenylglyoxal for 3 h at pH 10.5 (R_{DF} 0.34) and lane 8, porin treated with 50 mM-EDCD in the presence of 100 mM-aminobenzenesulphonate at pH 4.5 (R_{DF} 0.30); lane 9, native phosphoporin (R_{DF} 0.34); lane 10, phosphoporin treated with 50 mM-phenylglyoxal at pH 8.5 (R_{DF} 0.26) and, in lane 11, treated at pH 11.5 (R_{DF} 0.62); lane 12, phosphoporin treated with 50 mM-EDCD as described (R_{DF} 0.34 + 0.26); lane 13, native maltoporin (R_{DF} 0.27); lanes 14–17, maltoporin treated with 80 mM-phenylglyoxal at pH 8.5 (lane 14), pH 9.5 (lane 15), pH 10.5 (lane 16) and pH 11.5 (lane 17); lane 18, maltoporin treated with 50 mM-EDCD and 100 mM-aminobenzenesulphonate. The marker proteins in lane 1 are: β -galactosidase (116 kDa), phosphorylase (95 kDa), catalase (56 kDa), enolase (41 kDa), glyceraldehyde-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa) and trypsinogen (25.3 kDa). In the last lane (lane 19) are maltoporin (47.4 kDa), porin (37.2 kDa) and phosphoporin (36.8 kDa) dissociated by heating at 95 °C for 10 min in sample buffer. R_{DF} = R_{Dye} front.

Fig. 5. Quantitative changes in the electrophoretic behaviour of porin protein during chemical modification

Modification was performed with (a) 30 mM-phenylglyoxal at pH 10.5 and (b) 100 mM-EDCD + 250 mM-aminobenzenesulphonate at pH 4.5. SDS/polyacrylamide-gel electrophoresis was performed as described in Fig. 4 and gels stained with Coomassie Blue. After destaining, the protein bands were cut out and extracted with 1 ml of dimethyl sulphoxide for 24 h at room temperature. A sample was also cut out from a blank area of the gel and extracted in the same way. Each sample was filtered and the A_{595} was measured against the blank sample. Δ , Stain recovered at R_{DF} (see Fig. 4) 0.30; $\bullet$, stain recovered at R_{DF} 0.34 (native trimer); $\circ$, stain recovered at R_{DF} 0.57 (monomer).

Fig. 6. Time course of chemical modification of carboxy and tyrosine residues in porin

(a) Modification of carboxy residues with EDCD in the presence of aromatic amines at pH 4.5. EDCD was present at a concentration of 8 mM ($\blacktriangle$) or 80 mM ($\bullet$), whereas aminobenzene[^{35}S]sulphonate was present at a concentration of 250 mM. Open triangles represent modification with 80 mM-EDCD in the presence of 50 mM-aminofluorescein. (b) Modification of tyrosine residues with 10 mM-diazonium benzenesulphonate at pH 7.0 ($\triangle$), at 8.5 ($\bullet$) and at 9.6 ($\circ$).

carbon-hydrogen bonds on the α -carbon atom (Bayley & Knowles, 1977). This selectivity and the lipid solubility of the benzophenone moiety is expected to result in preferential labelling of those parts of the protein that are in contact with the hydrophobic core of the membrane. It is apparent from the distribution of label introduced by TID and BBPG that there are hydrophobic regions distributed throughout the proteins under study. The lack of clear segregation in the distribution of label from hydrophobic and hydrophilic probes is in agreement with the lack of clear segregation of apolar and polar residues observed in the amino acid sequences of these proteins (Chen *et al.*, 1979, 1982; Overbeeke *et al.*, 1980; Clement & Hofnung, 1981).

The precise location of some of the labels in the primary structure is now being determined by peptide mapping, but verification of the tentative interpretation of their location in the tertiary structure, suggested here on the basis of size and polarity of the reagents, will only be possible when the three-dimensional structure of the protein is known at high resolution. The analysis of porin crystals, which diffract to beyond 0.3 nm (3 Å) (Garavito *et al.*, 1983), should make this possible very soon. Meanwhile, the structure proposed for porin (Paul & Rosenbusch, 1985) can be compared with the results presented here. In that model, at least two arginine, 20 carboxy, 16 tyrosine and three lysine residues were suggested to be buried. All these values are similar to the numbers of groups inferred to be buried on the basis of their lack of reactivity with all but the smallest probes (if they react at all) in the native protein. Schlaeppli *et al.* (1985), who used hydrophilic reagents, have found that about eight of the lysine residues in porin are inaccessible to their smallest reagent, which, in view of the partition coefficients, is in accord with our results (Table 2). The observation that the experimental values for the number of buried groups are consistently higher than those inferred from the model suggests that segments linking

those presumed to be arranged in β -structure within the membrane boundary are also very tightly packed. The absence of large extramembrane domains is confirmed by electron microscopy of stained two-dimensional arrays and X-ray crystallography, which show that very little of the protein projects from the surface of the membrane and that much of the water-accessible surface lies within the pores (Steven *et al.*, 1977; Garavito *et al.*, 1983; Engel *et al.*, 1985).

With maltoporin, topographical constraints are available from the genetic mapping of mutations leading to phage resistance (Clement *et al.*, 1983), from the identification of the epitopes of monoclonal antibodies (Schenkman *et al.*, 1983), and from the location of sites that are the primary targets of limited proteolysis (Schenkman *et al.*, 1984). The model proposed by Charbit *et al.* (1984) implies membrane-buried sites which exhibit a similar relationship to the experimental values described above for porin. Once again, rather more residues appear to be located in the protein and membrane interior than are predicted from their model. These results thus reveal the potentials as well as the limitations of the models discussed. In any event, many more ionizable residues appear to be buried, at least partially, within the membrane domain and may there by involved in interactions that could explain the unusual stability of porin trimers by the formation of hydrogen-bonded networks and ion-pairs.

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Fig. 7. Labelling of porin with photoactivated probes

(a) Time course of labelling of porin by TID (●), BBPG (▲) and *N*-(nitrophenylazido)phosphatidylethanolamine (NAPE) (■). Radioactivity introduced with TID was measured with a Nuclear Enterprises NE 1600 γ -radiation counter, whereas radioactivity from BBPG and *N*-(nitrophenylazido)phosphatidylethanolamine was measured by liquid-scintillation counting. (b) Distribution of the radioactivity incorporated by photolysis of TID between the CNBr fragments derived from PhoE protein. A portion of a CNBr reaction mixture was neutralized with cold, concentrated NH_3 solution. The sample was evaporated to dryness under a stream of nitrogen and resuspended in sample buffer for SDS/polyacrylamide-gel electrophoresis. The separating gel contained 20% (w/v) acrylamide. After staining with Coomassie Blue and destaining, 2 mm slices were cut out, measured for radioactivity (○) in a γ -radiation counter and then for stain (●). The numbering of the fragments is that given by Overbeek *et al.* (1983). (c) Distribution of radioactivity incorporated by photolysis of BBPG between the CNBr fragments derived from phosphoporin. Cleavage and sample preparation were as described in (b) above, except that radioactivity was measured by liquid-scintillation counting after digestion of the slices with H_2O_2 . Of the radioactivity in the sample before CNBr treatment, 20% was recovered from the gel.

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