Chemical Modification of Matrix Porin from *Escherichia coli*: Probing the Pore Topology of a Transmembrane Protein

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ABSTRACT Chemical modification of amino groups in matrix porin solubilized and purified from outer membranes of *Escherichia coli* in β-octylglucoside was performed with eosin isothiocyanate and citraconic anhydride. At pH 8.5, the former reagent labeled a single amino group in the native protein, while more extensive derivatization was observed with increasing pH or upon denaturation. Citraconic anhydride modified ~12–14 residues in native porin and 15–16 of the total of 19 amino groups in the denatured state. Fluorescamine, another amine-specific reagent of intermediate size, derivatized 3 and 16 residues in the native and denatured states, respectively. These results indicate that reactive probes of various sizes may serve as indicators for the surface accessibility of reactive residues in matrix porin. The increased derivatization of lysyl residues at high pH (or in phosphate buffer) suggests the method's sensitivity to different conformational states of the protein. The extent of tyrosine modification (1–2 residues in the native, and ~22 in the denatured porin) depended on the state of protein folding, even with reagents of small size. The approach of using various probes with differing properties and specificities thus appears useful for the determination of membrane protein asymmetry, pore topology, and conformational states of transmembrane proteins.

Passive diffusion of small hydrophilic solutes (<700 daltons) across the permeability barrier of outer membranes of Gram-negative bacteria has been demonstrated to occur through transmembrane channels formed by a group of proteins called porins (1). A representative member of this group, called matrix porin (or matrix protein, M_r 36,500), has been isolated from *Escherichia coli* B^E and biochemically characterized (2). The following properties of this protein are relevant within the context of this study: (*a*) Conductance measurements have demonstrated that channels exist in at least two different states, open and closed (3, 4). (*b*) The equivalence diameter of the pores is ~1 nm (3, 5). Ultrastructural studies to a resolution of 18–20 Å have shown that the observed indentations, which most likely represent the orifices of the pores (6), have a diameter of 2 nm (7). (*c*) The amino acid composition of porin exhibits high polarity of the protein, a characteristic considered unusual for a protein almost entirely embedded within the hydrophobic environment of the membrane (8) and exhibiting an apparently highly hydrophobic surface (9). Sequence analysis of a closely related porin demonstrated the absence of obvious hydrophobic domains and revealed a random distribution of polar residues throughout the polypeptide chain (10). Its folding pattern, therefore, would have to be distinctly different from that found for soluble proteins, unless most of the polar residues were exposed towards the aqueous phase within the pore.

In an attempt to approach the problem of protein conformation, and to explore the topology of the pore in more details than ultrastructural studies have heretofore yielded, we have initiated an investigation employing chemical modification. The extent of labeling of amino groups, using both pore-permeant[1] and impermeant probes, and the degree of derivatization of tyrosyl residues indicate that the approach taken may contribute to our understanding of the questions posed.

MATERIALS AND METHODS

Materials

Eosin isothiocyanate (eosin-SCN), fluorescamine, and citraconic anhydride were the products of Molecular Probes (Plano, TX), Sigma Chemical Co. (St. Louis, MO), and Fluka Biochemicals (Basel, Switzerland). β-Octylglucoside was

[1] Pore-permeant probes are operationally defined as hydrophilic molecules with M_r <700. These molecules are capable of passive diffusion through matrix porin.

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purchased from Sigma Chemical Co. All other reagents were of the highest purity available commercially.

Methods

BUFFER SOLUTION: Bicarbonate buffer, pH 8.5, consisted of 2% $NaHCO_3$. Carbonate (0.2 M Na_2CO_3) and phosphate (50 mM Na_2HPO_4) buffers were adjusted to the pH indicated with NaOH and HCl. All buffers contained 1% β-octylglucoside, unless indicated otherwise.

PROTEIN ISOLATION: Matrix porin trimers were solubilized from cell envelopes (2) by repeated extractions with 3% β-octylglucoside (Sigma Chemical Co.) at 37°C for 1 h in 20 mM $NaPO_4$/0.1 M NaCl/3 mM NaN_3, pH 7.0 as described in references 4 and 6. The protein was pure, as attested to by PAGE. $^{32}P_i$ labeling of phospholipids and glycolipids demonstrated the absence of both lipopolysaccharide and phospholipids as contaminants after purification. The detergent concentration for all experiments was adjusted to 1% β-octylglucoside.

LABELING PROCEDURES (11): Eosin-SCN was prepared as 2 mg/ml solution in the particular buffers to be used for labeling. All labeling experiments were performed on nondenatured protein in detergent solution. Solutions were sonicated for 10 min. Aliquots of the label (2.5- to 5.0-fold molar excess over lysyl residues yielded similar results) were added to 0.5-ml matrix porin solutions (1 mg/ml) in the same buffers (5 mg of detergent to 0.5 mg of protein, or 10:1 wt/wt). Reactions proceeded overnight at room temperature and were subsequently terminated at room temperature with either alanine or lysine (final concentration, 33 mM). Labeling with 1 μl of undiluted citraconic anhydride (molar excess of 20) was carried out in 0.5 ml of bicarbonate buffer at pH 8.5. The reaction proceeded for 3 h at room temperature. Extent of modification of protein in the native state for all reagents did not vary from ratios of reagent to lysine of from 1:1 to 10:1. The mixture was then dialyzed overnight in bicarbonate buffer, containing 1% octylpolyoxyethylene (6) and 0.2 mM dithiothreitol. Fluorescamine assays (12) were performed in the same reaction buffers. Tyrosine residues were modified under the conditions and with the reagents given in Table I.

SPECTROPHOTOMETRIC ASSAYS: Eosin incorporation was estimated by the change in absorption of the protein-eosin complex in detergent solution (0.1 M NaCl, 1% β-octylglucoside, pH 7.0) at 525 nm ($\epsilon_{525} = 8.5 \times 10^4\ M^{-1}$ [13]) using a Zeiss PM Q II spectrophotometer. After labeling and quenching, the protein was subjected to gel filtration on a Sephadex G-25 column (1.5 × 25 cm) using 20 mM sodium phosphate, 0.1 M NaCl, 1% β-octylglucoside, pH 7.0, as eluant. Extensively dialyzed preparations of citraconylated protein in detergent solution were quantitated by monitoring the spectral change at 250 nm (ϵ_{250} = 2,180 M^{-1} [14]). For determining the extent of fluorescamine labeling, a Stoeffel spectrofluorimeter was employed to record fluorescence changes at λ (excitation) = 360 nm and λ (emission) = 475 nm. Lysozyme was used to establish a standard curve with the methodology following published procedures (12). The assay employed a range of protein concentrations from 5.8 to 17.4 nmol matrix porin. Extent of tyrosine modification for reaction with iodine was estimated using ϵ_{325} = 4,200 M^{-1} (15), while nitration was monitored λ = 427 nm (ϵ_{427} = 4,200 M^{-1} [16]).

AMINO ACID ANALYSIS: All samples were extensively dialyzed against H_2O before hydrolysis. Hydrolysis was carried out in 6 N HCl at 110°C *in vacuo* for 20 h. HCl was removed *in vacuo* over NaOH pellets, and the residue was dissolved in Durrum sample buffer (Dionex Corp., Sunnyvale, CA). Norleucine was added as an internal standard. The samples were analyzed on a Durrum amino acid analyzer.

SDS PAGE AND ULTRACENTRIFUGAL ANALYSIS: Aliquots of eosin-labeled reaction mixtures were electrophoresed as quoted in detail (2). Gels exhibiting purple bands were photographed directly and, subsequently, with illumination by UV light. The same gels were then stained with Coomassie Blue. Sedimentation velocity experiments were performed in an analytical ultracentrifuge (Beckman model E) as quoted (2).

RESULTS

ASSESSMENT OF NATIVE STATE: Detergent-solubilized matrix porin trimers demonstrated a preservation of biological activity as demonstrated by voltage dependence characteristics (3, 4), retention of conformational integrity as determined by circular dichroism and IR spectroscopy (Rosenbusch, J. P., manuscript in preparation), and a conservation of the protein's overall structure before and after removal from the outer membrane by a spectroscopic comparison of the predominantly anti-β-configuration of the protein in the outer membrane and detergent solution (Rosenbusch, J. P., A. Lustig, and M. Regenass, manuscript in preparation).

LABELING OF AMINO GROUPS WITH NONPERMEANT PROBES: At pH 8.5, eosin-SCN (M_r = 705) labeled matrix porin without causing denaturation. The native and denatured states of the protein were ascertained by their characteristic mobilities upon electrophoresis on polyacrylamide gels in SDS. The slow and fast moving bands represent the native and denatured states, respectively (8). These differences in mobility are well-correlated with resistance to proteolysis (2) and the ability to form channels in phospholipid bilayers (1, 3, 5). They thus may be used as criteria for the native state of the protein (8). Covalent modification of polypeptides resulted in the appearance of intense fluorescent bands (Fig. 1 *a–f*) on gels at positions where protein bands appeared upon gel staining (Fig. 1 *a*–f**). The band in the position of the denatured polypeptide appeared diffuse (Fig. 1 *b* and *b**). This was most likely due to its reaction with remaining free eosin-SCN during denaturation, since the addition of lysine or alanine before electrophoresis yielded sharp bands (Fig. 1 *d* and *d**) similar to those of untreated matrix porin (2). All subsequent reactions were

TABLE I

Modification of Lysyl and Tyrosyl Residues by Low and Intermediate Molecular Weight Probes

Residues modified	Reagent used	Conditions	Extent of derivatization
			mol/ mol polypeptide
Lysyl	Citraconic anhydride	pH 8.5 (bicarbonate) Native protein	12.4 ± 1.0
		pH 8.5 (bicarbonate) Denatured protein	15.0 ± 1.2
	Fluorescamine	pH 8.5 (bicarbonate) Native protein	2.9 ± 0.3
		pH 8.5 (bicarbonate) Denatured protein	16.4 ± 1.0
Tyrosyl	KI-I_2* (reference 15)	pH 8.5 (bicarbonate) Native protein	1.5 ± 0.4
		pH 11.0 (phosphate) Denatured protein	22.1
	Tetranitromethane‡ (reference 16)	pH 8.0 (50 mM Tris-HCl) Native protein	1.5

* Reactivity of free tyrosine demonstrated complete modification over the pH range used. The values shown are single measurements within a pH titration series (unpublished).

‡ Upon modification with tetranitromethane of matrix porin in the denatured state (100°C treatment in buffer for 5 min), protein samples remained at the top of the gel. This is most likely the result of cross-linkage of the protein (17) during reaction.

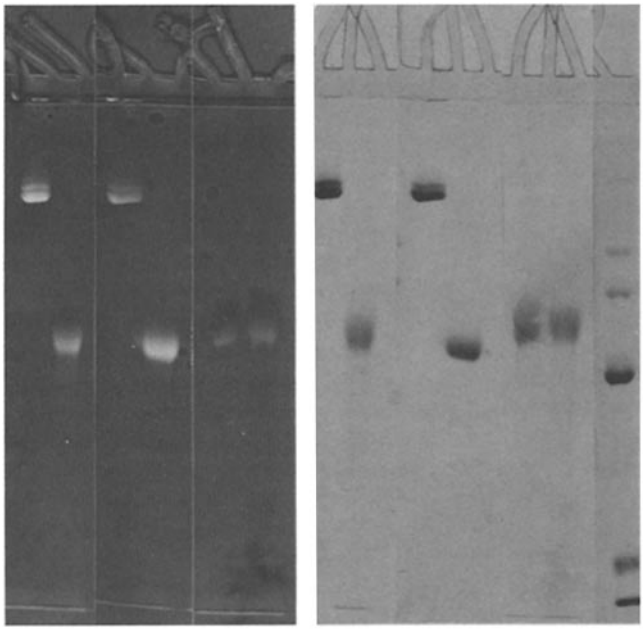

FIGURE 1 Labeling of matrix porin with eosin-SCN. Slots *a*, *a**, *b*, and *b** represent labeling with eosin-SCN (see Materials and Methods) at pH 8.5 in bicarbonate buffer containing 1% β-octylglucoside at room temperature for 2 h. Similar patterns were obtained after 24-h incubations. Slots *c*, *c**, *d*, and *d** represent identical reaction conditions, except that lysine (or alanine) was added to terminate the reaction before gel electrophoresis. Slots *e*, *e**, *f*, and *f** represent the results of labeling after denaturation (100°C treatment for 5 min). Slots *a*–*f* are from gels photographed during fluorescent excitation; slots *a**–*f** represent the Coomassie Blue–stained patterns of the same gel. Protein concentrations for all reactions were 1 mg/ml. Samples in slots *b*, *d*, and *f* were boiled in SDS sample buffer before application, while *a*, *c*, and *e* were applied in SDS sample buffer without boiling. The multiple bands, observed in the unboiled samples (*a* *c*), are indicative of minor contamination with lipopolysaccharides that is removed upon boiling in SDS (*b* and *d*). Standards (unlabeled slot) represent molecular weight polypeptides with masses of 98, 58, 49, 33, 17, and 14.3 kdaltons, respectively.

TABLE II

Stoichiometry of Lysyl Residues

pH	Lysyl modified
	mol/ mol polypeptide
7.0	0.6
8.5	0.7 [0.7–0.85*]
10.0	1.9
11.0	3.4 (5.0)
12.0	5.0

Stoichiometry of lysyl residues modified by eosin-SCN as a function of pH and buffer ions. Extent of modification at pH 8.5 was estimated by absorbance at 525 nm, while the protein concentrations were determined either by absorbance at 278 nm ($A_{278}^{0.1\%} = 1.41$ [2]) or by quantitative amino acid analysis (cf. Materials and Methods). The values thus obtained are given in brackets. All other values were obtained by subtractive amino acid analysis. The values indicated are those obtained in bicarbonate or carbonate buffers, except for the figure shown in parentheses which indicates the extent of labeling in phosphate buffer.

* Modification of lysyl residues at pH 8.5 has since been confirmed by extensive evaluation of substitution with acetylated β-alanine, activated in the carboxyl group (B. Erni and M. Bernstein, unpublished results).

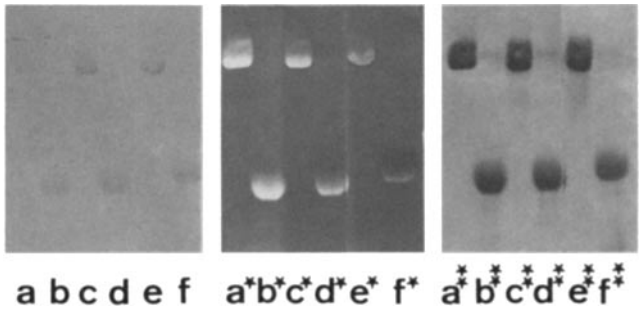

FIGURE 2 Modification of matrix porin at alkaline pH. The effect of pH and buffer ions is shown on a gel photographed before staining (left panel), upon fluorescence excitation of the same gel (central panel), and upon staining with Coomassie Blue (right panel). Labeling was performed as described in Materials and Methods, with eosin-SCN in carbonate buffer at pH 10 (all slots *a* and *b*), at pH 11 (all slots *c* and *d*), and in phosphate buffer at pH 11 (all slots *e* and *f*). The first slot of each pair represents the unboiled, the second the boiled sample.

therefore terminated by the addition of lysine or alanine. If matrix porin had been denatured before modification with eosin-SCN, the degree of substitution of the protein (Fig. 1 *e* and *e**) was enhanced despite an apparently decreased fluorescence (Fig. 1, compare *e* with *d*). This was also indicated by the appearance of a purple band visible before and after staining. It appears likely that the introduction of additional eosin groups led to the mutual quenching of their fluorescence emission. The stoichiometry of labeling, determined as described in the legend to Table II, yielded 0.7–0.85 residues modified per polypeptide at pH 8.5 (Table II). The protein remained native (Fig. 2) in buffers with pH up to 11 according to the criteria mentioned above. The influence of buffer ions was tested by the comparison of labeling in carbonate and phosphate buffers at pH 11. The extent of labeling with eosin-SCN appeared to be increased in phosphate buffer (Table II). The good correlation with the intensities of purple bands on gels before staining is shown in Fig. 2 *a*–*f*. High levels of modification resulted in an increase in quenching (Fig. 2, compare *f** with *b**) coupled with a decrease of mobilities of the denatured protein (Fig. 2, compare *d* and *e* with *b* in all three panels). When the pH was raised to 12, a pattern corresponding to that of heat-denatured protein (Fig. 1 *e** and *f**) was observed after modification. Even without labeling, the protein appeared irreversibly denatured (not shown) at this pH. The quantitation of the modified amino groups with eosin-SCN as a function of pH and buffer ions is shown in Table II.

MODIFICATION WITH PORE-PERMEANT PROBES: In all instances described, chemical modification was performed in buffers containing 1% β-octylglucoside or 1% octylpolyoxyethylene. Citraconic anhydride, a small polar amino group–reactive agent (M_r 112), labeled about two thirds of all amino groups present (18 ε- and 1 α-amino group [2]) at pH 8.5 (Table I). At pH 12, this value increased to 15. The extensive modification at the lower pH did not denature the protein according to our operational criteria. This was confirmed by analytical ultracentrifugation. Sedimentation velocity experiments of untreated trimers, and protein modified by citraconic anhydride at pH 8.5, yielded sedimentation coefficients (in phosphate buffer containing 1% β-octylglucoside) that were indistinguishable in the two cases ($s_{20}^{obs} = 5.2S$).

Substitution of amino groups with fluorescamine, a hydrophobic probe of intermediate size (M_r 280), yielded approximately three residues modified at pH 8.5 (protein in the native state), whereas nearly quantitative modification was observed after denaturation (Table I).

Modification of tyrosine, an example of a nonpolar side chain, was performed with KI-I_2 (15) and tetranitromethane (16). Approximately 1–2 tyrosine residues of the 24 residues per polypeptide chain in matrix porin were modified. The extent of derivatization was the same for both reagents, as shown in Table I. When attempts were made to modify the protein after denaturation (heat treatment in phosphate buffer at pH 11), essentially quantitative reaction was observed (Table I). Since both reagents presumably are capable of pore permeation, it appears significant that essentially complete modification of tyrosine occurred only after denaturation.

DISCUSSION

The results reported here suggest that in matrix porin of *E. coli* B^E a single amino group is available for modification with eosin-SCN, a reagent presumably excluded from pores. The definitive characterization of this proposed unique lysine awaits the isolation and analysis of labeled peptides, work that is currently in progress. When small polar probes are used, such as citraconic anhydride, about two thirds of the amino groups are substituted without denaturation of the protein. Thus, a large fraction of lysyl residues seem to be exposed to the aqueous phase, with all but one apparently contained within the pore. This result is consistent with the complete resistance of matrix-porin to trypsin proteolysis (2). Fluorescamine, a hydrophobic probe of intermediate size, modified about three residues in the native state.

The stoichiometries observed may represent slight overestimates, due to the presence of low levels of lipopolysaccharides. Contamination with this bacterial glycolipid is clearly indicated by the multiple bands (our unpublished results) for undenatured porin (Fig. 1). Since denaturation fully dissociates the protein-glycolipid complex, yet the position of the fluorescent band is that of the polypeptide upon heat treatment, the low degree of glycolipid labeling may be ignored in the present context. The increased labeling observed with eosin-SCN as a function of pH (and buffer ions) appears not to be uniquely related to the protonation of amino groups. This is suggested by the eventual irreversible denaturation (independent of labeling) above pH 11.0 and also by spontaneous pore activation at high pH in planar lipid bilayer studies (3). Preliminary experiments demonstrating greatly enhanced tyrosine modification as a function of increasing pH (our unpublished results) provide a clue that the conformational state of the protein may be linked rather to the ionization state of tyrosines. This is indicated by the nonreactivity of tyrosines in the native molecule, as opposed to nearly quantitative reaction in the denatured state (Table I).

Our results are valid only if the modifications of the side chains do not alter the native state of the protein. Four lines of evidence suggest that the native state is maintained after amino group substitution. First, the operational criteria of mobility in gels (see Results) indicated that the protein is not denatured by modification with citraconic anhydride at pH 8.5. Secondly, the comparison of the sedimentation coefficients of unmodified (6) and modified trimers show that matrix porin is not dissociated by this treatment. Thirdly, spectroscopic measurements of the amount of antiparallel β-configuration in the fully citraconylated protein agreed precisely with that determined for unmodified matrix-porin trimer (M. Schindler, unpublished results). And, finally, both proteins remained undigested in the presence of trypsin, whereas heat denaturation of matrix porin renders it completely susceptible to proteolysis (M. Schindler, unpublished results). Also, our results with tyrosine modifications demonstrate that with permeant probes essentially complete modification can be attained only with denatured protein. The low level of modification (1–2 tyrosines/polypeptide chain) under conditions that resulted in the extensive derivatization of amino groups by pore-permeant probes provides additional evidence of the structural integrity of the protein, while the high reactivity of tyrosines only in the denatured state indicates that these residues are not exposed in the pore but reside in the hydrophobic core of the protein.

Exposure of the majority of amino groups to the aqueous phase and the sequestering of most of the tyrosine residues within the hydrophobic core indicate that principles governing the internal structure of proteins are conserved also in transmembrane proteins presenting both hydrophobic surfaces (9) and high polarity (2, 10). Whether the amino groups that did not react with citraconic anhydride occur at contact areas between subunits, are less reactive due to steric or electrostatic hindrance, or form salt linkages within the hydrophobic core (18) remains to be determined.

The observation that a single reactive group is available for labeling with large, pore-impermeant reagents clearly demonstrates protein asymmetry. Reaction of whole cells with eosin-SCN has labeled matrix porin (our unpublished results) and exposure of whole cells to activated macromolecules removes porin (19). These observations suggest exposure of the reactive group on the outside of outer membranes. Although strain differences complicate comparison between porins, our finding may explain the low efficiency (J.-M. Neuhaus, personal communication) and the variability observed initially (20, 21) in cross-linkage experiments (20–23). The intermediate levels of substitution with fluorescamine may be related to its reactivity with residues near the pore orifice. To relate labeling to accessibility, a precise knowledge of the permeability properties of the reagent and the localization of the substituted polypeptide fragments is required.

In an attempt to explore pore topology and protein folding of matrix porin in more detail, the study of well-characterized activated peptides of various size is currently in progress, in conjunction with analysis of labeled peptides (E. Erni and M. Bernstein, unpublished studies). The approach described may also be extended to other reactive residues, with the goal of determining whether our results have a more general applicability. Selective modification of membrane proteins by probes of various size could be extended to other membrane proteins. The modification of the anion transport protein of erythrocytes by the amino-specific reagent 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS [24]) may be complemented by additional probes with different properties. Meanwhile, selective modification of lysyl or tyrosyl residues may prove useful for obtaining isomorphous derivatives of porin crystals (6, 25).

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