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Structural Comparison of *Neisseria gonorrhoeae* Outer Membrane Proteins

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Outer membranes from opaque colonial variants of *Neisseria gonorrhoeae* P9 contain a major outer membrane protein (protein I) together with one or more of a series of heat-modifiable proteins (proteins II). Proteins I, II, and IIa have been isolated by detergent extraction of outer membranes. Amino acid analysis showed proteins II and IIa to have a very similar composition. Cyanogen bromide cleavage of proteins II and IIa produced a pair of fragments with identical molecular weight and a pair which differed by an amount (0.5K) equivalent to the difference between the intact proteins. Tryptic peptide maps of ^{125}I-labeled proteins II, IIa, and IIb showed many similarities, with only a few peptides unique to any one protein. Peptide maps of protein IIa from cells which had been surface labeled showed that the unique peptides were exposed on the surface. The heat-modifiable proteins thus appear to form a family of proteins with closely related structure probably differing in that part which is exposed on the bacterial surface.

The proteins present in the outer membrane of gonococci are of considerable interest, being implicated in the host-bacteria interactions which determine the course of an infection. Recent studies have shown that even within a single strain, variations in surface protein composition can occur which have a considerable influence on the potential virulence of the organism (10, 12, 15).

The major proteins present in the outer membrane of colonial variants of a single strain may be divided into two classes. Protein I, or major outer membrane protein, is present in all colonial forms (14, 20), has a molecular weight in the range of 32,000 (32K) to 40K characteristic for that strain, and may be a major determinant of serotype specificity (11). In addition, variants which produce an opaque colonial phenotype may possess one or more additional outer membrane proteins (proteins II) in the molecular weight range of 24 to 30K (14, 20). One strain which has been extensively studied, P9, is particularly interesting in that as many as five additional proteins (II, IIa, IIb, IIc, and IId) may be produced, although no single variant has yet been isolated which produces more than two. Alterations in the content of these proteins can be correlated with variations in virulence properties such as susceptibility to the bactericidal activity of normal human serum, attachment to epithelial cells, and susceptibility to phagocytosis (15). Alterations in the host, such as variations in proteolytic enzyme levels during the menstrual cycle, can result in the selection of a variant which has the protein II phenotype with a selective advantage for that particular environment (10).

Protein II and the other variable proteins appear to form a closely related group with a number of shared properties in addition to their effect on virulence, including surface location (14, 20), sensitivity to proteolytic enzymes (10, 20), similarity of molecular weight, and variation in apparent molecular weight dependent on the temperature during solubilization for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6, 14). These observations suggest that they may be a family of proteins with substantial structural homology. The purpose of this study was to compare the structural characteristics of these proteins to further define the relationships between them and to gain further insight into the molecular organization of the outer membrane and the processes which might control it.

MATERIALS AND METHODS

Strains and growth conditions. *Neisseria gonorrhoeae* P9 colonial opacity variants were grown on the clear typing medium of James and Swanson (10). Colonial opacity variants were selected and stored in liquid N_2 as previously described (14). The variants used (and outer membrane proteins contained) were: P9-6 (I, II), P9-13 (I, IIa), and P9-16 (I, IIb) (15). For large-scale isolation of outer membranes bacteria were grown on trays (27 by 38 cm) of the same medium.

Isolation of outer membrane complex. Gonococci were harvested into 0.1 M phosphate-buffered saline (pH 7.2) and centrifuged at 12,000 × *g* for 20

min. The bacterial pellet was suspended in 0.2 M lithium acetate (pH 6.0; about 10 g [wet weight] per 100 ml) and shaken at 45°C for 2.5 h. The suspension was then homogenized for 1 min in a Vortex mixer (P. Silver, Hampton, England) with a blade clearance of 0.025 mm. Bacteria were removed by centrifugation at 15,000 × *g* for 20 min (three times), and crude outer membrane complex was obtained by centrifugation at 100,000 × *g* for 2 h (6). The pellet obtained was suspended in 6 M urea–0.2 M sodium acetate buffer (pH 6.0) at a protein concentration of 10 mg/ml. After incubation at 25°C for 30 min the suspension was diluted with an equal volume of buffer and centrifuged at 100,000 × *g* for 3 h. The supernatant solution, which contained contaminating non-outer membrane proteins (9), was discarded, and the pure outer membrane complex was suspended in water and washed three times at 100,000 × *g*. The final pellet was suspended in water and stored at −20°C until required.

SDS-PAGE. Samples were subjected to SDS-PAGE by using the discontinuous buffer system of Laemmli (13) in a slab gel apparatus. The gels contained a linear concentration gradient of acrylamide generated by mixing two stock solutions with a triple-channel peristaltic pump. The acrylamide-bisacrylamide ratio was constant at 38.5:1, and the more concentrated acrylamide solution contained 10% (vol/vol) glycerol to stabilize the gradient. Controlled polymerization of the gradient from the bottom of the gel was achieved by incorporating ammonium persulfate at 0.1 mg/ml in the higher-concentration acrylamide solution and at 0.05 mg/ml in the lower-concentration acrylamide solution (16). Gradients of 10 to 25% (wt/vol) acrylamide were used for outer membrane complex and protein preparations, and 10 to 30% gradients were used for fragments derived from them.

Samples were mixed with an equal volume of SDS-PAGE derivitization buffer containing 0.125 M Tris-hydrochloride buffer (pH 6.8), 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 0.002% (wt/vol) bromophenol blue. Samples were heated at 100°C for 5 min, and 50 μl (containing approximately 10 μg of protein) was applied to the slab gel and subjected to electrophoresis at 250 V for 20 h. Gels were stained and destained by the method of Fairbanks et al. (4).

Purification of outer membrane proteins. Proteins from variants P9-6 (I, II) and P9-13 (I, IIa) were purified by a development of the selective extraction procedure previously used (6). Membranes were extracted with 1% (wt/vol) sodium cholate in 0.1 M glycine-NaOH buffer (pH 9.5) at 37°C for 30 min, and after centrifugation at 100,000 × *g* for 2 h the supernatant was chromatographed on Sephadex G-200 to yield a pure protein II (or IIa) fraction (6). The residue, which contained protein I, was suspended in 1% Empigen-BB (Albright & Wilson, Whitehaven, Cumbria, England) in glycine buffer (pH 9.5) and incubated at 37°C for 1 h. After centrifugation at 100,000 × *g* the supernatant solution was applied to a column (640 by 15 mm) of Sephadex G-200 and eluted with Empigen-glycine buffer to yield a pure protein I fraction (L. T. James and J. E. Heckels, J. Immunol. Methods, in press). Detergent was removed from the protein preparations by extensive dialysis against 0.05 M sodium carbonate-bicarbonate buffer (pH 9.0) followed by dialysis against distilled water.

Amino acid analysis. Purified outer membrane proteins (50 μg) were heated in 6 M HCl (0.5 ml) at 105°C for 16 h in sealed evacuated glass tubes. HCl was removed by repeated evaporation, and amino acid analysis was performed on a Rank-Hilger Chromaspek amino acid analyzer (Margate, Kent, England). Tryptophan was determined by hydrolysis under vacuum in 4 M Methanesulfonic acid containing 0.2% tryptamine at 115°C for 24 h followed by neutralization with 4 M NaOH (19). Where necessary, individual amino acid destruction during hydrolysis was corrected for from a parallel hydrolysis of egg white lysozyme.

Isolation of ^{125}I-labeled outer membrane proteins by SDS-PAGE. Outer membrane proteins were labeled with ^{125}I and chloramine-T after denaturing in the presence of SDS (2). Membranes (500 μg of protein) were suspended in 250 μl of 0.05 M Tris-hydrochloride (pH 7.5) containing 1% (wt/vol) SDS and heated at 100°C for 5 min. Carrier-free ^{125}I (10 μl containing 100 μCi of ^{125}I) was added, followed by 12.5 μl of chloramine T solution (10 mg/ml). After 15 min the reaction was terminated by the addition of 100 μl of 2-mercaptoethanol, and protein was precipitated by the addition of 400 μl of 15% trichloroacetic acid. The mixture was centrifuged at 1,000 × *g* for 2 min, and the precipitate obtained was washed three times with acetone to remove the trichloroacetic acid. The final pellet was dissolved by heating at 100°C in 900 μl of SDS-PAGE derivitization buffer, followed by centrifugation at 1,000 × *g* for 2 min to remove any insoluble material. The supernatant solution was applied to a slab gel containing a linear gradient of 10 to 25% (wt/vol) acrylamide. After electrophoresis, staining, and destaining, the protein bands were excised with a razor blade, washed in water, and stored in 50% ethanol at −20°C until required.

Proteins labeled only in the region exposed on the bacterial surface were isolated in a similar manner. Gonococci were suspended to a concentration of 2 × 10^9 colony-forming units per ml and labeled with 100 μCi of ^{125}I by using the H_2O_2-lactoperoxidase system described previously (7). The labeled gonococci were recovered by centrifugation, boiled in derivitization buffer, and subjected to SDS-PAGE. After staining and destaining the bands corresponding to the major outer membrane proteins were excised and stored as above.

Cyanogen bromide cleavage of proteins. Purified membrane proteins (50 μg) were dissolved in 70% formic acid (100 μl) containing 50 mg of cyanogen bromide per ml, and the solutions were incubated at 37°C for 3 h (5). The reaction mixtures were diluted with water (1 ml) and lyophilized. The residues were dissolved in derivatization buffer, heated at 100°C for 5 min, and subjected to SDS-PAGE on a linear gradient of 10 to 30% acrylamide. Control samples treated in a similar manner, but omitting cyanogen bromide, showed no degradation of the protein.

Tryptic peptide maps of ^{125}I-labeled membrane proteins. Acrylamide gel slices containing ^{125}I-labeled protein (ca. 2 × 10^5 dpm) were lyophilized, suspended

in 0.5 ml of 50 mM ammonium bicarbonate (pH 8.0) containing 50 μg of trypsin (Sigma, type III) per ml, and incubated at 37°C for 16 h. The supernatant solutions were removed, lyophilized, and dissolved in water. Samples containing 10^5 dpm were applied to silica gel G-coated thin-layer plates (10 by 10 cm; Macherey-Nagel, Duren, Germany). The plates were subjected to electrophoresis (250 V for 45 min) at pH 3.5 in pyridine-acetic acid-water (1:10:89) in the first dimension followed by ascending chromatography in butan-1-ol-pyridine-acetic acid-water (15:10:3:12) in the second dimension (1). Radioactive peptides were detected by autoradiography with Kodak X-Omat H film exposed for 24 to 48 h.

In initial experiments tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Diagnostics) was used, which resulted in similar overall conclusions but produced rather fewer spots with poorer resolution of the peptides compared with untreated trypsin, presumably due to traces of chymotryptic activity present in the latter. Untreated trypsin was therefore used routinely.

RESULTS

Purification of proteins I, II, and IIa. The selective extraction procedure with cholate followed by gel chromatography produced pure protein II from P9-6 and protein IIa from P9-13 (6). Extractions of the insoluble residue with Empigen-BB followed by gel chromatography produced a pure protein I fraction. The advantage of this procedure over that previously used is that protein I is retained in an immunologically reactive form (James and Heckels, J. Immunol. Methods, in press).

Amino acid composition of purified outer membrane proteins. The amino acid composition of the purified proteins I, II, and IIa is shown in Table 1; the number of residues present per protein is calculated from the apparent subunit molecular weight on SDS-PAGE. Proteins II and IIa show very similar composition and have an almost identical proportion of hydrophobic amino acids. Protein I, however, also shows considerable similarity in overall composition and hydrophobic content.

Cyanogen bromide cleavage of purified outer membrane proteins. Since the overall amino acid composition of the proteins did not clearly reveal structural similarities and differences between them, advantage was taken of the presence of a single methionine residue in each protein. Cyanogen bromide cleavage of proteins II and IIa produced a very similar pattern (Fig. 1), giving as expected two peptides, one pair with identical molecular weight (13K) and the outer pair (17.5 and 17K) differing by an amount equivalent to that between the two original proteins (29 and 28.5K). The marked structural similarity between proteins II and IIa suggested by this observation was not shown with protein I, which produced fragments of 26 and 10K.

TABLE 1. *Amino acid analysis of outer membrane proteins*[a]

Amino acid	Mol% of protein:			Residues per mol of protein:		
	I	II	IIa	I	II	IIa
Asx	12.0	9.8	10.9	41	28	30
Thr	5.1	5.3	5.9	19	15	16
Ser	9.1	11.5	10.8	31	32	30
Glx	10.4	9.6	9.7	36	27	27
Pro	2.1	4.1	3.8	7	11	10
Gly	13.3	12.0	10.8	46	34	30
Ala	11.6	12.1	13.2	40	34	36
Cys (half)	0.5	ND[b]	ND	2	0	0
Val	8.4	6.4	6.2	29	18	17
Met	0.3	0.3	0.4	1	1	1
Ile	2.9	3.9	4.4	10	11	12
Leu	5.2	4.0	4.7	18	11	13
Tyr	3.6	5.0	4.1	13	14	11
Phe	3.9	3.1	2.9	13	9	8
His	1.7	1.8	1.9	6	5	5
Trp	0.4	0.4	0.3	1	1	1
Lys	6.4	6.1	5.8	22	17	16
Arg	3.3	4.6	4.0	11	13	11

[a] The content of the essentially hydrophobic amino acids (Val, Met, Ile, Leu, Tyr, Trp) (18) was: protein I, 24.6%; protein II, 23.1%, and protein IIa, 22.9%.
[b] ND, Not detected.

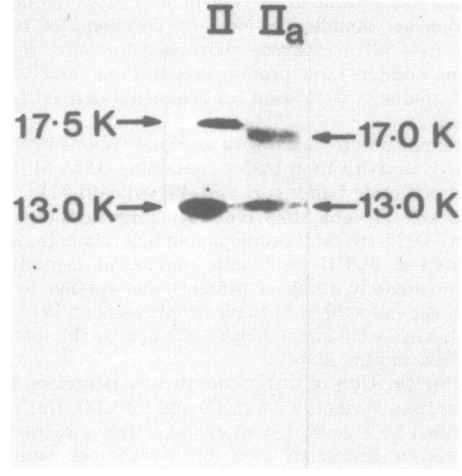

FIG. 1. *Cyanogen bromide cleavage of proteins II and IIa. SDS-PAGE of the fragments obtained after cyanogen bromide treatment of proteins II and IIa. The gel contained a linear gradient of 10 to 30% (wt/vol) acrylamide. The molecular weight standards used were protein II (29K) trypsin, myoglobin, and lysozyme.*

Tryptic peptide maps of outer membrane proteins. The structural homology between the outer membrane proteins was further investi-

gated by peptide mapping after ^{125}I labeling and trypsin digestion. Preliminary experiments were carried out by using protein bands cut from acrylamide gels followed by labeling within the slice (3), but high background levels of radioactivity were found with this method. More satisfactory results were obtained by treating membranes with ^{125}I and chloramine-T in the presence of SDS, followed by SDS-PAGE separation of the labeled protein (2). Autoradiographs of peptide maps obtained from proteins II, IIa, and IIb are shown in Fig. 2, together with composite drawings obtained from three separate experiments (Fig. 3). Most of the spots were common to two or three of the proteins with only two or three unique to any one protein. In addition, the unique spots were in the lower portion of the chromatography dimension, suggesting that they were among the more hydrophilic peptides obtained (22). Proteins IIc and IId also showed a pattern very similar to that of protein IIb, but protein I was quite different, showing little homology.

Tryptic peptide maps of proteins from lactoperoxidase-labeled cells. To examine the possibility that the unique peptides arose from the portion of the protein exposed on the surface, peptide mapping was carried out on proteins II and IIa isolated from SDS-PAGE gels of gonococci previously surface labeled with ^{125}I and lactoperoxidase. The autoradiographs obtained differed from those seen with the completely labeled proteins in having fewer spots, with altered relative intensities, showing a reduced degree of structural homology between proteins II and IIa (Fig. 4). Because of the gross differences in the patterns obtained by the two methods it was not possible to unambiguously relate every spot obtained on the lactoperoxidase map to the corresponding spot on the complete map. Nevertheless, it appeared that the major spots obtained included those which were unique. This was particularly true of protein IIa, in which the two unique spots were well separated from the others and could be identified on the lactoperoxidase map (Fig. 4, arrows).

Cyanogen bromide cleavage of proteins from lactoperoxidase-labeled cells. Proteins II and IIa from lactoperoxidase-labeled whole cells were also subjected to cyanogen bromide cleavage. In contrast to peptide mapping no difference was seen from the pattern obtained

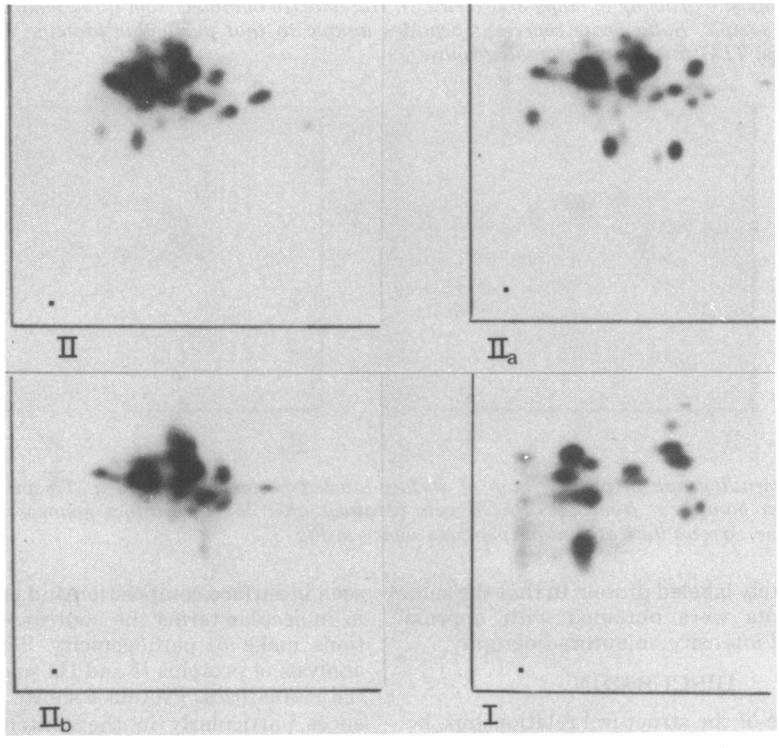

FIG. 2. *Autoradiographs of peptide maps of proteins I, II, IIa, and IIb. Trypsin digestion was carried out on bands cut from SDS-PAGE gels obtained after labeling outer membrane complex with ^{125}I and chloramine-T in the presence of SDS.*

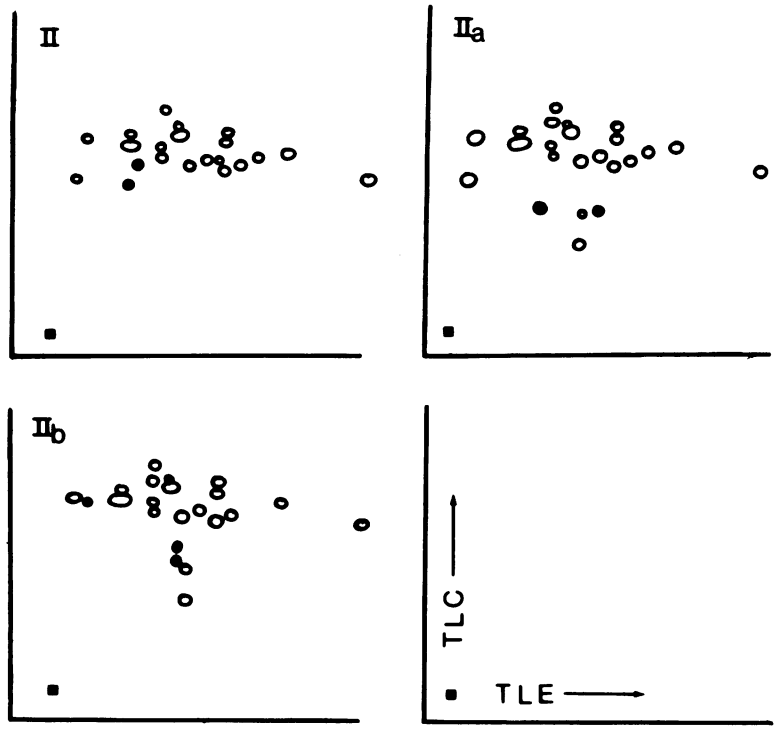

FIG. 3. *Composite drawing of maps of proteins II, IIa, and IIb obtained from three separate experiments.* ■, *Origin of sample. Solid spots represent peptides unique to that particular protein. TLE, Thin-layer electrophoresis; TLC, thin-layer chromatography.*

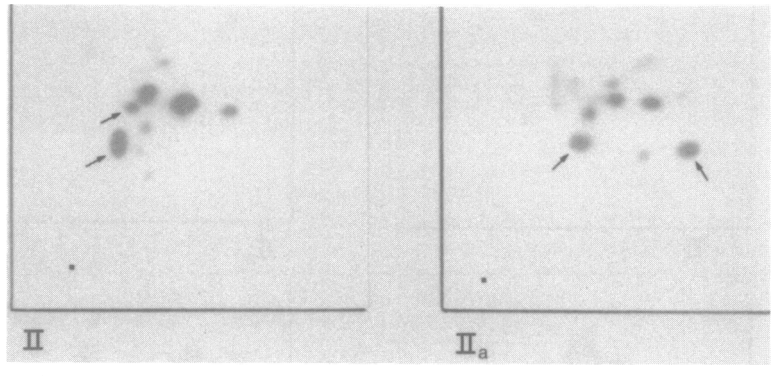

FIG. 4. *Autoradiograph of peptide map of surface-labeled proteins II and IIa. Tryspin digestion was carried out on bands cut from SDS-PAGE gels obtained after labeling intact gonococci with* ^{125}I *and lactoperoxidase. Arrows indicate unique peptides shown in Fig. 3.*

with completely labeled protein in that the same two fragments were obtained with approximately equal intensity on autoradiography.

DISCUSSION

Knowledge of the structural relationships between the outer membrane proteins of gonococci should lead to a further understanding of the factors which control the considerable variations seen in surface composition and perhaps explain in molecular terms the contribution the variations make to pathogenicity. The amino acid analysis of proteins II and IIa suggests substantial similarities. Protein I shows greater differences particularly in the content of cysteine, valine, and proline, although the overall content of hydrophobic amino acids is similar. This may, however, only reflect a general property of outer

membrane proteins since the hydrophobic content of meningococcal outer membrane protein is also similar (22). Indeed, even purified gonococcal pili also have a very similar content of hydrophobic amino acids (24 to 26%) (18). However, close structural homology between proteins II and IIa, but not protein I, is indicated by the patterns obtained on cyanogen bromide fragmentation. Thus, proteins II and IIa give a pair of fragments with identical molecular weight and a pair which differ only by an amount (0.5K) equal to the original difference between the intact proteins, suggesting that they may have a large degree of structural homology and differ only in one small region. This would be in agreement with a previous study, which showed close similarity on chymotryptic digestion not only between two proteins of this type produced by one gonococcal strain, but also between similar proteins from different strains (21). The tryptic peptide maps of ^{125}I-labeled proteins obtained in the current study show that all of the protein II species produced by strain P9 have considerable structural homology, with only a few peptides being unique to one protein. Although the total number of spots seen on autoradiography is in excess of the total number of tyrosine residues detected, this is not entirely unexpected since a similar phenomenon was observed with the chymotryptic digestion of protein II species and was attributed to the additional production of diiodotyrosine and iodohistidine residues (21). Indeed, the number of tyrosine residues detected in proteins II and IIa is entirely in agreement with the prediction made by Swanson of 10 to 15, based on the number of the most heavily labeled spots detected (21).

The position of the unique peptides among those with lowest mobility in the chromatographic dimension on peptide mapping suggests that they are among the more hydrophilic peptides and are, therefore, probably located on the bacterial surface (22). This view was strengthened by peptide mapping carried out on proteins from cells which had been surface labeled rather than completely labeled after unfolding. Although the total number of spots obtained was considerably reduced the two unique peptides from protein IIa were still labeled, demonstrating their surface location. It would therefore appear that the protein II species do indeed form a family with considerable structural homology, differing in a small portion probably exposed on the surface. Since both the fragments obtained on cyanogen bromide treatment of lactoperoxidase-labeled proteins were radiolabeled, then the tertiary structure of proteins II and IIa must be such that at least part of the common region is also exposed at the surface.

Previous studies with the same strain of gonococcus have shown that both protein I and all the protein II family can be surface labeled, but that only protein I together with a protein of molecular weight 60K (protein III) are exposed on the inner surface of the outer membrane (8). These observations, together with those reported here, suggest a model for the organization of the gonococcal outer membrane. Protein I spans the membrane, probably forming a hydrophilic diffusion pore, and does not alter in colonial variants of any one strain. The protein II species, in contrast, are located near the outer surface with a common region embedded in the membrane and modifications to the polypeptide occurring on the surface which contribute to variations in pathogenic properties.

The precise mechanism controlling the surface variations remains unclear. One possibility is that the proteins are derived by post-translational modification of a common gene product perhaps by variations in the proteolytic cleavage of the signal sequence associated with the initial membrane location of outer membrane proteins (17) or by subsequent additions to a common starting peptide. An answer to these questions must, however, await a detailed genetic study of the processes involved. Nevertheless, the potential for genetic variation influencing the surface composition of the gonococcus must play an important part in its success as a pathogen.

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