

ISOLATION AND PARTIAL CHARACTERIZATION OF THE REDUCTION-MODIFIABLE PROTEIN OF *NEISSERIA* *GONORRHOEAE*

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For several years investigators have examined and characterized some of the surface antigens of the bacterium *Neisseria gonorrhoeae*, including pili, lipopolysaccharide (LPS), and various proteins of the outer membrane (for review see references 1 and 2). The particular interest in our laboratory has been to understand how all of the components within the gonococcal outer membrane function to the advantage of the organism. In this effort, we have isolated and purified members of the protein I family and found them to be trimeric complexes that form voltage-dependent, anion-specific transmembrane porins (3–5). Furthermore, we have purified several members of the protein II family, one of which confers the opaque phenotype to gonococcal colonies (6) whereas another seems to be implicated in the association of gonococci with polymorphonuclear leukocytes (7). In order to complete our information about the outer membrane proteins and help us understand how these protein components of the gonococcal outer membrane might work in concert, we turned our attention to the last remaining major outer membrane protein family, the reduction-modifiable proteins, or proteins III.

Protein III is present in all strains of gonococci examined to date and differs from the other surface-exposed gonococcal outer membrane proteins by its high degree of intrastrain and interstrain homology in molecular weight, structure, and immunology (8, 9). These characteristics make protein III unique among the major outer membrane antigens of the gonococcus, as proteins I and II, as well as LPS and pili, all appear to display a considerable degree of variability either within a strain or between strains (1, 2). Because it has been hypothesized that this variability was in part due to the immunologic pressure of the human host, it was puzzling that gonococci would rigidly conserve such a surface antigen. For this reason it seemed that we should isolate and purify this protein in order to study its biochemical and immunochemical nature in more detail. Thus, this article will present a newly devised method of isolating and purifying protein III and a partial characterization of the structure and immunochemistry of the molecule based on our investigations that use the purified protein. The method used for isolating protein III in large quantities is a simple refinement of the

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technique used to purify proteins I and II as described in a previous report (6). The immunologic data obtained using protein III purified in this manner suggest that the original structure of the molecule is in no way destroyed by the purification process.

Materials and Methods

Bacteria and Culture Conditions. Most of the strains cited in this article have been cultivated in our laboratory for several years. Strain Pgh 3-2 was generously provided and used with the permission of Dr. Charles C. Brinton of the Department of Microbiology, University of Pittsburgh. The one *Neisseria meningitidis* strain referred to here as BNCV¹ is a group B nonencapsulated variant of strain M986, which was provided by Dr. Carl E. Frasch of the Bureau of Biologics, Washington, D.C. The bacteria were grown on solid typing medium defined by Swanson (10) and were identified as *N. gonorrhoeae* by Gram's stain, oxidase reaction, and carbohydrate fermentation ability. Nonpiliated organisms of both opaque and transparent colonial phenotypes were used. However, it should be noted that the protein III from the transparent variety was isolated with more ease.

For protein purification, the bacteria were grown in a liquid medium, the preparation of which has been described previously (3). A typical purification started with 12 liters of liquid medium dispersed in eight 2,800-ml Fernbach flasks. Each flask was inoculated with a 50-ml late log phase culture of gonococci. The Fernbach flask cultures were placed in a Brunswick rotary shaking incubator (New Brunswick Scientific Co., Inc., Edison, NJ) and incubated for 7 h at 37°C while shaking at 100 rpm. The bacteria were then harvested by centrifugation at 17,000 *g* for 10 min.

Purification. The harvested bacteria were extracted with 0.5 M CaCl₂/5% Zwittergent, *N*-tetradecyl-*N,N*-dimethyl-3-ammonia-1-propanesulfonate (Z3,14) as was described previously for the purification of the gonococcal opacity proteins (6). The precipitate that resulted from this extraction was then slowly resuspended in an equal volume of 0.65 M ethanolamine-HCl, pH 10.4. To this was added 6 vol of 0.5 M CaCl₂/5% Z3,14 and finally two volumes of absolute ethanol. This second extraction was then briefly sonicated into a smooth suspension in a bath sonicator (Branson Cleaning Equipment Co., Shelton, CT). The suspension was centrifuged at 17,000 *g* for 20 min. The supernatant was removed and additional absolute ethanol was added to bring the final concentration of ethanol to 80%, and the resultant precipitate was recovered by centrifugation. The precipitate was dissolved in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5% Z3,14 and clarified by centrifugation at 12,000 *g* for 10 min, and the soluble material was retained.

This solution was then applied to the first of two columns linked in tandem. The first (2.5 × 10 cm) was packed with DEAE-Sepharose 6B-CL (Pharmacia Fine Chemicals, Piscataway, NJ) and the second (0.9 × 10 cm) with CM-Sepharose 6B-CL (Pharmacia Fine Chemicals). Both columns had been equilibrated previously with 50 mM Tris-HCl, pH 8.0, with 10 mM EDTA and 0.05% Z3,14. The eluate from both columns was monitored for proteins at 280 nm absorption. After the sample was applied the columns were washed with the equilibration buffer until the 280-nm absorption fell to baseline. The columns were then separated and each eluted with a NaCl gradient between 0.0 and 0.5 M. The total volume applied to the CM-Sepharose column was 60 ml. Fractions containing protein by 280-nm absorption were analyzed by SDS-PAGE. The fractions containing the protein III were collected, pooled, and precipitated by the addition of ethanol to a final concentration of 80%. The precipitate was collected by centrifugation and redissolved in the Tris-HCl 5% Z3,14 buffer in ~1–2 ml. This material was applied to a Sephacryl S-200 (Pharmacia Fine Chemicals) column (1.5 × 100 cm). The elution buffer consisted of 100 mM Tris-HCl, 200 mM NaCl, 10 mM EDTA, 0.05% Z3,14 at pH 8.0 and the flow rate was 2 ml/h. Fractions of 2.0 ml were collected, monitored for 280 nm absorbance, and analyzed by SDS-PAGE.

¹ Abbreviations used in this paper: BNCV, group B nonencapsulated variant of strain M986 of *N. gonorrhoeae*; Z3,14, Zwittergent, *N*-tetradecyl-*N,N*-dimethyl-3-ammonia-1-propanesulfonate.

Amino Acid Analysis. The amino acid composition was determined using a Durrum model D500 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA) as previously described (3).

Determination of Amino-terminal Amino Acids. 3.0 mg of protein III was precipitated with 10% trichloroacetic acid, and the precipitate was washed with ethanol and acetone. The precipitate was then dissolved in 50% (vol/vol) acetic acid and introduced into a model 890B sequenator (Beckman Instruments, Inc.).

SDS-PAGE. The extract procedure for sample preparation and the electrophoresis have been described in detail elsewhere (3).

Isoelectric Focusing. The isoelectric point of protein III was determined using a modification of the method of Rosen et al. (11), which has been previously described (6).

Enzyme Digestion. α -chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4; Worthington Diagnostics, Freehold, NJ) were dissolved in 1 mM HCl at a concentration of 1 mg/ml and frozen in aliquots until used. 10 μ g of each of the enzymes per mg of protein III was added to separate tubes containing protein III and were incubated for 30 min at 37°C. Trichloroacetic acid was added with a final concentration of 10% which stopped the reaction. The samples were then processed for SDS-PAGE analysis as described.

Immunological Methods. Antisera to purified protein III were produced in the same manner as that described for the purified protein II (6). The ELISA technique used was also identical to that used previously (6). In the whole-cell absorption experiments, the bacteria from a certain strain were removed from solid agar medium with a dacron swab and suspended in sterile PBS. The organisms were then diluted with the PBS until the optical density at 500 nm reached 0.6. This suspension was then dispensed into 0.1-ml aliquots and centrifuged, and the supernate was removed. The bacteria were resuspended in 0.1 ml of the appropriate dilution of antiserum and incubated for 30 min at 4°C. The bacteria were then removed by centrifugation and the supernate was either applied directly to the ELISA plate or placed into a fresh aliquot of organisms and reabsorbed.

Results

Purification

CaCl₂/Z3,14 extraction. As described in a previous article (6), a solution of 5% Z3,14 in 0.5 M CaCl₂ solubilized several of the gonococcal outer membrane proteins when added to a suspension of bacteria. Because it was virtually impossible to separate protein I from protein III in a single extraction procedure, two such CaCl₂/Z3,14 extractions conducted at different pHs were required. The first extraction carried out at pH 4.0 released protein I and, when present, protein II from the outer membrane, while leaving protein III still associated with the membrane in the ethanol-precipitable fraction. When the pH was subsequently raised to 10.4 in the second extraction, protein III was solubilized. The solubilized fraction of the second CaCl₂/Z3,14 extraction containing protein III was prepared for ion-exchange chromatography by ethanol precipitation and resuspension in Tris-HCl, 5% Z3,14 buffer.

Ion-exchange chromatography. Upon chromatographing the protein III suspension on two ion-exchange columns linked together, it was found that protein III bound to the CM-Sepharose column. This isolated the protein from any residual nucleic acids not removed during the two extraction procedures, inasmuch as the nucleic acids adhered to the DEAE-Sepharose CL-6B column. Using a NaCl gradient from 0.0 to 0.5 M NaCl, protein III was eluted from the CM-Sepharose column in one peak between 6.4 and 7.4 mS as shown in Fig. 1.

Molecular sieve chromatography. Protein III eluted from a Sephacryl S-200 column (1.5 \times 100 cm) in a single peak with a K_{av} of 0.302 (Fig. 2). Thus the M_r

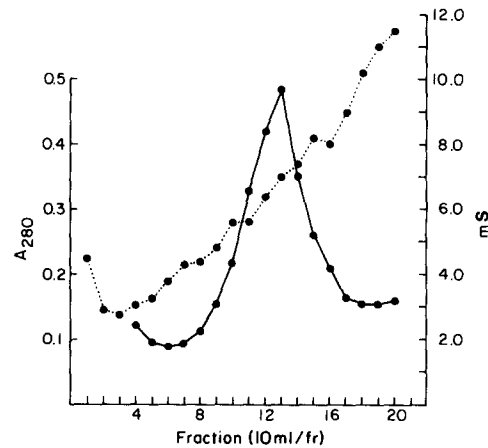


FIGURE 1. Elution profile of gonococcal protein III from a CM-Sepharose column (0.9×10 cm). The sample was applied in a buffer consisting of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5% Z3,14. The column was washed with the same buffer with 0.05% Z3,14 until the A_{280} fell to zero. The column was eluted with a NaCl gradient between 0.0 and 0.5 M. The elution was monitored by absorbance at 280 nm (*solid line*), conductivity (*dashed line*), and SDS-PAGE analysis. Protein III eluted from the column between 6.4 and 7.4 mS. The fractions containing protein III were collected and pooled.

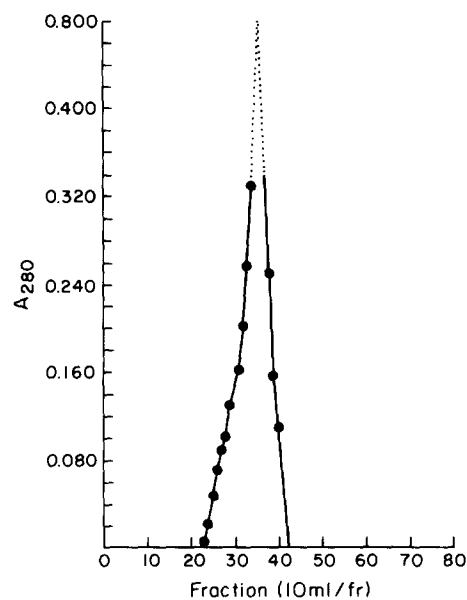


FIGURE 2. Protein III eluted from a Sephacryl S-200 column (1.5×100 cm) with a $-K_{av}$ of 0.302. The elution buffer consisted of 0.1 M Tris-HCl, pH 8.0, 0.2 M NaCl, and 0.05% Z3,14. The elution was monitored by absorbance at 280 nm (*solid line*) and SDS-PAGE analysis. Fractions containing protein III were collected and pooled.

of the protein III-Zwittergent micelle complex was calculated as being $\sim 46,000$. The protein profile upon SDS-PAGE analysis showed that the fractions in the elution peak contained essentially pure protein and that the protein was indeed

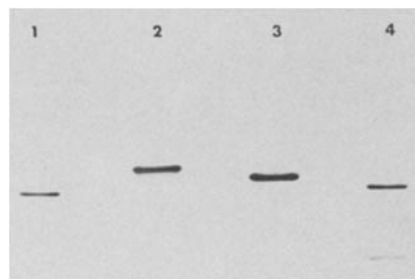


FIGURE 3. SDS-PAGE analysis of the purified protein III with 2-ME (lane 2) and without 2-ME (lane 3). Note that the purified protein maintains its modifiability. Lanes 1 and 4 contain the molecular weight standards: carbonic anhydrase (30,000) and soy bean trypsin inhibitor (21,500).

protein III, inasmuch as it exhibited the characteristic reduction-modifiability of that protein (Fig. 3).

Isoelectric Focusing

Using an agarose gel system of isoelectric focusing with a pH gradient of 7.0–10.0, it was found that protein III focused at a pH of ~8.6.

Amino Acid Analysis

As Table I indicates, protein III, like protein II, appears to contain more basic amino acids than does protein I, thus confirming the data obtained from ion-exchange chromatography and isoelectric focusing as to the basic nature of protein III. Also of particular interest are the two half-cystines, which could account for the variable migratory characteristics of protein III on SDS-PAGE in the presence or absence of 2-ME.

Amino-terminal Determination

Purified proteins III from two different gonococcal strains, Pgh 3-2 and R10, were precipitated with 10% trichloroacetic acid, and the precipitates were washed with ethanol and acetone and were then applied into a model 890B sequenator (Beckman Instruments, Inc., Fullerton, CA) in 50% acetic acid. The first 10 amino acids of the amino termini of both samples proved to have the identical sequence, as shown in Fig. 4. The purity of the sequence was >99%.

Proteolytic Enzyme Treatment

Samples of purified protein III were incubated separately with trypsin and α -chymotrypsin, and these preparations were then subjected to SDS-PAGE. In both cases, the protein appeared to be completely degraded, thus indicating extreme susceptibility to digestion by these endopeptidases.

Immunological Properties

All rabbits inoculated with purified protein III of one strain produced antibodies that reacted with the homologous purified protein III that was bound to the polystyrene in an ELISA assay. In addition, all rabbits injected with whole bacteria also produced antibodies to purified protein III as determined by ELISA. Western blot analysis in which a whole cell lysate of the homologous strain was

TABLE I
Amino Acid Composition of Gonococcal Protein III

Amino acids	Residue* (fraction) [‡]
	<i>mol</i>
Neutral	175 (0.63)
Aliphatic	138 (0.49)
Glycine	20
Alanine	34
Valine	29
Leucine	17
Isoleucine	9
Serine	18
Threonine	11
Aromatic	15 (0.05)
Phenylalanine	7
Tyrosine	8
Tryptophan	—
Sulfur-containing	6 (0.02)
Half cystine	2
Methionine	4
Imino acids/proline	16 (0.06)
Dicarboxylic amino acids	63 (0.22)
Aspartic/asparagine	26
Glutamic/glutamine	37
Basic amino acids	41 (0.15)
Histidine	4
Arginine	20
Lysine	17
Total	279

* Number of moles of residue per mole of protein.

[‡] Fraction of total number of residues represented in each class.

Strain	5	10
R10(pIII)	Gly-Glu-Ala-Ser-Val-Gln-Gly-Tyr-XXX-Val	
Pgh 3-2(pIII)	Gly-Glu-Ala-Ser-Val-Gln-Gly-Tyr-XXX-Val	

FIGURE 4. Amino-terminal amino acid sequence of gonococcal protein III.

reacted with antiserum raised against the purified protein showed that the protein isolated and purified by the method outlined above was indeed protein III, in that a very strong reaction with the antiserum was demonstrated by the 31,000 mol wt (reduced protein III) band. Western blot analysis using the same immune rabbit serum against whole-cell lysates of several different strains of gonococci, one group B nonencapsulated variant of *N. meningitidis* (BNCV), and one strain of *N. sicca* revealed a very high degree of crossreactivity among the proteins III of all strains of gonococci as well as with the class 4 (reduction-modifiable) protein of BNCV. No significant reactivity was detected for any protein of *N. sicca* (Fig. 5). Sequential absorptions of rabbit antiserum raised against purified protein III by homologous and heterologous whole gonococci and by BNCV confirmed this finding, because all of the strains tested exhibited considerable reactivity with the immune serum and almost identical patterns of absorption. These studies

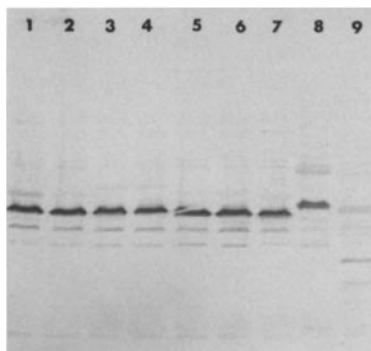


FIGURE 5. Western blot analysis of whole-cell lysates from several different strains of gonococci (lane 1, B2; lane 2, MAN 851; lane 3, MS11; lane 4, R10; lane 5, Pgh 3-2; lane 6, UU1; lane 7, 120176-2), one strain of meningococcus (lane 8, BNCV), and one *N. sicca* strain (lane 9). Hyperimmune rabbit serum raised against purified protein III was allowed to react with the proteins on the blot and these rabbit antibodies detected with a goat anti-rabbit alkaline phosphatase-conjugated antibody as described in Materials and Methods. Note that the protein detected by this antiserum is of identical molecular weight in each of the gonococcal strains. A protein of slightly higher molecular weight is detected in the meningococcal strain. No distinct reactive protein was found in the sicca lysate.

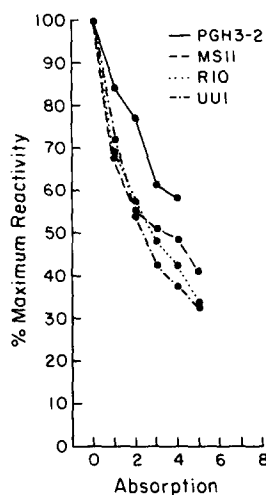


FIGURE 6. Absorptions of hyperimmune protein III antiserum by whole organisms (as described in Materials and Methods). The percentage of maximum reactivity was arrived at by taking the ELISA reading at 405 nm of an aliquot of antiserum that had been absorbed by whole cells and dividing it by the reading of an aliquot that had not been incubated with cells. Note the very rapid decline in reactivity in the first two to three cycles of absorption; thereafter, the reactivity declines much less rapidly. Also note that each of the four strains of gonococci absorb the hyperimmune antiserum raised against the purified protein III of Pgh 3-2.

also provided convincing evidence that protein III is indeed exposed on the surface of the outer membrane, in that the curves in Fig. 6 display a leveling off above baseline at later absorptions, a characteristic of partially surface-exposed antigens.

When the unreduced and reduced forms of protein III were compared by both Western blot analysis and ELISA, rabbit antiserum raised against protein

III appeared to react more strongly with the reduced protein. However, in an inhibition assay in which the antiserum was allowed to react with both reduced and unreduced protein III before exposure to the reduced protein that was bound to the polystyrene of a microtiter plate, the antiserum mixed with the reduced and with the unreduced protein III reacted virtually identically with the reduced protein on the plate, and the decrease in inhibition by the reduced form of protein III seen in the first assay was not observed. Therefore, the state of reduction of protein III does not appear to affect the immunogenicity of the protein.

Discussion

Our investigations using protein III purified by the method outlined above have provided new information regarding the structural and antigenic nature of this molecule and also served to confirm or clarify certain earlier hypotheses about the protein. We have demonstrated that protein III constitutes a discrete family of gonococcal outer membrane proteins that differs markedly from either protein I or protein II. We have also confirmed that protein III is unique among all outer membrane constituents in its extremely high degree of biochemical and immunogenic homogeneity. Whereas pili, LPS, and proteins I and II all vary considerably in their structure and behavior between and/or within strains, protein III exhibits little if any such variability. Furthermore, data obtained using rabbit antiserum raised to purified protein III have provided evidence that this reduction-modifiable molecule may be conserved in other *Neisseria* species as well.

In our efforts to purify protein III, we found it essential to perform two separate extraction procedures (versus the one extraction required for the purification of protein I and protein II). Earlier crosslinking and immunoprecipitation studies have indicated that protein I and protein III are very closely associated in vivo in the gonococcal outer membrane (12, 13), and as we discovered, it was virtually impossible to separate the two proteins from each other with only one extraction step. However, by performing the first extraction at a low pH, we succeeded in dissociating protein I from protein III and releasing protein I from the membrane. Raising the pH for the second extraction released protein III. It is unclear as to exactly why lowering the pH in the first extraction step disrupts the natural association of proteins I and III within the membrane. Based on the isoelectric points of the two proteins, it seems unlikely that the association of the two proteins is due to an ionic interaction. It is possible, however, that the reduction in pH alters the tertiary structure of either protein I or protein III, or both, in such a way as to interfere significantly with hydrophobic interactions between the two molecules.

Two unusual observations made during our investigations using purified protein III were (a) the unexpected behavior of protein III on gel filtration chromatography and (b) the different susceptibility to enzymatic degradation of protein III in the intact membrane and in its purified state. Unlike proteins I and II, which elute off molecular sieve columns at their predicted molecular weights, protein III eluted off the Sephacryl S-200 at a significantly lower K_{av} than expected. Based on its apparent molecular weight of 30,000 by SDS-PAGE,

the estimated size of protein III plus its Zwittergent micelle would be around 90,000 mol wt. Instead, we found that the protein-micelle complex migrated through the S-200 at the much lower molecular weight of 46,000. It would appear then that protein III interacts with the detergent molecules in a highly irregular manner; perhaps the detergent molecules associate with protein III in only a partial or intermittent way rather than forming a complete micelle around the protein. A possible interpretation of this data might be that protein III may contain only a very small hydrophobic region that is embedded in the lipid leaflets of the outer membrane, whereas the majority of the molecule, perhaps as much as two thirds, is hydrophilic and extends into the periplasm or past the outer surface of the membrane.

Earlier data obtained from proteolytic digests of whole gonococci and of outer membrane preparations suggest that very little if any of protein III is exposed on the surface of the bacterium, as no degradation of the protein is observed when analyzed by SDS-PAGE (14). This is in complete contrast to the results obtained when the purified protein is exposed to the same endopeptidases. The protein is degraded so completely by both trypsin and α -chymotrypsin that no peptides whatsoever could be seen on SDS-PAGE. These data would seem to indicate that protein III is not exposed on the surface of the outer membrane. Despite its apparent resistance to proteolysis in the intact membrane, our immunoabsorption studies clearly confirmed that at least some portion of the molecule is surface exposed, inasmuch as after several sequential absorptions with immune serum raised against protein III, a large amount of antibody reactivity is removed.

Two possible models for the orientation of protein III in the outer membrane could account for these observations, i.e., the protein's irregular behavior on gel filtration chromatography, its insusceptibility to enzymatic digestion in the intact membrane, its surface exposure, and its absorption patterns. One is that protein III might be embedded in the membrane in such a way that a large portion of the molecule extends out past the surface while a smaller hydrophilic portion extends into the periplasmic space. This model would seem to account well for the absorption data, because the majority of anti-protein III antibodies are absorbed out of the immune serum by the whole bacteria leaving a much smaller amount that still reacted with the purified protein on the microtiter plate (Fig. 6). This interpretation raises the question, however, of how so large a part of the protein can be exposed on the surface and yet be (a) insusceptible to at least some enzymatic degradation and (b) so highly conserved in light of the immunologic pressures to which it would be constantly exposed.

A second model might be that only a small segment of the protein III molecule is exposed on the outer surface of the membrane while the larger portion would extend into the periplasm. This would be more consistent with the enzymatic digestion data, because a small segment of a protein might not contain any trypsin or chymotrypsin sites. It also might fit the absorption data if one assumes that the shorter, exposed segment is more immunogenic than the larger unexposed portion and that there are a large number of protein III molecules on the surface of the gonococcus.

Recent work by Gotschlich et al. has resulted in the cloning of the gene for

gonococcal protein III. The DNA sequencing of this gene in the near future should provide much valuable information regarding the primary structure of the protein and further insight into the conformation, orientation, and antigenicity of protein III.

Given the high degree of structural and antigenic conservation of protein III and its known exposure on the surface of the outer membrane, the question arises as to how such an unvarying surface-exposed antigen could be of value to a pathogenic organism faced with the constant immune pressures of a host. This in turn raises the question as to the possible function(s) of protein III. Recent investigations by Rice et al. (15) may provide the answers to both of these questions. Using purified protein III isolated by our method, they have been able to demonstrate that protein III constitutes a major antigenic determinant for raising blocking antibodies in normal human sera that inhibit the efficient insertion into the bacterial membrane of the C5b-9 complement complex. If this hypothesis is correct, the immunogenic constancy of protein III becomes extremely beneficial to the gonococcus.

Summary

We have isolated and purified the reduction-modifiable protein (protein III) from several strains of *Neisseria gonorrhoeae*. We have found them to be basic proteins (pI 8.6) and virtually identical. A similar but not identical protein was isolated from a meningococcal strain. Antibodies raised against this purified protein would absorb to whole bacteria, confirming that the protein is exposed on the surface of the organism.

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