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One-Dimensional Peptide Mapping of the Major Outer Membrane Protein of *Neisseria gonorrhoeae*

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The major outer membrane protein (MOMP), or protein 1, of *Neisseria gonorrhoeae* is one of the immunodominant proteins of the gonococcal cell surface. It is at least partially responsible for imparting serotype specificity. This study attempted to compare the primary structure of MOMP molecules isolated from several gonococcal strains by preparative polyacrylamide gel electrophoresis. These isolated proteins were then subjected to enzymatic digestion with staphylococcal V8 protease and α-chymotrypsin. The generated peptides were separated by polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate. Of the eight strains analyzed, six exhibited distinct peptide maps after either staphylococcal V8 protease or α-chymotrypsin digestion. These six strains have also been shown to be immunologically distinct. The data suggest that the gene pool coding for the MOMP is diverse and reflect the complexity of the evolution of *N. gonorrhoeae*.

The surface antigens of *Neisseria gonorrhoeae* have been studied by many investigators (1, 2, 10–13, 19) who have attempted to better understand the host-parasite interaction and physicochemistry of gonococcal cell surface antigens and to develop a classification scheme.

In 1976, Johnston et al. (12) proposed a classification scheme for *N. gonorrhoeae* based on differences in the outer membrane proteins. These investigators used a mild chaotropic buffer containing lithium chloride and lithium acetate (LiCl-LiAc) to extract outer membrane antigens of *N. gonorrhoeae*. The extract was fractionated by Sepharose 6B-Cl gel filtration chromatography. By monitoring the presence of protein by the absorbance at 280 nm, two peaks were resolved. One peak was at the void volume and contained material having a molecular weight of more than 4×10^6. When different fractions were analyzed by Ouchterlony double immunodiffusion against homologous rabbit antiserum, a characteristic line of precipitation was formed from the interaction of the antiserum and the material in the void volume. Contents at the void volume from the LiCl-LiAc extracts of some gonococcal strains also exhibited this line of precipitation, whereas others did not. By using rabbit antisera raised against various extracts, hundreds of strains were classified into several serotypes based on the exhibition of this line of precipitation. The protein content in these extracts was suspected to be the basis of this classification scheme (10, 13).

By using transmission electron microscopy, McDade and Johnston found that the void volume of these extracts contain vesicles (18). Since the antigen(s) responsible for serotype specificity are contained in these vesicles, they are called serotype-specific vesicles (SSV). These investigators subsequently characterized the outer membrane protein content in the SSV (18). Within the SSV, there are three proteins of major interest; a reduction-modifiable protein (protein 3 [18]), a series of heat-modifiable proteins (protein [16, 18, 21], and a major outer membrane protein (MOMP), or protein 1 (18), which in many strains accounts for over 40% of the total protein content of the outer membrane (12). Protein 1 in association with protein 3 has been implicated in imparting serotype specificity (18). Protein 1 has also been implicated in serum resistance (7) and may function as porins which allow the passage of small molecules across the outer membrane (F. Greco, M. Blake, E. Gotschlich, and A. Mauro, Fed. Proc., p. 1813, 1980; F. A. Heasley, Abstr. EMBO Workshop, Hemavan, Sweden, 1980, p. 12).

We attempted to find out whether the antigenic diversity of protein 1 could be reflected in the primary structure of this protein from different strains by means of the one-dimensional peptide mapping technique of Cleveland et al. (3). We found that, of the eight gonococcal strains analyzed, six exhibited distinct peptide

patterns of protein 1; thus, it appears that the structural diversity of the MOMP is more diverse than previously reported (22).

MATERIALS AND METHODS

Bacteria and media. Strains of *N. gonorrhoeae* used in this study were obtained from the gonococcal collection maintained at the Department of Microbiology, University of Texas Health Science Center at Dallas. Strain F62 (*pro*, serotype *a*) was originally obtained from Douglas S. Kellogg, Jr., Centers for Disease Control, Atlanta, Ga. Strain DGI (serotype *f*) was isolated from synovial joint fluid of a patient suffering from disseminating gonococcemia by Robert S. Munford, Departments of Internal Medicine and Microbiology, University of Texas Health Science Center at Dallas. The remaining strains N033 (wild type, serotype *u*), N082 (*arg ser*, serotype *t*), N126 (*arg ile*, serotype *h*), N078 (*pro met*, serotype *b*), PW1 (*pro met*, serotype *g*), and N073 (*pro*, serotype *v*) were urogenital isolates collected in New York City by Kenneth H. Johnston. The amino acid requirements of strains were determined by Wong et al. (24). Serotypic designation of strains was described previously (12). All strains were of colony type 4 of mixed opaque/transparent variants as observed by the method of Swanson (21). They were cultured for 16 h on GC agar base plates (Difco Laboratories, Detroit, Mich.), supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.), at 35.5°C with 10% CO_2. Approximately 10^9 organisms were harvested from these plates and inoculated into 1 liter of SG-GC liquid medium (20) in a 3-liter baffled Fernbach flask. The liquid cultures were incubated at 35.5°C with shaking at 150 rpm. Cells were harvested at the mid-logarithmic phase of growth by centrifugation at 12,000 × *g* for 15 min at 4°C. Cell pellets were washed once with phosphate-buffered saline, pH 7.0.

Preparation of SSV. SSV of each strain were prepared as previously described (18). Briefly, organisms harvested from liquid medium were extracted with LiCl-LiAc buffer, pH 6.0, by vigorous shaking in the presence of glass beads at 45°C for 2 h. Cells were pelleted by centrifugation at 12,000 × *g* for 15 min at 4°C. The supernatant was fractionated by Sepharose 6B-Cl gel filtration chromatography. Fractions eluted at the void volume were pooled and concentrated by vacuum dialysis. The amount of total protein in SSV was determined by the method of Markwell et al. (17). SSV were stored at −76°C.

SDS-PAGE. The proteins in the SSV were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), using the discontinuous buffering system of Laemmli (15). A 100-μg amount of SSV protein was solubilized at 50°C without reducing agent for 15 min in 50 μl of digestion buffer which contained 2% (wt/vol) SDS, 20% (vol/vol) glycerol, and 0.001% (wt/vol) pyronin Y in 62.5 mM Tris-hydrochloride buffer, pH 6.8. They were loaded into the wells of a 12.5% acrylamide slab gel (12 by 16 by 0.15 cm), formed in a Bio-Rad model 220 slab gel unit. Electrophoresis was carried out at a constant 100 V until the dye marker reached the bottom of the resolving gel. The temperature of the gel was maintained at 10°C with a Haake model FK circulating cooling system. The gel was fixed and stained with 0.25% (wt/vol) Coomassie brilliant blue R250 in 25% (vol/vol) methanol–10% (vol/vol) glacial acetic acid overnight. It was destained in 10% (vol/vol) methanol–10% (vol/vol) glacial acetic acid by diffusion until the background was colorless. A photographic record of the gel was kept. The nomenclature of proteins in SSV was determined as described previously (18) based on their properties in the presence of a reducing agent or heat (100°C). The percent composition of protein 1 in the SSV was calculated from an integrated densitometric tracing of the protein profile in each lane of the slab gel.

Isolation of protein 1. Protein 1 was isolated by preparative SDS-PAGE with the discontinuous buffering system of Laemmli (15). A 5% acrylamide stacking gel was added on top of the 12.5% acrylamide preparative slab gel (10 by 16 by 0.15 cm) after it had polymerized. As the quantity of protein 1 in the SSV is strain dependent, the amount of SSV to be loaded onto the preparative gel was calculated from its percent composition in the whole SSV so that about 25 μg of protein 1 would be contained in each 0.5-cm wide gel slice (see below). The calculated amount of SSV to be used was lyophilized. It was solubilized in 1 ml of digestion buffer at 50°C without reducing agent for 15 min and then loaded onto the stacking gel. Electrophoresis was carried out at a constant 100 V until the dye front had run out for 30 min so as to maximize the separation of protein 1 from other proteins. During electrophoresis, the temperature was maintained at 10°C. Two guide strips were excised from both sides of the preparative gel, fixed, and stained for 5 h as described above to locate protein 1. During this process, the guide strips swelled and lengthened. Therefore, the rest of the preparative gel was also fixed in methanol-acetic acid solution without Coomassie brilliant blue to allow exact alignment with the guide strips to locate protein 1. The area in the preparative gel corresponding to protein 1 was excised and cut into slices 0.5 cm wide; these were soaked in 0.125 M Tris-hydrochloride buffer, pH 6.8, overnight at 4°C and then stored at −76°C in fresh buffer until ready to be used. For isolation of unfixed protein 1, the preparative gel was wrapped with Saran wrap and stored at 4°C while the guide strips were being stained. After alignment with guide strips, the corresponding gel area containing protein 1 was excised and treated as the fixed gel slices. The purity of protein 1 was confirmed by electrophoresis of representative gel slices.

Limited proteolysis of protein 1. The limited proteolysis procedure of Cleveland et al. (3) was used. A 15 to 20% linear acrylamide gradient gel (24 by 16 by 0.15 cm) was formed in a Bio-Rad model 221 slab gel unit (28 by 16 by 0.15 cm) with a Hoefer Scientific Inc. model 77 gradient maker. After polymerization, a 3.5% acrylamide stacking gel was added. Preparative gel slices containing protein 1 were pushed to the bottom of sample wells (one slice per well). Spaces around the slices were filled with an overlay buffer containing 0.125 M Tris-hydrochloride, pH 6.8, 1 mM EDTA, 20% glycerol, and 0.1% SDS (TEGS buffer). Staphylococcal V8 protease (500 U/mg of protein; Miles Laboratories) or α-chymotrypsin (type I-S, EC

3.4.21.1, 40 to 50 U/mg of protein; Sigma Chemical Co.) was added to the wells where desired with a Hamilton syringe. In some cases, trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) (EC 3.4.21.4, 241 U/ml of protein; Worthington Biochemical Corp. was used. The proteases were prepared at 0.1 or 1 μg/μl in TEGS buffer. Amounts (10 μg) of molecular weight markers at 1 mg/ml in TEGS buffer containing 0.001% pyronin Y were loaded into one or both of the outermost wells. Electrophoresis proceeded at constant power of 12 W at 10°C until the dye marker in the outermost lane(s) was about 0.5 cm from the bottom of the stacking gel. Power was turned off. The water in the cooling chamber of the slab gel unit was replaced with water at 45°C. The slab unit was incubated at 37°C for 30 min to allow degradation of protein 1 in the stacking gel. The peptides generated were separated by size with electrophoresis at constant power of 15 W at 10°C until the dye marker reached the bottom of the gradient gel. The gel was stained with Coomassie brilliant blue and then destained as described above.

Molecular weight markers. Molecular weight markers used in this study were bovine serum albumin (68,000 [68K]), hen egg albumin (43K), carbonic anhydrase (29K), lysozyme (14.4K), and insulin A chain (2.3K). All were purchased from Sigma Chemical Co.

RESULTS

Protein profiles of the SSV of eight gonococcal strains were analyzed with SDS-PAGE (Fig. 1). This gel was intentionally overloaded so that each 0.5-cm wide gel slice contained 25 μg of protein 1 and so that it would still be well isolated from other proteins. The molecular weights of the outer membrane proteins and the identification of heat-modifiable and reduction-modifiable proteins were determined by solubilizing SSV at different temperatures and in the presence or absence of 2-mercaptoethanol by the method of McDade and Johnston (18) in 5 to 25% linear acrylamide gradient gels. These strains possessed protein 1 molecules with molecular weights in the range of 35.5K to 37.5K. The apparent molecular weight of protein 1 of strains N126 and DGI was 35.5K; that of strains N033, N082, N078, and PW1 was 36.5K; that of strain F62 was 37K; and that of N073 was 37.5K. The variation in the apparent molecular weight of protein 1 molecules from different strains has been well documented (5, 12, 21). All strains possessed the reduction-modifiable protein, protein 3. Strains F62, N126, DGI, and N073 possessed one heat-modifiable protein, protein 2, whereas strains N033, N082, N078, and PW1 possessed two heat-modifiable proteins, 2a and 2b. Protein 1 molecules were isolated by preparative SDS-PAGE. Their primary structures were compared by one-dimensional peptide mapping technique in SDS-PAGE (3) in this study. The idea is that if two proteins are identical or very similar, they should generate an identical class of peptides of heterogeneous size upon proteolysis at specific sites. Conversely, if two proteins are different, they should generate different patterns of peptides upon proteolysis.

Titration of proteases. Two serine proteases, staphylococcal V8 and α-chymotrypsin,

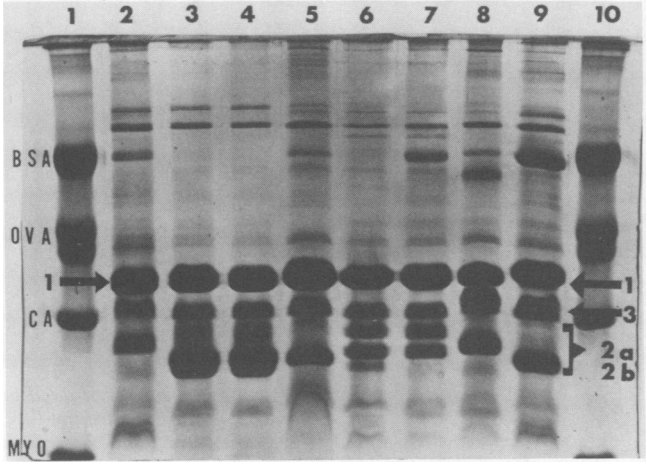

FIG. 1. *SDS-PAGE analysis of SSV from eight gonococcal strains. SSV were solubilized at 50°C without reducing agent for 15 min. Their protein 1 molecules, indicated by arrow 1, exhibited variations in apparent molecular weight as follows: N126 and DGI (lanes 5 and 8, respectively), 35.5K; N033, N082, N078, and PW1 (lanes 3, 4, 6, and 7, respectively), 36.5K; F62 (lane 2), 37K; N073 (lane 9), 37.5K. Protein 3 was reduction modifiable (arrow 3). Protein 2 (a and b) were heat modifiable. Molecular weight markers (lanes 1 and 10) were: bovine serum albumin (BSA), 68K; hen egg albumin (OVA), 43K; carbonic anhydrase (CA), 29K; myoglobin (MYO), 17K.*

were used. The former cleaves on the carboxyl side of glutamic acid and at a lower efficiency, i.e., that of aspartic acid residues (8). The latter cleaves on the carboxyl side of the amino acids with a bulky side chain, namely, tyrosine, tryptophan, and phenylalanine (6). Trypsin-TPCK, another serine protease with specific cleavage site on the carboxyl side of lysine and arginine (23), was used in some cases. A representative titration of staphylococcal V8 protease is shown (Fig. 2). A 25-μg amount of protein 1 from PW1 was digested with 0.2, 0.5, 1, 2, 5, and 10 μg of staphylococcal V8 protease in the 3.5% acrylamide stacking gel (lanes 3 through 8, Fig. 2). The protease could be seen in the stained gel as a double band only when 2 μg of enzyme or more was used (lanes 6 through 9 [arrow], Fig. 2). The variety of peptides decreased as the protease/substrate ratio increased to allow more complete digestion of protein 1. Since 0.5 μg of staphylococcal V8 protease generated a large variety of peptides to be compared without much native protein 1 left undigested, it was chosen to be the appropriate amount for use. In a similar fashion, 0.5 μg of α-chymotrypsin was determined to be the appropriate amount (data not shown). The maximum number of peptides remains constant for 0.2, 0.5, and 1 μg of α-chymotrypsin used despite some protein 1 remaining uncleaved. The titration of protein 1 from F62 to the proteases was similar to that of protein 1 from PW1 (data not shown). The proteases and their autoproteolytic products could not be seen in the gradient polyacrylamide gels because of the small amounts used in the following experiments, which were beyond the sensitivity of the stain used to visualize the protein 1 peptides.

To determine if the acid-alcohol fixing procedure of protein 1 altered its conformation and subsequent susceptibility to proteases, its peptide patterns were compared with those of unfixed protein 1 (Fig. 3). The results showed that the fixing procedure did not alter the susceptibility of protein 1 to proteolysis.

Limiting proteolysis of protein 1 molecules from gonococcal strains. Of the eight types of protein 1 molecules isolated from eight gonococcal strains chosen at random, six exhibited distinct peptide maps upon proteolysis by staphylococcal V8 protease (Fig. 4). Peptide patterns of protein 1 molecules from N033 and N082 were identical, and those from N078 and PW1 were identical. Protein 1 molecules from the remaining four strains possessed unique pep-

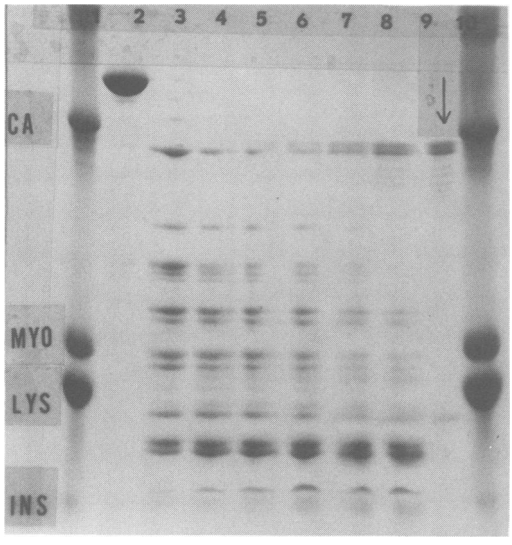

FIG. 2. *Staphylococcal V8 protease digestion of protein 1 from PW1. 25 μg of protein 1 was digested with 0, 0.2, 0.5, 1, 2, 5, and 10 μg of staphylococcal V8 protease (lanes 2 through 8, respectively). Lane 9 contained 15 μg of staphylococcal V8 protease alone. Lanes 1 and 10 contained molecular weight markers as for Fig. 3.*

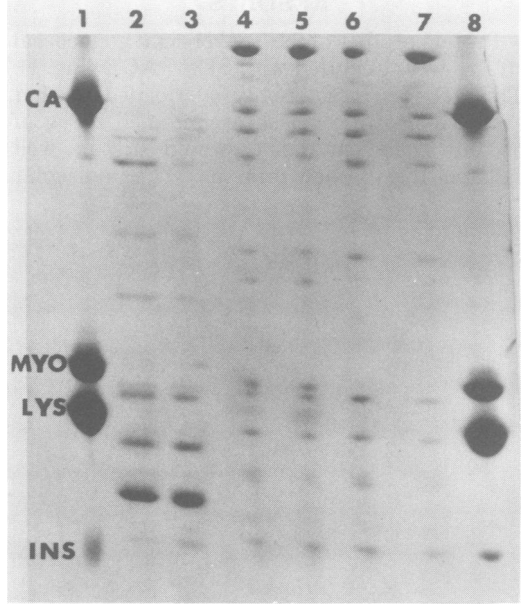

FIG. 3. *Proteolysis of fixed and unfixed protein 1. Lanes 2, 4, and 6 contained 25 μg of protein 1 from F62; lanes 3, 5, and 7 contained 25 μg of unfixed protein 1 from F62. Amounts of proteases added were: 0.5 μg of staphylococcal V8 protease (lanes 2 and 3); 40 μg of trypsin-TPCK (lanes 4 and 5); 0.5 μg of α-chymotrypsin (lanes 6 and 7). Molecular weight markers (lanes 1 and 8) were: carbonic anhydrase (CA), 29K; myoglobin (MYO), 17K; lysozyme (LYS), 14.4K; insulin A chain (INS), 2.3K.*

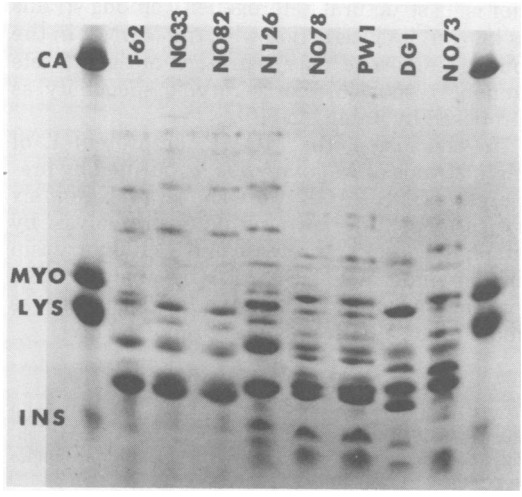

FIG. 4. *Peptide patterns from proteolysis of 25 μg of protein 1 molecules from eight gonococcal strains by 0.5 μg of staphylococcal V8 protease (lanes 2 through 9). See the legend to Fig. 3 for molecular weight markers (lanes 1 and 10).*

tide patterns. The relationship among peptide patterns of the eight strains was consistent when α-chymotrypsin was used (Fig. 5). The observation that identical amounts of protein 1 from different gonococcal strains treated with the same amount of α-chymotrypsin gave not only different peptide maps but also had different amounts of uncleaved substrate left supports our conclusion of the diversity among protein 1 molecules.

DISCUSSION

Of the eight gonococcal strains examined, there were six types of peptide patterns from protein 1 molecules. This indicated that the MOMP of *N. gonorrhoeae* was more diverse in its primary structure than previously reported by Swanson (22). The molecular weights of the MOMP molecules from the 10 strains he examined were 32K, 33K, and 34K. The ^{125}I-peptides generated by trypsin-TPCK and α-chmyotrypsin digestion of the MOMP molecules eluted from preparative SDS-PAGE were analyzed by the method of two-dimensional electrophoretic and chromatographic separations. Several ^{125}I-peptides were present in all of the MOMP molecules. The presence or absence of additional ^{125}I-peptides allowed the classification of the MOMP molecules into two groups. One group consisted of MOMP molecules having a molecular weight of 32K, and the other group consisted of MOMP molecules having molecular weights of 33K or 34K. In this study, differences among peptide patterns existed not only among protein 1 molecules having different apparent molecular weights (N126, 35.5K; N033, 36.5K; F62, 37K; and N073, 37.5K), but also among protein 1 molecules having the same apparent molecular weights (N126 versus DGI, 35.5K; N033 and N082 versus N078 and PW1, 36.5K). Multiple species of MOMP differing in primary structure have also been demonstrated in *Escherichia coli* (4, 9).

The two-dimensional electrophoretic and chromatographic technique used in the previous study (22) has the advantage of separating peptides by both size and charge. However, the effect of SDS on the chromatographic migration of ^{125}I-labeled proteolytic products of MOMP molecules isolated by SDS-PAGE was not clear. In addition, this technique can be used to analyze peptides from MOMP molecules of only one or two strains in a single two-dimensional analytical experiment. The one-dimensional peptide mapping technique of Cleveland et al. (3) used in this study allowed the comparison of peptide patterns of MOMP molecules from eight gonococcal strains in the same analytical polyacrylamide gel. This technique allowed the detection of peptides as small as 2,300 daltons (Fig. 3).

The discrepancy in the structural diversity of MOMP molecules between this and the previous study could be due to four things. First, the gonococcal strains chosen in this study were isolated from New York and Dallas over several

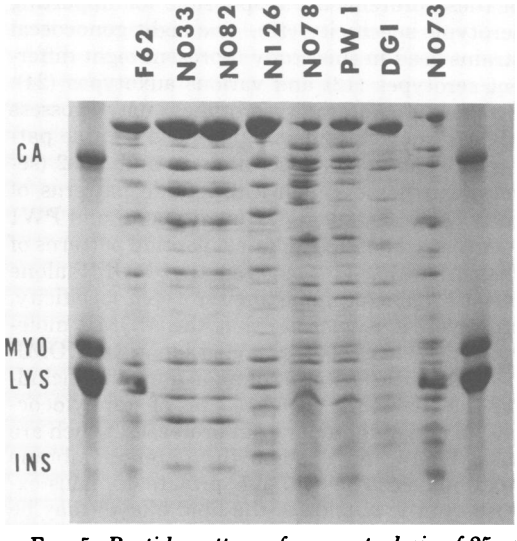

FIG. 5. *Peptide patterns from proteolysis of 25 μg of protein 1 molecules from eight gonococcal strains by 0.5 μg of α-chymotrypsin (lanes 2 through 9). See the legend to Fig. 3 for molecular weight markers (lanes 1 and 10).*

years. Therefore, they were more likely to be isolated from a more heterogeneous population. Second, the primary structure among protein 1 molecules having molecular weights between 35.5K and 37.5K (this study) may be more diverse than those having molecular weights between 32K and 34K (22). However, the difference in molecular weights of the MOMP molecules between the two studies could be due to technical variation. Third, the proteolytic conditions in the two studies were different. Proteolysis in this study was carried out in the stacking gel in the presence of 0.1% SDS, pH 6.8, and in the absence of dye. In the previous study, the MOMP molecules were eluted from in preparative polyacrylamide gels and were then digested by proteases in 0.05 M ammonium bicarbonate, pH 8.5. Fourth, peptides containing no tyrosine or histidine residues would not be detected by autoradiography in the previous study as chloramine T labels only tyrosine and histidine residues with ^{125}I (14).

McDade and Johnston (18) demonstrated that protein 1 and protein 3 (a reduction modifiable protein having an apparent molecular weight of 31K) could be immunoprecipitated with antiserum against homologous SSV or antiserum against electrophoretically purified homologous protein 1. These two proteins of the gonococcal outer membrane form a hydrophobically associated trimeric unit in situ and can be stabilized by selective cross-linking reagents (18). Hence, it was concluded that either or both of these proteins are responsible for imparting serotypic specificity (18). The eight gonococcal strains used in this study represent eight different serotypes (12) and various auxotypes (24). Strains of different serotypes may possess MOMP molecules having identical peptide patterns. That is, N033 (serotype *u*) and N082 (serotype *t*) possess identical peptide patterns of their MOMPs, and N078 (serotype *g*) and PW1 (serotype *f*) possess identical peptide patterns of their MOMPs. This suggests that MOMP alone is not capable of imparting serotypic specificity. However, it is possible that the MOMP molecules of two serotypes having identical MOMP peptide patterns share common domains, including the enzymatic cleavage sites of staphylococcal V8 and α-chymotrypsin proteases, which are most likely imbedded in the outer membrane lipid bilayer. Their unique, private domains exposed to the outside of the lipid bilayer may be similar in size but different in their amino acid compositions and subsequent antigenic conformations. Amino acid sequence analysis of the exposed peptide would allow us to scrutinize these two speculations more closely. Neither the role of protein 3 alone in serotypic specificity nor is its structural heterogeneity among strains is known. Its interaction with the MOMP in the outer membrane may represent antigenic sets and be responsible for serotypic specificity as proposed by Johnston (10).

In conclusion, the MOMP, or protein 1, of eight strains of *N. gonorrhoeae*, purified by preparative SDS-PAGE, were shown to exhibit six types of peptide patterns upon proteolysis by staphylococcal V8 protease and α-chymotrypsin in one-dimensional SDS-PAGE analysis. The primary structure of the MOMP molecules was more diverse than previously reported (22).

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