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Antigenic and Structural Differences among Six Proteins II Expressed by a Single Strain of *Neisseria gonorrhoeae*

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Gonococci express a family of related outer membrane proteins designated protein II (P.II), which undergo both phase and antigenic variation. Six P.II proteins have been identified in strain FA1090. We developed monoclonal antibodies specific for each P.II protein. Using these antibodies as probes, we purified the six different P.II proteins of this strain. Despite the relatedness of the proteins, we could not purify all of them by a single purification scheme. Four P.II proteins were purified by chromatofocusing, and the remaining two proteins were purified by hydrophobic interaction chromatography on phenyl-Sepharose. The N-terminal amino acid sequence of the proteins showed a high degree of sequence conservation. However, there was variability at specific amino acid residues, giving each P.II protein a unique N-terminal amino acid sequence. Thus P.II proteins of one strain differ among themselves not only in antigenic determinants and primary structure, but also in other characteristics affecting their properties in different chromatographic systems.

The outer membrane protein antigens of *Neisseria gonorrhoeae* are highly variable.These variations may be important in enabling the gonococcus to evade the host immune response and to adapt to differing host environments. One family of proteins that show such variation are the surface-exposed proteins II (P.II), which rapidly and reversibly alter expression within a single gonococcal strain and differ antigenically among different strains (1, 17, 32). The P.II proteins have apparent molecular weights of 24,000 to 30,000 and display a characteristic heat modifiability (an increase in apparent molecular weight when solubilized at 100°C versus 37°C) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7, 17, 30). A single gonococcal strain has been shown to express as many as seven P.II species, which differ in apparent molecular weight and antigenicity (23). A single organism may have zero to at least three P.IIs on its surface simultaneously (1), and spontaneous switches in P.II expression occur at a high frequency (19). There is a partial correlation of P.II expression with colonial phase-opacity phenotype (1, 30, 32, 34). Differences in colonial opacity or in P.II expression also correlate with differences in properties that may be related to virulence (6, 9, 10, 18). P.II proteins do vary in vivo, and gonococci lacking P.IIs are believed to be more virulent and invasive than those expressing the proteins (9, 23, 24).

P.II protein variants within the same strain or in different strains possess considerable structural homology, as revealed by tryptic peptide mapping (8, 12, 31) and immunological studies (33). However, different P.II proteins from one gonococcal strain possess several unique peptides, and these are primarily surface exposed (8, 12, 31). Antisera or monoclonal antibodies directed against presumed surface-exposed portions of P.IIs are highly specific for the homologous P.II variant (1, 5, 33).

Although procedures for the chromatographic purification of P.IIs have been reported (2, 8, 21), there has been no previous indication that the antigenic differences in individual P.II proteins of a single strain might be correlated with structural differences affecting the behavior of the proteins in different chromatographic systems. We report here that such structural heterogeneity does occur and that it necessitates the application of multiple purification schemes for the P.II proteins. The generation of a repertoire of monoclonal antibodies that could distinguish the P.II proteins was an essential first step in these studies.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* FA1090 is a serum-resistant prototrophic strain that was isolated from a patient with probable disseminated gonococcal infection (20). Other gonococcal strains were from the collection of P. F. Sparling, University of North Carolina School of Medicine. All neisserial strains were grown on GC medium base agar (Difco Laboratories) with Kellogg defined supplements or in GC broth plus supplements (13). Bacteria were incubated at 37°C in a 5% CO_2 atmosphere. Colonial opacity variants were identified by the criteria of Swanson (29).

Outer membrane preparation. Membranes used for P.II purification were obtained from liquid cultures (2 to 8 liters) of FA1090 gonococci expressing a single P.II as determined by SDS-PAGE and Western blotting (immunoblotting). Bacteria were grown to late log phase, harvested by centrifugation, and washed once in minimal broth, Davis (Difco). Outer membranes were prepared by lithium acetate extraction (11). For mouse immunizations, outer membranes were also prepared by Sarkosyl (CIBA-GEIGY Corp.) extraction (34) of gonococci grown on GC medium base agar.

Production of hybridomas. Procedures for immunization of BALB/c mice with gonococcal outer membranes (1), fusion of spleen cells with SP2/0-Ag14 myeloma cells (22), and screening of hybridomas by enzyme-linked immunosorbent assay for antibody production (1), were essentially as described previously. The antibody subclass was determined by enzyme-linked immunosorbent assay or Ouchterlony analysis with rabbit antibodies to mouse immunoglobulin subclasses (Litton Bionetics).

Complement-dependent bactericidal activity of monoclonal antibodies and colony blot radioimmunoassays, with

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hybridoma culture supernatant and radioiodinated protein A (Amersham Corp.) to probe filter blots of gonococci, were performed as described by Black et al. (1).

Electrophoresis and Western blotting. SDS-PAGE was performed as previously described (1) with 4 to 30% linear acrylamide gradient gels and the discontinuous buffer system of Laemmli (16). Samples (20 to 50 μg) were solubilized at 100°C for 5 min or at 37°C for 60 min. Gels were stained with Coomassie brilliant blue.

Western blotting and probing with monoclonal antibody culture supernatants (diluted 1:5) or mouse serum (diluted 1:500 to 1:1,000) and ^{125}I-labeled protein A have been previously described (1). Apparent molecular weights were determined by comparison with iodinated protein molecular weight standards (Pharmacia, Inc.), which were radioiodinated with Iodobeads (Pierce Chemical Co.).

Protein determination. Protein was quantitated by the Bio-Rad protein assay with ovalbumin as standard (3).

Chromatofocusing chromatography. Outer membranes were solubilized in 1% Nonidet P-40–25 mM triethylamine (pH 11) (Sigma) for 30 min at 37°C and then centrifuged at 100,000 x *g* for 2 h. The soluble fraction from 15 to 20 mg of outer membrane protein was subjected to PBE118 (Pharmacia) chromatofocusing chromatography essentially as described by Newhall et al. (21), except that 1% Nonidet P-40 replaced Triton X-100 in all buffers. Proteins were eluted with Pharmalyte 8-10.5 buffer (pH 7.0) (Pharmacia) diluted 1:45 (for P.IIb purification) or 1:30 (for all other P.II proteins). Fractions were assayed for the presence of P.II by SDS-PAGE and Western blotting, and the pH of column fractions was measured. P.II-containing fractions were dialyzed against 50 mM ammonium acetate (pH 9.5) at 4°C. Dialysates were then treated in a manner similar to that described by Blake and Gotschlich (2), involving precipitation in 10% trichloroacetic acid, precipitation in ethanol, and lyophilization. Pellets were washed three times in 85% acetone–5% triethylamine–5% glacial acetic acid–5% H_2O and lyophilized.

Phenyl-Sepharose chromatography. Hydrophobic interaction chromatography was performed at room temperature on 5-ml (1 by 6.5 cm) columns of phenyl-Sepharose CL-4B (Pharmacia). Columns were equilibrated with three bed volumes of appropriate buffer. Flow rates were 0.5 ml/min, and 1.5-ml fractions were collected.

For purification of P.IIc, outer membranes were solubilized for 1 h at 37°C in 1.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma)–50 mM sodium phosphate buffer (pH 7.5) at a protein concentration of 10 mg/ml and centrifuged at 100,000 × *g* for 2 h. A 1-ml portion of the soluble fraction was applied to a phenyl-Sepharose column equilibrated in solubilization buffer plus 2 mM EDTA (buffer A). The column was eluted in a stepwise fashion with 12 ml of each of the following buffers in succession: buffer A; buffer A plus 0.5% Tween 20 (Sigma); buffer A plus 1.5% Tween 20; and buffer A plus 3.0% Tween 20.

For purification of P.IId, outer membranes were solubilized for 1 h at 37°C in 1% sodium cholate (Sigma)–1 M NaCl–50 mM sodium phosphate buffer (pH 8.2) at a protein concentration of 2 mg/ml and then centrifuged as above. Triton X-100 (Research Products International) was added to the P.IId-containing soluble fraction to a final concentration of 0.75%. The soluble fraction was applied to a phenyl-Sepharose column equilibrated in 1% sodium cholate–0.75% Triton X-100–50 mM sodium phosphate buffer (pH 8.2). The column was then washed with 12 ml of equilibration buffer and eluted with a 50-ml buffer gradient of 1% cholate–0.75% Triton X-100–50 mM sodium phosphate buffer to 1% cholate–3.0% Triton X-100–10 mM sodium phosphate buffer.

P.IIc- or P.IId-containing fractions were identified by SDS-PAGE and Western blotting, and samples were processed as described for P.IIs purified by chromatofocusing.

Phenyl-Sepharose was regenerated by being washed with distilled water, being subjected to a step gradient of increasing ethanol concentration (5 to 95%), being treated with two bed volumes of *n*-butanol, being subjected to a step gradient of decreasing ethanol concentration (95 to 5%), being washed with distilled water, and being equilibrated in 50 mM sodium phosphate buffer. All columns were prepared from regenerated phenyl-Sepharose exclusively.

Amino-terminal amino acid sequence analysis. Sequencing was performed with an Applied Biosystems model 470A gas phase sequencer with standard programs and automated aqueous trifluoroacetic acid conversion to phenylthiohydantoin amino acids. Analysis of PTH amino acids was performed on a gradient high-performance liquid chromatograph (Waters Associates, Inc.).

RESULTS

Monoclonal antibodies. Six electrophoretically distinct forms of P.II proteins, designated P.IIa through P.IIf, have been identified for strain FA1090 (1). We previously reported the production of two monoclonal antibodies, one specific for P.IIa and one that binds to both P.IIb and P.IId (1, 28). We generated three additional monoclonal antibodies specific for the remaining P.II proteins of FA1090 by using outer membranes from colony variants expressing single P.II proteins to immunize mice. Antibody specificity was determined by ELISA of P.II colony variants and by Western blotting (Fig. 1; Table 1). The antibodies specific for P.IIc, P.IIe, and P.IIf each bound to only a single P.II protein and recognized both the heat-modified and unmodified form of the protein. None of these three antibodies was bactericidal for appropriate P.II-expressing variants of FA1090.

We were unable to produce a monoclonal antibody that could distinguish P.IIb from P.IId, despite several attempts with different immunogens and immunization strategies. No antibodies recognizing a common P.II epitope were generated in these fusions, suggesting that immunization with outer membranes may not present common immunogenic determinants to the mouse.

The FA1090 P.II-specific monoclonal antibodies were tested for binding to a panel of 23 gonococcal strains by colony blot radioimmunoassay. The strains included several different serotypes isolated from people with uncomplicated gonococcal infection or disseminated gonococcal infection. Twenty of the strains showed no binding with any of the antibodies; the remaining three strains bound one to three of the five antibodies. The epitopes recognized by these monoclonal antibodies were thus not common to all gonococcal strains, although they were not exclusively limited to strain FA1090.

Using this panel of monoclonal antibodies, we could unambiguously determine the P.II expression state of any colony variant of strain FA1090. Also, it was possible to isolate and maintain colony variants with verified predominant expression of a single P.II protein which facilitated purification of the different P.II proteins.

Purification of P.II proteins by chromatofocusing. We initially used a modification of the chromatofocusing method described by Newhall et al. (21), which takes advantage of

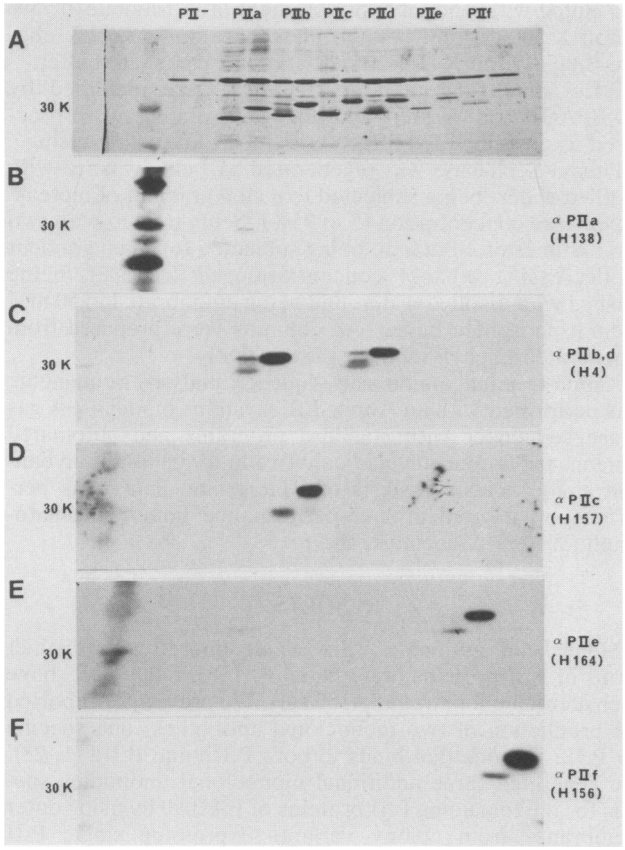

FIG. 1. Electrophoresis and Western blot analysis of P.II variants probed with P.II-specific monoclonal antibodies. (A) Coomassie-stained 4 to 30% SDS-PAGE of FA1090 P.II colony variants expressing no P.II (P.II$^-$) or a single P.II (P.IIa to P.IIf). Outer membranes (20 to 25 μg of protein) of each of the seven variants were solubilized at 37°C for 60 min (left lane of each pair) and at 100°C for 5 min (right lane of each pair). This gel was one of five replicates containing outer membranes of the same P.II variants prepared at different times. Each of the five gels was Western transferred and probed with one of the anti-P.II monoclonal antibodies. (B to F) Autoradiographs of each Western blot. The monoclonal antibodies used for each probe, and their specificities, are indicated to the right of each panel.

the high isoelectric point of P.II proteins, to attempt purification of the six P.IIs of FA1090. Four P.II proteins (P.IIa, P.IIb, P.IIe, and P.IIf) were purified to apparent homogeneity by this method. There were some differences in the behavior of the four proteins on the chromatofocusing column, since elution of P.IIa, P.IIe, and P.IIf required the use of a more concentrated solution of Pharmalyte elution buffer than elution of P.IIb did. Each of the P.II proteins was eluted at a pH of approximately 9.5. The purification of P.IIa is illustrated in Fig. 2. To demonstrate the purity of the preparation, it was subjected to SDS-PAGE and Western blotting and probed with a mixture of monoclonal antibodies directed at different proteins of FA1090 plus mouse antisera raised against outer membranes of all FA1090 P.II variants.

Two of the P.II proteins were refractory to purification by this method. Solubilized P.IIc and P.IId remained bound to the chromatofocusing column and could not be eluted under any conditions tested. Alternative purification schemes were necessary for these two proteins.

TABLE 1. Characteristics of P.II proteins and specificity of anti-P.II monoclonal antibodies

P.II variant	Mol wt[a]	Opacity phenotype[b]	Monoclonal antibody[c]	Immunoglobulin subclass[d]
P.IIa	30,000	Op^{++}	H138	IgG3
P.IIb	31,200	Tr	H4	IgG2a
P.IIc	31,700	Tr	H157	IgG3
P.IId	31,800	Op^{++}	H4	IgG2a
P.IIe	31,900	Tr	H164	IgG3
P.IIf	32,900	Op^{+}	H156	IgG3

[a] Apparent molecular weight on 4 to 30% SDS-PAGE when solubilized at 100°C for 5 min.
[b] Opacity phenotype of FA1090 colonies. Abbreviations: Op^{++}, very opaque; Op^{+}, moderately opaque; Tr, transparent.
[c] Monoclonal antibody specific for P.II protein.
[d] Immunoglobulin subclass of monoclonal antibodies. IgG, Immunoglobulin G.

Hydrophobic interaction chromatography. Successful purification of P.IIc and P.IId was achieved by using bile salt detergents to solubilize FA1090 outer membranes, followed by combinations of detergents and changes in ionic strength to selectively adsorb and elute P.II proteins on phenyl-Sepharose.

Extraction of FA1090 P.IIc outer membranes with 1.5% CHAPS (pH 7.5) buffer resulted in a soluble fraction enriched in P.IIc (30 to 40% of total membrane P.IIc) and lacking protein I. This fraction was applied to a phenyl-Sepharose column and eluted with a stepwise gradient of CHAPS buffer plus increasing concentrations of Tween 20. P.IIc eluted at a buffer composition of approximately 1.5% CHAPS–1.5% Tween 20. P.IIc-containing fractions were pooled, and the purity of the preparation was demonstrated by SDS-PAGE and Western blot (Fig. 3). The yield of purified protein was 1.65 mg from 20 mg of outer membrane protein.

In contrast to P.IIc, P.IId was not extracted from outer membranes by solubilization with bile salt detergents alone (Fig. 4). After solubilization in 1.5% CHAPS–50 mM sodium

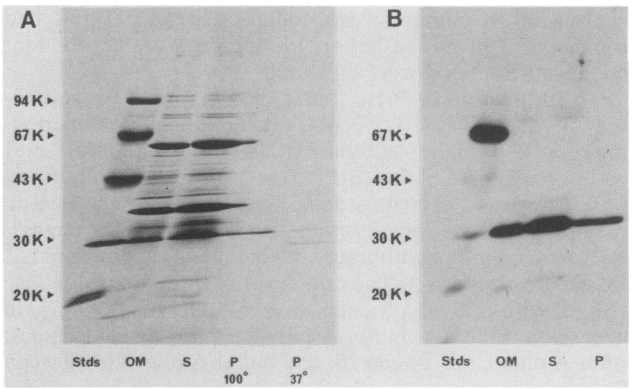

FIG. 2. Electrophoresis and Western blot analysis of P.IIa purification by chromatofocusing chromatography. (A) SDS-PAGE of P.IIa outer membranes (OM), solubilized membrane fraction (S), and purified P.IIa (P). Outer membranes (35 μg) and solubilized membrane fractions (50 μg) were solubilized for electrophoresis at 100°C for 5 min. P.IIa samples (45 μg in each lane) were solubilized at 100°C for 5 min or 37°C for 60 min. (B) Western blot of duplicate samples on the same gel, probed with a mixture of FA1090-specific monoclonal antibodies directed against all six P.II proteins, protein I, and the H.8 protein (4) and with mouse antisera raised against outer membrane preparations of the six FA1090 P.II variants.

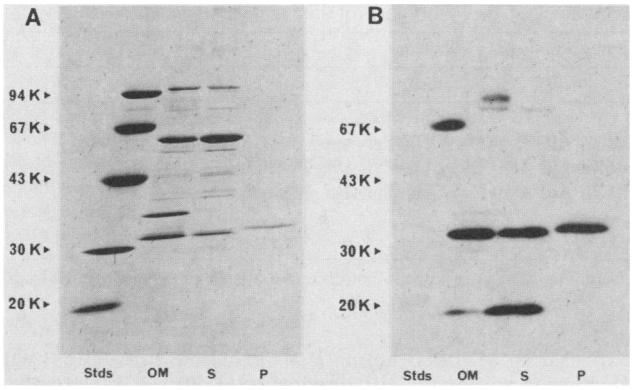

FIG. 3. Electrophoresis and Western blot analysis of the purification of P.IIc on phenyl-Sepharose. (A) SDS-PAGE of P.IIc outer membranes (OM), solubilized membrane fraction (S), and purified P.IIc (P). Samples were solubilized for electrophoresis at 100°C for 5 min. (B) Western blot of duplicate samples on the same gel probed with a mixture of antibodies as described in the legend to Fig. 2.

phosphate buffer (pH 7.5), no P.IId was detectable in the soluble fraction, whereas a substantial portion of P.IIc was extracted. The extraction of nearly all the other proteins was identical in the two membrane preparations. The inclusion of 1 M NaCl in a buffer containing 1% sodium cholate resulted in successful solubilization of P.IId. P.IId bound to phenyl-Sepharose in an apparently irreversible fashion when adsorbed in sodium cholate-NaCl buffer alone; it was eluted only when Triton X-100 was included in the equilibration and adsorption steps and the subsequent elution was done with a gradient of increasing Triton concentration in 1% sodium cholate. SDS-PAGE and Western blotting analyses of the purified preparation are shown in Fig. 5. The yield of purified P.IId was 1.63 mg from 8.0 mg of outer membrane protein. The P.IId doublet seen after Coomassie staining was occasionally present, for reasons we do not understand. It occurred with some solubilization regimens, was generally not present in the outer membrane samples, and appeared to have no effect on the ability to determine amino acid sequence.

All of the purified P.II proteins retained their characteristic heat modifiability, apparent molecular weight, and antibody-binding specificity on SDS-PAGE and Western blotting.

Amino-terminal amino acid sequence. The N-terminal amino acid sequence of each of the six P.II proteins is shown in Table 2. Although many residues were identical in all P.IIs, each of the six proteins had a unique amino acid sequence. Within the first 23 residues, there were differences in the amino acids at positions 2, 4, 5, 8, 15, and 19. Not all of the differences could be accounted for by mutations involving single base pair changes in P.II genes. The class of the variable amino acids, based on the polarity of their side chain groups, was not always conserved.

DISCUSSION

The objectives of this study were to characterize the extent of antigenic and structural variability among the P.II proteins made by one gonococcal strain. The occurrence of antigenic differences among P.II proteins has been documented (1, 17, 24, 32), but total number of antigenic variants in a strain has not been determined. Using the set of P.II-specific monoclonal antibodies, we showed that the six P.II proteins of strain FA1090 possessed at least five unique epitopes. These monoclonal antibodies cross-reacted with P.II proteins of only a limited number of other gonococcal strains. The antibodies probably recognized linear determinants, since they bound to P.II proteins in whole organisms or outer membranes and to the denatured proteins. The set of monoclonal antibodies specific for each of the FA1090 P.IIs proved extremely useful in identifying and maintaining colony variants of defined P.II expression state, which was essential for the purification of the individual P.II proteins. These antibodies should also be useful as tools in studies on the functions of different P.II proteins.

The N-terminal amino acid sequence of each of the six P.II proteins was unique, although there was substantial conservation of many residues. The residues conserved in the FA1090 P.II proteins were the same as those in other sequenced P.II proteins or P.II genes (2, 26, 27). Three of the four previously available P.II N-terminal sequences are from proteins or genes of strain MS11. Different strains within the species *N. gonorrhoeae* can vary considerably, and it has been suggested that strains causing disseminated gonococcal infection, such as FA1090, may in fact represent a distinct clone or subspecies of gonococci (14, 15). Therefore we

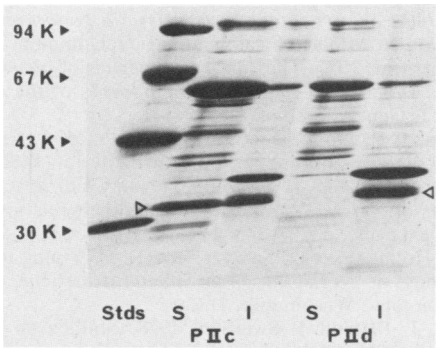

FIG. 4. Differential solubilization of P.IIc and P.IId. The SDS-PAGE profile of P.IIc and P.IId outer membranes solubilized at 37°C for 60 min in 1.5% CHAPS–50 mM sodium phosphate buffer (pH 7.5) is shown. Samples (30 to 45 μg) of soluble (S) and insoluble (I) fractions were solubilized for electrophoresis at 100°C for 5 min. Arrows indicate the location of P.II proteins.

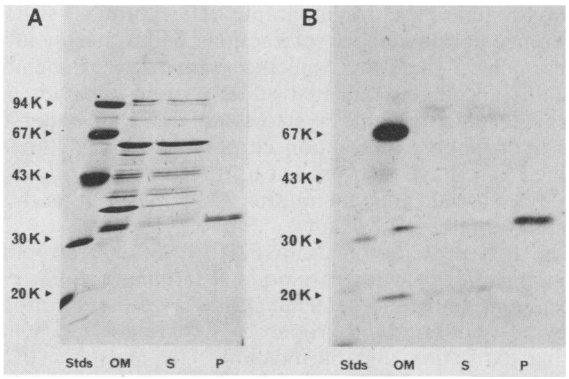

FIG. 5. Electrophoresis and Western blot analysis of the purification of P.IId on phenyl-Sepharose. (A) SDS-PAGE of P.IId outer membranes (OM), solubilized membrane fraction (S), and purified P.IId (P). Samples were solubilized for electrophoresis at 100°C for 5 min. (B) Western blot of duplicate samples of the same gel probed with a mixture of antibodies as described in the legend to Fig. 2.

TABLE 2. Amino-terminal amino acid sequences of FA1090 P.II proteins[a]

P.II variant	Amino-terminal amino acid sequence			
	5	10	15	20
P.IIa	**Ala**-Ser-**Glu**-Asp-Gly-**Gly**-**Arg**-Gly-**Pro**-**Tyr**-**Val**			
P.IIb	**Ala**-Thr-**Glu**-Gly-Asn-**Gly**-**Arg**-Arg-**Pro**-**Tyr**-**Val**-**Gln**-**Ala**-**Asp**-Leu-**Ala**-**Tyr**-**Ala**-Ala-**Glu**-( )-**Ile**-**Thr**			
P.IIc	**Ala**-Ser-**Glu**-Asp-Asn-**Gly**-( )-Gly-**Pro**-**Tyr**-**Val**-**Gln**-**Ala**-**Asp**-Lys-**Ala**-**Tyr**-**Ala**-Tyr-**Glu**			
P.IId	**Ala**-Ser-**Glu**-Gly-Asn-**Gly**-**Arg**-Gly-**Pro**-**Tyr**-**Val**-**Gln**-**Ala**-**Asp**-Leu-**Ala**-**Tyr**-**Ala**-Ala-**Glu**-**Arg**-**Ile**-**Thr**			
P.IIe	**Ala**-Gly-**Glu**-Asp-Asn-**Gly**-**Arg**			
P.IIf	**Ala**-Thr-**Glu**-Asp-Asn-**Gly**-**Arg**			

[a] Amino acids that were conserved in FA1090 P.II proteins and in P.II proteins of other strains are shown in boldface. All other amino acids were variable. Positions 21 of P.IIb and 7 of P.IIc were not determined.

believe that it is important to characterize the P.II proteins and the genes encoding them from more than a single gonococcal strain. The data on the P.II proteins of FA1090 are consistent with the identification of the extreme N-terminus of the P.II proteins as a semivariable region (25), the variation being sufficient to generate a unique N-terminal sequence for each P.II protein.

Our efforts to purify the six P.II proteins revealed differences in their behavior in chromatofocusing and hydrophobic interaction chromatography. Four of the P.II proteins (P.IIa, P.IIb, P.IIe, and P.IIf) were purified by chromatofocusing. The membrane-associated nature of P.IIs and the failure of P.IIc and P.IId to elute from the chromatofocusing matrix led to our application of hydrophobic interaction chromatographic techniques. We devised two efficient separation procedures that resulted in the purification of P.IIc and P.IId, in single steps, on phenyl-Sepharose. Requirements for extraction of P.IIc and P.IId from the membrane were different, as were the conditions required for adsorption to and elution from the phenyl-Sepharose column. The structural differences affecting the chromatographic properties of the proteins did not necessarily involve the unique epitopes recognized by the monoclonal antibodies. Proteins P.IIb and P.IId shared a common epitope, but were clearly distinct proteins differing in apparent molecular weight, N-terminal amino acid sequence, and opacity phenotype and bactericidal susceptibility of colonies expressing them. P.IIb was readily purified by chromatofocusing chromatography, whereas P.IId purification required development of a different method involving the use of phenyl-Sepharose chromatography.

We do not know whether the significant differences in behavior of FA1090 P.II proteins on chromatofocusing and phenyl-Sepharose chromatography reflect primary structural differences or secondary interactions with other membrane components, since the specific properties of membrane proteins and detergents that affect protein behavior in different chromatographic systems are not well understood. The hydrophobic interaction chromatography methods described here have also proved useful for the purification of other neisserial outer membrane proteins, such as the H.8 protein (unpublished results).

The P.II proteins of strain FA1090 differed structurally and antigenically, with six unique N-terminal sequences and five unique antigenic determinants among the six proteins. They also differed in properties affecting their behavior during extraction and purification. The existence of such heterogeneity within this family of closely related proteins reinforces the suggestion that specific P.II proteins of a gonococcal strain may have different functions. Some studies indicate that the expression of different P.II proteins of a strain correlates with differences in properties such as adherence or serum resistance (9, 18), and it is known that expression of only some P.II proteins correlates with changes in colony opacity phenotype (1, 30, 32, 34). It will be important to determine how the differences in primary structure of P.II proteins affect their organization in the membrane and possible function in gonococcal infection.

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