GONOCOCCAL PILI
Primary Structure and Receptor Binding Domain

By GARY K. SCHOOLNIK, ROSEMARY FERNANDEZ, JOSEPH Y. TAI, JONATHAN ROTHBARD, AND E. C. GOTSCHLICH

From the Departments of Medicine and Medical Microbiology, Stanford University; The Palo Alto Veterans Administration Hospital, Palo Alto, California, 94305; and The Rockefeller University, New York 10021

Gonococcal pili are surface appendages composed of identical polypeptide subunits termed pilin, which polymerize to form linear structures ~6 nm in diameter and 1,000–4,000 nm in length (1, 2). Pili bind epithelial cell receptor molecules and thereby promote mucosal colonization (3–5). They also interact with polymorphonuclear leukocytes and probably confer resistance to phagocytosis (6). Although pili from separate gonococcal strains and variants of the same strain exhibit similar functional and structural attributes (7), they may differ physically, chemically (8, 9), and antigenically (10–12). In order to elucidate the molecular basis for epithelial adherence and antigenic diversity, Schoolnik et al. (7) prepared cyanogen bromide (CNBr) fragments of pili from different gonococcal strains. CNBr-2 was found to be immunorecessive and to encompass a highly conserved region that mediates receptor binding function. CNBr-3 was found to be immunodominant and to comprise a variable region that confers type-specific antigenicity.

The complete amino acid sequence of gonococcal pilin from the transparent opacity variant (P+Tr, reference 8)1 of strain MS 11 is reported here and provides more detailed information about the topography of the receptor binding domain and the chemical basis for polymeric structure and antigenic diversity.

Materials and Methods

Gonococcal Strains and Growth Medium. Gonococcal strain R10 was collected by the Department of Microbiology, Cornell University School of Medicine, New York, from a case of male urethritis. Strain MS11 was obtained from Dr. John Swanson (Rocky Mountain Laboratories, Hamilton, Montana) and also came from a patient with uncomplicated disease. They were received on agar plates (New York City Medium) and the

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1 Abbreviations used in this paper: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; Eth buffer, 0.15 M ethanalamine-HCl buffer, pH 10.1; HPLC, high pressure liquid chromatography; LPS, lipopolysaccharide; MEM, minimal essential medium; P+Tr, gonococcal pilin from the transparent opacity variant; PBS, phosphate-buffered saline; Pth, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEL, steric exchange column; TFA, trifluoroacetic acid.
bacteria nonselectively removed with cotton-tipped swabs and stored frozen in Greaves' solution (13) at -70°C. Before pili purification, the organisms were identified as Neisseria gonorrhoeae by Gram stain, oxidase reaction, and sugar fermentation, propagated on solid typing medium (14) by selective daily passage of single colonies, and incubated at 36.5°C in 6.2% CO2 in air. The P+Tr opacity variants of each strain were used for pili production. They were selected according to their appearance when viewed through a dissecting stereomicroscope (15).

Purification of Pili. Single colonies with P+Tr morphology were inoculated onto each of five 100 × 15-mm disposable petri plates containing solid medium. 24 h later the organisms were harvested with a cotton swab into 10 ml of Protease Peptone No. 3 (Difco Laboratories, Detroit, MI) to an OD of 0.40 at 540 nm (16 × 125-mm tube). A bent glass rod was used to spread 0.5 ml of the bacterial suspension over the agar surface of 20 150 × 15-mm disposable petri dishes containing solid medium. After 24 h incubation the confluent growth was harvested with a glass slide into 100 ml of ice-cold 0.15 M ethanolamine-HCl buffer, pH 10.1 (Eth buffer). All subsequent operations were performed at 4°C.

Pili were purified by a modification of the method of Brinton et al. (16). The bacteria were homogenized for 10 min at 2,500 rpm in a Sorvall Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Newton, CT) and the sheared bacteria removed by centrifugation at 12,000 g for 30 min. Pilus filaments were precipitated from the supernatant by the addition of solid ammonium sulfate to 10% saturation, collected by centrifugation at 12,000 g for 45 min, and the pellet dissolved in Eth buffer. Insoluble contaminants were removed by centrifugation at 13,000 g for 60 min and the supernatant dialyzed against 0.05 M Tris-HCl buffer, pH 8.0 containing 0.15 M NaCl (Tris-saline buffer). The pilus crystals that formed were freed from soluble contaminants by centrifugation and the pellet dissolved in Eth buffer. Successive cycles of solubilization and crystallization by exposure to Eth buffer and Tris-saline buffer, respectively, resulted in a pilus preparation that was characterized by the presence of a single protein band on a Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO) stained polyacrylamide gel in which 50 µg of protein was electrophoresed according to the method of Laemmli (17) and by the absence of gonococcal lipopolysaccharide (LPS) by gas-liquid chromatography as determined by the failure to detect beta hydroxy fatty acids (18). The sensitivity of this method indicated that the percent by weight of contaminating LPS was <0.01. Amino acid and sequence analyses were performed on purified pili lyophilized from water.

Cyanogen Bromide Cleavage. Chemical cleavage at methionine residues with cyanogen bromide was performed according to the method of Gross and Witkop (19). 20 mg of lyophilized pili (~1 µmol) were dissolved in either 75% trifluoroacetic acid or 70% formic acid (N2-saturated) and 100 mg of cyanogen bromide (Pierce Chemical Co., Rockford, IL) were added. After 24 h at 22°C the reaction mixture was diluted 20-fold with distilled water and lyophilized.

Arginine-specific Hydrolysis. Pili were citraconylated by a modification of the method of Atassi and Habeeb (20) in 0.05 M sodium phosphate buffer, pH 9.0. A 15-fold molar excess of citraconic anhydride over total amino groups was added to the stirred solution in five aliquots over 60 min. The pH of the reaction was maintained at 9.0 by the addition of 2 M NaOH. The citraconylated protein was freed from salt by gel filtration with Sephadex G-15 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) equilibrated with 0.1 M ammonium bicarbonate, pH 8.0 and the arginyl bonds cleaved by the addition of TPCK-treated trypsin (Worthington Millipore Corp., Bedford, MA) to 2% (wt/wt). After 4 h at 37°C, the insoluble peptides were removed by centrifugation and the supernatant injected directly onto a high-pressure liquid chromatography (HPLC) TSK type SW steric exchange column (SEC) equilibrated in 0.1 M ammonium bicarbonate, pH 8.1 (side infra).

Iodosobenzoic Acid Cleavage of CNBr-3. 5 mg of lyophilized CNBr-3 were dissolved in 0.75 ml of 80% (vol/vol) acetic acid-3 M guanidine-HCl and mixed with 0.25 ml of 80% acetic acid containing 12 µg of o-iodosobenzoic acid (Pierce Chemical Co.) according to the method of Mahoney and Hermodson (21). Preincubation of the reagent with p-cresol
was not performed, since this fragment lacks tyrosine. The reaction was incubated under
\(N_2\) for 24 h at 22°C in the dark and CNBr-W-3 (Fig. 2) isolated by HPLC-SEC.

**Exopeptidase Hydrolysis.** The carboxy-terminal sequence of gonococcal pili was deduced
by hydrolysis with carboxypeptidase Y (Pierce Chemical Co.) as described by Hayashi (22).
5 mg of protein, 25 \(\mu\)g of carboxypeptidase Y, and 1.5 \(\mu\)mol of norleucine (internal
standard) were dissolved in 5 ml of 0.05 M pyridine/acetate buffer, pH 5.5 and incubated
at 25°C. The reaction was terminated at "0-time," 30 s, 1, 2, 10, 30, 60, and 120 min by
lyophilization. The hydrolysates were dissolved in 0.2 M citrate buffer, pH 2.2 and
injected into the amino acid analyzer.

**Reduction and Alkylation.** The presence of free sulfhydryl groups was determined with
Ellman's reagent (23), 5,5'-dithiobis (2-nitrobenzoic acid). Gonococcal pili (1.0 mg) were
dissolved in 0.3 ml of 6 M guanidine-HCl, pH 7.8 and incubated for 30 min at 50°C. 10
\(\mu\)l of Ellman's reagent (Pierce Chemical Co.) was added (10 mM in 0.1 M sodium-
phosphate buffer, pH 7.0) and the absorbance at 412 nm was measured.

Reduction and alkylation of pili preceded arginine-specific hydrolysis in some experi-
ments. Pili (0.25 \(\mu\)mol) and 2.5 \(\mu\)mol dithiothreitol were dissolved in 2.5 ml of 0.1 M
ammonium bicarbonate buffer, pH 8.2 containing 6 M guanidine-HCl and incubated
under \(N_2\) at 50°C for 1 h (24). Iodoacetic acid (3.0 \(\mu\)mol) was added and the reaction
allowed to proceed for 1 h at 25°C in the dark.

**Isolation of Peptides.** Cyanogen bromide fragments were separated by reverse-phase
HPLC. They were eluted from a Whatman Partisil-10 ODS-2 column in a linear gradient
of acetonitrile in water containing 0.05% (vol/vol) trifluoroacetic acid (TFA) at a flow
rate of 1.0 ml/min. Tryptic peptides of citraconylated pili and the iodosobenzoic acid
fragments of CNBr-3 were resolved by HPLC SEC in which one TSK G3000SW and two
TSK G2000SW 7.5-ram × 50-cm columns (Varian Associates, Inc., Walnut Creek, CA)
were connected in series and the peptides eluted in 0.1 M ammonium bicarbonate buffer,
pH 8.1 at a flow rate of 0.30 ml/min. Eluates were monitored by their absorbance at 218
nm and 280 nm, and the peptides identified by amino acid analysis and their purity
assessed by thin-layer chromatography and amino acid sequence analysis.

**Amino Acid Analysis.** The amino acid compositions of gonococcal pili and pilus peptides
were determined by hydrolysis in 4 N methane-sulfonic acid (25) in evacuated, sealed
tubes at 115°C for 22, 48, 72, and 108 h. The values for serine and threonine were
corrected for destruction during hydrolysis by extrapolation to 0 time. The values for
leucine, isoleucine, and valine were corrected for slow hydrolysis of the peptide bond by
extrapolation to infinite time. Half-cystine and methionine were determined as cysteic
acid and methionine sulfone, respectively after performic acid oxidation of the methane-
sulfonic acid hydrolysate. Homoserine was identified by the method of Adelstein and
Kuehl (26). The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer
using a one-column system.

**Carbohydrate Analysis.** Pili (1 mg) were hydrolyzed in 1.5 N methanolic-HCl at 80°C
for 16 h and the monosaccharides analyzed as their trimethylsilyl ethers (27) on a Varian
model 3700 gas liquid chromatography unit equipped with a CDS-111 integrator (Varian
Associates, Instrument Division, Palo Alto, CA).

**Analysis of Phosphorus and Phosphoamino Acids.** Phosphorus was determined by the
method of Chen et al. (28). Phosphoamino acid content was estimated from the complete
enzymatic hydrolysis of pili. Pili (2 mg) were incubated with trypsin (2% wt/wt) in 0.1 M
ammonium bicarbonate buffer, pH 8.1 at 37°C for 6 h and then pronase (Calbiochem-
Behring Corp., La Jolla, CA) and amino peptidase M (2% wt/wt; Boehringer Mannheim
Biochemicals Indianapolis, IN) were added. The reactants were incubated for an addi-
tional 10 h and lyophilized. Amino acid analysis of the hydrolysate indicated the presence
of phosphoserine or phosphothreonine. Identification of the phosphoamino acid was
accomplished by eluting the hydrolysate from an AG1-X50 anion ion exchange 0.9 × 15.0-cm column (Bio-Rad Laboratories, Richmond, CA) in 0.01 N HCl. The phosphoamino
acid was collected between 15 and 30 ml and lyophilized. Half of this material was
analyzed directly on the amino acid analyzer using phosphoserine and phosphothreonine
standards (Vega Biotechnologies, Inc., Tucson, AZ) and the other half was hydrolyzed in
Sequence Analysis. Automated Edman degradation was performed with a Beckman 890C sequencer (Beckman Instruments, Palo Alto, CA), using a modified Quadral program (No. 0011576) of Beckman Instruments in combination with polybrene. Thiazolinone derivatives of amino acids were converted to Pth-derivatives with aqueous 1.0 N HCl at 80°C for 10 min. Pth-derivatives were identified by HPLC with a Hewlett Packard 1084B HPLC (Hewlett Packard, Co., Cupertino, CA), using Dupont Zorbax ODS columns and confirmed by gas chromatography (GLC) and/or thin-layer chromatography (TLC). Sequenator stepwise yields were 92-96% and amino acids were assigned only when peak-to-background ratios were $>2:1$.

Pilus Peptide Receptor Binding Assay. The receptor binding properties of $^{125}$I-labeled peptide TC-2 (Fig. 2) was determined with human endocervical and buccal epithelial cells and with cultivated HeLa cells.

Endocervical cells were obtained from healthy, uninfected women of child-bearing age undergoing a routine pelvic examination. The cells were withdrawn from the endocervical canal with the Unimar Endocervical Aspirator or the ACCU-PEP T-Zone Sampler (United International Marketing Resources, Inc., Canoga Park, CA) according to the manufacturer's instructions, suspended in 10 ml of phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% (wt/vol) bovine serum albumin (BSA), 0.05% (wt/vol) sodium azide, and 5 mM EDTA and counted (cells/mm$^3$) in a Bright-Line hemocytometer (American Optical, Buffalo, NY). The cells were washed by low speed centrifugation in 50 mM Tris-acetate buffer, pH 6.5 containing 140 mM NaCl, 5 mM CaCl$_2$, 4 mM KCl, 2 mM MgCl$_2$, and 0.1% (wt/vol) BSA (receptor-binding buffer; reference 29) and used within 5 h. Epithelial cells were obtained from the buccal mucosa of healthy volunteers by the method of Pierce and Buchanan (3) and processed as described above. Monolayer cultures of HeLa cells were grown at 37°C in Earle's modified minimal essential medium (MEM; Grand Island Biological Co., Grand Island, NY). The cells were removed with 2 ml of 10 mM sodium phosphate buffer, pH 7.6 containing (wt/vol) 0.06% trypsin, 0.01% pancreatin, 0.05% EDTA, 0.8% NaCl, 0.04% KCl, and 0.001% phenol red. After removal, 10 ml of Earle's MEM containing 10% fetal bovine serum was added to inhibit the proteases, the reactants incubated for 1 h at 37°C, and the cells processed as described above.

$^{125}$I-labeled TC-2 was prepared by the chloramine-T procedure of Greenwood et al. (30) with carrier-free Na$^{125}$I (New England Nuclear, Boston, MA). Iodinated TC-2 was separated from the other reactants by Sephadex G25 (Pharmacia) gel filtration chromatography in PBS. The specific activity was calculated from the estimated protein concentration and by gamma counting (Beckman Model Gamma 5500) and was $\sim 1 \times 10^{7}$ cpm/µg. The protein-bound $^{125}$I fraction was estimated to be $>85\%$ by precipitation with 13% (wt/vol) trichloroacetic acid with 1% (wt/vol) BSA added as carrier. $^{125}$I-labeled TC-2 co-migrated as a single band with unlabeled TC-2 on SDS-PAGE (17) in which the gel was assessed by autoradiography and Coomassie Blue staining.

The receptor binding assay was conducted by combining $10^4$ washed cells (100 µl) and 900 µl of receptor binding buffer containing $10^3$ to $10^5$ cpm of $^{125}$I-labeled TC-2 in a 1.5-ml plastic microfuge tube (Beckman Instruments). The reactants were mixed for 60 min at 37°C and the cell-bound and -free TC-2 fractions were separated by centrifugation washes with receptor binding buffer in a Beckman microfuge (Model B). After the fifth wash the tip of the microfuge tube containing the cell pellet was removed with a razor blade and counted in the gamma counter. A negative (cell-free) control consisting of $10^3$ to $10^5$ cpm of $^{125}$I-TC-2 in 1.0 ml of receptor binding buffer was included in each experiment. This value was subtracted from the experimental values. The radioactivity of the thoroughly washed cell pellet was examined by SDS-PAGE (17). Autoradiography indicated the presence of a band that co-migrated with labeled TC-2.

Hydrophilicity and Secondary Structural Predictions. The local hydrophilicity using hexapeptide averages was predicted from the amino acid sequence according to the method of Hopp and Woods (31), except that the solvent parameter values of Levitt (32) were
used. Secondary structural predictions were calculated from the Chou-Fasman algorithm (38).

Computer Methods for Comparing Protein Sequences. The MS11 gonococcal and PAK pseudomonad (34) pilin sequences were analyzed by a program that compared all possible four-residue segments from one sequence with all segments of the same length from the other sequence.

30-residue segments of the MS11 gonococcal sequence were compared with all 30-residue segments of each of the 2,222 protein sequences stored at the National Biomedical Research Foundation, Georgetown University Medical Center by the computer program MATCH (35). The mutation data matrix proposed by Barker and Dayhoff (36) for the deduction of the evolutionary relatedness of proteins was not used in this analysis. Instead, all proteins with 10 identical positions per 30-residue segment were retrieved.

Results

Amino Acid Sequence Analysis. The proposed sequence of gonococcal pilin prepared from strain MS11 is shown in Fig. 1. A unique sequence was derived consisting of 159 amino acids in a single polypeptide chain. A minimal molecular weight of 17,497 has been calculated from the amino acid composition derived from the sequence data (Table I) and is in close agreement with the molecular weight of 17,500 established by SDS-PAGE (8).

The strategy used for the sequence analysis of gonococcal pilin entailed the selection of chemical and enzymatic cleavages resulting in large overlapping peptides. The isolated cyanogen bromide (CNBr) peptides used to determine the structure are indicated in Fig. 2. All peptides are numbered sequentially from the NH$_2$ terminus of either the intact protein or the parent peptide.

The amino acid sequences of uncleaved pilin prepared from gonococcal strains MS11 and R10 were determined through residues 59 and 57, respectively, by sequential degradation of 2 mg of protein. Identical sequences were found and these were homologous with the amino-terminal amino acid sequences of pilin from one meningococcal strain and four gonococcal strains determined by

MePhe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Ile-Ala-Ile-Gly-Ile-Leu-Ala-Ala-Val-Ala-20
Leu-Pro-Ala-Tyr-Gln-Asp-Tyr-Ala-Arg-Ala-Gin-Val-Ser-Glu-Ala-Ile-Leu-Leu-Ala-40
Glu-Gly-Gln-Lys-Ser-Ala-Val-Thr-Glu-Tyr-Leu-Leu-Asn-His-Gly-Lys-Trp-Pro-Glu-Aan-60
Aan-Thr-Ser-Ala-Gly-Val-Ala-Ser-Pro-Pro-Ser-Asp-Ile-Lys-Gly-Lys-Tyr-Val-Lys-Glu-80
Val-Glu-Val-Lys-Aan-Gly-Val-Val-Thr-Ala-Thr-Met-Leu-Ser-Ser-Gly-Val-Aan-Aan-Glu-100
Ile-Lys-Gly-Lys-Lys-Leu-Ser-Leu-Trp-Ala-Arg-Arg-Glu-Aan-Gly-Ser-Val-Lys-Trp-Phe-120

\[\text{Cycle: Gly - Gin - Pro - Val - Thr - Arg - Thr - Asp - Asp - Thr - Val - Ala - Asp - Ala - Lys - Asp - Gly - Lys} \quad 140\]

\[\text{Glu - Ile - Asp - Thr - Lys - His - Leu - Pro - Ser - Thr (Cycle) Arg - Asp - Lys - Ala - Ser - Asp - Ala - Lys (COOH)}\]

\text{FIGURE 1. Covalent structure of P+Tr MS11 gonococcal pilin. MePhe, N-methylphenylalanine.}
### Table 1

*Amino Acid Composition of Gonococcal Pilin and Pilus Peptides*

| Amino acid | MS11 Pilin Seq† | Ana‡ | CNBr-2 Seq | Ana§ | CNBr-3 Seq | Ana| TC-2 Seq | Ana|$  |
|------------|-----------------|------|-----------|------|-----------|-----|---------|-----|
| Lysine     | 15              | 15.4 | 6         | 6.7  | 9         | 8.5 | 9       | 9.2 |
| Histidine  | 2               | 2.4  | 1         | 1.1  | 1         | 1.1 | 0       | ND  |
| Arginine   | 5               | 5.3  | 1         | 1.7  | 4         | 3.4 | 1       | 0.8 |
| Aspartic (Ass) | 10          | 16.6 | 2         | 7.6  | 8         | 10.3| 1       | 7.4 |
| Asparagine | 7               |      |           |      | 3         |     | 6       |     |
| Threonine  | 11              | 10.4 | 5         | 4.5  | 5         | 4.9 | 4       | 3.5 |
| Serine     | 11              | 10.2 | 5         | 3.9  | 6         | 5.0 | 8       | 7.7 |
| Glutamic (Glx) | 10           | 15.1 | 6         | 10.0 | 3        | 4.6 | 7       | 9.2 |
| Glutamine  | 4               |      | 1         | 1.7  | 2         | 2.7 | 3       | 3.1 |
| Proline    | 6               | 7.0  | 4         | 2.9  | 2         | 2.7 | 3       | 3.1 |
| Glycine    | 11              | 11.5 | 6         | 6.4  | 5         | 5.1 | 7       | 7.3 |
| Alanine    | 18              | 18.2 | 13        | 12.4 | 5        | 6.0 | 8       | 8.0 |
| ½ Cystine¹ | 2               | 1.7  | 0         | ND*  | 2         | 1.8 | 0       | ND  |
| Valine     | 15              | 14.5 | 11        | 9.7  | 4        | 4.9 | 9       | 8.8 |
| Methionine³ | 2              | 2.0  | 1         | 1.1  | 0        | ND | 1       | 0.8 |
| Isoleucine | 9               | 7.9  | 6         | 5.4  | 2        | 2.2 | 3       | 2.9 |
| Leucine    | 11              | 12.3 | 5         | 6.1  | 4        | 4.0 | 6       | 5.9 |
| Tyrosine   | 5               | 5.4  | 5         | 4.5  | 0        | ND | 3       | 3.2 |
| Phenylalanine | 1          | 1.2  | 0         | ND  | 1        | 0.9 | 0       | ND  |
| Tryptophan | 3               | 2.9  | 1         | 1.1  | 2        | 1.8 | 2       | 2.3 |
| MePhe      | 1               |      |           |      | 0        |     | 0       |     |
| Total residues | 159          | 160.1 | 85       | 85.1 | 67      | 67.2| 81      | 80.1|

*Residues per subunit or peptide.
† Number of residues derived from the sequence shown in Fig. 1.
‡ Based on mol wts of 17,497 (pilin); 8,973 (CNBr-2); 7,676 (CNBr-3); and 8,517 (TC-2).
¹ Cysteine was analyzed as cysteic acid and methionine as methionine sulfone (pilin and TC-2) or as homoserine plus homoserine lactone (peptides CNBr-2 and CNBr-3; 25, 26). ND, no amino acid was detected.

Hermodson et al. (37) through 29 residues. High pressure liquid chromatography (HPLC) of the first phenylthiohydantoin (Pth) amino acid co-migrated with synthetic Pth-N-methylphenylalanine. N-methyl-phenylalanine also has been proposed as the amino-terminus of pilin from *Pseudomonas aeruginosa* strain PAK (34), *Moraxella nonliquefaciens* (38), and *Moraxella bovis* (G. Schoolnik, unpublished observation).

**Isolation and Alignment of Cyanogen Bromide Peptides.** Amino acid analysis of MS11 gonococcal pilin after performic acid oxidation indicated the presence of two methionine residues per subunit. Cyanogen bromide cleavage resulted in three fragments (Fig. 2) that were separated by reverse-phase HPLC (Fig. 3). Peptides were eluted in an ascending gradient of acetonitrile in water containing 0.05% (vol/vol) TFA and identified by amino acid analysis (Table I). CNBr-3 and CNBr-1 eluted as sharp peaks in 32% and 42% acetonitrile, respectively. The hydrophobic character of CNBr-2 was manifested by its elution as a broad peak between 52% and 55% acetonitrile (39). Alternatively, cyanogen bromide fragments could be partially purified by molecular sieve chromatography in aqueous buffers. CNBr-2 and uncleaved pilin eluted together in the void volume of a Sepharose 4B column equilibrated in 0.1 M NH₄HCO₃, pH 8.1. Since the...
exclusion limit of this gel for globular proteins is \( \sim 20 \times 10^6 \) daltons, CNBr-2 must exist as a macromolecular aggregate in water. In contrast, CNBr-3 eluted in the included volume with a \( K_w \) of 0.9.

Cyanogen bromide fragments were subjected to automated Edman degradation. CNBr-1 was sequenced in its entirety; the sequence of CNBr-2 was determined through residue 49 and was identical to the result from amino-terminal amino acid sequencing (Fig. 2). CNBr-3 peptides from pili purified from strains MS11 and R10 were sequenced through residues 143 and 138, respectively. When the first 46 residues of both CNBr-3 sequences were aligned as indicated in Fig. 4, 31 positions (67\%) were found to be homologous. 8 of the 15 nonidentical positions are clustered between residues 128 and 138. The amino acid sequence heterogeneity in this region is characterized by significant differences in the position of charged residues and may therefore be responsible for the antigenic diversity of the two pilus proteins from which these fragments were prepared (vide infra).

CNBr-1 and CNBr-2 were aligned by reference to the amino-terminal amino acid sequence.
FIGURE 3. Separation of cyanogen bromide fragments by reverse-phase HPLC. The cyanogen bromide digest (19) of MS11 gonococcal pilin was dissolved in 0.05% (vol/vol) TFA in water and applied to a Whatman Partisil-10 ODS-2 column equilibrated in the same solvent. The peptides were eluted in an ascending linear gradient of acetonitrile in water containing 0.05% TFA at a flow rate of 1.0 ml/min. Fractions (1.0 ml) were pooled according to their absorbance at 210 nm and concentrated in vacuo by rotary evaporation.

FIGURE 4. Amino terminal 46-residue sequences of P+Tr MS11 and R10 CNBr-3 pilus fragments. Residues in parentheses were identified by reverse-phase HPLC of the Pth derivative, only. Identical residues are boxed.

acid sequence (Fig. 2). CNBr-3 lacked homoserine (Table I) and was therefore placed in the carboxy-terminal portion of the molecule.

Trypsin Cleavage of Citraconylated Pilin. Amino acid analysis of MS11 gonococcal pilin indicated the presence of five arginine residues per subunit (Table I). Tryptic cleavage of reduced, carboxymethylated, and citraconylated pilin should result in six fragments (Fig. 2). TC-1 (residues 1–30) was insoluble and could be separated from the other fragments by centrifugation. No attempt was made to recover TC-3, a single arginine residue proven to be at position 112 by amino-terminal amino acid sequencing of CNBr-3 (Fig. 2). TC-2 (residues 31–111), TC-4 (residues 113–127), TC-5 (residues 128–152), and TC-6 (residues 153–159) were purified by sequential high pressure liquid, steric-exclusion chromatography performed in two steps. First, the citraconylated fragments were eluted in 0.1 M NH₄HCO₃, pH 8.1 from one TSK G3000SW and two TSK G2000SW
columns connected in series. Fractions under the resulting peaks were pooled, brought to pH 4.0 by the addition of formic acid, and the de-blocked fragments eluted in 0.1 M ammonium formate, pH 4.0 from the same columns. TC-2, a receptor binding peptide (vide infra), could be prepared in greater yield by performing the above operations on unreduced pilin. TC-4 and TC-5, joined by an intramolecular disulfide bond, then elute together as a single peak (Fig. 5).

TC-1 was identified by amino acid analysis, but was not independently sequenced since its structure had been confirmed by amino-terminal amino acid sequencing of uncleaved pilin and of CNBr-1 and CNBr-2 (Fig. 2). TC-2 was sequenced through residue 105 (74 steps) with one blank (residue 94). This provided a 13-residue overlap of Met92 (Fig. 2) and confirmed the alignment of the cyanogen bromide fragments. TC-4 and TC-5 were sequenced in their entirety and aligned by reference to the amino-terminal amino acid sequence of CNBr-3. The complete sequence of TC-6 was also determined; it was placed in the carboxy-terminal portion of the molecule by reference to the amino acids released by digestion of uncleaved pilin with carboxypeptidase Y (Fig. 2).

Iodosobenzoic Acid Peptides of CNBr-3. The alignment of peptides prepared by chemical and enzymatic cleavage of gonococcal pilin was deduced from overlaps of more than four residues with the exception of TC-5 and TC-6. Cleavage of CNBr-3 at tryptophan residues 109 and 119 was performed with ω-iodosobenzoic acid. CNBrW-3 (residues 120–159) was purified by high-pressure liquid steric-exclusion chromatography and sequenced through residue 154. This established a two-residue overlap for TC-5 and TC-6 (Fig. 2) and provided further evidence for the proposed alignment of these peptides.

Half-Cystine Residues. MS11 and R10 pilin contain a pair of half-cystine

![Figure 5](image-url)

**Figure 5.** Purification of TC-2. (A) Unreduced pili were citraconylated (20), digested with trypsin, and the TC fragments eluted in 0.1 M NH₄HCO₃, pH 8.1 from one TSK G3000SW and two TSK G2000SW high pressure liquid, size-exclusion 7.5-mm × 50-cm columns connected in series. Fractions under the bifid peak containing TC-2 and TC-4 plus TC-5 were pooled. (B) They were deblocked by the addition of formic acid to pH 4.0 and eluted from the same columns in 0.1 M ammonium formate, pH 4.0.
residues and both are encompassed by CNBr-3 and its carboxy-terminal trypto-
phenyl fragment, W-3. No sulfhydryl group was detected by titration of un-
cleaved pilin with 5,5'-dithiobis (2-nitrobenzoic acid) or by alkylation with
iodoacetic acid in 6 M guanidine-HCl. Therefore, the two half-cystine residues
exist in a disulfide bond.

Prosthetic Groups of Gonococcal Pilin. Phosphorus analysis of purified pili re-
vealed the presence of two phosphate residues per pilin subunit. To determine
whether phosphate was covalently bound to an amino acid, pilin was enzymati-
cally hydrolyzed by a combination of trypsin, pronase, and aminopeptidase M.
The hydrolysate was eluted from an ion exchange column to eliminate amino
acids that would otherwise interfere with phosphoamino acid analysis. Phospho-
serine was identified and confirmed by the regeneration of serine after acid
hydrolysis. MS11 gonococcal pilin therefore contains phosphate covalently bound
to serine residues.

~1.5 mol of galactose and 0.2 mol of glucose per pilin subunit were detected
by gas-liquid chromatography when pili were purified with aqueous solvents.
Lipid analysis indicated that the glucose content could not be attributed to
lipopolysaccharide contamination. However, after extraction in chloroform/
methanol 2:1, no covalently bound carbohydrate was detected by phenolsulfuric
acid. Sequence analysis also failed to indicate the presence of amino acid glyco-
sides. It is concluded therefore that gonococcal pilin is not a glycoprotein.

Conformational Analysis of Gonococcal Pilin. The predicted secondary structure
of MS11 gonococcal pilin was deduced by the method of Chou and Fasman (33).
Since this algorithm was derived from the tertiary structures of 29 globular
proteins, it may not be applicable to pili and the results must be cautiously
interpreted. Residues 3–12, 17–20, 30–41, 79–84, 106–112, 133–136, 141–
143, and 154–158 exhibit high alpha-helical potential (Fig. 6). Residues 8–20
exhibit beta-sheet potential, a segment that also encompasses two stretches of
alpha-helical potential (Fig. 6). Since both the alpha-helical and beta-sheet poten-
tials for this segment are high, the secondary structural predictions for this region
are ambiguous. This may indicate that the amino-terminal portion of the mole-
cule is unstable (40) and that its conformation is governed by environmental
conditions: alpha-helical in polymeric structure (vide infra) and beta-sheet during
synthesis or assembly when pilin may be membrane-bound (41). Residues 87–
93, 118–122, and 125–127 also exhibit beta-sheet potential (Fig. 6). A beta-turn
composed of residues 24–27 is predicted to link two helices and a series of turns
is predicted between residues 54 and 72.

Three segments of high beta-turn potential ($P_t \geq 1.5 \times 10^{-4}$) are predicted to
reside within the disulfide loop subtended by cysteines at positions 121 and 151.
These are composed of the tetrapeptides 129–132, 136–139, and 147–150 and
are predicted to flank two alpha-helices.

Hydrophilicity Analysis of Gonococcal Pilin. The local average hydrophilicity
along the polypeptide chain was determined as a moving average of hexapeptides
using the solvent parameter values of Levitt (32). The amino-terminal 27 residues
are distinctly hydrophobic and have been proposed to mediate subunit-subunit
interactions (vide infra). Although several regions of moderate average local
hydrophilicity exist in the first half of the molecule, CNBr-3 encompasses five
Figure 6. Predictive analysis of the secondary structure (33) and local average hydrophilicity (31, 32) of MS11 gonococcal pilin. The beta-turn, beta-sheet, and alpha-helix assignments are denoted by shaded areas and indicate a $P_t \geq 1.0 \times 10^{-4}$, $P_\beta \geq 1.0$, and $P_\alpha \geq 1.0$, respectively. The hydrophilicity values are derived from the hexapeptide averages along the length of the sequence plotted at the midpoint of the averaged group of residues. Hydrophilic regions appear as positive peaks above 0.
distinct stretches with hydrophilicity values $\geq 1.0$ (Fig. 6). Two (residues 127–133 and 137–145) reside within the intramolecular disulfide loop, coincide with segments of high beta-turn probability, and involve regions of amino acid sequence heterogeneity (Fig. 4) between serologically distinct gonococcal pili. The remaining hydrophilic segments encompass residues 100–106, 110–116, and 152–159. Both cysteines lie in hydrophobic troughs, a location that might favor their spatial juxtaposition and thereby the formation of the disulfide bond.

**Identification of a Receptor-binding Tryptic Peptide.** CNBr-2 was proposed by Schoolnik et al. (7) to contain functionally critical amino acids. The topography of the receptor binding domain was examined in this study with TC-2 (residues 31–111) a soluble, monomeric tryptic peptide that contains the last 62 amino acids of CNBr-2 (Fig. 2). TC-2 was labeled with $^{125}$I and incubated with endocervical, buccal mucosal, or HeLa cells.

After 60 min at 37°C, cell-bound and -free peptide were separated by centrifugation (Fig. 7). The relationship between counts added and counts bound was linear and saturable for endocervical cells; at 50% saturation, 88% of the added counts were cell associated. TC-2 did not bind HeLa or buccal mucosa cells (Fig. 7).

**Homology Studies.** Amino-terminal amino acid sequences of pili from *N. gonorrhoeae*, *N. meningitidis* (37), *M. nonliquefaciens* (38), and *P. aeruginosa* strain PAK (34) are highly homologous through residue 29 (vide supra). Amino acid substitutions in this stretch occur at only seven positions for pili of these genera, and four of these are conservative changes. The replacements include substitutions of valine for isoleucine (positions 10, 13, and 19), isoleucine for leucine (position 21), glutamine for alanine (position 23), asparagine for aspartic acid (position 26), and isoleucine or valine for threonine (position 28).

The entire PAK *P. aeruginosa* pilin sequence has been determined by Sastry et al. (34) and was compared with the MS11 gonococcal pilin sequence (Fig. 8). The number of amino acids per subunit is similar and both contain a second methionine residue approximately two-thirds from the amino-terminus and two cysteines in the third cyanogen bromide fragment. The carboxy-terminus for both is lysine. However, when the two structures were analyzed by a program
FIGURE 8. Comparison of the P+Tr MS11 gonococcal and the PAK pseudomonad (34) pilin sequences. All possible four-residue segments from one sequence were compared with all four-residue segments from the other sequence. A square is plotted when three or four residues of both segments are the same.

that compared all possible four-residue segments from the gonococcal sequence with all segments of the same length from the pseudomonad sequence, little homology was apparent after position 30 (Fig. 8).

The complete amino acid sequences of two E. coli pilus proteins also have been published (42). The K88 and CFA1 antigens mediate the adherence of enteropathogenic strains to porcine and human intestinal epithelia, respectively. Neither structure was found to be homologous with the gonococcal or pseudomonad sequence.

The similarity of the MS11 gonococcal pilin sequence to each of the protein sequences stored at the National Biomedical Research Foundation was determined. Table II lists the proteins in the data collection with 10 or more homologous positions per 30-residue segment. Five such proteins were identified for residues 1–30 including a group of five actin proteins, two proteins for residues 31–60, seven for residues 61–90, one for residues 91–150, and three for residues 130–159. Indian cobra nerve growth factor was selected for two segments (61–90 and 130–159). 20 positions are homologous between residues 18 and 87 of this protein and residues 61 and 159 of gonococcal pilin.

Discussion

The amino acid sequence of MS11 gonococcal pilin has been determined by automated Edman degradation of the intact protein and cyanogen bromide, citraconylated tryptic and iodosobenzoic acid peptides. Further, the existence of
TABLE II

Proteins with 10 or More Positions Per 30-Residue Segment Identical to the MS11 Gonococcal Pilin Sequence*

| Gonococcal segment | Corresponding segment |
|--------------------|-----------------------|
| 1–30               | Collagenolytic protease (fiddler crab) 73–102 |
| ATPas, protein 6 (human mitochondrion) | 188–217 |
| Hemoglobin beta major chain (mouse) | 103–132 |
| 50S ribosomal proteins L7/L12 (E. coli) | 20–59 |
| Tetracycline resistance protein (E. coli plasmid pBr322) | 97–126 |
| Actins            | Cytoplasmic beta, gamma (rat, mouse, bovine, human) 201–230 |
|                  | Sea urchin (S. purpuratus) 202–231 |
|                  | P. polypephalum, D. dissoideum, A. castellanii 201–230 |
|                  | Yeast (S. cerevisiae, S. carlsbergensis) 201–230 |
|                  | Soybean 202–231 |
| 31–60             | Anthranilate synthase, component II (N. crassa) 196–225 |
| Calcium-binding protein, intestinal (pig) | 2–31 |
| 61–90             | Amidophosphoribosyltransferase (E. coli) 303–332 |
| Subtilisin (B. amyloliquefaciens) | 123–152 |
|                   | Phosphoribosyl-AMP cyclohydrolase (bakers yeast) 507–536 |
|                   | Epidermal growth factor precursor (mouse) 1092–1121 |
|                   | Nerve growth factor (Indian cobra) 18–47 |
|                   | Internal virion protein D (bacteriophage T7) 649–678 |
|                   | Hypothetical protein 6 (human mitochondrion) 14–43 |
| 91–120            | None |
| 121–150           | Nonstructural protein NS1 (influenza B virus) 218–247 |
| 130–159           | Triosephosphate isomerase (bakers yeast) 175–204 |
|                   | Nerve growth factor (Indian cobra) 58–87 |
|                   | Colicin E1 protein (E. coli plasmid) 446–475 |

* 30-residue segments of the MS11 gonococcal pilin sequence were compared with all 30-residue segments of each of the 2222 protein sequences stored at the National Biomedical Research Foundation by the computer program MATCH (35). All proteins with 10 identical positions per 30-residue segment are denoted above.

two cysteine residues in disulfide linkage and serine-bonded phosphate residues, was demonstrated.

Sequence analysis was undertaken to determine the molecular basis for the functional and serologic attributes of gonococcal pili. Partially sequenced cyanogen bromide fragments were used by Schoolnik et al. (7) to test the hypothesis that receptor binding function and antigenic diversity are specified by separate domains. CNBr-2 and CNBr-3 (Fig. 2) were prepared from serologically distinct gonococcal pili. CNBr-2 was found to be highly conserved, immunorecessive, and to encompass the human erythrocyte binding domain. CNBr-3 was functionally inert but conferred type-specific antigenicity (vide infra).

The Receptor Binding Domain. Receptor-binding peptides were sought in the present study by arginine-specific tryptic cleavage of gonococcal pilin. TC-2 residue (31–111, Fig. 2), a relatively hydrophilic polypeptide that exists as a monomer in aqueous solvents, exhibited saturable binding of human endocervical, but not buccal epithelial or HeLa cells. The 62-amino acid segment (residues 31–92) common to TC-2 and CNBr-2 appears to encompass functionally critical
amino acids. These are presumed to be complementary to the pilus receptor, a cell surface glycoconjugate that has been partially characterized by its sensitivity to glycosidases (43) and by its release from plasma membranes by methods used to extract glycosphingolipids (G. Schoolnik, unpublished observation). The secondary structure and local average hydrophilicity predicted from the primary structure of this segment (Fig. 6) fail to disclose any unique feature that might be correlated with receptor binding function. Indeed, the structural basis for the sugar-binding capacity of lectin-like proteins is poorly understood and no algorithm has been advanced for this purpose. In the few examples where critical residues have been identified (44–46), two principles have emerged. First, the binding site is a hydrophobic cleft in the surface of the molecule (47). Second, critical residues are discontinuous in sequence but juxtaposed in space (44–46).

Smaller receptor binding segments may be identified by using smaller peptides, antibodies to synthetic peptides, receptor blocking monoclonal antibodies, and mutant pilus proteins. However, the liganded molecule's three-dimensional structure must be solved before critical residues can be unequivocally defined.

The differential binding of pill to endocervical and buccal epithelial cell surfaces (Fig. 7) indicates that the pilus receptor is likely to be tissue specific. It follows that receptor density and distribution may underlie the tissue tropism of gonococcal infections. These results are not consonant with Pearce and Buchanan's (3) finding that whole, iodinated pill bind buccal and endocervical cells equally well. This discrepancy might be explained by the observation of Lambert et al. (29) that some gonococcal pill appear to bind different receptor compounds. Alternatively, binding studies conducted with undenatured pill may lack specificity because the filaments readily associate with hydrophobic surfaces including glass, plastic, latex beads (48), and synthetic lipid bilayers (G. Schoolnik, unpublished observation). Although these interactions are not chemically specific they may be pertinent to the binding event. Hydrophobic bonding may serve to optimally position pilus filaments on cell surfaces, thereby facilitating receptor-mediated binding. The relative contributions of hydrophobic and receptor-specific binding to the pathogenic role of pill is currently under investigation.

**Type-specific Antigenicity.** Antigenic heterogeneity is characteristic of pill prepared from different gonococcal strains (10, 11) and even colonial variants of the same strain (9). The structural basis for this phenomenon was examined by Schoolnik et al. (7) with pill from gonococcal strains MS11 and R10, which bind the same erythrocyte receptor but exhibit <5% shared antigenicity. Antibody to their respective CNBr-3 fragments bound only the homologous pilus antigen (7). Further, type-specific pilus antibodies could be absorbed by the homologous CNBr-3 fragment (7). CNBr-3 therefore encompasses type-specific antigenic determinants responsible for serologic diversity.

The CNBr-3 R10 and MS11 sequences (Figs. 1 and 4) were scrutinized for regions that might constitute type-specific epitopes. These were predicted by three criteria: (a) high local average hydrophilicity (Fig. 6); (b) a probable beta-turn conformation (Fig. 6); and (c) amino acid sequence heterogeneity. Five regions between residues 93 and 138, each composed of four residues, were identified according to their Chou-Fasman (33) beta-turn probabilities ($P \approx 0.80 \times 10^{-4}$): 94–97, 101–104, 113–116, 129–132, and 136–139. All but one
(residues 94–97), are predicted to be hydrophilic; the last four entail amino acid substitutions that change the positions of charged residues. Remarkably, the amino acid replacements altered the predicted secondary structure at only one site (residues 136–139). This analysis suggests that the antigenic diversity of gonococcal pili involves surface projections of the molecule and entails amino acid substitutions that retain general features of the secondary structure but alter charge. The validity of this hypothesis has been confirmed by Rothbard et al. (submitted for publication) with synthetic peptide analogues of the predicted epitopes.

**Homology Studies. Implications for Polymeric Structure.** The N-terminal amino acid sequences of pili from the genera *Neisseria*, *Moraxella*, and *Pseudomonas* are highly homologous, indicating that these proteins may be derived from a common ancestral gene. Once established within a species, independent evolution of the gene might occur at rates that are a function of the mutation rate and constraints imposed by the structural and functional prerequisites of the organelle (49). Thus, N-terminal sequence homology might indicate that members of this pilus family recently diverged. However, comparison of the complete pseudomonad and gonococcal pilus sequences disclosed very little homology beyond residue 30 (Fig. 8). Therefore, it seems more likely that little variation is tolerable in this segment because it is functionally or structurally critical. Indeed, Watts et al. (50) studied the ionized state of tyrosine residues (positions 24 and 27, Fig. 1) during pH titrations of pilus filaments and after their dissociation by octylglucoside into monomers and dimers and concluded that this region of the pseudomonad sequence comprises a hydrophobic domain involved in subunit-subunit interactions. Their findings are supported by the observation that TC-1 (residues 1–30 Fig. 2) and CNBr-2 (residues 8–92) exist as supramolecular aggregates in aqueous solvents, whereas arginine-specific cleavage at residue 30 releases the remaining TC fragments as soluble, monomeric peptides.

This region of the gonococcal sequence and the homologous actin sequences (Table II) were examined in greater detail because aspects of the quaternary structure of this protein family and pili are similar: both exist as filamentous structures of approximately the same diameter and both are composed of identical subunits that polymerize to form helical strands (49). The 20-amino acid stretch of the actin sequence (residues 202–221) for the lower eucaryotic species *Dictyostelium discoideum*, *Physarum polycephalum*, and *Acanthamoeba castellanii* (51), most homologous to residues 2–21 of the gonococcal pilin sequence, is indicated in Fig. 9 and differs from rabbit skeletal muscle actin and beta and gamma cytoplasmic actins only by the substitution of cysteine for alanine at position 217 of the actin sequence. 10 of the 20 residues are identical. The secondary structure for this stretch of the gonococcal sequence is predicted by the Chou-Fasman algorithm to be either beta-sheet or alpha-helix (Fig. 6). Assuming an alpha-helical configuration with 3.6 residues per turn, this segment of each sequence was modeled according to the two-dimensional "helical-wheel" method of Schiffer and Edmundson (52) in which the amino acid side chains are separated by 100° of arc. Remarkably, the homologous residues are identically arranged and 8 of the 10 positions are clustered on the same side of the wheel (Fig. 9). Further, six of the eight clustered residues are hydrophobic, thus creating
FIGURE 9. Comparison of the gonococcal pilin and lower eucaryotic actin sequences. Residues 202-221 of the D. discoideum, P. polycephalum, and A. castellanii (51) actin sequences and residues 2-21 of the gonococcal sequence are indicated as a linear array and according to the two-dimensional "helical-wheel" method of Schiffer and Edmundson (52). Identical amino acids are enclosed.

A homologous nonpolar surface along one side of the helix. This topography might favor the interaction of this segment of one subunit with the identical segment of a second subunit of an opposing strand, thereby forming a thermodynamically stable double helical structure. These observations are not intended to suggest that gonococcal pili are evolutionarily related to actin filaments. Instead, a common physical feature (polymeric structure) of these proteins may have led to related sequences.

Summary

The complete amino acid sequence of pilin from gonococcal strain MS11 and the sequence of constant and variable regions from strain R10 pilin have been determined in order to elucidate the structural basis for adherence function, antigenic diversity, and polymeric structure. The MS11 pilin sequence consists of 159 amino acids in a single polypeptide chain with two cysteines in disulfide linkage and serine-bonded phosphate residues. TC-2 (31–111), a soluble monomeric pilus peptide prepared by arginine-specific digestion, bound human endocervical, but not buccal or HeLa cells and therefore is postulated to encompass the receptor binding domain. Variable regions of CNBr-3 appear to confer antigenic diversity and comprise segments in which changes in the position of charged residues occur in hydrophilic, beta-turns. Residues 2–21 and 202–221 of gonococcal pilins and lower eucaryotic actins, respectively, exhibit 50% homology. When these residues are arranged at intervals of 100 degrees of arc
on "helical wheels," the identical amino acids comprise a hydrophobic face on one side of the helix. This observation, the hydrophobic character of this region and the tendency for TC-1 (residues 1–30) to aggregate in water, suggest that this stretch interacts with other subunits to stabilize polymeric structure.

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