Biochemical Purification and Crystallographic Characterization of the Fiber-forming Protein Pilin from Neisseria gonorrhoeae*

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Pilus fibers are long protein filaments on many pathogenic bacteria that participate in attachment to host cells. Although the self-assembling protein pilin is the major structural component of the Neisseria gonorrhoeae pilus fiber, several other proteins co-purified with pilin through the repeated solubilization-reassociation steps of the biochemical purification. Pilin solubilized in the non-denaturing detergent n-octyl-β-D-glucopyranoside remained an aggregate of about 100 kDa at pH 9.5, but was reduced to a 40-kDa dimer at pH 10.5, suggesting that assembly involves electrostatic interactions of lysine, tyrosine, or other side chains with high pK₀ values. Pilin dimers and aggregates of higher molecular mass were partially stable even in the presence of sodium dodecyl sulfate and β-mercaptoethanol. Removal of pilus-associated proteins and stabilization of pilin multimers permitted the reproducible crystallization of pilin. Three-dimensional needle- and plate-shaped crystals of purified N. gonorrhoeae pilin (strain MS11 variant C30) grew from 36 to 40% polyethylene glycol 400, pH 8.0–9.0, in space group C222₁, with cell dimensions a = 126.4, b = 121.2, c = 26.7 Å and V = 2.84 Å³/dalton for one molecule per asymmetric unit. The best crystals diffracted to 2.4 Å resolution using synchrotron radiation, were stable to x-ray damage, and appear suitable for determination of the atomic structure. This approach of stabilizing and crystallizing an intermediate assembly state may be useful for other fiber-forming proteins, which have previously not been successfully crystallized in forms that diffract to atomic resolution.

Neisseria gonorrhoeae is a Gram-negative bacterium that causes the sexually transmitted disease, gonorrhea. For the gonococcus, as for many other infectious agents, the first step in infection is attachment to host cells. Although the self-assembling protein pilin is the major structural component of the Neisseria gonorrhoeae pilus fiber, several other proteins co-purified with pilin through the repeated solubilization-reassociation steps of the biochemical purification. Pilin solubilized in the non-denaturing detergent n-octyl-β-D-glucopyranoside remained an aggregate of about 100 kDa at pH 9.5, but was reduced to a 40-kDa dimer at pH 10.5, suggesting that assembly involves electrostatic interactions of lysine, tyrosine, or other side chains with high pK₀ values. Pilin dimers and aggregates of higher molecular mass were partially stable even in the presence of sodium dodecyl sulfate and β-mercaptoethanol. Removal of pilus-associated proteins and stabilization of pilin multimers permitted the reproducible crystallization of pilin. Three-dimensional needle- and plate-shaped crystals of purified N. gonorrhoeae pilin (strain MS11 variant C30) grew from 36 to 40% polyethylene glycol 400, pH 8.0–9.0, in space group C222₁, with cell dimensions a = 126.4, b = 121.2, c = 26.7 Å and V = 2.84 Å³/dalton for one molecule per asymmetric unit. The best crystals diffracted to 2.4 Å resolution using synchrotron radiation, were stable to x-ray damage, and appear suitable for determination of the atomic structure. This approach of stabilizing and crystallizing an intermediate assembly state may be useful for other fiber-forming proteins, which have previously not been successfully crystallized in forms that diffract to atomic resolution.

*This work was supported by the National Institutes of Health Grants AI-22160 (to J. A. T. and E. D. G.) and AI-20845 (to M. S.). Diffraction data collection was done at Stanford Synchrotron Radiation Laboratory, which is funded by the Department of Energy under Contract DE-AC03-89ER-13000, Office of Basic Energy Sciences, and the National Institutes of Health Biotechnology Resource Program Division of Research Resources. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

1 The abbreviations used are: SDS-PAGE, polyacylamide gel electrophoresis in the presence of sodium dodecyl sulfate; BOG, n-octyl-β-D-glucopyranoside; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(N-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; PEG, polyethylene glycol.
applied to studies of structure-function relationships for a fiber-forming protein. The bacterial pilus, constructed primarily of a single protein, self-assembles, and is implicated in virulence, epithelial cell binding, and transformation. The pilin sequence changes that generate the antigenic diversity of gonococcal pili (Hagblom et al., 1985; Swanson et al., 1987) must fit the constraints imposed by both structure and function; gonococci with nonassembled or disfunctional pilin will be avirulent and thus selected against (Tramont, 1989). From the standpoint of molecular biology, the gonococcal pilus presents intriguing questions relevant to understanding the structural basis for molecular assembly and recognition.

In order to study the structural biochemistry of the pilus, we have undertaken the purification and x-ray diffraction structure determination of the pilin protein. Here we report methods for large scale isolation and purification of gonococcal pilin, distinguish a set of pilus-associated proteins from monopolar anti-pilin and polyclonal anti-peptide antibody binding, isoelectric points, and dissociation characteristics. Using purified protein prepared by these methods, we have obtained crystals of N. gonorrhoeae pilin suitable for the determination of the three-dimensional atomic structure by x-ray diffraction and report here the growth conditions, space group determination, and diffraction characteristics of these crystals.

**EXPERIMENTAL PROCEDURES**

**Bacterial Growth and Pilus Preparation**—Initially, pili were isolated and partially purified from N. gonorrhoeae (strain MS11 variant C50; Segal et al., 1985) essentially as previously described (Brinton et al., 1978), but with minor modifications given below. Bacteria, frozen in 20% glycerol with Trypticase Soy Broth (BBL Microbiology Systems), were revived by plating onto solid media (GC Medium base and agar Noble from Difco) supplemented with 22.2 mm glucose, 0.7 mm L-glutamine, 0.6 mm thiamine, and 12.4 mm ferric nitrate. Cells were grown in 100-mm disposable Petri plates incubated at 37°C under 5% CO, for 18-22 h, and then centrifuged at 10,000 g for 20 min. The resultant protein pellet was suspended in one of two buffer solutions (0.5 g of BOG, 1.8 ml of distilled water, added to the focusing solution (0.5 g of BOG, 1.8 ml of Pharmalyte pH 3-10, 1.5 mm of Pharmalyte pH 4-6.5, distilled water to 50 ml, and 1 ml in DTT) and focused at 12 watts, 4°C. Peak fractions were collected, dialyzed into 50 mM CAPS, pH 10.5, 0.02% NaN, 1 mM DTT, and made 1% in BOG.

For the FPLC chromatofocusing procedure, partially purified pili were dialyzed overnight against 25 mM histidine (pH 7.1) in distilled water, added to the focusing solution (0.5 g of BOG, 1.8 ml of Pharmalyte pH 3-10, 1.5 mm of Pharmalyte pH 4-6.5, distilled water to 50 ml, and 1 ml in DTT) and focused at 12 watts, 4°C. Peak fractions were collected, dialyzed into 50 mM CAPS, pH 10.5, 0.02% NaN, 1 mM DTT, and made 1% in BOG.

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**Gel Filtration**—Final purification and characterization of the assembly state of solubilized pilin were achieved following size-exclusion chromatography. First, pilin fractions from chromatofocusing were run over a G-25 Sephadex column to change the buffer to 50 mM CAPS, pH 10.5, 0.02% NaN, 1 mM DTT, and made 1% in BOG. Peak fractions from the G-25 column were run in 1.0-5.0 ml samples at 20 ml/h on a Sephacryl S-200 gel filtration column (1 x 100 cm, total bed volume 180 ml). Optical density at 280 nm was monitored, and apparent molecular weights of proteins were estimated from Bio-Rad molecular weight standards for gel filtration. In addition to the major pilin-containing peak, a small peak was observed at the void volume, as well as a broad peak at low molecular weight containing the amphotolites. Fractions were checked for protein content with SDS-PAGE (Laemmli, 1970) using silver-stained (Tsai and Frasch, 1982) gels containing 12 or 15% polyacrylamide. Before crystallization, fractions containing the pilin peak were buffer-exchanged by gel filtration on a G-25 Sephadex column to yield purified pilin in 100 mM Tris, pH 8.0, 20 mM NaCl, 1 mM DTT, 0.02% NaN, and 1% (or 1.5%) in BOG. All antibodies were coupled as described previously (Brinton et al., 1978) to the immunogenic carrier protein keyhole limpet hemocyanin. Antibodies were immobilized in a course of three injections of 100 μg of coupled peptide (or about 100 μg of pilin) each. The coupled peptide was injected subcutaneously on day 0, in complete Freund's adjuvant and on days 11 and 14 (or 15) with incomplete Freund's adjuvant. The rabbits were bleed 10 days after the third injection. The rabbits were boosted 3 months from the last injection and bled again 7 and 14 days later. Antibodies against an outer membrane protein (approximately 24 kDa) isolated from non-piliated gonococci (strain MS11 variant C50) were purified by elution from the 24-kDa band of a two-dimensional Western blot with glycine buffer, pH 2.

Monoclonal antibodies were prepared against partially purified pilin. Spleen cells from immunized mice were fused to P3X63Ag8.653 murine myeloma cells as described previously (Fieser et al., 1987),
Ten days after fusion, tissue culture supernatants were screened for reactivity against chromotriauxpilin with a solid phase enzyme-linked immunosorbant assay. The immunoglobulin-containing fraction was precipitated from ascites fluid by addition of an equal volume of saturated ammonium sulfate solution. The monoclonal "2BE11 P12" was a gift from Dr. Wendell Zoller at the Walter Reed Army Institute of Research. Peptide mapping of the monoclonal antibodies verified that they recognize pilin sequences.  

For Western blots (Towbin et al., 1979), the protein was transferred from SDS-PAGE to a nitrocellulose filter and incubated with the antibodies. For some blots, proteins transferred to the filter were India ink-stained after the transfer and before the incubation with antisera. Antibody reactivity was detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Leary et al., 1983) and development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega Biotech).

**Electron Microscopy**—To scan several precipitating agents and identify initial crystallization conditions, microcrystalline precipitates were examined using the general procedure of Steven and Nava (1980) in which crystals were crushed, applied to grids, and washed for 10–30 s in distilled water, and stained with 1% uranyl formate for lo–30 s in distilled water, and stained with 1% uranyl formate for 1, 2, 3-triol, and 2-methyl-2,4-pentanediol as well as salts, including MgSO₄, MgCl₂, (NH₄)₂SO₄, LiBr, LiCl, NaCl, NaBr, CaCl₂, and ZnCl₂, were examined for their effects on crystal size, growth rate, and morphology.

**X-ray Diffraction**—The space group, unit cell dimensions, and diffraction characteristics of needle-shaped crystals of *N. gonorrhoeae* strain MS11 variant C30 pilin were determined from x-ray diffraction data, measured primarily from rotation photographs. Due to cryo-thinning, some strongly low resolution reflections were visible on low resolution precession photographs taken with CuKα radiation on CEA film using a Supper precession camera mounted on an Elliott GX20 rotating anode x-ray generator with a 200-µm fine focus cup, operating at 40 kV and 40 mA. More complete information was obtained from diffraction data sets collected by rotation photography using synchrotron radiation. At the Stanford Synchrotron Radiation Laboratory, diffraction data films were digitized on a P-1000 Optronics drum scanner linked to a VAX 11/750 computer. Films were scanned using a 50-µm raster, which provided good resolution of the diffraction spots. Digitized data were processed using a combination of programs: MOSCO (Greenhouzh and Helliwell, 1982) to provide an initial rough alignment, the determination of exact spindle value and refinement of unit cell dimensions, and the OSMGER package (Rossmann, 1985) for the refinement of orientation, integration of intensities, and merging and scaling of diffraction data.

### RESULTS

**Isolation, Purification, and Characterization**—Efficient isolation of pilin protein is most practically done using material obtained from about 200 plates. Each 100 plates commonly yielded 60 mg of pilin and a final yield of about 25 mg of pure pilin (overall 40% yield). The solubilization-reassociation steps remove much of the contaminating lipopolysaccharide (Fig. 1). Whole pilin purified by two or more cycles of solubilization-reassociation using pH and ionic strength changes appears to be about 96% pure: the major contaminants that remain are lipopolysaccharide (apparent molecular mass < 15 kDa), pilin aggregates, and degradation products (see below), and pilus-associated proteins with apparent molecular masses of approximately 24, 27, 29, 33, and 36 kDa, between 40 and 55 kDa, and greater than 60 kDa. Most of these proteins reassociate with pilin through large numbers of solubilization-reassociation steps (Fig. 2A). The 24-kDa protein appears to be more loosely associated, since it is gradually removed with repeated solubilization-reassociation steps. Polyclonal antibodies raised against this protein show that it is distinct from pilin, pilin aggregates, and pilin proteolytic fragments (Fig. 2B).

The isoelectric separation of the pilus-associated proteins, as well as any residual lipopolysaccharide, from partially purified pilin (solubilized in nondenaturing detergent) can be accomplished by preparative isoelectric focusing (Fig. 3) or, more completely, by FPLC chromatofocusing (Fig. 4). With a Mono P chromatofocusing column, pilin elutes over a range from pH 7 to 5, with major peaks at pH values 6.6, 6.1, and 5.5. Protein sequencing of three different isoelectric species of pilin obtained from isoelectric focusing (pH 3–10) revealed that each of the three species had the identical amino-terminal sequence for the first 20 amino acids. This N-terminal sequence corresponded to the previously determined sequence of the protein (Schoolnik et al., 1984), suggesting that the three isoelectric forms are all pilin, and the isoelectric variation may result from sequence variation in other regions of the protein, or from as yet unidentified differences in posttranslational modification. Consistent with these chromatographic results, anti-peptide antibodies used for Western blots of two-dimensional gels (isolectric focusing in the first dimension and 15% SDS-PAGE in the second) also show that pilin from a single bacterial strain is heterogeneous in isolectric point with about six different species in the pH 4–6.5 range examined (Fig. 5). Similar Western blots with antib-
FIG. 2. SDS-PAGE (A) and Western blots (B) of pilin and pilus-associated proteins. A, silver-stained SDS-PAGE shows that pilus-associated proteins and pilin multimers co-purify with pilin through multiple solubilization-reassembly steps. Positions of molecular mass standards are shown at left. Lane 1, pili as sheared from whole cells and purified by a single solubilization-reassembly cycle contain the major pilin band at about 20 kDa and many minor protein bands; lane 2, purified pili after six solubilization-reassembly cycles still contain proteins that appear to be strongly associated with the pilus. Note that the 24-kDa protein initially present is apparently removed by the six cycles. B, Western blots of G-75 sizing column fractions from partially purified pili indicate the presence of a 24-kDa non-pilin protein. Lane 1, affinity-purified, polyclonal antibodies against the 24-kDa protein show that the 24-kDa protein is “pilus-associated” (present in partially purified pili), but distinct from pilin; lane 2, polyclonal antibodies raised against partially purified pili recognize both pilin and the 24-kDa protein, suggesting that the 24-kDa protein is present in partially purified pili, but that it is only weakly pilus-associated.

FIG. 3. Silver-stained SDS-PAGE of isoelectric focusing fractions shows partial separation of pilus-associated proteins from pilin. Lane 1, partially purified pili before isoelectric focusing; lanes 2-10, all nine fractions of partially purified pili from a flatbed, preparative, Sephadex; isoelectric focusing gel with a range from pH 10 (left) to pH 3 (right). Protein staining with bromphenol blue on a paper replica identified two major pilin bands, shown in lanes 4 and 5. Pilus-associated proteins at apparent molecular masses of 33 and 36 kDa focus predominantly at pH 8.0-6.5 (lane 3), but also contaminate the major pilin fraction (lane 4). The 27-kDa pilus-associated protein focuses at pH 3.5-3.0 (lane 9).

FIG. 4. Silver-stained SDS-PAGE of chromatofocusing fractions shows almost complete separation of pilus-associated proteins from pilin. Positions of molecular mass standards are shown at left. Lane 1, partially purified pili before chromatofocusing; lanes 2-7, odd-numbered fractions from 9 (pH 7.1) to 19 (pH 6.6) of partially purified pili from a Mono P FPLC chromatofocusing column; lanes 8-14, all fractions from 20 (pH 6.5) to 26 (pH 5.7); lanes 15-25, odd-numbered fractions from 27 (pH 6.6) to 45 (pH 3.9). Pilin has been deliberately overloaded to show the pilin multimers (and some pilin degradation products) and the separation of the major pilin peak from apparent pilus-associated proteins at 24, 27, 29, 33, 36, 40-55, and greater than 80 kDa.

FIG. 5. Two-dimensional Western blot with anti-peptide antibody identifies pilin monomers and dimers with multiple isoelectric points. This anti-peptide antibody, raised against a peptide identical to pilin residues 37-56, identifies pilin monomers at multiple isoelectric points (horizontal line of spots at 20 kDa) consistent with those found by chromatofocusing and also identifies pilin dimers (40 kDa) with both the same and intermediate isoelectric points. This anti-peptide antibody also binds to small amounts of partially hydrolyzed pilin monomers and dimers (center). In the first dimension (horizontal), partially purified pili were isoelectrically focused in a polyacrylamide tube gel using a pH gradient from pH 3 (left) to pH 10 (right), expanded in the range between pH 4 and 6.5. The second dimension (vertical) was run in SDS-PAGE, 15% acrylamide, and transferred to nitrocellulose. Anti-peptide antibodies were labeled with alkaline phosphatase-conjugated goat anti-rabbit antibody.

ies to partially purified pili, before and after absorption with preparations of boiled nonpiliated gonococci (strain MS11 variant B2), show that all six isoelectric species are pilin (data not shown).

Another protein, with an apparent molecular mass of approximately 40 kDa, co-elutes with pilin from the chromatofocusing column, as does some material of lower molecular mass (less than 18 kDa). This lower molecular mass material was characterized as pilin degradation products, since it is recognized by both monoclonal anti-protein and polyclonal
anti-peptide antibodies (Fig. 6). Western blots using anti-peptide antibodies indicate that the lower molecular weight pilin bands on SDS-PAGE result from the loss of N- and C-terminal peptides (data not shown). Aggregates of pilin and pilin bound to pilus-associated proteins were removed by gel filtration. The smallest soluble aggregate of pilin under non-denaturing conditions is a dimer, as determined by gel filtration (Fig. 7). In these experiments, pilin runs at about 40 kDa at pH 10.5 at room temperature in the presence of 1–2% BOG. At pH 9.5, pilin runs as an aggregate with an apparent molecular mass of about 100 kDa. Monoclonal antibodies against pilin identify pilin dimers and higher multimers on SDS-PAGE (Fig. 6). Further evidence for a pilin dimer comes from the same set of isoelectric points for the 40-kDa species as for the 20-kDa pilin monomer (Fig. 5). Antibodies raised against the 40-kDa species also bind strongly to pilin bands on SDS-PAGE gels (data not shown). The monoclonal antibody 7BE11-E12 binds to both strain MS11 variant C30 and strain PGH 3–2 pilin, but appears to recognize the 40-kDa species only in the PGH 3–2 strain, suggesting that these two pilin dimers may retain distinct conformational epitopes even on Western blots, although individual subunits do not.

Crystallization—Three-dimensional micro-crystals of strain MS11 pilin were grown at 5 °C in glass vials from solutions containing about 2 mg/ml protein in 0.125 M ammonium sulfate. Negatively stained electron micrographs of the thin edges of these small plate-like crystals showed spacings of about 30 Å along one direction and over 100 Å along a perpendicular direction (data not shown). The formation of these microcrystals, as opposed to the normal assembly into fibers, was apparently allowed by the decreased stability of the fiber interactions at alkaline pH.

Macroscopic needle-shaped crystals (Fig. 8) suitable for x-ray diffraction data collection were obtained using partially purified pilin or purified pilin (strain MS11 variant C30, 8–10 mg/ml) solubilized by basic pH (8–9.5) and 1–2% BOG and grown at room temperature in hanging drops by vapor equilibration against 40% PEG 400. The presence of the detergent allowed higher concentrations of protein to be achieved without formation of fibers or microcrystalline precipitate. Similar appearing needle-shaped crystals were also obtained from N. gonorrhoeae strains PGH3-2 and ML1 pilin and from P. aeruginosa pilin, but these were not characterized further due to their small size and limitations on available synchrotron radiation beam time. Because pilin runs as an assembled aggregate of about 100 kDa at pH 9.5 (on a Sephacryl S-200 gel filtration column in the presence of 1% BOG), pilin size was also checked on a Sephacryl S-200 gel filtration column under the initial conditions used in the hanging drop for crystallization (20% PEG 400, 1.5% BOG, pH 8.0). The crystallization agents did not decrease the aggregation size (data not shown) suggesting that crystallization may proceed by the addition of assembled pilin multimers.

The presence of various salts and alcohols was found to

![Fig. 6. India ink-stained Western blot of chromatofocusing fractions visualized with A, a monoclonal anti-pilin antibody, and B, a polyclonal antibody against partially purified pilin.](image)

![Fig. 7. Gel filtration of detergent-solubilized pilin at pH 10.5 on an S-200 column.](image)

![Fig. 8. Three-dimensional gonococcal pilin crystals from which diffraction data were obtained.](image)
Pilin Purification and Crystallization

Influence both the solubility and the crystallization of pilin. Some salts, especially ZnCl₂ and MgCl₂, lowered the precipitation point in PEG. Pilin is not soluble in ethanol, and the addition of small percentages (1-10%) of alcohol also lowered the precipitation point in PEG. A more important effect, resulting from the addition of heptane, 1,2,3-triol (Fig. 8), was increased order in the crystals, as indicated by clean, sharp-edged crystals, and improved diffraction. Crystals obtained with 2-methyl-2,4-pentanediol (1-10% v/v) were morphologically similar needles, but they showed no birefringence under polarized light and did not diffract x-rays.

Although a number of different small amphiphiles were tested (see “Experimental Procedures”), the best crystals for diffraction were grown at room temperature in hanging drops by vapor equilibration against PEG 400 solutions including 3-6% heptane-1,2,3-triol. Although a number of techniques to increase crystal size were attempted, including seeding, these pilin crystals remain thin needles (about 1500 × 80 × 80 μm) or plates (about 800 × 100 × 20 μm). To date, crystal size has limited investigations using standard rotating anode radiation and required the space group determination to be primarily accomplished with synchrotron radiation at Stanford Synchrotron Radiation Laboratory using rotation geometry.

**Diffraction Results**—The unit cell dimensions (refined using the MOSFLM package of film integrating programs) are a = 128.4, b = 121.2, c = 26.7 Å, with α = β = γ = 90°, defining the molecular packing as orthorhombic. The systematic absences (h + k = 2n + 1, with all reflections present along the l-axis) define the space group as C222. The molecular mass of 13,000, unit cell dimensions, and crystal symmetry give a Vₐ = 2.84 Å³/dalton for one pilin subunit in the asymmetric unit, which is in the middle of the range (Vₐ = 1.7-3.5 Å³/dalton) expected for proteins (Matthews, 1968). Thus, the possibility of there being one dimer per asymmetric unit (corresponding to Vₐ = 1.42 Å³/dalton) is unlikely.

With synchrotron radiation, the best crystals of pilin diffract to about 2.9 Å resolution using 1.54 Å radiation (Fig. 9) and to about 2.4 Å resolution using 1.08 Å radiation. The crystals withstand irradiation well, allowing the collection of six to eight rotation films at a single crystal position. The quality of these small crystals should allow the determination of an atomic structure of pilin using synchrotron radiation. Native data sets using 1.54 and 1.08 Å radiation have been collected to 3.5 Å resolution using rotation geometry at Stanford Synchrotron Radiation Laboratory. Data collection of potential heavy atom derivatives is in progress.

**DISCUSSION**

The results reported here identify several pilus-associated proteins (apparent molecular masses of approximately 27, 29, 33, and 36 kDa, between 40 and 55 kDa, and greater than 80 kDa). These proteins are present in very low amounts (less than 5% of the overall pilus composition) compared to the pilin protein, and we have called them pilus-associated proteins solely based upon their co-purification with pilin through multiple solubilization-aggregation steps (Fig. 2A). A 24-kDa protein also appears to be associated with the isolated pilus, but it is gradually removed by repeated solubilization-reassociation steps (Fig. 2B). Thus our biochemical studies suggest that there are both strongly pilus-associated and weakly pilus-associated proteins. Proteins in the pilus other than pilin have also been identified by other types of experiments. Five proteins (masses of about 22, 52, 70, and two greater than 94 kDa) were found to copurify with pilus fractionated in deoxycholate-urea buffer (Muir et al., 1988).

Dimensional gel techniques have been used to identify increased expression of 26 proteins in the pilated (versus non-piliated) phase of N. gonorrhoeae (Klimpel and Clark, 1988). Although these studies suggest that there may be several proteins associated with the pilus besides pilin, the complete biochemical purification and characterization of individual pilus-associated proteins has not yet been reported. Definitive evaluation of the presence and role of such pilus-associated proteins in the assembled pilus will require corroboration from molecular genetics and antibody labeling experiments. Currently, proteins characterized as pilus-associated are operationally defined: their presence, apparent molecular masses, and other characteristics may depend in part on the purification procedure and on the strain of N. gonorrhoeae used.

Our results show that at least some of the higher molecular weight pilus-associated proteins are actually pilin multimers that are primarily dimers, although smaller amounts of trimer, tetramer, and higher multimers are also sometimes found (Figs. 5 and 6). The existence of dimeric forms even in the presence of SDS argues that dimeric interactions are strong and that the dimer may represent a building block for the pilus assembly. Gel filtration and Western blots show the presence of a pilin dimer at pH 10.5, at room temperature in the presence of 1-2% B(OH)₃ (Fig. 7), and higher molecular weight pilin multimers in similar conditions at pH 9.5 and below (Figs. 5 and 6). Protein environment can affect the actual pKₐ of a given residue, but the most likely amino acid side chains that could mediate this pH-controlled assembly below pH 10.5 are lysine (side chain pKₐ 10.5) and tyrosine (side chain pKₐ 10.1).

To find conserved residues (with appropriate side chain pKₐ values) that could participate in pilus assembly, we aligned 40 N. gonorrhoeae pilin amino acid sequences (data not shown). There are 12 positions of invariant positive charge (lysine or arginine), and six positions with invariant lysine...
protein dimensions may be considerably smaller, with the 60 Å dimension being the most likely to include bound detergent. Concentration and assembly state. Thus, in contrast to membrane proteins such as porin (Garavito et al., 1983; Zulauf et al., 1986), where the protein/detergent micelle is crystallized, and the photosynthetic reaction center (Deisenhofer et al., 1985), where the hydrophobic core is protected by detergent and crystal contacts are made between detergent-free protein regions, the spontaneous assembly of pilin into fibers has been inhibited by the presence of detergent (and high pH), while the fluidity of the detergent in the micelle has apparently allowed some of those same contacts to be made upon crystallization. The effect of heptane-1,2,3-triol on pilin crystallization was similar to that found with the photosynthetic reaction center (Michel, 1982; Deisenhofer et al., 1985; Allen et al., 1986); heptane-1,2,3-triol significantly improved order in the crystals, so that higher resolution diffraction data could be obtained.

In the light of our results, a similar methodology employing stabilization of an assembly intermediate to promote crystallization rather than fiber formation, may be applicable to a wide variety of fiber-forming proteins, once their assembly conditions are adequately established. Although crystals have been obtained for fiber-forming proteins such as tropomyosin (White et al., 1987; Phillips et al., 1986), their diffraction resolution has been relatively low (about 15 Å) and structural studies have been restricted to the determination of the overall molecular shape and identification of helical secondary structural arrangement. Whether or not our approach to obtaining high resolution three-dimensional crystals of pilin can be successfully applied to crystals of other fiber-forming proteins must await experimental tests with other systems. The results from pilin suggest that these tests will require a thorough knowledge of the biochemical and self-assembly characteristics of the protein subunits, as well as techniques for obtaining subunits in a homogeneous state. Hopefully, new cloning and expression techniques will allow these conditions to be achieved even with fiber-forming proteins such as tubulin (which self-assembles into microtubules) where the biochemical isolation of a single subunit form has proven difficult. It is interesting to note that the rod-shaped tobacco mosaic virus coat protein was crystallized (Bloomer et al., 1978) by stabilizing and crystallizing an assembled complex (the disk) in a manner analogous to the techniques reported here for the fiber-forming protein pilin. Whether or not the approach reported here is general for all fiber-forming proteins, the crystallization of the pilin rods may well be the future crystallization of other fiber-forming proteins.

**REFERENCES**


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Biochemical purification and crystallographic characterization of the fiber-forming protein pilin from Neisseria gonorrhoeae.

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