Formation of 9-nm Filaments from Pilin Monomers Obtained by Octyl-Glucoside Dissociation of Pseudomonas aeruginosa Pili

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Pili isolated from Pseudomonas aeruginosa PAK were solubilized in the detergent octyl-glucoside. Subsequent removal of the detergent by dialysis resulted in the formation of short rods of about 9 nm in diameter and various lengths. The aggregation process was followed by ultracentrifugation, viscometry, and electron microscopy to show that the aggregates produced in this way are distinct from pili produced by the bacteria, both in diameter, as measured by electron micrographs, and in their inability to compete in a biological assay for pili.

Pseudomonas aeruginosa PAK possesses polar pili which mediate susceptibility to infection by the pilus-specific bacteriophages PO4 and Pfl (5). The pili also confer on the bacteria a primitive form of motility known as twitching motility (4). Recently, the polar pili of P. aeruginosa were implicated in the adhesion of Pseudomonas sp. to mammalian buccal epithelial cells (19), suggesting that the pili may play a role in Pseudomonas pathogenesis.

Pseudomonas pili appear to be able to retract into the cell to bring attached bacteriophages into contact with the bacterial cell surface (3). This reversible assembly of pilin may play an important role in its various functions. Hence, it is of interest to study the nature of subunit/subunit interactions with respect to both dissociation and reassembly.

PAK pili consist of a single subunit, pilin, with a molecular mass of 18,100 daltons (9). X-ray diffraction studies have revealed that the pilin subunits are arranged in a helix with a diameter of 5.4 nm and a pitch of 4.1 nm, with approximately four subunits in each turn (8). Until recently, it has not been possible to dissociate pili into subunits without resorting to strong denaturants, such as sodium dodecyl sulfate (SDS). However, using the nonionic detergent octyl-glucoside, it has been possible to obtain a mixture of pilin monomers and dimers without disrupting protein secondary structure, as determined by circular dichroism (T. H. Watts, C. M. Kay, and W. Paranchych, Can. J. Biochem., in press).

In this report, we describe the removal of detergent from the solubilized pilin-detergent complexes, resulting in the assembly of rods approximately 9 nm in diameter and about 10 to over 200 nm in length.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and pilus purification. PAK/2P1s is a multipiliated mutant of P. aeruginosa PAK that has been described previously (9). PAK pili were purified from the multipiliated strain (provided by D. E. Bradley) by the method of Paranchych et al. (16). PAO pilus purification was the same as for PAK pili. Pili encoded by the F1 lac plasmid, EDP208, were kindly provided by E. A. Worobec (Department of Biochemistry, University of Alberta, Edmonton, Alberta Canada).

Phage PO4 was assayed and grown on double-layer agar plates as described by Bradley (2). Both PO4 phage and the host strain P. aeruginosa PAK were kindly provided by D. E. Bradley, Memorial University, St. John's, Newfoundland, Canada.

Media. SSC is standard saline citrate buffer, pH 7.0. TSB agar is 30 g of tryptic soy broth (Difco Laboratories) per liter and 10.5 g of agar (Difco) per liter.

Pilus dissociation and reassembly. PAK pili were suspended at a concentration of 1 mg/ml in 30 mM octyl-glucoside (n-octyl-β-D-glucopyranoside; Sigma Chemical Co.)–50 mM sodium phosphate, pH 7.2. Solubilization was allowed to take place at room temperature for 30 min, followed by centrifugation for 5 min at 2,000 x g in a Fischer microfuge to remove any large insoluble material. Dialysis against distilled water to remove octyl-glucoside and produce reassembled pili was carried out at 4°C for 48 h. Identical results were obtained when dialysis was carried out against 50 mM sodium phosphate buffered at pH 7.2.

Viscometry. For pilus dissociation experiments, 1-mg/ml samples of pilin at various concentrations of detergent were dialyzed overnight against the same concentration of detergent to provide suitable blanks for viscometry. For assembly experiments, it was necessary to know the rate of detergent removal.
Therefore, a calibration curve of octyl-glucoside concentration versus time of dialysis at 4°C was calculated using n-octyl-β-D-[(U-14C)]glucopyranoside (New England Nuclear Corp.) to follow detergent removal. At 4°C, a 1-mg/ml solution of pili in octyl-glucoside (1 ml) dialyzed against 1 liter of distilled water resulted in equilibrium in 12 h. Because protein concentrations changed slightly during dialysis, the protein concentration was determined on each sample subsequent to the viscosity measurement.

Viscosity measurements were carried out in a 0.5-ml-capacity Oswald viscometer held at 20°C in a constant-temperature water bath. The reduced viscosity, \( \eta_r \), was used for comparison, c being the protein concentration determined on each sample after dialysis:

\[
\eta_r = \frac{\eta - \eta_0}{c} = \frac{1}{c} \int_0^1 \frac{dn}{d[1/n]} \, dn
\]

where \( \eta \) and \( \eta_0 \) are the flow times of protein-detergent and detergent alone, respectively. The approximation \( \eta_r = \eta_0/c \) was used throughout.

**Circular dichroism.** Circular dichroism spectra were obtained with a Cary model 60 recording spectrophotometer with a Cary 6001 CD attachment, using the methodology of Oikawa et al. (15). The percentage of \( \alpha \)-helix was calculated by the method of Chen et al. (6), as described in detail elsewhere (Watts et al., in press).

**Ultracentrifugation.** Sedimentation-velocity runs were carried out in a BeckmanSpinco model E ultracentrifuge equipped with UV optics. Sedimentation coefficients were corrected for solvent viscosity and density, using the relationship

\[
S_{20,w} = S_20 \left( \frac{\eta_s}{\eta_w} \right) \left( \frac{1 - \psi P_{20,w}}{1 - \psi P_{20,w}} \right)
\]

where \( S_{20,w} \) is the sedimentation coefficient measured at 20°C, \( \eta_s \) is the viscosity of solvent and water at 20°C, \( \psi \) is the protein partial specific volume, and \( \rho \) is the density of solvent or water. Solvent viscosity was measured relative to water as described above. Solvent density was measured in a Paar precision densitometer. Protein partial specific volume for pili has been determined by densitometry to be 0.77 ml/g (Watts et al., in press). All runs were carried out at 20°C and 44,000 rpm. For rapidly sedimenting material (whole pili and reassembled pili) runs were repeated at 30,000 and 22,000 rpm to permit an accurate determination of sedimentation coefficient.

**Protein determinations.** Protein determinations were performed by the method of Lowry et al. (11), using bovine serum albumin as a standard. The concentration determined in this way was multiplied by a correction factor of 0.67 calculated from a comparison of the protein concentration determined by amino acid analysis with the protein concentration determined by the method of Lowry et al. (11). The amino acid composition reported elsewhere (16) was used to calculate protein concentration from amino acid analysis. The residues Lys, Arg, Gly, Ala, and Glu were found to give the most consistent results for concentration determinations.

Amino acid analysis was carried out by the method of Moore (13), using a Durham D-500 amino acid analyzer. The hydrolysis period for concentration determinations was 24 h.

**Electron microscopy.** Samples were negatively stained with 1.5% (wt/vol) sodium phosphotungstate (pH 7.2) and photographed in a Philips EM300 at a magnification of ×62,000 (calibrated with paracrystalline troponyosin, kindly supplied by L. B. Smillie).

Pili and reassembled rod diameters were measured on the ×3 enlarged prints. Thirty measurements were carried out on each structural type.

**Competition plaque assay.** PO4 phage, at a concentration of 8.8 ± 0.9 × 1010 PFU/ml, was diluted into SSC. To 1-ml samples of 10-9 and 10-10 dilutions of PO4 various amounts of whole pili were added and incubated for 2 h at room temperature. After 2 h, 100 µl of a standing overnight culture of P. aeruginosa PAK (5 × 109 cells per ml) was added, and the entire contents of the tube were mixed with 2.5 ml of 0.7% water-agar and poured onto TSB plates. Plaques were counted after 12 to 16 h at 37°C.

**X-ray diffraction methods.** Fibers of PAK pili and reassembled PAK pili were prepared from aqueous solutions (20 mg of protein per ml) by placing 5 to 10 µl between the ends of two glass rods and allowing the droplets to dry slowly at 98% humidity. Diffraction patterns were recorded for 15 to 40 h on Kodak No Screen film, using a pinhole camera mounted on a Jarrell-Ash 80,000 X-ray generator run at 40 kV and 6 mA. The camera was flushed with helium that had been equilibrated with saturated NaCl to maintain the humidity at 75%. The specimen-to-film distance was 4.5 cm, as determined from a 0.329-nm calibration ring obtained by dusting the fibers with calcite.

**RESULTS**

Dissociation/reassociation of pili. Pili were dissociated into subunits with the detergent octyl-glucoside as described above. It has been determined by analytical ultracentrifugation (Watts et al., in press) that pilin–octyl-glucoside complexes prepared in this manner consist of an equimolar mixture of monomers and dimers at a protein concentration of 1 mg/ml in 30 mM octyl-glucoside.

Table 1 compares octyl-glucoside and a number of other agents in their ability to dissociate pili into subunits. The sedimentation coefficient was used as an indicator of the aggregation state of the pili, and circular dichroism was used as an index of the protein conformation state. As was previously reported (Watts et al., in press), SDS was effective in dissociating pili into monomers but resulted in a 6% increase in \( \alpha \)-helix. This increase in \( \alpha \)-helix in the presence of SDS at the expense of other secondary structures has been observed for a number of proteins, e.g., concanavalin A (10) and EDP208 pili (G. D. Armstrong, Ph.D. thesis, University of Alberta, Edmonton, Alberta, Canada, 1980).

Guanidine hydrochloride was not very efficient in dissociating pili and resulted in drastic changes in the secondary structure. The 11S species obtained in the presence of 4 M guanidine hydrochloride was rather heterogeneous. No whole pili were observed in the electron
TABLE 1. Effect of various agents on the aggregation state and secondary structure of 
Pseudomonas pili

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% α-helix</th>
<th>S20.w</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>47</td>
<td>10-30</td>
</tr>
<tr>
<td>1% SDS</td>
<td>53</td>
<td>1.6</td>
</tr>
<tr>
<td>4 M guanidine hydrochloride</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>pH &lt; 2 or &gt; 12</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>8 M urea</td>
<td>22</td>
<td>ND</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>47</td>
<td>10-15</td>
</tr>
<tr>
<td>10 mM deoxycholate</td>
<td>45</td>
<td>10-15</td>
</tr>
<tr>
<td>10 mM taurodeoxycholate</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>30 mM octyl-glucoside</td>
<td>47</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Pilus concentration was 1 mg/ml in 50 mM phosphate buffer (pH 7.2) plus the reagent in question. Samples were dialyzed overnight before ultracentrifugation or circular dichroism measurements. —, None.

1 Percent α-helix was determined as described in the text. The circular dichroism spectra of native, SDS-, guanidine hydrochloride-, and octyl-glucoside-treated pili have been published elsewhere (Watts et al., in press).

2 Sedimentation coefficients were determined and standardized as described in the text.

3 An exact sedimentation coefficient cannot be determined for native PAK pili due to their heterogeneity in length.

microscope, but large aggregates of an apparently nonspecific nature were observed.

Similarly, extremes of pH resulted in denaturation and (at low pH) precipitation before dissociation. NaCl (1 M) appeared to decrease the formation of longitudinal aggregates, resulting in a smaller observed sedimentation coefficient without a change in structure.

The most useful agents for dissociating pili into subunits appear to be nonionic detergents. Sodium taurodeoxycholate (10 mM) and octyl-glucoside (30 mM), when added to a 1-mg/ml solution of pili, resulted in the formation of small aggregates: one to four subunits in the case of taurodeoxycholate and one to two subunits in the case of octyl-glucoside. For the dissociation/reassociation experiments, octyl-glucoside was preferred due to the ease with which it is removed by dialysis (1).

Both dissociation of pili in the presence of increasing amounts of octyl-glucoside and the reassociation of subunits upon the removal of octyl-glucoside were monitored by viscometry (Fig. 1). Curve A shows the dissociation of native pili as isolated from Pseudomonas sp. with increasing detergent concentration. The large reduced viscosity of pilus preparations is probably due to the extreme asymmetry of the filaments (5.4 nm in diameter and up to 2 μm long) and their tendency to form longitudinal aggregates or "ropes."

The removal of detergent by dialysis (Fig. 1B) resulted in an increase in ηp/p, but the viscosity never returned to that of the original solution. No further changes in viscosity were observed after 48 h of dialysis.

Upon examining the preparations in the electron microscope (Fig. 2B), it was found that the reassembled pili consisted of short rods about 9 nm in diameter and of various lengths. The fact that the rods were shorter than whole pili is compatible with the observed lower viscosity of pilin solutions after dissociation and detergent removal.

Ultracentrifugation analysis showed that 1 mg of pilin per ml in 30 mM octyl-glucoside has an S20,w of 2.3S. It was difficult to measure a sedimentation coefficient for whole pili as isolated from the bacteria since there was considerable heterogeneity in the length of the pili. It appeared that about 70% of the material had a sedimentation coefficient in the range of 10 to 30S, whereas the rest was more rapidly sedimenting, probably due to the formation of pilus ropes (Fig. 2A). In the case of reassembled pili, about 90% of the material was accounted for by species of 10 to 30S. However, a comparison of the two preparations in the electron microscope suggested that the two structures are quite different.

Figure 2 shows a series of electron micrographs comparing the two structures. The pili isolated from Pseudomonas sp. were filaments 5.4 nm in diameter and, on the average, >1 μm in length (Fig. 2A). They appeared very straight,

![Graph showing reduced viscosity vs. concentration of octyl-glucoside (mM)](http://jb.asm.org/)

FIG. 1. Dissociation and reassociation of pili as measured by viscometry. A. Dissociation of PAK pili in the presence of increasing amounts of detergent; B, aggregation of pili as detergent is dialyzed away. Time of dialysis was converted to octyl-glucoside concentration by calibration with [14C]octyl-glucoside as described in the text.
and details of subunit orientation were not discernible in the electron microscope. Sometimes ropes of several pili, aggregated side to side along their long axes, were observed. In concentrated solutions of pili, these ropes diffract X rays if the solutions are dried between glass rods to form a fiber (8; Fig. 3).

The reassembled pili, on the other hand, were considerably wider (Fig. 2B). The diameter of each was measured, using whole pili as an internal standard (Fig. 2C), and found to be 9.0 ± 1.5 nm. A diameter of 5.2 ± 4 nm was obtained for native pili, in agreement with the X-ray results (8). Striations approximately 45° to the filament axis could be discerned in some of the reassembled rods, suggesting that a looser arrangement of subunits in these rods than in native pili permits stain penetration. X-ray diffraction of native pili has shown that the surfaces of Pseudomonas pili are very nearly that of a cylinder with no large grooves through which the stain can penetrate (8).

No aggregation of 9-nm rods was observed in the electron microscope, although concentrations of up to 1 mg of pilin per ml were used. Fibers prepared from reassembled pili diffracted only poorly, showing 0.5- and 1.0-nm reflections characteristic of α-helix and two weak crystalline reflections, which indicate a regular packing of filaments in the fiber (Fig. 3). Failure to obtain
well-oriented specimens of reassembled pili is most likely due to their short length.

**Biological assay for pili.** Bacteriophage PO₄ infects *P. aeruginosa* by adsorbing to the lateral surface of the pilus (3). If the attachment site for the phage is contributed by residues on more than one subunit, it is likely that phage PO₄ would not recognize monomers or wrongly assembled pilin aggregates. This reasoning allowed us to develop an assay for biologically active pili based on a measurement of their ability to adsorb PO₄. Figure 4 (curve A) shows the effect of incubating phage PO₄ with increasing amounts of native PAO pili before adding PAO cells and plating. About 80 ± 10% was the maximum adsorption obtained. The failure to obtain 100% adsorption most likely reflects the equilibrium between irreversibly adsorbed and reversibly adsorbed phages on the pili. That is, a small amount of phage may become detached from pili upon dilution into soft agar and either reattach to free pili or infect the *P. aeruginosa* indicator strain added. Curves B and C show the effect of increasing amounts of pilin subunits in detergent (B) or reassembled pili (C). Within experimental error, one cannot distinguish between the two. Thus, this experiment provides further evidence that we have assembled an alternate structural form of polymeric pilin.

Table 2 summarizes some control experiments which show that these effects are specific to *P. aeruginosa* PAO pili. The closely related pili from strain PAO had no effect on phage infection, nor did pili encoded by the *Escherichia coli* conjugative plasmid EDP208. PAO pili were selected as a control because of their similarity to PAO pili. The two types of pili possess approximately 50% sequence homology (P. A. Sastry and W. Paranchych, unpublished data) and give almost identical diffraction patterns (8). However, PAO pili adsorb phage PO₄ much less efficiently than do PAO pili (2). Pili which have been sonicated to reduce their length were still able to prevent phage infection as well as the longer filaments, suggesting that it is the different quaternary structure and not the shorter average length that prevents reassembled pili from binding phage PO₄. Furthermore, the amount of detergent added with the pilin was not sufficient to block phage infection, as shown by the addition of detergent alone.

**DISCUSSION**

Recently, Eshdat and collaborators (7) reported the dissociation and reassembly of *E. coli* type I pili, using guanidine hydrochloride to

**FIG. 3.** X-ray fiber diffraction patterns of (A) native PAO/2Pf pili (15.3 h of exposure at 40 kV, 6 mA, 75% relative humidity, 4.5-cm specimen-to-film distance); and (B) reassembled pili (46 h of exposure at 40 kV, 0.6 mA, 75% relative humidity, 4.5-cm specimen-to-film distance). The inner circle represents a spacing of 1.0 nm, and the larger one is at 0.5 nm.

**FIG. 4.** Reduction in the number of plaque-forming units upon incubation of pili with dilutions of phage PO₄ before carrying out a standard plaque assay as described in the text. A, Native PAO pili; B, pilin–octyl-glucoside; C, reassembled pili.

**TABLE 2.** Inhibition of phage attachment to *P. aeruginosa*

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<thead>
<tr>
<th>Addition</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>PAO pili (100 μg)</td>
<td>80 ± 11</td>
</tr>
<tr>
<td>PAO pili (100 μg)</td>
<td>−7 ± 14</td>
</tr>
<tr>
<td>EDP208 pili (100 μg)</td>
<td>10 ± 15</td>
</tr>
<tr>
<td>Pilin–octyl-glucoside</td>
<td>18 ± 23</td>
</tr>
<tr>
<td>(100 μg) protein</td>
<td></td>
</tr>
<tr>
<td>PAO pili sonicated 2 min</td>
<td>72 ± 20</td>
</tr>
<tr>
<td>to reduce length (100 μg)</td>
<td></td>
</tr>
<tr>
<td>Reassembled pili (100 μg)</td>
<td>37 ± 10</td>
</tr>
<tr>
<td>Octyl-glucoside control</td>
<td>0 ± 10</td>
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dissociate the pili into subunits. Although these workers have demonstrated that removal of guanidine hydrochloride leads to the formation of a pilus-like structure, they have not demonstrated that the rods have the characteristics of functional, properly assembled pili. In light of our results, we suggest that more rigorous criteria be used in the future to define pilus reassembly.

By analogy with the F-pilus system, which requires several gene products for the expression of mature pilus on the cell surface (18), it is likely that the proper assembly of biologically active pili requires additional factors. An analogy can also be drawn between pili and the filamentous phage M13 in that both of these consist of helical arrays of a protein subunit (8), and in both cases the filament is assembled from a pool of subunits in the cytoplasmic membrane (12, 17; unpublished data). Since it has been demonstrated that a proton motive force is required for the assembly of phage M13 from the cytoplasmic membrane (14), one might also expect pilus assembly to require energy.

The fact that we have obtained a population of aggregates of Pseudomonas pilin of uniform diameter suggests that there are at least two stable quaternary structures for this protein. Native pili are formed when pilin is assembled from the cytoplasmic membrane (perhaps in the presence of other protein factors and a proton motive force); an alternate quaternary structure, 9-nm pilin filaments, is formed spontaneously upon removal of detergent from octyl-glucoside-solubilized pilin.

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LITERATURE CITED


