

Dissociation and Reassembly of *Escherichia coli* Type 1 Pili

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Escherichia coli type 1 pili, which mediate the mannose-sensitive adherence of the bacterium to eucaryotic cells, are comprised of very stable arrays of pilin protein subunits (molecular weight, approximately 17,000). Previous methods for the dissociation of pili caused their irreversible denaturation. We have found that incubation of pili in saturated guanidine hydrochloride at 37°C led to their complete dissociation, as evidenced by nephelometry and electron microscopy. Gel chromatography of the dissociated pili on a Sepharose CL-6B column in the presence of saturated guanidine hydrochloride yielded a single protein peak with a molecular weight corresponding to that of pilin. Dialysis of this peak against 5 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0) and rechromatography in the same buffer afforded a major protein peak, probably consisting of pilin dimers. About 25% of the protein in this peak bound to a mannan-Sepharose column and could be eluted with methyl α -D-mannoside. The pilin dimer gave a single protein band upon polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (molecular weight, 16,600) or 10 M urea and penetrated completely into 7% gels in the absence of denaturants. Reassembly of the pilin dimers into pili was achieved upon dialysis against the tris(hydroxymethyl)aminomethane buffer containing 5 mM MgCl₂, as observed by electron microscopy. Thus, the conditions used allow renaturation of the dissociated subunits and may aid in further studies of the structure-function relationship of pili.

Type 1 pili (or fimbriae) of *Escherichia coli* are hairlike appendages 7 nm in diameter protruding from the outer surface of the bacterium and are comprised of protein subunits (pilin) of a molecular weight of approximately 17,000 (2, 16). There is a considerable body of evidence showing that these pili participate in the mannose-sensitive adherence of *E. coli* to a variety of eucaryotic cells (6, 15-17). A detailed study of the structure, organization, and biological properties of these pili is crucial for the understanding of the interaction between the bacteria and the host cell surfaces and of the role of pili in microbial pathogenicity. Studies of type 1 pili have been greatly hampered by difficulties encountered in their solubilization and dissociation into biologically functional subunits. For example, type 1 pili are not readily denatured by sodium dodecyl sulfate (SDS), even with boiling, and the bulk of the protein does not penetrate SDS-polyacrylamide gels (12). Furthermore, the large size of intact pili (molecular weight, >10⁶) and their filamentous shape have limited the use of gel filtration or of affinity chromatography for their purification and characterization. The dis-

sociation of the pili into their subunits can be achieved under such conditions as boiling at low pH or chemical modification (2, 12, 17), which usually lead to irreversible protein denaturation. However, the disruption of pili by low concentrations of agents which break hydrogen bonds was briefly reported (C. C. Brinton, Jr. and S. L. Huang, *Bacteriol. Proc.*, p. 60, 1962).

In this report, we provide evidence that type 1 pili can be completely dissociated by saturated guanidine hydrochloride (GndHCl) and that the pilin subunits retain, at least in part, their ability to bind mannosyl residues and to reassemble into structures resembling short intact pili.

MATERIALS AND METHODS

Materials. Ultra Pure GndHCl and urea were obtained from Schwarz/Mann, and methyl α -D-mannoside was obtained from Pfanstiehl. Phenylmethanesulfonyl fluoride, Trizma base, and yeast mannan were obtained from Sigma Chemical Co., Sepharose 4B and Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Inc., and SDS (specially pure) was obtained from BDH. Other chemicals, purchased from commercial sources, were of the highest purity available and were used without further purification.

Growth of bacteria. *E. coli* 346 (19), a clinical isolate which exhibits mannose-sensitive attachment

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to buccal epithelial cells and to yeast cells, was grown in stationary cultures in Trypticase soy broth (4 liters) for 48 h at 37°C. The bacterial cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. They were suspended in 700 ml of 0.5% NaCl and recentrifuged as above to yield 10 g (wet weight) of cells.

Purification of pili. Type 1 pili were isolated by mechanical agitation of the bacteria and repeated precipitations in 0.1 M MgCl₂ (3) by the procedure of McMichael and Ou (12), with the following modifications (Y. Eshdat and K. Jann, manuscript in preparation): (i) the washed bacteria were suspended in 35 ml of 5 mM Tris-hydrochloride buffer (pH 8.0) containing 0.02% NaN₃ and 30 nM phenylmethanesulfonyl fluoride to avoid possible proteolysis; (ii) the bacteria were agitated in a Sorvall Omnimixer (setting 5, 10 min, 0°C) to remove the pili from the bacteria; (iii) the pili were precipitated by MgCl₂ (0.1 M), but to increase the yield they were kept for 2 to 4 h at 0°C before centrifugation; (iv) the last precipitate of pili was suspended in 25 ml of 5 mM Tris-hydrochloride buffer with 0.02% NaN₃ and centrifuged at 200,000 × *g* for 2 h at 4°C, and the pellet of pili was stored at 4°C until used.

Electron microscopy. Preparations of pili were negatively stained with 0.5% uranyl acetate on copper grids coated with parlodion and carbon (18). Grids of samples containing GndHCl were rinsed with several drops of 5 mM Tris-hydrochloride (pH 8.0) before staining. Samples were examined in a Philips EM 300 electron microscope.

Protein analysis. Protein content was determined according to Markwell et al. (11), with bovine serum albumin (Pentex Biochemical) as the standard. Homogeneity was determined by polyacrylamide gel electrophoresis in the presence of SDS or urea (7) or in the absence of denaturants according to Davis (5). Molecular weights were determined by (i) equilibrium sedimentation of the protein (0.34 mg/ml of 5 mM Tris-hydrochloride, pH 8.0, and 0.1 M NaCl) in an analytical ultracentrifuge (model E; Beckman Instruments, Inc.) equipped with an electronic speed control and a monochromator with a double-sector cell (12-mm optical path), (ii) gel filtration, and (iii) gel electrophoresis in 0.1% SDS; in the latter two methods, appropriate protein standards were used. Amino acid analyses were performed as previously described (7).

Nephelometry. Solutions of pili (0.6 mg of protein per ml in 5 mM Tris-hydrochloride, pH 8.0) were examined in a Perkin-Elmer 1000M fluorimeter in cells of 5-mm optical path (18).

Column chromatography. Gel filtration was carried out on a column of Sepharose CL-6B (1.5 by 84 cm) equilibrated and eluted with 5 mM Tris-hydrochloride buffer (pH 8.0) or with the buffer containing either saturated GndHCl or 0.1 M NaCl; 2-ml fractions were collected. The optical density of the eluate was continuously monitored at 220 or 230 nm with the aid of a 1-ml flow cell inserted in a Beckman DB-G spectrophotometer.

Affinity chromatography was performed on a column (1 by 15 cm) of yeast mannan linked to Sepharose 4B, prepared by coupling 5 mg of mannan per g of swollen Sepharose, by the method of Miron et al. (14).

Sample application and washing were done at 23°C with 5 mM Tris-hydrochloride (pH 8.0), and elution of the bound material was with the same buffer containing 0.5 M methyl α -D-mannoside.

Mannose-binding activity. The mannose-binding activity of pili was determined by their capacity to agglutinate yeast cells (13).

RESULTS

Characterization of purified type 1 pili. The purified pili were found to be homogeneous and free of flagella and membrane vesicles when examined in the electron microscope (Fig. 1A), and they exhibited mannose-sensitive agglutination of yeast cells down to a concentration of 50 μ g/ml. The amino acid composition of the pili is given in Table 1; it is similar to that obtained for type 1 pili purified from other *E. coli* strains (10, 17). SDS-gel electrophoresis of the purified pili, which had been boiled for 5 min in SDS-sample buffer without any pretreatment, revealed a few protein bands which represented only a small portion of the total protein applied and may correspond to several aggregation forms of a 17,000-dalton subunit (Fig. 2B). Most of the protein (>90% as determined by scanning the Coomassie blue-stained gel in a Gilford 2400-S spectrophotometer) remained on the top of the stacking gel (data not shown).

Gel filtration of the purified pili on a Sepharose CL-6B column afforded a major protein peak (Fig. 3A, I) which emerged in the void volume of the column. Under the electron microscope, the eluted material had the same appearance as before gel filtration, and its amino acid composition was essentially the same as that of the prechromatographed pili (data not shown).

Dissociation of pili. After incubation for 2 h at 37°C in 10 M urea or 7.5 M GndHCl, intact pili in large numbers could still be observed under the electron microscope (Fig. 1B). Light scattering of pili incubated for 2 h at 37°C in different concentrations of GndHCl is shown in Fig. 4. The large increase in the light-scattering properties of pili in suspensions at low GndHCl concentrations was probably due to the salting out of aggregates of intact pili, which were indeed observed by dark-field microscopy. Only in concentrations as high as 8.3 M GndHCl was the light scattering practically the same as that of the solvent. Electron microscopy of pili incubated for 2 h at 37°C in saturated GndHCl (approximately 8.6 M) revealed their complete dissociation (Fig. 1C). Gel filtration on Sepharose CL-6B in 5 mM Tris-hydrochloride (pH 8.0) in saturated GndHCl yielded a major protein peak with a molecular weight of approxi-

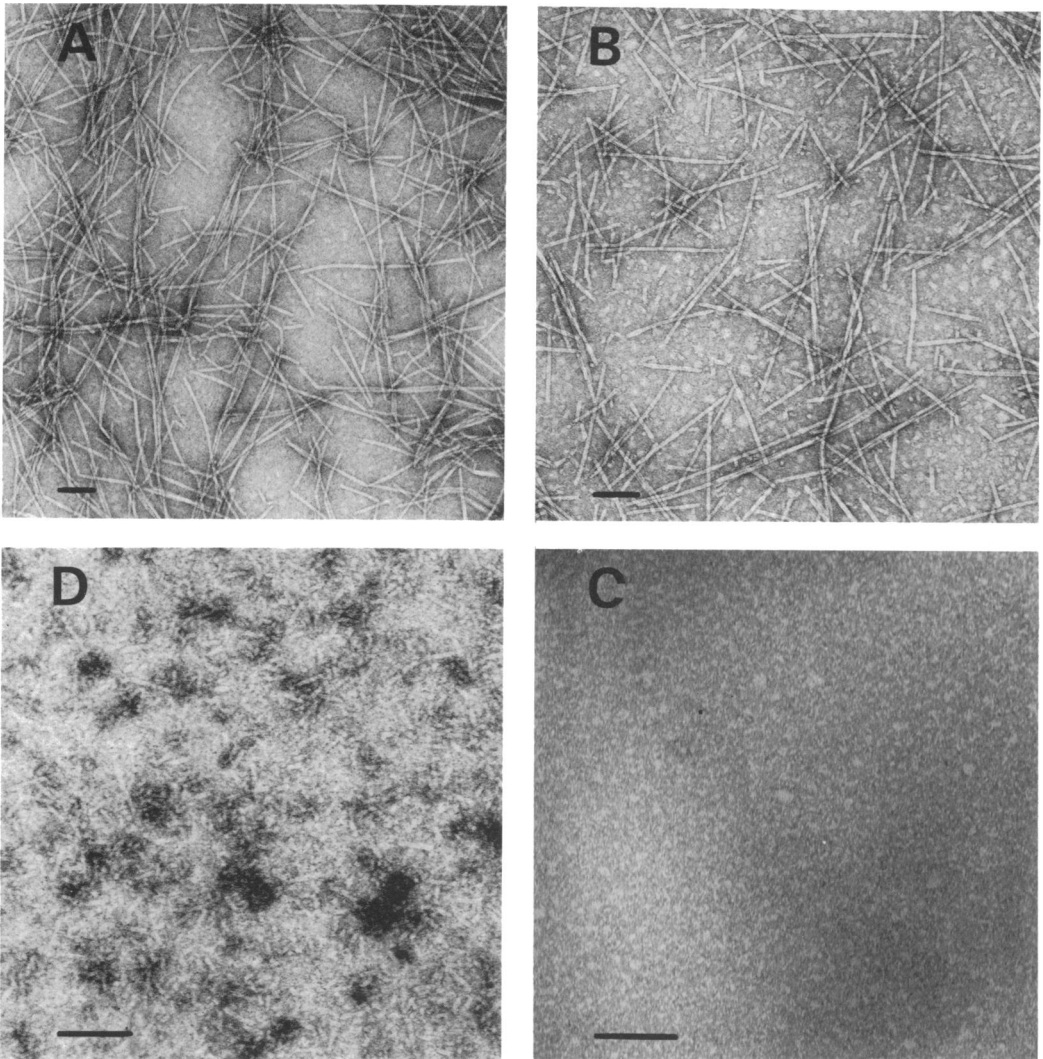


FIG. 1. Electron micrographs of type 1 pili from *E. coli* 346. (A) Purified pili; (B) pili incubated for 2 h at 37°C in 5 mM Tris buffer (pH 8.0)–7.5 M GndHCl; (C) pili incubated for 2 h at 37°C in 5 mM Tris buffer (pH 8.0) and saturated GndHCl; (D) reassembled pili in 5 mM Tris buffer (pH 8.0)–5 mM MgCl₂. The pili were negatively stained with uranyl acetate. Bar = 0.1 μm.

mately 16,000 (Fig. 3B, I), corresponding to that of pilin. No protein could be detected in the two minor peaks (Fig. 3B, II and III); these probably included a salt, such as NaN₃, which was present in the GndHCl-treated sample of pili, as well as other low-molecular weight contaminants. Dialysis of the dissociated pili (peak I) against 5 mM Tris buffer at 4°C and gel filtration of the dialyzed material on the Sepharose column with the Tris buffer in the absence or presence of 0.1 M NaCl gave one major protein peak (Fig. 3C, II). Samples taken from this protein peak were examined by gel electrophoresis in three different systems: (i) SDS-gel electrophoresis of a

sample which had been boiled with SDS-sample buffer for 5 min revealed essentially one band with an apparent molecular weight of approximately 16,600 (Fig. 2C); (ii) a sample which was mixed with 5 mM Tris-glycine buffer (pH 8.3) also completely penetrated 7% polyacrylamide gels in the absence of denaturant at pH 8.9 (Fig. 5A); (iii) a sample which had been preincubated with 5 mM 2-amino-2-methyl-1,3-propanediol hydrochloride (pH 9.4) and 8 M urea gave a single band upon electrophoresis in gels containing 10 M urea (Fig. 5B). From its elution volume on the Sepharose column (Fig. 3C), an apparent molecular weight of approximately 30,000 could

TABLE 1. Amino acid composition of *E. coli* 346 pili

Amino acid	mol%
Aspartic acid	13.5
Threonine	12.1
Serine	6.2
Glutamic acid	8.7
Proline	1.6
Glycine	9.8
Alanine	16.8
Cysteine	1.1
Valine	9.1
Methionine	<0.1
Isoleucine	3.2
Leucine	6.4
Tyrosine	2.2
Phenylalanine	3.6
Tryptophan	<0.1
Lysine	3.0
Histidine	1.1
Arginine	1.6

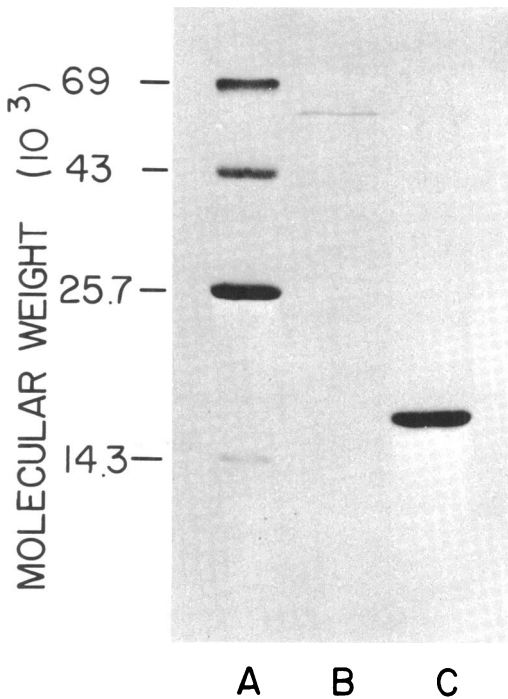


FIG. 2. SDS-gel electrophoresis in a 13% polyacrylamide slab gel of: (A) molecular weight markers lysozyme (14,300), α -chymotrypsinogen (25,700), ovalbumin (43,000), and serum albumin (69,000); (B) purified type 1 pili from *E. coli* 346 (120 μ g); and (C) pili (80 μ g) after their dissociation with saturated GndHCl and dialysis against 5 mM Tris-hydrochloride, pH 8.0. Gels were stained with Coomassie blue.

be assigned to the protein in peak II. Determination of its molecular weight by equilibrium sedimentation (partial specific volume, 0.74, calculated according to Cohn and Edsall [4] from

amino acid composition of the pili given in Table 1) showed that the dissociated pili in 5 mM Tris-hydrochloride (pH 8.0) were probably in the form of pilin dimers (molecular weight, approximately 30,000). No pili could be observed when this protein fraction was examined under the electron microscope, and its amino acid composition was the same (<4% difference) as that of the purified pili.

When the pilin dimer (0.4 mg) was applied to a column of mannan-Sepharose, the bulk of the protein was eluted with the buffer. The bound protein (approximately 25%) was eluted with 0.5 M methyl α -D-mannoside (Fig. 6). Both protein fractions gave SDS-polyacrylamide gel patterns

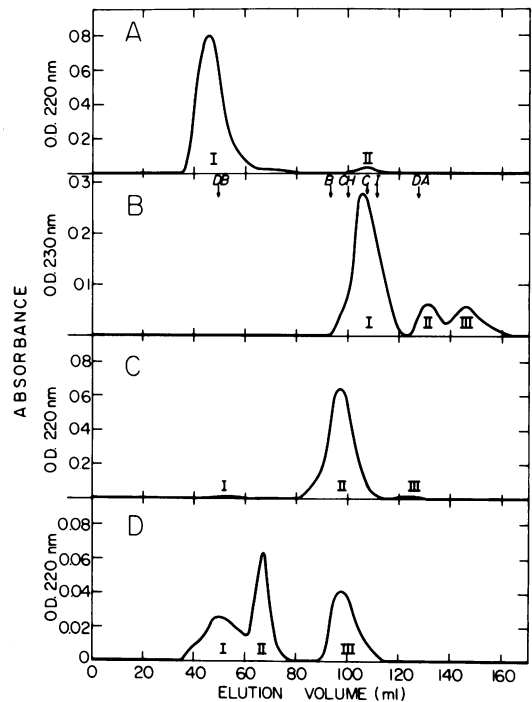


FIG. 3. Gel filtration on a Sepharose CL-6B column (1.5 by 84 cm). Elution patterns of (A) purified pili (3.5 mg) in 5 mM Tris buffer, pH 8.0; (B) dissociated pili (2.5 mg) in same buffer saturated with GndHCl (protein markers used: DB, dextran blue; BSA, bovine serum albumin; CH, α -chymotrypsinogen; C, cytochrome C; I, insulin; and DA, dinitrophenyl-D-alanine); (C) dissociated pili (2.5 mg) in 5 mM Tris buffer, pH 8.0, after dialysis of GndHCl against the Tris buffer (the same pattern was obtained when dialysis and elution were done with the Tris buffer in 0.1 M NaCl); and (D) reassembled pili (0.5 mg) in 5 mM Tris buffer, pH 8.0, obtained by dialysis of the dissociated pili against Tris buffer containing 5 mM $MgCl_2$ at 23°C. The optical density of the eluate was continuously monitored by a Beckman DB-G spectrophotometer. Fractions of 2 ml were collected. O.D., Optical density.

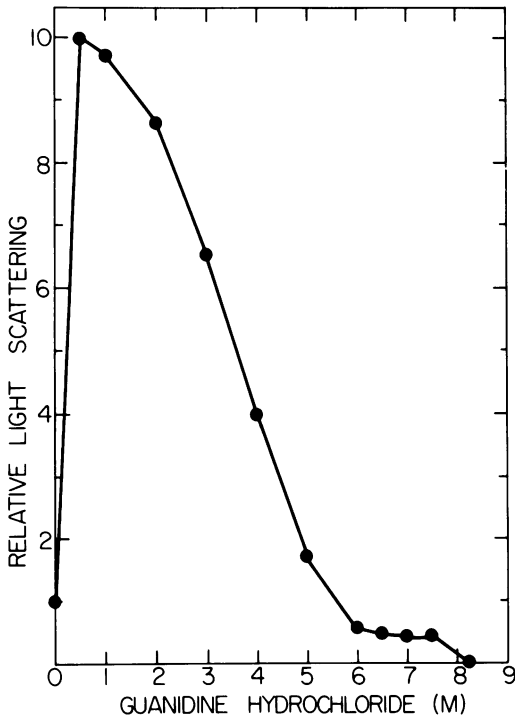


FIG. 4. Light scattering (at 550 nm) of purified type 1 pili from *E. coli* 346 (0.6 mg/ml) after incubation at 37°C for 2 h in different concentrations of GndHCl.

identical to that obtained for the pilin dimer (data not shown).

Reassembly of pili. Dialysis of the dissociated pili against 5 mM Tris buffer (pH 8.0)-5 mM MgCl₂ at room temperature afforded a preparation which under the electron microscope contained rodlike structures with diameters of approximately 7 nm resembling native pili (Fig. 1D). The reassembled pili were, however, shorter than the native ones. Gel filtration of the reassembled pili on Sepharose CL-6B in 5 mM Tris-hydrochloride (pH 8.0) suggested that about one-third of the dissociated protein reassembled into intact pili (Fig. 3D, peak I); the rest was probably in the form of pilin trimers (peak II) and dimers (peak III), as could be deduced from their elution volumes. The SDS-gel electrophoretic pattern of the protein in peak I was similar to that obtained from the native pili (see Fig. 2B), whereas the patterns of the proteins from peaks II and III were the same as that obtained for the pilin dimer (Fig. 2C).

DISCUSSION

The fact that type 1 pili are assemblies of identical protein subunits was recognized initially by Brinton (2), who also demonstrated

that the pilin monomers with a molecular weight of approximately 17,000 are aligned in an α -helical array with a diameter of about 7 nm and a hollow inner core of 2 nm.

In this study, we found that pili are unusually stable to high concentrations of reagents which readily denature other proteins. Similar findings have been reported by other laboratories (12, 17). Indeed, the selective resistance of pili to 6 M urea and to deoxycholate forms the basis of a new method of purifying pili from other bacterial surface proteins (10). It is possible that the *E. coli* strain examined by Brinton and Huang (Bacteriol. Proc., p. 62, 1962) possessed unusu-

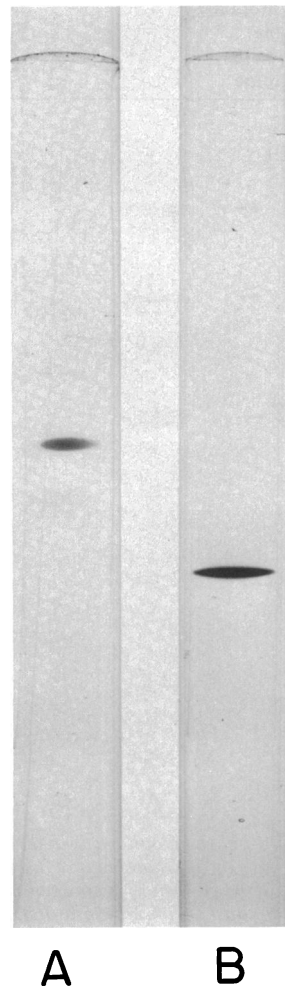


FIG. 5. Polyacrylamide disc electrophoresis of *E. coli* 346 pili after their dissociation with saturated GndHCl and dialysis against 5 mM Tris-hydrochloride, pH 8.0, on (A) 7% gel at pH 8.9 (5) and (B) 5% gel at pH 9.4 in the presence of 8 M urea (7). Gels were stained with Coomassie blue.

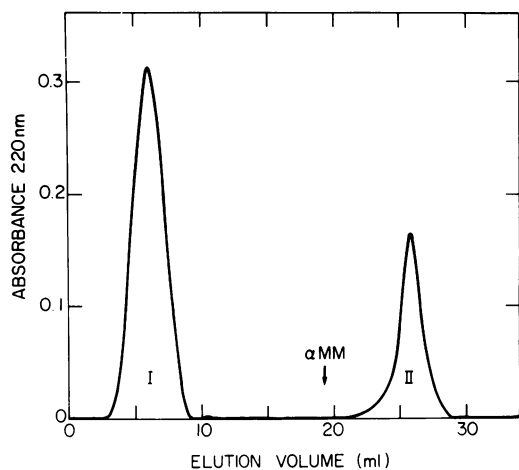


FIG. 6. Affinity chromatography of pilin dimer (0.4 mg) on a column (1 by 15 cm) of yeast mannan linked to Sepharose 4B. Washing was with 5 mM Tris-hydrochloride, pH 8.0, and elution was with 0.5 M methyl α -D-mannoside (α MM) in the same buffer. Fractions of 0.5 ml were collected.

ally labile pili which could be dissociated by a low concentration of hydrogen-breaking agents, in contrast to the strains used in subsequent studies (10, 12), including ours, the pili of which were highly stable. Our study demonstrates that complete dissociation of the pili occurred only upon their exposure to GndHCl at concentrations of 8.3 M or higher. This was shown by three independent observations: the absence of recognizable pili or fragments of pili by electron microscopy, the absence of light scattering by nephelometry, and the ability of the GndHCl-dissociated material to enter an acrylamide gel in the absence of SDS. Whenever tested, intact pili were still observed when incubated in the presence of ≤ 7.5 M GndHCl.

Our experiments demonstrate that pili can be dissociated by saturated solutions of GndHCl into pilin monomers, as revealed by their apparent molecular weight (approximately 16,000) determined by gel filtration in the presence of saturated GndHCl. After dialysis against 5 mM Tris buffer (pH 8.0), the monomers appeared to undergo aggregation to dimers, as evidenced by their behavior on gel filtration and ultracentrifugation. The finding that this preparation has no discernible structure in the electron microscope also suggests that it consists of dimers, since trimers might have been expected to resemble rings.

Exposure of pili to 8.6 M GndHCl was sufficiently mild so that the resultant subunits could reassemble into structures resembling short fragments of intact pili. No reassembly was ob-

tained from pili dissociated by acid treatment (2, 12). The reassembly required the presence of $MgCl_2$. From the proportion of protein in the incubation mixture that appeared in the void volume of a Sepharose CL-6B column, we estimated that about 30% of the subunits had reassembled when dialyzed against 5 mM $MgCl_2$ -5 mM Tris buffer (pH 8.0). It is of interest that a divalent cation is also required for the reassembly of tubulin and other self-assembling proteins (1).

Higher yields of reassembled pili (approximately 60%) were obtained if dialysis was done against 5 mM Tris buffer-50 mM $MgCl_2$, but this was accompanied by protein precipitation. Only trace amounts of reassembled pili (<5%) were detected if the dialysis was carried out in the absence of $MgCl_2$ or in the presence of 5 mM or 0.1 M NaCl for 20 h at 23°C, and none were detected at 4°C. However, we have not systematically explored the optimal conditions for reassembly, and it is possible that a higher yield could be obtained under other conditions. It is also possible that complete reassembly was not obtained because some subunits were irreversibly denatured by the treatment with GndHCl or because the concentrations of subunits fell below a critical level during the reassembly process.

The subunits formed by dissociation with 8.5 M GndHCl retained not only their ability to reassemble into intact pili but also their ability to bind to D-mannose. Thus, about 25% of the subunits obtained after removal of the GndHCl by dialysis were bound to immobilized mannan and could be eluted by methyl α -D-mannoside. We cannot adequately explain why only part of the subunits bound specifically to the mannan column. It is possible that the majority of the subunits were not renatured after removal of GndHCl to molecules capable of binding mannosyl residues. However, it is also possible that only a portion of the pilin monomers in each intact pilus has mannose-binding activity, implying biological heterogeneity of the individual pilus subunits.

Whatever the significance of the low proportion of binding activity, the fact that there was binding at all is additional and perhaps definitive proof that type 1 pili mediate mannose-binding activity of *E. coli*. Earlier attempts at establishing that intact pili bind to D-mannose could be criticized on the grounds of insufficient purity of the preparations used. In such preparations, the mannose-binding activity might have resided in contaminant proteins. The fact that other surface constituents, such as flagella (8) and outer membranes (9), also have mannose-binding ac-

tivity makes such an interpretation plausible. In the present study, the isolation of soluble pilus subunits, which behave chromatographically and electrophoretically as a single protein species and possess the ability to bind specifically and reversibly to immobilized mannan, provides strong evidence for the involvement of pili in the mannose-binding activity of *E. coli*.

We believe that the pilus subunits solubilized by GndHCl may also be useful for further study of these appendages. Solubilization and reassembly may provide a useful method of obtaining highly purified pili. The availability of low-molecular-weight aggregates (e.g., dimers and trimers) may permit evaluation of the influence of valence on surface phenomena mediated by pili. In addition, such highly purified, low-molecular-weight soluble proteins may be useful for immunochemical studies and the development of more specific vaccines and for the isolation of receptors of pili from eucaryotic cells. The procedure described here for the complete dissociation of pili can be used as an analytical tool for determination of the purity and homogeneity of preparations of pili and detection of contaminating pili in bacterial outer membrane preparations. Finally, a more detailed study of the reassembly process may lead to a greater understanding of the structure-function relationships of intact pili and of the processes involved in assembly of biological structures in general.

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