dehydration (Okamura et al., 1976). The spectral characteristics of 19-nor-10-keto derivatives will be useful in determining whether this class of vitamin D metabolites occurs systematically in ruminants and nonruminants. In this regard, we have observed 5(*E*)-19-nor-10-ketovitamin D_3, but not the 5(*Z*) isomer, in bovine blood, indicating that it is absorbed.

In preliminary assays of activity, 5-μg doses of 19-nor-10-ketovitamin D_3 did not stimulate bone calcium mobilization in rats. The same dose did stimulate intestinal calcium absorption, but the effect was less than that produced by 50 ng of vitamin D_3.[2] It therefore appears that these metabolites are in a pathway of vitamin D metabolic deactivation. The generation of these metabolites in rumen would appear to explain bovine tolerance to oral vitamin D.

This paper has reported the observation and structural characterization of a unique class of vitamin D metabolites and has reported the first example of microbial vitamin D metabolism. It appears that these metabolites are significant to ruminant detoxification of vitamin D. Work is under way to determine whether nonruminant mammals have 19-nor-10-ketovitamin D derivatives in circulation.

Registry No. Vitamin D_3, 67-97-0; vitamin D_2, 50-14-6; 25-hydroxyvitamin D_3, 19356-17-3; 27-nor-25-ketovitamin D_3, 77531-55-6; 5(*E*)-19-nor-10-ketovitamin D_3, 62743-72-0; 5(*E*)-19-nor-10-ketovitamin D_2, 85925-89-9; 5(*E*)-19-nor-10-keto-25-hydroxyvitamin D_3, 85925-90-2.

[2] J. L. Sommerfeldt, J. L. Napoli, R. Gardner, D. C. Beitz, E. T. Littledike, and R. L. Horst, unpublished results.

References

Barton, D. H. R., & Patin, H. (1976) *J. Chem. Soc., Perkin Trans. 1*, 829–831.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Harrison, I. T., & Lythgoe, B. (1958) *J. Chem. Soc.*, 837–842.

Hibbs, J. W., & Pounden, W. D. (1955) *J. Dairy Sci. 38*, 65–72.

Inhoffen, H. H., Irmscher, K., Hirschfeld, H., Stache, U., & Kreutzer, A. (1958) *Chem. Ber. 91*, 2309–2324.

Inhoffen, H. H., Irmscher, K., Hirschfeld, H., Stache, U., & Kreutzer, A. (1959) *J. Chem. Soc.*, 385–386.

Lawson, D. E. M. (1980) in *Vitamin D: Molecular Biology and Clinical Nutrition* (Norman, A. W., Ed.) pp 93–126, Marcel Dekker, New York.

Littledike, E. T., & Horst, R. L. (1982) *J. Dairy Sci. 65*, 749–759.

Okamura, W. H., Hammond, M. L., Jacobs, H. J. C., & Thuiyl, J. V. (1976) *Tetrahedron Lett. No. 52*, 4807–4810.

Sommerfeldt, J. L., Horst, R. L., Littledike, E. T., & Beitz, D. C. (1979) *J. Dairy Sci. 62 (Suppl. 1)*, 192, Abstr.

Sommerfeldt, J. L., Horst, R. L., Littledike, E. T., Beitz, D. C., & Napoli, J. L. (1982) in *Vitamin D: Endocrinological Aspects and Their Clinical Applications* (Norman, A. W., & Schaefer, K., Eds.) pp 23–26, de Gruyter, New York.

Spectral Properties of Three Quaternary Arrangements of *Pseudomonas* Pilin†

Tania H. Watts, Cyril M. Kay, and William Paranchych*

ABSTRACT: *Pseudomonas aeruginosa* possess multisubunit, filamentous appendages called pili which are involved in adhesion, twitching motility, and bacteriophage adsorption. The spectral properties of three forms of pili have been compared. These are native pili, pilin dimers in octyl glycoside, and an in vitro assembled form of pilin which we call reassembled pilin filaments. Alkaline pH titrations, solvent perturbation, quenching of tryptophan fluorescence with acrylamide, and circular dichroism were used to demonstrate that tyrosines-24 and -27 are at a dimer/dimer interface in both native pili and in the reassembled pilin filaments. Dissociation of pili by octyl glucoside results in exposure of the two tyrosines and in partial exposure of a least one tryptophan in pilin.

Pili are filamentous, multisubunit appendages distinct from flagella found on the surfaces of bacteria. *Pseudomonas aeruginosa* pili are involved in such processes as adhesion of the bacteria to host mucosal surfaces (Woods et al., 1980), twitching motility (Bradley, 1980), and bacteriophage adsorption (Bradley & Pitt, 1974). The pili appear to be able to retract into the bacterial cell, thereby bringing attached bacteriophage in contact with the cell surface (Bradley, 1974). To explain the mechanism of assembly and disassembly of pili, it is important to understand the interactions at the regions of subunit contact. To do this we have compared the spectral properties of three different arrangements of pilin subunit. As will be seen below, these are native pili, pilin dimers in octyl glucoside (Watts et al., 1982a), and reassembled pilin filaments (Watts et al., 1982b).

Native pili consist of a single subunit, pilin, of M_r 15 000, according to its sequence (Sastry et al., 1983). Previous publications (Frost & Paranchych, 1977; Paranchych et al., 1979) had suggested a molecular weight of 18 000 based on migration of pilin in sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels and upon its amino acid composition. This led Folkhard et al. (1981) to interpret from X-ray fiber diffraction data that native pili consist of 4.06–4.08 subunits in

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0006-2960/83/0422-3640$01.50/0

a 41-Å turn of helix. In light of the sequence data of Sastry et al. which suggest a smaller subunit, this must be reinterpreted as 5.06–5.08 pilin subunits per turn. Native pili have the overall appearance of a hollow cylinder of 5.2-nm diameter and a central channel of 1.2-nm diameter (Folkhard et al., 1981).

Pilin in octyl glucoside has been characterized previously (Watts et al., 1982a). On the basis of sedimentation–equilibrium data it was suggested that the pilin/octyl glucoside complex, at low protein concentrations, consists of a mixture of monomers and dimers. In fact, it will be shown below that the major species of pilin in octyl glucoside is a dimer.

Reassembled pilin filaments are flexible rods of twice the diameter of native pili. They are formed in vitro by removal of octyl glucoside from the pilin dimers in octyl glucoside (Watts et al., 1982b).

In the present study we have used absorption spectroscopy to follow the alkaline pH titration of two tyrosines in the three forms of pilin aggregate. In addition, solvent perturbation with dimethyl sulfoxide (Me_2SO) and acrylamide quenching of tryptophan fluorescence were used as a further probe of the environments of aromatic amino acids in the three forms of pilin.

Materials and Methods

Bacteria. The *Pseudomonas aeruginosa* strain used was PAK/2Pfs, a multipiliated mutant of PAK which has been shown to produce pili that are indistinguishable from wild-type PAK (Frost & Paranchych, 1977).

Reagents. Octyl glucoside (*n*-octyl β-D-glucopyranoside) was obtained from Sigma Chemical Co. Dimethyl sulfoxide (analytical grade) and acrylamide (electrophoresis pure) were obtained from BDH Chemicals Ltd. Unless otherwise stated, all samples were in 0.05 M Na_2HPO_4, pH 7.

Preparations of Three Forms of Pili. Purification of native pili has been described previously (Paranchych et al., 1979). The pilin dimers in octyl glucoside and the reassembled pilin filaments were prepared as described in Watts et al. (1982b).

Gel Exclusion Chromatography. The pilin molecular weight in octyl glucoside was determined by gel exclusion chromatography on a Sephadex G-200 column (Pharmacia Fine Chemicals) of dimensions 1 × 25 cm which had been equilibrated with 2 column volumes of octyl glucoside and 0.05 M Na_2HPO_4, pH 7.0. A calibration run was carried out under the same conditions with 0.5 mg each of bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), myoglobin (M_r 17 000), cytochrome *c* (M_r 13 000), and bacitracin (M_r 1411). Two separate calibration runs were carried out: one using BSA, myoglobin, and bacitracin and the other using ovalbumin, cytochrome *c*, and bacitracin. This allowed the standards which are close together in elution position to be resolved.

Octyl glucoside binding to the standard proteins was determined by equilibrium dialysis with ^{14}C-labeled octyl glucoside as described in Watts et al. (1982a). The specific activity of the [^{14}C]-octyl glucoside was 34 000 cpm/μmol. The samples contained 80–90 nmol of protein/mL, and the detergent concentration was 30 mM. The equilibrium dialysis cells consisted of two 1-mL compartments separated by M_r 6000–8000 cutoff dialysis tubing.

Protein Concentration. All protein concentrations were calculated from the extinction coefficient at 280 nm of 1.3_2 for a 1 mg/mL solution of pili in a 1-cm cell. A similar value was obtained for pilin in octyl glucoside. This value was determined by measuring the absorbance of samples whose concentrations were determined by quantitative amino acid analysis carried out by the method of Moore (1963). It should be noted that to eliminate scattering problems, longitudinal aggregates (Watts et al., 1982a) were removed by centrifugation for 5 min at 15600*g* in an Eppendorf microcentrifuge. The supernatant was then removed, its absorbance was measured, and aliquots were removed for amino acid analysis. Protein concentrations of standards were by the method of Lowry.

Circular Dichroism. Circular dichroism (CD) measurements were carried out on a Jasco J500C spectropolarimeter according to the methodology of Oikawa et al. (1968) with pantoyl lactone as an additional standard. Mean residue ellipticities were calculated by using a mean residue molecular weight of 107 [determined from the amino acid sequence of Sastry et al. (1983)]. Longitudinal aggregates of pili were removed, as mentioned above, to reduce the light scattering problems. During the run, the dynode voltage did not exceed 0.6 kV at 200 nm. The fraction α-helix and β-sheet were determined by using a program developed by Provencher & Glöckner (1981) which analyzes CD spectra as a sum of spectra of 16 proteins whose structures are known from X-ray crystallography. The input to the program was the molar ellipicities in 1-nm intervals from 190 to 240 nm. As a check on the calibration of the instrument, a CD spectrum was obtained for hen egg white lysozyme (one of the proteins in the reference set) and run through the program. We obtained 41% α-helix and 22% β-structure for lysozyme in agreement with the results of Provencher and Glöckner.

Alkaline pH Titration. Samples were prepared for measurement of absorbance and ellipticity as a function of pH as follows: 0.2 M buffers were prepared at each pH by using sodium phosphate or sodium bicarbonate buffers; 2 mg/mL solutions of each of the three forms of pilin aggregate were diluted 2-fold into each buffer solution, and the sample was used both for determination of the ellipticity at 222 nm and for a UV absorbance scan from 350 to 260 nm. In the case of pilin/octyl glucoside, the buffers were made up in octyl glucoside to maintain the detergent concentration. Absorption measurements were made on a Cary Model 219 spectrophotometer in 1-cm matched cells. Examination of the UV absorption spectra between 350 and 320 nm showed that light scattering was minimal provided that the samples had been centrifuged to remove large aggregates, as mentioned above.

Solvent Perturbation Measurements. Solvent perturbation experiments were carried out on a Cary 118C spectrophotometer operated in the autoslit mode. Difference spectra were recorded at a full scale absorbance range of 0.05 at a scan rate of 0.1 nm/s. The reference and sample cells were thermostated with a circulating water bath (Lauda K 2R thermoregulator). The experiments used split compartment tandem cells with a path length of 0.874 cm. The base line was generated with 2 mg/mL protein in both the reference and sample cells in one compartment and with 40% Me_2SO in the other compartment of both cells (the Me_2SO was made up in octyl glucoside for the experiments with pilin/octyl glucoside). The contents of the sample cell were mixed, and the spectrum was recorded. The contents of the reference cell were then mixed, and a second base line was recorded. If the second base line differed substantially from the first, the results were discarded. The perturbation spectra of *N*-acetyltryptophan and *N*-acetyltyrosine ethyl esters were determined separately and as a 1:1 molar ratio so that the contribution of tryptophan and tyrosine at each wavelength could be recorded. The effect of octyl glucoside on these spectra was also tested.

Fluorescence Measurements. Fluorescence measurements were carried out on a Perkin-Elmer MPF-44B spectrofluo-

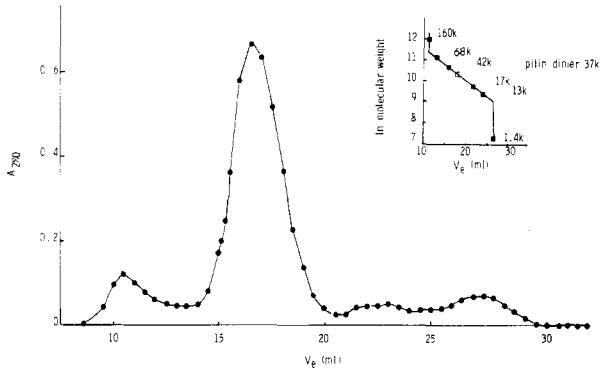

FIGURE 1: Gel exclusion chromatography of pilin in octyl glucoside on a Sephadex G-200 column as described under Materials and Methods. The inset shows a calibration run. The elution positions of the standard proteins are filled squares, and the open square is the elution position of the major pilin peak.

rometer operating in the ratio mode with 5-nm bandwidths for excitation and emission slits. A constant temperature of 20 °C was maintained in the cells by a circulating water bath. The excitation wavelength used was 295 nm to ensure that light absorption was primarily due to tryptophan. The initial A_{295} of the protein was ≤ 0.05 to avoid the inner filter effect. The fluorescence quenching was measured at the emission maximum of the sample and was initiated by the addition of 10-μL aliquots of 8 M acrylamide solutions to both the sample and a blank (to correct for acrylamide absorption). Cells of 1-cm path length were used, and stirring was by a magnetic "flea". The protein solutions were 2.0 mL. The results were plotted as F_0/F, where F_0 is the initial fluorescence and F is the fluorescence after addition of quencher, corrected for dilution of the protein.

Results and Discussion

Gel Exclusion of Pilin in Octyl Glucoside. Figure 1 shows the elution properties of pilin on Sephadex G-200 in the presence of 30 mM octyl glucoside. The inset shows a calibration of the same column in octyl glucoside by using protein standards. Since pilin in detergent has been shown to be globular (f/f_0 = 1.04; Watts et al., 1982a), the standards chosen were globular proteins. Octyl glucoside does not, in general, denature proteins, and therefore it is expected that the standards maintain their globular conformation during the calibration. It can be seen that the major peak (representing 87% of the material applied) migrates as an apparent molecular weight of 37 000. Equilibrium dialysis measurements have shown that a 1 mg/mL solution of pilin in 30 mM octyl glucoside binds 0.24 g of octyl glucoside/g of protein, or 12.5 ± 1 octyl glucoside per pilin. This results in a monomer/octyl glucoside molecular weight of 18 650 or a dimer of 37 300, assuming that the standards are not binding detergent. Octyl glucoside binding to bovine serum albumin, ovalbumin, and myoglobin was determined by equilibrium dialysis as described under Materials and Methods. No binding of octyl glucoside was observable for the three proteins under conditions where 5 mol of octyl glucoside/mol of protein would be readily detected. Therefore, it was concluded that any detergent binding to the standards was too small to effect its position on the standard curve. This finding is in agreement with the results of Helenius & Simons (1972), who have demonstrated that hydrophilic proteins do not, in general, bind nonionic detergents.

The apparent molecular weight obtained by gel exclusion chromatography in octyl glucoside (37 000) is consistent with sedimention-equilibrium experiments (Watts et al., 1982a) which show a molecular weight range of 38 000–24 000 at loading concentrations of 0.5 mg/mL. The small amount of material at the void volume of the column is aggregated pili, and the small peak eluting at about 22 mL may be the pilin monomer, while the last peak at 27 mL was ninhydrin negative.

The fact that the major species of pilin is a dimer in octyl glucoside raises the question of why a helical arrangement of identical subunits would form dimers upon dissociation. The most efficient way for the pilus to be constructed is to use the same packing arrangement repeatedly (Crick & Watson, 1956). Thus, one would expect all subunits to occupy an equivalent environment and be dissociated into monomers by breaking 100% of a specific set of bonds. However, if the basic building unit were a dimer of two identical subunits related by a 2-fold rotational axis of symmetry, then one could expect dissociation into dimers by disrupting a specific set of interactions while maintaining the interaction that relates the two halves of the dimer. This is shown schematically in Figure 2.

Figure 2 shows a model of the *Pseudomonas* pilus based loosely on X-ray diffraction (Folkhard et al., 1981) and hydrodynamic studies (Watts et al., 1982a). Figure 2A shows a scale model of the pilus as viewed by electron microscopy indicating its asymmetry. Figure 2B shows the dimensions of the pilus that have been established by X-ray fiber diffraction, namely, an outer diameter of 52 Å, a central channel of 12-Å diameter, and a pitch of 41 Å. Five hypothetical subunits are shown in each turn of the roughly cylindrical pilus. The subunit is fairly globular, but its exact shape and orientation are not known. The particular orientation shown in Figure 2B is consistent with low-resolution X-ray diffraction data as determined by computer model building (our unpublished results). The surface lattice representation in Figure 2C is useful in discussions of the symmetry of the structure. One can imagine that the cylindrical pilus has been cut open along a line that parallels its long axis and opened flat. Each dot in Figure 2C represents the origin of one subunit. One can see that if the basic building block were a dimer related by a 2-fold axis of symmetry as outlined in Figure 2, at low resolution the structure would be the same and should produce the same periodicity on the diffraction pattern.

Aromatic Residues in Pilin. The sequence of *Pseudomonas* pilin is shown in Figure 3 [reproduced from Sastry et al. (1983)]. It can be seen that there are two tyrosines at positions 24 and 27 and two tryptophans at positions 55 and 128. The tyrosines are found in an extremely hydrophobic N-terminal stretch of 29 residues containing only one charged group. Since octyl glucoside breaks hydrophobic interactions, we hypothesized that octyl glucoside binds in this region to disrupt subunit/subunit interactions. Since the only two tyrosines in the molecule are found in this region, it is of interest to examine the spectral properties of the tyrosines in the presence and absence of octyl glucoside.

Circular Dichroism. The far-UV circular dichroism of the three forms of pilin aggregate is shown in Figure 4. As has been shown previously (Watts et al., 1982a), the far-UV CD of native pili and pilin/octyl glucoside is indistinguishable. The far-UV CD of reassembled pilin filaments, however, shows a slight enhancement of the ellipicity at 222 nm. Previously (Watts et al., 1982a), the CD spectrum had only been obtained down to 210 nm for pilin. These data are extended to 190 nm, allowing the application of a program for calculating secondary structure (Provencher & Gloeckner, 1981) which uses the data in 1-nm intervals from 190 to 240 nm. Using this program, we calculate that native pili and pilin/octyl glucoside have 40%

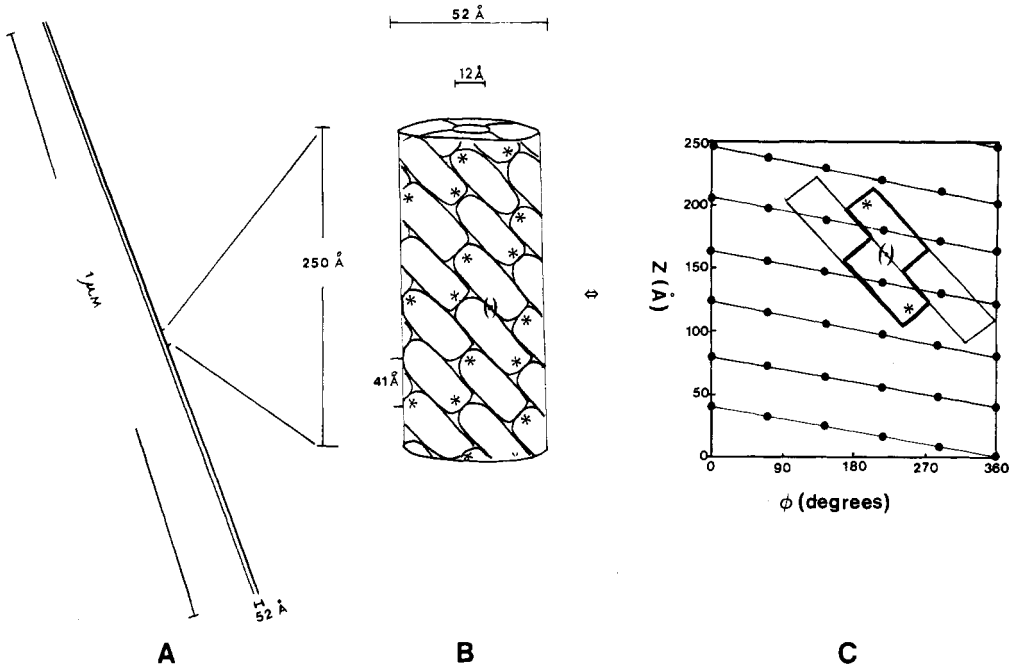

FIGURE 2: Model for pilus structure based on X-ray diffraction and hydrodynamic data. (A) A scaled representation of the intact pilus. (B) A schematic representation showing one of several possible subunit orientations and shapes. (■) indicates one of the 2-fold axes relating two halves of the dimer; each star can be rotated by 180° into the star in the other half of the dimer. (C) Surface lattice representation indicating the 5 unit per turn symmetry indicated by X-ray diffraction data. One dimer is outlined in heavy lines. z represents the distance along an axis parallel to the long axis of the pilus; ϕ is the rotation (using cylindrical polar coordinates).

```
                                                  10
N-MePhe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Val-Ala-Ile-Ile-Gly-Ile-

            20                                                30
Leu-Ala-Ala-Ile-Ala-Ile-Pro-Gln-Tyr-Gln-Asn-Tyr-Val-Ala-Arg-Ser-

                              40
Glu-Gly-Ala-Ser-Ala-Leu-Ala-Ser-Val-Asn-Pro-Leu-Lys-Thr-Thr-Val-

      50                                                60
Glu-Glu-Ala-Leu-Ser-Arg-Gly-Trp-Ser-Val-Lys-Ser-Gly-Thr-Gly-Thr-

                        70
Glu-Asp-Ala-Thr-Lys-Lys-Glu-Val-Pro-Leu-Gly-Val-Ala-Ala-Asp-Ala-

80                                      90
Asn-Lys-Leu-Gly-Thr-Ser-Ile-Ala-Leu-Lys-Pro-Asp-Pro-Ala-Asp-Gly-

                  100                                     110
Asp-(Thr,Ala)-Ile-Thr-Leu-Thr-Phe-Thr-Met-Gly-Gly-Ala-Gly-Pro-Gly

                              120
Lys-Asn-Lys-Lys-Ile-Ile-Thr-Leu-Thr-Arg-Thr-Ala-Ala-Asp-Gly-Leu-

      130                                               140
Trp-Lys-Cys-Thr-Ser-Asp-Gln-Asp-Glu-Gln-Phe-Ile-Pro-Lys-Gly-Cys-

Ser-Lys-COOH
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FIGURE 3: Primary structure of *Pseudomonas aeruginosa* pilin [reproduced from Sastry et al. (1983)]. The aromatic residues are underlined.

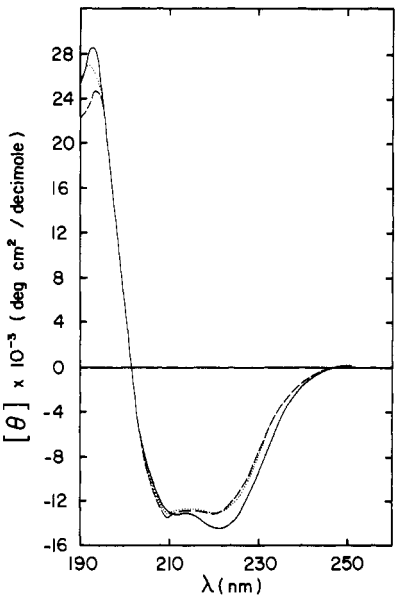

FIGURE 4: Far-UV circular dichroism of the three forms of pilin. (---) Native pili, (···) pilin dimers in octyl glucoside, and (—) reassembled pilin filaments. Spectra were obtained as described under Materials and Methods. [θ] = the mean residue ellipticity.

α-helix and 38% β-structure, in agreement with previous calculations based on less data (Watts et al., 1982a). We calculate a 1% increase in apparent α-helix and a 3% increase in β-structure for the reassembled pilin filaments. These results show that no gross changes in backbone conformation occur during the interconversion of the three forms of pili, so that changes in tyrosine and tryptophan accessibility can be attributed largely to dissociation rather than denaturation.

The near-UV CD of the three forms of pilin is shown in Figure 5. The noise in these spectra was quite high due to the low content of aromatic residues in pilin. However, one striking feature of the near-UV CD of native pili at neutral pH is the intense CD at 297 nm, which we attribute to tryptophan (Strickland, 1972). At pH 12.5, on the other hand, the ellipticity at 297 is the same for all three forms of pilin. At this point pH, considerable denaturation has occurred (Figure 6), and some dissociation is indicated by a reduction in sedimentation coefficient (not shown). At this point we cannot determine the cause of the unusually intense tryptophan CD at neutral pH in native pili. However, we can speculate that it arises from an interaction with another ring system that does not take place in dissociated pilin, perhaps across a subunit/subunit interface. In addition, it appears that this interaction cannot take place in reassembled pili as it is not apparent in the near-UV CD.

Alkaline pH Titration of Tyrosine Residues. One approach to determine the accessibility of tyrosines is to examine the titration behavior of the phenolic hydroxyl group (Donovan, 1964). The pK of this group may be altered if the hydrogen is involved in hydrogen bonding or if the tyrosine is buried in the protein and thus shielded from hydroxyl ions (Herskovits,

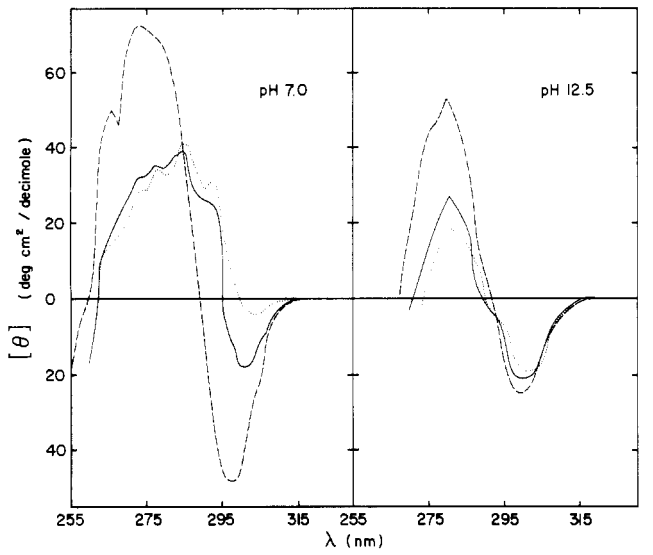

FIGURE 5: Near-UV circular dichroism of the three forms of pilin aggregate. (---) Native pili, (—) pilin/octyl glucoside, and (···) reassembled pilin filaments.

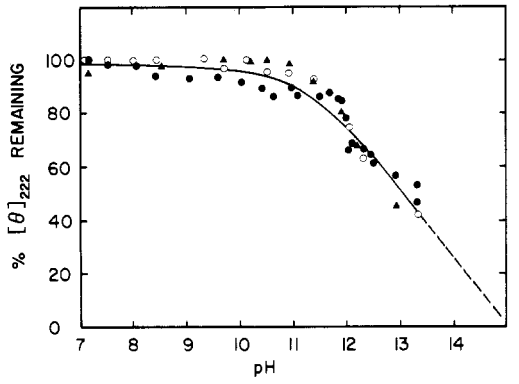

FIGURE 6: Effect of alkali on the mean residue ellipticity at 222 nm of three forms of pilin aggregate. (●) Native pili, (○) pilin dimers in octyl glucoside, and (▲) reassembled pilin filaments.

1968). In the latter case one expects titration to occur only after some denaturation has occurred. It is therefore necessary to determine the alkali stability of the protein in question. Figure 6 shows the effect of increasing pH on the ellipticity at 222 nm for the three forms of pilin. It can be seen that very little effect is seen until about pH 11 and that all three forms respond to alkali in a similar manner. Neutralization of pili which had been treated at pH 13 resulted in regeneration of the original CD spectrum, suggesting that the alkali denaturation was reversible up to this point. The protein appears to be very stable to alkali treatment. In addition, thermal denaturation or addition of guanidine hydrochloride to 5 M results in a loss of only 40–50% of the ellipticity at 222 nm (not shown). This suggests that there is a core of protein that is resistant to denaturation. This is not surprising since pili are extracellular proteins and therefore must be resistant to a varied environment.

Figure 7 shows the effect of pH on the absorbance at 290 nm for the three forms of pili. With free tyrosine, ionization of the hydroxyl usually results in the appearance of a peak at 293–295 nm (Herskovits, 1968). This maximum appears to be slightly blue shifted in the case of *Pseudomonas aeruginosa* pili. The pilin dimers in octyl glucoside show a titration with a pK of 9.9_5. This value is in the range of values published for free tyrosine [reviewed in Herskovits (1968)], suggesting that the tyrosines are on the surface in the pilin dimers. If one uses the published extinction coefficient for ionization of

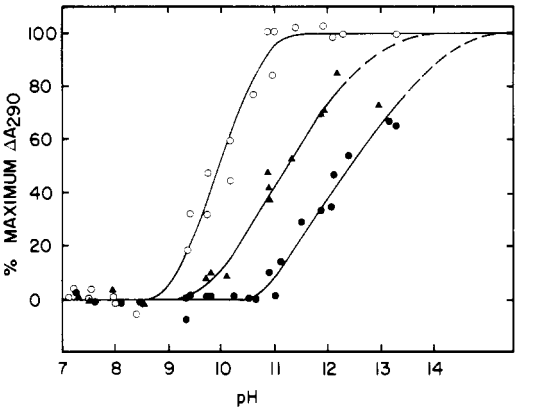

FIGURE 7: Alkaline pH titration of pili. (●) Native pili, (○) pilin dimers in octyl glucoside, and (▲) reassembled pilin filaments (see Materials and Methods for details).

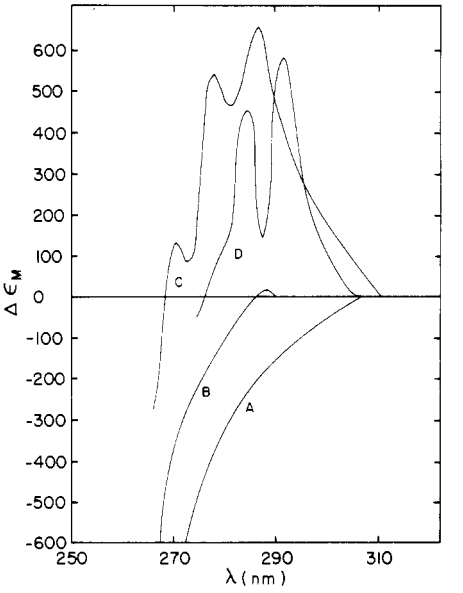

FIGURE 8: Solvent perturbation of pili with 20% Me_2SO. (A) Native pili, (B) reassembled pilin filaments, (C) pilin dimers in octyl glucoside, and (D) 1:1 molar ratio of *N*-acetyltryptophan and *N*-acetyltyrosine ethyl esters in octyl glucoside.

tyrosine of 2300 M^{-1} (Herskovits, 1968), one calculates that 1.97 tyrosines are titrating in the pilin dimers. This titration occurs over a range of 2 pH units, which is not unreasonably broad for a single transition. However, it is possible that there are two components to the titration due to differences in the pKs of tyrosines-24 and -27; however, these are not sufficiently different to be resolved in this experiment. Thus, it appears that tyrosines-24 and -27 are indistinguishable with respect to titration behavior in the case of the pilin dimers in octyl glucoside.

In the case of reassembled pilin filaments and native pili, the titration was not completed by pH 13. The change in molar extinction coefficient at 290 nm for these titrations suggests that 1.3 tyrosines have been titrated by pH 13.3 for native pili, while 1.4 tyrosines have been titrated in the reassembled filaments. By extrapolation to the full change of 4600 M^{-1} for the ionization of two tyrosines, one obtains a pK of 11.0 for reassembled pilin filaments and a pK of 11.9 for the tyrosine residues in native pili. Therefore, it is evident that some denaturation–dissociation must occur before the tyrosines in the filamentous forms of pilin are accessible to titration. The broadness of the transition, which occurs over a range of 3 pH units, is also indicative of this fact.

Solvent Perturbation. An alternate approach we have used to look at the tyrosine accessibility and to obtain information on the accessibility of tryptophan is the technique of solvent perturbation [as described by Herskovits & Laskowski (1962a)]. Figure 8 shows that perturbation spectrum of the three forms of pilin with 20% Me_2SO compared with the effect of Me_2SO on a 1:1 molar ratio of *N*-acetyltryptophan and *N*-acetyltyrosine ethyl esters in octyl glucoside. Me_2SO does not change the morphology of pili or reassembled pilin filaments as determined by electron microscopy (not shown). It was not possible, however, to obtain CD data below 230 nm with Me_2SO present. Nevertheless, Me_2SO is commonly used in this type of experiment because it causes a reasonably large perturbation without damaging protein structure (Herskovits & Laskowski 1962a,b). Curve C in Figure 8 shows the difference spectrum obtained for the pilin dimers in octyl glucoside. The difference spectrum is blue shifted with respect to the model compound spectrum, as was the case in the alkaline difference spectrum. If one solves two equations in two unknowns [as described in Herskovits (1968)] for the contribution of tyrosine and tryptophan to each of the two maxima using the extinction coefficients obtained for the model compounds in octyl glucoside and Me_2SO, one can calculate that 1.4 tyrosines and 0.9 tryptophan are fully exposed as compared to the model compounds. This could also be interpreted as two tyrosines 70% exposed and two tryptophans 45% exposed.

Curves A and B show the effect of Me_2SO on native pili and reassembled pilin filaments, respectively. It appears that the major effect here is a scattering effect. Pili being rather asymmetric tend to scatter light. In addition they have a tendency to aggregate along their long axes [see Watts et al. (1982b)]. If Me_2SO decreased or increased the aggregation of the particles, then the difference spectrum on exposing pili to Me_2SO would indicate this scattering. If tyrosines or tryptophans in pili or reassembled pilin filaments are perturbed by Me_2SO, the effect appears to be much smaller than that in the pilin dimers as it is not visible above the scattering. It should be mentioned that it was not possible to judge from electron microscopy whether or not there was a large change in the aggregation state of the pili.

Quenching of Tryptophan Fluorescence by Acrylamide. An additional approach we have used to gain information about tryptophan accessibility was to look at the quenching of the intrinsic fluorescence due to tryptophan by acrylamide. (Eftink & Ghiron, 1976). Acrylamide was chosen as the quenching agent to avoid problems with ionic interactions, especially since the sequence shows lysine residues close to both tryptophan residues. The excitation wavelength used was 295 nm so that only tryptophan fluoresces. The emission maxima obtained were as follows: L-tryptophan, 347 nm; L-tryptophan in octyl glucoside, 343 nm; native pili, 336 nm; pilin in octyl glucoside, 333 nm. Thus, octyl glucoside causes a slight blue shift in the emission maximum of both pili and free tryptophan. The shift in fluorescence maximum for pili compared to free tryptophan is suggestive of a more hydrophobic environment. Upon dissociation of pilin by octyl glucoside the emission maximum changes by 3 nm. However, a shift in emission maximum of 3 nm was also observed when octyl glucoside was added to free tryptophan, suggesting that there is no additional effect besides the direct detergent effect.

The effect of acrylamide on tryptophan fluorescence of free tryptophan, native pili, pilin/octyl glucoside, and tryptophan in octyl glucoside is shown in Figure 9. The upward curvature of the plot of F_0/F vs. acrylamide concentration for free

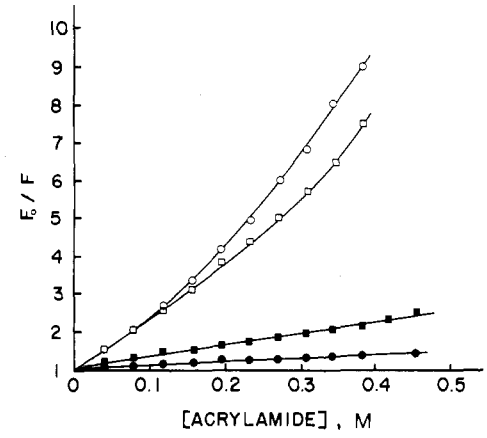

FIGURE 9: Acrylamide quenching of tryptophan fluorescence in pili. (○) L-Tryptophan, (●) native pili, (□) L-tryptophan in octyl glucoside, and (■) pilin/octyl glucoside.

tryptophan has been attributed to a static quenching component (Eftink & Ghiron, 1976). This upward curvature is also observed for single tryptophan-containing proteins, while proteins which show multicomponent quenching often give rise to plots which are linear or show downward curvature. Comparison of the curves for native pili and pilin/octyl glucoside suggests that at least one of the tryptophans in pili becomes more accessible to acrylamide quenching upon dissociation into dimers. However, comparison with the effect of acrylamide on free tryptophan suggests that the effect is less than that expected if a tryptophan were fully exposed to the medium. Since the quenching effect is small for both native pili and for the pilin dimers, it is difficult to assess whether the plots are linear or show an upward curvature, and hence, it is difficult to evaluate whether the quenching effect is due to one or both tryptophan residues in pilin.

Conclusions

On the basis of the above results, it appears that tyrosines-24 and -27 are at a subunit/subunit interface in pili and become exposed to the medium upon dissociation of pilin into dimers by octyl glucoside. The fact that the two tyrosines are close together in the sequence and that they are indistinguishable with respect to alkaline pH titration simplifies the data since one can assume that tyrosines-24 and -27 occupy a similar environment and hence can be treated as one residue. The unusually high p*K* of the tyrosines in native pili is attributed to their being buried at the subunit interface. An additional factor may be that the tyrosines are stacked either with each other (this is possible if they are at position *n* and *n* + 3 of an α-helix) or that they interact with another aromatic ring across the subunit interface. It is possible that the region around residues 24–27 interacts with an alternate region of pilin in making dimer/dimer contact. This possibility is shown schematically in Figure 10A. On the other hand, the region containing the two tyrosines might interact with the same region on another pilin subunit forming a head to head packing arrangement as shown in Figure 10B. In both these figures all solid lines represent interactions that are disrupted in octyl glucoside, leaving pilin dimers and exposing all tyrosines. In both cases, the symmetry dictated by the X-ray diffraction data (Folkhard et al., 1981) is obeyed.

In the case of the reassembled pilin filaments, the alkaline pH titration results suggest that roughly the same interface around tyrosines-24 and -27 is used in assembly but that the packing arrangement is less tight. This is in agreement with the appearance of reassembled pilin filaments in the electron

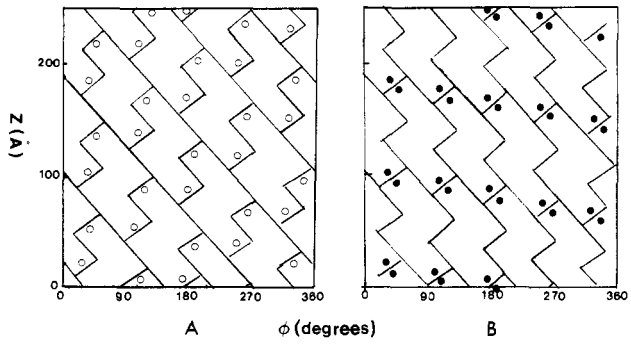

FIGURE 10: Schematic representation of two possible packing arrangements of pilin dimers in which all tyrosines (each pair of tyrosines-24 and -27 is represented by a circle) are exposed upon dissociation in octyl glucoside. The solid lines represent interactions between dimers that are broken in octyl glucoside. The surface lattice representation has been described in the text. (A) A head to tail packing of subunits and (B) a head to head packing of subunits with respect to tyrosine.

microscope (Watts et al., 1982b).

Our results are less conclusive for tryptophan residues. It appears that both tryptophans are buried in native pili and that dissociation into dimers results in increased exposure of at least one tryptophan. With reassembled pilin filaments it appears that at least one tryptophan occupies a different environment than its counterpart in native pili or pilin dimers.

It should be noted that in an assembly such as pili, one would expect a large portion of each subunit to be interacting with other pilin subunits, so that the hydrophobic region around tyrosines-24 and -27 is not sufficient to account for pilus assembly. Nevertheless, the fact that a detergent, octyl glucoside, disrupts most of these interactions suggests that the major forces holding pili together are hydrophobic interactions.

Acknowledgments

We thank Dr. W. McCubbin for advice on use of the fluorometer and for helpful discussions and K. Oikawa for carrying out the circular dichroism measurements.

Registry No. L-Tyrosine, 60-18-4; L-tryptophan, 73-22-3.

References

Bradley, D. E. (1974) *Virology 58*, 149–163.
Bradley, D. E. (1980) *Can. J. Microbiol. 26*, 146–154.
Bradley, D. E., & Pitt, T. L. (1974) *J. Gen. Virol. 24*, 1–15.
Crick, F. H. C., & Watson, J. D. (1956) *Nature (London) 177*, 473–475.
Donovan, J. W. (1964) *Biochemistry 3*, 67–74.
Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.
Folkhard, W., Marvin, D. A., Watts, T. H., & Paranchych, W. (1981) *J. Mol. Biol. 149*, 79–93.
Frost, L. S., & Paranchych, W. (1977) *J. Bacteriol. 131*, 259–269.
Helenius, A., & Simons, K. (1972) *J. Biol. Chem. 247*, 3656–3661.
Herskovits, T. T. (1968) *Methods Enzymol. 11*, 748–775.
Herskovits, T. T., & Laskowski, M. (1962a) *J. Biol. Chem. 237*, 2481–2492.
Herskovits, T. T., & Laskowski, M. (1962b) *J. Biol. Chem. 237*, 3418–3422.
Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.
Oikawa, K., Kay, C. M., & McCubbin, W. D. (1968) *Biochim. Biophys. Acta 168*, 164–167.
Paranchych, W., Sastry, P. A., Frost, L. S., Carpenter, M., Armstrong, G. D., & Watts, T. H. (1979) *Can. J. Microbiol. 25*, 1175–1181.
Provencher, S., & Glöckner, J. (1981) *Biochemistry 20*, 33–37.
Sastry, P. A., Pearlstone, J. R., Smillie, L. B., & Paranchych, W. (1983) *FEBS Lett. 151*, 253–256.
Strickland, E. H. (1972) *CRC Crit. Rev. Biochem. 2*, 113–175.
Watts, T. H., Kay, C. M., & Paranchych, W. (1982a) *Can. J. Biochem. 60*, 867–872.
Watts, T. H., Scraba, D. G., & Paranchych, W. (1982b) *J. Bacteriol. 151*, 1508–1513.
Woods, D. E., Strauss, D. C., Waldermar, G. J., Jr., Berry, V. K., & Bass, J. A. (1980) *Infect. Immun. 29*, 1146–1151.