Kinetics and Sequence Specificity of Processing of Prepilin by PilD, the Type IV Leader Peptidase of *Pseudomonas aeruginosa*

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PilD, originally isolated as an essential component for the biogenesis of the type IV pili of *Pseudomonas aeruginosa*, is a unique endopeptidase responsible for processing the precursors of the *P. aeruginosa* pilin subunits. It is also required for the cleavage of the leader peptides from the Pdd proteins, which are essential components of an extracellular secretion pathway specific for the export of a number of *P. aeruginosa* hydrolytic enzymes and toxins. Substrates for PilD are initially synthesized with short, i.e., 6- to 8-amino-acid-long, leader peptides with a net basic charge and share a high degree of amino acid homology through the first 16 to 30 residues at the amino terminus. In addition, they all have a phenylalanine residue at the +1 site relative to the cleavage site, which is N methylated prior to assembly into the oligomeric structures. In this study, the kinetics of leader peptide cleavage from the precursor of the *P. aeruginosa* pilin subunit by PilD was determined in vitro. The rates of cleavage were compared for purified enzyme and substrate as well as for enzyme and substrate contained within total membranes extracted from *P. aeruginosa* strains overexpressing the cloned *pilD* or *pilA* genes. Optimal conditions were obtained only when both PilD and substrate were contained within total membranes. PilD catalysis of *P. aeruginosa* prepilin followed normal Michaelis-Menten kinetics, with a measured apparent K_m of approximately 650 μM , and a k_{cat} of 180 min^{-1} . The kinetics of PilD processing of another type IV pilin precursor, that from *Neisseria gonorrhoeae* with a 7-amino-acid-long leader peptide, were essentially the same as that measured for wild-type *P. aeruginosa* prepilin. Quite different results were obtained for a number of prepilin substrates containing substitutions at the conserved phenylalanine at the +1 position relative to the cleavage site, which were previously shown to be well tolerated in vivo. Substitutions of methionine, serine, and cysteine for phenylalanine show that K_m values remain close to that measured for wild-type substrate, while k_{cat} and k_{cat}/K_m values were significantly decreased. This indicates that while the affinity of enzyme for substrate is relatively unaffected by the substitutions, the maximum rate of catalysis favors a phenylalanine at this position. Interestingly, PilD cleavage of one mutated pilin (asparagine) resulted in a lower K_m value of 52.5 μM , which indicates a higher affinity for the enzyme, as well as a lower k_{cat} value of 6.1 min^{-1} . This suggests that it may be feasible to design peptide inhibitors of PilD.

The opportunistic pathogen *Pseudomonas aeruginosa* causes high rates of morbidity and mortality in people with severe burns, cystic fibrosis, or other immunocompromising conditions (5). The organism produces a number of factors which have been postulated to contribute to virulence. These include extracellular appendages called pili, which, along with one or more nonpilus adhesins (35), mediate adherence of the organism to epithelial cells (39). *P. aeruginosa* also secretes a number of hydrolytic enzymes and exotoxins into the extracellular environment, including exotoxin A, elastase, and phospholipase C, which aid colonization of infected tissues (22).

Pili of *P. aeruginosa* belong to the type IV class (19), which includes pili from a diverse number of gram-negative prokaryotes, including *Neisseria gonorrhoeae* (25), *Moraxella bovis* (23), and *Dichelobacter* (formerly *Bacteroides*) *nodosus* (24). Pilins, the monomeric subunits of the type IV pili, are synthesized with a 6- or 7-amino-acid basic leader sequence (Fig. 1A). The more distantly related members of the type IV family, the toxin-coregulated pili of *Vibrio cholerae*, are synthesized with a longer leader sequence but share many of the structural features found in other type IV

pilins in the region around the cleavage site and the amino terminus of the mature polypeptide (34). These include an invariant glycine on the amino side of the scissile bond (at the -1 position), the presence of a phenylalanine (a methionine in toxin-coregulated pili) at the +1 position, and a stretch of hydrophobic amino acids in the amino-terminal region of the mature pilin, with an invariant glutamic acid at position +5. The first amino acid of the mature pilin subunit is in all cases N methylated.

Previous studies from this laboratory have identified three genes, *pilB*, *pilC*, and *pilD*, whose products are required for assembly of functional pili by *P. aeruginosa* (27). The *pilD* gene product is an endopeptidase, responsible for the cleavage of the 6-amino-acid leader peptide from the *P. aeruginosa* prepilin (29). The same enzyme is capable of efficiently removing the 7-amino-acid leader peptide from the *N. gonorrhoeae* prepilin as well. Moreover, a homolog of PilD, the *tcpJ* gene product, has been shown to process the toxin-coregulated pilin of *V. cholerae* in vivo (20).

In order to determine the recognition sequence for the PilD endopeptidase, we have previously introduced amino acid substitutions and deletions at various residues surrounding the cleavage site in the pilin precursor of *P. aeruginosa* by site-directed mutagenesis of the pilin gene (36). Expression of these altered pilins in a nonpilated

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A.

Type IV pilins

P. aeruginosa
 wild type MetLysAlaGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
 Phe3-1 MetLysAlaGlnLysGly MetThrLeuIleGluLeuMetIleValVal-
 Phe1-15 MetLysAlaGlnLysGly SerThrLeuIleGluLeuMetIleValVal-
 Phe1-18 MetLysAlaGlnLysGly AsnThrLeuIleGluLeuMetIleValVal-
 Phe4-1 MetLysAlaGlnLysGly CysThrLeuIleGluLeuMetIleValVal-

N. gonorrhoeae MetAsnThrLeuGlnLysGly PheThrLeuIleGluLeuMetIleValIle-

V. cholerae MetGln...16aa...LysLysThrGlyGlnGluGly MetThrLeuLeuGluValIleIleValLeu-▲

B.

Extracellular secretion proteins

P. aeruginosa
 PddA LeuGlnArgArgGlnGlnSerGly PheThrLeuIleGluIleMetValValVal-
 PddB MetArgAlaSerArgGly PheThrLeuIleGluLeuMetValValMet-
 PddC MetLysArgAlaArgGly PheThrLeuLeuGluValValAlaLeu-
 PddD MetArgLeuGlnArgGly PheThrLeuLeuGluLeuIleAlaIle-

K. oxytoca
 PulG MetGlnArgGlnArgGly PheThrLeuLeuGluIleMetValValIle-○
 PulH ValArgGlnArgGly PheThrLeuLeuGluMetMetIleLeu-
 PulI MetLysLysGlnSerGly MetThrLeuIleGluValMetValAlaLeu-
 PulJ MetIleArgArgSerSerGly PheThrLeuValGluMetLeuAlaLeu-

Consensus Phe
 -Gly or ThrLeuPheGlu- (Phe16-18)-
 Met

FIG. 1. Comparison of leader peptides and amino-terminal sequences of type IV pilins and proteins involved in extracellular secretion in *P. aeruginosa* and *K. oxytoca*. (A) Amino-terminal amino acid sequences of *P. aeruginosa* wild-type prepilin (19) and prepilins with substitutions for the +1 Phe residue (underlined) (37), *N. gonorrhoeae* prepilin (25), and *V. cholerae* prepilin (34). (B) Amino-terminal amino acid sequences of proteins involved in extracellular secretion from *P. aeruginosa* (30) and *K. oxytoca* (32). Symbols: ▼, leader peptide cleavage site; ●, cleavage demonstrated for PilD (28, 30, 36); ▲, cleavage demonstrated for TcpJ (20); ○, cleavage demonstrated for PulO (31).

mutant of *P. aeruginosa* demonstrated that the glycine residue at the -1 position relative to the cleavage site was required for processing by PilD, while the phenylalanine residue can be replaced with a variety of other amino acids without affecting in vivo processing. Substitutions and deletions of residues within the leader peptide demonstrated the overall necessity for the short peptide but not for the net positive charge. Little else is known about the sequence requirements existing in the mature portion of the prepilin substrate for cleavage by PilD.

In addition to its involvement in pilin precursor processing, PilD is also required for the extracellular localization of a number of exoenzymes of *P. aeruginosa* (38). The gene encoding PilD was independently isolated as *xcpA*, in which mutations also caused a pleiotropic export defect (2). The substrates of PilD (also known as XcpA) include precursors of four proteins (PddABCD, also called XcpTUVW) that are components of an apparatus of extracellular secretion in *P. aeruginosa* (3, 30). The amino-terminal regions of PddABCD share all of the sequence characteristics of the type IV pili, including a short, basic leader sequence, as well as the first 16 to 20 hydrophobic residues following the Gly-Phe cleavage site (Fig. 1B). Additional proteins homologous to type IV pilins, and to PddABCD, are involved in pullulanase export in *Klebsiella oxytoca* (32) (Fig. 1B) and in extracellular secretion of pectic enzymes in the plant pathogens *Erwinia chrysanthemi* (18) and *Xanthomonas campestris* pv. *campestris* (14). Interestingly, several proteins involved in DNA uptake by *Bacillus subtilis* have sequence similarities with type IV pilins near their amino termini (1, 7), while another component is a homolog of PilD as well (26, 29). Therefore, PilD and similar enzymes in other bacterial systems play a central role in biogenesis of a conserved macromolecular transport system.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
<i>E. coli</i> DH5α	<i>hsdR recA lacZYA</i> φ80 <i>lacZ</i> M15	BRL ^a
<i>P. aeruginosa</i> PAK 2B18	<i>pilD</i> mutant of PAK	27
PAK B30-NP	<i>pilD pilA</i> double mutant of PAK	29
Plasmids		
pMMB66EH	vector, <i>lacI^q tacp</i>	15
pMMB67HE	vector, <i>lacI^q, tacp</i>	15
pRBS-L	<i>pilD</i> , in pMMB66EH	29
pMStrac27PD	<i>pilA</i> , in pMMB66EH	36
pPhe3-1	<i>pilA</i> with F ⁺ M mutation	36
pPhe1-15	<i>pilA</i> with F ⁺ S mutation	36
pPhe1-18	<i>pilA</i> with F ⁺ N mutation	36
pPhe4-1	<i>pilA</i> with F ⁺ C mutation	This study
pMMB-NG	<i>N. gonorrhoeae</i> pilin gene, in pMMB66EH	29

^a BRL, Bethesda Research Laboratories.

In this study, we have determined the kinetic parameters of PilD cleavage of the wild-type prepilin substrate. These values are compared with those for selected mutated substrates containing substitutions for the phenylalanine residue at the +1 position relative to the cleavage site. The precursor of pilin from *N. gonorrhoeae* was also tested. The results show that substrate efficiency measured as k_{cat}/K_m decreases with substitutions at the +1 site in general, while substrate affinity, as measured by K_m , is less affected by these changes. One mutated pilin, however, exhibited a higher affinity for PilD but had a significantly lower k_{cat} value.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA modification enzymes.

All bacterial strains and plasmids used in this study are described in Table 1. All restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Plasmids were introduced into the various *P. aeruginosa* strains by conjugation as previously described (36).

Preparation of PilD. The purification of PilD was described previously by Nunn and Lory (29). Total membranes containing PilD were prepared by growing *P. aeruginosa* PAK 2B18(pRBS-L) to an A_{600} of 2 in minimal A medium (10) supplemented with 50 mM monosodium glutamate-1% (vol/vol) glycerol and containing 100 μg of carbenicillin and 200 μg of isopropyl-1-β-thiogalactopyranoside (IPTG) per ml. Membranes were prepared as previously described (29) and stored in small aliquots in 25 mM triethanolamine-HCl (pH 7.5)-10% glycerol at -20°C. The amount of PilD present in the membrane fraction was determined by comparison with known amounts of purified PilD on immunoblots. The concentration of PilD protein in the membrane fractions used in the kinetic experiments was determined to be ca. 2.7 mg/ml, which represented ca. 5% of the total membrane protein.

Expression and isolation of prepilin substrates. The construction of plasmids containing the wild-type pilin gene from *P. aeruginosa* PAK as well as the mutations in *pilA* and in the pilin gene from *N. gonorrhoeae* were described previously (29, 36), with the exception of mutant Phe4-1. This mutation in the *pilA* gene, which results in the substitution of a Cys residue for the Phe at the +1 position, was

constructed by site-directed mutagenesis similarly to the previously described constructs (36). All of the pilin constructs utilize the *tac* promoter in pMMB66EH (15) for overexpression of the cloned gene products.

For expression and purification of the precursor forms of these pilins, the plasmids were introduced into a *pilA pilD* double mutant of *P. aeruginosa* PAK, B30-NP, by conjugation. One liter of IPTG-induced cultures was grown in minimal A medium, and total membranes containing the overexpressed pilin precursors were prepared. Pilin constituted 50 to 75% of the total protein in the membrane fractions as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21).

Prepilin was further purified for use in the *in vitro* peptidase assay by solubilization of the total membrane fractions with 2% (wt/vol) SDS followed by fractionation on a Superose 12 HR10/30 FPLC gel filtration column (Pharmacia LKB Biotechnology, Piscataway, N.J.) equilibrated with 25 mM triethanolamine-HCl (pH 7.5)–1% SDS. Fractions containing the prepilin band were identified by SDS-PAGE, pooled, and precipitated with 5% sucrose (vol/vol) and 4 volumes of ice-cold acetone. The protein was resuspended in water and stored at -20°C . The concentration of the purified protein was determined by the Bradford method (6) (Bio-Rad Protein Assay Dye reagent; Bio-Rad Laboratories, Richmond, Calif.). In experiments in which the substrates were tested for PilD-mediated cleavage without purification from total membranes, the concentration of prepilin was determined by electrophoresing different amounts of membrane material side by side with purified prepilin at a known concentration on SDS-PAGE gels. After Coomassie staining of the gels, the individual lanes were scanned with an LKB model 2202 laser densitometer connected to an LKB model 2220 recording integrator, and the calculated areas of the peaks for the samples with unknown concentrations correlated to the areas obtained with the known concentrations.

Quantitation of cleavage products of PilD reactions. PilD peptidase cleavage reaction mixtures consisted of 1 to 6 μl of pilin substrate mixed with 1 μl of 0.5% (wt/vol) cardiolipin (Sigma Chemical Co., St. Louis, Mo.), 1 μl of PilD (purified or membrane associated), and water to a volume of 8 μl . The reactions were started with the addition of 2 μl of $5\times$ assay buffer (125 mM triethanolamine-HCl [pH 7.5]–2.5% [vol/vol] Triton X-100), and mixtures were incubated at 37°C . The concentration of PilD and time of incubation to use for each substrate were selected by carrying out cleavage reactions over several time intervals with different amounts of peptidase and substrate concentrations at saturation levels. Enzyme and substrate concentrations that gave cleavage reactions which were linear as a function of time and enzyme concentration were then used for the kinetic studies. After incubation, the reactions were stopped with 30 μl of sample buffer (0.125 M Tris-HCl [pH 6.8]–4% SDS–5% [vol/vol] 2-mercaptoethanol–10% glycerol–0.02% bromophenol blue). Samples were then boiled for 5 min, and 5- μl aliquots were electrophoresed in SDS–tricine–15% polyacrylamide gels (33). Gels were then stained with 50% ethanol–10% acetic acid–0.2% Coomassie blue R250 and then destained with 10% methanol–10% acetic acid.

The amount of precursor substrate converted to the processed form was determined from a densitometric scan of each lane, and the areas of the peaks were used to measure the fraction of precursor cleaved, a value which was then used to calculate the velocity of each reaction.

Analysis of enzyme kinetics. All analyses were carried out on measurements of initial reaction velocities with a mini-

FIG. 2. Kinetics of prepilin processing by PilD over time. Reaction conditions and determination of amount of cleavage of the substrate are described in Materials and Methods. Both enzyme and wild-type prepilin substrate were contained within total membrane fractions; the ratio of substrate to enzyme was ca. 500:1. (Inset) Coomassie-blue stained SDS-tricine-polyacrylamide gel from which the data for the graph were obtained; numbers on the left are positions of molecular weight standards, in thousands. The arrowheads mark the positions of prepilin and pilin.

mum of six different substrate concentrations and at least three separate reactions per concentration. Values for K_m (in micromolar units) and $V_{\max}$ (in micromoles per minute per micromole of enzyme) were derived from substrate concentrations and velocities by using the program Enzyme Kinetics, version 1.0c (Hypercard Stack for MacIntosh) (16), which calculates these values in a two-step procedure of first direct linear estimates (8) followed by a maximum likelihood estimation method (4). Using these units for $V_{\max}$ directly gives the substrate turnover rate, or k_{cat} , per minute.

RESULTS

Kinetics of cleavage of wild-type pilin precursor by the PilD endopeptidase. Previous work from this laboratory has demonstrated the cleavage of prepilin substrate by purified PilD endopeptidase provided that the reaction contained a mixture of nonionic detergents and acidic phospholipids (29). The requirement for a phospholipid-detergent mixture during *in vitro* cleavage most likely reflects the need for a specific conformation of the enzyme and its substrate for the enzymatic catalysis. Such a conformation is presumably attained in a mixed micelle of the detergent and phospholipid, which mimics the bacterial membrane and allows PilD and its substrate to interact. Therefore, it was interesting to compare the rates of cleavage obtained with the different combinations of purified enzyme, purified substrate, and total crude membrane preparations containing either PilD or wild-type substrate as expressed from the recombinant plasmids. In the latter preparations, the membrane-associated PilD and prepilin constituted approximately 5 and 75% of the total membrane protein, respectively.

Figure 2 shows results of a typical experiment demonstrating that processing of the pilin precursor by PilD was linear as a function of enzyme concentration and time when the substrate concentrations were at saturating levels. In this

FIG. 3. Kinetics of prepilin processing by PilD. Purified substrate at concentrations of 120, 240, 360, 480, 600, and 720 μM were reacted with 4.25 μmol of PilD at 37°C for 10 min. Velocity was determined as described in Materials and Methods. (Inset) Double-reciprocal (Lineweaver-Burk) plot of the same data, giving values of 459.2 μM for K_m and 54.4 min^{-1} for k_{cat} (16).

particular experiment involving both membrane-associated PilD and membrane-associated substrate, the molar ratio of substrate to enzyme was approximately 500:1. As can be seen, more than 75% of the prepilin is converted to the mature form in less than 10 min under the conditions used. Similar experiments were performed with all combinations of substrate and enzyme sources prior to determination of kinetics in order to select parameters within which the velocity of processing of substrate fell within the linear range.

PilD-catalyzed cleavage of prepilin substrate followed normal Michaelis-Menten kinetics, with the measured reaction velocity being dependent on substrate concentration. An example is shown in Fig. 3. To compare the effects of different sources of the enzyme and substrate on prepilin processing, kinetic constants were determined for the cleavage reaction by using different combinations of membrane-associated or purified prepilin or PilD. The respective k_{cat} and K_m values are shown in Table 2. In all cases, the affinity of PilD for its substrate, as measured by K_m , differed only by a factor of approximately 2, within the range of experimental error. However, the k_{cat} values were consistently 10-fold higher for substrate within membranes than for purified substrate when purified PilD was used, while the k_{cat} value

TABLE 2. Kinetics of PilD cleavage of wild-type prepilin

Preparation of ^a :		k_{cat} (min^{-1}) $\pm$ SE	K_m (μM) $\pm$ SE	k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
PilD enzyme	Wild-type prepilin substrate			
P	P	0.89 $\pm$ 0.28	184.0 $\pm$ 108.9	0.005
P	M	9.4 $\pm$ 2.2	392.9 $\pm$ 177.0	0.024
M	P	54.4 $\pm$ 4.1	459.2 $\pm$ 70.4	0.118
M	M	179.7 $\pm$ 103.4	654.6 $\pm$ 511.7	0.275

^a P, purified as described in Materials and Methods; M, component of total membrane fraction (PilD, ~5 to 10% of total membrane protein; prepilin, ~70% of total membrane protein).

obtained with membrane-associated PilD with purified substrate (54.4 min^{-1}) was 5-fold higher than the highest value obtained with purified enzyme. The highest k_{cat} value (~180 min^{-1}), as well as the highest k_{cat}/K_m ratio (0.275) (which is a measure of catalytic efficiency), was obtained when both PilD and prepilin remained as part of the total membrane fraction.

Effect of total phospholipids on the in vitro cleavage reaction. The more efficient rate of catalysis, as reflected in higher k_{cat} values obtained with membrane-associated prepilin substrate and PilD than with purified proteins, may be due to a number of factors. These could include alterations in the conformation of PilD during purification and failure to reform into a completely native conformation upon interaction with the detergent-phospholipid micelles during the assay. Similarly, the various purification steps of the prepilin substrate can lead to conformational alterations, which render it a less suitable substrate during the reaction. Alternatively, the removal of native phospholipids during the purification may prevent the reconstitution of enzyme and/or substrate into an optimal orientation in the lipid bilayer.

To determine whether phospholipids from bacterial membranes could provide a better medium for the cleavage reaction, the relative rates of cleavage of prepilin were determined in the presence of such phospholipids. Total phospholipids were extracted by methanol-chloroform extraction from *Escherichia coli* DH5 α and the *pilA pilD* double mutant *P. aeruginosa* B30-NP by established procedures (17) to determine their ability to substitute for crude membranes in the PilD in vitro assay. These results are shown in Table 3. Neither the phospholipids isolated from *E. coli* nor those from *P. aeruginosa* stimulated the cleavage of purified substrate with purified PilD in the absence of cardiolipin. In fact, the addition of phospholipids slightly decreased the level of precursor processing when cardiolipin was present. The addition of total membranes isolated from *P. aeruginosa* PAK B30-NP also did not stimulate prepilin processing with purified PilD in the absence of cardiolipin.

PilD-mediated cleavage of *P. aeruginosa* mutated pilin precursor substrates. The rates of processing of selected mutated prepilins were also determined. Because the highest k_{cat} values were obtained with crude enzyme and substrate, the same conditions were applied to the analysis of the kinetics of PilD cleavage of other substrates. The kinetic parameters for pilin precursors with methionine, serine, asparagine, and cysteine residues substituted for the phenylalanine at the +1 position were thus determined, as shown in Table 4. In all cases, the expression of these altered precursors in *P. aeruginosa* PAK-NP resulted in complete processing of prepilin (36, 37). The expression of all of the mutants, with the exception of the mutant Phe4-1 (which has a cysteine in place of phenylalanine at position +1 [$F^{+1}\text{C}$]), also restored the pilated phenotype to PAK-NP. The k_{cat} values obtained with these precursors are significantly lower in all cases, with the $F^{+1}\text{C}$ pilin precursor value being the lowest. Mutated precursor Phe3-1 ($F^{+1}\text{M}$) was closest to wild-type prepilin in turnover rate and, along with precursor Phe1-18 ($F^{+1}\text{N}$), in substrate efficiency. However, with the exception of the $F^{+1}\text{N}$ precursor, the K_m values for all are within a factor of 2, or within experimental error, of the wild-type prepilin value. Therefore, all of the mutated prepilins appear to have a similar affinity for PilD, but the resulting catalytic efficiencies (k_{cat}/K_m) for PilD cleavage of these precursors are lower than that seen with wild-type substrate. The $F^{+1}\text{N}$ pilin precursor is unusual in that it was determined to have a lower K_m , indicating a high affinity for

TABLE 3. Processing of purified wild-type *P. aeruginosa* prepilin substrate with purified PilD with different phospholipids^a

Time of incubation (min)	% Processing with ^b :									
	No phospholipid	0.05% CL	0.05% Ec-PL	0.1% Ec-PL	0.05% CL + 0.05% Ec-PL	0.05% CL + 0.1% Ec-PL	0.05% Pa-PL	0.1% Pa-PL	0.05% CL + 0.05% Pa-PL	0.05% CL + 0.1% Pa-PL
30	ND ^c	37.9	ND	ND	7.3	13.9	ND	ND	33.2	19.7
60	ND	63.1	ND	ND	14.4	26.7	ND	ND	51.4	35.6

^a Standard in vitro PilD cleavage reaction with a molar ratio of substrate to enzyme of 35:1.^b Phospholipids were added to the standard PilD in vitro reaction at the indicated concentrations (wt/vol). CL, cardiolipin; Ec-PL, total phospholipids extracted from *E. coli* DH5α; Pa-PL, total phospholipids extracted from *P. aeruginosa* PAK B30-NP.^c ND, none detected (limits of detection, ~1%).

the enzyme, but a low k_{cat} value, indicating that the rate of cleavage was decreased considerably with this substitution. However, these values give this substrate a raised k_{cat}/K_m value relative to the values for the other mutated precursors.

PilD-mediated cleavage of other type IV pilin precursors. Nunn and Lory (29) demonstrated that PilD efficiently processed the prepilin of *N. gonorrhoeae* in vitro. The kinetic analysis of processing of crude *N. gonorrhoeae* prepilin with PilD gave a K_m of 585 μ M and a k_{cat} of 199 min^{-1} (Table 4), values within experimental error of that obtained for wild-type *P. aeruginosa* prepilin (Table 2). Therefore, the affinity of PilD for *N. gonorrhoeae* prepilin as well as the turnover rate are essentially the same values measured for wild-type *P. aeruginosa* prepilin.

DISCUSSION

The product of the *P. aeruginosa pilD* gene is an endopeptidase responsible for the maturation of a number of protein precursors which have different functions. Both classes of substrates have short leader peptides with a net positive charge, a cleavage site lying between glycine and phenylalanine amino acid residues at the -1 and +1 positions, respectively, and highly conserved amino termini containing a predominance of hydrophobic residues (Fig. 1). The first class consists of the precursor of the pilin monomer, which, after processing, is polymerized into numerous pilus structures which protrude from the cell. Mutations in *pilD* lead to the absence of functional pili and the accumulation of unprocessed pilin precursors in the bacterial inner and outer membranes (27, 29, 36). The second class of PilD substrates, the Pdd protein precursors, make up part of a distinct protein extracellular secretion apparatus which is required for the normal translocation of a number of *P. aeruginosa* extracellular proteins, including exotoxin A, phospholipase C, alkaline phosphatase, and elastase, across both the cytoplasmic and outer membranes to the extracellular milieu (30, 38). Therefore, mutations in the *pilD* structural gene cause a pleiotropic export defect, resulting in the accumulation of normally extracellularly secreted enzymes in the periplasmic

space. Whether bacteria express additional polypeptides that require PilD processing for their maturation is not known at this time.

PilD exhibits typical Michaelis-Menten kinetics in its cleavage of the wild-type *P. aeruginosa* pilin precursor. The measured turnover rate of 180 min^{-1} (Table 2) is substantially higher than the k_{cat} values of 150 to 300 h^{-1} for leader peptidase I (13) but approximately 1/10 of that measured for leader peptidase II, which has a k_{cat} of 1,800 min^{-1} and a measured K_m of 6 μ M (11).

We have estimated that PilD represents less than 1% of the total cytoplasmic membrane protein. Yet even when the *pilA* gene is overexpressed from clones such as pMStac27PD, resulting in pilin levels making up 75% of the total membrane protein, complete processing is observed, and the same bacteria fail to show an extracellular protein secretion defect. Therefore, PilD must be capable of cleaving sufficiently large numbers of both prepilin monomers to be polymerized into pili and the Pdd protein precursors prior to assembly into an export apparatus. This can be most easily accomplished by an enzyme with high catalytic efficiency. The kinetic constants bear out the predictions for an enzyme that has a relatively low affinity for similar but not identical substrates, which is reflected in high values for K_m , but that is able to carry out a rapid hydrolysis of the Gly-Phe peptide bond, which is supported by very high k_{cat} values.

In the initial characterization of the endoproteolytic activity of PilD, in vitro processing of prepilin was shown to require the addition of acidic phospholipids (cardiolipin) and solubilization with low concentrations (<2%) of the nonionic detergents Triton X-100 and *n*-octyl glucoside (29). These requirements for phospholipids and detergents presumably reflect the topological requirements of the enzyme as well as its substrates in the cytoplasmic membrane of *P. aeruginosa*. Solubilization of PilD and substrate into detergent-lipid micelles may allow the presentation of substrate to the peptidase in the topological orientation optimal for cleavage. The importance of this is demonstrated by the fact that enzyme and substrate pretreated with Triton X-100 before being mixed in an in vitro reaction results in little or no cleavage of substrate, presumably because of the formation of micelles containing only one of the two reactants. Therefore, it is not surprising that mixtures of total membranes containing PilD and unprocessed substrate gave k_{cat} values consistently higher than those obtained with purified substrate. These conditions may more closely mimic those that exist in vivo. However, the addition of total membranes or purified phospholipids from *P. aeruginosa* or *E. coli* failed to stimulate cleavage of substrate by purified PilD (Table 3). Insertion of functional PilD as well as its substrate may require a local phospholipid environment which may be present only in the membranes of intact bacterial cells or

TABLE 4. Kinetics of PilD cleavage of other prepilin substrates

Prepilin designation	Mutation	k_{cat} (min^{-1}) $\pm$ SE	K_m (μ M) $\pm$ SE	k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
Phe3-1	F ⁺ 1M	46.8 $\pm$ 7.8	430.4 $\pm$ 92.0	0.109
Phe1-15	F ⁺ 1S	13.3 $\pm$ 4.4	165.5 $\pm$ 95.0	0.080
Phe1-18	F ⁺ 1N	6.1 $\pm$ 0.8	52.5 $\pm$ 14.4	0.116
Phe4-1	F ⁺ 1C	0.66 $\pm$ 0.35	175.6 $\pm$ 159.6	0.004
NG ^a	None	199.1 $\pm$ 36.0	584.9 $\pm$ 203.7	0.340

^a NG, prepilin from *N. gonorrhoeae*, expressed in *P. aeruginosa* PAK B30-NP.

which can occur most accurately in live, growing bacteria. Therefore, in vitro, the optimal topological orientation in the lipid micelles for enzyme activity may be difficult to attain.

We have previously investigated the role of amino acids surrounding the scissile bond of type IV prepilins by engineering a series of substitution mutations in *pilA* encoding the amino-terminal region of the pilin precursor polypeptide (36). Upon introduction of the mutated *pilA* gene into *P. aeruginosa* PAK-NP, we found that the conserved glycine residue at the -1 position to the cleavage site was necessary for processing of the pilin precursor, and substitutions at this position led to little or no processing and assembly of pili in *P. aeruginosa*. However, the highly conserved phenylalanine residue at the +1 position could be replaced with a number of other amino acids, with no appreciable difference in pilin processing or assembly into pili in *P. aeruginosa*. Because substitutions at the +1 position were apparently well tolerated in vivo despite the high degree of conservation of sequence around the cleavage site in the type IV pilins, it was interesting to determine whether evolutionary pressures to maintain the Gly-Phe cleavage site were reflected at the level of enzyme-substrate kinetics. As shown in Table 4, the K_m values obtained with the mutated prepilins containing a methionine, serine, or cysteine residue at the +1 position demonstrated that these substrates have about the same affinity for PilD as does the wild-type precursor. However, the apparent k_{cat} values are lower, ranging from approximately 20% of that obtained for the wild type (pPhe3-1 and F⁺1M) to <1% of that obtained for the wild type (pPhe4-1 and F⁺1C). Thus, the turnover rate is lower for these substitutions. These results also provide a possible explanation for conservation of phenylalanine at position +1 in the vast majority of type IV prepilins and their homologs. Any substitutions at this position may alter the efficiency of catalysis, without an immediate impact on the piliation phenotype. However, phenylalanine provides a substrate for maximal catalytic efficiency, which may be sufficient for favoring substrates with glycine-phenylalanine flanking the scissile bond of the leader peptide. It is interesting that the methionine substitution for phenylalanine gave kinetic values closest to those for the wild type, and this is the only amino acid naturally occurring as the first amino acid residue of mature proteins in some homologs of *P. aeruginosa* prepilin that are processed by PilD-like endopeptidases. Gly-Met is the cleavage site in the precursor of toxin-coregulated pili in *V. cholerae* (34), which is processed by the product of the *tcpJ* gene (20), and this site is also found in the *K. oxytoca* PulI precursor (31), which is a substrate of the peptidase PulO.

The overall rate of prepilin cleavage is apparently not affected by the length and net charge of the leader peptide. The type IV pilin from *N. gonorrhoeae* has the same sequence at the cleavage site and is identical to *P. aeruginosa* pilin in 19 of the first 20 residues of the mature protein, but it also has a 7-amino-acid-long leader peptide with a net charge of +1 instead of the 6-residue leader peptide of *P. aeruginosa* with a +2 net charge. The K_m and k_{cat} values obtained for cleavage of this substrate with PilD were essentially identical (within experimental error) to those obtained for the wild-type *P. aeruginosa* prepilin (Table 4).

The substitution of asparagine for phenylalanine also resulted in a lower k_{cat} value but, interestingly, had a lower apparent K_m , indicating a higher affinity of this substrate for PilD (Table 4). A peptide matching the sequence surrounding the cleavage site with a Phe-to-Asn substitution may have potential as a competitive inhibitor of PilD. Overall, these

results show that conservation of the Gly-Phe cleavage site in the *P. aeruginosa* pilin precursor, and in the Pdd proteins involved in extracellular transport, may simply reflect the most favorable sequence for the hydrolysis of the specific peptide bond between the mature protein and its leader sequence.

The results obtained show that PilD efficiently processes the pilin precursor, and presumably the precursors of the extracellular secretion proteins PddABCD, with a measured k_{cat} of approximately 180 min⁻¹. As a point of comparison, the k_{cat} value for leader (signal) peptidase I (SpaseI) of *E. coli* has been estimated to be 150 to 300 h⁻¹, with maltose-binding protein used as the substrate (13). SpaseI-catalyzed hydrolysis of peptides of various lengths which mimicked the cleavage site of maltose-binding protein gave K_m values between 1 and 4 mM with k_{cat} values ranging between 0.04 and 120 h⁻¹, depending on the length of peptide. Thus, processing of leader peptides by SpaseI also depends on the highly efficient cleavage of substrate bound with a relatively low affinity. As with PilD, this may reflect the rather wide range of substrates that are processed by SpaseI (9, 12), which certainly exceeds the relatively few substrates processed by PilD.

Specific inhibition of PilD function presents a possible method for designing novel therapeutic agents for treatment of *P. aeruginosa* infections, based on interference with extracellular secretion of a number of factors which contribute to virulence of the microorganism. Such inhibitors could be peptide-based pseudosubstrates, with high affinities for the active site of PilD but with low turnover rates. Kinetic studies with one mutated substrate, Phe1-18, in which the phenylalanine at position +1 is replaced with asparagine, shows processing of the mutated prepilin with a K_m an order of magnitude lower and a k_{cat} 30 times lower than those of the wild-type prepilin substrate. Similar high-affinity substrates that are hydrolyzed at a low rate could be designed by using the Phe1-18 mutated prepilin as a model.

ACKNOWLEDGMENTS

We thank David Nunn and David Simpson for helpful discussions and critical reading of the manuscript.

This work was supported by Public Health Service grant AI21451 from the National Institutes of Health.

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