Identification of Active-site Cysteines in the Conserved Domain of PilD, the Bifunctional Type IV Pilin Leader Peptidase/N-Methyltransferase of Pseudomonas aeruginosa*

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PilD is a bifunctional enzyme responsible for cleavage of the leader peptides from the precursors of the type IV pilin and four proteins with type IV pilin-like amino termini that are required for extracellular protein secretion in Pseudomonas aeruginosa. Following cleavage, PilD also catalyzes the secondary major posttranslational modification of these proteins, namely the N-methylation of the amino-terminal phenylalanine residues of the mature polypeptides. In this report, we demonstrate that the enzymatic activities of PilD involve cysteine residues that lie within a cytoplasmic domain that shows a high degree of similarity to other proteins postulated to perform the same function in other bacterial species. Both activities are reduced in the presence of sulfhydryl-reactive reagents such as N-ethylmaleimide and p-chloromercuribenzoate. Mutagenesis of pilD resulting in specific amino acid substitutions in all of the Cys residues in PilD show that the 4 conserved cysteines in the cytoplasmic domain are required for full peptidase activity in vivo and for complete peptidase and methyltransferase activities in vitro. Conversely, substitution for a Cys residue in a membrane spanning domain had no effect on PilD activity in vivo or in vitro. Evidence suggests that the peptidase and methyltransferase sites of PilD are adjacent, with the Cys residues in the cytoplasmic domain important for methyl donor binding, as prior reaction of PilD with the S-adenosyl-1-methionine analogue sinefungin afforded complete protection of peptidase activity from inactivation with N-ethylmaleimide.

The functions of pilus biogenesis and protein export in Pseudomonas aeruginosa are linked by the requirement for the type IV leader peptidase, PilD (Nunn and Lory, 1991; Strom et al., 1991) (also called XcpA) (Bally et al., 1992). This endopeptidase is responsible for proteolytic cleavage of short, 6-8 amino acids long, basic leader peptides from the precursors of the type IV pilin subunit of P. aeruginosa, as well as four proteins, PddABCD (also referred to as XcpTUWV), that are essential components of the extracellular protein secretion apparatus in P. aeruginosa (Nunn and Lory, 1992; Bally et al., 1992). The substrates of PilD are characterized by similar, short leader peptides and by extensive sequence conservation within the first 10-16 amino acids of the mature protein. The consensus sequence that forms the PilD recognition site is -Gly-\(\n\)-Phe-Thr-Leu/Ile-Glu-, where \(\n\) is the site of leader peptide cleavage. Following removal of the leader peptide, the amino-terminal Phe residue in type IV pilins and the Pdd proteins is methylated at the new amino terminus. N-Methylation is an unusual posttranslational modification of prokaryotic proteins demonstrated previously for only a handful of polypeptides in Escherichia coli, including CheZ (chemotaxis chemoreceptor), IF3 (initiation factor in protein synthesis), and the ribosomal proteins L16, S11, and L11 (Stock, 1988). This posttranslational modification appears to be essential for polymerization of the pilin monomers into pili, as mutations in the precursor which allow proteolytic processing but block this modification are not assembled into the mature organelle (Pasiloske and Paranchych, 1988; Strom and Lory, 1991).

Recently, we have demonstrated that PilD is a bifunctional enzyme, and as such, it not only catalyzes the removal of the leader peptides of its target substrates, it is also responsible for the subsequent N-methylation of the amino-terminal phenylalanine residue in both pilin and PddD precursor substrates (Strom et al., 1993). Type IV pilin are found in a wide variety of Gram-negative pathogens, including those of Neisseria gonorrhoeae (Meyer et al., 1984), Moraxella bovis (Marrs et al., 1985), Moraxella nonliquefaciens (Tonjum et al., 1991), Dichelobacter nodosus (McKern et al., 1983), and Vibrio cholerae (Shaw and Taylor, 1990). The pilin subunits in this class all have N-methylated residues at the amino terminus of the mature protein. We have also shown previously that PilD both efficiently cleaves the N. gonorrhoeae pilin precursor and methylates the mature protein (Strom and Lory, 1992; Strom et al., 1993), and it is likely that the type IV peptidases in these organisms have a bifunctional role as well.

Homologues of PilD have been isolated in a number of other genera. These include TcpJ in V. cholerae, in which mutations prevent the proteolytic cleavage of the leader peptide of the toxin-coregulated pilin (Kaufman et al., 1991), and PulO, which is necessary for extracellular secretion of pullulanase in Klebsiella oxytoca (Pugsley and Reyss, 1990). For the latter, PulO has been shown to be involved in the proteolytic processing of PulG, one of four proteins (PulGHIJ) in the pullulanase secretion pathway with amino-terminal sequences that contain the type IV peptidase consensus cleavage site (Pugsley and Dupuy, 1992). These four proteins are homologous counterparts to the P. aeruginosa secretion proteins PddABCD (Nunn and Lory, 1992). PulO has also been shown to cleave correctly the leader peptide from N. gonorrhoeae prepilin in vivo (Dupuy et al., 1992). ComC from Bacillus subtilis is also a PilD homologue and is a necessary
during the initial characterization of the peptidase activity of PilD homologue from N. gonorrhoeae has been cloned and sequenced and shown to have this same high level of homology (Lauer et al., 1993).

During the initial characterization of the peptidase activity of PilD, Nunn and Lory (1991) noted that the enzyme was sensitive to sulphhydril-blocking reagents and that the cleavage activity of purified PilD was stimulated by dithiothreitol. Moreover, a cluster of cysteines is found within a conserved region shared with other type IV peptidases (Fig. 1). This suggested that the enzyme may have cysteines in or near its active site.

To determine the involvement of the Cys residues in the enzymatic activities catalyzed by PilD, the inhibitory effects of several sulphhydril-reactive reagents on peptidase and methyltransferase activity were tested and compared. The cloned pilD gene was also subjected to oligonucleotide-specific site-directed mutagenesis to replace individually the Cys residues in each of the putative processing and glycosylation sites.

PilD mutants were made by site-directed mutagenesis and the activity of purified PilD was assayed as described with typical 10-µl reactions containing enzyme, substrate, and 0.05% cardiolipin. Preparations of PilD were made and used in 10-µl reactions in triplicate.

Preparation of membrane extracts made from the pilD mutants P. aeruginosa PAK-DR containing either the wild-type pilZD plasmid pRBS-L (Tompkins et al., 1991) or a corresponding pilZD plasmid with a cysteine to serine substitution (pRBS-L C253S) was followed by purification as described previously (Strom and Lory, 1992).

Materials and Methods

Bacterial Strains, Plasmids, and Reagents—All bacterial strains and plasmids used in this study are described in Table I. All restriction enzymes and T4 DNA ligase were purchased from Life Technologies, Inc. [3H]AdoMet was purchased from Amersham Corp. All other chemicals, including the sulphhydril inhibitors, were obtained from Sigma. Plasmids were introduced into P. aeruginosa strains by con-
The DNA sequence of one such fusion, pMS506, was infected with X-TnlacZ followed by selection on L-agar with 100 µg/ml ampicillin and 300 µg/ml kanamycin (Manoil, 1990) at 30 °C. After incubation for 2 days, plasmid DNA was isolated from pooled colonies by alkaline-SDS extraction and used to transform E. coli CC118. Selection for growth was done on L-agar containing kanamycin, and aliquots were analyzed either by SDS-Tricine-polyacrylamide gel electrophoresis or trichloroacetic acid precipitation. The latter reactions included 5 µCi of [3H]AdoMet and were performed in the presence and absence of 5 mM dithiothreitol. The reactions were stopped with the addition of 2 volumes of electrophoresis buffer, and aliquots were analyzed either by SDS-Tricine-polyacrylamide gel electrophoresis or trichloroacetic acid precipitation and liquid scintillation counting.

Construction of PilD-LacZ and PilD-PhoA Hybrid Proteins—To isolate pilD-lacZ fusions, E. coli CC118 carrying plasmid pRBS-L was infected with λ-TnlacZ followed by selection on L-agar with 100 µg/ml ampicillin and 300 µg/ml kanamycin (Manoil, 1990) at 30 °C. After incubation for 2 days, plasmid DNA was isolated from pooled colonies by alkaline-SDS extraction and used to transform E. coli CC118. Selection for growth was done on L-agar containing kanamycin and the β-galactosidase-chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside. Colonies that grew contained plasmids carrying TnlacZ insertions, whereas blue colonies were indicative of in-frame fusions between an open reading frame in a plasmid gene and lacZ. Plasmids from these blue colonies were then mapped by restriction endonuclease digestion to identify insertions in pilD. These were then sequenced (see below) to identify the pilD-lacZ fusion junction. The DNA sequence of one such fusion, pMS506, encoded a PilD-LacZ hybrid protein containing the first 92 residues of PilD (at Ala25) fused to LacZ, under tac promoter control. A negative control plasmid, pMS505, was constructed by ligating the 3.0-kilobase BamHI-EcoRI-containing TnlacZ fragment from pCM320 into the same sites in pMMB67EH.

To construct the complementary pilD-phoA fusion of the pilD-lacZ fusion in pMS506, it was first necessary to create a corresponding tac promoter signal sequence-deficient phoA vector. Plasmid pMS502 was constructed by inserting the 3.0-kilobase BamHI-HindIII fragment containing TpHpHaA from pCM35 into the same sites in pMMB67EH. Both TpHpHaA and TnlacZ have BamHI sites located just upstream of the respective reporter genes, and an in-frame gene fusion in one can be excised at this site with BamHI and ligated in-frame into the other. In addition, the pilD gene in pRBS-L has a single BamHI site located just upstream of the ribosome binding site. Therefore, the complementary pilD-phoA hybrid was constructed by subcloning the 0.3-kilobase BamHI fragment from pMS506 into the same site in pMS502, and designated pMS507. Both the orientation and maintenance of the correct reading frame were verified by DNA sequencing.

Levels of β-galactosidase and alkaline phosphatase activity produced by the various gene fusions were determined as described elsewhere (Brickman and Beckwith, 1975; Miller, 1972).

Substitution of Cys Residues in PilD by Oligonucleotide-directed Site-specific Mutagenesis—Mutations in pilD were constructed by oligonucleotide-directed site-specific mutagenesis of the M13mp19 derivative of pRBS-L (M13mpRBS-L) using the method of Kunkel et al. (1987) as described previously (Strom and Lory, 1981). The primers used to introduce changes in the codons specifying the Cys residues in PilD were as follows:

Cys2’, 5'-GGCGGCAAG[G,A]GCTCGTCCTGC-3'.
Cys97', 5'-GAATTCGGCG[G,A]GCCCGCGCTGC-3'.
Cys72', 5'-GCCTTTGTCTTG[G,A]GCGCCATCCTC-3'.
Cys75', 5'-GTGCTCGTCC[G,A]GCAAGGCCGCC-3'.

2 C. Manoil, personal communication.
The underlined bases indicate the position of the original Cys codon with the first position (in brackets) changed to encode Gly (GCC) or Ser (AGC) residues. After the mutagenesis reaction, individual plaques isolated from infected E. coli GW5180 were picked, and the single-stranded template was prepared and sequenced by the dideoxy method of Sanger et al. (1977) using the Sequenase version 2.0 kit (U. S. Biochemical Corp.). The replicative forms of derivatives of M13mp8BS-L containing the desired mutations were then used to subclone the region of DNA containing the pilD gene into the broad host range vector, pMMB67EH (Fürste et al., 1986), as SstI-HindIII fragments. This places the pilD gene under control of the tac promoter on a vector that also carries the lacIq repressor gene, allowing induction of expression with IPTG. These clones were then transferred into the P. aeruginosa PAK pilD mutant D10 by conjugation.

Phage Sensitivity Assays—For determination of phage PO4 sensitivity of P. aeruginosa pilD mutants containing the cloned wild-type and mutated pilD genes, cells were grown in L-broth with IPTG induction to an A660 of ~1.0 and then spread on a L-agar plate with a loop. After the culture dried, 5 μl of bacteriophage PO4 (about 1 × 108 plaque-forming units/ml) was spotted in the center of the streak. Phage sensitivity was scored as a zone of clearing where the phage was spotted and is indicative of the presence of pil.

RESULTS

Effect of Sulfhydryl Reagents on PilD-catalyzed Cleavage and Methylation—To assess the role of cysteine residues in PilD enzymatic activity initially, a determination of the sensitivity of PilD toward the sulfhydryl reagents NEM, p-chloromercuribenzoate, p-chloromercuriphenylsulfonate, and iodoacetamide was carried out. Incubation of PilD with any of these inhibitors substantially reduced both the peptidease and methyltransferase activity of the enzyme. The effects of p-chloromercuribenzoate and p-chloromercuriphenylsulfonate were readily reversible with the addition of dithiothreitol, further demonstrating that the sulfhydryl-blocking reagents are specifically acting on cysteine residues in PilD. It was not possible to determine whether some of the cysteines are more reactive than others because of a lack of sufficient amounts of purified PilD.

Site-directed Mutagenesis and Expression of PilD—In addition to the results demonstrating the sensitivity of PilD to sulfhydryl-blocking reagents, another factor pointing toward the involvement of the conserved Cys residues in the activities of the enzyme is the probable orientation of the domain containing these amino acids toward the cytoplasmic side of the inner membrane. There are several facts that support this topological model. We have isolated a pilD-lacZ fusion that encodes a hybrid protein consisting of the first 92 amino acids of PilD fused to β-galactosidase (Fig. 2). This fusion junction is in between the two Cys pairs at positions 72, 75, 97, and 100. As shown in Fig. 2, expression of this hybrid in P. aeruginosa PAK results in high levels of β-galactosidase activity, whereas expression of the complementary pilD-phoA fusion shows little alkaline phosphatase activity. This is strong evidence that the domain containing the Cys residues extends into the cytoplasm (Manoil, 1990). Proteolytic cleavage of leader peptides and subsequent methylation of PilD substrates is also likely to take place on the cytoplasmic face of the membrane, based on models developed from N. gonorrhoeae and P. aeruginosa pilin-PhoA hybrid data (Strom and Lory, 1987; Dupuy et al., 1991) and on the presence of the methyl donor AdoMet in the cytoplasm only.

Therefore, to assess the role of the Cys residues in PilD activity directly, oligonucleotide-directed site-specific mutagenesis of pilD was used to replace the Cys residues with Ser and/or Gly residues at positions 17, 72, 75, 97, and 100 of the 290 amino acid protein (Fig. 3 and Table I).

To assess the effect of cysteine mutations in PilD on in vivo processing of prepilin, PAK-DØ derivatives containing the different mutated pilD clones were tested for sensitivity to killing by the pilus-specific phage PO4. After IPTG induction of the altered pilD genes, the bacteria became completely phage-sensitive, whereas in the absence of IPTG they remained resistant to phage killing. This indicated that all of the mutants in PilD were processing prepilin to some extent, which allowed subsequent assembly of the subunits into pili.

To determine if the mutations in PilD altered the extent of processing of wild-type prepilin in vivo, the extracts from IPTG-induced cells grown overnight were subjected to immunoblot analysis using anti-pilin antisera (Fig. 4). In the PAK-DØ strain containing the cloning vector pMMB66EH, only uncleaved pilin is seen, whereas complete processing of pilin is seen in cells containing the wild-type parental
The differences seen in pilin processing with the mutated PilD forms could not be attributed to decreased levels of expression. Estimation of the amount of PilD made from various clones by quantitative immunoblot analysis showed that the levels of PilD expressed from the mutated genes are comparable or slightly exceed that obtained by expression of the wild-type gene (data not shown). The one exception was the Cys7→Ser-mutated PilD, where no PilD antigen was detectable on immunoblots. Furthermore, no peptidase or methylase activity was observed in in vitro using membrane extracts, even though all endogenous pilin was processed in vivo (Fig. 4). It is likely that this mutation resulted in an unstable form of PilD which is still able to cleave prepilin rapidly in growing cells for pilus assembly leading to phase PO4 sensitivity. However, PilD fails to accumulate in cell extracts to levels detectable by the immunoblot assay and is therefore not present in sufficient concentration to demonstrate peptidase and methylase activity above the limits of the detection by these assays.

Specific Endopeptidase and N-Methyltransferase Activities of Mutated PilD—To determine the effects of cysteine substitutions on PilD enzymatic activity, membranes were prepared, and peptidase and methylase activities were measured in vitro. Relative specific activities were determined by comparison with values obtained using membranes from PAK-DQ (pRBS-L) and PAK-DQ (pMMB66EH) as positive and negative controls, respectively. As shown in Table II, the Cys17→Gly and Cys17→Ser mutations had no effect on either activity. However, all single mutations in any of the remaining Cys residues resulted in a dramatic reduction in the ability of the enzyme to cleave prepilin and methylate pilin. The specific peptidase activity of the mutants as a whole was less attenuated than the decrease seen in methyltransferase activity.

Substitutions of either of the Cys residues at positions 72 and 75 appear to have a lesser effect on peptidase activity than the Cys residues at 97 and 100. No such differences were seen in the decrease in methyltransferase activity of the mutants. Substitutions for any of the conserved Cys residues resulted in a 98–99% reduction in methylase activity. The limit of detection for either activity was approximately 0.5%.

Protection of PilD Peptidase Activity from Alkylation by Sinefungin—We have shown previously that the methyltransferase activity of PilD is extremely sensitive to inhibition by the AdoMet analogue sinefungin, whereas the ability of the enzyme to bind and cleave substrate is unaffected (Strom et al., 1993). This suggests that the sites for prepilin/Pdd protein substrate binding to PilD may not necessarily overlap the methyl donor binding site. The decrease of both cleavage and methylase activities caused by sulfhydryl-reactive reagents and mutations in the cysteine residues, however, suggests that the active sites for both functions may be adjacent to each other. To determine whether the conserved cysteines are in a region of PilD which is shared by the peptidase and methylase sites, we examined the ability of sinefungin, which binds to the methylation site, to interfere with inactivation of the peptidase activity by NEM. Alkylation of PilD by NEM was carried out in the presence and absence of sinefungin, and the enzymatic activities were compared with untreated controls. The results of both peptidase and methylase assays on untreated, sinefungin alone, NEM alone, and sinefungin followed by NEM treatments are shown in Fig. 5. As can be seen, pretreatment with sinefungin afforded complete protection of PilD peptidase activity from inactivation with NEM. It is not possible to determine if this protection from NEM alkylation also extends to the methyltransferase activity.
since sinefungin appears to bind PilD very tightly and very likely cannot be removed without irreversibly inactivating the enzyme (Strom et al., 1993).

**DISCUSSION**

The product of the *P. aeruginosa pilD* gene is a bifunctional enzyme and is responsible for the maturation and posttranslational modification of type IV pil and four proteins which comprise the machinery for extracellular protein secretion. The PilD protein catalyzes the endoproteolytic cleavage of the leader peptide from the pilin subunit precursor and performs the same function for a group of four export determinants, PfdABCD. In addition, PilD catalyzes N-methylation of the amino-terminal phenylalanine residue after cleavage of the leader peptides of prepilins.

The 290-amino acid long PilD protein is largely hydrophobic, with four to six potential transmembrane spanning helices (Nunn et al., 1990) and is tightly associated with the cytoplasmic membrane (Nunn and Lory, 1991). The protein contains a large, 80-amino acid, relatively hydrophilic domain that is notable for the presence of 4 cysteine residues (Nunn et al., 1990). The arrangement of these 4 cysteines is in a pairwise fashion at positions 72 and 75, and 97 and 100, each pair separated by 2 amino acids (Fig. 1). The location of this domain in the cytoplasm was confirmed by engineering fusions to the cytoplasmic and periplasmic localization markers β-galactosidase and alkaline phosphatase, respectively (Fig. 2).

The environment in the bacterial cytoplasm is strongly reducing because of the high content of soluble thiols, primarily in the form of reduced glutathione (Fahey et al., 1978). It is therefore likely that the cysteines in the hydrophilic domain of PilD are not involved in disulfide bonds. Although added thiols are required for maximal activity of the N-methyltransferase, this requirement may reflect artificial formation of inter- or intrachain disulfide bonds during preparation of membranes and subsequent extraction of the enzyme.

Although the protease activity is modestly stimulated in the presence of thiols, the cysteine-containing region of PilD lacks the characteristic sequence surrounding the active-site cysteine of cellular serine proteases, QXXXG/E)XCW (Dufour, 1988), or viral proteases, G(Q,W,Y)CG(S,G) (Bazan and Fletterick, 1986). The enzyme therefore, may represent a new class of thiol proteases.

Alternatively, the cysteines may be involved in binding of metal cofactors. However, although the arrangement of cysteines resembles a zinc finger motif such as seen in the yeast transcriptional factor GAL4 (Pan and Coleman, 1969), there is no evidence that PilD requires any metals for activity and is not stimulated by the addition of divalent cations (Nunn and Lory, 1991).

Although PilD appears to be unrelated to any known proteases, a weak homology has been detected with two known methyltransferases. A search for homologies to thiol methyltransferases using Multiple Alignment Construction and Analysis Workbench (MACAW) (Schuler et al., 1991) found a conserved 6-amino acid region in PilD containing Cys^d7 (LGGKCS) and similar domains in the *E. coli EcoRII (INGKCP) (Greene et al., 1981)* and *EcoRII (INGKCS) (Som et al., 1987)* methyltransferases. Interestingly, an essential cysteine was identified in the CheR methyltransferase of *Salmonella typhimurium* by site-directed mutagenesis (Subbaranaiah et al., 1991) and photocross-linking of AdoMet (Subbaranaiah and Simms, 1992). However, the region surrounding this cysteine shows no similarity with the region flanking any 1 of the 4 conserved cysteines in PilD. Substitutions for the cysteines at positions 72, 75, 97, and 100 resulted in a striking reduction in both peptidase and methylase activities. The methylase activity of all of the mutated PilD proteins was less than 2% of wild-type, whereas the peptidase activity was reduced to between 20% and less than 0.6%. The substitution of glycine for the Cys residue at 72 or 75 resulted in the highest levels of peptidase activity at 20 and 11% respectively, whereas Cys^72 → Ser, Cys^100 → Ser, and Cys^72 → Gly were comparable at approximately 5% of wild-type. Cys^72 → Gly gave the lowest of the in vitro measured peptidase activities, possibly because of a deleterious conformational change around the glycine residue.

Replacement of the cysteine residues in the conserved cytoplasmic domain of PilD with serine or glycine results in a large decrease in peptidase activity in vitro, and in many cases a corresponding decrease in the levels of processed pilin in vitro, without affecting pilin (Fig. 4). One possible reason is that the measured turnover rate or K_cat of the wild-type enzyme at 180 min^-1 (Strom and Lory, 1992) is sufficiently high that as much as a 100-fold decrease in activity still results in enough processed pilin for assembly of functional pili to serve as receptors for PO4 phage.

Based on the observations that both the leader peptidase and N-methyltransferase activities are reduced by exposure to sulfhydryl-active reagents and by mutagenesis of any 1 of the Cys residues in the cytoplasmic domain of PilD, we postulate a model in which the catalytic sites for proteolysis and methylation are adjacent and share the region containing the 4 cysteine residues in the cytoplasmic domain. This hypothesis is supported by the experiment in which sinefungin, a competitive inhibitor of N-methyltransferase, protected 1 or several of the cysteines from alkylation, whereas binding of sinefungin did not interfere with leader peptide cleavage. The methyl donor in PilD, therefore, directly interacts with 1 or several cysteines. Peptidase activity is reduced in the mutants as a result of conformational changes in the methyl donor binding site, possibly affecting the substrate binding or the peptidase active site directly. Modification of cysteines may likewise inhibit PilD peptidase activity because of steric hindrance by the introduction of a bulky molecule bound to Cys residues at an adjacent methyl donor binding site. This result allows a prediction of the location of the enzyme active site to residues within a few amino acids upstream of the Cys^72,75 pair or downstream of the Cys^97,100 pair. Alternatively, the peptidase active site may lie within the two pairs of cysteines. However, it is also possible that the peptidase active site may be topologically close to this region because of protein folding but distant on the linear polypeptide. It is clear from the results that no single amino acid residue is involved in both activities of PilD. Isolation of mutants in PilD, which exclusively affect the leader peptidase or the methyltransferase activity, but not both, may allow a more precise localization of the catalytic site within this bifunctional enzyme.

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