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# A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family

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**ABSTRACT** **Precursors of the type IV pilins of a number of bacterial pathogens, as well as related proteins involved in extracellular protein export and DNA uptake, are synthesized with short basic leader sequences. Maturation of these proteins involves two consecutive posttranslational modifications. The leader sequence is first proteolytically removed by specialized endopeptidases, of which the prototype is encoded by the *pilD* gene of *Pseudomonas aeruginosa*. Subsequently, the amino termini of these proteins are methylated. Here we demonstrate that PilD, in addition to cleaving the amino-terminal leader sequences of prepilin, also catalyzes N-methylation of the amino-terminal phenylalanine of the mature pilin, using *S*-adenosyl-L-methionine as a methyl donor. Thus, to our knowledge, PilD is the first characterized bacterial *N*-methyltransferase. Complete inhibition of N-methylation, but not peptide cleavage, by structural analogues of *S*-adenosyl-L-methionine suggests that PilD is a bifunctional enzyme with proteolytic and methylation activities carried out within two distinct active sites.**

Methylation of proteins is an important posttranslational modification process in both prokaryotic and eukaryotic cells. The best characterized is the side-chain carboxyl methylation/demethylation of L-glutamic residues in bacterial chemoreceptors involved in chemotaxis (1, 2). In contrast, methylation of α-amino groups of polypeptides is rather rare in prokaryotes (3) and has been described only for *Escherichia coli* initiation factor 3 (IF-3) (4) and ribosomal proteins L16 (5), S11 (6), and L11 (7), the *E. coli/Salmonella typhimurium* chemoreceptor CheZ (8), and in type IV pili of bacterial pathogens (9). While several of the bacterial enzymes responsible for carboxyl methylation have been extensively studied, *N*-methyltransferases have remained unidentified.

Previous work in our laboratory has shown that type IV prepilin of *Pseudomonas aeruginosa* is processed by a specific endopeptidase, the product of the *pilD* gene (10). This enzyme cleaves a short basic leader sequence from prepilins, exposing a phenylalanine at the amino terminus of the pilin, which becomes subsequently N-methylated, prior to assembly of pilin subunits into pili. *N*-Methylphenylalanine is the first amino acid of pilins of most bacterial pathogens expressing type IV pili, including those of *Neisseria gonorrhoeae* (11), *Moraxella bovis* (12), *Moraxella nonliquifaciens* (13), and *Dichelobacter nodosus* (14). The toxin-coregulated pili of *Vibrio cholerae* are the only known exception; mature pilin from this organism contains methionine at its amino terminus, which is also N-methylated (15).

Functional PilD is also required for extracellular secretion (excretion) of several proteins from *P. aeruginosa* which contribute to virulence, including exotoxin A, phospholipase C, and elastase (16, 17). Four proteins homologous with type IV prepilins are components of the extracellular secretion apparatus of *P. aeruginosa* (18). The four proteins, PddABCD (also designated XcpTUVW; ref. 19), share a number of similarities with prepilin. Each is between 14 and 25 kDa and has a short leader sequence, a conserved region [-Gly▼Phe-Thr-(Leu/Ile)-Glu-] flanking the PilD cleavage site (▼), and a distinct hydrophobic character of the 16–18 amino acids immediately following the glutamate residue. As in pilin, the amino-terminal phenylalanines of the Pdd proteins are N-methylated (D.N.N. and S.L., unpublished work). A number of Gram-negative bacteria have an export machinery containing similar pilin homologues (20–24). Moreover, expression of natural competence and DNA uptake in *Bacillus subtilis* also utilizes proteins resembling type IV pilins (25, 26). Proteins homologous to PilD are required for pullulanase secretion (PulO) and DNA uptake (ComC) in *Klebsiella oxytoca* (27) and *B. subtilis* (26), respectively. The posttranslational modifications leading to assembly of the export machinery or DNA-uptake machinery in these bacteria therefore require not only processing by a PilD-like enzyme but very likely a concomitant modification by a conserved *N*-methyltransferase. The steps in the pathway leading to expression of pili in *P. aeruginosa* and assembly of a protein-excretion machinery are outlined in Fig. 1.

Here we show not only that the cleavage of the leader sequence from precursors of prepilin is catalyzed by PilD but also that the same enzyme is responsible for the subsequent N-methylation of the amino-terminal residue of its protein substrate. We also show that PilD is capable of methylating other substrates that it processes, including one of the precursors of components of the extracellular secretion apparatus and a prepilin from another bacterial species. Finally, we use inhibitors of methylation to demonstrate that the catalytic sites of this bifunctional enzyme are not overlapping, since inhibition of methylation does not interfere with proteolytic removal of the prepilin leader sequence.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Reagents.** All bacterial strains and plasmid constructs used in this study have been described (10, 28, 29). *S*-Adenosyl-L-[*methyl*-$^3$H]methionine ([$^3$H]AdoMet) was from Amersham. Unlabeled AdoMet and the analogues sinefungin, *S*-adenosyl-L-homocysteine, and *S*-adenosyl-L-ethionine were from Sigma.

**Enzyme and Substrate Purification.** Purified PilD was obtained by immunoaffinity chromatography of detergent-solubilized membranes from the wild-type *P. aeruginosa* strain PAK containing the overexpressing clone, pRBS-L (10), in which *pilD* is transcribed from the *tac* promoter. The purified enzyme migrated as a single band in SDS/PAGE.

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Abbreviation: AdoMet, *S*-adenosyl-L-methionine.

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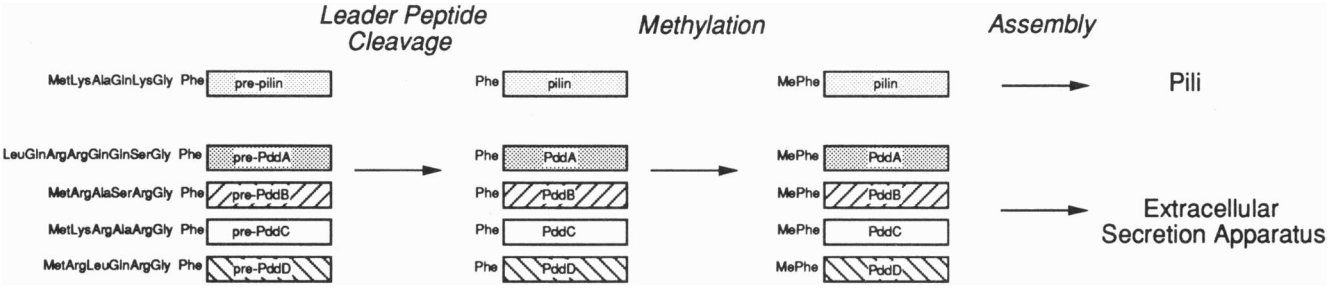


FIG. 1. Posttranslational modification and biogenesis of pili and components of the extracellular secretion apparatus in *P. aeruginosa*. The model depicts the posttranslational modifications of leader peptide cleavage and N-methylation of pilin subunits and subunits of the extracellular secretion apparatus that occur prior to polymerization into their final structures. PilD has previously been shown to be the type IV leader peptidase in *P. aeruginosa* that is responsible for removal of the leader peptides from the pilin and Pdd protein precursors. This paper demonstrates that PilD is the *N*-methyltransferase as well. Proteins involved in assembly functions have yet to be described for the type IV pili or extracellular secretion apparatus.

The purified protein had a specific activity of 400,000 units/mg (where 1 unit was arbitrarily defined as the amount of PilD required to cleave 50% of substrate in 30 min at 37°C), enriched 109 times over the activity found in total membranes.

The preparation of purified prepilin substrate was obtained by overexpressing *pilA*, also from the *tac* promoter, in pMStac27PD (29), in a *pilD* mutant of *P. aeruginosa*, PAK-2B18 (28), followed by total membrane extraction. SDS-solubilized membranes were fractionated through a Superose 12 HR10/30 FPLC gel filtration column (Pharmacia LKB) equilibrated with 25 mM triethanolamine·HCl, pH 7.5/1% SDS, to separate prepilin from other membrane proteins. Fractions containing prepilin were identified by SDS/PAGE, and the protein was precipitated with acetone and suspended at 10 mg/ml in water.

For experiments using preprocessed pilin, purified prepilin was cleaved with purified PilD in an *in vitro* reaction mixture containing substrate, enzyme, and cardiolipin (0.05%, wt/vol) in 25 mM triethanolamine, pH 7.5/0.5% Triton X-100. This mixture was incubated at 37°C for 2 hr, and complete processing of prepilin to its mature form was verified by PAGE. The PilD was inactivated by the addition of SDS to 1%, followed by acetone precipitation of the pilin and resuspension to ≈10 μg/μl.

***In Vitro* Prepilin Cleavage and Methylation Assays.** The *in vitro* PilD cleavage assay (10, 30) was modified for methylation assays to include [$^3$H]AdoMet, the addition of dithiothreitol to 5 mM, and an increase in triethanolamine concentration from 25 to 100 mM, pH 7.5, to buffer the AdoMet, which is stored in dilute acid for stability. Gel electrophoresis and analysis of reaction products included the use of SDS/tricine/15% polyacrylamide gels (31) to allow the separation of pilin and its precursor.

Methylation of the pilin substrate was analyzed by removing aliquots from the methylation assays, adding 2 volumes of sample buffer [0.125 M Tris·HCl, pH 6.8/4% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol/0.02% (wt/vol) bromophenol blue], and boiling. After SDS/tricine/PAGE the gels were stained in 50% ethanol/10% acetic acid/0.2% Coomassie blue R-250 and destained in 50% methanol/10% acetic acid. For fluorography, the gels were washed with water, treated with 0.5 M sodium salicylate/1.5% glycerol for 30 min (32), and then dried thoroughly under vacuum at 50°C, before exposure to x-ray film. For quantitative assay of methylation, aliquots from *in vitro* methylation assays were precipitated with 10% (wt/vol) trichloroacetic acid to precipitate protein, filtered on 0.22-μm cellulose filters (Millipore), and washed with 5% trichloroacetic acid. The filters were air-dried and dissolved in 5 ml of scintillation cocktail (NEN Liquiscint), and $^3$H was determined by scintillation counting.

**Amino-Terminal Sequencing of Pilin Methylated *in Vitro*.** Samples were prepared for amino-terminal sequencing by first incubating prepilin with purified PilD in the absence or presence of an excess of AdoMet. These samples, along with purified pilin isolated from the hyperpiliated *P. aeruginosa* PAK mutant PR1 (33), were then electrophoresed in SDS/tricine/polyacrylamide gels and electroblotted to poly(vinylidene difluoride) membranes (34). The membranes were stained with Coomassie blue, and the pilin bands were cut out and used for automated amino-terminal amino acid sequencing by sequential phenylisothiocyanate degradation in an Applied Biosystems model 477A gas-phase analyzer. Samples were analyzed by HPLC through a 5-μm $C_{18}$ column (220 × 2.1 mm) in a gradient of 5% tetrahydrafuran (A) and acetonitrile (B): 11% B, 0.4 min; 11% B to 35% B, 18 min; 35% B to 35% B, 27.1 min; and 36% B to 65% B, 35 min. The flow rate was 0.21 ml/min, at 35°C; detection was at 267 nm. Phenylthiohydantoin derivatives of phenylalanine and *N*-methylphenylalanine were used as standards. At least the first four residues were identified for each sample.

## RESULTS

**Mutants of *P. aeruginosa* with Decreased Levels of PilD Undermethylate Their Pilins.** In the initial studies of pilus biogenesis in this laboratory, transposon insertions in *pilC*, the gene adjacent to *pilD*, led to a decrease of PilD levels. The basis of this decrease is not clear, but it is not due to decreased transcription of *pilD* (T. Koga, K. Ishimoto, and S.L., unpublished work). The decrease in detectable PilD in the various mutants, however, resulted in significant undermethylation of the amino-terminal phenylalanine of the mature, processed pilin, without affecting the proteolytic removal of the amino-terminal leader peptide (Fig. 2). In each case, the extent of modification of phenylalanine was approximately proportional to the amount of PilD synthesized in each mutant background. One explanation that could account for the observed dependence of pilin methylation on the amount of PilD is that PilD has, in addition to leader peptidase activity, a methyltransferase activity. The incomplete methylation might reflect an inherently slower rate of methylation vs. peptidase activity by PilD, or some other rate-limiting step, such as interaction with a methyl donor prior to methylation. Alternatively, methylation of pilin may be carried out by a novel enzyme which requires PilD for expression of type IV pilin *N*-methyltransferase activity.

**Methylation of Pilin by PilD.** To determine whether PilD is involved in N-methylation of pilin, we developed an *in vitro* methylation system using purified prepilin or pilin as substrate and [$^3$H]AdoMet as a methyl donor. N-methylation of the prepilin substrate can presumably occur only after proteolytic removal of the prepilin leader peptide. In reaction

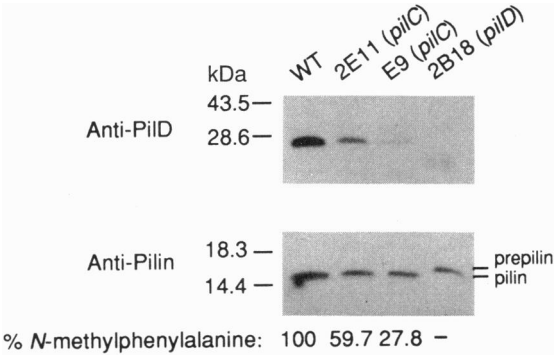


FIG. 2. Comparison of PilD and pilin levels in different *pilC* and *pilD* mutants of *P. aeruginosa* and the effects of these mutations on the level of *N*-methylphenylalanine. Western blots of whole extracts of wild-type *P. aeruginosa* PAK (WT), two *P. aeruginosa pilC* mutants (2E11 and E9), and one *pilD* mutant [2B18 (28)] were probed with PilD-specific antiserum (*Upper*) or pilin-specific antiserum (*Lower*). At the bottom are the percentages of *N*-methylphenylalanine found at the first position in pilin purified from wild-type PAK and each of the *pilC* mutants, as determined by automated Edman sequencing (—, not done for *pilD* mutant).

mixtures containing purified prepilin solubilized with Triton X-100, membranes from *P. aeruginosa* overexpressing PilD, and [$^3$H]AdoMet, detectable radioactivity was incorporated into mature pilin only (data not shown).

To investigate whether PilD plays a direct role in N-methylation of pilin, purified homogeneous PilD (10) was incubated with prepilin and [$^3$H]AdoMet. Incorporation of radioactivity was again detected. Fig. 3*A* shows kinetics of cleavage of the leader peptide and simultaneous labeling of mature pilin. This result directly demonstrates that PilD is indeed the enzyme responsible for methylation of pilin. To investigate whether cleavage and methylation are carried out simultaneously, prepilin was first cleaved in the absence of methyl donor, and mature pilin was reisolated and then incubated with purified PilD and [$^3$H]AdoMet. Efficient labeling of the pilin polypeptide was again detected (Fig. 3*B*). Cleaved but unmethylated pilin is a substrate which is as efficiently methylated by PilD as is pilin processed and concomitantly methylated. The similarity in the rates of methylation of prepilin and mature pilin suggests that the two reactions are not necessarily dependent on each other and that cleaved pilin can dissociate from the enzyme prior to methylation.

**Amino-Terminal Sequencing of Processed Pilin After Methylation by PilD.** To verify that the observed labeling of pilin in the presence of PilD and [$^3$H]AdoMet resulted in N-methylation of phenylalanine in the mature pilin, the amino terminus of the *in vitro* methylated pilin was identified. Prepilin was cleaved in the presence and absence of unlabeled AdoMet and subjected to amino-terminal sequencing. As expected, pilin cleaved by PilD in the absence of AdoMet had unmodified phenylalanine at the amino terminus (Fig. 4*B*). Pilin cleaved in the presence of AdoMet had amino-terminal *N*-methylphenylalanine (Fig. 4*C*), which is also the first amino acid of mature pilin isolated from assembled pili (Fig. 4*A*). This analysis confirms that, in the presence of AdoMet, PilD transfers a single methyl group onto the $\alpha$-amino group of the terminal phenylalanine, the reaction which it very likely carries out *in vivo*.

**Methylation of Substrates Other than Pilin by PilD.** Additional substrates known to be proteolytically processed by PilD were also examined for *in vitro* methylation. Prepilin of *N. gonorrhoeae*, another member of the type IV pilin family, is processed (10, 30) and methylated under the same conditions as *P. aeruginosa* prepilin (Fig. 5, lane 8). Similarly, PddD, one of the components of the protein-export machinery which is proteolytically cleaved by PilD, is also methylated by PilD (lane 10). The failure of PilD to methylate, *in vitro*, pilin isolated from mature pili (lane 6), which is already N-methylated in bacteria, demonstrates that there are no secondary sites on pilin for *in vitro* methylation, and PilD is unable to transfer a second methyl group onto *N*-methylphenylalanine.

**Inhibition of Methyltransferase Activity of PilD by AdoMet Analogues.** The similar rates of methylation by PilD of prepilin vs. mature pilin substrate (Fig. 3) suggest that cleavage and methylation are not obligatorily coupled. To examine whether prepilin and AdoMet compete for sites in PilD, we determined the inhibitory effects of structural analogues of AdoMet on cleavage and methylation. The most potent inhibitor of methylation was sinefungin (Fig. 6*A*), which inhibited the incorporation of the methyl group into pilin at a concentration ≈1500 times lower than AdoMet, whereas *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-ethionine were weaker inhibitors of methylation (Fig. 6*B*). In contrast to the efficient inhibition of methylation by sinefungin, cleavage of prepilin was unaffected at the highest concentration of sinefungin tested. These results suggest that the sites of prepilin binding and proteolysis in PilD do not overlap with the sites for AdoMet binding and methylation.

## DISCUSSION

We have described a second activity of the *P. aeruginosa* PilD enzyme, a previously described prepilin leader peptidase (10). Therefore, PilD represents a bacterial enzyme with *N*-methyltransferase activity, responsible for modification of type IV prepilin and components of the extracellular protein export pathway. It is likely that in other bacteria, enzymes analogous to PilD are also required for cleavage and methylation of pilin homologues required for protein export and DNA uptake. The amino acid sequence of the family of enzymes represented by PilD shares no detectable homology with any known protease or methyltransferase in protein data banks (Swiss-Prot, release 23.0; PIR, release 34; Prosite,

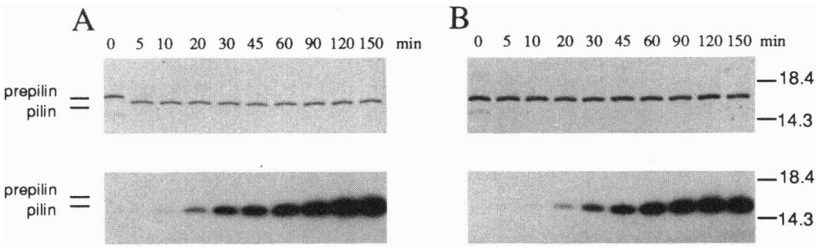


FIG. 3. Kinetics of PilD-mediated methylation of pilin, determined starting with either purified prepilin (30) (*A*) or mature pilin which was preprocessed *in vitro* (*B*). For each experiment, a Coomassie-stained gel (*Upper*) and fluorograph (*Lower*) are shown. In these experiments, 100 $\mu$g of each substrate (prepilin or pilin) was incubated with ≈5 $\mu$g of PilD in the presence of 50 $\mu$Ci (1.85 MBq) of [$^3$H]AdoMet in the *in vitro* cleavage and methylation assay mixture described in *Materials and Methods*. Aliquots (10 $\mu$l) were removed at the times indicated for gel electrophoresis and fluorography. Relative positions and molecular masses (kilodaltons) of protein standards are shown at right.

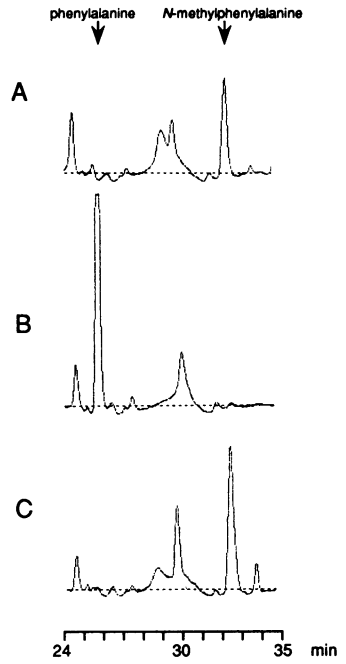


FIG. 4. Analysis of the amino-terminal amino acid residue of pilin isolated before and after *in vitro* methylation by PilD. Amino-terminal sequencing was performed on pilin isolated from a PilD wild-type *P. aeruginosa* strain and on prepilin processed *in vitro* by PilD in the absence or presence of AdoMet. The chromatograms show the first cycle of degradation of each phenylthiohydantoin derivative. (*A*) Pilin from assembled pili has *N*-methylphenylalanine as the amino-terminal residue. (*B*) Prepilin processed *in vitro* by purified PilD in the absence of AdoMet has only phenylalanine at the amino terminus. (*C*) Pilin processed *in vitro* by PilD in the presence of AdoMet has *N*-methylphenylalanine as the amino-terminal residue. Arrows represent the elution times of phenylalanine (25.8 min) and *N*-monomethylphenylalanine (32.6 min) phenylthiohydantoin standards.

release 9.10 and all available updates through November 1992).

While the role of N-methylation in the function of type IV pilins and related proteins is not known, it is possible that the modified amino terminus is essential for folding into a functional conformation, protection against proteolytic degradation, proper localization, or assembly into a macromolecular structure. An assessment of the requirement for N-methylated residues of proteins for their function will require isolation of mutants in PilD which are capable of cleaving the

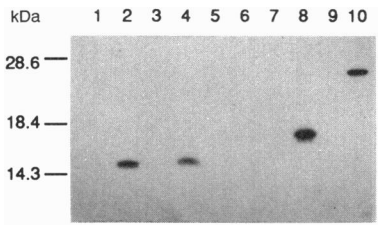


FIG. 5. Methylation of various substrates by PilD. The *in vitro* methylation reactions, SDS/tricine/15% PAGE, and fluorography were the same as for Fig. 3. For odd-numbered lanes, no purified PilD was added, whereas PilD was added for the even-numbered lanes. [$^3$H]AdoMet was included in all reactions. Substrates were as follows: lanes 1 and 2, purified *P. aeruginosa* prepilin; lanes 3 and 4, previously processed *P. aeruginosa* prepilin as described for Fig. 3; lanes 5 and 6, pilin isolated from sheared pili from the hyperpiliated *P. aeruginosa* PAK mutant PR1; lanes 7 and 8, prepilin from *N. gonorrhoeae*; lanes 9 and 10, purified PddD from *P. aeruginosa* PAK (D.N.N., unpublished work).

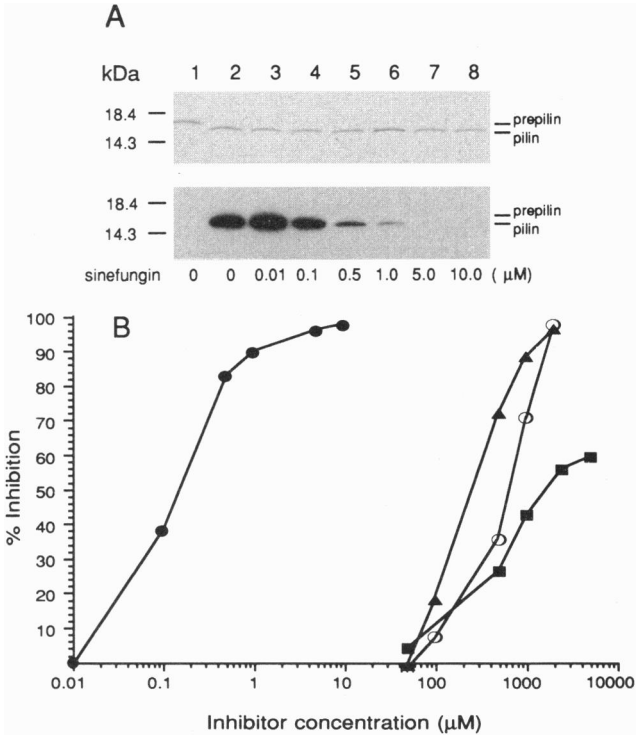


FIG. 6. Inhibition of *in vitro* methyltransferase activity of PilD by AdoMet analogues. (*A*) Coomassie-stained gel (*Upper*) and corresponding fluorograph (*Lower*) of the inhibition of PilD-mediated methylation of pilin by sinefungin. Lanes 1 (no PilD or sinefungin) and 2 (complete reaction without sinefungin) served as negative and positive controls for cleavage and methylation. For lanes 3–8, the reaction mixtures were as for lane 2 but containing sinefungin at the concentrations shown. In 10-μl mixtures, 20 pmol of purified PilD was preincubated for 5 min at 22°C with the concentrations of sinefungin shown. To this was added 60 nmol of purified prepilin, 50 pmol of [$^3$H]AdoMet, and 0.5% Triton X-100. After incubation at 37°C for 30 min, the reactions were stopped and analyzed by SDS/tricine PAGE followed by staining or fluorography. (*B*) Inhibition of methylation by sinefungin (●), AdoMet (▲), *S*-adenosyl-L-homocysteine (○), and *S*-adenosyl-L-ethionine (■). Values for inhibition were determined by comparisons of trichloroacetic acid-precipitable counts obtained for reaction mixtures containing the inhibitors to both positive and negative (background subtraction) controls.

leader peptide but defective in methylation of the respective substrates. Site-directed mutagenesis of cysteine residues in PilD yielded mutants that were defective in both proteolysis and methylation (M.S.S. and S.L., unpublished work). Our studies with methylation inhibitors, however, suggest that the methylation domain does not overlap the protease active site; hence a mutation affecting only one of the two activities of PilD should be possible to obtain.

Prokaryotic N-methylated proteins can be grouped based on the similarity of sequences and their amino termini, which led Stock *et al.* (3) to propose the existence of distinct methyltransferases responsible for N-methylation of proteins within each group. Type IV pili represent one such group of proteins that are N-methylated at terminal phenylalanines or methionines, with the substrate in each case generated by removal of a short amino-terminal leader sequence from the precursors of these proteins. Both the cleavage and N-methylation reactions in *P. aeruginosa* are catalyzed by the bifunctional enzyme PilD. Our results support the possibility that the cleavage and methylation reactions are not coupled but that they are carried out at two adjacent or distal domains containing the respective catalytic sites. Prepilin may bind at the protease domain by recognition of a sequence consisting

of the leader sequence and/or the mature terminus, and after hydrolysis of the Gly-Phe peptide bond, the exposed phenylalanine would be methylated from an adjacent methylation site containing bound AdoMet. Alternatively, cleaved pilin may dissociate from the catalytic site of the protease domain and bind to the methylation site located within a different domain of PilD. Recognition of the pilin substrate for methylation could also involve a distinct linear sequence within the mature polypeptide, or a conformation near the amino terminus attained by pilin after cleavage. Clearly, precise mapping of the proteolytic and methylating domains will require a better understanding of the three-dimensional structure of PilD bound to substrate.

Amino-terminal methylation of polypeptides differs from methylation of side chains in one important respect. The methyl-accepting group is not available in polypeptides initiated with formylmethionine, and therefore some form of amino-terminal processing is required before a protein can be N-methylated. Such processing can involve proteolytic removal of a specific leader sequence, as in the case of precursors of type IV pilins and related polypeptides. Other bacterial proteins which are N-methylated either at the initiating methionine or the adjacent amino acid also require modification to create a methylatable amino group. For example the *E. coli* CheZ, initiation factor 3, and L16 proteins can be methylated only following deformylation of the initiating methionine. In ribosomal proteins L11 and S11, the amino-terminal alanines are methylated after cleavage of the preceding initiating formylated methionine. These obligatory modifications preceding N-methylation may lead to evolution of bifunctional or multicomplex enzymes in which the modification steps are coupled to N-methylation.

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