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Purification of Peptide Synthetases Involved in Pristinamycin I Biosynthesis

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Several assays of pristinamycin I synthetases based on adenylate or thioester formation were developed. Purification to near homogeneity of these enzymatic activities from cell extracts of *Streptomyces pristinaespiralis* showed that three enzymes could activate all pristinamycin I precursors. SnbA, a 3-hydroxypicolinic acid: AMP ligase activating the first pristinamycin I residue, was purified 200-fold, using an ATP-pyrophosphate exchange assay. This enzyme was shown to be a monomer with an M_r of 67,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then a multifunctional enzyme, consisting of two identical subunits (SnbC) with M_rs of 240,000 and able to bind covalently L-threonine as a thioester, was purified 100-fold. This protein also activated L-aminobutyric acid, which is further epimerized to generate the third residue of the pristinamycin I macrocycle. A third protein, consisting of two identical subunits (SnbD) with M_rs estimated to be between 250,000 and 350,000, was purified 200-fold. This large enzyme catalyzed thioesterification and subsequent N-methylation of 4-dimethylamino-L-phenylalanine, the fifth pristinamycin I residue. SnbD could also activate L-proline, the fourth pristinamycin I residue, and some preparations retained a low but significant activity for the last two pristinamycin I precursors. Finally, a single polypeptide chain (SnbE) with an M_r of 170,000, catalyzing L-phenylglycine-dependent ATP-pyrophosphate exchange, was purified 3,000-fold and characterized. Stepwise Edman degradation of the entire polypeptides or some of their internal fragments provided amino acid sequences for the four isolated proteins. The purified SnbE protein was further shown to be a proteolytic fragment of SnbD.

Pristinamycins I (PI) are members of a family of closely structurally related branched cyclohexadepsipeptides produced by *Streptomyces pristinaespiralis* (24, 25) and belong to the streptogramin B group (2, 7, 40). The same molecules and some analogs are also synthesized by a large number of different streptomycetes and have been assigned several names, often related to the species from which they were isolated (7, 8, 40). The PI_A macrocycle (Fig. 1) consists of a characteristic 3-hydroxypicolinic acid chromophore and two common and four uncommon amino acid residues. Substitution of the typical PI_A amino acids, mainly in sites of D-aminobutyric acid, 4-dimethylamino-L-phenylalanine (L-DMPAPA), or 4-oxo-L-pipecolic acid, by structural analogs gives rise to a wide variety of minor components (2, 8), including PI_B and PI_C (Fig. 1).

The unique properties of streptogramin antibiotics lie in the synergy and the bactericidal effects exerted by mixtures of members of the A and B groups which individually are bacteriostatic components (2, 7, 40). Moreover, it was recently found (1) that two molecules of pristinamycin II_B (PII_B), belonging to the A group, could cocrystallize with one molecule of PI_A or some other members of the B group. These extraordinary features should have some implications for the regulation and organization of pristinamycin biosynthetic genes.

Unfortunately, PI_A and PII_A biosynthetic pathways are still poorly characterized, although several enzymes and genes involved in PII_A biosynthesis have been recently characterized (4, 39). PI_A biosynthesis could be schematically divided in three parts: precursor production, branched cyclohexadepsipeptide synthesis, and final macrocycle modifications. In this respect, incorporation studies of precursors labeled with radioactive or stable isotopes have provided insight into how the uncommon amino acid and chromophore residues of virginiamycin S_1, a PI_A analog having a αN-methyl-L-phenylalanine as residue 5, are built (22, 27, 28). More recently, isolation and characterization of a cluster of *pap* genes (3, 5, 6) led to a proposal for an L-DMPAPA biosynthetic pathway starting from chorismic acid. Some evidence that the L-pipecolic acid residue of PI_E is hydroxylated by the *snbF* gene product during the final conversion of PI_E into PI_A has also been obtained (5). In addition, L-pipecolic acid was proposed to be generated directly from lysine by a cyclodeaminase encoded by the *pipA* gene (5). The chromosomal region containing the preceding genes was also shown to include the *hpaA* gene required for 3-hydroxypicolinic acid formation (5).

As far as depsipeptide synthesis is concerned, only a 3-hydroxypicolinic acid:AMP ligase involved in the biosynthesis of etamycin has been isolated from *Streptomyces griseoviridus* (30), and a similar enzyme should operate in PI_A synthesis. Nevertheless, there has been a considerable and fruitful increase in the characterization of nonribosomal peptide synthesis at both the biochemical and the genetic levels (9, 20, 31, 36) since the initial discovery of the thiotemplate mechanism by Lipmann (21). As a result, it is now proposed that peptide synthetase enzymes are structurally organized in one or several 1,000-amino-acid modules (9, 20, 31). Each module, containing a phosphopantetheinyl arm attached to a serine residue, activates and incorporates one amino acid precursor into the peptide chain (31, 36). In addition, a larger choice of strategies

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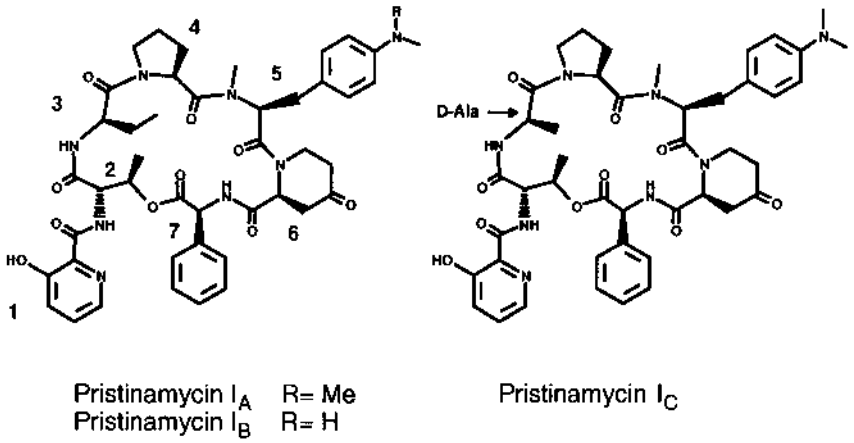

FIG. 1. Structures of PI_A, PI_B, and PI_C. PI_A is the main component of the PI family, while PI_B and PI_C are minor components arising respectively by substitution of the normal residue 5 by α*N*-methyl-4-methylamino-L-phenylalanine and of residue 3 by D-alanine. PI_A is also called mikamycin B, ostreogrycin B, streptogramin B, synergistin B1, vernamycin Bα, or PA-114 B1. 1, hydroxypicolinic acid; 2, L-threonine; 3, D-aminobutyric acid; 4, L-proline; 5, α*N*-methyl-4-(di)methylamino-L-phenylalanine; 6, 4-oxo-L-pipecolic acid; 7, L-phenylglycine residue.

may be used to isolate new peptide synthetases, and very promising possibilities for designing new peptide synthetases have emerged (35).

We report here the purification and a first characterization of several if not all enzymes involved in the peptide synthesis of the PI macrocycle. We also establish divergence points between the biosynthetic pathways of PI_A and of some minor components of the PI family.

MATERIALS AND METHODS

Materials. The following materials were obtained from commercial sources: leupeptin, Pefabloc SC, *S*-adenosyl-L-methionine (SAM), ATP disodium salt, and E-64 (Boehringer Mannheim Corp., Indianapolis, Ind.); ammonium sulfate, DNase I, phenylmethylsulfonyl fluoride (PMSF), EDTA, dithioerythritol (DTE), 1,4-dithiothreitol, soybean trypsin inhibitor (STI), tetrasodium pyrophosphate (PP_i), aprotinin, α_2-macroglobulin, L-pipecolic acid, L- and D-aminobutyric acid, L-phenylglycine, 4-amino-L-phenylalanine (L-PAPA), lysozyme from chicken egg white, and benzamidine (Sigma Co., St. Louis, Mo.); 3-hydroxypicolinic acid (Acros, Geel, Belgium); Sephadex G-25 Fine, Mono Q HR 5/5, HR10/10, and HR 16/10, Q-Sepharose Fast Flow, PD-10 columns, Superose 12 prep grade, phenyl-Sepharose, Superose 6 HR 10/30, Superdex 200 Hi-Load 16/60, and phenyl-Superose HR 10/10 (Pharmacia, Uppsala, Sweden); a Bio-Sil TSK 250 column (Bio-Rad, Richmond, Calif.); Centriprep, Centricon 10, and Centricon 30 (Amicon Co., Danvers, Mass.); Ultrafree MC GV (Millipore, Bedford, Mass.); [^{32}P]PP_i, [*methyl*-^{3}H]SAM (15 Ci/mmol), L-[2,3-^{3}H]alanine (57 Ci/mmol), L-[5-^{3}H]proline (34 Ci/mmol), DL-[2-^{3}H]phenylglycine (18 Ci/mmol), and L-[3-^{3}H] threonine (15 Ci/mmol) (Amersham, Buckinghamshire, England).

L-DMPAPA and 4-methylamino-L-phenylalanine (L-MMPAPA) were synthesized as described elsewhere (5, 13). 4-Oxo-L-pipecolic acid was prepared by hydrolysis of PI_A (12), and its structure was checked by mass spectrometry and nuclear magnetic resonance.

Bacterial strains and growth conditions. *S. pristinaespiralis* ATCC 25486 and *Streptomyces virginiae* ATCC 13161 were obtained from the American Type Culture Collection. *S. pristinaespiralis* SP92 was a subclone of ATCC 25486. Liquid cultures for pristinamycin or virginiamycin production were made as described previously (39).

General methods. Protein concentrations, native molecular weights, and N-terminal amino acid sequences were determined as previously described (39). Protein fragments were obtained either by overnight trypsin digestion or by cyanogen bromide treatment and were purified as previously described (39). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed either with 6% homogeneous gels stained with Coomassie blue or with 10-15 Phast Gel gradient gels, using a Phast system instrument (Pharmacia) and the Phast Gel Silver kit for staining.

Preparation of cell extracts. Cells for in vitro assays or for enzyme purification were harvested at the start of PI production by centrifugation of the culture broth. The previously described cell lysis procedure (39) was improved as follows to avoid protein degradation. One millimolar PMSF, 5 mM EDTA, 5 mM EGTA, and 500 mM KCl were included in the washing buffer. Lysozyme treatment was at 4°C for 90 min under vigorous agitation in 100 mM Tris-HCl buffer (pH 8.0) containing 20% (vol/vol) glycerol, 4 mM DTE, 2 mg of lysozyme per ml, and a pool of the following protease inhibitors: 5 mM benzamidine, 0.2 mM Pefabloc, 100 μg of E-64 per liter, 2 mg of leupeptin per liter, 1 mM EDTA, 1 mM EGTA, 2 mg of STI per liter, and 2 mg of aprotinin per liter. After a 30-min stirring period, 0.2 mg of DNase I per ml and 5 mM $MgCl_2$ were added to the suspension. Alternatively, the extract was prepared by passing the cell suspension in the same buffer through a French press at 4°C and a cell pressure of 5,000 lb/in^2. Cell debris was separated by centrifugation at 50,000 × *g* for 1 h. The supernatant was passed through a Sephadex G-25 Fine gel permeation column equilibrated with the cell lysis buffer.

Enzymatic assays. (i) ATP-PP_i exchange assay. A sample of enzyme was incubated at 27°C for 15 min with 250 nmol of the acid substrate, 500 nmol of ATP, 1.25 mmol of $MgCl_2$, and 50 nmol of [^{32}P]tetrasodium PP_i (100,000 dpm) in 250 μl of 50 mM *N*,*N*-methylenebisacrylamide (BIS)-Tris-propane buffer (pH 6.8). The reaction was terminated by the addition of 1 ml of a 1% suspension of activated charcoal in 4.5% perchloric acid and 75 mM sodium PP_i. The charcoal was collected by filtration through glass-fiber cotton and washed two times with 1 ml of the PP_i perchloric acid solution. The labeled organic molecules were eluted with 3 ml of a mixture of 50% methanol and 50% 1 N aqueous ammoniac solution, mixed with 12 ml of water, and counted. The radioactivity found in a control experiment made without the acid substrate was subtracted. One unit of activity was defined as the amount of enzyme necessary to label 1 μmol of ATP from [^{32}P]PPi in 1 h.

(ii) Thioester labeling assay. A sample of enzyme was incubated at 27°C for 15 min with 1 μCi of ^{3}H-labeled amino acid substrate, 500 nmol of ATP, and 1.25 mmol of $MgCl_2$ in 250 μl of 50 mM BIS-Tris-propane buffer (pH 6.8). The reaction was terminated by the addition of 150 μl of 25% trichloroacetic acid. Precipitated proteins were collected on an Ultrafree MC GV filter and washed three times with 400 μl of 7% trichloroacetic acid. Labeled products were eluted with 800 μl of 1 N sodium hydroxide, then mixed with 1 ml 1 N HCl and 12 ml of Ready Solv MP, and counted. Alternatively, the reaction was terminated by denaturation of the enzyme with SDS. The labeled polypeptides were separated by SDS-PAGE and identified either by autoradiography or by direct counting of sliced bands after Coomassie blue staining. One unit of activity was defined as the amount of peptide synthetase necessary to bind 1 pmol of the amino acid substrate as a thioester in 15 min.

(iii) Thioester N-methylation assay. The assay was performed exactly as the thioester labeling assay except that 250 nmol of the unlabeled amino acid substrate was used and 1 μCi of [*methyl*-^{3}H]SAM was included in the incubation mixture. One unit of activity was defined as the amount of peptide synthetase necessary to methylate 1 pmol of the amino acid thioester from [*methyl*-^{3}H]SAM in 15 min.

General enzyme purification procedures. All operations were carried out at around 4°C, and active fractions were kept at −70°C between purification steps. Several protecting agents specified below, glycerol, and 4 mM DTE were included in buffers for enzyme stability.

Anion-exchange chromatography was performed either at pH 8.0 in 100 mM Tris-HCl buffer or at pH 6.8 in 50 mM BIS-Tris-propane buffer. Proteins were eluted from Q-Sepharose or Mono Q columns with a linear gradient of 0 to 0.6 M KCl. When required for enzyme stability, PMSF, Pefabloc SC, EDTA, or EGTA was not included in the elution buffer but added directly in the collected fractions.

Gel permeation chromatography was performed at pH 6.8 on Superdex or Superose columns in 50 mM BIS-Tris-propane buffer containing 20% (vol/vol)

glycerol and 0.15 M NaCl or on a Bio-Sil TSK 250 column in 20 mM sodium dihydrogenphosphate buffer containing 50 mM sodium sulfate.

Phenyl-Sepharose chromatography and phenyl-Superose chromatography were performed at pH 8.0 in 100 mM Tris-HCl buffer containing 0.9 M ammonium sulfate, 15% (vol/vol) glycerol, 1 mM benzamidine, and 1 mM PMSF. Proteins were eluted from the column with a linear decreasing gradient of 0.9 to 0 M ammonium sulfate. DTE was not included in the buffer but added directly in the collected fractions.

Purification of 3-hydroxypicolinic acid:AMP ligase. For this enzyme, 15% (vol/vol) glycerol, 4 mM DTE, 1 mM PMSF, and 1 mM benzamidine were added in buffers or in fractions after chromatography (see above). The supernatant of a crude cell extract, obtained from 234 g (wet weight) of cells by lysozyme treatment, was applied first to an 80-ml Q-Sepharose Fast Flow column in Tris-HCl buffer at pH 8.0. Active fractions were pooled, diluted with 1 volume of Tris-HCl buffer (pH 8.0) containing 2 M ammonium sulfate, and loaded on a 50-ml phenyl-Sepharose column. The pool of fractions containing the activity was concentrated to a final volume of 5 ml with Centriprep 10 concentrators. The protein concentrate was applied to a 100-ml Superose 12 prep-grade column. The active fractions were pooled and applied in 6-mg aliquots onto a Mono Q HR 5/5 column in Tris-HCl buffer (pH 8.0). The active fractions were pooled, concentrated to a final volume of 1 ml with a Centricon 10 concentrator, and diluted with 3 ml of BIS-Tris-propane buffer (pH 6.8). The protein solution was loaded in 2-mg aliquots onto the same Mono Q HR 5/5 column but at pH 6.8. Best fractions were applied to a Bio-Sil TSK 250 column.

Purification of L-threonine thioesterification activity. For this enzyme, 20% (vol/vol) glycerol, 4 mM DTE, 1 mM PMSF or 0.2 mM Pefabloc SC, 1 mM EDTA, 1 mM EGTA, 2 mg of leupeptin per liter, 1 mg of E-64 per liter, and 1 mM benzamidine were added in buffers or in fractions after chromatography (see above). The supernatant of a crude cell extract, obtained from 150 g (wet weight) of cells by French press treatment, was applied first to a 200-ml Q-Sepharose Fast Flow column in Tris-HCl buffer at pH 8.0. Active fractions were pooled and concentrated to a final volume of 28 ml with Centriprep 30 concentrators. The protein concentrate was applied in 4-ml samples to a Superdex 200 Hi-Load 16/60 column. Fractions containing the activity were pooled and concentrated to a final volume of 15 ml with Centriprep 30 concentrators. The protein solution was desalted on PD-10 columns in Tris-HCl buffer (pH 8.0) and loaded in two aliquots onto a Mono Q HR 10/10 column in Tris-HCl buffer (pH 8.0). The active fractions were pooled and concentrated to a final volume of 1 ml with Centriprep and Centricon 30 concentrators. The protein solution was loaded in 2-mg aliquots onto a Superose 6 HR10/30 column.

Purification of L-DMPAPA thioester N-methylation activity. For this enzyme, the same set of protecting agents as for L-threonine-activating enzyme was used except that 2 mg of STI and 2 mg of aprotinin per liter were added in the buffer used for the preparation of the cell extract from 250 g (wet weight) of cells by French press treatment. The protein fraction precipitating at 4°C between 0 and 35% saturation with ammonium sulfate was collected by centrifugation, dissolved in the minimum volume of buffer, and desalted through a Sephadex G-25 column equilibrated with the buffer used for cell extract preparation. The desalted proteins were applied first to a 200-ml Q-Sepharose Fast Flow column in Tris-HCl buffer (pH 8.0). The proteins of the pool of active fractions were precipitated at 80% saturation with ammonium sulfate, dissolved in the minimum volume of buffer, and loaded on a Superdex 200 Hi-Load 16/60 column. Fractions containing the activity were pooled, concentrated to a final volume of 3 ml with Centriprep 30 concentrators, and diluted with 17 ml of Tris-HCl buffer (pH 8.0). The protein solution was applied in two aliquots onto a Mono Q HR 10/10 column in Tris-HCl buffer (pH 8.0).

Purification of L-phenylglycine: AMP ligase. For this enzyme, the same set of protecting agents as for L-threonine-activating enzyme was used except that 2 mg of STI, 2 mg of aprotinin, and 2 mg of α_2-macroglobulin per liter were added in the buffer used for the preparation of the cell extract from 250 g (wet weight) of cells by lysozyme treatment. The supernatant of the cell extract was desalted on a Sephadex G-25 column in the same buffer and applied first in 250-mg aliquots onto a Mono Q HR 16/10 column in Tris-HCl buffer (pH 8.0) containing the set of protecting agents and 2 mg of STI per liter. Active fractions were pooled, diluted with one-third of the pool volume of Tris-HCl buffer (pH 8.0) containing 3.4 M ammonium sulfate, and applied in five aliquots onto a phenyl-Superose HR 10/10 column. The pool of fractions containing the activity was concentrated to a final volume of 3.5 ml with Centriprep 30 concentrators. The protein concentrate was applied in two aliquots onto a Superdex 200 Hi-Load 16/60 column. The active fractions were pooled, diluted with 9 volumes of BIS-Tris-propane buffer (pH 6.8), and applied in 6-mg aliquots onto a Mono Q HR 5/5 column equilibrated with the same buffer. The active fractions were pooled, concentrated to a final volume of 0.6 ml with a Centricon 30 concentrator, heated for 10 min at 80°C with SDS and mercaptoethanol, and subjected to electrophoresis on 6% polyacrylamide gels. After Coomassie blue staining, the protein with an M_r of 170,000 was recovered by electroelution.

Steady-state kinetics. Peptide synthetase kinetic parameters were determined as follows. Apparent K_m values for the amino acid substrate were obtained at fixed concentrations of 2 mM ATP and 5 mM $MgCl_2$. For N-methylation activity, SAM was at 0.7 μM. L-Aminobutyric acid was shown to compete with L-[^{3}H]alanine in the thioester labeling assay, and its apparent K_m was calculated from the slope replot of the Lineweaver-Burk primary plot versus L-aminobutyric acid concentration. Concentrations used were 0 to 12 μM for L-[^{3}H]alanine and 0, 0.2, 0.4, and 1.0 μM for L-aminobutyric acid.

RESULTS

In vitro PI synthetase assays. Attempts to synthesize PI_A or PI_E with cell extracts and the appropriate precursors were unsuccessful. Therefore, several assays of the PI synthetase system were developed. They were based on acyl or aminoacyl adenylate formation measured by the rate of ATP-PP_i exchange or on thioester formation with the PI synthetase, using labeled precursors. These enzymatic activities were hardly detectable in crude extracts, but in the same assays, active fractions could be found after chromatography of the crude extract on a Mono Q column. Importantly, we checked that these PI synthetase activities were not found in extracts from cells harvested a few hours before the start of PI production. Thus, we showed that the ATP-PP_i exchange assay was appropriate only for the detection of 3-hydroxypicolinic acid or L-phenylglycine-activating enzyme, since a large contaminating activity due to aminoacyl-tRNA synthetases was found with threonine and proline and also with aminobutyric acid and pipecolic acid, which are analogs of alanine and proline. Concerning the thioesterification assay, we could label enzymes at the Mono Q step with [^{3}H]threonine or with [^{3}H]SAM in the presence of L-DMPAPA but not with [^{3}H]proline. SAM is the donor of the α*N*-methyl group of the fifth PI residue (Fig. 1). These four assays were used for further purification of PI synthetases.

Purification and characterization of 3-hydroxypicolinic acid: AMP ligase. 3-Hydroxypicolinic acid:AMP ligase (PI synthetase 1) was purified to homogeneity 200-fold from cell extracts of *S. pristinaespiralis* SP92 with an overall 3% yield, using a six-step procedure and ATP-PP_i exchange assay (Table 1). Pure PI synthetase 1 was eluted as a symmetrical peak from the gel filtration column, with an apparent M_r of 60,000, and the activity profile was exactly superimposable on this peak. SDS-PAGE revealed one band (Fig. 2A) with an M_r of 67,000 ± 6,000, indicating that this protein was a monomer. PI synthetase 1 was subjected to microsequencing and digested by trypsin to generate internal fragments. The NH_2-terminal sequence MLDGSVPWPEDVAAKYRAAGY ... and one internal amino acid sequence, VSA(−)EVEGHLGAHPDVQQAA, were obtained for 3-hydroxypicolinic acid AMP:ligase, which was called SnbA.

Purification and characterization of L-threonine-activating enzyme. L-Threonine-activating enzyme (PI synthetase 2) was purified to near homogeneity 100-fold from cell extracts of *S. pristinaespiralis* SP92 with an overall 3 to 6% yield, using a four-step procedure (Table 1). Purified PI synthetase 2 was eluted as a symmetrical peak from the gel filtration column, with an apparent M_r of 450,000, and the activity profile was exactly superimposable on this peak. SDS-PAGE revealed one main band (Fig. 2B) with an M_r of 240,000 ± 25,000, indicating that this protein was a homodimer. This band contained all of the radioactivity when the preparation was labeled with threonine prior to electrophoresis (slice 2 of lane c in Fig. 2B). The purity of the preparation could also be estimated by the ratio of labeled threonine to the protein (Table 2), assuming one covalent binding site per polypeptide chain. PI synthetase 2 was digested by trypsin to generate internal fragments. Microsequencing of some of these fragments yielded the sequences LAAFNDTARPVPR and VPAAFVPLDALPLTGNGVLD. PI synthetase 2 was called SnbC.

The size of SnbC strongly suggested that it could also activate aminobutyric acid, the third residue of the PI macrocycle. In this respect, ATP-PP_i exchange assays in the presence of

TABLE 1. Purification of PI synthetase components from *S. pristinaespiralis*

PI synthetase component	Purification step	Vol (ml)	Total amt (mg) of protein	Sp act[a] (U/mg)	Recovery (%)	Purification (fold)[b]
PI synthetase 1 (SnbA)	Crude extract	246	2,050	0.06		
	Q-Sepharose	40	188	0.47	100	1
	Phenyl-Sepharose	70	35	2.21	88	4.7
	Superose 12	16	17	2.03	39	4.3
	Mono Q, pH 8.0	4.5	9.0	2.09	21	4.5
	Mono Q, pH 6.8	1.0	2.0	2.9	6.6	6.2
	Bio-Sil 250	2.5	0.23	12.4	3.2	26
PI synthetase 2 (SnbC)	Crude extract	445	4,700	1		
	Q-Sepharose	308	834	7	100	1
	Superdex 200	120	105	22	40	3
	Mono Q HR	15	11.5	96	19	14
	Superose 6	7.5	2.8	122	6	17
PI synthetase 3 (SnbD)	Crude extract	800	8,100	4		
	35% ammonium sulfate	200	4,000	6		
	Q-Sepharose	132	498	46	100	1
	Superdex 200	45	39.5	417	71	9
	Mono Q HR	9	5.3	1,070	25	23

[a] 3-Hydroxypicolinic acid-dependent ATP-PP_i exchange, threonine labeling, and L-DMPAPA thioester N-methylation assays were used for purification of SnbA, SnbC, and SnbD, respectively. The activity in crude extracts could not be assayed precisely.
[b] The overall increase of specific activity could be estimated to 200-, 100-, and 200-fold for SnbA, SnbC, and SnbD, respectively.

either L-aminobutyric acid or D-aminobutyric acid were performed. Only L-aminobutyric acid-dependent ATP-PP_i exchange was observed, suggesting that epimerization, which is required to get the correct D configuration in the PI macrocycle, should occur at the thioester stage or after. Thioesterification with L-aminobutyric acid could not be directly tested because a radioactive form was not available. Nevertheless, since a D-alanine residue is found as the third residue in PI_C (Fig. 1), a minor component of the PI family, we showed that SnbC could be labeled by L-[^{3}H]alanine and that L-alanine and L-aminobutyric acid were competing substrates in this thioesterification assay. Thus, an apparent K_m value could be calculated for L-aminobutyric acid (Table 2).

Assay of total bound threonine by gel permeation on PD-10 columns, followed by trichloroacetic acid precipitation and determination of the thioesterified threonine (data not shown), indicated that SnbC had two binding sites for L-threonine: one noncovalent binding site thought to be the adenylate binding site (15, 29) and one covalent binding site thought to be the thioester site located on the 4-phosphopantetheinyl arm of the peptide synthetase (31, 36).

Purification and characterization of L-DMPAPA thioester N-methylating enzyme. L-DMPAPA thioester N-methylating enzyme (PI synthetase 3) was purified to near homogeneity 200-fold from cell extracts of *S. pristinaespiralis* SP92 with an overall 15 to 25% yield, using a four-step procedure (Table 1). Purified PI synthetase 3 was eluted as a symmetrical peak from the gel filtration column, with an apparent M_r of 700,000, and the activity profile was exactly superimposable on this peak. SDS-PAGE revealed one main band (Fig. 2B) with an estimated M_r of 275,000 ± 30,000, indicating that this protein could be a homodimer. Autoradiography of a gel run with labeled PI synthetase 3 showed that in addition to the main band, a faint band with an M_r of around 320,000 was also faintly labeled. PI synthetase 3 was cleaved with cyanogen bromide to generate internal fragments. Microsequencing of one of these fragments yielded the amino acid sequence P(−)VTPY RAYALAHLAG(−)DDD. PI synthetase 3 was called SnbD. Again, the size of SnbD indicated that this was a multifunctional protein. Purified SnbD could be labeled also by [^{3}H]proline and at a lesser extent by [^{3}H]phenylglycine (Table 2). In the case of phenylglycine, it was shown that the radioactivity was found in the main band at 275 kDa after electrophoresis. We also showed that ATP-PP_i exchange occurred with L-pipecolic acid but not with 4-oxo-L-pipecolic acid. Nevertheless, it could not be excluded that pipecolic acid was activated at the site of activation of proline, because the structures of L-proline and L-pipecolic acid are very similar. Thus, we showed that no competition occurred between L-pipecolic acid and [^{3}H]proline in the thioesterification assay. On the contrary, addition of L-DMPAPA resulted in a 50% increase of the amount of [^{3}H]proline bound to SnbD. Such binding stimulation has been observed for precursors of adjacent actinomycin residues by Stindl and Keller (37). They showed that partial peptide synthesis had occurred, leading to an enzyme-bound dipeptide at the site of the second amino acid in addition to the activated amino acid at its binding site (see also the model proposed for nonribosomal peptide synthesis in reference 10).

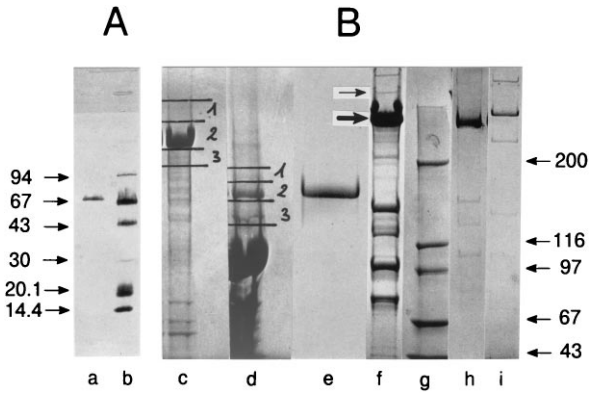

FIG. 2. SDS-PAGE of *S. pristinaespiralis* PI synthetases. (A) 10-15 Phast Gel (10 to 15% gradient polyacrylamide gel). Lane a, PI synthetase 1 (SnbA); lane b, standard marker proteins. (B) Six percent homogeneous gel. Lane c, PI synthetase 2 (SnbC); lanes f and h, PI synthetase 3 (SnbD) (arrows point at labeled bands); lane i, virginiamycin S synthetase 3 (from *S. virginiae*); lanes d and e, PI synthetase 4 (SnbE) before and after electroelution; lane g, standard marker proteins. The molecular weights (in thousands) of the standard marker proteins are shown at the sides of the gels.

TABLE 2. Substrate specificity of PI synthetases

Enzyme	PI residue no.	Substrate	K_m (μM)	ATP-PP_i exchange (U/mg)	Maximum binding[a] (molar ratio [%])
SnbC	2	L-Threonine	2.8	0.4	117
	3	L-Aminobutyric acid	0.5	2.0	ND[b]
	3	L-Alanine	8.4	0.5	85
SnbD	4	L-Proline	1.9	1.2	35
	5	L-DMPAPA	0.1	0.4	27[c]
	5	L-MMPAPA	6.0	ND	27[c]
	5	L-PAPA	160	<0.1	27[c]
	5	L-Phenylalanine	130	0.4	31[c]
	5	L-Tyrosine	1,400	0.1	35[c]
	6	L-Pipecolic acid	ND	0.8	ND
	7	L-Phenylglycine	0.3	1.4	3
SnbE	7	L-Phenylglycine	>10	6.0	1
SnbD from *S. virginiae*	5	L-Phenylalanine	0.2	ND	5

[a] Ratio of V_{max} in picomoles to the number of picomoles of peptide synthetase in the thioesterification assay. The value for the peptide synthetase amount was overestimated to the value of total protein amount in the assay.
[b] ND, not determined.
[c] Calculated as the number of picomoles of methyl groups transferred from SAM to the thioesterified amino acid.

Purification and characterization of L-phenylglycine:AMP ligase. L-Phenylglycine:AMP ligase (PI synthetase 4) was purified to homogeneity 3,000-fold from cell extracts of *S. pristinaespiralis* SP92 with an overall 10 to 25% yield, using a five-step procedure and an ATP-PP_i exchange assay (Table 3). The most active preparation of PI synthetase 4 was labeled with [^{3}H]phenylglycine, and proteins, treated with SDS but without mercaptoethanol, were further separated by electrophoresis. The labeled band at 170,000 ± 15,000 (slice 2 of lane d in Fig. 2B) was electroeluted and cleaved with cyanogen bromide to generate internal fragments. The internal amino acid sequence VTVFLNNTRLIQNFRPR(−)F(−)GD was obtained for the PI synthetase 4, which was called SnbE. Active SnbE protein was eluted from the gel filtration column with an apparent M_r of around 150,000, indicating that this protein was a monomer.

Careful reexamination of the profile of phenylglycine-dependent ATP-PP_i exchange activity from the first Mono Q chromatography showed that it was not symmetrical and that the size of labeled proteins increased gradually from 170 kDa, in fractions constituting the peak of activity, to 275 kDa, in fractions containing PI synthetase 3. This finding argued for PI synthetase 4 being a proteolytic fragment of PI synthetase 3 (SnbD). Such proteolysis has already been observed for flavin mononucleotide reductase isolated from the same strain (39) and for another peptide synthetase (23).

Substrate specificity of PI synthetases. In the conditions described by Schlumbohm and Keller (30), using [^{14}C]ATP, PI synthetase 1 was shown to form adenylates with 3-hydroxypicolinic acid, nicotinic acid, and 2-pyrazinecarboxylic acid. However, the adenylate formed with 3-hydroxypicolinic acid accounted only for 1% of the total radioactivity, compared with more than 10% for the adenylates formed with the two other substrates. This result might be explained by the instability of the 3-hydroxypicolinic acid adenylate previously reported by Schlumbohm and Keller (30). SnbC and SnbD affinities found for the normal amino acid precursor (Table 2) are in the micromolar range, while the affinities measured for analogs naturally occurring in place of aminobutyric acid or 4-dimethylamino-L-phenylalanine are always lower and in good agreement with the proportion of the corresponding PI components. Of special interest was the study of SnbD affinity for natural aromatic amino acids, since the L-DMPAPA residue (Fig. 1) may be substituted by some analogs such as L-MMPAPA in PI_B or phenylalanine in virginiamycin S_1. It must be emphasized first that the SnbD affinity for the L-PAPA derivatives increased markedly with the number of methyl groups attached to the amine function of the aromatic ring. This is in accordance with the recent finding that these two N-methylations occur at the amino acid level (3, 6). Besides, the weak SnbD affinity for L-PAPA, phenylalanine, and tyrosine is not too surprising since the corresponding PI analogs are not detected in *S. pristinaespiralis*. We also showed that the corresponding SnbD enzyme, which was purified from *S. virginiae*, the virginiamycin S_1 producer, by a protocol similar to that described for SnbD from *S. pristinaespiralis* (data not shown), had a very good affinity for phenylalanine (Table 2). Thus, it seems that even if the amino acid analog is an in vitro substrate for the peptide synthetase, the affinity must be in the micromolar range to effect efficient in vivo synthesis. This rule was verified when we tested several synthetic aromatic amino acids which could be activated in vitro and also incorporated in vivo by a mutant strain, in which the *papA* gene has been deleted, to generate new PI derivatives (5). This finding indicates not only that the SnbD specificity is large but also that such multifunctional enzymes are versatile enough to carry on peptide chain elongation when early residues of this chain have been substituted by misincorporation of some analogs.

TABLE 3. Purification of SnbE, a proteolytic fragment of PI synthetase 3 (SnbD) exhibiting L-phenylglycine:AMP ligase activity

Purification step	Total amt (mg) of protein	Sp act[a] (U/mg)	Recovery (%)	Purification (fold)
Crude extract	2,200			
Mono Q HR16/10	136	0.36	100	1
Phenyl-Superose	32.6	1.0	72	3
Superdex 200	3.1	5.2	34	15
Mono Q HR 5/5	2.0	6.0	25	18
SDS-PAGE	0.1			

[a] The activity in crude extracts cannot be assayed precisely since unspecific exchanges between PP_i and ATP occurred in control incubation mixtures in which L-phenylglycine was omitted. The overall increase of specific activity can be estimated to 3,000-fold (280-fold up to the Mono Q HR 5/5 step).

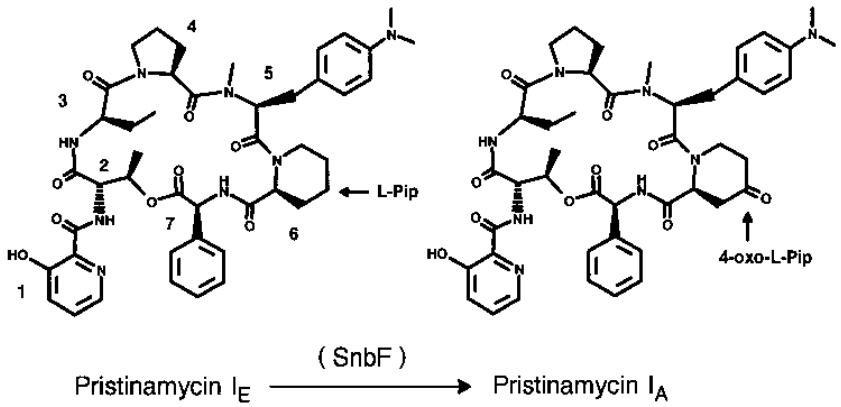

FIG. 3. PI_A macrocycle biosynthesis. The synthesis and cyclization of PI_E peptidic chain from 3-hydroxypicolinic acid, amino acid precursors, and SAM is catalyzed by a three-component PI synthetase. SnbA activates residue 1, SnbC activates residues 2 and 3, and SnbD activates residues 4 to 7. SnbF is involved in the oxidation of pipecolic acid residue of PI_E leading to PI_A. 4-oxo-L-Pip, 4-oxo-L-pipecolic acid.

DISCUSSION

PI is a branched cyclohexadepsipeptide believed to be nonribosomally synthesized. To get a first insight in the enzymology of the formation of the PI macrocycle, several assays of such peptide synthetases were developed. 3-Hydroxypicolinic acid- and L-phenylglycine-dependent ATP-PP_i exchange, L-threonine labeling, and L-DMPAPA αN-methylation assays were found to be relevant and used for further PI synthetase purification. Three enzymes, SnbA, SnbC, and SnbD, were shown to be able to activate all of the PI precursors and to catalyze adenylate or thioester formation in the presence of their substrates. They were purified to near homogeneity from cell extracts of *S. pristinaespiralis*. SnbE, another polypeptide-activating L-phenylglycine, was isolated but was shown to be a proteolytic fragment of SnbD (see below).

Until now, only one enzyme involved in streptogramin B synthesis, a 3-hydroxypicolinic acid:AMP ligase, had been isolated (30). The properties of SnbA are very close to those of the enzyme purified by Schlumbohm and Keller (30). SnbA belongs to the family of adenylate-forming enzymes with M_rs of 50,000 to 60,000 (16, 17, 19, 29–31, 34). This type of enzyme consists roughly of the activation domain present in the 120-kDa modules found in peptide synthetase (36) but lacks the elongation (9) and 4-phosphopantetheinyl arm (31, 36) domains. As a result, these enzymes catalyze adenylation of their substrates but do not bind them as a thioester. Stindl and Keller (37) recently reported on how such an enzyme could participate in the initiation of peptide synthesis in the case of actinomycin, and in agreement with their results, a new model for peptide synthesis initiation has been postulated (10). Another interesting feature of these enzymes is that their substrate is usually an aromatic acid which is often further acylated to the free N-terminal amino group of the peptide. However, enzymes of this type have also been found in some microorganisms producing peptides for which the adenylation step seems to be a prerequisite for esterification of the aromatic acid with the thiol group of a cysteine residue in the case of nosiheptide or with the alcohol group of a threonine residue in the case of thiostrepton (34).

We have shown that SnbC and SnbD are multifunctional enzymes. SnbC is responsible for the incorporation of the second and third residues of PI (Fig. 1). Interestingly, the size found for SnbC is in good agreement with the predicted size for a two-module peptide synthetase. It is a little bit less than the predicted size of the *snbC* gene product, which also includes an epimerization domain (10). In addition, SnbC activates the L form but not the D form of aminobutyric acid. A further epimerization step usually performed by the peptide synthetase (10, 18, 38) is thus required to get the correct configuration of this residue in the PI macrocycle. However, direct confirmation of this epimerization activity awaits characterization of the stereochemistry of the SnbC-thioesterified aminobutyric acid. SnbC protein is also able to activate L-alanine, which competes with the preferred L-aminobutyric acid substrate, and so it is not surprising to find a D-alanine as the third residue in PI_C (Fig. 1). PI_C is a minor member of the PI family, accounting for about 1% of the PI components produced by *S. pristinaespiralis*, whereas PI_A is the major one, accounting for more than 90% of PI components. Possible misincorporation of some residues is a feature of nonribosomal peptide synthetases, and such substitution of aminobutyric by alanine has also been found recently in antibiotics A21459 A and B (14, 33).

Characterization of SnbD protein and of its catalytic activities was not complete. On one hand, purified SnbD protein was not homogeneous and was sensitive to pronounced proteolysis during preparation of cell extracts and purification. As a result, the phenylglycine thioesterification activity can be found associated with, or separated from, SnbD during purification. Phenylglycine:AMP ligase activity was purified as a 170-kDa SnbE protein, which was itself even more sensitive to further proteolysis but could also be associated with polypeptides of intermediate size (see Results). Consequently, and also because it was somewhat surprising that SnbD protein had better catalytic properties for phenylglycine activation than SnbE protein did (Table 2), we suggest that this phenylglycine activation activity was originally associated only with SnbD protein. However, the molar ratio of thioesterified phenylglycine to SnbD (Table 2) is only 3% and about 10-fold less than the ratio found for proline and *N*-methyl-DMPAPA thioesters. Therefore, it cannot be excluded that a contaminating activity has been purified. On the other hand, the size of SnbD protein was not determined in the best conditions now used for such large peptide synthetases (26, 32), and it is probably underestimated. In fact, it could be close to 400 or 500 kDa, the expected size for a four-module peptide synthetase. Interestingly, reevaluation of the SnbD protein size would also improve the rather low thioester molar ratios found for activation of proline or L-DMPAPA by SnbD (Table 2), significantly lower than the ratio for activation of threonine or alanine by SnbC. Finally,

although it was shown that SnbD enzyme could catalyze ATP-PP_i exchange in the presence of pipecolic acid, the amount of SnbD thioesterified pipecolic acid could not be measured since radioactive pipecolic acid was not available. In addition, the specific activity found for peptide synthetase in the ATP-PP_i exchange seems unfortunately to be variable and not correlated with the molar binding ratio (Table 2). In conclusion, SnbD protein remains to be characterized in better conditions. But the finding of three peptide synthetases involved in PI biosynthesis is in agreement with a recently postulated rule for the number of peptide synthetase involved in peptide biosynthesis (9). According to this rule, a new enzyme is involved after each epimerization step. This has been once again verified since the activation and epimerization of L-aminobutyric acid are performed by SnbC and the activation of proline, which follows the D-aminobutyric acid residue in the PI amino acid sequence, is catalyzed by SnbD. Cloning and sequencing of the genes encoding SnbD and SnbE (11) revealed that SnbE is part of the four-module SnbD protein encoded by a unique *snbDE* gene and containing also the thioesterase activity necessary to effect the final cyclization of the PI macrocycle via a lactonic linkage between threonine and phenylglycine.

SnbC and SnbD substrate specificity studies led to the conclusion that PI_C and PI_B arose respectively by misincorporation of alanine as the third PI residue and of L-MMPAPA as the fifth PI residue. On the contrary, 4-oxo-L-pipecolic acid is not activated by SnbD, but this enzyme incorporates L-pipecolic acid as the sixth PI_E residue. This is in agreement with our previous observations (5) that PI_E is a precursor of PI_A (Fig. 3).

It must be emphasized that PI synthetases have been studied here separately and that the permanent covalent binding of the growing peptide chain to the peptide synthetase should require that the three components of PI synthetase, SnbA, SnbC, and SnbD, form a multienzyme complex for an efficient translocation of this growing peptide chain. No evidence for such a complex has been obtained, but the molar amounts of the various PI synthetases are interestingly in the same range and could be stoichiometric. The complete characterization of such an likely multienzyme complex will be possible when the proteolysis problem in *S. pristinaespiralis* has been solved. However, this pioneering work has opened the way to the cloning of the corresponding PI synthetase genes, and expression of these genes in a suitable host will make such mechanistic studies easier.

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