Phosphinothricin-Tripeptide Synthetases from *Streptomyces viridochromogenes*†

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ABSTRACT: Phosphinothricyl-alanyl-alanine (Pt tripeptide (Ptt), bialaphos) is a metabolite produced by *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*. It contains the unique phosphinoamino acid phosphinothricin (Pt), which after cleavage from Ptt is active as an inhibitor of glutamine synthetase. We have isolated three enzymes that assemble the building block of the Ptt peptide backbone in a nonribosomal mechanism. The first enzyme, named Ptt-synthetase I (PTTS I), activates *N*-acetyldemethylphosphinothricin (AcDMPt) as adenylate and thioester. Pt is not activated. PTTS I can also activate *N*-acetylphosphinothricin (AcPt) or *N*-acetylglutamate as structural analogues of AcDMPT. Native PTTS I has an estimated size of 62 kDa whereas the denatured form displays a size of 76 kDa. Immunoblot analysis and determination of its N-terminal protein sequence revealed that PTTS I is identical with the gene product of *phsA*. The *phsA* gene was previously identified near the Pt-resistance gene *pat* in the Ptt biosynthesis gene cluster in *S. viridochromogenes*. Besides PTTS I, two alanine-activating enzymes (PTTS II/III) were partially purified from *S. viridochromogenes* with estimated native sizes of ca. 120 kDa (enzyme 1) and ca. 140 kDa (enzyme 2). Both enzymes bind alanine as a thioester via the corresponding adenylate. Level of PTTS II/III and product formation were correlated with each other in several different strains of *S. viridochromogenes*. These results indicate that Ptt is synthesized by three peptide synthetases, each activating one single amino acid. The data also confirm previous genetic data, which suggest that AcDMPt-Ala-Ala is the precursor of Ptt.

Phosphinothricyl-alanyl-alanine (Pt tripeptide (Ptt)[1] bialaphos, Figure 1) is a linear tripeptide produced by *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus* (*1*, *2*). The compound is active both as an antibiotic and a herbicide due to the unique phosphinoamino acid phosphinothricin (Pt) (Figure 1). When Ptt enters the bacterial cell, Pt is cleaved from Ptt by peptidases and due to its structural similarity to glutamate Pt inhibits glutamine synthetase (*3*). In plant cells, the presence of Pt leads to an accumulation of ammonium ions that are toxic (*4*). This and the isolation of the gene determining self-resistance (*pat* or *bar*, respectively) from the producer organisms of Ptt and its transfer into the genomes of various crop plants make Pt a promising total herbicide for use in agriculture (*5*).

Studies of the biosynthesis of Pt tripeptide (Ptt) with blocked mutants of *S. hygroscopicus* revealed the steps of Pt formation leading from phosphoenolpyruvate to the Pt precursor demethylphosphinothricin (DMPt, Figure 1) (*6*−*10*). The involvement of *N*-acetylated Pt precursors such as *N*-acetyl-DMPt (AcDMPt, Figure 1) in the assembly of Ptt was subsequently elaborated (*11*, *12*). Gene cloning in these mutants by complementation of the corresponding defects revealed that Pt biosynthesis genes were located in a large gene cluster (*13*). Evidence was obtained that this gene cluster also harbors genes responsible for the alanylation step in Ptt synthesis (*12*). The Ptt biosynthesis gene cluster was also shown to be present in *S. viridochromogenes* in an identical arrangement of genes (*14*, *15*). Gene disruptions in *S. hygroscopicus* and *S. viridochromogenes* and analysis of accumulating intermediates in the corresponding mutants indicated that AcDMPt rather than DMPt or eventually Pt itself is the substrate in the process of peptide backbone assembly. The acetylation of DMPt was clearly assigned to the gene product of the Ptt self-resistance gene (*bar* or *pat*) (*5*, *11*) while deacetylation of the final product AcPtt was assigned to an ORF encoding a protein with similarity to *N*-acetyl hydrolases. Interestingly in that same region two ORFs were located that encode thioesterases (ORF1 and ORF2) presumed to catalyze the release of the peptide chain from a putative Ptt-synthetase (*16*).

In contrast to the biosynthesis of phosphinothricin, little is known about the enzymes catalyzing the assembly of Ptt or its suggested primary precursor AcDMPtt. Interestingly,

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[1] Abbreviations: Abu, α-aminobutyric acid; AcDMPtt, *N*-acetyldemethylphosphinothricyl-alanyl-alanine; AcDMPt, *N*-acetyldemethylphosphinothricin; AcPt, *N*-acetylphosphinothricin; AcPtt, acetylphosphinothricin tripeptide; cpm, counts per minute; DEAE, diethylaminoethyl; DMPt, demethylphosphinothricin; DTE dithioerythreitol; EDTA, ethylenediamineacetic acid; NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PAGE, polyacrylamide gel electrophoresis; Pt, phosphinothricin; Ptt, phosphinothricyl tripeptide; PTTS I/II/III, Ptt-synthetase I/II/III; TLC, thin-layer chromatography; PVDF, poly(vinylidene difluoride).

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the encoded gene product of an additional ORF closely linked to *pat* in *S. viridochromogenes* has similarity to some peptide synthetases, indicating that this gene product is directly involved in the assembly of the Ptt peptide backbone (*15*, *17*).

However, from its length of this gene (designated *phsA*), it would encode an enzyme consisting exclusively of one amino acid activation domain. It is therefore not clear whether this enzyme would activate DMPt, AcDMPt, or alanine. The nonribosomal assembly of a tripeptide requires three different amino acid activating domains, and an inactivation of any of the three domains would lead to an AcDMPt accumulating phenotype. To establish the enzymology of Ptt synthesis, we have isolated and characterized the enzymes involved in Ptt or AcDMPtt assembly of *S. viridochromogenes*. As will be shown, *S. viridochromogenes* harbors AcDMPt- and alanine-activating enzymes binding their substrates as thioesters, suggesting their participation in the nonribosomal synthesis of AcDMPtt.

EXPERIMENTAL PROCEDURES

Strains and Media. *Streptomyces viridochromogenes* Tü 494 was obtained from the Deutsche Sammlung für Mikroorganismen (DSM). Strain EK was derived from a single spore isolate of *S. viridochromogenes* Tü 494. Strain 333 was obtained by mutagenesis of strain EK with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (*18*). All Streptomyces strains were grown and maintained on a yeast/malt extract/glucose (HMG) medium as described (*19*). For liquid cultures, a 300-mL Erlenmeyer flask with 100 mL of HMG medium was inoculated with 100 μL of a *S. viridochromogenes* spore suspension (prepared by scraping the mycelial contents of one Petri dish into 20% glycerol). After 3 days of being shaken at 250 rpm at 30 °C (New Brunswick G 25 incubator), 1-mL portions of the culture served to inoculate 20−30 300-mL Erlenmeyer flasks each containing 100 mL of HMG (production stage). These flasks were incubated for 72−96 h with shaking as above. Cells were harvested by suction filtration and used for further enzyme preparation. *Bacillus subtilis* ATCC 6633 was used as a tester strain for Ptt production. It was maintained on either Difco Nutrient Agar or in Difco Nutrient Broth.

Radiochemicals and Chemicals. L-[U-^{14}C]Alanine (156 mCi/mmol) and L-[U-^{14}C]leucine (297 mCi/mmol) were purchased from Amersham International. [^{3}H-acetyl]Coenzyme A (4 Ci/mmol) and L-[U-^{14}C]valine (283 mCi/mmol) were from Du Pont New England Nuclear. DL-[^{14}C]-Aminobutyric acid (26.8 mCi/mmol) was from CEA (Gif sur Yvette, France). DL-[2,3-^{14}C]Phosphinothricin (8.7 Ci/mol), phosphinothricin, and acetylphosphinothricin were generously donated by Dr. H. Stumpff and Dr. Müllner (Hoechst AG, Frankfurt). Phosphinothricyl-alanyl-alanine was purified from a commercially available formulation (Herbiace, Meiji Seika) by repeated dissolving and precipitation of the compound in water and acetone, respectively. All other chemicals were of highest purity commercially available.

Syntheses. [^{3}H]Acetylphosphinothricin (AcPt) was synthesized from phosphinothricin and [^{3}H-acetyl]coenzyme A. The 5 μCi of [^{3}H-acetyl]coenzyme A, 50 nmol of Pt, and 10 μL of crude enzyme extract from *S. viridochromgenes* containing DMPt acetylase were incubated for 90 min in a total volume of 55 μL at 37 °C. The final buffer concentration was 55 mM potassium phosphate, pH 7.2. After incubation, the reaction mixture was heated for 5 min in a boiling water bath and then centrifuged shortly to remove precipitated protein, and the supernatant was applied to a silica gel plate (Merck), developed in solvent system II. Radioactive AcPt was detected by a TLC radioscanner, and the material was isolated by scraping off the radioactive region from the plate and extracting the silica gel with two 2-mL portions of water. The extracts were dried in a Speedvac centrifuge (Savant Instruments), and the radioactive AcPt was dissolved in water to a final concentration of 0.5 μCi/10 μL.

The crude enzyme extract used for AcPt synthesis was prepared from an extract of broken cells of *S. viridochromogenes* by DNase digestion and ammonium sulfate precipitation (the fraction precipitating between 0 and 65% saturation was taken). Before use, the pelleted protein was dissolved in 100 mM potassium phosphate buffer, pH 7.2, and desalted on a Sephadex G10 column (Pharmacia) previously equilibrated with the same buffer. Protein concentration was between 10 and 25 mg of protein/mL.

Buffers and Solvent Systems. Buffer A contained 100 mM Tris-HCl, pH 8.0, 20% (w/v) glycerol, 10 mM dithioerythritol (DTE), 1 mM benzamidine, 10 mM EDTA, pH 8.0, and 1 mM phenylmethanesulfonylfluoride (PMSF). The other buffers were modifications of buffer A as indicated: buffer B had 4 mM EDTA; buffer C had 4 mM DTE; buffer D had 4 mM EDTA, 4 mM DTE, and 0.1 M NaCl; buffer E had 4 mM EDTA and 4 mM DTE.

Thin-layer chromatography (TLC) of amino acids was done on silica gel sheets (Merck, Darmstadt) with BuOH:HOAc:water (4:1:1, by volume) (solvent system I), 2-propanol:HOAc:water (7:3:2; by volume) (solvent system II), ethanol:25% ammonia (7:3, by volume) (solvent system III), or ethanol:water (7:3, by volume) (solvent system IV).

Enzyme Assay. The ATP−pyrophosphate exchange was measured as described previously (*20*). Filter binding assays for measuring covalent binding of amino acid to enzyme were as described (*21*). For the enzymes described here, the assays contained aliquots of protein fractions (20−100 μL), 12 mM ATP, 16.5 mM $MgCl_2$, and varying amounts of ^{14}C- or ^{3}H-labeled amino acid substrates (up to 0.15 and 10 μCi, respectively) in a total volume of 120 μL. Incubation was at 30 °C for 20 min.

Purification of PTTS I (AcDMPt Activating Enzyme). All operations were carried out at 4 °C. A total of 20 g of *S. viridochromogenes* mycelium strains (freshly harvested or stored at −80 °C for up to 4 weeks) was suspended in 100 mL of buffer A, and 20 μg/mL trypsin inhibitor from egg white (Sigma) was added (this inhibitor was present in all stages of purification of PTTS I). The suspension was passed through a French press using a cell pressure of 12 000 psi. After the addition of 0.5 mg of DNase I and stirring for 45 min, the suspension was centrifuged 30 min at 15 000 rpm using a SS34 rotor of a Sorvall RC-2B centrifuge. The supernatant was applied to a Q-Sepharose (Pharmacia) Fast Flow column (8 × 3 cm bed dimensions) that had been previously equilibrated with buffer C. The protein was eluted with a linear gradient of 0−0.4 M NaCl in a total volume of 400 mL of buffer C. Fractions catalyzing the ATP−

pyrophosphate exchange, depending on AcPt, were pooled and concentrated to a final volume of 1 mL by ultrafiltration using an Amicon PM10 membrane. The concentrated protein was applied to a Superdex 200 gel filtration column (type prep grade 16/60, Pharmacia) (flow 0.5 mL/min, fraction size 2 mL) equilibrated before with buffer D. Fractions with enzyme activity were pooled, diluted with 3 vol of buffer E, and loaded to a Mono Q column (type HR 5/5, Pharmacia). Proteins were eluted with a 65-mL linear gradient in buffer E from 0 to 0.3 M NaCl (flow 1 mL/min, fraction size 1 mL).

Purification of PTTS II/III (Alanine Activating Enzymes). About 40 g (wet weight) of *S. virdochromogenes* mycelium (freshly harvested or stored at −80 °C for up to 4 weeks) was suspended in buffer B (200−250 mL final volume). The suspension was passed through a French press using a cell pressure of 12 000 psi. After the addition 0.5 mg of DNase I and stirring 45 min, the suspension was centrifuged 30 min at 15 000 rpm using a SS34 rotor. The supernatant was applied to a DEAE-cellulose column (8 × 4 cm bed dimensions) equilibrated before with buffer E. Proteins were eluted with a linear gradient of 0−0.3 M NaCl in a total volume of 500 mL of buffer E. Fractions showing alanine binding activity were pooled, and ammonium sulfate was added to give a saturation of 65%. This mixture was left on ice overnight. The precipitate was collected by centrifugation as above described. The pellets were dissolved in a minimum of buffer E and loaded to an Ultrogel AcA34 gel filtration column (40 × 3.5 cm bed dimensions) equilibrated with buffer E. Fractions containing activity were pooled and applied to an anion-exchange column (Resource Q, 6 mL, Pharmacia) equilibrated with buffer E. Proteins were eluted with a 36-mL linear gradient from 0 to 0.4 M NaCl in buffer E (flow 2 mL/min, fraction size 2 mL). From each alanine activating peak, after the Resource Q column, portions of 3 mL with the highest activity were taken for further purification to a Superdex 200 (type prep grade 16/60, Pharmacia) in buffer E (flow 0.5 mL/min, fractions size 2 mL). In the case of enzyme 1, purification was continued by chromatography on a Mono Q column (type HR 5/5, Pharmacia) equilibrated with buffer E. The enzyme was eluted with a 40-mL linear gradient from 0 to 0.3 M NaCl in buffer E (flow 1 mL/min, fraction size 1 mL/min).

Methods of Analyses. Protein concentration was determined according to Bradford (*22*). SDS−PAGE was done according to Laemmli (*23*). Serum containing antibodies against a truncated *phsA* gene product (*17*) was used without further purification. Western blot analysis was performed by standard techniques using 750-fold dilution of primary antibody, phosphatase-conjugated goat anti-rabbit antibody (Sigma), nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as reagents.

Radioactive bands on thin-layer plates were localized by autoradiography with Konica X-ray films. Exposure time was between 2 and 8 d. In the case of tritium-labeled material, silica gel plates were sprayed with En^{3}HANCE spray (Dupont) before exposure to X-ray film. Autofluorography of SDS gels containing labeled protein bands were essentially performed as described by Walzel et al. (*24*). Isolation of substrate−enzyme thioesters and cleavage with performic acid were as previously described (*21*).

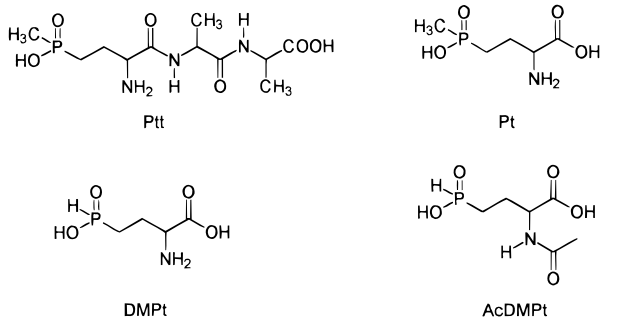

FIGURE 1: Structures of phosphino compounds produced by *S. virdochromogenes*. Ptt, phosphinothricin tripeptide (bialaphos); Pt, phosphinothricin; DMPt, demethylphosphinothricin; AcDMPt, *N*-acetyl-demethylphosphinothicin.

Molecular weights of native proteins were estimated by gel filtration on a calibrated Superdex 200 column. Marker proteins were β-amylase from sweet potato (200 000), aldolase (158 000), bovine serum albumin (66 000), and carbonic anhydrase (29 000). Determination of antibiotic activity in culture fluids of *S. viridochromogenes* or on solid medium was performed in paper disk or agar plug diffusion assays, respectively, using *Bacillus subtilis* ATCC 6633 as tester organism (*1*).

RESULTS

Acetyl-Pt Activation in S. viridochromogenes. Protein fractions from *S. viridochromogenes* obtained by anion-exchange chromatography or by gel filtration of cell extracts were found to contain a significant ATP−pyrophosphate exchange activity dependent on *N*-acetyl-Pt (AcPt, Figure 1) whereas Pt was not activated. The reason to use AcPt as substrate was that initially AcDMPt was not available, and we argued that AcPt due its structural similarity with AcDMPt (Figure 1) might be able to replace the putative natural substrate in the activation reaction. Purification of the AcPt-activating enzyme is shown in Table 1. Passage of cell extract through a Q-Sepharose column, fractionation by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography (Figure 2) afforded an 11-fold purification based on the Q-Sepharose step. If one assumes that the same number of units was present in the crude extract (which could not be determined due to strong nonspecific ATP−pyrophosphate exchange activity), the total purification was more than 520-fold. The yield of enzyme was rather low due to an unusual instability of the enzyme; e.g., the enzyme of the gel filtration step lost activity within 4−20 h depending on the quality of the preparation. This necessitated purication of the protein in less than 1 day. Freezing enzyme preparations abolished activity. SDS gel electrophoretic analysis of the enzyme from the last purification step showed one main band with an estimated size of 76 kDa (Figure 2). This band was considered as a possible candidate for the AcPt-activating enzyme.

Characterization of AcPt-Activating Enzyme (Ptt-Synthetase I, PTTS I). The native molecular mass of the AcPt-activating enzyme was estimated by gel filtration on a calibrated Superdex 200 column. The enzyme has a native size of 62 kDa in contrast to the 76 kDa determined for the denatured form of enzyme. From these size estimates, the enzyme would have the capacity to activate only one amino

Table 1: Purification of Phosphinothricin Tripeptide Synthetase I (PTTS I)[a]

purification step[a]	vol (mL)	total amt of protein (mg)	activity (pkat)[b]	specific act (pkat/mg)	recovery (%)	purification (fold)
crude extract	115	690	–[c]	–	–	–
Q-Sepharose FF	57	17.1	1069	63	100	1
$(NH_4)_2SO_4$ 70%	1.3	13.0	–	–	–	–
Superdex 200	7.5	2.3	244	106	23	1.7
MonoQ	6	0.2	143	715	13	11

[a] 17 g of cells from a 96-h-old culture of S. viridochomogenes was used. Purification is based on ATP−pyrophosphate exchange dependent on the presence of AcPt. [b] 1 pkat is the amount of enzyme catalyzing the exchange of 1 pmol of pyrophosphate into ATP per second. [c] Not determined.

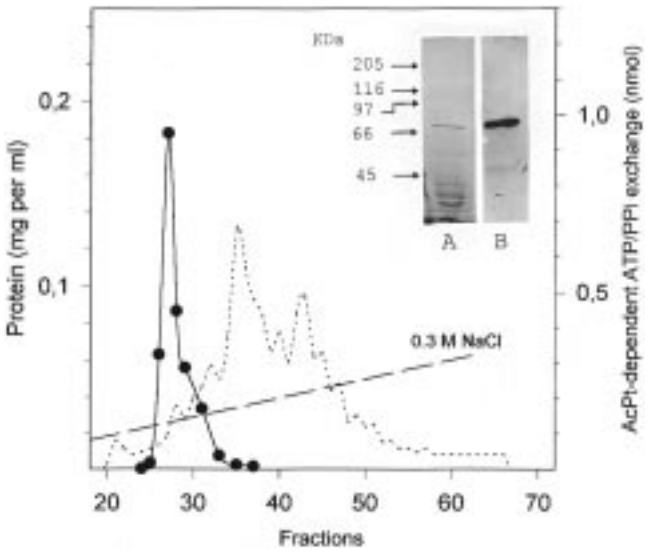

FIGURE 2: Separation of AcPt activating enzyme (PTTS I) from *S. viridochromogenes* by anion-exchange chromatography. Enzyme from the Superdex 200 separation (Table 2) was applied onto a Mono Q column as described in the Experimental Section. Assays were performed as described in the Experimental Section. (⋯) protein, (●) ATP−pyrophosphate exchange dependent on the presence of AcPt. The inset shows the SDS gel of the Mono Q fraction 27 stained with Coomassie Blue (lane A) and the corresponding immunoblotting experiment with polyclonal antibodies against PhsA (lane B).

Table 2: Substrate Specificity of the AcPt-Activating Enzyme (PTTS I)[a]

activation of various substrates catalyzed by PTTS I		
substrate added	nmol exchanged	rel acty (%)
AcDMPt	1.607	100
AcPt	1.194	74
AcGlu	0.168	11
AcAsn	0.028	2
Pt	0.028	2
Asp	0.023	1
AcAsp	0.019	1
Glu	0.018	1
AcGln	0.013	1
none	0.027	2

[a] Enzyme was tested in the ATP−pyrophosphate exchange rection dependent on the presence of variou substrates. 50 μL of the Mono Q step (see Table 1) was taken for each experiment. Assay conditions were as described in the Experimental Procedures.

acid residue (at least as adenylate) because it is known that one peptide synthetase domain activating one amino acid substrate would require a minimal length of 600 amino acid residues (*25*). Consistent with this, the enzyme does not activate alanine, indicating the need for additional enzymes involved in Ptt assembly distinct from the AcPt-activating enzyme (see below).

Table 2 shows the efficiency of the enzyme in activating various substrates besides AcPt. Importantly, the enzyme activates AcDMPt (generously donated by Dr. H. Seto, Meiji-Seika, Inc.) to a significantly higher extent than AcPt. This confirms that AcDMPt is most probably the natural substrate of the enzyme system responsible for Ptt assembly. Like Pt, the enzyme does not activate glutamate or aspartate. The fact that enzyme activates AcDMPt and AcPt but not Pt prompted us to test acetyl-glutamate (AcGlu) and acetyl-aspartate (AcAsp) in the ATP−pyrophosphate reaction. Enzyme activated AcGlu only—although to a much lesser extent than AcDMPt—which shows that the acetyl group attached to the amino group of DMPt, Pt, or glutamate is necessary for substrate recognition. Furthermore, these findings show that the enzyme can distinguish between the acidic side chains of AcGlu and AcAsp. Neither acetyl-glutamine nor acetyl-asparagine stimulated the ATP−pyrophosphate exchange, confirming the importance of the presence of the negative charge in the side chain of the substrate for substrate recognition. Thus, this enzyme has a very narrow substrate specificity in which it resembles a number of other activating enzymes involved in starter (i.e., amino terminal moiety) activation of several nonribosomal peptide synthesis systems (*24*).

The ability of the enzyme to bind its substrate covalently as thioester was measured by incubating enzyme with [^{3}H]-acetylphosphinothricin, which had been enzymatically prepared from [^{3}H-acetyl]coenzyme A and Pt as described in the Experimental Procedures. Enzyme was able to bind AcPt in an ATP-dependent manner as revealed by the formation of trichloroacetic acid-stable radioactivity. No enzyme-bound radioactivity was seen when radioactive Pt instead of [^{3}H]acetyl-Pt was used as substrate (not shown).

phsA is a gene located in the Ptt gene cluster of *S. viridochromogenes*. From its deduced amino acid sequence, it has sequence similarity with peptide synthetases from various organisms and has been assigned a function in the assembly of Ptt (*15*). Disruption of *phsA* leads to a phenotype unable to produce Ptt and accumulating AcDMPt (*17*). Antibodies that have been raised against the *phsA* gene product expressed in *Escherichia coli* (*17*) were used to analyze by Western blotting the enzyme fractions containing the AcPt-activating enzyme. These antibodies clearly recognized the 76-kDa band of the AcDMPT-activating enzyme (Figure 2) in the purified enzyme fraction as well as in the previous purification steps (the latter not shown). This lends support to the assumption that the AcDMPt-activating enzyme is the *phsA* gene product. Final proof for this came from microsequencing of the 76-kDa protein band after electroblot transfer to PVDF membrane. The sequence of the first seven amino acids of the enzyme was TPDTAPD, which is identical with residues 5−11 of the deduced *phsA*

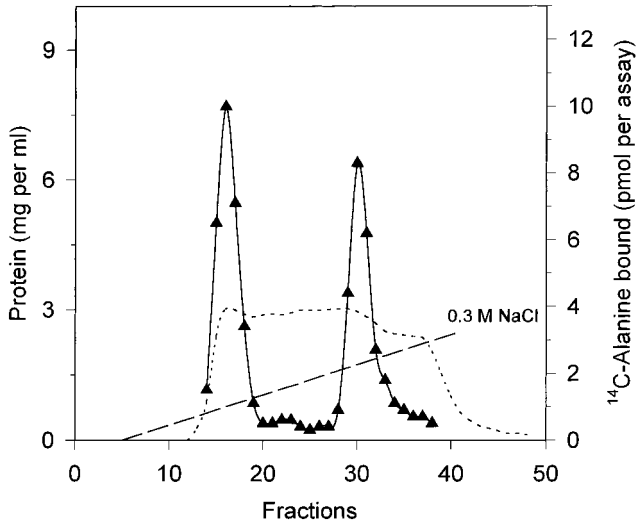

FIGURE 3: Separation of alanine-activating enzymes from *S. viridochromogenes* by anion-exchange chromatography. Enzyme from the Ultrogel AcA34 gel filtration (Table 3) was applied onto a Resource Q column (6 mL, Pharmacia) as described in the Experimental Section. Assaying the various fraction for alanine thioester formation reveals the presence of two enzyme species designated enzyme 1 and enzyme 2 (see text). (•••) protein, (▲) [^{14}C]alanine thioester formation. Assays were performed as described in the Experimental Section.

FIGURE 4: Alanine is bound to the alanine-activating enzyme (PTTS II, enzyme 1) as a thioester. The lanes show TLC separations of performic acid hydrolysis of alanine bound to PTTS II (lane A). Lane B show controls where bound alanine was treated with formic acid instead of performic acid. Lane C show an alanine control. Solvent system II was used.

gene product (MTAATPDTAPD). Apparently, the first four amino acids of the sequence are removed in a posttranslational modification step. The enzyme was designated Ptt-synthetase I (PTTS I).

Alanine Activation in S. viridochromogenes. *S. viridochromogenes* contains peptide synthetase activity capable of binding alanine covalently in an ATP-dependent manner. Alanine-binding activity was located always in one single peak after chromatography of extracts of broken cells on a DEAE-cellulose and subsequent gel filtration on Ultrogel AcA34. In the latter case, the enzymic activity appeared in the fractionation range between 100 and 150 kDa. Further chromatography of the alanine-binding enzyme on propylagarose columns and gradient elution with 0−0.1 M NaCl separated the enzyme peak of the Ultrogel AcA34 gel filtration into two alanine-activating protein species (eluting at ca. 10 and 30 mM NaCl, respectively, not shown). The resolution into two enzyme species could also be seen when alanine-binding enzyme was chromatographed on anion exchangers such as Resource Q (Pharmacia) instead on propylagarose (Figure 3). The two alanine-binding enzymes were designated enzyme 1 and enzyme 2 eluting at lower and higher salt strength, respectively.

Characterization of Alanine-Activating Enzyme. The nature of the covalent bond in which alanine becomes attached to the enzyme was analyzed by performic acid oxidation. Figure 4 shows that protein-bound amino acid attached to enzyme 1 could be liberated by treatment with performic acid but not with formic acid, indicating the thioester linkage in the enzyme−substrate complex. The same result was obtained with enzyme 2 (not shown). The charging of both enzymes with alanine was rapid and essentially complete within 5 min. Iodoacetamide at 1 mM concentration inhibited the thioester formation to some extent (40% inhibition in 40 min), indicating a rather insensitive sulfhydryl group. This probably represents a 4′-phosphopantetheine cofactor covalently bound to both enzymes and involved in substrate binding, which is a feature of peptide synthetases. Thus, from their substrate binding these two enzymes behave as peptide synthetases.

Enzyme 1 and enzyme 2 were never present in equal amounts in the enzyme preparation. Instead, enzyme 1 was the more abundant species making up 55−90% of the total alanine-binding activity. One reason for this deviation from a 1:1 molar ratio was the apparent instability of enzyme 2 (which is similar to the case of PTTS I). Losses of activity during purification precluded further purification of this enzyme; the purification after the Superdex 200 step was only 7-fold (Table 3). We noted that when EDTA was omitted from the buffers used in the purification procedure, enzyme 2 was completely absent in the separation step involving Resource Q anion exchanger. This indicated loss of the protein through action of metalloproteinases. By contrast, enzyme 1 was appreciably proteinase resistant and could be further purified to a final purification of 18-fold as calculated from the DEAE-cellulose step (Table 3). Assuming that the extract of broken cells contained the same number of units as in the DEAE-cellulose step, the total purification is at least 360-fold. SDS gel electrophoretic analysis of protein fractions obtained from the Mono Q step showed that the preparation was not pure. However, correlation of appearance of bands and alanine thioester forming activity indicated the largest band (116 kDa) in the preparation as the possible candidate for the enzyme.

The M_r values of enzymes 1 and 2 in their native forms were estimated by gel filtration on a calibrated Superdex 200. Values of ca. 120 000 and ca. 140 000 were obtained for enzymes 1 and 2, respectively. Analysis of the band of

Table 3: Purification of the Alanine-Activating Enzymes[a]

	vol (mL)		total protein (mg)		total activity (units)		specific activity (units/mg)		recovery (*x*-fold)		yields (%)	
step[b]	E1	E2	E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
crude extract	270		3038		–[c]		–		–		–	
DEAE-cellulose	108		345		9.5		0.028		1		100	
Ultrogel-AcA34	83		204		7.1		0.033		1.3		75	
Resource Q	10	10	33.3	27.2	2.5	2.1	0.075	0.077	3	3	26	22
Superdex 200	20	–	4.8	4.1	2.4	0.7	0.500	0.170	18	7	25	9
Mono Q	8	–	1.9	–	2.1	–	1.105	–	41	–	22	–

[a] Purification is based on thioester formation. For details see Experimental Procedures. One unit is the amount of enzyme that covalently binds 1 nmol of alnin/30 min. [b] 40 g of cells from a 96-h-old culture of *S. viridochomogenes* were used. [c] Not determined.

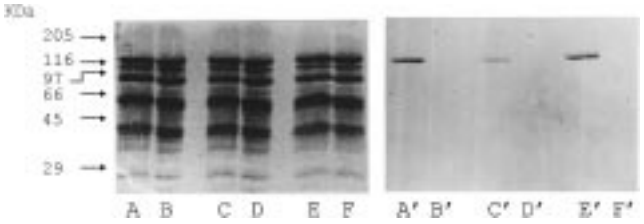

FIGURE 5: Autofluorogram of an electrophoretic separation of ^{14}C-labeled PTTS II 20 μL protein from the Mono Q separation was incubated in the presence of 9.5 mM ATP, 12.5 mM $MgCl_2$ and 0.25 μCi [^{14}C]Ala (lane A) or 0.5 μCi [^{14}C]Abu (lane C) or [^{14}C]-Val (lane E) in a total volume of 32 μL for 20 min at 30 °C. After incubation, 5 μL of 10% SDS was added, and the mixture was loaded on a 10% SDS gel. Lanes B, D, and F show the corresponding preparation in the absence of ATP. The corresponding autofluorogram is shown in lanes A′−F′.

enzyme 1 in SDS gels by autofluorography after substrate labeling was performed after chromatography on Mono Q (Pharmacia) ion exchanger. Alanine clearly labeled the 116-kDa protein presumed to represent the enzyme shown as shown in Figure 5. Both the amount and purification of enzyme 2 was too low for this kind of analysis. Interestingly, the size of the band represented by enzyme 1 differed in the various isolates of *S. viridochromgenes* isolates used in this study. While in *S. viridochromogenes* wild-type Tü 494 the alanine-labeled band had a size of 116 000 ± 5000 in other strains such as strain 333 it was larger (140 000 ± 7000), which indicates that, like enzyme 2, enzyme 1 is also subject to proteolysis and most likely is derived from a larger polypeptide. However, the 116-kDa form of enzyme 1 appears to be resistant to further proteolytic degradation. Attempts to detect high molecular weight bands (>200 kDa) by SDS−PAGE analysis of extracts of broken cells of *S. viridochromogenes* were not successful.

Substrate Specificity of Alanine-Activating Enzyme 1. Enzyme 1 was tested for whether it could activate substrates structurally related to alanine. Since enzyme 1 could not be purified to homogeneity, and therefore the possibility of accompanying contaminating activities could not be excluded, we tested the peak profiles of the enzyme either from the Resource Q or Mono Q columns in activation reactions with various substrates along with alanine as control. Table 4 shows that enzyme 1 activates alanine best in both the ATP−pyrophosphate exchange and thioester formation assays. Aminobutyric acid gave a substantial response both in the ATP−pyrophosphate exchange and in the formation of thioester (74% of that of alanine). Valine was less effective in enzyme charging (8%) and stimulated the ATP−pyrophosphate weakly. Figure 5 shows that both aminobutyric acid and valine labeled the same protein as did alanine.

Table 4: Substrate Specificity of the Alanine-Activating Enzyme (PTTS II, Enzyme 1)

activation of various substrates catalyzed by PTTS II				
	ATP−pyrophosphate exchange[a]		thioester formation[b]	
substrate added	nmol exchanged	rel acty (%)	pmol bound	rel acty (%)
L-Ala	1.078	100	32.0	100
L-Abu	0.442	41	23.8	74
NorVal	0.050	5	–[c]	–
L-Val	0.041	4	2.6	8
allylglycine	0.041	4	–	–
L-Leu	0.026	2	0.4	1
NorLeu	0.025	2	–	–
L-Ile	0.024	2	–	
β-Ala	0.022	2	–	–
H_2O	0.022	2	–	–

[a] 100 μL of a Resource Q-purified enzyme preparation was used for the ATP−pyrophosphate exchange reaction with various substrates. [b] Thioester formation was measured with 50 μL of a Mono Q-purified enzyme preparation. [c] Not determined.

This leaves no doubt that all of these substrates were activated by the same enzyme. Comparable levels to the activation of valine were seen also with allylglycine and norvaline in the ATP−pyrophosphate exchange. Activation of leucine [an amino acid present in a structural analogue of Ptt called phosalacine produced by *Kitasatosporia phosalacinae* (*26*)] as thioester was undetectable in autofluorograms. The activations of the amino acids mentioned in Table 4 indicate that the enzyme preferably accepts short-chain amino acids.

Correlation of Enzyme Level with Ptt Synthesis in Vivo. Attempts were undertaken to improve the productivity of *S. viridochromogenes* strain EK in respect of Ptt formation in order to achieve higher enzyme yields. Strain EK synthesized 5−15 mg/L of Ptt after 4 days of growth. Spores of strain EK were mutagenized with NTG. Analysis of ca. 1500 single colonies in a agar plug diffusion assay with *B. subtilis* ATCC 6633 as tester strain revealed two isolates with increased titer of Ptt in liquid culture. One of these, strain 333, produced 50−100 mg/L of Ptt after 4 days of growth, which is a ca. 10-fold increase of Ptt production when compared to the parent strain. Two determinations, each in parallel with the parent strain, of the alanine-activating enzyme in strain 333 revealed a 5- and 8-fold increase in specific activity of total alanine-activating activity. These results strongly suggest that this enzyme activity is part of the Ptt synthesizing enzyme system. We therefore designate enzymes 1and 2 Ptt-synthetases II and III, respectively (PTTS II/III).

DISCUSSION

Generally, the number of amino acid activation domains in a given nonribosomal multienzyme system equals the number of amino acid or acyl residues in the peptide product (*27*−*29*). In prokaryotes, it appears that peptide synthetase domains involved in the assembly of peptides are distributed over more than one polypeptide chain whereas in eukaryotes all domains of a biosynthesis system are assembled on one protein chain (*29*). From this, one had to expect that more than one enzyme was involved in Ptt assembly. Such enzymes can be detected by testing protein fractions for their ability to catalyze activation reactions dependent on the building blocks of the final peptide product, even if a total cell-free system is not available.

A central issue in the investigation of Ptt assembly is the question of which phosphinoamino acid would serve as the precursor of peptide-bound Pt. Mutants of *S. hygroscopicus* and *S. viridochromogenes* with gene disruptions in the neighboring region of the *bar* (*pat*) gene had an AcDMPt-accumulating phenotype (*12, 30*). This suggested that AcDMPT is the direct precursor in the assembly of Ptt. In agreement with these data, we could show here that a 62-kDa enzyme from Ptt-producing *S. viridochromogenes* designated PTTS I activates AcDMPt but not Pt as adenylate and thioester. Amino terminal sequencing of the enzyme revealed that it is identical with the gene product of *phsA* of *S. viridochromogenes*, a previously isolated peptide synthetase gene situated in the vicinity of the *pat* gene (*17*). *PhsA* would encode an enzyme of 622 amino acids, which contains one domain responsible for the activation and covalent binding of one amino acid. This is in accordance with the properties and size of PTTS I. Disruption of *phsA* has led to an AcDMPT-accumulating phenotype (*17*), but it had not been clear, previously, whether the gene coded for an alanine- or AcDMPT-activating enzyme because disruption in any peptide synthetase domain of the enzyme system catalyzing Ptt assembly would lead to this phenotype.

PTTS I has strict substrate specificity for the *N*-acetylated phosphinoamino acid AcDMPt and AcPt as well as for their structural analogue AcGlu. Pt is not activated and presumedly neither is demethylphosphinothricin (DMPt). This narrow substrate specificity may ensure that no Ptt is formed inside the cell because methylation of the phosphino group of DMPt cannot take place unless the latter is acetylated and incorporated into the tripeptide (*13*). AcPtt appears to be deacetylated later in the course of its export out of the cell (*13*). The importance of the substrate specificity in respect of residues involved in the initiation step in enzymatic peptide synthesis—as appears to be the case with AcDMPt—has been demonstrated also in other examples of peptide synthesis systems. By contrast, activation domains responsible for the activation of the amino acids next to the N-terminal one in the peptide peptide product often display broader substrate specificities (*24, 29*).

In addition to PTTS I, isolation of two alanine-activating enzymes (PTTS II/III) from *S. viridochromogenes* uncovered the residual missing of two amino acid activation domains necessary for AcDMPtt assembly. The two enzymes clearly have a broader substrate specificity than PTTS I and besides alanine can activate other short-chain amino acids such as aminobutyric acid or valine. They have coordinately elevated levels in *S. viridochromogenes* strains selected for Ptt high production when compared to the parent strain. Their sizes of ca. 120 and 140 kDa are in agreement with the length of activation domains of multidomain peptide synthetases including associated modules involved in acyl transfer and peptide bond formation, which leads to an average size of 125 kDa of a whole peptide synthetases unit (*29*). This indicates that PTTS II/III contains the necessary functions for acyl transfer and peptide bond formation in AcDMPtt assembly. By contrast, PTTS I exclusively consists of one activation domain including a thioester-forming ACP module.

The observed proteolytic processing of enzyme 1 and enzyme 2 leads to speculations whether the two enzymes are derived from one large two domain enzyme. Similar observations were observed in other nonribosomal peptide synthetase systems such as echinomycin (W. Schlumbohm, Laubinger, and U. Keller, unpublished) or pristinamycin synthesis (*31*) where enzymatically active fragments have been isolated that arose by proteolytic cleavage in the interdomain space creating two enzyme species from one larger multienzyme. Whether this is also the case in Ptt synthesis can only be answered when one or both genes of the enzymes 1 and 2 are cloned and sequenced. This will also eventually allow the heterologous expression of these genes for developing in vitro systems to study the mechanism of AcDMPtt formation.

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