Gramicidin S Synthetase 1 (Phenylalanine Racemase), a Prototype of Amino Acid Racemases Containing the Cofactor 4′-Phosphopantetheine†

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ABSTRACT: The biosynthesis of the decapeptide antibiotic gramicidin S in *Bacillus brevis* ATCC 9999 is catalyzed by a multienzyme system consisting of two multifunctional proteins, gramicidin S synthetase 1 and 2, encoded by the *grsA* and *grsB* genes, respectively. Gramicidin S synthetase 1 (phenylalanine racemase, EC 5.1.1.11, GS1) racemizes phenylalanine in the thioester-bound stage. The amount of 4′-phosphopantetheine liberated from highly purified GS1 was determined microbiologically using *Lactobacillus plantarum* as the test organism. It matches exactly with the amount of L-[^{14}C]phenylalanine covalently incorporated by GS1 as thioester. The reaction center of GS1 for L-phenylalanine thiolation and racemization was labeled with [^{3}H]iodoacetic acid. After tryptic fragmentation of the ^{3}H-carboxymethylated enzyme, the active site peptide for thioester binding and racemization of phenylalanine was isolated in pure form by multistep methodology and investigated by sequence, amino acid, and mass spectrometric analysis. A 4′-phosphopantetheine carrier was found to be attached to the active site serine of the consensus motif LGGDSI forming the thiolation site of phenylalanine. These specific properties establish GS1 as a prototype of amino acid racemases using 4′-phosphopantetheine as a cofactor and yield further evidence that multiple Pan carriers are involved in gramicidin S formation. Our results are strong evidence for the "multiple carrier model" as a new concept of nonribosomal peptide biosynthesis at protein templates as recently proposed [Stein, T., et al. (1994) *FEBS Lett. 340,* 39−44].

Microbial organisms produce a variety of low molecular mass linear and cyclic peptides, depsipeptides, and peptidolactones which often exhibit antibiotic effects. Structural features of these secondary metabolites are non-proteinogenic amino acids, hydroxy acids, and amino acids in D-configuration (Kleinkauf & von Döhren, 1990). With a few exceptions their biosynthesis is performed nonribosomally by multienzyme complexes consisting of multifunctional polypeptide chains which catalyze substrate selection, activation, modification (e.g., racemization, hydroxylation), and polymerization. Since the first gene sequences for peptide-forming multienzymes are available—*grsA* and *grsB* encoding gramicidin S synthetase 1 and 2, respectively (Krätzschmar et al., 1989; Turgay et al., 1992), and *pcbAB* and *acvA* encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Penicillium chrysogenum* (Smith et al., 1990; Díez et al., 1990) and *Aspergillus nidulans* (MacCabe et al., 1991), respectively—sequence alignment has shown that these enzymes are composed of highly conserved amino acid activating domains. Their number equals the number of substrate amino acids incorporated into the product. Approximately 120 kDa, or 1000 amino acid residues, comprise such an amino acid activation module. These findings are in good agreement with the polyenzyme model proposed by F. Lipmann (1954) in the preribosomal history of protein biosynthesis research. In particular, studies on the enzymology of gramicidin S and tyrocidine formation revealed the multienzymatic thiotemplate hypothesis for nonribosomal peptide biosynthesis (Kleinkauf et al., 1971; Lipmann, 1973; Laland & Zimmer, 1973; Kurahashi, 1974).

The biosynthesis of gramicidin S (D-Phe-L-Pro-L-Val-L-Orn-L-Leu)$_2$ produced by *B. brevis* ATCC 9999 is catalyzed by gramicidin S synthetase. It consists of two multifunctional enzymes, which activate their cognate amino acid substrates in a two-step process involving adenylation and thioester binding. Gramicidin S synthetase 1 (GS1,[1] M = 127 kDa) activates and racemizes phenylalanine (Tomino

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[1] Abbreviations: GS1, phenylalanine racemase, EC 5.1.1.11 (gramicidin S synthetase 1); GS2, gramicidin S synthetase 2 (condensing, peptide forming enzyme); Pan, 4′-phosphopantetheine; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CM-, carboxymethyl-; OPA, *o*-phthaldialdehyde; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; ESI MS, electrospray ionization mass spectrometry; *m/z*, mass to charge ratio; kV, kilovolt; PP_i, pyrophosphate; CI_{50}, inhibitor concentration at which 50% of an enzymic activity is left.

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et al., 1967; Vater et al., 1976). Racemization is performed in the stage of the thioester-bound substrate (Yamada & Kurahashi, 1968, 1969) in a D/L ratio of approximately 2 : 1 (Takahashi et al., 1971). A cofactor, like pyridoxal phosphate, which is used by certain other racemizing enzymes (Adams, 1972; Walsh, 1989, and references therein), has not been detected in GS1 (Kanda et al., 1978, 1989). Also 4′-phosphopantetheine could not be found as a constituent of this enzyme (Gilhuus-Moe et al., 1970; Lipmann et al., 1971; Kleinkauf et al., 1971). Specifically, D-Phe is transferred to gramicidin S synthetase 2 (GS2, $M = 510$ kDa), the condensing enzyme. It consists of four modules which activate the remaining four amino acids and catalyze the elongation of the growing peptide chain. Detection of peptide intermediates covalently bound to 4′-phosphopantetheine at GS2 (Kleinkauf et al., 1971) led to the hypothesis that a central 4′-phosphopantetheine swinging arm is involved in the assembly and transport of the growing peptide chain (Lipmann, 1973; Laland et al., 1972).

We have demonstrated by affinity labeling and analysis of active site peptides that an active site serine is required for covalent attachment of the substrate amino acids instead of a cysteine as originally proposed in the thiotemplate hypothesis (Schlumbohm et al., 1991). This serine is part of a strictly conserved LGG(H/D)S(L/I) core sequence that resembles the 4′-phosphopantetheine attachment site in acyl carrier proteins/domains of fatty acid and polyketide synthetases. Because the thiolation (Pan binding) motif is contained in each amino acid activation module of gramicidin S synthetase, each of them should be equipped with a separate 4′-phosphopantetheine cofactor.

In this article we demonstrate that the reaction center of gramicidin S synthetase 1 for thioester binding and racemization of phenylalanine is indeed equipped with such a 4′-phosphopantetheine cofactor. These are important results, because GS1 is not directly involved in peptide elongation, and no cofactor could be found as a constituent of this enzyme in former studies. Our data corroborate the "multiple carrier model" as a new concept of nonribosomal peptide biosynthesis and introduce GS1 as a prototype of 4′-phosphopantetheine-dependent amino acid racemases.

EXPERIMENTAL PROCEDURES

Materials. L-[U-^{14}C]phenylalanine and [^{3}H]iodoacetic acid were purchased from Amersham/Buchler (Braunschweig, Germany); tetrasodium [^{32}P]pyrophosphate was from DuPont-New England Nuclear (Bad Homburg, Germany). TPCK-treated trypsin, D-amino acid oxidase, catalase, bovine alkaline phosphatase, bovine serum albumin, and 2,4-dinitrophenylhydrazine were obtained from Sigma (Deisenhofen, Germany). L/D-Phenylalanine and calcium D(+)-dipantothenate were purchased from Merck (Darmstadt, Germany). The Mono Q HR 5/5 column was from Pharmacia (Freiburg, Germany). The C_{18} ODS Hypersil (5 μm) columns were obtained from Shandon (England) and Knauer (Germany).

Enzyme Preparation. Bacillus brevis ATCC 9999 was cultivated in a fumarate/phosphate medium, as reported by Chiu (1984). Phenylalanine racemase (gramicidin S synthetase 1, GS1) was purified as published previously (Vater et al., 1987). The protein concentration was measured using the procedures of Warburg and Christian (1941) and Bradford (1976).

Assays for the Reactions Catalyzed by GS1. (a) Gramicidin S Biosynthesis. The assay for gramicidin S biosynthesis was performed as described by Gevers et al. (1968) and Vater et al. (1987).

(b) Aminoacyl Adenylate Formation. The rate of the D-phenylalanine-dependent ATP−[^{32}P]pyrophosphate exchange was measured to analyze the aminoacyl adenylation reaction by GS1 as reported previously (Gevers et al., 1968; Vater et al., 1985). Reaction mixtures (200 μL) contained 2 mM ATP, 1 mM EDTA, 1 mM DTE, 10 mM $MgCl_2$, 0.5 mM $Na_4P_2O_7$, 0.2 μCi of [^{32}P]PP_i, and 1 mM D-phenylalanine in 20 mM sodium phosphate buffer (pH 7.2). After incubation for 10 min at 37 °C, the reaction was stopped by the addition of 0.5 mL of Norit A mixture.

(c) Thioester Binding of Phenylalanine. The covalent acid stable incorporation of L-phenylalanine by GS1 (Gevers et al., 1969; Vater et al., 1985) was measured in reaction mixtures of 200 μL containing 0.5−1 μM GS1, 20 μM L-phenylalanine (5 μM ^{14}C-labeled and 15 μM nonradioactive L-Phe), 2 mM ATP, 10 mM $MgCl_2$, and 1 mM EDTA in 20 mM sodium phosphate buffer (pH 7.2). After incubation for 10 min at 37 °C, the thioester complex of GS1 with phenylalanine was precipitated by adding 5% trichloroacetic acid (w/v), kept on ice for 30 min, and collected on membrane filters. The filters were washed with 5 mL of 5% (v/v) trichloroacetic acid as well as 5 mL of H_2O and were dried for 30 min at 110 °C. The quantity of formed thioester was determined by liquid scintillation counting. The data were corrected for the unspecific adsorption of the L-[^{14}C]phenylalanine tracer to the filter by assaying reference samples from which the enzyme was omitted.

(d) Phenylalanine Racemization Assay. For the analysis of the Phe racemization activity of GS1 a protocol similar to one described by several authors was followed (Yamada & Kurahashi, 1969; Zimmer & Laland, 1975; Kanda et al., 1978). One hundred microliters of a Mono Q fraction of GS1 was incubated in a final volume of 1 mL for 15 min at 37 °C in 100 mM triethanolamine/HCl buffer (pH 8.6) with 2.5 mM $MgCl_2$, 16 mM ATP, 1 mM AMP, 1 mM $Na_4P_2O_7$, 10 mM DTE, and 0.5 μCi of L-[U-^{14}C]phenylalanine. Five hundred microliters of the reaction mixture was incubated with 0.2 mM nonlabeled D-phenylalanine, 25 μg of D-amino acid oxidase, and 25 μg of catalase for 1 h at 37 °C. One hundred microliters of 0.1% 2,4-dinitrophenylhydrazine (w/v in 2 N HCl) was added, and after incubation for 5 min at 37 °C the formed 2,4-dinitrophenylhydrazone of phenylpyruvic acid was extracted twice with 1 mL of ethyl acetate. One hundred microliters of the ethyl acetate layer was mixed with 5 mL of scintillation cocktail, and the radioactivity was determined in a liquid scintillation counter (Packard). The measured radioactivity was corrected by subtraction of the counts obtained from references, which were treated in the same way as described above except that D-amino acid oxidase was omitted.

Determination of Pantothenic Acid Liberated from GS1. Two milligrams of bovine serum albumin was added to 800 μL of Mono Q fraction of GS1. The protein was precipitated by incubation with 1 mL of 3 M trichloroacetic acid for 30 min at 0 °C. After centrifugation the protein pellet was washed twice with 500 μL of 5% (w/v) trichloroacetic acid and 500 μL of a 1:1 (v/v) ethanol/diethyl ether mixture and dried in an desiccator. The protein was hydrolyzed with 1 N KOH for 1 h at 37 °C. After the pH was adjusted to 8.0,

the reaction mixture was incubated for 2 h at 37 °C and overnight at 4 °C with 1−3 units of bovine alkaline phosphatase. Pantothenic acid liberated from enzyme-bound 4′-phosphopantetheine was determined microbiologically using *Lactobacillus plantarum* ATCC 8014 as the test organism (Majerus et al., 1965; Gilhuus-Moe et al., 1970; Solberg & Hegna, 1979). Standard curves were measured with commercially available calcium D(+)-dipantothenate.

GS1 Inactivation by Iodoacetic Acid. One to 2 μg of GS1 was incubated with various concentrations of iodoacetic acid for 10 min at 37 °C. Reaction mixtures of 100 μL contained 20 mM sodium phosphate buffer (pH 8.2), 0.5 mM EDTA, and 1 μM to 1 M iodoacetic acid. The remaining D-Phe-dependent ATP−PP_i exchange and thioester binding of L-Phe of the carboxymethylated enzyme were measured as described above.

Labeling of the Reaction Center of GS1 for Thioester Binding and Racemization of Phe with [^{3}H]Iodoacetic Acid. One to 2 mg of GS1 in 2−4 mL of 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM DTE and 300 mM NaCl was dialyzed against 10 mM sodium phosphate buffer (pH 8.2) containing 0.25 mM EDTA (buffer P) which was degassed and saturated with nitrogen prior to usage. This enzyme solution was concentrated to a final volume of 1−2 mL. In two experiments GS1 was incubated with 0.98 mM [^{3}H]-iodoacetic acid for 10 min at 37 °C after protection with substrates by incubation with (A) 2 mM ATP and 10 mM $MgCl_2$ and (B) 2 mM ATP, 10 mM $MgCl_2$, and 20 μM L-phenylalanine for 10 min at 37 °C. In a reference experiment (C) the protecting substrates were omitted and GS1 was incubated with 0.98 mM [^{3}H]iodoacetic acid for 10 min at 37 °C. The resulting complexes were isolated by gel filtration on Sephadex G25 at 3 °C with buffer P as the eluent and concentrated to a final volume of 0.5−1.0 mL. For all concentration steps a Speed Vac concentrator (Savant) was used.

Digestion of ^{3}H-Carboxymethylated Gramicidin S Synthetase 1 with Trypsin. One to 2 mg of ^{3}H-carboxymethylated GS1 was digested with 150−200 μg of TPCK-treated trypsin in 0.5−1 mL of 100 mM phosphate buffer (pH 8.2) containing 1 mM EDTA, 0−2 M urea, and 10% acetonitrile (v/v) at 37 °C for 14 h and again for 4 h after addition of another 50−200-μg trypsin aliquot. Proteolysis was stopped by adding 500 μL of 0.1% (v/v) trifluoroacetic acid. The fragments were separated by HPLC.

Purification of Labeled Peptides by HPLC. C_{18} Hypersil (5 μm) columns (4 × 25 mm) were used for the separation of the tryptic fragments of the ^{3}H-carboxymethylated GS1. Peptide mixtures were dissolved in 300−500 μL of solvent A, loaded onto the columns, and eluted with linear gradients of acetonitrile. Two different systems were used: System 1 was 100 mM sodium phosphate in water, pH 2.5 (solvent A) and a 2:1 (v/v) mixture of acetonitrile and solvent A (solvent B). System 2 was composed of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in a 60% acetonitrile/40% water (v/v) mixture (solvent B). The flow rate was 0.3−0.5 mL/min. Fractions of 1−1.2 mL were collected. The peptides were detected by measuring the absorbance at 214 nm. ^{3}H-Labeled peptides were monitored by scintillation counting of 50−100 μL aliquots of each fraction.

Amino Acid Analysis. Fifty to 100 pmol of the dried peptide material was hydrolyzed with 6 N HCl at 110 °C for 20 h in evacuated tubes as described by Meltzer et al. (1987). Automatic precolumn derivatization of the amino acids with *o*-phthaldialdehyde (OPA) (Böhlen & Mellet, 1979) for 1 min was performed with a programmable multidimensional injection system (Spark, Holland). OPA-derivatized amino acids were separated by HPLC on a C_{18} column (ODS Hypersil, 5 μm, Knauer, Germany) using a multistep gradient. Eluent A was 12.5 mM Na_2HPO_4, pH 6.5. Eluent B was composed of 97% methanol and 3% tetrahydrofuran (v/v). Eluted OPA−amino acids were monitored with a Shimadzu RF-551 fluorescence detector (excitation, 350 nm; emission 455 nm). The β-alanine content was estimated using authentic β-alanine (Merck, Germany) as a standard.

Peptide Sequencing. Peptide sequences were determined with an Applied Biosystems Model 477A pulsed liquid phase sequencer. Phenylthiohydantoin−amino acids were separated on-line by an Applied Biosystems Model 120A analyzer. Thirty to 100 pmol of the dried peptide material was dissolved in 100% trifluoroacetic acid and adsorbed to a trifluoroacetic acid treated polybrene-coated glass filter that had been precycled as described by Hewick et al. (1981).

Electrospray Ionization Mass Spectrometry (ESI MS). ESI mass spectra were recorded on a Finnigan-MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source. The electrospray needle was operated at a voltage difference of 4 kV. Ten to 50 pmol of the dried peptide material was dissolved in 4−10 μL of 1% acetic acid (v/v) in a 1:1 mixture of methanol and water (v/v) and introduced into the source at a flow rate of 1 μL/min.

RESULTS

Determination of Pantothenic Acid Liberated from GS1. Gramicidin S synthetase 1 (GS1) was purified by high-resolution anion-exchange FPLC on Pharmacia Mono Q HR 5/5 (Vater et al., 1987) and tested for gramicidin S biosynthesis, D-phenylalanine-dependent ATP−[^{32}P]pyrophosphate exchange, covalent binding of L-[^{14}C]phenylalanine, and phenylalanine racemization. All these activities show their maximum in fraction 32 (Figure 1). In this fraction 0.3 mol of L-[^{14}C]Phe was covalently bound per mole of GS1 as thioester. We carefully reinvestigated the 4′-phosphopantetheine content of GS1 following the protocols of Majerus et al. (1965) and Gilhuus-Moe et al. (1970). Treatment of the enzyme with KOH and alkaline phosphatase resulted in liberation of pantothenic acid from enzyme-bound 4′-phosphopantetheine that was detected microbiologically using *Lactobacillus plantarum* as the test organism. The results are shown in Figure 1B. The amount of liberated pantothenic acid from GS1 found in the Mono Q fraction was equimolar with its capacity to bind L-phenylalanine covalently as thioester (0.3 mol of pantothenic acid per mole of GS1 in fraction 32). For the pantothenic acid determination we recommend investigating freshly prepared GS1 because the highly purified enzyme shows a rapid decrease of its activities upon aging. Most probably loss of the cofactor is the reason that only 30% of the GS1 reaction centers are able to incorporate L-[^{14}C]Phe as thioester. Presumably this may be the reason that Gilhuus-Moe et al. (1970) and Lipmann et al. (1971) did not find 4′-phosphopantetheine as a constituent of GS1 in their previous studies.

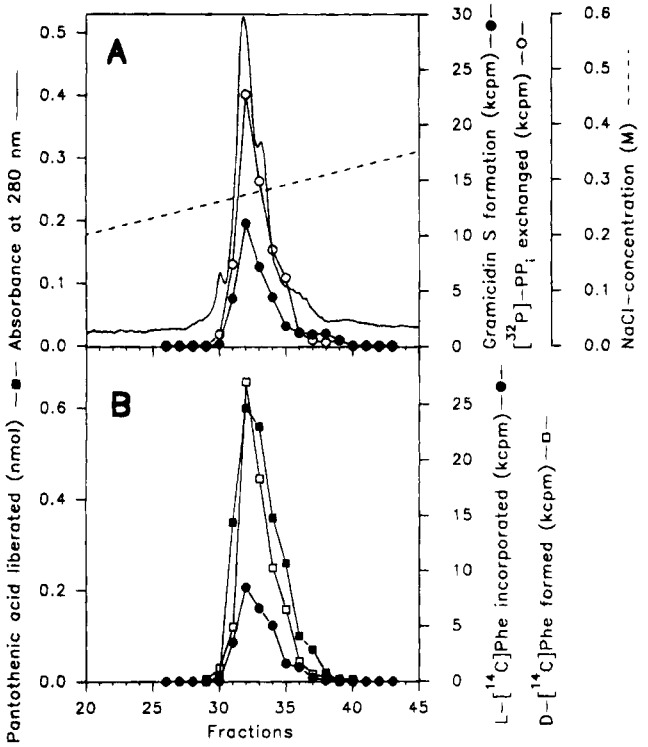

FIGURE 1: High-resolution anion-exchange FPLC of phenylalanine racemase (gramicidin S synthetase 1) on Pharmacia Mono Q HR 5/5. The enzyme was eluted from the column with a gradient from 0 to 1 M NaCl in 50 mM Tris/HCl buffer (pH 7.5) containing 2 mM DTE with a flow rate of 1 mL/min. Protein was detected by measuring the absorption at 280 nm. Fractions of 1 mL were collected. (A) GS1 was monitored by the assays for gramicidin S formation and D-phenylalanine-dependent ATP–[^{32}P]PP$_i$ exchange. (B) The active fractions were tested for covalent L-[^{14}C]Phe incorporation as thioester, racemase activity (D-phenylalanine formation), and pantothenic acid liberation as indicated under Experimental Procedures.

Labeling of the Reaction Center of GS1 for Thioester Binding and Racemization of Phe with [^{3}H]Iodoacetic Acid and Isolation of the Active Site Peptide. Kanda et al. (1981) identified a thiol group of GS1 which rapidly reacts with DTNB and which is essential for phenylalanine binding and racemization. This implies that the racemization reaction takes place in the thioester-bound stage of phenylalanine. In order to characterize this reaction center, we developed a technique to label the thiolation site of GS1 and isolated the active site peptide for Phe binding/racemization.

Affinity labeling of GS1 with radioactive L-phenylalanine as the tracer and isolation of the labeled active site peptide failed because of the instability of the L-Phe thioester in the course of protein fragmentation and peptide separation. A multistep indirect labeling procedure with *N*-[^{14}C]ethylmaleimide, which has been applied succesfully in the labeling of the valine thiolation site of GS2 (Stein et al., 1994), resulted only in low-picomolar yields of the active site peptide. Therefore its complete investigation, including sequencing and amino acid analysis, was not possible.

With the aim to develop a more efficient GS1 labeling technique, we studied the inhibition of its activation reactions with iodoacetic acid. As demonstrated in Figure 2, the covalent incorporation of L-Phe as thioester is blocked at 2 orders of magnitude lower inhibitor concentrations as the D-Phe-dependent ATP–PP$_i$ exchange in the first activation step. A CI$_{50}$ constant of 950 μM was obtained for thioester

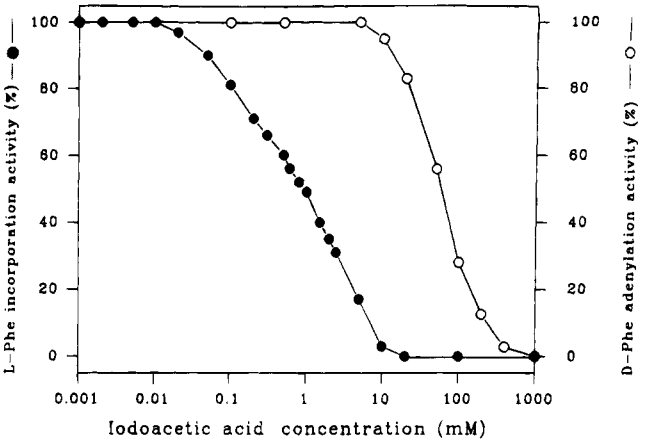

FIGURE 2: Inhibition of the D-Phe-dependent ATP–PP$_i$ exchange and the thioester binding of L-Phe by iodoacetic acid. Assays were performed as indicated under Experimental Procedures.

binding of L-Phe, while for the D-Phe-dependent ATP–PP$_i$ exchange a much higher CI$_{50}$ of 90 mM was calculated. Obviously the thiotemplate site of GS1 can be labeled by the modifier without affecting the primary adenylation reaction.

To identify the active site peptide that contains the thiol group essential for phenylalanine binding and racemization, we incubated GS1 with 0.98 mM [^{3}H]iodoacetic acid and analyzed the distribution of radioactivity in tryptic peptide maps of the GS1–inhibitor complex. [^{3}H]Carboxymethylation was performed at a concentration at which 50% of the thioester binding capacity for L-Phe was inhibited but the primary adenylation reaction was not affected. Prior to derivatization, GS1 was protected with substrates by incubation with (A) 20 μM L-phenylalanine, 2 mM ATP, and 10 mM $MgCl_2$ as well as (B) 2 mM ATP and 10 mM $MgCl_2$. In a reference experiment (C) the protecting substrates were omitted. GS 1 modified by the inhibitor was digested with trypsin, and the resulting peptide mixtures were separated on a C_{18} ODS Hypersil column (Figure 3, top) using a linear gradient of solvent system 1. No significant differences in the absorption patterns among the three experiments has been detected. To monitor [^{3}H]carboxymethylated peptides, aliquots of each fraction were analyzed by scintillation counting (Figure 3, bottom). In the case of experiment C (unprotected GS1) approximately 50% of the radioactivity eluted in fractions 43 and 44 (peak 2). The rest of the tracer was distributed among four minor peaks. Only peak 2 is clearly suppressed by substrate protection of GS1 prior to [^{3}H]-carboxymethylation (experiments A and B). In contrast, the portions of the minor labeled peaks even increased. Most likely a conformational change of GS1 is induced by incubation with the substrates L-Phe and Mg-ATP, exposing thiol groups to the surface of the enzyme which otherwise would be buried in the interior in the unprotected state. Under these conditions these thiols attain the ability to react more efficiently with iodoacetic acid, while the most reactive thiol group is protected from the reaction with the inhibitor. Therefore, we conclude that peak 2 contains the tryptic active site peptide of the thiolation/racemization site of GS1. The radioactively labeled peptide fraction of peak 2 was separated on a C_{18} ODS Hypersil column using a more shallow gradient from 32 to 63% eluent B (solvent system 2) in 300 min for elution.

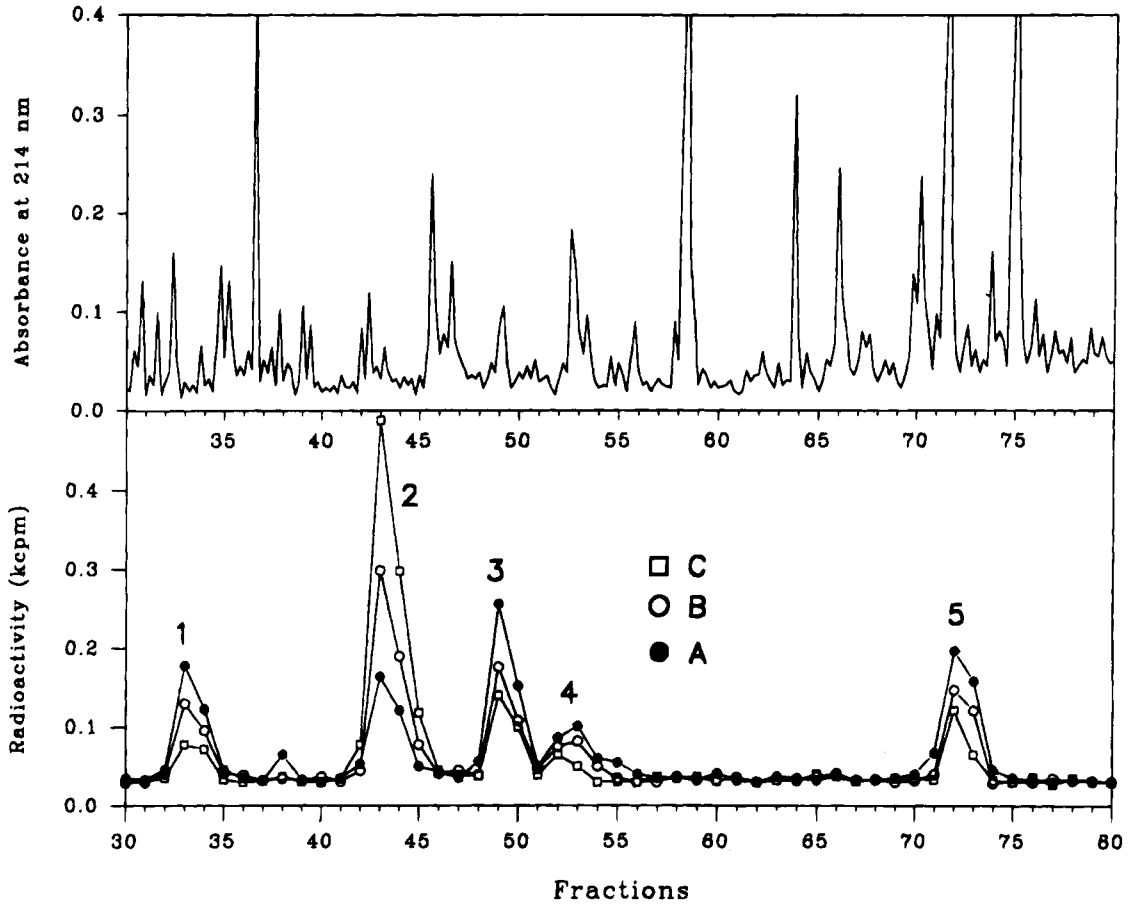

FIGURE 3: Peptide mapping of [^{3}H]carboxymethylated tryptic peptides of gramicidin S synthetase 1. GS1 was protected with substrates by incubation with (A) 20 μM L-Phe, 2 mM ATP, and 10 mM $MgCl_2$ or (B) 2 mM ATP and 10 mM $MgCl_2$. In a reference experiment (C) the substrates were omitted. The reaction mixtures were incubated with [^{3}H]iodoacetic acid to label the nonprotected thiol groups. The resulting complexes were digested with trypsin, and peptide mixtures were separated on a C_{18} ODS Hypersil column by HPLC using solvent system 1 as the eluent as indicated under Experimental Procedures. Peptides were eluted with a linear gradient from 20 to 100% eluent B in 160 min. The flow rate was 0.3 mL/min. Fractions of 1.2 mL were collected. The peptides were detected by measuring the absorbance at 214 nm. A part of the chromatogram between 30 and 60% B is shown in the top panel. Radioactively labeled peptide fractions were monitored by scintillation counting of 100-μL aliquots of each fraction (bottom panel).

Characterization of the Active Site Peptide of GS1 for Thioester Binding and Racemization of Phe. The pure radiolabeled tryptic peptide isolated by HPLC was analyzed by liquid-phase sequencing, mass spectrometry, and amino acid analysis. By liquid phase sequencing the following dodecapeptide was obtained:

1	2	3	4	5	6	7	8	9	10	11	12
D	N	F	Y	A	L	G	G	D	ΔA	I	K

This result is in accordance with the residues D (564)−K (575) (Krätzschmar et al., 1989) of the grsA-derived sequence with the exception that at position 10 a dehydroalanine was found instead of a serine as claimed by the gene sequence. Similar results have been obtained for the active site peptides of the L-valine and L-leucine thiolation sites of GS2 (Schlumbohm et al., 1991). Recently we provided evidence for the L-valine-activating module of GS2, that dehydroalanine orginated from elimination of a 4′-phosphopantetheine substituent together with the tracer from the serine residue of the active site peptide, due to the alkaline milieu in the first Edman degradation step (Stein et al., 1994).

To verify the hypothesis of an attached 4′-phosphopantetheine cofactor to the active site peptide of GS1 for L-Phe binding, we investigated this peptide by electrospray ionization mass spectrometry. A series of ions at m/z = 850.0, 860.9, 871.7, 882.9, 893.3, and 904.6 were detected which consecutively show a m/z difference of approximately 11. These species can be interpreted as the doubly charged H^+/Na^+ adducts of the active site peptide M starting with $[M + 2H]^{2+}$ (m/z = 850.0) followed by $[M + H + Na]^{2+}$ (m/z = 860.9) and ending with $[M - 3H + 5Na]^{2+}$ (m/z = 904.6). A molecular mass of 1697.5 Da was calculated for M. This mass is appreciably higher than the 1299.4-Da mass calculated from the gene-derived sequence for the tryptic active site peptide using average mass numbers. The mass difference of 398.1 Da exactly corresponds to a covalent substitution of the peptide with an *S*-([^{3}H]carboxymethyl)phosphopantetheinyl adduct. The nature of the substituent is supported by amino acid analysis of the active site peptide. One mole of the active site dodecapeptide contained approximately 1 mol of β-alanine, confirming the data obtained from mass spectrometry. Electrospray mass spectrometry of the tryptic active site peptide of GS1 for thioester binding and racemization of phenylalanine derivatized with *N*-[^{14}C]-ethylmaleimide resulted in a molecular mass of 1764.7 Da (data not shown). Substitution of the active site peptide with an *S*-(*N*-[^{14}C]ethylsuccinimido)phosphopantetheinyl adduct explains the 465.3-Da mass difference relating to the gene-derived mass of the peptide (Stein et al., 1994).

Table 1: Amino Acid Sequence Alignment of 4′-Phosphopantetheine Attachment Sites of Amino Acid Activating Modules of Peptide Synthetases

	gene[a]		4′-phosphopantetheine binding motif[b]		ref
1	*grs*A[c]	(564)	D N F Y A L G G D S I K A I Q V	(579)	Krätzschmar et al. (1989)
2	*grs*B1	(997)	D N F F S L G G H S L K A I T L	(1012)	Turgay et al. (1992)
3	*grs*B2	(2033)	D N F F E L G G H S L R A M T M	(2048)	
4	*grs*B3	(3079)	D D F F T I G G H S L K A M A V	(3094)	
5	*grs*B4	(4118)	D N F F E L G G H S L K A T L L	(4133)	
6	*tyc*A[c]	(553)	D N F Y S L G G H S I Q A I Q V	(568)	Weckermann et al. (1988)
7	*srf*AA1	(997)	D N F F E T G G H S L K A M T L	(1012)	Fuma et al. (1993)
8	*srf*AA2	(2034)	D H F F D I G G H S L A G M K M	(2049)	
9	*srf*AA3[c]	(3065)	D N F F S L G G D S I K G I Q M	(3080)	
10	*srf*AB1	(990)	D N F F M I G G H S L K A M M M	(1005)	
11	*srf*AB2	(1515)	D N F F D L G G H S L K G M M L	(1531)	
12	*srf*AB3[c]	(3064)	D N F F S L G G D S I K G I Q M	(3079)	
13	*srf*AC	(994)	D D F F A L G G H S L K A M T A	(1009)	
14	*ent*F	(997)	A D F F A L G G H S L L A M K L	(1013)	Rusnak et al. (1991)
15	*irp*2	(1430)	T D F F Q Q G G D S L I A T R L	(1445)	Guilvout et al. (1993)
16	*pcb*A	(846)	D N F F R L G G H S I T C I Q L	(861)	Díez et al. (1990) and
17	*pcb*B	(1930)	S D F F S L G G D S L K S T K L	(1945)	Smith et al. (1990)
18	*pcb*C[c]	(3017)	D D L F K L G G D S I T S L H L	(3032)	
19	*pcb*A	(818)	S N F F R L G G H S I A C I Q L	(833)	Gutiérrez et al. (1991)
20	*pcb*B	(1907)	S D F F S L G G D S L K S T K L	(1922)	
21	*pcb*C[c]	(2981)	D D L F A R G G D S I S S L R L	(2996)	
22	*pcb*A	(811)	D D F F R L G G Q S I S C I L L	(826)	Coque et al. (1991)
23	*pcb*B	(1887)	D D F F A L G G D S I R A M A L	(1902)	
24	*pcb*C[c]	(2935)	D D F F R C G G D S I S A L H L	(2950)	
25	*acv*A	(873)	D N F F R L G G H S I T C I Q L	(888)	MacCabe et al. (1991)
26	*acv*B	(1956)	S D F F T L G G D S L K S T K L	(1971)	
27	*acv*C[c]	(3041)	D D L F R L G G D S I T A L H L	(3056)	
28	*esyn*A	(1038)	A T F F E M G G N S I I A I K M	(1053)	Haese et al. (1993)
29	*esyn*B	(2529)	D H F F Q L G G H S L L A T K L	(2544)	
30	*cysyn*01	(1049)	D N F F D L G G H S L T A M K L	(1064)	Weber et al. (1994)
31	*cysyn*02	(2547)	D N F F N V G G H S L L A T K L	(2562)	
32	*cysyn*03	(4034)	E N F F D L G G H S L M A T K L	(4049)	
33	*cysyn*04	(5026)	D N F F N L G G H S L M A T K L	(5041)	
34	*cysyn*05	(7023)	D N F F D L G G H S L M A T K L	(7038)	
35	*cysyn*06	(8083)	D G F F D L G G H S L L A T K L	(8098)	
36	*cysyn*07	(9578)	D N F F D M G G H S L M A T K L	(9593)	
37	*cysyn*08	(11075)	D N F F E L G G H S L M A T K L	(11090)	
38	*cysyn*09	(12147)	D N F F D L G G H S L I A T K L	(12162)	
39	*cysyn*10	(13646)	D N F F E L G G H S L L A T K L	(13661)	
40	*cysyn*11	(14718)	D N F F E L G G H S L L A T K L	(14733)	
41	*hts*1A[c]	(809)	Q S F I H A G G D S I T A M Q V	(824)	Scott-Craig et al. (1992)
42	*hts*1B	(2420)	S D F F S S G G N S M A A I A L	(2435)	
43	*hts*1C	(3574)	D D F F S L G G S S I S A M R L	(3589)	
44	*hts*1D	(4076)	D N F L G I G G D S I A A I K L	(4721)	Morris & Jinks-Rob. (1991)
45	*lys*2	(872)	D S F F D L G G H S I L A T K M	(887)	Wohlleben et al. (1992)
46	*phs*A	(571)	D D F F S L G G T S I S A L R V	(586)	
47	*lys*B		D Q F F D L G G H S L L A L Q M		Bernhard, unpublished
	Consensus		**D N F F x L G G H S L x A x x L**		
			D I D I G/C V/M		

DISCUSSION

In a previous paper we provided evidence from chemical and genetic studies that an active site serine is involved in covalent binding of the substrate amino acids at each reaction center of gramicidin S synthetase 2 (Schlumbohm et al., 1991), instead of a cysteine proposed by the original version of the thiotemplate hypothesis (Kleinkauf et al., 1971; Lipmann, 1973; Laland & Zimmer, 1973; Kurahashi et al., 1974). This serine is part of a strictly conserved motif, GG-(H/D)S(L/I), which is included in each of at least 47 amino acid activating domains whose gene-derived sequences have been determined so far (Table 1). We proved for the first time that this motif is a 4′-phosphopantetheine binding site and that the thiol group of the serine-bound cofactor represents the thiotemplate site of the L-Val-activating module of GS2 (Stein et al., 1994). These findings imply that multiple peripheral 4′-phosphopantetheine carriers are involved in non-ribosomal peptide biosynthesis instead of a single central Pan arm. However, this conclusion is in contrast to the results of several authors which suggest that multienzymes which activate only one amino acid, like tyrocidine synthetase 1 (Lee et al., 1973), gramicidin S synthetase 1 (Lipmann et al., 1971; Gilhuus-Moe et al., 1970), and an L-proline-activating domain obtained by limited proteolysis of gramicidin S synthetase 2 (Kurotsu et al., 1991), do not contain 4′-phosphopantetheine.

To clarify this contradiction, we carefully reinvestigated the 4′-phosphopantetheine content of GS1 and characterized the active site peptide for Phe binding. Our results demonstrate that the amount of liberated pantothenic acid from GS1 is equimolar with its capacity to bind L-[^{14}C]phenylalanine covalently as thioester, supporting the hypothesis that the SH-group of the cofactor is the Phe thiotemplate site. The reason for the significantly less than stoichiometric Pan content of GS1 observed in our experiments and the failure to detect the cofactor in previous studies of Gilhuus-Moe et al. (1970) and Lipmann et al. (1971) most probably is the lability of the Pan binding to this enzyme. Therefore, we purified and assayed GS1 after isolation from *B. brevis* ATCC 9999 as quickly as possible. Usually enzyme preparations could be obtained within 2 days. Prolongation of the preparation procedure and storage of the enzyme caused a progressive decrease of its Pan content and thioester binding capacity. In analogy with proline racemase, the covalent attachment of approximately 0.3 mol of Pan per mole of GS1 might suggest a dimeric structure of this protein showing either a two-fold axis of symmetry of its reaction center or a half-site reactivity. This certainly can be excluded because GS1 shows a monomeric structure in aqueous solution (Vater et al., 1989).

In order to isolate the active site peptide for thioester binding and racemization of phenylalanine, we developed a technique to modify GS1 with [^{3}H]iodoacetic acid and studied the distribution of radioactivity in tryptic peptide maps of the resulting enzyme−inhibitor complexes. We purified the active site peptide for L-phenylalanine thiolation and investigated it by amino acid, sequence, and mass spectrometric analysis. It contained the thioester binding motif DSI (Table 1) and was substituted with a 4′-phosphopantetheine moiety esterified to the active site serine.

Our results demonstrate that the reactive thiol group of the Pan cofactor forms the thiotemplate site of GS1 for phenylalanine instead of a cysteine as proposed by the original thiotemplate hypothesis. This conclusion is consistent with studies of GS1 from various gramicidin S nonproducing mutants deficient in thiolation and racemization of phenylalanine but not in Phe adenylation (Shimura et al., 1974; Kanda et al., 1981; Hori et al., 1994). Recently Hori et al. (1994) have reported that some of the mutant genes coding for GS1 show entirely the same sequence as the wild-type gene. These findings indicate that the mutant proteins were lacking the Pan cofactor presumably because of a deficiency in the transferase enzyme that introduces 4′-phosphopantetheine into gramicidin S synthetase. GS1 wild-type and mutant enzymes cloned in *E. coli* as, for example, the pG 309 gene product (Kanda et al., 1989; Hori et al., 1994) show a similar reaction pattern. Also here most probably the low specific racemization activity is due to the insufficient posttranslational Pan modification of GS1 expressed in the heterologous host organism. Furthermore, our results are corroborated by active site mutagenesis studies of tyrocidine synthetase 1 bearing an S → A mutation in its Pan binding motif, which abolished the thiolation and racemization activity of this enzyme (Gocht & Marahiel, 1994). In addition, these authors succeeded in labeling tyrocidine synthetase 1 with β-[^{3}H]alanine, which is a substituent of 4′-phosphopantetheine.

Our studies support the multiple carrier model of nonribosomal peptide biosynthesis at protein templates proposed by us in a previous article (Stein et al., 1994). According to this hypothesis peptide synthetases are equipped with multiple Pan cofactors, one at each thiotemplate reaction center. These carrier arms interact in the assembly of the peptide products in a series of transpeptidation reactions. A detailed discussion of this new concept of non-ribosomal peptide biosynthesis will be published elsewhere (T. Stein and J. Vater, unpublished results).

Amino acid racemases/epimerases (EC 5.1.1) catalyze the α-proton abstraction from an amino acid, stabilize the carbanion intermediate, and re-protonate the substrate at the opposite face of the molecule (Adams, 1972). Cardinale and Abeles (1968) categorized racemization enzymes by the number of bases involved for the exchange of the substrate protons in this process.

[a] The numbers at the left margin represent the following gene products: (1) gramicidin S synthetase 1 (phenylalanine racemase) and (2−5) gramicidin S synthetase 2 (*Bacillus brevis* ATCC 9999); (6) tyrocidine synthetase 1 (*Bacillus brevis* ATCC 8185), (7−9) surfactin synthetase 2 (E1A, SRFS2), (10−12) surfactin synthetase 3 (E1B, SRFS 3), and (13) surfactin synthetase 4 (E2, SRFS 4) (*Bacillus subtilis* OKB105); (14) enterobactin synthetase F (Ent F) (*Escherichia coli*); (15) high molecular weight protein 2 (HMWP2) (*Yersinia enterocolitica*); ACV synthetase from (16−18) *Penicillium chrysogenum*, (19−21) *Acremonium chrysogenum*, (22−24) *Nocardia lactamdurans*, and (25−27) *Aspergillus nidulans*; (28−29) enniatin synthetase (*Fusarium scirpi*); (30−40) cyclosporin A synthetase (*Tolypocladium niveum*); (41−42); HC-toxin synthetase 1 (HTS-1); (43−44) HC-toxin synthetase 2 (HTS-2) (*Cochliobolus carbonum*); (45) LYS2 (*Saccharomyces cerevisiae*); (46) phosphinotricin synthetase 1 (*Streptomyces viridochromogenes*); and (47) lysobactin synthetase 2 (*Lysobacter sp.* ATCC 53042). [b] The numbers in parentheses represent the positions of the first and last aligned amino acids of the Pan binding consensus motif in the sequence of the corresponding peptide synthetase. Only for the L-valine activating module of gramicidin S synthetase 2 (*grs*B2) was the 4′-phosphopantetheine attachment to the active site serine demonstrated (Stein et al., 1994). [c] Peptide synthetase modules catalyzing racemization reactions.

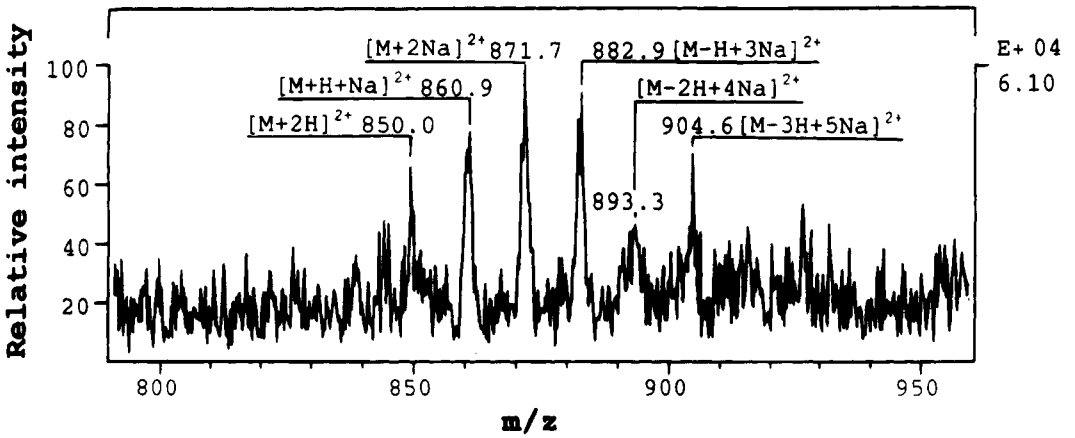

FIGURE 4: Electrospray ionization mass spectrum (partial) of the [^{3}H]carboxymethylated active site peptide of gramicidin S synthetase 1 for thioester binding and racemization of phenylalanine. The signals at m/z = 850.0, 860.9, 871.7, 882.9, 893.3, and 904.6 correspond to the doubly charged Na^+ adduct series of the dodecapeptide starting with $[M + 2H]^{2+}$, followed by $[M + H + Na]^{2+}$, and ending with $[M - 3H + 5Na]^{2+}$. A molecular mass M = 1697.5 Da was calculated.

The one-base mechanism is characteristic for all racemases containing pyridoxal phosphate. The cofactor is bound via an aldimine linkage to the ϵ-amino group of a lysine, which is highly conserved in the VVKANAYGHG motif (Tanizawa et al., 1988). Representative examples are the alanine racemases from *Bacillus subtilis* (Ferrari et al., 1985), *Bacillus stearothermophilus* (Tanizawa et al., 1988), and *Salmonella typhimurium* (Walsh, 1989, and references herein) or the threonine racemase of *Pseudomonas putida* (Lim et al., 1993). A pyridoxal phosphate dependent alanine racemase, which provides the D-Ala portion in the biosynthesis of cyclosporin A, is the first amino acid racemase isolated from a eukariotic organism (*Tolypocladium niveum*; Hoffmann et al., 1994). Enzymes of this class contain one cofactor molecule per 39−50 kDa subunit. They form a pyridoxal phosphate substrate complex via transaldimination. The α-hydrogen of the substrate amino acid is abstracted by a single base. The resonance-stabilized carbanion intermediate is kept at the active site until the proton is translocated from the acceptor to the opposite face, where re-protonation occurs. In no case was the base responsible for this reaction was identified in the racemase structure.

None of the known racemases using the two-base mechanism contain a cofactor. One enzymic base removes the α-proton from the substrate, and the conjugate acid of a second delivers a proton to the opposite face. Homodimeric enzymes like proline racemase from *Clostridium sticklandii* (Rudnick & Abeles, 1975), hydroxyproline epimerase from *P. putida* (Ramaswamy, 1984), and 2,6-diaminopimelic acid epimerase from *E. coli* (Wiseman & Nichols, 1984) contain highly conserved cysteine residues, one per 30−40 kDa subunit. They have been labeled with [^{14}C]iodoacetamide and were identified by active site peptide sequencing in proline racemase (Rudnick & Abeles, 1975). Obviously they are part of a conserved CGXG motif (Higgins et al., 1989). Aspartate racemase from *Streptococcus thermophilus* (Yohda et al., 1991) as well as the glutamate racemases from *E. coli* (Yoshimura et al., 1993) and *Lactobacillus fermenti* (Gallo & Knowles, 1993) with molecular masses of approximately 28−31 kDa also follow the two-base pathway. Amino acid sequence alignment revealed that they contain two conserved cysteine residues as part of the VXVCNTA and GCT motifs. Conversion of both conserved cysteine residues to alanine residues by site-directed mutagenesis of glutamate racemase (*L. fermenti*) resulted in a loss of the racemization activity (Tanner et al., 1993), corroborating the assumption that these cysteines represent the two bases essential for the racemization process.

Much mechanistic information about the racemization reaction was obtained from analyzing the 3-D structure of mandelate racemase (EC 5.1.2.2, *Pseudomonas putida*, 38.5 kDa) which has beeen crystallized by Neidhart et al. (1988). This enzyme racemizes mandelate following the two-base mechanism (Powers et al., 1991). The bases lysine (166) and histidine (297) are involved in hydrogen abstraction and re-protonation. An acid/base charge-transfer system, lysine (164)/glutamic acid (317), stabilizes the enol tautomer of mandelate prior to deprotonation and is directly involved in the dispersion of the negative charge of the intermediate (Gerlt & Gassman, 1993; Landro et al., 1994).

Sequence alignment of amino acid activating modules of peptide synthetases indicates that only modules which catalyze racemization reactions are equipped with at least five highly conserved motifs (Table 2) in their C-terminal region (Fuma et al., 1993). Most probably these consensus motifs represent essential structural elements in the racemization process. They are absent in nonracemizing modules and do not show significant sequence homologies to other racemizing enzymes.

Kanda et al. (1981) suggested that a conserved cysteine residue of gramicidin S synthetase 1 is involved in phenylalanine binding and racemization. The racemization reaction is performed in the thioester-bound stage of phenylalanine and exhibits the characteristics of the one-base pathway (Kanda et al., 1989). Because no conserved cysteine residue is included in the putative racemase region of peptide synthetases (Table 2), we suppose that one of the strictly conserved arginine, histidine (motif R2), or tyrosine residues (motif R4) is involved in proton abstraction and re-protonation. Presumably an acid/base charge-transfer system similar to mandelate racemase is involved in the stabilization of the intermediate structure.

Taking all these results together, we categorize gramicidin S synthetase 1 (phenylalanine racemase) as a prototype of a new class of racemases/epimerases. Their common feature is the 4′-phosphopantetheine cofactor dependent racemization activity. The thiol group of the cofactor represents the

Table 2: Amino Acid Sequence Alignment of Multifunctional Peptide Synthetase Modules Catalyzing Racemization Reactions

	gene[a] (amino acid racemized)	motif R1	*b*	motif R2	*b*	motif R3	motif R4	*b*	motif R5	ref
1	*grs*A (Phe)	D I L L T A L G F A	-14-	E G H G R E	-9-	R T V G W F T S Q Y P	E N L R R I P N K G I G Y	-20-	P E I N F N Y L G Q F	Krätzschmar et al. (1989)
6	*tyc*A (Phe)	D L L L A A L G L A	-14-	E G H G R E	-9-	R T V G W F T S Q Y P	E N M R K I P R K G I G Y	-20-	P E V T F N Y L G Q F	Weckermann et al. (1988); Gocht et al. (1994)
9	*srf*AA3 (Leu)	D I L L T A F G L A	-14-	E G H G R E	-9-	R T V G W F T S M Y P	E D I R H V P N K G V G Y	-20-	P D V S F N Y L G Q F	Fuma et al. (1993)
12	*srf*AB3 (Leu)	D I L L S A L G L T	-14-	E G H G R E	-9-	R T V G W F T A Q Y P	E N L R R I P D K G V G Y	-16-	P E I S F N Y L G Q F	Fuma et al. (1993)
18	*pcb*C (Val)	D D L L T S V G L A	-14-	E G H G R E	-9-	R T M G W F T T M Y P	E R F R Q V P A R G V G Y	-11-	P Q V T V N Y L G Q L	Díez et al. (1990);
21	*pcb*C (Val)	E I L L M A V G S A	-14-	E G H G R E	-10-	R T V G W F T S M Y P	E A M R R V P N R G V G Y	-11-	P A V S F N Y L G R L	Gutíerrez et al. (1991)
24	*pcb*C (Val)	D L L L T A T G F A	-14-	E G H G R E	-9-	D T V G W F T T M H P	A N R R R V P H H G I G Y	-10-	P A V S F N Y L G R L	Coque et al. (1991)
27	*acv*C (Val)	D L L L A A V G L A	-14-	E G H G R E	-10-	R T L G W F T S M Y P	E C L R Q V P A R G I G F	-11-	P Q V T F N Y L G Q L	MacCabe et al. (1991)
41	*hts*1A (Pro)	V L L A A V L V S F	-14-	E G H G R E	-10-	R T V G W F T T I S P	D Y R W A T P N N G F D Y	-19-	A E I L F N Y E G R Y	Scott-Craig et al. (1992)
	Consensus	**D L L L x A x G x A**		**E G H G R E**		**R T V G W F T S Q Y P**	**E x x R x V P x x G I G Y**		**P x V x F N Y L G Q F**	
				↑ ↑		T M	I V		I ↑ R L	

[a]Genes encoding amino acid activating modules, which catalyze racemization reactions. In relation to the 4′-phosphopantetheine binding motif (Table 1), the putative racemase region is positioned 300−315 amino acid residues toward the C-terminus. The numbers at the left margin represent the same gene products as indicated in Table 1. [b] Number of amino acid residues between two adjacent consensus motifs. The arrows indicate highly conserved amino acid residues which may be essential structural elements in the racemization process.

thiolation site (thiotemplate). In the case of gramicidin S and tyrocidine synthetase 1, as well as that of HC-toxin synthetase, which catalyze the initial step of the peptide-forming system, the racemization reaction occurs in the stage of the activated single amino acid. It can be detected using the racemase assay applied by Yamada and Kurahashi (1969), Zimmer and Laland (1975), and Kanda et al. (1978). In contrast to these enzymes, surfactin (Menkhaus et al., 1993), δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (Schwecke, 1993), and actinomycin D synthetase (Stindl & Keller, 1994) catalyze epimerization of the amino acid portion in the stage of the thioester-bound peptide intermediate (Stindl & Keller, 1994). However, both species of peptide synthetases contain the strictly conserved 4′-phosphopantetheine binding site and the putative racemization region. Therefore, the mechanism of the racemization process should be identical in both cases.

To understand the mechanism of the racemization/epimerization reaction in detail, more information about the structure−function relationship is needed. Enzymological studies of synthetases with amino acid substitutions in the highly conserved putative racemase region and, finally, determination of the three−dimensional structure of a crystallized amino acid activating module are promising approaches.

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