The Multiple Carrier Model of Nonribosomal Peptide Biosynthesis at Modular Multienzymatic Templates*

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Gramicidin S synthetase 1 and 2 were affinity-labeled at their thiolation centers either by thioesterification with the amino acid substrate or by specific alkylation with the thiol reagent N-ethylmaleimide in combination with a substrate protection technique. The labeled proteins were digested either chemically by cyanogen bromide or by proteases. An efficient multistep high pressure liquid chromatography methodology was developed and used to isolate the active site peptide fragments of all five thiolation centers of gramicidin S synthetase in pure form. The structures of these fragments are investigated by N-terminal sequencing, mass spectrometry, and amino acid analysis. Each of the active site peptide fragments contains the consensus motif LGG(H/D)LS(1/L), which is specific for thioester formation in nonribosomal peptide biosynthesis. It was demonstrated that a 4'-phosphopantetheine cofactor is attached to the central serine of the thiolation motif in each amino acid-activating module of the gramicidin S synthetase multitemplate system forming the thioester binding sites for the amino acid substrates and catalyzing the elongation process. Our data are strong support for a "multiple carrier model" of nonribosomal peptide biosynthesis at multifunctional templates, which is discussed in detail.

Microbial organisms produce a variety of structurally diverse, low molecular weight bioactive peptides, depsipeptides, peptide lactones, and lipopeptides (1). These secondary metabolites exhibit valuable properties qualifying them for biotechnological and medical uses as antibiotics, antiviral and antitumor agents, immunomodulators, as well as biosurfactants, for example. In general, the biosynthesis of these compounds is accomplished nonribosomally by large multienzyme systems. More than 20 years ago, the multienzymatic thiotemplate model was proposed by several groups (2–5). According to this model, amino acid activation occurs in a two-step process including (a) aminoacyl adenylation similar to the amino acid activation process catalyzed by tRNA ligases, and (b) aminoacyl thioesterification at specific reactive thiol groups of the multienzyme (thiotemplates). In analogy to fatty acid synthase, peripheral cysteines have been proposed as the thiotemplate sites. A central thiol-group of an intrinsic 4'-phosphopantetheine carrier was assumed to interact with the thioesterified substrate amino acids managing a step-by-step elongation of the peptide product in a series of transeptidation and transthiolation reactions (2–5).

The biosynthesis of the cyclo-decapeptide gramicidin S, cyclo-(d-Phe-L-Pro-L-Val-L-Orn-L-Leu), occurs by the interaction of two multifunctional proteins (for a review, see Ref. 6). Gramicidin S synthetase 1 (GS1, 1 phenylalanine racemase, EC 5.1.1.11, coded by the grsA gene; Ref. 7) activates and racemizes phenylalanine. Specifically the d-Phe enantiomer is transferred to the condensing enzyme gramicidin S synthetase 2 (GS2, coded by the grsB gene; Ref. 8), which activates L-Pro, L-Val, L-Orn (L-ornithine), and L-Leu and catalyzes the synthesis of a pentapeptide. Cyclization is performed by two head to tail condensations between two pentapeptides.

Sequence alignments of peptide synthetases revealed that they are composed of modular homologous building blocks, which comprise 1000–1500 amino acid residues. Each of these modules acts as an independent enzyme, which catalyzes the selection, activation and, in some cases, modification of its specific amino acid substrate (for recent reviews, see Refs. 9 and 10, and references herein). In agreement with the polyenzyme model proposed by Lipmann in 1954 (11), the specific linkage of the modules defines the amino acid sequence of the peptide product. To evaluate structure/function relationships of these multienzymes, our research is focused on the investigation of the thioester binding sites of gramicidin S synthetase 1 and 2. To characterize these essential structural elements in detail in previous studies, we affinity-labeled both enzymes either with a radioactive substrate amino acid (12) or the thiol inhibitor N-ethylmaleimide (13). The resulting complexes were digested with cyanogen bromide or proteases. The radioactively labeled thiotemplate site peptide fragments were isolated in pure form by multistep reversed phase HPLC. All of them contained the highly conserved thioester binding motif Leu-Gly-Gly-(His/Asp)-Ser-(Leu/Ile). Apparently the serine residue of this motif is involved in the thioester formation of gramicidin...
S synthetase with its amino acid substrates (12). The core of the thioester binding motif Asp-Ser-Leu has been identified as a binding site for a 4'-phosphopantetheine (Pan) cofactor in acyl carrier proteins/domains of fatty acid and polyketide synthases (12), implying that each module of gramicidin S synthetase would be equipped with a separate Pan-prosthetic group. Recently we provided evidence by mass spectrometric and amino acid analysis that such a cofactor is indeed attached to the active serine of the thioester binding sites of GS2 for l-valine (13) and GS1 for phenylalanine (14). In this paper we present for the first time a thorough study of the structures of all thiotemplate sites of a peptide-forming multienzyme. Our results demonstrate that each amino acid-activating module of gramicidin S synthetase is equipped with a separate 4'-phosphopantetheine prosthetic group forming the thioester binding site for the amino acid substrate and catalyzing the elongation process. These data are strong support for the hypothesized "multiple carrier model" of nonribosomal peptide biosynthesis at multifunctional protein templates, which is discussed in detail.

**EXPERIMENTAL PROCEDURES**

Materials—N-[3H]Ethylmaleimide (44.4 Ci/mmol) was purchased from DuPont (Dreieich, Germany (FRG)); l-[14C]leucine (317 mCi/mmol) was from Amersham/Buchler (Braunschweig, FRG). Endoproteinase GluC from Staphylococcus aureus V8, pepsin, and l-1-lysylamido-2-phenylethyl chloromethyl ketone-treated trypsin were obtained from Miles/Bayer Diagnostics (München, FRG) and Sigma (Deisenhofen, FRG). Cyanogen bromide was a product of Fluka (Neu-Ulm, FRG). The C$_8$ODS Hypersil (5 μm) columns (4.3 × 25 mm) were obtained from Shandon (United Kingdom) and Knauer (Berlin, FRG). The preparative C$_8$EnCaPharm column (25 × 16 mm) was purchased from Molnar (Berlin, FRG). Bacillus brevis ATCC 9998 (new ATCC number Bacillus subtilis milarus) was cultivated in a fumarate/phosphate medium, as reported previously (15).

Enzyme Purification and Assays—Gramicidin S synthetase 1 and 2 were purified as published by Vater et al. (16). Assays for thiotemplate formation of these enzymes with substrate amino acids (16, 17) and biosynthesis of gramicidin S (16, 18) were performed as described previously. The protein concentration was measured using the procedure of Warburg and Christian (19) and Bradford (20).

Specific Labeling of the Thioester Binding Sites of GS1 and 2 with N-[3H]Ethylmaleimide—5-30 mg of the multienzyme in 3 ml of 20 mM phosphate buffer (pH 7.2) containing 1 mM EDTA (buffer P) were specifically labeled at their thiotemplate sites by incubation with 2 mM ATP, 10 μM MgCl$_2$, and a saturating substrate amino acid concentration of 15 μM for 10 min at 37°C. Reactive groups at the bulk of the synthetase-substrate-thioester complex were saturated by incubation with 2 mM NEM for 30 min at 37°C. The resulting complexes were isolated by gel filtration on Sephadex G-25 at 3°C with 3% buffer P as the eluent and concentrated to a final volume of 3 ml. Incubation with 2 mM dithioerythritol for 60 min at 37°C, the substrate amino acid was removed from the synthetase. The enzymes were again isolated by gel filtration (Sephadex G-25; 3°C; eluent: buffer P) and concentrated by ultrafiltration to 3 ml. For all concentration steps, an Amicon Ultrafiltration XM 50 was used. Finally, the respective thioester binding sites of the synthetase complexes were specifically labeled by incubation of the deprotected multienzyme with 23 μM [3H]NEM at 37°C for 30 min. The labeled protein was isolated by gel filtration on Sephadex G-25 at 3°C with buffer P (pH 8.2) as the eluent, concentrated in a Speed Vac concentrator until a final protein concentration of approximately 2 mg/ml, and digested with trypsin.

Affinity Labeling of the L-Leucine Thioester Site of Gramicidin S Synthetase 2—GS2 was specifically labeled at the thioester binding site for l-Leu by acid-stable incorporation of l-[3H]leucine using the procedure of Schlumpbier et al. (12). Five milligrams of highly purified GS2 was incubated with 2 mM ATP, 10 μM MgCl$_2$, 1 μM EDTA in 20 mM phosphate buffer (pH 7.2) and saturating [3H]leucine concentrations of 15 μM for 10 min at 37°C. The residual cyanogen bromide of the GS2-substrate complex were alkylated by incubation with 5 mM N-ethylmaleimide. The labeled and modified synthetase was isolated by gel filtration on Sephadex G-25 at 3°C. In the eluent a pH of 2 was adjusted by addition of formic acid, because the GS2-substrate amino acid complexes were stable in acidic medium only. Approximately 0.8 mol of l-[3H]Leu/mol of GS2 were bound.

Cleavage of the [3H]NEM-alkylated GS1/2 and the GS2-l-[3H]Leu Complexes—For cleavage of the GS2-l-[3H]Leu complex with cyanogen bromide, proteolysis of [3H]NEM- labeled GS1 and GS2 with trypsin, as well as for the proteolysis of [3H]NEM-labeled tryptic fragments with endoproteinase GluC from Staphylococcus aureus V8 and for digestion of the l-[3H]leucine-labeled CNBr fragments of GS2 with pepsin, the procedures were used as reported previously (12-14).

Separation of Peptides by Reversed Phase HPLC—A preparative C$_8$EnCaPharm as well as the analytical C$_8$ODS Hypersil (5 μm) columns from Shandon and Knauer were used for separation of the peptide fragments of the synthetases.

Peptide mixtures were dissolved in 300-500 μl of 10-20% eluent B, loaded onto the columns which were equilibrated in 10-20% B and eluted with linear gradients of acetonitrile.

Eluent system 1 consisted of: A, 100 mM sodium phosphate (pH 2.5); B, 60% acetonitrile in 40% eluent A (v/v). Eluent system 2 consisted of: A, 100 mM ammonium acetate (pH 6.8); B, 60% acetonitrile in 40% eluent A (v/v). Eluent system 3 consisted of: A, 0.1% trifluoroacetic acid in H$_2$O (v/v: pH 2.0); B, 0.1% trifluoroacetic acid in 80% acetonitrile and 19.9% H$_2$O (v/v). Eluent system 4 consisted of: A, 0.1% trifluoroacetic acid in H$_2$O (v/v: pH 2.0); B, 0.1% trifluoroacetic acid in 60% acetonitrile and 39.9% H$_2$O (v/v).

The peptides were detected measuring the absorbance at 280 or 214 nm. [H]- and [14C]-labeled active site peptide fragments were monitored by scintillation counting of 5-40 μl aliquots of each fraction.

Characterization of the Radioactively Labeled Thiolation Site Peptide Fragments of Gramicidin S Synthetase—The investigation of the active site peptides by N-terminal sequencing, amino acid analysis, and mass spectrometry (fast atom bombardment and electrospray ionization MS) was performed as described previously by Stein et al. (13).

**RESULTS**

The thioester binding sites of gramicidin S synthetase 2 for l-Pro-, l-Val-, and l-Orn as well as gramicidin S synthetase 1 for Phe were specifically labeled with N-[3H]Ethylmaleimide using a substrate protection technique (13). In the first step, the thiolation center of the peptide synthetase was protected by its cognate amino acid substrate, followed by alkylation of the reactive residues at the bulk of the multienzymes with high concentrations of non-radioactive NEM (2 mm). After removal of the substrate amino acid from the thiolation site by incubation with dithioerythritol, the reactive thiol of this reaction center was specifically labeled with low concentrations of [3H]NEM (23 μM). After removal of the resulting modified synthetase complexes were isolated from the substrates by G-25 gel filtration. To obtain the radioactively labeled, active site peptide fragments of the thiolation sites of gramicidin S synthetase, the [3H]NEM-alkylated enzymes were digested with trypsin. In the case of GS2, this proteolysis should result in a very complex mixture of 391 peptides, 27 single lysine, and 14 arginine residues assuming quantitative fragmentation of the Lys-Xaa or Arg-Xaa peptide bonds. To purify the labeled active site peptide fragments from these mixtures to homogeneity, high resolution separation procedures were required. As summarized in Fig. 1, we developed a multistep reversed phase HPLC methodology using preparative and analytical C$_8$ODS columns and acetonitrile-containing eluent systems with different composition and pH-value of their aqueous components. If necessary this procedure was repeated after additional cleavage(s) with other proteases. This technique can be utilized as a very efficient general strategy for the isolation of a target peptide from very complex peptide mixtures containing several hundred contaminants.

As indicated in Fig. 2A, 5-10-mg portions of the tryptic peptide mixtures of the [3H]NEM-alkylated GS2 complexes were separated in the first step by reversed phase chromatography on a C$_8$EnCaPharm column applying an linear acetonitrile gradient from 10 to 80% eluent B in 350 min of eluent system 1. As a representative example of the developed separation procedure, the purification of the peptide fragment of
the l-ornithine thiolation site of GS2 is shown in Fig. 2 (B and C). The radioactively labeled peptide fraction containing this active site peptide fragment with a retention time of 250–260 min (60–62% eluent B, Fig. 2A) was rechromatographed by reversed phase HPLC on Hypersil ODS with an acetonitrile gradient of eluent system 3. As is apparent from the chromatogram, the radioactivity eluted in one peak with a retention time of 119–123 min (42% eluent B, Fig. 2B). Because the absorbance profile of this chromatography implies that the radioactive peptide fraction contained at least 2–3 contaminant tryptic peptides of GS2, it was subsequently digested with endoproteinase Glu-C from Staphylococcus aureus V8 and fractionated again by reversed phase HPLC on Hypersil using an acetonitrile gradient of eluenet system 3. The fraction in Fig. 2C with the main portion (80%) of the 3H activity was detected at a retention time of 217–227 min (42% B). Two minor peaks eluted at retention times of 172–175 (37% B) and 185–188 min (38% B) each with 10% of the radioactivity. Liquid phase sequencing of these three radioactive peptide fractions demonstrated that all of them contained an active site peptide fragment of GS2 for L-Orn thiolation.

Fraction 1: VGIHDDFTIGGHDSLK (main product at 42% B)
Fraction 2: DFFTIGGHDSLK (peptide fraction at 38% B)
Fraction 3: FFTIGGHDSLK (peptide fraction at 37% B)

The active site peptide of GS2 for l-Orn thiolation contained no glutamic acid residue. Therefore, a fragmentation of the peptide by Glu-C proteolysis at pH 4.0 in ammonium acetate buffer did not occur (main product). However, unspecific fragmentation of the Asp-Xaa peptide bonds of this peptide led to the minor products. In addition, the contaminating peptides were cut into smaller pieces which could efficiently be removed by HPLC.

After the initial preparative EnCaPharm chromatography, the tryptic fragment of the l-Val thiolation site of GS2 was purified to homogeneity in only one additional reversed phase HPLC step (Fig. 1). This strategy allowed a much more efficient preparation of the active site peptide than the previously reported four- to five-step purification procedure including two or three fragmentation steps (13). The l-Leu thiolation site of GS2 was affinity-labeled with L-[[14C]Leu. Here the purification procedure described by Schlumbohm et al. (12) was optimized (Fig. 1).

Each of the isolated active site peptide fragments of the thiolation sites of gramicidin S synthetase was investigated by N-terminal sequencing using a liquid phase sequencer (Edman degradation). The obtained sequences of the thiolate sites of GS 1 for L-Phe (D564-K575) and GS2 for L-Pro (I983-K1008), L-Val (I2029-R2044), L-Orn (V3075-K3090, main radioactive product), and L-Leu (F4120-L4132) are in accordance with the corresponding sequences derived from the grsA and grsB1–4 gene segments (7, 8). All peptide fragments contained the highly conserved thioester binding motif LGG(H/D)S(L/I). In each case the radioactivity was eliminated from the peptide during the first Edman degradation step. Instead of the invariant serine residue of the thiolation motif claimed by the gene derived sequence, a dehydroalanine was found at this position. To prove our hypothesis that the radioactive tracer is not directly bound at the reactive serine and that an additional structural element, a 4'-phosphopantetheine carrier, is attached to the reactive serine of the (H/D)S(L/I) core, all isolated thiolate site peptide fragments of gramicidin S synthetase were investigated by mass spectrometric techniques.

As an example the extensive mass spectrometric analysis of the active site hexadecapeptide fragment of GS2 for L-Orn thiolation is demonstrated in Fig. 3. A quasimolecular ion [M + H]+ was determined for this fragment by high field fast atom bombardment mass spectrometry (FAB-MS) at m/z = 2207.9 daltons, as shown in Fig. 3A. This mass is appreciably higher than 1742.9 daltons, the mass calculated for the free hexadecapeptide derived from the grsB3-derived DNA sequence (7), assuming tryptic cleavages at Lys-2028 and Arg-2044. The mass difference of 465 daltons corresponds exactly to a covalent substitution of the peptide with a S-(N-ethylsuccinimido)-4'-phosphopantetheinyl adduct (NES-Pan). The much weaker quasi-molecular ion signal at m/z 2083 is attributed to the 4'-phosphopantetheinyl peptide itself, indicating that a portion of molecules does not carry the NES label. The structure of the
The signals at \( m/z \) 1104.9; 737.1, and 553.2 that can be interpreted as the doubly, triply, and quadruply charged active site hexadecapeptide \([M + H]^+\), \([M + 2H]^2+\), and \([M + 4H]^4+\). A molecular mass, \( M \), of 2208.0 ± 0.3 Da was calculated using a deconvolution algorithm (23) and average mass numbers which matches exactly with the \( m/z \) value obtained by FAB-MS.

The triply charged ion at \( m/z = 737.1 \) was collision-activated. The ESI tandem MS spectrum is shown in Fig. 4B. Fragment ions at \( m/z = 912.6 \) and 863.4 were detected. They can be attributed to the doubly charged phosphorylated heptapeptide \([M_p + 2H]^2+\) and the dehydrated species showing a dehydroalanine in position 14 \([M_x + 2H]^2+\). In addition, fragment ions were obtained at \( m/z = 484.0 \) and 386.2 representing the eliminated carrier ion with and without phosphate, respectively. By increasing the sensitivity of the mass spectrometric detection in the \( m/z \) range between 250 and 1400, N- and C-terminal fragments (single and doubly charged a, b, c, and x, y, z series) of the phosphorylated hexadecapeptide as well as the dehydrated species were observed. From these data the structures shown in Fig. 4C were derived, which are consistent with the amino acid sequence determined for this active site peptide fragment by gas phase sequencing and FAB-MS. The electrospray data demonstrate that the phosphoryl group is located at the serine residue in position 14 of the hexadecapeptide corroborating the attachment of the Pan carrier at this position.

The interpretation of an attached Pan prosthetic group to the active site peptide fragments is supported by investigation of their amino acid content after total hydrolysis. A total of 1–1.3 mol of 3-aminopropionic acid (β-alanine), a constituent of 4'-phosphopantetheine were found per mole of the active site peptides.
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DISCUSSION

In a previous paper (11), we provided evidence from chemical and genetic studies that an active serine is involved in covalent binding of the substrate amino acids at each reaction center of gramicidin S synthetase 2, instead of a cysteine as proposed in the original version of the thiotemplate mechanism (2–5). This serine is part of a strictly conserved LGG(H/D)S(L/I) motif, which has been detected as an essential structural element of each amino acid-activating domain of multifunctional peptide synthetases the gene sequences of which have been determined so far. However, the chemical features of the reactive intermediates are consistent with the existence of thioester bond (2–5, 11). Therefore, it seems unlikely that the substrate amino acids of GS2 are directly bound to these serine residues at the reaction centers. This conclusion is supported by the strong similarity of the (H/D)S(L/I) core of the thiotemplate motifs of GS2 with 4'-phosphopantetheine binding sites of fatty acid and polyketide synthetases. To clarify this fundamental question in detail, we isolated the thiolation site peptide fragments of gramicidin S synthetase and investigated them by N-terminal sequencing, mass spectrometry, and amino acid analysis.

Each of the five thiotemplate site peptide fragments of gramicidin S synthetase was investigated by FAB- and ESI-MS. As is apparent from Table I, the molecular masses of all labeled thiolation site peptide fragments are appreciably higher than the values calculated from the amino acid sequence of GS1 and GS2 (7, 8). Each of the observed mass differences indicate a covalent substitution of the peptide moiety with a 4'-phosphopantetheine cofactor, which is either alkylated with [3H]NES or thioesterified with the amino acid substrate in the case of the L-Leu thiolation site fragment of GS2. The nature, covalent linkage, and site of location of the Pan substituent attached to the serine residue of the thiolation motif was proven by interpretation of the fragmentation data obtained from FAB-MS as well as collision-induced dissociation ESI-MS, as demonstrated for the active site peptide of GS2 for the thioesterification of L-ornithine in Figs. 3 and 4. The nature of the substituent is supported by amino acid analysis. One mole of each active site peptide fragment of gramicidin S synthetase contained approximately 1–1.3 mol of β-alanine, which is a constituent of 4'-phosphopantetheine.

Our results, summarized in Table I, give evidence that each of the five amino acid-activating modules of gramicidin S synthetase is equipped with a separate 4'-phosphopantetheine prosthetic group esterified to the active site serine in the (H/D)S(L/I) core of their thiolation motifs. The cysteamine thiol groups of the cofactors represent the thiolating binding sites for the substrate amino acids, instead of cysteine residues as proposed in the original version of the thiotemplate hypothesis (2–5). Our study of the structure of all thiotemplate sites of gramicidin S synthetase demonstrates for the first time that peptide-forming multienzymes contain multiple Pan-cofactors, one at each reaction center of their amino acid-activating modules. Our data are strong support for the multiple carrier model of nonribosomal peptide biosynthesis.

Since the first gene sequences of peptide-forming multienzymes have been elucidated within the last 6 years, important progress has been achieved concerning the analysis of their
structure-function relationships (for recent reviews, see Refs. 9–10). Peptide synthetases are composed of homologous building blocks comprising 1000–1500 amino acid residues, which are distinguished by a linear array of highly conserved sequence motifs representing the reactive structures of functional domains for substrate binding and catalysis of all intermediate steps in peptide biosynthesis as demonstrated for gramicidin S synthetase in Fig. 5. Each of these modules functions as an independent enzyme, which catalyzes the selection, activation, and in some cases modification of its amino acid substrate. By affinity labeling of peptide-forming multienzymes as well as by site-directed mutagenesis and specific dissection of gene structures coding for these enzymes, followed by functional analysis, some of these motifs could be attributed to specific functions. As illustrated in Fig. 5 for gramicidin S synthetase, each of the amino acid-activating modules is organized into specific functional domains for amino acid adenylation (500–600 amino acid residues; gray) (24–30), thioester binding (80–100 amino acid residues, black) (12–14, 30), and elongation of the peptide product (E, 300–400 amino acids; white) (31–35). Epimerizing/racemizing modules as in the case of GS1 contain an elongation domain with significant differences in comparison to non-epimerization modules (E-Epi, 300–400 amino acids; light gray) (14, 31–35). For interacting protein components of peptide-forming multienzymes as GS1 and GS2, a specific domain at the N terminus of the acceptor enzyme is observed showing significant homologies to the elongation domains (EN; light gray) (10, 31, 33). At the C-terminal end of each peptide-forming multienzyme system like GS2, a sequence homologous to thioesterase II (T) is found instead of the elongation domain, which is observed in all other modules (34, 35).

From the present knowledge of structure-function relationships of peptide synthetases, we imply that three specific sites for the Pan carrier within an amino acid-activating module are involved in the biosynthetic process: a charging position for thioester formation with the substrate amino acids as well as a peptidyl-acceptor and a peptidyl-donor site. Most probably the elongation domain contains the structural elements for interaction between their Pan carriers, including the peptidyl-donator site for the first one and the peptidyl-acceptor site for the subsequent cofactor. This transpeptidation reaction resembles the peptidyl-transfer process between aminoacyl-tRNA and peptidyl-tRNA within the ribosomal A and P site. This model allows a much simpler and straightforward description of the biosynthetic process than the old version (2–5), because the assumption of a central carrier and the transthiolation reactions required for the transport of the peptide intermediates could be omitted. In addition, the charging of a multienzyme with all intermediates of the growing peptide chain, not understandable by the former hypothesis, can be easily explained by the multiple Pan carrier concept. As a representative example for the multiple carrier model, the first steps of the biosynthesis of gramicidin S leading to the formation of the tripeptide D-Phe-L-Pro-L-Val are shown in Fig. 6. The amino acid-activating modules catalyze aminoacyl adenylation and thioesterification of their cognate amino acids to their Pan carrier (Fig. 6A) at the charging position within the adenylation domain (C<sub>amino acid</sub>). The energy for this process is provided by hydrolysis of an ATP α-β linkage resulting in the release of

**Table I**

| Enzyme | Position of the Thiopeptide site peptide fragments |
|--------|---------------------------------------------------|
| GS1    | D 564 – K 575 | DNFYALGDSIK, Pan-[H]NES |
| GS2    | I 983 – K1008 | IWEVELGISQIGIQDNFFSLGHSKL, Pan-[H]NES |
| L-Val  | I2029 – R2044 | IGVLDNFELGHSKL, Pan-[H]NES |
| L-Orn  | V3075 – K3090 | VGIHDDFTIGHSKL, Pan-[H]NES |
| L-Leu  | F4120 – L4132 | FELGHSKLATL, Pan-[C]Leu |

*The molecular masses of the peptides were calculated from the gene-derived sequence (7, 8).  
*Mass was calculated as the sum of the molecular masses of the peptide moiety, the 4'-phosphopantetheine substituent that is covalently attached to the serine residue, and the radioactively labeled tracers (shaded boxes, NES: N-ethylsuccinimido and L-leucine, respectively) bound to the reactive thiol group of the Pan cofactors.  
*Results of the investigation of the active site peptide fragments by electrospray mass spectrometry (ESI-MS).
AMP and pyrophosphate (PP\textsubscript{i}). As illustrated in Fig. 6B, the elongation cycle starts with the interaction of gramicidin S synthetase 1 (GS1) and gramicidin S synthetase 2 (GS2). The Pan carrier of GS1 transports phenylalanine to the elongation site (E\textsubscript{1}-Epi), where epimerization of Phe also occurs. The dipeptide D-Phe-L-Pro (F-P) is formed in E\textsubscript{2} in a transpeptidation reaction by nucelophilic attack of the imino group of L-Pro (Pan\textsubscript{pro} carrier in its acceptor site) at the thioester-activated carboxyl C-atom of D-Phe (Pan\textsubscript{phe} carrier in its donor site). Most probably the domain E\textsubscript{2} of GS2 is involved in this process. A free thiol group of the Pan\textsubscript{phe} carrier is recovered in this reaction, which is able to bind Phe again and to start a new cycle of elongation. As demonstrated in Fig. 6C, the Pan carrier of the L-Pro module of GS2 thioesterified with the dipeptidyl intermediate (F-P) is translocated to its donor site. By interaction with the L-valyl-Pan\textsubscript{val}-carrier in its acceptor site, the tripeptide D-Phe-L-Pro-L-Val is formed in a second transpeptidation reaction. In a similar manner the tetra- and pentapeptide intermediates D-Phe-L-Pro-L-Val-L-Orn and D-Phe-L-Pro-L-Val-L-Orn-L-Leu are assembled involving the individual Pan carriers of the ornithine- and leucine-activating domains of gramicidin S synthetase 2 (data not shown). Finally the decapeptide gramicidin S is formed by cyclization of two pentapeptide moieties.

For the experimental verification of the multiple carrier model, more detailed investigations of structure-function relationships of peptide synthetases are necessary. In particular, the three sites attributed by us to the functions of the Pan carrier have to be localized within an amino acid-activating module and investigated. For this purpose the functional characteristics of the highly conserved consensus motifs, especially in the putative elongation domain, will provide the clue to understand the process of peptide bond formation. The existence of specific recognition elements for the growing peptide chain within this part of a module is a possible explanation for the unidirectional assembly of the peptide product, presumably in combination with conformational changes induced during the transpeptidation reactions. Approaches to clarify these essential questions, such as site-directed mutagenesis of putative functional amino acids within the conserved motifs in combination with elongation studies as well as the determination of the three-dimensional structure of peptide-forming multienzymes, are in progress.

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