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Detection of 4′-phosphopantetheine at the thioester binding site for L-valine of gramicidinS synthetase 2

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Abstract

Biosynthesis of gramicidinS in *Bacillus brevis* is catalysed by a multienzyme system consisting of two multifunctional proteins, gramicidinS synthetase 1 and 2 codified by the grsA and grsB genes, respectively. GramicidinS synthetase 2 shows a modular architecture of four amino acid-activating domains each containing a thioester binding motif LGG H/D S L/I highly conserved in its C-terminal region, as demonstrated by sequence analysis of the grsB gene [W. Schlumbohm et al. (1991) J. Biol. Chem. 266, 23135–23141]. This multienzyme was specifically labeled at the thioester binding site of L-valine with [^{3}H]*N*-ethylmaleimide using a substrate protection technique. After enzymatic digestion a labeled active site peptide was isolated in pure form by multistep methodology. This fragment was identified by gas-phase sequencing as the active site peptide of the thiotemplate site for L-Val by comparison with the grsB gene sequence. By mass spectrometry in combination with amino acid analysis it was demonstrated that a 4′-phosphopantetheine carrier was attached to the active serine in this motif. Our results give evidence that multiple peripheral 4′-phosphopantetheine carriers are involved in the formation of gramicidinS in contrast to a central carrier arm as assumed in the original version of the thiotemplate mechanism. A 'Multiple Carrier Model' of nonribosomal peptide biosynthesis is proposed.

Key words: GramicidinS synthetase; Thioester binding site; Affinity labeling; Active site peptide; 4′-Phosphopantetheine; Multiple carrier model

1. Introduction

GramicidinS is a cyclic decapeptide produced by *Bacillus brevis* ATCC 9999.

D-Phe – Pro – Val – Orn – Leu
| |
Leu – Orn – Val – Pro – D-Phe

Its biosynthesis is catalysed by gramicidinS synthetase which is one of the best characterized peptide forming multienzyme systems consisting of two multifunctional enzymes which activate their substrate amino acids in a two-step process involving aminoacyl adenylate and thioester formation [1–5]. GramicidinS synthetase 1 (GS1) activates and racemizes phenylalanine and transfers D-Phe to gramicidinS synthetase 2 (GS2) which activates the other 4 amino acids and catalysis the elongation of the growing peptide chain by the participation of 4′-phosphopantetheine as a carrier. Recently the entire nucleotide sequence of the *B. brevis* grsB gene encoding GS2 has been determined [6].

The mechanism of nonribosomal biosynthesis of numerous microbial peptides is compatible with the polyenzyme model suggested by F. Lipmann [7] and the thiotemplate mechanism [1–5]. These models propose that (a) amino acid activating domains are arranged in series along multifunctional polypeptide chains, (b) each reaction center of a peptide synthesizing multienzyme is equipped with a specific sulfhydryl group to which the amino acid substrates are attached as thioesters (thiotemplates), and (c) the growing peptide chain is assembled by the action of 4′-phosphopantetheine (Pan) as a carrier. In accordance with the polyenzyme model [7] four conserved and repeated gene segments grsB1–grsB4 have been identified in the grsB structure. Analyses of

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Abbreviations: GS, gramicidinS synthetase; GS1, gramicidinS synthetase 1 (phenylalanine racemase, EC 5.1.1.11); GS2, gramicidinS synthetase 2 (condensing, peptide forming multienzyme); Pan, 4′-phosphopantetheine; NEM, *N*-ethylmaleimide; NES, *N*-ethylsuccinimido; x, *S*-(*N*-ethylsuccinimido)4′-phosphopantetheine; DTE, dithioerythritol; OPA, *o*-phthaldialdehyde; HPLC, high-performance liquid chromatography; FPLC, fast protein (peptide) chromatography; HF-FAB MS, high-field fast-atom bombardment mass spectrometry; ESI MS, electrospray ionization mass spectrometry; *m/z*, mass to charge ratio; kV, kilovolt.

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active site peptides of the reaction centers of GS2 for thiolation of the substrate amino acids showed that they could be attributed to the activation domains of Pro, Val, Orn and Leu of this multienzyme [8]. Surprisingly an active serine is required for covalent attachment of the substrate amino acids instead of a cysteine as originally proposed for the thiotemplate mechanism. These serine residues are part of a LGGH/DSL/I motif which is highly conserved in the C-terminal region of each amino acid activating domain of the peptide synthetases whose gene sequences have been determined so far [6,9–13]. The H/DSL/I core of this motif has been identified as the 4′-phosphopantetheine binding site of acyl carrier proteins/domains of fatty acid and polyketide synthetases [8].

In this publication we show for the first time by mass spectrometric analysis that 4′-phosphopantetheine is indeed attached to the reactive serine of the LGGHDSL motif at the thioester binding site of L-Val in the second domain of gramicidinS synthetase 2. Our data provide evidence that peptide synthetases contain multiple Pan-carriers instead of one central Pan-arm as postulated in the original version of the thiotemplate model [5] and form the basis of a 'Multiple Carrier Model' as a new concept of the mechanism of nonribosomal peptide biosynthesis.

2. Materials and methods

2.1. Materials

[^{3}H]*N*-Ethylmaleimide was purchased from DuPont (Dreieich, FRG). *Staphylococcus aureus* V8-protease and TPCK-treated trypsin were obtained from Miles/Bayer Diagnostics (München, Germany) and Sigma (Deisenhofen, Germany). *Bacillus brevis* ATCC 9999 was cultivated in a fumarate/phosphate medium, as reported by Chiu [14].

2.2. Enzyme purification and assays

GS2 was purified as published by Vater et al. [15]. Assays for thioester formation of GS2 with substrate amino acids [16,17] and biosynthesis of gramicidinS [15,18] were performed, as described previously.

2.3. Specific labeling of the thioester binding site of GS2 for L-valine with [^{3}H]N-ethylmaleimide

30 mg GS2 in 50 ml 400 mM phosphate buffer, pH 7.2, containing 2 mM DTE and 1 mM EDTA were concentrated to 3 ml by ultrafiltration. This enzyme solution was dialyzed against a 20 mM phosphate buffer, pH 7.2, containing 1 mM EDTA (buffer P) which prior to usage was degassed and saturated with nitrogen.

GS2 was specifically protected at the thiotemplate site of L-valine by incubation with 2 mM ATP, 10 mM $MgCl_2$ and a saturating L-valine concentration of 15 μM for 10 min at 37°C. Reactive groups at the bulk of the GS2–L-Val–thioester complex were saturated by incubation with 2 mM NEM for 30 min at 37°C. The resulting complex was isolated by gel filtration on Sephadex G-25 at 3°C with buffer P as the eluent and concentrated to a final volume of 3 ml by ultrafiltration. By incubation with 2 mM DTE for 60 min at 37°C L-valine was removed from GS2. The enzyme was 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 ultrafilter XM 50 was used. Finally the thioester binding site for L-valine was specifically labeled by incubation of the deprotected multienzyme with 23 μM [^{3}H]NEM at 37°C for 30 min. The labeled protein was isolated by gel filtration on Sephadex G25 at 3°C with buffer P, pH 8.2 as the eluent and concentrated in a Speed Vac concentrator until a final protein concentration of approx. 2 mg/ml.

2.4. Digestion of the [^{3}H]NEM-labeled GS2 with trypsin

1–2 mg [^{3}H]NEM-labeled GS2 were digested with 33–66 μg TPCK-treated trypsin in 0.5–1 ml 100 mM phosphate buffer, pH 8.2, containing 1 mM EDTA, 10% acetonitrile (v/v) at 37°C for 3 × 60 min. The reaction was stopped by adding 500 μl 0.1% trifluoroacetic acid (v/v). The resulting fragments were separated by FPLC/HPLC procedures.

2.5. Digestion of the [^{3}H]NEM-labeled tryptic fragments of GS2 with Staphyloccus aureus *V8 protease*

Tryptic peptides of GS2 labeled with [^{3}H]NEM were dissolved in 200 μl ammonium acetate buffer, pH 4.0, containing 1 mM EDTA and 10% acetonitrile (v/v). The peptides were digested for 20 h at 37°C with *S. aureus* V8 protease using a molar ratio of protease to substrate of approx. 1:100. The reaction mixture was dried in a Speed Vac concentrator and separated by reversed phase HPLC.

2.6. Separation of peptides by reversed phase FPLC/HPLC

Pharmacia Pro RPC HR 10/10 and Pep RPC HR 5/5 FPLC columns as well as a Shandon ODS Hypersil (5μ) column were used for separation of the tryptic fragments. After further digestion with *S. aureus* V8-protease an ODS Hypersil (5μ) column was applied for peptide purification.

Peptide mixtures were dissolved in 300 μl 0.1% trifluoroacetic acid, pH 2.0, loaded onto the columns and eluted with linear gradients of acetonitrile. Solvent A was 0.1% trifluoroacetic acid in H_2O (v/v). Eluent B was composed of 0.1% trifluoroacetic acid in 80% acetonitrile/20% H_2O (v/v). The flow rate was 0.75 ml/min. The peptides were detected measuring the absorbance at 280 or 214 nm. [^{3}H]NEM labeled active site peptides were monitored by scintillation counting of 5–20 μl aliquots of each fraction.

2.7. Peptide sequencing

Peptide sequences were determined on an Applied Biosystems pulsed-liquid phase sequencer, model 477 A. Phenylhydantoin amino acids were separated on line in an Applied Biosystems model 120 A analyzer. Samples were 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. [19].

2.8. Amino acid analysis

50–100 pmol of the active site peptide were hydrolyzed with 6 N HCl at 110°C for 24 h in sealed, evacuated tubes. The Programmable Multidimensional Injection System (Spark, Holland) with automatic pre-column derivatization (t = 1 min) was applied for the reaction of the amino acids with o-phthaldialdehyde (OPA). OPA-amino acids were separated by HPLC on a C18-column (ODS Hypersil (5μ), Knauer, Germany) using a multistep gradient. Eluent A was 12.5 mM Na_2HPO_4, pH 6.5. Eluent B was 97% methanol and 3% tetrahydrofurane (v/v). OPA-amino acids were detected with a Shimadzu-RF-551 Fluorescence-Detector (excitation: 350 nm; emission: 455 nm).

2.9. Mass spectrometric analysis of the active site peptide

2.9.1. Fast atom bombardement mass spectrometry (FAB-MS). The active site peptide was dissolved in 5% acetic acid and 1 μl aliquots loaded onto a sample probe treated with 1 μl of meta-nitrobenzyl alcohol or glycerol/thioglycerol 5/1 (v/v) in separate experiments. The peptide was then characterized by high-field fast atom bombardment mass spectrometry (HF-FABMS) using a Fisons ZAB 2SE2FPD Focal Plane Detector instrument equipped with a VG cesium ion gun (30 kV), the spectra being acquired on an Opus Data System and calibrated using CsI clusters, giving molecular masses based on accurate atomic numbers. Initial spectra were recorded on a ZABHF instrument equipped with an M-Scan xenon ion gun (10 kV) with spectra recorded on oscillographic chart paper, giving molecular masses based on integer atomic numbers. Sample aliquots (approx. 50 pmol) were further subdigested with carboxypeptidase B in 50 mM ammonium bicarbonate buffer pH 8.5 for 30 min at 37°C followed by reanalysis in the mass spectrometer.

2.9.2. Electrospray ionization mass spectrometry (ESI-MS). ESI mass spectra were recorded on a Finnigan-MAT triple stage quadrupole mass spectrometer TSQ 700 equipped with an electrospray ion source. The electrospray needle was operated at a voltage difference of 4 kV. The peptide material was dissolved in 50:50 mixture of 1% acetic acid and methanol (v/v) and introduced into the source at a flow rate

of 1 μl/min. The sample concentrations were in the low pmol/μl range. Collision activated dissociation experiments were conducted at an energy of 17 eV. Argon at a pressure of 3 mtorr was used as the collision gas.

3. Results

In a previous paper [8] we affinity-labeled GS2 with L-[^{14}C]valine and L-[^{14}C]leucine at its thioester binding sites, isolated active site peptides and showed that a serine is involved in the covalent attachment of the amino acid substrates to the reaction centers of this multienzyme. This direct labeling procedure which is highly specific shows some disadvantages. Because the thioester complexes of GS2 with its substrate amino acid are stable only in acidic media the label was lost in the first Edman degradation step during sequencing of the active site peptides. Therefore, it was difficult to obtain precise information on the nature of the thioester binding sites by chemical analysis. On the other hand the choice of the techniques for protein fragmentation and separation of the complex fragment patterns by multistep HPLC/FPLC methodology is restricted to those techniques which can be applied in acidic media.

To circumvent these obstacles in this paper we labeled the thioester binding site of GS2 for L-valine by an indirect technique which comprises the following steps: (a) substrate protection of the reaction center; (b) saturation of the reactive residues at the bulk of the multienzyme with high concentrations of nonlabeled NEM; (c) removal of the substrate amino acid from the thiolation site under mild conditions, and finally (d) specific labeling of the thiotemplate site at low concentrations of [^{3}H]NEM. In this way a stable GS2–[^{3}H]NEM adduct was obtained that allowed the application of the full spectrum of fragmentation and separation techniques.

In order to isolate the active site peptide of the thiotemplate site of GS2 for L-valine we digested the GS2–[^{3}H]NEM complex with trypsin. The resulting peptide mixture was separated by reversed-phase FPLC/HPLC methodology in 3 steps. In the first one the tryptic peptides were fractionated by reversed phase FPLC on a Pharmacia Pro RPC HR 10/10 column (C1/C8 alkyl chains, pore size: 300 Å) in a preparative scale using a linear acetonitrile gradient from 0 to 100% eluent B. The fractions containing the radioactivity were rechromatographed by reversed-phase FPLC using a Pharmacia Pep RPC HR 5/5 column (C2/C18 alkyl chains, pore size: 100 Å) and a more shallow gradient from 20 to 50% eluent B for elution. The radioactive material was separated in the third step by reversed-phase HPLC on ODS Hypersil applying an acetonitrile gradient from 30–60% eluent B for 300 min. The radioactively labeled tryptic peptide fraction was subsequently digested with *Staphylococcus aureus* V8-protease and fractionated by reversed-phase HPLC on ODS Hypersil using an acetonitrile gradient from 20 to 50% eluent B. The pure radiolabeled peptide isolated in this way was analyzed by gas phases sequencing, amino acid analysis and mass spectrometry. By gas phase sequencing the following heptapeptide was obtained:

$$\begin{array}{l} 1\ \ 2\ \ 3\ \ 4\ \ \ 5\ \ 6\ \ 7 \\ \mathrm{L-G-G-H-\Delta A-L-R} \end{array} \qquad (1)$$

It is identical with the fragment that we have previously identified as the active site peptide of the thioester binding site for L-valine by direct labeling of GS2 with L-[^{14}C]valine. This is confirmed by sequence comparison of this fragment with the corresponding segment in the grsB2 domain of this multienzyme [8]. However, the gene sequence claims a serine at position 5 which is in accordance with the amino acid analysis data shown in Table I. Surprisingly also in the case of alkali-stable modification of the thioester binding site of L-Val with [^{3}H]NEM the radioactivity was eliminated from the peptide in the first Edman degradation step and a dehydroalanine appeared in position 5. This implies that the tracer is not directly bound to the amino acid residue at this position and corroborates our previous hypothesis [8] that an additional structural element, possibly a 4′-phosphopantetheine carrier, is attached to the reactive serine of the HSL core. This is confirmed by our amino acid analysis data showing that one mole of the active site heptapeptide contains approximately one mole β-alanine (see Table I). To clarify this fundamental question in detail

Table 1
Amino acid analysis of the active site peptide for L-valine thiolation of GS2

Amino acid	Residue per peptide molecule	
	(a) Experimental	(b) derived from gene sequence
Leu	2.0	2
Gly	2.0 ± 0.1	2
Ser	0.9 ± 0.1	1
His	0.9 ± 0.1	1
Arg	1.0 ± 0.1	1
Ala	0.30 ± 0.05	–
Asx	0.08 ± 0.02	–
Glx	0.13 ± 0.02	–
Val	0.10 ± 0.02	–
Ile	0.08 ± 0.02	–
Thr	0.03 ± 0.01	–
Tyr	0.01	–
Met	0.01	–
Phe	–	–
Lys	–	–
β-Ala*	1.3 ± 0.2	–

The content of the constituent amino acids was referred to the amount of L-leucine. Amino acid analysis was performed as indicated under Methods. *β-Ala is a constituent of the 4′-phosphopantetheine substituent.

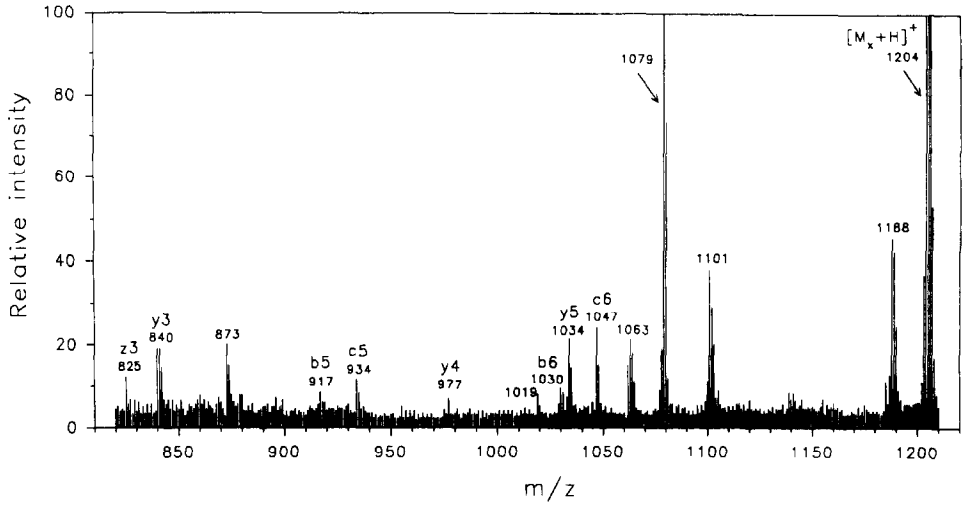
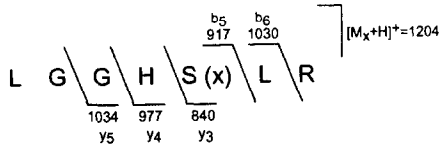


Fig. 1. HF-FABMS spectrum of the active site peptide for L-valine thiolation of GS2 showing an intense quasi-molecular $[M_x+H]^+$ ion at *m/z* = 1204 together with related fragment ions. The spectrum is drawn from an oscillographic recording. For interpretation see the text.

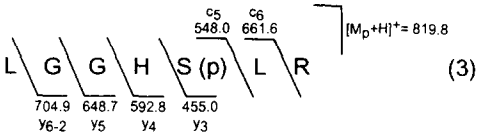
it was investigated by mass spectrometric techniques. A quasi-molecular ion $[M_x + H]^+$ was determined for the active site peptide by HF-FABMS at *m/z* = 1204 Da, as shown in Fig. 1. This mass is appreciably higher than the 739 Da as was calculated from the gene-derived sequence. The mass difference of 465 Da corresponds exactly to a covalent substitution of the peptide with a *S*-(*N*-ethylsuccinimido)-4′-phosphopantetheine adduct (NES-Pan). The much weaker quasi-molecular ion signal at *m/z* 1079 corresponds to the 4′-phosphopantetheinyl peptide itself, i.e. a proportion of molecules not carrying the NES label. The nature, covalent linkage and site of location of the NES-Pan substituent on the serine residue was proven by interpretation of the fragmentation data in Fig. 1 according to rules defined by us in earlier work [20,21] on both known and unknown peptide sequences. The fragmentation pattern observed for the active site peptide adduct is a series of N- and C-terminal sequence ions (b, c, y and z) at *m/z* 825, 840; 917; 934; 977; 1030; 1034 and 1047 which, together with MS data on the carboxypeptidase B-treated sample showing removal of a free C-terminal arginine residue (156 Da) via a signal at *m/z* 1048, determine the structure of the active site heptapeptide summarised in (2) below. These results clearly demonstrate that the NES-Pan substituent must be attached at the serine residue of the thioester binding motif.

Results of electrospray mass spectrometry measurements are shown in Fig. 2. They are complementary to the HF-FABMS data. The ESI mass spectrum (Fig. 2A) shows signals at *m/z* = 603.0 and 402.3 that can be interpreted as the doubly and triply charged active site heptapeptide $[M_x + 2H]^{2+}$ and $[M_x + 3H]^{3+}$. A molecular mass M_x of 1204.0 ± 0.3 was calculated using a deconvolution algorithm [22] and average mass numbers. M_x matches exactly with the m/z value obtained by FABMS. The doubly charged ion at *m/z* = 603.0 was collision activated. The ESI tandem MS spectrum is shown in Fig. 2B. Fragment ions at *m/z* 819.3 and 721.5 were detected. They can be attributed to the phosphorylated heptapeptide and the dehydrated species showing a dehydroalanine in position 5 which originate from partial or complete elimination of the NES-4′-phosphopantetheine substituent. In addition fragments were obtained at *m/z* = 484.5 and 386.0 that represent the eliminated carrier ion with and without phosphate, resp. By increasing the sensitivity of the mass spectrometric detection in the *m/z* range between 400 and 710 N-terminal and C-terminal fragments (y and c series) of the phosphorylated heptapeptide ion $[M_p+H]^+$ were observed. From these data structure 3 was derived which is consistent with the amino acid sequence determined for the active site peptide by gas phase sequencing (1) and FABMS (2). The electrospray data demonstrate that the phosphoryl group is located on the serine residue corroborating the attachment of the Pan-carrier at this position.



(2)

x = *S*-(*N*-ethylsuccinimido)4′-phosphopantetheine



(3)

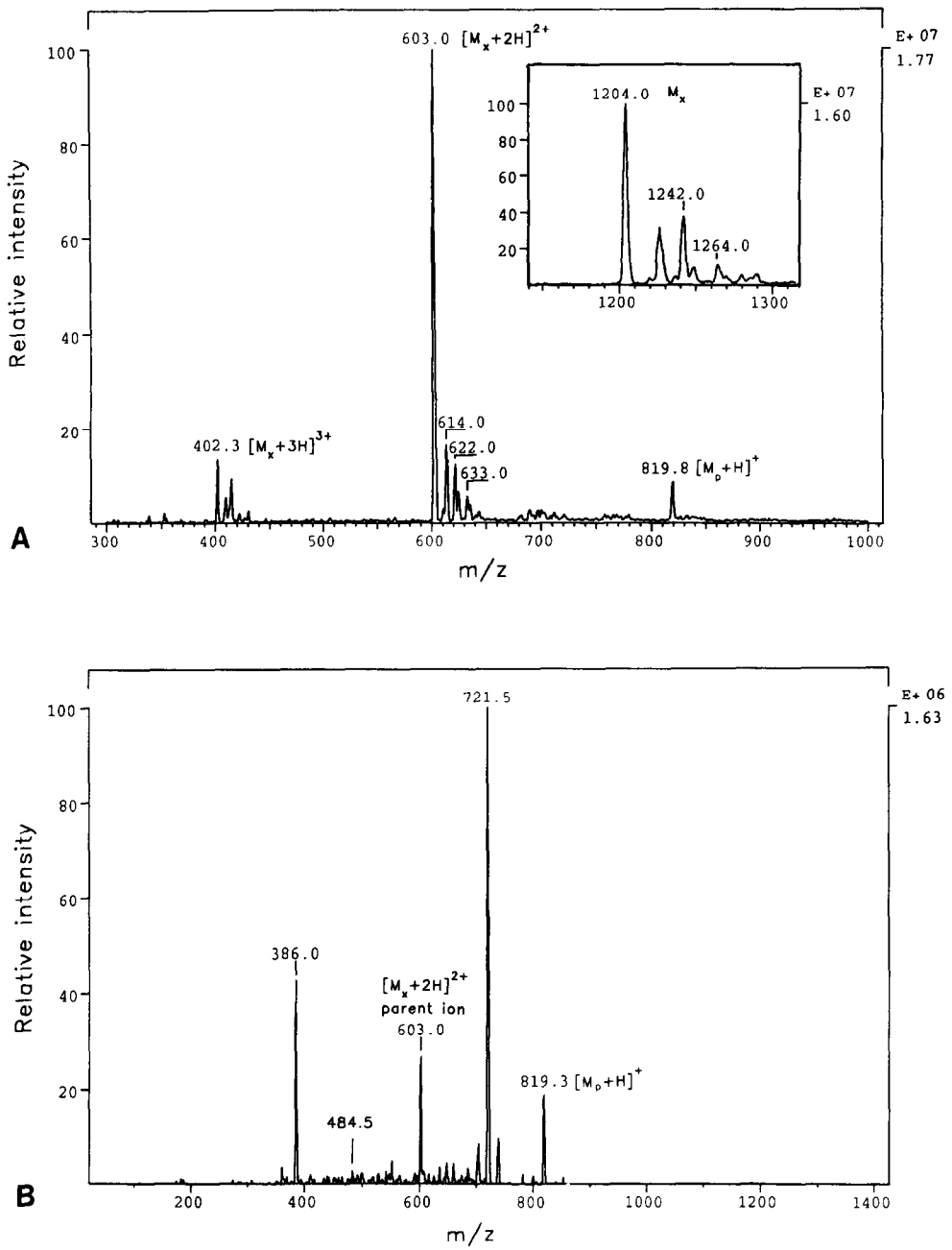


Fig. 2. (A) Electrospray ionization mass spectrum of the active site peptide for L-valine thiolation of GS2. The ions at *m/z* = 603.0 and 402.3 correspond to the doubly and triply charged heptapeptide $[M_x+2H]^{2+}$ and $[M_x+3H]^{3+}$. This interpretation is supported by the appearance of the minor signals at *m/z* = 614, 625, 633 and 410/415 which represent doubly or triply charged Na^+/K^+ adducts of the heptapeptide M_x, as $[M_x+Na+H]^{2+}$, $[M_x+K+H]^{2+}$, $[M_x+2Na]^{2+}$, $[M_x+Na+K]^{2+}$ and $[M_x+Na+2H]^{3+}$ as well as $[M_x+K+2H]^{3+}$, for example. Insert: Computer analysis of the ESI mass spectrum. A molecular mass of 1204.0 ± 0.3 was calculated by a deconvolution algorithm [22]. (B) ESI-tandem mass spectrum of the active site peptide for L-valine thiolation of GS2. A collision induced fragment ion spectrum of the doubly charged peptide at *m/z* = 603.0 is shown. The enhancement of the minor signals at a higher sensitivity of the instrument allowed a nearly complete assignment of the structure relevant ions (see the text).

4. Discussion

In a previous paper [8] 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 gramicidinS synthetase 2 instead of a cysteine as proposed by the original version of the thiotemplate mechanism [1–5]. This serine is part of a strictly conserved LGGH/DSL/I motif which has been detected at the C-terminal region 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 bonds [1–5,8]. 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/DSL core of the thiotemplate motifs of GS2 with 4′-phosphopantetheine binding sites of fatty acid and polyketide synthetases [8].

In this paper we present experimental evidence for this hypothesis for the first time by sequence-, amino acid- and mass spectrometric analysis of the active site peptide of the thioester binding site of GS2 for L-valine which has been labeled with [^{3}H]NEM using the substrate protection technique. Our data which prove that a Pan-carrier is indeed attached to the active site serine represent a fundamental contribution to the elucidation of the mechanism of enzymatic peptide biosynthesis. From our results we conclude that multiple 4′-phosphopantetheine residues are involved in the biosynthesis of gramicidinS and numerous other bioactive peptides. These carriers are attached to the central serine of the H/DSL core of the thioester binding motif at the reaction centers of peptide synthetases forming the thiolation sites for the substrate amino acids. Therefore, the original version of the thiotemplate mechanism has to be replaced by a 'Multiple Carrier Model'. Kleinkauf et al. [5] originally postulated that bioactive peptides would be assembled in a series of transpeptidation and transthiolation reactions by interaction of the sulfhydryl group of a central 4-phosphopantetheine swinging arm with the peripheral cysteines at the reaction centers. Our results demonstrate that these thiols have to be replaced by peripheral Pan-carriers which are the essential mobile elements in the dynamics and catalysis of peptide biosynthesis.

If peptide synthetases are equipped with multiple Pan-carriers the postulation of a central swinging arm would not be necessary. In this case a simpler model could be suggested assuming that the peptide chain is formed in a series of transpeptidation steps by interaction of the Pan-carriers at the reaction centers. The transthiolation reactions which had to be adopted in the original version of the thiotemplate model for the transport of the peptide intermediates could be omitted. Also the charging of a multienzyme with all intermediates of the growing peptide chain simultaneously could be easily understood under these conditions. For the experimental verification of the 'Multiple Carrier Hypothesis' the precise determination of the number of the 4′-phosphopantetheine carriers of GS2 by specific labeling of all reaction centers followed by analysis of the active site peptides is needed. Also the factors determining the unidirectional assembly of the peptide product are still unknown. Here specific recognition elements at the elongation sites and/or conformational changes in concert with the transpeptidation steps are discussed. The investigation of these questions, answers to which are essential for the elucidation of the mechanism of nonribosomal peptide biosynthesis, is in progress.

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References

[1] Lipmann, F. (1973) Accts. Chem. Res. 6, 361–367.
[2] Laland, S.G. and Zimmer, T.-L. (1973) Essays Biochem. 9, 31–57.
[3] Kurahashi, K. (1974) Annu. Rev. Biochem. 43, 445–459.
[4] Kleinkauf, H. and von Döhren, H. (1990) Eur. J. Biochem. 192, 1–15.
[5] Kleinkauf, H., Roskoski, R., Jr., and Lipmann, F. (1971) Proc. Natl. Acad. Sci. USA 68, 2069–2072.
[6] Turgay, K., Krause, M. and Marahiel, M.A. (1992) Mol. Microbiol. 6, 529–546.
[7] Lipmann, F. (1954) in: The Mechanism of Enzyme Action (Mc Elroy, W.D. and Glass, B., Eds.) p. 599, John Hopkins University Press, Baltimore.
[8] Schlumbohm, W., Stein, T., Ullrich, C., Vater, J., Krause, M., Marahiel, M.A., Kruft, V. and Wittmann-Liebold, B. (1991) J. Biol. Chem. 266, 23135–23141.
[9] Krätzschmar, J., Krause, M. and Marahiel, M.A. (1989) J. Bacteriol. 171, 5422–5429.
[10] Weckermann, R., Fürbaß, R. and Marahiel, M.A. (1988) Nucleic Acids Res. 16, 11841.
[11] MacCabe, A.P., van Liempt. H., Palissa, H., Unkles, S.E., Riach, M.B.R., Pfeifer, E., von Döhren, H. and Kinghorn, J.R. (1991) J. Biol. Chem. 266, 12646–12654.
[12] Marahiel, M.A. (1992) FEBS Lett. 307, 40–43.
[13] Cosmina, P., Rodriguez, F., de Ferra, F., Grandi, G., Perego, M., Venema, G. and van Sinderen, D. (1993) Mol. Microbiol. 8, 833–841.
[14] Chiu, C.-W. (1984) Thesis, Technical University of Berlin.
[15] Vater, J., Schlumbohm, W., Palacz, Z., Salnikow, J., Gadow, A. and Kleinkauf, H. (1987) Eur. J. Biochem. 163, 297–302.
[16] Vater, J., Mallow, N., Gerhardt, S., Gadow, A. and Kleinkauf, H. (1985) Biochemistry 24, 2022–2027.
[17] Vater, J., Schlumbohm, W., Salnikow, J., Irrgang, K.-D., Miklus, M., Choli, T. and Kleinkauf, H. (1989) Biol. Chem. Hoppe-Seyler 370, 1013–1018.
[18] Gevers, W., Kleinkauf, H. and Lipmann, F. (1968) Proc. Natl. Acad. Sci. USA 60, 269–276.
[19] Hewick, R.M., Hunkapillar, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990–7997.
[20] Morris, H.R., Panico, M., Barber, M., Bordoli, R.S., Sedgwick, R.D. and Tyler, A.N. (1981) Biochem. Biophys. Res. Commun. 101, 623–631.
[21] Morris, H.R., Panico, M., Karplus, A., Lloyd, P.E. and Riniker, B. (1982) Nature 300, 643–645.
[22] Mann, M., Meng, C.K. and Fenn, J.B. (1989) Anal. Chem. 61, 1702–1708.