Characterization of the Binding Site of the Tripeptide Intermediate d-Phenylalanyl-L-Prolyl-L-Valine in Gramicidin S Biosynthesis*

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The tripeptide intermediate d-Phe-Pro-Val in the biosynthesis of gramicidin S was labeled by incorporation of either L-[1-14C]phenylalanine or L-[1-14C]valine in an in vitro biosynthetic assay. The gramicidin S synthetase 2-tripeptide complex was first digested with CNBr and subsequently by Staphylococcus aureus V8 protease. The active site peptide carrying the radioactively labeled tripeptide was isolated in pure form by reversed phase high performance liquid chromatography technology and analyzed by liquid phase sequencing, mass spectrometry, and amino acid analysis. It was demonstrated that d-Phe-Pro-Val is attached to the 4'-phosphopantetheine cofactor at the thiolation center for valine of gramicidin S synthetase 2. In this way the attachment site of a peptide intermediate in nonribosomal peptide biosynthesis was identified for the first time. Our results are in full agreement with the multiple carrier model of nonribosomal peptide biosynthesis (Stein, T., Vater, J., Kruft, V., Otto, A., Wittmann-Liebold, B., Franke, P., Panico, M., McDowell, R., and Morris, H. R. (1996) J. Biol. Chem. 271, 15426–15435), which predicts that the growing peptide chain in the elongation process should always be bound to the thiotemplate site specific for its C-terminal amino acid component.

Multifunctional peptide synthetases like gramicidin S synthetase from Bacillus brevis ATCC 9999 catalyze the biosynthesis of their peptide products by a thiotemplate mechanism (1–5). They activate their amino acid substrates in a two-step process involving aminoacyl adenylate formation and subsequent thioesterification at specific thiol groups (thiotemplates). Such multienzymes show a modular organization (6–9). They are composed of homologous biosynthetic units (modules) consisting of 1000–1500 amino acid residues. Their arrangement along the multifunctional polypeptide chain is usually colinear to the sequence of the amino acid components in the peptide product. Such modules can be subdivided into three main domains responsible (a) for amino acid recognition, binding, and the primary activation as aminoacyl adenylates, (b) for the thiolation of the amino acid substrates, and (c) for peptide elongation, in some cases in concert with amino acid epimerization.

Recently we demonstrated by affinity labeling of gramicidin S synthetase at its thiolation centers and analysis of isolated active site peptides that each module of this multienzyme is equipped with its own 4'-phosphopantetheine (4'-PPan) cofactor (5). The sulfhydryl group of the cofactors cysteine component functions as the thiotemplate site for the specific amino acid substrate. Each of these mobile 4'-PPan carriers is connected in a phosphodiester linkage with a specific serine that is part of a strictly conserved consensus motif LGG/H/D/S(L/I). On the basis of these results we developed a multiple carrier model of nonribosomal peptide biosynthesis (5). This new concept replaces the old version of the thiotemplate mechanism, which was proposed by several groups in the early seventies (1–4). The old model postulated a single central 4'-PPan carrier for peptide elongation that was assumed to interact during the elongation cycle with peripheral thiols charged with the amino acid substrates in thioester linkage.

According to our new concept the growing peptide chain is assembled in a series of transpeptidation reactions by successive unidirectional interaction of the individual 4'-PPan carriers thioesterified with substrates or peptide intermediates. After transpeptidation the elongated peptide chain should be attached to the 4'-PPan cofactor specific for the binding of the C-terminal amino acid. This means that in the case of gramicidin S synthetase, for example, the di-, tri-, and tetrapeptide intermediate would be bound as thioesters to the 4'-PPan cofactor at the proline, valine, and ornithine reaction centers, respectively. Unfortunately, the d-Phe-Pro-dipeptide and the d-Phe-Pro-Val-Orn-tetrapeptide complexes of gramicidin S synthetase 2 (GS2) are unstable because of internal cyclization reactions (10–12). On the other hand the GS2-d-Phe-Pro-Va-tripeptide intermediate shows an exceptionally high stability (13). Therefore, this species is optimally qualified to solve this question. In this communication we have identified the d-Phe-Pro-Val-attachment site of gramicidin S synthetase 2. This research is of general interest to understand the mechanism of nonribosomal peptide biosynthesis on the molecular level.

EXPERIMENTAL PROCEDURES

Materials—L-[1-14C]Valine and L-[1-14C]phenylalanine were purchased from Amersham/Buchler (Braunschweig, Germany). Staphylococcus aureus V8 protease and cyanogen bromide were obtained from Miles/Bayer Diagnostics (München, Germany) and Sigma (Deisenhofen, Germany). B. brevis ATCC 9999 was cultivated in a fumurate/medium, as reported by Chiu (14).

Enzyme Purification and Assays—GS1 and GS2 were purified as published by Vater et al. (12). Assays for thioester formation of GS2
with substrate amino acids (11, 15) and biosynthesis of gramicidin S (12, 16) were performed as described previously. The protein concentration was determined according to Bradford (17).

Specific Labeling of the Binding Site of GS2 for the Tripeptide Intermediate D-Phe-Pro-Val—For labeling experiments 500 μg of GS2 and 160 μg of GS1 were used in a volume of 2 ml. The reaction mixture contained 10 μM phenylalanine, 10 μM proline, and 10 μM valine, 2 mM ATP, 1 mM EDTA in 10 mM sodium phosphate buffer, pH 7.2. For labeling either [14C]phenylalanine or [14C]valine was used. The reaction mixture was incubated for 30 min at 37 °C. The protein was precipitated during 60 min on ice at a trichloroacetic acid concentration of 7% (w/v). The precipitated protein was collected by centrifugation, and nonbound radioactivity was removed by consecutive washing three times with 1 ml of 5% (w/v) trichloroacetic acid and 1 ml 100% ethanol, respectively. The precipitate was then dried in a Speed Vac concentrator.

Analysis of Enzyme-bound Peptide Intermediates by Thin Layer Chromatography—For TLC analysis of the enzyme-bound peptide, the dried precipitate was dissolved in 250 μl of 0.1 N NaOH. The mixture was incubated 15 min at room temperature. After neutralization with 250 μl of 0.1 N HCl, the released peptide intermediates were extracted three times with 350 μl of n-butanol. The butanolic extract was dried in a Speed Vac concentrator and dissolved in 20 μl of n-butanol. 10 μl of this solution were applied to a silica gel DC 60 plate. Butanol:acetic acid:water (4:1:1) was used as mobile phase. Radioactive spots were detected by radio scanning with an automatic TLC linear analyzer TraceMaster 20 (Berthold, Germany).

Separation and Analysis of 14C-Labeled Peptides by Reversed Phase HPLC—Fragmentation of 500 μg of the GS2-[14C]tripeptide complex with cyanogen bromide was carried out as described by Schlumberg et al. (18). Subsequently 14C-labeled cyanogen bromide fragments of GS2 were digested with S. aureus V8 protease as reported previously (19). Peptide fragments were separated by reversed phase HPLC using a LKB system.

Cyanogen bromide fragments were dissolved in 50% (v/v) acetic acid loaded onto a Bio-Rad BioSil 304-10 column (C4-alkyl chains) and eluted with a linear gradient of acetonitrile. Solvent A was 0.1% trifluoroacetic acid in H2O (v/v). Eluent B was acetonitrile containing 0.1% trifluoroacetic acid (v/v). The flow rate was 0.75 ml/min. S. aureus V8 protease fragments were dissolved in 500 μl of 10 mM sodium phosphate, pH 2.5, and loaded onto a Bio-Rad BioSil 318-10 column (C18-alkyl chains). Fractions were eluted with a linear gradient of acetonitrile. Solvent A was 10 mM sodium phosphate in H2O, pH 2.5; Eluent B contained 40% solvent A and 60% acetonitrile (v/v). The flow rate was 0.75 ml/min. In the final purification step S. aureus V8 protease fragments were dissolved in 0.1% trifluoroacetic acid in H2O, loaded onto a μRPC SC 2.1/10 column (C2/C18-alkyl chains), and eluted with a linear gradient of acetonitrile on a Amersham Pharmacia Biotech Smart System. Solvent A was 0.1% trifluoroacetic acid in H2O (v/v). Eluent B was acetonitrile containing 0.08% trifluoroacetic acid in acetonitrile (v/v). The flow rate was 50 μl/min. Peptides were detected by measuring the absorbance at 214 nm. 14C-Labeled peptides were monitored by scintillation counting of 4–10 μl aliquots of each fraction.

Peptide sequences were determined on an Applied Biosystems Protein Sequencer. Samples were dissolved in 100% methanol and adsorbed to a polybrene-coated glass filter treated with trifluoroacetic acid that had been precycled as described by Hewick et al. (20). Amino acid analysis of 80 pmol of the active site peptide was performed as described elsewhere (19).

Matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded on a Bruker Reflex MALDI-TOF instrument containing a 337-nm nitrogen laser for desorption and ionization. Active site peptides were analyzed in a saturated α-cyano-4-hydroxycinnamic acid matrix solubilized in 30% acetonitrile in water containing 0.1% trifluoroacetic acid (v/v). Ions were accelerated with a voltage of 20 kV. The positive ion detection and reflector mode was used.

RESULTS AND DISCUSSION

As demonstrated in our previous studies each amino acid activating module of a peptide synthetase using the thiotemplate mechanism is equipped with its own 4'-PcPan cofactor that functions as the thiolation site for the cognate amino acids substrate (5, 19). On the basis of these results a multiple carrier model was proposed that claims that the growing peptide chain is assembled during the elongation cycle in a series of transpeptidation steps. Each peptide intermediate should be bound to the 4'-PcPan carrier of that module, which is responsible for the thiolation of the C-terminal amino acid component. To verify these predictions we investigated the binding site of GS2 for the tripeptide D-phenylalanyl-prolyl-valine. This intermediate is well suited for such experiments, because of the high stability of its thioester bond with the corresponding reaction center of GS2 (13). For this purpose we labeled gramicidin S synthetase with the radioactive tripeptide as outlined under “Experimental Procedures.” In control experiments L-proline was omitted from the tripeptide reaction mixture. In this case elongation was interrupted and L-[14C]Phe and L-[14C]Val were incorporated into GS1 and GS2 as thioesters, respectively (A and C). The free amino acids were analyzed under the same conditions as the tripeptide.

Peptide formation was monitored by TLC on silica gel DC 60 plates at Rf 0.72 with butanol:acetic acid:water (4:1:1) as the mobile phase (Fig. 1). Control samples contained the same reaction mixture from which L-proline was omitted. Under these conditions phenylalanine and valine were incorporated into GS as thioesters. After cleavage with alkali, they were detected in free form on the TLC plates instead of the tripeptide at Rf 0.62 and Rf 0.52, respectively. In the case of [14C]phenylalanine as tracer free phenylalanine was detected in addition to the tripeptide, which was released from GS1 by hydrolysis.

To investigate the binding site of the tripeptide intermediate to gramicidin S synthetase 2, the GS2-4'-PcPan-thioester complex was digested with CNBr and after fractionation of the CNBr peptides subsequently by S. aureus V8 protease. The active site peptide bearing the radiolabeled tripeptide was isolated in pure form by a three-step reversed phase HPLC purification protocol as described under “Experimental Procedures.” Because the thioester bond is unstable at neutral and alkaline pH, all cleavage and purification steps had to be performed in acidic medium (Fig. 2).

By liquid phase sequencing of the purified radiolabeled ac-
The sequence shown in the upper row corresponds to the active site peptide of the thiolation center of GS2 for valine, which can be discriminated from the other thioester binding sites of gramicidin S synthetase by its arginine in position 7 instead of a lysine. This site was previously characterized by affinity labeling and analysis of the isolated peptide fragment (5, 19) with the exception that in position 5 a dehydroalanine was found instead of a serine as derived from the gene sequence (21). In previous experiments it has been demonstrated that this modification originates from an elimination reaction at the active site serine because of the alkaline conditions during Edman degradation (5, 19). Furthermore we would expect to find a methionine in position 9 of this peptide, which we could not detect. This is probably because of its conversion to the homoserine lactone during CNBr fragmentation.

In the first two Edman degradation steps, in addition to L-\[14C\]phenylalanine and G, F and P were detected, indicating that the FPV tripeptide was in fact attached to our active site peptide. If L-\[14C\]phenylalanine was used, the tracer quantitatively eluted in the first Edman degradation step. In the case of L-\[14C\]valine the radiolabel appeared in the third step. However, the degradation product was not identical with the phenylthiohydantoin derivative of valine. Presumably valine was linked to a 4'-PPan carrier via a thioester bond.

The structure of the active site peptide was determined by interpretation of the fragmentation data (Fig. 3) according to MALDI mass spectrometry (Fig. 3A). In the linear mode we found an intensive signal at \(m/z = 1587.0\) together with the fragment ions. Representative signals were found at \(m/z = 974.1\) and 875.9, which could be attributed to the phosphorylated and the dephosphorylated, dehydrated active site peptide. The structure of the dephosphorylated, dehydrated active site nonapeptide \(M_2\) derived from the fragment signals that represent series of N- and C-terminal sequence ions. Masses of histidine-directed internal fragment ions indicated by an asterisk were also used for sequence determination.

To identify the mode of attachment of the phenylalanyl-prolyl-valine intermediate at the thiolation site of GS2 for L-valine, we investigated the isolated active site peptide by MALDI mass spectrometry (Fig. 3A). In the linear mode we found an intensive signal at \(m/z = 1587.0\) for the quasi-molecular ion \(M + H^+\) of this peptide containing L-\[14C\]valine as tracer. Its mass is consistent with the sum of the masses calculated (a) for the active site peptide comprising Leu-2037 to Met-2045 (m/z = 1587.0) and (b) for a 4'-PPan cofactor (m/z = 340.3), and (c) for the tripeptide intermediate phenylalanyl-prolyl-valine (m/z = 352.4 with L-\[14C\]valine as the label). A second signal appeared at \(m/z = 876.4\) that corresponds to the mass of a valine active site peptide of GS2 from which the 4'-PPan-tripeptide adduct was eliminated, converting the serine at position 2041 to a dehydroalanine.

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rules defined by Morris et al. (22, 23) and Biemann (24). The fragmentation pattern of the parent ion at \( m/z = 1587.0 \) comprised a few series of N- and C-terminal (A, B, C, X, Y, and Z ions) as well as histidine-directed internal sequence ions (A*, B*, C* ions), resulting in the amino acid sequence shown in Fig. 3B. The dominant fragments are found at \( m/z \) ratios of 974.1 and 875.9. They correspond to the mass values of the phosphorylated and the dephosphorylated, dehydrated form of the active site peptide, respectively, with a dehydroalanine in position 5. The mass at \( m/z = 893.8 \) can be assigned to the dephosphorylated form of the active site peptide with serine in position 5. Furthermore the mass at \( m/z = 120.6 \) was attributed to the immonium ions of phenylalanine. The B ion of the dipeptide phenylalanyl-proline was detected at an \( m/z \) ratio of 245.8. The mass at \( m/z = 18014 \) can be assigned to the dephosphorylated Pan-valyl-prolyl-phenylalanine adduct. We also analyzed the fragment pattern of the ion at \( m/z = 876.4 \). It is remarkably similar to that of the parent ion at \( m/z = 1587.0 \) and yields the same amino acid sequence (Fig. 3).

The results obtained by amino acid analysis agree well with our sequence data. The active site peptide contained 1 mol each of \( \beta \)-alanine and taurine, the latter being an oxidation product of cysteamine corroborating the presence of an attached 4'-phosphopantetheine cofactor of the thio-methylase for one of its peptide intermediates for the first time. Our results are in full agreement with the prediction of the multiple carrier model that the growing peptide chain in the elongation process is attached to the thio-template site of its C-terminal amino acid component. This work represents a fundamental contribution to the understanding of the mechanism of nonribosomal peptide biosynthesis.

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