Synthesis of Linear Gramicidin Requires the Cooperation of Two Independent Reductases†

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ABSTRACT: The linear pentadecapeptide gramicidin has been reported to be assembled by four large multimodular nonribosomal peptide synthetases (NRPSs), LgrABCD, that comprise 16 modules. During biosynthesis, the N-formylated 16mer peptide is bound to the peptidyl carrier protein (PCP) of the terminal module via a thioester bond to the carboxyl group of the last amino acid glycine$_{16}$. In a first reaction the peptide is released from the protein template in an NAD(P)H-dependent reduction step catalyzed by the adjacent reductase forming an aldehyde intermediate. Here we present the biochemical proof that this aldehyde intermediate is further reduced by an aldoreductase, LgrE, in an NADPH-dependent manner to form the final product gramicidin A, *N*-formyl-pentadecapeptide-ethanolamine. To determine the potential use of the two reductases in the construction of hybrid NRPSs, we have tested their ability to accept a variety of different substrates in vitro. The results obtained give way to a broad spectrum of possible use.

Linear gramicidin is one of the most intensivley studied peptide antibiotics as it forms an ion channel which is widely used as a model system. The peptide consists of 15 hydrophobic amino acids with alternating L- and D-configuration. The primary sequence of gramicidin A is formyl-Val$_1$-Gly$_2$-Ala$_3$-D-Leu$_4$-Ala$_5$-D-Val$_6$-Val$_7$-D-Val$_8$-Trp$_9$-D-Leu$_{10}$-Trp$_{11}$-D-Leu$_{12}$-Trp$_{13}$-D-Leu$_{14}$-Trp$_{15}$-ethanolamine (*1*). The tryptophan at position 11 is variable, leading to the isoforms gramicidin B (tryptophan → phenylalanine, 5%) and gramicidin C (tryptophan → tyrosine, 15%; (*2*)). In all three isoforms, an isoleucine instead of a valine at position 1 has been described (∼5%; (*1*)). Striking features are the modifications of the N- and C-termini which presumably prevent degradation of the linear peptide by proteases in vivo. The N-terminal amino acid valine is N-formylated, and the C-terminus of the peptide is composed of ethanolamine. The secondary structure of the peptide is a β-helix where all residues point outward, forming an ion channel. Gramicidin forms helical or head-to-head homodimers and inserts itself into lipid bilayers where it allows singly-charged cations to pass through with a speed close to diffusion in water (*3*). As a result, the transmembrane potential collapses, leading to cell death.

Recently, a nonribosomal peptide synthetase of *Bacillus brevis* ATCC 8185 has been identified, sequenced, and shown to be responsible for the synthesis of gramicidin (*4*). Nonribosomal peptide synthetases (NRPSs)[1] have been extensively studied in the past decades, as many of their products possess important bioactive properties, e.g. penicillin, cyclosporine, or bleomycin. NRPSs are multifunctional enzymes that carry out several reactions in a specific and coordinated manner. As substrates, a wide variety of carboxyl or amino acids including nonproteinogenic ones are accepted and incorporated into the peptide chain (*5, 6*). As these building blocks are sometimes additionally modified during and after the nonribosomal peptide synthesis, e.g. by N-methylation, epimerization into the D-form or heterocyclization, the products possess an extremely broad structural diversity (*7*). This diversity is often crucial for the bioactivity of the compounds, prominent examples are vancomycin and bacitracin.

In linear types of NRPSs as defined by Mootz et al., the number of modules and thus incorporated residues is identical to the number of building blocks found in the final product (*8*); this mechanism is comparable to an assembly line. Modules can be subdivided into domains that harbor the catalytic activities for substrate recognition and activation (adenylation domain; A domain (*9, 10*)), covalent loading (peptidyl carrier protein; PCP (*11, 12*)), and peptide bond formation (condensation domain; C domain (*13, 14*)). Sometimes modifying domains are included in a module, e.g. epimerization domains are commonly found in modules that are responsible for the incorporation of D-amino acids (*15, 16*); N-methylation, heterocycle formation, and oxidations have been described as well (*17, 18*). In most cases, a thioesterase domain attached to the last module is responsible for product release either as cyclic, branched-cyclic, or linear product (*19, 20*). The gramicidin NRPS consists of 16 modules distributed on four multienzymes (LgrABCD) that harbor 56 domains including a putative N-formyltransferase domain at the N-terminus of LgrA and a rare reductase domain (R domain) instead of a thioesterase at the C-terminus of LgrD (see Figure 1; (*4*)). So far, only three R domains of

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[1] Abbreviations: NRPS, nonribosomal peptide synthetase; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PCP, peptidyl carrier protein; R domain, reductase domain; ORF, open reading frame; 4′-Ppant, 4′-phosphopantetheinyl.

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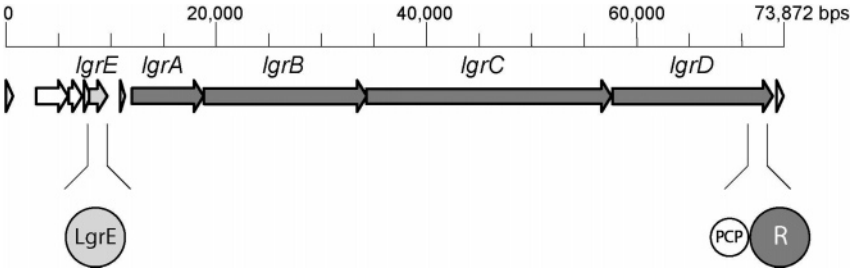

FIGURE 1: The *lgr*-gene cluster (accession no. AJ566197) from *Bacillus brevis* ATCC 8185. The ORF *lgrE*, encoding a putative oxidoreductase, is located about 3 kbps upstream of the *lgrABCD* genes that encode the gramicidin NRPS. The enzymes used in this study, PCP-R and LgrE, are indicated.

NRPS have been described, namely SafA from *Myxococcus xanthus* (*21*), MxcG from *Stigmatella aurantiaca* Sg a 15 (*22*), and Lys2 from fungi, e.g. from *Saccharomyces cerevisiae* (*23*). Lys2 and presumably SafA perform a one-step reduction to an aldehyde intermediate whereas MxcG has been shown to perform a two-step reduction to the alcohol product. The R domain shows high homology to all three enzymes, namely 36% identity, 51% similarity to SafA, 32% identity, 48% similarity to MxcG and 32% identity, 50% similarity to Lys2 (*4*).

We previously showed that the premature gramicidin is released from the terminal module of the NRPS by this R domain (*4*). However, this reductive release lead only to the formation of the aldehyde intermediate (2-aminoethanal). The question remains how this intermediate is transformed into the final product carrying a C-terminal 2-aminoethanol. We wondered if a second reductase might be encoded close to the *lgr*-gene cluster which is responsible for this product conversion.

Here, we clearly demonstrate that the aldoreductase LgrE is responsible for the reduction of the aldehyde intermediate to the final product gramicidin in a reaction that is NADPH-dependent. In addition, we have synthesized a variety of modified substrates to test the ability of both reductases to accept other peptides.

EXPERIMENTAL PROCEDURES

General Methods. All oligonucleotides used in this study were purchased from Operon (Cologne, Germany). Cells were usually grown in LB-Medium at 37 °C overnight for plasmid and chromosomal DNA preparations. Plasmid preparations were carried out using the QIAprep spin Miniprep Kit (Qiagen, Germany). Chromosomal DNA was prepared using the QIAGEN Genomic tip 100/G columns according to the manufacturer's manual. Blast searches were carried out using either the blastx or blastp function at the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/, (*24*)). Multiple sequence alignments were made using MegAlign (DNAStar, GATC Biotech, Germany).

Cloning, Overproduction, and Purification of PCP-R and LgrE. Primers used for the PCR reactions were 5′-cac cca aac gcc agt gga aat cat g-3′ and 5′-gtt ccg tgt att ttg tgt cac-3′ for PCP-R and 5′-cac cat gat tac agg aaa agc gac ac-3′ and 5′-cct cgc gcc aat gac ggc-3′ for LgrE. Chromosomal DNA from *Bacillus brevis* ATCC 8185 was used as template. Both constructs were cloned using the pBAD directional TOPO Kit (Invitrogen) as described in the manual. The corresponding vectors were transformed into *E.* coli BL21 (DE3), and the desired proteins were overproduced as described in the manual, using LB-medium supplied with kanamycin (50 μg/mL final concentration). The recombinant proteins carried an N-terminal thioredoxin-fusion protein and a C-terminal V-epitope with His_6-Tag and were purified using Ni^{2+}-NTA affinity chromatography as described before. Purity of the proteins was controlled via SDS−PAGE and PCP-R and LgrE were concentrated using Vivaspin 20 mL concentrators (membrane 50 000 MWCO PES; purchased from Vivascience/Sartorius) to a concentration of about 3−5 mg/mL.

Substrate Synthesis. The peptidyl-CoA substrate were prepared as follows. Peptide synthesis was carried out on an Advanced ChemTech APEX 396 synthesizer (0.1 mmol scale) by using 2-chlorotrityl resin (IRIS biotech, Marktredwitz, Germany) as described before (*25*). All 9-fluorenylmethoxycarbonyl (Fmoc)-amino acids and coupling reagents were purchased from IRIS biotech and Novabiochem (Schwalbach, Germany). 25 μmol peptide was used during CoA-modification. 1 equiv of CoA, 4 equiv of K_2CO_3, and 1.5 equiv of PyBop were added in 2 mL of 50% tetrahydrofuran and stirred for 4 h at RT. The reaction was then frozen in liquid N_2 and lyophilized overnight. Afterward a mixture of 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% H_2O was added, and the reaction was stirred for 3 h at RT to deprotect the side groups. The reaction mixture was precipitated by the addition of 30 mL of ice cold diethyl ether and centrifuged for 5 min at 4.000 rpm/4 °C. The pellet was resolved in 20−35% MeCN depending on the solubility of the substrate and purified using an Agilent 1100 preparative system. The purified products were analyzed by MALDI-TOF mass spectrometry and lyophilized overnight. Substrates were then dissolved in dimethyl sulfoxide to a concentration of 10 mM.

Photometric Assay with the R Domain. The purified PCP-R enzyme was dialyzed against a buffer containing 50 mM Hepes, 50 mM NaCl, pH 7. The assay contained the following ingredients: 30 μM PCP-R, 2.5 μM Sfp, 50 μM substrate, 150 μM NADPH, 5 mM $MgCl_2$, 5 μM $MnCl_2$. The volume was adjusted to 200 μL using a buffer containing 50 mM Hepes, 50 mM NaCl, pH 6 in a quarz cuvette. The control lacked both Sfp and the substrate. Standard solutions with NADPH concentrations varying from 0 to 200 μmol were used for comparison of the ΔE values. All measurements were carried out at 340 nm in a photometer.

R Domain and LgrE Assays. The purified PCP-R and LgrE enzymes were dialyzed against a buffer containing 50 mM Hepes, 50 mM NaCl, pH 7. The assay contained the following ingredients: 60 μM PCP-R, 10μM LgrE, 5 μM Sfp, 100 μM substrate, 300 μM NAD(P)H, 10 mM $MgCl_2$,

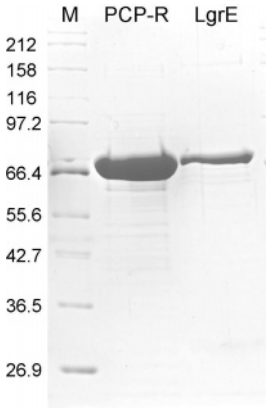

FIGURE 2: PCP-R (70.1 kDa) and LgrE (76 kDa) after Ni^{2+}-NTA-purification. M, marker (kDa).

10 μM $MnCl_2$. The volume was adjusted to 100 μL using a buffer containing 50 mM Hepes, 50 mM NaCl, pH 6. Controls lacked either PCP-R, LgrE, Sfp, substrate or NAD(P)H. The assay of LgrE with the purified aldehyde substrate contained 10 μM LgrE, 100 μM aldehyde, and either 300 μM NADH, 300 μM NADPH, or no cofactor in a buffer containing 50 mM Hepes, 50 mM NaCl, pH 6. All assays were thoroughly mixed and incubated at 25 °C for 30 min. The reactions were stopped by addition of 1 mL MeOH, kept on ice for >30 min, and then centrifuged at maximum speed in a conventional tabletop centrifuge for 30 min. The solvent from the supernatant was then removed under vacuum at 30 °C to dryness and the pellet resuspended in 100 μL of 50% methanol containing 0.05% formic acid and analyzed using HPLC (1100 series, Agilent, Germany) coupled with a 1100 MSD-A ESI-Quadrupol mass spectrometer (Agilent, Germany). Samples (95 μL each) were applied to a 250/2-Nucleodur-C18−Pyramid column (Macherey-Nagel, Germany) with a particle diameter of 5 μm. The gradient of solvent A (water, 0.05% formic acid) and solvent B (acetonitrile, 0.045% formic acid) used was as follows: Linear from 15% B to 70% B within 40 min, increasing to 95% B within 5 min and holding 95% B for additional 5 min at a flow rate of 0.3 mL/min and a column temperature of 50 °C. UV detection was carried out at 215 nm. MSD parameters were as follows: Mass range was set from 650 to 850 amu in positive ion mode, the gain was set to 3.0 and the fragmentor to 60. The drying gas flow (N_2) was 13 L/min, the nebulizer pressure 35 psig, the drying gas temperature 350 °C, and the capillary voltage 4300 V.

RESULTS

The R Domain. Following the experiment carried out by us before, we synthesized the natural substrate mimic Ac-DLeu$_{12}$-LTrp$_{13}$-DLeu$_{14}$-LTrp$_{15}$-Gly$_{16}$-S-CoA and tested both NADPH and NADH as potential electron donors. We cloned, overproduced, and purified the last two domains of LgrD, PCP-R, for further biochemical investigation (see Figure 2).

The 4′-phosphopantetheinyl (4′Ppant)-transferase Sfp uses Coenzyme A (CoA) to convert PCPs from the *apo*- to the *holo*-form by attaching the 4′-Ppant moiety onto a highly conserved serine residue, thereby releasing 3′,5′-AMP. It has been shown that Sfp is rather unspecific and that it accepts various peptidyl-CoAs as substrates (*26*). In the assays, we substituted CoA for the desired peptidyl-CoA substrates, which resulted in preloaded PCPs upon conversion into the *holo*-form. The preloaded PCPs can now serve directly as substrates for the adjacent R domain, mimicking the natural environment of this enzyme.

The assays showed that the R domain is capable of using both NADPH and NADH without detectable differences. Analysis of the assays using LCMS showed the same peaks in all assays (see Figure 3). Using positive mode, we detected a peak with mass 700.4 (aldehyde, calculated M + H^+ 700.2), 716.4 (hydrolysis of CoA-substrate, calculated M + H^+ 716.2) and 730.4 (methanolysis of excess CoA-substrate, calculated M + H^+ 730.2). We repeated the assay several times, and in all cases we have been unable to detect any alcohol formation.

Even though our experimental setup was based only on a single turnover reaction, we determined the time-dependency of the reaction. As NADPH is converted into $NADP^+$ during the reaction, we could observe a change in extinction at 340 nm in photometric assays and calculated the amount of NADPH used by comparison to standard solutions. When only the enzyme PCP-R and NADPH were present (no substrate and Sfp), we observed a decrease of NADPH of 32 μmol, corresponding to a molar ratio of enzyme: NADPH usage of 1:1.07 (see Figure 4a). This showed the saturation of each enzyme molecule with one molecule of NADPH. When we performed the assay with substrate and Sfp added, we detected a decrease of NADPH of 68 μmol, and the molar ratio of enzyme: NADPH usage was 1:2.27 (see Figure 4b). This showed the usage of 1 molecule of NADPH per enzyme molecule in the reaction in addition to the re-saturation of the protein with NADPH afterward.

To furthermore investigate the substrate specificity of the R domain, we then synthesized several other substrates to test the influence of the C-terminal amino acid upon selectivity (see Table 1). We exchanged the glycine$_{16}$ residue of the peptidyl-CoA-substrate for L-leucine, L-alanine, L-aspartate, L-lysine, and L-phenylalanine. In the experiments, we observed that all substrates despite their chemical diversity were accepted by the R domain and reduced to the corresponding aldehydes (see Figure 5). In addition, we have also synthesized the substrates Ac-DLeu$_{12}$-**D**Trp$_{13}$-DLeu$_{14}$-**D**Trp$_{15}$-Gly$_{16}$-S-CoA and Ac-**L**Leu$_{12}$-LTrp$_{13}$-**L**Leu$_{14}$-LTrp$_{15}$-Gly$_{16}$-S-CoA to determine the importance of the L-D configuration for substrate recognition. Again, our assays showed that both substrates were reduced to the aldehyde intermediates.

When we substituted the C-terminal glycine$_{16}$ residue with D-alanine$_{16}$, D-phenylalanine$_{16}$, or D-leucine$_{16}$, we found that in the assay containing Ac-DLeu$_{12}$-LTrp$_{13}$-DLeu$_{14}$-LTrp$_{15}$-**D**Ala$_{16}$-S-CoA, the substrate bound on the PCP was accepted and reduced to the corresponding aldehyde. We were unable to detect any aldehyde production in the case of the other two substrates, Ac-DLeu$_{12}$-LTrp$_{13}$-DLeu$_{14}$-LTrp$_{15}$-**D**Phe$_{16}$-S-CoA and Ac-DLeu$_{12}$-LTrp$_{13}$-DLeu$_{14}$-LTrp$_{15}$-**D**Leu$_{16}$-S-CoA.

Surprisingly, in the cases of all aldehyde mass spectra (also for the native substrate mimic) we observed an additional mass signal differing from the singly protonated aldehyde (M + H^+) by 40 mass units and from the M + Na^+ signal by 18 mass units (see Figures 3 and 5). Tandem mass spectrometry of this compound revealed the neutral loss of water at low collision energies. The signal disappeared altogether with the aldehyde upon addition of LgrE and a

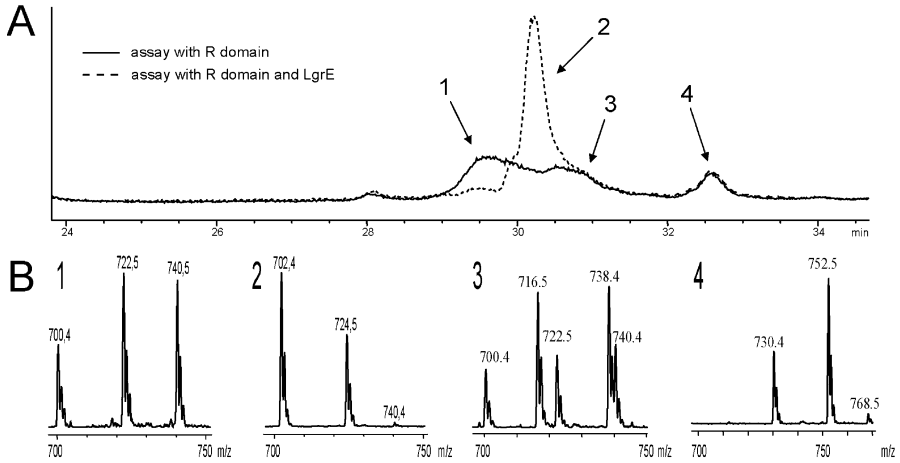

FIGURE 3: The total ion chromatogram (A) and the corresponding mass spectra (B) of the R domain assay with and without LgrE (straight and dotted lines, respectively). The mass spectra shown correspond to (1) aldehyde, calculated M + H^+ 700.2; (2) alcohol, calculated M + H^+ 702.2; (3) hydrolysis of substrate, calculated M + H^+ 716.2; (4) methanolysis of substrate, calcuclated M + H^+ 730.2. Peak 2 is only present upon addition of LgrE whereas peak 1 disappears under these conditions.

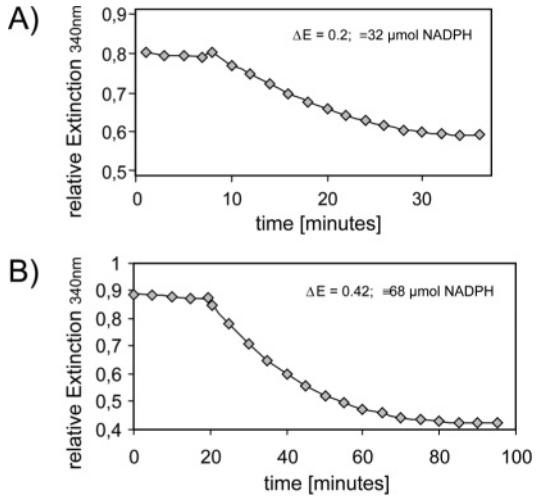

FIGURE 4: Photometric measurements of the R domain reaction at 340 nm. A. Without substrate and Sfp, approximately 1 molecule of NADPH per molecule enzyme is used. B. Upon addition of substrate and Sfp, about two molecules of NADPH per molecule enzyme are used during the course of the reaction.

comparable mass shift was not observed for the corresponding alcohols. Therefore, we conclude that a water molecule is to some extent reversibly added to the aldehyde function at the C-termini of the aldehydes during ionization as has been described before for peptide aldehydes (*27*).

The Second Reductase LgrE. As the R domain reduces the substrate mimic and its derivatives only to the aldehyde intermediate, it appeared logical that a second reductase would perform the last step, reducing the aldehyde intermediate to the alcohol. We found a putative oxidoreductase of the aldo-/ketoreductase family encoded by an open reading frame approximately 3 kbps upstream of the gene cluster *lgrABCD*, the four NRPS genes responsible for the biosynthesis of gramicidin (see Figure 1). A blastP search of the NCBI database showed high sequence homology to many putative or predicted oxidoreductases of the aldo-/ketoreductase family (28−33% identity, 47−52% similarity), but none of these has been characterized biochemically before.

We cloned, overproduced, and purified the putative oxidoreductase for further biochemical investigations (see Figure 2). At first, we used a coupled assay where we added LgrE to the assay described above for the first reductase. We expected the aldehyde intermediate to be completely converted into the corresponding alcohol. We found that LgrE was capable of performing this reaction as predicted when NADPH was present in the coupled reaction assay (see Figure 3). LCMS analysis in positive mode showed that the peak with mass 700.4 (aldehyde, calculated M + H^+ 700.2) disappeared, whereas a new peak with mass 702.4 (alcohol, calculated M + H^+ 702.2) appeared.

When we used NADH as sole electron donor in the assay, the level of aldehyde produced by the R domain remained unaltered in the presence of LgrE with no traces of the alcohol product. These results were strong hints toward a strict NADPH-dependence of the aldoreductase. In addition, these findings demonstrate that it is not a possible binding of LgrE to the R domain that facilitates the reduction of the aldehyde to the alcohol by the R domain itself; instead this shows that the catalytic activity of the aldoreductase LgrE is needed for the conversion of the aldehyde to the alcohol.

To test LgrE independently of a coupled reaction assay, we tried to chemically synthesize the aldehyde intermediate. Even though we tried different methods, we were unsuccessful in synthesizing the desired aldehyde substrate. Therefore, we performed a 75-fold upscale of the reaction with the R domain and the native substrate mimic and purified the aldehyde intermediate via HPLC. Using this enzymatic method, the total amount was very low (~95 μg of pure aldehyde) but sufficient for the following assay. We added LgrE to the purified aldehyde and either NADPH or NADH as described in Experimental Procedures. LCMS analysis showed that only in the presence of NADPH was the aldehyde completely reduced to the alcohol, whereas in the presence of NADH the substrate remained unaltered, giving final proof to the assumption that LgrE is strictly NADPH-dependent. Furthermore, this clearly demonstrates that LgrE recognizes the peptide-aldehyde precursor also in the absence of the NRPS template.

In a second step, we tested the other substrates we had synthesized in coupled assays (see Figure 5 and Table 1). We found that LgrE converted most of the produced aldehydes completely when the substrates Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-**LLeu_{16}**-S-R, Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-

Table 1: Substrates Used in This Study. Summary of the Results of the Assays

substrates bound on PCP	acceptance by NRPS reductase	aldehyde substrates[a]	acceptance by LgrE
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-Gly_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-Gly_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LAla_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LAla_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LLeu_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LLeu_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LAsp_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LAsp_{16}*	~5%
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LLys_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LLys_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LPhe_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-LPhe_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DAla_{16}-S-PCP	+	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DAla_{16}*	+
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DLeu_{16}-S-PCP	−	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DLeu_{16}*	n.d.[b]
Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DPhe_{16}-S-PCP	−	Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-DPhe_{16}*	n.d.
Ac-LLeu_{12}-LTrp_{13}-LLeu_{14}-LTrp_{15}-Gly_{16}-S-PCP	+	Ac-LLeu_{12}-LTrp_{13}-LLeu_{14}-LTrp_{15}-Gly_{16}*	+
Ac-DLeu_{12}-DTrp_{13}-DLeu_{14}-DTrp_{15}-Gly_{16}-S-PCP	+	Ac-DLeu_{12}-DTrp_{13}-DLeu_{14}-DTrp_{15}-Gly_{16}*	~50%

[a] * C-terminal amino acid is reduced to the corresponding aldehyde. [b] n.d., not determined.

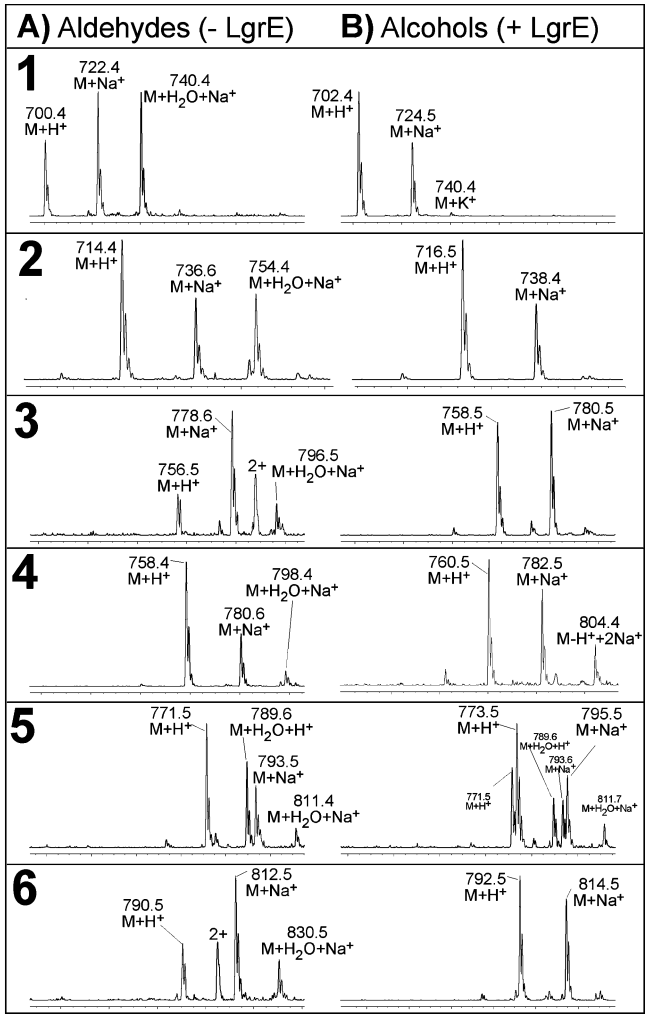

FIGURE 5: Mass spectra of the aldehyde (A) and alcohol (B) products formed by the R domain only and the R domain with LgrE, respectively. Substrates used were Ac-LLeu_{12}-LTrp_{13}-LLeu_{14}-LTrp_{15}-X_{16}-S-CoA with X being **1** − Gly, **2** − LAla, **3** − LLeu, **4** − LAsp, **5** − LLys, **6** − LPhe. 2^+, doubly charged impurity.

L$\mathbf{Lys}_{16}$-S-R, Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-**L**$\mathbf{Phe}_{16}$-S-R, Ac-**L**$\mathbf{Leu}_{12}$-LTrp_{13}-**L**$\mathbf{Leu}_{14}$-LTrp_{15}-Gly_{16}-S-R, Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-**L**$\mathbf{Ala}_{16}$-S-R, or Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-**D**$\mathbf{Ala}_{16}$-S-R were used. However, two substrates were not accepted efficiently. One was the substrate where $glycine_{16}$ had been replaced by L-aspartate, Ac-DLeu_{12}-LTrp_{13}-DLeu_{14}-LTrp_{15}-**L**$\mathbf{Asp}_{16}$-S-CoA. In this assay, we detected only a minor alcohol peak and still high levels of aldehyde. Peak comparisons showed that only about 5% of the aldehyde was converted into alcohol, indicating a major sterical or electronic hindrance caused by the negatively charged residue. The second exception was the substrate where the two L-tryptophan residues had been converted into the D-form, Ac-DLeu_{12}-**D**$\mathbf{Trp}_{13}$-DLeu_{14}-**D**Trp_{15}-Gly_{16}-S-CoA. Here, we found that LgrE converted only about 50% of the substrate aldehyde into the alcohol.

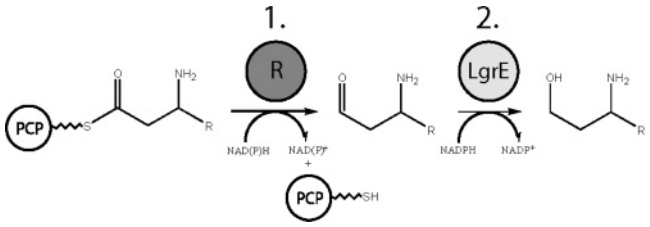

FIGURE 6: Results of this study. First step: the R domain reduces the peptide chain off its adjacent PCP in an NAD(P)H-dependent reaction. Second step: LgrE accepts the aldehyde intermediate and reduces it strictly NADPH-dependent to the final alcohol product.

DISCUSSION

In this study we have shown a two step reductive mechanism of product release in NRPSs which has not been reported before. Gramicidin is reduced off the terminal PCP of the assembly line by the C-terminal R domain attached to module 16 and is released from the NRPS assembly line as an aldehyde intermediate. In a second step, the external aldoreductase LgrE converts the peptide-aldehyde intermediate into the final product gramicidin harboring the alcohol 2-aminoethanol at its C-terminus (see Figure 6). Interestingly, the internal R domain accepts both NADPH and NADH as reducing cofactor, while LgrE is specific for NADPH.

The R domain appeares to be rather unspecific as neither the C-terminal amino acid nor the L-D configuration of the other residues had any detectable influence on the reducing ability of the enzyme. An exception appears to be the stereochemistry of the C-terminal amino acid as only DAla_{16} was accepted in contrast to the larger residues DLeu_{16} and DPhe_{16}. We infer that the stereochemistry is important for the nucleophilic attack which might only be possible if the C-terminal residue is glycine, in L configuration or in D configuration with a small side group.

However, we are not able to determine distinct rates because we are dealing with a coupled single turnover process, as the enzyme did not accept the peptidyl-SNAC

(peptidyl-S-N-acetylcysteamine) as a substrate. Peptidyl-SNACs mimic the 4′phosphopantetheine moiety of the PCP loaded with a substrate and have been sucessfully used to characterize NRPS domains (*28*, *29*). Upon the basis of our experiments, we propose that the R domain recognizes the adjacent PCP in combination with its peptidyl-S-pantetheine moiety rather than the nature of the peptide chain bound onto it as thioester. As in general the A domains are responsible for substrate selectivity in NRPSs (*30*), there is no need for the last domain of an assembly line to exhibit a strong specificity toward the peptide chain it releases. The observed relaxed release mechanism makes the R domain an ideal candidate for the construction of hybrid NRPSs where stable, linear products with a blocked C-terminus could be generated both in vitro and in vivo.

Of the four described R domains Lys2, MxcG, SafR, and LgrD, only Lys2 and MxcG have been biochemically characterized. Lys2 reduces its substrate α-aminoadipate to α-aminoadipate semialdehyde at C_6. The α-aminoadipate semialdehyde then cyclizes rapidly to the cyclic imine, Δ^1-piperidine carboxylate, or is processed further enzymatically to lysine (*23*). In contrast to this reaction, MxcG performs a two-step reduction of the carboxyl group of the peptide 2,6-bis-*N*-dihydroxybenzoate-lysine bound as thioester onto the PCP to the alcohol myxochelin A. However, even though MxcG is capable of performing a two-step reduction, it obviously also releases aldehyde intermediates which are then transaminated supposedly by MxcL to myxochelin B (*22*, *31*). Another R domain, SafA, is thought to reduce its substrate once to the aldehyde intermediate L-Ala-Gly-L-Tyr-L-Tyr-CHO which then forms intramolecular Schiff bases and thus cyclizes to a six-ring hemiaminal structure (*23*). The R domain of LgrD shows remarkable functional similarity to all of these and therefore fits into this class of enzymes.

This study also proves LgrE to be an external aldoreductase responsible for the post-NRPS assembly step of gramicidin biosynthesis. This result confirms the prediction based upon the blastP searches where LgrE showed high similarity to putative, so far uncharacterized oxidoreductases. The enzyme was shown in both the coupled and independent assays to accept the peptide-aldehyde intermediate and reduce it to the corresponding alcohol. LgrE was also capable of accepting various derivatives of the native mimic with few exceptions.

In the examples of MxcG, SafA, and Lys2, the final product is never the aldehyde intermediate, as it is quickly processed further either enzymatically or by self-cyclization. This is due to the fact that free aldehydes are more thermodynamically activated and act as uncontrollably reactive carbonyl groups in biological milieus. Therefore, we find that peptide-aldehydes are either reduced (MxcG), transaminated (MxcL), or cyclized intramolecularly (Lys2 and SafA) or they can be oxidized to the carboxyl acid (U26; a reverse Lys2 pathway in mice (*32*)). As a result, all of the compounds are far less reactive. LgrE fits into this scheme perfectly, reducing the gramicidin-CHO intermediate to the unreactive alcohol. We do not know why gramicidin is released by two reductases where one R domain could theoretically be sufficient, but this also shows that there are many ways how unusal and diverse bioactive peptides are generated and released by NRPSs.

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