Synthesis of a Linear Gramicidin by a Combination of Biosynthetic and Organic Methods†

Karl Bauer, Robert Roskoski, Jr., Horst Kleinkauf,‡ and Fritz Lipmann*

ABSTRACT: Linear gramicidin is a pentadecapeptide with the sequence N-formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. Ribosome-free extracts from Bacillus brevis (ATCC 8185), on Sephadex G-200 filtration, yielded fractions free of aminoacyl-tRNA ligases which catalyzed ATP-PPi exchanges and covalent trichloroacetic acid stable binding of all amino acids incorporated in linear gramicidin. From these fractions Cl,COOH-stable protein-bound peptides were prepared and, after alkali (pH 11) or peroxidative liberation, separated by thin-layer chromatography. Labeled valine, glycine, alanine, leucine, and tryptophan used alternatively with phenylalalnine, i.e., all the amino acids present in linear gramicidin, were incorporated into one of the peptides. The amino acid stoichiometry found in this peptide by double labeling as described in the text was Gly:Ala:Leu:Val:Ph, 1:2:4:4:4. Moreover, in agreement with the structure of linear gramicidin, alanine was found only in L configuration, valine in 50% D and 50% L, and leucine in the D configuration; all derived from L-amino acids used as biosynthetic precursors. Aminoethanolation of this enzyme-bound intermediate, followed by organic formylation, yielded a product which comigrated with linear gramicidin in four solvent systems. These results indicate that biosynthetically a pentadecapeptide is formed which remains thioester linked to the enzyme and presumably is released enzymatically by aminoethanolation. Crude extracts yielded a formylated peptide analogous to the one analyzed. The experiments suggest for the biosynthesis of linear gramicidin a mechanism analogous to that of gramicidin S and tyrocidine (Lipmann, F. (1971), Science 173, 875). However, a complete biosynthesis of linear gramicidin could not be achieved due to our inability to obtain extracts that linked the C-terminal aromatic amino acid to ethanolamine.

The linear gramicidins are antibiotics produced by the same strains of Bacillus brevis (ATCC 8185 or Dubos strain ATCC 10068) that produce tyrocidine (Hotchkiss, 1944). Figure 1 shows the structures of these pentadecapeptides, which differ only in the alternative incorporation of tryptophan, tyrosine, and phenylalanine as aromatic amino acids similar to the differences in the tyrocidines. The amino-terminal valine is formylated, and the carboxyl-terminal tryptophan is peptidically linked to ethanolamine. If glycine is considered an equivalent of a D-amino acid, the linear gramicidins consist throughout of alternating L- and D-amino acid residues, thereby placing constraints on the secondary structure (Urry et al., 1971).

The development of cell-free systems for the synthesis of GS† and Ty made it possible to decipher the mechanism of biosynthesis of these cyclic decapeptides by nonribosomal systems (Saito et al., 1970; Kurahashi et al., 1969; Bredesen et al., 1968; Lipmann et al., 1971). These studies showed that the amino acids are activated by ATP on complementary enzymes of a molecular weight roughly proportional to the number of amino acids activated. From the resulting acyladenylate the amino acid is then transferred to an enzymic sulfhydryl where it is bound covalently as thioester (Kleinkauf and Gevers, 1969). In both GS and Ty the N-terminal phenylalanine is activated and racemized by the smallest enzyme, mol wt 100,000. Reaction between it and the larger enzyme carrying the other amino acids initiates polymerization to peptide intermediates that remain thioester linked to enzymes until released by cyclization (Gevers et al., 1969; Ljones et al., 1968). Pantetheine, covalently bound to the larger enzyme proteins (Kleinkauf et al., 1970; Gilhuus-Moe et al., 1970), appears to mediate, by alternating thiolation and transpeptidation, the elongation of successive peptidyl additions to enzyme-thioester-linked amino acids (Kleinkauf et al., 1971).

The present experiments describe a soluble enzyme system that performs a partial LG biosynthesis. The mode of amino acid activation parallels that for GS and Ty biosynthesis. Thioester-linked glycine and alanine, exclusively present in LG, were already found in trichloroacetic acid precipitates during studies of Ty biosynthesis in the same extracts used here for studying LG biosynthesis (Roskoski et al., 1970a). We have been able to identify an enzyme-bound pentadecapeptide intermediate of LG. After chemical ethanolamination from its thioester link to denatured enzyme and chemical formylation, a compound was isolated that appeared to be identical with authentic LG. In crude extracts enzymatic N-terminal formylation was obtained; however, enzymatic conjugation of the carboxyl terminal with ethanolamine to complete the biosynthesis was not achieved.

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* Abbreviations used are: LG, linear gramicidin; GS, gramicidin S; Ty, tyrocidine.
Experimental Section

Growth of \(B.\) brevis. \(B.\) brevis (ATCC 8185) was cultured by the general method of Fujikawa et al. (1968). Spores from a single potato agar slant were suspended in sterile water, and equal portions were transferred to two 2-l. flasks containing 500 ml of milk-yeast extract medium. After incubation in a New Brunswick rotatory shaker (18 hr, 37°C), about 300 ml of the culture was used to inoculate 10 l. of the meat extract-salt medium. The cells were grown at 37°C with mechanical stirring (350 rpm) in a New Brunswick MMF-14 fermentation apparatus. The rate of sparging with air was 9 l./min until the \(A_{600}\) reached 0.5, when the rate was increased to 12 l./min. A decreased rate of stirring or aeration decreased the yield of cells and enzyme activity. The cells were harvested by the general method of Fujikawa previously described (Gevers et al., 1968). Fractions 60-87 (1.1-1.6 exclusion volumes) were used as the source of the "Sephadex enzyme." The amino acid dependent exchanges after fraction 90 are associated with amino acid-tRNA ligase activity measured as previously described (Roskoski et al., 1970a).

Results

Amino-Acid Dependent ATP-[\(^{32}\)P]PP\(_{i}\) Exchanges of Sephadex G-200 Fractions. Previous studies in this laboratory indicated that exclusion chromatography is an effective way to resolve the enzyme fractions required for GS and Ty biosynthesis. The location of the biosynthetic enzymes was monitored by their amino acid dependent ATP-[\(^{32}\)P]PP\(_{i}\)-exchange activities. Figure 2 shows the Sephadex elution profile obtained by monitoring the exchange activities dependent upon the LG constituent amino acids. These exchanges eluted between 1.1 and 1.6 exclusion volumes. Moreover, these fractions were devoid of amino acid-tRNA ligase activity which eluted between 1.8 and 2.5 exclusion volumes (Roskoski et al., 1970a).

The following experiments indicate that the early eluting fractions contain the enzymes that are activating the amino acids incorporated in LG. There were two peaks of activity for alanine, valine, and tryptophan, one main peak for glycine, and only one for leucine (Figure 2). The tryptophan and leucine peaks overlap with the ones seen in extracts synthesizing Ty since the Ty heavy enzyme (Roskoski et al., 1970a),...
which catalyzes valine, leucine, and tryptophan activity, is also found in this early eluting region. The glycine and alanine activation activities are exclusively related to LG biosynthesis (Roskoski et al., 1971).

In view of the instability of the LG-synthesizing system, its resolution into complementary enzymes was postponed.

FIGURE 4: Recovery of the precursor amino acids from the 0.45 peptide after acid hydrolysis. The peptide, prepared as described in Figure 3 using labeled glycine, alanine, valine, and phenylalanine precursors, was treated with 6 N HCl for 84 hr at 110°, and the hydrolysate was chromatographed on EM silica gel plates (from Brinkmann) with phenol-H2O (75:25, w/w) in a chamber atmosphere saturated with 37% NH3. Since tryptophan is destroyed by acid hydrolysis, phenylalanine was used as the precursor amino acid.

TABLE 1: Optical Configuration of Amino Acids Obtained after Hydrolysis of the Rf 0.45 Peptide.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>L-Amino Acid (%)</th>
<th>D-Amino Acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Valine</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>Alanine</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

The present study concentrated on the isolation of a large, enzyme-bound polypeptide that could be identified as the pentadecapeptide corresponding to LG and on its chemical conversion to the antibiotic.

Isolation of Enzyme-Bound Peptide Intermediates. Since peptide intermediates thioester linked to enzyme have been isolated in GS and Ty biosynthesis (cf. Lipmann, 1971), we tried to isolate similar intermediates in LG biosynthesis. The pooled Sephadex fractions were incubated with ATP, Mg2+, and the LG amino acids (one or more of which was radio-labeled). After Sephadex G-50 gel filtration to remove low molecular weight precursors, the protein in the eluant was precipitated with trichloroacetic acid and washed to remove noncovalently bound material as previously described (Roskoski et al., 1970b). The covalently bound products were liberated by alkali (pH 11.0) and resolved by two successive thin-layer chromatographic systems (Figure 3). It was surmised that the product with the highest Rf would be the most hydrophobic and consequently the longest intermediate. Therefore the Rf 0.45 peptide (Figure 3b) was further characterized.

All the LG constituent amino acids were incorporated into the Rf 0.45 peptide. With different LG amino acids labeled with 14C and 1H, radioactivity comigrated in a single zone. Moreover, after elution and rechromatography in a third solvent system (1-butanol-2-butanone-H2O, 2:2:1), a single zone containing the double label was obtained with Rf 0.40. To show that the Rf 0.45 peptide product is an amino acid derivative, it was treated with 6 N HCl at 110° for 84 hr. In these experiments, as in the study of Ty biosynthesis (Roskoski et al., 1970b), only phenylalanine was used as the aromatic amino acid. This was essential since tryptophan would have been decomposed by acid hydrolysis. The results show that, as found with Ty biosynthesis, phenylalanine substitutes well for tryptophan in the isolated enzyme system. The hydrolysate was chromatographed and the amino acids were recovered (Figure 4). This phenylalanine incorporation shows that analog substitution occurs to a greater extent in vitro than in vivo, which parallels the findings of the Ty-enzyme system (Roskoski et al., 1970b).

To further substantiate the identity of the Rf 0.45 peptide as an intermediate in LG biosynthesis, the optical configuration of the amino acids in the hydrolysate was determined (Table I). In agreement with the primary structure (Figure 1), all the leucine from the Rf 0.45 peptide had the D configuration although l-leucine was used as the precursor;
TABLE II: Stoichiometry of Amino Acids in the RF 0.45 Peptide.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Leucine</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>[4C]</td>
<td>1:4</td>
</tr>
<tr>
<td>Alanine</td>
<td>[3H]</td>
<td>1:2</td>
</tr>
<tr>
<td>Valine</td>
<td>[4C]</td>
<td>1:1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>[3H]</td>
<td>1:1</td>
</tr>
</tbody>
</table>

RF 0.45 peptide for each experiment was prepared as described in Figure 3 using the specified labeled amino acids. After resolution, the peptides were eluted from the thin layers and radioactivity was determined by liquid scintillation spectrometry.

Discussion

B. brevis (ATCC 8185) produces the pentadecapeptide linear gramicidins (A–C) and the cyclic decapetide tyrocidine. However, the extract prepared from cells after lysis in hypotonic buffer, although active in Ty biosynthesis, did not

FIGURE 5: Isolation of the RF 0.6 peptide after liberation from enzyme in the crude extract. The reaction was carried out as described in Figure 3 using 4 mg of protein from crude extract and [4C]formate and [3H]leucine markers. After chromatography on silica gel thin layer in ethyl acetate-pyridine-HOAc-H2O (90:30:9:16) the plate was segmented and the radioactivity measured by liquid scintillation spectrometry.

INTERMEDIATES IN LINEAR GRAMICIDIN BIOSYNTHESIS
produce LG. Supplementation of the extracts with ethanolamine, ethanolamine phosphate, CDP-ethanolamine, in addition to ATP, Mg2+, and amino acids, failed to activate extracts for LG bioformation. Furthermore, it was considered that serine might be incorporated at the carboxyl terminus and undergo decarboxylation to produce peptidically linked ethanolamine. However, labeled serine was not incorporated into LG in vitro even when the incubation medium was supplemented with pyridoxal phosphate which might have been required for decarboxylation. During LG biosynthesis in vitro, labeled ethanolamine was not incorporated, nor was it possible to find it inside the cells. When the cells were incubated with labeled serine, which is transported into them, label spread into both LG and Ty with no increased specific activity in ethanolamine.

As previously reported (Roskoski et al., 1970a), the early eluting Sephadex G-200 filtrate contained enzymes that catalyze ATP-[32P]PP exchanges and that are dependent upon the LG and Ty constituent amino acids. These enzymes were not related to the amino acid-tRNA ligase activities, which elute later. As previously demonstrated (Roskoski et al., 1970a), the amino acids in Ty and LG bind to the Sephadex enzyme covalently as thioester. Thus the mode of amino acid activation appears to be the same in the two systems and parallels that of the GS system (Gevers et al., 1968). The number of enzyme fractions required for LG biosynthesis is unknown. In GS two fractions and in Ty three fractions are required. The GS and Ty heavy fractions contain 4'-phosphopantetheine (Kleinkauf et al., 1970). We suspect that this cofactor also participates in LG bioformation but have not yet examined this point.

The crude cell-free extracts catalyzed the formation of the formylated pentadecapeptide, and the early eluting Sephadex G-200 fractions were active in the biosynthesis of the unformylated intermediate. The formylation reaction has not yet been identified in detail; however, aminopterin in vitro inhibited LG synthesis more than 95% and stimulated Ty synthesis twofold.

The evidence that the RF 0.45 peptide, an enzyme-bound pentadecapeptide, is an intermediate in LG biosynthesis includes: (1) incorporation of all the LG amino acids with the stoichiometry found in LG; (2) conversion of the peptide into the precursor amino acids by acid hydrolysis; (3) the optical configuration of the amino acids in the pentadecapeptide is the same as that in LG; (4) organic ethanolaminolysis of enzyme-bound product followed by organic formylation and deamidiformation of the O-formyl ethanolamine derivative gave a product not distinguishable from LG after thin-layer chromatography on four different solvent systems.

### References


Dissociation of Ribosomal Complexes


Effect of Polypeptide Chain Length on Dissociation of Ribosomal Complexes†

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**Abstract:** The selectivity of Na⁺ in distinguishing free ribosomes from those complexed with peptidyl-tRNA and mRNA (Beller, R. J., and Davis, B. D. (1971), *J. Mol. Biol.*, 55, 477) suggested its usefulness in assessing the possible effect of the length of the nascent peptide on ribosome stability. Accordingly, ribosomes from *Escherichia coli* bearing peptides of defined length and composition were prepared in vitro with phage R17 RNA as messenger. Ribosomes bearing tripeptides were completely dissociated under these conditions. However, gradients containing a high K⁺ concentration showed that the completed initiation complex was more stable than free 70S ribosomes. Ribosomes complexed with tripeptidyl-tRNA (Met-Ala-Ser-tRNA), prepared in the presence of fusidic acid, showed intermediate stability; when such ribosomes were analyzed in a Na⁺ gradient the majority of the ribosomes was dissociated, but the tripeptide remained associated with the 50S subunit peak. Thus the degree of resistance to dissociation by salt, conferred on ribosomes by complexed peptidyl-tRNA, increases with increasing peptide length, and tripeptidyl-tRNA seems to have a greater affinity than fMet-tRNA for the peptidyl binding site on the 50S subunit. In contrast to the normal initiation complexes, those prepared with a nonhydrolyzable analog of GTP could not be distinguished in their stability from free ribosomes.

It is known that ribosomes complexed with mRNA and peptidyl-tRNA are not dissociated under various conditions that do cause dissociation of free ribosomes. These conditions include the addition of the ribosome dissociation factor (Subramanian et al., 1969; Albrecht et al., 1970), replacement of K⁺ by Na⁺ in sucrose gradient buffers (Beller and Davis, 1971), lowering of the Mg²⁺ concentration (Ron et al., 1968; Oppenheim et al., 1968; Kelly and Schaechter, 1969), elevation of the K⁺ concentration (Edelman et al., 1960; Martin et al., 1969), and exposure of ribosomes to air in the absence of sulphydryl compounds (Miyazawa and Tamaoki, 1967; Beller and Davis, 1970). This stabilization against dissociation might depend only on the presence of a bound tRNA, or it might also depend on the nature of the nascent peptide chain. Accordingly, we have prepared ribosomal complexes carrying nascent peptides of various defined lengths in extracts of *Escherichia coli*, with R17 phage RNA as messenger, and have compared them for their ability to survive gradient centrifugation under various ionic conditions. Furthermore, we have compared the stability of initiation complexes prepared with GTP or with the nonhydrolyzable analog, GMP-PCP.†‡

**Materials and Methods**

Preparation of Ribosomes and Factors. All subcellular components were prepared from *E. coli* strain MRE600 (Cam-mack and Wade, 1965), grown at 37° in minimal medium A (Davis and Mingioli, 1950), supplemented with 0.2% glucose and 0.2% Casamino Acids.

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*Abbreviations used are: GMP-PCP, 5′-guanylylmethylene phosphonate; IF, initiation factors; TKM, 10 mM Tris·HCl (pH 7.6)-50 mM KCl-5 mM magnesium acetate; TNA, TKM with NaCl instead of KCl.*