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MEMBRANOUS PHOSPHOGLYCERIDE-LINKED BIOSYNTHESIS OF PENTADECA PEPTIDE, LINEAR GRAMICIDIN, BY Bacillus brevis ATCC 8185*

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SUMMARY: A peptide antibiotic, linear gramicidin A, from Bacillus brevis ATCC 8185 was biosynthesized with a cell-free preparation. An ethanolamine donor required for masking of a carboxyl terminal in this linear peptide was detected. Phosphatidylethanolamine, one of phosphoglycerides and a major structural element of membranes in bacterial cells, was verified to be the primary donor of terminal ethanolamine in the total synthesis of the peptide. This paper suggests that one of the non-ribosomal peptidyl products undergoes tight linkage to a component of cellular membranes.

Linear gramicidin A is produced by Bacillus brevis ATCC 8185 and is composed of 15 amino acids arranged in an alternative L,D,L,D sequence with a formylated N-terminal valine residue, while the other end of tryptophan is masked with ethanolamine **N-formyl-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-ethanolamine** (1-6). Some attempts at biosynthesis of this peptide with a cell-free extract have not yet achieved complete success in total synthesis (7-12), as reviewed by Fritz Lipmann in 1982 and 1984 (13,14). It was, however, determined that the serine residue was closely associated with the biosynthesis of this linear peptide (12). It is interesting to note that the serine residue, which is not a constitutive amino acid in the peptide, strongly generated formic acid for formylation of the N-terminal and also derived ethanolamine for inductive aminoethanolysis of another terminal tryptophan. However, activation of L-serine with ATP into aminoacyl adenylate could not be induced by an amino acid dependent ATP-^{32}PPi exchange reaction in a subcellular system (15), though serine was incorporated into linear gramicidin which was recovered as ethanol-

*I would like to dedicate this paper to the late Dr. Fritz Lipmann deceased on July 24, 1986.

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amine and glycine residues by hydrolysis (12). Little has been clarified in the detail of the mechanism of linear gramicidin production, with the exception of a couple of already published observations on the initial production process (9-12) and involvement of cytidine diphosphoethanolamine (CDP-EA) which is a chemical donor of phospholipids in cell membranes (22).

In the unsolved processes of the total biosynthesis of the peptide, it was finally clarified by this presentation that phosphatidylethanolamine (L-3-phosphatidyl(2-^{14}C)ethanolamine, 1,2-dioleoyl), a commercial product recently introduced, was the ethanolamine donor in the synthesis of linear gramicidin with a cell-free preparation. An enzymatic reaction needed for biosynthesis was well induced only when phosphatidylethanolamine was provided as a terminal in place of ethanolamine itself, phosphorylethanolamine, CDP-EA or phosphatidylserine in the presence of L-form constitutive amino acids, L-serine, ATP and an enzyme protein prepared from cytosol lysed by sonication and precipitated with ammonium sulfate. This approach to the explication of total biosynthesis has also revealed that phosphorylethanolamine was transferred from phosphatidylethanolamine to the protein, because it could be recovered by alkalization of the residual protein of the trichloroacetic acid-precipitate after alcohol-extraction of formed peptides.

MATERIALS AND METHODS

Cells and Enzyme Preparation:

Seven grams of cells were harvested from 500 ml of the culture fluid at the late log. phase 7 hours after inoculation (12) and lysed by sonication for a min with a generating power of 90W in 10 ml of 50 mM phosphate buffer (pH 7.7) containing 1 mM each of DTT and $MgCl_2$. The enzyme protein was precipitated with ammonium sulfate between 35% and 60% saturation in the supernatant obtained by centrifugation of the lysate, and dialysed against the same buffer solution as used in lysis.

Cell-Free Reaction for Biosynthesis and Partial Purification of Product:

Zero point five microcurie of each labelled compound as a terminator was incubated with 1 umole of Val, Gly, Ala, Leu, Trp, Ser, ATP and $MgCl_2$ and 0.1 umole of tetrahydrofolic acid. Five milligrams of the enzyme protein in 50 mM phosphate buffer were added to 0.6 ml of the reaction mixture which was readjusted to pH 7.7 with 0.5N of KOH just before incubation. Reactions were allowed to proceed at 36°C for 10 min and stopped in boiling water. The resultant mixture was then precipitated with 5% chilled trichloroacetic acid (TCA) containing 3% sodium chloride, and 0.1 mg of commercial linear gramicidin (Sigma) in 80% aqueous ethanol was added to promote sedimentation. Five milliliters of alcoholic extractions from the protein precipitate were subjected to a column of basic aluminum oxide for partial purification of the product. The radioactivity in 0.5 ml al-

iquots of the eluate was determined using a liquid scintillation counter.

Linear Gramicidin from Growing Cells:

Zero point five milliliter aliquots of the growing cell culture were placed in a test tube 7.5 hours after inoculation to prepare ^{14}C-labelled linear gramicidin as a marker for autoradiographical identification by thin layer chromatography. The cells were incubated at 33°C for 30 min with 0.5 uCi of L-(U-^{14}C)leucine with shaking and harvested by centrifugation. Labelled linear gramicidin was extracted with 2.5 ml of hot (60°C) ethyl alcohol from the cells precipitated. The eluate was evaporated and applied to a silica gel plate which was developed with ethylacetate/pyridine/water (12/3/1.5).

Alkaline Liberation of Ethanolamine and Phosphorylethanolamine from Residual Trichloroacetic Acid Precipitate:

Ethanolamine and phosphorylethanolamine residues were recovered by alkaline liberation of the resultant reaction protein which was precipitated with the product by trichloroacetic acid. The TCA-precipitate was thoroughly washed with ethyl alcohol for extraction of products prior to alkaline liberation with 0.5N KOH. After neutralization by perchloric acid to pH 7, the aqueous phase obtained by centrifugation was concentrated, applied to a silica gel plate and chromatographed with isobutyric acid/water/28% ammonia water (66/33/1).

Radioisotopes:

The following radioisotope compounds were used;
(1) L-3-Phosphatidyl(2-^{14}C)ethanolamine, 1,2-dioleoyl. Amersham
(2) L-3-Phosphatidyl-L-(U-^{14}C)serine, 1,2-dioleoyl. Amersham
(3) Cytidine diphosphate(1,2-^{14}C)ethanolamine. ICN Radiochemicals
(4) Phosphoryl(1,2-^{14}C)ethanolamine. New England Nuclear
(5) Ethan-1-ol-2-amine-2-^{14}C. Amersham and
(6) L-(U-^{14}C)leucine. Amersham.

RESULTS

Total Synthesis of Linear Gramicidin A by Cell-Free Enzyme Protein:

Table 1 shows the total synthesis of linear gramicidin A with a cell-free extract using phosphatidylethanolamine as a terminator. The synthesis was carried out in the reaction mixtures that contained L-form constituent amino acids including serine, ATP, $MgCl_2$ and tetrahydrofolic acid in phosphate buffer pH 7.7. Phosphatidylserine, cytidine diphosphoethanolamine, phosphorylethanolamine and ethanolamine itself were all inactive to be the C-terminal donor for the peptide biosynthesis.

Autoradiogram of Biosynthesized Linear Gramicidin A by Cell-Free Enzyme System and from Growing Cell Culture:

Both the alcoholic extractions from the TCA-precipitate dependent on cell-free synthesis with ^{14}C-phosphatidylethanolamine and from the growing cells labelled with ^{14}C-leucine were applied to a column of basic aluminum oxide separately, and aliquots of the eluates were evaporated. The residue finally obtained was subjected to thin layer

Table 1. **Total Synthesis of Linear Gramicidin A**

^{14}C-labelled Compounds as a Presumable C-terminal Donor	Complete System	Without ATP
(1) Phosphatidylethanolamine	58,000 CPM	3,500 CPM
(2) Phosphatidylserine	BKG	BKG
(3) Cytidine diphospho-ethanolamine	BKG	BKG
(4) Phosphorylethanolamine	BKG	BKG
(5) Ethanolamine	BKG	BKG

BKG: Background (approx. 40 CPM)

Refer to **MATERIALS AND METHODS** for experimental procedures. Table 1shows the total activity in the eluate from a column.

chromatography and developed in ethylacetate/pyridine/water (12/3/1.5). Figure 1 shows the total synthesis of linear gramicidin as autoradiographically identified. Linear gramicidin represents twin

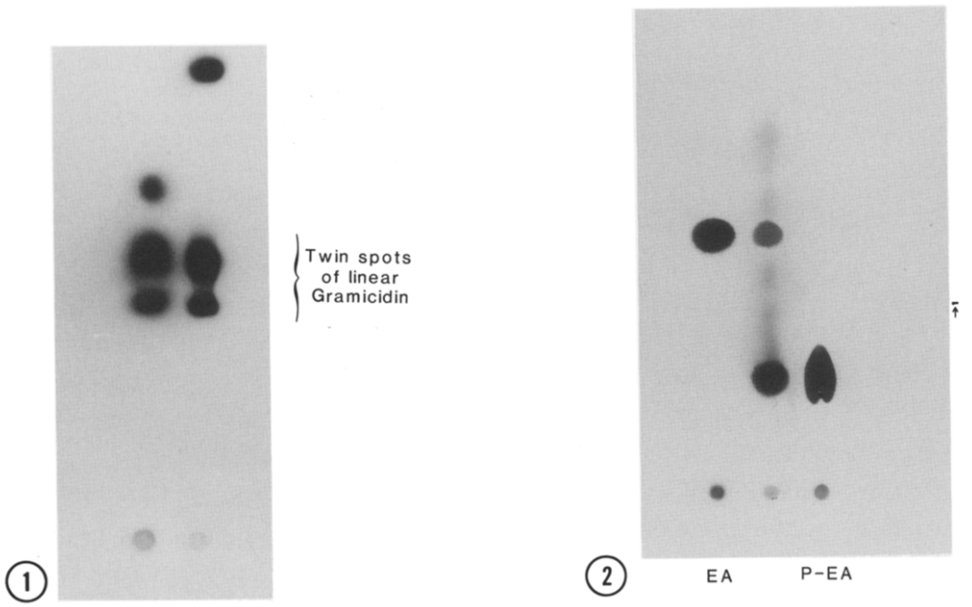


Figure 1. Biosynthesis of Linear Gramicidin A, its Autoradiographical Identification.
Refer to **MATERIALS AND METHODS** for experimental procedure. Left side represents a proof of cell-free synthesis, and right side is a marker of linear gramicidin produced by growing cells. Linear gramicidin shows twin spots in dimer formation (12).

Figure 2. Liberation of Ethanolamine and Phosphorylethanolamine from the Residual Protein.
Refer to **MATERIALS AND METHODS** for experimental procedure. Left (ethanolamine) and right (phosphorylethanolamine) are authentic markers respectively.

spots in a dimer formation on this solvent system as described in (12).

Liberation of Ethanolamine and Phosphorylethanolamine from Trichloroacetic Acid Precipitate:

Autoradiographical identification confirmed alkaline liberation of ethanolamine and phosphorylethanolamine residues from the protein precipitated by trichloroacetic acid of the reaction mixture which contained ^{14}C-phosphatidylethanolamine (Figure 2).

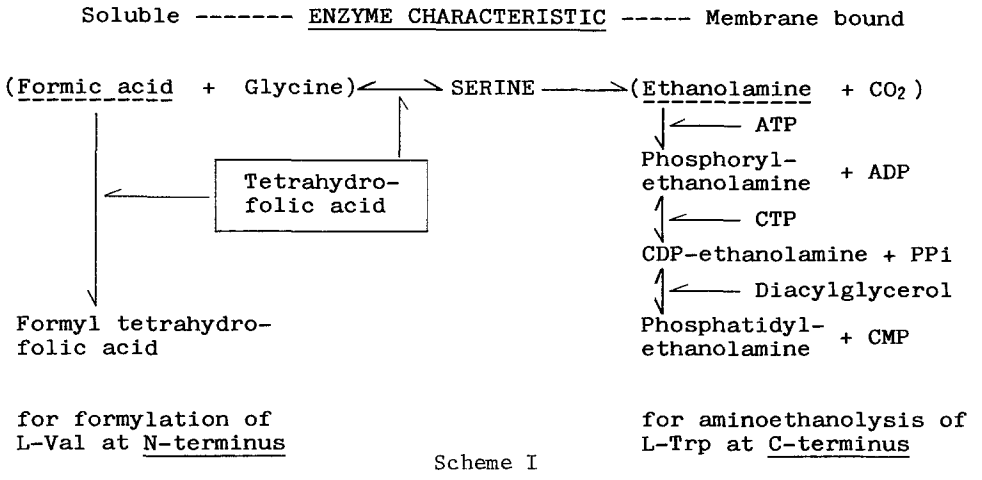
DISCUSSION

It is already known that amino acids are activated by ATP into aminoacyl adenylates and transferred to an enzyme-bound thiol group to be polymerized into a polypeptide instead of tRNA in the ribosomal protein synthesis. Thus the SH-group in enzyme-bound 4'-phosphopantetheine is particularly involved in the biosynthesis of antibiotic polypeptides in various microorganisms (16-19). The biosynthetic mechanism of some of these peptides such as another cyclic tyrocidine from the present strain, cyclic gramicidin S from *B. brevis* ATCC 9999, cyclic and linear chain-attached polymixin E from *B. polymixa* and branched cyclic bacitracin from *B. licheniformis* have been extensively studied (20,21). It was proven that all these peptides were synthesized with soluble enzymes alone in cytosol. There still remain unsolved problems in the mechanism of linear gramicidin biosynthesis. The elucidation of its unknown aspect was challenged by an elaborate attempt at the total synthesis dependent on phosphatidylethanolamine (L-3-phosphatidyl(2-^{14}C)ethanolamine, 1,2-dioleoyl) using a cell-free enzyme preparation.

Further attempt with phosphoglyceride phosphatidylserine (L-3-phosphatidyl-L-(U-^{14}C)serine, 1,2-dioleoyl) which may undergo decarboxylation to yield phosphatidylethanolamine residue resulted in failure to synthesize linear gramicidin with the same reaction system. The present findings indicate distinctly that the process of serine residue taking part in the peptide biosynthesis begins with decarboxylation of serine to produce ethanolamine at first, followed by phosphorylation of ethanolamine by a catalysis of ethanolamine kinase into phosphorylethanolamine. In subsequent processes nascent phosphorylethanolamine may react with CTP to produce CDP-EA, and finally in a usual manner CMP of CDP-EA is cleaved off and phosphorylethanolamine is partially transferred to diacylglycerol to form phosphatidylethanolamine. Enzymes catalyzing these reactions are

found in cell membranes, to which they are tightly bound. Presumably, enzymes involved in the synthesis of these two peptides, linear gramicidin and tyrocidine, seem likely to be independently localized apart in bacterial cells. The former may require both soluble and membrane-bound proteins in a final step to synthesis, while the latter demands only one enzyme dissolved in cytoplasm.

Metabolic pathways of serine residue involved in the biosynthesis of linear gramicidin in the present strain may be summarized as below (Scheme I).



Scheme I

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