Peptides from a Mycobacillin-Synthesizing Cell-Free System

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In a cell-free system from *Bacillus subtilis* B₃, ATP-P_i exchange was catalysed by L-proline at a pH optimum of 7.2. Further stimulation by component amino acids of mycobacillin was inhibited by deprivation from the synthesizing system of even a single amino acid occurring at any point of the cyclic peptide. This inhibition, however, decreased with the distance in the molecule of the given amino acid from L-proline. Peptides containing respectively two, three, four, five and six amino acids were isolated from the mycobacillin-synthesizing system by an amino acid-deprivation technique. The amino acid composition of these peptides and also their *N*- and *C*-terminal amino acid residues were the same as those of peptides that would be obtained if mycobacillin synthesis occurred starting from L-proline and was interrupted at various points along the polypeptide chain.

It is now generally accepted that small peptides and peptide antibiotics, which are usually found to contain D-amino acids, unusual amino acids and non- α -peptide linkages, are synthesized by a non-ribosomal mechanism. Studies on the biosynthesis of small peptides, such as GSH (Snoke & Bloch, 1952) or ophthalmic acid (Lane & Lipmann, 1961), indicate that none of their constituent amino acids becomes activated and catalyses any ATP-P_i or ATP-PP_i exchange individually, although they are added sequentially from their respective *N*-terminal amino acids with concomitant breakdown of ATP into ADP and P_i. A carboxyl-activated phosphorylated peptide intermediate was isolated from the enzyme system in both of the above cases (Strumeyer & Bloch, 1960; Nishimura *et al.*, 1964). However, in the biosynthesis of cyclic peptides such as gramicidin S or tyrocidine all the constituent amino acids are activated individually, as indicated by ATP-PP_i exchange, and are then incorporated into peptides for antibiotic synthesis (Gevers *et al.*, 1969; Roskoski *et al.*, 1970). Isolation of a series of peptides (Holm *et al.*, 1966; Tomino *et al.*, 1967) from enzyme systems incubated with suitable mixtures of amino acids, and elucidation of their structures, have made it possible to suggest D-phenylalanine as the point of initiation and subsequent growth leading to the formation of these cyclic peptides.

Mycobacillin, a cyclic peptide antibiotic produced by *Bacillus subtilis* B₃, contains both D-amino acids and γ -peptide linkages in the molecule (Sengupta *et al.*, 1971). An effective cell-free system catalysing its synthesis has been reported (Banerjee & Bose, 1967) and the enzyme system is localized in the soluble supernatant of the producer cells (Sengupta & Bose,

1971). The synthesizing system, although it preferentially incorporates D-isomers into the antibiotic (Banerjee & Bose, 1967), fails to catalyse any D-amino acid-dependent ATP-PP_i (Banerjee & Bose, 1968) or ATP-P_i exchange (S. Sengupta & S. K. Bose, unpublished work). However, the latter authors have observed that the enzyme system catalyses an L-proline-dependent ATP-P_i exchange and that this exchange is significantly stimulated only by other mycobacillin amino acids if they are added sequentially from L-proline. This stimulation is, however, inhibited by the deprivation from the amino acid mixture of D-aspartic acid, the amino acid next to L-proline (S. Sengupta & S. K. Bose, unpublished work).

In the light of these observations, we have studied the conditions of the inhibition of stimulation, caused by mycobacillin amino acids, on the ATP-P_i exchange initiated by L-proline and also on the fate of mycobacillin synthesis by using a sequential amino acid-deprivation technique starting from L-proline.

Materials and Methods

Materials

Hydrazine hydrate (80%, w/v) and 2,4-dinitro-1-fluorobenzene were purchased from E. Merck A.-G., Darmstadt, Germany. Hydrazine hydrate was distilled to 99% (w/v) as described by Locker (1954). L-Glutamate decarboxylase (*Clostridium welchii*) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. DL-[5-¹⁴C]Glutamic acid, U-¹⁴C-labelled algal protein hydrolysate and H₃³²PO₄ were purchased from Bhabha Atomic Research Centre, Trombay, India. L-[U-¹⁴C]Proline was isolated from U-¹⁴C-labelled algal protein hydrolysate by two-dimensional

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paper chromatography. D-[5- 14 C]Glutamic acid was prepared from DL-[5- 14 C]glutamic acid by destruction of the L-isomer with L-glutamate decarboxylase (*Cl. welchii*). The incubation mixture for the decarboxylation contained 0.5 ml of 0.2 M-sodium acetate buffer, pH 5.0, 0.5 ml of L-glutamate decarboxylase (26 mg/ml) and 50 μ mol of labelled DL-glutamic acid in acetate buffer (2.0 ml). The reaction was conducted in a Warburg apparatus at 37°C for 4 h and terminated by the addition of 2 vol. of ethanol. The clear supernatant was then deionized on a column of Dowex 50 (H^+ form) and dried *in vacuo*. The residue was dissolved in water and was purified by paper chromatography in two dimensions with (a) butan-1-ol-acetic acid-water (4:1:1, by vol.) and (b) water-saturated phenol. Unchanged D-glutamic acid was thus separated from 4-aminobutyric acid (the decarboxylated product of L-glutamic acid) and was eluted from the chromatogram.

Preparation of enzyme

The producer organism *B. subtilis* B₃ was grown in nutrient broth supplemented with 1% glucose for 40 h at 30°C and a 10000g supernatant of the producer cells was prepared as described by Sengupta & Bose (1971). The supernatant was then precipitated with $(NH_4)_2SO_4$, and the precipitate that was collected between 30 and 75% saturation was dissolved in 0.1 M-sodium phosphate buffer (pH 7.2) and dialysed for 2 h against 0.1 M-sodium phosphate buffer (pH 7.2) containing 0.1 mM-2-mercaptoethanol, and used as a source of enzyme.

ATP- P_i exchange study

The exchange was studied as described by Stulberg & Novelli (1960). The incubation mixture contained 100 μ mol of tris-HCl buffer (pH 7.2), 5 μ mol of ATP, 5 μ mol of $MgCl_2$, 10 μ mol of each amino acid, 50 μ mol of KF, 5 μ mol of $^{32}P_i$ (0.1 mCi) and 2.5 mg of enzyme. The total volume was 2.0 ml and the reaction mixture was incubated at 30°C for 20 min. The reaction was terminated by the addition of an equal volume of 5% (w/v) trichloroacetic acid and the precipitate was removed by centrifugation. Labelled ATP was recovered from the supernatant by adsorption with activated charcoal and elution with 1 M-HCl as described by Stulberg & Novelli (1960). P_i was determined by the method of Fiske & SubbaRow (1925).

Identification of radioactive mycobacillin formed by the enzyme system

The incubation mixture and the method of isolation of the radioactive mycobacillin were as described by Sengupta & Bose (1971).

Isolation of labelled peptides

The incubation mixture used for the isolation of peptides contained 5 mg of enzyme, 100 μ mol of tris-HCl buffer (pH 7.2), 10 μ mol of ATP, 5 μ mol of mercaptoethanol and mixtures of two, three, four, five, six and seven mycobacillin amino acids (10 μ mol each; only one or two were ^{14}C -labelled and each had a radioactivity of 10 μ Ci/ μ mol) starting sequentially from L-proline, as in the molecule. The total volume was 2.0 ml. The mixture was incubated for 20 min and the reaction was terminated with 4 vol. of chloroform-butan-1-ol (1:4, v/v). Each set of 25 tubes was extracted three times with the chloroform-butan-1-ol mixture and centrifuged. The clear solvent layer was then dried under a cold air stream and dissolved in a minimum volume of ethanol [containing 10% (v/v) of 10 M-HCl] and was chromatographed in two dimensions in solvent systems (i) 70% (v/v) ethanol and (ii) butan-1-ol-acetic acid-water (4:1:1, by vol.) and sprayed with a solution of 0.05% ninhydrin in acetone. Ninhydrin-positive spots, other than those for residual amino acids added initially to the incubation mixture, were eluted from an unsprayed chromatogram and re-chromatographed in the same solvent systems to test their homogeneity. Each of the spots obtained in the chromatogram was then eluted with 50% (v/v) ethanol, plated on a planchet and radioactivity was measured in a windowless gas-flow counter.

Identification of amino acids, including C- and N-terminal residues, in each peptide and determination of their molar proportion

Each of the new spots (spots other than those for mycobacillin amino acids added in the incubation mixture) eluted from the chromatogram was then hydrolysed with 10 M-HCl in a sealed tube at 110°C for 5 h. The hydrolysate was dried *in vacuo* and chromatographed in two dimensions in the solvent systems (i) and (ii) as above. The amino acids appearing in the chromatogram were then identified and their molar proportions were determined by a ninhydrin method (Giri *et al.*, 1952).

The C-terminal amino acid of each peptide was determined as described by Akabori *et al.* (1956). In practice each peptide fraction isolated from the chromatogram was dried in a vacuum desiccator and then incubated at 60°C for 2 h with two drops of anhydrous hydrazine in a sealed tube. The tubes were opened and excess of hydrazine was removed in a vacuum desiccator over H_2SO_4 . The dried mass was then dissolved in water and shaken overnight with the carboxylic resin Amberlite IRC-50 (H^+ form). After removal of the residue the supernatant (2 ml) was shaken with benzaldehyde (0.2 ml) and pyridine (0.1 ml) overnight in a stoppered cylinder. The benzaldehyde layer was discarded and the aqueous

layer was dried and chromatographed in two dimensions in the solvent systems (i) and (ii) described above. Amino acids were identified by comparison with an authentic sample.

The *N*-terminal amino acid of each peptide was determined by the fluorodinitrobenzene method (Sanger, 1945). In practice each peptide eluted from the chromatogram was dissolved in 1 ml of 0.1M-NaHCO₃ solution. To the solution were added 0.1 ml of fluorodinitrobenzene and 2 ml of ethanol. The mixture was shaken for 2 h and transferred to a separating funnel and the volume made up to 10 ml with water. The mixture was then shaken with 10 ml of peroxide-free ether to remove unchanged fluorodinitrobenzene and was acidified with 1 ml of 5.7M-HCl. The acidified solution was extracted with 5 × 10 ml of ethyl acetate. The ethyl acetate layer was then washed with 3 × 10 ml of water and acidified with two drops of 5.7M-HCl. The solvent was evaporated off under reduced pressure to give a Dnp-peptide. The Dnp-peptide was hydrolysed with 5.7M-HCl for 3 h at 110°C and extracted with ether. The ether layer was then dried and the residue subjected to two-dimensional paper chromatography in the dark in the solvent systems chloroform-benzyl alcohol-acetic acid (70:30:3, by vol.) and benzene-pyridine-acetic acid (40:1:1, by vol.). Each Dnp-amino acid was identified by comparison with an authentic sample.

Results

Inhibition of stimulation by mycobacillin amino acids of the L-proline-catalysed ATP-P_i exchange

Table 1 shows that the stimulation by mycobacillin amino acids of L-proline-initiated ATP-P_i exchange

was inhibited by deprivation not only of D-aspartic acid, as observed by S. Sengupta & S. K. Bose (unpublished work), but also of any one of the amino acids occurring at any part of the polypeptide chain of mycobacillin. Thus the deprivation of D-aspartic acid, D-glutamic acid, L-tyrosine and L-serine from the mycobacillin amino acid mixture decreased the stimulation. The decrease in stimulation was not the same in all cases and the effect was maximum with D-aspartic acid and minimum or practically nil with L-leucine or L-alanine. This perhaps agrees with the observation (S. Sengupta & S. K. Bose, unpublished work) that the stimulation reaches its maximum value with the addition of the fourth different amino acid. Thus the inhibitory effect on stimulation is more marked by the omission of an amino acid at the beginning of the chain than of one at the latter part, where the chain has already grown to the length of at least five amino acid units. The pH optimum for the exchange was found to be 7.2 (Fig. 1).

Effect of pyrophosphate on mycobacillin synthesis

Fig. 2 shows that pyrophosphate, which normally inhibits ATP-PP_i exchange, stimulates mycobacillin synthesis. The maximum stimulation takes place at a concentration of 50 mM, after which it slightly inhibits synthesis.

Isolation of peptides from the mycobacillin-synthesizing cell-free system

Chromatography of the six incubation mixtures containing two, three, four, five, six and seven amino acids in sequence starting from L-proline showed that all except one contained (in addition to spots for

Table 1. *Effect of mycobacillin amino acids on L-proline-catalysed ATP-P_i exchange*

The incubation mixture contained 100 μmol of tris buffer (pH 7.2), 5 μmol of ATP, 5 μmol of MgCl₂, 10 μmol of each amino acid, 50 μmol of KF, 5 μmol of H₃³²PO₄ (0.1 mCi) and 2.5 mg of enzyme. Total volume was 2.0 ml and the reaction mixture was incubated at 30°C for 20 min. The reaction was terminated by the addition of an equal volume of 5% (w/v) trichloroacetic acid and the precipitate was removed by centrifugation. Labelled ATP was recovered as described by Stulberg & Novelli (1960). P_i was determined by the method of Fiske & SubbaRow (1925). Mycobacillin is cyclo(-L-Pro-D-Asp-D-Glu-L-Tyr-L-Asp-L-Tyr-L-Ser-D-Asp-L-Leu-D-Glu-D-Asp-L-Ala-D-Asp-).

Mycobacillin amino acids added	Amount in radioactivity in ATP (c.p.m./nmol of P _i)
L-Pro	17021
Mycobacillin amino acids	24614
Mycobacillin amino acids except D-aspartic acid	17110
Mycobacillin amino acids except D-glutamic acid	18120
Mycobacillin amino acids except L-tyrosine	19411
Mycobacillin amino acids except L-serine	20998
Mycobacillin amino acids except L-leucine	24518
Mycobacillin amino acids except L-alanine	24428

Fig. 1. Optimum pH for L-proline-catalysed ATP-³²P_i exchange

The incubation mixture contained 5 μ mol of ATP, 5 μ mol of MgCl₂, 10 μ mol of L-proline, 50 μ mol of KF, 5 μ mol of ³²P_i (0.1 mCi), 2.5 mg of enzyme and 100 μ mol of tris-HCl buffers of the pH range 6.0–9.0. Total volume was 2.0 ml. The reaction mixture was incubated at 30°C for 20 min and the reaction was terminated by adding an equal volume of 5% (w/v) trichloroacetic acid. Labelled ATP was recovered as described by Stulberg & Novelli (1960).

residual amino acids added in the incubation mixture) a single ninhydrin-positive spot, which for each mixture possessed a specific *R_F* value. In all, five such spots were obtained. These, on hydrolysis, showed the presence of only those amino acids that were added in the incubation mixture (Table 2). Peptides containing respectively two, three, four, five and six amino acids were obtained. However, no peptide containing all the seven different mycobacillin amino acids could be identified in the chromatogram from the complete reaction mixture.

Amino acid composition, C-terminal and N-terminal amino acid residues of the isolated peptides

The amino acid composition of these peptides (Table 2) indicated that the peptides containing two, three, four, five and six amino acids were respectively di-, tri-, hexa-, octa- and undeca-peptides. Each of these peptides had proline as its N-terminal residue and aspartic acid, glutamic acid, tyrosine, aspartic

Fig. 2. Effect of pyrophosphate ion on mycobacillin synthesis

Sodium pyrophosphate was added at various concentrations to a mycobacillin-synthesizing incubation mixture containing 5 mg of enzyme, 100 μ mol of tris-HCl buffer (pH 7.2), 10 μ mol of ATP, 5 μ mol of mercaptoethanol and 10 μ mol of each mycobacillin amino acid. The reaction mixture was incubated for 1 h at 30°C and the reaction was terminated by adding 3 vol. of butan-1-ol. Non-labelled mycobacillin (2 mg) was added as carrier and labelled mycobacillin was recovered from the incubation mixture as described previously (Sengupta & Bose, 1971).

acid and aspartic acid as its C-terminal amino acid respectively.

Discussion

The present work sought to find conditions for inhibiting stimulation caused by mycobacillin amino acids, of the L-proline-catalysed ATP-P_i exchange and to isolate intermediate peptides in the biosynthesis of the antibiotic. The stimulation of L-proline-catalysed ATP-P_i exchange was inhibited when even a single amino acid occurring at any part of the polypeptide chain was omitted. The optimum pH for the exchange reaction was 7.2, which is also the optimum pH for mycobacillin synthesis. Pyrophosphate, usually an inhibitor of ATP-P_i exchange, stimulated mycobacillin synthesis. This stimulation may be accounted for by the prevention of ATP loss caused by the synthesis of aminoacyl adenylates not required for mycobacillin synthesis.

Table 2. *Amino acid composition, C-terminal and N-terminal amino acids of peptides*

For experimental details see the text.

Amino acids added in the incubation mixture	<i>R_F</i> values of peptides in solvents		Radioactivity of peptides (c.p.m.)	C-terminal amino acid	N-terminal amino acid	Amino acid composition
	Butan-1-ol- acetic acid- water (4:1:1, by vol.)	Ethanol (70%, v/v)				
L-[U- ¹⁴ C]Pro, DL- Asp	0.59	0.72	1249	Asp	Pro	Pro ₁ , Asp ₁
L-[U- ¹⁴ C]Pro, DL- Asp, D-[5- ¹⁴ C]Glu	0.65	0.71	1051	Glu	Pro	Pro ₁ , Asp, Glu ₁
L-[U- ¹⁴ C]Pro, DL- Asp, D-[5- ¹⁴ C]Glu, L-Tyr	0.56	0.84	1148	Tyr	Pro	Pro ₁ , Asp ₂ , Glu ₁ , Tyr ₂
L-[U- ¹⁴ C]Pro, DL- Asp, D-[5- ¹⁴ C]Glu, L-Tyr, L-Ser	0.61	0.74	1149	Asp	Pro	Pro ₁ , Asp ₃ , Glu ₁ , Tyr ₂ , Ser ₁
L-[U- ¹⁴ C]Pro, DL- Asp, D-[5- ¹⁴ C]Glu, L-Tyr, L-Ser, L-Leu	0.41	0.82	1824	Asp	Pro	Pro ₁ , Asp ₄ , Glu ₂ , Tyr ₂ , Ser ₁ , Leu ₁
L-[U- ¹⁴ C]Pro, DL- Asp, D-[5- ¹⁴ C]Glu, L-Tyr, L-Ser, L- Leu, L-Ala	—	—	—	—	—	—

With these results it was logical to follow mycobacillin synthesis inhibited at any point in the chain by suitable amino acid deprivation. Accordingly the amino acid composition of the incubation mixture for mycobacillin synthesis was so adjusted as to include two, three, four, five, six and seven amino acids in sequence as in the molecule starting from L-proline. On analysis the incubation mixtures were found to contain not only unchanged amino acids but also, in every case except one, only a single peptide which, depending on the mixture used, was a di-, tri-, hexa-, octa- or undeca-peptide containing two, three, four, five or six amino acids respectively (Table 2). No straight-chain tridecapeptide containing seven amino acids was obtained from the incubation mixture, which may mean that immediate cyclization may occur when such a peptide is formed. The constituent amino acids and their molar ratios in these peptides were the same as those in the peptides that one can obtain theoretically from mycobacillin, if its synthesis is stopped at various points along the polypeptide chain, starting from L-proline.

The C-terminal and N-terminal amino acids of these peptides were also determined. The amino acid composition of the peptides and also their N- and C-terminal amino acid analysis were sufficient to

indicate, in the absence of complete sequence and stereoisomeric configuration of their constituent amino acids, that they may be fitted into mycobacillin, if synthesis starts from L-proline. It is now possible to suggest that these peptides are not randomly synthesized in the incubation mixture, but are intermediates of a sequence of reactions starting from L-proline and may actually be the intermediates of mycobacillin synthesis, although the elucidation of their exact role requires further experiments.

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