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L-α,γ-DIAMINOBUTYRATE-ACTIVATING ENZYME FROM *BACILLUS POLYMYXA*

PROPERTIES AND DISTRIBUTION*

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SUMMARY

1 An enzyme which catalyses the L-α,γ-diaminobutyrate-dependent exchange of ATP with PP_i was partially purified from sonic extracts of the polymyxin B-producing organism, *Bacillus polymyxa* 2459. This enzyme could be separated from most other amino acid-activating enzymes and exhibited an apparent K_m for L-α,γ-diaminobutyrate of 0.6 mM.

2 Treatment of sonic extracts with streptomycin sulfate led to an alteration in the properties of the activating enzyme This appeared to be due to the conversion of the enzyme from polydisperse aggregates of average molecular weight 300 000 to units of 100 000 molecular weight

3 In lysed protoplast preparations, the L-α,γ-diaminobutyrate-activating enzyme was predominantly associated with the particulate fraction, in contrast with other amino acid-activating enzymes The K_m for L-α,γ-diaminobutyrate of the particulate preparation was the same as that of the soluble enzyme

4 The specific activity of the L-α,γ-diaminobutyrate-activating enzyme rose about 10-fold during vegetative growth, beginning at the time of the first appearance of polymyxin in the growth medium

5 Mutants of *B polymyxa* that had lost the ability to produce polymyxin also lacked the L-α,γ-diaminobutyrate-activating enzyme On the other hand, other polymyxin-producing organisms, such as *B polymyxa* ATCC 10401 and *B circulans* ATCC 14040, were found to have significant levels of this enzyme These observations suggest that the L-α,γ-diaminobutyrate-activating enzyme plays a role in the biosynthesis of polymyxin

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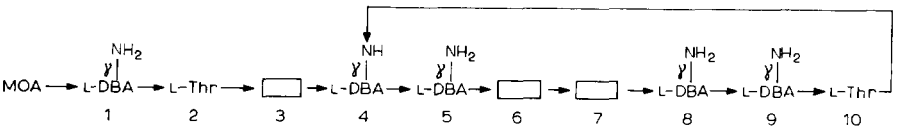
INTRODUCTION

Although ample evidence has accumulated, both from experiments *in vivo*[1–5] and *in vitro*[6–13] that the biosynthesis of polypeptide antibiotics proceeds by a mechanism which differs from that of protein synthesis, little information is yet available on the nature of the reactions involved However, attention has been drawn to the activation of amino acids by the finding that the biosynthesis of tyrocidines by *Bacillus brevis* is mediated by two enzyme fractions (presumably multienzyme complexes) that also catalyse an exchange of PP_i with ATP in the presence of the constituent amino acids[6,10,14]

In this report, we describe some studies relating to the biosynthesis of the antibiotic polymyxin B by *Bacillus polymyxa* This branched cyclic decapeptide is terminated by a fatty acid and contains six residues of L-α,γ-diaminobutyrate (Fig 1) Previous reports by CIFERRI *et al* [15] and from this laboratory[16] have described the



Antibiotic	Residue 3	Residue 6	Residue 7
Polymyxin B_1	L-DBA	D-Phe	L-Leu
Polymyxin D_1	L-Ser	D-Leu	L-Leu
Circulin A	L-DBA	D-Leu	L-Ile

Fig 1 Structures of some polymyxins MOA, (+)-6-methyloctanoate, DBA, α,γ-diamino butyrate

presence of an enzyme in extracts of *B polymyxa* that catalyzes an L-α,γ-diaminobutyrate-dependent exchange of PP_i with ATP Some of the properties of this enzyme will be described, and evidence will be presented which implicates the L-α,γ-diaminobutyrate-activating enzyme in the biosynthesis of polymyxin

MATERIALS AND METHODS

Chemicals

L-α,γ-Diaminobutyrate was prepared by the Schmidt degradation of L-glutamate as described previously[3] The product was recrystallized 6 times as the monohydrochloride from aqueous ethanol All other amino acids were A-grade materials from Calbiochem [^{32}P]PP_i was purchased as the sodium salt from New England Nuclear or Tracerlab $(NH_4)_2SO_4$ (enzyme grade) and streptomycin sulfate were obtained from Mann, lysozyme (egg white) from Armour, pancreatic deoxyribonuclease from Worthington, and alcohol dehydrogenase (yeast) from Calbiochem

Bacterial strains

The polymyxin B-producing organism *B polymyxa*, strain Pfizer 2459, was obtained through the courtesy of Dr I A Solomons and was used in earlier portions of this work Subsequently, a well-sporulating derivative of this strain, designated

B polymyxa 2459s, was isolated which had relatively higher levels of the L-α,γ-diaminobutyrate-activating enzyme This strain was maintained at −15° as a suspension of heat-treated spores Polymyxin-negative mutants were isolated from *B. polymyxa* 2459s after treatment with ethane methane sulfonate, ultraviolet light or acridine orange by scoring for absence of antibiotic production both on solid medium and in liquid culture *B polymyxa* ATCC 10401 (polymyxin D producing) and *B circulans* ATCC 14040 (circulin producing) were obtained as lyophylized cultures from the American Type Culture Collection

Growth of organisms

B polymyxa 2459 was cultured at 37° in the synthetic medium described previously[3], supplemented with 4 2% $(NH_4)_2SO_4$ *B polymyxa* 10401 was grown at 30° in the same medium at low (1 5%) $(NH_4)_2SO_4$ concentration. *B circulans* 14040 was cultured at 25° in the medium of MURRAY *et al* [17], containing (per l) KCl (4 g), KH_2PO_4 (0 2 g), yeast extract (20 g), $(NH_4)_2SO_4$ (5 g), $CaCO_3$ (8 g, enclosed in dialysis tubing) and sucrose (2 g; added separately after sterilization) All cultures were grown on a rotary shaker in erlenmeyer flasks filled with one-fifth of their volume of medium

Antibiotic assay

Polymyxin was assayed by the turbidimetric method of WINTERMERE *et al* [18] in samples of the growth medium from which the cells had been removed by centrifugation 1 unit of polymyxin was defined as the antibiotic activity of 0 1 μg of polymyxin B·HCl

Assay of amino acid-activating enzymes

Amino acid-activating enzymes were assayed by measuring the amino acid-dependent exchange of [^{32}P]PP_i with ATP (ref 19) The reaction mixtures contained in a final volume of 0 5 ml the following (unless otherwise stated) 10 mM potassium phosphate buffer (pH 7 5), 2 mM [^{32}P]PP_i, 2 mM ATP, 5 mM $MgCl_2$, 1 mM amino acid and enzyme The incubations were carried out for 30 min at 37° (unless otherwise stated) and were terminated by the addition of 1 ml of 1 M $HClO_4$ and 1 ml of an aqueous suspension of Norit A charcoal (10 mg/ml) After 2 h at 0°, the charcoal was collected on Millipore filters and was washed with 5 × 10 ml of water The filters were transferred to glass vials, dried at 100° and counted in a liquid scintillation spectrometer using 10 ml of toluene containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis-2′-(5′-phenyloxazolyl)benzene (0 1 g/l) 1 unit of activity is defined as the amount of enzyme catalyzing the incorporation of 1 μmole of [^{32}P]PP_i into ATP under the conditions of the assay

Partial purification of the enzyme from sonic extracts

Washed cells (14 g) of *B polymyxa* 2459, harvested in late exponential phase, were suspended in 40 ml of 5 mM potassium phosphate buffer (pH 7 5) and sonicated in 10-ml portions for 10 min each with an MSE Model 60 ultrasonic disintegrator The extract was centrifuged at 100 000 × *g* for 90 min, and the supernatant solution (crude extract) was diluted to 100 ml with 5 mM potassium phosphate buffer (pH 7 5).

Procedure A Solid $(NH_4)_2SO_4$ (12 5 g) was added to the crude extract (50 ml) The precipitate was collected by centrifugation at 25 000 × *g* for 30 min and resuspended in 5 ml of potassium phosphate buffer (pH 7 5)

Procedure B A solution of streptomycin sulfate (900 mg) in 5 mM potassium phosphate buffer (pH 7 5) was added dropwise with stirring to the crude extract

TABLE I

PURIFICATION OF ENZYME FROM SONIC EXTRACTS OF *B polymyxa* 2459

Fraction	*Protein*	*Activation of* L-α,γ-*Diamino-butyrate*		L-*Met (total units)*	L-*Leu (total units)*
		Total units	*Specific activity (units/mg)*		
Procedure A					
Crude extract	950	37	0 039	403	1220
$(NH_4)_2SO_4$	465	39	0 084	63	159
Procedure B					
Crude extract	950	37	0 039	403	1220
Streptomycin	360	25	0 070	404	960
$(NH_4)_2SO_4$	108	15	0 14	250	605

(50 ml) The stirring was continued for 12 h and the precipitate was removed by centrifugation at 25 000 × *g* for 30 min The supernatant solution (50 ml) was treated with solid $(NH_4)_2SO_4$ (14 g), the precipitate was discarded, and the resulting supernatant solution was then treated with 2 5 g of $(NH_4)_2SO_4$ The precipitate was collected by centrifugation at 25 000 × *g* for 30 min and redissolved in 5 ml of potassium phosphate buffer (pH 7 5).

The results of these procedures are summarized in Table I

Preparation of crude particulate fraction

Washed cells (1 g) of *B polymyxa* 2459s, harvested in middle exponential phase, were suspended in 50 ml of Buffer A (10 mM Tris buffer (pH 7 5), 10 mM KCl, 2 mM EDTA and 2 mM 2-mercaptoethanol) and treated with lysozyme (25 mg) at 25° for 20 min The lysate was centrifuged at 30 000 × *g* for 20 min to yield a soluble fraction and a particulate fraction which was resuspended in half the original volume of Buffer A

Protein determination

Protein was estimated spectrophotometrically[20] or by the biuret method of GORNALL *et al.*[21]

RESULTS

Purification of enzyme from sonic extracts

Fractionation of crude sonic extracts with $(NH_4)_2SO_4$ effected a partial separation of the L-α,γ-diaminobutyrate-activating enzyme from other amino acid-activating enzymes (Table I and Fig 2A) Further purification could be achieved by gel filtration The L-α,γ-diaminobutyrate-activating enzyme emerged from columns of Sephadex G-200 before the L-methionine-activating enzyme, which in turn preceeded most of the other amino acid-activating activities (Fig 3A) Procedure B provided preparations of higher specific activity than did Procedure A (Table I) However, prior streptomycin treatment led to an increase in the amount of $(NH_4)_2SO_4$ required to precipitate the enzyme and thus interfered with the separation from the

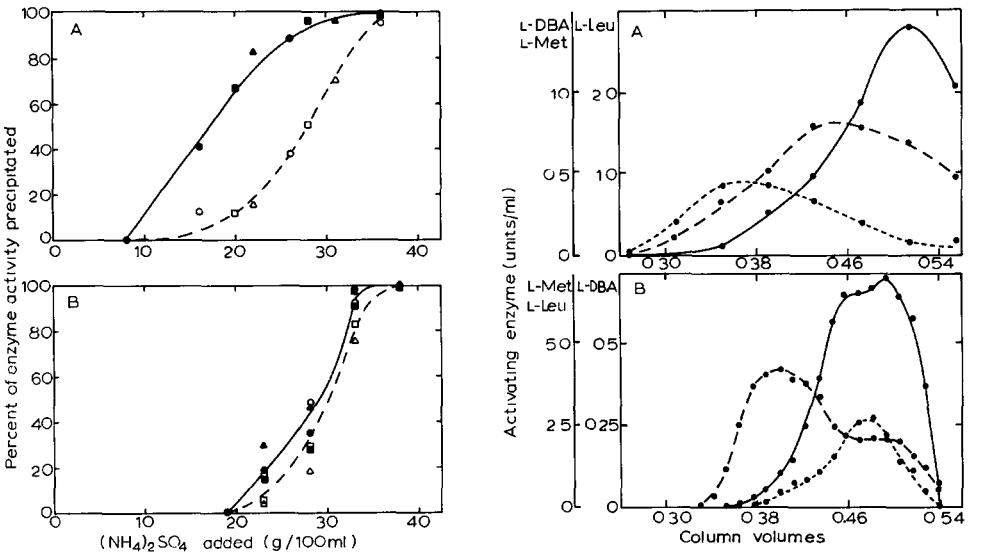


Fig 2 Precipitation of L-α,γ-diaminobutyrate-activating enzyme with $(NH_4)_2SO_4$ Enzyme fractions were treated with successive amounts of $(NH_4)_2SO_4$ as indicated, and the L-α,γ-diaminobutyrate-activating activity (solid symbols) and the L-leucine-activating activity (open symbols) were assayed in each of the precipitated fractions The different sets of symbols refer to separate experiments A Crude extract (Procedure A) B Streptomycin supernatant fraction (Procedure B)

Fig 3 Gel filtration on Sephadex G-200 A The $(NH_4)_2SO_4$ fraction prepared by Procedure A was chromatographed on a column (7 5 cm × 25 cm) of Sephadex G-200 in 5 mM potassium phosphate buffer (pH 7 5) Fractions of 9 ml were collected and the exchange of [^{32}P]PP_i with ATP dependent on L-α,γ-diaminobutyrate (L-DBA) (· ·), on L-methionine (— — —), and on L-leucine (———) was determined B The $(NH_4)_2SO_4$ fraction prepared by Procedure B was chromatographed on a column (5 cm × 60 cm) of Sephadex G-200 as above

L-leucine-activating enzyme (Fig 2B) Moreover, the L-α,γ-diaminobutyrate-activating enzyme in such preparations emerged from columns of Sephadex G-200 together with the L-leucine-activating activity (Fig 3B) The altered behavior of the enzyme after treatment with streptomycin sulfate appeared to be related to a reduction in molecular weight, as shown by sucrose gradient centrifugation While the untreated enzyme was polydisperse with an average molecular weight of about 300 000, the streptomycin-treated enzyme gave a sharp peak, coincident with the L-leucine-activating enzyme, at a position corresponding to a molecular weight of about 100 000 (Fig. 4)

Properties of the soluble enzyme from sonic extracts

The streptomycin-treated preparations were relatively unstable, as indicated by low recoveries of activity upon $(NH_4)_2SO_4$ fractionation and gel filtration on Sephadex G-200. On the other hand, the activating enzyme prepared by Procedure A was stable under these conditions and could be stored in 40% ethylene glycol at −15° for a year without loss of activity After repeated gel filtration, such preparations were relatively specific for the activation of L-α,γ-diaminobutyrate. The rates of exchange of PP_i with ATP, dependent on L-leucine and on L-methionine, were

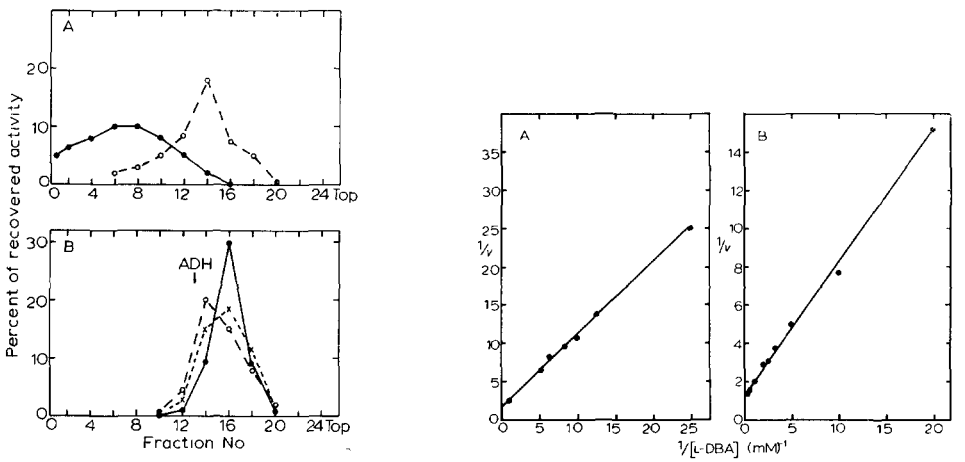


Fig 4 Sucrose gradient centrifugation of soluble enzyme Fractions containing L-α,γ-diaminobutyrate-activating activity from the Sephadex G-200 columns in Fig 3 were pooled and concentrated by precipitation with $(NH_4)_2SO_3$ Samples (0 2 ml) of the concentrated fractions were layered onto 4 6 ml of linear gradients of sucrose (5–20% by wt) in 5 mM potassium phosphate buffer (pH 7 5) and centrifuged at 39 000 rev /min in the Spinco SW-39 rotor for 12 h at 4° Fractions (0 2 ml) were collected from the bottom of the tubes and assayed for exchange of [^{32}P]PP_i with ATP, dependent on L-α,γ-diaminobutyrate (●—●), on L-methionine (○— — —○), and on L-leucine (× ×) Yeast alcohol dehydrogenase (ADH, molecular weight 150 000) was used as a molecular weight marker A Fractions from Fig 3A, containing 1 0, 0 34, and 0 09 units respectively, of L-α,γ-diaminobutyrate-, L-methionine-, and L-leucine-activating enzyme B Fractions from Fig 3B, containing 1 0, 8 5, 33 units, respectively, of L-α,γ-diaminobutyrate-, L-methionine, and L-leucine-activating enzyme

Fig 5 Effect of L-α,γ-diaminobutyrate (L-DBA) concentration on the activating enzyme A Soluble enzyme (0 23 mg), prepared from *B polymyxa* 2459 by Procedure A and purified further by gel filtration on Sephadex G-200 B Particle-bound enzyme from *B polymyxa* 2459s, washed twice with Buffer A as described in Table III, and assayed at 25°

reduced to 15 and 35%, respectively, of the L-α,γ-diaminobutyrate-dependent exchange, while the rate of exchange due to L-lysine was only 1% and that due to other basic amino acids (L-histidine, L-arginine, and L-ornithine) and to D-α,γ-diaminobutyrate could not be detected The specificity of the activating enzyme for L-α,γ-diaminobutyrate was also supported by the low apparent K_m (0 6 mM) for this amino acid (Fig 5A)

Subcellular distribution of the enzyme

Upon centrifugation of extracts of *B polymyxa* at 20 000 × *g*, a slightly larger proportion of the L-α,γ-diaminobutyrate-activating enzyme was sedimented than of the L-leucine- and L-methionine-activation activities, regardless of whether the extracts were prepared by sonication, Hughes' press treatment, or grinding with alumina (Table II) Moreover, when extracts were produced by a very mild treatment, such as osmotic lysis of protoplasts produced by lysozyme, a substantial fraction (60%) of the L-α,γ-diaminobutyrate-activating enzyme sedimented at 20 000 × *g* while most of the L-leucine-activating activity remained soluble Successive washes and treatment with deoxyribonuclease of the particulate fraction obtained by the use of lysozyme led to the progressive removal of other amino acid-activating enzymes (except that for L-threonine) and thus to a relative enrichment in the L-α,γ-diamino-

TABLE II

SUBCELLULAR DISTRIBUTION OF ACTIVATING ENZYMES IN *B polymyxa* 2459S

Sonication Sonic extracts, prepared as described in MATERIALS AND METHODS, were centrifuged at 20 000 × *g* for 30 min to yield a soluble and particulate fraction Hughes' press Cells (2 g) were crushed in a Hughes' press at −70°, suspended in 10 ml of 0 05 M potassium phosphate buffer (pH 7 5) and centrifuged as above Alumina grinding Cells (2 g) were ground in a mortar at 4° with 4 g of levigated alumina, suspended in 10 ml of 0 05 M potassium phosphate buffer (pH 7 5), centrifuged at 2000 × *g* to eliminate most of the alumina, and then centrifuged as above Lysozyme lysis Cells (1 g) were treated with lysozyme as described in MATERIALS AND METHODS and then centrifuged as above Enzyme assays were carried out at 37° for 15 min, except for the lysozyme lysate which was assayed at 25° for 30 min

Method of disruption	*(^{32}P)PP$_i$ exchange with ATP (μmoles/g of cells)*					
	Particulate fraction dependent on			*Soluble fraction dependent on*		
	L-α,γ-*Diamino-butyrate*	L-*Leu*	L-*Met*	L-α,γ-*Diamino-butyrate*	L-*Leu*	L-*Met*
Sonication	22	34	10	131	400	266
Hughes' press	33	16	4	208	460	236
Alumina grinding	37	14	8	128	382	243
Lysozyme lysis	82	27	—	56	240	—

butyrate-activating activity (Table III) Upon isopycnic centrifugation in a sucrose gradient, this activity formed a somewhat disperse band at a density of 1 20–1 28 g/ml, which was devoid of all other amino acid-activating enzymes except that for L-threonine (Fig 6)

Properties of the particle-bound activating enzyme

The apparent K_m for L-α,γ-diaminobutyrate of the particle-bound enzyme preparation was 0 7 mM (Fig 5B) and thus not significantly different from that of the soluble enzyme On the other hand, the particle-bound enzyme was distinguished by its lability at elevated temperatures, its half-life at 37° being about 5 min (Fig 7).

TABLE III

PURIFICATION OF PARTICULATE ENZYME FROM *B polymyxa* 2459S

Lysozyme lysis was carried out as described in MATERIALS AND METHODS to yield a soluble fraction and a crude particulate fraction (P-1) The latter was washed twice with half the original volume of Buffer A to yield Fraction P-2 This was suspended in half the original volume of Buffer B (10 mM Tris buffer (pH 7 5), 10 mM KCl, 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol) and treated with 1 mg of pancreatic deoxyribonuclease for 10 min at 25° The sample was then centrifuged at 30 000 × *g* for 20 min and washed once with Buffer B to yield particulate Fraction P-3 Amino acid–activating activities were measured with 2 mM amino acid at 25° for 30 min

Fraction	*Activation of (units/g of cells)*					
	L-α,γ-*Diamino-butyrate*	L-*Leu*	*L-Thr*	L-*Phe*	L-*Lys*	L-*Met*
Soluble	48	186	14	3 5	3 8	5 7
P-1	53	39	13	2 6	5 0	20
P-2	49	12	8 0	2 8	1 7	8 1
P-3	32	1 9	5 3	1 1	0 6	1 6

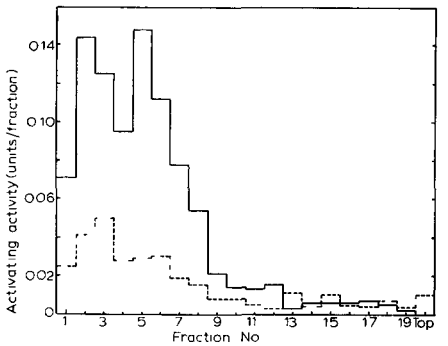
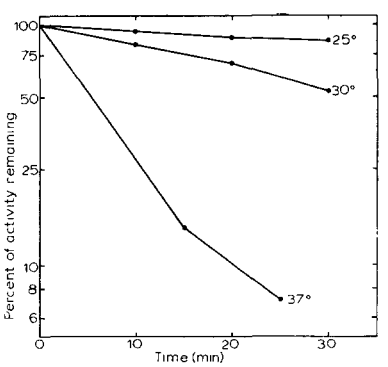


Fig 6 Isopycnic sucrose gradient centrifugation of particle-bound activating enzyme A particulate preparation, corresponding to Fraction P-3 in Table III, was further purified to eliminate any possible contamination by unbroken cells, as follows A sample (1 ml) was layered onto 4 ml of 60% sucrose in Buffer B and centrifuged at 39 000 rev /min in a Spinco SW-39 rotor for 1 h The material remaining at the top of the tube was diluted with 10 vol of Buffer B and centrifuged at 40 000 × *g* for 30 min The particulate materials was resuspended in Buffer B, and a sample (0 2 ml) containing 1 74, 0 56, and 0 05 units of activating activity for L-α,γ-diamino-butyrate, L-threonine, and L-leucine, respectively was layered onto 4 8 ml of a linear gradient of sucrose (20–60% by wt) in Buffer B After centrifugation for 48 h at 39 000 rev /min in a Spinco SW-39 rotor at 4°, fractions (0 25 ml) were collected from the bottom of the tube These were assayed at 25° for exchange of [^{32}P]PP_i with ATP in the presence of 5 mM L-α,γ-diamino-butyrate (———) or with 5 mM L-threonine (— — —)

whereas the soluble enzyme, when assayed at 37°, exhibited linear kinetics over a period of 45 min This instability necessitated that the particle-bound enzyme preparation be assayed at 25° where the rate of inactivation was markedly lower The particle-bound enzyme lost no activity when stored for a week at 4°



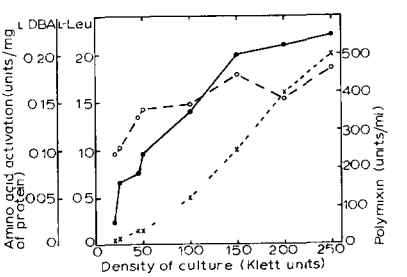


Fig 7 Stability of the particle-bound activating enzyme A washed particulate preparation from *B polymyxa* 2459s was suspended in a buffer containing 10 mM each of Tris buffer (pH 7 5), KCl, $MgCl_2$, and 2-mercaptoethanol and was incubated at the temperatures indicated Samples were removed at various times and assayed for the activation of L-α,γ-diaminobutyrate at 25°

Fig 8 Relation of amino acid activation to polymyxin production Cultures of *B polymyxa* 2459s were harvested at various densities and lysed with lysozyme as described in MATERIALS AND METHODS The activation of L-leucine (○— — —○) in the soluble fraction and that of L-α,γ-diaminobutyrate (L-DBA) (●—●) in the washed particulate fraction was assayed at 25° with 5 mM amino acid Polymyxin (×— — —×) was determined in the culture medium

Relation of L-α,γ-*diaminobutyrate activation to polymyxin production*

While the specific activity of the L-leucine-activating enzyme increased less than 2-fold during vegetative growth, that of the particle-bound L-α,γ-diaminobutyrate-activating enzyme rose 10-fold, beginning at the time of the first appearance of polymyxin in the growth medium (Fig 8) The soluble fraction of the L-α,γ-diaminobutyrate-activating enzyme increased in a similar fashion except that a slight decrease was observed towards the end of exponential growth

Absence of the enzyme from polymyxin-negative strains

A particulate and soluble fraction was prepared by the use of lysozyme from *B. polymyxa* 63, an asporogenic and polymyxin-negative mutant derived from *B polymyxa* 2459s The levels and subcellular distribution of the various activating enzymes resembled that of the parent strain, except that no activation of L-α,γ-diaminobutyrate could be observed The L-α,γ-diaminobutyrate-activating enzyme was also absent from sonic extracts of this strain and of four other polymyxin-negative and asporogenic strains derived from *B polymyxa* 2459s

Activation of L-α,γ-*diaminobutyrate in other polymyxin-producing organisms*

The presence of the L-α,γ-diaminobutyrate-activating enzyme was also investigated in *B polymyxa* ATCC 10401 and *B circulans* ATCC 14040, producers of polymyxin D and circulin, respectively Table IV shows that these strains exhibited L-α,γ-diaminobutyrate-activating activity but that the method of disruption of the cells was critical In general, sonication resulted in negligible activity, while methods that generate less heat yielded activities comparable to those observed in *B polymyxa* 2459s This probably reflects the labile nature of the enzyme in these strains, since the activity in extracts of *B polymyxa* 10401 was almost completely lost upon storage at 4° overnight or upon storage a week in 40% ethylene glycol at −15° Like with *B polymyxa* 2459, these methods of disruption resulted in the solubilization of most of the L-α,γ-diaminobutyrate-activating activity Unfortunately, protoplast

TABLE IV

L-α,γ-DIAMINOBUTYRATE ACTIVATION BY *B polymyxa* 10401 AND *B circulans* 14040

Extracts were prepared in Buffer B as described in Table II, and centrifuged at 30 000 × *g* for 20 min The supernatant solution was assayed by the standard procedure

Organism	*Method of extraction*	*Activation of (units/mg of protein)*	
		L-α,γ-*Diamino-butyrate*	L-*Leu*
B polymyxa 10401	Hughes' press	0 16	1 2
B circulans 14040	Alumina grinding	0 35	3 1
B circulans 14040	Sonication	<0 01	3 1

formation with lysozyme was not successful with these organisms, and the subcellular distribution of the activating enzyme could therefore not be studied

DISCUSSION

The specificity of the activating enzyme for L-α,γ-diaminobutyrate is indicated by the relatively low apparent K_m for this amino acid (0 6 mM) and by the fact that preparations which are considerably enriched for the activation of L-α,γ-diaminobutyrate relative to that of other amino acids can be obtained by simple purification steps Moreover, the particle-bound L-α,γ-diaminobutyrate-activating enzyme can be separated from all other amino acid-activating activities (except that for L-threonine) by mere washing of the particulate fraction That the L-α,γ-diaminobutyrate-activating activity is distinct from the particle-bound L-threonine-activating enzyme is suggested by the fact that extracts of *B polymyxa* 63 lack the former but have normal levels of the latter

The activating enzyme in sonic extracts of *B polymyxa* 2459 appears to exist as aggregates of relatively high molecular weight which can be dissociated into subunits of molecular weight 100 000 by treatment with streptomycin sulfate This is reminiscent of the behaviour of membrane proteins which, when solubilized, frequently tend to form high molecular weight aggregates (*e g* ref 24) Indeed, when lysed protoplasts of *B polymyxa* were examined, a large fraction of the L-α,γ-diaminobutyrate-activating enzyme was found to be associated with the particulate fraction, presumably the cytoplasmic membrane This association seemed to be quite specific, since no other amino acid-activating enzyme (except that for L-threonine) was observed to be particle bound Unlike the solubilized enzyme, the particle bound L-α,γ-diaminobutyrate-activating enzyme was found to be quite unstable at temperatures above 25° This difference in stability of the soluble and particulate enzyme appears to be an example of allotypy[26] Another example of such behavior is the adenosine triphosphatase activity of coupling factor F_1, which becomes subject to inactivation by cold upon solubilization from the mitochondrial membrane[27]

Several observations suggest that the enzyme which activates L-α,γ-diaminobutyrate may play a role in the biosynthesis of polymyxin (a) The specific activity of the enzyme increased markedly during vegetative growth, beginning just prior to the first appearance of polymyxin B in the growth medium An analogous observation has been made by OTANI *et al* [22] with the ornithine-activating enzyme from *B brevis*, which is first detected when gramicidin S formation begins (b) The activation of L-α,γ-diaminobutyrate could not be observed in mutants that have lost the ability to form polymyxin (c) Organisms which produce the related antibiotics polymyxin D and circulin are also able to activate L-α,γ-diaminobutyrate The failure of DIGIROLAMO and co-workers[15,23] to detect the activation of L-α,γ-diaminobutyrate in extracts of *B polymyxa* 10401 and *B circulans* 14040 is probably due to the instability of the enzyme in these organisms Our results confirm that little activity can be expected under the conditions used by these workers, which involved disruption by sonication or shaking with glass beads and 24-h dialysis prior to assay

The nature of the reaction catalyzed by the L-α,γ-diaminobutyrate-activating enzyme is still obscure Since the amino acid is required for the exchange of PP_i with ATP, it is likely that an L-α,γ-diaminobutyryl adenylate is the initial product

of the reaction Such a compound would be expected to be very unstable on account of its tendency to cyclyze to 3-aminopyrrolidone-2 (*e g* ref 25). The adenylate must either be stabilized by the enzyme or react immediately with an acceptor. A transfer of L-α,γ-diaminobutyrate by the activating enzyme to another compound, such as transfer RNA, a peptide, or an amino acid, has, however, not yet been detected (K JAYARAMAN AND H PAULUS, unpublished observations) It is thus not possible to define the role of the L-α,γ-diaminobutyrate-activating enzyme in polymyxin biosynthesis, especially since a cell-free system capable of mediating the biosynthesis of this antibiotic has not yet been developed A similar failure to detect an intermediate exists in tyrocidine biosynthesis, where one of the enzyme fractions catalyzes the exchange of PP_i with ATP in the presence of the amino acids that constitute tyrocidine[6,10,14,22] but mediates neither a formation of aminoacyl RNA (refs 10 and 22) nor an amino acid-dependent exchange of AMP with ATP[10].

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