ORNITHINE ACTIVATING ENZYME FROM BACILLUS BREVIS*

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Current studies on biosynthesis of gramicidin S have revealed that the peptide is synthesized by cell-free system from Bacillus brevis and the mechanism of its formation is different from that in usual protein synthesis, i.e. there is no participation of any RNA or influence of any inhibitor on protein biosynthesis (Yukioka et al., 1965; Bhagavan et al., 1966; Spaeren et al., 1967; Tomino et al., 1967). The activations of the five constituent amino acids were each shown to occur by amino acid dependent PPi-ATP exchange reactions, but amino acyl-tRNA formation was not observed (Gevers et al., 1968). Among these reactions, it is most interesting to study the mechanism of activation of ornithine, since ornithine is not involved in usual protein biosynthesis.

In this paper, a procedure for partial purification of the ornithine activating enzyme from extracts of Bacillus brevis Nagano, some properties of the enzyme and also the relation of this enzyme to the formation of gramicidin S are presented.

MATERIALS AND METHODS

Bacillus brevis Nagano was used throughout. Nutrient broth containing 10 g polypeptone, 10 g meat extract, 5 g NaCl and 0.5 g $MgSO_4$ in 1 liter of distilled water was used. The organism was grown in this

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medium with aeration and agitation at 37°C. About 100 g wet weight of cells were obtained from 15 liter of culture at the late logarithmic phase of growth and stored at -15°C. Hydroxylapatite was prepared by the procedure of Tiselius (Tiselius et al., 1956) and stored in 0.001 M phosphate buffer, pH 6.8. DEAE-Sephadex A-25 was obtained from Pharmacia. L-Phenylalanine-U-^{14}C (specific activity 7.0 mC/mmole) and L-arginine-U-^{14}C (specific activity 6.4 mC/mmole) were purchased from Radiochemical Centre, Amersham. L-Ornithine-U-^{14}C was prepared from L-arginine-U-^{14}C by treatment with arginase. ^{32}PPi was produced from ^{32}Pi by heating at 400°C for 60 minutes. The reaction system for gramicidin S formation and isolation of this peptide were the same as in the previous report (Yukioka et al., 1965). The incorporation of L-phenylalanine-U-^{14}C into gramicidin S was measured as de novo synthesis of peptide using a Nuclear Chicago automatic gasflow counter and sample of infinite thinness. Ornithine activation was determined by measuring PPi-ATP exchange dependent upon ornithine, according to the method of Calendar (Calendar et al., 1966), with some modification. The reaction mixture contained Tris:HCl buffer, pH 8.0, 10 µmoles, $MgCl_2$ 5 µmoles, L-ornithine 2.5 µmoles, ATP 2.5 µmoles, ^{32}PPi 2.5 µmoles (40,000-50,000 cpm), potassium fluoride 35 µmoles, 2-mercaptoethanol 15 µmoles and enzyme 0.2-2.0 mg protein in a final volume of 1.5 ml. Incubation was carried out at 37°C for 15 minutes. The reaction was stopped by addition of 1.0 ml of 12 % of trichloroacetic acid solution. Fifty mg of charcoal were added to the deproteinized reaction mixture. The charcoal suspension was filtered on a carbon filter (Schleicher & Shuell) and the residue was washed 5 times with 10 ml volumes of distilled water. The charcoal and carbon filter were transfered together to a planchet, dried under a lamp and radioactivity was counted in an endwindow counter. The values were corrected for the value of a blank run without ornithine, but no correction was made for self absorption. One unit of enzyme was

defined as the amount of enzyme incorporating 1 μmole of ^{32}PPi into ATP in 15 minutes at 37°C. The specific activity of the enzyme was defined as enzyme units per milligram of protein. Ornithyl-tRNA formation was measured by the method described by Calendar (Calendar et al., 1966). Bacillus brevis tRNA was prepared by the method of Zubay (Zubay, 1966). The radioactivity of the ornithyl-tRNA synthesized was estimated with a Nuclear Chicago Liquid Scintillation Counter using toluene scintillation medium. Protein was measured by the method of Kalkar (Kalkar, 1947).

RESULTS AND DISCUSSION

Frozen cells were thawed and suspended in 0.01 M Tris:HCl buffer, pH 8.0, containing 0.01 M of $MgCl_2$ and 2-mercaptoethanol. Cell disruption, ammonium sulfate precipitation and fractionation by hydroxylapatite column chromatography were carried out as described in the previous paper (Otani et al., 1966). The ornithine activating enzyme was found in the final fraction (Fr-0.15) from the hydroxylapatite column which was eluted with 0.15 M potassium phosphate buffer, pH 6.8. The enzyme was precipitated by 50 % saturation of ammonium sulfate. The precipitate was dissolved in a minimum amount of 0.1 M Tris:HCl buffer, pH 8.0, and dialyzed against the same buffer. The dialyzed enzyme solution was applied on a DEAE-Sephadex column (3 x 50 cm). After washing with 0.1 M Tris:HCl buffer, pH 8.0, containing 0.2 M potassium chloride, the column was developed with a linear concentration gradient of potassium chloride made from 400 ml of 0.2 M potassium chloride and 400 ml of 0.5 M potassium chloride in the same buffer. Fractions 70 through 80, inclusive, were combined and represented the most purified enzyme fraction. The elution pattern from the column is shown in Fig. 1. The results of purification are summarized in Table 1. The enzyme was purified about thirty fold in specific activity.

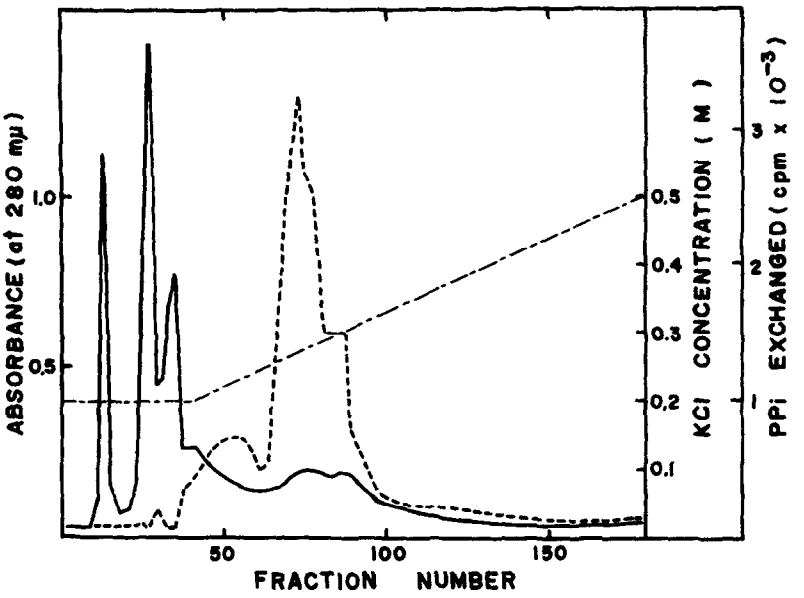

Fig. 1. DEAE-Sephadex column chromatography.
The column was developed as described in the text at a flow rate of 30 ml per hour at 0-3°C. Fractions of 5.5 ml were collected per tube.

——— Absorbancy at 280 mμ
- - - - - - Ornithine dependent incorporation of PPi into ATP
— · — · — KCl concentration

Table 1. Purification of ornithine activating enzyme.

Fraction	Volume (ml)	Protein (mg/ml)	Specific Activity (units/mg)	Total Units	Yield (%)
Supernatant of 44,330 x g centrifugation	550	49.3	0.037	1003	100
Precipitate with 36-45 % ammonium sulfate	84	52.2	0.121	531	53
Hydroxylapatite (Fr-0.15)	17	33.0	0.591	332	33
DEAE-Sephadex	50	0.2	1.180	12	1.2

The effects of ornithine analogues on the PPi-ATP exchange reaction are shown in Table 2. When Fr-0.15 was used as the enzyme preparation, L-lysine caused a slight increase in the exchange reaction. Other analogues had less than 10 % of the effect of ornithine. Using the most purified enzyme fraction in a similar experiment, none of these analogues stimulated the exchange reaction. The Km value was 6.7×10^{-5} M for ornithine. These results show that the PPi-ATP exchange reaction was not a false reaction of arginyl or lysyl tRNA synthetase.

Table 2. Effect of ornithine analogues.

Analogue added (2.5 µmoles)	* PPi exchanged (mµmoles)	** PPi exchanged (mµmoles)
L-Ornithine	746.2	64.3
L-Arginine	33.8	3.5
L-Lysine	97.5	2.0
DL-α-Amino-n-butyric acid	31.9	—
γ-Guanidinobutyric acid	5.3	0
L-Argininic acid	53.4	3.8
L-Canavanine	3.1	0

* Hydroxylapatite fraction (Fr-0.15) was used as enzyme source.
** The most purified enzyme fraction was used as enzyme source.
The activities in the two columns cannot be compared, because different batches of enzyme were used to prepare the fractions.

Next, the relation of enzyme activity to gramicidin S formation during cell growth was studied. Aliquots of culture were removed at appropriate intervals. The cells were homogenized and the fraction precipitating at 0-50 % saturation of ammonium sulfate was used to assay ornithine activation and gramicidin S formation. As shwon in Fig. 2,

the highest activity was found in the late logarithmic phase of growth. The activity of the ornithine activating enzyme was almost parallel to that of gramicidin S formation. No formation of ornithyl-tRNA was observed.

These results strongly suggested that the ornithine activating enzyme is responsible for gramicidin S formation and ornithyl adenylate seems to be intermediate.

With the most purified enzyme fraction, the activations of the other four constituent amino acids were observed as well as that of ornithine but resolution of the former enzymes was not achieved. It was not decided whether the enzymes activating the other four amino acids were amino acyl tRNA synthetases or not. The relation of the present enzyme fraction to Fraction I reported by Tomino et al.(1967) and Gevers et al. (1968) is not clear.

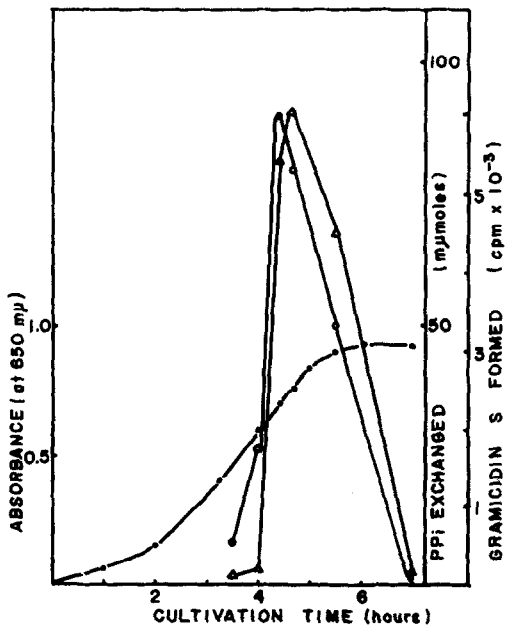

Fig. 2. Changes of ornithine activation and gramicidin S formation during cell growth.
●——● Cell growth, absorbancy at 650 mμ
○——○ Activation of ornithine, mμmoles of PPi incorporated into ATP
△——△ Gramicidin S formation, radioactivity of phenylalanine incorporated into gramicidin S

Some properties and the kinetics of the ornithine activating enzyme were also studied. Complete loss of activity was observed in the presence of 1 x 10^{-3} M of p-chloromercuribenzoate or mercuric chloride and partial loss of activity in the same concentration of 5,5'-dithio-bis-2-nitrobenzoic acid or N-ethylmaleimide. The most purified enzyme fraction required 2-mercaptoethanol for full activity of the PPi-ATP exchange reaction.

These results suggested that the ornithine activating enzyme required a sulfhydryl group for activity.

In the absence of Mg^{++} ions, no PPi-ATP exchange was observed. The optimal Mg^{++}/ATP ratio was 2.0-3.0. A high concentration of Mg^{++} ions markedly inhibited the exchange reaction.

The pH optimum of the reaction was between 7.6 and 8.3.

Further purification of the enzyme and detailed studies on the activation mechanism are now in progress.

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