ACTIVATION OF L-ORNITHINE BY CELL-FREE EXTRACTS OF *BACILLUS BREVIS* CATALYZING THE SYNTHESIS OF GRAMICIDIN S

T.SAND, J.C.SIEBKE and S.LALAND
Department of Biochemistry, University of Oslo, Blindern, Norway

Received 6 June 1968

1. Introduction

The formation of the peptide bonds of the cyclic decapeptide gramicidin S involves the activation of the constituent amino acids. The availability of a cell-free system [1] makes possible an investigation on the activation of the amino acids present in gramicidin S. A study on the activation of L-ornithine seemed a good choice as it is probably not present in bacterial proteins. Hence the present report describes experiments on the activation of this amino acid.

It has been found that cell-free extracts of *Bacillus brevis* contain enzymatic activity which in the presence of L-ornithine catalyzes an ATP-[^{32}P] pyrophosphate exchange reaction and the formation of ornithyl hydroxamate in the presence of hydroxylamine. The experiments suggest that ornithine is activated by the formation of ornithyl-AMP. However, the ornithyl-group is not transferred to t-RNA.

2. Materials and methods

2.1. *Preparation of cell-free extracts*

The *B. brevis* strain ATCC 9999 was grown under aeration in the synthetic medium described previously [2]. Cultures were harvested at a density of 0.38 - 0.40 measured at 650 μm in 1 cm cells in a Coleman Junior spectrophotometer. The 11000 × *g* supernatant used as crude extract was prepared as described by Spaeren et al. [3], except that the cells were suspended in 0.1 M Tris HCl, pH 7.5, containing 0.005 M $MgCl_2$ and 0.005 M 2-mercaptoethanol, and then submitted to ultrasonication for 1.5 min.

2.2 *Fractionation of crude extract*

In order to remove RNA, 1 M $MnCl_2$ (4.5 ml) was added to the 11000 × *g* supernatant (30 ml). The precipitate was centrifuged down after 10 min and solid $(NH_4)_2SO_4$ added to the supernatant. Fractions at 30% and 40% saturation were removed by centrifugation after standing for 30 min each time. The precipitates were dissolved in 0.1 M Tris-HCl buffer, pH 7.5 (10 ml) containing 0.005 M $MgCl_2$ and 0.005 M 2-mercaptoethanol. All manipulations were performed at 0 - 5°. In one experiment the 40% $(NH_4)_2SO_4$ fraction was prepared directly without prior precipitation of the 30% fraction.

2.3. *Formation of ornithyl hydroxamate*

The hydroxamate was estimated as described by Conway, Lansford and Shive [4]. An incubation mixture (1 ml) with a final pH 7.3 containing 1 mmole salt-free NH_2OH [5], 10 μmole ATP, 5 μmole L-ornithine, 10 μmole $MgCl_2$, 3 μmole 2-mercaptoethanol, and 0.5 ml enzyme solution (in 0.1 M Tris-HCl containing 0.005 M $MgCl_2$ and 0.005 M 2-mercaptoethanol) was incubated for 50 min at 37°. The mixture was then added to an aqueous solution of TCA (10% w/v) containing HCl (5% w/v) and $FeCl_3$ (10% w/v), the precipitate removed by centrifugation and the hydroxamate complex was determined spectrophotometrically at 540 mμ in a Unicam Spectrophotometer. An incubation mixture containing no ornithine served as a blank. The amount of hydroxamate formed was calculated using a synthetic amino acyl hydroxamate as a standard.

Standard ornithine hydroxamate was synthesized by the method of Safir and Williams [6]. The orni-

thyl hydroxamate formed as a result of ornithine activation in the incubation mixture was isolated by the method of Hoagland, Keller and Zamecnik [7]. The ornithyl hydroxamate was identified on thin layer plates (MN-cellulose powder 300, Macherey, Nagel and Co., Germany) developed in sec.-butanol-formic acid – water (15 : 15 : 10, v/v/v) and sprayed with a mixture of $FeCl_3 \cdot 6H_2O$ (5 g), conc. HCl (5 ml) and water (95 ml).

2.4. *Ornithine-dependent ATP pyrophosphate exchange*

The procedure described by McCorquodale and Mueller [8] was used. An incubation mixture (1 ml) with a final pH of 7.3 containing 5 μmole of ATP, 10 μmole $MgCl_2$, 3 μmole 2-mercaptoethanol, 5 μmole ornithine, 10 μmole KF, 2 μmole [^{32}P]-pyrophosphate (prepared from [^{32}P]orthophosphate by the method of Conway et al. [4] and 0.5 ml of enzyme solution (in 0.1 M Tris HCl containing 0.005 M $MgCl_2$ and 0.005 M 2-mercaptoethanol) was incubated at 37° for 15 min. TCA (1 ml, 10% w/v) was added and the precipitate removed by centrifugation. To the supernatant was added 100 mg acid washed charcoal and 0.2 M sodium acetate (2 ml) and a solution containing 0.125 M $Na_4P_2O_7$ and 0.125 M KH_2PO_4, pH 4.5 (2 ml). The charcoal was centrifuged down after 15 min and washed four times with 0.2 M sodium acetate, pH 4.5, 3 ml each time and finally twice with distilled water, 3 ml each time. The charcoal was suspended in N HCl (4 ml) and the mixture heated at 100° for 15 min. Aliquots of the supernatant were counted.

2.5. *Preparation of t-RNA fraction*

The method of Hall, Sedat, Adiga, Uemura and Winnick [9] for *B. brevis* t-RNA was used. The t-RNA fraction was precipitated by ethanol in the presence of potassium acetate and the t-RNA stripped of amino acids by the method of Lipmann [10]. The t-RNA was dissolved in 0.1 M tris HCl buffer, pH 7.5, containing 0.005 M $MgCl_2$ (2 mg t-RNA per 0.6 ml).

2.6. *Formation of amino acyl t-RNA*

The procedure was similar to that described by Mach and Tatum [11]. An incubation mixture (0.9 ml) with a final pH adjusted to 7.5 with KOH containing 5 μmole ATP, 5 μmole $MgCl_2$, 2 μmole 2-mercaptoethanol, 0.05 μmole of [^{14}C]-amino acid (0.5 μC), 0.2 ml enzyme solution and 0.6 ml t-RNA solution (2 mg) was incubated for 15 min at 37°. The reaction was terminated by the addition of icecold 0.5 M $HClO_4$ (1.5 ml) containing the corresponding [^{12}C]-amino acid (1 mg/ml). The precipitate was washed three times with ice-cold 0.25 N $HClO_4$ solution, 3 ml each time, then with ethanol-ether and finally ether. The precipitate was dissolved in 0.1 N NaOH (0.2 ml) and an aliquot counted. An incubation mixture without t-RNA was used as a blank.

2.7. *Estimation of protein*

The method used was that described by Zamenhof [12]. Egg albumin was used as a standard.

2.8. *Measurement of radioactivity*

Radioactivity was measured with a Frieseke and Hoepfner Gas Flow counter.

2.9. *Radioactive amino acids*

The following amino acids were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.: L[5-^{14}C] ornithine (9.8 mC/m-mole), L[U-^{14}C]-phenylalanine (360 mC/m-mole), L[U-^{14}C] leucine (222 mC/m-mole).

3. Results and discussion

The activation of ornithine was studied using the [^{32}P] pyrophosphate-ATP exchange reaction and hydroxamate formation both in the crude 11000 × *g* supernatant of *B. brevis* and in fractions (of the 11000 × *g* supernatant) obtained after precipitation, first at 30% $(NH_4)_2SO_4$, and subsequently at 40% $(NH_4)_2SO_4$ saturation. The results are recorded in table 1. The [^{32}P] pyrophosphate-ATP exchange found without the addition of L-ornithine is due to the presence of amino acids in these comparatively crude fractions. It is seen (table 1) that the addition of L-ornithine results in a significant increase in the radioactivity of ATP. When [^{32}P] orthophosphate replaced [^{32}P] pyrophosphate in the exchange experiments, addition of L-ornithine gave no significant increase in the radioactivity of ATP.

Table 1
The activation of ornithine in fractions of *B. brevis* measured by ^{32}P-pyrophosphate ATP exchange reaction and hydroxamate formation (for a description of the fractions see section 2).

Fraction	Protein in incubation mixture	Amino acid added	Radioactivity in ATP (counts/min)	Hydroxamate formed as result of ornithine addition (μmole)
11000 × *g* supernatant	3	None	24840	
11000 × *g* supernatant	3	L-ornithine	28400	1.0
30% $(NH_4)_2SO_4$ fraction	0.8	None	3920	
30% $(NH_4)_2SO_4$ fraction	0.8	L-ornithine	13760	2.0
40% $(NH_4)_2SO_4$ fraction	1.3	None	14280	
40% $(NH_4)_2SO_4$ fraction	1.3	L-ornithine	29860	3.5

Table 2
Formation of amino acyl t-RNA using the 40% $(NH_4)_2SO_4$ fraction as the enzyme source. The 40% $(NH_4)_2SO_4$ fraction was prepared without previous precipitation of the 30% $(NH_4)_2SO_4$ fraction (see Methods). Each incubation mixture contained 0.65 mg protein.

^{14}C-amino acid added	Incorporation in t-RNA (counts/min)
L-ornithine	0
L-phenylalanine	7160
L-leucine	21800

The presence of hydroxylamine, L-ornithine and ATP results in the formation of hydroxamate (table 1). Ornithyl hydroxamate in the incubation mixture was demonstrated by thin layer chromatography in sec. butanol-formic acid – water.

Hence it appears (table 1) that L-ornithine activation occurs in the 11000 × *g* supernatant and that activating enzymes precipitate at a concentration of 30-40% $(NH_4)_2SO_4$ saturation. The results indicate that L-ornithine is activated by the following reaction:

$$\text{L-ornithine} + \text{ATP} \leftrightharpoons \text{L-ornithyl-AMP} + PP_i .$$

L-ornithine seems to be activated by a mechanism similar to that of the amino acids in protein synthesis. However, it is seen from table 2 that the 40% $(NH_4)_2SO_4$ fraction which activates L-ornithine, will not transfer this amino acid to t-RNA whereas the same fraction will activate and charge t-RNA with L-phenylalanine and L-leucine. In this respect the activation of L-ornithine differs from that found for the amino acids involved in protein synthesis. Earlier studies on the biosynthesis of gramicidin S have indicated that RNA does not participate [1,3]. The present result is therefore in agreement with this earlier finding.

Since the fractions which have been found to activate L-ornithine also catalyze the formation of gramicidin S, it seems likely that the activation of L-ornithine described participates in the biosynthesis of gramicidin S.

The mechanism of activation of L-ornithine is similar to that suggested for the activation for L-phenylalanine in the biosynthesis of gramicidin S [13]. This mechanism differs from that suggested for the synthesis of peptides like glutathione [14], ophthalmic acid [15] and the cell wall peptide in *Staphylococcus aureus* [16]. During synthesis of these peptides ADP and inorganic phosphate are formed from ATP.

References

[1] J.E.Bredesen, T.L.Berg, K.J.Figenschou, L.O.Frøholm and S.G.Laland, European J. Biochem., in press.
[2] T.S.Eikhom, J.Jonsen, S.G.Laland and T.Refsvik, Biochim. Biophys. Acta 76 (1963) 648.
[3] U.Spæren, L.O.Frøholm and S.G.Laland, Biochem. J. 105 (1967) 451.
[4] T.M.Conway, E.M.Lansford and W.J.Shive, J. Biol. Chem. 237 (1962) 2850.
[5] H.Beinert, D.E.Green, P.Hele, H.Hift, R.W.von Korff and C.V.Ramahrishman, J. Biol. Chem. 203 (1953) 35.
[6] S.R.Safir and J.H.Williams, J. Org. Chem. 17 (1952) 1298.
[7] M.B.Hoagland, E.B.Keller and P.Zamecnik, J. Biol. Chem. 218 (1956) 348.

[8] D.J.McCorquodale and D.C.Mueller, J. Biol. Chem. 232 (1958) 51.
[9] J.B.Hall, J.W.Sedat, P.R.Adiga, I.Uemura and T.Winnick, J. Mol. Biol. 12 (1965) 162.
[10] G.Van Ehrenstein and F.Lipmann, Proc. Nat. Acad.. Sci. U.S. 47 (1961) 941.
[11] B.Mach and T.L.Tatum, Proc. Nat. Acad. Sci. U.S. 52 (1964) 876.
[12] S.Zamenhof, in: Methods in Enzymology, Vol. 3, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York, 1957) p. 762.
[13] M.Yamada and K.Kurahashi, J. Biochem. (Jap.) 63 (1968) 59.
[14] S.Mandeles and K.Bloch, J. Biol. Chem. 214 (1956) 639.
[15] J.S.Nishimura, E.A.Dodd and A.Meister, J. Biol. Chem. 239 (1964) 2553.
[16] S.T.Nathenson, J.L.Strominger and E.Ito, J. Biol. Chem. 239 (1964) 1773.