BIOSYNTHESIS OF VALINOMYCIN

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1. Introduction

Valinomycin is a cyclododecadepsipeptide consisting of three residues each of L-valine, α-D-hydroxyisovaleric acid, D-valine and L-lactic acid joined together by alternating amide and ester linkages [1]. The results on the biosynthesis of gramicidin S [2], tyrocidine [3] and alamethicin [4] suggest that valinomycin is formed by a similar non-ribosomal peptide synthesizing mechanism. In this report we describe the isolation of an enzyme complex that is capable of synthesizing the complete molecule of valinomycin from the precursors L-valine and L-alanine or L-threonine.

2. Materials and methods

2.1. *Growth of the organism and identification of [^{14}C] valinomycin*

A valinomycin producing strain of *Streptomyces fulvissimus* was grown at 37°C with vigorous stirring and aeration in 10 l of medium containing 200 g soy bean peptone (Merck) and 100 g yeast extract (Merck). When the optical density at 600 nm was 1.5, the cells (about 15 g) were harvested at room temperature, and reincubated in 10 l synthetic medium (200 g sodium glutamate, 100 g glucose, 10 g KH_2PO_4, and 25 mg each of $FeSO_4 \cdot 7\,H_2O$, $CaCl_2$, $MgSO_4 \cdot 7\,H_2O$, and $ZnSO_4 \cdot 7\,H_2O$) for 1 hr. The cells were collected by centrifugation and kept frozen at −90°C.

For in vivo experiments 20 ml of cell suspension (optical density = 1.5) were centrifuged, washed twice with synthetic medium and incubated overnight in 20 ml of the same medium containing 1 μCi of L-[^{14}C]-valine (228 mCi/mM) or of L-[^{14}C]alanine (128 mCi/mM), or of L-[^{14}C]threonine (180 mCi/mM). The cells were centrifuged and extracted twice with 80% methanol. The supernatant was concentrated and extracted with *n*-butanol. The combined extracts were reextracted with ethyl acetate and finally chromatographed on silica thin layer plates in 50% (v/v) *n*-propanol in H_2O or cyclohexane:pyridine:acetic acid = 100:15:5 (v/v/v) and valinomycin was detected by autoradiography. Authentic valinomycin as reference was visualized by the chlorination–iodide/starch reaction. For quantitation the radioactive valinomycin was eluted with ethyl acetate and the radioactivity estimated in a Nuclear Chicago Isocap scintillation counter. Hydrolysis of the cyclic peptide was performed according to Brockmann et al. [1]. [^{14}C]Valine, [^{14}C]hydroxyisovaleric acid and [^{14}C]lactic acid were identified by high voltage electrophoresis in a Savant apparatus on Whatman 3 MM paper at pH 1.9 and pH 6.4, respectively. The paper strip was cut into segments and radioactivity was estimated in a scintillation counter. D-Valine and L-valine were determined with L- and D-amino acid oxidase. Occasionally the peptide antibiotic was identified, in addition, by its ionophoretic properties in chloroplast membranes [5].

2.2. *Enzyme preparation and purification*

Batches of cells (about 100 g) were suspended in a buffer containing: 0.02 M Tris–HCl, pH 7.8; 0.00025 M EDTA; 0.01 M magnesium acetate; 0.001 M DTT, and 5% glycerol (buffer A) and disrupted in a glass bead homogenizer (Bühler, Tübingen) in the presence of 10 mg of lysozyme. The extract was treated with 500 μg of DNAse I. Cell debris was removed by centrifugation at 10 000 *g* and ribosomes were pelleted at

100 000 *g*. Protein was concentrated by ammonium sulfate precipitation (60%). The enzyme preparation was passed through a Sephadex G-200 column equilibrated with buffer A. The enzyme-containing fractions were located according to Gevers et al. [6] by the ATP–PP_i exchange reaction. Appropriate fractions were pooled and concentrated by ammonium sulfate precipitation. After dialysis the enzyme fraction was loaded onto a DEAE column, equilibrated with buffer A. The proteins were fractionated with a 900 ml linear gradient of 0–0.4 M KCl in buffer A. To demonstrate the formation of AMP during amino acid activation, aliquots of the enzyme fraction were incubated with 0.04 μCi [^{14}C]ATP (spec. act. 418 mCi/mM) in the presence of TRA, pH 7.8 (0.025 M), magnesium acetate (0.01 M), EDTA (0.0025 M), DTT (0.0001 M) and the appropriate amino acids (0.005 M) at 37°C for 20 min. The samples were chromatographed on PEI plates in 1.2 M LiCl. Radioactivity was detected with a Berthold Thin Layer Scanner, System BF 210.

2.3. *Synthesis of valinomycin in vitro*

The reaction mixture contained 1 ml of enzyme purified by DEAE-chromatography in a total volume of 2 ml of TRA, pH 7.8 (0.025 M), magnesium acetate (0.01 M), KCl (0.01 M), DTT (0.0005 M), ATP (0.002 M), L-[^{14}C]val (0.001 mM = 1 μCi), L-ala (0.005 M), NADH (0.005 M). For ATP regeneration 250 μg phosphoenolpyruvate kinase was added. To label the lactyl portion, unlabelled alanine was replaced by an equivalent amount of L-[^{14}C]alanine or L-[^{14}C]threonine. When threonine was used in the system, NADH was replaced by NAD and pyridoxalphosphate (0.005 M) as well as glutathione (0.005 M) were added. Occasionally the synthesis was performed in the presence of RNAse (50 μg/ml) or puromycin (20 μg/ml). The reaction mixture was incubated for 1 hr at 37°C and valinomycin was extracted with butanol and ethyl ester as described above.

3. Results

3.1. *Production of valinomycin in Streptomyces fulvissimus*

The valinomycin production in *Streptomyces fulvissimus* was measured by short term incorporation of [^{14}C]valine. It was found that the formation of valinomycin occurs only within a limited time span at the end of the logarithmic phase of growth.

L-Valine forms three quarters of the valinomycin backbone as the precursor of both D- and L-valine, as well as of the D-hydroxy-iso-valeroyl moiety [7]. Incorporation studies with [^{14}C]alanine identify this amino acid in addition as a possible precursor of the lactyl portion. Lactate can be formed from alanine via deamination and reduction. Also threonine is incorporated into the lactyl portion and apparently more efficiently than alanine. At the moment, however, the mechanism by which threonine is transformed to lactic acid is not understood.

When valinomycin was labelled in vivo by [^{14}C]-valine and [^{14}C]alanine quantitative chemical analysis after acid hydrolysis showed a molar ratio of L-valine: D-valine: hydroxyisovaleric acid: lactic acid as 1.0:1.0:0.6:0.1–0.2. The low incorporation into the hydroxy acids might be due to the existence of an internal pool as suggested by MacDonald [7]. The value for lactic acid might reflect incomplete recovery of this compound. The labelled valinomycin is active as an ionophore in chloroplast membranes proving its molecular integrity.

3.2. *Isolation of the valinomycin synthesizing enzyme*

To identify the valinomycin synthesizing enzyme after Sephadex G-200 filtration, the fractions were tested for amino acid activation by the ATP–PP_i exchange reaction (fig. 1). Of the constituents of valinomycin, only L-valine was activated. No activation of D-valine, D-hydroxyisovaleric acid, lactate or of L-alanine, a known precursor of lactate, was found. A significant background activation without amino acids, however, was observed. The L-valine activating peak is quite broad and may contain additional activating species such as tRNA synthetases. Another possibility is that the broad peak reflects aggregated or degraded enzyme species which are still able to activate L-valine.

Within this L-valine activating peak a threonine activating enzyme was localized (fig. 1). This enzyme exhibits a remarkable high background activity. The fractions containing the threonine activating enzymes were further purified by DEAE-cellulose chromatography. In table 1 the activation of the constituents as well as of L-alanine and L-threonine are listed, demonstrating that only L-valine and – to a much smaller

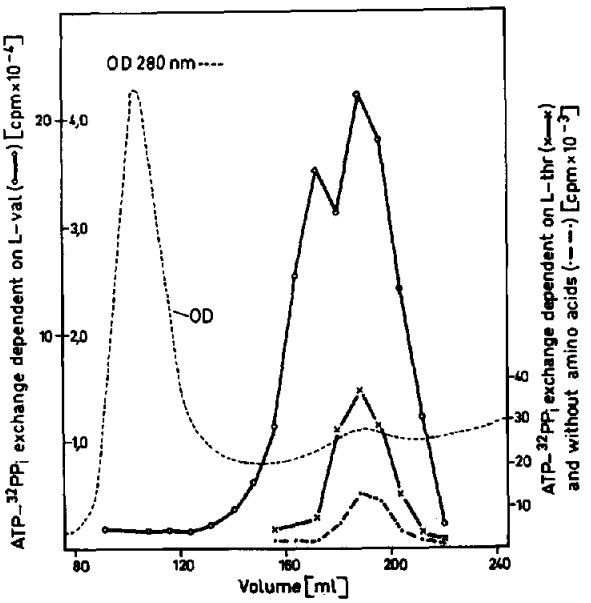

Fig. 1. Sephadex G-200 filtration of a ribosome free extract. Fractions of 4 ml were collected and aliquots of 50 μl were tested for L-valine- and L-threonine-activation as well as for 'background activation' by the ATP–PP_i exchange reaction.

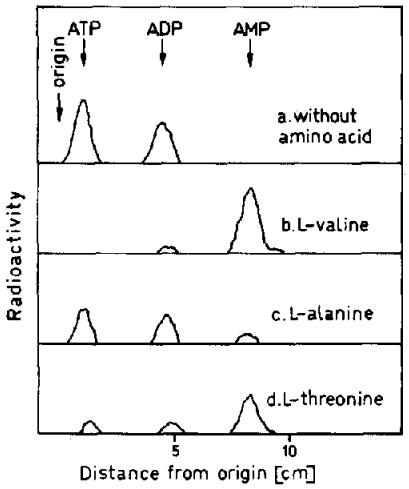

Fig. 2. Formation of AMP and ADP from [^{14}C] ATP during amino acid activation by the enzyme fraction after purification on DEAE cellulose. Samples were chromatographed on PEI plates and radioactivity monitored with a Thin Layer Scanner.

extent – L-threonine are activated. The 'background activity' of this fraction is remarkably high, which can not be explained at the moment. In addition pyruvate, pyruvate plus NADH, phosphoenolpyruvate and propionic acid were tested for activation without positive results. The amino acids L-phenylalanine, glycine, L-glutamic acid and L-lysine were also not activated. L-Leucine, however, showed activation comparable to L-valine suggesting a certain unspecificity of the enzyme. More information about the activation of amino acids by the enzyme fraction derives from experiments following the fate of [^{14}C] ATP during the reaction. The enzyme incubated with [^{14}C] ATP but without amino acids hydrolizes ATP to ADP and P_i as shown in fig. 2a. It is not yet clear whether this hydrolysis is due to the amino acid-activating enzymes or to a contaminant. No hydrolysis occurs without enzyme. In the presence of L-valine [^{14}C] ATP is converted to AMP and PP_i as expected from the ATP–PP_i exchange reaction. The formation of ADP by the enzyme is almost completely suppressed (fig. 2b). In the presence of L-alanine some AMP is formed from ATP (fig. 2c). This is in contrast to the finding that L-alanine is not activated by the ATP–PP_i exchange reaction. In the presence of L-threonine most of the ATP is converted to AMP (fig. 2d). With phosphoenolpyruvate, pyruvate, lactate and serine very little or no AMP is formed. The results demonstrate that the enzyme fraction activates the amino acids L-valine, L-threonine and L-alanine, probably by forming an amino acyl-AMP complex.

Table 1
Amino acid activation tested with the ATP–$^{32}PP_i$ exchange reaction

Amino acid	ATP–$^{32}PP_i$ exchange (cpm)*
– aa	3180
L–Valine	59830
D–Valine	3650
L–Hydroxyisovaleric acid	3020
L–Lactic acid	2980
L–Alanine	3160
L–Threonine	7310
–aa, –enzyme	250

* Twenty μl of the DEAE purified enzyme were used.

3.3. *Valinomycin synthesis in vitro*

Complete synthesis of valinomycin was achieved with the enzyme fraction purified by DEAE-cellulose chromatography. As expected, L-[^{14}C]valine is incorporated into the product and is partially racemized to D-valine as found after acid hydrolysis and treatment with L- and D-amino acid oxidase. Very little L-[^{14}C]alanine was incorporated, whereas the incorporation of L-[^{14}C]threonine is significant. That the product is in fact valinomycin is shown by the following experiments:

1. The product cochromatographs with authentic valinomycin in two different systems (50% propanol and cyclohexane/pyridine/acetic acid).

2. The product contains D-valine and L-valine in the proportion 1:1.

3. The product acts as a potassium carrier through isolated chloroplast membranes qualitatively in the same way as authentic valinomycin.

The production of valinomycin is not affected by pancreatic ribonuclease or puromycin, suggesting a synthesizing mechanism which does not involve messenger ribonucleic acid or ribosomes.

4. Discussion

The results described above demonstrate that valinomycin is synthesized in a similar way to gramicidin, tyrocidine and other antibiotics [8]. That means that the constituents are linked together without the participation of messenger RNA, ribosomes and transfer RNA, but by one or more enzymes. In the case of gramicidin S and tyrocidine the constituents are only amino acids whereas valinomycin contains in addition the hydroxy acids D-hydroxyisovaleric acid and L-lactate, which derives in our experiments from L-valine and L-alanine or L-threonine, respectively. The mechanism by which the amino acids are converted to hydroxy acids is still unknown, especially the conversion of L-threonine into lactic acid is a completely unsolved problem. Moreover the formation of ester- and amide-bonds are not understood. However, the isolation of the valinomycin synthesizing enzymes allows further studies on these problems.

Acknowledgements

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