STUDIES ON THE BIOSYNTHESIS OF VALINOMYCIN

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1. Introduction

For some time, this laboratory has been exploring the in vitro synthesis of antibiotic polypeptides on polyenzymes; the latter catalyze the reaction between amino acids + ATP yielding aminoacyl adenylates. Eventually, the amino acid residues are transferred to a thioester linkage and a pantetheine carrier protein attached to each polyenzyme collects the amino acids into polypeptides [1,2]. In view of these results, we proposed approaching the biosynthesis of valinomycin, a cyclododecadepsipeptide, which contains the thrice-repeating sequence of the tetradepsipeptide: L-lactyl–L-valyl–D-α-hydroxyisovaleryl–D-valyl. Earlier reports on its biosynthesis in vegetative cells by MacDonald and Slater [3] suggested that D-α-hydroxyisovaleric acid is the direct precursor of the D-α-hydroxyisovaleric acid moiety in valinomycin. The D- and L-valyl parts of valinomycin were shown to be derived from L-valine. In 1974, Ristow et al. [4] published briefly on the cell-free synthesis of valinomycin from L-valine using L-threonine or L-alanine as the precursors of lactic acid.

We will describe here experiments where, under appropriate conditions, radioactively marked lactic acid is easily, but alanine poorly, incorporated into valinomycin by living cells. Since we were unable, using the same organism as Ristow et al. [4], to obtain in vitro synthesis in our cell homogenates, after disruption of the bacteria by means of a sonifier or a French-press, we prepared protoplasts which showed a considerably greater activity in forming valinomycin than the original bacteria. We hoped to obtain biosynthesis of the antibiotic from lysates but did not succeed. However, we found the protoplast lysates contained hydroxy acid-activating enzymes with lactate and α-hydroxyisovaleric acid as substrates, and we tend to consider these enzymes to be part of the enzymatic equipment responsible for valinomycin synthesis. Since Ristow et al. used alanine or threonine as precursors of the lactic acid in their biosynthesis rather than the seemingly natural lactic acid, we hope that their results will be more explicitly confirmed.

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2. Materials and methods

L-Lactic acid and D,L-α-hydroxyisovaleric acid were purchased from Sigma, L-α-hydroxyisovaleric acid and valinomycin from Calbiochem. L-[U-^{14}C]-Lactic acid was obtained from Amersham/Searle and L-[3-^{14}C]lactic acid from New England Nuclear.

Submerged cultivation of *Streptomyces* sp. (ATCC 23836) was done in 5 liters medium (2.5% yeast extract, 2.5% glucose, pH 7.5–8.0) in a New Brunswick MMF 114 fermentor (400 rev/min, 10 liters air/min) at 28°C. The inoculum was 2% of a 2 day old culture. Under these conditions, production of valinomycin in the cells began about 15 h after inoculation and stopped after 60–70 h at 60–100 mg/liter culture.

Valinomycin was extracted from the cells with acetone and purified by thin-layer chromatography on silica gel plates in a cyclohexane/ethyl acetate/formic acid solvent system (120:40:5). Hydrolysis of valinomycin was done with 6 N HCl–acetic acid [5]; lactic acid, valine, and D-α-hydroxyisovaleric acid were

separated by thin-layer chromatography on silica gel plates with *n*-butanol/acetic acid/water (4:1:1) as solvent.

ATP–$^{32}PP_i$ exchange was measured using the following incubation mixture in total vol. 0.1 ml: 4 mM substrate, 2 mM ATP, 2 mM $^{32}PP_i$ (350 000 cpm), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 20 mM Tris–HCl buffer, pH 8.1 and enzyme. After reaction for 15 min at 27°C, the exchange was measured as described [6].

The protein determination was done by a modified method of Lowry et al. as described by Ross and Schatz [7].

3. Results

For in vivo experiments, cells from a 50 ml culture sample were washed with water and incubated with the radioactive precursors in 50 ml water for 1 h at 27°C on a rotary shaker. The results of these experiments are outlined in table 1. L-Valine was readily incorporated into the valine and D-α-hydroxyisovaleric acid moieties of valinomycin but not into the lactic acid parts. The incorporation of alanine was rather low. When lactic acid was added to the medium, no significant uptake and incorporation of radioactivity into valinomycin could be observed. By suspending the cells in 20 ml 0.1 M KH_2PO_4, pH 4.5, containing 10 μCi of the radioactive precursor, we succeeded in introducing 3 μCi of L-[^{14}C]lactic acid into the cells. After resuspending these cells in 5 mM Tris–HCl buffer, pH 7.5, incorporation of lactic acid into valinomycin could be observed, which compared favorably with that of valine considering the multiple incorporation of the latter.

To convert vegetative cells into protoplasts, the cells from 1 liter fermentor culture (15 h after inoculation, 5 g wet wt) were washed, divided into four portions, and incubated with 100 ml medium containing 80 mg lysozyme, 0.25% yeast extract, 0.25% glucose, 20% sucrose and 30 mM $MgCl_2$. The pH was adjusted to 7.6. After shaking for 120 min at 27°C, all cells were converted to protoplasts. Incorporation of valine into valinomycin was nearly five times higher in protoplasts than in vegetative cells.

For purification of the hydroxy acid-activating enzymes, the protoplasts from 1 liter fermentor culture were collected by centrifugation, suspended in 40 ml 20 mM Tris–HCl buffer, pH 7.6, 2 mM EDTA and passed through a cooled French press at very low pressure. The protoplast residue was removed by centrifugation (30 min, 30 000 × *g*) and the supernatant containing 1.1 g protein was applied to a Whatman DE-52 (4 × 4 cm) column and washed with 200 ml 20 mM Tris–HCl buffer, pH 7.6, contain-

Table 1
Incorporation into vegetative cells of radioactively marked L-lactic acid, L-alanine and L-valine[a]

Precursor	Incorporation (dpm)			
	Valinomycin	L-Lac[b]	D-α-Hiv	D-Val + L-Val
L-[3-^{14}C]lactic acid 1 μCi, 175 nmol	165			
L-[U-^{14}C]lactic acid[c] 3 μCi, 50 nmol	16 800	5650	2800	5300
L-[U-^{14}C]alanine 1 μCi, 6.5 nmol	2800	881	323	502
L-[U-^{14}C]valine 1 μCi, 3.8 nmol	38 900	0	7020	18 180

[a] To test for incorporation into individual constituents, the valinomycin was acid-hydrolyzed and the fragments separated as described in Materials and Methods
[b] Lac, lactic acid; D-α-Hiv, D-α-hydroxyisovaleric acid
[c] After preincubation in acidic medium as described in the text

ing 0.1 M KCl and 1 mM dithiothreitol. The protein was eluted with the same buffer with KCl increased to 0.3 M and concentrated by means of a Diaflo apparatus to 19 ml containing 290 mg protein. The concentrated eluate was applied to a Sephadex G-150 column (70 × 2.6 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.6, containing 0.1 M KCl, 1 mM EDTA and 1 mM dithiothreitol and eluted with the same buffer. The fractions, which showed slightly higher ATP–$^{32}PP_i$ exchange in the presence of L-lactic acid and D-α-hydroxyisovaleric acid as compared to the water blank (from 1.4–1.7 exclusion vol.), were combined. These fractions, containing 160 mg of protein, were immediately applied for a second DEAE-cellulose chromatography to a column with Whatman DE-52 (5.6 × 1.5 cm) equilibrated with 20 mM Tris buffer, pH 7.6, containing 2% glycerol and 1 mM dithiothreitol (buffer A). The column was eluted with a linear KCl gradient (0.1–0.3 M) in buffer A; the hydroxy acid-activating enzymes were both eluted at 0.2 M KCl, pooled, concentrated to 7 ml containing 29.4 mg protein, then stored in liquid nitrogen. The specific activity of this enzyme preparation was 76 pmol exchange/μg protein in 15 min in the presence of L-lactic acid and D-α-hydroxyisovaleric acid.

For glycerol-gradient centrifugation, 100 μl concentrated enzyme solution from the second DEAE-cellulose chromatography were layered on 4 ml glycerol gradients of 8–32% in 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol and 0.5 mM EDTA, and were centrifuged for 11.5 h at 50 000 rev/min using the SW-56 rotor of the Beckman L-2 centrifuge. The fractions were tested for ATP–$^{32}PP_i$ exchange; the results are shown in fig.1. Exchange activities for both hydroxy acids overlapped. By comparison with bovine serum albumin and γ-globulin, the average molecular weight of the enzymes was estimated to be approx. 80 000. In most experiments, the exchange in the presence of lactic acid was between 50% and 80% of the one obtained with D-α-hydroxyisovaleric acid. This difference varied from batch to batch. In the peak fractions after glycerol-gradient centrifugation, ATP–$^{32}PP_i$ exchange was observed only in the presence of the D-isomer of α-hydroxyisovaleric acid; pyruvic acid and L-α-hydroxyisovaleric acid were not activated, the observed exchange being below control. A high ATP–

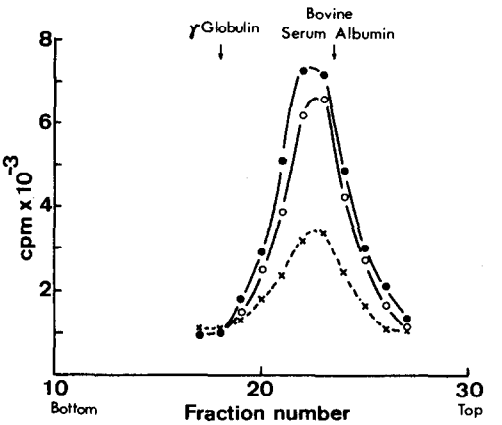

Fig.1. Glycerol-gradient centrifugation of the enzyme preparation after the second DEAE-cellulose chromatography. 10 μl of each fraction was assayed for ATP–$^{32}PP_i$ exchange in the presence of (○——○) L-lactic acid, (●——●) D,L-α-hydroxyisovaleric acid, (×- -×) water added instead of substrate. The specific activity of the enzyme preparation from fractions 22 and 23 was 330 pmol exchange/μg protein in 15 min in the presence of D-α-hydroxyisovaleric acid.

$^{32}PP_i$ exchange in the presence of L-valine was due to the presence of large amounts of valyl-tRNA synthetase, detected by the method of Muench and Berg [8]. Ristow et al. [4] also tested for ATP–$^{32}PP_i$ exchange in the presence of valine and, without evaluating the presence of valyl-tRNA synthetase, considered it indicative of valine activation for valinomycin synthesis. We were unable to eliminate or explain a still considerable blank (water added instead of substrate) which, in all chromatograms and in glycerol gradient centrifugation, always parallelled the ATP–$^{32}PP_i$ exchange in the presence of the hydroxy acids.

4. Discussion

Our experiments with vegetative cells show that once lactic acid has entered the cell it is easily incorporated into the lactic acid moieties of valinomycin. The incorporation into the other parts is thought to be due to prior conversion to pyruvic acid, which is a direct precursor of valine and thus of α-hydroxyisovaleric acid. Because the valine activating enzyme present in the protoplast lysates were found to

coincide with an abundantly present valyl-tRNA synthetase, we concentrated on the purification of the hydroxy acid-promoted ATP–$^{32}PP_i$ exchange, presumably an unique feature of the valinomycin synthesizing system. The best enzyme preparations supporting ATP–$^{32}PP_i$ exchange in the presence of lactic acid and D-α-hydroxyisovaleric acid were found to have an approximately equal molecular weight of about 80 000, which is very near the average molecular weight of about 75 000 found for the subunit fragments of the polyenzymes that activate constituent amino acids in tyrocidine biosynthesis [2]. This makes it likely that the hydroxy acid activating enzymes are fragments of a large polyenzyme presumably responsible for the synthesis of the three tetradepsipeptides; the latter then presumably cyclize to valinomycin in a manner similar to that found with the two pentapeptides that cyclize form gramicidin S [9].

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