BIOSYNTHESIS OF ACTINOMYCIN D : PURIFICATION AND PROPERTIES OF AN ENZYME WHICH ACTIVATES L-VALINE

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1. Introduction

Biogenetic studies on actinomycin D (fig. 1), an antibiotic produced by *Streptomyces antibioticus*, have shown that L-valine is a precursor of the D-valine and N-methyl-L-valine residues [1], that L-proline and L-threonine are the precursors of these amino acids in the antibiotic [2], and that glycine is a precursor of the sarcosine moiety [1, 3, 5]. The methyl groups of sarcosine [4, 5], N-methyl-L-valine [1], and of the phenoxazinone nucleus are donated from L-methionine [6]. We have studied the biogenesis of this antibiotic at an enzymatic level and here describe the purification and some of the properties of an enzyme which is probably involved in the activation of L-valine prior to incorporation into actinomycin D.

2. Materials and methods

2.1. *General*

L-valine-^{14}C-U (260 mCi/mM) and sodium orthophosphate-^{32}P (63 Ci/mg P) were purchased from Amersham, Searle, and sodium pyrophosphate-^{32}P (835 mCi/mM) from New England Nuclear Co. Radioactivity was measured in a Packard scintillation counter (model 2002) using Bray's solution [7] or a toluene solution of the same scintillator.

Protein concentration was estimated by Lowry's method [8] or from the ratio of optical densities at 280 and 260 nm [9].

Isotopic exchange between pyrophosphate or phosphate and ATP was measured as described by Calendar and Berg [10] except that the buffer was Tris-HCl, pH 7.5. The reaction mixture was incubated at 30° for 30 min and the reaction stopped by the addition of 0.7 ml of 15% perchloric acid containing 0.4 M cold pyrophosphate or phosphate. After removal of the precipitate by centrifugation (5,000 *g*, 10 min) a 15% suspension of acid-washed Norit A (0.1 ml) was added to the supernatant. The mixture was stirred vigorously, filtered through glass fibre discs (Reeve Angel, no. 934 AH) the filter washed with water (4 × 5 ml) and ATP eluted under a slight suction with 50% ethanol containing 0.3 M ammonia (4 × 1 ml). The radioactivity of the eluate was measured.

One unit of enzyme was defined as the amount incorporating 1 μmole of pyrophosphate into ATP in 30 min at 30°.

L-valine-tRNA formation was estimated by the Calendar and Berg method [10]. The incubation mixture contained 100 μM cacodylate buffer, pH 7.0, 5 μmoles $MgCl_2$, 0.04 mg bovine serum albumin, 1 μmole dithiothreitol, 2 μmoles ATP, 200 μmoles L-valine-^{14}C-U (10^6 cpm), 1 mg tRNA from *S. antibioticus* ATCC 14888 [11] and enzyme in a total vol of 0.5 ml.

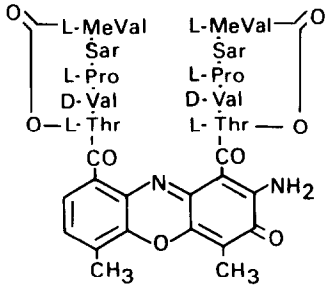

Fig. 1. Actinomycin D.

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Table 1
Amino acid dependence of ATP-pyrophosphate exchange catalysed by crude enzyme extract of cells of *S. antibioticus*.

Amino acid added	ATP-pyrophosphate exchange (μmoles)
None	18.0
L-Valine	61.2
D-Valine	20.9
L-Proline	39.0
L-4-Hydroxyproline	22.0
Sarcosine	22.0
Glycine	24.1
L-Threonine	38.7
N-Methyl-DL-Valine	22.6

The exchange reaction was performed as described in Materials and methods. To each reaction mixture was added the appropriate amino acid (20 μM) and crude enzyme (0.315 mg protein).

The incorporation of ^{14}C-L-valine into actinomycin D and *S. antibioticus* protein was measured by the methods of Weissbach and Katz [1, 12] using 65 hr cells grown in Katz' media [13, 14] in a rotary shaker at 30°.

2.2. *Enzyme purification*

All operations were performed at 4°. All buffers contained 1 mM dithiothreitol and 1 mM $MgCl_2$.

S. antibioticus cells (65 hr, washed in 5 mM $MgCl_2$) in 100 ml 0.1 M Tris-HCl, pH 7.5, containing 20% glycerol were sonicated (4 × 15 sec) and centrifuged (13,000 *g*, 30 min). Streptomycin sulfate (1.2% final concentration) was added to the supernatant and the precipitate removed by centrifugation (10,000 *g*, 20 min). Solid $(NH_4)_2SO_4$ (70%) was added to the supernatant and the precipitate dissolved in 20 ml 0.05 M phosphate buffer and dialysed against this buffer (2 l) for 8 hr. The retentate (referred to as crude enzyme) was used in preliminary experiments or subjected to the following chromatographic procedures.

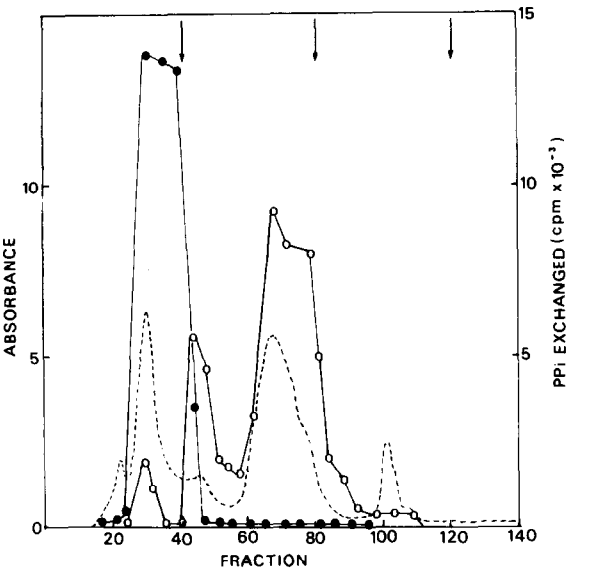

Fig. 2. Chromatography of crude enzyme preparation on hydroxylapatite. Fractions (5 ml) were collected at 30 min intervals. The column was eluted successively with 0.1 M, 0.15 M, 0.25 M and 0.3 M potassium phosphate buffer, pH 6.8; the arrows indicate the points at which the buffer was changed. (- - - -): Absorbance, A, at 280 nm; (o-o-o): L-valine dependent ATP-pyrophosphate (PPi) exchange; (•-•-•): amino acid independent ATP-pyrophosphate exchange.

Crude enzyme solution (20 ml) prepared as above was applied to a column of hydroxylapatite (Biorad; 3 × 30 cm) packed in 0.1 M potassium phosphate buffer, pH 6.8, and eluted as described in fig. 2.

Table 2
Summary of purification of L-valine activating enzyme.

Fraction	Volume (ml)	Protein (mg)	Total units	Specific activity (units/mg protein)	Yield (%)
Crude enzyme	20	73.0	620	8.5	100
Hydroxylapatite (Frac. 60–86)	160	35.4	450	12.7	72.5
Sephadex G-200 (Frac. 8–24)	82	22.5	332	14.7	53.5
DEAE-Sephadex (Frac. 27–32)	30	3.3	88	26.9	14.2

L-valine dependent ATP-pyrophosphate exchange was measured as described in the text.

Table 3
Requirements for ATP-pyrophosphate exchange reaction by purified enzyme preparation.

Omission from system	ATP-pyrophosphate exchange (mμmoles)
None	40.4
L-Valine	3.4
ATP	2.2
$MgCl_2$	2.1
KF	42.5
Dithiothreitol	44.3
EDTA	44.2
Enzyme	0.3

The reaction was performed as described in the text; 1 mg of purified protein was added to each reaction mixture.

Fractions 60–86 were pooled, the protein precipitated by $(NH_4)_2SO_4$ addition to 70% and redissolved in 10 ml 0.05 M Tris-HCl buffer, pH 7.5 (buffer 1) and dialyzed against this buffer (2 l) for 5 hr. The enzyme solution was then applied to a Sephadex G-200 column (3 × 40 cm) and eluted with the same buffer (fig. 3). Fractions 8–24 were pooled, the protein precipitated by $(NH_4)_2SO_4$ (70%) redissolved in 5 ml buffer 1, dialyzed against this buffer (2 l, 5 hr) and further purified by chromatography on a DEAE-Sephadex A-25 column (3 × 42 cm) using buffer 1 containing a KCl gradient ranging from 0–1.0 M as eluting solution (fig. 4). Fractions 27–32 were pooled, glycerol added to 50% concentration and the solution stored at –20°.

3. Results

3.1. *Properties of crude enzyme*

Of the amino acids tested L-valine stimulated the exchange of pyrophosphate into ATP best (table 1). Also the preparation contained a large amount of amino acid independent ATP-pyrophosphate exchange activity. It had no detectable amino acid dependent ATP-phosphate exchange activity, but amino acid independent activity was detected.

3.2. *Purification of L-valine dependent enzyme*

Chromatography of the crude enzyme on hydroxylapatite (fig. 2) completely separated the amino acid independent activity from the L-valine dependent activity. The latter activity was further chromatographed on Sephadex G-200 (fig. 3) and DEAE-Sephadex (fig. 4), giving a product which contained < 0.7% nucleic acid as judged by the ratio of absorbancies at 280 and 260 nm. The purification is summarised in table 2.

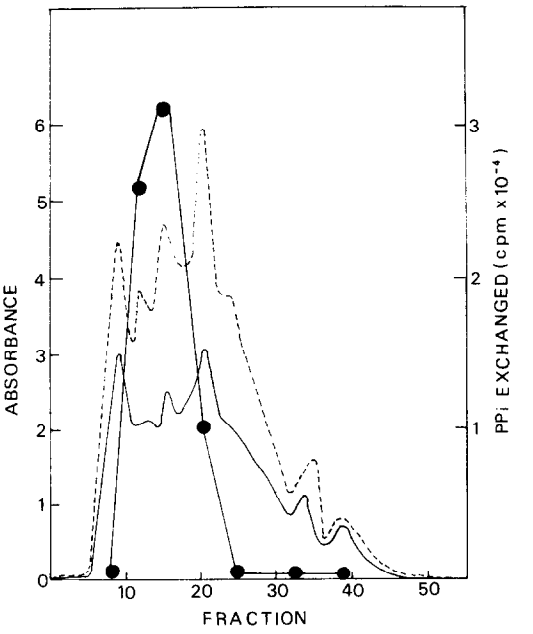

Fig. 3. Chromatography on Sephadex G-200. The flow rate was 15 ml/hr; fractions were collected every 20 min. (——): A 280 nm; (- - -): A 260 nm; (•-•-•): L-valine dependent ATP-pyrophosphate (PPi) exchange.

3.3. *Properties of purified enzyme*

ATP-pyrophosphate exchange increased linearly with time up to 20 min and was proportional to the amount of enzyme added. The exchange reaction required L-valine, Mg^{2+}, and ATP (table 3). None of the other constituent amino acids of actinomycin D nor L-leucine, L-isoleucine, glycine or L-4 hydroxyproline added singly stimulated the exchange. The optimal Mg^{2+}: ATP ratio was 2:5. Complete loss of activity was noted when either 1 mM *p*-chloromercuribenzoate or 1 mM $HgCl_2$ were added and 50% loss when 1 mM thio-bis-2-nitrobenzoate was added. In a study of the effect of L-valine concentration on the ATP-pyrophosphate exchange a K_m value of 0.44 mM was obtained. The enzyme had no catalytic effect on the formation of L-valyl tRNA. This latter reaction was catalyzed by the enzyme fraction eluted

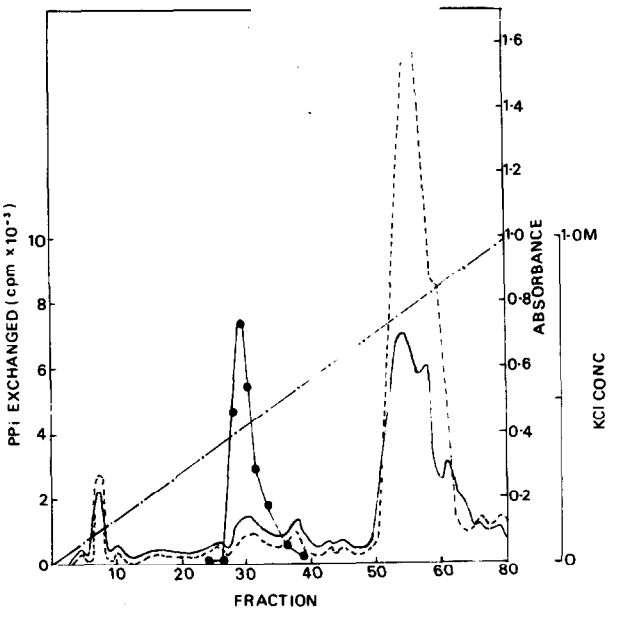

Fig. 4. Chromatography on DEAE Sephadex A-25. The flow rate was 15 ml/hr; fractions were collected at 20 min intervals. (——): A 280 nm; (- - -): A 260 nm; (●-●-●): L-valine dependent ATP-pyrophosphate (PPi) exchange; (•—•): KCl gradient.

Table 4
Formation of L-valyl-tRNA and ATP-pyrophosphate exchange.

	L-valyl-tRNA (cpm)	Pyrophosphate exchanged (cpm)
Experiment I		
Complete system	1,037	6,595
−tRNA	68	–
−enzyme	26	–
Experiment II		
Complete system	45	5,547
−tRNA	33	–
−enzyme	26	–

The abilities of 2 different enzyme fractions to catalyse L-valyl-tRNA formation and ATP-pyrophosphate exchange were compared. In experiment I 0.5 mg of enzyme protein eluted by 0.1 M phosphate from hydroxylapatite column was used and in experiment II 0.05 mg of enzyme protein from DEAE-Sephadex column.

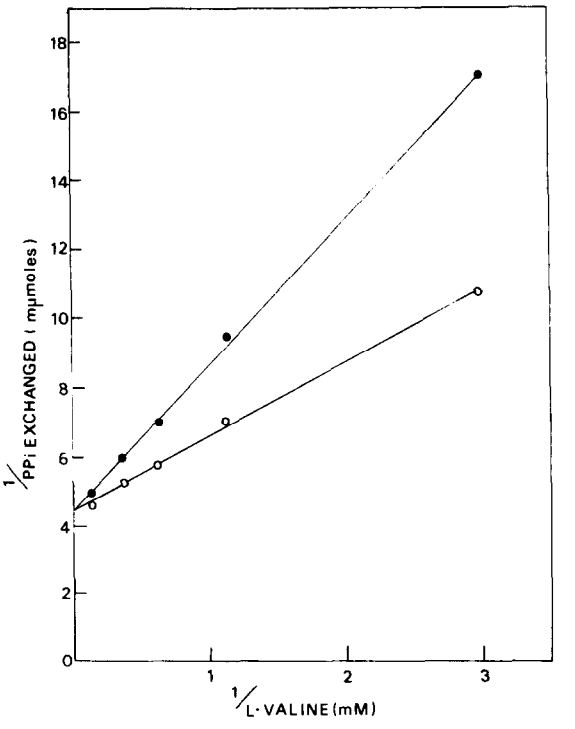

Fig. 5. Effect of D-valine on L-valine dependent ATP-pyrophosphate (PPi) exchange. The exchange reaction was carried out as described in the text with varying amounts of L-valine in the absence (○-○-○), and in the presence (●-●-●) of 10 μmoles D-valine.

Table 5
Effect of D-valine on actinomycin biosynthesis by washed cells.

D-valine added (μmoles)	Actinomycin (cpm)	Protein (cpm/mg)
0	1,605	14,300
10	1,105	18,300
20	1,030	18,000
50	726	13,800

by 0.1 M phosphate buffer from the hydroxyl-apatite column (table 4).

D-valine competitively inhibited L-valine dependent ATP-pyrophosphate exchange (fig. 5) and also inhibited incorporation of ^{14}C-L-valine into actinomycin D by washed cells (table 5; see also [15]). It had no effect on incorporation of L-valine into cell protein or

on L-valyl-tRNA formation by the enzyme fraction eluted from the hydroxylapatite column by 0.1 M phosphate.

4. Discussion

The above describes an enzyme which can cause the exchange of pyrophosphate into ATP in the presence of L-valine and which does not catalyse the transfer of L-valine to tRNA. Its action probably requires an active -SH group. Similar enzymes are believed to be involved in the biogenesis of other peptide antibiotics [16]. The facts that this enzyme is present in *S. antibioticus* at a time when actinomycin D is being actively synthesized [17] and that both the enzyme and actinomycin D production in whole cells are inhibited by D-valine, suggest that the enzyme is involved in actinomycin biosynthesis. However, the ultimate proof of this suggestion will come if the enzyme is shown to be involved in the cell-free synthesis of actinomycin D from known precursors. Extracts prepared under the above and other conditions cannot perform such a synthesis efficiently [18].

Acknowledgements

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References

[1] E. Katz and H. Weissbach, J. Biol. Chem. 238 (1963) 666.
[2] E. Katz, Lloydia 31 (1968) 364.
[3] E. Katz and H. Weissbach, Biochem. Biophys. Res. Commun. 8 (1962) 186.
[4] O. Ciferri, A. Albertini and G. Cassini, Biochem. J. 96 (1965) 853.
[5] O. Ciferri, A. Albertini and G. Cassini, Biochem. J. 90 (1964) 82.
[6] A.J. Birch, D.W. Cameron, P.W. Holloway and R.W. Rickards, Tetrahedron Letters (1960) 26.
[7] G.A. Bray, Analyt. Biochem. 1 (1960) 279.
[8] O. Lowry, N. Rosebrough, L. Fair and R. Randall, J. Biol. Chem. 193 (1951) 265.
[9] H.M. Kalckar, J. Biol. Chem. 167 (1947) 461.
[10] R. Calendar and P. Berg, in: Procedures in Nucleic Acid Research, eds. G.L. Cantoni and D.R. Daub (Harper and Row, New York, 1966) p.455.
[11] G. Zubay, in: Procedures in Nucleic Acid Research, eds. G.L. Cantoni and D.R. Daub (Harper and Row, New York, 1966) p.375.
[12] H. Weissbach, B. Redfield, V. Beaven and E. Katz, Biochem. Biophys. Res. Commun. 19 (1965) 524.
[13] W.A. Goss and E. Katz, Appl. Microbiol. 5 (1957) 95.
[14] E. Katz, P. Pienta and A. Sivak, Appl. Microbiol. 6 (1958) 236.
[15] L.A. Salzman and E. Katz, J. Biol. Chem. 239 (1964) 1864.
[16] F. Lipmann, Science 173 (1971) 875.
[17] E. Katz and W.A. Goss, Biochem. J. 73 (1959) 458.
[18] J.E. Walker, S. Otani and D. Perlman, unpublished observations.