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Purification of the Polyenzymes Responsible for Tyrocidine Synthesis and Their Dissociation into Subunits†

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ABSTRACT: The biosynthesis of the antibiotic tyrocidine, a cyclic decapeptide →(NH_2)DPhe-Pro-Phe-DPhe(Trp,Tyr)Asn-Gln-Phe(Trp,Tyr)Val-Orn-Leu→ in extracts of *Bacillus brevis* (ATCC 8185), was shown to require three complementary enzyme fractions of mol wt 100,000, 230,000, and 440,000. As described (Roskoski *et al.*, *Biochemistry 9*, 4839, 4846 (1970)), these fractions activate the component amino acids with ATP to become thio ester linked to the enzymes. On combination, they polymerize sequentially, beginning from the N-terminal phenylalanine, in the direction indicated by the arrows to enzyme-bound peptidyl thio esters; addition of the last amino acid, leucine, causes release of cyclic decapeptide. The two larger enzyme fractions have now been purified to near homogeneity, and their enzymatic functions have been reassessed. The polyenzyme of mol wt 230,000 activates and binds, in addition to proline (*cf.* Roskoski *et al.*, *Biochemistry 9*, 4839, 4846 (1970)), L- and D-phenylalanine (*cf.* Kambe *et al.*, *J. Biochem.* (*Tokyo*) *69*, 1131 (1971)); the 440,000 molecular weight enzyme binds the last six amino acids in the sequence. Both polyenzymes contain 1 mol of pantetheine each; division of their molecular weights by the number of amino acids activated, *i.e.*, 230,000/3 and 440,000/6, yields uniform figures of 70,000–75,000. This seemed to indicate that the polyenzymes may be composed of amino acid activating subunits of such size, which is now confirmed by dissociation of the 230,000 and 440,000 molecular weight enzymes into subunits using two independent procedures. (1) Incubation of cell lysates with DNase for 40 min at 37° yields, on Sephadex G-200 chromatography or sucrose gradient centrifugation, a mixture of *ca.* 70,000 molecular weight fragment enzymes that respond to all amino acids present in the tyrocidine with ATP–PP_i exchange. (2) Sodium dodecyl sulfate gel electrophoresis causes partial dissociation of the 230,000 and 440,000 molecular weight enzymes to subunits of a similar size.

Our interest in the mechanism of biosynthesis of a group of bacterial antibiotic polypeptides was aroused by reports that such could take place in cytosol fractions that were exhaustively treated with RNase. We could confirm (Roskoski *et al.*, 1970a) that a ribosomal type of synthesis could be excluded, and that this synthesis involved polyenzymes which activated and bound the amino acids to be incorporated. The initial activation by ATP followed the scheme

$$\text{amino acid} + \text{ATP} \rightleftharpoons \text{AMP}\sim\text{amino acid} + PP_i$$

which is analogous to that in amino acid ligase activity. However, in contrast to the ligase reaction, the amino acid here is transferred to an enzyme-bound thiol to form an amino acid thio ester.

In the synthesis of gramicidin S, the first to be analyzed in detail, two complementary fractions are involved. Gramicidin S is a cyclic decapeptide composed of two identical pentapeptides, DPhe-Pro-Val-Orn-Leu, which cyclize head to tail. One enzyme fraction of mol wt 100,000 thioesterifies and racemizes phenylalanine, the second fraction of mol wt 280,000 thioesterifies the four amino acids following D-phenylalanine; these four can be bound without polymerization, and are initiated by reaction with the small enzyme that carries D-phenylalanine. Polymerization may be followed by the single addition of amino acids in sequence, which leads to the formation of enzyme-bound peptidyl thio esters of increasing chain length up to the pentapeptide. Addition of the fifth, the leucine, is rapidly followed by release of gramicidin S through cyclization; omission of an amino acid in the sequence stops polymerization. The size of the enzymes activating the amino acids appears to be approximately proportional to the number of amino acids activated. This was a first indication that we might be dealing here with polyenzymes composed of vectorially arranged amino acid specific binding subunits of similar molecular size.

Progressing to the study of tyrocidine biosynthesis, we hoped to find there an analogous mechanism on a larger scale. Tyrocidine, although a cyclic decapeptide like gramicidin S, is composed of a specific sequence of ten amino acids (Figure 1). Fujikawa *et al.* (1968) were the first to succeed in preparing from a tyrocidine-producing *Bacillus brevis* strain by DEAE-cellulose chromatography, two complementary cytosol fractions that synthesized the antibiotic; they isolated a 100,000 molecular weight enzyme analogous to the small one in gramicidin S that racemized and bound D-phenylalanine, and a larger fraction.

As reported (Roskoski *et al.*, 1970a), using ammonium sulfate fractionation and chromatography on Sephadex G-200, we obtained from a similar extract of *B. brevis* (ATCC 8185) three fractions of molecular weight 100,000, 230,000, and 460,000, and assumed that Kurahashi's heavier fraction

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TABLE I: Enzyme Activities in the Biosynthesis of Gramicidin S and Tyrocidine.

Decapeptide Synthesized	No.	Mol Wt of Enzymes	Amino Acid Activated and Fixed in Sequence	Mol Wt/ Amino Acid $\times 10^3$	Pantetheine Content (mol/Enzyme)
Gramicidin S[a]	1	100,000	DPhe	100	None
	2	280,000	Pro,Val,Orn,Leu	70	1
Tyrocidine	1	100,000	DPhe	100	None
	2	230,000	Pro,Phe,DPhe	76	1
	3	440,000	Asn,Gln,Phe,Val,Orn,Leu	74	1

[a] The decapeptide in this case is synthesized by antiparallel cyclization of two identical pentapeptides.

was a mixture of the last two. The 230,000 molecular weight fraction seemed predominantly to activate proline. The molecular weight of 230,000, however, impressed us as being comparatively too high for an enzyme activating a single amino acid (Roskoski *et al.*, 1970a) when compared with the 280,000 molecular weight gramicidin S enzyme that activates four amino acids.

Because of the difficulties encountered previously in obtaining sufficient material to purify the tyrocidine-producing enzymes, the earlier experiments were made with impure fractions. In the meantime, with the help of Dr. Novelli and his colleagues, Ed Tharies and Mary Lawn, at Oak Ridge Biological Laboratories, we have obtained several pounds of tyrocidine-producing *B. brevis*, which has now enabled us to purify the tyrocidine system.

Results obtained with a nearly homogeneous fraction of the 230,000 molecular weight enzyme indicate that it binds not only proline but also L- and D-phenylalanine, *i.e.*, the second, third, and fourth amino acids in the sequence. The six remaining amino acids, it will be shown, are bound by the large polyenzyme. Kurahashi's group recently confirmed (Kambe *et al.*, 1971) the separation of the tyrocidine-synthesizing system into three complementary fractions, and in addition, reported that the intermediate size polyenzyme activated three amino acids.

The comparison in Table I of the molecular weights of polyenzymes taking part in gramicidin S and tyrocidine synthesis with the number of amino acids activated now confirms very strikingly the proposition of a composition of the polyenzymes of subunits of *ca.* mol wt 70,000 for each amino acid activated. This proposition is experimentally verified by data reported in the second part of this paper, where the tyrocidine-synthesizing polyenzymes are shown to yield subunits of the expected size and number, either by prolonged incubation of crude extracts, or by sodium dodecyl sulfate gel electrophoresis. The table shows that 1 mol of 4′-phosphopantetheine is also part of IE.[1]

Experimental Section

Isolation of Enzyme-Bound Amino Acids. Using essentially the procedure of Roskoski *et al.* (1970a), enzyme fractions were incubated for 20 min at 37° in an incubation volume of 0.1 ml containing 20 mM triethanolamine buffer (pH 7.4), 10 mM $MgCl_2$, 20 mM KCl, 2 mM ATP, 1 mM dithiothreitol, 0.5 mM EDTA, and the ^{14}C-labeled amino acids. At the end of the incubation period, protein was precipitated with 2 ml of 5% trichloroacetic acid, and after standing for 30 min at room temperature, 0.1 mg of bovine serum albumin was added. The precipitate was collected on Millipore filters, 0.45 μ pore size, and washed with seven 3-ml portions of 5% trichloroacetic acid containing 0.1% of cold amino acid; the radioactivity was determined as described (Roskoski *et al.*, 1970a).

For determination of the racemization of phenylalanine, the procedure described by Yamada and Kurahashi (1968) was used.

Enzyme-Bound Nascent Peptides. LE and IE were incubated at 37° for 1 hr with [^{14}C]proline and [^{3}H]phenylalanine. IE was separated from the incubation mixture by sucrose gradient centrifugation. The nascent peptides were liberated from IE by alkali hydrolysis, and the peptides were separated by thin layer chromatography as described by Roskoski *et al.* (1970b).

Gel Electrophoresis. A 5% gel was prepared by mixing the following solutions: (1) 9 ml of 10% acrylamide (10 g of acrylamide and 0.5 g of methylenebisacrylamide in 100 ml of distilled water); (2) 6 ml of 0.09 M triethanolamine buffer (pH 8.1) containing 20 μl of *N,N,N′,N′*-tetramethylethylenediamine, 3 mM EDTA, and 3 mM dithiothreitol; and (3) 15 mg of ammonium persulfate in 3 ml of distilled water. The gel was polymerized in tubes of 0.75 × 7 cm. A prerun electrophoresis for 1 hr at 4 mA/gel using 0.03 M triethanolamine buffer (pH 8.1) was followed by electrophoresis of the enzyme samples for 5 hr using the same buffer at the same current. Staining and destaining of the gel were done according to the method described by Weber and Osborn (1969).

Sodium Dodecyl Sulfate Gel Electrophoresis of IE and HE. About 150 μg each of purified IE and HE was incubated for 5 hr at 37° in an incubation volume of 0.2 ml containing 0.5%

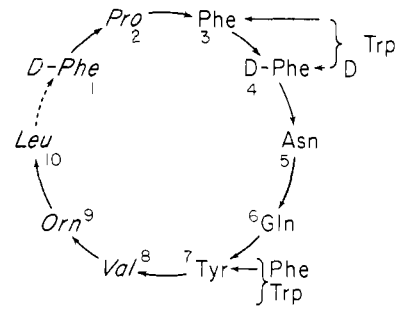

FIGURE 1: Tyrocidine.

[1] Abbreviations used are: HE, heavy enzyme; IE, intermediate enzyme; LE, light enzyme.

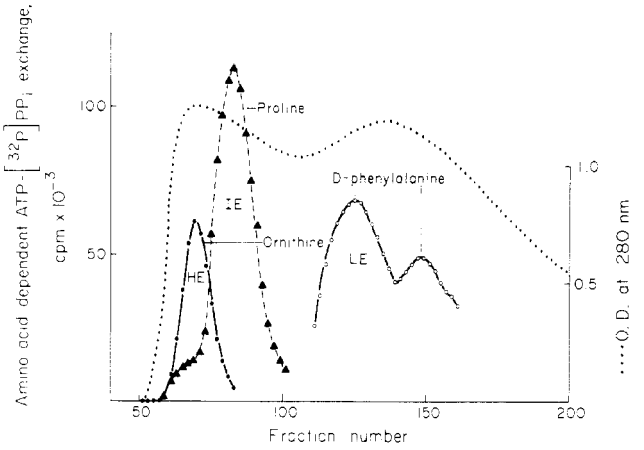

FIGURE 2: Separation of the three complementary fractions HE, IE, and LE on Sephadex G-200. About 2.3 g of protein from the 33–45% ammonium sulfate fraction was applied to a column (52 × 7 cm) equilibrated with buffer B containing 0.1 M KCl. It was eluted with buffer B containing 0.1 M KCl and 7-ml fractions were collected. From each fraction, 20 μl was assayed for L-ornithine-dependent (●), L-proline-dependent (▲), and D-phenylalanine-dependent (○) ATP–$^{32}PP_i$ exchange activities. The reaction mixture for the exchange contained in a reaction volume of 0.12 ml: 4 mM designated amino acids, 2 mM ATP, 2 mM $^{32}PP_i$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM EDTA, 18 mM KCl, and 20 mM triethanolamine buffer (pH 7.5). After reaction for 15 min at 37°, the exchange was measured as described (Gevers *et al.*, 1968).

sodium dodecyl sulfate and 0.05 M mercaptoethanol in 0.01 M phosphate buffer (pH 7.2). The enzymes were then precipitated by adding 0.8 ml of acetone and the precipitates collected by centrifugation. The pellets were dissolved in 0.1 ml of gel buffer (3.9 g of $NaH_2PO_4 \cdot H_2O$, 19.3 g of $Na_2HPO_4 \cdot 7H_2O$, and 1 g of sodium dodecyl sulfate per l. of distilled water). A 7% gel containing the gel buffer was prepared as described above. The gel buffer was given a prerun electrophoresis for 1 hr at 4 mA/gel, and the enzyme samples were electrophoresed for 8 hr. The staining and destaining of the gel were carried out as described above.

Pantetheine Assay. The pantetheine assays for the purified IE and HE were carried out as described by Pugh and Wakil (1965).

Protein Determination. The protein content of crude enzyme samples was determined by the method described by Lowry *et al.* (1951), and that of purified enzyme samples by the Biuret method described by Gornall *et al.* (1949).

Purification Procedures. PREPARATION OF EXTRACTS. As mentioned, several pounds of *B. brevis* (ATCC 8185) harvested at the peak of tyrocidine-synthesizing activity were kindly supplied by the Oak Ridge Biology Laboratory. A batch of 250 g of frozen cake was broken up and thawed by blowing air over it at room temperature. The procedure, in important details, was modified from that described by Roskoski *et al.* (1970a). The paste was suspended in 700 ml of 0.02 M triethanolamine buffer (pH 7.7) containing 0.5 mM EDTA and 1 mM dithiothreitol (buffer B), and to this was added 250 mg of lysozyme in 50 ml of buffer B. After incubation for 10 min at 37°, the cells were lysed and 150 μg of DNase and 3 ml of 1 M $MgCl_2$ were added to optimize DNase action. Hydrolysis of DNA was completed after 1–2 min as indicated by the disappearance of the viscosity. The lysate was chilled immediately to 4–6°, and centrifuged for 15 min at 20,000*g*. To the supernatant, solid ammonium sulfate was added to 33% saturation and the precipitate was removed by centrifugation for 10 min at 20,000*g*. More ammonium sulfate was added to the supernatant to 45% saturation and the precipitate was collected by centrifugation at 20,000*g* for 10 min. The pellets, which contained about 7 g of protein, were dissolved in 40 ml of buffer B.

SEPHADEX G-200 FILTRATION; PRELIMINARY SEPARATION OF ENZYME FRACTIONS. The 33–45% ammonium sulfate fraction was divided into three equal portions which were carried separately through the following steps. They were applied to a Sephadex G-200 column (52 × 7 cm) equilibrated with buffer B containing 0.1 M KCl, and were eluted with the same solution. Fractions of 7 ml were collected. L-Ornithine-, L-proline-, and D-phenylalanine-dependent ATP–$^{32}PP_i$ exchanges were measured (Figure 2) as described by Gevers *et al.* (1968) to identify the HE, IE, and LE fractions. The D-phenylalanine-linked ATP–$^{32}PP_i$ exchange assay yielded a smaller peak at lower molecular weight level, about 75,000, in addition to the earlier formed peak at approximately 100,000, due possibly to a fragment of IE. It will be shown later that on autolysis of crude extracts a rather rapid breakdown of IE and HE to fragments of this size occurs, which catalyzes exchange activities for single tyrocidine constitutive amino acids.

To the HE, IE, and LE peak fractions, solid ammonium sulfate was added to 50% saturation and the precipitates were collected by centrifugation. They were then dissolved in 5 ml of buffer B and passed through Sephadex G-50 (40 × 2 cm) equilibrated with the same buffer. The enzymes were eluted with the same buffer to which sucrose had been added to a final concentration of 5%, and were stored in liquid nitrogen until used for further purification.

Purification of LE. The LE from G-200 chromatography was concentrated to about 20 ml by means of a Diaflo apparatus, and the concentrated enzyme (about 25 mg of protein per ml) was applied to a DEAE-cellulose column (12 × 1.8 cm) equilibrated with buffer B containing 5% sucrose. The column was eluted with 100 ml of buffer B containing 5% sucrose and 0.1 M KCl, then further eluted with 500 ml of KCl gradient (0.1–0.4 M) in buffer B containing 5% sucrose. The LE, which was eluted between 0.15 and 0.18 M KCl, was assayed by measuring D-phenylalanine-dependent ATP–$^{32}PP_i$ exchange activity. The enzyme was concentrated by means of the Diaflo apparatus to about 12 mg of protein/ml.

Purification of IE. HYDROXYLAPATITE STEP. The IE fractions from three runs were combined (780 mg) and about 620 mg was applied to a hydroxylapatite column (50 × 2.2 cm) equilibrated with 0.01 M phosphate buffer (pH 7.3) containing 5% sucrose and 1 mM dithiothreitol. The column was eluted with 1 l. of a gradient of 0.01–0.15 M phosphate buffer containing 5% sucrose and 1 mM dithiothreitol. Fractions of 7 ml were collected and the IE fraction was located by measuring L-proline-dependent ATP–$^{32}PP_i$ exchange. These fractions were concentrated by means of the Diaflo apparatus to about 5 ml, and this was passed through a Sephadex G-50 column (40 × 2 cm) equilibrated with buffer B. Sucrose was added to 5% to the enzyme solution eluted from the column, which was then stored in liquid nitrogen until used.

DEAE-CELLULOSE CHROMATOGRAPHY. The IE from the hydroxylapatite step was applied to a DEAE-cellulose column (12 × 1.8 cm) equilibrated with buffer B containing 5% sucrose. The column was eluted with 50 ml of buffer B containing 5% sucrose and 0.1 M KCl, and then further eluted with KCl gradient (0.1–0.5 M) in buffer B and 5% sucrose. Fractions of 7 ml were collected and those with IE activity concentrated by means of the Diaflo apparatus to about 5

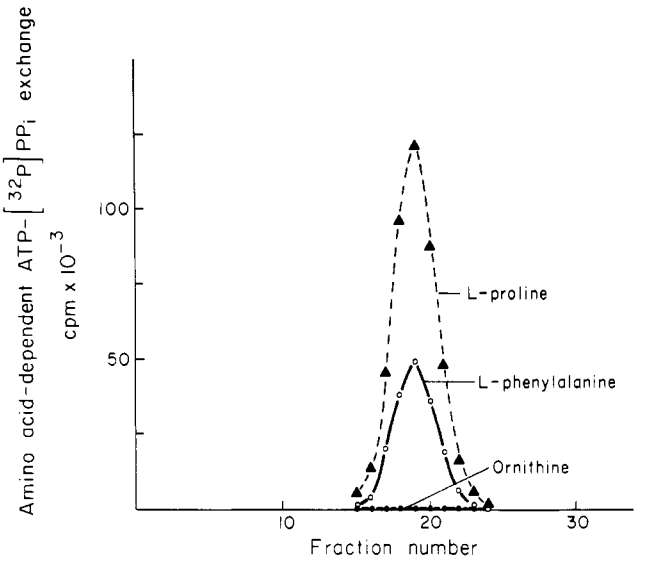

FIGURE 3: Last step of purification of IE; sucrose gradient centrifugation. IE (0.1 ml) from DEAE-cellulose chromatography (7 mg of protein/ml) was layered on three 5.5-ml 10–30% sucrose gradients in buffer B containing 0.1 M KCl, and was centrifuged for 7 hr at 50,000 rpm using the SW-50 rotor. Fractions of 0.13 ml were collected as described in the Experimental Section, and L-proline-dependent (▲), L-phenylalanine-dependent (○), and L-ornithine-dependent (●) ATP-$^{32}PP_i$ exchange activities were assayed with 2 μl from each fraction (top left, right bottom). The reaction mixture contained in a volume of 0.1 ml: the above enzyme, 4 mM each of the above amino acids, 2 mM ATP, 2 mM $^{32}PP_i$ (0.32 μCi), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM KCl, and 20 mM triethanolamine buffer (pH 7.4). The reaction was carried out at 37° for 15 min.

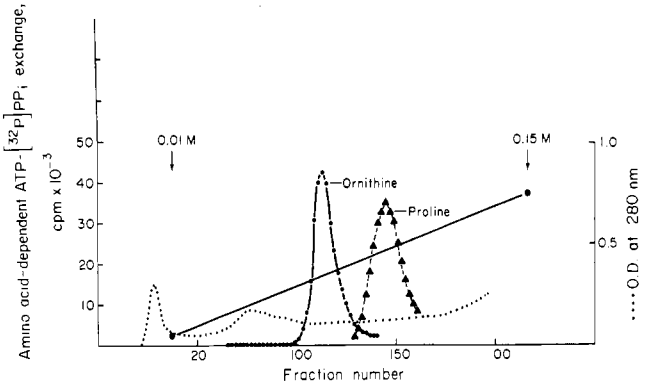

FIGURE 4: Hydroxylapatite chromatography of HE. About 320 mg of HE from fraction 61–74 (see Figure 1) was applied to a hydroxylapatite column (50 × 2.2 cm) equilibrated with 0.01 M phosphate buffer (pH 7.3) containing 1 mM dithiothreitol and 5% sucrose. It was eluted with 1 l. of phosphate buffer gradient (0.01–0.15 M, pH 7.3) containing 1 mM dithiothreitol and 5% sucrose. Fractions of 7 ml were collected, and 10 μl from each was assayed for HE by L-ornithine-dependent (●) and for IE by L-proline-dependent (▲) ATP-$^{32}PP_i$ exchange activities.

ml. The enzyme was precipitated by adding ammonium sulfate to 50% saturation, and the precipitate collected by centrifugation and dissolved in 0.5 ml of buffer B.

SUCROSE GRADIENT CENTRIFUGATION. From the concentrated eluate of IE from DEAE-cellulose chromatography, 0.1 ml was layered on three 5.5-ml sucrose gradients of 10–30% in buffer B containing 0.1 M KCl, and centrifuged for 7 hr at 50,000 rpm using the SW-50 rotor of the Spinco centrifuge. The gradient was fractionated by forcing gravity-fed 40% sucrose from the bottom and collecting the fractions from the top (Figure 3).

Purification of HE. The HE fraction that eluted between tube 61 and 74 from Sephadex G-200 (Figure 2) was purified by hydroxylapatite chromatography in a manner identical to that described for IE (Figure 4). As may be seen, this step removed sizeable amounts of IE. The HE eluted from the hydroxylapatite column was concentrated by means of the Diaflo apparatus and precipitated with ammonium sulfate (50% saturation). The precipitate was collected by centrifugation, dissolved in 1.0 ml of buffer B, and without DEAE column fractionation, submitted to sucrose gradient centrifugation in a manner identical with that described for IE (Figure 5). The progress of purification is summarized in Table II.

Comments on the Revised Procedure for Purification. In the extracts used earlier for Sephadex G-200 filtration, a relatively high viscosity caused by undigested DNA was responsible for the incomplete separation of the three component enzymes in various stages of purification. Neither streptomycin nor protamine sulfate were found to be practical in selectively removing DNA from the crude enzyme. However, addition of higher concentrations of Mg^{2+} considerably reduced the time of incubation with DNase to obtain low viscosity; furthermore, KCl was substituted for $MgCl_2$ (Roskoski *et al.*, 1970a) in Sephadex G-200 chromatography. Even though the elution pattern of the three complementary enzymes remained similar to that reported previously, the change of electrolytes resulted in a severalfold reduction of the amount of protein eluted at the void volume, whereby the contamination of HE by IE was considerably reduced. In hydroxylapatite chromatography, the length of column seemed to be critical for the separation of IE and HE. HE could be eluted from the column at 0.05–0.06 M phosphate regardless of column length; however, IE was eluted from a column of 12 cm at 0.05–0.06 M, of 20 cm at 0.07–0.08 M, and of 50 cm at 0.09–0.11 M. The yields and purity of IE and HE at the various purification steps are shown in Table II.

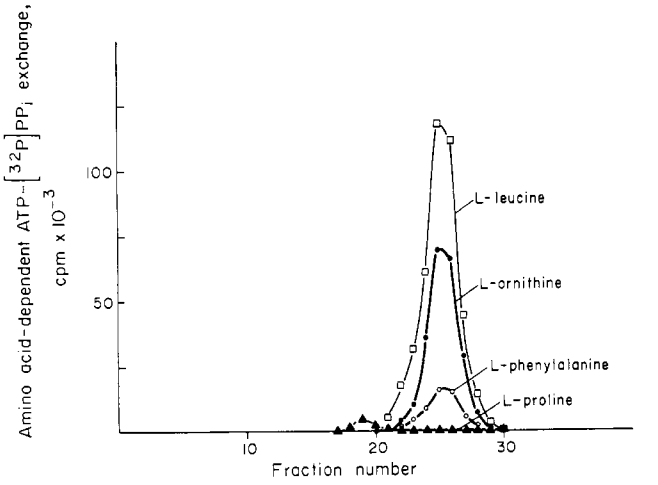

FIGURE 5: Last step of purification of HE; sucrose gradient centrifugation. HE (0.13 ml) from hydroxylapatite chromatography was layered on three sucrose gradients and centrifuged in a manner identical with that described in Figure 3. From each fraction 2 μl was assayed for L-leucine-dependent (□), L-ornithine-dependent (●), L-phenylalanine-dependent (○), and L-proline-dependent (▲) ATP-$^{32}PP_i$ exchange activities.

TABLE II: Purification of the Intermediate and the Heavy Polyenzymes.

Stages	Protein[a] (mg)	Amino Acid Dependent ATP–$^{32}PP_i$ Exchanges (cpm)[b]	Sp Act. (cpm/mg of Protein)	Yield
Intermediate		Proline Dependent		
1. 20,000*g* supernatant	26,000	3.1×10^9	1.2×10^5	
2. Ammonium sulfate (33–45% saturation)	7,400	2.4×10^9	3.2×10^5	77
3. Sephadex G-200	780	1.8×10^9	2.3×10^6	58
4. Hydroxylapatite	72	9.3×10^8	1.3×10^7	30
5. DEAE-cellulose	11	5.1×10^8	4.6×10^7	16
6. Sucrose gradient	3.8	2.8×10^8	7.4×10^7	9
Heavy		Ornithine Dependent		
1. 20,000*g* supernatant	26,000	1.1×10^9	4.2×10^4	
2. Ammonium sulfate (33–45% saturation)	7,400	7.2×10^8	9.7×10^4	65
3. Sephadex G-200	460	5.9×10^8	1.3×10^6	54
4. Hydroxylapatite	26	3.1×10^8	1.2×10^7	28
5. Sucrose gradient	5.8	1.5×10^8	2.6×10^7	14

[a] Protein content was determined by the method of Lowry *et al.* (1951) in stages 1–5, and by the Biuret method (Gornall *et al.*, 1949) in stage 6. [b] In stages 1 and 2 a small amount of the enyzme samples was centrifuged on sucrose gradients to separate the IE from low molecular weight fragments that catalyze proline-dependent or ornithine-dependent ATP–$^{32}PP_i$ exchange. The low molecular weight fragments included proline tRNA ligase and subunits of IE and HE (see Figures 8 and 9).

To test the final preparations of IE and HE for purity, they were submitted to acrylamide gel electrophoresis. The result is seen in Figure 6. The photograph indicates a single band for HE; in IE the major band was accompanied by a small impurity running ahead, which faded on washing. We assess the purity of these preparations to be approximately 90%.

Results Obtained with Purified Enzymes

Amino Acid Activation of Purified IE and HE. As mentioned in the introduction, our earlier results with rather impure preparations had seemingly indicated that only proline was activated and fixed by IE, whereas Kurahashi's results suggested that IE activates the second to fourth amino acids, proline, phenylalanine, D-phenylalanine. This has been confirmed using our purified enzyme. Table III shows the amino acid dependent ATP–$^{32}PP_i$ exchange with IE and HE. In addition to proline, IE activates L- and D-phenylalanine and L- and D-tryptophan, which can interchangeably fill the third and fourth positions after proline. HE shows an activation of the six following amino acids, including phenylalanine and tryptophan, but only the L forms. However, tyrosine reacts considerably better with HE than with IE, as is to be expected

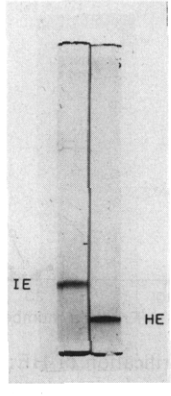

FIGURE 6: Gel electrophoresis of purified IE and HE. From the sucrose gradient centrifugation 12 μg each of IE and HE were subjected to electrophoresis using 5% acrylamide gel. The electrophoresis was carried out at 4 mA/gl for 5 hr with 0.03 M triethanolamine buffer (pH 8.1).

TABLE III: Amino Acid Dependent ATP–$^{32}PP_i$ Exchange with IE and HE.[a]

Amino Acids	[^{32}P]ATP Formed: Intermediate (nmol)	[^{32}P]ATP Formed: Heavy (nmol)
L-Proline	30.2	0
L-Phenylalanine	12.3	2.2
D-Phenylalanine	2.1	0
L-Tryptophan	25.6	6.8
D-Tryptophan	5.3	0
L-Asparagine	0	20.0
L-Glutamine	0	11.6
L-Tyrosine	2.7	13.6
L-Valine	0	38.2
L-Ornithine	0	15.4
L-Leucine	0.1	24.8

[a] The last stage purified IE (2.4 μg) and the last stage purified HE (4.1 μg) were incubated for 15 min at 37° with 4 mM of the designated amino acids and 2 mM ATP, 2 mM $^{32}PP_i$ (0.32 μCi), 1 mM dithiothreitol, 20 mM triethanolamine buffer (pH 7.4), and 5 mM KCl in a reaction volume of 0.1 ml. The ATP–$^{32}PP_i$ exchanges were assayed with charcoal as described (Gevers *et al.*, 1968).

TABLE IV: Binding of Amino Acids to IE in Thio Ester Linkage.[a]

Amino Acids	Amino Acid Concn (mM)	mol of Amino Acid Bound/mol of Enzyme
L-Proline	0.08	0.049
	0.16	0.078
	0.30	0.135
	0.60	0.233
L-Phenylalanine	0.08	0.094
	0.16	0.154
	0.30	0.266
	0.60	0.460
L-Asparagine	0.12	0

[a] From the sucrose gradient centrifugation 62 μg of IE (see Table I) was incubated in a reaction volume of 0.1 ml for 20 min at 37° with the ^{14}C-labeled amino acids shown above (0.2 Ci/mol), 2 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 mM triethanolamine buffer (pH 7.4). The amino acids bound to the enzyme were determined in the trichloroacetic acid precipitate as described in the Experimental Section. The number of moles of enzyme in the reaction mixture was computed on the assumption that the enzyme was 90% pure.

since it is preferentially incorporated into position seven where it is found in tyrocidine A, B, and C.

The Covalent Binding of Amino Acids to HE and IE. As has been extensively shown in the previous communications on the biosynthesis of tyrocidine and gramicidin S (Roskoski *et al.*, 1970a,b, 1971; Gevers *et al.*, 1968, 1969; Kleinkauf *et al.*, 1969–1971), the amino acid is transferred to an enzyme-bound SH group after initial activation with ATP to aminoacyl adenylate. This is most easily determined by fixation of the radioactivity in the trichloroacetic acid precipitate. The binding to this precipitate was assayed with purified IE and

TABLE V: Binding of Amino Acids to HE in Thio Ester Linkage.[a]

Amino Acids (0.12 mM)	mol of Amino Acid Bound/mol of Enzyme
L-Proline	0
L-Asparagine	0.157
L-Glutamine	0.138
L-Phenylalanine	0.080
L-Valine	0.092
L-Ornithine	0.085
L-Leucine	0.162

[a] From the sucrose gradient (see Table I), 31 μg of HE was incubated with 0.12 mM of ^{14}C-labeled amino acids and the other ingredients shown in Table IV, and covalently bound amino acids were determined in the trichloroacetic acid precipitate as described in the Experimental Section. The number of moles of enzyme in the reaction mixture was computed on the assumption that the enzyme was 90% pure.

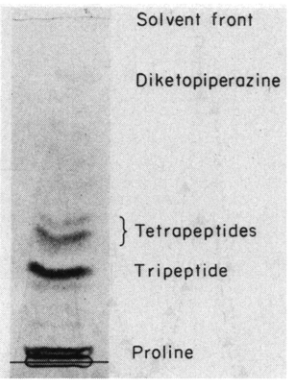

FIGURE 7: Nascent peptides bound to IE. From the sucrose gradient centrifugation (see Table I) 18 μg of IE and 31 μg of LE from DEAE-cellulose chromatography were incubated for 1 hr at 37° in a reaction volume of 0.1 ml with 1.5×10^{-4} M [^{14}C]proline (0.4 μCi), 1.5×10^{-4} M [^{3}H]phenylalanine (2 μCi), 2 mM ATP, 10 mM $MgCl_2$, and 20 mM triethanolamine buffer (pH 7.4). After 1 hr, IE was separated from the incubation mixture by sucrose gradient centrifugation in the manner described in Figure 3. The nascent peptides were liberated from IE by alkali hydrolysis and the peptides separated by thin-layer chromatography as described by Roskoski *et al.* (1970b).

the results are shown in Table IV. With increasing concentrations of proline and phenylalanine added, the binding of the amino acids was found to be strongly concentration-dependent and saturation was not yet reached at 0.6 mM. However,

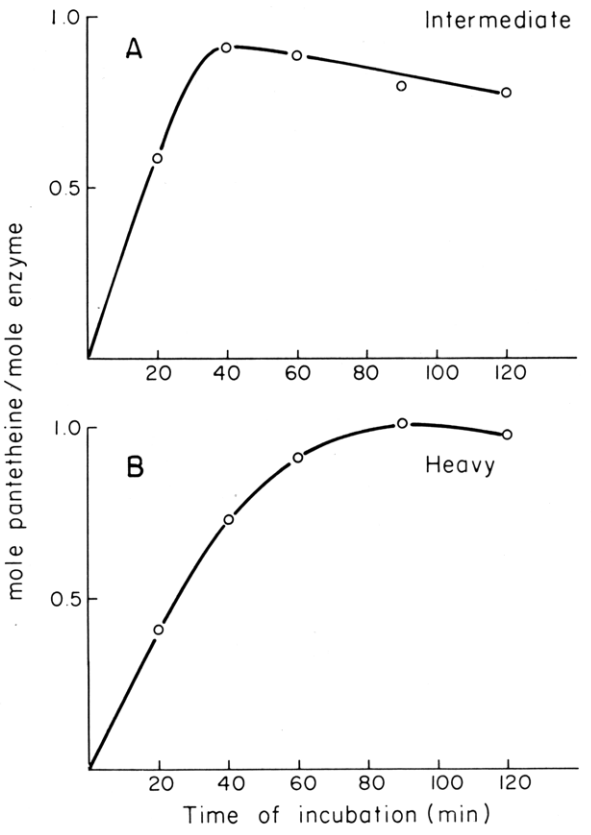

FIGURE 8: Pantetheine content of IE (A) and HE (B). From the sucrose gradient centrifugation, 18 μg of IE or 12 μg of HE was incubated for varying lengths of time with 1 N KOH in a volume of 0.2 ml at 100°. The pH of the incubation mixture was adjusted to about 8, and it was subjected to alkali phosphatase. The pantothenic acid liberated was then assayed microbiologically as described by Pugh and Wakil (1965).

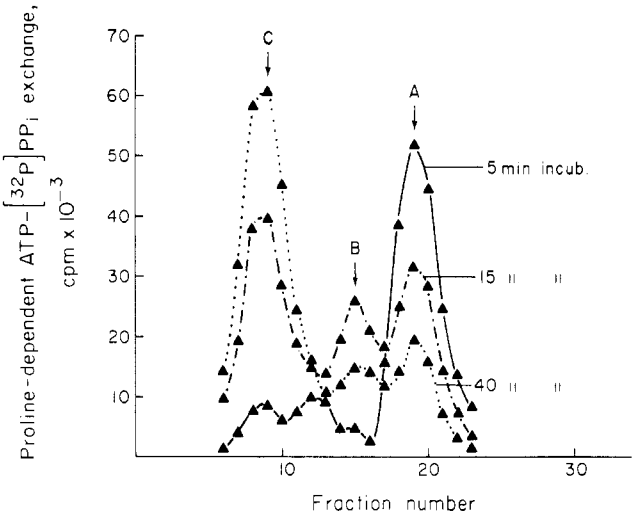

FIGURE 9: Dissociation of IE. To 5 g of cells suspended in 15 ml of buffer B was added 10 mg of lysozyme in 5 ml of the same buffer. The mixture was lysed for 10 min at 37°. To the lysate, 0.1 ml of 1 M $MgCl_2$ and 10 μg of DNase in 0.1 ml of buffer B were added, and lysate was further incubated at the same temperature. At intervals of 5, 15, and 40 min after the addition of DNase, 5-ml aliquots were drawn off and centrifuged for 10 min at 20,000*g*. Ammonium sulfate was added to 50% saturation to the supernatant, and the precipitates were collected by centrifugation. The pellets were dissolved in buffer B to give about 20 mg of protein/ml, and 0.2 ml of this enzyme solution was layered on a 5.5-ml 10–30% sucrose gradient and centrifuged for 7 hr at 50,000 rpm using a SW-50 rotor. Forty-three fractions of 0.13 ml were obtained from the gradient (top left, bottom right) and 50 μl from each were assayed for L-proline-dependent ATP-$^{32}PP_i$ exchange activity indicating derivatives of IE. The reaction mixture for the exchange contained in a reaction volume of 0.3 ml: 2 mM ATP, 2 mM $^{32}PP_i$ (0.96 μCi), 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM triethanolamine buffer (pH 7.4), 20 mM KCl, and the enzyme from the sucrose gradient. After 30 min at 37°, the exchanges were assayed with charcoal as described (Gevers *et al.*, 1968). Peaks A, B, and C corresponded to approximate molecular weights of 230,000, 150,000, and 75,000, respectively.

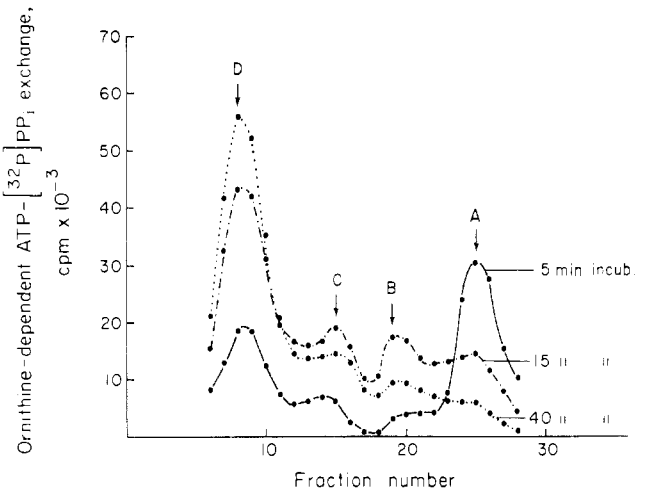

FIGURE 10: Dissociation of HE. The same fractions collected from the sucrose gradient were assayed for L-ornithine-dependent ATP-$^{32}PP_i$ exchange activities indicating derivatives of HE. Peaks A, B, C, and D corresponded to approximate molecular weights of 440,000, 230,000, 15,000, and 70,000, respectively.

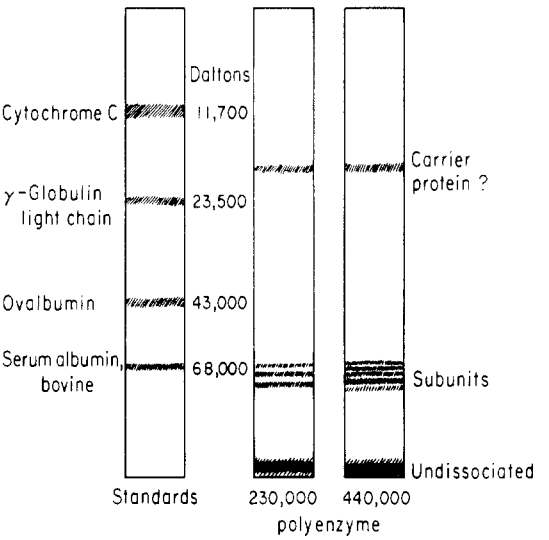

FIGURE 11: Sketch of sodium dodecyl sulfate gel electrophoresis of IE and HE. Conditions for electrophoresis are described in Experimental Section.

in all cases, the amount of both amino acids fixed was proportional to the concentration added, and approximately twice as much phenylalanine was fixed as proline, implying that for every proline, two phenylalanines were bound. Using L-phenylalanine and assaying for enzyme-bound D form, it was found that part of the L-phenylalanine was converted to the D configuration. Thus, IE, like LE, contains a racemase for aromatic amino acids. The absence of HE from this preparation is indicated by no incorporation of asparagine, one of the amino acids specifically activated by HE.

In Table V, the binding of the six other amino acids by HE is shown. It appears that their affinities differ; using the same concentration, they are not bound in equimolar proportions.

Initiation of Polymerization by LE. A combination of LE and IE yielded enzyme-bound proline and a tripeptide and a tetrapeptide, the Pro-Phe-diketopiperazine being lost during trichloroacetic acid precipitation (Figure 7). Two bands appeared in the tetrapeptide region; comparison of the ratios of [^{3}H]phenylalanine and [^{14}C]proline in several experiments indicated in the lighter, upper band a ratio of Phe:Pro of 3, and in the heavier, lower band, of 2.7. The explanation for this may be found in the fact that two of the phenylalanine residues are D and therefore have undergone racemization. A pyridoxal-independent racemase of β-hydroxylbutyryl-SCoA has been found in animal tissues (Stern, 1961), and another pyridoxal-independent and apparently SH-dependent racemase for hydroxyproline has been described (Cardinale and Abeles, 1968). With the latter, deuterium exchange parallels racemization. Since we know that the racemizing D-phenylalanine is thio ester linked to the enzyme (Gevers *et al.*, 1969; Takahashi *et al.*, 1971) it should have lost tritium, which would explain the lower ratio of $^{14}C:^3H$ in one of the bands. The reason for their differential chromatography, however, is not quite clear. We are planning to study this aspect of the racemization in antibiotic synthesis.

Pantetheine Content of IE and HE

Since it now appears that IE synthesizes a sequence of three amino acids, purified IE was analyzed for pantetheine and found to contain 1 mol/mol of enzyme (Figure 8A). Using the nearly pure HE (Figure 8B), the earlier reported pantetheine content of 1 mol/mol of enzyme was confirmed. Thus, in the well-studied cases of this series of antibiotics, namely,

gramicidin S and tyrocidine, where longer peptide chains are synthesized on polyenzymes, pantetheine takes part in the polymerization reaction probably in a manner previously discussed (Roskoski *et al.*, 1970b, 1971; Gevers *er al.*, 1969; Kleinkauf and Gevers, 1969; Kleinkauf *et al.*, 1970, 1971).

Disaggregation of the Polyenzymes IE and HE

Incubation of Crude Extracts. On incubation of cell lysate containing membranes, ribosomes, and debris, at 37° with DNase, a progressive split of the polyenzymes occurred, as shown by sucrose gradient centrifugation (Figures 9 and 10). Shorter incubation at 37° led to the appearance of split products with activating capacities for two or one amino acids only; after 40 min, nearly all IE and HE polyenzymes had disappeared and a large peak appeared at the region of mol wt 70,000–75,000 which seemed to represent the overlapping 70,000 molecular weight subunits for single amino acid activation.

While IE (A of Figure 9) binds proline and phenylalanine in a ratio of 1:2, the fragment designated as B was found to bind proline and phenylalanine in thio ester linkage in a ratio of 1:1. The fragment designated C presumably contains a subunit that binds only with proline or phenylalanine; the latter was not assayed for. The possibility that either peak B or peak C represents proline tRNA ligase is ruled out since the synthetase activity was found in fraction 11, as indicated in the figure. HE (A of Figure 10) was converted to three low molecular weight fractions (B, C, and D of Figure 10). The peak designated B had the same sedimentation value as IE, about one-half the molecular weight of HE. The C peak had a sedimentation value similar to the fragment from IE which binds proline and phenylalanine in 1:1 ratio, whereas the D peak's sedimentation value was slightly lower than peak C from IE. The mode of action of the series of low molecular weight fractions from HE thus confirms the suggestion that HE consists of six subunits, one for every amino acid bound, and that peaks B, C, and D represent the fragments which consist of three, two, and one subunits respectively.

Similar results were obtained by Sephadex G-200 chromatography. Upon prolonged incubation of crude extracts, nearly all IE and HE polyenzymes disappeared and the activating activities of all constituent amino acids of IE and HE appeared at the 70,000 molecular weight region. Incubation of purified enzyme with DNase failed to produce the low molecular weight fractions from IE or HE, ruling out the possibility that DNase has any direct effect on the fragmentation of these enzymes. This makes it likely that the fragmentation is due to an activity present in the crude extract. Experiments towards the identification of this activity are in progress.

Separation of Subunits by Sodium Dodecyl Sulfate Gel Electrophoresis. Independent evidence that IE and HE are polyenzymes composed of subunits was obtained by this method. As shown in Figure 11, on incubation with sodium dodecyl sulfate, IE yielded three closely spaced bands each with a molecular weight of about 70,000, while HE yielded five closely spaced bands each with a molecular weight of about 70,000. One of the five bands that originated from HE was more heavily stained than the remaining four, suggesting that it contained two subunits of similar molecular weights. Both IE and HE also yielded a band at the region of 20,000 molecular weight, which could correspond to a carrier protein containing pantetheine (Kleinkauf *et al.*, 1970); this remains to be confirmed by analysis.

The amount of purified enzymes available for these experiments was too small to give bands strong enough to be photographed and therefore the results are represented by a drawing. Although weak, the bands were rather sharply defined, and are thus presented as preliminary data.

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