Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2343–2347, June 1977
Biochemistry

Isolation of amino acid activating subunit–pantetheine protein complexes: Their role in chain elongation in tyrocidine synthesis

(antibiotic peptides/nonribosomal peptide synthesis/peptide bond formation/multienzymes)

SUNG G. LEE AND FRITZ LIPMANN

The Rockefeller University, New York, New York 10021

Contributed by Fritz Lipmann, March 24, 1977

ABSTRACT **Dissociation of the multienzymes of tyrocidine synthesis by prolonged incubation of crude extracts of *Bacillus brevis* (Dubos strain, ATCC 8185) has yielded, on Sephadex G-100 chromatography, two fractions of amino acid activating subunits, a larger one of 70,000 daltons and a smaller one of 90,000 daltons; the latter was a complex consisting of the 70,000 dalton subunit and the pantetheine-carrying protein of about 20,000 daltons. When it dissociated, the intermediate enzyme, which activates three amino acids, contained two-thirds of the subunits in the 70,000 dalton and one-third in the 90,000 dalton fraction; the heavy enzyme, which activates six amino acids, contained five-sixths of the subunits in the former fraction and one-sixth in the latter. Both fractions showed ATP-PP_i exchange with all amino acids that are activated by the respective polyenzymes. With proline as an example, the 70,000 dalton subunit exhibited a single low-affinity binding site, which should correspond to the peripheral thiol acceptor site, whereas the 90,000 dalton subunit showed both a low-affinity binding site and an additional high-affinity site for proline; the high-affinity site is attributed to the pantetheine present on the pantetheine-carrying protein, and suggests that amino acids are translocated from the peripheral SH to the pantetheine-carrying moiety during chain elongation. This was confirmed by the observation that the 90,000 dalton complex, when incubated with the light enzyme in the presence of phenylalanine and proline, produced DPhe-Pro dipeptide that cyclized into DPhe-Pro diketopiperazine, but the 70,000 dalton activating subunit, when similarly incubated, did not. After subunit dissociation, however, no further elongation occurred after the transfer from phenylalanine to proline.**

Various microorganisms have now been found to produce antibiotic peptides nonribosomally (1–18). Much of our present knowledge and our formulation (1) of nonribosomal peptide synthesis has been obtained from the study of the biosynthetic mechanisms of gramicidin S and tyrocidine, which are decapeptide antibiotics produced by the *Bacillus brevis* strains ATCC 9999 and ATCC 8185, respectively. The biosynthesis of gramicidin S requires two complementary enzymes, that of tyrocidine, three. Of these complementary enzymes, one for gramicidin S and two for tyrocidine biosynthesis are multienzymes containing pantetheine (7–13, 15). The peptide synthesis proceeds with the ATP-driven activation of amino acids followed by attachment of the activated amino acids to the peripheral enzymic thiol groups; then, with the aid of enzyme-linked pantetheine, condensation of the thioesterified amino acids ensues (1, 7–9). Both the involvement of two or more enzymes, including the pantetheine-containing multienzymes, and the ATP-driven activation of amino acids now appear to be common features of the nonribosomal synthesis of many peptide antibiotics (4–6, 13–18).

The three complementary enzymes of tyrocidine synthesis (9, 12, 16), termed the light, intermediate, and heavy enzymes, have been shown to have molecular weights of 100,000, 230,000, and 440,000, respectively. The intermediate and heavy enzymes are multienzymes composed of three and six amino acid activating subunits of about 70,000 daltons each (16). We have shown (17) that these multienzymes contain 1 mole of a 17,000–20,000 dalton pantetheine-containing protein per mole of multienzyme in addition to the amino acid activating subunits, and that the nascent peptides are exclusively bound to this protein. This confirms the previously suggested role of pantetheine in the polymerization of the enzyme-bound amino acids (18).

Although the essential role of the pantetheine-containing protein in the polymerization of amino acids cannot be disputed, one unresolved question remained: the actual site of peptide bond formation in the multienzyme complex. This could occur between the carboxyl terminus of the pantetheine-bound nascent peptide and the amino group of the amino acid bound to the peripheral thiol site located on the activating subunit, in which case the site of condensation would have to be on the activating subunit. In an alternative scheme that is quite analogous to the donor and acceptor sites on the ribosome, the presence of two sites on the pantetheine-carrying protein may be envisaged, with pantetheine being one of them. In this scheme, the amino acid bound to the peripheral thiol site located on the 70,000 dalton subunit is presumably translocated to an acceptor on the pantetheine-carrying protein complex. The carboxyl group of the nascent peptide is then transferred from the donor site to the amino group of the amino acid bound to the acceptor site, thereby increasing the chain by one amino acid. This new nascent peptide is then translocated back to the donor site, making the acceptor site available for the oncoming next amino acid.

With the isolation, to be described here, of both the amino acid activating subunits and the complexes composed of the amino acid activating subunits and pantetheine-containing protein, we have been able to approach more closely the site of amino acid condensation. Our experimental results detailed in the present report indicate the pantetheine-carrying protein complex acts as a peptidyl transferase to the newly adding amino acid.

METHODS

Growth and Harvest of Cells. Cells of the tyrocidine-producing strain of *B. brevis* (ATCC 8185) were grown and harvested as described (15) at the peak of tyrocidine synthesis. This peak occurs for a brief period during the early stationary phase of cell growth and is determined by monitoring β-alanine uptake, which develops slightly ahead of the induction of tyrocidine synthesis enzymes, as reported earlier (14).

Preparation of Subunits of the Multienzymes of Tyrocidine Synthesis. The cells were lysed and the lysate was allowed to stand at room temperature for 20–30 min as described (16) to ensure autolysis of the multienzymes of tyrocidine synthesis. Cytosol was then prepared from the lysate by centrifugation

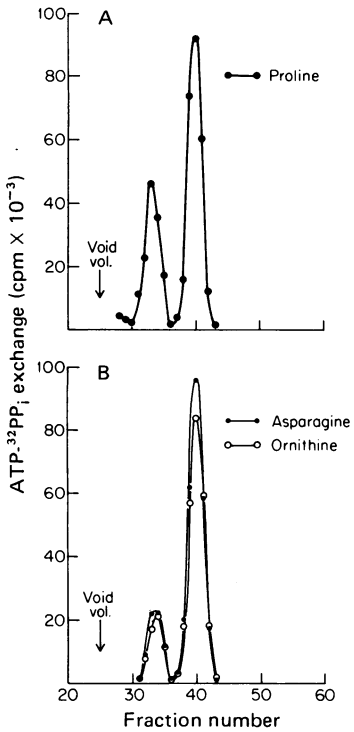

FIG. 1. Resolution by Sephadex G-100 chromatography of two forms of subunits from the intermediate (*A*) and the heavy (*B*) enzymes of tyrocidine synthesis. A subunit fraction of about 120 mg of protein, purified to the Sephadex G-200 stage as described (16), was applied to a Sephadex G-100 column (2.8 × 85 cm) equilibrated with 10 mM triethanolamine buffer, pH 7.5, containing 100 mM KCl, 1 mM dithiothreitol, and 0.5 mM EDTA, and the column was eluted with the same buffer solution. Subunits were located by measuring amino acid-dependent ATP-$^{32}PP_i$ exchange activity (11, 15).

at 20,000 × *g* for 20 min, and therefrom an ammonium sulfate fraction precipitating between 33% and 50% saturation was prepared and subjected to Sephadex G-200 chromatography as described (16). The subunit region (70,000–90,000 molecular weight) from the G-200 chromatography was concentrated by means of a Diaflo apparatus, and was further purified by chromatography on Sephadex G-100, DEAE-cellulose, and hydroxylapatite, as described in the figure legends. Throughout the purification, the subunit fractions were located by determining amino acid activation.

Assay for Amino Acid Activation. Amino acid activation was assayed as described (11, 15) by measuring the ATP-$^{32}PP_i$ exchange activity dependent on corresponding amino acids.

RESULTS

Resolution of Two Forms of Subunit from the Multienzymes of Tyrocidine Synthesis. The initial steps for preparing the amino acid activating subunits of the two multienzymes of tyrocidine synthesis were carried out as described (16). When rechromatographed on Sephadex G-100, the broad peak of the subunit region from Sephadex G-200 resolved into two distinct peaks, one of 90,000 daltons and one of 70,000 daltons, that activated all the constituent amino acids of tyrocidine (Fig. 1 *A* and *B*). This indicated that all of the amino acid activating subunits of the two multienzymes could be dissociated into these two forms. The relative amount of the 70,000 molecular weight form was always higher than that of the 90,000 molecular weight form in all the subunits examined.

The 70,000 dalton form originating from the intermediate enzyme invariably had twice as much amino acid activating

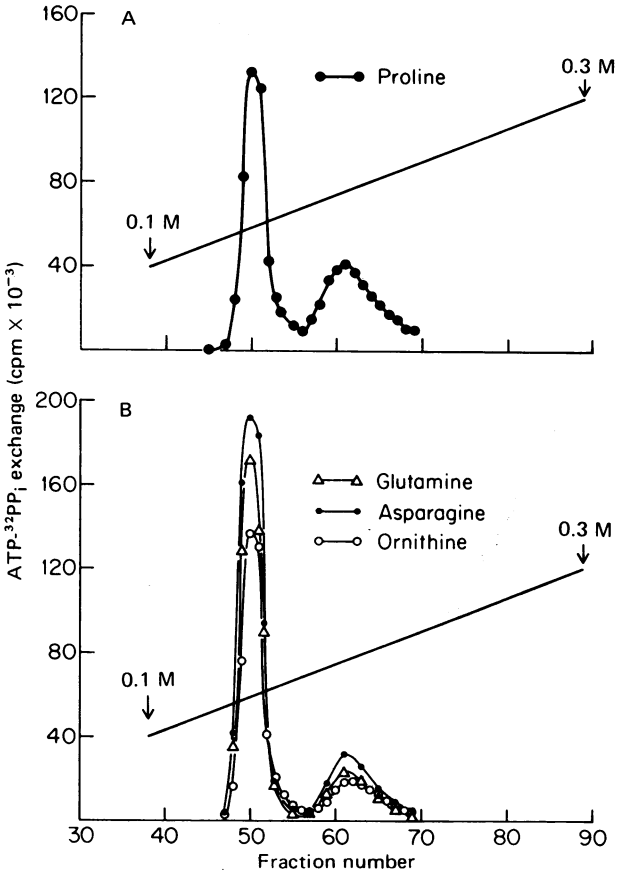

FIG. 2. Resolution of two forms of subunits from the intermediate (*A*) and the heavy (*B*) enzymes of tyrocidine synthesis by DEAE-cellulose chromatography. A subunit fraction of about 180 mg of protein, purified to the Sephadex G-200 stage, was applied to a DEAE-cellulose column (2.2 × 14 cm) equilibrated with 20 mM triethanolamine buffer, pH 7.5, containing 5% sucrose, 1 mM dithiothreitol, and 0.5 mM EDTA. The column was eluted with 150 ml of 0.1 M KCl in the same buffer solution and then with 500 ml of linear KCl gradient (0.1–0.3 M) in the same buffer solution. Subunits were located by measuring amino acid-dependent ATP-$^{32}PP_i$ exchange activity.

activity as the 90,000 dalton form (Figs. 1*A* and 2*A*), whereas in the subunits from the 440,000 dalton heavy enzyme there was about five times the activity in the former compared to the latter. The significance of the proportion of the subunits distributed in these two forms will be discussed further.

The subunit region from Sephadex G-200 could also be resolved into two distinct peaks by DEAE-cellulose chromatography (Fig. 2). The molecular weights of the first and second peaks obtained by such treatment were likewise 70,000 and 90,000, respectively, when determined by rechromatography on Sephadex G-100. The two peaks also contained all of the amino acid activating subunits, and the proportion of the activity of the two was again 2:1 for the subunits of intermediate enzyme origin, and 5:1 for subunits from the heavy enzyme. Fig. 2 *A* and *B* shows that the elution profiles of the different subunits from DEAE-cellulose chromatography are indistinguishable from one another, indicating that all of them have nearly identical physicochemical properties. Their similarity was also seen in the nearly identical elution profiles of the 70,000 dalton subunits for proline, ornithine, and phenylalanine, i.e., for the subunits from both the intermediate and heavy enzymes, from the hydroxylapatite column (Fig. 3).

Identity of the 90,000 Molecular Weight Subunits. Because

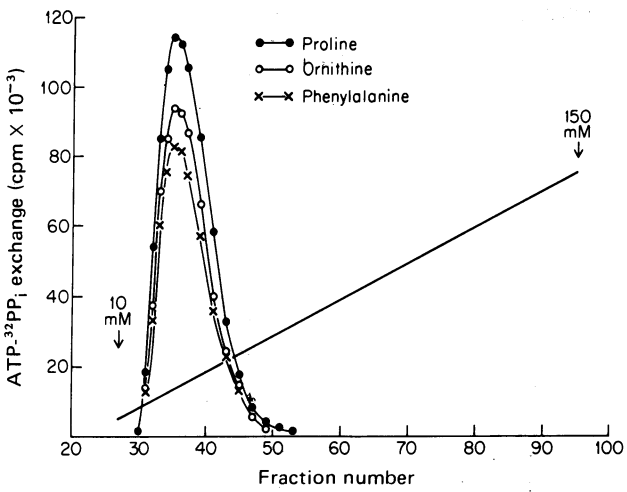

FIG. 3. Hydroxylapatite chromatography of 70,000 dalton subunits. The 70,000 dalton subunit fraction, purified by Sephadex G-100 and DEAE-cellulose chromatography as described in Figs. 1 and 2, was applied to a hydroxylapatite column (2.2 × 45 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.5, containing 5% sucrose and 1 mM dithiothreitol. The column was eluted first with 200 ml of the same buffer solution, then with 1 liter of phosphate buffer gradient (10–150 mM) containing sucrose and dithiothreitol.

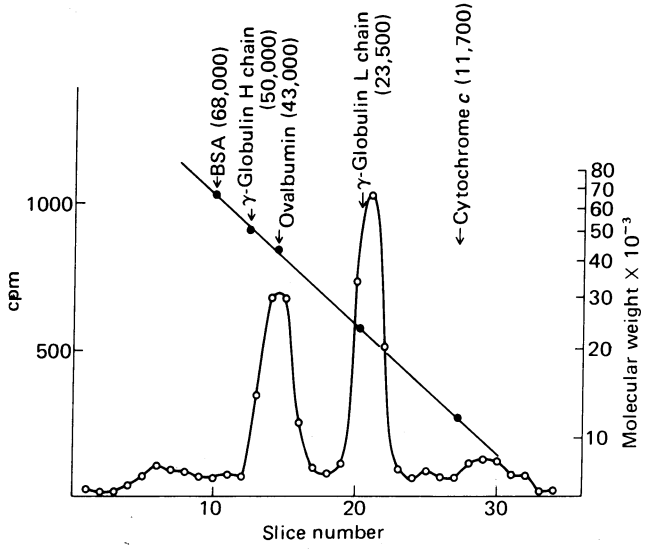

FIG. 4. Sodium dodecyl sulfate gel electrophoresis of the 90,000 dalton subunits. The 90,000 dalton subunit fraction was purified by chromatography on Sephadex G-100, DEAE-cellulose, and hydroxylapatite as described in Figs. 1–3. The purified 90,000 dalton subunit fraction was heated for 1 hr at 90° with 100 mM dithiothreitol and 2% sodium dodecyl sulfate, and then subjected to electrophoresis using 7% polyacrylamide gel containing 0.5% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer, pH 7.0. BSA, bovine serum albumin; H, heavy; L, light.

we have reported (16, 17) that each of the multienzymes of tyrocidine synthesis contains 1 mole of a 17,000–20,000 dalton pantetheine-carrying protein per mole of multienzyme in addition to the amino acid activating subunit of about 70,000 daltons, it was tempting to test to see whether the 90,000 molecular weight forms were composed of 70,000 dalton amino acid activating subunits and pantetheine-carrying protein. To examine this possibility, we labeled the multienzymes as described (17) with β-[^3H]alanine, a component of pantetheine, and, after dissociating them by autolysis, purified the 90,000 dalton subunits as described in the legend of Fig. 4; these subunits were then subjected to sodium dodecyl sulfate electrophoresis. As can be seen in Fig. 4, a high peak of radioactivity due to β-alanine content at 21,000 daltons and another peak about two-thirds as high between 40,000 and 44,000 daltons were observed, indicating that the decomposition of the 90,000 dalton form yielded mainly a pantetheine-carrying protein of 21,000 daltons; judging from the strong tendency of the pantetheine protein to form aggregates (17), the 40,000–44,000 dalton peak is presumably a dimer of this protein.

Amino Acid Binding Affinity to the Two Subunits and to Intact Multienzymes. It has been shown in this laboratory, first with the enzymes of gramicidin S synthesis (7, 8) and later with the enzymes of tyrocidine synthesis (9, 13), that the amino acids activated by these enzymes are subsequently translocated to enzymic thiol groups, and that the enzyme-bound amino acids undergo polymerization with the aid of 4′-phosphopantetheine linked to a low-molecular-weight carrier protein (17). In the present experiments, the binding of proline to the two different forms of subunits was examined and compared with its binding to undissociated intermediate enzyme.

As shown in Fig. 5*A*, the 70,000 molecular weight subunit revealed a single low-affinity binding constant for proline, indicating that each 70,000 dalton subunit has a single amino acid binding site for the respective amino acid. Thus, the present observation is proof of the previous assignment (7–9, 13) of a single "peripheral thiol group" for the binding of each of the tyrocidine constituent amino acids. In contrast to the 70,000 dalton subunit, the 90,000 dalton subunit exhibited one low-affinity binding constant and an additional high-affinity one (Fig. 5*B*). These results indicate that the high-affinity binding site has to be assigned to the pantetheine-containing protein, and that the amino acids bound to the 70,000 dalton subunits are translocated to the pantetheine-containing protein prior to the polymerization process. The undissociated intermediate enzyme, which contains three amino acid activating subunits, including the proline activating subunit and a pantetheine-containing protein, likewise showed the two binding constants for proline, one low and one high affinity.

DPhe-Pro Dipeptide Synthesis with a Pantetheine-Containing Subunit. In the normal course of tyrocidine synthesis, elucidated largely from the work done in this laboratory (9, 16), the 100,000 dalton light enzyme activates the first amino acid of tyrocidine, phenylalanine, which is then translocated to the enzymic thiol site, where it is racemized into its D isomer; it is then transferred to the intermediate enzyme and condenses with the enzyme-bound proline, the second amino acid of tyrocidine, to form the DPhe-Pro dipeptide, which, normally, in turn condenses with the next amino acid and the polymerization process continues if multienzymes are used. Although the participation of pantetheine in the amino acid polymerization process was shown earlier (13, 18), it has not yet been determined whether polymerization occurs on the amino acid activating subunits, i.e., the 70,000 dalton subunit, or on the pantetheine-containing protein complex of 90,000 daltons.

To approach this question experimentally, we incubated the 70,000 and 90,000 dalton fractions with the light enzyme in the presence of phenylalanine and [^{14}C]proline. As shown in Fig. 6, the 90,000 dalton subunit produced the DPhe-Pro dipeptide, which easily cyclized to DPhe-Pro diketopiperazine, while the 70,000 dalton subunit failed to produce the condensation. These results show that dipeptide formation proceeds only with a proline present on the pantetheine-containing protein, indicating this protein to be the site of amino acid polymerization. However, as was to be expected, no larger peptides were produced from the subunits, while in a mixture of intact light en-

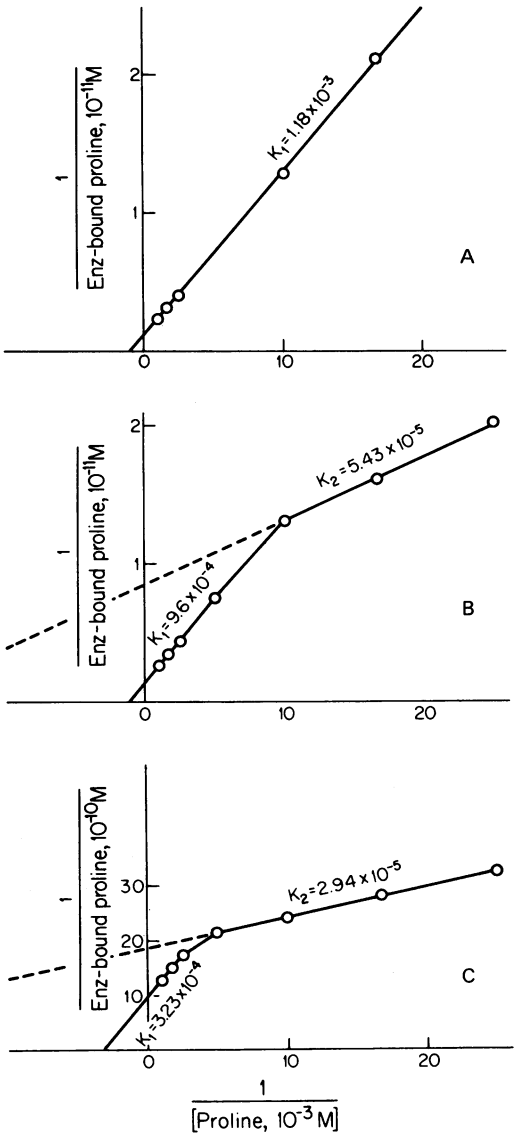

FIG. 5. Binding of proline to the 70,000 dalton (*A*) and 90,000 dalton (*B*) subunits and to the undissociated intermediate enzyme (*C*) in thioester linkage as indicated by trichloroacetic acid precipitability. The 70,000 and 90,000 dalton subunit fractions, purified as described in the legends of Figs. 3 and 4, and the intermediate enzyme purified as described (15), were incubated for 20 min at 37° in a 0.2-ml reaction volume with 0.04–1 mM [^{14}C]proline (5 μCi/μmol)/2 mM ATP/10 mM $MgCl_2$/100 mM KCl/1 mM dithiothreitol/0.5 mM EDTA/20 mM triethanolamine buffer, pH 7.5. At the end of the reaction, the enzymes were precipitated with 5% trichloroacetic acid and the precipitates were collected on Millipore filters. The filters were washed with five 5-ml portions of trichloroacetic acid and dried, and the radioactivity was determined. Binding constants, *K*, are in units of M^{-1}.

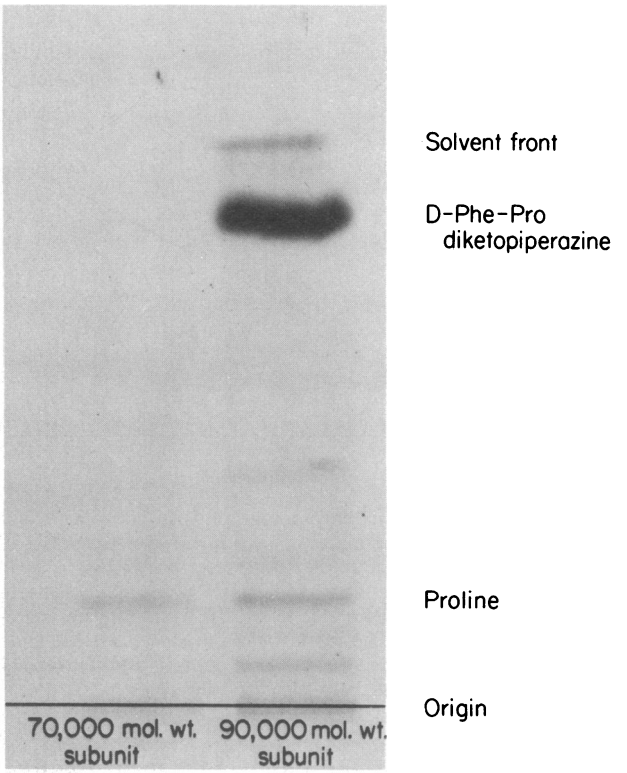

FIG. 6. Synthesis of DPhe-Pro diketopiperazine by the 70,000 and 90,000 dalton subunits. Both the 70,000 dalton and the 90,000 dalton subunit fractions were incubated with the light enzyme of tyrocidine synthesis for 1 hr at 37° in a reaction volume of 0.5 ml in the presence of 0.02 mM [^{14}C]proline (2.6 μCi)/0.2 mM phenylalanine/2 mM ATP/10 mM $MgCl_2$/100 mM KCl/1 mM dithiothreitol/0.5 mM EDTA/20 mM triethanolamine buffer, pH 7.5. At the end of the incubation, 2 ml of water and 3 ml of 1-butanol/chloroform (4:1 vol/vol) were added to the reaction vessels, and the mixture was vigorously shaken and centrifuged; the top organic solvent layer containing the diketopiperazine if formed was collected and shaken with 2 ml of 0.1 M NaCl and then centrifuged to extract residual water-soluble materials. The top organic solvent layer was collected and vacuum dried, and the residue was resolved in a small volume of 90% ethanol and was banded on a silica gel thin-layer plate. The chromatogram was developed with ethyl acetate/pyridine/acetic acid/water (90:30:9:16 vol/vol) and the radioautogram was made from it. The light enzyme used was prepared as described (15) and the two subunit fractions were prepared as described in the legends of Figs. 3 and 4.

zyme and intermediate enzyme, the tetrapeptide DPhe-LPro-LPhe-DPhe would have been formed.

DISCUSSION

As shown in Figs. 1 and 2, dissociation of the multienzymes of tyrocidine synthesis yielded 90,000 dalton amino acid activating subunit–pantetheine protein complexes in addition to 70,000 dalton amino acid activating subunits. All of the latter were remarkably similar in physicochemical properties, and were equally capable of forming a complex with the pantetheine protein. As mentioned, the ratio of the two subunits was 2:1 for those of intermediate enzyme origin and 5:1 for those from the heavy enzyme. A simple explanation for the observed ratios may be that the pantetheine protein is shared by three amino acid activating subunits in the intermediate enzyme and by six in the heavy enzyme. Thus, when the former dissociates, one-third of all the subunits carry the pantetheine protein and the remaining two-thirds have none. Likewise, when the heavy enzyme dissociates, one-sixth of all subunits will carry a pantetheine protein and the remaining five-sixths will be without it. Therefore, all of the amino acid activating subunits should be equally capable of associating with the pantetheine protein, and thus dissociation of a large population of the intermediate and heavy enzymes would result in the observed ratios for the 70,000 and 90,000 dalton forms.

In our present experiments, we obtained a value of approximately 21,000 daltons for the pantetheine protein. A similar value was obtained previously when this protein was separated from highly purified multienzymes by sodium dodecyl sulfate electrophoresis (16). However, when the multienzymes were dissociated by prolonged incubation with crude

cell lysate and the pantetheine protein was subsequently purified, the molecular weight was only 17,000 (17). One possible explanation for this discrepancy may be that the 21,000 dalton pantetheine protein is the native form and the 17,000 dalton form arises from partial cleavage of the native pantetheine protein by the protease present in the cell lysate. An analogous situation has been reported for the acyl carrier protein for fatty acid synthesis (19).

This work was supported by Grants GM-13972 from the National Institutes of Health and PCM75-21165 from the National Science Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Lipmann, F. (1973) "Nonribosomal peptide synthesis on polyenzyme templates," *Acc. Chem. Res.* **6**, 361–367.
2. Jayaraman, K., Moreal, J. & Paulus, H. (1969) "L-α-γ-Diaminobutyrate-activating enzyme from *Bacillus polymxa*," *Biochim. Biophys. Acta* **185**, 447–457.
3. Laland, S. G., Frøyshov, Ø., Gilhuus-Moe, C. & Zimmer, T-L. (1972) "Gramicidin S synthetase, an enzyme with an unusually large number of catalytic functions," *Nature New Biol.* **239**, 43–44.
4. Frøyshov, Ø. & Laland, S. G. (1974) "On the biosynthesis of bacitracin by a soluble enzyme complex from *Bacillus licheniformis*," *Eur. J. Biochem.* **46**, 235–242.
5. Ishihara, H. & Shimura, K. (1974) "Biosynthesis of bacitracin. III. Partial purification of a bacitracin-synthesizing enzyme system from *Bacillus licheniformis*," *Biochim. Biophys. Acta* **338**, 588–600.
6. Kurylo-Borowska, Z. (1975) "Edeine synthetase," in *Methods in Enzymology*, ed. Hash, J. H. (Academic Press, New York), Vol. 43, pp. 559–567.
7. Kleinkauf, H., Gevers, W. & Lipmann, F. (1969) "Interrelation between activation and polymerization in gramicidin S biosynthesis," *Proc. Natl. Acad. Sci. USA* **62**, 226–233.
8. Gevers, W., Kleinkauf, H. & Lipmann, F. (1969) "Peptidyl transfers in gramicidin S biosynthesis from enzyme-bound thioester intermediates," *Proc. Natl. Acad. Sci. USA* **63**, 1335–1342.
9. Roskoski, R., Jr., Gevers, W., Kleinkauf, H. & Lipmann, F. (1970) "Tyrocidine biosynthesis by three complementary fractions from *Bacillus brevis* (ATCC 8185)," *Biochemistry* **9**, 4839–4851.
10. Tomino S., Yamada, M., Itoh, H. & Kurahashi, K. (1967) "Cell-free synthesis of gramicidin S," *Biochemistry* **6**, 2552–2560.
11. Itoh, H., Yamada, M., Tomino, S. & Kurahashi, K. (1968) "The role of two complementary fractions of gramicidin S synthesizing enzyme system," *J. Biochem. (Tokyo)* **64**, 259–261.
12. Kambe, M., Sakamoto, Y. & Kurahashi, K. (1971) "Biosynthesis of tyrocidine by a cell-free enzyme system of *Bacillus brevis* ATCC 8185," *J. Biochem. (Tokyo)* **69**, 1131–1133.
13. Roskoski, R., Jr., Ryan, G., Kleinkauf, H., Gevers, W. & Lipmann, F. (1971) "Polypeptide biosynthesis from thioesters of amino acids," *Arch. Biochem. Biophys.* **143**, 485–492.
14. Lee, S. G., Littau, V. & Lipmann, F. (1975) "The relation between sporulation and the induction of antibiotic synthesis and of amino acid uptake in *Bacillus brevis*," *J. Cell Biol.* **66**, 233–242.
15. Lee, S. G. & Lipmann, F. (1975) "Tyrocidine synthetase system," in *Methods in Enzymology*, ed. Hash, J. H. (Academic Press, New York), Vol. 43, pp. 585–600.
16. Lee, S., Roskoski, R., Jr., Bauer, K. & Lipmann, F. (1973) "Purification of polyenzymes responsible for tyrocidine synthesis and their dissociation into subunits," *Biochemistry* **12**, 398–405.
17. Lee, S. G. & Lipmann, F. (1974) "Isolation of a peptidyl-pantetheine-protein from tyrocidine-synthesizing polyenzymes," *Proc. Natl. Acad. Sci. USA* **71**, 607–611.
18. Kleinkauf, H., Roskoski, R., Jr. & Lipmann, F. (1971) "Pantetheine-linked peptide intermediates in gramicidin S and tyrocidine biosynthesis," *Proc. Natl. Acad. Sci. USA* **68**, 2069–2072.
19. Fall, R., Claser, M. & Vagelos, P. R. (1976) "Acetyl coenzyme A carboxylase," *J. Biol. Chem.* **251**, 2063–2069.