PEPTIDYL TRANSFERS IN GRAMICIDIN S BIOSYNTHESIS FROM ENZYME-BOUND THIOESTER INTERMEDIATES*

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Abstract.—The biosynthesis of the peptide antibiotic gramicidin S involves successive peptidyl transfer reactions between intermediates bound in thioester linkages to two active enzyme fractions, I and II. Fraction II activates and racemizes phenylalanine, and then initiates peptidyl transfer by catalyzing a reaction between the carboxyl group of D-phenylalanine, bound to an enzymic sulfhydryl group, and the free imino group of L-proline, one of four L-amino acids all linked by their carboxyl functions to separate sulfhydryl groups on fraction I. Successive reactions of this type in the active centers of the multienzyme complex of fraction I lead to the formation of thioester-bonded nascent peptide chains and, ultimately, of the antibiotic product.

We have previously reported the 100-fold purification, from extracts of *B. brevis* (ATCC 9999), of two nucleic acid-free complementary enzyme fractions, I and II, sufficient for gramicidin S synthesis *in vitro*.[1, 2] Fraction I (molecular weight 280,000) is a complex of enzymes capable of activating the four constituent L-amino acids of the antibiotic. Fraction II (molecular weight 100,000) activates, racemizes, and incorporates phenylalanine into the product where this residue has the D-configuration. Gramicidin S, a cyclic decapeptide, contains the repeating sequence D-Phe-L-Pro-L-Val-L-Orn-L-Leu.

Our previous experiments have led us to presume that the primary amino acid-activation reactions of gramicidin S biosynthesis, in each of which ATP is cleaved to form inorganic phosphate,[1] are followed by transfers of the individual amino-acyl moieties from enzyme-associated adenylates to some other functional groups on the involved proteins before ordered polymerization.[1, 2] Thus, enzyme-substrate complexes resulting from ATP-linked activation, when isolated by filtration through Sephadex G-50, contain an excess of bound amino acid over bound AMP despite the absence of peptide bonding.[2] In the case of at least one amino acid substrate tested, phenylalanine, all protein-bound material reacts with hydroxylamine at neutral pH, suggesting that carboxyl activation is retained after transfer.[2] Furthermore, fraction II catalyzes a relatively rapid ATP-AMP exchange dependent on phenylalanine;[1] as shown below, this exchange is also carried out, but less rapidly, by fraction I in the presence of its amino acid substrates.

Free sulfhydryl groups are essential for the activity of gramicidin S-synthesizing enzymes. Reports describing cell-free preparations capable of antibiotic synthesis have alluded to the requirement of the enzyme systems for thiol-reducing agents, both for activity during short-term incubations,[3] and for stability during storage.[1, 4] Overall synthesis is inhibited by *p*-chloromercuribenzoate,[5] as is the activity of an L-ornithine activating enzyme.[6] Recently, Ljones and

coworkers have reported *in vitro* pulse-chase experiments with labeled amino acids which indicate that protein-bound intermediates participate in gramicidin S synthesis.[7]

We now present evidence that the secondary acceptors of amino acids on the individual enzymes are sulfhydryl groups. These are reversibly esterified by transfer of the aminoacyl moieties from adenylates. The resulting thioesters appear to be the reactive intermediates in successive peptidyl transfers that lead to formation of protein-bound nascent chains and, ultimately, of the antibiotic product. A striking feature of this process is that the function of the phenylalanine-charged fraction II is to initiate peptide bonding, which is followed by chain elongation on the multienzyme fraction I. The sequence of amino acid addition appears to be a function of the order in which the active centers of the enzyme system allow the peptidyl thioester units to react with the amino groups of adjacent aminoacyl thioesters; omission of one amino acid charge interrupts elongation.

Of relevance to the mechanism by which L-phenylalanine is converted into the D-phenylalanine that appears in the peptide is the observation that the thioester form of this amino acid linked to fraction II is a racemic mixture of the two isomers.

Methods and Materials.—Enzyme preparations and assays: Fractions I and II were purified and stored as previously described.[1, 2] Exchanges between ATP and PP_i^{32}, and between ATP and AMP, were measured as described.[1] Gramicidin S formation was estimated by a modification of our Millipore filtration technique[1] (see also Table 5).

Isolation of protein-bound intermediates: Reaction mixtures containing enzymes, amino acids, and ATP, as well as other constituents to be described later in connection with individual experiments, were applied to columns of Sephadex G-50 and eluted with buffer A. The excluded material (containing protein and bound substrates in about 2 ml) was collected, a sample removed for measurement of radioactivity, and the remainder treated with TCA to give a final concentration of 7%. Carrier bovine serum albumin (0.5 mg) was added. The sedimented material was washed once with 7% TCA, and then extracted twice with cold ether to remove the precipitant. Further treatments of the pellet will be outlined later.

Determination of D- and L-amino acids: The amounts of C^{14}-labeled D-amino acids in certain samples were determined using hog kidney D-amino acid oxidase (obtained from Calbiochem).[8] Radioactivity in L-amino acids was measured by the same technique, except that venom from *Crotalus atrox* (supplied by Sigma) was used as an L-specific amino acid oxidase at a final concentration of 160 μg/ml (see Kearney and Singer[9]).

High-voltage electrophoresis and paper chromatography of amino acid and peptidyl hydroxamates: Electrophoresis was carried out for 2.5 hr in 20% acetic acid[10] on a cooled plate, at 50 v/cm and 40 ma, using Whatman 3 MM paper. Sample volumes applied were 50 to 100 μl. Free amino acids and amino acid hydroxamates were also isolated by ascending chromatography for 6 hr on Whatman no. 1 paper in *n*-butanol:formic acid:water (75:15:10, v/v). Radioactivity in paper strips was determined in a Packard 7200 radiochromatogram scanner.

Results.—ATP-AMP exchanges catalyzed by fraction I: An L- or D-phenylalanine-dependent ATP-AMP exchange is catalyzed by fraction II under appropriate conditions.[1] When relatively large amounts of purified fraction I were incubated in the same medium with one or more of its amino acid substrates, a similar exchange was elicited, and the rate was proportional to the number of amino acids present (Table 1). In the presence of one amino acid, the quantity

TABLE 1. *Amino acid-dependent ATP-AMP exchanges and other reactions catalyzed by fractions I and II.*

Activity	Amino acids added	Fraction I (25 μg)	Fraction II (10 μg)
ATP-PP_i^{32} exchange (cpm)	L-Orn	318,000	380
	L-Phe	625	301,000
Amino acid binding (μμmoles)	L-Orn	35	0
	L-Phe	0	34
ATP-AMP-C^{14} exchange (cpm)	L-Phe	0	7,015
	L-Orn	370	0
	L-Val	350	0
	L-Orn, L-Val	720	0
	L-Orn, L-Val, L-Pro	1,080	0
	L-Orn, L-Val, L-Pro, L-Leu	1,450	0

Samples of fractions I and II were assayed under identical conditions for the listed activities, as described in *Methods and Materials*.

of fraction I required to catalyze ATP-AMP exchange at the same rate as a given amount of fraction II was about 20 times more active in the amino acid-dependent ATP-PP_i^{32} exchange reaction and in the capacity to bind substrates as measured on Sephadex columns (Table 1). Although no explanation can yet be offered for the difference in the rates of exchange between the two fractions, the fact that there is ATP-AMP exchange in the presence of each L-amino acid is consistent with the notion that each of the aminoacyl moieties is transferred to another energy-rich bonding on the enzyme.

Properties of isolated amino acid-protein complexes: Complexes between individual labeled amino acids and enzymes, the formation of which absolutely requires ATP, can be isolated by Sephadex filtration, and contain an approximately twofold excess of bound amino acids over AMP.[2] We found that precipitation of the protein in such solutions, by addition of TCA, discharged all the bound AMP and about half the bound amino acids into solution (Table 2). The remainder of the labeled amino acids remained associated with the precipitates, despite repeated washings with dilute TCA; the latter was then removed by extraction of the pellets with cold ether. Resuspension of the precipitates in dilute NaOH solutions (pH 9) and heating at 100° for five minutes released all the bound amino acids, as could be demonstrated by paper chromatography. The

TABLE 2. *Isolation of protein-bound amino acids free of aminoacyl adenylates.*

Materials	C^{14}-amino acid (μμmoles)	H^3-AMP (μμmoles)
(*A*) Sephadex eluate	35.0	18.1
TCA-precipitable material, dissolved in dilute alkali	16.2	0.0
(*B*) Sephadex eluate	34.0	19.0
TCA-precipitable material, dissolved in dilute alkali	17.0	0.0

Samples of (*A*) fraction I (25 μg) and (*B*) fraction II (10 μg) were incubated separately for 5 min at 37° with C^{14}-L-leucine (2 μM, 0.5 μc/tube) and C^{14}-L-phenylalanine (1.3 μM, 0.5 μc/tube), respectively. The reaction mixtures also contained in 0.2 ml: 10 μmoles of triethanolamine, pH 7.8; 5 μmoles of magnesium acetate; 0.5 μmole of dithiothreitol; 80 μg of inorganic pyrophosphatase, and 12.8 mμmoles of H^3-ATP (2.4 μc/tube). The mixtures were separately applied to Sephadex G-50 columns, eluted with buffer A, and the excluded protein collected.[2] Details of further treatments listed are given in *Methods and Materials*, and in the text.

procedure described, which was suitable for fractions I and II and all their respective substrates, depends on the stability of the complexes at acid pH and on their marked lability in dilute alkali (Table 3).

When the resuspended pellets of TCA-precipitable material were incubated at 37° for 20 minutes in 0.1 *M* sodium maleate buffer, pH 6.6, in the presence of 1 per cent mercuric or methylmercuric acetate (gift of Merck and Co., Agricultural and Industrial Division), the bonds between the protein and labeled amino acids were quantitatively cleaved, but controls were not hydrolyzed (Table 3). On incubation of the resuspended materials in 3 *M* salt-free hydroxylamine, both at pH 6.1 and at pH 7.9, the bound amino acids were completely converted to the respective hydroxamates. Treatment of the complexes with sodium borohydride led to liberation of the amino acids as the amino alcohols (Table 3). These properties of the amino acid-protein complexes indicate that one is dealing with covalent thioester bonds between the individual amino acids and the specific

TABLE 3. *Stability of bonds between amino acids and denatured enzyme proteins.*

	Cleavage (%)			
	Leucine-protein		Phenylalanine-protein	
Conditions	(37°)	(60°)	(37°)	(60°)
(*A*) pH 2.5	0	0	0	0
pH 4.5	0	0	0	0
pH 6.6	5	20	8	27
pH 7.9	30	69	35	70
pH 10	61	90	64	90
(*B*) Blank, pH 6.6	5	...	8	...
With mercuric acetate	84	...	80	...
With methylmercuric acetate	71	...	65	...
(*C*) 3 *M* hydroxylamine, pH 6.1	100	...	100	...
3 *M* hydroxylamine, pH 7.9	100	...	100	...
(*D*) With borohydride (25°)	100	...	100	...

Protein precipitates containing C^{14}-Phe and others with C^{14}-Leu, were prepared as outlined in Table 2 and in the text. Samples of the materials were resuspended and incubated for 10 min in 0.2 ml of the following media: (*A*) 0.1 *M* buffers at pH 2.5 (phthalate), pH 4.5 (acetate), pH 6.6 (maleate), pH 7.9 (triethanolamine HCl), and pH 10 (glycine-NaOH); (*B*) 0.1 *M* sodium maleate buffer, pH 6.6 with and without 1% mercuric salts; (*C*) Buffer A, containing salt-free hydroxylamine; (*D*) Buffer A, containing 7.5 mM freshly dissolved sodium borohydride. The percentage cleavage in each case was determined by paper chromatography of the soluble products.

enzymes involved. The important points are the stability in acid (even at elevated temperatures[11]), the lability in dilute alkali, the susceptibility to cleavage by salts of mercury at neutral pH,[12] the reaction with hydroxylamine at pH 6.1 as well as pH 7.9, and the reductive cleavage by borohydride.[13] (See also the general summary by Jaenicke and Lynen.[14]) We have also carried out these tests on amino acid-enzyme complexes before acid denaturation, and have not detected any properties different from those described. Characterization of the acceptor functions on the individual activating enzymes as sulfhydryl groups is consistent with the energy-rich nature of the amino acid-enzyme bonds, as suggested by the reversibility of the transfer reactions between the adenylates and the "second forms." The retention of carboxyl activation also indicates that the thioesters are likely intermediates of the polymerization process.

Racemization of phenylalanine on fraction II: The products of dilute alkaline cleavage of the precipitated Phe-S-fraction II were tested for the relative quan-

tities of D- and L-phenylalanine. The isomers were present in approximately equal amounts, irrespective of whether the complex had originally been formed by reaction of the enzyme with C^{14}-D- or L-phenylalanine. In similar experiments with leucine bound in thioester linkage to fraction I, it was found that only L-leucine was present (Table 4). It is likely that racemization occurs while phenylalanine is bound to fraction II in this manner. A symmetrical coplanar intermediate may well be formed, as appears to be the case in the racemization of methylmalonyl-CoA.[15]

Formation of protein-bound nascent peptide chains: Experiments were carried out in which fractions I and II were incubated with ATP, Mg^{++}, and various combinations of amino acids including C^{14}-L-phenylalanine. The enzyme-bound radioactivity was isolated by filtration through Sephadex G-50 columns, and the TCA-precipitable materials were then obtained as described in the previous section. The amount of phenylalanine radioactivity released from the protein in alkali increased in a regular fashion as more amino acids were added to the original incubation medium, but only if this was done in the order L-Phe, L-Pro, L-Val, L-Orn (Table 5). For example, the omission of L-proline from the mixture cut down the bound radioactivity to that obtained with L-phenylalanine alone even

TABLE 4. *Configuration of amino acids bound to fractions I and II.*

Fraction	Initial labeled substrate	Configuration of protein-bound amino acid (%) D	L
I	L-leucine	0	100
II	L-phenylalanine	46	54
III	D-phenylalanine	55	45

Complexes of leucine with fraction I and of phenylalanine with fraction II were prepared and isolated in the form of protein precipitates, as outlined in Table 2. The pellets were taken up in 0.5 ml of 0.01 *N* NaOH (final pH about 9), heated to 100° for 10 min, and analyzed for content of D- and L-amino acids as described in *Methods and Materials.*

TABLE 5. *Formation of protein-bound nascent peptide chains and gramicidin S.*

	Radioactivity in Sephadex eluate (cpm)	Non-TCA-precipitable radioactivity (cpm)	TCA-precipitable radioactivity (cpm)	Gramicidin S (cpm)
C^{14}-L-Phe	2,959	1,410	1,549	265
C^{14}-L-Phe, L-Pro	7,671	1,670	6,001	375
C^{14}-L-Phe, L-Pro, L-Val	11,460	1,455	10,005	1,531
C^{14}-L-Phe, L-Pro, L-Val, L-Orn	16,160	1,835	14,325	1,005
C^{14}-L-Phe, L-Pro, L-Val, L-Orn, L-Leu	3,531	1,502	2,029	25,409
C^{14}-L-Phe, L-Val, L-Orn	3,101	1,491	1,610	376

Duplicate tubes were set up, containing, in 0.2 ml: 10 μmoles of triethanolamine, pH 7.8; 5 μmoles of magnesium acetate; 0.1 μmoles of ATP; 0.5 μmole of dithiothreitol; 80 μg of inorganic pyrophosphatase; 5 μg each of fractions I and II; 0.5 mμmole of C^{14}-L-phenylalanine (0.2 μc); and 10 mμmoles of other amino acids where indicated. Incubation was for 10 min at 37°. One tube of each pair was used for isolation of protein-bound materials by filtration through Sephadex G-50 columns;[2] samples were applied to Millipore filters previously treated with 7% TCA and dried. The remainder of the eluates was treated with TCA, and washed pellets of denatured protein were obtained (see Table 2); these were solubilized in dilute NaOH (pH 9) and samples applied to Millipore filters. Salt-free hydroxylamine (pH 7.9, final concentration 3 *M*) was added to the second tube of each pair to solubilize bound chains; after incubation for 20 min at 60°, 7% TCA was added in excess and the mixtures passed through Millipore filters which were washed with TCA. Radioactivity on all the filters was determined as described.[1]

when L-valine and L-ornithine were provided. The amount of radioactivity discharged from the precipitated proteins by TCA treatment of the Sephadex eluates represents phenylalanyl adenylate and was the same in each case (Table 5). This suggested that the chains that were being formed were linked to the enzymes by acid-stable bonds. When the labeled materials were stripped from the precipitated proteins by incubation with hydroxylamine, radioactive peptidyl hydroxamates were released and could be demonstrated by high-voltage paper

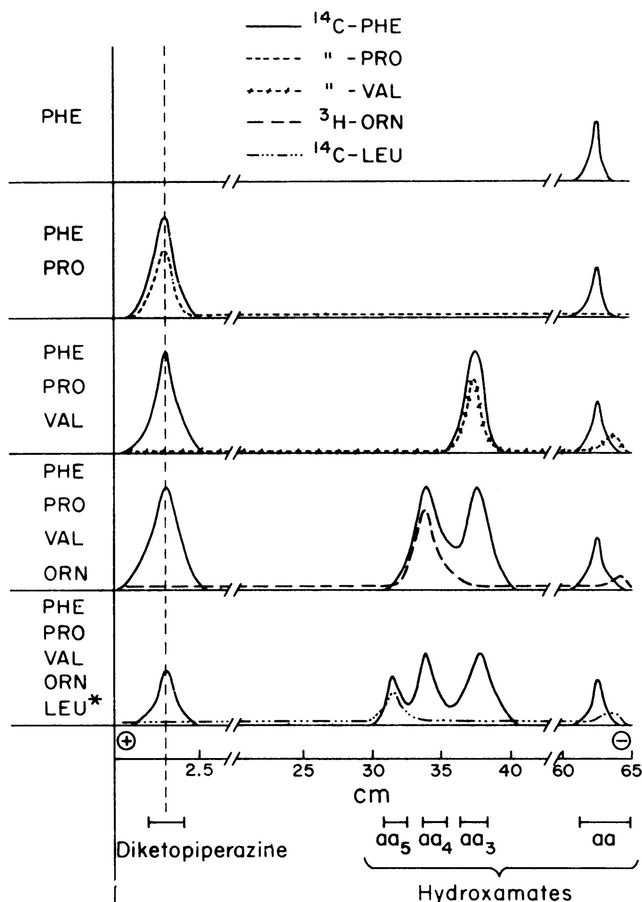

FIG. 1.—Separation of amino acid and peptidyl hydroxamates by high-voltage paper electrophoresis. Incubation mixtures were constituted as described in Table 5, except that the duplicate tubes contained either C^{14}-L-phenylalanine or a different labeled amino acid as indicated, other amino acids being unlabeled. The incubations were for 10 min at 37°, except in the last system where only phenylalanine, proline, valine, and ornithine were initially present, and L-leucine was added for a further 2 min (Leu*). The protein-bound materials were isolated by Sephadex filtration and TCA treatment as described in the text, and the radioactivity was liberated by heating with 3 *M* hydroxylamine as outlined in Table 5. The electrophoretic and counting procedures are described in *Methods and Materials*. Further cross-tests for identification of amino acid components of the isolated peptidyl hydroxamates were carried out but are not shown; these compounds are designated aa_n-hydroxamates according to this general identification procedure.

electrophoresis (Fig. 1). The chain length corresponded to the number of amino acids added, in the right order, to the original incubation mixtures. The various peaks were identified by carrying out duplicate experiments with different labeled amino acids. Of interest was the appearance of Phe-L-Pro-diketopiperazine near the origin of the electropherogram, instead of dipeptidyl hydroxamate; the conditions of isolation appear to favor ring closure almost exclusively. The relative amounts of the various chains were similar, indicating that the enzyme system is charged with chains, and confirming our previous observations as to the 1:1:1:1 stoichiometry between the activating sites of fraction I.[2] The obligatory order of amino acid addition to growing peptides is clearly demonstrated by these experiments, as is the strong likelihood that, after the first peptide bond has been formed, the individual peptidation reactions involve two specific fraction I-bound thioesters in each case, one being peptidyl-S-enzyme, and the other (incoming) aminoacyl-S-enzyme. Peptidyl adenylates are clearly absent from the system.

When all five amino acids were present in incubation systems such as those described above, protein-bound chains were also formed, but the total protein-bound radioactivity was much less than when L-leucine was omitted (Table 5). Gramicidin S was formed in large amounts under these conditions; it was retained on Millipore filters but did not appear in the isolated protein pellet (Table 5). To detect the presence of chains longer than four residues, a mixture containing the first four amino acids only was incubated for ten minutes; unlabeled L-leucine was then added, and the incubation continued for two minutes before further treatment of the sample to isolate peptidyl hydroxamates. Electrophoresis showed that an additional smaller peak was now present, migrating short of tetrapeptidyl hydroxamate (Fig. 1). This peak contained label when C^{14}-L-leucine was used. There were no peaks in the area proximal to the peak of pentapeptidyl hydroxamate, and there is at present no evidence for chains between six and nine residues long in this system. It is possible that a doubling reaction between two antiparallel pentapeptide units occurs to form gramicidin S.

Discussion.—The participation of thioester intermediates in the biosynthesis of gramicidin S by a multienzyme system in some manner parallels the two-carbon addition mechanism in the fatty acid synthetase of yeast[16] and mammalian liver.[17] We have not yet explored whether the reactive sulfhydryl groups in our case might likewise be provided by enzyme-bound 4′-phosphopantotheine.[18] The mechanism of polymerization that we propose demands a unique arrangement of the active sites of the participating enzymes, in that four different amino acids bound to separate active centers on a polyenzyme fraction have to be aligned in correct order to permit sequential peptidyl transfer reactions, each of which is specific for the amino group of an incoming bound amino acid and for the activated carboxyl of a peptidyl unit bound to an adjacent site. The phenylalanine reacts from a separate activating-racemizing enzyme, fraction II.

The type of polymerization system described here is one that displays "head-growth." [19] Both reactive molecules are carboxyl-activated, and the incoming residue retains the activation when the stable peptide bond has been formed. This is very much like ribosomal polypeptide synthesis, although that is where most of the resemblance ends. The sequence of amino acids is not determined by

"adaptors" but by the specific aggregation of enzymes in a complex and unique architectural pattern, and there are obvious limits to the size of the polypeptide products.

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