The ergot fungus *Claviceps purpurea* produces the medically important ergopeptines, which consist of a cyclol-structured tripeptide and D-lysergic acid linked by an amide bond. An enzyme activity capable of non-ribosomal synthesis of D-lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam, the non-cyclol precursor of the ergopeptine ergotamine, has been purified about 18-fold from the ergotamine-producing *C. purpurea* strain D1. Analysis of radioactively labeled enzyme-substrate complexes revealed a 370-kDa lysergyl peptide synthetase 1 (LPS 1) carrying the amino acid activation domains for alanine, phenylalanine, and proline. The activation of D-lysergic acid is catalyzed by a 140-kDa peptide synthetase (LPS 2) copurifying with LPS 1. LPS 1 and LPS 2 contain 4′-phosphopantetheine and bind their substrates covalently by thioester linkage. Kinetic analysis of the synthesis reaction revealed a $K_m$ of $\sim 1.4 \, \mu M$ for both D-lysergic acid and its structural homolog dihydrolysergic acid, which is one to two orders of magnitude lower than the $K_m$ values for the other amino acids involved. The $K_m$ values for the amino acids reflect their relative concentrations in the cellular pool of *C. purpurea*. This may indicate that in *in vivo* conditions D-lysergyl peptide formation is limited by the D-lysergic acid concentration in the cell. *In vitro*, the multienzyme preparation catalyzes the formation of several different D-lysergyl peptide lactams according to the amino acids supplied. Specific antiserum was used to detect LPS 1 in various *C. purpurea* strains. In *C. purpurea* wild type, the enzyme was expressed at all stages of cultivation and in different media, suggesting that it is produced constitutively.

The ergot fungi growing on their host plants develop characteristic sclerotia containing ergot alkaloids. The basic structure of these compounds is a characteristic tetracyclic ring system (ergoline unit). The various ergolines are distinguished from each other by various side groups and modifications, and the most important among them is D-lysergic acid (for review, see Refs. 1 and 2). Natural and synthetic D-lysergic acid-derived compounds have various pharmacological activities that are used for human therapy (3). Knowledge about the biosynthesis of ergot alkaloids may help the development of new variants, and it may help in the suppression of their formation in the environment where they pose a serious risk for human and animal health.

Ergot peptide alkaloids, also called ergopeptines, are produced by *Claviceps purpurea* and consist of cyclol-structured tripeptides attached by amide linkage to D-lysergic acid (Fig. 1a). Variations in the structures of naturally occurring ergopeptines arise by substitutions of the amino acid positions I and II, while amino acid III is always L-proline. Related to the ergopeptines are the ergopeptams (4), which accumulate as by-products of ergopeptines in saprophytic cultures of some *C. purpurea* strains (5) (reviewed in Refs. 2 and 4). They contain L-proline, and their tripeptide chain is a non-cyclol lactam (Fig. 1b). It has been postulated that the cyclol in the ergopeptines arises by introduction of a hydroxyl group to the α-C of the amino acid I of a putative D-lysergyl (L,L,L)tripeptide lactam (6, 7). The reaction product is proposed to undergo ring closure to yield the corresponding ergopeptine most probably in a non-enzymatic reaction (8). Ergopeptams arise by spontaneous epimerization of D-lysergyl (L,L,L)tripeptide lactams in the proline residue (8). Because of the D-configuration of proline, they cannot be converted into the corresponding ergopeptines and ergopeptams thus accumulate in cultures (4).

We have previously reported a cell-free system capable of forming the novel D-lysergyl peptides D-lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam and the corresponding D-proline containing stereoisomer (9). They are synthesized from free D-lysergic acid, L-alanine, L-phenylalanine and L-proline with consumption of ATP (9). The formation of the (L,L,L) compound has not been detected in *in vivo* conditions as yet, because it appears to be converted rapidly to the corresponding ergopeptine or ergopeptam. The *in vitro* formation, therefore, confirms the previous hypotheses on ergopeptine formation from the D-lysergyl peptide lactam as well as the intermediacy of free D-lysergic acid in the biosynthetic process. In the present report we describe the purification and characterization of the more than 500-kDa enzyme system catalyzing D-lysergyl peptide lactam synthesis. It will be shown that the enzyme system has four peptide synthetase activities. They activate and incorporate into product D-lysergic acid and the three amino acids of the tripeptide moiety. A thiol template mechanism is used as for other non-ribosomal peptide syntheses (10).

**MATERIALS AND METHODS**

**Strains and Cultures**—*C. purpurea* wild type ATCC 20102, its mutant derivative D1 selected for high production of ergotamine, and strain 1029, were described previously (11). *C. purpurea* strain Cc93 (kindly supplied by Dr. H. Kobel, Sandoz AG) is a high producer of ergocristine. Maintenance and culture conditions for ATCC 20102-derived strains were as described previously (11, 12). Strain Ecc93 was cultivated according to Kobel and Sangier (13). Inoculum medium and medium T25 were used for alkaloid fermentations (14, 15). In some occasions a modified Vogel’s medium was used (12, 16).

**Radiochemicals and Chemicals**—[9,10-3H]-9,10-Dihydroergocrypt...
tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as reagents. 4'-Phosphopantetheine, a protein fraction (liquid samples) were done by the method of Pugh and Wakil (22) modified according to Zocher et al. (23). Alternatively determinations of 4'-phosphopantetheine in protein bands were done according to a method of Stindl (24). To this end, enzyme, concentrated after the DEAE-cellulose step (Table I) was electrophoresed in 1.5-mm thick SDS-PAGE gels and blotted onto glassy bond membranes. After brief staining with Coomassie Blue, bands of interest were cut out (in general from 5 to 10 lanes run in parallel), and transferred to Eppendorf tubes. After treatment with 1 M NaOH in a boiling water bath for 1 h, samples were neutralized in 1 M Tris-HCl, pH 8. Alkaline phosphatase was added, and incubation was resumed for a further hour at 37 °C. After this, the sample was subjected to a microbiological assay for the presence of pantetheinase based on growth dependence of Lactobacillus plantarum DSM 20205. Controls involved treatment of glassy bond strips from the same lanes but devoid of protein bands and strips with protein bands known to contain no 4’-phosphopantetheine.

**Enzyme Assays**—Amino acid and ergoline carboxylic acid dependent ATP-ryophosphate exchange reactions were performed as described previously (25, 26). Assays for measuring t-lysergyl peptide lactam synthesis are that used elsewhere (9). In the case of final electrophoresis eluting from column separations, 1-ml portions from fractions of Ultrogel separations were concentrated about 10-fold on small DEAE-cellulose columns in Pasteur pipettes, equilibrated in buffer B (see “Buffers and Solvent Systems”). After elution with 0.2 M NaCl (in buffer B), enzyme was desalted on small AcA 202 Pasteur pipette columns equilibrated with buffer B without diithioerythritol and assayed immediately. In the case of DEAE-cellulose fractions, the same procedure was used except that prior to concentration, samples were diluted 2-fold with buffer B to allow enzyme to adsorb to DEAE-cellulose. Reaction products were extracted into ethyl acetate and, after evaporation of solvent to dryness, analyzed by TLC in various solvent systems (see below). The assay for measuring thioester formation was as described previously (25).

**Buffers and Solvent Systems**—Buffer A for the preparation of extracts of broken cell of *C. purpurea* was described previously (9). Buffer B was 0.1 M Tris-HCl, pH 8.0, 15% (w/v) glycerol, 10 mM diithioerythritol, 1 mM benzamidine, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride. For the preparation of buffers, bidistilled water was used that had been previously autoclaved at 121 °C for 20 min.

TLC was performed on silica gel plates using the following solvent systems for the indicated purposes: ethyl acetate: methanol:water (100: 5:5, by volume, solvent system I); ethyl acetate: methanol:water (100: 10:5, by volume, solvent system II); ethyl acetate: dimethylformamide: ethanol (75:15.5, by volume, solvent system III) for chromatography of t-lysergyl peptides and ergopeptines; isopropanol acetone (70:30:20, by volume, solvent system IV), 1-butanol:acetic acid:water (4:1:1, by volume; solvent system V) for chromatography of amino acids; ethanol:water (8:2, by volume, solvent system VI) for chromatography of t-lysergic acid and dihydrolysergic acid.

**Enzyme Purification**—All operations were performed at 2–4 °C. Approximately 50 g of freshly harvested mycelium of *C. purpurea* were suspended in 150 ml of buffer A and passed through a French press at 10,000 p.s.i. This method of cell disintegration was less time-consuming than the previously used procedure (9) and yielded more units of enzyme. The resultant homogenate was centrifuged at 15,000 rpm for 30 min in a SS34 rotor. To the supernatant was added a 15% Polymin P solution to give 0.3% final concentration. After 30 min, the sample was centrifuged as above. The clear supernatant was precipitated by fractionated ammonium sulfate from 35 to 55% saturation for 3 h standing on ice. After centrifugation as above, the pellet was dissolved in buffer B (designated crude extract). 3–4 ml portions of crude extract were subjected to Ultrogel AcA 34 gel chromatography on a column of bed dimensions 48 cm × 3 cm that had been previously autoclaved at 121 °C for 20 min.
then proceeded fairly linearly for about 10 min at 25 °C, then continued at a gradually reducing rate for up to 30 min. Further fractionation of crude extract was done with small portions (3–4 ml) on relatively small, fast-running Ultrogel AcA 34 columns. D-Lysergyl peptide-synthesizing activity appeared immediately behind the void volume, indicating that the protein was larger than 300 kDa, as was to be expected for a multifunctional protein involved in a non-ribosomal peptide synthesis (Fig. 2). The activity profile of D-lysergyl peptide formation showed a single peak which coincided with the peak for D-lysergic acid activation, measured by ATP-pyrophosphatase exchange, and the binding of the three amino acids alanine, phenylalanine, and proline as thioesters (curves for alanine and proline are not shown). Interestingly, in these fractions dihydrolysergic acid was activated to nearly the same level as D-lysergic acid, which is consistent with the in vivo incorporation of this compound into the corresponding dihydroergopeptines (27). Further purification of enzyme was achieved by chromatography on DEAE-cellulose from which it eluted as a single peak between 130 and 150 m arbitrary units NaCl (not shown). Finally, the enzyme preparation was subjected to gel filtration on Ultrogel AcA 22. The overall purification was about 18-fold and the yield was 1.7%. The data for the individual steps are listed in Table I. Attempts to achieve higher specific activity were unsuccessful because of severe activity losses. It was noted that phenylmethylsulfonyl fluoride improved enzyme stability at all stages of purification. SDS-PAGE revealed that the preparation contained at all stages multiple protein bands. The two strongest bands in the Mr range higher than that of myosin (205,000) can be seen in Fig. 3 (indicated by arrows). They appear to become enriched in the course of the activity purification and were therefore considered as candidates for the putative d-lysergyl peptide synthetase.

Identification of d-Lysergyl Peptide Synthetase—To see which one of the two prominent large proteins seen in Fig. 3 was responsible for synthesis of the d-lysergyl peptide lactam, concentrated enzyme purified on Ultrogel AcA 22 was incubated with radioactively labeled phenylalanine, proline, alanine, or dihydrolysergic acid each in the presence of ATP. The samples were fractionated by SDS-PAGE, and the radioactive bands were identified by autodensitometry. Fig. 4 shows that [14C]phenylalanine labeled the slowest migrating protein (des-
D-Lysergyl Peptide Synthesis in C. purpurea

TABLE I

| Step | Volume | Protein | Units | Spec. activity | Yield | Purification |
|------|--------|---------|-------|---------------|-------|-------------|
| 1. Extract from broken cells | 142 | 2,800 | 43.3 | 0.0154 | 100 | 1 |
| 2. Polymin P fractionation | 124 | 1,600 | ND | ND | ND | ND |
| 3. Fractionation with (NH4)2SO4 (35–55%) “crude extract” | 5.6 | 630 | 8.4 | 0.013 | 18 | 0.87 |
| 4. Ultrogel AcA 34 gel filtration | 32 | 64 | 4.3 | 0.067 | 10 | 4.35 |
| 5. DEAE-cellulose chromatography | 70 | 26 | ND | ND | ND | ND |
| 6. DEAE-cellulose peak concentrated | 5 | 18.5 | 3.1 | 0.168 | 7 | 10.9 |
| 7. Ultrogel AcA 22 | 31 | 2.6 | 0.75 | 0.288 | 1.7 | 18.7 |

* One unit is the amount of enzyme catalyzing the formation of 1 nmol of d-lysergyl-alanyl-phenylalanyl-proline lactam at 26°C under the conditions described under “Materials and Methods.”

** ND, not determined.

Fig. 3. SDS-PAGE and Western blot analysis analysis of various steps in the purification of d-lysergyl peptide synthetase. Samples from the various purification steps indicated in Table I were subjected to SDS-PAGE (left panel) and Western blotting (right panel). Each lane contains 10 μg of protein. Lane 1, marker from 20 to 205 kDa; lanes 2 and 2’, extract from broken cells; lanes 3 and 3’, crude extract; lanes 4 and 4’, pool from Ultrogel AcA 34; lane 5 and 5’, DEAE-cellulose concentrated enzyme; lanes 6 and 6’, Ultrogel AcA 22. Arrow a indicates LPS 1, arrow b the 250-kDa protein that copurifies with LPS (see text). Immunostaining (lanes 2–6’) was performed as described under “Materials and Methods.”

Fig. 4. Autofluorogram of an electrophoretic separation of radiolabeled d-lysergyl peptide synthetase. Ultrogel AcA 22-purified protein (20 μg in each experiment) was incubated with [14C]phenylalanine or [14H]dihydrolysergic acid and MgATP in a total volume of 70 μl (for conditions, see “Materials and Methods”). After incubation, 50 μl of acetonewere added, and the precipitate was collected by a short spin in an Eppendorf centrifuge. After solubilization in 10 μl of buffer B, 10 μl of 10% SDS were added, and the mixture was fractionated by 5% SDS-PAGE. a, radioactively labeled proteins were visualized by autofluorography; b, Coomassie-stained gel.

Ignored LPS 1) and a protein of ~120 kDa which was presumed to be a degradation product of the larger protein. Interestingly, labeling with [14C]dihydrolysergic acid revealed a 140-kDa protein, LPS 2, which is responsible for d-lysergic acid activation and incorporation into the peptide.

Labeling of LPS 1 by [14C]proline and [14C]alanine yielded only a faint band in the autofluorograms. Besides the weak response of [14C]-labeled compounds to the fluorophor used in these experiments, the faint labeling may be due to the high Km values for alanine and proline (see below) and the fact that purified enzyme preparations usually contain varying amounts of amino acids already bound to the enzyme. This can also explain uneven product labeling (9). Furthermore, instability of these bands during isolation (Fig. 3, lane 3’) suggests that LPS 1 is, at least in part, cleaved after cell disruption. Interestingly, the antibodies show no significant cross-reaction with the strong band copurifying with LPS 1 and migrating between myosin and LPS 1, suggesting that this band represents a protein unrelated to LPS 1.

Molecular Mass Determination of d-Lysergyl Peptide Synthetase—The molecular mass of LPS 1 was determined using 4% SDS-PAGE gels using peptide synthetases of known molecular mass as reference markers. Fig. 5 shows that LPS 1 has a molecular mass of 370 kDa, which should be sufficient for three amino acid activation domains (28, 29). From these considerations it is unlikely, however, that the 370-kDa protein could contain all four amino acid domains required for synthesis of the whole d-lysergyl peptide lactam. LPS 2, which was found in the same protein fraction, is most probably carrying the residual fourth domain required for tetrapeptide synthesis.

Characteristics of the Substrate Binding Reaction—Covalent binding of substrate amino acids to peptide synthetases in thioster-linkage is a prerequisite for peptide formation in non-ribosomal peptide synthesis (28, 29). Accordingly, when testing LPS 1 we saw thioster formation of alanine, phenylalanine, and proline (as measured by conversion of substrates into a trichloroacetic acid-precipitable form) which was very rapid and required ATP and Mg2+. Analysis of these covalent enzyme-substrate complexes showed that they could be cleaved by performic acid but not by formic acid. Cleavage was also seen upon treatment with mild alkali such as 0.1 NaOH for 10 min at room temperature.2 Fig. 6 shows the results of performic acid oxidations in the case of phenylalanine and proline (alanine not shown).

Presence of 4’-Phosphopantetheine in LPS—Portions of enzyme fractions purified on Ultrogel AcA 34 or DEAE-cellulose were subjected to determination of 4’-phosphopantetheine, which is a covalently bound cofactor in every amino acid activation domain of all peptide synthetases (30). The analysis showed that the peak of LPS activity coincided with that of 4’-phosphopantetheine contents of the various fractions (results not shown). To test whether LPS 1 and LPS 2 contained 4’-phosphopantetheine, concentrated enzyme from the DEAE-cellulose step was subjected to SDS-PAGE, and the separated proteins were transferred to membranes by electroblotting. After isolation, bands of LPS 1 and LPS 2 were subjected to determination of 4’-phosphopantetheine using a microbiological assay with L. plantarum. Hydrolysates of LPS 1 and LPS 2 stimulated growth of the Lactobacillus and therefore contained 4’-phosphopantetheine (not shown). Controls using empty
absence of externally added D-lysergic acid when using enzyme preparations from cultures with exceptionally high alkaloid yields. The apparent Km values for proline and alanine were ~125 and ~190 μM, respectively. The Km for phenylalanine was ~15 μM, reflecting the lower concentration of this amino acid in the free amino acid pool of C. purpurea ATCC 20102 strains, at least in part, membrane strips or with strips with bovine serum albumin or lysozyme gave no growth of the test organism.

**Kinetic Properties of d-Lysergyl Peptide Synthetase**—Determinations of Km values of the various substrates of the multienzyme were partly hampered by the fact that the partially purified enzyme preparations contain varying (small) amounts of free amino acids associated with the protein. We occasionally observed the enzyme by treatment with performic acid, but not by formic acid. Note that the additional band in lane d is the formylation product of phenylalanine.

**Substrate Specificity of d-Lysergyl Peptide Synthetase—**C. purpurea D1 produces ergotamine together with minor amounts of Leu-ergokryptine. Besides the ergotamine group of ergopeptides with alanine in amino acid position I, there exist also the ergoxine and ergotoxine groups which contain aminobutyric acid or valine in position I, respectively (1,4). To test whether our enzyme preparation could also synthesize these peptide lactams, it was incubated with [3H]dihydrolysergic acid, phenylalanine, proline, alanine, amnobutyric acid, or valine. Fig. 7 (lanes 1–3) shows TLC separations of ethyl acetate extracts from these reactions each of which had produced the expected dihydrolysergyl peptide lactam. Moreover, when phenylalanine was replaced by leucine, dihydrolysergyl peptide lactam homologs of Leu-ergokryptine and Leu-ergoptine were formed (lane 4 and 5, respectively). The identity of all of these compounds was confirmed by alkaline and acid hydrolysis after labeling with [3H]dihydrolysergic acid, phenylalanine, proline, alanine, amnobutyric acid, or valine. The formation of these compounds was ATP-dependent (lane 6). The results shown here suggest that LPS 1 and LPS 2 can synthesize various different d-lysergyl peptides and that the diversity of structures elaborated by the various C. purpurea strains, at least in part, reflects the different actual concentrations of substrate amino acids in their free cellular pools.

**Immunological Screening**—Western blots were performed of extracts of broken cells of a number of peptide alkaloid-produc-
ing \textit{C. purpurea} strains from this laboratory such as ATCC 20102 (wild type), strain D1, strain 1029, and \textit{C. purpurea} strain Ecc93 cultured under different conditions and for different times. Antibody to LPS 1 recognized a 370-kDa band in all cases together with lower bands (raised by proteolysis), which were present in appreciable amounts when the strains were grown in media favoring ergot peptide alkaloid synthesis (such as inoculum medium, medium T25, or production medium of Ecc93) (not shown). Despite the different ergotamine productivities of the \textit{C. purpurea} strains (e.g. wild type strain ATCC 20102 produces $10^{-15}$ mg/liter, and strain D1 produces $700-1000$ mg of ergotamine/liter after 14 days of cultivation), the amount of immunoreactive material in all of the strains was fairly comparable (not shown). Obviously, from these results all strains would have the ability to synthesize appreciable amounts of ergopeptides. A possible reason for the different productivities may be the limited production of \textit{D}-lysergic acid. In fact, short term productivity measurements in \textit{C. purpurea} wild type ATCC 20102 revealed a 4–5-fold stimulation of ergotamine formation by the addition of \textit{D}-lysergic acid to protoplasts or intact mycelium (31). Similar experiments with strain D1 did not show a response upon addition of \textit{D}-lysergic acid, which may indicate that in this strain the intracellular level of \textit{D}-lysergic acid was at saturation and therefore considerably higher than in its parent.\footnote{U. Keller, unpublished data.}

**Correlation of Ergotamine Production and Levels of LPS**

When grown in a modified Vogel’s medium (12, 16) with a high phosphate content, \textit{C. purpurea} wild type strain or strain 1029 developed long, slim vegetative-type mycelium, which did not produce ergot peptide alkaloid (12). In production medium where phosphate is limited, productivity is associated with a type of morphology called sclerotia-like cells (12) (for review, see Ref. 32). Surprisingly, Western blots with cell extracts of wild type strain grown in the two different media showed the presence of comparable levels of LPS 1 in both vegetative and sclerotia-like cells (not shown). Thus, LPS 1 is synthesized constitutively and thus responsible for growth-linked repression of peptide alkaloid synthesis by phosphate (33, 34).

**DISCUSSION**

The work presented here characterizes the enzyme system catalyzing the formation of \textit{D}-lysergyl-alanlyl-phenylalanlyl-proline lactam, the non-cyclic peptide precursor of ergotamine in the ergot fungus \textit{C. purpurea}. Conditions of isolation and purification were established that enabled us to identify two proteins responsible for the activation and condensation of the building blocks of the peptide alkaloid. As it appears, LPS 1 is a multifunctional polypeptide chain of 370 kDa that activates the amino acids of the tripeptide portion of the peptide alkaloid (alanine, phenylalanine, and proline) in covalent thioester linkage. LPS 2 is a 140-kDa protein-activating \textit{D}-lysergic acid only. The presence of 4’-phosphopantetheine covalently bound to enzyme in peptide alkaloid-synthesizing fractions has been demonstrated, and the cofactor could be detected unambiguously in both proteins. This identifies them as the thiol template of ergot peptide alkaloid synthesis. Such thiol templates involved in the biosynthesis of various antibiotic peptides from bacteria and fungi have been shown to consist of repeating units of approximately 1000-amino acid length (equivalent to 120 kDa each) (28, 29). These units contain regions with homology to acyl carrier proteins containing 4’-phosphopantetheine as prosthetic groups and are referred to as peptide synthetase domains. After activation as adenylates, the amino acids become attached to the 4’-phosphopantetheine of the peptide synthetase domains in thioester linkage, thus serving as carriers in amino acid and peptidyl transfer during their polymerization (10).

Enzymes LPS 1 and LPS 2 comprise a total length of more than 500 kDa, which should be sufficient to activate and polymerize four acyl or aminocarboxyl residues. Indeed, the purified enzyme catalyzes the formation of the whole \textit{D}-lysergyl peptide lactam containing four peptide bonds. Similar sizes are seen in the case of gramicidin S synthetase 2 (510 kDa, activating four and polymerizing five amino acids) (35); HC toxin synthetase (550 kDa, activating, modifying, and polymerizing four amino acid residues) (36); or aminoadipyl-cysteine-valine synthetase (421 kDa, activating, modifying, and polymerizing three amino acids) (37).

The attachment of \textit{D}-lysergic acid to the tripeptide portion in the ergopeptides is through an amide bond which requires activation of the carboxyl group at least as adenylate. Conflicting results have been obtained previously because of the occurrence of a \textit{D}-lysergic acid-activating enzyme in extracts of \textit{C. purpurea}, which catalyses the formation of \textit{D}-lysergyl adenylate but not of dihydrolysergyl adenylate and which does not form thioester (9, 26). On the basis of these findings it was presumed that ergot peptide synthesis would proceed in a similar fashion as in the case of the class of acylpeptide lactones from streptomyces (38). Here, aromatic carboxylic acids are activated by relatively small enzymes (45–60 kDa) as adenylates but not as thioesters. The adenylate reacts later with the next amino acid in the reaction chain on the surface of a large multifunctional peptide synthetase that carries this amino acid as thioester. The reaction product is the corresponding acylamino acid that can react further with the next amino acid of the peptide lactone ring as in the case of actinomycin (39–41). The data described here clearly show that this type of reaction will not occur in \textit{D}-lysergyl peptide formation because LPS 2 activates \textit{D}-lysergic acid (or dihydrolysergic acid) as a thioester and thus resembles other single amino acid-activating enzymes with a known acyl carrier-like module such as gramicidin S synthetase 1 (42). Furthermore, recent data from this laboratory indicate that the \textit{D}-lysergic acid-activating enzyme could be separated from the peptide-synthesizing multienzyme, proving their independence from each other.\footnote{U. Keller, unpublished data.}

An important difference between bacterial and fungal peptide synthetases is that the latter contain all domains on one single polypeptide chain, such as in the case of enniatin synthetase (43) or cyclosporin synthetase (44), while in the bacterial ones they are contained in more than one protein. Because of the strong degradation of LPS 1 during isolation, LPS 1 and LPS 2 could be produced from a larger polypeptide by proteolytic cleavage. The gene sequence for the HC-toxin synthetase of \textit{Cochliobolus carboneum} predicts a 550-kDa protein, but two separate proteins always have been isolated (36). These proteins activated three of the four amino acid constituents of the toxin. They arose by fragmentation of the 550-kDa polypeptide that was barely visible in SDS-PAGE gels (36). No >500-kDa protein has been detected yet in cell extracts from \textit{C. purpurea}, and the sequence of the LPS gene is not yet available.

The constitutive expression of LPS and finely tuned dependence of enzyme activity on the supply of \textit{D}-lysergic suggests that the regulation may occur at the earlier steps, namely at the synthesis of the ergoline ring carboxylic acid. Dimethylallyl thryptophan synthetase, an enzyme catalyzing the first step in the ergoline ring synthesis, has been described to be inducible through thryptophan and repressible through phosphate as is alkaloid production (33). It would therefore be an appropriate target for the observed repression of ergotamine synthesis in \textit{C. purpurea} wild type in Vogel’s medium (12). In the cases of
Enniatin synthetase in Fusarium scirpi, the producer of the cyclohexadepsipeptide enniatin B and of other toxins of phytopathogenic fungi, constitutive peptide synthetase expression has also been demonstrated (45). It may be indicated that the constitutive expression of peptide synthetases may allow rapid conversion of key metabolites such as D-lysergic acid into the peptide alkaloids. D-Lysergic acid, in contrast to clavines or ergot peptides, may be too unstable to be deposited safely in the growing sclerotium.

An interesting insight into the enzymatic regulation of ergot peptide synthesis came from kinetic characterization of the enzyme complex, which indicates that D-lysergyl peptide synthesis is strongly controlled by the available free D-lysergic acid due to the low apparent $K_m$ value, which is $-1.4 \mu M$. Limitation of ergotamine production by low D-lysergic acid concentration of ergotamine production was seen in C. purpurea wild type (31) but not in the ergotamine high producing derivative strain D1. Furthermore, the rate of synthesis and the spectrum of products is controlled by the nature and concentration of the amino acids present in the cell-free incubations, which is consistent with earlier in vivo data concerning the role of amino acids in the cellular pool of C. purpurea (31, 46). From the data presented here it follows that LPS has a broad amino acid substrate specificity giving it the capacity to synthesize naturally many different ergopeptine structures, probably according to fluctuations in the free amino acid pool. These properties make LPS a promising tool for the development of novel D-lysergic acid-containing compounds in the future.

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