D-Lysergic acid activation and cell-free synthesis of D-lysergyl peptides in an enzyme fraction from the ergot fungus Claviceps purpurea

ARTICLE *in* BIOCHEMISTRY · SEPTEMBER 1988

Impact Factor: 3.02 · DOI: 10.1021/bi00416a051 · Source: PubMed

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D-Lysergic Acid Activation and Cell-Free Synthesis of D-Lysergyl Peptides in Enzyme Fractions from the Ergot Fungus *Claviceps purpurea*†

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Received September 30, 1987; Revised Manuscript Received February 23, 1988

ABSTRACT: The D-lysergic acid activating enzyme from the ergot fungus *Claviceps purpurea* was purified to near homogeneity. It has a native M_r of about 245 000 and in its denatured form is a single polypeptide chain of M_r 62 000. The enzyme catalyzes the ATP–pyrophosphate exchange reaction dependent on D-lysergic acid and, though much less, that dependent on dihydrolysergic acid. Western blot analysis of SDS electropherograms of crude protein extracts from *C. purpurea* using monospecific antibodies directed against the D-lysergic acid activating enzyme revealed the immunostaining of one particular band which was identical with that of the D-lysergic acid activating enzyme. No significant immunoreactive band with higher molecular weight was seen, which precludes the possibility that the enzyme had arisen from the proteolysis of a high molecular weight ergot peptide synthetase. An ammonium sulfate fractionated enzyme fraction was prepared from *C. purpurea* strain C1 that catalyzed the incorporation of D-lysergic acid into two peptides which besides D-lysergic acid contained alanine, phenylalanine, and proline. Dihydrolysergic acid was efficiently incorporated into the corresponding dihydrolysergic acid containing analogues of the two compounds. Radiochemical analysis and degradation studies suggest that the two D-lysergic acid containing peptides most probably are *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-L-proline lactam and *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-D-proline lactam, respectively. *N*-[*N*-(D-Lysergyl)-L-alanyl]-L-phenylalanyl-L-proline lactam is considered to be the immediate precursor of ergotamine. These data support the idea that free D-lysergic acid is a free intermediate in the biosynthesis of ergopeptines. The role of the D-lysergic acid activating enzyme is discussed.

Ergopeptines are a family of cyclopeptides produced by the ergot fungus *Claviceps purpurea*. They consist of three different amino acids that form a bicyclic ring system by means of peptide and lactone bonds. This ring system is attached via its amino terminus to the carboxy group of D-lysergic acid in an amide bond (Figure 1). In vivo studies on the biosynthesis of ergotamine, the most prominent representative of this class of compounds, indicate that the peptide portion of the alkaloid is synthesized from the free amino acids (Bassett et al., 1973). Free D-lysergic acid stimulates ergotamine synthesis in whole cells and protoplasts of *C. purpurea* when added externally (Keller et al., 1980), and feeding *C. purpurea* cells with dihydrolysergic acid results in the biosynthesis of dihydroergotamine (Anderson et al., 1979). These data suggest that free D-lysergic acid is the direct precursor of the D-lysergic acid portion in the ergopeptines.

On the other hand, previously published data on the cell-free synthesis of ergopeptines indicate that biosynthetic precursors of D-lysergic acid such as elymoclavine or agroclavine rather than free D-lysergic acid serve as the precursors of peptide-bound D-lysergic acid (Maier et al., 1981). Instead of free D-lysergic acid a D-lysergyl coenzyme A thioester is proposed to play a role in the biosynthetic process (Maier et al., 1972).

In a preliminary paper from our laboratory, however, a D-lysergic acid activating enzyme from *C. purpurea* was described which is capable of catalyzing the D-lysergic acid dependent ATP–pyrophosphate exchange and the synthesis of ATP from chemically synthesized D-lysergic acid adenylate and pyrophosphate (Keller et al., 1984a). In addition, the enzyme activates, though less than D-lysergic acid, dihydrolysergic acid. These data suggest that in *C. purpurea* D-lysergic acid is activated via the adenylate and may directly become incorporated into the peptide chain of ergotamine.

In this contribution we describe the purification of the D-lysergic acid activating enzyme to near homogeneity and its further characterization. Moreover, studies on the total cell-free synthesis of D-lysergic acid peptides catalyzed by an enzyme fraction from *C. purpurea* will give evidence that free D-lysergic acid is indeed incorporated into such peptides.

Materials and Methods

Strains and Cultures. *C. purpurea* strain ATCC 20102 and derivatives of it such as strains 1029, C1 (nic-1), and D1 (nic-2) were described previously (Keller, 1983). Strains C1 and D1 are auxotrophs requiring nicotinic acid. They produce 500–600 mg L^{-1} ergotamine after growth for 2 weeks in the inoculum medium of Amici et al. (1966) in the additional presence of 3 mM tryptophan. The strains were maintained and grown as described previously (Keller et al., 1980, 1984b; Keller, 1983). Modified Vogel medium was as described (Keller et al., 1984b). *C. purpurea* strain Ecc 93 was kindly provided by Dr. H. Kobel, Sandoz AG (Basle, Switzerland). It was maintained and cultivated according to Kobel and Sanglier (1976). The strain produces about 200 mg L^{-1} ergopeptines mainly consisting of ergocristine (75–80%) after growth for 2 weeks in the production medium.

Chemicals and Radioisotopes. Tetrasodium [^{32}P]pyrophosphate (2.6 Ci/mmol) was obtained from NEN. L-[U-^{14}C]alanine (111 Ci/mol), L-[U-^{14}C]phenylalanine (509 Ci/mol), L-[U-^{14}C]proline (232 Ci/mol), [9,10(n)-^{3}H]-9,10-

† This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 9, Teilprojekt D5).

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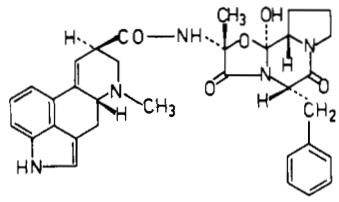

FIGURE 1: Structural formula of ergotamine. Replacement of the α-hydroxyalanyl moiety by α-hydroxyvaline gives ergocristine.

dihydroergocryptine (16.2 Ci/mmol), *N*-ethyl-[2,3-^{14}C]-maleimide (10 Ci/mol), and the preformed streptavidin–biotin–peroxidase complex system were from Amersham International. Diaminobenzidine, goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, biotinylated goat anti-rabbit IgG (whole molecule) antibody, L-amino acid oxidase, D-amino acid oxidase, and ergotamine tartrate were from Sigma. Dihydrolysergic acid was kindly provided by Dr. H. Kobel, Sandoz AG, Basle, Switzerland. L-Phenylalanyl-L-proline (L-Phe-L-Pro) and L-phenylalanyl-D-proline (L-Phe-D-Pro) were from Bachem, Bubendorf, Switzerland.

Nitrocellulose sheets (BA 85) were from Schleicher & Schuell, (Dassel, Federal Republic of Germany), and silica gel plastic sheets 60 were from Merck, Darmstadt, FRG. All other chemicals were of the highest purity commercially available.

Syntheses. D-Lysergic acid was prepared from ergotamine tartrate as described (Stoll et al., 1943). [9,10(n)-^{3}H]-9,10-Dihydrolysergic acid was prepared by treating 200 μCi of [9,10(n)-^{3}H]-9,10-dihydroergocryptine in a total volume of 1 mL of 1 M NaOH at 90 °C under an atmosphere of nitrogen for 1 h. After neutralization of the mixture with 2 M HCl at room temperature, the sample was freeze-dried and the residue was dissolved in 200 μL of 70% ethanol. This was applied to a silica gel plate and chromatographed in solvent system VI. The radioactive zone of dihydrolysergic acid was detected with a radioscanner, scraped off the plate, and finally extracted 5 times with 8-mL portions of ethanol. The combined ethanol extracts were evaporated to dryness at 40 °C, and the product was kept in a minute volume of ethanol at −20 °C. The product was identical with authentic dihydrolysergic acid as revealed by cochromatography in solvent systems V, VI, and VIII. Besides this it has a R_f value which differed from that of the starting material dihydroergocryptine. Dihydrolysergic acid was also analyzed by HPLC on a Lichrosorb RP18 (5-μm) column. The mobile phase was $MeOH/H_2O/H_3PO_4$ (60:40:0.01 v/v). A Knauer HPLC system was used. *cyclo*-(L-Phe-L-Pro) and *cyclo*-(L-Phe-D-Pro) were prepared from L-Phe-L-Pro and L-Phe-D-Pro, respectively, according to Nitecki et al. (1968). Alternatively, *cyclo*-(L-Phe-D-Pro) was synthesized from *cyclo*-(L-Phe-L-Pro) according to Hofmann et al. (1963): *cyclo*-(L-Phe-L-Pro) (10 mg) was dissolved in 1 mL of 2 M NaOH and left at room temperature for 30 min. After acidification of the mixture with 6 N HCl to pH 2, the cyclopeptide was extracted 3 times with 2-mL portions of chloroform. After evaporation of the organic phase the residue represented almost exclusively *cyclo*-(L-Phe-D-Pro) as revealed by chromatographic comparison with the product obtained by cyclization of L-Phe-D-Pro. R_f values for *cyclo*-(L-Phe-L-Pro) were 0.44 (solvent system I), 0.92 (solvent system II), 0.2 (solvent system III), 0.88 (solvent system IV), and 0.44 (solvent system V), and those for *cyclo*-(L-Phe-D-Pro) were 0.40 (I), 0.73 (II), 0.22 (III), 0.84 (IV), and 0.29 (V). The identity of the two cyclopeptides was checked by mass spectrometry which revealed a mole peak of 244.

Buffer and Solvent Systems. Buffer A consisted of 0.2 M Tris-HCl, pH 8.0, 40% (w/v) glycerol, 10 mM DTE,[1] and 0.3 M NaCl. Buffer B consisted of 0.1 M Tris-HCl, pH 8.0, 15% glycerol, 10 mM DTE, and 2 mM phenylmethanesulfonyl fluoride. Buffer C consisted of 50 mM potassium phosphate, pH 7, 1 mM DTE, and 1 mM phenylmethanesulfonyl fluoride. Buffer D consisted of 20 mM Tris-HCl, pH 8, and 1 mM DTE. Buffer E was 25 mM Pipes, pH 6.3, and 1 mM DTE.

TLC was performed on silica gel by using the following solvent systems: $EtOAc/MeOH/H_2O$ (100:5:5 v/v, solvent system I), $CHCl_3/EtOH$ (10:1 v/v, II), diisopropyl ether/$CHCl_3$/AcOH (6:3:1 v/v, III), $CHCl_3$/MeOH/AcOH (14:2:1 v/v, IV), $CHCl_3$/EtOH (10:0.5 v/v, V), $EtOH/H_2O$ (8:2 v/v, VI), $CHCl_3/MeOH/H_2O$ (65:35:4 v/v, VII), BuOH/AcOH/H_2O (4:1:1 v/v, VIII), and $CHCl_3$/MeOH (7:3 v/v, IX).

Methods of Analysis. Acid hydrolysis of radioactively labeled peptides and analysis of the hydrolysates was performed as described (Keller & Kleinkauf, 1977). Methanolysis of enzymatically formed *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-L-proline lactam and *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-D-proline lactam was done according to Stütz et al. (1973): Material scraped off from TLC plates was allowed to stand in methanol for 3 days at room temperature. After this period the methanol was evaporated and the dry residue was taken up in a minute volume of a mixture of chloroform/methanol (1:1 v/v), applied to silica gel TLC plates, and chromatographed in various solvent systems.

Radioactivity measurements of enzymatically formed ergot peptides were done either by liquid scintillation counting of EtOAc extracts of reaction mixtures in a toluene-based scintillation fluid or by radioscanning of TLC separations. For visualization of radioactive bands on TLC plates, autoradiography was performed with Kodak X-ray film X-Omat. For detection of tritium-labeled compounds, plates were treated with EN^{3}HANCE spray (NEN) according to the manufacturer's instructions. Protein concentrations were determined according to Bradford (1976). The buffer for D- and L-amino acid oxidase was 0.02 N NH_4HCO_3. Hydrolysates [(2–10) × 10^4 cpm] were taken up in a total of 20 μL of buffer containing 5 μg of L-amino acid oxidase or 1 μg of D-amino acid oxidase per microliter. Each assay contained 30 μg of catalase. Incubation was at 37 °C overnight. Reaction products were subjected to TLC.

Purification of D-*Lysergic Acid Activating Enzyme.* Some 13 g of freeze-dried mycelium of *C. purpurea* strain 1029 or Ecc 93 was ground in a mortar together with 300 mg of phenylmethanesulfonyl fluoride for 5–10 min at room temperature. The resultant fine powderous material was stirred into about 300 mL of ice-cold buffer C. The subsequent steps all were performed at 2–4 °C. After the suspension was stirred for 1 h, it was centrifuged for 20 min at 10000 rpm in the GSA rotor of a Sorvall RC-2B centrifuge (step 1). To the supernatant was added a 14% (w/v) Polymin P (BASF, Ludwigshafen, FRG) solution to give 0.2% final concentration. After 45 min, the suspension was centrifuged as above. The supernatant (step 2) was then passed through a DEAE-cellulose column (bed dimensions 6 × 5.5 cm) that had been equilibrated previously with buffer C, and 10-mL fractions were collected. Fractions containing protein were combined (step 3), and a saturated ammonium sulfate solution was added gradually to give 30% saturation. After standing 45 min on

[1] Abbreviations: DTE, dithioerythritol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

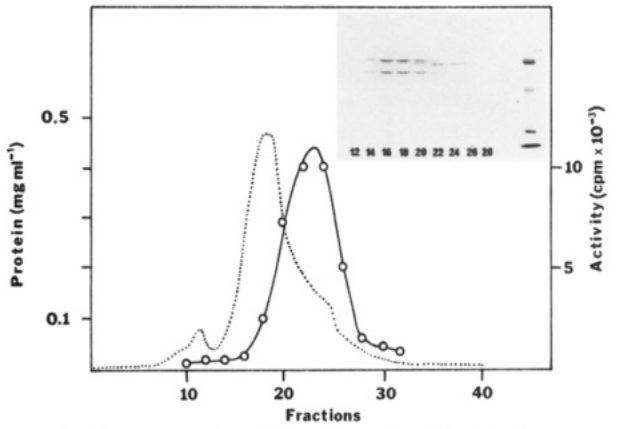

FIGURE 2: Chromatography of the D-lysergic acid activating enzyme on aminohexyl-Sepharose. The enzyme was eluted from the column with a 100-mL linear gradient ranging from 0 to 0.4 M NaCl in 0.025 M Pipes, pH 6.3. Fractions (2.5 mL) were collected. (···) Protein concentration; (○) ATP–pyrophosphate exchange dependent on D-lysergic acid. The inset shows SDS–PAGE separations of 25-μL aliquots from the indicated fractions. Each fraction was assayed for activity by incubating 25 μL of enzyme with 20 nmol of pyrophosphate (10^5 cpm) in a total volume of 200 μL as described.

ice, the suspension was centrifuged as above. The precipitate was discarded, and the supernatant was brought to 55% saturation with saturated ammonium sulfate solution. After standing 1 h on ice, the precipitate was collected by centrifugation as above.

The supernatant was discarded, and the pellet was taken up in a minute volume of buffer C (step 4) and applied onto a Ultrogel AcA 34 column (bed dimensions 42 × 3 cm) that had been equilibrated previously with buffer C.

Seven-milliliter fractions were collected. Fractions containing the D-lysergic acid activating enzyme were pooled (step 5), and the whole pool was diluted with 1 volume of water containing 1 mM DTE to give a final concentration of 25 mM potassium phosphate. The enzyme solution was applied to a DEAE-cellulose column (bed dimensions 6 × 1 cm) that had been equilibrated previously with 25 mM potassium phosphate, pH 7, 1 mM DTE, and 1 mM phenylmethanesulfonyl fluoride. The column was then washed with 100 mL of the same buffer, and enzyme was eluted from the column with 15 mL of buffer C (step 6).

The eluate was then applied to an aminohexyl-Sepharose (Sigma) column (bed dimensions 6 × 1 cm) that had been equilibrated previously with buffer C. Enzyme was washed onto the column with 100 mL of buffer D and was eluted with a linear 200-mL gradient of 0–0.4 M NaCl in buffer D (2.8-mL fractions). Enzyme activity eluted between 0.22 and 0.27 M NaCl, and active fractions were pooled (step 7). To this pool was added 2 volumes of buffer C, and the resulting solution was applied again to an aminohexyl-Sepharose column (bed dimensions 8 × 1 cm) previously equilibrated with buffer D. Enzyme was washed onto the column with 100 mL of buffer E and subsequently eluted with a linear 200-mL gradient ranging from 0 to 0.4 M NaCl (Figure 2). Fractions (2.5 mL) were collected. Fractions containing enzyme activity were subjected to SDS–PAGE, and the fractions containing the enzyme with more than 95% purity (i.e., fractions 22–27) were pooled (step 8). The pool of enzyme was concentrated by means of microconcentrators (Amicon) to a final concentration of about 1.5 mg/mL. Enzyme was then frozen in the presence of 15% glycerol at −80 °C.

Preparation of Antiserum. Enzyme was dispensed in 100 μg/250 μL aliquots in buffer C, and one aliquot was injected subcutaneously with Freund's complete adjuvant into a rabbit. After 2 weeks the same procedure was repeated five times in weekly intervals in the presence of incomplete adjuvant. Serum was prepared from blood removed 2 months after the first injection.

Purification of Antibodies. Purified enzyme (3 mg in 2 mL of buffer E) was mixed with 0.5 g of freshly prepared cyanogen bromide activated Sepharose 4B (Pharmacia) previously equilibrated in 0.1 M sodium carbonate buffer (pH 8.8). After being gently shaken for 24 h at 4 °C, the gel was stripped with 0.1 M ethanolamine at pH 8.8. After washing the gel 3 times with 10 mM NaH_2PO_4, pH 7.25, and 150 mM NaCl (PBS), it was layered on top of a Ultrogel AcA 202 column (12 × 1 cm) that had been previously equilibrated with PBS. Serum (1 mL) was applied to the column and was allowed to react with the affinity gel matrix at room temperature overnight. After the column was washed with 50 mL of PBS, antibodies were eluted with 2 mL of 0.1 M glycine, pH 2.8, and were immediately desalted on the Ultrogel AcA 202. A total of 80–120 μg of antibody/mL of serum was obtained.

Enzyme-Linked Immunosorbent Assay (ELISA). Polystyrene microtiter plates were coated with 100 μL of D-lysergic acid activating enzyme in 50 mM potassium phosphate, pH 7 (0.1 μg per well), overnight at 4 °C. The wells were subsequently incubated with appropriate dilutions of antibody (0.02–0.1 μg mL^{-1}), goat anti-rabbit antibody alkaline conjugate, and 4-nitrophenyl phosphate.

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS slab gel electrophoresis with 10% gels was performed according to Laemmli (1970). Samples to be analyzed were heated in a boiling water bath for 5 min in the presence of 1% SDS and 10% mercaptoethanol. Portions of gels were stained with Coomassie Blue G 250; parallel runs were used for electrophoretic transfer of protein bands to nitrocellulose (Towbin et al., 1979). After protein transfer nitrocellulose strips were processed according to the biotin–streptavidin brochure of Amersham International by using 1000–5000-fold dilutions of primary antibody, biotinylated goat anti-rabbit antibody, and the biotin–streptavidin–peroxidase preformed complex.

Preparation of Cell-Free Extract Catalyzing Ergot Peptide Lactam Synthesis. Some 10 g of 3 day old freeze-dried mycelium of *C. purpurea* strain C1 was ground in a mortar with 100 mg of phenylmethanesulfonyl fluoride for 15 min at room temperature. The resultant fine powderous material was stirred into 200 mL of ice-cold buffer A. Stirring was continued for 1 h (all operations were carried out at 0–4 °C). After this period the suspension was centrifuged in a GSA rotor of a Sorvall RC-2B centrifuge at 10000 rpm for 30 min. To the supernatant was added a 14% Polymin P solution to give a final concentration of 0.3%. After being stirred for 30 min, the suspension was centrifuged as above. To the supernatant was gradually added a saturated ammonium sulfate solution until 30% saturation was reached. After 1 hour on ice the mixture was centrifuged as above. The pellet was discarded, and the supernatant was brought to 45% saturation with saturated ammonium sulfate solution. This mixture was left on ice for a further 1 h and then centrifuged as above. The supernatant was discarded, the pellet was dissolved in a minute volume of buffer B, and aliquots of 1 mL were frozen at −80 °C.

For enzyme assay 1 mL was passed through a Ultrogel AcA 202 column (bed dimensions 12 × 1 cm) that had been equilibrated previously with buffer B but without DTE.

Enzyme Assays. The ATP–pyrophosphate exchange reaction dependent on D-lysergic acid was measured as described

Table I: Purification of D-Lysergic Acid Activating Enzyme[a]

step	volume (mL)	protein (mg)	units[c] (nkat)[d]	sp act.[c] (nkat/mg)[d]	purification (x-fold)
(1) crude extract	202	2020			
(2) Polymin P precipitation	192	1644			
(3) DEAE-cellulose (50 mM potassium phosphate)	326	391			
(4) $(NH_4)_2SO_4$ fractionation (30–55%)	10.3	103			
(5) Ultrogel AcA 34 gel filtration	52	23.5	3.72	0.159	1
(6) DEAE-cellulose (25 mM potassium phosphate)	20	8.2	3.09	0.38	2.5
(7) aminohexyl-Sepharose (20 mM Tris, pH 8)	40	2.5	2.67	1.09	7
(8) aminohexyl-Sepharose (25 mM Pipes, pH 6.4)	15	0.6[b]	1.17	1.95	12

[a] A total of 12 g freeze-dried mycelium of *C. purpurea* strain 1029 was used. [b] In this step only the rear half of the peak contained almost pure enzyme. [c] Enzymatic activity was determined by measuring the ATP–PP_i exchange reaction dependent on D-lysergic acid. [d] One nanokatal (1 nkat) is the amount of enzyme catalyzing the incorporation of 1 nmol of pyrophosphate into ATP per second.

previously (Keller et al., 1984a). The assay mixture for enzymatic ergot peptide lactam synthesis contained the following in a total of 300 μL: D-lysergic acid or dihydrolysergic acid (0.05 μmol), 0.25 μCi of [U-^{14}C]phenylalanine, 0.5 μmol of alanine, 0.5 μmol of proline, 3 μmol of $MgCl_2$, 1.5 μmol of ATP, and 200 μL of enzyme. In experiments with other radioactive amino acids, alanine or proline was replaced by 0.25 μCi of ^{14}C-labeled material or D-lysergic acid was replaced by 0.5 μCi of [^{3}H]dihydrolysergic acid. The assay mixture was incubated at 26 °C for 30 min. After the reaction was stopped with 1 mL of H_2O, radioactive ergot peptide lactams were extracted from the mixture with two 2-mL portions of ethyl acetate. The combined EtOAc extracts were evaporated to dryness at reduced pressure at 30 °C and subjected to TLC in solvent system I.

The assay for measuring thioester formation was as described previously (Keller, 1987).

Analytical Protein Separation Procedures. p*I* determinations were done by isoelectric focusing by using Servalyt Precotes sheets (pH range 5–8) (Serva, Heidelberg, FRG) according to the manufacturer's instructions. At the end of each run the gel matrix was cut into zones of 0.5-cm width and each zone was scraped with a spatula off the sheets. The gel strips were immersed in 200-μL aliquots of buffer C. After they were shaken gently for 1 h at 0 °C, the ATP–pyrophosphate exchange dependence of D-lysergic acid was measured as described (Keller et al., 1984a). Chromatofocusing was performed with a column (bed dimensions 25 × 1.2 cm) containing chromatofocusing gel PBE 94 (Pharmacia) equilibrated with 25 mM imidazole, pH 7.4. After application of the sample (4 mL) containing the D-lysergic acid activating enzyme in the same buffer, elution was performed with polybuffer 74 (Pharmacia) according to the manufacturer's instructions. FPLC of the D-lysergic acid activating enzyme was done by using a Pharmacia Mono Q HR 5/5 anion-exchange column. Enzyme was eluted from the column with a linear gradient of 0–0.5 M NaCl in 0.01 M Tris-HCl, pH 7.5, and 1 mM DTE. A Pharmacia FPLC system was used. The FPLC runs were performed at room temperature.

Results and Discussion

Purification of D-Lysergic Acid Activating Enzyme. In Table I are shown the various steps of purification of the D-lysergic acid activating enzyme from *C. purpurea* 1029. The procedure differs from the previously published one (Keller et al., 1984a) but still contains several of its basic steps. Passage of the Polymin P treated cell extract through DEAE-cellulose in 50 mM potassium phosphate buffer removed all ATP–pyrophosphate exchange activity which is independent of D-lysergic acid (step 3). After ammonium sulfate precipitation and fractionation on Ultrogel AcA 34,

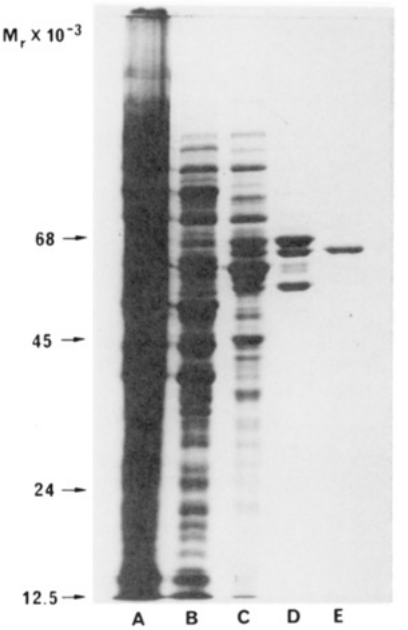

FIGURE 3: SDS–PAGE of various purification steps in the purification procedure for the D-lysergic acid activating enzyme. (A) Crude enzyme (step 1); (B) DEAE-cellulose-fractionated enzyme (step 3); (C) enzyme after Ultrogel AcA 34 gel filtration (step 5); (D) enzyme after aminohexyl-Sepharose, pH 8 (step 7); (E) enzyme after aminohexyl-Sepharose, pH 6.3 (step 8). Fifty microliters of each step was treated as described under Materials and Methods and was loaded on a 10% slab gel. Staining was with Coomassie Blue.

enzyme was adsorbed to DEAE-cellulose in the presence of 25 mM potassium phosphate, pH 7, and eluted with 50 mM potassium phosphate, pH 7 (step 6). Two chromatographic separations on aminohexyl-Sepharose at pH 8 and 6.3 finally afforded a purification of the enzyme to near homogeneity (step 8). This last step is presented in Figure 2, and from the inset showing the gel electrophoretic analysis of the active fractions it can be seen that the rear half of the activity peak contains almost pure enzyme. Subsequent separation of the purified enzyme by FPLC on Mono Q or chromatography on DEAE-cellulose in 50 mM Tris, pH 8, did not result in a significant increase in the specific activity (data not given). The degree of purification from step 5 to 8 was about 12-fold. The yield of enzyme is between 1–2 mg per 15 g of mycelial dry weight.

The various steps of purification of enzyme were routinely checked by SDS–PAGE (Figure 3). The analysis of samples from each step shows the progress of purification which finally results in a preparation containing one single band. Gel scans of the Coomassie Blue stained gel lane E gave a purity of 96%.

Molecular Weight Determination. By use of several molecular weight markers, the molecular weight of denatured

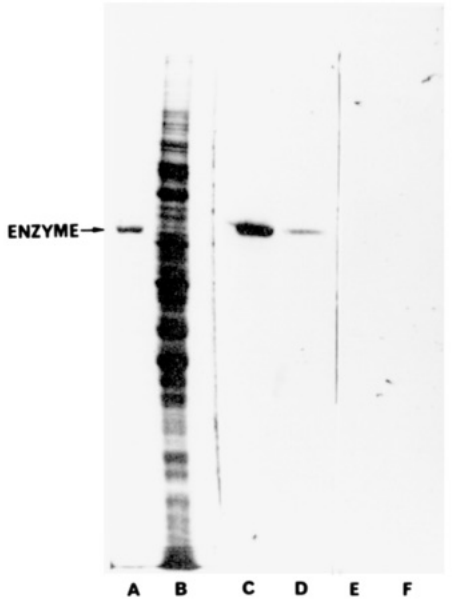

FIGURE 4: Immunoblot of purified D-lysergic acid activating enzyme and the crude protein extract (step 1, Table I) using antibodies directed against the D-lysergic acid activating enzyme. (A) Stained gel with purified enzyme; (B) stained gel with crude extract; (C) Lane A blotted on nitrocellulose and reacted with first antibody; (D) lane B blotted on nitrocellulose and reacted with first antibody; (E) same as lane C without first antibody; (F) same as lane D without first antibody. About 8 μg of purified enzyme and 50 μL of crude extract were used. Antibody dilution was 1:1500 of a solution containing about 80 μg/mL antibody.

enzyme was determined to be 62 000. Isoelectric focusing of the purified native enzyme showed that the activity resided in one single band and thus made sure that the band in Figure 3, lane E, represented the D-lysergic acid activating enzyme (not shown).

The apparent discrepancy between the previously reported molecular weight of the native enzyme of 135 000–140 000 (Keller et al., 1984a) and that of the denatured enzyme led us to repeat the determination of the native molecular weight by gel filtration on Sephadex G-200 and Ultrogel AcA 34. In these experiments we always found the native enzyme to possess an M_r of 245 000–250 000. These data suggest that the enzyme is a tetramer of four similar subunits. The reason for the difference to the earlier finding could not be clarified as yet. However, it is noteworthy that in a number of gel filtration experiments the lower molecular weight form could be detected to a low but significant extent.

In order to see whether the enzyme originally was the integral part of a larger multifunctional peptide synthetase that had arisen through proteolysis during preparation of the enzyme, freshly prepared crude extracts of *C. purpurea* were separated by SDS–PAGE and checked for the presence of enzyme in Western blots. Figure 4 shows that purified antibodies reacted almost exclusively against one particular band in the protein extract which had the same electrophoretic mobility as the enzyme. No significant immunoreactive band with higher molecular weight than the enzyme was seen in these blots.

Further Properties of the Enzyme. The enzyme possesses a p*I* of about 5.5 as revealed by isoelectrofocusing and chromatofocusing. No inhibition of activity was observed when iodoacetamide (0.5 mM) or *p*-(hydroxymercuri)benzoate (0.1 mM) was added. By contrast, *N*-ethylmaleimide (NEM) inhibited the enzyme completely when added at a concentration higher than 0.1 mM. However, enzyme could be completely protected against NEM when D-lysergic acid (or dihydrolysergic acid) was added before addition of the inhibitor. Incubation of enzyme with [^{14}C]NEM yielded no detectable covalent labeling, which suggests that NEM may occupy a site involved in the entry of D-lysergic acid without reacting with amino acid residues of the protein. Thiol group protecting agents such as DTE had no measurable influence on the activity of enzyme. Apparently, the enzyme does not possess a catalytic active SH group. Also, attempts to charge the purified enzyme with [^{3}H]dihydrolysergic acid via thioester linkage failed. The enzyme did not activate the other constituent amino acids of ergotamine and did not catalyze the synthesis of D-lysergic acid containing peptides. It appears therefore, that the enzyme portion responsible for the assembly of the amino acids has been lost during the purification of the D-lysergic acid activating enzyme. If the enzyme is part of the ergot peptide synthesizing enzyme system, it would resemble in its role that of actinomycin synthetase I in the biosynthesis of actinomycin (Keller et al., 1984c; Keller, 1987). Actinomycin synthetase I possesses no other detectable activity than to activate the chromophore precursor of the peptide antibiotic, 4-methyl-3-hydroxyanthranilic acid (4-MHA), via the adenylate and is involved in some way in the attachment of the aromatic carbonic acid to the peptide chains of actinomycin. However, in the case of ergot peptide synthesis there is no possibility to examine the D-lysergic acid activating enzyme for its involvement in D-lysergyl peptide formation unless a cell-free system can be separated into its components and reconstituted to full activity (see below). Therefore, it cannot be excluded that the D-lysergic acid activating enzyme described here may be involved in an as yet unknown reaction in D-lysergic acid metabolism.

The presence of the enzyme in cell extracts of ergopeptine high producers of *C. purpurea* such as ECC 93 and D1 was checked by immunoblotting of SDS–PAGE electropherograms of crude protein extracts (step 1, Table I) at various stages of cultivation. Enzyme was significantly present already after the first day of cultivation in the production media, and a 2–3-fold expression was seen during the phase of maximum ergopeptine production in these two strains (data not shown). This behavior is unusual because work in this laboratory on other microbial peptide synthetase systems such as that of actinomycin (*Streptomyces*) or enniatin (*Fusarium*) revealed that the relevant enzymes had their highest levels (specific activities) just prior to the phase of maximum synthesis of secondary metabolite (A. Stindl and U. Keller, unpublished results; A. Billich and R. Zocher, personal communication).

D-Lysergic acid activating activity was detected at comparable levels in alkaloid-producing strains ECC 93, 1029, D1, and C1. Surprisingly, in the wild-type strain ATCC 20102 (producing only 5–10 mg of ergotamine/L) it was present at the same level. However, when all of these strains were grown in modified Vogel medium which does not support the formation of sclerotia-like cells and alkaloid production (Keller et al., 1984b), the enzyme was nearly undetectable. This dependence on the growth conditions that favor a morphology characteristic for alkaloid production in *C. purpurea* provides additional evidence that the enzyme is linked to the secondary metabolism in the ergot fungus. In this context, it may be noted that strain ATCC 20102 is an alkaloid low producer due to its reduced capacity in D-lysergic acid synthesis (Keller et al., 1984a) and it may contain more D-lysergic acid activating enzyme than would be expected from its low productivity.

Incorporation of D-*Lysergic Acid and Dihydrolysergic Acid into Ergot Peptides.* In order to provide more direct evidence for the intermediacy of free D-lysergic acid in ergot peptide

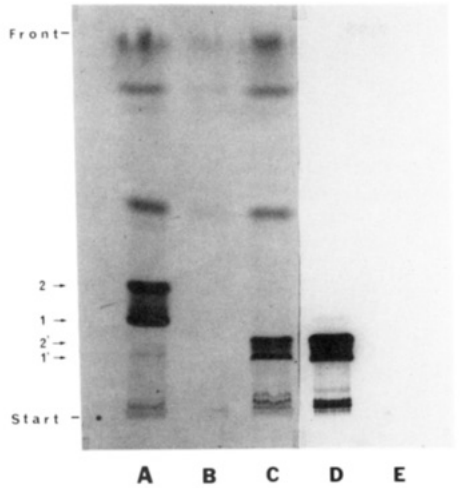

FIGURE 5: Enzymatic synthesis of D-lysergic acid peptides. EtOAc extracts of various reaction mixtures containing [^{14}C]phenylalanine and the other constituents of ergotamine were chromatographed on silica gel by using solvent system I. The experiments were modified as indicated: (A) with D-lysergic acid; (B) without D-lysergic acid; (C) with dihydrolysergic acid instead of D-lysergic acid; (D) with [^{3}H]dihydrolysergic acid instead of D-lysergic acid, and with [^{14}C]-phenylalanine replaced by unlabeled material; (E) same experiment as in lane D but with the omission of ATP.

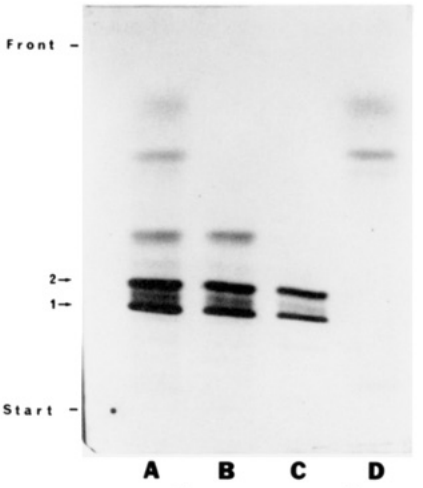

FIGURE 6: Incorporation of [^{14}C]alanine and [^{14}C]proline into D-lysergic acid peptides. EtOAc extracts of reaction mixtures containing [^{14}C]phenylalanine (A), [^{14}C] proline (B), and [^{14}C]alanine (C) were chromatographed on silica gel by using solvent system I. Lane D shows a control of a [^{14}C]phenylalanine-containing reaction mixture with the omission of ATP.

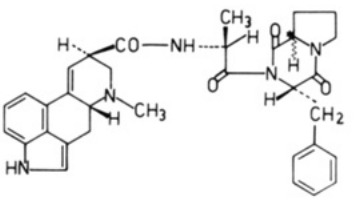

FIGURE 7: Structural formula of *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-L-proline lactam and *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-D-proline lactam. The asymmetric α-C atom of proline is indicated by an asterisk.

formation, cell-free extracts were prepared from various *C. purpurea* strains available in this laboratory by using buffer systems that had been proven suitable for the isolation of peptide lactone synthetase from streptomycetes (Keller, 1987).

Incorporation studies in such extracts revealed that D-lysergic acid or dihydrolysergic acid was incorporated into D-lysergic acid or dihydrolysergic acid containing peptides. Most suitable for the preparation of such enzyme extracts were mycelia from strains C1 and D1. In Figure 5 are presented thin-layer chromatograms of EtOAc extracts of reaction mixtures containing D-lysergic acid (or dihydrolysergic acid) and the other constituents of ergotamine. Lane A shows the incorporation of [^{14}C]phenylalanine in the presence of D-lysergic acid into several compounds of which two (designated 1 and 2) were missing in a parallel experiment without D-lysergic acid (lane B). These two bands were therefore tentatively considered as D-lysergyl peptides. When in the same reaction mixture D-lysergic acid was replaced by dihydrolysergic acid, the [^{14}C]phenylalanine was incorporated into two new compounds (designated 1′ and 2′) (Figure 5, lane C). It is obvious that these two new compounds have coordinately reduced R_f values when compared to compounds 1 and 2 and thus may be considered as the dihydrolysergic acid analogues of compounds 1 and 2, because dihydrolysergic acid has a lower R_f value than D-lysergic acid in various solvent systems. Accordingly, [^{3}H]dihydrolysergic acid was incorporated into compounds 1′ and 2′ when [^{3}H]dihydrolysergic acid was used as the radiolabel (lane D). Lane E shows that no [^{3}H]dihydrolysergic acid incorporation took place when ATP was missing in the reaction mixture. The formation of all of the compounds was not observed when D-lysergic acid (or dihydrolysergic acid) was replaced by elymoclavine or agroclavine (not shown).

Analysis of Radioactive Lysergyl Peptides. Besides [^{14}C]phenylalanine, [^{14}C]alanine or [^{14}C]proline was efficiently incorporated into compounds 1 and 2 (Figure 6). Acid hydrolysis of both compounds after labeling with [^{14}C]phenylalanine, [^{14}C]proline, or [^{14}C]alanine gave back each of the radioactive amino acids. These findings indicate that the compounds were not ergopeptines; otherwise, alanine would not have been recovered. The optical configuration of the radioactive proline in the acid hydrolysates determined by using amino acid oxidase was the D configuration as was to be expected from the acid hydrolysis of an ergopeptine or ergot peptide lactam (Hofmann, 1961). When [^{14}C]phenylalanine- or [^{14}C]proline-labeled compounds 1 and 2 were treated with absolute methanol at room temperature for 3 days, they were converted into new radioactive compounds. Cochromatography with authentic material in various solvent system revealed that compound 2 was completely converted into *cyclo*-(L-Phe-D-Pro) whereas compound 1 gave a mixture of 95–99% *cyclo*-(L-Phe-D-Pro) and 1–5% *cyclo*-(L-Phe-L-Pro). Treatment with 1 N NaOH at 90 °C of substances 1′ and 2′, labeled with [^{3}H]dihydrolysergic acid, gave back [^{3}H]dihydrolysergic acid.

Earlier work of Stütz et al. (1973) revealed the presence of *N*-[*N*-(D-lysergyl)-L-valyl]-L-phenylalanyl-L-proline lactam in the alkaloid mixture of an ergocristine-producing strain of *C. purpurea*. This alkaloid differs from ergocristine in that it is missing the cyclol bridge and the proline moiety has the D configuration. In ergopeptines the proline moiety always has the L configuration (for comparison see Figures 1 and 7). It was speculated that the ergot peptide lactam was the product of a biosynthetic dead-end route (Stadler, 1982) which could not be hydroxylated at the peptide-bound valine residue as is the case with the L-proline-containing peptide. L-Proline-containing acyldioxopiperazines in polar solvent systems have a high tendency to undergo epimerization into the D-proline-containing isomers (Hofmann et al., 1963; Ott at al., 1963), and this would explain why Stütz et al. (1973) found exclusively the D-proline-containing peptide lactam.

Methanolysis of the *N*-[*N*-(D-lysergyl)-L-valyl]-L-phenylalanyl-D-proline lactam yielded *N*-(D-lysergyl)-L-valine methyl

Table II: Dependence of Ergot Peptide Lactam Synthesis on the Presence of Various Substrates in the Cell-Free System

cell-free system	ergot peptide lactams (cpm)	cell-free system	ergot peptide lactams (cpm)
complete[a]	47 080	-alanine	2 180
-D-lysergic acid	nd[b]	-ATP	nd
-D-lysergic acid, +dihydrolysergic acid	37 260	$-Mg^{2+}$	nd
		+10 mM DTE	3 250
-proline	3 860		

[a] The complete system contained 0.25 μCi of [^{14}C]phenylalanine, 0.05 μmol of D-lysergic acid, and all other ingredients in unlabeled form as described under Materials and Methods. [b] nd, not detected.

ester and L-phenylalanyl-D-proline lactam (Stütz et al., 1973). In addition, studies of the chemical synthesis of ergotamine revealed that dioxopiperazines with the L configuration in the proline portion are readily epimerized to the D configuration when dissolved in polar solvents (Hofmann et al., 1963; Ott et al., 1963). Thus, under the light of the findings of these groups, our results strongly suggest that compounds 1 and 2 are *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-L-proline lactam and *N*-[*N*-(D-lysergyl)-L-alanyl]-L-phenylalanyl-D-proline lactam, respectively (Figure 7).

Initially, we were concerned about the possibility that compound 2 was the isolysergic acid form of compound 1. However, the finding that also with dihydrolysergic acid the cell-free system synthesized two compounds provides indirect evidence against this possibility, because dihydrolysergic acid is much more stable against isomerization than D-lysergic acid. Accordingly, this fact is a further substantiation of the above conclusions.

Further Properties of the Cell-Free System of Ergot Peptide Synthesis. Enzyme activity was lost after 1 day of standing on ice. For the stability of the enzyme a 6–10 mM concentration of DTE was necessary; otherwise the activity disappeared within 2 h. Enzyme could be stored frozen at −80 °C in the presence of DTE for 2 weeks with a 30% reduction of activity.

Besides depending on the presence of the various amino acids present in the peptide chain of ergotamine, synthesis of the ergot peptide lactams depended on the presence of Mg^{2+} (Table II). Surprisingly, under assay conditions the synthesis of ergot peptide lactams was strongly reduced when DTE was present (Table II). Thus, DTE always had to be removed by gel filtration prior to incubation. This finding may indicate that intact disulfide bonds are necessary for the activity of the peptide-synthesizing enzyme system.

In summary, the data reveal that free D-lysergic acid (or dihydrolysergic acid) is a free intermediate in D-lysergyl peptide synthesis. The question whether the D-lysergic acid activating enzyme participates in ergot peptide lactam synthesis remains open until conditions are established that allow the purification of the enzyme system responsible for ergot peptide synthesis. This enzyme system is at present very unstable.

It may be noted that work on the total cell-free synthesis of ergopeptines (Maier et al., 1981, 1983, 1985) yielded results that are quite different from those presented here. The most striking difference is that these authors found ergopeptine synthesis dependent on the biosynthetic precursors of D-lysergic acid, namely, agroclavine or elymoclavine, rather than on D-lysergic acid itself. In addition, their enzyme system appears to synthesize exclusively ergopeptines, the final products of the alkaloid biosynthetic pathway. The reason for the different results of these authors when compared to ours is as yet not clear. It is expected that future work on the enzymes involved in peptide alkaloid biosynthesis will help to clarify this question.

Acknowledgments

We thank Dr. Rainer Zocher for valuable discussions. We are indebted to Dr. H. Kobel, Sandoz AG, Basle, Switzerland, for supplying us with dihydrolysergic acid and *C. purpurea* strain Ecc 93.

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