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Molecular Cloning and Analysis of the Ergopeptine Assembly System in the Ergot Fungus *Claviceps purpurea*

Telmo Correia,[1,3] Nicolas Grammel,[2,3,4] Ingo Ortel,[2] Ullrich Keller,[2,*] and Paul Tudzynski[1,*]
[1]Institut für Botanik
Westfälische Wilhelms-Universität
Schlossgarten 3
D-48149 Münster
[2]Institut für Chemie
Fachgebiet Biochemie
Technische Universität Berlin
Franklinstrasse 29
D-10587 Berlin-Charlottenburg
Germany

Summary

Claviceps purpurea **produces the pharmacological important ergopeptines, a class of cyclol-structured alkaloid peptides containing D-lysergic acid. These compounds are assembled from D-lysergic acid and three different amino acids by the nonribosomal peptide synthetase enzymes LPS1 and LPS2. Cloning of alkaloid biosynthesis genes from *C. purpurea* has revealed a gene cluster including two NRPS genes, *cpps 1* and *cpps 2*. Protein sequence data had assigned earlier *cpps1* to encode the trimodular LPS1 assembling the tripeptide portion of ergopeptines. Here, we show by transcriptional analysis, targeted inactivation, analysis of disruption mutants, and heterologous expression that *cpps 2* encodes the monomodular LPS2 responsible for D-lysergic acid activation and incorporation into the ergopeptine backbone. The presence of two distinct NRPS subunits catalyzing formation of ergot peptides is the first example of a fungal NRPS system consisting of different NRPS subunits.**

Introduction

D-lysergic acid ($\Delta^{9,10}$-6-methyl-ergolene-8-carboxylic acid) is an important pharmacophore that in its amidated form exerts useful activities for therapy for the treatment of migraine, hypertension, or prolactine disorders [1, 2]. The specific effects of the various amides of D-lysergic acid depend on the amide substituents which confer to D-lysergic acid structural similarity to adrenaline, serotonine, or dopamine, causing agonist or antagonist behavior against the corresponding neurotransmitter families [3]. Most prominent among the D-lysergic amides are the ergopeptines, in which the amide component is a tripeptide chain in the modified form of a bicyclic cyclol-lactam structure (Figure 1). Simpler D-lysergic acid derivatives are ergometrine or D-lysergic acid α-hydroxyethylamide (Figure 1), where D-lysergic acid is attached to small amino alcohols [4, 5].

Main producers of ergopeptines and the D-lysergic acid amides are members of the genus *Claviceps* [6]. Whereas *Claviceps purpurea* can produce both groups of D-lysergic acid derivatives, *Claviceps paspali* produces simple D-lysergic acid amides or paspalic acid, the immediate precursor of D-lysergic acid [7, 8]. Other species, notably *Claviceps fusiformis*, lack the ability to synthesize D-lysergic acid, producing clavine alkaloids instead, which have still simpler structures [9]. The main representatives of the clavines represent intermediates in the ergot alkaloid biosynthesis (for review see [4, 5]). Figure 2 shows the various steps of the ergolene pathway from the formation of dimethylallyl tryptophan (DMAT) to the conversion of paspalic acid to D-lysergic acid. DMAT synthase (DMATS) has been the first enzyme of the ergot alkaloid pathway that was purified to homogeneity [10, 11]. The DMATS-encoding gene (*dmW*) was the first cloned alkaloid biosynthesis gene from the clavine-producing species *Claviceps fusiformis* [12]. Importantly, an ortholog of *dmW* named *cpd1* was shown to be present in ergopeptine-producing *Claviceps purpurea*, which proved the common biosynthetic origin of the clavines and the D-lysergic acid-derived group of alkaloids [13].

Previously, investigations of the assembly of D-lysergyl tripeptide lactams, the immediate precursor of ergopeptines, in *C. purpurea* have shown that they are formed by a large nonribosomal peptide synthetase (NRPS) multienzyme that, during purification, separated in two enzyme activities [14]. The two enzymes, D-lysergyl peptide synthetase 1 and 2 (LPS1 and 2) have sizes of 370 and 140 kDa, respectively. Synthesis starts with binding of D-lysergic acid to LPS2 as thioester followed by transfer to LPS1, where three successive condensations into the D-lysergyl mono-, di-, and tripeptide thioester intermediate take place, with final release of the end product D-lysergyl tripeptide lactam [15]. These data indicated that LPS1 and LPS2 would contain four modules with individual adenylation (A) domains, peptidyl carrier protein (PCP or T) domains, condensation (C), domains and a putative domain catalyzing release of the lactam product, presumably a thioesterase (Te) domain [16]. While LPS1 would harbor the three amino acid modules, LPS2 would represent the D-lysergic acid module (Figure 2).

Sequencing the flanking regions of the recently cloned DMAT synthase gene *cpd1* from *C. purpurea* led to the identification of further genes most probably involved in ergoline ring synthesis (Figure 3). Importantly, downstream to *cpd1* the gene encoding the trimodular enzyme LPS1, *cpps1*, was identified, indicating the presence of an ergot alkaloid gene cluster [17]. This finding suggested that further chromosome walking on the flanking regions of *cpd1* would disclose the gene of LPS2, for which protein sequence data were not available. In this report, we describe the genetic organization of an as yet unidentified region of the ergot alkaloid

*Correspondence: ullrich.keller@tu-berlin.de (U.K.), tudzyns@uni-muenster.de (P.T)
[3]These authors contributed equally to this work.
[4]Present address: ActinoDrug Pharmaceuticals GmbH, Neuendorfstrasse 24a, D-16761 Hennigsdorf, Germany.

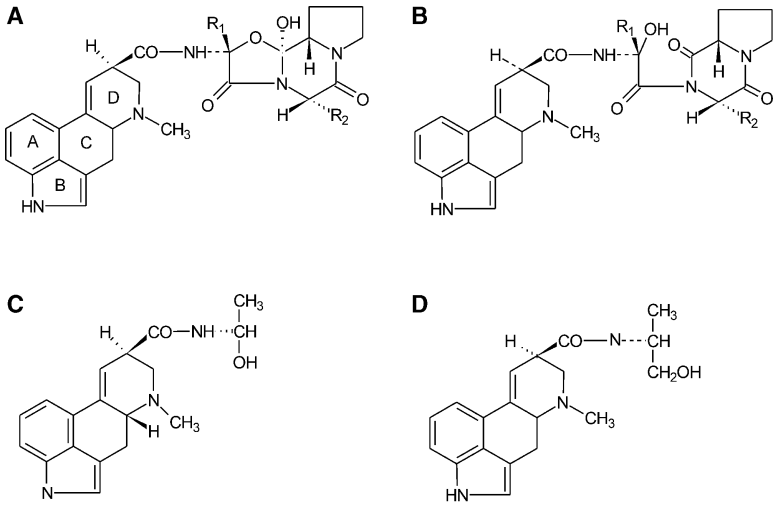


Figure 1. Natural D-Lysergic Acid Derivatives

(A) General structure of ergopeptines [R_1 = $-CH_3$, R_2 = $-CH_2C_6H_5$, ergotamine; R_1 = $-CH(CH_3)_2$, R_2 = $-CH_2CH(CH_3)_2$, ergocryptine; R_1 = $-CH_3$, R_2 = $-CH_2CH(CH_3)_2$ ergosine]. (B) Structure of D-lysergyl-tripeptide lactams, the immediate precursors of ergopeptines. (C and D) (C), D-lysergyl-α-hydroxyethylamide; (D), ergometrine (syn. ergobasine).

cluster with a novel NRPS gene *cpps2*, which by genetic and biochemical characterization was shown to encode LPS2.

Results

Sequence Analysis of LPS1, Prediction of the Structure of LPS2, and Cloning of *cpps 2*

Detailed analysis of the amino acid sequence deduced from the gene sequence of *cpps1* had shown that LPS1, as expected from previous biochemical work, contains three A and three PCP domains (also called T domains) (Figure 3A). However, the sequence also revealed that LPS1 has only two C domains, i.e., in module 2 and module 3. No such C domain was seen aminoterminally to the first A domain in module 1. This suggested that the C domain for formation of the peptide bond between D-lysergic acid and the first amino acid of the cyclol peptide chain was located either on LPS2 or on another as yet unknown protein component of the LPS multienzyme complex. Another interesting feature of the deduced amino acid sequence of *cpps1* is that LPS1 has a carboxy-terminal domain with no similarity to the typical release domain of NRPS, such as the thioesterase (Te) domain [18, 19]. Instead, this domain has some similarity to C domains as well as to heterocyclization domains (Cy domains) of NRPS [16]. However, it is ~70 amino acids shorter than the typical C and Cy domains and shows significant differences in the signature sequences (C1 to C6 and Cy1 to Cy6, respectively [16]) of C and Cy domains. In particular, the C3 motif of the LPS1 carboxy-terminal domain, Q-R-A-Q-D-G-V-S, differs from both the C3 consensus H-H-x-I-S-D-G-W and Cy3 consensus D-x-x-x-x-D-x-x-S of the C and Cy domains, respectively (Figure 3B). The double H of the C3 motif as well as the first D of the Cy3 motif are missing, which suggests a different mechanism of this domain. Therefore, this domain cannot be regarded as a regular NRPS C domain. In view of the release of the D-lysergyl tripeptide chain from LPS1 as an acyl-diketopiperazine, it is most likely involved in product release catalyzing the lactam formation step from D-lysergyl tripeptidyl-PCP thioester. We designated this domain as the cyclization domain (Cyc domain)

In order to identify the gene of LPS2, which from the biochemical data should be composed at least of an A and a PCP domain, a chromosome walking was performed downstream of the gene *cpox2*. During this, several ORFs could be detected [4]; sequence analyses led to the identification of a putative P450 mono-oxygenase gene (*cpP450-1*), a putative catalase gene (*cpcat2*), a putative oxidase gene (*cpox3*), and, interestingly enough, a gene showing significant homology to fungal NRPS, which was named *cpps2* (Figure 3A). This gene (GenBank accession number AJ439610) has a coding sequence of 3924 bp interrupted by an intron of 65 bp, leading to a putative protein of 1308 amino acids with a calculated Mr of 140 kDa.

Since *cpps2*, according to the size of the encoded protein and its vicinity to other ergot alkaloid biosynthesis genes in *C. purpurea* strain P1, was a possible candidate gene for LPS2, its deduced amino acid sequence was analyzed in respect to the overall structure and domain composition. Alignments of Cpps2 with various NRPS sequences in the database revealed that it is composed of four domains (Figure 3A). An A domain in the protein sequence commences at a distance of 280 amino acids from the amino-terminal end. The A domain is followed by a typical PCP domain (T) of about 100 amino acid residues, whereas in the carboxy-terminal portion of Cpps2 a domain of 500 amino acids is present, showing high similarity to the regular C domains of peptide synthetases. Interestingly, the amino-terminal domain of Cpps2 with about 280 amino acids has no similarity to known proteins except for a stretch of ~100 amino acids between the amino acid positions 40 to about 140 with weak homology to the carboxy-terminal half of C domains. Based on these findings, *cpps2* was considered the gene of LPS2, particularly because of the presence of the carboxy-terminal C domain that exactly matches what would be expected from the biochemical investigations of the LPS system and the structure and size of LPS1.

Expression Analysis of *cpps2* in *C. purpurea*

In order to show the significance of Cpps2 for D-lysergyl peptide synthesis in *C. purpurea*, Northern blot analyses of total RNA using the *cpps2* gene were performed. It

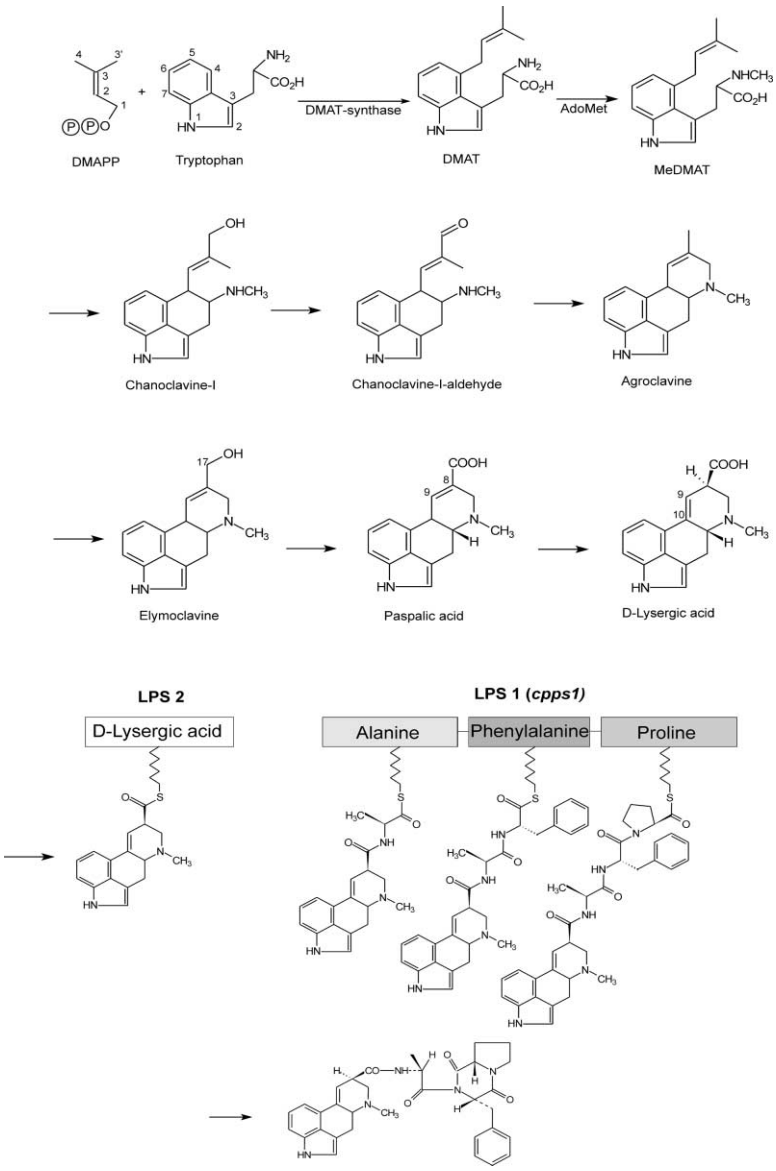


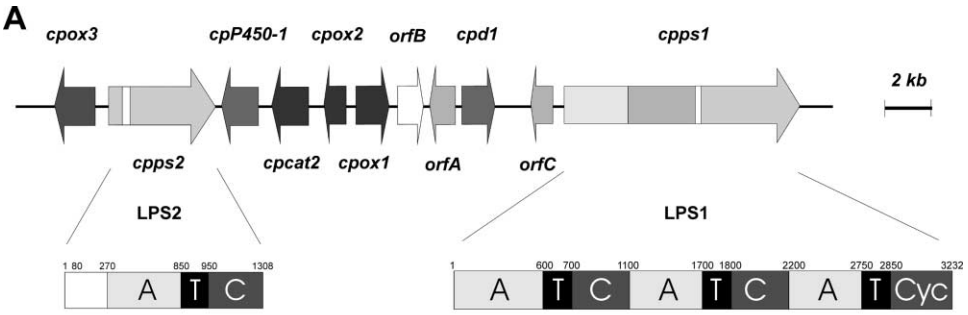
Figure 2. The Ergoline Ring System Pathway

Steps leading from dimethylallylpyrophosphate (DMAPP) and tryptophan to D-lysergic acid [5] and assembly of D-lysergyltripeptide in the LPS system [15].

is known that ergot alkaloid synthesis is regulated by the presence of phosphate and that repression by high phosphate concentrations can be relieved by tryptophan, an inducer of ergot alkaloid synthesis [20, 21]. Indeed, we could show recently that several genes of the ergot alkaloid cluster (Figure 3A), including the gene encoding DMATS (*cpd1*) and the oxidase genes (*cpox1*, *cpP450-1*), are coregulated in terms of their dependence of expression on the phosphate concentrations in the medium, which confirms their potential role in alkaloid biosynthesis (Y. Lübbe, T.C., and P.T., unpublished data). Expression analysis of the *cpps2* gene therefore was performed with mycelia grown under low- and high-phosphate conditions (see Experimental Procedures). The data presented in Figure 4 clearly show a transcript of *cpps2* of the expected size which is upregulated under low phosphate condition, comparable to the behavior of *cpd1* and *cpps1* as controls. This regulation pattern correlated with alkaloid production of the cultures (data not shown).

Functional Analysis of *cpps2*

To show the direct involvement of *cpps2* in ergopeptine assembly, gene inactivation was performed by a gene replacement approach: *cpps2* was interrupted by a phleomycin-resistance cassette as shown in Figure 5. From the 5′ and 3′ genomic region of *cpps2*, two fragments were cloned upstream and downstream, respectively, of the phleomycin-resistance cassette of vector pAN8-1UM. The ApaI/BamHI fragment carrying the whole replacement construct was used to transform *C. purpurea* strain P1. Phleomycin-resistant transformants obtained were individually checked for the presence of the replacement fragment in the genomes (generated by double crossover events) by PCR as described in Experimental Procedures (Figure 5A). From a total of 98 primary transformants, 26 showed the correct "diagnostic" PCR fragments of 1692 bp (generated with primers p2HUX and P2HLX) and 1829 bp (primers P2HUCP/P2HLCP). This is a rather high frequency (27%) when compared to the previous values of 1%–2% observed in



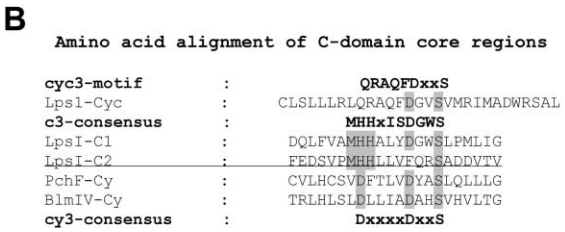


Figure 3. Map of the Ergot Alkaloid Biosynthesis Gene Cluster in *C. purpurea* Strain P1 and Section of an Amino Acid Alignment of Cyc, C, and Cy Domain Regions of NRPS

(A) Location of *cpps*1 and *cpps*2 in the ergot alkaloid biosynthesis gene cluster and the deduced structures of LPS 1 and LPS 2. *Cpcat2*, *cpP450*, *cpox3*, and *cpps2* have been sequenced during this study, whereas the other genes were sequenced previously [17]. The narrow white boxes in *cpps*1 and 2 denote the introns in these genes.

(B) Conserved motif HHxxxDxxS from regular C domains of LPS1, the corresponding Cyc domain motif QRAQFDxxS from the carboxy-terminal domain of LPS1, and the corresponding Cy motif DxxxxDxxS from heterocyclization domains of NRPS. Sequences are from the two internal C domains of LPS1 (GenBank accession number CAB39315), the Cy domains of pyochelin synthetase (PchF1) (GenBank AAD55801), of bleomycin synthetase (BlmIV) (GenBank AAG02364), and from the Cyc domain of LPS1 (GenBank CAB39315).

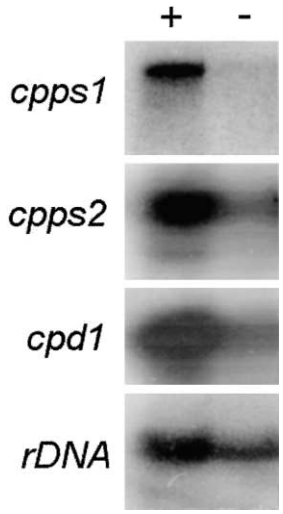


Figure 4. Northern Analysis of *cpps2* in High- and Low-Phosphate Conditions and Coregulation of Other Genes of the Ergot Alkaloid Cluster

Wild-type mycelia were grown under low- and high-phosphate conditions (see Experimental Procedures), RNA was extracted and, after separation in a agarose gel, was blotted on a nylon membrane. Probes used for hybridization were as follows: 4.0 kb HindIII fragment (*cpps1*, 17), a 0.8 kb SalI fragment (*cpd1*, 17), and a 3.5 kb SalI fragment (*cpps2*, Figure 3). A plus sign denotes RNA under low-phosphate conditions, and a minus sign indicates high-phosphate conditions

knockout experiments using the pathogenic field isolate *Claviceps purpurea* 20.1 [17, 22, 23]. The identity of the PCR fragments was confirmed by sequence analysis (data not shown). It is known that *C. purpurea* P1 and its parent strain ATCC 20102 are polykaryotic, harboring more than 10 nuclei per cell compartment [24]. From this, it is clear that single integration events always lead to heterokaryons. In fact, all of our positive primary transformants also showed the wild-type fragment. To obtain homokaryotic segregants, a positive transformant was propagated from which single-cell units had to be prepared by protoplast subculturing and hyphal tip isolation because of the inability of P1 to produce conidia (see Experimental Procedures). From a total of 600 subcultures thus generated, one homokaryotic mutant could finally be isolated. This transformant was lacking the wild-type PCR fragment containing the diagnostic band only (shown for the left flank of disruption construct in Figure 5B); it was named Δcpps2-1. Southern analyses confirmed the PCR data (Figure 5C) Final proof for the inactivation of *cpps2* in the Δcpps2-1 mutant came from Northern analysis which showed that no *cpps2* transcript could be detected in the mutant under conditions that stimulate expression of *cpps2* in strain P1 (Figure 5D).

Analysis of the Alkaloid Spectrum of Δcpps2-1

Since strain Δcpps2-1 represents a bona fide deletion mutant, it was analyzed for its ability to form ergopep-

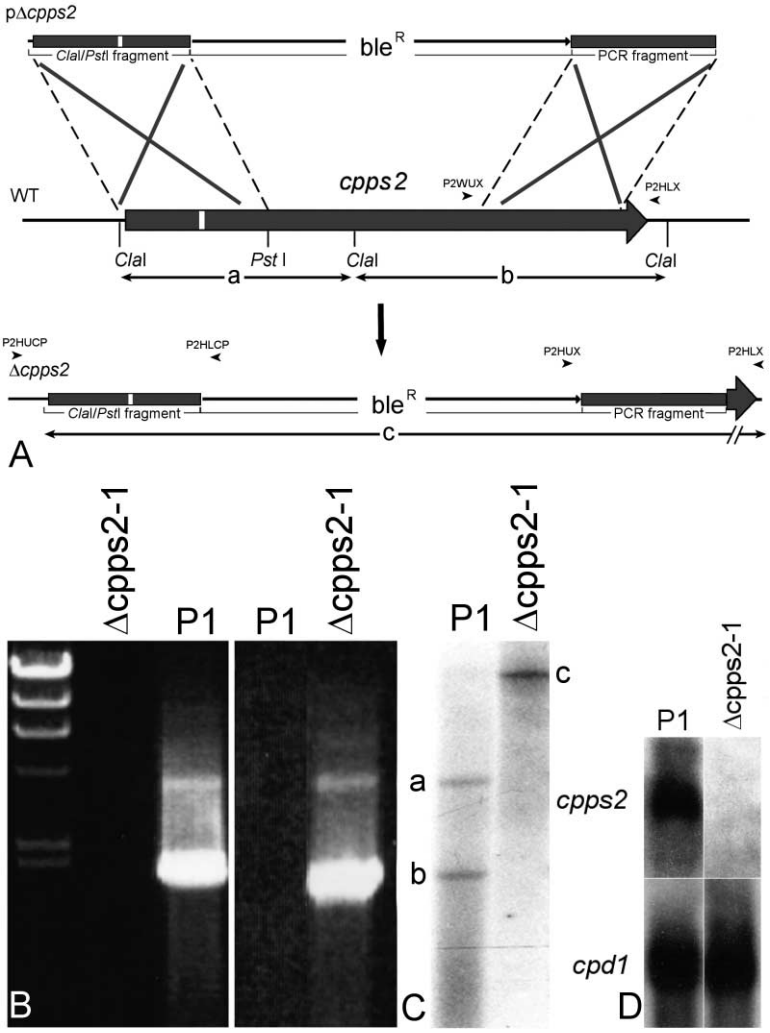


Figure 5. Functional Analysis of *cpps2*

(A) Gene replacement strategy: the design of the replacement fragment (upper part) and the genomic situation after a successful gene replacement by double crossover (lower part) are presented. Primer binding sites are indicated by arrow heads (for details see Experimental Procedures). The narrow white box indicates the intron in *cpps2*.

(B) PCR analysis of a Δcpps2 mutant and the wild-type strain (P1) using diagnostic primers for the left flank (HI) and wild-type primers (WT). Left panel, wild-type primers; right panel, with "left flank" primers. For details, see Experimental Procedures.

(C) Southern analysis of a replacement transformant: genomic DNA of the wild strain and mutant Δcpps2-1 was digested with ClaI, transferred to a nylon membrane, and hybridized to a 3.5 kb SalI fragment of the *cpps2* region (see Figure 3). The labels a, b, and c denote the hybridizing ClaI fragments expected to be present in Δcpps2-1 and wild-type P1, respectively.

(D) Northern analysis: total RNA from wild-type and mutant Δcpps2-1 was extracted from mycelia grown under alkaloid production conditions, blotted on a nylon membrane, and hybridized to the *cpps2* probe (see C).

tines and other alkaloids. Cultivation of the mutant, along with an ectopic integration mutant and the recipient strain P1 as controls, was in standard alkaloid production medium with limiting phosphate concentrations. Analysis of the individual components of the alkaloid mixtures was performed by TLC, HPLC, or electrospray mass spectrometry. Figure 6 shows that strain P1 produces ergotamine, ergocryptine, and ergosine as main alkaloids. Moreover, from the clavines, elymoclavine was detected in significant amounts (data not shown). By contrast, the Δcpps2-1 mutant did not produce any ergopeptine on solid or in liquid medium but accumulated D-lysergic acid, which is absent from strain P1, indicating accumulation of that compound due to blockage of ergopeptine synthesis. Radioisotope labeling of ergopeptines using radioactive amino acids in short-term incubations of washed mycelium and TLC separations of the radioactive alkaloid mixtures in P1 or the Δcpps2-1 mutant confirmed these results (data not shown). The fact that strain Δcpps2-1 accumulated D-lysergic acid instead of forming ergopeptines clearly indicated that ergoline ring synthesis was unaffected by gene disruption in *cpps2* (Figure 6).

Enzymatic Analysis of Δcpps2-1

Protein fractions of the Δcpps2-1 mutant along with protein fractions from the parent strain P1 were tested for the presence of LPS1 and LPS2 by measuring the ability to form enzyme thioester with ^{14}C-valine or ^{14}C-phenylalanine and ^{3}H-dihydrolysergic acid, respectively. To this end, protein from extracts of broken cells of the two strains was fractionated by ammonium sulfate precipitation and subjected to gel filtration on Ultrogel AcA34. Testing fractions from the gel filtrations from both strain P1 and the mutant gave a peak of valine or phenylalanine thioester formation shortly behind the void volume of the column, which represented LPS1 activity (Figure 7). The peak of activity of dihydrolysergyl enzyme thioester of LPS2 in strain P1 could be detected separately from that of LPS1 in the fractionation range of about Mr 150,000. In SDS-gel electrophoretic separations of the fraction containing dihydrolysergic thioester-forming activity, a ~140 kDa band could be visualized by autofluorography after incubation with radioactive dihydrolysergic acid and ATP (Figure 7) . By contrast, no dihydrolysergic acid thioester formation was detected in protein extracts of the Δcpps2-1 mutant, which con-

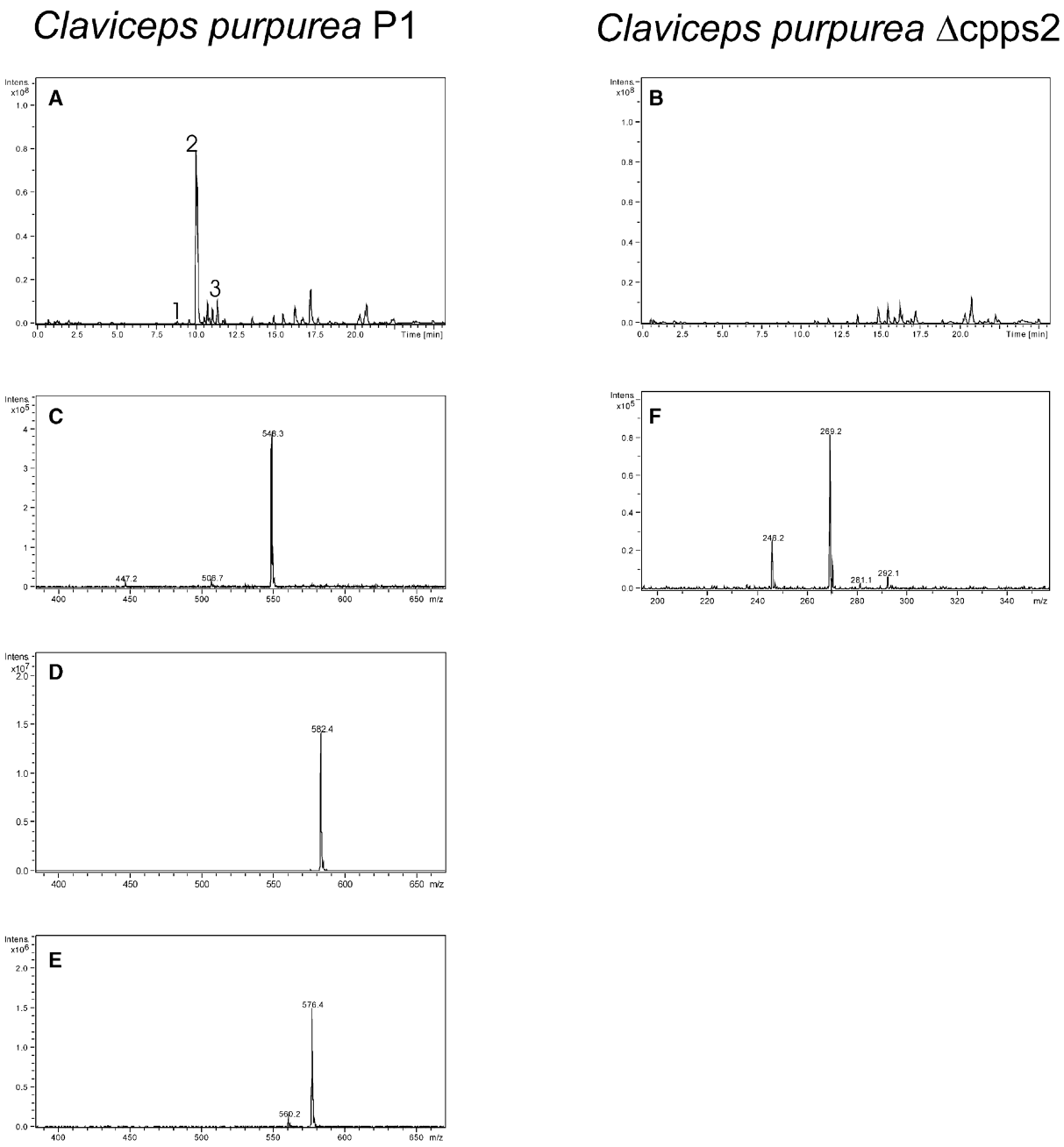


Figure 6. Mass Spectrometric Analysis of Alkaloid Extracts from Strain *C. purpurea* Strain P1 and the Δcpps2 Mutant

Extracts from each strain grown on solid or liquid media were subjected to electrospray mass spectrometry as described in Experimental Procedures.

(A and B) HPLC separations of extracts from strain P1 and the Δcpps2 mutant, respectively.

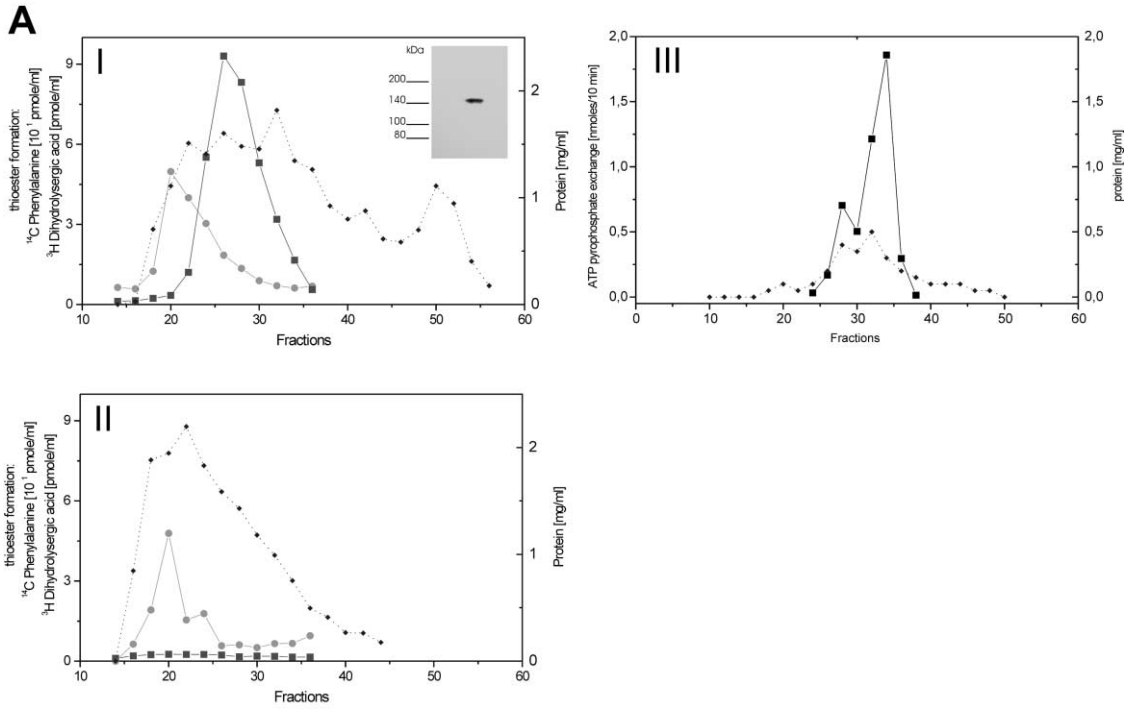
(C–E) Mass peaks of the indicated peaks from (A). (C), ergosin m/z = 548 [M + H]; (D), ergotamin m/z = 582 [M + H]; (E), ergocryptin m/z = 576 [M +H]).

(F) Mass peaks of compounds accumulated in (B) (D-lysergic acid m/z = 269 [M + H]).

tained solely LPS1 at a level comparable to strain P1 (Figure 7). This represents good evidence that *cpps 2* is encoding LPS2.

Heterologous Expression of *cpps2* in *E. coli*

To characterize the gene product of *cpps2* further, *cpps2* was engineered for expression as a hexaHis fusion protein in *E. coli*. After verification of the intron of *cpps2* by RT-PCR, which revealed the correctness of the intron-exon junctions inferred from analysis of the genomic DNA sequence (data not shown), a cDNA was constructed via PCR amplification of fragments of the exon-encoding regions of *cpps2*, allowing the two exons to join via an EcoRV restriction site (see Experimental



B

Amino acid position in A-domain (PheA numbering)

	235	236	239	278	299	301	322	330	substra
LPS 2	**D**	**V**	**F**	**S**	**V**	**G**	**L**	**V**	D-LSA*
CepB(I)	**D**	**A**	**V**	**H**	**L**	**G**	**L**	**L**	HPG
CdaI(VI)	**D**	**V**	**Y**	**H**	**L**	**G**	**L**	**L**	HPG
SafAI(I)	**D**	**I**	**L**	**Q**	**L**	**G**	**L**	**V**	3h4mPh
CdaI(III)	**D**	**A**	**W**	**S**	**V**	**G**	**S**	**V**	Trp
SnbA	**N**	**F**	**C**	**S**	**Q**	**G**	**V**	**L**	HPic
YbtE	**N**	**F**	**C**	**A**	**Q**	**G**	**V**	**L**	Sal.
ACMSI	**N**	**M**	**T**	**Y**	**V**	**G**	**V**	**L**	4-MHA
EntE	**N**	**Y**	**S**	**A**	**Q**	**G**	**V**	**V**	DHB

Figure 7. Comparative Functional Analysis of LPS2 in Protein Fractions from *C. purpurea* Strains P1 and Δcpps2-1 and from *E. coli*

(A) Analysis of protein extracts of *C. purpurea* strains P1, Δcpps2 mutant, and *E. coli* carrying plasmid cpps2_pNG5 (*cpps2*) for the presence of LPS2 activity. I and II show gel filtration on Ultrogel AcA 34 of an ammonium sulfate-fractionated protein extract from *C. purpurea* strain P1 (I) producing ergopeptines and *C. purpurea* Δcpps2 (II). Each fraction was assayed for the presence of LPS1 and LPS2 by the thioester formation assay with ^{14}C-phenylalanine (or ^{14}C-valine) (circles) and ^{3}H-dihydrolysergic acid (squares), respectively. Protein is denoted by diamonds. The inset in I shows an autofluorograph of an SDS polyacrylamide gelelectrophoretic separation of the peak fraction containing LPS2 activity after incubation enzyme with ^{3}H-dihydrolysergic acid and ATP. No LPS2 activity is present in *C. purpurea* Δcpps2. III shows gel filtration on Superdex 200 of recombinant LPS2-32 partially purified from *E. coli* after expression from plasmid (cpps2_pNG5). Fractions were tested for the ATP-pyrophosphate exchange dependent on D-lysergic acid (squares); protein is denoted by diamonds.

(B) Aligment of substrate specificity determining amino acid residues in binding pockets of LPS2 and several NRPS adenylation domains with known substrates. Shown are the extracted residues from the LPS2 A domain and the A domains of NRPS A domains and Aryl-AMP-ligases (arylcarboxylate activating enzymes). D-LSA, D-lysergic acid. Numbers refer to the corresponding residues in the PheA domain of gramicidin synthetase 1 [34, 35]. CepB, chloroeremomycin synthetase (GenBank accession number CAA11795); CdaI, CDA peptide synthetase I (GenBank CAB38518); SafAI, saframycin Mx1 synthease A (GenBank AAC44129); SnbA, pristinamycin synthetase I (GenBank CAA67140); YbtE, yersiniabactin synthetase (GenBank AAC69591); ACMS I, actinomycin synthetase I (GenBank AAD30111); EntE, enterobactin synthetase (GenBank AAN79156); HPG, dihydroxyphenylglycine; 3h4mPhe, 3-hydroxy-4-methyl-phenylalanine; HPic, 3-hydroxypicolinic acid; 4-MHA, 4-methyl-3-hydroxyanthranilic acid; DHB, 2,3-dihydroxybenzoic acid, Sal, salicylic acid.

Procedures). Since the intron is located in the region of the gene encoding the amino-terminal domain, the resultant change of the amino acid sequence (LF→DI) was not expected to disturb the activity of the A domain.

Two constructs were prepared on the basis of expression vectors pQE 32 (LPS2-32) and pQE 70 (LPS2-70) with a hexaHis-encoding sequence at the 5′ and the 3′ ends, respectively. Expression was in *E. coli* M15.

Attempts to purify the soluble fraction of either enzyme on Ni-NTA matrix failed due to lack of binding to the affinity matrix. Using the D-lysergic acid-dependent ATP-pyrophosphate exchange as assay, LPS2-32 was partially purified by a classical purification protocol [25] involving ammonium sulfate precipitation and several steps of gel filtration and anion exchange chromatography (Figure 7). In each separation step, there was a peak of D-lysergic acid activation which was absent from the nontransformed strain (data not shown). Like the native LPS2 enzyme, LPS2-32 did not activate any of the amino acids present in the peptide chain of D-lysergyl peptide lactam or tryptophan (which, like D-lysergic acid, contains an indole ring as characteristic structural element). Attempts to demonstrate dihydrolysergic acid enzyme-thioester formation with LPS2-32 failed. Most probably, LPS2-32 does not contain a 4′-phosphopantetheine cofactor necessary for covalent binding of the substrate as thioester. Incubations of the protein with two different 4′-phosphopantetheine transferases such as Sfp [26] or OrfC [27] in the presence or absence of coenzyme A with ^{3}H-dihydrolysergic acid did not show any increase in bound radioactivity.

Discussion

The results presented here show that the ergot alkaloid gene cluster from *Claviceps purpurea* harbors two NRPS genes, *cpps1* and *cpps2. Cpps1* had been identified earlier as the gene encoding the nonribosomal peptide synthetase LPS1 (370 kDa), which is responsible for the assembly of the tripeptide portion of the D-lysergyl tripeptides in *C. purpurea*. Detailed analysis of the deduced amino acid sequence of LPS1 showed that it has a domain arrangement (A-PCP-C-A-PCP-C-A-Cyc) with three A and three PCP domains, two C-domains, and a terminal domain called the Cyc domain. The Cyc domain has limited similarity to both C and Cy domains of NRPS but is most different in the so-called C3 and Cy3 motif of the latter domains (Figure 3B), suggesting a special mechanism in acyl diketopiperazine formation, which is the final step of D-lysergyl peptide lactam synthesis (Figure 1). Formation of peptide lactam formation requires abstraction of a proton from the amide nitrogen of the peptide bond between the first and second amino acid in the D-lysergyl peptide intermediate, nucleophilic attack of the resulting enimine electron pair onto the carbonyl C of the thioester group, and cyclization. Proton abstraction from a peptide nitrogen group appears to rely on a mechanistic basis different from that of abstraction from amino groups of amino acids catalyzed by regular C domains or by thioesterase domains [31].

The analysis of LPS1 also revealed that, unexpectedly, it was missing an amino-terminal C domain in the first module. This led us to conclude that this C domain is located on another protein such as LPS2, the d-lysergic acid module. In the course of this study, *cpps2* was then definitely shown by genetic and biochemical criteria to encode LPS2. This also proved that LPS1 and LPS2 are distinct proteins and not the degradation products of a larger NRPS multienzyme consisting of a single polypeptide chain. These findings demonstrate for the first time a fungal NRPS system consisting of several subunits. The systems known previously have always consisted of one polypeptide chain [28].

The analyses showed that LPS2 is a stand-alone initiation module analogous to bacterial NRPS systems such as the phenylalanine recruiting module for initiation of gramicidin S or tyrocidine in *Bacillus brevis* [16] or the aryl carboxylic acid recruiting composite EntE/EntB in the initiation of siderophore synthesis in *E. coli*, *Yersinia pestis*, or in *Vibrio cholerae* [29]. Such initiation modules or units consist at least of an A and a PCP domain, and they normally interact *in trans* with their cognate C domain located on the subunit harboring the next module of the biosynthetic sequence catalyzing the elongation step following activation of the starter residue. In bacterial NRPS systems, even such C domains can occur as autonomous proteins, e.g., the stand-alone VibH, which condenses enzyme thioester-activated aryl carboxylic acids with a free amine during the biosynthesis of the siderophore vibriobactin in *V. cholerae* [30]. However, the analysis of the deduced amino acid sequence of *cpps2* revealed that in contrast to the integral or, even more, to the composite bacterial initiation modules, LPS2, in addition to its A and PCP domain, does contain an integral "regular" C domain at its carboxyl terminus in the arrangement (A-PCP-C). From the deduced domain arrangement and calculated size (140 kDa, 1308 aa), the *cpps2* gene product meets all criteria required to fulfil the role of LPS2 [14]. In particular, the presence of the C domain at the carboxyl terminus of LPS2 readily points to a role attributed to the C domain originally believed to reside at the amino-terminal end of LPS1 necessary for condensation of D-lysergic acid with the amino acid activated by the first module of LPS1. The domain arrangement (A-PCP-C) of the *cpps2* gene product is nonetheless unusual in NRPS systems, because C domains are usually *cis*-acting domains in respect to their downstream A and PCP domains [19]. This may be a consequence of the observed substrate specificity of C domains, which has been reported to be high for the substrate bound to the downstream PCP-bound substrate and low for the substrate from the upstream module [32]. Accordingly, elongation subunits in NRPS very often commence at their amino-terminal ends with a C domain, which reflects the observed functional intimacy between C domains and their downstream A domains. In the LPS system, the D-lysergic acid amidating C domain would therefore act *in trans* to its dowstream A domain. The significance of the carboxy-terminal *trans*-acting C domain of LPS2 is not clear. Whether it is a mere exception to the rule or may have significance in the light of the very high substrate specificity of LPS2 for D-lysergic acid [14] remains to be seen in the future, when swapping of that C domain between LPS2 and LPS1 will become possible. The only known example of a stand-alone module having a carboxy-terminal C domain is the monomodular VibF of vibriobactin synthesis in *Vibrio cholerae* [33]. Like Cpps2, VibF (domain arrangement Cy1-Cy2-A-C1-PCP-C2) has the carboxy-terminal C domain (termed C2), which is the only catalytically active one (in contrast to C1) in acylating a free small diamine, dihydroxybenzoyl-norspermidine, at both its primary and secondary amino groups with a small peptide gener-

ated by the Cy1-Cy2-A-(C1)-PCP domain assembly upstream of C2. However, the difference to LPS2 lies in the fact that VibF acts *in trans* acylating a free substrate, whereas LPS2 acts *in trans* acylating the amino acid covalently tethered to the first module of LPS1, which may require specific protein-protein interactions. Comparison of the C domain sequence of Cpps2 (LPS2) with the internal C domain sequences of LPS1 gave no hint for characteristic peptide sequences possibly involved in protein-protein interactions (data not shown).

Direct and unequivocal evidence for the identity of Cpps2 and LPS2 was obtained by gene replacement of *cpps2* in the *C. purpurea* genome by a phleomycin-resistance cassette that created phenotype accumulating D-lysergic acid instead of ergotamine and other ergopeptines. This clearly indicated a blockade in the assembly of D-lysergyl tripeptide lactam. Comparative enzymatic studies of protein extracts derived from the Δcpps2 mutant and its parent strain P1 showed that the ergopeptine-producing strain P contained both LPS1 and LPS2, whereas extracts from the Δcpps2 mutant were devoid of any LPS2-related activity (Figure 7). Moreover, Northern analysis of RNA obtained from cultures grown in low and high concentrations of phosphate indicated the same regulation pattern for *cpps2* as for *cpps1* and other genes located in the gene cluster that show growth-linked repression by phosphate (e.g., the DMAT synthase gene *cpd1*) concomitantly with lowered alkaloid production in cultures of *Claviceps* sp. [20] (Y. Lübbe, T.C., and P.T., unpublished data). These findings indicate a clear involvement of the gene product of *cpps2* in D-lysergyl peptide assembly and also coregulation with expression of *cpps1* by phosphate. This is particularly important in view of the fact that the known fungal NRPS consist of single polypeptide chains, which warrants equimolarity of the various modules in these multienzymes. In order to warrant such conditions of balanced levels of the different modules for the LPS system, coordinate transcription of *cpps1* and *cpps2* is obviously necessary.

Final proof for the identity of Cpps2 with LPS came from expression experiments with *cpps2* cDNA in *E. coli* which yielded a protein of the expected size of 140 kDa that specifically catalyzed the D-lysergic acid- (and dihydrolysergic acid-)dependent ATP-pyrophosphate exchange (Figure 7). Like wild-type enzyme, the enzyme did not activate any of the amino acids of ergopeptines or tryptophan. In contrast to its ability to catalyze the ATP-pyrophosphate exchange dependent on D-lysergic acid, the LPS2 from *E. coli* did not catalyze the enzyme-thioester formation with dihydrolysergic acid, as did the wild-type enzyme. We argue that this is probably due to the lack of 4′-phosphopantetheine cofactor. Possibly, the LPS2-apoenzyme may be a specific substrate for a Ppant transferase from the ergot fungus *C. purpurea* that is not yet known.

The inspection of the specificity-determining region of the A domain (amino acid binding pocket) of LPS2 by comparison with the amino acid pocket of the PheA domain of tyrocidine synthetase I according to previously published procedures [34, 35] indicated uniqueness of the D-lysergic acid binding pocket (Figure 7B). It showed similarity to some A domains activating hydrophobic amino acids (best 50% score with the p-hydroxyphenylglycine-activating module of chloroeremomycin synthetase [36]). Furthermore, no convincing similarity of the LPS2 A pocket residues was seen with the corresponding residues characterizing the A domain pockets of aryl-AMP ligases [37]. Strikingly, there was also no evident similarity with the specificity-determining residues of tryptophan-activating A domains of various NRPS, suggesting that the indole portion may not play a role in the recognition of D-lysergic acid by LPS2 (Figure 7B). Since the best matches were observed with the pocket residues of A domains catalyzing hydrophobic amino acids, it may be suggested that the LPS2 A domain substrate binding pocket resembles typical A domains binding amino acids (e.g., the ones of LPS1) rather than the binding pockets of A domains activating aryl carboxylic acids. Possibly, therefore, ring C and D of D-lysergic acid, which have no aromatic character, would contribute to recognition by the A domain (Figure 1). The indole ring system of D-lysergic (ring A and B) as recognition element would play a minor role, being too distant from the carboxyl and the methylamino group in ring D. Unfortunately, no substrate analogs of D-lysergic acid were available to test this hypothesis, and one must therefore await structure data of the LPS2 A domain in the future that will give insight into the molecular details of D-lysergic acid recognition and binding.

The fact that LPS1 and 2 are distinct enzymes raises the question of whether this exception to the hitherto described fungal NRPS systems is of vital importance for *Claviceps purpurea* and ergot fungi in general. It is known that D-lysergic acid is constituent not only of ergopeptines but also of the simpler D-lysergic acid peptides and amides such as ergosecaline or ergometrine and D-lysergic acid α-hydroxyethylamide, respectively [4]. A number of *C. purpurea* strains can produce both ergopeptines and ergometrine simultaneously [6], which indicates the presence in one strain of two different biosynthesis systems, both using D-lysergic acid as substrate. Ergometrine is derived from D-lysergyl alanine [38], for which a D-lysergic acid module like LPS2 and an alanine module with an additional releasing domain would be required. In strains producing both ergopeptines and simple D-lysergic acid amides, LPS2 could play a central role by interacting either with LPS1-type enzymes or alanine modules in some kind of natural combinatorial biosynthesis. This could at least partly explain why the D-lysergic acid module stands alone and is not part of a multimodular NRPS, because its standing alone would easily permit its integration and participation in different enzyme systems. Moreover, the presence or absence of such genes of LPS2- and LPS1-like enzymes may facilitate the understanding of the basis of diversity in product formation in various species of ergot fungi.

Significance

The results presented here demonstrate unequivocally that alkaloid peptides are assembled by an NRPS system consisting of two distinct multienzymes (LPS1 and LPS2) harboring three and one modules, respectively.

This is in contrast to known fungal NRPS systems which harbor all their modules on one polypeptide chain. Cloning of the ergot alkaloid gene cluster of *C. purpurea* revealed the presence of the corresponding genes, *cpps1* and *cpps2*, in an alkaloid biosynthesis gene cluster. Analysis of *cpps2* along with *cpps1* by transcription analysis and targeted inactivation in *C. purpurea* revealed coordinate expression with other genes in the cluster upon induction of alkaloid synthesis in cultures. Enzymatic analysis of a Δcpps2 mutant and of the heterologously expressed protein gave clear evidence that *cpps2* encodes LPS2, the D-lysergic acid-activating module which, together with LPS1, assembles the D-lysergyl tripeptide precursors of ergopeptines. The existence of LPS2 as a monomodular enzyme points to its role in other D-lysergyl peptide assemblies in ergot fungi such as the D-lysergyl-alkylamides which may determine the spectrum of products formed. Thus, the observed accumulation of D-lysergic acid in Δcpps2 mutants points to the possibility that the ergot alkaloid gene cluster in different ergot fungi may differ from each other by the presence or absence of genes involved in steps of ergoline ring synthesis or peptide assemblies. The availability of the independent D-lysergic acid module LPS2 and its gene *cpps2* opens new and interesting perspectives for the understanding of the biosynthesis of the wide spectrum of naturally occurring D-lysergic acid amides and for design of new pharmacological lysergic acid derivatives by combinatorial biosynthesis.

Experimental Procedures

Strains and Cultures

C. purpurea strain P1 (1029/N5) was described previously [21]. It produces mainly ergotamine, with minor amounts of ergocryptine and ergosine. Maintenance and culture conditions for strain P1 and the replacement mutant Δcpps2-1 (see Results) were as described [14, 17].

Chemicals and Radiochemicals

[9,10-^{3}H]-9,10-dihydroergocryptine (17.5 Ci/mmol) was from Hartmann Analytics, Braunschweig (Germany). L-[U-^{14}C]-phenylalanine (443 mCi/mmol), L-[U-^{14}C]-alanine (148 mCi/mmol), and L-[U-^{14}C]-valine (260 mCi/mmol) were from Amersham International. [9,10-^{3}H]-9,10-dihydrolysergic acid was prepared from [9,10-^{3}H]-9,10-dihydroergocryptine [14]. All other materials were of the highest purity commercially available.

Methods of Analyses

Radioactive determinations were as described [14]. Protein concentrations were determined according to [39]. SDS-polyacrylamide gel electrophoresis was performed according to [40]. Autofluorography of radiolabeled samples in SDS-poylacrylamide gels was as described [14]. Immunoblot analyses were performed by standard procedures. Enzyme assays were based on the ATP-pyrophophate reaction dependent on D-lysergic acid and amino acids or on enzyme-thioester formation from dihydrolysergic acid or the relevant amino acid substrates of LPS1 [14]. TLC for separation of alkaloid mixtures was performed on silica gel plates using ethyl acetate:methanol:water (100:5:5, by volume) or ethyl acetate:dimethylformamide:ethanol (75:15:5, by volume) [14].

Mass Spectrometry

Mass spectrometric detection was carried out using an Esquire3000+ ion trap instrument (Bruker Daltonik GmbH, Germany) equipped with a API-ESI interface. Ions were scanned with a scan speed of 13,000 Da/s. Using ESI in positive ion mode, mass spectra were acquired from m/z = 100 to 700. For spectra acquisition, a total of 10 scans were summarized. The HPLC system consisted of a HP series 1100 solvent delivery system (Waldbronn, Germany). HPLC separation was carried out on a C18 column (100 × 2 mm, phenomenex LUNA 3μ) at a flow rate of 300 μl/min, operated at 30°C. Compounds were eluted using a gradient (A = H_2O, 0.05% formic acid, B = acetonitril, 10 to 100% B in 30 min).

Nucleic Acid Extraction and Analysis

Standard recombination DNA methods were performed according to [41]. The plasmids used for subcloning fragments were pUC19, pTZ18 U (Amersham Pharmacia Biotech), or pSP72 (Promega). Expression plasmids were pQE32 and pQE70 (Qiagen). *Echerichia coli* strains used for cloning were TOP10F′ (Invitrogen) and, for expression, M15 (Qiagen). All of these strains were grown according to standard protocols. Extraction of genomic DNA and of RNA, Southern and Northern analyses, and DNA sequencing were performed as described [23]. Sequence comparisons, multiple sequence alignments, and identity scores were computed with Megalign (DNASTAR), BESTFIT (HUSAR), or CLUSTAL X [42]. For PCR analyses, REDTaq (Sigma-Aldrich), Bio Therm (Genecraft), and Vent DNA polymerase (BioLabs) were used according to the manufacturer's instructions.

Cloning of *cpps2* and Generation of a Replacement Vector

A genomic λEMBL3 library of *C. purpurea* strain P1 (1029/N5 [17]) was probed with a 1.8 kb fragment of the religation plasmid p25S7ReHindIII containing the 3′ end of the *cpps2* gene using the plaque filter hybridization technique [41]). Of 69,000 screened plaques, 4 hybridizing λ clones were obtained and purified. One of them, λ63, was digested with several restriction enzymes (BamHI, EcoRI, HindIII, SalI, and XbaI), and the resulting fragments were further cloned into the pUC19 cloning vector. The overlapping subclones were sequenced to obtain the complete coding sequence of *cpps2*. For construction of the *cpps2* replacement vector, a 1.35 kb ClaI/PstI fragment of plasmid p63SmaI6,4 (containing the whole coding region of *cpps2*) was subcloned into the pAN81 UM [43] to yield pΔcpps2α. Digestion with ApaI and NotI gained a 4.6 kb fragment including the 5′ region of cpps2 and the phleomycin-resistance cassette (phle). The 3′ region of cpps2 located between 2589 bp to 3794 bp (respective to the start codon) was amplified by PCR using primers PS2U and PS2L and cloned into the PCR2.1 TOPO vector to yield pΔcpps2β. This plasmid construction was linearized with ApaI and NotI and ligated with the ApaI/NotI fragment of pΔcpps2α, resulting in the circular replacement vector pΔcpps2. The replacement fragment was excised with ApaI/BamHI and used to transform *C. purpurea* (see Figure 3).

Transformation of *C. purpurea* and Molecular Characterization of Transformants

Transformation of *C. purpurea* strain P1 was performed as follows: a mycelial supension of strain P1 was spread on the surface of cellophane sheets on BII agar [44] and grown for 2–3 days at 28°C. The cellophane sheets were placed on petri dishes containing 15 ml of lytic solution corresponding to SMac buffer (0.2 M potassium maleate [pH 5.2]) containing 15 mg/ml β-D-Glucanase Enzyme (Interspex, USA), so that the mycelia faced downwards. The mycelia could then be easily detached and were incubated for about 2 hr at 80 rpm and 28°C. The protoplasts were filtered through sterile filter funnels (POR1 and POR2). They were further pelleted by centrifugation 10 min at 4500 × g, washed twice with STC buffer (0.85 M sorbitol, 10 mM Tris, 50 mM $CaCl_2$), and brought to a final concentration of 10^8 protoplasts/ml STC. 10^7 protoplasts were transformed as described [22].

After 8 hr of regeneration at 28°C, protoplasts were overlaid with 10 ml BII/8 containing 100 μg/ml phleomycin (Cayla, final concentration 33 μg/ml). Positive transformants were screened by transferring them to 100 μg/ml bleomycin selection plates. Due to the inability of the production strain P1 to sporulate, the purification of the mutants was performed by protoplasting (see above) or hyphal tip dissection. Phleomycin-resistant transformants carrying a homologous integration of the replacement construct were identified by PCR using primers P2HUX (5′-TGAATGCTCCGTAACACCCAATA 3′), P2HLX (5′-AAAGGCCAAAGAAAATCACG-3′) (shown in Figure

5A) for the right flank, P2HUCP (5′- CAAGAGGGCGTGCTGAAT-3′), P2HLCP (5′ GCGGAACTGGCCCTTATCGT 3′) for the left flank, and 10–100 ng of genomic DNA. The predicted 1692 bp (right) and 1829 bp (left) fragments were amplified from DNA of strain Δcpps2-1. The lack of the wild-type gene copy in the Δ*cpps2* mutants was checked using the primers P2WUX (5′-ACGCCGCAATGCCACTTCAG-3′) and P2HLX (5′-AAAGGCCAAAGAAAATCACG-3′), which gave rise to a 1922 bp fragment with the wild-type strain. Southern analysis was performed in order to verify the homologous integration of the replacement vector. Genomic DNA from knockout mutants and wild-type was restricted with ClaI, seperated via gel electrophoresis (1%), and transferred to a nylon filter (HybondN$^+$, Amersham). The filter was probed with a 3.5 kb SalI fragment from the p63SalI3,5 plasmid.

Transcription of *cpps2* and Δ*cpps2* was tested by Northern analysis. Mycelia from strains P1 and Δcpps2-1 were incubated at 24°C under alkaloid biosynthesis conditions (T25N production medium, 0.5 g/l phosphate, 300 g/l sucrose) for 7 days. The RNA extraction was performed according to [24]. The RNA was electrophoretically separated and afterwards transferred to a nylon filter. The filter was hybridized with a 3.5 kb SalI fragment (p63SalI3,5; *cpps2*) and a 0.8 kb SalI fragment (p21SalI0,8; *cpd1*)

Engineering *cpps2* for Expression in *E. coli* as an Amino-Terminal HexaHis Fusion Protein (LPS2-32)

Exon 1 in the *cpps2* sequence (Genbank accession number AJ439610) between nt 545 and nt 1054 was engineered via PCR using genomic *cpps2* as template to carry a single SphI site encompassing the ATG-start codon and an EcoRV site encompassing the 3′ end of the exon. The resulting SphI-EcoRV gene fragment was ligated for subcloning into plasmid pTZ18 (Promega), resulting in plasmid cpps2_pNG1. Primers were 5′-TAGCAAGCATGCCAACCC CCGAG-3′ (forward) and 5′-CCGCAACGCTCTCAATGATATCAGT GAT-3′ (reverse). Exon 2 was engineered first via PCR by amplifying nt 1116 to nt 3015 with the introduction of an EcoRV site at the 5′ end (representing the junction with exon 1) and conservation of the natural EcoRV site located at nt 3012 of cpps2. The resultant 1.9 kb EcoRV PCR fragment was subcloned into plasmid pTZ18 for sequencing and later cloned into cpps2_pNG1, resulting in cpps2_pNG2. Primers were 5′-GTGATAGATATCGATCTTGTTACCC GTGTC-3′ (forward) and 5′-CTTCCTCGATATCATTACTTGGCG-3′ (reverse). Next, for completion of c*cpps2*, cpps2_pNG2 was linearized with PstI at nt position 1747. Into this site, a natural genomic 3.7 kb PstI fragment comprising the rest of *cpps2* ranging from nt 1747 to 4534 together with ~1 kb of its 3′ region was cloned. The resultant plasmid was named cpps2_pNG3.Trimming the 3′ region of *cpps2* in the plasmid cpps2_pNG3 was done by introducing a HindIII cleavage site 219 bp downstream of the stop codon. This was by synthesizing via PCR an ~300 bp fragment using cpps2_pNG3 as template with primers TTTGGGTAAGAGCATGCAAGAG-3′ (forward). and 5′-CCAAAACAAGCTTTGCAGATG-3′ (reverse). This Sph1-HindIII fragment had in its middle the stop codon of *cpps 2*. After subcloning and sequencing, the fragment was cloned into Sph1-HindIII-cleaved expression vector pQE32 (Quiagen), which resulted in plasmid cpps2_pNG4. Into the Sph1 site of cpps2_pNG4 was cloned the 3855 bp Sph1 fragment obtaind from Sph1 cleavage of cpps2_pNG3. This gave cpps2_pNG5 with the engineered *cpps2* gene carrying 5′ hexaHis encoding sequence under the control of the lacZ promoter stemming from the pQE32 vector. Engineering of *cpps2* for expression in *E. coli* as an carboxy-terminal hexaHis fusion protein (LPS2-70) involved introduction of a BglII site into the *cpps2* sequence of cpps2_pNG2 encompassing the last two codons of the gene, including the stop codon. This was done by PCR using the primers 5′-CGG TGC CTT GTA GAA TAA CAC TCG-3′ (forward) and 5′-GTC AAG ATC TAGAGT GAA GAG ATT TGA-3′ (reverse) with pNG5 as template. Several rounds of subcloning with various fragments derived from cpps2_pNG5 resulted in cpps2_pIO4 carrying the engineered *cpps2* gene with a 3′-hexaHis-encoding sequence in the original stop codon of Cpps 2 under the control of the lacZ promoter of pQE70.

Heterologous Expression of *cpps2*

Expression of *cpps2* carried by plasmids cpps2_pNG5 (LPS2-32) or pps2_pIO4 (LPS2-70) was in *E. coli* strain M15. Cultures of M15/pNG5 (1.6 liters of 2× YT medium, 100 μg/ml ampicillin, 25 μg/ml kanamycin) were grown at 30°C to an A_{600} of 0.9 and then induced with 0.1 mM isopropylthiogalactoside. Cells were harvested after a further 14 hr of incubation at 30°C.

Enzyme Purifications

The procedure to isolate LPS1 and LPS2 from extracts of *Claviceps purpurea* strains was as described previously. Seven-day-old mycelia were used in each case, and the amount of starting material was 30 g wet weight [14]. For the purpose of the present work, the first Ultrogel AcA 34 step was sufficient to show the presence or absence of LPS2 in extracts of strain P1 and the Δcpps2-1 mutant, respectively [14]. Purification of recombinant LPS2 from *E. coli* by Ni-NTA matrix was not possible with the hexaHis-LPS2 versions of LPS2 encoded by cpps2_pNG5 or pps2_pIO4. Therefore, LPS2-30 was chosen for partial purification from *E. coli*, following some of the steps of purification of the protein from *C. purpurea*. The various steps involved cell disintegration by rupture in the French press and ammonium sulfate precipitation of the polymin P-cleared supernatant of cell extract (60% saturation). This was followed by gel filtration on Ultrogel Aca 34 (detection of enzyme activity by the D-lysergic acid-dependent ATP-pyrophophate exchange). Gel filtration was followed by two rounds of anion exchange chromatography on resource Q and Mono Q (Pharmacia). Finally, enzyme was subjected to gel filtration on Superdex 200 (Figure 7). Buffer conditions were as described previously [14]. Enzyme activity in all separations eluted as a single peak.

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Accession Numbers

The sequence for *cpps2* has been deposited in GenBank under the accession number AJ439610.