Arch Microbiol (2002) 178 : 267–273
DOI 10.1007/s00203-002-0451-1

ORIGINAL PAPER

Mirko Glinski · Claus Urbanke · Till Hornbogen
Rainer Zocher

Enniatin synthetase is a monomer with extended structure: evidence for an intramolecular reaction mechanism

Received: 14 March 2002 / Revised: 16 May 2002 / Accepted: 27 May 2002 / Published online: 12 July 2002

Abstract Enniatin synthetase (Esyn), a 347-kDa multienzyme consisting of two substrate activation modules, is responsible for the nonribosomal formation of the cyclohexadepsipeptide enniatin. The synthesis follows the so-called thiol template mechanism. While this process is basically well established, no substantial insight into the 3-dimensional arrangement of these enzymes and possible interactions between them exists to date. To find out whether enniatin synthesis is an intramolecular process or the result of three interacting Esyn molecules (intermolecular), analytical ultracentrifugation equilibration studies were carried out. The molecular mass of Esyn was determined by ultracentrifugation and is in good agreement with that calculated from the ORF of the encoding gene, indicating that Esyn exists in solution as a monomer. This strongly suggests that synthesis of the cyclohexadepsipeptide enniatin follows an intramolecular reaction mechanism in which all three reaction cycles are catalyzed by a single Esyn molecule. This finding was supported by in vitro complementation studies in which [^{14}C]-methylvalyl Esyn, upon incubation with the second substrate D-2-hydroxyisovaleric acid (D-Hiv) and ATP, did not yield radioactive enniatin. This confirms our previous assumption of an iterative reaction mechanism similar to that for fatty acid synthase. Furthermore, the sedimentation rate constant evaluated from analytical ultracentrifugation was lower ($S_{20,w}$=14.1S) than expected ($S_{20,w}$=16.9S) for a globular protein, indicating that Esyn has an extended structure.

M. Glinski · T. Hornbogen · R. Zocher (✉)
Fakultät II, Institut für Chemie,
Arbeitsgruppe Biochemie und Molekulare Biologie,
Technische Universität Berlin,
Franklinstrasse 29, 10587 Berlin, Germany
e-mail: razzo@chem.tu-berlin.de,
Tel.: +49-30-31424168, Fax: +49-30-31473522

C. Urbanke
Biophysikalische Chemie, Medizinische Hochschule Hannover,
Carl-Neuberg-Straße 1, 30623 Hannover, Germany

Keywords Analytical ultracentrifugation · Enniatin synthetase · *Fusarium scirpi* · *N*-methylcyclopeptides · Nonribosomal peptide synthesis · Thiol template mechanism

Abbreviations *AdoMet S*-adenosyl-L-methionine · *D-Hiv* D-2-Hydroxyisovaleric acid · *Esyn* Enniatin synthetase · *NRPS* Nonribosomal peptide synthetase · *PFsyn* PF1022A synthetase · *PKS* Polyketide synthase

Introduction

Enniatins are *N*-methylated cyclohexadepsipeptides produced by various *Fusarium* species and with manifold biological properties (Tomoda et al. 1992). They are composed of alternating residues of D-2-hydroxyisovaleric acid (D-Hiv) and an *N*-methylated branched-chain amino acid such as L-valine, L-leucine or L-isoleucine (Plattner et al. 1948). The biosynthesis of enniatins is catalyzed by the multifunctional enzyme enniatin synthetase (Esyn), which belongs to the class of nonribosomal peptide synthetases (NRPSs). NRPSs are organized into coordinated groups of active sites termed modules (Zocher and Keller 1997; Glinski et al. 2001; Walsh et al. 2001). Each module is responsible for catalysis of one cycle of polypeptide chain elongation and associated functional group modifications (von Döhren et al. 1997; Konz et al. 1999). Biochemical investigations and analysis of the Esyn-encoding gene *esyn1* from *Fusarium scirpi* revealed that the enzyme consists of one polypeptide chain with a molecular mass of 347 kDa (Zocher et al. 1982; Haese et al. 1993). Like other NRPS systems, Esyn follows a so-called thiol template mechanism, in which the monomer constituents are activated as thioesters mediated by enzyme-bound 4′-phosphopantetheine residues (Zocher et al. 1982). In contrast to other NRPSs, the enzyme consists of only two activation modules (EA and EB), which contain the catalytic binding sites for the substrates D-Hiv and the branched-chain L-amino acid, respectively (Haese et al. 1993). The modules can be further divided into single do-

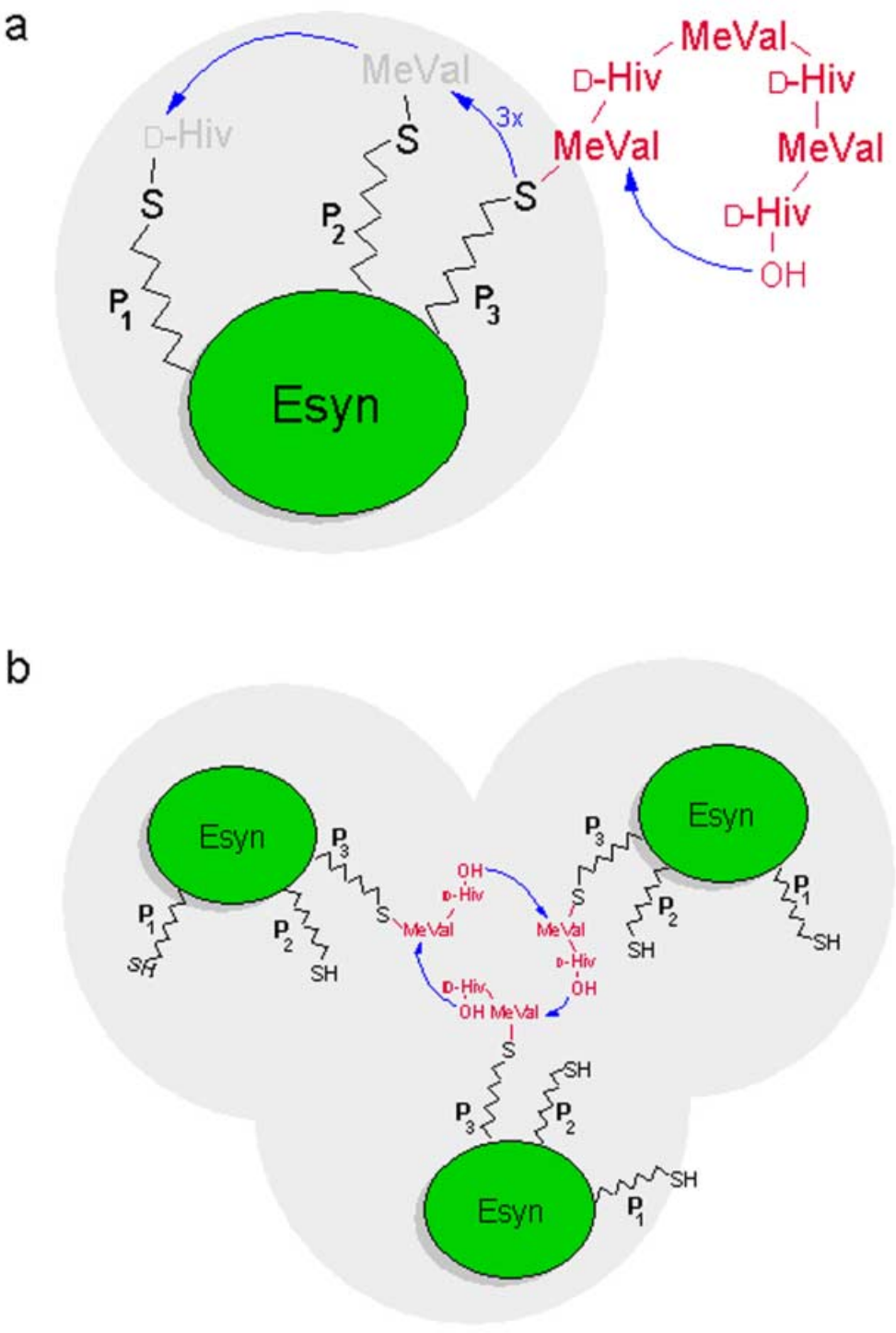


Fig. 1a, b Possible mechanisms of enniatin biosynthesis depending on the quarternary structure of Esyn in solution. **a** Intramolecular process catalyzed by monomeric Esyn. **b** Intermolecular process catalyzed by trimeric Esyn. P_1, P_2, P_3 4′-phosphopantetheine cofactor; P_3 represents the waiting position; *MeVal* *N*-methyl-L-valine

mains each of which has a specific function in the biosynthetic process, such as adenylation, thiolation, condensation, and *N*-methylation (Billich and Zocher 1987; Hacker et al. 2000; Haese et al. 1994; Pieper et al. 1995). In enniatin biosynthesis the depsipeptide chain is formed by three successive condensations of dipeptidol building blocks (Zocher and Keller 1997). One dipeptidol is formed in each reaction cycle by nucleophilic attack of the amino group of the branched-chain amino acid substrate at the carboxy group of D-Hiv and is then transferred to a third 4′-phosphopantetheine (C-terminal to module EB), which has been suggested to act as a waiting position to allow a new reaction cycle (Glinski et al. 2001).

Two possible mechanisms of enniatin formation are discussed (Fig. 1):

1. An intramolecular process in which three dipeptidol building blocks are condensed in an iterative manner by a single Esyn molecule to give a hexapeptidol that is finally cyclized in a putative cyclization cavity on the enzyme. Peptide chain growth proceeds via di-, tetra- and hexapeptidol formation.
2. An intermolecular process catalyzed by three Esyn molecules (trimer state), each of which catalyzes dipeptidol formation only. As a result of the three-dimensional arrangement of trimeric Esyn, a central cyclization cavity is formed. Enniatin formation then occurs by condensation of the three thioesterfied dipeptidol units followed by the terminating cyclization reaction.

According to the second model, no cyclization cavity on the enzyme is needed for enniatin biosynthesis.

The homologous PF1022A synthetase (PFsyn) is responsible for the synthesis of cyclooctadepsipeptides of the PF1022 type. These peptides consist of four alternating residues of *N*-methyl-L-leucine and four residues of D-lactate, D-phenyllactate or D-hydroxyphenyllactate (Weckwerth et al. 2000). Like Esyn, this multienzyme comprises two peptide synthetase modules with an integrated *N*-methyltransferase domain (Futamura et al. 2001; Haese et al. 1993). The molecular size of PFsyn in SDS-PAGE is identical with that of Esyn (350 kDa), although PFsyn assembles four dipeptidol units instead of three (Weckwerth et al. 2000). PFsyn is encoded by an ORF of 9,633 nucleotides with a calculated molecular mass of 354 kDa (Futamura et al. 2001).

In previous reports on enniatin biosynthesis, we favored an iterative reaction mechanism; however, no experimental evidence had been presented. Here we report the results of a further analysis of enniatin biosynthesis, elucidating how the programming of enniatin assembly is related to the structural organization of Esyn.

Material and methods

Strain, growth media, culture conditions, and materials

Fusarium scirpi ETH 1536/J5 was grown in FCM liquid medium (3% molasses, 1% corn steep) as described by Madry et al. (1982). All chemicals used were obtained from Merck (Darmstadt, Germany) and Sigma (Taufkirchen, Germany) and were of the highest purity commercially available.

Enzyme purification, protein determination, SDS-PAGE

Esyn was isolated from *F. scirpi* mycelium as previously described (Pieper et al. 1992). After gel filtration on Ultrogel AcA 34 (Biosepra, Frankfurt, Germany, column size: 60×6 cm) Esyn-containing fractions were subjected to hydrophobic interaction chromatography on pentyl agarose (Sigma, 1.5×4 cm). Esyn was eluted by a linear gradient of NaCl (0–0.3 M) in Tris buffer (100 mM Tris-HCl, 4 mM dithioerythritol, 1 mM benzamidine, 1 mM EDTA, 10% (w/v) glycerol), pH 7.8. Active fractions were pooled and rechromatographed on pentyl agarose. Fractions catalyzing enniatin B formation were concentrated using Centrifree Micropartition Devices (Millipore, Bedford, Mass., USA) and stored at –80 °C.

Protein concentrations were determined via the dye-binding method of Bradford as described previously (Bradford 1976), using bovine serum albumin as a standard.

SDS-PAGE was carried out according to the method of Laemmli (1970). Gels contained 5% acrylamide.

Determination of covalent enzyme-substrate complexes

Covalent enzyme-substrate complexes were assayed according to the method of Kleinkauf and Gevers (1969). Purified Esyn was incubated in the presence of 5 mM ATP, 10 mM MgCl2, and 1 µCi of L-[U-^{14}C] valine (260 Ci/mol) for 10 min at 26 °C. Two ml of 7% trichloroacetic acid were added and the mixture was left to stand on ice for 10 min. Radioactive protein was subsequently isolated by suction filtration on membrane filters ME 25 (Schleicher and Schuell, Dassel, Germany). After two washes with 10 ml of water, filters were dried at 60 °C for 15 min and radioactivity was measured by scintillation counting. The scintillation counter was calibrated by measuring defined amounts of radioactivity on membrane filters. This allowed the corresponding dpm values of experimental measurements to be calculated. Ten µg (0.028 nmol) of Esyn charged with L-[U-^{14}C] valine (1 µCi) for 10 min resulted in a value of 18,540±952 dpm (0.032 nmol). The reaction reached saturation after 2 min and could not be further stimulated by increasing concentrations of labeled valine.

In vitro complementation studies

For in vitro synthesis of enniatin B, 10 µg of purified Esyn were incubated in the presence of 5 mM ATP, 1 mM D-Hiv, 10 mM $MgCl_2$, 5 mM *S*-adenosyl-L-methionine (AdoMet), and 1 µCi of L-[U-^{14}C]-valine in a total volume of 225 µl for 10 min at 26 °C. After addition of 2 ml of water, the product was extracted with 2 ml of ethyl acetate. The organic phase was evaporated, and the residue was subjected to thin layer chromatography. Products were separated on silica-gel plates (Merck) with ethyl acetate/methanol/H_2O (100:5:1 v/v/v) as the solvent system. Enniatin B was identified by co-chromatography with the authentic substance. The silica-gel plates were subjected to autoradiography using Konica medical A2 X-ray films. For complementation studies Esyn was incubated in the presence of 5 mM ATP, 10 mM $MgCl_2$, 5 mM *S*-adenosyl-L-methionine (AdoMet), and 1 µCi of [^{14}C]-valine in a total volume of 225 µl for 10 min at 26 °C to form covalently bound *N*-methyl valine. The reaction mixture was then applied to an Ultrogel AcA 54 column (Biosepra, 0.5×6 cm). Proteins were eluted with Tris buffer. Fractions of 200 µl were collected. To check the stability of the covalent enzyme-substrate complexes (i.e. thioesterified *N*-methyl valine) trichloroacetic-acid-stable radioactivity (as described above) was measured before and after gel filtration. There was no significant loss of enzyme-bound radioactivity after gel filtration, indicating the stability of the covalent complexes (results not shown). For complementation experiments, radioactive protein fractions were incubated with 5 mM ATP, 10 mM $MgCl_2$, and 5 mM D-Hiv for a further 10 min at 26 °C. In parallel experiments, additional L-valine (5 mM) was added. After addition of 2 ml of water, labeled products were extracted with 2 ml of ethyl acetate and separated by thin-layer chromatography as described above.

Analytical ultracentrifugation

Analytical ultracentrifugation was carried out in a Beckman/Coulter XL-A analytical ultracentrifuge equipped with UV absorption optics. Protein concentration was monitored at 280 nm. For sedimentation rate experiments, between 0.2 and 1 µM Esyn was sedimented in double sector centerpieces filled to a height of approximately 1 cm at 20 °C and 47,000×*g* acceleration (25,000 rpm).

Equilibrium experiments were carried out in six channel equilibrium centerpieces (Beckman /Coulter, Fullerton, USA) with 120 µl of sample filling to a height of approximately 3 mm. Equilibrium was attained after 30 h at an acceleration of 1,830×*g* (5,000 rpm) as judged from the temporal stability of the concentration gradient

after that time. For a single species of molar mass M, partial specific volume $\bar{v}$ forming an equilibrium in a solvent of density ρ at an angular velocity ω, the absorption $A(x)$ at any place x inside the cell will be described by:

$$A(x) = A_o + A(x_0)\exp\left(\frac{M(1-\bar{v}\rho)}{RT}\omega^2\left(x^2 - x_0^2\right)\right)$$

where A_0 is the buffer absorption and $A(x_0)$ the absorption of the molecules at distance x_0. By fitting $A(x_0)$ and M this function can be used to evaluate an apparent molar mass from the measured concentration gradients.

Results and discussion

There is little information regarding the quaternary structure of NRPSs and polyketide synthases (Schmidt et al. 1992; Staunton et al. 1996). The PKS 6-desoxyerythronolide B synthase (DEBS) contains six modules, two housed within each multienzyme DEBS1, DEBS2 and DEBS3. Limited proteolysis, mutational analysis, cross-linking and analytical ultracentrifugation experiments with DEBS have led to the suggestion that in modular PKSs each module forms a homodimer in a possibly helical structure with the subunits arranged in a head-to-tail assembly (Staunton et al. 1996; Kao et al. 1996).

The only example for NRPS systems is cyclosporin synthetase. This protein is a multifunctional enzyme comprising 11 activating modules, seven of which have an integrated *N*-methyltransferase domain (Weber et al. 1994). In contrast, the two-module NRPS Esyn catalyzes the formation of a cyclohexadepsipeptide. Each of the 11 modules of cyclosporin synthetase is responsible for the recognition and activation of one constitutive amino acid of the cycloundecapeptide cyclosporin. The sequence of the final product is determined by the collinear arrangement of the modules. Peptide chain growth proceeds stepwise in the N- to C-terminal direction. Considering the function of the enzyme as determined by sedimentation velocity analysis experiments, an oblate ellipsoid shape of the multienzyme has been suggested (Schmidt et al. 1992). An average molecular mass of 1.4 MDa with a maximum error of ±160 kDa was reported (Schmidt et al. 1992), corresponding to a 45.8-kb ORF encoding a peptide with a calculated molecular mass of 1,689,243 Da (Weber et al. 1994). A reasonable interpretation in structural terms could be achieved only for a monomer (Schmidt et al. 1992). Electron microscopy of cyclosporin synthetase showed at least two types of structures: globular complexes of a number of particles (up to 11) and chain-like complexes (Hoppert et al. 2001). The globular structure was described as a wound-up chain-like complex. In the chain-like structure, a single polypeptide chain of 11 separate particles connected by intermodule sections could be observed, probably representing the 11 modules of cyclosporin synthetase (Hoppert et al. 2001).

The siderophore enterobactin synthetase system comprises a two-module NRPS like Esyn. The modules are spread over three proteins, EntE, EntB, and EntF, with EntE contributing an adenylation domain, EntB a thiolation domain and EntF a condensation, adenylation, thiolation and thioesterase domain. The module composition of Esyn differs from the two-module enterobactin system, because of its additional thiolation domain at the C-terminus of module EB, the so-called waiting position, and an additional C-terminal condensation domain. Reconstitution gel-filtration experiments with the three purified enzymes EntE, EntB and EntF were used to detect oligomerization, but there was no evidence for an enterobactin synthetase complex (Gehring et al. 1998).

Enniatin synthesis proceeds by a stepwise condensation of three dipeptidol building blocks. To gain structural insights into the mechanism of enniatin biosynthesis and the 3D-organization of active Esyn, two strategies were followed: (1) equilibrium analytical ultracentrifugation, which is a very efficient tool to determine the quaternary structure (and therefore the molecular mass) of macromolecules in aqueous solutions. Because the experiments were carried out in free solution, there were no complications due to matrices interactions which can obscure interpretation of certain types of experiments, including gel filtration. (2) Biochemical complementation studies in vitro to elucidate the reaction mechanism of enniatin biosynthesis.

1. In equilibrium analytical ultracentrifugation, more than 80% of the enzyme showed a molecular mass of 330± 40 kDa with no apparent concentration dependence (Fig. 2). Since analysis of the Esyn-corresponding gene *esyn1* (Haese et al. 1993) predicts a molar mass of

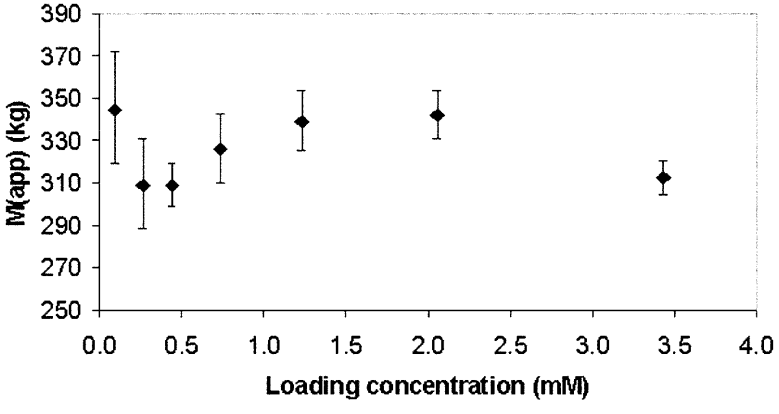


Fig. 2 Apparent molar mass of enniatin synthetase as determined from analytical equilibrium ultracentrifugation at an acceleration of 1,830×*g* (5,000 rpm). Several different loading concentrations in 100 mM Tris buffer containing 1 mM dithiothreitol, pH 7.8, were applied. *Error bars* Individual statistical error of each determination

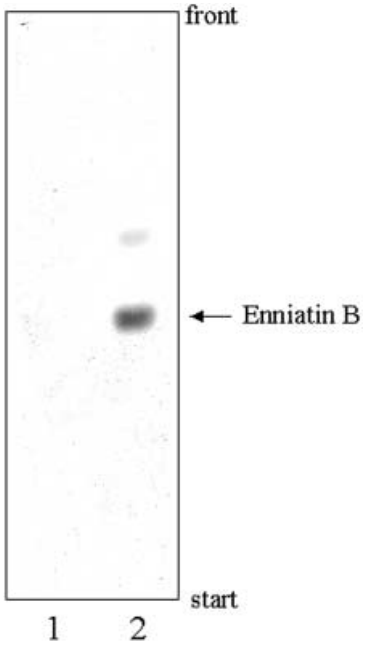
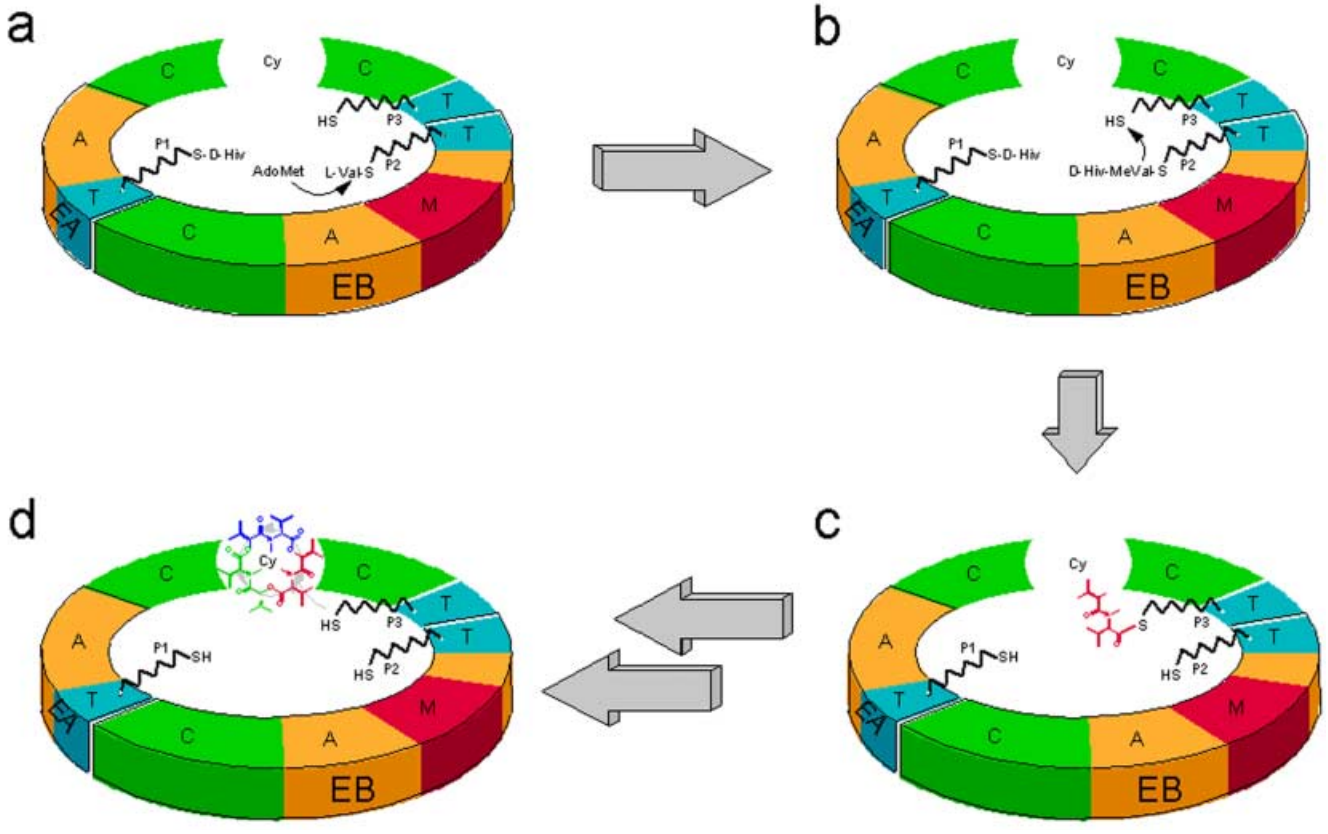


Fig. 3 Complementation experiment: in vitro synthesis of enniatin B (autoradiograph of thin layer chromatography separation of ethyl-acetate-extractable compounds). *Lane 1* [^{14}C]-methylvaline thioesterfied Esyn from *Fusarium scirpi* was incubated in the presence of 5 mM D-Hiv, 5 mM ATP, and 10 mM $MgCl_2$. *Lane 2* Control: in vitro synthesis of enniatin B upon incubation of [^{14}C]-methylvalyl Esyn with 5 mM D-Hiv, 5 mM L-valine, 5 mM AdoMet, 5 mM ATP, 10 mM $MgCl_2$. The solvent system was ethyl acetate/methanol/H_2O (100:5:1 v/v/v). As can be seen in *lane 2*, enniatin formation is observed

347 kg for the covalent protein chain, these results indicate that Esyn under these conditions is present as a monomer. To obtain more information about the shape of the enzyme, purified Esyn was subjected to analytical ultracentrifugation in order to determine the sedimentation rate constant. A value of $S_{20,w}$=14.1S was determined. For a globular protein with a molar mass of 341 kg and a partial specific volume of 7.36×10^{-4} m^3 kg^{-1} $S_{20,w}$=16.9S would be expected. From this deviation we conclude that Esyn has a somewhat extended structure.

2. The finding of a monomeric structure of Esyn could be supported by an in vitro complementation experiment with purified enzyme. This experiment took advantage of the ability of Esyn to form stable enzyme-substrate complexes in which the corresponding D-hydroxy acid or L-amino acid moieties are covalently bound. When the enzyme was incubated with radiolabeled valine the labeled enzyme could be separated by gel filtration

Fig. 4 Suggested mechanism of enniatin biosynthesis. Order and organization of Esyn domains as deduced from gene sequence and biochemical characterization. *Green-colored segment* Condensation domain: catalyzes peptide bond formation (elongation). *Orange-colored segment* Adenylation domain: recognizes and activates corresponding substrate. *Blue-colored segment* Thiolation domain: catalyzes covalent binding of substrate in thioester linkage; P_1, P_2, P_3 4′-phosphopantetheine cofactor (P_3 represents the waiting position). *Red-colored segment N*-methyltransferase domain: responsible for *N*-methylation of the L-amino acid; *Cy* cyclization cavity; *EA* D-Hiv-activating module; *EB* L-valine-activating module; *MeVal N*-methyl-L-valine



without any loss of covalently bound radioactivity (see Materials and methods). In the complementation experiment Esyn was incubated with [^{14}C]-valine, AdoMet, and ATP yielding Esyn with covalently bound (1 mol valine per mol Esyn) [^{14}C]-methylvaline. Separation of [^{14}C]-methylvalyl-Esyn from unbound substrates was then achieved by gel filtration on Ultrogel AcA 54. The addition of D-Hiv and ATP to [^{14}C]-methylvalyl-Esyn leads to peptide bond formation between thioester-bound [^{14}C]-methylvaline and D-Hiv, resulting in one thioesterfied dipeptidol unit per Esyn molecule. In the case of three interacting Esyn molecules arranged in a trimer state, the formation of radioactively labeled enniatin by condensation of the three dipeptidol building blocks would therefore be expected. However, separation by thin-layer chromatography and subsequent autoradiography showed that no radioactively labeled enniatin B was synthesized upon further incubation in the presence of D-Hiv and ATP (Fig. 3). In a control experiment [^{14}C]-methylvalyl Esyn was incubated in the presence of D-Hiv, L-valine, AdoMet, and ATP under saturating conditions. The formation of enniatin B clearly shows that the enzyme was catalytically active (Fig. 3).

Taken together, these results suggest that Esyn, like cylosporin synthetase, exists in solution as a monomer and that product assembly is achieved in an intramolecular manner. Figure 4 shows the proposed mechanism of enniatin biosynthesis. The reaction sequence leading to the cyclohexadepsipeptide includes the recognition and adenylation of D-Hiv and the L-amino acid by the corresponding A-domains. The acyladenylates are then covalently bound to the thiol groups of the 4′-phosphopantetheine arms P_1 and P_2. 4′-Phosphopantetheine facilitates the ordered shift of carboxy-thioester-activated substrates between the two modules. Characteristically, the thioesterified L-amino acid is methylated with AdoMet, and thus *N*-methylation takes place prior to peptide bond formation and subsequent cyclization reactions After *N*-methylation the amino acid and D-Hiv form a dipeptidol. Peptide bond formation is catalyzed by the condensation domain C of module EB. The dipeptidol unit remains covalently attached to the N-terminal T-domain of module EB and is then transferred to the waiting position P_3 in order to reinitiate a new reaction cycle. The hydroxyl group of the newly formed dipeptidol then attacks the carboxy group of the dipeptidol in the waiting position in a nucleophilic reaction yielding a tetrapeptidol covalently bound to the waiting position. In a third condensation reaction, the next dipeptidol attacks the tetrapeptidol to form a hexapeptidol, which yields enniatin in the terminating cyclization reaction. We therefore conclude that a trimeric structure is not necessary for the catalytic function of Esyn.

Acknowledgements Financial support of this work by the Deutsche Forschungsgemeinschaft (grant Zo 43/5–3) is gratefully acknowledged.

References

Billich A, Zocher R (1987) *N*-Methyltransferase function of the multifunctional enzyme enniatin synthetase. Biochemistry 26: 8417–8423

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Futamura T, Aihara S, Midoh N, Okakura K, Kleinkauf H, Miyamoto K, Watanabe M, Yanai K, Murakami T, Yasutake T (2001) Cyclic depsipeptide synthases, genes thereof and mass production system of cyclic depsipeptide. Meiji Seika Kaisha, patent AU6874100

Gehring AM, Mori I, Walsh CT (1998) Reconstitution and characterization of the *Escherichia c*oli enterobactin synthetase from EntB, EntE, and EntF. Biochemistry 37:2648–59

Glinski M, Hornbogen T, Zocher R (2001) Enzymatic synthesis of fungal *N*-methylated cyclopeptides and depsipeptides. In: Kirst H, Wu-Kuang Y, Zmijewski M (eds) Enzyme technologies for pharmaceutical and biotechnological applications. Dekker, New York, pp 471–497

Hacker C, Glinski M, Hornbogen T, Doller A, Zocher R (2000) Mutational analysis of the *N*-methyltransferase domain of the multifunctional enzyme enniatin synthetase. J Biol Chem 275: 30826–30832

Haese A, Schubert M, Hermann M, Zocher R (1993) Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalysing *N*-methyl-depsipeptide formation in *Fusarium scirpi*. Mol Microbiol 7:905–914

Haese A, Pieper R, von Ostrowski T, Zocher R (1994) Bacterial expression of catalytically active fragments of the multifunctional enzyme enniatin synthetase. J Mol Biol 247:116–122

Hoppert M, Gentzsch C, Schörgendorfer K (2001) Structure and localization of cyclosporin synthetase, the key enzyme of cyclosporin biosynthesis in *Tolypocladium inflatum*. Arch Microbiol 176: 285–293

Kao CM, Pieper R, Cane DE, Khosla C (1996) Evidence for two catalytically independent clusters of actice sites in a functional modular polyketide synthase. Biochemistry 35: 12363–12368

Kleinkauf H, Gevers W (1969) Nonribosomal polypeptide synthesis: the biosynthesis of a cyclic peptide antibiotic, gramicidin S. Cold Spring Harbor Symp Quant Biol 34:805–13

Konz D, Marahiel MM (1999) How do peptide synthetases generate structural diversity? Chem Biol 6:39–48

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Madry N, Zocher R, Kleinkauf H (1992) Enniatin production by *Fusarium oxysporum* in chemically defined media. Eur J Appl Microbiol Biotechnol 17:75–79

Pieper R, Kleinkauf H, Zocher R (1992) Enniatin synthetases from different Fusaria exhibiting distinct amino acid specificities. J Antibiot 45:1273–1277

Pieper R, Haese A, Schröder W, Zocher R (1995) Arrangement of catalytic sites in the multifunctional enzyme enniatin synthetase. Eur J Biochem 230:19–126

Plattner PA, Nager U, Boller A (1948) Werkstoffe und Antibiotika. Siebte Mitteilung über die Isolierung neuartiger Antibiotika aus *Fusarien*. Helv Chim Acta 31:594–602

Schmidt B, Riesner D, Lawen A, Kleinkauf H (1992) Cyclosporin synthetase is a 1.4 MDa multienzyme polypeptide, re-evaluation of the molecular mass of various peptide synthetases. FEBS Letters 307:355–360

Staunton J, Caffrey P, Aparicio JF, Roberts GA, Bethell SS, Leadlay PF (1996) Evidence for a double-helical structure for modular polyketide synthases. Nat Struct Biol 3:188–192

Tomoda H, Nishida H, Huang XH, Masuma R, Kim YK, Omura S (1992) New cyclodepsipeptides, enniatins D, E and F produced by *Fusarium* sp. FO-1305. J Antibiot 45:2167–2170

Von Döhren H, Keller U, Vater J, Zocher R (1997) Multifunctional peptide synthetases. Chem Rev 97:2651–2673

Walsh CT, Chen H, Keating TA, Hubbard BK, Losey HC, Luo L, Marshall CG, Miller DA, Patel, HM (2001) Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. Curr Opin Chem Biol 5:525–534

Weber G, Schörgendörfer K, Schneider-Scherzer E, Leitner E (1994) The peptide synthetase catalysing cyclosporin production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. Curr Genet 26:120–125

Weckwerth W, Miyamoto K, Iinuma K, Krause M, Glinski M, Storm T, Bonse G, Kleinkauf H, Zocher R (2000) Biosynthesis of PF1022 and related cyclooctadepsipeptides. J Biol Chem 275:17909–17915

Zocher R, Keller U (1997) Thiol template peptide synthesis systems in bacteria and fungi. Adv Micr Phys 38:85–131

Zocher R, Keller U, Kleinkauf H (1982) Enniatin synthetase: A novel type of multifunctional enzyme catalyzing depsipeptide synthesis in *Fusarium oxysporum*. Biochemistry 21:43–48