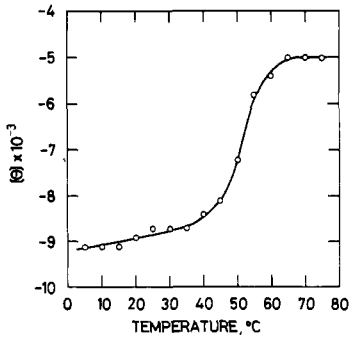

FIGURE 8: Thermal unfolding transition of HEWL in 0.5 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6, as measured by the static CD measurements.

2.3 and 17.6 kcal/mol for RNase A at 0.1 M and 2.0 M GdmCl, respectively, at pH 6 (Nall et al., 1978).

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Enniatin Synthetase, a Novel Type of Multifunctional Enzyme Catalyzing Depsipeptide Synthesis in *Fusarium oxysporum*†

Rainer Zocher, Ullrich Keller, and Horst Kleinkauf*

ABSTRACT: Enniatin synthetase, a multifunctional enzyme catalyzing depsipeptide formation in *Fusarium oxysporum* was purified to 98% homogeneity as judged by analytical disc gel electrophoresis. The enzyme consists of a single polypeptide chain of a molecular weight of about 250 000. Similar to a number of peptide synthetases and to fatty acid synthetase the enzyme contains 4′-phosphopantetheine as a prosthetic group. Studies on substrate specificity revealed that the enzyme is capable of synthesizing enniatins A–C and also mixed-type enniatins containing more than one species of amino acid. A linear dependence of rate of enniatin synthesis on enzyme concentration was observed, indicating that depsipeptide formation is an intramolecular process. Omission of the methyl donor *S*-adenosyl-L-methionine resulted in the formation of unmethylated enniatins with a reaction rate of about 10% of that observed in the case of enniatins. Sulfhydryl-directed reagents generally had an inhibitory influence on enniatin synthesis. However, inhibition studies with iodoacetamide revealed that it behaves differently toward the hydroxy acid site(s) and amino acid site(s). This indicates the presence of chemically distinct thiol groups in the active sites for the two substrates.

Enniatins constitute a class of depsipeptides produced by several strains of *Fusarium* (Plattner et al., 1948). They consist of three residues of one of the branched-chain amino acids L-valine, L-leucine, or L-isoleucine and three residues of D-2-hydroxyisovaleric acid. (D-Hyiv).[1] The constituents of the depsipeptides are linked together by amide and ester bonds in an alternating fashion. In addition, amide bonds are

† From the Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, 1000 Berlin 10, West Germany. *Received June 2, 1981.* Parts of this work have been presented at the VIth Fermentation Symposium, London, Canada, 1980. This research was supported by Grant No. KL 148/13 from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Hyiv, hydroxyisovaleric acid; SAM, *S*-adenosyl-L-methionine; PEI, poly(ethylenimine).

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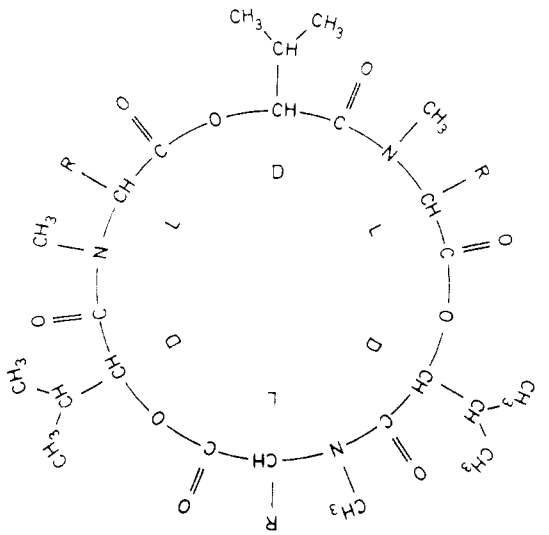

FIGURE 1: Enniatin A, R = $-CH(CH_3)CH_2CH_3$; enniatin B, R = $-CH(CH_3)_2$; enniatin C, R = $-CH_2CH(CH_3)_2$.

methylated as shown in Figure 1. Enniatins A–C are reported to act as efficient cationophors (Wipf et al., 1968; Tosteson, 1968; Ivanov et al., 1973) and thus resemble valinomycin functionally as well as structurally. It is believed that they contribute to the wilt toxic character exerted by the fungus which produces them. Previous work indicates that enniatins of various types may act as an immunomodulator in mice (Simon-Lavoine & Forgeot, 1979) and may eventually have some importance as therapeutic agents in the future.

We have reported the cell-free synthesis of enniatin B previously (Zocher et al., 1976; Zocher & Kleinkauf, 1978). In this cell-free system, the formation of enniatin B from L-valine, D-Hyiv, and *S*-adenosyl-L-methionine (SAM), at the expense of ATP, could be demonstrated. The enzyme responsible for the formation of the depsipeptide is also capable of catalyzing ATP–PP_i exchange reactions dependent on L-valine and D-Hyiv. The process of N-methylation takes place at the stage of thioesterified L-valine. In this paper evidence will be presented that this enzyme also is capable of catalyzing the formation of enniatins A and C and mixed-type enniatins as well. Furthermore, the purification to homogeneity and further properties of the enzyme now called enniatin synthetase will be described.

Materials and Methods

Chemicals and Radioisotopes. L-[^{14}C]Valine (285 Ci/mol), L-[^{14}C]isoleucine (350 Ci/mol), L-[^{14}C]leucine (350 Ci/mol), DL-[^{14}C]valine (60 Ci/mol), and L-[^{3}H]valine (56 Ci/mmol) were obtained from Radiochemical Centre, Amersham. ^{14}C-Labeled and unlabeled D-2-Hyiv were prepared from DL-[^{14}C]valine and D-valine, respectively, by reaction with HNO_2 (Fischer & Scheibler, 1908). The *N*-methyl derivatives of L-valine, L-leucine, and L-isoleucine were obtained from Bachem. Synthetic enniatins A and B were generously supplied by Dr. R. Benz (Universität Konstanz, West Germany) and Dr. Studer (Hoffmann-La Roche, Basel, Switzerland). Enniatin C was isolated from *Fusarium oxysporum* ETH 1536/9 as described (Audhya & Russell, 1973b). Its authenticity was confirmed by the methods described below. All other reagents were of highest purity commercially available.

Analysis of Reaction Products. Reaction products of enniatin synthetase were extracted with EtOAc and separated by thin-layer chromatography on silica gel plates (Merck) using EtOAc–MeOH–H_2O (100:4:1) for development (solvent system I). Another thin-layer technique on cellulose plates (Macherey & Nagel, Düren, Germany) in formamide–*n*-heptane (solvent system II) was used according to Simon-Lavoine & Forgeot (1979). EtOAc extracts were applied to the cellulose plates. The upper part of each plate was then carefully dipped into a mixture of acetone–formamide (3:1 v/v) so that only the part above the origin was soaked (contact of the formamide with the origin must be avoided). Then the plates were developed in *n*-heptane (formamide saturated). Radioactive products were localized by radioscanning or autoradiography, scraped off the plates, and extracted with EtOAc. The analysis of the compounds was carried out as described previously (Zocher et al., 1976) except that for mild alkaline hydrolysis the method of Audhya & Russell (1974) was used.

Radioactive Measurements. These were carried out as described (Zocher & Kleinkauf, 1978). For double-labeling experiments, counts were corrected for counting efficiency with standard solutions of the labeled amino acids in the ^{3}H/^{14}C channel.

Protein Determinations. Protein concentrations were determined by a modified Bradford procedure (Spector, 1978) using bovine serum albumin as a standard.

Molecular Weight Determinations. The molecular weight of denatured enniatin synthetase was determined by NaDodSO$_4$–polyacrylamide gel electrophoresis according to the method of Weber & Osborn (1969) with the modification that 6% acrylamide gels were used. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in methanol–AcOH–H_2O (45:10:45) and destained in an aqueous solution of 10% methanol and 6% AcOH.

The molecular weight of enniatin synthetase was estimated from the linear calibration curve obtained from gramicidin S synthetase (heavy enzyme) GS II (280 000), myosin (200 000), β-galactosidase (130 000), bovine serum albumin (68 000), phosphatase (93 000), and bovine serum albumin dimer (136 000). The molecular weight of the native enzyme was estimated by gel filtration on Ultrogel AcA 34 (LKB) (column dimensions 1.6 × 100 cm) using 0.1 M potassium phosphate buffer, pH 7.0 (4 mM dithiothreitol). The column was calibrated with gramicidin S synthetase (light enzyme) GS I (100 000), gramicidin S synthetase GS II (280 000), and catalase (250 000).

Determination of 4′-Phosphopantetheine. Pantothenic acid was liberated from protein-bound 4′-phosphopantetheine and determined microbiologically with *Lactobacillus plantarum* (ATCC 8014) as a test organism (Pugh & Wakil, 1965). For this purpose, enzyme fractions were heated with KOH (final concentration 1 N) in a steam bath for 1 h to separate 4′-phosphopantetheine from the protein. Then the solution was adjusted to pH 8.4 with concentrated HCl and exposed to alkaline phosphatase (Sigma Chemical Co.) for 3 h at 37 °C to liberate free pantothenate for the microbiological assay.

Enzyme Assay. This was done as described previously (Zocher & Kleinkauf, 1978). The incorporation of L-[^{14}C]-valine (0.5 μCi/assay) was used routinely for the detection of the enzyme during preparation.

In addition, the reaction mixture contained SAM (0.2 mM), D-Hyiv (0.2 mM), ATP (4 mM), and $Mg(OAc)_2$ (4 mM) in a total volume of 100 μL. However, under these conditions there was no substrate saturation with respect to valine. For measurement under saturating conditions, L-[^{3}H]valine (2 μCi) was used in the presence of unlabeled valine (1 mM). Incubation was carried out at 25 °C for 10 min.

Determination of Covalent Enzyme–Substrate Complexes. This was carried out according to Kleinkauf & Gevers (1969).

Table I: Purification of Enniatin Synthetase[a]

purification step	total protein (mg)	total act. (nkat)	sp act.[b] (nkat/mg)	yield (%)
crude extract	1300	2.1	0.0016	100
PEI precipitation	930	1.9	0.0020	90
$(NH_4)_2SO_4$ precipitation	84	1.2	0.014	57
Ultrogel AcA 34	16	0.8	0.05	38
propylagarose	5.8	0.52	0.09	25
sucrose gradient	1.7	0.17	0.10	9.5

[a] 10 g of lyophilized cells was used. [b] Compared with the purification table in our previous paper (Zocher & Kleinkauf, 1978), the specific activities in the present work are different due to the fact that they were measured under saturating conditions. The reaction was followed by the incorporation of [^{3}H]valine in the presence of excessive unlabeled valine as described under Materials and Methods. This was not the case in the previous paper, where units were measured under defined conditions in order to determine the degree of purification. Specific activity was expressed in nanokatals (1 kat is defined as the amount of enzyme catalyzing the formation of 1 mol of enniatin B/s).

ATP/PP_i Exchange. Amino or hydroxy acid dependent ATP–PP_i exchange was carried out as described by Lee & Lipmann (1975).

Growth of Organism. F. oxysporum ETH 1536/9 strain C 7/5 was grown in a submerged culture as described previously (Zocher & Kleinkauf, 1978). However, this high-producing strain reached its maximum activity, measured by an increase of the enniatin titer (Audhya & Russell, 1973a), after about 45 h and was harvested at this time. The strain was obtained by treatment of *F. oxysporum* conidia with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine).

Enzyme Purification. All operations were carried out at 4 °C. For all preparation steps 0.05 M potassium phosphate buffer (pH 6.8) containing 4 mM dithiothreitol was used (buffer A).

Preparation of the crude extract from 10 g of lyophilized mycelium of *F. oxysporum* ETH 1536/9 strain C 7/5, poly(ethylenimine) precipitation, fractional ammonium sulfate precipitation (35–45%), and gel filtration on Ultrogel AcA 34 (column dimensions 3 × 80 cm) were carried out as described earlier (Zocher & Kleinkauf, 1978).

Hydrophobic Chromatography. Active fractions from the Ultrogel AcA 34 chromatography were pooled and applied to a propylagarose column (1 × 6 cm) previously equilibrated with buffer A. After the column was washed with a 3-fold column volume of the same buffer, the enzyme was eluted with a linear KCl gradient between 0.0 and 0.35 M (2 × 70 mL). Fractions containing enzyme were pooled and dialyzed against buffer A. The enzyme was concentrated by repeated adsorption on propylagarose and stepwise elution with 0.25 M KCl.

Sucrose Gradient Centrifugation. After dialysis of the concentrated enzyme, 0.5-mL portions were layered on 36-mL sucrose gradients between 5 and 20% (buffer A). It was then centrifuged for 26 h at 27 000 rpm in a Beckman centrifuge with an SW-27 rotor. The gradient was eluted from the bottom. Fractions of 1 mL were collected.

Active fractions were mixed with glycerol (15%) and stored at −20 °C.

Results

Purification of Enniatin Synthetase. Enniatin synthetase from *F. oxysporum* ETH 1536/9 strain C 7/5 was purified to near homogeneity in six steps (Table I). Steps 1–3 are identical with the procedure described previously (Zocher & Kleinkauf, 1978) except that 50 mM potassium phosphate buffer (pH 6.8) instead of 25 mM of the same buffer was used. In comparison with our previous work, the yield of total units and also the specific activity of the enzyme were now considerably higher (about 4-fold). This is obviously due to the fact that the high-producer strain C 7/5 of ETH 1536/9 was used. This strain produces about 1 g/L enniatin B in contrast to the parent strain which is less active (80 mg/L).

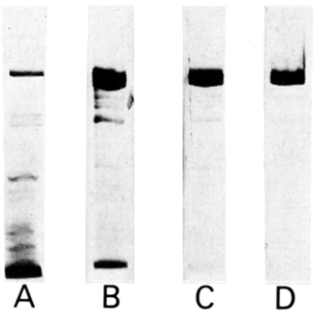

FIGURE 2: $NaDodSO_4$–acrylamide gel electrophoresis of different stages of purification. (A) Protein (30 μg) from the amonium sulfate step. (B) Protein (25 μg) from the Ultrogel AcA 34 step. (C) Protein (22 μg) from the propylagarose step. (D) Protein (18 μg) from the sucrose gradient step (bottom fractions of 70% of the activity curve). Electrophoresis was performed on a 6% acrylamide gel according to Laemmli (1970) for 8 h at 80 mA. Gels were stained with Coomassie Blue.

Analytical disc gel electrophoresis (Laemmli, 1970) was used to follow the course of purification (Figure 2). After hydrophobic chromatography on propylagarose, the enzyme was about 94% homogeneous as judged by gel scans. Enniatin synthetase activity was eluted as a single peak coincident with the peak of protein (not shown). In the final step (sucrose gradient centrifugation) the enniatin synthetase peak still contained traces of contaminating proteins. However, the purity of the synthetase was greater than 98% if only the fractions from the lower 70% (bottom) of the peak were combined. The purified enzyme could be preserved for several months by storage at −20 °C in the presence of 15% glycerol without significant loss of activity.

Deviations from this procedure, especially omission of dithiothreitol or events causing aeration or mechanical stress, always resulted in a rapid loss of activity.

Molecular Weight Determination. Molecular weight determinations of native enzyme by gel filtration chromatography with several standard proteins (Andrews, 1965) gave a value of about 250 000, assuming a globular protein. The molecular weight of denatured enzyme was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). The value obtained by this procedure was the same as that for native enzyme, even after treatment with reducing agents such as mercaptoethanol or dithiothreitol at concentrations of 0.1 M (100 °C; 5 min). From these findings it is concluded that the enzyme consists of a single polypeptide chain.

Presence of 4'-Phosphopantetheine in Enniatin Synthetase. Analysis of fractions from a sucrose gradient centrifugation of enzyme from the propylagarose step in 50 mM Tris-HCl (pH 7.0) was done in order to determine the 4'-phosphopantetheine content. As shown in Figure 3, the synthetic activity of enniatin synthetase comigrates with pantothenate in the gradient. The fact that most of the pantothenate was released after alkaline phosphatase treatment proves that it is present as 4'-phosphopantothenate. A determination of the amino acid composition of the enzyme revealed the existence of β-alanine in the acid hydrolysate (not shown). The exact amino acid

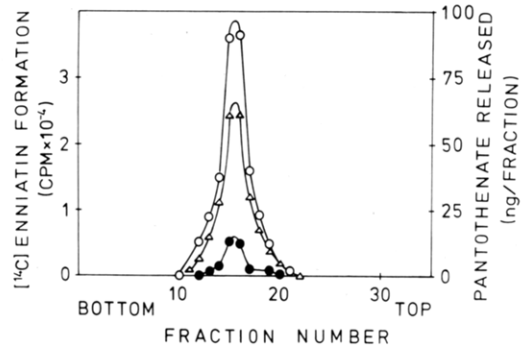

FIGURE 3: Sucrose gradient centrifugation of enzyme (200 μg) from the propylagarose step. A 5–20% linear sucrose gradient containing 50 mM Tris-HCl buffer (pH 7.2) and 4 mM dithiothreitol was used. Fractions were assayed for enniatin synthetase activity (△), pantothenate content after treatment with alkaline phosphatase (○), and pantothenate content without alkaline phosphatase treatment (●).

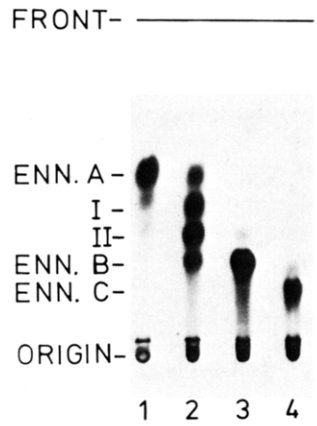

FIGURE 4: Products of enniatin synthetase. Thin-layer chromatogram (solvent system II) of EtOAc extracts from reaction mixtures containing all substrates necessary for enniatin synthesis [amino acid(s) in labeled form] and purified enniatin synthetase. (1) [^{14}C]Isoleucine yielded mainly enniatin A. (2) A mixture of [^{14}C]isoleucine (1 μCi) and [^{14}C]valine (0.5 μCi) yielded besides enniatin A and B two other compounds (I and II) which are obviously hybrid enniatins. (3) [^{14}C]Valine yielded enniatin B. (4) [^{14}C]Leucine (1 μCi) yielded enniatin C.

composition and β-alanine content per mole of enzyme are under investigation.

Influence of pH and Temperature. Incubation of the enzyme at different pH values showed an optimum at pH 6.5–6.8 with respect to synthesis of all enniatins. The optimum temperature for the reactions was 25–26 °C. With increase of temperature above 30 °C, there was a sharp decrease of synthetic activity of enzyme, whereas when the temperature was lowered, only a gradual decrease in the rate of synthesis was observed.

Substrate Specificity of Enniatin Synthetase. F. oxysporum ETH 1536/9 strain C 7/5 produces enniatin B as the main product together with a minor amount of enniatins A and C. In order to examine the possibility that the enzyme also could synthesize the other enniatins, we designed a set of different experiments. The purified enzyme was incubated separately with radioactively labeled L-valine, L-isoleucine, or L-leucine in the presence of all other substrates necessary for enniatin synthesis under optimal conditions (Zocher & Kleinkauf, 1978). In each of the three experiments, a radioactive product was formed which after isolation and subsequent analysis could be identified as the corresponding enniatin (Figure 4, lanes 1, 3, and 4). From this finding it was supposed that mixed-type enniatins, i.e., enniatins containing more than one species of amino acid, also should be formed under appropriate conditions. Figure 4 (lane 2) shows the result of such an experiment. Incubations of enzyme were done in an identical fashion as above with the exception that two instead of one of the amino acids were added (for details see Figure 4). Without any doubt the purified enzyme is capable of synthesizing the three enniatins and mixed-type enniatins as well. The presumed mixed-type enniatins were analyzed as follows: Each compound was isolated and hydrolyzed in 6 N HCl (110 °C; 21 h). Amino acid analysis of the hydrolysates by high-voltage paper electrophoresis (pH 1.9) revealed that compounds I and II both contained valine and isoleucine. In addition the two compounds could be isolated in labeled form, when the enzyme was incubated with DL-[^{14}C]Hyiv, unlabeled valine, isoleucine, and the other components necessary for enniatin synthesis. Analysis of mixed-type enniatins was also done by double-labeling experiments with L-[^{3}H]valine and L-[^{14}C]isoleucine in the case of enniatins B and A, respectively. Table II shows the distribution of radioactivity in the four bands shown in Figure 4 (lane 2) which were derived from a separate experiment using the two radioisotopes. As enniatins A and B carry exclusively the ^{14}C and ^{3}H label, respectively, the two bands between them carried both labels in molar ratios for L-valine:L-isoleucine L-isoleucine of 1:2 (compound I) and 2:1 (compound II). These findings unambiguously indicate that formation of mixed-type enniatins had occurred. Similar results with respect to formation of enniatins mixed with L-leucine and L-isoleucine were also obtained (not shown).

Table II: Double Labeling of Hybrid Enniatins[a]

compound	pmol incorporated	
	[^{3}H]valine	[^{14}C]isoleucine
enniatin A	0.0	5.8
I	7.1	13.6
II	21.8	11.3
enniatin B	28.4	0.0

[a] The enzyme was preincubated in the presence of 50 μCi of L-[^{3}H]valine and the other substrates necessary for enniatin synthesis (for concentrations, see Materials and Methods) for 5 min at 25 °C. After this period unlabeled valine still convalently attached to the enzyme had completely reacted. 2 μCi of [^{14}C]isoleucine was then added, and the reaction was continued for 30 min. Radioactively labeled enniatins were isolated as described and characterized by alkaline and acid hydrolysis. In order to prove the molar ratios of amino acids in the different mixed-type enniatins, we examined a sample of each compound for the presence of ^{3}H and ^{14}C label.

When the enzyme was incubated in the presence of L-isoleucine and L-leucine, respectively (Figure 4, lanes 1 and 4), the formation of traces of hybrid enniatins was also observed. This finding may be explained by the fact that the enzyme was still loaded with L-valine. After preincubation of the enzyme with the corresponding unlabeled amino acid, the exclusive formation of the homogeneous enniatins was observed.

A detailed analysis of the kinetics of enniatin synthetase has not yet been performed. However, experiments with respect to K_m values of the different amino acids and D-Hyiv by initial rate studies have been done (Zocher et al., 1980). The initial reaction rate was determined at varying concentrations of one substrate with fixed concentrations of the others. K_m values were determined from double-reciprocal plots. Such experiments should provide knowledge about the affinity of the enzyme for the various substrates and should permit conclusions about the role of amino acid concentrations and the formation of different enniatins in the cell. The K_m value with

respect to L-valine is lowest (8×10^{-5} M), whereas those of L-isoleucine and L-leucine were higher (1.2×10^{-4} M and 2.6×10^{-4} M, respectively). This finding (as well as varying amino acid concentrations in the cell) may explain the fact that enniatin B is a main component of the total enniatin fraction from *F. oxysporum* ETH 1536/9.

Some further amino acids were tested for their ability to serve as substrates for enniatin synthetase. In these experiments ^{14}C-labeled Hyiv was used as the radiolabel and the relevant amino acids were added to the incubation mixtures containing enzyme and the other necessary substrates. The results revealed that after addition of D-valine no radioactive enniatin was formed. Also, L-phenylalanine, the amino acid constituent of beauvericin, a structural homologue of enniatins, did not yield any product. The same observation was made in the case of L-alanine and 2-methyl-L-alanine.

Previous experiments (Zocher et al., 1980) revealed that substitution for D-Hyiv in enniatins was not possible for other analogous branched-chain D-2-hydroxy acids. Determination of the K_m value for D-Hyiv gave 4×10^{-6} M, which is 20 times less than that of valine. The optical isomer of D-Hyiv was not incorporated. From these findings it was concluded that D-Hyiv was the sole substrate of the hydroxy acid site(s) of enniatin synthetase. However, during the course of the present investigation, where enzyme was obtained from a high producer mutant of *F. oxysporum* with much higher specific activity compared to that from earlier experiments, a low but significant incorporation of DL-2-hydroxyisocaproic acid and a higher incorporation of DL-2-hydroxy-3-methylvaleric acid with high substrate concentrations (2 mM) (8 and 45%, respectively, compared to Hyiv) were observed.

Role of Methylation in the Process of Depsipeptide Formation. During our previous work we found that *S*-adenosyl-L-methionine acts as the donor of the methyl group in enniatin B. Methylation takes place at the stage of thioesterified valine, yielding *N*-methylvaline, which could be isolated by alkaline or performic acid cleavage from the enzyme.

Further experiments were done to determine the mechanism of methylation and its place in the process of enniatin synthesis. When enniatin synthetase was incubated with L-[^{14}C]valine, D-Hyiv, and ATP but without SAM, a compound could be isolated from the incubation mixture which apparently was unmethylated enniatin B (R_f 0.55, solvent system I). Acid hydrolysis (5.6 N HCl, 110 °C; 22 h) of the compound yielded exclusively [^{14}C]valine; mild alkaline hydrolysis yielded a compound with electrophoretic behavior identical with that of D-hydroxyisovaleryl-D-valine, a depsipeptide obtained by alkaline cleavage of valinomycin. The presence of D-Hyiv in the unknown compound was proven in a parallel experiment using [^{14}C]Hyiv as the radiolabel (valine in unlabeled form). A compound with the same R_f value (solvent system I) was formed, which yielded after acid hydrolysis exclusively [^{14}C]Hyiv. The cyclic structure of the unknown compound was deduced from its inability to move in the electric field at different pH values. These findings strongly suggest that the compound is unmethylated enniatin B. Besides the unmethylated enniatin at least four other products with higher R_f values (solvent system I) could be isolated in smaller amounts. The structure of these latter compounds is still unknown. More work is required to elucidate the structures of all unmethylated products of enniatin synthetase.

Initial velocity measurements of the reaction rate of enniatin formation in the presence and absence of SAM were done. The results clearly demonstrate the effect of SAM on the biosynthetic process. In the presence of the methyl donor, the rate of depsipeptide formation is about 10-fold higher than when the methyl donor is absent (not shown). In connection with the above unknown compounds, one may speculate about a regulatory role of SAM which leads to possible random disorder of the reaction sequence in the absence of the methyl donor.

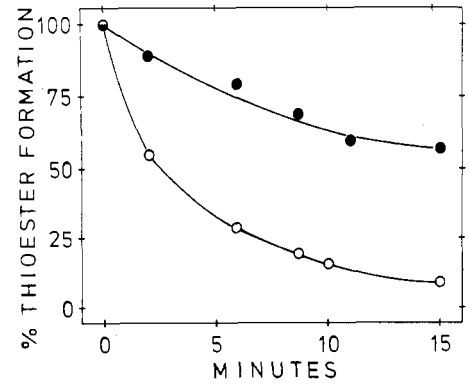

FIGURE 5: Influence of iodoacetamide on the extent of thioester formation with respect to DL-[^{14}C]-2-Hyiv (●) and [^{14}C]valine (○). After various times of preincubation of enzyme (25 μg) in the presence of the inhibitor (2 mM), the enzyme was assayed for the capability to form covalent enzyme–substrate complexes (see Materials and Methods). The amount of covalently bound substrate at 100% was 0.095 nmol for [^{14}C]valine and 0.102 nmol for [^{14}C]Hyiv.

Enzyme Concentration and Reaction Rate. Enniatins may be considered to be composed of repeated sequences of didepsipeptides or esters between *N*-methyl L-amino acids and D-2-hydroxyisovaleric acid. This fact may raise the question whether elongation and cyclization of the depsipeptide chain occur on the surface of one enzyme molecule or are the result of an intermolecular condensation. The latter mechanism should result in a nonlinear dependence of the rate of synthesis on enzyme concentration. When the dependence of enniatin synthesis on enzyme concentration was measured, in all cases a linear dependence of the rate of synthesis on enzyme concentration was observed.

Influence of Thiol-Directed Reagents on Enzymatic Activity. Thiol-group blocking agents generally had an inhibitory influence on enniatin synthesis. These were iodoacetamide, iodoacetate, *N*-ethylmaleimide, *p*-chloromercuribenzoate, and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. It was noteworthy that the ATP–PP$_i$ exchanges dependent on the two substrates L-valine and D-Hyiv proceeded nearly unaffected in the presence of these inhibitors. Since the activation reaction via acyl adenylates is not affected by thiol group specific reagents, it may be supposed that the subsequent transfer of aminoacyl and hydroxylacyl residues is inhibited. This was confirmed by the finding that the ability to form covalent enzyme–substrate complexes was reduced after incubation of the enzyme in the presence of thiol group specific agents. Furthermore, this reduced capability was accompanied by a concomitant decline of total synthetic activity.

Figure 5 shows the time course of covalent substrate binding in the presence of iodoacetamide.

When the enzyme was preincubated for different times in the presence of inhibitor, a decrease of thioester formation between enzyme and L-[^{14}C]valine of D-[^{14}C]Hyiv was observed. The site(s) of Hyiv binding was (were) less affected than that of valine binding. After 15 min only 6% of valine sites were accessible for valine, whereas more than 60% of Hyiv sites were still unoccupied. This indicates the presence of chemically distinct SH groups in the active sites for the two substrates.

Discussion

Enniatin synthetase was purified from *F. oxysporum* (ETH 1536/9 strain C 7/5). At the last step of purification, the protein was at least 98% homogeneous as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis. Further attempts to improve the purification always resulted in the same specific activity. In contrast to other antibiotic synthetases, e.g., gramicidin S or tyrocidine synthetase, the enzyme activity for total synthesis of enniatins resided in one single polypeptide chain with a molecular weight of about 250000. This finding was established by the impossibility of finding any subunit from the enzyme after excessive treatment with NaDodSO$_4$ and in the presence of reducing agents even under drastic conditions. Therefore, this enzyme represents a novel type of multifunctional enzyme among peptide and fatty acid synthetases.

The reactions catalyzed by the enzyme are as follows: (1) activation of branched-chain L-amino acids and D-2-hydroxyisovaleric acid via adenylate; (2) transfer of amino- and hydroxyacyl residues to specific thiol groups; (3) methylations of thioesterified amino acids with SAM; (4) condensation reactions between thioesterified *N*-methyl amino acids and D-2-hydroxyisovaleric acid; (5) elongation and cyclization of depsipeptides. Thus, the enzyme resembles other peptide synthetases in some features as activation via adenylate and thioester and perhaps also in the elongation process. The latter similarity may lie in the presence of 4′-phosphopantetheine as a prosthetic group involved in a process commonly referred to as the thiotemplate mechanism. However, N-methylation and ester bond formation have not yet been observed in multifunctional systems. The relative low substrate specificity of the enzyme toward branched-chain amino acids leads under appropriate conditions to the formation of different enniatins.

K_m values for the three relevant branched chain amino acids were lowest in the case of L-valine and highest in the case of L-leucine. These values were in agreement with the finding that under identical substrate concentrations the amounts of enniatins formed in the presence of each of the three different amino acids were highest in the case of enniatin B (valine) and lowest in the case of enniatin C (leucine). Since the organism from which the enzyme was isolated mainly produces enniatin B and smaller amounts of enniatin A, it can be concluded that these enniatins are produced during different periods of the cultivation. This holds especially true if one considers the ability of the enzyme to form mixed-type enniatins which were not observed in the strain used for this investigation. However, we have evidence that formation of mixed-type enniatins was possible when the strain was fed with additional L-isoleucine during the enniatin B formation period (O. Kocian, R. Zocher, and U. Keller, unpublished experiments). This finding clearly indicates that the extent of formation of enniatins in the cell is dependent on (1) the affinity of the enzyme for its substrates (amino acids) and (2) the concentration of the amino acids in the cellular pool.

Such internal regulation of depsipeptide formation seems also to take place in *Fusarium equiseti*, which synthesizes cyclic depsipeptides consisting of *N*-methyl amino acids and D-2-hydroxyisovaleric acid (Simon-Lavoine & Forgeot, 1979). In this organism mixed-type depsipeptide as well as homogeneous depsipeptides (apparently enniatins A and B) have been found. Similar findings were described by Kiryushkin et al. (1968).

The N-methylation of L-valine and also the other substrates for the amino acid site(s) of enniatin synthetase is of considerable importance for the synthetic process. In the absence of SAM, the reaction rate is about 10% of that observed in the presence of the methyl donor. It may be supposed that the N-methylation of the amino group of the substrate brings about an alteration in the molecular structure so that an optimum of substrate juxtaposition can be achieved. It is noteworthy that omission of SAM during synthesis of unmethylated enniatins provokes synthesis of compounds, the structure of which is still unknown. These compounds may be the result of disordered reactions.

The results presented indicate the presence of a highly specialized reaction chain on a multifunctional enzyme consisting of a single polypeptide chain. So far established are the relative broad specificity for amino acid substrates and also the determination of product formation by different K_m values and particularly through the N-methylation step.

The role of the 4′-phosphopantetheine residue(s) covalently attached to the enzyme is still unclear but may be part of the elongation system involved in depsipeptide synthesis.

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