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MECHANISM OF DEPSIPEPTIDE FORMATION CATALYZED BY ENNIATIN SYNTHETASE

Rainer Zocher, Ullrich Keller and Horst Kleinkauf

Institut für Biochemie und Molekulare Biologie,
Technische Universität Berlin, 1000 Berlin 10,
Franklinstr. 29, West Germany.

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Covalently bound intermediates of enniatin B synthesis could be isolated from enniatin synthetase by treatment with performic acid. By comparison with products of mild alkaline cleavage of authentic enniatin B they could be identified as the dipeptide D-2-hydroxyisovaleryl-N-methylvaline and the corresponding tetrapeptide. Synthesis of enniatins apparently proceeds via condensation of dipeptides. This was confirmed by the use of the substrate analogue isovaleric acid, which has shown to be a strong inhibitor for enniatin synthesis by formation of N-isovaleryl-N-methyl valine.

Enniatins are cyclic hexadepsipeptides consisting of three residues of N-methyl amino acids and three residues of D-2-Hyiv, which are arranged in an alternating fashion (1) (fig. 1). They are synthesized by enniatin synthetase, a multifunctional enzyme. This enzyme has been purified to near homogeneity (2) from Fusarium oxysporum. It has been shown that the enzyme activates the residues of enniatins as thioesters via the corresponding adenylates and after the methylation of the amino acid with SAM condenses the various residues to form the depsipeptides (3). Whereas the activation and methylation of residues during enniatin synthesis is now quite well understood (2,3), elongation and cyclisation reactions were still unclear. In this paper experiments are described which deal with the reaction mechanism of depsipeptide formation.

Abbreviations:
SAM, S- adenosyl- L- methionine; Hyiv, hydroxyisovaleric acid; NMeVal, N- methyl- L- valine; HVEP, high voltage electrophoresis.

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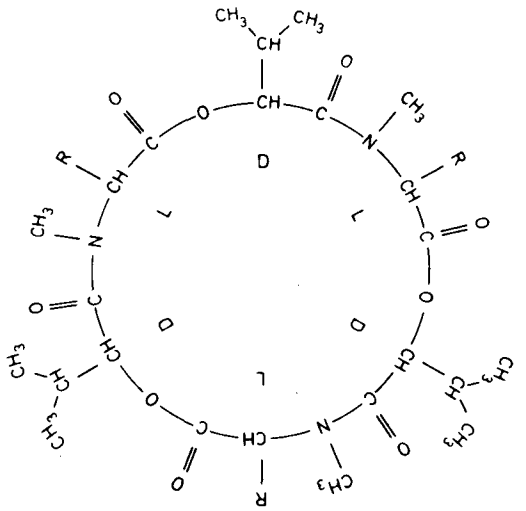

Fig. 1. R= $-CH(CH_3)CH_2CH_3$ Enniatin A
R= $-CH(CH_3)_2$ Enniatin B
R= $-CH_2CH(CH_3)_2$ Enniatin C

Materials and Methods

Radioactive DL-2-hydroxyisovaleric acid and unlabelled D-Hyiv were prepared from DL-[1- ^{14}C]valine (spec. act.: 60 Ci/mole) and D-valine respectively as described (5). Spec. act. of L-[U-^{14}C]valine was 285 Ci/mole. Radioactive compounds were purchased from Amersham. All other reagents were of highest purity commercially available.

Labelled compounds on thinlayer plates were detected with a Berthold thin layer scanner, system BF 210. Quantitative measurements were done with a Packard TRI-CARB liquid scintillation spectrometer C 2425 using toluene based scintillation liquid.

Enzyme purification and enzyme assay were performed as described (2).

Covalently bound intermediates were liberated by performic acid oxidation as described (6).

The following tlc systems on silicagel plates (Merck, 0.2mm) were used: Solvent system I: EtOAc: MeOH:H_2O=100:5:1 (v/v/v), Solvent system II: BuOH:AcOH:H_2O=4:1:1 (v/v/v). Thinlayer high voltage electrophoresis was carried out on cellulose plates (Macherey & Nagel, Düren, Germany).

Determination of covalently bound radioactivity was carried out as described by Roskoski et al. (7).

ATP/pyrophosphate exchange was tested as described (8).

Isovaleryl valine was prepared from isovaleryl chloride and valine by Schotten Baumann reaction.The authenticity of the compound was checked by mass spectrometry.

L-2-bromoisovaleric acid and D-2-bromoisovaleric acid were prepared from D-valine and L-valine, respectively, by reaction with HNO_2 in the presence of KBr (9).

Mild alkaline cleavage of enniatin B was done according to Plattner et al. (10). Hydrolysis was stopped after 15 min by the addition of 0.1 N H_2SO_4 (final pH=1). The ether extract was separated by tlc using solvent sytem I. Products of hydrolysis were detected by spraying with aqueous 0.02% bromophenol blue solution. Bands were extracted with acetone and analyzed by mass spectrometry.

Results and Discussion

The formation of thioesters (3) and the presence of 4'- phosphopantetheine in enniatin synthetase suggest that covalently bound intermediates are present as in the case of gramicidin S (11). To verify this assumption enniatin synthetase was incubated together with all ingredients necessary for enniatin B formation ([^{14}C] valine as the radiolabel). After a brief incubation period (2 min) the reaction was stopped by the addition of 7% trichloroacetic acid. Protein was centrifuged and washed several times with trichloroacetic acid and alcohol. Finally, it was treated with performic acid. In a control experiment formic acid was used instead of performic acid. Thin layer chromatographic analysis of reaction products on silica gel (fig. 2a, lane A) revealed the presence of three radioactive compounds (besides [^{14}C] N- methyl valine), designated I, II and III. When [^{14}C] DL- Hyiv was used as a radiolabel, compounds with identical Rf- values were obtained (not shown). The compounds

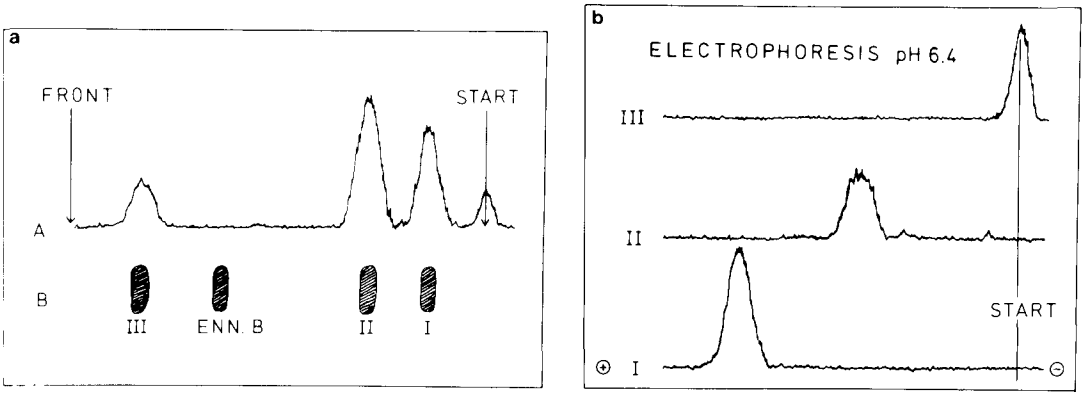

Fig. 2a. Separation of radioactive intermediates by tlc (solvent system I) after performic acid treatment (lane B) and products of limited mild alkaline cleavage of enniatin B (lane A). The same pattern was observed. Compounds I, II and III could be identified as the dipeptide, tetrapeptide and the cyclo dipeptide. The bands were visualized with bromophenol blue.

Fig. 2b. Separation of radioactive intermediates I, II and III by high voltage thin layer electrophoresis on cellulose plates at pH 6.4. Compounds I and II are moving with different mobilities towards the anode, whereas compound III shows no electrophoretic migration.

were isolated from the plates and separately subjected to thin layer electrophoresis at pH 6.4 on cellulose plates. Compounds I and II migrated toward the anode, whereas compound III remained at the origin. At pH 1.9 none of the compounds moved in the electric field. Further analyses revealed that mild alkali treatment of compounds II and III converted them into compound I. Dilute acid converted compound I to compound III. The interconversion of compounds indicates that they must contain the same structural unit in different arrangements. Acid hydrolysis (22h, 105°C, 6N HCl) of each of the radioactive compounds yielded exclusively [^{14}C] NMeVal or [^{14}C] D- Hyiv when [^{14}C] L- Val or [^{14}C] DL- Hyiv was used as the radiolabel. Thus the structural unit must be a dipeptide between D- 2- Hyiv and NMeVal.

Saponification of authentic enniatin B followed by acidification and extracion with ether and separation of the products by tlc yielded three bands with Rf- values on tlc and HVEP plates identical to those obtained by performic acid treatment of enzyme bound radioactive intermediates (fig. 2a, lane B). Mass spectrometric determinations revealed that compound I was identical with the dipeptide 2- hydroxyisovaleryl- N- methyl valine ($M-H_2O$)$^+$ = 213. Compound II gave a molepeak corresponding to the tetrapeptide ($M-H_2O$)$^+$ = 426. Compound III had a molepeak corresponding to the cyclic dipeptide 2,5,- dioxo- 3,6,- diisopropyl- morpholine (M^+ = 213). The results can be interpreted as follows: Limited alkaline cleavage of enniatin B yields the dipeptide 2- hydroxyisovaleryl- N- methyl valine and to a minor extent the corresponding tetrapeptide. Under acidic conditions the dipeptide is able to undergo intramolecular condensation to give the corresponding dioxomorpholine. The hexapeptide could only be detected in traces ($M-H_2O$)$^+$ = 639.

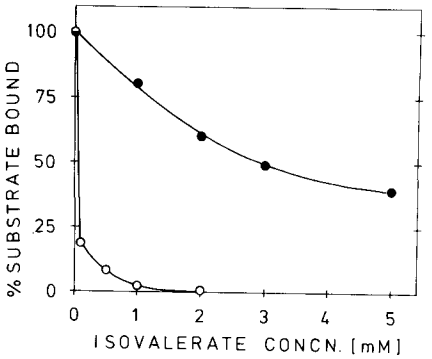

Fig. 3. Inhibition of thioester formation by isovaleric acid. The enzyme (20 μg) was preincubated in the presence of different concentrations of isovaleric acid (10 min). Then 0.5 μCi of [^{14}C] valine or [^{14}C]Hyiv were added. After 10 min the reaction was stopped by addition of trichloroacetic acid. Covalently bound radioactivity was determined as decribed (6). The capability of the enzyme to form active thioesters is nearly completely abolished at concentrations of 1-2 mM isovaleric acid for Hyiv O——O . In the case of valine ●——● total inhibition was observed at about 20 mM of isovaleric acid.

We conclude that enniatin B synthesis proceeds via the condensation of three identical dipeptides consisting of D- Hyiv and NMeVal. To confirm these findings, several analogues of D- Hyiv and L- valine lacking the OH or NH_2- group were tested with respect to their ability to compete with the two substrates during enniatin synthesis. Among D- bromoisovaleric acid, L- bromoisovaleric acid and isovaleric acid the last was found to be most effective inhibiting enniatin synthesis completely when present in concentrations of about 1 mM. The data presented in figure 3 clearly indicate the inhibition by isovaleric acid of active thioester formation. The different behaviour toward Hyiv and valine binding can be explained by the different affinities of the enzyme for its substrates (2). The Km- value of valine has been found to be about 20- fold higher than that of D-2-Hyiv. Therefore isovaleric acid can be considered as an inhibitor for both amino acid and hydroxyacid binding site(s). Since isovaleric acid lacks the OH or NH_2- group there is no possibility of chain elongation and hence enniatin synthesis stops.

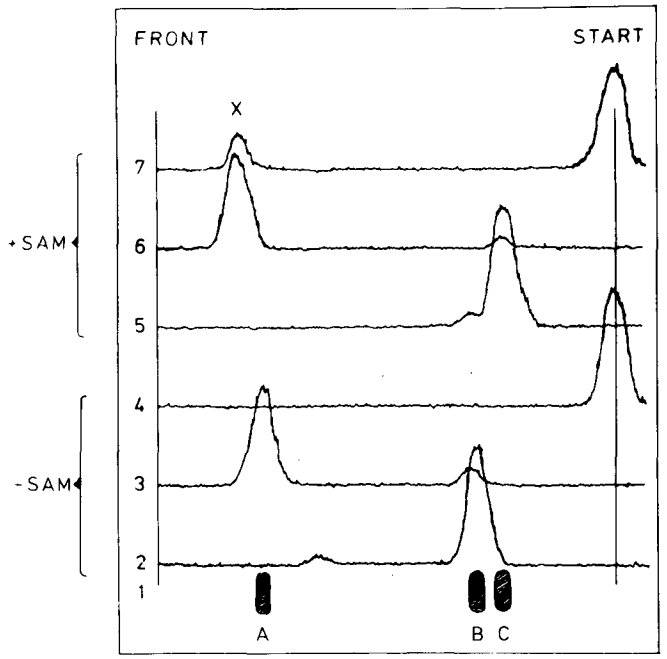

Fig. 4. Influence of isovaleric acid on the formation of covalently bound intermediates (separation by tlc using solvent system II). First the enzyme was loaded in triplicate with [^{14}C] valine. In parallel experiments the same conditions were used with the additional presence of SAM. After 5 min to two of the incubations isovalerate was added (final concn. 10 mM) and after a further 5 min the reaction was stopped by addition of 7 % trichloroacetic acid. Lane 1: Marker substances: A= isovaleryl valine, B= valine, C= NMeVal. Lane 2: Val-enzyme-complex + performic acid. Lane 3: Val-enzyme-complex + isovalerate + performic acid. Lane 4: Val-enzyme-complex + isovalerate + formic acid. Lane 5: NMeVal-enzyme-complex + performic acid. Lane 6: NMeVal-enzyme-complex + isovalerate + performic acid. Lane 7: NMeVal-enzyme-complex + isovalerate + formic acid. (Compound X is obviously isovaleryl-N-methyl valine).

Measurements of ATP/PPi- exchange reactions dependent on L- valine, D- Hyiv and isovaleric acid revealed that the latter compound is activated by the enzyme via adenylate. However, the rate of reaction was less than in the case of L- valine (35%) and D-2-Hyiv (22%) at concentrations of 5 mM of the individual substrates. These results suggest the use of isovaleric acid as a probe for the elucidation of the reaction mechanism of peptide or ester formation during enniatin synthesis.

The influence of isovaleric acid on the formation of covalently bound intermediates during enniatin synthesis was also measured.

First the enzyme was incubated in triplicate with [^{14}C]valine, ATP and Mg^{2+}. In parallel experiments the same conditions were used with the additional presence of SAM (fig. 4). Without the addition of isovaleric acid performic acid treatment of enzyme yielded exclusively [^{14}C]valine or [^{14}C]NMeVal (lane 2 and 5). In the presence of isovaleric acid a second compound was released from the enzyme which in the absence of SAM was identified as isovaleryl valine by chromatographic comparison with the authentic product (lane 4). This compound was not released by formic acid treatment indicating the presence of an isovaleryl valyl thioester. In the presence of SAM a compound could be released by performic acid treament with a slightly higher Rf- value which is obviously the corresponding iso- valeryl- N- methyl valine. Acid hydrolysis of the latter product yielded NMeVal. Additional experiments using [^{14}C-methyl] SAM revealed the exclusive labelling of this compound and NMeVal (not shown). Interestingly, isovaleryl- N- methyl valine was partially released by treatment with formic acid, indicating that the corresponding thioester is sensitive against the action of acid. Apparently the methylated peptide residue renders the SH- group more labile. This is in good agreement with the earlier finding that SAM stimulates the formation of enniatin B 8 to 10- fold compared to the unmethylated product (2). When the same series of experiments was repeated with [^{14}C]Hyiv only Hyiv could be isolated after performic acid treatment (not shown).

These data demonstrate that enniatin synthesis proceeds via the dipeptide between D- Hyiv and NMeVal. Thus the reaction sequence for enniatin synthesis involves the formation of a dipeptide between D- Hyiv and NMeVal (fig. 5) and its subsequent condensation to give the cyclic hexapeptide. The results also reveal that the synthesis of depsipeptides proceeds via a

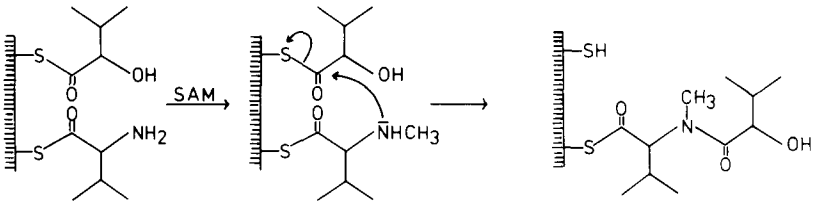

Fig. 5. Scheme of dipeptide formation

reaction chain different from that of other peptide synthetases like gramicidin S where the succesive addition of amino acids to the growing peptide chain has been described (11). However, it is not clear whether the tetrapeptide observed after performic acid treatment of the enzyme is a real intermediate or an artifact due to the action of the acid.

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