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Biosynthesis of Cyclosporin A: Partial Purification and Properties of a Multifunctional Enzyme from *Tolypocladium inflatum*†

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Received May 3, 1985

ABSTRACT: An enzyme fraction most probably involved in the biosynthesis of cyclosporin A was purified 540-fold from *Tolypocladium inflatum*. The enzyme was capable of forming covalent enzyme–substrate complexes and catalyzed the ATP–pyrophosphate exchange reactions dependent on the unmethylated constituent amino acids of cyclosporin A. Evidence was obtained that covalent binding of substrate amino acids occurred via thioester linkage. Furthermore, the N-methylation of thio-esterified valine, leucine, and glycine residues with *S*-adenosyl-L-methionine was demonstrated. De novo synthesis of cyclosporin A was not observed but the formation of the diketopiperazine *cyclo*-(D-Ala-MeLeu) from D-alanine and L-leucine under the consumption of ATP and *S*-adenosyl-L-methionine. This cyclodipeptide represents a partial sequence of cyclosporin A. Molecular mass determinations revealed the enzyme activity to be lying in the range of about 700 kDa.

Cyclosporin A is a cyclic undecapeptide with immunosuppressive properties (Borel et al., 1977) produced by the fungus *Tolypocladium inflatum* (Traber et al., 1977). Its structure is shown in Figure 1. Besides the unusual amino acids 2-aminobutyric acid, D-alanine, and the (2*S*,3*R*,4*R*,6*E*)-2-amino-3-hydroxy-4-methyloct-6-enoic acid (C_9-acid), it also contains a number of N-methylated peptide bonds similar to the fungal depsipeptides enniatin and beauvericin. The latter compounds have been shown to be synthesized by large multienzymes from their primary precursors L-amino acid and D-hydroxy acid under consumption of ATP (Zocher et al., 1978, 1982; Peeters et al., 1983). Depsipeptide synthesis involves activation of the different substrates by thioester linkage via the corresponding adenylates, N-methylation of the covalently bound amino acid residues, and elongation and cyclization reactions (Zocher et al., 1983).

The biosynthesis of cyclosporin A is also likely to proceed by a nonribosomal process as indicated by the cyclic structure and the presence of several unusual amino acids in this compound. Studies so far have been made by feeding experiments with ^{13}C (Kobel et al., 1983) and ^{14}C-labeled precursors (Zocher et al., 1984). The results suggest that the *N*-methyl groups originate from L-methionine and the relevant amino acids from their naturally occurring precursors. This paper describes the partial purification and characterization of a multienzyme from *T. inflatum* most probably involved in cyclosporin biosynthesis.

Materials and Methods

Chemicals. Chemicals were usually of the highest purity commercially available. L[U-^{14}C]Leucine (specific radioactivity 337 Ci/mol), L-[U-^{14}C]alanine (165 Ci/mol), D-[U-^{14}C]alanine (40 Ci/mol), L-[U-^{14}C]valine (290 Ci/mol), and $Na_4{}^{32}P_2O_7$ were obtained from Amersham. [U-^{14}C]Glycine (100 Ci/mol) and DL-2-amino[3,4-^{14}C]butyric acid were from CEA (Sacley, France). The N-methylated derivatives of L-valine, L-leucine, and glycine were obtained from Bachem (Bubendorf, Switzerland). Authentic cyclosporin A, C_9-acid, and *N*-methyl-C_9-acid were generously supplied by Dr. H. Kobel (Sandoz Ltd., Basle, Switzerland).

Organism and Growth Conditions. Tolypocladium inflatum (deposited as *Trichoderma polysporum* DSM 915) was maintained on agar slants (3% molasses, 1% cornsteep liquor, 1.5% agar). Strain 29-1 used in this study was selected after nitrosoguanidine mutagenesis as described (Madry et al., 1983). Spore suspensions were obtained by filtering 21 day old submerged cultures (3% molasses, 1% cornsteep liquor) through a double layer of Cleenex cloth. All submerged cultures were run on a rotatory shaker (115 rpm, 27 °C) with 500-mL Erlenmeyer flasks containing 100 mL of medium. The cultures were harvested by suction filtration about 72 h after inoculation. The mycelial cake was washed with deionized water and stored at −80 °C.

Enzyme Preparation. All operations were carried out at 4 °C. Buffer A was 50 mM potassium phosphate buffer (pH 7.3) containing 10% glycerol, 1 mM EDTA,[1] and 5 mM dithioerythritol. Freeze-dried mycelium was homogenized with sand in a mortar and extracted with 20 volumes per gram dry weight of buffer A containing 0.3 M KCl. After 40 min of gentle stirring, the homogenate was centrifuged for 20 min at 20000*g*. A neutral solution of Polymin P (BASF, Ludwigshafen, FRG) (5%) was added to give a final concentration of 0.2% in order to remove nucleic acids. After 20 min the extract was centrifuged as described above. Saturated ammonium sulfate solution in buffer A was gradually added to the supernatant. Precipitates were pelleted by centrifugation (20 min, 25000*g*). The precipitate between 30 and 50% saturation was dissolved in a minimal volume of buffer A. The enzyme solution was applied to an Ultrogel AcA 22 column (2.3 × 47 cm) previously equilibrated with buffer A. Fractions of 5.4 mL were collected. Active fractions were stored at −80 °C.

† This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 9, Teilprojekt C 3).

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; Me-Leu, *N*-methyl-L-leucine.

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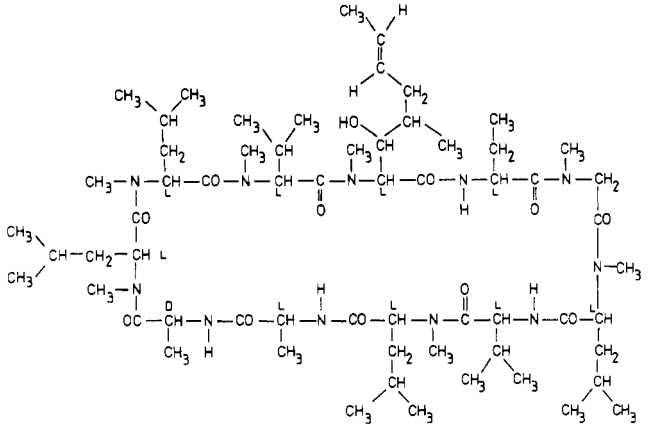

FIGURE 1: Structure of cyclosporin A.

Assay of cyclo-(D-*Ala-MeLeu*) *Synthesizing Activity in Cell-Free Extracts.* The assay mixture contained ATP (7.3 mM), $MgCl_2$ (7.3 mM), AdoMet (3.7 mM), D-alanine (3.7 mM), and 0.5 μCi of L-[U-^{14}C]leucine in a total volume of 250 μL. After 30-min incubation at 25 °C, 2 mL each of H_2O and EtOAc was added to the assay mixture. EtOAc-extractable compounds were separated by different TLC systems on silica gel (see below). The amount of diketopiperazine formed was calculated by measuring the peak areas of scanning curves obtained with a Berthold thin-layer scanner system BF 210. A calibration curve was made with L-[U-^{14}C]leucine as a standard.

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

ATP–PP$_i$ Exchange. Amino acid dependent ATP–PP$_i$ exchange was carried out as described (Gevers et al., 1969).

Molecular Weight Determinations. The molecular weight of the native enzyme was determined by gel filtration on Sepharose 6B (bed dimensions 2.4 × 53 cm) in buffer A. The column was calibrated with apoferritin (430 000), bovine thyroglobulin (670 000), human IgG (150 000), and bovine serum albumin (68 000).

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

Thin-Layer Chromatographic Analyses. Separation of amino acids and their *N*-methyl analogues was carried out on silica gel plates (Merck) with BuOH/AcOH/H_2O (4:1:1). *cyclo-*(D-Ala-MeLeu) was separated on silica gel plates with EtOAc/MeOH/H_2O (100:5:5) (Keller & Kleinkauf, 1977) and diisopropyl ether/$CHCl_3$/AcOH (6:3:1) (Nitecki et al., 1968).

High-Pressure Liquid Chromatography. HPLC of *cyclo-*(D-Ala-MeLeu) was performed on a Knauer HPLC system with a Lichrosorb RP-18 (Pharmacia) column, which was run with 0.1 M potassium phosphate (pH 2.1)/MeOH (50:50).

Synthesis of cyclo-(D-*Ala-MeLeu*). This was carried out according to Nitecki et al. (1968) with *N*-methyl-L-leucine methyl ester and *N*-Boc-D-alanine as the starting substances. The authenticity of the compound was checked by mass spectrometry.

Determination of the Optical Configuration of Alanine. This was done according to Gevers et al. (1969). The amounts of ^{14}C-labeled D- and L-alanine in acid hydrolyzates were determined with D-amino acid oxidase (porcine kidney) and L-amino acid oxidase (*Crotalus atrox* venom), respectively. Reaction mixtures were then separated by ion-exchange column chromatography (Dowex cation exchanger, 50W-X2). Samples were applied at pH 2, and after the column was washed with water the amino acids were eluted by increasing the pH (2 M NH_4OH).

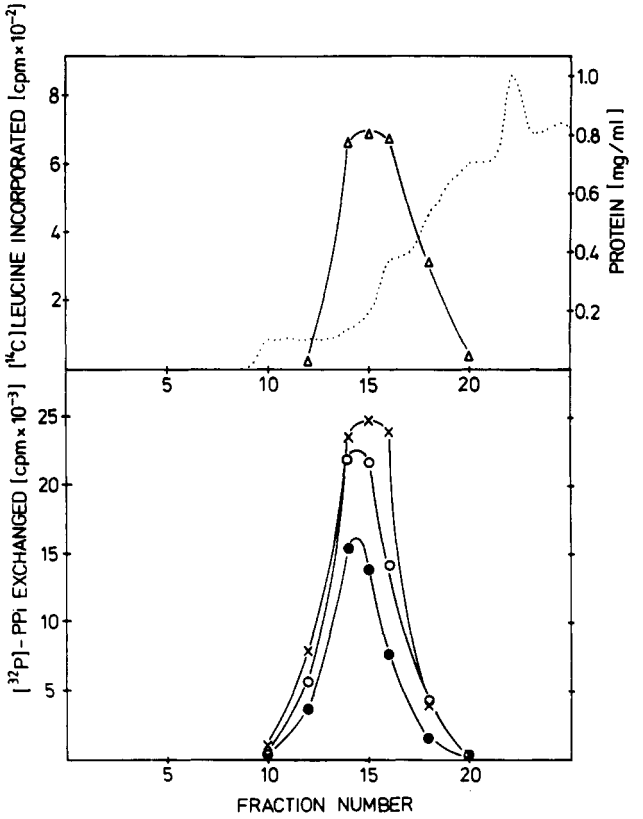

FIGURE 2: Ultrogel AcA 22 gel filtration of ammonium sulfate fractionated crude extracts of *T. inflatum* (5.2-mL fractions were collected): (···) absorbance at 280 nm; (●) C_9-acid-dependent ATP–PP$_i$ exchange; (○) D-Ala-dependent ATP–PP$_i$ exchange; (×) L-Leu-dependent ATP–PP$_i$ exchange; (△) L-[^{14}C]Leu incorporation into *cyclo-*(D-Ala-MeLeu).

RESULTS

Activation Studies of Amino Acid Residues of Cyclosporin A. Attempts were made to establish a cell-free system of total cyclosporin A synthesis using crude extracts from *T. inflatum.* However, no evidence was obtained for the in vitro formation of this compound. Therefore, we tried to isolate partial activities of the hypothetical cyclosporin A synthesizing complex by screening protein fractions obtained by gel filtration on Ultrogel AcA 22 by measuring their capability to catalyze the ATP–pyrophosphate exchanges dependent on the constituent amino acids of cyclosporin A. It was expected from previous findings with enniatin synthetase (Zocher et al., 1976, 1978) that the nonmethylated species were activated instead of the N-methylated residues.

Figure 2 shows the separation of a protein extract fractionated by poly(ethylenimine) and ammonium sulfate precipitation. As can be seen, an ATP–pyrophosphate exchange activity dependent on D-Ala, L-Leu, and the unusual C_9-acid could be detected in fractions 12–18. The other amino acids of cyclosporin A also exhibited a significant ATP–pyrophosphate exchange in contrast to the N-methylated analogues or amino acids not related to those present in the peptide chain of cyclosporin A, e.g., L-proline or L-aspartate (not shown). The optimal pH for the pyrophosphate exchange was found to lie between 7.0 and 7.3 in the potassium phosphate buffer system.

It was of interest to see whether these compounds were bound to the enzyme by thioester linkage as has been shown

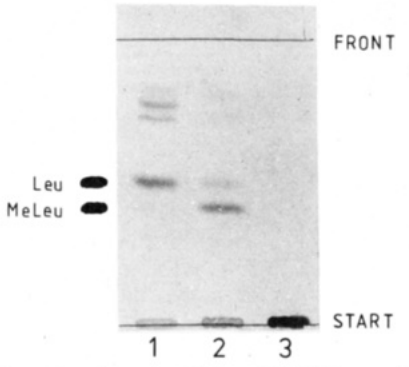

FIGURE 3: Liberation of covalently bound [^{14}C]Leu or [^{14}C]MeLeu by treatment with performic acid after labeling of the enzyme with [^{14}C]Leu (lane 1) or with [^{14}C]Leu in the additional presence of AdoMet (lane 2). Lane 3 represents a control experiment with formic acid instead of performic acid using the [^{14}C]Leu-labeled enzyme. TLC was carried out as described under Materials and Methods. Exposure of the chromatogram to X-ray film was for 4 days.

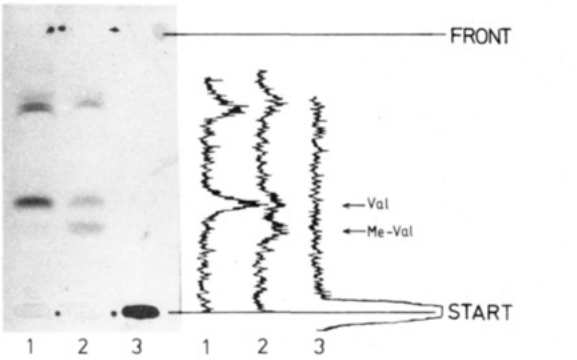

FIGURE 4: Liberation of covalently bound [^{14}C]Val and [^{14}C]MeVal by treatment with performic acid after labeling of the enzyme with [^{14}C]Val (lane 1) or with [^{14}C]Val in the additional presence of AdoMet (lane 2). Lane 3 represents a control experiment with formic acid instead of performic acid using the [^{14}C]Val-labeled enzyme. TLC was carried out as described under Materials and Methods. Exposure of the chromatogram to X-ray film was for 4 days.

in numerous cases in nonribosomal peptide synthesis (Kleinkauf & von Döhren, 1981). Figures 3 and 4 show the results of experiments where covalently bound leucine or valine was split off the enzyme with performic acid (lane 1) but not with formic acid (lane 3). Glycine, L-aminobutyric acid, and L-alanine were also bound to the enzyme via thioester linkage (not shown). The C_9-acid was not tested because it was not available in labeled form. Further evidence for the thioester bond between the substrates and the enzyme came from the findings that thiol group blocking agents such as *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, iodoacetic acid, or iodoacetamide prevented its formation (not shown).

Mechanism of N-Methylation. Cyclosporin A contains a number of N-methylated amino acids, and it was assumed that their synthesis was accomplished by a mechanism similar to that of the previously described enniatin biosynthesis. From Figure 3 (lane 2) and 4 (lane 2) it turns out that the N-methylation takes place at the stage of the thio-esterified amino acid, because the *N*-methyl amino acid can be split off the protein by performic acid treatment, when the enzyme had been incubated in the additional presence of AdoMet. It is obvious that in the case of leucine (Figure 3) most of the radioactivity is localized in a band corresponding to *N*-methylleucine. This would fit with the exclusive presence of *N*-methylleucine residues in cyclosporin A. On the other hand, the same experiment performed with radioactive valine revealed an almost equal distribution of the radioactivity between

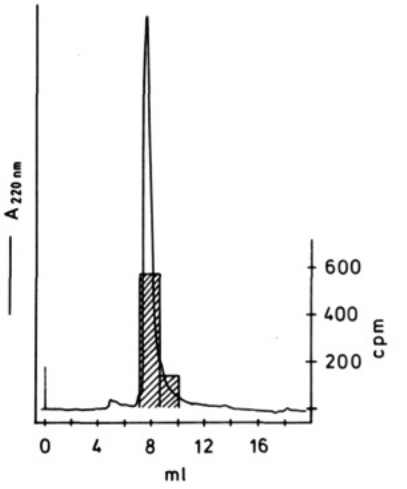

FIGURE 5: HPLC separation of a mixture of synthetic *cyclo*-(D-Ala-MeLeu) and [^{14}C]Leu-labeled enzymic reaction product on a Lichrosorb RP 18 column (flow rate 1.4 mL/min). Dashed area represents radioactivity eluted.

valine and *N*-methylvaline as shown in Figure 4 (lane 2). This latter finding is in agreement with the presence of one residue of each valine and *N*-methylvaline in the peptide chain of cyclosporin A.

Formation of cyclo-(D-Ala-MeLeu). Incubation of the enzyme fraction with D-Ala, [^{14}C]Leu, AdoMet, and ATP/Mg^{2+} resulted in the formation of the diketopiperazine *cyclo*-(D-Ala-MeLeu). Evidence for the identity of this compound came from chromatographic comparison on various TLC systems and HPLC with the chemically synthesized compound (Figure 5). Furthermore, acid hydrolysis of the radioactive product obtained after using D-[^{14}C]Ala or L-[^{14}C]Leu as radiolabels yielded exclusively D-[^{14}C]Ala and [^{14}C]MeLeu, respectively. In the absence of AdoMet no product formation could be observed. The other constituent amino acids of cyclosporin A had no influence on the extent of formation of the cyclodipeptide. It is noteworthy that the rate of the synthesis of the diketopiperazine was favored by a relatively high pH value with an optimum of about 9.0–9.5 in contrast to the activation reactions. The time course of *cyclo*-(D-Ala-MeLeu) formation followed a linear dependence up to 90 min. Similarly, a linear dependence of diketopiperazine formation on enzyme concentration was observed (not shown).

A possible explanation for the formation of the *cyclo*-(D-Ala-MeLeu) and the lack of cyclosporin A total synthesis could lie in the origin of the D- and L-Ala portion in the cyclosporin A molecule. If free L-Ala is not the direct precursor of the enzyme-bound L-Ala portion, i.e., formed by a cofactor-dependent epimerase function of the enzyme from D-Ala, a growing peptide chain would be terminated by D-Ala in the absence of cofactor(s). A number of possible racemaze cofactors such as NAD^+, $NADP^+$, FAD, pyridoxal phosphate, Fe^{3+}, and glutathione were tested in the presence of all other constituent amino acids of cyclosporin A, ATP/Mg^{2+} and AdoMet. However, in no case was formation of cyclosporin A observed.

The hypothetical existence of an enzyme-bound D-Ala epimerase would lead to an equal distribution of radioactive D- and L-Ala in cyclosporin A during feeding experiments of *T. inflatum* with D-[^{14}C]Ala. However, D-[^{14}C]Ala was found to be almost exclusively incorporated into the D-Ala position of cyclosporin A (not shown) as meausred after acid hydrolysis of cyclosporin by the D-amino acid oxidase method (see Materials and Methods).

Table I: Purification of *cyclo*-(D-Ala-MeLeu) Synthesizing Enzyme

purification step	total act. (units)[a]	total protein (mg)	sp act. (units/mg of protein)	purification (*x*-fold)
crude extract	557	1555	0.36	(1.00)
$(NH_4)_2SO_4$ precipitation (30–50% saturation)	870	110	7.9	22
Ultrogel AcA 22	966	5	193	540

[a] One unit is defined as the amount of enzyme that incorporates 1 pmol of L-[^{14}C]leucine into *cyclo*-(D-Ala-MeLeu) in 30 min under the conditions described (see Materials and Methods).

Protein Purification, Molecular Weight, and Enzyme Stability. The purification of *cyclo*-(D-Ala-MeLeu) synthesizing activity is shown in Table I. After the different purification steps, a 540-fold enrichment was obtained. The apparent molecular weight of the enzyme activity in gel filtrations monitored by the D-Ala-dependent ATP–pyrophosphate exchange strongly depended on the starting material used. In the case of freeze-dried mycelium, nearly all of the activity resided in a fraction corresponding to a molecular weight of about 700 000. When wet mycelium was used, most of the activity eluted in a fraction with a molecular weight of about 200 000. With respect of the capability to catalyze the formation of the *cyclo*-(D-Ala-MeLeu), only the high molecular weight enzyme fraction was active. In addition, only this enzyme fraction was able to form covalent enzyme–substrate complexes. On the other hand, both enzymes were able to catalyze the ATP–pyrophosphate exchanges dependent on all of the amino acid constituents of cyclosporin A. After the Ultrogel AcA 22 step, the heavy enzyme could be stored in the presence of 20% glycerol at −80 °C without any loss of activity.

Discussion

An enzyme fraction with an apparent molecular weight of about 700 000 was purified from crude extracts of *T. inflatum*, a cyclosporin A producer. The results suggest evidence that this enzyme is involved in the biosynthesis of the cycloundecapeptide cyclosporin A. Such evidence came from the findings that the enzyme catalyzes the activation of the amino acids present in the peptide chain of cyclosporin A as thioesters via the corresponding adenylates. Furthermore, the enzyme was shown to catalyze the N-methylation of thio-esterified amino acids present in cyclosporin A.

These results illustrate another example of an amino acid N-methylation as shown previously in the case of cyclodepsipeptide synthesis (Zocher et al., 1982; Peeters et al., 1983). The formation of the diketopiperazine *cyclo*-(D-Ala-MeLeu) strongly resembles that of *cyclo*-(D-Phe-Pro) in the gramicidin S system (Kurahashi, 1961). Significant amounts of the latter diketopiperazine are detectable when only D-Phe and L-Pro are present as the substrate amino acids of gramicidin S synthetase (Otani et al., 1966). Apparently in the case of "cyclosporin synthetase", a similar situation can be observed. It may be speculated that due to the inability of the enzyme to synthesize the whole undecapeptide the synthesis of *cyclo*-(D-Ala-MeLeu) takes place. Compared with the *cyclo*-(D-Phe-Pro) in the gramicidin S system, it must be assumed that the *cyclo*-(D-Ala-MeLeu) synthesis is the result of a nonenzymatic intramolecular cyclization of an enzyme-bound thio-esterified dipeptide. This is consistent with the finding that cyclodipeptide formation is favored at high pH conditions (9.0–9.5) under which free amino or imino groups are most reactive.

The reason for the lack of the overall synthesis of cyclosporin A is still unclear. A possible explanation may be the absence of an essential factor (protein, substrate) in our cell-free system.

Acknowledgments

E.P. thanks the Erwin-Riesch-Stiftung (Lorch, FRG) for a fellowship. We are indebted to Dr. H. Kobel (Sandoz Ltd., Basel) for the supply of various chemicals and to K. Eckart for mass spectrometric analyses.

Registry No. ATP, 56-65-5; PP_i, 14000-31-8; AdoMet, 29908-03-0; *cyclo*-(D-Ala-MeLeu), 99560-20-0; cyclosporin A, 59865-13-3.

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