A specific alanine racemase, which is a key enzyme in the biosynthesis of the undecapeptide cyclosporin A, was purified to electrophoretic homogeneity from the fungus *Tolypocladium niveum*. This is the first enzyme of this kind isolated from an eucaryotic organism. The enzyme catalyzes the reversible racemization of alanine and requires pyridoxal phosphate as the exclusive co-factor. *Km* values for L- and D-alanine were found to be 38 and 2 mM, respectively. Maximal reaction velocity was observed at 42 °C and pH 8.8 for the L to D direction. Molecular mass determinations of the denatured enzyme by SDS-polyacrylamide gel electrophoresis gave a value of 37 kDa, whereas gel filtration calibration studies yielded a value between 129 and 150 kDa, indicating an oligomeric native structure.

Cyclosporin A is a cyclic undecapeptide (Fig. 1) with anti-inflammatory, antifungal, and antiparasitic properties (1). As a potential immunosuppressive agent, cyclosporin A profoundly represses the cellular immune response to foreign antigens (2) and is used worldwide to prevent allograft rejection. Cyclosporin A is produced in a non-ribosomal manner by the fungus *Tolypocladium niveum*. The biosynthesis involves at least 40 different reaction steps (5, 6) and is catalyzed by the cyclosporin synthetase, a multifunctional enzyme with a molecular mass of about 1,500 kDa (3, 4). This enzyme activates the constituent amino acids of cyclosporin A to amino acyl adenylates and binds them covalently via thioester linkages. At this stage, seven of the substrate amino acids become N-methylated by a methyltransferase function, an integral activity of the cyclosporin synthetase, using AdoMet as a methyl group donor (3). Finally peptide bond formation is facilitated by a prothetic 4'-phosphophantetheine group, and the cyclosporin molecule is released from the enzyme (6). In addition to L-valine, L-leucine, and glycine, 2-aminoabutyric acid, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine, and D-alanine are precursors for this process. Whereas first insights into the biosynthetic pathways of (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine have recently been achieved (7), the origin of D-alanine in *T. niveum* is not understood so far. This paper describes the identification and purification of an alanine racemase from *T. niveum*. We discuss the function of the racemase within the biosynthesis of cyclosporin A and the diketopiperazine cyclo(d-alanyl-N-methyl-lylleucine) (d-DKP, Fig. 2), which is a further product of cyclosporin synthetase, and we compare the properties of the enzyme with its procarboxylic counterparts.

**Experimental Procedures**

**Materials**—Chemicals were of the highest purity commercially available. Radiolabeled compounds were purchased from Amersham Corp. Amino acids were obtained from Fluka Chemie AG. D-Amino acid oxidase, L-alanine dehydrogenase, and lactate dehydrogenase are products of Boehringer Mannheim. EHC peptone was procured from Amber, and casein peptone was obtained from Difco. Cyclosporin synthetase and cyclosporin A were isolated and purified using the procedures described by Zocher et al. (3).

**Growth of Organisms**—*T. niveum* strain 7339/45 was donated by Sandoz Ltd. and maintained on agar slants (1% malt extract, 0.5% yeast extract, 1.5% agar). For purifications we used chemically defined medium (MCP 75) as described by Billich and Zocher (6). MCP 75 contains the following ingredients per liter of distilled water: 75 g of maltose, 25 g of casein peptone, 1 g of KH₂PO₄, and 2.5 g of KCl; pH of the medium was 5.5. After 48 h 10 ml of the preculure were used as inoculum for 10 flasks with 200 ml of COPA medium (same composition as MCP 75, but containing EHC peptone instead of casein peptone). Cultures were maintained on a rotary shaker (180 rpm) at 26 °C and harvested after 145 h by suction filtration. The mycelial cake was washed with distilled water, shock-frozen at −80 °C, and lyophilized.

**Purification of Alanine Racemase**—All operations were carried out at 4 °C. Buffer A used throughout the purification procedure was 50 mM Tris, pH 8.5, containing 4 mM EDTA, 20 mM dithiothreitol, and 50 μM pyridoxal phosphate unless otherwise stated. The lyophilized mycelium was suspended in buffer A with 300 mM KCl and 200 mM Tris. After disruption by French press (16,000 p.s.i.), cell debris was removed by centrifugation at 20,000 × g for 30 min. The resultant extract was dialyzed against buffer A and loaded onto a 60-ml QAE-Sepharose column (Pharmacia LKB Biotechnology Inc.) with a flow rate of 2 ml/min. The column was washed with buffer A and eluted with the same buffer containing 150 mM NaCl. Active fractions were pooled and applied to hydrophobe interaction chromatography on a 30-ml phenyl-Sepharose column (Pharmacia). After washing with buffer A the protein was eluted by a linear gradient of 0-7% Triton X at 1 ml/min. Active fractions were concentrated by ultrafiltration (Cenitom 30 from Amicon, Inc.) to give a final volume of 5 ml and subjected to fast performance liquid chromatography on a Superox 200 16/60 column (Pharmacia), using buffer B (50 mM HEPES, pH 7.5, 200 mM NaCl, 20 mM dithiothreitol, and 50 μM pyridoxal phosphate). The flow rate was 0.5 ml/min with a fraction size of 2 ml. After addition of glycerol (10% final concentration), protein was loaded on a 2-ml cellulose phosphate column (E. Merck, Darmstadt, Germany) and washed extensively with buffer C (buffer B, pH 7.5, containing 10% glycerol). The enzyme was eluted by a linear NaCl gradient from 0 to 220 mM in 5 min and from 220 to 400 mM in 20 min (flow rate, 1 ml/min; fraction size, 2 ml). Active fractions were diluted with 1 volume of distilled water and bound to a MonoQ anion exchange column (Pharmacia) on fast performance liquid chromatography. The protein was used for elution with 0–70 mM NaCl (buffer B) in 10 min and 70–120 mM in 60 min at 1 ml/min. Active fractions were collected and concentrated by lyophilization.

**Enzyme Assays**—The standard alanine racemase assay mixture contained 50 mM Tris (pH 8.7), 25 mM L- or D-alanine, 30 μM pyridoxal phosphate, and 20 μM dithiothreitol. The reaction was initiated by

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‡The abbreviations used are: AdoMet, S-adenosyl-L-methionine; DKP, diketopiperazine; PAGE, polyacrylamide gel electrophoresis; Ala-P, (1-aminoethyl)phosphonate.

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the enzymatic biosynthesis of was used for calculation of enzyme activity.

The change of absorbance at of enzyme catalyzing the formation of 1 mol of epimerized product!

The reaction mixture for the reverse direction contained 0.1 unit of L-alanine dehydrogenase. In the volume of NADH was followed by a Uvicon

After consumption of NADH was detected by the

Enzymatic Properties of Alanine Racemase from T. niveum

Racemase activity was measured spectrophotometrically using either α-amino acid oxidase coupled to lactate dehydrogenase or L-alanine dehydrogenase. In the L to D direction we used 0.3 units of o-amino acid oxidase, 25 units of lactate dehydrogenase, and 0.2 ml of NADH in a final volume of 350 µl. The reaction mixture for the reverse direction contained 0.1 unit of L-alanine dehydrogenase and 10 µm NAD⁺ in the same volume. The change of absorbance at 340 nm due to the formation or consumption of NADH was followed by a Uvicon 930 recording spectrophotometer. A molar extinction coefficient of 6.22 cm²/pmol of NADH was used.

To demonstrate the formation of α-amino acid, the alanine racemase assay was carried out with [14C]-labeledprecursors (120-180 mCi/mmol). After incubation, the proteins were precipitated by adding acetone. The supernatant was evaporated and redissolved in 0.1 ml of HCl/methanol (1:1). For separation of the isomers, enantioselective TLC was used.

At the nanomolar range, formation of α-keto acids, derived from α-amino acids by D-amino acid oxidase, 25 units of lactate dehydrogenase, and 0.2 ml of NADH in a final volume of 350 µl. The reaction mixture for the reverse direction contained 0.1 unit of L-alanine dehydrogenase and 10 µm NAD⁺ in the same volume. The change of absorbance at 340 nm due to the formation or consumption of NADH was followed by a Uvicon 930 recording spectrophotometer. A molar extinction coefficient of 6.22 cm²/pmol of NADH was used.

Protein Determination—Protein concentrations were determined by a modified Bradford procedure (10) with bovine serum albumin as a standard.

RESULTS

Purification of Alanine Racemase—T. niveum was harvested at various times, and the racemase activities were measured in crude extracts. Racemase activity reached its maximum after 142 h and decreased slowly after 150 h. For purification of the enzyme, 145-h cultures were used. Among several disruption techniques, French press gave the best results. Ammonium sulfate precipitation, usually a good method for protein fractionation, resulted in loss of activity. A similar behavior was reported for the dad B alanine racemase from Salmonella typhimurium (11). Ion exchange chromatography on QAE-Sepharose was found to be much more effective, giving about a 10-fold purification. The total procedure is summarized in Table I. QAE-Sepharose was followed by hydrophobic interaction chromatography on phenyl-Sepharose, Superdex 200 gel filtration, and cation exchange chromatography on cellulose phosphate.
Anion exchange chromatography on MonoQ was used as the last step in the purification procedure and yielded a single band in SDS-PAGE (Fig. 3, lane A). Starting with 25 g of lyophilized mycelium, the typical yield was 1 µg of homogenous enzyme in SDS-PAGE (Fig. 3, lane A). Starting with 25 g of lyophilized mycelium, the typical yield was 1 µg of homogenous enzyme in SDS-PAGE (Fig. 3, lane A). Assuming a globular structure, this indicated a molecular mass of about 150 kDa (Fig. 4). A similar result was obtained by gel filtration on a high pressure liquid chromatography column (TSK 3000-SWG), where the maximum activity of the enzyme eluted according to a molecular mass of about 120 kDa (data not shown).

The different $M_r$ values from SDS-PAGE and gel filtration suggest a trimeric or tetrameric structure of the native racemase, composed of identical subunits.

**Cofactor Requirement**—Pyridoxal phosphate could be identified as the exclusive cofactor of the racemase. The $K_m$ value was estimated to be 12 µM. When incubated with 2 mM NH$_4$OH at pH 7.5, all activity was lost but could be partly restored by the addition of 30 µM pyridoxal phosphate. After Superdex gel filtration with pyridoxal phosphate-free buffer A, activity of racemase decreased by as much as 10%. Similar results were obtained after dialyzing the enzyme sample overnight. Activity was restored to 100% by addition of 30 µM pyridoxal phosphate.

**Kinetic Parameters**—A wide range of amino acids, including 2-aminobutyric acid, was used to investigate the substrate specificity of the racemase under saturating conditions. The enzyme exhibits high specificity with respect to $L$- and $D$-alanine. Highest relative activities toward alternative substrates measured at pH 8.0 were found with $L$-serine (23%), $L$-aminobutyric acid (15%), and $L$-leucine (13%) ($L$-alanine = 100%).

**Formation of Diketopiperazines by a Coupled Reaction of Cyclosporin Synthetase and Alanine Racemase**—It has been demonstrated in our laboratory that cyclosporin synthetase is able to synthesize cyclo($D$-alanyl-$N$-methylleucine) (D-DKP) (3). Here we demonstrate the formation of cyclo($L$-alanyl-$N$-methylleucine) (L-DKP) using $L$- instead of $D$-alanine as a precursor. Offering both $L$- and $D$-alanine as substrates, we obtained a product mixture of D-DKP and L-DKP. In contrast to D- and

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**Table I**

| Step           | Total activity | Protein | Specific activity | Activity recovery | Purification |
|----------------|----------------|---------|-------------------|-------------------|--------------|
|                | milliunits     | mg      | units/mg          | %                 | fold         |
| Crude extract  | 17,462         | 1,230.000 | 0.014           | 100.0            | 1.0          |
| QAE            | 14,630         | 180.000  | 0.082            | 83.0             | 5.8          |
| Phenyl-Sepharose | 4,135        | 16.800   | 0.250            | 24.0             | 17.9         |
| Superdex 200   | 2,150          | 1.720    | 1.250            | 12.0             | 89.3         |
| Cellulose phosphate | 630         | 0.300    | 2.100            | 3.6              | 150          |
| Mono-Q         | 143            | 0.001    | 143.000          | 0.8              | 10,214.3     |

**Fig. 3.** SDS-PAGE of protein samples from different purification steps of alanine racemase. Lane A, pooled active fractions after Superdex 200 gel chromatography (4 µg); lane B, pooled active fractions of cellulose phosphate cation exchange chromatography (3 µg; lane C, purified alanine racemase after MonoQ ion exchange chromatography (300 ng).

**Fig. 4.** Molecular mass of alanine racemase determined by Superdex 200 size exclusion gel chromatography.
L-alanine, these are stereoisomers and can easily be separated by TLC as described under “Experimental Procedures.” Interestingly D-DKP formation is very sensitive. Using 14C-labeled leucine, we could detect the formation of D-DKP up to 10 pmol of D-alanine. Furthermore we could demonstrate the formation of D-DKP by the coupled reaction from alanine racemase and cyclosporin synthetase after incubation with l-alanine, AdoMet, and 14C-labeled l-leucine. In the control reaction without alanine racemase only l-DKP was formed (not shown).

**DISCUSSION**

Alanine racemases (EC 5.1.1.1) are well known as typical procaryotic enzymes catalyzing the interconversion between L-and D-alanine (for review see Refs. 16, 17, and 19). Because of their involvement in cell wall biosynthesis (20), they are essential for bacteria and potential targets for antibiotics (21). The kinetic and structural properties of the T. niveum enzyme indicate a close relationship to the procaryotic enzymes. Alanine racemases are typically monomers or dimers of identical subunits with molecular masses of about 40 kDa (22). The subunit of the T. niveum enzyme has a similar molecular mass of 37 kDa, but the holoenzyme seems to be composed of at least three subunits as indicated by gel filtration. This is similar to the alanine racemase from Pseudomonas striata, where Roise et al. (18) observed a concentration-dependent assembling of three or more subunits.

All known alanine racemases utilize pyridoxal phosphate to build an intermediary Schiff’s base with the substrate (19). We demonstrate that the activity of T. niveum racemase also depends on pyridoxal phosphate as the sole cofactor. Pyridoxal phosphate is loosely bound to the enzyme and can be removed by gel filtration or dialysis. We also have tested several possible racemase inhibitors that target the pyridoxal phosphate group. Strong inhibition was caused by hydroxylamine (K_i = 60 μM), probably by formation of an oxime with the pyridoxal phosphate aldehyde group. Additional well known inhibitors of alanine racemases are D- and L-(1-aminoethyll-phosphonate (Ala-P) (11). Incubation of the racemase for 30 min with 2 mM Ala-P resulted in decreased activities (60% for D-Ala-P and 40% for L-Ala-P). The inhibition is time-dependent and similar to the racemases from the Gram-positive organisms *Staphylococ-*
Enzymatic Properties of Alanine Racemase from T. niveum

The enzyme plays a key role in the biosynthesis of cyclosporins. The current knowledge of procaroytic non-ribosomal peptide synthesis is mainly based on studies focusing on the building mechanisms of two procaroytic decapetides, Gramicidin S (14) and Tyrocidine (15). Both compounds contain n-phenylalanine, and it has been shown that integrated domains of the peptide synthetases are responsible for the formation of these α-amino acids. L-Phenylalanine is accepted as a substrate and is isomerized after it is bound as a thioester. Previous attempts to find a similar behavior in the case of cyclosporin showed that cyclosporin synthetase is not able to isomerize L-alanine and did not accept L-alanine as a substrate for the D-alanine position (3). Here we identify an alanine racemase responsible for the formation of D-alanine, the first example of a distinct racemase involved in non-ribosomal biosynthesis of peptides.

Recently linear peptides of different length were isolated from the cyclosporin synthetase by performic acid treatment (24). These peptides have been identified as thioester-bound acid. For these reasons production of D-alanine seems to be a means to isomerize L-alanine and did not accept L-alanine as a substrate and is the main reaction product only if D-alanine and L-leucine are available as amino acid substrates. We used and optimized this method as an in vitro assay and could show that cyclosporin synthetase is also able to synthesize cycloL-alanyl-N-methylleucine (L-DKP), dependent on whether D- or L-alanine is offered as a substrate amino acid. When both isomers are offered, cyclosporin synthetase prefers the D-form. Even with a 10,000-fold molar surplus of L-alanine, D-DKP was the main reaction product reflecting the higher affinity of the synthetase for D-alanine. This method also provides a very sensitive and selective enzymatic assay for the detection of D-alanine at a nanomolar level.

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