In vitro biosynthesis of ring-extended cyclosporins

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Cyclosporin synthetase, a multifunctional polypeptide, catalyses the biosynthesis of the set of natural cyclosporins. We report that this enzyme is also capable of introducing a β-alanine into position 7 or 8 of the ring instead of the α-alanines present at these positions in cyclosporin A. This leads to 34-membered rings in contrast to the 33-membered ring of the cyclo-undecapeptide cyclosporin A. Both [βAla8]CyA and [βAla8]CyA show immunosuppressive activity. The cyclosporin synthetase-related enzyme peptolide SDZ 214-103 synthetase, on the other hand, does not incorporate either β-alanine into position 7 or β-hydroxy acids into position 8, confirming the previously described higher substrate specificity of this enzyme compared with cyclosporin synthetase [Lawen and Traber (1993) J. Biol. Chem. 268, 20452-20465].

INTRODUCTION

Cyclosporin A (CyA; Figure 1), a cyclic undecapeptide with a 33-membered ring, exerts several biological properties, including anti-fungal, anti-parasitic, anti-inflammatory and immunosuppressive properties (Borel, 1986). Because of its immunosuppressive potential, it is widely used in human transplantation surgery (Kahan, 1984) and in the treatment of autoimmune diseases (Schindler, 1985). CyA is produced by the fungus Beauveria nivea (previously designated as Tolypocladium inflatum; see Lawen and Zocher, 1990) as the main product of at least 25 so-called 'natural' cyclosporins (Traber et al., 1987). With the exception of cyclosporin H (CyH; [d-MeVal11]CyA, i.e. CyA with d-methylvaline at position 11) all of these cyclosporins are produced by cyclosporin synthetase (Lawen and Zocher, 1990), which possesses rather broad substrate specificity at most of its active sites (Lawen et al., 1989; Lawen and Traber, 1993). This enzyme is a very complex multifunctional polypeptide, consisting of a single polypeptide chain with a molecular mass of at least 1.4 mDa (Schmidt et al., 1992). It catalyses all 40 reaction steps necessary for the biosynthesis of cyclosporin A starting from the unmethylated constituent amino acids (Lawen and Zocher, 1990).

In 1988, a novel cyclosporin-related peptolide, with several substitutions compared with CyA, called SDZ 214-103 was discovered. It is produced by the fungus Cylindrotrichum oligospermum (Corda) bonorden (Dreyfuss et al., 1989). The main structural difference is a 2-hydroxy acid instead of an amino acid at position 8. According to the conventional nomenclature of cyclosporins, it can be designated as [Thr8,Leu5,D-Hiv8,Leu10]cyclosporin (d-Hiv, 2-hydroxyisovaleric acid). This novel drug exhibits properties similar to those of CyA, including immunosuppressive, anti-inflammatory, anti-fungal and anti-parasitic activities. SDZ 214-103 is also produced by a multifunctional enzyme, called peptolide SDZ 214-103 synthetase (Lawen et al., 1991b). The molecular mass of this enzyme is similar to that of cyclosporin synthetase (Lawen et al., 1991a) but its substrate specificity appears, as far as it has been analysed, to be much narrower than that of cyclosporin synthetase (Lawen and Traber, 1993).

We show in this paper that [βAla8]CyA, a cyclosporin with a 34-membered ring and a minor product of fermentations of [d-Ser8]CyA (Traber et al., 1989), is a product of direct enzymic biosynthesis. The position 7 analogue, [βAla8]CyA, was also obtainable with cyclosporin synthetase in vitro. Peptolide SDZ 214-103 synthetase, on the other hand, did not synthesise any ring-extended peptolides.

MATERIALS AND METHODS

Organisms and culture conditions

B. nivea, strain 7939/45, was maintained and grown in submerged culture as previously described for enzyme production (Lawen et al., 1989) and for the fermentation of [d-Ser8]CyA (Traber et al., 1989). Maintenance and culture of C. oligospermum (Corda) bonorden, strain 85-22651/F, isolate 5.1.15 was carried out as reported earlier (Lawen et al., 1991b; Lawen and Traber, 1993).

Purification of [βAla8]cyclosporin from fermentation broth

Chromatographic conditions

Column I: Chromasil RP 18 (5 µm bead diameter, 250 mm x 20 mm) at 80 °C. Column II: Raynin silica gel (5 µm bead diameter, 250 mm x 20 mm) at room temperature. Mobile phases: A, water/10% (w/v) tetramethylammonium hydroxide/85% orthophosphoric acid (1000:20:6.6, by vol.); B, acetonitrile; C, water/85% (w/v) orthophosphoric acid (1000:1, v/v); D, acetonitrile/r-butyl methyl ether (290:60, v/v); E, diethyl ether/methanol (95:5, v/v). Flow rate, 20 ml/min; detection wavelength, 210 nm.

Isolation of [βAla8]CyA

[d-Ser8]CyA was produced by fermentation and [2-hydroxyethyl-d-Ser8]CyA (SDZ IMM-125) was prepared from [d-Ser8]CyA by
Figure 1 Structure of CyA

Abu, L-2-aminobutyric acid; Bmt, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine; Sar, sarcosine.

Semisynthesis as described previously (Eberle, 1991). A 760 mg chromatographic side fraction of SDZ IMM-125 containing ~ 15 % (w/w) [βAla]CyA was separated by preparative h.p.l.c. on column I using mobile phase A/B (45:55, v/v) by repetitive injection of 80 mg portions in each run. The enriched material (60 mg) was further chromatographed by using column I and mobile phase C/D (50:50, v/v) and subsequently mobile phase C/D (53:47, v/v), yielding 10 mg of almost pure compound. After final purification using column II and mobile phase E, 8 mg of [βAla]CyA of 92 % purity, as determined by analytical h.p.l.c., was obtained.

After each chromatographic step using column I and a system containing water as a mobile phase, the enriched fractions were concentrated by removing the organic solvent under reduced pressure, extracting the resulting water phase with dichloromethane and subsequently evaporating the extract in vacuo to dryness.

Enzyme preparations and in vitro peptide formation

Preparations of cyclosporin synthetase and peptidase SDZ 214-103 synthetase from mycelia of B. nivea or C. oligospernum respectively were performed according to previously described procedures (Lawen and Zocher, 1990; Lawen et al., 1991b; Lawen and Traber, 1993). In vitro cyclosporin and peptidolide formation and extraction of the products were also carried out as reported previously (Lawen et al., 1989; Lawen and Traber, 1993).

Analytical methods

T.l.c.

T.l.c. analyses were carried out on Merck high-performance silica gel 60 plates using water-saturated ethyl acetate as the mobile phase. After drying, the plates were subjected to autoradiography using β-max X-ray film (Amersham). The Rf values were determined from the autoradiograms (Lawen et al., 1989).

H.p.l.c.

This was performed on a Shandon Hypersil column (5 μm bead diameter; 250 mm x 4.6 mm) at 75 °C using acetonitrile/water/orthophosphoric acid (630:370:0.1, by vol.) as the mobile phase and a flow rate of 1.2 ml/min. Cyclosporins were detected by their u.v. absorption at 210 nm (Lawen et al., 1989).

Mass spectroscopy

Fast atom bombardment m.s. (f.a.b.-m.s.) was performed using a VG 70-SE mass spectrometer (Vacuum Generator, Manchester, U.K.) at 8 keV using nitrobenzyl alcohol as the liquid matrix.

1H-n.m.r. spectroscopy

1H-n.m.r. spectra were recorded in CD3HCl at 24 °C with a Bruker AMX-400 spectrometer. All 1H-resonance lines of CyA had been assigned previously (Kessler et al., 1985). Correlation spectroscopy (COSY) and rotating-frame Overhauser enhancement spectroscopy (ROESY) data were recorded with a spectral width of 3601 Hz in t2 and 1610 Hz in t1; 32 scans of 256 increments in t1 were acquired. The mixing time for the ROESY spectrum was 150 ms. The data were processed using standard Bruker software.

Biological assays

The in vitro immunosuppressive activity of the cyclosporins was determined as described (Schreier et al., 1992). Growth inhibition of lymphoid cells in a mixed lymphocyte reaction was quantified by the incorporation of tritiated thymidine, starting from a concentration of 10 μg/ml cyclosporin and applying 10-fold dilution steps. To distinguish between immunosuppressive and cytotoxic effects, inhibition of proliferation of murine bone marrow cells stimulated by haemopoetic growth factors was measured in a parallel assay. For cyclosporins, no cell-toxic effects were observed with the concentrations used.
RESULTS AND DISCUSSION

Isolation of [\(\beta\text{Ala}^8\)]CyA

SDZ IMM-125 (12-hydroxyethyl-\(\beta\)-Ser\(^8\))CyA, a cyclosporin derivative which is in clinical development as an immunosuppressive drug (Hiestand et al., 1992; Donatsch et al., 1992; Baumann et al., 1992), was prepared by semisynthesis from natural [\(\beta\)-Ser\(^8\)]CyA which itself is a fermentation product. In the course of the chromatographic purification process of SDZ IMM-125, several side fractions were obtained which contained, according to h.p.l.c. analyses, an unknown minor product amounting to \(\sim 15\%\). Its separation from the more polar SDZ IMM-125 was achieved by repetitive chromatography using preparative h.p.l.c. techniques. After the final purification step, a sample of 92\% pure [\(\beta\text{Ala}^8\)]CyA was obtained as a white amorphous powder.

Elucidation of the structure of [\(\beta\text{Ala}^8\)]CyA

The f.a.b.-m.s. spectrum with a molecular ion peak at \(m/z\) 1202 (MH\(^+\)) gave the first indication that the unknown component was an isomer of CyA (C\(_{44}H_{115}N_{11}O_{12}\)). Upon comparison of its \(^1\)H-n.m.r. spectrum (Figure 2) with the spectral data of CyA (Kessler et al., 1985), the differences in the chemical structures became evident (Table 1). Whereas in the \(^1\)H-n.m.r. spectrum of CyA the amide proton of \(\alpha\)-Ala\(^8\) is found as a doublet at 7.18 p.p.m., in the spectrum of [\(\beta\text{Ala}^8\)]CyA the corresponding signal is shifted to 6.73 p.p.m. and appears as a doublet. In the region of the \(\alpha\)-protons, the signal at 4.84 p.p.m. (H-C\(_{\alpha}\) of \(\alpha\)-Ala\(^8\)) in CyA is replaced by a new multiplet at 2.46 p.p.m. (2H) originating from the new \(\alpha\)-methylene protons. The doublet at 1.26 p.p.m. which is characteristic of the \(\beta\)-CH\(_2\) group of [\(\beta\text{Ala}^8\)]CyA in CyA is missing; two multiplets at 3.20 (1H) and 3.87 p.p.m. (1H) respectively are observed, corresponding to the \(\beta\)-methylene protons of the [\(\beta\text{Ala}^8\)] unit. The final assignments of the proton n.m.r. signals of [\(\beta\text{Ala}^8\)]CyA have been corroborated by n.m.r. COSY experiments (Figure 3) and its amino acid sequence has been confirmed by n.m.r. ROESY experiments (results not shown).

In vitro synthesis of [\(\beta\text{Ala}^8\)]CyA

One of the so-called natural cyclosporins (Traber et al., 1987), namely CyH ([\(\beta\)-MeVal\(^{11}\)]CyA), is not a product of direct biosynthesis. Cyclosporin synthetase strictly discriminates between the \(\delta\)- and \(\lambda\)-substrates (Lawen and Traber, 1993). Current understanding of CyH formation is that during the extraction of CyA with acidic alcoholic solution, a small portion isomerizes to form isocyclosporin A (Rüegg et al., 1976). This can then either be converted into CyH or back into CyA. Although no mechanism is known by which one could explain the isomerization of CyA to [\(\beta\text{Ala}^8\)]CyA or the generation of the latter compound from [\(\beta\)-Ser\(^8\)]CyA during the extraction process, it seemed unlikely that the enzyme would have the flexibility to form either a 33- or a 34-membered ring. We therefore examined whether [\(\beta\text{Ala}^8\)]CyA could be a product of direct enzymic synthesis by using our previously described in vitro cyclosporin biosynthesis system (Lawen et al., 1989; Lawen and Traber, 1993). Replacement of \(\delta\)-Ala in the CyA assay by \(\beta\)Ala led to the formation of a new product which co-chromatographed with [\(\beta\text{Ala}^8\)]CyA (Figure 4a, lane 5).

In a second experiment, we endeavoured to synthesize microgram amounts of the putative [\(\beta\text{Ala}^8\)]CyA to enable further characterization. The extraction of a 25 ml incubation mixture formulated for [\(\beta\text{Ala}^8\)]CyA biosynthesis yielded six different cyclosporins, as judged from chromatographic behaviour and from u.v. spectra. Besides 100 mg of each of CyA and CyV ([\(\text{Alb}^2\)]CyA; Albu, 1-2-aminobutyric acid), which could be synthesized from amino acids bound to the enzyme, 100 mg of a new

Table 1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>[(\beta\text{Ala}^8)]CyA</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-NH</td>
<td>6.73 (H, dd, (J = 6, 8) Hz)</td>
<td>7.18 (H, d, (J = 8) Hz)</td>
</tr>
<tr>
<td>8-H-((\alpha))</td>
<td>2.46 (2H, m)</td>
<td>4.84 (1H, q, (J = 7) Hz)</td>
</tr>
<tr>
<td>8-H-((\beta))</td>
<td>3.20 (1H, m)</td>
<td>1.26 (3H, q, (J = 7) Hz)</td>
</tr>
<tr>
<td>3.87 (1H, m)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
compound with an $\alpha$-value of 6.79 and which co-eluted with authentic [flAla8]CyA was isolated. As outlined in Table 2, the f.a.b.-m.s. spectrum showed a molecular ion peak at 1202 ($M+H^+$) and the in vitro immunosuppressive activity was measured to be $\sim$10-fold less than that of CyA. All the data support the interpretation that this compound is [flAla8]CyA. Three minor compounds were also separated in this experiment, but only one of them could be identified. 10 $\mu$g of [Gly7]CyA with an $\alpha$-value of 5.36 and a molecular ion peak at 1174 ($M+H^+$) was isolated. It had been shown previously that cyclosporin synthetase produces as the major product [flGly7]CyA, when D-Ala is omitted from the incubation mixture, and also produces [flGly7]CyA when Ala is omitted from incubation mixture (Lawen and Traber, 1993). The two further separated cyclosporins (10 $\mu$g, $\alpha = 2.90$, no interpretable f.a.b.-m.s. spectrum, and 50 $\mu$g, $\alpha = 4.01$, 1174 ($M+H^+$)) could not be identified.

Figure 3 1H-1H COSY n.m.r. spectrum of [flAla8]CyA (400 MHz, CDCl3)

Table 2  Selected properties of products from incubations designed to form fAla-containing cyclosporins

<table>
<thead>
<tr>
<th>Cyclosporin</th>
<th>T.I.C. $R_2$</th>
<th>H.p.l.c. $\alpha$-factor</th>
<th>F.a.b.-m.s. ($M+H^+$)</th>
<th>Biosynthesis</th>
<th>Immunosuppressive activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyA</td>
<td>0.37</td>
<td>10.00</td>
<td>1202</td>
<td>Natural</td>
<td>++</td>
</tr>
<tr>
<td>CyA</td>
<td>0.37</td>
<td>10.00</td>
<td>1202</td>
<td>Enzymic</td>
<td>++</td>
</tr>
<tr>
<td>CV</td>
<td>0.48</td>
<td>11.97</td>
<td>1216</td>
<td>Enzymic</td>
<td>++</td>
</tr>
<tr>
<td>[flAla8]CyA</td>
<td>0.34</td>
<td>8.64</td>
<td>1202</td>
<td>Natural</td>
<td>+</td>
</tr>
<tr>
<td>[flAla8]CyA</td>
<td>0.20</td>
<td>6.66</td>
<td>1202</td>
<td>Enzymic</td>
<td>++</td>
</tr>
<tr>
<td>[flAla8]CyA</td>
<td>0.20</td>
<td>6.79</td>
<td>1202</td>
<td>Enzymic</td>
<td>++</td>
</tr>
<tr>
<td>[Gly7]CyA</td>
<td>n.d.</td>
<td>5.41</td>
<td>1174</td>
<td>Enzymic</td>
<td>++</td>
</tr>
</tbody>
</table>

Figure 4  In vitro synthesis of ring-extended CyA analogues

The enzyme (100 $\mu$g) (cyclosporin synthetase from A, peptolide SDZ 214-103 synthetase in B) was incubated together with ATP, Mg$^{2+}$, S-adenosyl-l-[35S]-methylmethionine and all constitutive amino (hydroxy) acids of CyA or peptolide SDZ 214-103 respectively for 2 h at 25 °C (lane 1). Autoradiograms of the high-performance t.l.c. separation of ethyl acetate-extractable reaction products are shown. (a) Lane 2, D-Ala omitted from reaction mixture. Lane 3, Ala replaced by fl-Ala. Lane 4, D-Ala omitted. Lane 5, D-Ala replaced by fl-Ala. The position of CyA is indicated on the left, the position of the reference [fAla8]CyA is indicated on the right. In lane 3, the position of [flAla8]CyA is indicated by an arrow. The major band in lanes 2 and 3 represents [Abu7]CyA, the major band in lanes 4 and 5 represents [Gly7]CyA. For the identity of the other minor bands, refer to Lawen and Traber (1993). (b) Lane 2, D-Hiv was omitted. Lane 3, D-Hiv was replaced by O$_2$-mevalonic acid lactone. Lane 5, D-Hiv replaced by $\beta$-hydroxybutyric acid. Lane 6, D-hiv replaced by $\beta$-propiolactone. Lane 7, Ala omitted. Lane 8, Ala replaced by fl-Ala. The position of SDZ 214-103 is marked on the left.

Search for biosynthesis of further ring-expanded cyclosporin homologues

A new product could also be detected with Ala was replaced with fl-Ala in the incubation mixture (Figure 4A, lane 3). Because no reference [flAla8]CyA was available, we had to perform three preparative incubations to identify this cyclosporin. We could isolate up to five different cyclosporins, four of which could be identified. Experiment 1 yielded 10 $\mu$g of cyclosporin (I), 5 $\mu$g of cyclosporin (II), 25 $\mu$g of cyclosporin (III), 70 $\mu$g of cyclosporin (IV) and 70 $\mu$g of cyclosporin (V); experiment 2 yielded 10 $\mu$g of cyclosporin (I), 10 $\mu$g of cyclosporin (II), 20 $\mu$g of cyclosporin (III), 50 $\mu$g of cyclosporin (IV) and 80 $\mu$g of cyclosporin (V), and experiment 3 yielded 20 $\mu$g of cyclosporin (III), 70 $\mu$g of cyclo-
sporin (IV) and 100 μg of cyclosporin (V). Cyclosporin (IV) coeluted with CyA, showed a molecular ion peak at 1202 ([M + H\(^+\)]) and exerted high immunosuppressive activity. It was therefore identified as CyA. Cyclosporin (V) (α = 11.93, 1216 [M + H\(^+\)]) clearly could be characterized as CyV. Cyclosporin (I) (α = 5.41, 1174 [M + H\(^+\)]) moderate immunosuppressive activity comparable to that of [βAla\(^+\)]CyA is supposed to be [Gly\(^+\)]CyA. Cyclosporin (II) (α = 6.34) could not be identified. Cyclosporin (III) shows an α-value of 8.64, a molecular ion peak at 1202 (M + H\(^+\)) and only moderate immunosuppressive activity and is therefore clearly distinct from CyA. From these data, summarized in Table 2, and from our assay conditions, we concluded that this compound is the expected [βAla\(^+\)]CyA.

β-Ala at position 8 of CyA has been shown to be the starting amino acid in the biosynthetic process (Lawen et al., 1992; Dittmann et al., 1994). Thus, ring extension occurs at the cyclization site. To clarify whether the capability to extend the peptide ring by one carbon at positions 7 or 8 is a general phenomenon for this family of multienzyme polypeptides, we also examined the cyclosporin synthetase-related peptolide SDZ 214-103 synthetase (Lawen et al., 1991b). The product of this enzyme, peptolide SDZ 214-103, has a δ-2-hydroxysisovaleric acid at position 8 (instead of the β-Ala in cyclosporin A). At position 7, SDZ 214-103 carries, like CyA, an Ala moiety. We tested the capability of SDZ 214-103 synthetase to introduce into position 8 a series of 3-hydroxy acids (or the appropriate lactones), namely mevalonic acid lactone, δ(-(−))β-hydroxybutyric acid and β-propiolactone. According to its previously published substrate specificity (Lawen and Traber, 1993), at least the latter two compounds should be substrates of the enzyme, if only the side chain is restricting and the enzyme allows ring extension. However, the synthetase did not incorporate any of these compounds or β-Ala (Figure 4b). Therefore, it remains an open question, whether the ability to produce peptides with a ring extension at the cyclization site is a specific feature of cyclosporin synthetase, or whether the failure to do that is an unusual property of peptolide SDZ 214-103 synthetase. It would therefore be of interest to examine further multienzyme poly-

peptides, e.g. SDZ 90-215 synthetase (Lee and Lawen, 1993), for their capability to synthesize ring-extended products.

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