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ORIGINAL PAPER

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Structure and localization of cyclosporin synthetase, the key enzyme of cyclosporin biosynthesis in *Tolypocladium inflatum*

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Abstract The electron microscopic image of native cyclosporin synthetase molecules showed large globular complexes of 25 nm in diameter, built up by smaller interconnected units. Compartmentation of cyclosporin synthetase and the functionally interconnected D-alanine racemase was revealed after sucrose density gradient centrifugation of subcellular fractions and immunoelectron microscopy. A considerable proportion of cyclosporin synthetase and D-alanine racemase was detected at the vacuolar membrane. The product cyclosporin was localized in the fungal vacuole.

Keywords Cyclosporin A · Cyclosporin synthetase · *Tolypocladium inflatum* · Compartmentation · Immunoelectron microscopy

Introduction

The fungus *Tolypocladium inflatum* is of major biotechnological interest due to its ability to synthesize cyclosporins, a group of cyclic undecapeptides. Cyclosporin A is the most important molecule of this group, with unique immunosuppressive properties. Cyclosporin A was introduced into clinical use in the late 1970s to reduce graft rejection after organ transplantation, a property based upon interference of the agent with lymphokine biosynthesis (Randak et al. 1990; Brabletz et al. 1991; Schreiber and Crabtree 1992).

Synthesis of cyclosporin is catalyzed by the cyclosporin synthetase, a nonribosomal peptide synthetase with a molecular mass of 1.6 MDa. The enzyme is composed of eleven modules, each being responsible for the recognition, activation and modification of one substrate (Lawen and Zocher 1990; Weber et al. 1994) and a small "twelfth module" putatively responsible for cyclization. According to the analysis of the deduced sequence from the gene and referring to the established model for nonribosomal peptide synthetases (Marahiel et al. 1997), each module of cyclosporin synthetase essentially consists of a central adenylation domain (A-domain; recognition, activation), thiolation domain (T-domain; covalent binding of adenylated amino acid on phosphopantetheine) and condensation domain (C-domain; elongation step). Seven modules harbor an additional methyltransferase domain (M-domain; *N*-methylation). Presently it is not clear whether the domains form a structurally compact module or if they act as somehow independent beads on a chain. Nevertheless, based on the fact that the amino acid is bound on the phosphopantetheine, all the domains of one module have to be in close vicinity to each other. Substrates for cyclosporin are L-valine, L-leucine, L-alanine, glycine, the unusual amino acids 2-aminobutyric acid, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-threonine (butenyl-methyl-Thr), and D-alanine. Cyclosporin synthetase activates the amino acids to amino acyl adenylates and binds them covalently via thioester linkages at prosthetic phosphopantetheine groups. Seven of the substrate amino acids become *N*-methylated by *S*-adenosylmethionine via respective methyltransferase activities of cyclosporin synthetase. After a final cyclization step, cyclosporin is released from the enzyme. Aminobutyric acid is provided by main metabolic pathways of the cell. D-Alanine and butenyl-methyl-Thr are synthesized exclusively for the biosynthesis of cyclosporin A. Thus, the action of two other enzymes providing butenyl-methyl-Thr and D-Ala is essential for cyclosporin synthesis: the butenyl-methyl-Thr polyketide synthase (Offenzeller et al. 1993) and D-alanine racemase (Hoffmann et al. 1994). D-Ala is synthesized by racemization of L-Ala, catalyzed by alanine racemase with pyridoxal phosphate as cofactor. Biosynthesis of the undecapeptide starts with D-Ala, and a close structural association between alanine racemase and cyclosporin synthetase may be reasonable.

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Though essential data on the structure and catalytic mechanisms of the enzymes involved in cyclosporin biosynthesis and its function are available, localization of these enzymes inside the cell, and possible compartmentation of the cyclosporin biosynthetic pathway remain to be elucidated. Here, we present insight into the overall structure and cellular localization of cyclosporin synthetase, the large enzyme complex involved in cyclosporin biosynthesis, and alanine racemase.

Material and methods

Strains, growth media and culture condtions

The wild-type strain of *Tolypocladium inflatum* (ATCC 34921) and an adapted strain (*T. inflatum* Cy 65457, provided by Biochemie GmbH, Austria) were grown in maltose-casein peptone medium (contents per liter distilled water: 50 g maltose, 10 g caseine (tryptic digest), l.5 g KH_2PO_4, 2.5 g KCl, pH 5.6) and incubated at 25 °C under continuous aeration.

Embedding procedures

Cells were harvested by centrifugation at 10,000×*g*, resuspended in 50 mM potassium phosphate buffer, chemically fixed in 0.5% (w/v) formaldehyde and 0.3% (w/v) glutardialdehyde solution for 90 min at 0 °C, dehydrated in a graded methanol series and embedded in Lowicryl K4M resin (Roth et al. 1981; Hoppert and Holzenburg 1998).

Cryopreparation was performed essentially according to the procedure of Tokuyasu (1986). A chemically fixed cell suspension was embedded in a 10% (w/v) gelatin solution. The sample was cut into small, pyramid-shaped pieces and incubated in 0.4 M sodium phosphate-buffer containing 1.6 M sucrose and 25% (w/v) polyvinylpyrrolidone for 24 h, mounted on an aluminum support, frozen in liquid nitrogen and subjected to cryosectioning.

Antisera and immunolocalization

Catalytically active cyclosporin synthetase was purified to apparent electrophoretic homogeneity as described (see Lawen and Zocher 1990; Schmidt et al. 1992). Antisera of the purified cyclosporin synthetase were raised in rabbits according to established procedures (Harlow and Lane 1988). Specificity of the antisera was tested by Western-blotting and by immunolocalization control experiments (Hoppert and Holzenburg 1998).

Antigenic determinants in the primary structure of alanine racemase from *Tolypocladium inflatum* (Schörgendorfer et al. 1994) were predicted by use of the software PROSITE/PCGENE (Intelligenetics, Oxford, UK). The peptide sequence SPLRSRAERAGLERL was selected. Oligopeptide and antiserum were prepared by Eurogentec (Seraing, Belgium). Removal of fungal cell-wall antibodies was achieved by one-step chromatography of the antisera on washed *Tolypocladium* mycelia (Hoppert and Holzenburg 1998). The selectivity of the antiserum was tested by Western blots using purified enzyme and crude cell extract.

Resin sections of 80–100 nm thickness were cut with glass knives. Immunolocalization was performed with specific antisera and Protein-A-linked colloidal gold (10 nm in diameter, Aurion, Wageningen, The Netherlands) according to established procedures (Roth et al. 1978; Hoppert and Holzenburg 1998).

Preparation of subcellular fractions by density-gradient centrifugation

A pellet of *Tolypocladium inflatum* cells was suspended in K/NaP buffer (66 mM KH_2PO_4/Na_2HPO_4 buffer), pH 6.2. One pellet volume was suspended in three volumes of K/NaP buffer containing Novozym 234 (2 mg/ml), Cytohelicase (0.5 mg/ml) and Zymolyase (0.1 mg/ml) and incubated overnight at room temperature under gentle shaking. A 1-ml aliquot of the resulting protoplast suspension was loaded onto a 35% (w/v) sucrose solution (4 ml) and centrifuged in a swinging-bucket rotor at 400×*g* and 20 °C for 20 min. After centrifugation, the protoplast fraction was concentrated at the boundary layer of the K/NaP buffer and the sucrose solution. This fraction was withdrawn with a syringe and centrifuged for 15 min at 320×*g*. The resulting pellet was resuspended in 2 ml K/NaP buffer. After addition of 4 ml of an ice-cold solution of mannitol (0.6 M), EDTA (1 mM) and Triton X100 (0.025%, v/v) in K/NaP buffer, the suspension was incubated for 30 min at room temperature. The resulting suspension of lysed protoplasts was centrifuged at 900×*g* to remove debris. The supernatant was layered onto a sucrose density gradient (40 ml, 20–50%, w/v, sucrose in K/NaP buffer). The gradient was loaded with 1–2 ml of the lysed protoplasts and centrifuged overnight at 80,000×*g* and 4 °C (Keller et al. 1984). Samples of the fractionated gradient were subjected to dot-blot analysis (Hawkes 1986) for detection of cyclosporin synthetase and alanine racemase antigens as well to enzyme assays for the respective enzymes and for α-mannosidase.

Enzyme assays

The catalytic activities of cyclosporin synthetase and alanine racemase were determined as described (Lawen and Zocher 1990; Offenzeller et al. 1993; Hoffmann et al 1994). α-Mannosidase was assayed according to Levvy and Conchie (1966).

Negative staining and electron microscopy

Negative staining with a 4% (w/v) aqueous solution of uranyl acetate (pH 4.5) was performed according to Valentine et al. (1968). Electron micrographs were taken at calibrated magnifications with a Zeiss EM 902 instrument (Zeiss, Oberkochen, Germany), operated in the conventional bright-field mode or in the dark-field mode, by "electron spectroscopic imaging" (energy loss ΔE =114 eV, specific for uranium) for samples negatively stained with uranyl acetate (Bauer 1988) or with a Philips EM 301 (Philips, Eindhoven, The Netherlands) operated in the conventional bright-field mode. Particle numbers were determined in 100 protein complexes per complex type. In order to confirm the number of units per particle, image averaging with data sets obtained from 70 particles (per projection form) was performed essentially as described (Birkenhäger et al. 1995; see also Hoppert and Holzenburg 1998).

Results

Overall structural features of cyclosporin synthetase

Electron spectroscopic imaging of negatively stained cyclosporin synthetase samples showed two types of structures: globular complexes 25–30 nm in diameter (see Fig. 1A, arrows) and smaller particles around 7 nm long (Fig. 1A, circles). These particles were not uniformly scattered over the carbon film. They appeared to occur in groups, as depicted in Fig. 1A, E, and seemed to be lined up like beads on a chain (Fig. 1E). These particles were also visible in the larger, globular complexes as highlighted by arrowheads in Fig. 1D. In Fig. 1B, C, the distribution of particle numbers visible in globular and chain-like complexes is depicted. The globular complexes did not contain more than 11 particles. In most of the com-

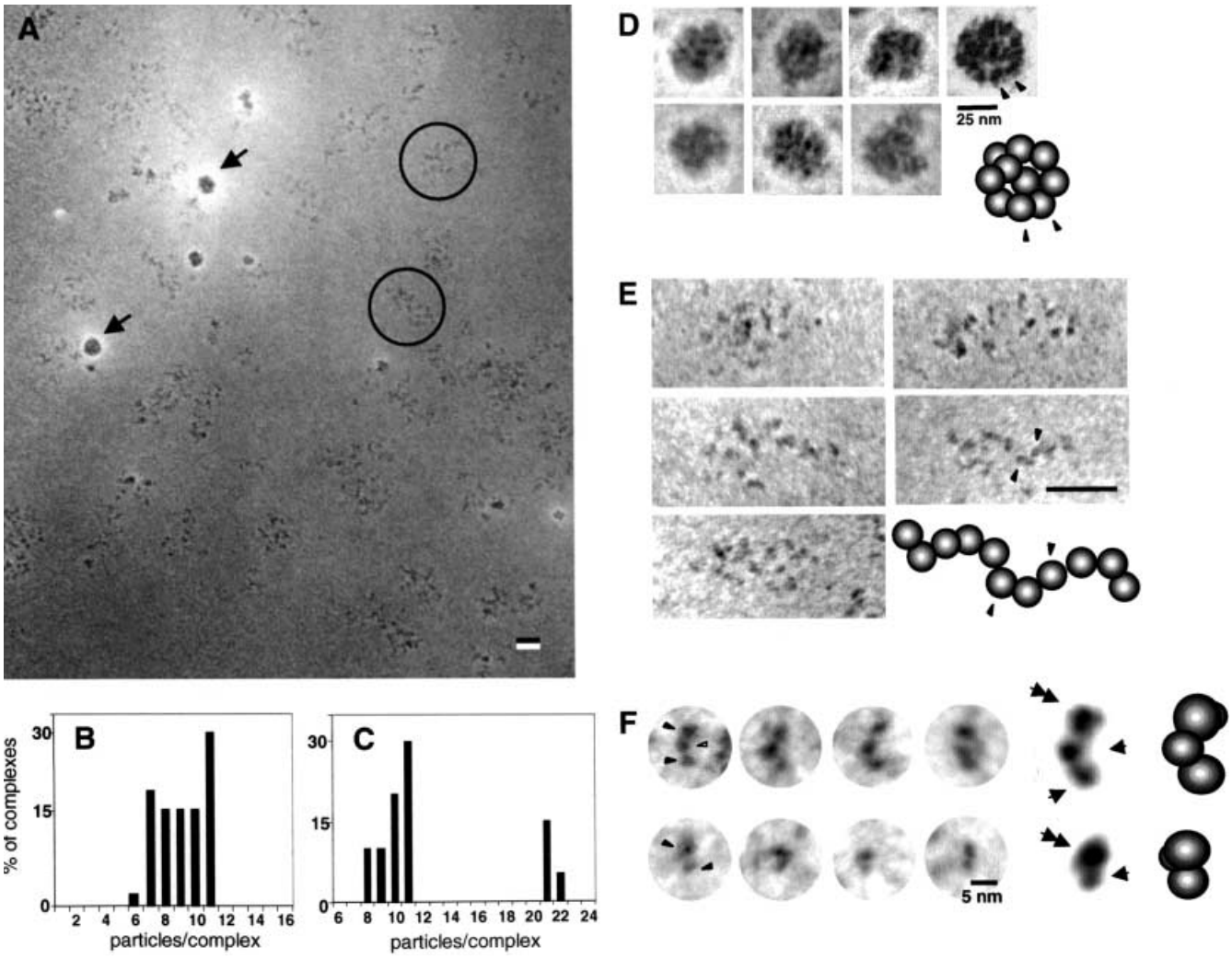

Fig. 1A–F Electron micrographs (dark-field mode) of negatively stained cyclosporin synthetase complexes. **A** Overview depicting globular complexes (*arrows*) and chain-like complexes (*circles*). **B** Distribution of the numbers of visible particles in globular complexes (all projection forms). No more than 11 particles per complex could be detected**. C** Distribution of the numbers of visible particles in chain-like complexes. Two peaks could be observed: chains with 11 particles and a minor peak around 21–22. This indicates aggregation of two chains. **D** Globular complexes. *Arrowheads* mark distinct particles. **E** Chain-like complexes. *Arrowheads* mark distinct particles. The models in **D** and **E** depict the overall structures of globular and chain-like cyclosporin synthetase complexes. *Arrowheads* mark distinct particles. **F** Structural details of single particles. The gallery shows individual particles exhibiting either three (*upper row*) or two (*lower row*) substructures. *Small arrowheads* mark the substructures. Image averaging of projection forms confirms the presence of three or two substructures, as marked by *large arrowheads*. The *double arrowheads* mark the largest substructure in a particle. The models illustrate arrangement and overall size of the substructures. *Bars* 50 nm if not indicated otherwise

plexes, however, fewer particles were visible. Most of the chain-like complexes exhibited 11 particles. Fewer complexes were found with double the number of particles, indicating the presence of aggregates of two chains.

The structural details of the particles in chain-like complexes, were studied. Some 24% of all particles could not be processed because they were densely aggregated with other neighboring particles. In the remaining group of particles, 44% exhibited three substructures and 32% two substructures as depicted in Fig. 1F. In 24% of the particles, the number of substructures could not be counted. The number of substructures in the two largest groups of particles was confirmed by image averaging (Fig. 1F).

Specificity of antibodies

Western blotting of crude extracts revealed that antibodies to all of the enzymes were monospecific for their respective enzymes (not shown).

On ultrathin sections, purified antisera did not cross-react with cell walls or cellular constituents (especially the vacuole of another ascomycete containing no cyclosporin). Specific labeling of vacuoles could be suppressed by co-incubation with the respective antigen (data not shown), indicating specificity of the binding affinities of the antibodies.

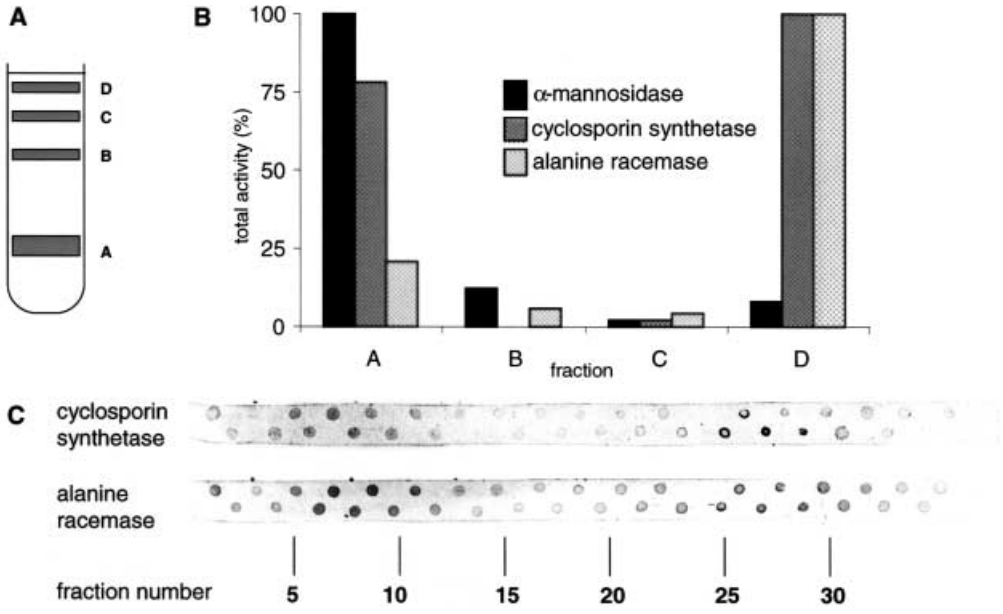

Fig. 2A–C Alanine racemase and cyclosporin synthetase activities after sucrose density gradient centrifugation. **A** Appearance of cell lysate in a centrifuge tube after density gradient centrifugation. Four visible bands approximately correspond to four distinct protein peaks and the resulting pool fractions *A–D*. *Pool A* Fraction 5–10, *B* 13–15, *C* 17–19, *D* 25–30. **B** Alanine racemase and cyclosporin synthetase enzyme activities in pool fractions (*A–D*) of four distinct protein peaks of the density gradient. Peak activities were set to 100%, respectively and represent 190 U for α-mannosidase, 0.5 μU for cyclosporin synthetase and 4.1 mU for alanine racemase. **C** Dot blot of the fractionated gradient. Antigenic determinants of cyclosporin synthetase and alanine racemase were detected in fraction 5–10 (*pool A*) and 25–30 (*pool D*)

Localization studies

For the localization studies, samples of cells were harvested 10 days after inoculation. After sucrose density gradient centrifugation, a suspension of lysed protoplasts could be separated into four bands (Fig. 2). Following determination of enzyme activity and the presence of antigenic determinants, the fractions obtained from the gradient were combined into four pools approximately representing these bands (Fig. 2B, C). Pool A contained the highest α-mannosidase activity, indicating the presence of vacuoles or vacuolar fragments. A considerable proportion of the cyclosporin synthetase and alanine racemase activity could be detected in this peak. The majority of both activities was detected in fraction D (without α-mannosidase activity), representing the cytosolic fraction of the lysed protoplasts. The distribution of antigenic determinants of cyclosporin synthetase and alanine racemase correlated with the activity pattern of the gradient (Fig. 2C).

Immunolocalization studies in cells harvested after 4 and 10 days of growth revealed the important role of vacuolar membranes in compartmentation of the cyclosporin biosynthetic pathway. Ten days after inoculation, 79% of the cyclosporin synthetase-specific immunogold markers and 83% of the alanine racemase markers were associated with the vacuole. The remaining markers were located in the cytosol; 45% of the vacuolar cyclosporin synthetase markers and 60% of the vacuolar alanine racemase markers were located at the membranes of vacuoles (Figs. 3 and 4). The remaining markers were located at dark-stained vacuolar inclusion bodies.

For detection of the product cyclosporin A, the cells were processed by cryofixation and cryosectioning in order to avoid extraction or dislocation of the peptide by organic solvents or the resin during the embedding procedure. Cyclosporin was located in the vacuolar lumen, without any obvious aggregation or association with particulate vacuolar constituents such as the membrane or dark-stained vacuolar inclusion bodies (Fig. 5).

Discussion

Cyclosporin synthetase is one of the largest enzymes in nature. Its structure is of special interest, because the enzyme molecule is composed of one polypeptide chain organized in (at least functionally) separate modules instead of separate polypeptides (i.e. subunits) (Weber et al. 1994). The corresponding coding region of cyclosporin synthetase contains an ORF of 45.8 kb that encodes a peptide with a calculated molecular mass of 1,689,243 Da. The predicted gene product contains 11 amino-acid-activating modules that are very similar to one another and to the domains of other peptide synthetases (Weber et al. 1994): each module is responsible for the recognition, activation and modification of a substrate amino acid. Seven of these modules harbor *N*-methyltransferase functions localized on methyltransferase domains (Husi et al. 1997). Some of these features are reflected in the overall structure of the cyclosporin synthetase molecules, as depicted in Fig. 1.

In negatively stained preparations, two types of complexes were visible: irregular globules of 25–30 nm and chain-like complexes. The globular as well as the chain-

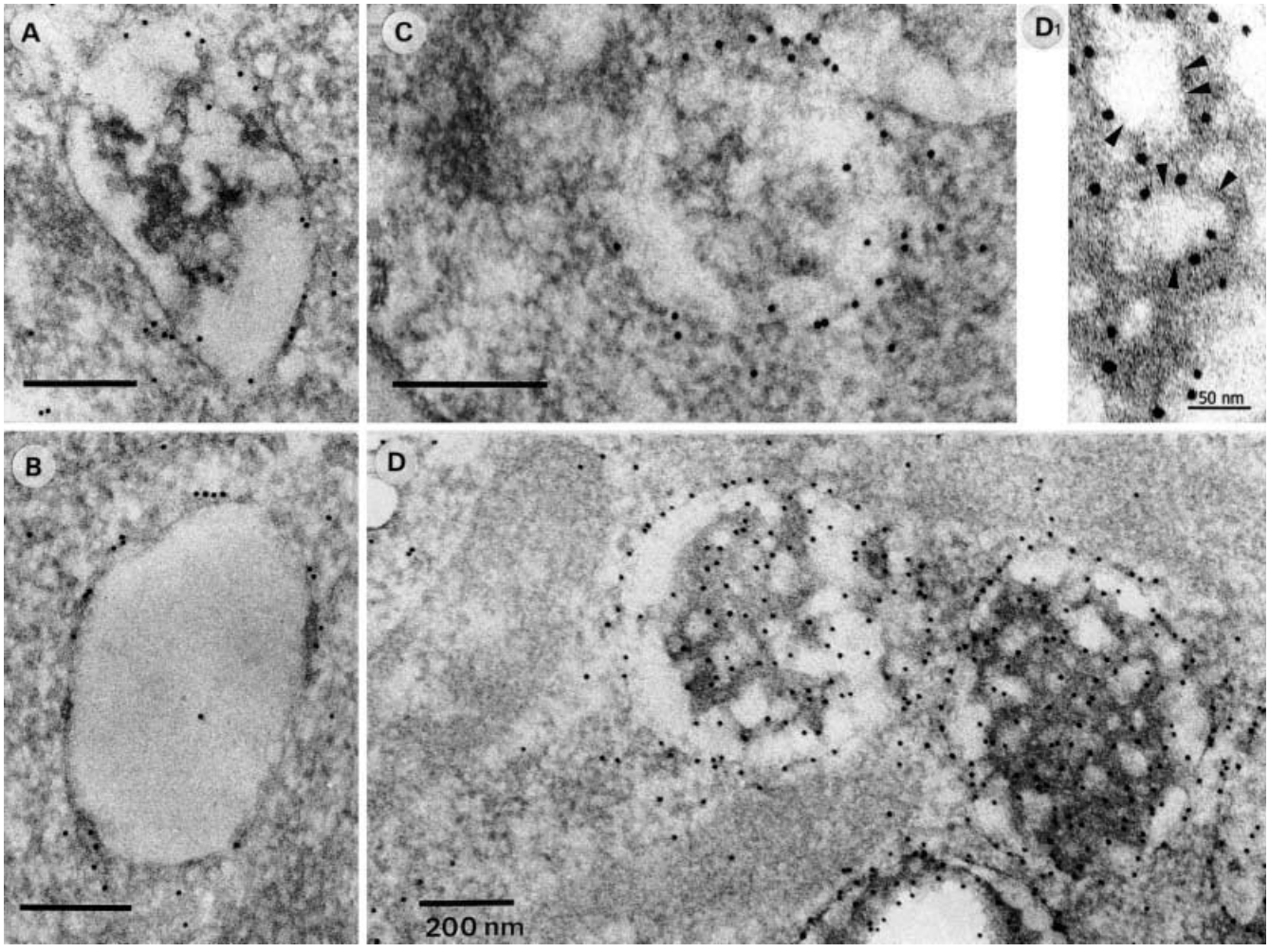

Fig. 3A–D Immunogold localization of cyclosporin synthetase in *Tolypocladium* cells. **A, B** After 4 days of fermentation. **C, D, D1** After 10 days of fermentation. Immunogold markers are predominantly located at the vacuolar periphery or bound to dark stained inclusion bodies. The *upper right figure* (**D1**) shows a portion of the intravacuolar inclusion body. Inside these bodies, especially the borderlines (*arrowheads* in **D1**) of small vesicles are decorated with gold markers

like complexes are composed of condensed, structurally separate units. In chain-like complexes, a connection between two units was not visible, but they seemed to be arranged like beads on a chain, indicating the presence of (invisible) linkers. One globule consists of a wound-up chain-like complex. Globular complexes exhibited no more than 11 units, but in numerous particles fewer units were visible. In the globules, some particles were obscured by others, and only complexes that appeared to be spread out (and thus flattened) on the carbon support film showed all particles. Similar observations have been made for the cellulosome, where only slightly decomposed ("open") complexes were attached to the carbon support film suchthat all units were visible (Mayer et al. 1987). The presence of less than 11 (and 22) particles in chain-like complexes may also be explained by: (1) the fact that sometimes the particles were obscured by their neighbors in the flexible chain, and (2) partial decomposition of the chain. In fact, a small number of single particles and short chains could be observed on the carbon film. The particles appeared to be composed of smaller substructures (Fig. 1F), with most projection forms showing two or three (Fig. 1F, arrowheads) of these substructures. In order to confirm the number of substructures, image averaging of these particles (all of them taken from chain-like complexes) was performed. In Fig. 1F, averaged images of all particles in the chain are shown. It should be kept in mind that averaging was performed with structurally similar, but not identical particles. The average image confirmed the number of substructures (either two or three) and their overall size, but did not allow resolution of further structural details.

The structurally separate particles may very well represent the described, functionally distinct 11-amino-acid modules, connected by intermodule sections of the polypeptide chain representing the linkers. Neither in globular complexes nor in chain-like complexes (Fig. 1D, E), was any obvious symmetrical arrangement visible. This would only have been expected for identical units, but in the case of cyclosporin synthetase, the sizes of the modules differed with respect to the lengths of their polypeptide

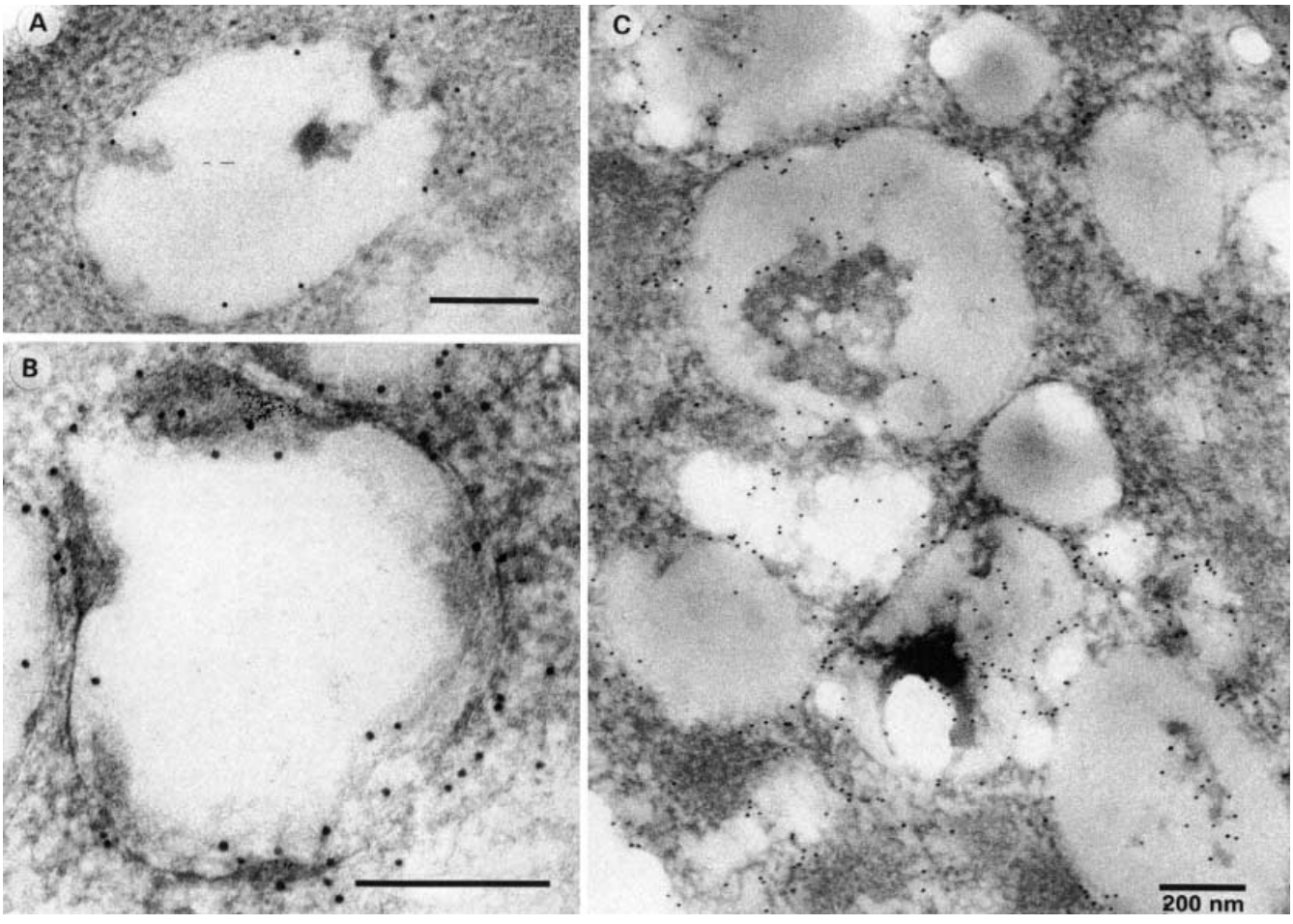

Fig. 4A–C Immunogold localization of alanine racemase in *Tolypocladium* cells. **A** After 4 days of fermentation. **B, C** After 10 days of fermentation. Immunogold markers are predominantly located at the vacuolar periphery

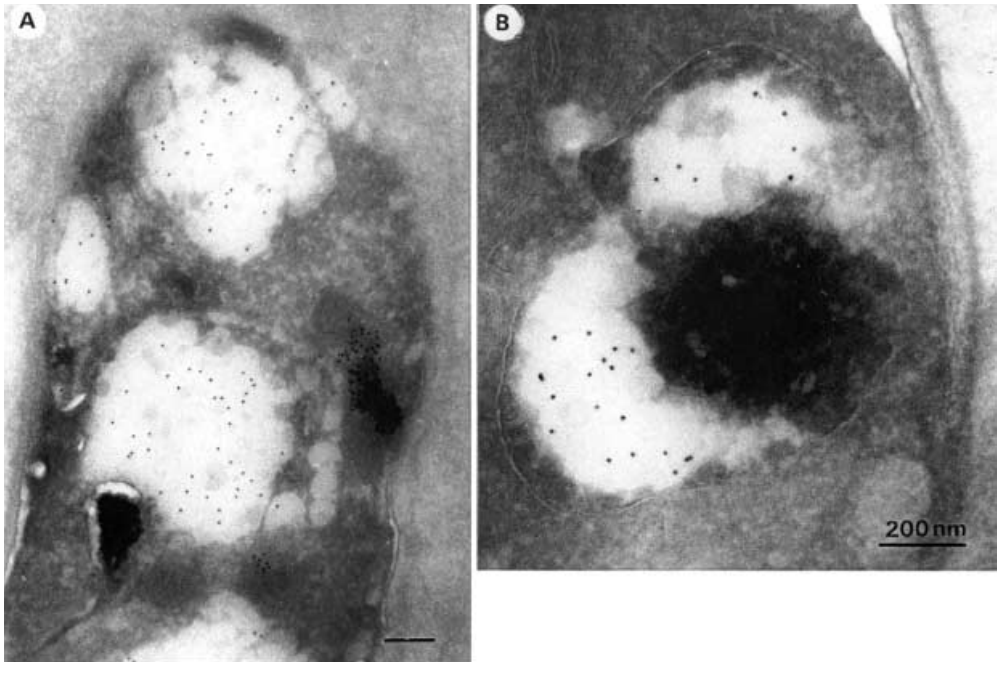

Fig. 5A, B Immunogold localization of cyclosporin in ultrathin cryosections of *Tolypocladium* cells. **A** Overview, **B** detailed view. Immunogold markers are almost exclusively located inside the vacuole. The vacuolar membrane and dark stained inclusion bodies are not labeled

chains. For modules as parts of a single polypeptide chain, the possibilities for arrangement in a complex are reduced as compared to non-covalently linked subunits. Thus, a large globular complex composed of eleven different modules (of similar size) would show numerous projection forms of very similar appearance. This seems to be true for the cyclosporin synthetase molecule. Cellulosomes, which are also composed of similar but not identical units, share some overall structural features with cyclosporin synthetase complexes. Also, cellulosomes appear as large, slightly irregular globular complexes ("closed" complexes) and slightly decomposed "open" complexes, where units with different enzyme activities are arranged in rows, but not ideally symmetrically (Mayer et al. 1987). In cellulosomes, however, the units are individual polypeptide chains interconnected by a scaffolding structure (Bayer et al. 1998).

The chain-like complexes could be observed independent of the age and specific activity of the preparations and the pH value of the staining salt. Spreading the preparation on the carbon support film may also have induced conversion of globular complexes to chain-like ones. This accounts for a certain structural instability of the whole complex.

Most particles in the chain like complexes consisted of three or two visible substructures (Fig. 1F). Actually, as deduced from alignment procedures of the amino acid sequence, seven of the eleven modules contain three large domains, composed of 450–460 amino acids, respectively (Husi et al. 1997), and representing the condensation, adenylation and methylation domains. Two smaller domains, one responsible for thioester formation (75 amino acids) and the other of unknown function (54 amino acids), must be also present but they could not be resolved in the electron microscopic image. Three of the eleven modules do not contain a methyltransferase domain, i.e. they consist of two larger and two smaller domains (the eleventh module contains an additional smaller domain). The larger domains (either two or three) were detectable as separate units in the negatively stained specimens. The smaller domains were invisible as separate units, but they may have contributed to the size of the largest unit in each particle (marked by a double arrow in Fig. 1F).

The proximity of single domains in a module allows defined channeling of the peptide. If, however, the presence of the chain-like complexes accounts for a loose interaction between modules, transfer of the growing peptide chain to the next-following module does not require a continuously defined spatial arrangement of the modules in the complex. This finding supports the fact that peptide synthetases with recombinant domains are often inactive, i.e. the interaction between domains in a module appears to be highly specific. In contrast, the exchange of complete modules may be more tolerable for the enzyme (Doekel and Marahiel 2000; Mootz et al. 2000).

Subcellular compartmentation of β-lactam antibiotic biosynthesis has been revealed by immunoelectron microscopy and monitoring of activities of the relevant enzymes in subcellular fractions (Müller et al. 1991; Lendenfeld et al. 1993; reviewed in Van der Kamp 1999). In penicillin biosynthesis, vacuoles, microbodies (or structural equivalents), and the cytosol are involved in compartmentation. According to the model developed by Lendenfeld et al. (1993), the tripeptide ACV (δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine) is synthesized from intravacuolar amino acids (valine, cysteine and L-α-aminoadipate) by the action of ACV synthetase. According to the distribution of intracellular marker enzymes (e.g. α-mannosidase), the activity of this enzyme co-localizes with vacuole fractions in a Percoll density gradient. Nevertheless, sequence analysis of either ACV or cyclosporin synthetase did not reveal obvious membrane-spanning domains and purification procedures did not require solubilization steps (Zocher et al. 1986; Smith et al. 1990; Weber et al. 1994; Theilgaard et al. 1997). Moreover, maximum catalytic activities in vitro were measured around neutral pH, indicating that the enzymes are not active in acidic compartments such as lysosomal vacuoles (Lawen and Zocher 1990; Offenzeller et al. 1993; Hoffmann et al 1994; Theilgaard et al. 1997 and Schneider-Scherzer, Biochemie GmbH, personal communication). In this study, catalytic activity and antigenic determinants of cyclosporin synthetase were detected in two peaks of the density gradient (see Fig. 2). Peak D may very well have represented the soluble, cytosolic fraction of the cells, whereas peak A contained the vacuolar fraction. Though density gradient centrifugation only allows a crude separation of cellular constituents, the presence of cyclosporin synthetase and alanine racemase in two peaks shows that the enzymes are not exclusively soluble and cytosolic. A more refined view on the distribution of both enzymes was achieved using immunoelectron microscopy. Immunolocalization experiments revealed that cyclosporin synthetase and alanine racemase are located in association with vacuoles, especially the membrane region (Figs. 3, 4). Since only about 20% of the cyclosporin synthetase and alanine racemase markers were located in the cytosol, it must be assumed that, due to artificial disruption and rearrangement of vacuolar membranes before and during density gradient centrifugation, most of the enzymes were sheared off the vacuolar membrane. The enzymes would have thus been purified from the cytosolic fractions as reported previously (Hoffmann et al. 1994; Zocher et al. 1986). The results indicate that the enzymes are only loosely attached to the vacuolar membranes, as also assumed for the ACV synthetase in *Penicillium chrysogenum* (Lendenfeld et al. 1993). Especially in cells after 10 days of growth, a considerable part of the cyclosporin synthetase and alanine racemase markers bound to dark-stained condensed areas resembling particulate vacuolar inclusions that are known from several fungal cells (see e.g. Wiemken et al. 1979). The co-localization of α-mannosidase with cyclosporin synthetase and alanine racemase and the observed morphology were similar to the features and distribution patterns of (lysosomal) vacuoles from other fungal cells. Thus, it may be excluded that these vacuoles are specialized compartments (such as microbodies involved in compartmentation of the penicillin

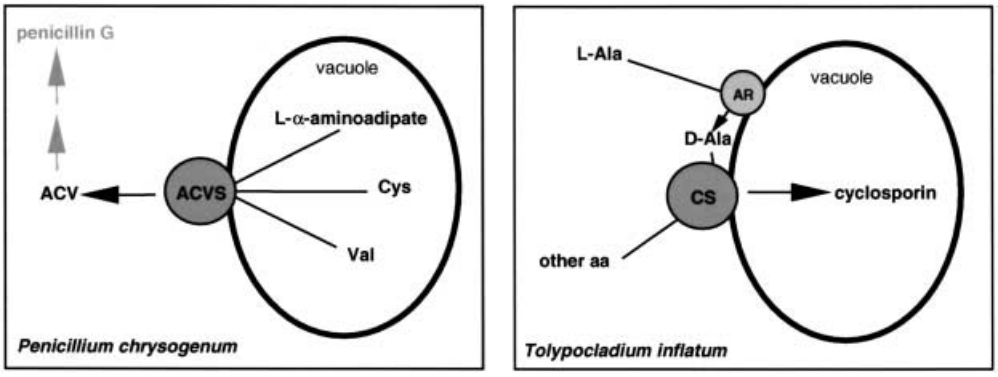

Fig. 6 Compartmentation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthesis in *Penicillium* and cyclosporin synthesis in *Tolypocladium*. In both organisms, a close interaction between the peptide synthetase and the vacuolar compartment is deduced from immunolocalization studies. In *Tolypocladium inflatum*, D-Ala is synthesized by alanine racemase and passed over to cyclosporin synthetase. *ACVS* ACV synthetase, *AR* alanine racemase, *CS* cyclosporin synthetase, *aa* amino acids

biosynthetic pathway). Intravacuolar enzymes exhibit pH optima below 7. In contrast, the in vitro maximum activity of cyclosporin synthetase and alanine racemase is around neutral pH (see above). Therefore, intravacuolar antigenic determinants may indicate the presence of inactive enzymes that have been imported into the vacuole to become hydrolyzed by proteases. Similar distribution patterns of markers have been described for stages during the yeast lysosomal degradation of cytosolic fructose-1,6-bisphosphatase (Chiang et al. 1996). Upon induced fructose-1,6-bisphosphatase degradation, intravacuolar markers were detected inside vacuoles, either free or engulfed by membranes. In our study, the intravacuolar inclusions showed a large number of vesicles, surrounded by darker material. Especially the cyclosporin synthetase markers seemed to decorate the borderline of these intravacuolar vesicles (see Fig. 3D1). It may be assumed that this borderline represents remnant invaginated vacuolar membranes with bound antigenic determinants of cyclosporin synthetase. Indications for peroxisomal delivery of cyclosporin synthetase and alanine racemase to the vacuole or the presence of autophagic bodies could not be observed (compare Baba et al. 1994; Chiang et al. 1996).

Taking these facts and considerations together, the following model for compartmentation of the cyclosporin biosynthetic pathway may be proposed. Cyclosporin synthetase and alanine racemase are attached to the outside of the vacuolar membrane (presumably via an additional linker) and synthesize the cyclic undecapeptide cyclosporin from single amino acids. Cyclosporin is subsequently deposited in the vacuolar lumen (Fig. 5). Alanine racemase and cyclosporin synthetase seem to be located in close vicinity to each other, since D-alanine is the leading amino acid of the polypeptide chain synthesized by cyclosporin synthetase. The schematic drawing in Fig. 6 depicts the analogy to penicillin biosynthesis. There, ACV synthetase is located at the vacuolar membrane and synthesizes ACV into the cytoplasm, presumably using the intravacuolar pool of amino acids. Because ACV has to be processed by subsequent steps, transfer from the cytoplasm to another compartment, the microsome, may be reasonable. In contrast to ACV, cyclosporin is the final product of the pathway and needs no further processing. Compartmentation is, therefore, quite simple: only the cytoplasm and the vacuole are involved and the product is synthesized into the vacuolar lumen. Immunolocalization data did not reveal any transport of cyclosporin outside of the cell; rather, high concentrations of cyclosporin are stored inside vacuoles and may be released slowly via vacuolar and cytoplasmic membranes or rapidly upon cell lysis.

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