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δ -(L- α -Aminoadipyl)-L-cysteinyl-D-valine synthetase, that mediates the first committed step in penicillin biosynthesis, is a cytosolic enzyme

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Abstract

Penicillin biosynthesis by *Penicillium chrysogenum* is a compartmentalized process. The first catalytic step is mediated by δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase), a high molecular mass enzyme that condenses the amino acids L- α -aminoadipate, L-cysteine, and L-valine into the tripeptide ACV. ACV synthetase has previously been localized to the vacuole where it is thought to utilize amino acids from the vacuolar pools. We localized ACV synthetase by subcellular fractionation and immunoelectron microscopy under conditions that prevented proteolysis and found it to co-localize with isopenicillin N synthetase in the cytosol, while acyltransferase localizes in microbodies. These data imply that the key enzymatic steps in penicillin biosynthesis are confined to only two compartments, i.e., the cytosol and microbody. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: β -Lactam antibiotics; Compartmentalization; Penicillin biosynthesis; Peptide synthetase; Secondary metabolism; Vacuole

1. Introduction

Several Gram-positive bacteria and filamentous fungi produce β -lactam antibiotics (e.g., cephamycins, cephalosporins, and penicillins) (Martín and Liras, 1989). Since the discovery and biochemical characterization of the enzymes involved in the biosynthesis of these secondary metabolites, much effort has been made to understand the molecular basis of antibiotic biosynthesis and its regulation. Regulation of the synthesis of the penicillin biosynthetic enzymes is tightly interlinked with the regulatory networks controlling primary metabolism

of the cell. In filamentous fungi such as *Penicillium chrysogenum*, penicillin biosynthesis is compartmentalized (Van de Kamp et al., 1999). The first committed step in penicillin biosynthesis is the condensation of the amino acids L- α -aminoadipate, L-cysteine, and L-valine to the tripeptide δ -L- α -aminoadipyl-L-cysteinyl-D-valine (ACV). This reaction is catalyzed by a 420-kDa ACV synthetase (Martín and Liras, 1989; Zhang and Demain, 1992). During penicillin biosynthesis the amino acid precursors are thought to be withdrawn from storage pools in the vacuole (Affenzeller and Kubicek, 1991; Lendenfeld et al., 1993). Next, a cytosolic enzyme, isopenicillin N synthase, catalyzes the formation of the β -lactam backbone from the ACV tripeptide yielding isopenicillin N (Müller et al., 1991; Ramos et al., 1985). Finally, isopenicillin N:acyltransferase exchanges the L- α -aminoadipyl side-chain of isopenicillin N for a more hydrophobic (CoA-activated) side chain resulting in biologically active penicillin. This step occurs within a

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microbody (Müller et al., 1991, 1992, 1995). During penicillin G synthesis, the side-chain phenylacetic acid is activated by a CoA ligase that also is localized to the microbody (Gledhill et al., 1995).

Subcellular compartmentalization of the catalytic steps enables penicillin biosynthetic enzymes to act in distinct and optimized environments, but also requires intracellular transport of precursors, intermediates and products. Consequently, the exact nature of the compartmentalization is important. ACV synthetase is a 420-kDa, multi-domain enzyme with multi-enzymatic activities (Aharonowitz et al., 1993). It has been associated with or confined to membrane structures. Initially, these membranes were identified as Golgi-like organelles (Kurylowicz et al., 1987; Kurzltkowski and Kurylowicz, 1991). A more recent cell fractionation experiment suggests that part of the cellular ACV synthetase is localized in, or associated with, vacuoles (Lendenfeld et al., 1993). However, the intrinsic instability and susceptibility of ACV synthetase for proteolysis has hampered an unambiguous localization of the intact enzyme using cell lysis and fractionation techniques. The *in vitro* ACV synthetase activity shows a pH optimum of pH 8.4 (Theilgaard et al., 1997) which is well above that of the vacuolar pH. Since the vacuole is a storage and degradation organelle, the observed ACV synthetase localization may be an artifact of the vacuole isolation procedure which was dependent on a protoplast formation and sucrose gradient centrifugation steps that required up to 24 h (Lendenfeld et al., 1993). Therefore, we used in addition to a rapid biochemical analysis, immuno-electronmicroscopy to localize the AVC synthetase in the cell under conditions in which the enzyme remains intact.

2. Materials and methods

2.1. Strains, media, and growth conditions

Penicillium chrysogenum strains DS04825, Wisconsin 54–1255 (Wis54–1255) *wt* and *npe10* (Cantoral et al., 1993) were kindly supplied by DSM-Anti-Infectives (Delft, The Netherlands) and J.F. Martín (Léon, Spain). Strains were grown for 64–70 h in batch cultures (25 °C and 250 rpm) on a penicillin-production medium (pH 6.3) containing lactose, glutamate, and phenylacetate as the side-chain precursor (Hillenga et al., 1994).

2.2. Materials

To raise antibodies against AVC synthetase, a 1.7-kb fragment encoding the valine-activating domain of the large *pcbAB* gene was PCR amplified using the FWD-ValD (5'-CCCTTCGAGCTCGGCGACCAG

CCTATTCAACAAAAGCACGATCCAGGG-3') and REV-ValD (5'-CTCAGCCCCGGG TCACTGCACGAATCCGACTTCCTCGCGCTTGAGTCCG-3') primers. The resulting fragment was sequenced and cloned in frame to the *malE* gene, encoding the maltose binding protein (MBP), for rapid purification from *Escherichia coli* cell-free extract. For this, the fragment was digested with *SacI* and *SmaI* (bold and underlined in the primers), cloned into pAJL104 (Little, 1997) digested with the same enzymes. The resulting plasmid pAJL104ValD was transformed to TOP10F cells (Invitrogen).

For expression and purification of IPN synthase, also a fusion with MBP was constructed. The *pcbC* gene was amplified by PCR, using the FWD-IPNS (5'-CCC GGGGAATTCACCATGGCTTCCACCCCAAGGC C-3') and REV-IPNS (5'-CCCGGGGTCGACTTCCA TCCCGGTCCCATCCATGGGCC-3') primers, introducing *EcoRI* and *SalI* sites. The PCR fragment was digested with *EcoRI* and *SalI* and subsequently ligated to the digested pAJL104 vector, resulting in pAJL-*pcbC*.

Both plasmids contain the *lacI^q* gene and the fusion protein is under control of the *tac* promoter. After induction by IPTG, cells of 100 ml culture were suspended in 2 ml extraction buffer (5 mM DTT, 0.5 mM PMSF, 50 mM Tris-HCl, pH 7.5). The suspension was sonicated (6 times 10 s, cooled on ice). After centrifugation, the proteins were isolated from the supernatant fraction via an amylose column (Biolabs, New England) and eluted with a buffer containing 100 mM maltose, 50 mM Tris-HCl, pH 7.5. The fractions containing purified fusion proteins were collected and stored at -80 °C. Rabbits were immunized intradermally with MBP-ACVS or MPB-IPNS and two subsequent boosts were given (Eurogentec, Belgium). Approximately 600 µg of peptide was used for both during the entire immunization procedure. Antibodies against acyltransferase were described previously (Müller et al., 1991).

2.3. Stabilization of ACVS in cellular extracts

Mycelium from a 150-ml culture of *P. chrysogenum* Wis54–1255 or DS04825 grown for 64–70 h was harvested by suction filtration, washed twice with 50 ml of an ice-cold solution of 0.9% NaCl (typical yield of 10 g of wet mycelium), and immediately resuspended in 50 ml ice-cold disruption buffer D1 (0.25 M sorbitol, 50 mM MOPS/KOH buffer, pH 7.5, 25 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂) or D2 (same as D1 but with 1.5 M instead of 0.25 M sorbitol). Subsequently, 30 g of 1.0-mm diameter glass beads were added, and cells were disrupted either for 3 min (D1) or 7.5 min (D2) using a Braun MSK cell homogenizer (Braun GmbH, Melsungen Germany) operating under semi-continuous cooling with a cold air-stream of expanding CO₂-gas (Cramer et al., 1983; Hillenga et al., 1994) until

disruption was >90%. The suspension was cleared from undisturbed mycelium by centrifugation (5 min, 3000g, 2°C). The supernatant fraction was distributed over various tubes, so that they contained buffer D1 or D2 with all possible combinations of the following additions (final concentrations): glycerol (25% [v/v]); NaN₃ (10 mM); Complete (Boehringer, Mannheim, Germany) (1 tablet/50 ml); DTT (5 mM); L- α -amino adipate (Aad) (4 mM), L-Cys (1 mM), and L-Val (4 mM). Suspensions were incubated under mild shaking conditions at 4°C (D1-buffer) or 25°C (D2-buffer). Samples were taken after 1, 2, 4, 8, 24, 60, and 96 h of incubation, precipitated with 12.5% TCA (Wessel and Flügge, 1984), solubilized in sample buffer, and analyzed by SDS-PAGE and Western blotting.

2.4. Sedimentation of organelles from disrupted protoplasts

Mycelia were harvested by suction filtration and washed with water and 0.8 M KCl-citrate buffer, pH 6.2. For protoplasting, the mycelium was incubated (2 h, 25°C, 100 rpm) in the same buffer supplemented with 10–15 mg/ml caylase (Cayla, France), and subsequently diluted with one volume of 1.2 M sorbitol, 50 mM MOPS/KOH, pH 7.5. Protoplasts were collected by centrifugation (2000g, 4°C, 20 min), washed three times using 1.2 M sorbitol, 50 mM MOPS/KOH, pH 7.5 containing a protease inhibitor cocktail (Complete, Boehringer), and finally lysed by resuspending the pellet in 1 M sorbitol, 25% glycerol, 50 mM MOPS/KOH, pH 7.5, 5 mM DTT and 2 tablets of Complete/50 ml, and homogenization. The material was centrifuged (500g, 20 min, 4°C) after which the resulting large pellet, consisting mainly of intact protoplasts, cellular debris and nuclei, was discarded. The post-nuclear supernatant was immediately subjected to centrifugation (100,000g, 4°C, 30 min), and the supernatant was collected and precipitated using 12.5% TCA. Pellets were solubilized in an equal volume of the same buffer containing 0.5% Triton-X 100 and immediately precipitated using 12.5% TCA. All TCA pellets were washed using ice-cold acetone, resuspended in SDS sample buffer and analyzed by SDS-PAGE.

2.5. Electron microscopy

Hyphae of *P. chrysogenum* Wisconsin 54–1255 and *npe10*, grown for three days on penicillin-production medium, were fixed in a mixture of glutaraldehyde (0.5% [v/v]) and formaldehyde (2.5% [v/v]) as described previously (Waterham et al., 1994). Immuno-labeling was performed on ultrathin sections of unicryl-embedded hyphae, using specific antibodies against various proteins and gold-conjugated goat anti-rabbit antibodies (Waterham et al., 1994).

2.6. Other methods

The vacuolar V₀V₁-ATPase was detected by immunoblotting using an antibody against the β -subunit of the V₀V₁-ATPase from *Saccharomyces cerevisiae*.

3. Results

3.1. Stability of ACV synthetase

ACV synthetase is unstable in crude cellular extracts (Theilgaard et al., 1997; data not shown). By means of immuno-blotting, we observed little if any, degradation of ACV synthetase and the vacuolar membrane marker, the V₀V₁-ATPase, when mycelium of *P. chrysogenum* Wisconsin 54–1255 was incubated (up to 24 h) with cell-wall lytic enzymes to prepare protoplasts provided that the protoplasts were transferred directly to standard SDS-sample buffer. ACV synthetase was rapidly degraded if, prior to SDS-PAGE, the cellular extract was prepared (at 4 or 25°C) by a disruption method involving glass-beads (Hillenga et al., 1994). Various additives were tested for their ability to stabilize the enzyme in cellular extracts, including the addition of NaN₃, dithiothreitol (DTT), substrate amino acids, glycerol, protease inhibitors such as EDTA, PSMF, and Complete (Boehringer, Mannheim, Germany), and sorbitol in different combinations and concentrations as exemplified in Fig. 1. ACV synthetase remained stable up to 96 h when the cell lysate was prepared in the presence of 25% glycerol and 0.25 or 1.5 M sorbitol at 4 or 25°C, respectively (Fig. 1, and data not shown). In the absence of glycerol, about 50% of the enzyme was degraded after 8 h incubation at 4°C. Optimal stabilization was observed in a buffer containing 25% glycerol, 1 M sorbitol, 50 mM MOPS/KOH, pH 6.3, 5 mM DTT,

ACV synthetase	Western blot analysis															
Fraction	S	P	S	S	P	S	S	P	S	S	P	S				
	3	100	3	100	3	100	3	100	3	100	3	100				
Stabilizer	NaCl				sorbitol				NaCl				sorbitol			
	-								glycerol							

Fig. 1. Stability and sedimentation of ACV synthetase. Mycelium from Wis54–1255 was resuspended in 50 ml ice-cold disruption buffer (50 mM MOPS/KOH buffer, pH 7.5, 25 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂) supplemented with either 0.25 M sorbitol or 0.15 M NaCl. The cells were disrupted as described in Section 2, and the suspension was cleared from undisturbed mycelium by centrifugation (5 min, 3000g, 2°C). The post-nuclear supernatant was distributed into the disruption buffer with or without 25% (v/v) glycerol and Complete (1 tablet/50 ml). The suspensions (S3) were incubated for 24 h under mild shaking conditions at 4°C, and subsequently fractionated into a pellet (P100) and supernatant (S100) fraction by centrifugation (100,000g, 4°C, 30 min). The various fractions were analyzed by SDS-PAGE and Western blotting using polyclonal antibodies directed against ACVS.

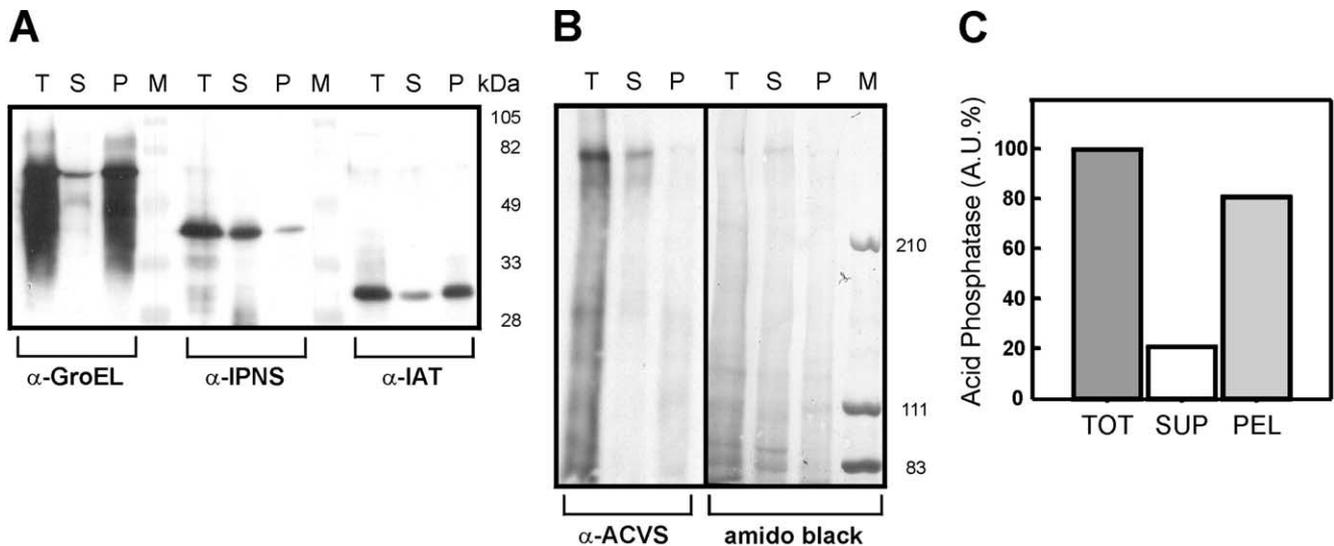


Fig. 2. Subcellular fractionation and sedimentation of organelles. Analysis of proteins present in the post-nuclear supernatant fraction of disrupted protoplasts (T) of *P. chrysogenum* DS04825. After centrifugation at 100,000g, the cytoplasmic material was recovered in the supernatant (S) fraction whereas organellar and membranous material was pelleted (P) and resuspended to the same volume as the original total extract. For comparison, aliquots of identical volume of the three fractions were loaded on the SDS-PAGE. Markers (M) are indicated. (A) Western analysis after 12% SDS-PAGE using antibodies against IPNS, AT, and *E. coli* GroEL. (B) Western analysis after 5% SDS-PAGE using an anti-ACV synthetase antibody. As a control, the blot was stained with amido black showing intact enzyme as a high molecular mass protein. (C) Activity of the vacuolar marker acid phosphatase in the different fractions.

and 1 tablet of the protease inhibitor cocktail Complete/25 ml. This buffer was used for the cell-fractionation experiments.

3.2. Subcellular fractionation of ACV synthetase

To localize ACV synthetase, we prepared protoplasts from *P. chrysogenum* DS04825 or Wisconsin 54–1255 (data not shown) by incubation of the hyphae with the cell-wall degrading enzyme caylase. Protoplasts were disrupted by homogenization in stabilization buffer to protect the ACV synthetase against degradation. The post-nuclear supernatant was fractionated by ultracentrifugation (100,000g, 4°C, 30 min) to yield a pellet (P) and soluble (S) fraction that represent the organelles and cytosol, respectively. The fractions were analyzed by enzyme assays, SDS-PAGE and immuno-blotting. Acyltransferase was immuno-detected primarily in the 100,000g pellet (Fig. 2A) in line with earlier observations that this enzyme is present in the microbody matrix (Müller et al., 1991). The mitochondrial marker GroEL, a chaperone molecule detected by an antibody against *E. coli* GroEL (Fig. 2A) as well as the activity of the vacuolar marker acid phosphatase (Fig. 2C) were detected in the pellet under these conditions. Isopenicillin N synthase (Fig. 2A) and ACV synthetase (Fig. 2B) were immuno-detected in the soluble fraction. Since ACV synthetase and the cytosolic isopenicillin N synthase (Kurztkowski and Kuryłowicz, 1991; Kurztkowski et al., 1991; Müller et al., 1991) were observed in the same fraction under conditions at which vacuolar,

mitochondrial, and peroxisomal markers were found to sediment, we hypothesize that ACV synthetase is localized in the cytosol.

3.3. Immuno-gold electron microscopy localization

To further analyze the localization of ACV synthetase, the enzyme was immuno-detected on ultrathin sections of *P. chrysogenum* Wisconsin 54–1255 hyphae, using antibodies against ACV synthetase and gold-conjugated goat anti-rabbit antibodies. The α-ACVS specific labeling was randomly distributed over the cytosol, and invariably absent in the vacuole and any other organelle (Fig. 3B). In control experiments, using mycelium of the Wis54–1255 *npe10* mutant strain that lacks the complete penicillin biosynthesis gene cluster (Cantoral et al., 1993), no labeling was observed (Fig. 3A). Using the same immuno-localization method, we found that the two other enzymes directly involved in penicillin biosynthesis, isopenicillin N synthase and acyltransferase, localize in the cytosol and microbody, respectively (Figs. 3C–F) confirming previous findings (Müller et al., 1991). Taken together, these data indicate that ACV synthetase is a soluble cytosolic protein that is neither attached to nor located in vacuoles.

4. Discussion

Previously, it has been suggested that ACV synthetase, a key enzyme in the biosynthetic route of β-lactam

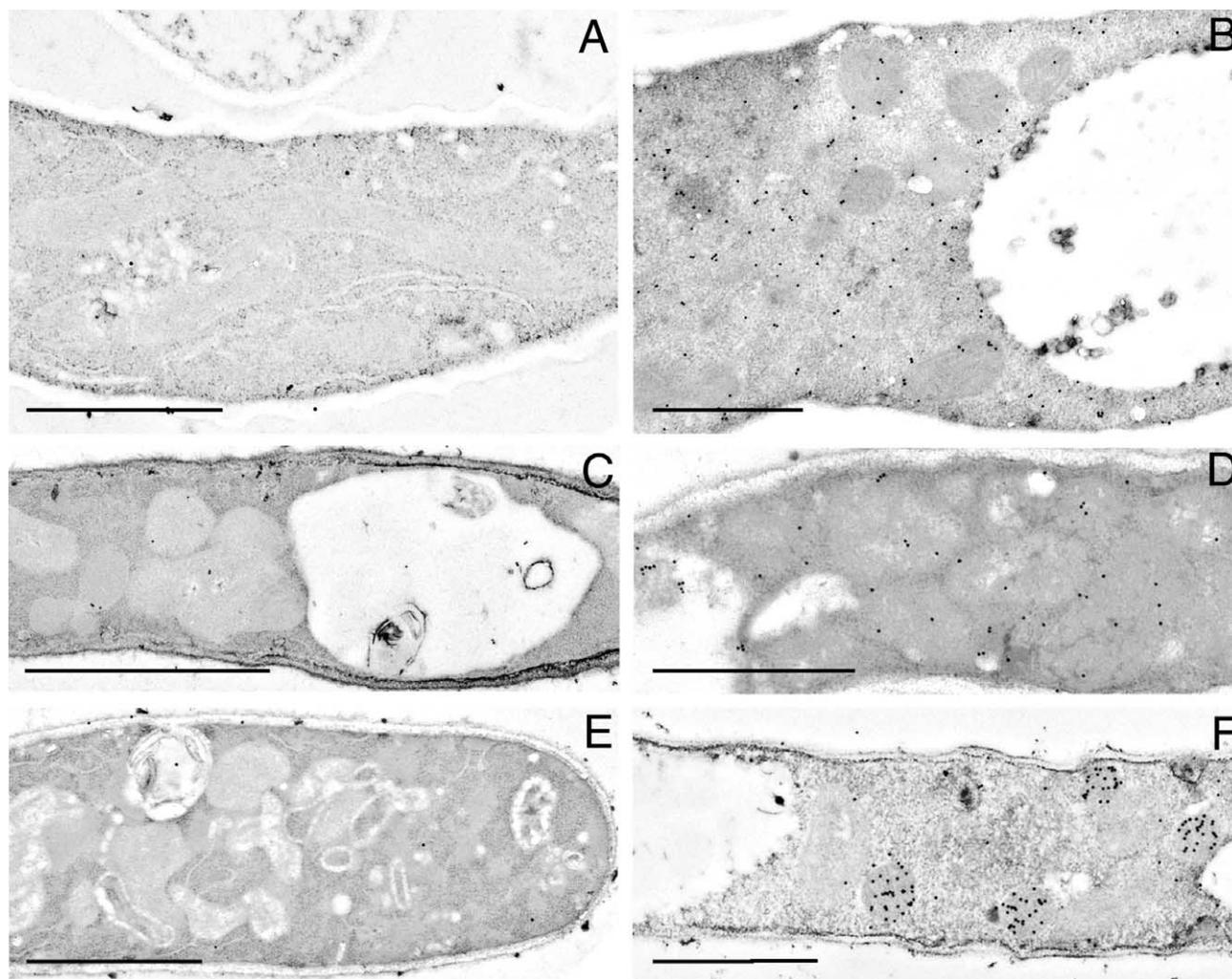


Fig. 3. Immuno-gold labeling of ACV synthetase (A and B), IPN synthase (C and D) and acyltransferase (E and F) in electron micrographs of mycelium of *P. chrysogenum*. For immuno-localization, strain Wis54-1255 was used (B, D, and F), and the *npe10* (A and C) or *npe6*, an acyltransferase mutant (E). The strains were grown under penicillin producing conditions. Labeling experiments were carried out as described in the text. The drawn marker represents 1 μm . M, mitochondrion; V, vacuole.

synthesis, is a vacuolar enzyme (Lendenfeld et al., 1993). Here we show by means of combined cell fractionation under stabilizing conditions and immuno-localization experiments using specific antibodies, that ACV synthetase is a cytosolic enzyme in *P. chrysogenum*. This localization is consistent with the pH optimum of the enzyme, which is close to pH 8.4, and the known high protease susceptibility of this large multi-domain enzyme which would render it highly unstable in the proteolytic acidic vacuolar environment (Theilgaard et al., 1997). In this respect, ACV synthetase does not have sequence motifs known to predict a membrane-bound location, or an identifiable signal sequence, which would direct the enzyme to a specific membrane or organelle. Our results contrast earlier findings that ACV synthetase is localized in or with vacuoles (Lendenfeld et al., 1993). However, in the latter study substantial proteolysis of ACV synthetase occurred, and the possibility

exists that these fragments localize to the vacuole. Moreover, a preliminary report on a GFP-ACV synthetase fusion protein in a related filamentous fungus producing penicillin, *Aspergillus nidulans*, confirms our finding that the enzyme localizes to the cytosol (Vousden and Turner, 2001). Using immuno-chemistry, we also confirmed that the two other enzymes directly involved in penicillin biosynthesis, IPN synthase and acyltransferase, are located in the cytosol and microbody, respectively, in line with previous findings (Müller et al., 1991).

The localization of ACV synthetase in the cytosol has important implications for our current understanding of β -lactam biosynthesis. First, the results imply that the microbody is the only subcellular compartment in which part of penicillin biosynthesis takes place (Fig. 4). The presumed vacuolar localization of ACV synthetase is inconsistent with the pH optimum of the enzyme which

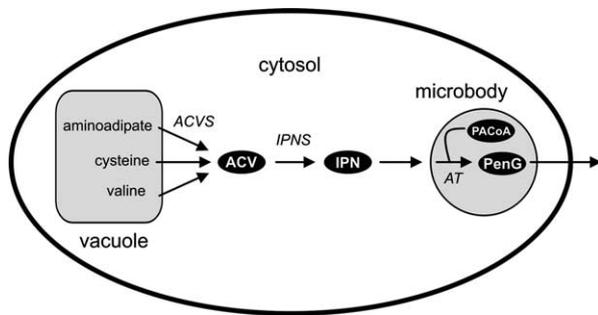


Fig. 4. Scheme for the compartmentalization of penicillin biosynthesis in *P. chrysogenum*. ACVS, ACV synthetase; ACV, δ -L- α -aminoadipyl-L-cysteiny-D-valine; AT, acyltransferase; IPNS, IPN synthase; IPN, isopenicillin N; PACoA, phenylacetyl-Coenzyme A; PenG, penicillin G.

would render the enzyme nearly inactive in the acidic vacuolar environment. In the neutral cytosolic environment such restriction does not exist. Second, the tripeptide product ACV is formed in the same compartment (cytosol) as where IPN synthase is localized. In principle this implies that ACV can be immediately oxidized to isopenicillin N, which is important as ACV synthetase appears to be a relatively inefficient enzyme with a low turnover (Aharonowitz et al., 1993) while IPN synthetase is rather instable in the presence of ACV and during the turnover (Perry et al., 1988). An immediate conversion which is possible due to the co-localization of ACV and IPN synthetase will therefore facilitate the biosynthetic flux towards penicillin. Third, ACV synthetase is a multidomain enzyme with a complex co-factor requirement. A vacuolar localization would require some means of transport of such molecules into the organelle. With a cytosolic localization of the enzyme, there is no need to take such processes into account for metabolic flux analysis and strain improvement for the industrial production of penicillin. The vacuole may, however, play an important role in amino acid retention and, likely, the regulated supply of the amino acids to the cytosol where the ACV synthetase resides. It is believed that during penicillin biosynthesis the amino acid precursors are withdrawn from the vacuolar lumen (Affenzeller and Kubicek, 1991; Lendenfeld et al., 1993). Recently, the bidirectional amino acid transport systems in the vacuolar membrane of *S. cerevisiae* have been analyzed genetically (Rusnak et al., 2001). The availability of these genes now allows the identification of the transporters of *P. chrysogenum* that are involved in α -aminoadipate, valine, and cysteine transport in and out of the vacuole, and an assessment of their control on the flux through the β -lactam biosynthetic pathway. In this respect, α -aminoadipate originates from the lysine biosynthetic pathway (Casqueiro et al., 1999) and is not derived from the protein proteolysis in the vacuole. On the other hand, vacuolar influx of α -aminoadipate is likely prevented by a homolog of the permease AVT6 that in *S. cerevisiae*

efficiently mediates the efflux of the acidic amino acid glutamate and aspartate out of the vacuole (Kitamoto et al., 1988). It therefore seems unlikely that the vacuole fulfils a central role in α -aminoadipate supply. With the supply of the amino acids L-valine and L-cysteine, a different situation may exist and vacuolar partitioning may well influence the biosynthetic flux leading to the formation of penicillin and other β -lactams in filamentous fungi.

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