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

Lende, Ted R. van der; Kamp, Mart van de; Berg, Marco van den; Sjollema, Klaas; Bovenberg, Roel A.L.; Veenhuis, Marten; Konings, Wil N.; Driessen, Arnold J.M.

Published in:
Fungal Genetics and Biology

DOI:
10.1016/S1087-1845(02)00036-1

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase, that mediates the first committed step in penicillin biosynthesis, is a cytosolic enzyme

Ted R. van der Lende, Mart van de Kamp, Marco van den Berg, Klaas Sjollema, Roel A.L. Bovenberg, Marten Veenhuis, Wil N. Konings, and Arnold J.M. Driessen

1 Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands
2 DSM Anti-Infectives, P.O. Box 425, 2600 AK Delft, The Netherlands
3 Department of Eukaryotic Microbiology and Electron Microscopy, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Received 28 November 2001; accepted 21 May 2002

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., cephemycins, 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
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’-CCCTTCGAGCTCGGCCGACCAG

| CCTATTCACAAAAAGCACGATCCAGGGG-3’ | and REV-ValD (5’-CTCACCGCCGGG TCACTGACGA ATCCGACCTTCGCCGCTGATCG-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 Smal (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 GGGGAATTTCACCATGGGCTTCCACCCCCCAAGG C-3’) and REV-IPNS (5’-CCCGGGGTCGACCTTCCA TCCGGGTCATCCATGGGC-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 lacIq 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 MBP-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 undisrupted 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-aminoadipate (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 Flugge, 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 (500 g, 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 unicyl-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 immuno-blotting 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 vacular 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, and 0.25 M KCl.

![Fig. 1. Stability and sedimentation of ACV synthase. Mycelium from Wn54–1255 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 undisrupted 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 (S1) 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.](image-url)
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 (100,000 *g*, 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 100,000 *g* 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 Kurylowicz, 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 invariantly 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...
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 localize 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 implication 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
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 the 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; Lenfenfeld et al., 1993). Recently, the bidirectional amino acid transport systems in the vacuolar membrane of S. cerevisiae have been analyzed genetically (Russnak 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 fulfills 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.

Acknowledgments

This work was supported by grants from the European Union (BIOT CT 94-2100, and EUROFUNG Cell-Factory-RTD BIO4CT96-0535). Antibodies against ACV synthetase, isopenicillin N synthase, and acyltransferase were kindly provided by DSM Anti-Infectives (Delft, the Netherlands). We thank S. Tuinman for the purification of the fusion proteins and antibody formation. Antibodies against the β-subunit of the V₀V₁-ATPase from S. cerevisiae were a gift from Dr. Anraku (Tokyo University, Japan). P. chrysogenum strains DS04825, Wisconsin 54–1255 (Wis54–1255), npe6 and npe10 were kindly supplied by DSM Anti-infectives, Delft, The Netherlands, and Prof. J.F. Martín, Léon Spain.

References


