The cellular localization of the origin of \( \alpha \)-aminoadipate used in penicillin biosynthesis and the first enzymatic step in *Penicillium chrysogenum* involved, \( \delta \)-(\( \alpha \)-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), has been studied. Subcellular fractions were obtained from protoplasts of a high penicillin-producing strain upon lysis by Triton X-100, and vacuoles purified from protoplasts prior to subcellular fractionation. 15.6 and 26.5%, respectively, of \( \delta \)-labeled \( \alpha \)-aminoadipate, and 8.5 and 10.3%, respectively, of \( \delta \)-labeled valine added accordingly were also found in the vacuole, and the higher proportion was found in vacuoles isolated from penicillin-producing mycelia. ACVS protein was detected in the membrane as well as the soluble fraction of the purified vacuoles. We propose therefore that ACVS is located either within or bound to the vacuolar membrane, and that the precursor amino acids for penicillin biosynthesis are withdrawn from the vacuolar amino acid pool.

The considerable industrial potential of the \( \beta \)-lactam antibiotic penicillin has prompted intensive research on its biosynthesis. Although the enzymes and structural genes involved in penicillin biosynthesis have been isolated (Ramos et al., 1985; Carr et al., 1986; Whitman et al., 1990; Veenstra et al., 1988; Diez et al., 1990; Smith et al., 1990), knowledge of the regulation and subcellular compartmentation of the penicillin biosynthetic pathway is still incomplete and in part controversial. With regard to control of channeling of amino acid precursors into the penicillin biosynthetic pathway, the cellular concentration of \( \alpha \)-aminoadipate, an intermediate of lysine biosynthesis in fungi and the starting amino acid for penicillin formation (Fig. 1), has been shown to be critical; higher penicillin-producing strains were shown to accumulate higher intracellular concentrations of \( \alpha \)-aminoadipate (Jaklitsch et al., 1986). Raising the intracellular concentration by addition of exogenous \( \alpha \)-aminoadipate increased the rate of \( \delta \)-(\( \alpha \)-aminoadipyl)-cysteinyl-valine synthesis (Honlinger et al., 1988; Honlinger and Kubicek, 1989a). These findings suggest a limitation of \( \delta \)-(\( \alpha \)-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS)\(^1\) by one of its substrates in *vivo*

Some attempts to analyze the metabolic regulation of the \( \alpha \)-aminoadipate pool size in *Penicillium chrysogenum* have been published (Friedrich and Demain, 1978; Revilla et al., 1986; Jaklitsch et al., 1987). We have recently recognized that the \( \alpha \)-aminoadipate pool used for penicillin biosynthesis is apparently sequestered from the cytosol (Honlinger and Kubicek, 1989b). Exchange of \( \alpha \)-aminoadipate between the cytosol and this yet unidentified compartment could be an important factor controlling the size of the pool used for penicillin biosynthesis. The identification of this compartment is thus necessary to understand \( \alpha \)-aminoadipate accumulation and its role in penicillin biosynthesis in *P. chrysogenum*.

Kurylowitz, Kurzatkowski, and co-workers previously reported on the localization of the whole penicillin biosynthetic pathway in Golgi-like organelles (reviewed by Kurylowitz et al. (1987)). In contrast, Müller et al. (1991, 1992) most recently provided evidence that the final phenylacetyl-transfase step is localized in microbodies, whereas the synthesis of isopenicillin **N** occurs in the cytoplasm. The localization of the enzyme catalyzing the first step in the pathway (ACVS) remained unclear from their study; it appeared to be associated with a "light membraneous" structure, sedimenting at 100,000 \( \times \) g, which was not further identified.

We present data showing that a large portion of cellular \( \alpha \)-aminoadipate is contained in the vacuoles. ACVS is also associated with the vacuoles. Hence the vacuole appears to be the compartment containing the precursor amino acids for \( \beta \)-lactam biosynthesis in *P. chrysogenum*.

**EXPERIMENTAL PROCEDURES**

*Organism and Cultivation—*The *Penicillium chrysogenum* strain used throughout the present study was BC 1505, which is proprietary to Biochemie GmbH (Kundl, Tyrol, Austria). It was obtained bi-monthly as a freshly prepared conidial suspension and kept at 4 °C in sterile physiological saline until used. It was grown in a seed medium consisting of (g/liter): glucose, 30; (NH\(_4\))\(_2\)SO\(_4\), 10; KH\(_2\)PO\(_4\), 10; and a trace element stock solution (MgSO\(_4\), 7H\(_2\)O, 25; FeSO\(_4\), 7H\(_2\)O, 10; CuSO\(_4\), 7H\(_2\)O, 0.5; ZnSO\(_4\), 7H\(_2\)O, 2; Na\(_2\)MoO\(_4\), 50; MnSO\(_4\), H\(_2\)O, 2; CaCl\(_2\), 2H\(_2\)O, 5; 10 ml/liter). The pH was adjusted to 6.5. 200 ml portions of this medium were distributed into 1-liter wide-mouth Erlenmeyer flasks and sterilized (120 °C, 20 min). After cooling, 400 \( \mu \)l of the respective conidial suspension were added to give a final density of 10° conidia/liter. The flasks were then incubated on a rotary shaker (240 rpm, amplitude 70 mm) for 72 h at 30 °C. To

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\(^1\) The abbreviations used are: ACVS, \( \delta \)-(\( \alpha \)-aminoadipyl)-L-cysteinyl-D-valine synthetase; IPNS, isopenicillin **N** synthetase; MOPS, 4-morpholino-propanesulfonic acid; TES, Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
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produce penicillin, the whole biomass from a single flask (1-2 g, damp wet weight) was harvested on a sterile G 1 porosity sinter funnel and transferred to a previously sterilized and cooled 500-ml Erlenmeyer flask containing 30 ml of penicillin production medium. The medium consisted of (g/liter): lactose, 80; maltose, 20; CaSO₄ (precipitated), trace element stock solution (CuSO₄·5H₂O, 0.1; phenoxyacetate acid, 1; chloramphenicol, 0.1; and a trace element stock solution (CuSO₄·5H₂O, 0.5; ZnSO₄·7H₂O, 2; MnSO₄·H₂O, 2; Na₂SO₄, 50), 10 ml/liter. pH was 6.5. The culture was then incubated as above for another 24-120 h.

**Extraction of ACVS from Mycelia**—To extract ACVS, approximately 3-4 g of mycelia (wet weight) were suspended in about a 10-fold weight of extraction buffer (0.1 M MOPS, pH 7.5, containing 0.2 M KCl, 10 mM MgCl₂, 40% (v/v) glycerol, and 10 mM dithiothreitol), mixed with 20 ml of glass beads (0.45-0.5-mm diameter), and homogenized in a Bead Beater (Biospec Products, Bartlesville, OK), using a small-size (40 ml) homogenization chamber. Homogenization was carried out with cooling by ice-water in six 20-ml treatments, interspersed with 2-min cooling periods. Thereafter the beads were decanted, the supernatant fluid were centrifuged for 60 min at 12,000 × g, and washed twice with buffer B (1.2 M sorbitol, 50 mM CaCl₂). The final protoplast pellet (2000 X g, 10 min, 4 °C) was collected by centrifugation and resuspended in 10 ml of buffer B (1.2 M sorbitol, pH 7.5, containing 50 mM CaCl₂, and 0.025% (w/v) Triton X-100) and incubated for up to 15 min at room temperature. Lysis of protoplasts was observed under the microscope, and subcellular fractionation was initiated as soon as 50% of the protoplasts had already lysed. Subcellular fractionation was carried out by several subsequent centrifugation steps (400 × g, 6 min; 1000 × g, 12 min; 3000 × g, 18 min; 12,000 × g, 30 min), yielding four pellet fractions (P₁₀₀₀, P₂₀₀₀, P₄₀₀₀, and P₈₀₀₀) and the 12,000 g supernatant fluid (S₁₂₀₀₀). The pellets were suspended in 1-2 ml of buffer B. All fractions were kept at -20 °C until analysis. To purify vacuoles, the P₈₀₀₀ was loaded on a Percoll gradient (20 ml of 1.2 M sorbitol in 15% (v/v) Percoll, containing 10 mM Tris-HCl, pH 7.5; prepared by centrifugation for 25 min at 18,000 × g), and centrifuged for 10 min at 2000 × g, followed by 10 min at 7800 × g. Distribution of organelles in individual fractions was analysed by harvesting 1.2-ml fractions starting with the top of the tube.

**Isolation of Vacuolar Membranes**—Vacuoles prepared as described above were osmotically lysed by mixing of 0.5 ml of a vacuolar suspension with 0.7 ml of 10 mM Tris-HCl, pH 7.2, and incubation for 5 min at room temperature with occasional vortexing. Thereafter, the mixture was centrifuged in an Eppendorf centrifuge (10 min, full speed), and the supernatant as well as the pellet kept for further analysis.

**Uptake of ³⁵Cl-Labeled Amino Acids by Protoplasts and Vesicles**—In some experiments, the mycelia were incubated with 6-³⁵Cl-aminoadipate (Centre Energie Atomic (Saclay, France), 55 mCi/mmole), ³⁵Cl-valine (Amersham, 250 mCi/mmole), or ³⁵Cl-lysine (Amersham, 300 mCi/mmole). For this purpose, the washed protoplasts were suspended in 2 ml of buffer B and incubated with 0.5 µCi of either amino acid for 90 min at 30 °C with gentle shaking. Thereafter the suspension was diluted with 2 ml of buffer B, and centrifuged (400 × g, 12 min, 4 °C). This washing procedure was repeated twice. Thereafter the protoplasts were taken up in 3-4 ml of buffer C and lysed as described above.

**Enzyme Assays**—α-Mannosidase was assayed by incubating 50 µl of the respective enzyme sample with 0.5 ml of 50 mM Tris-HCl buffer, pH 7.2, and 0.1 ml of the respective p-nitrophenyl substrate (100 mM in the respective buffer) for 60 min at 50 °C. The reaction was terminated by addition of 2 ml of 1 M Na₂CO₃, and the A₄₅₀ measured against a blank, treated accordingly, but in which the Na₂CO₃ solution had been added prior to the substrate. AMPase was determined as P released from AMP as described by Bowman and Bowman (1982). The activities of glucose-6-phosphate dehydrogenase and citrate synthase were assayed according to Laemmli and Stahelin (1970) and Sere (1969), respectively. 1 unit is defined as the amount of enzyme which releases 1 µmol of p-nitrophenol/min under these conditions. Specific activities are given as units/mg protein. Protein was determined by the dye-binding procedure (Bradford, 1976).

**Electrophoresis—Denaturing SDS-PAGE was used to detect the presence of ACVS in subcellular fractions from gel permeation chromatography (see "Results") and centrifuged for 10 min at 48,500 × g, and washed twice with buffer B (1.2 M sorbitol, pH 7.5, containing 50 mM CaCl₂). The final protoplast pellet was suspended in 3-4 ml of buffer C (10 mM Tris-HCl, pH 7.5, 1 M sorbitol, 50 mM CaCl₂, and 0.025% (w/v) Triton X-100) and incubated for up to 15 min at room temperature. Lysis of protoplasts was observed under the microscope, and subcellular fractionation was initiated as soon as 50% of the protoplasts had already lysed. Subcellular fractionation was carried out by several subsequent centrifugation steps (400 × g, 6 min; 1000 × g, 12 min; 3000 × g, 18 min; 12,000 × g, 30 min), yielding four pellet fractions (P₁₀₀₀, P₂₀₀₀, P₄₀₀₀, and P₈₀₀₀) and the 12,000 g supernatant fluid (S₁₂₀₀₀). The pellets were suspended in 1-2 ml of buffer B. All fractions were kept at -20 °C until analysis. To purify vacuoles, the P₈₀₀₀ was loaded on a Percoll gradient (20 ml of 1.2 M sorbitol in 15% (v/v) Percoll, containing 10 mM Tris-HCl, pH 7.5; prepared by centrifugation for 25 min at 18,000 × g), and centrifuged for 10 min at 2000 × g, followed by 10 min at 7800 × g. Distribution of organelles in individual fractions was analysed by harvesting 1.2-ml fractions starting with the top of the tube.
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**Table I**

Distribution of vacuolar markers during cell fractionation.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lysate</th>
<th>P_{400}</th>
<th>P_{1000}</th>
<th>P_{1200}</th>
<th>S_{12000}</th>
<th>Vacuoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (µg)</td>
<td>5550</td>
<td>840</td>
<td>260</td>
<td>930</td>
<td>3280</td>
<td>27</td>
</tr>
<tr>
<td>α-Mannosidase Specific activity</td>
<td>0.170</td>
<td>0.31</td>
<td>0.58</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>6.3</td>
</tr>
<tr>
<td>Total activity</td>
<td>1.1</td>
<td>0.26</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Polyphosphate mg/mg</td>
<td>10.6</td>
<td>&lt;1.0</td>
<td>7.60</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>168.3</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>269</td>
<td>407</td>
<td>984</td>
<td>75</td>
<td>100</td>
<td>7037</td>
</tr>
</tbody>
</table>

*Vacuoles refers to the fraction of the Percoll gradient containing the highest activity of α-mannosidase.

Applied to silica gel TLC plates (Merck, Darmstadt, Germany) and chromatographed using methylacetate:2-propanol:chloroform: methanol:KCl (0.25, w/v) (25:25:25:10:7, v/v) as mobile phase. Phospholipids were detected by spraying with a commercial phosphomolybdate spray (Merck) and identified by the aid of phospholipid standards (Sigma).

**RESULTS**

Isolation of Vacuoles from Penicillin-producing and -non-producing Mycelia of P. chrysogenum—To isolate vacuoles from *P. chrysogenum*, we attempted various published methods (Cramer et al., 1983; Keller et al., 1984; Vaughan and Davia, 1981). Preliminary experiments showed that the preparation of protoplasts and their lysis under isotonic conditions by the aid of Triton X-100 produced best results. Our primary vacuolar markers were α-mannosidase and AMPase, two enzymes predominantly localized in vacuoles of both Neurospora crassa (Vaughn and Davies, 1981; Bowman and Bowman, 1982) and Saccharomyces cerevisiae (Wiemken et al., 1979). In addition, we followed the ratio of sedimentable (nonprotein) lysine to protein, and the content of polyphosphate, which both monitor only intact vacuoles and hence avoid misinterpretations of enzyme markers due to latency or multiple locations. Upon differential centrifugation of the crude lysates so obtained, all vacuolar markers (polyphosphate, α-mannosidase, AMPase, and the majority of cellular lysine) were concentrated in the 400 and 1000 g pellet. All this suggested that the 400 and 1000 g pellets contained vacuoles, but were still contaminated with enzymes, which were either cytosolic (glucose-6-phosphate dehydrogenase) or mitochondrial (citrate synthase) (Table I). This was due to the presence of intact protoplasts, as detected by microscopic examinations especially in the 400 g pellet. Purification of the vacuolar fraction could be obtained by layering the 1000 g fraction on a Percoll gradient (Table I). This procedure resulted in the separation of all putative vacuolar markers from glucose-6-phosphate dehydrogenase and citrate synthase activity. Less than 3% of total protein of the crude lysate were obtained in the peak fraction, and the activity of α-mannosidase, [14C]lysine radioactivity, and polyphosphate content were enriched 20-40-fold. Electron microscopical examinations proved the absence of other organelar material as nuclei or mitochondria or of small protoplasts (data not given). Larger protoplasts were pelleted under these conditions, as indicated by the simultaneous appearance of vacuolar as well as other marker enzymes in a second peak (see Fig. 3a).

Penicillin Precursor Amino Acids Are Contained in the Vacuole—Having a reliable procedure in hand to isolate vacuoles from *P. chrysogenum*, we analyzed the subcellular location of the presence of precursor amino acids of penicillin. However, the yield of pure vacuoles was too low (usually 20–40 µg of total protein) for the detection of α-aminoacidipate, valine, or cysteine by HPLC. Therefore, protoplasts were incubated with either 6-[14C]α-aminoadipate and [14C]valine, respectively, prior to the isolation of vacuoles, and the radioactive labeling of the individual fractions during purification of the vacuoles analyzed (Table II). It is essential to note that no metabolism of these two amino acids occurred during isolation of the vacuoles, and hence the radioactivity recovered was actually due to the presence of α-aminoacidipate and valine, as shown by HPLC and measuring of radioactivity in individual fractions (data not given). Both amino acids occurred in a sequestered as well as soluble pool, as shown by peak radioactivity occurring in the vacuolar and in the cytosolic fraction. The rise in [14C]valine and [14C]α-aminoacidipate radioactivity recovered in the vacuolar fraction under penicillin-producing conditions is especially noted. It must be considered, however, that the labeling of amino acid distribution as carried out in this study does not reflect the true cellular concentrations and instead reflects the ability of the cells to sequester these amino acids under the given conditions.

Identification of ACVS in Cell-free Extracts of *P. chrysogenum*—The detection of the amino acid precursors of penicillin in extracts of *P. chrysogenum* and the comparison of the results with those obtained from extracts of *P. chrysogenum* indicates that the biosynthesis of penicillin occurs in the vacuole. The presence of α-aminoacidipate and valine in the vacuolar fraction under penicillin-producing conditions is especially noted. It must be considered, however, that the labeling of amino acid distribution as carried out in this study does not reflect the true cellular concentrations and instead reflects the ability of the cells to sequester these amino acids under the given conditions.

**Table II**

Recovery of radioactive label from 6-[14C]α-aminoacidipate and [14C]valine in subcellular fractions obtained from *P. chrysogenum*

Percent values (%) are given as recovery of originally present dpm (cf. first line). PNP and PP indicate penicillin-non-producing conditions and penicillin-producing conditions, respectively. The value given for vacuoles indicates the percentage of original activity present in the peak fraction of α-mannosidase activity of the Percoll gradient centrifugation of combined fraction P_{400} and P_{1200}.

<table>
<thead>
<tr>
<th>Amino acid condition</th>
<th>6-[14C]α-aminoacidipate</th>
<th>[14C]Valine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate (total dpm)</td>
<td>9801</td>
<td>1672</td>
</tr>
<tr>
<td>P_{400} (%)</td>
<td>28.1</td>
<td>27.8</td>
</tr>
<tr>
<td>P_{1000} (%)</td>
<td>13.9</td>
<td>22.4</td>
</tr>
<tr>
<td>P_{12000} (%)</td>
<td>2.9</td>
<td>2.0</td>
</tr>
<tr>
<td>S_{12000} (%)</td>
<td>35.2</td>
<td>42.9</td>
</tr>
<tr>
<td>Vacuoles (%)</td>
<td>15.6</td>
<td>25.5</td>
</tr>
</tbody>
</table>
Localization of Penicillin Biosynthesis

cillin biosynthesis mainly in the cytosolic and the vacular fraction favors the assumption, that the “stable amino acid pool, involved in penicillin biosynthesis” described earlier (Hönlinger and Kubicek, 1989b; Affenzeller and Kubicek, 1991) is identical with the vacuole. This assumption would be greatly strengthened by detection of a similar location of the enzyme using these amino acids as substrates, i.e. ACVS. In order to identify the cellular location of this step in the penicillin biosynthetic pathway, a reliable method to detect ACVS was required first. Measurement of ACVS activity is tedious and requires high amounts of enzyme protein, and hence was unsuitable for the purpose of this study. We have thus purified ACVS from *P. chrysogenum* and raised polyclonal antibodies against it, but we were unable to obtain such with sufficient specificity. 

Hence, we decided to identify ACVS according to its outstanding size (the nucleotide sequence of its gene (Diez et al., 1990) predicts a subunit molecular mass of the corresponding ACVS protein of 428 kDa), which enables its visualization in low percentage SDS-PAGE gels upon protein staining. Initial attempts to demonstrate the presence of such a protein in cell-free extracts by SDS-PAGE in 5% gels were unsuccessful, however. It was subsequently found that ACVS penetrated into the polyacrylamide gel only when it was first denatured and precipitated by trichloroacetic acid and then solubilized by alkali prior to heating in SDS-sample buffer. Under these conditions, a high molecular mass protein band of about 270 (± 15) kDa was identified; it was only formed in the mycelia after transfer to a medium promoting penicillin biosynthesis (Fig. 2a). The identity of this band with ACVS was established by comparing its mobility in 5% SDS-PAGE gels with that of an ACVS sample obtained from other authors (Fig. 2b), and by its ability to covalently bind [14C]valine via its thiotemplate arm (Fig. 2c). It should be noted that the approximate molecular mass of about 270 kDa given in this paper may probably not be the correct value for ACVS, since it has been obtained by extrapolation of the marker protein calibration curve only, and the peculiar shape of this protein (Martin, 1991) questions the relationship between migration in SDS-PAGE and *M*. Hence our data do not necessarily contradict the molecular mass of 428 kDa of ACVS, as derived from the gene sequence.

**Localization of ACVS at the Vacuolar Membrane**—Using the method for assaying the presence of ACVS as developed above, samples from subcellular fractionation and vacuole purification were analyzed (Fig. 3, a and b): both the 1000 × *g* pellet and the peak vacuolar fraction during Percoll gradient centrifugation clearly showed the presence of the ACVS protein band. In order to study the vacuolar location of ACVS in more detail, we employed severe osmotic shock to lyse the vacuoles and to isolate a vacuolar membrane fraction. By this procedure we obtained a membrane fraction containing 17% of the vacular protein and no more than 1% of the radioactive [14C]lysine. More than 70% of α-mannosidase was recovered in the supernatant, which is in accordance with results from *N. crassa* (Bowman and Bowman, 1982). ACVS, however, was distributed between the supernatant and the membranes in comparable amounts. From these data, we suggest that ACVS is only loosely associated with the vacuolar membrane in *P. chrysogenum*.

IPNS, the subsequent enzyme in penicillin biosynthesis, for which a cytosolic location has recently been established, was investigated as a control in these experiments. Our results essentially confirmed its cytosolic location (Fig. 3c).

**Identification of ACVS in Vesicles Probably Derived from Vacuoles**—In order to explain the difference in the location of ACVS, as reported in this study and previously by Müller et al. (1991), we attempted to analyze the nature of the light membraneous fraction described by them. When cell-free extracts for the isolation of subcellular fractions were prepared under the conditions used by Müller et al. (1991), we could confirm that ACVS was associated with very little, yet sedimentable (centrifugation at 50,000 × *g*) cellular structures, which exhibited a size of 2-5 × 10^6 Da upon gel permeation chromatography (Fig. 4, a and b). A closer inspection of this material revealed that it contained α-mannosidase (Fig. 4c), was capable of taking up 14C-labeled α-aminoacidipate (Fig. 4d), and contained polyphosphate (Fig. 4e). Mitochondrial (citrate synthase) and cytosolic (glucose-6-phosphate dehydrogenase) markers eluted clearly separated from this peak, and their position correlated with their putative native molecular mass, indicating that the ACVS-containing vesicles did not include other cellular compounds. Qualitative TLC revealed that its membraneous material contained phosphatidylserine (data not shown). The latter compound has been reported to occur in vacuolar membranes only (Bowman et al., 1987). It was thus concluded that the light membraneous fraction represents fragments and vesicles from the vacuoles.
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which were not stable under the conditions of the homogenization procedure used by Müller et al. (1991).

DISCUSSION

In this paper, we have studied the localization of the initial step of the pathway of penicillin biosynthesis by cell-fractionation experiments. We identified the vacuole as the cellular compartment in P. chrysogenum, which contains the precursor amino acids for penicillin biosynthesis. Since no other amino acid-containing compartments were detected during this study, and the amino acids used for penicillin biosynthesis are not derived from a cytosolic pool (Affenzeller et al., 1991), the vacuole is very likely the source of amino acids for β-lactam biosynthesis. The vacuole has earlier been shown to be involved in the provision of amino acid substrates for alkaloid biosynthesis in Penicillium cyclopium (Roos and Luckner, 1986). Fungal vacuoles are known as storage compartments of amino acids, but acid and neutral amino acids are usually strongly underrepresented (Matile, 1978; Horak, 1986). The present finding of a high portion of cellular α-aminoadipate in P. chrysogenum vacuoles is the first report on vacuolar sequestering of this amino acid. Differences in the kinetics of exchange of valine between the “penicillin precursor pool” and the cytosol in high and low producing strains of P. chrysogenum have been recently reported (Affenzeller and Kubicek, 1991). It is tempting to speculate that the ability of superior penicillin producer strains of this fungus to accumulate a higher pool of α-aminoadipate (Jaklitsch et al., 1986) may be due to the improved ability of these strains to transport α-aminoadipate into the vacuole.
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The importance of the vacuolar amino acid pool for penicillin biosynthesis was underlined by our demonstration of a vacuolar location of the first enzyme in penicillin biosynthesis, i.e., ACVS. Previous cell fractionation studies by others (Fawcett and Abraham, 1975, 1976; Abraham et al., 1981; Müller et al., 1991) indicated that ACVS was associated with vesicles or small organelles. According to the present results, these findings were obviously due to the disruption of vacuoles during isolation of subcellular fractions. Difficulties in isolation of intact vacuoles have also been reported by others (Vaughn and Davis, 1981; Cramer et al., 1983; Keller et al., 1984) and were observed during this study. Only Kurylowitz et al. (1987) reported on the occurrence of ACVS activity in various subcellular fractions sedimenting between 400 and 30,000 × g, which they interpreted as Golgi vesicles. As the size and density of isolated vacuoles are heavily influenced by centrifugation and polyphosphate content (Vaughn and Davis, 1981), they can display a very heterogeneous organelle population. It is likely that Kurylowitz et al. (1987) also isolated vacuoles containing ACVS.

Upon lysis of the vacuoles, ACVS appeared distributed between the vacuolar membranes and the supernatant, suggesting that it is a protein which is only loosely bound to the vacuolar membrane. A membrane spanning of ACVS was also reported by others (Vaughn and Davis, 1981; Cramer et al., 1983; Keller et al., 1984; Eisenberg et al., 1984). The mechanism by which ACVS is associated with the vacuolar membrane and, particularly, whether it is associated to the cytosolic or inner side of the membrane is an attractive subject for further study. Since the subsequent step in penicillin biosynthesis, IPNS, has been shown clearly to occur in the cytosol (Müller et al., 1991; this study), either the ACV tripeptide must traverse the vacuolar membrane, or ACVS must have direct access to the amino acids transported out.

Based on the present studies we therefore propose the following organization model of the penicillin biosynthetic pathway in P. chrysogenum (Fig. 5). An intrinsic feature of this model is that penicillin biosynthesis obviously involves a number of transport steps whose nature has so far been neither considered nor dealt with. This topic presents a challenge for further studies, especially with respect to the understanding of how penicillin production can be further improved.

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REFERENCES


Müller, W. H., Bovenberg, R. A. L., Groothuis, N. H., Kattevilder, F., Smaal,