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Purification and characterization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Penicillium chrysogenum*

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δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) from *Penicillium chrysogenum* was purified to homogeneity by a combination of $(NH_4)_2SO_4$ precipitation, protamine sulphate treatment, ion-exchange chromatography, gel filtration and hydrophobic interaction chromatography. The molecular mass of ACVS was estimated with native gradient gel electrophoresis and SDS/PAGE. The native enzyme consisted of a single polymer chain with an estimated molecular mass of 470 kDa. The denatured enzyme had an estimated molecular mass of 440 kDa. The influence of different reaction parameters such as substrates, cofactors and pH on the activity of the purified ACVS was investigated. The K_m values for the three precursor substrates L-α-aminoadipic acid, L-cysteine and L-valine were determined as 45, 80 and 80 μM respectively, and the optimal assay concentration of ATP was found to be 5 mM (with 20 mM $MgCl_2$). The dimer of the reaction product bis-δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (bisACV) gave feedback inhibition of the purified ACVS; the inhibition parameter K_{bisACV} was determined as 1.4 mM. Furthermore dithiothreitol was shown to inhibit the purified ACVS. From the addition of a glucose pulse to a steady-state glucose-limited continuous culture of *P. chrysogenum* it was found that there is glucose repression of the synthesis of ACVS and that there must be a constant turnover of ACVS owing to synthesis and degradation.

INTRODUCTION

The first step in the biosynthesis of β-lactam antibiotics is the condensation of the three amino acids L-α-aminoadipic acid, L-cysteine and L-valine with the concomitant epimerization of L-valine to D-valine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This ATP- and Mg^{2+}/Mn^{2+}-dependent reaction is catalysed by a single multifunctional enzyme ACV synthetase (ACVS). As the reaction is at the connection point between primary and secondary metabolism it represents a possible regulatory point in the biosynthetic pathways to β-lactams.

Because of the extreme instability of ACVS its purification has been difficult. Until now the *Penicillium chrysogenum* ACVS has not been reported purified, whereas the ACVS of *Aspergillus nidulans* [1,2], *Cephalosporium acremonium* [3,4] and *Streptomyces clavuligerus* [4–7] have been purified and characterized. However, because more than 65% of the β-lactams produced worldwide are derived from penicillins synthesized by *P. chrysogenum*, the *P. chrysogenum* ACVS is the most interesting.

In this paper we report on the purification and characterization of ACVS from *P. chrysogenum* and the results of its biochemical characterization including the investigation of the putative feedback inhibition of ACVS by the dimer of the reaction product ACV [8]. Finally, we have studied the effect of glucose on the ACVS activity during a glucose pulse experiment in a continuous culture.

MATERIALS AND METHODS

Strain

The high-penicillin-yielding strain of *Penicillium chrysogenum* used in this study was kindly donated by Novo Nordisk A/S (Bagsværd, Denmark).

Chemicals

L-[U-^{14}C]Valine (10.1 GBq/mmol) was purchased from DuPont–NEN® (Dupharma A/S, Kastrup, Denmark), bisACV was a gift from Gist-Brocades (Delft, The Netherlands). Sephadex G-25®, Q-Sepharose FF®, Sephacryl S-300 HR®, butyl-Sepharose 4B® and PD-10® columns were purchased from Pharmacia Biotech (Allerød, Denmark). All chemicals were of analytical or HPLC grade (Sigma, St. Louis, MO, U.S.A., and Merck, Darmstadt, Germany).

Medium composition

A batch cultivation (used to start up the continuous cultivation) was performed on a chemically defined medium containing: 7.0 g/l $(NH_4)_2SO_4$, 25.0 g/l sucrose, 0.04 g/l $FeSO_4,7H_2O$, 0.1 g/l $MgSO_4,7H_2O$, 0.5 g/l KCl, 1.6 g/l KH_2PO_4, 0.005 g/l $CuSO_4,5H_2O$, 0.02 g/l $ZnSO_4,7H_2O$, 0.02 g/l $MnSO_4,H_2O$, 0.05 g/l $CaCl_2,2H_2O$ and 0.5 ml/l Pluronic® F68 (Fluka, Buchs, Switzerland) all sterilized together. In the feed medium the sucrose was replaced by 15 g/l glucose, sterilized separately; phenoxyacetic acid was added to a final concentration of 6.5 g/l. The pH was adjusted to 6.50 with 4 M NaOH.

Cultivation conditions

The continuous cultivation was performed in a 7 litre Chemap bioreactor operated as a constant-mass chemostat [9] at a dilution rate of 0.05/h. The temperature was kept at 25 °C. The aeration rate was 1 v/v per min, the headspace pressure was 1.5 bar (150 kPa) and the dissolved oxygen level (pO_2) was kept above 100% saturation. The pH was controlled at 6.50 by the addition of 4 M NaOH.

Abbreviations used: ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; ACVS, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase; DTT, dithiothreitol.

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Standard ACVS assay

The activity of soluble enzyme preparation was measured in an assay system with a total volume of 50 μl containing 0.1 M Mops/HCl, pH 7.5, 20 % (v/v) glycerol, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF, 5 mM ATP, 20 mM $MgCl_2$, 2 mM L-α-aminoadipic acid, 1 mM L-cysteine, 2.5 μCi (192 μM) L-[U-^{14}C]valine (10.1 GBq/mmol), and 5 μl of crude enzyme extract or purified enzyme. The reaction mixture was incubated at 26 °C for 30 min and terminated by the addition of 15 μl of 20 % (w/v) trichloroacetic acid. Precipitated proteins were removed by centrifugation (20000 ***g*** for 20 min) and the supernatant was analysed for [^{14}C]ACV by HPLC. PMSF and DTT were omitted from the assay for the purified enzyme.

Preparation of crude extract

To measure the ACVS activity in crude cell-free extracts, samples withdrawn from the culture medium were collected in a cold flask and quickly filtered on a cold Büchner funnel equipped with a large-pore filter, washed with cold 0.9 % aq. NaCl and frozen by immersion in liquid nitrogen. Approx. 2 g of frozen mycelium was ground to a fine powder either in a mortar previously cooled with liquid nitrogen or in a Krups KM75 coffee mill. The powder was suspended in 4 ml (4 °C) of 0.1 M Mops/HCl buffer, pH 7.5, containing 50 % (v/v) glycerol, 5 mM DTT, 50 mM KCl, 20 mM EDTA and 1 mM PMSF, and centrifuged at 20000 ***g*** for 20 min. A 2.5 ml sample of the supernatant was then applied to a PD-10 column (Pharmacia Biotech) previously equilibrated with 25 ml (4 °C) of 0.1 M Mops/HCl buffer, pH 7.5, containing 20 % (v/v) glycerol, 5 mM DTT, 1 mM EDTA and 1 mM PMSF. The column was eluted with 3.5 ml of the same buffer. The eluate was used as the enzyme source. The protein content of the eluate was measured by the method of Bradford [10] with the Bio-Rad dye reagent, using BSA as standard. The eluate generally contained approx. 5 mg/ml protein.

HPLC

The HPLC system was from Waters Associates and consisted of a Nova-Pak C_{18} column (300 mm × 3.9 mm) with 4 μm packing and a Nova-Pak C_{18} precolumn, one pump (type 510), a WISP 712 autosampler equipped with a built-in cooling unit, and a TCM column oven. Results acquisition and pump control were performed by the Waters Maxima 820 Baseline software. The radioactivity was detected with an EG&G Berthold Radioactivity Monitor LB 507 B equipped with a 400 μl yttrium silica flow cell (type YG-400 U4).

The injection volume was 10 μl and the column was eluted isocratically at 35 °C with 10 % (v/v) methanol, 90 % (v/v) 50 mM KH_2PO_4, pH 6.0, for 15 min at a flow rate of 1 ml/min. The elution times were: valine 4.0 min and ACV 7.1 min.

Purification procedure for ACVS

Buffer A was 0.1 M Mops/HCl (pH 7.5)/50 % (v/v) glycerol/5 mM DTT/50 mM KCl/20 mM EDTA/1 mM PMSF. Buffer B was similar to buffer A but lacked glycerol. Buffer C was 0.1 M Mops/HCl (pH 7.5)/20 % (v/v) glycerol/5 mM DTT/1 mM EDTA. Buffer D was buffer C plus 150 mM NaCl. Buffer E was buffer C plus 400 mM NaCl. Buffer F was buffer C plus 1 M $(NH_4)_2SO_4$. Buffer G was buffer C plus 20 % (v/v) ethylene glycol.

Mycelium from a steady-state continuous cultivation was harvested and washed with ice-cold 0.9 % aq. NaCl and immediately frozen in liquid nitrogen; 222 g of frozen mycelium was homogenized to a fine powder in several batches with a Krups KM75 coffee mill. The frozen powder was suspended in 444 ml of buffer A and left on ice for 30 min, then centrifuged for 20 min at 20000 ***g***. The following steps were performed at 4 °C. The supernatant was diluted 1:1 with buffer B to decrease the viscosity. Then the diluted crude extract was treated with protamine sulphate by adjusting the concentration to 0.07 % (w/v) with a 3 % stock solution. The preparation was left with slow stirring for 20 min and then centrifuged at 20000 ***g*** for 20 min. $(NH_4)_2SO_4$ was gradually added to the supernatant with slow stirring, to reach 35 % saturation. It was left for 20 min, then centrifuged for 20 min at 20000 ***g***. $(NH_4)_2SO_4$ was added to the supernatant as before to reach 45 % saturation; after 20 min the preparation was centrifuged at 20000 ***g*** for 20 min. The supernatant was discarded and the pellet that precipitated between 35 % and 45 % $(NH_4)_2SO_4$ saturation was taken up in a minimal volume of buffer D. The proteins were desalted on a Sephadex G-25 column (2.6 cm × 34 cm) equilibrated and eluted with buffer D at a flow rate of 125 ml/h. The standard ACVS assay was used to determine which protein peak contained the ACVS activity; these fractions were pooled. Desalted proteins (43 ml) were applied to a Q-Sepharose FF ion-exchange column (2.6 cm × 36 cm) equilibrated with buffer D, the column was washed with 2 column vol. of buffer D to eliminate non-bound protein and eluted with a 1000 ml linear gradient of buffer D (150 mM NaCl) to buffer E (400 mM NaCl) at a flow rate of 125 ml/h. Fractions of 7 ml were collected and assayed for ACVS activity and protein content. Fractions with high ACVS activity were analysed for purity by SDS/PAGE before pooling. Pooled fractions (77 ml) were concentrated to 16 ml by ultrafiltration with an Amicon cell equipped with a 10 kDa cut-off membrane and applied to a Sephacryl S-300 HR gel-filtration column (2.6 cm × 94 cm) equilibrated with buffer D. The column was eluted with buffer D at a flow rate of 20 ml/h; fractions of 1.8 ml were collected and assayed for ACVS activity and protein content. Fractions with high enzyme activity were analysed for purity by SDS/PAGE before pooling. $(NH_4)_2SO_4$ was added to the pooled fractions (20 ml) to reach 25 % saturation, and the preparation was applied to a butyl-Sepharose 4B column (1.6 cm × 2.5 cm) equilibrated with buffer F for hydrophobic interaction chromatography. The column was washed with approx. 10 column vol. of buffer F and eluted with a 200 ml linear gradient of buffer F to buffer G at a flow rate of 30 ml/h. Fractions of 4.2 ml were collected and assayed for ACVS activity and protein content. Fractions with high ACVS activity were analysed for purity by SDS/PAGE and pooled as the purified enzyme. The pooled fractions (55 ml) were concentrated to 4.4 ml by ultrafiltration with an Amicon cell equipped with a 10 kDa cut-off membrane, and taken up in buffer C.

Gel electrophoresis

During the purification of ACVS, SDS/PAGE was performed by the procedure of Laemmli [11] with 5 % (w/v) slab gels and silver staining. To determine the molecular mass of ACVS the PhastSystem of Pharmacia Biotech was used with a native PAGE gradient gel of 4–15 % (w/v). Phast gels were silver-stained by the method of Heukeshoven and Dernick [12] and Blum et al. [13]. The standard proteins used were those of the high-molecular-mass (HMW) Kit (Pharmacia Biotech).

Glycosylation of ACVS

To hydrolyse possible N-glycan chains, ACVS was treated with N-glycosidase F (Boehringer Mannheim, Germany) for 15 min

at room temperature (2.6 units/mg ACVS) before native Phast gradient PAGE.

RESULTS AND DISCUSSION

Linearity of the enzymic assay

The standard ACVS assay was linear with respect to the protein concentration up to approx. 25 μg, which corresponds to 5 μl of crude enzyme extract. With 5 μl of enzyme extract the assay was linear with time up to 60 min, at which point it levelled off. This was not caused by the depletion of valine, because only 15 % of this substrate was incorporated into ACV. An incubation time of 30 min was used as standard except where saturating levels of valine were required. By the addition of unlabelled valine the concentration was raised to 0.5 mM (corresponding to 10 K_m) and the incubation time was increased to 1 h.

Purification of ACVS

The cells for the ACVS purification were harvested from a constant-mass continuous cultivation during the period of high penicillin productivity [9]. The nucleic acids were precipitated with protamine sulphate and removed by centrifugation. The ACVS activity was recovered from the $(NH_4)_2SO_4$ pellet, precipitating between 35 % and 45 % saturation. The dissolved pellet was desalted and applied to a Q-Sepharose FF column. The purification was followed by incubation of the collected fractions as described above. The enzyme activity resided in a single peak eluting during the salt gradient at approx. 245 mM NaCl; 54 % of the enzyme activity was recovered.

The pooled fractions were concentrated and applied to a Sephacryl S-300 HR column. The chromatography resulted in a single activity peak coinciding with the protein profile. Fractions with high enzyme activity were collected; 23 % of the enzyme activity was recovered. This preparation was treated with $(NH_4)_2SO_4$ before applying it to the butyl-Sepharose 4B. The ACVS activity eluted in an asymmetrical peak during the gradient of ethylene glycol. The protein peak almost coinciding with the ACVS peak could not be detected by UV monitoring but was determined by the Bio-Rad protein assay. The pooled fractions were concentrated by ultrafiltration, yielding the purified enzyme.

Data from the ACVS purification are presented in Table 1. From the $(NH_4)_2SO_4$ step the ACVS was purified 6-fold with a yield of 9 %, resulting in 8.5 mg of protein and a specific activity of 41.4 nkat/g of protein. The specific activity of ACVS was not measured in the crude extract used for the purification, but in other crude extracts the specific activity was typically 6 nkat/g protein, which would correspond to a 7-fold purification.

The level of ACVS activity in a frozen (at −80 °C) biomass sample declined fast and generally the cells used for a purification or an assay should not be stored for more than 1 day. There are several reasons for the success of this purification method compared with the previous attempts to purify ACVS from *P. chrysogenum*. The use of a high-yielding production strain combined with a cell disintegration method that worked readily at −80 °C resulted in a sufficient amount of enzyme in the crude extract to start the purification. Further, a high concentration of glycerol was used in the extraction buffer to stabilize the crude enzyme; EDTA, PMSF and DTT seemed to be vital for its stability as well. Proteases and other contributors to the enzyme's instability were removed by the $(NH_4)_2SO_4$ and protamine sulphate precipitation, and during the subsequent procedure the enzyme retained its stability. The activity of the purified ACVS in buffer C was unchanged for several months when stored at −80 °C.

Except for the cell-opening technique and the use of PMSF to inhibit proteases, the primary steps of this purification strategy are no different from the previously employed procedures to purify ACVS from other micro-organisms. In these reported purifications the resulting specific activity of ACVS ranged from 0.8 [1] to 641 nkat/g of protein [4], most often below 20 nkat/g of protein. The resulting high specific activity of 41.4 nkat/g of protein in this work is thus a result of employing four chromatographic steps, compared with only two or three steps normally. Further, as each chromatographic step can lead to inactivation of the enzyme because of conformational changes when adsorbing and desorbing from the gel matrices, the yield of 9 % is very satisfying, the inactivation being reflected in the high protein content of the purified fraction.

Molecular mass

During the purification the ACVS was identified on the 5 % (w/v) SDS/PAGE gels as a single band larger than both the thyroglobulin subunit of 330 kDa and the ferritin subunit of 220 kDa. To estimate the molecular mass of ACVS, native PAGE was performed by using the PhastSystem with a 4–15 % (w/v) gradient gel. When native PAGE was used the proteins were separated according to charge density, size and shape, but the molecular mass calibration kit covered a more adequate interval (140–669 kDa) than with denaturing conditions (60–330 kDa). Figure 1 shows the ACVS and the high-molecular-mass calibration kit run on a 4–15 % (w/v) Phast gradient gel. Thus the ACVS of *P. chrysogenum* is a homogeneous monomeric protein. When the R_F value of the ACVS is marked in the standard curve of the high-molecular-mass proteins (results not shown), the size of the native ACVS is estimated as 470 kDa. By SDS/PAGE [3.3 % (w/v) gel] (results not shown), the molecular mass of the denatured ACVS is extrapolated to 440 kDa. The reason for the discrepancy between the two size estimations is probably that the ratio between molecular size and molecular mass of the ACVS is different from those of the globular protein standards, which will be reflected on a native gel.

The estimated molecular mass of ACVS isolated from

Table 1 Yield and degree of purification of ACVS from *P. chrysogenum*

Step	Volume (ml)	Protein (mg)	Total activity (pkat)	Specific activity (nkat per g of protein)	Yield (%)	Degree of purification (fold)
$(NH_4)_2SO_4$ pellet	38.0	547.2	3885	7.1	100	1
Q Sepharose FF	15.5	76.7	2106	27.4	54	4
Sepharose S300 HR	20.0	30.0	879	29.3	23	4
Butyl-Sepharose 4B-concentrated fractions	4.4	8.5	352	41.4	9	6

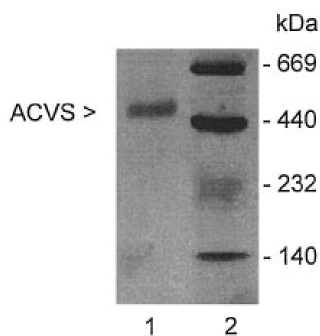

Figure 1 Native Phast gradient electrophoresis [4–15% (w/v) gel] of purified ACVS from *P. chrysogenum*

Lane 1, 10 ng of purified ACVS in buffer C; lane 2, 5 ng of high-molecular-mass calibration kit. The marker proteins were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and lactate dehydrogenase (140 kDa).

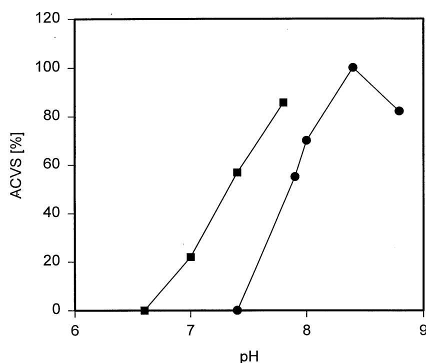

Figure 2 Effect of pH and buffer system on ACVS activity

Symbols: ■, Mops; ●, Tris. Both buffers were at 0.1 M with otherwise standard assay conditions.

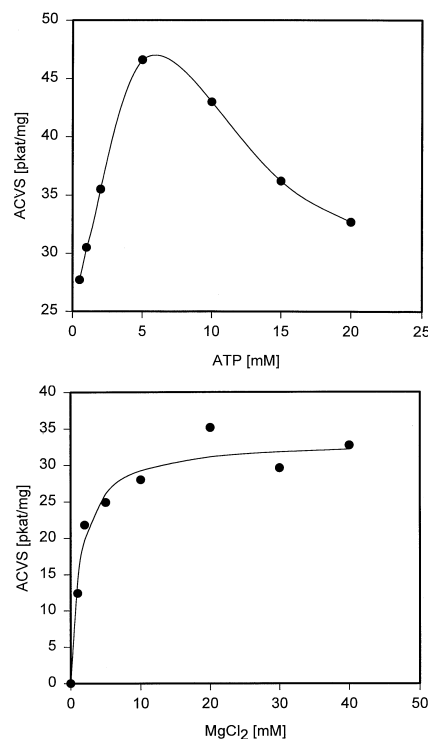

Figure 3 ACVS activity as function of ATP concentration (upper panel) and $MgCl_2$ concentration (lower panel)

In the upper panel the $MgCl_2$ concentration was fixed at 20 mM; in the lower panel the ATP concentration was 5 mM.

A. nidulans [1,2], *C. acremonium* [3,4] and *S. clavuligerus* [4–7] have varied between 230 and 500 kDa but have rarely been in accordance with the values determined from the gene sequence. Electrophoretic estimates can, however, easily be hampered by the lack of appropriate size markers as described above or by the use of a polyacrylamide gel concentration in which the linear relationship between log$M$ and R_F of the protein is not maintained for all components.

On the basis of the gene sequence of ACVS from *P. chrysogenum* the mass of the encoded polypeptide is 424.051 kDa [14,15]. An explanation for the variation in molecular mass could be that the enzyme is glycosylated, which would lead to a lower mobility and a greater size during electrophoresis. To examine whether ACVS from *P. chrysogenum* is glycosylated, the purified enzyme was treated with N-glycosidase F and the treated fractions were run on a native Phast PAGE gel together with untreated fractions (results not shown). Both fractions migrated exactly the same distance, so ACVS was not identified as a glycoprotein by these means.

Influence of pH and buffer system

The effect of pH on the ACVS reaction was determined in both a Mops and a Tris buffer (0.1 M) with otherwise standard conditions (Figure 2). With a MOPS buffer the highest activity was achieved at pH 7.8; the pH optimum was 8.4 in the Tris buffer. For the standard conditions the Mops buffer at pH 7.5 was chosen because the stability of ACVS from crude extracts was much higher in this buffer and pH 7.5 is closer to the physiological conditions.

Zhang et al. [16] also found Mops buffer to be superior to both Tris and Mes for ACVS from *S. clavuligerus*, although with a pH optimum at 8.5. For the standard assay pH 7.5 was chosen for the same reasons as above. Baldwin et al. [4] found a pH optimum of 7.5 for partly purified ACVS from *S. clavuligerus* and *C. acremonium*.

Effect of the cofactors ATP and Mg^{2+}

ACVS requires ATP and Mg^{2+} to activate and bind the three precursor amino acids. The dependence of ACVS activity on the two cofactors was investigated under normal assay conditions (Figure 3). The optimal concentration of ATP was 5 mM (20 mM $MgCl_2$). When ATP was at 5 mM, the saturating concentration of Mg^{2+} was approx. 20 mM. The fact that the optimum concentration of Mg^{2+} is higher than that of ATP could be due to the fact that at high ratios of Mg^{2+} to ATP, ATP will predominantly exist in the complexed form $MgATP^{2-}$, which is the actual substrate for the enzyme [16].

Determination of K_m values

To determine the K_m for the three precursor amino acids, they were added in a series of concentrations directly to the reaction mixture just before the reaction was started, with the two other amino acids kept at a saturating level. There was no obvious

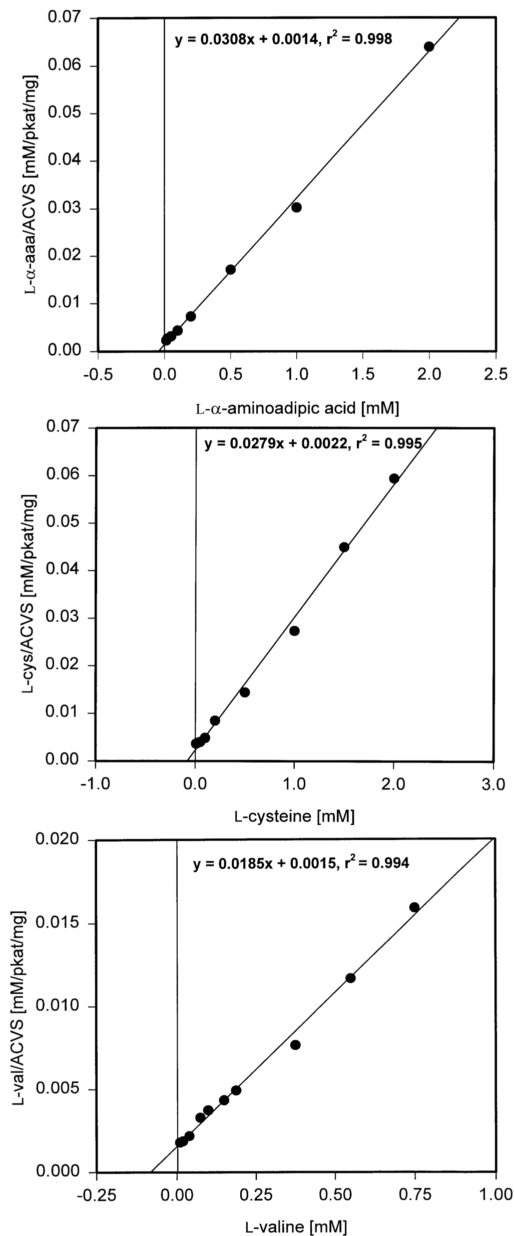

Figure 4 Dependence of ACVS activity on the concentrations of the three precursor amino acids

The influence of the amino acids on the ACVS activity is depicted in Hanes plots, i.e. c_i/V is plotted as function of c_i, where V is the enzyme activity and c_i is the amino acid concentration (mM).

substrate inhibition at the concentrations tested. The results are shown in Figure 4. The enzyme obeyed Michaelis–Menten kinetics with respect to the individual amino acids. A Hanes plot gave the following K_m values: L-α-aminoadipic acid, 46 ± 2 μM; L-cysteine, 83 ± 4 μM; L-valine, 80 ± 4 μM. In Table 2 these K_m values are compared with reports for the ACVS from other micro-organisms. The K_m values for L-α-aminoadipic acid and L-valine for the *P. chrysogenum* enzyme are almost an order of magnitude smaller than those determined for the other synthetases. Whereas the *P. chrysogenum* ACVS has the highest affinity for L-α-aminoadipic acid, the other ACVS forms have highest affinity for L-cysteine.

Table 2 K_m values of ACVS from various micro-organisms for the three precursor amino acids

	K_m (μM)			
Micro-organism	L-α-Aminoadipic acid	L-Cysteine	L-Valine	Reference
P. chrysogenum	46±2	80±4	83±4	This study
C. acremonium	170	26	340	[24]
S. clavuligerus	560	70	1140	[25]
S. clavuligerus	630	120	300	[16]
S. clavuligerus	630	430	380	[26]

Feedback inhibition by bisACV

With the purified ACVS obeying Michaelis–Menten kinetics with respect to the individual amino acids [16], the kinetics can be specified as:

$$r_{ACV} = V_{max}(X)/(1+K_{AAA}\cdot c_{AAA}^{-1}+K_{Cys}\cdot c_{Cys}^{-1}+K_{Val}\cdot c_{Val}^{-1}) \quad (1)$$

where c_i is the intracellular concentration of amino acid i (AAA being L-α-aminoadipic acid) and V_{max} is specified as a function of a state vector $\boldsymbol{X}$, which accounts for the various effectors on the maximum reaction rate including ATP, AMP, pyrophosphate, phosphate and Mg^{2+} [16,17]. The kinetic expression eqn. (1) is empirical and does not indicate anything about the mechanism of the ACVS-catalysed reaction. Another proposed effector is ACV, which might provide feedback inhibition of ACVS [18]. Zhang et al. [16] found no inhibition of ACVS by ACV at a concentration of 0.035 mM. However, this value is at less than one-tenth of the measured intracellular concentration of ACV of 0.25–4.0 mM during fed-batch cultivations of a high-yielding strain of *P. chrysogenum* [19]. If feedback inhibition is included, the expression for the overall kinetics of the formation of ACV by ACVS becomes:

$$r_{ACV} = \frac{V_{max}(X)}{1+K_{AAA}\cdot c_{AAA}^{-1}+K_{Cys}\cdot c_{Cys}^{-1}+K_{Val}\cdot c_{Val}^{-1}}\cdot\frac{1}{1+c_{ACV}\cdot K_{ACV}^{-1}} \quad (2)$$

where K_{ACV} is an inhibition parameter.

With the purified ACVS the proposed feedback inhibition by ACV was investigated by adding bisACV to the standard ACVS assay to a final concentration in the range 0–4 mM. The dimer of ACV was not reduced to ACV before the assay addition because the reducing agent DTT (even at high concentrations) was not able to reduce bisACV completely. Furthermore DTT was found to inhibit ACVS. Another reducing agent, $NaBH_4$, was too strong and destroyed the enzyme.

To estimate the inhibition parameter for bis-ACV, K_{bisACV}, eqn. (2) is rearranged and the specific rate of ACV formation r_{ACV} is replaced with the integrated peak area in the HPLC chromatogram because they are proportional. The first term in the equation is assumed constant, which gives the expression:

$$\text{Peak area} = K/(1+K_{bisACV}\cdot c_{bisACV}^{-1}) \quad (3)$$

In Figure 5 the peak area is shown as a function of the final concentration of bisACV. In the corresponding Lineweaver–Burk plot a straight line is found and from the linear relationship the inhibition parameter K_{bisACV} is determined as 1.4 ± 0.34 mM.

One can speculate on the mechanism for the inhibitory effect of bisACV on ACVS. The bisACV might bind to ACVS and act as an allosteric effector. In view of the high value of K_{bisACV} this is, however, unlikely; the inhibitory effect is therefore believed to be a consequence of mass-action effects. Thus, if the last step in

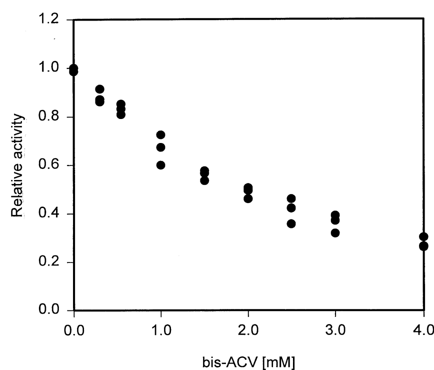

Figure 5 Inhibition of the purified ACVS from *P. chrysogenum* by bisACV

The bisACV was added to the assay mixture together with the three substrates. The activity, relative to the activity at zero concentration of bisACV, is plotted as function of the bisACV concentration.

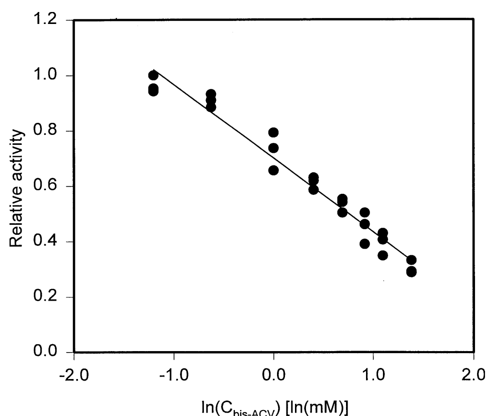

Figure 6 Relative activity of ACVS as function of ln(c_{bisACV})

The activity is given relative to the activity at zero concentration of bisACV.

the ACVS-catalysed reaction is an equilibrium process, e.g. diffusion of the formed ACV out of the catalytic site, then traces of reduced ACV in the bisACV solution might result in a shift in the equilibrium of this step. The overall result could be an increasing fraction of the enzyme holding ACV in the active site and therefore being unavailable for catalysis. If the inhibition of bisACV is a consequence of mass-action effects the reaction rate should decrease linearly with the natural logarithm to the concentration of the product [20], and as seen in Figure 6 this seems to be so.

In vivo the newly formed ACV is probably oxidized to bisACV and other mixed dithiols, and here also the concentration of reduced ACV might be low. As only the reduced form of ACV is a substrate for the subsequent enzyme of the penicillin biosynthesis pathway (isopenicillin N synthetase) [21] it is, however, necessary for the cell to maintain a certain level of ACV in its reduced form, and recently a broad-range disulphide reductase has been identified in *P. chrysogenum* [22]. Thus *in vivo* the concentration of the reduced form of ACV is likely to be much higher than in the present inhibition experiments *in vitro*, and the inhibitory effect of ACV *in vivo* might consequently be substantial.

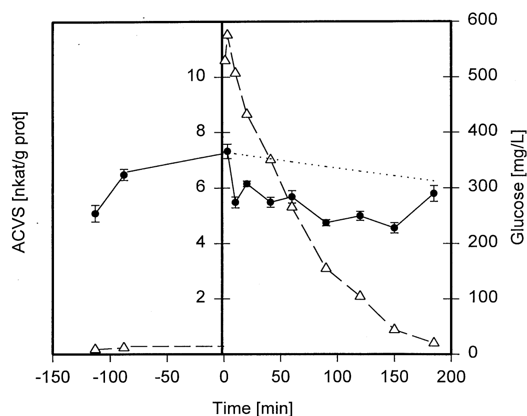

Figure 7 ACVS activity during a glucose pulse experiment in a glucose-limited chemostat culture of *P. chrysogenum*

At zero time a pulse of glucose was added to an initial concentration of approx. 0.5 g/l. The added glucose was metabolized by the cells and washed out of the bioreactor after 2.5 h, at which point the concentration was again very low. The high glucose concentration did not affect the activity of ACVS. Symbols: ●, ACVS activity (average of three); ▲, extracellular glucose concentration. The dotted line represents the ACVS activity that would hold if its enzymic synthesis and degradation were zero, i.e. ACVS activity would decrease only as a consequence of dilution due to cell growth.

Inhibition of ACVS by DTT

During the preliminary inhibition experiments bisACV seemed to have a stimulatory effect on ACVS. The enzyme activity was 126 % on average compared with the activity without the addition of bisACV. Zhang et al. [16] measured in a corresponding experiment with 0.035 mM ACV an enzyme activity of 112 %. It turned out that the stabilizing agent DTT affected the inhibition experiments, itself being an inhibitor at the standard assay concentration of 5 mM. When bisACV was added with DTT to the standard assay, the DTT was consumed by bisACV. This was reflected in a rise in the enzyme activity as the inhibition by bisACV was much smaller than the inhibitory effect of DTT. The succeeding experiments to estimate K_{bisACV} were performed without the addition of DTT or PMSF. Zhang et al. [16] found that an increase in the DTT concentration from 3 to 8 mM led to a 10 % higher enzyme activity but found no further increase at 13 mM. They added DTT to keep L-cysteine reduced. Instead of adding DTT, a fresh solution of L-cysteine was used by us, to maintain a high ratio of L-cysteine to L-cystine.

Glucose pulse experiment

The inhibitory effect of glucose on the activity of crude and purified ACVS from *C. acremonium* has been investigated *in vitro* [23]. Glucose seemed strongly inhibitory to crude ACVS but the effect was reversed by doubling the concentration of ATP; the 'glucose' effect is therefore most probably a consequence of ATP drain, e.g. by hexokinase present in the crude extract. Furthermore purified ACVS was not inhibited by glucose [23]. To investigate the influence of glucose on the ACVS activity in growing cells, a glucose pulse was added to a steady-state continuous culture of *P. chrysogenum* where the glucose concentration was very low, i.e. less than 10 mg/l. After the pulse addition the glucose concentration increased rapidly to 550 mg/l and thereafter the glucose concentration decreased owing to

consumption and wash out from the bioreactor. The ACVS activity was measured before the pulse addition and it was followed in a series of assays during the consumption of glucose (Figure 7). The ACVS concentration (X_{ACVS}) in the cells is given by the balance equation:

$$dX_{ACVS}/dt = r_{ACVS,syn} - r_{ACVS,deg} - \mu X_{ACVS} \quad (4)$$

where $r_{ACVS,syn}$ is the rate of formation of ACVS and $r_{ACVS,deg}$ is the rate of degradation of ACVS. The last term accounts for the dilution of the ACVS due to growth of the cells. If there is no degradation of ACVS and the ACVS synthesis stops immediately after the glucose pulse addition because of glucose repression, the ACVS concentration (which is linearly correlated with the measured activity *in vitro*) will decrease owing to dilution when the cells grow. The dotted line in Figure 7 indicates the decrease in the ACVS concentration resulting from dilution due to cell growth if $r_{ACVS,syn} = r_{ACVS,deg} = 0$ after the glucose pulse. From the measurements it is clear that there is a drastic effect of the glucose pulse on the ACVS activity. The results indicate strongly that there is degradation of ACVS (probably also under derepressed conditions, i.e. before the pulse is added) and that there is repression by glucose of the synthesis of ACVS. This glucose repression is most probably at the gene level, i.e. there is glucose repression of expression of the *penAB* gene, which encodes ACVS.

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