ISOLATION AND PARTIAL CHARACTERISATION OF ACV SYNTHETASE FROM *Cephalosporium acremonium* AND *Streptomyces clavuligerus*

EVIDENCE FOR THE PRESENCE OF PHOSPHOPANTOTHENATE IN ACV SYNTHETASE

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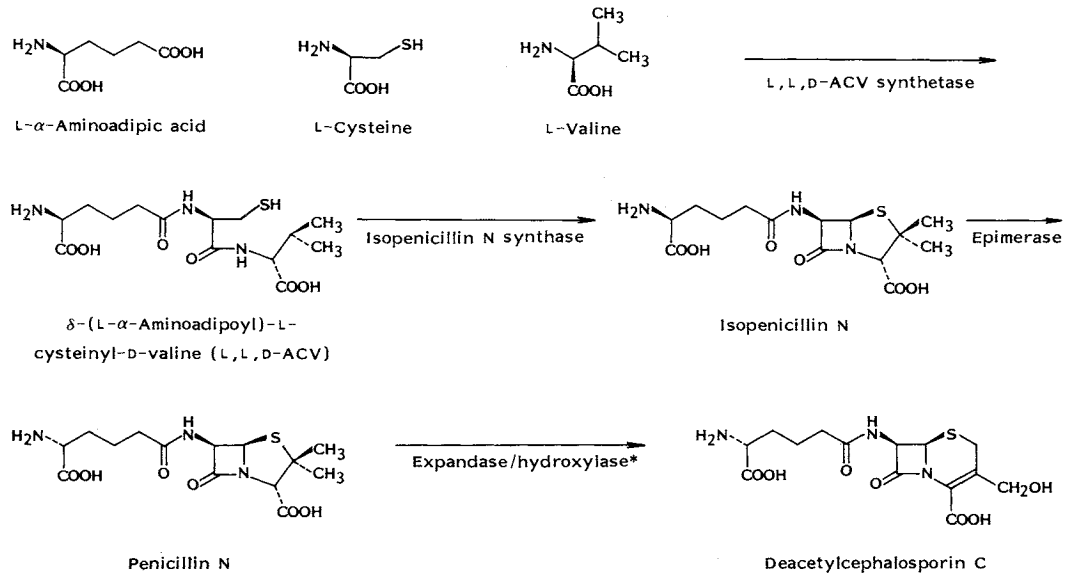
δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase was isolated and partially characterised from *Cephalosporium acremonium* CO728 and *Streptomyces clavuligerus*. The purification procedure resulted in a 745- and 277-fold increase in specific enzyme activity, respectively. Both enzymes had similar apparent molecular masses of *ca.* 300 kdaltons by SDS-polyacrylamide electrophoresis, under reducing and denaturing conditions, and in excess of 600 kdaltons in the native state by gel filtration. Attempts to obtain an *N*-terminal amino acid sequence of ACV synthetase from *C. acremonium* were unsuccessful, hence internal amino acid sequence data were obtained after tryptic digestion of the protein. Phosphopantothenic acid was shown to be associated with the enzyme from both sources, which suggests the possible involvement of pantothenate as a 'swinging arm' in the formation of the tripeptide ACV.

A number of enzymes in the biosynthetic pathway leading to the penicillins and cephalosporins have been purified and partially characterised[1]. In addition, the genes coding for some of these enzymes have been cloned and overexpressed in *Escherichia coli*[1]. This report concerns δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase, which catalyses the formation of the tripeptide ACV from the corresponding L-amino acids, the initial step in the biosynthesis of penicillins and cephalosporins (Fig. 1). At present, little structural or mechanistic information about this enzyme is available. In view of the possibility that the first step in this pathway is rate determining[2], and consequently the regulatory step, further characterisation of ACV synthetase is clearly of interest.

ACV synthetase has been isolated from *Aspergillus nidulans*[2] and the cell-free synthesis of ACV has been demonstrated with crude extracts from *Cephalosporium acremonium* CO728[3] and *Streptomyces clavuligerus* SC 2[4]. Here, we report the isolation and partial characterisation of the enzymes from both *C. acremonium* and *S. clavuligerus* and amino acid sequences of fragments obtained by tryptic digestion of ACV synthetase from the former. It has been established that 4′-phosphopantotheine is present in a number of peptide synthetase multienzyme complexes[5] and is involved in the transfer and reaction of activated amino acids enabling peptide bond formation. Evidence is presented here for the presence of phosphopantothenate in ACV synthetase preparations, which indicates that this enzyme may be the most simple peptide synthetase described so far that operates by the so called thiol template mechanism[5],††.

†† A preliminary account of this work has been previously communicated[6].

Fig. 1. Biosynthesis of penicillins and cephalosporins in *Cephalosporium acremonium*.



* Two enzymes perform these functions in streptomycetes, deacetoxycephalosporin C/deacetylcephalosporin C synthases.

Materials and Methods

ACV was prepared as described previously[7]. Protein concentrations were determined by the method of BRADFORD[8] using bovine serum albumin as a standard. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of LAEMMLI[9], using 5% or 7.5% polyacrylamide gels. Gels were stained with Coomassie Blue and MW's were estimated by comparison with standard MW markers. Total carbohydrate was measured by the method of DUBOIS *et al.*[10] with glucose and sweet almond β-glucosidase as standards. SDS-PAGE gels were stained for carbohydrate using the method of ZACHARIUS *et al.*[11] Amino acid sequence analysis was carried out using either an Applied Biosystems 470A or 473A protein sequencer with on-line analysis. Quantitation and assignment was performed using a digital Microvax 3100 and Waters Expert-Ease software.

C. acremonium CO728 (ATCC 60777) was grown in a defined medium according to SHEN *et al.*[12], except that $CuSO_4 \cdot 5H_2O$ was substituted for $CaSO_4 \cdot 5H_2O$. Growth was monitored by optical density measurements at 600 nm after a 20-fold dilution of the culture in water. Mycelia were harvested in early log phase of growth ($OD_{600\,nm}=0.5$), stored at −70°C and freeze-dried prior to extraction. When the fungus was grown in the presence of radiolabelled pantothenate, D-[1-^{14}C]pantothenic acid sodium salt (57 mCi/mmol), was added in a single portion to the culture at $OD_{600\,nm}=0.42$ to a concentration of 25 μCi/liter and mycelia were harvested 4 hours later. *S. clavuligerus* (ATCC 27064) was grown according to JENSEN *et al.*[4] and harvested after 48 hours. Mycelia were stored at −70°C until required.

Freeze-dried *C. acremonium* mycelia were ground with sand (3 g/g mycelia) in a mortar and pestle for 10 minutes, resuspended in buffer A (17 ml/g of dry mycelia) and stirred for 60 minutes at 4°C (buffer A: 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10 mM DTT, 1 mM EDTA, 45% (w/v) glycerol). Thawed *S. clavuligerus* mycelia were suspended in buffer A (1.7 ml/g) and sonicated for 8 × 30 seconds intervals.

Both extracts were then treated similarly at 4°C unless otherwise stated. Cell debris was removed by centrifugation (10,000 × *g* for 20 minutes) and nucleic acids were precipitated from the resulting supernatant by the addition of polyethylenimine to a final concentration of 0.1%. After centrifugation, (10,000 × *g* for 20 minutes) the supernatant was fractionated by the addition of various amounts of a saturated solution of ammonium sulfate. The precipitates obtained between 35~50% saturation (*C. acremonium*) and

30～50% saturation (*S. clavuligerus*) were collected by centrifugation (10,000 × *g*, 30 minutes) and dissolved in buffer B (buffer B: 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA, 9% (w/v) glycerol). Any remaining particulate material was removed by centrifugation at 100,000 × *g* for 90 minutes. The supernatant was either applied to a gel filtration column (AcA 34; 2.8 × 60 cm), eluted in buffer B at a flow rate of 12 ml/hour at 4°C, or, more efficiently and conveniently, gel filtration was performed using a Superdex 200 35/600 FPLC column, at 2.0 ml/minute at room temperature. Active fractions were pooled (elution volume: 0.3～0.35 × bed volume) and applied to a Mono Q 16/10 anion exchange column, (equilibrated in buffer B), and elution was carried out using a linear gradient between 0.15 M～0.4 M NaCl at room temperature. Fractions displaying ACV synthetase activity normally eluted at approximately 0.27 M NaCl. Further purification was obtained by chromatography on Mono Q 5/5, using a salt gradient between 0.2 M～0.32 M NaCl.

ACV synthetase activity was routinely measured according to the method of VAN LIEMPT *et al.*[2] except that equal amounts of each amino acid (1 mM) were present in the incubation. Redissolved ammonium sulfate precipitates and fractions obtained after anion-exchange chromatography were desalted on Sephadex G-25 prior to measurement of ACV synthetase activity.

Pantothenate was released from the purified enzyme preparations (～95% purity), by hydrolysis with KOH[13] or NaOH[14], incubated with alkaline phosphatase[13] and the released pantothenate/phosphopantothenate was measured using the microbiological assay as described by GILHUUS-MOE *et al.*[13].

N-Terminal amino acid sequencing of ACV synthetase from *C. acremonium* was attempted by electroblotting the protein onto immobilon (after electrophoresis on 5% polyacrylamide gels) according to the method of MATSUDAIRA[15], by adsorbing the protein directly onto the same solid support, and by conventional sequencing on a Polybrene glass-fibre filter.

Thiol groups of ACV synthetase from *C. acremonium* were reduced and alkylated prior to tryptic digestion. Guanidinium HCl (in 0.1 M Tris-HCl, pH 8.0) was added to a solution of 126 μg of protein (in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA) to a final concentration of 6 M and dithiothreitol was added to a concentration of 1 mg/ml. After incubation for 2 hours at 37°C, 4-vinyl pyridine was added to a final concentration of 1%. The reaction mixture was incubated for a further hour at 37°C and dialysed against 0.1 M Tris-HCl, pH 8.2 for 48 hours.

At an enzyme: substrate ratio of 1 : 10 (w/w), ACV synthetase was digested with trypsin overnight at 37°C. A control digestion with trypsin and buffer was included. Peptides were separated on an Applied Biosystems Spheri-5 C18 reverse phase HPLC column (220 × 4.6 mm) in 0.1% TFA using a linear gradient of acetonitrile from 5～90% over 90 minutes at a flow rate of 1.0 ml/minute. Pure peptides were sequenced directly; others were rechromatographed in the same solvents using a shallower acetonitrile gradient on an Applied Biosystems Aqua Pore 300 C8 column (100 × 2.2 mm) at 0.2 ml/minute.

Results and Discussion

ACV synthetase was purified from both *S. clavuligerus* and *C. acremonium* by similar procedures involving ammonium sulfate fractionation, gel filtration and anion-exchange chromatography. This protocol was similar to that used for purification of ACV synthetase from *A. nidulans*[2]. Incorporation of L-[^{14}C]valine into [^{14}C]ACV was measured at each stage (Table 1). This fractionation procedure resulted in a 745- and 277-fold increase in specific activity for the enzymes from *C. acremonium* and *S. clavuligerus*, respectively. Final preparations appeared to be homogenous when analysed by SDS-PAGE. The MW of both enzymes (under reducing and denaturing conditions) was estimated to be *ca.* 300 kdaltons by SDS-PAGE (Fig. 2). This is considerably larger than the reported molecular weight of the enzyme from *A. nidulans* (220 kdaltons)[2]. Gel filtration chromatography indicated that the MW of the native enzymes from *C. acremonium* and *S. clavuligerus* were greater than 600 kdaltons, indicating aggregation.

In order to determine if any carbohydrate was associated with the protein from either source, total carbohydrate was measured in purified preparations and SDS-polyacrylamide gels were stained for

Table 1. Purification of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*.

Fraction	Protein (mg)	Specific activity[a]	Recovery (%)	Fold purification
C. acremonium[b]:				
Crude[d]	700	—	—	—
Ammonium sulfate	77	3.1	100	1
Superdex 200	25	66.5	59	22
Mono Q	0.27	2,308	34	745
S. clavuligerus[c]:				
Crude[d]	344	—	—	—
Ammonium sulfate	133	2.21	100	1
AcA 34	3.1	20.8	22	9.4
Mono Q	0.05	610	10.4	277

[a] Specific activities are expressed as nmol ACV formed per mg protein per hour.
[b] Extract obtained from 3 g of freeze dried mycelia.
[c] Extract obtained from 12 g of damp mycelia.
[d] No reliable activity measurements could be obtained using crude extracts.

Fig. 2. SDS-PAGE (5%) of purified ACV synthetase isolated from 1. *Cephalosporium acremonium* 2. *Streptomyces clavuligerus*.

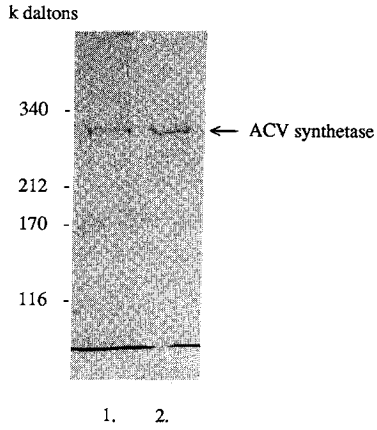
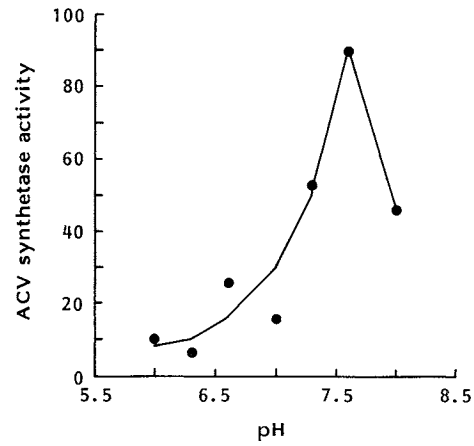


Fig. 3. Effect of pH on ACV synthetase activity.

ACV synthetase activity, expressed as nmol ACV produced/hour/mg protein, is plotted against pH of incubation.



glycoprotein. No carbohydrate could be detected by either method under conditions for which positive results were obtained with an equivalent weight of sweet almond β-glucosidase which has a carbohydrate content of 3.5% (w/w)[16].

Partial characterisation of ACV synthetase from *C. acremonium* was performed using purified preparations which had been subjected to ion-exchange chromatography. The rate-pH profile for the ACV synthetase reaction was determined using the standard assay procedure except that 50 mM sodium phosphate buffer was substituted for 50 mM Tris-HCl at pH 6.0~pH 7.0, and indicated that the optimum pH for the reaction was pH 7.5 (Fig. 3).

The effect of ferrous iron on ACV synthetase from *C. acremonium* was also determined. MARTIN and LIRAS[1] have reported that δ-L-α-aminoadipoyl-L-cysteine (AC) formation by cell-free extracts of *C. acremonium*, presumably by the action of ACV synthetase, is not inhibited by ferrous ions. In contrast, JHANG and co-workers have reported that ACV synthetase activity in cell-free extracts of *S. clavuligerus*

is strongly inhibited by Fe^{2+} with an IC_{50} of 0.2 mM[17]. In view of the requirement for iron by isopenicillin N synthase and subsequent enzymes of the β-lactam pathway, we investigated the effect of Fe^{2+} on the activity of purified ACV synthetase from *C. acremonium*. Ferrous ions were found to be a potent inhibitor of ACV synthetase with an IC_{50} of approximately 0.2 mM, supporting the data of JHANG and co-workers. It is not known whether the observed inhibition is due to direct interaction of Fe^{2+} ions with ACV synthetase or complexation of Fe^{2+} with either a substrate or a cosubstrate, therefore reducing its availability.

Attempts at obtaining an *N*-terminal amino acid sequence for ACV synthetase from *C. acremonium* proved unsuccessful, even though a number of methods were attempted. Electroblotting of material of such high MW proved inefficient and long blotting times were necessary. No *N*-terminal sequence was visible from electroblotted material or homogeneous protein absorbed directly onto an immobilon membrane. 200 picomol of material (60 μg) were subjected to conventional sequencing and this also failed to produce an *N*-terminal sequence, but a very high background was observed in this run. These results suggest that the *N*-terminal amine of ACV synthetase is probably chemically blocked.

To obtain internal amino acid sequence data, homogeneous ACV synthetase from *C. acremonium* was digested with trypsin after reduction and alkylation of the thiol groups as described in the Materials and Methods section. The resulting peptides (at least 75) were separated by reverse phase HPLC on a C18 column. A number of pure peptides were sequenced directly (No. 57, No. 69 and No 72). Others were repurified and five peptides were sequenced (No. 37.5, No. 39.3, No. 42.8, No. 43.9 and No. 63.4). The amino acid sequence data are presented in Table 2. These peptide sequences have been compared with amino acid sequence predicted from preliminary DNA sequence covering the majority of the *pcb*AB gene which codes ACV synthetase from *C. acremonium*. Peptides No. 72, No. 37.5, No. 42.8 and No. 63.4 showed 90%, 100%, 80% and 100% homology, respectively, with the translated DNA sequence. The other sequences showed no homology to the translation of the available DNA sequence (P. L. SKATRUD, Eli Lilly and Co.; personal communication).

Two possible mechanisms were considered for the *in vivo* formation of ACV. It has been proposed that ACV synthetase is one of the simplest enzymes described so far that belongs to the group of enzymes forming peptide bonds through a thiol template type mechanism[2]. This group includes the peptide antibiotic synthetases: gramicidin S synthetase, bacitracin synthetase and tyrocidine synthetase. These are large multienzymes containing phosphopantotheine, associated with one or more of the enzyme subunits, acting as a swinging arm in the transfer of activated amino acids[18]. Alternatively it was envisaged that ACV formation may be by a mechanism analogous to the biosynthesis of the ubiquitous metabolite glutathione (γ-glutamylcysteinylglycine). In this case, one enzyme would be anticipated to form the dipeptide AC and a second

Table 2. Amino acid sequence data obtained from tryptic digestion of ACV synthetase from *Cephalosporium acremonium*.

Peptide No.	Amino acid sequence	Homology[a] (%)
57	FLMHPVFNAVIGDNDIMY	0
69	ISLTIVDTPGFGDQIDNEASF-AEIVGYLER	0
72	ILNEYGFTES[DDGHE]LNIFE[b]	80
37.5	QDHWQLSVR	100
39.3	SGLSGLS	71
42.8	WIHNANGPG (G)-E	80
43.9	QYPWGVVEVDNP	0
63.4	LPHLHMVTAAR	100

[a] Sequences have been compared with the amino acid sequence predicted from the DNA sequence of the ACV synthetase gene from *C. acremonium*.

[b] In a second analysis a sequence of [AFVTA] was obtained for the bracketed portion of peptide sequence No. 72. Insertion of this sequence into peptide No. 72 and comparison of the resultant sequence with the predicted amino acid sequence gave 100% homology.

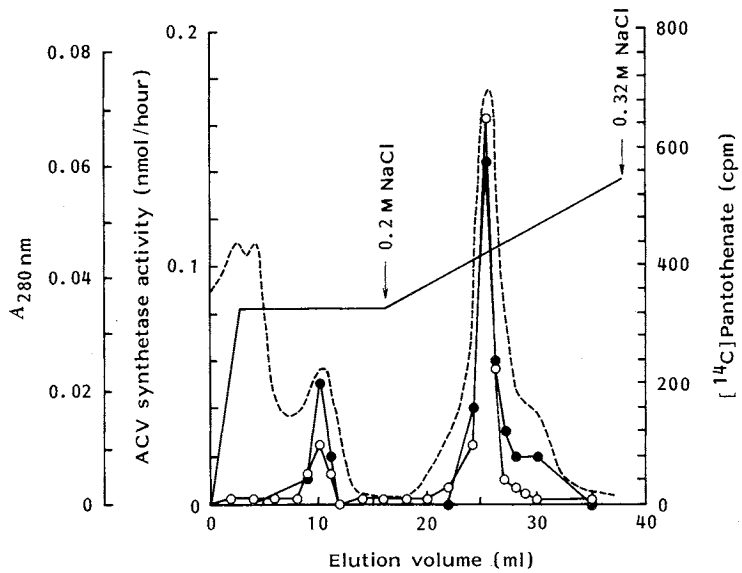
Table 3. Release of pantothenic acid from ACV synthetase preparations.

Hydrolysis	Alkaline phosphatase	ACV synthetase *Cephalosporium acremonium*	ACV synthetase *Streptomyces clavuligerus*	BSA
KOH/100°C	+	1.0	0.95	0
	−	0	0	0
NaOH/37°C	+	1.1	nd	0
	−	0	nd	0

Pantothenate was released from the protein by hydrolysis with KOH[11] or NaOH[12] as described in the Materials and Methods section. Results are expressed in mol of pantothenate detection per mol of protein.
BSA: bovine serum albumin.
nd: not determined.

Fig. 4. Purification of ACV synthetase from *Cephalosporium acremonium* by chromatography on Mono Q after growth of the organism in the presence of [^{14}C]pantothenate.

--- $A_{280\,nm}$, ○ ACV synthetase activity (nmol/hour), ● [^{14}C]pantothenate (cpm).



to catalyse the formation of ACV. However, incubation of the cell-free extracts of *C. acremonium* with the dipeptide AC and valine showed less ACV formation than incubations with the free amino acids[19]. Furthermore, a single polypeptide was shown to be responsible for ACV formation in *A. nidulans*[2] suggesting that ACV synthetase operates in a manner similar to other peptide antibiotic synthetases.

Therefore, it was of interest to determine if phosphopantothenic acid could be detected in purified preparations of ACV synthetase from both sources. Two methods were utilised. Firstly phosphopantothenate was liberated from the protein by alkaline hydrolysis[13,14] and subsequently digested with alkaline phosphatase to release pantothenic acid. The amount of released pantothenate was then assayed using the pantothenate requiring organism *Lactobacillus plantarum*. The results are summarised in Table 3. These results indicate that approximately 1 mol of pantothenate per mol of protein is associated with ACV synthetase isolated from *C. acremonium* and *S. clavuligerus*. Growth was not observed when preparations were not treated with alkaline phosphatase, indicating the presence of pantothenate in the form of phosphopantothenate.

In order to confirm these results, *C. acremonium* was grown in the presence of [^{14}C]pantothenate as

described in the Materials and Methods section. ACV synthetase was extracted and purified as before. After chromatography on Mono Q, radioactivity was shown to coincide with ACV synthetase activity (Fig. 4). A small proportion of ACV synthetase activity (~10%) was seen to elute before gradient elution began. SDS-PAGE followed by autoradiography of the fraction highest in ACV synthetase activity and highest in radioactivity indicated that the radioactivity was associated with the protein of 300 kdaltons (not shown). SDS-PAGE showed that all fractions which displayed enzyme activity contained a protein with a MW of 300 kdaltons. These results clearly establish that pantothenic acid (in the form of phosphopantothenate) is associated with ACV synthetase which support the model of ACV formation[1] involving pantotheine as a swinging arm in the transfer of activated amino acids.

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