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Exchange of the Valine 2-H in the Biosynthesis of *L*-δ-(α-Aminoadipoyl)-*L*-Cysteinyl-*D*-Valine

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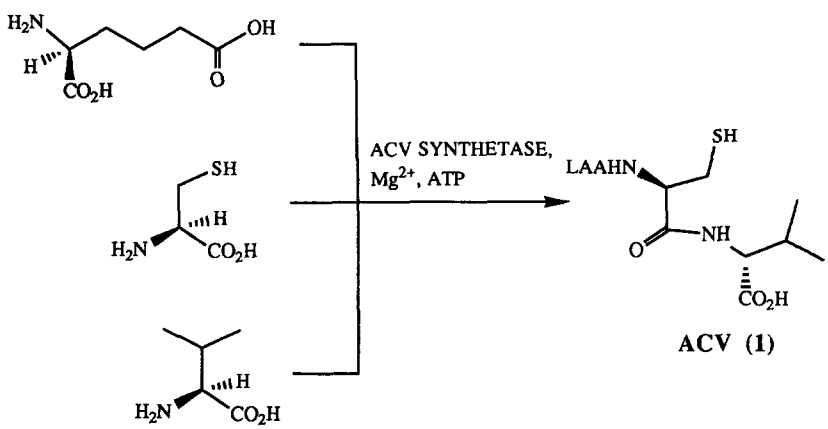
Abstract: Incubation of [2-^{2}H]-valine with purified ACV synthetase from both *Cephalosporium acremonium* and *Streptomyces clavuligerus* produced *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV), determined by the essentially complete (>95%) loss of deuterium from the α position of the incorporated valine Incubations with deuterium oxide/water as solvent produced ACV with significant incorporation of deuterium into the valinyl residue These observations confirm the prior proposal that a single multifunctional enzyme is responsible for both the formation of the peptide bonds of ACV and the epimerisation of the valinyl residue



Scheme 1,
Abbreviations ACV = *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (1),
LAAHN = *L*-δ-(α-aminoadipoyl)

The committal step in the biosynthesis of both penicillin and cephalosporin antibiotics is the formation of the tripeptide *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV, 1) with

concomitant inversion of stereochemistry at the valinyl α-centre This tripeptide is oxidatively cyclized to isopenicillin N by isopenicillin N synthase which has an absolute stereochemical requirement for the *D*-stereochemistry of the valinyl residue

By contrast to the other enzymes of the β-lactam biosynthetic pathway, elucidation of the mechanism of ACV synthesis has been hampered by the difficulty in developing a reliable assay for ACV synthesis and the lability of the enzyme(s) concerned The conversion of the individual amino acids to ACV is much faster than the conversion of the *L*-δ-(α-aminoadipoyl)-*L*-cysteine dipeptide plus *L*-valine to the tripeptide[1] This, along with several other lines of evidence[2,3,4] led to the proposal[1] that ACV is synthesized by a single multifunctional enzyme similar to other antibiotic peptide synthetases ACV synthetase was first isolated from *Aspergillus nidulans* [5] in an active form stabilized by the addition of high concentrations of glycerol to the extraction and column buffers This enzyme (molecular weight approx 220 kDa) catalysed the formation of ACV from the *L*-enantiomers of the substrate amino acids in the presence of MgATP It also catalysed MgATP pyrophosphate exchange in the presence of each of the three amino acid substrates This observation is consistent with an enzyme mechanism in which the reversible formation of an enzyme-bound aminoacyladenylate is the initial step in the reaction pathway The presence of pantothenate covalently bound to ACV synthetase[6] and detection of [^{14}C]-valinoylated ACV synthetase in which the label was labile to performic acid oxidation[5] suggest a multi-enzyme "thiol-template" mechanism for the synthetase The exchange of one and both valine oxygen atoms when a *C acremonium* mutant was fed exogenous L-[$^{18}O_2$] valine *in vivo*[7] and exchange of only one valine oxygen *in vitro* in the formation of ACV catalysed by purified *C acremonium* ACV synthetase[8] are consistent with the irreversible formation of the putative enzyme/substrate thioester intermediate Significant sequence identity of the deduced amino acid sequence of ACV synthetase from several sources with other "thiol template" peptide synthetases[9-14] further supports the conclusion that a single multifunctional enzyme is responsible for ACV synthesis

Here we demonstrate both the essentially complete loss of ^{2}H from the substrate *L*-[2-^{2}H]-valine during the formation of ACV and significant incorporation of ^{2}H from solvent ^2H_2O into the product valinyl residue catalysed by purified ACV synthetase from both *C acremonium* and *S clavuligerus* We also report modified procedures for isolating ACV synthetase from these sources

Results and Discussion

Elucidation of the mechanism of ACV synthetase has been hampered by the difficulty in purifying sufficient amounts of stable, active enzyme We have modified our previous protocol to improve routine isolation of ACV synthetase (Table 1) Determinations of ACV synthetase activities in crude extracts and in the presence of high concentrations of ammonium sulphate were variable but ACV synthetase specific activities from *C acremonium* consistently of approx 15 nkat/g for pooled material from the Superdex S-200 column have been routinely achieved This corresponds to relative specific activities of approx 350 pkat/g at the final concentration of valine used previously in this laboratory (1 5 μM) Subsequent chromatography on a MonoQ ion-exchange column results in apparently essentially (>95%) homogeneous enzyme on SDS-polyacrylamide gel electrophoresis The enzyme showed no loss of peptide synthetase

activity after 6 weeks at -80 °C Similar purifications have been achieved for *S clavuligerus* but our recoveries of total activity have been consistently much lower for the enzyme from this source[6] The preparations from both *S clavuligerus* and *C acremonium* were highly active in the ATP pyrophosphate exchange assay[5] in the presence of each of the amino acid substrates The routine availability of active, stable enzyme has made more detailed mechanistic studies of ACV synthetase feasible

The inversion of the stereochemistry of the valine α-centre by ACV synthetase is suggested by the observations that although the *D*- configuration of the valine residue in ACV is absolutely required by isopenicillin N synthase[15], *D*-valine is not a substrate for ACV synthetase[6] We have not been able to detect significant ATP pyrophosphate exchange in response to *D*-valine, indicating that this enantiomer is discriminated against in the initial step of the reaction pathway (not shown) Although no *L,L,L*-ACV has been detected either *in vivo* or *in vitro*, the required epimerisation of valine has not explicitly been shown thus far to be catalysed by ACV synthetase Although the carboxyl group of the valine in ACV is free, *L*-valine stimulates the ATP pyrophosphate exchange reaction[5] and is probably irreversibly transferred to an enzyme bound thiol [5,7,8] One rationale for the activation of the valine carboxylate is that it is required to drive the epimerisation reaction and that this step in the biosynthesis is also catalysed by the enzyme

Table 1 Purification of ACV synthetase from *C. acremonium*

Step	protein (mg)	activity (pkat)	specific activity (nkat/g)
50% satn $(NH_4)SO_4$	300	- [a]	- [a]
Superdex S-200	51	715	14
MonoQ 5/5	2 9	203	70

[a]assays of the ammonium sulphate fraction were too variable to be included

Synthesis of ACV by purified ACV synthetase *in vitro* with [2-^{2}H]-valine as substrate resulted in effectively complete washout of deuterium from the valine α-centre, as demonstrated by the isolation of a tripeptide from the incubation shown by electrospray mass spectrometry to have m/z 364, identical to that of authentic ACV (Table 2, entry 3) Essentially analogous results were obtained with ACV synthetase from *S clavuligerus* (Table 2, entry 4) No such loss of deuterium from *L*-[2-^{2}H]-valine was observed when it was recovered from incubations with ACV synthetase in the absence of the other amino acid substrates (not shown) Biosynthesis of ACV from unlabelled *L*-valine by the *C acremonium* enzyme in buffer solution with approximately 1 1 D_2O H_2O led to isolation of ACV containing a significant proportion of ACV containing a single deuterium atom, presumably resulting from exchange at the α-position of valine There was no evidence for a significant amount of ACV in which two deuterium atoms were present, suggesting that epimerisation at the α-positions of cysteine or α-aminoadipic acid does not occur freely Thus far, the lower yields of activity in the isolation of ACV synthetase from *S clavuligerus* have precluded the

analogous experiment (Table 2, entry 5) By observing the exchange of deuterium between the valine α centre and solvent in both directions *in vitro* with purified enzyme, we have demonstrated explicitly that the epimerisation of the valine residue is catalysed by ACV synthetase

Table 2. Electrospray mass spectrometry of tripeptides isolated from incubations with ACV synthetase (Tripeptides were analyzed after overnight reduction with 10 mM dithiothreitol, for details see experimental section)

Entry	Tripeptide source	m/z	361	362	363	364	365	366	367
1	Authentic ACV (1)	%obs	0	1	1	100	23	7	2
2	Authentic AC-[2-^{2}H]-valine	%obs	0	1	0	17	100	18	7
3	*C acremonium* ACVS with [2-^{2}H] valine	%obs	0	0	0	100	17	7	1
4	*S clavuligerus* ACVS with [2-^{2}H] valine	%obs	0	0	0	100	25	8	7
5	*C acremonium* ACVS with D_2O/H_2O (*ca* 1 1)	%obs	0	0	0	100	84	19	6

Experimental

Assay of ACV synthetase activity

The reported radiochemical assay of ACV synthetase has previously measured the incorporation of 1 - 3 μM [^{14}C] -valine into ACV [5,6] The low specific radioactivity of the available [^{14}C]-valine precludes using saturating concentrations (approx 1 mM) of this substrate, resulting in both consistent underestimation of total enzyme activity and the determination of relative specific activities Using high specific activity [3,4-^{3}H]-valine we have routinely assayed V_{max} for the enzyme This method is useful for determining the amount of enzyme to use for the synthesis of relatively large amounts of ACV suitable for experiments utilising isotopic label in large scale incubations (as below) where the concentration of valine is saturating Enzyme assays typically contained Tris-HCl (40 mM), pH 7 5, $MgCl_2$ (70 mM), *L*-α-aminoadipate, *L*-cysteine, *L*-valine (1 3 mM), ATP (70 mM), dithiothreitol (1 mM), glycerol (4%[v/v]) and 0 75 μCi *L*-[3,4-^{3}H] valine (61 Ci/mmol, NEN) The synthesised tritium labelled tripeptide was recovered by Porapak Q chromatography as described below, and the incorporated radioactivity determined by liquid scintillation counting

Isolation of ACV synthetase

This was performed with several modifications to the previous protocol[6] Routinely, 200 g wet weight *C acremonium* mycelia were suspended in 200 ml of Tris-HCl(100 mM), pH 7 5, KCl (100 mM), EDTA (1 mM), dithiothreitol (10 mM), glycerol [45% (v/v)] , $MgCl_2$ (1mM), DNase I (4 μg/ml), and the protease inhibitors pepstatin, benzamidine, soyabean trypsin inhibitor and leupeptin (2 μg/ml each) The cells were homogenized by ultrasonication, centrifuged and the cell-free extract brought to 0 1% (v/v) with polyethylenimine-HCl After removal of the precipitated DNA by centrifugation, solid ammonium sulphate was added to 50% saturation After centrifugation, the precipitate was redissolved in Tris-HCl (50 mM),

EDTA (0 1mM), dithiothreitol (0 1 mM), glycerol [9% (v/v)], pH 7 5 and applied at 1 5 ml/min to a Superdex 200 Prep Grade 35/600 column (Pharmacia), equilibrated in the same buffer The column was eluted at 3 ml/min Fractions containing ACV synthetase activity (eluting in the void volume of the column) were pooled and applied to a MonoQ 5/5 anion exchange column equilibrated in the Superdex column buffer containing 150 mM NaCl The column was washed with 20 ml of this buffer and then eluted with a 20 ml linear gradient of 150-200 mM NaCl in the starting buffer The flow rate was 0 5 ml/min Fractions were desalted prior to assay, and those with high specific activity were frozen at -80 °C For *S clavuligerus* the procedure was similar except the fraction precipitated by 35-50% saturation ammonium sulphate was collected for Superdex S-200 chromatography

Incubation of [2-^{2}H]-valine with ACV synthetase

Cephalosporium acremonium ACV synthetase

Incubations of [2-^{2}H]-valine with ACV synthetase from *C acremonium* were typically carried out in a total volume of 1 4ml containing $MgCl_2$ (72 7 mM), *L*-α-aminoadipic acid (5 5 mM), *L*-cysteine (5 5 mM), *L*-[2-^{2}H]-valine (5 5 mM), dithiothreitol (10 9 mM), ATP (27 3 mM), and ACV synthetase (approx 5 5 pmol/s total activity) in Tris-HCl buffer (50 mM), pH 7 5 After incubation at 27 °C for 4h the reaction was terminated by addition of trichloroacetic acid to a final concentration of 5% (w/v) The precipitate was removed by centrifugation (13 000 rpm, 5 min) The supernatant was loaded onto a Porapak Q column (2 ml), which had been pre-equilibrated with 20 ml of aqueous 8% (v/v) acetic acid, 2% (v/v) formic acid After washing with this solution (10 ml) the column was eluted with methanol (5 ml) The methanol was removed *in vacuo* and the remaining eluate freeze-dried Tripeptides were isolated by reverse phase h p l c as described below When the incubation was done in the presence of *L*-[2-^{2}H] valine only, the incubation was for 16 h

Streptomyces clavuligerus ACV synthetase

Incubation of [2-^{2}H]-valine with ACV synthetase from *S clavuligerus* was typically carried out in a total volume of 7ml containing $MgCl_2$ (44mM), *L*-α-aminoadipic acid (3 2mM), *L*-cysteine (3 2mM), *L*-[2-^{2}H]-valine (3 9mM), dithiothreitol (4 3mM), ATP (16 2mM), and ACV synthetase (approx 0 45 pmol/s total activity) in Tris-HCl buffer (50mM), pH 7 5 After incubation at 27 °C for 16h the tripeptide product was isolated as above

Incorporation of ^{2}H from ^2H_2O into ACV

Incubations were carried out as above with ACV synthetase from *C acremonium* using unlabelled *L*-valine and an approx 1 1 (v/v) ratio of deuterium oxide to water Tripeptides were isolated as before

Isolation of reaction products by h.p.l.c.

Product tripeptides from the Porapak Q column were further purified by reverse phase h p l c on an octadecylsilane column (4 6 x 250mm) employing isocratic elution with a mixture of aq NH_4HCO_3 (25mM) and methanol (4 1[v/v]) as the mobile phase at 1 ml/min The tripeptides isolated in this way were the reduced (thiol) form, which gave a retention time of approx 4 3 mins Valine eluted with a retention time of approx 3 2 min

Mass spectrometry

ACV samples were analysed as the thiol form of the tripeptide following reduction of neutral samples of ACV with dithiothreitol (10 mM) overnight Electrospray mass spectra were run using 1 1 MeOH/H_2O 1% AcOH at typically 50 pmol ml^{-1} on a VG Biotech VG Bio-Q mass spectrometer

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