Substrate specificity of L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase from *Cephalosporium acremonium*: demonstration of the structure of several unnatural tripeptide products

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Potential substrates for L-δ-(α-aminoadipoyl)-L-(cysteinyl)-D-valine (ACV) synthetase were initially identified using both the amino-acid-dependent ATP⇌pyrophosphate exchange reaction catalysed by the enzyme and the incorporation of ^{14}C-radiolabelled cysteine and valine into potential peptide products. *S*-Carboxymethylcysteine was an effective substitute for α-aminoadipate and both allylglycine and vinylglycine could substitute for cysteine, indicating that the thiol group of cysteine is not essential for peptide formation. L-*allo*-Isoleucine but not L-isoleucine substituted effectively for valine. The structures of the presumed peptide products derived from these amino acids were confirmed by combined use of electrospray-ionization m.s. (e.s.m.s.) and ^{1}H n.m.r. These results clearly indicate that, in common with other peptide synthetases, but in contrast with ribosomal peptide synthesis, ACV synthetase has a relatively broad substrate specificity.

INTRODUCTION

The initial enzyme in the biosynthetic pathway leading to the penicillin and cephalosporin antibiotics is L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase, which catalyses the formation of the tripeptide ACV from the L-enantiomers of the individual amino acids. Investigations into the substrate specificity and mechanism of this enzyme have been hampered by its large size and apparent instability [1]. However, it is presumed that, as with other 'thiol template' peptide synthetases with which ACV synthetase shares significant sequence identity, the initial step is amino acid activation by the formation of the respective aminoacyladenylate. Subsequently, the aminoacyl group is thought to be transferred to an enzyme-bound thiol [2,3]. Translocation of the incomplete peptide is believed to be by a phosphopantetheine 'swinging arm' [1].

The peptide synthetases are of considerable interest because they represent a route for peptide-bond formation which is independent of the ribosome and thus permit the incorporation of amino acids not normally found in proteins [3]. ACV synthetase is the simplest peptide synthetase described so far which is thought to use the 'thiol template' mechanism. In view of the relative simplicity of the enzyme compared with other enzymes of this class and the likelihood that the first step in the β-lactam pathway is rate-determining, several groups have published work on the purification and partial characterization of ACV synthetase from several sources [1,2,4–8]. Various structural analogues have been used in preliminary investigations of the substrate specificity of the enzyme [4,7,9]. However, the chemical structures of the putative products formed with substrates shown to be active by various assay methods have not hitherto been unequivocally determined. We are interested in studying the substrate specificity of ACV synthetase with regard to the potential development of specific chemical probes for the mechanism of the enzyme and also for the enzymic synthesis of unnatural peptides. Additionally, it is possible that novel β-lactams can be synthesized enzymically from analogues of appropriate precursor amino acids via ACV synthetase, since it has been shown that isopenicillin N-synthase can cyclize numerous unnatural tripeptides and deacetoxy/deacetylcephalosporin C synthase can expand unnatural penicillins to cephalosporins [10,11].

Here we report results for several substrate analogues which were active in the ATP⇌PP$_i$ exchange reaction catalysed by ACV synthetase and which gave significant amounts of a putative peptide product as determined by the incorporation of ^{14}C-labelled natural substrates into potential ACV analogues [2]. For several of the analogues active in these assays we have isolated sufficient product tripeptide to confirm their chemical structures by both electrospray ionization m.s. (e.s.m.s) and ^{1}H n.m.r. spectroscopy.

MATERIALS AND METHODS

Chemicals

L-[U-^{14}C]Cystine (322.2 mCi/mmol) and tetrasodium [^{32}P]pyrophosphate (20–40 Ci/mmol) were purchased from NEN–du Pont, L-[U-^{14}C]valine (266 mCi/mmol) from Amersham International, and Porapak Q from Waters Associates (Milford, MA U.S.A.). (2*R*,3*S*)- and (2*R*,3*R*)-α-Amino-β-fluorobutyric acid were synthesized from L-threonine by a minor modification of the reported methodology [12,13]. DL-α-Methylvaline was synthesized from methyl isopropyl ketone [14]. DL-Allenylglycine was prepared by a novel methodology [15,16]. DL-2-Amino-3-methylbuten-3-oic acid was synthesized from methyl α-nitrodimethylacrylate [17]. L-Homocysteine was produced *in situ* by reduction of L-homocystine with dithiothreitol. L-δ-(α-Aminoadipoyl)-L-(allylglycinyl)-D-valine was synthesized using a standard methodology [18]. Inorganic pyrophosphatase (baker's yeast) was from Sigma Chemical Co. Other chemicals were purchased from Aldrich or Sigma Chemical Co.

Abbreviations used: ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; e.s.m.s., electrospray-ionization m.s.
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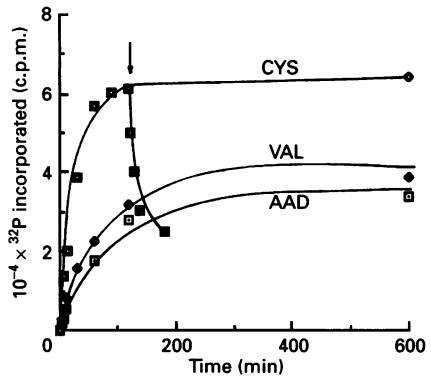

Figure 1 Time course of equilibration of [^{32}P]PP_i into ATP catalysed by ACV synthetase in response to the natural amino acid substrates

The data points for 600 min (essentially complete equilibration) were determined at 120 min with 5 times more enzyme preparation in the incubations than at the other data points. Inorganic pyrophosphatase (approx. 0.5 unit) was added to a parallel incubation containing cysteine after 120 min (arrowed). Abbreviation: AAD, α-aminoadipate.

Enzyme preparation

ACV synthetase was prepared from *Cephalosporium acremonium* CO728 and *Streptomyces clavuligerus* as previously reported [19] routinely through the Superdex S-200 stage. ACV synthetase from *C. acremonium* purified in this manner showed no detectable loss of peptide synthetase activity after 6 weeks at −80 °C, and retained > 15% of its activity after 10 months at −80 °C.

ATP ⇌ PP_i exchange assay

This was performed as in [2] with minor modifications. Typically, ATP (1.9 mM), $MgCl_2$ (1.9 mM), dithiothreitol (1.9 mM), substrate or analogue (1.5 mM), enzyme (5–10 μl, 12.5–25 μg of protein) were incubated in Mops buffer [50 mM, pH 7.8, including 10% (v/v) glycerol, 1 mM EDTA] in a final volume of 260 μl. The exchange reaction was followed by addition of [^{32}P]PP_i (8 mM, 6.0–8.0 × 10^5 d.p.m.) and incubated at 28 °C for 20 min. The reaction was terminated by a mixture (300 μl) containing 1% (w/v) activated charcoal, aq. 6% (v/v) perchloric acid and tetrasodium pyrophosphate (10 mM). The charcoal was spun down in a Microfuge (13000 rev./min, $r_{av.}$ 6.5 cm, 5 min) and washed with 600 μl of aq. 6% (v/v) perchloric acid containing 10 mM tetrasodium pyrophosphate. The amount of ^{32}P incorporated into ATP was determined from the Čerenkov radiation remaining bound to the charcoal. Assays were performed in triplicate and control incubations contained active enzyme but no exogenously added amino acids. Apparent K_m values were determined from double-reciprocal plots of data from assays performed under initial-rate conditions (< 10% fractional attainment of isotopic equilibrium, Figure 1).

Tripeptide formation assay

This procedure was modified from [2]. The reaction mixture contained ATP (6.9 mM), $MgCl_2$ (34.5 mM), dithiothreitol (1.03 mM), glycerol (10%, w/v), amino acid analogue or natural substrate (1.29 mM of each) and enzyme preparation (200 μl, approx. 300–500 μg protein, up to 1.5–2.5 pkat peptide synthetase activity) in Tris/HCl buffer (50 mM, pH 7.5) in a final volume of 290 μl and was incubated at 28 °C for 90 min. The reaction was terminated by a mixture (300 μl) containing ACV disulphide form (83 mM), L-cysteine or L-valine, depending on the radiolabelled amino acid used (250 μM), dithiothreitol (0.33 mM), trichloroacetic acid (10%, w/v). The protein was removed by centrifugation in a Microfuge (13000 rev./min, $r_{av.}$ 6.5 cm, 5 min) and 500 μl of the supernatant was passed through a Porapak Q column (0.5 ml packed vol. in a Pasteur pipette), which had been pre-equilibrated with 10 ml of water/acetic acid/formic acid (45:4:1, by vol.). After washing with 4 ml of this solution, the bound putative radioactive tripeptides were eluted with methanol (2 ml) and the extent of product formation was assayed by liquid-scintillation counting of 200 μl of the eluate. When L-α-aminoadipate or L-cysteine analogues were screened in place of the natural substrates, L-[U-^{14}C]valine (3.24 μM, 250 nCi) was used and the reaction terminated after 90 min. When potential L-valine analogues were screened in place of L-valine, L-[U-^{14}C]cysteine was generated from L-[U-^{14}C]cystine (2.14 μM, 200 nCi) *in situ* in the presence of the dithiothreitol and the reaction was terminated after 60 min.

Preparative-scale enzymic synthesis of tripeptides

Incubations were carried out at 27 °C for 16 h in a mixture containing: $MgCl_2$ (65.6 mM), natural substrates or analogues (5 mM each), dithiothreitol (3.8 mM), ATP (26.6 mM) and ACV synthetase (total peptide synthetase activity up to 100–150 pkat) in Tris/HCl buffer (50 mM), pH 7.5, in a final volume of 1.5 ml and were stopped by addition of aq. trichloroacetic acid to a final concentration of 5% (w/v). The protein was removed by centrifugation (13000 rev./min, 5 min). The supernatant was loaded on to a Porapak Q column (2 ml packed vol., pre-equilibrated with 20 ml of water/acetic acid/formic acid (45:4:1, by vol.). The column was washed with this solution (10 ml) and eluted with acetone (5 ml). The acetone was removed *in vacuo* and the rest of the eluate freeze-dried.

Isolation and identification of tripeptide products

Product peptides were isolated by reverse-phase h.p.l.c. [Hypersil C_{18}, 4.6 × 250 mm column, 1 ml/min of 20 mM NH_4HCO_3 in aq. 20% (v/v) methanol], and characterized by proton n.m.r. (500 MHz) and e.s.m.s. using a BioQ mass spectrometer (VG Biotech, Altrincham, Cheshire, U.K.). If the putative product contained a thiol group it was incubated with dithiothreitol (10 mM) overnight before analysis.

The tripeptide analogues isolated were characterized as follows.

For L-(*cysteine-S-acetyl*)-L-cysteinyl-D-valine: retention vol. 4.3 ml; δ_H (500 MHz, ^2H_2O, referenced to residual $^1H^2HO$) 0.80, 0.84 [2 × 3H, 2 × d, *J* 7.0 Hz, $CH(CH_3)_2$], 1.95–2.15 [1H, m, $CH(CH_3)_2$], 2.93–3.21 (4H, m, 2 × SCH_2CH), 3.40 (2H, s, SCH_2CO), 3.81–3.90 (1H, m, $C_\alpha H$), 4.01 [1H, d, *J* 5.5Hz, $C_\alpha HCH(CH_3)_2$], 4.48–4.55 (1H, m, $C_\alpha H$); *m/e* (e.s.m.s.) 381 (MH^+)

For L-δ-(aminoadipoyl)-L-*allylglycinyl*-D-valine: retention vol. 6.8 ml; δ_H (500 MHz, ^2H_2O, referenced to residual $^1H^2HO$) 0.85, 0.87 [2 × 3H, 2 × d, *J* 7.0 Hz, $CH(CH_3)_2$], 1.56–1.67 (2H, m, CH_2CH_2), 1.80–1.85 (2H, m, CH_2CH_2), 2.08–2.12 [1H, m, $CH(CH_3)_2$] 2.31 (2H, distorted t, *J* 8.0 Hz, CH_2CO), 2.40–2.44 and 2.45–2.50 (2 × H, 2 × m, $CH_2CH{=}CH_2$), 3.74 (1H, t, *J* 6.5 Hz, $C_\alpha HCH_2CH_2$), 4.03 [1H, d, *J* 6.0 Hz, $C_\alpha HCH(CH_3)_2$], 4.37–4.41 (1H, m, $C_\alpha HCH_2CH{=}CH_2$), 5.10 (2H, m, $CH_2CH{=}CH_2$), 5.70

(1H, m, $CH_2CH{=}CH_2$); *m/e* (e.s.m.s) 358 (MH^+); fragmentation experiment (% intensities in parentheses): *m/e* 358 (70), 241 (82, MH^+ − valine), 215 (68, MH^+ − aminoadipoyl), 143 (95, aminoadipoyl$^+$), 118 (93, valine + H^+), 98 (65, allylglycinyl + H^+), 84 (100)

For L-δ-(aminoadipoyl)-L-cysteinyl-D-*isoleucine*; retention vol. 9.0 ml; δ_H (500 MHz, ^2H_2O, referenced to residual $^1H^2HO$) 0.77 (3H, t, *J* 7.5 Hz, CH_2CH_3), 0.82 (3H, d, *J* 7.0 Hz, $CHCH_3$), 1.00–1.16 and 1.21–1.34 (2H, 2 × m, CH_2CH_3), 1.58–1.68 and 1.73–1.78 (5H, 2 × m, $CHCH_2CH_2$ and $CHCH_3$), 2.30–2.35(2H, m, CH_2CO), 2.79–2.86 (2H, eight lines, *ABX*, CH_2S), 3.58–3.66 (1H, m, $C_xHCH_2CH_2$), 4.02 (1H, d, *J* 6.0 Hz, C_xHCHCH_3), 4.44–4.54 (1H, distorted t, *J* 6.5 Hz, C_xHCH_2); *m/e* (e.s.m.s) 377 (MH^+).

For L-δ-(aminoadipoyl)-L-*vinylglycinyl*-D-valine: retention vol. 4.3 ml; *m/e* (e.s.m.s) 343 (MH^+).

RESULTS AND DISCUSSION

In order to establish rigorously that tripeptide analogues of ACV could be synthesized by ACV synthetase it was first necessary to identify potential substrate analogues quickly. We therefore initially screened candidate structural analogues using two established assays: [^{14}C]substrate-amino-acid incorporation into putative final peptide products and amino-acid-stimulated ATP⇌PP_i exchange [2]. Rather than laboriously refine the assays for each potential substrate, we reasoned that those analogues showing relatively high activities in both assays would be the most likely alternative substrates to explore further, since the assays are prone to potential artifacts that are essentially independent. Only after an initial selection of candidate substrate analogues did we attempt to isolate and characterize the putative tripeptide products.

ATP⇌PP_i exchange activities of *C. acremonium* ACV synthetase

In common both with other peptide synthetases and the aminoacyl tRNA synthetases, ACV synthetase catalyses a substrate-amino-acid-dependent ATP⇌PP_i exchange reaction [2] demonstrating activation of the amino acids as enzyme-bound aminoacyladenylates. The exchange reaction at chemical equilibrium can be followed by inclusion of [^{32}P]PP_i followed by determination of the incorporation of [^{32}P] into ATP [2]. We have been able to detect this partial reaction of ACV synthetase with enzyme from *C. acremonium* in response to all three amino acid substrates (Figures 1 and 2) and some potential analogues (Figure 2 and Tables 1–3). The initial rate of the incorporation of ^{32}P into ATP was proportional to the amount of enzyme used (results not shown). Addition of inorganic pyrophosphatase (approx. 0.5 units) after equilibration of the ^{32}P label caused hydrolysis of the PP_i formed by the forward reaction to P_i, as demonstrated by the loss of ^{32}P from ATP (Figure 1), confirming the existence of the exchange equilibrium. The apparent K_m value for PP_i at the cysteine site was 1.5 mM. We determined the activities of other nucleotide triphosphates as co-substrates at the cysteine site. At 2 mM nucleotide triphosphate the initial rates were ordered ATP > GTP = TTP > CTP = dATP = dGTP > dTTP = dCTP = ITP = UTP = 0; GTP and TTP showed approx. 20% and CTP, dATP and dGTP approx. 15% the rate of exchange seen with ATP.

By contrast with the enzyme from *Streptomyces clavuligerus* [3] we have been able to detect ATP⇌PP_i exchange (Figures 1 and 2) in response to L-α-aminoadipate (apparent K_m 100 μM) in most of our preparations of ACV synthetase from *C. acremonium* although the activity was rather labile. We have also determined apparent K_m values for the other natural substrates cysteine (210 μM) and valine (90 μM). Subsequently we determined apparent K_m values for the unnatural substrates which were clearly incorporated into tripeptides (see below): L-allylglycine (0.8 mM) L-vinylglycine (2.8 mM), *S*-carboxymethylcysteine (3.0 mM) and L-*allo*-isoleucine (0.3 mM). The values for the natural substrates are approx. 10-fold lower than those reported for ACV synthetase from *S. clavuligerus* [3] and are similar to those reported for the aminoacyl-tRNA synthetases (e.g. 0.17 mM for yeast valine aminoacyl-tRNA synthetase [19]). Analogues lacking carboxy groups (cysteamine and valinol) were used as controls and were unable to stimulate the exchange reaction (Tables 2 and 3).

In our initial substrate screening trials with amino acid substrates the amount of ACV synthetase used resulted in

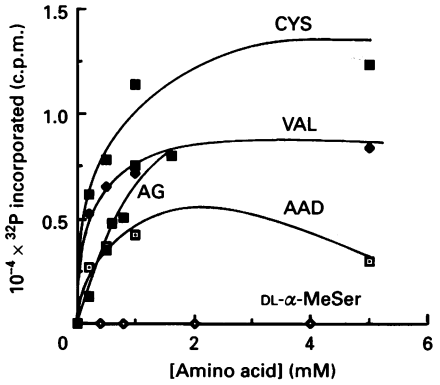

Figure 2 Amino-acid-concentration-dependence of the ATP⇌PP_i exchange reaction

Assays were carried out at less than 10% fractional attainment of equilibrium, using twice the specific activity of [^{32}P]PP_i than used in Figure 1. Data for two potential analogues DL-α-methylserine (no exchange observed) and allylglycine (which was incorporated into a product tripeptide) are also shown. Abbreviations: AAD, α-aminoadipate; AG, allylglycine; DL-α-MeSer, DL-α-methylserine.

Table 1 Identification of potential analogues of α-aminoadipate in the ACV synthetase reaction

Analogues were included in the tripeptide formation assay in place of α-aminoadipic acid and the extent of [^{14}C]valine incorporation into potential tripeptide product was measured. The rate of formation of authentic ACV was 2.0 pkat; values are given as percentages of this activity. Analogues were also assessed for their ability to stimulate the ATP⇌[^{32}P]PP_i exchange reaction with sufficient enzyme to equilibrate the included ^{32}P within 2 min in the presence of the natural substrate. The data given are percentages of the ^{32}P incorporation observed at equilibrium for the natural substrate that were observed for the analogue after 90 min. Entries are divided into three groups: (i) those which gave characterized tripeptides, (ii) those which were apparently active in both assays and (iii) others.

Part	Substrate	Relative rate of ^{14}C incorporation (%)	ATP⇌PP_i exchange (%)
(i)	L-α-Aminoadipic acid	100	100
	L-*S*-Carboxymethylcysteine	99	95
(ii)	L-Glutamic acid	19	93
(iii)	D-α-Aminoadipic acid	14	0
	Adipic acid	2	0
	5-Aminovaleric acid	2	0
	L-Aspartic acid	2	0

Table 2 Identification of potential analogues of cysteine in the ACV synthetase reaction

Analogues were included in place of cysteine, and (iv) a control incubation lacking cysteine was performed. Other details are as in the legend to Table 1.

Part	Substrate	Relative rate of [^{14}C] incorporation (%)	ATP$\rightleftharpoons$PP$_i$ exchange (%)
(i)	L-Cysteine	100	100
	L-Allylglycine	122	29
	L-Vinylglycine	101	3
(ii)	DL-*O*-Methylserine	76	2
	L-*S*-Methylcysteine	60	52
	L-*N*-Acetylcysteine*	44	2
	D-Cysteine	21	4
	L-β-Chloroalanine	12	16
	L-Homocysteine	9	4
	D-β-Chloroalanine	8	6
	L-Serine	4	3
	L-*O*-Methylthreonine	16	< 1
(iii)	5-Aminovaleric acid	12	0
	Cysteamine	11	0
	DL-α-Methylserine	10	0
	L-*allo*-Threonine	9	0
	L-Methionine	9	0
	L-Threonine	6	0
	3-Mercaptopropionic acid	2	0
	Adipic acid	0	0
(iv)	No cysteine control	7	

* The possibility of partial hydrolysis to cysteine in the incubation cannot be eliminated.

Table 3 Identification of potential analogues for valine in the ACV synthetase reaction

Analogues were included in place of valine, and [^{14}C]cysteine incorporation into potential tripeptide analogues was measured. A control incubation lacking valine (iv) was also performed. Other details are as for Table 1.

Part	Substrate	Relative rate of ^{14}C incorporation (%)	ATP$\rightleftharpoons$PP$_i$ exchange (%)
(i)	L-Valine	100	100
	L-*allo*-Isoleucine	100	38
(ii)	DL-2-Amino-3-methylbuten-3-oic acid	34	15
	L-t-Leucine	22	3
	L-Isoleucine	20	3
	L-Allylglycine	15	154
	L-Threonine	5	1
	L-Vinylglycine	5	15
	(2*S*)-α-Aminobutyric acid	5	158
	L-β-Chloroalanine	4	355
	DL-β-Fluorovaline	3	3
	(2*R*,3*S*)-α-Amino-β-fluorobutyric acid	2	24
	(2*R*,3*R*)-α-Amino-β-fluorobutyric acid	2	5
(iii)	DL-Allenylglycine	38	0
	DL-α-Methylvaline	15	0
	(2*S*)-Valinol	14	0
	L-*allo*-Threonine	7	0
	Isovaleric acid	0	0
	D-Valine	0	0
(iv)	No valine control	6	

complete isotopic equilibration of the added ^{32}P within 2 min of addition of the label in the presence of the natural substrates. The large amount of active enzyme used in these experiments was to facilitate detection of any weak isotope exchange in response to the large range of potential analogues initially screened. The results are shown in Tables 1, 2 and 3. Importantly, several potential analogues which showed zero ATP$\rightleftharpoons$PP$_i$ exchange activity in the preliminary screening (e.g. DL-α-methylserine, Figure 2) also failed to show any incorporation of ^{32}P into ATP at substrate concentration of up to 4 mM, even when the amounts of enzyme and [^{32}P]PP$_i$ were raised by a factor of 5 and the total incubation time was increased by a factor of 4 compared with the conditions of the initial assays. In the case of the analogues it is important to note that any of them could potentially act at any of the aminoacyladenylation sites. For example both allylglycine and vinylglycine in particular could be activated at either or both the cysteine and valine sites.

Screening of potential ACV synthetase substrates

The rate of ACV formation in the routine [^{14}C]valine incorporation assay [2] is linear up to 2 h and is useful for identifying active fractions during the purification of ACV synthetase. The amino acid dependent ATP$\rightleftharpoons$PP$_i$ exchange reactions catalysed by ACV synthetase from *C. acremonium* were described in detail in the preceding section. However, it is important to note that both assays are potentially prone to artifacts when used to screen substrate analogues. Firstly, the product ACV analogues may not bind well to the Porapak Q resin, leading to apparent weak activities in the tripeptide formation assay. Secondly, some apparent ^{14}C incorporation was seen for several analogues which were completely inactive in the ATP$\rightleftharpoons$PP$_i$ exchange assay [part (iii) of Tables 1, 2 and 3]. Cysteamine and valinol were included as controls for this phenomenon, since they lack carboxy groups and thus could not have been active in ATP$\rightleftharpoons$PP$_i$ exchange (Tables 2 and 3). Thus at least some of the lower apparent activities in [^{14}C]tripeptide formation are artifacts of the assay protocol. They might reflect detection of the synthesis of trace amounts of dipeptides or 'shunt' products. For example, synthesis of the doubly labelled tripeptide L-δ-(α-aminoadipoyl)-L-valine-D-valine, which has been isolated as a minor natural product from *C. acremonium* fermentations [21], would explain the low ^{14}C incorporations in Table 2 [part (iii)]. The results in Table 3, part (iii), might similarly reflect formation of trace amounts of δ-(α-aminoadipoyl)cysteinylcysteine. The low, but readily detectable, incorporation of [^{14}C]cysteine in the absence of valine and [^{14}C]valine incorporation seen in the absence of cysteine into putative tripeptides is consistent with this hypothesis [parts (iv) of Tables 2 and 3]. Thirdly, high activities in the ATP$\rightleftharpoons$PP$_i$ exchange assay need not necessarily represent peptide synthesis, since an activated analogue might be discriminated against in the next stage of the catalytic cycle. Fourthly, low exchange activity contrasting with apparent high activity in the tripeptide-formation assay might reflect different final equilibrium positions reached between the amino acid and the enzyme-bound aminoacyladenylate, since we did not perform complete kinetic analyses for all the analogues. There are differences between the final equilibrium positions reached in response to the natural substrates [3], where the equilibrium amount of ^{32}P incorporated into ATP is ordered cysteine > valine > aminoadipate (Figure 1). Finally, it was important to ensure sufficient incorporation of the relatively low specific radiochemical activity ^{14}C-labelled substrates into the putative peptides for their detection by the assay. Thus, the final concentration of the labelled substrates (3–5 μM) did not saturate the ACV synthetase.

The results in Tables 1–3 show that both a carboxy and amino group were essential for incorporation into putative product.

That the D analogues of aminoadipate, cysteine or valine (Tables 1, 2 and 3) had little relative activity in the tripeptide formation assay indicates that as expected, the D-enantiomers were discriminated against by the enzyme. The low observed activities in the ATP$\rightleftharpoons$$PP_i$ exchange assay further indicate that the D-enantiomers were discriminated against in the initial step in the overall reaction sequence. We would speculate that the apparent weak activity of the D-enantiomers of the substrate amino acids detected in the assays is due to small amounts of the L forms contaminating the commercially supplied products.

α-Aminoadipate analogues

Of the analogues of α-aminoadipate tested (Table 1) only *S*-carboxymethylcysteine (sulphur in place of the γ-CH_2) was an apparently good substrate in both assays, substantiating the result described previously [4]. L-Glutamate was a good substrate for aminoacyladenylate formation, but an apparently weak substrate for tripeptide synthesis. L-Aspartate showed negligible activity in both assays. These observations indicate that two carboxylate groups an optimal distance apart might be important for substrate recognition at this site.

Cysteine analogues

The results obtained for the cysteine analogues were more interesting. L-Homocysteine (Table 2) was first investigated since previously [4] it had been shown to substitute for cysteine in the reaction catalysed by ACV synthetase from *S. clavuligerus*. This conclusion was partly based on cyclisation of the putative product by isopenicillin N-synthase to yield an apparently antibiotically active product. We were surprised by this result, since previous work in this laboratory had shown unequivocally that the expected resultant tripeptide [i.e. L-δ-(α-aminoadipoyl)-L-homocysteinyl-D-valine] gave an antibiotically inert monocyclic product upon incubation with isopenicillin N- synthase [22]. The data in Table 2 indicate that L-homocysteine is at best a weak substrate for ACV synthetase from *C. acremonium*. Subsequently we failed to recover any δ-(α-aminoadipoyl)homocysteinylvaline from a preparative scale incubation (see below). The observation of an apparently antibiotically active substance being formed from a potential analogue by this linked assay might result from small amounts of cysteine produced by degradation of the ACV synthetase preparation. Indeed, we have observed that our *S. clavuligerus* ACV synthetase preparations can sometimes produce very considerable ATP$\rightleftharpoons$$PP_i$ exchange in the absence of exogenously added amino acids. This high background activity, which can be partly reduced by prior dialysis of the enzyme preparation, could account for up to 35% of the total ATP$\rightleftharpoons$$PP_i$ exchange seen at saturating concentrations of added amino acid substrates under initial-rate conditions. This is one reason why we have concentrated on the enzyme isolated from *C. acremonium* where the background exchange activity in the absence of added amino acid is typically not more than 1.5%. It was also suggested by Zhang et al. [4] that L-*S*-methylcysteine was a substrate for ACV synthetase, but this conclusion was based on the observation that the tripeptide product gave an antibiotically active product upon incubation with isopenicillin N-synthase. It seems unlikely that the resultant tripeptide [L-δ-(α-aminoadipoyl)-L-*S*-methylcysteinyl-D-valine] would yield a β-lactam product on incubation with isopenicillin N-synthase as this would obviously require the unprecedented removal of the *S*-methyl group either by ACV synthetase, isopenicillin N-synthase or an uncharacterized contaminating activity. Again, the observation of an apparently bioactive product might result from incorporation of cysteine derived from degradation of the enzyme preparation into authentic ACV. However, L-*S*-methylcysteine was a reasonably good substrate in both enzyme assays (Table 2), but we were unable to recover sufficient tripeptide from a large-scale incubation to analyse the anticipated product. We also found (Table 2) that L-*O*-methylserine had an even better activity (75% of cysteine) but that L-serine was effectively devoid of activity. L-Vinylglycine and L-allylglycine also were apparently effective substitutes for cysteine in both assays (Table 2), demonstrating that the thiol group of cysteine is not essential for recognition by the enzyme.

Valine analogues

Both L-vinyl- and L-allyl-glycine also showed significant activity when substituted for valine in the ^{14}C incorporation assay (Table 3) as did DL-allenylglycine and DL-2-amino-3-methylbuten-3-oic acid, which are also characterized by a double bond in the β- or γ-position. However, it is noteworthy that allylglycine (and vinylglycine) was a preferential substrate (see below) for the cysteine site. We have substantiated (Table 3) the result in [4] that L-*allo*-isoleucine is an effective substitute for valine, whereas L-isoleucine is at best a much weaker substrate. This observation is informative with regard to the specificity of the putative methyl-binding pockets at the valine binding site. We would anticipate that the stereochemical configuration of the α-centre of the incorporated isoleucine has been inverted by the ACV synthetase, since previous work [7] showed that the resultant tripeptide was cyclized by isopenicillin N-synthase to a product capable of β-lactamase induction.

Characterization of several unnatural tripeptide products of ACV synthetase

On the basis of our assay data and results presented previously in [4] we attempted preparative-scale incubations with analogues as follows: (1) α-aminoadipate replaced by *S*-carboxymethyl cysteine; (2) cysteine replaced by allylglycine, vinylglycine, *O*-methylserine, *S*-methylcysteine and homocysteine; (3) valine replaced by *allo*-isoleucine, allylglycine, vinylglycine, DL-allenylglycine and DL-2-amino-3-methylbuten-3-oic acid. We then attempted to isolate the anticipated tripeptide products in sufficient amounts for characterisation by both n.m.r and e.s.m.s. Incubations with L-*S*-carboxymethylcysteine in place of α-aminoadipate, both L-vinylglycine and L-allylglycine in place of cysteine and L-*allo*-isoleucine in place of valine produced sufficient tripeptide for isolation and characterization as detailed in the Materials and methods section.

L-Allylglycine was further shown to yield the tripeptide L-δ-(α-aminoadipoyl)-L-allylglycinyl-D-valine. The tripeptide products were co-eluted with the authentic synthetic sample when they were co-chromatographed on h.p.l.c. using the described system (see the Materials and methods section). We observed a single set of resonances when a mixture of biosynthetic and authentic synthetic product was analysed by n.m.r. The fragment molecular ions observed in the mass spectrum of the product of the incubations containing L-allylglycine in place of cysteine demonstrated unequivocally that this tripeptide had the expected sequence δ-(α-aminoadipoyl)allylglycinylvaline (see the Materials and methods section). This result indicates that, at least for the *C. acremonium* ACV synthetase, L-allylglycine is not an effective substitute for valine, in contrast with the work of Zhang et al. [4] with *S. clavuligerus* ACV synthetase, although it clearly effectively

substituted for cysteine. We have inferred the sequences of the vinylglycine-, *allo*-isoleucine- and *S*-carboxymethylcysteine-containing peptides from this observation, although currently we have no direct evidence that these sequence assignments are entirely unambiguous. However, on the basis of the available results, any alternative assignments would seem most unlikely. For the enzyme incubations containing L-*S*-methylcysteine and DL-*O*-methylserine instead of cysteine and L-allylglycine, L-vinylglycine and DL-allenylglycine as substitutes for valine, we were unable to isolate significant amounts of tripeptide product(s) after screening fractions from the h.p.l.c effluent using 500 MHz n.m.r. One possible reason for our failure to isolate the anticipated *tri*peptides from these incubations is that some of the analogues were incorporated into incomplete *di*peptide 'shunt' products. This intriguing possibility is currently under investigation, since it may provide information on the mechanism of the synthetase, for example identification of the dipeptide intermediate in the synthesis.

In the case of preparative experiments containing L-*allo*-isoleucine and L-*S*-carboxymethylcysteine as replacements for L-valine and L-δ-(α-aminoadipate) respectively the amounts of tripeptides obtained were typically approx. 50 % of that obtained for the natural substrate. In the case of the L-allylglycine and L-vinylglycine analogues as replacement for L-cysteine, the yields of tripeptide were much lower (< 5 %). Absolute yields varied considerably, owing to differences in the peptide synthetase activity of the enzyme preparation used for the incubations. Although *S*-carboxymethylcysteine substituted for α-aminoadipic acid, and vinyl- and allyl-glycine for cysteine in the screening assays, and we were able to recover enough product from the incubations to determine the structure of the tripeptides, we recovered only authentic ACV from large-scale incubations when these analogues were included at the same concentration as the natural substrates. This is consistent with the higher apparent K_m values (approx. 3-, 12- and 30-fold for allylglycine, vinylglycine and *S*-carboxymethylcysteine respectively) for the analogues compared with the natural substrates.

The activity of various potential substrate analogues of ACV synthetase has been reported previously using an h.p.l.c. separation system with pre-column derivatization of the product peptide [4,23–25] or a bioassay using isopenicillin N- synthase to cyclise the putative products to antibiotically active compounds [4]. Preliminary substrate specificities were also communicated [7] from this laboratory using the β-lactamase induction assay coupled with conversion of the putative product tripeptide into a β-lactam product by isopenicillin N-synthase. We have demonstrated unequivocally by a combination of n.m.r. and e.s.m.s. that, for several analogues, the apparent activities in two independent assays was indeed due to the synthesis of ACV analogues and that each of the amino acids in ACV could be replaced with a structural analogue.

The relatively broad substrate specificity of ACV synthetase illustrated by this work is a property shared with other enzymes of the β-lactam synthetic pathway. Both isopenicillin N-synthase and deacetoxycephalosporin C synthase are 'promiscuous' enzymes [10,11]. Additionally, the lax specificity of ACV synthetase might explain the recovery of various unusual tripeptides from fermentations or cell-free extracts of several organisms [26]. In fact, the expected product of cyclization of L-δ-(*S*-carboxymethylcysteinyl)-L-cysteinyl-D-valine by isopenicillin N-synthase was identified as an antibiotically active compound isolated from *C. acremonium* fermentations done in the presence of *S*-carboxymethylcysteine [27]. The present study would indicate that this compound arose from the incorporation of *S*-carboxymethylcysteine into the precursor tripeptide rather than by transfer catalysed by an acyltransferase after formation of the β-lactam ring.

The results presented here are consistent with the view (see [28] for a review) that the peptide synthetases in general exhibit relatively lax substrate specificities. This is in contrast with the high fidelity of ribosomal peptide synthesis. Presumably, the low specificity of enzymic synthesis of peptides reflects a lack of the 'proof-reading' mechanisms which characterise the ribosomal route of peptide bond formation. Possibly the lax specificity of the peptide synthetases, like the other enzymes of secondary metabolism, constitutes an evolutionary asset in terms of the generation of novel structures which might confer a competitive advantage.

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