δ-L-(α-Aminoadipoyl)-L-cysteinyl-D-valine synthetase: isolation of L-cysteinyl-D-valine, a 'shunt' product, and implications for the order of peptide bond formation

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Abstract **L-Cysteinyl-D-valine was isolated from incubations of L-glutamate, L-cysteine and L-valine with δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase and identified by ^{1}H NMR and electrospray ionization MS. This is entirely consistent with our prior proposal (Shiau, C.-Y., Baldwin, J.E., Byford, M.F., Sobey, W.J. and Schofield, C.J. (1995) FEBS Lett. 358, 97–100) that the α-peptide bond between cysteine and valine is formed before the δ-peptide bond between α-aminoadipate and cysteine. The inclusion of L-glutamate, an analogue of L-α-aminoadipate, did not result in a detectable amount of tripeptide product, but did increase apparent yields of L-cysteinyl-D-valine. Conceivably, formation of the L-glutamyladenylate stimulates synthesis of the cysteinyl-valine dipeptide indirectly via a conformational change in the enzyme.**

Key words: Antibiotic; Biosynthesis; β-Lactam; Peptide synthetase; *Cephalosporium acremonium*

1. Introduction

A single multifunctional enzyme, ACV synthetase, catalyses the biosynthesis of δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV), the common precursor of the penicillin and cephalosporin antibiotics. It is evident on the basis of several properties of the enzyme and sequence identity with other peptide synthetases that it is a representative of this class of enzyme [1–4]. A general 'thiol template' mechanism has been proposed for the peptide synthetases [5] in which the carboxyl groups of the substrate amino acids are activated by formation of their respective aminoacyladenylates [1,6] and the aminoacyl groups are transferred to an enzyme-bound thiol. The thiol template mechanism also invokes the translocation of the enzyme-bound amino acid thioesters and the dipeptide intermediate by an indeterminate number of 4′-phosphopantetheine cofactors [5]. The proposed thiol template mechanism for the synthesis of ACV postulates the formation of the peptide bond between the δ-carboxyl of L-α-aminoadipate and the amino group of L-cysteine prior to that formed between L-cysteine and valine. Since ACV synthetase is relatively small (420 kDa, [4]) compared with most other members of the peptide synthetase family and catalyses comparatively few reactions, it is a useful model system for non-ribosomal peptide synthesis.

Recently, we reported the isolation of two diastereoisomeric dipeptides, L-(*O*-methylserinyl)-L-valine and L-(*O*-methylserinyl)-D-valine from incubations of ACV synthetase with L-α-aminoadipate and the cysteine analogue L-*O*-methylserine and L-valine [7]. In this communication we report the isolation of L-cysteinyl-D-valine itself from incubations of ACV synthetase with L-cysteine and L-valine. We propose this dipeptide is a 'shunt' product arising from the incomplete biosynthesis of ACV in the absence of α-aminoadipate. Detection of this dipeptide synthesized from natural substrates provides further evidence for a revised mechanism for ACV biosynthesis, in which the cysteinyl-valine amide bond is formed prior to the aminoadipoyl-cysteinyl amide bond, which had previously been suggested by results obtained using the cysteine analogue, *O*-methylserine.

2. Experimental

2.1. General

Enzyme preparation, ACV synthetase assays, ^{1}H NMR (500 MHz) and electrospray ionization MS analyses were done as described in [6]. Yields of isolated cysteinyl-valine were variable and dependent upon the enzyme preparation used. L-Cysteinyl-L-valine, L-cysteinyl-D-valine, L-δ-(α-aminoadipoyl)-L-cysteine and L-(γ-glutamyl)-L-cysteinyl-D-valine were synthesized by standard solution-phase methods and their structures confirmed by spectroscopic analyses and electrospray ionisation MS [8].

2.2. Enzymic preparation of L,D-cysteinyl-valine dipeptide

Incubations (final volume: 1 ml) typically contained $MgCl_2$ (42 mM), L-cysteine (5 mM), L-valine (5 mM), L-glutamate (5 mM), dithiothreitol (17 mM) and 100–150 pkat ACV synthetase in 50 mM Tris-HCl buffer pH 7.5. and were carried out for 16 h at 27°C. An equal volume of acetone was added and the precipitated protein was removed by centrifugation. The acetone was removed in vacuo and the residue dissolved in 0.8 ml of water.

2.3. Isolation of L-cysteinyl-D-valine dipeptide and attempted isolation of L-δ-(α-aminoadipoyl)-L-cysteine dipeptide

These protocols were developed using authentic synthetic standards of L-cysteinyl-D-valine and L-δ-(α-aminoadipoyl)-L-cysteine [8]. The enzymic product L-cysteinyl-D-valine was isolated by reverse-phase h.p.l.c. (Hypersil C_{18} 4.6 × 250 mm) with 20 mM NH_4HCO_3 in 20% (v/v) methanol at 1 ml/min. Retention volume: 5.6 ml. Prior to analysis, it was converted to the disulphide form, L-cystinyl-(D-valine)$_2$, by bubbling O_2 through the fraction collected from the HPLC after adjustment of the pH to approximately 10. We were unable to detect formation of L-δ-(α-aminoadipoyl)-L-cysteine in analogous incubations using L-cysteine and L-α-aminoadipate. We therefore experimentally established a detection limit for this dipeptide. Acetone supernatants (200 μl) from enzyme incubations obtained as detailed above were doped with authentic L-δ-(α-aminoadipoyl)-L-cysteine at 0.016 μg/μl. The acetone was removed in vacuo and the supernatant freeze-dried. The residue was dissolved in water and then loaded on to a reverse phase HPLC column (Hypersil C_{18} 4.6 × 250 mm). The mobile phase (A) was triethylamine hydrochloride (4.3 g/l) in 20 mM $NH_4H_2PO_4$, pH 6.5. The modifier (B) was obtained by adding 10% (v/v) methanol to the mobile phase. The flow rate was 1 ml/min. Isocratic elution was employed for 3 min with solution A, and then 16 min with solution B. Retention volume of

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L-δ-(α-aminoadipoyl)-L-cysteine: 6.5 ml. Contaminating salts in the sample from the first HPLC purification were removed by a second HPLC step (Hypersil C_{18} 4.6 × 250 mm). The flow rate was 1 ml/min and the mobile phase was 25 mM NH_4HCO_3. A peak could readily be detected in the HPLC chromatogram at the expected retention volume for δ-(α-aminoadipoyl)-L-cysteine, indicating that a total yield 4 μg (<0.5% yield) of dipeptide could be detected by HPLC. We were unable to detect any such material in incubations of ACV synthetase with α-aminoadipate and L-cysteine.

2.4. Quantification of L-cysteinyl-D-valine dipeptide formation

Incubations contained $MgCl_2$ (35 mM), dithiothreitol (1 mM) cysteine (2 mM), valine (2 mM), 9% (v/v) glycerol, 33 nkat inorganic pyrophosphatase, 0.5 μCi [3,4 ^{3}H] valine (2.3 TBq/mmol, Amersham), a range of concentrations of glutamate and 20–30 pkat ACV synthetase in a final volume of 300 μl. Reactions were initiated by addition of ATP to a final concentration of 7 mM and proceeded for 2 h at 27°C. Control incubations contained active enzyme but either no ATP or no cysteine. The reactions were terminated by the addition of acetone to 40% by volume and the protein removed by centrifugation in a microfuge. Sufficient 10 M NaOH was added to the recovered supernatants to raise the pH to approximately 10. O_2 was bubbled through the supernatants for 10 min. The acetone was removed in vacuo and sufficient 10% (w/v) aq. trichloroacetic acid, containing 400 μM cystine as carrier, was added to bring the volume of the residue back to that of the original incubation. Portions (250 μl) of this were chromatographed on Porapak Q columns as previously described [1,6,9] The methanol eluate (10%) was taken for liquid scintillation counting. Background counts for the incubations done either in the absence of ATP or the absence of cysteine were very similar (< ± 2.0%).

3. Results

The recent isolation of both L-*O*-methylserinyl-D-valine and L-*O*-methylserinyl-L-valine from incubations of ACV synthetase with L-α-aminoadipate, L-*O*-methylserine and L-valine prompted us to attempt to isolate cysteinyl-valine itself from incubations with the natural substrates L-cysteine and L-valine. Initially, incubations were carried out under the usual conditions used for the enzymic production of ACV [6]. Authentic ACV and cysteinyl-valine were difficult to separate under the conditions used so biosynthetic samples of ACV were collected and analysed by electrospray ionization m.s. No cysteinyl-valine was detected in the ACV fraction within the limits of the instrument. However, it is not possible to rule out entirely the production of cysteinyl valine (<10% relative to ACV) under these conditions. In contrast, when L-α-aminoadipate was omitted from incubations, HPLC analysis revealed a peak at a similar retention volume to authentic cysteinyl-valine. ^{1}H NMR analysis indicated the presence of an isopropyl group in the putative product, but insufficient material was isolated from the incubation for unambiguous identification of the peptide.

L-Glutamate is a substrate for the ATP-PP_i exchange reaction catalysed by *C. acremonium* ACV synthetase [6], presumably because it is an analogue of α-aminoadipate. We speculated that inclusion of this analogue might enhance production of cysteinyl-valine by the enzyme. We could accrue no evidence for the production of γ-glutamyl-cysteinyl-valine during our original attempts to synthesize novel tripeptides using ACV synthetase, indicating that this analogue is discriminated against in a step subsequent to activation as its aminoacyladenylate [6]. Inclusion of 5 mM L-glutamate in the incubation mixture enhanced the production of the putative dipeptide product sufficiently such that a final yield of 50 (±5) μg (5.5%) of L-cysteinyl-D-valine (analysed as L-cystinyl-(D-valine)$_2$) could be isolated from the incubation. The yield was determined by

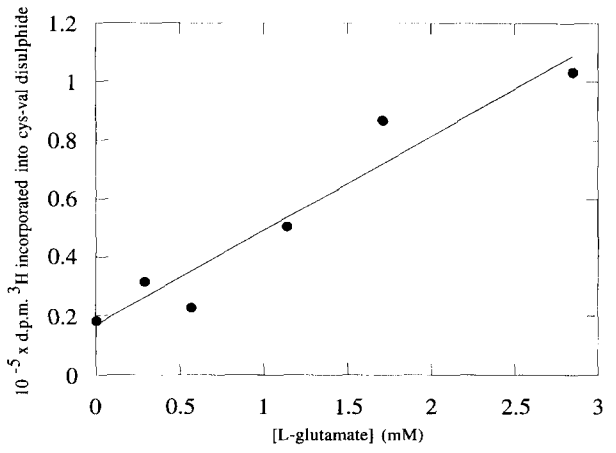


Fig. 1. Dependence of L-cysteinyl-D-valine dipeptide formation on L-glutamate concentration. The cysteinyl-[^{3}H]valine dipeptide was converted to its disulphide form and isolated by Porapak Q chromatography as described in section 2. It was quantified by liquid scintillation counting. The values shown are the means of triplicate determinations.

integration of the product resonances versus those of an external standard [(2,2′,3,3′)-[$^2H_4$]-(trimethylsilyl)p ropanoic acid] in the ^{1}H NMR (500 MHz) spectrum. The identity of the dipeptide as the disulphide form of cysteinyl valine was further substantiated by electrospray ionization MS: m/z (MH^+), % relative abundancies in parentheses, 438 (0) 439 (MH^+, 100) 440 (25) 441 (10) 442 (0). The stereochemistry of the recovered dipeptide was assigned by doping with an authentic standard. We were unable to detect any biosynthesis of γ-glutamyl-cysteinyl-valine during these experiments despite the availability of an authentic standard.

Incubations were also carried out using authentic L,D- and L,L-cysteinyl-valine (5 mM) and α-aminoadipate (5 mM) with ACV synthetase. Since cysteinyl-valine and δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine were poorly separated by the HPLC system used, the recovered cysteinyl-valine starting material, potentially containing ACV was analysed by both ^{1}H NMR and electrospray ionization MS. No evidence for the production of ACV was accrued, indicating that neither L,D nor L,L cysteinyl-valine are substrates for peptide synthesis. However production of a small amount of δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine (<5% that of recovered cysteinyl-valine) during these experiments cannot be ruled out. Neither L,D nor L,L cysteinyl-valine were able to stimulate ATP-PP_i exchange, indicating no formation of dipeptide aminocyladenylates.

The effect of L-glutamate on the production of L-cysteinyl-D-valine by ACV synthetase was quantified by modifying the assay for tripeptide formation [1,6,9] to detect L-cysteinyl-D-valine. We found by using ^{1}H NMR analysis that L-cysteinyl-D-valine did not substantially bind to the Porapak Q resin in its thiol form. However, conversion of authentic L-cysteinyl-D-valine to its disulphide form, as expected, resulted in a sufficient increase in hydrophobicity to cause retention of the dipeptide on the Porapak Q column. We then measured the incorporation of ^{3}H from [3,4 ^{3}H] valine into L-cystinyl-(D-valine)$_2$ at various concentrations of L-glutamate and saturating concentrations of the other two amino acids. That there is a significant effect of L-glutamate concentration on the production of cysteinyl-valine is evident (Fig. 1).

In contrast, when L-δ-α-aminoadipic acid and L-cysteine were incubated with ACV synthetase, we were unable to detect any L-δ-(α-aminoadipoyl)-L-cysteine by on the HPLC, or by ^{1}H NMR analysis of the portion of the HPLC effluent expected to contain the dipeptide. Control experiments (see section 2), involving doping of acetone supernatants with authentic L-δ-(α-aminoadipoyl)-L-cysteine indicated that a yield of <4 μg (< 0.5%) of dipeptide in the incubation would have been detected in the expected portion of the HPLC chromatogram on the scale on which the incubations were carried out. The possibility that inclusion of L-glutamate might also stimulate the production of ACV and free L-δ-(α-aminoadipoyl)-L-cysteine was investigated. Inclusion of L-glutamate in the routine assay for ACV synthesis led to significant inhibition of ACV formation by the synthetase (Fig 2). Aspartate, which is not a significant substrate for ATP-PP_i exchange [6], failed to inhibit ACV formation at concentrations of up to 4 mM at which L glutamate inhibited ACV formation by approximately 85%. These observations show that glutamate, but not aspartate, inhibits ACV formation, presumably by competing for the activation site for α-aminoadipate. Since it is a reasonable assumption that any putative production of L-δ-(α-aminoadipoyl)-L-cysteine must be by a related mechanism as the L-δ-(α-aminoadipoyl)-L-cysteine moiety found in the complete product ACV, glutamate cannot enhance production of a hypothetical L-δ-(α-aminoadipoyl)-L-cysteine intermediate, in contrast to its ability to stimulate significantly L-cysteinyl-D-valine production.

4. Discussion

Recently, we proposed a revised mechanism for the biosynthesis of ACV as a result of our detection of the diastereoisomers L-(*O*-methylserinyl)-L-valine *and* L-(*O*-methylserinyl)-D-valine as novel *di*peptide products of ACV synthetase [7] (Scheme). We also discussed the implications of this observation for the timing of peptide bond formation and the epimerisation of the valine residue during the catalytic cycle. The results substantiate the proposal that epimerisation in the 'peptide-bound' state might be a common mechanism for this reaction catalyzed by some of the peptide synthetases e.g. actinomycin synthetase I, in which the D-enantiomer in the final product is not the

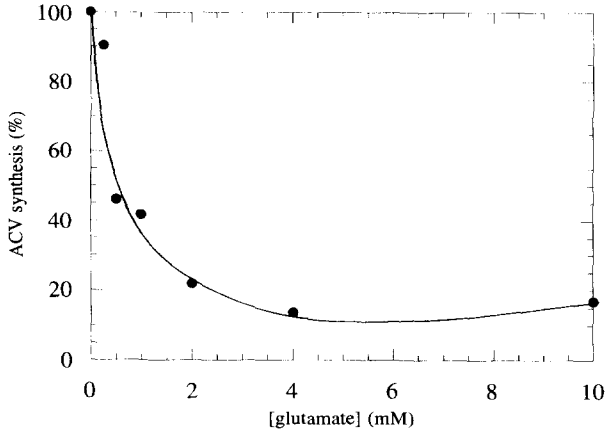
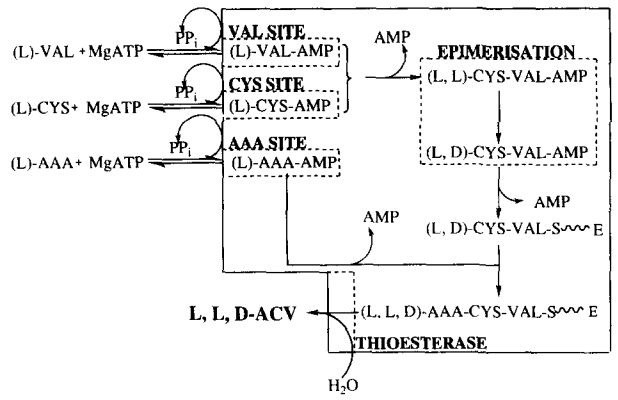


Fig. 2. Inhibition of ACV synthesis by L-glutamate. Various concentrations of L-glutamate were included in the assay for ACV synthesis [13]. Determinations of incorporation of radiolabel were done in triplicate.



Scheme 1. Proposed mechanism for ACV synthetase. AAA: α-aminoadipate; (Xaa-AMP): aminoacyladenylate of amino acid Xaa. E ~: covalently bound pantetheinate cofactor. Independent sites for the aminoacyladenylation, epimerisation, and thioesterase partial reactions are shown boxed (dashed lines). Note thioester formation is shown as occurring at the dipeptide stage, but in fact may occur at amino acid or even tripeptide stages.

N-terminal residue [10]. This is in contrast to e.g. tyrocidin synthesis [11], where the freely reversible conversion of L- to D-phenylalanine by a phenylalanine racemase occurs prior to synthesis of a peptide in which the D-phenylalanine is the N-terminal residue. A caveat of these results was that the use of a cysteine analogue might have grossly perturbed the normal enzyme reactions, and that detection of the production cysteinyl-valine dipeptide analogues was a result of aberrant enzyme function.

In this paper we have clearly demonstrated that small but detectable amounts of L,D-cysteinyl-valine (but not L,L-cysteinyl-valine) are produced by ACV synthetase in the absence of L-α-aminoadipate. Furthermore, this dipeptide was detected in solution as a 'shunt product', in the same manner as the *O*-methylserinylvaline products we identified previously and not as an enzyme-bound intermediate. It is noteworthy that inversion of the stereochemical configuration of the valinyl α-centre is apparently complete in the case of the dipeptide produced from the natural substrates. This would indicate that the enzyme bound L-*O*-methylserinyl-L-valine is a less effective substrate for the epimerisation reaction than L-cysteinyl-L-valine. We also observed that production of L-cysteinyl-D-valine was significantly enhanced in the presence of L-glutamate; in fact unequivocal identification of this dipeptide was not possible with the amounts produced in the absence of L-glutamate. In contrast L-glutamate, but not L-aspartate, significantly inhibited the production of the tripeptide product ACV, indicating competition for the L-α-aminoadipate activation site by L-glutamate. This observation indicates that L-glutamate would be unable to stimulate the production of a putative L-δ-(α-aminoadipoyl)-L-cysteine intermediate since this would be formed by the same mechanism as the L-δ-(α-aminoadipoyl)-L-cysteine portion of the complete tripeptide. A stimulatory effect of glutamate on the production of penicillins ascribed to activation of the then postulated L-α-aminoadipoyl-L-cysteinyl synthetase has been previously reported [12]. More intriguingly, the observed stimulation of formation of the L-cysteinyl-D-valine peptide bond by the α-aminoadipate analogue glutamate might indicate a regulatory effect on the rate of production and/or

release of the dipeptide intermediate by the α-aminoadipate activation site. Potentially, aminoacyladenylate formation at the α-aminoadipate site might be required for efficient formation of the L-cysteinyl-D-valine peptide bond. Only relatively small amounts of dipeptide product (Fig. 1) were produced in the absence of exogenous L-glutamate. This basal activity might simply reflect adventitious weak activation at the α-aminoadipate site by cysteine, valine, or trace amounts of amino acids resulting from degradation of ACV synthetase under the incubation conditions. Alternatively, there may be residual tightly-bound α-aminoadipoyladenylate at this site. In contrast to cysteine and valine, α-aminoadipate is not a proteinogenic amino acid. Regulation of the production of the L,D cysteinyl-valine intermediate by L-α-aminoadipate might have physiological relevance, ensuring that there is minimal futile synthesis and release of L,D cysteinyl-valine in the absence of L-α-aminoadipate. To our knowledge, there are no reports of the detection of L,D cysteinyl-valine in vivo as a natural product.

In summary, although the kinetic viability of an enzyme-bound cysteinyl-valine intermediate has not yet been proved for the biosynthesis of ACV, the observation that ACV synthetase is capable of catalysing both the formation of the cysteinyl-valine peptide link and the C-terminal epimerisation of the valinyl residue (in the 'peptide-bound' state) strongly supports the proposal that the cysteinyl-valine bond is formed both before epimerisation at the valinyl position and before the amide bond with L-α-aminoadipate (Scheme 1).

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