

δ -L-(α -Aminoadipoyl)-L-cysteinyl-D-valine synthetase: the order of peptide bond formation and timing of the epimerisation reaction

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Abstract δ -L-(α -Aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase catalyses the formation of the common precursor tripeptide of both the penicillin and cephalosporin antibiotics from the L-enantiomers of its constituent amino acids. Replacement of cysteine with L-O-methylserine in preparative-scale incubations led to the isolation of both L-O-methylserinyl-L-valine and L-O-methylserinyl-D-valine dipeptides. The dipeptides were characterized with the aid of authentic synthetic standards by both ^1H NMR and electrospray ionization MS. A revised mechanism for ACV biosynthesis involving formation of the cysteinyl-valine peptide bond before the epimerisation of valine and subsequent condensation with the δ -carboxyl of L- α -aminoadipate is therefore proposed.

Key words: Biosynthesis; β -Lactam; Peptide; Synthetase; Epimerisation; *Cephalosporium acremonium*

1. Introduction

The biosynthesis of δ -L-(α -aminoadipoyl)-L-cysteinyl-D-valine (ACV), the common precursor of the penicillin and cephalosporin antibiotics, is catalysed by a single multifunctional enzyme, ACV synthetase, which has been isolated from several sources [1–4]. On the basis of several properties of the enzyme and its sequence identity to previously described peptide synthetases it is evident that it is a member of this class of large multifunctional enzymes [5]. A general 'thiol template' mechanism has been proposed for the peptide synthetases [6]. In this proposal the carboxyl groups of the substrate amino acids are activated by formation of their respective aminoacyladenylates [2] and the aminoacyl groups are then proposed to be transferred to an enzyme-bound thiol. Indeed ACV synthetase from *Cephalosporium acremonium* has been shown to catalyse substrate amino acid-dependent ATP-PP_i exchange reactions in response to all three natural substrates and some substrate analogues [5,7]. In the case of L- α -aminoadipate activation the activity was rather labile and could not be detected at all for the enzyme isolated from *Streptomyces clavuligerus* ([5]; Byford, M.F. and Shiau, C.-Y., unpublished observations). The thiol template mechanism also postulates the translocation of the enzyme-bound amino acid thioesters and the dipeptide intermediate by (a) 4'-phosphopantetheine cofactor(s) [6] and ACV synthetase contains 4'-phosphopantetheine [8]. For ACV

synthesis, the thiol template mechanism invokes 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. Epimerisation of the valine residue is postulated to occur at the valinyl thioester stage as this is a rationale for the observed activation of the valine carboxyl which is not involved in peptide bond formation [2]. Evidence for aminoacyl groups covalently bound to the enzyme via thioesters was qualitatively assessed by release of labelled valine from the enzyme after treatment with performic but not formic acid [2]. A summary scheme for the thiol template mechanism for ACV synthesis is shown in Scheme 1. Compared with most of the members of the peptide synthetase family, ACV synthetase is relatively small (420 kDa, based on translation of the nucleotide sequence [9]) and catalyses comparatively few reactions: minimally the formation of two peptide bonds and the epimerisation of the valine residue. Thus, ACV synthetase may serve as a useful model system for non-ribosomal peptide synthesis.

Recently, we reported results on the substrate specificity of ACV synthetase from *C. acremonium* [7]. Potential substrates were initially screened using both the ATP-PP_i exchange assay and the incorporation of [^{14}C]cysteine and [^{14}C]valine into potential tripeptide products. Based on these results, we attempted the preparative-scale conversion of several analogues of L- α -aminoadipate, L-cysteine, and L-valine into tripeptides. 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 the isolation and characterization of L-(cysteine-S-acetyl)-L-cysteinyl-D-valine, L- δ -(aminoadipoyl)-L-vinylglycinyl-D-valine, L- δ -(aminoadipoyl)-L-allylglycinyl-D-valine and L- δ -(aminoadipoyl)-L-cysteinyl-D-isoleucine, respectively. In several other cases in which the ^{14}C incorporation assay had indicated the formation of potential peptide product and for which there was detectable substrate analogue-dependent ATP-PP_i exchange, we were unable to detect any of the anticipated L,L,D tripeptide product when the HPLC effluent was screened by ^1H NMR (500 MHz). In particular, when O-methyl serine was used as a substitute for cysteine, assays for peptide formation using [^{14}C]valine incorporation showed an apparent rate that was 75% that observed for cysteine itself. Despite the availability of authentic δ -L-(α -aminoadipoyl)-L-O-methylserinyl-D-valine we were unable to detect the biosynthesis of this tripeptide. Although, as discussed in detail in [7], other explanations for these apparently conflicting results are possible, we postulate that the O-methylserinyl-[^{14}C]valine dipeptide(s) might be produced in the incubations.

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2. Experimental

2.1. General

C. acremonium ACV synthetase was prepared as described previously [10], routinely through the Superdex S-200 stage. ATP-PP_i exchange assays, ¹H NMR (500 MHz) and electrospray ionization MS analyses were done as in [7]. δ-L-(α-Aminoadipoyl)-L-O-methylserinyl-D-valine, L-(O-methylserinyl)-L-valine and L-(O-methylserinyl)-D-valine were synthesized by standard solution-phase methods and their structures confirmed by ¹H NMR (500 MHz) and electrospray ionization MS (Table 1).

2.2. Enzymic preparation of O-methylserine-valine dipeptides

Incubations including the cysteine analogue O-methylserine (5 mM) in the presence of L-α-aminoadipic acid (5 mM) and L-valine (5 mM) were carried out as in [7] for the preparative scale enzymic synthesis of tripeptides, except that the incubations were terminated by addition of acetone (40% v/v, final concentration). After removal of the protein by centrifugation (13,000 rpm, 5 min) the acetone was removed in vacuo and the supernatant dried in vacuo. The residue was dissolved in water (approx. 1 ml). L-[2-³H]Valine or [1-¹³C]valine replaced L-valine when required.

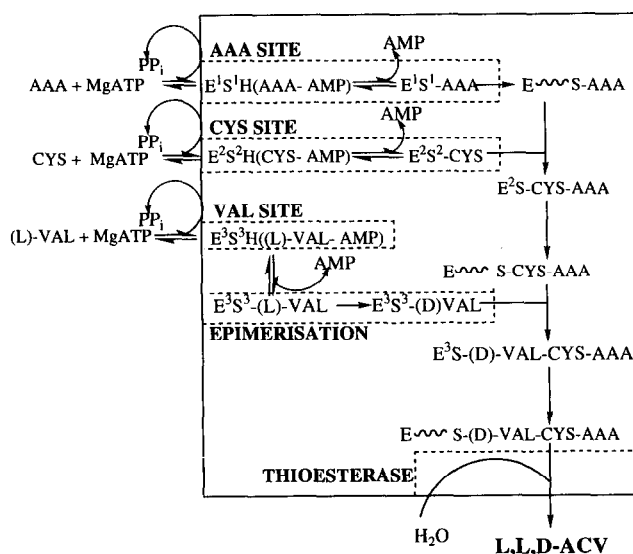
For incubations containing L,L-O-methylserinyl-valine the reaction mixture contained MgCl₂ (65.6 mM), L-α-aminoadipic acid (5.0 mM), L,L-O-methylserinyl-valine (trifluoroacetate salt) 1.1 mM, valine (5.0 mM), dithiothreitol (3.0 mM), ATP (26.6 mM), enzyme preparation (400 μl, total synthetase activity up to 100–150 pkat) in Tris-HCl buffer (50 mM, pH 7.5) in a final volume of 800 μl and was carried out at 27°C for 16 h. The isolation of both diastereoisomers followed the protocol described below.

2.3. Isolation of O-methylserinyl-valine dipeptides

The protocols were developed using authentic synthetic standards. Appropriate aliquots of the aqueous fraction obtained as detailed above were injected onto a reverse-phase ODS column (Hypersil C₁₈ 4.6 × 250 mm) and eluted with 21 mM NH₄HCO₃ in 15% (v/v) aq. methanol. The flow rate used was 1 ml/min. The fraction containing the desired dipeptide (retention vol. 5.9 ml) was freeze-dried. The residue was dissolved in water (1 ml) and acidified to pH 2–3 with a minimum amount of approx. 0.1 M aqueous HCl. To remove residual glycerol, the acidic solution was loaded to a pre-washed Dowex 50W-X8 column (1 ml). The loaded column was washed with water (20 ml) and then eluted with 2 M NH₄OH (25 ml). The NH₄OH fraction was freeze-dried and the residue was dissolved in water (0.3 ml). This sample was re-chromatographed on reverse phase HPLC with an ODS column (Hypersil C₁₈, 4.6 × 250 mm), using a linear gradient. The procedure was developed using authentic synthetic standards. The mobile phase consisted of two eluants: solvent (A) 25 mM aq. NH₄HCO₃ and solvent (B) was obtained by the addition of 15% (v/v) methanol to solvent (A). The linear gradient used was: 0% B at zero time to 60% (v/v) B at 20 min returning to 0% B at 23 min. The flow rate was 1 ml/min. Retention vol. of L,L-O-methylserinyl-valine 18.7 ml; retention vol. of L,D-O-methylserinyl-valine 19.5 ml.

3. Results

Protocols for the isolation and separation of the diastereoisomers L-O-methylserinyl-L-valine and L-O-methylserinyl-D-valine were developed with the aid of authentic synthetic standards. Fig. 1a shows clearly that instead of the anticipated tripeptide, both the L,L dipeptide (L-O-methylserinyl-L-valine) and the L,D-dipeptide (L-O-methylserinyl-D-valine) were produced by ACV synthetase in incubations containing L-α-aminoadipate, L-O-methylserine and L-valine. Although yields of the dipeptides were low (<0.25%), sufficient material was isolated from a 16 h incubation to determine the anticipated structures by both ¹H NMR (500 MHz) and electrospray ionization MS (Table 1). The low final yield of the separated diastereoisomers reflects a strategy pooling the HPLC effluent for maximum purity of each isomer rather than for maximum yield (Fig. 1b).



Scheme 1. The 'thiol-template' mechanism for ACV synthetase. AAA, α-aminoadipate; (Xaa-AMP), aminoacyladenylate of amino acid Xaa; E~S, covalently bound pantetheinate cofactor. Independent sites for the aminoacyladenylation, epimerisation and thioesterase partial reactions are shown boxed (dashed lines).

The identities of the two products were confirmed unambiguously by doping the biosynthetic products with the authentic standards followed by ¹H NMR (500 MHz) analysis. Any possibility of contamination of the biosynthesized samples by trace amounts of the authentic compounds in the HPLC system was eliminated by biosynthesis of the dipeptides from [1-¹³C]valine and confirmation of its incorporation by electrospray MS (Table 1).

The ratios of the formation of the L,L- and L,D-dipeptides were somewhat variable depending on the enzyme preparation used but were typically approximately 1:1. Biosynthesis of the dipeptides from [2-³H]valine resulted in the formation of deuterated L,L dipeptide which could be characterized. Insufficient L,D dipeptide was obtained for characterization, as in this case the ratio of the L,L to L,D diastereoisomer was estimated from the HPLC analysis as >10:1 (Fig. 1c). Since the diastereoisomers were formed in approximately equal yield by the same enzyme preparation with unlabelled valine as a substrate, a primary deuterium kinetic isotope effect would appear to be operating in the epimerisation process. Yields of both diastereoisomers were decreased significantly when L-α-aminoadipate was omitted from the incubation. No conversion of authentic L,L-dipeptide to the L,D form was seen when it was incubated with ACV synthetase, indicating that the L,L form is not a free intermediate in the synthesis of the L,D dipeptide. Neither the L,L nor L,D O-methylserinyl-valine dipeptide stimulated the ATP-PP_i exchange reaction (not shown), indicating that neither dipeptide was able to be reactivated as a weak analogue of valine and form a dipeptide aminoacyladenylate.

4. Discussion

We have demonstrated previously [7] that the substrate specificity of ACV synthetase is comparatively broad, since the enzyme can synthesize tripeptides where each of the natural

Table 1
Electrospray ionization MS analysis of authentic and biosynthesized *O*-methylserinyl-valine dipeptides

Entry	Dipeptide	% observed at m/z (MH^+)					
		218	219	220	221	222	223
^a	Synthetic (L,L)- <i>O</i> -methylserinyl-valine	0	100	13	0	0	0
^a	Synthetic (L,D)- <i>O</i> -methylserinyl-valine	0	100	11	2	4	0
^b	Biosynthetic (L,L) + (L,D) <i>O</i> -methylserinyl-valine	0	100	12	2	0	0
^c	Biosynthetic (L,L) + (L,D) <i>O</i> -methylserinyl-[1- ¹³ C]valine	0	4	100	14	3	1
^d	Biosynthetic (L,L) <i>O</i> -methylserinyl-[2- ² H]valine	1	4	100	17	3	4

^a Synthetic standards.

^b Biosynthesized dipeptides (analysed as a mixture of diastereoisomers) from the incubation of L-valine and L-*O*-methylserine.

^c Biosynthesized dipeptides (analysed as a mixture of diastereoisomers) from the incubation of L-[1-¹³C]valine and L-*O*-methylserine.

^d Biosynthesized L,L-dipeptide from the incubation of L-[2-²H]valine and L-*O*-methylserine.

substrates is replaced by a structural analogue. However, during the initial screening process for potential substrate analogues we noticed that some analogues significantly stimulated ATP-PP_i exchange but we were unable to isolate a tripeptide product. We reasoned that this could be due to the analogue being discriminated against in a subsequent step in the catalytic sequence. Similarly, we reasoned that the apparently significant incorporation of [¹⁴C]valine into putative peptide products in incubations done in the presence of substrate analogues (e.g. *O*-methylserine), which also stimulated ATP-PP_i exchange, might be due to the formation of dipeptide product(s), which could reflect an inability of the enzyme to complete tripeptide formation from an analogue of the natural dipeptide intermediate.

The results presented in this paper demonstrate clearly that, at least in the case of L-*O*-methylserine, a dipeptide is indeed produced in detectable amounts by ACV synthetase even in the presence of L- α -aminoadipate. Furthermore, both the L,L and L,D diastereoisomers were produced. This presumably reflects incomplete epimerisation of the valine residue at the dipeptide stage, particularly since recovery of the epimerised product decreased significantly when [2-²H]valine was used as the substrate. Thus, the epimerisation of the valine residue occurs after the formation of the first peptide bond (although with substrates forming tripeptides it may occur at the tripeptide stage). Surprisingly, the recovered dipeptide is an analogue of cysteinyl-valine and not an analogue of α -aminoadipoyl-cysteine as would be expected from the conventional thiol template mechanism. All attempts to isolate detectable amounts of L- α -aminoadipoyl-L-cysteine from preparative incubations have thus far been unsuccessful.

These two key observations are inconsistent with previous mechanistic proposals for ACV synthetase. Previously, it has been suggested that the epimerisation occurs on a postulated enzyme-bound valinyl thioester intermediate in accord with studies on e.g. gramicidin and tyrocidin biosynthesis [11] where the inversion of configuration is completely reversible and occurs prior to the insertion of the D-residue into the peptide chain. Work on actinomycin D, which contains an internal D-valine residue, has shown that the enzyme actinomycin synthetase II assembles the first three residues of the peptide lactone chain of 4-methyl-3-hydroxyanthraniloyl-L-threonyl-D-valinyl-L-prolyl-L-(*N*-methyl)glycyl-L-(*N*-methyl)valine. Very recently, Stindl and Keller [12] have shown that both 4-methyl-3-hydroxyanthraniloyl-L-threonyl-L-valine and 4-methyl-3-hydroxyanthraniloyl-L-threonyl-D-valine are enzyme-bound in-

termediates, and they suggest that the former is an obligatory intermediate in the synthesis of the latter. These authors also proposed that epimerisation at the peptide stage may be a general mechanism for peptide synthetases which catalyse formation of peptides containing D-amino acids at positions other than at the N-terminus. The production of L,L- and L,D-dipeptides by ACV synthetase when incubated with *O*-methylserine provides independent support for this proposal, derived from another peptide synthetase and determined using different tech-

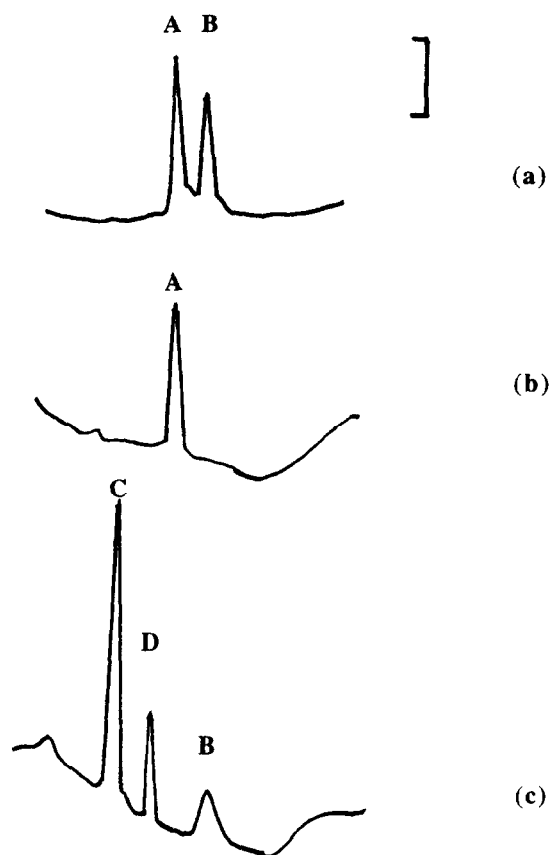
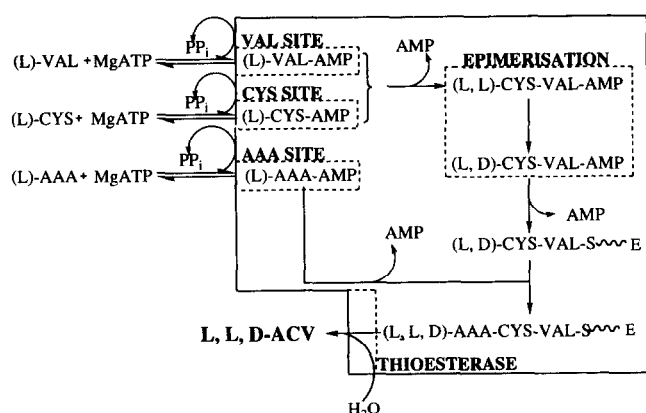


Fig. 1. Separation of L-*O*-methylserinyl-L-valine and L-*O*-methylserinyl-D-valine by reverse-phase HPLC. Chromatography was performed as described in section 2. The detector was set at 210 nm. The bar represents an absorbance of 0.01. (a) Separation of the biosynthesized diastereoisomers. A, L,D isomer; B, L,L isomer; (b) demonstration of homogeneity of isolated B (L,D product); (c) decreased yield of B (L,D product) when [2-²H]valine was used as a substrate (peaks C and D are 'impurities' sometimes derived from the Dowex column chromatography).



Scheme 2. A revised mechanism for ACV synthetase. Abbreviations as for Scheme 1.

niques. Furthermore, we have recovered the dipeptides from solution, whereas the dipeptides in actinomycin D biosynthesis were detected as enzyme-bound intermediates. This indicates that the enzyme can release the 'incorrect' dipeptide. The results described clearly imply that the biosynthesis of ACV itself proceeds via an enzyme-bound cysteinyl-valine intermediate. Previous results (based on very sensitive radiochemical and bio-assays) [8,13] reporting very low conversion of L- δ -(α -aminoadipoyl)-L-cysteine and L-valine with ACV synthetase may seem to argue against the intermediacy of cysteinyl-valine. However, in this case δ -L-(α -aminoadipoyl)-L-cysteine may act as a weak analogue of L-cysteine with epimerisation occurring at the tripeptide stage. Consistent with this hypothesis, we have observed that the authentic dipeptide L- δ -(α -aminoadipoyl)-L-cysteine stimulated ATP-PP_i exchange significantly (36% the rate seen in response to cysteine) whereas authentic L,L- and L,D-cysteinyl-valine were effectively unable to stimulate the exchange (<0.8% the rate seen with cysteine).

This is the first report of incubations *in vitro* with a peptide synthetase leading to the isolation and unambiguous characterization of an incomplete peptide product containing an L-amino acid residue at the same position as that occupied by a D-amino acid residue in the complete natural product. The isolation of both L-O-methylserinyl-L-valine and L-O-methylserinyl-D-valine indicates that the biosynthesis of ACV proceeds from the C- to the N-terminus with epimerisation occurring at either the di- or tripeptide stage. As a result of our observations we wish

to propose a simpler revised mechanism for ACV synthetase (Scheme 2). This incorporates (a) formation of the α -peptide bond between cysteine and valine prior to (b) epimerisation of the valine residue in the intermediate dipeptide, followed by (c) formation of the peptide bond to the δ -carboxyl of α -aminoadipate, and finally (d) release of the tripeptide from the enzyme-bound 4'-phosphopantetheine by a thioesterase activity. Our results indicate also that the individual partial reactions in the synthesis of ACV have subtly distinct substrate specificities. They may thus act as a series of 'gates' which prevent incorporation of some analogues into complete tripeptide products. That the ACV synthetase can release an 'incorrect' dipeptide intermediate into solution might indicate a form of editing by the peptide synthetase.

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