# The Mechanism of ACV Synthetase

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## Contents



## I. Introduction

Despite the development of many new antibiotics, the penicillins and cephalosporins still represent a considerable fraction of antibiotics in clinical use. The bicyclic nuclei of all penicillins and cephalosporins used currently are derived from natural fermentation products. Extensive studies have resulted in a clear picture of the enzyme catalyzed steps involved in their biosynthesis (Figure 1). An essential step in penicillin biosynthesis is the initial irreversible commitment of metabolic carbon to the secondary metabolic pathway. This is achieved by the biosynthesis of the tripeptide L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) from its constituent L-amino acids. This initial step has now been shown conclusively to be performed in a way that was unexpected a decade ago. Rather than using two separate enzymes, as was assumed initially by analogy to glutathione biosynthesis (Figure 2), the first intermediate in the pathway is synthesized by a single multifunctional enzyme: L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase (EC 6.*x*.*y*.*z*.). The enzyme is structurally and functionally related to other large multifunctional enzymes known to catalyze nonribosomal peptide bond formation (sections III and IV). The biosynthesis of the tripeptide precursor of the penicillin and cephalosporin antibiotics is the subject of this review, although other peptide synthetases will also be discussed to place the mechanism in the context of peptide synthetases as a class of enzymes.

Interest in the mechanism of ACV synthetase arises because, since it synthesizes only a tripeptide, it is probably the simplest known peptide synthetase with regard to the number of reactions catalyzed. It is noteworthy that the tripeptide product (Figure 2) contains a nonproteinogenic amino acid (α-aminoadipate), in which the δ-carboxyl participates in peptide bond formation, and a D-configured amino acid (D-valine). The incorporation of nonproteinogenic and modified residues typifies nonribosomally synthesized peptides. Protein engineering of the peptide synthetases might ultimately be used to generate new enzymes with altered substrate selectivities enabling the efficient synthesis of peptides of choice. However this application may require a more detailed picture of the complete sequence of partial reactions needed for the synthesis of the product peptides than is currently available.

## II. Early Observations on ACV Synthesis

ACV was established as the immediate precursor of isopenicillin N when Abraham and co-workers demonstrated that a crude *Cephalosporium acremonium* cell lysate converted labeled ACV into penicillin N and isopenicillin N.[1] Subsequently, the latter was identified as the major product, and it was established that the carbon skeleton of ACV remained intact during conversion to isopenicillin N.[2,3] That ACV was synthesized by a mechanism independent of ribosomes was demonstrated by enhancement of ACV synthesis in the presence of either anisomicin or cycloheximide, which selectively inhibit ribosomal peptide synthesis.[4]

Bauer reported the biosynthesis of a tripeptide using a cell-free extract of *Penicillium chrysogenum*, however, the stereochemistry of the residues was not determined.[5] Cell-free extracts from *C. acremonium* capable of biosynthesizing ACV were demonstrated by Abraham and Loder,[6,7] who also reported that the biosynthesis of ACV from L-δ-(α-aminoadipoyl)-L-cysteine (AC) and L-valine was MgATP-dependent. They were unable to demonstrate ACV formation from L-α-aminoadipate plus L-cysteinyl-L-valine, D-α-aminoadipate plus L-cysteinyl-L-valine or AC plus D-valine. A soluble activity was detected in cell-free

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Mike Byford was born and raised on the Sussex coast of southern England. He received both his B.Sc. (1980) and Ph.D. (1983, with Dr. David Bloxham) degrees from the University of Southampton. After postdoctoral work at the University of Washington in Seattle, he returned to Southampton to initiate a research project into the neural differentiation response of human neuroblastoma cells. He has been researching at the Oxford Centre for Molecular Sciences, University of Oxford, into ACV synthetase, among other things, for several years. His diverse interests include enzymology, protein structure and function, protein chemistry, and signal transduction mechanisms.

Jack Baldwin was an undergraduate at Imperial College and received a Ph.D. under the direction of Professor, Sir Derek Barton in 1964. He was assistant lecturer and lecturer at IC until 1967 when he moved to Pennsylvania State University. Subsequently he moved to MIT (1969) and in 1978 to Oxford as Waynflete Professor of Chemistry and Head of the Dyson Perrins Laboratory. His reesarch interests are broadly organic chemistry and its application to biology as a source of understanding and intervention. He has specifically contributed to areas of synthesis including, sigmatropic reactions, (alkoxyvinyl)lithium reagents, $\beta$-lactam chemistry, free-radical reagents in organic synthesis, and also the total synthesis of complex natural products, including the fungal isonitriles and the kainoids. His group synthesized the first reversible iron–porphyrn oxygen carriers as well as the then unknown "activated" forms of the carcinogenic nitrosoamines. Biosynthesis has been a major interest which has included the fungal isonitriles, the plant hormone ethylene and the sponge-derived alkaloids, for which the latter he proposed a biosynthetic theory which revealed the structural relationships of this new and expanding class of natural products. The biosynthesis origin of the antibiotics penicillin and cephalosporins has been a major theme of his research, which recently culuminated in the elucidation of the three-dimensional structure of the key enzyme isopenicillin N synthase combined with its substrate and analogue of dioxygen. This has led to a new insight into the remarkable reaction mechanism. He has received the Corday Morgan Medal, Paul Karrer Medal, and the Davy Medal of the Royal Society. He has given the Tilden, Simonsen, Hugo Muller, and Pedler lectureships of the Royal Society of Chemistry and has been elected Foreign Member of the American Academy of Arts and Sciences.

extracts of a $\beta$-lactam defective mutant which showed MgATP-dependent ACV synthesis from the L enantiomers of the three constituent amino acids.[8] Small

Chia-Yang Shiau was born in Taiwan, ROC, in 1958. He received his B.S. in Pharmacy in 1981 from the National Defence Medical Centre (NDMC) and was a teaching assistant for two years in the Department of Pharmacy. He received his M.S. in Pharmaceutical Chemistry with Professor An-Rong Lee in 1985 synthesizing derivatives of oxadiazole as potential monoamine oxidase inhibitors. He worked in research and development at the Department of Pharmaceutical Research Institute, NDMC, for five years before commencing his doctoral work. He received his D. Phil. from the University of Oxford in 1994 for mechanistic studies on ACV synthetase under the supervision of Professor Jack E. Baldwin and Dr. Christopher J. Schofield. He returned to Taiwan as Associate Researcher in Pharmaceutical Research Institute and Associate Professor in Department of Biochemistry at NDMC in 1995. His current interests include nonribosomal peptide synthesis, synthesis of novel antihypertensive agents, and development of a chemical nuclease.

Chris Schofield was born in the Wirral peninsula in northwest England in 1960. His undergraduate studies were in Chemistry at the University of Manchester Institute of Science and Technology. In 1982 he undertook his D. Phil. studies on the chemical and enzymatic syntheses of antibiotics with Professor Jack E. Baldwin at the Dyson Perrins Laboratory, University of Oxford. Subsequently he has remained at the Dyson Perrins Laboratory and, latterly, the Oxford Centre for Molecular Sciences. In 1990 he was appointed a University Lecturer and Fellow of Hertford College. His research interests center on the biosynthesis of antibiotics, antibiotic resistance mechanisms, and the structures and mechanisms of hydrolytic enzymes and oxygenases/oxidases those catalyzing synthetically impossible reactions.

amounts of AC were also detected but no evidence was accrued for the biosynthesis of L-cysteinyl-D-valine (CV). Partial purifications of putative AC synthetases from both *P. chysogenum*[9] and *C. acremonium*[10] were also reported. Banko *et al.* reported that the ACV-forming activity in *C. acremonium* was significantly stabilized by the addition of a high concentration of glycerol.[11] Significantly, the same authors reported that conversion of the individual amino acids to ACV was faster than conversion of AC plus L-valine to ACV. The identity and yield of ACV was determined by HPLC analysis using an

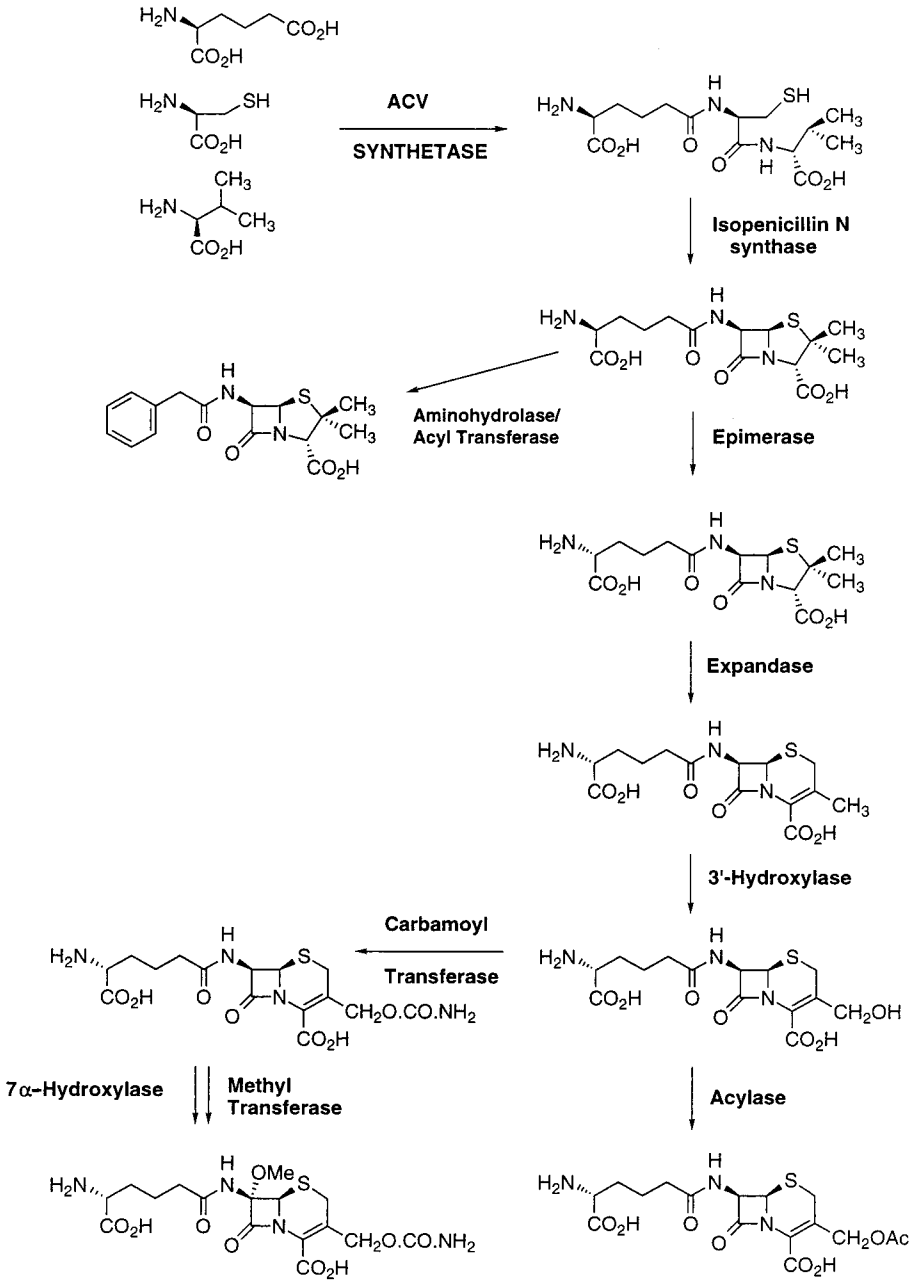


**Figure 1.** The $\beta$-lactam biosynthetic pathway.

authentic standard and by conversion of the product ACV to an antibiotically active compound (isopenicillin N) using isopenicillin N synthase. No isopenicillin N was detected in this way when L,L,L-ACV was incubated with the purified extracts. Similar results were obtained with ACV synthetase isolated from *Streptomyces clavuligerus* which also catalyzed ACV production when immobilized on an anion exchange resin.[12,13] These important observations prompted Banko *et al.* to be the first to propose that ACV synthesis was catalyzed by a single multifunctional polypeptide, similar to those previously characterized catalyzing the formation of peptide antibiotics.[11,14,15] It is noteworthy however that, in this case, the peptide product itself has no known antibiotic activity.

## III. Isolation of ACV Synthetase

Firm evidence for the proposal that ACV was synthesized in a manner similar to the peptide antibiotics came in 1989. In a pioneering piece of work, isolation of a large enzyme from *Aspergillus nidulans* capable of the synthesis of ACV, ACV synthetase, was reported by von Döhren and coworkers in Berlin.[16] A grinding method was used to disrupt the fungal mycelium and again the tripeptide-synthesizing activity was stabilized by using an extraction buffer with a high glycerol concentration. The isolation of the large and apparently unstable enzyme was facilitated by the development of a means of reliably assaying the initial rate of production of radiolabeled ACV from $^{14}$C-labeled valine. This exploited selective retention of the labeled product tripeptide, but not the valine substrate, on a hydrophobic Porapak Q column under acid conditions. The isolated enzyme formed ACV from the L enantiomers of its constituent amino acids in the obligatory presence of MgATP (Figure 2). The enzyme also catalyzed ATP−$PP_i$ exchange reactions (section IV.B)

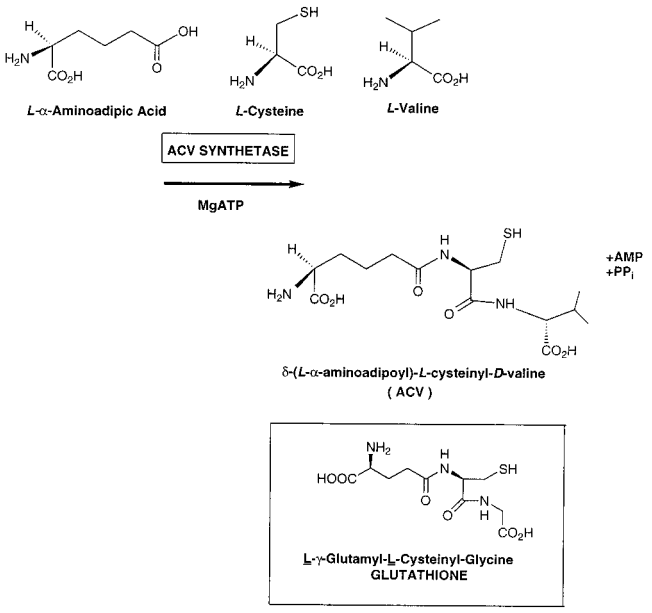


**Figure 2.** The ACV synthetase reaction. The structure of glutathione is shown for comparison (boxed).

in response to the individual substrate amino acids. This observation indicates the freely reversible formation of enzyme-bound aminoacyl adenylates, an initial partial reaction that the peptide synthetases share with the aminoacyl-tRNA synthetases.[17] In the absence of cysteine and α-aminoadipic acid, [$^{14}$C]valine bound to the enzyme in an MgATP-dependent manner. This enzyme-bound radiolabel was preferentially released from the protein by performic acid oxidation rather than formic acid hydrolysis. This lability of enzyme-bound label to performic acid oxidation has long been used as a qualitative test for attachment of substrate amino acids to peptide synthetases by a thioester link[18] (section IV.C). Thus it was concluded that ACV biosynthesis was catalyzed by a single polypeptide probably using a "thiol-template" mechanism (section VII.A) as previously proposed for other large multifunctional synthetases synthesizing antibiotically active peptides, including gramicidin S and tyrocidin (Figure 3).[14,15]

The isolation of ACV synthetase from *A. nidulans* prompted the isolation of ACV synthetases from other organisms producing $\beta$-lactams, including another eukaryote (*C. acremonium*) and the prokaryote *S. clavuligerus*.[19−22] The relative molecular masses reported for isolated ACV synthetases are somewhat variable. The *A. nidulans* enzyme was reported to have a molecular mass of 220 kDa based both on gel electrophoresis in the presence of detergent and size-exclusion chromatography.[16] The sizes of ACV synthetases from *C. acremonium* (283 kDa) and *S. clavuligerus* (360 kDa) were estimated as considerably larger under denaturing conditions.[19,22] Other members of the peptide synthetase class are also very large proteins.[14,15] It is possible that the variations seen in the size of ACV synthetase are due to inherent inaccuracies in the methods used when applied to very large proteins. Alternatively, there may be processing of the primary translation product *in vivo* or artifactual degradation *in vitro* during isolation.

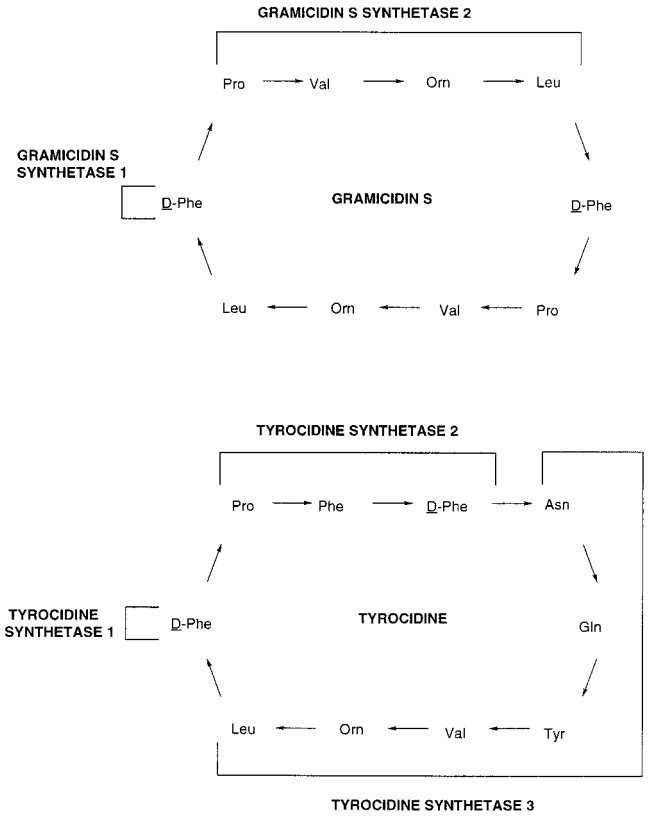


**Figure 3.** Representative antibiotics produced by other peptide synthetases.

## IV. Reactions Catalyzed by ACV Synthetase

### A. Initial Considerations

The multistep biosynthesis of even a tripeptide and modification of a single residue (valine) by a single multifunctional enzyme is a nontrivial mechanistic problem. An important factor is the coordination of the individual partial reactions required for elaboration of the correct product. The formation of the two requisite peptide bonds and the necessary inversion of the valine α center are processes which require activation of the amino acid carboxylate groups. Additionally, there must be means to sequester the intermediates to prevent them from being lost from the enzyme during translocation between the presumably distinct and possibly distant active sites. There must also be a way to release the completed tripeptide into solvent at the end of the catalytic cycle. The enzyme must, therefore, minimally catalyze the following steps: (i) binding of substrates; (ii) activation of the $\delta$-carboxylate of L-α-aminoadipate and the carboxylate of L-cysteine; (iii) formation of the amide bonds between L-α-aminoadipate and L-cysteine, and L-cysteine and L- (or D-) valine; (iv) inversion of the configuration of the valine C$^{\alpha}$ atom; and (v) product release.

A means of transferring intermediates between individual active sites is probably also required.

### B. ATP–PP$_i$ Exchange Reactions

A common feature shared between the peptide synthetases and the ribosomal system for peptide bond formation is that amino acid substrates are activated as aminoacyl adenylates (Figure 4). These

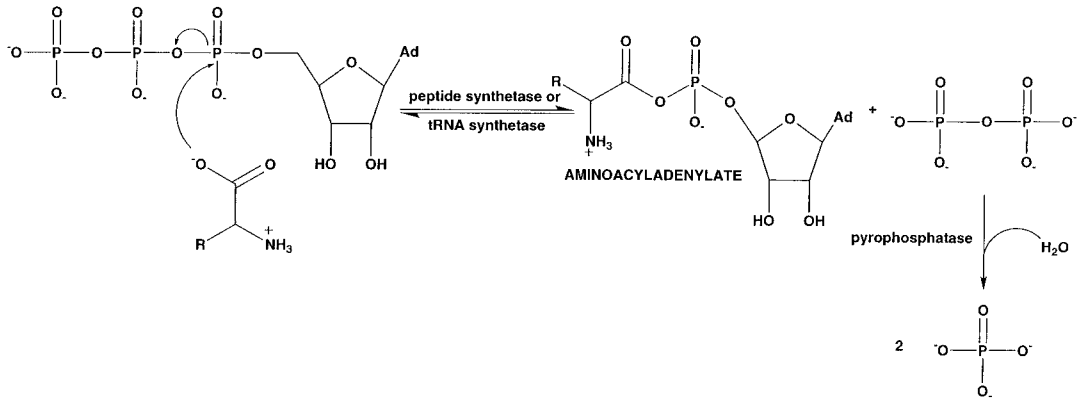


**Figure 4.** Activation of amino acids for peptide bond synthesis by formation of aminoacyl adenylates.

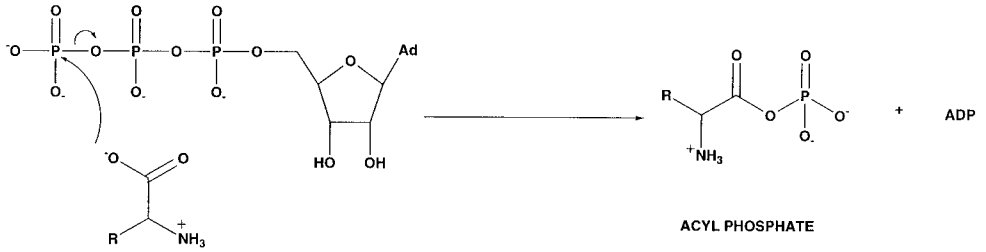


**Figure 5.** Activation of substrates for glutathione biosynthesis by formation of acyl phosphates.

are enzyme-bound intermediates which are sequestered from the aqueous phase due to their hydrolytic lability. They are bound to the aminoacyl-tRNA synthetase enzymes for aminoacyl tRNA synthesis prior to translation but to the multienzymes themselves in the nonribosomal system. Despite the similarity of the reactions catalyzed there are no obvious similarities between the sequences of any of the aminoacyl-tRNA synthetases and peptide synthetases[23] (section V). It should be noted that aminoacyl adenylate formation is not the only strategy employed in nature for the activation of amino acids for peptide bond formation. For example, in the biosynthesis of glutathione the amino acid substrates are activated at the expense of ATP as their respective acyl phosphates[24] (Figure 5).

The aminoacyl adenylation reaction is believed to be the same in both the aminoacyl-tRNA synthetases and the peptide synthetases (E, peptide synthetase or aminoacyl-tRNA synthetase):

$$E + \text{amino acid} + MgATP \rightleftharpoons [E\cdot\text{aminoacyl adenylate}] + PP_i$$

Since there is no dissociation of the enzyme-bound aminoacyl adenylate the enzyme must considered to be a reactant, and the complex a product. Therefore their concentrations must appear in the equation for the equilibrium constant for the reaction. This property of the reaction means that chemical equilibrium is reached virtually instantaneously. Addition of $[^{32}P]PP_i$ can be used to assay the reaction as it approaches isotopic equilibrium[25] (Figure 6). At low (<5−10%) fractional attainment of isotopic equilibrium the reaction is virtually linear with respect to the amount of enzyme and can be used to determine apparent $K_m$ values for substrates and substrate analogues in these partial reactions.[26] The apparent $K_m$ values for the natural substrates of ACV

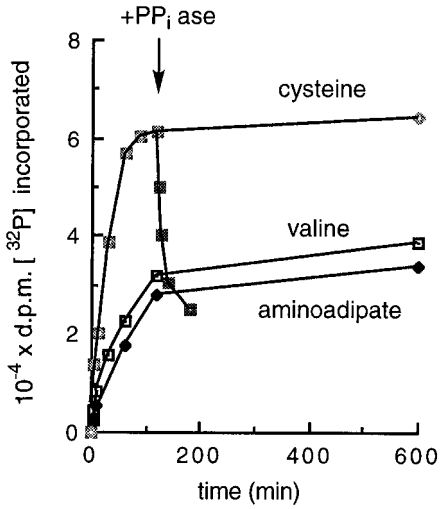


**Figure 6.** Reaction progress curves for the three ATP−$PP_i$ exchange reactions catalyzed by ACV synthetase in response to the three amino acid substrates. The effect of adding inorganic pyrophosphatase ($PP_i$ase) to one incubation is also shown.[26]

synthetase from *C. acremonium* in this reaction are similar to those reported for the aminoacyl tRNA synthetases and their substrates.[26] The exchange reactions of *C. acremonium* ACV synthetase are readily detected in response to cysteine and valine, but are more difficult to detect reliably in response to α-aminoadipate. Assays with this substrate can sometimes require a larger amount both of enzyme and higher specific activity $^{32}PP_i$ for reliable data to be obtained.[26] Hitherto there has been no success in detecting significant ATP−$PP_i$ exchange in response to α-aminoadipate in ACV synthetase isolated from *S. clavuligerus*.[27,28] The reasons for this apparent difference in the ACV synthetase isoenzymes are unclear.

Although the ATP−$PP_i$ exchange reactions are close to equilibrium as written above, the ubiquity

of the enzyme inorganic pyrophosphatase (EC 3.6.1.1) *in vivo* ensures that the $PP_i$ produced by the reaction is rapidly hydrolyzed to orthophosphate, thus aminoacyl adenylate formation is favored *in vivo*. Addition of inorganic pyrophosphatase to the exchange reactions *in vitro*, as expected, causes rapid collapse (Figure 7) of the exchange equilibrium as demonstrated by rapid loss of $^{32}P$ accumulated in the ATP pool during the exchange reaction.[26]

The selectivity of ACV synthetase for the nucleotide substrate in these initial partial reactions is comparatively broad. The nucleotide selectivity of the ATP−$PP_i$ exchange reactions stimulated by cysteine were measured at 2 mM nucleotide.[26] GTP and TTP showed approximately 20% and CTP, dATP, and dGTP approximately 15% the initial rate seen with ATP. Negligible activity was seen with dTTP, dCTP, ITP, or UTP. The substrate selectivity of the exchange reactions for amino acids is interesting in that some amino acids appear to be substrates for this initial activation reaction but do not participate detectably in any further reactions in the catalytic sequence. These observations will be discussed in section V. However, note that there is evidence for the weak activation of L,L-α-aminoadipoylcysteine, presumably as an analogue of cysteine at the cysteine aminoacyl adenylation site.[27,29] This observation may explain early results demonstrating low but detectable conversion of L,L-α-aminoadipoylcysteine plus valine to ACV. Such unexpectedly low selectivity of the cysteine activation site in this instance may explain the initial confusion regarding the identity of the enzyme system responsible for the synthesis of ACV (section II).

One feature of the initial rates of the individual ATP−$PP_i$ exchange reactions in response to the three natural substrates is that they are not additive. That is, apparent initial isotope exchange rates in response to the presence of two or all three amino acids substrates is less than the sum of the rates seen when the amino acids are included in the assays separately at the same concentrations. This effect has been seen in independent laboratories.[26,28] One explanation is that the presence of two or more aminoacyl adenylates could result in initiation of irreversible reactions leading ultimately to peptide bond formation. Alternatively, the aminoacyl adenylation reactions might not be completely independent and the presence of an aminoacyl adenylate at one site could inhibit maximum formation of an aminoacyl adenylate at another site, possibly in order to ensure the correct reaction sequence.

## C. Enzyme Aminoacylation

Available evidence for protein aminoacylation during catalysis by ACV synthetase is indirect. In the thiol-template and multi-pan carrier models for ACV synthetase (sections VII.A and VII.B) the aminoacyl groups derived from the substrate amino acids by aminoacyl adenylation are transferred to enzyme-bound thiols. The chemical nature of the proposed essential thiols has been revised recently (section VII.B). Early work with gramicidin S synthetase and tyrocidin synthetase indicated that there were significant amounts of radiolabeled enzyme-bound amino acids recovered in trichloroacetic acid-precipitated preparations after incubation in the obligatory presence of MgATP.[18] The interpretation that these results are consistent with substrate aminoacyl groups covalently binding via thioesters to the enzyme is based on three lines of evidence. Firstly, since "free" aminoacyl adenylates are labile to acid hydrolysis, it was assumed that any enzyme-bound aminoacyl adenylates should be hydrolyzed by the trichloroacetic acid. Secondly, free thioesters are relatively acid stable therefore any remaining enzyme bound amino acid was more likely to be linked via a thioester to the enzyme. Thirdly, free thioesters are labile to performic acid oxidation; therefore, that the bound aminoacyl groups were released by treatment with performic acid was consistent with a thioester linkage between the aminoacyl group and the protein.

ACV synthetase retained small amounts of performic acid-labile radioactivity when precipitated after incubation with L-U-[$^{14}C$]valine. That there was covalent linkage between the radiolabeled valine and the enzyme was demonstrated by the detection of some labeled material in the band corresponding to ACV synthetase after electrophoresis in the presence of sodium dodecyl sulfate.[16] Stoichiometries for the binding of the labeled valine to the ACV synthetase preparations were not given but can be approximated from the presented data subject to specific assumptions, principally that no unlabeled carrier valine was added to the pool of valine in the incubations (none is mentioned in the paper). Calculations indicate that the peak fraction eluted from the gel filtration column represented binding of 6.6 mmol valine/mol total protein and the final material purified by DEAE-Sepharose, 2.9 mmol valine/mol total protein (value given corrects for the purification factor achieved by this final step). This would indicate that the thiol templates were not stoichiometrically aminoacylated with valinoyl groups under the conditions used for their detection. It is unlikely that there was significant loss of any essential enzyme aminoacylation activity during purification, since ACV synthesis and valine-dependent ATP−$PP_i$ exchange activities were recovered in good yield, as were the exchange activities for the other two amino acid substrates. Alternative explanations for these results will be discussed in section VII.C.

Schwecke *et al*. reported that ACV synthetase from *S. clavuligerus* formed a thioester complex with radiolabeled α-aminoadipate.[28] However, as mentioned in section IV.B, there is no detectable ATP−$PP_i$ exchange in response to this substrate catalyzed by ACV synthetase from this source. Aminoacylation of the enzyme from this source by α-aminoadipate is paradoxical since aminoacylation without prior activation at the expense of ATP is unfavorable thermodynamically. It was also demonstrated that MgATP-dependent binding of radioactivity occurred when *S. clavuligerus* ACV synthetase was incubated with $^{14}C$-labeled cysteine. However, after performic acid oxidation, the recovered radioactivity did not correspond chromatographically to either cysteine or cysteic acid indicating conversion of this substrate to some other labeled product by the enzyme.

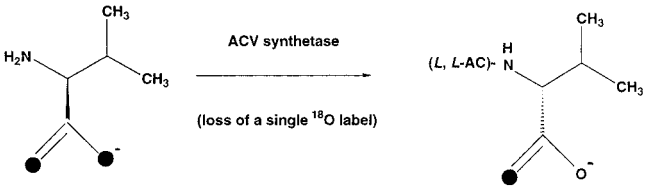


**Figure 7.** Loss of one (only) $^{18}O$ label from di-[$^{18}O$]-valine during the biosynthesis of ACV. The filled circles denote the $^{18}O$ isotope.

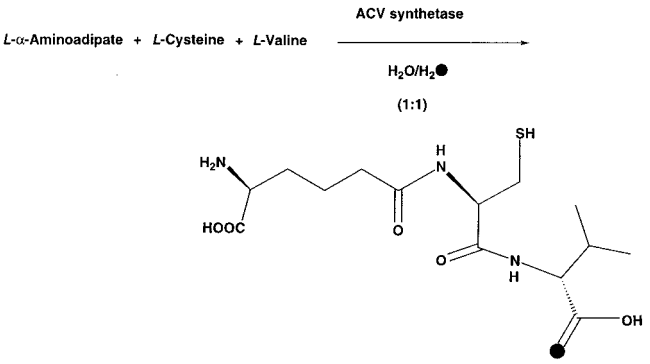


**Figure 8.** Incorporation of one (only) $^{18}O$ label into the valine carboxylate from $^{18}O$-enriched water during the biosynthesis of ACV. The filled circles denote the $^{18}O$ isotope.

An alternative approach to investigating the role of aminoacylated enzyme in the reaction mechanism is to utilize amino acid substrates selectively labeled with $^{18}O$ in their carboxyl groups. If there is a single irreversible transfer of the aminoacyl group to the enzyme formation of any (thio)ester to the valine carboxylate must remove one of the labeled oxygen atoms. Reversible aminoacylation of the enzyme will result in further loss of label. By contrast to the evidence from the trace radiolabeling of the enzyme, no evidence for the transfer of di[$^{18}O$]valine to the enzyme was seen since there was no detectable loss of label when the labeled valine was incubated with the ACV synthetase alone or in the presence of the other two amino acid substrates.[30] However, loss of one valine $^{18}O$ must occur at some point since the recovered ACV product had lost one of the labeled oxygen atoms[31] (Figure 7). In the converse experiment, detecting transfer of $^{18}O$ from $^{18}O$-enriched water, a single $^{18}O$ is incorporated detectably only into the valine carboxylate[32] (Figure 8). These experiments demonstrate that there is transfer of the valine residue at some stage during the catalytic cycle, but do not give information on the timing of this partial reaction relative to the complete catalytic cycle. The exchange of only one of the valine $^{18}O$ atoms, as determined in both directions, demonstrates that this process is irreversible or that it occurs after an irreversible step.

In principle, the single turnover aminoacylation reaction should be detectable by transfer of $^{18}O$ from di[$^{18}O$]valine to AMP, the other postulated product of the aminoacyl adenylation reaction. ACV synthetase produces AMP during ACV synthesis at a stoichiometry approaching 3 mol AMP/mol ACV (M. F. Byford and C.-Y. Shiau, unpublished observations). Attempts to detect incorporation of any $^{18}O$ from the valine substrate into AMP as result of enzyme aminoacylation failed although comparatively large activities of synthetase were used for the experiments.[33] This failure could have resulted from technical problems, or the fact that the equilibrium of the transfer reaction lies too far to the left for efficient detection of the low yield of labeled AMP, or that the presence of (an)other amino acid(s) is required for the transfer of the valine carboxyl to the enzyme (section IV.D).

## D. Peptide Bond Formation

Peptide bond formation involves the nucleophilic attack by the α-amino group of one amino acid or peptide on the activated carboxyl group of the other participant amino acid or peptide in the bond. This step must involve recognition of the two participant amino acids and their condensation, one as amino group donor and the other as amino group acceptor.[18] In the thiol template mechanism for nonribosomal peptide bond synthesis (section VII.A), the activated thioester to 4′-phosphopantetheine (Figure 9) is the amino group acceptor and this is condensed with the amino group of the next amino acid in sequence which has previously been thioesterified to its respective thiol template. This sequential process is repeated leading to chain elongation in a specific sequence determined by the substrate selectivity of each of the thiol templates.

In 1982 it was reported[9] that AC synthetase activity could be detected in cell-free extracts from *P. chrysogenum*, and later a similar activity was reported in *C. acremonium*.[11,34] In these reports the identification of the AC dipeptide was based on comigration of the enzymic product with an authentic standard in various chromatographic systems. Subsequently it was reported that AC dipeptide synthesis could not be detected in *S. clavuligerus*.[19] No evidence was seen for any production of AC from L-α-aminoadipate and L-cysteine using partially purified *C. acremonium* ACV synthetase either in the presence or absence of valine using HPLC isolation of products coupled with electrospray ionization MS and $^1H$ NMR.[27]

Having failed to detect any significant formation of AC, data previously collected was reexamined.[26] There was significant incorporation of radiolabeled substrates into material retained by Porapak Q columns when ACV production assays were done with cysteine analogues most notably *S*-methylcysteine and *O*-methylserine. Paradoxically, no production of either α-(aminoadipoyl)-*S*-(methylcysteinyl)valine valine or α-(aminoadipoyl)-*O*-(methylserinyl)valine could be detected. Additionally, ACV synthesis assays performed with some "aged" preparations of enzyme failed to show significantly reduced incorporation of radiolabeled valine into material retained on Porapak Q even though the these preparations were less than 10% as active as recently isolated ACV

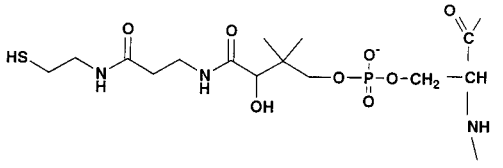

**Figure 9.** Structure of 4′-phosphopantetheinylserine.

synthetase in the synthesis of ACV itself.[35] These observations, taken together, led to the evaluation of the hypothesis that *di*peptides containing valine were synthesized from these cysteine analogues and valine by ACV synthetase, even in the presence of α-aminoadipate.

With the aid of synthetic L,L- and L,D-*O*-(methylserinyl)valine and L,L- and L,D-*S*-(methylcysteinyl)valine standards isolation and characterization (by electrospray ionization MS and $^1$H NMR) of the anticipated dipeptide products was attempted. *O*-(Methylserinyl)valine was indeed produced by ACV synthetase incubations containing α-aminoadipate, *O*-methylserine, and valine.[27] Similar results were obtained with the *S*-methylcysteine analogue but apparent yields were lower and the isolation of the peptide was more difficult, possibly due to the tendency of the sulfur to oxidize. It was also shown, in the case of the *O*-methylserine analogue, that both the L,L and L,D diastereoisomers were synthesized. This observation has been useful in analyzing the timing of the epimerization reaction (see section IV.E).

Attempts to synthesize cysteinylvaline itself from incubations of ACV synthetase with L-cysteine and L-valine failed to yield enough material for unequivocal characterization by $^1$H NMR spectroscopy or mass spectrometry. Possibly this was due to the absence of α-aminoadipate activation since reduced synthesis of *O*-(methylserinyl)valine was observed in the absence of α-aminoadipate. It had already been observed that L-glutamate stimulated ATP−$PP_i$ exchange but was not detectably incorporated into a tripeptide product (section V).[26] It was possible that its presence might enhance production of cysteinylvaline sufficiently to enable its identification and determination of the stereochemistry of the valine residue. Incubation of L-glutamate, L-cysteine, and L-valine led to the synthesis of sufficient L,D-cysteinylvaline for identification.[36] The stereochemistry was assigned by doping experiments with synthetic L,D- and L,L-cysteinylvaline. Notably no L,L-cysteinylvaline could be detected, in contrast to the observations with the diastereoisomers of *O*-(methylserinyl)valine (see section IV.E). Furthermore, no tripeptide product, i.e., glutamylcysteinylvaline could be detected. L-Glutamate, but not L-aspartate, acts as a weak inhibitor of ACV synthesis itself, presumably by competing for the α-aminoadipate aminoacyl adenylation site, indicating that the addition of L-glutamate acts to enhance cysteinylvaline formation by acting at the site normally activating α-aminoadipate.[36] The effect of this analogue on the production of cysteinylvaline could be quantified and was apparently concentration dependent.[36] These observations would be consistent with catalytic turnover at the α-aminoadipate aminoacyl adenylation site causing a conformational change resulting in enhanced production of (a) cysteinylvaline intermediate(s). Futile production of cysteinylvaline would be inhibited in the absence of the nonproteinogenic amino acid α-aminoadipate since significant stimulation by L-glutamate was seen at unphysiologically high concentrations.

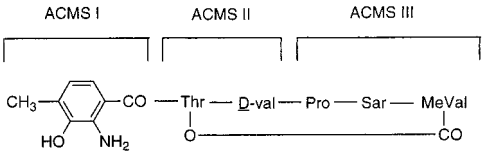


**Figure 10.** Actinomycin D biosynthesis. The polypeptides ACMS I-III synthesize the peptides indicated. ACMS I activates the first residue; ACMS II activates the second and third residues and forms the peptide bond between them and that to the first residue. The last three residues are activated and polymerized by ACMS III, which also synthesizes the peptide bond to proline and catalyses lactone formation.[37]

The experiments leading to the production of dipeptides containing valine were done at near saturating concentrations of amino acid substrates to maximize formation of the products. The physiological concentrations of these substrates are unknown but are probably considerably lower than those employed in the experiments. However it is unlikely that the mechanism of the synthetase would be variable and dependent on the concentration of the available amino acid substrates. The production of dipeptides containing valine, but none containing α-aminoadipate, by ACV synthetase suggests that the synthesis of ACV proceeds through a cysteinylvaline intermediate.

A similar reaction sequence is seen in the synthesis of actinomycin D. In a multistep synthesis actinomycin D is elaborated by the action of distinct polypeptides (Figure 10). One of these, actinomycin synthetase II synthesizes L-threonyl-D-valine from L-threonine and L-valine (section IV.E).[37] In the additional presence of another enzyme, actinomycin synthetase I and its substrate 4-methyl-3-hydroxyanthranilic acid, the tripeptide 4-methyl-3-(hydroxyanthraniloyl)-L-threonyl-D-valine is formed. The biosynthesis of this tripeptide therefore has features in common with that of ACV. These include epimerization of the C-terminal valine residue and incorporation of a nonproteinogenic component (4-methyl-3-hydroxyanthranilic acid) at the N-terminus. This peptide is further elaborated to the final product by a further peptide synthesizing polypeptide (actinomycin D synthetase III). An interpretation of the interesting results presented is that the L,D-threonylvaline is in fact formed prior to the peptide bond to the nonproteinogenic substrate, since there was no evidence presented that peptide bond formation between 4-methyl-3-hydroxyanthranilic acid and threonine occurs in the absence of valine.[37] ACV synthetase could represent an analogous enzyme, particularly since the mechanism for the inversion of the valine α-center appears to be similar in both enzymes (section IV.E). However, in ACV synthetase, the activities responsible for activation of the nonproteinogenic amino acid and formation of the peptide bond to this substrate have become fused with those synthesizing the initial dipeptide in a single trifunctional chain.

Peptide bond formation between the cysteine analogue *O*-methylserine and valine catalyzed by ACV synthetase was extended to investigate the timing of the loss of $^{18}$O from di[$^{18}$O]valine during the synthesis of this novel dipeptide product. Previously

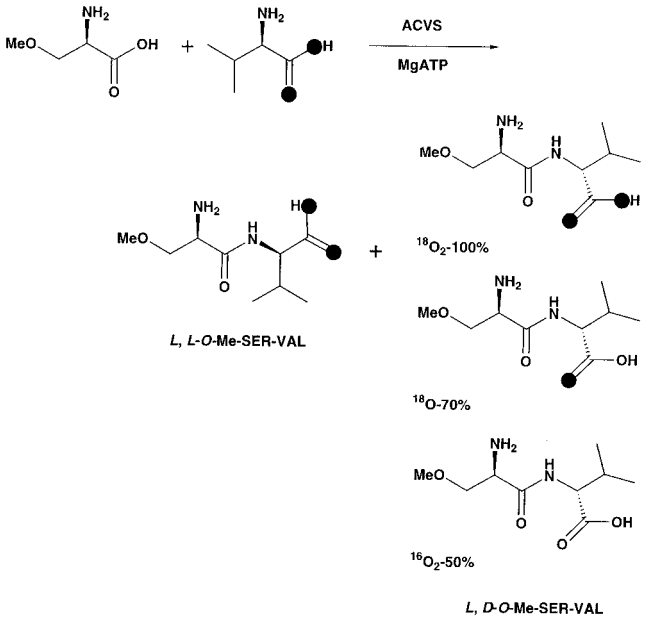


**Figure 11.** Loss of $^{18}O$ label from di-[$^{18}O$]-labeled valine during the synthesis of L,L and L,D-*O*-methylserinylvaline by ACV synthetase. The filled circles denote the $^{18}O$ isotope.

(see section IV.D) we had failed to detect transfer of $^{18}O$ from the valine carboxylate to AMP when this substrate was incubated with the ACV synthetase in the presence of MgATP. An explanation for this failure was furnished by the unexpected observation that the L,L-*O*-(methylserinyl)valine dipeptide was synthesized without significant loss of label from the valine carboxylate. That is, the peptide bond between these two amino acids was formed without prior (thio)esterification of the valine residue. Moreover, the L,D-*O*-(methylserinyl)valine dipeptide was recovered with all three possibilities for the labeling of the D-valine carboxylate, i.e., $^{18}O^{18}O$, $^{18}O^{16}O$, and $^{16}O^{16}O$ patterns (Figure 11).[33] One explanation for these observations is that some of the L,D diastereoisomer (but not the L,L diastereoisomer) is transferred to the 4′-phosphopantetheine cofactor, then lost from the enzyme with reesterification of a small proportion of the intermediate prior to a further release from the cofactor. This process might reflect part of a "proofreading" mechanism by which the enzyme is able to reject an "incorrect" dipeptide intermediate which cannot participate in the formation of the second ($\delta$-) peptide bond. Thus the dipeptide "shunt" products accumulate in solution. The implications of these observations on the timing of thioester formation relative to the timing of peptide bond formation for the thiol-template mechanism are discussed in section VII.C.

## E. Epimerization of the Valine Residue of ACV

A common feature of nonribosomally synthesized peptides is the occurrence of D-amino acid residues in the peptide products. There are three known ways in which these nonproteinogenic amino acids may arise in the product peptide. First, free preformed D-amino acids from a specific intracellular pool may be incorporated into the peptide by the synthetase. This would appear to be the case in the biosynthesis of cyclosporin and in the incorporation of D-amino acids into the cell-wall polymers of some bacteria.[38−40] Second, amino acid racemase subunits, which are noncovalently associated with the peptide synthetases gramicidin S synthetase and tyrocidin synthetase (Figure 3), are known which specifically convert the L enantiomer precursor amino acids (L-phenylalanine) of the N-terminal ("starter") amino acid to the D configuration (D-phenylalanine) prior to their participation in peptide-bond formation.[41,42] Both the D and L enantiomers can serve as precursors of the D residue found in the peptide product. Considerable evidence exists in these enzymes for a 4′-phosphopantetheinyl cofactor (Figure 9) covalently attached to a specific serine residue within a common consensus sequence (section VI). This modification appears to be essential for the racemization reaction.[43] It has been proposed therefore that racemization occurs when the aminoacyl group is thioesterified to the 4′-phosphopantetheinyl group. Third, inversion of the configuration of the $\alpha$-center can occur after the peptide bond(s) to the amino acid have been formed. This is the means by which D-amino acid residues occasionally appear in peptides which are initially synthesized ribosomally containing the corresponding L-amino acid residue. This process is seen in the lantibiotic antibiotics and in the dermorphin opioid peptides.[44,45] Incorporation of D-amino acids into positions other than at the N-terminus is a feature of numerous nonribosomally synthesized peptides. In these cases precursor studies in whole cells or cell-free extracts have demonstrated that only the L enantiomer of the free amino acid is incorporated into the positions of the D residue in the peptide products. This has been demonstrated for actinomycin, etamycin, bacitracin and penicillin biosynthesis.[46−49]

Purified preparations of ACV synthetase rigorously discriminate against D-valine as a substrate since D-valine does not significantly stimulate ATP−$PP_i$ exchange.[19,26] This selectivity for the L enantiomer differentiates the ACV synthetase epimerization mechanism from the distinct racemase subunits used in gramicidin S or tyrocidin synthetases. Inversion of the valine $\alpha$-center during its thioesterification to ACV synthetase has been postulated by analogy to gramicidin S and tyrocidin synthesis (Figure 13).[15] This hypothesis is one way of rationalizing the activation of the valine carboxyl as a thioester via its aminoacyl adenylate by ACV synthetase even though no peptide bond is formed to the valine carboxyl group.

The inversion of the stereochemistry of the valine $\alpha$-center during ACV biosynthesis provides a convenient model system for studying this process in those peptide synthetases which invert the configuration of amino acids at the C-terminus of complete peptides or intermediates. Epimerization of the valine residue is essential for the further conversion of ACV to antibiotically active compounds since L,L,L-ACV is not a substrate for isopenicillin N synthase.[50] Although no L,L,L-ACV has been detected *in vitro* or *in vivo* the possibility remained that the epimerization occurred after peptide bond formation, possibly even by a distinct enzyme activity not associated with ACV synthetase. Synthesis of ACV by purified ACV synthetase *in vitro* using [2-$^2$H]valine as a probe

resulted in complete (>95%) loss of deuterium from the valine α-center since isolation of the ACV produced and its analysis by electrospray ionization MS gave a $MH^+$ *m*/*z* identical to authentic ACV. Both ACV synthetases from *C. acremonium* and *S. clavuligerus* gave qualitatively identical results. No loss of deuterium was seen from [2-$^2$H]valine when it was incubated with ACV synthetase in the absence of the other two amino acid substrates. This indicates that epimerization is either dependent on the presence of one or other or both of the amino acid cosubstrates or alternatively that epimerization occurs only after peptide bond formation. Incubation of *C. acremonium* enzyme in buffer solution approximately 50% enriched in $D_2O$ led to isolation of ACV of which a significant proportion contained a single deuterium atom only at the valine residue. This indicates that there is no significant epimerization of cysteine or α-aminoadipate.[51]

The observation that incubation of ACV synthetase (*C. acremonium*) with valine and the cysteine analogue *O*-methylserine produced *O*-(methylserinyl)valine dipeptides was exploited to investigate the timing of the epimerization reaction. Intriguingly, the dipeptide was isolated as an approximately 1:1 mixture of L,L- and L,D-*O*-(methylserinyl)valine diastereoisomers.[27] When these dipeptides were synthesized using labeled [2-$^2$H]valine substrate as a probe only the L,L diastereoisomer was produced in sufficient yield for spectroscopic characterization. The ratio of L,L to L,D was estimated by HPLC analysis to be >10:1. This significant change in relative yield of the diastereoisomers indicates a primary deuterium kinetic isotope effect operating in the epimerization process. It also suggests that the L,L diastereoisomer is an obligatory intermediate in the synthesis of the L,D diastereoisomer. No conversion of authentic standard L,L diastereoisomer to the L,D form by ACV synthetase was seen, indicating that the L,L dipeptide is not a free intermediate in the synthesis of the L,D dipeptide. Neither dipeptide stimulated significant enzyme-catalyzed ATP−$PP_i$ exchange, indicating that their activation as bound acyl adenylates does not occur. The results demonstrate that at least in the case of these dipeptides epimerization of the valine occurs after participation of the valine amino group in peptide bond formation. It is important to note, however, that in the case of ACV synthesis itself, the results do not completely eliminate the possibility of the epimerization occurring at the tri- rather than the dipeptide stage.

Stindl and Keller have studied the related epimerization reaction catalyzed by the enzyme actinomycin synthetase II (section IV.D).[37] Like ACV synthetase there is no epimerization of the valine α-carbon in the absence of other amino acid substrates.[52] In this study *p*-toluic acid was used as a convenient substitute for the natural substrate (4-methyl-3-hydroxyanthraniline). Incubation of L-threonine and L-valine together with actinomycin synthetase II and MgATP resulted in the recovery of both L-threonyl-L-valine and L-threonyl-D-valine. Incubation of the *p*-toluic acid analogue with L-threonine and L-valine together with actinomycin synthetase I (required to activate the 4-methyl-3-hydroxyanthraniline or *p*-toluic acid), actinomycin synthetase II and MgATP resulted in the formation of both *p*-toluyl-L-threonyl-L-valine and *p*-toluyl-L-threonyl-D-valine. The dipeptide intermediates were liberated from the enzyme using performic acid and analyzed by thin-layer chromatography followed by fluorography. Their stereochemistry was determined by analysis on chiral thin-layer plates and by analysis of digested peptides with L- and D-amino acid oxidases. Experiments using [2,3-$^3H_2$]valine revealed that the L,D-diastereoisomer had lost approximately 50% of the tritium label, and incorporation of tritium into solvent water was detected. Loss of tritium from the valine was much reduced in the absence of L-threonine or MgATP. Thus it seems likely that during actinomycin synthesis the peptide bond to valine was formed prior to the inversion of the valine α-center, since both L,L and L,D diastereoisomers were liberated in approximately equal amounts on performic acid oxidation. It was concluded that the L,L diastereoisomer was an obligatory intermediate in the synthesis of the L,D diastereoisomer, and thus the valine residue was inverted after incorporation into the intermediate dipeptide. It was proposed that epimerization in the "peptide-bound" state might be a common mechanism by which amino acids not at the N-terminus were converted into their D-amino acid residue counterparts. The observations regarding ACV and actinomycin biosynthesis used different methodologies and a different peptide synthetases and are thus mutually supportive. A difference in the protocols is noteworthy in that the dipeptide intermediates in the actinomycin synthetase studies were released by performic acid oxidation. By contrast, in the ACV synthetase experiments the "incorrect" L,L- and L,D-*O*-(methylserinyl)valine (and L,D-cysteinylvaline, section IV.D) "shunt" products accumulated in solution.

It therefore seems likely that epimerization in the "peptide bound" state is a common mechanism by which the L substrate amino acids are converted to their D residue counterparts in those peptide synthetases in which the stereochemically inverted residue is not at the N-terminus of the peptide product.

## F. Thioester Hydrolysis

Assuming that the ACV synthetase mechanism involves formation of a (thio)ester link between L,L,D-ACV and the enzyme, a mechanism must exist for its hydrolysis. Evidence for the existence of a thioesterase activity comes from observation of some sequence identity (section VI) between a small C-terminal region in ACV synthetase and vertebrate thioesterases. It is noteworthy that a similar region is found in the predicted product of the *grs*T open reading frame found in the operon-encoding gramicidin S synthetase.[53] The *grs*T product is a small polypeptide which is probably noncovalently associated with the complete gramicidin S synthetase multisubunit complex. In ACV synthetase it would appear that this functional domain has become fused to the multifunctional polypeptide. The observed incorporation of $^{18}O$ from $^{18}O$-enriched water (Figure 9) into the valine residue in ACV is probably due to

the thioesterase activity of the synthetase (section IV.B).[32]

## V. Amino Acid Substrate Selectivity of ACV Synthetase

### A. Initial Observations

A feature of nonribosomal peptide synthesis is that the substrate selectivity of the peptide synthetases is less than that of the nucleotide-directed translation machinery.[54] This can be attributed to a lack of the sophisticated "multiple sieving" selectivities of the aminoacyl tRNA synthetases and the ribosome itself.[55] The enormous selective pressure to develop very high fidelity translation for proteins early in evolution would not be expected to operate as effectively for nonribosomal peptide synthesis. This would simply be because errors in the latter are much less likely to prove fatal to the organism since, by definition, they would not affect primary metabolism or other key cellular functions. It is also possible that the relatively lax specificities of the peptide synthetases, like both of the other enzymes of the $\beta$-lactam pathway (e.g., isopenicillin N synthase[56]) and enzymes of secondary metabolism in general, may constitute an evolutionary asset rather than a handicap.

Initial qualitative assessments of some aspects of the substrate selectivity of ACV synthetase isolated from both *C. acremonium* and *S. clavuligerus* were undertaken using a coupled assay. Potential product tripeptides were converted to isopenicillin N by isopenicillin N synthase and the products in turn were assayed by their ability to induce $\beta$-lactamase expression.[19] A clear limitation of this approach is its dependence on the product tripeptide being a substrate for isopenicillin N synthase. However it was shown that L-α-aminobutyrate could substitute for valine in the case of both *S. clavuligerus* and, to a lesser extent, *C. acremonium* ACV synthetase. The substrate selectivity of ACV synthetase from *C. acremonium* was examined also using an assay based on isolation, derivatization to yield a fluorescent product and quantitation of tripeptides using HPLC. The ability of L-α-aminobutyrate to substitute for valine was confirmed by direct assay and it was reported that L-*allo*-isoleucine was an even better substituent.[57] Additionally it was shown that L-(carboxymethyl)cysteine could substitute for L-α-aminoadipic acid, with L-glutamic acid being a very much weaker substituent. It has also been reported that both L-norvaline and L-allylglycine could replace valine.[11] A drawback of the HPLC assay is that it depends on derivatization of the free thiol group of the cysteinyl residue using monobromobimane to yield a fluorescent product. Therefore the effect of any potential cysteine substituents cannot be directly assayed in this manner.

### B. Screening of Potential Substrates for ACV Synthetase

A wider substrate selectivity screen for ACV synthetase (from *C. acremonium*) has been undertaken.[26] The aims of this study were twofold. The first was to determine by mass spectrometry and NMR spectroscopy that novel unnatural tripeptides could be produced by ACV synthetase. Such peptides might be substrates for isopenicillin N synthase, producing novel $\beta$-lactams. The second was to identify any substrate analogues which might later be exploited as mechanistic probes for the sequence of partial reactions required for ACV synthesis and, by implication, peptide synthetases in general.

A range of candidate substrate analogues were screened in two established assays: [$^{14}$C] substrate amino acid incorporation into putative peptide products retained on Porapak Q columns and amino acid-dependent ATP$-$PP$_i$ exchange (section IV.A and references cited therein).[16] Both assays are prone to potential artifacts when used to screen analogues.[26] It is particularly important to note that high activity in the ATP$-$PP$_i$ exchange assay need not necessarily reflect any peptide formation since the analogue may be discriminated against in subsequent steps during catalysis. It is also important to note that complete kinetic analyses in the assays were not undertaken for all the analogues. However, since the artifacts of the two assays are largely independent it seemed likely that those analogues showing activity in both assays were likely candidates for the more time-consuming procedure of isolating and characterizing potential unusual tripeptide products. The salient conclusions of this study can be summarized as follows. In each assay both a carboxyl and an amino group were essential for incorporation into putative products, thus validating the assays. The D enantiomers of the natural substrates were not significantly incorporated into peptide products and their inability to stimulate ATP$-$PP$_i$ exchange indicates that they are discriminated against in this initial step in the catalytic sequence. Of the analogues of α-aminoadipate explored only *S*-(carboxymethyl)cysteine (sulfur in place of the $\gamma$-CH$_2$ ) was a good substrate in both assays. This finding acted as a positive control for the assays used to conduct the screening trial since the tripeptide product with this substitution is known to be converted all the way through the $\beta$-lactam pathway to an antibiotically active cephalosporin.[58] L-Glutamate was a good substrate for ATP$-$PP$_i$ exchange but at best a much weaker substrate for peptide formation. L-Aspartate was not a significant substrate in either assay. These observations indicate that a key feature for substrate recognition at the α-aminoadipate site is the distance between the two carboxylates. L-*S*-Methylcysteine and L-*O*-methylserine were substrates in both assays, whereas L-serine was not a significant substrate. L-Vinylglycine and L-allylglycine also were apparently good substitutes for cysteine in both assays, demonstrating that the thiol group is not important for peptide formation. L-Vinylglycine and L-allylglycine were also good substitutes for valine as were D,L-allenylglycine and D,L-2-amino-3-methyl-3-butenoic acid which are also characterized by a double bond in the side chain. That L-*allo* isoleucine is an effective substitute for valine was consistent with a previously reported result.[57] L-Isoleucine was at best a much less effective substrate. This observation

provides information regarding the selectivity of the putative methyl binding pockets at the valine site.

## C. Synthesis of Novel Tripeptide Products

For several of the analogues giving positive results in both assays sufficient product tripeptide was isolated to enable characterization.[26] Acetone eluates of the Porapak Q columns loaded with preparative scale amounts of putative tripeptides derived from those substituents identified in the screening assays were isolated by reverse-phase HPLC and characterized by both electrospray ionization MS and $^1$H NMR. This led to the identification of L-(cysteinyl-*S*-acetyl)-L-cysteinyl-D-valine, L-(δ-aminoadipoyl)-L-(allylglycinyl)-D-valine, L-(δ-aminoadipoyl)-L-cysteinyl-D-isoleucine, and L-(δ-aminoadipoyl)-L-(vinylglycinyl)-D-valine as tripeptide products of ACV synthetase (Figure 12). The apparent $K_m$ values for the analogues were considerably higher than those for the natural substrates, consistent with the observation that when natural substrates were included with the analogues only authentic ACV was formed in a detectable amount.[26]

Some substitutions did not result in isolatable amounts of tripeptide product although the screening data indicated peptide formation. Also L-vinylglycine and L-allylglycine were preferential substitutes for cysteine rather than valine since it was not possible to isolate tripeptides with these amino acids incorporated in place of valine. An initial hope had been that incorporation of L-allylglycine in place of valine would be successful since conversion of the anticipated tripeptide product to six- and seven-membered ring structures by isopenicillin N synthase has been demonstrated.[56] Some of the analogues, however, had potential for being further analyzed as mechanistic probes. For example *O*-methylserine and *S*-methylcysteine might be incorporated into *di*peptide products, highlighting the ability of the enzyme to produce novel peptide products and, as proved to be the case, give information on the reaction sequence required for their synthesis. The observation that, e.g., L-glutamate and L-β-chloroalanine were probably forming an enzyme-bound aminoacyl adenylate although not being detectably incorporated into a tripeptide was also noteworthy. It is clear that compared with ribosomal peptide synthesis, ACV synthetase is comparatively "promiscuous" in its choice of substrate although some partial reactions are clearly less selective than others.

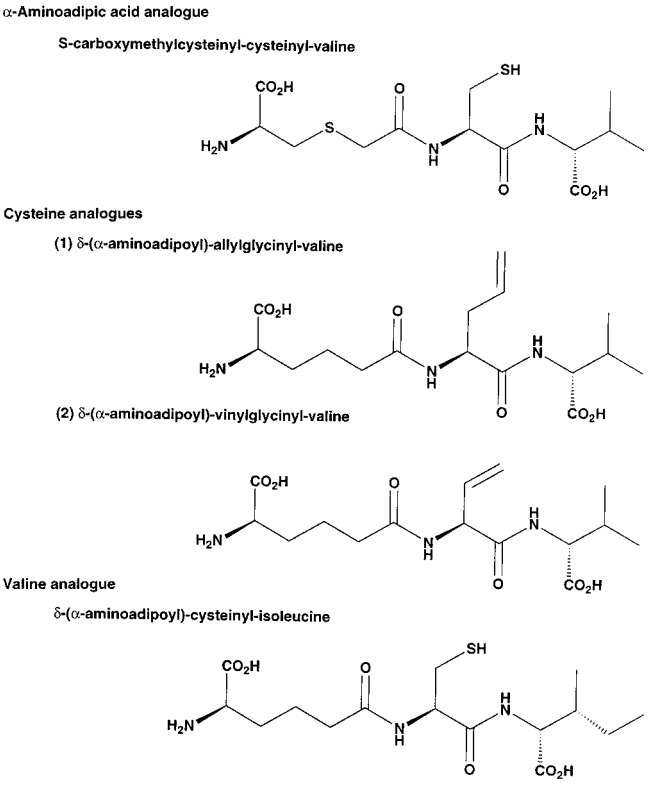


**Figure 12.** Novel tripeptide products of ACV synthetase.

## VI. Primary Structure of ACV Synthetase

The power of modern DNA sequencing methodology has been demonstrated by the efficiency with which several groups were able to establish the inferred primary structure of ACV synthetase and even larger peptide synthetases. The calculated molecular masses of ACV synthetase from the inferred primary translation products from several sources are therefore as follows: from *A. nidulans* molecular mass 422 428 Da, 3 770 residues;[59] from *C. acremonium* molecular mass 414 767 Da, 3 712 residues;[60] from *Nocardia lactamdurans* molecular mass 404 079 Da, 3 649 residues;[61] from *P. chrysogenum* two slightly variant sequences giving molecular masses of 421 068 Da, 3 746 residues and 425 914 Da, 3 791 residues.[62,63] Clearly the molecular masses of the inferred primary translation products are much more consistent than estimates of the molecular mass of the isolated enzymes, although as noted in section III there might be significant differences caused by post-translational processing or artifactual degradation during isolation. The inferred primary structures show a number of features in common with other peptide synthetases. The sequence reveals a 3-fold internal homology with three distinct domains, A, B, and C.[59−63] The other peptide synthetases are also comprised of very similar concatenenated homologous domains, typically one for each of the amino acids in the peptide product of the enzyme. It was therefore proposed that these structural repeats correspond to the functional entities responsible for the activation and incorporation of each amino acid substrate into the growing peptide chain. Evidence that this hypothesis is in fact correct comes from an elegant series of experiments in which a specific structural domain (C, the valine domain) of ACV synthetase was incorporated into another peptide synthetase, that producing the lipopeptide antibiotic surfactin in *Bacillus subtilis*.[64] Functional domains from a third peptide synthetase (the gramicidin S synthetase from *B. brevis*) were also successfully incorporated into the expected functional chimera. The chimeric synthetases synthesized the expected novel peptide products.

ACV synthetase also shares consensus sequence motifs around specific serine residues which are thought to be recognized by the enzyme responsible for 4′-phosphopantetheinylation[65] (section V). There is one such site in the C-terminal region of each

substructural domain in ACV synthetase. A small C-terminal domain may correspond to the postulated thioesterase region (section IV.F). They also share sequence identity in a highly conserved region with two other enzymes which activate substrates by acyladenylation, namely 4-coumarate CoA ligase (parsley) and firefly luciferase.[62] There is no significant homology between the peptide synthetases and the aminoacyl-tRNA synthetases despite the similarity of the partial reaction catalyzed[23] (section IV.B).

## VII. Mechanistic Proposals for ACV Synthetase

### A. The "Thiol-Template" Mechanism

The paradigm for the mechanism of the peptide synthetases for over two decades has been the so-called "thiol-template" mechanism. This was proposed by Lipmann in 1971 (and developed by Laland and Zimmer).[18,66] The mechanism represents a modified form of Lipmann's preribosomal "polyenzyme" model for peptide bond synthesis and is derived largely by analogy to the mechanism of fatty acid biosynthesis.[67] The model is supported by the key observation that the trace amounts of radiolabeled substrate amino acids recovered in precipitated synthetases are released preferentially by performic acid oxidation (section IV.C) implying a thioester bond between the substrate and the enzyme.[68] The thiol-template proposal (depicted as it relates to ACV synthesis in Figure 13) postulates the initial activation of the substrate amino acids as enzyme-bound aminoacyl adenylates. This is demonstrated by the observation that the enzymes catalyse substrate amino acid-dependent ATP−$PP_i$ exchange reaction (section IV.B). The activated aminoacyl groups are then transferred to enzyme-bound thiols, the "thiol templates" of the title, which act somewhat analogously to the 2′- or 3′-hydroxyl groups of tRNA in ribosomal peptide synthesis. The aminoacyl group is then trans-thioesterified to a second enzyme-bound thiol, that of a covalently bound 4′-phosphopantetheine cofactor. The peptide bond is then formed by nucleophilic displacement of the thiol group by the amino group of the amino acid C-terminal to the initial amino acid, which has also similarly been previously thioesterified at its respective thiol template. The enzyme-bound dipeptide thus formed is then trans-thioesterified to the thiol of the thus recently liberated 4′-phosphopantetheine cofactor and subsequently displaced by the amino group of the next amino acid to be incorporated. Thus the 4′-phosphopantetheine cofactor was postulated to act as a "swinging arm" (Figure 14) responsible for transporting the intermediate peptides between distant active sites and sequestering them from the aqueous medium. This sequence is repeated at each of the thiol templates, synthesizing the peptide product in the N- to C-terminal direction. Ultimately the complete peptide product is released from the enzyme by a thioesterase activity which cleaves the final thioester between the product and the enzyme. In this model only one 4′-phosphopantetheine cofactor was invoked as only one could be detected in several peptide synthetases catalyzing formation of peptides of different lengths.[68]

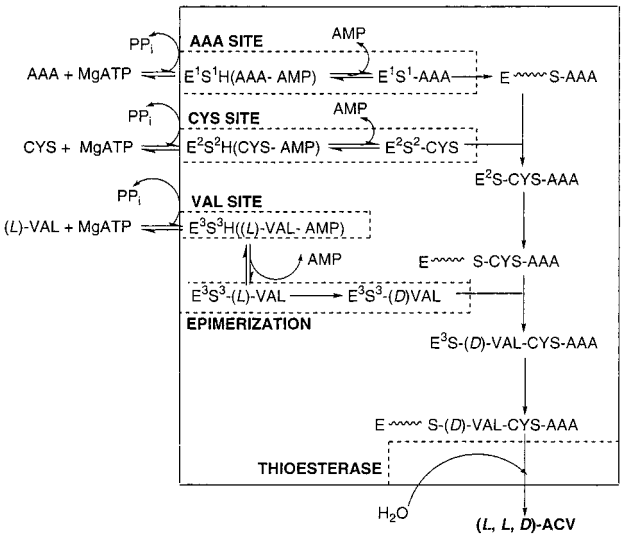


**Figure 13.** The "thiol-template" mechanism for ACV synthetase: (Xaa-AMP), aminoacyl adenylate of amino acid, Xaa. E〰, covalently bound 4′-phosphopantetheinyl cofactor. Individual sites for the activation, epimerization and thioesterase partial reactions are shown boxed (dashed lines).

The presence of the 4′-phosphopantetheine cofactor (Figure 9) in ACV synthetase has been verified experimentally with an apparent stoichiometry of ~1 mol pantothenic acid per mol protein.[19] Isolation of ACV synthetase from *C. acremonium* grown in the presence of [$^{14}$C]pantothenate showed that radioactivity was associated with the electrophoretic band corresponding to ACV synthetase.[20]

### B. The "Multi-Pan Carrier" Model

The availability of inferred amino acid sequences for several peptide synthetases prompted a reappraisal of some details of the thiol-template mechanism. In the original proposal, the thiol templates were proposed to be cysteinyl residues in the polypeptide chain itself. However there are, in fact, no obviously conserved cysteinyl residues in any of the peptide synthetases for which inferred sequences are available. Given the significant amounts of internal sequence identity between individual synthetase domains and the considerable sequence identity between all members of this class of enzymes so far sequenced this was something of a surprise.[69] If key cysteinyl residues are essential to the mechanism of nonribosomal peptide bond formation it is reasonable to suppose that these would be highly conserved in such homologous proteins. However, the C-terminal portions of the substructural domains contain conserved serine residues in an apparent consensus sequence motif: Leu-Gly-Gly-His/Asp-Ser-Leu/Ile in all synthetases thus far sequenced.[70] The serine residue at the "core" of this common sequence (His/Asp-Ser-Leu/Ile) is thought to be the site of attachment of 4′-phosphopantetheine of both the polyketide synthetases and acyl carrier proteins.[69] Complete structural analysis of any peptides derived from gramicidin S synthetase 2 containing a thioesterified $^{14}$C-labeled amino acid by the Edman degradation was precluded by the lability of this link to the basic conditions used during the sequencer cycle to couple

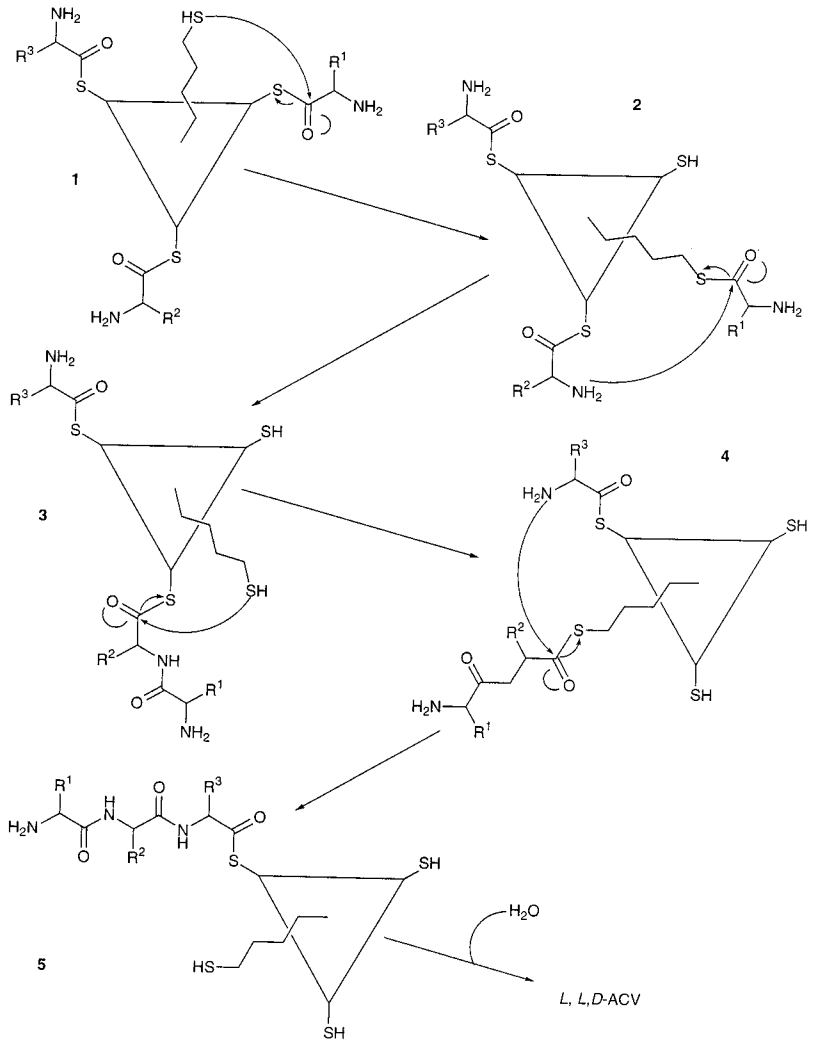


**Figure 14.** Proposed role of 4′-phosphopantetheinyl cofactor as a "swinging arm" in ACV biosynthesis.

the phenylisothiocyanate to the N-terminal of the peptide.[69] Isolation of any labeled peptide was also hampered due to apparent lability of the thioester to prolonged exposure to the acid media traditionally used in peptide isolation protocols (although, paradoxically, stability to acid is also used as an argument that any covalent link formed is, in fact, a thioester).[18] However, the [$^3$H]-*N*-ethylmaleimide−peptide adduct anticipated on the basis of the available sequence data was recovered for analysis from gramicidin S synthetase using a multistep protocol.[71] Isolation and Edman degradation of the recovered labeled peptide yielded dehydroalanine instead of the serine predicted from the DNA sequence, consistent with $\beta$-elimination of 4′-phosphopantetheine from the serine residue in the Edman chemistry. Acid hydrolysis followed by amino acid analysis showed stoichiometric (1 mol/mol peptide) recovery of $\beta$-alanine as expected from acid hydrolysis of 4′-phosphopantetheine. Analysis by mass spectrometry gave a molecular mass and sequence ions consistent with both a *S*-(*N*-ethylsuccinimido)-4′-phosphopantetheinyl−peptide adduct and underivatized 4′-phosphopantetheinyl−peptide.

That the apparent thiol template for valine was in fact a 4′-phosphopantetheinyl−serinyl residue and not a cysteinyl residue prompted the proposal of the "multi-pan carrier model" for nonribosomal peptide biosynthesis which supercedes the thiol-template proposal. In this modified mechanism the key thiols are provided by post-translational modification by 4′-phosphopantetheinylation of conserved serine residues in specific motifs, presumably those sequences recognized by the 4′-phosphopantetheinylating enzyme(s). This enhanced role of the pantetheine cofactor may explain why recombinant peptide synthetases are apparently synthesized with much reduced enzyme activity compared with their "wild-type" counterparts (see section VIII).[43] Clearly significant absence of these apparently important cofactors would result in less active enzyme. The identification of the enzyme probably responsible for carrying out the phosphopantetheinylation of proteins *in vivo* may help to overcome this problem (section VIII).[72]

It is not clear from this modification of the thiol-template mechanism what mechanisms are invoked to replace the 4′-phosphopantetheine "swinging arm" of the original proposal. There are no supernumerary potential phosphopantetheinylation sites which might harbor an additional 4′-phosphopantetheine to function as a "swinging arm" detected in excess over those required to replace the "thiol templates" with "pan carriers".

## C. The "Direct Acyl-Transfer" Mechanism

One drawback of the "thiol-template" and "multipan carrier" models is the large number of acyl-transfer steps required for the synthesis of even a tripeptide. Recent observations have led to a re-evaluation of the evidence for the "aminoacyl transfer to enzyme thiols" proposal, in both its formulations, for the biosynthesis of ACV in the light of various observations on the mechanism gleaned from the use of small-molecule probes. The working hypothesis is that the individual partial reactions of the synthetase, in common with the overall reaction sequence, have comparatively lax substrate selectivities. In consequence, they act as a series of "gates" in the overall catalytic cycle through which some, but not all, small molecule probes can pass to yield complete tripeptide products.[27] Other substrate analogues can pass through only some of the "gates" and are thus "dead-ended" at various specific points in the overall reaction sequence. Analysis of the these "incomplete" products resulting from a truncated reaction sequence thus provides information on the timing and sequence of partial reactions required for their enzymic synthesis. Selected analogues can therefore act effectively as (sometimes ready-made) mechanistic probes for nonribosomal peptide synthesis by ACV synthetase. By implication, an analogous approach could also be exploited for similar mechanistic studies on related peptide synthetases. This would help to establish or not the generality of the mechanism of non ribosomal peptide bond synthesis proposed in this section.

Observations made using this approach are apparently at variance with the obligatory occurrence of enzyme−substrate thioesters in the thiol-template and multi-pan carrier mechanisms. The implicit assumption, by analogy to the ribosomal system, that nonribosomal peptide synthesis must *a priori* always occur in the N- to C-terminal direction is additionally cast in some doubt. The relevant observations detailed in previous sections may be summarized as follows.

(a) The recovery of both L,L- and L,D-*O*-(methylserinyl)valine from incubations containing the ACV synthetase, MgATP, L-α-aminoadipate, L-*O*-(methylserinyl)valine, and L-valine implies that the α-peptide bond between cysteine and valine is formed before the δ-peptide bond between cysteine and α-aminoadipate. This was supported by the observation that incubations of L-glutamate, L-cysteine, and L-valine with the synthetase resulted in the synthesis of L,D-cysteinylvaline. In this case, the L-glutamate would seem to act as an analogue of L-α-aminoadipate for the aminoacyl adenylation reaction but the γ-carboxyl is unable to participate in peptide bond formation. An L-glutamate concentration-dependent enhancement of the rate of L,D-cysteinylvaline synthesis can be quantified (section IV.D). Amounts of *O*-(methylserinyl)valine-containing dipeptides synthesized in the absence of L-α-aminoadipate, and similarly L,D-cysteinylvaline synthesized in the absence of L-glutamate were comparatively low.

(b) The recovery of both L,L- and L,D-*O*-(methylserinyl)valine implies that inversion of the valine α-center occurs after peptide bond formation. That the L,L-*O*-(methylserinyl)valine is an intermediate in the synthesis of the L,D-*O*-(methylserinyl)valine is indicated by the isotope effect observed when the peptides are synthesized using 2-[$^2$H]valine which reduces the yield of the L,D diastereoisomer.

(c) L,L-*O*-(Methylserinyl)valine is produced in experiments using [$^{18}O_2$]-labeled valine in which the valine residue retains the two $^{18}$O labels of the valine substrate. This demonstrates that participation of the valine carboxyl group in a thioester bond to the enzyme, which occurs during the synthesis of ACV itself at some point (see point e, below), occurs after peptide bond formation and that peptide bond formation does not (obligatorily) require prior thioesterification of at least one participant amino acid in the bond. This is consistent with the negative results obtained for both $^{18}$O transfer from labeled $^{18}O_2$ valine to AMP and failure also to detect any loss of $^{18}$O from $^{18}O_2$ valine (section IV.C).

(d) The bulk of L,D-*O*-(methylserinyl)valine produced in experiments using [$^{18}O_2$]-labeled valine is also synthesized with the retention of both $^{18}$O labels of the substrate. This implies that the epimerization of the valine residue in the peptide-bound state also occurs before there is any thioesterification of the valine residue. Therefore, this partial reaction is also independent of any thioester formation to the enzyme.

(e) Notwithstanding the results summarized in c and d there is loss of (only) one $^{18}$O label from $^{18}O_2$ valine in the biosynthesis ACV and some loss of one $^{18}$O label from $^{18}O_2$ valine in the synthesis of L,D-*O*-(methylserinyl)valine. This is probably due to (thio)ester formation to the valine residue at some point in the catalytic cycle not obligatory for peptide bond formation or epimerization. The observed loss of the $^{18}$O label from ACV is therefore proposed to occur when the dipeptide intermediate is translocated by the 4′-phosphopantetheine to the site at which δ-peptide bond formation to the α-aminoadipoyl adenylate occurs. Incorporation of significant $^{18}$O label from $H_2{}^{18}O$ specifically into (only) the valine residue probably arises from final cleavage of the thioesterified complete tripeptide from the enzyme. Conversely, there is no significant incorporation observed of $^{18}$O label from $H_2{}^{18}O$ into the valine substrate when it is incubated alone with ACV synthetase, MgATP and valine in $^{18}$O-enriched water. Thus either (i) thioesterification of the valine substrate to the enzyme is effectively irreversible under these conditions or (ii) thioesterification of the valine residue occurs after an irreversible step. This latter option is attractive since the irreversible step could represent peptide bond formation consistent with the observations in c, above.

These observations have prompted the proposal of a new mechanism[27,33,36] for ACV biosynthesis. In the "direct acyl-transfer" mechanism (Figure 15) no thioesters are formed between the individual amino acid substrates and the enzyme. Rather, the aminoacyl adenylates themselves donate the activated aminoacyl groups directly to the α-amino group of the other participant amino acid in the peptide bond to be formed. Energetically there is no need for any intermediate acyl-transfer steps since amide bond

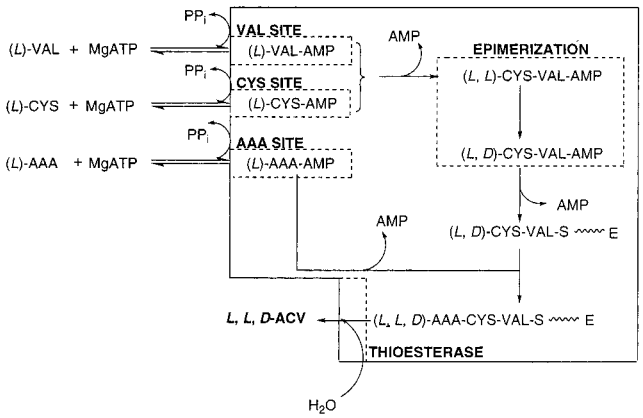


**Figure 15.** Direct acyl transfer mechanism for ACV biosynthesis. Details are the same as in Figure 13.

formation from the aminoacyl adenylate is thermodynamically favorable. In the ribosomal system an intermediate acyl-transfer step is obviously essential due to the function of the tRNA as an adapter molecule. In enzymic peptide synthesis each peptide is synthesized by a specific multifunctional enzyme and so aminoacylation of an additional nucleophile could be superfluous. One consequence of this reduction in the number of acyl-transfer steps however is that, assuming linear synthesis, the elaboration of the peptide necessarily occurs from the C- to the N-terminus. Although the reported "shunt" products produced by gramicidin S synthetase[18] imply synthesis of the product peptide in the N- to C-terminal direction this does not necessarily preclude synthesis of a short peptide effectively in the C- to the N-terminal direction. Precedent exists for the direct donation of aminoacyl groups from enzyme bound aminoacyl adenylates to amino groups of another substrate with no intermediate acyl transfer step(s). For example the *bir*A gene product in *Escherichia coli* catalyzes a similar reaction in which biotin is activated as an enzyme-bound biotinyl 5′-adenylate prior to direct transfer to the primary $\epsilon$-amino groups of specific lysine residues in enzymes requiring post-translational 5′-biotinylation.[73]

Esterification must occur between the valine residue of ACV at some stage in the its synthesis (see lines of evidence reviewed in section IV.C). These observations should be incorporated in to the direct acyl-transfer proposal. Similarly the occurrence of at least one 4′-phosphopantetheine group in ACV synthetase (section VII.A) must also be accounted for. In the direct acyl-transfer model thioesterification is not required for synthesis of the peptide bonds but prevents dipeptide intermediates from being lost to the aqueous medium and translocates them between active sites (cf., the thiol-template mechanism). This is consistent with the observed loss of the dipeptide shunt products from the enzyme as this step acts as a "gate" in the complete catalytic cycle with a potential "proof-reading" capacity. It is proposed that this is achieved by thioester formation between the valine carboxylate of a dipeptide intermediate (only) and the thiol of the detected 4′-phosphopanthetheine cofactor. This accounts for the observed loss of a single $^{18}O$ atom from the valine carboxylate during ACV synthesis and rationalizes activation of the valine carboxylate. This step sequesters the dipeptide intermediate prior to translocation to the active site at which the formation of the $\delta$-peptide bond to $\alpha$-aminoadipate occurs.

Such a thioesterification reaction may help to explain the observed binding of [$^{14}C$]valine to ACV synthetase during electrophoresis in the presence of sodium dodecyl sulfate (section IV.C).[16] Potentially, small amounts of tightly bound residual cysteinyladenylate might result in peptide bond formation to the labeled valine to give a dipeptide that then reacts with the enzyme bound 4′-phosphopantetheinyl cofactor to give a thioester complex. Alternatively, weak activation of the labeled valine itself at the *cysteine* activating site might have resulted in the synthesis of the doubly labeled dipeptide L-valinyl-D-valine which is subsequently covalently bound to the enzyme bound thiol. Some evidence has been accrued in analogue screening assays which is apparently consistent with the formation of small amounts of ($\alpha$-aminoadipoyl)valinylvaline.[26] Furthermore, small amounts of L-($\alpha$-aminoadipoyl)-L-valinyl-D-valine have been isolated from large-scale fermentations of *C. acremonium*, indicating the ability of valine to substitute weakly for cysteine at the cysteine activating site in ACV synthetase from this source.[74]

Ultimately in the revised proposal, the complete ACV tripeptide product is released from the enzyme bound 4′-phosphopantetheinyl moiety by a thioesterase activity, presumably corresponding to the C-terminal region in the sequence with sequence identity to thioesterases (section IV.F). This ester hydrolysis would account for the observed incorporation of $^{18}O$ from $H_2{}^{18}O$ into the valine residue (only) of the product, ACV.[27]

In summary, this alternative proposal (Figure 15) incorporates the following sequential steps: (1) activation of the amino acid substrates as enzyme-bound aminoacyl adenylates; (2) formation of the $\alpha$ peptide bond between cysteine and valine; (3) epimerization of the valine residue in the synthesis of dipeptide shunt products (this would also seem likely to occur in the synthesis of ACV, but the possibility that it occurs in the tripeptide stage in this case cannot explicitly be ruled out); (4) transfer of the dipeptide intermediate to the thiol of the 4′-phosphopantetheine cofactor, which is required for translocation of the intermediate dipeptide to another active site (this may also act as a "proof-reading" step); (5) formation of the $\delta$-peptide bond to $\alpha$-aminoadipate followed by a further translocation; and (6) release of the complete tripeptide product from the enzyme by a thioesterase activity.

ACV synthetase has been shown to contain (minimally) one phosphopantetheine group per polypeptide chain but contains three potential sites for 4′-phosphopantetheinylation.[19,20,65] If indeed the catalytic cycle of ACV synthetase involves the formation of a single thioester intermediate a question arises as to why the apparently redundant sites and possibly any attached 4′-phosphopantetheine groups are conserved. The currently available evidence does not explicitly rule out *irreversible* thioester formation to the $\alpha$-aminoadipate or cysteine carboxyl groups. If this really does not occur it may be that the conserved

regions have structural roles or are evolutionary "fossils" currently retained in all ACV synthetase modules.

If the direct acyl transfer mechanism could be completely validated for ACV synthetase it is likely to apply, at least in part, to nonribosomal peptide bond formation by other peptide synthetases since sequence comparisons indicate that they constitute a closely related class of enzymes.

## VIII. Summary and Perspectives

The initial uncertainty regarding the nature of the enzyme system responsible for ACV biosynthesis, derived by analogy to glutathione biosynthesis, has given way to the universally accepted viewpoint that a large peptide synthetase is responsible for the first step in the $\beta$-lactam biosynthetic pathway, the synthesis of the Arnstein tripeptide, ACV. The energy cost to the organism of synthesizing over three-and-half thousand peptide bonds ultimately to catalyze formation of only two such bonds and also synthesize a large mRNA and replicate such a large gene must be considerable. At some point there must have been selective pressure preferentially to recruit and adapt peptide synthetase modules to the synthesis of the $\beta$-lactams rather than adapt the smaller enzymes synthesizing glutathione, a tripeptide similar to ACV. Possibly mutations in the glutathione synthesizing enzymes are almost invariably lethal to the organism due to the crucial role of this metabolite thus precluding easy adaptation of these enzymes to the synthesis of a similar tripeptide.

The realization that ACV synthetase was a single multifunctional enzyme (section III) and subsequent application of modern DNA sequencing methodology (section VI) led to a clear picture of a tridomain structure. The individual domains are homologous to the same domains featured in other enzymes synthesizing peptides. That these structural similarities between peptide synthetases are reflected in functional relationships between members of this class of enzymes has been shown elegantly by the construction of functional chimaeric synthetases (section VI).

Isolation of ACV synthetases from a variety of $\beta$-lactam-producing organisms followed by the evaluation of several properties of the isolated enzyme led to the proposal that the enzyme shared the same thiol-template mechanism for nonribosomal peptide synthesis already proposed for its homologues synthesizing antibiotically active peptides (section IV). These properties include substrate amino acid-dependent ATP$-$PP$_i$ exchange and incorporation of radiolabeled valine into the enzyme. It was shown that ACV synthetase shares with other peptide synthetases a comparatively lax substrate specificity: each amino acid in the final tripeptide can be replaced with a structural analogue (section V). These studies also revealed that not all potential alternative substrates lead to the anticipated tripeptide product. Analogues were identified which stimulated ATP$-$PP$_i$ exchange but failed detectably to participate in peptide bond formation. Other analogues were identified, most notably *O*-methylserine, that were able to participate in further, but not all, partial reactions in the catalytic cycle. These observations led to the proposal that the individual partial reactions in the catalytic cycle are analogous to a series of "gates" which allow the passage of some substrates through some, but not all, steps in the synthesis. The release of dipeptide products formed from some analogues of the natural substrates indicates an ability of the ACV synthetase to abort the catalytic sequence when an "incorrect" dipeptide is synthesized. That these "incorrect" intermediates can be released from the enzyme could reflect a primitive form of "proofreading" capacity in the synthetase. That the dipeptides formed from these analogues always contained valine but never $\alpha$-aminoadipate is consistent with the view that the intermediate in the synthesis of ACV itself is cysteinylvaline. That the dipeptides could be recovered in both diastereoisomeric forms showed that the epimerization of the valine residue occurred after peptide bond formation. Synthesis of one peptide bond by the synthetase, that between L-*O*-methylserine and L-valine, without prior thioesterification of the valine has led to a new mechanistic proposal for ACV synthetase. This is clearly distinct from the thiol-template proposal, since it has to incorporate several features of the synthesis found inconsistent with the application of this mechanism to ACV synthesis.

The modular construction of the peptide synthetases from homologous domains suggests immediately the possibility of expressing the individual modules which might retain catalytic competence in some of the reactions of the overall synthetic sequence. Thus far this approach has apparently been of limited usefulness.[75] This might be due to the fact that the individual domains are not in a fully functionally active conformation when expressed separately. That there is a considerable degree of conformational signaling between distinct structural domains would seem likely in the coordinated operation of the several partial reactions required for peptide synthesis. The effect of activating the L-$\alpha$ aminoadipate aminoacyl adenylation site with the analogue L-glutamate to cause significant enhancement in the production of L,D-cysteinylvaline by ACV synthetase would appear to be a case in point (section IV.C). Similar effects have been documented in other large enzymes carrying out coordinated multistep reactions at distinct distant active sites, e.g., tryptophan synthase.[76]

With regard to establishing the mechanism of ACV synthetase it is obviously desirable to determine its three-dimensional structure. Such an effort would be facilitated by the cloning and overexpression of fully functional ACV synthetase. Overproduction of fully functional complete peptide synthetases has been a somewhat difficult endeavor. One possible explanation for the lack of active synthetase is that there may be incorrect folding of such a large polypeptide in *E. coli*. Alternatively there may be a lack of the 4′-phosphopantetheine cofactor.[43] Whatever the functions of this moiety may in fact be (section VII) it would appear that inefficient 4′-phosphopantetheinylation of the synthetases in *E. coli* results in inactive "apoenzyme" product. Incomplete post-translational 4′-phosphopantetheinylation

in *E. coli* could be due to the fact there are several distinct 4′-phosphopantetheinylating enzymes modifying different protein substrates.[77] It is noteworthy that the *E. coli* pantetheinylating enzyme can pantetheinylate the polyketide synthetase acyl carrier proteins in yeast but not apparently the peptide synthetases.[78] Similar problems led to the ultimate successful expression of active mammalian fatty acid synthase in the insect baculoviral system enabling site-directed mutagenesis studies of this multienzyme.[79] This enzyme shares some features with the peptide synthetases in that it is a large polypeptide requiring phosphopantetheinylation of a specific serine residue for activity. This suggests that baculoviral expression might also be a fruitful avenue for the expression of fully active peptide synthetases. Alternatively cooverexpression of the enzyme responsible for the phosphopantetheinylation of the peptide synthetases with various peptide synthetases in *E. coli* might enable more complete phosphopantetheinylation of the enzymes to be achieved leading to fuller activity.[72,77,78]

The elucidation of the precise series of mechanistic steps of ACV synthetase, aided by the complete three-dimensional structure of the multifunctional enzyme would be a significant step toward the ultimate goal of engineering peptide synthetases for the synthesis of novel peptides of choice.

## IX. Acknowledgments

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