L-δ-(α-Aminoadipoyl)-L-cysteinyl-D-valine Synthetase: Thioesterification of Valine Is Not Obligatory for Peptide Bond Formation†

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ABSTRACT: L-δ-(α-Aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase is probably the simplest known peptide synthetase in terms of the number of reactions catalyzed. In the "thiol-template" proposal for nonribosomal peptide synthesis, a key step is transfer of aminoacyl groups derived from the substrates to enzyme-bound thiols prior to peptide bond formation. No incorporation of ^{18}O was seen in AMP isolated from the reaction mixture when di[^{18}O]valine was incubated with relatively large amounts of active synthetase and MgATP. We therefore utilized di[^{18}O]valine as a substrate for the biosynthesis of the diastereomeric dipeptides L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine [Shiau, C.-Y., Baldwin, J. E., Byford, M. F., Sobey, W. J., & Schofield, C. J. (1995) *FEBS Lett. 358*, 97−100]. In the L-*O*-(methylserinyl)-L-valine product, no significant loss of ^{18}O was observed. However, in the L-*O*-(methylserinyl)-D-valine product, a significant loss of one or both ^{18}O labels was observed. Thus, both peptide bond formation and the epimerization of the valine residue can both occur before formation of any thioester bond to the valine carboxylate in the biosynthesis of these dipeptides. The usual qualitative test for thioesterification of substrates to the synthetase, lability of enzyme-bound radiolabeled amino acid to performic acid, proved inconclusive in our hands. These results require a new mechanism for the enzymic synthesis of L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine and imply that a revised mechanism for ACV synthesis is also required.

Apart from the well-characterized ribosomal route, synthesis of peptide bonds by enzymes is also of importance in nature. Individual enzymes catalyze the formation of peptide bonds in, for example, glutathione and peptidoglycan synthesis, whereas a diverse range of biologically active peptides are synthesized by large multifunctional peptide synthetases which share some features in common with polyketide synthetases [for a review, see Kleinkauf and von Döhren (1990)]. The peptide synthetases are of considerable interest as they are involved in the synthesis of several secondary metabolites of clinical importance (e.g. β-lactam antibiotics and cyclosporin) and they allow the incorporation of nonproteinogenic amino acids into peptides. The peptide synthetases might ultimately be rationally designed to synthesize efficiently peptides of choice. This potential application of these enzymes, however, initially requires a more detailed knowledge of the catalytic mechanism of the synthetases than is available currently.

The complete primary structures of several peptide synthetases have been inferred from DNA sequencing (Díez et al., 1990; Smith et al., 1990; MacCabe et al., 1991; Gutíerrez et al., 1991; Turgay et al., 1992; Marahiel, 1992; Pavela-Vrancic et al., 1994). They are clearly a related group of enzymes and are comprised of concatenated homologous domains, one for each amino acid incorporated into the final peptide product. Domains from one synthetase have been incorporated into another, resulting in a chimeric synthetase capable of synthesizing the expected peptide product (Stachelhaus et al., 1995). Despite the wealth of sequence information available on the peptide synthetases, the precise coordination and molecular mechanism of the individual partial reactions required to synthesize even a small peptide have not so far been unequivocally determined. The conventional mechanistic proposal for the peptide synthetases is the "thiol-template" mechanism proposed by Lipmann (1971) and developed by Laland and Zimmer (1973) and is derived largely from analogy to the mechanism of fatty acid biosynthesis. The proposal is supported by the observation that small amounts of ^{14}C-radiolabeled substrate amino acids are recovered in trichloroacetic acid precipitates of purified peptide synthetases when the enzyme and substrate are incubated together in the presence of MgATP. The radiolabel is preferentially released from the protein precipitate by performic rather than formic acid, and thus, it is concluded that a thioester bond exists between the amino acid and the enzyme. In the thiol-template proposal (Scheme 1), the amino acid substrates are initially activated as their enzyme-bound aminoacyladenylates. This can be demonstrated by the ability of the synthetases to catalyze an amino acid-dependent ATP−PP_i exchange reaction in response to substrate amino acids (van Liempt et al., 1989; Vater, 1990). The activated aminoacyl groups are then transferred to specific enzyme-bound thiols (the thiol templates). Next, the N-terminal aminoacyl group is transferred to the thiol of a 4′-phosphopantetheine cofactor covalently bound to the enzyme. The peptide bond is then proposed to be formed by nucleophilic displacement of the thiol group by the amino group of the amino acid C-terminal to the first amino acid,

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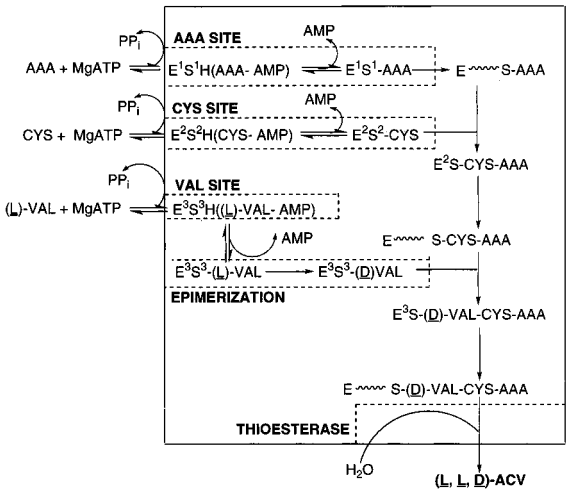
Scheme 1: Thiol-Template Mechanism for ACV Synthetase[a]



[a] Note that the enzyme-bound cysteine thiols have recently been replaced with further 4′-phosphopantetheinate moieties in the multipan carrier model (Stein et al., 1994). Note also that recently it has been shown that in peptide synthetases containing epimerized residues not at the N terminus the epimerization occurs in the "peptide-bound" state (Stindl & Keller, 1994; Shiau et al., 1995a). Independent sites for the aminoacyladenylation, epimerization, and thioesterase partial reactions are shown boxed (dashed lines).

thus forming an enzyme-bound dipeptide. This intermediate is then relocated to the thiol of the liberated 4′-phosphopantetheine cofactor and subsequently displaced by the amino group of the next amino acid to be incorporated. This reaction sequence is repeated at each of the thiol templates until the complete peptide product is released from the enzyme by a thioesterase activity. Although the thiol templates themselves were proposed originally to be cysteinyl residues, there are no obviously conserved cysteines in the available inferred sequences. In the revised multipan carrier model (Stein et al., 1994), multiple covalently bound phosphopantetheine cofactors are invoked. These are proposed to be attached to conserved serinyl residues found in common sequence motifs and apparently now replace the key cysteinyl residues of the original thiol-template proposal.

We have been investigating the enzyme mechanism of ACV[1] synthetase (Banko et al., 1987; van Liempt et al., 1989), which is responsible for the biosynthesis of the Arnstein tripeptide [L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine], the common tripeptide precursor of the penicillin and cephalosporin antibiotics. Because the enzyme synthesizes only a tripeptide, it represents a simple example of the mechanism of nonribosomal peptide bond formation, since comparatively few individual reactions are required for formation of only two peptide bonds. Recently, we observed that incubation of the cysteine analogue L-*O*-methylserine, L-α-aminoadipate, and L-valine with ACV synthetase and MgATP resulted in the unexpected synthesis of both L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine (Shiau et al., 1995a). This presumably reflects an inability of the synthetase to (a) complete tripeptide synthesis and (b) fully epimerize the valine residue when an unnatural analogue is incorporated in place of L-cysteine. By contrast, incubation of L-cysteine, L-valine, and the L-α-aminoadipate analogue L-glutamate resulted in the recovery of only L-cysteinyl-D-valine (Shiau et al., 1995b). These observations, taken alone, do not explicitly prove that L-cysteinyl-L-valine and L-cysteinyl-D-valine dipeptides themselves are viable kinetic intermediates in the synthesis of ACV. Additionally, it is important to note that the epimerization could occur at the tripeptide stage during the synthesis of ACV. However, the results clearly merit further work regarding determination of the timing of thioesterification of the valine residue relative to the timing of peptide bond synthesis to the valine α-amino group catalyzed by the enzyme. We have shown unequivocally that there is indeed esterification of the valine carboxylate at some stage in the ACV synthetase catalytic cycle since there is incorporation of a single ^{18}O from ^{18}O-enriched water specifically into the valine residue of the tripeptide product (Shiau et al., 1995c). However, we have been unable to detect any loss of ^{18}O from [$^{18}O_2$]valine when it was incubated with ACV synthetase in the presence or absence of other amino acid substrates (Baldwin et al., 1991, 1992). In this paper we describe an investigation into the timing of the loss of the ^{18}O label from di[^{18}O]valine in both dipeptide products L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine. Our results demonstrate clearly that formation of the peptide bond between L-valine and L-*O*-methylserine occurs before there is any formation of a covalent thioester bond to the valine carboxyl group. Thus, thioesterification of at least the valine residue is not obligatory for peptide bond formation to the valine amino group by the peptide synthetase.

EXPERIMENTAL PROCEDURES

General. ACV synthetase was prepared from *Cephalosporium acremonium* as described previously (Baldwin et al., 1993) routinely through the Superdex S-200 stage. Protein determinations were as described by Bradford (1976). Electrospray ionization mass spectroscopy (MS) was done as described earlier (Baldwin et al., 1994). L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine were isolated from preparative scale incubations as described before (Shiau et al., 1995a). Homogeneity of the L,L diastereoisomer was achieved and established by a second hplc run using the described protocol, whereas homogeneous L-*O*-(methylserinyl)-D-valine required a third hplc run to achieve and establish removal of any contaminating L,L diastereoisomer.

Incubations of DL-[$^{18}O_2$]valine, MgATP, and ACV Synthetase. The incubations contained $MgCl_2$ (7.5 mM), ATP (2.5 mM), DL-[$^{18}O_2$]valine (5 mM), dithiothreitol (1.5 mM) in Tris-HCl (50 mM, pH 7.5), and ACV synthetase (6 mL, 1.5 nkat of ACV synthetase activity in 50 mM Tris-HCl at pH 7.5) in a final volume of 6.5 mL. After termination of the reaction with an equal volume of ice-cold acetone, the sample was held on ice for 5 min and the protein was removed by centrifugation in a microfuge (13 000 rpm, 5 min). The supernatant fraction was dried *in vacuo,* and the residue was dissolved in water (1.5 mL). ATP, AMP, and valine were isolated from the mixture using ion pair reverse phase chromatography (Hypersil C_{18} column, 4.6 × 250 mm). The chromatographic procedure was modified from a published procedure (Stocchi et al., 1985) and was optimized using authentic standards. The mobile phase was

[1] Abbreviations: ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; AAA, α-aminoadipic acid; Xaa-AMP, aminoacyladenylate of amino acid Xaa; ~~ E, covalently bound 4′-phosphopantetheinate cofactor; Ad, adenosine.

3 mM $NH_4H_2PO_4$ (pH 6.5) containing 1.5% (v/v) methanol. The modifier was obtained by bringing the methanol concentration to 5% (v/v). The column was developed using a step gradient to 100% modifier after 4.5 min, and isocratic elution was continued for 25.5 min before returning to initial conditions. The flow rate was 1 mL/min. Retention volumes are in parentheses: valine (3.8 mL), AMP (18.9 mL), and ATP (26.3 mL). Fractions containing these components were freeze-dried to a minimal residue and redissolved in water. The fractions were desalted using a reverse phase Hypersil C_{18} column (4.6 × 250 mm). For fractions containing valine, the mobile phase was 20 mM NH_4HCO_3 in 20% (v/v) aqueous methanol at a flow rate of 1 mL/min and the retention volume was 3.3 mL. For fractions containing ATP and AMP, the mobile phase was 25 mM aqueous NH_4HCO_3 at a flow rate of 1 mL/min. The retention volumes of these nucleotides varied from 5 to 7.5 mL depending on the injection volume and the content of the residual triethylamine salt. The AMP was further purified by running the column with a mobile phase of 24 mM NH_4HCO_3 in 3.5% (v/v) aqueous methanol at 1 mL/min, with a retention volume of 3.7 mL.

Analysis of Radiolabeled Amino Acids Remaining Bound to Protein Preparations after Trichloroacetic Acid Precipitation. Incubations were done in 50 mM Tris-HCl (pH 7.5) containing 0.5 μCi of labeled amino acid, 2 mM dithiothreitol, 2 mM ATP, 5 mM magnesium acetate, and routinely 45 μg of protein preparation (approximately 90 pkat of peptide synthetase activity). Aliquots (routinely 25 μL) of incubation mixtures were taken at the required times, spotted onto 2 cm^2 squares of Whatman 3MM filter paper, and dropped immediately into ice-cold 10% (w/v) trichloroacetic acid (approximately 5 mL/filter). After 5 min, the acid was replaced with a fresh solution. After 5−10 min, the squares were washed with the same volume of water for 5−10 min followed by ethanol (5 min) and finally a small volume of diethyl ether. The residual ether was evaporated in a fume hood. When dry, the papers were counted in toluene-based scintillant to a preset counting error of ±2.0%. Performic acid oxidation of the precipitates on the filters was done at 4 °C by saturating the squares with 200 μL of performic acid, freshly prepared as described in Hirs (1967). The performic acid treatment was not significantly deleterious to the integrity of the filter squares over the time courses used. The performic acid oxidations were quenched at the required times by dropping the filters into 1 L of water. The papers were dried and counted as described above.

Materials. [$^{18}O_2$]Valine was synthesized essentially as described by O'Donnell and Ekrich (1978). Authentic L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine were synthesized by standard solution phase methods (Shiau, 1994) and their structures confirmed by electrospray ionization MS and ^{1}H NMR spectroscopy. Alcohol dehydrogenase (yeast) was from Boehringer Mannheim (Mannheim, Germany). The liquid scintillation fluid was BetaMax (ICN, Thame, Oxon, U.K.). [U-^{14}C]Valine (265 mCi/mmol), [U-^{14}C]proline (260 mCi/mmol), [3,4-^{3}H]valine (57.3 Ci/mmol), and [U-^{14}C]cystine (260 mCi/mmol) were from Amersham (Little Chalfont, Bucks, U.K.). [U-^{14}C]Cystine generated [U-^{14}C]cysteine *in situ* in the incubations due to the presence of dithiothreitol.

RESULTS

Incubations Using ACV Synthetase, DL-[$^{18}O_2$]*Valine and MgATP.* In principle, if incubation of di[$^{18}O_2$]valine, MgATP, and ACV synthetase results in irreversible thioester formation between the enzyme and the valine, there should be incorporation of a single ^{18}O atom into the AMP released from the aminoacyladenylate, in a yield stoichiometric with the amount of valinoylated enzyme formed during the duration of the incubation. If thioester formation is freely reversible under these conditions, there is clearly scope for the incorporation of further ^{18}O atoms. The possibility of detecting ^{18}O incorporation into AMP as a consequence of the postulated thiolation reaction was specifically investigated. ACV synthetase was incubated with DL-[$^{18}O_2$]valine and MgATP. The use of racemic labeled valine does not affect the outcome of any of the experiments since D-valine is not a substrate for the initial obligatory aminoacyladenylation reaction (Baldwin et al., 1994). As much ACV synthetase as possible was committed to these experiments (1.5 nkat of peptide synthetase activity per incubation) since the enzyme itself is a reactant in this proposed partial reaction and thus any irreversible transfer of the labeled oxygen to AMP would result from a single-turnover reaction. After termination of the reaction with acetone, the valine, ATP, and AMP present in the supernatant were separated by ion pair reverse phase hplc. Desalted fractions containing AMP were further purified by reverse phase hplc (see Experimental Procedures). The three recovered compounds were analyzed for their ^{18}O content by electrospray ionization MS. We failed to observe any incorporation of ^{18}O into either AMP or ATP or any loss of ^{18}O from valine. Clearly, this observation does not explicitly exclude the possibility of thioester formation to the enzyme under these conditions since the aminoacyl transfer reaction might be occurring in a yield too low to be easily detected with the approach and time scale used. We therefore decided to determine the pattern of ^{18}O labeling after the formation of a peptide bond to the valine residue.

Incubations Using ACV Synthetase, DL-[$^{18}O_2$]*Valine,* L-*O-Methylserine,* L-α-*Aminoadipate, and MgATP.* Incubations of L-valine, L-*O*-methylserine, L-α-aminoadipate, and MgATP with ACV synthetase result in the formation of both L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine dipeptides in high enough yield (approximately 5% that seen with the natural tripeptide product) to allow their analysis by electrospray ionization MS and ^{1}H NMR spectroscopy. The presence of L-α-aminoadipate is necessary to enhance significantly production of the unnatural dipeptide products (Shiau et al., 1995a). These dipeptides are produced catalytically by the enzyme and released to accumulate in solution. We therefore sought to examine the labeling pattern of the valine oxygen atoms after the catalytic turnover required for the synthesis of these dipeptide products. Preparative scale incubations were performed with DL-[$^{18}O_2$]valine, L-α-aminoadipate, and L-*O*-methylserine with ACV synthetase and MgATP. Both the L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine diastereoisomers were isolated (Shiau et al., 1995a). The effluent from the hplc runs containing the desired peptides was pooled for maximum chromatographic resolution of the diastereoisomers rather than maximum yield of the dipeptides. Homogeneity of the diastereoisomers was further improved and analyzed

Table 1[a]

	% observed							
	$^{16}O_2$		$^{18}O^{16}O$			$^{18}O_2$		
entry	*m/z* 218	*m/z* 219	*m/z* 220	*m/z* 221	*m/z* 222	*m/z* 223	*m/z* 224	*m/z* 225
authentic dipeptide								
synthetic L,L-*O*-(methylserinyl)valine	0	100	13	0	0	0	0	0
synthetic L,D-*O*-(methylserinyl)valine	0	100	11	2	4	0	0	0
dipeptide after first hplc								
biosynthetic L,L-*O*-(methylserinyl)valine	0	3	3	15	0	100	11	1
biosynthetic L,D-*O*-(methylserinyl)valine	0	32	3	77	0	100	11	5
dipeptide after second hplc								
biosynthetic L,L-*O*-(methylserinyl)valine	3	8	0	17	3	100	9	1
biosynthetic L,D-*O*-(methylserinyl)valine	0	22	2	75	9	100	10	1
dipeptide after third hplc								
biosynthetic L,D-*O*-(methylserinyl)valine	0	21	0	51	0	100	17	0

	% observed							
	$^{16}O_2$		$^{18}O^{16}O$			$^{18}O_2$		
entry	*m/z* 117	*m/z* 118	*m/z* 119	*m/z* 120	*m/z* 121	*m/z* 122	*m/z* 123	*m/z* 124
authentic synthetic [$^{18}O_2$]valine	0	5	0	7	0	100	7	1

[a] Incubations were done in the presence of α-aminoadipic acid. In the absence of α-aminoadipic acid, the results were virtually identical within the limits of experimental error, although the apparent yields of dipeptides were lower.

by a second hplc run for the L,L diastereoisomer and also a final third run for the L,D diastereoisomer.

Analysis of the product diastereoisomers by electrospray ionization MS gave unexpected results clearly at variance with those anticipated by application of the thiol-template mechanism to the synthesis of these dipeptides (Table 1). The L,L diastereoisomer retained both the ^{18}O atoms showing unequivocally that the peptide bond to the valine amino group was formed prior to participation of the valine carboxylate in any thioester bond. Even more interesting was the observation that the L,D diastereoisomer was recovered with all three possibilities for labeling of the valine carboxyl oxygens. Most of this isomer retained both the ^{18}O atoms; another species resolved by MS had lost one ^{18}O label, and the third minor species detected had lost both ^{18}O atoms. These labeling patterns were consistent, within experimental error, in three independent experiments. A similar experiment in which L-α-aminoadipate was omitted from the incubation produced qualitatively identical results, although the yield of the products was significantly lower (not shown). This observation is consistent with the presence of the α-aminoadipoylacyladenylate being required for the maximum rate of dipeptide synthesis. Some possibilities for the labeling pattern observed for the L,D diastereoisomer will be suggested in the Discussion.

Binding of Radiolabeled Amino Acids to ACV Synthetase. Having failed to detect the postulated incorporation of ^{18}O from di[^{18}O]valine into AMP expected to result from ACV synthetase valinoylation and having failed also to observe any loss of ^{18}O from di[^{18}O]valine prior to peptide bond formation to the valine during the synthesis of L-*O*-(methylserinyl)-L-valine, we reassessed the available evidence for the occurrence of thioester formation between individual amino acid substrates and ACV synthetase. To facilitate rapid handling and rigorous washing of trichloroacetic acid precipitates, aliquots of the incubation mixtures were spotted on filter paper squares and the ACV synthetase was precipitated into the filter squares by dropping them into trichloroacetic acid (see Experimental Procedures). Bound radiolabeled amino acids were quantified by liquid scintillation counting of the extensively washed, dried filter papers using a toluene-based scintillant.

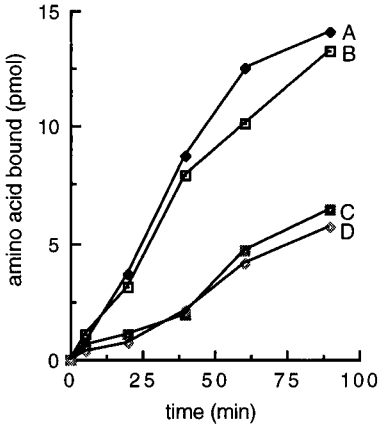


FIGURE 1: Binding of radiolabeled substrates to ACV synthetase. [^{14}C]Cysteine and [^{3}H]valine were incubated over time with ACV synthetase (150 μg/mL, 45 μg) and the radioactivity bound to the trichloroacetic acid-precipitated material determined: (A) [^{14}C]-cysteine with MgATP, (B) [^{3}H]valine with MgATP, (C) [^{14}C]-cysteine without MgATP, and (D) [^{3}H]valine without MgATP. Control values (not shown for clarity) for incorporation of both radiolabeled substrates into alcohol dehydrogenase and active ACV synthetase in the absence of MgATP were essentially identical to the heat-inactivated controls. Values shown are the means of triplicate determinations.

Both [^{14}C]cysteine and [^{3}H]valine (diluted to the same specific activity as the [^{14}C]cysteine with unlabeled valine) were recovered in trichloroacetic acid precipitates when incubated with ACV synthetase and MgATP. The binding of the labeled amino acids was time-dependent (Figure 1). There was also substantial binding of the labeled substrates in the absence of MgATP which was also time-dependent and accounted for up to 40−50% of the total binding observed. We investigated this effect specifically using heat-inactivated (5 min, 95 °C) ACV synthetase and alcohol dehydrogenase as control protein preparations at the same concentration of protein as the active ACV synthetase. Both the control preparations bound also approximately 50% the total labeled valine that was seen with the active enzyme.

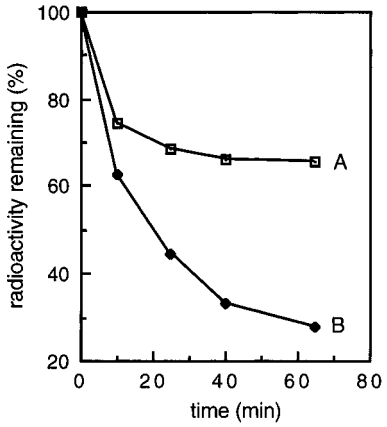


FIGURE 2: Release of ^{14}C-labeled cysteine from ACV synthetase by formic and performic acids. ACV synthetase (150 μg/mL, 45 μg) was incubated with [^{14}C]cysteine for 2 h. Aliquots of the reaction mixture were spotted onto filter squares and dropped into 10% (w/v) trichloroacetic acid. The filters were washed, dried and subsequently saturated with the same amount of either (A) formic or (B) performic acid. The reaction was quenched with a large excess of water and the residual radioactivity determined. Values shown are the means of triplicate determinations. The mean initial radioactivity recovered in the trichloroacetic acid precipitates was 3.23×10^3 dpm (5.58 pmol) for the formic acid-treated material and 2.93×10^3 dpm (5.05 pmol) for the performic acid-treated material.

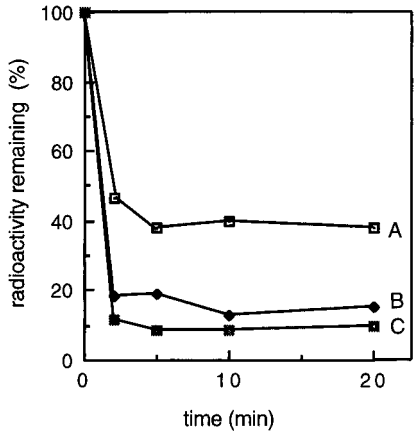


FIGURE 3: Effect of performic acid treatment on binding of radiolabeled amino acids recovered with trichloroacetic acid-precipitated proteins. ^{14}C-labeled valine and proline were incubated with samples of alcohol dehydrogenase and ACV synthetase (50 μg of each per incubation at 170 μg/mL) for 2 h. The mean total bound radioactivity recovered in each incubation is in parentheses: (A) ACV synthetase and valine (4.25×10^3 dpm, 7.21 pmol), (B) ACV synthetase and proline (2.05×10^3 dpm, 3.55 pmol), and (C) alcohol dehydogenase and valine (2.30×10^3 dpm, 3.9 pmol). The protein preparations were trichloroacetic acid-precipitated and treated with performic acid as in Figure 2. Virtually identical results (omitted for clarity) were seen for labeled proline and alcohol dehydrogenase (2.85×10^3 dpm, 4.93 pmol initially bound). Values given and plotted are the means of triplicate determinations.

This effect was time-dependent but independent of the presence of MgATP. The binding of [^{3}H]valine to ACV synthetase was highly labile to performic acid treatment but comparatively stable to the same treatment with formic acid at the same concentration (Figure 2) as has been previously reported (van Liempt et al., 1989); virtually identical results were also seen for [^{14}C]cysteine (not shown). However in our hands, nonspecific binding of the ACV synthetase substrate [^{14}C]valine to alcohol dehydrogenase was also highly labile to performic acid after trichloroacetic acid precipitation as was the binding of [^{14}C]proline (not known to be a substrate of ACV synthetase) to ACV synthetase (Figure 3). Further control incubations of [^{14}C]cysteine with alcohol dehydrogenase also showed similar amounts of amino acid bound to the trichloroacetic acid precipitates, 70% of which could be released by performic acid treatment within 1 h (not shown). Thus, on performic acid oxidation, similar results are seen with ACV synthetase substrates and a non-peptide synthetase enzyme as are seen with the substrate and ACV synthetase. Conversely, with a non-substrate amino acid and ACV synthetase, we also obtained results very similar to those obtained with ACV synthetase and valine.

Specific Binding of [^{3}H]Valine to ACV Synthetase Is Rapidly and Fully Reversible. When the MgATP-independent (nonspecific) binding of [^{3}H]valine was carefully corrected for by inclusion of control time courses which contained alcohol dehydrogenase and/or time courses containing heat-inactivated ACV synthetase as well as those lacking MgATP, considerable additional MgATP-dependent binding remained which was time-dependent (Figure 1). Addition of a large molar excess of unlabeled valine led to rapid loss of the specific component of the radiolabel bound to the protein, indicating that the amino acid bound in the presence of MgATP is in free equilibrium with the free amino acid substrate (Figure 4). The nonspecific binding of valine was unaffected by this treatment, indicating that the ATP-

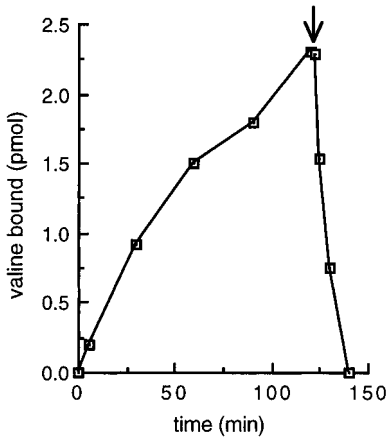


FIGURE 4: Reversibility of specific binding of [^{3}H]valine to ACV synthetase. Aliquots of the incubation mixture [containing ACV synthetase (33 μg/mL, 30 μg)] were taken at the indicated times and precipitated into filter squares as before. Replicate incubations had unlabeled valine added to a final concentration of 0.25 mM at the point indicated by the arrow. Control experiments with mixtures containing the same concentration of heat-inactivated enzyme (see Figure 1) were also run, and this nonspecific component was subtracted to reveal the specific component plotted in the figure. Values shown are the means of triplicate determinations.

independent binding is not enzymic. If the MgATP-dependent binding component were due to formation of a thioester to the enzyme subsequent to aminoacyladenylate formation, this result could not occur as this postulated thiolation partial reaction should be effectively irreversible in the presence of an excess of valine and MgATP. Analogous results were seen using [^{14}C]cysteine (not shown). Both cysteine and valine have been shown to stimulate rapid ATP$-$PP$_i$ exchange by ACV synthetase (van Liempt et al., 1989; Baldwin et al., 1993), consistent with their freely reversible activation as enzyme-bound aminoacyladenylates. The rapid loss of the MgATP-dependent binding of a single individual radiolabeled substrate from the enzyme in the

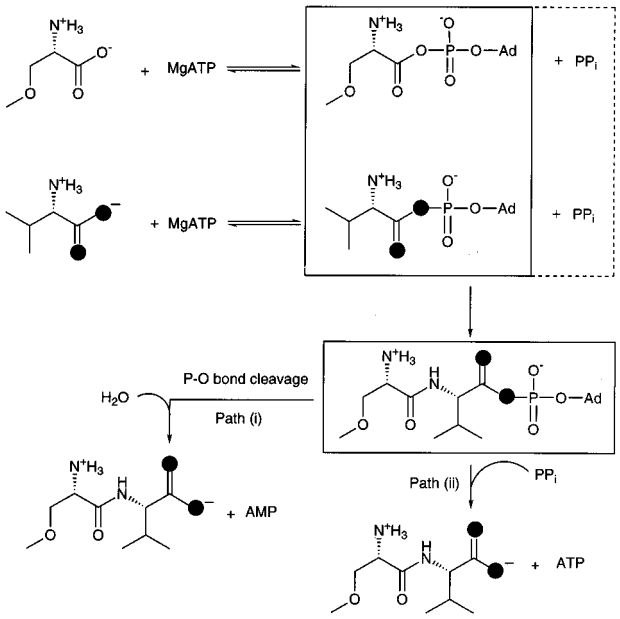
presence of excess unlabeled amino acid is consistent with rapid isotopic dilution of the amino acid in the enzyme-bound aminoacyladenylate.

DISCUSSION

Investigations into the peptide synthetases have recently largely focused on the structure of the enzymes inferred from the deduced amino acid sequences. The concept of concatenated homologous domains thereby derived is well founded and amply demonstrated by the incorporation of a domain from one synthetase into another to generate the expected functional chimera (Stachelhaus et al., 1995). However, the precise sequence of reactions required to synthesize the peptide products has never explicitly been shown. This is despite fact that the thiol-template mechanism has been a paradigm for nonribosomal peptide synthesis for nearly a quarter of a century. We have been using small-molecule probes in an attempt at least partially to dissect and hence analyze the reaction sequence involved in nonribosomal peptide synthesis, using ACV synthetase as a model system. Our working hypothesis is that individual partial reactions in the complete reaction sequence have subtly different substrate specificities and therefore act as a series of "gates" (Shiau et al., 1995a) which some, but not all, substrate analogues can pass through to yield complete tripeptides (Baldwin et al., 1994). Analysis of the products of incomplete reaction sequences can thus probe the sequence of partial reactions required for their synthesis and release. They therefore provide information on the validity of the thiol-template mechanism proposed for the synthetase.

We have shown previously that incubation of ACV synthetase with the cysteine analogue *O*-methylserine, α-aminoadipate, and valine results in the synthesis of the diastereomeric pair of peptides L,L and L,D *O*-(methylserinyl)-valine, presumably reflecting an inability of the synthetase to complete tripeptide synthesis from these unnatural dipeptides (Shiau et al. 1995a). No evidence of any formation of a *δ*-peptide bond between α-aminoadipate and *O*-methylserine has been accrued (Baldwin et al., 1994). Presumably, this failure to complete tripeptide synthesis causes these dipeptides to be "shunted" off the enzyme. Similarly, it is possible to detect synthesis of L,D (but not L,L) cysteinylvaline under specific conditions by ACV synthetase (Shiau et al., 1995b). It is important to note however that none of the synthetic dipeptides when included at substrate concentrations with the ACV synthetase stimulate significant ATP−PP_i exchange. This demonstrates that these dipeptides are not reactivated by the enzyme as dipeptide aminoacyladenylates. Consistent with this observation, none of the synthetic dipeptides when incubated with the synthetase and α-aminoadipate produce any detectable ACV (Shiau et al., 1995a,b). By contrast, there is some evidence that there is some weak activation of α-(aminoadipoyl)cysteine as a dipeptide aminoacyladenylate (Shiau et al., 1995b). This is consistent with the observation that some conversion of α-(aminoadipoyl)cysteine plus valine to ACV does occur, although at a very much lower rate than the synthesis of ACV from the individual amino acids (Banko et al., 1986). Thus, it is not possible to probe all the potential partial reactions of the synthetase that might be desired using dipeptides. However, the fortuitous synthesis of both L,L and L,D *O*-(methylserinyl)valine dipeptides in amounts that can be isolated and characterized can be exploited. Synthesis of these dipeptide products using di[$^{18}O_2$]valine serves as a useful probe of the timing of the thioester formation to valine relative to both peptide bond formation and epimerization.

Scheme 2: Potential Pathways for the Synthesis of L-*O*-(Methylserinyl)-L-[$^{18}O_2$]valine[a]



[a] The filled circles represent the ^{18}O isotope. Enzyme-bound intermediates are shown boxed.

The investigation of the timing of thioester formation during the synthesis of *O*-(methylserinyl)valine gave results which were most surprising since they were clearly at variance with those expected from application of the thiol-template mechanism to the synthesis of these dipeptides. The retention of both ^{18}O labels in the L,L diastereoisomer demonstrates unequivocally that thioesterification of the valine carboxyl group occurs after formation of the peptide bond to the valine amino group. This would completely explain our failure to detect any incorporation of ^{18}O label into AMP or loss of ^{18}O from the labeled valine substrate when it was incubated with the synthetase and MgATP. That peptide bond formation is independent of thioesterification of one of the substrate amino acids is not postulated in the thiol-template mechanism, and thus, a new mechanism must be formulated at least for the synthesis of these dipeptides.

On the basis of our results, the simplest mechanism for L-*O*-(methylserinyl)-L-valine synthesis is as follows (Scheme 2). Both L-valine and L-*O*-methylserine are activated as their aminoacyladenylates, consistent with their participation in ATP−PP_i exchange reactions (Baldwin et al., 1994). Peptide bond formation between the L-valine amino group and the L-*O*-methylserine carboxyl group then occurs, resulting in loss of AMP from the L-cysteinyl adenylate. This is followed by loss of AMP from the L-valine adenylate. It is noteworthy that this occurs by P−O bond cleavage rather than by C−O bond cleavage, leading to retention of both labeled oxygens. This could occur by regiospecific hydrolysis (path i) or conceivably by pyrophosphorolysis (path ii), although this is less likely due to the very low concentration of enzymically produced PP_i in the incubations. Formation of the valine aminoacyladenylate initially seems unnecessary as no peptide bond is formed to the valine carboxylate. The valine aminoacyladenylate could conceivably be an evolutionary artifact that is formed solely for recognition purposes and

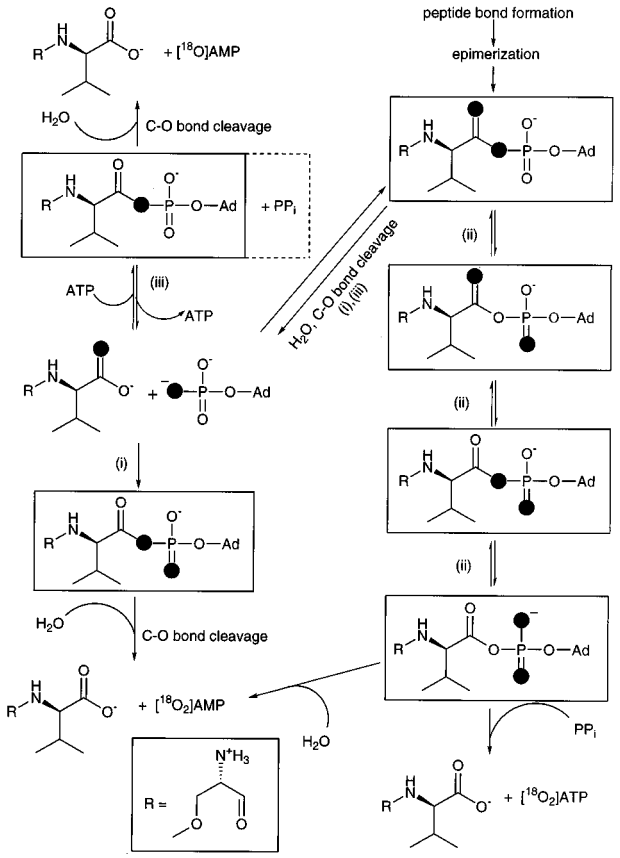
cleaved prior to the formation of the peptide bond. Alternatively, activation of the valine carboxylate as the aminoacyladenylate could be required for inversion of the valine α-center in the "peptide-bound" state (Stindl & Keller, 1994; Shiau et al., 1995a).

The formation of the L-*O*-(methylserinyl)-D-valine retaining both ^{18}O labels demonstrates clearly that epimerization occurs after formation of the peptide bond but before thioester formation. As with the L,L diastereoisomer, cleavage of the P−O bond in the dipeptide adenylate might occur by hydrolysis (or pyrophosphorolysis). The formation of the L-*O*-(methylserinyl)-D-[$^{18}O^{16}O$]valine and L-*O*-(methylserinyl)-D-[$^{16}O_2$]valine peptides is more difficult to account for. L-*O*-(Methylserinyl)-D-[$^{18}O^{16}O$]valine in principle could be formed by cleavage of the C−O bond of the dipeptide adenylate by water. This seems unlikely since the L-*O*-(methylserinyl)-L-[$^{18}O_2$]valine is cleaved at the P−O bond, but the possibility cannot be completely eliminated since the L,D and L,L diastereoisomers might behave differently. Similarly, although loss of one or both labels by positional isotope exchange clearly does not occur with the L,L diastereoisomer, this potential mechanism in the case of loss from the L,D diastereoisomer cannot explicitly be ruled out.

We have shown previously that one ^{18}O atom is lost from ACV during its synthesis (Baldwin et al., 1988) and that one ^{18}O atom is incorporated specifically into the valine residue from $H_2{}^{18}O$ (Shiau et al., 1995c), consistent with the formation and subsequent cleavage of a thioester bond between the valine carboxylate and the enzyme at some point during the catalytic cycle. We hypothesize therefore that the ^{18}O is lost from the L,D dipeptide by formation of a thioester to the carboxyl group and appears as [^{18}O]AMP, although we have not yet performed experiments to confirm this by isolation of the labeled product. Subsequent hydrolysis of the dipeptide thioester would release the L-*O*-(methylserinyl)-D-[$^{18}O^{16}O$]valine dipeptide to accumulate in solution.

Enzymic synthesis of comparatively small amounts of L-*O*-(methylserinyl)-D-[$^{16}O_2$]valine dipeptide was unexpected since it represents a clear departure from the catalytic sequence involved in the synthesis of ACV in which only one ^{18}O is lost (Baldwin et al., 1988, 1991). For the purpose of further discussion we shall assume that L-*O*-(methylserinyl)-D-[$^{18}O_2$]valine and L-*O*-(methylserinyl)-D-[$^{18}O^{16}O$]-valine are obligatory intermediates in the synthesis of the L-*O*-(methylserinyl)-D-[$^{16}O_2$]valine. Thus, some possibilities for the synthesis of this dipeptide from the initially formed L,D-aminoacyladenylate are summarized in Scheme 3. Path i can be excluded since formation of the aminoacyladenylate from L-*O*-(methylserinyl)-D-[$^{18}O^{16}O$]valine and [^{18}O]AMP is thermodynamically unfavorable. Path ii represents possibilities for positional isotope exchange and seems unlikely since, again, no such reactions were seen to occur with the L,L diastereoisomer. In path iii, hydrolysis of L-*O*-(methylserinyl)-D-[$^{18}O_2$]valine adenylate occurs by C−O bond cleavage and the dipeptide is reactivated by a second ATP molecule. Neither authentic synthetic L-*O*-(methylserinyl)-L-valine nor L-*O*-(methylserinyl)-D-valine stimulates ATP−PP_i exchange significantly (Shiau et al., 1995a). This observation does not rigorously exclude this possibility since if activation by ATP of the enzyme-bound dipeptide were effectively irreversible this would not cause significant isotope exchange. This mechanism seems unlikely however

Scheme 3: Some Potential Pathways for the Synthesis of L-*O*-(Methylserinyl)-D-[$^{18}O^{16}O$]valine and L-*O*-(Methylserinyl)-D-[$^{16}O_2$]valine[a]

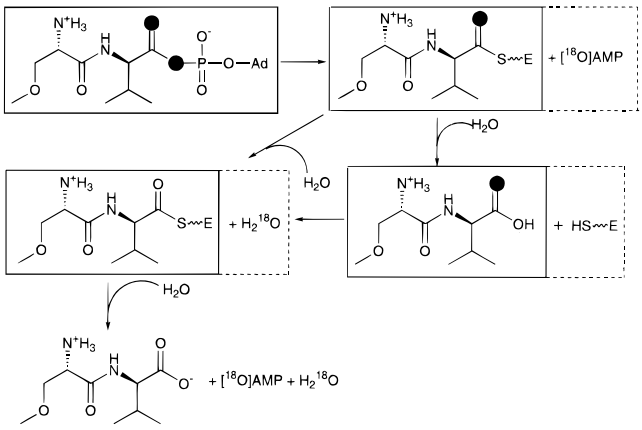


[a] Details as for Scheme 2.

due to the precedent above that P−O bond cleavage occurs in the synthesis of the L,L diastereoisomer, although it is again conceivable that the L,D diastereoisomer behaves differently.

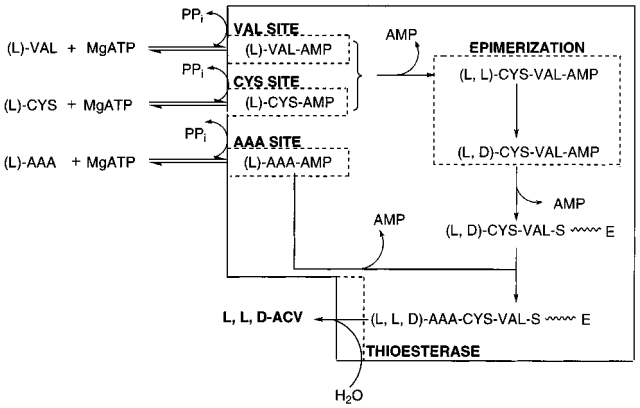
For these reasons, we favor a mechanism by which reversible formation of a thioester bond between the enzyme and the enzyme-bound intermediates occurs. (Direct exchange of the thioester oxygen is also a possibility.) Our results show that this occurs after peptide bond formation and epimerization of the valine residue. We hypothesize that thioesterification involves the participation of (a) 4′-phosphopantetheine cofactor(s) that have previously been detected in ACV synthetase preparations (Baldwin et al., 1990). This formation of a covalent adduct between the dipeptide and the enzyme might thus be required for the translocation of the natural intermediate L-cysteinyl-D-valine to a distant active site on the multienzyme for completion of ACV synthesis. In this scenario, the loss of both the ^{18}O labels from some of the unnatural dipeptide product would result from two thioester hydrolyses incorporating two oxygens from solvent water with an intermediate re-esterification (Scheme 4). This might reflect a novel mechanism by which the enzyme aborts the catalytic cycle at either of two points to release the "incorrect" dipeptide L-*O*-(methylserinyl)-D-valine which is unable to participate in the formation of the δ-peptide bond to α-aminoadipate. We postulate that the L-*O*-(methylserinyl)-L-valine isomer is a poorer substrate for transfer to the enzyme-bound thiol than L-*O*-(methylserinyl)-D-valine and thus is released as a single species with both carboxyl oxygens retained.

Scheme 4: Proposed Mechanism for the Synthesis of L-*O*-(Methylserinyl)-D-[$^{18}O^{16}O$]valine and L-*O*-(Methylserinyl)-D-[$^{16}O_2$]valine[a]



[a] Details as for Scheme 2.

Scheme 5: Revised Mechanism for ACV Synthesis[a]



[a] Details as for Scheme 1. Note that in this scheme epimerization is shown occurring at the dipeptide stage, although the possibility that it occurs at the tripeptide stage during tripeptide synthesis cannot explicitly be ruled out.

Given the pivotal role previously ascribed to the thioesterification of amino acid substrates to peptide synthetases in the thiol-template mechanism, we re-evaluated the evidence for covalent binding of radiolabeled amino acids to ACV synthetase. In our hands, the binding observed was not entirely a specific effect since a substantial component was independent of exogenous MgATP. We also observed considerable binding of labeled proline to ACV synthetase preparations and conversely significant binding of labeled ACV synthetase substrates to control protein preparations catalytically inert with regard to peptide synthetase activity. In these experiments, the "binding" was highly labile to performic acid, a previously proposed qualitative test for the existence of a thioester link between the labeled amino acid and ACV synthetase (van Liempt et al., 1989). We corrected for the binding of labeled cysteine and valine to ACV synthetase using parallel control time courses containing no MgATP and additional parallel control time courses containing catalytically inert protein preparations at the same protein concentration. This revealed an additional MgATP-dependent component of the binding of the substrate amino acids to the synthetase. This additional labeled component, however, was rapidly and completely washed out from the enzyme by addition of a large excess of unlabeled amino acid. We concluded that this specific labeling of the enzyme represents the tightly bound aminoacyladenylate as excess amino acid would not wash out labeled substrate attached by a thioester to the enzyme under these conditions. That this binding component indeed represents the tightly bound aminoacyladenylate is currently under investigation. Prolonged performic acid treatment in any case is a rather drastic chemical modification when applied to proteins. Other than oxidative cleavage of any thioesters present, the treatment rapidly oxidizes sulfur-containing amino acids and also converts tryptophan to a variety of products. Serine, threonine, and tyrosine are also oxidized on prolonged exposure (Hirs, 1967). We have chosen therefore to disregard performic acid treatment as a qualitative test for thioester formation between the labeled amino acid and the synthetase because we found it insufficiently selective.

In conclusion, we have specifically addressed the validity of the thiol-template mechanism for the synthesis of L-*O*-(methylserinyl)-L-valine and L-*O*-(methylserinyl)-D-valine by ACV synthetase using [$^{18}O_2$]valine as a probe. Clearly, the synthesis of the peptide bond and the epimerization of the valine α-carbon occurred prior to the esterification of the valine carboxylate to the enzyme. Thus, thioesterification of at least one of the amino acid substrates is not obligatory for peptide bond formation to the amino group of that substrate. It is evident that the use of an unnatural substrate has perturbed the catalytic path seen in ACV synthesis since, in addition to the recovery of a di- rather than a tripeptide, a small proportion of the recovered L-*O*-(methylserinyl)-D-valine had lost both labeled oxygens. This might in fact reflect a "proof-reading" ability of the enzyme, resulting in release of an incorrect dipeptide intermediate. It is arguable that synthesis of the dipeptides observed results itself from a gross perturbation, caused by the use of a substrate analogue, of the catalytic cycle used to synthesize ACV. However, it is equally plausible that an analogous mechanism (Scheme 5) is used to synthesize an L-cysteinyl-D-valine intermediate in the catalytic sequence leading to ACV since we have isolated this dipeptide as a product of ACV synthetase under specific conditions (Shiau et al., 1995b). The formation of a thioester between 4′-phosphopantetheine and the valine in this scheme serves to sequester the dipeptide intermediate and prevent it from being lost from the enzyme during translocation to a distant active site. This simpler scheme is attractive as it obviously would not require the numerous acyl transfer steps required by the thiol-template mechanism. Precedent exists for enzymic formation of amide bonds between an enzyme-bound acyladenylate and an amino group with no postulated intermediate acyl transfer step. For example, the post-translational biotinylation of specific lysine residues is proposed to be carried out by the *Escherichia coli bir*A gene product using an enzyme-bound biotinyl adenylate directly as an activated biotinyl donor (Eisenberg et al., 1982). It would obviously be most interesting to determine the labeling pattern in the valine carboxyl group in L-cysteinyl-D-valine (Shiau et al., 1995b) which is synthesized from natural substrates of the enzyme. Although there are technical difficulties due to the lower yield of this dipeptide compared with that obtained for the *O*-(methylserinyl)valine analogues, we are attempting currently to produce enough ^{18}O-labeled L-cysteinyl-D-valine for analysis. In terms of the potential exploitation of peptide synthetases for enzymic synthesis of any dipeptides of choice, it is important to note that this does not necessarily require

an intermediate acyl transfer step. Thus, post-translational modification by phosphopantetheinylation of ACV synthetase could be unnecessary for substrate activation, epimerization, and *di*peptide synthesis in contrast to the essential role proposed recently (Stein et al., 1995) for peptide synthetases in general.

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