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Epimerization of the D-Valine Portion in the Biosynthesis of Actinomycin D†

Antje Stindl and Ullrich Keller*

Institut für Biochemie, Technische Universität Berlin, Franklinstrasse 29, 10587 Berlin-Charlottenburg, Germany

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ABSTRACT: In the biosynthesis of actinomycin, the multifunctional actinomycin synthetase II (ACMS II) assembles 4-methyl-3-hydroxyanthranilic acid (4-MHA), L-threonine and D-valine, the first three residues of the 4-MHA peptide lactone chain. ACMS II activates L-threonine and L-valine but not D-valine as thioesters via their adenylates, and there is no epimerization of the covalently bound L-valine. When L-threonine and L-valine are presented to the enzyme together with the 4-MHA analogue *p*-toluic acid and the 4-MHA-activating enzyme ACMS I, ACMS II forms the two diastereomers *p*-toluyl-L-Thr-L-Val and *p*-toluyl-L-Thr-D-Val in equal amounts along with *p*-toluyl-L-Thr in a cofactor-independent manner. Studies with [2,3-$^3H_2$]valine revealed that *p*-toluyl-L-Thr-D-Val contained approximately 50% of the tritium label found in the LL-diastereomer. Concomitantly, radioactive water was formed due to enzyme-catalyzed hydrogen exchange with the solvent during epimerization. In the absence of threonine (or MgATP), however, the amount of radioactive water formed from [^{3}H]valine was significantly less, which suggests that the peptide bond between L-threonine and L-valine is formed prior to the epimerization at C-2 of valine. The facts that both LL- and LD-acyldipeptides are equally present on the enzyme's surface—as revealed by using ^{14}C-labeled threonine or valine as precursors—and that the L-valine in the LL-diastereomer apparently has not lost hydrogen strongly suggests that the LL-diastereomer is an obligatory intermediate in the formation of the LD-dipeptide. Accordingly, the loss of hydrogen most likely is the result of epimerization of a peptide rather than of a single amino acid. This mechanism could apply to a large number of peptides containing D-amino acids at positions other than the N-terminus where the corresponding L-amino acids rather than the free D-amino acids are incorporated.

Most peptides containing D-amino acids are synthesized enzymatically in a thiol template mechanism (Kleinkauf & von Döhren, 1990). This mechanism involves ATP-dependent activation of amino acid residues and their modification and polymerization on multifunctional enzymes via enzyme-bound aminoacyl and peptidyl intermediates in thioester linkage (Lipmann, 1971, 1973). On the one hand, free D-amino acids, preformed somewhere in the cell, may be directly activated and incorporated into the peptide chain, as in the biosynthesis of cyclosporin (Zocher et al., 1986; Lawen & Zocher, 1990) and the incorporation of D-amino acids into some bacterial cell wall polymers (Heaton & Neuhaus, 1992). On the other hand, racemases that are part of the peptide synthetase systems themselves activate equally well the L- or D-enantiomers of the relevant amino acids and racemize them while still covalently bound to the activating enzyme without the need of a cofactor, as in the case of gramicidin S or tyrocidin (Takahashi et al., 1971; Vater & Kleinkauf, 1976). In this case, incorporation of the D-enantiomer is a later step in the reaction sequence of peptide synthesis (Pass et al., 1973). Thus, in all of these cases epimerization occurs prior to peptide bond formation and may be considered as an α-epimerization of starter (N-terminal) amino acids in peptide synthesis.

By contrast, D-amino acids in internal positions of some ribosomally synthesized peptides arise by posttranslational processing of the corresponding propeptides, as in the case of the lantibiotic group of antibiotics (Jung, 1991) or the dermorphin opioid peptides (Erspamer & Melchiorri, 1980). The unusual nonproteinogenic D-amino acids in the lantibiotics most probably arise via the intermediacy of dehydro derivatives of serine, threonine, and cysteine residues in several positions of the corresponding propeptides (Schnell et al. 1988). Less clear is the origin of the single D-amino acid residue in the dermorphins (Mor et al., 1991). Current hypothesis favors a model of dehydroenamine intermediacy in the conversion of the L- to the D-enantiomer of the amino acid residue in the peptide chain based on stereospecific de/rehydrogenation (Mor et al., 1992).

A large number of non-ribosomally made peptides have D-amino acids in their chains located at positions other than the N-terminus. In these cases, precursor studies—mostly done with whole cells—have revealed that, as in the case of ribosomally made peptides, the L- rather than the D-enantiomers are incorporated into the relevant D-amino acid positions. This has been shown for bacitracin (Kurahashi, 1974), actinomycin (Salzman et al., 1964), etamycin (Hook & Vining, 1973), and penicillins (Huang et al., 1975) and has led to the assumption that their mechanism of epimerization must differ from that catalyzed by the racemases involved in phenylalanine activation of gramicidin S or tyrocidin biosynthesis, where both enantiomeric forms can serve as precursors of the peptide-bound D-enantiomer.

In the actinomycins, position 2 contains D-valine or D-*allo*-isoleucine (see Figure 1) (Katz, 1967). Previous *in vivo* studies on actinomycin D synthesis indicated that L-valine rather than D-valine is the precursor of peptide-bound D-valine and that the epimerization proceeds via loss of hydrogen at C-2 of valine (Salzman et al., 1964; Mason et al., 1977). The multifunctional enzyme actinomycin synthetase II (ACMS II,[1] 280 kDa) assembles the amino acids L-threonine and L-valine in positions 1 and 2 of actinomycin in the precursor peptide (Figure 1) by activating them as enzyme-bound

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* Corresponding author.

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[1] Abbreviations: 4-MHA, 4-methyl-3-hydroxyanthranilic acid; ACMS II, actinomycin synthetase II; ACMS I, actinomycin synthetase I; DTE, dithioerythritol; TLC, thin-layer chromatography; OBz, *O*-benzyl; RT, room temperature; FAB-MS, fast atom bombardment mass spectrometry.

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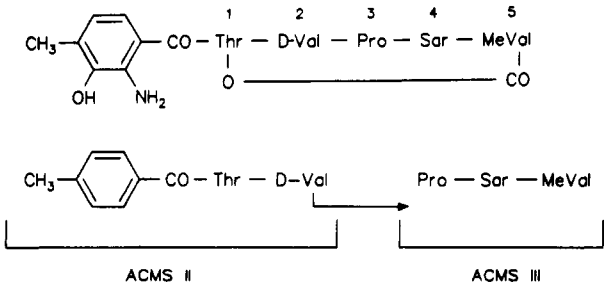

FIGURE 1: Structure of 4-MHA-pentapeptide lactone and sequence of that portion of the molecule assembled by actinomycin synthetase II (ACMS II). Sar = sarcosine (*N*-methylglycine); MeVal = *N*-methyl-L-valine.

thioesters via their corresponding adenylates. We also have shown that the enzyme N-acylates the enzyme-bound threonine with 4-methyl-3-hydroxyanthranilic acid (4-MHA) with consumption of 4-MHA-adenylate delivered by actinomycin synthetase I (4-MHA-activating enzyme) (Stindl & Keller, 1993). Investigations of the enzyme-bound valine with D-amino acid oxidases revealed that it was exclusively in the L-form (Keller, 1987). Here we report on further catalytic activities of ACMS II. This enzyme catalyzes the formation of the peptide bond between threonine and valine and epimerizes in a cofactor-independent manner L-valine to D-valine, most probably in the peptide-bound state.

MATERIALS AND METHODS

Radioisotopes and Chemicals. L-[U-^{14}C]Threonine (228 Ci/mol), L-[U-^{14}C]valine (285 Ci/mol), L-[U-^{14}C]isoleucine (324 Ci/mol), and ^3H_2O (100 mCi/mL) were from Amersham International. L-[2,3-$^3H_2$]Valine (29 Ci/mmol) was from ICN. [1-^{14}C]-*p*-Toluic acid (8.3 Ci/mol), D-valine, L-isoleucine, D-*allo*-isoleucine, L-valine benzyl ester *p*-toluenesulfonate salt, and D- and L-amino acid oxidases were from Sigma. The following chemicals were obtained as indicated: *N*-Boc-threonine hydroxysuccinimide ester, H-Thr(Bzl)-OH·HCl, and L-threonyl-L-valine (Bachem, Bubendorf, Switzerland); *p*-toluic acid (Aldrich Chemical Co.); chiral thin-layer plates (Macherey and Nagel, Düren, FRG); silica gel 60 plates (Merck, Darmstadt, FRG). All other chemicals were of the highest purity commercially available.

Buffers and Solvent Systems. Buffer A contained 200 mM Tris-HCl, pH 8.0, 15% (w/v) glycerol, 30 mM $MgCl_2$, 10 mM dithioerythritol (DTE), and 1 mM benzamidine. Assay buffer was buffer B containing 100 mM Tris-HCl, pH 8.0, 15% (w/v) glycerol, 4 mM DTE, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 1 mM EDTA, pH 8.0. TLC was performed either on silica gel with the solvent systems 1-butanol/acetic acid/water (4:1:1 w/w/w) (I), di-*n*-butyl ether/ethyl acetate/acetic acid (50:50:10 w/w/w) (II), *n*-hexane/ethyl acetate/acetic acid (50:50:10 w/w/w) (III), or chloroform/methanol/acetic acid (19:5:1 w/w/w) (IV) or on chiral plates using methanol/acetonitrile/water (50:50:200 w/w/w) (V).

Enzyme Purification. Actinomycin synthetases I and II were prepared as described previously (Keller & Schlumbohm, 1992; Stindl & Keller, 1993) from *Streptomyces chrysomallus* strain X2-18 (Keller & Schlumbohm, 1992).

Preparation of Crude Cell Extract from S. chrysomallus. Freshly harvested cells were suspended in ice-cold buffer A in a ratio of 1:5 (w/v). After rupture of the cells in a French press at 10 000 psi, 20 μg/mL DNase and 1 mM $MgCl_2$ were added to the homogenate. After stirring for 30 min and subsequent centrifugation at 10000*g* (20 min), saturated ammonium sulfate solution (in water) was added to the supernatant to give 60% saturation. After incubation on ice for 2 h, the precipitate was collected by centrifugation at 30000*g* (20 min). The pellet was dissolved in buffer B at a protein concentration of 20–25 mg/mL and could be stored frozen at −80 °C for 2 months. Prior to use in cell-free studies of peptide synthesis, this crude extract was desalted on a small Sephadex G-25 column.

Methods of Analysis. Protein determinations were performed according to Bradford (1976). Radioactivity determinations and autoradiography were performed as described previously (Keller, 1987). Determination of the configuration of radioactive amino acids split off the enzyme or from acid hydrolysates of enzyme-bound peptides was done by digestion with L- or D-amino acid oxidase in the additional presence of catalase according to the manufacturer's manual. Completeness of the reactions was checked by chromatographic analysis of the reaction product ketoisovaleric acid using solvent system I. Acid hydrolysis was performed with 6 N HCl under reduced pressure for 20 h at 105 °C.

Isolation of Aminoacyl and Peptidyl Intermediates Covalently Bound to Actinomycin Synthetase II. The formation of covalently bound reaction intermediates on ACMS II was followed either in the crude extract or with purified ACMS II in the absence or presence of ACMS I.

In the case of the crude extract, the assay contained in a total volume of 300 μL 0.5 μCi of ^{14}C-labeled amino acid (either *p*-toluic acid, Thr, Val, or Ile), 20 mM $MgCl_2$, 10 mM ATP, 2 mM nonlabeled *p*-toluic acid, Thr, Val, or Ile, respectively, and 250 μL of enzyme. After incubation for 1 h at 28 °C, 2 mL of 7% trichloroacetic acid was added to the reaction mixture. Reaction intermediates covalently bound as thioesters to ACMS II were isolated by performic acid oxidation and thin-layer chromatography as described (Stindl & Keller, 1993). In the case of experiments with purified ACMS II, the enzyme (ca. 50 pmol) was incubated with 0.5 μCi of [^{14}C]threonine or [^{14}C]valine, 20 mM $MgCl_2$, 10 mM ATP, and 2 mM nonlabeled amino acid in a total volume of 150–250 μL. In the case of labeling with [2,3-$^3H_2$]valine, 30 μCi of the tritium-labeled amino acid was used in each experiment. Incubation and isolation of covalently bound intermediates were done as described above.

Syntheses: L-Threonyl-D-valine. *N*-Boc-L-Threonine hydroxysuccinimide ester (316 mg, 1 mmol) was reacted in 5 mL of dioxane/water (1:1 v/v) with 117 mg (1 mmol) of D-valine in the presence of 249 mg (3 mmol) of $NaHCO_3$ with stirring for 3 days at RT. After evaporation of the reaction mixture to dryness under reduced pressure and deprotection with formic acid, purification was performed by preparative TLC (solvent system I). The yield was 45%. Threonyl-D-valine had a R_f value of 0.55, which differed from those of threonine, D-valine, and the reaction product *N*-Boc-threonyl-D-valine. In contrast to the latter, L-Thr-D-Val reacted with ninhydrin and was further analyzed by acid hydrolysis, which yielded L-threonine and D-valine. The configuration of the D-valine was confirmed by chromatography on chiral plates (solvent system V) and by digestion with L- and D-amino acid oxidases.

D-Valine Benzyl Ester. D-Valine (1.4 g, 12 mmol) was refluxed in 25 mL of benzylic alcohol in the presence of 5 g of *p*-toluenesulfonic acid monohydrate for 4 h. The oily reaction mixture was then poured into a mixture of 200 mL of crushed ice und 10 mL of concentrated HCl. The mixture separated into two phases. The aqueous phase was adjusted to pH 10 with solid Na_2CO_3 and extracted three times with

diethyl ether. The ether phase was dried and evaporated to dryness. TLC (solvent system IV) revealed one single spot which upon saponification with NaOH yielded D-valine.

p-Toluyl-threonyl-D-valine and p-Toluyl-threonyl-L-valine. *p*-Toluylthreonine(OBz)OH (Stindl & Keller, 1993) (654 mg, 2 mmol) and 607 mg (2.5 mmol) of D-valine(OBz) were reacted with 413 mg (2 mmol) of DCCD in the presence of 202 mg (2 mmol) of triethylamine in a total volume of 20 mL of 1,4-dioxane with stirring for 24 h at RT. Dicyclohexylurea was filtered off and the mixture was evaporated to dryness. The residue was dissolved in EtOAc and the organic phase was washed two times with water and dried. The organic phase was applied onto a column filled with alumina oxide (ICN, Type I). The *p*-toluyl-threonyl(OBz)-D-valine benzyl ester was chromatographed off the column with *n*-hexane containing increasing amounts of EtOAc. The identity of *p*-toluyl-threonine(OBz)-D-valine(OBz) was checked by FAB-MS. The molecular ion peak was $(M + H)^+$ 517. The compound was deprotected by hydrogenation in MeOH with Pd/C as catalyst. The configuration of the D-valine portion of the compound after acid hydrolysis was confirmed by chromatography on chiral plates (Macherey and Nagel) using solvent system V. Synthesis of *p*-toluyl-threonyl-L-valine was performed in the same way as that of *p*-toluyl-threonyl-D-valine except that L-valine benzyl ester *p*-toluenesulfonate salt instead of D-valine(OBz) was used.

Determination of Radioactive Water. Partially purified ACMS II (40–45 pmol) was incubated with 30 μCi of [2,3-$^3H_2$]valine and 20 mM $MgCl_2$ in the presence or absence of 10 mM ATP and 1 mM nonlabeled threonine in a total volume of 50 μL in an Eppendorf test tube. After incubation for 1 h, the protein was denatured by addition of HCl (final pH 1.5). The Eppendorf test tube (with lid removed) was placed into a scintillation vial containing at its bottom 1 g of freshly molten calcium chloride. The scintillation vial was firmly sealed and left overnight at room temperature. After this, the test tube was removed, the protein was analyzed for covalently bound reaction intermediates, and the radioactive water absorbed to the calcium chloride was determined by liquid scintillation counting. Quantitation was done by using standard curves obtained with calcium chloride and various known amounts of tritium oxide.

RESULTS AND DISCUSSION

ACMS II Individually Catalyzes Formation of L-Thr-L-Val and L-Thr-D-Val from L-Thr and L-Val. ACMS II activates threonine and L-valine as thioesters (Keller, 1987) and it also catalyzes the transfer of 4-methyl-3-hydroxyanthranilic acid to the amino group of threonine at the expense of adenylyl-4-MHA previously formed by ACMS I (Stindl & Keller, 1993). This latter reaction represents the initiation reaction of the peptide chain formation. To identify additional reactions catalyzed by ACMS II, the purified enzyme was incubated with mixtures containing both L-threonine and L-valine and MgATP. Figure 2 shows the TLC separation of the radioactive material covalently bound to the enzyme after incubation with [^{14}C]-L-threonine or [^{14}C]valine and their nonlabeled counterparts, respectively. Besides each of the two amino acids, two new labeled compounds in approximately equal amounts with R_f values higher than those of the amino acids were seen. Acid hydrolysis and chromatographic analysis of the two compounds obtained after [^{14}C]threonine labeling revealed that both contained [^{14}C]-L-threonine. In the case of labeling with [^{14}C]valine, the upper band yielded radioactive L-valine, while the lower band yielded

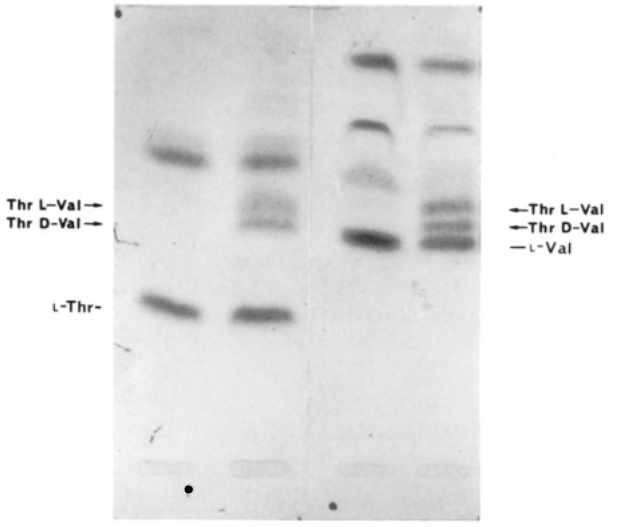

FIGURE 2: Formation of L-Thr-L-Val and L-Thr-D-Val catalyzed by ACMS II in the absence of ACMS I. Reaction intermediates covalently bound to ACMS II after incubation with L-threonine and L-valine in the presence of MgATP were isolated and chromatographed as described in Materials and Methods. Lane 1, ACMS II + [^{14}C]threonine; lane 2, ACMS II + [^{14}C]threonine + L-valine; lane 3, ACMS II + [^{14}C]-L-valine; lane 4, ACMS II + [^{14}C]-L-valine + threonine. Bands other than indicated are due to formylation through performic acid which was performed at 60 °C for 1 h. Chromatography was performed on TLC silica gel plates using solvent system I.

D-valine. The configuration of all of the amino acids was determined by single and double digestions of the acid hydrolysates of the dipeptides with D- and L-amino acid oxidase, respectively. In addition, chromatography of the hydrolysates on chiral thin-layer plates confirmed the results of the amino acid oxidase treatments. Final confirmation of the radiochemical analyses came from the chromatographic comparison of the enzymically synthesized compounds with the authentic dipeptides L-Thr-L-Val and L-Thr-D-Val (see Materials and Methods), which unambiguously revealed the two compounds to be L-Thr-L-Val and L-Thr-D-Val.

Besides the two covalently bound dipeptides, the enzyme carried significant amounts of covalently bound threonine (Figure 2) or valine which could also be split off the enzyme by performic acid. Control digestions of the valine with D- and L-amino acid oxidases or chromatography on chiral plates revealed that it was always in the L-configuration (this work; Keller, 1987). Thus, these data identify ACMS II as the enzyme responsible for peptide formation between the threonine and valine residues in the peptide chains of actinomycin. Moreover, the results clearly show that ACMS II harbors the epimerization function for epimerization of the D-valine residue from L-valine. However, its mechanism differs from previously described epimerizations in enzymatic peptide synthesis in that D-valine itself does not appear as the product of the epimerization reaction but L-Thr-D-Val, which is formed along with its L-Thr-L-Val diastereomer.

In the Presence of ACMS I and p-Toluic Acid, ACMS II Catalyzes Formation of p-Toluyl Dipeptides. When the above experiments with ACMS II, threonine and valine were repeated in the presence of ACMS I and *p*-toluic acid, a model substrate used instead of the natural substrate 4-MHA (Stindl & Keller, 1993), the formation of the two diastereomeric peptides was abolished and other new products were formed on ACMS II which were analyzed by acid hydrolysis and chromatographic comparison with chemically synthesized peptides (Figure 3). The new compounds formed in the presence of *p*-toluic acid and its activating enzyme have much higher R_f values than

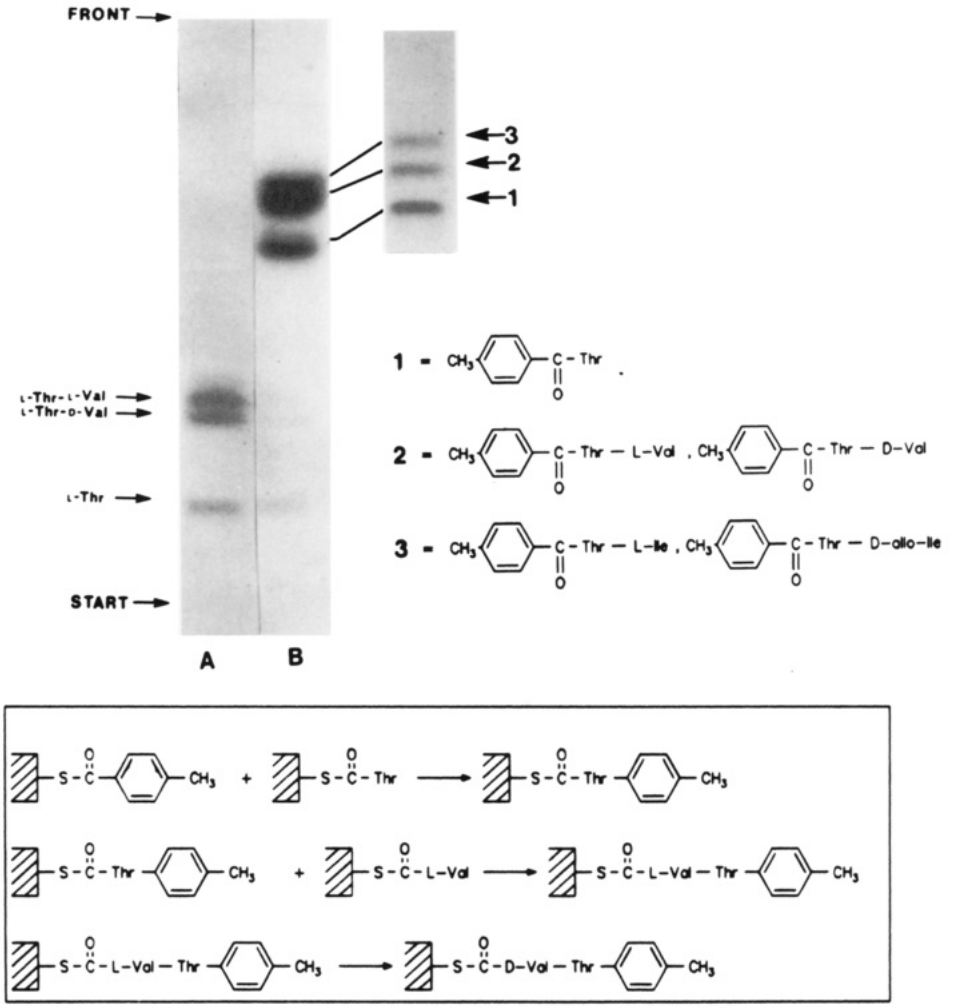

FIGURE 3: Formation of *p*-toluyl-L-Thr-L-Val and *p*-toluyl-L-Thr-D-Val catalyzed by ACMS II in the additional presence of ACMS I. Reaction intermediates covalently bound to ACMS II after incubation with L-threonine, L-valine, *p*-toluic acid, MgATP, and ACMS I were isolated and chromatographed as described in Materials & Methods. Lane A, ACMS II incubated with [^{14}C]Thr, L-Val, and ACMS I; lane B, ACMS II incubated with [^{14}C]Thr, L-Val, ACMS I, and *p*-toluic acid. The chromatography was performed on silica gel plates using solvent system I. The inset (upper right) shows rechromatography of the experiment of lane B in solvent system II. The bands designated 1, 2, and 3 were analyzed and were assigned the indicated structures. The sequence of events on ACMS II leading to the formation of acyldipeptides is given in the lower part of the figure.

L-Thr-L-Val and L-Thr-D-Val in the same solvent system (Figure 3, lanes A and B, respectively). Labeling with [^{14}C]-*p*-toluic acid in the reaction mixtures showed after acid hydrolysis that all compounds designated 1, 2 and 3 (and for ease of distinction rechromatographed in a second solvent system) contained radioactive *p*-toluic acid (not shown). Furthermore, acid hydrolysis and radioanalysis of appropriately labeled compounds revealed L-threonine to be present in all three bands. Band 2 contained a 1:1 mixture of D- and L-valine. Cochromatography with authentic chemically synthesized *p*-toluyl-L-threonine, *p*-toluyl-L-threonyl-L-valine, and *p*-toluyl-L-threonyl-D-valine revealed that band 1 is *p*-toluyl-L-threonine and band 2 is a 1:1 mixture of *p*-toluyl-L-threonyl-L-valine and *p*-toluyl-L-threonyl-D-valine.

Amino Acid Thioester Activation Sites on ACMS II Are Most Probably Identical with Those to Which Are Bound the Peptides p-Toluyl-L-threonine and p-Toluyl-L-threonyl-(D,L)-valine. A comparison of data in Figures 2 and 3 reveals that, in the presence of *p*-toluic acid and ACMS I, not only the dipeptides L-Thr-L-Val and L-Thr-D-Val are absent on ACMS II but also the amino acids threonine and valine (valine only partly shown). From these results it appears that in the presence of *p*-toluic acid the amino acid residues become completely acylated with *p*-toluic acid and *p*-toluyl-L-threonine, respectively. This implies that the amino acid thioester activation sites are the same ones that harbor the peptides *p*-toluyl-L-threonine and *p*-toluyl-L-threonyl-(D,L)-valine. If there were distinct sites for aminoacyl and peptidyl residues, one would expect an unchanged level of amino acid in both the presence and absence of *p*-toluic acid. This observation fits with our previous finding that ACMS II contains 4′-phosphopantetheine and that *p*-toluic acid thioester binding on ACMS II is accomplished via a 4′-phosphopantetheine cofactor rather than through a peripheral sulfhydryl group (Stindl & Keller, 1993). Correspondingly, in the activation of other residues catalyzed by ACMS II, individual 4′-phosphopantetheine residues would be involved not only as acceptors but also as carriers of individual partial sequences of the actinomycin peptide chain. This conclusion is supported by recent data concerning the sequence of an active-site peptide fragment of the valine binding domain of gramicidin synthetase II (Schlumbohm et al., 1991; Turgay et al., 1992), which indicated the presence of a serine instead of cysteine in the catalytic centers of various peptide synthetases. These serines most probably represent binding sites for 4′-phosphopantetheine (Marahiel, 1992). Thus each individual amino acid-activating domain of a peptide synthetase would harbor a 4′-phosphopantetheine arm.

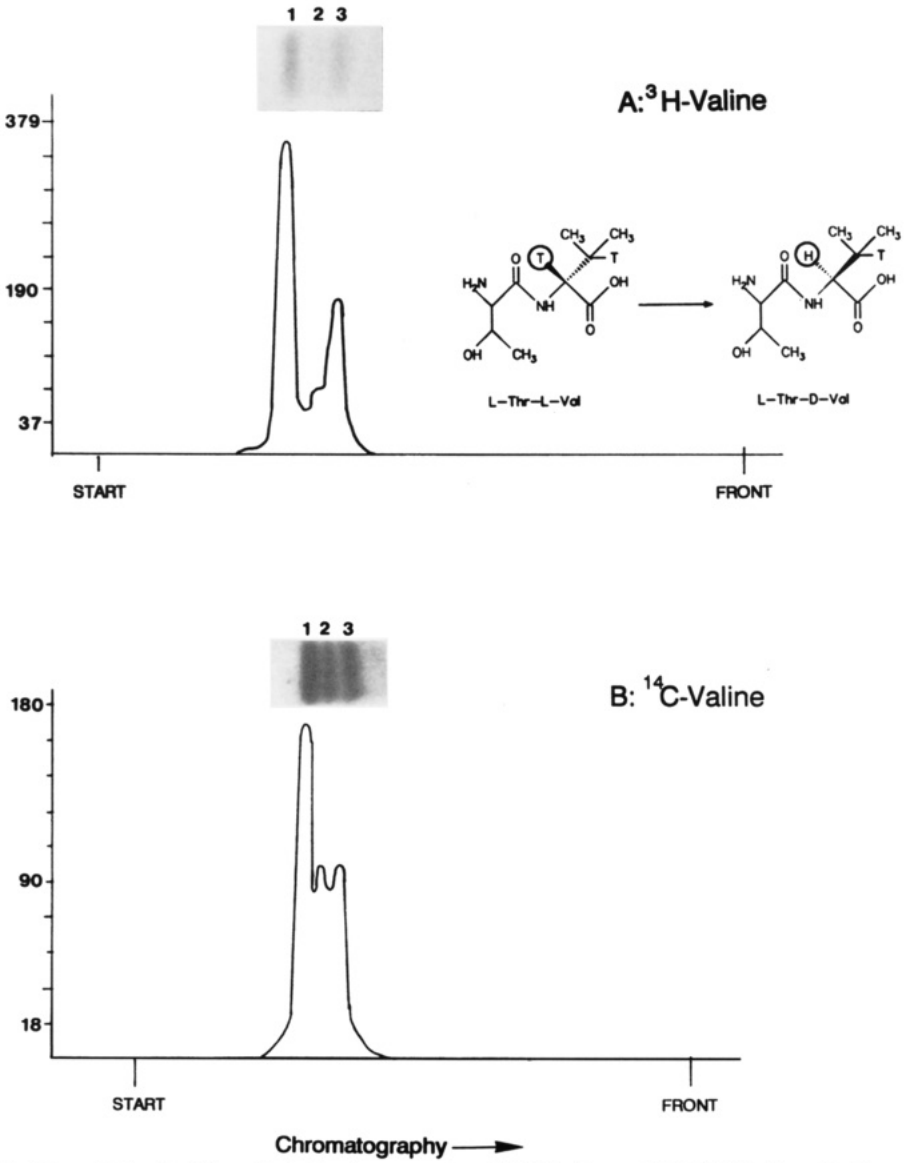

FIGURE 4: Labeling of L-Thr-L-Val and L-Thr-D-Val after incorporation of [^{14}C]valine and [2,3-$^{3}H_2$]valine. Radioscan and autoradiograph of TLC separation of reaction intermediates cleaved from purified ACMS II after incubation with (A) [2,3-$^{3}H_2$]valine and threonine or (B) [^{14}C]valine and threonine. Each chromatogramm was developed on silica gel using solvent system I. Time of exposure to X-ray film was (A) 14 days or (B) 1 week. (1) L-Valine; (2) L-Thr-D-Val; (3) L-Thr-L-Val.

As yet, it cannot be decided whether *p*-toluyl-L-Thr-L-Val and *p*-toluyl-L-Thr-D-Val are located at the same sulfhydryl group as a racemic mixture or whether they are located at different sites. Since ACMS II can only catalyze the partial reaction steps—initiation, elongation, and epimerization—up to amino acid position 2 (D-Val, Figure 1), we must await future information about the structure of that multifunctional enzyme and a system for cell-free synthesis involving the participation of ACMS III. However, from the results it should be clear that it is exclusively the *p*-toluyl-threonyl-D-valine which is transferred from ACMS II to ACMS III. Taken together, the data presented suggest that *p*-toluyl-L-Thr and *p*-toluyl dipeptide formation is the normal route for acylpeptide lactone formation, while synthesis of L-Thr-L-Val and L-Thr-D-Val is only observed in the absence of 4-MHA (or *p*-toluic acid). If 4-MHA is absent, considerable amounts of valine and threonine would be attached to the enzyme (Figure 2). This indicates that peptide bond formation on the enzyme is greatly enhanced by the presence of *p*-toluic acid.

ACMS II Also Catalyzes Formation of p-Toluyl-L-Thr-L-Ile and p-Toluyl-D-allo-Ile. Actinomycin-producing *S. chrysomallus* produces a mixture of actinomycins designated C_1, C_2, and C_3, C_2 and C_3 being the main compounds of the mixture. These differ from each other by the substitution of one (C_2) or both (C_3) D-valine residues in the two peptide chains of the antibiotic by D-*allo*-isoleucine. To show that ACMS II is also responsible for the synthesis of the latter actinomycins, incubation of the enzyme was performed with ACMS I, *p*-toluic acid, threonine, and [^{14}C]isoleucine. As was to be expected, TLC analysis of enzyme-bound reaction intermediates revealed one radiolabeled band, which upon acid hydrolysis yielded a mixture of both radioactive L-isoleucine and D-*allo*-isoleucine (not shown). When [^{14}C]-threonine was used as radiolabel with nonlabeled isoleucine, the same band became labeled with threonine. This band subsequently turned out to be identical with the band designated 3 in Figure 3, lane B. These findings suggest that the band represents a mixture of *p*-toluyl-threonyl-L-Ile and *p*-toluyl-threonyl-D-*allo*-Ile like in the case of the valine-containing band 2 (Figure 3, lane B). Interestingly, in the experiment of Figure 3, lane B, the diastereomer pair was formed from [^{14}C]threonine in the absence of externally added L-isoleucine, indicating that the purified ACMS II preparation contains isoleucine available for incorporation into the peptides.

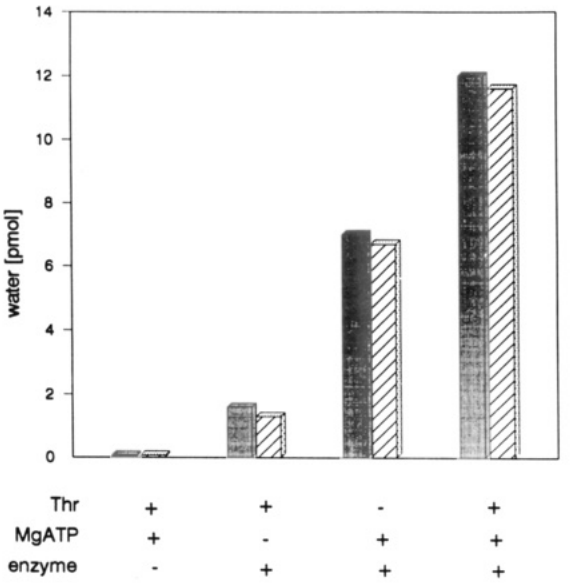

FIGURE 5: Dependence of formation of tritiated water from [2,3-$^3H_2$]valine on the presence of MgATP and threonine. Results from two independent experiments are shown. Solid bars, 45 pmol of ACMS II; hatched bars, 40 pmol of ACMS II.

On the other hand, in the absence of *p*-toluic acid, endogenous isoleucine does not interfere with the synthesis of Thr-L-Val and Thr-D-Val from L-threonine and L-valine (see Figure 2), which may indicate that the enzyme has a high preference for isoleucine against valine as substrate when *p*-toluyl-L-threonine can be formed. By contrast, valine more effectively reacts with threonine than isoleucine when *p*-toluic acid is absent.

Loss of Hydrogen during Epimerization of L-*Valine.* Mason et al. (1967) showed that [2,3-$^3H_2$]-L-valine after in vivo incorporation into actinomycin D had lost the tritium at C-2, while that at C-3 was completely retained. We therefore compared the extent of incorporation of [2,3-$^3H_2$]-L-valine into L-Thr-L-Val and L-Thr-D-Val with that of [U-^{14}C]-L-valine. In all cases, when [^{14}C]valine was used both diastereomers contained radioactivity in a ratio of 1:1 (with a mean deviation of 7% in three determinations). By contrast, with [2,3-$^3H_2$]valine we found L-Thr-D-Val contained only 45% and 50% (as measured in two independent experiments) of the radioactivity found in L-Thr-L-Val. This result is illustrated in Figure 4 and is consistent with previous analyses of Mason et al. (1967). These findings rule out the intermediacy of a dehydrovalyl moiety during epimerization as well as a mechanism of epimerization in which the hydrogen at C-2 is retained in the antipodal product, as in lactate racemase (Cantwell & Dennis, 1974).

The epimerization reaction was shown to be independent of added cofactors such as pyridoxal phosphate, nicotine adenine dinucleotide, and flavin adenine dinucleotide. The absorbance spectrum of the purified enzyme revealed that ACMS II did not contain any tightly bound cofactor, and the activity of the enzyme was not affected by extensive dialysis in the absence or presence of 0.1 mM pyridoxal phosphate.

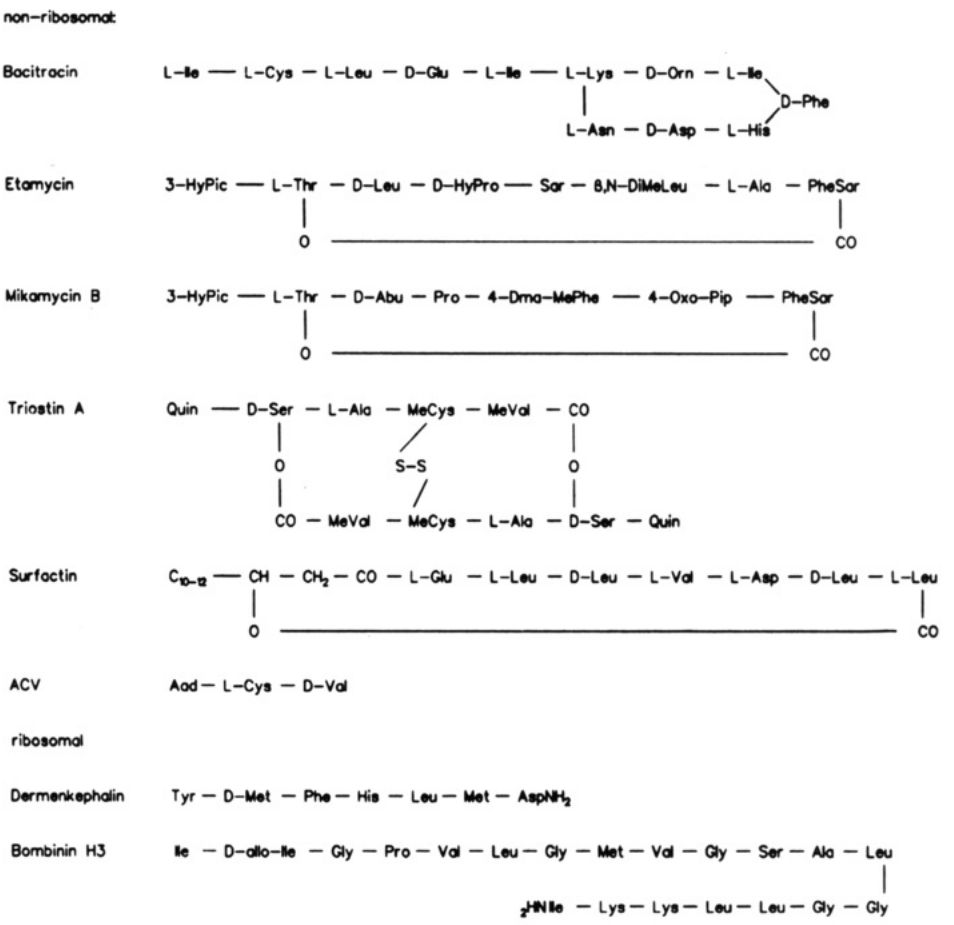

FIGURE 6: Representative naturally occurring peptides containing D-amino acids in internal and carboxy-terminal positions. 3HyPic = 3-hydroxypicolinic acid; D-HyPro = D-hydroxyproline; Sar = sarcosine; β,*N*-DiMeLeu = β,*N*-dimethylleucine; PheSar = phenylsarcosine; D-Abu = D-aminobutyric acid; 4-Dma-MePhe = 4-(dimethylamino)-*N*-methylphenylalanine; 4-OxoPip = 4-oxopipecolinic acid; MeCys = *N*-methylcysteine; MeVal = *N*-methylvaline; Quin = quinoxaline-2-carbonic acid, C_{10-12} = 3-hydroxydecanoic acid; ACV = α-aminoadipyl-cysteinyl-valine; Aad = α-aminoadipinic acid.

Also, addition of 1 mM semicarbazide to enzyme assays had no inhibitory effect on the reaction.

The Hydrogen Lost during Inversion Is Exchanged with Solvent. In order to determine the nature of the hydrogen lost during inversion, dipeptide formation was run with [2,3-$^3H_2$]valine as radiolabel and the formation of radioactive water was measured (Figure 5). In the two experiments, approximately 15% and 20% of radioactivity bound to the enzyme was recovered as tritium-labeled water. These results suggest the involvement of a carbanion at C-2 of valine during epimerization because it is unlikely that [^{3}H]water is formed as result of a hydride shift.

The exchange of tritium with water was dependent on ATP, which indicates that the valine must be activated prior to epimerization, and was stimulated by the addition of L-threonine. The fact that the effect of threonine addition was less pronounced than that of ATP was clarified by control experiments which revealed that the enzyme preparation always contained free threonine that resulted in the formation of some dipeptide in the absence of externally added threonine (not shown).

From these results it can be inferred that prior to hydrogen release from L-valine the peptide bond between threonine and valine is formed. Accordingly, the L-Thr-L-Val (*p*-toluyl-L-Thr-L-Val) intermediate would be the precursor of the corresponding LD-dipeptide. This model also provides an explanation for the existence of both LL- and LD-dipeptides on the enzyme even though the final product of the biosynthetic sequence, actinomycin, has D-valine or D-isoleucine exclusively in that position. Attempts to isolate tritium-labeled L-Thr-D-Val from ACMS II by incubating the enzyme with its nonlabeled substrates in tritium-labeled water failed. One reason might be that ACMS I and ACMS II catalyze only one round of the biosynthetic sequence and stop at the formation of *p*-toluyl-L-Thr-D-Val. Therefore, the enzyme from cells grown in normal water is not able to fill up its donor site with tritium in a next round. It will be interesting to follow this reaction in more detail when a cell-free system of pentapeptide lactone biosynthesis is available.

The data presented here are strong evidence for a mechanism of epimerization of amino acids in the peptide-bound state. The L-valine epimerization in the peptide lactone actinomycin D appears to take place after formation of the peptide bond with the N-neighboring L-threonine via a carbanion structure at C-2 of valine. This mechanism is supported by the finding that the enzyme converts L-isoleucine to D-*allo*-isoleucine and not D-isoleucine. If the epimerization would proceed via the intermediacy of a dehydro amino acid structure, it is unlikely that subsequent rehydrogenation should leave the C-3 stereochemistry of isoleucine unaffected. An unknown proton acceptor group on ACMS II may participate in this C-2 epimerization. This could be an active sulfhydryl group, as in the case of diaminopimelic acid epimerase or proline racemase (Wiseman & Nichols, 1984; Cardinale & Abeles, 1968).

On comparing the structures of actinomycin peptide (Figure 1) with a number of other peptides containing D-amino acids at positions other than the N-terminus (Figure 6), it is tempting to anticipate that the mechanism of epimerization described here also works in the biosyntheses of virginiamycins, mikamycins, quinoxaline antibiotics, and linear peptides with D-amino acids at carboxy-terminal positions, such as δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, the immediate precursor of penicillin N (Huang et al., 1975). It is also noteworthy to point out that the D-amino acids in opioid peptides arise by posttranslational epimerization of the corresponding L-residues in the propeptides. In the dermorphins (Mor et al., 1991, 1992; Erspamer & Melchiorri, 1980) and the bombinins H3–H5 (Mignogna et al., 1993) it is always the second amino acid in the mature peptide chain. This indicates a strong specificity of the enzyme involved in the epimerization of the amino acid in position 2. In light of our findings with the actinomycin peptide chain epimerization, a mechanism of amino acid epimerization involving a carbon C-2 carbanion could also be possible in these cases of the ribosomally synthesized opioid peptides from amphibian skin.

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