Chirality of Peptide Bond-Forming Condensation Domains in Nonribosomal Peptide Synthetases: The C_5 Domain of Tyrocidine Synthetase Is a $^{D}C_{L}$ Catalyst†

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Received June 24, 2003; Revised Manuscript Received August 16, 2003

ABSTRACT: Nonribosomal peptides (NRP) such as the antibiotic tyrocidine have D-amino acids, introduced by epimerase (E) domains embedded within modules of the enzymatic assembly lines. We predict that the peptide bond-forming condensation (C) domains immediately downstream of E domains are D-specific for the peptidyl donor and L-specific for the aminoacyl acceptor ($^{D}C_{L}$). To validate this prediction and establish that the C_5 domain of tyrocidine synthetase is indeed $^{D}C_{L}$, the apoT (thiolation) forms of module 4 ($TycB_3$ AT_4E) and module 5 ($TycC_1$ C_5AT_5) were expressed. T_5 was posttranslationally primed with CoASH to introduce the HS-pantetheinyl group and autoaminoacylated with radiolabeled L-Asn* or L-Asp*. Alternate donor substrates were introduced by priming apo AT_4E with synthetically prepared tetrapeptidyl-CoA's differing in the chirality of Phe-4, D-Phe-L-Pro-L-Phe-L-Phe-CoA, and D-Phe-L-Pro-L-Phe-D-Phe-CoA. The tetrapeptidyl-*S*-T_4 and L-Asp*-*S*-T_5 were studied for peptide bond formation and chain translocation by C_5 to yield pentapeptidyl*-*S*-T_5, whose chirality (D-L-L-D-L- vs D-L-L-L-L-) was assayed by thioester cleavage and chiral chromatography of the released pentapeptides*. Only the D-Phe-4 pentapeptidyl-*S*-T_5 was generated, implying that only D-L-L-D-*S*-T_4 was utilized, proving C_5 is indeed a $^{D}C_{L}$ catalyst. Furthermore, a mutant with an inactive E domain transferred tetrapeptide only when loaded with D-Phe-4 tetrapeptidyl donor, not L-Phe-4, confirming that in the wild-type assembly line C_5 only transfers D-L-L-L-tetrapeptidyl-*S*-T_4 after in situ epimerization by the E domain. These results contrast the observation that C_5 can make both L-Phe-L-Asn and D-Phe-L-Asn when assayed with Phe as the donor substrate. Hence, utilizing an aminoacyl-*S*-T_4 versus the natural peptidyl-*S*-T_4 donor produced misleading information regarding the specificity of the condensation domain.

D-Amino acid residues are hallmarks of bioactive peptides assembled by nonribosomal peptide synthetase (NRPS)[1] multimodular enzymes (*1*−*3*). Occasionally, the D-amino acids are directly selected and activated by an adenylation (A) domain in an NRPS module. For example, this is the strategy for recognition of D-alanine by module 1 of the cyclosporin synthetase (*4*). In that biosynthetic cluster there is a dedicated alanine racemase to provide the requisite D-amino acid from the L-amino acid pool (*5*). However, most of the time D-amino acid residues in nonribosomal peptides arise in situ after selection and covalent tethering of the L-amino acids by A domains onto the thiolation (T) domains via thioester linkages to phosphopantetheine prosthetic groups (*2*, *6*, *7*). The L- to D-C_α-configurational equilibration is affected by 50 kDa epimerase (E) domains embedded within NRPS modules. Thus, almost all NRPS modules responsible for incorporating a D-amino acid residue into the growing peptide have the domain composition CATE, where C is the condensation domain, the peptide-bond forming catalyst, A is the amino acid-recognizing adenylation domain, and T is the thiolation domain that is posttranslationally primed with phosphopantetheine and is the site of covalent tethering of the growing chain in each module.

Tyrocidine (Figure 1A) is a cyclic decapeptide antibiotic produced by *Bacillus brevis* that acts as an antibiotic by membrane perturbation (*8*, *9*). Tyrocidine has two D-amino acids, D-Phe-1 and D-Phe-4, and the tyrocidine synthetase correspondingly has two E domains in its 10 modules, one in the AT_1E initiation module, and one in the fourth module, CAT_4E. The tyrocidine synthetase has its 10 modules arrayed over three separate subunits, one in TycA, three in TycB,

† This work was supported in part by a postgraduate fellowship from NSERC (Canada) to S.L.C., Studienstiftung des deutschen Volkes (S.A.S.), Deutsche Forschungsgemeinschaft (M.A.M.), and the National Institutes of Health (Grant GM 20011 to C.T.W.).

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[1] Abbreviations: A, adenylation domain; C, condensation domain; CoA, coenzyme A; DCM, dichloromethane, DIPCDI, diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; E, epimerase domain; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); HOBt, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IPTG, isopropyl *β*-D-thiogalactopyranoside; LB, Luria-Bertani; LCMS, liquid chromatography mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NRP, nonribosomal peptide; NRPS, nonribosomal peptide synthetase; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; T, thiolation domain; TCEP, tris(2-carboxyethyl)phosphine; Te, thioesterase domain; TFA, trifluoracetic acid; TFE, trifluoroethanol; TIS, triisopropylsilane; TLC, thin-layer chromatography.

10.1021/bi035090+ CCC: $25.00
Published on Web 09/25/2003

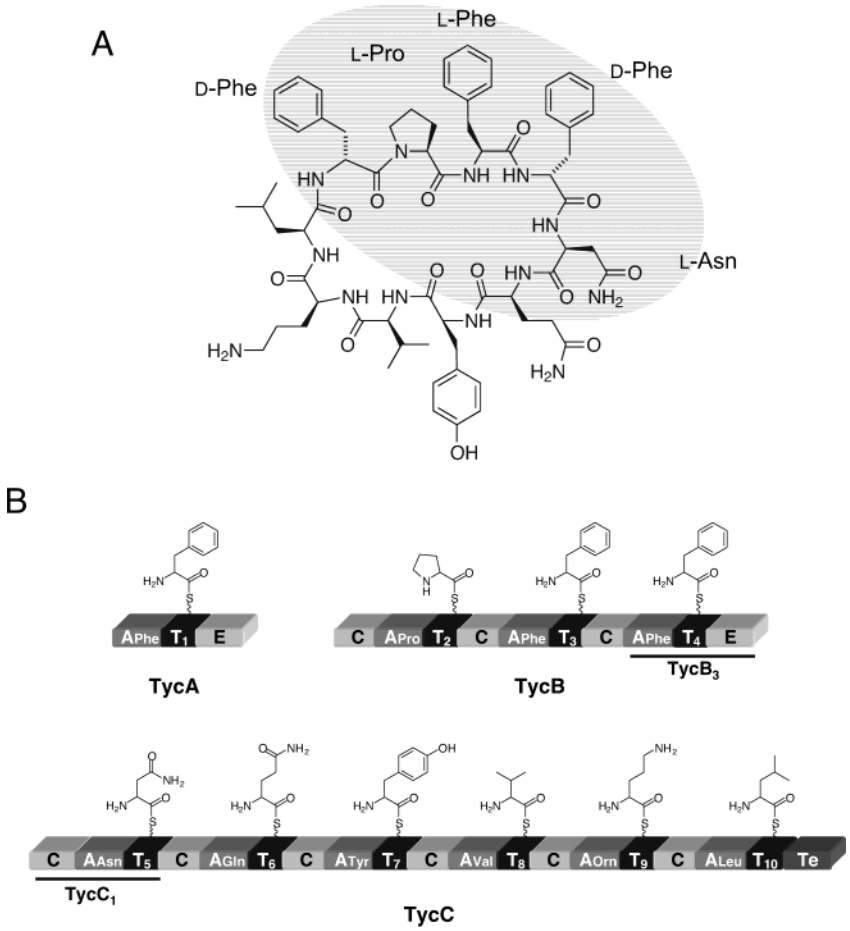

FIGURE 1: (A) Structure of the cyclic decapeptide tyrocidine with the pentapeptide product of the C_5-mediated reaction under analysis highlighted. (B) Schematic representation of tyrocidine synthetase, which is composed of 3 proteins, 10 modules, and 32 domains. The phosphopantetheine arm on a conserved serine in the thiolation domain is represented by a wavy line. The excised domains of interest in this study are underlined.

and six in TycC, so the CAT_4E module is termed the $TycB_3$ module (Figure 1B). Correspondingly, the fifth module in the 10-module NRPS assembly line is the first in the TycC subunit and has been designated $TycC_1$. The domains of this fifth module are referred to as C_5AT_5, even though the initiation module, TycA, contains modules AT_1E, but no C domain.

When an E domain is in the initiation module of an NRPS, as in tyrocidine synthetase and gramicidin S synthetase, the initial L-Phe-*S*-T_1 aminoacyl enzyme intermediate is equilibrated to the D-Phe-*S*-T_1 enzyme before condensation (*2*, *10*, *11*). It follows that the C domain of the downstream module, in this case $TycB_1$ CAT_2, should be D-specific for the upstream donor and L-specific for the downstream acceptor, designated $^{D}C_{L}$ (*12*), and this selectivity was experimentally validated (*13*, *14*). When an E domain is in an NRPS chain elongation module, the timing of the E domain action relative to the C domains immediately upstream and downstream should be inextricably connected. For the $TycB_3$ module example, C_4AT_4E, the A domain activates and covalently loads L-Phe onto T_4. Then the C_4 domain acts *before* the E domain, making D-Phe-L-Pro-L-Phe-L-Phe-*S*-T_4 tetrapeptidyl enzyme (*12*, *15*). The C_4 domain has $^{L}C_{L}$ selectivity. Then the E domain acts, at the peptidyl- rather than the aminoacyl-*S*-enzyme stage, and produces an equilibrated mixture of tetrapeptidyl-*S*-T_4 enzyme, with L-Phe-4 and D-Phe-4 diastereomeric tetrapeptidyl enzyme forms (*12*, *15*). Despite our insights into upstream C domains, confirmation of the model for stereoselectivity in C domains awaits the study of C domains downstream of elongation E domains, the subject of this report.

Given that the cyclic decapeptide antibiotic product tyrocidine contains only D-Phe at position 4, the immediate downstream C_5 domain in $TycC_1$ should be a $^{D}C_{L}$ peptide bond-forming catalyst to impose the observed chiral specificity on the growing pentapeptidyl chain. To validate this expectation and establish that the C_5 domain of the $TycC_1$ module is indeed doubly stereospecific ($^{D}C_{L}$) for tetrapeptidyl-*S*-enzyme donor and aminoacyl-*S*-enzyme acceptor is challenging experimentally for the following reasons. First, the C_5AT_5 tridomain $TycC_1$ module has to be excised from its 720 kDa TycC subunit and expressed in active form in *Escherichia coli* (*15*), and the aminoacyl acceptor substrate, L-Asn-*S*-T_5, must be covalently attached in this module. More problematic is provision of the upstream 124 kDa AT_4E module from $TycB_3$, suitably derivatized as the tetrapeptidyl-*S*-T_4 acyl enzyme donor substrate, with either D- or L-chirality at Phe-4 of the tetrapeptidyl-*S*-enzyme (Figure 2). We report results describing the synthesis of tetrapeptidyl-*S*-CoA's, the enzymatic loading of the tetrapeptidyl-*S*-pantetheinyl phosphate moieties by the promiscuous phosphopantetheinyl transferase Sfp (*16*−*18*), and the generation of pentapeptidyl-*S*-T_5 acyl enzyme by the action of C_5. Cleavage of the thioester to release pentapeptide product identified as the D-L-

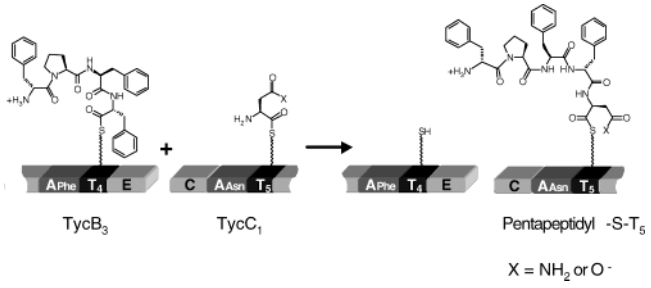

FIGURE 2: Enzymatic reaction between $TycB_3$ AT_4E and $TycC_1$ C_5AT_5 under analysis.

L-D-L diastereomer established the ^DC_L double stereospecificity of the tyrocidine synthetase C_5 domain. In contrast, if only dipeptide formation, Phe-Asn, is monitored with L-Phe-T_4 or D-Phe-T_4 as the donor, both the L-L- and D-L-dipeptides are detected (*15*). Using this chemoenzymatic approach, we have circumvented the problem of natural substrate loading on an excised protein module from a large multimodular system. This technique of loading elongated peptidyl intermediates could be generally applicable to the study of many NRPS systems, such as engineered synthetases.

EXPERIMENTAL PROCEDURES

General. Radiolabeled L-[^{14}C]-Asn (220 mCi/mmol) and L-[2,3-^{3}H]-Asp (37 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. (ARC), and L-[^{14}C]-Asp (207.2 mCi/mmol) was from New England Nuclear (NEN). L-Phe-Asp was purchased from Bachem. Tris(2-carboxyethyl)-phosphine (TCEP) was from BioVectra dcl (PE, Canada). HOBt, HBTU, PyBop, and Boc- and Fmoc-protected amino acids were purchased from NovaBiochem. Ampicillin, kanamycin, coenzyme A (CoA), and *N*,*N*-diisopropylehtylamine (DIPEA) were from Sigma, and triisopropylsilane (TIS) from Aldrich. All other chemicals were of reagent grade and used without further purification.

Preparative HPLC was performed on a Beckman Coulter System Gold instrument with a Vydac Proteins and Peptides C18 column (10 μm, 22 $\times$ 250 mm). LCMS identification was performed utilizing a Shimadzu LCMS-QP8000α, equipped with two LC-10AD*VP* liquid chromatography pump modules, a SPD-10AV*VP* UV−vis detector, and a SIL-10AD*VP* autosampler module, and a Vydac C18 Mass Spec column (5 μm, 2.1 $\times$ 250 mm). MALDI-TOF mass spectrometry was accomplished using a PerSeptive Biosystems Voyager-DE STR mass spectrometer. Radio-HPLC separation employed a Beckman Coulter instrument, with a Beckman 171 radioisotope detector and a Vydac C18 Proteins and Peptides column (5 μm, 4.6 $\times$ 250 mm) or a Chirobiotic T column (5 μm, 4.6 $\times$ 250 mm) from Advanced Separation Technologies Inc. TLC plates were exposed to a BAS-TR2040 or BAS-IIIs image plate for approximately 12−24 h, and then the images were read by a Bio-Imaging Analyzer BAS1000 (Fuji) and the results analyzed using Image Gauge Ver. 3.45 and L Process Ver. 1.95 (Science Lab 99; Fuji Photo Film Co., Ltd).

E. coli M15/pREP4 was purchased from Qiagen, and electrocompetent cells were prepared with a Bio-Rad Gene Pulser electroporator. *E. coli* TOP10 chemically competent cells were purchased from Invitrogen. Protein samples were concentrated using Centriprep YM50 (Millipore). Enzyme assays were performed in 50 mM HEPES, 1 mM TCEP, pH 7.5. Oligonucleotide primers were purchased from Integrated DNA Technologies. DNA sequencing was performed by the Dana-Farber Cancer Institute Molecular Biology Core Facilities.

Protein Overproduction and Purification. The pQE (Qiagen)-based plasmids encoding the C-terminal histidine$_6$-tagged proteins of interest, $TycB_3$ AT_4E, and $TycC_1$ C_5AT_5 (*15*), were transformed into *E. coli* M15/pREP4 electrocompetent cells. Cells were grown at 37 °C in Luria-Bertani (LB) media supplemented with 10 mM $MgCl_2$, 100 μg/mL ampicillin, and 25 μg/mL kanamycin to an OD_{600} of 0.5 when the temperature was reduced to 28 °C, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) added, and the culture grown for 5−6 h more. The cells were harvested by centrifugation (4000*g*, 15 min) and stored at −80 °C. Cells were thawed in buffer A (50 mM Tris, 0.5 M NaCl, 5 mM imidazole, pH 8.0) and disrupted by French Press (Thermo Spectronic), two passes at 16 000 psi. Cell debris was removed by ultracentrifugation (95000*g*, 30 min).

The clarified cell extract was incubated with Ni-NTA resin (Qiagen; ~1 mL of 50% suspension/L of cell culture) for 3−4 h at 4 °C in a batch-binding format. The suspension was then loaded into a gravity column and washed with buffer A. $TycB_3$ ATE or $TycC_1$ CAT was then eluted with a stepwise gradient of imidazole (20−300 mM) in buffer A. The fractions containing the purified protein of interest, as determined by SDS−PAGE with Coomassie staining, were pooled and dialyzed against 2 $\times$ 1 L of 50 mM HEPES, 1 mM TCEP, 10% glycerol, pH 7.5. The concentration of the proteins was determined spectrophotometrically at 280 nm using the calculated extinction coefficients, 135.8 mM^{-1} cm^{-1} for $TycB_3$ AT_4E and 116.7 mM^{-1} cm^{-1} for $TycC_1$ C_5AT_5. The proteins were flash frozen in liquid nitrogen and stored at −80 °C. The yield for $TycB_3$ AT_4E was approximately 6 mg/L and for $TycC_1$ C_5AT_5 25 mg/L.

Mutagenesis and Purification of $TycB_3$ ATE H736A. Mutation of the active site histidine to alanine (residue no. 736 in this construct) in the E domain of the $TycB_3$ AT_4E protein was accomplished in a one-step QuikChange site-directed mutagenesis protocol (Strategene), using the recommended conditions. The plasmid encoding the wild-type protein, pQE70-$TycB_3$ATE, was used as the template, and the oligonucleotide primers (codon mutating His to Ala in bold) were 5′-GGTCGCGATCCAC**GCG**CTTGTCGTG-GATGG-3′ and 5′-CCATCCACGACAAG**CGC**GTGGAT-CGCGACC-3′. Following *Dpn*I treatment to remove the methylated, wild-type DNA, the PCR reaction product mixture was transformed into *E. coli* TOP10 cells.

The mutant $TycB_3$ ATE enzyme was purified as described for the wild-type enzyme, with a yield of approximately 6 mg/L of cell growth.

Synthesis of Peptide Standards: D-Phe-L-Asp, D-Phe-L-Pro-L-Phe-L-Phe-L-Asp and D-Phe-L-Pro-L-Phe-D-Phe-L-Asp, D-Phe-L-Pro-L-Phe-L-Phe, D-Phe-L-Pro-L-Phe-D-Phe. Peptide synthesis was performed using a PerSeptive Biosystems 9050 synthesizer (0.3 mM scale) using diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt) chemistry on H-Asp(OtBu)-2-ClTrt (chlorotrityl), or H-Phe-2-ClTrt resin (NovaBiochem), or Fmoc-D-Phe-2-ClTrt prepared by coupling Fmoc-D-Phe to 2-chlorotrityl chloride resin using standard solid-phase synthesis protocols. The D-Phe-L-Asp dipeptide was coupled by hand using comparable protocols.

The peptide was cleaved from the resin, Asp side-chain protecting group, and the N-terminal Boc-D-Phe was deprotected in a single treatment with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triiospropylsilane (TIS) at room temperature for 3 h. The solution was then added to cold ether dropwise, and the precipitated peptide was collected by centrifugation. The purified peptides were lyophilized to yield a white powder, and the identity and purity were established by analysis on LCMS: D-Phe-L-Asp calculated 281.1 [$(M + H)^+$], 280.9 observed; D-Phe-L-Pro-L-Phe-L-Phe-L-Asp 672.3 [$(M + H)^+$] calculated, 672.5 observed; D-Phe-L-Pro-L-Phe-D-Phe-L-Asp 672.3 [$(M + H)^+$] calculated, 672.5 observed; D-Phe-L-Pro-L-Phe-L-Phe 557.3 [$(M + H)^+$] calculated, 557.1 observed; and D-Phe-L-Pro-L-Phe-D-Phe 557.3 [$(M + H)^+$] calculated, 557.2 observed.

Synthesis of D-Phe-L-Pro-L-Phe-L-Phe-CoA and D-Phe-L-Pro-L-Phe-D-Phe-CoA. Following peptide synthesis as described above, the peptidyl-CoA's were prepared based on a previously reported protocol (*14*). The peptide was cleaved from the resin with 1:1:3 acetic acid/trifluoroethanol (TFE)/dichloromethane (DCM) and incubated at room temperature for 3 h. The resin was removed by filtration, and *n*-hexane was added to precipitate the fully protected peptide. Following rotary evaporation to remove the solvent, the peptide was redissolved in DCM and precipitated with *n*-hexane; this was repeated twice. The CoA coupling to the protected peptide was accomplished by the addition of 1 equiv of Coenzyme A (Li^+ salt; Sigma), 4 equiv of potassium carbonate, and 1.5 equiv of PyBOP in 1:1 THF/water. The reaction was mixed by tipping for 2 h at room temperature, and then the solvent was removed by rotary evaporation followed by lyophilization. Removal of the N-terminal Boc protecting groups was achieved by treatment with 95% TFA, 2.5% water, and 2.5% TIS at room temperature for 3 h. The solution was then added to cold ether dropwise, and the precipitate was removed by centrifugation following overnight incubation at −20 °C. The peptidyl-CoA was then dissolved in acetonitrile/water and purified by preparative HPLC on a reversed-phase C18 column with a gradient of 0−100% acetonitrile in 0.1% TFA/water over 35 min. The purified compounds were lyophilized and the identity and purity established by analysis in negative ion mode on LCMS and MALDI-TOF mass spectrometry: D-Phe-L-Pro-L-Phe-L-Phe-CoA calculated 1304.4 [$(M - H)^-$], 1303.3 observed by LCMS; and D-Phe-L-Pro-L-Phe-D-Phe-CoA 1304.4 [$(M - H)^-$] calculated, 1303.4 observed. To verify the thioester linkage in the products, a sample of each peptidyl-CoA was incubated with 0.1 M KOH to liberate the tetrapeptide or under milder conditions by the addition of 10 μM TycC thioesterase (Te) (*19*) and a further 2 h incubation, after which the product was analyzed by LCMS and MALDI-TOF MS.

Enzymatic CoA Loading. The apo-$TycC_1$ CAT was primed to the holo form by incubation with 0.5 μM *Bacillus subtilis* phosphopantethienyl transferase Sfp (*17*, *18*), 100 μM CoASH, 10 mM $MgCl_2$, and 1 mM TCEP in 50 mM HEPES, pH 7.5, at 30 °C for 1 h. Radiolabeled L-Asp (either ^3H or ^{14}C) was then loaded onto the holo-protein by the addition of 5 equiv of amino acid to protein, 4 mM ATP, and further incubation at 30 °C for 1 h. For ^{14}C-labeled Asp, 100% uniformly labeled amino acid was utilized, whereas for ^3H-labeled Asp, 90% unlabeled amino acid was used.

For the dipeptide product-forming reactions, apo-$TycB_3$ ATE was primed as described for $TycC_1$ CAT and loaded with L-Phe or D-Phe by adding 3 equiv and 4 mM ATP to the protein, incubating at 30 °C for 1 h further to ensure complete loading and to allow the epimerization reaction to reach equilibrium.

Apo-$TycB_3$ ATE, both wild-type and the E domain mutant proteins, were loaded with peptidyl-CoA by incubation of the protein (40 μM) with 100 μM peptidyl-CoA, 10 μM Sfp, 10 mM $MgCl_2$, and 1 mM TCEP at room temperature for 30 min.

Enzymatic Reaction and Product Identification. Analysis of the C_5-catalyzed reaction was performed by incubating 10 μM $TycB_3$ AT_4E with 2 μM $TycC_1$ C_5AT_5 at 30 °C. Twenty-microliter samples were quenched at various time intervals with 80 μL of 10% trichloroacetic acid (TCA; w/v). Mixtures were vortexed, incubated on ice, and then centrifuged for 30 min at 4 °C (16060*g*) to remove the precipitated protein. The pellet was then washed with 500 μL of ether and spun again for 6 min. The product was hydrolyzed from the protein with the addition of 100 μL of 0.1 M KOH and incubation at 60 °C for 15 min. To remove the protein following the product hydrolysis, 500 μL of methanol was added, and the sample was centrifuged for 30 min at 4 °C. The supernatant was removed and dried under vacuum in a speed-vac system. The samples were taken up in 10 μL of aqueous solution and separated by TLC or HPLC.

The products from the dipeptide-forming reaction were separated by reversed-phase HPLC, eluting with 0% acetonitrile for 5 min and then shifting to 5% acetonitrile/0.1% TFA, isocratic, monitoring both ^3H radioactive counts and at 220 nm. For TLC separation, 1-μL samples were applied to silica gel TLC plates (Silicagel 60 F_{254}; EM Science) and developed in 1:1:1:1 (v/v) butanol/acetic acid/ethyl acetate/water. The ratio of the product, D-Phe-L-Asp and L-Phe-L-Asp pentapeptide or the pentapeptide D-Phe-L-Pro-L-Phe-D-Phe-L-Asp, and the radioactive L-Asp starting material was calculated from the phosphorimage of the TLC plate.

Confirmation of the chirality of the pentapeptide product was achieved by HPLC separation on a Teicoplanin-based Chirobiotic T column, using 60% methanol, isocratic, monitoring at 220 nm and radioactive counts. Samples from reactions utilizing L-[^3H]-Asp were co-injected with synthetically prepared pentapeptide standards.

To confirm that the reaction of tetrapeptidyl-*S*-(AT_4E) and Asp-*S*-(C_5AT_5) was indeed producing the desired pentapeptide product, a large-scale reaction (1 mL reaction, 20 μM $TycB_3$ AT_4E and 4 μM $TycC_1$ C_5AT_5) without radioactive amino acids was performed with the D-Phe-4-tetrapeptidyl-CoA (D-Phe at the fourth position in the tetrapeptide; 100 μM) loaded for 30 min, and the enzymatic reaction was allowed to proceed for 5 min. The reaction was quenched, the product was liberated in the manner described above, and the product was desalted using a C_{18} reversed-phase ZipTip (Millipore) and then analyzed by LCMS.

Analysis of E-Domain Activity. To ensure that the E domain in the $TycB_3$ AT_4E construct was epimerizing the loaded tetrapeptide, the enzyme was loaded with tetrapeptidyl-CoA containing L-Phe-4 or D-Phe-4 under the standard conditions described above, and the reaction was quenched after 30 min (500 μL, 40 μM enzyme). The tetrapeptide product was released by the addition of 10 μM TycC Te

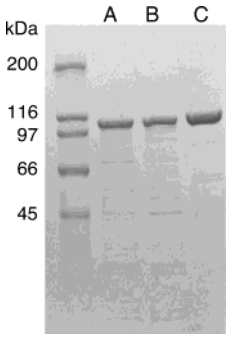

FIGURE 3: Separation of (A) the purified 124-kDa wild-type and (B) E domain mutant $TycB_3$ ATE and (C) the 117-kDa $TycC_1$ CAT on a 4−15% SDS−PAGE gel with Coomassie staining.

(*19*) and a further 2-h incubation or KOH hydrolysis as described above. The protein was methanol precipitated, and the product was analyzed by LCMS and compared to the elution of synthetically prepared standards, D-Phe-L-Pro-L-Phe-D-Phe and D-Phe-L-Pro-L-Phe-L-Phe. This analysis was repeated with the $TycB_3$ ATE E domain mutant protein to confirm that the substrate was not epimerized.

RESULTS

Modules 4 and 5 of the Tyrocidine Synthetase Assembly Line. $TycC_1$ CAT and $TycB_3$ ATE wild-type and the E domain mutant proteins were successfully expressed in *E. coli* M15/pREP4 and purified by Ni-NTA chromatography (Figure 3).

Amino Acid Activation and Loading. As difficulties were encountered during the analysis of the reaction products with a C-terminal Asn, as discussed below, the ability of $TycC_1$ C_5AT_5 to load alternate amino acids was investigated. Pyrophosphate exchange assays (*20*) with ^{32}PPi suggested that, in addition to activating L-Asn, $TycC_1$ CAT will activate L-Asp but to a lesser degree, L-Asp/L-Asn = 0.3 after 10 min and 0.7 after 30 min of incubation. D-Asn was activated to an ever lower extent. The activation of L-Gln, L-Ala, L-Phe, and D-Asp was negligible (data not shown). Further studies using radiolabeled amino acids indicated that maximal loading was observed with 2 mole equiv of Asn to enzyme, but 5 equiv of Asp was required to reach comparable levels. Under these conditions, greater than 75% of the protein is loaded with the desired amino acid. Hence, these levels were used to load holo-$TycC_1$ CAT in all subsequent studies.

Dipeptide Product Formation. Previous studies suggested that the C domain (C_5) of $TycC_1$, when assayed with Phe-*S*-T_4 rather than the physiologic tetrapeptidyl-*S*-T_4, was selective but not exclusively specific since both L-Phe-L-Asn and D-Phe-L-Asn were formed (*15*). The L-L peptide predominated 2:1. This is in contrast to the expected D-selectivity (-D-Phe-4-L-Asn-5-) based on the chirality of the tyrocidine peptide product. Initially attempts were made to monitor dipeptide formation with the natural L-Asn on $TycC_1$. This proved problematic during the basic hydrolysis of the Phe-Asn dipeptide product off the protein after the reaction. The C-terminal Asn was unstable under these conditions. It is postulated that during the hydrolysis of the product under basic conditions, the Asn β carboxamido nitrogen reacts in an internal displacement to attack the thioester carbonyl and lead to deacylation. There is precedent

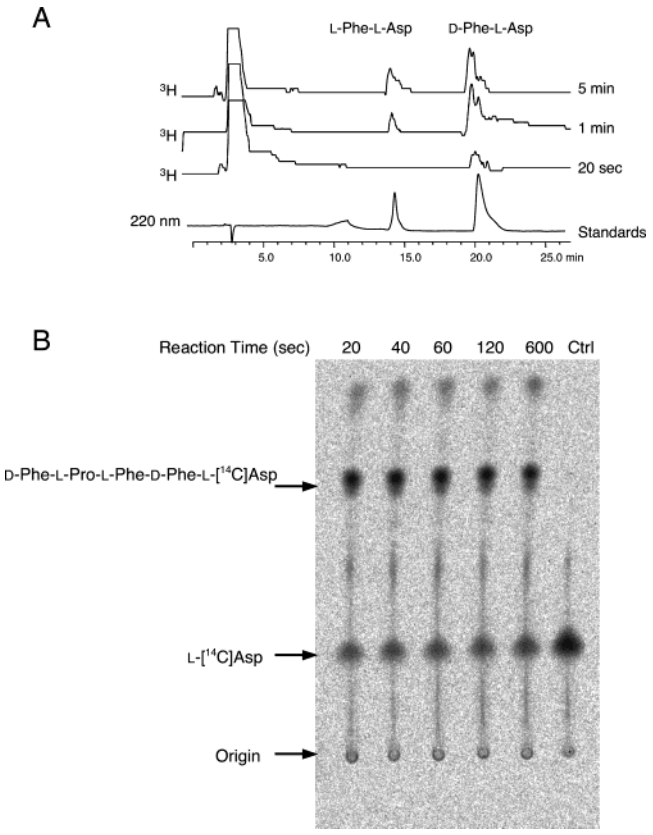

FIGURE 4: (A) Reversed-phase HPLC separation of the dipeptide products from the reaction between D-Phe-*S*-(AT_4E) (10 μM) and [^{3}H]-Asp-*S*-(C_5AT_5) (2 μM). (B) Phosphorimage of the silica TLC plate separation of the single-turnover reaction products from the condensation reaction between D-Phe-L-Pro-L-Phe-D-Phe-*S*-(AT_4E) (10 μM) and [^{14}C]-Asp-*S*-(C_5AT_5) (2 μM).

for such Asn internal nucleophilic participation, for example in intein splicing as an essential deconvolution step for the last step of protein splicing (*21*, *22*). The previous work utilized an engineered construct with the TycC Te fused at the C-terminus of the $TycC_1$ CAT construct (*15*). This avoided the Asn side reactions observed with KOH hydrolysis but precluded determination of the rate of the condensation reaction alone. To avoid this problem, L-Asp was loaded onto $TycC_1$ CAT. Asp did not appear to have the same side reactions during the product workup as observed for Asn, and hence Asp was used in the subsequent analyses. With L-Asp loaded, a mixture of L-Phe-L-Asp and D-Phe-L-Asp was also observed (Figure 4A). Examination of the early time points suggests that although both L- and D-Phe are accepted, the rate of D-Phe-L-Asp formation is quicker. Therefore, although the enzyme appears to have relaxed substrate specificity for aminoacyl donors, it is still somewhat selective. Under the reaction conditions, 10 μM $TycB_3$ AT_4E and 2 μM $TycC_1$ C_5AT_5, the reaction appears to be complete within 120 s. Although no additional product is formed after 2 min, only ~50% of the aspartate loaded onto $TycC_1$ CAT has reacted, based on quantitation of the intensity of the Asp versus dipeptide (both containing [^{14}C]-Asp) spots on the phosphorimage of the TLC (not shown). Perhaps the A domain can activate and load the amino acid but the C domain is not functionally active to catalyze the condensation reaction in all of the protein molecules.

Tetrapeptidyl CoA's: Preparation and Utilization by Sfp. The synthetically prepared peptidyl-CoA substrates were

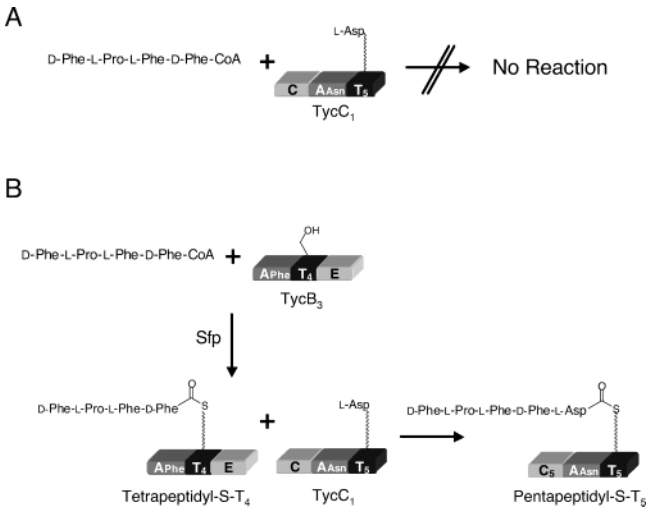

FIGURE 5: (A) C_5 does not catalyze the condensation reaction with the tetrapeptidyl-CoA soluble substrate. However, when the tetrapeptidyl-CoA is loaded onto T_4 of the $TycB_3$ protein, the $TycC_1$ condensation domain (C_5) catalyzes the reaction to form a pentapeptidyl product (B).

successfully loaded onto apo-$TycB_3$ AT_4E using 2.5-fold excess over protein and 10 μM Sfp. Attempts to further prime with radiolabeled CoA suggest that the enzyme was greater than 75% loaded when either the D-Phe-4 or L-Phe-4 tetrapeptidyl-CoA was used.

Pentapeptide Product Formation. Analysis of the reaction product from $TycC_1$ C_5AT_5-Asp* (* indicates radioactive label) and tetrapeptidyl-CoA-loaded $TycB_3$ AT_4E, followed by separation on silica TLC, indicated that the pentapeptide product was formed (Figures 4B and 5B). Coelution with authentic standards on HPLC (Figure 6) and identification by ESMS confirmed that the pentapeptide was produced (Figure 7C; calculated 672.3 [$(M + H)^+$], 672.5 observed). To clearly identify the chirality of the product at the fourth position, a Chirobiotic T HPLC column was utilized, in which Teicoplanin is linked to silica gel. This method produced excellent separation of the synthetically prepared pentapeptide standards with D-Phe-4 (t_R = 11.1 min) or L-Phe-4 (t_R = 21.5 min). Separating the product of the reaction of [^{3}H]-Asp on $TycC_1$ CAT and the tetrapeptide-CoA's with either D-Phe-4 or L-Phe-4 on wild-type $TycB_3$ ATE clearly established that the pentapeptide product had D-Phe at position four. There was no pentapeptide observed containing L-Phe-4 (Figure 6), suggesting that C_5 is D-specific. Kinetics of the condensation reaction were monitored using [^{14}C]-Asp and separation of the pentapeptide product from the Asp starting material on silica TLC (Figure 4B). Under the reaction conditions examined, the reaction was complete in less than 20 s. Precise determination of the condensation rate constant will require rapid quench analyses. As in the dipeptide-forming reaction, this reaction appears to proceed to only 50% completion, suggesting that only half of the enzyme is functional. Large-scale analysis indicated that both the D-Phe-4 and L-Phe-4 tetrapeptide were still present on the $TycB_3$ AT_4E enzyme (Figure 7C); hence, substrate availability was not an issue. In addition, no pentapeptide product was formed in the control reactions containing tetrapeptidyl-CoA and *Asp-*S*-(C_5AT_5), suggesting that C_5 will not accept soluble peptidyl-CoA substrates under the conditions examined (Figure 5A).

$TycB_3$ ATE E Domain Mutant Analysis. To validate the D-specificity for the upstream donor in C_5, a $TycB_3$ ATE E domain mutant was constructed in which the active site histidine (His736) was mutated to an alanine. Previous work on the bimodule protein, $TycB_3$ AT_3CAT_4E, demonstrated

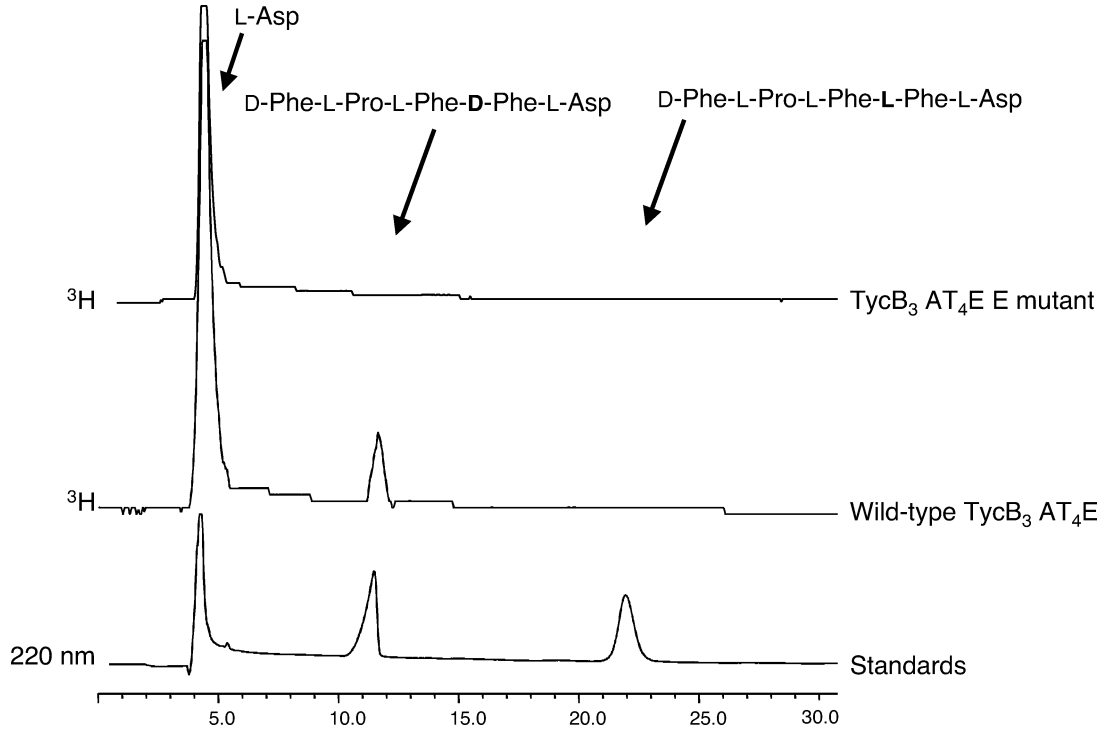

FIGURE 6: Chiral HPLC separation on a Chirobiotic T column showing coelution of the nonradioactive pentapeptide standard, D-Phe-L-Pro-L-Phe-D-Phe-L-Asp, with the product for the reaction of 10 μM D-Phe-L-Pro-L-Phe-D-Phe-*S*-(AT_4E) and 2 μM [^{3}H]-Asp-*S*-(C_5AT_5) (middle elution profile). Note that there is no D-Phe-L-Pro-L-Phe-L-Phe-L-Asp formed. No pentapeptide is formed when the D-Phe-L-Pro-L-Phe-L-Phe-CoA tetrapeptide is loaded on the $TycB_3$ AT_4E E domain mutant enzyme (top).

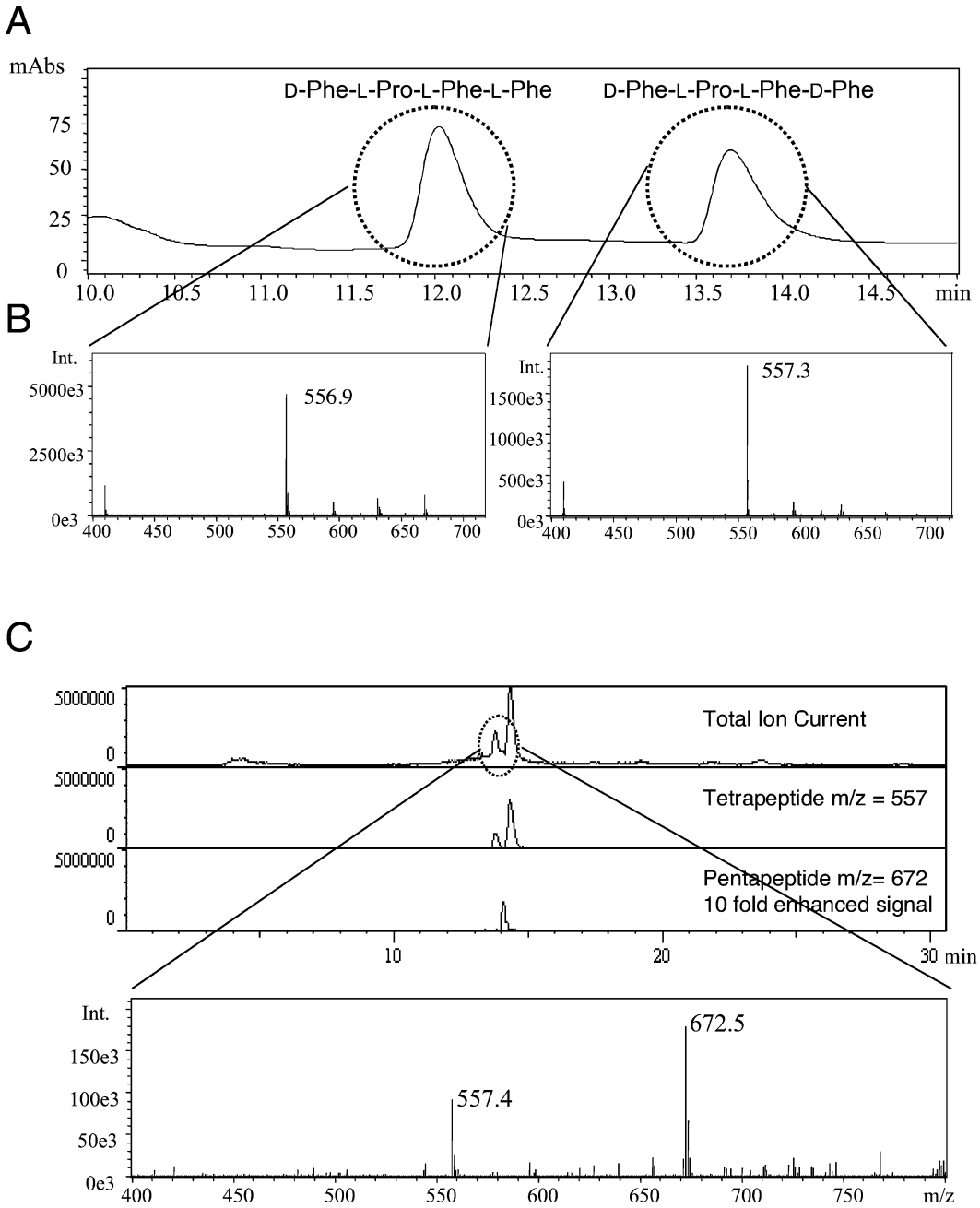

FIGURE 7: LCMS illustrating (A) LC separation and (B) MS identification of both the D-Phe-L-Pro-L-Phe-D-Phe and D-Phe-L-Pro-L-Phe-L-Phe tetrapeptides in a ~1:1 mixture following loading and epimerization on $TycB_3$ AT_4E. (C) Identification of the pentapeptide product of the correct mass following the reaction of 10 μM D-Phe-L-Pro-L-Phe-D-Phe-*S*-(AT_4E), 2 μM Asp-*S*-(C_5AT_5), and the tetrapeptide substrate remaining following the reaction. The total ion current is shown with the signal from the masses of interest highlighted below. These data were collected in positive ion mode; hence, the pentapeptide, with the negatively charged Asp residue, does not ionize as well as the tetrapeptide, and therefore its signal was amplified 10-fold for visualization.

that mutation of this histidine (residue 1773 in the didomain construct) abolished the E domain activity (*12*).

The $TycB_3$ ATE E domain mutant protein was expressed and purified under the same conditions employed for the wild-type enzyme. When the D-Phe-4 tetrapeptide-CoA was utilized, pentapeptide with only D-Phe-4 was observed, as seen for the wild-type enzyme. However, when the tetrapeptide-CoA containing L-Phe-4 was loaded onto the mutant enzyme, no product was observed (Figure 6). This E domain mutant enzyme cannot epimerize the Phe-4, as was validated by a large-scale reaction and LCMS analysis of the released tetrapeptide. Hence, this is further confirmation that C_5 is D-specific for the donor substrate.

Confirmation of the Epimerization Activity in the Wild-Type Enzyme. The ratio of D/L-Phe-4 in the tetrapeptidyl chain on $TycB_3$ AT_4E was determined following a 30-min reaction to load the peptidyl-CoA and epimerize the Phe-4. The peptide was released from the protein by the addition of the TycC Te and a further 2-h incubation, or by standard KOH hydolysis. Figure 7A illustrates the chromatographic separation of the D-Phe-4 and L-Phe-4 tetrapeptides following a reaction to load the L-Phe-4 peptidyl-CoA and KOH release. The ESMS identification of these tetrapeptides (calculated 557.3 [$(M + H)^+$], L-Phe-4 556.9 observed, D-Phe-4 557.3 observed) is shown in Figure 7B. In each of these reactions the ratio of D-Phe-L-Pro-L-Phe-D-Phe to

D-Phe-L-Pro-L-Phe-L-Phe was close to 1:1, based on the LC elution profile (Figure 7A). This was independent of the chirality at Phe-4 in the tetrapeptidyl-CoA loaded.

DISCUSSION

As noted in Figure 1, the 10 modules of the tyrocidine synthetase assembly line are distributed over three subunits, TycA−B−C, with one, three, and six modules, respectively. The D-L- peptide bond formation under investigation (-D-Phe-4-L-Asn-5-) is carried out as the growing peptide chain is translocated across the TycB/TycC subunit boundaries. Thus, the C_5 domain is in the TycC subunit while the tetrapeptidyl chain donor is on the most downstream thiolation domain (T_4) of the TycB subunit (Figure 1B and 2).

To study the chirality of the C_5 domain, which is responsible for the Phe-4-Asn-5 peptide bond during chain elongation, requires that modules four and five be available. In prior efforts it has been established that one can express recombinant module four, AT_4E, in *E. coli* in soluble form with the T_4 domain in the apo form (*12*, *15*). This protein is referred to as $TycB_3$ ATE. Likewise, it has been possible to express the C_5AT_5 module in *E. coli* in apo form, referred to as $TycC_1$ CAT. Hence, these enzyme fragments were available as his-tagged proteins that could be efficiently produced and purified (Figure 3). To convert the inactive, apo forms of modules four and five to the holo, phosphopantetheinyl forms capable of covalently loading aminoacyl and peptidyl chains, the phosphopantetheinyl transferase Sfp from *B. subtilis* can be used in coexpression studies in vivo or as a purified catalyst in vitro (*18*, *23*).

At this point, both modules could be evaluated for component domain activity by demonstrating that $TycB_3$ AT_4E will activate and load L-[^{14}C]-Phe or D-[^{14}C]-Phe and $TycC_1$ C_5AT_5 will activate and load L-[^{14}C]-Asn (data not shown) (*15*). When dipeptide formation is measured in this and prior studies, a surprising lack of chiral selection is detected with both L-L- and D-L-dipeptidyl-*S*-T_4 detected (Figure 4A) (*15*). While this assay has convenience in its favor, it appears to lead to erroneous conclusions about the C_5 chirality extrapolated to the natural tetrapeptidyl chain donor.

To evaluate the chirality of the C_5 condensation domain in peptide bond formation with the natural tetrapeptidyl chain as donor required installation of the tetrapeptidyl chain (D-Phe-Pro-Phe-Phe) on the phosphopantetheinyl prosthetic group on T_4. Since the AT_4E had been excised and separated from the three upstream modules normally generating the tetrapeptidyl chain that docks on T_4, a separate approach for site-specific introduction of the tetrapeptidyl chain was required.

We turned to Sfp, which normally functions to add phosphopantetheinyl groups to a serine side chain in T domains of the NRPS assembly line catalyst surfactin synthetase (*17*) by cleaving cosubstrate CoASH and releasing the 3′,5′-ADP fragment. We have shown that Sfp is usefully promiscuous in that it will accept acyl-*S*-CoA thioesters in place of free CoASH and transfer acyl-*S*-pantetheinyl phosphate in place of pantetheinyl phosphate to the serine side chain of many apo-T domains of noncognate NRPS modules (*14*, *24*). We put the promiscuity of Sfp to transfer acyl-*S*-pantetheinyl groups to a rigorous test by synthesis of tetrapeptidyl-*S*-CoA's, specifically the D-Phe-L-Pro-L-Phe-D-Phe-CoA and D-Phe-L-Pro-L-Phe-L-Phe-*S*-CoA, corresponding to the diastereomers that are thought to accumulate on the T_4 domain of module $TycB_3$ during tyrocidine chain growth. Remarkably, Sfp is efficient with both diastereomeric tetrapeptidyl-CoA's, transferring the tetrapeptidyl-*S*-pantetheinyl moieties onto apo-T_4 (Figure 5B and 7). This chemoenzymatic approach will allow synthesis and then installation of diverse peptidyl-*S*-pantetheinyl groups onto many apo-T domains and so should prove generally useful to evaluate specificity and mechanism in different NRPS contexts, for example in the cyclization of peptidyl-*S*-enzyme intermediates by T-Te didomain constructs (*25*).

Using this method, the donor tetrapeptidyl-*S*-T_4 form of $TycB_3$ AT_4E was made available in substrate quantities. The acceptor substrate, L-Asn-*S*-T_5 could be generated in more straightforward fashion by allowing the Sfp-primed, holo-C_5AT_5 protein module to select, activate, and auto-load L-[^{14}C]-Asn on its pantetheinyl T_5 domain in cis. The radioactive label would be useful for evaluation of subsequent pentapeptide product. During the initial studies between D-Phe-*S*-(AT_4E) and L-Asn-*S*-(C_5AT_5), it was observed that the *Asn label on T_5 appeared to be unusually unstable during the reaction workup and is postulated to undergo deacylation. This side reaction made studies with L-Asn-*S*-T_5 intractable. L-Asp was accepted by the A domain of C_5AT_5, and L-Asp-*S*-T_5 did not undergo the same intramolecular deacylation reaction. Thus, all subsequent studies to evaluate C_5 chirality were performed with L-[^{14}C]-Asp-*S*-(C_5AT_5) as acceptor substrate.

The reaction between tetrapeptidyl-*S*-(AT_4E) and L-Asp-*S*-(C_5AT_5) is a single-turnover event because the product pentapeptidyl-*S*-(C_5AT_5) stays covalently attached to the $TycC_1$ protein bearing the catalytic C_5 domain. This presents both signal-to-noise issues and product detection challenges. The signal-to-noise was dealt with by using substrate quantities of both donor peptidyl-*S*-module four and acceptor aminoacyl-*S*-module five and by a radioactive label in L-Asp* (^{14}C or ^{3}H). The product detection for both pentapeptide identity and chirality at residue four required hydrolytic release of pentapeptide from its covalent thioester tether to T_5. For this, KOH-mediated hydrolysis was employed. Radioactive pentapeptide diastereomers (D-L-L-L-L- and D-L-L-D-L-) could be separated on a teicoplanin column and compared with authentic standards (Figure 6).

The results of this analysis were clear. Only the D-Phe-L-Pro-L-Phe-D-Phe-L-Asp pentapeptide was detected, validating that C_5 is stereospecific for the tetrapeptidyl donor. We have designated superscript and subscript shorthand for upstream donor and downstream acceptor chirality, respectively (*12*), and thus describe C_5 of tyrocidine synthetase as a $^{D}C_{L}$ peptide bond condensation catalyst.[2] This is in contrast to what is observed when only Phe is present on $TycB_3$ as the donor substrate. Both L-Phe-L-Asn and D-Phe-L-Asn are observed in a 2:1 mixture (*15*). Formation of both D-L- and L-L dipeptides was also observed here with L-Asp on $TycC_1$ (Figure 4A). Perhaps, as the active site of C_5 has evolved to

[2] We have demonstrated that C_5 catalyzes a $^{D}C_{L}$ peptide bond condensation reaction, but we have not eliminated the possibility that a D-amino acid could also be accepted in the downstream acceptor position.

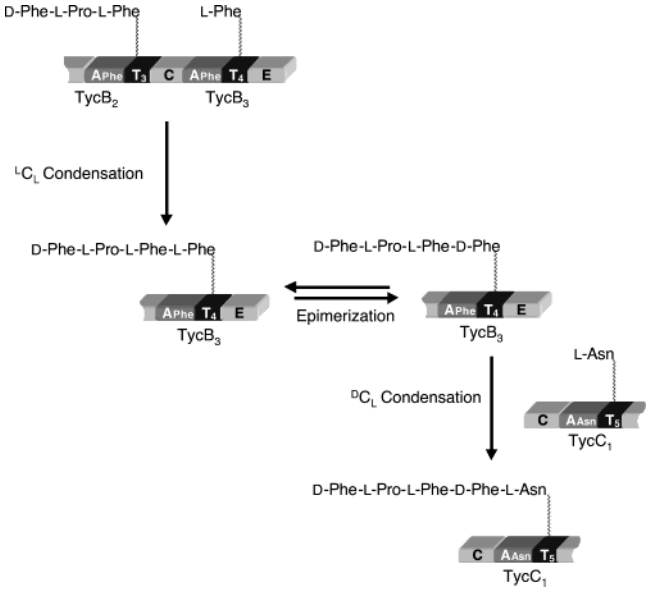

FIGURE 8: Model for neighboring C domain action for the generation of D-amino acid-containing peptides. Outline of the chemical pathway during peptide chain elongation for tyrocidine and the chirality of the C_4 and C_5 domains. Condensation of the tripeptidyl chain at $TycB_2$ with L-Phe on $TycB_3$ occurs prior to epimerizaton of Phe-4, making C_4 $^{L}C_L$-specific, and then condensation of the D-Phe-4-tetrapeptidyl chain with $TycC_1$, yielding a pentapeptide with C_5 being $^{D}C_L$-specific.

accommodate a peptidyl donor substrate, the decrease in substrate size when an aminoacyl donor, Phe, is employed permits more flexibility, resulting in both D- and L-Phe isomers being accepted. Precisely why the presence of the peptidyl-*S*-enzyme confers such chiral selectivity not observed with the aminoacyl donor is currently unknown but stresses an ambiguity avoided by the peptidyl-CoA T domain priming approach described here to evaluate the natural chain donor.

We have previously shown that the immediately preceding C domain, C_4 in the $TycB_3$ subunit (Figure 1), is an $^{L}C_L$ catalyst (*12*). This accords with the timing of E domain action with respect to C_4 and C_5. The tripeptidyl D-Phe-L-Pro-L-Phe donor chain is condensed with L-Phe-*S*-T_4 *before* the epimerase domain acts (*12*, *15*) to yield the D-Phe-L-Pro-L-Phe-L-Phe-*S*-T_4 tetrapeptidyl-*S*-enzyme as the initial condensation product (Figure 8**)**. Then the epimerase domain acts on the D-L-L-L-tetrapeptidyl-*S*-enzyme to equilibrate C_α configuration of Phe-4 and yield an ~1/1 mix of D-L-L-D-/D-L-L-L-*S*-T_4 acyl enzymes. At this juncture, C_5 comes into action. It selectively transfers only the D-Phe-4 tetrapeptidyl diastereomer. The L-Phe-4 tetrapeptidyl-*S*-T_4 left behind is rapidly epimerized, and then all the tetrapeptidyl chain can be brought forward as D-L-L-D-diastereomer out of the preequilibrium mix attached to T_4. For wild-type module 4, both D-L-L-L- and D-L-L-D-tetrapeptidyl CoA's, loaded by Sfp, gave D-L-L-D-L-pentapeptidyl-*S*-T_5 products, consistent with epimerase function. In an inactive epimerase mutant form of $TycB_3$ AT_4E, the L-Phe-4 tetrapeptidyl-*S*-T_4 acyl enzyme is not processed by C_5 while the D-Phe-4 version is, further confirming the C_5 selectivity.

We suggest that this will be the general strategy for C/E/C coordination in any elongation module of nonribosomal peptide synthetase assembly lines. When D-amino acid residues show up in the natural products, the available L-isomers will be selected by A domains in a four-domain CATE module. The C domain of that module will generally be an $^{L}C_L$ catalyst, transferring the growing peptidyl$_n$ chain to the L-aminoacyl-*S*-T intermediate and generating a nascent $(n + 1)$ peptidyl-*S*-T acyl enzyme. The E domain will epimerize this peptidyl$_{n+1}$-*S*-T enzyme, equilibrating the C_α chirality. This D-/L-peptidyl-*S*-enzyme mix will be presented to the next downstream C domain, and that will be a $^{D}C_L$ catalyst, as exemplified in the tyrocidine synthetase case. Bioinformatics analysis of C domain primary sequences has previously led to the suggestion that two subcategories, in our nomenclature $^{L}C_L$ and $^{D}C_L$, can be divided by sequence alignment (*26*).

Two additional points are then worth making. First, for swapping C domains in and out of modules in combinatorial biosynthesis approaches, it will be important to know that there will be two chiral populations, $^{L}C_L$ and $^{D}C_L$, readily identifiable from their placement upstream and downstream of epimerase domains. It should become possible to swap $T/^{L}C_L$ domains by triads of $T/E/^{D}C_L$ domains and insert a D-residue at any desired point in an NRPS assembly line. Second, it is unclear if there will be any $^{D}C_D$ domains, even in cases such as vancomycin where the heptapeptide chirality is D-D-L-D-D-L-L and one might a priori have assumed that C_2 and C_5 of that assembly line (*27*) would be D-specific for donor peptidyl and acceptor aminoacyl-*S*-T domains. If the E domain acts after the upstream C domain action, then the $^{L}C_L$ and $^{D}C_L$ chirality patterns shown here should be the rule.

ACKNOWLEDGMENT

Uwe Linne is gratefully acknowledged for providing the plasmids for the production of $TycB_3$ and $TycC_1$. Lusong Luo and Rahul Kohli are thanked for helpful discussions and careful reading of the manuscript.

REFERENCES

1. Konz, D., and Marahiel, M. A. (1999) *Chem. Biol. 6*, R39−48.
2. Stachelhaus, T., and Walsh, C. T. (2000) *Biochemistry 39*, 5775−5787.
3. Walsh, C. T., Chen, H., Keating, T. A., Hubbard, B. K., Losey, H. C., Luo, L., Marshall, C. G., Miller, D. A., and Patel, H. M. (2001) *Curr. Opin. Chem. Biol. 5*, 525−534.
4. Dittmann, J., Wenger, R. M., Kleinkauf, H., and Lawen, A. (1994) *J. Biol. Chem. 269*, 2841−2846.
5. Hoffmann, K., Schneider-Scherzer, E., Kleinkauf, H., and Zocher, R. (1994) *J. Biol. Chem. 269*, 12710−12714.
6. Marahiel, M. A., Stachelhaus, T., and Mootz, H. D. (1997) *Chem. Rev. 97*, 2651−2674.
7. Stein, T., Kluge, B., Vater, J., Franke, P., Otto, A., and Wittmann-Liebold, B. (1995) *Biochemistry 34*, 4633−4642.
8. Mootz, H. D., and Marahiel, M. A. (1997) *J. Bacteriol. 179*, 6843−6850.
9. Katz, E., and Demain, A. L. (1977) *Bacteriol. Rev. 41*, 449−474.
10. Luo, L., and Walsh, C. T. (2001) *Biochemistry 40*, 5329−5337.
11. Luo, L., Burkart, M. D., Stachelhaus, T., and Walsh, C. T. (2001) *J. Am. Chem. Soc. 123*, 11208−11218.
12. Luo, L., Kohli, R. M., Onishi, M., Linne, U., Marahiel, M. A., and Walsh, C. T. (2002) *Biochemistry 41*, 9184−9196.
13. Ehmann, D. E., Trauger, J. W., Stachelhaus, T., and Walsh, C. T. (2000) *Chem. Biol. 7*, 765−772.
14. Belshaw, P. J., Walsh, C. T., and Stachelhaus, T. (1999) *Science 284*, 486-489.
15. Linne, U., and Marahiel, M. A. (2000) *Biochemistry 39*, 10439−10447.
16. Reuter, K., Mofid, M. R., Marahiel, M. A., and Ficner, R. (1999) *EMBO J. 18*, 6823−6831.
17. Lambalot, R. H., and Walsh, C. T. (1995) *J. Biol. Chem. 270*, 24658−24661.

18. Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) *Biochemistry 37*, 1585−1595.
19. Trauger, J. W., Kohli, R. M., Mootz, H. D., Marahiel, M. A., and Walsh, C. T. (2000) *Nature 407*, 215−218.
20. Keating, T. A., Suo, Z., Ehmann, D. E., and Walsh, C. T. (2000) *Biochemistry 39*, 2297−2306.
21. Geiger, T., and Clarke, S. (1987) *J. Biol. Chem. 262*, 785−794.
22. Shao, Y., Xu, M. Q., and Paulus, H. (1995) *Biochemistry 34*, 10844−10850.
23. Lau, J., Cane, D. E., and Khosla, C. (2000) *Biochemistry 39*, 10514−10520.
24. O'Connor, S. E., Chen, H., and Walsh, C. T. (2002) *Biochemistry 41*, 5685−5694.
25. Sieber, S. A., Walsh, C. T., and Marahiel, M. A. (2003) *J. Am. Chem. Soc. 125*, 10862−10866.
26. von Dohren, H., Dieckmann, R., and Pavela-Vrancic, M. (1999) *Chem. Biol. 6*, R273−279.
27. van Wageningen, A. M., Kirkpatrick, P. N., Williams, D. H., Harris, B. R., Kershaw, J. K., Lennard, N. J., Jones, M., Jones, S. J., and Solenberg, P. J. (1998) *Chem. Biol. 5*, 155−162.

BI035090+