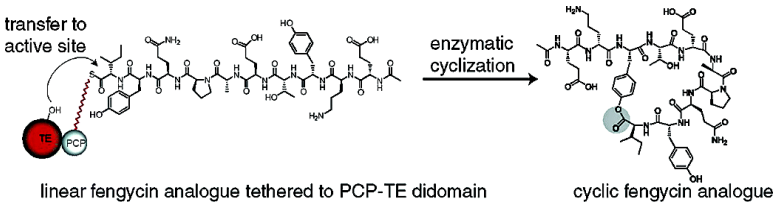
Article

Loading Peptidyl-Coenzyme A onto Peptidyl Carrier Proteins: A Novel Approach in Characterizing Macrocyclization by Thioesterase Domains

Stephan A. Sieber, Christopher T. Walsh, and Mohamed A. Marahiel

J. Am. Chem. Soc., **2003**, 125 (36), 10862-10866• DOI: 10.1021/ja0361852 • Publication Date (Web): 16 August 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 8 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 08/16/2003

Loading Peptidyl-Coenzyme A onto Peptidyl Carrier Proteins: A Novel Approach in Characterizing Macrocyclization by Thioesterase Domains

Stephan A. Sieber,[†] Christopher T. Walsh,[‡] and Mohamed A. Marahiel*,[†]

Contribution from Fachbereich Chemie/Biochemie, Philipps-Univerität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

Received May 16, 2003; E-mail: marahiel@chemie.uni-marburg.de

Abstract: Here we report a new experimental approach to characterize recombinant nonribosomal peptide cyclases that do not show activity with conventional *N*-acetylcysteamine (SNAC) substrates. To explore the great potential of these domains for the catalysis of cell-free cyclization reactions, the new strategy takes advantage of the direct interaction between the natural substrate where the peptide chain is attached to the phosphopantetheine arm of the peptidyl carrier protein (PCP) and the peptide cyclase. A prerequisite for this reaction is the promiscuity of the *Bacillus subtilis* phosphopantetheinyl transferase Sfp for loading chemically synthesized peptidyl-coenzyme A substrates instead of the smaller natural substrate coenzyme A (CoASH) onto apoPCP. With this novel method we were able to characterize the regioselectivity of branched-chain cyclization catalyzed by the fengycin cyclase, which displays no activity with peptidyl-SNAC substrates.

1. Introduction

A large and diverse set of natural products is synthesized by nonribosomal peptide synthetases (NRPS). In contrast to ribosomally synthesized proteins, these short, often cyclic peptides are structurally diverse and feature high pharmacological and biological activities.[1,2] Their targets are essential components of the cell, like the protein- and the cell wall-biosynthetic apparatus, reflecting specific interactions between a bioactive peptide and dedicated molecular targets. To achieve this aim, the peptide is often constrained in its biologically active conformation by macrocyclization to display a sterically fixed functionality that is specifically recognized by its target. In nature the regio- and stereoselective cyclization of a NRPS-tethered linear peptide precursor is catalyzed by thioesterase domains (TE, cyclase), which are the terminal catalytic units of the NRPS assembly lines.[3] Since regiospecific macrocyclization can be difficult to achieve by synthetic methods, there is a utility in applying these enzymes for cell-free synthesis of new cyclic peptide libraries with improved or altered activities.[4−7] In the natural NRPS biosynthetic template the amino acid substrates as well as all peptide intermediates are covalently tethered via a thioester bond on the phosphopantetheinyl arm (ppan) of each corresponding peptidyl carrier protein (PCP). For artificial substrate mimics, the PCP-phoshopantetheine moiety was successfully replaced by *N*-acetylcysteamine (SNAC), which represents the terminal part of the cofactor ppan[4] to which the synthetic peptide is attached. Studies with such peptidyl-SNAC substrates for surfactin and tyrocidine peptide cyclases revealed a relaxed substrate specificity for the replacement of amino acids in distinct regions of the peptide sequence.[8] Recombinant peptide cyclases from various systems are being explored, but for fengycin and mycosubtilin cyclases we observed no activity when peptidyl-SNACs were used (data not shown), indicating a limitation in the application of this method.

The crystal structure of surfactin TE domain revealed a channel on the surface that can accommodate the tethering phosphopantetheine.[9] This channel might be crucial in directing and placing the cofactor-bound linear peptide into the active site of the enzyme. With the peptide attached to the natural cofactor phosphopantetheine-PCP, a suitable positioning of the substrate in the catalytic center of the TE should be ensured. An upstream peptidyl-S-PCP donor is the natural substrate for TE domains. The task was then to synthesize peptidyl-ppan-PCP. In vivo, ppan-PCP is synthesized by the covalent attachment of the phosphopantetheine part of CoASH to an invariant serine residue of the apoPCP, leaving 3′,5′-ADP as a byproduct.

[†] Philipps-Univerität Marburg.
[‡] Harvard Medical School.

(1) Walsh, C. T. *Antibiotics: Actions, Origins, Resistance*; ASM Press: Washington, DC, 2003.
(2) Mootz, H. D.; Schwarzer, D.; Marahiel, M. A. *Chembiochem* **2002**, *3*, 490−504.
(3) Kohli, R. M.; Walsh, C. T. *Chem. Commun.* (*Cambridge*) **2003**, 297−307.
(4) Trauger, J.; Kohli, R.; Mootz, H.; Marahiel, M.; Walsh, C. *Nature* **2000**, *407*, 215−218.
(5) Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092−7098.
(6) Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099−7108.
(7) Tseng, C. C.; Bruner, S. D.; Kohli, R. M.; Marahiel, M. A.; Walsh, C. T.; Sieber, S. A. *Biochemistry* **2002**, *41*, 13350−13359.
(8) Kohli, R. M.; Walsh, C. T.; Burkart, M. D. *Nature* **2002**, *418*, 658−661.
(9) Bruner, S. D.; Weber, T.; Kohli, R. M.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T.; Stubbs, M. T. *Structure* (*Cambridge*) **2002**, *10*, 301−310.

10.1021/ja0361852 CCC: $25.00

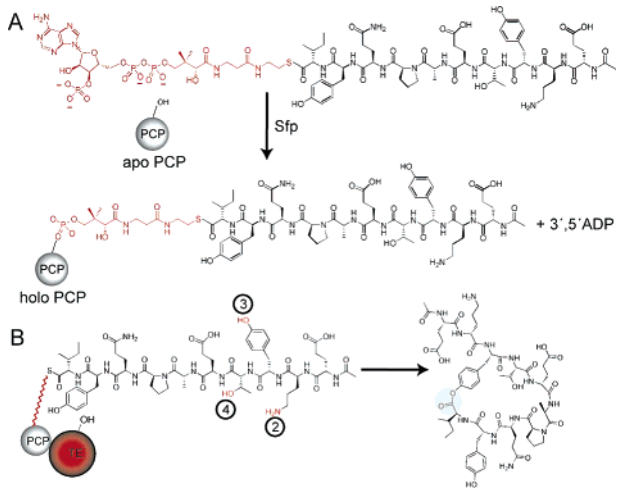


Figure 1. Loading peptidyl-CoA on PCP and interaction of peptidyl-PCP with the TE domain. (A) Sfp catalyzes the nucleophilic attack of the invariant serine residue of apoPCP onto the phosphodiester bond of fengycin-CoA (FLP) to give peptidyl-holoPCP. (B) Natural substrate interaction between FLP tethered to PCP in the PCP-TE didomain with subsequent TE-mediated cyclization via tyrosine at position 3.

This reaction is catalyzed by dedicated phosphopantetheinyl transferases such as Sfp, which was previously described to tolerate also acetyl-CoA and aminoacyl-CoA.[10,11]

Here we report a new experimental approach, capable of fully characterizing peptide cyclases that do not show activity with conventional SNAC surrogates and suitable to study direct interaction between the natural substrate peptidyl-ppan-PCP and the peptide cyclase. A prerequisite of this method is the promiscuity of the *Bacillus subtilis* phosphopantetheinyl transferase Sfp for loading chemically synthesized peptidyl-CoA substrates onto apoPCP (Figure 1A) instead of coenzyme A (CoASH). We show that Sfp tolerates peptidyl-CoAs, makes peptidyl-ppan-PCPs, and allows us to successfully characterize the regioselectivity of the fengycin cyclase (Figure 1B), which was inactive with peptidyl-SNACs. This approach can in general also be useful for in vitro characterization of other NRPS domains such as epimerization and condensation domains, which also interact in the natural system with peptidyl substrates.

2. Experimental Section

2.1. Cloning, Site-Directed Mutagenesis, Expression, and Purification. The surfactin and fengycin gene fragments encoding Srf PCP (ATTC 21332) and Fen PCP-TE[12] were amplified from chromosomal DNA by polymerase chain reaction (PCR) with Pfu polymerase (Stratagene) with the following oligonucleotides: PCP, 5′-AAA CCA TGG AAT GGA TTG GAC CGC GGA AC-3′ and 5′-AAA GGA TCC GTT TTT CAA ATA CGC TGA AAT GC-3′; Fen PCP-TE, 5′-AAA AAA AAC ATA TGC GTC AGG ACC TCA CAC CGC-3′ and 5′-AAA AAA CTC GAG ATG CTT ATT TGG CAG CAC TTT TTG-3′. The PCR product of Srf PCP was cloned into the *Nco*I/*Bam*HI site of pQE60 (Qiagen), and the PCR product of Fen PCP-TE was cloned into the *Nde*I/*Xho*I site of pET37b (Novagen). The plasmids were directly used to produce proteins with a C-terminal hexahistidine tag.

The gene fragment encoding the Fen PCP-TE site-directed mutant was constructed by use of the Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The construct was obtained by PCR amplification of Fen PCP-TE containing pET37b plasmid with the following oligonucleotides (modified sequences underlined): 5′-CTA TTA GGC TAC GCC GCG GGC GGA ACT T-3′ and 5′-TCC GCC CGC GGC GTA GCC TAA TAG AAC GT-3′. *Escherichia coli* TOP10 (Novagen) was used for preparation of recombinant plasmids. All constructs were confirmed by sequencing.

Overproduction of recombinant proteins was carried out in *E. coli* BL21 by standard protocols.[13] Purification of the proteins was carried out as described previously[14] and analyzed by SDS−polyacrylamide gel electrophoresis. After dialysis against standard assay buffer [25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and 50 mM NaCl, pH 7.0], all proteins were shock-frozen in liquid nitrogen. Concentration of the purified proteins were determined photometrically at 280 nm (milligrams per milliliter) by use of calculated extinction coefficients (for PCP, 1.28; Fen PCPTE, 1.09).

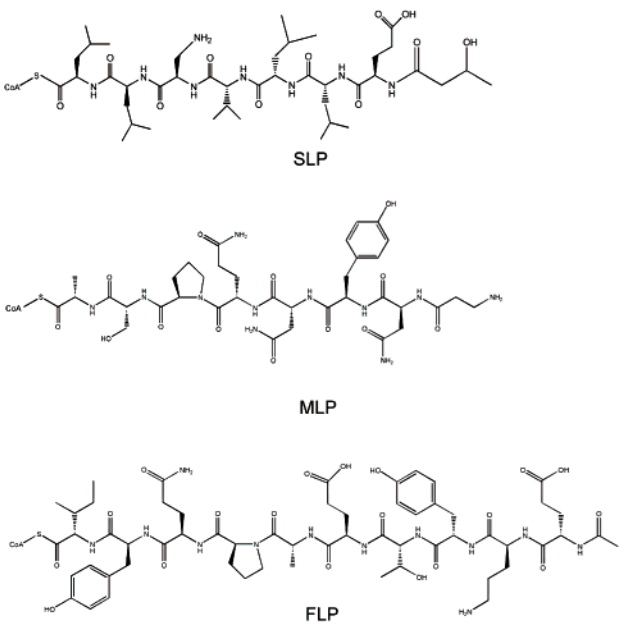
2.2. Synthesis of Peptidyl-CoA Substrates. The linear peptides were produced by solid-phase peptide synthesis as described previously.[6] Protected amino acids were purchased from Novabiochem and Bachem Bioscience. All other compounds were purchased from Sigma−Aldrich.

The general procedure for the synthesis of peptidyl-CoA substrates was based on a synthesis described previously.[10] To 1 equiv of protected peptide were added 1−2 equiv of coenzyme A, 1.5 equiv of PyBOP, and 4 equiv of potassium carbonate, dissolved in a 1:1 THF/water mixture. The mixture was agitated for 2 h at room temperature and the solvent was removed. Cleavage of the acid-labile side-chain protecting groups was carried out by using trifluoracetic acid (TFA), trifluoroethanol, and water in a ratio of 95:2.5:2.5. The deprotected peptidyl-CoA was purified by preparative high-performance liquid chromatography (HPLC) on a Äkta purifier (Pharmacia) HPLC system with a reversed-phase C18 Nucleodur (Macherey and Nagel) column. The identities of peptidyl-CoA thioesters were verified by high-performance liquid chromatography−mass spectrometry (HPLC−MS) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. To confirm the thioester linkage all product peaks, identified by their mass, were additionally incubated with 0.1% KOH to hydrolyze the thioester bond, which was again verified by LC−MS.

2.3. Assays. Loading reactions of apoPCPs were carried out in standard assay buffer (25 mM HEPES and 50 mM NaCl, pH 7.0) in a total volume of 100 μL at room temperature. For the kinetic characterization of the PCP loading reaction, the concentration of PCP was kept constant at 50 μM (K_M = 1.3−1.8 μM).[15] Initial rates were determined at various peptidyl-CoA concentrations ranging from 5 to 100 μM with two time points (7 and 14 min) at each concentration. A time course showed that modifications were linear up to 30 min. Reactions were initiated by addition of Sfp to give a final concentration of 2 μM and quenched by the addition of 30 μL of 4% TFA/H_2O. The products were analyzed by analytical HPLC−MS (Agilent 1100) with a reversed-phase C_{18} Nucleosil (Macherey and Nagel, 250/3, pore diameter 120 Å, particel size 3 μm) column. The identities of the products were confirmed by HPLC−MS and MALDI-TOF MS. Different gradients were applied according to resolution of the substrates (0−26 min, 55−70% acetonitrile/0.1% TFA in water/0.1% TFA, or 0−26 min, 40−57% acetonitrile/0.1% TFA in water/0.1% TFA; 0.9 mL/min, 45 °C).[16] The concentration of apoPCP was calculated from experimentally determined extinction coefficients at 220 nm, which were assumed to be the same for the peptidyl holoPCPs.

PCP-TE cyclization assays were carried out in 100 μL of standard assay buffer at room temperature. The peptidyl-CoA concentration

(10) Belshaw, P. J.; Walsh, C. T.; Stachelhaus, T. *Science* **1999**, *284*, 486-489.
(11) Chen, H.; O'Connor, S.; Cane, D. E.; Walsh, C. T. *Chem. Biol.* **2001**, *8*, 899−912.
(12) Steller, S.; Vater, J. *J. Chromatogr. B: Biomed. Sci. Appl.* **2000**, *737*, 267−275.
(13) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(14) Stachelhaus, T.; Mootz, H. D.; Bergendahl, V.; Marahiel, M. A. *J. Biol. Chem.* **1998**, *273*, 22773−22781.
(15) Quadri, L. E.; Weinreb, P. H.; Lei, M.; Nakano, M. M.; Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585−1595.
(16) Schwarzer, D.; Mootz, H. D.; Linne, U.; Marahiel, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14083−14088.



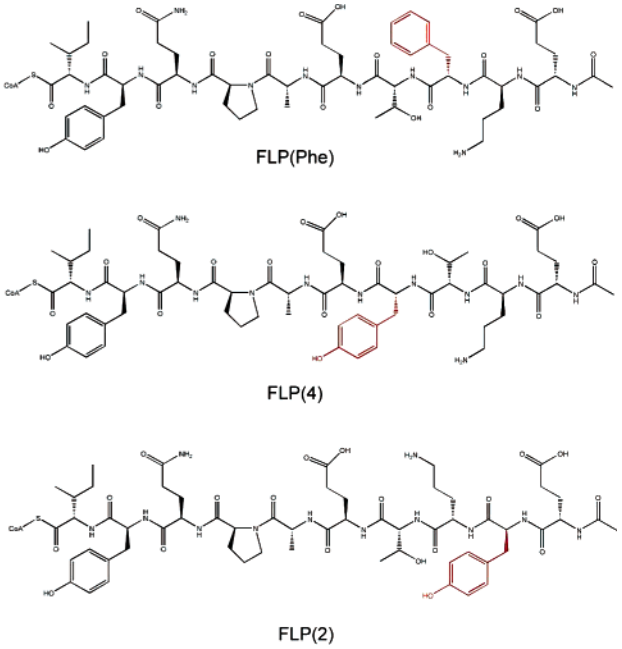


Figure 2. Structures of all CoA substrates.

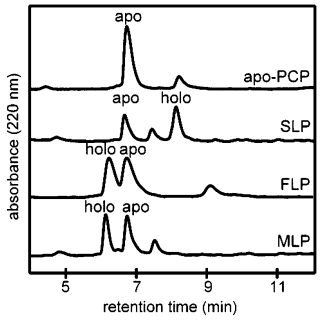


Figure 3. Results of PCP loading. HPLC traces (UV 220 nm) of 14 min Sfp loading reactions with surfactin-CoA (SLP), fengycin-CoA (FLP), and mycosubtilin-CoA (MLP). The peaks corresponding to the apoPCP (apo) and peptidyl-PCP (holo) are labeled.

ranged from 20 to 60 μM, and the PCP-TE concentration was 60 μM. Reactions were initiated by addition of Sfp to give a final concentration of 5 μM, ensuring a fast modification reaction. Reactions were quenched at various time points by addition of 40 μL of 4% TFA/H_2O and were analyzed by HPLC−MS on a reversed-phase C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter 100 Å, particle size 3 μm) with the following gradient: 0−35 min, 30−60% acetonitrile/0.1% TFA in water/0.1% TFA; 0.4 mL/min, 40 °C. Identities of the products were confirmed by ESI-MS and MALDI-TOF MS, and the cyclic products were additionally verified by MS−MS analysis (Table 2) (API Qstar Pulsar I, Applied Biosystems). Cyclic product hydrolysis assays were carried out in 100 μL of standard assay buffer under the same conditions as mentioned above with 30 μM PCP-TE and 50 μM fengycin cycle.

3. Results and Discussion

To load peptidyl-CoA substrates with Sfp, we cloned and overexpressed a PCP domain from module 7 of the surfactin synthetase in its apo form.

Three different N-acylpeptidyl-CoA substrates were synthesized. The structures of all peptidyl-CoA substrates are shown in Figure 2. The peptide sequences are derived from surfactin (SLP), mycosubtilin (MLP), and fengycin (FLP). In comparison with all three wild-type sequences, the long fatty acid moiety was replaced with shorter acyl-chain mimics to increase solubility. In the surfactin sequence, Asp at position 5 was replaced with 2,3-diaminopropionic acid as described before,[7] and in the mycosubtilin sequence, Asn at position 7 was replaced by Ala for synthetic reasons. The subsequent coupling with CoA yielded 50−60% product.[10] The peptidyl-CoAs were characterized by mass analysis as shown in Table 1.

Table 1. Characterization of Substrates by MS

compound	species	ionization method	obsd (calcd) mass (Da)
SLP	$[M + H]^+$	ESI	1620.4 (1620.6)
MLP	$[M + H]^+$	ESI	1613.5 (1613.5)
FLP	$[M + H]^+$	ESI	2018.5 (2018.7)
FLP(Phe)	$[M + H]^+$	ESI	2003.6 (2003.7)
FLP(2)	$[M - H]^-$	MALDI	2016.6 (2016.7)
FLP(4)	$[M - H]^-$	MALDI	2016.6 (2016.7)

Table 2. Characterization of Products by MS

compound	species	ionization method	obsd (calcd) mass (Da)
apoPCP	$[M + H]^+$	MALDI	9876.0 (9877.3)
SLP-PCP	$[M + H]^+$	MALDI	11 069.0 (11 068.9)
MLP-PCP	$[M + H]^+$	MALDI	11 063.0 (11 061.7)
FLP-PCP	$[M + H]^+$	MALDI	11 467.0 (11 466.9)

			obsd (calcd) mass (Da)	
compound	species	ionization method	cyclized product	hydrolyzed product
FLP	$[M + H]^+$	ESI	1251.2 (1251.6)	1269.4 (1269.6)
FLP (Phe)	$[M + H]^+$	ESI	N/D[a]	1253.4 (1253.6)
FLP (2)	$[M + H]^+$	ESI	1251.2 (1251.6)	1269.4 (1269.6)
FLP (4)	$[M + H]^+$	ESI	N/D	1269.4 (1269.6)

[a] N/D = no product detected.

Incubation of apoPCP with the three peptidyl-CoA substrates and Sfp revealed a substantial conversion into peptidyl holoPCPs (Figure 3, Table 2). The K_M values of the loading reactions were higher for SLP-CoA (17.2 μM), FLP-CoA (18.1 μM), and MLP-CoA (11.3 μM) than that of CoASH alone (0.7 μM) as described previously.[15] Also, a strong reduction of the k_{cat} values for the loading of the three peptidyl-CoAs was observed (SLP-CoA, 0.92 min^{-1}; FLP-CoA, 0.94 min^{-1}; MLP-CoA, 0.69 min^{-1})

Table 3. MS−MS Fragmentation Data

compound	species	obsd (calcd) mass of fragments (Da) cyclization via Orn	cyclization via Tyr	cyclization via Thr
FLP cycle[a]	$[M + H]^+$	1080.538[b] (1080.537)	966.459 (966.457)	N/D (803.394)
FLP cycle[c]	$[M + H]^+$	N/D[d] (1063.510)	949.443 (949.431)	N/D (786.367)
FLP(2)cycle[a]	$[M + H]^+$	N/D (917.473)	1080.542 (1080.537)	N/D (803.394)
FLP(2)cycle[c]	$[M + H]^+$	N/D (900.447)	1063.502 (1063.510)	N/D (786.367)

[a] Fragmentation of the peptide bond at the proposed cyclization position. [b] The observed mass belongs to a peptide fragment that carries Orn but is cyclized via Tyr. If cyclization would occur via Orn, no cyclic fragment would have been obtained with the apparent mass of Tyr cyclization. [c] Fragmentation of the adjacent NH−CH bond. [d] N/D = not detected.

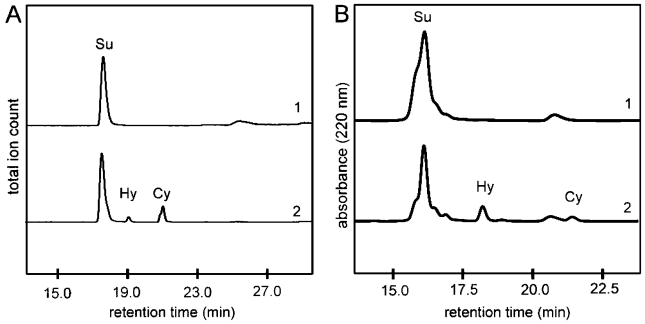


Figure 4. Results of the cyclization reactions with PCP-TE. Peaks corresponding to the substrate (Su), hydrolyzed product (Hy), and cyclic product (Cy) are labeled. (A). LC-MS traces (TIC) of 30-min reactions containing fengycin PCP-TE, FLP-CoA, and Sfp. Trace 1 shows the reaction with FLP-CoA and a mutated enzyme (Ser to Ala in the active site of the TE). Trace 2 shows the same reaction with the wild-type enzyme. (B) LC-MS traces (UV 220 nm) of 30-min reactions containing fengycin PCP-TE and a fengycin-CoA derivative (FLP-2) with Tyr at position 2 and Orn at postion 3 without Sfp (trace 1) and in the presence of Sfp (trace 2).

when compared with CoASH alone (102 min^{-1}).[15] Sfp is promiscuous enough to tolerate dramatically larger derivatives of CoA with a reduced kinetic efficiency but is preparatively useful. This is in good agreement with the crystal structure of Sfp, which shows a specific recognition of the 3′-phospho-5′-ADP moiety of CoA but shows no coordination for the terminal part of the pantetheine arm.[17] It is now possible to convert an apoPCP into peptidyl holoPCP and study its interaction with TE domains in cis or in trans.

One such TE domain is derived from fengycin synthetase. Fengycin is a potent antifungal branched cyclic lipopetide that inhibits filamentous fungi but does not act on yeast and bacteria.[18] To understand principles of cyclization, fengycin TE was cloned and overexpressed as an apoPCP-TE didomain. The PCP-TE didomain should first ensure a proper folding of the TE and second allow the loading of FLP-CoA substrate onto apoPCP to mimic the natural substrate for the TE (Figure 1B). Pure PCP-TE was first incubated with a linear fengycin SNAC substrate for several hours but did not show any cyclization or hydrolysis (data not shown). In contrast, when the new FLP-CoA substrate, PCP-TE, and Sfp were used, cyclization and hydrolysis were observed while a PCP-TE mutant enzyme with a Ser to Ala mutation in the TE active site did not show any activity under the same conditions (Figure 4A, Table 2). Also, a control reaction with substrate and apo PCP-TE but without Sfp treatment did not reveal activity, indicating that the covalent loading of the lipopeptidyl substrate onto the PCP is required for both cyclization and hydrolysis reactions. This shows that soluble SNAC or peptidyl-CoA substrates do not find the correct way into the TE active site by diffusion. Covalent loading of CoA substrates onto PCP seems to direct the peptide substrate via the phosphopantetheine arm into the active site and ensures appropriate alignment for the catalytic reaction. The observed cyclization-to-hydrolysis ratio was 2:1, validating that the TE in this PCP-TE didomain retains cyclase activity. A time course showed maximum cyclization after approximately 1 h. With increasing time the amount of cyclic product decreased while hydrolyzed product increased. Incubation of isolated cyclic peptide with PCP-TE showed progressive hydrolysis to linear lipopeptide. Thus the fengycin TE can run backward to a linear peptidyl-O-TE that can be captured by water.

The identity of the cyclic peptidolactone was confirmed by MS−MS sequencing (Table 3). Masses of two characteristic cyclic fragments [(a) fragmentation of the peptide bond at the proposed cyclization position and (b) fragmentation of the adjacent NH−CH bond] were calculated for all possible nucleophiles and compared with the observed fragments.

The results show that cyclization occurred regioselectively via nucleophilic attack of Tyr at position 3, discriminating two other nucleophiles at position 2 (Orn) and 4 (Thr). This high selectivity raised the question whether the TE specifically recognizes position 3 or if the identity of the amino acid residue (Tyr) is important. To answer this question, three new fengycin-CoA substrate analogues were synthesized (Table 1). The first carried a Phe residue instead of Tyr at position 3 (FLP-Phe). The enzymatic reaction with PCP-TE showed only hydrolysis but no cyclization as judged by LC−MS, indicating that neither of the other two nucleophiles could replace tyrosine. In additional reactions, substrates with an exchange of positions Tyr 3 and Orn 2 (FLP-2) as well as Tyr 3 and Thr 4 (FLP-4) were used. The relocation of Tyr at position 4 revealed only hydrolysis, but Tyr relocation at position 2 showed hydrolysis and cyclization (Figure 4B, Table 2). The identity of Tyr 2 as the nucleophile for this cyclization was again confirmed by MS−MS sequencing (Table 3). These results show that fengycin TE specifically recognizes Tyr as the nucleophile in position 3 and 2 and an increase of the peptidolactone ring size by one residue (from 29 to 32 atoms) is possible. Fengycin is the first cyclic branched-chain peptidolactone where regioselectivity of enzymatic macrocyclization has begun to be explored.

We conclude that this new approach, enabled by the ability of promiscuous phosphopantetheinyl transferase Sfp to covalently tether peptidyl-ppan of chemically synthesized peptidyl-CoA substrates onto PCP-TE, is a powerful method to explore and characterize new TE domains of systems that show no

(17) Reuter, K.; Mofid, M. R.; Marahiel, M. A.; Ficner, R. *EMBO J.* **1999**, *18*, 6823−6831.
(18) Vanittanakom, N.; Loeffler, W. *J. Antibiot.* **1986**, *39*, 888−901.

activity with soluble substrate surrogates. With the fengycin PCP-TE didomain, principles of enzymatic regioselectivity were successfully investigated, and new substrate variants with improved or altered activities can now be created. Moreover, the technique could in principle be used to bypass specificity of NRPS by loading peptidyl-CoA on any apoPCP within an assembly line.

Acknowledgment. Funding for this work was provided by the Studienstiftung des deutschen Volkes (S.A.S.), Deutsche Forschungsgemeinschaft (M.A.M.), and NIH Grant GM20011 (C.T.W.). We thank Uwe Linne for MS−MS sequencing and Junhua Tao for his help with synthesis.

JA0361852