Heterocycle Formation in Vibriobactin Biosynthesis: Alternative Substrate Utilization and Identification of a Condensed Intermediate†

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ABSTRACT: The iron-chelating peptide vibriobactin of the pathogenic *Vibrio cholerae* is assembled by a four-subunit nonribosomal peptide synthetase complex, VibE, VibB, VibH, and VibF, using 2,3-dihydroxybenzoate and L-threonine as precursors to two 2,3-dihydroxyphenyl- (DHP-) methyloxazolinyl groups in amide linkage on a norspermidine scaffold. We have tested the ability of the six-domain VibF subunit (Cy-Cy-A-C-PCP-C) to utilize various L-threonine analogues and found the β-functionalized amino acids serine and cysteine can function as alternate substrates in aminoacyl-AMP formation (adenylation or A domain), aminoacyl-S-enzyme formation (A domain), acylation by 2,3-dihydrobenzoyl- (DHB-) S-VibB (heterocyclization or Cy domain), heterocyclization to DHP-oxazolinyl- and DHP-thiazolinyl-S-enzyme forms of VibF (Cy domain) as well as transfer to DHB-norspermidine at both N_5 and N_9 positions (condensation or C domain) to make the bis(oxazolinyl) and bis(thiazolinyl) analogues of vibriobactin. When L-threonyl-S-pantetheine or L-threonyl-S-(*N*-acetyl)cysteamine was used as a small-molecule thioester analogue of the threonyl-S-VibF acyl enzyme intermediate, the Cy domain(s) of a CyCyA fragment of VibF generated DHB-threonyl-thioester products of the condensation step but not the methyloxazolinyl thioesters of the heterocyclization step. This clean separation of condensation from cyclization validates a two-stage mechanism for threonyl, seryl, and cysteinyl heterocyclization domains in siderophore and antibiotic synthetases. Full heterocyclization activity could be restored by providing CyCyA with the substrate L-threonyl-S-peptidyl carrier protein (PCP)-C2, suggesting an important role for the protein scaffold component of the heterocyclization acceptor substrate. We also examined heterocyclization donor substrate specificity at the level of acyl group and protein scaffold and observed intolerance for substitution at either position.

Vibriobactin, **1**, is the low molecular weight, iron-chelating peptide produced by *Vibrio cholerae* in conditions of iron limitation (e.g., during infections in vertebrates) where it likely functions as a virulence factor (*1*). This siderophore is a tris catecholic iron chelator, produced by two iron-regulated gene clusters encoding the proteins VibA−H (*2*−*5*). The VibE, -B, -H, and -F proteins function as a four-subunit nonribosomal peptide synthetase (NRPS) to produce vibriobactin from 2,3-dihydroxybenzoate (DHB),[1] two molecules of L-threonine, three ATPs, and the *Vibrio* triamine norspermidine (Figure 1). The three iron-chelating catechols in **1** are provided by three DHBs, one directly connected in amide linkage to a norspermidine scaffold and the other two in amide linkage to threonyl groups that have been heterocyclized to methyloxazolinyl (mOx) moieties, which are in turn connected to the remaining two amines of norspermidine.

We have previously established the biosynthetic pathway to vibriobactin (Figure 1) (*6*, *7*). The VibE subunit activates each DHB as DHB-AMP and installs it onto the aryl carrier protein (ArCP) domain of HS-phosphopantetheinyl-VibB (holo-VibB), yielding the thioester DHB-S-VibB. The core of the vibriobactin assembly line is contained within the 270 kDa VibF subunit, with six domains (Cy-Cy-A-C-PCP-C) predicted by bioinformatic analysis to be heterocyclization (Cy), adenylation (A), peptidyl carrier protein (PCP), and two amide-bond-forming condensation (C) domains. Analogous to the activity of VibE, the VibF A domain makes L-Thr-AMP and installs it on the holo form of the PCP domain. Next, one or both of the Cy domains function in heterocyclization, which is thought to occur as follows: (a) condensation of the DHB donor from DHB-S-VibB with the amino group of the L-threonyl-S-VibF acyl enzyme, presumably generating DHB-Thr-S-VibF as an intermediate; (b) cyclization to the oxazoline by attack of the β-OH of the threonyl group onto the amide carbonyl of the DHB; and

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[1] Abbreviations: A, adenylation domain; ArCP, aryl carrier protein; C, condensation domain; CoA, coenzyme A; Cy, heterocyclization domain; DHB, 2,3-dihydroxybenzoate; DHB-NS, N^1-(2,3-dihydroxybenzoyl)norspermidine; DHP, 2,3-dihydroxyphenyl; DTT, dithiothreitol; I, isochorismate lyase; IPTG, isopropyl β-D-thiogalactopyranoside; L-2-NHbut, L-2-aminobutyrate; Me, methyl; mOx, 5-methyloxazoline; NAC, *N*-acetylcysteamine; NRPS, nonribosomal peptide synthetase; Pant, D-pantetheine; PCP, peptidyl carrier protein, PP_i, inorganic pyrophosphate; PPTase, phosphopantetheinyl transferase; *R*-3-OHbut, *R*-3-hydroxybutyrate; TCEP, tris(carboxyethyl)phosphine; TCA, trichloroacetic acid.

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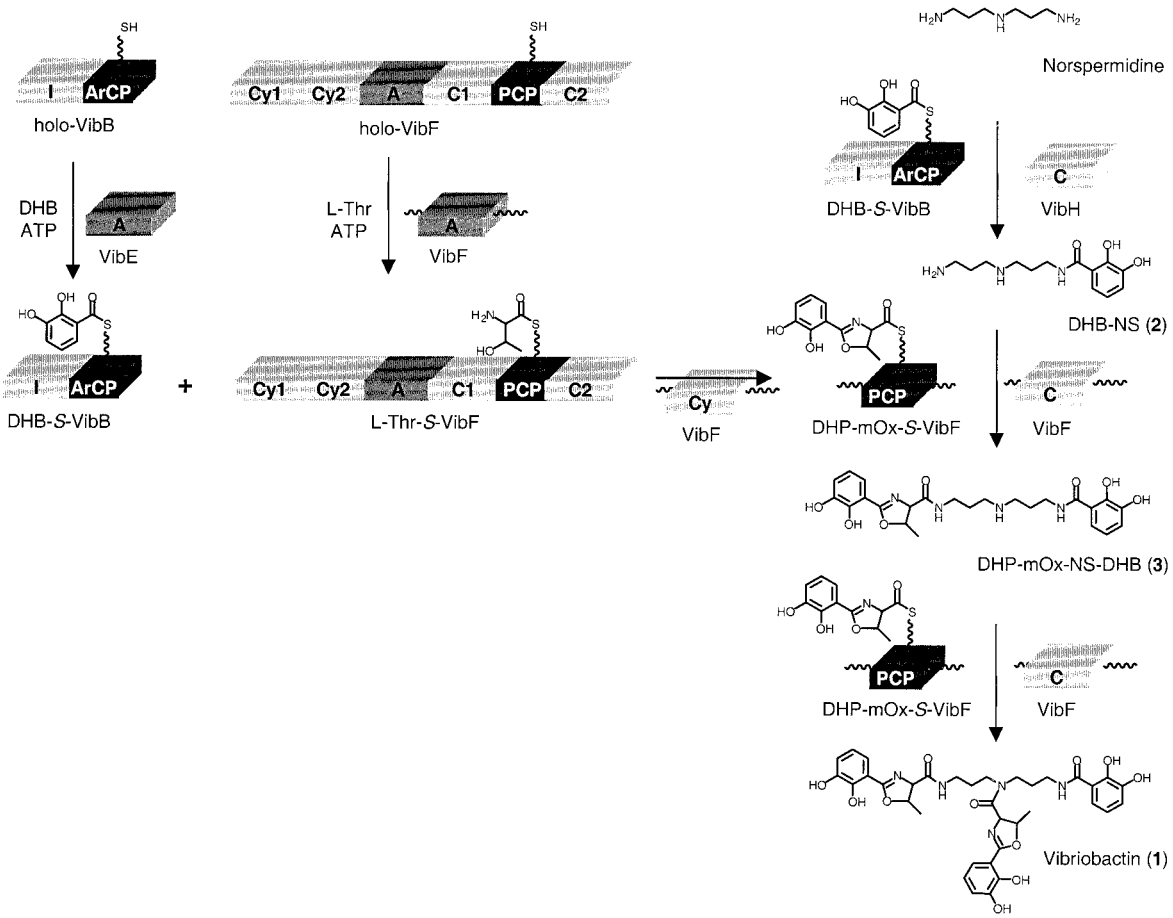


FIGURE 1: Biosynthesis of vibriobactin from DHB, L-Thr, norspermidine, and ATP by VibE, -B, -H, and -F. DHB-NS, the product of VibH, is acylated twice by VibF with activated DHP-mOx generated by VibE, VibB, and VibF.

(c) dehydration to yield the (dihydroxyphenyl)methyloxazolinyl-S-VibF acyl enzyme (DHP-mOx-S-VibF). This heterocyclic acyl group is transferred to the primary amine of DHB-norspermidine (DHB-NS, **2**), the product of VibH, by one or both of the two C domains in VibF, generating a free intermediate, **3,** that can capture a second DHP-mOx-S-VibF acyl enzyme at the secondary amine to yield vibriobactin, **1**.

In this study we have focused on three aspects of VibF catalysis. First, we have probed whether other amino acids can replace L-threonine for one or more steps in the VibF assembly line including adenylation, covalent loading onto the VibF PCP, heterocyclization, and transfer to DHB-NS. We found that only amino acids with β-functionalities (i.e., Ser and Cys) could act as alternate substrates and that these were processed completely to the bisheterocyclic vibriobactin analogues **7** and **9**. Second, we utilized L-threonyl-S-pantetheine (Thr-S-Pant) and L-threonyl-S-*N*-acetylcysteamine (Thr-S-NAC) as small-molecule surrogates for the native heterocycle acceptor substrate L-threonyl-S-VibF (Thr-S-VibF) with a PCP-free CyCyA fragment of VibF. We were able to detect condensation but not heterocyclization, establishing at least two discrete steps in Cy domain heterocyclization activity. Full heterocyclization activity was restored by supplying the acceptor substrate as an L-Thr-S-PCPC2 fragment of VibF. Third, we have probed heterocyclization donor substrate specificity by comparing the native DHB-S-VibB to the protein homologue DHB-S-EntB, to the acyl-group homologue Sal-S-VibB and to the small-molecule homologue DHB-S-NAC. All but the last were rejected as acyl donors to a Thr-S-Pant acceptor.

EXPERIMENTAL PROCEDURES

Materials and General Methods. 2,3-DHB, coenzyme A, and all amino acids were purchased from Sigma−Aldrich Chemical Co. ATP was purchased from Boehringer Mannheim. TCEP was purchased from Fluka. DHP-mOx-NS-DHB was obtained as described previously (*6*). Standard recombinant DNA techniques and microbiological procedures were performed as described elsewhere (*8*). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Plasmid vectors were purchased from Novagen. Pfu polymerase and competent *Escherichia coli* was purchased from Stratagene. Oligonucleotide primers were purchased from Integrated DNA Technologies, and DNA sequencing to verify the fidelity of amplification was performed on double-stranded DNA by the Molecular Biology Core Facilities of the Dana-Farber Cancer Institute (Boston, MA). Ni-NTA Superflow resin was purchased from Qiagen.

HPLC Sample Preparation and Analysis. Reactions to be analyzed by HPLC were quenched with 9 volumes of ice-cold methanol, and precipitant was removed by centrifugation at 11600*g* for 30 min at 4 °C. Samples were dried under vacuum at 32 °C and suspended in 150 μL of 15% acetonitrile for injection onto a C18 Vydac small-pore column on a Beckman System Gold. Peaks were eluted at 1 mL/min in a two-phase gradient from 12.5% to 17.5% to

87.5% acetonitrile in 0.08% TFA over 23 min/phase. Product elution was monitored at 254 nm. Peak integration values were converted to nanomoles of product on the basis of standard curves generated with DHB-octylamine and DHP-mOx-NS-DHB as described previously (*6*).

MALDI-TOF Mass Spectrometry. Samples were collected from HPLC eluant, immediately frozen, lyophilized for 16 h, and suspended in 5 μL of 30% acetonitrile. Sample was spotted with an equal volume of 10 mg/mL 2,5-DHB matrix. Samples were ionized with a laser intensity of 2000−2400 units on a Perceptive Biosystems Voyager-DE STR spectrometer in positive ion detection, reflector mode.

Purification of VibB, VibE, VibF, and VibH. VibB, VibE, VibF, and VibH were purified as described previously (*6*, *7*)

Assay of VibF. Substrate-dependent ATP−PP_i exchange assays (100 μL) containing 10−100 nM VibF were performed as described previously (*6*). Heterocyclization assays (50 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 1 mM DHB, 5 μM holo-VibB, 10 mM ATP, 0.25−0.5 μM holo-VibF, 2 μM VibE, 1 mM DHB-NS (for primary amine acylation) or 50 μM DHP-mOx-NS-DHB (for secondary amine acylation), and a concentration of amino acid equal to the ATP−PP_i exchange K_m. Reactions were incubated at 30 °C for a time determined to be in the linear range of turnover activity and then analyzed by HPLC. Holo-VibB and VibF were generated as described previously (*6*). DHP-thiazolinyl-NS-DHB and DHP-oxazolinyl-NS-DHB were purified for use in secondary amine acylation assays by HPLC purification from large-scale primary amine acylation assays. Upon peak collection, samples were immediately frozen, lyophilized for 16 h, and suspended in 10 mM Tris, pH 7.5, to 250 μM.

Construction of CyCyA, PCP_xC2, and PCPC2. DNA encoding CyCyA and PCPC2 were amplified from pVibF (*7*) by use of the following primer pairs: CyCyAf, 5′-GGAATTCCATATGAAAGAAATGACCGCAATG-3′, and CyCyAr, 5′-GGCGCCCGCTCGAGTGATGCTGACGAAGT-GAT-3′; or PCPC2f, 5′-GGAATTCCATATGAGCGCTG-TAAATTCCTCTGCG-3′, and PCPC2r, 5′-GTGGTGCTC-GAGCCCCATGG-3′. The gene for PCP_xC2 was created by SOE mutagenesis (*9*) using the following primer pairs: (PCPC2f, above, and PCP_xC2r, 5′-CGTTGCGATCAG**GGC**-ATGTCCGCCAAA-3′; or PCP_xC2f, 5′-TTTGGCGGACAT-**GCC**CTGATCGCAACG-3′, and PCPC2r, above). Restriction sites are underlined and the Ser to Ala mutation of PCP_xC2 is shown in boldface type. Amplified products were digested with *Nde*I and *Xho*I, ligated to similarly digested pET37b (CyCyA) or pET29b (PCPC2 and PCP_xC2) and transformed into competent *E. coli* BL21 (DE3). The resulting plasmids pETCyCyA, pETPCPC2, and pETPCP_x-C2 each express a translational fusion of a C-terminal histidine tag with the respective protein.

Purification of CyCyA, PCP_xC2, and PCPC2. Cultures of *E. coli* containing the plasmids encoding CyCyA, PCP_xC2, and PCPC2 were grown in Luria−Bertani broth containing 40 μg/mL kanamycin and 2 mM $MgCl_2$ at 37 °C to an OD_{600} of 0.6 and then induced for 5 h at 22 °C with 1 mM IPTG. Cells were harvested by centrifugation (10 min at 2000*g*) and suspended in 20 mM Tris, 300 mM NaCl, and 5 mM imidazole, pH 8.0 (buffer A). Cells were lysed by passage through a French pressure cell and the lysate was clarified by centrifugation (30 min at 95000*g*). The proteins were batch-bound to Ni-NTA Superflow resin for 2 h and eluted with a step gradient of increasing concentrations of imidazole in buffer A. Eluted proteins were analyzed by SDS−PAGE, and pooled fractions were dialyzed against 25 mM Tris, 50 mM NaCl, 1 mM DTT, and 10% glycerol, pH 8.0, and then flash frozen and stored at −80 °C.

Synthesis of L*-Thr-S-Pant and DHB-S-NAC*. Synthesis of L-Thr-S-Pant is described elsewhere (manuscript in preparation). 2,3-DHB (308 mg) and 1-hydroxybenzotriazole hydrate (HOBt) (306 mg) were dissolved in 4 mL of dry tetrahydrofuran (THF). *N*-Acetylcysteamine (266 μL) was added to the stirred solution, followed by dicyclohexylcarbodiimide (DCC) (413 mg). After 1 h at 23 °C, solid potassium carbonate (132 mg) was added and stirring was continued for another hour. Ethyl acetate (5 mL) was added to quench, and then the mixture was filtered and the filtrate concentrated to dryness. The residue was dissolved in ethyl acetate, washed twice with 10% sodium bicarbonate, dried over sodium sulfate, and concentrated to a white solid insoluble in water, methanol, or small volumes of ethyl acetate. The product was purified by dissolving in dimethyl sulfoxide (DMSO) followed by preparative HPLC (20−40% acetonitrile over 20 min). ^{1}H NMR (200 MHz, DMSO) δ 10.20 (s, 1H); 9.68 (s, 1H); 8.11 (t, 1H, $J = 5.3$ Hz); 7.23 (dd, 1H, $J = 8.0$ and 1.4 Hz); 7.03 (dd, 1H, $J = 8.0$ and 1.4 Hz); 6.77 (t, 1H, $J = 7.8$ Hz); 3.27 (q, 2H, $J = 6.2$ Hz); 3.08 (t, 2H, $J = 6.4$ Hz); 1.80 (s, 3H).

Assay of CyCyA. Unless specified otherwise, heterocyclization assays (50 μL) using small-molecule substrates contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 1 mM DHB, 5 μM holo-VibB, 10 mM ATP, 0.5−5 μM CyCyA, and various amounts of small-molecule acceptor substrate. Reactions were incubated at 30 °C for a time determined to be in the linear range of turnover activity and then analyzed by HPLC. Aminoacylation assays (50 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 10 mM ATP, 5 mM [^{3}H]-L-Thr (1 μCi), 12.5 nM CyCyA, and various amounts of holo-PCPC2. Protein was TCA-precipitated and analyzed for ^{3}H content as described previously (*6*). Holo-PCPC2 was formed in reactions (50 μL) containing contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 100 μM coenzyme A, 40 μM apo-PCPC2, and 3 μM Sfp.

RESULTS

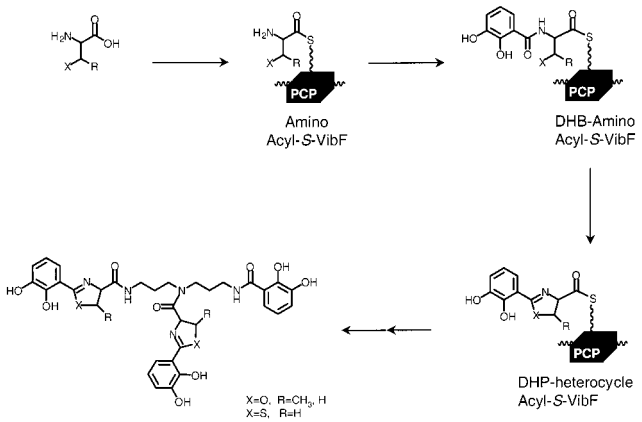
Use of Alternative Amino Acids in Heterocycle Formation by VibF. *(a) Aminoacyl-AMP Formation by the A Domain of VibF*. To evaluate the use of alternative acceptor amino acids in VibF heterocyclization, it was necessary to determine the efficiency with which these amino acids could be activated as amino acyl adenylates. The kinetic parameters of ATP−PP_i exchange on VibF bound to various L-Thr analogues are shown in Table 1. L-2-NHbut, L-Ser, and L-Cys were utilized 50−100-fold less efficiently than L-Thr, with L-Ser displaying a 25-fold elevation in K_m. While L-Ala and L-Val supported a reasonable k_{cat}, K_m values were sufficiently raised to result in a decrease in catalytic efficiency of three log units. *R*-3-OHbut, lacking an amino group, was not detectably converted to the acyl-AMP by the VibF A domain.

(b) Amino Acid Utilization by the Cy and C Domains of VibF. Assays for heterocyclization and first transfer to the

Table 1: VibF Adenylation Domain ATP−PP_i Exchange with Various Acyl Groups

acyl substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
L-Thr	980 ± 43	1.7 ± 0.2	575
2-NHbut	80 ± 1	6.9 ± 0.4	11.6
L-Ser	260 ± 9	43 ± 3	6.0
L-Cys	26 ± 1	3.3 ± 1.2	7.9
L-Ala	68 ± 1	77 ± 1	0.9
L-Val	16 ± 0.2	21 ± 1	0.8
3-OHbut	<0.6	>500	<0.001

Scheme 1



norspermidine scaffold were performed. VibF-bound aminoacyl-AMPs were assayed for progression through the catalytic cycle of VibF to an untethered, detectable product. As shown in Scheme 1, the expectation is that after the adenylate is installed on the PCP, the aminoacyl-S-VibF intermediate should be acylated by DHB, heterocyclized if it contains a β-OH or -SH, and then transferred to the primary amino group of DHB-NS to give the released product. Holo-VibF was incubated with DHB-S-VibB (formed in situ by DHB, ATP, holo-VibB, and VibE), all of the amino acids of Table 1, and DHB-NS and analyzed for turnover by HPLC (Figure 2). No products were detected when L-Ala, L-Val, L-2-NHbut, and 3-OHbut were used in place of L-Thr; however, both L-Cys and L-Ser yielded new products. Mass spectrometric analysis (Table 2) of the novel product peaks from incubations with L-Ser confirmed the formation of the oxygen-containing heterocycle DHP-oxazolinyl-NS-DHB, **4**, while incubations with L-Cys gave the corresponding sulfur heterocycle DHP-thiazolinyl-NS-DHB, **5**. Oxazoline and thiazoline product formation rates were linear and saturation behavior was observed for L-Ser and L-Cys at concentrations approximating the K_m values of the ATP−PP_i exchange reaction (data not shown), indicating that aminoacyl activation is not rate-limiting. The k_{cat} of 74 min^{-1} for L-Thr was reduced 2.2-fold for L-Ser and 7.4-fold for L-Cys, a much smaller change than the almost 40-fold rate reduction seen for L-Cys in the ATP−PP_i exchange (Table 3). Thus the first transfer of DHP-oxazolinyl-S-VibF or DHP-thiazolinyl-S-VibF to the primary amine of DHB-NS was reasonably efficient with these alternate heterocyclic donors.

(c) Second Transfer of a DHP-Heterocyclic Acyl Group from VibF to the Secondary Amine of the Norspermidine Scaffold. To assay the second acylation step (Scheme 1) that completes the production of a bisheterocyclic analogue of vibriobactin required purification of the above three products,

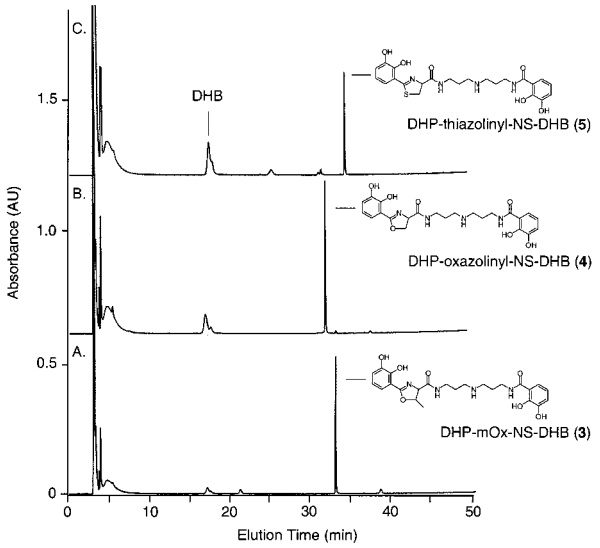


FIGURE 2: HPLC elution profiles of VibF primary amine acylation products containing various heterocycles. Reactions contained VibE, holo-VibB, VibF, DHB, ATP, DHB-NS, and the following heterocycle acceptor substrates: (A) L-Thr, (B) L-Ser, and (C) L-Cys. Unused DHB and reaction products are indicated. See Scheme 1.

Table 2: Mass Spectrometric Analysis in This Study

reaction product	predicted $[M + H]^+$	observed $[M + H]^+$
DHP-mOx-NS-DHB (**3**)	487.5	487.3
DHP-oxazolinyl-NS-DHB (**4**)	473.5	473.3
DHP-thiazolinyl-NS-DHB (**5**)	489.6	489.4
6	692.7	692.4
7	678.7	678.4
8	708.8	708.4
9	710.8	710.4
DHB-Thr-O-Tris	359.3	359.1
DHB-Thr-OH	256.2	256.1
DHB-Thr-O-Me	270.3	270.1
DHB-Thr-S-Pant (**10**)	516.6	516.3
DHB-Thr-NS-DHB (**11**)	505.5	505.2
DHP-mOx-OH	238.2	238.1
DHP-mOx-O-Tris	341.3	341.2

Table 3: Rates of Use of L-Thr Analogues in Primary and Secondary Amine Acylation by VibF

amine class	amine acceptor	rate of use (min^{-1})		
		L-Thr	L-Ser	L-Cys
primary	DHB-NS (**2**)	74 ± 3	33 ± 0.5	10 ± 0.2
secondary	DHP-mOx-NS-DHB (**3**)	55 ± 4		
	DHP-oxazolinyl-NS-DHB (**4**)	40 ± 2	21 ± 1	
	DHP-thiazolinyl-NS-DHB (**5**)	74 ± 3		2.2 ± 0.2

3−**5**, by HPLC and reincubating them as acceptor substrates for secondary amine acylation. Again Ser, Cys, or Thr was utilized to generate the heterocycle-containing donor substrates in reactions with DHB-S-VibB, holo-VibF, and the various acceptor substrates. In each instance a new peak was detected by HPLC (Figure 3) and confirmed by mass spectrometry (Table 2) to be a tris-acylated norspermidine with one DHB acyl group and two DHP-heterocyclic acyl groups, **6**−**9**. The methyloxazoline, oxazoline, or thiazoline ring in the acceptor **3, 4,** or **5** had very little effect on rates, while the switch from the natural DHP-mOx-S-VibF donor to the corresponding oxazolinyl- or thiazolinyl-S-VibF donors had a 1.9-fold and a 34-fold reduction in rate, respectively (Table 3). These results clearly show a combination of

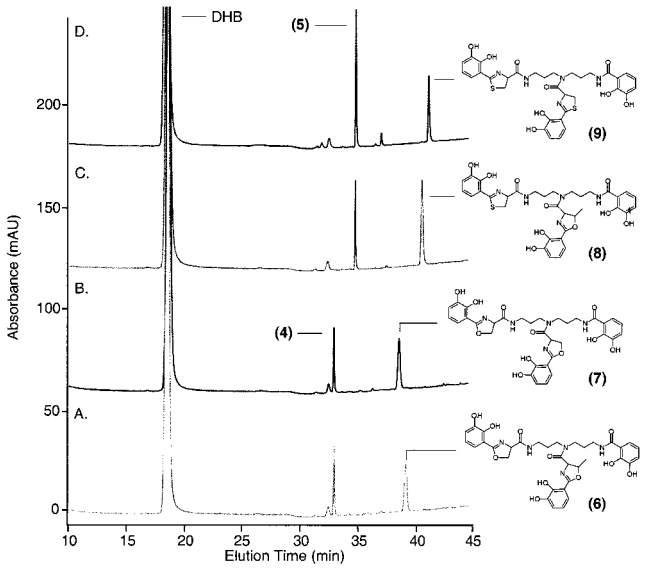


FIGURE 3: HPLC elution profiles of VibF secondary amine acylation products containing various heterocycles. Reactions contained VibE, holo-VibB, VibF, DHB, ATP, either **4** (panels A and B) or **5** (panels C and D), and (A) L-Thr, (B) L-Ser, (C) L-Thr, or (D) L-Cys. See Scheme 1.

oxazolinyl/methyloxazolinyl (**6**) and thiazolinyl/methyloxazolinyl (**8**) variants and that the bisoxazolinyl (**7**) and bisthiazolinyl (**9**) variants of vibriobactin can be made by VibF.

Utilization of Small-Molecule L*-Threonyl Thioesters To Assay the Heterocyclization Function of VibF*. To isolate the heterocyclization step of VibF we replaced the native acceptor substrate Thr-S-VibF, tethered covalently in cis to the Cy domains, with small-molecule threonyl thioesters Thr-S-NAC and Thr-S-Pant, which function as in trans analogues of Thr-S-VibF and allow turnover independent of releasing agents such as DHB-NS. Use of these substrates with VibF resulted in the production of the corresponding small-molecule heterocycles at very low rates (data not shown). However, experiments on these substrates with a VibF S1891A mutant, in which the VibF PCP is not competent for posttranslational modification with phosphopantetheine, resulted in loss of heterocycle formation, suggesting that this activity was due to slow thiol exchange of the thioester substrates with the holoenzyme form of VibF. As a result, we replaced the full-length, six-domain (Cy-Cy-A-C-PCP-C) VibF with the 160 kDa three-domain fragment CyCyA, which lacks a PCP domain, eliminating the possibility for thiol exchange. The 1−1402 CyCyA fragment of VibF was expressed in *E. coli* as a C-terminal His-tag fusion protein and purified by nickel affinity chromatography (Figure 4) with a high yield of 22 mg of soluble protein/L of culture.

HPLC analysis (Figure 5) revealed that incubation of DHB-S-VibB, CyCyA, and either Thr-S-NAC or Thr-S-Pant did indeed give new products. From both incubations, the free acid *N*-DHB-threonine (DHB-Thr-OH) and the esters *N*-DHB-Thr-O-Tris and *N*-DHB-Thr-O-Me, all derivatives of the unstable condensation products *N*-DHB-Thr-S-NAC and *N*-DHB-Thr-S-Pant, were observed. The intact *N*-DHB-Thr-S-Pant thioester, **10**, was also detected (Scheme 2). No methyloxazoline-containing products were detected. Thus CyCyA was capable of producing a distinct condensation

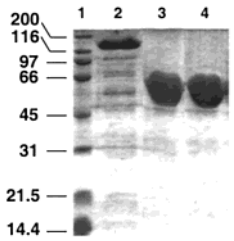


FIGURE 4: Coomassie-blue stained SDS−12% polyacrylamide gel of purified VibF protein fragments used in this study. Lane 1, molecular weight standards; lane 2, CyCyA; lane 3, PCPC2; lane 4, PCP_XC2.

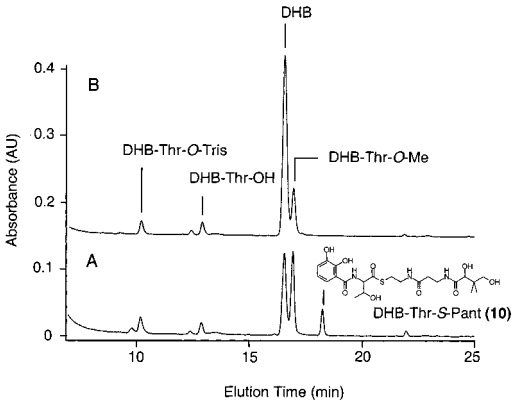


FIGURE 5: HPLC elution profiles of CyCyA products derived from small-molecule substrates. Reactions contained VibE, holo-VibB, CyCyA, DHB, ATP, and (A) L-Thr-*S*-Pant or (B) L-Thr-*S*-NAC. See Scheme 2.

intermediate along the path to heterocycle formation. However, neither small-molecule substrate could be processed fully to the heterocycle. The threonyl thioester substrates showed saturation kinetics (Table 4), with both giving a maximal turnover near 15 min^{-1}, about 5-fold less than the maximal turnover observed with full-length VibF in the normal catalytic sequence. The CyCyA catalytic fragment showed 2-fold higher affinity for the -S-Pant side chain (K_m of 3.9 mM) than the -S-NAC. The L-Ser-S-NAC was also a substrate for acylation by DHB-S-VibB, but now with a 6.5-fold higher K_m than Thr-S-NAC and a net 10-fold drop in k_{cat}/K_m. L-Ala-*S*-NAC was essentially inactive as a surrogate acceptor for acylation by DHB-S-VibB, consistent with the previously observed requirement for a β-functionality in the heterocycle acceptor.

To address the possibility that the PCP domain might be necessary as an allosteric activator for CyCyA to heterocyclize DHB-Thr-S-Pant after condensation, a PCP_xC2 fragment of VibF (consisting of residues 1852−2415 with a Ser to Ala mutation at position 1891) was expressed and purified from *E. coli* (at 16 mg/L, Figure 4) for use in assays containing CyCyA and Thr-S-Pant. No heterocyclization to methyloxazoline products were detected, but PCP_xC2 inhibited DHB-Thr-S-Pant formation with an IC_{50} of 12.5 μM (data not shown).

Reconstitution of Heterocyclization and Primary Amine Acylation by the CyCyA and PCPC2 Fragments of VibF. To determine if the three-domain CyCyA fragment retained the intrinsic capacity for complete heterocyclization activity, we supplied the nativelike two-domain substrate fragment L-Thr-S-PCPC2. The holo form of PCPC2 was able to function in trans as a substrate for covalent threonyl loading

Scheme 2

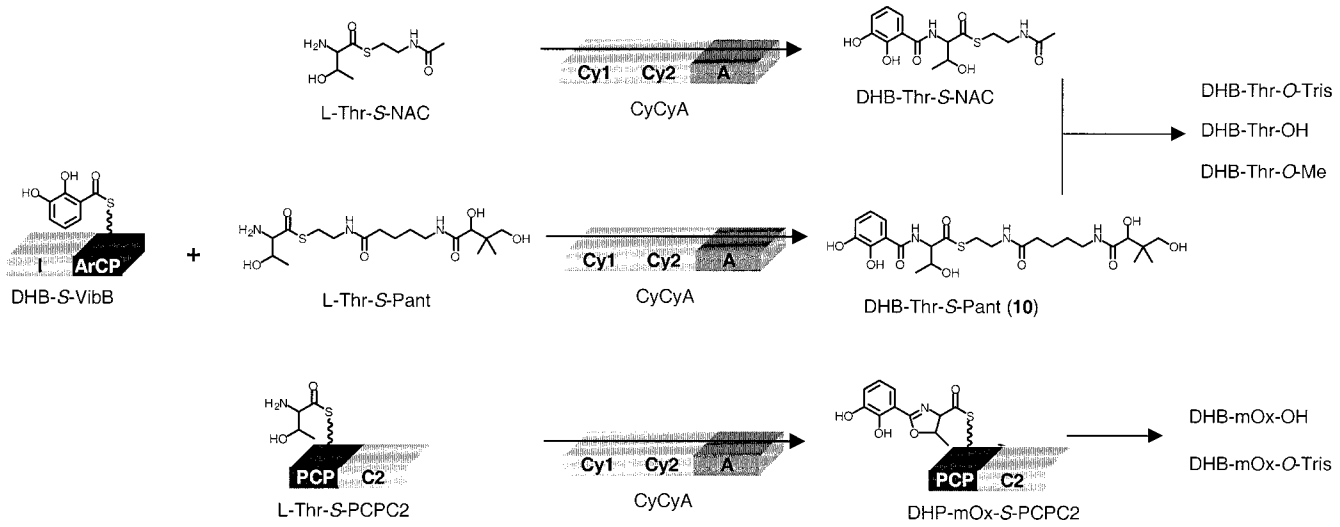


Table 4: Small-Molecule Acceptor Substrates Condensed with DHB-S-VibB by CyCyA

acceptor substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
L-Thr-*S*-NAC	15 ± 1	8.0 ± 0.7	1.9
L-Thr-*S*-Pant	13 ± 2	3.9 ± 1.6	3.3
L-Ser-*S*-NAC	11 ± 2	52 ± 17	0.2
L-Ala-*S*-NAC	<0.1		

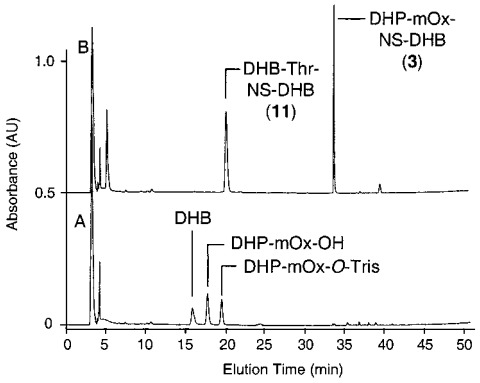


FIGURE 6: HPLC elution profiles of CyCyA products derived from L-Thr-*S*-PCPC2. Reactions contained VibE, holo-VibB, CyCyA, DHB, ATP, L-Thr, PCPC2, and (A) no amine acceptor or (B) DHB-NS. See Scheme 3.

by the A domain of CyCyA, with a K_m of 21.4 μM and a k_{cat} of 163 min^{-1}, to produce L-Thr-S-PCPC2. This aminoacyl-S-enzyme was further incubated with CyCyA and the DHB-S-VibB acyl enzyme and assessed for the formation of DHB-Thr-S-PCPC2 and/or DHP-mOx-S-PCPC2, with turnover of products dependent on either hydrolysis or C2-catalyzed transfer to DHB-NS. In the absence of DHB-NS, the heterocycle-containing products DHP-mOx-OH (Figure 6A) and DHP-mOx-O-Tris ester were produced at slow rates of 1.7 min^{-1} and 1.1 min^{-1}, respectively, as assessed by HPLC and MS analysis (Table 2). Thus, CyCyA was capable of fully processing to methyloxazoline when provided L-threonine tethered to the holo-PCP scaffold.

In the presence of the amine acceptor DHB-NS, both the uncyclized DHB-Thr-NS-DHB, **11**, and the cyclized DHP-mOx-NS-DHB, **3**, amide products were produced, at rates of 8.5 min^{-1} and 2.8 min^{-1}, respectively (Figure 6B, Scheme 3). These findings establish C2 as catalytically competent for primary amine acylation and that the aminolysis flux is about 5-fold higher than the hydrolysis flux under the experimental conditions. When PCPC2 concentration was increased up to 20 μM, the rate of product formation increased linearly, with no effect on partition between the uncyclized threonyl and the cyclized methyloxazolinyl amide products (Figure 7A). Instead the partition ratio between condensation and condensation/heterocyclization was dependent on CyCyA concentration (Figure 7B). An 8-fold increase in CyCyA from 1 to 8 μM had a 2.5-fold effect in favor of cyclization.

Heterocyclization Donor Substrate Specificity of CyCyA in Condensation Assays with Thr-S-Pant Acceptor. To probe the requirements for the upstream donor species in condensation with the Thr-S-Pant acceptor by the CyCyA fragment, we varied this substrate at the level of acyl group and protein scaffold. The native substrate, DHB-S-VibB (maintained in constant concentration via an excess of VibE, DHB, and ATP), exhibited apparent constants of $k_{cat} = 69 \pm 8$ min^{-1} and $K_m = 20 \pm 4$ μM in formation of DHB-threonyl species

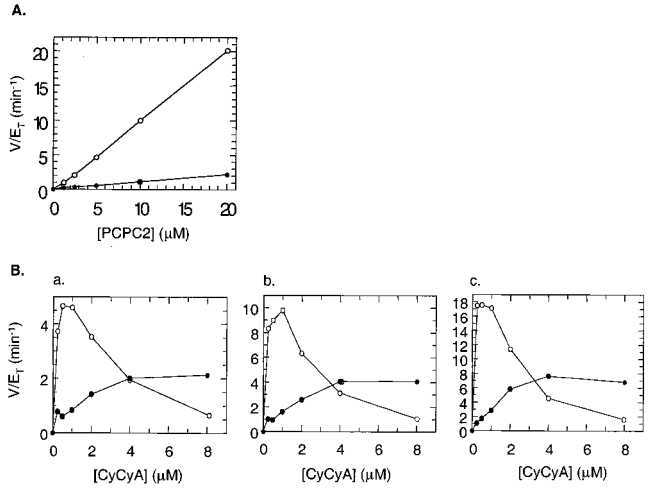
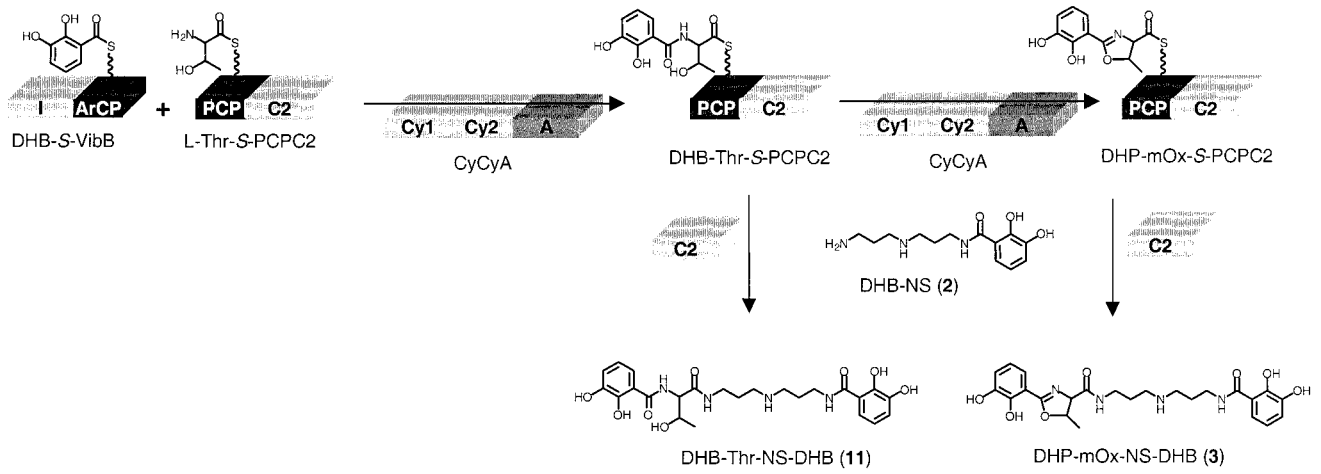


FIGURE 7: (A) Effect of PCPC2 concentration on formation of (○) DHB-Thr-NS-DHB and (●) DHP-mOx-NS-DHB. (B) Effect of CyCyA concentration on formation of (○) DHB-Thr-NS-DHB and (●) DHP-mOx-NS-DHB in reactions containing (a) 5 μM, (b) 10 μM, or (c) 20 μM PCPC2.

Scheme 3



with Thr-S-Pant fixed at 4 mM. In contrast, both salicyl-S-VibB (generated by VibE) and DHB-S-EntB (generated by EntE) failed to support amide bond formation by CyCyA. The small molecule surrogate DHB-S-NAC, however, did support turnover, and while saturation kinetics could not be measured due to limited substrate solubility, a k_{cat}/K_m of 0.60 $(min \cdot mM)^{-1}$ was estimated, 5800-fold below the catalytic efficiency of DHB-S-VibB.

DISCUSSION

The *Vibrio cholerae* iron chelator vibriobactin, **1**, has three catecholic rings, one in a dihydroxybenzoyl group and the other two as (dihydroxyphenyl)methyloxazolinyl (DHP-mOx) moieties, assembled on a triamine scaffold provided by norspermidine (*1*). The DHB group is attached in direct amide linkage to one of the primary amines of norspermidine. The two DHP-mOx groups arise biosynthetically from DHB-threonyl (DHB-Thr) enzyme intermediates that have undergone enzymatic cyclization to DHP-mOx enzyme intermediates prior to transfer to N_5 and N_9 of norspermidine (Figure 1). Analogous (2-hydroxyphenyl)thiazolines are found in other siderophores such as yersiniabactin and pyochelin, where they are thought to be iron-coordination sites and arise by analogous heterocyclization of salicyl-cysteinyl-enzyme intermediates by NRPS enzymes (*10, 11*). The biosynthetic machinery in NRPS assembly lines responsible for construction of the DHB-Thr- or salicyl-cysteinyl-enzyme intermediates that then undergo heterocyclization comprise a sequential array of five domains: an aryl acid-activating A domain; the holo form of an aryl carrier protein domain (ArCP); a heterocyclization domain (Cy); a Thr-, Ser-, or Cys-activating A domain; and the holo form of a peptidyl carrier protein (PCP). These five domains can be distributed over several subunits. In the vibriobactin system, VibE is the aryl acid-activating A domain, VibB contains the ArCP domain, and the VibF subunit begins with a tandem pair of Cy domains and also has the aminoacyl A and PCP domains. The heterocyclization step requires (a) condensation of the donor substrate with the thioesterified aminoacyl acceptor, (b) cyclization to generate the five-membered heterocyclic ring and (c) dehydration to yield the final dihydro heterocyclic product.

In this work we have addressed the ability of the VibF subunit to utilize alternate heterocyclization acceptor and donor substrates. In the first component of the study, amino acid acceptors, both with and without β-functional groups, were assessed for processing by VibF. Although VibF could activate several amino acids lacking a β-functional group, it refused to process them further, even to the stage of a condensed intermediate. This argues for an important role for these groups in substrate binding or catalytic mechanism. The processing of Thr, Ser, and Cys to their respective methyloxazoline-, oxazoline-, and thiazoline-containing products establishes that a single Cy domain (or set of Cy domains) can take the three common β-substituted amino acids and produce the full range of dihydroheteroaromatic rings found in bacterial siderophores.

The observed promiscuity indicates that all three catalytic domains of NRPS enzymes found in VibF (A, Cy, and C) can process the alternate monomers Ser and Cys. While the A domain made L-Ser-AMP and L-Cys-AMP about 50−100-fold less efficiently than L-Thr-AMP according to the PP_i−ATP exchange assays, aminoacyl activation was not the rate-limiting process; thus improvement of VibF as an alternative heterocycle catalyst may require multidomain modification. As the gatekeeper and the sole determinant of substrate flux from the bulk solvent into the pathway, mutagenesis of the A domain (*12*) in favor of Ser or Cys recognition would be required to improve the efficiency of alternative heterocycle production. Here Ser would benefit more than Cys, with a K_m 25-fold higher than that of Thr. Once loaded onto VibF, these alternative substrates were processed by the Cy domain(s) (to the DHP-heterocyclic acyl-S-VibF intermediates) and by the C domain(s) (to the corresponding *N*-acylated-NS-DHB) with almost equal efficiency; the k_{cat} values for this first acylation of DHB-NS were within 2−7-fold of the natural intermediate. An improvement here would largely benefit Cys.

In the last acylation step on the norspermidine skeleton, in which the secondary amine of these N^9-heterocycle-containing intermediates served as acceptor to a second DHP-methyloxazolinyl/thiazolinyl/oxazolinyl acylation, no preference was observed for the native heterocycle in the acceptor substrate (less than 2-fold decrease). For the donor, however, the net k_{cat} was down 2-fold for Ser and 32-fold for Cys utilization. Again, Cys bears the weight of the discrimination in postactivation events, more so in the acylation of the secondary amine than the primary.

By assaying each of the iterated norspermidine acylations separately, it was possible to coax VibF into making not only the bis(oxazolinyl) analogue **7** of vibriobactin and the bis(thiazolinyl) analogue **9** but also either mixed regioisomer, the thiazolinyl-mOx (**6**) or oxazolinyl-mOx (**8**) forms of iron chelator. This demonstrates the versatility of the VibF assembly line as a double heterocyclization catalyst and suggests that such Cy domains would be useful in the conversion of *N*-acylated Ser, Thr, and Cys peptides into aryl-capped dihydroheterocyclic structures.

In the second part of this study, the effect of omitting the protein component from the heterocyclization acceptor substrate was examined by use of small-molecule threonyl-thioesters that mimic the native Thr-S-VibF. This has the advantage of isolating the heterocyclization step kinetically from downstream events normally required to effect enzyme turnover. Previous experiments with -S-NAC surrogates with the C domain of the enterobactin NRPS enzyme EntF have resulted in successful condensation, albeit with reduced efficiency (*13*). When full-length VibF was assayed with Thr-S-NAC, a mixture of (mostly) uncyclized DHB-Thr-OH and cyclized DHP-mOx-OH was released by hydrolysis. The small amount of heterocyclic product arose from trans-thiolation of the threonyl group from the S-NAC sulfur to the sulfur of the S-Pant arm of the PCP domain in VibF, as proven by the lack of such activity in the Ser to Ala mutant, which is incapable of being converted to the holoenzyme. To remove this background reaction, we turned to the half-molecule fragment of VibF, the 160 kDa CyCyA fragment as catalyst. The PCP-free VibF truncate CyCyA did in fact use the small-molecule surrogates Thr-S-NAC and Thr-S-Pant with saturation kinetics and reasonable k_{cat} values, however, not to produce the expected DHP-mOx-thioester heterocycles but only the DHB-Thr thioester condensation products. The two implications then are, first, that the PCP protein scaffold to which the acceptor acyl group is attached plays an important role in achieving full heterocyclization activity, and second, that a distinct condensation intermediate lies along the pathway to the dihydroheterocyclic final product.

The efficiency of use of L-Ser-S-NAC as an acceptor substrate in CyCyA-catalyzed condensation allows an examination of heterocyclization domain substrate specificity independent of substrate activation (A domain) or product release (C domain) constraints. That the 10-fold decrease in efficiency is borne almost entirely by the increase in K_m attests to the importance of a single methyl group in substrate binding, providing more evidence that A domains are not the only source of selectivity in NRPS modules. That L-Ala-S-NAC is refused outright, while the *E. coli* enterobactin NRPS EntF will accept it as a condensation acceptor substrate with a mere 2-fold decrease in rate relative to the native L-Ser (*13*), reinforces the importance of the β-functionality in the heterocyclization process.

Given that the presentation of the threonyl thioester on an -S-NAC or -S-Pant chain was insufficient to provide enough affinity for heterocyclization, we put the threonyl group back on the PCP domain by combining CyCyA and a holo-PCPC2 fragment of VibF, resulting in an in trans loading of normally in cis PCP. L-Thr-S-PCPC2 was converted to DHP-mOx-S-PCPC2 in the presence of DHB-S-VibB, as evidenced by the slow release of DHP-mOx hydrolytic products and tris esters. This observation confirms the importance of the PCP scaffold in presentation of the thioesterified acyl group for full heterocyclization activity.

The relatively rapid production of primary aminoacylated products when PCPC2 thioesters are released by DHB-NS argues for a catalytic role for PCPC2 in primary amine acylation, a known activity but previously unassigned function in VibF (*7*). These thioesters were of two varieties, the uncyclized DHB-Thr-S-PCPC2 and the cyclized DHP-mOx-S-PCPC2, indicating that the in trans reconstitution of VibF activity by holo-PCPC2, while more successful than small-molecule thioester acceptor substrates, is not as efficient as the native in cis system. The partition of the observed uncyclized and cyclized norspermidine containing products (**11** and **3**, respectively) results from a competition between the rate of DHB-Thr-S-PCPC2 heterocyclization and the rate of its dissociation from CyCyA, where it is susceptible to C2-mediated capture by the primary amine. An increase in PCPC2 increases the rate of production of both products, suggesting an increase in the level of DHB-Thr-S-PCPC2, the branch point for both pathways. However increased PCPC2 does not increase flux to **11**, arguing for saturation of primary amine acylation at all concentrations tested, which might be expected for an enzyme whose substrate is provided in cis. When CyCyA is increased however, not only is overall product formation increased (the result of increasing A domain dependent L-Thr-*S*-PCPC2) but also the flux to **3** is dramatically increased. This argues for the pathway presented in Scheme 3, in which formation of **3** is dependent on the ability of Cy to recapture the condensation product DHB-Thr-S-PCPC2 and finish the job to DHP-mOx-S-PCPC2, which is released for primary amine acylation in cis by the C2 domain. This supports the ability of the heterocyclization domain to process a condensation intermediate to the final dihydroheterocyclic product.

In a recent study of thiazoline formation in yersiniabactin synthesis, both Cy domains of HMWP2 [each of which contributes one ring of a tandem bis(thiazolinyl) intermediate] responded differently to perturbations of the heterocycle acceptor substrate (*14*). When supplied their normally in cis acyl-S-PCP substrates in trans, one Cy was fully functional, processing completely to the thiazoline, while in the other, heterocyclization activity was reduced to simple condensation. Taken together with the findings in this study, it is clear that distinct condensation intermediates form along the path to the dihydroheterocycle final product. The inability of the one Cy to fully heterocyclize its acyl substrate despite presentation on the protein scaffold may indicate that, in some cases, the contribution of the protein component to the binding energy of the interaction is sufficiently low that it must be supplied in cis.

The kinetic isolation of the heterocyclization step by use of a small-molecule thioester allows the examination of donor substrate specificity, an important consideration in evaluating heterocyclization domains as candidates for NRPS engineering. C and Cy domain donor substrate specificity has been examined in cyclic peptide and siderophore NRPS, respectively (*15*, *16*), with evidence suggesting relaxed specificity at the level of acyl group. Condensation assays with the Thr-S-Pant acceptor and analogues of the DHB-S-VibB donor reveal a disruption in this trend by the refusal to use either a salicyl acyl surrogate or an EntB ArCP surrogate. The

5800-fold decrease in efficiency suffered by DHB-S-NAC attests to the crucial role played by the protein component of the donor substrate.

These results on the VibF subunit in vibriobactin synthetase reveal much of the catalytic capacity that most likely underlies the assembly of all the aryl-N-capped siderophores and antibiotics [e.g., actinomycin D (*17*)]. The function of the heterocyclizing Cy domains as a subset of NRPS C domains with a gain of function, cyclodehydration, is deciphered as a second chemical transformation that has to be enacted before the growing chain is transferred downstream to the next module in these assembly lines. It also suggests Cy domains have potential to function in the synthesis of biosynthetic libraries of domains able to cyclize β- thiol and β-hydroxyamino acyl thioesters, although limitations imposed by rigid donor substrate requirements may have to be overcome.

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