Excision of the Epothilone Synthetase B Cyclization Domain and Demonstration of in Trans Condensation/Cyclodehydration Activity†

Wendy L. Kelly, Nathan J. Hillson, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: The epothilones are potent anticancer natural products produced by a polyketide synthase (PKS)−nonribosomal peptide synthetase (NRPS) hybrid involving proteins EpoA−F. The single NRPS module of the epothilone assembly line, EpoB, is a distinct subunit of approximately 160 kDa and consists of four successive domains: cyclization, adenylation, oxidation, and peptidyl carrier protein (Cy−A−Ox−PCP). The cyclization domain is responsible for introduction of the thiazoline heterocycle into the growing polyketide/nonribosomal peptide chain from the precursors malonyl-CoA and cysteine through the multiple steps of condensation, cyclization, and dehydration. This enzyme-bound thiazoline intermediate is subsequently oxidized to a thiazole by the EpoB Ox domain. The EpoB module was dissected to provide 57 kDa EpoB(Cy) and 102 kDa EpoB(A−Ox−PCP) as subunit fragments to evaluate Cy as a free-standing domain. EpoB was reconstituted by these fragments in trans to generate the methylthiazole product. Using this system, apparent kinetic constants for the upstream acyl donor EpoA(ACP) and EpoB(Cy) were determined, providing a measure of affinity for the naturally occurring interface of the amino terminus of EpoB and the EpoA carboxy terminus. Site-directed mutants in excised EpoB(Cy) were prepared and used to examine residues involved in condensation and heterocycle formation. This work demonstrates the ability to define a functional Cy domain by excision from its native NRPS module, and examine both its protein−protein interactions and mechanism of activity.

Nonribosomal peptide synthetases (NRPSs)[1] and polyketide synthases (PKSs) are large, multimodular enzymes responsible for the biosynthesis of a number of clinically relevant natural products, such as vancomycin and rapamycin (*1*, *2*). The NRPSs, and, in an analogous fashion, the PKSs, employ a multiple-carrier thiotemplate mechanism for elongation of the growing peptide chain (*3*, *4*). Each amino acid incorporated into the final product arises from one module of the NRPS. The modules themselves are further demarcated into discrete structural and catalytic domains, with a minimal module consisting of three fundamental domains. The first of these, the adenylation (A) domain, selects an amino acid from the available metabolic pool and utilizes ATP to activate the substrate as the aminoacyl adenylate, releasing inorganic pyrophosphate. After adenylate formation, the amino acid is covalently tethered as a thioester to the 4′-phosphopantetheinyl cofactor of the peptidyl carrier protein (PCP). The final requisite core domain is the condensation (C) domain, which facilitates the attack of the α-amino group of one thioesterified amino acid on the activated acyl group of an upstream amino acid or peptide in forming a new amide bond (Figure 1A).

Introduction of a thiazoline or oxazoline ring into the peptide-based metabolite is mediated through the action of a cyclization (Cy) domain, a variant of the classical C domain, on amino acid substrates possessing a β-heteroatom, such as cysteine, serine, and threonine (*5*−*7*). The heterocycle fundamentally alters chain connectivity, since a hydrolyzable peptide bond is replaced with a heterocycle resistant to subsequent protease action (Figure 1A).

It has recently been possible to dissect the four-domain NRPS subunit EntF (C−A−PCP−TE) of enterobactin synthetase to generate the N-terminal C domain as a separate 50 kDa fragment that was functional with the small-molecule acceptor substrate mimic seryl-*S*-NAC and the enzyme-bound acyl donor 2,3-dihydroxybenzoyl-*S*-EntB (*8*). Such separate C domains can then be assessed for catalytic function and ultimately structural information. In this work, we have addressed the parallel question of whether an excised Cy domain can be studied as an autonomous catalytic domain. We have chosen the EpoB NRPS subunit because of the medicinal interest in epothilones (*9*, *10*) and because we have previously shown the oxidation (Ox) domain of EpoB can be excised (*11*) and then reinserted into a distinct NRPS assembly line (*12*).

The initial steps of epothilone biosynthesis involve formation of a methylthiazole ring through the action of EpoB, the single NRPS module of the PKS/NRPS pathway (*13*, *14*). The loading module, EpoA, activates malonyl-CoA and forms acetyl-*S*-EpoA as the acyl donor for the Cy domain

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* To whom correspondence should be addressed. E-mail: christopher_walsh@hms.harvard.edu. Phone: (617) 432-1715. Fax: (617) 432-0438.

[1] Abbreviations: Epo, epothilone; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; Cy, cyclization; A, adenylation; Ox, oxidation; PCP, peptidyl carrier protein; ACP, acyl carrier protein; C, condensation; NAC, *N*-acetylcysteamine; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; TCEP, tris(2-carboxyethyl)phosphine; TCA, trichloroacetic acid; TE, thioesterase; 2,3-DHB, 2,3-dihydroxybenzoic acid.

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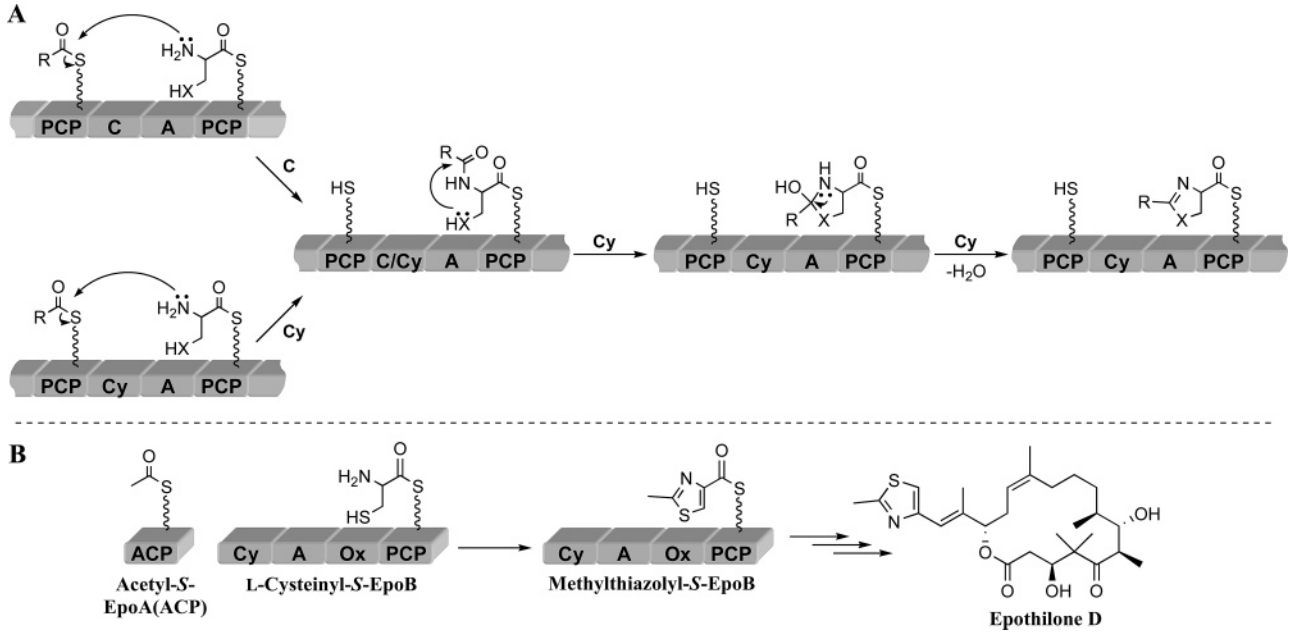

FIGURE 1: (A) General mechanism of condensation domain- and cyclization domain-catalyzed amide bond formation and cyclodehydration. (B) Methylthiazole formation in the initial stages of epothilone D biosynthesis.

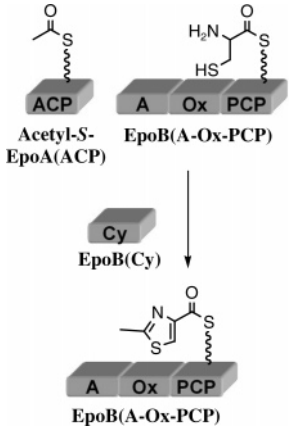

FIGURE 2: Dissection of EpoB into functional Cy and A−Ox−PCP fragments.

of EpoB (Figure 1B) (*15*). In turn, the EpoB subunit provides L-cysteinyl-*S*-PCP, and the amino-terminal Cy domain directs condensation and subsequent formation of the intermediate thiazoline ring, which is next oxidized to the thiazolyl-*S*-PCP by the EpoB Ox domain (*11, 15*). The methylthiazole moiety is then transferred to the EpoC PKS subunit for further elongation and ultimate progression through the EpoD−F PKS system to the final macrolactone ring structure (*16*).

In this work, we have dissected EpoB to provide two folded fragments: EpoB(Cy), as a single free-standing Cy domain, and the complementary three-domain fragment EpoB(A−Ox−PCP) (Figure 2). Both constructs were expressed efficiently and, when incubated together with acetyl-*S*-EpoA(ACP) as an acyl donor, were able to effect methylthiazole formation. This demonstrates that EpoB(Cy), normally acting in cis with the remaining NRPS subunit, retains enough affinity for EpoB(A−Ox−PCP) in trans to preserve functional integrity. This system was utilized to obtain kinetic constants under single-turnover conditions for the Cy domain using the upstream acyl donor, acetyl-*S*-EpoA(ACP), and the downstream acyl acceptor, cysteinyl-*S*-EpoB(A−Ox−PCP), as cosubstrates. Additionally, a number of site-directed mutants of EpoB(Cy) were prepared and tested to investigate the catalytic mechanism of the excised protein.

MATERIALS AND METHODS

General. Chemically competent *Escherichia coli* BL21-(DE3) was purchased from Invitrogen. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and Pfu Ultra was purchased from Stratagene. The pET28b, pET28b, and pET30a expression vectors were purchased from Novagen. DNA primers for polymerase chain reaction (PCR) amplification were purchased from Integrated DNA Technologies. DNA sequencing was performed by the Dana Farber Cancer Institute Molecular Biology Core Facility. [^{35}S]-L-Cysteine was purchased from PerkinElmer. All chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich. High-pressure liquid chromatography (HPLC) analysis was performed on a Vydac small-pore C18 column (4.6 mm × 250 mm) with a Beckman System Gold instrument and an in-line radioactivity detector tuned to ^{35}S (IN/US Systems β-RAM model 3), employing a gradient of 0 to 60% solvent B (acetonitrile) in solvent A (0.1% TFA in water) over 25 min at 1 mL/min. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (*17*).

Construction of EpoB(Cy) Variants and EpoB(A−Ox−PCP). The EpoB(Cy) fragment was amplified by PCR from pET28b/epoB (*15*) at nucleotides 1−1476 or 1−1491 of the *epoB* coding sequence. The forward primer (EpoBCyF, Table 1) possessed a *Nco*I restriction site, and the second codon was mutated from alanine (ACG) to threonine (GCG) to accommodate the restriction site. The reverse primers (EpoBCyER and EpoBCyQR) contained a TAA stop codon following nucleotides 1476 and 1491, respectively, and a *Not*I restriction site. The PCR product was digested and ligated into linearized pET30a to provide pET30a/epoB-CyE492 and pET30a/epoBCyQ497, and the constructs were confirmed by DNA sequencing.

Table 1: DNA Primers Used in This Study

Primer Name	Nucleotide Sequence[a]
EpoBCyF	5′-GCCGCGCGGCAGCCCCATGGCGATCAATCA-3′
EpoBCyER	5′-GCATCTGTGCGGCCGCTTATTCCTCAGTGAGCCGCCGGA-3′
EpoBCyQR	5′-GCGGAAGCGCGGCCGCTTACTGTTCACTCCATGGTTCCTCAGT-3′
EpoBRPst	5′-GAGCCTCTGCAGCGAGGTGACACCAAC-3′
EpoBFNde	5′-CGCGGCAGCCATATGACGATCAATCAG-3′
EpoBRKpn	5′-TCCTCACGGGGTACCCGATGGACC-3′
EpoBCyFNco	5′-AACCATGGCGATCAATCAGCTTCTGAACGAGC-3′
EpoBCyRNco	5′-TTCACTCCATGGTTCCTCAGTG-3′
H122AF	5′-TCGCGCGG**GCC**GACATGCTTCGGGCC-3′
H122AR	5′-AAGCATGTC**GGC**CCGCGCGACGACTTTCC-3′
D201AF	5′-CGTGCTCAGTATC**GCT**CTCATTAACGTTGACCT-3′
D201AR	5′-AACGTTAATGAG**AGC**GATACTGAGCACGAGACGG-3′
D206NF	5′-CTCATTAACGTT**AAC**CTAGGCAGCCTGTCCATC-3′
D206NR	5′-GGACAGGCTGCCTAG**GTT**AACGTTAATGAGATCGAT-3′
S209AF	5′-GTTGACCTAGGC**GCC**CTGTCCATCATCTTCAAGG-3′
S209AR	5′-GAAGATGATGGACAG**GGC**GCCTAGGTCACCGTTTAATG-3′
S418AF	5′-GTGCTCACG**GCC**GCGCTCAACCAGCAAGTC-3′
S418AR	5′-GTTGAGCGC**GGC**CGTGAGCACCACGGG-3′
S418NF	5′-GTGCTCACG**AAC**GCGCTCAACCAGCAAG-3′
S418NR	5′-TTGAGCGC**GTT**CGTGAGCACCACGGG-3′
EpoB-S-F	5′-AACATATGAGTGAACAGATGCGCTGTTCGCTTCCGCC-3′
EpoB-M-F	5′-AACATATGCGCTGTTCGCTTCCGCCTGC-3′
EpoB-L-F	5′-GCCATATGCTTCCGCCTGCCCAGCTAGAAGC-3′
EpoBNotCHR	5′-AAGCGGCCGCGCTACGTCTCCTGCCCTTGCG-3′

[a] Restriction sites are underlined, and the alanine or asparagine codon is in bold.

Table 2: PCR Primer Pairs Used in Construction of EpoB Fragments and Mutants

construct	5′ primer	3′ primer
pET30a/epoBCyE492	EpoBCyF	EpoBCyER
pET30a/epoBCyQ497	EpoBCyF	EpoBCyQR
pET28b/epoB H122A	H122AF	EpoBRPst
	EpoBFNde	H122AR
pET30a/epoBCy H122A	EpoBCyFNco	EpoBCyRNco
pET28b/epoB D201A	D201AF	EpoBRPst
	EpoBFNde	D201AR
pET30a/epoBCy D201A	EpoBCyFNco	EpoBCyRNco
pET28b/epoB D206N	D206NF	EpoBRPst
	EpoBFNde	D206NR
pET30a/epoBCy D206N	EpoBCyFNco	EpoBCyRNco
pET28b/epoB S209A	S209AF	EpoBRPst
	EpoBFNde	S209AR
pET30a/epoBCy S209A	EpoBCyFNco	EpoBCyRNco
pET28b/epoB S418A	S418AF	EpoBRKpn
	EpoBFNde	S418AR
pET30a/epoBCy S418A	EpoBCyFNco	EpoBCyRNco
pET28b/epoB S418N	S418NF	EpoBRKpn
	EpoBFNde	S418NR
pET30a/epoBCy S418N	EpoBCyFNco	EpoBCyRNco
pET29b/ΔCyEpoB S495	EpoB-S-F	EpoBNotI-CHR
pET29b/ΔCyEpoB M498	EpoB-M-F	EpoBNotI-CHR
pET29b/ΔCyEpoB L502	EpoB-L-F	EpoBNotI-CHR

Site-directed mutants of EpoB(Cy) were constructed by the extension overlap method (*18*) from pET28b/epoB using the appropriate DNA primers for the desired mutant (Table 2). The reverse primers employed a native restriction site in the *epoB* nucleotide sequence, either *Pst*I or *Kpn*I. The extension-overlap PCR products were ligated into linearized pET28b/epoB to provide Cy domain mutants of full-length EpoB. The region of the Cy domain containing the mutation was again PCR-amplified from each full-length gene (Table 2). The forward primer, EpoBCyFNco, contained a *Nco*I restriction site and a mutation for the second codon from ACG (Ala) to GCG (Thr). The reverse primer, EpoBCyRNco, contained the *Nco*I restriction site found in the native nucleotide sequence. The products were digested with *Nco*I and ligated into linearized pET30a/epoBCyQ. The correct orientation of the fragment containing the mutation was checked by restriction digestion, and DNA sequencing confirmed the absence of undesired mutations.

The fragments for EpoB(A−Ox−PCP) designed with C-terminal hexahistidine tags were PCR-amplified from pET28b/epoB using the primers from Table 2. The amino-terminal boundaries, Ser495, Met498, and Leu502, were defined in the forward primers at nucleotides 1483, 1492, and 1504 of *epoB*, respectively. An *Nde*I restriction site was present in the forward primers, and the reverse primers possessed a *Not*I restriction site. The PCR products were digested with *Nde*I and *Not*I and then ligated into linearized pET29b. DNA sequencing confirmed the absence of any PCR-induced mutations.

Expression and Purification of EpoB(Cy) and Site-Directed Mutants of EpoB(Cy). E. coli BL21(DE3) cells harboring the expression plasmid for the EpoB(Cy) variant of interest were grown in 6 × 1 L of Luria Burtani (LB) medium supplemented with 50 μg/mL kanamycin at 20 °C for 48 h, in the absence of added IPTG. Harvested cells were resuspended in 120 mL of lysis buffer [20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 μg/mL deoxyribonuclease I] and lysed by homogenization (Avestin EmulsiFlex-C5 homogenizer). The lysates were clarified by ultracentrifugation. The cell-free extract was incubated with 6 mL of Ni−NTA resin (Qiagen) for 1 h at 4 °C. The resin was loaded onto a column and washed with 45 mL of wash buffer [20 mM Tris (pH 8.0), 500 mM NaCl, and 5 mM imidazole]. Protein was eluted with a step gradient of wash buffer containing 25, 50, 75, 100, and 200 mM imidazole (20 mL of each). The fractions containing the EpoB(Cy) protein were pooled together and dialyzed against 2 × 1 L of storage buffer [20 mM Tris (pH 7.5), 10% glycerol, 1 mM DTT, and, for the site-directed mutants, 200 mM NaCl]. Proteins were flash-frozen in liquid nitrogen and stored at −80 °C.

Expression and Purification of EpoB(A−Ox−PCP). E. coli BL21(DE3) cells containing the expression plasmid for EpoB(A−Ox−PCP) were grown in 6 × 1 L of Luria Burtani medium for 72 h at 15 °C in the absence of IPTG. Harvested cells were resuspended in 160 mL of lysis buffer [25 mM Tris (pH 8.0), 500 mM NaCl, 2.5 mM imidazole, 2 mM $MgCl_2$, 15% glycerol, 1 mM PMSF, and 10 μg/mL deoxyribonuclease I] and lysed by homogenization. The lysates were clarified by centrifugation. The cell-free extract was incubated with 24 mL of Ni−NTA resin (Qiagen) for 1.5 h. The resin was loaded onto a column, and the resin was washed with 250 mL of wash buffer [25 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 2 mM $MgCl_2$, and 15% glycerol]. Protein was eluted with 100 mL of elution buffer (wash buffer containing 250 mM imidazole), collecting 8 mL fractions. Fractions containing the protein were pooled together and dialyzed against 2 × 3 L of storage buffer [25 mM Tris (pH 7.5), 2 mM $MgCl_2$, 500 mM NaCl, 15% glycerol, and 1 mM DTT]. The proteins were flash-frozen in N_2(l) and stored at −80 °C.

Cysteine-Dependent ATP−PP_i Exchange Assay for EpoB-(A−Ox−PCP). The 100 μL reaction mixtures incubated at 24 °C for 5 min contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM TCEP, 3 mM ATP, 10% glycerol, 1 mM [^{32}P]-pyrophosphate (2.32 μCi/μmol), 200 nM EpoB(A−Ox−PCP), and varying concentrations of L-cysteine. Reactions were initiated by addition of enzyme and quenched with 500 μL of quench solution [1.6% (w/v) charcoal, 4.5% (w/v) tetrasodium pyrophosphate, and 3.5% perchloric acid in

water]. The charcoal suspensions were pelletted by centrifugation and then washed twice with 500 μL of quench solution lacking charcoal. The pellet was resuspended in 1 mL of water, and the ^{32}P content was measured by liquid scintillation counting.

Preparation of Acetyl-S-EpoA(ACP). Priming of EpoA(ACP) with acetate was achieved by incubating the carrier protein with surfactin phosphopantetheinyl transferase (Sfp) (*19*) and acetyl-CoA. A typical priming reaction mixture (20 μL) was incubated at 24 °C for 30 min and contained 48 μM EpoA(ACP), 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM TCEP, 800 μM acetyl-CoA, and 1.5 μM Sfp. The appropriate aliquot was added directly to the methylthiazole reaction mixture (see below).

Preparation of [^{35}S]Cysteinyl-S-EpoB(A−Ox−PCP). A typical reaction mixture of 90 μL contained 8 μM EpoB(A−Ox−PCP), 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM TCEP, 150 μM CoA, 1.5 μM Sfp, 3 mM ATP, and 1.5 mM [^{35}S]-L-cysteine (70 μCi/μmol). Reactions were initiated in the absence of ATP and [^{35}S]-L-cysteine, and permitted to progress for 30 min at 24 °C. Following addition of ATP and [^{35}S]-L-cysteine, the reaction mixtures were incubated at 24 °C for an additional 40 min. The appropriate aliquot was added to the methylthiazole production reaction mixture (see below).

Cyclization Domain Activity Assay. Reaction mixtures (110 μL) for detecting methylthiazole production were preincubated with acetyl-*S*-EpoA(ACP) and [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP) and aliquoted from the above reaction mixtures for 2 min at 24 °C, and the reactions were initiated by the addition of EpoB(Cy). The final reaction mixtures containing 4 μM [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP), 4 μM acetyl-*S*-EpoA(ACP), 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM TCEP, and 9 μM EpoB(Cy) were incubated at 24 °C for 120 min. Reactions were quenched with 900 μL of 10% TCA, and protein-bound products were pelletted by centrifugation. The pellet was washed twice with 800 μL of 10% TCA, resuspended in 200 μL of 0.1 M KOH, and heated at 60 °C for 10 min. Protein was precipitated with 20 μL of 50% TFA and removed via centrifugation, and the supernatant was analyzed by radio-HPLC.

Kinetics of Heterocycle Formation by EpoB(Cy). To obtain saturation kinetics for heterocycle production by EpoB(Cy), 110 μL reaction mixtures contained 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM TCEP, 6.5 μM [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP) (70 μCi/μmol), 54 nM EpoB(Cy), and varying concentrations of acetyl-*S*-EpoA(ACP). Reaction mixtures for the rate of heterocycle formation as a function of [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP) concentration contained 8.6 μM acetyl-*S*-EpoA(ACP) while varying the concentration of the aminoacyl-*S*-EpoB(A−Ox−PCP) in the presence of 54 nM EpoB(Cy). The reaction mixtures were incubated at 24 °C for 5 min, and the reactions were quenched with 900 μL of 10% TCA. The protein pellet was washed twice with 800 μL of 10% TCA, resuspended in 100 μL of 0.1 M KOH, and heated at 60 °C for 10 min. Protein was precipitated with 10 μL of 50% TFA, and the supernatant was analyzed by radio-HPLC.

Rate of Heterocycle Formation by EpoB(Cy) and Variants. The rate (k_{obs}) of methylthiazole formation was measured using 110 μL reaction mixtures containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM TCEP, 6.5 μM [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP) (70 μCi/μmol), and 8.6 μM acetyl-*S*-EpoA(ACP). Reactions were initiated with the EpoB(Cy) variant and mixtures incubated at 24 °C as indicated: 1 μM wild-type EpoB(Cy) for 0.5 min, 6.4 μM EpoB(Cy) H122A for 8 min, 3 μM EpoB(Cy) D201A for 8 min, 2 μM EpoB(Cy) S209A for 5 min, 2 μM EpoB(Cy) S418A for 5 min, and 4 μM EpoB(Cy) S418N for 8 min. Reactions were quenched and mixtures prepared for radio-HPLC analysis as described above.

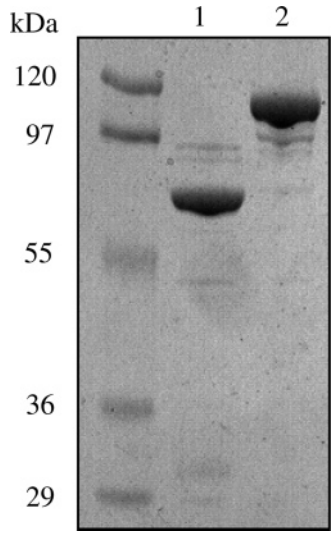

FIGURE 3: SDS−PAGE analysis of purified (1) EpoB(Cy) and (2) EpoB(A−Ox−PCP).

RESULTS

Construction and Purification of EpoB(Cy). The size of the full-length four-domain EpoB module is 158 kDa (*8*, *13*, *14*). The boundary between the 57 kDa amino-terminal Cy domain and the subsequent internal A domain was assigned to a loop identified by secondary structure prediction, as undertaken for the EntF C domain (*8*). Since the initial 56 amino acids of the N-terminus of EpoB have previously been shown to be required for communication with the upstream subunit EpoA(ACP), this amino-terminal region was retained in its entirety (*20*). Two possible sites for the EpoB(Cy) C-terminus were designated Glu492 and Gln497. The coding sequences were amplified by PCR and ligated into pET30a. The proteins were heterologously expressed as N-terminal hexahistidine tag fusions in *E. coli* BL21(DE3) under control of the T7 promoter and purified by nickel chelate chromatography (Figure 3). The Glu492 variant proved to be largely insoluble, and therefore, the Gln497 construct was chosen for further analysis due to improved yields of soluble protein, at 4 mg/L of culture.

Construction, Purification, and Characterization of EpoB(A−Ox−PCP). Using the same loop identified as the boundary region between the Cy and A domains of EpoB, three different sites were chosen for the N-terminus for construction of the 102 kDa fragment EpoB(A−Ox−PCP): Ser495, Met498, and Leu502. The constructs were expressed as C-terminal hexahistidine-tagged proteins to ensure that an N-terminal fusion would not interfere with recognition of EpoB(Cy). While the construct containing Leu502 as the amino-terminal boundary was only sparingly soluble, the other two truncation sites produced protein in good yield.

The C-terminal hexahistidine fusion of Ser495 EpoB-(A−Ox−PCP) was then chosen for further analysis because it is more soluble than its Met498 counterpart, providing 20 and 11 mg/L of culture, respectively.

To test whether the three-domain 102 kDa fragment EpoB-(A−Ox−PCP) adopted the proper protein fold relative to the full-length module, first the A domain was assessed for its ability to activate L-cysteine to L-cysteinyl-AMP, as examined with the amino acid-dependent $^{32}PP_i$−ATP exchange reaction. The kinetic constants obtained for L-cysteine activation by the truncated EpoB construct ($K_m = 0.14 \pm 0.01$ mM, $k_{cat} = 41.0 \pm 0.7$ min^{-1}) were comparable to those obtained for the full-length system ($K_m = 0.46$ mM and $k_{cat} = 54.9$ min^{-1}) (*15*). Next, the native apo-PCP domain architecture was verified by phosphopantetheinylation using the surfactin phosphopantetheinyltransferase Sfp (*19*) and radiolabeled CoA substrate (data not shown). The loading of cysteinyl-AMP onto the terminal thiol of the phosphopantetheinyl arm of the holo-PCP domain was measured by A domain-dependent covalent attachment of [^{35}S]-L-cysteine to the PCP domain via TCA precipitation of the assay mixture. It was observed that transfer was complete in less than 20 min (data not shown). Finally, the purified protein possessed an absorption spectrum consistent with the presence of a flavin cofactor in the Ox domain, similar to full-length EpoB (*15*). Using these as benchmarks for the proper structure and activity of the three domains in EpoB(A−Ox−PCP), it was inferred that in the absence of the N-terminal Cy domain the remaining domains of EpoB were likely to have adopted their native conformations.

Heterocyclization Activity of EpoB(Cy). Evaluation of the free-standing EpoB(Cy) domain requires both the nucleophilic acceptor (cysteinyl-loaded A−Ox−PCP) as detailed above and the electrophilic acyl donor, in this case, the acetyl-*S*-ACP domain excised from the four-domain EpoA subunit (*15*). Transfer of the acyl moiety of acetyl-*S*-EpoA-(ACP) to [^{35}S]-L-cysteinyl-*S*-EpoB(A−Ox−PCP) would validate that the free-standing Cy domain recognizes both the ACP and A−Ox−PCP proteins in a three-protein complex as it catalyzes acetyl transfer. Initially, activity was monitored using a stoichiometric ratio of the three protein components (ACP, Cy, and A−Ox−PCP). The methylthiazolyl-*S*-PCP, detected by radio-HPLC as the free acid after base hydrolysis of the thioester linkage, was indeed readily generated under these conditions (Figure 4A), establishing the functional integrity of EpoB(Cy) in recognizing both the ACP donor and cysteinyl-*S*-EpoB(A−Ox−PCP) acceptor (Figure 2). Note that detection of the five-membered methylthiazole carboxylate confirms that the three reactions have been effected: acetyl transfer to the cysteinyl-*S*-A−Ox−PCP, cyclodehydration to methylthiazolinyl-*S*-PCP, and oxidation to the methylthiazolyl-*S*-PCP.

Reduction of the EpoB(Cy) concentration to substoichiometric ratios (54 nM) relative to the other two protein components (the ACP subunit was held at 3 μM and the A−Ox−PCP fragment at 6 μM) still provided a detectable amount of the methylthiazole product (Figure 4B). With the Cy domain as a catalyst at a level 50−100-fold lower than those of the ACP and A−Ox−PCP partners, it was then possible to obtain the kinetic constants for acetyl-*S*-EpoA-(ACP) and L-cysteinyl-*S*-EpoB(A−Ox−PCP) as substrates of EpoB(Cy). In time course analysis of methylthiazole formation, it became apparent that enzyme activity rapidly declined after 10 min with an inactivation half-life of 12.6 min (data not shown), and all measurements were taken within the first 10 min, when the rate of product formation was linear with respect to time. The cause for this decline is currently undetermined, and possibly could be attributed to loss of function in either EpoB(Cy) or the Ox domain of EpoB(A−Ox−PCP). On an initial attempt, acetyl-*S*-EpoA-(ACP) was held at 8.6 μM (160-fold higher than that of the Cy domain catalyst) while varying the concentration of the nucleophilic acceptor L-cysteinyl-*S*-EpoB(A−Ox−PCP) up to 30 μM. While a linear dependence on A−Ox−PCP fragment concentration was observed, saturation was not achieved (Figure 5); thus, kinetic data are reported in the k_{cat}/K_m regime (0.187 min^{-1} μM^{-1}). Holding the EpoB-

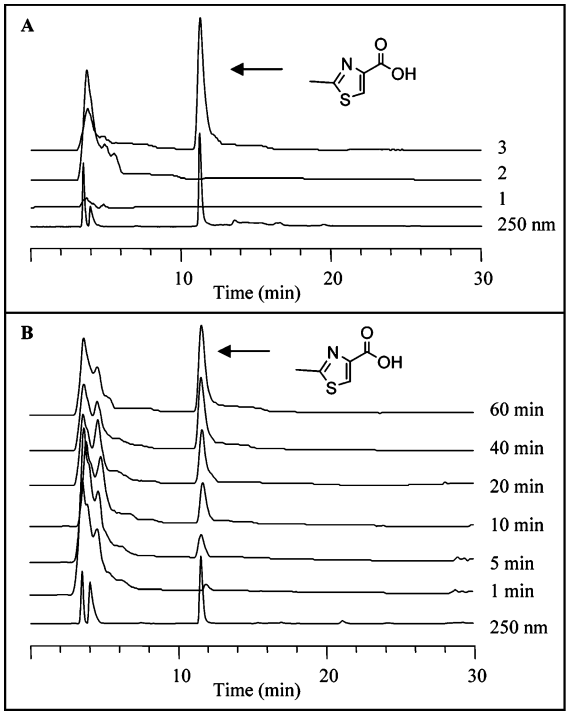

FIGURE 4: (A) ^{35}S radio-HPLC analysis of methylthiazole formation by acetyl-*S*-EpoA(ACP), EpoB(Cy), and EpoB(A−Ox−PCP). The absorbance of the nonradioactive standard of 2-methylmethylthiazolyl-4-carboxylic acid was monitored at 250 nm: (1) reaction mixture without ATP, (2) reaction mixture without EpoB(Cy), and (3) full reaction mixture. (B) Time course of methylthiazole formation by EpoB(Cy), acetyl-*S*-EpoA(ACP), and EpoB(A−Ox−PCP).

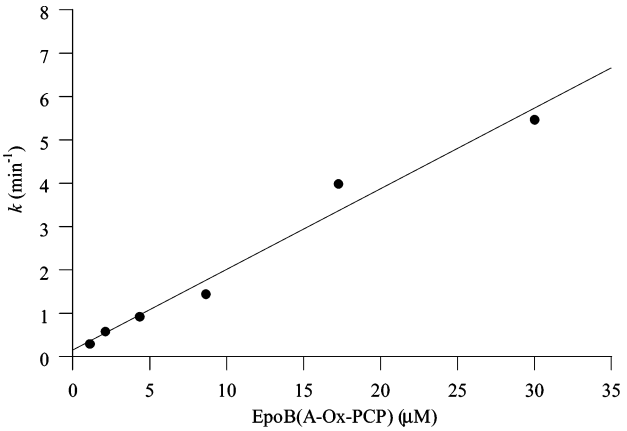

FIGURE 5: Substrate-dependent methylthiazole formation for EpoB-(Cy) and EpoB(A−Ox−PCP).

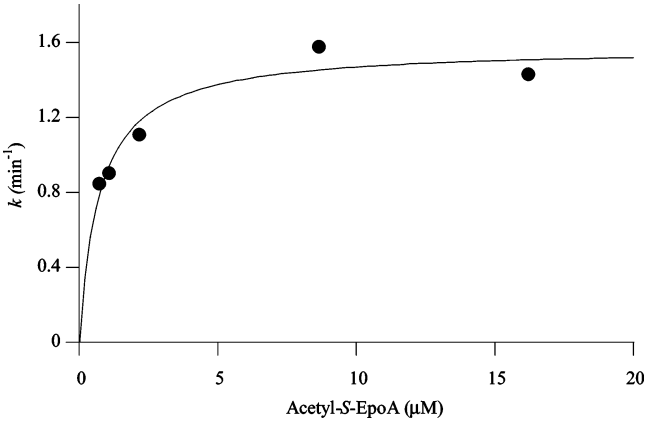

FIGURE 6: Michaelis−Menten analysis used to determine kinetic parameters for EpoB(Cy) with acetyl-*S*-EpoA(ACP).

(A−Ox−PCP) concentration at 6.5 μM did facilitate saturation conditions for the acetyl-*S*-EpoA(ACP) donor, enabling measurement of the apparent kinetic constants for acetyl-*S*-EpoA(ACP): $K_{m,app} = 730 \pm 180$ nM, $k_{cat,app} = 1.6 \pm 0.1$ min^{-1} (Figure 6).

The heterocyclization activity of EpoB(Cy) was also examined by the ability to utilize an alternate aminoacyl-*S*-NAC acceptor in the presence of acetyl-*S*-EpoA(ACP). Serine was chosen over the preferred EpoB substrate, cysteine, because of the likelihood of a transthioesterification side reaction of an L-cysteine-*S*-NAC. Previously, it was demonstrated that full-length EpoB activated L-serine in lieu of L-cysteine, ultimately producing the methyloxazole (*21*). Furthermore, the EpoB Ox domain, both as the excised protein and in full-length EpoB, readily accepted both thiazolinyl- and oxazolinyl-*S*-NAC substrates (*11*). Generation of the methyloxazole, however, was not detected under the assay conditions when EpoB(Cy) was presented with either L-Ser-*S*-NAC or *N*-acetyl-L-Ser-*S*-NAC (data not shown).

Analysis of EpoB(Cy) Site-Directed Mutants. To evaluate the EpoB(Cy) domain as a prototypic amide condensation/heterocyclization catalyst, a set of site-directed mutants was constructed to evaluate the effect of particular residues in thiazole formation. The Cy domain signature motif, DxxxxDxxS (*5*), was the initial target, and the D201A, D206N, and S209A site-directed mutants of EpoB(Cy) were prepared. While the D201A and S209A mutants were expressed with yields similar to that of the wild-type construct, the D206N mutant was significantly less soluble, and yielded a preparation with a purity of ~10% following nickel chelate chromatography, and no activity was detected in the protein extract. Both the D201A and S209A mutants produced the methylthiazole in the three-protein component reconstituted system, yet the rate was sufficiently diminished that initial rate conditions could not be measured due to detection limitations of the assay. Instead, we report a k_{obs} for the reaction. EpoB(Cy) S209A was 1 order of magnitude slower than the wild-type construct [0.055 ± 0.017 and 0.77 ± 0.23 min^{-1}, respectively (Table 3)]. The D201A variant was also noticeably impaired in activity relative to the wild type, providing a 100-fold slower k_{obs} at 0.0073 ± 0.0018 min^{-1} (Table 3).

In addition to the DxxxxDxxS motif, a recent analysis identified residues conserved in typical Cy domains, but absent in the second Cy domain of VibF, which was demonstrated to mediate only acylation, and lacks significant cyclodehydration activity (*22*, *23*). One of these conserved residues, Ser984 of the BacA Cy domain (corresponding to Ser418 of the EpoB Cy domain), was found to be essential for the cyclodehydration half of the reaction (*23*). An alignment of 17 known Cy domains (Figure 7) also revealed a conserved histidine residue, His122 of EpoB, not fully conserved in the Cy2 domains of vibriobactin and leinamycin synthetases. The S418A, S418N, and H122A mutants of EpoB(Cy) were therefore prepared. The solubility of the H122A mutant, similar to that observed for D206N, was diminished relative to those of the other constructs, making it difficult to obtain protein that was >10% pure following nickel chelate purification. However, it still facilitated production of the methylthiazole with a k_{obs} of 0.0019 ± 0.0007 min^{-1}, a greater than 300-fold reduction from the wild-type value (Table 3). The S418A variant was still capable of generating the methylthiazole with a k_{obs} of 0.042 ± 0.006 min^{-1}, a decrease of ~15-fold relative to that of the wild-type Cy domain. Although the methylthiazole could be detected with prolonged incubation with EpoB(Cy) S418N, the k_{obs} for the S418N mutant could not be measured due to the sensitivity limits of the assay (Table 3), allowing an estimate of <0.0034 min^{-1}.

Table 3: Methylthiazole Formation Rates

enzyme	k_{obs} (min^{-1})
wild-type EpoB(Cy)	0.77 ± 0.23
EpoB(Cy) H122A	0.0019 ± 0.0007
EpoB(Cy) D201A	0.0073 ± 0.0018
EpoB(Cy) D206N	not determined
EpoB(Cy) S209A	0.055 ± 0.017
EpoB(Cy) S418A	0.042 ± 0.006
EpoB(Cy) S418N	$<0.0034 \pm 0.0003$

DISCUSSION

In this work, we have demonstrated that the Cy domain of EpoB can be extracted from its native NRPS module and expressed as a soluble, functional 57 kDa fragment. Unlike a classical C domain, which is only responsible for amide bond formation, the Cy domain must retain integrity for catalysis of three distinct chemical steps: acylation of the downstream amino acid substrate by the upstream acyl-*S*-ACP donor, cyclization of the β-heteroatom-containing amino acid side chain, and dehydration to afford the dihydroaromatic heterocycle. For the reconstituted EpoB system, detection of the methylthiazole product resulting from oxidation of the intermediate methylthiazoline by the internal Ox domain of EpoB is used to infer competence of EpoB(Cy) for all three Cy domain-catalyzed reactions.

In addition to maintaining chemical fidelity, the free-standing EpoB(Cy) must successfully interact with two separate protein fragments. The association with the acyl-*S*-EpoA subunit is a naturally occurring in trans interface between the N-terminus of EpoB and the ACP domain of EpoA, and it was anticipated this association should not suffer following excision of EpoB(Cy). The EpoB N-terminal leader sequence and C-terminal residues of EpoA were shown, in large part, to direct protein−protein recognition between the upstream carrier protein and EpoB (*20*, *24*). Indeed, this is reflected in the $K_{m,app}$ for acyl-*S*-EpoA(ACP)

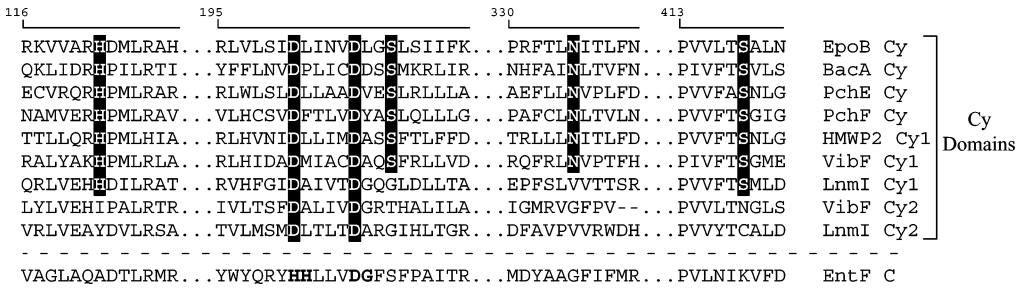

FIGURE 7: Alignment of selected regions of nine Cy domains. The numbering is that of the EpoB Cy domain sequence. Cy domains from epothilone synthetase B (EpoB), bacitracin synthetase A (BacA), pyochelin synthetase E (PchE), pyochelin synthetase F (PchF), yersiniabactin synthetase 2 (HMWP2), vibriobactin synthetase F (VibF), and leinamycin synthetase I (LnmI) are shown. The C domain of enterobactin synthetase (EntF) is also displayed. Conserved residues are highlighted.

of 0.73 μM, which is in good agreement with K_m values observed for other C domains and their respective acyl-*S*-carrier protein partners. The free-standing C domain and subunit of vibriobactin synthetase, VibH, exhibits a K_m of 0.88 μM for its upstream acyl donor, 2,3-DHB-*S*-VibH (*25*). Evaluation of the reconstituted enterobactin synthetase activity reveals that half-maximal velocity for the amino-terminal C domain of EntF is achieved at 0.73 μM acyl donor 2,3-DHB-*S*-EntB (*26*). Corresponding measurements are also observed for PKS subunit interactions: the K_D values between subunits of the pikromycin (PikAIII/PikAIV) and erythromycin (DEBS2/DEBS3) complexes are 95 nM and 1 μM, respectively (*27*, *28*). It therefore appears there is no detrimental effect upon the ability of EpoB(Cy) to bind its acyl donor substrate, acetyl-*S*-EpoA(ACP).

Dissection of the EpoB module and successful reconstitution of heterocyclization activity also rely upon the ability of the resulting free-standing Cy domain to recognize the A−Ox−PCP fragment, now presented artificially in trans. Although the two fragments retain sufficient affinity to support measurable methylthiazole formation, the observed absence of saturation kinetics with EpoB(Cy) and EpoB-(A−Ox−PCP) implies this recognition is impaired relative to the intact module where the Cy and A domains are tethered in cis. Excision of the EntF C domain comparably generates a C domain that is unable to recognize the remaining three domains (A−PCP−TE) of the EntF module (E. D. Roche and C. T. Walsh, unpublished results). The recently reported bisection of EntF supports a similar requirement for in cis interactions for the A and PCP domains contained in a naturally occurring subunit. The C−A and PCP−TE fragments of EntF did each retain structural and functional integrity, yet the EntF C−A fragment is rendered incapable of transferring the activated seryl-AMP to the severed carrier protein partner PCP-TE (*29*). In full-length EpoB, it is possible the Cy and PCP domains are joined in cis, presumably in a conformation that optimizes the proximity of these two domains for acetylation and cyclodehydration, precisely because they do not retain enough affinity if presented in trans. The enforced covalent tethering of the catalytic and carrier domains in NRPS and PKS subunits in assembly lines may overcome a lack of affinity that may have developed during evolution of interdomain boundaries to low-affinity linkers.

Relatively little is known with regard to the catalytic mechanism of the Cy domains. Secondary structure predictions indicate a similar overall fold for C and Cy domains, despite weak primary sequence similarity between the two (*30*). The C domains possess a highly conserved motif, HHxxxDG, wherein the aspartate is essential for both activity and the conformation of the active site (*8*, *31*, *32*). The second histidine residue, based on the similarity of C domains to the acetyltransferase superfamily, is thought to be the catalytic base required for deprotonation of the amine of the downstream aminoacyl-*S*-PCP to facilitate nucleophilic attack on the thioesterified upstream acyl donor (*33*). While mutation of this residue to alanine generally leads to diminished activity, the impact of this varies from negligible (*22*, *30*) to drastic (*8*, *22*, *31*, *32*), depending on the context of the specific C domain and the corresponding condensation reaction.

The HHxxxDG sequence element of the C domains has been directly replaced by a DxxxxDxxS motif at the corresponding location in the Cy domain homologues (*5*). The proposed similarity in the structure of the C and Cy domains, and the overlap of the respective sequence elements, imply a potential key role for those residues in Cy domains, as well. Although the second aspartate has been shown to be essential in all cases studied to date (*22*, *34*), the role of the first aspartate is less clear. Mutation of the first aspartate to alanine resulted in elimination of the activity in the first Cy domain of the yersiniabactin synthetase subunit HMWP2, while the comparable mutation in the Cy2 domain of the unusual vibriobactin NRPS module VibF diminished activity only 10-fold (*22*, *34*). Recent mutational studies with the BacA Cy domain (*23*) further implicated two residues with critical roles for the cyclodehydration half of the reaction sequence, N900 and S984 (numbering based on BacA sequence). The alanine variants of these two residues in BacA failed to generate the thiazoline, while retaining production of the acylated intermediate (*23*).

The importance of the residues comprising the DxxxxDxxS motif was examined in the extracted EpoB Cy domain. These mutants were initially introduced into the full-length EpoB protein; however, the solubility of several mutants suffered, preventing analysis of these proteins (data not shown). The serine residue of this motif is not strictly conserved among Cy domains, and the serine to alanine mutant in the HMWP2 Cy1 domain revealed no effect on heterocycle formation, having a rate nearly identical to that of the wild type (*34*). The corresponding mutation in EpoB(Cy), S209A, imparted a 10-fold decrease in activity, suggesting at best a small role in EpoB(Cy) catalysis. Mutation of the second of the conserved aspartate residues, D206, to asparagine resulted in both a substantial loss of both protein solubility and detectable heterocyclization ability, in agreement with earlier

studies identifying this as a critical residue for catalysis and likely for maintaining the proper fold for the protein (*8*, *22*, *34*). The importance of the first aspartate of the consensus motif was also examined through an alanine mutation. In this case, activity diminished 2 orders of magnitude, implicating this residue as critical, though not essential, for heterocycle formation. If this first conserved aspartate were to act as a catalytic base for deprotonation of the amine nucleophile, its importance appears to vary according to the synthetase and substrate in question, as observed for the second histidine of the C domain HHxxxDG motif.

In addition to mutants of the DxxxxDxxS consensus element, the cyclodehydration role of the serine residue corresponding to BacA S984 was examined in two mutants of the corresponding position in EpoB(Cy): S418A and S418N. Unlike BacA S984A, both variants of EpoB(Cy) yield the methylthiazole product in the reconstituted EpoB system, albeit at reduced rates: ~1 order of magnitude slower for S418A and at least 2 orders of magnitude slower for the S418N mutant. The corresponding S418N mutation in full-length EpoB provides a k_{cat} at 0.037 min^{-1} (data not shown), a reduction of at least 1700-fold relative to the rate estimated for full-length wild-type EpoB (k_{cat} estimated to be 60 min^{-1}) (S. E. O'Connor and C. T. Walsh, unpublished results). It is possible the asparagine side chain, larger than that of both the alanine mutant and the wild-type serine, is sufficient to disrupt the proper orientation of the β-heteroatom side chain and hinder Cy domain catalysis. The BacA S984A mutant examined by Duerfahrt et al. was constructed by fusion of the first two BacA modules to the thioesterase (TE) domain of tyrocidine synthetase C to facilitate multiple turnovers of product (*23*). While the BacA system certainly suggests an impairment of cyclodehydration activity for the S984A and N900A variants, it is possible hydrolysis of the thioester-bound acylated intermediate by the attached TE domain could mask any remaining cyclodehydration ability. Detailed structural studies of a Cy domain, coupled with site-directed mutagenesis, will be necessary before the roles of conserved Cy domain residues with condensation and cyclodehydration can be fully elucidated.

The activity of EpoB(Cy) reconstituted with EpoB(A−Ox−PCP) is measured through detection of the methylthiazole, the final product of the EpoB module, providing an overall measurement of the extent of thiazole ring formation. This includes not only the condensation and cyclodehydration reactions of the Cy domain but also oxidation of the thiazoline by the EpoB Ox domain. Precise assertions concerning the roles of these various residues with condensation or cyclodehydration will require quantification of all three intermediates of the reaction: *N*-acetyl-L-cysteine, the methylthiazoline, and the methylthiazole. Efforts to simultaneously detect both the methylthiazole and the condensation product, *N*-acetyl-L-cysteine, were unsuccessful. The dihydroaromatic thiazoline ring is susceptible to hydrolysis (*21*), further complicating analysis and obscuring detection of this intermediate following base-catalyzed cleavage of the methylthiazolinyl-*S*-PCP.

Cy domains are unusual in that they effect two separate reactions for the NRPS assembly line: acylation of the downstream amino acid by the upstream donor and subsequent cyclodehydration. The heterocycle, once formed, can meet with one of three fates. It can be incorporated unmodified in its current dihydroaromatic oxidation state. Alternatively, it can be oxidized by an oxidase (Ox) domain in cis or in trans to the heteroaromatic thiazole/oxazole (*15*, *35*), or the heterocycle can be reduced in trans by a discrete reductase to the tetrahydro thiazolidine/oxazolidine (*36*). The oxidation state of the ring can dictate the pharmacological properties of the metabolite: thiazolines and thiazolidines (as well as their oxygen-containing counterparts) are competent as iron(III) chelators (*37*), while the planar thiazole ring has lost metal chelating ability and often imparts useful structural restraints vital to the potency of an antibiotic or antitumor agent, such as bleomycin (*38*).

There has been much interest in engineering biosynthetic assembly lines to guide the biosynthesis of novel metabolites, and the PKS and NRPS machinery appears to be amenable to this approach (*39*, *40*). Already, the EpoB Ox domain has proven to be amenable to both excision from the full-length protein and insertion into a heterologous NRPS module (*11*, *21*). Here, we have examined the initial questions for portability for another of the EpoB domains, the Cy domain. Before addressing whether the domain can properly function in a foreign module, we must identify the domain boundaries and show that it can retain activity as a discrete protein. This investigation opens the door for further studies examining whether the Cy domain (and Cy−Ox combinations) can serve as a portable domain to be placed in a non-native module and direct heterocycle formation of a rationally designed metabolite where a hydrolyzable amide bond has been heterocyclized.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE analysis of purified wild-type EpoB(Cy) and EpoB(Cy) mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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