BlmIII and BlmIV Nonribosomal Peptide Synthetase-Catalyzed Biosynthesis of the Bleomycin Bithiazole Moiety Involving Both in Cis and in Trans Aminoacylation†

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ABSTRACT: Cloning and sequence analysis of the bleomycin (BLM) biosynthetic gene cluster predicted that the two nonribosomal peptide synthetases (NRPSs), BlmIV and BlmIII, are responsible for the biosynthesis of the BLM bithiazole moiety. BlmIV is a seven domain (C_2-A_2-PCP_2-Cy_1-A_1-PCP_1-Cy_0) NRPS, and BlmIII is a three domain (A_0-PCP_0-Ox) NRPS. The three domains of Cy_1-A_1-PCP_1 residing on the BlmIV subunit, the four domains of Cy_0 residing on the BlmIV subunit, and A_0-PCP_0-Ox residing on the BlmIII subunit constitute the two thiazole-forming NRPS-1 and NRPS-0 modules, respectively. BlmIII-A_0 was predicted to be nonfunctional, raising the question of how the NRPS-0 module activates and loads the Cys substrate to its cognate BlmIII-PCP_0. The NRPS-0 module consists of domains residing on two different subunits, requiring precise protein−protein interaction. Here, we report the production of the BlmIV and BlmIII NRPSs as an excised domain(s), module, or intact subunit form and biochemical characterizations of the resultant enzymes in vitro for their roles in BLM bithiazole biosynthesis. Our results (a) confirm that BlmIII-A_0 is a naturally occurring nonfunctional mutant, (b) demonstrate that BlmIV-A_1 activates Cys and catalyzes both in cis aminoacylation of BlmIV-PCP_1 (for NRPS-1) and in trans aminoacylation of BlmIII-PCP_0 (for NRPS-0), and (c) reveal that the C-terminus of the BlmIV subunit, characterized by the unprecedented AGHDDD(G) and PGHDDG repeats, is absolutely required for in trans aminoacylation of BlmIII-PCP_0. These findings underscore the flexibility and versatility of NRPSs in both structure and mechanism for natural product biosynthesis and provide an outstanding opportunity to study the molecular recognition and protein−protein interaction mechanism in NRPS assembly line enzymology.

The five-membered heterocycles, thiazole and oxazole as well as their reduced or oxidized variants, are common structural features of many biologically important natural products. The heterocycle-containing siderophores imbue a growth advantage to the microbial pathogens by sequestering iron from the iron-limited host environment (Figure 1A) (*1*−*3*). These molecules are widely distributed, including yersiniabactin from *Yersinia pestis*, pyochelin from *Pseudomonas aeruginosa*, acinetobactin from *Acinetobacter baumannii*, mycobactin from *Mycobacterium tuberculosis*, vibriobactin from *Vibrio cholerae*, and anguibactin from *Vibrio anguillarum*. The antitumor drug bleomycin (BLM)[1] from *Streptomyces verticillus* contains a bithiazole moiety that is believed to play an important role in DNA recognition when the drug exerts its oxidative DNA-cleavage activity (*4*). Other heterocycle-containing peptide or hybrid peptide-polyketide natural products include antitumor antibiotics such as epothilone from *Sorangium cellulosum* (*5*, *6*) and leinamycin from *Streptomyces atroolivaceus* (*7*, *8*), the electron transport inhibitor myxothiazol from *Stigmatella aurantiaca* (*9*), and antibacterial antibiotics such as bacitracin from *Bacillus licheniformis* (*10*) and pristinamycin IIB from *Streptomyces pristinaespiralis* (Figure 1B) (*11*).

Two mechanisms for heterocycle biosynthesis are known. One is exemplified by the microcin B17 synthetase that catalyzes the heterocycle-forming steps posttranslationally in microcin B17 biosynthesis (*12*, *13*), and the other is exemplified by nonribosomal peptide synthetases (NRPSs) that catalyze the heterocycle-forming steps processively in peptide and hybrid peptide-polyketide biosynthesis (*1*−*3*, *14*−*16*). NRPSs have a modular structure, employing an assembly line enzymology for nonribosomal peptide biosynthesis (*3*, *14*−*16*). A heterocycle-forming NRPS module minimally consists of three domains—(a) an adenylation (A) domain that recognizes and activates an amino acid (Cys, Ser, or Thr) as an aminoacyl adenylate, (b) a peptidyl carrier protein (PCP) domain that accepts the activated amino acid to form an aminoacyl thioester, and (c) a condensation/

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[1] Abbreviations: A, adenylation; ATP, adenosine triphosphate; BLM, bleomycin; CoA, coenzyme A; C, condensation; Cy, condensation/cyclization; DTT, dithiothreitol; MBP, maltose binding protein; NRPS, nonribosomal peptide synthetase; Ox, oxidation; P-pant, 4′-phosphopantetheine; PCP, peptidyl carrier protein; PKS, polyketide synthase; PPi, pyrophosphate; PPTase, 4′-phosphopantetheinyl transferase; TCA, trichloroacetic acid.

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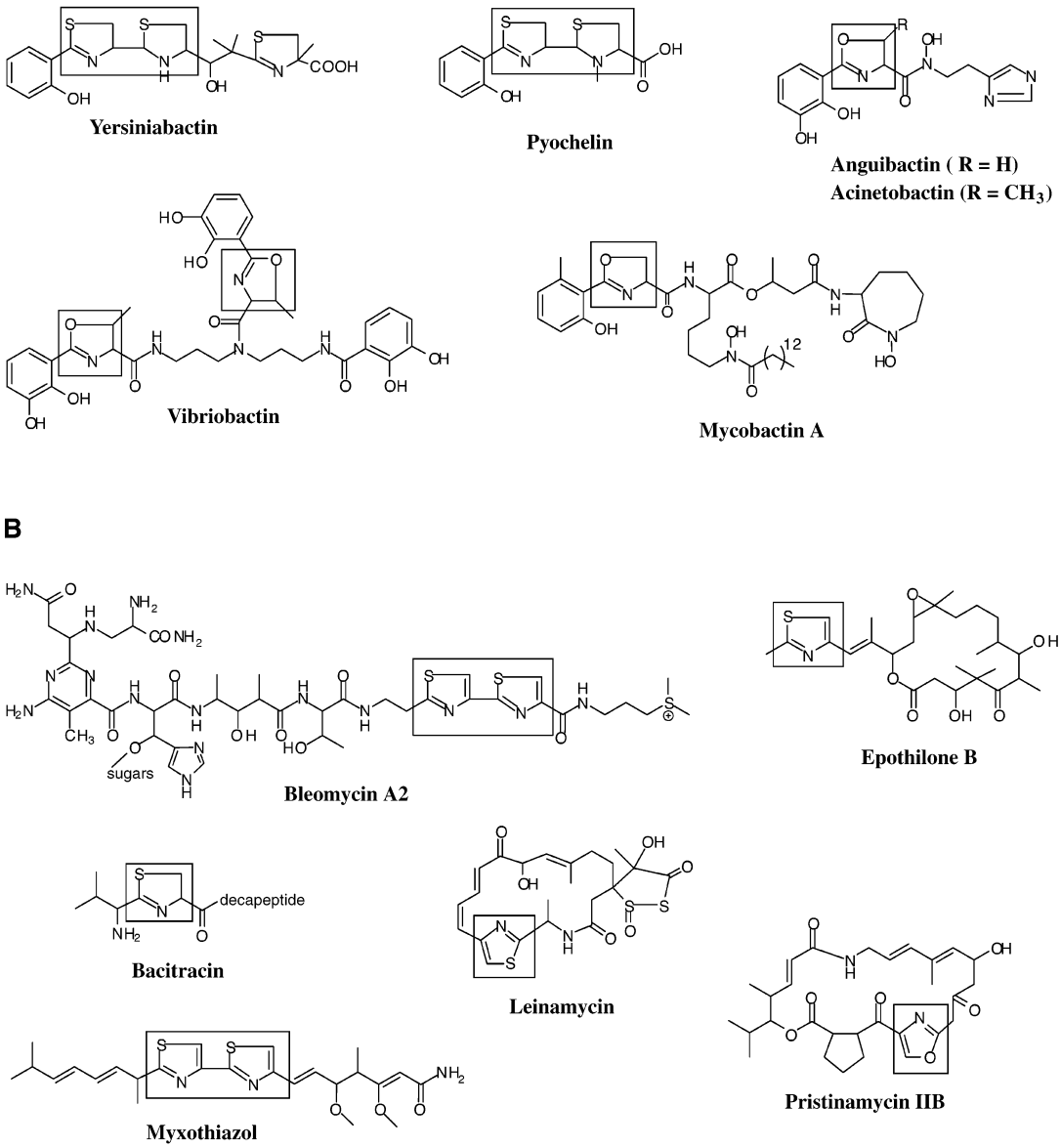

FIGURE 1: Examples of five-membered, heterocycle (boxed)-containing natural products: (A) siderophores and (B) antibiotics.

cyclization (Cy) domain that catalyzes peptide elongation and cyclodehydration to form a thiazoline (from Cys) or oxazoline (from Ser or Thr) intermediate. The PCP domain is posttranslationally modified by the 4′-phosphopantetheinyl transferase (PPTase) that catalyzes the covalent attachment of the 4′-phosphopanetheine (P-pant) moiety of coenzyme A (CoA) to the conserved Ser residue (*17*, *18*). The growing peptidyl intermediates remain covalently attached to PCP via a thioester linkage to the terminal thiol of the P-pant prosthetic group during the entire peptide biosynthesis process. The nascent thiazoline or oxazoline intermediate could be further elongated directly, leaving the thiazoline or oxazoline moiety in the final natural products as seen in yersiniabactin (*19*−*26*) and vibriobactin (*27*−*30*), or undergo oxidative or reductive modification prior to the subsequent cycle of chain elongation. Oxidative modification is catalyzed by the NRPS oxidase (Ox) domain that converts the thiazoline or oxazoline intermediate into a thiazole or an oxazole as seen in bleomycin (*31*−*33*), epothilone (*33*−*35*), and myxothiazol (*9*). Reductive modification converts the thiazoline or oxazoline intermediate into a thiazolidine or an oxazolidine as seen in pyochelin (*36*−*38*) and yersiniabactin (*19*−*26*), and as in the case of pyochelin, this modification appears to be catalyzed by a discrete reductase (*37*, *38*).

We have been studying the biosynthesis of antitumor drug BLM in *S. verticillus* (*31*−*33*, *39*−*42*). Feeding experiments with isotope-labeled precursors and isolation of biosynthetic intermediates suggested that the BLM aglycone is derived from nine amino acids, one acetate, and two methionines (*39*). Cloning and characterization of the *blm* biosynthetic gene cluster confirmed that BLM biosynthesis from the amino acid and carboxylic acid precursors proceeds according to the BLM hybrid NRPS-polyketide synthase (PKS) assembly line enzymology with the BlmIV and BlmIII NRPS subunits responsible for the biosynthesis of the BLM bithiazole moiety (*31*, *41*, *42*). The BlmIV and BlmIII subunits are characterized with seven- and three-NRPS domains, respectively, and together these domains constitute three NRPS modules, NRPS-2, NRPS-1, and NRPS-0 (Figure 2A). The NRPS-1 and NRPS-0 modules catalyze the activation and incorporation of two Cys residues into BLM

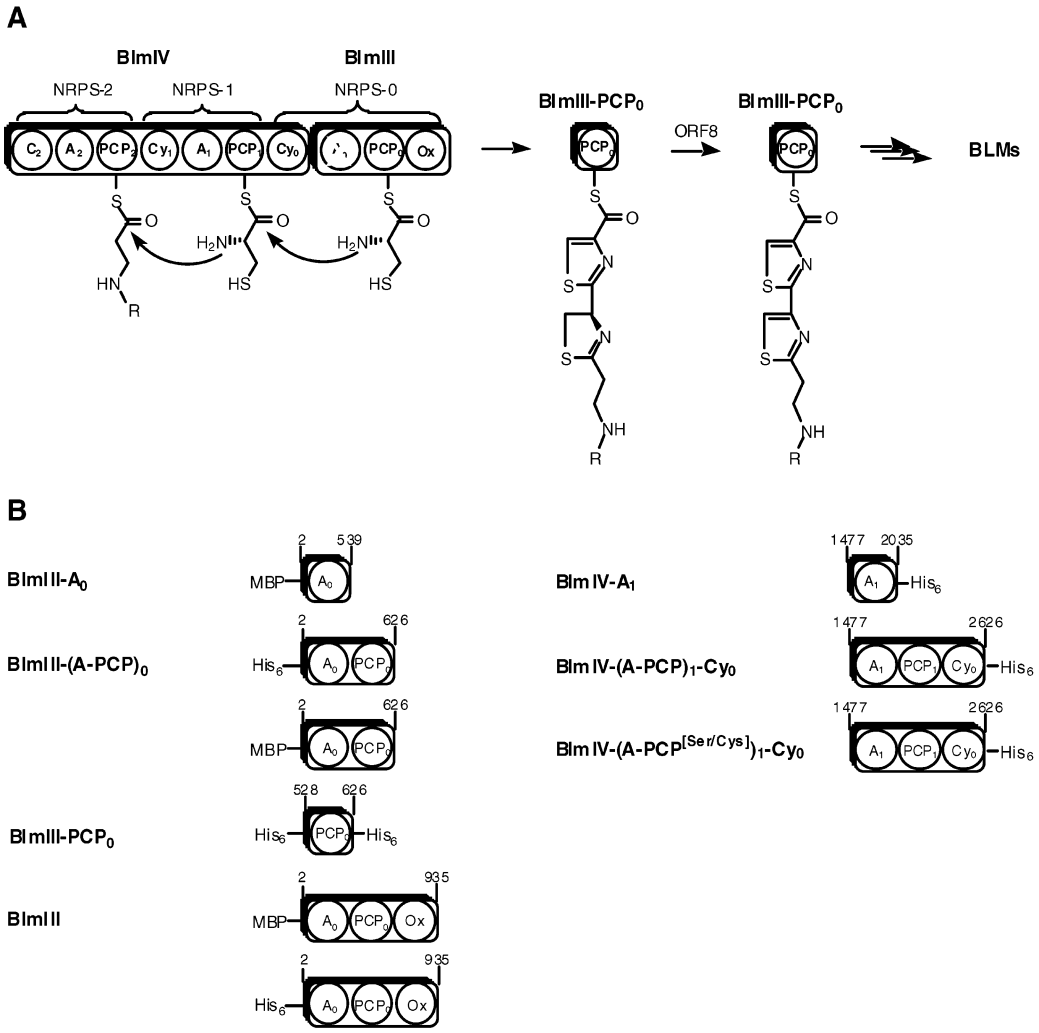

FIGURE 2: (A) Proposed mechanism for BlmIV and BlmIII NRPS-catalyzed biosynthesis of the BLM bithiazole moiety. (B) BlmIII and BlmIV domains, subunits, and mutants overproduced in and purified from *E. coli* and used in this study. The numbers shown above the rectangles indicate amino acid residues of the corresponding native subunits. A, adenylation; BLMs, bleomycins; C, condensation; Cy, condensation/cyclization; MBP, maltose binding protein; Ox, oxidation; PCP, peptidyl carrier protein.

and their subsequent cyclodehydration and regioselective oxidation to yield a thiazolinylthiazole intermediate that is further oxidized, most likely by a discrete oxidase such as ORF8, to afford the BLM bithiazole moiety (Figure 2A) (*31*, *41*, *42*). In this paper, we expressed *blmIII* and *blmIV* as an excised domain(s), module, or subunit in *Escherichia coli* and purified the resultant proteins for biochemical characterization in vitro (Figure 2B). Our results demonstrated that BlmIV-A_1 activates and loads Cys to both BlmIV-PCP_1 (in cis) and BlmIII-PCP_0 (in trans), and the latter in trans aminoacylation requires specific protein−protein interaction between the BlmIV and the BlmIII subunits. Demonstration and biochemical characterization of BlmIII catalyzed thiazoline-to-thiazole oxidation, using thiazolinyl-*S*-(*N*-acetyl)-cysteamine thioesters as surrogate substrates in vitro, can be seen in the accompanying paper (*33*).

EXPERIMENTAL PROCEDURES

General DNA Manipulations. Plasmid preparation and DNA extraction were carried out by using commercial kits (Qiagen, Santa Clarita, CA), and all other manipulations were carried out according to standard methods (*43*). *E. coli* DH5α strain was used as the host for general DNA propagations.

Overexpression of blmIII and blmIV as Excised Domain-(s) or Intact Subunit in E. coli. Two strategies were used to express the intact *blmIII* gene in *E. coli*. Overproduction of the BlmIII subunit as an N-terminal His_6-tagged fusion protein was accomplished in pBS15 as described previously (*32*). Alternatively, *blmIII* was moved as a 2.8-kb *Bam*HI/*Sal*I fragment from pBS15 and cloned into the same sites of pMAL-c2x (New England BioLabs) to yield pBS26. The latter resulted in the production of the BlmIII subunit as an N-terminal maltose binding protein (MBP) fusion protein.

To express *blmIII-A_0*, a 1614-bp fragment encoding the BlmIII-A_0 domain was amplified from *S. verticillus* ATCC 15003 genomic DNA by PCR using a forward primer of 5′-A TAT GGA TCC ACG AGC GCC CGG CCC ACG CCG ACA-3′ (*Bam*HI site underlined) and a reverse primer of 5′-GTC AAG CTT TCA GAG TTC GGC CTC GTC CCG CGC-3′ (*Hind*III site underlined). The resultant product was sequenced to confirm PCR fidelity and cloned into the *Bam*HI/*Hind*III sites of pMAL-c2x to yield pBS27. The latter resulted in the production of BlmIII-A_0 as an N-terminal MBP fusion protein.

To express *blmIII-(A-PCP)$_0$*, an 1875-bp fragment encoding the BlmIII-(A-$PCP)_0$ domains was amplified from *S.*

verticillus ATCC 15003 genomic DNA by PCR using the same forward primer as for *blmIII-A_0* and a reverse primer of 5′-GTC AAG CTT TCA TGC GAA CGC TAC GGA CGC GGG-3′ (*Hind*III site underlined). The resultant product was sequenced to confirm PCR fidelity and cloned into the *Bam*HI/*Hind*III sites of pET28a and pMAL-c2x to yield pBS28 and pBS29, respectively. pBS28 resulted in the production of BlmIII-(A-PCP)$_0$ as an N-terminal His$_6$-tagged fusion protein, while pBS29 resulted in the production of BlmIII-(A-PCP)$_0$ as an N-terminal MBP fusion protein.

To express *blmIII-PCP_0*, a 297-bp fragment was amplified from *S. verticillus* ATCC 15003 genomic DNA by PCR using a forward primer of 5′-CCG GAA TTC GCG GCG TCG GCA CCC GCG-3′ (*Eco*RI site underlined) and a reverse primer of 5′-GAA AAG CTT TGC GAA CGC TAC GGA CGC-3′ (*Hind*III site underlined). The resultant product was sequenced to confirm PCR fidelity and cloned into the *Eco*RI/*Hind*III sites of pET28a to yield pBS30. The latter resulted in the production of BlmIII-PCP$_0$ as a fusion protein with a His$_6$-tag at both the N- and the C-termini.

To express *blmIV-(A-PCP)$_1$-Cy_0*, a 0.9-kb fragment was amplified from an *Not*I subclone of the *blm* gene cluster, pBS31 that contains a 1.0-kb *Not*I fragment in pGEM11zf, by PCR using the M13 forward primer and 5′-GGC AAG CTT CTC CGG TCC ACC TCC CTC TC-3′ (*Hind*III site underlined) as the reverse primer. The resultant product was sequenced to confirm PCR fidelity and recovered as a 766-bp *Not*I/*Hind*III fragment. This fragment encodes the C-terminus of the intact BlmIV subunit and was cloned into the same sites of pANT841 (*44*) to yield pBS32. A 6229-bp *Xho*I/*Not*I fragment of the *blm* gene cluster, which encodes most of the BlmIV subunit except the N- and C-termini (*31*), was cloned into the same sites of pBS32 to yield pBS33, completing the C-terminus of the BlmIV subunit. We have previously constructed a *blmIV-A_1* expression vector in pET29a, pBS13 (*31*). To complete the *blmIV-(A-PCP)$_1$-Cy_0* expression construct, we finally moved a 2915-bp *Pst*I/*Hin*dIII fragment that contains the C-terminal portion of *blmIV-(A-PCP)$_1$-Cy_0* from pBS33 into the same site of pBS13 to yield pBS34. The latter resulted in the production of BlmIV-(A-PCP)$_1$-Cy$_0$ as a fusion protein with an S-tag at the N-terminus and a His$_6$-tag at the C-terminus, respectively.

Purification of the Overproduced BlmIII and BlmIV Subunits or Domains. *E. coli* BL21(DE3) (Novagen) or TB1 (BioLabs) strain was used for expression from pET-based or pMAL-based constructs, respectively. The standard conditions for cell growth and expression induction were followed as recommended by the manufacturers. If protein solubility became an issue, the incubation temperature was lowered to 16−22 °C, and the cells were grown overnight without IPTG induction (*23*). Soluble fractions of the overproduced proteins were purified by affinity chromatography on Ni-NTA resin (Qiagen) or amylose resin (BioLabs), depending on the fusion tag. The purified proteins were desalted on a PD-10 column (Pharmacia Biotech, Piscataway, NJ) into 50 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol and stored at − 80 °C for in vitro assays.

Construction and Overproduction of the BlmIV-(A-$PCP^{[Ser/Ala]}$)$_1$-Cy_0 and BlmIV-(A-$PCP^{[Ser/Cys]}$)$_1$-Cy_0 Mutants. Two BlmIV-PCP$_1$ mutants were generated by site-directed mutagenesis with the following pairs of primers 5′-C CTC GGC GGC ACC GCC CGG GTC GCG ATC-3′/3′-G GAG CCG CCG TGG CGG GCC CAG CGC TAG-5′ (for Ser-to-Ala mutation with the Ala codon underlined) and 5′-C CTC GGC GGC ACC TGC CGG GTC GCG ATC-3′/3′-G GAG CCG CCG TGG ACG GCC CAG CGC TAG-5′ (for Ser-to-Cys mutation with Cys codon underlined), respectively, using the QuickChange kit from Stratagene (La Jolla, CA) according to the manufacturer's instruction. The resultant mutant fragments, *blmIV-$PCP_1^{[Ser/Ala]}$* or *blmIV-$PCP_1^{[Ser/Cys]}$*, were cloned into pBS34 to replace the wild-type *blmIV-PCP_1* fragment to yielded pBS35 and pBS36, respectively. Introduction of pBS35 or pBS36 into *E. coli* BL21(DE3) resulted in the overproduction of BlmIV-(A-$PCP^{[Ser/Ala]}$)$_1$-Cy$_0$ or BlmIV-(A-$PCP^{[Ser/Cys]}$)$_1$-Cy$_0$, respectively, as fusion proteins with an S-tag at the N-terminus and a His$_6$-tag at the C-terminus. Although both mutants were moderately overproduced, BlmIV-(A-$PCP^{[Ser/Ala]}$)$_1$-Cy$_0$ was completely insoluble under all conditions tested. However, BlmIV-(A-$PCP^{[Ser/Cys]}$)$_1$-Cy$_0$ was partially soluble, and the soluble fraction was subsequently purified and used for in vitro assays.

In Vitro 4′-Phosphopantetheinylation of PCPs. Sfp was used for in vitro 4′-phosphopantetheinylation of all the PCP domains of the BlmIII and BlmIV enzymes before biochemical characterization (*17*, *18*). A typical assay solution (100 μL) contained 7.1 μM apo-protein, 1.5 μM Sfp, 25 μM [^{3}*H*-pantetheine]CoA (0.9 μCi, 40 Ci/mM), 10 mM $MgCl_2$, and 5 mM DTT in 75 mM Tris-HCl buffer, pH 8.0. After 30 min incubation at 37 °C, the assays were stopped by the addition of 0.9 mL of cold 10% (v/v) trichloroacetic acid (TCA). The precipitated proteins were collected by centrifugation at 14 000 rpm, 20 min, 4 °C; washed with 10% TCA three times; and resolved by SDS−PAGE. The ^{3}H-activity incorporated into holo-proteins was determined by autoradiography as described previously (*40*).

ATP-PPi Exchange Assay for BlmIII and BlmIV Subunits or Domains. Amino acid-dependent adenosine triphosphate (ATP)-pyrophosphate (PPi) assays were performed following the same procedures described previously (*40*). A typical assay solution (100 μL) contained 180 nM BlmIII or BlmIV enzyme, 1 mM ATP, 0.1 mM [^{32}*P*]PPi (0.2 μCi, 20 mCi/mmol; NEN Life Science Products, Inc., Boston, MA), 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM L-amino acid in 50 mM sodium phosphate buffer, pH 7.8. After 15 min incubation at 30 °C, the assays were stopped by addition of 0.9 mL of cold 1% (w/v) activated charcoal in 3% (v/v) perchloric acid. The precipitates were collected on glass fiber filters (2.4 cm, G-4, Fisher, Pittsburgh, PA); washed successively with 10 mL of 0.2 M sodium phosphate buffer, pH 8.0, 4 mL of water, and 1 mL of ethanol; and dried in air. The filters were mixed with 7 mL of scintillation fluid (ScintiSafe Gel, Fisher) and counted on a Beckman LS-6800 scintillation counter to determine the radioactivity.

Aminoacylation of BlmIII and BlmIV Subunits or Domains. The aminoacylation of BlmIII or BlmIV enzyme was carried out according to essentially the same procedure as previously described (*40*). A typical assay solution (100 μL) contained 0.5−7.1 mM BlmIII or BlmIV enzyme, 1.5 μM Sfp, 0.1 mM CoA, 2 mM ATP, 2 mM $MgCl_2$, and 0.1 μM L-[^{35}*S*]Cys (1.6 μCi, 160 Ci/mmol; NEN Life Science Products, Inc., Boston, MA) in 75 mM Tris-HCl buffer, pH 8.0. After 10 min

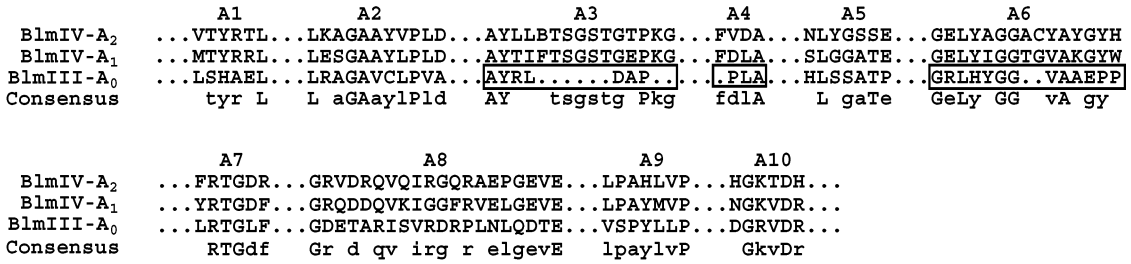

FIGURE 3: Comparison of the conserved motifs found in BlmIV-A_2, BlmIV-A_1, and BlmIII-A_0 showing that BlmIII-A_0 lacks several conserved motifs (boxed).

preincubation at room temperature, the assays were initiated by the addition of Cys and incubated at 37 °C for 30 min. The reactions were terminated by the addition of 0.9 mL of cold 7% (v/v) TCA. The precipitated proteins were collected by centrifugation at 14 000 rpm, 20 min, 4 °C and resolved by SDS−PAGE. The radioactivity incorporated was similarly determined by autoradiography as described above.

RESULTS

Domain Organization of the BlmIII and BlmIV Subunits. The *blmIV* and *blmIII* genes encode the last two NRPS subunits of the BLM NRPS/PKS/NRPS megasynthetase (*31*). BlmIV and BlmIII were predicted to contain seven (C_2-A_2-PCP_2-Cy_1-A_1-PCP_1-Cy_0) and three (A_0-PCP_0-Ox_0) domains, respectively, and together they constitute three NRPS modules of NRPS-2, NRPS-1, and NRPS-0 (Figure 2A). The BlmIII-A_0 domain lacks the highly conserved A3 motif, which is the ATP-binding site critical for adenylation of the amino acid substrate and exhibits poor sequence homology at several other conserved motifs (Figure 3) (*31*). Among the three PCP domains, BlmIV-PCP_2 and BlmIV-PCP_1 contain the typical core-VI motif of FFxLGGH(T)S at the conserved 4′-phosphopantetheinylation site, but BlmIII-PCP_0 deviates from this consensus by having a motif of LLDA-GATS (*31*). While the domains constituting modules NRPS-1 and NRPS-2 reside on the same subunit, BlmIV, the NRPS-0 module consists of domains residing on two separate subunits, Cy_0 on BlmVI and A_0, PCP_0, and Ox on BlmIII, a rare architecture among known NRPSs (Figure 2A) (*15*, *16*, *31*). Sequence analysis of the substrate binding pocket of the three A domains according to the so-called nonribosomal code predicted that these three NRPS modules are responsible for selecting, activating, and incorporating β-Ala, Cys, and Cys, respectively, into the bithiazole moiety of BLM during its biosynthesis (*31*).

Overproduction and Purification of the BlmIV and BlmIII Subunits or Domains. To study the biosynthesis of the bithiazole moiety in vitro, we overproduced BlmIV and BlmIII in *E. coli* as either intact subunit (for BlmIII) or excised domain(s) or module (for both BlmIII and BlmIV) (Figure 2B). The overproduction and purification of the His_6-tagged BlmIV-A_1 (pBS13, calculated molecular weight of 64 227 Da) and the His_6-tagged intact BlmIII (pBS15, calculated molecular weight of 101 020 Da) have been described previously (*31*, *32*). We also overproduced the BlmIII as a MBP fusion protein (pBS26, calculated molecular weight of 140 427 Da). The intact seven-domain BlmIV subunit was expressed with low yield but was completely insoluble. The di-domain BlmIV-$(A\text{-}PCP)_1$ was overproduced with a reasonable yield but in a totally insoluble form under all conditions tested; lower growth temperature, coexpression with GroEL/S (*45*), and change of expression hosts or vectors did not circumvent the problems. However, the tri-domain BlmIV-$(A\text{-}PCP)_1$-Cy_0, which includes the intact C-terminus of the BlmIV subunit, was overproduced (pBS34) and partially soluble. BlmIV-$(A\text{-}PCP)_1$-Cy_0 was purified by affinity chromatography using Ni-NTA column and migrated with the predicted size of 129 Da (calculated 129 260 Da) upon SDS−PAGE (Figure 4A).

For *blmIII-A_0* and *blmIII-$(A\text{-}PCP)_0$* expression, initial attempts were made in pQE60 and pET28a or pET29a to produce His_6-tagged fusion proteins. However, pQE-based constructs resulted in very poor expression, and pET-based constructs, such as pBS28 for producing BlmIII-$(A\text{-}PCP)_0$ as an N-terminal His_6-tagged fusion protein, while well-expressed, yielded essentially insoluble proteins. Subsequently, these two proteins were produced as an MBP-fusion protein as in pBS27 and pBS29, respectively. The MBP-fused proteins were partially soluble and could be purified by affinity chromatography using an amylose resin column. Upon SDS−PAGE, the purified MBP-BlmIII-A_0 and MBP-BlmIII-$(A\text{-}PCP)_0$ migrated with the expected size of 99 kDa (calculated 99 655 Da) and 108 kDa (calculated 108 721 Da), respectively (Figure 4B). The His_6-tagged BlmIII-PCP_0 (pBS30) was produced with a very high yield and mostly soluble. Upon SDS−PAGE, BlmIII-PCP_0 migrated with the expected size of 16 kDa (calculated 15 607 Da) (Figure 4C).

BlmIV-A_1 Activates Cys But BlmIII-A_0 Is a Naturally Occurring Nonfunctional Mutant. As demonstrated previously, BlmIV-A_1 specifically activated Cys among all the amino acids tested in the ATP-PPi exchange assay (*31*). When the purified BlmIII-A_0 was tested under the identical condition, no activation was observed from any amino acids tested, including Cys. This result is consistent with the sequence-based prediction that BlmIII-A_0 lacks the key motifs of a functional A domain, thereby being a naturally occurring nonfunctional mutant. A nonfunctional domain is very rare among all known NRPSs. While a nonfunctional condensation (C) domain has been reported for VibF (*28*), this is the first time to our knowledge that an A domain in NRPS is found to be nonfunctional because of natural mutations.

Both BlmIV-PCP_1 and BlmIII-PCP_0 Can Be 4′-Phosphopantetheinylated. The observation that BlmIII-A_0 is inactive prompted us to examine whether the adjacent BlmIII-PCP_0 domain is functional. When incubated with Sfp, a promiscuous PPTase (*17*, *18*), both BlmIII-$(A\text{-}PCP)_0$ and the intact BlmIII subunit were labeled by [^{3}*H*-pantetheine]CoA (Figure 5A, lanes 1 and 7). These results showed that BlmIII-PCP_0 is not affected by the presence of an adjacent nonfunctional

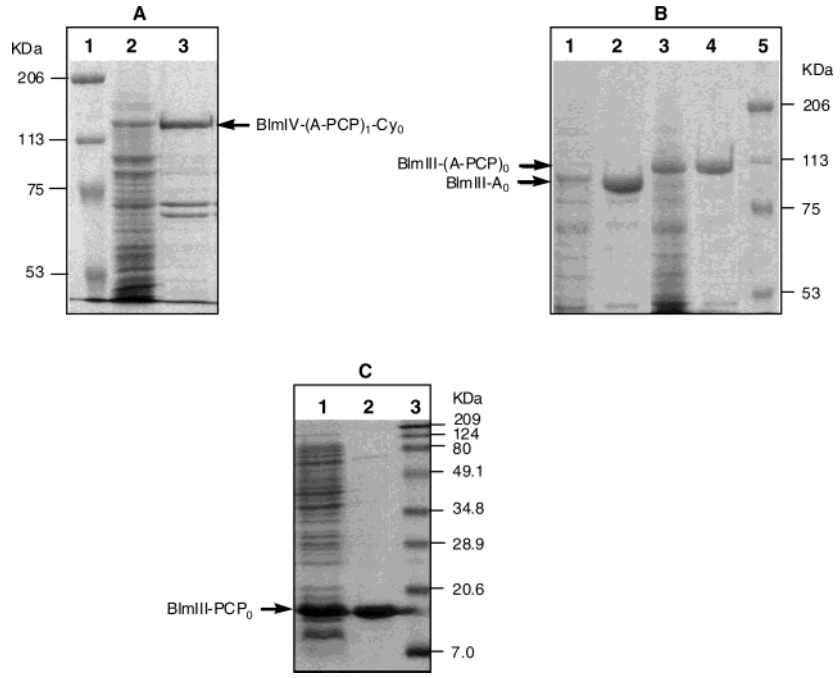

FIGURE 4: Overexpression and purification of BlmIV and BlmIII as excised domains. (A) BlmIV-(A-PCP)$_1$-Cy$_0$: lane 1, molecular weight markers; lane 2, total soluble proteins; and lane 3, Ni-NTA-purified protein. (B) BlmIII-(A)$_0$ and BlmIII-(A-PCP)$_0$: lane 1, total soluble proteins for BlmIII-A$_0$; lane 2, Ni-NTA purified BlmIII-A$_0$; lane 3, total soluble proteins for BlmIII-(A-PCP)$_0$; lane 4, Ni-NTA purified BlmIII-(A-PCP)$_0$; and lane 5, molecular weight markers. (C) BlmIII-PCP$_0$: lane 1, total soluble proteins; lane 2, Ni-NTA-purified protein; and lane 3, molecular weight markers.

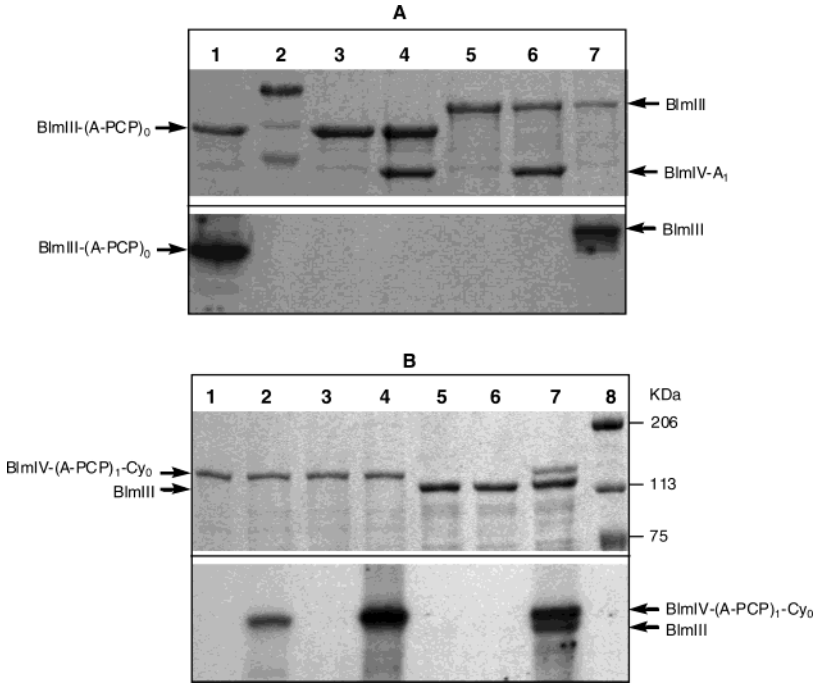

FIGURE 5: In vitro phosphopantetheinylation and aminoacylation of the BlmIII and BlmIV NRPSs. (A) Phosphopantetheinylation of BlmIII-(A-PCP)$_0$ and BlmIII by Sfp and in trans aminoacylation by BlmIV-A$_1$: lane 1, BlmIII-(A-PCP)$_0$, Sfp, [^{3}*H*-pantetheine]CoA; lane 2, molecular weight markers (206, 113, and 75 kDa); lane 3, BlmIII-(A-PCP)$_0$, Sfp, CoA, ATP, [^{35}*S*]Cys; lane 4, BlmIII-(A-PCP)$_0$, Sfp, CoA, ATP, [^{35}*S*]Cys, BlmIV-A$_1$; lane 5, BlmIII, Sfp, CoA, ATP, [^{35}*S*-Cys]; lane 6, BlmIII, Sfp, CoA, ATP, [^{35}*S*]Cys, BlmIV-A$_1$; and lane 7, BlmIII, Sfp, [^{3}*H*-pantetheine]CoA. (B) In trans aminoacylation of the intact BlmIII subunit by BlmIV-(A-PCP)$_1$-Cy$_0$: lane 1, BlmIV-(A-PCP)$_1$-Cy$_0$, [^{3}*H*-pantetheine]CoA; lane 2, BlmIV-(A-PCP)$_1$-Cy$_0$, [^{3}*H*-pantetheine]CoA, Sfp; lane 3, BlmIV-(A-PCP)$_1$-Cy$_0$, Sfp, CoA, [^{35}*S*]Cys; lane 4, BlmIV-(A-PCP)$_1$-Cy$_0$, Sfp, CoA, [^{35}*S*]Cys, ATP; lane 5, BlmIII, Sfp, CoA, [^{35}*S*]Cys; lane 6, BlmIII, Sfp, CoA, [^{35}*S*]Cys, ATP; lane 7, BlmIII, Sfp, CoA, [^{35}*S*]Cys, ATP, BlmIV-(A-PCP)$_1$-Cy$_0$; and lane 8, molecular weight markers. The upper panels of each figure are SDS−PAGEs, and the lower panels are the corresponding autoradiograms.

BlmIII-A$_0$. The successful phosphopantetheinylation of both BlmIII-(A-PCP)$_0$ and the intact BlmIII subunit also suggested that the inability for BlmIII to activate Cys is unlikely to be due to an improper folding of the enzyme upon overexpression in the hetelogous host but rather because of the structural defect in BlmIII-A$_0$, a finding that is consistent with its lack

of several core motifs conserved among all known A domains (Figure 3) (*31*). BlmIV-(A-PCP)$_1$-Cy$_0$ could also be efficiently phosphopantetheinylated with [^{3}*H*-pantetheine]CoA by Sfp as evidenced by the autoradiogram (Figure 5B, lanes 1 and 2), suggesting that BlmIV-(A-PCP)$_1$-Cy$_0$ has also been overproduced in the functional form.

Neither BlmIII-A$_0$ nor BlmIV-A$_1$ Alone Could Load Cys to BlmIII-PCP$_0$. Two Cys residues are incorporated into the peptide backbone, which are cyclized, dehydrated, and oxidized to form the bithiazole moiety of BLM (*31*−*33*, *39*, *41*, *42*). Incubation of either BlmIII-(A-PCP)$_0$ or the intact BlmIII subunit (both as MBP-fusion proteins) with [^{35}*S*]Cys in the presence of CoA, Sfp, and ATP, however, failed to load Cys to the BlmIII-PCP$_0$ domain as evidenced by the autoradiogram (Figure 5A, lanes 3 and 5). To exclude the possibility that the MBP moiety might interfere with the assay, the same assays were repeated with the His$_6$-tagged version of BlmIII-(A-PCP)$_0$ or the intact BlmIII subunit. Again, no Cys-loading was detected upon autoradiogram detection (Figure 5B, lanes 5 and 6, shown data only for the intact BlmIII subunit). Thus, amino acid activation (as tested by the ATP-PPi exchange assay) and aminoacylation (as tested by Cys-loading to PCP) confirmed that BlmIII-A$_0$ lacks both activities characteristic to a functional A domain and is incapable of activating and loading Cys to its cognate PCP. Therefore, there must be an alternative mechanism for Cys-loading to BlmIII-PCP$_0$. Given the fact that BlmIV-A$_1$ is known to activate and load Cys to its cognate BlmIV-PCP$_1$, BlmIV-A$_1$ serves as the most likely candidate for this reaction, should it be able to recognize and communicate to the BlmIII-PCP$_0$ domain in trans. To test this hypothesis, BlmIII-(A-PCP)$_0$ or the intact BlmIII subunit were incubated with BlmIV-A$_1$ in the presence of [^{35}*S*]Cys. However, no Cys-loading to either BlmIII-(A-PCP)$_0$ or the intact BlmIII subunit was observed as evidenced upon autoradiogram analysis (Figure 5A, lanes 4 and 6). The latter results suggested that the BlmIII-A$_1$ domain in the excised form is unable to catalyze in trans Cys-loading to BlmIII-PCP$_0$.

BlmIV-(A-PCP)$_1$-Cy$_0$ Catalyzes Both in Cis Aminoacylation of Its Cognate BlmIV-PCP$_1$ Domain and in Trans Aminoacylation of the BlmIII-PCP$_0$ Domain of the BlmIII Subunit. Since BlmIII-A$_0$ was nonfunctional and BlmIV-A$_1$ alone cannot load Cys to BlmIII, yet BlmIII-A$_0$ and BlmIV-A$_1$ are the only two domains identified within the *blm* cluster that could possibly activate Cys, we reasoned that there might be critical protein−protein recognition elements missing while the BlmIV or BlmIII domain was assayed in its excised form. To test this hypothesis, we overproduced a three-domain protein, BlmIV-(A-PCP)$_1$-Cy$_0$, which has the intact C-terminus of the native BlmIV subunit. Upon incubating with Sfp, CoA, ATP, and [^{35}*S*]Cys, BlmIV-(A-PCP)$_1$-Cy$_0$ was specifically and efficiently labeled by [^{35}*S*]Cys as evidenced by autoradogram (Figure 5B, lanes 3 and 4). The latter results confirmed that BlmIV-(A-PCP)$_1$-Cy$_0$ is competent for in cis aminoacylation of the cognate BlmIV-PCP$_1$ domain, as predicted previously on the basis of sequence analysis (*31*). We then carried out the assays under the identical conditions but with the addition of the intact BlmIII subunit. Specific and efficient cysteinylation was observed, as evidenced by the autoradiogram, not only for BlmIV-(A-PCP)$_1$-Cy$_0$ but also for the BlmIII subunit (Figure 5B, lane 7). These data unambiguously demonstrated that BlmIV-(A-PCP)$_1$-Cy$_0$ indeed catalyzed Cys-loading of the BlmIII subunit, and in order for this in trans aminoacylation to occur, the intact C-terminus of the BlmIV-(A-PCP)$_1$-Cy$_0$ subunit is required.

Cysteinylation of BlmIII-PCP$_0$ Was Catalyzed by the BlmIV-A$_1$ Domain Directly Rather than Involving the BlmIV-Cy$_0$ Domain of the BlmIV-(A-PCP)$_1$-Cy$_0$ Subunit. BlmIV-(A-PCP)$_1$-Cy$_0$ contains not only the native C-terminus of the BlmIV subunit but also the BlmIV-Cy$_0$ domain that, together with the BlmIII subunit, constitutes the NRPS-0 module (Figure 2A). Thus, there might be two possibilities to account for BlmIV-(A-PCP)$_1$-Cy$_0$-catalyzed loading of Cys to the BlmIII subunit. BlmIV-A$_1$ could activate and load Cys directly to BlmIII-PCP$_0$, and this would be in a mechanistic analogy to the yersiniabactin synthetases, in which the HMWP2-A$_{cys}$ domain activates and loads Cys to all three PCPs of HMWP2-PCP$_1$, HMWP2-PCP$_2$, and HMWP1-PCP$_3$ (*20*, *21*). Alternatively, after BlmIV-A$_1$ activates and loads Cys to BlmIV-PCP$_1$, BlmIV-Cy$_0$ could act as an acyltransferase and transfer Cys from BlmIV-PCP$_1$ to BlmIII-PCP$_0$, and this would be consistent with the sequence similarity between C domains and acyltransferases (*15*) and the recent finding of VibF whose Cy domains act as aminoacyltransferase in vibriobactin biosynthesis (*28*). To distinguish these two possibilities, we mutated the PCP domain of BlmIV-(A-PCP)$_1$-Cy$_0$ by changing the P-pant attachment site Ser to Ala or Cys. However, only the Ser-to-Cys mutant was partially soluble that was subsequently purified and used in this study. Upon incubation with [^{35}*S*]Cys, Sfp, CoA, and ATP, the BlmIV-(A-PCP$^{[Ser/Cys]}$)$_1$-Cy$_0$ mutant indeed lost its ability of self-cysteinylation, as would be expected for a nonfunctional BlmIV-PCP$_1$ domain, but retained its ability to cysteinylate the BlmIII subunit. These results excluded the participation of BlmIV-Cy$_0$ in Cys-loading to BlmIII and supported the proposal that BlmIV-A1 activates and loads Cys directly to the BlmIII subunit. A similar conclusion has been reached previously for the yersiniabactin synthetases—introduction of a Ser-to-Ala mutation to HMWP2-PCP$_1$ had no effect on HMWP2-A$_{cys}$'s ability to cysteinylate both NMWP2-PCP$_2$ and HMWP1-PCP$_3$ (*23*).

Intact Termini of the BlmIII Subunit Are not Required for the in Trans Aminoacylation by BlmIV-(A-PCP)$_1$-Cy$_0$. The importance of the intact C-terminus of the BlmIV subunit in the in trans aminoacylation of the BlmIII subunit prompted us to explore whether the terminal sequences of the BlmIII subunit are also critical in this reaction. When the Ox domain was removed from BlmIII, BlmIII-(A-PCP)$_0$ was efficiently cysteinylated by BlmIV-(A-PCP)$_1$-Cy$_0$ (Figure 6A). This suggests that the C-terminus of the BlmIII subunit is not absolutely essential in the in trans aminoacylation by BlmIV. Furthermore, when both the N-terminal domain (A$_0$) and the C-terminal domain (Ox) of BlmIII were removed, the resulted protein, BlmIII-PCP$_0$, was still able to serve as a substrate for BlmIV-(A-PCP)$_1$-Cy$_0$ (Figure 6B). These results showed that both termini of the BlmIII subunit are unlikely to play a critical role in the in trans aminoacylation by the BlmIV subunit. This is also consistent with the fact that either the intact BlmIII subunit or its various combinations of domains were functional in the in trans aminoacylation assay, although they were expressed as fusion proteins with either the His$_6$-tag or the MBP at the N- or C-terminus (Figure 2).

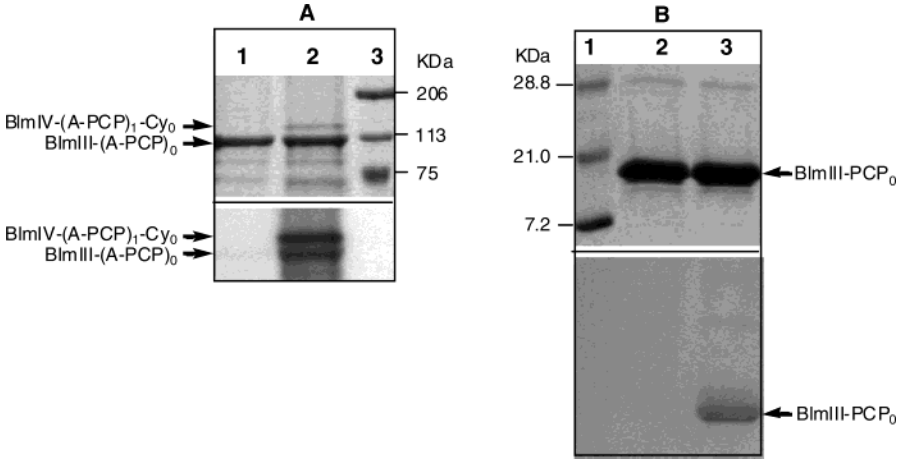

FIGURE 6: In trans aminoacylation of the excised or heterologously fused BlmIII NRPSs by BlmIV-(A-PCP)$_1$-Cy$_0$. (A) BlmIII-(A-PCP)$_0$: lane 1, BlmIII-(A-PCP)$_0$, Sfp, CoA, ATP, [^{35}S]Cys; lane 2, BlmIII-(A-PCP)$_0$, Sfp, CoA, ATP, [^{35}S]Cys, BlmIV-(A-PCP)$_1$-Cy$_0$; and lane 3, molecular weight markers. (B) BlmIII-PCP$_0$: lane 1, molecular weight markers; lane 2, BlmIII-PCP$_0$, Sfp, CoA, [^{35}S]Cys, BlmIV-(A-PCP)$_1$-Cy$_0$; and lane 3, BlmIII-PCP$_0$, Sfp, CoA, [^{35}S]Cys, BlmIV-(A-PCP)$_1$-Cy$_0$, ATP. The upper panels of each figure are SDS−PAGEs, and the lower panels are the corresponding autoradiograms.

DISCUSSION

The anticancer drug BLMs are a family of glycopeptides differing structurally at the C-terminal amines, and the BLM hybrid peptide/polyketide/peptide backbone is characterized with a bithiazole moiety. This moiety provides the majority of the BLM-DNA affinity and may contribute to polynucleotide recognition and DNA cleavage selectivity (*4*). The knowledge of biosynthetic insights into this moiety is of fundamental importance to future genetic manipulation of the BLM hybrid NRPS/PKS/NRPS megasynthetase to develop novel BLM congeners with better clinical efficacy and lower toxicity. Here, we characterized the mechanism of how the BlmIV and BlmIII NRPS subunits activate and load the Cys substrates onto the BLM megasynthetase to synthesize the BLM biathiazole moiety. A single A domain, BlmIV-A$_1$ residing on the BlmIV subunit, is responsible for the activation and loading of Cys to two PCP domains, in cis for BlmIV-PCP$_1$ residing on the same subunit as BlmIV-A$_1$ and in trans for BlmIII-PCP$_0$ residing on a separate subunit of BlmIII, and the in trans aminoacylation is necessary because of natural mutations of the BlmIII-A$_0$ domain that render it to be nonfunctional.

We used intact subunit or excised domain(s) or module of the BlmIV and BlmIII NRPSs to characterize BLM bithiazole biosynthesis in vitro. These proteins were overproduced in and purified from *E. coli*. The expression of *blm* NRPS subunits or domains in *E. coli* has proven to be very difficult (*18*, *31*−*33*, *40*−*42*), and frequently the recombinant proteins were either produced in very low yields or totally insoluble. By using such conditions as coexpression with GroEL/S (*45*) or prolonged growth of cells at very low temperature (16 °C) without IPTG induction (*23*), we were able to produce and purify enough amounts of active proteins for the current studies.

Sequence analysis of the *blm* biosynthetic gene cluster had previously predicted that BlmIII-A$_0$ might be nonfunctional because of its lack of several highly conserved motifs, especially the ATP-binding motif A3 (Figure 3) (*31*). This has now been confirmed biochemically by both the Cys-dependent ATP-PPi exchange assay and the aminoacylation of the BlmIII-PCP$_0$ domain—BlmIII-A$_0$ could neither activate nor load Cys to the BlmIII-PCP$_0$ domain. Since the PCP$_0$ domain of the BlmIII subunit could be 4′-phosphopantetheinylated by Sfp and the Ox domain of the BlmIII subunit is functional in catalyzing thiazoline-to-thiazole oxidation (*32*, *33*), it is unlikely that the lack of Cys activation and aminoacylation activities of the BlmIII subunit resulted from its misfolding upon expression in the heterologous host. Therefore, BlmIII-A$_0$ represents the first characterized naturally occurring nonfunctional A domain.

The inability of BlmIII-A$_0$ to activate and load Cys to its cognate BlmIII-PCP$_0$ led us to search for an alternative mechanism to load Cys to the NRPS-0 module. BlmIV-A$_1$ is an apparent candidate since it is fully functional as evidenced in both the Cys-dependent ATP-PPi exchange assay and cysteinylation of its cognate BlmIV-PCP$_1$. However, our data showed that the excised BlmIV-A$_1$ alone, while functional in activating Cys, could not transfer the resultant cysteinyl adenylate onto BlmIII-PCP$_0$. Since it is known in PKS that linkers, residing at both N- and C-termini of the interacting subunits, play a critical role in both subunit recognition and communications and intermediate channeling along the megasynthase assembly line (*46*), we reasoned that the intact C-terminus of the BlmIV subunit might be essential for BlmIV-A$_1$ to interact with the BlmIII subunit. Using a three-domain protein, BlmIV-(A-PCP)$_1$-Cy$_0$ that contains the native C-terminus of the BlmIV subunit, we demonstrated that BlmIV-A$_1$ can efficiently cysteinylate the BlmIII subunit in trans. Introduction of a Ser-to-Cys mutation into the conserved phosphopantetheinylation site of BlmIV-PCP$_1$ afforded the BlmIV-(A-PCP$^{[Ser/Cys]}$)$_1$-Cy$_0$ mutant. While the latter is nonfunctional for in cis cysteinylation as would be expected for an apo-BlmIV-PCP$_1$, it retains its in trans cysteinylation activity to BlmIII, excluding the involvement of BlmIV-Cy$_0$ in the BlmIV-(A-PCP)$_1$-Cy$_0$-catalyzed loading of Cys to BlmIII-PCP$_0$, via cysteinyl-*S*-BlmIV-PCP$_1$ as an intermediate. Taken together, these results have at least three implications. First, it demonstrated that the BlmIV-A$_1$ domain of the BlmIV subunit is responsible for cysteinylation of both the BlmIV-PCP$_1$ (the NRPS-1 module) and the BlmIII-PCP$_0$ (the NRPS-0 module) domains, accounting for the missing activity of the NRPS-0 module because of its nonfunctional

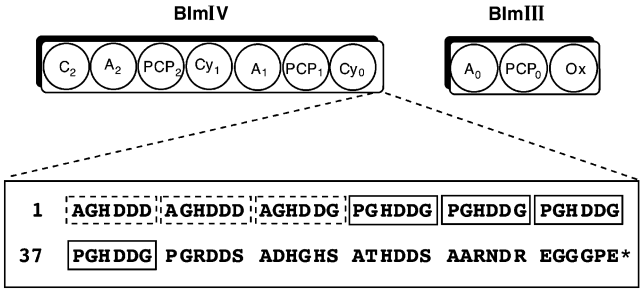

FIGURE 7: C-terminal amino acid sequence of the BlmIV subunit with the AGHDDD(G) and PGHDDG repeats boxed in broken- and solid-line, respectively.

BlmIII-A_0 domain. Second, the BlmIV-A_1 domain directly loads Cys to BlmIII-PCP_0 without the participation of the BlmIV-PCP_1 domain, and the intact C-terminus of the BlmIV subunit is required for this in trans cysteinylation, facilitating specific protein−protein recognition and communication. Third, it demonstrated once again the flexibility both in structure and mechanism for NRPSs to catalyze natural product biosynthesis. The modular organization of NRPSs, consisting of functionally independent domains, provides the structural basis for such flexibility. When a mutation occurred and rendered a domain catalytically inactive, a functionally equivalent domain residing in the neighboring module could compensate the lost activity in trans. However, to do so efficiently, a specific protein−protein interaction is required as has been demonstrated here for the BlmIV and BlmIII subunits.

The BlmIV subunit has a unique C-terminus that might play a critical role in protein−protein interaction, facilitating the in trans aminoacylation. The 72-amino acid C-terminus following the BlmIV-Cy_0 domain is highly rich in acidic (22 D and 2 E) and basic (10 H and 3 R) amino acids (Figure 7). Strikingly, this sequence has three repeats of AGHDDD-(G) and four perfect repeats of PGHDDG. Database searches did not reveal any possible function of these repeats, and we propose that they represent a new motif for protein−protein recognition and interaction. In contrast, no noticeable feature could be identified for the termini of the BlmIII subunit. In fact, neither the C- nor the N-terminus of the BlmIII subunit was essential for the in trans aminoacylation, and the excised BlmIII-PCP_0 domain lacking either the N-, the C-, or both termini were all cysteinylated in trans by BlmIV-$(A\text{-}PCP)_1$-Cy_0. It should be noted that there might be a kinetic difference in BlmIV-$(A\text{-}PCP)_1$-Cy_0-catalyzed in trans cysteinylation of BlmIII-PCP_0 when the latter was presented within the intact subunit or as various excised forms. However, protein−protein interactions clearly exist between the subunits without which the in trans aminoacylation is not possible.

The NRPS-1 and NRPS-0 modules have been proposed to be responsible for the biosynthesis of the BLM bithiazole moiety. After BlmIV-A_1 loads the two Cys substrates to BlmIV-PCP_1 and BlmIII-PCP_0, the two Cy domains, BlmIV-Cy_1 and BlmIV-Cy_0, presumably catalyze the condensation and cyclodehydration to form a thiazoline intermediate that is finally oxidized to afford the characteristic bithiazole moiety of BLM (Figure 2A). Among all heterocycle-forming NRPS modules known to date, the Cy domain invariably resides on the same subunit with its cognate A and PCP domains in the order of Cy-A-PCP as exemplified by those for the biosynthesis of yersiniabactin (*19*−*26*), leinamycin (*7*, *8*), epothilone (*5*, *6*, *33*−*35*), bacitracin (*10*), and myxothiazol (*9*). While this architectural feature is kept for the NRPS-1 module, it is not true for the NRPS-0 module whose Cy (BlmIV-Cy_0) and A, PCP, and Ox (BlmIII) domains reside on two physically separated subunits (Figure 2A). This suggests that while the NRPS-1 module catalyzes in cis condensation and cyclodehydration steps, NRPS-0 has to do so in trans, requiring precise protein−protein interaction between the BlmIV and the BlmIII subunits. Furthermore, only one Ox domain has been identified for the two thiazole-forming NRPS modules (*31*, *32*), the involvement of which in catalyzing the thiazoline-to-thiazole oxidation has only been demonstrated for the second (C-terminal) thiazole of BLM (*33*). A discrete oxidase, ORF8, was proposed to catalyze the thiazoline-to-thiazole oxidation for the first thiazole of BLM (*31*−*33*, *39*, *41*, *42*). Therefore, the formation of the BLM bithiazole moiety is also likely to involve both in cis (NRPS-0 for the second thiazole) and in trans (between NRPS-1 and ORF8 for the first thiazole) oxidation. While deviations from the archetype model of NRPS featuring the collinearity between domain and modular organization and the structure of the resultant products are constantly emerging (*15*, *16*), BLM bithiazole biosynthesis is certainly exceptional both in NRPS structure and mechanism, employing both in cis and in trans steps for substrate activation and loading, condensation and cyclodehydration, and oxidation. These findings provide an outstanding opportunity to study the molecular recognition and protein−protein interaction mechanism in assembly line enzymology and underscore once again the flexibility and versatility of NRPS in combinatorial biosynthesis for natural product structural diversity.

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