

## Oxidase Domains in Epothilone and Bleomycin Biosynthesis: Thiazoline to Thiazole Oxidation during Chain Elongation<sup>†</sup>

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Received May 14, 2003; Revised Manuscript Received June 20, 2003

**ABSTRACT:** The natural products epothilone and bleomycin are assembled by hybrid polyketide/nonribosomal peptide synthetases. Of note in these assembly lines is the conversion of internal cysteine residues into thiazolines and their subsequent oxidation to heteroaromatic thiazole rings. We have excised the EpoB oxidase domain, EpoB-Ox, proposed to be responsible for thiazoline to thiazole oxidation in epothilone biosynthesis, and expressed it in soluble form in *Escherichia coli*. The purified domain is an FMN-containing flavoprotein that demonstrates thiazoline to thiazole oxidase activity when incubated with thioester substrate mimics. Kinetic parameters were determined for both thiazoline and oxazoline substrates, with  $k_{\text{cat}}$  values ranging between 48.8 and 0.55 min<sup>-1</sup>. While the physiological electron acceptor is not yet known, molecular oxygen is needed in these in vitro assays to mediate reoxidation of reduced FMN. Additionally, the oxidase domain-containing BlmIII from the bleomycin assembly line was heterologously expressed and purified. BlmIII is also an FMN-containing protein with activity similar to EpoB-Ox. This work marks the first direct characterization of nonribosomal peptide synthetase oxidase domain activity and will lead to further exploration of these flavoproteins.

Nonribosomal peptides such as the antibiotic vancomycin and the immunosuppressant cyclosporin A are assembled by multimodular nonribosomal peptide synthetases (NRPS)<sup>1</sup> (1). Analogously, natural products such as the bleomycins and epothilones that are hybrids of polyketides (PK) and nonribosomal peptides (NRP) are biosynthesized by hybrid PK/NRP synthetase assembly lines (2–5). NRP moieties often contain D-amino acid residues, epimerized during chain elongation, as well as nonproteinogenic amino acids (6, 7). Some of the nonproteinogenic amino acids, such as 4-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine in vancomycin, are created as dedicated monomers by enzymes in the antibiotic biosynthetic cluster (8), while others are generated during the elongation process (9).

Of particular note in NRPS and hybrid NRPS/PKS assembly lines is the conversion of internal cysteine, serine, and threonine residues into thiazolines and oxazolines and their subsequent oxidation to the heteroaromatic thiazole and

oxazole rings. The presence of such heterocycles embedded in peptide chains of natural products suggests an NRPS origin, and the transformation of peptide bonds to heterocycles has profound consequences on the shape and stability of the molecules. Such heterocycles can occur once in the product (as in the epothilones (10)), as tandem dimers (as in the bleomycins (11) and myxothiazol (12)), and as up to eight tandem five-membered rings as seen in the potent telomerase inhibitor telomestatin (13) (Figure 1).

We have previously studied the tandem enzymatic thiazoline-forming cyclodehydrations of bis-cysteinyll-S-pantotheinyl enzyme intermediates during the elongation phase of yersiniabactin synthetase to create the iron-chelating yersiniabactin siderophore in *Yersinia pestis* (14). The conversion of the acetyl-cysteinyll-S-pantotheinyl enzyme on the EpoB subunit of epothilone synthetase to the methylthiazolyl-S-pantotheinyl enzyme intermediate has also been characterized (15). Similarly, the vibriobactin siderophore of *Vibrio cholerae* contains methyloxazolinyll rings that arise during chain elongation by the vibriobactin synthetase VibF subunit (16). In all of these cases, the cyclodehydrations are catalyzed by cyclization (Cy) domains that are variants of the normal condensation (C) domains that carry out peptide bond formation during NRP chain elongation (17, 18). Thus, the thiazoline- and oxazoline-forming Cy domains carry out three steps—peptide bond condensation, cyclization, and dehydration—as they transform peptide bond connectivity into heterocycle connectivity in the growing peptide chain.

Some dihydroheterocycles persist during subsequent chain translocations and elongations to appear in the final products, such as in vibriobactin and yersiniabactin. Others are reduced by two electrons to form tetrahydroheterocycles, thiazolidines, as found in the *Pseudomonas aeruginosa* siderophore

<sup>†</sup> This work was supported by NIH Grants GM20011 to C.T.W. and CA94426 to B.S. B.S. is a recipient of NSF CAREER Award (MCB 9733938) and NIH Independent Scientist Award (AI51687). T.L.S. is supported by Postdoctoral Fellowship Grant PF-02-023-01-CDD from the American Cancer Society.

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<sup>1</sup> Abbreviations: NRPS, nonribosomal peptide synthetase; PK, polyketide; Cy, cyclization; C, condensation; Ox, oxidase; FMN, flavin mononucleotide; HPLC, high-pressure liquid chromatography; MALDI-MS, matrix assisted laser desorption ionization-time-of-flight mass spectrometry; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; NAC, N-acetylcysteine; TE, thioesterase; A, adenylation; T, thiolation; FAD, flavin adenine dinucleotide; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

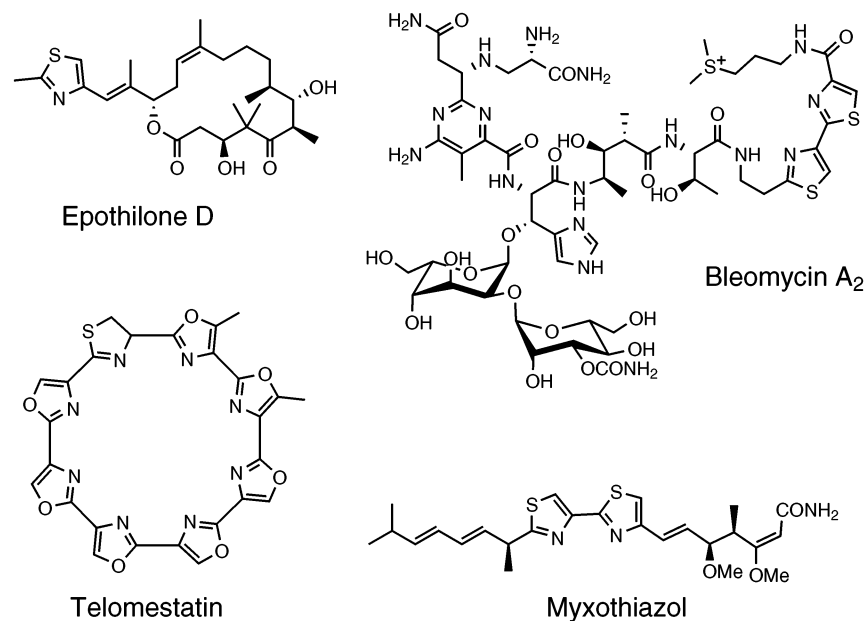


FIGURE 1: NRPS-derived thiazole- and oxazole-containing natural products.

pyochelin and in one of the rings of yersiniabactin (19, 20). A third fate for dihydroheterocycles is two electron oxidation to yield heteroaromatic thiazoles and oxazoles, as seen in the epothilones and bleomycins. This two electron oxidation is thought to be catalyzed by multimodular NRPS assembly lines, and gene sequencing in the epothilone (4, 5), bleomycin (3), and myxothiazol (12) clusters has revealed an additional 30 kDa domain in Cy domain-containing modules. This additional domain could function in the conversion of thiazoline-*S*-enzyme to thiazole-*S*-enzyme intermediates and has been termed an oxidase (Ox) domain. In this study, we demonstrate that such an Ox domain, excised from the epothilone EpoB module, can be expressed heterologously in *Escherichia coli*, folds autonomously as an FMN-containing domain, and has thiazoline to thiazole oxidase activity when small molecule thioester surrogate substrates were evaluated. The bleomycin BlmIII module, which contains an Ox domain, showed similar activity when overexpressed in *E. coli* and evaluated in parallel assays. Biochemical characterization of BlmIII and BlmIV in bleomycin bithiazole biosynthesis can also be seen in the accompanying paper (45).

## MATERIALS AND METHODS

**General.** Chemically competent *E. coli* TOP10 and BL21-(DE3) cell strains were purchased from Invitrogen. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. The pET24b overexpression vector was purchased from Novagen. Oligonucleotides for PCR amplification were prepared by Integrated DNA Technologies. Reverse phase HPLC analysis of enzyme reaction products was performed on a Beckman System Gold with a Vydac C18 column (300 Å, 4.6 × 250 mm). MALDI-MS (matrix assisted laser desorption ionization-time-of-flight mass spectrometry) was performed at a Voyager-DE STR BioSpectrometry Workstation (PerSeptive Biosystems) with predicted accuracy to 0.1%. All samples were prepared using 2,5-dihydroxybenzoic acid as matrix. UV-vis spectra were collected on a Hewlett-Packard 8453 spectrophotometer.

For synthesis of enzyme substrates and HPLC standards, all chemicals and solvents were purchased from Sigma-Aldrich, unless otherwise noted, and used without further purification. Chiral TLC was performed on glass plates precoated with chiral-silica gel (60 Å, 250 μM, Sigma-Aldrich). Preparative reverse phase HPLC was carried out on a Beckman System Gold with a Vydac C18 column (300 Å, 22 × 250 mm). NMR spectra were recorded on Varian Mercury 200 MHz and Bruker Spectrospin 400 MHz spectrometers.

**Cloning of *epoB*-Ox.** The *epoB*-Ox fragment (nucleotides 2917–3666 of *epoB*) was amplified by PCR from a plasmid containing the *epoB* gene (15). The forward primer was designed to incorporate an *Nde*I site, and the reverse primer incorporated a *Not*I site into the amplified sequence. Amplified products were digested and ligated into a linearized pET24b vector to give pOx1. DNA sequencing confirmed the correct insertion of the gene into the plasmid.

**Overexpression and Purification of *EpoB*-Ox.** BL21(DE3) cells were transformed with pOx1 plasmid and grown in LB media (3 × 1L) supplemented with 30 μg/mL kanamycin. Cultures were grown to OD<sub>600</sub> = 0.6 at 37 °C, protein expression was induced by the addition of IPTG to 100 μM, and growth continued for an additional 3 h at 25 °C. Harvested cells were resuspended in 25 mL of lysis buffer (25 mM Tris, pH 8.0, 200 mM NaCl, 10% (v/v) glycerol) and lysed by French Press. Cell lysate was clarified by centrifugation (30 min at 35 000g), and the resulting supernatant was incubated with 1 mL of Ni-NTA resin (Qiagen) for 2 h at 4 °C. The resin was decanted into a column and washed with 20 mL of 5 mM imidazole in lysis buffer. C-terminally His<sub>6</sub>-tagged *EpoB*-Ox protein was eluted with a step gradient of 30, 60, 100, 250, and 500 mM imidazole in lysis buffer. Fractions containing *EpoB*-Ox as judged by SDS-PAGE were pooled and dialyzed against lysis buffer (2 × 2 L). A solution of purified *EpoB*-Ox was bright yellow. Enzyme concentration was calculated based on the known extinction coefficient for FMN, 12 200 cm<sup>-1</sup> M<sup>-1</sup> at 450 nm (21). *EpoB*-Ox samples were heat denatured

for 2 min at 90 °C, and the supernatant was subjected to UV–vis analysis. The total yield of protein, calculated based on flavin content, was typically 5 mg/L. For comparison assays, the full-length EpoB module was prepared as described previously (15) but dialyzed into the same lysis buffer described above. EpoB concentration was also based on FMN content.

**Overexpression and Purification of BlmIII.** The overproduction of the full-length BlmIII module as an N-terminally His<sub>6</sub>-tagged fusion protein was reported previously (22). Here, BL21(DE3) cells were transformed with the *blmIII* expression plasmid pBS15 and grown in LB media (6 × 1 L) supplemented with 30 µg/mL kanamycin. Cultures were grown to OD<sub>600</sub> = 0.6 at 37 °C, protein expression was induced by the addition of IPTG to 100 µM, and growth continued for an additional 5.5 h at 25 °C. Harvested cells were resuspended in 50 mL of lysis buffer (25 mM Tris, pH 8.0, 200 mM NaCl, 10% (v/v) glycerol) and lysed by French Press. Cell lysate was clarified by centrifugation (30 min at 35 000g), and the resulting supernatant was incubated with 3 mL of Ni-NTA resin (Qiagen) for 3 h at 4 °C. The resin was decanted into a column and washed with 30 mL of 10 mM imidazole in lysis buffer. BlmIII was eluted with a step gradient of 30, 60, 100, 250, and 500 mM imidazole in lysis buffer. Fractions containing BlmIII as judged by SDS–PAGE were pooled and dialyzed against lysis buffer (2 × 2 L). Purified BlmIII was also yellow. Enzyme concentration was calculated based on FMN content as described above. Protein yield was approximately 2 mg/L.

**Chemical Synthesis of Enzyme Substrates and Standards.**  
**Methylthiazolyl-S-NAC.** Sodium 2-methylthiazolyl-4-carboxylate (23.4 mg, 140 µmol; Synchem) was combined with PyBOP (0.1 g, 210 µmol; Invitrogen), *N*-acetylcysteamine (NAC; 23.4 µL, 210 µmol), and triethylamine (14 µL, 210 µmol) in a mixture of 200 µL of DMF and 500 µL of THF. Similar coupling methods were used for all other thioester preparations. The reaction mixture was stirred at 25 °C for 2 h, and the desired thioester-linked product was purified using preparative HPLC. A linear gradient of acetonitrile in water from 0 to 50% over 30 min gave the desired product at *t*<sub>R</sub> = 22 min. <sup>1</sup>H NMR (400 MHz, 10% THF-*d*<sub>8</sub> in D<sub>2</sub>O): 5.6 (m, 1H), 3.52 (m, 1H), 3.44 (m, 1H), 3.26 (t, 2H), 2.98 (m, 2H), 2.19 (s, 3H), 1.83 (s, 3H). [M + H]<sup>+</sup>: observed 247.1, calculated 247.3.

**Methylthiazolyl-S-NAC.** 2-Methylthiazolyl-4-carboxylic acid ethyl ester (Maybridge) was subjected to ester hydrolysis, and thioester coupling proceeded as described above. A linear gradient of acetonitrile in water with 0.1% TFA from 0 to 100% over 30 min gave the desired product at *t*<sub>R</sub> = 14 min. [M + H]<sup>+</sup>: observed 245.2, calculated 245.3.

**Phenylthiazolyl-S-NAC.** A reported procedure (23) for the preparation of hydroxyphenylthiazoline carboxylic acid was modified to give 2-phenylthiazoline-4-carboxylic acid. Benzonitrile (3 mL, 29.1 mmol) was combined with L-cysteine hydrochloride (9.15 g, 58.5 mmol) in 100 mL of degassed 1:1 MeOH/0.1 M sodium phosphate, pH 6. The reaction was stirred under nitrogen at 40 °C for 3 days. Precipitate was removed through filtration, and MeOH was removed using rotary evaporation. The remaining solution was placed on ice, and phosphoric acid was added to pH 4. The resulting precipitate was extracted into CH<sub>2</sub>Cl<sub>2</sub>, dried, and concentrated to yield a yellow oil (73%) that was used

without further purification. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 7.77 (m, 2H), 7.40 (m, 3H), 5.21 (t, 1H), 3.63 (d, 2H). Enantiopurity was greater than 95% as measured by chiral TLC (MeCN/H<sub>2</sub>O/MeOH, 4:1:1); *R*<sub>f</sub> = 0.6 while *R*<sub>f</sub> = 0.5 for the D enantiomer. The thioester was prepared using standard PyBOP coupling methods, and the reaction mixture was purified using a linear HPLC gradient of acetonitrile in water with 0.1% TFA from 20 to 80% over 30 min. Product *t*<sub>R</sub> = 20 min. [M + H]<sup>+</sup>: observed 309.2, calculated 309.1.

**Phenylthiazolyl-S-NAC.** 2-Phenylthiazoline-4-carboxylic acid was oxidized using established methods (15) with a yield of 62%. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 8.27 (s, 1H), 8.00 (m, 2H), 7.49 (m, 3H). The related thioester was prepared as described, and the reaction mixture was purified using a linear HPLC gradient of acetonitrile in water with 0.1% TFA from 20 to 80% over 30 min. Product *t*<sub>R</sub> = 22 min. [M + H]<sup>+</sup>: observed 307.3, calculated 307.1.

**Phenylloxazolyl-S-NAC.** 2-Phenylloxazoline-4-carboxylic acid methyl ester was prepared following literature protocol (24) with a 44% yield. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 7.92 (m, 2H), 7.32 (m, 3H), 4.96 (m, 1H), 4.65 (m, 2H), 3.82 (s, 3H). Stoichiometric base hydrolysis gave free 2-phenylloxazoline carboxylate, which was coupled with *N*-acetylcysteamine to yield the desired thioester product. The reaction mixture was purified using a linear HPLC gradient of acetonitrile in water from 20 to 80% over 30 min. Product *t*<sub>R</sub> = 15 min. [M + H]<sup>+</sup>: observed 293.4, calculated 293.4.

**Phenylloxazolyl-S-NAC.** 2-Phenylloxazoline-4-carboxylic acid methyl ester was oxidized following established protocols (25), giving a 78% yield of the desired oxazole. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 8.26 (s, 1H), 8.08 (m, 2H), 7.44 (m, 3H), 3.92 (s, 3H). Base hydrolysis of the ester followed by coupling with *N*-acetylcysteamine gave the desired thioester product. The reaction mixture was purified using a linear HPLC gradient of acetonitrile in water with 0.1% TFA from 20 to 80% over 30 min. Product *t*<sub>R</sub> = 19 min. [M + H]<sup>+</sup>: observed 291.2, calculated 291.4.

**Evaluating Enantiopurity of Thioester-Linked Substrates.** Under the coupling conditions described above, a mixture of thioester enantiomers resulted in each case. Reactions carried out in the absence of triethylamine, or with other coupling agents including DCC alone or with HOBT, also yielded a mixture of enantiomers. Efforts to separate the SNAC-linked enantiomers with chiral TLC or HPLC proved unsuccessful. Therefore, thioester bonds were cleaved under neutral conditions using the excised EpoF thioesterase (TE) domain (26). TE (38 µM) was combined with 25 mM phenylthiazolyl-S-NAC in 75 mM sodium phosphate buffer, pH 7, with a total reaction volume of 42 µL. The reaction mixture was spotted onto a chiral TLC plate, and the presence of both carboxylic acid enantiomers (*R*<sub>f</sub> = 0.5 and *R*<sub>f</sub> = 0.6) in equal amounts was detected within 30 min.

**Oxidase Activity Assays.** Reactions containing EpoB-Ox or BlmIII were carried out in 75 mM sodium phosphate, pH 7, at ambient temperature. Reactions that contained phenylthiazolyl-S-NAC or phenylloxazolyl-S-NAC also included 10% THF for substrate solubility. Reactions were initiated by the addition of oxidase and quenched by heat denaturation at 90 °C for 2 min. Reaction products were detected by analytical HPLC using linear gradients of acetonitrile in water with 0.1% TFA that varied by substrate: for methylthiazolyl-S-NAC, 0–67% over 20 min;



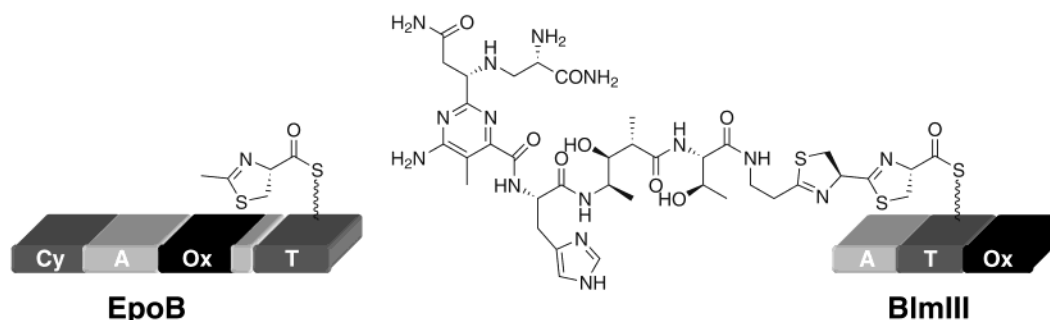


FIGURE 2: Domain organization of EpoB and BlmIII. Ox domains are highlighted in black. The Ox domain in EpoB is sandwiched into the adenylation (A) domain, while the Ox domain in BlmIII follows the thiolation (T) domain. The probable natural substrate for each Ox domain is shown tethered to the proximal T domain.

for phenylthiazolynyl-*S*-NAC, 40–70% over 15 min; and for phenyloxazolynyl-*S*-NAC, 20–70% over 22 min. Products were quantified by integrating peak area and comparing it with a standard curve prepared with synthetic standards.

Kinetic parameters for reactions with EpoB-Ox were determined with 3  $\mu$ M enzyme and a range of substrate concentrations in a total volume of 50  $\mu$ L. Reactions were quenched by heat denaturation at times within the linear range for each substrate: 5 min for methylthiazolynyl-*S*-NAC and 45 min for phenylthiazolynyl-*S*-NAC and phenyloxazolynyl-*S*-NAC. Each experiment was carried out in triplicate, and the reported error denotes the standard deviation among the three trials.

Kinetic parameters for reactions with BlmIII were determined with 3  $\mu$ M BlmIII for assays with methylthiazolynyl-*S*-NAC and 0.75  $\mu$ M BlmIII for assays with phenylthiazolynyl-*S*-NAC and phenyloxazolynyl-*S*-NAC in a total volume of 50  $\mu$ L. Reactions were quenched by heat denaturation at times within the linear range for each substrate: 15 min for methylthiazolynyl-*S*-NAC, 5 min for phenylthiazolynyl-*S*-NAC, and 10 min for phenyloxazolynyl-*S*-NAC. Each experiment was carried out in triplicate, and the reported error denotes the standard deviation among the three trials.

**Determination of Oxidase Enantioselectivity.** Phenylthiazolynyl-*S*-NAC (6.4 mM) was incubated with 54  $\mu$ M EpoB-Ox or buffer alone for 10 min in a total volume of 233  $\mu$ L. Remaining phenylthiazolynyl-*S*-NAC in each case was separated from other reaction components using HPLC and lyophilized to dryness. Each sample was resuspended in 5  $\mu$ L of THF and incubated with 33  $\mu$ M TE in phosphate buffer for 10 min at ambient temperature in a total reaction volume of 40  $\mu$ L. Samples were spotted on chiral TLC plates and resolved (MeCN/H<sub>2</sub>O/MeOH, 4:1:1). TLC plates were visualized by UV at 254 nm.

Phenylthiazolynyl-*S*-NAC (6.4 mM) was incubated with 6.5  $\mu$ M BlmIII or buffer alone for 15 min in a total volume of 1 mL. The remainder of the experiment proceeded as described for EpoB-Ox.

**Oxidase Activity under Anaerobic Conditions.** A total of 1 mL of 31.2  $\mu$ M EpoB-Ox and 15.6  $\mu$ L of 100 mM methylthiazolynyl-*S*-NAC (50-fold excess) were placed in separate chambers of an anaerobic cuvette. The cuvette was sealed and repeatedly evacuated and flushed with argon 12 times. UV-vis spectra were collected before and after mixing the two reactants and after the cuvette was reopened to air.

## RESULTS

**Expression and Purification of the EpoB-Ox Fragment and the Full-Length BlmIII Module.** We have previously described the heterologous expression of the four domain EpoB protein from the epothilone biosynthesis gene cluster (15). Here, we describe work done with the excised Ox domain from EpoB that examines the mode of action of this flavoprotein in greater detail. The domain organization of EpoB and BlmIII is shown in Figure 2. The EpoB-Ox domain was identified based on sequence alignment with other proposed Ox domains found in BlmIII from bleomycin (3) and in MtaC and MtaD from myxothiazol (12) and contains the highly conserved Ox-1 and Ox-2 motifs (22). The 249-residue EpoB-Ox fragment (residues 973–1222 of EpoB) was expressed with a C-terminal His<sub>6</sub> tag in *E. coli* in good yield (5 mg/L). The 29 kDa protein was soluble and readily purified using nickel affinity chromatography (Figure 3A). Purified EpoB-Ox was yellow, indicating that the FMN cofactor identified in EpoB (15) was also bound by the excised Ox domain. As shown in Figure 3, the presence of FMN was confirmed by UV-vis spectroscopy, HPLC analysis after heat denaturation of the protein, and MALDI-MS (for FMN, [M + H]<sup>+</sup> observed, 458.2; calculated, 458.3). The A<sub>270/445</sub> ratio for purified EpoB-Ox is 6.9:1.

BlmIII from the bleomycin biosynthesis gene cluster is a three domain protein also containing an Ox domain. Its expression in *E. coli* as an N-terminally His<sub>6</sub>-tagged 101 kDa protein has been described (22). BlmIII also contains a noncovalently bound FMN cofactor (22), and the A<sub>270/445</sub> ratio for purified BlmIII is 10.9:1. The sequences of the Ox domains from EpoB and BlmIII display approximately 40% amino acid identity with 50% similarity.

**Preparation and Characterization of Soluble Substrates for EpoB-Ox and BlmIII.** The normal substrate for EpoB-Ox is proposed to be methylthiazolynyl-*S*-pantotheinyl-EpoB, tethered in cis on the thiolation (T) domain just downstream of Ox domain (Figure 2). Similarly, the expected substrate for the BlmIII Ox is predicted to be the more elongated acylbithiazolynyl chain tethered in cis on the BlmIII T domain, as shown in Figure 2. Acyl enzyme intermediates are difficult to obtain in substrate quantities, and these systems would generate only a single turnover since the oxidized products remain attached to the protein. Since single turnover reactions are challenging to measure because of low sensitivity of product detection, we chose to use soluble small molecule thioester surrogates as substrates for EpoB-Ox and

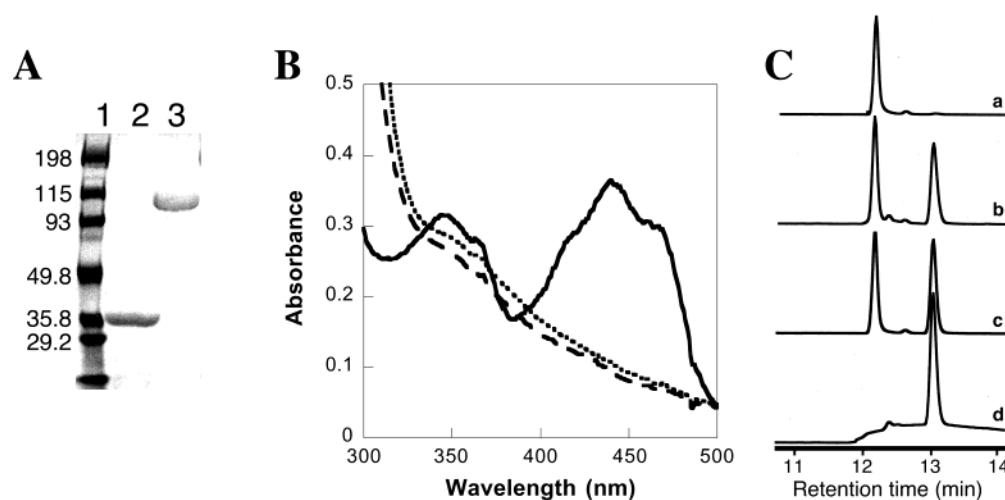


FIGURE 3: (A) SDS-PAGE analysis of the purification of proteins by nickel affinity chromatography. Lane 1, molecular weight markers (kDa); lane 2, purified EpoB-Ox; lane 3, purified BlmIII. (B) UV-vis spectrum of 30  $\mu$ M purified EpoB-Ox (solid line), showing the absorption maxima at 349 and 445 nm that are characteristic of flavin-containing proteins. Also shown is the UV-vis spectrum of the protein after addition of a 50-fold excess of methylthiazolanyl-S-NAC under anaerobic conditions (dashed line). Loss of signal at 445 nm persisted 10 min after mixing occurred (dotted line). (C) HPLC characterization of EpoB-Ox flavin cofactor after heat denaturation of protein. Traces shown are authentic FAD (a), authentic FAD and FMN (b), authentic FAD and EpoB-Ox flavin (c), and authentic FMN and EpoB-Ox flavin (d).

BlmIII. *N*-Acetylcysteamine (NAC) has been used successfully as a mimic for the phosphopantetheinyl prosthetic group of T domains (27, 28) and was linked to substrates of interest here to give SNAC thioesters.

Methylthiazolanyl-S-NAC and phenylthiazolanyl-S-NAC were prepared synthetically as soluble substrates for EpoB-Ox and BlmIII. Full-length EpoB has shown tolerance for unnatural substrates, including phenyl-substituted substrates and even oxazoline-containing substrates (15, 29). Thus, we also prepared phenyloxazolanyl-S-NAC to investigate this tolerance further. These substrates were prepared in moderate yield, but the methylthiazoline ring, in particular, was very susceptible to hydrolytic ring opening, necessitating storage in neutral pH buffer. Additionally, it was not possible to maintain enantiopurity while coupling the dihydroheterocyclic carboxylic acids to SNAC. Unfortunately, thioester-linked enantiomers of phenylthiazoline could not be separated using chiral TLC or HPLC methods. The related carboxylates, though, could be separated on chiral TLC following mild hydrolysis of the thioester bonds at neutral pH catalyzed by the excised EpoF TE domain (26). Such an analysis of phenylthiazolanyl-S-NAC revealed that we had prepared a racemic mixture of the substrate. Further experiments with EpoB-Ox, detailed below, confirmed that only approximately 50% of the substrate was consumed even when the molar ratio of EpoB-Ox to substrate was 5:1. Therefore, we make the assumption in our kinetic analysis of EpoB-Ox and BlmIII activity that only 50% of the substrate is actually available for oxidase-catalyzed oxidation.

Analysis was further complicated by the fact that phenylthiazolanyl-S-NAC readily undergoes nonenzymatic epimerization at the  $\alpha$ -carbon under the neutral pH buffer conditions used for enzyme assays. The half-life of this process ( $127 \pm 2$  min) was measured by monitoring deuterium exchange in  $D_2O$  by NMR (data not shown). Similar experiments with methylthiazolanyl-S-NAC revealed that exchange for this molecule was almost undetectable, with a half-life greater than 14 h. Additionally, no exchange was detected with

phenyloxazolanyl-S-NAC in a 3 h experiment under identical conditions. On the basis of this information, all enzyme kinetic data were collected at times equal to or less than 45 min.

**Enzymatic Activity of Excised EpoB-Ox Domain.** Purified EpoB-Ox (3  $\mu$ M) was incubated with 2.5 mM methylthiazolanyl-S-NAC at ambient temperature. After 3 h, the reaction products were evaluated by HPLC. In the presence of EpoB-Ox or full-length EpoB, a new peak was observed that coeluted with chemically prepared methylthiazolanyl-S-NAC (Figure 4A). No new peak was observed in the absence of catalyst or when an equivalent amount of free FMN was added to methylthiazolanyl-S-NAC. Oxidation of phenylthiazolanyl-S-NAC and phenyloxazolanyl-S-NAC was also observed in the presence of EpoB-Ox (Supporting Information). In each case, the enzymatic oxidation product peak was collected and confirmed to be the expected thiazole or oxazole by MALDI-MS. No EpoB-Ox-catalyzed oxidation of either phenylthiazoline carboxylic acid or phenylthiazolanyl-*O*-NAC was observed, revealing the importance of the thioester linkage for enzyme activity.

The activity of EpoB-Ox was compared to that of full-length EpoB in parallel time course assays. The turnover rate of EpoB-Ox was very similar to EpoB, as seen in Figure 4B. Excision of Ox from EpoB does not appear to hinder its efficacy as a catalyst.

Kinetic parameters for EpoB-Ox were determined with all three substrates and are listed in Table 1.  $K_m$  values were in the low millimolar range for all substrates, not surprising since SNAC substrate mimics of acyl-S-pantotheinyl enzyme were used. The fastest turnover of substrate occurred with methylthiazolanyl-S-NAC, the closest model of the natural substrate ( $k_{cat} = 48.8 \pm 0.1 \text{ min}^{-1}$ ). The rate constant measured for phenylthiazolanyl-S-NAC was almost an order of magnitude lower ( $k_{cat} = 7.7 \pm 0.8 \text{ min}^{-1}$ ), while phenyloxazolanyl-S-NAC was still lower by over an order of magnitude ( $k_{cat} = 0.55 \pm 0.1 \text{ min}^{-1}$ ). While alternate

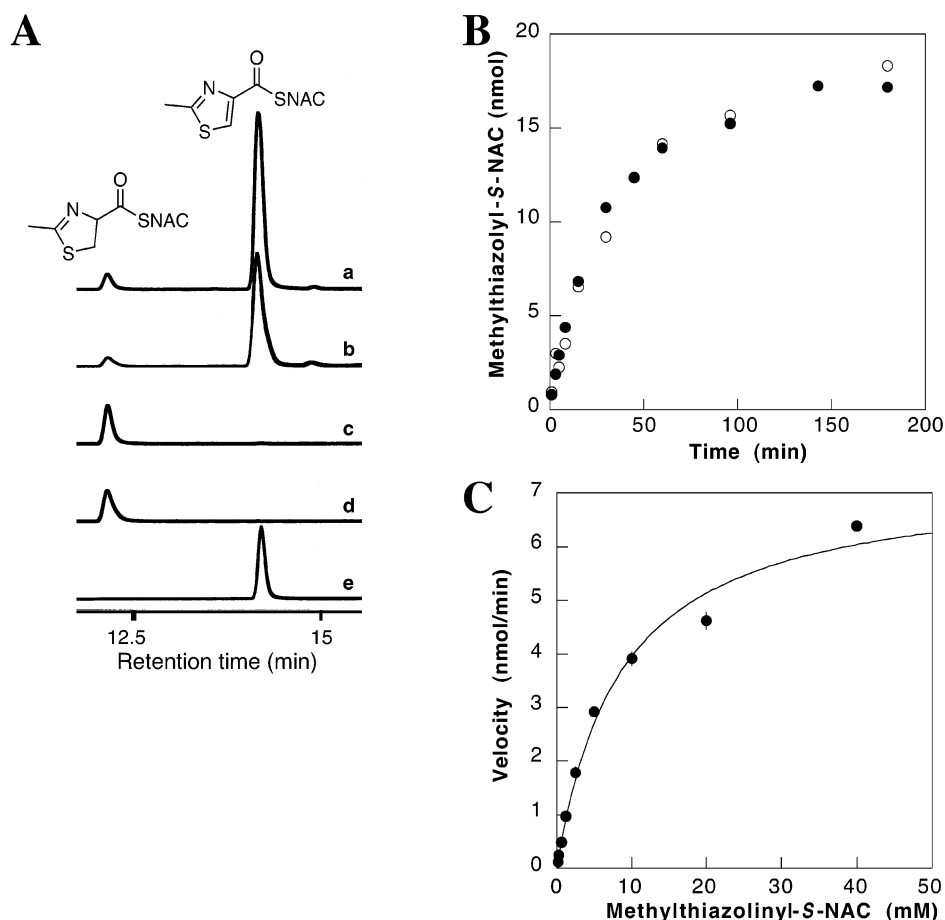
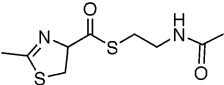
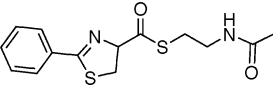
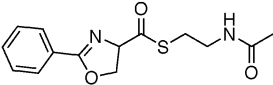


FIGURE 4: Oxidase activity of purified EpoB-Ox protein. (A) Methylthiazolyl-S-NAC combined with EpoB-Ox (a), EpoB (b), FMN (c), or no catalyst (d) and incubated for 3 h at 25 °C prior to HPLC analysis. Authentic methylthiazolyl-S-NAC (e) is shown. Formation of methylthiazolyl-S-NAC catalyzed by Ox ( $t_R = 14.2$  min) was validated by MALDI-MS ( $[M + H]^+$ : observed, 245.3; calculated, 245.3). (B) Formation of methylthiazolyl-S-NAC over time as catalyzed by 3  $\mu$ M EpoB (○) or 3  $\mu$ M EpoB-Ox (●). (C) Michaelis-Menten analysis used to determine kinetic parameters for EpoB-Ox with methylthiazolyl-S-NAC. 3  $\mu$ M EpoB-Ox was combined with increasing concentrations of methylthiazolyl-S-NAC in a total volume of 50  $\mu$ L. Error bars denote standard deviation.

Table 1: Kinetic Parameters for Thioester-Linked Substrate Mimics

Substrate	$K_m$ (mM)	$k_{cat}$ ( $\text{min}^{-1}$ )	$k_{cat}/K_m$ ( $\text{mM}^{-1}\text{min}^{-1}$ )	oxidase
	$4.3 \pm 0.3$	$48.8 \pm 0.1$	$11.3 \pm 0.8$	EpoB-Ox
Methylthiazolyl-S-NAC	$2.9 \pm 0.2$	$22.8 \pm 0.5$	$7.9 \pm 0.6$	BlmIII
	$2.1 \pm 0.4$	$7.7 \pm 0.8$	$3.7 \pm 0.8$	EpoB-Ox
Phenylthiazolyl-S-NAC	$1.5 \pm 0.3$	$313 \pm 20$	$209 \pm 44$	BlmIII
	$1.0 \pm 0.3$	$0.55 \pm 0.1$	$0.55 \pm 0.2$	EpoB-Ox
Phenylloxazolyl-S-NAC	$6.6 \pm 0.3$	$18.4 \pm 0.8$	$2.8 \pm 0.2$	BlmIII

substrates are tolerated by EpoB-Ox, they are processed less efficiently.

**Enantioselectivity of EpoB-Ox.** When incubated with a racemic mixture of methylthiazolyl-S-NAC, EpoB-Ox catalyzes only about 50% conversion to thiazole product, even with 5:1 excess of EpoB-Ox over substrate, suggesting

that only one enantiomer is oxidized (Figure 5A). Similar results were observed with phenylloxazolyl-S-NAC (data not shown). EpoB-Ox catalyzes further conversion of phenylthiazolyl-S-NAC (Figure 5B), but this is likely due to concomitant nonenzymatic substrate epimerization as discussed above. We were able to identify which phenylthi-

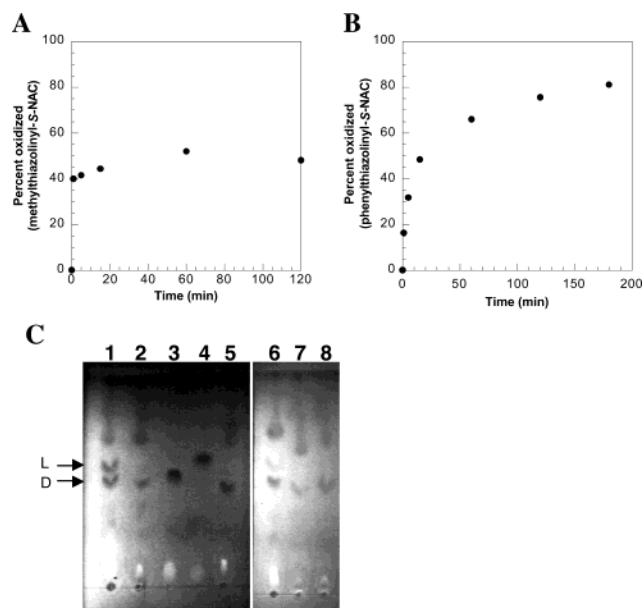


FIGURE 5: (A) Percent of methylthiazoliny-S-NAC oxidized over time when incubated with a 5-fold excess of EpoB-Ox. (B) Percent of phenylthiazoliny-S-NAC oxidized over time when incubated with a 5-fold excess of EpoB-Ox. (C) Chiral TLC analysis of EpoB-Ox enantioselectivity. Lane 1, phenylthiazoliny-S-NAC incubated without catalyst, HPLC purified, and then incubated with TE to cleave thioester; lane 2, phenylthiazoliny-S-NAC incubated with EpoB-Ox, purified, and then incubated with TE to cleave thioester; lane 3, authentic D-phenylthiazoline carboxylic acid; lane 4, authentic L-phenylthiazoline carboxylic acid; lane 5, co-spotting of lane 2 sample with authentic D-phenylthiazoline carboxylic acid; lane 6, phenylthiazoliny-S-NAC incubated without catalyst, HPLC purified, and then incubated with TE to cleave thioester; lane 7, phenylthiazoliny-S-NAC incubated with BlmIII, purified, and then incubated with TE to cleave thioester; and lane 8, co-spotting of lane 7 sample with authentic D-phenylthiazoline carboxylic acid.

azoliny-S-NAC enantiomer was favored by EpoB-Ox in a two-step process. First, phenylthiazoliny-S-NAC was incubated for 15 min in the presence or absence of EpoB-Ox. Remaining phenylthiazoliny-S-NAC was separated from oxidized product using HPLC, lyophilized to dryness, and then subjected to mild thioester cleavage at neutral pH, catalyzed by the EpoF TE domain. The resulting carboxylates could then be resolved on chiral TLC. In reactions with EpoB-Ox present, only D-phenylthiazoline carboxylate was detected while both L- and D-phenylthiazoline carboxylate remained when EpoB-Ox was absent (Figure 5C). Thus, we find that EpoB-Ox preferentially catalyzes the oxidation of L-phenylthiazoliny-S-NAC. This result is expected since the adenylation domain of EpoB is specific for L-cysteine, and L-phenylthiazoliny-S-EpoB is the likely cyclodehydration product from L-cysteine.

**Role of FMN Cofactor.** We expect that the FMN bound by the Ox domain undergoes a two electron reduction when the substrate is oxidized, giving FMNH<sub>2</sub>. While the physiological electron acceptor is not yet known, it is likely that molecular oxygen would mediate reoxidation of FMNH<sub>2</sub>, giving FMN and H<sub>2</sub>O<sub>2</sub> or superoxide in these *in vitro* assays. To test these hypotheses, we executed the EpoB-Ox assay under anaerobic conditions. EpoB-Ox and methylthiazoliny-S-NAC were placed in separate parts of an anaerobic cuvette. The cuvette was repeatedly evacuated and flushed with argon. EpoB-Ox was combined with excess methylthiazoliny-S-

NAC under anaerobic conditions, resulting in a complete loss of signal at 445 nm in the UV-vis spectrum of the sample, corresponding to reduction and related loss of conjugation within the isoalloxazine ring system of FMN (Figure 3B). This loss of signal persisted while the cuvette was kept closed to air. Once the cuvette was reopened, UV-vis spectra collected over time indicated a gradual increase in signal at 445 nm, reflecting reoxidation of FMN that was contingent on the presence of molecular oxygen. These results confirm our proposal that electrons flow from the substrate to FMN and that FMN is regenerated through the reduction of molecular oxygen.

Using a tandem horseradish peroxidase assay (30), we were unable to detect H<sub>2</sub>O<sub>2</sub> release although substrate oxidation did occur under these assay conditions as measured by HPLC. We also assessed the production of superoxide using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT), a tetrazolium dye that is reduced by superoxide (31). Reduced XTT was detected spectrophotometrically in EpoB-Ox assays, and the formation of reduced XTT was inhibited by approximately 63% in the presence of superoxide dismutase (data not shown). These results suggest that a significant fraction of oxygen reoxidation is mediated through superoxide production in this *in vitro* system.

**Enzymatic Activity of BlmIII.** The oxidase activity of BlmIII had not been detected during prior investigations of the enzyme (22). With the thioester-linked substrates prepared for EpoB-Ox activity measurements, we were able to measure the activity of BlmIII as well. BlmIII also catalyzed the oxidation of the three substrates discussed above, as detected by HPLC and confirmed by MALDI-MS (Supporting Information). In assessing the kinetic parameters of the enzyme, we found some different substrate preferences between BlmIII and EpoB-Ox (Table 1). Here, the fastest turnover was observed with phenylthiazoliny-S-NAC ( $k_{\text{cat}} = 313 \pm 20 \text{ min}^{-1}$ ), while methylthiazoliny-S-NAC was over an order of magnitude slower ( $k_{\text{cat}} = 22.8 \pm 0.5 \text{ min}^{-1}$ ). Perhaps the phenyl substituted thiazoline is a better mimic of the proposed natural acylbithiazoline moiety bound to BlmIII. Further experiments indicated that BlmIII, like EpoB-Ox, shows substrate preference for the L-phenylthiazoliny-S-NAC enantiomer (Figure 5C).

## DISCUSSION

Five-membered heterocycles are inserted into peptide chains in a number of NRP-derived natural products. This is also true for PK/NRP hybrids, including the antitumor agents epothilone and bleomycin. In general, the conversion of acyclic amide (PK/NRP hybrids) or peptide (NRP) structures to their heteroaromatic counterparts is in part utilitarian as it radically decreases the susceptibility of these natural products to proteolysis and degradation. The presence of heterocycles can also impact the bioactivity of natural products. In epothilone, the thiazole ring is an important determinant of its microtubule-stabilizing activity and thereby its antitumor action (32–34). The bithiazole moiety in bleomycin is thought to be a DNA intercalator, accounting for the binding of the drug to DNA (35, 36). Additionally, telomestatin, thiostrepton, and GE 2270 are known polythiazole- and polyoxazole-containing NRP natural products



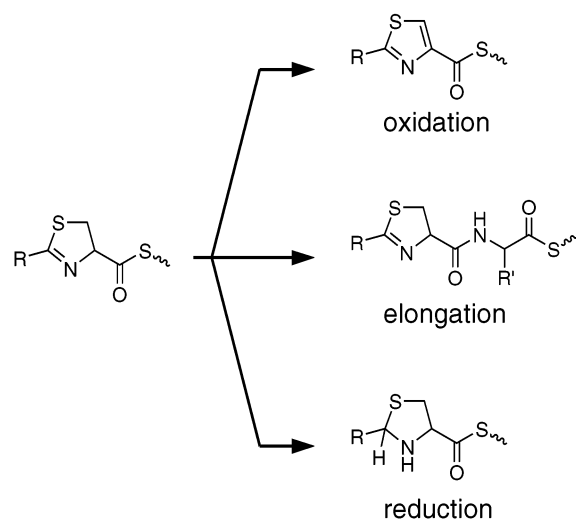


FIGURE 6: Possible redox states for a thiazoline ring during NRPS chain elongation.

that target telomeres, ribosomes, and the EF-Tu complex, respectively (37–39). All of these examples suggest that the introduction of oxazole and thiazole rings creates important steric constraints and pharmacophores in many natural products.

Because of the functional roles for these heterocycles in natural product bioactivity, it is important to study their mode of biosynthesis. While a ribosomal peptide, Microcin B17, has eight such heterocycles, most examples of these heterocyclic compounds are made by multimodular NRPS assembly lines (40). The primary cyclic product is at the dihydro oxidation state, with thiazolines derived from cysteine cyclization and oxazolines from serine or threonine cyclization. The dihydroheterocycle can then be elongated by subsequent modules or be reduced or oxidized enzymatically (Figure 6). Reduction by tailoring enzyme domains uses NADPH, while oxidation requires the FMN cofactor. The thiazoline to thiazolidine reductions of thiazolanyl-*S*-pantetheinyl intermediates by NADPH-mediated hydride transfer are catalyzed by reductase domains acting in trans, as seen with YbtU in the production of yersiniabactin (20) and PchG in pyochelin formation (19). The thiazoline to thiazole oxidations of thiazolanyl-*S*-pantetheinyl intermediates are catalyzed by oxidase domains acting in cis. While the mechanism of this oxidation process remains to be addressed, the reaction has a clear analogy to the oxidation of dihydroorotate to orotate observed in pyrimidine biosynthesis, where an FMN cofactor-mediated redox step goes via a proton/hydride mechanism (41). It is also analogous to reactions of FAD-containing acyl CoA dehydrogenases in which the  $\alpha$ -hydrogen of the substrate thioester is activated for proton abstraction, preparing the system for  $\beta$ -hydride transfer to the flavin (42). Indeed, incubation of methylthiazolanyl-*S*-NAC with EpoB-Ox under anaerobic conditions produces the FMNH<sub>2</sub>-EpoB-Ox form of the catalyst. The FMNH<sub>2</sub> is then regenerated by O<sub>2</sub>, although it is not known if O<sub>2</sub> is the physiological electron acceptor in vivo.

The goal of this study was to deconvolute thiazolanyl-*S*-enzyme oxidation. To this end, the Ox domain from EpoB of epothilone synthetase was expressed in a form that folds autonomously and retains bound FMN coenzyme. Another Ox-containing NRPS module, full-length BlmIII from bleo-

mycin synthetase, was expressed in a soluble, active form as well. BlmIII is composed of an inactive adenylation domain, thiolation domain, and an Ox domain with significant homology to the EpoB Ox domain. Additional efforts focused on the generation of thiazolanyl-thioester substrates for the two oxidases. In epothilone biogenesis, the thiazole is formed early in chain elongation, in module 2 of 10, so the acyl chain at that point is simply methylthiazolanyl, as shown in Figure 2 (15). In bleomycin, though, the bithiazole is formed late in the multistage cascade of elongating acyl enzyme intermediates (3), and the relevant bithiazolanyl acyl chain is not readily available. Perhaps more challenging than the formation of the acyl chain portion of the substrate would be tethering these acyl groups via pantotheinyl prosthetic groups to T domains embedded in multidomain subunits. In EpoB, a 150 kDa protein, the substrate for the Ox domain is the methylthiazolanyl-*S*-pantetheinyl-T domain within the same subunit, just downstream of the Ox domain. Similarly, in BlmIII, a covalently attached acylbithiazolanyl-*S*-pantetheinyl-T domain substrate would form part of a 100 kDa protein. Even if catalysis were detectable, the thiazole products would still be covalently connected to the T domain in the enzyme modules, and only single turnover assays could be achieved.

Our approach toward more tractable substrates was to simplify the *S*-pantetheinyl-protein portion of the substrate to *N*-acetylcysteamine, which has been used as a surrogate in several other NRPS and PKS studies (26, 28, 43). We were able to make methylthiazolanyl-, phenylthiazolanyl-, and phenyloxazolanyl-*S*-NACs and show that they functioned as multiple turnover substrates for the isolated Ox domain of EpoB as well as full-length BlmIII. While one cannot expect to learn much about affinity with such truncated thiazolanyl and oxazolanyl thioesters, the  $k_{\text{cat}}$  values and the  $k_{\text{cat}}/K_m$  catalytic efficiency ratios should be good indicators of oxidase domain capacity. Preliminary data suggest that the full-length EpoB module is able to form a methylthiazolyl-*S*-EpoB product at a rate of about one per second (personal communication, S. E. O'Connor). This turnover number is in the same range as that measured for EpoB-Ox with methylthiazolanyl-*S*-NAC ( $k_{\text{cat}} = 48.8 \pm 0.1 \text{ min}^{-1}$ ).

These results validate that the excised Ox domain from EpoB catalyzes thiazolanyl-thioester to thiazolyl-thioester conversions. In epothilone fermentations, one of the minor variants shows the thiazole replaced by oxazole, so one would anticipate that the EpoB Ox domain should be able to oxidize oxazolanyl-thioesters. This was observed here at reduced catalytic efficiency, consistent with low yields of oxazole product in vivo. The relative energy barriers for thiazoline versus oxazoline oxidation are not known, although it is established that thiazoles are more stable than oxazoles, exhibiting a higher degree of aromaticity (44). Also, the lower kinetic acidity of the phenyloxazolanyl-*S*-NAC  $\alpha$ -hydrogen as compared with phenylthiazolanyl-*S*-NAC is consistent with a higher energy barrier to carbanion formation, the proposed initial step in the oxidase mechanism. Further work is needed to fully deconvolute the reason for the observed difference in product formation.

BlmIII was likewise shown to have thiazoline to thiazole and oxazoline to oxazole activity when assayed with SNAC-linked substrates. The question of whether BlmIII works to oxidize both of the tandem thiazolanyl rings anticipated



during bleomycin chain elongation is an interesting one. Sequence analysis of the bleomycin gene cluster has suggested that bleomycin biosynthesis proceeds via a tandem bithiazolanyl-*S*-BlmIII intermediate (3). Since BlmIII contains the only NRPS Ox domain in the gene cluster, it seems possible that it would catalyze the dual oxidations. It has also been proposed, though, that a second oxidase exists within the gene cluster with high homology to coproporphyrinogen III oxidases rather than NRPS oxidases (3). It is therefore conceivable that the second oxidation in bleomycin formation is catalyzed in a later step, following release from the NRPS assembly line. It would be interesting to probe this question that has relevance to the timing of multiple ring heteroaromatizations in polythiazole natural products.

The initial studies described here, validating flavoprotein oxidase activity on thiazolanyl thioester model substrates presented in trans, form the basis for subsequent analysis of how Ox domains work on thiazolanyl-*S*-pantotheinyl-T domains installed in cis. The EpoB and BlmIII domain organizations suggest that oxidase domains can be upstream or downstream of the T domains presenting thiazolanyl-*S*-pantotheinyl chains for oxidation. This indicates that Ox-T and T-Ox pairs may be portable to other Cy-containing modules in NRPS assembly lines, thereby routing thiazolanyl- and oxazolanyl-*S*-enzyme intermediates to aromatic heterocycle oxidation states.

## ACKNOWLEDGMENT

The authors gratefully acknowledge Huawei Chen for his preparation of the pOx1 plasmid and initial work on EpoB-Ox expression and Liangcheng Du for pBS15 and initial work on BlmIII expression and purification. We also thank Frédéric Vaillancourt for helpful discussions on superoxide detection and Kosan Biosciences for providing the *epoB* gene.

## SUPPORTING INFORMATION AVAILABLE

HPLC traces illustrating the oxidase activity of EpoB-Ox and BlmIII and MALDI-MS product characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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BI034792W