Enzymatic Production of (−)-Indolactam V by LtxB, a Cytochrome P450 Monooxygenase

Minh U. Huynh,[†] Matthew C. Elston,[†] Nick M. Hernandez,[†] David B. Ball,[†] Shin-ichiro Kajiyama,[⊥] Kazuhiro Irie,[§] William H. Gerwick,[‡] and Daniel J. Edwards*[,†]

Department of Chemistry and Biochemistry, California State University, Chico, 400 W. 1st Street, Chico, California 95929

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The P450 cytochrome monooxygenase gene, *ltxB*, was cloned and overexpressed in *Escherichia coli* as a 6xHis-tagged protein. The resulting recombinant LtxB was purified by Ni-NTA affinity chromatography and characterized biochemically. Purified LtxB demonstrated typical cytochrome P450 spectroscopic properties including substrate-induced transition from a low-spin ($\lambda_{max} = 414$ nm) to high-spin state ($\lambda_{max} = 386$ nm) upon incubation with *N*-methyl-L-valyl-L-tryptophanol. The catalytic activity of LtxB was verified by demonstrating the oxidation/cyclization of *N*-methyl-L-valyl-L-tryptophanol to (−)-indolactam V. LtxB shows a relaxed specificity for analogue substrates in which the valyl group is substituted for other aliphatic groups. The relaxed substrate specificity of LtxB, along with the relaxed specificity of the prenyltransferase, LtxC, allowed for the enzymatic production of a series of (−)-indolactam V and lyngbyatoxin analogues.

(−)-Indolactam V (ILV)[1] (Figure 1) is a potent activator of various protein kinase C (PKC) isozymes and is the main pharmacophoric component of the lyngbyatoxin[2,3] and teleocidin[4−6] natural products. Over the years ILV and analogues have been extensively studied as synthetic targets for structure−activity studies.[7−11] Of particular importance is the recent identification of ILV as a specific inducer of human embryonic stem cell differentiation to pancreatic cell types.[12] This newly identified activity for ILV and related compounds has refocused attention on developing efficient synthetic and/or biosynthetic routes to these compounds.

Since the isolation of the lyngbyatoxin biosynthetic gene cluster,[13] we have been investigating the biosynthetic enzymes involved in the generation of lyngbyatoxin and its biosynthetic precursor, ILV. The lyngbyatoxin biosynthetic gene cluster was isolated from a Hawaiian strain of *Lyngyba majuscula* and comprises three genes.[13] The first gene, *ltxA*, encodes for a nonribosomal peptide synthetase (NRPS) that condenses *N*-methyl-L-Val and L-Trp and releases *N*-methyl-L-valyl-L-tryptophanol (NMVT) via an NADPH-dependent reductive cleavage.[14] The second gene product, LtxB, is a cytochrome P450 monooxygenase. Although this activity is consistent with the oxidation and cyclization of NMVT to ILV, biochemical verification of this activity has yet to be reported. The last gene in the lyngbyatoxin pathway, *ltxC*, encodes for a prenyltransferase that has been shown to attach a geranyl group at C-7 of ILV to generate a quaternary carbon center.[13]

Here, we report on the function of LtxB as an *N*-arylation catalyst in the generation of the nine-membered ring of ILV (Figure 1). We also explored the substrate permissiveness of LtxB versus a series of NMVT analogues with substitutions in the valyl position. The relaxed substrate specificity of LtxB allowed for the production of ILV analogues. Subsequently all ILV analogues produced by LtxB were found to act as substrates for LtxC, leading to the production of a series of new lyngbyatoxin analogues.

To experimentally verify the role of LtxB, we cloned the entire *ltxB* gene into an *E. coli* overexpression plasmid and overproduced the recombinant C-terminal His-tagged LtxB protein. LtxB was purified in a single step by Ni-NTA chromatography to yield 3 mg/L of culture volume of purified protein. Purity was assessed as being greater than 90% by SDS-PAGE (Supporting Information). LtxB showed the characteristic orange-red color of heme-containing proteins and demonstrated a Soret band with $\lambda_{max} = 414$ nm. Upon reduction of the heme Fe^{3+} to Fe^{2+} with dithionite, LtxB was able to bind CO, generating a new Soret band with a $\lambda_{max} = 444$ nm, characteristic of CO-bound ferrous heme (Supporting Information). Small amounts of the P420 form of the enzyme were also observed and varied slightly from batch to batch.

Additional spectroscopic studies with LtxB verified that LtxB underwent the expected transition from a low-spin ($\lambda_{max} = 414$ nm) to high-spin state ($\lambda_{max} = 386$ nm) upon incubation with the NMVT substrate. To determine the specificity of the NMVT-LtxB interaction, we performed active site titration of LtxB with NMVT, allowing the calculation of the dissociation constant as $K_d = 0.50 \pm 0.04$ μM (Supporting Information). NMVT analogues substituted in the valyl position were also evaluated for their ability to bind to LtxB. Dipeptides with conservative substitutions at Val bound with similar affinity to LtxB (Supporting Information). Other possible substrates including L-tryptophanol, L-Trp, and *N*-methyl-L-phenyl-Gly-L-Trp-ol were also tested for their ability to induce a spin-state transition with LtxB, but did not induce spectroscopic shifts at concentrations up to 200 μM. Because the binding of substrate preceded electron transfer in the reaction cycle of cytochrome P450s,[15] the type I substrate binding behavior of LtxB toward NMVT and close structural analogues was consistent with a catalytically active cytochrome P450.

To explore the catalytic activity of LtxB, we tested the enzymatic conversion of NMVT to ILV. In the assay we used NADPH as an electron source and spinach ferredoxin and spinach ferredoxin reductase as electron transfer agents. Incubation of LtxB with NMVT led to the clear production of ILV, as demonstrated by HPLC coelution and ESIMS $[M + H]^+$ signals identical to an authentic ILV standard (Figure 2B). The production of ILV was absolutely dependent on the presence of LtxB, NADPH, spinach ferredoxin, and spinach ferredoxin reductase (Figure 2B−E). A control reaction with boiled LtxB also showed no activity (data not shown). In addition, we tested the ability of LtxB to convert alternative substrates to cyclized ILV analogues. Various *N*-methyl-L-X-L-Trp-ol substrates were tested with LtxB, and small amounts of cyclized products could be detected by HPLC analysis with UV-diode array detection followed by ESIMS analysis. *N*-Methyl-L-norvalyl-L-Trp-ol (Figure 2G) and *N*-methyl-L-X-L-Trp-ol (X = Leu, Ile, norleucine) all gave cyclized product (see Table 1 for a

* Author for correspondence. Tel: (530) 898-5226. Fax: (530) 898-5234. E-mail:djedwards@csuchico.edu.

[†] California State University, Chico.

[⊥] School of Biology-Oriented Science and Technology, Kinki University, 930 Nishimitani, Kinokawa, Wakayama, 649-6493, Japan.

[§] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan.

[‡] Scripps Institution of Oceanography and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093.

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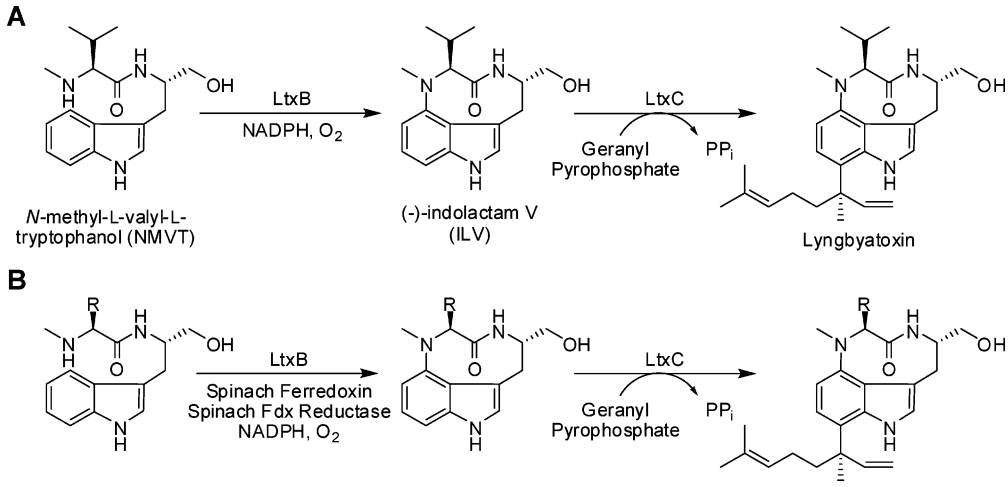

Figure 1. (A) Last two biosynthetic steps in the production of lyngbyatoxin. (B) Production of ILV and lyngbyatoxin analogues from synthetic *N*-Me-L-X-L-Trp-ol compounds (norvaline analogue, R = $CH_2CH_2CH_3$; Leu analogue, R = $CH_2CH(CH_3)_2$; Ile analogue, R = $CH(CH_3)CH_2CH_3$; norleucine analogue, R = $CH_2CH_2CH_2CH_3$; phenylglycine analogue, R = phenyl group).

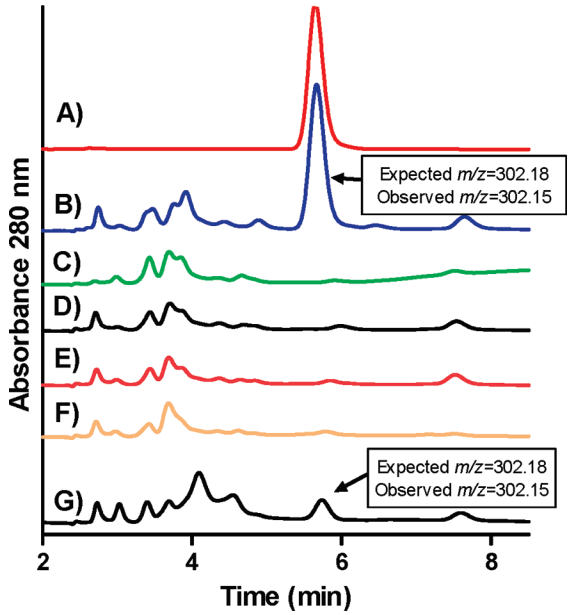

Figure 2. HPLC chromatograms demonstrating the enzymatic activity of LtxB: (A) (−)-indolactam V chemical standard, (B) enzymatic reaction of LtxB with NMVT, (C) control reaction without LtxB, (D) control reaction without NADPH, (E) control reaction without spinach ferredoxin reductase, (F) control reaction without spinach ferredoxin, (G) enzymatic reaction of LtxB with *N*-methyl-L-norvalyl-L-Trp-ol.

Table 1. Relative Reaction Yields for LtxB and LtxB/LtxC Combination Reactions with *N*-Me-L-X-L-Trp-ol Substrates[a]

Substrate (R group)	Relative Reaction Yield (%)	
	LtxB only	LtxB/LtxC
Val	100	100
Leu	30	31
Ile	71	47
NorLeu	74	83
NorVal	65	86

[a] The relative yield was defined as the ratio of the conversion of a substrate to that of *N*-Me-L-Val-L-Trp-ol.

comparison of the relative yields). No cyclized product could be detected with *N*-methyl-L-phenylglycyl-L-Trp-ol.

We next tested the ability of an LtxB/LtxC coupled enzyme system to convert NMVT and NMVT analogues to lyngbyatoxin-like compounds (Figure 1B). When NMVT was incubated with LtxB and LtxC, the production of lyngbyatoxin could be clearly demonstrated by HPLC (Figure 3B). In the single enzyme system LtxB showed conversion of various *N*-methyl-L-X-L-Trp-ol substrates to the corresponding products; however, it was not clear if the corresponding cyclized products would also act as substrates for LtxC. HPLC analysis of reaction products in the LtxB/LtxC enzyme system showed that all *N*-methyl-L-X-L-trp-ol compounds that acted as substrates for LtxB were prenylated to the corresponding lyngbyatoxin analogue by LtxC (Figure 3C−F). Reversed-phase HPLC gave chromatographic peaks with retention times that corresponded well with the expected structural changes imposed by the varying R groups on the NMVT analogues, and verification by ESIMS supported the gross structures of these new products. Successful generation of lyngbyatoxin and lyngbyatoxin analogues was absolutely dependent on the presence of both LtxB and LtxC (data not shown). Reactions in which the valine residue of NMVT was substituted with leucine, isoleucine, norvaline, and norleucine all gave similar or only slightly lower yields of lyngbyatoxin analogues as compared to NMVT (Figure 3, Table 1). However, the overall inefficient turnover of our in vitro P450 reaction system did not allow us to perform detailed structural characterization or biological testing of either the ILV or lyngbyatoxin analogue reaction products.

In this report we confirm LtxB as the enzyme catalyzing *N*-arylation of NMVT to form ILV, thus verifying its role in lyngbyatoxin biosynthesis. We also show that both LtxB and LtxC exhibit relaxed substrate specificity, allowing for the production of several ILV and lyngbyatoxin analogues from synthetic dipeptide

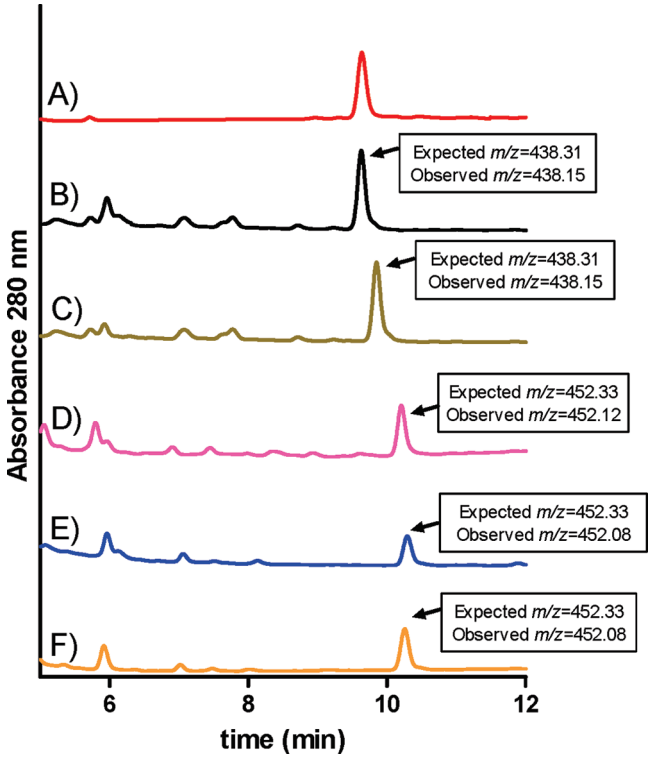

Figure 3. Reversed-phase HPLC analysis of LtxB/LtxC chemoenzymatic reactions (see Experimental Section for complete description of reaction mixture): (A) lyngbyatoxin standard, (B) reaction with *N*-methyl-L-Val-L-Trp-ol, (C) reaction with *N*-methyl-L-norvalyl-L-Trp-ol, (D) reaction with *N*-methyl-L-norleucyl-L-Trp-ol, (E) reaction with *N*-methyl-L-Leu-L-Trp-ol, (F) reaction with *N*-methyl-L-Ile-L-Trp-ol.

compounds. The ILV analogues proposed to be produced in this study have already been reported from microbial conversion experiments in *Streptoverticillium blastmyceticum* NA34-17.[16] Although detailed chemical and biological testing of the reaction products could not be performed due to the inefficient turnover of LtxB, this study demonstrates proof-of-concept that the LtxB/LtxC coupled system can in principle be used to generate new lyngbyatoxin analogues. We are currently exploring several refinements of our system, such as construction of an LtxB/cytochrome P450 reductase protein fusion system as described for RhFRED.[17,18] An LtxB/RhFRED reductase fusion system may improve the conversion efficiency, as well as eliminate the need for expensive spinach ferredoxin/ferredoxin reductase. In addition, an NADPH generation system could be adapted to lyngbyatoxin chemoenzymatic synthesis to provide an efficient and lower cost reducing agent for the cytochrome P450 reaction. These improvements may lead to more efficient chemoenzymatic routes to the ILV and lyngbyatoxin class of compounds.

Experimental Section

Reagents and Chemicals. All *N*-methyl-L-X-L-Trp-ol substrates were synthesized according to the literature procedure.[16] δ-Aminolevulinic acid, spinach ferredoxin, spinach ferredoxin reductase, NADPH, and His-Select chromatography resin were obtained from Sigma Life Sciences (St. Louis, MO). Geranyl pyrophosphate was obtained from Echelon Biosciences Incorporated (Salt Lake City, UT). Authentic (−)-indolactam V was obtained from Axxora, LLC (San Diego, CA). Authentic lyngbyatoxin A was isolated from *Lyngbya majuscula* collected from Kahala Beach, Oahu, HI. Lyngbyatoxin A was purified by HPLC, and its structure was verified by ^{1}H NMR data.

Expression of LtxB, Purification of Recombinant Protein. The *ltxB* gene was amplified from fos-DE3-86[13] by PCR with the following primers: 5′-GGA ATT CAT ATG ACA AAT CCT TTT GCA GA-3′ and 5′-CCG CTC GAG CCA CTC AGC AGG TAA CT-3′ with the *Nde* I and *Xho* I cloning sites underlined, respectively. The resulting product was cloned into the *Nde*I-*Xho*I sites of pET20a. The ensuing construct pET20a-LtxB was transformed into *E. coli* BL21(DE3). The BL21(DE3) cells containing pET20a-LtxB were grown until an OD 0.6, at which time the growth temperature was lowered to 25 °C and δ-aminolevulinic acid (0.5 mM) was added to the culture broth to increase the production of the heme-containing LtxB. LtxB was produced as a recombinant His_6-tagged protein and purified by Ni-NTA affinity chromatography using His-select resin according to the manufacturer's directions to yield an orange-red protein.

Spectroscopic Characterization of LtxB and Substrate Binding Studies. The absorption spectra of purified LtxB with and without substrate were obtained on a Hewlett-Packard 8453 spectrophotometer. A reduced CO spectrum of LtxB was obtained by placing LtxB (5 μM) in a quartz cuvette (100 μL) with dithionite (5 mM) and bubbling CO gas through the sample.[19]

The interaction of potential LtxB substrates was examined by perturbation of the heme Soret band. LtxB (1 μM) in 50 mM Tris-HCl (2.0 mL, pH 8.0) was placed in a 2 mL cuvette. After thermal equilibration at 25 °C, a baseline was established between 350 and 450 nm. Next, sequential additions of a concentrated solution (1−3 μL) of *N*-methyl-L-X-L-Trp-ol dissolved in EtOH were added to the sample cuvette to give a final ligand concentration in the range 0.2−20 mM. To a reference cuvette was added an equal volume of 100% EtOH to account for any effect the solvent might have on the absorption spectra. The LtxB spectrum without substrate was subtracted from each spectrum with added substrate. From the resulting difference spectra the $\Delta A_{386-418}$ values were calculated for each concentration of peptide. The K_d values were estimated by fitting the $\Delta A_{386-418}$ versus [*N*-methyl-L-X-L-Trp-ol] to a saturation curve in GraphPad.

Enzymatic Activity of LtxB. The production of ILV from NMVT was carried out in a 500 μL reaction: LtxB (1 μM), *N*-methyl-L-X-L-Trp-ol (0.5 mM), spinach ferredoxin (3.5 μM), spinach ferredoxin reductase (0.1 units/mL), and NADPH (1 mM), in Tris-HCl buffer (50 mM, pH 8.0). For the production of lyngbyatoxin and its analogues from *N*-methyl-L-X-L-Trp-ol, geranyl pyrophosphate (90 μM) and LtxC (0.6 μM) were also added to the reaction mixture. Reactions were incubated at 30 °C for 4 h. Reaction products were isolated by extraction with EtOAc (3 × 500 μL). The EtOAc extractions were combined, evaporated to dryness with a Savant SpeedVac system, and resuspended in 50% MeOH/H_2O solution. The products were analyzed by analytical HPLC on a Hewlett-Packard Series 1100 instrument with a UV diode array detector. Separation of (−)-indolactam products was carried out on an Adsorbosphere Phenyl column (Alltech, 150 × 4.6 mm, 5 μm pore size) with 40% MeOH in H_2O with a flow rate of 0.9 mL/min over 15 min. Separation of lyngbyatoxin products was done with a 50% MeOH/H_2O to 100% MeOH gradient over 15 min with a flow rate of 0.9 mL/min. Lyngbyatoxin and lyngbyatoxin analogue products were detected by their absorbance at 280 nm. Product peaks were collected and their masses determined by direct infusion in positive ion mode on a ThermoFinnigan LCQ Advantage mass spectrometer.

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Supporting Information Available: SDS PAGE gel of LtxB purification, absorption spectra, and active site titration plots of LtxB are available free of charge via the Internet at http://pubs.acs.org.

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