SyrB2 in syringomycin E biosynthesis is a nonheme Fe^{II} α-ketoglutarate- and O_2-dependent halogenase

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Contributed by Christopher T. Walsh, May 27, 2005

The nine-residue lipodepsipeptide syringomycin E, elaborated as a phytotoxin by *Pseudomonas syringae* pv. *syringae* B301D contains a 4-Cl-L-Thr-9 moiety where failure to chlorinate results in a 3-fold drop in biological activity. The proteins SyrB1 and SyrB2 encoded by the biosynthetic cluster are shown to act as a substrate and enzyme pair for SyrB2-mediated chlorination of the aminoacyl-*S*-enzyme L-Thr-*S*-SyrB1. SyrB2 is a member of the nonheme Fe^{II} α-ketoglutarate-dependent enzyme superfamily, and requires O_2 and α-ketoglutarate as well as chloride ion to carry out monochlorination of the -CH_3 group of L-Thr-*S*-SyrB1. Chlorination of L-Thr-*S*-SyrB1 was validated by thioesterase-mediated release of L-Thr and 4-Cl-L-Thr, *N*-derivatization as fluorescent isoindoles, and HPLC separation compared with authentic standards. Incubations with L-[^{14}C]Thr and [$^{36}Cl^-$] as well as MS of the released products further validated identification. Enzymatic oxidative halogenation is a previously uncharacterized reaction type for nonheme Fe^{II} enzymes and may be the general mode for biosynthetic halogenation of aliphatic carbons of natural products.

natural product biosynthesis | enzymology | organohalogen

A large variety of natural products from both terrestrial and marine microorganisms contain covalently attached halogen substituents, predominantly chlorine or bromine (1). These products include chlorinated nonribosomal peptides such as the vancomycin and teicoplanin family of glycopeptides (2) and aromatic polyketides such as chlorotetracycline (3). Most of the organochlorine linkages are in aromatic or heteroaromatic rings, such as the 3-Cl-β-OH-Tyr of the glycopeptides, the chloro substituent in the aminocoumarin antibiotics (2–4), and the 4,5-dichloropyrrole in pyoluteorin (5) (Fig. 1*A*). There are also aliphatic carbons bearing one, two, or three chlorine atoms in natural products, such as the 4-Cl-L-Thr at residue nine of the peptide scaffold of the phytotoxic syringomycin E from *Pseudomonas syringae* pv. *syringae* B301D (6), the dichlorinated β-hydroxy acid of lyngbyabellin A (7), and the trichloroleucine-derived moiety in barbamide (8) (Fig. 1*B*). Both the aliphatic and aromatic C-Cl linkages are generated enzymatically.

The timing and mechanism for covalent incorporation of chlorine into these diverse groups of natural products has been unclear, but two lines of information have clarified some of the molecular logic of enzymatic halogenation. The first line has come largely from the isolation and characterization of regioselective tryptophan halogenases from the pyrrolnitrin pathway (9, 10), the rebeccamycin pathway (11), and the pyrroindomycin B pathway (12). This type of halogenase was shown to have a requirement for $FADH_2$ and O_2 as cofactors (9, 11). More recently, we have characterized a pyrrole-dichlorinating flavoprotein PltA in the pyoluteorin pathway (P. C. Dorrestein, E. Yeh, S. Garneau, N. L. Kelleher, and C.T.W., unpublished data). Microbial genome sequencing has turned up homologous 50-kDa ORFs with FAD signature binding sites as predicted halogenases in the biosynthetic gene clusters for several nonribosomal peptide and polyketide halogenated metabolites. The general view now is that aromatic and heteroaromatic ring halogenations are effected by such flavoprotein halogenases, although both mechanistic understanding of the active chlorinating species and timing of halogenation relative to peptide chain elongation is still rudimentary.

Halogenation at aliphatic carbon centers of amino acid constituents of nonribosomal peptides appears to use different enzymatic machinery. For example, neither the barbamide synthetase assembly line gene cluster nor the syringomycin synthetase cluster (6, 8) contain obvious flavoprotein halogenase homologs. Instead, they have ORFs predicted to be nonheme Fe^{II}, α-ketoglutarate (α-KG)-requiring enzymes. In this study, we have purified the SyrB1 and SyrB2 components of the syringomycin synthetase from *P. syringae* (6) in active form. We demonstrate that L-Thr, tethered in thioester linkage to the peptidyl carrier protein domain of SyrB1, is chlorinated to 4-Cl-L-Thr-*S*-SyrB1 by the purified Fe^{II}-containing SyrB2 enzyme in the presence of O_2, α-KG, and chloride ions, providing direct evidence of aliphatic halogenase activity for a nonheme Fe^{II} enzyme.

Materials and Methods

Chemicals. L-[^{14}C]Thr [175 mCi/mmol (1 Ci = 37 GBq)] and [^{36}Cl]NaCl (16 mCi/g Cl) were from American Radiolabeled Chemicals. α-[1-^{14}C]Ketoglutaric acid, sodium salt (54.5 mCi/mmol), and [^{32}P]pyrophosphate were from PerkinElmer. Thrombin was from Novagen. 4-Cl-L-Thr was synthesized by following the method of Webb and Mathews (13). All other chemicals were of analytical grade.

Strains, Media, and Growth. *Escherichia coli* Top10 (Invitrogen) was used for DNA propagation. *E. coli* BL21 (DE3) (Invitrogen), transformed with derivatives of pET28b (Novagen), was used for overexpression in LB medium, supplemented with a potassium phosphate buffer described for Terrific Broth (14). An HCl-solubilized solution of minerals (15) was added for overexpression of the SyrB2 protein. Plasmid pJZ514 (16) containing the *syrB1* and *syrB2* genes was a gift from Dennis C. Gross (Texas A & M University, College Station, TX).

Construction of Plasmids and Overexpression of Proteins. DNA was purified, manipulated, and transformed according to standard protocols (14). The *syrB1* and *syrB2* genes were amplified from the pJZ514 plasmid. The oligo pairs used for PCR amplification are the following: oSyrB1-for-NdeI 5′-GGAATTCCATATGCCGATTACGAACACT-3′; oSyrB1rev-EcoRI 5′-CGGAATTCATCAATGGGCTTGAACAAG-3′ and oSyrB2for-NdeI 5′-GGAATTCCATATGAGCAAAAAATTCGCC-3′ oSyrB2rev-EcoRI 5′-CGGAATTCATCAGACCGCTTCGAATTT-3′. PCR reactions were performed by using the *Pfu*Turbo DNA polymerase (Stratagene) according to the manufacturer's instructions. The resulting amplicons were digested with NdeI and EcoRI and cloned into pET28b (Novagen), yielding the different expression plasmids. The cloned DNA was sequenced to confirm that it contained no errors.

Abbreviation: α-KG, α-ketoglutarate.

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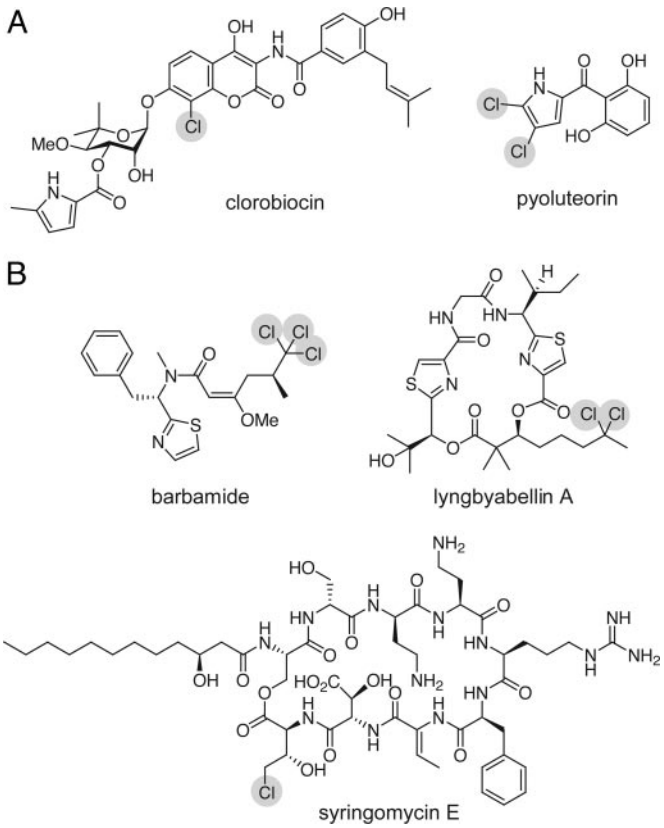

Fig. 1. Structures of chlorine-containing natural products. (*A*) Aromatic and heteroaromatic natural organohalogens. (*B*) Aliphatic natural organohalogens containing one, two, or three chlorines at a single carbon center.

His-tagged Syr proteins were overexpressed in *E. coli* BL21 (DE3) transformed with their respective plasmids. Two-liter cultures were inoculated with 20 ml of an overnight culture grown at 37°C. The cultures were incubated for 9 h at 25°C, then cooled to 15°C for 1 h before the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The cultures were incubated for another 16–17 h, then harvested.

Protein Purification. For SyrB1, a cell pellet from 6 liters of culture was resuspended in buffer A [20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, pH 8.0/300 mM NaCl/5 mM imidazole]. The cells were disrupted by two successive passages through a French press operated at 12,500 psi (1 psi = 6.89 kPa) and 4°C. The cell debris was removed by centrifugation at 27,000 × *g* for 30 min. The supernatant was carefully removed. This "raw extract" was incubated with 4 ml Ni-nitrilotriacetic acid agarose resin (Qiagen) for 1 h and poured in a low-pressure chromatography column (1.5-cm diameter; Bio-Rad). The resin was washed successively with 5 column volumes of buffer A and 4 column volumes of buffer B [20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, pH 8.0/300 mM NaCl/30 mM imidazole] to remove nonspecifically bound contaminants. SyrB1 was eluted by using buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid pH 8.0/100 mM NaCl/200 mM imidazole]. The eluate was concentrated by using Amicon Ultra centrifugal devices (Millipore) and immediately applied to a 26/60 Superdex 200 column (GE Healthcare) equilibrated with buffer D (20 mM Hepes, pH 7.5). The protein fractions were pooled, concentrated, flash-frozen in liquid N_2 in 10–100-μL aliquots, and stored at −80°C until further use.

For SyrB2, all cell-free preparations were manipulated under an inert atmosphere by using an Mbraun Labmaster glove box (Stratham, NH) maintained at 2 ppm O_2 or less. Chromatography was performed by using an ÄKTA Explorer 100 (GE Healthcare) configured to maintain an anaerobic atmosphere during purification, as described in ref. 15. Buffers were sparged with Argon and equilibrated in the glove box for 24 h before use. A cell pellet from 6 liters of culture was resuspended and subjected to the French press as described for SyrB1. The broken cells were then sparged with argon for 20 min before transfer in gas-tight tubes in the glove box and centrifugation at 27,000 × *g* for 30 min. The Ni-nitrilotriacetic acid column was performed as described for SyrB1 except that it was performed in the glove box. The Ni-nitrilotriacetic acid eluate was concentrated by using Amicon stirred ultrafiltration cells (Millipore) under N_2 pressure. SyrB2 was exchanged into buffer E (20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid pH 8.0/80 mM NaCl) by a series of concentrations and dilutions in the ultrafiltration cell. Two-millimolar $CaCl_2$ was added, and the solution was incubated for 3 h at 19°C with thrombin (1 units:10 mg, thrombin:SyrB2). The protein solution was then diluted 1:1 with buffer F (20 mM Tris, pH 8.0) and loaded onto a MonoQ 10/100GL column (GE Healthcare), equilibrated with buffer F (multiple runs were performed). SyrB2 was eluted by using a NaCl gradient of 150–300 mM over 15 column volumes at a flow rate of 4 ml/min. Fractions of 2.5 ml were collected and checked for purity by SDS polyacrylamide gels. SyrB2 eluted at ≈230 mM NaCl. The fractions were pooled, concentrated, and injected on a 26/60 Superdex 75 column equilibrated in buffer D. SyrB2 eluted as a monomer. The fractions were pooled, concentrated, flash-frozen in liquid N_2 in 50–100-μl aliquots, and stored at −80°C until further use. Protein concentrations were determined by the Bradford method (17).

Metal Analysis. The metal content of SyrB2 was investigated by using inductively coupled plasma MS performed by Phytronix Technologies (Quebec). In reconstitution experiments, iron concentrations were determined colorimetrically by using FERENE S (18).

***In Vitro* Reconstitution of SyrB2.** Apo-SyrB2 was reconstituted anaerobically by using two different approaches. In the first approach, SyrB2 was incubated with 1 mM DTT/0.75 mM $Fe(NH_4)_2(SO_4)_2$ for 30 min before desalting on a Bio-Gel P6DG column (Bio-Rad) equilibrated in 20 mM Hepes (pH 7.5) to remove DTT and excess iron. In the second approach, SyrB2 was incubated with 1 mM DTT/0.75 mM $Fe(NH_4)_2(SO_4)_2$/2 mM α-KG for 30 min before desalting on a Bio-Gel P6DG column equilibrated in 20 mM Hepes, pH 7.5/2 mM α-KG.

ATP-[^{32}P]PPi Exchange Assay of the A Domain of SyrB1. The ATP-[^{32}P]PPi exchange assay was performed as described in ref. 19 except that 20 mM Hepes (pH 7.5) was used and no Tris(2-carboxyethyl)phosphine was added.

Priming and Self-Loading Assay of SyrB1. The thiolation domain of SyrB1 was posttranslationally modified with the phosphopantetheinyl group by using the purified Sfp protein (20). For the self-loading assay, SDS polyacrylamide gels of reactions performed in the presence and absence of ATP were done before autoradiography. [^{14}C]Methylated protein standards (GE Healthcare) were coloaded with the broad range standards (Bio-Rad) in the molecular weight standards lanes.

SyrB2 Activity Assays. The reaction catalyzed by SyrB2 was investigated by incubating the enzyme with loaded SyrB1. Loaded SyrB1 was prepared by incubating holo-SyrB1 (100 μM) with 2.5 mM L-[^{14}C]Thr (20 Ci/mol) or 2.5 mM L-Thr and 2.5 mM ATP for 60 min, followed by incubation with 2 mM α-KG and SyrB2 (6.5 μM) for 60 min. Chloride (2.5 mM) was present in the reaction mixture. The resulting reactions were transferred to 0.5 ml Ultrafree cen-

trifugal devices equipped with a 10-kDa membrane cutoff (Millipore). Excess amino acids were removed by using four wash steps with 0.5 ml of 20 mM Hepes, pH 7.5. The resulting protein solution was incubated with 5 μM of the TycF type II thioesterase (21) to release the loaded amino acids. The amino acids were separated from the proteins by centrifuging the filter devices. The amino acids were then derivatized by using *o*-phthalaldehyde and 3-mercaptopropionate as described in amino acid analysis (22) and analyzed by using HPLC on a Beckman System Gold HPLC equipped with a Beckman 171 radioisotope detector or a Jasco FP-2020 fluorescence detector ($\lambda_{excitation}/\lambda_{emission}$, 340 nm/450 nm) and compared with authentic standards. The activity of SyrB2 was also investigated on L-Thr and L-Thr-*S*-*N*-acetylcysteamine (23) at substrate concentrations of 0.1, 1, and 10 mM with α-KG, oxygen, and $MgCl_2$ present.

α-KG and Fe Dependency of the SyrB2 Reaction. The α-KG dependency of the SyrB2 reaction was investigated by using the Fe-reconstituted form of SyrB2 in the absence of α-KG. The reaction was performed with L-[^{14}C]Thr-*S*-SyrB1 as described in the previous section with 15 μM SyrB2 in the absence and presence of 2 mM α-KG. For assessing the Fe dependency of the SyrB2 reaction, the apoprotein (14 μM) supplemented with 2 mM α-KG was used in incubations.

Oxygen Dependency of the SyrB2 Reaction. The oxygen dependency of the SyrB2 reaction was investigated by preparing L-Thr-*S*-SyrB1 as previously described. L-Thr-*S*-SyrB1 was brought into the glove box and desalted on a BioGel P6-DG desalting column equilibrated in 20 mM Hepes, pH 7.5. The fractions containing proteins were pooled and divided in 4 aliquots. Two millimolar α-KG, 1 mM NaCl and 2.6 μM SyrB2 were added to each tube. Two of the tubes were brought out of the box and exposed to air; the other two tubes remained in the glove box for a 60-min incubation. The aerobic and anaerobic reactions were then quenched with the addition of 2 mM 1,10-phenanthroline.

Chloride Dependency of the SyrB2 Reaction. The chloride dependency of the SyrB2 reaction was investigated by using two methods: radio-HPLC and SDS polyacrylamide gels autoradiography by using [^{36}Cl]NaCl. In the first approach, 0.5 ml of 100 μM L-Thr-*S*-SyrB1 was incubated with 1.5 mM α-KG, 8 mM [^{36}Cl]NaCl (16 mCi/g Cl), and 14 μM SyrB2 and exposed to air for 60 min.

In the second approach, 100 μM L-Thr-*S*-SyrB1 and 100 μM holo-SyrB1 were brought into the glove box and desalted on a BioGel P6DG desalting column equilibrated in 20 mM Hepes, pH 7.5. The fractions containing proteins were pooled and incubated with 2 mM α-KG, 35 mM [^{36}Cl]NaCl (16 mCi/g Cl), and 15 μM SyrB2. A control experiment in which L-Thr-*S*-SyrB1 was incubated with α-KG and [^{36}Cl]NaCl, but no SyrB2 was also performed. The reaction mixtures were incubated in air for 60 min before gel loading and autoradiography. [^{14}C]Methylated protein standards (GE Healthcare) were coloaded with the broad range standards (Bio-Rad) in the molecular weight standards lanes.

Monitoring of the SyrB2 Reaction by Using α-[1-^{14}C]KG. The consumption of α-KG by SyrB2 was monitored by following the loss of radioactivity in reactions performed with α-[1-^{14}C]KG. Reactions were performed with 100 μM L-Thr-*S*-SyrB1, 4 mM $MgCl_2$, 1 mM α-[1-^{14}C]KG (18 mCi/mmol), and 7 μM SyrB2 for 1 h. The remaining radioactivity was monitored by using liquid scintillation counting and used to calculate the number of turnovers performed by SyrB2 before inactivation. Control reactions were performed by omitting L-Thr-*S*-SyrB1 or SyrB2 in the mixture.

Liquid Chromatography (LC)-MS Analysis. The masses of the released L-Thr and 4-Cl-L-Thr from SyrB1 by using TycF were monitored after derivatization with *o*-phthalaldehyde and 3-mercaptopropionate. Three hundred-microliter reactions of 100 μM SyrB1 were performed with or without SyrB2. The amino acids were hydrolyzed as previously described and analyzed by LC-MS (positive mode).

Results

Expression and Purification of SyrB1 and SyrB2. The *syrB1* and *syrB2* genes from the *P. syringae* syringomycin biosynthetic cluster were expressed in *E. coli* as His-tagged fusions and purified as soluble proteins, with yields of 30 mg for SyrB1 and 10–12 mg for SyrB2 per liter of bacterial culture. SDS polyacrylamide gels indicated >95% and 99% purity, respectively.

Because we anticipated SyrB2 would be an oxygen-labile Fe^{II} enzyme, the crude extract was immediately kept under N_2 gas and transferred to an anaerobic glove box for purification and storage. The purified SyrB2 was an apoprotein and the His-tag was removed by proteolytic cleavage and chromatography to yield an enzyme that was then subjected to anaerobic reconstitution with Fe^{II}. In the absence of added α-KG, SyrB2 had 0.3–0.4 equivalents of bound Fe^{II} after anaerobic gel filtration. When α-KG was included in the reconstitution mixture, SyrB2 contained 0.85 to 0.95 equivalents of

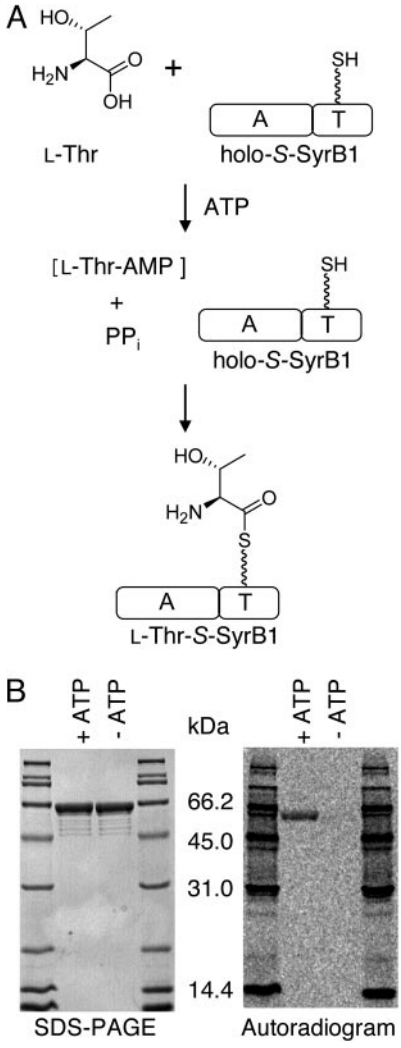

Fig. 2. L-Thr activation and self-loading reaction catalyzed by SyrB1. (*A*) Reaction catalyzed by SyrB1. A, adenylation domain of SyrB1; T, thiolation domain of SyrB1. (*B*) Self-loading reaction catalyzed by SyrB1. Lane 1 and 4, molecular mass standards. Lane 2, holo-*S*-SyrB1, L-[^{14}C]Thr, ATP. Lane 3, holo-*S*-SyrB1, L-[^{14}C]Thr, no ATP.

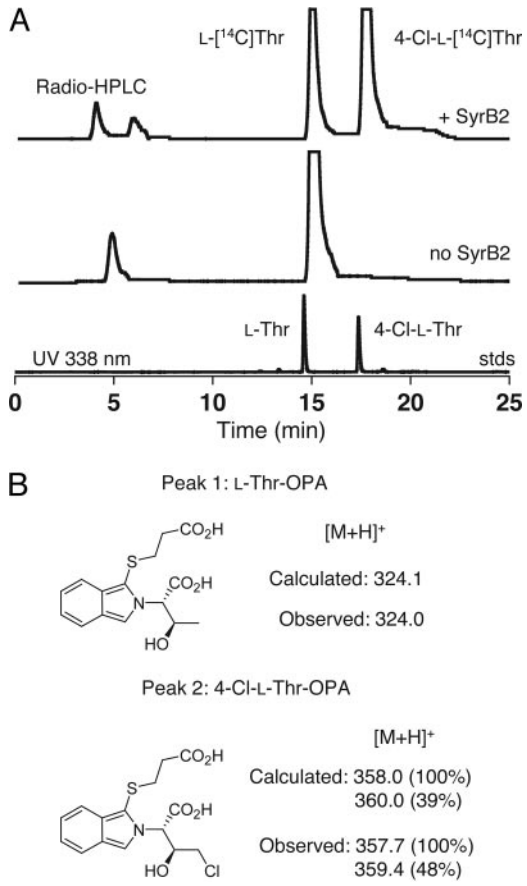

Fig. 3. Formation of 4-Cl-L-Thr by SyrB2. (*A*) HPLC traces of hydrolyzed amino acid obtained after incubation of L-[^{14}C]Thr-*S*-SyrB1 ± SyrB2. (*B*) Mass spectrometric analysis of the isoindole derivatives of L-Thr and 4-Cl-L-Thr hydrolyzed from SyrB1.

Fe^{II} after gel filtration, consistent with the prediction it is an α-KG- and Fe^{II}-requiring enzyme.

Characterization of SyrB1 Activity. SyrB1 is a 66-kDa didomain protein, consisting of an adenylation (A) domain and a thiolation (T) domain. Previous studies had indicated it could activate L-Thr (6) and led to the conclusion that SyrB1 selects and activates Thr-9 in the syringomycin backbone. For our purposes, we wanted to confirm that SyrB1 would show selectivity for L-Thr over 4-Cl-L-Thr as would be anticipated if chlorination occurred after covalent loading and formation of L-Thr-*S*-SyrB1. The first half-reaction of the A domain, reversible formation of L-Thr-AMP, was followed by amino acid-dependent exchange of [^{32}PPi] into ATP. In the presence of 5 mM amino acid, the relative rates were obtained as follows: L-Thr (100 ± 3.6%), L-Ser (3.1 ± 0.1%), 4-Cl-L-Thr (1.14 ± 0.01%), D-Thr (1.01 ± 0.01%), L-Ile (0.40 ± 0.01%), L-Leu (0.33 ± 0.01%), and D-Ser (0.24 ± 0.04%). All other proteinogenic L-amino acids were tested and showed a rate of 0.25% or less when compared with L-Thr. The high specificity of SyrB1 for L-Thr was confirmed by a 60-fold selectivity in the apparent kinetic parameters for L-Thr (K_m = 3.1 ± 0.2 mM; k_{cat} = 29.1 ± 0.9 min^{-1}; k_{cat}/K_m = 9.4 ± 0.4 $mM^{-1}min^{-1}$) vs. L-Ser (K_m = 7.7 ± 0.5 mM; k_{cat} = 1.23 ± 0.05 min^{-1}; k_{cat}/K_m = 0.161 ± 0.006 $mM^{-1}min^{-1}$). Most notably, SyrB1 shows low ability to activate 4-Cl-L-Thr, consistent with chlorination downstream of amino acid activation.

The second half-reaction of SyrB1 should be the transfer of the activated threonyl moiety in L-Thr-AMP to the phosphopantetheinyl moiety on the T domain (Fig. 2*A*). The holo form (phosphopantetheinylated) of the T domain was generated posttranslationally *in situ* through action of Sfp (20) on purified SyrB1. Autoaminoacylation of holo SyrB1 in an ATP-dependent manner is then demonstrated by the autoradiogram in Fig. 2*B* with L-[^{14}C]-

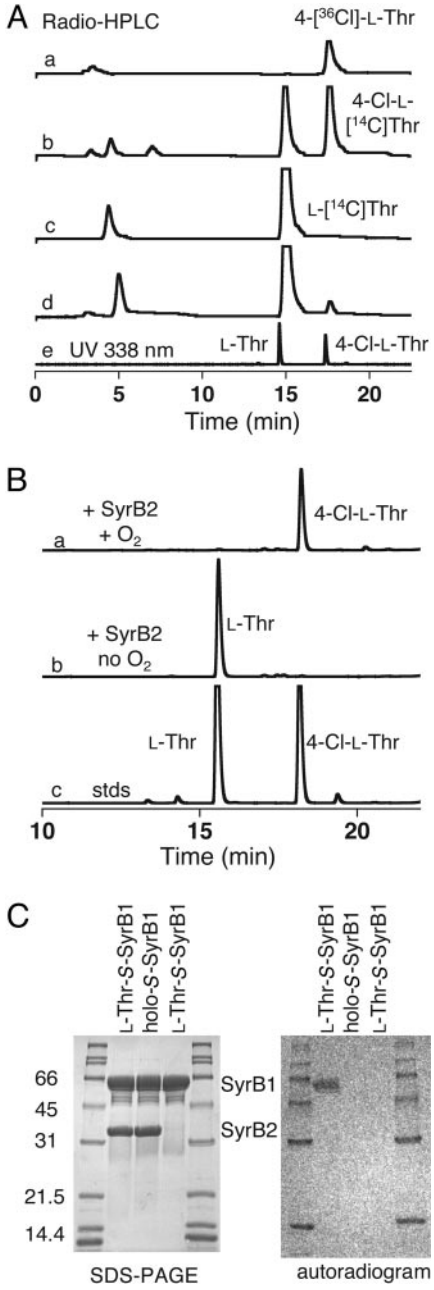

Fig. 4. Dependencies of the SyrB2 reaction. (*A*) HPLC traces of hydrolyzed amino acid obtained after incubation of L-Thr-*S*-SyrB1 with SyrB2. (*Aa*) Reaction performed with L-Thr-*S*-SyrB1, α-KG and SyrB2 in presence of Na[^{36}Cl]. (*Ab*) Reaction performed with L-[^{14}C]Thr-*S*-SyrB1, chloride, α-KG, and SyrB2. (*Ac*) Reaction performed with L-[^{14}C]Thr-*S*-SyrB1, chloride, and SyrB2 in the absence of α-KG. (*Ad*) Reaction performed with L-[^{14}C]Thr-*S*-SyrB1, α-KG, chloride, and apo-SyrB2 (< 5% Fe^{II}). (*Ae*) UV trace of L-Thr and 4-Cl L-Thr standards. (*B*) Fluorescence HPLC traces showing the oxygen dependency of the SyrB2 reaction. (*Ba*) Reaction performed with L-Thr-*S*-SyrB1, chloride, α-KG, and SyrB2 in air. (*Bb*) Reaction performed with L-Thr-*S*-SyrB1, chloride, α-KG, and SyrB2 in the absence of oxygen. (*Bc*) UV trace of L-Thr and 4-Cl L-Thr standards. (*C*) Incorporation of [^{36}Cl]chloride into L-Thr-*S*-SyrB1 by SyrB2. Lane 1 and 5, molecular mass standards. Lane 2, L-Thr-*S*-SyrB1, Na[^{36}Cl], and SyrB2. Lane 3, holo-*S*-SyrB1, Na[^{36}Cl], and SyrB2. Lane 4, L-Thr-*S*-SyrB1 and Na[^{36}Cl]. α-KG and O_2 were present in all assays (lanes 2–4).

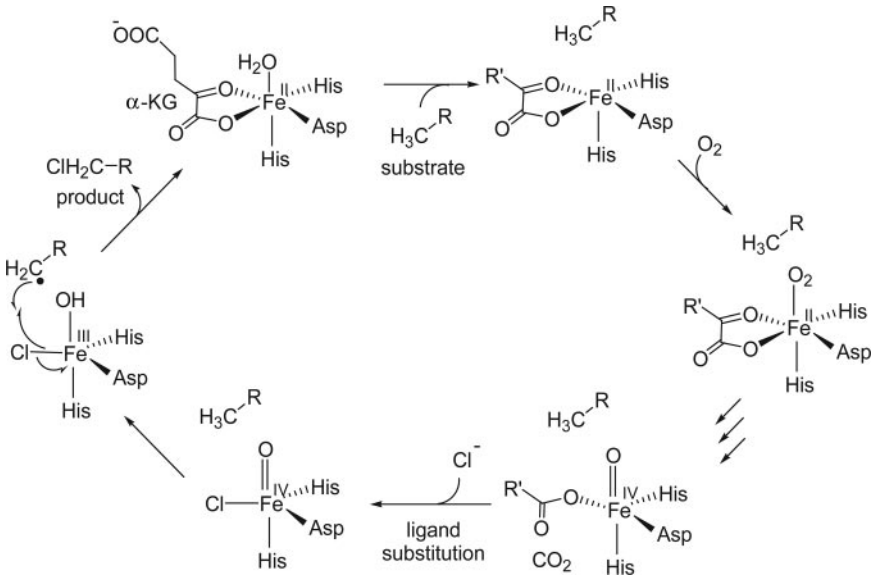

Fig. 5. Proposed mechanism of halogenation catalyzed by SyrB2 involving formation of the Fe^{IV}═O intermediate characteristic of this enzyme class but its diversion for chlorination rather than hydroxylation. R-CH_3 is the C_4 methyl group of the L-Thr-*S*-pantetheinyl-SyrB1 protein substrate.

Thr as substrate. This L-Thr-*S*-SyrB1 enzyme thioester was used as the substrate for SyrB2.

Chlorination Activity of SyrB2. To evaluate the activity of SyrB2, the enzyme was removed from anaerobic storage just before incubations and added to L-Thr-*S*-SyrB1. In the presence of the three cosubstrates (O_2, α-KG, and chloride ions), halogenation activity was observed. Given that the amino acid is covalently tethered to the T domain of SyrB1, detection of product involved the release of the aminoacyl moieties by enzymatic treatment with a thioesterase, TycF. The amount of 4-Cl-L-Thr product is, at best, stoichiometric with the amount of SyrB1 (10 nmol) present in the incubations as protein substrate. To facilitate detection, the amino acids were derivatized with *o*-phthalaldehyde and 3-mercaptopropionate to yield the highly fluorescent isoindole derivatives (22). As shown in Fig. 3*A*, the enzymatic incubation with SyrB2 gave both L-[^{14}C]-Thr substrate-derived isoindole and 4-Cl-L-[^{14}C]Thr product-derived isoindole. Product formation depended on SyrB2. In addition to the radio-HPLC cochromatography with authentic isoindole standards (Fig. 3*A*), the SyrB1/B2 incubations were scaled up with nonradioactive L-Thr as substrate and subjected to MS to confirm identification. As noted in Fig. 3*B*, peak 1 from the HPLC of the enzymatic reaction workup gave the L-Thr-derived isoindole while peak 2 had the mass of the 4-Cl-L-Thr adduct, containing the isotopic signature of a chlorine atom.

Validation of the dependence of SyrB2 on the three small molecule cosubstrates is shown in Fig. 4 *A* and *B*. In the absence of α-KG (trace c of Fig. 4*A*), there is no chlorination. Likewise, in trace b of Fig. 4*B*, the dependence on O_2 as cosubstrate is demonstrated. Incorporation of [$^{36}Cl^-$] is shown in trace a of Fig. 4*A*. Additionally, it was possible to demonstrate incorporation of radioactive chloride ion into the SyrB1 protein by using SDS polyacrylamide gels (Fig. 4*C*) that depends on the presence of L-Thr, interpreted as formation of [^{36}Cl]-L-Thr-*S*-SyrB1. In studies not shown, we demonstrated that SyrB2 would not chlorinate free L-Thr or the small molecule surrogate for L-Thr-*S*-SyrB1, L-Thr-*S*-*N*-acetylcysteamine (23).

Many nonheme Fe^{II} enzymes that use O_2 and α-KG catalyze their own autooxidative destruction after a small number of turnovers, presumably due to the decomposition of high-valent oxoiron species (24). SyrB2 was similarly labile. By monitoring the consumption of α-[1-^{14}C]KG, SyrB2 was shown to catalyze 7 ± 2 turnovers before inactivation. Kinetic analysis of the chlorination reaction remains to be undertaken, given the difficulties in determining the conversion of a 66-kDa aminoacyl-*S*-protein substrate to a chloro-aminoacyl-*S*-protein product.

Discussion

This study demonstrates that SyrB2 is a nonheme Fe^{II}-containing enzyme that has a previously uncharacterized catalytic activity, the ability to halogenate an aliphatic methyl group on a protein-bound threonyl thioester. In the course of this initial work, we repeated the earlier demonstration that SyrB1 activated L-Thr and proved in addition that it would install the threonyl moiety on its thiolation domain. This reaction was enabled by *in vitro* phosphopantetheinylation of SyrB1 to convert it from the apo form to the holo form of the T domain, able to engage in aminoacyl-*S*-T domain formation. In turn, this L-Thr-*S*-SyrB1 constitutes the substrate for SyrB2.

SyrB2 is thus a tailoring enzyme working *in trans* on the threonyl group presented by the SyrB1 T domain scaffold. Our anaerobic purification and reconstitution of SyrB2 with Fe^{II} was designed to minimize any untoward oxidative damage of the active site from adventitious uncoupled reaction with O_2. The successful reconstitution with Fe^{II} validated bioinformatics predictions that SyrB2 would be an Fe^{II} enzyme. Further, the increase to full stoichiometry of Fe^{II} reconstitution in the presence of α-KG indicated that α-KG is a stabilizing ligand for Fe^{II}, as noted for other enzymes in this enzyme superfamily (24).

The previously described members of the α-KG-dependent nonheme Fe^{II} enzymes bind α-KG as a ligand to iron. After decarboxylation in the presence of cosubstrate O_2, succinate and CO_2 are generated together with a high-valent Fe^{IV}═O oxoiron species, which is a potent oxidizing and hydroxylating species toward bound substrate (25). Indeed, assays of SyrB2 show a comparable requirement for both O_2 and α-KG for catalytic turnover. The product outcome, however, is divergent. SyrB2 chlorinates but does not detectably hydroxylate L-Thr-*S*-SyrB1. The incorporation of chloride into the threonyl scaffold was validated by cochromatography of the released product as the isoindole derivative, incorporation of radioactive [$^{36}Cl^-$], and MS with the signature isotopic doublet for a monochlorinated compound.

Mechanistic studies remain for this transformation, but the proposed pathway in Fig. 5 is likely. As with other O_2 cleaving, α-KG-decarboxylating enzymes, one can find conserved histidines

and aspartate as putative ligands for Fe^{II} (26–27). The formation of the Fe^{IV}═O ferryl species is likely to be a common intermediate in both the oxygenase and halogenase mechanism. The high-valent oxoiron species can break unactivated C-H bonds in substrates homolytically to produce the substrate radical and an Fe^{III}-OH as donor of an OH· by radical rebound to complete hydroxylation (24, 25, 28). We anticipate that there will be a chloride ion bound to the Fe^{IV} intermediate of the SyrB2 active site. After C-H homolysis at carbon 4 of the threonyl moiety of L-Thr-*S*-SyrB1, there could be a competition between transfer of OH· or Cl· to the threonyl CH_2·. Because no hydroxylation is detected (data not shown), we assume an orientation favoring Cl· transfer. A favored reactivity toward chlorination could also be due to the lower potential of Cl· vs. OH· (29). This proposed enzyme chemistry has precedent in two model systems where transfers of metal-coordinated Cl· have been formulated: chromyl chloride-mediated chlorination of cyclohexane (30, 31) and, perhaps of even closer relevance, the chlorination of cyclohexane by a nonheme Fe-Cl reagent (29). The SyrB2 enzyme is generating reactive intermediates because the enzyme is inactivated after approximately seven catalytic cycles under the reaction conditions.

SyrB2 will not chlorinate free L-Thr or the threonyl moiety presented as the model thioester, L-Thr-*S*-*N*-acetylcysteamine, suggesting a strong recognition of the presenting T domain scaffold. This preference suggests the *in vivo* timing of SyrB2 is indeed to tailor the threonyl moiety while on SyrB1. Presumably, 4-Cl-L-Thr-*S*-SyrB1 is then the immediate donor to the growing octapeptidyl-*S*-SyrE and incorporated as the Cl-Thr-9 found in mature syringomycin (6). We have recently reported that PltA is analogously a tailoring halogenase during elongation of a pyrrolyl-*S*-T domain during pyoluteorin formation by the hybrid nonribosomal peptide synthetase/polyketide synthase assembly line (P. C. Dorrestein, E. Yeh, S. Garneau, N. L. Kelleher, and C.T.W., unpublished data). In that case, PltA is an $FADH_2$- and O_2-dependent halogenase rather than an α-KG- and O_2-dependent nonheme Fe^{II} halogenase. The timing of chlorination, of acyl and aminoacyl substrates presented on carrier domain proteins, may be general for other halogenated nonribosomal peptide and polyketide natural products, e.g., in the vancomycin family of chlorinated glycopeptide antibiotics (2).

Although attention has focused in recent years on the $FADH_2$- and O_2-dependent class of halogenases as the biosynthetically relevant halogenases for natural products because they are encoded within many biosynthetic gene clusters, the flavoprotein halogenases work primarily on activated aromatic and heteroaromatic rings of natural product scaffolds (9–12). They may not generally have the oxidizing/halogenating power to functionalize unactivated carbon centers.

In this regard, nature usually turns to high-valent oxoiron, both in heme and nonheme contexts (24, 26, 32), to hydroxylate unactivated methyl and methylene groups. This type of reaction may be the chemical niche for nonheme Fe^{II} halogenases. It is very likely that a second example of this nonheme Fe^{II} halogenase class is the BarbB1/B2 pair, implicated in functionalizing the unactivated methyl group of a L-Leu moiety during trichloroleucine formation as a building block in barbamide biosynthesis (8). Thus, a subclass of the nonheme Fe^{II} α-KG-dependent superfamily may use α-KG and O_2 to generate high-valent oxoiron species but divert them for halogenation, rather than hydroxylation, of many unactivated carbon centers in the >4,000 chlorinated and brominated natural products. Deciphering how the diversion from hydroxylation to halogenation occurs during natural product maturation remains a significant challenge.

We thank Dennis C. Gross (Texas A & M University) for providing plasmid pJZ514 containing the *syrB1* and *syrB2* genes and David A. Vosburg and Ellen Yeh for carefully reading the manuscript. This work was supported in part by National Institutes of Health Grants GM 20011 and GM 49338 (to C.T.W.), a Merck-sponsored Fellowship of the Helen Hay Whitney Foundation (to F.H.V.), and a Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship (to F.H.V.).

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