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SyrB2 in syringomycin E biosynthesis is a nonheme Fe\textsuperscript{II} \(\alpha\)-ketoglutarate- and \(O_2\)-dependent halogenase

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Contribution by Christopher T. Walsh, May 27, 2005

The nine-residue lipodepsipeptide syringomycin E, elaborated as a phytotoxin by \textit{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 aminocycl-S-enzyme L-Thr-S-SyrB1. SyrB2 is a member of the nonheme Fe\textsuperscript{II} \(\alpha\)-ketoglutarate-dependent enzyme superfamily, and requires \(O_2\) and \(\alpha\)-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 [\(^{35}\)Cl]NaCl as well as MS of the released products further validated identification. Enzymatic oxidative halogenation is a previously uncharacterized reaction type for nonheme Fe\textsuperscript{II} enzymes and may be the general mode for biosynthetic halogenation of aliphatic carbons of natural products.

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-\(\beta\)-OH-Tyr of the glycopeptides, the chloro substituent in the aminocoumarin antibiotics (2–4), and the 4,5-dichloroppyrrole in pyoluteorin (5) (Fig. L4). 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 \textit{Pseudomonas syringae pv. syringae} B301D (6), the dichlorinated \(\beta\)-hydroxy acid of lyngbyabellin A (7), and the trichloroleucine-derived moiety in barbamide (8) (Fig. 1B). 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 pyrrolinitrin 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 \(O_2\) for enzyme in the presence of \(O_2\), \(\alpha\)-KG, and chloride ions, providing direct evidence of aliphatic halogenase activity for a nonheme Fe\textsuperscript{II} enzyme.

Materials and Methods

Chemicals. L-\([^{14}\text{C}]\)Thr [175 mCi/mmol (1 Ci = 37 GBq)] and \([^{35}\text{Cl}]\)NaCl (16 mCi/g Ci) were from American Radiolabeled Chemicals. \(\alpha\)-[\(^{1}\text{H}\)-Ketoglutaric acid, sodium salt (54.5 mCi/mmol), and \([^{32}\text{P}]\)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. \textit{Escherichia coli} Top10 (Invitrogen) was used for DNA propagation. \textit{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 \textit{syrB1} and \textit{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 \textit{syrB1} and \textit{syrB2} genes were amplified from the pJZ514 plasmid. The oligo pairs used for PCR amplification are the following: oSyrB1for-NDel 5\'\'-GGAATTCATATGCGAT-TACGAAACACT-3\'; oSyrB1rev-EcoRI 5\'\'-CGGAATTCTCAT-CATGGGCTTGAACAGAAG-3\'; oSyrB2for-NDel 5\'\'-GGAATTTCAATGAGCAGAAAATCGCC-3\'; oSyrB2rev-EcoRI 5\'\'-GGAATTCTCAGACCCATTGTCATTT-3\'. PCR reactions were performed by using the PfuTurbo DNA polymerase (Stratagene) according to the manufacturer’s instructions. The resulting amplicons were digested with NDel 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: \(\alpha\)-KG, \(\alpha\)-ketoglutarate.

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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 (Millipore) under N2 pressure. SyrB2 was exchanged into 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 N2 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 Phytotronix Technologies (Quebec). In reconstitution experiments, iron concentrations were determined colorimetrically by using FEREN® 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(NH4)2(SO4)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(NH4)2(SO4)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-[32P]PPi Exchange Assay of the A Domain of SyrB1. The ATP-[32P]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 phosphopantetheinyll 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. [14C]Methylated protein standards (GE Healthcare) were loaded 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-[35S]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-
centrations of 0.1, 1, and 10 mM with omitting L-Thr-
by SyrB2 before inactivation. Control reactions were performed by
SyrB2. A control experiment in which L-Thr-
was performed with L-[14C]Thr-
incubations.

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-[^14C]Thr-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-
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 [56Cl]NaCl. In the first approach, 0.5 μl of 100 μM L-Thr-
S-SyrB1 was incubated with 1.5 mM α-KG, 8 mM [56Cl]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-SyrB2 were 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 incubated with 2 mM α-KG, 35 mM [56Cl]NaCl (16 mCi/g Cl), and 15 μM SyrB2. A control experiment in which L-Thr-S-SyrB1 was incubated with α-KG and [56Cl]NaCl, but no SyrB2 was also performed. The reaction mixtures were incubated in air for 60 min before gel loading and autoradiography. [14C]Methylated protein standards (GE Healthcare) were coloaded with the broad range standards (BioRad) in the molecular weight standards lanes.

Monitoring of the SyrB2 Reaction by Using α-[1-14C]KG. The consumption of α-KG by SyrB2 was monitored by following the loss of radioactivity in reactions performed with α-[1-14C]KG. Reactions were performed with 100 μM L-Thr-S-SyrB1, 4 mM MgCl2, 1 mM α-[1-14C]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 FeII
enzyme, the crude extract was immediately kept under N2 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 FeII. In the absence of added α-KG, SyrB2 had 0.3–0.4 equivalents of bound FeII after anaerobic gel filtration. When α-KG was included in the reconstitution mixture, SyrB2 contained 0.85 to 0.95 equivalents of

![Fig. 2.](image-url)

**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-SyrB1-L-[14C]Thr, ATP. Lane 3, holo-
SyrB1-L-[14C]Thr, no ATP.
FeII after gel filtration, consistent with the prediction it is an α-KG- and FeII-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-SyrB1. The first half-reaction of the A domain, reversible formation of L-Thr-AMP, was followed by amino acid-dependent exchange of [32PPi] into ATP. In the presence of 5 mM amino acid, the relative rates were obtained as follows: L-Thr (100%), L-Ser (3.1%), 4-Cl-L-Thr (1.14%), D-Thr (1.01%), L-Ile (0.40%), L-Leu (0.33%), and D-Ser (0.24%). 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 (K_m = 9.4 ± 0.4 mM, k_cat/K_m = 1.23 ± 0.05 min^{-1}; k_cat/K_m = 0.161 ± 0.006 min^{-1}) vs. L-Ser (K_m = 7.7 ± 0.5 mM; k_cat/K_m = 0.291 ± 0.09 min^{-1}; k_cat/K_m = 9.4 ± 0.4 mM). 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 phosphopantetheinyln moity on the T domain (Fig. 2A). The holo form (phospho-pantetheinylated) 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. 2B with l-[14C]-
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 (O₂, 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 α-phthalaldehyde and 3-mercaptopropionate to yield the highly fluorescent isoindole derivatives (22). As shown in Fig. 3A, the enzymatic incubation with SyrB2 gave both l-[¹⁴C]-Thr substrate-derived isoindole and 4-Cl-l-[¹⁴C]Thr product-derived isoindole. Product formation depended on SyrB2. In addition to the radio-HPLC chromatography with authentic isoindole standards (Fig. 3A), the SyrB1/SyrB2 incubations were scaled up with nonradioactive l-Thr as substrate and subjected to MS to confirm identification. As noted in Fig. 3B, 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. 4A and B. In the absence of α-KG (trace c of Fig. 4A), there is no chlorination. Likewise, in trace b of Fig. 4B, the dependence on O₂ as cosubstrate is demonstrated. Incorporation of [³⁵Cl] is shown in trace a of Fig. 4A. Additionally, it was possible to demonstrate incorporation of radioactive chloride ion into the SyrB1 protein by using SDS polyacrylamide gels (Fig. 4C) that depends on the presence of l-Thr, interpreted as formation of [³⁵Cl]-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 O₂ and α-KG catalyze their own autoxidative 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-[¹³C]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 O₂. The successful reconstitution with Fe(II) validated bioinformatics predictions that SyrB2 would be an Fe(II) enzyme. Further, the increase to full stoichiometry with Fe(III) reconstitution in the presence of α-KG indicated that α-KG is a stabilizing ligand for Fe(III), 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 O₂, succinate and CO₂ 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 O₂ 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 chlorine into the threonyl scaffold was validated by chromatography of the released product as the isoindole derivative, incorporation of radioactive [³⁵Cl⁻], 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 O₂ cleaving, α-KG-decarboxylating enzymes, one can find conserved histidines
In that case, PltA is an FADH$_2$- and O$_2$-dependent halogenase (Yeh, S. Garneau, N. L. Kelleher, and C.T.W., unpublished data).

SyrB2 will not chlorinate free l-Thr or the threonyl moiety presented as the model thioester, l-Thr-S-N-acetylcyesteamine, 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 synrgomycin (6). We have recently reported that PtaA 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).

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.

This in 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$^{III}$ halogenases. It is very likely that a second example of this nonheme Fe$^{III}$ 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$^{III}$-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.

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