Dichlorination of a pyrrolyl-*S*-carrier protein by $FADH_2$-dependent halogenase PltA during pyoluteorin biosynthesis

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The antifungal natural product pyoluteorin contains a 4,5-dichloropyrrole moiety. The timing of dichlorination in the heteroaromatic ring is now shown to occur after proline is tethered by thioester linkage to the carrier protein PltL and enzymatically desaturated to the pyrrolyl-*S*-PltL. Surprisingly, the $FADH_2$-dependent halogenase PltA catalyzes chlorination at both positions of the ring, generating the 5-chloropyrrolyl-*S*-PltL intermediate and then the 4,5-dichloropyrrolyl-*S*-PltL product. PltA activity strictly depends on a heterologous flavin reductase that uses NAD(P)H to produce $FADH_2$. Electrospray ionization–Fourier transform MS detected five covalent intermediates attached to the 11-kDa carrier protein PltL. Tandem MS localized the site of covalent modification on the carrier protein scaffold. HPLC analysis of the hydrolyzed products was consistent with the regiospecific chlorination at position 5 and then position 4 of the heteroaromatic ring. A mechanism for dichlorination is proposed involving formation of a FAD-4a-OCl intermediate for capture by the electron-rich C_4 and C_5 of the heteroaromatic pyrrole moiety.

electrospray ionization–Fourier transform MS | clorobiocin | coumermycin | flavin reductase | pyrrole biosynthesis

There are >4,000 halogenated natural products, over half of which contain carbon-chlorine bonds (1). Genes encoding halogenases responsible for forming these bonds are embedded in many polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) biosynthetic gene clusters (2–6). The antibiotics vancomycin **1** (6, 7) and clorobiocin **2** (5) and the antitumor agent rebeccamycin **3** (8) are regiospecifically chlorinated at one or more positions on aromatic or heteroaromatic rings. Antifungal agents pyrrolnitrin **4** (9) and pyoluteorin **5** (4) also carry chlorine atoms on pyrrole moieties. Addition of electronegative chlorine often has significant effects on bioactivity. For example, the deschloro analog of the antibiotic clorobiocin was 8-fold less active against *Bacillus subtilis* (5). Removal of the chlorine atoms from balhimycin, a glycopeptide antibiotic, resulted in an 8- to 16-fold reduction in the activity against a variety of pathogenic bacteria, whereas bromobalhimycin in which chlorine is replaced by bromine groups was twice as active against *Enterococcus faecium* but 8-fold less active against *Staphylococcus aureus* (10). Characterization of the chlorination step, including timing, substrate tolerance, regiospecificity, and mechanism, is critical for unlocking the potential for natural product diversification for these biologically important compounds.

The antifungal natural product pyoluteorin is a hybrid polyketide-nonribosomal peptide molecule. Ten biosynthetic genes, *pltLABCDEFGMR*, are clustered in a 24-kb genomic region of *Pseudomonas fluorescens* Pf-5 (4). We have previously demonstrated the formation of the pyrrolyl ring in pyoluteorin (Fig. 1*B*): PltF, a prolyl-AMP ligase, activates proline and installs it as a thioester on the phosphopantetheinyl arm of carrier protein PltL. Tandem four-electron oxidation of the prolyl-*S*-PltL by flavoprotein PltE yields the pyrrolyl-*S*-PltL (11). Subsequently, type I PKSs PltB and PltC are responsible for construction of the resorcinol ring (4). Of the remaining *orf*s, PltR is a transcriptional regulator for *plt* gene transcription, and PltG is a thioesterase (4). Finally the remaining gene products, PltA, PltD, and PltM, are homologous to $FADH_2$-dependent halogenases found in other NRPS and PKS biosynthetic gene clusters. PltD lacks a highly conserved FAD-binding sequence and is likely not functional (4), raising the prospect that PltA and PltM could be responsible for each of two chlorination steps in pyoluteorin formation.

After formation of the pyrrolyl-*S*-PltL by the action of PltE or equivalent flavoprotein dehydrogenases (11, 12), we assessed the ability of the putative halogenases, PltA and PltM, to carry out chlorination of the pyrrole ring. We used electrospray ionization–Fourier transform MS (ESI-FTMS) analysis to observe the different acyl species tethered to the intact PltL. Surprisingly, a single halogenase, PltA, carries out both chlorination steps on the acylated protein, pyrrolyl-*S*-PltL, in the presence of $FADH_2$ and chloride ions. Dichlorination by PltA demonstrates $FADH_2$-dependent halogenase activity in a NRPS/PKS pathway and opens the way for further characterization of this tailoring function in these carrier protein systems.

Experimental Procedures

Detailed procedures for cloning (Table 1, which is published as supporting information on the PNAS web site), expression, and purification of PltA, PltM, PltL, and SsuE, preparation of ^{13}C-^{15}N-depleted PltL, HPLC separation of the acyl-PltL species, PltA reaction time course, and synthesis of the chlorinated pyrrole-2-carboxylate standards are described in *Supporting Text*, which is published as supporting information on the PNAS web site.

MS of Acyl-PltL Species. For MS analysis, a custom 8.5-tesla ESI-FTMS mass spectrometer was used that was equipped with a front-end quadrupole (13) and automated Nanospray (Advion Biosciences, Ithaca, NY).

Formation of the Substrate Pyrrolyl-*S*-PltL. To generate the ^{13}C-^{15}N-depleted pyrrolyl-*S*-PltL, 3 μl of 126 μM Sfp, 3 μl of 800 mM $MgCl_2$, and 30 μl of 3.3 mM CoA were added to 300 μl of 58 μM apo ^{13}C-^{15}N-depleted apo PltL and allowed to incubate for 1 h. Holo-PltL (300 μl) was diluted with 300 μl of Tris·Cl (pH 7.4) and 1 mM Tris-(2-carboxyethyl)phosphine. To acylate the holo-PltL with proline, 12 μl of CouN4 (5.04 mg/ml), 6 μl of 500 mM L-proline, and 24 μl of 100 mM ATP were added. The pyrrolyl-*S*-PltL was generated by adding 60 μl of 32 μM CloN3 and 18 μl

Abbreviations: ESI-FTMS, electrospray ionization–Fourier transform MS; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase.

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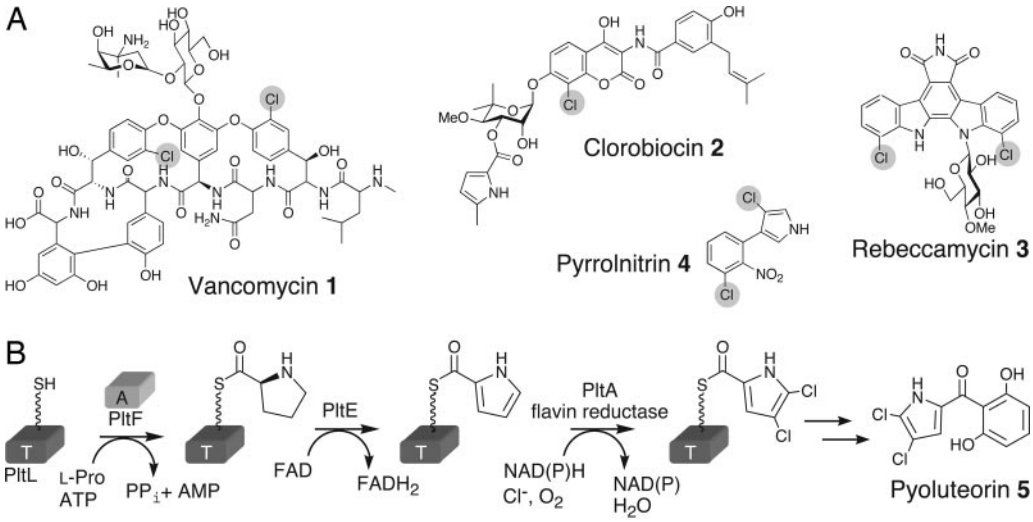

Fig. 1. Flavin-dependent halogenases in natural product biosynthesis. (*A*) Natural products containing chlorinated aromatic and heteroaromatic rings. (*B*) Formation of the dichloropyrrole moiety during pyoluteorin biosynthesis

of 6 mM FAD to 600 μl of the above reaction mixture and incubated for 3 h at room temperature.

Formation of the Dichloropyrrolyl-*S*-PltL. To 110 μl of the pyrrolyl-*S*-PltL reaction mixture prepared above, 10 μl of 262 μM PltA, 10 μl of 129 μM PltM, 10 μl of 142 μM SsuE, 10 μl of 56 mM NADH, and 10 μl of 5M NaCl were added and allowed to incubate for 1 h before quenching 1:1 with 160 μl of 10% formic acid. The reaction mixture was analyzed by HPLC, and fractions from 18.0 to 19.6 min as 0.1- to 0.2-min increments were collected. Samples were lyophilized and redissolved in 60 μl of 78% acetonitrile and 0.2% acetic acid and subsequently analyzed by MS.

Radio-HPLC Analysis for Identification of PltA Reaction Products. Holo-PltL was prepared by *in vitro* phosphopantetheinylation using Sfp as described above. The holo-protein was then loaded with 0.25 mM L-[^{14}C]proline (100 Ci/mol; PerkinElmer) in the presence of 3 mM ATP, 5 mM $MgCl_2$, and 8 μM CouN4 for 1 h. Addition of 24 μM CloN3 and 100 μM FAD allowed formation of [^{14}C]pyrrolyl-*S*-PltL as a substrate for subsequent chlorination reactions. The substrate was incubated with 4 mM NADPH, 12 μM SsuE, and 30 μM PltA and/or PltM for 10–60 min (final reaction volume 40 μl). At the indicated times, the reaction was diluted 15-fold in cold buffer (75 mM Tris, pH 7.5) and centrifuged in a 5-kDa cut-off Ultrafree filter device (Millipore). Unreacted amino acid and cofactors were removed in three additional wash steps. The resulting protein was incubated with 10 μM TycF thioesterase (14) to release protein-bound products. Protein was removed by centrifugation through filter devices. The filtrant was analyzed on a C18 column (Vydac, Hesperia, CA) using gradient 100% A for 2 min, then increasing 0–15% B over 2 min, and finally 15–50% B over 16 min (A = H_2O, 0.1% trifluoroacetic acid; B = MeCN). The instrument used for analysis was a HPLC (Beckman) equipped with a flow-through radiodetector (β–RAM model 3; IN/US, Tampa, FL).

Chloride and Bromide Competition. To 110 μl of the pyrrolyl-*S*-PltL reaction mixture as prepared above (which contains ≈40 mM chloride from the buffer), 10 μl of 262 μM PltA, 10 μl of 129 μM PltM, 10 μl of 142 μM SsuE, 10 μl of 56 mM NADH, and 10 μl of 5 M NaBr were added. Samples were prepared and analyzed as described above for the reaction forming dichloropyrrolyl-*S*-PltL.

Results

Purification of PltA, PltM, and SsuE. After protein overproduction in *Escherichia coli* host, both PltA and PltM were purified to homogeneity; PltD was insoluble. PltM was purified as the apo protein and did not contain bound flavin. Attempts to bind FAD to PltM did not yield holo-protein. In contrast, purified PltA contained tightly bound FAD.

It has been shown previously that $FADH_2$-dependent halogenases require a separate flavin reductase for catalysis (15). Flavin reductases from heterologous systems may be used to fulfill this function. Because no flavin reductase is encoded in the pyoluteorin cluster, a previously characterized NAD(P)H-dependent flavin reductase from the two-component alkanesulfonate monooxygenase system was used instead (16). The reductase SsuE derives reducing equivalents from NAD(P)H to carry out reduction of FAD to $FADH_2$. The reduced cofactor then diffuses from the active site of the reductase and is available for subsequent oxidation reactions carried out in the active site of a second protein.

Formation of Pyrrolyl-*S*-PltL, the Substrate for Halogenation. As previously demonstrated in the pyoluteorin, coumermycin, and clorobiocin system (11, 12), pyrrolyl-*S*-PltL was generated in a three-step reaction that was followed by HPLC and ESI-FTMS (Fig. 6, which is published as supporting information on the PNAS web site). Apo-PltL [retention time (RT) = 19.7 min] was incubated with Sfp, a broad specificity phosphopantetheinyltransferase from *B. subtilis* (17), and CoA to generate the phosphopantetheinylated holo-PltL (RT = 18.8 min). Holo-PltL was then acylated with proline by using the adenylating enzyme CouN4 from the coumermycin biosynthetic pathway (12). Although CouN4 is not the authentic adenylating enzyme, stoichiometric conversion of holo-PltL to prolyl-*S*-PltL (RT = 18.4 min) was observed by HPLC. Subsequently, prolyl-*S*-PltL underwent a four-electron oxidation to pyrrolyl-*S*-PltL (RT = 19.0 min) catalyzed by the flavoprotein dehydrogenase CloN3 from the clorobiocin biosynthetic pathway. Judged by the shift in retention time on HPLC analysis, only 10–40% conversion of the prolyl-*S*-PltL to the pyrrolyl-*S*-PltL was observed during the 3-h incubation. Each step in the formation of pyrrolyl-*S*-PltL was also monitored by high-resolution MS, and the expected mass changes of +340 Da upon phosphopantetheinylation, +97 Da upon acylation with proline, and −4 Da upon oxidation to the pyrrolyl-*S*-PltL were observed. Tandem MS using collisionally

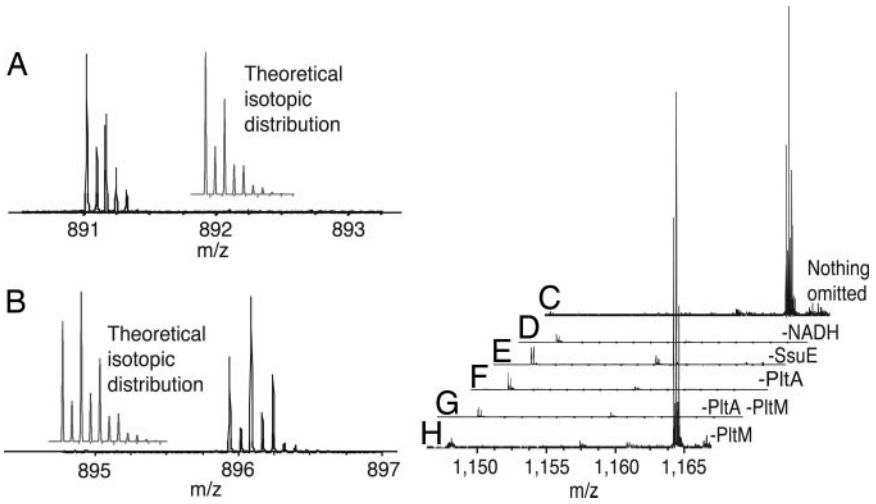

Fig. 2. Chlorination of pyrrolyl-*S*-PltL by PltA. (*A*) Comparison of the theoretical isotopic distribution to the experimental data for prolyl-*S*-PltL. (*B*) Comparison of the theoretical isotopic distribution to the experimental data for dichloropyrrolyl-*S*-PltL. (*C*) Acyl-PltL species resulting from a reaction containing pyrrolyl-*S*-PltL, FAD, chloride, SsuE, PltA, PltM, and NADH were separated by HPLC and analyzed by ESI-FTMS. MS analysis of the fraction eluting between 19.2 and 19.4 min is shown and contains a product whose mass corresponds to the dichloropyrrolyl-*S*-PltL (11,634.2 Da). (*D*) The same reaction as in *C* but with NADH omitted. (*E*) The same reaction as in *C* but with SsuE omitted. (*F*) The same reaction as in *C* but with PltA omitted. (*G*) The same reaction as in *C* but with both PltA and PltM omitted. (*H*) The same reaction as in *C* but with PltM omitted. The charge of the ions in *A* and *B* is 13+; the charge state for all other ions shown is 10+.

activated dissociation (18) of the final pyrrolyl-*S*-PltL species localized the site of covalent modification to three amino acids, NSM, in which Ser-42 is predicted to be posttranslationally modified by the phosphopantetheinyl arm (Fig. 6).

Generation of ^{13}C-^{15}N-Depleted PltL. To unambiguously detect the ion of a chlorinated species covalently linked to the carrier protein PltL, ^{13}C-^{15}N-depleted PltL was prepared by overproduction in minimal media containing ^{13}C-depleted glucose as the carbon source and ^{15}N-depleted ammonium sulfate as the nitrogen source (Fig. 7, which is published as supporting information on the PNAS web site). Depletion of the ^{13}C and ^{15}N isotope in analytical samples simplifies the complex mass envelope resulting from the incorporation of these isotopes at their natural abundance throughout the protein and improves the detection limits by high-resolution MS by 5- to 10-fold (19). In addition, because chlorine (^{35}Cl) has a highly abundant heavy isotope (^{37}Cl) comprising 24.23% of the total natural abundance, the simplified mass distribution of ^{13}C-^{15}N-depleted protein allowed observation of the chloride isotopic signature on the intact protein. The distinctive isotopic distribution (e.g., Fig. 2*B*) provides a direct marker for the incorporation of chloride into the intact protein substrate when compared with a nonchlorinated PltL species like the proline acylated PltL in Fig. 2*A*.

Formation of Dichloropyrrolyl-*S*-PltL by PltA. The substrate ^{13}C-^{15}N-depleted pyrrolyl-*S*-PltL was incubated with NADH and Cl^- (FAD was present from the reaction with flavoprotein CloN3) in the presence of putative halogenases PltA and PltM and the flavin reductase SsuE. After 1 h, the reactions were quenched and analyzed by HPLC. In a reaction containing PltA, PltM, and reductase SsuE, HPLC analysis indicated that the majority (>90%) of the pyrrolyl-*S*-PltL was no longer present and that a new peak eluting at 19.3 min had appeared. Subsequent FTMS analysis of the HPLC region from 18.2 to 19.7 min in 0.1-min intervals indicated that this new peak at 19.3 min was 67.96 Da larger than the pyrrolyl-*S*-PltL (Fig. 2*C* and Fig. 8, which is published as supporting information on the PNAS web site). The dichlorinated species is predicted to be 67.92 Da heavier (gain of two chlorides and loss of two protons). The observed dichloropyrrolyl-*S*-PltL had a nearly identical isotopic distribution as would be predicted from its isotopic content (Fig. 2*B*). Furthermore, a small amount of a +33.97 Da was observed (Fig. 8). A monochlorinated species is expected to be 33.96 Da heavier than pyrrolyl-*S*-PltL species. No mass corresponding to monochlorinated or dichlorinated product derived from nonspecific chlorination of holo-PltL or prolyl-*S*-PltL was detected in any of the samples.

Unexpectedly, when PltM was omitted from the reaction, the new chlorinated product masses were again observed (Figs. 2*H* and 8). In contrast, chlorinated product formation was abolished when PltA was omitted from the reaction (Fig. 2*F*). The reaction products formed by PltA alone were the same as those formed in the presence of PltA and PltM (Fig. 2*C*). Radio-HPLC analysis of reaction products (derived from L-[^{14}C]proline), which were released from the protein after incubation with PltA and/or PltM, confirmed the dependency of dichlorination on the single halogenase PltA without involvement of PltM. Although PltM could not replace PltA and did not seem to affect PltA activity, formation of monochloropyrrolyl-*S*-PltL and dichloropyrrolyl-*S*-PltL depended on SsuE, NADH, and PltA because product formation was lost when these components were omitted (Fig. 2 *D*–*G*). An unidentified, nonchlorinated PltL species (mass 11,607.3, +37 Da compared with prolyl-*S*-PltL) was also observed in these reactions when both SsuE and NADH were present (Fig. 8). Formation of this species did not depend on the activity of PltM or PltA, and it is likely to be an off-pathway side product.

Localization of the Dichlorination on the Pyrrolyl-*S*-PltL by Tandem MS. Although the absence of chlorinated holo-PltL and prolyl-*S*-PltL indicates that halogenation by PltA was selective, it was important to localize the site-specific incorporation of chlorine on the protein. Therefore, the dichloropyrrolyl-*S*-PltL was analyzed by tandem MS, resulting in 10 *b* and 12 *y* fragment ions (*b* ions are N-terminal fragments truncated from the C terminus of the protein and *y* ions are C-terminal fragments truncated from the N terminus of the protein). As in the pyrrolyl-*S*-PltL species described above, Ser-42 is the site of covalent modification on PltL by the phosphopantetheinyl arm. None of the *b*

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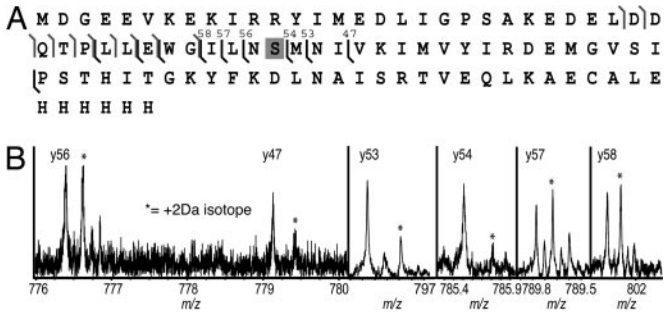

Fig. 3. Localization of the dichlorination on PltL by tandem MS. (*A*) The protein sequence of PltL is shown in which Ser-42 (shaded) is covalently modified by a phosphopantetheinyl arm acylated with dichloropyrrole. *b*-fragments (gray brackets) and *y*-fragments (black brackets) resulting from collisionally activated dissociation of the dichloropyrrolyl-*S*-PltL species are indicated. Numbers designate specific *y*-fragments whose mass spectra are shown below. (*B*) Mass spectra of fragments *y*56, *y*57, and *y*58 that contain Ser-42 (and the dichloropyrrolyl modification) have the characteristic isotopic pattern where the +2-Da isotope is as large or larger than the parent isotope because of the incorporation of two chlorines. Fragments *y*54, *y*53, and *y*47 lacking this residue do not display this characteristic pattern. For all fragments shown, the +2-Da isotope is indicated (*).

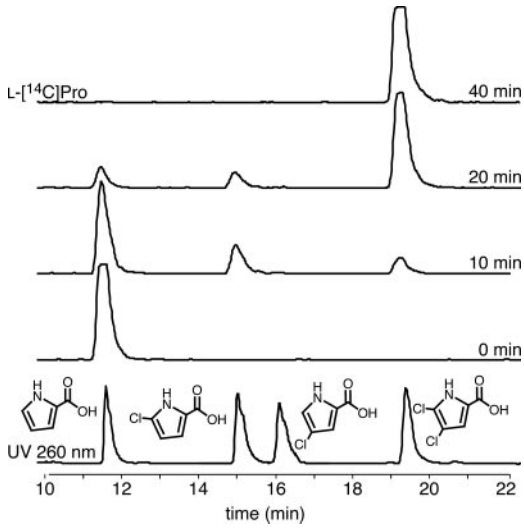

Fig. 4. Regiospecific chlorination at C5 on the pyrrole ring followed by chlorination at C4. [^{14}C]pyrrolyl-*S*-PltL substrate (derived from L-[^{14}C]proline) was prepared. After reaction with PltA and SsuE from 0 to 40 min, reaction products were released from the protein by thioesterase treatment and analyzed by radio-HPLC. The observed products were compared with chemical standards of pyrrole-2-carboxylic acid, 5-chloropyrrole-2-carboxylic acid, 4-chloropyrrole-2-carboxylic acid, and 4,5-dichloropyrrole-2-carboxylic acid, which were also separated by HPLC (bottom trace; monitored at 260 nm).

fragments observed contained the +501-Da modification (resulting from mass increases +340 for the phophopantetheinyl arm, +93 for the pyrrolyl group, and +68 for addition of two chlorine atoms) as they do not include the Ser-42 residue. On the other hand, a mass increase of +501 Da was detected for the *y* fragments, *y*56 to *y*62 and *y*64 containing Ser-42. Because of the abundance of the M + 2-Da isotope of chlorine, these *y* fragments containing the dichloropyrrolyl modification are easily recognized by the characteristic isotopic pattern in which the M + 2-Da isotope is larger than the monoisotopic peak (*y*56, *y*57, and *y*58 shown in Fig. 3*B*). On the other hand, the fragments, *y*47, *y*53, and *y*54, which do not contain Ser-42, lack this characteristic pattern and therefore do not carry the dichloropyrrolyl modification (Fig. 3*B*). As a result of this analysis, the site of dichloropyrrolyl modification was localized to the two residues, NS, in which Ser-42 carries both the dichlorinated acyl group and phosphopantetheinyl arm (Fig. 3*A*).

Time Course for the Formation of Dichloropyrrolyl-*S*-PltL from Pyrrolyl-*S*-PltL. To follow the temporal sequence of product formation, a reaction mixture containing pyrrolyl-*S*-PltL, PltA, SsuE, Cl^-, NADH and FAD was quenched at various times by the addition of formic acid and analyzed by HPLC and ESI-FTMS (Fig. 9, which is published as supporting information on the PNAS web site). A lag period was observed before build-up of the monochlorinated pyrrole intermediate, shown below to correspond to the 5-chloropyrrolyl-*S*-PltL. Shortly after the formation of the 5-chloropyrrolyl-*S*-PltL intermediate, the 4,5-dichloropyrrolyl-*S*-PltL product began to accumulate. At 64 min, the final data point in the time course, nearly all of the monochlorinated intermediate had been converted to the 4,5-dichloropyrrolyl-*S*-PltL product.

Regiospecific Chlorination by PltA at Position 5 and then Position 4 of the Pyrrole Ring. Chlorine groups are found at positions 4 and 5 of the pyrrole ring in the product pyoluteorin. As MS cannot determine the regiochemistry of the monochlorinated and dichlorinated products formed in the PltA reaction, radiolabeled reaction products (derived from L-[^{14}C]proline) were hydrolyzed from the carrier protein PltL by treatment with a thioesterase (14). The hydrolyzed products were analyzed by radio-HPLC and verified by comigration with chemically synthesized standards of 4-chloropyrrole-2-carboxylic acid, 5-chloropyrrole-2-carboxylic acid, and 4,5-dichloropyrrole-2-carboxylic acid. Fig. 4 shows the initial formation of the 5-chloropyrrolyl intermediate within 10 min of reaction, which was fully converted to the 4,5-dichloropyrrolyl product after 40 min. The 4-chloropyrrolyl intermediate was not observed in the PltA reaction.

Bromide Can Replace Chloride for Halogenation. Bromide ions were able to compete with and replace chloride in the PltA reaction. The use of excess bromide ions resulted in a mixture of monochloropyrrolyl-*S*-PltL, monobromopyrrolyl-*S*-PltL, bromochloropyrrolyl-*S*-PltL, and dibromopyrrolyl-S-PltL as evidenced by MS analysis (Figs. 10 and 11, which are published as supporting information on the PNAS web site). Like chlorine, bromine (^{79}Br; 50.6%) has a highly abundant isotope (^{81}Br; 49.3%), which gives brominated compounds a distinctive isotopic distribution. In conjunction with the observed masses that were consistent with each product, comparison of the experimental isotopic distributions of the new brominated products with their theoretical distributions were also similar (Fig. 11). The addition of the sterically larger bromide, however, did slow the reaction as only an estimated 10–20% of the pyrrolyl-*S*-PltL was converted to halogenated species in 1 h as opposed to >90% when chloride was the only halide present.

Discussion

The catalytic activity of several $FADH_2$-dependent halogenases has been demonstrated during the biosynthesis of halogenated natural products (15, 20–22). Halogenation is carried out by a halogenase/reductase pair and depends on O_2, Cl^-, and the cofactor $FADH_2$. The formation of the dichloropyrrolyl moiety in pyoluteorin is unique in several aspects. First, a single halogenase PltA catalyzes chlorination at both positions of the pyrrole ring of pyoluteorin. Given that PltA alone carries out dichlorination of the pyrrolyl substrate and purified PltM showed no activity, it is not clear what the physiological role of the remaining two protein homologs, PltD and PltM, are in pyoluteorin assembly. Second, the pyrrolyl substrate is presented on the carrier protein PltL via thioester linkage to a phosphopantetheinyl prosthetic group. PltA specifically recognized the

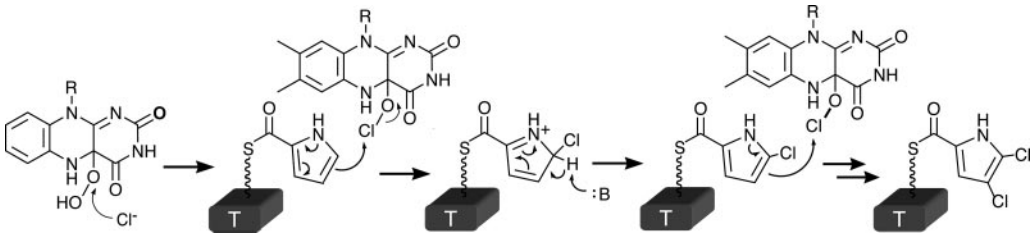

Fig. 5. Mechanistic proposal for the formation of dichloropyrrolyl-*S*-PltL. A proposed FAD-4a-OCl intermediate is formed from the attack of Cl^- on FAD-4a-OOH. The FAD-4a-OCl intermediate then reacts with substrate pyrrolyl-*S*-PltL in a two-electron electrophilic aromatic substitution to first form the 5-chloropyrrolyl intermediate and then the 4,5-dichloropyrrolyl product.

pyrrolyl substrate on the carrier protein scaffold because the free pyrrole-2-carboxylate was not accepted for halogenation by PltA (data not shown). Halogenation of a substrate bound to a carrier protein is likely a common strategy among NRPS and PKS assembly lines. An aminoacyl-*S*-PCP may be relevant for the timing of chlorination of amino acid residues during vancomycin, balhimycin, and related glycopeptide antibiotics (7).

The mechanism of dichlorination by PltA is likely similar to that outlined for monochlorinating $FADH_2$-dependent halogenases. The requirement for a separate flavin reductase was first demonstrated by van Pee and coworkers (15) in studies of the tryptophan-7-halogenase PrnA from the pyrrolnitrin biosynthetic cluster. The reductase generates $FADH_2$, which then reacts with O_2 and Cl^- in the halogenase active site. The strategy of using two enzymes, one to form reduced flavin and the other to catalyze substrate oxidation, is also seen in two-component monooxygenases during pristinamycin and actinorhodin biosynthesis (23, 24). In the current study, $FADH_2$ is formed by an *E. coli* NAD(P)H reductase, SsuE (16), such that the reductase SsuE and halogenase PltA comprise a heterologous two-component halogenase. The identity of the flavin reductase in the *Pseudomonas* producer is not yet known but is not encoded in the *plt* cluster. Because $FADH_2$ is thought to be released by the reductase and diffuse to the active site of the halogenase (15, 21), candidates for such a reductase in the genome of this pseudomonad could include those that function in two-component flavin-dependent monooxygenase systems. Once $FADH_2$ is bound in the active site of PltA, it is proposed to be reoxidized by O_2 to the prototypical FAD-4a-OOH intermediate.

The nature of the reaction pathway after formation of the FAD-4a-OOH intermediate in this large class of biosynthetic halogenases is not yet clear. Whereas alternative mechanisms include formation of an epoxide intermediate proposed for the tryptophan-7-halogenase PrnA (15), we favor attack by chloride ion on the proximal oxygen of the FAD-4a-OOH to yield a FAD-4a-OCl intermediate as we proposed for the $FADH_2$-dependent halogenase RebH (ref. 21 and Fig. 5). This intermediate could react via a one-electron or a two-electron mechanism to provide either radical $Cl^{\cdot}$ or an electrophilic chlorine species, respectively, for the chlorinating reaction as model reactions indicate (25, 26). The reaction of the putative FAD-4a-OCl intermediate with the pyrrolyl substrate in a two-electron mechanism is shown in Fig. 5 as one example. In the case of dichlorination by PltA, the order of chlorination in the PltA reaction is first at position C5 followed by position C4 of the pyrrole moiety. The reaction with a FAD-4a-OCl intermediate (in either a one- or two- electron mechanism) would involve a minimal reorganization of the active site for the second chlorination reaction to occur.

Detailed kinetic analysis of monochlorination and dichlorination will be complex because one is measuring the conversion of the third acyl-*S*-PltL intermediate to the fourth and fifth intermediates. Kinetics is further complicated by side reactions such as the generation of an off-pathway species, such as the 11,607-Da PltL-derived species observed in this study. Nonetheless, the time course (Fig. 9) shows a build-up of the monochlorinated intermediate, which was shown to be 5-chloropyrrolyl-*S*-PltL, followed by conversion to the 4,5-dichloropyrrolyl-*S*-PltL product (Fig. 4).

Dichlorinations catalyzed by PltA are the fourth and fifth enzymatic transformations occurring on an acyl moiety tethered to the 11-kDa carrier protein PltL. Starting from the apo form of PltL, the five steps of phosphopantetheinylation, prolylation, oxidation to the pyrrolyl thioester, and then monochlorination and dichlorination change the initial molecular mass from 11,133 Da for the apo PltL to a molecular mass of 11,634 Da for the dichloropyrrolyl-*S*-phosphopantetheinyl PltL. All of these species have been directly detected by HPLC and ESI-FTMS. Chlorination is specific for the pyrrolyl-*S*-PltL as chlorination of holo-PltL or prolyl-*S*-PltL was not observed. Furthermore, tandem MS localized the site of acyl modification on the PltL protein scaffold. The regiochemistry of the PltA products, as verified by comigration with authentic chloropyrrole-2-carboxylic acid standards, was consistent with the pattern of chlorine incorporation observed in the product pyoluteorin.

Finally, pyoluteorin is a hybrid NRPS/PKS natural product composed of the NRPS-derived pyrrole moiety and the PKS-derived resorcinol ring. The sequence of reactions ending in the formation of dichloropyrrolyl-*S*-PltL by the action of PltA reconstitutes the NRPS pathway and provides the likely substrate for the PKS steps catalyzed by PltB and PltC. Further steps completing the biosynthesis of pyoluteorin could involve the capture of the dichloropyrrole moiety and its extension by malonyl-CoA monomers followed by cyclization to the resorcinol ring.

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