

# Robust *in vitro* activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis

Ellen Yeh, Sylvie Garneau, and Christopher T. Walsh\*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

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The indolocarbazole antitumor agent rebeccamycin is modified by chlorine atoms on each of two indole moieties of the aglycone scaffold. These halogens are incorporated during the initial step of its biosynthesis from conversion of L-Trp to 7-chlorotryptophan. Two genes in the biosynthetic cluster, *rebF* and *rebH*, are predicted to encode the flavin reductase and halogenase components of an FADH<sub>2</sub>-dependent halogenase, a class of enzymes involved in the biosynthesis of numerous halogenated natural products. Here, we report that, in the presence of O<sub>2</sub>, chloride ion, and L-Trp as cosubstrates, purified RebH displays robust regiospecific halogenating activity to generate 7-chlorotryptophan over at least 50 catalytic cycles. Halogenation by RebH required the addition of RebF, which catalyzes the NADH-dependent reduction of FAD to provide FADH<sub>2</sub> for the halogenase. Maximal rates were achieved at a RebF/RebH ratio of 3:1. In air-saturated solutions, a *k*<sub>cat</sub> of 1.4 min<sup>-1</sup> was observed for the RebF/RebH system but increased at least 10-fold in low-pO<sub>2</sub> conditions. RebH was also able to use bromide ions to generate monobrominated Trp. The demonstration of robust chlorinating activity by RebF/RebH sets up this system for the probing of mechanistic questions regarding this intriguing class of enzymes.

enzymology | natural product biosynthesis | flavoenzyme

To date, >3,800 halogenated natural products have been described (1). These natural products include important antibiotics such as vancomycin and chloramphenicol as well as the antitumor agent rebeccamycin (2–4). In addition to these chlorine-containing compounds, a variety of brominated products has been isolated from marine environments that provides a rich and largely untapped resource for novel compounds (5). In mammals, iodinated T3 and T4 forms of thyroid hormone are halogenated metabolites with key physiological functions (6).

Given the prominence of halogenation among natural products, there has been intense interest in understanding the mechanisms by which these functional groups are incorporated during natural product biosynthesis. The first FADH<sub>2</sub>-dependent halogenase was cloned from a strain producing chlortetracycline by complementation of a chlorination-deficient mutant (7). Since then, sequencing of numerous gene clusters involved in the biosynthesis of halogenated natural products has identified many additional members of this class. These halogenases mainly carry out chlorination of electron-rich aromatic rings (e.g., β-OH-tyrosine in vancomycin) but are also involved in formation of the dichloroacetyl moiety in chloramphenicol and iodination of orsellinic acid in calicheamicin biosynthesis (2, 3, 8).

Despite the many representatives of this halogenase class and its role in the biosynthesis of medicinally important halogenated aromatic natural products, only a few of these catalysts have been characterized (9–11). PrnA, the Trp-7-halogenase involved in the formation of pyrrolnitrin, is foremost among FADH<sub>2</sub>-dependent halogenases that have been described (9, 10). In these studies, van Pée and coworkers (9, 10) established the requirement for a separate flavin reductase to provide reduced FADH<sub>2</sub> for the halogenase, in analogy with two-component flavin

monooxygenase systems (12–14). However, low *in vitro* activity of this system has precluded more detailed studies, so even a parameter as fundamental as catalytic efficiency has yet to be reported for a FADH<sub>2</sub>-dependent halogenase, and little is known about the mechanism of halogenation by these enzymes.

Rebeccamycin is an indolocarbazole natural product containing chlorine atoms on each of two indole rings of the scaffold. Because of its inhibition of DNA topoisomerase, it has been explored as a potential therapy against various tumors (15–18). Halogenation is functionally significant because removal of the chlorine atoms results in loss of activity in cell antiproliferative assays (19). The fused ring structure of rebeccamycin arises from multistep oxidative condensation of two molecules of L-Trp (20–22). Chlorination occurs early in the biosynthetic pathway and involves two genes identified in the biosynthetic cluster: *rebF*, a predicted NAD(P)H-dependent flavin reductase, and *rebH*, a putative FADH<sub>2</sub>-dependent halogenase that shares 55% identity with PrnA (4). In this study, we demonstrate that, in the presence of RebF, RebH catalyzes the regiospecific chlorination of L-Trp to 7-chlorotryptophan during the initial step of rebeccamycin biosynthesis (Fig. 1). The robust activity of this two-component RebF/RebH system provides the first kinetic characterization of a FADH<sub>2</sub>-dependent halogenase and sets the stage for further investigation of this remarkable class of halogenases as well as subsequent steps in rebeccamycin assembly.

## Experimental Methods

**Materials.** L-[<sup>14</sup>C]Trp was purchased from New England Nuclear. [<sup>36</sup>Cl]NaCl was purchased from American Radiolabeled Chemicals (St. Louis). Authentic 7-chlorotryptophan was provided by Robert S. Phillips (University of Georgia, Athens) (23). Other chemicals used in this study were purchased from Sigma-Aldrich.

**Cloning, Expression, and Purification of RebF and RebH.** The *rebF* and *rebH* genes were amplified from genomic DNA isolated from *Lechevalieria aerocolonigenes* (strain 39243; The American Type Culture Collection). Primers for RebF (5'-GAGGACCATATG-ACGATCGAGTTCGACAGACCC-3' and 5'-CGATGTAA-CTTTCATCCCTCTGTCCACACGGC-3') and RebH (5'-GTACGTCATATGTCCGGCAAGATTGACAAG-3' and 5'-GTCAGCAAGCTTTTCAGCGGCCGTGTGTGCC-3') contained *NdeI* and *HindIII* restriction sites (italicized) and were cloned into corresponding *NdeI*/*HindIII* sites of pET28a (Novagen). The cloned expression vectors were confirmed by DNA sequencing. *Escherichia coli* BL21(DE3) (Invitrogen) overexpressing RebF or RebH were grown in Luria-Bertani medium supplemented with kanamycin (50 μg ml<sup>-1</sup>). Cells were grown at 30°C to OD<sub>600</sub> = 0.5 and then induced with 100 μM isopropyl β-D-thiogalactoside (IPTG) and grown for an additional 16 h at

\*To whom correspondence should be addressed at: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115. E-mail: christopher\_walsh@hms.harvard.edu.

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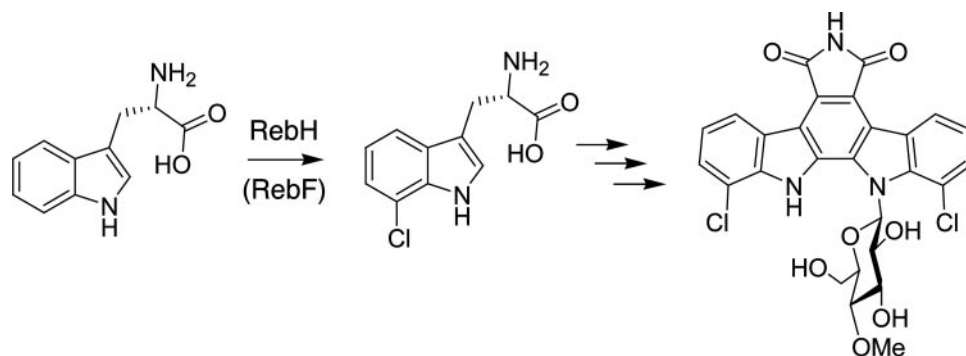


Fig. 1. Chlorination of Trp to 7-chlorotryptophan as the initial step in the biosynthesis of rebeccamycin.

15°C. *N*-His-tagged proteins were purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography. For preparation of apo-RebF, an additional 2 M potassium bromide/2 M urea wash was performed before elution from Ni-NTA (24). Eluted proteins were further purified by gel-filtration chromatography on HiLoad 26/60 Superdex 200 (GE Healthcare). The final storage buffer contained 25 mM Hepes (pH 7.5) and 10% glycerol.

**NADH Oxidation by Flavin Reductase RebF.** Oxidation of NAD(P)H to NAD(P)<sup>+</sup> was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm ( $\Delta\epsilon_{340} = -6,220 \text{ M}^{-1}\text{cm}^{-1}$ ). For kinetic characterization, 1.0  $\mu\text{M}$  RebF was incubated with 0.025–1.0 mM NADH and 50  $\mu\text{M}$  FAD or FMN in 25 mM Hepes (pH 7.5) and 10 mM NaCl.  $K_m$  values for FAD and FMN were obtained by varying concentrations from 0.02 to 12.5  $\mu\text{M}$  in the presence of 0.5 mM NADH. Reactions were monitored at 340 nm by Spectra Max Plus microplate spectrophotometer by using SOFTMAX PRO 3.1 for data analysis.

**Anaerobic Reaction Preparation and Analysis.** For the data shown in Fig. 24, reactions were prepared in a Unilab glove box (Mbraun, Stratham, NH) maintained at <2 ppm O<sub>2</sub>, as described in ref. 25. Protein samples and all reagents were prepared in O<sub>2</sub>-free buffer (25 mM Hepes, pH 7.5). L-[<sup>14</sup>C]Trp (0.5 mM; 10 Ci/mol; 1 Ci = 37 GBq) was incubated with 25  $\mu\text{M}$  RebF and RebH in the presence of 10 mM NADH, 100  $\mu\text{M}$  FAD, and 10 mM NaCl. For labeling with <sup>36</sup>Cl, the reaction was prepared with unlabeled L-Trp and 1 mM [<sup>36</sup>Cl]NaCl (1 mM; 0.5 Ci/mol). Reactions were then removed from the glove box and exposed to O<sub>2</sub>. Reactions were quenched by addition of one equivalent MeOH and then centrifuged to remove precipitated protein. The supernatant was then analyzed by reversed-phase HPLC on a Vydac (Hesperia, CA) C18 (200 Å, 4.6 × 200 mm) column (1 ml/min; 0–20 min, 0–50% B) (A, H<sub>2</sub>O/0.1% TFA; B, acetonitrile/0.1% TFA) on a System Gold HPLC (Beckman). Flow-through radioactivity was monitored by using a Radioisotope Detector 171 (Beckman).

**MALDI MS and NMR Analysis of Product 7-Chlorotryptophan.** For large-scale preparation of reaction product, a 5-ml reaction containing 2 mM L-Trp, 20  $\mu\text{M}$  FAD, 50 mM NADH, 30  $\mu\text{M}$  RebH, 120  $\mu\text{M}$  RebF, and 50 mM NaCl was incubated overnight with agitation. The product-TFA salt was purified by HPLC and lyophilized. Authentic 7-chlorotryptophan was purified similarly. The final product and the chemical standard were analyzed by MALDI-TOF MS (0.2 mg/ml with saturated 2,5-dihydroxybenzoic acid; 1:1 dilution) and <sup>1</sup>H-NMR (500 MHz; D<sub>2</sub>O).

**RebF/RebH Enzymatic Assays.** Rates for the RebF/RebH reaction were determined by monitoring the conversion of 0.5–20  $\mu\text{M}$

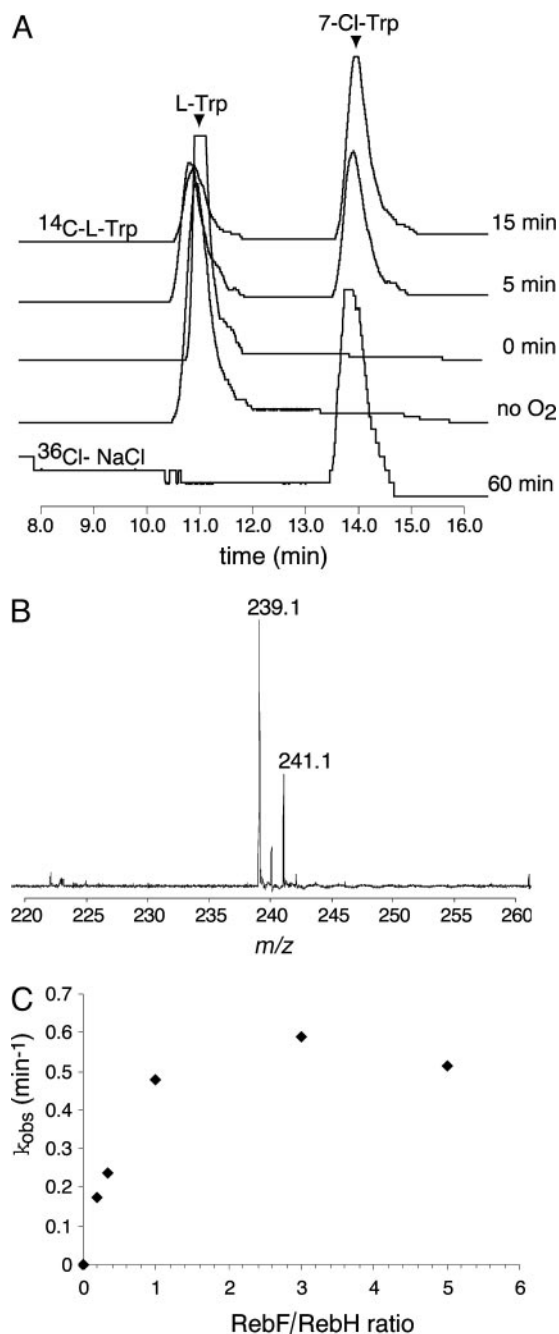
L-[<sup>14</sup>C]Trp (53 Ci/mol) in the presence of 10 mM NADH, 100  $\mu\text{M}$  FAD, 10 mM NaCl, 1  $\mu\text{M}$  RebH, and 3  $\mu\text{M}$  RebF. Reactions were quenched at 1–10 min with 1 eq MeOH, and protein was removed by centrifugation. For bromination reactions, NaCl was replaced with 100 mM NaBr. The supernatant was developed on C18-silica TLC plates (Sigma) in 10% acetonitrile and exposed 12–16 h on BASIII imager plates (Fuji). Plates were analyzed on a Typhoon 9200 (GE Healthcare). The ratio of L-Trp ( $R_f = 0.59$ ) and 7-chlorotryptophan ( $R_f = 0.2$ ) was calculated to determine reaction rates.

## Results

**Characterization of Flavin Reductase RebF.** During the sequencing of the *reb* cluster, *rebF* was annotated as a putative flavin: NAD(P)H reductase (4). Enzymes that require reduced flavin cofactors for activity must first generate FADH<sub>2</sub> or FMNH<sub>2</sub> from reducing equivalents of NADH or NADPH. Although most flavin monooxygenases carry out flavin reduction and substrate oxidation in a single active site, two-component flavin-dependent monooxygenases have been described that use a separate flavin reductase to catalyze the first step of the catalytic cycle (12, 14). The reduced flavin is believed to diffuse into the active site of the monooxygenase component, because this catalytic cooperation does not appear to depend on protein–protein interactions (12–14). In analogy to these two-component flavin monooxygenases, RebF and RebH were proposed to form a two-component halogenase.

RebF was cloned and heterologously expressed in *E. coli*. The resulting 19.9-kDa protein was purified with 23% bound FAD (data not shown). Apo-protein was prepared by washing with 2 M urea/2 M potassium bromide to release the bound cofactor before elution from the Ni affinity column (24). Oxidation of NAD(P)H to NAD(P)<sup>+</sup> was monitored by the decrease in absorbance at 340 nm. In the presence of FAD or FMN, RebF oxidized NADH with  $k_{\text{cat}}$  of 108 min<sup>−1</sup> ( $K_m = 0.7 \mu\text{M}$  for FAD and 1.3  $\mu\text{M}$  for FMN). NADPH was not accepted for oxidation by RebF. The characterization of RebF as a NADH-dependent flavin reductase then allowed for the investigation of its participation in the halogenase reaction.

**Formation of 7-Chlorotryptophan by RebH in Presence of RebF.** In addition to the requirement for a flavin reductase to provide reduced flavin cofactor, previous studies of FADH<sub>2</sub>-dependent halogenases indicated that the reaction also depended on O<sub>2</sub> (9). For that reason, RebF/RebH reactions were prepared anaerobically, and O<sub>2</sub> was subsequently introduced to initiate the reaction. L-[<sup>14</sup>C]Trp was used as the substrate to detect the appearance of new products derived from that amino acid. Analysis by radio-HPLC revealed a new product peak that was formed over time when RebF/RebH was incubated with substrate L-Trp, FAD, and NADH (Fig. 2A). Neither FMN nor



**Fig. 2.** Formation of 7-chlorotryptophan by RebF/RebH. (A) Radio-HPLC traces of RebF/RebH reaction products derived from L-[<sup>14</sup>C]Trp showing time-dependent formation of 7-chlorotryptophan. Product formation requires O<sub>2</sub>. When chloride ion was provided as [<sup>36</sup>Cl]NaCl, L-Trp was converted to product containing <sup>36</sup>Cl label (trace at the bottom). (B) MALDI MS analysis of the [M + H]<sup>+</sup> reaction product is consistent with formation of a monochlorinated Trp with the characteristic 3:1 M/M + 2 isotopic distribution of chlorine. (C) *k*<sub>obs</sub> for RebH (6 μM) varies with RebF concentration (0–30 μM). Optimal activity for RebF/RebH is achieved at a RebF/RebH ratio of 3:1.

NADPH supported product formation (data not shown). In the absence of O<sub>2</sub>, product formation was also abolished, suggesting that molecular oxygen is a substrate for the halogenase as well. <sup>36</sup>Cl was incorporated into the product when chloride ion was provided as [<sup>36</sup>Cl]NaCl. Mass analysis by MALDI MS gave a [M + H]<sup>+</sup> of 239 and 241 in ≈3:1 ratio, consistent with a monochlorinated Trp compound (Fig. 2B). Furthermore, the product

coeluted with an authentic standard of 7-chlorotryptophan (23). Comparison of <sup>1</sup>H-NMR data of the product and authentic 7-chlorotryptophan also confirmed the site of chlorination at position 7 of the indole ring: (D<sub>2</sub>O, 500 MHz) 7.5 (dd, *J* = 1, 8 Hz, 1H, *H*-4), 7.25 (s, 1H, *H*-2), 7.25 (d, *J* = 8 Hz, 1H, *H*-6), 7.02 (t, *J* = 8 Hz, 1H, *H*-5), 4.1 (dd, *J* = 5, 8 Hz, 1H, *H*<sub>α</sub>), 3.3 (dd, *J* = 5, 15.2 Hz, 1H, *H*<sub>β</sub>), and 3.2 (dd, *J* = 8, 15.2 Hz, 1H, *H*<sub>β</sub>′).

In the absence of RebF, RebH showed low-level chlorinating activity that may have resulted from low-level contamination by highly active *E. coli* flavin reductases (26, 27) during heterologous expression and purification. However, rates of product formation were dramatically enhanced with addition of RebF. By varying the ratio of RebF and RebH components, a RebF/RebH ratio of 3:1 was found to be optimal for activity, as shown in Fig. 2C. Subsequent kinetic characterization of RebF and RebH was conducted at this optimal ratio of protein components.

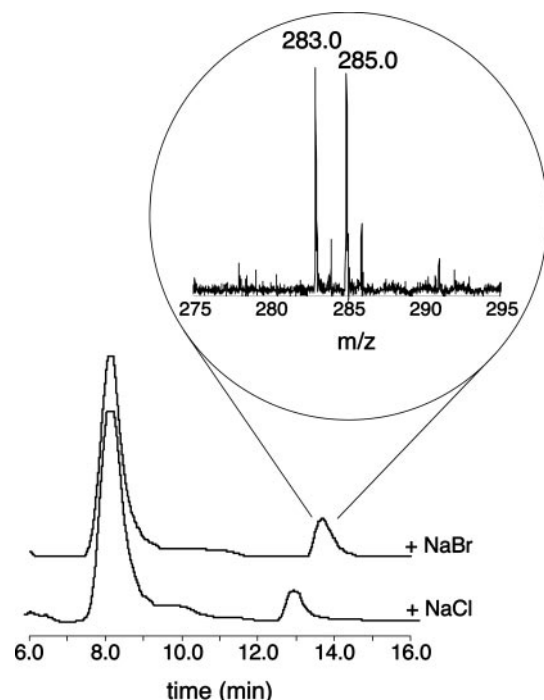
**Kinetic Characterization of RebH.** Kinetic parameters were determined for the conversion of L-Trp to 7-chlorotryptophan by the halogenase RebH. Reactions were prepared under aerobic conditions and assayed in the presence of FAD, NADH, and various L-Trp concentrations. RebH catalyzed chlorination of L-Trp with a *k*<sub>cat</sub> of 1.4 min<sup>-1</sup> and a *K*<sub>m</sub> for L-Trp of 2.0 μM. A *K*<sub>m</sub> for Cl<sup>-</sup> could not be measured because of an undetermined source of chloride contamination in reaction solutions. Because the reaction depends on both FADH<sub>2</sub> and O<sub>2</sub> as substrates, enzyme activity may be sensitive to the amount of O<sub>2</sub> in solution. As shown in Fig. 2A, 54% of the available L-Trp was converted to 7-chlorotryptophan within 5 min when the reaction was prepared anaerobically and O<sub>2</sub> introduced slowly. When the same reaction was prepared in air-saturated solutions, product formation decreased by 10-fold so that only 5% of the substrate was converted in 5 min of reaction (data not shown). The higher reaction rate observed at the lower pO<sub>2</sub> condition likely reflects a decrease in adventitious air oxidation of FADH<sub>2</sub> released by the reductase RebF. This decreased air oxidation would increase the availability of the reduced cofactor for diffusion into and binding in the halogenase active site. A low pO<sub>2</sub> environment may reflect the reducing environment within the cell more accurately and be more favorable for observing the activity of this two-component system that depends on the diffusion of FADH<sub>2</sub>.

**Formation of 7-Bromotryptophan by RebF/RebH.** Brominated rebeccamycin products have also been detected in culture broths from the producer *L. aerocolonigenes* and in genetically reconstituted hosts (28, 29), indicating the ability of RebH halogenase to accept Br<sup>-</sup> in lieu of Cl<sup>-</sup> as the halide substrate. When RebF/RebH activity was assayed in presence of excess bromide, a Trp-derived product was observed with [M + H]<sup>+</sup> of 283 and 285 in a 1:1 ratio characteristic of a monobrominated species (Fig. 3). The rate of formation of the brominated product was found to be 0.4 min<sup>-1</sup>. Neither F<sup>-</sup> nor I<sup>-</sup> were competent for halogenation by RebH (data not shown). In fact, I<sup>-</sup> appeared to inhibit the formation of 7-chlorotryptophan.

## Discussion

Nature employs several classes of enzymes to catalyze specific halogenation of organic compounds. A recurrent theme in biological halogenation is the use of a peroxide functionality and halide ions (Cl<sup>-</sup>, Br<sup>-</sup>, or I<sup>-</sup>) to introduce halogen atoms. Vanadium-dependent bromoperoxidases, isolated from marine algae, use H<sub>2</sub>O<sub>2</sub> to activate bromide ions abundant in seawater, generating an electrophilic bromine species that is likely coordinated to the active site V<sup>5+</sup> (5, 30–32). Similarly, organification of iodide in the thyroid gland during thyroxine biosynthesis requires H<sub>2</sub>O<sub>2</sub> to generate an enzyme-bound



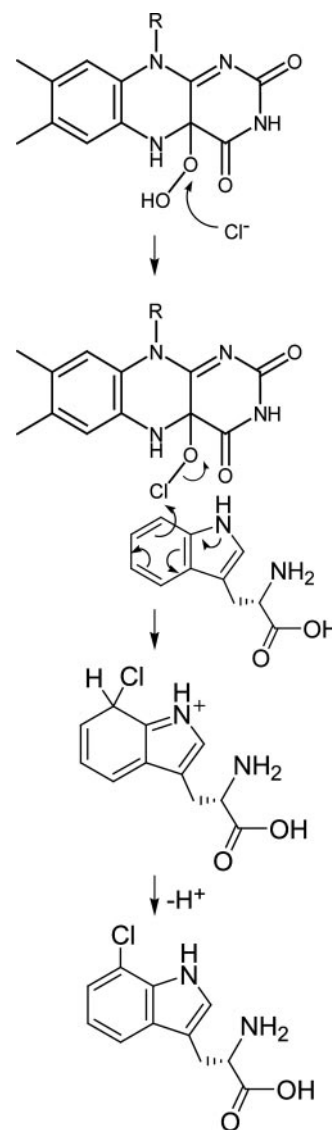


**Fig. 3.** Bromination of L-Trp by RebF/RebH. Radio-HPLC of the RebF/RebH reaction supplemented with excess NaBr shows a new L-[<sup>14</sup>C]Trp-derived product with a retention time greater than that of 7-chlorotryptophan. The [M + H]<sup>+</sup> compound mass and a 1:1 M/M + 2 mass ratio observed by MALDI-MS are indicative of a monobrominated Trp.

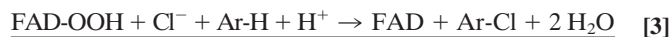
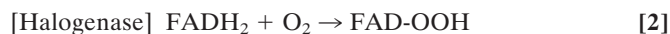
hypoiodite (33–35). Once the reactive oxidized halogen species is formed, the specificity of the subsequent reaction must be controlled. Until the cloning of the *chl* gene from chlortetracycline (7), heme-dependent haloperoxidases were believed responsible for the halogenation of many natural products now known to involve FADH<sub>2</sub>-dependent halogenases. After breakdown of a heme Fe-O-Cl intermediate, free HOCl was released (36–40). Chlorination by HOCl lacked both substrate specificity and regiospecificity (36, 40, 41). Gene disruption of the haloperoxidase from a number of clusters showed no loss of natural product formation, providing definitive proof against the role of haloperoxidases in the biosynthesis of these halogenated organic compounds (42, 43).

RebH is a member of the FADH<sub>2</sub>-dependent halogenase superfamily found in scores of NRPS/PKS biosynthetic clusters. Pioneering work in the pyrrolnitrin system has established several basic features of these halogenases (9). First, they depend on the FAD form of flavin cofactors, in particular FADH<sub>2</sub> in the reduced state. Second, a separate flavin reductase is required to catalyze the initial reduction of flavin by NAD(P)H. In rebecamycin, the flavin reductase RebF is encoded within the biosynthetic cluster along with the halogenase RebH. For most FADH<sub>2</sub>-dependent halogenases, a separate flavin reductase cannot be found in the gene cluster and likely is encoded elsewhere in the genome. A third feature is the requirement for molecular oxygen, consistent with the formation of an FAD-OOH intermediate in the halogenase reaction from FADH<sub>2</sub> and O<sub>2</sub>. This is the key intermediate in the reaction of flavin monooxygenases, in which it acts as an electrophile to hydroxylate aromatic substrates (44).

The reactions leading to chlorination by a two-component halogenase are set forth below.



**Fig. 4.** Proposed mechanism for halogenation by FADH<sub>2</sub>-dependent halogenases. After formation of a FAD-OOH intermediate, the reactive chlorine is generated as a proposed FAD-O-Cl intermediate. Chlorination of L-Trp proceeds by attack of the aromatic  $\pi$  electrons on the FAD-O-Cl intermediate in a two-electron mechanism. Abstraction of a proton then generates the final product, 7-chlorotryptophan.



Overall, a four-electron reduction of O<sub>2</sub> is coupled with four-electron oxidation of both NADH and the aromatic substrate. Flavin frequently acts as an electron carrier between NADH and O<sub>2</sub>. In two-component flavin monooxygenases, FADH<sub>2</sub> shuttles electrons from a reductase that oxidizes NAD(P)H to a second protein that ultimately donates electrons from both FADH<sub>2</sub> and substrate to O<sub>2</sub> forming H<sub>2</sub>O. Because complex formation between the components has not been detected in any of these systems, the mechanism is thought to involve the transfer of freely diffusing FADH<sub>2</sub> between the two active sites (12–14).

However, the use of two proteins, one to generate FADH<sub>2</sub> and the other to catalyze oxidation of the substrate, creates the liability that diffusing FADH<sub>2</sub> will be intercepted by O<sub>2</sub> adventitiously. Given the  $k_{\text{cat}}$  values reported here for RebF and RebH in air-saturated solutions and the 3:1 ratio of the components required for optimal activity,  $\approx 230$  molecules of FADH<sub>2</sub> are generated by the reductase for a single chlorination reaction, a very inefficient usage of the reduced cofactor. Efficient coupling *in vivo* may be achieved in the lower pO<sub>2</sub> environment of the cell because a 10-fold increase in RebH rates was seen under low pO<sub>2</sub> conditions.

The paucity of information regarding biosynthetic FADH<sub>2</sub>-dependent halogenases is in part due to the uncertainty of timing of the halogenation reaction and, consequently, what substrate should be assayed. But it may also be due to the need for both FADH<sub>2</sub> and O<sub>2</sub> as substrates, which creates competition between nonenzymatic oxidation of FADH<sub>2</sub> and productive generation of FAD-OOH by the halogenase. Clearly, kinetic characterization of this two-component halogenase will require maximizing the flux toward productive formation of FAD-OOH in the halogenase active site by conducting reactions at fixed, low pO<sub>2</sub> conditions. Evaluation of the affinity of RebF for RebH may also indicate whether transfer of FADH<sub>2</sub> depends on free diffusion, as in two-component monooxygenase systems.

After formation of the FAD-OOH intermediate, the mechanism of regiospecific halogenation at C7 of Trp, as represented by reaction 3 above, is not fully understood. It has been suggested that chlorination could result from a typical monooxygenase-type reaction to generate an epoxide intermediate, followed by nucleophilic addition of Cl<sup>−</sup> and dehydration to rearomatize the ring (9). However, as evidenced by heme- and vanadium-dependent haloperoxidases, nature seems to favor formation of an electrophilic R-OX species from R-OOH and halide ions. Free HOCl is unlikely to be the active chlorinating agent generated by RebH as such a reaction would lack the specificity to form 7-chlorotryptophan as the sole reaction product (typically, other positions on the indole ring are more activated for

electrophilic aromatic substitution). Also, we did not observe any new products when 1 mM NaOCl was reacted with L-Trp in the presence or absence of RebH (data not shown). Thus, under the enzymatic reaction conditions, <sup>−</sup>OCl/HOCl could not directly chlorinate Trp, nor could it be used by the halogenase to catalyze product formation. Instead, we propose that an FAD-O-Cl could be formed from nucleophilic attack of Cl<sup>−</sup> on the FADH-OOH intermediate (Fig. 4). The unusual regioselectivity demonstrated by both RebH and PrnA could be achieved through a restricted orientation of the bound Trp toward this FAD-O-Cl intermediate in the RebH active site. Detection of such an intermediate would be an exciting addition to the list of chemistries of which this versatile redox cofactor is capable.

Flavin cofactors undergo both one-electron and two-electron chemistry (44–46), so either reaction manifold could be involved in halogen transfer. An FAD-O-Cl intermediate could react via a one-electron mechanism to form <sup>•</sup>Cl as the chlorinating species. Photolysis of ROCl to <sup>•</sup>Cl and <sup>•</sup>RO has been observed (47, 48). However, in vanadium-dependent haloperoxidases, a radical-generating reaction has been ruled out in favor of a mechanism generating cationic bromine (30).

More likely, the formation of 7-chlorotryptophan by RebH occurs via a two-electron mechanism through attack of the indole  $\pi$  electrons on an FAD-O-Cl intermediate. This route, as shown in Fig. 4, would lead to a resonance-stabilized iminium- or imine-like transition state and could account for the unusual regiochemistry of chlorination. Abstraction of the proton at C7 could then regenerate the aromaticity of the indole. The demonstration of robust activity in the purified RebF/RebH system provides a starting point for addressing mechanistic questions regarding this biosynthetically important class of FADH<sub>2</sub>-dependent halogenases.

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