Chlorination by a Long-Lived Intermediate in the Mechanism of Flavin-Dependent Halogenases[†,||]

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ABSTRACT: The flavin-dependent halogenase RebH catalyzes the formation of 7-chlorotryptophan as the initial step in the biosynthesis of antitumor agent rebeccamycin. The reaction of $FADH_2$, Cl^-, and O_2 in the active site generates the powerful oxidant HOCl, which was presumed to carry out the chlorination reaction. Herein, we demonstrate the formation of a long-lived chlorinating intermediate ($t_{1/2}$ = 63 h at 4 °C) when RebH, $FADH_2$, Cl^-, and O_2 react in the absence of substrate tryptophan. This intermediate remained on the enzyme after removal of FAD and transferred chlorine to tryptophan with kinetically competent rates. The identity of this intermediate is suggested by the X-ray crystal structure of RebH, which revealed an active site Lys79 located in a central position between flavin and tryptophan binding sites and just 4.1 Å above C7 of tryptophan. The chlorinating species is proposed to be a Lys-ϵNH-Cl (lysine chloramine) from reaction of enzyme-generated HOCl with the active site Lys79. This covalent enzyme chloramine likely plays a key role in directing regiospecific chlorination of substrate in this important class of biosynthetic enzymes.

The modification of organic scaffolds by addition of halogen atoms is a prominent feature of many bioactive small molecules. Flavin-dependent halogenases are a major class of enzymes required for the biosynthesis of numerous halogenated natural products, including the antibiotics vancomycin and chloramphenicol as well as chemotherapeutic agent rebeccamycin. These enzymes catalyze the formation of carbon−halogen bonds at electron-rich carbon positions using $FADH_2$[1] provided by a partner flavin reductase, halide ion (usually Cl^-), and O_2 (*1*).

Insight into the chemical mechanism of flavin-dependent halogenases has emerged both from the structure of the halogenase PrnA from the pyrrolnitrin biosynthetic pathway and from kinetic characterization of RebH involved in rebeccamycin biosynthesis (*1*, *2*). PrnA and RebH share 55% sequence identity and, within their respective pathways, catalyze the identical overall reaction to chlorinate tryptophan at its C7 position (*3*, *4*). The reaction of $FADH_2$ and O_2 in the active site generates a FAD(C4a)-OOH intermediate, which was detected during the RebH reaction (*2*). Both structural and kinetic evidence supports the subsequent formation of hypochlorous acid (HOCl) in the active site when FAD(C4a)-OOH is captured by Cl^- (Scheme 1) (*1*, *2*). It has been presumed that HOCl would then act as the proximal chlorinating agent, providing an electrophilic Cl^+ equivalent to directly chlorinate the indole ring of tryptophan (*1*).

A potent oxidant, HOCl reacts indiscriminately with many biological molecules, including the side chains of several protein residues. To fulfill its proposed function as the chlorinating agent in the active site of flavin halogenases, it would need to be protected from other reactive functionalities in these enzymes and quickly and specifically consumed in the substrate chlorination reaction. However, two observations from the RebH reaction kinetics and the PrnA structure seemed to challenge these assumptions. First, during stopped-flow studies to monitor formation of flavin intermediates in RebH, flavin reactions leading to HOCl production were observed with or without L-Trp present, suggesting that this potent oxidant is formed in the active site without available substrate for reaction (*2*). Second, in the PrnA structure, an active site Lys79 residue is located adjacent to the bound tryptophan (*1*). Studies of protein oxidation by HOCl show that the ϵNH_2 of lysine reacts rapidly with HOCl to form a long-lived chloramine, Lys-ϵNH-Cl ($t_{1/2}$ > 25 h) (*5*, *6*). Chloramines can also carry out chlorination reactions, albeit with reduced reactivity compared to HOCl (*7*−*9*), and may play a role in the flavin halogenase mechanism (*2*, *10*, *11*). These observations led us to ask whether HOCl itself or the stable product of an enzyme reaction with HOCl might persist in the active site if flavin reactions were carried out

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[||] The coordinates have been deposited within the Protein Data Bank (PDB codes 2OAM, 2E4G, and 2OAL).

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[1] Abbreviations: FAD, flavin adenine dinucleotide, oxidized form; $FADH_2$, flavin adenine dinucleotide, reduced form; HOCl, hypochlorous acid; HDL, high-density lipoprotein; HSQC, heteronuclear single-quantum correlation; Lys-ϵNH-Cl, lysine chloramine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TCA, trichloroacetic acid.

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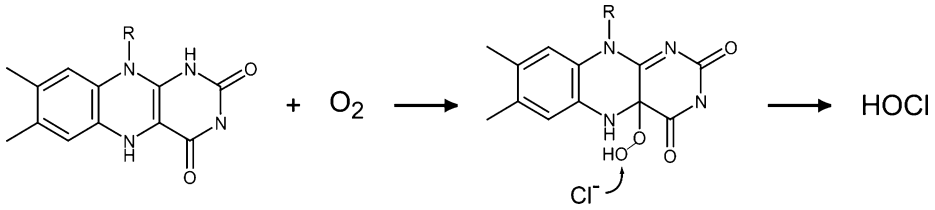
Scheme 1: Generation of HOCl in the Active Site of Flavin-Dependent Halogenases



in the absence of substrate. Furthermore, would such a stable species in the active site still be competent for reaction once substrate was added?

In the following study, we describe the formation of a long-lived intermediate in the RebH active site when the reaction of $FADH_2$, Cl^-, and O_2 was catalyzed in the absence of tryptophan. Upon addition of tryptophan, this stable species carried out the chlorination reaction with kinetically competent rates, suggesting that this enzyme-chlorinating species is an important intermediate on the normal reaction pathway. In the crystal structure of RebH with bound flavin and tryptophan solved at 2.1 Å, Lys79 occupies a key position between the binding pockets for flavin and substrate tryptophan. Furthermore, the structure of the apoenzyme reveals an open active site, unsuitable for protection of a reactive HOCl. On the basis of the extended half-life of the intermediate ($t_{1/2}$ = 63 h at 4 °C) as well as the central position of Lys79 in the RebH structure, we propose that the chlorinating intermediate exists as a stable covalent Lys79-ϵNH-Cl, the proximal chlorinating agent in the chemical mechanism of flavin-dependent halogenases.

EXPERIMENTAL PROCEDURES

Expression Constructs and Protein Overproduction. Construction of pET28a RebH and preparation of *N*-His_6-RebH was previously reported (*4*). Site-directed mutagenesis of pET28a RebH was performed by overlap extension using primers from previous cloning of RebH (*4*) and primers containing mutations to introduce the amino acid change (bold) and an *Nhe*I site, 5′-GCGGGAGTGCAACGCTAGCTAC**GC**GGTCGCCATCAAGTTC-3′ (K79A) and 5′-GCGGGAGTGCAACGCTAGCTACA**T**GGTCGCCATCAAGTTC-3′ (K79M). Fragments containing the desired mutation were cloned into *Nde*I/*Hin*dIII sites of pET28a. Enzyme variants were overexpressed and purified using the same procedure as for the wild-type enzyme (*4*).

Product Formation Assays for Intermediate Detection. Reactions were performed as described (*2*) by mixing equal volumes of $FADH_2$ solution and oxygenated solution containing RebH and NaCl. After 10 min incubation, flavin was removed by a desalting step on a Bio-Rad P-6DG column. L-[^{14}C]Tryptophan (Perkin-Elmer; 53 Ci/mol) was added in a separate reaction. Protein was precipitated by addition of trichloroacetic acid (final concentration 5%). Reaction products were separated on silica-C18 TLC (Aldrich) in 10% acetonitrile/90% H_2O and detected by storage phosphor autoradiography (*2*). To determine the product formation rate, enzyme containing the chlorinating intermediate was purified on large scale following reaction with $FADH_2$, NaCl, and O_2. Reactions were initiated by addition of L-[^{14}C]Trp and quenched between 3 and 1200 s. The product yield was plotted against time, and the data were fitted to an exponential equation to derive the rate constant for 7-chlorotryptophan formation.

^{36}Cl Labeling of the Reaction Intermediate and Products. Equal volumes of $FADH_2$ solution and oxygenated solution containing RebH and [^{36}Cl]NaCl (0.5 Ci/mol; American Radiolabeled Chemicals) were mixed as described (*2*). The protein was purified by desalting on a Bio-Rad P-6DG column to remove flavin and excess [^{36}Cl]NaCl. If indicated, L-tryptophan was added. The reaction was analyzed by size exclusion chromatography on a silica-based Bio-Sep SEC-S 300 column (Phenomenex) in 20 mM sodium phosphate (pH 7.5) buffer (native conditions) or with addition of 0.5% SDS or 6 M urea (denaturing conditions). A Beckman System Gold HPLC instrument with an in-line IN/US β-RAM 3 radiodetector was used. A standard curve of the radioactivity detected with different amounts of [^{36}Cl]NaCl was calculated and used to quantify the amount of ^{36}Cl label on the protein.

Crystallization and Data Collection. Crystals of apo-RebH appeared within 48 h using the hanging-drop vapor diffusion method at 4 °C. Apo-RebH (2 μL of 8 mg mL^{-1} in 20 mM HEPES, pH 7.5) was mixed with an equal volume of reservoir solution (1.2 M sodium/potassium phosphate, pH 7.0). Crystals were cryoprotected by transferring to reservoir solution augmented with 10%, 20%, and then 30% glycerol and flash frozen in liquid nitrogen. To obtain the Trp-bound RebH structure, crystals were soaked in reservoir solution containing 5 mM FAD, 5 mM NaCl, and saturating L-tryptophan. They were cryoprotected in this soaking solution augmented with 10%, 20%, and 30% glycerol. FAD was clearly present in this structure but was too disordered to model. To attempt to generate the chlorinating intermediate in the crystal, crystals of apo-RebH were soaked in anaerobic reservoir solution with 10 mM $FADH_2$ and 3 mM NaCl, and the $FADH_2$ was allowed to oxidize to FAD. This soak was repeated with 10%, 20%, and 30% glycerol, and then the crystals were flash frozen in liquid nitrogen. The resulting structure is referred to here as FAD-RebH.

Native data sets for apo-RebH and Trp-RebH were collected at the Stanford Synchrotron Radiation Light Source (SSRL) beamline 9-1, and the FAD-RebH data set was collected at SSRL beamline 9-2. All data were collected at a wavelength of 0.98 Å and at a temperature of 100 K. Data were processed and scaled in HKL2000 (*12*). The crystals belonged to the spacegroup $P6_2$ with two RebH molecules per asymmetric unit. Data collection statistics are summarized in Table 1.

Structure Determination and Refinement. The structure of apo-RebH was solved by molecular replacement with data to 3.0 Å resolution using the CCP4 (*13*) program PHASER (*14*) and the structure of PrnA (2AQJ) as a search model. PrnA has 54% identity and 82% homology with RebH. The top solution gave a *Z*-score of 57.0. The model was built

Table 1: Data Collection and Refinement Statistics (Molecular Replacement)

	apo-RebH	Trp-RebH	FAD-RebH
data collection			
space group	$P6_2$	$P6_2$	$P6_2$
cell dimensions			
a, *b*, *c* (Å)	114.81, 114.81, 230.63	114.75, 114.75, 231.23	114.72, 114.72, 230.88
α, β, γ (deg)	90.00, 90.00, 120.00	90.00, 90.00, 120.00	90.00, 90.00, 120.00
resolution (Å)	41−2.30 (2.38−2.30)[a]	42−2.08 (2.15−2.08)	42−2.10 (2.18−2.10)
R_{sym}	7.9 (49.5)	8.2 (49.7)	4.2 (17.6)
$I/\sigma I$	18.2 (2.4)	19.6 (2.6)	36.3 (6.6)
completeness (%)	99.7 (99.6)	99.7 (99.5)	99.6 (97.2)
redundancy	4.5 (4.5)	4.5 (4.5)	3.9 (3.8)
refinement			
resolution (Å)	2.30	2.08	2.10
no. of reflections	75897	96465	75285
R_{work}/R_{free}	19.9/22.6	19.2/21.7	20.1/23.1
no. of atoms	8964	9268	9040
protein	8386	8452	8432
ligand/ion		30	106
water	578	786	502
B-factors			
protein	38.9	30.8	36.7
ligand		28.7	57.5
water	43.5	41.0	41.8
rms deviations			
bond lengths (Å)	0.0064	0.011	0.011
bond angles (deg)	1.31	1.75	1.63

[a] Highest resolution shell is shown in parentheses.

using Xfit (*15*) and Coot (*16*) and refined in CNS (*17*). The Trp-RebH and FAD-RebH structures were solved by rigid body refinement using the apo-RebH model. The same set of test reflections (extended for higher resolution structures) was used to calculate R_{free} for all models. Simulated annealing composite omit maps calculated in CNS were used to verify structures throughout refinement. The final model of apo-RebH contains residues 2−528 out of 530 with one chain break per molecule (from residue 451 to residue 454). In the Trp-RebH structure, residues 2−528 were observed in the electron density. The FAD-RebH structure contains residues 2−528 with one chain break (residue 451 to residue 452 in chain B). The *B*-factors for the FAD in this structure are higher than those of the surrounding protein, indicating that the FAD is not present at full occupancy. This is unsurprising given that the natural substrate of RebH is $FADH_2$. The topology and parameter files for FAD were obtained from the HIC-Up server (*18*). A summary of the final refinement statistics is available in Supporting Information, Table 1. Assessment of the models in PROCHECK (*19*) revealed no unfavorable geometries, with 90.3%, 90.8%, and 89.7% of the residues in the "most favored" conformation for the apo-RebH, Trp-RebH, and Fad-RebH structures, respectively. Figures were generated in PyMOL (*20*).

RESULTS

Formation of a Chlorinating Species. The overall reaction of RebH to form 7-chlorotryptophan during the biosynthesis of rebeccamycin is shown in Figure 1A. Formation of 7-chlorotryptophan by RebH was previously demonstrated in single-turnover assays (*2*) by mixing enzyme, NaCl, and L-[^{14}C]tryptophan in oxygenated buffer with an anaerobic solution of $FADH_2$ (Figure 1B, lane 1). To detect the formation of a stable enzyme species following flavin reactions, product formation was again monitored by single-turnover assays, except that enzyme was first reacted with $FADH_2$, Cl^-, and O_2 in the absence of L-tryptophan. The reaction was complete when colorless $FADH_2$ was fully oxidized to yellow FAD. The enzyme was isolated on a desalting column, resulting in nearly complete removal of FAD, and incubated at 25 °C for 30 min. L-[^{14}C]Trp was then added in a second reaction. Reaction products were separated by thin-layer chromatography and analyzed by storage phosphor autoradiography. The yield of 7-chlorotryptophan by this procedure was 70% (ratio of enzyme to L-[^{14}C]Trp = 1; Figure 1B, lane 3) and comparable to the reaction in which enzyme, substrate, and reactants were combined in a single step (lane 1).

By monitoring the incorporation of radioactive ^{36}Cl into the enzyme, it was determined that the detected enzyme species contains radioactivity derived from Cl^-, consistent with its activity to chlorinate the substrate. The initial flavin reactions were performed as described above, substituting [^{36}Cl]NaCl as the source of Cl^-. Protein and small molecule components of the reaction were then separated by silica-based size exclusion chromatography, and each fraction was monitored for radioactivity. Incorporation of 0.6 equiv of ^{36}Cl was observed on the enzyme following the flavin reactions (Figure 1C, trace 2). Upon addition of L-Trp, ^{36}Cl label from the enzyme appeared in new peak corresponding to 7-[^{36}Cl]chlorotryptophan (trace 3). Notably, ^{36}Cl labeling of the enzyme was lost when reactions were analyzed under denaturing conditions using 0.5% SDS or 6.0 M urea.

Taken together, these results demonstrate the formation of an enzyme-chlorinating species during the reaction of RebH with $FADH_2$, Cl^-, and O_2. This species remains on the enzyme after removal of FAD and is fully competent to transfer chlorine to L-Trp in a second reaction forming 7-chlorotryptophan product. Neither product formation nor ^{36}Cl labeling of the enzyme was detected in control reactions when RebH was initially reacted with oxidized FAD (Figure 1B, lane 2, and Figure 1C, trace 1). The enzyme variants, K79A (Figure 1B, lane 4, and Figure 1C, trace 4) and K79M (data not shown), were also inactive in both product formation and enzyme labeling assays. The species formed in the active site may be tightly bound or a covalent modification of the protein but is either released or labile upon denaturation of the enzyme.

Product formation and ^{36}Cl labeling assays also indicate formation of a stable chlorinating species in the flavin-dependent halogenase PltA, which catalyzes the dichlorination of a pyrrole ring presented on a carrier protein scaffold during biosynthesis of pyoluteorin (*21*) (see Supporting Information, Figure 1). Curiously, PltM, another halogenase found in the pyoluteorin gene cluster but whose function in the biosynthetic pathway is unknown, also reacts with $FADH_2$, [^{36}Cl]NaCl, and O_2 to form the enzyme containing the ^{36}Cl label. Because of the difficulty of accessing substrate in this system and lack of kinetic information, we did not carry out further characterization of the chlorinating species in these enzymes. However, the identity of the intermediate is likely the same as in RebH.

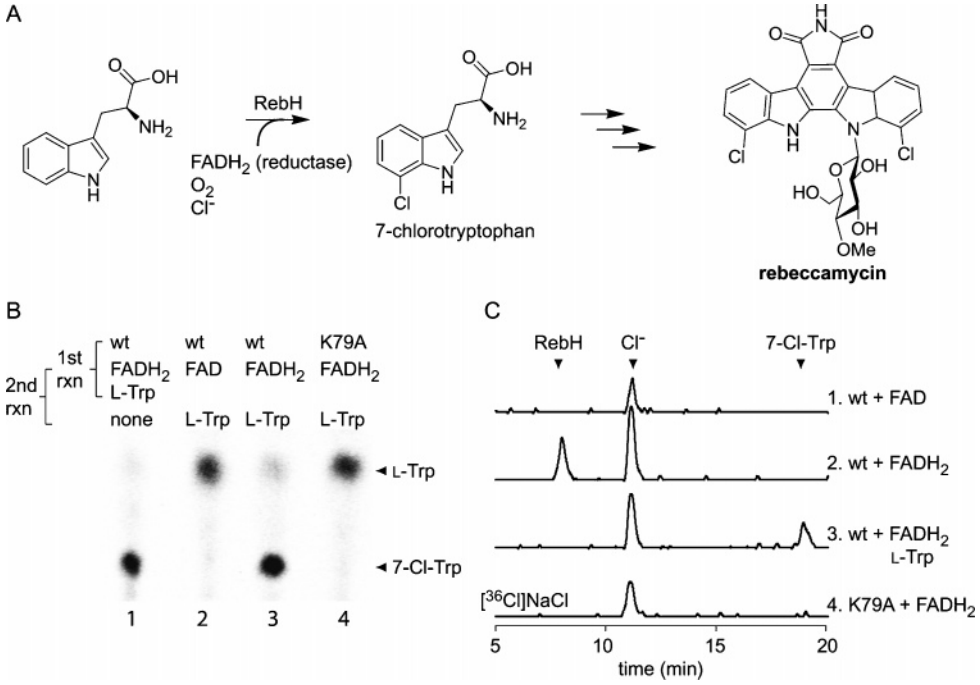


FIGURE 1: Detection of a stable chlorinating intermediate in RebH. (A) Formation of 7-chlorotryptophan by RebH as the initial step in biosynthesis of rebeccamycin. (B) Formation of 7-chlorotryptophan when RebH, isolated after reaction with $FADH_2$, O_2, and NaCl, was incubated with L-[^{14}C]Trp in a separate reaction (lane 3). The same product formed in a control reaction in which all reactants were combined in a single reaction (lane 1). No product is formed when reactions are carried out with oxidized FAD (lane 2) or using the K79A enzyme variant (lane 4). Final reactions contained 30 μM RebH, 120 μM $FADH_2$, 5 mM NaCl, and 30 μM L-[^{14}C]Trp. (C) Incorporation of ^{36}Cl into RebH following reaction with $FADH_2$, O_2, and [^{36}Cl]NaCl (trace 2). Upon addition of L-Trp, 7-[^{36}Cl]chlorotryptophan was formed (trace 3). ^{36}Cl labeling is not observed when reactions are carried out with oxidized FAD (trace 1) or using the K79A enzyme variant (trace 4). Final reactions contained 50 μM RebH, 150 μM $FADH_2$, and 10 mM [^{36}Cl]NaCl $\pm$ 100 μM L-Trp.

Stability of the Chlorinating Species in the Active Site. During experiments to detect the chlorinating species in RebH, we noted above that the species was sufficiently stable to allow isolation of the enzyme and incubation at 25 °C before carrying out the substrate chlorination reaction. The half-life of decay of the chlorinating species was determined by monitoring its substrate chlorination activity from 0 to 288 h following its formation. Loss of substrate-chlorinating activity was attributed to decay of the species in the active site. This analysis showed that the chlorinating species is remarkably long-lived with $t_{1/2}$ of 63 h at 4 °C and 28 h at 25 °C (Figure 2A). The stability of the enzyme-associated intermediate suggests that, once formed, it can remain in the active site for an extended time until substrate becomes available for reaction. RebH can also catalyze bromination of tryptophan by substituting Cl^- with Br^- when NaBr is present in solution (*4*). The $t_{1/2}$ of the corresponding brominating species was approximately 60 min at 4 °C. The brominating species may have lower intrinsic chemical stability. Alternatively, the active site may not be as well-suited for protection of the brominated analogue.

I^- was found to be a noncompetitive inhibitor of the RebH reaction in steady-state assays (see Supporting Information, Figure 2). In particular, I^- completely inhibits formation of the chlorinating species when present in solution during flavin reactions. The addition of I^- after flavin reactions also increased the rate of decay of the chlorinating species by >200-fold such that complete loss of activity was noted within 20 min of NaI treatment (see Supporting Information, Figure 2). Thus, I^- is able to access and eliminate the species in the active site once it is formed.

Kinetic Characterization of a Reaction Intermediate. The activity of the detected enzyme-chlorinating species to form the 7-chlorotryptophan product suggests that it is a critical intermediate in the enzymatic mechanism, rather than the product of an off-pathway side reaction. We determined the product formation rate for the active site chlorinating species to establish its kinetic competence as a reaction intermediate. RebH was first reacted with $FADH_2$, Cl^-, and O_2 to isolate enzyme with the chlorinating species preformed in the active site. L-[^{14}C]Trp was then added to the isolated enzyme sample, and the reaction was quenched at time points from 0 to 1800 s. The rate of 7-chlorotryptophan formation by the enzyme-chlorinating species was 0.29 s^{-1} at 25 °C and 0.17 s^{-1} at 4 °C (Figure 2B).

These rates were compared with those obtained previously for the RebH reaction, specifically those for flavin reactions generating HOCl and overall product formation (*2*) (Figure 2C). In the reaction in which RebH is reacted with $FADH_2$, Cl^-, O_2, and tryptophan, the rate of 7-chlorotryptophan formation was 0.05 s^{-1} at 25 °C (0.009 s^{-1} at 4 °C). This overall reaction rate corresponds to the steady-state k_{cat} value and the rate-determining slow step in the mechanism (*2*, *4*). Any individual step in the mechanism (and any intermediate that catalyzes this step) must proceed at a rate equal to or faster than this slowest rate. The detected chlorinating species in RebH catalyzes product formation at a rate significantly faster than in the overall reaction (6-fold increase at 25 °C and 20-fold increase at 4 °C) and, therefore, is a kinetically competent intermediate on the RebH reaction pathway. Indeed, it likely represents the proximal chlorinating agent in the active site which reacts directly with the substrate.

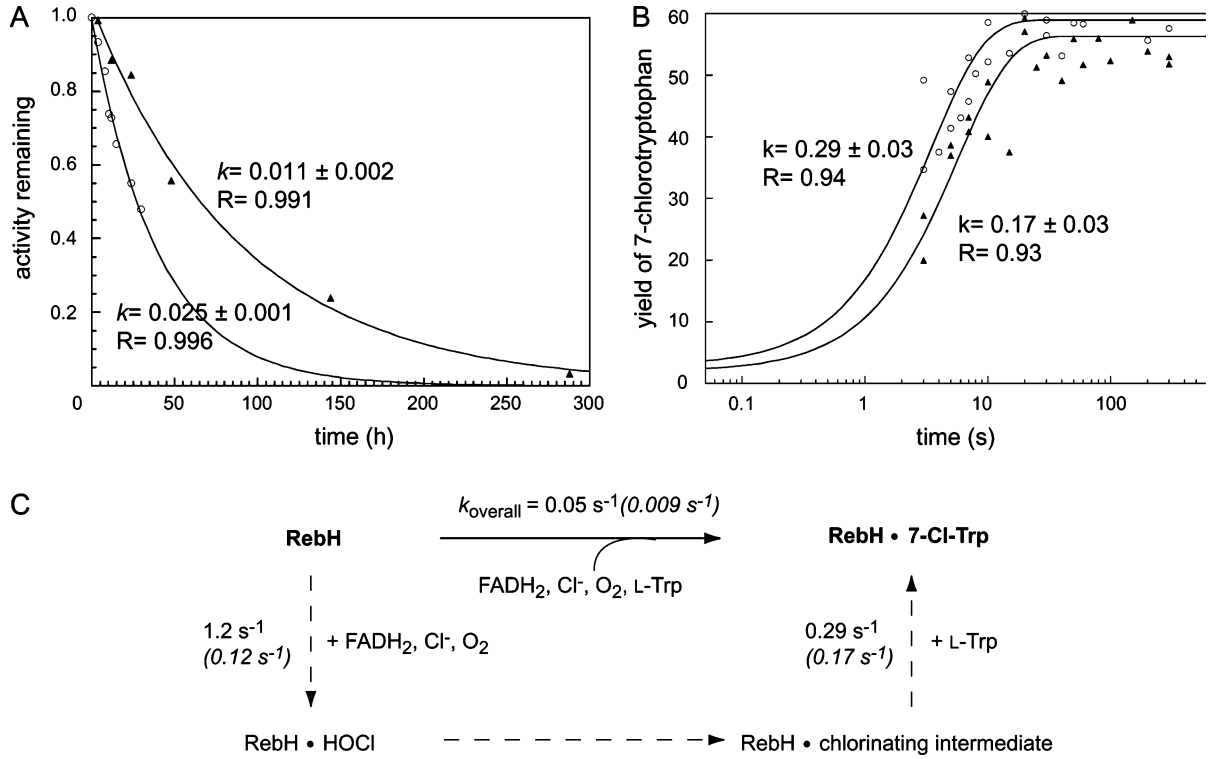


FIGURE 2: Kinetic characterization of the chlorinating intermediate. (A) Decay of the chlorinating intermediate was monitored by incubating enzyme isolated following reaction with $FADH_2$, O_2, and NaCl at 4 °C (▲) and 25 °C (○). The activity of the enzyme sample was determined by adding L-[^{14}C]Trp at $t = 0-288$ min. The yield of product after each incubation period was compared with that from the initial time point. Due to protein precipitation for $t > 30$ h at 25 °C, data from these time points were not used in derivation of rate constants. (B) The rate of 7-chlorotryptophan formation was determined at 4 °C (▲) and 25 °C (○) by isolating RebH after reaction with $FADH_2$, O_2, and NaCl to yield enzyme containing chlorinating intermediate in the active site. Reactions were initiated by adding L-[^{14}C]Trp to the enzyme sample and quenched at $t = 3-1800$ s. Final reactions contained 30 μM RebH, 120 μM $FADH_2$, 5 mM NaCl, and 60 μM L-[^{14}C]Trp. Data for yield of 7-chlorotryptophan versus time were fitted to an exponential equation to derive rate constants for product formation. (C) Kinetic scheme for the RebH reaction showing the overall reaction (top; solid arrow) as well as individual steps for formation of HOCl and the detected chlorinating species (dashed arrows). Rates are indicated at 25 °C (4 °C). Rates for HOCl formation [equal to rates for formation of FAD(C4a)-OH] and overall product formation were determined in previous studies.

Furthermore, the observation that both flavin reactions forming HOCl and product formation by the chlorinating intermediate are fast implies that the rate-determining step in the reaction is likely to be the formation of this chlorinating intermediate.

Central Position of an Active Site Lysine. We solved the structure of apo-RebH and of enzyme with bound FAD or tryptophan to obtain structural evidence for the identity of the chlorinating intermediate formed in the RebH active site. The structure of PrnA, which shares 55% sequence identity with RebH, was previously determined (*1*). As expected, in the overall fold and binding of flavin and tryptophan, RebH closely resembles PrnA with an rmsd of 0.99 Å for superimposition of the α carbons of 483 residues (see Supporting Information, Figure 3). Though we were unable to obtain a structure of the enzyme containing the actual chlorinating intermediate, the apo, tryptophan-bound, and flavin-bound RebH structures were evaluated in light of the new evidence for formation of a stable chlorinating intermediate in the active site.

A key finding from the structure of halogenase PrnA was that flavin and tryptophan are separated by over 10 Å in the active site (*1*). It was concluded that HOCl generated in the flavin binding site would have to traverse this distance in order to catalyze the chlorination of tryptophan. Residue K79 in PrnA was highlighted as having a potential role in orientation of HOCl within the active site. We were also intrigued by the key position of K79 in the RebH structure (corresponding to the same residue in PrnA). In both PrnA and RebH, K79 is located directly between the flavin and tryptophan binding sites. Using the program CASTp[3] and a standard probe size of 1.4 Å, we searched for cavities in the tryptophan-bound RebH structure. This analysis showed that flavin and tryptophan bind in two separate cavities and that there is no open pathway between the two binding sites (Figure 3a). In fact, it would appear that HOCl generated in the flavin binding site would first encounter K79 before it could access the Trp binding pocket. In studies of protein oxidation, HOCl reacts readily with the ϵNH_2 of lysine ($k = 7900\ M^{-1}\ s^{-1}$) to form a stable chloramine, Lys-ϵNH-Cl (*5*, *6*). Notably, the side chain of K79 is ordered, and its position and interactions are conserved in all of the structures solved thus far of RebH and PrnA. In both enzymes, K79 sits just 4.1 Å from C7 of tryptophan and forms a tight hydrogen-bonding interaction with the hydroxyl of S347 (Figure 3b and Supporting Information, Figure 4). This interaction may be critical to the overall structure of the enzyme, as RebH K79A and K79M variants are affected in flavin oxidation reactions as well as overall halogenation activity (data not shown). Therefore, residue K79 occupies a key position in the RebH (and PrnA) active site and is a potential site for covalent modification by HOCl.

An alternative to the formation of a lysine chloramine as the chlorinating intermediate is the sequestration of a small

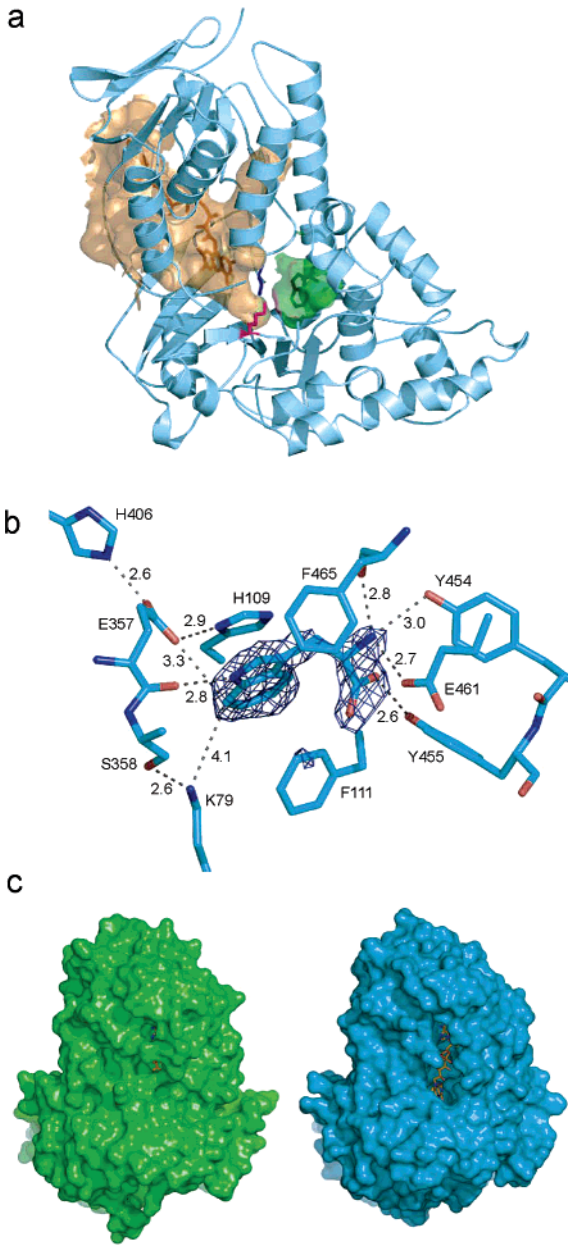


FIGURE 3: Structure of RebH. (a) The ribbon structure of RebH with bound tryptophan (green sticks) shows the central position of Lys79 (pink sticks) between flavin and tryptophan binding sites. FAD (orange sticks) is superimposed from the flavin-bound structure. Lys79 forms an H-bond with Ser347 (blue sticks). The interior surface of the flavin (orange) and the tryptophan (green) binding pockets were mapped using a standard probe size of 1.4 Å. No open pathway exists between these two cavities. (b) An unbiased $F_o - F_c$ map for tryptophan bound in RebH contoured to 3σ shows protein interactions with the substrate (average distances are indicated in angstroms). Lys79 sits 4.1 Å from C7 of tryptophan and forms a tight H-bond pair with S347. The remainder of the tryptophan binding site is well conserved with the PrnA structure, including the position of E357 proposed to be involved in stabilization of the protonated Cl-Trp intermediate. In both RebH and PrnA structures, the side chain of E357 forms hydrogen bonds to two histidine residues, including one very short interaction with H406. (c) Surface representation of PrnA (green) with bound FAD showing closure of the flavin binding pocket (left). Surface representation of apo-RebH (cyan) superimposed on FAD from PrnA showing opening of the flavin binding pocket (right). Structures are in the same orientation.

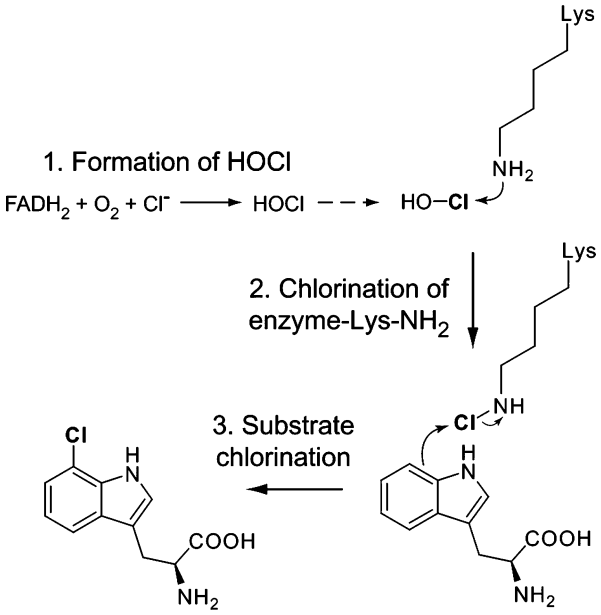
molecule, HOCl, in the active site to carry out the chlorination reaction. Since the form of the enzyme that contains the chlorinating intermediate does not require either bound FAD or L-Trp, we compared the structure of apo-RebH to that of PrnA containing both FAD and tryptophan to assess the potential for a small-molecule binding pocket in the apoenzyme. Notably, when flavin and tryptophan binding sites are unoccupied, the active site becomes exposed to solvent. A loop comprised of residues 40−49 is shifted 6 Å away from the opening of the flavin binding site, allowing solvent access to the entire flavin binding pocket (Figure 3c and Supporting Information, Figure 3). Residues 468−475 also become disordered, allowing both substrate and solvent access to the tryptophan binding site (see Supporting Information, Figure 3). Thus, in the active site of the apo-RebH structure, we were unable to identify a binding pocket that could protect a small-molecule intermediate, particularly one as reactive as HOCl. Moreover, addition of tryptophan to the FAD-bound enzyme also causes FAD to become disordered, so that the flavin binding site becomes exposed when substrate is in position to react. Lacking a covalent attachment, the available structures suggest that it would be exceedingly difficult to retain a chlorinating agent in the active site.

DISCUSSION

HOCl generated from capture of FAD(C4a)-OOH by Cl^- during flavin redox reactions (*1*, *2*) was previously proposed to function as the chlorinating agent that reacts directly with bound substrate during the halogenase reaction (*1*) (Scheme 1). This mechanism now appears unlikely given the remarkable stability of the enzyme-bound chlorinating intermediate detected in RebH ($t_{1/2}$ = 63 h or 2.6 days at 4 °C). It seems highly improbable that HOCl could be sequestered within the active site for such an extended time (up to 2 days) to prevent its diffusion away from the enzyme and reaction with other biological molecules. In the RebH structure and previously reported PrnA structure (*1*), no binding site for protection of such a reactive intermediate could be identified. In fact, the RebH active site becomes relatively exposed to solvent when flavin and tryptophan are absent. The ability of I^- to access the chlorinating intermediate in RebH and accelerate its decomposition also suggests that the active site which contains this intermediate is solvent-accessible and unlikely to harbor a noncovalently associated, reactive small molecule.

Rather, the RebH structure and closely related PrnA structure (*1*) show Lys79 located between the binding pockets for FAD and tryptophan in the active site. In this arrangement, HOCl generated from flavin redox reactions would encounter this lysine residue before reaching the substrate tryptophan. We propose that HOCl reacts with the active site lysine to form a stable chloramine. Indeed, the stability of the detected active site chlorinating species ($t_{1/2}$ = 28 h at 25 °C and 63 h at 4 °C) is consistent with that of Lys-ϵNH-Cl, which has $t_{1/2}$ > 25 h at 37 °C (*5*, *22*). As a covalently bound lysine chloramine, the chlorinating intermediate can be tethered via the N−Cl bond in the active site for prolonged periods. Neither HOCl nor an enzyme chloramine is expected to be stable in the unfolded enzyme as both react readily with Met and Cys residues that become exposed upon protein denaturation. Similarly, both are decomposed upon reaction with I^-.

Scheme 2: Proposed Mechanism of RebH (PrnA) with Formation of an Enzyme Lys-ϵNH-Cl Intermediate as the Proximal Chlorinating Agent



Unfortunately, attempts to characterize this intermediate by X-ray crystallography, NMR spectroscopy, and mass spectrometry were unsuccessful. Although the apoenzyme crystallized in 2 days at 4 °C, crystallization of the intermediate form of the enzyme required 2 weeks, by which time all of the intermediate had decayed. Conformational dynamics which occur during the flavin reactions may also prevent in situ generation of the chlorinating intermediate, as it could not be detected in the crystal of the apoenzyme following soaking with $FADH_2$. A distinct chemical shift change of the ϵC of lysine is associated with formation of free lysine chloramine, but we were unable to observe the same change by ^{13}C-HSQC or direct ^{13}C detection of enzyme containing lysine-specific labeling. The sensitivity by NMR may be limited by the large size of the enzyme, which likely exists as the dimer (120 kDa). Finally, the decay of the intermediate upon protein denaturation precluded proteolytic digestion and detection of modified protein fragments by mass spectrometry.

Despite the lack of direct detection of the proposed enzyme chloramine intermediate, the biochemical and structural evidence presented herein provides a basis for proposal of the following mechanism with Lys-ϵNH-Cl as the proximal chlorinating agent. As shown in Scheme 2, HOCl is formed as a product of flavin-based redox reactions and reacts with the active site lysine to form Lys-ϵNH-Cl. In the RebH structure, the ϵN of Lys79 sits 4.1 Å away from C7 of tryptophan (*1*). Thus, a Lys79-ϵNH-Cl may be ideally oriented for delivering a Cl^+ equivalent for electrophilic aromatic substitution of the indole ring at C7. A candidate lysine corresponding to Lys79 can be identified in other known flavin-dependent halogenases (see Supporting Information, Figure 5). Furthermore, a similar chlorinating species has also been detected in the halogenase PltA and PltM from pyoluteorin biosynthesis (*21*) (see Supporting Information, Figure 1). Hence, we anticipate that formation of an enzyme chloramine intermediate will be a common feature in the mechanism of flavin-dependent halogenases.

The available kinetic data for RebH suggest that formation of this chlorinating intermediate is the rate-limiting step (Figure 2C**)**. Because the chemical reaction between HOCl and lysine to form Lys-ϵNH-Cl is fast (*8*), the slow rate could be attributed to protein conformational changes that occur upon formation of this covalent enzyme intermediate. Protein dynamics was already shown to be critical for formation of flavin intermediates in RebH (*2*), and structural changes in the enzyme following formation of the chlorinating intermediate are indicated by NMR studies (data not shown). Notably, the K_D for tryptophan in the unreacted enzyme was >100 μM as determined by equilibrium dialysis and ultrafiltration (data not shown) despite a steady-state K_m value of 2.0 μM (*4*). Structural changes following intermediate formation could increase the affinity of the enzyme (now in the reactive form) for the substrate. In this way, substrate tryptophan only binds to the enzyme once the chlorinating intermediate is formed and the active site is set up for the chlorination reaction, possibly to prevent inappropriate reaction of substrate with HOCl that is also generated in the active site.

The sequence of reactions proposed in the halogenase active site has precedent in protein oxidation carried out by HOCl and its chloramine products during the inflammatory response. Myeloperoxidase-generated HOCl is released by neutrophils and macrophages as a chemical weapon against invading microbes, where oxidative damage to cellular proteins leads to cell death. In addition to direct rapid oxidation by HOCl, chloramine products formed from the reaction of HOCl with biological amines have been shown to be important secondary mediators of further oxidative reactions (*23*, *24*). The relatively stable chloramines remain active long after the initial immune response and can also diffuse away from the local site to inflict oxidative damage. As oxidizing agents, chloramines are milder and more selective for their target (*7*, *23*), since their reactivity can be influenced by local structure and environment. Following HOCl treatment of high-density lipoprotein (HDL), formation of a chloramine on an adjacent lysine directs chlorination of a specific Tyr192 residue in HDL (*25*−*27*). Chlorination of a catalytic Tyr in D-amino acid oxidase also requires specific binding of D-amino acid chloramine but does not occur with L-amino acid derivatives (*28*, *29*). In this biological setting, the stability and selectivity of chloramines distinguish them from more potent and indiscriminate HOCl.

The conversion of HOCl to a covalent chloramine in the active site of flavin-dependent halogenases offers distinct advantages for carrying out the regiospecific halogenation of small molecules for natural product construction. First, the oxidative potential of reactive HOCl is stored in a long-lived chloramine intermediate that is primed to react upon availability of the substrate. Because reactions with monochloramines RNHCl are typically 10^5 slower compared with HOCl (*7*−*9*), precise tuning of the reactivity of the chloramine intermediate must occur in the active site in order to provide the oxidative potential required for chlorination of substrates such as tryptophan. Both the protonated monochloramine RNH_2Cl^+ ($pK_a \sim 1.5$) and dichloramine $RNCl_2$ (stable at pH <5) are more reactive, though significant perturbation of the local protein environment would be required to stabilize these forms of the intermediate at physiologic pH (*22*, *30*−*32*). Efficient delivery of a Cl^+ equivalent may also be facilitated

by presentation of the substrate in optimal orientation and van der Waals distance from the N−Cl bond. Second, covalent catalysis by a reactive modification on a protein side chain provides for substrate and regiospecificity essential to the reaction by facilitating the orientation of substrate to the chloramine. This precise arrangement may be especially important since C7 is not the most activated position for nucleophilic substitution on the indole ring. Orientation effects may also explain the regiospecificity of halogenases that chlorinate at C5 or C6, rather than C7 (*33*, *34*). In contrast, perhydrolases and haloperoxidases that generate free HOCl lack the specificity required for utility in biosynthetic reactions (*35*−*39*). Detection of the long-lived chlorinating intermediate in the flavin-dependent halogenase mechanism suggests nature's ingenious solution to the chemical problem of controlling a reactive and potentially destructive oxidant, HOCl, for C−Cl bond construction.

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SUPPORTING INFORMATION AVAILABLE

Formation of the chlorinating intermediate in halogenase PltA and PltM, inhibition of RebH by iodide, structural homology of RebH and PrnA, electron density map of the tryptophan binding site in RebH, and sequence alignment of flavin-dependent halogenases from various organisms. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Dong, C., Flecks, S., Unversucht, S., Haupt, C., van Pee, K. H., and Naismith, J. H. (2005) Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination, *Science 309*, 2216−2219.
2. Yeh, E., Cole, L. J., Barr, E. W., Bollinger, J. M., Ballou, D. P., and Walsh, C. T. (2006) Flavin redox chemistry precedes substrate chlorination in the reaction of the flavin-dependent halogenase RebH, *Biochemistry 45*, 7904−7912.
3. Keller, S., Wage, T., Hohaus, K., Holzer, M., Eichhorn, E., and van Pee, K. H. (2000) Purification and partial characterization of tryptophan 7-halogenase (PrnA) from *Pseudomonas fluorescens*, *Angew. Chem., Int. Ed. 39*, 2300−2302.
4. Yeh, E., Garneau, S., and Walsh, C. T. (2005) Robust in vitro activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 3960−3965.
5. Hazen, S. L., Hsu, F. F., d'Avignon, A., and Heinecke, J. W. (1998) Human neutrophils employ myeloperoxidase to convert alpha-amino acids to a battery of reactive aldehydes: a pathway for aldehyde generation at sites of inflammation, *Biochemistry 37*, 6864−6873.
6. Nightingale, Z. D., Lancha, A. H., Jr., Handelman, S. K., Dolnikowski, G. G., Busse, S. C., Dratz, E. A., Blumberg, J. B., and Handelman, G. J. (2000) Relative reactivity of lysine and other peptide-bound amino acids to oxidation by hypochlorite, *Free Radical Biol. Med. 29*, 425−433.
7. Peskin, A. V., and Winterbourn, C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate, *Free Radical Biol. Med. 30*, 572−579.
8. Pattison, D. I., and Davies, M. J. (2001) Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds, *Chem. Res. Toxicol. 14*, 1453−1464.
9. Hawkins, C. L., Pattison, D. I., and Davies, M. J. (2003) Hypochlorite-induced oxidation of amino acids, peptides and proteins, *Amino Acids 25*, 259−274.
10. Vaillancourt, F. H., Yeh, E., Vosburg, D. A., Garneau-Tsodikova, S., and Walsh, C. T. (2006) Nature's inventory of halogenation catalysts: Oxidative strategies predominate, *Chem. Rev. 106*, 3364−3378.
11. Naismith, J. H. (2006) Inferring the chemical mechanism from structures of enzymes, *Chem. Soc. Rev. 35*, 763−770.
12. Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode, *Methods Enzymol. 276*, 307−326.
13. Collaborative Computational Project, No. 4 (1994) The CCP4 suite: Programs for protein crystallography, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 50*, 760−763.
14. Read, R. J. (2001) Pushing the boundaries of molecular replacement with maximum likelihood, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 57*, 1373−1382.
15. McRee, D. E. (1999) XtalView/Xfit−A versatile program for manipulating atomic coordinates and electron density, *J. Struct. Biol. 125*, 156−165.
16. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 60*, 2126−2132.
17. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., PAnnu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 54*, 905−921.
18. Kleywegt, G. J., and Jones, T. A. (1998) Databases in protein crystallography, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 54*, 1119−1131.
19. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK−a program to check the stereochemical quality of protein structures, *J. Appl. Crystallogr. 26*, 283−291.
20. Delano, W. L. (2002) The PyMOL molecular graphics system, DeLano Scientific, San Carlos, CA.
21. Dorrestein, P. C., Yeh, E., Garneau-Tsodikova, S., Kelleher, N. L., and Walsh, C. T. (2005) Dichlorination of a pyrrolyl-S-carrier protein by $FADH_2$-dependent halogenase PltA during pyoluteorin biosynthesis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 13843−13848.
22. Thomas, E. L., Grisham, M. B., and Jefferson, M. M. (1986) Preparation and characterization of chloramines, *Methods Enzymol. 132*, 569−585.
23. Weiss, S. J., Lampert, M. B., and Test, S. T. (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity, *Science 222*, 625−628.
24. Test, S. T., Lampert, M. B., Ossanna, P. J., Thoene, J. G., and Weiss, S. J. (1984) Generation of nitrogen-chlorine oxidants by human phagocytes, *J. Clin. Invest. 74*, 1341−1349.
25. Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995) Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils, *J. Biol. Chem. 270*, 16542−16548.
26. Bergt, C., Fu, X., Huq, N. P., Kao, J., and Heinecke, J. W. (2004) Lysine residues direct the chlorination of tyrosines in YXXK motifs of apolipoprotein A-I when hypochlorous acid oxidizes high density lipoprotein, *J. Biol. Chem. 279*, 7856−7866.
27. Pattison, D. I., and Davies, M. J. (2005) Kinetic analysis of the role of histidine chloramines in hypochlorous acid mediated protein oxidation, *Biochemistry 44*, 7378−7387.
28. Rudie, N. G., Porter, D. J., and Bright, H. J. (1980) Chlorination of an active site tyrosyl residue in D-amino acid oxidase by *N*-chloro-D-leucine, *J. Biol. Chem. 255*, 498−508.
29. Ronchi, S., Galliano, M., Minchiotti, L., Curti, B., Rudie, N. G., Porter, D. J., and Bright, H. J. (1980) An active site-tyrosine-containing heptapeptide from D-amino acid oxidase, *J. Biol. Chem. 255*, 6044−6046.
30. Weil, I., and Morris, J. C. (1949) Equilibrium studies on *N*-chloro compounds, *J. Am. Chem. Soc. 71*.
31. Gray, E. T., Jr., Margerum, D. W., and Huffman, R. P. (1978) Chloramine equilibria and the kinetics of disproportionation in aqueous solution, *ACS Symp. Ser. 82*, 264−277.
32. Snyder, M. P., and Margerum, D. W. (1982) Kinetics of chlorine transfer from chloramines to amines, amino acids, and peptides, *Inorg. Chem. 21*, 2545−2550.

33. Zehner, S., Kotzsch, A., Bister, B., Sussmuth, R. D., Mendez, C., Salas, J. A., and van Pee, K. H. (2005) A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005, *Chem. Biol. 12*, 445−452.
34. van Pee, K. H. (2001) Microbial biosynthesis of halometabolites, *Arch. Microbiol. 175*, 250−258.
35. Geigert, J., Neidleman, S. L., and Dalietos, D. J. (1983) Novel haloperoxidase substrates. Alkynes and cyclopropanes, *J. Biol. Chem. 258*, 2273−2277.
36. Libby, R. D., Shedd, A. L., Phipps, A. K., Beachy, T. M., and Gerstberger, S. M. (1992) Defining the involvement of HOCl or Cl_2 as enzyme-generated intermediates in chloroperoxidase-catalyzed reactions, *J. Biol. Chem. 267*, 1769−1775.
37. Picard, M., Gross, J., Lubbert, E., Tolzer, S., Krauss, S., van Pee, K. H., and Berkessel, A. (1997) Metal-free bacterial haloperoxidases as unusual hydrolases: Activation of H_2O_2 by the formation of peracetic acid, *Angew. Chem., Int. Ed. 36*, 1196−1199.
38. Kirk, O., and Conrad, L. S. (1999) Metal-free haloperoxidases: Fact or artifact?, *Angew. Chem., Int. Ed. 38*, 977−979.
39. Puk, O., Huber, P., Bischoff, D., Recktenwald, J., Jung, G., Sussmuth, R. D., van Pee, K. H., Wohlleben, W., and Pelzer, S. (2002) Glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908: Function of a halogenase and a haloperoxidase/perhydrolase, *Chem. Biol. 9*, 225−235.

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