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Understanding and Improving the Activity of Flavin Dependent Halogenases via Random and Targeted Mutagenesis

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Abstract

Flavin dependent halogenases (FDHs) catalyze the halogenation of organic substrates by coordinating reactions of reduced flavin, molecular oxygen, and chloride. Targeted and random mutagenesis of these enzymes has been used to both understand and alter their reactivity. These studies have led to insights into residues essential for catalysis and FDH variants with improved stability, expanded substrate scope, and altered site selectivity. Mutations throughout FDH structures have contributed to all of these advances. More recent studies have sought to rationalize the impact of these mutations on FDH function and to identify new FDHs to deepen our understanding of this enzyme class and to expand their utility for biocatalytic applications.

Keywords

halogenase; flavin; C-H functionalization; mutagenesis; directed evolution

1. Introduction

Over 4,500 halogen-containing natural products have been reported to date (1, 2). The biological activity of many of these compounds has led to widespread use of halogenated compounds, including the antibiotic vancomycin and the antitumor agent rebeccamycin, as pharmaceuticals (3, 4). The halogenated antibiotics laurinterol and isolaurinterol have even demonstrated potent activity against penicillin- and vancomycin-resistant bacterial strains, which have become increasingly problematic as bacteria continue to evolve to withstand commonly administered drugs (5). Structure-activity relationship studies have demonstrated that halogen substitution often directly affects the biological activity of compounds (6–10). For example, the efficacy of vancomycin (**1**) as an antibiotic and that of desformylflustrabromine (**2**) as a biofilm inhibitor are both significantly diminished upon halogen removal (Fig. 1A) (6, 7). Halogen substitution is also required for isoform-specific serotonin receptor activation by the anti-obesity drug Lorcaserin (Fig. 1A, **3**) (8). Without

halogenation, the specificity of this drug is diminished and harmful side effects can occur. Additionally, halogen substitution has been shown to raise the potency of insecticides, herbicides, and fungicides, making it invaluable in agrochemical design (11). While halogen substitution was initially thought to non-specifically impact bioactivity by increasing small molecule lipophilicity and hydrophobic interactions with target proteins, the importance of specific halogen bonding interactions with both nucleophiles and electrophiles is increasingly appreciated (Fig. 1B) (12–14).

In addition to possessing a wide range of biological activities, organohalogen compounds are also important building blocks for organic synthesis (15). Biaryl motifs commonly found in pharmaceutical compounds (16) can be accessed through cross-coupling reactions, which typically involve aryl halide starting materials (17–20). Although organic compounds can be halogenated via a wide range of methods, including electrophilic aromatic substitution, radical substitution, and electrophilic addition, controlling the site selectivity of these reactions remains challenging. In most cases, site selectivity is determined by substrate control (e.g. substrate electronics). This review focuses on catalyst-controlled halogenation using flavin-dependent halogenases (FDHs), which proceeds through an electrophilic aromatic substitution (EAS) mechanism.

Non-enzymatic halogenation proceeding via EAS typically involves the reaction of substrates at their most electronically activated sites (21). When a molecule has several electronically activated sites, such the ortho and para positions of anilines or phenols, product mixtures are usually observed. This lack of site selectivity between electronically activated sites limits the overall yield and efficiency of the reaction (21). Furthermore, most halogenation strategies cannot access less electronically activated sites on an aromatic compound in the presence of more activated sites. For example, the benzo sites of indoles (C4–7) are difficult to halogenate in the presence of one or both pyrrolo sites (C2–3, Fig. 2A) (22). Thus, halogenation catalysts capable of overriding substrate electronics to selectively halogenate organic compounds are highly desirable.

FDHs accomplish this challenging task by binding a substrate and directing a reactive halogenating species to a single site on that substrate, even if it is not the most electronically activated site (Fig. 2A). Our group has used halonium affinity (HalA) calculations (23) to quantify the nucleophilicity of different sites on a molecule and thus identify cases in which FDH selectivity is different than that expected from substrate electronics alone. In analogy to calculated proton affinities (24), the HalA of a sp^2 hybridized carbon is simply the energy associated with addition of a halonium ion (X^+) to that site, thus producing an sp^3 hybridized C-X adduct (Fig. 2B). Higher HalA values correspond to sites on a molecule that are more nucleophilic and therefore more reactive toward electrophilic halogenation (Fig. 2C). Calculated HalA values correlate well with experimentally observed product ratios for reactions proceeding via EAS and previous computational work describing the relative nucleophilicity of different compounds (25).

Fully harnessing the potential of FDHs to serve as site selective biocatalysts requires a thorough understanding of their mechanism. While numerous classes of halogenases (e.g. haloperoxidases, nonheme iron-dependent halogenases, *S*-adenosyl-L-methionine-dependent

halogenases) have been the focus of much exciting work (26–31), this review will focus on FDHs. We will address advances in our understanding of FDHs obtained through structural and biochemical studies, with an emphasis on targeted mutagenesis and directed evolution experiments. Additionally, FDH applications in biocatalysis and FDH substrate-activity profiling will be discussed.

2. Flavin-dependent halogenases (FDHs)

2.1 FDH mechanism

Much of our current understanding of FDHs comes from studies of tryptophan-FDHs (Trp-FDHs), which halogenate the benzo positions of the indole moiety in L-tryptophan. Through these studies, it was discovered that FDHs closely resemble flavin-dependent oxygenases both mechanistically and structurally. In flavin-dependent oxygenases, bound FADH₂ reacts with O₂ to form a FAD(C4a)-OOH intermediate (34, 35). This intermediate can subsequently oxidize organic substrates. For example, the flavin-dependent oxygenase *p*-hydroxybenzoate hydroxylase (PHBH) uses FAD(C4a)-OOH as an "OH⁺" source that is used to hydroxylate the proximally bound aromatic substrate via electrophilic aromatic substitution (Fig. 3A, blue) (35, 36).

Walsh and coworkers used stopped-flow kinetics to characterize the FAD(C4a)-OOH intermediate in the 7-tryptophan FDH (Trp-FDH) RebH as well (38); however, in contrast to monooxygenases such as PHBH, the FAD(C4a)-OOH intermediate does not directly oxidize substrate. As shown in Fig. 3A, PHBH positions the C4a of the isoalloxazine ring 4.2 Å from the functionalized site of substrate, close enough for subsequent electrophilic aromatic substitution to occur directly (39). The crystal structures of Trp-FDHs show much longer isoalloxazine-substrate distances of ~10 Å, indicating the FAD-peroxide cannot interact directly with substrate without large conformational changes (Fig. 3A, green) (40–42). No such conformational changes are apparent from crystal structures with and without substrate and/or FAD bound (40–42). When chloride is present in FDH solutions during crystallization and/or soaked into FDH crystals, a chloride-binding site proximal to the isoalloxazine is observed (40–42). Whether this chloride-binding site is relevant to FDH halogenation activity or simply an artifact of crystallization is still uncertain (43). From these structural studies, it is proposed that, instead of organic substrate, chloride (perhaps that bound near the isoalloxazine ring) reacts with the FAD(C4a)-OOH intermediate to form hypochlorous acid (HOCl, Fig. 3A, green). This HOCl species is thought to travel through a tunnel to the active site where it interacts with an active site lysine residue, either via simple hydrogen bonding (44) or to form a chloramine intermediate (41). The proximally bound L-tryptophan can subsequently undergo electrophilic aromatic substitution (EAS) with either the bound HOCl or chloramine species. In Trp-FDHs, an active site glutamate residue has also been found to be important for catalysis (Fig. 3B). Several functions have been proposed for this residue and will be discussed in greater detail in section 3.1.

Whether a covalent or non-covalent interaction is occurring between HOCl and Lys79, the importance of this residue for site selective halogenation is crucial. By anchoring the halogenating species within the enzyme active site, Trp-FDHs can halogenate a single C–H bond on L-tryptophan. In RebH and PrnA, L-tryptophan binds in the active site such that the

C7–H bond is positioned proximal to the "Cl⁺" species and undergoes electrophilic aromatic substitution (41, 42). In addition to 7-Trp-FDHs (45, 46), both 5- (47–49) and 6-Trp-FDHs (50–54) have been characterized. As previously noted, site-selectivity for a single benzo-position, especially in the presence of the more electronically activated pyrrolo position, is remarkable. A crystal structure of the 5-Trp-FDH PyrH with bound L-tryptophan shows that the active site lysine residue is positioned proximal to C5–H bond of L-tryptophan, consistent with the observed selectivity (55). To achieve this differential L-tryptophan binding with respect to the 7-Trp-FDHs RebH and PrnA, L-tryptophan is flipped in the active site (Fig. 3B). Structures of 6-Trp-FDHs SttH (56) and Th-Hal (53) have also been elucidated; however, neither contains bound L-tryptophan. Further structural characterization or computational analysis will be necessary to determine the mode of substrate binding in 6-Trp-FDHs.

2.2 Structural and functional diversity of known FDHs

In addition to Trp-FDHs, FDHs that chlorinate substrates containing phenol (57–65), pyrrole (66–70), and even alkyl (71, 72) moieties have also been identified. Several of these FDHs have been shown to halogenate free substrates; however, many accept only substrates tethered to acyl carrier proteins (ACP) (58, 66, 67, 73). Walsh and coworkers reported the first example of such an FDH (66), PltA, which was determined to be the halogenase responsible for chlorination of pyoluteorin. *In vitro* activity of PltA was reconstituted only when the pyrrolyl substrate was tethered to an ACP (Fig. 4A, 4). Similarly, the FDH SgcC3 was found to chlorinate CP-acylated β -tyrosine (Fig. 4A, 5), whereas no chlorination activity was observed using the free amino acid (73).

On the other hand, many free substrates are also viable substrates for FDH-catalyzed halogenation. Unlike PltA, for example, the FDH PrnC halogenates the pyrrole ring of monodechloroaminopyrrolnitrin (Fig. 4A, 7) without the need for a ACP (68). The FDH MibH was recently discovered and found to chlorinate a lanthipeptide (~1kD) (74). Several FDHs, including Rdc2/RadH (59), CazI (61), and Bmp5 (58), have been found to halogenate free phenol-containing compounds (63) (Fig. 4A, 6, 11, 12). Interestingly, Bmp5 is the first and, prior to work later performed in the Lewis lab (75), only reported single component FDH. Like single component flavin-dependent oxygenases, Bmp5 is capable of generating reduced flavin, removing the need for a separate flavin reductase (58). Also unique to FDHs currently characterized, Bmp5 catalyzes a two-step reaction, in which bromination occurs at the 3-position of 4-hydroxybenzoate, followed by subsequent decarboxylative-bromination.

Interestingly, FDHs capable of halogenating alkyl substrates have recently been described (71, 72). The FDH CmlS was identified as crucial for the production of the dichloroacetyl moiety of chloramphenicol (Fig. 4A, 8); however, the substrate of CmlS has not been established (71). It is thought that a CoA thioester form of the dichloroacetyl moiety is added to a chloramphenicol precursor by other enzymes in this cluster (76), but it remains unclear whether halogenation or thioester formation occurs first. The identity of the acyl group of this compound prior to halogenation is also unknown. Although acetate, acetoacetate, and malonate could serve as the acyl source, the latter two would serve as more reactive substrates, should chlorination proceed via an enolate intermediate (71). Should

these dicarbonyl substrates be involved instead of acetate directly, a retro-Claisen reaction (for acetoacetyl-CoA) or decarboxylation (for malonyl-CoA) could form the acyl donor dichloroacetyl-CoA. Regardless of the exact substrate chlorinated by CmlS, this FDH marks an interesting case of non-aromatic halogenation. The pK_a for malonate (13.5) and acetoacetate (8.5) are such that deprotonation followed by chlorination with hypochlorous acid or chloramine are reasonable, thus CmlS could indeed follow a similar mechanism as Trp-FDHs (71).

A fungal FDH was also reported to chlorinate an unactivated aliphatic carbon (72). Hertweck and coworkers have established *in vitro* halogenation activity of FDH AoiQ (Fig. 4A, **13**). For *in vitro* reactions, the des-chloro precursor of **13** (orthosporin) was added as substrate to buffer containing purified AoiQ, flavin reductase, FAD, NaCl, and NADH. Formation of chlorinated products was monitored by LCMS, but product from these reactions was not isolated. Instead, authentic standards for the expected mono and dichlorinated products were synthesized and shown to co-elute with reaction product peaks when analyzed by LCMS. Furthermore, when mono chlorinated orthosporin is used as substrate, higher yields of **13** are observed. These results were used to suggest that AoiQ is halogenating an unactivated, sp³-hybridized carbon. The authors note that the mechanism of this FDH likely deviates from previously characterized FDHs (perhaps proceeding through a radical pathway), but further studies will be required to determine how this unique reactivity is achieved. In addition to substantially expanding upon the known scope of FDHs, AoiQ is also the first reported FDH to contain a functional O-methylation domain.

As mentioned in section 2.1, FDHs are strikingly similar to flavin-dependent monooxygenases. When the structures of CmlS (71), CndH (77) (an FDH thought to halogenate a phenolic substrate), PltA (78), MibH (74), and RebH (40) are compared, many structurally conserved regions are also found in the oxygenase PHBH (71). Structural analysis displays high conservation of the FAD-binding domain and halogenation active site; however, the C-terminus varies between CmlS, CndH, PltA, MibH, and Trp-FDHs (Fig. 4C). This region is thus thought to impart substrate specificity between FDHs (71). In the crystal structure of CndH, the C-terminus is less structured than Trp-FDHs and does not completely envelop the active site as is the case for Trp-FDHs (Fig. 4C). This observation led to the hypothesis that CndH halogenated a ACP-bound substrate, and that a more unstructured C-terminus and open active site was common to this type of FDH. Based on the constricted active site in the CmlS crystal structure reported in 2010, CmlS was originally hypothesized to function on free substrate; however, the structure of PltA, reported in 2015 (78), demonstrated that this need not be the case. The C-terminal region of PltA was well ordered and constructs the putative active site. Because PltA is known to halogenate a ACP-bound substrate (66), the C-terminus must undergo a large conformational change to allow substrate access. Further studies are necessary to identify the nature of these conformational changes, as well as whether CndH and CmlS function on free or ACP-bound substrate.

On the other hand, the crystal structure of MibH, which halogenates a lantipeptide (~1 kDa), does indeed have a much larger substrate binding pocket than other FDHs (718.5 Å² larger), even in the absence of bound substrate (74). Although it appears that MibH, like Trp-FDHs, does not need to undergo large conformational changes to halogenate this peptide,

crystallization of substrate bound structure will be necessary to confirm this. Another FDH, which appears to halogenate a precursor of Ammosamide B (Fig. 4A, **10**), has recently been identified and demonstrates the highest sequence homology to MibH (79). It was originally thought that chlorination of L-Trp by this FDH, Amm3, was the first biosynthetic step for Ammosamide B; however, evidence suggests that Amm3 chlorinates a downstream precursor (79).

2.3 Biocatalysis with FDHs

Detailed mechanistic and structural studies of known halogenases, in addition to the discovery of new, diverse FDHs described above, reveals the exciting potential of FDHs as site-selective, environmentally-friendly halogenation catalysts. Expanding the substrate scope of these enzymes and increasing the reaction scales on which they can be used is important for their use in many synthetic applications. To increase reaction sizes, high enzyme expression yields and turnover numbers are necessary. In 2013, Lewis and coworkers developed a method to express RebH at concentrations 10 times higher than previously reported conditions (80). Using this method, sufficient enzyme for multiple preparative-scale reactions (10 mg) could be rapidly obtained. Through these reactions, it was discovered that RebH selectively halogenates several aromatic compounds, including various tryptamines, tryptophol, and D-tryptophan. Following this report, Frese et al. developed a method for the immobilization of FDHs into cross-linked enzyme aggregates (CLEAs). Using this methodology, CLEAs containing RebH and necessary cofactor regeneration enzymes were used as catalyst to produce 1.813 g of 7-bromotryptophan using enzyme obtained from 5 L of RebH culture (81).

Like RebH (33), several other FDHs, including the Trp-FDHs SttH (56), Thal (33), PrnA (82), PyrH (82), and KrmI (83) as well as the phenol FDHs Rdc2 and Gsfl (33, 59, 84), have demonstrated broad substrate scope and interesting site-selectivities (Fig. 4B, **14–20**). As preparative methods became more established and substrate scope broadened, interest arose in using FDH-catalyzed halogenation products as intermediates in cross-coupling reactions to achieve site-selective arylation. Goss and coworkers were able to chlorinate the peptide antibiotic pacidamycin and use this as a functional handle to selectively arylate this site (85). As a more general method, Lewis and coworkers were able to selectively arylate several biologically active aromatic compounds from crude FDH-catalyzed reaction mixtures (86). In addition, this system was used to demonstrate formation of new C–N and C–O bonds from FDH-generated aryl chlorides. Even more recently, Micklefield and coworkers devised a method for conducting site-selective FDH-halogenation and subsequent cross-coupling reactions in one pot using membrane compartmentalization (87).

In addition to their utility for *in vitro* biocatalysis, FDHs have also proven valuable for *in vivo* halogenation (88). Trp-FDHs, when introduced into *Streptomyces* species, can produce chlorinated pacidamycin (85) and indolocarbazole derivatives (89). Similarly, Rdc2 and RadH have been used to halogenate several phenol-containing compounds *in vivo* in *E. coli*, making use of the native *E. coli* reductases necessary to provide reduced flavin (37, 59). Lewis and coworkers have demonstrated that by using FDH-reductase fusions *in vivo*, higher products titers of both native and non-native substrates can be obtained in *E. coli*

BL21 (75). Expanding even past bacterial hosts, Trp-FDHs, co-expressed with a reductase partner, have been used *in planta* to generate halogenated alkaloid derivatives (90, 91). Recently, it has been shown that FDHs can be targeted to the chloroplasts *in planta*. Because chloroplasts contain naturally occurring reductases, there was no need for heterologous reductases (92).

3. Structure-guided mutagenesis of FDHs

3.1 Active site mutations to probe mechanism

The range of biocatalytic applications reported for FDHs to date has established these enzymes as useful tools for site-selective, green halogenation. Targeted mutagenesis of several FDHs has been used to probe their mechanism and alter their catalytic properties. Crystal structures are required for these efforts, however, and the first FDH to be characterized crystallographically was PrnA. As noted above, this structure led to the hypothesis that an active site lysine residue directly interacts with a chlorinating agent to enable site selective halogenation (42). This active site lysine residue is conserved in all known FDHs. Mutation of this residue to alanine completely abolished halogenation activity in RebH (K79) (41), PrnA (K79) (42), Rdc2 (K74) (93), and RadH (K74) (37). While the importance of this residue in catalysis and site-selectivity remains unquestioned, the nature of the interaction between HOCl and the active site lysine residue is still debated (Fig. 3A). Work from the Walsh and Drennan labs has demonstrated the existence of a long-lived chlorine-containing form of RebH (41). This species was formed when RebH, O₂, reduced flavin, and chloride react in the absence of L-tryptophan. Flavin was subsequently removed through standard desalting procedures, and the resulting RebH form retained chlorinating ability for up to 63 hours (41). Lys79 was found to be crucial to the formation of this species, and thus it was proposed that HOCl reacts with Lys79 to form the more stable (with respect to HOCl) chloramine (41), which was found to be a kinetically competent halogenating species.

On the other hand, work from the van Pée lab suggests that HOCl may be the active chlorinating species involved in FDH catalysis. This claim is based on the observation that active site residue Glu346 in PrnA, another 7-Trp-FDH, is crucial for high rates of catalysis (42, 44). When the point mutation E346Q is introduced into PrnA, halogenation rates drop by two orders of magnitude (42). This glutamate residue has been proposed to deprotonate the Wheland intermediate involved in arene halogenation proceeding via EAS thus restoring aromaticity (Fig. 2B). No KIE is observed for halogenation of L-tryptophan (32), confirming that deprotonation is facile and occurs after the rate-limiting step (presumably formation of the Wheland intermediate), as is commonly observed for EAS reactions (94). Given the acidity of such an intermediate, it is unlikely that a dedicated general base would be required for its deprotonation. Glu346 could instead lower the barrier associated with electrophilic attack of substrates by the active halogenating species via electrostatic stabilization of the positive charge that develops during formation of the Wheland intermediate. van Pée and coworkers have demonstrated that E346D mutation in PrnA, which remains negatively charged, is inactive (44). It was discovered upon structural characterization of the E346D

variant that this mutation adopts a different conformation than wild-type. Thus, van Pée and coworkers conclude that localization of negative charge is crucial to its function.

In light of these mutational studies with the active site glutamate of PrnA, van Pée and coworkers propose another role in catalysis - one in which direct interaction between glutamate and the chlorinating species occurs. Instead of Lys-εNH-Cl serving as the chlorinating species, they propose that it could act as a stable Cl⁺ reservoir that, upon substrate binding, could regenerate HOCl. Subsequently, HOCl could interact with both Lys79 and Glu346 to increase its electrophilicity (44). Interestingly, although this active site glutamate is conserved among Trp-FDHs, it is absent in pyrrole and phenol FDHs. It has been proposed that the absence of this residue in the latter cases is due to the greater electronic activation of these substrates relative to the indole benzo ring; further activation by the enzyme is not needed for EAS (44). HalA calculations (23) and nucleophilicity studies (25) indicate that pyrroles are indeed more activated than the benzo positions of indole, but neutral phenols are not (Fig. 2C). It is possible that phenol FDHs deprotonate the relatively acidic hydroxyl group of these substrates to increase their nucleophilicity (Fig. 3C) (37, 58). This is known to occur in monooxygenases (35), but it has not yet been established in FDHs, as there is currently no substrate-bound crystal structure of a phenol FDH. Further experiments and, likely, simulations will be necessary to clarify the nature of the active chlorinating species in FDHs as well as the role of the active site glutamate in Trp-FDHs.

Recently, work from the Micklefield lab provides the first support of this proposed deprotonation event in phenol FDHs (37). A homology model was built for RadH (77% sequence identity to Rdc2) based on a geranylgeranyl reductase flavoprotein. The native substrate of RadH was docked into this model and active site residues that could potentially interact with this substrate were identified. Alanine mutations were individually introduced into the RadH gene at these residues. N98, S329, S330, and Q413 are within H-bonding distance of substrate, and alanine mutations of these residues did indeed reduce the activity of RadH. K74, F328, and D325 are highly conserved among phenol FDHs. When these were mutated to alanine in RadH, F328A, which is thought to be involved in π-stacking interactions with substrate, demonstrated reduced activity; however, K74A and D325A abolished activity. The role of K74 in catalysis is discussed above. According to the homology model, D325 is positioned proximal to the phenol of substrate. This residue could be deprotonating substrate, and thus increasing the electronic activation of substrate. This hypothesis is supported by the observation that halogenation is always observed ortho to the phenol hydroxyl substituent (33, 37, 59), since D325 and K74 are positioned such that deprotonation and chlorination would always occur with this regioselectivity.

3.2 Non-active site mutations to probe mechanism

The mechanism of FDHs involves two separate binding pockets - one for the substrate to be halogenated and one for flavin/halide. As described previously, hypohalous acid (HOX) is formed within the flavin binding pocket. To get to the substrate active site, HOX is thought to travel through a tunnel approximately 10 Å long that connects these two pockets within the FDH. Upon reaching the active site, HOX can interact with the crucial, active site lysine

residue (Fig. 3A). The structure of CmlS, an FDH involved in the biosynthesis of chloramphenicol, has provided an interesting view of this tunnel in which a hydrogen bonding network extends from the FDH surface to the substrate active site (Fig. 5A) (71). Within this network, a water molecule is located where the peroxide of FAD(C4a)-OOH is thought to reside. To reach the active site, it appears that newly generated HOX first forms a hydrogen bond with a serine residue highly conserved among all FDHs (S305 in CmlS). Indeed, van Pée and coworkers proposed this mechanism of HOX transfer based on X-ray crystal structures of PrnA. Interestingly, when this seemingly crucial residue is mutated to alanine in PrnA (S347A), the resulting variant only displays a 2.5-fold loss of activity (44). Even more surprising, mutation of this conserved residue can potentially be advantageous (95) (see section 4.1). A more thorough understanding of substrate, flavin, and chloride entry and movement through these enzymes will perhaps be necessary to elucidate the role of this serine residue in catalysis.

On the other end of the tunnel, a highly conserved glutamate residue is the last residue in this hydrogen-binding network and links the protein surface with the active site (Fig. 5A, E44 in CmlS, Fig. 5B, E46 in PrnA). It has been proposed that this network can function as a proton shuttle (71). When this residue is mutated in PyrH (E46Q and E46D), k_{cat} is greatly diminished, thus supporting its importance to catalysis (k_{cat} of wtPyrH = 3.56 min^{-1} ; k_{cat} of PyrH-E46D/Q = 0.06 min^{-1}) (55). It is unclear why mutation of this residue, if it acts as a proton shuttle, is more detrimental to catalysis than mutation of the serine residue located at the active site end of the tunnel. Interpreting the function of this glutamate residue is further complicated by its proximity to the FAD binding pocket. van Pée and coworkers note that this residue undergoes a large, 180 degree conformational change upon FAD bound vs. apo structures (Fig. 5B), and thus hypothesize that this residue is involved in regulating the exchange of reduced and oxidized FAD (55).

Another highly conserved motif in FDHs is the sequence $\text{W}\times\text{W}\times\text{IP}$, located near the isoalloxazine ring of FAD (Fig. 5B). It has been proposed that the bulky Trp residues (W272 and W274 in PrnA) prevent substrate binding proximal to FAD and thus inhibit monooxygenase activity (42). In the monooxygenase PHBH, these residues correspond to F208 and L210, and in PHBH these residues are positioned further from FAD. Surprisingly, mutation of either of the Trp residues in PrnA to Phe does not affect halogenation activity (44). Additionally, although both W residues are highly conserved, mutation of W274A completely abolishes halogenation activity, while W272A appears to have no effect on activity. It would be interesting to know whether variants W272F/A and W274F do produce other oxidative products as well as halogenated product. Overall, the conservation of this motif remains enigmatic.

Also within the FAD binding domain is the conserved sequence $\text{D}(\text{W/Y})\text{SY}$, which is only absent in Trp-FDHs. In Trp-FDHs, FAD is non-covalently bound within the enzyme and is thought to be exchanged during the catalytic cycle; however, structural characterization of CmlS led to the discovery that covalent attachment of FAD is also possible within FDHs (71). In CmlS, a covalent bond between residue D277 ($\text{D}(\text{W/Y})\text{SY}$) and the isoalloxazine ring is apparent in the crystal structure (Fig. 5A). Jia and coworkers discovered that upon purification of CmlS, flavin could not be easily washed away, further supporting

crystallographic evidence of a covalent attachment. When the mutation D277N is introduced into CmlS; however, all flavin can be removed upon washing. Notably, after purification, similar amounts of flavin-bound CmlS are observed for wtCmlS and the D277N variant, suggesting that non-covalent interactions are very important to flavin binding within this enzyme as well.

3.3 Mutations to alter functionality

As insight into the structure and mechanism of FDHs continues to increase, so to do efforts aimed at engineering these enzymes using site-directed point mutagenesis. These efforts have led to both altered function and further mechanistic understanding of FDHs. Targeted mutations to alter substrate specificity, enzyme activity, and site-selectivity of FDHs will be discussed in this section.

As noted in section 2.3, FDHs have been used *in planta* to create new chlorinated monoterpene indole alkaloids (90). Although chlorinated alkaloids were synthesized using wt RebH, a large build-up of 7-chlorotryptophan was observed. This was due to the reduced activity of tryptophan decarboxylase, the first step in alkaloid formation, on 7-chlorotryptophan. The product of this decarboxylation step, tryptamine, was found to also be selectively halogenated at the 7-position by RebH but at a much lower rate (80). To increase the efficiency of alkaloid biosynthesis using this system, O'Connor and coworkers used site-directed mutagenesis of the substrate-binding pocket to change RebH specificity from L-tryptophan to tryptamine (91). Seventeen active site variants were individually cloned and evaluated; however, only variants L113G and Y455W retained activity. Of these, Y455W led to a 10-fold reduction in L-Trp halogenation and a 3-fold increase in tryptamine halogenation. Using this variant *in planta* removed accumulation of 7-chlorotryptophan. Based on the crystal structure of RebH, it was proposed that the larger Trp residue at site 455 sterically hinders carboxylic acid binding, which would be detrimental to Trp but not tryptamine. It is unclear why this variant also demonstrates increased activity on tryptamine.

The ability to understand and control the site-selectivity of FDHs has also been pursued through targeted mutagenesis. To gain insight into which active site residues contribute to substrate binding and regioselectivity, van Pée and coworkers introduced three point mutations into PrnA (96). Large, aromatic residues were chosen and mutated to alanine (H101A, F103A, and W455A – note that this last residue corresponds to W466 in RebH, not the W455 targeted by O'Connor). Both H101A and W455A significantly decreased substrate binding (from $K_m \sim 50 \mu\text{M}$ to $K_m \sim 1800 \mu\text{M}$) and turnover number. F103A also decreased enzyme efficiency, but another halogenated isomer of Trp was also observed using this variant. Characterization of the halogenated product mixture showed that a 1:2 mixture of 5- and 7-bromotryptophans was produced using this variant; the product ratio for the corresponding chlorination reaction was not reported (Fig. 6A). Analysis of the wtPrnA crystal structure led to the hypothesis that when the bulky F103 residue was mutated to Ala, L-Trp could rotate within the active site to a similar conformation observed in the 5-Trp-FDH PyrH. These studies thus demonstrated the importance of these 3 residues in substrate binding as well as the potential to alter the site-selectivity of halogenation of FDHs, though the native isomer was still favored by a significant margin.

In 2015, Micklefield and coworkers explored the activity of Trp-FDHs PyrH and PrnA toward several benzoic acids, including anthranilic acid (82). PyrH catalyzed halogenation of this substrate exclusively at the position para to the aniline substituent while PrnA provided primarily the ortho isomer (84:16 ortho/para). Kinetic characterization of PrnA indicated that its decreased activity on anthranilic acid relative to L-Trp results primarily from poor substrate binding. In an attempt to improve substrate binding and anthranilic acid halogenation efficiency, the authors introduced several mutations into the PrnA active site. Residues Y443, Y444, E450, and F454 were all mutated to both Arg and Lys with the reasoning that introducing a positive charge into the active site could improve binding with the benzoic acid moiety of anthranilic acid. Variants Y443R/K and Y444R/K had little impact on the activity and selectivity of halogenation; however, E450R/K and F454R/K did indeed improve conversion. Of these single point mutations, E450K demonstrated the highest increase in conversion (8-fold) and increased the site selectivity for ortho halogenation from 84:16 to 93:7. The double mutant E450K+F454R switched the selectivity to 38:62, so that the major isomer was halogenated at the para position (Fig. 6B).

Following this report, Lewis and coworkers demonstrated that the E450K mutation in PrnA to improve anthranilic acid activity and selectivity carried over to RebH (E461K) (97). This variant was then used as a starting point for mutagenesis studies that targeted the FAD-binding pocket for improved activity. Unlike typical site-directed mutagenesis, the method employed in these studies targeted 25 sites within the FAD-binding pocket and introduced the degenerate NNN codon at these sites with tunable frequency. 1,000 variants from a library containing 1–2 mutations per gene at the targeted sites were screened for activity on anthranilic acid. As expected when mutating such a highly conserved domain like the FAD-binding pocket, most of the variants in this library showed no conversion of anthranilic acid; however, one mutation – R231K – increased the k_{cat} 1.7-fold. It is unclear why this mutation increases rate.

As more FDH structures are deposited into the PDB, alignments of these structures are informing mutational studies. Recently, the structure of the 6-Trp-FDH SttH was solved (56). When SttH is aligned with the 5-Trp-FDH PyrH, the key differences between the active sites of these structures are three residues located in the amino acid binding pocket (L460, P461, P462 in SttH; F451, E452, T453 in PyrH). When these residues are mutated in PyrH (F451L, E452P, T453P), the resulting variant is inactive; however, the triple mutant in SttH (L460F, P461E and P462T) remains active and alters the site-selectivity of L-Trp halogenation from 100% at the 6-position to a mixture of 68% 6-halogenation and 32% 5-halogenation. Moreover, the triple SttH mutant produces a 3:1 mixture of the 5- and 6-chloro isomers of 3-indolepropionic acid whereas SttH gives a 1:9 mixture (Fig. 6C). Interestingly, all three mutations are necessary to alter the selectivity of SttH – individual mutation did not affect selectivity – highlighting the beneficial epistasis often necessary to confer change in enzyme function.

4. Directed evolution of FDHs

Although much information about FDHs has been gained by targeted mutagenesis and in some cases, desirable biocatalysts can be engineered, it is obvious that we do not yet have

sufficient understanding of FDH function to reliably predict beneficial mutations. Additionally, targeted engineering has focused on the active site, but it is well-established that mutations distal to the active site can significantly improve enzyme activity. Directed evolution, involving iterative rounds of targeted or random mutagenesis and screening, can be used to identify mutations that would not be apparent with our current understanding of FDHs and thus alter the function of these enzymes in a systematic manner (98–100).

4.1 Mutations to improve thermostability

The first example of using directed evolution to alter FDH function made use of error-prone PCR (ep-PCR) and screening with UPLC to increase the thermostability of the 7-Trp-FDH RebH (101). Following heat treatment, FDH cell lysates were tested for residual activity on L-Trp. After only three rounds of mutagenesis and screening, a variant (3LSR) with an 18 °C higher melting temperature (T_m) than RebH was identified. This variant was found to produce higher yields of halogenated products due to increased catalyst lifetime. The crystal structure of 3LSR was solved (PDB ID: 4LU6) and, as is often observed with thermostable variants, most mutations were located distal to the active site and near the surface of the enzyme (102, 103). One such mutation – Q494R – increases surface charge, which is reported to deter protein aggregation (104). Interestingly, this same mutation was independently identified via ep-PCR of a different RebH variant, and it increased conversion in this case also, likely for the same reason (see section 4.3) (32). The mutation S2P, which alone increases the T_m of RebH by 2 °C, could be adding extra rigidity to the polypeptide chain, thereby preventing thermal unfolding via “fraying” at the N-terminus (105). Additionally, the mutation K145M, located nearby two arginine residues, could improve the stability of RebH by decreasing local positive charge. It was later found that when K145 is present, mutation of one of these arginine residues to glutamine (R509Q) also provides higher conversion (32).

As more successful examples of FDH engineering emerged, both through site-directed mutagenesis and directed evolution, novel strategies have been developed to increase throughput of library screening (32, 37, 95, 106). The use of UPLC in screening is general and effective; however, faster methods are desirable. A high-throughput colorimetric screen was developed by Micklefield and coworkers in which aryl amines are added to catechol through a Michael addition (106). These compounds are highly colored and, in several cases, significant differences are observed between the UV/Vis spectra of their chlorinated analogues. Chlorination of these aryl amines can be quantitated through spectrophotometric analysis. Although this approach could prove very valuable for screening larger libraries of FDHs, it has only been established for reactions using purified enzyme. In order for this to be a practical method for directed evolution of FDHs, it will have to function using products in cell lysates. Additionally, this method is inherently limited to aryl amines that have different UV/Vis spectra of chlorinated analogs, which is frequently not the case.

Work from the Sewald lab has also produced a novel, rapid screening method for FDH halogenation reactions (95). In this screen, FDH-generated tryptophan bromides are coupled with boronic acids to produce biaryls with strong fluorescence emission that tryptophan lacks. Moreover, it was demonstrated that this method can be used in crude cell lysates.

Using this screen and ep-PCR, Schnepel et al. were able to identify a more thermostable variant of the 6-Trp-FDH Thal, containing two mutations – S359G and K374R (95). This variant, termed Thal-GR, has a T_m 10 °C higher than that of wild-type and displays higher activity on L-Trp. It is not clear if only one or both of these mutations is responsible for improved function. K374R is a surface mutation; however, S359 corresponds to S347 in PrnA – a highly conserved residue among FDHs that is located along a hydrogen bonding network that extends from the surface to the active site (Fig. 5). Previously, as discussed in section 3.2, van Pée and coworkers introduced the mutation S347A into PrnA and displayed a 2.5-fold loss in activity (44). If S359G in Thal-GR is responsible for increased stability and activity, it would be interesting to know if the advantages of this mutation are FDH-dependent or whether the difference between residue (A vs. G) is important.

Complementing these engineering approaches to stabilize known halogenases, Micklefield and coworkers used genome mining to identify a thermostable FDH, Th-Hal (53). This enzyme was reported to possess a higher melting temperature (49 °C) than several other FDHs (T_m =30–40 °C for RebH, SttH, PrnA, PyrH). While these T_m values suggest that Th-Hal is relatively stable, the absolute melting temperatures are significantly lower than those previously reported for RebH (T_m = 52 °C) (101) and Thal (T_m = 47 °C) (95).

4.2 Mutations to alter activity and substrate scope

FDHs are powerful biocatalysts for halogenation because of their unique site selectivities (Fig. 2A); however, FDHs typically suffer from relatively low (107) kinetic efficiency ($k_{cat} \sim 0.5\text{--}3 \text{ min}^{-1}$). Additionally, although the substrate scope has been demonstrated to be quite broad for many FDHs, limitations still exist. To address these issues, directed evolution has been employed to increase FDH activity as well as broaden substrate scope (37, 108).

The Trp-FDH RebH was found to possess broad substrate scope on smaller substrates (~200 Da), but it did not accept larger indole-containing structures such as those found in a range of biologically active molecule and natural products. The first example in which directed evolution was used to expand FDH substrate scope was reported by Lewis and coworkers. These authors used a substrate walking approach to engineer RebH variants 3SS and 4V, which could halogenate large, biologically active substrates such as the biofilm inhibitor **34** and yohimbine (Fig. 7A, **35**) (108). Both variant 3SS and 4V contains 6 mutations. Of these mutations, three were previously found to increase thermostability (101). Mutations N467T and N470S are also found in both variants and are located near the active site. Both mutations were found to increase conversion of tryptoline. These smaller residues, which are near the amino acid binding pocket of L-Trp, could perhaps allow more space in the active site for the increased rigidity of tryptoline relative to tryptophan. Interestingly, the N470S mutation also increases conversion of the smaller substrate tryptamine, suggesting that opening the binding pocket may not be the function of N470S--a finding discussed further in section 4.3. Mutation G112S is also near the active site and was found to increase conversion of tryptoline derivatives but was detrimental to the activity of many of the substrates tested. For this reason, G112S was removed from 4V. Lastly, 4V contains the mutation A442V, which was found to expand the scope of RebH to much larger substrates such as yohimbine and evodiamine. This particular mutation is notable in that its location distal to the active site

and the fact that it increases residue size (from A to V) make its conferring improved activity on larger substrates difficult to rationalize.

Very recently, the phenol halogenase RadH, a close homologue of Rdc2 (59, 60) was the subject of a directed evolution effort. Micklefield and coworkers used a high-throughput screen that detects differences in fluorescence emissions of 7-hydroxycoumarins and their chlorinated counterparts (37). This screen worked in crude lysate and was used to identify a RadH variant with improved expression and 1.4-fold improved activity on 7-hydroxycoumarin (37). This variant contains mutations D465E and T501S, both of which appear distal to the active site according to a homology model of RadH; however, structural characterization is necessary to confirm residue location.

4.3 Mutations to alter site-selectivity

In addition to low kinetic parameters, a fundamental challenge facing these catalysts (and C–H functionalization catalysts in general) is changing their site-selectivity (109). Site selectivity is only useful if it happens to be the selectivity one is interested in; if it is not, robust means to alter the observed selectivity are needed. Examples of altering site selectivity of FDHs through site-directed mutagenesis are discussed in section 3.3, however, these methods do not provide a general and systematic approach to tuning selectivity--mutations were introduced, and altered selectivity was sometimes observed. Moreover, only modest changes in selectivity were reported (Fig. 6A–B), and this was true for other enzymes for which similar efforts had been undertaken. In response to this problem, Lewis and coworkers designed a MALDI-MS screen that makes use of deuterated probe substrates to systematically tune FDH selectivity for targeted product isomers (32). Starting from the 7-Trp-FDH RebH, which was found to chlorinate C7 of tryptamine with 99% selectivity, variants capable of selectively chlorinating at C6 (90% sel., 8F) and C5 (95% sel., 10S) were engineered (Fig. 6D). These levels of altered selectivity are far higher than any previously reported using targeted mutagenesis. Additionally, introducing point mutation N470S into RebH (variant 0S) increased total conversion of tryptamine without affecting selectivity.

Further mutagenesis studies, including individual reversion mutations of these three variants (0S, 8F, and 10S), have shed light on the underlying molecular mechanisms by which activity and/or site-selectivity is altered (110). In both 8F and 10S, mutations at residue 52 (I52M and I52H, respectively) and 465 (F465L and F465C, respectively) affected selectivity the most. Mutations at these sites were first identified following ep-PCR and optimal substitutions were subsequently established by screening degenerate codon libraries. Structural characterization of 10S revealed that H52 is pointed into the active site. Native tryptamine binding would cause a steric clash between the His side chain and the pyrrole of tryptamine. To mitigate this interaction, docking studies suggest that in 10S, tryptamine is flipped in the active site such that the C5 position is proximal to the catalytic Lys, much like 5-Trp-FDH PyrH (Fig. 3B). Mutation of H52F in 10S also leads to high selectivity for the C5 position. When the identity of this residue is compared between native Trp-FDHs, the broad importance of this residue for selectivity becomes apparent - all three known 5-Trp-FDHs contain a Phe residue at this position while the 7-Trp-FDHs PrnA and RebH both contain an Ile residue. Crucially, mutation of this residue alone to either His, Phe, or Tyr in

RebH leads to inactive enzymes (32, 91). Residue 465 is near the active site, however, it is still unclear how mutation to Leu in 8F and Cys in 10S specifically alter activity and selectivity. In 8F, the mutation S110P was found to increase both activity and selectivity. This residue is flanked by two aromatic residues – H109 and F111 – which are thought to π -stack with tryptamine. By introducing a proline residue between these two, extra rigidity could be helping to correctly position tryptamine for reaction (111).

In addition to site selectivity, several residues were found to influence activity. The mutation N470S was found to increase total conversion in all three variants. In 0S, a 6-fold improvement in k_{cat} is observed. As discussed briefly in section 4.2, N470S is located near the active site and could be improving activity by opening up the binding pocket; however, N470A in the variant 0S does not improve activity. Mutation R509Q was determined to improve total conversion in 8F and 10S. This residue is located in the same area of high local positive charge as the K145 residue discussed in section 4.1. It would be interesting to determine whether only one of these mutations improves activity/stability at a time, which would support that they are acting via a similar mechanism. The mutation S448P was found to substantially reduce the activity of 8F but not 10S. This mutation is distal to the active site, in a flexible loop that is often disordered in crystal structures of RebH (41, 110). It is unclear how this is affecting activity, as this loop is also disordered in all crystal structures of 8F. Interestingly, the mutation F111L, which corresponds to F103 in PrnA (discussed in section 3.2), was found to modestly alter site selectivity early in the evolutionary lineage. However, upon later reversion, higher activities and, in the case of 8F, higher selectivity was observed. To gain further insight into the role of these mutations, the crystal structures of 0S, 10S and 8F were solved and are currently under analysis.

5. FDH substrate activity profiling

5.1 Substrate scope and site-selectivity of FDHs on diverse substrate panels

To further assess and understand FDH substrate scope, Lewis and coworkers generated substrate activity profiles for a broad set of arenes by evaluating the activity of native FDHs (RebH, Thal, Rdc2, and GsfI) and engineered FDHs on 93 substrates. The 93 substrates selected for evaluation were grouped into two panels based on their structures. One panel consisted of 86 *N*-containing aromatic substrates (indoles, pyrroles, azoles, anilines, and anilides). The second panel contained 7 phenols and anisoles, taking into consideration the native substrates of the fungal FDHs. Because FDH halogenation proceeds through electrophilic aromatic substitution (41, 44), all substrates in both panels contained at least one electronically activated site, and most had multiple potentially reactive sites. Substrates were also chosen to probe significant steric and electronic variation, as well as functional group substitution. Moreover, the compounds in these panels were acquired from a fragment compound library from the Novartis Institute for Biomedical Research and are therefore representative of those commonly used in fragment-based drug design (112). These studies were thus expected to provide valuable insight into FDH reactivity toward motifs common in pharmaceuticals.

Analysis of these substrate activity profiles indicated that 69% of panel 1 (59 substrates) was halogenated by at least one of the Trp-FDHs, clearly establishing that FDHs can possess

broad substrate scope. Remarkable functional group tolerance was observed, with substrates containing amines, alcohols, amides, esters, nitriles, thioesters, sulfonamides, pyridines, azoles, quinolones, and pyrroles all undergoing halogenation. Interestingly, the FDH with the broadest substrate scope on panel 1 substrates was RebH variant 4V, which had been previously engineered for increased substrate scope to larger, biologically active indole-containing compounds (see section 4.2) (108). It appears that widening the substrate scope to certain target compounds enabled a broader, more general scope as well.

Although the Trp-FDHs chlorinated the majority of substrates in panel 1, very few were accepted as substrates by the fungal FDHs (8%, 7 substrates). For this reason, a second panel of phenols and anisoles, substrates more closely resembling these enzymes native substrates, was analyzed with all 8 FDHs. The Trp-FDHs were not able to halogenate these less nucleophilic substrates (25) to as great of an extent as the more electronically activated aniline, indole, pyrrole, and azole compounds; however, all panel 2 substrates were chlorinated by at least one of the fungal FDHs. This complementarity in substrate scope between the Trp-FDHs and fungal FDHs was particularly interesting.

The site selectivity of FDH halogenation was analyzed after evaluating the substrate scope for 8 FDHs. Despite the fact that, as noted above, many of these compounds possess multiple sites that could undergo electrophilic aromatic substitution, a single chlorinated isomer was typically observed in RebH-catalyzed chlorination reactions. For example, the deschloro analog of **14** (Fig. 4) is highly activated for EAS at the positions ortho and para to its aniline substituent; however, only chlorination at a single ortho position (**14**) is observed.

Given that site-selective RebH-catalyzed chlorination was observed even on non-native substrates with structures significantly different than that of L-tryptophan, this selectivity was then compared with that of the small-molecule chlorinating agent *N*-chlorosuccinimide (NCS). Several substrates for which RebH-catalyzed chlorination provided a single isomer were chlorinated at several positions with NCS. Remarkable examples of this include substrates **38** and **39** (Fig. 7B), which have three and two unique, electronically activated positions, respectively. Although RebH chlorinates nearly exclusively (>95% selectivity) at a single position on each substrate, NCS gives product mixtures of all expected chlorinated isomers (Fig. 7B).

Excitingly, one case arose for which both NCS and RebH produced a single chlorinated product; however, NMR characterization revealed that different isomers were produced. NCS halogenated 1-(4-hydroxyphenyl)pyrrole (Fig. 2A) selectively at the 2-position of the pyrrole (i.e. the most electronically activated site) while RebH only catalyzed chlorination of the less electronically activated 3-position. Although complete overriding of substrate electronics has been observed with Trp-FDHs for 3-substituted indoles, such high levels of catalyst control on a substrate so different from L-tryptophan is notable.

The deschloro form of **19** (Fig. 4), a long-acting β_2 agonist known as formoterol (113), contains three sites sufficiently activated for EAS. Rdc2-catalyzed chlorination of this substrate exclusively at the position ortho to the phenol hydroxyl substituent. On the other hand, halogenation of formoterol using NCS led to a complex mixture of mono- and di-

halogenated compounds. Like formoterol, phenol (Fig. 7C, **41**) has more than one electronically activated site and is halogenated by Rdc2. It was found that Rdc2 halogenates the ortho position of phenol; however, only para chlorination is observed with RebH variant 4V. This particular example highlights the power of FDH catalyst control for aromatic halogenation, whereby even a simple non-native substrate can be site selectively halogenated.

Following this effort, Micklefield and coworkers demonstrated that the fungal halogenase RadH, which has high sequence similarity to Rdc2, functions on several other phenolic substrates as well (37). As in the study of Rdc2 substrate scope by Lewis and coworkers, ortho selectivity was observed for all phenols that were successfully halogenated. The importance of this finding will be discussed more in section 5.2.

5.2 Substrate electronics and binding contribute to FDH site-selectivity

From the results described in sections 5.1, it is clear that substrate binding within FDHs influences halogenation site selectivity, even for non-native substrates, since the results were not consistent with substrate electronics alone. Nonetheless, because FDH halogenation proceeds through an EAS mechanism (41, 42), substrate electronics should have some effect on specificity and selectivity as well. The electronic activation of each sp^2 -hybridized C-H bond on a given compound was determined via calculation of halonium affinity (HalA) values to look for correlations between halogenase activity and electronic effects (23). Similar methods have been used to rationalize site selectivities of electrophilic aromatic substitution reactions for small molecule reagents (25, 114–116).

HalA values were calculated for all sp^2 -hybridized C-H bonds on all substrates for which RebH site selectivity had been determined. These values were subsequently plotted against the observed chlorination at each site. As expected from site selectivity comparisons with NCS, several sites that were highly electronically activated (175–185 kJ/mol) were not chlorinated by RebH. Although no clear trend existed between conversion at a particular site and HalA, there appeared to be a HalA cutoff, below which trace to no conversion to product was observed for RebH (~160 kJ/mol). Taken together, these results suggest that a given C-H bond must be sufficiently electronically activated for RebH halogenation ($\text{HalA} > 160 \text{ kJ/mol}$) as well as able to access a position in the active site proximal to the catalytic Lys79.

When HalA values for the phenol substrates described in section 5.1 were calculated, lower HalA values were observed than the anilines, indoles, pyrroles, and azoles. Rdc2 is able to halogenate these less nucleophilic phenol substrates to a much greater extent than RebH. It is striking that Rdc2 is able to accomplish this, but halogenates very few of the substantially more activated aniline substrates. This interesting substrate specificity could be due to differences in substrate binding of phenol vs. aniline substrates within the Rdc2 active site or due to a slightly different mechanism for Rdc2 chlorination. For example, if the phenol hydroxyl substituent was deprotonated during chlorination, the resulting phenolate character would dramatically increase the nucleophilicity of these substrates beyond what would be expected based on HalA values calculated for the neutral compounds. As discussed in section 3.1, a recently reported homology model of RadH supports this notion; it was suggested that RadH is able to partially or fully deprotonate the alcohol substituent of a

phenol substrate using residue D325 (37). In addition to Rdc2, engineered RebH variants were able to chlorinate phenol substrates to a greater extent than RebH. These results suggest that both directed evolution and genome mining for novel, natural FDHs are viable methods for discovering FDHs with the ability to halogenate compounds with lower HalA values.

To further understand how substrate specificity and selectivity is conferred by FDHs, substrate binding in RebH through docking simulations was explored using both ICM Molsoft docking (117) and ROCS pharmacophore overlay (118). Neither method was found to reliably predict substrate specificity - catalytically relevant poses were returned for several compounds that were not accepted as substrates by RebH. In addition, many poses from the ICM Molsoft docking method were not catalytically relevant (i.e. no sp^2 -hybridized C-H bond proximal to Lys 79). On the other hand, although ROCS pharmacophore overlay did not provide insight into substrate specificity, it did provide a number of interesting results concerning the selectivity on substrates that were halogenated. For example, several substrates, including **38**, were all selectively halogenated at a site other than the most electronically activated. When docked into the RebH active site using the pharmacophore model, the site for which halogenation was known to occur was positioned proximal to Lys79. Polar functional groups distal to the site of halogenation appear to bind in the same pocket as the amino acid moiety of L-tryptophan (Fig. 7D). The unique site selectivities observed for these substrates could result from this anchoring effect (119).

Although these cases provide insight into manner in which binding could be influencing selectivity, many substrates docked so that the C-H bond proximal to Lys79 was not consistent with experimentally determined site selectivity. Thus, although this computationally inexpensive docking method can provide insight into FDH selectivity, more computationally rigorous methods such as molecular dynamics simulations (120) could improve predictive abilities for FDH substrate specificity and selectivity.

6. Summary and Outlook

As emphasized throughout this review, a great deal of progress has been made on both understanding the mechanism and improving the utility of FDHs since they were first functionally characterized in 1997. Many of these studies have focused on or benefited from targeted or random mutations to alter FDH function. This has led to the identification of residues intimately involved in FDH catalysis, such as the active site lysine residue conserved in all known FDHs. Other mutations, found throughout the structures of different FDHs, have led to significant changes in substrate binding and thus the selectivity and scope exhibited by the resulting enzyme variants. Of course, identifying mutations that alter enzyme function does not typically provide a clear picture of the origin by which altered function arises. Ongoing studies involving enzyme kinetics, X-ray crystallography, MD simulations, and QM/MM calculations could shed light on these details and ultimately aid in further improvements to FDH function for practical applications

It is also clear from initial efforts to delineate the scope of known FDHs and to identify new FDHs that much reactivity remains to be discovered within this enzyme class. Expanding

efforts into FDH genome mining will therefore be hugely valuable from a biocatalysis perspective, and the sequence-function data obtained from these efforts will provide further insights into the impact of different residues on catalysis of different halogenation reactions. Finally, while this review has focused on FDHs, the successes realized for this class of enzymes will likely inspire similar efforts with other classes of halogenases, particularly Fe(II) α -ketoglutarate dependent halogenases. As has been the case for FDHs, targeted and random mutagenesis of these enzymes, coupled with efficient screening methods, will surely lead to exciting advances for selective halogenation of a wide range of organic substrates.

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References

1. Gribble GW. Naturally occurring organohalogen compounds. *Acc. Chem. Res.* 1998; 31(3):141–52.
2. Chen X, van Pée K-H. Catalytic mechanisms, basic roles, and biotechnological and environmental significance of halogenating enzymes. *Acta Biochim Biophys Sinica.* 2008; 40(3):183–93.
3. Herrera-Rodriguez LN, Khan F, Robins KT, Meyer H-P. Perspectives on biotechnological halogenation Part I: Halogenated products and enzymatic halogenation. *Chimica Oggi-Chemistry Today.* 2011; 29(4):31–33.
4. Lu Y, Liu Y, Xu Z, Li H, Liu H, Zhu W. Halogen bonding for rational drug design and new drug discovery. *Expert Opin Drug Discov.* 2012; 7(5):375–83. [PubMed: 22462734]
5. Vairappan CS, Kawamoto T, Miwa H, Suzuki M. Potent Antibacterial Activity of Halogenated Compounds against Antibiotic-Resistant Bacteria. *Planta med.* 2004; 70(11):1087–90. [PubMed: 15549668]
6. Harris CM, Kannan R, Kopecka H. The role of the chlorine substituents in the antibiotic vancomycin: preparation and characterization of mono- and didechlorovancomycin. *J. Am. Chem. Soc.* 1985; 107(23):6652–58.
7. Bunders CA, Minvielle MJ, Worthington RJ, Ortiz M, Cavanagh J, Melander C. Intercepting Bacterial Indole Signaling with Flustramine Derivatives. *J. Am. Chem. Soc.* 2011; 133(50):20160–63. [PubMed: 22091927]
8. Smith BM, Smith JM, Tsai JH, Schultz JA, Gilson CA, et al. Discovery and Structure–Activity Relationship of (1R)-8-Chloro-2,3,4,5-tetrahydro-1-methyl-1H-3-benzazepine (Lorcaserin), a Selective Serotonin 5-HT_{2C} Receptor Agonist for the Treatment of Obesity. *J. Med. Chem.* 2008; 51(2):305–13. [PubMed: 18095642]
9. Groll M, Huber R, Potts BCM. Crystal Structures of Salinosporamide A (NPI-0052) and B (NPI-0047) in Complex with the 20S Proteasome Reveal Important Consequences of β -Lactone Ring Opening and a Mechanism for Irreversible Binding. *J. Am. Chem. Soc.* 2006; 128(15):5136–41. [PubMed: 16608349]
10. Pereira ER, Belin L, Sancelme M, Prudhomme M, Ollier M, et al. Structure–Activity Relationships in a Series of Substituted Indolocarbazoles: Topoisomerase I and Protein Kinase C Inhibition and Antitumoral and Antimicrobial Properties. *J. Med. Chem.* 1996; 39(22):4471–77. [PubMed: 8893841]
11. Jeschke P. The unique role of halogen substituents in the design of modern agrochemicals. *Pest. Manag. Sci.* 2009; 66(1):10–27.
12. Cavallo G, Metrangolo P, Milani R, Pilati T, Priimagi A, et al. The Halogen Bond. *Chem. Rev.* 2016; 116(4):2478–2601. [PubMed: 26812185]

13. Wilcken R, Zimmermann MO, Lange A, Joerger AC, Boeckler FM. Principles and Applications of Halogen Bonding in Medicinal Chemistry and Chemical Biology. *J. Med. Chem.* 2013; 56(4): 1363–88. [PubMed: 23145854]
14. Auffinger P, Hays FA, Westhof E, Ho PS, Van Holde KE. Halogen Bonds in Biological Molecules. *Proc. Natl. Acad. Sci.* 2004; 101(48):16789–94. [PubMed: 15557000]
15. Nicolaou KC, Bulger PG, Sarlah D. Palladium-Catalyzed Cross-Coupling Reactions in Total Synthesis. *Angew. Chem. Int. Ed.* 2005; 44(29):4442–89.
16. Horton DA, Bourne GT, Smythe ML. The Combinatorial Synthesis of Bicyclic Privileged Structures or Privileged Substructures. *Chem. Rev.* 2003; 103(3):893–930. [PubMed: 12630855]
17. Littke AF, Fu GC. A Convenient and General Method for Pd-Catalyzed Suzuki Cross-Couplings of Aryl Chlorides and Arylboronic Acids. *Angew. Chem. Int. Ed. Engl.* 1998; 37(24):3387–88. [PubMed: 29711304]
18. Old DW, Wolfe JP, Buchwald SL. A highly active catalyst for palladium-catalyzed cross-coupling reactions: room-temperature Suzuki couplings and amination of unactivated aryl chlorides. *J. Am. Chem. Soc.* 1998; 120(37):9722–23.
19. Han F-S. Transition-metal-catalyzed Suzuki–Miyaura cross-coupling reactions: a remarkable advance from palladium to nickel catalysts. *Chem. Soc. Rev.* 2013; 42(12):5270–29. [PubMed: 23460083]
20. Li L, Zhao S, Joshi-Pangu A, Diane M, Biscoe MR. Stereospecific Pd-Catalyzed Cross-Coupling Reactions of Secondary Alkylboron Nucleophiles and Aryl Chlorides. *J. Am. Chem. Soc.* 2014; 136(40):14027–30. [PubMed: 25226092]
21. Podgoršek A, Zupan M, Iskra J. Oxidative Halogenation with “Green” Oxidants: Oxygen and Hydrogen Peroxide. *Angew. Chem. Int. Ed.* 2009; 48(45):8424–50.
22. Maddox SM, Nalbandian CJ, Smith DE, Gustafson JL. A Practical Lewis Base Catalyzed Electrophilic Chlorination of Arenes and Heterocycles. *Org. Lett.* 2015; 17(4):1042–45. [PubMed: 25671756]
23. Ashtekar KD, Marzijarani NS, Jaganathan A, Holmes D, Jackson JE, Borhan B. A new tool to guide halofunctionalization reactions: the halenium affinity (HalA) scale. *J. Am. Chem. Soc.* 2014; 136(38):13355–62. [PubMed: 25152188]
24. Smith BJ, Radom L. Assigning absolute values to proton affinities: a differentiation between competing scales. *J. Am. Chem. Soc.* 1993; 115(11):4885–88.
25. Pratihari S, Roy S. Nucleophilicity and Site Selectivity of Commonly Used Arenes and Heteroarenes. *J. Org. Chem.* 2010; 75(15):4957–63. [PubMed: 20670024]
26. Latham J, Brandenburger E, Shepherd SA, Menon BRK, Micklefield J. Development of Halogenase Enzymes for Use in Synthesis. *Chem. Rev.* 2017; acs.chemrev.7b00032–acs.chemrev.7b00038.
27. Agarwal V, Miles ZD, Winter JM, Eustáquio AS, Gamal El AA, Moore BS. Enzymatic Halogenation and Dehalogenation Reactions: Pervasive and Mechanistically Diverse. *Chem. Rev.* 2017; 117(8):5619–74. [PubMed: 28106994]
28. Weichold V, Milbredt D, van Pée K-H. Specific Enzymatic Halogenation-From the Discovery of Halogenated Enzymes to Their Applications In Vitro and In Vivo. *Angew. Chem. Int. Ed. Engl.* 2016; 55(22):6374–89. [PubMed: 27059664]
29. Neumann CS, Fujimori DG, Walsh CT. Halogenation Strategies In Natural Product Biosynthesis. *Chem. Biol.* 2008; 15(2):99–109. [PubMed: 18291314]
30. Vaillancourt FH, Yeh E, Vosburg DA, Garneau-Tsodikova S, Walsh CT. Nature's Inventory of Halogenation Catalysts: Oxidative Strategies Predominate. *Chem. Rev.* 2006; 106(8):3364–78. [PubMed: 16895332]
31. Smith DR, Grischow S, Goss RJ. Scope and potential of halogenases in biosynthetic applications. *Curr. Opin. Biotechnol.* 2013; 17(2):276–83.
32. Andorfer MC, Park HJ, Vergara-Coll J, Lewis JC. Directed evolution of RebH for catalyst-controlled halogenation of indole C–H bonds. *Chem. Sci.* 2016; 7:3720–29. [PubMed: 27347367]
33. Andorfer MC, Grob JE, Hajdin CE, Chael JR, Siuti P, et al. Understanding Flavin-Dependent Halogenase Reactivity via Substrate Activity Profiling. *ACS Catal.* 2017; 7(3):1897–1904. [PubMed: 28989809]

34. Massey V. Activation of molecular oxygen by flavins and flavoproteins. *J. Biol. Chem.* 1994; 269(36):22459–62. [PubMed: 8077188]
35. Walsh CT, Wencewicz TA. Flavoenzymes: Versatile catalysts in biosynthetic pathways. *Nat. Prod. Rep.* 2013; 30(1):175–200. [PubMed: 23051833]
36. Entsch B, van Berkel WJ. Structure and mechanism of para-hydroxybenzoate hydroxylase. *FASEB J.* 1995; 9(7):476–83. [PubMed: 7737455]
37. Menon B, Brandenburger E, Sharif H, Klemstein U, Shepherd S, et al. RadH a Versatile Halogenase for Integration into Synthetic Pathways. *Angew. Chem. Int. Ed. Engl.* 2017
38. Yeh E, Cole LJ, Barr EW, Bollinger JM, Ballou DP, Walsh CT. Flavin Redox Chemistry Precedes Substrate Chlorination during the Reaction of the Flavin-Dependent Halogenase RebH †. *Biochemistry.* 2006; 45(25):7904–12. [PubMed: 16784243]
39. Schreuder HA, van der Laan JM, Hol WG, Drenth J. Crystal structure of p-hydroxybenzoate hydroxylase complexed with its reaction product 3,4-dihydroxybenzoate. *J. Mol. Biol.* 1988; 199(4):637–48. [PubMed: 3351945]
40. Bitto E, Huang Y, Bingman CA, Singh S, Thorson JS, Phillips GN Jr. The structure of flavin-dependent tryptophan 7-halogenase RebH. *Proteins.* 2007; 70(1):289–93.
41. Yeh E, Blasiak LC, Koglin A, Drennan CL, Walsh CT. Chlorination by a Long-Lived Intermediate in the Mechanism of Flavin-Dependent Halogenases. *Biochemistry.* 2007; 46(5):1284–92. [PubMed: 17260957]
42. Dong CJ, Flecks S, Unversucht S, Haupt C, van Pee KH, Naismith JH. Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination. *Science.* 2005; 309(5744):2216–19. [PubMed: 16195462]
43. Blasiak LC, Drennan CL. Structural Perspective on Enzymatic Halogenation. *Acc. Chem. Res.* 2009; 42(1):147–55. [PubMed: 18774824]
44. Flecks S, Patallo EP, Zhu X, Ernyei AJ, Seifert G, et al. New Insights into the Mechanism of Enzymatic Chlorination of Tryptophan. *Angew. Chem. Int. Ed.* 2008; 47(49):9533–36.
45. Yeh E, Garneau S, Walsh CT. Robust in vitro Activity of RebF and RebH, a Two-Component Reductase/Halogenase, Generating 7-Chlorotryptophan during Rebeccamycin Biosynthesis. *Proc. Natl. Acad. Sci.* 2005; 102(11):3960–65. [PubMed: 15743914]
46. Keller S, Wage T, Hohaas K, Hölzer M, Eichhorn E, van Pée KH. Purification and Partial Characterization of Tryptophan 7-Halogenase (PrnA) from *Pseudomonas fluorescens*. *Angew. Chem. Int. Ed. Engl.* 2000; 39(13):2300–2302. [PubMed: 10941070]
47. Zehner S, Kotsch A, Bister B, Süßmuth RD, Méndez C, et al. A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005. *Chem. Biol.* 2005; 12(4):445–52. [PubMed: 15850981]
48. Chang F-Y, Brady SF. Cloning and Characterization of an Environmental DNA-Derived Gene Cluster That Encodes the Biosynthesis of the Antitumor Substance BE-54017. *J. Am. Chem. Soc.* 2011; 133(26):9996–99. [PubMed: 21542592]
49. Ryan KS. Biosynthetic Gene Cluster for the Cladoniamides, Bis-Indoles with a Rearranged Scaffold. *PLoS ONE.* 2011; 6(8):e23694–12. [PubMed: 21876764]
50. Chang FY, Brady SF. Discovery of indolotryptoline antiproliferative agents by homology-guided metagenomic screening. *Proc. Natl. Acad. Sci.* 2013
51. Seibold C, Schnerr H, Rumpf J, Kunzendorf A, Hatscher C, et al. A flavin-dependent tryptophan 6-halogenase and its use in modification of pyrrolnitrin biosynthesis. *Biocatalysis and Biotransformation.* 2006; 24(6):401–8.
52. Zeng J, Zhan J. Characterization of a tryptophan 6-halogenase from *Streptomyces toxytricini*. *Biotechnol Lett.* 2011; 33(8):1607–13. [PubMed: 21424165]
53. Menon BRK, Latham J, Dunstan MS, Brandenburger E, Klemstein U, et al. Structure and biocatalytic scope of thermophilic flavin-dependent halogenase and flavin reductase enzymes. *Org. Biomol. Chem.* 2016; 14(39):9354–61. [PubMed: 27714222]
54. Milbredt D, Patallo EP, van Pée K-H. A Tryptophan 6-Halogenase and an Amidotransferase Are Involved in Thienodolin Biosynthesis. *ChemBioChem.* 2014; 15(7):1011–20. [PubMed: 24692213]

55. Zhu X, De Laurentis W, Leang K, Herrmann J, Ihlefeld K, et al. Structural Insights into Regioselectivity in the Enzymatic Chlorination of Tryptophan. *J. Mol. Biol.* 2009; 391(1):74–85. [PubMed: 19501593]
56. Shepherd SA, Menon BRK, Fisk H, Struck A-W, Levy C, et al. A Structure-Guided Switch in the Regioselectivity of a Tryptophan Halogenase. *ChemBioChem.* 2016; 17(9):821–24. [PubMed: 26840773]
57. Chankhamjon P, Boettger-Schmidt D, Scherlach K, Urbansky B, Lackner G, et al. Biosynthesis of the Halogenated Mycotoxin Aspirochlorine in Koji Mold Involves a Cryptic Amino Acid Conversion. *Angew. Chem. Int. Ed.* 2014; 53(49):13409–13.
58. Agarwal V, Gamal El AA, Yamanaka K, Poth D, Kersten RD, et al. Biosynthesis of polybrominated aromatic organic compounds by marine bacteria. *Nat. Chem. Biol.* 2014; 10(8): 640–47. [PubMed: 24974229]
59. Zeng J, Zhan J. A Novel Fungal Flavin-Dependent Halogenase for Natural Product Biosynthesis. *ChemBioChem.* 2010; 11(15):2119–23. [PubMed: 20827793]
60. Wang S, Xu Y, Maine EA, Wijeratne EMK, Espinosa-Artiles P, et al. Functional Characterization of the Biosynthesis of Radicol, an Hsp90 Inhibitor Resorecylic Acid Lactone from *Chaetomium chiversii*. *Chem. Biol.* 2008; 15(12):1328–38. [PubMed: 19101477]
61. Sato M, Winter JM, Kishimoto S, Noguchi H, Tang Y, Watanabe K. Combinatorial Generation of Chemical Diversity by Redox Enzymes in Chaetoviridin Biosynthesis. *Org. Lett.* 2016; 18(6): 1446–49. [PubMed: 26959241]
62. Ferrara M, Perrone G, Gambacorta L, Epifani F, Solfrizzo M, Gallo A. Identification of a Halogenase Involved in the Biosynthesis of Ochratoxin A in *Aspergillus carbonarius*. *Appl. Environ. Microbiol.* 2016; 82(18):5631–41. [PubMed: 27422838]
63. Wick J, Heine D, Lackner G, Misiek M, Tauber J, et al. A Fivefold Parallelized Biosynthetic Process Secures Chlorination of *Armillaria mellea* (Honey Mushroom) Toxins. *Appl. Environ. Microbiol.* 2015; 82(4):1196–1204. [PubMed: 26655762]
64. Xiao Y, Li S, Niu S, Ma L, Zhang G, et al. Characterization of Tiacumicin B Biosynthetic Gene Cluster Affording Diversified Tiacumicin Analogues and Revealing a Tailoring Dihalogenase. *J. Am. Chem. Soc.* 2011; 133(4):1092–1105. [PubMed: 21186805]
65. Neumann CS, Walsh CT, Kay RR. A flavin-dependent halogenase catalyzes the chlorination step in the biosynthesis of *Dictyostelium* differentiation-inducing factor 1. *Proc. Natl. Acad. Sci.* 2010; 107(13):5798–5803. [PubMed: 20231486]
66. Dorrestein PC, Yeh E, Garneau-Tsodikova S, Kelleher NL, Walsh CT. Dichlorination of a pyrrolyl-S-carrier protein by FADH(2)-dependent halogenase PltA during pyoluteorin biosynthesis. *Proc. Natl. Acad. Sci.* 2005; 102(39):13843–48. [PubMed: 16162666]
67. Yamanaka K, Ryan KS, Gulder TAM, Hughes CC, Moore BS. Flavoenzyme-Catalyzed Atropo-Selective N,C-Bipyrrole Homocoupling in Marinopyrrole Biosynthesis. *J. Am. Chem. Soc.* 2012; 134(30):12434–37. [PubMed: 22800473]
68. Hohaus K, Altmann A, Burd W, Fischer I, Hammer PE, et al. NADH-Dependent Halogenases Are More Likely To Be Involved in Halometaolite Biosynthesis Than Haloperoxidases. *Angewandte Chemie International Edition in English.* 1997; 36(18):2012–13.
69. Mantovani SM, Moore BS. Flavin-Linked Oxidase Catalyzes Pyrrolizine Formation of Dichloropyrrole-Containing Polyketide Extender Unit in Chlorizidine A. *J. Am. Chem. Soc.* 2013; 135(48):18032–35. [PubMed: 24246014]
70. Walsh CT, Garneau-Tsodikova S, Howard-Jones AR. Biological formation of pyrroles: Nature's logic and enzymatic machinery. *Nat. Prod. Rep.* 2006; 23(4):517–15. [PubMed: 16874387]
71. Podzelinska K, Latimer R, Bhattacharya A, Vining LC, Zechel DL, Jia Z. Chloramphenicol Biosynthesis: The Structure of CmlS, a Flavin-Dependent Halogenase Showing a Covalent Flavin–Aspartate Bond. *J. Mol. Biol.* 2010; 397(1):316–31. [PubMed: 20080101]
72. Chankhamjon P, Tsunematsu Y, Ishida-Ito M, Sasa Y, Meyer F, et al. Regioselective Dichlorination of a Non-Activated Aliphatic Carbon Atom and Phenolic Bismethylation by a Multifunctional Fungal Flavoenzyme. *Angew. Chem.* 2016; 128(39):12134–38.

73. Lin S, Van Lanen SG, Shen B. Regiospecific Chlorination of (S)- β -Tyrosyl- S-Carrier Protein Catalyzed by SgcC3 in the Biosynthesis of the Eneidyne Antitumor Antibiotic C-1027. *J. Am. Chem. Soc.* 2007; 129(41):12432–38. [PubMed: 17887753]
74. Ortega MA, Cogan DP, Mukherjee S, Garg N, Li B, et al. Two Flavoenzymes Catalyze the Post-Translational Generation of 5-Chlorotryptophan and 2-Aminovinyl-Cysteine during NAI-107 Biosynthesis. *ACS Chem. Biol.* 2017; 12(2):548–57. [PubMed: 28032983]
75. Andorfer MC, Belsare KD, Girlich AM, Lewis JC. Aromatic Halogenation Using Bifunctional Flavin Reductase-Halogenase Fusion Enzymes. Submitted.
76. Pacholec M, Sello JK, Walsh CT, Thomas MG. Formation of an aminoacyl-S-enzyme intermediate is a key step in the biosynthesis of chloramphenicol. *Org. Biomol. Chem.* 2007; 5(11):1692–93. [PubMed: 17520135]
77. Buedenbender S, Rachid S, Müller R, Schulz GE. Structure and Action of the Myxobacterial Chondrochloren Halogenase CndH: A New Variant of FAD-dependent Halogenases. *J. Mol. Biol.* 2009; 385(2):520–30. [PubMed: 19000696]
78. Pang AH, Garneau-Tsodikova S, Tsodikov OV. Crystal structure of halogenase PltA from the pyoluteorin biosynthetic pathway. *Journal of Structural Biology.* 2015; 192(3):349–57. [PubMed: 26416533]
79. Jordan PA, Moore BS. Biosynthetic Pathway Connects Cryptic Ribosomally Synthesized Posttranslationally Modified Peptide Genes with Pyrroloquinoline Alkaloids. *Cell Chemical Biology.* 2016; 23(12):1504–14. [PubMed: 27866908]
80. Payne JT, Andorfer MC, Lewis JC. Regioselective Arene Halogenation using the FAD-Dependent Halogenase RebH. *Angew. Chem. Int. Ed.* 2013; 52(20):5271–74.
81. Frese M, Sewald N. Enzymatic halogenation of tryptophan on a gram scale. *Angew. Chem. Int. Ed. Engl.* 2015; 54(1):298–301. [PubMed: 25394328]
82. Shepherd SA, Karthikeyan C, Latham J, Struck A-W, Thompson ML, et al. Extending the biocatalytic scope of regiocomplementary flavin-dependent halogenase enzymes. *Chem. Sci.* 2015; 6:3454–60. [PubMed: 29511510]
83. Smith DRM, Uria ARR, Helfrich EJM, Milbredt D, van Pée K-H, et al. An unusual flavin-dependent halogenase from the metagenome of the marine sponge *Theonella swinhoei* WA. *ACS Chem. Biol.* 2017; 12(5):1281–87. [PubMed: 28198609]
84. Zeng J, Lytle AK, Gage D, Johnson SJ, Zhan J. Specific chlorination of isoquinolines by a fungal flavin-dependent halogenase. *Bioorg. Med. Chem. Lett.* 2013; 23(4):1001–3. [PubMed: 23312946]
85. Roy AD, Grischow S, Cairns N, Goss RJM. Gene Expression Enabling Synthetic Diversification of Natural Products: Chemogenetic Generation of Pacidamycin Analogs. *J. Am. Chem. Soc.* 2010; 132(35):12243–45. [PubMed: 20712319]
86. Durak LJ, Payne JT, Lewis JC. Late-Stage Diversification of Biologically Active Molecules via Chemoenzymatic C–H Functionalization. *ACS Catal.* 2016; 6(3):1451–54. [PubMed: 27274902]
87. Latham J, Henry J-M, Sharif HH, Menon BRK, Shepherd SA, et al. Integrated catalysis opens new arylation pathways via regiodivergent enzymatic C-H activation. *Nat Commun.* 2016; 7:11873. [PubMed: 27283121]
88. Brown S, O'Connor SE. Halogenase Engineering for the Generation of New Natural Product Analogues. *ChemBioChem.* 2015; 16(15):2129–35. [PubMed: 26256103]
89. Sánchez C, Zhu L, Braña AF, Salas AP, Rohr J, et al. Combinatorial biosynthesis of antitumor indolocarbazole compounds. *Proc. Natl. Acad. Sci.* 2005; 102(2):461–66. [PubMed: 15625109]
90. Runguphan W, Qu X, O'Connor SE. Integrating carbon–halogen bond formation into medicinal plant metabolism. *Nature.* 2010; 468(7322):461–64. [PubMed: 21048708]
91. Glenn WS, Nims E, O'Connor SE. Reengineering a Tryptophan Halogenase To Preferentially Chlorinate a Direct Alkaloid Precursor. *J. Am. Chem. Soc.* 2011; 133(48):19346–49. [PubMed: 22050348]
92. Fräbel S, Krischke M, Staniek A, Warzecha H. Recombinant flavin-dependent halogenases are functional in tobacco chloroplasts without co-expression of flavin reductase genes. *Biotechnology Journal.* 2016:1–25.
93. Unpublished results from the Lewis lab

94. Hubig SM, Kochi JK. Structure and Dynamics of Reactive Intermediates in Reaction Mechanisms. σ - and π -Complexes in Electrophilic Aromatic Substitutions. *J. Org. Chem.* 2000; 65(21):6807–18. [PubMed: 11030996]
95. Schnepel C, Minges H, Frese M, Sewald N. A High-Throughput Fluorescence Assay to Determine the Activity of Tryptophan Halogenases. *Angew. Chem. Int. Ed.* 2016; 55(45):14159–63.
96. Lang A, Polnick S, Nicke T, William P, Patallo EP, et al. Changing the Regioselectivity of the Tryptophan 7-Halogenase PrnA by Site-Directed Mutagenesis. *Angew. Chem. Int. Ed.* 2011; 50(13):2951–53.
97. Belsare KD, Andorfer MC, Cardenas FS, Chael JR, Park HJ, Lewis JC. A Simple Combinatorial Codon Mutagenesis Method for Targeted Protein Engineering. *ACS Synth. Biol.* 2017; 6(3):416–20. [PubMed: 28033708]
98. Romero PA, Arnold FH. Exploring protein fitness landscapes by directed evolution. *Nat. Rev. Mol. Cell Biol.* 2009; 10(12):866–76. [PubMed: 19935669]
99. Packer MS, Liu DR. Methods for the directed evolution of proteins. *Nat. Rev. Genet.* 2015; 16(7):379–94. [PubMed: 26055155]
100. Payne JT, Andorfer MC, Lewis JC. Engineering Flavin-Dependent Halogenases. *Meth. Enzymol.* 2016; 575:93–126. [PubMed: 27417926]
101. Poor CB, Andorfer MC, Lewis JC. Improving the Stability and Catalyst Lifetime of the Halogenase RebH By Directed Evolution. *ChemBioChem.* 2014; 15(9):1286–89. [PubMed: 24849696]
102. Zhao H, Arnold FH. Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng. Des. Sel.* 1999; 12(1):47–53.
103. Giver L, Gershenson A, Freskgard PO, Arnold FH. Directed evolution of a thermostable esterase. *Proc. Natl. Acad. Sci.* 1998; 95(22):12809–13. [PubMed: 9788996]
104. Lawrence MS, Phillips KJ, Liu DR. Supercharging Proteins Can Impart Unusual Resilience. *J. Am. Chem. Soc.* 2007; 129(33):10110–12. [PubMed: 17665911]
105. Blake PR, Park JB, Bryant FO, Aono S, Magnuson JK, et al. Determinants of protein hyperthermostability: purification and amino acid sequence of rubredoxin from the hyperthermophilic archaeobacterium *Pyrococcus furiosus* and secondary structure of the zinc adduct by NMR. *Biochemistry.* 1991; 30(45):10885–95. [PubMed: 1932012]
106. Hosford J, Shepherd SA, Micklefield J, Wong LS. A High-Throughput Assay for Arylamine Halogenation Based on a Peroxidase-Mediated Quinone-Amine Coupling with Applications in the Screening of Enzymatic Halogenations. *Chem. Eur. J.* 2014; 20(50):16759–63. [PubMed: 25319801]
107. Bar-Even A, Noor E, Savir Y, Liebermeister W, Davidi D, et al. The Moderately Efficient Enzyme: Evolutionary and Physicochemical Trends Shaping Enzyme Parameters. *Biochemistry.* 2011; 50(21):4402–10. [PubMed: 21506553]
108. Payne JT, Poor CB, Lewis JC. Directed Evolution of RebH for Site-Selective Halogenation of Large Biologically Active Molecules. *Angew. Chem.* 2015; 127(14):4300–4304.
109. Hartwig JF. Catalyst-Controlled Site-Selective Bond Activation. *Acc. Chem. Res.* 2017; 50(3):549–55. [PubMed: 28945414]
110. Andorfer MC, Yang S, He CQ, Garcia-Borras M, Lam Y, Girlich AM, Vergara-Coll J, Sukumar N, Houk KN, Lewis JC. Understanding Flavin Dependent Halogenase Selectivity via X-ray Crystallography and Molecular Dynamics Simulations of Laboratory-Evolved Halogenase Variants.
111. Zhang C, Welborn M, Zhu T, Yang NJ, Santos MS, et al. Π -Clamp-mediated cysteine conjugation. *Nat. Chem.* 2015; 8(2):120–128. [PubMed: 26791894]
112. Erlanson DA, McDowell RS, O'Brien T. Fragment-Based Drug Discovery. *J. Med. Chem.* 2004; 47(14):3463–82. [PubMed: 15214773]
113. Löfdahl CG, Svedmyr N. Formoterol fumarate, a new β_2 -adrenoceptor agonist. *Allergy.* 1989; 44(4):264–71. [PubMed: 2544117]
114. Tishchenko O, Pham-Tran N-N, Kryachko ES, Nguyen MT. Protonation of Gaseous Halogenated Phenols and Anisoles and Its Interpretation Using DFT-Based Local Reactivity Indices. *J. Phys. Chem. A.* 2001; 105(38):8709–17.

115. Schwabe T, Grimme S. Theoretical Description of Substituent Effects in Electrophilic Aromatic Substitution Reactions. *Eur. J. Org. Chem.* 2008; 2008(35):5928–35.
116. Aoki S, Matsuo Y, Ogura S, Ohwada H, Hisamatsu Y, et al. Regioselective Aromatic Substitution Reactions of Cyclometalated Ir(III) Complexes: Synthesis and Photochemical Properties of Substituted Ir(III) Complexes That Exhibit Blue, Green, and Red Color Luminescence Emission. *Inorg. Chem.* 2011; 50(3):806–18. [PubMed: 21214169]
117. Abagyan R, Totrov M, Kuznetsov D. ICM—A new method for protein modeling and design: Applications to docking and structure prediction from the distorted native conformation. *Journal of Computational Chemistry.* 1994; 15(5):488–506.
118. Grant JA, Gallardo MA, Pickup BT. A fast method of molecular shape comparison: A simple application of a Gaussian description of molecular shape. *Journal of Computational Chemistry.* 1996; 17(14):1653–66.
119. Negretti S, Narayan ARH, Chiou KC, Kells PM, Stachowski JL, et al. Directing Group-Controlled Regioselectivity in an Enzymatic C–H Bond Oxygenation. *J. Am. Chem. Soc.* 2014; 136(13):4901–4. [PubMed: 24627965]
120. Narayan ARH, Jiménez-Osés G, Liu P, Negretti S, Zhao W, et al. Enzymatic hydroxylation of an unactivated methylene C–H bond guided by molecular dynamics simulations. *Nat. Chem.* 2015; 7(8):653–60. [PubMed: 26201742]

Figure 1. Structure-activity relationship studies show halogenation directly affects the bioactivity of compounds (A). This is in many cases due to unique halogen-bonding interactions with target molecules (B, PDB ID: 3KRI).

Figure 2.

A. Halonium affinities (kJ/mol) for all sp^2 -hybridized C-H bonds are displayed for tryptamine and 1-(4-hydroxyphenyl)pyrrole. Halogenation site selectivity using NCS or FDH is denoted with an arrow (32, 33). B. Chemical equation from which HalA is derived. C. Maximum HalA values associated with aromatic compounds correlate with compound nucleophilicity (25).

Figure 3.

A. A peroxyflavin species is formed in PHBH (blue, PDB ID: 1PHH) and FDHs (green, RebH, PDB ID: 2OA1). B. Selectivity of Trp-FDHs is controlled by positioning substrate in the active site. 7-Trp-FDH RebH (green, PDB ID: 2OA1) and 5-Trp-FDH PyrH (gray, PDB ID: 2WEU) are aligned to show the difference in L-Trp positioning. C. FDHs that halogenate phenols are proposed to partially or fully deprotonate substrate in the active site (37).

Figure 4.

A. Natural products involving FDH in the biosynthetic halogenation step. B. Examples of non-native substrates found to be halogenated by FDHs. C. The FAD-binding domain of FDHs is well conserved (FAD shown as red spheres). The C-terminal domain (shown in different colors for different FDHs) is thought to impart substrate specificity.

Figure 5.

A. Hydrogen bonding network from surface to active site in CmlS (PDB ID: 3I3L). B. Structures of PrnA, PyrH+FAD, and PyrH-FAD (PDB ID: 2AQJ, 2WET, and 2WEU, respectively) with sites of structure-guided mutagenesis shown as sticks. Sites that significantly affect activity are shown in magenta, those that do not are shown in yellow. Note the conformational change of E46 between PyrH+FAD and PyrH-FAD.

Figure 6.

Altering the site-selectivity of FDHs has been demonstrated on several substrates using structure-guided mutagenesis (A–C) and directed evolution (D).

Figure 7.

A. Substrates halogenated by engineered RebH variants that are not halogenated by wtRebH (33, 108). If halogenation site is known, it is denoted with an arrow. B. Examples of substrate that RebH halogenates at a single position when several isomers are observed with N-chlorosuccinimide. C. Orthogonal selectivity is observed for some substrates using different FDHs, such as 40 and 41. D. Docking of non-native substrates within RebH reveals an anchoring effect that is thought to account for the site selectivity observed for substrates such as 38.