CHAPTER FIVE

Engineering Flavin-Dependent Halogenases

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Abstract

In the two decades since the discovery of the first flavin-dependent halogenase (FDH), great strides have been made in elucidating the diversity of enzymes in this class as well as their structures and functions. More recently, efforts to engineer these enzymes for synthetic applications have yielded their first successes. FDH variants with improved stability, expanded substrate scope, and altered regioselectivity have been developed. Here, we review these efforts and provide representative protocols that have been employed for FDH engineering. Random and structure-guided mutagenesis approaches and screening methods, including HPLC, mass spectrometry, and spectrophotometric methods, are discussed. The protocols described herein should be generalizable to many FDHs and a wide variety of engineering goals.

1. INTRODUCTION

Halogenation is an important process in the production of pharmaceuticals and agrochemicals; an estimated one-quarter of all such

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compounds possess a halogen substituent (Herrera-Rodriguez, Khan, Robins, & Meyer, 2011). The importance of halogenation is in part due to the profound effect that halogen substituents can have on the biological activity of a compound (Hernandes, Cavalcanti, Moreira, de Azevedo Junior, & Leite, 2010; Sun, Keefer, & Scott, 2011), and examples of these effects can be seen in antibiotics (Bunders et al., 2011; Harris, Kannan, Kopecka, & Harris, 1985), anticancer compounds (Williams et al., 2005), and psychoactive compounds (Smith et al., 2008). Despite the importance of halogenation, current halogenation methods often rely on harsh reaction conditions, produce mixtures of products, and are unable to selectively halogenate compounds at electronically disfavored positions (Smith & El-Hiti, 2004).

In contrast to halogenation methods employing chemical reagents, which have been used for over a century, the importance and power of biological halogenation has only more recently come to light. Through the 1960s, halogenated natural products were thought to be infrequently occurring "chance products of nature" (Petty, 1961), but the number of known halogenated natural products has since increased dramatically to nearly 5000 (Gribble, 2010). Along with this increased inventory of structures has come an increased understanding of the enzymes responsible for their biosynthesis.

The first characterized halogenases were haloperoxidases (van Pée, 2013), and the first of these came from investigations of the *Caldariomyces fumago* caldariomycin biosynthetic pathway (Fig. 1A) (Morris & Hager, 1966). This enzyme was suspected to halogenate an electron-rich enol substrate to afford the product caldariomycin, although its role in caldariomycin biosynthesis was never definitively shown (van Pée & Patallo, 2006). Despite this, monochlorodimedone, an analog of the precursor to caldariomycin, has been used as a reliable spectrophotometric assay for haloperoxidase activity (Fig. 1A) (van Pée, 1990). This activity is believed to involve oxidation of halide anion (X^-, X = I, Br, Cl) to the corresponding hypohalous acid (HOX), the active halogenating species, which is released from the enzyme active site such that halogenation occurs freely in solution (Manoj, 2006). As such, haloperoxidases tend to halogenate electron-rich substrates with the same regioselectivity as conventional halogenation reagents (ie, that resulting from the chemoselectivity of HOX) (Wagner & König, 2012). Only relatively recently have haloperoxidases capable of enantioselective halogenation been discovered, indicating that in at least some cases halogenation occurs within the enzyme (Bernhardt, Okino, Winter, Miyanaga, & Moore, 2011; Carter-Franklin & Butler, 2004; Diethelm, Teufel, Kaysser, & Moore, 2014).

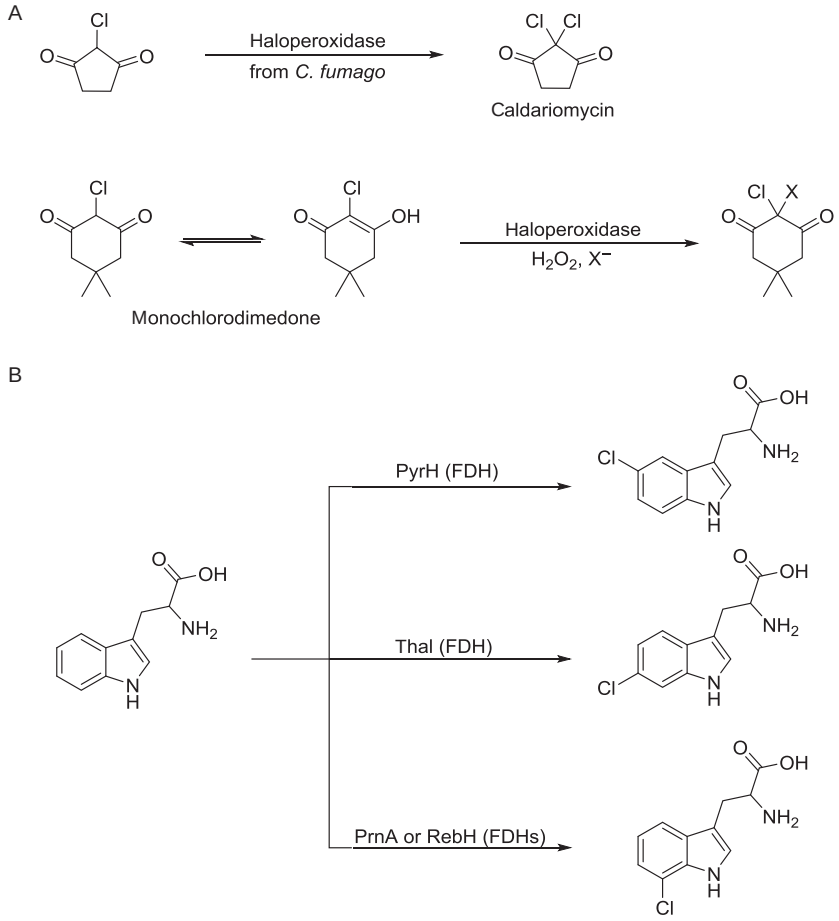


Fig. 1 (A) Reaction catalyzed by the first discovered haloperoxidase and the spectrophotometric assay using monochlorodimedone that was subsequently developed and (B) regioselective halogenations catalyzed by different FDHs on the same substrate, L-tryptophan, affording three unique products (Keller et al., 2000; Seibold et al., 2006; Yeh, Garneau, & Walsh, 2005; Zehner et al., 2005). *Panel (A): Adapted from van Pée, K. -H. (1990). Bacterial haloperoxidases and their role in secondary metabolism.* Biotechnology Advances, 8, *185–205.*

Prior to the discovery of these enantioselective haloperoxidases, characterization of natural products with halogen substitution at electronically disfavored positions led to speculation that other classes of halogenases must be responsible for their biosynthesis since free HOX would not lead to such selectivity (van Pée, 2013). Indeed, flavin-dependent halogenases (FDHs) were discovered in the late 1990s (Dairi, Nakano, Aisaka,

Katsumata, & Hasegawa, 1995), and one of these, PrnA, was found to be responsible for the selective indole 7-chlorination of L-tryptophan in the course of pyrrolnitrin biosynthesis (Fig. 1B). This finding clearly showed how an FDH could catalyze halogenation at an electronically disfavored site on a substrate (Kirner et al., 1998). Later structural analysis of PrnA revealed that reduced FAD is bound in one area of the enzyme, which then reacts with O_2 to generate a flavin peroxide intermediate (Vaillancourt, Yeh, Vosburg, Garneau-Tsodikova, & Walsh, 2006). This flavin peroxide then reacts with halide to generate a HOX, which is believed to travel to a second enzyme active site in which the L-tryptophan substrate is bound. The HOX is then either hydrogen bonded to (Flecks et al., 2008) or reacts with (Yeh, Blasiak, Koglin, Drennan, & Walsh, 2007) a specific lysine residue, such that it is proximal to only a single C–H bond of the bound substrate. The FDH is thus able to accomplish C–H halogenation selectively at a single position of the substrate, even if that position is significantly disfavored electronically relative to other positions on the substrate (Lewis, Coelho, & Arnold, 2011).

In subsequent years, numerous other FDHs have been discovered, some of which have been found to selectively halogenate L-tryptophan at either the 5-, 6-, or 7-position, demonstrating the significant power of these enzymes for regioselective halogenation (Fig. 1B) (Keller et al., 2000; Seibold et al., 2006; Yeh et al., 2005; Zehner et al., 2005). Other FDHs have been found to halogenate additional substrate classes, including phenols, pyrroles, and even unactivated hydrocarbons (Wagner & König, 2012), but in many of these cases the substrate is bound to a carrier protein (van Pée, 2013), thus complicating synthetic applications. As of Dec. 2015, BLAST searches of these characterized FDHs return hundreds of additional putative halogenases.

To date, tryptophan halogenases are the best-characterized FDHs and have thus been most extensively explored for biotechnological applications. For example, three L-tryptophan FDHs were integrated into heterologously expressed biosynthetic pathways for indolocarbazole compounds to generate numerous chlorinated and brominated natural product analogs (Sánchez et al., 2005). Two of these FDHs were incorporated into the medicinal plant *Catharanthus roseus* to produce novel chlorinated alkaloids *in planta* (Runguphan, Qu, & O'Connor, 2010). Similarly, L-tryptophan FDHs were integrated into the pacidamycin biosynthetic pathway in order to produce chlorinated pacidamycin derivatives (Roy, Grüschow, Cairns, & Goss, 2010). All of these examples relied on wild-type FDHs halogenating their

native substrate, L-tryptophan. Despite this limitation, the novel chlorinated products from two of the aforementioned metabolic engineering efforts were also employed for subsequent transition metal catalyzed cross-coupling reactions to create further novel products (Roy et al., 2010; Runguphan & O'Connor, 2013). This chemoenzymatic methodology has been further expanded upon to allow access to an even greater diversity of products (Durak, Payne, & Lewis, 2016).

A number of studies have also focused on exploring the activity of wild-type FDHs on nonnative substrates. One of the earliest such explorations was performed using the L-tryptophan 7-halogenase PrnA (Hölzer, Burd, Reißig, & van Pée, 2001). The authors reported that numerous nonnative indoles, including 5- and *N*-methyltryptamine, as well as 3- and 5-methylindole, were halogenated by PrnA, while others, including indole itself, gramine, and indole-3-acetic acid were not. More significantly, the authors reported that the nonnative substrates that were accepted were invariably halogenated at the electronically favored 2-position, rather than at the relatively electronically disfavored 7-position as seen with the native substrate. This was nonetheless an encouraging first demonstration that nonnative substrates could be halogenated by an FDH and led to later work showing that PrnA can halogenate kynurenine and a range of nonnative anthranilic acids and anthranilamides (Shepherd et al., 2015).

In more recent years, several reports have focused on the substrate scope of another L-tryptophan 7-halogenase, RebH (Yeh et al., 2005). In 2011, Glenn et al. reported that RebH could functionalize the nonnative substrate tryptamine selectively at the indole 7-position (Glenn, Nims, & O'Connor, 2011). This work was a follow-up to the authors' earlier work in which wild-type RebH and an L-tryptophan 5-halogenase, PyrH, were integrated into the biosynthetic pathway of several catharanthine alkaloids to afford halogenated derivatives of these alkaloids (Runguphan et al., 2010). Selective tryptamine, rather than L-tryptophan, halogenation helped to overcome a bottleneck in these biosynthetic pathways. In 2013, Payne et al. reported that wild-type RebH halogenated a significantly wider range of nonnative substrates, including tryptamine derivatives, the tricyclic tryptoline, indole, and substituted naphthalenes (Payne, Andorfer, & Lewis, 2013). Notably, many of these substrates were halogenated selectively at the indole 6- and/or 7-positions, although these substrates were still all comparable to or smaller than the native substrate, L-tryptophan. Frese et al. later reported that a range of L-tryptophan derivatives with methyl, amino, fluoro, or hydroxyl substituents at the indole 5- or 6-positions could also

be selectively halogenated away from the preferred indole 2-position (Frese, Guzowska, Voß, & Sewald, 2014). One additional report showed that a fungal FDH, Rdc2, which natively halogenates monocillin II, also accepted two hydroxyisoquinoline substrates (Zeng, Lutle, Gage, Johnson, & Zhan, 2013). As was seen with the L-tryptophan FDHs, only substrates comparable in size or smaller than the native substrate are accepted.

The activities and/or conversions reported for PrnA, RebH, and Rdc2 on nonnative substrates tend to be significantly lower than those observed on the native substrate. With RebH, for example, the total turnover number observed with tryptoline was nearly 50-fold lower than that observed with L-tryptophan (Payne et al., 2013). In order to obtain FDHs useful for biotechnological applications, including metabolic engineering and in vitro biocatalysis, engineering efforts have thus been performed to increase the activities observed on nonnative substrates and to further expand FDH substrate scope. While most efforts at FDH protein engineering are relatively recent, significant progress has been made in improving the stability, expanding the substrate scope, and altering the regioselectivity of these enzymes (vide infra). These efforts have furnished protocols that, while initially applied to an individual FDH to accomplish a specific task, can be generalized to a wide range of FDHs and potential engineering efforts. The recent work that has been done on engineering FDHs and the protocols employed are thus discussed in this work, with care taken to describe how the protocols may be tailored toward novel efforts. Protocols for activity assays and purification of FDHs were described in a recent work (van Pée, 2012) and are thus not described herein.

2. IMPROVING THE STABILITY OF FDHs

2.1 Improving FDH Stability via Directed Evolution

The stability of an enzyme is an extremely important factor in determining its synthetic utility. More stable enzymes tend to have longer lifetimes and are easier to store and handle (Liao, 1993; Polizzi, Bommarius, Broering, & Chaparro-Riggers, 2007; Wu & Arnold, 2013), and it has been demonstrated that the thermostability of an enzyme can improve its evolvability (Bloom, Labthavikul, Otey, & Arnold, 2006). As mutations are introduced to the parent enzyme to expand its substrate scope or increase its activity, these mutations will tend to be destabilizing. The more thermostable the parent enzyme is, the greater the mutational load it will likely tolerate over

the course of subsequent evolution. The use of directed evolution to optimize enzymes for use in industrial processes is increasingly common, and more thermostable enzymes will thus make better candidates for subsequent tailoring to industrial applications (as well as for their ability to tolerate higher reaction temperatures) (Liao, 1993; Wu & Arnold, 2013; Zhao & Arnold, 1999). Furthermore, it has been suggested that higher thermostability of an enzyme facilitates protein crystallography efforts (Derewenda, 2010), which are an invaluable resource for structural details that can guide targeted mutagenesis efforts. The stability of enzymes toward organic solvents is also important, as organic solvents are frequently employed when reaction components are not fully soluble in aqueous media (Liao, 1993).

Some of the earliest examples of directed evolution were focused on improving the thermostability (Liao, 1993) and organic solvent tolerance (You & Arnold, 1994) of enzymes. In the case of halogenases, both the organic solvent and pH tolerance of haloperoxidases have been improved via directed evolution (Hasan et al., 2006; Yamada, Higo, Yoshikawa, China, & Ogino, 2014). In both of these examples of haloperoxidase engineering, colorimetric assays were employed for high-throughput screening; a similar screen for FDHs is discussed in Section 2.3. The first example of FDH directed evolution was focused on improving the thermostability of RebH (Poor, Andorfer, & Lewis, 2014). In three rounds of mutagenesis and UPLC screening, eight nonsynonymous mutations were introduced (Scheme 1) that resulted in a nearly 20°C increase in the T_m of the protein relative to wild-type RebH. It was also demonstrated that the resultant RebH variants demonstrated increased activity at higher reaction temperatures and longer enzyme lifetime in reactions with several nonnative substrates. One of these variants served as the starting point for a future directed evolution effort to expand the substrate scope of RebH, and a crystal structure was obtained of another. This work demonstrated the application of directed evolution to improve the thermostability of an FDH to facilitate biocatalysis, crystallization, and further engineering efforts, and the procedure employed should be generalizable to improve the thermostability of any other FDH.

Scheme 1 Mutations incorporated via error-prone PCR into RebH to improve its thermostability.

2.1.1 Procedure for Evolving FDH Thermostability

While the procedure described here should be generalizable to any FDH, the specific examples given in parentheses for each step indicate the specific conditions used by Poor et al. (2014).

1. Select a parent FDH (eg, RebH) and obtain either plasmid or genomic DNA.
2. Generate a mutant library by error-prone PCR: with RebH, 1-2 non-synonymous mutations were obtained on an average using *Taq* polymerase from New England Biolabs with the manufacturer's recommended conditions and 100–150 μ*M* $MnCl_2$. A 50 μL reaction volume was used for PCR with the following procedure: 95°C 30 s (95°C 30 s, 55°C 30 s, 72°C 90 s) for 20 cycles, 72°C 10 min. Primers should be constructed to generate restriction digest sites (eg, *Nde*I and *Hind*III sites) to allow for integration into the expression plasmid in step 7.
   a. *Note*: a range of $MnCl_2$ concentrations should be tested prior to library screening in order to determine the proper concentration to elicit the desired mutation rate. Sequencing 24 colonies from step 10 was used to determine the average mutation rate.
   b. *Note*: $MnCl_2$ supplementation need not be used to generate the mutant library. For example, random libraries may be generated through the use of the Agilent GeneMorph random mutagenesis kit following the manufacturer's protocol. Site-directed libraries may be generated using sequence-overlap extension (SOE) to integrate point mutations, NNK codons, NDT codons, etc., or via codon mutagenesis methods. Employing one of these alternative methods may alter steps 2 through 8.
3. Using a QIAgen Gel Extraction kit, isolate the mutant library DNA by agarose gel purification and extraction.
4. Digest the mutant library DNA using the restriction digests appropriate for the sites generated in step 2. Also digest the desired expression plasmid (eg, pET28) using the same restriction digests.
5. Isolate the digested mutant library DNA and digested expression plasmid DNA by agarose gel purification and extraction.
6. Treat the digested expression plasmid DNA with Antarctic Phosphatase to minimize self-ligation in the following step. Heat inactivation of the Antarctic Phosphatase without subsequent purification provides sufficient ligation efficiency.
7. Ligate the digested mutant library DNA with the digested expression plasmid DNA using T4 DNA Ligase. For RebH, 10 μL ligation

reaction volumes, with an insert:vector molar ratio of 6–10:1, and 10–100 ng/μL total DNA concentration have been found to give the most reliable results.

8. Using a Zymo Clean & Concentrate kit, desalt the ligation reaction prior to transformation, eluting with 8 μL of molecular biology grade water.
9. Transform 4 μL of the desalted ligation reaction into BL21 (DE3) *Escherichia coli* containing the pGro7 chaperone plasmid via electroporation.
   a. *Note*: coexpression with the pGro7 chaperone plasmid gives an approximate sevenfold increase in the titer of soluble RebH obtained in crude lysate (Payne et al., 2013), and similar or even greater increases have been seen with other FDHs (data unpublished). In order to obtain electrocompetent cells containing pGro7, the commercially obtained pGro7 plasmid was itself transformed into BL21 (DE3) *E. coli*, selected for on agar plates containing chloramphenicol, and then used to prepare electrocompetent cells following the procedure described in Sambrook and Russell.
10. After allowing the electroporated cells to recover for 45–60 min in SOC media, plate the cells onto LB/agar plates containing chloramphenicol (for the pGro7 chaperone plasmid) and the appropriate antibiotic for the FDH expression plasmid (eg, kanamycin for pET28). Plating 100–200 μL of the transformation mixture has consistently provided 100–300 colonies per plate. Allow the colonies to grow for 16 h until they are large enough to be easily picked by hand or using a colony picker.
11. Pick (either using a colony picker or by hand) the desired number of colonies (~1000 were screened per round of evolution by Poor et al. (2014)) and array them into 1 mL 96-well plates containing 300 μL LB with the appropriate antibiotics. Leave 2–4 wells in each plate uninoculated to monitor for potential contamination and an additional 2–8 wells in each plate should be inoculated solely with cells containing the parent plasmid (and pGro7 if employed) to determine the parent activity. Significant plate-to-plate variation has been observed, so it is essential that wells containing only parent are used in *each* plate.
12. Incubate the 1-mL plates overnight at 37°C and 250 rpm.
13. Using 50–100 μL of the culture from the 1-mL plates (noting that the wells left uninoculated have not grown, indicating no contamination), inoculate 2 mL 96-well plates containing 1 mL TB with the appropriate antibiotics. Save the remaining culture in the 1-mL plates at 4°C.

14. Incubate the 2-mL plates at 37°C and 250 rpm until they reach an OD_{600} of 0.9–1.0, then induce enzyme expression. For pET28/RebH coexpressed with pGro7, final concentrations of 10 μ*M* IPTG and 0.2 mg/mL L-arabinose were employed.
15. Incubate the induced 2 mL plates at 30°C and 250 rpm for 20 h, then spin the plates down at 3600 rpm for 15 min at 4°C and decant the supernatant.
16. Resuspend the cell pellets by gently vortexing in 200 μL HEPES buffer (25 m*M*, pH 7.4), then spin the plates down again 3600 rpm for 15 min at 4°C and decant the supernatant. This wash step removes residual L-tryptophan and chloride from the growth media that could otherwise interfere with library screening.
17. Resuspend the cell pellets by gently vortexing in 100 μL HEPES buffer (25 m*M*, pH 7.4) containing 0.75 mg/mL lysozyme. Incubate at 37°C and 250 rpm for 30–45 min, then flash freeze in liquid nitrogen and thaw in a 37°C water bath. Add 10 μL of HEPES buffer (25 m*M*, pH 7.4) containing 1 mg/mL DNase I and then incubate at 37°C and 250 rpm for 15 min. Spin down the plates at 3600 rpm for 15 min at 4°C and transfer 50 μL of the supernatant to a microtiter plate.
18. In order to screen for increasing thermostability, the crude lysate is at this point subjected to a heat pretreatment step prior to subsequent reaction screening. The exact temperature and duration of the heat pretreatment can be determined empirically by taking samples of crude lysate and pretreating at different temperatures until only ~20% activity of the parent is observed. For the evolution of thermostability of RebH, this resulted in a 2 h, 42°C pretreatment for the first round, a 2 h, 51°C pretreatment for the second round, and a 3 h, 54°C pretreatment for the third round. The microtiter plates containing crude lysate were sealed using AeraSeals from Research Products International, incubated in a water bath at the temperature and for the duration described, and then immediately cooled in an ice-water bath.
19. Add solutions of substrate (eg, L-tryptophan for RebH, 10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 0.5 m*M* final concentration), halide (eg, NaCl, 1.5 *M* in HEPES buffer (25 m*M*, pH 7.4), 10 m*M* final concentration), FAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), NAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), FAD reductase (eg, MBP-RebF, 50–100 μ*M* in HEPES storage buffer (25 m*M*, pH 7.4, 10% glycerol), 2.5 μ*M* final concentration—other reductases have been employed for

other FDHs), and glucose dehydrogenase (eg, GDH-105 from Codexis, 1800 U/mL in HEPES buffer (25 m*M*, pH 7.4), 9 U/mL final concentration) to the pretreated crude lysate. Add additional HEPES buffer (25 m*M*, pH 7.4) such that the final reaction volume is 75 μL and then add a solution of glucose (1 *M* in HEPES buffer (25 m*M*, pH 7.4), 20 m*M* final concentration) to initiate the reaction. Reseal the microtiter plates with AeraSeals and then shake overnight at 600 rpm at room temperature.

20. Quench the reactions with 75 μL of methanol to precipitate out the proteins, add an internal standard to control for differences in handling (phenol at a final concentration of 0.5 m*M* is commonly employed, but a different standard may be required depending on the substrate and analysis method used), and spin the microtiter plates down at 3600 rpm for 15 min at 4°C.

21. Filter the supernatant using a 0.45-μm filter plate placed directly on a 96-well microtiter plate prior to UPLC analysis. 96-well microtiter plates can then be rearrayed into 384-well plates to increase analysis throughput.

22. Seal the plates with aluminum foil using a plate sealer to minimize evaporation and prevent particulate contamination, then analyze the reactions (eg, by UPLC) for halogenated product production. For RebH, reactions were analyzed for 7-chlorotryptophan production using an Agilent 1200 UPLC with an Agilent Eclipse Plus C18 2.1×50 mm column, 1.8 μ*M* particle size; solvent A = H_2O/0.1% TFA, solvent B = CH_3CN; 0–0.5 min, B = 16%; 0.5–1.5 min, B = 16–80%.

23. Analyze the reaction data to determine the relative improvement in halogenated product production relative to the parent wells added to each plate in step 11. The wells that show the greatest degree of improvement should be grown on a larger scale and purified as described in steps 25–34 (using the saved primary culture in the 1-mL plates in step 13), plasmid isolated and sequenced to confirm the presence of nonsynonymous mutations, and halogenase variants analyzed in vitro as described in step 35 (for FPLC purification of either non-His-tagged or His-tagged halogenases, see van Pée (2012)).

24. If multiple improved variants are found displaying different nonsynonymous mutations, it is recommended that the beneficial mutations be analyzed both individually and in combination. Mutations can be recombined via SOE PCR; for RebH, PCR conditions were as follows: 98°C 30 s (98°C 10 s, 72°C 50 s) for 35 cycles, 72°C 10 min. Once

plasmids are obtained containing the combined mutations, the halogenase variants should be immediately assayed in vitro as described in steps 25–36. DNA shuffling can also be used to combine mutations; various methods exist with individual advantages and disadvantages, which have been reviewed elsewhere (Fox & Huisman, 2008).

25. Use the saved primary culture in 1 mL plates from step 13 to generate fresh primary cultures by inoculating LB with the appropriate antibiotics and growing overnight at 37°C and 250 rpm.
26. Add 500 μL of the fresh primary culture to 50 mL TB with the appropriate antibiotics in a 250-mL Erlenmeyer flask covered with aluminum foil then incubate at 37°C and 250 rpm.
27. Once the cultures reach an OD_{600} of 0.6–0.8, induce enzyme expression. For pET28/RebH coexpressed with pGro7, final concentrations of 100 μ*M* IPTG and 2.0 mg/mL L-arabinose were employed (both are 10-fold higher than the concentrations used for 96-well plates).
28. Incubate the induced 50 mL cultures at 30°C and 250 rpm for 20 h, then spin the cultures down at 3600 rpm for 15 min at 4°C and decant the supernatant.
29. Resuspend the cell pellets in 10 mL cold HEPES buffer (20 m*M*, pH 7.4, 150 m*M* NaCl).
30. Lyse the resuspended pellets by sonication (Qsonica S-4000 with a 0.5″ horn; 8 × 30 s with 45 s rests, 20% duty cycle delivering 40–50 W), keeping the lysing cultures cold with a circulating ice-water bath.
31. Clarify the lysed cultures by centrifugation at 15,000 rpm for 45 min at 4°C.
32. Purify the clarified lysate. For FPLC purification of either non-His-tagged or His-tagged halogenases, see van Pée (2012). His-tagged halogenases can also be purified by Ni-NTA affinity chromatography using a peristaltic pump or centrifugation following the resin manufacturer's recommended protocol. Verify the presence of purified halogenase by SDS-PAGE.
33. Combine the pure protein fractions and exchange into HEPES storage buffer (20 m*M*, pH 7.4, 150 m*M* NaCl, 10% glycerol) using dialysis or a spin filter (eg, Amicon 30 kDa 15 mL filters, spun at 4000 × *g* for 15 min at 4°C).
34. Determine the protein concentration using a BCA assay or using A_{280} and extinction coefficients calculated based on amino acid composition (Protein Calculator v3.3, http://www.scripps.edu/~cdputnam/protcalc.html).

35. Determine the residual activity of each halogenase variant using the reaction conditions described in step 19, substituting purified halogenase from step 33 (25 μ*M* final concentration) for the crude lysate and performing the reactions in 1.5-mL microcentrifuge tubes. Quenching and analysis steps are the same as steps 20–23 described earlier for crude lysate reactions.
36. To determine the T_m of each halogenase variant, monitor the thermal denaturation at 222 nm by circular dichroism spectroscopy in 2°C increments from 20°C to 90°C with 2 min equilibration at each temperature. The halogenase variant should be at a concentration of 20 μ*M* in HEPES storage buffer (20 m*M*, pH 7.4, 150 m*M* NaCl, 10% glycerol). For RebH, an AVIV 202 CD Spectrometer with Peltier temperature controller was used. The midpoint of the denaturation curve was determined with SigmaPlot after fitting to a four-parameter sigmoid.

The steps described earlier may be repeated as many times as necessary until a sufficiently thermostabilized FDH is obtained.

2.1.2 Procedure for Evolving FDH Organic Solvent Tolerance

Poor et al. also observed that several of the thermostabilized variants of RebH displayed significantly increased tolerance to organic solvents (Lewis, Poor, & Payne, 2014). In particular, one of the intermediate variants generated containing two nonsynonymous mutations displayed 2.5-fold higher activity in the presence of 30% DMSO than wild-type RebH did. While RebH was not explicitly evolved for increased organic solvent tolerance, the procedure described in Section 2.1.1 can be easily modified to do so. Step 18 is simply modified as follows:

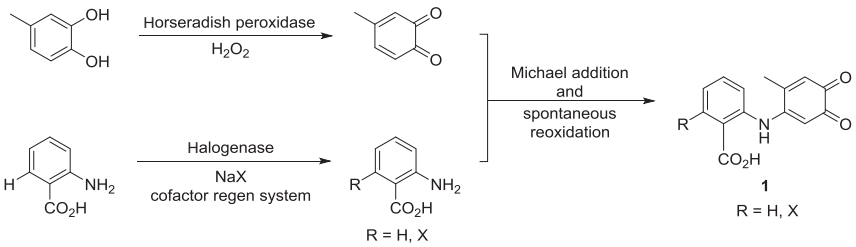
18. In order to screen for increasing organic solvent tolerance, a quantity of the desired organic solvent is added to the crude lysate prior to subsequent reaction screening. The exact quantity of organic solvent to add can be determined empirically by taking samples of crude lysate and adding different volumes until only ~20% activity of the parent is observed. Adjust the amount of HEPES buffer added in step 19 to accommodate the organic solvent added in this step while still maintaining a 75 μL final reaction volume.

2.2 Alternative Screening Methods for FDH Evolution

The procedure described in Section 2.1.1 to evolve RebH for enhanced thermostability involved UPLC analysis to analyze the halogenation

reactions (step 22) (Poor et al., 2014). Chromatographic separations can be used to screen enzymes for essentially any reaction even (or especially) when multiple products are formed, the products have only minor structural differences (eg, regioisomers), or impurities are present. It is important to note that enzymes are increasingly used to address reactions that suffer from precisely these complications, making chromatographic separation an important tool for directed evolution. The cost of this generality, however, is screening time. The UPLC method developed to monitor chlorination of L-tryptophan, for example, was optimized to 90 s per sample, with an additional ~50 s per sample of pre- and post-run time required. This results in approximately 2 days of UPLC analysis time for a ~1000 member mutant library. While this is a manageable amount of time if a dedicated UPLC is available and can be reduced further using SFC, it does place a limit on the number of enzyme variants that can be examined.

In some cases, colorimetric or fluorimetric assays could potentially be used to increase the size of the mutant libraries that can be evaluated and to decrease screening times. For example, haloperoxidase engineering efforts have utilized the monochlorodimedone assay described in Fig. 1A (Yamada et al., 2014). Hosford et al. recently reported the development of a colorimetric method for the analysis of arylamine halogenation (Scheme 2) (Hosford, Shepherd, Micklefield, & Wong, 2014). This method requires only UV/vis analysis to provide a quantitative measure of arylamine halogenation, and the authors demonstrated its consistency with the results obtained from HPLC analysis of the RebH catalyzed chlorination of



Scheme 2 Method for peroxidase-mediated quinone–amine coupling for colorimetric scheme for arylamine halogenation. *Adapted from Hosford, J., Shepherd, S. A., Micklefield, J., & Wong, L. S. (2014). A high-throughput assay for arylamine halogenation based on a peroxidase-mediated quinone-amine coupling with applications in the screening of enzymatic halogenations.* Chemistry A European Journal, 20, *16759–16763.*

2-aminonaphthalene. This substrate undergoes a marked decrease in the absorbance at 516 nm upon chlorination. Thus, the response at a given wavelength can be inversely correlated to the percent conversion of the halogenation reaction, and if a response factor is calculated, this correlation can be quantified.

Such assays offer several distinct advantages when applied to enzyme engineering. Each measurement requires only seconds, allowing a 96-well plate of halogenase variants to be analyzed in a matter of minutes. This should allow access to much larger library sizes, thus allowing for the discovery of more beneficial mutations per round of evolution. Furthermore, it only requires a UV/vis plate reader for analysis, which is more frequently available than UPLC or SFC instruments. The key limitation of such methods, however, is the requirement that the target reaction result in a unique spectroscopic observable, which is not typical for most substrates or for instances in which isomeric products are formed.

Researchers have also explored different methods to replace enzyme screening altogether with selection processes in which higher performing variants have an increased rate of survival and are thus enriched (Lin & Cornish, 2002; Taylor, Kast, & Hilvert, 2001). Selections have been widely used to engineer enzyme properties that can be readily linked to the genetic circuitry of the host organism (Collins, Leadbetter, & Arnold, 2006). For example, Esvelt et al. reported the development of a phage-assisted continuous evolution system that could enable the continuous directed evolution of any enzyme that can be directly linked to the host genetic circuitry and demonstrated its use by rapidly evolving RNA polymerase (Esvelt, Carlson, & Liu, 2011). Unfortunately, the requirement to link a trait of interest to cell survival can be difficult to satisfy when the trait of interest is selective functionalization of a small molecule. Three-hybrid systems have been developed to enable selections for chemical reactions, but these systems also impose limitations on the types of reactions that can be examined (Lin, Tao, & Cornish, 2004).

Selections can also be enabled by differential biological activities of potential reaction products. For example, a selection was used to improve the enantioselectivity of a lipase for hydrolysis of a mixture of the acetate ester of (*S*)-isopropylidene glycerol and the fluoroacetate ester of (*R*)-isopropylidene glycerol, since the product of the hydrolysis of the acetate is an energy source while that of the fluoroacetate is toxic (Reetz, Höbenreich, Soni, & Fernández, 2008). While fortuitous, this activity, like the cases noted earlier, is not readily generalizable to the vast majority of

substrates, enzymes, or reactions. Neither the evolution of RebH substrate scope (Section 4.2) nor the evolution of RebH regioselectivity (Section 3.1) involves products that can be readily linked to *E. coli* survival. No selection has, as of yet, been reported to have been successfully employed for the evolution of FDHs.

Despite the limitations of high-throughput screens and selections, the high-throughput nature of these processes makes them attractive options for halogenase engineering, particularly for applications involving a single substrate that does not change over the course of the engineering project. Efforts to improve the thermostability, organic solvent tolerance, activity at a particular pH, or to optimize turnover frequency/number on a particular substrate may potentially be compatible with these screens. The full procedure for the colorimetric assay described by Hosford et al. (2014) or any other high-throughput assay would be substituted for steps 20–22 of the procedure described in Section 2.1.1.

2.3 Improving Enzyme Stability Through Immobilization

Enzyme immobilization has been reported to increase enzyme stability without significantly compromising activity, for example, in improving the organic solvent tolerance (Truppo, Strotman, & Hughes, 2012) and pH range (Koszelewski, Müller, Schrittwieser, Faber, & Kroutil, 2010) of transaminases. Frese and Sewald recently reported on the enzymatic halogenation of L-tryptophan on a gram scale by forming cross-linked enzyme aggregates (CLEAs) from crude RebH lysate (Frese & Sewald, 2015). The authors reported that RebH combiCLEAs had significantly increased retention of activity relative to free, purified RebH after extended storage at 4°C, and that RebH combiCLEAs also displayed significantly increased catalyst lifetime in an active biohalogenation reaction (although relative total turnover numbers were not reported). Furthermore, RebH combiCLEAs could be reused up to 10 times while still displaying significant halogenation activity. CombiCLEAs, which have been demonstrated to be used successfully with RebH, and other enzyme immobilization methods, which as of yet lack specific demonstration with FDHs, are most likely to be useful in preparative-scale halogenation reactions, rather than high-throughput screening applications. The reported procedure for the particular immobilization strategy employed would thus be substituted for steps 31–34 of the procedure described in Section 2.1.1.

3. ALTERING THE REGIOSELECTIVITY OF FDHs

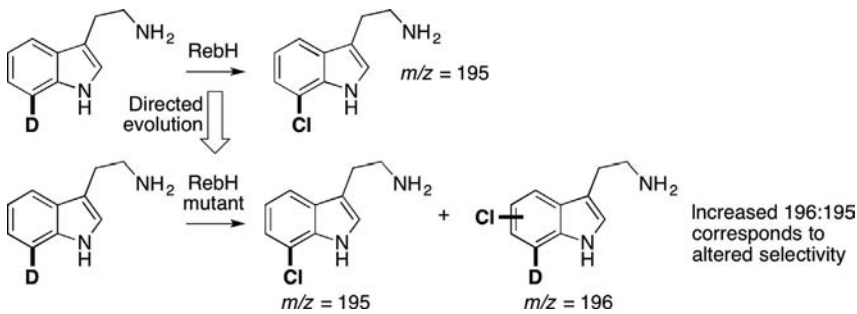
The ability to change the site on a substrate that an enzyme functionalizes is a powerful tool in protein engineering. Functionalizing specific C–H bonds is a particularly challenging problem (Lewis et al., 2011). Fishman et al. demonstrated that structure-guided mutagenesis of toluene *para*-monooxygenase TpMO could be used to generate variants for *ortho*-, *meta*-, and (improved) *para*-hydroxylation of toluene (Fishman, Tao, Rui, & Wood, 2005). Cytochromes P450 have also been engineered to catalyze hydroxylation of different C–H bonds on a given substrate (Fasan, 2012; Lewis et al., 2009). The unique selectivities of tryptophan FDHs have led researchers to investigate the possibility of altering the functionalization site of these halogenation catalysts as well. For example, the crystal structure of a tryptophan 5-halogenase, PyrH (Zehner et al., 2005) was used to identify a single residue that, when introduced into the tryptophan 7-halogenase PrnA (Keller et al., 2000), imparted some tryptophan 5-halogenation ability to PrnA (Lang et al., 2011). However, this variant still produced a 2:1 ratio of regioisomers with mostly 7-halotryptophan produced and displayed severely decreased activity (from 0.316 to 0.020 min/μM).

As mentioned in Section 1, Shepherd et al. demonstrated that the tryptophan 7-halogenase PrnA and the tryptophan 5-halogenase PyrH display activity on the tryptophan metabolite kynurenine and several anthranilamides and anthranilic acid (Shepherd et al., 2015). Halogenated anthranilic acids are present in many pharmaceuticals and agrochemicals so the authors sought to increase the halogenation activity of PrnA on anthranilic acid through protein engineering. By examining an available crystal structure of PrnA (Dong et al., 2005), the authors reasoned that selected residues could be mutated to the positively charged residues lysine or arginine to create a salt bridge contact with the carboxylate moiety of anthranilic acid. PrnA E450K not only increased anthranilic acid conversion eightfold (from less than 2% to 14% conversion after 1 h at 2 mol% enzyme loading) but also increased the ratio of 3- to 5-halogenation relative to wild-type PrnA (93% 3-halogenation compared with 86% for wild-type PrnA). Conversely, F454K increased conversion fourfold on the same substrate but had the opposite effect on regioselectivity, increasing the ratio of 5- to 3-halogenation (38% 5-halogenation compared with 14% for wild-type PrnA). A double mutant incorporating both of these mutations furnished a 16-fold increase in conversion (to 27% conversion) and an even greater preference for 5-halogenation (54% 5-halogenation).

The work by Payne et al. discussed in Section 4.2 (vide infra) led to the RebH variant 3-SS which not only had significantly improved activity on the substrate tryptoline but also changed the regioselectivity from a nearly 1:1 mixture of 6- and 7-chlorotryptoline with the wild-type enzyme to >95% selectivity for 6-chlorotrypotoline. While these examples demonstrate the ability to tune enzyme selectivity via rational design and directed evolution, respectively, no screening pressure was applied to enable discovery of variants with a specific regioselectivity. Libraries were screened for activity improvements and selectivity was determined through further characterization of activity hits. Directly screening for a specific change in regioselectivity can greatly expedite the development of halogenase variants that produce a specific halometabolite; a procedure for doing so is discussed later.

3.1 Altered Regioselectivity via Iterative Mutagenesis and Screening

Recently, Andorfer et al. demonstrated that selectivity of FDHs could be systematically tuned using a MALDI-MS assay (Scheme 3) (Andorfer et al., 2016). By using a combination of random and targeted mutagenesis, variants capable of halogenating the substrate tryptamine with 90% selectivity for C-6 and 95% selectivity for C-5 were developed from the 7-halogenase RebH. Starting from the point mutant RebH-N470S, 10 rounds of iterative mutagenesis and screening were performed to obtain these 5- and 6-halogenases (named 10S and 8F, respectively, Fig. 2). Libraries were screened using a selectively deuterated tryptamine probe as substrate and analyzed by MALDI-MS. In this way, variants were directly screened for



Scheme 3 Deuterium labeling of substrates to determine regioselectivity by mass spectrometry. *Adapted from Andorfer, M. C., Park, H. J., Vergara-Coll, J., & Lewis, J. C. (2016). Directed evolution of RebH for catalyst-controlled halogenation of indole C–H bonds,* Chemical Science, *Advance article.*

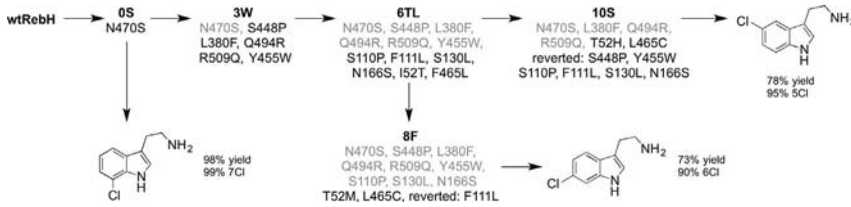


Fig. 2 Mutations introduced and relative conversions seen along lineage of altered regioselectivity RebH variants. *Adapted from Andorfer, M. C., Park, H. J., Vergara-Coll, J., & Lewis, J. C. (2016). Directed evolution of RebH for catalyst-controlled halogenation of indole C–H bonds,* Chemical Science*, Advance article.*

altered selectivity. The 5- and 6-halogenases developed in this work were found to change the selectivity on a variety of substrates, including tryptamine derivatives, tryptophol, simple arenes, and some of the indole and carbazole drugs discussed in Section 4.2.

The benefits of using random mutagenesis alongside targeted libraries are highlighted in this work. In the first rounds (1–5) of evolution, random mutagenesis was used to identify sites that led to large changes in selectivity. These sites found via random mutagenesis were targeted through libraries of variants containing the degenerate NNK codon. These targeted libraries led to the rapid identification of highly selective variants for C-5 and C-6 of tryptamine. Interestingly, when key mutations from targeted libraries were introduced into wild-type RebH, little to no change in selectivity was observed, suggested the advantageous mutagenesis of certain sites is template dependent.

3.1.1 Procedure for Evolving FDH Regioselectivity via Random Mutations

1. Follow steps 1–17 in the procedure described in Section 2.1.1 to generate the crude lysate of the FDH mutant library. A similar mutation rate and library size to those described for the evolution of increased thermostability were employed for each of the rounds of random mutagenesis.
2. Select a suitable substrate for screening. The primary considerations for selecting the proper substrate are:
   a. Sufficient activity on the substrate. For the MALDI-MS screening described later, at least 10% conversion on the substrate greatly decreased the quantity of false positives obtained (and thus the workload necessary for steps 25–35). In contrast to screening for increased activity on a particular substrate for which conversion

should be kept relatively low so as to highlight activity improvements, higher conversion is desirable when screening for altered selectivity. In many cases, variants with altered selectivity on a substrate also have significantly lowered activity (Andorfer et al., 2016; Lang et al., 2011).

b. Solubility in a buffer:cosolvent system tolerated by the FDH. RebH shows very little loss of activity with 95% HEPES buffer: 5% organic solvent (MeOH/EtOH/iPrOH/acetone/DMSO), providing ample options for a suitable solvent system.

c. Substrate can be selectively deuterated. Deuterium incorporation can be introduced either at the native site of halogenation or at the desired halogenation site. If halogenation at more than one site on a substrate is of interest, deuterium incorporation at the native halogenation site is an effective method to screen for altered selectivity at any position. However, if a particular site is of interest, deuteration at this site is most effective.

3. Add solutions of substrate (eg, 7-deutero-trypamine, 30 m*M* in ethanol, 1.5 m*M* final concentration), halide (eg, NaCl, 1.5 *M* in HEPES buffer (25 m*M*, pH 7.4), 10 m*M* final concentration), FAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), NAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), FAD reductase (eg, MBP-RebF, 50–100 μ*M* in HEPES storage buffer (25 m*M*, pH 7.4, 10% glycerol), 2.5 μ*M* final concentration—other reductases have been employed for other FDHs), and glucose dehydrogenase (eg, GDH-105 from Codexis, 1800 U/mL in HEPES buffer (25 m*M*, pH 7.4), 9 U/mL final concentration) to the pretreated crude lysate. Add additional HEPES buffer (25 m*M*, pH 7.4) such that the final reaction volume is 75 μL and then add a solution of glucose (1 *M* in HEPES buffer (25 m*M*, pH 7.4), 20 m*M* final concentration) to initiate the reaction. Reseal the microtiter plates with AeraSeals from Research Products International and then shake overnight at 600 rpm at room temperature.

4. Quench the reactions with 75 μL of methanol to precipitate out the proteins. Add dilute acid to each reaction mixture to slightly acidify it (eg, for reactions with tryptamine probes, 10 μL of 75 m*M* HCl was sufficient). *Note*: Acidification is necessary when the substrate is basic. Without this step, the reaction mixture dissolves the prespotted matrix on the MALDI target. However, adding significantly more acid could cause loss of the deuterium label due to proton exchange.

5. Spin the quenched microtiter plates down at 3600 rpm for 15 min at 4°C.
6. Filter the supernatant directly into a 96-well microtiter plate using a 0.45-μm filter plate.
7. Spot matrix onto a 384-well MALDI target plate. Conditions can vary by substrate. For tryptamine, 2 μL of a solution of α-cyano-4-hydroxycinnamic acid was used per spot (7.5 mg/mL of α-cyano-4-hydroxycinnamic acid in 1:1 THF:H_2O). This can then be dried on the benchtop or in a vacuum oven.
8. Once the MALDI target is dry, spot 2 μL of filtered reaction mixture onto each spot using a liquid handling robot. Dry on the benchtop or in a vacuum oven.
9. Seal the 96-well plates containing the remaining reaction mixtures with aluminum foil using a plate sealer. These can be stored at 4°C and used as a secondary screen to eliminate false positives from the primary MALDI-MS screen.
10. Collect spectra for MALDI-MS. For tryptamine, spectra were collected on a Bruker Ultraflextreme MALDI-TOF-TOF using an automated method (AutoXecute tool of the Flex Control acquisition software). The spectra were generated in the positive ion reflector mode over a detector range of 160–200 Da (m/z of deutero-tryptamine = 162, m/z of chloro-deutero-tryptamine = 196). Final spectra constituted the average of 500 raster shots taken at 50 random positions.
11. Set all peaks besides those corresponding to starting material and products as background. Export data into Microsoft Excel. Use an Excel macro to insert "0" for all reactions that did not have product peak. Array these data into 96-well plate format using Excel and calculate the conversions and selectivities for each reaction.
12. The reactions that show the greatest degree of change in selectivity or the greatest degree of improvement in conversion for all products while maintaining parent selectivity can be rescreened on a UPLC using the procedure outlined in Section 2.1.1, step 22. This will often not show resolution of product isomers, but can remove false positives for improved conversions.
13. The reactions that show the greatest degree of improvement with MALDI-MS and UPLC screening should be grown on a larger scale and purified as described in steps 25–34 of Section 2.1.1 (using the saved primary culture in the 1 mL plates), plasmid isolated and sequenced to confirm the presence of nonsynonymous mutations,

and halogenase variants analyzed in vitro as described in step 35 (for FPLC purification of either non-His-tagged or His-tagged halogenases, see van Pée (2012)).

3.1.2 Procedure for Altering FDH Regioselectivity via Targeted Mutations

1. Generate a mutant library by introducing the degenerate NNK codon at the desired site via overlap extension PCR: for RebH, PCR conditions were as follows: 98°C 30 s (98°C 10 s, 72°C 50 s) for 35 cycles, 72°C 10 min.
2. Follow steps 3–17 in the procedure described in Section 2.1.1 to generate the crude lysate of the FDH mutant library.
   a. *Note*: For step 11, the number of colonies that should be picked for each library depends on the number of degenerate NNK codons that are introduced as well as the desired library coverage. For RebH, enough colonies were picked to ensure 95% library coverage. The CASTer tool was used to determine these numbers (http://www.kofo.mpg.de/en/research/biocatalysis).
3. Follow steps 2–13 in the procedure described in Section 3.1.1.

4. EXPANDING THE SUBSTRATE SCOPE OF FDHs

4.1 Expanding FDH Substrate Scope via Targeted Mutations

The narrow structural range of substrates known to be halogenated by wild-type FDHs, as described in Section 1, was a significant barrier to the application of FDHs in chemical synthesis. Glenn et al. determined that the L-tryptophan 7-halogenase RebH could also accept the nonnative substrate tryptamine, producing solely 7-chlorotryptamine as its product (Glenn et al., 2011). The authors had previously integrated RebH and another halogenase into biosynthetic pathways to produce halogenated metabolites (Runguphan et al., 2010), but found that the subsequent enzyme in the biosynthetic pathways, tryptophan decarboxylase, accepted 7-chlorotryptophan at only 3% of the efficiency with which it accepted L-tryptophan. They accordingly sought to instead halogenate tryptamine, the product of tryptophan decarboxylase, thus bypassing this bottleneck. Wild-type RebH was found to accept L-tryptophan and tryptamine roughly equally (although a later report indicated wild-type RebH prefers for L-tryptophan significantly over tryptamine), so the authors sought use the available crystal structure of RebH

(Yeh et al., 2007) to guide a protein engineering effort to increase selectivity for tryptamine over L-tryptophan.

Six residues were selected proximal to the carboxylate moiety of L-tryptophan with the intention to mutate these residues to larger amino acids. It was hoped that this would disrupt binding of L-tryptophan in the RebH active site without impacting the binding of tryptamine, which lacks this carboxylate moiety. A total of 17 mutants were explored, only two of which retained any activity for either L-tryptophan or tryptamine. One of these mutants, RebH Y455W, displayed significantly increased preference for tryptamine over tryptophan. In a competition assay, RebH Y455W displayed a 10-fold decrease in production of 7-chlorotryptophan, with a simultaneous threefold increase in the production of 7-chlorotryptamine, resulting in a net 30-fold increase in selectivity for tryptamine. More recently, Shepherd et al. performed point mutagenesis of select residues of PrnA based on its crystal structure with the intent of generating a salt bridge contact with the carboxylate moiety of anthranilic acid (Shepherd et al., 2015). Several of these point mutants displayed increased conversion on nonnative substrates; these variants were discussed in detail in Section 3.

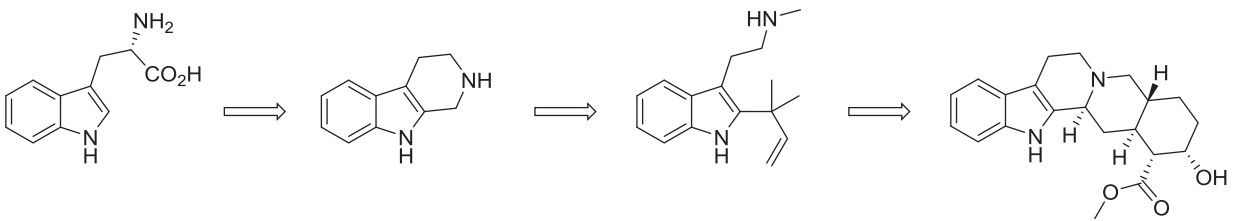
4.2 Expanding FDH Substrate Scope via Random Mutations

The availability of crystal structures of several FDHs affords great potential for structure-guided protein engineering efforts. However, the effects of structure-guided mutations can often be surprising. For example, in the work of Glenn et al. (2011), a total of 17 variants were designed, for all of which it was intended that the enzyme would prefer tryptamine over L-tryptophan, but only one of these variants had this preference. A general engineering approach that can be applied to any FDH to improve activity on any particular substrate would also be very useful. In particular, all the FDHs, wild type or engineered, discussed thus far only accept substrates comparable in size to or smaller than the native substrate and do so with significantly diminished activities. An engineered FDH capable of site-selective halogenation of large, bioactive substrates would be of great utility in both synthetic chemistry and metabolic engineering applications like those described earlier. The engineered FDH would then act as the last step in the biosynthetic sequence, thus avoiding any disruption of the intermediate steps by creating bottlenecks from poor processing of halogenated intermediates (Glenn et al., 2011).

The FDHs discussed so far were not known to accept any particularly large, nonnative substrates, making an engineering effort to directly increase activity on a particular large substrate difficult. However, the activity of an FDH can be improved on a known substrate with structural homology to a target substrate, thus identifying FDH variants with improved activity on the target substrate, and then repeating this process iteratively in order to gradually evolve the substrate scope of the enzyme. This technique is known as substrate walking, and examples have been reported of its use to expand the substrate scopes of cytochrome P450 monooxygenases (Fasan, Chen, Crook, & Arnold, 2007), transaminases (Savile et al., 2010), tRNA synthetases (Xie, Liu, & Schultz, 2007), and monoamine oxidases (Ghislieri et al., 2013).

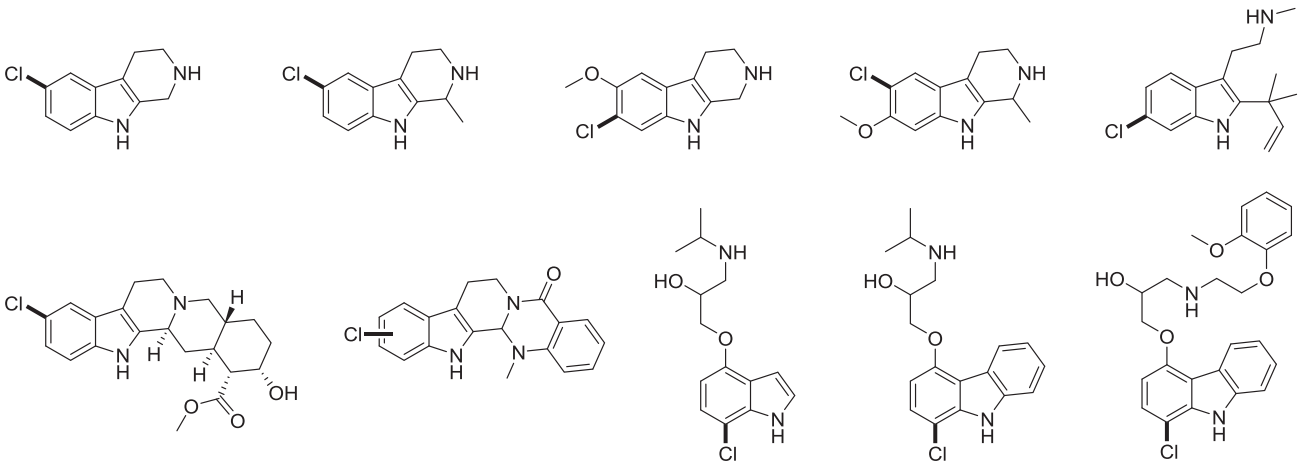
Payne et al. applied this technique to expand the substrate scope of RebH to a range of large, bioactive indoles (Scheme 4) (Payne, Poor, & Lewis, 2015). As described in Section 1, RebH showed some initial activity on the tricyclic indole tryptoline, though its conversion of this substrate is 50-fold lower than that seen with L-tryptophan (Payne et al., 2013). Starting with a thermostabilized RebH variant generated in the work described in Section 2.1, error-prone PCR was used to introduce mutations at random throughout the entirety of the RebH sequence, following the procedure described in Section 2.1.1. This first library was screened for increased activity on L-tryptophan, and one of the resulting variants also demonstrated nearly twofold increased activity on tryptoline. This increase provided sufficient activity to allow direct UPLC screening on tryptoline, and the subsequent round of evolution afforded a nearly 10-fold increase in conversion on tryptoline. This variant, 3-SS, possesses a total of six nonsynonymous mutations relative to wild-type RebH and displays over 60-fold increased activity on tryptoline. In addition, site-selective halogenation was afforded on a range of bioactive substituted tryptoline derivatives (Table 1).

The authors had chosen an analog of an inhibitor of bacterial biofilm formation, deformylflustrabromine, as a target for FDH halogenation. Deformylflustrabromine is an inhibitor of bacterial biofilm formation when the bromine substituent is present at the indole 6-position, but when this bromine is removed, no such activity is seen (Bunders et al., 2011). However, analogs with the bromine present at the indole 4-, 5-, or 7-positions have not been assayed, nor have any of the chlorinated analogs. Performing site-selective halogenations at these other positions would be extremely challenging using conventional chemical techniques, but FDHs seem well



Scheme 4 Substrate walking used to expand the substrate scope of RebH. *Adapted from Payne, J. T., Poor, C. B., & Lewis, J. C. (2015). Directed evolution of ReBH for site-selective halogenation of large biologically active molecules.* Angewandte Chemie International Edition, 54, *4226–4230.*

Table 1 Substrates Halogenated (with Selectivities Shown) by Engineered RebH Variants with Expanded Substrate Scope



equipped for this task. However, wild-type RebH displayed no quantifiable activity on unhalogenated deformylflustrabromine. Gratifyingly, the RebH variant engineered to have increased activity on tryptoline, 3-SS, displayed low activity on this substrate. The authors found that reverting one of the mutations present in 3-SS provided a fivefold increase in activity, enough to screen directly for further improvements on unhalogenated deformylflustrabromine.

An additional round of error-prone PCR furnished variant 4-V, which possesses a single addition A442V mutation and displays nearly twofold increased activity on unhalogenated deformylflustrabromine. Furthermore, it was found that 4-V provides significant increases in activity on a range of large, bioactive substrate, including the pentacyclic compounds yohimbine and evodiamine as well as a range of indole and carbazole drugs used clinically as beta blockers, which possess significant substituents at the indole 4-position. One of these substrates, carvedilol, has nearly twice the molecular weight of the native substrate of RebH, L-tryptophan. Especially remarkable is the fact that the A442V mutation that enabled these large substrates to be functionalized is distal from the enzyme active site, and thus would not have been a likely target of structure-guided engineering efforts. Such mutations have been reported to occur in other enzyme classes as well (Fasan et al., 2007; Romero & Arnold, 2009; Shimotohno, Oue, Yano, Kuramitsu, & Kagamiyama, 2001; Spiller, Gershenson, Arnold, & Stevens, 1999) and these examples highlight a unique advantage of random mutagenesis efforts.

4.2.1 Procedure for Evolving FDH Substrate Scope via Random Mutations

1. Follow steps 1–17 in the procedure described in Section 2.1.1 to generate the crude lysates of the FDH mutant library. A similar mutation rate and library size to those described for the evolution of increased thermostability was employed for each of the three rounds of evolution used to expand the substrate scope of RebH.
2. Select a suitable substrate for screening. The primary considerations for selecting the proper substrate are:
   a. Structural homology to the ultimate target substrate. For example, if the target substrate possesses an additional ring system or bulky substituent relative to the native substrate, an intermediate substrate might possess methyl groups in these locations. One reported example that sought to accommodate an additional bipyridyl group first

engineered activity on an intermediate biphenyl analog (Xie et al., 2007). They thus developed an enzyme variant that could accommodate the added steric bulk prior to engineering a final enzyme capable of recognizing the increased hydrophilicity of the bipyridyl group relative to the biphenyl group.

b. Sufficient activity on the substrate. For the UPLC screening described later, at least 10% conversion on the substrate greatly decreased the quantity of false positives obtained (and thus the workload necessary for steps 25–35) and 20% conversion was optimal.

c. Solubility in a buffer:cosolvent system tolerated by the FDH. RebH shows very little loss of activity with 95% HEPES buffer: 5% organic solvent (MeOH/EtOH/iPrOH/acetone/DMSO), providing ample options for a suitable solvent system.

3. Add solutions of halide (eg, NaCl, 1.5 *M* in HEPES buffer (25 m*M*, pH 7.4), 10 m*M* final concentration), FAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), NAD (10 m*M* in HEPES buffer (25 m*M*, pH 7.4), 100 μ*M* final concentration), FAD reductase (eg, MBP-RebF, 50–100 μ*M* in HEPES storage buffer (25 m*M*, pH 7.4, 10% glycerol), 2.5 μ*M* final concentration—other reductases have been employed for other FDHs), and glucose dehydrogenase (eg, GDH-105 from Codexis, 1800 U/mL in HEPES buffer (25 m*M*, pH 7.4), 9 U/mL final concentration) to the pretreated crude lysate. Add additional HEPES buffer (25 m*M*, pH 7.4) such that the final reaction volume is 75 μL, a solution of substrate in the organic cosolvent (eg, tryptoline for RebH 2-T, 10 m*M* in methanol), 0.5 m*M* final concentration), and then add a solution of glucose (1 *M* in HEPES buffer (25 m*M*, pH 7.4), 20 m*M* final concentration) to initiate the reaction. Reseal the microtiter plates with AeraSeals from Research Products International and then shake overnight at 600 rpm at room temperature.

a. *Note*: the substrate in the organic cosolvent is added near the end of the procedure such that the enzymes are not subjected to a transiently higher than desired concentration of organic cosolvent.

4. Quench the reactions with 75 μL of methanol to precipitate out the proteins, add an internal standard to control for differences in handling (phenol at a final concentration of 0.5 m*M* is commonly employed, but a different standard may be required depending on the substrate and analysis method used), and spin the microtiter plates down at 3600 rpm for 15 min at 4°C.

5. Filter the supernatants into a 96-well plate using a 96-well 0.45-μm filter plate prior to UPLC analysis.
6. Seal the plates with aluminum foil using a plate sealer to minimize evaporation and prevent particulate contamination, then analyze the reactions (eg, by UPLC) for halogenated product production. The following methods were used for the different substrates employed in the expansion of the substrate scope of RebH:
   a. L-tryptophan: Agilent 1200 UHPLC with an Agilent Eclipse Plus C18 2.1 × 50 mm column, 1.8 μ*M* particle size, 0.550 mL/min flow rate; solvent A = H_2O/0.1% TFA, solvent B = CH_3CN; 0–0.5 min, *B* = 16%; 0.5–1.5 min, *B* = 16–80%.
   b. Tryptoline: Agilent 1200 UHPLC with an Agilent Eclipse Plus C18 2.1 × 50 mm column, 1.8 μ*M* particle size, 0.550 mL/min flow rate; solvent A = H_2O/0.1% TFA, solvent B = CH_3CN; 0–0.5 min, *B* = 15%; 0.5–1.5 min, *B* = 15–40%; 1.5–2.1 min, *B* = 40%.
   c. Unhalogenated deformylflustrabromine: Agilent 1200 UHPLC with an Agilent Eclipse Plus C18 2.1 × 50 mm column, 1.8 μ*M* particle size, 0.550 mL/min flow rate; solvent A = H_2O/0.1% TFA, solvent B = CH_3CN; 0–0.5 min, *B* = 15%; 0.5–1.5 min, *B* = 15–40%; 1.5–2.5 min, *B* = 40%.
7. Analyze the reaction data to determine the relative improvement in halogenated product production relative to the parent wells added to each plate in step 11 in Section 2.1.1. The wells that show the greatest degree of improvement should be grown on a larger scale and purified as described in step 9 (using the saved primary culture in the 1-mL plates in step 13 in Section 2.1.1), plasmid isolated and sequenced to confirm the presence of nonsynonymous mutations, and halogenase variants analyzed in vitro as described in step 9 (for FPLC purification of either non-His-tagged or His-tagged halogenases, see van Pée (2012)).
8. If multiple improved variants are found displaying different nonsynonymous mutations, it is recommended that the beneficial mutations be analyzed both individually and in combination. Mutations can be recombined via SOE PCR; for RebH, PCR conditions were as follows: 98°C 30 s (98°C 10 s, 72°C 50 s) for 35 cycles, 72°C 10 min. Once plasmids are obtained containing the combined mutations, the halogenase variants should be immediately assayed in vitro as described in step 9.
9. To analyze the halogenase variants in vitro, follow steps 25–35 from Section 2.1.1.

5. CONCLUSIONS

While protein engineering of FDHs is a relatively new practice, significant progress has already been achieved. Using directed evolution techniques that should be generalizable to any FDH, the thermostability (Poor et al., 2014), substrate scope (Payne et al., 2015), and regioselectivity (Andorfer et al., 2016) of RebH have all been significantly altered. In addition to random mutagenesis, crystal structures are available for several FDHs that should assist in structure-guided mutagenesis efforts. Continued evolution of enhanced FDH variants will facilitate further biotechnology efforts, including expansions on the work that has already been done on metabolic engineering (Roy et al., 2010; Runguphan et al., 2010; Wang et al., 2015), combinatorial biosynthesis (Sánchez et al., 2005), and preparative-scale in vitro biocatalysis (Frese & Sewald, 2015) using wild-type FDHs.

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