Identifying and Engineering Flavin Dependent Halogenases for Selective Biocatalysis

Jared C. Lewis*

Department of Chemistry, Indiana University, Bloomington, Indiana 47405, USA

Conspectus

Organohalogen compounds are extensively used as building blocks, intermediates, pharmaceuticals, and agrochemicals due to their unique chemical and biological properties. Installing halogen substituents, however, frequently requires functionalized starting materials and multistep functional group interconversion. Several classes of halogenases evolved in nature to enable halogenation of a different classes of substrates; for example, site-selective halogenation of electron rich aromatic compounds is catalyzed by flavin-dependent halogenases (FDHs). Mechanistic studies have shown that these enzymes use $FADH_2$ to reduce O_2 to water with concomitant oxidation of X^- to HOX (X = Cl, Br, I). This species travels through a tunnel within the enzyme to access the FDH active site. Here, it is believed to interact with an active site lysine proximal to bound substrate, enabling electrophilic halogenation with selectivity imparted via molecular recognition, rather than directing groups or strong electronic activation.

The unique selectivity of FDHs led to several early biocatalysis efforts, preparative halogenation was rare, and the hallmark catalyst-controlled selectivity of FDHs did not translate to non-native substrates. FDH engineering was limited to site-directed mutagenesis, which resulted in modest changes in site-selectivity or substrate preference. To address these limitations, we optimized expression conditions for the FDH RebH and its cognate flavin reductase (FRed), RebF. We then showed that RebH could be used for preparative halogenation of non-native substrates with catalyst-controlled selectivity. We reported the first examples in which the stability, substrate scope, and site selectivity of an FDH were improved to synthetically useful levels via directed evolution. X-ray crystal structures of evolved FDHs and reversion mutations showed that random mutations throughout the RebH structure were critical to achieving high levels of activity and selectivity on diverse aromatic substrates, and these data were used in combination with molecular dynamics simulations to develop predictive model for FDH selectivity. Finally, we used family-wide genome mining to identify a diverse set of FDHs with novel substrate scope and complementary regioselectivity on large, three-dimensionally complex compounds.

The diversity of our evolved and mined FDHs allowed us to pursue synthetic applications beyond simple aromatic halogenation. For example, we established that FDHs catalyze enantioselective reactions involving desymmetrization, atroposelective halogenation, and halocyclization. These results highlight the ability of FDH active sites to tolerate different substrate topologies. This utility was further expanded by our recent studies on the single component FDH/FRed, AetF. While we were initially drawn to AetF because it does not require a separate FRed, we found that it halogenates substrates that are not halogenated efficiently or at all by other FDHs and provides high enantioselectivity for reactions that could only be achieved using RebH variants after extensive mutagenesis. Perhaps most notably, AetF catalyzes site-selective aromatic iodination and enantioselective iodoetherification. Together, these studies highlight the origins of FDH

engineering, the utility and limitations of the enzymes developed to date, and the promise of FDHs for an ever-expanding range of biocatalytic halogenation reactions.

Key References

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• Jiang, Y.; Snodgrass, H. M.; Zubi, Y. S.; Roof, C. V.; Guan, Y.; Mondal, D.; Honeycutt, N. H.; Lee, J. W.; Lewis, R. D.; Martinez, C. A.; Lewis, J. C. The Single-Component Flavin Reductase/Flavin-Dependent Halogenase AetF Is a Versatile Catalyst for Selective Bromination and Iodination of Arenes and Olefins. *Angew. Chem. Int. Ed.* **2022**, *61* (51), e202214610.[4] The first report of a single component FDH catalyzing non-native transformations with catalyst controlled site- and enantioselectivity.

Introduction

Enzyme catalysis often involves activation of a functional group to affect a chemical transformation. For example, serine proteases contain an "oxyanion hole" containing backbone amide N-H groups that activate carboxylic acid derivatives toward nucleophilic attack by a serine residue in a Ser-His-Asp catalytic triad (Figure 1A).[5] Other enzymes avoid the need for functional group activation on one substrate by generating reactive intermediates via activation of another.[6] Cytochromes P450, for example, react with O_2 to generate compound I, a potent Fe(IV)-oxo radical cation that can abstract sp^3 C-H bonds to enable aliphatic hydroxylation via a radical rebound mechanism (Figure 1B).[7] Such intermediates can often react with different functional groups; P450s can catalyze epoxidation of olefins and heteroatom demethylation[8]. In such cases, the enzyme controls substrate binding and orientation relative to the reactive intermediate, but functional group activation is typically not required.[6]

Harnessing this flexibility for synthetic purposes requires methods to control substrate and transition state binding orientation. The enzyme must accommodate these species in a manner that situates a sufficiently reactive substituent proximal to the reactive intermediate. General and rapid means to tune binding so that different substrates and sites/functional groups on those substrates can react selectively must be developed.[9] When I started my independent research group, several features of flavin dependent halogenases (FDHs) suggested that they could be amenable to these requirements (Figure 1C).[10–13] The tryptophan halogenases PyrH,[14] Thal,[15] and PrnA[16] were known to selectively halogenate tryptophan at its 5-, 6-, or 7-position, respectively. Because these

sites on the benzene ring of tryptophan are less electronically activated than the pyrrole ring (*vide infra*), this capability indicated that FDHs had the potential to override substrate-controlled reactivity.

Structural analysis of these enzymes revealed that their tryptophan and FAD binding sites are separate but linked by a tunnel,[17–19] and mutagenesis studies indicated that conserved active site lysine and glutamate residues in the tryptophan binding site are essential for catalysis[17,20]. Based on these observations, it was hypothesized that reduced flavin could react with O_2 to form a flavin hydroperoxide intermediate and that this species could react with halide anions to form HOX (X = Cl, Br). This species could be funneled to the active site where it is activated via H-bonding to lysine to enable selective electrophilic aromatic substitution of proximally bound tryptophan.[21] Kinetic studies involving the FDH/flavin reductase (FRed) pair RebH/F[22] suggested that a long-lived lysine-derived haloamine could be responsible for halogenase catalysis,[23] though this study could not rule out the haloamine serving as a reservoir for HOX following reaction with water. Both mechanistic proposals suggest the formation of an electrophilic halogen intermediate within the FDH active site that could engage substrates in catalyst-controlled halogenation. Given the impact of halogenation on the physical and biological properties of molecules,[24–26] the utility of halogenated compounds for synthesis, and the challenges often associated with conventional halogenation methods,[27–29] we set out to identify and engineer FDHs for selective biocatalysis.

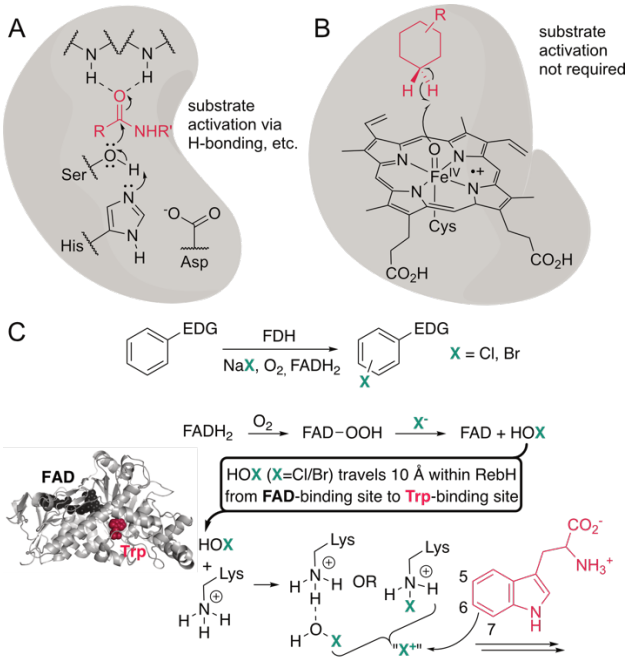


Figure 1. A) Enzyme catalysis proceeding via substrate activation vs. reactive intermediate formation. B) Simplified mechanism of FDH catalysis.

Early FDH Engineering Efforts

Our first efforts to use FDHs and FReds for preparative biocatalysis were complicated by low enzyme yields from gene expression in *E. coli*. We found that co-expressing RebH with the GroEL/GroES chaperonin system[30] improved its yield from ~15 mg/L culture to >100 mg/L.[1] We

later found that this improvement translated to FDHs from a wide range of organisms, including fungi.[2,31] Similar improvements were not observed for RebF, but fusing maltose binding protein (MBP)[32] to the RebF N-terminus improved protein yield from 3 mg/L to 33 mg/L.[1] The fusion enzyme displayed similar kinetics to wt RebF, so it was used without cleaving the MBP tag in the studies discussed below.

A more fundamental issue that we faced was that the only non-native substrate scope reported for an FDH, a tryptophan 7-halogenase from *P. fluorescens* BL915, suggested that the unique selectivity of FDHs did not extend even to structurally related substrates.[33] The enzyme halogenated a variety of indoles but only on the activated pyrrole ring. Suspecting that these results might not reflect the capabilities of other FDH/substrate pairs, we examined the activity of RebH toward a variety of indoles.[1] Several 3- and 2,3-disubstituted indoles were halogenated on their benzene ring, but that simpler substrates, like indole, 2-aminonaphthalene, and 1-hydroxynaphthalene were halogenated at their most reactive site (Figure 2A). The latter result showed that molecular recognition would have to be improved to enable catalyst-controlled halogenation even for modest changes in substrate structure. This study also established that a cofactor regeneration system comprising a glucose dehydrogenase[34] and MBP-RebF could be used to supply FDHs with reduced flavin, and that reactions could be conducted using enzyme in crude lysate to facilitate reactions on up to 100 mg scale (Figure 2B).

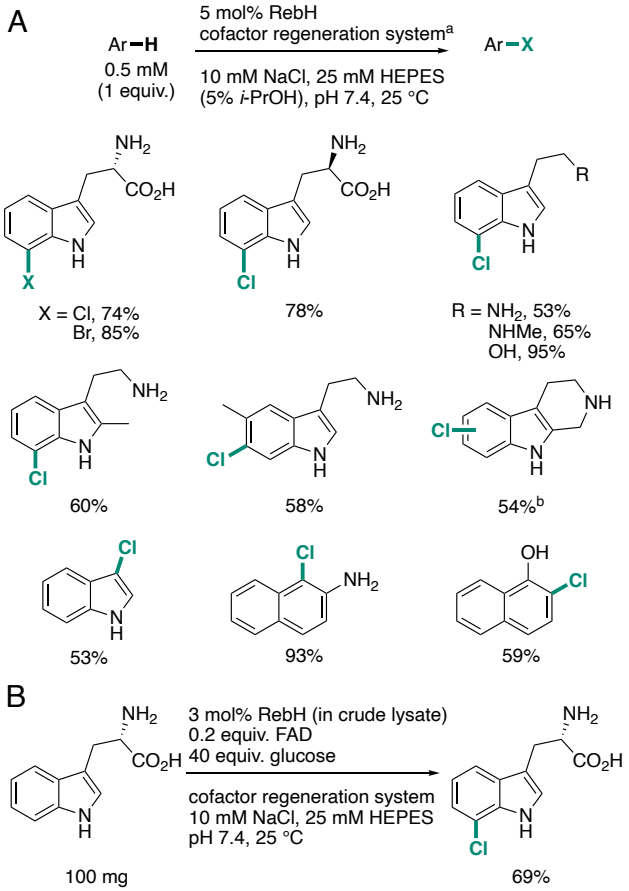
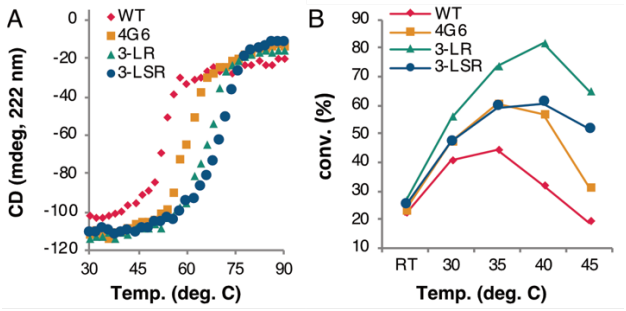
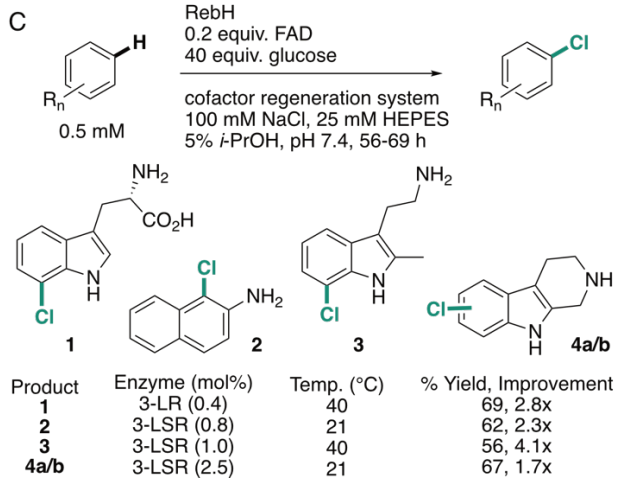


Figure 2. A) Representative RebH substrate scope with isolated yields. [a]The cofactor regeneration system consists of 0.5 mol % RebF and 50 U mL^{-1} glucose dehydrogenase. [b]10 mol % RebH loading was used. A nearly 1:1 mixture of 5- and 6- halogenation was observed. B) Preparative chlorination using RebH in cell lysate.

We next sought to improve the stability of RebH.[35] Thermophilic FDHs had not yet been reported,[36,37] and we worried that the modest stability of RebH, which has a melting temperature of only 50 °C and typically precipitated during biocatalysis,[1] would be compromised by active site mutations.[38] Optimized RebH/F expression and cofactor regeneration provided sufficient product yields using lysates produced in 96 well plates to screen using UPLC, which enabled directed evolution of stabilized RebH variants. Libraries were generated using error-prone PCR, and the activity of these variants on tryptophan was evaluated following heat treatment at increasing temperatures to give variants 3-LR and 3-LSR. These variants possessed melting temperatures 5 °C and 18 °C higher than RebH (Figure 3A), gave higher substrate conversions at their optimal reaction temperatures than RebH at its lower optimal temperature (Figure 3B), and provided 1.7-4.1-fold improved yields for the other substrates examined (Figure 3C).[35] Reaction progress and kinetic analysis established that these improvements resulted from extended catalyst lifetime. This effort was the first example of FDH directed evolution and provided stabilizing variants that were used in subsequent evolution efforts.





Product	Enzyme (mol%)	Temp. (°C)	% Yield, Improvement
1	3-LR (0.4)	40	69, 2.8x
2	3-LSR (0.8)	21	62, 2.3x
3	3-LSR (1.0)	40	56, 4.1x
4a/b	3-LSR (2.5)	21	67, 1.7x

Figure 3. A) CD melting curves for evolved RebH variants. B) Improved tryptophan chlorination yields for evolved RebH variants at increased temperatures. C). Improved yields of substrates using stabilized RebH variants.

Concurrent with stability evolution, we began to probe whether RebH substrate scope could be expanded using directed evolution.[39] One report had established that a single point mutation could switch RebH substrate preference from tryptophan to tryptamine, albeit with significantly reduced activity.[40] We envisioned that substrate walking,[41] which involves screening for activity on probe substrates increasingly similar to a target substrate,[42] could be used to generate variants with activity on indole-containing drugs and natural products. We selected 2-methyltryptamine (**5**), tryptoline (**6**), and indole **7** as probe substrates to achieve this goal (Figure 4A). Because variant RebH S2P from the first round of our (then) ongoing stability evolution effort provided improved yields on several substrates,[35] site saturation libraries of this enzyme were generated using degenerate NDT codons at ten active site residues (52, 109, 111, 454, 455, 461, 465, 466, 467, and 470). The activity of these variants was evaluated on 2-methyltryptamine, which provided similar conversion as tryptophan. None of these libraries led to variants with improved conversion following purification (unpublished results), consistent with a previous report that point mutations at several of these residues led to inactive variants[40].

By this time, however, stability evolution using error prone PCR had led to the variant 1-PVM.[35] This variant contained a single active site mutation, N467T, which provided a sufficient increase in activity on tryptoline to screen on this substrate.[39] A library generated via error prone PCR led to variant 3-SS, which contained active site mutations G112S and N470S. Random mutagenesis of this variant and screening on indole **7** led to variant 4-V, which contained the non-active site mutation A442V. Together, 3-SS and 4-V were capable of halogenating a variety of bulk indole-containing compounds (Figure 4B). Notably, residues 467 and 470 were included in our site saturation libraries, and while N467T is not accessible using an NDT codon, N470S is. These libraries were created using RebH S2P rather than the more evolved variant in which they ultimately proved useful. This result mirrors a common finding in protein engineering: designs and libraries may be "rational", but it is difficult to predict how the enzyme used to test those designs may affect outcomes.[43] These issues were mitigated by directed evolution via random mutagenesis and substrate walking.

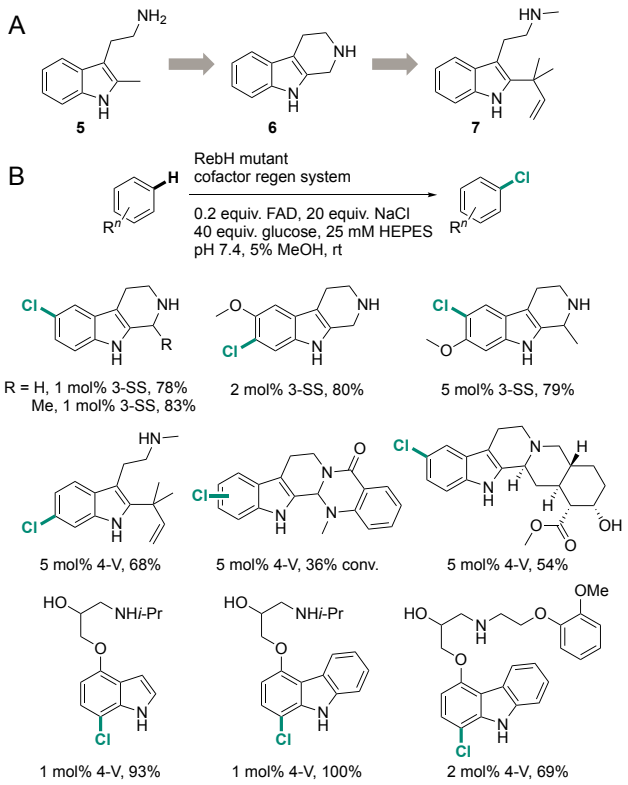


Figure 4. A) Substrates used for evolution of RebH substrate scope. B) Representative substrate scope of evolved RebH variants with isolated yields.

We next sought to establish whether directed evolution could be used to systematically change the site selectivity of FDHs.[44] While structure-based methods had been used to alter FDH selectivity,[45–47] modest selectivities were typically observed,[48] though a later studied showed that grafting active site residues from the 7-selective RebH into 6-selective Thal imparted high 7-selectivity to the latter[49]. Because crystal structures are available for relatively few FDH/substrate pairs, this approach would not be viable for the expanding range of substrates that we had identified for different FDHs.[48] No changes in selectivity were observed during our substrate scope evolution,[39] indicating that either such changes were not occurring or our UPLC assays could not detect them. More broadly, efforts to alter the site selectivity of enzymatic C-H functionalization were rare at the time we initiated our efforts,[6,9] and the successful examples in the literature did not involve direct screens for altered selectivity[50].

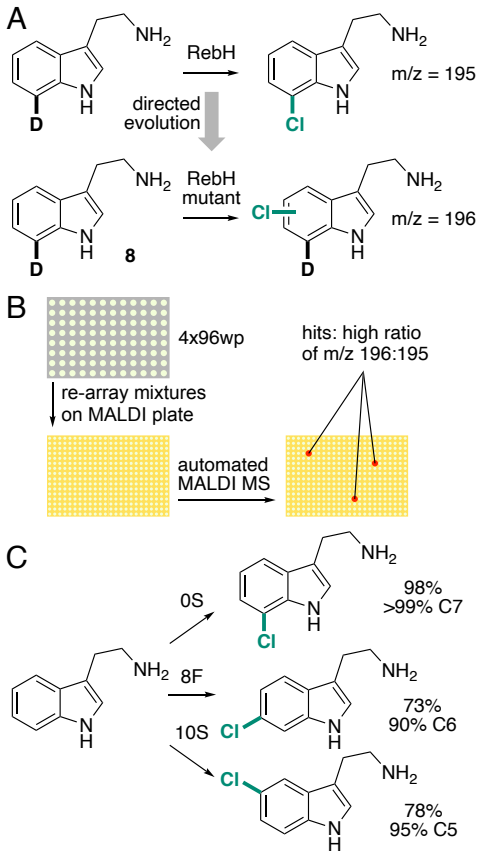


Figure 5. A/B) MALDI-MS screen for altered site selectivity using deuterated probe substrates **8** and **9** (not shown). C) Yields and site selectivities of evolved RebH variants 0S, 8F, and 10S.

We envisioned that deuterated probe substrates[51] could enable a general screen for site-selective C-H functionalization (Figure 5A/B).[44] Tryptamine was selected as a substrate because the required deuterated derivatives could be readily synthesized, and the sites on its benzene ring possess similar reactivity based on halenium affinity (HalA) calculations[52] that compare the enthalpy for electrophilic attack of X^+ on different sites to form the corresponding cationic adducts (*vide infra*). The screen was implemented by screening the 7-selective FDH RebH for activity on 7-deuterotryptamine (**8**) using MALDI MS. Halogenating the 7-position of **8** gives a product with m/z=195 while halogenating other sites gives products with m/z=196, allowing for rapid identification of variants with altered selectivity. Variant 6TL, obtained after six rounds of evolution, provided similar amounts of 5-, 6-, and 7-chlorotryptamine, so starting in this round, 5-deuterotryptamine (**9**) was used to directly screen for 5-selective variants. Variants that maintained high activity with low 5-selectivity on **9** were then screened by UPLC to identify 6-selective variants since 7- and 5/6-chlorotryptamine are chromatographically separable and the latter are mass differentiable. Eight and ten rounds of mutagenesis, including random mutagenesis, site saturation mutagenesis, point mutations, and recombination, were required to obtain 6- and 5-selective (>90% selective; 73% and 78% yield, respectively) halogenase variants 8F and 10S (Figure 5B), which also provided altered selectivity toward a number of different substrates. Combined with our ability to expand substrate scope,[39] this capability provides a means to engineer FDHs with altered site selectivity on diverse substrates, provided suitable deuterated probes can be synthesized.

During this effort, screening for altered selectivity alone led to variants with dramatically reduced activity, so some rounds involved increasing overall activity to enable continued high throughput screening. Several mutations that increased selectivity were located in the active site, and many of these were included in our initial NDT libraries (e.g. 52, 111, and 465). Some mutations that increased 5/6-selectivity by either increasing 5/6-halogenation or decreasing 7-halogenation could be removed from the final 5- and 6-selective variants to improve their activity and/or selectivity. Key mutations that led to high 5- and 6-selectivity during the course of directed evolution (e.g. I52M and L111F in 8F; T52H and F465C in 10S) led to dramatically reduced yields and minimal change in selectivity when introduced directly into RebH. These findings again emphasize how the variant into which mutations are introduced plays a large role in their effects.

Synthetic Applications of Engineered FDHs

As our library of engineered FDHs expanded, we began to explore the synthetic utility of these enzymes. Several groups had shown that tryptophan FDHs could be used in chemoenzymatic C-H halogenation/Suzuki coupling sequences using tryptophan.[53–56] With access to enzymes with high activity on non-native substrates, we showed that this approach could be used on crude extracts of halogenated compounds, and that C-C, C-O, and C-N bond formation was possible (Figure 6A).[57] These conditions were also used in conjunction with enzymes engineered in efforts outlined below to enable functionalization of chiral halogenated compounds,[58,59] and similar efforts further highlighted the generality of this approach[60].

To demonstrate the utility of FDHs for halogenation of fragment compounds commonly used in drug development, we collaborated with researchers at Novartis to profile the activities of tryptophan halogenases (RebH and Thal), engineered RebH variants (0-K, 4-V, 6-TL, and 10-S), and fungal halogenases (Rdc2 and GsfI)[61,62] using a panel of 93 aromatic compounds, each containing at least one electronically activated site.[31] Collectively, the tryptophan halogenases chlorinated 67% of the anilines, indoles, pyrroles, and azoles evaluated with >1% conversion (the conversion from which our selectivity and substrate walking evolution commenced). Only 7% of these compounds were halogenated by Rdc2 or GsfI, but these enzymes halogenated all seven of the phenols and anisoles evaluated, which were not well tolerated by the tryptophan FDHs. Variant 4-V from our substrate walking lineage exhibited the broadest scope of the enzymes evaluated. High site selectivity was typically observed when multiple electronically activated sites were present, and in several cases, less electronically activated sites were halogenated over more activated sites (Figure 6B). Directly comparing the selectivity of different FDHs to that of *N*-chlorosuccinimide showed that the former provided higher or unique selectivity compared to the latter.

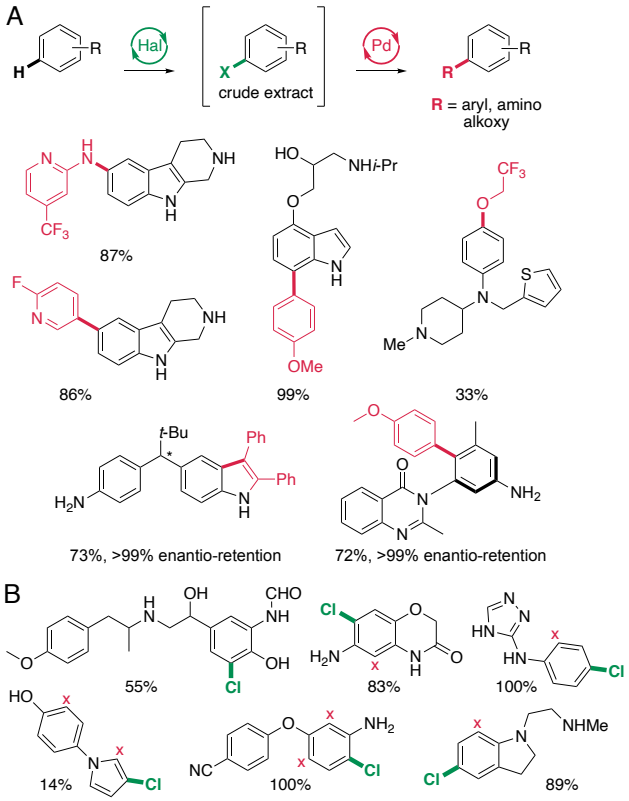


Figure 6. A) Chemoenzymatic halogenation/cross-coupling. B) Representative substrate scope of FDHs from activity profiling with "x" noting electronically activated sites that are not halogenated.

FDH Genome Mining

Activity profiling showed that while FDHs possess broad scope, over a third of the compounds evaluated were not halogenated, and the complementary reactivity of tryptophan and fungal halogenases suggested that distinct classes of FDHs could help to address this issue. We envisioned that family-wide genome mining using probe substrates could be used to systematically evaluate FDH sequence space for useful biocatalysts with unique reactivity.[2] Known FDH substrates were mapped onto a sequence similarity network[63] with e-values set to generate subnetworks containing enzymes with like substrate scope, and sequences from these subnetworks were selected to sample functionally distinct enzymes (Figure 7A-C). Based on this analysis, 128 putative FDH genes were synthesized, 87 of which provided sufficient protein for activity assays following expression in *E. coli*.

Because the substrates for these enzymes were unknown, a panel of 12 probe substrates, each containing multiple reactive sites, was used to increase the probability that activity could be measured on at least one substrate. This approach also screens for enzymes with useful levels of expression, stability, and substrate scope. Control studies established that screening reactions using enzyme in cell lysate or containing multiple substrates led to false negatives, so reactions were conducted using purified enzyme acting on individual substrates. Quenched reactions were pooled so that four reactions were screened in each MS run to improve screening throughput.

Analysis of chlorination and bromination of 12 probe substrates by 87 enzymes confirmed 39 of these as FDHs (45% of the soluble enzymes, Figure 7D/E). All active FDHs catalyzed bromination but only 16% catalyzed chlorination. The activity profiles of the enzymes clustered largely along lines anticipated based on the subnetworks within the SSN, but the native substrate that defined the subnetwork had little predictive ability. For example, while the largest "activity cluster" was almost exclusively comprised of enzymes from the "indole subnetwork", most of these enzymes halogenated anilines and phenols. Notably, the mined enzymes provided many benefits over previously reported FDHs. Site-selective halogenation of several large, biologically active compounds and site-complementary halogenation were both achieved (Figure 7F). An FDH with high expression yield and a melting temperature nearly as high as our evolved 3-LSR variant was also identified. These results significantly expanded the range of FDHs available for biocatalysis and provided a map for ongoing iterative genome mining for different applications.

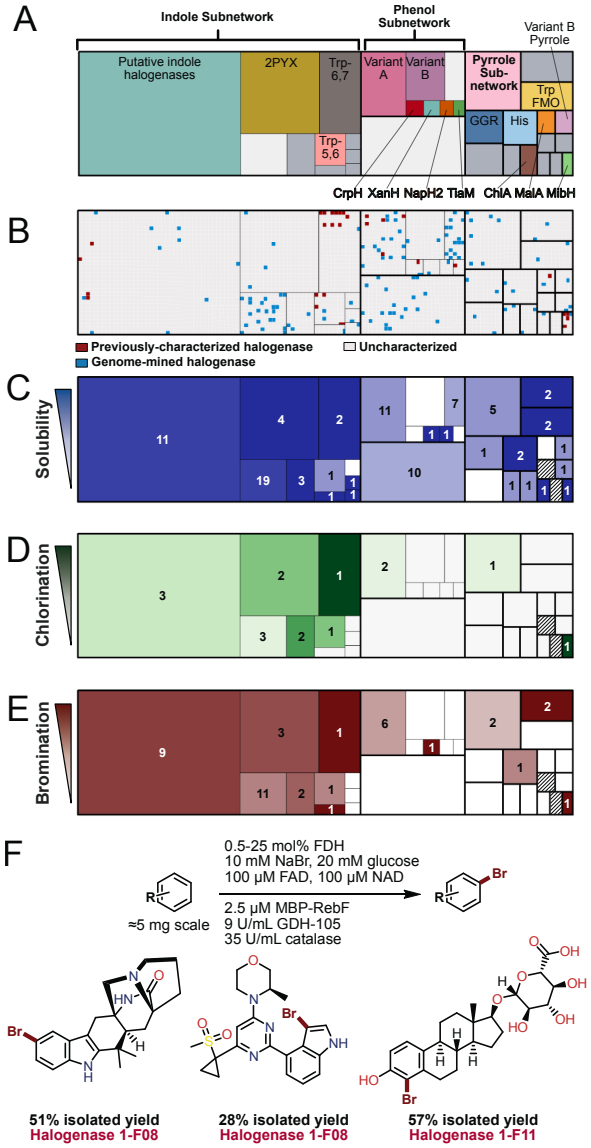


Figure 7. Treemap of FDH sequence space showing A) reported substrate preferences, B) characterized and mined sequences, C) enzyme solubility, D/E) chlorination and bromination activity. F) Representative scope of mined FDHs.

Selectivity Models and Mechanism

During the studies outlined above, we attempted to develop models to predict FDH selectivity and substrate scope. While substrate docking is not necessarily a good predictor of reactivity in general, it can be useful when reactant state binding resembles transition state binding. We therefore analyzed whether substrates could bind in an orientation that projected reaction sites toward the catalytically essential active site lysine of different FDHs as had been established for native FDH/substrate pairs.[17–19,37] For example, we analyzed tryptamine orientation in models variants 8-F and 10-S, but poses consistent with both the observed and other selectivities were obtained.[44] We used different docking methods to analyze substrate preferences of enzymes from our activity profiling effort, but no correlation between those preferences and docking scores was apparent.[31]

These approaches ignore whether a given site on a substrate is sufficiently activated to undergo electrophilic aromatic halogenation within the FDH active site. For example, the calculated HalA[52] values (enthalpy for electrophilic attack of X^+ to form the corresponding cationic adduct) for the 4-7 sites on tryptamine are 161-166 kcal/mol, while that for the 2-position is 177 kcal/mol,[44] showing that tryptophan halogenases overcome significant electronic activation of the 2-position.[44] In our activity profiling study, we calculated HalA values for all sites on all substrates, which showed that only substrates with a HalA above ~150 kcal/mol are reactive.[31] We also showed that several FDHs can override electronic preferences of non-native substrates in both our activity profiling and genome mining studies.[2,31]

We next used a combined experimental/computational approach to develop a model capable of addressing the importance of both substrate binding and electronics on FDH reactivity.[64] We solved the crystal structures of variants 0-S, 8-F, and 10-S from the selectivity lineage discussed above and used these structures in combination with reversion mutations to identify key residues involved in altering FDH selectivity. We then used DFT calculations and MD simulations to establish that only hypohalous acid activated by the active site lysine residue and not a chloramine intermediate involving that residue form near attack conformations (NACs)[65] consistent with the observed site selectivity of these enzymes. Finally, we used this MD/NAC model in combination with calculated halogenation transition state energies to recapitulate the site selectivity of several other FDH/substrate pairs. While successful, this approach is computationally expensive, highlighting the challenges associated with predicting FDH substrate scope and selectivity using conventional computational methods. Related efforts have come to different conclusions about the intermediacy of hypohalous acid and haloamine intermediates in different FDHs.[21,66–68]

Enantioselective FDH Catalysis

All known FDHs natively catalyze halogenation of arenes, enols, or alkynes.[10–13] Chiral intermediates form during some of these reactions but are not known to impact their outcomes, and rate differences for arenes bearing chiral moieties (e.g. the amino acid moiety of tryptophan) have not been exploited for chiral resolution. Nonetheless, we reasoned that the diversity of FDH active sites that we and others had identified and engineered, coupled with the range of synthetic transformations induced by electrophilic halogen reagents,[27] could enable a variety of enantioselective halogenation reactions.[69] We were particularly inspired in these efforts by studies

showing how peptides could catalyze a variety of enantioselective processes involving halogenation.[70]

Our first efforts toward this end focused on desymmetrization of 4,4'-methyelenedianilines since this reaction only requires differentiation of chirality distal to a reactive aniline moiety (Figure 8A).[58] RebH variant 4-V from our substrate walking lineage[39] exhibited high enantioselectivity toward a 4,4'-methyelenedianiline bearing a bulky *tert*-butyl group, but lower selectivity was observed for other substrates. Site-directed mutagenesis aimed at favoring binding such that one enantiotopic aniline projected toward the active site lysine was conducted, and variants with improved enantioselectivity for benzylic substrates were obtained. Other variants evolved for different halogenation reactions ultimately provided good enantioselectivity on substrates with smaller substituents.

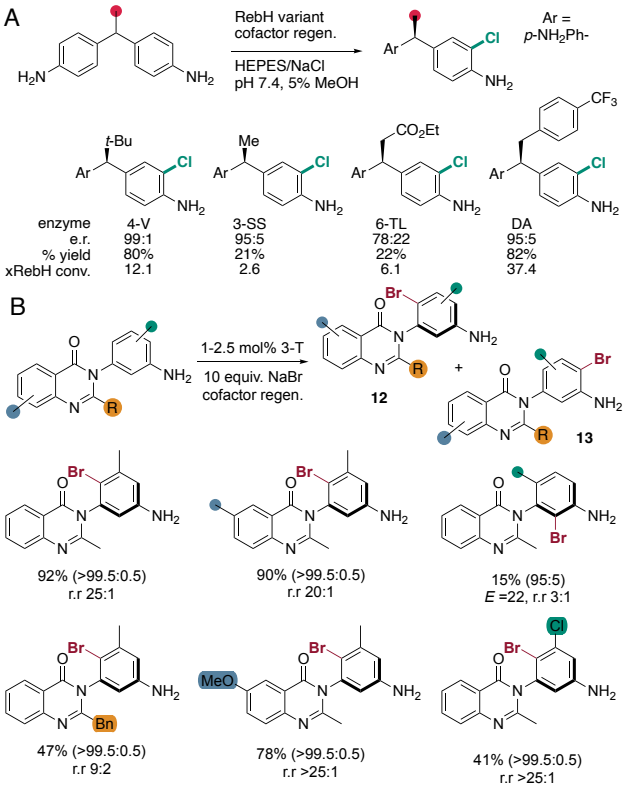


Figure 8. Representative substrate scope for enantioselective FDH catalysis involving A) desymmetrization and B) atroposelective halogenation.

We next wondered if FDHs might be able to catalyze atroposelective halogenation.[59] This class of reactions is more challenging than our initial desymmetrization since the site of halogenation is proximal to steric bulk along the axis of chirality in the substrate. We targeted halogenation of 3-(3-aminophenyl)-4(3H)-quinazolinones using **10** and **11** (Figure 8B, R = H and Me, respectively) as probe substrates due to the potential utility of the resulting compounds and the fact that this substrate class would demand both site- and atroposelectivity of our enzymes[71]. From a panel of nearly 150 engineered and wild type FDHs, we found that RebH variant 6-TL F465P from our selectivity lineage catalyzed halogenation of 10 to give ~5% assay yield of isomers **12** and **13** and >99% enantioselectivity for **12**. Three rounds of directed evolution involving an initial screen for

conversion and site-selectivity using **10** or **11** followed by analysis of enantioselectivity led to the identification of variant 3-T, which gave 14- and 25-fold increases in the conversion of these substrates and 35- and 91-fold increases in site selectivity over 6-TL F465P. The high enantioselectivity observed for atroposelective halogenation show that FDHs can accommodate substrate orientations proximal to the site of halogenation that different significantly from that associated with typical aromatic halogenation, albeit with significant evolution.

Given the apparent geometric tolerance of FDH active sites, we hypothesized that these enzymes might also be capable of reacting with alkenes to enable halosubstitution involving an external or pendant nucleophile. These reactions generate three-dimensional complexity via formation of adjacent stereogenic centers yet remain challenging for many substrate classes using small molecule catalysts.[72] Unlike native aromatic halogenation, however, these reactions involve attack of a π system by nucleophile either *cis* or *trans* to the site of attack by HOX. We reasoned that 4-methoxyphenyl-4-pentenoic acid (**14**) would serve as a good model substrate to explore the feasibility of FDH-catalyzed halocyclization since it possesses a planar, aromatic core and a pendant carboxylate analogous to features present in tryptophan (Figure 9A).[3]

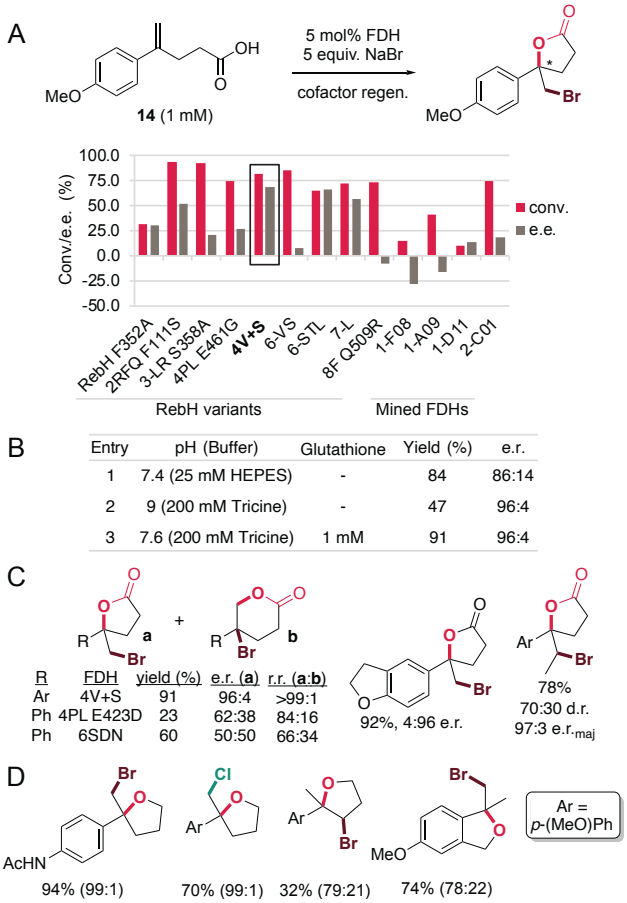


Figure 9. A) Halocyclization of **14** catalyzed by engineered and mined FDHs. B) High pH or glutathione increases the e.r. for halocyclization of **14** by 4V+S. Representative products for halocyclization involving C) carboxylate nucleophiles and D) alcohol nucleophiles.

Several evolved RebH variants catalyzed halocyclization of **14** with promising yield and enantioselectivity, and a few mined variants provided the opposite product enantiomer. Optimizing reaction conditions improved the enantioselectivity and yield of the model reaction and ultimately enabled halocyclization of a variety of substrates with good-to-high selectivity and yields. Adding glutathione to scavenge HOX that led to racemic cyclization was particularly important to achieve high enantioselectivity. While HOX was previously believed to remain localized in FDH active sites, this study and one other[73] showed that release from FDHs is possible and can compromise selectivity. Further studies established that substrates baring alcohol nucleophiles also undergo selective halocyclization and that different cyclization modes are possible (Figure 9B).[74] Analysis of FDHs with halocyclization activity showed that this reaction is enabled by a key mutation in the RebH active site, which originally appeared in the substrate walking lineage but also provided room for approach of the pendant nucleophile. A key limitation of FDH-catalyzed halocyclization to date, however, is the need for electron rich styrene substituents for high yields and selectivities. Carboxylate nucleophiles react poorly and alcohols not at all with electron neutral or deficient alkenes, though the apparent formation of 5- and 6-member rings in the former case suggests that FDHs could be evolved to control the regioselectivity of these processes. Directed evolution aimed at correcting these issues is ongoing.

Single Component FDHs

As shown in Figure 1C, FDH catalysis requires a source of reduced flavin. The reactions above used MBP-RebF[1] for this purpose, and other reports of FDH catalysis use related FReds[36,75]. Because FReds are not commercially available, they must be expressed in house or produced on demand, which limits use of FDHs. As FDHs are evolved to suit different process conditions, FRed evolution could also be required. We were therefore interested in reports that the single component FDH/FRed AetF could di-brominate the benzene ring of tryptophan.[76,77] This selectivity distinguishes AetF from a previously reported single component FDH, Bmp5,[78] which generates halogenated phenols that can be accessed using chemical halogenation reagents. We had engineered fusions of RebH and RebF that improved halogenation yields in *E. coli*,[79] but AetF comprises a single polypeptide chain with domains for FAD, NADP, and substrate binding.

We showed that AetF halogenates a wide range of aromatic substrates, including relatively electron deficient compounds that were not halogenated by FDHs that we had previously evaluated (Figure 10B).[4] More impressively, this enzyme catalyzes enantioselective halogenation reactions that proceed via desymmetrization, atroposelective halogenation, and halocyclization without the extensive evolution required for RebH. Finally, AetF catalyzes selective iodination of aromatic substrates and enantioselective iodolactonization. These findings clearly show that catalyst-controlled selectivity was possible for FDH-catalyzed iodination, a feature that has been disputed for other FDHs[80–82].

While exploring additional substrates for AetF-catalyzed halocyclization reactions, we found that this enzyme catalyzes alkene C-H functionalization on substrates lacking a pendant nucleophile (Figure 10C).[83] Both bromination and iodination proceed with high stereoselectivity, favoring halogenation *trans* to an electron rich arene, even on 1,1-disubstituted substrates with aromatic groups of similar size. Notably, AetF also catalyzes bromination and iodination *p*-methoxyphenylacetylene. Given this unique reactivity, we investigated the activity of several AetF

homologues toward a small panel of alkenes and alkynes, revealing two additional single component FDHs with activity on electron rich styrenes and unactivated alkynes. Mutagenesis experiments and kinetic isotope experiments were used to suggest a mechanism for halogenation of unactivated alkynes involving covalent catalysis. Efforts are underway to validate this mechanism and to explore its practical implications.

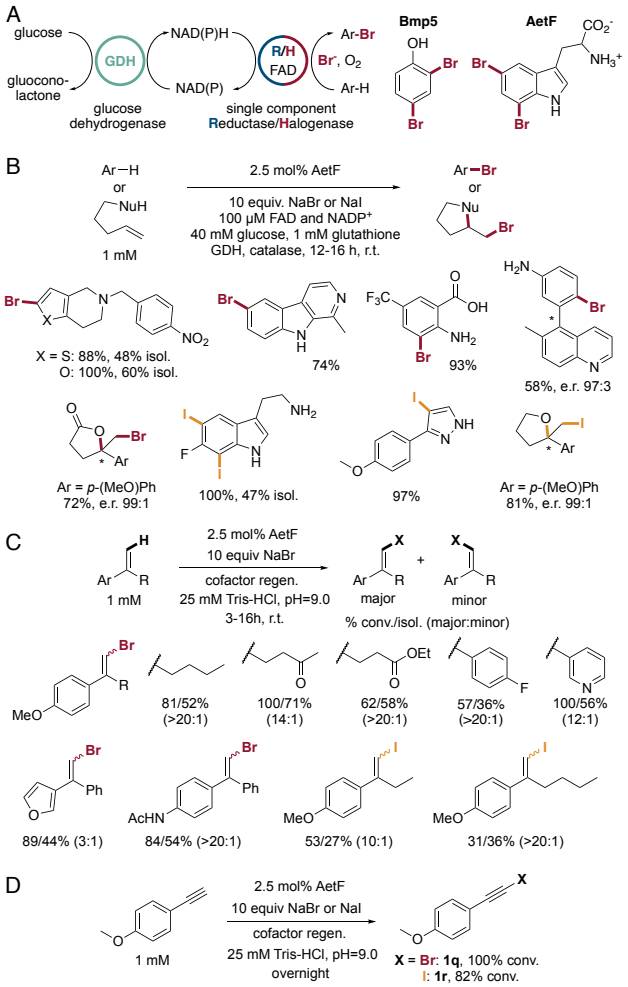


Figure 10. A) Native products of the single component FDHs Bmp5 and AetF. B) Representative substrate scope of AetF. C/D) Selective alkene and alkyne halogenation by AetF.

Conclusion and Outlook

The studies outlined above and those cited from many other groups highlight the utility of FDHs for selective halogenation of arenes, alkenes, and alkynes, and for enantioselective halocyclization of alkenes bearing pendant nucleophiles. This catalytic flexibility stems from the reactivity of a unique halogen intermediate generated within the FDH active site toward diverse π systems and the ability of mutations throughout the FDH structure to tune substrate/transition state binding proximal to this intermediate. Most evidence suggests that the reactive intermediate in conventional FDHs is HOX activated via H-bonding to a conserved active site lysine residue.[17,20,21,64,66,67] Single component FDHs also possess a conserved active site lysine,[4,83] but its distance from the substrate binding site is too great for H-bonding to HOX situated proximal to

substrate, suggesting that the role of the lysine and/or the nature of the halogen intermediate may be different in these systems. Our initial modeling incorrectly assumed substrate binding proximal to this lysine in the flavin binding site,[4] but a subsequently published crystal structure showed substrate binding distal to this site[84]. Our recent study showing different responses of single component FDHs to isotope effects also hint at interesting mechanistic complexity to be unraveled.[83]

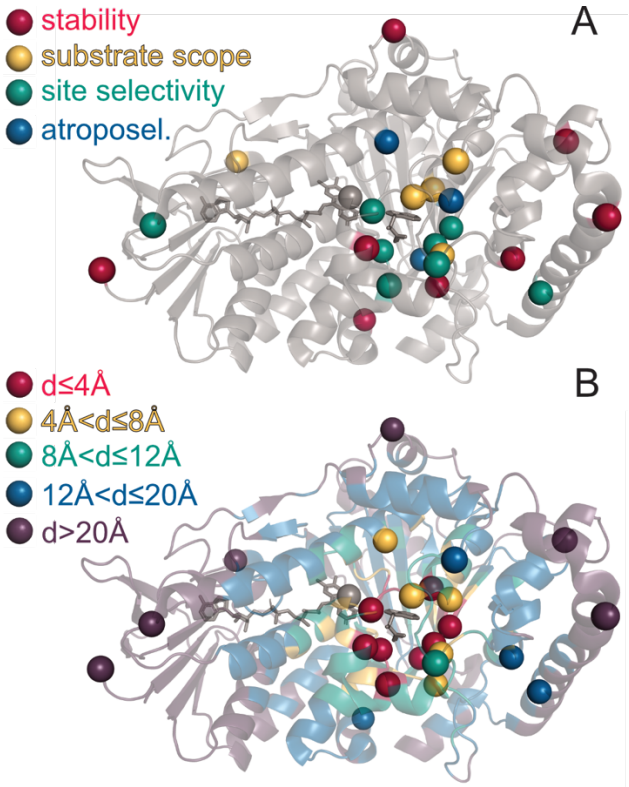


Figure 11. Structure of RebH (PDB ID 2OA1) with C_α of mutated residues shown as spheres and colored based on A) goal of directed evolution campaign in which mutations were identified or B) distance (d) from bound tryptophan.

Nearly all of the reactions we have developed using conventional halogenases have required extensive mutagenesis. The selectivity, substrate scope, and atroposelectivity evolution efforts noted above started from enzymes that provided only ~1-5% of the desired product, so directed evolution of FDHs even from these low conversions using enzyme in cell lysate is possible with careful optimization of screening conditions and analytical methods. Initial site saturation mutagenesis proximal to the active site did not lead to improved variants. Over the course of evolution efforts aimed at increasing stability, improving activity on different substrates, altering site selectivity, and enabling atroposelective halogenation, however, mutations at 7/10 of these originally targeted sites ultimately proved beneficial (Figure 11A). Mutations distal to the active site, all identified via random mutagenesis methods, were required to reap the benefits of active site mutations. Together, our published variants contain 24 mutations distributed from the active site (8), to the second sphere (6), to the periphery (10) of RebH (Figure 11B).[35,39,44,59,85] Given the inefficiency of random mutagenesis/screening to identify critical distal mutations, these findings emphasize the need for improved methods to predict such mutations, and the sequence/activity

data in these studies could help in the development of machine learning methods toward this end.[86] SSN-guided genome mining led to the identification of FDHs with improved properties and a map of FDH sequence space to search for additional enzymes with novel activity, but it can only identify sequences contained within the initial search space. It is therefore notable that AetF, the most catalytically versatile FDH we have studied, was not in our SSN due to its low sequence and structural similarity to RebH and other conventional FDHs. Identification of novel enzymes capable of catalyzing halogenation reactions thus remains important.

Improved protein engineering and genome mining tools will be critical to address the related issues of low rates, total turnover numbers, and reactivity toward electron deficient substrates that hinder broader adoption of FDHs. Conventional FDH rates, typically ~1 $s^{-1}\mu M^{-1}$ for both aromatic halogenation and halocyclization, are middling for enzymes in general[87] but low relative to those typically used for biocatalysis, and though we have established that rates on poor substrates can be increased to this level, we have not exceeded it. These low rates coupled with modest stability lead to low total turnover numbers in preparative reactions. Low rates presumably include contributions from the complicated mechanism of HOX formation, the modest electrophilicity of that species, and non-productive decomposition pathways, some of which likely compromise FDH stability.[73] AetF and homologues[83,88] could offer improvements in these regards. Their single component nature simplifies HOX formation. Their unique reactivity suggests that they may have the capability to exceed typical rates of FDHs; for example, alkene C-H functionalization with AetF exceeds 10 $s^{-1}\mu M^{-1}$, and this enzyme acted on less electronically active substrates.[4,83] Directed evolution of AetF and related enzymes therefore hold great promise for improving the synthetic utility of FDHs.

Author Information

Corresponding Author
*E-mail: jcl3@iu.edu

ORCID
0000-0003-2800-8330

Biographical Information
Jared C. Lewis was born and raised in Effingham, IL. He obtained his B.S. in chemistry from the University of Illinois (2002, Prof. Eric Oldfield), earned his Ph.D. in chemistry from the University of California, Berkeley (2007, Profs. Jonathan Ellman and Robert Bergman), and conducted postdoctoral studies at Caltech (2010, Prof. Frances Arnold). He started his independent career at the University of Chicago in 2011 and, in 2018, moved to Indiana University, where he is currently a Professor of Chemistry.

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