Analysis of Laboratory-Evolved Flavin-Dependent Halogenases Affords a Computational Model for Predicting Halogenase Site Selectivity

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Abstract

Flavin-dependent halogenases catalyze selective halogenation of electron-rich aromatic compounds without the need for harsh oxidants required by conventional oxidative halogenation reactions. Predictive models for halogenase site selectivity would greatly improve their utility for chemical synthesis. Toward this end, we analyzed the structures and selectivity of three halogenase variants evolved to halogenate tryptamine with orthogonal selectivity. Crystal structures and reversion mutations revealed key residues involved in altering halogenase selectivity. Density functional theory calculations and molecular dynamics simulations are both consistent with hypohalous acid as the active halogenating species in FDH catalysis. This model was used to

accurately predict the site selectivity of halogenase variants toward different synthetic substrates, providing a valuable tool for implementing halogenases in biocatalysis efforts.

Introduction

Flavin-dependent halogenases (FDHs) natively catalyze selective halogenation of electron-rich aromatic compounds.¹ The utility of these enzymes stems from their unique mechanism, which involves the reaction of FDH-bound reduced flavin with O₂ to form a peroxyflavin species that reacts with proximally-bound chloride or bromide to generate HOX (X = Cl, Br).² This species migrates through the enzyme to the substrate binding site, where it forms a persistent intermediate thought to be a lysine-derived haloamine.³ It is not known if this intermediate directly halogenates substrates³ or serves as a reservoir of X⁺ that can be used to reform HOX upon reaction with water⁴. In either case, halogenation proceeds via electrophilic aromatic substitution (EAS). Although conventional EAS requires harsh oxidants to generate reactive halonium (X⁺) formal equivalents,⁵ FDHs use atmospheric oxygen and halide salts. Moreover, whereas EAS is typically subject to substrate-controlled site selectivity, substrate binding within an FDH can override substrate-based selectivity (Figure 1A/B),^{6,7} providing remarkable examples of catalyst control on a range of substrates^{8,9}.

Site selectivity is only useful if it involves the site of interest. To assess the feasibility of tuning FDH selectivity, a number of groups have used targeted mutagenesis to alter the site of halogenation.¹⁰ We used directed evolution to engineer variants of the FDH RebH that halogenate the C7, C6, and C5 positions of tryptamine with >90% selectivity (0S, 8F, and 10S, Figure 1C).¹¹ We also established that these enzymes possess high activity and selectivity on a range of non-native substrates and therefore became interested in developing a general approach to predict FDH

selectivity. Docking calculations revealed multiple substrate binding modes, only some of which were consistent with observed selectivity.⁸ This inability to reliably predict relevant binding modes indicated that a more sophisticated approach was required to model FDH selectivity.

Figure 1. A) Substrate positioning within FDHs controls EAS selectivity. B) Selective EAS catalysis requires an active site lysine residue thought to form a reactive chloramine intermediate or activate HOCl. C) Directed evolution was used to engineer RebH variants that halogenate tryptamine at C5, C6, or C7 with high site selectivity.

We reasoned that 0S, 8F, and 10S, which possess only 5-10 mutations relative to one another, would facilitate comparative analysis of FDH selectivity since wild type enzymes with analogous selectivity preferences differ by hundreds of residues.¹ Steady state kinetic parameters, crystal structures, and reversion mutations were used to identify mutations responsible for the observed changes in 0S, 8F, and 10S activity and selectivity. To accurately model the selectivity of these mutations, we computationally evaluated the energetic feasibility of both chloramine and HOCl halogenating agents and then established whether FDH selectivity could be reliably predicted using molecular dynamics (MD) simulations involving energetically relevant species in analogy

to recent studies on native FDH/substrate pairs^{12,13}. This approach provided insight into the mechanisms by which mutations in the RebH scaffold alter binding and selectivity on tryptamine and added support to the case that HOCl is the active halogenating species in FDH catalysis. Moreover, we established that DFT calculations paired with docking calculations and MD simulations involving this species constitute a framework for accurately predicting FDH site selectivity on non-native substrates, improving the utility of these enzymes for biocatalysis.

Results

Reversion of Mutations in Evolved RebH Variants

Relative to RebH, variants 0S, 8F, and 10S possess 1-11 total mutations that increase k_{cat} by 1.2-5.9 fold but significantly increase K_{M} for 8F and 10S (Table 1).¹¹ Each of the mutations in 10S and 8F was individually reverted to the wild type residue, and the activity and selectivity of each reversion variant toward tryptamine was evaluated (Figure 2).

Table 1. Kinetic parameters of RebH and variants.¹¹

Enzyme	K_{M} (μM)	k_{cat} (min^{-1})	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min } \mu\text{M})^{-1}$	Number of Mutations
RebH	9	0.023	2.6×10^{-3}	0
0S	10.6	0.135	2.6×10^{-2}	1
8F	1747	0.037	2.1×10^{-5}	11
10S	160	0.028	1.8×10^{-4}	6

The simplest RebH variant in the selectivity lineage, 0S, contains a single point mutation (N470S) that gives rise to a 6-fold increase in k_{cat} with minimal change in K_{M} and selectivity for C7 (>99%). To establish if the beneficial effects of S470 only result from its smaller size, S470A was introduced into 0S. Although an increase in conversion compared to RebH was observed for this

variant, 10S still outperformed S470A by ~3-fold. The N470S mutation is present in all three enzymes studied, and its reversion also led to reduced yields in 10S and 8F (Figure 2). N470A was also introduced into 10S and 8F, and reduced activity relative to 10S (Figure 2, ~7-fold reduced) and 8F (~3.5-fold reduced) were again observed.

Variant 10S contains 6 mutations that enable it to halogenate tryptamine with 95% selectivity for C5. Reverting these mutations (Figure 2) showed that active site residues H52 and C465 are primarily responsible for the observed selectivity of 10S. H52F led to a modest shift in selectivity relative to 10S, indicating that H52 acts as more than a simple aromatic residue. The loss in activity for the C465S variant similarly indicates that C465 acts as more than just a small replacement for the native Phe residue. Previous mutagenesis studies revealed that introducing only I52H and F465C into RebH abolished its activity.¹¹ By increasing the activity of 10S, N470S and R509Q, the latter of which is not in the active site, enable detection of chlorinated product.

Variant 8F, which catalyzes 6-chlorination of tryptamine with 90% selectivity, contains 11 mutations. Individually reverting each of these mutations (Figure 2) again revealed that residues 52 and 465 influence selectivity to the greatest extent, but P110S also affects the site selectivity of this variant. Interestingly, the S110P mutation provided improved conversion, not altered selectivity, when it was originally identified. While several reversions reduced 8F activity, the largest effect was observed for P448S, which was identified because it altered selectivity at the expense of conversion. The effects of S110P and S448P on 8F activity and selectivity are all the more notable since both were in a progenitor to 10S but were removed after it was found that they had no effect on 10S itself.

Figure 2. The locations of the 11 sites mutated during the evolutionary campaign to alter site selectivity are shown as spheres within the RebH crystal structure (PDB ID: 2OA1)¹⁴. Dark grey residues were found to have no effect on enzyme activity or selectivity. Black residues were found to impact activity and/or selectivity. FAD and L-Trp are shown as red sticks. Zoom-in on active site is shown in the boxed panel to the right. Graphs indicate activity and selectivity of reversion mutations. All variants are reversions except 10S-S470A, 10S-H52F, and 10S-C465S. N=3.

Crystal Structures of the 0S-Tryptamine-FAD, 10S-FAD, and 8F-FAD Complexes

The structures of 0S, 8F, and 10S were solved to resolutions of 2.6, 2.25, and 2.55 Å (PDB IDs: 7JU0, 6P00, and 6P2V), respectively. Aligning each structure to that of RebH (PDB ID: 2OA1)¹⁴ revealed RMS values of 0.167, 0.316, and 0.181 Å² for 0S, 8F, and 10S, respectively, indicating that subtle changes in structure are responsible for the observed changes in selectivity. Consistent with previous structures, the asymmetric unit contained two protein molecules. Occupancy of different ligands typically observed in FDH structures (FAD and halogenation substrate) varied in the structures obtained. In all structures, electron density for the adenine base and phosphate moieties of FAD was observed, but the isoalloxazine ring showed very little density. The occupancy of the loop comprised of residues 449-455, often disordered in previous RebH structures,^{3,14} also varied in the structures obtained.

In the structure of 0S, residues 2-528 were included in chain A and residues 2-529 in chain B with no missing regions in between (530 residues for full length enzyme). Density in the composite omit map was observed for the loop consisting of residues 449-455. Though crystals of 0S, 8F, and 10S were soaked in a solution containing tryptamine, occupancy for this substrate was only observed for 0S. This finding is consistent with the much lower K_M for tryptamine of 0S (10.6 μM) relative to 10S and 8F (160 and 1747 μM , respectively). Tryptamine was found to bind in the active site of 0S in a manner analogous to how L-tryptophan binds to RebH (Figure 3A). A hydrogen-bonding network between S470, H109, and E357, which is reminiscent of the catalytic triad found in serine hydrolases¹⁵ and could play a role in ordering the active site, is also observed in the 0S structure but not the RebH structure, which contains N470.

Figure 3. Crystal structures of variants 0S (red), 10S (blue), and 8F (green) overlaid with RebH (grey, PDB ID: 2OA1). A) Tryptamine bound in active site of 0S. B) H52 in 10S clashes with L-Trp in RebH. C) Mutations affecting 8F selectivity are shown as darker green spheres. Mutations affecting only 8F activity are shown as light green spheres. D) 8F mutation P110 alters 8F selectivity and activity, perhaps by adding rigidity to neighboring aromatic residues.

The 10S structure consisted of residues 2-528 in chain A and residues 2-529 in chain B with the loop between residues 451-455 omitted in both chains. Although density is observed within this

region, the main chain cannot be reliably modeled within this density. Numerous attempts were made to soak tryptamine into 10S crystals; however, even with high concentrations of tryptamine (>10 mM), no clear density for tryptamine was observed within the active site. Overlaying the structures of 10S and RebH containing L-Trp substrate shows a steric clash between H52 in 10S and L-Trp, suggesting that the combination of H52 and C465 must significantly alter substrate binding (Figure 3B).

The 8F structure consisted of residues 1-527 in chain A and residues 2-529 in chain B, but the loop between residues 447-456 was omitted in both chains. Seven mutations played a significant role in 8F (Figure 3C). Similar to 10S, when the structure of RebH containing L-Trp is overlaid with 8F, a steric clash is observed between N1 and C2 of the indole moiety of substrate and the sulfur atom of M52 (3.1 and 2.6 Å, respectively). L465 opens the active site, potentially allowing substrate to rotate or flip to accommodate the M52 mutation. P110 is flanked by two aromatic residues, H109 and F111, which sandwich tryptophan bound in the overlaid RebH structure (Figure 3D).

Computational Comparison of Possible Halogenating Species

As previously noted, a lysine-derived chloramine or bound HOCl, both of undetermined protonation state, have been proposed as the active halogenating species during FDH-catalyzed arene halogenation.^{3,4} In either case, aromatic halogenation is believed to occur via EAS involving rate-limiting electrophilic attack to form a Wheland intermediate followed by low-barrier proton loss to form the aromatic product. The lack of a deuterium KIE for RebH-catalyzed tryptophan halogenation is consistent with this mechanism.¹¹ The ΔG° for formation of the Wheland intermediate ($\Delta G^\circ_{\text{Whel}}$) has been found to correlate strongly with $\Delta G^\ddagger$ for this reaction.¹⁶ We

therefore reasoned that $\Delta G^\circ_{\text{Whel}}$ values calculated by density functional theory (DFT) could provide insight into feasibility of different proposed halogenating agents within FDHs. Scheme 1 and Table 2 show the calculated $\Delta G^\circ_{\text{Whel}}$ using several possible halogen donors for EAS at the the 7-position of indole.

Very high $\Delta G^\circ_{\text{Whel}}$ values were calculated for reactions involving neutral methylchloramine and HOCl (72.4 and 21.2 kcal/mol; Table 2, Entries 1 and 2), making chlorination by these species unlikely. Protonation of either lowers these $\Delta G^\circ_{\text{Whel}}$ values considerably (Table 2, Entries 3 and 4) due to the increased electrophilicity of the chlorine. The extremely negative $\Delta G^\circ_{\text{rxn}}$ value calculated for H_2OCl^+ calls into question the existence of such a highly reactive species in an enzyme. Instead, it has been suggested that K79 transiently protonates HOCl during the formation of the Wheland intermediate,¹² resulting in a slightly negative $\Delta G^\circ_{\text{Whel}}$ (Table 2, Entry 5). Taken together, these energies support two possible chlorinating agents: a positively charged lysine-bound chloramine at K79, and HOCl with general acid catalysis by K79^{2,4,12}.

Scheme 1. Electrophilic attack of different halonium donors on the 7-position of indole.

Table 2. Thermodynamic parameters for electrophilic attack of different Cl^+ donors on indole.

Entry	Donor-Cl	Donor ⁻	$\Delta G^\circ_{\text{Whel}}$
1	CH_3NHCl	CH_3NH^-	72.4
2	HOCl	HO^-	21.2
3	$\text{CH}_3\text{NH}_2\text{Cl}^+$	CH_3NH_2	1.7
4	H_2OCl^+	H_2O	-57.8
5	$\text{HOCl} + \text{CH}_3\text{NH}_3^+$	$\text{H}_2\text{O} + \text{CH}_3\text{NH}_2$	-3.3

Figure 4. MD simulations with different chlorinating species. A) RebH (blue, PDB ID: 2OA1) with a chlorine covalently bound to K79. Different C-Cl distances are shown (right) over the course of a representative 500 ns simulation. Long distances are observed for each carbon, and these distances are highly variable between replicas. B) RebH with a HOCl docked into the active site and loosely constrained to K79. Much shorter distances are observed, and these are consistent across replicas.

Because DFT calculations suggested that two species were plausible halogenating intermediates, we next sought to compare these models using molecular dynamics (MD) simulations of tryptamine binding to RebH. Two systems were prepared to represent the two possible reactant complexes determined by DFT: one with the chlorine covalently bound to K79 (K79-Cl, Figure 4a), and one with HOCl held close to K79 (K79-HOCl, Figure 4b)¹³ using restraints in AMBER (see methods). Each simulation was run in pentaplicate, and the distances between the chlorine and substrate carbons were measured (Figure S1). Consistently long C-Cl distances are observed with K79-Cl, and these distances are highly variable between replicas. In contrast, much shorter C-Cl distances are observed with K79-HOCl, and these distances were consistent across all replicas, as observed in simulations of tryptophan binding in PrnA and PyrH¹³. In the current simulations, the chlorine of HOCl remains closer to C7 than either C6 or C5, which is consistent with the experimentally observed selectivity of the enzyme. These simulations suggest that FDH

catalysis likely involves halogenation by HOCl rather than a chloramine and that accounting for this distinction could improve predictive models of FDH selectivity.

Modeling Selectivity of RebH Variants

With a model for the reaction mechanism in hand, we next sought to investigate how mutations in 0S, 10S, and 8F give rise to the observed site selectivity of these variants. Transition state structures were calculated using DFT for the acid-catalyzed electrophilic attack at the 5-, 6- and 7-positions of indole (Figure 5). These transition states are all structurally similar. A proton is shared between the methylammonium nitrogen and the HOCl oxygen with a nearly linear N–H–O angle. Simultaneously, the chlorine is shared between the HOCl oxygen and indole carbon with a nearly linear O–Cl–C angle. Energetically, TS6 is most favorable with an activation energy of 18.2 kcal/mol with respect to the complex. TS5 was slightly higher in energy (19.0 kcal/mol) and TS7 was higher still (20.1 kcal/mol). The ability of RebH and 0S to chlorinate the least favorable C7 highlights the importance of substrate binding (i.e. catalyst control) for halogenation of indole-containing substrates¹⁰.

Figure 5. DFT calculated transition state structures for the acid-catalyzed chlorination of indole at the 5-, 6- and 7-positions. Geometric similarities between structures are highlighted. Chlorination at C6 is most favorable according to the calculated activation energy. Chlorination at C7 is least favorable.

Crystal structures of other FDH-tryptophan complexes show that each enzyme binds its substrate with the carbon to be halogenated closest to the active site lysine.^{2,3,14,17–19} RebH is no exception,

as C7 of tryptophan is closest to K79 in this structure.³ The indole ring is held in place by pi stacking with H109 and F111, and the amine extends upward to hydrogen bond with Y454, E461, and F465 (Figure 6). The crystal structure of the 0S-tryptamine complex shows that the N470S mutation does not affect this binding orientation, and the two substrates overlay well (Figure 6A). The 10S and 8F variants were not co-crystallized with their substrates, so tryptamine was docked into the active site of these crystal structures. Docked structures show that tryptamine binds to 10S and 8F in different orientations than that observed in RebH and 0S (Figure 6B, C). In these variants, the indole ring flips to hydrogen bond with the backbone of S110 (P110 in 8F), and different carbons are projected towards the catalytic K79. In 10S, C5 is closest to K79 with a N-C5 distance of 4.3 Å; in 8F, C6 is closest to K79 with a N-C6 distance of 3.9 Å. These orientations are consistent with solved crystal structures of PyrH, which selectively chlorinates the 5-position of tryptophan (Figure 6D)¹⁷. Thus, the crystal structure of the 0S-tryptamine complex and structures of tryptamine docked into 10S and 8F show that the evolved variants all follow the established trend that substrates bind with the halogenation site closest to the active site lysine.

Figure 6. Substrate binding poses in crystal structures and docking calculations. The wild type RebH is shown in all images (blue) bound to tryptophan (green). A) Overlay of 0S binding tryptamine. B) Overlay of 10S binding tryptamine. C). Overlay of 8F binding tryptamine. D) Overlay of PyrH binding tryptophan.

As previously noted, a correlation between halogenation site and proximity of that site to the active site lysine of RebH was not observed in docking simulations of more structurally diverse substrates. We therefore sought to determine if MD simulations using HOCl-bound models of FDH variants could provide improved predictive value in this regard. Simulations of RebH in complex with HOCl and tryptamine show that the chlorine remains closer to C7 than either C5 or C6 for most of the simulation (Figure S2, Table S2), and simulations of 0S show similar behavior. Likewise, previously reported simulations of tryptophan binding in PrnA and PyrH in complex with HOCl show that chlorine remains closer to C7 and C5, respectively, in these systems.¹³ These results show that FDHs bind their substrates in an orientation that not only places halogenation sites proximal to the active site lysine, but also appears consistent with a near attack conformation (NAC),²⁰ a reactant state conformation that converts directly to the transition state. Simulations of 8F and 10S were less clear; the carbon closest to chlorine changed frequently, and considerably longer C-Cl distances were observed. These observations are consistent with the higher K_M values experimentally measured for these variants. We reasoned that these C-Cl distances, which are significantly longer than those in the calculated transition state structures, are likely not relevant to the observed selectivity.

To precisely define a NAC for halogenation at each site of tryptamine, intrinsic reaction coordinate calculations were performed on the respective calculated transition state structures, and the reactant complexes were optimized by DFT. The resulting complexes show that a C-Cl distance of 3.0-3.1 Å is ideal for NAC formation. Distances this short were rarely, if ever, observed in MD simulations using conventional force fields, so this threshold was extended to include any distance below 3.6 Å. The free energy of NAC formation (ΔG_N°) in the enzyme can then be estimated by calculating the relative mole fraction of enzyme-NAC relative to enzyme-ground state in each

simulation. Calculated ΔG_N° values provide a quantitative comparison to intrinsic $\Delta G_{TS}^\ddagger$ calculated by DFT.

Table 3. Substrate ($\Delta G_{TS}^\ddagger$) vs catalyst (ΔG_N°) control over site selectivity of tryptamine in RebH and mutants

FDH	C _n	$\Delta G_{TS}^\ddagger$ ^a (kcal/mol)	ΔG_N° ^b (kcal/mol)	$\Delta \Delta G_{TS}^\ddagger$ (kcal/mol)	$\Delta \Delta G_N^\circ$ (kcal/mol)	Obs. ^c (%)
RebH	5	19.0	-	0.8	-	0
	6	18.2	2.2	0.0	1.9	0
	7	20.1	0.3	1.9	0.0	99
0S	5	19.0	4.2	0.8	3.5	0
	6	18.2	2.8	0.0	2.1	0
	7	20.1	0.7	1.9	0.0	99
8F	5	19.0	2.2	0.8	0.0	11
	6	18.2	2.3	0.0	0.1	89
	7	20.1	4.0	1.9	1.8	0
10S	5	19.0	4.2	0.8	0.0	94
	6	18.2	4.2	0.0	0.0	6
	7	20.1	-	1.9	-	0

^aTransition state free energy barrier for chlorination in solution calculated by DFT. ^bFree energy of NAC formation in enzyme estimated by MD simulations. Empty values indicate no NAC is formed at this site. ^cPercent of different isomers as determined by ¹H NMR spectroscopy

Using this approach, preferential NAC formation can be observed at C7 for RebH and 0S (Table 3 and S3). This site is favored by roughly 2 kcal/mol in these enzymes, which contrasts with the 1.9 kcal/mol the site is disfavored from DFT-calculated $\Delta G_{TS}^\ddagger$ values. This remarkable similarity between values obtained by MD simulations and DFT calculations show that differences in NAC formation can be used to gauge the enzymes' ability to override intrinsic site selectivity of the reaction. In the variants 8F and 10S, there is a clear shift away from NAC formation at C7. Instead, NAC formation is roughly equivalent for both C5 and C6 in these enzymes. While preferential NAC formation at C5 is slightly underestimated in 10S, this metric provides a useful explanation for why these variants lack selectivity for the original halogenation site and correctly identifies two other sites where NAC formation is preferential.

Predicting Site Selectivity in Non-native Substrates Beyond Tryptamine

Given successful recapitulation of RebH selectivity toward tryptamine using the methods described above, we next sought to evaluate their efficacy toward a subset of the compounds explored in our previous substrate-activity profiling effort.⁸ Relative free energies of Wheland intermediate formation ($\Delta\Delta G_{\text{whel}}$) were calculated at each sp^2 hybridized carbon to gauge the intrinsic site selectivity for chlorination (Table 4). As previously noted, Wheland intermediate energies have been shown to correlate strongly with transition state barrier heights, while being easier to determine computationally. Comparison of $\Delta\Delta G_{\text{whel}}$ on various sites of the indole ring confirm this to be the case (Figure S3). For many of the substrates, including **1**, **2**, **4**, **6**, and **7**, chlorination is observed at the most reactive carbon (lowest $\Delta\Delta G_{\text{whel}}$), suggesting that the enzyme has little catalyst control over most non-native substrates. This is not always the case (e.g. substrates **3** and **5**), so further modeling of the enzyme is needed to reliably predict chlorination sites.

Docking the substrates into the RebH active site yielded several low-energy poses for each (Figures S4-S10). These different binding orientations often projected different halogenation sites towards the catalytic K79, making it difficult to predict the halogenation site based on these structures alone. In every case, docking poses that were consistent with experimentally-determined selectivity were observed, where the carbon to be halogenated is closest to K79. In the docking poses inconsistent with observed selectivity, a carbon predicted to be unreactive by DFT ($\Delta\Delta G_{\text{whel}} > 15$ kcal/mol) was closest to K79. Although such poses may exist in the enzyme, it is unlikely that substrate binding can overcome these differences in reactivity. Selectivity on the native substrate, tryptamine, occurs on a site disfavored by 11.2 kcal/mol (C7 vs. C2), and it is unlikely RebH has a higher degree of catalyst control over any non-native substrate. Limiting our search to

only include reactive poses (where a site with $\Delta\Delta G_{\text{whel}} < 11$ kcal/mol is closest to K79) the observed halogenation site was always the site closest to K79.

Table 4. Substrate ($\Delta\Delta G_{\text{whel}}$) vs catalyst ($\Delta\Delta G_{\text{N}}^{\circ}$) control over site selectivity of non-native substrates in RebH

Entry	Compound	C _n	$\Delta\Delta G_{\text{whel}}^{\text{a}}$ (kcal/mol)	$\Delta\Delta G_{\text{N}}^{\circ \text{b}}$ (kcal/mol)	Obs. ^c (%)
1		1	5.8	-	0
		2	20.9	2.4	0
		3	0.0	0.0	100
		8	2.7	-	0
2		1	0.0	-	100
		3	0.4	0.0	0
		4	29.1	4.1	0
3		1	1.8	0.0	100
		3	5.1	-	0
		5	0.0	-	0
		6	34.4	1.6	0
		8	28.7	-	0
		10	48.0	-	0
4		1	0.0	0.3	100
		3	18.7	0.0	0
		4	26.6	2.3	0
		7	20.6	-	0
		8	29.7	-	0
		9	15.2	-	0
5		1	25.2	-	0
		5	26.0	-	0
		7	0.0	-	0
		9	6.2	-	100
6		1	0.0	0.0	93
		3	0.7	-	7
		6	20.8	2.5	0
7		1	1.7	-	6
		3	0.0	0.0	94
		4	35.4	-	0

^aRelative free energy of Wheland intermediate formation at the given site calculated by DFT.

^bRelative free energy of NAC formation in RebH estimated by MD simulations. Empty values indicate no NAC is formed at this site. ^cPercent of different isomers as determined by ¹H NMR spectroscopy

Finally, MD simulations were conducted to quantify catalyst control in the HOCl-bound enzyme (Table 4 and S4). For many of the substrates, including **1**, **3**, **6** and **7**, NAC formation was favored at the observed halogenation site. Moreover, in substrate **3**, preferential NAC formation correctly predicts chlorination at C3 over C5, the most reactive site calculated by DFT. Substrate **4** shows similar NAC formation for both C1 and C3; however, differences in intrinsic reactivity make C1 the only possible halogenation site for this substrate. Halogenation could not be correctly predicted with this method for two of the substrates tested. In substrate **2**, NAC formation predicts chlorination at C3, yet no chlorination is experimentally observed at this site. Instead, chlorination is observed at C1, which is calculated to be the most reactive site. For substrate **5**, poor substrate binding was observed, and no NACs were formed at any site. In this case, chlorination is observed at C9, which is less reactive than C7 but closer to K79 in docking calculations.

Discussion

Molecular Insight into the Altered Selectivity of Evolved FDHs

The crystal structures of 0S, 8F, and 10S, reversion data for mutations in these enzymes, and our NAC approach to model selectivity was used to rationalize the effects of mutations identified via directed evolution. The crystal structure of 0S, for example, shows that the only mutation in this enzyme (N470S) is located within the active site but does not contact the substrate. Though the smaller size of serine relative to asparagine could facilitate substrate entry, it is unclear why this would increase k_{cat} nearly 6-fold relative to WT RebH. Mutating this residue to alanine did not fully account for the increase in conversion, suggesting that the hydroxyl group of serine, rather than its small size, is important for the improved function of 0S. As previously noted, this residue forms a hydrogen-bond network with H109 and E357 that is geometrically similar to that found in

serine hydrolases. This same triad is present in 10S and 8F, thus providing a rationale for why the benefit of N470S could carry over to those enzymes as well. MD simulations show that H109 forms π -stacking interactions with the indole core of tryptamine and E357 hydrogen bonds to the indole N-H. By helping to order these residues, S470 could better orient the substrate relative to bound HOCl to facilitate halogenation.

Four of the six mutations in 10S are critical to its altered activity (N470S and R509Q) and selectivity (I52H and F465C). A potential explanation for the role of N470S was noted above, but the importance of R509Q cannot be gleaned from either the crystal structure or the MD simulations for 10S. Overlaying the structures of 10S and RebH with bound L-tryptophan (PDB ID: 2OA1, Figure 3B) suggests that I52H sterically restricts one region of the active site whereas F465C expands a different section. Docking calculations show that 10S binds the indole moiety in a different orientation than RebH (Figure 6B). Moreover, H-bonding of H52 to E357 leads to closer proximity of these residues, which provides space for the substrate to rotate into an orientation that enables C5 halogenation (Figure 7A). Consistent with this hypothesis, variant H52F possesses reduced selectivity for C5 (85.5% vs 93.7%) perhaps due to its inability to H-bond to E357. The activity of variant 10S C465S was also examined to establish if the H-bonding capability of C465 is responsible for the improvement it conveyed. Site selectivity is not affected by this mutation, but a substantial decrease in conversion is observed relative to 10S (nearly 8-fold, Figure 2A). It is possible that both C465 and S465 open the active site, allowing for new tryptamine binding poses which do not clash with H52 (presumably which place the C5 proximal to K79).

Figure 10. Tryptamine binding for A) 10S and B) 8F obtained from docking calculations.

Although several residues play a significant role in the activity and selectivity of 8F, reversions P110S and P448S have the largest effects on 8F activity. P110 could mediate π -stacking between bound substrate and adjacent residues H109 and F111 as well as hydrogen bond with the indole N-H of tryptophan to favor the flipped substrate orientation. Both P110 and P448 could be rigidifying the active site when substrate is bound, which has been proposed to increase activity in FDHs.¹³ Mutations at residues 52 and 465 again largely determine the observed selectivity of 8F (and M52I and L465F). Just as in 10S, mutation of these residues to methionine and leucine would be expected to favor the flipped substrate binding conformation based on their sizes relative to the WT residues, and this is supported by docking calculations (Figure 10B). In 8F, however, E357 is pointing more to the 6-position of bound tryptamine due steric repulsion from the bulky methionine residue.

Comparing the effects of critical mutations in 10S and 8F to those present in natural halogenases reveals interesting parallels. First, the three reported 5-tryptophan halogenases possess F52,^{21–23} which, as noted above, was nearly as effective as H52 in the engineered tryptamine 5-halogenase 10S. Notably, O'Connor and coworkers showed that introducing I52F into RebH results in an inactive variant.²⁴ Introducing I52H both alone and in combination with F465C into RebH also resulted in inactive variants in our hands, indicating that while an aromatic residue at this position

can lead to a 5-halogenase, other mutations are required to enable its effectiveness in RebH. Previously reported 6-tryptophan halogenases possess either V52 and greater sequence similarity to 7-tryptophan halogenases^{18,19} or F52 and more sequence similarity to 5-tryptophan halogenases²⁵⁻²⁷. Here, 8F adopted a different solution (I52M) than that observed in nature. Similarly, all known 5- and 6-Trp-FDHs contain proline at the residue corresponding to residue 110 in RebH. While this solution was also used in the tryptamine halogenase 8F, it was not in the 5-halogenase 10S. On the other hand, all known 5- and 6-Trp-FDHs contain serine at the site corresponding to residue 470 in RebH, and all 7-Trp-FDHs contain asparagine, yet S470 increases the catalytic activity of 0S without changing its selectivity. In short, FDHs can find many solutions to selectivity problems, some of which are similar to those found via natural selection and some of which are not,²⁸ and the natural solutions do not necessarily work in an unnatural context.

A Computational Model for FDH Selectivity

Both HOCl and an active site lysine-derived haloamine have been proposed as the active halogenating species in FDH catalysis.^{3,4} DFT calculations suggest halogenation by either of these species, when protonated, is exergonic. Calculated transition state energies show that K79 can activate HOCl by general acid catalysis, and this finding is consistent with mutagenesis experiments that show K79 is essential⁴. Furthermore, the catalytic lysine of many halogenases has been calculated to be much more acidic than free lysine in solution^{29,30}. We calculate pK_a values of 7.1, 7.0, 6.9, and 6.9 for K79 in RebH, 0S, 10S, and 8F, respectively. These low pK_a values result largely from hydrophobic residues, including A50, I52, I82, and F111, that are proximal to K79, and suggest that at physiological pH, K79 can readily donate a proton to HOCl to assist in halogenation. MD simulations of wild type RebH using K79-HOCl also give much

shorter C-Cl distances than K79-Cl, and these distances are consistent with the observed selectivity. Taken together, these data suggest that chlorination by HOCl provides a better model for site selectivity in RebH than a lysine-derived chloramine.

Calculated transition state energies show that chlorination of tryptamine at C6 is most favored and chlorination at C7 is least favored. Nevertheless, RebH and 0S chlorinate C7 with high selectivity, which highlights the importance of substrate binding. Indeed, all tryptophan FDHs crystalized with substrates to date bind their substrates in orientations that project the C_{sp2}-H bond to be halogenated proximal to the active site lysine residue.^{2,3,14,17-19} Although 10S and 8F were not crystallized with substrate, docking calculations show these enzymes also bind tryptamine following this trend. MD simulations using HOCl show that each enzyme preferentially forms near attack conformations for the observed halogenation site. Comparison of $\Delta\Delta G_{N^\circ}$ values determined by MD simulations to $\Delta\Delta G_{TS}^\ddagger$ values calculated by DFT shows that preferential NAC formation accounts for almost all the catalyst control over the reaction.

Predicting Selectivity on Other Substrates

Beyond providing insights into the selectivity of 0S, 8F, and 10S toward tryptamine, computational tools also provided improved ability to predict the selectivity of other non-native FDH-substrate pairs. We previously attempted to use docking calculations alone to model tryptamine binding in 0S, 10S, and 8F¹¹ and to model the binding poses of a panel of substrates in RebH⁸. In both cases, poses consistent with the observed halogenation selectivity were observed, but so too were poses inconsistent with the observed selectivity and scope.

Calculating the relative energies of Wheland intermediates provided a wealth of predictive ability. Similar metrics, like RegioSQM,³¹ and HalA,³² have been used previously, and we find that these

methods perform comparably (Figure S12). For almost every substrate, the site identified as most reactive was the experimentally observed halogenation site. This is perhaps unsurprising, as these enzymes would not necessarily be expected to exhibit high catalyst control over reactions involving non-native substrates. Docking provided additional insight into substrate-enzyme interactions. As with our previous studies,^{8,11} many docking poses could be identified, and each projected a different halogenation site proximal to K79. Including the $\Delta\Delta G_{\text{whel}}$ values calculated from DFT allowed us to exclude docking poses that do not represent catalytically active binding conformations (i.e. NACs) and provided a much better predictor for site selectivity. Furthermore, MD simulations show that NAC formation can provide a quantitative comparison between the intrinsic site selectivity of the substrate and the catalyst control of the enzyme.

Density functional theory calculations, docking, and molecular dynamics simulations all provided insight into site selectivity, but no one method was able to reliably predict the halogenation site every time. Instead, the three methods used together provide a more complete picture for substrate and catalyst control over the reaction to identify sites where halogenation is likely. Perfect predictive ability, while ideal, is not completely necessary for a model to provide value. The ability to predict one or a few sites out of many can identify regions of the substrate where chlorination is possible and can be paired with subsequent directed evolution to engineer highly selective catalysts for the desired site.

Conclusions

Improved models for rationalizing and predicting FDH selectivity toward aromatic substrates would substantially improve the utility of these enzymes for biocatalysis. Our recent finding that FDHs can also catalyze non-native olefin halocyclization suggests that such models would have

utility beyond even the native function of these enzymes.³³ We therefore evaluated the energetics of electrophilic aromatic substitution (EAS) by hypohalous acid and chloramine species that have been proposed as active halogenating intermediates in FDH catalysis. The first step of EAS is highly endergonic unless these species are protonated. A low pKa value was calculated for K79, which is more consistent with its role as a proton donor than chloramine source. Moreover, MD simulations showed that HOCl provided a better model for site selectivity in RebH.

Crystal structures and reversion mutations for three previously evolved RebH variants with orthogonal selectivity were next used to identify mutations that altered variant activity and selectivity. Notably, RebH variants containing only those mutations that led to large changes in selectivity in the evolved variants were inactive, highlighting the importance of permissive mutations, identified via random mutagenesis, for achieving the high selectivities observed. Because these enzymes only possess a handful of mutations, they constitute ideal test cases for predictive models of FDH selectivity. Indeed, docking calculations and MD simulations show that each enzyme binds tryptamine in an orientation that places the carbon to be chlorinated closest the catalytic lysine. This binding orientation allows the enzyme to overcome the intrinsic site selectivity that favors chlorination at the 6-position of tryptamine. Similar calculations also recapitulated the selectivity for several non-native FDH/substrate pairs, which provides a general framework for modeling and predicting FDH selectivity for biocatalysis efforts.

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‡These authors contributed equally. D.E., S.Y. and C.Q.H. carried out all computations, including DFT calculations, docking, and MD simulations with guidance from K.N.H. All experimental work was performed by M.C.A, A.M.G., J.V.C., and N.S under the direction of J.C.L. Manuscript was written by J.C.L, M.C.A., and D.E. with contributions from all other authors.

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