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Aromatic Halogenation by Using Bifunctional Flavin Reductase–Halogenase Fusion Enzymes

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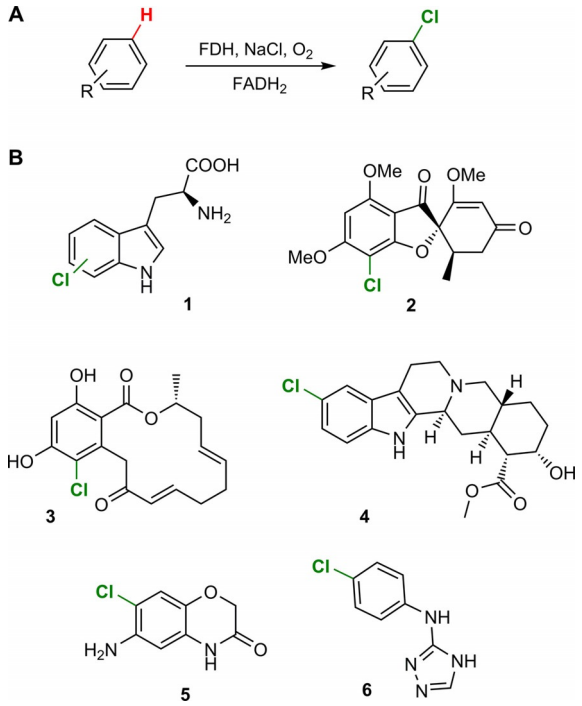
The remarkable site selectivity and broad substrate scope of flavin-dependent halogenases (FDHs) has led to much interest in their potential as biocatalysts. Multiple engineering efforts have demonstrated that FDHs can be tuned for non-native substrate scope and site selectivity. FDHs have also proven useful as in vivo biocatalysts and have been successfully incorporated into biosynthetic pathways to build new chlorinated aromatic compounds in several heterologous organisms. In both cases, reduced flavin cofactor, usually supplied by a separate flavin reductase (FR), is required. Herein, we report functional synthetic, fused FDH-FR proteins containing various FDHs and FRs joined by different linkers. We show that FDH-FR fusion proteins can increase product titers compared to the individual components for in vivo biocatalysis in *Escherichia coli*.

Halogenated aromatic compounds often exhibit unique biological activities and are thus commonly used as pharmaceutical drugs and agrochemicals.[1] Aryl halides are also valuable building blocks for synthetic chemistry, particularly because of their centrality to a range of powerful cross-coupling reactions.[2,3] Despite the importance of aromatic halogenation, however, common methods for aromatic halogenation, perhaps most notably electrophilic aromatic substitution, often suffer from poor regioselectivity.[4] More recent efforts have therefore explored the ability of directing groups to enable selective halogenation of proximal C–H bonds on suitably prefunctionalized substrates.[5,6]

Complementing these traditional synthetic methods, flavin-dependent halogenases (FDHs) have been shown to halogenate a range of electron-rich hetero(arenes) with high selectivity (Scheme 1).[7–9] FDH catalysis proceeds through an electrophilic halogen species (both a lysine-derived haloamine[10] and HOX[11] have been proposed), which, owing to its orientation relative to the bound substrate, can override electronic biases of different substrates to catalyze aromatic halogenation with novel regioselectivity.[12,13] Notably, FDH catalysis proceeds in aqueous solution at ambient temperature and requires only reduced flavin cofactor ($FADH_2$), sodium chloride as a halide source, and oxygen from air as a terminal oxidant. A cofactor regeneration system (CRS) comprising a flavin reductase, a NAD(P) oxidoreductase (e.g., glucose dehydrogenase), FAD, NAD(P), and a terminal reductant (e.g., glucose, the only stoichiometric reagent in the CRS) can be used to supply $FADH_2$.



Scheme 1. A) General scheme for chlorination by FDHs. B) Representative products from the chlorination of native FDH substrates **1**–**3** and non-native FDH substrates **4**–**6**.[14–17]

A number of efforts involving directed evolution and targeted mutagenesis have been used to engineer FDH variants with increased stability,[18,19] expanded substrate scope,[16,20] and altered regioselectivity.[21,22] In all of these efforts, a CRS analogous to that described above was used to ensure maximum product formation, which necessitates the purification of a suitable flavin reductase. Typically, *Escherichia coli* flavin reductase, Fre, or the native RebH (a 7-tryptophan FDH from the native organism *Lechevalieria aerocolonigenes*) partner RebF is used for in vitro halogenation assays.[20] Owing to low solubility of RebF if overexpressed in *E. coli*, a fusion of maltose binding protein and RebF (MBPF) is often used in place of RebF.[23] The requirement of flavin reductase (FR) can be tedious for directed evolution efforts, as sufficient reductase for thousands of reactions must be regularly prepared, purified, and quality tested. Of course, this requirement can be eliminated by co-(over)expressing genes for the reductase and halogenase either individually or as fusion enzymes. Genetic fusion of the flavin reductase and halogenase could also improve halogenation efficiency, particularly for in vivo applications, for which a

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high local concentration of reduced $FADH_2$ cannot necessarily be guaranteed.

The utility of in vivo FDH-catalyzed halogenation has been established in a number of different organisms.[15,24–29] For example, Rdc2 was used to halogenate phenolic compounds in *E. coli* without co-expressing a flavin reductase, as this organism contains naturally occurring flavin reductases.[15,30] Likewise, targeting FDH expression to plant chloroplasts (which have high levels of $FADH_2$) was shown to be sufficient to enable FDH catalysis in planta, but co-expression of a reductase was required if cytosolic expression was desired.[26] Regardless of whether endogenous reductases may be able to supply $FADH_2$, many studies have shown that increasing the local concentration of enzymes can increase flux through multistep enzymatic pathways.[31,32] Previous work demonstrated that Baeyer–Villiger monooxygenases could be genetically fused with $NADP^+$ reductases, which simplified cofactor regeneration.[33] Ferrodoxin and flavodoxin reductase-type domains could also be fused to cytochrome P450 heme domains to generate self-sufficient hydroxylation catalysts.[34–38] We therefore envisioned that an FDH-FR fusion enzyme could be useful for a wide range of in vitro and in vivo applications.

In vitro characterization of FDH-FR fusion enzymes

The genes encoding wild-type RebH and RebF were genetically fused by using three linkers based on sequences used to create the functional P450-reductase fusion enzymes noted above.[34,35] These linkers consisted of 10, 16, and 22 residues (Figure 1 A), and the corresponding fusion enzymes are referred to as H-10-F, H-16-F, and H-22-F. The fusion constructs were co-expressed with the pGro7 chaperone system in *E. coli* to afford 10–30 mg L^{-1} soluble protein following purification (Figure S2 in the Supporting Information). These yields are similar to those observed with the MBP-RebF fusion (33 mg L^{-1}) often used in FDH bioconversions; however, higher yields were observed upon expressing RebH with the chaperone system.[39]

The activities of both the reductase and halogenase domains of the fusion enzymes were next examined. Halogenase activity was established by comparing the yield of L-tryptophan chlorination catalyzed by RebH in the presence of MBPF to the yields for the same reaction catalyzed by H-10-F, H-16-F, and H-22-F (Figure 1 B). All three fusion enzymes retained substantial halogenase activity (Table 1, entries 2–4; 38–56% yield); however, the observed chlorination yields were lower than those obtained with RebH/MBPF (Table 1, entry 1; 90% yield). Chlorination yields were unaffected by differences in linker length and amino-acid composition between linkers 10 and 16 (55 and 56% yield, respectively), but the longest linker, 22, led to a lower yield (38%). Reductase activity was established by measuring NADH oxidation,[40] which occurred at similar rates for H-16-F and MBPF (turnover number, k_{cat} = 206 and 197 min^{-1}, respectively; Figures S3 and S4).

Table 1. Aromatic chlorination catalyzed by different FDH or FDH-FR fusion enzymes.[a]

	FDH	Substrate	[FDH] [μM]	Yield [%]
1	RebH	**7**	1.5	90.3
2	H-22-F	**7**	1.5	38.3
3	H-16-F	**7**	1.5	56.2
4	H-10-F	**7**	1.5	54.6
5	H-16-Fre	**7**	1.5	30.3
6	3SS	**8**	1	23.4
7	3SS-16-F	**8**	1	14.8
8	1K	**9**	1	38.2
9	1K-16-F	**9**	1	22.5
10	10S	**10**	25	26.3
11	10S-16-F	**10**	25	17.2

[a] Reaction conditions: 0.5 mM substrate, 1–25 μM FDH, 9 U mL^{-1} GDH, 10–100 mM NaCl, 20 mM glucose, 100 μM NAD and FAD, 25 mM HEPES buffer pH 7.4, 25 °C, 75 μL final reaction volume. Reductase (2.5 μM) was added to reactions that did not contain a fusion enzyme. Reactions were quenched with one volume MeOH. 0.5 mM phenol (for **7**, **8**, and **10**) or 0.5 mM benzoic acid (for **9**) was added as an internal standard, and reactions were analyzed by HPLC.

The apparent melting temperature (T_m) of H-16-F was also compared with that of RebH to determine if decreased stability, in addition to modestly decreased activity, might hinder its performance. Apparent T_m values of 49.5 and 44.9 °C were obtained for RebH and H-16-F, respectively, which indicated that reduced stability of the fusion could indeed be compromising H-16-F performance (Figure S5). Previously, a thermostable flavin reductase from *Bacillus subtilis* (Fre) was found to be compatible with FDH halogenation systems.[41] This thermostable Fre was therefore fused to RebH by using the 16-amino-acid linker with the goal of generating a fusion enzyme with increased stability. This resulting fusion enzyme, RebH-16-Fre, also expressed as a soluble protein, but it provided a lower yield for L-tryptophan chlorination (Table 1, entry 5; 30% yield), and its melting temperature (apparent T_m = 45 °C, Figure S5) was comparable to that of H-16-F.

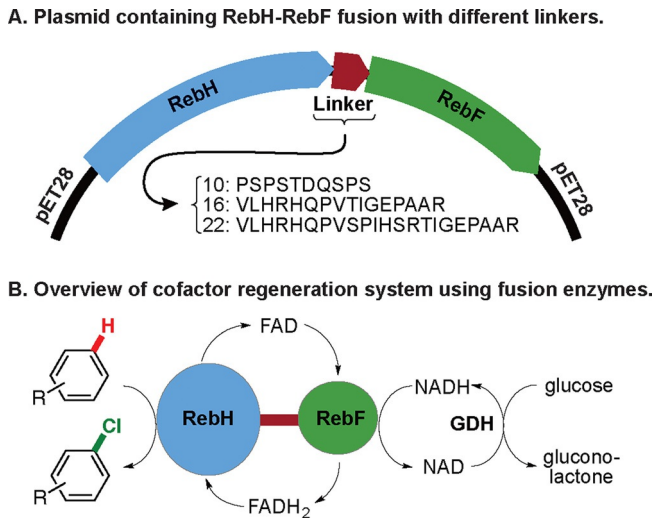
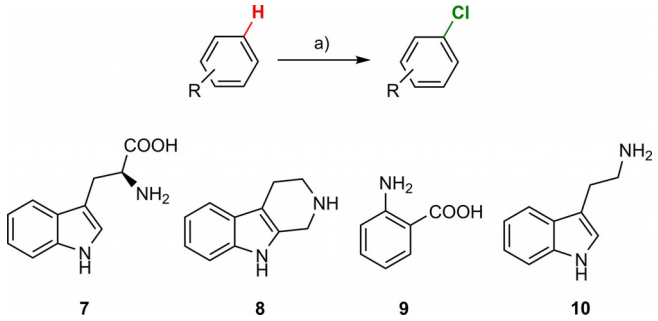


Figure 1. A) Fusion constructs encoded on a pET28 vector. B) Overview of cofactor regeneration by using fusion enzymes. GDH = glucose dehydrogenase.

Three RebH variants previously engineered in our laboratory, 1K,[42] 3SS,[16] and 10S,[22] were also fused to RebF through the 16-residue linker described above. These variants were engineered for altered substrate scope (1K-E461K + R231K and 3SS-S2P + M71V + G112S + K145M + N467T + N470S) and site selectivity (10S-I52H + L380F + F465C + N470S + Q494R + R509Q). Soluble protein was obtained for all FDH fusion enzymes, and bioconversions were conducted with the purified enzymes. As observed for H-16-F, all three FDH fusion enzymes retained activity for their respective substrates (Table 1, entries 7, 9, and 11), but the conversions observed were lower than those of the individual enzymes. Bioconversions using H-16-F, 1K-F, 3SS-F, and 10S-F were scaled up, and the site selectivity of chlorination was found to be the same as that for the corresponding two-component FDH system in all cases (see the Supporting Information and Scheme 2).



Scheme 2. General reaction scheme for in vitro reactions on substrates **7**–**10**. a) 0.2–5 mol% FDH cofactor regeneration system, 10–100 mM NaCl, 25 mM HEPES (5% DMSO), pH 7.4.

We sought to understand further the reduced yields of the fusion enzyme in vitro. No significant change in reductase activity was observed (see above), and only a slight decrease in the apparent melting temperature of H-16-F was observed relative to that of RebH. Although this difference in T_m could be responsible for the decreased yield, the time courses revealed that decreased catalyst lifetime was not observed. On the basis of these observations, we hypothesized that reduced kinetic parameters of halogenase activity of H-16-F was primarily responsible for the reduced yield. We therefore measured the steady-state halogenase kinetics for RebH and H-16-F. Because the Michaelis constant (K_m) of the RebH-catalyzed chlorination of L-tryptophan was low ($\approx$2 μM),[40] low substrate concentrations and correspondingly low FDH concentrations (0.007 μM) were required to ensure that the rates were measured at <10% conversion. For preparative FDH-catalyzed reactions, we generally found that 2.5 μM FR provided satisfactory product yields regardless of FDH concentration on the reaction scales we investigated.[16–18,22,39] Whereas this concentration represents an enormous excess amount of FR relative to the FDH concentrations noted above, we reasoned that supplementing bioconversions involving either RebH or H-16-F with 2.5 μM FR would allow for analysis of halogenase activity under conditions for which halogenase activity (rather than $FADH_2$ supply) limited overall product formation.[40,43] Under these conditions, similar K_m values were observed for both halogenases ($K_{m\text{-RebH}}$ and $K_{m\text{-H-16-F}} = 0.7$ μM); however, the k_{cat} observed for the H-16-F fusion was nearly two times lower than that observed for RebH ($k_{cat\text{-RebH}} = 4.26\ min^{-1}$ and $k_{cat\text{-H-16-F}} = 2.34\ min^{-1}$; Table 2, entries 1 and 2).

Table 2. Kinetic parameters for aromatic chlorination catalyzed by RebH and H-16-F.[a]

	FDH	[H-16-F] [μM]	[RebH] [μM]	[RebF] [μM]	k_{cat} [min^{-1}]	K_m [μM]
1	RebH	–	0.007	2.5	4.26 ± 0.13	0.7 ± 0.1
2	H-16-F	0.007	–	2.5	2.34 ± 0.07	0.7 ± 0.1
3	RebH	–	0.03	0.03	0.18 ± 0.01	0.7 ± 0.2
4	H-16-F	0.03	–	–	0.14 ± 0.01	0.9 ± 0.3

[a] Reaction conditions: 0.5–15 μM L-tryptophan, 0.007–0.03 μM FDH, 0.03–2.5 μM reductase, 9 U mL^{-1} GDH, 10 mM NaCl, 20 mM glucose, 100 μM NAD and FAD, 25 mM HEPES buffer pH 7.4, 25 °C, 75 μL final reaction volume. Reactions were quenched with MeOH 5–20 min after reaction initiation. Phenol (0.5 mM) was added as an internal standard, and reactions were analyzed by HPLC. Saturation plots used to calculate values can be found in the Supporting Information (Figures S8–S11).

Kinetic parameters were also obtained for H-16-F with no added reductase, conditions under which H-16-F was the only source of $FADH_2$. Interestingly, a much lower k_{cat} was observed under these conditions (0.14 min^{-1}; Table 2, entry 4) relative to those noted above. Presumably, because the concentration of FR was >80 times lower than that in previous experiments, the supply of $FADH_2$ to RebH, which involves both the rate of FAD reduction and uncatalyzed $FADH_2$ oxidation prior to RebH binding, could become rate limiting.[40,43] It is possible that the lower concentration of $FADH_2$ is insufficient to saturate RebH under the conditions used, which would lead to the lower rates of chlorination observed. To test whether the k_{cat} of the two-component system was also significantly affected by the concentration of reductase, steady-state kinetic parameters were obtained for RebH under analogous conditions, 1:1 RebH/MBPF. A lower k_{cat} was again observed (0.18 min^{-1}; Table 2, entry 3). Surprisingly, at this lower concentration of reductase (and thus lower concentration of $FADH_2$), there appeared to be little difference between the chlorination rates of RebH and H-16-F. This would suggest that the local concentration of reductase is important in the comparative halogenase activity of the single- and two-component systems; however, further detailed analysis will be necessary to understand this observation fully.

In addition to using FDH-FR fusion enzymes to facilitate directed evolution efforts, we also envisioned that they could provide higher product yields in whole-cell bioconversions. Our kinetic data support the hypothesis that as the reductase concentration decreases, the use of fusion to generate a higher local concentration of $FADH_2$ becomes more relevant. Within a cell, the local concentration of $FADH_2$ could greatly impact FDH activity. To test whether the fusion would outperform the two-component system in vivo, we examined the hal-

ogenase activity of *E. coli* BL21 pGro7 cells transformed with H-16-F, RebH, RebH + RebF, and RebH + MBPF (note that RebH + RebF and RebH + MBPF cells contain these two enzymes on two separate plasmids). Cultures were grown to an OD_{600} of 0.85–0.9, isopropyl-β-D-thiogalactopyranoside (IPTG; 100 μM) and L-arabinose (2 mg mL^{-1}) were added to induce halogenase expression, and solutions of L-tryptophan (**7**, 1 mM) and NaCl (100 mM) were added to initiate the halogenation reactions. High titers of 7-chlorotryptophan were obtained following incubation at 30 °C for 24 h, and a 2.5-fold increase in product concentration was observed for cells containing H-16-F relative to cells containing RebH, RebH + RebF, or RebH + MBPF (Figure 2). Excited by this result, we sought to demonstrate in

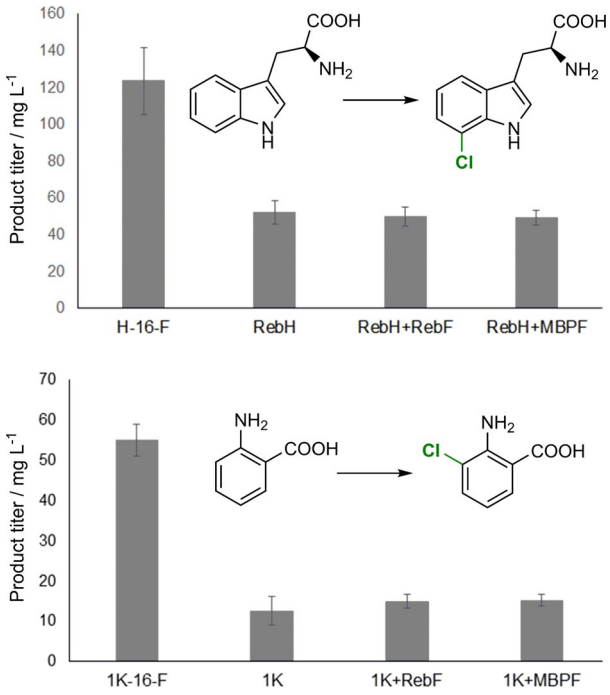


Figure 2. In vivo biocatalysis with H-16-F and 1K-16-F to afford chlorinated L-tryptophan (**7**) and anthranilic acid (**9**), respectively. Upon induction of expression of 50 mL cultures in terrific broth (TB) medium, 1 mM substrate and 100 mM NaCl were added. Cultures were expressed for 24 h at 30 °C, and aliquots of the supernatant were analyzed by HPLC. Three independent trials of triplicate cultures were performed for each cell line, and resulting standard deviations are shown as error bars ($n = 9$).

vivo chlorination on a non-native substrate. Because *E. coli* cells contain significant quantities of L-tryptophan, we used an engineered FDH that does not halogenate L-tryptophan. Variant 1K was found to have greatly reduced activity on L-tryptophan, and in competition reactions between L-tryptophan and anthranilic acid (**9**), no conversion of tryptophan was observed (see the Supporting Information). Significantly higher titers (55.01 mg L^{-1} with 1K-16-F, 15.21 mg L^{-1} with 1K + MBPF) were obtained with this enzyme in vivo (Figure 2).

Because lower yields of purified, soluble enzyme were produced for in vitro studies by using the fusion FDH-FRs, we anticipated that the observed increase in product formation in vivo was the result of a higher local concentration of $FADH_2$, not increased fusion expression relative to the corresponding FDHs. An SDS-PAGE gel and a western blot for the soluble fractions of in vivo bioconversions confirmed low levels of soluble fusion expression for H-16-F compared to RebH (Figures S12–S13). Although this does not unequivocally confirm our hypothesis, it does qualitatively suggest that increased enzyme expression is not responsible for high product titers in vivo by using the fusion system.

In summary, we demonstrated that functional FDH-FR fusion enzymes could be engineered by using different linkers, FDHs, and reductases. Although a slight reduction in activity was observed for these enzymes relative to that observed for their corresponding two-component systems in vitro, the use of fusion enzymes could simplify FDH engineering efforts by eliminating the need for added FR. In addition, higher product titers were observed if FDH-FR fusion enzymes were used for in vivo biocatalytic transformations. These systems could, therefore, serve as valuable tools for in vivo chlorination in several different organisms, and efforts are currently underway in our laboratory to engineer systems that provide increased product titers for large-scale halogenation in *E. coli*.

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