Communication

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Enantioselective Hydrogen Atom Transfer: Discovery of Catalytic Promiscuity in Flavin-Dependent 'Ene'-Reductases

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Supporting Information Placeholder

ABSTRACT: Flavin has long been known to function as a single electron reductant in biological settings, but this reactivity has rarely been observed with flavoproteins used in organic synthesis. Here we describe the discovery of an enantioselective radical dehalogenation pathway for α-bromoesters using flavin-dependent 'ene'-reductase. Mechanistic experiments support the role of flavin hydroquinone as a single electron reductant, flavin semiquinone as the hydrogen atom source, and the enzyme as the source of chirality.

Hydrogen atom transfer (HAT) is a pervasive termination event in radical mediated reactions.[1] While the ubiquity of this mechanistic step has fostered the discovery of many reagents capable of HAT, methods for controlling the stereoselectivity of HAT events remain rare.[2] The lack of reports is in stark contrast to the number of methods for stereoselective protonation.[3] This gap in the synthetic literature reflects the difficulty of controlling neutral radical intermediates. As a consequence, considerable effort is still required to develop methods that address this limitation.

Enzymes are known to be powerful catalysts for synthetic chemistry, offering unparalleled levels of selectivity for even the most challenging substrates.[4] Our group is interested in developing enzymatic catalysts to address selectivity challenges in radical mediated reactions. Unfortunately, enzymes that are known to use radical mechanisms present technical challenges, which have prevented their application in organic synthesis. We hypothesized that this limitation can be overcome by utilizing the inherent redox promiscuity of biological cofactors localized within highly evolvable and broadly substrate permissive enzyme active sites.[5,6,7] This general idea was reduced to practice in our seminal studies exploring the enantioselective radical dehalogenation of α-halolactones using NADPH dependent ketoreductases under visible light irradiation (Figure 1a).[8] In that report, experimental evidence supports the role of NADPH as both a single electron reductant and a hydrogen atom source. While this report represents one of the only highly enantioselective hydrogen atom transfer reactions,[9] the scope is limited to lactone substrates due to the requirement for the formation of an electron donor-acceptor (EDA) complex for electron transfer to occur. To expand the scope of this unique reactivity, we questioned if other enzymes might be capable of electron transfer without requiring the formation of an EDA complex.[10]

Flavin is a ubiquitous redox cofactor in nature, capable of both 2-electron and 1-electron chemistry.[11] Yet, commonly used flavoenzymes in organic synthesis, such as 'ene'-reductases (EREDs) and Baeyer-Villiger monooxygenase (BVMOs), react with organic substrates via 2-electron mechanisms.[12] Given the redox promiscuity of flavin cofactors, we hypothesized that these enzymes may also be capable of generating radical species if one-electron pathways are available.

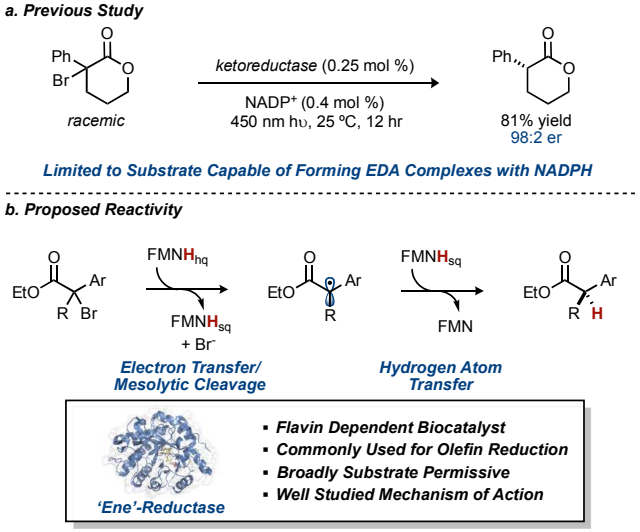
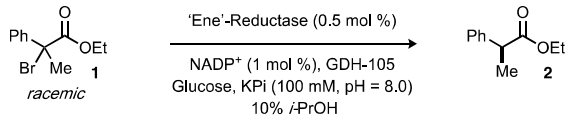


FIGURE 1. a. Previous studies using ketoreductases. b. The proposed enantioselective radical dehalogenation using an 'ene'-reductase.

We targeted the enantioselective dehalogenation of α-bromoesters as an ideal model reaction to investigate the ability of flavoenzymes to catalyze radical reactions. Enantioselective hydrogen atom transfer to this class of substrate is a challenging reaction for small molecule catalysts, typically providing low levels of enantioselectivity with stoichiometric reagents.[13] EREDs were selected as an ideal enzyme platform because they are known to be highly evolvable, broadly substrate permissive, and capable of providing high levels of enantioselectivity for enoate reduction. Furthermore, the mechanism of this reduction has been extensively studied and is well understood to proceed via a hydride transfer mechanism.[14] However, early studies by Miura found that old yellow enzyme (OYE1) is able to reduce menadione to the corresponding radical anion via a single electron pathway.[15] Mechanistically, we hypothesized that a dehalogenation could occur via electron transfer from $FMNH_{hq}$ to the substrate, followed by rapid mesolytic cleavage to form α-acyl radical (Figure 1b). This species could abstract a hydrogen atom from $FMNH_{sq}$ (calc N–H BDE = 59.9 kcal/mol) to form the reduced product and FMN.[16] Mechanistically, this proposed reactivity is reminiscent of flavin-dependent iodotyrosine deiodinase where $FMNH_{hq}$ is proposed to reduce the keto tautomer of iodotyrosine to form a phenoxy radical and $FMNH_{sq}$.[17]

TABLE 1. Screen of Structurally Diverse Flavin-Dependent Oxidoreductases



entry[a]	'ene'-reductase[b]	yield (%)[c]	enantiomeric ratio (er)
1	GluER	57%	88:12
2[d]	MorB	62%	68:32
3	OPR-1	75%	59:41
4	OYE-1	76%	59:41
5	OYE-2	30%	56:44
6	OYE-3	19%	65:35
7	PETNr	68%	56:44
8	Xen A	51%	49:51
9	YqjM	40%	51:49
10	PAMO	47%	72:28
11	GluER-Y177F	69%	97:3
12[e]	GluER-Y177F	89%	97:3

[a] 1 (20 μmol, 5 mg), 'ene'-reductase (0.1 μmol), $NADP^+$ (0.2 μmol), GDH-105 (0.5 mg/rxn), glucose (49.5 μmol), KPi (100 mM, pH=8.0, 450 μl), *i*-PrOH (50 μl), 24 hours, 25 °C, anaerobic. [b] Purified 'ene'-reductase, sequence information found in SI. [c] ^1H NMR Assay Yield. [d] NAD^+ instead of $NADP^+$. [e] 'ene'-reductase (0.75 mol %).

We began our investigation by subjecting α-bromo-α-aryl ester **1** to a panel of nine structurally diverse EREDs (Table 1). To our surprise, all of the enzymes tested provided the desired dehalogenated product **2** with OYE-1 providing product in the highest yield (Table 1, entry 4). However, the ERED from *G. oxydans* (GluER) afforded the highest levels of enantioselectivity favoring the (*S*)-enantiomer (Table 1, entry 1).[18] Importantly, the unreacted starting material from the GluER catalyzed reaction was found to be racemic, suggesting that the enzyme does not preferentially react with one enantiomer over the other. It is also interesting to note that an engineered Baeyer-Villiger monooxygenase, which uses the FAD cofactor to perform flavin-catalyzed oxidations, is also capable of this reactivity, providing product in modest yield and enantioselectivity (Table 1, entry 10).[19]

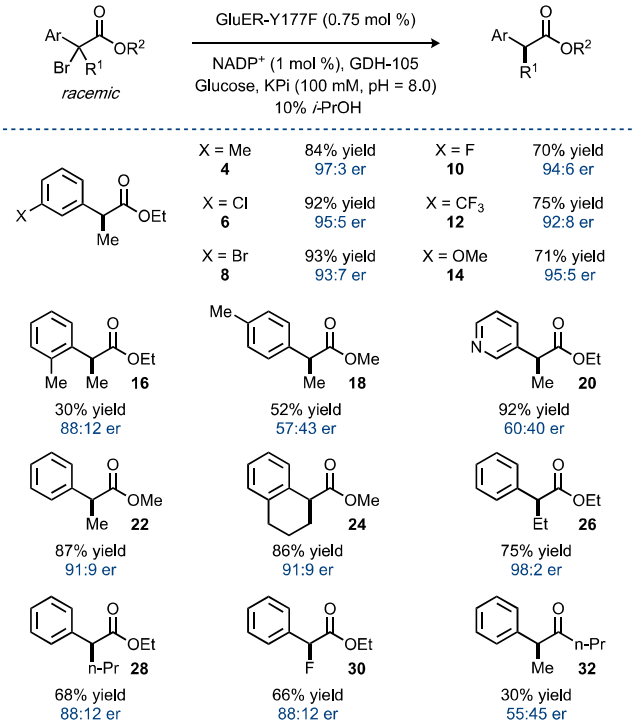
In an effort to improve the enantioselectivity of the observed dehalogenation, we targeted the conserved tyrosine residue position at 177 for mutagenesis. Given the role of tyrosine in the natural reaction, we hypothesized that it could also serve as a hydrogen source, which would diminish the overall selectivity of the transformation. To our delight, mutation to phenylalanine (Y177F) provided product in 69% yield and 97:3 er (Table 1, entry 11) with the remaining mass balance being unreacted starting material. Interestingly, while mutation of the conserved tyrosine residues in PETNr and YqjM provided a similar increase in yield, no change in the enantioselectivity was observed (see supplemental information). The reaction yield could be further improved by increasing the catalyst loading to 0.75 mol % with no observed change in the enantioselectivity (Table 1, entry 12). The reaction is tolerant of cell free lysates, furnishing product with good enantioselectivity albeit in modest yield (see supplemental information).

With ideal conditions in hand, we explored the scope and limitation of this reaction with the evolved *G. oxydans* variant GluER-Y177F. The reaction is broadly tolerant of substituents appended to the *meta* position of the arene, with both electron rich (Table 2, **4** and **14)** and electron poor (Table 2, **6**, **8**, **10**, **12**) substituents providing product with good to excellent enantioselectivity. *Ortho* substituents are also accepted, albeit in modest yield and enantioselectivity (Table 2, **16**). Intriguingly, *para* substituents are poorly tolerated with ethyl esters, providing less than 10% of the desired product with the remaining mass balance being unreacted starting material. The *para* substituted methyl ester variants afford the product in higher yield but again with low levels of enantioselectivity (Table 2, **18**). Testing other variants failed to provide product in superior enantioselectivity (see supplemental information). Heterocyclic arenes are reactive substrates, but unfortunately provide low levels of enantioselectivity presumably due to an altered binding mode afforded by the Lewis basic nitrogen (Table 2, **20**).

Mechanistic experiments suggest that the low levels of enantioselectivity are due to altered substrate binding rather than a mechanistic change (see supplemental information). Methyl esters are also competent substrates, providing product in good yields and enantioselectivity (Table 2, **22**). A variety of substituents at the α-position of the ester are tolerated by the enzyme, furnishing the desired products in promising yields and enantioselectivities (Table 2, **24**, **26**, **28, 30**). While ketones are reactive substrates, the dehalogenation is less efficient and occurs with poor selectivity (Table 2, **32**).

A set of mechanistic experiments were conducted to elucidate the mechanism of this novel reactivity. At the onset, we imagined four possible mechanisms for dehalogenation. The first involves halide elimination to generate an α,β-unsaturated ester, which could engage in an ERED catalyzed reduction. However, when the putative enoate intermediate **33** was subjected to the reactions conditions, it was found to be completely unreactive (Scheme 1a). This is consistent with previous observations that variants lacking the conserved tyrosine are poor catalysts for olefin reduction.[20] Furthermore, dehalogenation of substrates lacking β-hydrogens occur, conclusively discounting the intermediacy of an enoate intermediate (Table 2, **30**, and supplemental information).

TABLE 2. Substrate Scope

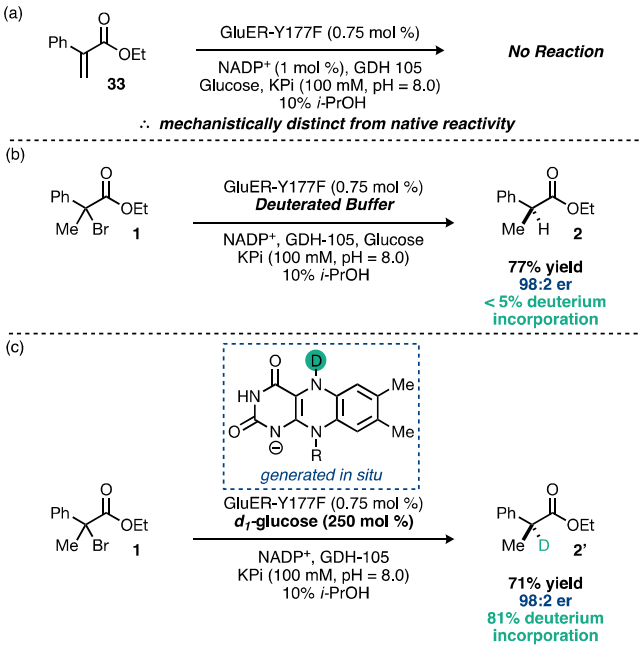


[a] 1 (19.5 μmol, 5 mg), 'ene'-reductase (0.098 μmol), NADP+ (0.2 μmol), GDH-105 (0.5 mg/rxn), glucose (49.5 μmol), KPi (100 mM, pH=8.0, 450 μl), *i*-PrOH (50 μl), 24 hours, 25 °C, anaerobic.

A second mechanistic hypothesis involves electrophilic bromination of the $FMNH_{hq}$ by **1** to afford an intermediate enolate that would be rapidly protonated by solvent or a protic side chain. We discount this mechanism because the requisite orbital overlap between $FMNH_{hq}$ and the C–Br σ* would suggest one enantiomer would react preferentially which is inconsistent with our experimental observations.

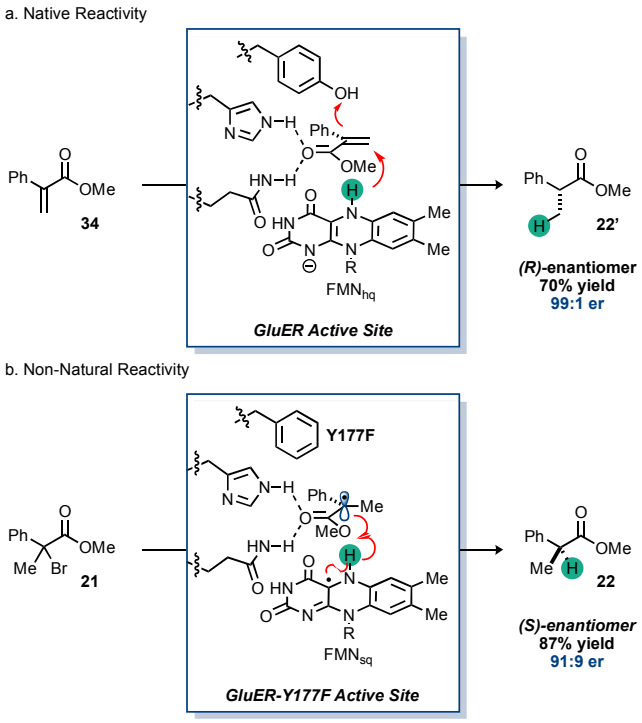
The final mechanistic possibilities involve an initial electron transfer and mesolytic cleavage to form an α-acyl radical. In one termination pathway, a second electron transfer occurs from $FMNH_{sq}$ to form an enolate, which is rapidly protonated (pathway A). Alternatively, the α-acyl radical can engage in HAT with $FMNH_{sq}$ to form product (pathway B). As radical clock experiments are poorly suited to distinguish between these two possibilities, we elected to use an isotopic labeling strategy. In deuterated buffer, we would expect to see deuterium incorporation for pathway A, but not for pathway B. Alternatively, if isotopically labeled flavin were used, we would expect to observe a large degree deuterium incorporation for pathway B, but little to no deuterium incorporation for pathway A. Consistent with pathway B, when a D_2O based buffer is used, <5% deuterium incorporation is observed, suggesting ionic intermediates are not formed in this reaction (Scheme 1b). Furthermore, when d_1-glucose is used to generate $FMND_{hq}$ *in situ*, 81% incorporation is observed for isolated product **2** (Scheme 1c).[21] These data strongly support pathway A, where flavin functions as both a single electron reductant and as a hydrogen atom source. However, mechanistic experiments suggest pathway B is operative in OYE-1, potentially accounting for the low levels of enantioselectivity observed with this variant. Light irradiation is not required in this system by comparison to our previous report because of the increased reducing ability of FMN_{hq} (E_{ox}=-490 mV vs SCE) by comparison to NADPH (E_{ox}=570 mV vs SCE).[22] While this mechanism necessitates an initial endergonic electron transfer, the irreversible mesolytic cleavage makes the overall process thermodynamically feasible. The endergonic electron transfer is potentially the reason for the modest catalysts efficiency and substrate scope.

SCHEME 1. Mechanistic Experiments



The newly described reactivity provides the means of accessing both enantiomers of aryl propionates using a single point mutation. When enoate **34** is subjected to wild type GluER, the *(R)*-enantiomer of phenyl propionate **2** is isolated in 70% yield with 99:1 er. In contrast, when bromoester **21** is subjected to GluER-Y177F, the same product **2** is isolated 87% yield with 91:9 er favoring the *(S)*-enantiomer. The observed selectivity fits with our mechanistic hypothesis. In the case of enoate reduction, hydride transfer from $FMNH_{hq}$ to the β-position of the enoate forms an enzyme bound enolate, which is rapidly protonated by the conserved tyrosine residue in the stereodetermining step (Scheme 2a).[20] This is in contrast to dehalogenation where HAT from flavin to the same position is enantiodetermining (Scheme 2b). As tyrosine and flavin reside on opposite enantiotopic faces of the prochiral olefin or radical, the observed selectivities are consistent with our mechanistic hypothesis.

SCHEME 2. Stereochemical Outcome



In conclusion, we have discovered that flavin-dependent oxidoreductases are capable of effecting an enantioselective radical dehalogenation reaction. This work represents novel reactivity for a well-studied family of enzymes. Efforts to expand this reactivity to complexity building reactions are ongoing.

ASSOCIATED CONTENT

Supporting Information.

Experimental procedures and characterization data is available free of charge via the Internet at http://pubs.acs.org.

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