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Communication

Photoenzymatic Reductions Enabled by Direct Excitation of Flavin-Dependent "Ene"-Reductases

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ABSTRACT: Non-natural photoenzymatic reactions reported to date have depended on the excitation of electron donor−acceptor complexes formed between substrates and cofactors within protein active sites to facilitate electron transfer. While this mechanism has unlocked new reactivity, it limits the types of substrates that can be involved in this area of catalysis. Here we demonstrate that direct excitation of flavin hydroquinone within "ene"-reductase active sites enables new substrates to participate in photoenzymatic reactions. We found that by using photoexcitation these enzymes gain the ability to reduce acrylamides through a single electron transfer mechanism.

Photoinduced electron transfer (PET) is a common mechanism to facilitate electron transfer events that would be inaccessible to molecules in their ground state. This activation mode has been broadly applied in fields as impactful and diverse as water splitting, organic synthesis, and photovoltaics.[1] In the realm of biological systems, PET is responsible for the activities associated with photosynthetic complexes and light-oxygen-voltage sensing domains, yet it is rarely employed in biocatalytic transformations. Indeed, only one of the three classes of photoenzymes found in nature has shown any synthetic potential.[2,3]

Our group has targeted PET as a mechanism to expand the synthetic capabilities of substrate promiscuous enzymes.[4] In previous studies, we found that flavin-dependent "ene"-reductases (EREDs) can catalyze radical dehalogenations and cyclizations with high levels of stereoselectivity.[5] In these studies, radical formation occurred via photoexcitation of ternary charge-transfer (CT) complexes formed between the flavin hydroquinone (FMN_{hq}) and electron deficient substrates within the protein active site (Figure 1A).[6] While this mechanism of PET localizes the radical within the enzyme, it also limits the types of substrates that can function as radical precursors. Specifically, we have only observed radical formation using alkyl chlorides, bromides, iodides, and redox-active esters. To overcome this limitation, new photoenzymatic mechanisms for radical formation need to be developed. For instance, direct excitation of the flavin cofactor would enable high reduction potentials to be accessed. DNA photolyase, a photoenzyme that repairs pyrimidine dimer lesions formed on DNA, relies on the photoexcited state of flavin hydroquinone to initiate DNA repair via single electron reduction of its substrate. While this mechanism is, in theory, available to all flavoproteins, no others are known to utilize it. We recognized that if this mechanism could be achieved with EREDs, it could function as a substrate agnostic approach for generating a variety of radical intermediates (Figure 1B).[7]

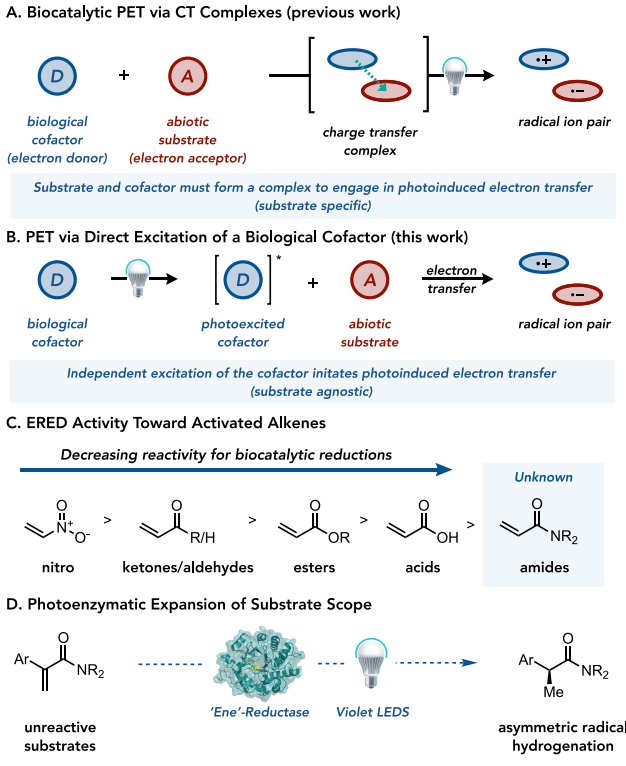

Figure 1. Photoenzymatic catalysis and biocatalytic reductions.

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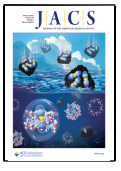

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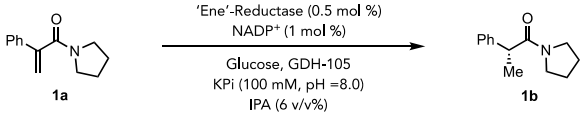
The challenge inherent to all chiral photocatalysts is ensuring that radical formation only occurs within the chiral environment of the catalyst.[8] To ensure binding, we targeted substrates that closely resemble established substrates for EREDs. Inspired by Pac et al.'s pioneering study of single electron reduction of enones using photoredox catalysts,[9] we questioned whether EREDs could reduce α,β-unsaturated amides via direct photoexcitation of FMN_{hq}.[10] While EREDs are known for their ability to reduce electronically activated alkenes using a hydride transfer mechanism, they are not known to reduce moderately electrophilic amides (Figure 1C).[11,12] We hypothesized that a PET mechanism would unlock this elusive reactivity, allowing for the generation of radical intermediates from simple olefin precursors (Figure 1D).

We explored the viability of this mechanism on the asymmetric reduction of acrylamide **1a** with a panel of six different EREDs (Table 1). In the absence of light, none of these enzymes were able to reduce the amide, consistent with our understanding of the substrate preference for these enzymes.[13]

Table 1. Enzyme Screen



entry[a]	ERED[b]	dark[c]	violet LEDs[c]
1	GluER	N.R.	93%, 27:73 er
2	MorB	N.R.	96%, 31:69 er
3	NostocER	N.R.	92%, 81:19 er
4	PETNr	N.R.	79%, 80:20 er
5	OYE1	N.R.	68%, 91:9 er
6	YqjM	N.R.	66%, 57:43 er
7	FMN	N.R.	19%, 50:50 er
8[d]	OYE1+FMN	N.R.	90%, 89:11 er
9[d]	OYE1-F269G	N.R.	97%, 90:10 er

[a]**1a** (10.0 μmol, 2.0 mg), "ene"-reductase (0.050 μmol), NADP+ (0.10 μmol), GDH-105 (0.25 mg/rxn), glucose (40 μmol), KPi (100 mM, pH = 8.0, 470 μL), *i*-PrOH (30 μL), total volume (0.5 mL), 24 h, 25 °C. [b]Purified "ene"-reductase, full sequence information found in the Supporting Information. [c]LCMS assay yield, enantiomeric ratio determined by HPLC. [d]Reaction run with FMN (1 mol%) in tricine buffer (100 mM, pH 9.0).

Next, we irradiated the same panel of enzymes with a 52 W violet LED whose emission window overlaps with the absorption of FMN_{hq}. Under these conditions, all enzymes provided the reduced product, with OYE1 providing the highest levels of enantioselectivity (Table 1, entry 5). While control experiments confirmed that each component of the reaction was essential for reactivity, they did reveal that FMN in the absence of protein could catalyze the reaction, albeit in low yield and with no enantioselectivity (Table 1, entry 7). Optimization of the reaction with OYE1 resulted in improved yield upon addition of FMN (1 mol%) in tricine buffer at pH 9.0 (Table 1, entry 8). Site-saturation mutagenesis of residues lining the active site revealed that mutation of phenylalanine 296 to glycine (OYE1-F296G) increased catalyst efficiency, providing product in 97% yield with 90:10 er (Table 1, entry 9).[14]

We conducted mechanistic experiments to gain a better understanding of the reaction. The intermediacy of a CT complex was ruled out due to a lack of spectral changes in the UV−vis spectrum upon addition of **1a** to reduced OYE1-F269G (Supplemental Figure S7). Transient absorption spectroscopy was conducted to probe the mechanism and determine the lifetime of radical intermediates. These studies were conducted using OYE1-F269G and allyl amide **7a** due to the superior solubility of this substrate. As an initial control, we tested whether amide **7a** quenched the excited state of free FMN_{hq} in the absence of protein. When FMN is reduced with sodium dithionite and excited at 370 nm, the excited state hydroquinone lifetime is 55 ps. This lifetime decreased to 50 ps in the presence of substrate indicating that substrate reduction can occur in solution, consistent with the control experiments (Supplemental Figure S9).

Similar studies were conducted with OYE1-F269G. When reduced with NADPH and excited, a biexponential decay of 9.9 and 323 ps is observed in the absence of amide **7a** (Supplemental Figure S16). In the presence of **7a**, the initial hydroquinone excited state decays within 6.6 ps to one of two states shown in Supplemental Figure S18. Physically, these two states most likely arise from enzyme with or without substrate bound. The unbound protein decays to the ground state with the same 323 ps lifetime as that observed without substrate, whereas the substrate bound protein decays to a spectrum corresponding to the neutral semiquinone ($FMN_{sq}H$). This species further decays with a lifetime of 47 ps to an initial combination of

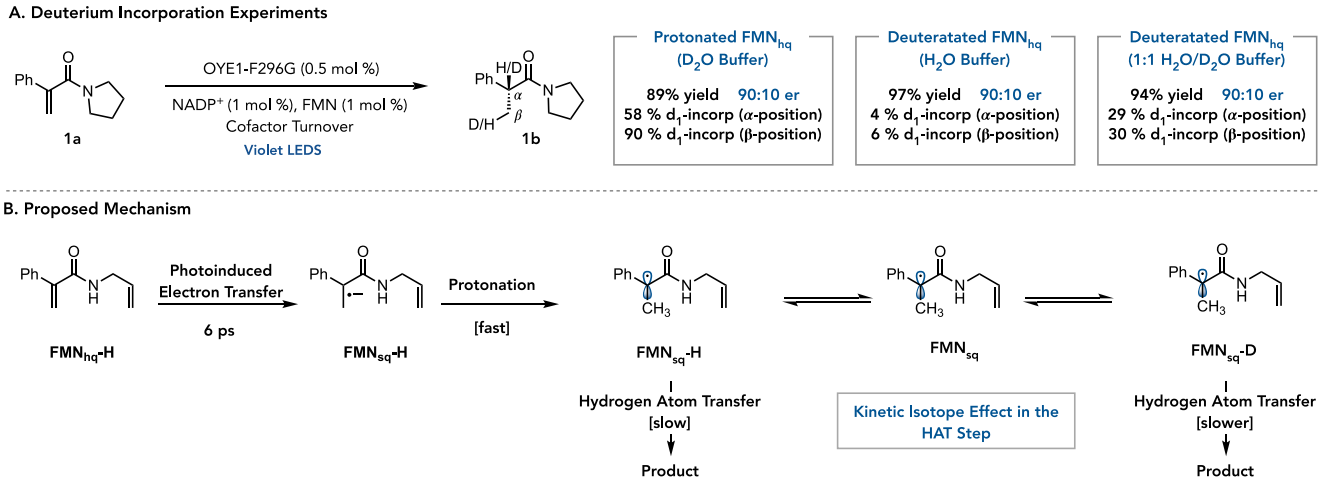


Figure 2. Mechanistic studies.

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$FMN_{sq}H$ and anionic semiquinone (FMN_{sq}). Finally, these species decay over 2.5 ns into a long-time component that represents an equilibration of flavin semiquinones (Supplemental Figures S22 and S25). This combination of the two radical flavin species survives for a period greater than 80 ns. Collectively, these experiments indicate that the excited state hydroquinone is capable of electron transfer through PET enabled by direct excitation of the flavin cofactor.

The results from the transient absorption spectroscopy experiments suggest a unique radical mechanism. Electron transfer is the only feasible mechanism for forming $FMN_{sq}H$, which simultaneously results in the formation of a substrate-centered radical anion. We anticipate this species to be rapidly quenched via regioselective protonation to afford an α-acyl radical. Consistent with this mechanistic hypothesis, when we run a reaction in buffer containing 95% D_2O, we observe 90% deuterium incorporation at the β-position (Figure 2A). Our previous studies with these enzymes indicate that radical termination of α-acyl radicals occurs via enantioselective hydrogen atom transfer from flavin. Surprisingly, when flavin is isotopically labeled using d_7-glucose, only 4% deuterium incorporation is observed at the α-position (Figure 2A). In contrast, reactions run in 95% D_2O provide product with 58% deuterium incorporation at that position. These results suggest two possible mechanistic scenarios: (i) radical termination occurs via an electron transfer/proton transfer (ET/PT) mechanism or (ii) radical termination occurs via HAT with the isotopic label at the N-5 position being exchanged during the reaction. Incomplete deuterium incorporation at the α-position supports the second mechanistic hypothesis.[15] The high degree of enantioselectivity observed for this reaction suggests a single reaction mechanism, as competing mechanisms would presumably afford decreased selectivity. A decreased rate of conversion to product when using d_7-glucose is further evidence of a HAT radical termination mechanism rather than ET/PT (Supporting Information S6). Finally, when a reaction is run with a 1:1 ratio of H_2O/D_2O and d_7-glucose, 29% deuterium incorporation is observed at the α-position. This experiment suggests that the degree of deuterium incorporation at the α-position is a function of kinetic effects in the HAT step rather than kinetic effects that change the termination mechanism.

These experiments suggest that radical termination occurs via hydrogen atom transfer from flavin, but that the isotopic label at the N-5 position is lost over the course of the reaction. As isotope exchange is slow in the hydroquinone oxidation state, we hypothesize that exchange must be occurring at the semiquinone oxidation state. The N-5 position of $FMN_{sq}H$ is known to be acidic. Reversible protonation of the FMN_{sq} would provide a mechanism for isotopic label exchange on flavin. In our previous studies, we observed radical lifetimes of <250 ps, providing insufficient time for complete label exchange. In this reaction, spectroscopic evidence for FMN_{sq} and $FMN_{sq}H$, coupled with radical lifetimes in excess of 80 ns, provides a rationale for exchanging the label on flavin. We hypothesize that this extended lifetime is due to the nature of the radical intermediate being formed. Related species are known to have persistent radical character, making them significantly less reactive.[16]

In summary, we propose the following reaction mechanism. Photoexcitation of FMN_{hq} yields a highly reducing singlet excited state of the anionic hydroquinone. Electron transfer to the substrate occurs within picoseconds to generate a radical anion with anionic character primarily at the β-position. Pulse radiolysis measurements indicate the reduction potential of the substrate to be approximately −2.7 V vs Fc/Fc+ (see Supplemental Figures S27−S29 and discussion). Based on the excited state potential of FMN_{hq} in DNA photolyase, electron transfer is likely modestly endergonic.[17] Protonation of the radical anion results in the formation of a stabilized α-acyl radical which can undergo enantioselective hydrogen atom transfer from $FMN_{sq}H$ to generate the reduced product and reform oxidized FMN.[11] Reduction of the cofactor with NADPH returns flavin to the hydroquinone state and restarts the catalytic cycle.

With an understanding of the reaction mechanism in hand, we explored the scope and limitations of this novel activation mode. We found that the reaction is tolerant to a variety of cyclic and acyclic amide substituents affording products in good yields and enantioselectivities (Figure 3, **1b**−**6b**). A secondary allyl amide was reactive but afforded product with more modest levels of

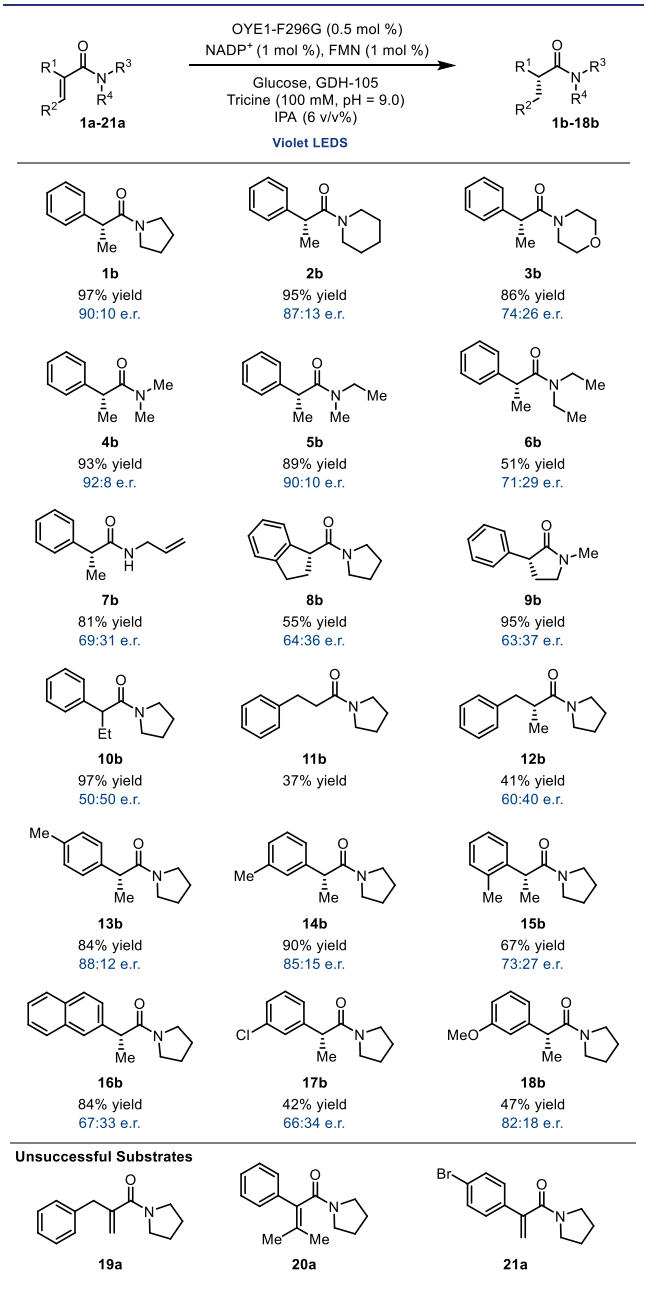

Figure 3. Scope exploration.

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enantioselectivity (**7b**). Trisubstituted alkenes were reactive, but afforded products with poor enantioselectivities (**8b**−**10b**). Interestingly, a tetra-substituted alkene (**20a**) provided only trace product. The presence of an aryl substituent was essential for achieving reactivity (**19a**), but the substituent could be located at either the α- or β-position (**11b** and **12b**).[18] With regard to aromatic substituents, the reaction is tolerant of aliphatic substituents (**13b**−**15b**) and a surprising amount of steric bulk (**16b**). However, heteroatom substituents significantly decrease reactivity and selectivity (**17b**, **18b**, and **21a**).

Next, we explored whether this PET mechanism could be applied to other types of difficult reductions. In particular, we were interest in determining whether these enzymes could catalyze defluorination reactions. C−F bonds represent some of the strongest bonds in organic molecules, making them challenging to cleave using traditional small molecule strategies. Highly reducing photocatalysts have been demonstrated to reduce these bonds, enabling them to function as radical precursors.[19] To explore whether EREDs could catalyze this transformation, we prepared two α-fluoroamides. When subjected to the photoenzymatic reaction conditions, these amides were defluorinated in good yields and with enantioselectivities that closely mirror those observed in the related alkene reduction reaction (Figure 4). Interestingly, defluorination did

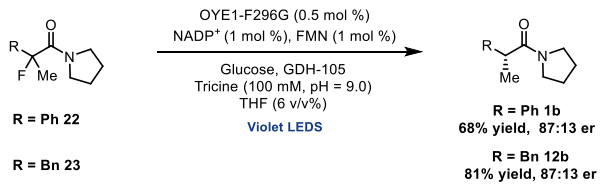

Figure 4. Defluorinations.

not require adjacent aromatic substituents, suggesting that fluorine itself is sufficiently activating for reduction. As the enantioselectivities closely mirror the selectivities observed for related alkene reduction, we hypothesize that these reductions similarly terminate via HAT from $FMN_{sq}H$.

Finally, we looked to expand the synthetic applicability of this reactivity to C−C bond formation. We previously found that EREDs are capable of catalyzing cyclizations of α-chloroamides. To our delight, α,β-unsaturated amides bearing a pendent alkene can afford γ-lactams in good yields (Figure 5). These

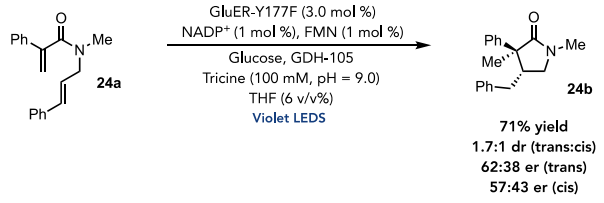

Figure 5. Radical cyclization.

types of highly substituted lactams are challenging to prepare using traditional synthetic methods, particularly considering the stability of the benzylic α-acyl radical. Moreover, this approach avoids the need for organohalide functionalities that might be unstable with certain substitution patterns.

In conclusion, we have demonstrated that direct photoexcitation of FMN_{hq} in EREDs gives rise to a potent, enzyme-bound, single electron reductant that can be used to carry out asymmetric radical transformations using minimally functionalized starting materials. Harnessing this neglected activation mode allows enzymatic reduction of α,β-unsaturated amides, which were previously considered to be an unreactive substrate class for enzymatic reductions. We anticipate that this new method for generating radical intermediates in ERED enzymes could lead to a host of novel enzymatic transformations, thereby fueling momentum in utilizing biocatalytic promiscuity for organic synthesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c11494.

Experimental procedures and characterization data, including supplemental Figures S1−S29 (PDF)

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Notes

The authors declare no competing financial interest.

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(15) We would expect radical terminal via ET/PT to provide 90% deuterium incorporation at the α-position, consistent with the observed deuterium incorporation at the β-position in 90% D_2O buffer.

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