Engineering a Non-Natural Photoenzyme for Improved Photon Efficiency

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ABSTRACT: Photoenzymes are biological catalysts that use light to convert starting materials to products. These catalysts require photon absorption for each catalyst turnover, making quantum efficiency an important optimization parameter. Flavin-dependent 'ene'-reductases (EREDs) display latent photoenzymatic activity for synthetically valuable hydroalkylations; however, protein engineering has not been used to optimize this non-natural function. Here, we describe a protein engineering platform for the high throughput optimization of photoenzymes. A single round of engineering results in improved catalytic function toward the synthesis of γ , δ , ϵ -lactams, and acyclic amides. Mechanistic studies indicate that key mutations can alter the enzyme's excited state dynamics, enhance its photon efficiency, and ultimately increase catalyst performance. Transient absorption spectroscopy reveals that engineered variants display dramatically decreased radical lifetimes – indicating a shift toward a concerted mechanism. Overall, this platform enables the development and optimization of photoenzymes for tailored applications in chemical synthesis.

Photoenzymes are a burgeoning class of biocatalysts that utilize photoinduced electron transfer to facilitate chemical transformations.^{1,2,3} As each catalyst turnover requires a photon of light, efficient use of the photoexcited state is necessary for high catalytic activity. Natural photoenzymes, having been subject to eons of natural selection, display high quantum yields ($\Phi = 0.85$ for DNA Photolyase and 0.80 for Fatty Acid Photodecarboxylase), favoring productive processes over undesired pathways (Figure 1a).^{4,5} In contrast, non-natural photoenzymes, like flavin-dependent 'ene'-reductases (EREDs),^{6,7} display much lower quantum efficiencies. For instance, the radical intramolecular hydroalkylation of α -chloroamides catalyzed by the ERED from *Gluconobacter oxydans* (GluER) occurs with a quantum yield of $\Phi = 0.024$.⁸ In this reaction, the photoexcited species is a charge-transfer (CT) complex composed of α -chloroamide and flavin hydroquinone (FMN_{hq}). The low quantum yield is due to back electron transfer (BET) proceeding faster than mesolytic cleavage of the C–Cl bond.⁹ To increase the catalyst efficiency, we questioned whether protein engineering could enhance the excited state dynamics of the system.

Protein engineering is a powerful tool for modifying the properties of biocatalysts.^{10,11,12} Among existing strategies, directed evolution is notable in its capacity to optimize enzymes in the absence of mechanistic and structural information.¹³ We recognized that protein engineering could be a powerful strategy for optimizing quantum efficiency where factors determining excited-state dynamics are not readily apparent. However, there are no examples of directed evolution being utilized to optimize the activity of photoenzymes (natural or non-natural).^{5d} In contrast to 'dark' reactions where sources of reaction variability are minimal in a 96-well plate, photoenzymatic reactions present unique challenges. For instance, photoenzymatic reactions are photon-limited thus product formation is proportional to the number of photons introduced into the reaction. Consequently, consistent irradiation in each reaction well is

essential.¹⁴ While this could be potentially achieved by dedicating an individual LED to each well, crosstalk from well-to-well (where the LED inadvertently irradiates neighboring wells) introduces an additional source of variability.^{15,16} Finally, thermal differences across the plate caused by constant irradiation can denature proteins in wells which exhibit increased temperature (Figure 1b).¹⁷ These challenges have previously made the power of directed evolution and protein engineering inaccessible to photoenzymatic catalysis.

A. Photon Efficiency of Photoenzymes

B. Challenge - Screening Assay for Photoenzymatic Reactions

Figure 1. Engineering for Improved Quantum Yields

We selected the radical cyclization of α -chloroamides to form γ -lactams by GluER as a model reaction on which to develop a novel photoenzyme engineering platform.⁸ GluER-T36A was used as the parent enzyme and the reactions were irradiated in a 96-well plate using a Lumidox® GenI Blue LED array. Despite the maximum emission ($\lambda_{\text{max}} = 464$ nm) having

poor spectral overlap with the CT complex, the desired product is formed in good yield.⁸ Using clear microtiter plates resulted in inconsistent results across the wells due to the interior wells having increased illumination by comparison to exterior wells. Moving to white microtiter plates with clear bases cooled by small fans provided consistent irradiation and temperature across the plate – providing reproducible results. (Figure 2a)

Figure 2. Engineered Variants

In the absence of a clear mechanistic strategy for improving the photon efficiency of the enzyme, we elected to conduct error-prone mutagenesis to prepare a library of 1000 GluER-T36A variants.¹⁸ We identified an improved variant, GluER-T36A-K317M-Y343F, that contained two mutations over the parent (henceforth referred to as GluER-G6 or G6 Variant). Position K317 is located at the surface of the protein, while Y343 is located within the enzyme active site (Supplemental Figure S29). For the 5-exo substrate (**1a**), the variant afforded a greater than three-fold increase in activity (600 TTN) by comparison to wild-type (126 TTN) in addition to improving the enantioselectivity of the transformation (Figure 2b). Having identified an improved catalyst, we prepared a series of variants where individual mutations are removed to determine their impact on GluER-G6. These studies revealed Y343F mutation to be critical for improved catalytic function. Interestingly, the T36A mutation was less important in the evolved variant than the parent, pointing toward the non-additivity of mutational effects on performance (Figure 2b). Finally, further optimization of the reaction conditions, enabled the radical cyclization to be run at 25 g/L substrate loading with only 0.15 mol% of GluER-G6, affording product in 86% yield with 97:3 er. When the same conditions are used with the wild-type enzyme, product is formed in only 30% yield with 91:9 er (Supplemental Figure 31).

With an improved variant in hand, we investigated elucidate the origin of the improved performance of GluER-G6. We used ultrafast transient absorption spectroscopy to determine the quantum yield of our GluER variants, where Φ is defined as the number of photons that lead to product over the total number of excitation events.¹⁹ Using transient absorption spectroscopy, we can monitor the amount of FMN_{ox} (~480 nm) formed per excitation pulse. We can then use that as a proxy for quantum yield (Figure 3a). Wild-type GluER has a quantum yield of $\Phi = 0.024$ with 2.4% of excited states leading to product with the remaining undergoing back electron transfer. GluER-T36A, the parent for the error-prone library had a quantum yield of $\Phi = 0.068$,⁸ representing a roughly 2-fold increase in quantum efficiency. GluER-G6 has a quantum yield of $\Phi =$

0.105, a 4-fold improvement over the wild-type indicating a significant change in the excited state dynamics (Figure 3a).

A. Quantum Yield and Excited State Dynamics

B. Promotion of Rate Determining Step

Figure 3. Evolutionary Impact on Excited State Dynamics

To determine the mechanistic origin of the improved quantum efficiency, global analysis was performed on the model substrate comparing GluER-WT and the evolved G6 variant.²⁰ First, the excited state dynamics of the enzymes were determined in the absence of substrate (Supplemental Figure S17 and S21). This information was used to deconvolute the excited state dynamics of enzyme in the presence of substrate. The model used to capture both bound and unbound dynamics (Supplemental Figure S17-S24) involved restraining the excited decay of unbound protein and allowing other time constants to fluctuate to minimize error.²⁰ Using this analysis, we were able to compare the lifetimes and relative abundance of various reactive intermediates. We began by looking at the photoexcitation event and the relative absorbance of the enzyme complex containing the radical ion pair (flavin semiquinone (FMN_{sq}) and the radical anion involved in mesolytic cleavage). This ion pair has a three-fold stronger absorbance in GluER-G6 than in GluER-WT (Supplemental Table S3). Since both GluER-WT and GluER-G6 have similar affinities for the starting material (Supplemental Figure S7 and S8) and the unbound species associated difference spectra (SADS) show similar relative intensities (Supplemental Figure S20 and S23), the increase in the absorption indicates that, per photon absorbed, the evolved variant forms more radical anion than the wild-type enzyme – indicating a decrease in the rate of back electron transfer. The greater abundance of this intermediate increases the likelihood of mesolytic cleavage and thus increases quantum efficacy.

The most dramatic change between these variants is in the lifetime of the radical intermediates. In our previous report, we propose that after mesolytic cleavage, the resulting α -acyl-radical cyclizes into the pendant alkene to form a benzylic radical, which it ultimately quenched through hydrogen atom transfer from FMN_{sq} – resulting in product and fully oxidized FMN_{ox}. Based on this mechanism, we can use the FMN_{sq} signal to determine the lifetime of radical intermediates. Using GluER-WT, radical intermediates persist within the protein active site for 700 ps before FMN_{ox} is formed (Figure 4a). With GluER-G6, we were surprised that we did not observe FMN_{sq}. Instead, we find the transient spectral signature of the radical ion pair directly decays to a species assigned to FMN_{ox} in only 20 ps (Figure 4a). The formation of oxidized flavin on this time-scale was unexpected, potentially indicating a change in

reaction mechanism. As, the transient absorption spectroscopy data suggest that the mechanism by which photoexcitation initiates the reaction is similar for the two enzymes, we conducted isotopic labelling experiments to determine if there is a change in the termination mechanism. Our previous studies indicated that flavin terminates radical intermediates via hydrogen atom transfer from FMN_{sq} . Indeed, when conducting the reaction with GluER-WT, where FMN_{hq} is labelled with deuterium at the N5 position, product is formed with 80% deuterium incorporation at the benzylic position. When the same experiment is conducted using GluER-G6, 89% of the product is isotopically labelled suggesting similar radical termination mechanisms between the two variants (Figure 4a).

Based on these studies, we believe that the differences in radical lifetime are indicative of a subtle change in mechanism. With GluER-WT, a stepwise mechanism is operative

Figure 4. Evolutionary Effect on Radical Lifetimes

where discrete radical intermediates are formed within the active site prior to radical termination (Figure 4b). In contrast, we believe that the reaction catalyzed by GluER-G6 occurs via a concerted reaction mechanism where mesolytic cleavage, C-C bond formation, and hydrogen atom transfer occur within a single transition state (Figure 4c). Evidence for this concerted mechanism can be observed in the lifetime of the radical ion pair involved in mesolytic cleavage with the model substrate and a structurally related analog lacking the alkene (**2a**). For the substrate lacking the alkene, the radical ion pair exists for 3.2 ps in GluER-G6, while the substrate containing the alkene exists for only 1.1 ps. In GluER-WT this lifetime is more consistent, increasing from 3 ps to 4 ps in the alkene and π -alkene, respectively. This change is potentially due to the π -electrons of the alkene hyperconjugatively interacting with the $\sigma^*_{\text{C-Cl}}$ to facilitate mesolytic cleavage (Supplemental Table S3).

With evidence for a concerted reaction mechanism in hand, we questioned whether GluER-G6 would have a similar effect for other substitution patterns or cyclization modes. This variant was effective for a 5-exo-trig cyclization involving *t*-butyl substituted alkenes (**3a**), providing a 3-fold improvement over the wild-type (Figure 5a). Similar changes were observed for both quantum yield and radical lifetime (Supplemental Table S2). Interestingly, GluER-G6 is ineffective for 6-exo-trig (**4a**) and 7-exo-trig (**5a**) cyclizations suggesting the

improvements can only be translated to alternative substitution patterns within the same cyclization mode.

Figure 5. Active Site Engineering to Optimize Photoenzymatic Hydroalkylations

Using insights from the engineering campaign to access a more effective catalyst for the 5-exo-trig cyclization, we set out to identify improved catalysts for 6-exo-trig (**4a**) and 7-exo-trig (**5a**) cyclizations. Recognizing that active site mutations were important in changing active site dynamics, we analyzed the crystal structure of GluER-T36A to identify positions that would be expected to directly interact with the substrate. We targeted sites W66, Y177, Q232, F269, and Y343 for mutagenesis and mutated each position to alanine, leucine, phenylalanine, and aspartic acid to capture distinct sizes and electrostatic characteristics (Figure 5). These 24 variants were transformed into a 96-well plate to enable rapid screening.

We began by testing the 24 active site variants on the 6-exo-trig cyclization. While GluER-WT formed product in

28% yield, a variant where tryptophan at position 66 was mutated to alanine (GluER-T36A-W66A), proved to be more effective, affording product in 95% yield (Figure 5b). The increase in yield can be attributed, in part, to a 5.5-fold increase in the quantum yield. This mutant was also effective for increasing the yield of a 7-exo trig cyclization, forming product in 50% yield while GluER-WT afforded only 13% yield (Figure 5c). Interestingly, the quantum yield of the wild-type enzyme was very low (0.06%), potentially accounting for the low yield. GluER-T36A-W66A displayed a significantly higher quantum yield (2.4%), representing a 40-fold increase in photon efficiency.

Finally, we questioned whether protein engineering would have a similar effect on the intermolecular hydroalkylation. We previously found that α -chloroamides (**6a**) form quaternary CT complexes with the protein, flavin hydroquinone, and alkenes.²¹ In this study, we found that electron-deficient vinyl pyridines (**6b**) can function as coupling partners but in modest yield. Upon screening the collection of active site variants, we found that GluER-T36A-W66L afforded product with 3-fold higher yield (92%), enabling the enzyme loading to be decreased to 0.5 mol% (Figure 5d). As observed previously, a 25-fold increase in the quantum yield of the reaction rationalizes the significant increase in the activity of the protein.

In conclusion, through the development of a novel platform, we demonstrated the first report of protein engineering used to enhance latent photoenzymatic reactivity. Moreover, transient absorption spectroscopy revealed that these improvements are the result of modulating the dynamics of the photoexcited CT-complex within the enzyme active site. We also significantly increased the quantum efficiency of EREDS in the synthesis γ , δ , ϵ -lactams, and acyclic amides. Having established a screening platform for photoenzymes, we anticipate protein engineering and directed evolution to be applied more broadly to challenges associated with photoenzymatic catalysis.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge.

Detailed experimental procedures, characterization data (NMR spectra and HPLC traces)

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Notes

The authors declare no competing financial interest.

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