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Bioorthogonal Enzymatic Activation of Caged Compounds

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Abstract: *Engineered cytochrome P450 monooxygenase variants are reported as highly active and selective catalysts for the bioorthogonal uncaging of propargylic and benzylic ether protected substrates, including uncaging in living E. coli. observed selectivity is supported by induced-fit docking and molecular dynamics simulations. This proof-of-principle study points towards the utility of bioorthogonal enzyme/protecting group pairs for applications in the life sciences.*

Bioorthogonal chemistry allows the execution of chemical reactions inside living cells without interfering with native biochemical processes.[1] Originally mainly focusing on stoichiometric and uncatalyzed reactions, recent efforts aim at identifying catalysts to selectively recognize specific functional groups uncommon in nature and facilitate their transformation within a complex biological system.[2–6] Despite the advantages of such systems, balancing high reactivity at low substrate concentrations and inertness towards countless cellular molecules is a formidable challenge for the design of small-molecule catalysts, and reported examples of artificial bioorthogonal catalysts with high turnovers are rare.[2]

The undeniable advantage of signal amplification through catalytic turnover has been successfully exploited in the area of enzyme-based bioimaging and sensing.[7] Enzymes have evolved over millions of years to be efficient biocatalysts, working optimally and selectively in diverse biological environments. Furthermore, protein engineering provides powerful tools such as directed evolution[8] to improve enzyme properties, including thermal or chemical stability, enhanced activity, and stereo- and regioselectivity towards native and non-native substrates. We thus reasoned that engineered enzymes could expand the toolbox of bioorthogonal chemistry by selectively catalyzing the cleavage of an artificial protecting group in living cells (Figure 1). Such protecting group/enzyme pairs could address the performance issues observed in chemical- or light-induced uncaging reactions.[2,9]

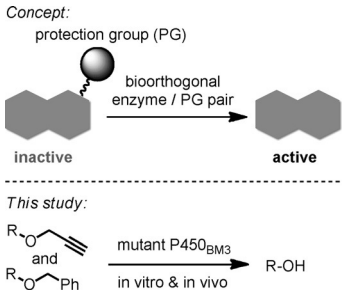

Figure 1. The concept of bioorthogonal enzymatic deprotection of caged compounds realized through the P450-catalyzed oxidative cleavage of propargylic and benzylic ethers.

Initially, suitable enzyme/protecting group pairs were identified, ideally featuring bioorthogonality, which means that the protecting group is completely stable in a biological environment and is cleaved efficiently and selectively solely by the engineered enzyme of choice. Based on these criteria, we chose non-native propargylic ethers as alcohol-protecting groups to be cleaved by a member of cytochrome P450 monooxygenase (CYP) family (see Scheme S1 in the Supporting Information for mechanistic details). We hypothesized that propargylic ethers would not be recognized as substrates by natural enzymes but only by engineered CYPs. As a model system, we used $P450_{BM3}$ (CYP102A1), a soluble fatty acid hydroxylase from *Bacillus megaterium*,[10] which is a highly active, selective, and versatile biocatalyst that has been demonstrated to be tunable for many different applications.[11,12] As a fusion protein carrying its own reductase domain, $P450_{BM3}$ is self-sufficient and depends only on oxygen and its cofactor NADPH. As model reactions, we used ether-protected fluorophores **1**–**3**, which are nonfluorescent but recover their fluorescence upon deprotection, so that ether cleavage could be detected in a straightforward fashion through fluorescence measurements. Based on known methods[13,14] we screened cytochrome $P450_{BM3}$ libraries compris-

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ing more than 1000 enzyme variants capable of oxidizing hydrophobic compounds (see the Supporting Information).

First, a bis-propargylic ether derivative of fluorescein (**1a**), together with the related bis-allyl- (**1b**) and bis-benzyl-protected derivatives (**1c**; Figure 2) was screened for fluorescence development induced by the $P450_{BM3}$ variants. As shown in Figure 3, only a few enzyme variants were found to convert the allyl derivative **1b**, while no mutants were found to cleave the benzyl diether **1c**. However, a number of enzyme variants displayed remarkably high activity for cleavage of the propargylic diether **1a**, the most prominent example being mutant TFFIS (mutations R47T/S72F/A82F/F87I/L437S). Importantly, mutant TFFIS preferentially activates the propargylic diether **1a** over **1b** (weak activation) or **1c** (no activation), while wild type (wt) $P450_{BM3}$ does not activate **1a**, thus demonstrating a compelling degree of selectivity. HPLC analysis revealed the mono-deprotection of **1a** (see Figure S1 in the Supporting Information). The distinct suitability of the propargylic protecting group was confirmed by experiments with the alkyne derivatives **1d–f**. Whereas a large number of $P450_{BM3}$ mutants display weak activity towards the 2-butynyl diether **1d**, no active variants could be found for the alkynes **1e** and **1f** (Figures 2 and 3).

Encouraged by these results, the propargylic ether derivatives (**2a** and **3a**) of two coumarins (**2** and **3**) were investigated and the respective 2-butynyl (**2d** and **3d**) and benzyl (**2c** and **3c**) ethers were used as references. 4-Methylumbelliferone (**2**) is an established drug used in bile therapy[15] and known for its activity as an anticancer agent that is effective against various cancer types.[16] Its ethers are closely related to a known probe for CYP levels in clinical samples.[17] The second compound, 3-carboxycoumarin (**3**), is a known substrate for similar reactions catalyzed by P450 variants.[18] Upon screening, mutant M01 (R47L/F87V/L188Q/E267V/G415S)[19] was capable of activating the propargylic ethers **2a** and **3a**, whereas mutant WMV (R47W/A82M/F82V) displayed very high activity towards the benzylic ethers **2c** and **3c**. These results are particularly noteworthy since alkynes are known to act as suicide inhibitors, irreversibly inactivating CYPs through alkylation of the heme group.[20]

The most prominent variants were purified and characterized biochemically by using defined enzyme concentrations and the same reaction conditions used for the screening procedure (see the Supporting Information). It was thus possible to cancel out differences in expression levels present in lysates, thereby allowing the comparison of our most promising hits (Figure 4). In the case of substrate **1a**, the most active mutant TFFIS yields a 52-fold higher fluorescence response than the wt, corresponding to a turnover number (TON) of 425. The Michaelis–Menten parameters K_M and k_{cat} were determined to be 3.7 μM and 0.26 s^{-1}, respectively ($k_{cat}/K_M = 70.3\ mM^{-1}s^{-1}$). None of the other hits was able to convert any of the fluorescein substrates. While the highly active mutant WMV (for **3d**: TON > 5000, $K_M = 7.2$ μM, $k_{cat} = 550\ s^{-1}$; $k_{cat}/K_M = 7.6 \times 10^4\ mM^{-1}s^{-1}$) activated all of the coumarin-derived substrates (**2a**, **2c**, **3c**, and **3d**), the more selective mutant M01 showed activity towards substrates **2a** and **2c** only, at about 4-fold higher activity than the wt (TON = 47). This means that even compounds with very similar core structures can be differentiated by various mutants, but this gain in selectivity comes with an activity penalty, for example, WMV shows 16-fold higher activity than M01 towards substrate **3d**. Previous reports for **3c**[18c] with other $P450_{BM3}$ mutants achieved only a 7-fold increase at similar concentrations in a different buffer[21] after one round of random directed evolution, which differs from our library

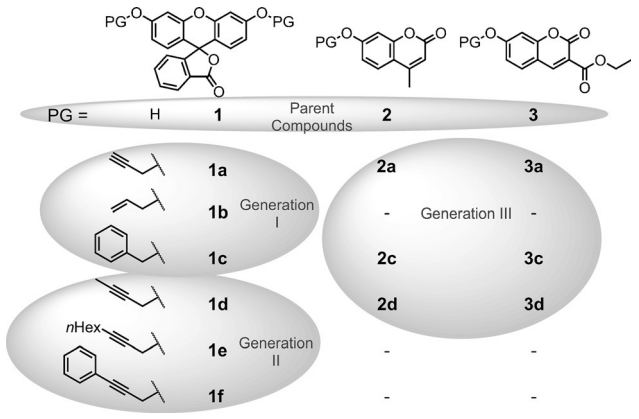

Figure 2. Model compounds screened. The first- and second-generation compounds are fluorescein derivatives, whereas the third-generation compounds are based on coumarins.

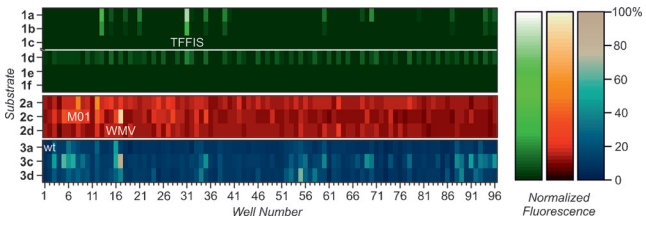

Figure 3. Activity of the $P450_{BM3}$ mutants towards different caged compounds arranged in a 96-well microtiter plate (MTP) format. Fluoresceins **1a–f** (green) and coumarin-based substrates **2a,c,d** (red) and **3a,c,d**, (blue) were used for library screening. Fluorescence intensities are normalized to the lowest/highest values included in the plates, with brighter colors showing higher activity (see the Supporting Information for original values).

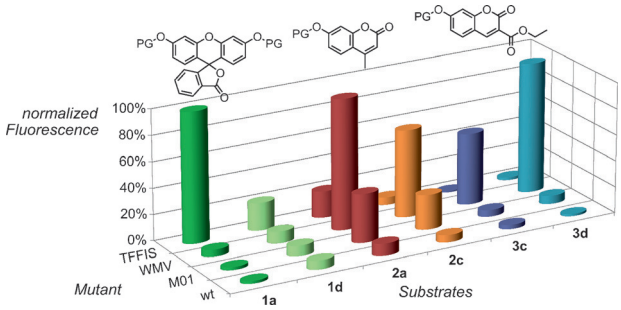

Figure 4. In vitro characterization. Enzymatic activity of $P450_{BM3}$ variants towards caged compounds at fixed enzyme concentrations (generally 100 nM), normalized to the largest observed fluorescence response for each substrate (see Appendix 2 in the Supporting Information for original data).

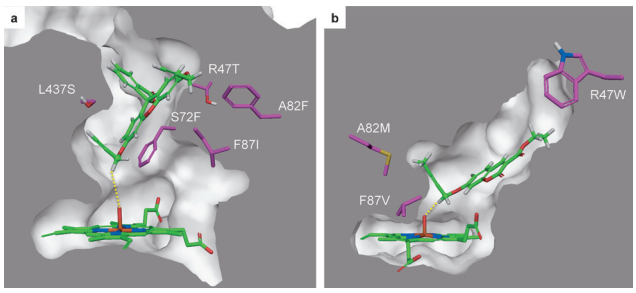

Figure 5. Modeling results. Docking poses for a) substrate **1a** in the TFFIS mutant, and b) substrate **3d** in the WMV mutant. Distances *r*(O–H) between the substrate and active center are 3.64 Å and 2.29 Å, respectively. Highlighted are the oxidized prosthetic heme groups, substrate, and mutated residues.

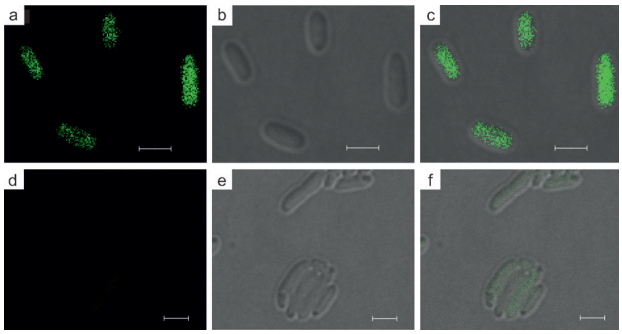

Figure 6. Live-cell experiments. *E. coli* expressing TFFIS (panels a–c) show a high fluorescence signal, thus indicating deprotection of substrate **1a.** No significant signal level is observed in the wt (panels d–f). Shown are the fluorescence channel (left), the optical channel (middle), and an overlay of the two channels (right). Scale bars: 2 μm.

mutants where the active site was targeted according to the recommended guidelines.[22]

To provide structural insight into binding modes and substrate selectivity of the most active substrate/mutant combinations, docking and molecular dynamics (MD) simulations were performed (Figure 5 and the Supporting Information). Briefly, substrates **1a** and **3d** were modeled in the TFFIS and WMV mutants, respectively. Since there are no published crystal structures of the mutants studied here, mutations were computationally modeled in the wt crystal structure (PDB code 1BU7).[23] In both cases, stable binding modes of the substrates were obtained.

The computational results exhibit substrate binding orientations in the active site of the corresponding variants that enable hydroxylation of the protecting group by the oxidizing iron–oxo species (Compound I). Binding of **1a** in the TFFIS mutant (Figure 5a) involves a π-stacking interaction with the S72F side chain, which positions the protecting group for transformation. On the other hand, substrate **3d** can enter deep into the pocket of the WMV mutant, thereby placing the methylene hydrogen atom close to the heme group and allowing fast turnover, in accordance with the kinetic profile (see Table S3). All of the found hits have a mutation at position R47, which has been proposed to play a crucial role in substrate binding.[24] Since this residue is the only charged residue around the binding pocket, substitution for a polar but neutral or even aliphatic amino acid, as in this case, has a large effect on catalytic efficiency.

The ability of the mutants to deprotect the model compounds in living *E. coli* was explored (Figure 6). High fluorescence intensity within the cells and low efflux were observed for **1a**, as can be seen from supernatant and lysate analyses (Figure S3). Contrary to expectations based on previous results by Ruff et al.,[18c] substrates **3c** and **3d** could not be monitored in this way. High efflux of **3** raised the background to a level too high for imaging, even after repeated washing of the cells. Thus, unlike fluorescein derivative **1a**, the coumarin-based substrates were not suitable for imaging, but nevertheless the reaction in living cells was successful in all tested cases.

In summary, we report the identification of engineered CYP variants that are able to efficiently and selectively uncage compounds protected with propargylic and benzylic ethers. We also explain the basis of the selectivity of the most promising mutants by using docking and molecular dynamics simulations. Finally, we show that these P450 mutants successfully catalyze the uncaging reaction in living *E. coli*. Such bioorthogonal engineered enzyme/protecting group pairs may find applications in the life sciences, for instance, for the release of imaging agents or the catalytic activation of prodrugs at their site of action.

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