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Biocatalysis

Cofactor-Free Light-Driven Whole-Cell Cytochrome P450 Catalysis**

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Abstract: *Cytochromes P450 can catalyze various regioselective and stereospecific oxidation reactions of non-functionalized hydrocarbons. Here, we have designed a novel light-driven platform for cofactor-free, whole-cell P450 photobiocatalysis using eosin Y (EY) as a photosensitizer. EY can easily enter into the cytoplasm of Escherichia coli and bind specifically to the heme domain of P450. The catalytic turnover of P450 was mediated through the direct transfer of photo-induced electrons from the photosensitized EY to the P450 heme domain under visible light illumination. The photoactivation of the P450 catalytic cycle in the absence of cofactors and redox partners is successfully conducted using many bacterial P450s (variants of P450 BM3) and human P450s (CYPs 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) for the bioconversion of different substrates, including marketed drugs (simvastatin, lovastatin, and omeprazole) and a steroid (17β-estradiol), to demonstrate the general applicability of the light-driven, cofactor-free system.*

Cytochromes P450 (P450 or CYP) belong to a superfamily of multifunctional monooxygenases that contain heme molecules (i.e., Fe-porphyrin) as a prosthetic group. They can catalyze various oxidative metabolic reactions of endogenous and exogenous compounds in living organisms.[1] Their catalytic diversity and vast substrate range with regio- and stereo-specificity have high potential in applications to drug metabolism as well as in the fine chemical synthesis of steroids, lipids, vitamins, and natural products.[2] P450s function as a part of multi-component electron-transfer chains. Microsomal P450s (class II) receive electrons directly from membranous NADPH-P450 reductase (CPR) that contains diflavins (FAD and FMN). Bacterial/mitochondrial P450s (class I) rely on a shuttle of ferredoxin, an iron–sulfur protein, and NAD(P)H-dependent ferredoxin reductase. The oxidation of NAD(P)H provides electrons to the diflavins of the reductase, followed by the transfer of electrons to the catalytic P450 heme domain through an intermolecular electron transfer, resulting in the generation of a reactive ferryl–oxo species.[3] In most cases, P450s and their reductase partners are synthesized as separate polypeptides. However, some self-sufficient class II P450s were found,[4] where the P450 heme domain is fused at the carboxyl terminus with a CPR. P450 BM3 (CYP102A1) from *Bacillus megaterium* is an entire class II P450 system in a single polypeptide and it has served as a model for the microsomal, especially mammalian, class II P450 system.[3] Efficient and continuous supplementation of electrons to P450s is required to sustain the catalytic activity of the P450s.

The industrial application of P450s has remained a challenge mainly due to their low catalytic activity, low stability, and the dependence on a NAD(P)H and a reductase. To surmount the dependence of the NAD(P)H and the reductase, many alternative approaches had been reported: cofactor regeneration systems,[5] surrogate oxygen atom donors,[6] direct chemical,[7] electrochemical,[8] and light-driven reduction approaches.[9] In particular, light-driven P450 reactions were established by using different mediators. Briefly, the whole photosystem I (PSI) extract from plant cells was combined with P450 and ferredoxin as an electron carrier to enable light-driven hydroxylation of tyrosine.[9a]

Deazaflavin was also used to recycle the electron donor cofactor.[9b] Recently, Ru^{II}-linked P450 BM3 without a reductase domain had been reported as a photosensitized P450 system.[9e–h] The hybrid P450 BM3 heme domain containing a covalently attached Ru^{II} photosensitizer at L407C residue catalyzed the light-driven hydroxylation of lauric acid without the need of a reductase and NADPH.[9h] However, the hybrid P450 needs an extra step for post-translational modification: covalent attachment of Ru^{II} to the specific cysteine residue. This modification may be specific to each P450 and the application of this strategy to other P450s (bacterial or human) does not guarantee the success of catalytic reaction. Furthermore, the reactions catalyzed by Ru^{II}-linked P450 had been limited to the conversion of few natural substrates, such as lauric acid[9b,f–h] and tyrosine.[9a] Although these alternative

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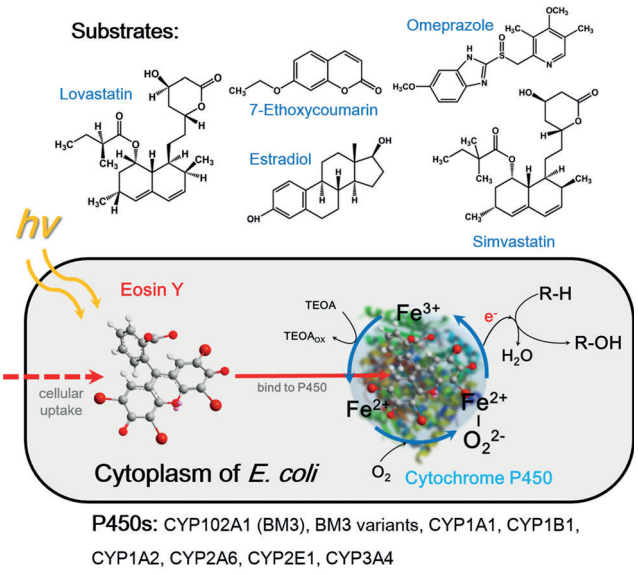
approaches may resolve the problems with the electron supply and provide the perspectives of P450-catalyzed reactions, each system worked with only one case, mainly bacterial P450, or showed relatively low and slow catalytic activities. Therefore, broader applications with existing cofactor-free P450 systems are still a challenge.

Herein, we report on cofactor-free in vivo photoreduction of P450s using eosin Y ($C_{20}H_6O_5Br_4Na_2$, 2,4,5,7-tetrabromofluorescein) (EY) as a photosensitizer that mediates direct electron transfer to the heme domain of P450. EY is an anionic fluorescent dye that has been widely used for biomedical and diagnostic purposes.[10] It has also been reported that photosensitization of EY induces photocatalytic reactions such as photoenzymatic hydrogen evolution[11] and indirect cofactor regeneration.[12] Scheme 1 illustrates the concept of a cofactor-independent, visible light-driven photocatalytic reaction through direct photoinduced electron transfer to P450 heme domain expressed in the cytoplasm of *Escherichia coli*. We found that EY and triethanolamine (TEOA, an electron donor) simultaneously enter into the cytoplasm of *E. coli* and EY injects photoexcited electrons to P450. In this way, P450 continues to catalyze oxidative reactions under visible light illumination without NADPH and a redox partner. We successfully demonstrate the proof of this concept by using BM3m2 (Y51F/F87A), a variant of CYP102A1. P450 BM3 has been extensively studied for a long time and many redesigned variants by rational design and directed evolution have been developed. These variants are known to catalyze various catalytic oxidations of fine chemicals, pharmaceuticals, and steroids to produce the desired products.[13] We investigated the specific interaction of the BM3m2 heme domain with EY using multiple methods such as electrochemical/photochemical analyses. Light-induced catalytic activities of whole P450 BM3m2 and its domains were examined using 7-ethoxycoumarin (7-EC) as a substrate. We then applied the platform of the light-driven whole-cell catalysis to the generation of valuable human metabolites using other BM3 variants (BM3m8 and BM3m10) and human P450s (CYPs 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) with various substrates, including drugs (simvastatin, lovastatin, and omeprazole) and a steroid (17β-estradiol).



Scheme 1. Cofactor-free, visible light-driven photo-biocatalytic reaction by P450 expressed in *E. coli*. The catalytic turnover of the heme domain in P450 is mediated by photosensitized EY that transfers electrons from the sacrificial electron donor (TEOA) to the heme domain of P450 in cytoplasm of *E. coli*. Various substrates were used in this visible light-driven photo-biocatalytic reaction with several P450s from bacteria and humans.

The BM3m2 consists of two domains: 1) heme domain (BM3-H, 1-472 residues) responsible for catalytic reaction and 2) diflavin-reductase domain (BM3-R, 473-1049 residues) responsible for the transfer of electrons to the heme domain.[3] We constructed expression systems for each domain (i.e., BM3-H and BM3-R) and whole BM3m2 (Figure S1 a in the Supporting Innformation), and confirmed that all proteins were produced in the cytoplasm of *E. coli* with high solubility (Figure S1 b). After the supplementation of NADPH (0.10 mM), we examined the enzymatic activity of each protein in cell lysate on 7-EC. As expected, only the whole BM3m2 exhibited high activity. Neither the heme domain (BM3-H) nor the reductase domain (BM3-R) showed any detectable activities due to the lack of reductase function and catalytic function (Figure S2).

Cells producing whole BM3m2 and BM3-H accumulated high level of EY in their cytoplasm, which indicates EY's affinity to the P450 heme domain. To investigate cell staining with EY, *E. coli* cells producing whole BM3m2 or each domain were mixed with EY and cellular fluorescence was analyzed using a confocal microscope and a flow cytometer. The *E. coli* cells producing whole BM3m2 and BM3-H gave a high fluorescence in the confocal microscopic analyses, but the other *E. coli* cells producing BM3-R and *E. coli* host-only (i.e., no plasmid) did not give any detectable fluorescence (Figure S3 a). We also analyzed the EY-staining cells that produce a superfolder green fluorescent protein (sfGFP)-fused BM3-H to confirm the binding between P450 and EY by the confocal microscope. When the cells producing sfGFP-fused BM3-H were incubated with EY, two different fluorescent probes (green and red) showed identical signals (Figure S3 b). The binding of EY to other P450s used in the followed experiments was also confirmed with the confocal microscope (Figure S3 c). Flow cytometric analysis further confirmed the result; *E. coli* producing whole BM3m2 and BM3-H showed much higher fluorescence intensities than other cells producing BM3-R or no P450 (Figure 1 a). We also observed that the degree of accumulation of EY in cytoplasm of *E. coli* producing BM3-H was highly proportional to the concentration of supplemented EY (Figure 1 b). Furthermore, we examined in vitro binding of EY to each domain of P450 BM3m2 by spectrofluorometric analysis (Figure S4).

To investigate the interaction of EY with the P450 heme domain, we analyzed possible fluorescence quenching of EY by fluorescence energy transfer from EY to the heme domain. For this investigation, each protein (i.e., whole BM3m2, BM3-H, and BM3-R) was purified to a high purity by using affinity

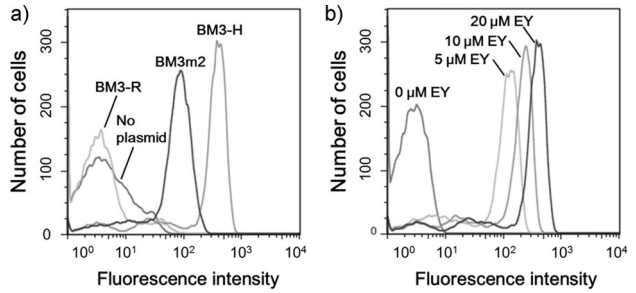


Figure 1. Fluorescent analysis of *E. coli* cells staining with EY. a) Flow cytometry analysis of EY (20 μM)-stained cells. b) Flow cytometry analysis of cells harboring pBM3-H. The cells were stained with various concentrations of EY (0–20 μM).

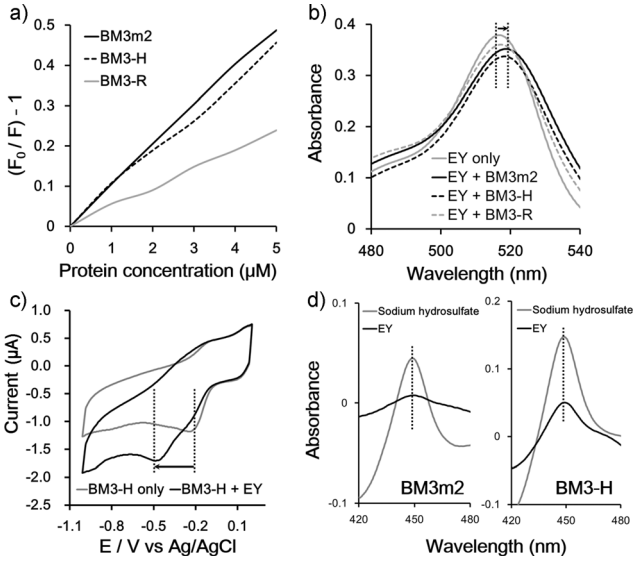


Figure 2. Analysis of the interaction between EY and purified P450s. a) Stern–Volmer relationship between EY and each protein. b) Spectroscopic analysis of EY-P450 interaction. c) Voltammetric measurement of BM3-H modified electrode in the absence and presence of EY. d) CO-difference spectra of BM^3 m2 and BM3-H reduced by sodium hydrosulfate or EY.

chromatography (Figure S5). The Stern–Volmer constants (K_{SV}) of whole BM3m2 and BM3-H were approximately twice higher than that of BM3-R, which indicates that the rate of fluorescence energy transfer from EY is higher for the heme-containing proteins (Figure 2a). Under the presence of whole BM3m2, BM3-H, and BM3-R, the temporal changes in absorption spectrum of EY were also analyzed by spectroscopic analysis. In the cases of whole P450 and BM3-H, the absorption peaks of EY at 517 nm were red-shifted (ca. 2 nm), while BM3-R did not affect the absorption spectrum of EY (Figure 2b). According to the literature,[14] the binding of EY with other proteins such as bovine serum albumin (BSA) affects the spectrophotometric property (e.g., fluorescence quenching, red-shifted absorption spectrum). To investigate electrochemical relationship between EY and P450 heme domain, we analyzed redox properties of each protein using cyclic voltammetry. In the absence of EY, reduction and oxidation of BM3-H occurred at the potential around −0.2 V (Figure 2c), which should be caused by the Fe^{III}/Fe^{II} redox couple of heme molecule (i.e., Fe-porphyrin) in the domain.[15] In the presence of EY, however, the reduction potential of BM3-H shifted significantly from −0.23 V to −0.48 V (Figure 2c). This new redox peak was observed between the reduction potentials of EY (ca. −0.95 V vs Ag/AgCl, 3 M NaCl)[16] and BM3-H. The negative shift of redox potential is due to the co-reduction of the two components,[12a] suggesting the presence of an intermediate species of EY-BM3-H. The reduction potential of BM3-R was measured at −0.51 V and no potential shift was observed with the addition of EY (Figure S6). This implies that BM3-R does not produce an electrochemical intermediate species that was observed from the BM3-H and EY couple.

Spectral binding titrations with EY were performed with P450 BM3 variants and human P450s to confirm the binding of EY to the active site of P450. The addition of EY to a solution with BM3m8 (A74G/F87V/L188Q) and human CYP3A4 produced a typical Type I conversion in the heme Soret band (Figure S7a,d). In the case of BM3m9 (R47L/L86I/L188Q) and BM3m10 (R47L/F87V/L188Q), EY binding resulted in a Type II shift (Figure S7b,c).[17] This result indicates that the EY can bind directly to the active site of the P450s including P450 BM3 and human P450s.

We also studied the activation of the P450 heme domain through the EY-mediated electron transfer from TEOA to the heme domain by using CO-binding analysis. It is well known that reduced P450 heme domain reacts with CO to exhibit a characteristic absorbance peak at 450 nm.[18] We monitored the Fe^{II}-CO absorption band at 450 nm in the CO difference spectra before and after light illumination for 5 min to the solutions containing EY, TEOA, and each purified protein (i.e., whole BM3m2, BM3-H, or BM3-R). After the light illumination in the presence of CO, we observed the strong appearance of a 450 nm absorption band for both EY-BM3-H and EY-P450, while there was no change in the absorbance for EY-BM3-R (Figure S8). In the photoreduction experiment, light is essential for the whole system and TEOA plays the role of electron donor to EY to sustain the catalytic turnover. Along these lines, we examined the effect of light and TEOA on the reduction of the P450 heme domain and observed that a reduction of heme domain occurs only in the presence of light and TEOA (Figure S9). Under photoreduction with EY, 17.43% of BM3m2 and 34.18% of BM3-H were reduced compared to the reduction of heme by sodium hydrosulfite (Figure 2d).

We conducted light-driven, cofactor-free, whole-cell P450 catalysis under visible light by combining the cellular uptake of EY and photoinduced activation of the P450 heme domain. Each of *E. coli* cells producing whole BM3m2, BM3-H, or BM3-R were incubated in a photochemical reaction solution containing EY, TEOA, and substrate (i.e., 7-EC) to analyze the conversion yield according to fluorescent intensity of the product (i.e., 7-hydroxycoumarin (7-HC)). Under light illumination, *E. coli* cells producing BM3m2 and BM3-H efficiently catalyzed the 7-EC *O*-dealkylation reaction, while the cells producing BM3-R or no P450 showed no substantial reaction yield (Figure 3a). The turnover number

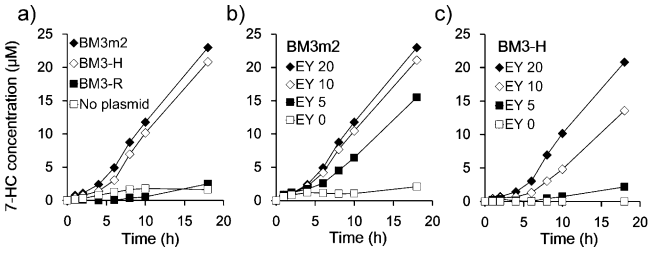


Figure 3. Whole cell photo-biocatalytic reactions by EY and P450. a) Photo-biocatalytic reactions of cells producing whole P450 or each domain under light irradiation. b,c) Photosynthetic reaction catalyzed by cells producing BM3m2 (b) or BM3-H (c) with various EY concentrations.

(concentration of product/concentration of enzyme) of BM3m2 was approximately 16 at 18 h. Under dark conditions, reaction conversions were negligible for all cases (Figure S10a). In addition, the productivity of light-driven reaction decreased drastically in the absence of TEOA, the electron donor (Figure S10b). We also confirmed that the reaction rate catalyzed by *E. coli* cells producing whole BM3m2 and BM3-H was proportional to the concentration of EY (Figure 3b,c) as well as the concentration of proteins expressed in *E. coli* cytoplasm (Figure S11). In order to confirm the role of EY, the kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) were calculated by using purified BM3m2, BM3-H and BM3m10 (Table S1, Figure S12). EY-sensitized photosynthesis in *E. coli* also depended on the wavelength of the irradiated light source. Note that EY exhibits a sharp absorption peak near 517 nm.[5e] When red ($\lambda_{max} \approx 640$ nm), green ($\lambda_{max} \approx 525$ nm), and blue ($\lambda_{max} \approx 465$ nm) light emitting diodes (LEDs) were employed as light sources, the photoenzymatic conversion by *E. coli* producing BM3-H showed the highest efficiency with green LED (Figure S13), the emission of which best fits the EY absorption spectrum.

In addition to BM3m2, we analyzed other BM3 variants (BM3m8 and BM3m10) and human P450s (CYPs 1A1, 1A2, 1B1, 2A6, and 2E1) with 7-EC as a substrate for light-driven catalysis. *E. coli* cells producing P450 were illuminated by visible light using the same experimental conditions as BM3m2 (Figure S14 and S15). Figure S16 shows all the P450s tested in this study successfully converted the substrate to 7-HC with a different conversion yield. Furthermore, we performed a EY-based P450 catalysis with practical substrates such as drugs (simvastatin, lovastatin, and omeprazole) and a steroid (17β-estradiol). The recombinant cells expressing P450s showed apparent oxidation activity toward typical human P450 substrates (Figure 4). Co-expression of rat CPR gene (shown as + CPR) did not show any apparent effect on the human P450 activity driven by EY and light. Time profiles and HPLC spectrum for each P450-catalyzed photoreaction are shown in the Figure S17–20. The maximum turnover number of P450 for each substrate is summarized in Table S2. These results indicate that the light-driven cofactor-free reaction induced by EY is generally applicable to different types of P450 enzymes including human P450s.

When the EY-P450 platform is compared to other cofactor-free systems described above,[6–9] it has significant merits that are sufficient to make the system a more valuable and powerful tool in the P450 reactions. First, with the EY-P450 platform, any pretreatments that generally require labor-intensive and time-consuming works, including purification, amino acid substitution in P450, genetic fusion, and other electron shuttle systems, are not necessary. As shown clearly, the simple mixing of EY with a suspension of cells producing P450s, is enough to drive the P450 catalyzed reactions in the whole-cells producing specific P450s. Second, the EY-P450 platform can be generally applied to different types of P450s. So far, most light-driven P450 systems could not be applied to different P450s in the same experimental condition. In contrast, we successfully demonstrated that our EY-P450 platform could be applied to various P450s including bacterial P450 BM3 variants and several human P450s (Figure 3 and 4). The point we thought most important is that EY can mediate the oxidative reaction of human drugs and steroids with human P450s, which have never been demonstrated before by other light-driven P450 platforms. Especially, the results of EY-derived CYP3A4 catalysis to generate chiral metabolites of simvastatin and lovastatin are very encouraging (Figure 4). CYP3A4 is involved in the metabolism of ca. 50% of the currently marketed drugs, and so CYP3A4 has received much attention because of its importance in drug development.[19] As the human metabolites of drugs and steroids should be very valuable and useful in the pharmaceutical field,[20] the light-driven whole cell system provides a powerful and economic tool for pharmaceutical industry. Another merit of the EY-P450 platform is that our light-driven system is based on whole-cell reaction

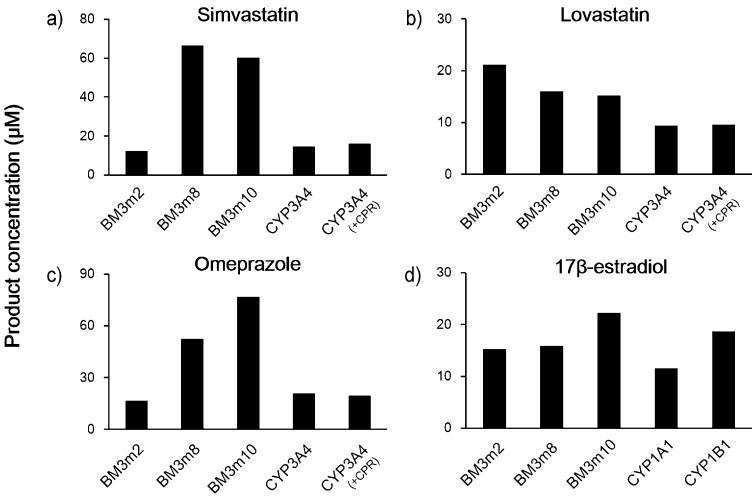


Figure 4. Productivity of whole-cell photo-biocatalytic P450 reactions by EY toward marked drugs and a steroid. a) Simvastatin 6′β-hydroxylation. b) Lovastatin 6′β-hydroxylation. c) Omeprazole 5′-hydroxylation. d) 17β-Estradiol 4′-hydroxylation. The values presented are means of results of duplicate determinations. See Figures S17–S20 for time profiles and HPLC spectrum for each light-driven P450 reaction.

unlike as other P450 platforms that are mainly cell-free systems and require massive P450 to be purified for industrial-scale reactions.

In summary, we have designed a new platform for P450-catalyzed reactions: cofactor-free, reductase-independent, visible light-driven, whole-cell P450 photocatalysis. Our analyses clearly revealed that EY can transfer photoexcited electrons directly to the heme domain of P450 for the photoactivation of P450 catalytic cycle. We have demonstrated the general and potential utility of our platform using various P450-catalyzed reactions: *O*-dealkylation of 7-EC by P450 BM3 variants and catalytic conversions of marketed drugs including simvastatin, lovastatin, 17β-estradiol, and omeprazole by different human P450s. The platform, in which photoexcited EY can activate P450 heme domain directly without the need for a redox partner and a cofactor, provides a simple and powerful tool for P450-catalyzed reactions. The whole-cell P450 system is driven by visible light with inexpensive photosensitizer and without the need for an expensive cofactor and a redox partner, thus it will become a powerful platform for developing cost-effective, scalable, and sustainable processes for P450-catalyzed reactions.

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