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COMMUNICATION

Solar-Powered Whole-Cell P450 Catalytic Platform for C-Hydroxylation Reactions

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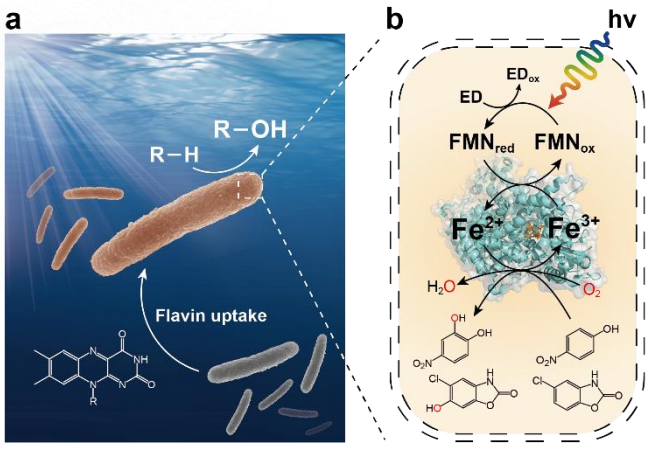
Abstract: Photobiocatalysis is a green platform for driving redox enzymatic reactions using solar energy, not needing high-cost cofactors and redox partners. Here, we present a visible light-driven whole-cell platform for human P450 photobiocatalysis using natural flavins as a photosensitizer. Photoexcited flavins mediate NADPH/reductase-free, light-driven biocatalysis by human CYP2E1 both *in vitro* and in the whole-cell systems. Our *in vitro* tests demonstrate that the photobiocatalytic activity of CYP2E1 is dependent on the substrate type, the presence of catalase, and the acid type used as a sacificial electron donor. We found a protective effect of catalase against the inactivation of CYP2E1 heme by H_2O_2 and the direct transfer of photo-induced electrons to the heme iron not by peroxide shunt. Furthermore, the P450 photobiocatalysis in whole cells containing human CYPs 1A1, 1A2, 1B1, and 3A4 demonstrates the general applicability of the solar-powered, flavin–mediated P450 photobiocatalytic system.

Introduction

Cytochrome P450s (P450 or CYP) are a superfamily of heme-containing monooxygenases that catalyze direct C-H functionalization of various endogenous and exogenous compounds using molecular oxygen and the electrons delivered by redox partners, such as diflavin-containing NADPH-P450 reductase (CPR).[1] Regioselective and stereospecific oxygenation activities of P450s toward a variety of substrates are of considerable importance in drug development and the synthesis of fine chemicals.[2] The electron transfer cascade in the P450 system begins with the CPR that delivers electrons from a cofactor [reduced nicotinamide adenine dinucleotide (phosphate), NAD(P)H] to flavin adenine dinucleotide (FAD); then, the reduced FAD transfers the electrons to flavin mononucleotide (FMN), which finally supplies the electrons to the heme of the P450.[3] Thus, efficient and continuous supplementation of electrons to the P450 heme is critical to sustain the catalytic turnover of P450s. The dependence on NAD(P)H and the redox partners limits the potential of P450s as industrial biocatalysts.[2, 4]

Here, we report whole-cell photobiocatalysis of human P450s (CYPs 2E1, 1A1, 1A2, 1B1, and 3A4) using natural flavins as a photosensitizer to accomplish direct electron transfer from the photosensitizer to the P450 heme. Scheme 1 illustrates the concept of NADPH/reductase-free, light-driven P450 reactions through photo-induced electron transfer from photoexcited flavins to the P450 heme irons in whole cells. For the light-driven P450 catalysis not relying on NADPH and reductase, three components are required: (i) a photosensitizer as a photoexcitable redox mediator, (ii) a sacrificial electron donor, and (iii) a reductase-free P450 as a biocatalyst. Flavins, which are vital compounds for diverse redox reactions in living organisms, undergo reversible redox conversion. Furthermore, they are excellent photosensitzers that absorb visible blue light.[5] As a model P450 system, we tested reductase-free CYP2E1 that was expressed in *Escherichia coli*.[6] The current study pursued two specific goals. First, we performed a set of appropriate experiments to investigate the influence of sacrificial electron donors on the catalytic activity of light-driven CYP2E1 using *in vitro* systems. This information is critical for defining the design prerequisites of a successful flavin-sensitized P450 system in whole cells and thus the scope of the concept. Second, on the basis of the gained knowledge, we applied the solar-powered whole-cell P450 catalytic platform to the synthesis of valuable metabolites using other human P450s with various substrates, including drugs (simvastatin and lovastatin)[7] and a steroid (17β-estradiol).[8]



Scheme 1. A possible mechanism for FMN as a photosensitizer to transfer electrons to CYP2E1 in the presence of sacrificial electron donor (ED) (EDTA or TEOA) and light. Direct electron transfer occurs from reduced FMN, which are made by light and sacrificial electron donors, to P450 heme without reductase. The heme can directly hydroxylate the substrate (R-H) with yielding H_2O as a side product. On the other hand, the reduced FMN quickly reacts with O_2 to yield H_2O_2 and superoxide.

Results and Discussion

To test the effect of sacrificial electron donors on the light-driven CYP2E1 biocatalysis, we used FMN as a photosensitizer both *in vitro* and in the whole-cell systems. We carried out reactions by adding catalase or superoxide dismutase (SOD) to verify whether light-driven biocatalysis occurs via the heme iron (Fe^{2+}) directly reduced by the photoexcited FMN or via the intrinsic peroxygenase activity of P450 using H_2O_2 produced by FMN and sacrificial electron donor under light. We tested a set of different sacrificial electron donors for 4-nitrophenol (4-NP) hydroxylation and chlorzoxazone (CHZ) 6-hydroxylation as marker activities of CYP2E1 (Fig. S1). 4-Nitrophenolis a typical substrate for CYP2E1. 4-Nitrophenol 2-hydroxylation has been widely used as a measure of CYP2E1 catalytic activity.[9] CHZ is a centrally acting muscle relaxant used to treat muscle spasms, which is a typical substrate for measuring CYP2E1 activity both *in vivo* (clinic) and *in vitro* human studies.[10] According to our results (Table S1), ethylenediaminetetraacetic acid (EDTA) was the best sacrificial electron donor to support photobiocatalytic 4-NP hydroxylation by CYP2E1. The presence of catalase increased the 4-NP hydroxylation activity with EDTA by 2.2-fold. On the other hand, triethanolamine (TEOA) was the most efficient donor to drive CHZ 6-hydroxylation by CYP2E1 (Table S2). For all tested acids, the enhancing effect of catalase on CHZ 6-hydroxylation activity was lower than that on 4-NP hydroxylation. We attribute the distinguishing effect of catalase on CHZ 6-hydroxylation (compared to 4-NP hydroxylation) to the protective effect of CHZ against the CYP2E1 deactivation by H_2O_2. According to the literature,[11] H_2O_2 produced by FMN/EDTA under light can inhibit the CYP2E1 activity or inactivate the CYP2E1. The heme destruction of CYP2E1 during reaction is prevented by catalase and/or by the substrate.[11] In this study, the CYP2E1 samples without catalase showed much lower total turnover numbers (TTNs) of 12-13 at 8 h incubation for 4-NP hydroxylation than the samples with catalase (TTNs of 37-38) (Fig. 1a). However, the effect of catalase on CHZ 6-hydroxylation is less pronounced than 4-NP hydroxylation (Fig. 1b). Although the presence of catalase/SOD shows the highest activity of CHZ 6-hydroxylation during the reaction with TTN of 4.1 at 12 h incubation, the enhancing effect of catalase was not apparent when compared to the case of 4-NP hydroxylation. Other samples showed similar TTNs of ~3.0 at 12 h incubation. These results indicate that the sensitivity of CYP2E1 heme to H_2O_2 is dependent on the type of substrate.

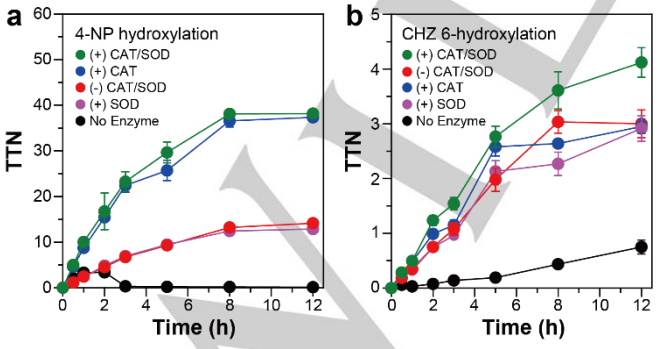


Figure 1. Time profiles of photobiocatalytic 4-NP hydroxylation (a) and CHZ 6-hydroxylation (b) catalyzed by human CYP2E1 in the presence and absence of catalase (CAT) and/or SOD. TTNs of photobiocatalytic CYP2E1 (0.4 μM) by FMN (200 μM) toward 0.5 mM substrate were measured with catalase (blue), SOD (purple), and catalase/SOD (green), or without catalase/SOD (red) for 0.5-12 h incubation. EDTA (50 mM) and TEOA (50 mM) were used as sacrificial electron donors for 4-NP hydroxylation and CHZ 6-hydroxylation assays, respectively. Concentrations of catalase and SOD were 1000 and 10 units per mL, respectively. Only P450 was excluded for negative control experiments (black). The value presented are means of results of triplicate determinations with S.E.M.

We examined the effect of substrate concentration on photobiocatalytic hydroxylation of 4-NP and CHZ by CYP2E1 (Fig. S2 and S3**)**. The production of 4-nitrocatechol (4-NC) from 4-NP was enhanced with the increasing substrate concentration up to 500 μM, in which TTNs were 38 and 14 in the presence and absence of catalase/SOD for 8 h incubation under light, respectively (Fig. S2). The addition of catalase and SOD to the reaction mixture increased the TTNs by 2.7-fold. This result clearly shows that photobiocatalytic 4-NP hydroxylation occurred via direct electron transfer to the heme iron by reduced FMN, not by the peroxide shunt. It is well known that H_2O_2 inhibits P450 activities via heme modification.[12] The CYP2E1 activities in the absence of catalase should come from either (1) the reduced heme iron by a direct electron transfer from activated FMN or (2) the peroxygenase activity of CYP2E1 supported by H_2O_2 that is generated by photoexcited FMN/EDTA. The enhancement of the CYP2E1 activity by catalase indicates that CYP2E1 has very low or no peroxygenase activity. According to the literature,[12] cumene hydroperoxide supported the peroxygenase activities of CYP2E1 toward ethanol and alkyl isonicotinic acid esters. However, CYP2E1 peroxygenase activity supported by H_2O_2 has not been reported yet.

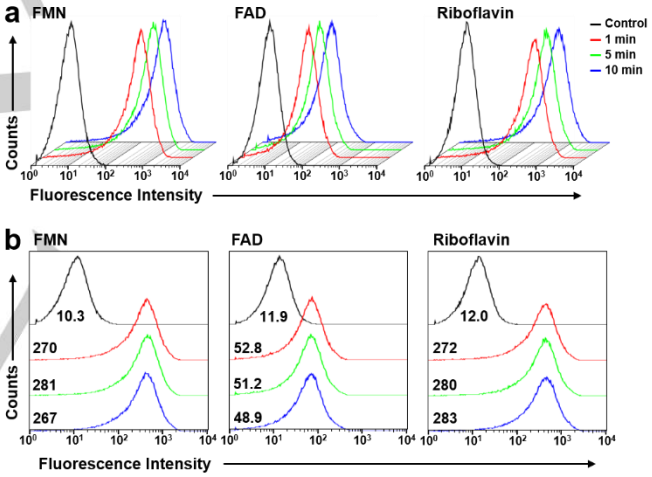


Figure 2. Cytometry analysis to measure the transport of flavins into *E. coli* cells. (a) Combined histograms of cytometry showing the transport of each flavin into the *E. coli* cells with time course. (b) Comparison of cytometric histograms with mean fluorescence intensity of each analysis. Cytometry experiments to check the time course of 1, 5, and 10 min were performed for FMN, riboflavin, and FAD to enter into *E. coli* cells. Twenty μM of each flavin was used. Absorbance of *E. coli* cells at 660 nm was 1.0.

To validate the photocatalytic activity of CYP2E1 by direct reduction of heme through FMN-mediated electron transfer from EDTA, we conducted CO-binding analysis.[14] After light irradiation to the solutions containing FMN, EDTA, and CYP2E1, the characteristic Fe^{2+}·CO absorption peak at 450 nm appeared, which evidences substantial reduction of the heme moiety (Fig. S4). Photoexcited FMN reduced 21% and 12% of the CYP2E1 in the presence and absence of catalase, respectively, when compared to the complete reduction of the CYP2E1 heme by sodium hydrosulfite. The increase of heme reduction by catalase shows the protective effect of the catalase on heme destruction. This result supports that photoexcited FMN induced the reduction of the CYP2E1 heme iron using EDTA as an electron donor. Note that the characteristic P450 peak was not observed when any

component (i.e., EDTA, FMN, or light) was missing. Our *in vitro* tests demonstrate that the photobiocatalytic activity of CYP2E1 is dependent on the substrate type, the presence or absence of catalase, and the acid type used as a sacificial electron donor. The enhanced product formation of the photoactivated CYP2E1 in the presence of catalase suggests a protective effect of catalase against the inactivation of CYP2E1 heme by H_2O_2. The inconsistent effect of substrate type and catalase on the light-driven activity of CYP2E1 is attributed to the distinct sensitivity of each P450 system on H_2O_2.

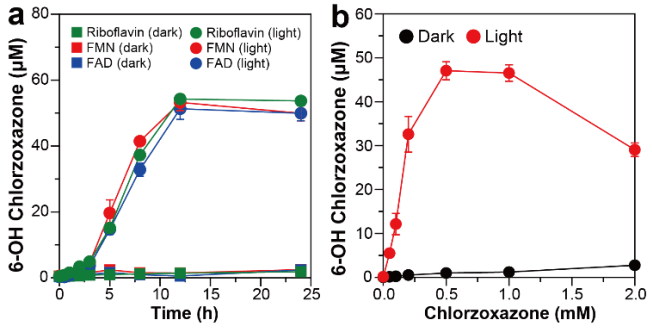


Figure 3. Whole-cell photobiocatalytic reactions by flavins and human CYP2E1. (a) Time profiles of photobiocatalytic CHZ 6-hydroxylation in whole cells expressing CYP2E1. The product concentrations were determined with 0.5 mM CHZ, 200 μM flavin, and 50 mM TEOA in whole cells (15 g cells L^{-1}). Negative control experiments (squares marked with dark) with 200 μM flavin, in which only light was excluded. (b) Dependence of photobiocatalytic CHZ 6-hydroxylation on substrate concentrations in whole cells expressing CYP2E1. The product concentration was determined with 0.05–2 mM CHZ, 200 μM FMN, and 50 mM TEOA in whole cells (15 g cells L^{-1}) after 12 h incubation. Negative control experiments (black circle) with 200 μM FMN, in which only light was excluded. The value presented are means of results of triplicate determinations with S.E.M.

For whole-cell photocatalysis (Table S3, Fig. S5), we observed the transport of flavins into the *E. coli* cells producing CYP2E1 by cytometric analysis (Fig. 2). Utilization of whole-cell catalysis has more application potential than enzyme catalysis, because the whole-cell catalysis not only simplifies the downstream processing, but also enzymes are significantly more stable in the protected cell environment.[15] The redox system of *E. coli* was not omitted but a light-dependent regeneration system was used for the whole-cell photocatalysis. When each flavin (20 μM) was added to the cells, the florescent intensities at three time intervals (1, 5, and 10 min) and even at 6 h were similar. The population of the *E. coli* cells with EDTA or TEOA was not apparently changed up to 12 h by analysis of the cell debris using the flow cytometry. This result indicates that the transport of flavins is saturated within a minute. The fluorescent intensities of flavins were in order of FMN ≈ riboflavin > FAD. This result shows that flavins simultaneously enter into the cytoplasm of *E. coli.* The combination of the cellular uptake of flavins and the photo-induced activation of the CYP2E1 increased the 6-OH CHZ production of *E. coli* cells producing CYP2E1 up to 51-53 μM (10-11% yields) for all three tested flavins (i.e., riboflavin, FMN, and FAD) with 0.5 mM CHZ at 12 h incubation (Fig. 3a). The productivity of 6-OH CHZ at 0.2 mM and 0.5 mM CHZ was 32 μM (16% yield) and 47 μM (9.4% yield), respectively, at 12 h incubation (Fig. 3b). Decreasing the product formation at 1 and 2 mM substrate is considered as substrate inhibition. The productivity of 6-OH CHZ increased with the increasing FMN concentration (Fig. S6).

To demonstrate general applicability of solar-powered P450 catalysis in whole cells, we tested other human P450s (CYPs 1A1, 1A2, 1B1, and 3A4) in addition to CYP2E1 using drugs (lovastatin and simvastatin)[7] and a steroid (17β-estradiol)[8] as substrates. The recombinant cells expressing human CYPs 1A1, 1A2, 1B1, and 3A4 showed apparent product formation toward corresponding substrates such as lovastatin, simvastatin, and 17β-estradiol (Fig. 4). These results indicate that the reductase/cofactor-free solar reactions sensitized by flavins are generally applicable to different types of human P450 enzymes expressed in whole cells. According to the literature,[16] *in vivo* overproduction of riboflavin, FMN, and FAD is possible by modular engineering of the flavin pathway in bacteria such *E. coli* and *Bacillus subtilis*. Engineered *E. coli* strain by overexpression of *ribF* gene encoding the bifunctional riboflavin kinase/FMN adenylyltransferase could increase the intracellular concentrations of FMN and FAD up to 481 and 213 μM, respectively.[16c] We envision that the engineered cells may generate the natural photosensitizer intracellularly for driving P450 photocatalysis in human P450- expressing recombinant bacterial cells.

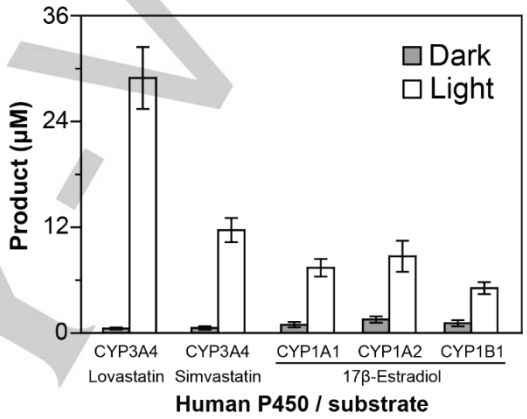


Figure 4. Whole-cell photobiocatalytic reactions by FMN and human CYPs. Product concentrations of 6'β-OH lovastatin, 6'β-OH simvastatin, and 4-OH 17β-estradiol obtained from lovastatin, simvastatin, and 17β-estradiol were shown, respectively. Corresponding human P450 enzymes to catalyze each hydroxylation reaction were indicated. The product concentrations were determined with 1 mM (lovastatin, simvastatin) or 0.5 mM (17β-estradiol), 200 μM FMN, and 50 mM TEOA in whole cells expressing each P450 (15 g cells L^{-1}) after 5 h incubation for lovastatin and simvastatin, and after 12 h incubation for 17β-estradiol. Negative control experiments (filled bar), in which only light was excluded. The value presented are means of results of triplicate determinations with S.E.M.

To overcome the dependence of P450 catalysis on high-cost cofactor NAD(P)H and the redox partners that limited the employment of P450 enzymes in the industry[2, 4], many attempts have been made in the past, such as NAD(P)H regeneration and the use of oxygen surrogates (i.e., H_2O_2).[17] Industrial-scale cofactor regeneration is an enzymatic method that uses secondary enzymes (e.g., formate dehydrogenase, glucose dehydrogenase).[18] Various other cofactor regeneration methods include chemical,[19] (photo)electrochemical,[20] and photocatalytic[21] routes.[22] The electrochemical approach has some advantages, such as (i) low-cost electricity, (ii) transfer of quasi mass-free electrons, and (iii) easy control of regeneration kinetics by tuning electrons' Fermi level.[18, 23] However, the reaction rates and productivities of electrochemical platforms are generally lower than those of enzymatic platforms.[24]

For industrial fine chemical production, the economic feasibility of a given enzymatic reactions requires minimal productivity of 133 (kg product/kg enzyme) and minimal TTN of 26,666. In other words, a minimum space-time yield of 0.1 g L^{-1} h^{-1} and a minimal final product concentration of 1 g L^{-1} are required.[25]

Recently, we have shown that flavins mediate light-driven catalysis by bacterial CYP102A1 heme domain using *in vitro* systems.[26] In the previous study, the flavin-sensitized CYP102A1 activities mainly resulted from H_2O_2-supported peroxygenase activity of the CYP102A1 heme domains. In this study, we found that human CYP2E1 catalyzes light-driven catalysis with the direct transfer of photo-induced electrons to the heme iron without apparent peroxygenase activity. The photobiocatalytic activity of CYP2E1 was also dependent on the substrate type and the acid type used as a sacificial electron donor. Futhermore, flavins could mediate light-driven catalysis by human CYP2E1 and other various human P450s in whole-cell systems. The current study is a proof of concept, so the level of productivity is lacking for industrial applications yet. Future follow-up studies are expected to improve the productivity through *de novo* synthesis of photosensitizers (i.e., FMN and FAD),[16] engineering of P450s through directed evolution,[27] and reaction optimization of photochemo-enzymatic whole-cell process.[28]

Conclusion

We have devised a catalytic scheme for whole-cell-based flavin-sensitized P450 catalytic reactions that are free of NADPH and a reductase. The current study demonstrates that the need for reductase (i.e., CPR) and cofactor NADPH (or its complicated regenerating system) can be avoided using visible light as a source of energy and EDTA (or TEOA) as an electron donor. We have shown that natural flavins mediate light-driven catalysis by human CYP2E1 both *in vitro* and in the whole-cell systems. The photoactivation of flavins was productively coupled with the direct transfer of photo-induced electrons to CYP2E1 heme iron to boost the photobiocatalytic C-hydroxylation reactions of P450. Furthermore, the FMN-sensitized P450 biocatalysis in whole cells expressing human CYPs 1A1, 1A2, 1B1, and 3A4 for the bioconversion of marketed drugs and a steroid was conducted to demonstrate general applicability of the photobiocatalytic system. The light-driven, flavin-sensitized P450 catalysis paves a cost-effective and eco-friendly way to create a promising discipline of green and sustainable chemistry with high potential for P450 applications.

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Keywords: biocatalysis • C-H activation • cytochrome P450 • hydroxylation • photocatalysis

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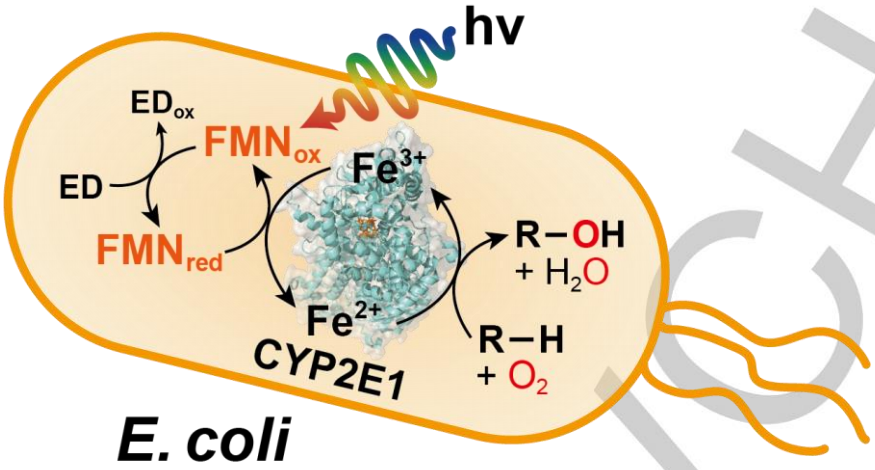

Schematic illustration of whole-cell photobiocatalytic reaction by CYP2E1 with FMN as a photosensitizer. The catalytic turnover of human CYP2E1 is mediated by photosensitized FMN that directly transfers electrons from the sacrificial electron donor to the heme of CYP2E1. The FMN-sensitized P450 biocatalysis in whole cells expressing human CYPs 1A1, 1A2, 1B1, and 3A4 for the bioconversion of marketed drugs and a steroid demonstrates general applicability of the photobiocatalytic system.