Electronic Supplementary Information

Solar-Driven Biocatalytic C-Hydroxylation through Direct Transfer of Photoinduced Electrons

Thien-Kim Le,[a,‡] Jong Hyun Park,[b,‡] Da Som Choi,[c,‡] Ga-Young Lee,[a] Woo Sung Choi,[b] Ki Jun Jeong*,[b], Chan Beum Park,*,[c] and Chul-Ho Yun*,[a]

[a] School of Biological Sciences and Technology, Chonnam National University, 77 Yongbong-ro, Buk-gu, Gwangju 61186, Republic of Korea.

[b] Department of Chemical and Biomolecular Engineering, KAIST Institute for BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 335 Science Road, Daejeon 34141, Republic of Korea.

[c] Department of Materials Science and Engineering, KAIST, 335 Science Road, Daejeon 34141, Republic of Korea.

‡These authors contributed equally to this work: Thien-Kim Le, Jong Hyun Park, Da Som Choi

*Correspondence and requests for materials should be addressed to K.J.J. (kjjeong@kaist.ac.kr), C.B.P. (parkcb@kaist.ac.kr), or C.H.Y. (chyun@jnu.ac.kr).

Table of Contents

Table S1. Variation in the amino acid sequences of the CYP102A1 variants used in this study.

Variant	Mutated amino acid residues	reference
Wild-type		(1)
M1	F87A	(2)
M8	A74G/F87V/L188Q	(3)
M10	R47L/F87V/L188Q	(3)
M16	R47L/F81I/F87V/E143G/L188Q/E267V	(3)
M16V2	R47L/F81I/F87V/E143G/L188Q/E267V/A474V/E558D/ T664A/P675L/A678E/E687A/A741G/K813E/R825S/ R836H/E870N/I881V/E887G/P894S/S954N/M967V/ Q981R/ A1008D/ H1021Y/Q1022E[a]	(4)
F2	R47L/Q73R/F81I/F87V/S108N/E143G/L188Q/I220M/ E267V/A474V/E558D/T664A/P675L/A678E/E687A/ A741G/K813E/R825S/R836H/E870N/I881V/E887G/ P894S/S954N/M967V/Q981R/A1008D/ H1021Y/Q1022E[a]	(4)

[a]Variant M16V2 and F2 have additional 20 mutations at the reductase domain (underlined).

***Table S2*.** Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Sources or Refs
E. coli strain		
XL1-Blue	*F'::Tn10 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96 (Nal*^{r}*) thi hsdR17(*$r_K^-m_K^+$*) gln V44relA1 lac*	Stratagene Co. (Santa Clara, CA)
Plasmid		
pTac99A	pTrc99A derivative, *tac* promoter, Amp^{r}	(5)
pBM3-H	pTac99A derivative, heme domain of BM3	This study
pBM3m8-H	pTac99A derivative, heme domain of BM3m8	This study
pBM3m10-H	pTac99A derivative, heme domain of BM3m10	This study
pBM3m16-H	pTac99A derivative, heme domain of BM3m16	This study
pBM3F2-H	pTac99A derivative, heme domain of BM3F2	This study

***Table S3*.** Oligonucleotides used in PCR.

Name	Sequences (5′ → 3′)
BM3F-EcoRI	GGGAATTCATGACAATTAAAGAAATGCCTCAGCC (*Eco*RI)*
HemeR-NcoI	GCCCATGGCTATTAATGGTGGTGATGATGGTGGCGTACTTTTTTA GCAGACTGTTCAG (*Nco*I)*

* The restriction enzyme sites are underlined and described in parentheses.

Table S4. Light-driven 4-NP hydroxylation by CYP102A1 variants[a]

Variant	Total turnover numbers of 4-Nitrocatechol formation (mol products/mol enzymes)					
	Riboflavin		FAD		FMN	
	(–) Catalase	(+) Catalase	(–) Catalase	(+) Catalase	(–) Catalase	(+) Catalase
Wild-type	9.6 ± 0.4	27 ± 2	3.7 ± 1	6.4 ± 0.1	15 ± 1	33 ± 1
heme wild-type	2.6 ± 0.5	18 ± 1	0.90 ± 0.05	1.7 ± 0.5	14 ± 1	20 ± 1
BM3 M8	5.6 ± 0.4	16 ± 2	1.8 ± 0.3	2.3 ± 0.4	8.2 ± 2.3	18 ± 1
heme BM3 M8	31 ± 1	27 ± 6	3.7 ± 0.7	1.7 ± 0.5	53 ± 10	25 ± 1
BM3 M10	9.1 ± 0.3	25 ± 1	2.9 ± 0.9	1.7 ± 0.9	13 ± 1	28 ± 1
heme BM3 M10	14 ± 1	23 ± 1	1.1 ± 0.8	2.4 ± 0.7	27 ± 5	24 ± 3
BM3 M16	13 ± 1	20 ± 1	5.1 ± 0.8	8.7 ± 1.0	15 ± 2	23 ± 1
heme BM3 M16	99 ± 11	46 ± 6	67 ± 10	3.8 ± 1.3	139 ± 15	54 ± 11
BM3 F2	76 ± 1	39 ± 1	68 ± 6	11 ± 1	94 ± 3	47 ± 4
heme BM3 F2	50 ± 5	31 ± 1	10 ± 3	2.9 ± 0.7	60 ± 4	27 ± 1

[a]The photo-biocatalytic reactions by CYP102A1 variants and their heme domains were conducted and analyzed by HPLC as described at the Experimental. The values presented are means of results of duplicate determinations with standard errors.

Table S5. Distribution of hydroxylated products of lauric acid by CYP102A1 variants[a]

	Light-driven hydroxylation				NADPH supported reaction	
	F2 holoenzyme		F2 heme domain		F2 holoenzyme	M1
Position of hydroxylation	(–) catalase	(+) catalase	(–) catalase	(+) catalase		
C-7	9	10	24	15	7	17
C-8	7	8	11	8	5	20
C-9	51	55	41	55	60	45
C-10	21	20	12	15	17	11
C-11	11	8	11	7	10	8

[a]Regioselectivity of the hydroxylated products of fatty acids at positions C-7 through C-11 was determined. Values are the means of two independent experiments. All standard errors were less than 10% of the average.

Table S6. Summary of the light-driven hydroxylation of 4-NP and lauric acid by CYP102A1 heme domain under different reaction compositions

Substrate (μM)	BM3 (heme domain)	Mediator (flavin, μM)	Source of reducing equivalents (EDTA, mM)	Incubation time (h)	Catalase	SOD	TTN	% yield
4-NP								
500	16	Riboflavin, 200	50	5	+	–	46	7.3
500	16	FAD, 200	50	5	+	–	3.8	0.61
500	16	FMN, 200	50	5	+	–	54	8.7
500	F2	Riboflavin, 200	50	5	+	–	31	4.9
500	F2	FAD, 200	50	5	+	–	2.9	0.46
500	F2	FMN, 200	50	5	+	–	27	4.3
500	F2	FMN, 200	50	5	+	+	24	3.9
500	F2	FMN, 100	50	5	+	+	14	2.2
500	F2	FMN, 100	20	5	+	+	10	1.7
1000	F2	FMN, 200	50	5	+	+	57	4.6
1000	F2	FMN, 200	50	24	+	+	52	4.1
Lauric acid								
50	16	Riboflavin, 200	50	8	+	–	5.1	4.1
50	16	FAD, 200	50	8	+	–	0.83	0.67
50	16	FMN, 200	50	8	+	–	5.6	4.5
50	F2	Riboflavin, 200	50	8	+	–	4.6	3.6
50	F2	FAD, 200	50	8	+	–	N.D.	N.D.
50	F2	FMN, 200	50	8	+	–	5.5	4.3
50	F2	FMN, 200	50	8	+	+	4.9	4.0
50	F2	FMN, 200	50	24	+	+	9.9	7.9
500	F2	FMN, 200	50	8	+	+	10	0.81

N.D. not detectable

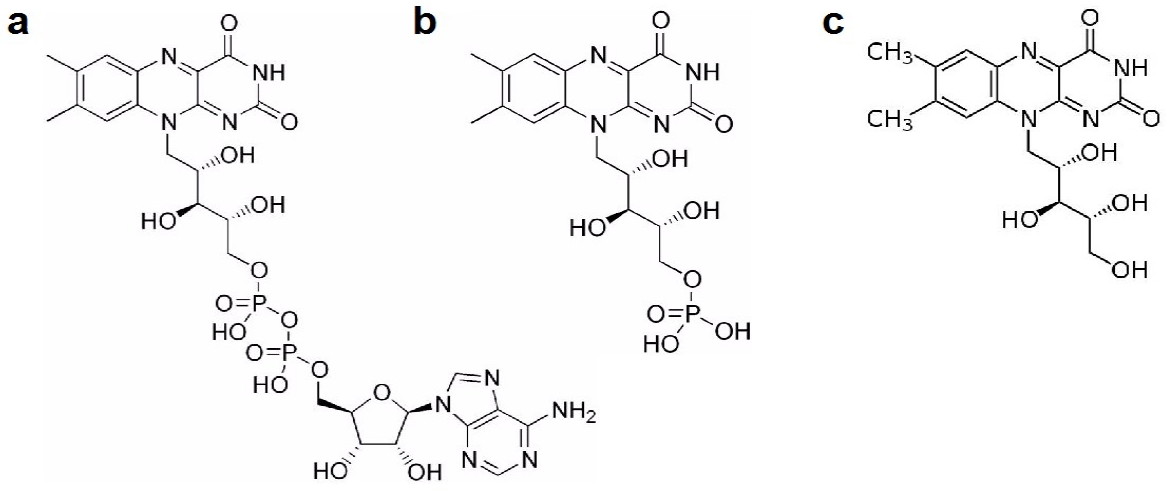


Figure S1. Structure of flavins. FAD (**a**), FMN (**b**), and riboflavin (**c**).

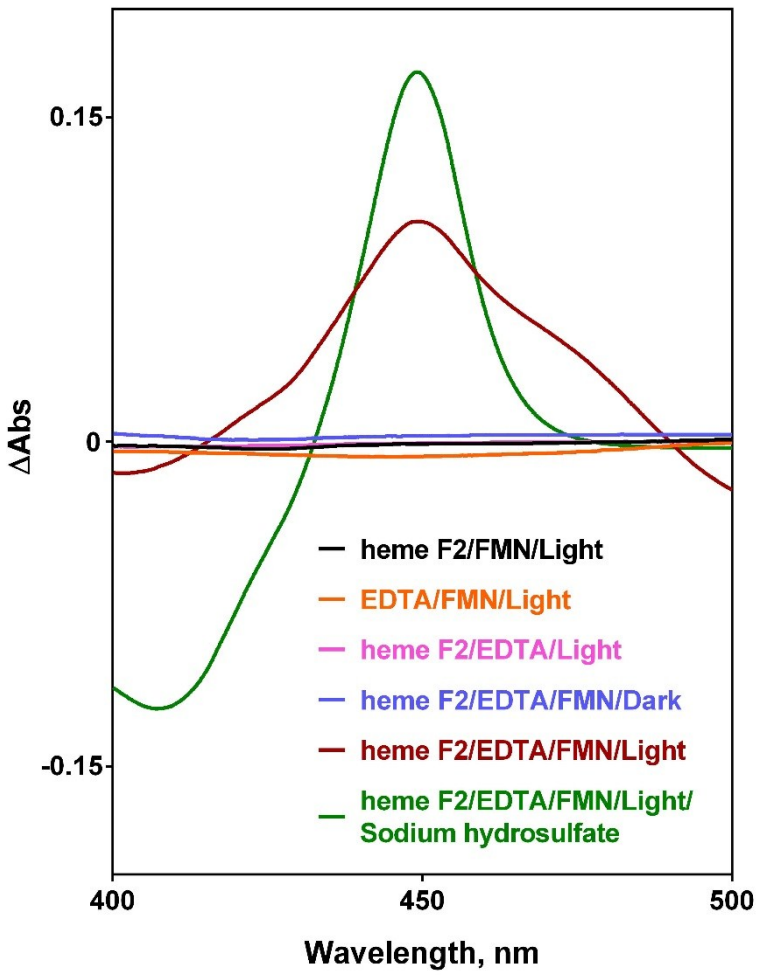


Figure S2. Light-induced, direct reduction of P450 heme by flavin. Formation of the characteristic CO difference spectra were obtained by light-driven reduction of the heme domain of CYP102A1 F2 variant in the presence of FMN as a photosensitizer. Complete reduction of the P450 was performed using sodium hydrosulfite. For negative control experiments, each component (EDTA, FMN, and light) was excluded. In contrast, the apparent Fe^{2+}·CO absorption band at 450 nm was not observed for the heme domain of WT CYP102A1 (results not shown). Photoexcited FMN reduced 59% of the F2 heme domain when compared to the complete reduction of the heme domain by sodium hydrosulfide.

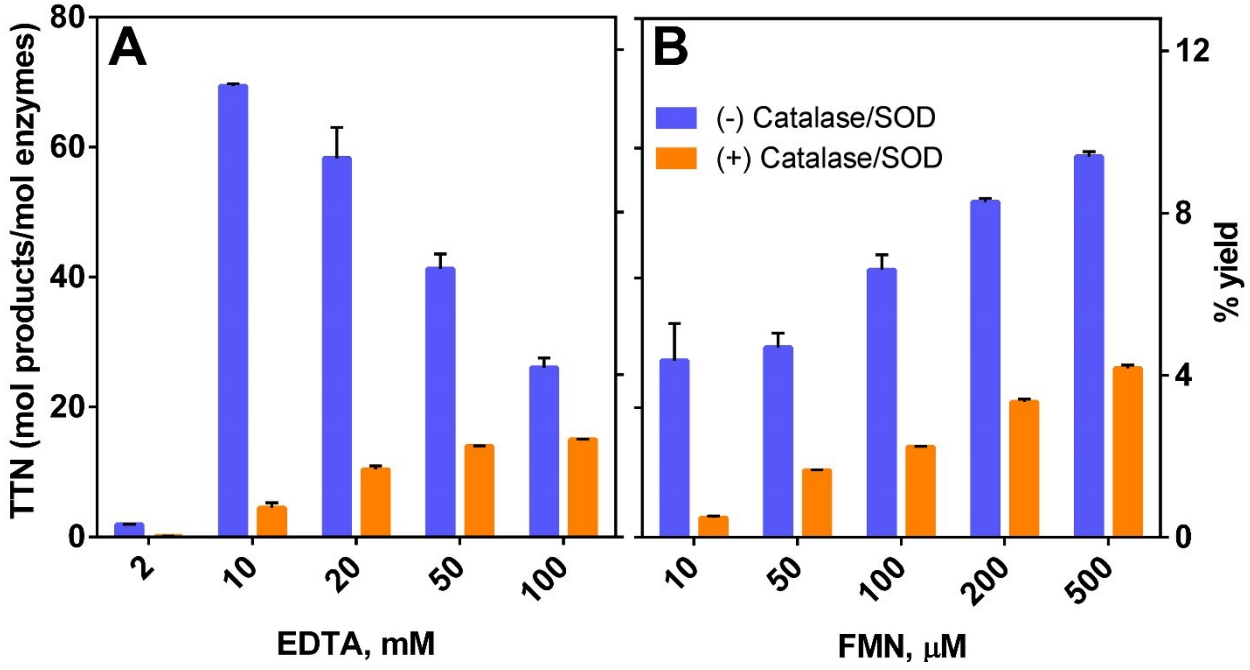


Figure S3. The concentration effect of EDTA (A) and FMN (B) on 4-NC formation (TTNs) and yield (%) using F2 heme domain/flavin/EDTA/light system in the presence and absence of catalase (2,000 units/ml) and SOD (10 units/ml). (A) Each reaction mixture was consisted of F2 heme domain (0.80 μM), FMN (100 μM), 4-NP (0.5 mM), and in the range of 2~100 mM EDTA. (B) Each reaction mixture was consisted of F2 heme domain (0.80 μM), EDTA (50 mM), 4-NP (0.5 mM), and in the range of 10~500 μM FMN. For control experiments, each of FMN or enzyme was excluded from the full set of reaction mixture. Reaction was performed for 5 h in the presence of light. The values presented are means of results of duplicate determinations with standard errors.

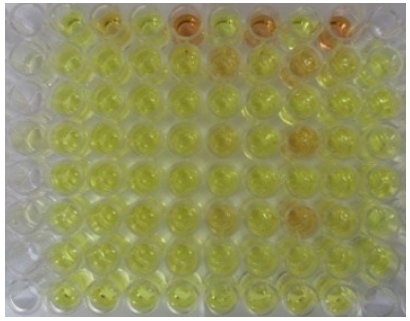
a)

Component	*composition*	Final concentration
Enzyme	P450	0.4 μM
H_2O_2 Scavenger	Catalase	20,000 U/mL
Substrate	4-NP	500 μM
Electron donor	EDTA	50 mM
Photosensitizer	Flavin	50 μM
Buffer	Tris-HCl	100 mM (pH 7.4)

b)

Flavin only	#8	#8	#16	#16	M16V2	M16V2	F2	F2	No enzyme
	(-) NADPH	(+) NADPH	(-) NADPH	(+) NADPH	(-) NADPH	(+) NADPH	(-) NADPH	(+) NADPH	
Riboflavin	EDTA + Riboflavin	EDTA + Riboflavin + Catalase	EDTA + Riboflavin	EDTA + Riboflavin + Catalase	EDTA + Riboflavin	EDTA + Riboflavin + Catalase	EDTA + Riboflavin	EDTA + Riboflavin + Catalase	EDTA + Riboflavin
	Riboflavin	Riboflavin + Catalase	Riboflavin	Riboflavin + Catalase	Riboflavin	Riboflavin + Catalase	Riboflavin	Riboflavin + Catalase	EDTA + Riboflavin + Catalase
FAD	EDTA + FAD	EDTA + FAD + Catalase	EDTA + FAD	EDTA + FAD + Catalase	EDTA + FAD	EDTA + FAD + Catalase	EDTA + FAD	EDTA + FAD + Catalase	EDTA + FAD
	FAD	FAD + Catalase	FAD	FAD + Catalase	FAD	FAD + Catalase	FAD	FAD + Catalase	EDTA + FAD + Catalase
FMN	EDTA + FMN	EDTA + FMN + Catalase	EDTA + FMN	EDTA + FMN + Catalase	EDTA + FMN	EDTA + FMN + Catalase	EDTA + FMN	EDTA + FMN + Catalase	EDTA + FMN
	FMN	FMN + Catalase	FMN	FMN +Catalase	FMN	FMN + Catalase	FMN	FMN + Catalase	EDTA + FMN + Catalase
	EDTA	EDTA + Catalase	EDTA	EDTA + Catalase	EDTA	EDTA + Catalase	EDTA	EDTA + Catalase	

c)



d)

Flavin only	#8	#8	#16	#16	M16V2	M16V2	F2	F2	No enzyme
	0.1106	0.1845	0.0939	0.4007	0.1167	0.3494	0.0907	0.3315	
0.0956	0.0879	0.1152	0.1384	0.1232	0.2035	0.1178	0.2138	0.1463	0.0898
	0.139	0.1171	0.1262	0.1322	0.1088	0.1203	0.1227	0.1269	0.1038
0.1215	0.0976	0.1093	0.1125	0.117	0.148	0.1121	0.2204	0.1367	0.0975
	0.0985	0.1098	0.1208	0.1257	0.1049	0.1163	0.1089	0.1208	0.1044
0.0907	0.0965	0.1087	0.128	0.1234	0.1712	0.118	0.2282	0.1377	0.0889
	0.1011	0.1121	0.1232	0.1342	0.1077	0.1239	0.111	0.1437	0.1595
	0.0914	0.0986	0.0945	0.0976	0.1046	0.129	0.1078	0.1054	

Figure S4. The photo-biocatalytic reactions by CYP102A1 variants (whole enzyme) conducted in 96-microwell plates. (**a**) Typical reaction mixture consisted of P450 holoenzymes, flavin, and 4-NP in 0.25 ml of Tris-HCl buffer (pH 7.4) containing EDTA. (**b**) Summary of components of each reaction. Positive and negative control samples were prepared by adding NADPH generating system (shown as NADPH) and emitting each component of the whole system, respectively. (**c**) The reaction mixture was irradiated for 5 h with visible light using a white-light emitting plate. (**d**) The conversion of 4-NP to catechol product was monitored by a microplate reader at 510 nm as described at the Experimental.

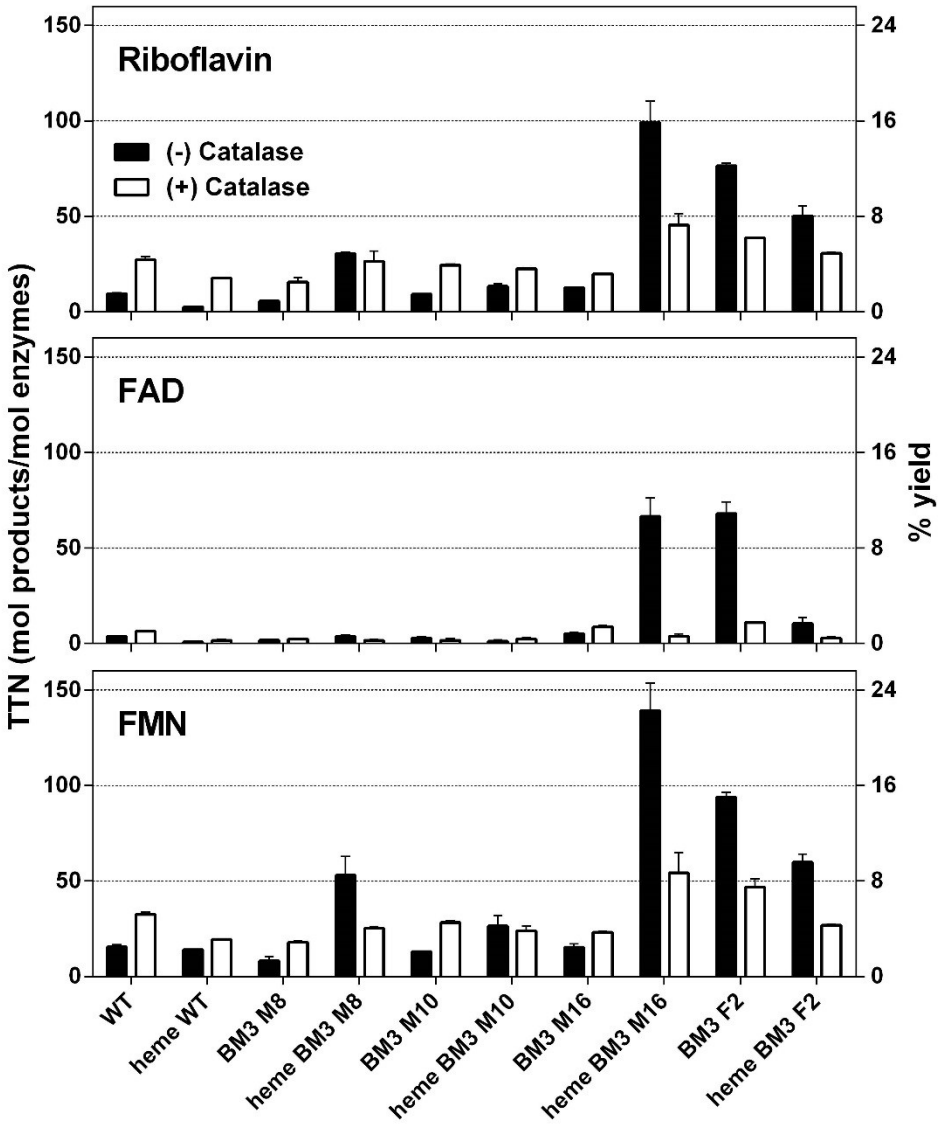


Figure S5. Total turnover numbers and yields (%) of photo-biocatalytic CYP102A1 reactions of variant holoenzymes and their heme domains by flavins toward 4-NP. 4-NP hydroxylation to make a catechol product was measured in the presence of riboflavin (**a**), FAD (**b**), and FMN (**c**) as a photosensitizer. Reaction mixture contained 0.80 μM P450, 200 μM flavin, and 50 mM EDTA with (closed square) or without (open square) of catalase (2,000 units/ml) in Tris-HCl buffer (100 mM, pH 7.4). The reaction was performed in the presence of light for 5 h. The values presented are means of results of duplicate determinations with standard errors.

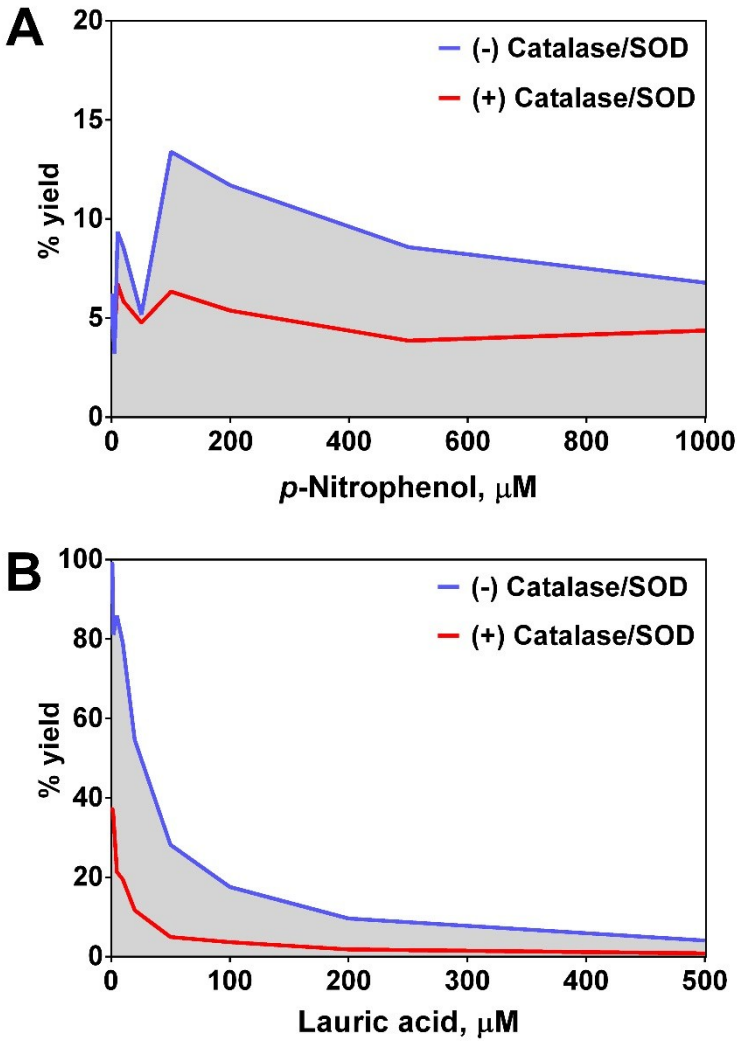


Figure S6. Yield (%) of photobiocatalytic productivity of 4-NP hydroxylation (A) and lauric acid hydroxylation (B) dependent on substrate concentration. Each reaction mixture was consisted of F2 heme domain in the presence or absence of catalase and SOD. Reactions were performed for 4 and 8 h toward 4-NP (A) and lauric acid (B), respectively, in the presence of light. The concentrations deployed for 4-NP (A) and lauric acid (B) were from 1 μM to 1 mM in the presence (red circles) or absence (blue circles) of catalase/SOD. Negative control experiments, in which only P450 was excluded, showed that the product concentration of 4-NP was less than 0.1% of substrate and that the product concentration of lauric acid was not detectable.

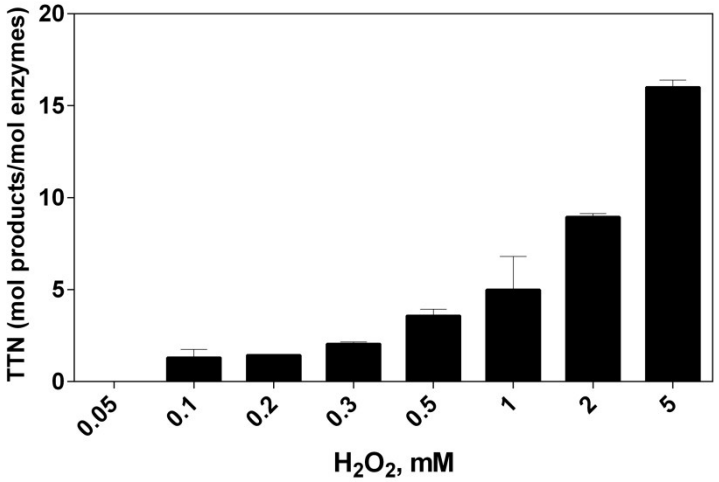


Figure S7. Dependence of H_2O_2 concentration on lauric acid hydroxylation. H_2O_2 at the indicated concentration was externally added to the reaction mixture of 50 μM lauric acid and 0.40 μM F2 heme domain in 0.50 ml of 100 mM KPi buffer (pH 7.4) at 23 °C. The reaction proceeded for 1 h.

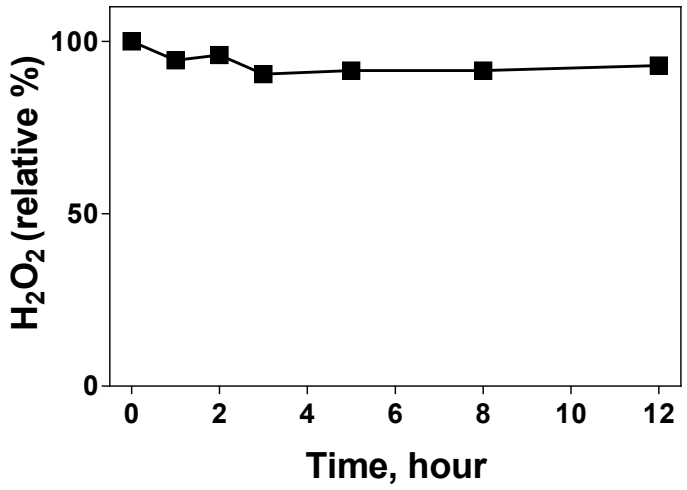


Figure S8. Stability of H_2O_2 up to 12 h. After 1 μM H_2O_2 was externally added to the reaction buffer of 100 mM Tris-HCl buffer (pH 7.4) at 23 °C, H_2O_2 concentration were determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit at indicated times. The values presented are means of results of duplicate determinations with standard errors. During 5-12 h incubations, approximately 92% H_2O_2 remained.

References for Electronic Supplementary Information

1. R. T. Ruettinger, L. P. Wen and A. J. Fulco, *J. Biol. Chem*., 1989, **264**, 10987.

2. A. B. Carmichael and L. L. Wong, *Eur. J. Biochem*., 2001, **268**, 3117.

3. D. H. Kim, K. H. Kim, D. H. Kim, K. H. Liu, H. C. Jung, J. G. Pan and C. H. Yun, *Drug Metab. Dispos.*, 2008, **36**, 2166.

4. J. Y. Kang, S. H. Ryu, S. H. Park, G. S. Cha, D. H. Kim, K. H. Kim, A. W. Hong, T. Ahn, J. G. Pan, Y. H. Joung, H. S. Kang and C. H. Yun, *Biotechnol. Bioeng*., 2014, **111,** 1313.

5. S. J. Park and S. Y. Lee, *Biotechnol. Tech.,* 1998, **12**, 815.