Supporting Information

Tryptophan-96 in cytochrome P450 BM3 plays a key role in enzyme survival

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Contents

Materials

Unless otherwise noted, all chemicals and reagents for chemical reactions were obtained from commercial suppliers (Sigma-Aldrich) and used without further purification.

Site-directed mutagenesis

Plasmid pET22 was used as cloning vector. Site-directed mutagenesis was accomplished by a QuikChange Site-Directed Mutagenesis kit (Qiagen) using primers bearing desired mutations (Invitrogen). Forward primer: 5'-ACGCATGAAAAAAATCACAAAAAAGCGCATAAT-3'. Respective reverse primer: 5'-ATTATGCGCTTTTTTGTGATTTTTTTCATGCGT-3'. Samples were prepared on ice, containing 2 µL buffer stock (10X), 2 µL dNTP, 25 ng of parent plasmid, 50 ng of forward primer, 50 ng of reverse primer, 0.4 µL Pfu DNA polymerase and milli-Q water to a total volume of 20 µL. The Polymerase Chain Reaction (PCR) protocol was performed on a MJ Research PT150 Minicycler for 18 cycles of 30 s at 95°C, 30 s at 95°C, 60 s at 55°C, and 9 min at 68°C. Digestion of methylated DNA (parent plasmid) was achieved by addition of 1 µL dpn1 enzyme and incubation at 37 °C for one h. The PCR mixtures were stored at –20 °C until needed.

The amino acid sequence for WT (holoprotein) is as follows:

>SEQ ID NO:1: gi|142798|gb|AAA87602.1| cytochrome P-450:NADPH-P-450 reductase precursor [*Bacillus megaterium*]

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDKNLSQALKFVRD
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VSEADARLWLQQLEEKGRYAKDVWAGHHHHHH

The nucleotide sequence for WT (holoprotein) is as follows:

ATGACAATTAAAGAAATGCCTCAGCCAAAAACGTTTGGAGAGCTTAAAAATTTACCGTTATTAAACACAGATAAAC
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CAGCAGCTAGAAGAAAAAGGCCGATACGCAAAAGACGTGTGGGCTGGGCTCGAGCACCACCACCACCACCACTG
AGATCCGGCTGCTAACAAAGC

The primers for site-directed mutagenesis:

W96H-forward: ACGCATGAAAAAAATCACAAAAAAGCGCATAAT

W96H-reverse: ATTATGCGCTTTTTTGTGATTTTTTTCATGCGT

W90F-forward: GGGTTATTTACAAGCTTCACGCATGAAAAAAAT

W90F-reverse: ATTTTTTTCATGCGTGAAGCTTGTAAATAACCC

Y334F-forward: GCGTTTTCCCTATTTGCAAAAGAAGAT

Y334F-reverse: ATCTTCTTTTGCAAATAGGGAAAACGC

Y256F-forward: GAGAACATTCGCTTTCAAATTATTACA

Y256F-reverse: TGTAATAATTTGAAAGCGAATGTTCTC

W325F-forward: GCGCTGCGCTTATTTCCAACTGCTCCT

W325F-reverse: AGGAGCAGTTGGAAATAAGCGCAGCGC

All sequences were verified by Laragen (Culver City, California).

Theoretical Isoelectric Point (pI)

The theoretical pI is 5.34 for both the wild type $P450_{BM3}$, calculated using the Expasy ProtParam tool (https://web.expasy.org/protparam/).

Transformation Protocol

Plasmid for the desired mutant (2 µL) was incubated on ice with NovaBlue competent cells (100 µL) for 5 min, followed by 60 s of heat shock at 42 °C. Following an additional 2 min of incubation on ice, 200 µL Super Optimal broth with Catabolite repression (S.O.C) broth were added and the transformation mixture was incubated for 45 min at 37°C, 250 rpm. The transformation mixture was plated on LB/Agar culture plates containing 100 µg/mL ampicillin and incubated overnight at 37 °C.

Amplification and purification of plasmid DNA

To amplify plasmid DNA, the purified plasmid DNA or PCR mixture was transformed into NovaBlue competent cells. The cultures containing 5 mL Luria Bertani (LB) broth, 100 µL/mL ampicillin, and a single E. coli colony are grown for 16 hours at 37°C, 250 rpm. Cells were pelleted by centrifugation (10 min, 13200 rpm), and supernatant was discarded. Plasmid DNA was extracted using a Qiagen miniprep kit. To verify successful mutagenesis, 20 µL samples were submitted to Laragen for sequencing along with sequencing primers.

Enzyme expression and purification

The $P450_{BM3}$ protein was expressed and purified with modification based on the previously reported protocol [1]. The pET22b(+) plasmid (0.5 µL), encoding the full length $P450_{BM3}$ under the control of the *tac* promoter, was transformed into *Escherichia coli* BL21(DE3) competent cells (100 µL) and grown for 16 hours at 37°C on a Lysogeny Broth (LB) plate supplemented with 100 mg/mL Ampicillin. A single colony was then grown in 5 mL of LB media for 6 to 7 hours at 37°C while shaking at 250 rpm and subsequently used to inoculate 100 mL of Terrific Broth supplemented with 100 mg/mL Ampicillin (TB_{amp}), which was then grown overnight at 37°C with shaking at 250 rpm. TB_{amp} (0.5 L) was inoculated with the overnight culture (10 mL) and were shaken at 200 rpm at 37°C. Thiamine (0.5 mM) and power mix (5 mL per 0.5 L culture) were added after 1.5 h of growth at 37°C, and the cultures were continued to grow up to 4 h until an optical density of 1.2–1.8 was reached. The cultures were cooled down to room temperature on ice water bath and the shaker temperature was reduced to 22°C, then the cultures were induced by adding IPTG (0.5 mM), aminolevulinic acid (1 mM) and extra trace metal mix (500 µL per 0.5 L of culture). The ×1000 trace metal mix was prepared using 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnSO_4$, 10 mM $ZnSO_4$, 2 mM CoSO4, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na2MoO_4 and 2 mM H_3BO_3 and sterile filtered. The cultures were allowed to continue for another 20 hours at 22°C and 200 rpm. Cells were harvested by centrifugation (4°C, 15 min, 3000xg), and the cell pellet was stored at -80°C.

For the purification, the cell pellet was resuspended in Ni-NTA buffer A (25 mM Tris HCl, 200 mM NaCl, 25 mM imidazole, pH 8.2, 0.5 mL/g of pellet) and lysed by sonication (10 minutes at 30 seconds ON/30 seconds OFF pulse mode and 70% power) on the ice bath. The lysate was centrifuged at 27,000xg for 20 min at 4°C to remove cell debris. The collected supernatant was first subjected to a Ni-NTA chromatography step using HisPur™ Ni-NTA Resin (Cat# 88222, Thermo Fisher Scientific). The enzyme was eluted from the Ni-NTA column using 25 mM Tris HCl, 200 mM NaCl, 300 mM imidazole, pH 8.2. Ni-purified protein was buffer exchanged into 20 mM Tris HCl buffer (pH 8.2) using a 30 kDa molecular weight cut-off centrifugal filter. The enzyme was then subjected to HiTrap™ Q HP (5 mL, Cytiva) equilibrated and washed with 10 column volume of exchange buffer and eluted by elution buffer (20 mM Tris, 1 M NaCl, pH 8.2). The protein was buffer exchanged into Tris–HCl buffer (0.1 M, pH 8.2) using a 30 kDa molecular weight cut-off centrifugal filter. The protein purity and weight were confirmed using LCMS (Figure S1). For storage, proteins were portioned into 100 µL aliquots containing 20% glycerol and stored at -80°C.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS was used to obtain and confirm the molecular weight of the protein. LC-MS experiments were performed using a Waters UPLC chromatography system interfaced with a Waters LCT Premier XE Electrospray Time-of-flight mass spectrometer operated in the positive ion mode. The UPLC column was a 2.1 x 50 mm i.d. BioResolv RP column from Waters using water with 0.1% formic acid and acetonitrile with 0.1% formic acid as eluents.

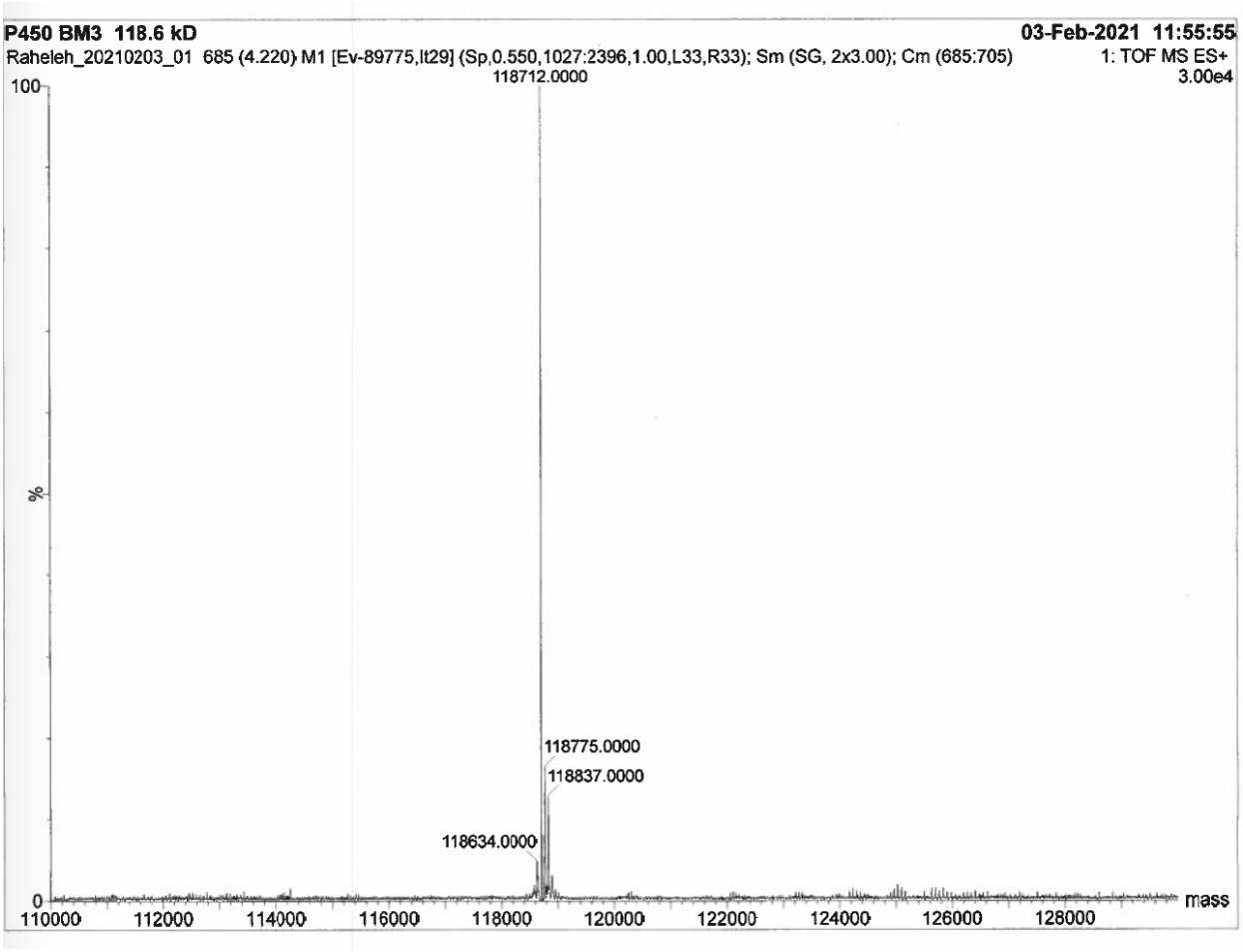

Figure S1. Mass spectrum of $P450_{BM3}$ wild type.

Synthesis of the 12-p-Nitrophenoxycarboxylic Acid (12-pNCA)

The 12-pNCA substrate was synthesized in three steps. Carboxylic acid esterification was performed by adding acetyl chloride (12.5 mmol) to a solution of 12-bromododecanoic acid (2.75 mmol) in ethanol (50 mL). The solution was stirred at room temperature for 4 hours, then concentrated using rotary evaporator. The residue was dissolved in diethyl ether (70 mL) and washed with sat. $NaHCO_3$ (3 x 70 mL), water (70 mL) and brine (70 mL). The organic phase was dried with Na_2SO_4 and concentrated [2]. After adding excess of 25 mmol sodium p-nitrophenolate to the 12-bromododecanoic acid ester dissolved in 100 mL of DMSO, the reaction was continued for 8 h at 120°C. After cooling the solution to room

temperature, distilled water was added dropwise, precipitating the pNCA esters which were filtered and washed with cold distilled water. Recrystallization in DMSO gave a white powder [3]. The hydrolysis of the pNCA ester was performed by dissolved into THF, mixed with an equal volume of an aqueous solution of 1 M KOH, and allowed to reflux with stirring for 6 hours. Following hydrolysis, 12-pNCA in THF phase was crystallized by addition of H_2SO_4 and then filtered and washed with distilled water [4]. The final product was verified by ^{1}H-NMR, recorded on a Bruker 400 MHZ spectrometer (Figure S2).

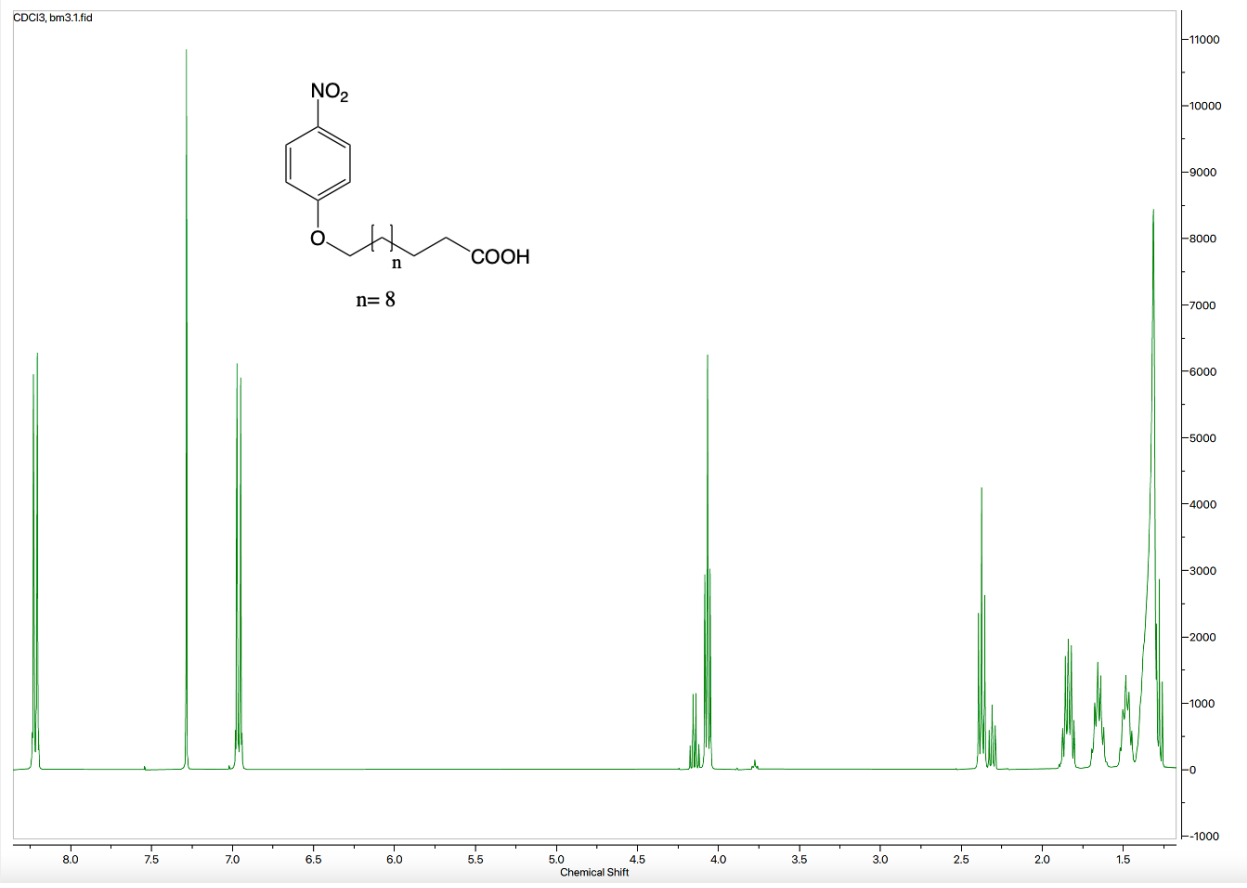

Figure S2. ^{1}H NMR spectra of p-Nitrophenoxydodecanoic acid (12-pNCA).

$P450_{BM3}$ Activity Assay using 12-pNCA

A spectrophotometric-based assay was used to measure enzyme activity. The synthetic 12-pNCA was applied as substrate for the $P450_{BM3}$. UV-visible absorbance of the chromophore p-nitrophenolate (p-NP) formation was monitored on an Agilent 8453 diode array spectrophotometer under aerobic condition. Figure S3 illustrates the reaction scheme of the pNCA activity assay.

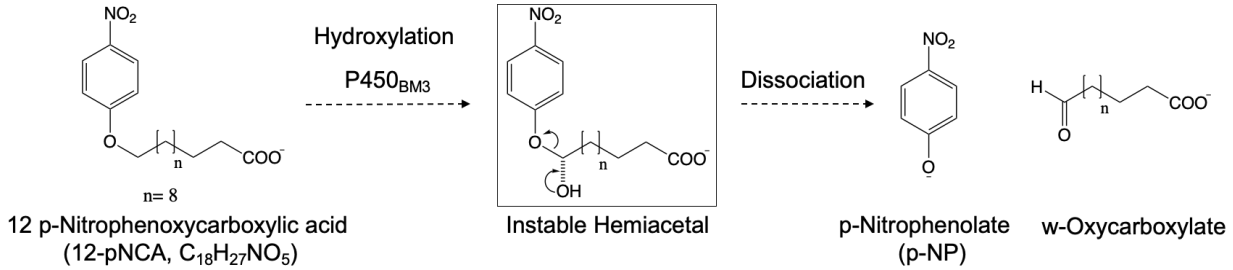

Figure S3. Reaction scheme of the conversion of *p*-nitrophenoxycarboxylate (*p*NCA) by $P450_{BM3}$. Terminal hydroxylation of *p*NCA froms the instable hemiacetal, dissociating into the chromophore p-nitrophenolate and ω-oxycarboxylate. The formation of p-nitrophenolate can be directly monitored using a spectrophotometer at a wavelength of 400 nm.

ω-Hydroxylation of pNCA by $P450_{BM3}$ forms an unstable hemiacetal intermediate which dissociates into the yellow p-nitrophenolate and ω -oxycarboxylic acid. Calculations according to the Henderson–Hasselbalch equation indicate that at pH 8.2, 90 and 92% of all p-nitrophenolate molecules are deprotonated and exhibit a yellow color with the absorption maximum at 400 nm [3]. Under the conditions of our assay at pH 8.2 and detection at 400 nm, the extinction coefficient was calculated as 14,100 $M^{-1}cm^{-1}$ (Figure S4)

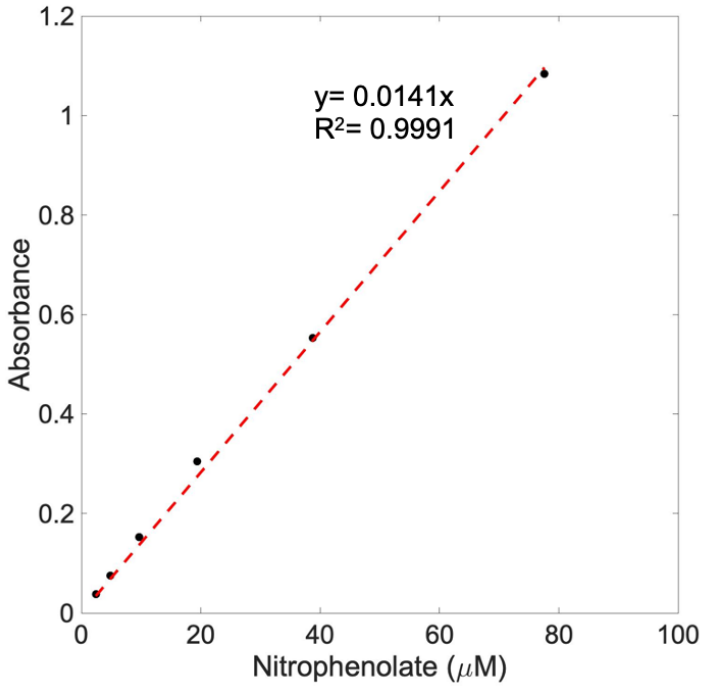

Figure S4. The standard curve to convert absorbance to the p-nitrophenolate concentration upon the substrate hydroxylation by $P450_{BM3}$ and calculated extinction coefficient for p-nitrophenolate at 400 nm.

For all assay procedures, a 6 mM solution of pNCA in DMSO was diluted using Tris–HCl buffer (0.1 M, pH 8.2) to prepare 7 different substrate concentrations (2, 4, 8, 16, 32, 64, 128 µM). After addition of 0.1 µM $P450_{BM3}$ to the DMSO–pNCA solution, the reaction was started by the addition of 100 µM NADPH prepared in Tris–HCl buffer (0.1 M, pH 8.2) in a final volume of 200 µL reaction mixture. The formation of nitrophenolate product of pNCA was monitored over 300 seconds.

Enzyme kinetics parameters were evaluated from three replicate measurements of p-nitrophenolate absorbance as a function of time after mixing the substrate and NADPH with $P450_{BM3}$ (Figures S5-S9). Absolute p-nitrophenolate concentrations were determined using the standard calibration curve (Figure S4). Specific reaction velocities (V_0) were extracted from the slopes of linear fits to [p-NP] versus time

plots, scaled by the total enzyme concentration, at different substrate concentrations (Figure S10). Rate constants were extracted from the plots of V0 versus substrate concentration (Figure S11).

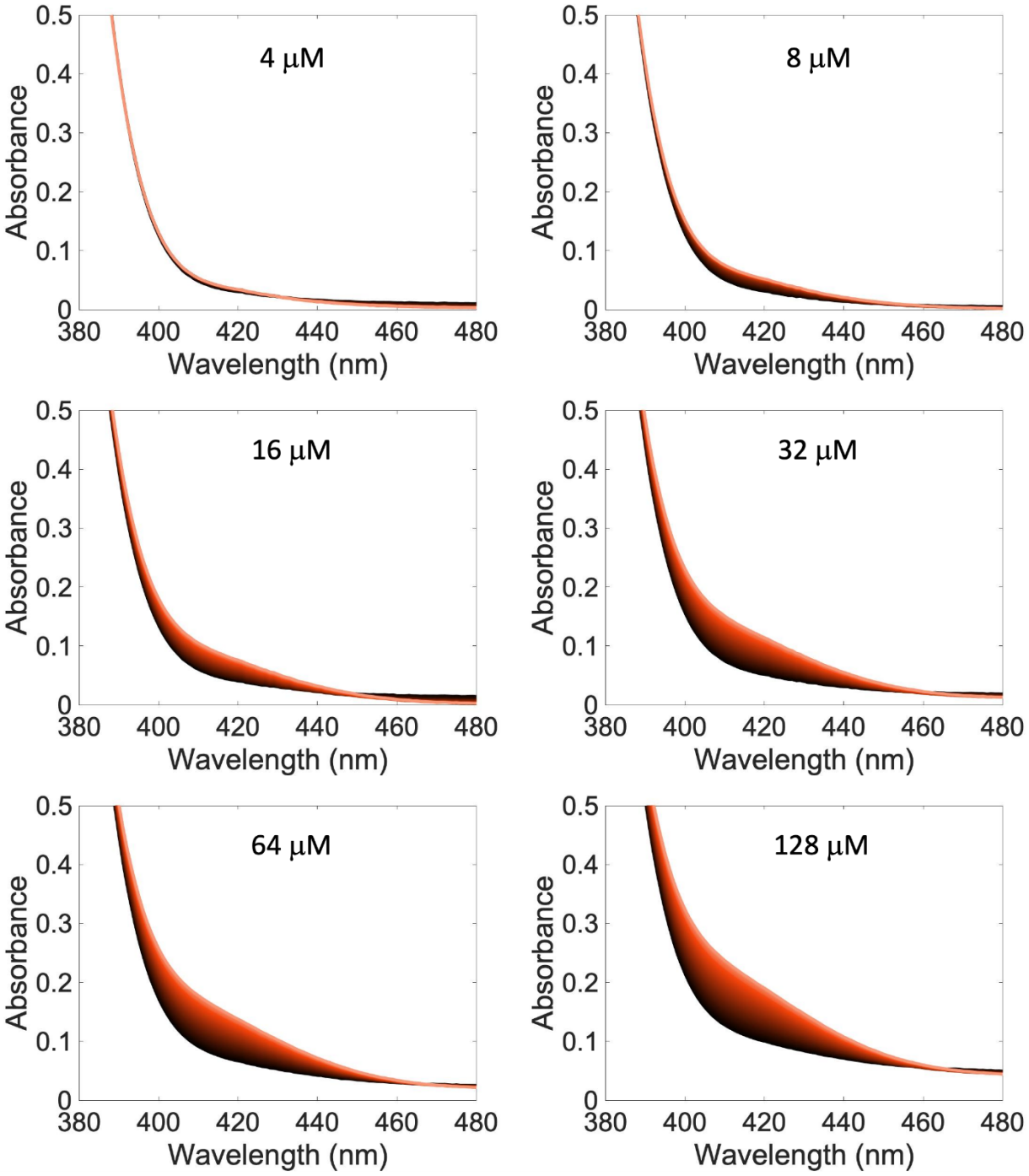

Figure S5. The formation of p-nitrophenolate after the reaction of 12-pNCA with the wild type $P450_{BM3}$, which results in an increased absorbance at 400 nm at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM).

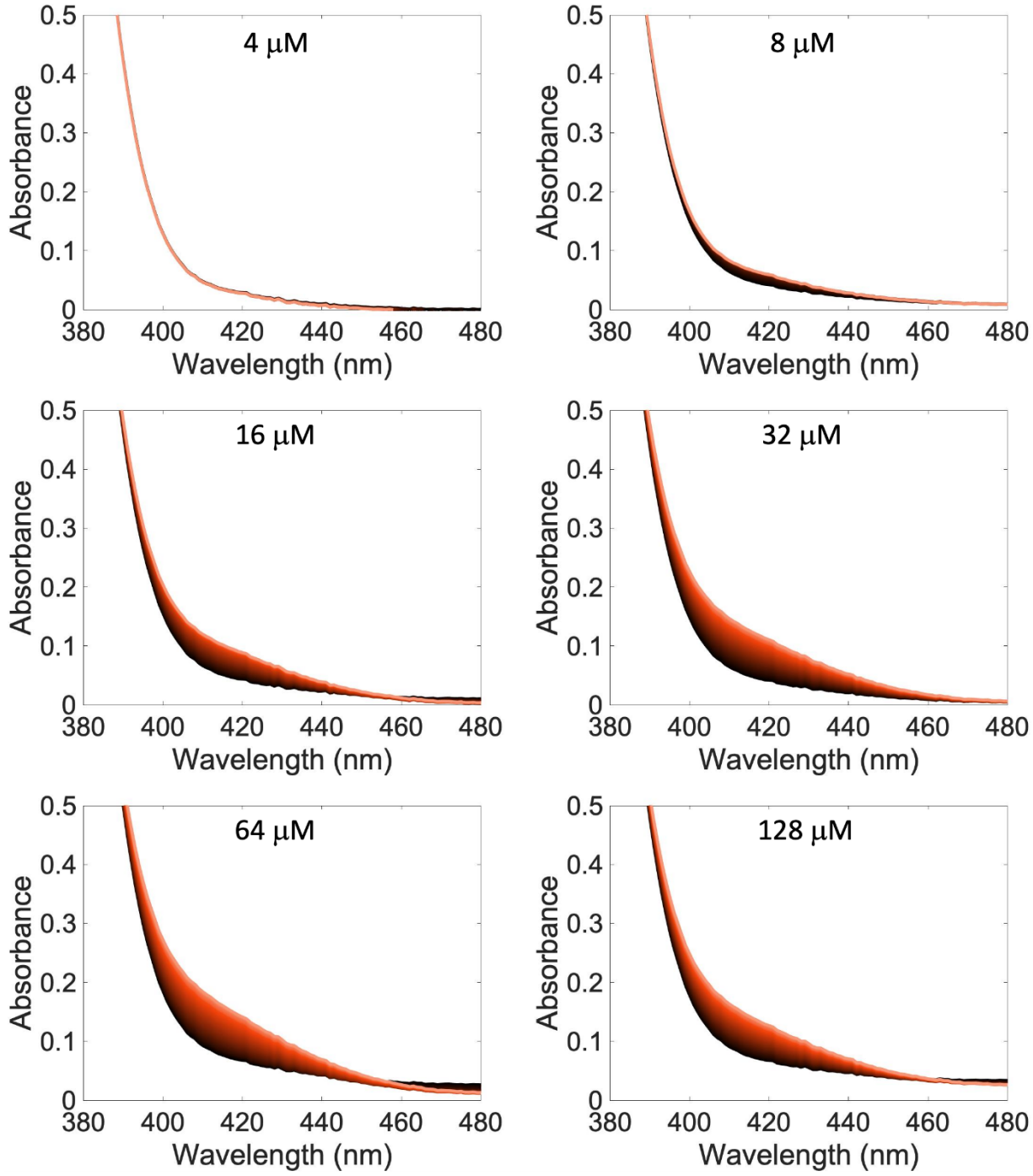

Figure S6. The formation of p-nitrophenolate after the reaction of 12-pNCA with W96H single mutant, which results in an increased absorbance at 400 nm at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM).

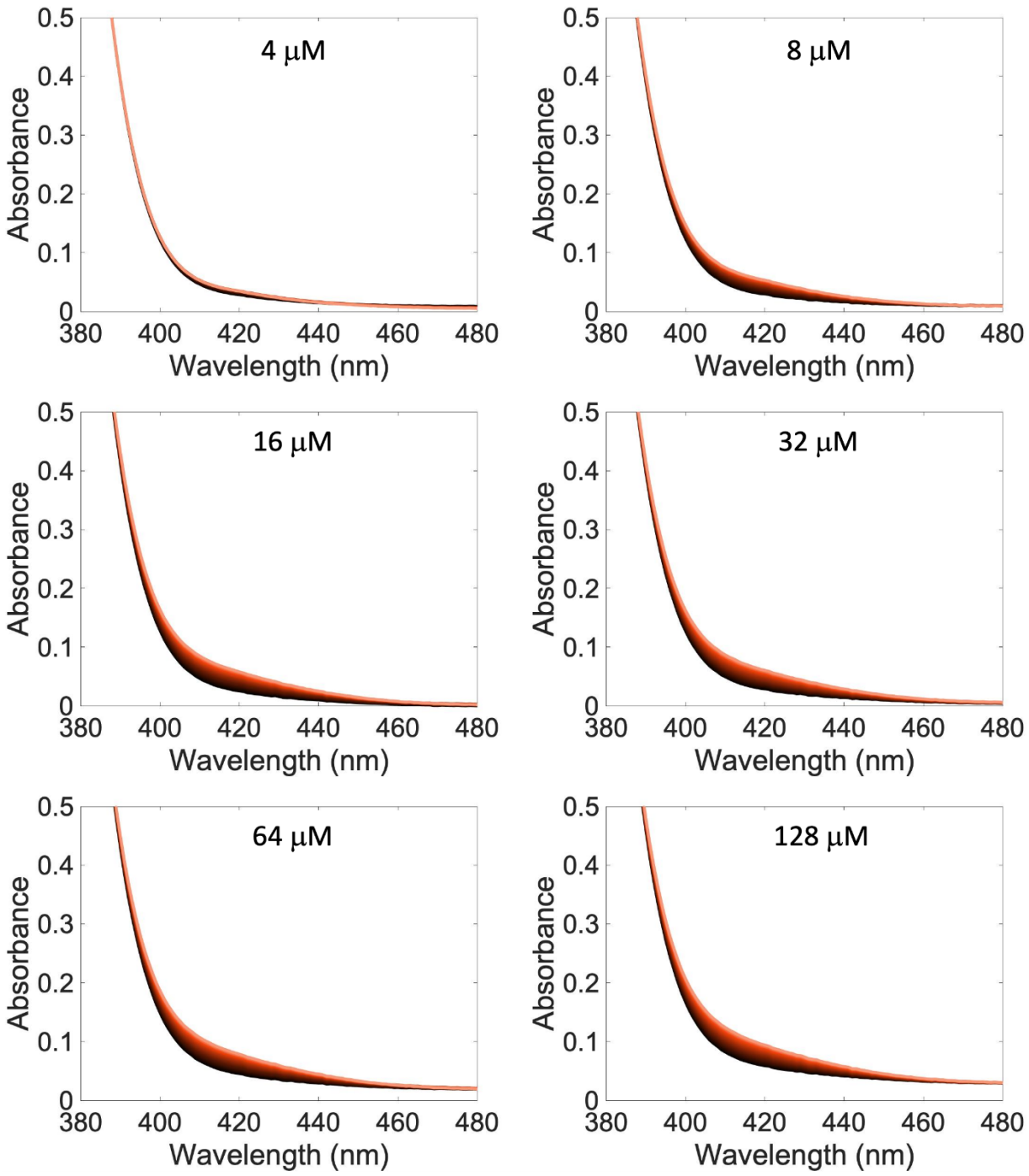

Figure S7. The formation of p-nitrophenolate after the reaction of 12-pNCA with W90F/W96H double mutant, which results in an increased absorbance at 400 nm at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM).

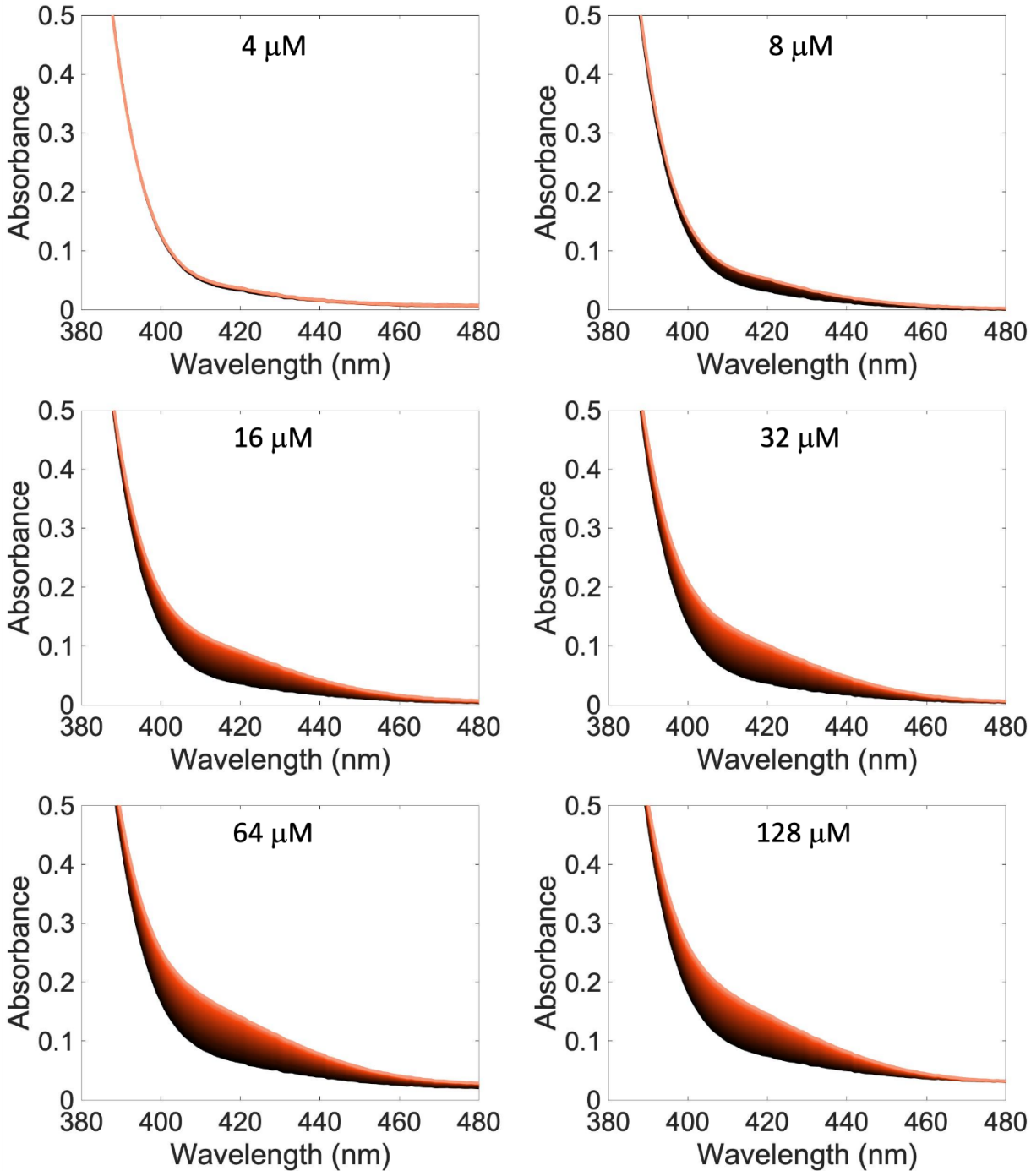

Figure S8. The formation of p-nitrophenolate after the reaction of 12-pNCA with W90F/W96H/Y334F Triple mutant, which results in an increased absorbance at 400 nm at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM).

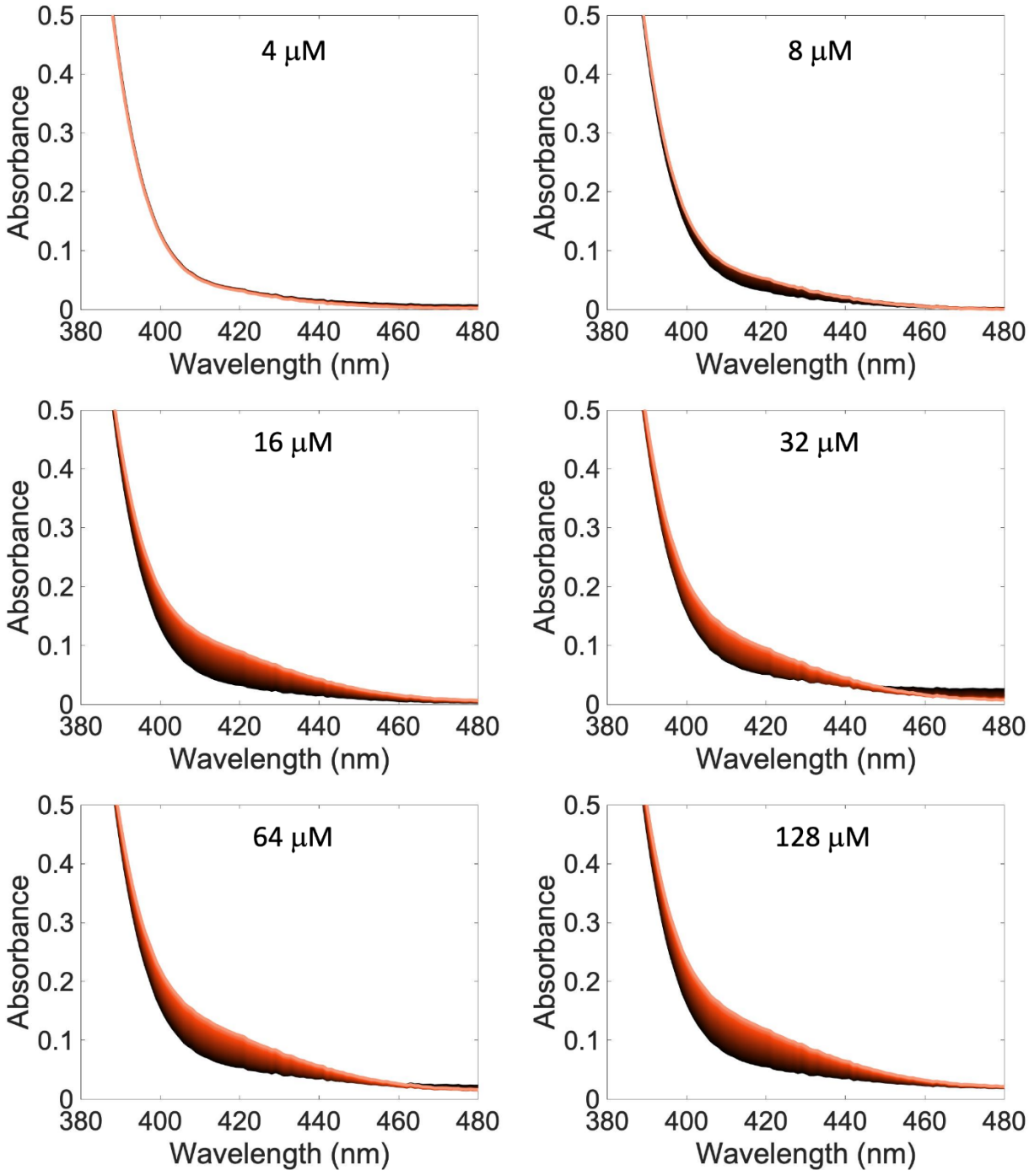

Figure S9. The formation of p-nitrophenolate after the reaction of 12-pNCA with W96H/Y256F/W325F Triple mutant, which results in an increased absorbance at 400 nm at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM).

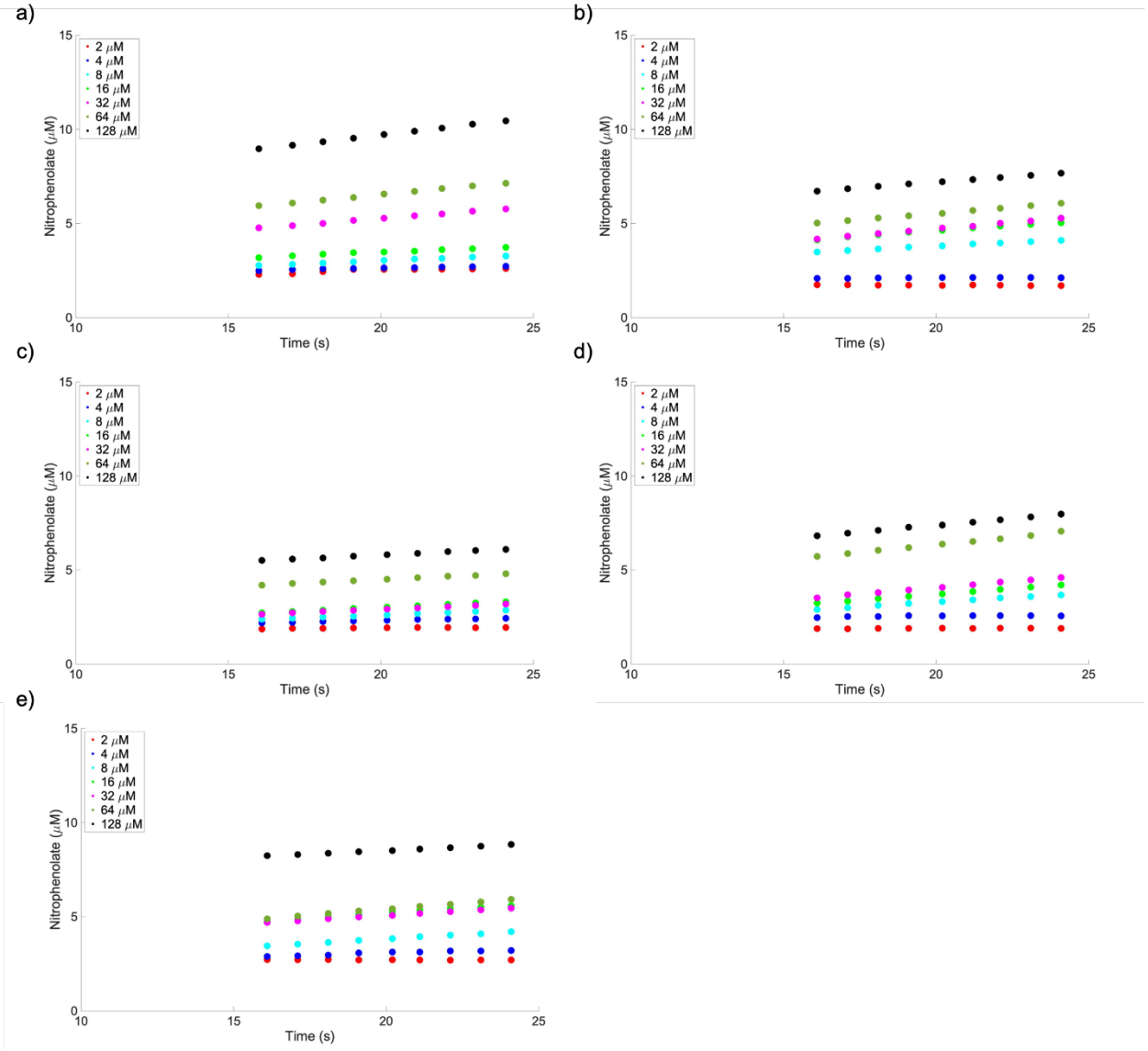

Figure S10. The scatter plot of nitrophenolate product (μM) formation versus time at different substrate concentrations (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM) for (a) $P450_{BM3}$ wild type, (b) W96H single mutant, c) W90F/W96H double mutant, d) W90F/W96H/Y334F Triple mutant, and e) W96H/Y256F/W325F Triple mutant.

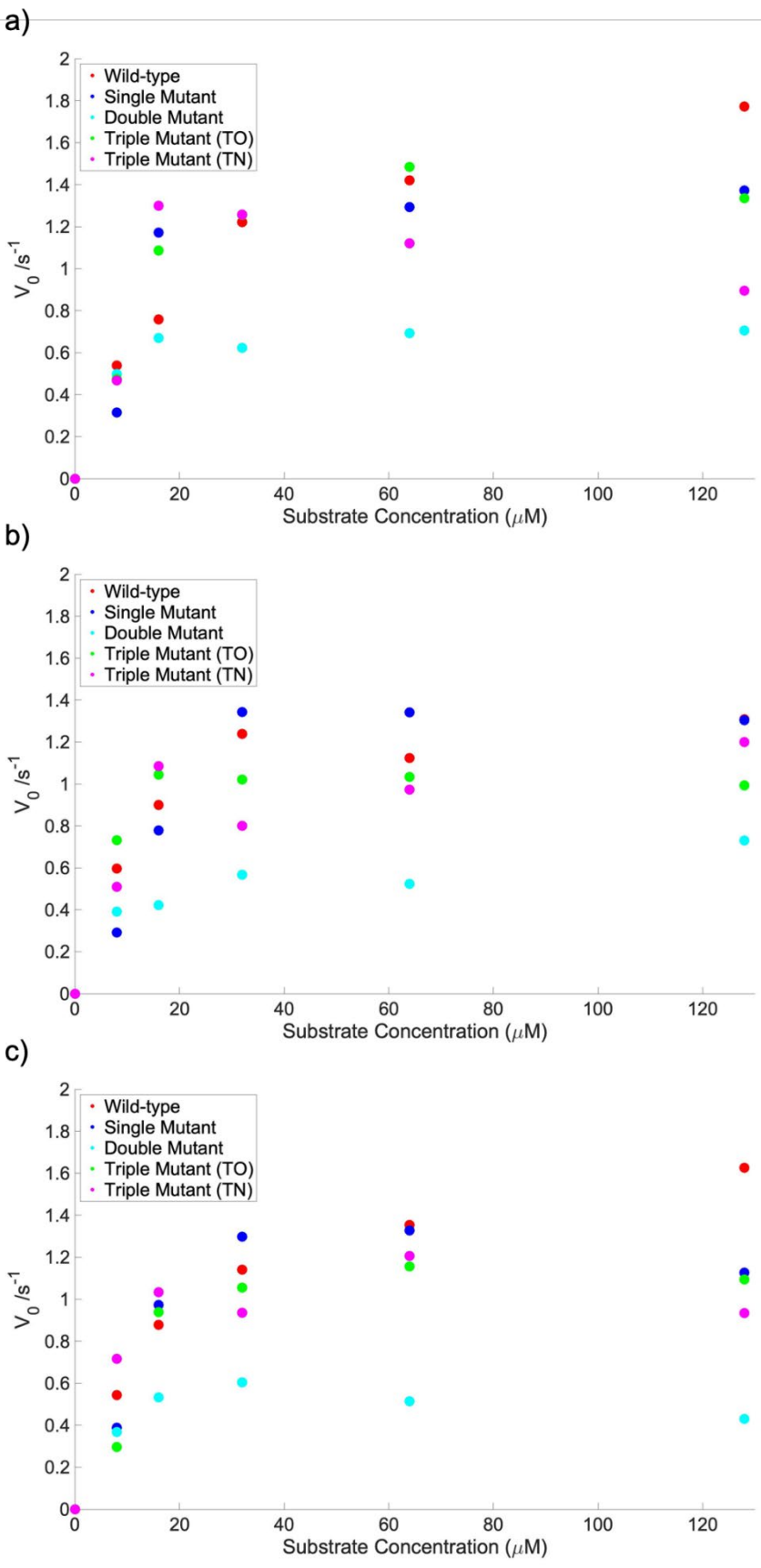

Figure S11. Plots of specific reaction velocity (V_0) at pH 8.2 as functions of substrate concentration: wild type (red), W96H single mutant (blue), W90F/W96H double mutant (pink), W90F/W96H/Y334F Triple mutant (TO) (green), and W96H/Y256F/W325F Triple mutant (TN) (black).

Total Turnover Numbers (TTN)

The TTN are determined as mole of hydroxylated products per mole of enzymes until no further turnover was observed. Briefly, 0.1 µM $P450_{BM3}$ was reacted with 100 µM NADPH and 30 µM pNCA substrate in 10 mL total reaction volume for 30 minutes. Then, the enzyme was separated from the other components of reaction mixture using Amicon Ultra-15 centrifugal filter with a mass cutoff at 30 kDa (Millipore, Bedford, MA). The enzyme was supplemented with more substrate and NADPH source to continue the reaction cycles for 4 times, after which no further significant turnover was observed. TTN was calculated based on total µmoles of product formed per µmoles of enzyme. The coupling efficiency was calculated based on total µmoles of product formed per µmoles of NADPH consumed in the reactions.

CO binding assay

One cuvette containing $P450_{BM3}$ was prepared. Carbon monoxide (CO) was gently bubbled through the solution for 30 seconds and sodium dithionite (2 mg) was added immediately. The absorbance at Soret band red-shifted from 418 nm to 449 nm (Figure S12).

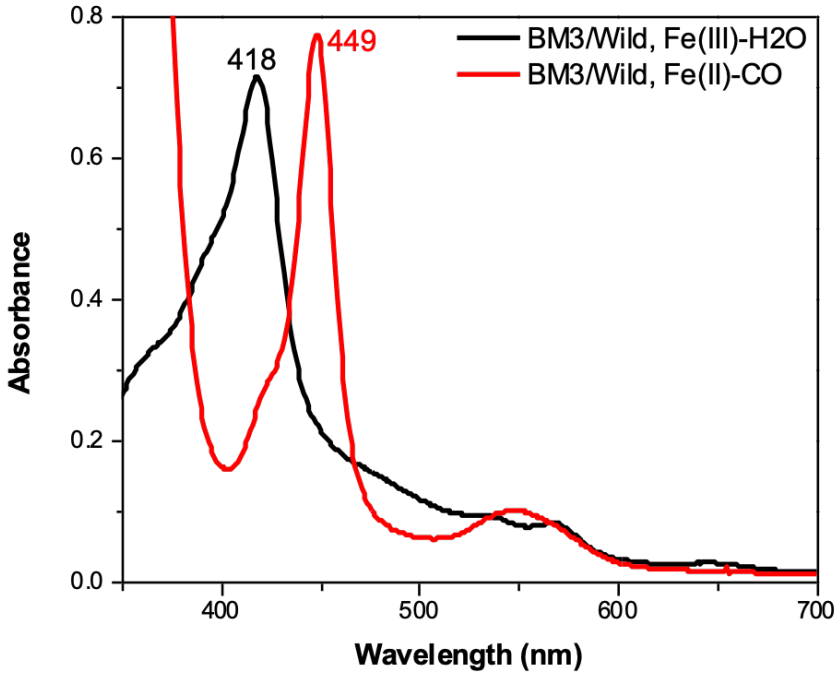

Figure S12. UV/vis spectra of wild type $P450_{BM3}$ (black) and its reduced form coordinated with CO (red).

Kinetics Modeling

The model outlined in Figure 2 of the main manuscript is described by set of coupled first-order differential equations. If the concentrations of NADPH, O_2, and RH are high enough to assume pseudo-first-order conditions for the initial step in catalysis, the differential equations become linear, but require the solution of a 4th order polynomial to obtain an analytical solution.

Relative yields of products provide some insight into the factors that determine TTN and Coupling values. Equation 1 in the main text defines TTN as follows:

$$TTN = \frac{\Phi_{ROH}}{\Phi_{DEAD}} = \frac{\Phi_{C\text{-}I}\Phi_{k_P}}{\Phi_{C\text{-}I'}\Phi_{D}} \qquad (S1)$$

The yield of Cmpd-I formation ($\phi_{C\text{-}I}$) is given by the ratio of the rate constant for Cmpd-I formation ($k_{C\text{-}I}$) to the sum of all other rate constants for decay of $[(Por)(^{Cys}S)Fe^{III}(O_2H)]^-$:

$$\Phi_{C\text{-}I} = \frac{k_{C\text{-}I}}{k_{C\text{-}I} + k_{C\text{-}I'} + k_H} \quad \text{(S2)}$$

The yield of product formation from Cmpd-I (ϕ_P) is given by:

$$\Phi_P = \frac{k_P}{k_P + k_R} \quad \text{(S3)}$$

The yield of Cmpd-I′ formation ($\phi_{C\text{-}I'}$) is given by the ratio of the rate constant for Cmpd-I formation ($k_{C\text{-}I'}$) to the sum of all other rate constants for decay of $[(Por)(^{Cys}S)Fe^{III}(O_2H)]^-$:

$$\Phi_{C\text{-}I'} = \frac{k_{C\text{-}I'}}{k_{C\text{-}I} + k_{C\text{-}I'} + k_H} \quad \text{(S4)}$$

The yield of deactivated enzyme from Cmpd-I′ (ϕ_D) is given by:

$$\Phi_D = \frac{k_D}{k_D + k_R} \quad \text{(S5)}$$

Substitution of these expressions into eq. S1, rearranging, and using the definitions of $R_{C\text{-}I}$, R_D, and R_P from the main text gives the expression in eq 1.

The ROH coupling efficiency is given by the product of the yield of Cmpd-I formation ($\phi_{C\text{-}I}$) times the yield of product formation from Cmpd-I (ϕ_P). Substitution of the expressions for these quantities, rearranging, and using the definitions of $R_{C\text{-}I}$, R_H, and R_P from the main text gives the expression in eq 2.

Heme hole survival kinetics were estimated by numerical solution of the coupled first-order linear differential equations describing electron-transfer among the heme and participating Trp and Tyr residues. The system of differential equations is given by the eigenvalue problem in eq. S6. Eigenvalues and eigenvectors were calculated in Matlab using the *eig* function.

$$\mathbf{x}' = \mathbf{A}\mathbf{x} \quad \text{(S6)}$$

Individual rate constants in **A** for electron-transfer between components *i* and *j* (k_{ij}) were given by the semi-classical expression (eq. S7) [5].

$$k_{ij} = 10^{13} e^{-\beta(r_{ij}-r_0)} e^{\frac{-(\Delta G^o_{ij}+\lambda_{ij})^2}{4\lambda_{ij}RT}} \quad \text{(S7)}$$

The Gibbs free-energy change for electron transfer from component *i* to component *j* is given by eq. S8 and the reorganization energy (λ_{ij}) is given by eq. S9.

$$\Delta G^o_{ij} = -\Delta E^o_{ij} = -\left(E^o_j - E^o_i\right) \quad \text{(S8)}$$

$$\lambda_{ij} = \frac{1}{2}\left(\lambda_{ii} + \lambda_{jj}\right) \quad \text{(S9)}$$

The 15-Å cutoff Trp/Tyr hole transfer network included the heme and 16 Trp and Tyr residues. Formal potentials, reorganization energies, and electron-transfer distances used in the rate calculations are given in the following tables.

Site	$E°_i$	λ_{ii}
Heme	1.00	1.50
W90	0.95	0.80
W96	1.10	0.80
W130	1.10	0.80
W325	0.95	0.80
W367	1.10	0.80
Y51	0.90	1.00
Y115	1.20	1.00
Y160	0.90	1.00
Y166	0.90	1.00
Y256	0.90	1.00
Y278	0.90	1.00
Y305	0.90	1.00
Y313	1.20	1.00
Y334	0.90	1.00
Y345	0.90	1.00
Y429	1.50	0.80

r_{ij} (Å)	W90	W96	W130	W325	W367	Y51	Y115	Y160	Y166	Y256	Y278	Y305	Y313	Y334	Y345	Y429	Heme
W90	0	8.35	38.30	26.11	27.74	15.83	25.32	29.20	38.81	13.17	34.42	32.85	39.80	4.45	22.03	35.56	13.79
W96		0	33.17	26.09	24.61	22.02	16.71	24.67	34.13	12.12	32.28	23.06	33.38	10.71	27.43	32.85	7.32
W130			0	18.40	26.66	32.63	21.70	21.29	13.39	31.35	13.27	21.53	17.38	39.53	44.15	13.93	18.54
W325				0	13.27	13.05	26.81	31.18	31.12	29.85	6.17	27.13	16.42	25.51	23.55	9.61	12.78
W367					0	16.88	26.59	37.40	38.85	34.44	17.34	23.21	13.74	25.22	19.20	22.73	15.57
Y51						0	32.52	35.60	40.23	26.33	23.06	35.25	31.05	15.13	12.81	22.68	15.01
Y115							0	14.41	19.85	18.31	28.20	8.55	24.51	28.19	40.60	30.26	10.20
Y160								0	11.62	16.47	31.15	23.34	34.90	34.49	48.15	29.81	16.79
Y166									0	27.24	27.97	24.29	31.50	42.92	53.30	26.87	22.43
Y256										0	34.34	28.76	39.56	20.33	37.56	32.41	12.80
Y278											0	27.64	14.38	34.20	32.43	36.92	17.24
Y305												0	18.90	33.39	41.06	31.02	15.64
Y313													0	38.75	36.57	19.48	20.33
Y334														0	17.07	34.21	14.79
Y345															0	34.74	24.27
Y429																0	18.80
Heme																	0

The value of r_0 was taken to be 3 Å. Concentration versus time profiles were calculated from the eigenvectors and tested for accuracy by numerically differentiating and comparing to the profiles calculated by substitution into eq. S6. Excellent agreement was found for all components except Y429 which is not substantially populated due to its high estimated potential (no proton acceptor is near the buried residue).

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