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# Supporting Information for
## Tryptophan Extends the Life of Cytochrome P450

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**This PDF file includes:**



**Other supporting materials for this manuscript include the following:**

Dataset S1

**Supporting Information Text**

**Materials and Methods**

**Protein Expression, Isolation, and Purification.** The cloning vector employed in this study for cytochrome $P450_{BM3}$ was plasmid pET22. Site-directed mutagenesis was carried out utilizing the QuikChange Site-Directed Mutagenesis kit from Qiagen, with primers designed to introduce the desired mutations, sourced from Invitrogen. The forward primer sequence was 5'-ACGCATGAAAAAAATCACAAAAAAGCGCATAAT-3', and the corresponding reverse primer sequence was 5'-ATTATGCGCTTTTTTGTGATTTTTTTCATGCGT-3'. Experimental samples were prepared on ice, containing 2 µL of 10X buffer stock, 2 µL of dNTP, 25 ng of the parent plasmid, 50 ng of the forward primer, 50 ng of the reverse primer, 0.4 µL of Pfu DNA polymerase, and milli-Q water to reach a total volume of 20 µL. The Polymerase Chain Reaction (PCR) protocol was executed on a MJ Research PT150 Minicycler, comprising 18 cycles of 30 s at 95°C, 30 s at 95°C, 60 s at 55°C, and 9 min at 68°C. Methylated DNA (parent plasmid) was digested by the addition of 1 µL of Dpn1 enzyme and subsequent incubation at 37 °C for one hour. The PCR mixtures were stored at –20 °C until required (*1*).

The amino acid sequence for the wild-type $P450_{BM3}$ (holoprotein) is as follows:

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

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEAC
DESRFDKNLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQ
LVQKWERLNADEHIEVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQ
RANPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDEN
IRYQIITFLIAGHETTSGLLSFALYFLVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNE
ALRLWPTAPAFSLYAKEDTVLGGEYPLEKGDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAI
PQHAFKPFGNGQRACIGQQFALHEATLVLGMMLKHFDFEDHTNYELDIKETLTLKPEGFVVKAKS
KKIPLGGIPSPSTEQSAKKVRKKAENAHNTPLLVLYGSNMGTAEGTARDLADIAMSKGFAPQVAT
LDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQASADEVKGVRYSVFGCGDKNWATTY
QKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFNLDIENSEDNKSTL
SLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQEGDHLGVIPRN
YEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAAKT
VCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRV
DEKQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGP
GTGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRM
PNQPKTYVQHVMEQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARL
WLQQLEEKGRYAKDVWAGHHHHHH

The nucleotide sequence for WT $P450_{BM3}$ (holoprotein) is as follows:

ATGACAATTAAAGAAATGCCTCAGCCAAAAACGTTTGGAGAGCTTAAAAATTTACCGTT
ATTAAACACAGATAAACCGGTTCAAGCTTTGATGAAAATTGCGGATGAATTAGGAGAAATCTT
TAAATTCGAGGCGCCTGGTCGTGTAACGCGCTACTTATCAAGTCAGCGTCTAATTAAAGAAG
CATGCGATGAATCACGCTTTGATAAAAACTTAAGTCAAGCGCTTAAATTTGTACGTGATTTTG
CAGGAGACGGGTTATTTACAAGCTGGACGCATGAAAAAAATTGGAAAAAAGCGCATAATATC
TTACTTCCAAGCTTCAGTCAGCAGGCAATGAAAGGCTATCATGCGATGATGGTCGATATCGC
CGTGCAGCTTGTTCAAAAGTGGGAGCGTCTAAATGCAGATGAGCATATTGAAGTACCGGAA
GACATGACACGTTTAACGCTTGATACAATTGGTCTTTGCGGCTTTAACTATCGCTTTAACAGC
TTTTACCGAGATCAGCCTCATCCATTTATTACAAGTATGGTCCGTGCACTGGATGAAGCAATG
AACAAGCTGCAGCGAGCAAATCCAGACGACCCAGCTTATGATGAAAACAAGCGCCAGTTTC
AAGAAGATATCAAGGTGATGAACGACCTAGTAGATAAAATTATTGCAGATCGCAAAGCAAGC
GGTGAACAAAGCGATGATTTATTAACGCATATGCTAAACGGAAAAGATCCAGAAACGGGTGA
GCCGCTTGATGACGAGAACATTCGCTATCAAATTATTACATTCTTAATTGCGGGACACGAAAC
AACAAGTGGTCTTTTATCATTTGCGCTGTATTTCTTAGTGAAAAATCCACATGTATTACAAAAA
GCAGCAGAAGAAGCAGCACGAGTTCTAGTAGATCCTGTTCCAAGCTACAAACAAGTCAAACA
GCTTAAATATGTCGGCATGGTCTTAAACGAAGCGCTGCGCTTATGGCCAACTGCTCCTGCGT

TTTCCCTATATGCAAAAGAAGATACGGTGCTTGGAGGAGAATATCCTTTAGAAAAAGGCGAC
GAACTAATGGTTCTGATTCCTCAGCTTCACCGTGATAAAACAATTTGGGGAGACGATGTGGA
AGAGTTCCGTCCAGAGCGTTTTGAAAATCCAAGTGCGATTCCGCAGCATGCGTTTAAACCGT
TTGGAAACGGTCAGCGTGCGTGTATCGGTCAGCAGTTCGCTCTTCATGAAGCAACGCTGGT
ACTTGGTATGATGCTAAAACACTTTGACTTTGAAGATCATACAAACTACGAGCTCGATATTAA
AGAAACTTTAACGTTAAAACCTGAAGGCTTTGTGGTAAAAGCAAAATCGAAAAAAATTCCGCT
TGGCGGTATTCCTTCACCTAGCACTGAACAGTCTGCTAAAAAAGTACGCAAAAAGGCAGAAA
ACGCTCATAATACGCCGCTGCTTGTGCTATACGGTTCAAATATGGGAACAGCTGAAGGAACG
GCGCGTGATTTAGCAGATATTGCAATGAGCAAAGGATTTGCACCGCAGGTCGCAACGCTTG
ATTCACACGCCGGAAATCTTCCGCGCGAAGGAGCTGTATTAATTGTAACGGCGTCTTATAAC
GGTCATCCGCCTGATAACGCAAAGCAATTTGTCGACTGGTTAGACCAAGCGTCTGCTGATGA
AGTAAAAGGCGTTCGCTACTCCGTATTTGGATGCGGCGATAAAAACTGGGCTACTACGTATC
AAAAAGTGCCTGCTTTTATCGATGAAACGCTTGCCGCTAAAGGGGCAGAAAACATCGCTGAC
CGCGGTGAAGCAGATGCAAGCGACGACTTTGAAGGCACATATGAAGAATGGCGTGAACATA
TGTGGAGTGACGTAGCAGCCTACTTTAACCTCGACATTGAAAACAGTGAAGATAATAAATCTA
CTCTTTCACTTCAATTTGTCGACAGCGCCGCGGATATGCCGCTTGCGAAAATGCACGGTGC
GTTTTCAACGAACGTCGTAGCAAGCAAAGAACTTCAACAGCCAGGCAGTGCACGAAGCACG
CGACATCTTGAAATTGAACTTCCAAAAGAAGCTTCTTATCAAGAAGGAGATCATTTAGGTGTT
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GCGCTATTACTCGATTTCTTCATCACCTCGTGTCGATGAAAAACAAGCAAGCATCACGGTCA
GCGTTGTCTCAGGAGAAGCGTGGAGCGGATATGGAGAATATAAAGGAATTGCGTCGAACTA
TCTTGCCGAGCTGCAAGAAGGAGATACGATTACGTGCTTTATTTCCACACCGCAGTCAGAAT
TTACGCTGCCAAAAGACCCTGAAACGCCGCTTATCATGGTCGGACCGGGAACAGGCGTCGC
GCCGTTTAGAGGCTTTGTGCAGGCGCGCAAACAGCTAAAAGAACAAGGACAGTCACTTGGA
GAAGCACATTTATACTTCGGCTGCCGTTCACCTCATGAAGACTATCTGTATCAAGAAGAGCTT
GAAAACGCCCAAAGCGAAGGCATCATTACGCTTCATACCGCTTTTTCTCGCATGCCAAATCA
GCCGAAAACATACGTTCAGCACGTAATGGAACAAGACGGCAAGAAATTGATTGAACTTCTTG
ATCAAGGAGCGCACTTCTATATTTGCGGAGACGGAAGCCAAATGGCACCTGCCGTTGAAGC
AACGCTTATGAAAAGCTATGCTGACGTTCACCAAGTGAGTGAAGCAGACGCTCGCTTATGGC
TGCAGCAGCTAGAAGAAAAAGGCCGATACGCAAAAGACGTGTGGGCTGGGCTCGAGCACC
ACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC

The primers for site-directed mutagenesis:

W96H-forward: ACGCATGAAAAAAATCACAAAAAAGCGCATAAT

W96H-reverse: ATTATGCGCTTTTTTGTGATTTTTTTCATGCGT

W90F-forward: GGGTTATTTACAAGCTTCACGCATGAAAAAAAT

W90F-reverse: ATTTTTTTCATGCGTGAAGCTTGTAAATAACCC

Y334F-forward: GCGTTTTCCCTATTTGCAAAAGAAGAT

Y334F-reverse: ATCTTCTTTTGCAAATAGGGAAAACGC

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

**Transformation, Amplification and Purification of Plasmid DNA.** For the transformation, 2 µL of plasmid for the desired mutant were chilled on ice and then mixed with 100 µL of NovaBlue competent cells for 5 minutes, followed by a 60-second heat shock at 42°C. After an additional 2 minutes of incubation on ice, 200 µL of Super Optimal broth with Catabolite repression (S.O.C) broth were added to the mixture, and the transformation mixture was then incubated for 45 minutes at 37°C with shaking at 250 rpm. Subsequently, the transformation mixture was plated on LB/Agar culture plates containing 100 µg/mL ampicillin and incubated overnight at 37°C.

To amplify plasmid DNA, either the purified plasmid DNA or the PCR mixture was introduced into NovaBlue competent cells. Cultures containing 5 mL of Luria Bertani (LB) broth supplemented with 100 µg/mL ampicillin and a single *E. coli* colony were grown for 16 hours at 37°C with shaking at 250 rpm. The cells were then pelleted by centrifugation (10 minutes, 13200 rpm), and the supernatant was discarded. Plasmid DNA was subsequently extracted using a Qiagen miniprep kit. To confirm the success of mutagenesis, 20 µL samples were sent to Laragen for sequencing, along with the required sequencing primers (*1*).

**Protein 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 $CoSO_4$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM $Na_2MoO_4$ 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 theoretical isoelectric point (pI) for the wild-type $P450_{BM3}$ is 5.34, calculated using the Expasy ProtParam tool (https://web.expasy.org/protparam/). 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 LC-MS. 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. For storage, proteins were portioned into 100 µL aliquots containing 20% glycerol and stored at -80°C.

**Total Turnover Number (TTN) Measurements.** TTN values were assessed under varying NADPH concentrations, both in the presence and absence of ascorbate, by quantifying the moles of yellow *p*-nitrophenolate generated from 12-p-Nitrophenoxycarboxylic Acid (12-*p*NCA) per mole of enzyme until no additional turnover was detected. The 12-*p*NCA substrate was synthesized following a previously reported procedure (*1*). In brief, 0.1 µM $P450_{BM3}$ was allowed to react with 25 µM of the pNCA substrate and 50 µM, 100 µM, and 300 µM NADPH in a total reaction volume of 10 mL for a duration of 30 minutes. The impact of 100 µM ascorbate on TTN was also investigated. Subsequently, the enzyme was separated from the other components of the reaction mixture using an Amicon Ultra-15 centrifugal filter with a mass cutoff of 30 kDa (Millipore, Bedford, MA). Additional substrate, NADPH and ascorbate were then supplied to the enzyme to

enable further reaction cycles, after which no significant additional turnover was observed. TTN was calculated by considering the total micromoles of product formed per micromoles of enzyme.

**Kinetics of Oxygen and NADPH Consumption.** The kinetics of oxygen and NADPH consumption (Supplementary Figures 1, 2, and 3) were determined by recording the reaction of 0.1 μM $P450_{BM3}$, 50 μM pNCA substrate and 400 μM NADPH in 4700 μL total reaction volume for a duration of 20 minutes. Oxygen consumption was measured using an Ocean Optics NeoFox oxygen sensing system with FOXY oxygen sensor probe. The system uses a fiber optic fluorescence probe with proprietary oxygen-sensing thin-film coating on the tip, designed for monitoring oxygen partial pressure in aqueous solution. NADPH consumption was monitored on an Agilent 8453 diode array spectrophotometer at 340 nm. Tables S1 and S2 demonstrate the kinetic results of oxygen consumption and NADPH consumption, respectively.

**Hydrogen Peroxide ($H_2O_2$) Assay.** $P450_{BM3}$ (0.1 μM) was allowed to react with 100 μM NADPH and 50 μM pNCA substrate in a total reaction volume of 10 mL for a duration of 30 minutes. The enzyme was then supplemented with additional substrate and a source of NADPH to enable more reaction cycles, after which no significant further turnover was observed. Subsequently, the enzyme was separated from the other components of the reaction mixture using an Amicon Ultra-15 centrifugal filter with a mass cutoff of 30 kDa (Millipore, Bedford, MA). $H_2O_2$ concentration was measured by the peroxide assay kit (ab272537, Abcam, Cambridge, UK). This assay kit is specifically designed to determine peroxide concentrations in samples without the need for any prior treatment. The method relies on the chromogenic $Fe^{3+}$-xylenol orange reaction, wherein a purple complex form as a result of the oxidation of $Fe^{2+}$ provided in the reagent by peroxides present in the sample. The intensity of this color, measured within the range of 540-610 nm, serves as a precise indicator of peroxide levels in the sample. The assay's detection range spans from 0.2 μM to 30 μM $H_2O_2$. In brief, a fresh set of standards was prepared and serially diluted immediately before use. The premix standard was created by combining 5 μL of Standard (3% $H_2O_2$) with 495 μL of $H_2O$, resulting in a 1:100 dilution within a 1.5 mL Eppendorf tube. Subsequently, 1470 μL of a 30 μM Premix was prepared by mixing 5 μL of $H_2O_2$ (1:100 dilution) with 1465 μL of distilled water. Finally, the standards were diluted in 1.5 mL centrifuge tubes according to the Table S3.

The $H_2O_2$ detection reagent was prepared for all samples and standards by combining 2 μL of Reagent A with 200 μL of Reagent B, both of which were provided in the peroxide assay kit. Subsequently, 200 μL of the detection reagent was added separately to 80 μL of both the standards and the samples. The reactions were then incubated at room temperature for 30 minutes. After this incubation period, the optical density was measured at the wavelength range of 540-610 nm, with the peak at 585 nm. To calculate the sample peroxide content, the optical density value of standard #8 ($H_2O$) was subtracted from the optical density values of the other standards. These corrected values were then plotted against known $H_2O_2$ concentrations to generate a standard curve (Supplementary Figures 4 and 5). The sample's peroxide content was subsequently determined by referring to this standard curve.

**Solvent Exposure of $P450_{BM3}$ Residues.** Estimates of solvent exposure for P450BM3 residues were determined using the Biovia Discovery Studio Visualizer program with the x-ray crystal structure coordinates from PDB ID 2IJ2. Parameters used in the analysis: 240 grid points per atom; probe radius 1.40 Å.

**CYP102 Sequence Alignments.** CYP102 amino acid sequences were aligned using the Clustal Omega multiple sequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/). This list of CYP102 sequences was taken from Parvez et al. (*2*). The results are provided in the accompanying Supplementary Information file: CYP102_alignment_sorted.pdf

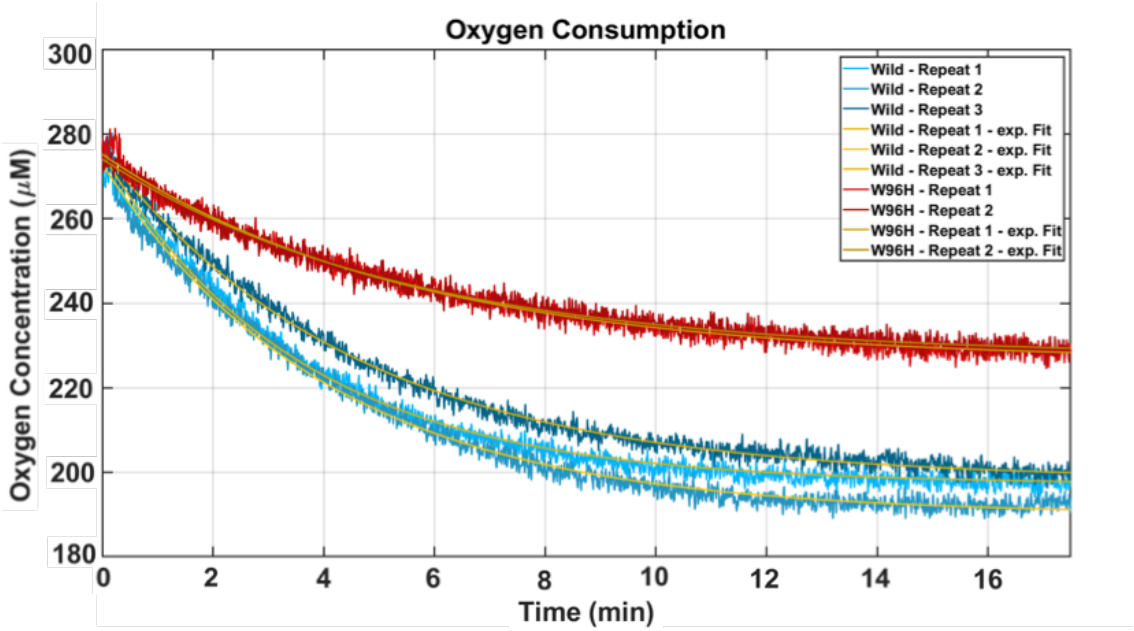


**Fig. S1.** Kinetics of oxygen consumption by WT and W96H $P450_{BM3}$. Fitting parameters are set out in Table S1. The relative rates of $O_2$ and NADPH consumption are used to identify the uncoupling pathways. The in the oxidase shunt pathway, the NADPH consumption rate (Figures S2, S3; Table S2) is twice that of the $O_2$ consumption rate.

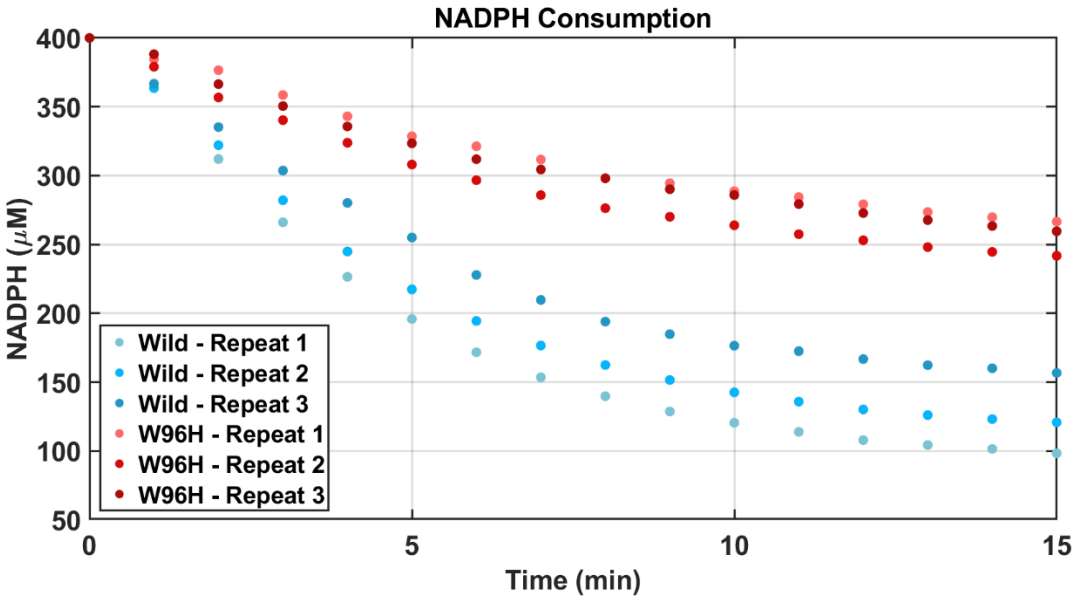


**Fig. S2.** Kinetics of NADPH consumption by WT and W96H $P450_{BM3}$. The relative rates of $O_2$ and NADPH consumption are used to identify the uncoupling pathways. The in the oxidase shunt pathway, the NADPH consumption rate is twice that of the $O_2$ consumption rate (Figure S1; Table S1).

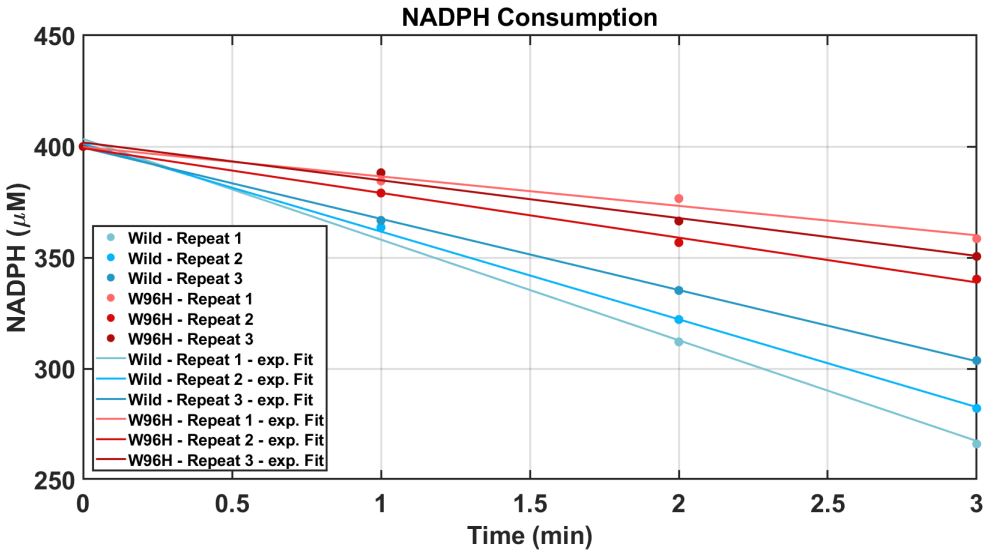


**Fig. S3.** Initial rates of NADPH consumption. Fitting parameters are set out in Table S2. The relative rates of $O_2$ and NADPH consumption are used to identify the uncoupling pathways. The in the oxidase shunt pathway, the NADPH consumption rate is twice that of the $O_2$ consumption rate (Figure S1; Table S1).

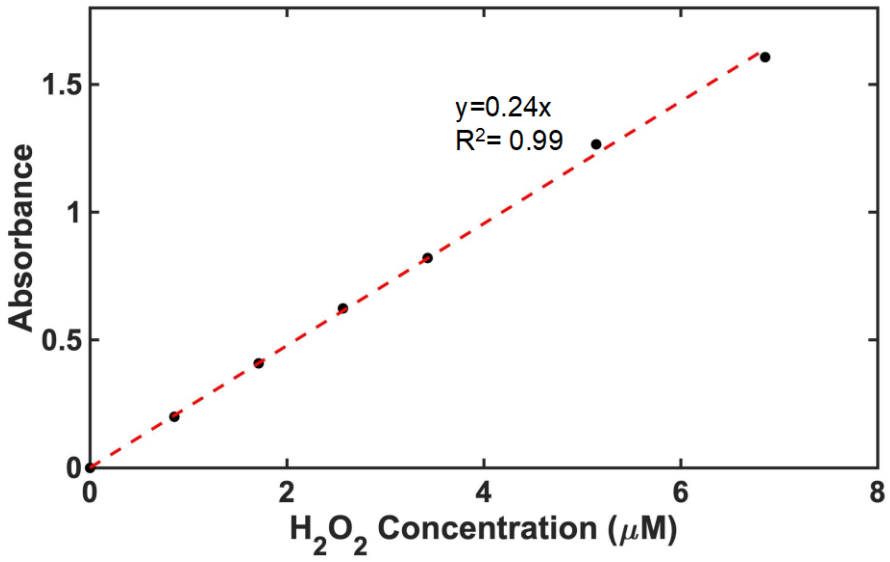


**Fig. S4.** Calibration curve for $H_2O_2$ assay.

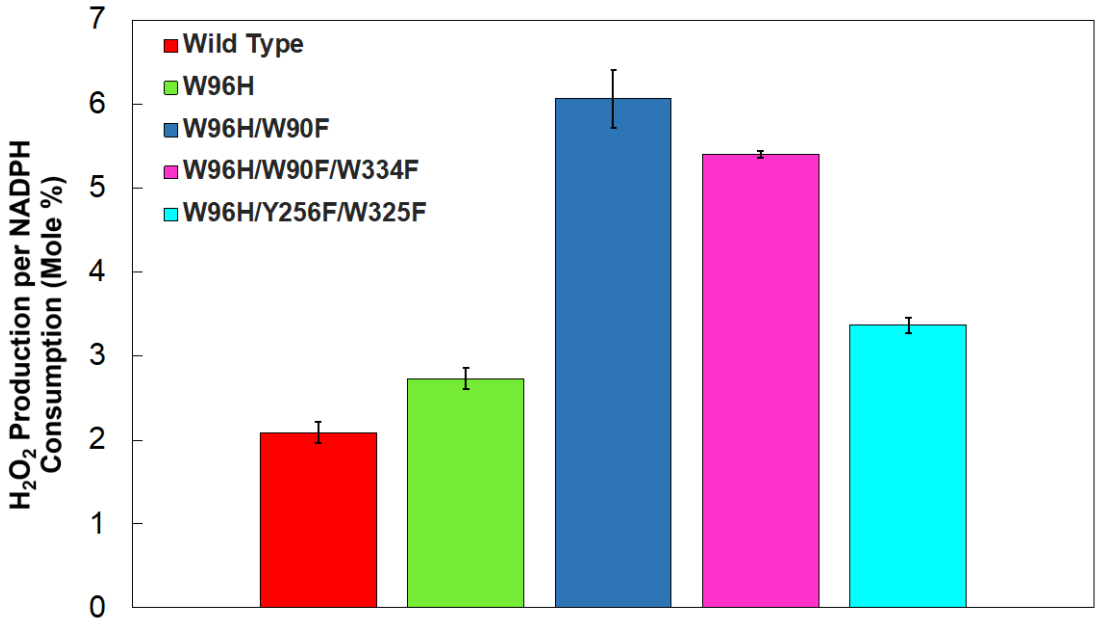


**Fig. S5.** $H_2O_2$ produced during $P450_{BM3}$ turnover in the presence of pNCA and NADPH.

**Table S1.** Results of fits to oxygen consumption kinetics.

| Parameters | | Wild | W96H |
|---|---|---|---|
| A | Repeat 1 | 197.3 μM | 226.3 μM |
| | Repeat 2 | 190.1 μM | 227.5 μM |
| B | Repeat 1 | 78.0 μM | 48.8 μM |
| | Repeat 2 | 82.8 μM | 46.6 μM |
| τ | Repeat 1 | 3.6 min | 5.5 min |
| | Repeat 2 | 4.1 min | 5.5 min |
| Initial rate | Repeat 1 | -21.7 $\mu M.min^{-1}$ | -8.9 $\mu M.min^{-1}$ |
| | Repeat 2 | -20.2 $\mu M.min^{-1}$ | -8.5 $\mu M.min^{-1}$ |
| | **Average** | **-20.9 ± 1.0 $\mu M.min^{-1}$** | **-8.7 ± 0.2 $\mu M.min^{-1}$** |

Fitting curve equations:

$M_{O2} = A + B \times \exp(-t/\tau)$

$M_{O2}(t=0) = A + B$

$dM_{O2}/dt = -(B/\tau) \times \exp(-t/\tau)$

$dM_{O2}/dt\ (t=0) = -B/\tau$

$M_{O2}$: Oxygen concentration (μM)

t: time (min)

τ: Oxygen consumption time constant (min)

$dM_{O2}/dt\ (t=0) = -B/\tau$: Initial rate of oxygen consumption ($\mu M.min^{-1}$)

$M_{O2}\ (t=0) = A+B$: Initial oxygen concentration (μM)

**Table S2.** Results of fits to NADPH consumption kinetics.

| Parameters | | Wild | W96H |
|---|---|---|---|
| Initial concentration (B) | Repeat 1 | 403.4 μM | 399.8 μM |
| | Repeat 2 | 401.2 μM | 399.2 μM |
| | Repeat 3 | 399.5 μM | 401.8 μM |
| Initial rate (A) | Repeat 1 | -45.3 $\mu M.min^{-1}$ | -13.2 $\mu M.min^{-1}$ |
| | Repeat 2 | -39.5 $\mu M.min^{-1}$ | -20.1 $\mu M.min^{-1}$ |
| | Repeat 3 | -32.0 $\mu M.min^{-1}$ | -17.0 $\mu M.min^{-1}$ |
| | **Average** | **-39.0 ± 6.6 $\mu M.min^{-1}$** | **-16.8 ± 3.4 $\mu M.min^{-1}$** |

Fitting curve equations:
$M_{NADPH} = At + B$
$M_{NADPH}\ (t=0) = B$
$dM_{NADPH}/dt = A$
$dM_{NADPH}/dt\ (t=0) = A$

$M_{NADPH}$: NADPH concentration (μM)
t: time (min)
$dM_{NADPH}\ /dt\ (t=0) = A$: Initial rate of NADPH consumption ($\mu M.min^{-1}$)
$M_{NADPH}\ (t=0) = B$: Initial NADPH concentration (μM)

**Table S3.** Table of calibration standard for $H_2O_2$ production assay.

| Standard # | Premix (μL) | $H_2O$ (μL) | $H_2O_2$ (μM) |
|---|---|---|---|
| 1 | 100 | 0 | 30 |
| 2 | 80 | 20 | 24 |
| 3 | 60 | 40 | 18 |
| 4 | 40 | 60 | 12 |
| 5 | 30 | 70 | 9 |
| 6 | 20 | 80 | 6 |
| 7 | 10 | 90 | 3 |
| 8 | 0 | 100 | 0 |

**Dataset S1 (CYP102_alignment_sorted.pdf).** Clustal omega alignment of 245 CYP102 amino acid sequences.

## SI References

Sample References:


1. Ravanfar R, Sheng Y, Gray HB, & Winkler JR (2023) Tryptophan-96 in cytochrome P450 BM3 plays a key role in enzyme survival. *FEBS Lett.* 597(1):59-64. DOI: 10.1002/1873-3468.14514.
2. Parvez M*, et al.* (2016) Molecular evolutionary dynamics of cytochrome P450 monooxygenases across kingdoms: Special focus on mycobacterial P450s. *Scientific Reports* 6(1):33099. DOI: 10.1038/srep33099