Supporting Information

Whole-cell microtiter plate screening assay for terminal hydroxylation of fatty acids by P450s

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Material and Method

1. Chemicals, bacterial strains and plasmids

Yeast extract, agar and tryptone were purchased from Roth (Germany), fatty acids (substrate and product ω-OHFA) and all other chemicals were acquired from Sigma-Aldrich (Germany). *E. coli* strain BL21 (DE3) was purchased from Novagen (Madison, WI, USA). The plasmid pET-30 containing the galactose oxidase mutant $M_{3\text{-}5}$ ($GOase_{M3\text{-}5}$) gene was kindly provided by the Manchester Institute of Biotechnology, University of Manchester.[1] The plasmid pET-28a(+) (Novagen, Madison, WI, USA) with $P450_{BM3}$ from *Bacillus megaterium* and pET-28a(+) harbouring the fusion construct between the heme domain of CYP153A from *Marinobacter aquaeolei* and the reductase domain of $P450_{BM3}$ ($CYP153A_{M.aq}$-CPR_{BM3}) wild type and each variant from the mutant library were generated at the University of Stuttgart.[2]

2. Sequence alignments and designed primers

The substitutions selected for mutagenesis were based on amino acids frequencies deriving from conservation analysis in CYPs families.[3,4] Site-directed mutagenesis was performed by QuikChange method to generate the small focussed library.

Table S1. Designed primers for the generation of a small-focussed mutant library

Variants	Primers	Sequences (5'--> 3')
V306I	Forward Reverse	ctg ctc ata ATT ggc ggc aac gat acg acg cgt cgt atc gtt gcc gcc AAT tat gac cag
G307R	Forward Reverse	ctg ctc ata gtc CGT ggc aac gat acg acg cgt cgt atc gtt gcc ACG gac tat gag cag
F455V	Forward Reverse	cgg gtg cag tcc aac GTG gtg cgg ggc tat ata gcc ccg cac CAC gtt gga ctg cac ccg
D134V	Forward Reverse	ccg caa atc att ctc ggt GTG cct ccg gag ggg ctg tcg cga cag ccc ctc cgg agg CAC acc gag aat gat ttg cgc
I145L	Forward Reverse	gaa atg ttc CTG gcg atg gat ccg ccg cgg cgg atc cat cgc CAG gaa cat ttc
S453A	Forward Reverse	cgg gtg cag GCG aac ttc gtg cgg ggc tat ata gcc ccg cac gaa gtt CGC ctg cac ccg

The PCR mixture contained: DNA polymerase buffer with $MgSO_4$ 10X (1X, 5 µL), forward/reverse primer (10 pmol, 1 µL each), DNTP mix (50 mM, 1 µL), plasmid template (100 ng, 2 µL), *pfu* Ultra II polymerase enzyme (1 µL) and sterile water to a final volume of 50 µL. The PCR program included:

1) Denaturation: 95 °C, 2 min
2) Denaturation: 95 °C, 30 sec
3) Annealing: 55 °C, 60 sec
4) Extension: 72°C, 4 min (30 sec/kb of plasmid length)

x 18 cycles

5) Final extension: 72°C, 7 min
6) Storage at 8 °C

Following the PCR, the mixtures were treated for 2 h at 37 °C (digestion step) with 1 µL of *Dpn I* restriction enzyme (Fermentas, Darmstadt, Germany), and subsequently purified and concentrated by a ZymoClean DNA concentrator kit prior to a chemical transformation into *E. coli* DH5α cells.

3. Fermentation, galactose oxidase expression and purification

$GOase_{M3-5}$ was transformed into *E. coli* BL21 Star™ (DE3) cells (Invitrogen) according to manufacturer's specifications. A single colony was picked from an overnight LB plate containing 1 µL of kanamycin of a 30 mg mL^{-1} stock solution per mL of agar and used to inoculate 5 mL LB medium supplemented with 5 µL kanamycin and grown overnight at 37 °C and 250 rpm. 500 µL of the overnight culture was used to inoculate 250 mL of an auto-induction medium (8ZY-4LAC)[5] and supplemented with 250 µL of kanamycin in a 2-L-baffled Erlenmeyer flask. The cells were grown at 26 °C and 250 rpm for 60 h. Cells were harvested by centrifugation at 8967 *g* and 4 °C for 20 min and subsequently prepared for protein purification. The protein purification was accomplished on ÄKTA purifier 10 (GE Healthcare Biosciences, Uppsala, Sweden) using a Strep-Tag-II column as described elsewhere.[5]

4. $CYP153A_{M.aq}$-CPR_{BM3} expression, whole resting cell preparation and biocatalytic performance

4.1. Bacterial cell fermentation and P450 expression

For overnight cultures, a fresh *E. coli* transformant was used to inoculate 5 mL of LB medium (30 µg mL^{-1} of kanamycin) and 100 µL were used to carry out a protein expression in 2 mL TB medium containing 2 µL kanamycin in 24-deep well plates. The cells were grown at 37 °C and 180 rpm on a plate shaker until an OD_{600nm} of 0.8-1 and the plates were covered with a plate sealer (Greiner Bio-One, Frickenhausen, Germany). For protein induction, 0.1 mM of IPTG, 0.5 mM of 5-aminolevulinic acid and 0.5 mM of $FeCl_3$ were added and the protein expression performed at 25 °C and 180 rpm for 10-16 h.

4.2. Lysate preparation and *in vitro* performance

For the preparation of cell extract subsequently after the protein expression, the cells were centrifuged (2752 *g*, 4 °C, 20 min) and the pellet resuspended in potassium phosphate buffer, 100 mM pH 7.4. The cells were lysed by sonication on ice (3x2 min, 1 min interval, 40 % amplitude, 0.35 s cycle time (Branson Sonifier 250, Schwäbisch Gmünd, Germany)) and centrifuged for removal of cells debris (34864 *g*, 45 min, 4 °C). A screen of activities in lysate was performed in 100 mM potassium phosphate buffer pH 7.4 with 1 mM of dodecanoic acid (C_{12}), 5 % DMSO (v/v), 1 mM NADPH, cofactor regeneration, and 0.5 µM of lysate for 2 h at 30 °C.

4.3. Whole resting cell biotransformation

To perform whole resting cell biotransformations consequently after the protein expression the plates were centrifuged for 10 min at 2752 *g* and 4 °C. The medium was discarded and the cell pellets washed once with 2 mL of 100 mM potassium phosphate buffer pH 7.4. After centrifugation at 3000 *g* for 3 min, the cell pellets were resuspended in the same buffer to reach 100 g_{cww} L^{-1} (corresponding to 1-2 mL buffer added). The biotransformations were performed with 1 mL of 50 g_{cww} L^{-1} resting cells in the presence of C_{12} and product 12-hydroxydodecanoic acid (ω-OHC_{12}), 5 % DMSO (v/v, not exceeding the solubility limit), for 2 h at 25 °C and 500 rpm. Negative controls were accomplished in the same fashion with the same concentration of organic co-solvent added. The reactions were stopped by centrifugation for 3 min at 3000 *g* and the supernatant was stored at -20 °C until further analysis.

5. Galactose oxidase assay

Prior to the GOase based assay, the cell-free supernatants from P450-catalysed reactions were treated at 90 °C for 30 min in order to deactivate potential additional metabolic activity. The GOase assay was accomplished in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in a final volume of 200 µL. To each well was added: 118 µL of the heat-treated supernatant, 1 µL of commercially available horseradish peroxidase (HRP, from 9000 units μL^{-1}), 8 µL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, from 10 mM stock), 10 µL of $GOase_{M3-5}$ (stock solution of 3.5-8 µM mL^{-1}) and 100 mM potassium phosphate buffer pH 7.4. Plates were read immediately for up to 4 h using a plate reader (POLARstar Omega BMG Labtech, Ortenberg, Germany). The formation of $ABTS^{ox}$ ($\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}) was monitored at $\lambda = 420$ nm, taking the linear and early part of the graphs (V_{max}, where there is no limiting substrate). One unit of $GOase_{M3-5}$ is defined as the amount of protein that oxidises 1 µmoL of substrate per minute at 30 °C.

6. CO spectra

The P450 protein concentration in whole cells was determined as described previously.[6,7] One spatula of sodium dithionite was added to the sample prior to incubation on ice for 30 min ensuring a complete reduction of the heme domain. Subsequently, CO gas (except for the reference well) was applied to the reduced samples followed by incubation at 4 °C for 1 h. Plates were read on a plate reader in a range of λ = 400-500 nm.

7. GC-FID analysis

The samples were extracted twice with the same volume of methyl *ter*-butylether (MTBE). The organic phases were combined and evaporated (GeneVac EZ 2 Plus, Ipswich England). For derivatization, to each sample 45 µL of MTBE and 45 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1 % trimethylchlorsilane were added and samples incubated at 70 °C for 30 min. Decanoic acid was used as internal standard for quantification. The

reaction progress was monitored by GC-FID (Shimadzu, Japan) using a Elite-5 column (PerkinElmer, Waltham, MA, USA) and H_2 as carrier gas. For analysis of C_{12} fatty acids, the column oven was set at 140 °C for 2 min, then raised to 250 °C at a rate of 10 °C min^{-1} for 1 min, held isotherm for 1 min, and then raised to 310 °C at 65 °C min^{-1}.

Supporting tables and figures

1. Galactose oxidase substrates panel

Table S2. Panel of primary alcohols and hydroxylated fatty acids tested as potential substrates for the GOase based assay.

Substrate	OD_{420nm} (after 10 min incubation)
ω-OHC_6	0.03 ± 0.022
ω-OHC_8	0.24 ± 0.019
ω-OHC_9	0.04 ± 0.0001
ω-OHC_{10}	0.18 ± 0.005
ω-OHC_{12}	0.10 ± 0.018
1-hexanol	0.55 ± 0.069
1-octanol	0.40 ± 0.056
1-dodecanol	0.15 ± 0.009

2. Heating step

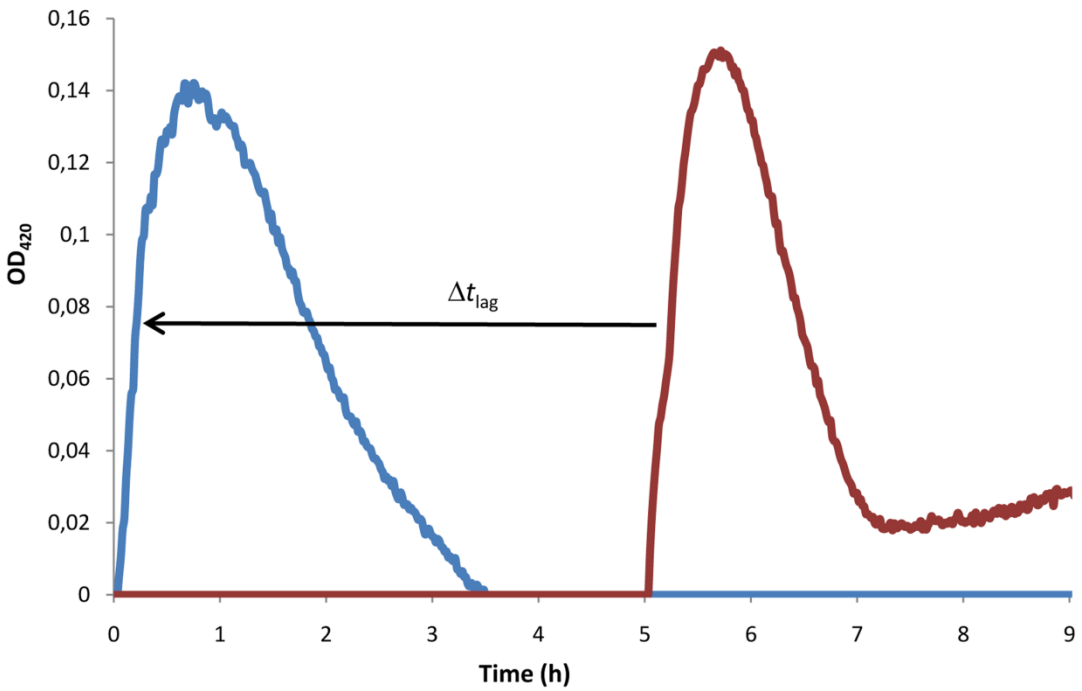

Figure S1. Heating step was applied to the supernatant following a 2 h biotransformation of 2 mM of C_{12} substrate with resting *E. coli* cells (50 g_{cww} L^{-1}). This crucial step enables a maximum of absorption displayed after 1 h incubation via the GOase assay (blue curve) compared to 6 h in the absence of the heating step (red curve).

3. ω-OHC_{12} detection limit

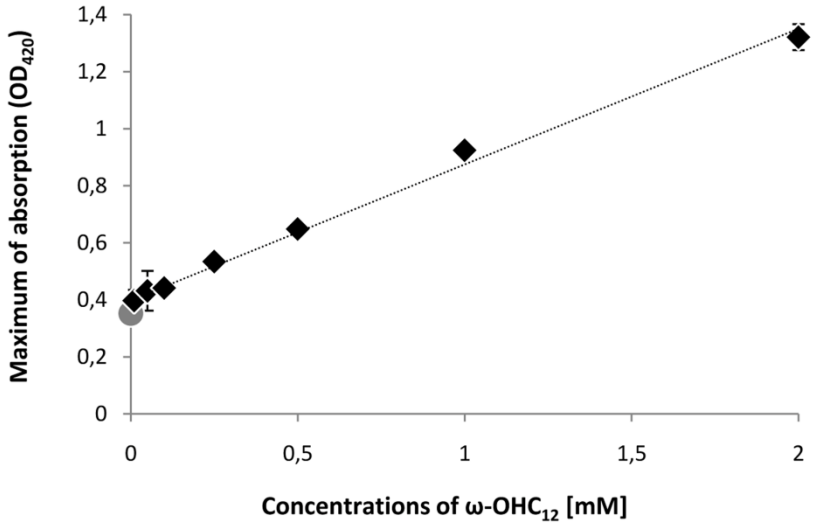

Figure S2. Increased concentrations of ω-OHC_{12} applied to whole resting cell experiments after 2 h. A control assay (replacement of the product by the same volume of DMSO) was also implemented (grey circle). The intensity of the signal recorded via the assay correlates with the GC-FID analysis: a higher intensity of signal equals to a large amount of product formed.

4. Controls

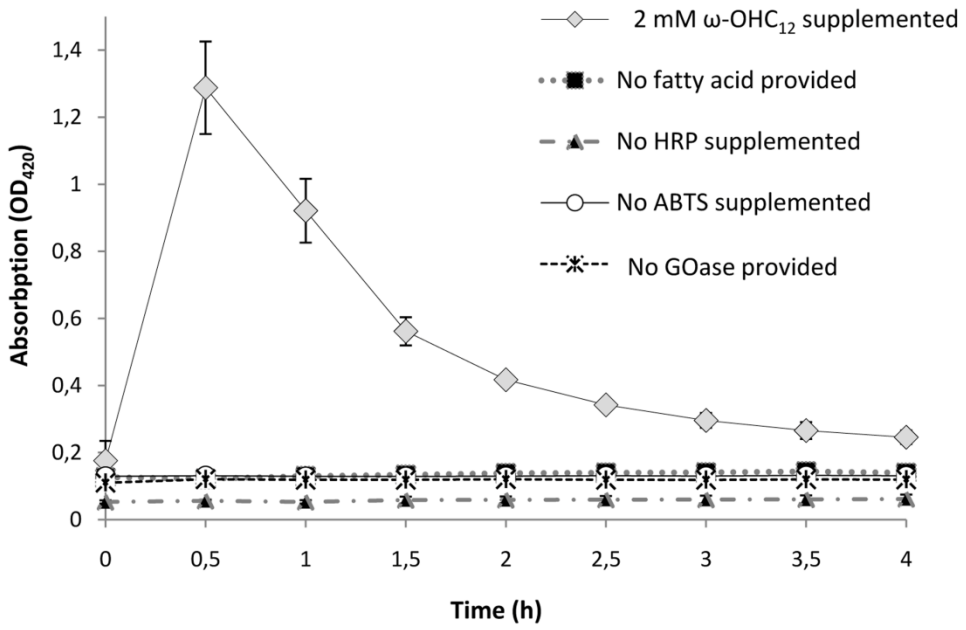

Figure S3. Control reactions were tested in the absence of one of the following components: GOase, HRP, ABTS or ω-OHC_{12} in comparison with a positive control containing each of the above mentioned ingredients. The GOase assay is only valid with the presence of each component.

5. Selectivity of the GOase$_{M3-5}$

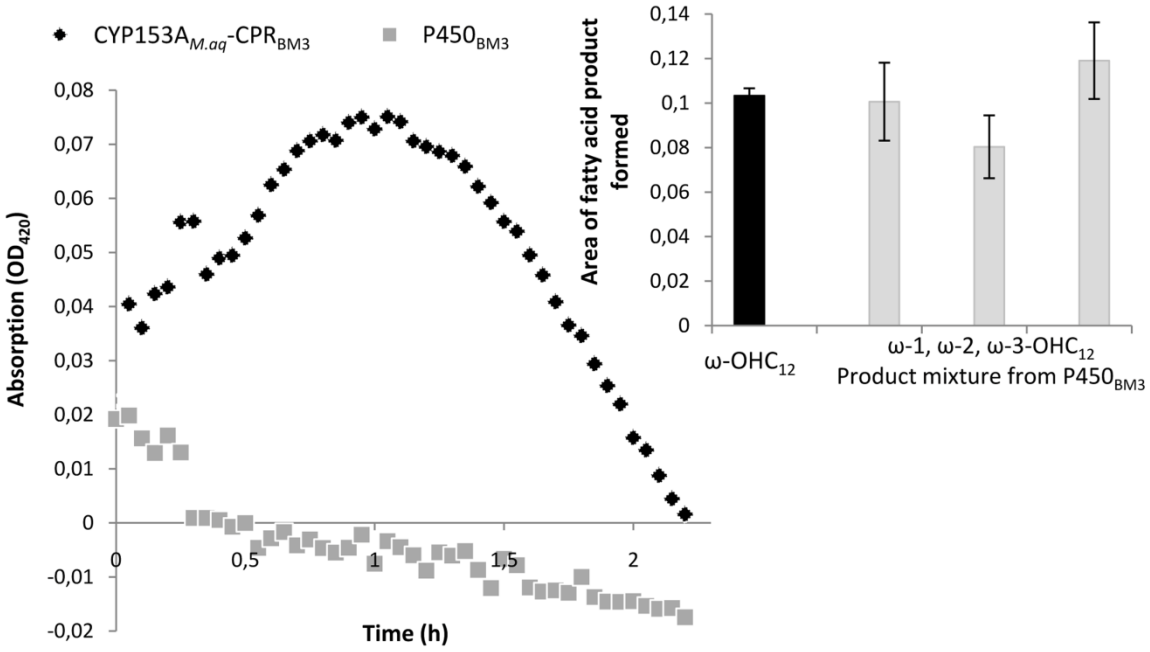

Figure S4. Comparison of the product formation between CYP153A$_{M.aq}$-CPR$_{BM3}$ and P450$_{BM3}$ measured by the GOase assay and after extraction and analysis on GC-FID. Specific activity of the GOase towards ω-OHC$_{12}$ as a signal of absorption is displayed during the assay only for the product of CYP153A$_{M.aq}$-CPR$_{BM3}$. P450$_{BM3}$ was shown active after GC-FID analysis, however the assay does not enable the detection of the product mixture ω-1, ω-2 and ω-3-OHC$_{12}$.

6. Small focussed mutant library

Table S3. Positions selected for site-directed mutagenesis. The substitutions for the positions located in the active site were selected based on the results from a previous small focussed library targeting the binding pocket only.[8] To expand the diversity three positions from the substrate access tunnel were also addressed for mutation.

Positions	Location	Selected substitution and frequency	Remarks
V306I	Active site (I –Helix)	14.98 %	Position involved in the anchoring of the carboxyl group of the substrate. Substitutions tested in the past: V306T
G307R	Active site (I –Helix)	0.71 %	Substitution G307A previously tested leading to the most active variant. Other substitution evaluated: G307V
F455V	Active site (C-terminal β-sheet loop)	6.73 %	Position influencing the substrate orientation to the heme centre. Previous substitutions tested: L, I, Y
D134V	Substrate entrance	3.18 %	Influence of the substrate access tunnel evaluated by replacing with hydrophobic residues
I145L	Substrate entrance	24.40 %	
S453A	Substrate entrance	4.33 %	

Table S4. The relative conversion and specific conversion of C_{12} from MTP and GC-FID as well as the specific activity were calculated for the parent enzyme (WT). The table displays comparisons to G307A set at 100 % and S453A mutants. (AS: active site; SE: substrate entrance, MTP: microtiter plate)

Mutants	Mutation locations	Rel. conversion MTP-Assay [%]	P450 conc. [µM]	Rel. specific conversion MTP-Assay [%]	Rel. specific conversion GC-FID [%]	Specific activity [µM min^{-1} $µM^{-1}$]
WT	-	76 ± 0.01	1.2 ± 0.03	60 ± 0.01	95 ± 0.04	2.50 ± 0.36
G307A	AS	100	1.3 ± 0.03	100	100	2.62 ± 0.57
S453A	SE	**86** ± 0.02	0.8 ± 0.14	**119** ± 0.03	**116** ± 0.03	**3.06** ± 0.28

References

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