Chapter 8

Materials and Methods

A. Reagents

All chemicals, including liquid alkanes and product standards, were purchased from Sigma-Aldrich (St. Louis, MO). Solvents (hexanes, chloroform, methylene chloride) were purchased from EMD (Gibbstown, NJ). Ethane (99.95% or 99.99%), propane (99.5 %), dimethyl ether (99.8%), and methane (99.5%) were purchased from Special Gas Services (Warren, NJ) and Airgas (El Monte, CA). Methane (99.0%), ethane (99.99%), propane (99.975), butane (99%), and gaseous halomethanes, chloromethane (99.8%), and bromomethane (99.5%) were purchased from Sigma-Aldrich. NAD^+, DL-isocitric acid trisodium salt, and recombinant isocitrate dehydrogenase used as an NADPH regeneration system in experiments described in Chapters 2 – 4 were purchased from Sigma-Aldrich. $NADP^+$ and NADPH were also purchased from Codexis, Inc. (Redwood City, CA). Recombinant isocitrate dehydrogenase was also purchased from OYC, Inc. (Azusawa Japan). Alcohol oxidase, horse radish peroxidase, and ABTS used in ethanol and methanol detection assays in experiments described in Chapters 3 and 5, were purchased from Sigma-Aldrich. Restriction enzymes (*Bam*HI, *Sac*I, *Eco*RI, *Nde*I, *Xho*I, *Kpn*I) and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA), Taq DNA polymerase from Roche (Indianapolis, IN), Pfu turbo DNA polymerase from Stratagene (La Jolla, CA), and Phusion DNA polymerase from Finnzymes (Woburn, MA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from MP Biomedicals, Inc. (Aurora, OH).

B. Expression of P450s

B.1. BM3 holoenzymes (Chapters 2, 3, 5, and 7)

The P450 BM3 gene was cloned downstream of a double *tac* promoter of the expression vector pCwori using the flanking restriction sites *BamH*I and *EcoR*I. Silent mutations were used

to introduce a *Sac*I site 130 bp upstream of the end of the heme domain and a *Kpn*I site at 81 bp downstream of the end of the heme domain to facilitate cloning of heme libraries. *Escherichia coli* Dh5α cells transformed with the plasmids containing P450 BM3 and its derived variants were used for the expression on both 50 – 500 mL scales as well as in 96-well plates. For protein production, terrific broth (TB) medium containing 100 μg/ml ampicillin was inoculated with an overnight culture at 1:500 dilution for shake flask cultures and 1:20 dilution for 96-well plates. The cultures were initially incubated at 37 ^{o}C and 250 rpm for 4.5 hours, and after cooling to 25 ^{o}C, expression was induced by the addition of a mixture of IPTG (1 mM) and δ-aminolevulinic acid hydrochloride (δ-ALA; 0.5 mM). The cultures were harvested by centrifugation at 4 ^{o}C and 5,000 rpm for 10 min 20 to 24 hours after induction and stored at -20 ^{o}C. The protein yields varied from about 10 to 100 mg/L depending on the variant identities.

B.2. Expression of P450$_{cam}$, CYP153A6, fdrA6, fdxA6, and BM3 heme domain (Chapter 6)

The genes of P450$_{cam,}$ CYP153A6, CYP153A6 ferredoxin, and CYP153A6 ferredoxin reductase were cloned into the pET-22b(+) expression vector downstream of a T7 promoter using flanking restriction sites *Nde*I and *Xho*I. A his$_6$ affinity tag along with the amino acid pair of leucine and glutamic acid corresponding to the *Xho*I site were also introduced to the C-terminus of the P450cam and CYP153A6 constructs. The heme domain of P450 BM3 and its derived variants (amino acids 1-463) were cloned into a modified pCwori vector with flanking restriction sites *BamH*I and *Xho*I. The same his$_6$-tag with the leucine and glutamic acid appendage was also introduced to the C-terminus. *E. coli* BL21(DE3) cells transformed with these plasmids were used for expression in 50 – 500 mL scales. For expression, supplemented TB medium (500 mL, 100 μg/ml ampicillin, trace metal mix: 50 μM $FeCl_3$, 20 μM $CaCl_2$, 10

μM $MnSO_4$, 10 μM $ZnSO_4$, 2 μM $CoSO_4$, 2 μM $CuCl_2$, 2 μM $NiCl_2$, 2 μM $NaMoO_4$, and 2 μM H_3BO_3) was inoculated with TB overnight culture to an initial optical density at 600 nm (OD_{600}) of 0.5. After 3.5 hours of incubation at 37 ^oC and 250 rpm, the cultures were cooled to 25 ^oC, and expression was induced with the addition of IPTG (1 mM). The cells were harvested by centrifugation at 4 ^oC, 5,000 rpm for 10 mins, 20 to 24 hours after induction and stored at -20 ^oC. For the expression of fdrA6 and fdxA6, the cultures were cooled to 30 ^oC for induction and allowed to express for 24 to 30 hours at 30 ^oC.

C. Purification of P450s and Reductase Components

C.1. Single-step purification of P450 BM3 (Chapters 2, 3, 5,and 7)

A single-step purification protocol was developed based on a published procedure (*1*). During the purification, all samples were kept on ice or at 4 ^oC. Cell pellets of BM3 and its derived variants were resuspended in Tris-HCl (25 mM, pH 8.0, 0.5 ml/gram cell weight) and lysed by sonication (2 x 30 s, output control 5, 50% duty cycle; Sonicator, Heat Systems–Ultrasonic, Inc.). The lysate was centrifuged at 20,000 rpm for 30 min, and the supernantent was filtered with a 22 μM filter (Millipore, Billerica, MA) before loading on a pre-equilibrated Toyopearl® Super Q-650M column for ion exchange chromatography. After washing with five column volumes of 25 mM Tris-HCl and five column volumes of 0.15 M NaCl in 25 mM Tris-HCl, the protein was eluted with 0.34 M NaCl in 25 mM Tris-HCl. The P450 fractions were collected and concentrated using a 30 kDa molecular weight cut-off centrifugal (mwco) filter (Millipore, Billerica, MA). The protein solution was then buffer-exchanged with potassium phosphate buffer (100 mM, pH 8.0) by either using PD-10 desalting columns (GE heathcare, Piscataway, NJ), or through three dilution and concentration cycles in Amicon spin columns. The

P450 enzyme concentrations were quantified by CO-binding difference spectra as described (*2*), using 91 mM^{-1} cm^{-1} as the extinction coefficient. The protein solutions were flash-frozen with dry ice and stored at -80 ^{o}C.

C.2. Three-step purification of P450cam, CYP153A6, and BM3 heme domains (Chapter 6)

To obtain highly pure protein preparations for terminal oxidant reactions described in Chapter 6, a three-step purification was employed. During the purification, all samples were kept on ice or at 4 ^{o}C. Cell pellets were resuspended in Ni-NTA buffer A, (25 mM Tris-HCl, 100 mM NaCl, 30 mM imidazole, pH 8.0, 0.5 ml/gcw) and lysed by sonication (2 x 30 s, output control 5, 50% duty cycle; Sonicator, Heat Systems–Ultrasonic, Inc.). The lysate was centrifuged at 20,000 rpm for 30 min, and the supernantent was filtered with a 22 μM filter before loading on a pre-equilibrated Ni-NTA column.

After washing with five column volumes of Ni-NTA buffer A, the P450 was eluted with buffer B (25 mM Tris-HCl, 100 mM NaCl, 100 mM imidazole, pH 8.0). After buffer-exchange with 25 mM Tris-HCl, the desalted protein solution was loaded onto a pre-equilibrated Q sepharose^{TM} column (HiTrap^{TM} Q HP, GE healthcare, Piscataway, NJ). A wash step with five column volumes of 25 mM Tris-HCl was followed by elution of the P450 with a linear gradient of NaCl from 0 to 500 mM over 10 column volumes.

The P450 fractions were collected and concentrated using a 30 kDa mwco centrifugal filter. Following buffer exchange with 20 mM HEPES buffer, the protein solution was loaded onto a pre-equilibrated HiPrep 16/60 Sephacryl S-100 HR column (GE healthcare, Piscataway, NJ). The protein was eluted with a flow rate of 0.5 ml/min; purified fractions were concentrated to 2 mL using a 30 kDa mwco centrifugal filter. To remove potential alcohol contaminants, the purified

protein was flash-frozen with dry ice and lyophilized overnight on a bench-top lyophilizer (Millrock Technology, Kingston, NY). P450 concentration was determined in triplicate after resuspension in potassium phosphate buffer (100 mM pH 8.0) by CO-difference spectroscopy.

C.3. Two-step purification of fdrA6 and fdxA6 (Chapter 6)

To obtain the A6 reductase components for NADH/O_2-supported alkane reactions described in Chapter 6, a two-step purification was employed. During the purification, all samples were kept on ice or at 4 ^{o}C. Cell pellets were resuspended in 25 mM Tris-HCl at 0.5 ml/gcw and lysed by sonication (2 x 30 s, output control 5, 50% duty cycle; Sonicator, Heat Systems–Ultrasonic, Inc.). The lysate was centrifuged at 20,000 rpm for 30 min, and the supernantent was filtered with a 22 μM filter before loading on a pre-equilibrated Q sepharose^{TM} column (HiTrap^{TM} Q HP, GE healthcare, Piscataway, NJ). A wash step with five column volumes of 25 mM Tris-HCl was followed by elution of the P450 with a linear gradient of NaCl from 0 to 500 mM over 10 column volumes.

The protein fractions were collected and concentrated using a 10 and 30 kDa mwco centrifugal filter for fdxA6 and fdrA6, respectively. Following buffer exchange with 20 mM HEPES buffer, the protein solution was loaded onto a pre-equilibrated HiPrep 16/60 Sephacryl S-100 HR column (GE healthcare, Piscataway, NJ). The proteins were eluted with a flow rate of 0.5 ml/min; purified fractions were concentrated and flash-frozen with dry ice before lyophilization with a bench-top lyophilizer (Millrock Technology, Kingston, NY).

D. Mutagenesis and Library Construction of P450 BM3 Variants

D.1. Thermostabilization of variant 35E11 (Chapter 2)

In our effort to thermostabilize variant 35E11, we grafted known stabilizing mutations of the P450 heme domain (*3*). The following mutations L52I, S106R, M145A, L324I, V340M, I366V, and E442K in conjunction were found to stabilize a P450 BM3-derived peroxygenase by 18 ^{o}C. Each of these mutations was first individually introduced into the pCwori_35E11 plasmid by site-directed overlap extension PCR (SOE-PCR) (*4*). BamHI_Fwd and SacI_rev served as flanking primers. For all primer sequences see section D.10. For each mutation, two separate PCRs were performed with *Pfu* turbo (Stratagene), each using a perfectly complementary flanking primer and a mutagenic primer. The two resulting overlapping fragments containing the nucleotide substitutions were then annealed in a second PCR to amplify the complete mutated gene. The amplified gene was then digested with *BamH*I and *Sac*I restriction enzymes and ligated with T4 DNA ligase into pCwori_WT, previously digested with *BamH*I and *Sac*I to remove the wild-type gene. The ligation mixtures were used to transform electro-competent *E. coli* DH5α cells. The transformed cells were plated onto Luria-Bertani (LB) (*5*) agar plates containing ampicillin to form single colonies. Colonies from each of the seven variants were expressed and purified as described above. Mutations that resulted in an improved T_{50} (see Section J) and less than 20% loss of propane turnover number (TON) (see Section F.1) were recombined in a pair-wise fashion using SOE-PCR. The best variant identified in this thermostabilization effort was ETS8 with mutation L52I and I366V.

D.2. Random mutagenesis of ETS8 (Chapter 2)

A random mutagenesis library of the heme domain of variant ETS8 (aa 1-433) was created by error-prone PCR based on published protocols (*6*). Four different concentrations of $MnCl_2$ (50, 100, 200, 300 μM) were used to induce mutations with *Taq* DNA polymerase (Roche) in the mutagenic PCR containing approximately 50 ng of plasmid DNA as template, and BamHI_Fwd and SacI_rev serving as end primers. The PCR product was then digested with *BamH*I and *Sac*I restriction enzymes and ligated with T4 DNA ligase into pCwori_WT, previously digested with *BamH*I and *Sac*I to remove the wild-type gene. The ligation mixtures were then electoporated into electro-competent *E. coli* DH5α cells. The transformed cells were plated onto LBamp agar plates to form single colonies. For each Mn^{2+} concentration, a small library of 88 mutants in a 96-well plate (four wells containing parent ETS8, four blank wells) was picked and screened for both folding by CO-binding difference spectroscopy (E.2) as well as activity toward dimethyl ether as described below (E.3). The library prepared with a Mn^{2+} concentration of 200 μM yielded ~ 50% inactive mutants, and thus it was selected for screening of 2,500 members for activity towards dimethyl ether. Variant 19A12 was selected from the DME screening with roughly twice the parental activity.

D.3. Site-saturation mutagenesis and recombination of variant 19A12 (Chapter 2)

Using pCwori_19A12 as template, 17 residues along the active site channel, identified from the P450 BM3 crystal structure 1JPZ, were selected for saturation mutagenesis. NNK libraries were constructed individually at positions 74, 75, 78, 82, 87, 88, 181, 184, 188, 260, 264, 265, 268, 328, 401, 437, and 438 by SOE-PCR. The mutagenic primer for each library contained an NNK (N = A, T, G or C, K = T or G) codon at the mutation site, which encoded all 20 possible amino acids and only one stop codon out of the possible 32 codons. For each library, two separate PCRs were performed, each using a perfectly complementary end primer and a

mutagenic primer. The two resulting overlapping fragments were then annealed in a second PCR to amplify the complete mutated gene. For the libraries at residue 437 and 438, BamHI_Fwd and EcoRI_rev were used as the end primers, and digestion with *BamH*I and *EcoR*I was employed. For all other libraries BamHI_Fwd and SacI_Rev were used. End primers and the subsequent cloning into pre-digested pCwori_WT occurred as previously described (D.1).

For each library, 88 mutants in a 96-well plate (four wells containing parent 19A12, four blank wells) were picked and screened for folding and activity toward dimethyl ether. The five beneficial mutations A74S, A74Q, V184S, V184T, and V184A were recombined using degenerate primers or a mixture of primers. A second recombination library was also constructed that allowed for 74NNK, 82S/G, and 184NNK. Screening these two libraries produced variants 1-3 (19A12-A74S, V184A) and 7-7 (19A12-A74E, S82G) with comparable propane TTN.

D.4. Reductase libraries construction (Chapter 2)

The FMN and FAD sub-domains of the BM3 reductase were mutated separately by error-prone PCR with *Taq* Polymerase (Roche). Using pCwori_35E11 as template, the primer pairs FMN_for/ FMN_rev and FAD_for/FAD_rev were used in the PCR relying on $MnCl_2$ (100, 200, 300 μM) as the mutation inducer. The amplified genes were cloned into pre-digested pCwori_35E11 using restriction sites *Sac*I and *Nsi*I for FMN libraries and *Nsi*I and *Eco*RI for the FAD libraries. Screening for improved DME activity yielded beneficial mutations at positions 443, 445, 515, 580, 654, 664, 698, and 1037.

Using pCwori_11-3 as template, site-saturation libraries were constructed at all eight positions identified from the 35E11 random reductase libraries using NNK primers and SOE PCR as described (D.1). FMN_for and EcoRI_rev end primers were used to amplify the entire

reductase. Following *Sac*I and *EcoR*I digest, the mutagenized genes were ligated into a pre-digested pCwori_11-3 and electoporated into *E. coli* Dh5α. Screening of 88 colonies from each library identified beneficial mutations G443A, P654K, T664G, D698G, and E1037G. A recombination library was constructed using degenerate or mixed primers allowing for both the wild-type amino acid and mutated amino acid at each position. This reductase library was fused to pCwori_7-7 through the *Sac*I and *EcoR*I restriction sites. Screening of the resulting library yielded $P450_{PMO}$ R1 (7-7-G443A, P654K, and E1037G) and $P450_{PMO}$ R2 (7-7-G443A, D698G).

D.5. Targeted mutagenesis of the P450 BM3 active site (Chapter 3)

Ten residues along the active site channel, identified from the P450 BM3 crystal structure 1JPZ, were selected for saturation mutagenesis. Using pCwori_WT as template, NNK libraries were constructed individually at positions 74, 75, 78, 82, 87, 181, 184, 188, 328, and 330 by SOE-PCR as described (D.1). At least 91 colonies of each library were screened for folding by CO-binding difference spectroscopy and activity for dimethyl ether.

Combinatorial active site saturation test with a reduced set of allowed amino acid (reduced CASTing) libraries targeting two and three sites simultaneously were constructed at positions 78, 82, and 328 with degenerate codons that encoded for L, I, F, V, A, M, and W. To obtain the desired amino acid restriction at each site, three primers containing the codons GYN (encodes A and V), WTS (encodes L, I, M and F), and TGG (encodes W) were mixed to a 2:4:1 ratio to achieve equal representation of each amino acid. A modified SOE-PCR strategy was used for two-site focused libraries, where the target residues were more than 20 nucleotides apart, A328+V78 and A328+A82. Three overlapping fragments were first generated using the primer pairs, BamHI_Fwd/Site1_rev, Site1_fwd/Site2_rev, and Site2_fwd/SacI_Rev in three

separate reactions. A second PCR annealed these three fragments and amplified the full length gene with the end-primers BamHI_Fwd and SacI_Rev. The two-site focused library with target residues less than 20 nucleotides apart, V78+A82, was constructed with a sequential SOE-PCR strategy. Standard SOE-PCR was used to introduce the first mutagenic codon into the full-length gene, which then served as template for a second round of SOE-PCR. The three-site focus library, A328+V78+A82, was constructed similarly by sequential SOE-PCRs.

D.6. Random mutagenesis of P450 BM3 (Chapter 3)

A random mutagenesis library of the heme domain of P450 BM3 (aa 1-433) was created by error-prone PCR as described (D.2). Four different concentrations of $MnCl_2$ (50, 100, 150, and 200 μM) were used to induce mutations with *Taq* DNA polymerase (Roche). From screening 88 colonies of each of the four libraries for both folding and activity toward dimethyl ether, the library prepared with a 150 μM Mn^{2+} concentration, which contained ~ 50% inactive mutants, was selected for full screening. Ten variants were randomly selected for sequencing, which revealed an average error rate of 2.1 amino acids/protein. In the subsequent full screening, 1,408 members of this library were assayed for activity towards dimethyl ether.

D.7. Construction of CRAM- and C^{orbit}- designed libraries (Chapter 3)

The CRAM and C^{orbit} libraries were constructed to allow for two possible amino acids at ten selected residues. The allowed amino acids were determined for each design following the algorithms detailed in Appendix B. The selected amino acids and the degenerate codons or mixed codons that were used in library construction are summarized below in Table 8.1.

Table 8.1 CRAM and C^{orbit} designs; allowed amino acids and codon usage. (K = T or G, S = C or G, W = A or T, Y = C or T, R = A or G)

	CRAM Design		C^{orbit} Design	
Target Residue	Allowed Amino Acid	Codon	Allowed Amino Acid	Codon
A74	L/W	TKG	A/V	GYC
L75	L/F	TTS	L/F	TTS
V78	F/I	WTT	L/V	STG
A82	L/V	STG	A/S	KCG
F87	F/A	TTC/GCA	F/A	TTC/GCA
L181	L/W	TKG	L/F	TTS
A184	A/V	GYC	A/T	RCG
L188	L/W	TKG	L/W	TKG
A328	F/V	KTC	A/F	TTC/GCA
A330	L/W	TKG	A/V	GYC

For both libraries, the ten target positions were clustered into three different nodes for ease of primer design and PCR. The first node included residues 74, 75, 78, 82, and 87. A single mutagenic primer was able to span this entire node and introduce the desired codon mixture to all five positions. For the remaining targeted positions, residues 181, 184, and 188 were grouped into a second node, and residues 328 and 330 were grouped into a third node. Four overlapping fragments were first generated using BamHI_Fwd and SacI_Rev in conjunction with forward and reverse mutagenic primers at each node. These four fragments were annealed in a second PCR to amplify the full-length gene. Digestion with *BamH*I and *Sac*I was followed by ligation with T4 DNA ligase into a pre-digested pCwori_WT. The ligation mixture was then electroporated into electro-competent *E. coli* Dh5α cells and plated on LBamp. Single colonies

were picked into 96-well plates and screened for protein folding and activity for dimethyl ether as described above.

D.8. Random mutagenesis of AlkB and Cyp153A6 (Chapter 4)

Mutagenesis of pCom plasmids was performed in *E. coli* XL1-Red strains according to the manufacturer's manual (Stratagene) and in *E. coli* JS200 (pEPPol I) as described previously (*7*). Mutated *alkB* genes or the CYP153A6 gene along with the *fdrA6* and *fdxA6* operons were cloned into the original pCom10 or into pCom8* plasmids as *Eco*RI-*Hind*III or *Kpn*I-digested fragments, respectively. Plasmid pCom8_alkBFG was constructed by amplifying the *alkBFG* operon from plasmid pblaP4_*alkJBFG-luxAB* (*8*) using the primers alkBFG_1 and alkBFG_2 in a standard PCR. The resulting fragment was cloned into the pCom8 vector using the *Nde*I and *Xma*I restriction sites introduced by the primers. The resulting plasmid was digested with *Spe*I, and the *alkB*-containing 3.7 -kb fragment was replaced with the appropriate fragment from *Spe*I-digested pCom10_alkB, pCom10_alkB-BMO1, and pCom10_alkB-BMO2 by cloning, resulting in constructs pCom8_alkBFG, pCom8_alkB-BMO1_alkFG, and pCom8_alkBBMO2_alkFG.

D.9. Site-saturation and random mutagenesis of variant E31 (Chapter 5)

Ten residues along the active site channel, identified from the P450 BM3 crystal structure 1JPZ, were selected for saturation mutagenesis. Using pCwori_E31 identified from the CRAM and C^{orbit} screening as template, NNK libraries were constructed individually at positions 74, 75, 78, 82, 184, 263, 264, 328, 436, and 437 by SOE-PCR as described (D.1). At least 88 colonies of each library were screened for protein folding by CO-binding difference spectroscopy and activity for ethane hydroxylation (E.4).

The first-generation random mutagenesis library targeting the heme domain of variant E31 (aa 1-490) was created by error-prone PCR using the Genemorph II kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Approximately 100 ng of plasmid DNA were used as template along with primers Heme_fwd_1 and KpnI_rev in the PCR. Sequencing of ten randomly selected variants revealed an average nucleotide substitution rate of 3.7/protein was obtained for this library. After screening 3,000 members of this library, we obtained variants 24F8 and 22F11 with improved ethane hydroxylation activity with three and two mutations, respectively. SOE-PCR was used to generate a recombination library with these five mutations. From screening 90 colonies of this library for improved ethane hydroxylation activity, we obtained variant RD2 with only two of the five mutations.

The second-generation random mutagenesis library targeting the heme domain was constructed using pCwori_RD2 as the template at three different concentrations of $MnCl_2$ (175, 200, and 300 μM) to induce mutations with *Taq* DNA polymerase (Roche). Sequencing of ten randomly selected variants revealed that an average nucleotide substitution rate of 4.3/protein was obtained for this library. A pre-screen of 88 colonies from each library led us to choose the 175 μM Mn^{2+} library for screening. After screening 3,000 members of this library for improved ethane hydroxylation activity, we obtained variants 20D4.

D.10. Primer list

Table 8.2 Primers used in P450 library construction

#	Primer name	SEQUENCE
1	L52I_for	5'-CGCGCTACATATCAAGTCAGC-3'
2	L52I_rev	5'-GCTGACTTGATATGTAGCGCG-3'
3	M145A_for	5'-GTATCGGAAGACGCGACACGTTTAACG-3'
4	M145A_rev	5'-GTATCGGAAGACGCGACACGTTTAACG-3'
5	V340M_for	5'-GAAGATACGATGCTTGGAGGAG-3'

6	V340M_rev	5’-CTCCTCCAAGCATCGTATCTTC-3’
7	I366V_for	5’-CGTGATAAAACAGTTTGGGGAGACG-3’
8	I366V_rev	5’-CGTCTCCCCAAACTGTTTTATCACG-3’
9	E442K_for	5’-CGTTAAAACCTAAAGGCTTTGTGG-3’
10	E442K_rev	5’-CCACAAAGCCTTTAGGTTTTAACG-3’
11	L324I_for	5’-CGAAGCGCTGCGCATCTGGCCAACTT-3’
12	L324I_rev	5’-AAGTTGGCCAGATGCGCAGCGCTTCG-3’
13	S106R_for	5’-CTTACTTCCAAGGTTCAGTCAGCAGG-3’
14	S106R_rev	5’-CCTGCTGACTGAACCTTGGAAGTAAG-3’
15	BamHI_fwd	5’-CACAGGAAACAGGATCCATCGATGCTTAGG-3’
16	SacI_rev	5’-CTAGGTGAAGGAATACCGCCAAGCGGA-3’
17	L437NNK_for	5’-CGATATTAAAGAAACTNNKACGTTAAAACC-3’
18	L437NNK_rev	5’-GGTTTTAACGTMNNAGTTTCTTTAATATCG-3’
19	T438NNK_for	5’-CGATATTAAAGAAACTTTANNKTTAAAACC-3’
20	T438NNK_rev	5’-GGTTTTAAMNNTAAAGTTTCTTTAATATCG-3’
21	EcoRI_Rev	5’-CCGGGCTCAGATCTGCTCATGTTTGACAGC-3’
22	L181NNK_for	5’-GGTCCGTGCANNKGATGAAGTAATG-3’
23	L181NNK_rev	5’-CATTACTTCATCMNNTGCACGGACC-3’
24	A82NNK_for	5’-CGTGATTTTNNKGGAGACGGGTTA-3’
25	A82NNK_rev	5’-TAACCCGTCTCCMNNAAAATCACG-3’
26	A74NNK_for	5’-AACTTAAGTCAANNKCTTAAATTC-3’
27	A74NNK_rev	5’-GAATTTAAGMNNTTGACTTAAGTT-3’
28	L75NNK_for	5’-GTCAAGCGNNKAAATTCTTTCGTG-3’
29	L75NNK_rev	5’-CACGAAAGAATTTMNNCGCTTGAC-3’
30	V78NNK_for	5’-GTCAAGCGCTTAAATTCNNKCGTGATTTT-3’
31	V78NNK_rev	5’-AAAATCACGMNNGAATTTAAGCGCTTGAC-3’
32	A328NNK_for	5’-GGCCAACTNNKCCTGCGTTTTCC-3’
33	A328NNK_rev	5’-GGAAAACGCAGGMNNAGTTGGCC-3’
34	A184NNK_for	5’-GCACTGGATGAANNKATGAACAAG-3’
35	A184NNK_rev	5’-CTTGTTCATMNNTTCATCCAGTGC-3’
36	L188NNK_for	5’-GAACAAGNNKCAGCGAGCAAATCC-3’
37	L188NNK_rev	5’-GGATTTGCTCGCTGMNNCTTGTTC-3’
38	I401NNKfwd	5’-GCGTGCGTGTNNKGGTCAGCAG-3’
39	I401NNKrev	5’-CTGCTGACCMNNACACGCACGC-3’
40	T268NNKfwd	5’-GCGGGACACGAANNKACAAGTGGTC-3’
41	T268NNKrev	5’-GACCACTTGTMNNTTCGTGTCCCGC-3’
42	G265NNKfwd	5’-CATTCTTAATTGCGNNKCACGAAACAACAAGTG-3’
43	G265NNKrev	5’-CACTTGTTGTTTCGTGMNNCGCAATTAAGAATG-3’
44	A264NNKfwd	5’-CATTCTTAATTNNKGGACACGAAACAACAAGTG-3’
45	A264NNKrev	5’-CACTTGTTGTTTCGTGTCCMNNAATTAAGAATG-3’

46	T260NNKfwd	5’-CAAATTATTNNKTTCTTAATTGCGGGAC-3’
47	T260NNKrev	5’-GTCCCGCAATTAAGAAMNNAATAATTTG-3’
48	L75NNKfwd	5’-GTCAAGCGNNKAAATTTGTACG-3’
49	L75NNKrev	5’-GTCCCGCAATTAAGAAMNNAATAATTTG-3’
50	T88NNKfwd	5’-GACGGGTTATTTNNKAGCTGGACGCATG-3’
51	T88NNKrev	5’-GTCCCGCAATTAAGAAMNNAATAATTTG-3’
52	F87NNKfwd	5’-GACGGGTTANNKACAAGCTGG-3’
53	F87NNKrev	5’-CCAGCTTGTMNNTAACCCGTC-3’
54	A82G_for	5’-CGTGATTTTGGTGGAGACGGGTTA-3’
55	A82G_rev	5’-TAACCCGTCTCCACCAAAATCACG-3’
56	FMN_for	5’-GCTGGTACTTGGTATGATGCT-3’
57	FMN_rev	5’-CCAGACGGATTTGCTGTGAT-3’
58	FAD_for	5’-CGTGTAACAGCAAGGTTCGG-3’
59	FAD_rev	5’-CTGCTCATGTTTGACAGCTTATC-3’
60	G443NNK_for	5’-CGTTAAAACCTGAANNKTTTGTGG-3’
61	G443NNK_rev	5’-CCACAAAMNNTTCAGGTTTTAACG-3’
62	V445NNK_for	5’-CCTGAAGGCTTTNNKGTAAAAGCA-3’
63	V445NNK_rev	5’-TGCTTTTACMNNAAAGCCTTCAGG-3’
64	T480NNK_for	5’-CGCTCATAATNNKCCGCTGCTTGTG-3’
65	T480NNK_rev	5’-CACAAGCAGCGGMNNATTATGAGCG-3’
66	T515NNK_for	5’-CCGCAGGTCGCANNKCTTGATTCAC-3’
67	T515NNK_rev	5’-GTGAATCAAGMNNTGCGACCTGCGG-3’
68	P654NNK_for	5’-GCGGATATGNNKCTTGCGAAAATG-3’
69	P654NNK_rev	5’-CATTTTCGCAAGMNNCATATCCGC-3’
70	T664NNK_for	5’-GGTGCGTTTTCANNKAACGTCGTAGCA-3’
71	T664NNK_rev	5’-TGCTACGACGTTMNNTGAAAACGCACC-3’
72	D698NNK_for	5’-CAAGAAGGANNKCATTTAGGTG-3’
73	D698NNK_rev	5’-CACCTAAATGMNNTCCTTCTTG-3’
74	E1037NNK_for	5’-CAGCAGCTAGAANNKAAAGGCCG-3’
75	E1037NNK_rev	5’-CGGCCTTTMNNTTCTAGCTGCTG-3’
76	BMfor_1504	5’-GCAGATATTGCAATGAGCAAAGG-3’
77	BMrev1504	5’-CCTTTGCTCATTGCAATATCTGC-3’
78	BMfor2315	5’-CGGTCTGCCCGCCGCATAAAG-3’
79	BMrev2315	5’-CTTTATGCGGCGGGCAGACCG-3’
80	Heme fwd 1	5'-CAGGAAACAGGATCAGCTTACTCCCC-3'
82	74NNK fwd	5'-GATAAAAACTTAAGTCAANNKCTTAAATTTGTACGTG-3'
83	74NNK rev	5'-CACGTACAAATTTAAGMNNTTGACTTAAGTTTTTATC-3'
84	75NNK fwd	5'-GATAAAAACTTAAGTCAAGCGNNKAAATTTGTACGTG-3'
85	75NNK rev	5'-CACGTACAAATTTMNNCGCTTGACTTAAGTTTTTATC-3'
86	78NNK fwd	5'-GCGCTTAAATTTNNKCGTGATTTTGCAGG-3'

87	78NNK rev	5'-CCTGCAAAATCACGMNNAAATTTAAGCGC-3'
88	81NNK fwd	5'-GTACGTGATNNKGCAGGAGACGGG-3'
89	81NNK rev	5'-CCCGTCTCCTGCMNNATCACGTAC-3'
90	82NNK fwd	5'-GTACGTGATTTTNNKGGAGACGGG-3'
91	82NNK rev	5'-CCCGTCTCCMNNAAAATCACGTAC-3'
92	87NNK fwd	5'-GACGGGTTANNKACAAGCTGGACGC-3'
93	87NNK rev	5'-GCGTCCAGCTTGTMNNTAACCCGTC-3'
94	88NNK fwd	5'-GACGGGTTATTTNNKAGCTGGACGC-3'
95	88NNK rev	5'-GCGTCCAGCTMNNAAATAACCCGTC-3'
96	181NNK fwd	5'-GTCCGTGCANNKGATGAAGCAATGAAC-3'
97	181NNK rev	5'-GTTCATTGCTTCATCMNNTGCACGGAC-3'
98	184NNK fwd	5'-GCACTGGATGAANNKATGAACAAGCTG-3'
99	184NNK rev	5'-CAGCTTGTTCATMNNTTCATCCAGTGC-3'
100	188NNK fwd	5'-CAATGAACAAGNNKCAGCGAGCAAATCC-3'
101	188NNK rev	5'-GGATTTGCTCGCTGMNNCTTGTTCATTG-3'
102	263NNK fwd	5'-AATTATTACATTCTTANNKGCGGGACACGAAACAAC-3'
103	263NNK rev	5'-GTTGTTTCGTGTCCCGCMNNTAAGAATGTAATAATT-3'
104	264NNK fwd	5'-AATTATTACATTCTTAATTNNKGGACACGAAACAAC-3'
105	264NNK rev	5'-GTTGTTTCGTGTCCMNNAATTAAGAATGTAATAATT-3'
106	328NNK fwd	5'-GGCCAACTNNKCCTGCGTTTTCCC-3'
107	328NNK rev	5'-GGGAAAACGCAGGMNNAGTTGGCC-3'
108	330NNK fwd	5'-GCCAACTGCTCCTNNKTTTTCCCTATATG-3'
109	330NNK rev	5'-CATATAGGGAAAAMNNAGGAGCAGTTGGC-3'
110	V78VA fwd	5'-GCGCTTAAATTTGYNCGTGATTTTGCAGG-3'
111	V78VA rev	5'-CCTGCAAAATCACGNRCAAATTTAAGCGC-3'
112	V78LIMF fwd	5'-GCGCTTAAATTTWTSCGTGATTTTGCAGG-3'
113	V78LIMF rev	5'-CCTGCAAAATCACGSAWAAATTTAAGCGC-3'
114	V78W fwd	5'-GCGCTTAAATTTTGGCGTGATTTTGCAGG-3'
115	V78W rev	5'-CCTGCAAAATCACGCCAAAATTTAAGCGC-3'
116	A82VA fwd	5'-GTACGTGATTTTGYNGGAGACGGG-3'
117	A82VA rev	5'-CCCGTCTCCNRCAAAATCACGTAC-3'
118	A82LIMF fwd	5'-GTACGTGATTTTWTSGGAGACGGG-3'
119	A82LIMF rev	5'-CCCGTCTCCSAWAAAATCACGTAC-3'
120	A82W fwd	5'-GTACGTGATTTTTGGGGAGACGGG-3'
121	A82W rev	5'-CCCGTCTCCCCAAAAATCACGTAC-3'
122	A328VA fwd	5'-GGCCAACTGYNCCTGCGTTTTCCC-3'
123	A328VA rev	5'-GGGAAAACGCAGGNRCAGTTGGCC-3'
124	A328LIMF fwd	5'-GGCCAACTWTSCCTGCGTTTTCCC-3'
125	A328LIMF rev	5'-GGGAAAACGCAGGSAWAGTTGGCC-3'
126	A328W fwd	5'-GGCCAACTTGGCCTGCGTTTTCCC-3'

127	A328W rev	5'-GGGAAAACGCAGGCCAAGTTGGCC-3'
128	V78A82MNYY fwd	5'-GCGCTTAAATTTATGCGTGATTTTNYYGGAGACGGG-3'
129	V78A82MNYY rev	5'-CCCGTCTCCRRNAAAATCACGCATAAATTTAAGCGC-3'
130	V78A82NYYM fwd	5'-GCGCTTAAATTTNYYCGTGATTTTATGGGAGACGGG-3'
131	V78A82NYYM rev	5'-CCCGTCTCCCATAAAATCACGRRNAAATTTAAGCGC-3'
132	V78A82KGGNYY fwd	5'-GCGCTTAAATTTKGGCGTGATTTTNYYGGAGACGGG-3'
133	V78A82KGGNYY rev	5'-CCCGTCTCCRRNAAAATCACGCCMAAATTTAAGCGC-3'
134	V78A82NYYKGG fwd	5'-GCGCTTAAATTTNYYCGTGATTTTKGGGGAGACGGG-3'
135	V78A82NYYKGG rev	5'-CCCGTCTCCCCMAAAATCACGRRNAAATTTAAGCGC-3'
136	V78A82KGG2 fwd	5'-GCGCTTAAATTTKGGCGTGATTTTKGGGGAGACGGG-3'
137	V78A82KGG2 rev	5'-CCCGTCTCCCCMAAAATCACGCCMAAATTTAAGCGC-3'
138	V78A82NYY2 fwd	5'-GCGCTTAAATTTNYYCGTGATTTTNYYGGAGACGGG-3'
139	V78A82NYY2 rev	5'-CCCGTCTCCRRNAAAATCACGRRNAAATTTAAGCGC-3'
140	V78A82M2 fwd	5'-GCGCTTAAATTTATGCGTGATTTTATGGGAGACGGG-3'
141	V78A82M2 rev	5'-CCCGTCTCCCATAAAATCACGCATAAATTTAAGCGC-3'
142	F87T88MNYY fwd	5'-GACGGGTTAATGNYYAGCTGGACGC-3'
143	F87T88MNYY rev	5'-GCGTCCAGCTRRNCATTAACCCGTC-3'
144	F87T88NYYM fwd	5'-GACGGGTTANYYATGAGCTGGACGC-3'
145	F87T88NYYM rev	5'-GCGTCCAGCTCATRRNTAACCCGTC-3'
146	F87T88KGGNYY fwd	5'-GACGGGTTAKGGNYYAGCTGGACGC-3'
147	F87T88KGGNYY rev	5'-GCGTCCAGCTRRNCCMTAACCCGTC-3'
148	F87T88NYYKGG fwd	5'-GACGGGTTANYYKGGAGCTGGACGC-3'
149	F87T88NYYKGG rev	5'-GCGTCCAGCTCCMRRNTAACCCGTC-3'
150	F87T88KGG2 fwd	5'-GACGGGTTAKGGKGGAGCTGGACGC-3'
151	F87T88KGG2 rev	5'-GCGTCCAGCTCCMCCMTAACCCGTC-3'
152	F87T88NYY2 fwd	5'-GACGGGTTANYYNYYAGCTGGACGC-3'
153	F87T88NYY2 rev	5'-GCGTCCAGCTRRNRRNTAACCCGTC-3'
154	F87T88M2 fwd	5'-GACGGGTTAATGATGAGCTGGACGC-3'
155	F87T88M2 rev	5'-GCGTCCAGCTCATCATTAACCCGTC-3'
156	A328KGG fwd	5'-GGCCAACTKGGCCTGCGTTTTCCC-3'
157	A328KGG rev	5'-GGGAAAACGCAGGCCMAGTTGGCC-3'
158	A328NYY fwd	5'-GGCCAACTNYYCCTGCGTTTTCCC-3'
159	A328NYY rev	5'-GGGAAAACGCAGGRRNAGTTGGCC-3'
160	N1F fwd	5'-CTTAAGTCAATKGTTSAAATTTWTTCGTGATTTTS TGGGAGACGGGTTATTCACAAGCTGG-3'
161	N1F rev	5'-CCAGCTTGTGAATAACCCGTCTCCCASAAAATCACG AAWAAATTTSAACMATTGACTTAAG-3'
162	N1A fwd	5'-CTTAAGTCAATKGTTSAAATTTWTTCGTGATTTTSTGG GAGACGGGTTAGCAACAAGCTGG-3'
163	N1A rev	5'-CCAGCTTGTTGCTAACCCGTCTCCCASAAAATCACGA AWAAATTTSAACMATTGACTTAAG-3'

164	N2 fwd	5'-GTCCGTGCATKGGATGAAGYCATGAACAAGTKGCAGCGAGCAAATC-3'
165	N2 rev	5'-GATTTGCTCGCTGCMACTTGTTCATGRCTTCATCCMATGCACGGAC-3'
166	N3 fwd	5'-GCTTATGGCCAACTKTCCCTTKGTTTTCCC-3'
167	N3 rev	5'-GGGAAAACMAAGGGAMAGTTGGCCATAAGC-3'
168	O N1F fwd	5'-CTTAAGTCAAGYCTTSAAATTTSTGCGTGATTTTKCGGGA GACGGGTTATTCACAAGCTGG-3'
169	O N1F rev	5'-CCAGCTTGTGAATAACCCGTCTCCCGMAAAATCACGCASAA ATTTSAAGRCTTGACTTAAG-3'
170	O N1A fwd	5'-CTTAAGTCAAGYCTTSAAATTTSTGCGTGATTTTKCGGGAG ACGGGTTAGCAACAAGCTGG-3'
171	O N1A rev	5'-CCAGCTTGTTGCTAACCCGTCTCCCGMAAAATCACGCASA AATTTSAAGRCTTGACTTAAG-3'
172	O N2 fwd	5'-GTCCGTGCATTSGATGAARCGATGAACAAGTKGCAGCGAGCAAATC-3'
173	O N2 rev	5'-GATTTGCTCGCTGCMACTTGTTCATCGYTTCATCSAATGCACGGAC-3'
174	O N3F fwd	5'-GCTTATGGCCAACTTTCCCTGYCTTTTCCC-3'
175	O N3F rev	5'-GGGAAAAGRCAGGGAAAGTTGGCCATAAGC-3'
176	O N3A fwd	5'-GCTTATGGCCAACTGCACCTGYCTTTTCCC-3'
177	O N3A rev	5'-GGGAAAAGRCAGGTGCAGTTGGCCATAAGC-3'
178	N1 NNK fwd	5'-CTTAAGTCAANNKNNKAAATTTNNKCGTGATTTTNNKG GAGACGGGTTANNKACAAGCTGG-3'
179	N1 NNK rev	5'-CCAGCTTGTMNNTAACCCGTCTCCMNNAAAATCACGM NNAAATTTMNNMNNTTGACTTAAG-3'
180	N2 NNK fwd	5'-GTCCGTGCANNKGATGAANNKATGAACAAGNNK CAGCGAGCAAATC-3'
181	N2 NNK rev	5'-GATTTGCTCGCTGMNNCTTGTTCATMNNTTCATC MNNTGCACGGAC-3'
182	N3 NNK fwd	5'-GCTTATGGCCAACTNNKCCTNNKTTTTCCC-3'
183	N3 NNK rev	5'-GGGAAAAMNNAGGMNNAGTTGGCCATAAGC-3'
184	alkBFG_1	5'-GATCTACATATGCTTGAGAAACACAGAGTTCTGGATTC-3'
185	alkBFG_2	5'-GATCTACCCGGGTCACTTTTCCTCGTAGAGCACATAGTC-3'
186	E31-74NNK fwd	5'-GATAAAAACTTAAGTCAANNKCTGAAATTTGTACGTG-3'
187	E31-74NNK rev	5'-CACGTACAAATTTCAGMNNTTGACTTAAGTTTTTATC-3'
188	E31-75NNK fwd	5'-GATAAAAACTTAAGTCAACTGNNKAAATTTGTACGTG-3'
189	E31-75NNK rev	5'-CACGTACAAATTTMNNCAGTTGACTTAAGTTTTTATC-3'
190	E31-78NNK fwd	5'-CTGCTGAAATTTNNKCGTGATTTTTTGGG-3'
191	E31-78NNK rev	5'-CCCAAAAAATCACGMNNAAATTTCAGCAG-3'
192	E31-82NNK fwd	5'-ATTCGTGATTTTNNKGGAGACGGG-3'
193	E31-82NNK rev	5'-CCCGTCTCCMNNAAAATCACGAAT-3'
194	E31-184NNK fwd	5'-GCACTGGATGAANNKATGAACAAGTGG-3'
195	E31-184NNK rev	5'-CCACTTGTTCATMNNTTCATCCAGTGC-3'

196	E31-263NNK fwd	5'-AATTATTACATTCTTANNKGCGGGACACGAAACAAC-3'
197	E31-263NNK rev	5'-GTTGTTTCGTGTCCCGCMNNTAAGAATGTAATAATT-3'
198	E31-264NNK fwd	5'-AATTATTACATTCTTAATTNNKGGACACGAAACAAC-3'
199	E31-264NNK rev	5'-GTTGTTTCGTGTCCMNNAATTAAGAATGTAATAATT-3'
200	E31-328NNK fwd	5'-GGCCAACTNNKCCTTGGTTTTCCC-3'
201	E31-328NNK rev	5'-GGGAAAACCAAGGMNNAGTTGGCC-3'
202	E31-436NNK fwd	5'-CGATATTAAAGAANNKTTAACGTTAAAACC-3'
203	E31-436NNK rev	5'-GGTTTTAACGTTAAMNNTTCTTTAATATCG-3'
204	E31-437NNK fwd	5'-CGATATTAAAGAAACTNNKACGTTAAAACC-3'
205	E31-437NNK rev	5'-GGTTTTAACGTMNNAGTTTCTTTAATATCG-3'
206	E31-140 fwd	5'-GATGAGCATATTGAWGTACCGGAAGAC-3'
207	E31-140 rev	5'-GTCTTCCGGTACWTCAATATGCTCATC-3'
208	E31-215 fwd	5'-GGTGATGAACGACCYAGTAGATAAAATTATTGCAG-3'
209	E31-215 rev	5'-CTGCAATAATTTTATCTACTRGGTCGTTCATCACC-3'
210	E31-454 fwd	5'-CGAAAAAAATTYCGCTTGGCGGTATTCC-3'
211	E31-454 rev	5'-GGAATACCGCCAAGCGRAATTTTTTTCG-3'
212	E31-222 fwd	5'-GATAAAATTATTGCAGAKCGCAAAGCAAGCGG-3'
213	E31-222 rev	5'-CCGCTTGCTTTGCGMTCTGCAATAATTTTATC-3'
214	E31-289 fwd	5'-GTATTACAAAAAGMAGCAGAAGAAGCAGC-3'
215	E31-289 rev	5'-GCTGCTTCTTCTGCTKCTTTTTGTAATAC-3'
216	RD2-432 fwd	5'-CTACGAGCTCGRTATTAAAGAAAC-3'
217	RD2-432 rev	5'-GTTTCTTTAATAYCGAGCTCGTAG-3'
218	RD2-74 fwd	5'-GATAAAAACTTAAGTCAACKGCTGAAATTTGTACGTG-3'
219	RD2-74 rev	5'-CACGTACAAATTTCAGCMGTTGACTTAAGTTTTTATC-3'
220	RD2-78I fwd	5'-CTGCTGAAATTTATTCGTGATTTTTTGGG-3'
221	RD2-78I rev	5'-CCCAAAAAATCACGAATAAATTTCAGCAG-3'
222	RD2-78W fwd	5'-CTGCTGAAATTTTGGCGTGATTTTTTGGG-3'
223	RD2-78W rev	5'-CCCAAAAAATCACGCCAAAATTTCAGCAG-3'
224	RD2-436 fwd	5'-CGATATTAAAGAAASATTAACGTTAAAACC-3'
225	RD2-436 rev	5'-GGTTTTAACGTTAATSTTTCTTTAATATCG-3'

E. High-Throughput Screening of P450 BM3 Libraries

E.1. Lysate preparation of high-throughput screening (Chapters 2, 3, 5, and 7)

96-well plates with 1-mL wells containing LB medium (300 μL, supplemented with 100 mg/mL ampicillin) were inoculated with single colonies either by hand or using a Qpix colony

picking robot (Genetix, Beaverton, OR). Two to four wells were also inoculated with the parent by hand, and one to four wells was left uninoculated as sterility and negative control for high-throughput screening. After overnight incubation (at least 12 hrs) at 37 ^{o}C, 250 rpm, and 80% relative humidity in a Kühner shaker (Kühner, Birsfeden, Switzerland), 50 µL of the LB culture was used to inoculate 96-well plates with 2-mL wells containing TB medium (600 – 800 µL, supplemented with 100 mg/mL ampicillin). The TBamp expression culture was incubated at 37 ^{o}C, 250 rpm, and 80% relative humidity for four hours before the temperature was reduced to 25 ^{o}C. After thirty minutes at the reduced temperature, protein expression was induced with 0.5 mM IPTG and 0.5 mM δ-ALA. After 20 – 24 hours of expression at 25 ^{o}C, the cultures were harvested by centrifugation at 5,000 rpm and 4 ^{o}C, and stored at -20 ^{o}C.

The frozen cell pellets were resuspended in 0.1 M phosphate buffer, pH 8.0, containing 10 mM $MgCl_2$, 0.5 mg/ml lysozyme (Sigma), and 2.0 U/mL DNaseI. After lysis at 37 ^{o}C for 60 minutes, the lysates were centrifuged at 5,000 rpm and 4 ^{o}C for 10 min, and the supernatants were transferred to 96-well microtiter plates for high-throughput assays.

E.2. High-throughput P450 protein folding assay (Chapters 2 – 7)

The CO-binding difference spectroscopy has been well established for determination of folded P450 concentrations. Following a modified protocol (*9*) for the CO-binding assay in 96-well microtiter plates, 40 – 100 µL of 0.4 M sodium hydrosulfite were added to 100 – 160 µL of lysate in each well of a 96-well microtiter plate. The UV/Vis absorbance from 400 to 500 nm was taken using a microtiter plate reader before exposure to carbon monoxide. The microtiter plates were placed in a vacuum chamber, which was evacuated to 20 mmHg. Carbon monoxide was then used to fill the chamber to atmosphere pressure. After a 15 minute incubation period,

the 400 – 500 nm spectrum was obtained and subtracted from the pre-incubation spectrum. The extinction coefficient of 91 mM cm^{-1} was used to obtain the P450 concentration of each well.

E.3. High-throughput demethylation assay using Purpald (Chapters 2, 3 and 7)

For quantifying the demethylation reaction of P450 with substrates such as dimethyl ether, purpald was used to detect the resulting aldehydes (*10*). In a 96-well microtiter plate, 120 µL of 0.1 M, pH 8.0 phosphate buffer pre-saturated with dimethyl ether were added to 30 µL of cell lysate, and the reaction was initiated with 50 µL of 4 mM NADPH. The reaction was allowed to proceed for 15 – 30 minutes, after which 50 µL of 168 mM purpald in 2 M NaOH were added to quench the reaction and develop the purple adduct with the released aldehydes. After 15 minutes, the purple color was quantified by absorbance at 550 nm using a microtiter plate reader.

E.4. High-throughput ethane hydroxylation assay (Chapters 3 and 5)

The ethanol product from the ethane hydroxylation reaction was quantified with a coupled enzyme assay utilizing alcohol oxidase and horseradish peroxidase. The alcohol oxidase converted the ethanol product from the P450 reaction into one molecule of acetaldehyde and one molecule of hydrogen peroxide. The horseradish peroxidase was then able to oxidize ABTS into a green soluble end-product using hydrogen peroxide.

To minimize ethanol fermentation from intact cells, the lysate was first filtered through a 0.2 micron filter plate (Pall, Ann Arbor, MI). In a 96-well Symx reactor (Symx, Miami, FL), 20 µL of lysate were added to each well along with 440 µL of 0.1 M phosphate buffer, pH 8.0, pre-saturated with ethane, and the reaction was initiated with 40 µL of an NADPH regeneration system consisting of 20 mM DL-isocitric acid trisodium salt, 400 µM $NADP^+$, and 0.5 U/mL

isocitrate dehydrogenase. The reactor was then pressurized to 30 psi with ethane and allowed to react for 24 – 48 hours at 4 ^{o}C. After the reactor was vented to atmospheric pressure, the reaction mixture was transferred to a 2-mL 96-well plate. To each well, 15 μL of 5 M HCl were added to oxidize the remaining NADPH to $NADP^{+}$ to prevent the NADPH from reducing the oxidized ABTS product. The 96-well plate was then shaken to mix at 150 rpm, 25 ^{o}C for 10 minutes. After centrifugation to pellet the denatured protein, 150 μL of the reaction mixture were transferred to a 96-well microtiter plate. Another 3 μL of 5 M HCl were added followed by 75 μL of 1 M phosphate buffer, pH 8.0, to raise the solution to a neutral pH. In the final color development setup, 50 μl of ABTS (1.3 mg/mL), 10 μL of HRP (3 mg/ml), and 10 μL of AOX (0.5 U/ml) were added in this sequence, and after 10 minutes of incubation, the green color was quantified at 420 nm.

E.5. Determination of NAD(P)H consumption rates (Chapter 2 – 7)

The P450 enzymes were purified and quantified by CO-binding as described above. Initial rates of NADPH consumption were determined by monitoring the absorption decrease at 340 nm measured with a 1 cm pathlength cuvette on a Cary 100 UV/VIS spectrophotometer (Varian, Walnut Creek, CA) or with a 96-well microtiter plate (pathlength 0.48 cm for 150 μL sample volume) on a Tecan plate reader (Tecan, Durham, NC). For liquid substrates such as alkanes, substrate stock solutions in ethanol (50-fold) were added to a protein solution (50 to 200 nM) in 0.1 M, pH 8.0 phosphate buffer and incubated for at least 30 seconds. The reaction was initiated by the addition of NAD(P)H varying from 200 μM to 1 mM depending on the rate of consumption and instrument range. For gaseous substrates, such as propane or ethane, the protein

solution was made in pre-saturated buffer. The consumption rates were determined using an extinction coefficient of ε_{340nm} = 6,210 M^{-1} cm^{-1}. Each rate was determined at least in triplicate.

E.6. Growth selection of AlkB and CYP153 libraries (Chapter 4)

Luria-Bertani broth and modified M9 medium with 1.5% yeast extract (*11*) supplemented with appropriate antibiotics, or E2 (*12*) and M9 (*13*) minimal media supplemented with carbon sources, were used for growth. All cultures were grown aerobically at 30 °C (*P. putida*) or 37 °C (*Escherichia coli*). Antibiotic concentrations were 100 μg/ml ampicillin, 15 μg/ml tetracycline, 20 μg/ml chloramphenicol, and 10 μg/ml gentamicin for *E. coli* cultures and 50 μg/ml for *Pseudomonas*. Bacterial strains were grown on solid E2 minimal medium with liquid alkanes provided through the gas phase as described previously (*14*). Solid minimal medium growth tests on gaseous alkanes were conducted in gas-tight plastic containers (GasPak 150 large anaerobic vented system; VWR), pressurized at 20 psi for 20 s (ethane and propane) or 10 psi for 6 s (butane). Liquid minimal medium cultures growing on alkanes were shaken in custom-made gas-tight flasks with 1% liquid alkane (pentane and octane) in a reservoir or in gas-tight serum bottles (Alltech), pressurized with gaseous alkanes as described above. For growth tests of *P. putida* GPo12(pGEc47ΔB) on alkanols, cells from an LB preculture were washed three times with M9 medium and used to inoculate the 5-mL M9 main cultures in 14-mL tubes to an optical density (OD_{600}) of 0.1, then grown at 30 °C with continuous shaking. Libraries of *P. putida* GPo12(pGEc47ΔB) strains expressing AlkB or CYP153A6 variants were precultured on E2 minimal medium plates with antibiotics and 0.2% (wt/vol) citrate as carbon source and then enriched for improved strains through continuous growth in liquid E2 minimal medium with small-chain-length alkanes as the sole carbon source

F. P450 Reactions (Chapters 2 – 7)

F.1. Gaseous alkane hydroxylation with P450 BM3 variants (Chapter 2 and 3)

Gaseous alkane reactions were carried out in 10 or 20 mL crimp-top head-space vials (Agilent, Santa Clara, CA). The 1.0 – 5.0 mL reaction mixture contained 20 – 200 μM P450 in 0.1 M phosphate buffer, pH 8.0, saturated with alkane and oxygen. The buffer was saturated with the gaseous alkane through vigorous bubbling for at least 10 minutes. After the addition of the P450 mixture, the crimp-top vial was sealed and stirred moderately at 25 ^{o}C. The reaction was then initiated by the addition of 0.5 mL of a NADPH regeneration system consisting of 10 mg/mL isocitrate, 0.2 mg/mL $NADP^{+}$, and 10 U/mL of isocitrate dehydrogenase. For determination of the TON, the reaction was carried out at 4 ^{o}C over 24 hrs. For initial rate of formation determination, the reaction was initiated by the addition of 500 μM NADPH and was quenched with 200 μL of concentrated H_2SO_4 after an appropriate time depending on the enzyme activity (20 second to 10 minutes) at 25 ^{o}C.

The alcohol product was initially analyzed as alkyl nitrites using a published method (*15*). To a 5.0 mL reaction mixture, 0.3 g of sodium nitrite and 2 mL of hexanes were added along with 1-butanol as an internal standard, and the mixture was cooled on ice. The reaction was initiated with the addition of 0.2 mL concentrated sulfuric acid and quenched by dilution with 5 mL of deionized water after 15 minutes. The reaction mixture was further washed with 20 mL of water in a separatory funnel. The organic phase was collected and analyzed with GC-ECD (see H.1). Alternatively, the alcohol products were also analyzed by GC-FID using Supelco SPB-1 column and 1-pentanol as an internal standard (see H.2). All measurements were carried out in triplicate.

F.2. Alkane hydroxylation with CYP153A6 (Chapter 6)

For alkane reactions with CYP153A6, 0.15 – 0.3 mL reaction mixtures contained 0.5 – 2.0 μM A6 and 0.5 – 2.0 μM FDR and 5.0 – 20.0 μM FDX at a 1:1:10 ratio in 0.1 M phosphate buffer, pH 8.0. In the case of liquid alkanes, a stock solution in ethanol was used to add 25 μM to 10 mM alkane in a final solution containing 2% ethanol. For gaseous alkanes, the reaction was carried out in crimp-top head-space vials pressurized to 20 psi with the alkane. The reaction was initiated with the addition of 1 – 2 mM NADH. For determination of the initial rate, the reactions were quenched with 20 μL of 3.0 M HCl after 5 minutes.

Reactions with liquid alkanes were extracted with 100 – 150 μL of chloroform after the addition of 2-nonanol as an internal standard. The mixture was then vortexed for 30 seconds before centrifuging at 14,000x*g* for 2 minutes. The bottom organic phase was collected and analyzed by GC-FID, see H.2 for details. Reactions with gaseous alkanes were quenched with 20 μL of 3.0 M HCl and neutralized with 75 μL of 1.0 M phosphate buffer, pH 8.0. This acidification and neutralization sequence resulted in the desired precipitation of the enzymes in solution. After centrifuging at 14,000x*g* for 2 minutes to pellet the denatured protein, the clarified solution was analyzed by GC-MS (see H.3 for details).

F.3. Alkane hydroxylation with terminal oxidants (Chapter 6)

For alkane reactions with P450s using terminal oxidants, 0.3 mL reaction mixture contained 50 – 250 μM of highly pure enzyme, as described in C.2, in 0.1 M phosphate buffer, pH 8.0. The liquid alkanes were added from an ethanol stock solution, to yield a final solution containing 2.5 mM alkane and 2% ethanol. For gasesous alkanes, lyophilized protein was added directly to a 10 mL crimp-top headspace vial. The vial was then sealed and flushed with the

alkane substrate for 2 minutes before the addition of 0.27 mL of pre-saturated 0.1 M phosphate buffer, pH 8.0. The reaction was then pressurized to 20 psi with the gaseous alkane. For reactions with PhIO, the reaction was initiated with the addition of 0.03 mL of 5 mM PhIO, solubilized in deionized water by sonication. For reactions with MCPBA, the reaction was initiated with the addition of 5 mM MCPBA in an ethanol or isopropanol stock solution. Reactions with H_2O_2 were similarly initiated with the addition of 5 mM H_2O_2. All reactions were allowed to proceed for 10 minutes at 25 ^{o}C. The work-up and analysis of the alcohol products followed the same protocol as described in Section F.2.

F.4. Iodomethane reaction with CYP153A6 (Chapter 6)

For iodomethane reactions with CYP153A6, 0.15 – 0.3 mL reaction mixtures contained 0.5 μM A6 and 0.5 μM FDR and 5.0 μM FDX at a 1:1:10 ratio in 0.1 M phosphate buffer, pH 8.0. Iodomethane in an ethanol stock solution was used to add 25 μM to 10 mM alkane in a final solution containing 2% ethanol. The reaction was initiated with the addition of 1 mM NADH and quenched after 5 minutes with either 168 mM purpald in 2 M NaOH or 20 μL of 3.0 M HCl. Reactions quenched with purpald were quantified by absorbance at 550 nm after 10 minutes of color development. For reactions quenched with acid, a neutralization step followed with 75 μL of 1.0 M phosphate buffer, pH 8.0, to precipitate the proteins in solution. After centrifuging at 14,000x*g* for 2 minutes, the clarified solution was analyzed by GC-MS (see H.3 for details).

F.5. Metabolite production with P450s (Chapter 7)

Reactions of verapamil, astemizole, and LY294002 with a panel of 120 P450s were conducted in 96-well microtiter plates. For P450 holoenzymes, 60 μL of lysate, generated as described in E.1, were added to 110 μL of 0.1 M phosphate buffer, pH 8.0, and 10 μL of

substrate (5 mM). The reaction was initiated with the addition of 20 μL NADPH (20 mM). For P450 peroxygenases, 50 μL lysate were added along with 100 μL of 0.1 M EPPS buffer, pH 8.2, and 10 μL of substrate (20 mM). The reaction was initiated with the addition of 40 μL of H_2O_2 (5 mM). The plates were shaken briefly after the addition of NADPH or H_2O_2, and incubated for two hours at 25 ^oC. After this time, 200 μL of acetonitrile were added to quench the reaction. The resulting mixture was centrifuged, and the supernatant was used for subsequent HPLC and LC/MS analysis.

G. Absorption Spectra of P450s (Chapter 6)

The absorption spectra over the range from 350 to 500 nm were taken using a 5 μM solution of purified P450 in 0.1 M, pH 8.0, phosphate buffer with 1 cm pathlength cuvette on a Cary 100 UV/VIS spectrophotometer. Liquid substrate in an ethanol stock solution was used to add 10 mM substrate in a final solution containing 2% ethanol. For gaseous substrates, the protocol for alkane hydroxylation with terminal oxidants (see F.3) was used to expose the enzymes to substrates. The differences in absorption spectra at A_{418nm} corresponding to a low-spin ferric state and A_{390nm} corresponding to a high-spin state were used to determine the extent of the induced spin-shift.

H. Gas Chromatography

All peak assignments in our gas chromatographs were determined by comparison to authentic standards. Analyte peaks were quantified using calibrations curves containing at least four standard concentrations. Unless stated otherwise, every analysis was done with a 1 μL sample injection.

H.1. GC-ECD analysis of alkane reactions

Analysis of the ethanol and propanol products as their alkyl nitrite derivatives followed published procedures (*15*), using a Hewlett-Packard 5890 Series II Plus gas chromatograph (Agilant, Santa Clara, CA) with a HP-1 column (30 m length, 0.32 mm ID, 0.25 μm film thickness) connected to an electron capture detector (ECD). A typical temperature program used was: 60 °C injector, 150 °C detector, 35 °C oven for 3 min, 10 °C/min gradient to 60 °C, 25 °C/min gradient to 200 °C, and then 200 °C for 5 min.

H.2. GC-FID analysis of alkane reactions

Analysis of hydroxylation products of small alkanes was performed on a Hewlett Packard 5890 Series II Plus gas chromatograph with a flame ionization detector (FID) and fitted with a HP-7673 autosampler system. Direct analysis of ethane, propane, butane, and pentane, hydroxylation products was performed on a Suplerco SPB-1 column, (60 m length, 0.32 mm ID, 0.25 μm film thickness). A typical temperature program used for separating the alcohol products was 250 °C injector, 300 °C detector, 50 °C oven for 3 minutes, then 10 °C/minute gradient to 200 °C, 25 °C/minute gradient to 250 °C, then 250 °C for 3 minutes.

Analysis of hydroxylation products of hexane and octane was similarly performed as described above using a DB-624 column, (30 m length, 0.53 mm ID, 3.0 μm film thickness) connected to a FID detector. A typical temperature program used for separating the alcohol products was 250 °C injector, 300 °C detector, 40 °C oven for 5 minutes, then 10 °C/minute gradient to 260 °C, then 260 °C for 3 minutes.

H.3. GC-MSD analysis

Analysis of hydroxylation products of methane, ethane, propane, and iodomethane was performed on Hewlett Packard 6890 Series II Plus gas chromatograph with a 5975C mass selective detector (MSD). Direct analysis of the alcohol products in buffer were performed using a DB-624 column, (30 m length, 0.53 mm ID, 3.0 μm film thickness) with a 0.5 μL sample injection. A typical temperature program used for separating the alcohol products consisted of 250 °C injector, 300 °C detector, 45 °C oven for 5 minutes, then 10 °C/minute gradient to 150 °C, then 25 ^{o}C/minute gradient to 250 °C, and a hold for 3 minutes at 250^{o}C.

I. HPLC/ LCMS

Analysis of the reactions of drug compounds verapamil, astemizole, and lead compound LY294002 with a panel of P450s was performed using a Supelco Discovery C18 column (2.1 x 150 mm, 3 μm) on a Waters 2690 Separation module in conjunction with a Waters 996 PDA detector. Clarified supernantant (25 μL) was analyzed with 0.2% formic acid (solvent A) and acetonitrile (solvent B) at the following conditions: 0 – 3 min, A/B 90:10; 3 – 25min, linear gradient to A/B 30:70; 25 – 30 min, linear gradient to A/B 10:90. LCMS and MS/MS spectra were obtained with a ThermoFinnigan LCQ classic (San Jose, CA), using identical conditions to the HPLC method detailed above for the LC conditions. The MS was operated in positive ESI mode, and the MS/MS spectra were acquired for the most abundant ions.

J. Half-Denaturation Temperature (T_{50}) Determination

Samples of purified enzyme (3 – 5 μM) were incubated for 15 minutes at various temperatures from 25 ^{o}C at the bench top to 65 ^{o}C using a PCR thermocycler or a thermoblock. After centrifugation at 14,000xg to pellet the denatured protein fraction, 160 μL of the

supernatant were mixed with 40 μL of 0.4 M sodium hydrosulfite one a microtiterplate for CO-difference spectroscopy, see E.2 for details. The T_{50} values were determined by data fitting to a 2-state denaturation model using Sigmaplot (Systat, San Jose, CA).

K. Whole-Cell Bioconversion with P450 BM3 Variants (Chapters 2 and 5)

Propane bioconversions were carried out at 80 mL and 300 mL scale using temperature controlled 100-mL (Ochs-Labor, Bovenden, Germany) and 1-L fermenter (DasGip, Jülich, Germany), respectively. Freshly transformed *Escherichia coli* Dh5α cells were grown in modified M9 medium supplemented with 0.4% glucose, and 1.5% yeast extract. In addition to the standard salts, the modified M9 contained additional nutrients: calcium pantothenate, p-aminobenzoic acid, p-hydroxybenzoic acid, thiamine, and trace metals (CoCl2, CuSO4, MnCl2, ZnSO4). After reaching an OD_{600} of 1.2, the cells were induced with 0.25 mM IPTG and 0.25 mM δ-aminolevulininc acid, and harvested after 10 – 12 hours of expression. The cells were centrifuged at 5,000 rpm and 4 ^{o}C for 10 minutes.

The resulting pellet was resuspended in nitrogen-free modified M9 medium supplemented with 1% glucose. A gas mixture of propane and air at 1:2 ratio was bubbled through the resting cells at a flow rate of 15 L/hr. A bubbler filled with an equal volume of water as the reaction volume was connected to the reactor's gas outlet to retain any alcohol product removed by the gas flow. At defined time intervals, 1-mL samples of the cell suspension and the bubbler fraction were removed for GC analysis of the alcohol product. The cell suspension samples were centrifuged and filtered prior to GC analysis. In addition, the P450 concentration was also determined by CO-binding difference spectroscopy on cell lysate obtained by sonication of the cell suspension pellet.

L. Whole-cell bioconversion with AlkB and CYP153 (Chapter 4)

Freshly transformed *E. coli* BL21(DE3) cells expressing AlkB variants were precultured in LB medium supplemented with the appropriate antibiotic at 37 ^{o}C and shaken at 250 rpm for 24 hours. LB cultures (12 mL) in 1-L flasks were then inoculated to a staring OD_{600} of 1.0 and incubated at 37 °C, 250 rpm, for 2.5 h. These cultures were then cooled to 25 °C and induced with 0.4 mM dicyclopropylketone (Sigma-Aldrich) after 30 minutes. After 20 hours of protein expression, the cell cultures were centrifuged at 3,300x*g*, 10 min, and 25 ^{o}C. CYP153A6 variants were similarly cultured, with the exception of using modified M9 medium supplemented with 1.5% yeast extract for only 14 hours of expression.

The cell pellets were resuspended in equal volume of either 0.1 phosphate buffer, pH 7.0, for AlkB or nitrogen-free modified M9 medium for CYP153. For bioconversion of liquid alkanes, 250 μL of alkane, 1% glycerol were added to 1-mL cell suspensions in a glass vial. The reaction was capped and shaken at 200 rpm for 1 hour at 25 ^{o}C. The addition of 200 μL of 1 M HCl and 250 μL of hexanes and vigorous vortexing quenched the reaction and extracted the alcohol products to the organic phase. After centrifuging at 14,000x*g* for 5 minutes, the organic layer was collected for GC analysis.

For bioconversion of gaseous alkanes, 80 mL of the cell suspension and 15 μL of antifoam (Sigma-Aldrich) were stirred in a 100-mL fermenter at 25 ^{o}C. The gaseous alkane was bubbled through the reaction mixture with air at 10 L/hr and at a 1:3 ratio. The reaction was initiated with the addition of 1% final concentration of glycerol for AlkB and 20 mM glucose for CYP153A6. Similar to the bioconversion of P450 BM3, at defined time intervals, 1-mL samples of the cell suspension and the bubbler fraction were removed for GC analysis of the alcohol

product. The P450 concentration was also determined by CO-binding difference spectroscopy on cell lysate obtained by sonication.

M. References

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