Advanced Synthesis & Catalysis

Supporting Information

Supporting Information

Utilizing Terminal Oxidants to Achieve P450-Catalyzed Oxidation of Methane

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Chemical compounds:

All chemicals, including liquid and gaseous alkanes and product standards, were purchased from Sigma-Aldrich (St. Louis, MO). NADH was purchased from Codexis, Inc. (Redwood City, CA). Iodosylbenzene was prepared according to published protocols.[1]

Plasmid:

The genes encoding the heme domains (AA 1-465) of cytochrome P450 BM3 (GenBank number J04832) and $P450_{PMO}$ (ref. 2) were cloned in the expression vector pCWori (pBM3_WT18-6) downstream of the double *tac* promoter with flanking *BamH*I and *EcoR*I sites carrying an N-terminal poly-histidine tag. The genes encoding CYP101 (GenBank number M12546), CYP153A6, CYP153A6 ferredoxin, CYP153A6 ferredoxin reductase (GenBank number AJ), and CYP153A6 BMO-1[3] were cloned into expression vector pET-22b(+) downstream of the T7 promoter with flanking *NdeI* and *XhoI* sites carrying an N-terminal poly-histidine tag. These plasmids were used for all subsequent cloning procedures and protein expression.

Protein expression and purification:

E. coli Dh5α cells were transformed with the plasmids containing P450 BM3 and PMO, and E. coli BL21(DE3) cells (Novagen) were transformed with plasmids containing P450cam, CYP153A6, CYP153A6 BMO-1, CYP153A6 ferredoxin, and CYP153A6 ferredoxin reductase. An aliquot of an overnight culture in LB medium, supplemented with ampicillin (100 μg/ml) was used to inoculate TB medium supplemented with 100 μg/ml ampicillin, 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 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 shaking, 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 min, 20 to 24 hours after induction and stored at -20 ^oC. For lysis of bacterial cells, 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 supernatant was filtered with a 22-μM filter.

A three-step purification was performed using an AKTA purifier FPLC system (GE Healthcare). The collected lysate was first subjected to a Ni-NTA chromatography step using a Ni sepharose column (HisTrap^{TM} HP, GE healthcare, Piscataway, NJ) followed by anion exchange chromatography step using a Q sepharose column (HiTrap^{TM} Q HP, GE healthcare, Piscataway, NJ) and a final gel-filtration chromatography step using a HiPrep 16/60 Sephacryl S-100 HR column (GE healthcare, Piscataway, NJ). The P450 was eluted from the Ni sepharose column using 25 mM Tris-HCl, 100 mM NaCl, 100 mM imidazole, pH 8.0, the Q sepharose column using 25 mM Tris-HCl, 340 mM NaCl, pH 8.0, and the gel-filtration column with 20 mM HEPES. In between each step, the P450 fractions were collected and concentrated using a 30 kDa molecular weight cut-off centrifugal filter and buffer exchanged with the loading buffer of the subsequent step. 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.[4]

Terminal oxidant supported reactions:

For reactions with liquid alkanes, the substrates were added from an ethanol stock solution to a 0.27-mL reaction mixture containing 50-250 μM P450, in 0.1 M phosphate buffer, pH 8.0, to yield a final solution containing 2.5 mM alkane, 2% ethanol. For gaseous alkanes, lyophilized protein was added to a 10-mL crimp-top headspace vial. The vial was sealed and flushed with the substrate for 2 minutes before the addition of 0.27 mL of pre-saturated 0.1 M phosphate buffer, pH 8.0. The head-space 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 stock solution for all alkanes expect ethane, and an isopropanol stock was used for the ethane reactions. 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. After ten minutes, reactions with liquid alkanes were extracted with 150 µL of chloroform with the addition of 2-nonanol as an internal standard, the organic phase was collected and analyzed by GC-FID. 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 14,000*g* for 2 minutes to pellet the denatured protein, the clarified solution was analyzed by GC-MS.

A6 *in vitro* hydroxylation reactions:

For the *in vitro* A6 alkane hydroxylation reactions, purified reductase components, ferredoxin reductase (fdrA6) and ferredoxin (fdxA6) were quantified using known extinction coefficients for their FAD and [Fe_2-S_2] cofactors.[5] An optimum ratio of reductase components of 1:1:10 for A6:fdrA6:fdxA6 was determined from octane hydroxylation reactions and used for subsequent experiments. The 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 substrates, additions from stock solutions in ethanol were used to reach final solutions with 25 µM to 10 mM substrate and 2% ethanol. For gaseous alkanes, the reaction was carried out in crimp-top head-space vials pressurized to 20-60 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. The post reaction work-up and analysis were performed as described above for the terminal oxidant reactions.

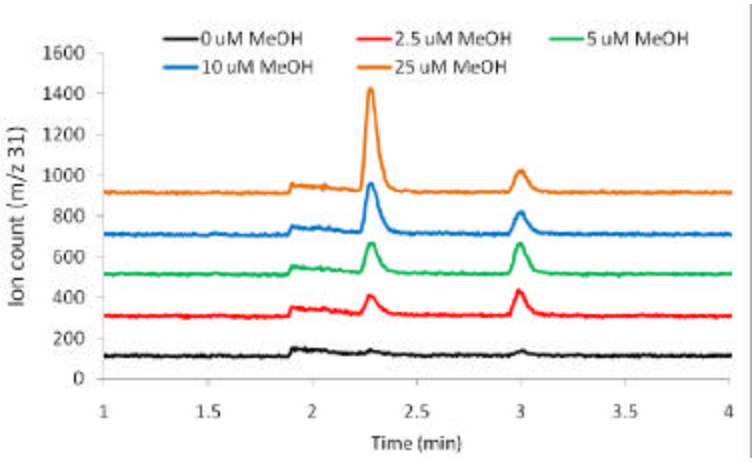
GC analyses:

Analysis of hydroxylation products of hexane and octane reactions 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 using a DB-624 column (30 m length, 0.53 mm ID, 3.0 µm film thickness). 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. For quantification, ratios of product peak area relative to the standard peak area were compared to standard curves generated with authentic alcohol standards.

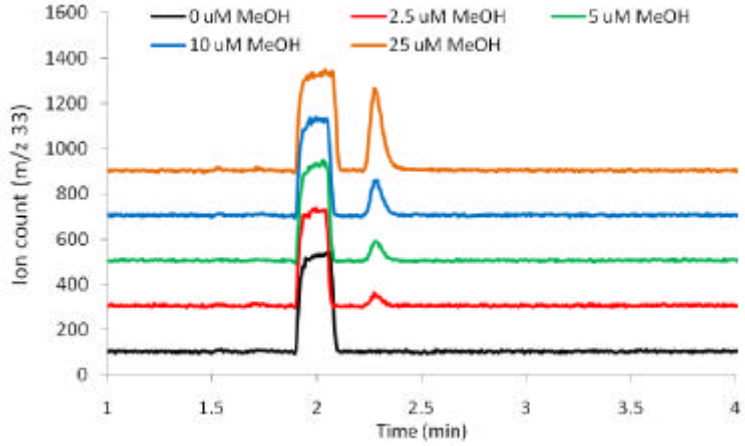
Analyses of hydroxylation products of methane, ethane, propane, and iodomethane were performed on Hewlett Packard 6890 Series II Plus gas chromatograph with a 5975C mass selective detector (MSD) operating in selective ion monitoring (SIM) mode. Direct analyses of the alcohol products in buffer were performed 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.

GC/MS-SIM chromatogram of methanol standards and P450 reactions

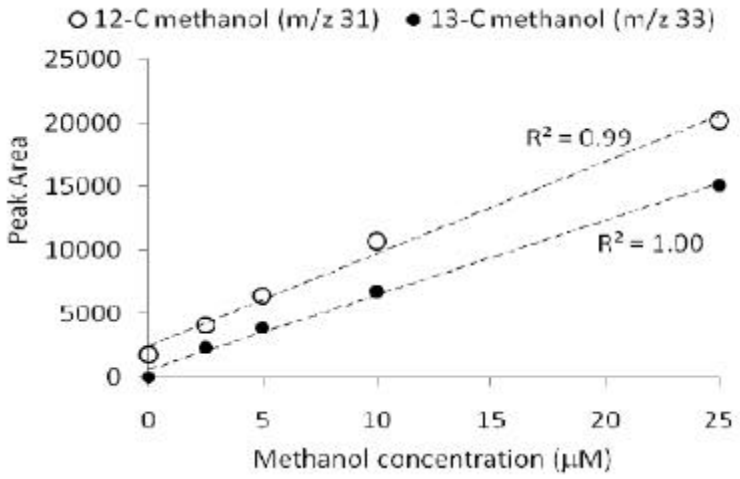
(a)



(b)



(c)



(d)

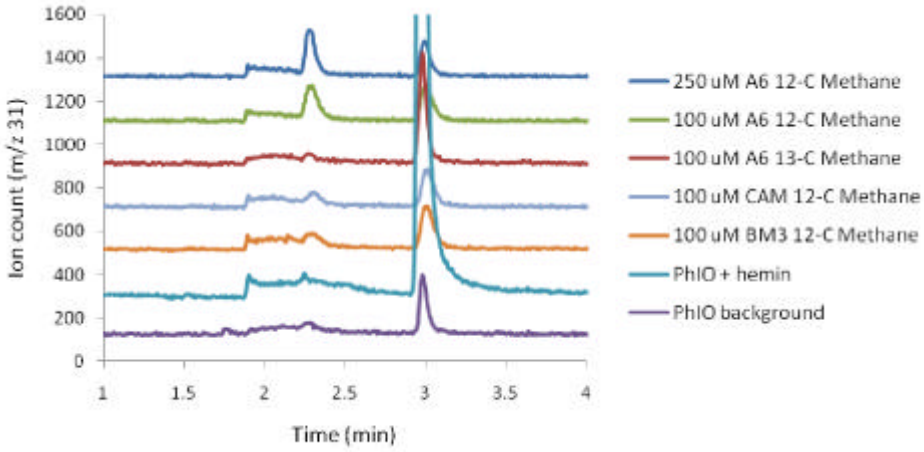


Figure S1: GC/MS-SIM chromatogram of ^{12}C and ^{13}C methanol calibration standards and P450 reactions (a) ^{12}C methanol standards quantified by m/z 31 ion with peak retention at 2.26 min, shown with a vertical offset. Peak at 3.0 min corresponds to background ethanol. (b) ^{13}C methanol standards quantified by m/z 33 ion. (c) Calibration curves for ^{12}C and ^{13}C methanol used for methanol quantification. (d) GC/MS-SIM chromatogram of PhIO-supported ^{12}C-methane reactions with BM3, CAM, and A6 with controls reactions containing PhIO, PhIO and hemin dissolved in ethanol, and PhIO-supported ^{13}C-methane reaction with A6, shown with a vertical offset.

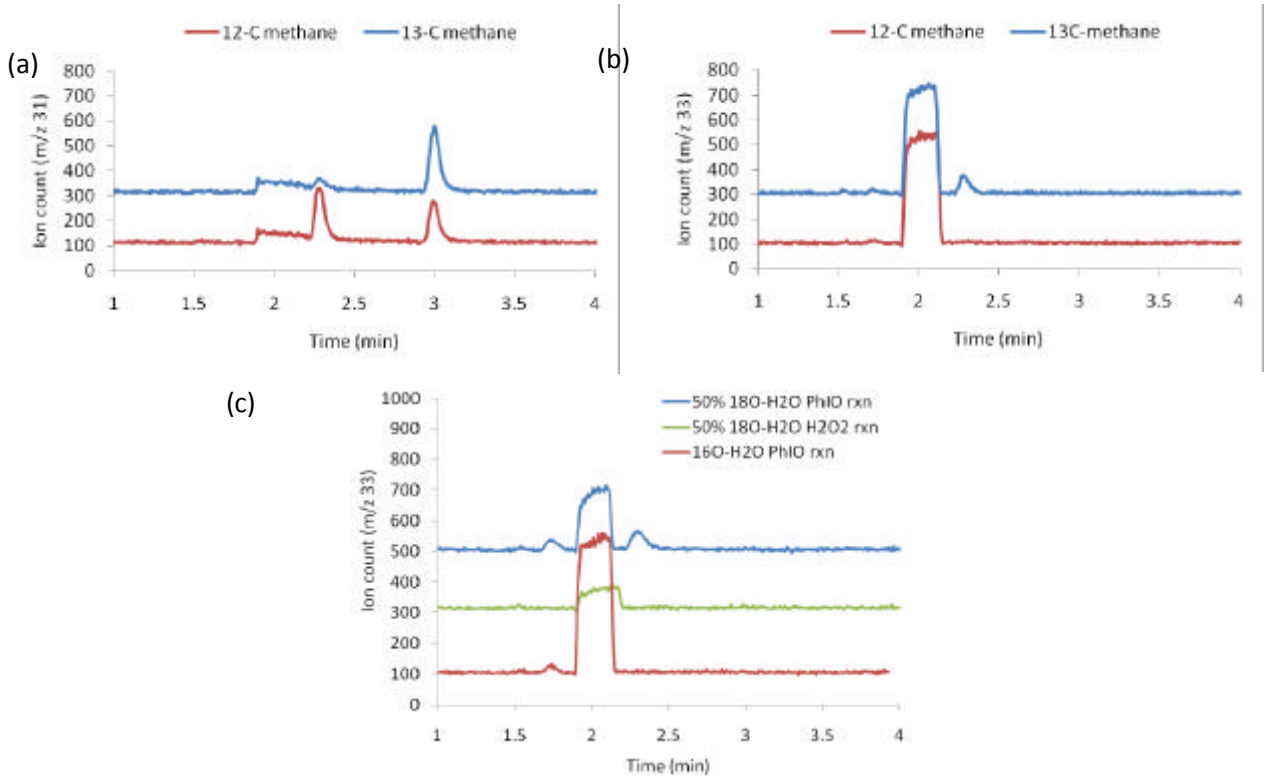


Figure S2: GC/MS-SIM chromatogram of PhIO-supported A6 methane reactions with ^{12}C-and ^{13}C-methane and 50% $H_2{}^{18}O$ (a) m/z 31 ion chromatogram of PhIO-supported A6 methane reactions with ^{12}C-and ^{13}C-methane, shown with a vertical offset (b) m/z 33 ion chromatogram of PhIO-supported A6 methane reactions with ^{12}C-and ^{13}C-methane, shown with a vertical offset (c) m/z 33 ion chromatogram of terminal oxidant-supported A6 methane reactions with ^{16}O-and ^{18}O-water, shown with a vertical offset.

UV-VIS spectroscopy

UV/VIS spectra recorded 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. All measurements were performed at 25 ^{o}C. Liquid substrate in an ethanol stock solution was used to add 10 mM substrate in a final solution containing 2% ethanol. For gaseous alkanes, P450 solutions were expose to substrates in 10mL crimp-cap vials with a head-space pressure of 40-psi.

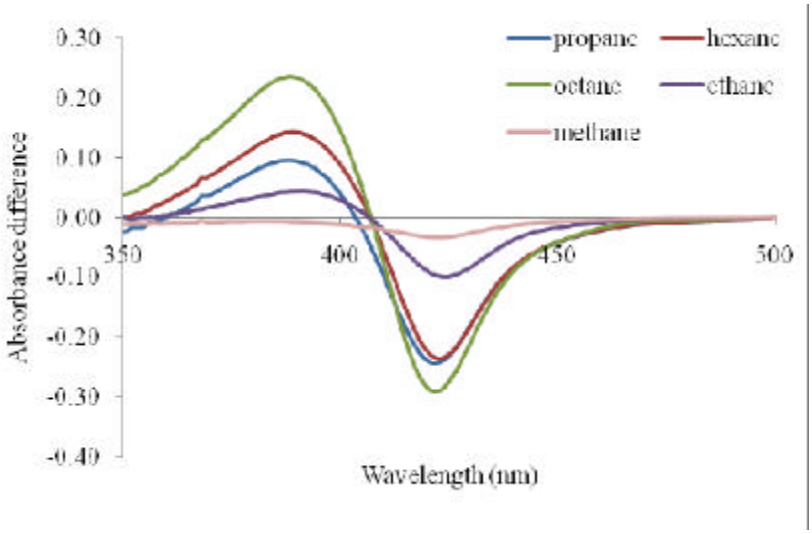
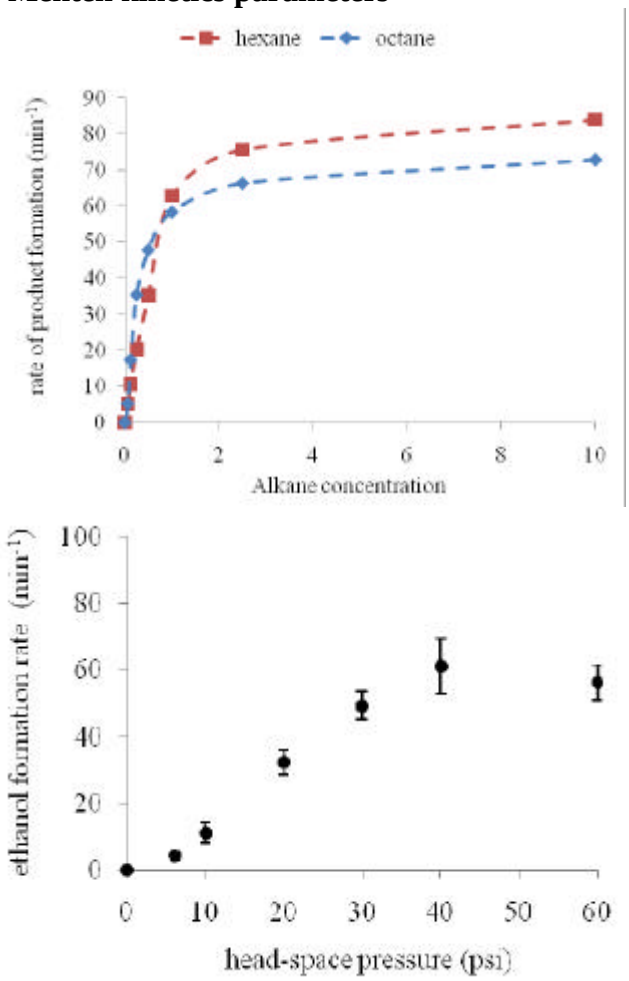


Figure S3: Difference UV/Vis spectra of CYP153A6 in the presence of different alkanes. Hexane and octane were present at 1 mM in 1% ethanol and referenced against an A6 solution containing 1% ethanol. Gaseous alkanes were present at 40-psi headspace pressure and referenced against an A6 solution.

Determination of Michaelis-Menten kinetics parameters



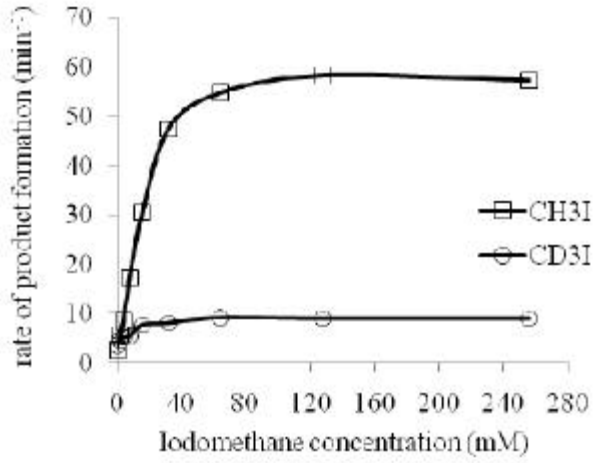


Figure S3: Plots of initial product formation rates at various substrate concentrations. (a) Octane and hexane reactions; (b) Ethane reactions; (c) Iodomethane and d_3-Iodomethane reactions;

References for Supporting Information

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3. D. Kock, M. M. Chen, J. B. van Beilen, F. H. Arnold, *AEM* **2009**, 75, 337-344.
4. T. Omura, R. Sato, J. Biol. Chem. **1964**, *239*, 2370-2378.
5. I.C. Gusalus, G.C. Wagner, *Methods Enzymology* **1978**, *52*, 166-188.