Analysis of the oxidation of short chain alkynes by flavocytochrome P450 BM3

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Supplementary data

Materials and Methods

Purification of WT and F87G mutants of flavocytochrome P450 BM3

Briefly, the P450 BM3 WT and F87G proteins were purified from *E. coli* transformant cell extracts by fractionation using ammonium sulfate precipitation (BM3 was enriched in proteins precipitated in a fraction between 30-60% saturation with ammonium sulfate), followed by pellet resuspension in 50 mM Tris.HCl buffer (pH 7.2) plus 1 mM EDTA (buffer A) and dialysis into a large volume of the same buffer to desalt the protein. Thereafter, BM3 was further purified by two successive anion exchange chromatography steps. First, the BM3-containing fractions were loaded onto a DEAE Sephacel column (Amersham-Pharmacia) pre-equilibrated in buffer A and eluted in a gradient of 0-500 mM KCl in the same buffer (500 ml). The fractions with the highest total P450 to protein (Reinheitzahl or Rz) ratio were pooled based on A_{418}/A_{280} measurements and concentrated by ultrafiltration, and then dialysed into salt free buffer A before being loaded onto a Q-Sepharose column (Amersham-Pharmacia) and eluted with a 500 ml gradient of 0-500

mM KCl in the same buffer (500 ml). As above, the fractions with the highest Rz ratio were pooled and concentrated by ultrafiltration, before extensive dialysis into buffer B (25 mM potassium phosphate, pH 6.5). The final step was fractionation on a hydroxyapatite column pre-equilibrated in buffer B. Protein was loaded in buffer B and eluted with a 500 ml gradient of buffer B to 250 mM potassium phosphate, pH 6.5. The fractions with the highest Rz ratio were once again pooled and concentrated by ultrafiltration (using a Millipore Amicon stirrer, 30 kDa cut-off) to a volume of ~10 ml, and then further concentrated to >500 μM using a Millipore Centriprep YM30 concentrator. Protein was then dialysed into 50% glycerol in buffer A prior to storage of BM3 protein in small aliquots at -80 ºC. All purifications steps were done at 4 ºC, and protease inhibitors (benzamidine hydrochloride and phenylmethanesulfonylfluoride, both at 1 mM) were present at all stages.

Results

Establishment of the chirality of 1-alkyne oxidation by WT flavocytochrome P450 BM3

Chirality of the 1-octyn-3-ol products derived from 1-octyne turnover by WT P450 BM3 was established by product separation on a chiral GC column, as described in the *Materials and Methods* section of the main paper, and with reference to (*S*)-(−)-1-octyn-3-ol and (*R*)-(+)-1-octyn-3-ol standards. As shown in Figure S1, the major product derived from WT BM3 was (*S*)-(−)-1-octyn-3-ol (~90%), with ~10% of product assigned to the (*R*)-(+)-1-octyn-3-ol enantiomer. While the relevant enantiomers of 1-hexyn-3-ol could not be obtained pure in either the *R* or *S* forms, the identification of a similar chiral GC elution pattern and peak volume ratio to that observed for the 1-octyn-3-ol products allowed the WT BM3-generated products from 1-hexyne to be assigned as (*S*)-(−)-1-hexyn-3-ol (~90%) and (*R*)-(+)-1-hexyn-3-ol (10%).

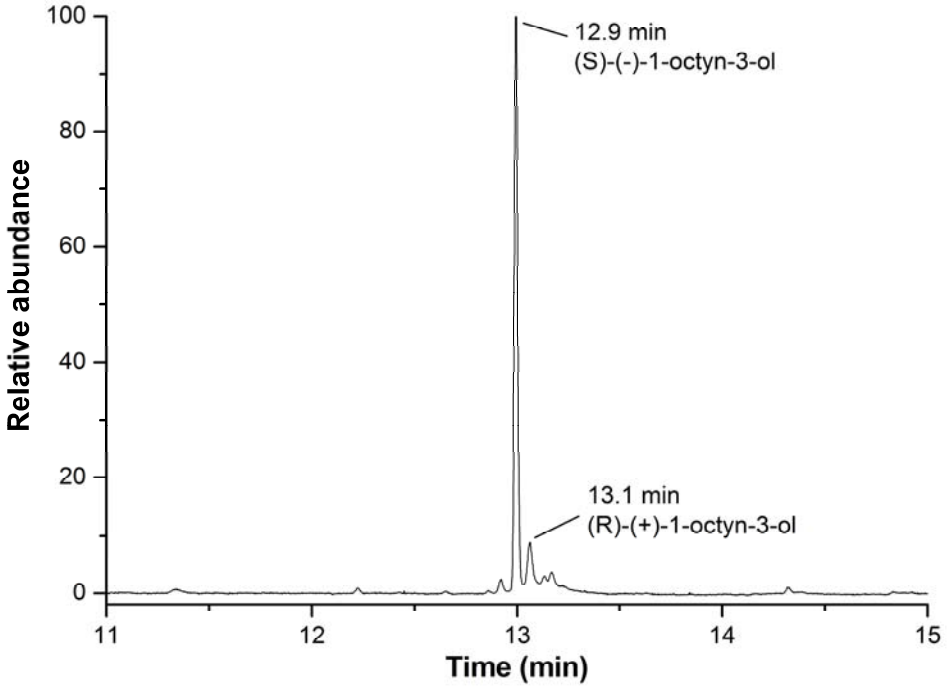

Figure S1. **Analysis of chiral products from oxidation of 1-octyne by WT P450 BM3**. The chirality of the 1-octyn-3-ol products was determined by GC separation using a chiral column, as described in the *Materials and Methods* section of the main paper. Standards were run to ascertain the retention time of the *R* and *S* enantiomers. (*R*)-(+)-1-octyn-3-ol eluted with a retention time of 13 min 6 s and the *S* enantiomer ((*S*)-(−)-1-octyn-3-ol) with a retention time of 12 min 54 s, as indicated in the figure. Curve fitting gave a product ratio of ~90% (*S*)-(−)-1-octyn-3-ol and ~10% (*R*)-(+)-1-octyn-3-ol.

Oxidation of octane and hexane by WT flavocytochrome P450 BM3

The WT P450 BM3-mediated oxidation of octane and hexane, and the isolation and analysis of oxidized products was done as described in the *Materials and Methods* section of the main paper. Figure S2 shows the separation of oxidized products from unconverted octane substrate by gas chromatography, with assignments of products made by fragment library comparisons, as described for the analysis of the oxidized alkynes in the main paper text. For octane, the major

products obtained were octan-4-ol (~24% of starting material) and octan-3-ol (~14%). In the case of hexane, residual substrate was not resolved from the solvent, but two products were clearly observed as hexan-3-ol (~98%) and hexan-2-ol (~2%).

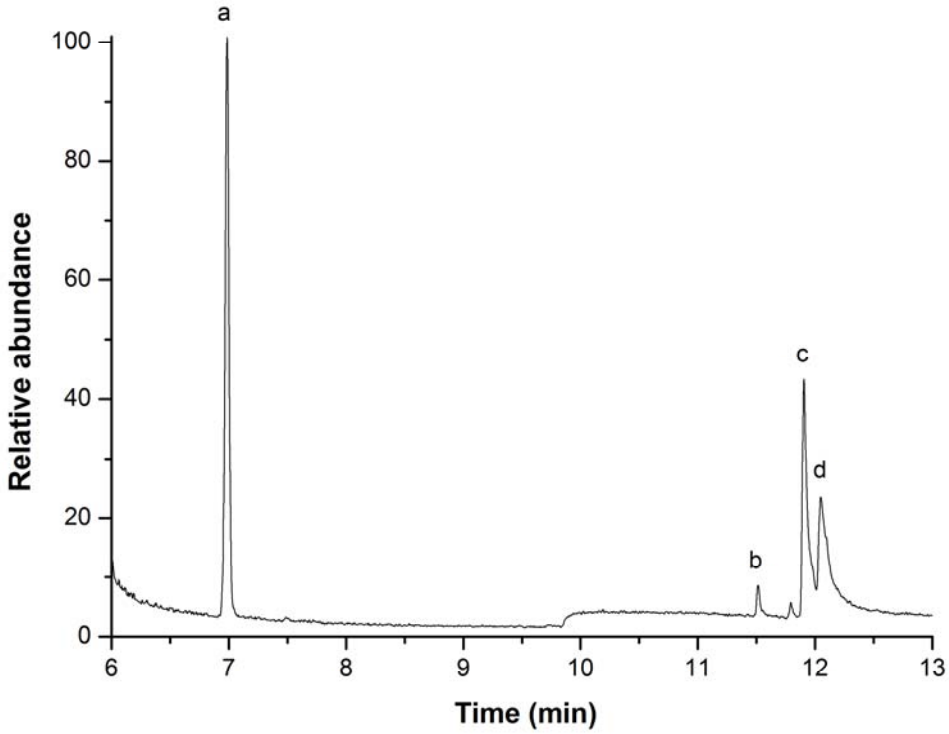

Figure S2. GC trace for octane turnover by flavocytochrome P450 BM3. The Figure shows the gas chromatogram from an octane turnover reaction using WT P450 BM3. Octane was incubated with WT P450 BM3, NADPH and a glucose-6-phosphate/glucose-6-phosphate dehydrogenase NADPH coenzyme regeneration system, with reaction details given in the *Materials and Methods* section of the main paper. Products and unconverted substrate were extracted from the aqueous phase using MTBE and resolved by gas chromatography. Peak (a) is assigned to unreacted substrate (octane, ~60% of the total material) and elutes after 6 min 59 s. The initial (minor) product peak (b) at 11 min 30 s is assigned to octan-4-one. The major product peak (c) which elutes after 11 min 54 s is assigned to octan-4-ol (~24% of starting material). The final product peak (d) with an elution time of 12 min 6 s is assigned to octan-3-ol (~14% of starting material).