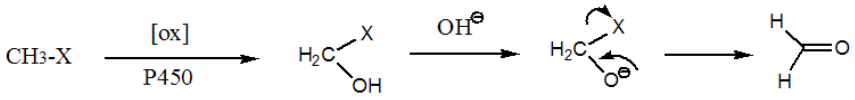
Appendix C

Candidate High-Throughput Screens for Small Alkane Hydroxylation

C.1 Halomethane Dehalogenation Screen

Halomethanes (CH_3X) are an obvious surrogate for methane and ethane as they share similar molecular size and C-H bond dissociation energies. The P450 catalyzed dehalogenation of halomethanes ultimately yields formaldehyde (see equation (C.1)), which can be quantified calorimetrically with the use of Purpald®, similarly as the DME screen detailed in Chapter 8.E.3.



(C.1)

After assaying BM3 variants for CH_3I, CH_3F, CH_3Br, and CH_3Cl dehalogenation, we determined CH_3Cl to be the most amenable substrate for high-throughput screening as it had the highest activity (20 – 70 TON) and assay signal (A_{550nm} = 0.2 – 0.5). While the assay captured differences in dehalogenation activity of BM3 variants (see Figure C.1), when it was used to evaluate mutant libraries, the variants with improved dehalogenation activity did not frequently exhibit improved ethane hydroxylation activity.

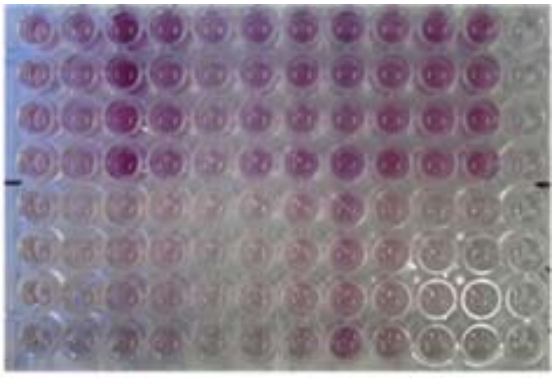
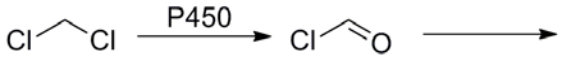


Figure C.1: Colorimetric screen for chloromethane dehalogenation; each column contains eight reactions with the same variant–the top four wells contain reactions with chloromethane, the bottom four wells are control reactions without chloromethane. Column one contains variant 35E11, columns two through eleven contain BM3 variants derived from 35E11, and column 12 contains wild-type BM3.

C.2 Dichloromethane Dehalogenation Screen

In addition to using chloromethane dehalogenation as surrogate for ethane or methane hydroxylation, we also attempted to use dehalogenation of dichloromethane as a high throughput screen. The P450 catalyzed dehalogenation of dichloromethane results in the *in situ* formation of carbon monoxide (see equation (C.2)), which when bound to P450 heme generates the characteristic 450 nm soret peak.



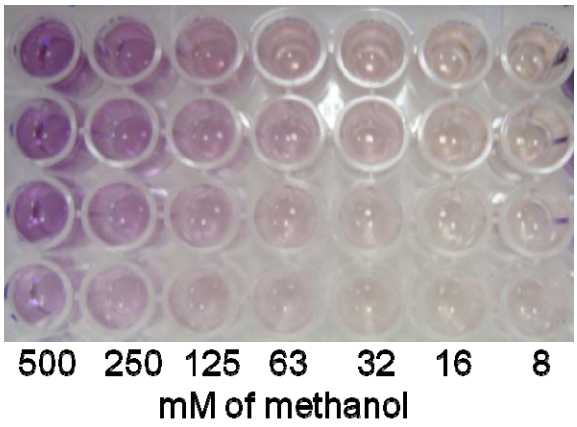
(C.2)

Despite having a weaker C-H bond as compared to chloromethane (93 kcal/mol vs. 98 kcal/mol), the activity of BM3 variants for dehalogenation of dichloromethane was equally poor, 20 – 50 TON. In order to quantify the catalytic release of CO, purified P450 heme domains are needed in excess. The final drawback of this assay is the inhibitory nature of CO of the P450 reaction.

C3. Methanol Oxidation Screen

The small size of methane and ethane significantly limits the molecules that can serve as a suitable surrogate. In addition to halomethanes, methanol is another compound with an intermediate size between methane and ethane. While methanol oxidation activity is non-ideal for a selective methane oxidizing catalyst, it is present in MMOs. Since ethanol is a known P450 substrate, pursuing methanol oxidation as a high-throughput screen was appealing. The P450 catalyzed methanol oxidation resulted in the production of formaldehyde, which can be quantified by Purpald® (see Figure C.2).

(a)



(b)

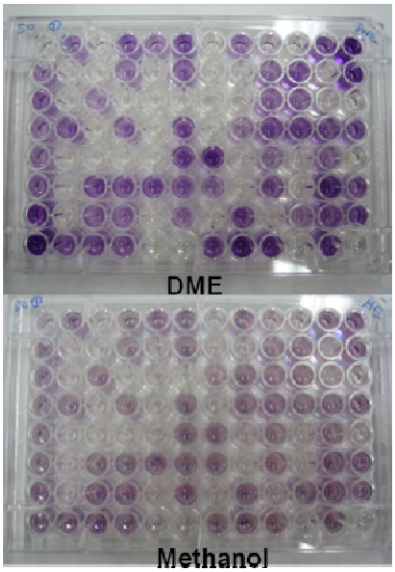


Figure C.2: High-throughput methanol oxidation screen: (a) the methanol hydroxylation activity of 7-7 AB2, a $P450_{PMO}$-derived BM3 variant, at various methanol concentrations; (b) a sample screening plate from 7-7 AB2 random heme library assayed for DME demethylation and methanol oxidation activity

Methanol oxidation activity was not observed in wild-type BM3 and most variants in the $P450_{PMO}$ lineage. $P450_{PMO}$ and its derived variants were able to oxidize methanol with detectable activity with more than 50 mM methanol present in the reaction. From screening of a random heme domain library for both DME demethylation and methanol oxidation it was evident that these two activities were well-correlated (see Figure C.2 (b)). As a result, there was not a significant difference between variants identified from these two assays.