Evolution of cytochrome P450 BM3 towards aromatic hydroxylation

Alexandra Weingartner, Anna Gärtner, Alexander Dennig, Anna Joëlle Ruff, Ulrich Schwaneberg

Institute of Biotechnology, RWTH Aachen University, Germany

a.weingartner@biotec.rwth-Aachen.de

Introduction

The monooxygenase P450 BM3 from *Bacillus megaterium* has gained great interest within the last decades due to its extraordinary nature to catalyze the CH-activating oxidative hydroxylation of organic compounds. After two rounds of evolution by site saturation mutagenesis the highly improved P450 BM3 variant M2 (R47S/Y51W/I401M) was found to catalyze the specific *o*-hydroxylation of several mono- and di-substituted benzenes[1]. Recently, a new P450 BM3 variant with an additional amino acid substitution was generated showing improved activity towards aromatic substrates and increased formation of dihydroxylated products. The synthesis of dihydroxylated products from benzene derivatives in a one-pot reaction employing P450 BM3 as a sole catalyst demonstrates a novel production route for attractive aromatic building blocks such as tri-methyl-hydroquinone (TMHQ).

P450 BM3 variant M2 (R47S, Y51W, I401M)[1] was used as starting variant for the saturation of position A330 to all 20 canonical amino acids and screened for improved NADPH oxidation.

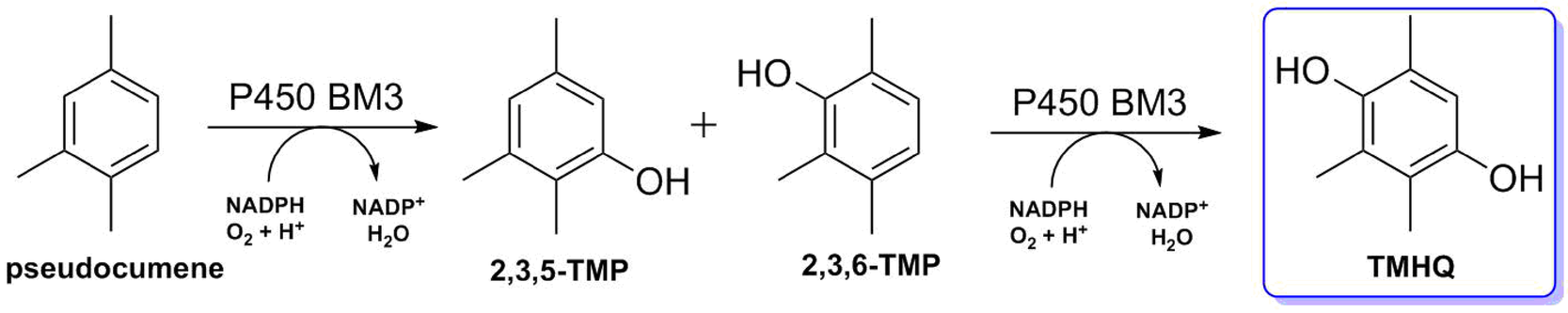
The new P450 BM3 variant M3 was identified showing a 2-fold increased coupling efficiency compared to M2 and a 13-fold improved turnover frequency (Table 1).

Table 1 Catalytic data for conversion of pseudocumene with purified P450 BM3 enzymes.

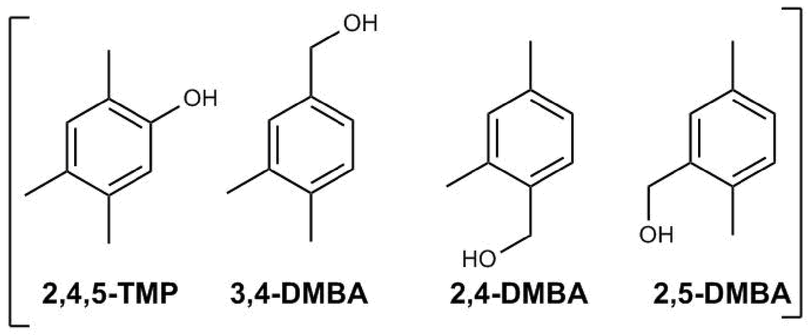
Catalyst	NADPH ox rate [min-1]	Coupling efficiency [%]	TOF [min -1]
WT	22 ± 4	15 ± 2	3
M2	87 ± 3	19 ± 2	17
M3	499 ± 91	45 ± 6	226

NADPH oxidation rate ($mol_{cofactor}$ mol $_{P450-1}$ min^{-1}) was determined spectrophotometrically; Coupling efficiency (%) = ratio between product formation [µM] and cofactor oxidation [µM]; Turnover frequency ($mol_{product}$ mol_{P450-1} min^{-1}). WT = wild type P450 BM3; M2 and M3 = P450 BM3 variants

P450 BM3 variants are able to produce TMHQ under continuous NADPH-recycling directly from pseudocumene (Fig 1)..



Pseudocumene can be hydroxylated by P450 BM3 at all six carbon atoms resulting in six mono-hydroxylated products, trimethylphenols (TMP) and dimethylbenzylalcohols (DMBA).



The intermediates 2,3,5-TMP and 2,3,6-TMP can be further hydroxylated to TMHQ.

Fig.1 Hydroxylation of pseudocumene by P450 BM3

Substrate binding and chemo-selectivity of P450 BM3

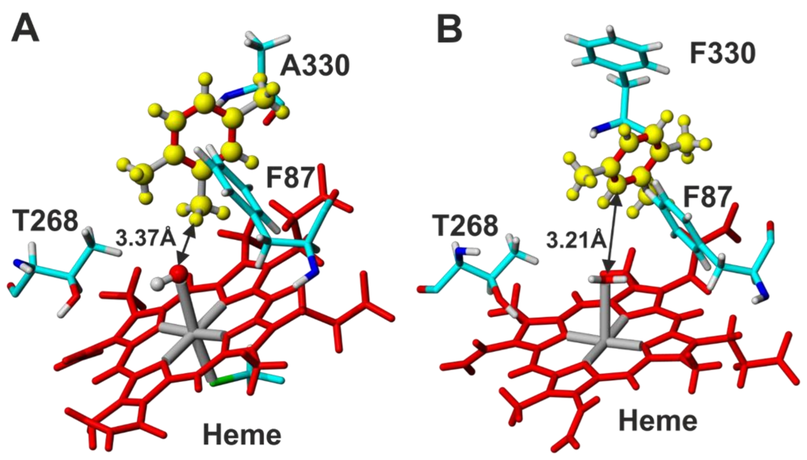


Fig. 2 Docking of pseudocumene into the active site of P450 BM3 WT (A) and variant M3 (B)

Conversion of pseudocumene by P450 BM3 WT leads predominantly to benzyl alcohols.

The engineered P450 BM3 variants M2 and M3 displayed altered chemoselectivity compared to P450 BM3 WT with increased formation of phenols as well as further secondary hydroxylations (Table 2) [2].

Different chemo-selectivity for pseudocumene of P450 BM3 variants indicates varying bindings mode of the substrate in the active site.

Table 2. Selectivity and productivity of the P450 BM3-catalyzed hydroxylation using cell-free lysates

Catalyst	2,5-DMBA	2,4-DMBA	2,3,6-TMP	2,3,5-TMP	3,4-DMBA	2,4,5-TMP	TMHQ	TMHQ [mg L-1]
WT	14 %	27 %	9 %	8 %	5 %	34 %	3 %	< 10
M1	12 %	31 %	4 %	5 %	2 %	34 %	12 %	80
M2	7 %	17 %	7 %	4 %	2 %	31 %	32 %	190
M3	6 %	17 %	7 %	4 %	2 %	29 %	35 %	180

Different chemo-selectivity for pseudocumene of P450 BM3 variants indicates varying bindings mode of the substrate in the active site.
The mutation A330F provides additional aromatic interactions (‘π-X-π’) that improve binding and conversion of benzenes (Fig. 2).

Acknowledgements

This work is part of the ROBOX project. The ROBOX project has received funding from the European Union (EU) project ROBOX (grant agreement n°635734) under EU’s Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. Any statements made herein reflects only the author’s views. The European Union is not liable for any use that may be made of the information contained herein.

References

[1] A. Dennig et al. in *Angew. Chemie - Int. Ed.* Vol 52, 2013, pp. 8459–8462.
[2] A. Dennig and A. Weingartner *et al.* 2017 submitted to ACS catalysis.