Engineering of P450 BM3 using OmniChange multi-site saturation mutagenesis

Alan Mertens[a], Anna Joëlle Ruff[a] and Ulrich Schwaneberg[a,b]

[a] *Institute of Biotechnology, RWTH Aachen University, Aachen, Germany;* [b] *DWI – Leibniz Institute for Interactive Materials, Aachen, Germany*

a.mertens@biotec.rwth-aachen.de

Introduction

Focused mutagenesis is a valuable tool for rational or semi-rational protein engineering as it enables improvement of mainly localizable enzyme properties such as selectivity or activity[1]. In order to explore synergistic substitutions multi-site saturation mutagenesis approaches are of high interest as they facilitate the simultaneous exchange of multiple residues[2]. Here we present the application of the OmniChange multi-site saturation mutagenesis method for engineering of P450 BM3 from *Bacillus megaterium*. P450 BM3 is the best studied redox self-sufficient cytochrome P450 monooxygenase and of particular biotechnological interest as it exhibits very high catalytic rates in comparison to other P450s[3].

OmniChange[4]: Simultaneous saturation of up to five individual positions

OmniChange (see Figure 1) is a sequence-independent multi-site mutagenesis method which enables simultaneous saturation of up to five individual positions in fast and efficient manner[4]. Consequently, the OmniChange method is eminently suited for recombining multiple positions.

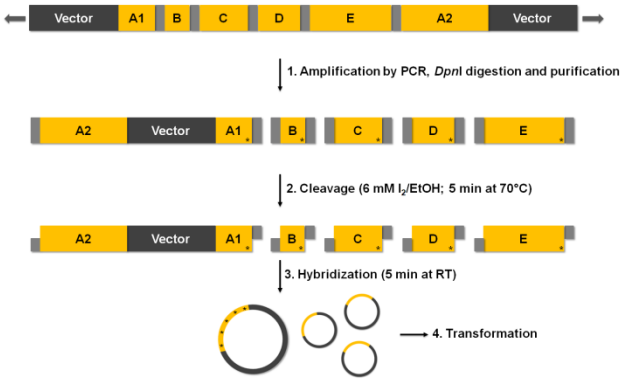

Figure 1[4]. Schematic depiction of the generation of an OmniChange library with five saturated codons. Step 1: Amplification of five DNA fragments bearing a NNK-saturated codon (indicated with *) and approx. 12 phosphorothioated nucleotides on 5'-ends. Step 2: Chemical cleavage to generate complementary single-stranded 3'-overhangs. Step 3: Hybridization of all fragments to a circular plasmid containing ten DNA nicks. Step 4: Transformation and nick-repair in *E. coli*.

Targeting multiple key-residues in P450 BM3

In this study the OmniChange method was applied to improve the activity of P450 BM3 towards mono-substituted benzenes. Positions for recombination were selected based on reported positions in literature. Key residues R47, Y51, A330 & I401 were selected for simultaneous SSM by OmniChange (see Figure 2).

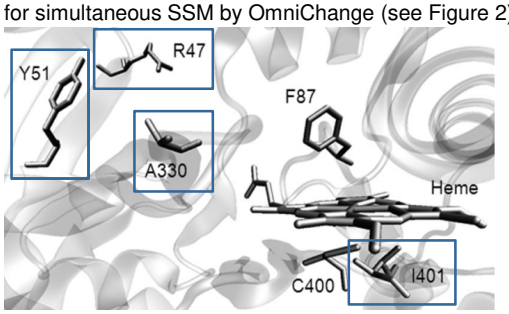

Figure 2[5]. View on the active site of P450 BM3. The residues targeted for mutagenesis (R47, Y51, A330, and I401) are highlighted in blue boxes.

Generation and Screening of P450 BM3 OmniChange library

An OmniChange library containing four simultaneously saturated codons at selected positions was generated (theoretical protein sequence space: 64,000 variants) and diversity was verified by sequencing. About thousand clones of the OmniChange library were screened using the 4-AAP assay[6] in presence of chlorobenzene as substrate (see reaction scheme in Figure 3).

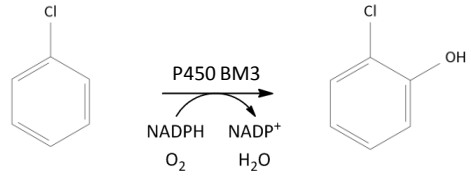

Figure 3. Conversion of chlorobenzene to chlorophenol by P450 BM3. The catalyzed hydroxylation reaction is very regioselective (> 95% o-hydroxylation)[7].

More than 20 variants exhibiting at least doubled product formation in comparison to P450 BM3 wildtype were found in the library. The best variant Omni-1 was compared with several other variants derived from iterative saturation mutagenesis (ISM). It turned out that Omni-1 was most active among the tested variants and approx. 20 times more active than wildtype (see Figure 4).

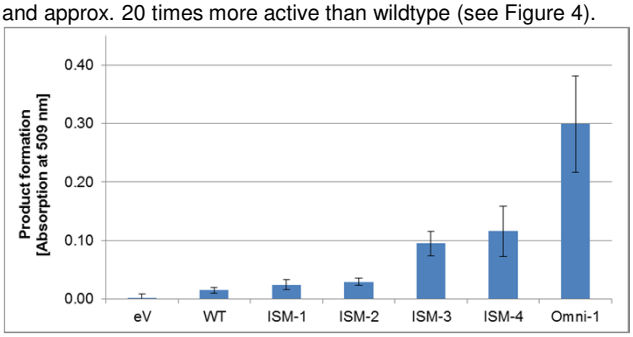

Figure 4. Product formation (4-AAP assay, reaction was stopped after first variant depleted NADPH) of several P450 BM3 variants. eV = empty vector control, WT = wildtype, ISM-1 = R47D, ISM-2 = R47D/A330Y, ISM-3 = R47D/Y51V/A330Y/I401M, ISM-4 = R47D/Y51V/A330Y/I401Y, Omni-1 = R47Y/Y51V/A330Y/I401V.

Conclusion and future prospects

- OmniChange is a valuable tool for recombining key residues
- A P450 BM3 OmniChange library containing four simultaneously saturated codons was successfully generated
- Screening of less than 1% of the theoretical protein sequence space yielded a variant that is superior in comparison to several ISM variants and approx. 20 times more active than wildtype
- Currently screening of another P450 BM3 OmniChange library for improving the activity towards cyclic alkenes is in progress

References

[1] Reetz M. T., Prasad, S., Carballeira, J. D., Gumulya, Y., Bocola, M., J. Am. Chem. Soc. (2010), 132: 9144-9152.
[2] Ruff, A. J., Dennig, A., Schwaneberg, U., FEBS J. (2013), 280: 2961-2978.
[3] Whitehouse, C. J. C., Bell, S. G., Wong, L.-L., Chem. Soc. Rev. (2012), 41: 1218-1260.
[4] Dennig, A., Shivange, A. V., Marienhagen, J., Schwaneberg, U., PLoS ONE (2011), 6: e26222.
[5] Dennig, A., Marienhagen, J., Ruff, A.J., Guddat, L., Schwaneberg, U., ChemCatChem (2012), 4, 771-773.
[6] Wong, T. S., Wu, N., Roccatano, D., Zacharias, M., Schwaneberg, U., J. Biomol. Screen. (2005), 10, 246-252.
[7] Dennig, A., Lülsdorf, N., Liu, H., Schwaneberg, U., Angew. Chemie (2013), 53, 8459-8462.

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.