Redesign of Baeyer–Villiger Monooxygenases for Synthetic Applications
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Chapter 7

Conclusions and outlook
The research described in this thesis comprises a representative case of a protein engineering cycle, which includes enzyme characterisation, development of suitable screening methods, design and screening of libraries, ultimately leading to the isolation of variants with improved properties. Detailed knowledge of the active-site architecture and the catalytic mechanism, coming from structural studies, modelling, kinetic analyses, and characterisation of mutants allowed the design of a high quality library while efficient screening methods enabled successful fishing of mutants with improved properties. Importantly, the presented approach, which has been shown to be successful for PAMO, can be applied in engineering of Baeyer–Villiger monoxygenases or other NAD(P)H dependent enzymes thanks to the generic nature of the developed screening method.

**BVMOs are biocatalysts with industrial potential**

The enzymatic Baeyer–Villiger reaction represents an attractive alternative to traditional chemical methods because it utilises molecular oxygen, which is a mild and cheap oxidant, and it offers high selectivity of the catalysed oxidations. In previous years, applications of BVMOs were hampered by the limited number of available enzymes, their poor stability, need for using expensive coenzymes, and the lack of structural information, which held back improvements of these enzymes by rational engineering. As discussed in Chapter 2, the research on BVMOs has intensified over the last decade, and several milestones have been reached. A few crystal structures have been solved, which greatly improved our understanding on how BVMOs function. The available structural information and insights into the catalytic mechanism have facilitated engineering of BVMOs. Bioinformatic analyses of the continuously increasing number of sequenced genomes have provided access to new enzymes coming from diverse kingdoms and environments. Advances have also been made in the field of coenzyme regeneration by using electrochemical, enzymatic, and photochemical methods. One of the remaining limitations is the relatively low activity of these enzymes on industrially relevant compounds. Thus, there is a need for substantial improvements in activity of BVMOs, and related to this, efficient engineering methods for this class of monoxygenases. The research described in this thesis has contributed to our knowledge on functioning of BVMOs and provided tools for modifying their properties through protein engineering.

**BVMOs present strict specificity for NADPH as an electron donor**

BVMOs require nicotinamide coenzymes for the flavin reduction. Strikingly, most BVMOs use NADPH for this purpose and show very low activity with NADH. In Chapter 3, we tried to change the coenzyme specificity of PAMO in favour of
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NADH by using rational enzyme engineering. We used the structure of CHMO with NADP\(^+\) to model the coenzyme in the active site of PAMO. Structural as well as sequence analyses disclosed several positions which are potentially involved in the interactions with the coenzyme. We performed site-directed mutagenesis on these positions and characterised the mutants with respect to their kinetic parameters with NADPH and NADH. Although only minor improvements in the activity of PAMO with NADH could be achieved, this study has proved the importance of R217 in the interaction with NADPH and, therefore, improved our understanding of the discrimination between NADPH and NADH in BVMOs (Figure 1).

Recent structural studies on PAMO have provided an explanation for why relaxing the strict preference of PAMO for NADP\(^+\) is not straightforward (Orru et al., 2011). The elucidated structure of PAMO in complex with NADP\(^+\) has revealed that NADP\(^+\), together with R337, takes a central position in the network of interactions stabilising two crucial enzyme intermediates: the peroxylflavin and the Criegee intermediate.

**Figure 1.** Structure of PAMO (PDB: 2YLT) with the positions critical for the coenzyme recognition and the substrate specificity. R217, which interacts with NADPH, is displayed as a dark blue sphere. P253, G254, R258, and L443, mutated in the PAMO\(_{15-p3}\) variant with expanded substrate range, are shown as red spheres. Positions identified in the mutational analysis are presented as orange spheres (V54, I67, Q152, A435, S441, A442, and M446). FAD is shown in yellow, NADP\(^+\) in blue, and MES in purple. The picture was prepared using the PyMol software.
The importance of NADP+ in the catalytic mechanism has been demonstrated also for other flavin-containing monooxygenases. *Aspergillus fumigatus* siderophore A (SidA) is an N-hydroxylating monooxygenase that catalyses the hydroxylation of ornithine. Stability of the C4a-hydroperoxyflavin in SidA was two times lower in the presence of NADH than in the case of NADPH. Furthermore, biochemical studies including flavin fluorescence measurements and limited proteolysis suggested different binding modes for NADPH and NADH in SidA (Romero et al., 2012). Structural studies on SidA indicated that C4a-hydroperoxyflavin stabilisation is probably achieved by H-bonds between the carboxamide of the cofactor and the protonated N5 atom of the reduced flavin (Franceschini et al., 2012a).

Structural and kinetic analyses of several mutants of flavin-containing monooxygenase from *Methylophaga* sp. strain SK1 (mFMO) as well as mFMO with NADP+ analogues led to the conclusion that NADP+ plays multiple roles in the catalytic cycle (Orru et al., 2010). Except for providing electrons to reduce the flavin, the coenzyme forms part of an oxygen binding site. Moreover, NADP+ is crucial for the oxygen reactivity of the enzyme. H-bonds between the ribose 2’-OH group and the hydroperoxyflavin as well as between the carboxamide group and the N5 and O4 atoms of the flavin stabilise the hydroperoxyflavin, thereby promoting the monooxygenase activity.

Overall, the data on flavin-containing monooxygenases suggest that preference for NADPH is well conserved in the subfamily of class B flavoprotein monooxygenases and explain the limited success of our attempts of changing the coenzyme specificity of PAMO. Due to the complex role of the coenzyme in the catalytic cycle, coenzyme specificity of PAMO could not be modified by simple, rationally designed substitutions in the NADP-binding domain. Perhaps more radical reconstruction of the NADP-binding domain, for instance, simultaneous mutagenesis of several positions would enable activity with NADH. The new assay described in Chapter 5 could be used for screening libraries in order to evolve PAMO towards increased activity with NADH. Alternatively, one may focus on a newly discovered group of class B flavoprotein monooxygenases members of which were found to act as NADH-dependent BVMOs (Riebel et al., 2013).

**Site-directed mutagenesis identified hot-spots for the substrate specificity of PAMO**

It has been proposed that careful selection of target residues for mutagenesis can increase the chance of finding improved enzyme variants. In particular, when aiming at altered specificity or selectivity, it is reasonable to restrict the introduction of mutations to the active site of an enzyme (Morley and Kazlauskas,
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2005). Obviously, this is only possible when detailed structural information on the enzyme is available.

In the case of PAMO, the first solved structure did not contain a substrate or an inhibitor. Therefore, it was difficult to infer any information on how a substrate binds in the enzyme. For this reason, we followed an indirect approach based on comparative analysis of the structure of PAMO and a model of CPMO, a BVMO with an impressively broad substrate scope. Upon comparison of the active sites of these two enzymes, we selected 15 positions to be probed. Site-directed mutagenesis of these 15 positions and characterisation of the mutants in terms of activity and enantioselectivity with a range of substrates led us to the conclusion that V54, I67, Q152, A435, S441, A442, L443, and M446 are involved in interactions with the substrate and are promising targets for future, more extensive mutagenesis studies (Figure 1). These results made an important contribution to the design of the library described in Chapter 6. Moreover, some of the mutants, for example, I67T, A442G, or L443F, showed increased activity and improved enantioselectivity in biotransformations of sulfides and constitute a valuable addition to the collection of BVMO-based biocatalysts.

This mutagenesis study had been carried out before the structures of PAMO (mutants) with 2-(N-morpholino)ethanesulfonic acid (MES) bound became available (Orru et al., 2011). These new structures of PAMO revealed two sites which can potentially bind the substrate. While I67, A442, L443, and M446 form parts of the MES binding sites, other residues identified in the screening of mutants (V54, Q152, A435, and S441) do not directly interact with the ligand. The latter residues belong to the second shell of the active site, and they can influence the enzyme selectivity by affecting the positions of FAD or substrate-binding-site amino acids. Thus, the results of the mutagenesis study, which identifies the role of several residues in binding/positioning of the substrate in the active site, and the structures of PAMO (mutants) in complex with MES indicating positions occupied by the substrate, bring complementary conclusions about the determinants of the substrate specificity of PAMO. These insights form a strong basis for redesign studies aimed at altering the specificity of PAMO and related enzymes.

Comparison of putative substrate binding sites of PAMO, CHMO, and STMO does not provide clear explanation for differences in substrate specificity between these enzymes. The conserved elements of the active sites (FAD, NADPH, arginine and aspartate residues respective to R337 and D66 in PAMO) are important for reduction of the flavin by NADPH, formation and stabilisation of the peroxyflavin and the Criegee intermediate. Apparently, the size and hydrophobicity of the residues lining the active site dictate the substrate specificity and only general trends in substrate acceptance can be defined (Franceschini et al., 2012b; Orru et al., 2011). A similar active-site organisation is observed in FMOs, which also
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feature a relatively broad substrate scope (Alfieri et al., 2008). On the contrary, in active sites of ornithine hydroxylases (PvdA and SidA), a clear pocket for binding ornithine can be observed, and the structures reveal multiple H-bond interactions and salt bridges between the substrate and the active-site residues (Franceschini et al., 2012a; Olucha et al., 2011). This explains the narrow substrate specificity of these $N$-hydroxylating monooxygenases which is limited to ornithine and lysine.

**The phosphate-based assay is a powerful tool for screening BVMO libraries**

In Chapter 5, a novel method for screening libraries of BVMOs is presented (Figure 2). In this method, the Baeyer–Villiger reaction is coupled to coenzyme regeneration by using phosphite dehydrogenase which leads to the production of phosphate. Phosphate is used as a reporter of the Baeyer–Villiger activity, and it is quantified in reaction ammonium molybdate. Since the method is based on an indirect assay of the Baeyer–Villiger activity via NADPH consumption, it can be applied in combination with any desired target substrate. This is further facilitated by the periplasmic expression of PAMO. The periplasm of *E. coli* is accessible to molecules up to 1 kDa big, which can diffuse through outer membrane pores. Therefore, potential substrates can easily reach the periplasmic enzyme. Thus, the activity detection based on NADPH usage and the periplasmic expression make this screening method a generic approach for testing activity of BVMOs. Isolation of false positive hits, namely the uncoupling mutants, is prevented thanks to the detection of hydrogen peroxide, a by-product of the uncoupling reaction. Furthermore, the phosphate method allows screening whole cells, which eliminates the need for the preparation of cell extracts. Overall, the phosphate screening method offers a satisfactory throughput of a few thousands of clones per week without compromising the reliability of the results. The throughput of the method is relatively high when compared to alternative methods for testing BVMO activity (for example, GC or HPLC).

![Figure 2. Schematic representation of the principle of the phosphate-screening method. *E. coli* cells express PAMO in the periplasm. Phosphite dehydrogenase (PTDH) regenerates reduced coenzyme used by PAMO and produces phosphate, which is measured in a colorimetric assay. Hydrogen peroxide, a product of the uncoupling reaction, is detected in reaction with a fluorogenic probe.](image-url)
This new and versatile assay may be applied in screening libraries of NADPH/NADH-dependent monooxygenases for activity with new substrates as well as for increased stability or altered coenzyme preference. In Chapters 5 and 6, we have used this method in screening libraries for activity with an aromatic sulfide and cyclic ketones, which proves that this screening method can be applied for different substrate classes.

It would also be valuable to extend the phosphate-based approach for screening on agar plates. This would require overcoming several issues, for instance, control of the protein expression and periplasmic translocation of PAMO expressed in cells growing as colonies on solid medium. Furthermore, the phosphomolybdate product is soluble in water, and it could diffuse in agar. Therefore, an alternative phosphate detection assay, leading to an insoluble product, might be necessary. Adaptation of the phosphate method for agar plate screening could significantly increase the throughput and allow screening libraries of $10^4$–$10^5$ mutants. Presumably, such an assay would work as a pre-screen and require verification of the hits in 96-well plate format.

Growth selection assays represent one of the most powerful ways of screening libraries. One could envision a selection method in which growth of E. coli would depend on phosphate produced by PTDH upon regeneration of NADPH used by a BVMO. Previously, Woodyer et al. used a selection system to engineer enhanced activity of PTDH (Woodyer et al., 2006). They applied toxic concentrations of phosphite which inhibited reactions with phosphate and based their selection on removing phosphite toxicity by improved PTDH variants. It remains to be tested whether phosphate formation in the coupled BVMO/PTDH system could be sufficient to support growth of E. coli and, therefore, used as a selection marker for engineering BVMOs.

**PAMO can be engineered for activity with new compounds by multi-site mutagenesis**

Equipped with the knowledge on the active site of PAMO and the developed screening method, we proceeded with the design, preparation, and screening of a library of PAMO mutants. As previous studies showed that introducing single mutations usually brings only small changes in the catalytic properties of PAMO, we simultaneously modified 11 positions in the active site of the enzyme. Screening 1,500 clones of the library yielded a mutant active on cyclopentanone which contained four substitutions: P253F, G254A, R258M, and L443F (Figure 1). Further characterisation of this mutant revealed that its substrate scope represents a hybrid of the substrate ranges of PAMO and cycloketone monooxygenases. Remarkably, the stability of the mutant was the same as that of wild-type PAMO.
With these results, we have confirmed that the hypothetical substrate binding site marked by the presence of MES in the crystal structure is a valid hot-spot region for modifying the specificity of PAMO. We have successfully applied the OmniChange mutagenesis method for generating a high-quality library. This method is fast and simple as only a single PCR amplification step is required for each fragment before they are assembled into full size plasmids. The OmniChange method allows flexibility with regard to number and position of the residues to be mutated. As we have shown, multiple sites can be targeted in each fragment.

We have also demonstrated that targeting multiple positions in PAMO is required to change substrate specificity of this enzyme. This shows that cooperative effects of mutations can have a huge influence on the enzyme properties. Similar results were obtained by Sandström et al., who engineered Candida antarctica lipase A for improved activity and enantioselectivity on an ibuprofen ester (Sandström et al., 2012). By targeting 9 positions and allowing 1–3 mutations at each of them, they obtained an enzyme variant containing five substitutions which displayed remarkable improvements for the target biotransformation. Reversing each of these substitutions resulted in a decrease in the activity and enantioselectivity. Moreover, combinations of each two mutations found in the best mutant did not lead to any significant improvements in the properties of the enzyme. These results indicate that in some cases, many mutations are necessary to find an improved variant. Therefore, the approach of combinatorial introduction of a limited set of mutations at many positions at the same time should not be underappreciated.

Lastly, we showed that the phosphate screening method can be successfully applied in screening libraries of PAMO mutants, and, therefore, it is a powerful tool for engineering BVMOs. The phosphate-based screening method could be used to further increase the activity of the PAMO_{15-F5} mutant identified in Chapter 6 with aliphatic compounds. Our results on the characterisation of the quadruple mutant point to the new hot-spots for the substrate specificity of PAMO. Site-saturation mutagenesis on P253, G254, R258, or L443 could result in new mutants with interesting properties.

**Future directions**

Enzyme-based processes have to be competitive with traditional chemical approaches and economically feasible. The use of oxidoreductases and, in particular, of BVMOs on industrial scale is limited by several factors. These enzymes feature complex catalytic mechanisms involving multiple substrates and redox equivalents, and they require cofactors for their activity. On the other hand, chemo- and stereoselective oxyfunctionalisations catalysed by BVMOs are of
great synthetic potential. Therefore, it is worthwhile to invest efforts in bringing these enzymes closer to applications.

A large number of genes that can be annotated as BVMO-encoding genes await characterisation. While most enzymes studied so far come from bacterial sources, nowadays, many putative BVMOs can be found in sequenced eukaryotic genomes. Thus, discovery and characterisation of new BVMOs may bring novel biocatalysts with interesting properties. Until now, PAMO is the only thermostable BVMO known. Exploration of enzymes from extremophiles will hopefully expand the toolbox of stable BVMOs. The work presented in this thesis focused on improving activity and expanding substrate scope of the robust and thermostable PAMO. Alternatively, stability of other BVMOs, like CPMO- and CHMO-type of enzymes, which display substrate ranges much wider than PAMO, could be improved. The phosphate-based screening method could be of use for this purpose.

Production of a biocatalyst is an important factor contributing to a cost of a bioprocess. So far, BVMOs were expressed in *E. coli* and *Saccharomyces cerevisiae*. It could be advantageous to produce BVMOs, especially from Eukaryotes, in other expression systems in order to increase expression levels. Secretion of enzymes into the growth medium is a strategy often used to increase the production yield. However, in the case of BVMOs, the requirement for the FAD cofactor makes extracellular expression of BVMOs difficult. Most of the secretion systems transport a polypeptide chain in an unfolded state which causes the enzyme to lose the cofactor. Export of a BVMO to the periplasm via the Tat export system proved to be a useful approach for screening purposes (Chapter 5), but the limited capacity of the Tat secretion machinery would most probably prevent the use of *E. coli* cells expressing Tat-PAMO as an efficient whole-cell catalyst. Lastly, in the case of extracellular expression, reduced coenzyme for BVMO has to be provided by an auxiliary enzyme and sacrificial substrate, by a cascade reaction (for example, in combination with a dehydrogenase), or by chemical methods.

The phosphate-based screening method comprises a generic approach for engineering activity of BVMOs on new substrates. So far, this method has been applied in screening for activity with ketones and sulfides, but it is likely that other substrate classes will be compatible with the screening as well. This is particularly important because setting up a screening method is a time-consuming process. Furthermore, methods based on detection of specific compound often employ surrogate or labelled substrates which can lead to artefacts. The phosphate screening method is also compatible with harsh conditions, for example, the presence of cosolvents. Relatively low activity of BVMOs as well as substrate/product inhibition are serious issues which also could be tackled be screening mutant libraries with the developed assay. Finally, it would be worthwhile to demonstrate the use of the screening assay with other enzymes: BVMOs, FMOs, or cytochrome P450 monooxygenases.
As of now, crystal structures of four Type I BVMOs are available. Mechanistic studies, protein engineering efforts, and biocatalytic exploration of BVMOs have gathered a substantial amount of information on this class of flavoproteins. The generic phosphate-based screening method in combination with available mutagenesis techniques and with the current wealth of information on BVMOs forms a solid platform for engineering BVMOs towards new applications. Increasing the throughput of the phosphate method by applying it for screening colonies on solid medium or by implementing the same principle in a selection assay would further facilitate redesign of BVMOs.

A significant part of the experiments described in this thesis concerned the redesign of BVMOs using rational or semi-random mutagenesis. Of the two approaches, knowledge-guided semi-random mutagenesis allowed the identification of a mutant with expanded substrate scope while the attempts to change substrate or coenzyme specificity by rationally designed mutations resulted in only minor improvements. This indicates that in-depth knowledge of the active-site architecture and the mechanism of the enzyme is required to tailor the enzyme in a rational way and accurately predict effects of mutations. Meanwhile, randomisation of selected positions based on the structural and mechanistic studies increases the chance of finding an improved variant and reduces screening effort required for this as compared to purely random mutagenesis. With biochemical and bioinformatic data on different classes of enzymes accumulating and a growing number of methods for preparing and screening libraries, protein engineering for creating new biocatalysts will become more efficient.

References

