Chapter 8

Summary and outlook

8.1 Introduction

Enzymes are known to perform highly chemo-, regio- and enantioselective reactions and therefore can be applied in the (industrial) preparation of a.o. food and pharmaceutical products. Nowadays, a wide variety of amino acids, carboxylic acids, amines, alcohols and epoxides can be produced using enzymes in biotechnological processes.[1,2]

Enzymes that have received quite some attention in the last few years are oxygenases.[3] Members that belong to this subgroup perform oxidative reactions using molecular oxygen as oxidant and incorporate either one or two oxygen atoms in an organic substrate. Monooxygenases are enzymes that catalyze the insertion of a single oxygen atom from O_2. In order to perform this type of reaction, these enzymes need to activate molecular oxygen to overcome the spin-forbidden reaction of O_2 with the organic substrate. For this, monooxygenases often require (in)organic cofactors.

Monooxygenases can be subdivided based on the type of cofactor that is used, as described in **Chapter 1**. The majority of these enzymes contain either a heme or a flavin as cofactor,[4,5] but also copper-, pterin- and non-heme iron-dependent monooxygenases have been identified.[6-8] Additionally, enzymes have been found that catalyze the insertion of a single oxygen atom without utilizing a cofactor.[9] Monooxygenases are able to catalyze a wide variety of reactions, e.g. hydroxylations, epoxidations, Baeyer-Villiger oxidations, (de)halogenations, heteroatom dealkylations and sulfoxidations (Figure 8.1).

Besides molecular oxygen, monooxygenases require two electrons to reduce their cofactor. In most cases, these electrons are provided by reduced nicotinamide coenzymes, i.e. NADH and NADPH. However, as stoichiometric amounts of these expensive coenzymes are required, the biotechnological application of monooxygenases is limited by the impact of coenzyme costs on the production costs. Various methods have been developed to regenerate reduced nicotinamide coenzymes.[10-13] Of all available methods, the regeneration of NAD(P)H using either isolated enzymes or whole cells is the most appealing, as this process is quite cost-effective, selective and efficient.

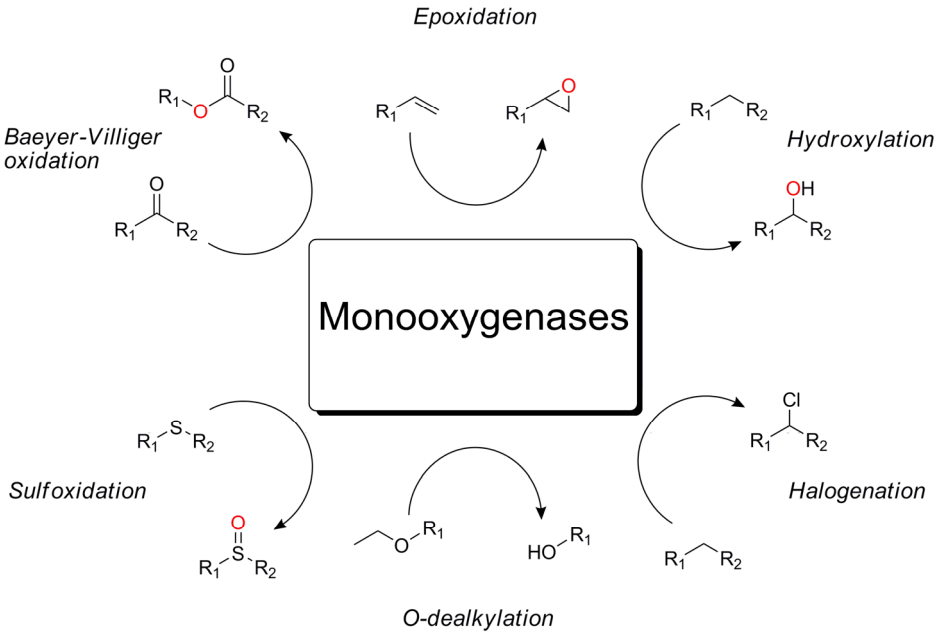

Figure 8.1 Examples of reactions that are catalyzed by monooxygenases.

8.2 Baeyer-Villiger monooxygenases

Baeyer-Villiger monooxygenases (BVMOs) represent valuable oxidative biocatalysts (for a review, see **Chapter 2**). A special feature of these atypical monooxygenases is that they do not only catalyze Baeyer-Villiger oxidations, i.e. the conversion of a ketone to an ester, but also sulfoxidations and a number of other oxidation reactions. Except for this promiscuity in reactivity, BVMOs are often very chemo-, regio- and/or enantioselective while accepting a broad range of substrates. These monooxygenases can be subdivided into at least four subclasses, of which Type I BVMOs are by far the most abundant.[14]

Type I BVMOs consist of a single polypeptide chain, contain FAD as tightly bound cofactor, and utilize NADPH as electron donor. They contain two Rossmann-fold motifs, GxGxxG, which indicates that these enzymes bind NADPH and FAD using separate dinucleotide binding domains. In addition, these monooxygenases contain a BVMO-specific sequence motif (FxGxxxHxxxW$^{D}/_{P}$), which has been used to identify novel Type I BVMOs in (meta)genome databases.[15] So far, all identified

(putative) BVMO genes originate from bacteria or fungi while none have been found in archaea and higher eukaryotes. On average, roughly one out of two microbial genomes contains a BVMO gene. This suggests that at present ~ 400 novel Type I BVMOs genes are present in the genome sequence database. While analyzing the sequence regions flanking a BVMO gene, we found that many of these genes are flanked by an esterase/hydrolase gene. This suggests that BVMOs often play a role in a catabolic pathway, which is in agreement with the fact that most reported BVMOs are involved in such pathways.[16]

Over the past years the list of heterologously expressed Type I BVMOs has increased significantly. Besides for BVMOs acting on small cyclic ketones (cyclopentanone and cyclohexanone),[17-19] variants that are active with larger cyclic ketones (e.g. cyclopentadecanone),[20,21] aromatic ketones (4-hydroxy-acetophenone),[22] aliphatic ketones [23-25] and steroids have been discovered.[26] Substrate profiling studies indicate that these enzymes have a broad substrate range and often display overlapping substrate specificities. The best-studied BVMO is cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO, EC 1.14.13.22).[17] This enzyme has not only been subjected to various substrate profiling studies (over 100 substrates are accepted),[27-31] but also to site-directed mutagenesis and directed evolution studies.[32-34] In addition, pre-steady-state kinetic studies have elucidated the mechanism of action of this BVMO.[35,36] Other well-studied Type I BVMOs include cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO, EC 1.14.13.16), 4-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescence* ACB (HAPMO, EC 1.14.13.81) and phenylacetone monooxygenase from *Thermobifida fusca* (PAMO, EC 1.14.13.92).[18,19,22,37] The latter enzyme was successfully purified and crystallized, resulting in the first and so far only three-dimensional structure of a Type I BVMO.[38] This structure has triggered several structure-inspired enzyme redesign studies on PAMO and sequence-related BVMOs.[39-42]

Racemic bicyclo[3.2.0]hept-2-en-6-one is often used to probe the biocatalytic potential of BVMOs, as both the enantiopure ketones and the corresponding enantiopure lactones are highly interesting for synthetic purposes. As indicated in Figure 8.2, various lactones can be obtained when applying different BVMOs as

biocatalysts.[37,43-45] These lactones can be used as chiral starting materials for preparation of e.g. prostaglandins.

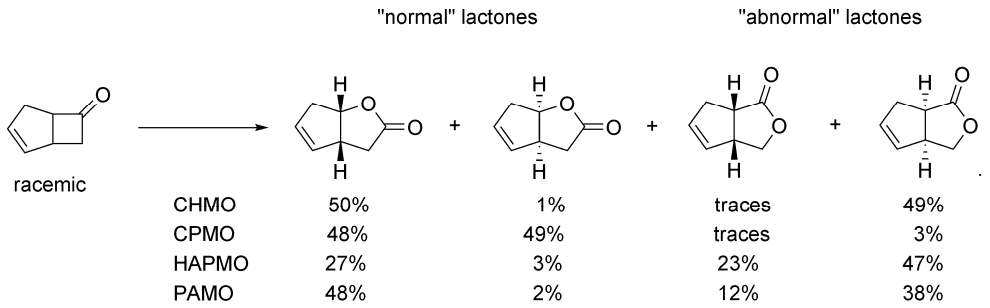

Figure 8.2 BVMO-catalyzed Baeyer-Villiger oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one.

Studies on the applicability of BVMO-mediated catalysis on a large scale have thus far only been performed with CHMO from *Acinetobacter* as model system. The main focus has been on using whole cells expressing CHMO. By this approach the problem of NADPH usage by the enzyme can be circumvented. In combination with resin-based *in situ* substrate feeding and product removal (SFPR) and a tuned oxygen supply, a highly productive process was developed. By this method, one kilogram of bicyclo[3.2.0]hept-2-en-6-one was converted into two nearly enantiopure regioisomeric lactones in a good yield. This example nicely illustrates that BVMOs can be applied on a scale that is relevant for synthesis of fine chemicals.[46]

8.3 Synthesis of optically pure sulfoxides using BVMOs

HAPMO and PAMO are Type I BVMOs that act on aromatic ketones and sulfides, e.g. 4-hydroxyacetophenone and benzyl methyl sulfide. Some enantiopure products that are obtained upon oxidation are known to have various interesting properties for pharmaceutical applications and therefore the activity and selectivity of these enzymes towards such compounds were studied (**Chapter 3**).

HAPMO is able to catalyze the sulfoxidation reaction of a large number of aromatic sulfides. In general, phenyl sulfides seem to be the best substrates for the enzyme, yielding (*S*)-sulfoxides with high enantioselectivities. Low enantiomeric

excesses were obtained with benzyl sulfides, and inversion of enantiopreference from (*S*) to (*R*) was observed for benzyl sulfides with alkyl chains longer than ethyl. Reversal in enantiopreference, with moderate selectivities, was also found when the sulfur atom was located further away from the aromatic ring. With *para*-substituted phenyl methyl sulfides, the enzyme showed high selectivity for substrates bearing electron-donating groups on the aromatic ring, while electron-withdrawing ones had a negative effect on the enantioselectivity. The biocatalyst was not significantly affected in terms of selectivity by changing the position of the chloro-substituent on the aromatic ring. It was also found that HAPMO can oxidize aromatic sulfoxides but with no enantioselectivity. Finally, the enzyme is also able to convert 3-phenyl-penta-2,4-dione with high enantiopreference and to catalyze the oxidation of phenylboronic acid. This shows that HAPMO can be applied for a wide variety of selective oxidation reactions resulting in formation of, for example, optically active sulfoxides or aromatic esters.

By studying the biocatalytic properties of PAMO, we revealed the enzyme's activity with a large range of aromatic ketones, sulfides and sulfoxides. Remarkably, the catalytic activity of the enzyme towards these substrates was rather constant (k_{cat} = 1.2 - 3.6 s^{-1}). On the other hand, more variation was found in the K_M values, suggesting differences in affinity, while the rate of catalysis is probably restricted by a common substrate-independent kinetic step. Interestingly, the enzyme complements HAPMO in terms of enantioselective oxidation of the sulfides tested; PAMO shows good enantioselectivity towards the benzyl sulfides, whereas HAPMO shows to be an excellent biocatalyst for the enantioselective oxidation of phenyl sulfides. The broad substrate range and reactivity makes this biocatalyst a valuable tool for performing selective oxidations at either the β- or γ-position of phenyl compounds, whereas oxidation at the α-position was either rather poor or did not occur at all. The low apparent affinity of PAMO for the tested sulfoxides in comparison with their corresponding sulfides indicates that a substrate with an oxygen-substituted sulfur is poorly accepted by the enzyme. In addition, PAMO has difficulties accepting bulky aromatic ketones.

8.4 Mutagenesis of PAMO for an altered substrate specificity and enantioselectivity

A comparison of the PAMO structure and a homology-built model of CPMO revealed that the active sites are remarkably similar. Indeed, most residues that surround the flavin cofactor and form the active site are identical. Only three residues could be identified as different: residues Q152, L153 and M446 in PAMO align with residues F156, G157 and G453 in CPMO. It was postulated that these 'first shell' residues determine the two widely different substrate specificities of PAMO and CPMO. We replaced these residues in PAMO by the corresponding residues found in CPMO, thereby yielding three PAMO mutants (**Chapter 4**). Surprisingly, two of these mutants were found to be inactive (Q152F/L153G and Q152F/L153G/M446G). Although the bound FAD cofactor in these two mutants was readily reduced by NADPH, they had lost the ability to perform oxygenation reactions. Apparently, the simultaneous mutation of Q152 and L153 resulted in an altered active site in which substrates are not able to be oxidized by the C4a-peroxyflavin.

The third PAMO mutant (mutant M446G) showed several interesting novel catalytic features while retaining the thermostability of the wild type enzyme. Although the mutation appears mild, it has a dramatic effect on the substrate specificity and enantioselectivity. Several new compounds were identified as substrate for this PAMO variant (e.g. indole and benzaldehyde). The mutant was more selective towards substrates containing the carbonyl group or the heteroatom in close proximity to the aromatic ring. A different positioning of the substrate's aromatic ring in the active site might cause this observed shift in regioselectivity. Such an altered substrate binding pocket also explains the substantial changes in enantioselectivity observed towards sulfides and ketones. This confirms the role of M446 in modulating the substrate binding pocket. Surprisingly, the changes in enantioselectivity observed for this mutant are similar to those observed for wild type PAMO in the presence of 30 % methanol (Figure 8.3).[47] The identification of residue M446 to be crucially involved in determining the substrate specificity and enantioselectivity complements the known hotspots that influence BVMO specificity.[40,41]

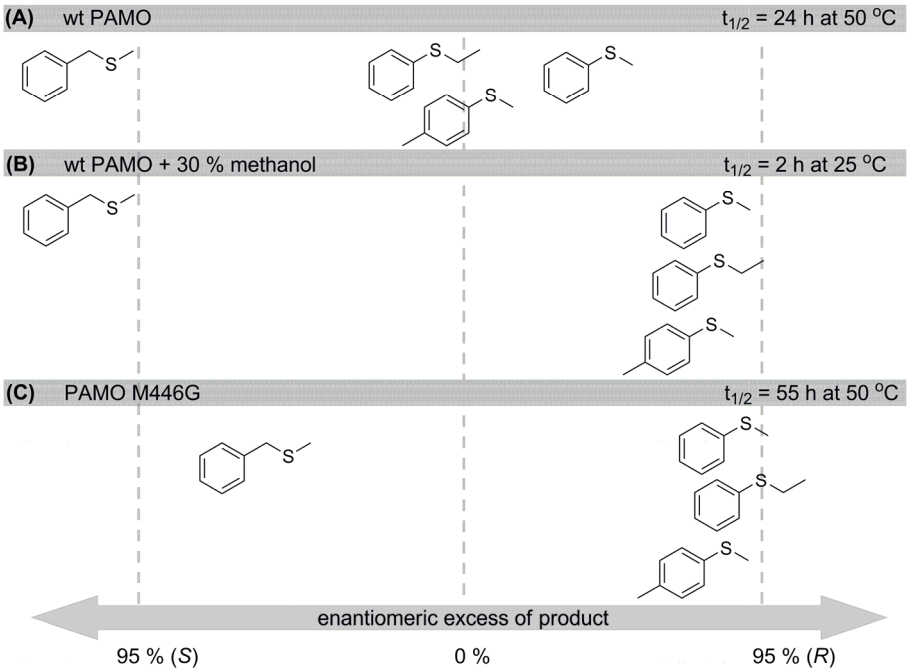

Figure 8.3 Enantioselective sulfoxidations by (A) wild type PAMO, (B) wild type PAMO in the presence of 30 % (v/v) methanol and (C) PAMO M446G.

8.5 Kinetic studies on PAMO

As mentioned above, elucidation of the crystal structure of PAMO has provided valuable structural insight as it represents the only available BVMO structure. This structure shows that a strictly conserved arginine in Type I BVMOs (residue R337 in PAMO) adopts two alternate conformations (Figure 8.4). It is proposed that the arginine shifts conformations upon domain rotation in order to facilitate catalysis.[38] By performing steady-state and pre-steady-state kinetic studies on wild type PAMO and two arginine mutants, R337A and R337K, we have elucidated the kinetic mechanism of this BVMO (**Chapter 5**).

Reductive half-reaction: Our study shows that PAMO binds NADPH with high affinity ($K_{d,\ NADPH}$ = 0.7 μM) after which reduction of the flavin cofactor takes place by transfer of the nicotinamide (*R*)-hydrogen (k_{red} = 12 s^{-1}). This illustrates that binding and correct positioning of NADPH in PAMO is crucial. Replacing the

strictly conserved R337 by an alanine or lysine residue drastically reduces the rate of flavin reduction ($k_{red,\ mutants} \sim 0.1\ s^{-1}$), while the affinity for NADPH is not altered. This indicates that R337 is involved in e.g. proper alignment of the reduced nicotinamide moiety with respect to the isoalloxazine moiety of the flavin cofactor and/or may modulate the redox properties of the flavin cofactor.

Oxidative half-reaction: The stopped-flow experiments also showed that the formation of the C4a-peroxyflavin intermediate upon oxygenation of reduced PAMO is a relatively fast process (k_{ox} = 870 $mM^{-1}.s^{-1}$). The reaction of the peroxyflavin intermediate with phenylacetone is also relatively fast (k_1 = 73 s^{-1}, K_{PA} = 730 µM) while a kinetic event following this oxygenation reaction is relatively slow and limits the rate of catalysis (k_2 = 4.1 s^{-1}). The spectral features of the enzyme intermediate that is formed upon oxygenation of phenylacetone are unusual. The exact nature of this intermediate could not be verified and awaits future study. Two possible scenarios for the observed intermediate are suggested (Figure 5.11); the peroxyflavin intermediate reacts with phenylacetone and yields a Criegee intermediate. Thereafter, rearrangement of this intermediate results in the oxygenated product (benzylacetate), water and oxidized flavin cofactor (*route A*). Alternatively, a hydroxyflavin intermediate is formed and observed, yielding benzylacetate in the first step. Subsequently, this hydroxyflavin intermediate decays and water is formed in the rate-limiting step (*route B*). $NADP^+$ release is relatively fast and represents the final step of the catalytic cycle. In the absence of substrate, the peroxy form of PAMO slowly decays to yield oxidized PAMO and hydrogen peroxide (k_{unc} = 0.01 s^{-1}). Additionally, we identified that the conserved active site arginine, R337, is essential for the catalytic activity of PAMO. This residue is required for not only proper reduction and oxygenation of the flavin cofactor ($k_{red} \sim 0.1\ s^{-1}$ and $k_{ox} \sim 50\ mM^{-1}.s^{-1}$ for both mutants), but also for the reaction of the C4a-peroxyflavin with organic substrates. Surprisingly, the arginine was not required for the stabilization of the formed C4a-peroxyflavin, which was found to have a 10-fold longer life-time than wild type in the absence of a substrate. Replacement of the conserved arginine in HAPMO also resulted in inactivation of the enzyme.[48]

8.6 Bifunctional BVMOs

BVMOs require stoichiometric amounts of NADPH as electron donor for the oxygenation of various organic compounds. However, due to the expensive nature of the reduced nicotinamide coenzyme, it is too costly to apply these enzymes on a large scale. To overcome this problem, several (electro)chemical and photochemical approaches have been explored.[10-12] However, the efficiency of these approaches is typically poor. Furthermore, it has been shown that BVMOs require $NADP^+$ for stability and enantioselective catalysis.[47,49] An efficient and commonly used method for coenzyme regeneration employs whole cells or isolated enzymes.

We have engineered self-sufficient redox biocatalysts by fusing two independent enzymes to form a new bifunctional biocatalyst. These self-sufficient BVMOs are also referred to as CRE/BVMOs (CRE; coenzyme regeneration enzyme). **Chapter 6** demonstrates the feasibility of this novel concept for coenzyme regeneration with three distinct BVMOs and a phosphite dehydrogenase (PTDH, EC 1.20.1.1) from *Pseudomonas stutzeri* WM88 for orthogonal coenzyme recycling.[50,51] As model BVMOs, we selected the thermostable PAMO and the well-studied CHMO and CPMO. The genes of these enzymes were cloned in modified pBAD vectors containing the *ptxD* gene. PAMO was linked to both the N- and C-terminus of PTDH, respectively, whereas CHMO and CPMO were only linked to this dehydrogenase at their N-terminus. All four bifunctional enzymes showed excellent expression levels when *E. coli* TOP10 was used as expression host. The bifunctional enzymes PAMO−CRE, CRE−PAMO and CRE−CHMO were purified by column chromatography to yield 10-50 mg pure and soluble fusion enzymes from 1 L culture broth.

The fusion enzymes were successfully applied for bioconversions using whole cells, cell-free cell extracts and purified enzyme. They are complementary in their substrate profiles (PAMO accepts aromatic ketones, CHMO and CPMO convert aliphatic cycloketones) as well as in their stereoselectivity (CHMO and CPMO display production of antipodal lactones in a large variety of examples). The three monooxygenases are sufficiently different in sequence and phylogenetic relationship to suggest general applicability of this coenzyme regeneration concept

at least among the family of BVMOs. Considering the diverse reactivity of novel members of this family, our work may contribute to the further proliferation of this highly interesting biotransformation platform.

However, the application of the above-mentioned bifunctional BVMOs as biocatalysts was limited by the instability of the PTDH subunit.[52] Therefore, we choose to create a new generation of these self-sufficient fusion BVMOs by replacement of the current PTDH subunit by a thermostable variant of this dehydrogenase (**Chapter 7**).[53] Compared to the first generation of bifunctional BVMOs (CRE/BVMOs), a thermostable PTDH mutant with 16 additional mutations was used, while the fused enzymes also contained an N-terminal histidine tag to facilitate purification of these new bifunctional biocatalysts (CRE2/BVMOs). Additionally, the nucleotide sequence of the mutant *ptxD* gene was codon optimized for better expression in *E. coli*. The stability of these CRE2/BVMOs was indeed shown to be significantly improved. Moreover, due to their increased stability, more efficient bioconversions were achieved with these CRE2/BVMOs. Presently, we are conducting additional studies to further optimize the efficiency of these newly developed self-sufficient BVMOs for ultimate application in large-scale production of chiral intermediates for the synthesis of bioactive compounds. Additionally, we are expanding our biocatalyst library of CRE2/BVMOs by fusing other members of the Type I BVMO family to the thermostable His-tagged PTDH.

8.7 Outlook

The work presented in this thesis concerns a special family of oxidative enzymes; Type I Baeyer-Villiger monooxygenases. These enzymes are considered to be interesting biocatalysts, as they perform oxidative reactions using mild conditions in comparison to the traditional chemical approaches. Until recently, the application of these enzymes in biocatalytic processes was mainly limited due to the poor availability, the instability of known BVMOs, and the fact that these enzymes require stoichiometric amounts of the costly reduced nicotinamide coenzyme NADPH. Additionally, no dedicated enzyme redesign studies could be performed, as no three-dimensional structure of a Type I BVMO was available.

The latter limitation was overcome in 2005, when the X-ray crystal structure of the thermostable phenylacetone monooxygenase from *Thermobifida fusca* was elucidated.[37,38] Although various structure-inspired mutagenesis studies have been performed on this BVMO,[39,52,54] still little is known of the exact mode of substrate binding in PAMO. Active site residues of PAMO that have been subjected to these studies are shown in Figure 8.4. Active PAMO mutants obtained so far, contain only mutations on or near the active site loop, i.e. residues 441 – 446. As described in **Chapter 4**, mutant M446G accepts indole and benzaldehyde as new substrates and shows a higher enantioselectivity towards phenyl sulfides. Other active mutants contain deletions of one or two residues of this active site loop. Best results were obtained with mutant ΔS441/ΔA442, which efficiently converts the bulky substrate 2-phenylcyclohexanone with high enantioselectivity.[39] This indicates that the binding of the organic substrate is

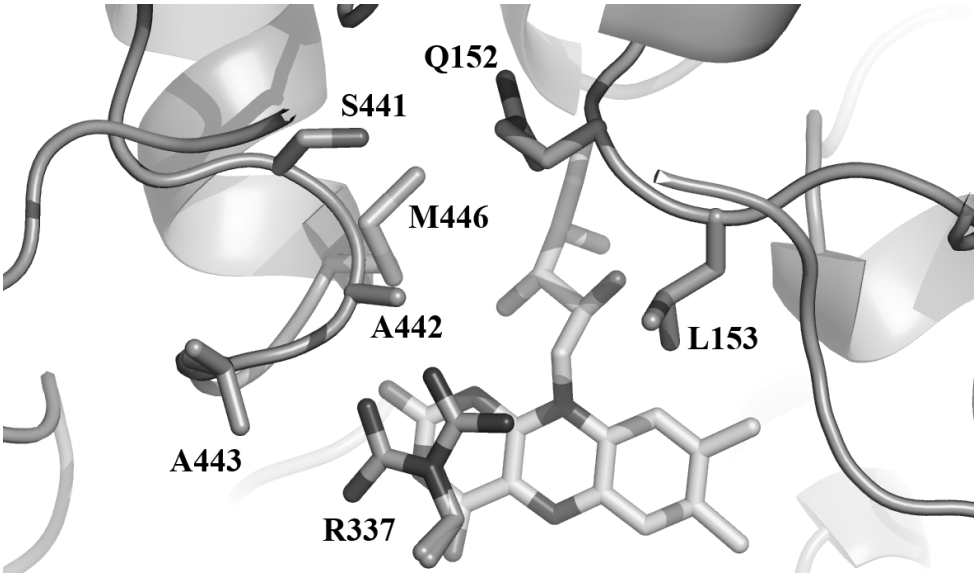

Figure 8.4 Representation of the active site of phenylacetone monooxygenase. Active site residues that have been subjected to the various mutagenesis studies are shown in sticks, while also the FAD cofactor is highlighted. For R337, both side chain orientations that are observed in the crystal structure are shown (PDB: 1W4X). The figure was prepared using the PyMol software (www.pymol.org).

coordinated by these residues. In order to expand the substrate specificity and change the enantioselectivity of PAMO, it will be necessary to understand the role of these and other active site residues. For this, it would be informative to solve the three-dimensional structure of this enzyme with one or more bound ligands. In particular, a structure of PAMO complexed with NADP(H) should shed some light on active site residues that are involved in substrate binding and catalysis. By redesigning the active site, thermostable PAMO mutants with high affinity towards new substrates, e.g. bridged ketones, can be obtained and its enantioselectivity towards these and other substrate can be fine-tuned. Alternatively, a novel structure of a Type I BVMO with a bound ligand should also provide some indication of active site residues that are involved in substrate binding.

By engineering a set of self-sufficient BVMOs, we have shown that BVMOs can be used as efficient biocatalysts that require only catalytic amounts of the expensive coenzyme. Obviously, we would like to expand our library of self-sufficient BVMOs by fusing other members of the Type I BVMO family to phosphite dehydrogenase. Candidate BVMOs with interesting biocatalytic properties are HAPMO,[22] M446G PAMO,[42] ethionamide-activating monooxygenase (EtaA, EC 1.14.13.92) from *Mycobacterium tuberculosis* H37Rv,[55] cyclopentadecanone monooxygenase (CPDMO) from *Pseudomonas* HI-70,[21] methylethylketone monooxygenase (MekA) from *Pseudomonas veronii* MEK700,[56] cyclohexanone monooxygenase ($CHMO_{Xantho}$) from *Xanthobacter* sp. ZL5.[57-59] As PTDH can accept both NAD^+ and $NADP^+$, it can also function as a coenzyme regeneration partner for other NAD(P)H-dependent enzymes (e.g. flavin-dependent monooxygenases, Type II BVMOs, alcohol dehydrogenases). Furthermore, by fusing these NAD(P)H-dependent enzymes to the C-terminus of PTDH containing an N-terminal His-tag, the dehydrogenase can also function as an expression and purification tag for (novel) enzymes. Analysis of the genomes of *Rhodococcus* RHA1,[60] *Thermobifida fusca* [61] and *Nocardia farcinica* IFM 10152 [62] has shown that these microorganisms contain a wide variety of putative $NAD(P)^+$-dependent enzymes and are excellent targets for exploration. Future studies on the engineered CRE2/BVMOs will reveal whether these bifunctional enzymes can be effectively applied in biocatalytic processes on a large scale, like for instance the synthesis of (-)-modafinil (a psychostimulant drug) by the thermostable CRE2−PAMO.[63]

Overall, the data reported in this thesis shows that Type I BVMOs form a family of oxidative enzymes with interesting biocatalytic properties. While still many challenges lie ahead, e.g. enzyme redesign, these enzymes show to be promising oxidative biocatalysts that can be applied in future biotechnological processes.

8.8 References

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