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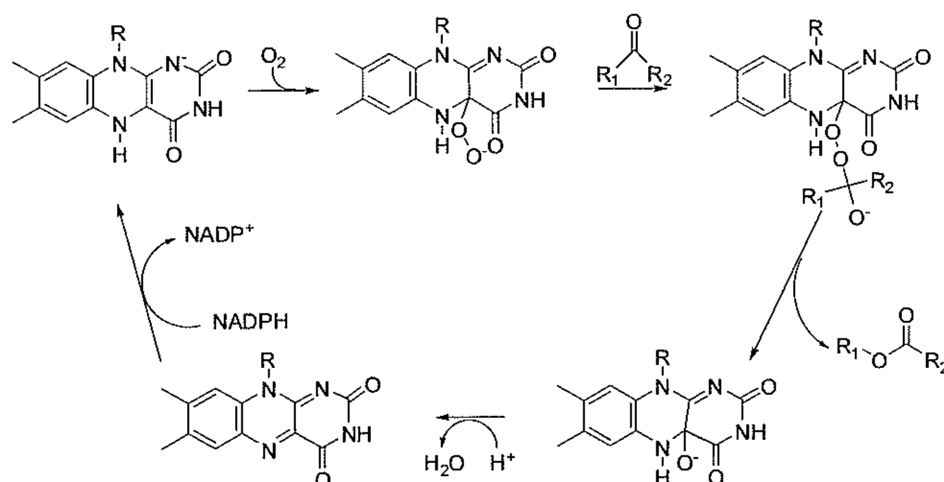
The Development of Monooxygenases into Biocatalysts for Selective Partial Oxidations in Organic Synthesis

Introduction

Biocatalysis is more and more recognized by synthetic chemists as a valuable tool for the synthesis of complex organic compounds with high specificity and under environmentally benign conditions.[1, 2] In many cases biocatalysis can complement partial weaknesses of methods from classic organic synthesis. In particular, biocatalytic redox reactions seldom possess a chemical counterpart showing comparable stereo- and regiospecificities together with the virtual absence of competing side reactions and high turnover frequencies of up to $10^3 s^{-1}$.[3] This extraordinary potential of redox biocatalysts however is not reflected by the current number of synthetic applications using redox active enzymes.[4] This apparent discrepancy derives from a variety of different factors, as there are the often low stability of the enzymes under operational conditions, their dependency on redox cofactors (for example nicotinamides, NAD(P)H and $NAD(P)^+$), and the still limited availability of redox enzymes showing the desired specificities to perfom a particular reaction.

In order to overcome these limitations, two different classes of monooxygenases, a currently underdeveloped family of redox enzymes, were studied within the framework of this thesis. Baeyer-Villiger Monooxygenases (BVMOs) were subjected to enzyme engineering by directed evolution and rational design techniques and subsequently used for synthetic applications. In the second project line, novel Cytochrome P450s were cloned from a number of bacterial strains, expressed in *E. coli*, purified and characterized in terms of their biocatalytic potential.

In Baeyer-Villiger monooxygenases (BVMOs) dioxygen from air reacts with the enzyme bound reduced flavin cofactor (FAD) to form a reactive intermediate flavin peroxide species which in its deprotonated form initiates the Baeyer-Villiger reaction by transferring one oxygen atom to the ketone substrate while ultimately reducing the other one to water. Finally, the flavin cofactor is regenerated to its reduced state by NADPH. In its protonated form the flavin peroxide species can carry out heteroatom oxidations such as sulfoxidations in a similar fashion.[5-7]



Scheme 1: Simplified reaction mechanism of a Baeyer-Villiger Monooxygenase.

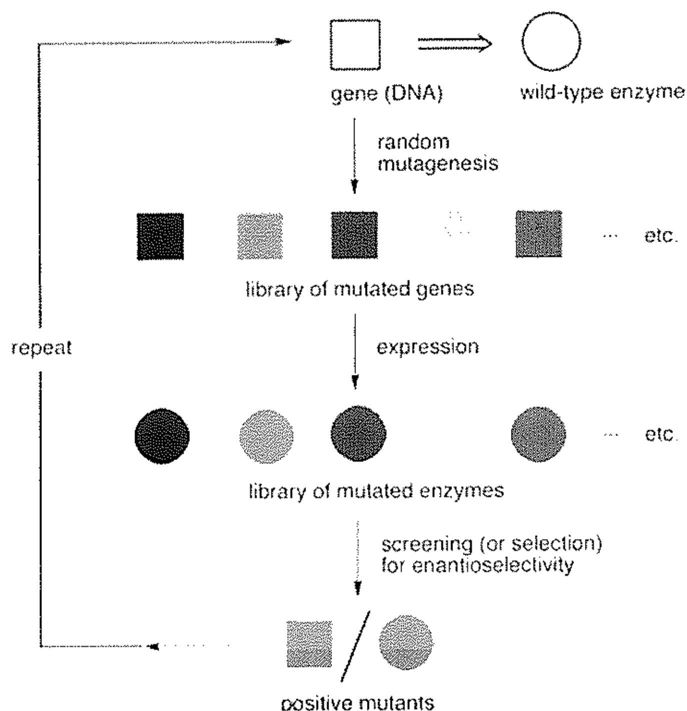
Cytochrome P450s became most known for their efficiency in hydroxylation of unactivated alkanes as only few oxygenases possess the requisite "active oxygen" state. With equal efficiency, P450s can carry out a wide variety of about twenty different biotransformations.[8-10] The catalytically active species is an iron heme complex, which catalyzes the reductive activation of molecular oxygen to carry out the reactions. The mechanism of P450 is a complex cascade of individual steps involving the interaction of protein redox partners and consumption of reducing equivalents, most commonly in the form of NAD(P)H.[10] Usually P450 require the presence of coupled enzymes (P450 reductases plus electron shuttle enzymes) which transfer the reducing equivalents from the reduced nicotinamides to the iron heme center. This hinders the applicability of most P450, since these multicomponent enzyme cascade reactions slow down the turnover frequencies and render the heterologous expression of the biocatalyst difficult. However, a special subclass of P450, the most prominent member being P450 BM-3 from *Bacillus megaterium*, has its redox partner protein directly fused to its heme domain, which enables high turnover frequencies together with a highly simplified expression. The drawback of P450 BM-3 lies in its narrow substrate scope which in order to be broadened requires intensive enzyme engineering efforts. This work focused on finding new homologous P450 BM-3 like enzymes with new and interesting substrate specificities.

Both classes of enzymes, Baeyer-Villiger monooxygenases and Cyctochrome P450, have in common, that they show a great potential for synthetic use, since for the reaction scope of both classes, the equivalent methods in synthetic chemistry are

weak. But at the same time, both classes of enzymes possess challenges to be met for research and development.

Engineering of Baeyer-Villiger Monooxygenases

The milestone was to engineer the enantioselectivity of a BVMO by means of directed evolution.[11, 12] For our study we chose the desymmetrization of 4-hydroxycyclohexanone as the model reaction and Cyclohexanone Monooxygenase (CHMO) from *Acinetobacter calcaoaceticus* NCIMB 9871 (EC 1.14.13.22) as the "Baeyer–Villigerase". CHMO catalyzes the oxidation of a range of different ketones, often with high enantioselectivity, but inevitably many non natural substrates fail to react with sufficiently high *ee*. Since no structural information was available, we chose error prone PCR as mutagenesis method for the creation of enzyme libraries. Over two rounds of directed evolution we achieved to increase the enantioselectivity of CHMO in the model reaction from 9% *ee* (*R*) to 90% *ee* (*R*). In addition, other mutants of CHMO yielded the opposite enantiomer of the product lactone.[13] These results represent the first successful engineering of a BMO.



Scheme 2: Schematic description of a directed evolution process. Starting from the gene which encodes a wild-type enzyme, a library of mutated genes is generated and the corresponding enzymes are screened or selected for improved properties. The best enzyme mutant resulting from this screening is used as template for a second round of mutagenesis and screening, which exerts evolutionary pressure on the biological system and leads over several cycles to significantly improved enzymes.

Further characterization of several hit mutants revealed that the increase of selectivity was not restricted to the model substrate, but that these mutated CHMOs could catalyze the oxidation of a variety of structurally complex ketones with high enantioselectivity and again in many of these cases, depending on the mutant used, both enantiomers of the product could be isolated.[14] In addition, a number of mutants catalyzed the oxidation of substrates which were rejected by the wild-type and yielded products with a high degree of enantioselectivity. Overall a hot spot in the sequence of CHMO was identified, which enabled the control of enantioselectivity of CHMO and contributes to the substrate scope of the enzyme, not only for a variety of ketones but also for sulfoxidation reactions.[14, 15]

To provide a means for the rationalization of these results, a project was started in collaboration with the protein crystallography group of Prof. Dr. I. Schlichting to purify and crystallize CHMO and its mutants. During the course of this project, a high level expression system of CHMO was established and a procedure for the purification and *in vitro* stabilization of this highly sensitive enzyme was developed and handed over to the crystallographers.

Recently the first crystal structure of a BVMO was published. The enzyme of choice was Phenylacetone Monooxygenase (PAMO).[16] PAMO represented the first described thermostable BVMO and was therefore promising for biocatalytic applications, especially since the applicability of established enzymes such as CHMO was hampered by their low stability.[17] However, the drawback of PAMO was its comparably narrow substrate scope and its limited enantioselectivtiy in Baeyer-Villiger oxidations.[18]

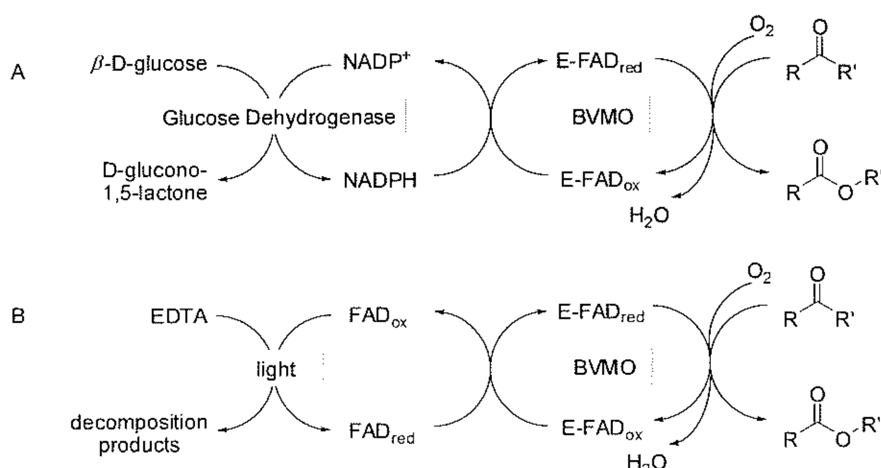
We used the structure of PAMO to build a homology model of CHMO and in this model we located the hot spot that controls the enantioselectivity of CHMO and contributes to the substrate scope. The information gained in this theoretical work was used to devise an approach for the rational design of PAMO to increase its substrate scope and enantioselectivity.[19] The catalytic profile of PAMO was shifted close to CHMO, without loss of thermostability. This provided us with a potentially practical biocatalyst for enantioselective synthesis.

The Application of mutated Enzymes for *in vitro* Biocatalysis

To evaluate the synthetic applicability of the new PAMO mutants, a setup for preparative scale biocatalytic Baeyer-Villiger oxidations was developed. By means of buffer additives we could protect PAMO and its mutants from deactivation by organic

solvents, which enabled the use of a second liquid phase in *in vitro* experiments. This way, the usual limitations of aqueous phase biocatalysis such as low substrate solubility and substrate or product inhibition of the enzyme could be circumvented. A protocol for gram scale biocatalytic Baeyer-Villiger oxidations with high turnover numbers for the enzyme (TN > 30,000) in combination with high substrate concentrations (20 g/l) was developed.[20] Regeneration of the NADPH cofactor was in this case achieved by standard methodology using a coupled enzyme.

In a following project, a new scheme for a much more simplified regeneration of the enzyme was developed to bypass the need for the highly expensive NADPH or a complex NADPH regeneration system. In detail, light was used as the driving force for the BVMO catalyzed reaction. Hereby, free FAD, which was added in catalytic amounts, was photochemically activated for its reduction by simple electron donors such as EDTA. The resulting reduced free FAD replaced the usual NADPH and brought about the reduction of its enzyme bound counterpart, which initiated the Baeyer-Villiger oxidation of the substrates.[21]



Scheme 3: Comparison of conventional regeneration of a Baeyer-Villiger monooxygenase (BVMO, e.g., PAMO) for catalysis via enzymatic regeneration of NADPH (A) and the novel simplified light driven pathway via a flavin (B).[21] A shows an enzyme coupled regeneration system employing the common glucose dehydrogenase to catalyze the regeneration of the reduced nicotinamide cofactor. The use of this coupled enzyme can be replaced by visible light, which reduces the complexity of the setup by eliminating the need for a coupled enzyme. E-FAD = enzyme bound FAD.

This light driven system was found to not only work for the regeneration of Baeyer-Villiger Monooxygenases, but also for other classes of flavin dependent redox enzymes.[22] It therefore represents a tool of broad applicability in biocatalytic redox reactions.

Cloning, Expression and Characterization of Novel Cytochrome P450

Since only a small number of P450 BM-3 like enzymes were known, altogether with a limited substrate scope,[23] a project was initiated aiming on the one hand at the finding of novel BM-3 homologs and on the other hand at the directed evolution of BM-3 itself.

P450 BM-3 was cloned from *B. megaterium* and a new expression system in *E. coli* was set up for this enzyme, which yielded more than 80 mg of purified enzyme per liter of culture. To enable a directed evolution of the substrate scope and regioselectivity of this enzyme in collaboration with an industrial partner, a high-troughput screening system was developed.

In another project, novel BM-3 homologs were identified by genome mining in several microbes. Using a small number of actinomycetes as sources for the genes, we cloned and expressed these enzymes in *E. coli*. Purification of the enzymes and ensuing characterization revealed considerably different substrate scopes of these enzymes compared to BM-3, while maintaining other properties such as the high turnover frequencies.[24]

The P450 project line was completed by the genetic screening of microbial libraries for the presence of BM-3 homologs. A number of strains were found to possess such enzymes. Cloning and characterization of them is currently under study.

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