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Mutability-landscape guided enzyme engineering

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SUMMARY AND FUTURE PERSPECTIVES

EXPLOITING ENZYME PROMISCUITY FOR DEVELOPING NEW ENZYMATIC ACTIVITIES

Enzymes are a sustainable and efficient alternative to traditional catalysts used in synthetic organic chemistry. Therefore, their use as biocatalysts in the production of valuable chemicals and pharmaceuticals has become increasingly popular over the past decades. However, some of the reactions which are widely used in organic chemistry have not been observed in biological systems, and therefore no enzymes are known which naturally catalyze these reactions. To provide a biocatalytic alternative for these reactions, the development of enzymes with new activities is required. A promising source of new transformations is the catalytic promiscuity of existing enzymes. Once such a promiscuous enzyme activity has been identified, protein engineering can be used to enhance the activity and/or selectivity of the enzyme, leading to new biocatalysts with unnatural enzymatic activities. In the protein engineering field, there is a trend towards generating large amounts of mutational data to guide the engineering efforts. Mutational data which includes beneficial, neutral and detrimental mutational effects can be used to chart a mutability landscape of an enzyme. Such a mutability landscape gives detailed insight into sequence-function relationships, and can be used to guide protein engineering to improve various enzymatic properties, including promiscuous activities. Chapter 1 provides an overview of recent studies in which mutability landscapes of enzymes have been generated and used for engineering the enzyme's catalytic performance.

MUTABILITY-LANDSCAPE GUIDED ENGINEERING OF 4-OT'S 'MICHAELASE' ACTIVITY

4-Oxalocrotonate tautomerase (4-OT) is an enzyme with several peculiar features. First of all, it is one of the smallest enzymes known. Although it functions as a homohexamer, its monomer size is only 62 amino acid residues. Secondly, 4-OT has a characteristic N-terminal proline, which functions as a catalytic base (pK_a ~6.4) in the tautomerization reaction naturally catalyzed by 4-OT. Since proline and its derivatives are widely used as organocatalysts for carbon-carbon bond-forming reactions, Poelarends and coworkers anticipated and discovered promiscuous catalysis of carbon-carbon bond-forming Michael-type addition and aldol reactions by 4-OT. The aim of the work described in this thesis was to improve these promiscuous carbon-carbon bond-forming activities of 4-OT.

4-OT's promiscuous Michael-type addition activity is of particular interest since enzymes that naturally catalyze such a reaction are extremely rare. Furthermore, the 4-OT-catalyzed Michael-type additions of acetaldehyde to nitroalkenes yield enantioenriched γ-nitroaldehydes, which are valuable building blocks for the production of gamma-aminobutyric acid (GABA) derivatives, which represent an important class of pharmaceuticals acting on the central nervous system. To improve 4-OT's promiscuous Michael-type addition activity and to gain insight into

the functionally important residues, we generated mutability landscapes of 4-OT for its soluble expression and natural tautomerase and promiscuous Michael-type addition activities (Chapter 2). For this, we constructed a defined collection of genes, which covered nearly all possible single mutants of 4-OT, and used this set of mutants to determine the effect of each individual single amino acid substitution on soluble expression and the above mentioned activities. Based on the mutability landscape of 4-OT's Michael-type addition activity, using acetaldehyde and *trans*-β-nitrostyrene as substrates, we identified a single mutant (A33D) with a significantly enhanced activity in this reaction. Further characterization of this mutant revealed that besides its improved activity, it also had an improved enantioselectivity compared to that of wild-type 4-OT. Mutant A33D can catalyze the addition of acetaldehyde to a range of aromatic and aliphatic nitroalkenes, producing chiral γ-nitroaldehydes with enantiomeric ratios (e.r.) in the range of 95:5 to >99:1. Although these e.r. values are good to excellent, the γ-nitroaldehyde enantiomers produced are precursors for the biologically inactive enantiomers of the GABA derivatives. Hence, the production of the desired enantiomers of the γ-nitroaldehydes would require the inversion of 4-OT's enantioselectivity. Therefore, we have generated a mutability landscape of 4-OT for its enantioselectivity in the Michael-type addition of butanal to *trans*-β-nitrostyrene. From this landscape, we identified several single mutations which led to the inversion of enantioselectivity. By making combinations of these single mutations, we designed the double mutant M45Y/F50A which had good enantioselectivity in the acetaldehyde addition to *trans*-β-nitrostyrene (product e.r. = 96:4), producing the opposite product enantiomer compared to wild-type 4-OT and mutant A33D. M45Y/F50A did not only have the desired enantioselectivity, it also had an enhanced catalytic rate compared to wild-type 4-OT. Substrate scope analysis revealed that M45Y/F50A could accept the same range of aromatic and aliphatic nitroalkenes as A33D and consistently produced the pharmaceutically relevant enantiomer of the resulting γ-nitroaldehydes. To determine the structural effects of these mutations, we determined the crystal structures of unliganded M45Y/F50A as well as M45Y/F50A in complex with *trans*-β-nitrostyrene. This revealed the opening of a hydrophobic pocket in the active site of 4-OT, which was able to bind the phenyl group of *trans*-β-nitrostyrene. It is likely that this active-site remodeling is responsible for the observed switch in enantioselectivity and enhanced activity.

IMPROVING 4-OT'S PROMISCUOUS ALDOLASE ACTIVITY

The other promiscuous carbon-carbon bond-forming activity of 4-OT is the aldolase activity. To enhance this activity, we first systematically scanned the whole amino acid sequence of 4-OT to identify residue positions at which mutations lead to an improved aldolase activity for the cross-condensation of acetaldehyde with benzaldehyde (Chapter 3). We identified three 'hotspot' positions (His-6, Met-45 and Phe-50), which are all in close proximity to the active site, indicating that single mutations closer to the active site improve 4-OT's promiscuous aldolase activity more effectively than

distant ones. The most active single mutant was F50V, which displayed a 636-fold improved catalytic efficiency (k_{cat}/K_m) for this cross-condensation reaction. To further improve 4-OT's aldolase activity, combinatorial mutagenesis was performed on these 'hotspot' positions. This resulted in the identification of a double mutant (M45T/F50A) and a triple mutant (H6F/M45T/F50A), which displayed a 3300-fold and a 5300-fold improvement in catalytic efficiency, respectively. To obtain further insight in the catalytic performance of these mutants, we tested their ability to process 3-hydroxy-3-phenyl-pronanal, which is the aldol intermediate that is formed during the cross-condensation of acetaldehyde with benzaldehyde. Interestingly, both wild-type 4-OT and mutant F50V mainly catalyzed the dehydration of this aldol compound to yield cinnamaldehyde, whereas mutants M45T/F50A and H6F/M45T/F50A mainly catalyzed a retro-aldol reaction yielding acetaldehyde and benzaldehyde. This might be an important discovery in view of our long-term goal to design 4-OT variants which are highly active aldolases but that lack dehydration activity.

To investigate whether we could improve 4-OT's promiscuous aldolase activity for the self-condensation of aliphatic aldehydes, we systematically scanned the whole amino acid sequence of 4-OT to identify residue positions at which mutations lead to an improved aldolase activity for the self-condensation of propanal to yield 2-methyl-2-pentenal (**Chapter 4**). Interestingly, His-6, Met-45 and Phe-50 were again identified as 'hotspot' positions. Simultaneous randomization of these 'hotspot' positions and subsequent screening of the resulting library, led to the identification of a mutant with a strongly enhanced activity for this self-condensation reaction. This 4-OT mutant (M45Y/F50V) catalyzes the self-condensation of acetaldehyde, propanal or butanal with a strongly enhanced activity when compared with that of wild-type 4-OT or mutant F50A, which is a previously constructed 4-OT mutant with a highly improved aldolase activity for cross-condensations. Interestingly, when M45Y/F50V was incubated with two different aldehydes, such as propanal and benzaldehyde, we mainly observed the self-condensation product of propanal (2-methyl-2-pentenal). In contrast, wild-type 4-OT and mutant F50A mainly produced the cross-aldol coupling product (α-methylcinnamaldehyde) under identical experimental conditions. This indicates that M45Y/F50A has an altered substrate specificity compared to wild-type 4-OT and mutant F50A and prefers the self-condensation over the cross-coupling of aldehydes. Taken together, the results presented in **Chapters 3** and **4** of this thesis indicate that 4-OT can be tailored to catalyze specific aldol reactions (either cross- or self-condensations of aldehydes) by mutagenesis of positions Met-45 and Phe-50.

FUTURE PERSPECTIVES

As mentioned above, the promiscuous 'Michaelase' activity of 4-OT is of particular interest since enzymes which naturally catalyze carbon-carbon bond-forming Michael-type additions are extremely rare. In the work described in this thesis, we have exploited this promiscuous C-C bond-forming activity of 4-OT to develop two enantiocomplementary 'Michaelases'. One of these 'Michaelases' (mutant M45Y/

F50A) can be used as a catalyst for the production of the desired enantiomers of γ-nitroaldehydes, which are important precursors for pharmaceutically active GABA derivatives. Although the acetaldehyde additions to nitroolefins catalyzed by M45Y/F50A afford various enantioenriched γ-nitroaldehydes with e.r. values ranging from 62:38 to 97:3, this enantioselectivity requires further improvement to develop an efficient biocatalytic process. Improvement of the enantioselectivity of the M45Y/F50A-catalyzed Michael-type additions would especially be attractive for those reactions which lead to pharmaceutically relevant γ-nitroaldehydes such as 5-methyl-3-(nitromethyl)hexanal, which is a precursor for the marketed GABA derivative Pregabalin.

One possible approach to improve the enantioselectivity of the M45Y/F50A catalyzed Michael-type additions is by (co-)solvent engineering. Currently, aqueous reaction media consisting of water/ethanol or water/DMSO mixtures are used in preparative-scale reactions using engineered 4-OT enzymes. These solvent systems have, however, been optimized for reactions with wild-type 4-OT and the effect of different solvent systems on the activity and enantioselectivity of M45Y/F50A has not been investigated yet. Additionally, it would be highly desirable to replace DMSO with a more environmentally friendly (co-)solvent. Also, the removal of DMSO from an aqueous medium is laborious. Optimization of the (co-)solvent system for M45Y/F50A-catalyzed Michael-type additions would require systematic experimentation on more environmentally friendly (co-)solvents such as co-solvents with a low boiling point, ionic or deep-eutectic liquids, or co-solvents which can be easily removed by liquid/liquid extraction. For this (co-)solvent optimization, the solubility of the starting material (nitroalkene) should also be taking into account. The low solubility of the nitroalkenes in aqueous solutions prevents high substrate loadings and is therefore a limiting factor for up-scaling. Alternatively, the use of whole cell biocatalysts or biphasic systems can be explored to improve the performance of the M45Y/F50A-catalyzed Michael-type additions.

Another approach to improve the enantioselectivity of the M45Y/F50A-catalyzed Michael-type reactions is to optimize the biocatalyst by protein engineering. This can be done by using mutagenesis of residues lining the active site of M45Y/F50A. For this, the crystal structure of M45Y/F50A in complex with *trans*-β-nitrostyrene (**Chapter 2**) can be used to select residue positions at which mutations are likely to improve the enantioselectivity of 4-OT. Alternatively, target positions for mutagenesis can be identified based on the mutability landscape of 4-OT for enantioselectivity in the addition of butanal to *trans*-β-nitrostyrene. For example, several mutations in the C-terminal 'GIGGEL'-motif lead to improved enantioselectivity towards the pharmaceutically relevant enantiomers of γ-nitroaldehydes. Targeting these residues for mutagenesis with the M45Y/F50A mutations in the background might yield new 4-OT variants with the desired improvement in enantioselectivity. During these enzyme engineering projects, it would be attractive to simultaneously screen for activity, since improvement of both activity and enantioselectivity is highly desirable.

In the end, a combination of co-solvent engineering and enzyme engineering may be required to generate an efficient 4-OT-based biocatalytic process for the production of enantiopure γ-nitroaldehydes.

Besides activity and (enantio-)selectivity, enzyme stability is another important feature which needs to be addressed to develop an efficient 4-OT-based biocatalytic process. Although 4-OT is a rather stable enzyme, we have observed loss of enzyme activity in the presence of high concentrations of aldehyde substrates and co-solvents (e.g. ethanol). To address these issues, it would be highly interesting to chart mutability landscapes of 4-OT for chemo- and solvent-stability. These landscapes might not only guide the engineering of more stable 4-OT mutants but may also yield valuable information on residues important for substrate and solvent resistance.

In addition to engineered 4-OT variants with enhanced promiscuous 'Michaelase' or aldolase activities, the work described in this thesis provided important knowledge on how to efficiently generate protein mutability landscapes, providing a large amount of mutational data as well as valuable insights in structure-function relationships. In collaboration with the SME Bio-Prodict B.V., we aim to use the mutational data and machine learning algorithms to generate mutation prediction models for 4-OT as well as closely related tautomerase superfamily members. Ultimately, we aim to design fully automated modules for the prediction of mutations that improve activity and enantioselectivity.