# Mutability-landscape guided enzyme engineering

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# MUTABILITY-LANDSCAPE GUIDED ENZYME ENGINEERING

## Improving the promiscuous C-C bond-forming activities of 4-oxalocrotonate tautomerase

Jan Ytzen van der Meer

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# AIM AND OUTLINE

## AIM AND OUTLINE OF THIS THESIS

Enzymes are Nature's catalysts which facilitate the biosynthesis of the vast number of intricate molecules found in the biotic world. To do so, enzymes need to be highly active and selective for the chemical transformations they catalyze. Indeed, millions of years of evolution has provided us with a seemingly inexhaustible collection of highly active and selective enzymes. Applying these wonderful natural catalysts in organic synthesis is called biocatalysis, which can be an efficient and sustainable alternative to the use of traditional catalysts in synthetic chemistry. However, enzymes have not evolved to be used as biocatalysts. Therefore, enzyme engineering is applied to tailor the properties of an enzyme, before it can be used as a suitable biocatalyst. Moreover, some of the reactions that are widely used in synthetic chemistry have not been observed in Nature. To provide a biocatalytic alternative for those reactions, the generation of enzymes with new and unnatural activities is needed. One way of generating these new enzymatic activities is by exploiting existing enzymes that exhibit catalytic promiscuity. Catalytic promiscuity is the ability of an enzyme to catalyze one or more chemical transformations different from its biologically relevant one. These promiscuous activities, which have been suggested to play an important role in the natural evolution of new enzymatic functions, can be used as a starting point to generate biocatalysts for those chemical reactions that do not have a biological counterpart.

A prime example of an enzyme with catalytic promiscuity is 4-oxalocrotonate tautomerase (4-OT). Owing to its catalytic N-terminal proline, which resembles proline used in organocatalysis, several promiscuous carbon-carbon bond-forming activities have been anticipated and found. The aim of the work described in this thesis was to improve these promiscuous carbon-carbon bond-forming activities of 4-OT. Because of the strong advantage of working with an enzyme with a small monomer size (homohexameric 4-OT only has 62 residues per monomer), we used a systematic mutagenesis approach to map the neutral, beneficial and detrimental effects of nearly all possible single amino acid substitutions on soluble expression, catalytic activities and enantioselectivity of 4-OT. These maps, which represent 4-OT's mutability landscapes, highlight the functionally important regions, as well as mutational robustness and evolvability of 4-OT. We used these mutability landscapes to guide the engineering of 4-OT, with the aim to improve its promiscuous carbon-carbon bond-forming activities.

In Chapter 1, the available studies on mutability landscapes of enzymes are reviewed. Mutability landscapes can be generated either by using a deep-mutational scanning approach or by analyzing a defined collection of single mutants of an enzyme. Both these methods are discussed in this chapter with a specific focus on the usefulness of mutability landscapes for enzyme engineering.

In Chapter 2, we describe the generation of mutability landscapes for the soluble expression, tautomerase and Michael-type addition activities, and enantioselectivity of 4-OT. The promiscuous, carbon-carbon bond-forming Michael-type addition

activity of 4-OT is of particular interest, because Michael-type addition reactions are widely used in synthetic chemistry and enzyme-catalyzed carbon-carbon bond-forming Michael-type additions are rare. Moreover, the 4-OT-catalyzed Michael-type additions can be exploited to produce chiral γ-nitroaldehydes. These compounds are valuable precursors for γ-aminobutyric acid analogues (GABAs), which are important pharmaceuticals acting on the central nervous system. Therefore, it would be very attractive to enhance this promiscuous "Michaelase" activity of 4-OT and to improve and invert its enantioselectivity. Guided by the mutability landscapes of 4-OT, we generated a 4-OT variant (H6M/A33E/F50V) with a strongly enhanced "Michaelase" activity. Moreover, we obtained a single mutant (A33D) with improved enantioselectivity and a double mutant (M45Y/F50A) with an inverted enantioselectivity. This set of enantiocomplementary ''Michaelases'' was used in asymmetric addition of acetaldehyde to various nitroalkenes, providing convenient access to both enantiomers of γ-nitroaldehydes.

In **Chapter 3**, we describe the systematic scanning of the whole 4-OT protein to identify residue positions at which mutations lead to an improved aldolase activity for the cross-condensation of acetaldehyde with benzaldehyde. Interestingly, all identified ''hotspots'' (His-6, Met-45 and Phe-50) were in close proximity to the active site, providing strong support for the notion that for evolving new catalytic activities in a promiscuous enzyme, mutations closer to the active site improve the enzyme more effectively than distant ones. By performing combinatorial mutagenesis on these "hotspots", we obtained two 4-OT mutants with significantly enhanced cross-condensation activities and decreased tautomerase activities, leading to a remarkable $>10^7$-fold change in reaction specificity.

In **Chapter 4**, we report our efforts to enhance 4-OT's promiscuous aldolase activity for the self-condensation of aliphatic aldehydes. First, we applied the systematic mutagenesis approach to identify residue positions in 4-OT at which mutations lead to improved aldolase activity for the self-condensation of propanal. Interestingly, His-6, Met-45 and Phe-50 were again identified as "hotspot" positions. Simultaneous diversification of these positions and subsequent screening of the resulting libraries, led to the identification of 4-OT variant M45Y/F50V. Although this enzyme was engineered for improved aldolase activity for the self-condensation of propanal, it also had improved activity for the self-condensation of acetaldehyde and butanal. Interestingly, the M45Y/F50V mutant enzyme is highly selective for performing self-condensations, rather than cross-condensations, when it is incubated with two different aldehydes.

In **Chapter 5**, we provide a summary of the work described in this thesis and some future perspectives of this work.