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Aldolase Activities and Enzyme Redesign

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PhD thesis
University of Groningen, The Netherlands

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CONTENTS

Aim and outline of this thesis

Enzymes reduce the timescales of the chemical reactions that drive biological processes from millions of years to fractions of seconds. Traditionally, textbooks have highlighted the exquisite specificity of enzymes for the reactions they have evolved to catalyze, and have largely ignored the promiscuous behavior of enzymes. However, recent years have produced ever-increasing evidence that most enzymes are in fact highly nonspecific, not just processing different substrates (*i.e.*, substrate promiscuity) but in many cases even catalyzing chemically distinct reactions (*i.e.*, catalytic promiscuity). Enzyme promiscuity has been suggested to be crucial to the evolution of new protein functions, and interest in this area has exploded, both due to a desire to understand natural enzyme evolution, but also because catalytically promiscuous enzymes provide a highly promising starting point for laboratory evolution of new biocatalysts.

Enzymes that can efficiently catalyze carbon-carbon (C-C) bond-forming reactions are of great interest for synthetic applications. Earlier studies on enzyme promiscuity for C-C bond-forming reactions have mainly focused on hydrolytic enzymes. In the work described in this thesis, we have focused our attention on the enzyme 4-oxalocrotonate tautomerase (4-OT), which naturally catalyzes an enol-keto tautomerization reaction as part of a catabolic pathway for aromatic hydrocarbons, but is also able to promiscuously catalyze synthetically useful C-C bond-forming reactions.

In **Chapter 1**, we highlight recent advances in enzyme promiscuously for a number of important C-C bond-forming reactions with a focus on enzymes that possess high enantioselectivities.

It has previously been reported that 4-OT can promiscously catalyze the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde, and the Michael-type addition of acetaldehyde to a wide variety of nitroolefins to yield valuable γ-nitroaldehydes. To gain insight into how 4-OT catalyzes these unnatural reactions, we performed H-D exchange and crystallographic studies in the presence of acetaldehyde. In **Chapter 2**, we report that H-D exchange within acetaldehyde is 4-OT-catalyzed and that the active site Pro-1 residue is crucial for this activity. X-ray crystallography studies confirmed that Pro-1 of 4-OT reacts with acetaldehyde to give an enamine species. A reaction between this enamine intermediate and an electrophilic substrate such as benzaldehyde or trans-β-nitrostyrene results in carbon-carbon bond formation.

In **Chapter 3**, we describe that 4-OT promiscuously catalyzes different types of aldol reactions, including the self-condensation of propanal, the cross-coupling of propanal and benzaldehyde, the cross-coupling of propanal and pyruvate, the intramolecular cyclization of hexanedial, and the intramolecular cyclization of heptanedial.

It has been suggested that enzyme promiscuity is related to the natural evolvability of proteins. Similarly, a promiscuous enzyme can be used as a starting point for laboratory evolution, using (semi)-rational or directed evolution approaches, to develop a novel

enzyme with a new function. We therefore performed mutagenesis studies with the aim to improve the promiscuous aldolase activity of 4-OT.

In **Chapter 4**, we report the use of a systematic mutagenesis strategy to identify 'hotspot' positions at which mutations give a strong improvement in 4-OT's activity for the aldol cross-condensation of acetaldehyde with benzaldehyde. By exploring focused libraries in which only 'hotspot' positions are varied, a 4-OT variant with a $>$5000-fold improvement in catalytic efficiency for this aldol-condensation reaction was obtained. This large increase in promiscuous aldolase activity is accompanied by a large decrease in natural tautomerase activity, which results in a $>10^7$-fold change in reaction specificity, indicating a strong negative tradeoff between evolving and existing activity.

In **Chapter 5**, we describe the use of the same engineering strategy to improve the promiscuous activity of 4-OT for the aldol self-condensation of propanal. This led to the discovery of a 4-OT variant with enhanced activity in the self-condensation of linear aldehydes such as acetaldehyde, propanal and butanal. Notably, in the presence of both propanal and benzaldehyde, this 4-OT variant (unlike wild-type 4-OT and the previously constructed mutant F50A) mainly catalyzes the self-condensation of propanal rather than the cross-condensation of propanal and benzaldehyde, indicating that it has an altered reaction specificity.

In **Chapter 6**, we summarize the work described in this thesis and provide some suggestions for future research.