Catalytic promiscuity of a proline-based tautomerase

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CHAPTER 6

SUMMARY AND FUTURE PERSPECTIVES

6.1 Exploring enzyme promiscuity for carbon-carbon bond formation

In many biochemistry textbooks enzymes are described to be highly specific, both in the reaction that they catalyze and in their choice of substrates. However, recent years have produced ever-increasing evidence that most enzymes are in fact highly non-specific, not just processing different substrates but in many cases even catalyzing reactions other than their biologically relevant one. This latter phenomenon is defined as catalytic promiscuity. Promiscuous enzyme activities are usually low-level relative to the main activity and are under neutral selection. Although these secondary activities are physiologically irrelevant, under new selective pressures they may confer a fitness benefit (to the organism), thereby prompting the evolution of the promiscuous activity to become the new primary activity. As such, enzyme promiscuity is thought to be crucial to the natural evolution of new protein functions. In view of that, catalytically promiscuous enzymes may provide a promising starting point for laboratory evolution of new biocatalysts.

The construction of carbon-carbon bonds stands out in organic synthesis as an indispensable step for building up new molecules. However, reported examples of enzymes that catalyze important carbon-carbon bond-forming reactions such as Michael(-type) additions, Henry reactions, Mannich reactions, or Knoevenagel condensations as their natural activity are rare, while aldolases, which catalyze aldol reactions as their natural activity, often exhibit limited substrate acceptance. In recent years, several promising enzymes have been described that are able to promiscuously catalyze these important C-C bond-forming reactions, showing good to excellent enantioselectivity (reviewed in **Chapter 1**). The discovery of these promiscuous enzyme activities is an important step

on the way to develop efficient biocatalysts for synthetically useful C-C bond-forming reactions.

6.2 Formation of a covalent enamine species between Pro-1 of 4-OT and aldehyde substrates

The enzyme 4-oxalocrotonate tautomerase (4-OT) is a member of the tautomerase superfamily, a group of homologous proteins having a β-α-β structural fold and a unique catalytic amino-terminal proline (Pro-1) in common. 4-OT takes part in a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate into 2-oxohexa-3-enedioate. In this tautomerization reaction, Pro-1 acts as a general base (pK_a of Pro-1 $\sim$6.4) abstracting the 2-hydroxyl proton of 2-hydroxyhexa-2,4-dienedioate and transferring it to the C5-position to give 2-oxohexa-3-enedioate.

4-OT has attracted our attention because it exhibits promiscuous carbon-carbon bond-forming activities, including the Michael-type addition of linear aliphatic aldehydes to a wide variety of aliphatic and aromatic nitroolefins to yield valuable γ-nitroaldehydes and the cross-aldolization of acetaldehyde and benzaldehyde to give cinnamaldehyde. To gain insight into how 4-OT catalyzes these unnatural reactions, we performed exchange studies in D_2O and X-ray crystallography studies **(Chapter 2)**. The former studies showed that H-D exchange within acetaldehyde is 4-OT-catalyzed and that the Pro-1 residue is crucial for this activity. The structural studies showed that Pro-1 of 4-OT had reacted with acetaldehyde to give an enamine species. These results provide evidence for a mechanism of the 4-OT-catalyzed aldol and Michael-type addition reactions in which acetaldehyde is activated for nucleophilic addition via Pro-1 dependent formation of an enamine intermediate. A reaction between this nucleophilic intermediate and an electrophilic substrate such as benzaldehyde or *trans*-β-nitrostyrene results in carbon-carbon bond formation.

To obtain further mechanistic insight into the promiscuous carbon-carbon bond-forming activities of 4-OT, it will be important to determine crystal structures of 4-OT in complex with other donor (*i.e.* aliphatic aldehydes) or acceptor (*e.g.* benzaldehyde or nitroolefins) substrates, or products (*e.g.* cinnamaldehyde or γ-nitroaldehydes). Such crystallographic results would also provide an important guide for future engineering experiments, aiming to enhance 4-OT's activity and selectivity in carbon-carbon bond-forming reactions.

6.3 Different types of aldol reactions promiscuously catalyzed by 4-OT

As we described earlier, 4-OT catalyzes the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde. In the work described in **Chapter 3**, we set out to investigate the substrate scope of 4-OT in both inter- and intramolecular aldol reactions. We demonstrated 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. Mutation of the catalytic amino-terminal proline (P1A) greatly reduced 4-OT's aldolase activities, whereas mutation of another active site residue (F50A) strongly enhanced 4-OT's aldolase activities, providing convincing evidence that aldolization is an active site process. Further systematic screening of 4-OT and closely related tautomerase superfamily members, in which an active-site proline is present as nucleophile, may prove to be a useful approach to discover completely new promiscuous aldolase activities. These non-natural activities could be exploited as starting point to create novel aldolases for synthetically useful self- and cross-coupling reactions.

6.4 Enhancement of the promiscuous aldolase activity of 4-OT by enzyme engineering

After we established that 4-OT is able to catalyze various types of aldol reactions, we focused on enhancing these promiscuous activities by enzyme engineering. As described in **Chapter 4**, we have used a systematic mutagenesis strategy to identify three 'hotspots' positions (His-6, Met-45 and Phe-50) in 4-OT at which single mutations give a marked improvement in aldolase activity for the condensation of acetaldehyde with benzaldehyde. Notably, all beneficial mutations were found to be near the active site of 4-OT, providing support for the notion that for new catalytic activities in a promiscuous enzyme, mutations closer to the active site improve the enzyme more effectively than distant ones. Activity screening of a focused library in which the three 'hotspots' positions were randomized simultaneously, led to the discovery of a 4-OT variant (H6F/M45T/F50A) with a >5000-fold improvement in catalytic efficiency for the aldol condensation of acetaldehyde with benzaldehyde. The large increase in promiscuous aldolase activity for this mutant is accompanied by a large decrease in natural tautomerase activity, resulting in a $> 10^7$-fold change in reaction specificity, indicating a strong negative tradeoff between evolving and existing activity.

We also demonstrated that the promiscuous enzyme 4-OT can be engineered into a more efficient aldolase (variant M45Y/F50V) for self-condensations of small aliphatic aldehydes (*i.e.*, acetaldehyde, propanal and butanal) by exploring small libraries in which only two identified 'hotspots' positions (Met-45 and Phe-50) were varied (**Chapter**

5). Indeed, the same residue positions were identified as 'hotspots' for improving 4-OT's aldolase activity for the condensation of acetaldehyde with benzaldehyde, but combinatorial mutagenesis of these positions followed by activity screening yielded a different 4-OT variant (M45T/F50A; see **Chapter 4**) for this cross-condensation. Hence, 4-OT can be tailored to process different carbonyl substrates and catalyze a specific aldol reaction. In future work, it will be highly interesting to test whether variant M45Y/F50V (or perhaps a further evolved derivative), is able to catalyze aldolizations using formaldehyde or substituted aldehydes such as glycolaldehyde and chloroacetaldehyde as substrates, potentially affording new aldol compounds (*e.g.* aldose carbohydrates).

6.5 Concluding remarks and future challenges

The major part of the work described in this thesis focused on the discovery and engineering of promiscuous aldolase activities of 4-OT. For most of the 4-OT catalyzed aldol reactions, it was shown that both the aldol coupling and dehydration steps are enzyme catalyzed, yielding non-chiral products. In future work, it will therefore be important to engineer 4-OT variants that only catalyze the formation of the aldol compound without facilitating its dehydration. For instance, the development of 4-OT variants that are able to catalyze the cross-coupling of acetaldehyde and chloroacetaldehyde or the intramolecular cyclization of hexanedial or heptanedial, without being able to catalyze the dehydration of the corresponding aldol products, would be highly interesting because these aldol compounds are valuable building blocks for pharmaceutical synthesis.

An example of a 4-OT-catalyzed aldol reaction in which the aldol product does not undergo enzymatic or chemical dehydration, is the cross-coupling of propanal and pyruvate to yield the chiral compound 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (see **Chapter 3**). To the best of our knowledge this compound has not been reported in the literature before, illustrating that enzyme promiscuity has indeed great promise as a source of synthetically useful catalytic transformations. In future work, it would be interesting to establish the absolute configuration of this enzymatic product and determine the enantiomeric excess. If the 4-OT catalyzed aldol reaction turns out to yield highly enantioenriched 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid, it will be of interest to enhance the activity of 4-OT for this reaction by using the engineering procedure described in **Chapters 4 and 5**. If successful, it may lead to a biocatalytic process for the synthesis of this novel compound.

Finally, it would be interesting to expand the reaction scope of 4-OT with regard to carbon-carbon bond formation. So far the enzyme has been shown to promiscuously catalyze various Michael-type additions and different types of aldol reactions (see **Chapter 1**). It would be fascinating to investigate the possibilities of 4-OT catalyzed carbon-carbon bond-forming Mannich reactions. In a Mannich reaction an amine reacts with a carbonyl compound to form an electrophilic imine, which is subsequently attacked by a carbon nucleophile, generating a new carbon-carbon bond. The Mannich reaction is a popular reaction for the synthesis of many pharmaceuticals because of the ubiquitous

presence of nitrogen in these compounds. Our initial experiments showed that the main challenge in studying 4-OT catalyzed Mannich reactions is the generation of soluble and stable imines in aqueous buffers. Our mechanistic and structural studies confirmed the formation of an enamine species between the Pro-1 residue of 4-OT and acetaldehyde. Thus, chemical or enzymatic formation of imines that are stable in aqueous solutions (either pre-formed or *in situ*), and potent to react with the enamine species generated in 4-OT's active site, may lead to new biocatalytic Mannich reactions that are synthetically useful.