Exploiting Catalytic Promiscuity for Biocatalysis

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7 Summary and future perspectives

Switch on the "dark side" of an enzyme

As described in many biochemistry textbooks, enzymes are highly specific catalysts, both for the reactions that they catalyze and the substrates that they transform. However, many enzymes have been shown to catalyze reactions or act on substrates other than the ones they have evolved for. This phenomenon is defined as enzyme promiscuity. For a long time, enzyme promiscuity has been considered as the "dark side" of an enzyme since secondary activities may lead to the formation of undesired by-products. Nowadays, enzyme promiscuity has become an important source of new enzyme activities for challenging and synthetically useful chemical transformations. Many enzymes have attracted considerable attention for their unexpected promiscuous activities. For instance, trypsin, which is a classical example of a highly specific enzyme as it cleaves only peptide bonds on the carboxyl side of lysine and arginine residues, has been used as a promiscuous biocatalyst for carbon-carbon (C-C) bond-forming reactions. Enzyme promiscuity has greatly contributed to enlarging the enzyme toolbox for practical applications. Switching on the "dark side" of promiscuous enzymes by protein engineering, using either rational or random mutagenesis procedures, has become an applied strategy for the development of new biocatalysts.

Recently, many enzyme promiscuity-based biocatalytic procedures have been reported for prominent types of C-C bond-forming reactions such as the aldol coupling, Michael(-type) addition, Mannich, Henry, and Knoevenagel condensation reactions. Although the majority of promiscuous enzymes exhibit limited substrate scope and poor enantioselectivity, in the last five years a few promising enzymes have been described that show excellent enantioselectivity in promiscuously catalyzing C-C bond-forming reactions. These promiscuous enzymes can be used as starting templates for developing efficient biocatalysts for the synthesis of valuable chiral products (**Chapter 1**).

An N-terminal proline residue: the beauty of 4-oxalocrotonate tautomerase (4-OT)

Most of the enzyme promiscuity-based biocatalytic procedures were established by screening a collection of commercially available enzymes for the desired activities; however, the non-natural C-C bond coupling activity of 4-oxalocrotonate tautomerase (4-OT) was discovered on the basis of an envisioned catalytic mechanism. 4-OT is a member of the tautomerase superfamily and is part of a catabolic pathway for aromatic compounds in *Pseudomonas putida* mt-2, in which it catalyzes the conversion of 2-hydroxymuconate to 2-oxo-3-hexenedioate (Figure 1A). Homohexameric 4-OT is a small enzyme (62 residues per monomer) and is characterized by a catalytic N-terminal proline (Pro-1) embedded in the active site.

Inspired by the success of proline and its derivatives as organocatalysts in asymmetric enamine catalysis, we were motivated to investigate whether the Pro-1 residue of 4-OT, which has the correct protonation state ($pK_a \sim 6.4$) to be able to act as a nucleophile at neutral pH, can form enamines with carbonyl compounds. The enzymatically generated enamine then

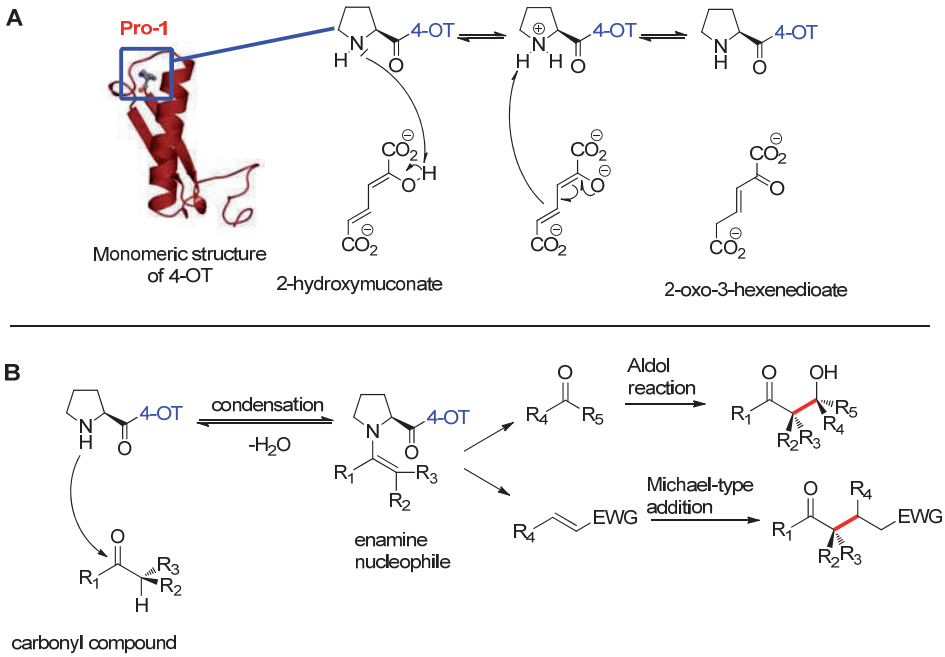

Figure 1. The natural tautomerization reaction (A) and proposed C-C bond-forming reactions (B) catalyzed by 4-OT. For clarity, the β-α-β building block carrying the N-terminal proline is shown (rather than the functional homohexameric structure). EWG, electron withdrawing group.

could undergo a range of C-C bond formation reactions by using different electrophiles (Figure 1B). $NaCNBH_3$ trapping and mass spectrometry experiments demonstrated that the Pro-1 residue of 4-OT indeed can react with aldehydes and ketones to give reactive enamines. After screening a number of potential electrophiles, we have found that 4-OT catalyzes C-C bond-forming reactions including the Michael-type addition of acetaldehyde to *trans*-nitrostyrene and the aldol condensation between acetaldehyde and benzaldehyde.

4-OT is the new "Michaelase" from the tautomerase superfamily

Encouraged by the discovery of the 4-OT catalyzed Michael-type addition of acetaldehyde to *trans*-nitrostyrene, we focused on the substrate scope of 4-OT in this synthetically useful reaction. First, we investigated the enamine donor scope and found that linear aldehydes ranging from acetaldehyde to octanal are accepted by 4-OT as donor substrates for the addition to *trans*-nitrostyrene. In all relevant cases, the *syn*-isomer was obtained as the major diastereomer, which is in agreement with the transition state model proposed for asymmetric Michael-type addition of aldehydes to nitroolefins. As a general trend, we observed that increasing the chain length of the linear aldehyde donor slows down the reaction rate and reduces the enantioselectivity of 4-OT (**Chapter 2**). Next, we investigated the enamine acceptor (i.e. electrophilic substrate) scope by examining a series of aromatic

and aliphatic nitroolefins. Surprisingly, a wide range of nitroolefins are taken by 4-OT as Michael acceptors in reactions with acetaldehyde to give γ-nitroaldehydes with high yields (up to 74%) and good to excellent enantiopurities (up to 98% *ee*). These γ-nitroaldehydes are valuable precursors of several marketed GABA-based pharmaceuticals such as phenibut (tranquilizer), baclofen (anti-alcoholic), and pregabalin (anticonvulsant) (**Chapters 3** & **4**). Hence, we have developed a unique biocatalytic approach for the Michael-type addition of aldehydes to nitroolefins in aqueous solvent systems. The reactions are promiscuously catalyzed by a tautomerase, include a wide range of donor and acceptor substrates, and proceed with high stereoselectivity. To further improve the 4-OT methodology for synthesizing useful γ-nitroaldehydes, we performed substrate engineering by using different *ortho*-, *meta*-, and *para*-substituted nitrostyrene derivatives in the asymmetric Michael-type addition of acetaldehyde. Intriguingly, all examined nitrostyrene derivatives are accepted by 4-OT as substrates for reactions with acetaldehyde to form highly enantioenriched (*R*)- or (*S*)-γ-nitroaldehydes (up to 97 and 96% *ee*, respectively) with good to excellent yields (up to 96%). The presence of electron donating and electron withdrawing groups on the aromatic ring of the substrate seems to strongly affect the rates of the 4-OT-catalyzed reactions. In general, electron donating substituents such as methoxyl and hydroxyl groups on the aromatic ring of the substrate increase the catalytic rate and thus lower the reaction time, whereas electron withdrawing substituents have the opposite effect. The enantioselectivity was found to be influenced by the position of the substitutions. Interestingly, substituents at the *ortho*-position of the nitrostyrene derivatives invert the enantioselectivity of the 4-OT-catalyzed reaction, yielding enantioenriched (*R*)-γ-nitroaldehydes instead of the corresponding (*S*)-enantiomers that are obtained with *para*- and *meta*-substituted β-nitrostyrene derivatives, which is probably caused by a stereo-facial shielding effect (**Chapter 5**).

Another showcase of enzyme promiscuity: the aldolase activity of 4-OT

The aldol condensation between acetaldehyde and benzaldehyde yielding cinnamaldehyde is another 4-OT-catalyzed C-C bond-forming reaction that we have discovered previously. We have recently confirmed that both the aldol coupling and the subsequent dehydration step are catalyzed by 4-OT. This low-level aldolase activity of 4-OT can be enhanced 600-fold in terms of catalytic efficiency (k_{cat}/K_m) by one single mutation (F50A). As the aldol reaction is one of the most widely used reactions in organic chemistry for C-C bond formation, we set out to investigate the substrate scope of the 4-OT-catalyzed aldol reactions. We found that 4-OT catalyzes several types of aldol reactions including the self-condensation of propanal, cross-coupling of propanal and benzaldehyde, cross-coupling of propanal and pyruvate, as well as intramolecular cyclization of hexanedial and heptanedial. In all cases, the mutant F50A showed improved aldolase activity when compared to that of wild-type 4-OT. Additionally, we also observed H-D exchange of the acidic protons of the examined aldehyde and ketone substrates, which is catalyzed by 4-OT and critically depends on its Pro-1 residue. This H-D exchange activity indicates that the active site of 4-OT can

deprotonate carbonyl compounds, thereby providing further evidence for a mechanism for the 4-OT-catalyzed aldol and Michael-type reactions in which the aldehyde substrate is activated for nucleophilic addition via Pro-1 dependent formation of an enamine (or enolate) intermediate (**Chapter 6**).

Concluding remarks and future challenges

In summary, the results included in this thesis demonstrate a new biocatalytic methodology for C-C bond formation in aqueous media using the enzyme 4-OT, which displays high stereoselectivity and a broad substrate scope. This enzymatic methodology has potential to be implemented into industrial applications for the following reasons: 1) biocatalytic and enantioselective C-C bond-forming Michael(-type) addition reactions are extremely rare; 2) enzymes that can perform both an aldol addition and the subsequent dehydration are not abundant; and 3) the γ-nitroaldehyde products from the 4-OT-catalyzed Michael-type additions are valuable precursors of several marketed GABA-based pharmaceuticals, including pregabalin, the active ingredient of Lyrica (global sales: 4.6 billion USD in 2013). Nevertheless, there are several challenges that need to be considered in order to develop 4-OT into an industrially applicable biocatalyst. Firstly, the catalytic rates of 4-OT for both the aldol and Michael-type addition reactions are still lower (k_{cat} << 1 s^{-1}) than the standard for industrial biocatalysts, which is not cost effective for industrial operations. Enhancing these non-natural activities of 4-OT by protein engineering is therefore absolutely necessary. Random mutagenesis tools, such as error-prone PCR, or the construction of entire protein mutability landscapes can be used to identify functional hotspots in 4-OT. Site-saturation mutagenesis or combinatorial libraries based on identified hotspots can then be constructed and screened to yield 4-OT variants with improved catalytic activity. Also, structure-based mutagenesis can be applied if crystal structures of 4-OT in complex with any of these unnatural substrates become available in the future. Secondly, many of the GABA-based pharmaceuticals are chiral γ-amino acids and the biological activity of these molecules is often dependent on the configuration of their chiral centers. Unfortunately, the γ-nitroaldehydes that we produce using wild-type 4-OT do not have the correct stereochemistry to be converted into the pharmaceutically active GABA-analogous. Thus, in order to synthesize these valuable pharmaceutical precursors, we need to invert the stereoselectivity of 4-OT to obtain the desired enantiomers. Enzyme engineering would be a good option to achieve this, as many successful examples of altering the stereoselectivity of enzymes by directed evolution techniques have been reported in literature. Thirdly, to simplify the procedures and improve the efficiency of the downstream processing, it is crucial to optimize the co-solvent and pH conditions of the reaction media. Currently, DMSO (up to 50%, v/v) is used as a co-solvent in several 4-OT catalyzed Michael-type addition reactions, which enables the use of higher concentrations of the starting materials (i.e., the nitroalkenes) in aqueous media. Since DMSO is relatively expensive and the removal of this high-boiling point solvent (bp: 189 °C) from aqueous media is laborious, finding more environmentally and economically friendly (low-boiling) co-solvents should be addressed

in the further development of the 4-OT methodology for practical applications. If needed, enzyme engineering, enzyme immobilization and/or the use of biphasic systems are good options to improve the stability and compatibility of 4-OT for newly selected co-solvents. If the catalytic rate, enantioselectivity and operational stability of 4-OT can be successfully improved in the future to meet the industrial requirements for large-scale application, we will not only have a practical biocatalyst for synthetically useful C-C bond formation reactions, but also have gained valuable knowledge about how to identify and enhance promiscuous activities of enzymes, which can be used in developing new biocatalysts.