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Catalytic Promiscuity and the Emergence of New Enzymes

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Background: turqoise blue crackle glaze by the Dutch ceramic artist Bert Nienhuis, dated 1921, from the collection of the author of this thesis.
Graphic: 3D crystal structure of RhCC (see chapter 4).

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“Nature does not hurry, yet everything is accomplished”
Lao Tzu

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Table of Contents

Aim and outline of this thesis

Unraveling the underlying principles that govern the evolution of enzymatic activities in nature has evoked great scientific interest during the last few decades. The concept has been postulated that catalytic promiscuity might play a central role in the divergent evolution of enzyme function. Identification and characterization of shared promiscuous activities of enzymes belonging to the same superfamily may provide insight into this hypothesis.

Catalytic promiscuity, i.e. the ability of an enzyme to catalyze different chemical transformations, is a property found in most members of the tautomerase superfamily that have currently been studied. Tautomerase superfamily members are characterized by a catalytic amino-terminal proline and a structural β-α-β-fold. The identification of new superfamily members that are closely or more distantly related to well-studied members based on sequence similarity, and characterization of the structural and catalytic properties displayed by these newly identified homologues, may provide valuable insights into the possible role that catalytic promiscuity plays in the divergent evolution of enzymatic activities within the tautomerase superfamily.

The aim of the work described in this thesis was to explore the catalytic diversity within the tautomerase superfamily. Characterization of the catalytic abilities of both known and newly identified superfamily members, and detailed analysis of the structural features of these enzymes and the mechanistic aspects of their catalytic activities, has provided insights into the chemical versatility displayed by members of the tautomerase superfamily, as well as the important role of catalytic promiscuity in the divergent evolution of function within this superfamily.

Recently identified examples of catalytic promiscuity in the tautomerase superfamily are reviewed in **Chapter 1**. The role which catalytic promiscuity might play in the divergent evolution of enzyme function is discussed, centered on the notion that a catalytically promiscuous enzyme might serve as a starting template for both natural and laboratory evolution of new enzymatic functions.

The enigmatic existence of dehalogenases (CaaD and *cis*-CaaD, respectively) acting on *trans*- and *cis*-3-chloroacrylic acid, the persistent products of a man-made nematocide introduced into the environment in 1946, raises the intriguing question how nature has evolved these enzymes in a short timeframe. The low-level promiscuous phenylenolpyruvate tautomerase (PPT) activity of *cis*-CaaD, which may be a vestige of the function of its progenitor, prompted us to clone and characterize the more distantly related homologue MsCCH2 (annotated as a putative tautomerase) from *Mycobacterium smegmatis* strain MC2 155, which is described in **Chapter 2**. MsCCH2 was found to be a robust PPT that displays low-level promiscuous *cis*-CaaD as well as CaaD activity. Based on its properties, MsCCH2 could be characteristic of an evolutionary intermediate along the past route for the divergence of *cis*-CaaD from an unknown superfamily tautomerase. A likely scenario for the evolution of *cis*-CaaD is postulated in which catalytic promiscuity plays a central role. Hence, it is likely that a

tautomerase enzyme with promiscuous low-level *cis*-CaaD activity has been recruited as a starting template for the evolution of a full-fletched *cis*-CaaD enzyme.

A fascinating example of catalytic promiscuity was discovered in mouse macrophage migration inhibitory factor (MIF), which is described in **Chapter 3**. MIF is a member of the tautomerase superfamily and functions as a cytokine. Interestingly, this mammalian cytokine also displays PPT activity. Reasoning that the bacterial dehalogenases CaaD and *cis*-CaaD display promiscuous PPT activity, we investigated whether mouse MIF exhibits promiscuous dehalogenase activity. Indeed, MIF was found to act as a dehalogenase towards *trans*-3-chloroacrylic acid. Intriguingly, the low-level dehalogenase activity of the wild type enzyme could be enhanced 200-fold by introducing just two mutations (I64V/V106L) in the active site pocket. This clearly demonstrates that the evolution of dehalogenase activity towards a xenobiotic compound can be surprisingly facile, which supports the notion that catalytic promiscuity might play a central role in the divergent evolution of function within the tautomerase superfamily.

A striking example of the chemical versatility of the β-α-β-fold is described in **Chapter 4**. The enzyme RhCC from *Rhodococcus jostii* RHA1, which belongs to the *cis*-CaaD family of the tautomerase superfamily, was found to catalyze a direct reaction between molecular oxygen (dioxygen, O_2) and 4-hydroxyphenylenolpyruvate without the use of a redox-cofactor. Hence, RhCC was identified as a rare example of a cofactor-independent oxygenase. The cofactor-independent activation of molecular oxygen has attracted much scientific interest in recent years; however, the mechanistic details are still under investigation. The identification of RhCC provides an opportunity to study this intriguing process within the unusual context of the tautomerase superfamily.

The study of catalytic promiscuity has provided a wealth of mechanistic insights into the catalytic abilities of various members of the tautomerase superfamily. A key issue being the different catalytic roles which an active site residue can fulfill. These insights gathered for 4-oxalocrotonate tautomerase (4-OT) has allowed us to predict a new promiscuous activity of this enzyme based on the properties of its active site residues. In **Chapter 5**, the identification by mechanistic reasoning of a promiscuous aldolase activity of 4-OT (the aldol-condensation of acetaldehyde and benzaldehyde yielding cinnamaldehyde, in which Pro-1 functions as a nucleophile rather than a base), and the characterization thereof are described.

The role of Pro-1 as a key catalytic residue in 4-OT has previously been investigated by the characterization of a limited number of Pro-1 variants of 4-OT, such as P1A and P1G. The post-translational removal of the translation-initiating methionine is a prerequisite in the study of Pro-1 variants of 4-OT. In *E. coli*, the enzyme methionine aminopeptidase (MetAP) is responsible for this crucial step; however, it only accepts peptide substrates of which the residue following the N-terminal methionine is small and uncharged. Hence, 4-OT variants with proline or alanine at the penultimate position (the first position after the initiating methionine) are accepted as substrates by MetAP, but variants of 4-OT with a bulky or charged residue at the penultimate position will not be processed, resulting in proteins with an N-terminal methionine instead of

the desired N-terminal residue. Therefore, the possibility to produce demethionylated Pro-1 variants of 4-OT (P1S, P1H and P1Q) by co-expression with an engineered methionine aminopeptidase (MetAP-*TG) with an expanded substrate scope was investigated, which is described in **Chapter 6**. The degree of demethionylation was established for these variants, and their activity towards 2-hydroxymuconate, the natural substrate of 4-OT, was determined. In addition, the promiscuous oxaloacetate decarboxylase activity of the P1S variant was characterized.

Finally, in **Chapter 7** a summary of the work described in this thesis is provided, as well as suggestions for future research.