Artificial Metalloenzymes

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7 Conclusions and Perspectives

This chapter summarizes the key findings of the research presented in this thesis, followed by future perspectives for the field of artificial metalloenzymes.

7.1 Introduction

Hybrid catalysts comprising a transition metal (complex) in combination with a bio-scaffold, such as a protein, are regarded as artificial metalloenzymes. Over the last few decades artificial metalloenzymes have evolved from a curiosity to a field that is slowly taking on challenges that cannot be accomplished with traditional homogenous catalysis or biocatalysis alone.[1-3] The concept of artificial metalloenzymes uses the broad reaction scope of traditional homogenous catalysis and places it in an environment exploited by natural enzymes, *i.e.* the second coordination sphere, to generate novel and versatile catalysts. However, over the years only a limited number of bioscaffolds have been successfully applied in artificial metalloenzymes. This limited choice of bioscaffolds slows down progress in the field of artificial metalloenzymes. The aim of the research presented in this thesis was to take up this challenge and introduce new design concepts for the construction of new artificial metalloenzymes exploiting dimer interfaces.

The main achievements described in this thesis are; i) the development and construction of an artificial metalloenzyme based on covalently grafting of a Cu(II) complex at the dimer interface of the protein LmrR and its successful application in the catalytic enantioselective Diels-Alder reaction, ii) successful application of these novel artificial metalloenzymes in the asymmetric catalytic hydration of α,β-unsaturated carbonyl compounds, iii) residues important for catalysis in the scaffold of the artificial metalloenzyme LmrR were identified, and iv) the development and construction of an artificial metalloenzyme by supramolecular anchoring of a Cu(II) complex on the dimer interface of LmrR and successful application in the enantioselective catalytic Friedel-Crafts alkylation reaction.

Based on these accomplishments it can be concluded that a new design concept for artificial metalloenzymes has been developed succesfully. A new active site was created at the dimer interface of LmrR by three strategies, *i.e.* covalent and supramolecular anchoring of a Cu(II) complex and biosynthetic incorporation of a ligand for Cu(II).

In this chapter a short overview of the key findings reported in this thesis is provided and future prospects discussed.

7.2 Research Overview

The selection of scaffolds for the construction of artificial metalloenzymes is based on a few important factors, as described in more detail in chapter 1. A scaffold should have a relative large pocket that is large enough to accommodate the transition metal (complex) and still leave space for the reactants in the catalyzed reaction. The most famous example of a successful scaffold that meets these criteria is streptavidin. This protein has a large pocket in which biotinylated metal complexes can bind and the resulting hybrid has been applied in a wide variety of late transition metal-

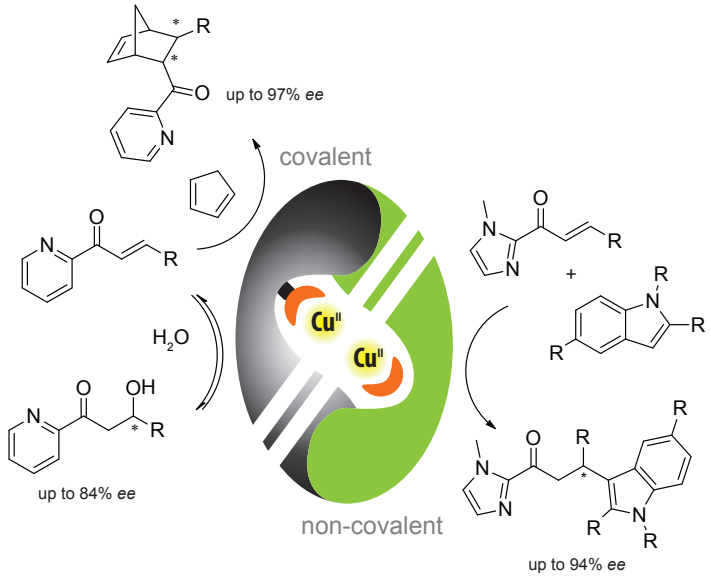

FIGURE 1: *Schematic representation of LmrR based artificial metalloenzymes. LmrR with the covalent anchored Cu(II) complex was applied in the enantioselective Diels Alder reaction (CHAPTER 2 and 4) and enantioselective conjugate addition of water reaction (CHAPTER 3 and 4). Enantioselective Friedel-Crafts alkylation reactions were employed by the LmrR with a non-covalent anchored Cu(II) complex (CHAPTER 5).*

catalyzed transformations.[4] However, the number of available protein scaffolds with such a pocket are limited. In an alternative approach, a new active site can be created at a site in a bioscaffold that is not necessarily an existing active site or binding pocket. A particularly appealing microenvironment for the construction of such a new active site is the dimer interface of proteins, since hydrophobic interactions play a key role in dimerization of proteins. This makes such regions a potentially good environment for binding of small organic molecules. Together with the chiral second coordination sphere provided by this interface, this environment was envisioned to give rise to high activity and enantioselectivity in a catalyzed reaction.

This novel concept was first demonstrated by construction of an artificial metalloenzyme using the dimeric protein Lactococcal multidrug resistance Regulator (LmrR), as described in CHAPTER 2.[5] A phenanthroline Cu(II) complex was grafted covalently onto the dimer interface by a selective cysteine conjugation strategy. The resulting artificial metalloenzyme catalyzed a Diels-Alder reaction with excellent enantioselectivities, *i.e.* up to 97% *ee* of the (+)-enantiomer. Interestingly, the introduction of a Cu(II) bipyridine complex resulted in the preferential formation of the other enantiomer in 66% *ee*. In a mutagenesis study it was proven that the catalyzed reaction indeed takes place in the newly created active site. In addition to the observed enantioselectivity of the catalyzed reaction, a protein accelerating effect

was observed as well. Moreover, a correlation between the enantioselectivity and the rate of the catalyzed reaction was observed, *i.e.* the most enantioselective enzyme gives rise to the highest reaction rates.

With the concept established, a more challenging reaction was explored. The enantioselective addition of water to enones gives direct access to chiral alcohols. This transformation is difficult to achieve with traditional homogenous catalysis or biocatalysis alone, owing to the poor nucleophilicity of water and the narrow substrate scope of natural hydratases. On the basis of the concept described above, an artificial metallo-hydratase was constructed that catalyzed the hydration of α,β-unsaturated ketones with enantioselectivities up to 84% *ee* (CHAPTER 3).[6] A relationship was observed between the steric bulk of substituents at the β-position of the substrate and the enantiomeric outcome of the reaction. The highest *ee* values were observed with a bulky substituent, *e.g.* a *t*-butyl group.

The effect of the second coordination sphere provided by the dimer interface of LmrR on the catalysis was probed by a mutagenesis study (CHAPTER 2, 3 and 4). Residues in proximity to the covalently anchored Cu(II) complex were selected on the basis of a manually docked structure. Two residues important for catalysis of both the Diels-Alder reaction and enantioselective hydration reaction were identified. The phenylalanine at position 93 appears to be essential for achieving good enantioselectivities in both reactions. Mutagenesis revealed that steric bulk at this position is especially important. In addition, a precisely located carboxylate moiety at position 100 is essential for achieving high activities and enantioselectivities. Replacing the aspartic acid at position 100 by the structurally similar but non-charged asparagine resulted in no enantioselectivity. It is hypothesized that F93 and D100 play a role in positioning and/or activating the copper complex instead of having a direct effect on catalysis itself. This is supported by the observation that the fundamentally different Diels-Alder and hydration reactions, respond similarly to mutations at these positions.

Residues located at the back entrance of the hydrophobic pocket at the dimer interface had no significant effect on catalysis, whereas residues located in the front had an effect. Thus, the catalyzed reactions indeed take place in the newly created active site and the catalytic site is situated near the front entrance of the hydrophobic pocket. At this moment it is unclear whether both Cu(II) complexes that were introduced due to the homo-dimeric nature of the protein (one per monomer) take part in the reaction. It is possible that the Cu(II) complex near the front entrance of the hydrophobic pocket is involved in catalysis and the second Cu(II) complex is not. The latter can still have an important structural effect on the pocket. However, more structural information will be needed to confirm this statement.

As mentioned above, residues located in the newly created active site (the inner sphere residues) have similar effects on the two catalyzed reactions (CHAPTER 4). However, a mutagenesis study performed on residues located away from the front

entrance, referred to as outer sphere mutants, gave rise to different effects in catalysis. The Diels-Alder reaction is not affected by mutation, *i.e.* replacing charged lysine residues by alanine did not significantly affect the results of catalysis. In contrast, the enantioselective hydration was affected by outer sphere mutations. The transition state of the hydration reaction is charged, which is not the case in the Diels-Alder reaction, and removal of charged residues can affect the hydration reaction in a positive or negative manner depending on the mutation (CHAPTER 4). Combined, it is hypothesized that the reactant upon binding to the Cu(II) complex has interactions, *e.g.* electrostatic and/or via hydrogen bonding, with the residues located on the outside of the hydrophobic pocket in the outer sphere. On the other hand, residues located inside the hydrophobic pocket mainly seem to effect the position of the Cu(II) complex.

In addition to the covalent anchoring strategy, the LmrR scaffold was used for the construction of an artificial metalloenzyme by supramolecular assembly by binding a catalytically active Cu(II) complex to the scaffold (CHAPTER 5). The resulting artificial metalloenzyme successfully catalyzed a Friedel-Crafts alkylation reaction with excellent enantioselectivities, *i.e.* up to 94% *ee*. Two tryptophan residues, *i.e.* W96 and W96', located at the dimer interface play a key role in catalysis. No induction of enantioselectivity was observed when these tryptophans were replaced by alanines. The quenching of fluorescence of these tryptophans by the Cu(II) complex demonstrates that the complex binds in proximity to W96, and thus in the hydrophobic pocket. Combined, these results clearly demonstrate that the catalyzed reaction takes place in the newly created active site. It was suggested that the substrates for the Friedel-Crafts reaction, *i.e.* an enone and an indole, are bound between the tryptophan residues via π stacking and positioned correctly towards the Cu(II) complex to achieve the observed enantioselectivities.

In a third design strategy, an artificial metalloenzyme based on LmrR was constructed containing genetically encoded 7-azatrypthophan (7-ATrp) residues (CHAPTER 6). Two 7-ATrp residues were placed in the LmrR scaffold to achieve bidentate coordination to Cu(II) ions. The resulting artificial metalloenzyme was tested in the Diels-Alder reaction. Preliminary results showed minor, albeit significant, enantioselective induction. However, the incorporation efficiency of 7-ATrp was low, *i.e.* 50%, and should be improved.

In conclusion, the novel concept of grafting a new active site on a dimer interface of a protein for the construction of an artificial metalloenzyme was demonstrated. The different design strategies used, *i.e.* the covalent and supramolecular anchoring of a Cu(II) complex, underscore the versatility of this scaffold and the concept of using dimer interfaces. Furthermore, to date this scaffold is the first that has been applied successfully in both the covalent and supramolecular anchoring strategies.

7.3 Perspectives

The concept of creating artificial metalloenzymes by grafting a new active site on the dimer interface of proteins introduced in this thesis greatly expands the number of potential scaffolds for artificial metalloenzyme design. The second coordination sphere, provided by the dimer interface, plays a key role in achieving good selectivityies in catalysis. However, the exact role of this second coordination sphere in the LmrR based artificial metalloenzymes is not fully understood yet. Mutagenesis studies revealed residues important for catalysis, but this was mainly the result of trial and error. Therefore, on the basis of structural information obtained from X-ray structures, supplemented with computational and mutagenesis studies, a better understanding of the second coordination sphere should be provided. This information might then be used for optimization of the current LmrR based artificial metalloenzyme and serve as a basis for the design of new ones. Until a few years ago, a majority of artificial metalloenzyme designs, including LmrR, were based on introducing a metal complex into a chiral environment that is provided by the second coordination sphere. However, as in natural metalloenzymes, the second coordination sphere activates and/or positions the catalytically active metal as well as provides interactions with potential substrates to achieve high activity and selectivity. In a recent example, the catalytically active metal ion in the artificial biotin-streptavidin system was activated by an extra coordinating residues that was introduced. As a result, the resulting catalyst showed increased activity.[7] Moreover, the carboxylate residues in the covalent LmrR based hybrid catalysts were proposed to have the same role in catalysis, *i.e.* providing an interaction with the metal complex to achieve high selectivities (chapter 3 and 4).[6] Thus, more emphasis should be given to the second coordination sphere in designing these hybrid catalysts, considering coordination to the catalytically metal as well. Indeed, recent new designs of artificial metalloenzyme have begun to take these considerations into account.[1]

Optimization and/or identification residues important for catalysis in the LmrR based-artificial metalloenzymes can also accomplished by performing directed evolution of the hybrid catalysts, as has been demonstrated for the streptavidin-based systems.[8] Together with structural information, this would be a valuable tool to construct new artificial metalloenzymes. Evolution of these catalysts requires a hybrid catalyst that is readily available and can be produced and screened for activity in a high-throughput manner. Hence, post modification of the LmrR scaffold as described in chapter 2 and 3 is not suitable. In contrast, LmrR based hybrid catalysts constructed by self-assembly, as described in chapter 5, or containing a biosynthetically incorporated ligand for a metal, as described in chapter 6, would be suitable since no chemical modification and subsequent purification is necessary. However, in the latter case, the method for the incorporation of metal-binding unnatural amino acids in recombinant LmrR has thus far not been efficient enough.

In addition, the method described in CHAPTER 6 is limited with respect to the number of unnatural amino acids that can be incorporated. The expanded genetic code methodology introduced by Schultz has a broader scope of unnatural amino acids, *e.g.* bipyridyl alanine for bidentate coordination of Cu(II) ions, and is more selective compared to the method described in CHAPTER 6.[9] For that reason, the construction of a LmrR based artificial metalloenzyme having a genetically encoded bipyridyl alanine, introduced by the Schultz methodology, is a potentially better candidate for directed evolution experiments.[10] This is currently being explored in the Roelfes group. The resulting libraries should be screened for enantioselectivity, relying on a high-throughput HPLC or GC methods since no color assays exists for screening on *ee*. This method places a constraint on the library size, *i.e.* only relative small libraries can be screened effectively, and focused libraries based on iterative saturation mutagenesis should be used.[11]

The field of artificial metalloenzymes is moving away from proof-of-principle studies and is slowly focusing on new challenges as described in a recent review.[1] These challenges include exploring novel reactivity that has no equivalent in traditional homogenous catalysis or biocatalysis alone. *In vivo* catalysis is another challenge that will be addressed with artificial metalloenzymes. Artificial metalloenzymes based on LmrR can be used to tackle these challenges.

Artificial metalloenzymes have been shown to catalyze enantioselective hydration reactions of enones[6,12] and asymmetric C-H activation reactions[13] that otherwise could not be performed using traditional methods. Interactions between the substrate and the second coordination sphere, *e.g.* activating the substrate by electrostatic and hydrogen bond interactions, *etc*., play a key role in these transformations. Following the same strategy, the addition of ammonia to enones to create chiral amines is a potentially attractive transformation for LmrR based artificial metalloenzymes. To date, only Lewis acid-catalyzed reactions have been catalyzed by LmrR-based artificial metalloenzymes. However, a vast majority of transition metal-catalyzed reactions in organic synthesis are organometallic in nature. Therefore, organometallic catalysis with LmrR-based artificial metalloenzymes has to be explored. A particularly attractive class of reactions in this regard are metal-catalyzed carbene reactions.[14,15]

Catalysis by artificial metalloenzymes in cells is both fundamentally important and has potentially practical applications. For example, whole cell catalysis is a convenient way to perform bio-catalysis on a large scale, as the catalyst is prepared *in vivo*. The introduction of an artificial metalloenzyme would provide biocatalysis with a broader scope of possible reactions that do not exist in the normal repertoire of cells. Directed evolution experiments on artificial metalloenzymes would benefit from the introduction of these hybrid catalysts in live cells, as selection can be coupled to the survival of the cell. Also the introduction of artificial metalloenzymes in biosynthetic pathways can open the possibility of synthesizing complex molecules in conjunction

with existing bio routes.

Many challenges lie ahead for achieving *in vivo* catalysis of artificial metalloenzymes. However the first steps have already been taken. It was shown that the streptavidin-based artificial metalloenzymes are biocompatible and can work in cascade reactions with natural enzymes.[16] In addition, it has been demonstrated that abiotic reactions, *i.e.* asymmetric carbene transfer reactions to generate cyclopropane products, can be performed in live cells.[15] LmrR-based artificial metalloenzymes bearing the genetically encoded bipyridyl alanine are potentially suitable candidates to explore *in vivo* catalysis. As cells do not possess high levels of endogenous copper but do possess iron, iron based catalysis should be pursued as well. Therefore, the asymmetric carbene reaction would be a good candidate as it was shown to be biocompatible, *vide supra*.[14, 15]

In conclusion, LmrR based artificial metalloenzymes have proven to be versatile catalysts and together with other promising classes of hybrid catalysts, the field of artificial metalloenzymes is ready to take on new challenges.

7.4 References

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