CHAPTER 1

General introduction

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1.1 INTRODUCTION

Chirality is one of the most important as well as intriguing phenomena in nature, starting from chirality of amino acids and sugars all the way to the chiral DNA and proteins. Chirality has also important influence on our daily life; chiral compounds are needed as pharmaceuticals, agrochemicals, flavours, fragrances and other functional materials.[1,2] However, the synthesis of pure enantiomers remains a challenge in the field of organic chemistry. Homogenous transition metal, organocatalysis and enzymatic catalysis are the most important approaches for the selective synthesis of optically pure compounds. Homogeneous transition metal complexes catalyze a broad range of challenging chemical transformations. In general, they consist of a metal ion bound to one or more ligand molecules. These catalysts have broad reaction scope and they can be readily modulated, however selectivity can be a problem with these catalysts. Enzymes differ from homogenous catalysts in the sense that they have evolved to catalyze a specific transformation, of a specific compound, in an aqueous environment at physiological conditions, with high activity and selectivity, within a complex mixture of molecules. Enzymes contain a specific active site pocket to bind substrates with high affinity. New technologies such as rational computational modelling, together with directed evolution and combinatorial techniques have greatly improved properties of the enzymes and increased their use in the industrial processes.[3–5]

1.2 ARTIFICIAL METALLOENZYMES

Artificial metalloenzymes (AMEs) have emerged as an attractive approach for the design of *de novo* biocatalysts that aim to combine the broad substrate and catalytic scope of transition metal catalysis with high activity and selectivity typical for enzymatic catalysis. Inspired by the early reports of Kaiser in 1976[6] and Whitesides in 1978[7], the search for novel metal-enzyme hybrid catalysts has become a very active area. Over the past two decades, many new and impressive results have been reported on the development of new approaches. An artificial metalloenzyme consists of a catalytically active metal complex that is embedded into biomolecular scaffold (**Figure 1**).[8,9] The second coordination sphere of the biomolecular host plays a key role in providing the chiral microenvironment where the catalysis takes place. While the first coordination sphere of the metal complex plays major role in the catalytic activity, the second coordination sphere interactions are responsible for the enantioselectivity of the reaction. Three major

strategies have been described for the formation of AMEs: i) the redesign of naturally occurring metalloenzymes[10–12], ii) creation of new metalloenzymes from metal-free biomolecular scaffolds[7,13] iii) *in silico* or *de-novo* metalloenzyme design[14–17].

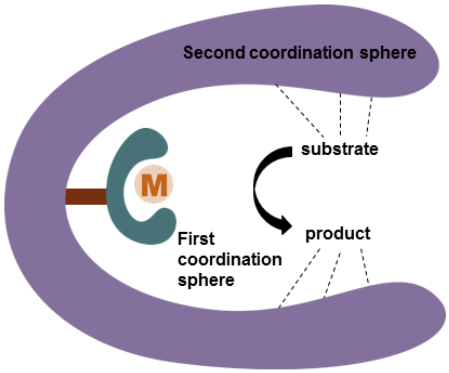

Figure 1. Schematic representation of an artificial metalloenzyme consisting of a transition metal complex (*first coordination sphere*) and a biomolecular scaffold (*second coordination sphere*).

Since the aim of this thesis falls into the second category, further description of the design approaches will focus on that. When designing novel metalloenzymes, the choice of both metal complex and biomolecular scaffold are important. The choice of metal is in principle dependent on the desired reaction. However, it still should fulfil some criteria such as being orthogonal to the biomolecular scaffold and being tolerant to the aqueous environment. There are several important criteria for the choice of a biomolecular scaffold. Preferably, structural information from X-ray crystallography or NMR should be available. The second important parameter is the presence of a cavity of sufficient size to accommodate the whole active complex, that is, the metal complex and the substrate/substrates. Optimal biomolecular scaffolds should be readily available in sufficient amounts and high purity, and ideally stable at higher temperatures, tolerant to organic solvents and different pH values. When the desired scaffold and metal are chosen, four different approaches for linking them together can be considered: covalent, supramolecular, dative and the unnatural amino acid (UAA) approach (**Figure 2**).

Figure 2. Current anchoring approaches applied in the preparation of artificial metalloenzymes. Starting from the left: covalent, supramolecular, dative and unnatural amino acid approach.

1.2.1 Anchoring strategies

In the covalent approach, the metal complex is incorporated into the biomolecule *via* a covalent chemical bond. During this process, the biomolecular scaffold has to undergo post-biosynthetic modifications. The most commonly used bioconjugation strategy is utilization of the nucleophilicity of cysteine. The advantage of using cysteines is that they are not frequently occurring amino acids in the sequences of natural proteins, but they can be easily introduced at the desired positions.[18] A variety of partner molecules have been described for these bioconjugations, such as α-halogenated carbonyl compounds or maleimide-substituted ligands. DiStefano and coworkers were the first to utilize artificial metalloenzymes created via covalent anchoring of Cu(II) complexes. Iodoacetamido-1,10-phenanthroline was linked through a unique cysteine residue in adipocyte lipid binding protein (ALBP) to produce the conjugate ALBP-Phen. The resulting hybrid catalyst was tested in a copper catalyzed enantioselective hydrolysis of esters and activated amides. Hydrolysis reactions were performed with racemic amino acid esters resulting in up to 86% *ee* of diastereomeric products. These results were further improved in the follow-up study where the enantioselectivity was increased up to 94% by re-positioning of the complex within the protein scaffold (mutant variant ALBP-L72C).[19,20]

Other amino acids, like lysine or serine can be used for covalent anchoring, however such examples are scarce. The tryptophan gene repressor of *E. coli* has been converted into a site-specific nuclease by covalently attaching the 1,10-phenanthroline-copper complex *via* lysine residues.[21] In the report of van Koten[22,23,] a phosphonate ester that is functionalized with an organometallic moiety acts as an inhibitor of the enzymatic family of serine hydrolases and irreversibly forms a covalent bond with the serine residue of the catalytic triad. Following the same approach, an artificial metalloenzyme has been described by Klein Gebbink and co-workers in which a Rh(NHC) phosphonate complex was introduced into the

lipases cutinase and *Candida Antarctica* lipase B (CAL-B). This new hybrid catalyst was shown to catalyze cross metathesis reactions, albeit the E/Z ratio was similar to the one found for the catalyst alone.[24,25]

Covalent anchoring allows for the precise control over the localization of the complex. However, utilizing the natural amino acid means that it has to be uniquely present in the scaffold and removing additional instances of these amino acids may lead to the structural perturbations. Use of unnatural amino acids with a unique chemical functionality represents a possible solution. Recently, Lewis and co-workes introduced a methodology in which a bicyclononyne-substituted metal complexes was coupled *via* strain-promoted azide–alkyne cycloaddition to the thermostable protein tHisF containing a genetically encoded L-4-azidophenylalanine residue.[26] The resulting artificial metalloenzymes have shown potential in cyclopropanation and Si-H insertion reactions, although no enantioselectivity was observed. In a follow-up study, the same methodology was used with a prolyl oligopeptidase scaffold to generate dirhodium tetracarboxylate artificial metalloenzymes, enabling olefin cyclopropanation with up to 92% *ee*.[27]

The supramolecular anchoring strategy relies on self-assembly of the AME by noncovalent interactions between the biomolecular scaffold and a metal complex. The metal complex is incorporated into the protein structure *via* various protein-ligand interactions, such as hydrogen bonding, π–π stacking, hydrophobic and electrostatic interactions. The first example of this strategy was the biotin-(strept)avidin technology, in which the metal complex is attached to biotin, which displays a very strong affinity towards the protein avidin. This was first pioneered by Whitesides in 1976[7] and further explored by the group of Ward, who changed the protein scaffold from avidin to streptavidin. In an early report, this AME displayed up to 96 % *ee* in the hydrogenation of acetamidoacrylic acid.[28] Later on, the biotin-streptavidin technology has been widely used in different reactions, for example in the asymmetric C-H activation and in redox cascades. Some specific examples will be described later.[29–32]

Another variant of AMEs in the category of the supramolecular approach takes advantage of serum albumins and their high affinity towards a wide variety of hydrophobic ligands. Gross and co-workers prepared range of artificial metalloenzymes introducing manganese or iron corroles to the serum albumins, successfully catalyzing asymmetric sulfoxidation up to 74% *ee*.[33] Another example using albumins was described by the group of Reetz.[34] It involved enantioselective artificial metalloenzymes based on a commercially available copper(II)phthalocyanine complex anchored supramolecularly to various serum albumines, namely, bovine serum albumin (BSA), human serum albumin, sheep serum albumin and rabbit serum albumin. These hybrid catalysts proved to be

effective in the Diels-Alder reaction of cyclopentadiene with azachalcone derivatives showing good conversions, high *endo* selectivities, and high enantioselectivities. The best results were obtained with BSA; the products were obtained with *ee´*s ranging from 85–98%. DNA-based asymmetric catalysis represents another very successful and broadly-applied example of the supramolecular approach. Roelfes and co-workers introduced Cu^{II}-bipyridine complex into the helical structure of DNA and achieved high enantioselectivities in various C-C bond forming reactions in water; *ee's* up to 99%, 93% and 98% were obtained in Michael addition, Friedel-Crafts alkylation and Diels-Alder reactions, respectively.[35]

Dative anchoring strategy represents methodology that involves supramolecular interactions, in which the metal is coordinated directly to the amino acid residues, such as aspartates, glutamates and histidines, already present in the protein structure. For example, Okrasa and Kazlauskas described a manganese-substituted carbonic anhydrase with peroxidase activity, giving rise up to 67% *ee* in the epoxidation of *p*-chlorostyrene.[36] Carbonic anhydrase represents a typical protein host in the dative approach, since the zinc ion present in its active site be easily replaced by another metal ion. In another example, the group of Ward used this methodology with a streptavidin-based artificial metalloenzyme, in which biotinylated Rh piano stool complex was localized into the pocket of streptavidin with two histidine residues and the resulting construct was shown to catalyze an asymmetric reduction of prochiral imines.[37]

The most recent addition introduced by our group to the anchoring strategies is the construction of artificial metalloenzymes by *in vivo* incorporation of an unnatural amino acid into the structure of a protein. Here, the noncanonical metal-binding amino acid is introduced into the protein during the biosynthesis of the protein and the artificial metalloenzyme is conveniently achieved by addition of the transition metal salt. The major part of this thesis describes the development and utilization of this approach, for more details see *chapters 2,3* and *4*.

1.2.2 Reaction scope

In the past two decades, the reaction scope of artificial metalloenzymes has significantly expanded. These hybrid catalysts are no longer being tested only with the model reactions, but are living up to their potential and are capable of catalyzing multiple challenging reactions. In **Table 1**, the reactions catalyzed by artificial metalloenzymes are summarized, together with examples of the catalysts and anchoring strategies.

Table 1. Reaction scope of artificial metalloenzymes.

Type of reaction	Catalyst (scaffold/cofactor)	Type of anchoring	Reference
C-H activation	Streptavidin/ $[RhCp^{*biotin}Cl_2]_2$	Supramolecular	[31]
Cross Methathesis/ Ring-closing methathesis	*M. jannaschii* small heat shock protein/ Ru Hoveyda Grubbs–catalyst	Covalent	[38]
	Streptavidin/biotinylated Ru Hoveyda-Grubbs catalyst	Supramolecular	[39]
	Human carbonic anhydrase II/ Hoveyda Grubbs–catalyst	Dative	[40]
	FhuA/Hoveyda–Grubbs catalyst	Covalent	[41]
	Cutinase, CAL-B/ Rh(NHC) phosphonate complex	Covalent	[23,24]
	α-chymotrypsin/Ru Hoveyda–Grubbs catalyst	Covalent	[42]
Cyclopropanation	*st*-DNA/Cu(I)dipyridophenazine	Supramolecular	[43]
	st-DNA/Fe(III)porphyrins	Supramolecular	[44]
	POP/$Rh_2(OAc)_4$-BCN	Covalent, UAA	[26]
	tHisF/$Rh_2(OAc)_4$-BCN	Covalent, UAA	[27]
	Myoglobin/Rh, Ru and Ir porphyrins	Supramolecular	[45]
Diels-Alder reaction	*st*-DNA/Cu(II) metal-binding ligands	Supramolecular	[46]
	G-quadruplex/$Cu(NO_3)_2$	Supramolecular	[47]
	LmrR/Cu(II)-Phen	Covalent	[13]
	tHisF(His-His-Asp)/Cu(II)	Dative	[48]
	Albumins/Cu(II)phthalocyanine	Supramolecular	[34]
	Sterol carrier protein type 2 like domain/Cu(II)-Phen	Covalent	[49]
	Papain/Ru(II)-Phen	Covalent	[50]
	Neocarzinostatin /Cu(II)-Phen-testosterone	Supramolecular	[51]
Epoxidation	Carbonic anhydrase/Mn(II)	Dative	[36]
	Xylanase 10A/[Mn(TpCPP)	Supramolecular	[52]
	Nitrobindin/Mn(II)terpyridine	Covalent	[53]
Fluorination	*st*-DNA/$[Cu(dmbipy)(NO_3)_2]$	Supramolecular	[54]
Friedel-Crafts reaction	*st*-DNA/$[Cu(dmbipy)(NO_3)_2]$	Supramolecular	[55]
	LmrR_BpyAla/$Cu(NO_3)_2$	UAA	[56]
	LmrR/$[Cu(phen)(NO_3)_2]$	Supramolecular	[39]

Heck coupling	CAL-B/Pd pincer	Covalent	[58]
Hydroformylation	Carbonic anhydrase/$[Rh(CO)_2(acac)]$	Dative	[59]
Hydrogenation	Carbonic anhydrase/$Rh(cod)_2BF_4$	Dative	[60]
Hydrolysis	*st*-DNA/$[Cu(dmbipy)(NO_3)_2]$	Supramolecular	[61]
	$[(gp5\beta f)_3]_2$/Sc(III)-bpy	Covalent/Dative	[62]
	ALBP/Cu(II)-Phen	Covalent	[19]
	Neocarzinostatin/Testo-BisPyPol-Zn(II)	Supramolecular	[51]
Hydroxylation	Myoglobin/Mn(II)-porphycene	Supramolecular	[63]
Michael addition	*st*-DNA/$[Cu(dmbipy)(NO_3)_2]$	Supramolecular	[64]
	G-quadruplex/$[Cu(dmbipy)(NO_3)_2]$	Supramolecular	[65]
Oxidation	Nitrobindin/Mn(II)-terpyridine	Covalent	[53]
Polymerization	Nitrobindin/Rh(I)(Cp)(cod)	Covalent	[66]
	Ferritin/Rh(I)(norbornadiene)	Supramolecular	[67]
Sulfoxidation	Streptavidin/VOSO4	Dative	[68]
	Phytase/VO_4^{3-}	Dative	[69]
	Myoglobin/$[Cr(II)(5,5'$-*t*Bu-salophen$)]^+$	Supramolecular	[70]
	Myoglobin/Fe(II)-porphycene	Supramolecular	[63]
	Serum albumins/Fe(III), Mn(III) corroles	Supramolecular	[33]
	Neocarzinostatin/Fe(III)Porfyrin-testosterone	Supramolecular	[71]
Suziky-Miyuara	Streptavidin/PdCl(cinnamyl)	Supramolecular	[72]
Trans hydrogenation	Papain/Ru(II)/Rh(III) d6-piano stool complexes	Covalent	[50]
	bovine β-lactoglobulin/Ru(II) and Rh(III) complexes	Supramolecular	[73]
	Streptavidin/$[\eta^6$-(arene)Ru(Biot-*p*-L)Cl]	Supramolecular	[74]
	Streptavidin/His/Rh[Cp*Ir(Biot-*p*-L)Cl]	Supramolecular, dative	[37]
Water-addtion	*st*-DNA/ Cu(II)-2-(aminomethyl)pyridine-based ligands	Supramolecular	[75]
	LmrR/Cu(II)-Phen	Covalent	[76]

1.2.3 New Developments

Cascades

Nature has evolved a highly efficient approach to multistep synthesis in the form of multistep cascade, commonly working without the separation of intermediates. Inspired by nature, application of cascade reactions in organic synthesis potentially offers a lot of advantages over the classical step-by-step approach. Biocatalytic cascades are easier to perform since they benefit from the fact that most enzymes reach their catalytic optimum at similar temperature and pH in aqueous conditions. Chemo-enzymatic combinations encounter problems since biocatalysts are often unstable in organic solvents or the organo- or metallo-catalyst are inactivated in an aqueous environment or by the protein. Several examples of successful chemo-enzymatic cascades have been described utilizing nanoparticles or immobilization. The use of AME represents an attractive approach, since the metal-catalysts are already incorporated within the biomolecular scaffold, avoiding mutual inhibition with the other enzymes.

Kohler *et al*. established several multi-enzymatic cascades, employing incorporated biotinylated Ir-organocatalyst into a streptavidin protein scaffold. This artificial transfer hydrogenase proved to be compatible with several cofactor dependent enzymes, namely flavin-dependent monooxygenase, heme dependent enzymes (catalase and peroxidase), a flavin dependent amine oxidase, and amino acid oxidases. The scope of the performed cascade reactions includes reductions of prochiral imines with subsequent deracemization, the stereoinversion of nicotine, the synthesis of L-pipecolic acid and a regioselective oxidation.[32]

In a report by Okamoto *et al*., a novel NAD(P)H-dependent artificial transfer hydrogenase which involves an Cp^*Ir cofactor, containing a biotin moiety, and 4,7-dihydroxy-1,10-phenanthroline was described. This novel artificial metalloenzyme catalyzes imine reduction with milimolar concentrations of NADPH, which can be regenerated by a glucose dehydrogenase. A four-enzyme cascade consisting of this artificial transfer hydrogenase, glucose dehydrogenase, a monoamine oxidase, and a catalase was used for the production of enantiopure amines.[77]

NADH mimics (mNADHs) have been described to accelerate and activate ene reductase-catalyzed reactions. However, the methods known for regeneration of NAD(P)H fail for mNADHs. The first efficient regeneration system described for mNADHs is based on AMEs with streptavidin variants and a biotinylated iridium cofactor. The regeneration was enabled with formate and coupled with an ene reductase-catalyzed asymmetric reduction of α,β-unsaturated substrates. With

10 mol% $mNAD^+$, a preparative scale reaction gave full conversion (TTN> 2000) with 98% *ee*.[78]

In vivo catalysis

One of the biggest challenges of the field of AME is how to transport this catalysis to cells. This was first reported by Song and Tezcan. They described a new artificial metallo-β-lactamase, constructed by metal-directed self-assembly of a monomeric redox protein into a tetrameric complex that possesses catalytic zinc sites at its interfaces.[17] This was performed using a strategy called 'metal templated interface redesign' in which a small number of surface mutations are introduced to create a metal binding site at the interface between two proteins. This newly prepared hybrid protein (Zn_8:$^{A104}AB3_4$) showed hydrolytic activity against substrates such as *p*-nitrophenylacetate and ampicillin. Most intriguingly, *in vivo* catalytic activity in *Escherichia coli* cells was described, using a variant of the enzyme that is directed to the periplasm. It was found that *E. coli* cells containing the artificial zinc enzyme were able to grow despite the presence of ampicillin, whereas controls containing variants without the catalytic Zn(II) sites could not grow under the same conditions. This clearly demonstrated that the designed enzyme was active *in vivo*.

Recently, a streptavidin-biotin based artificial metalloenzyme was shown to perform catalysis *in vivo*.[79] This is following up on previous studies described by the group of prof. Ward in which an artificial metalloenzyme for olefin metathesis was developed. Since the metal cofactor used for the reaction, biot-Ru, is inhibited by cellular components, a new variant of streptavidin was designed, allowing the periplasmic expression of streptavidin (SAV^{peri}) in *E. coli*. The corresponding artificial metalloenzyme biot-Ru–SAV^{peri} was active *in vivo,* with activity similar to the complex with purified enzyme. This design allows easier screening in directed evolution studies (**Figure 3**).

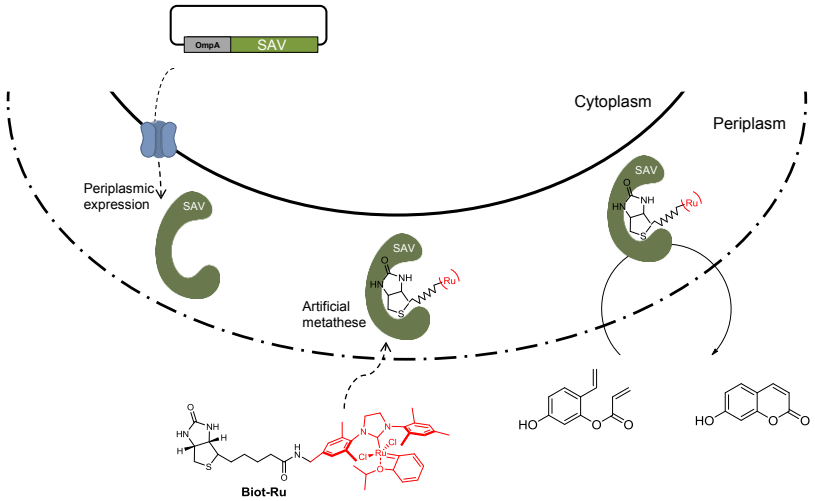

Figure 3. Schematic representation of streptavidin-biotin based *in vivo* catalysis (adapted from 79).

1.3 TRANSCRIPTION FACTOR LmrR

Lactococcal multidrug resistance Regulator (LmrR) (**Figure 4**) is a transcription repressor from the Gram-positive bacterium *Lactococcus lactis*. In its natural function, LmrR controls the expression of the ABC multidrug transporter LmrCD via an induction mechanism that involves direct binding of transporter ligands to LmrR.[80,81] LmrR belongs to PadR subfamily 2 (PadR-s2), a protein family of homodimeric transcription regulators that are structurally related to each other. The structure of LmrR is divided into two functional domains: a typical wHTH DNA-binding domain, consisting of helices α1, α2, the DNA recognition helix α3 and wing-forming strands β1 and β2 and a dimerization domain containing the C-terminal helix α4 (**Figure 4**). Helix α4 forms an arm, in a nearly antiparallel orientation against helix α1′ (the prime indicates the other monomeric subunit), as well as an interaction with the C-terminal region of helix α2′ and the loop connecting helices α2′ and α3′. There is no interaction between the C-terminal helices α4 and α4′, nor between N-terminal helices α1 and α1′. Remarkably, this arrangement results in the formation of a large flat-shaped pore (approximately 22 Å in width and 6 Å in height). The pore serves as a multidrug-binding site.[82] It was shown that two tryptophan residues (W96 and W96') in the centre of pore are essential for the binding of ligands, which are mostly planar heterocyclic compounds. The crystal structures of apo and drug-bound complex with Hoechst 33342 and daunomycin and recently also in complex with riboflavin have been elucidated.[82,83]

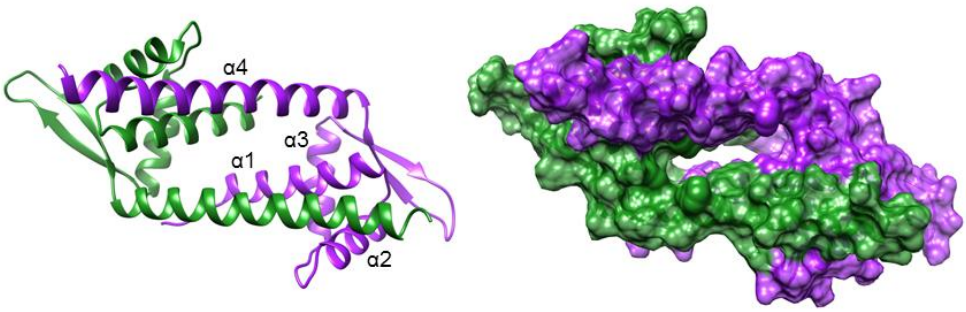

Figure 4. Cartoon representation of the dimeric structure of LmrR in (left) the space-filling model (right). PDB of LmrR in drug-free state: 3F8B.

1.3.1 LmrR as artificial metalloenzyme

Inspired by the fascinating structure of LmrR, Bos *et al.* introduced a novel class of artificial metalloenzymes, created by grafting a novel active site on the dimer interface of this protein.[84] The large hydrophobic core on the dimer interface was functionalized with two phenanthroline- or bipyridine-based cofactors (one per monomer), by alkylation of cysteine residues with bromoacetamide-substituted 1,10-phenanthroline (Phen) or 2,2′-bipyridine (Bpy) metal-binding moieties (**Figure 5a**). Based on the crystal structure, the positions N19 and M89, which are at the far ends of the pore, were selected as anchor positions. Since the structure does not naturally contain cysteine, site-directed mutagenesis was used to introduce cysteines at the chosen positions. This artificial metalloenzyme was first tested in the Diels-Alder reaction and gave rise to remarkable results. The mutant LmrR-M89C-Cu(II)-Phen achieved excellent *ee*'s (up to 97%) with high conversions (up to 93%). On the other hand, the LmrR-M89C-Cu(II)-Bpy variant yielded the opposite enantiomer of the product in 66% *ee*, suggesting that in this case the active complex is in a very different microenvironment. This nicely demonstrates that both enantiomers of the product can be obtained using the same chiral scaffold by judicious choice of the introduced synthetic moiety. The same artificial metalloenzyme was also successfully employed in a more challenging reaction, the enantioselective addition of the water to C=C double bond (**Figure 5a**).[76] This reaction is highly appealing, however remains difficult for synthetic organic chemistry due to the challenges associated with water acting as a nucleophile[75]. Up to this date, this represents the only example of an artificial metallohydratase. In this report, the artificial metalloenzyme variant LmrR-M89C-Cu(II)-Phen with covalently anchored Cu(II)-phen complex was used and tested on a range of α,β-unsaturated 2-acyl pyridines with varying substituents (R) at the β-position. The best results were obtained with R=*tert*-butyl, giving rise to *ee*'s up to 84% and high conversion (up to 80%). Additionally, a mutagenesis study showed that, in order to achieve high enantiomeric excess, a residue with a large side chain at position 93 is important. Moreover, it was found that the aspartate residue at position D100 was essential to achieve good and selective catalysis.

More recently, artificial metalloenzymes were created by supramolecular assembly of LmrR with Cu-(II)-phenanthroline complexes.[57] This approach takes the most advantage of the natural function of the protein to bind planar aromatic molecules on the dimer interface between two tryptophan moieties. The K_d of the Cu(II) complex to LmrR was determined to be 2.6 μM. The catalytic potential of the artificial metalloenzyme was evaluated in the aforementioned Friedel-Crafts reaction of 1-(1-methyl-1*H*-imidazol-2-yl)but-2-en-1-one with a variety of indoles.

High conversions and excellent *ee*'s were observed: up to 94% *ee* for indole and 93% for 2 methyl-indole (**Figure 5b**). The mutant variant W96A, where the tryptophans that are assumed to be responsible for binding were replaced with alanine, gave rise to poor conversion and complete loss of *ee*. This confirms the key role played by these residues in positioning of the Cu(II)-phenathroline complex within the pore of the protein scaffold.

Noteworthy, LmrR represents the first example of a scaffold that can be used in multiple anchoring approaches.

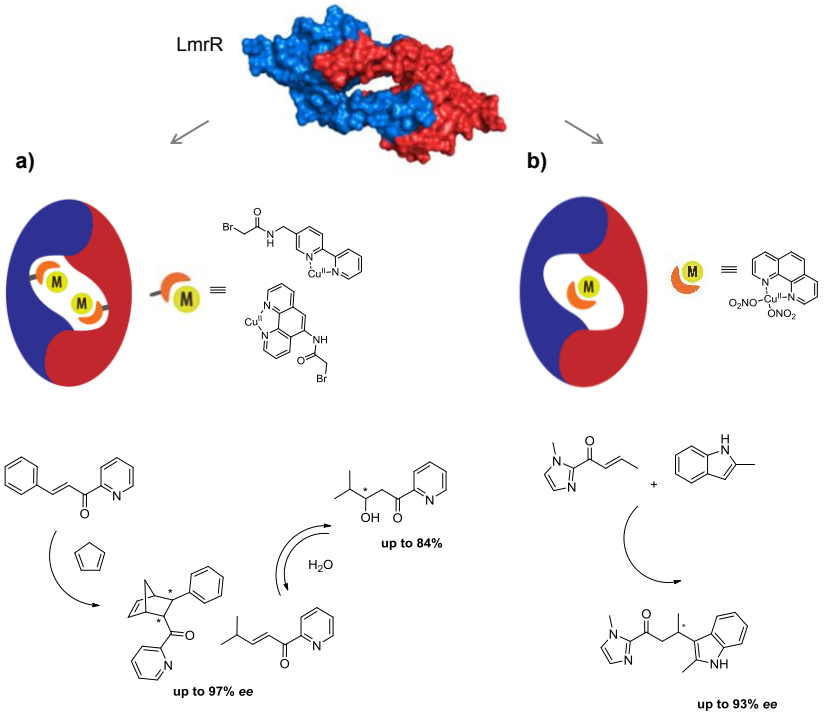

Figure 5. Overview of the artificial metalloenzymes prepared based on the LmrR scaffold with **a)** covalent and **b)** supramolecular anchoring and the reactions catalyzed**.**

1.4 EXPANDED GENETIC CODE METHODOLOGY

The genetic code of all organisms encodes the same 20 common amino acids, with the rare exceptions of selenocysteine[85] and pyrrolysine[86]. The natural amino acids cover a relatively limited chemistry with their functional groups like hydroxyl, carboxyl, thiol, amine or amide and aromatic side chains so additional post-translational modifications and cofactors are often required for protein to gain full functionality. Therefore, addition of new building blocks to the genetic code should provide a powerful strategy for the construction of proteins with new and useful chemical or biophysical properties and functionalities. Unnatural amino acids represent a diverse array of structural modifications in the side chain of amino acids.

A number of different methods have been explored for the incorporation of UAAs in protein structures. First, chemical modification of amino acid side chains was used. However nonselective modification together with the limited number of residues that can be chemically modified represents a drawback of this method.[87] Next, solid phase peptide synthesis allows for a large number of modifications in the protein structures, however it is generally limited to peptides and smaller proteins due to the decreased yield and purity associated with the synthesis of larger proteins. Solid phase peptide synthesis and chemical ligation remain a valuable option with amino acids analogues that are toxic or incompatible to cell environment. Another method uses nonsense or frameshift suppressor tRNAs that are chemically misacylated with a UAA in a cell free system. This method can be used to produce proteins of different sizes with UAAs located at any preferred position. Over 80 novel amino acids have been incorporated into proteins using this methodology. However, the protein yields are low and the creation of aminoacyl-tRNA involves a complex multistep process.

The possibility to incorporate UAAs directly *in vivo* offers considerable advantages over the previous strategies, including higher yields, technical ease and additionally also allows the study of the protein structure and function *in vivo.* In the earliest reports, the UAAs could be incorporated *in vivo*, replacing natural amino acids with close analogues using appropriate auxotrophic cells.[88,89] Later on, site-specific incorporation has been introduced, described as expanded genetic code methodology.[90] Here, the amber stop codon, UAG, is generally reassigned to incorporate UAAs. This codon is rarely used in nature, which makes it the most suitable triplet to be reassigned. Upon binding of specific tRNA with an anticodon, it is possible to change the stop signal for the UAA of choice. Two components are

necessary to incorporate an unnatural amino acid *in vivo*. First a specific tRNA ($tRNA_{CUA}$) which binds the unusual codon and secondly an aminoacyl tRNA synthese (aaRS) (**Figure 6**). The aaRS catalyzes the covalent attachment of unnatural amino acid to the orthogonal tRNA. The aaRS has a pocket that is selective for a single amino acid and recognizes tRNA with the correct anticodon. The aaRS has to be modified to be selective for every new unnatural amino acid. In order to minimize crosstalk with the natural translational system of the host cells, the suitable orthogonal tRNA/aaRS pair were derived from various species, such as *Methanocaldococcus jannaschii* or *Pyrococcus horikoshii.* Nowadays, other signals to encode desired unnatural amino acids are also considered, like rare sense and four base codons.[91,92]

In this thesis, we make use of the pEVOL plasmid[93], a new vector prepared for the specific incorporation of several different unnatural amino acids. This vector represents an enhanced system for the introduction of UAA in proteins in *E.coli* and was developed for obtaining higher yields. It makes use of an evolved aaRS/$tRNA_{CUA}$ pair derived from *Methanocaldococcus jannaschii* and employs two promoters, constitutive and inducible, to drive the transcription of two aaRS genes. Due to toxicity of foreign aaRS to the endogenous translation machinery, pEVOL makes use of an inducible aaRS promotor together with constitutive expression of basal levels of aaRS. $tRNA_{CUA}$ is not toxic and is expressed continuously.

Using expanded genetic code methodology, more than 150 unnatural amino acids have been incorporated in proteins.[94] While initially mostly *E.coli* has been used, currently the unnatural amino acids can also be incorporated in other bacteria, yeast, plant and mammalian cells.[95,96]

Many proteins containing amino acids with novel side chains including fluorophores, metal chelators, photocrosslinking moieties, unique reactive functional groups, as well as NMR, IR, and x-ray crystallographic probes have been described. Recently, engineering of natural enzymes through the use of the UAAs has become an emerging technique for the improvement of their stability or activity.[97,98] Several UAAs are depicted and their functions are given in **Figure 7**.[99]

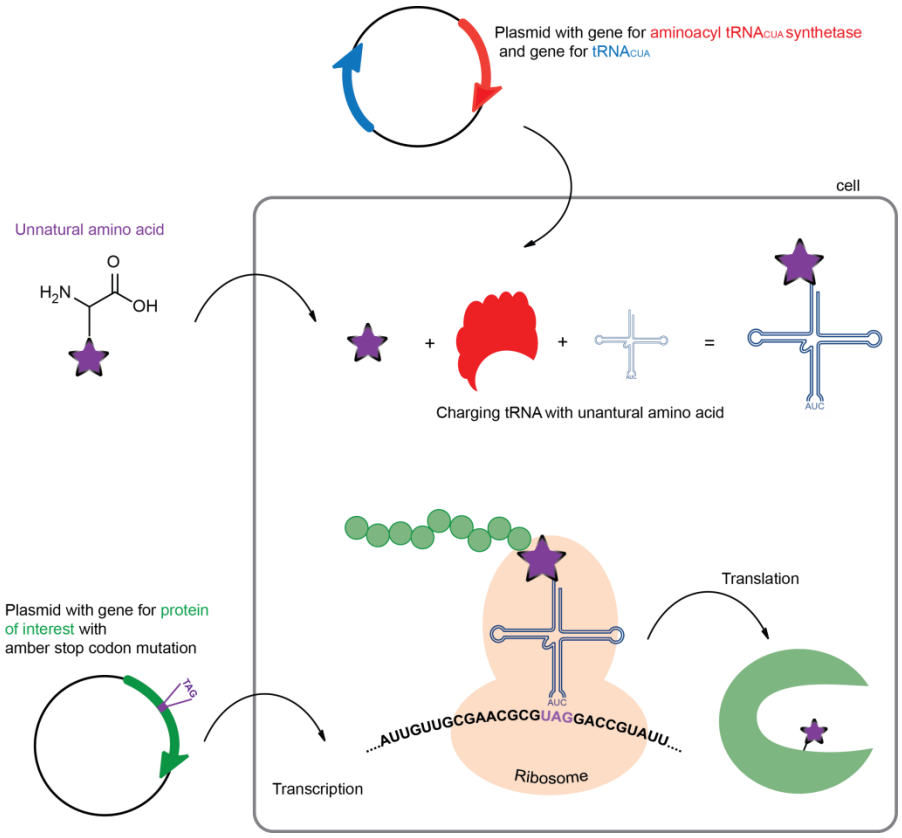

Figure 6. Schematic representation of the expanded genetic code methodology.

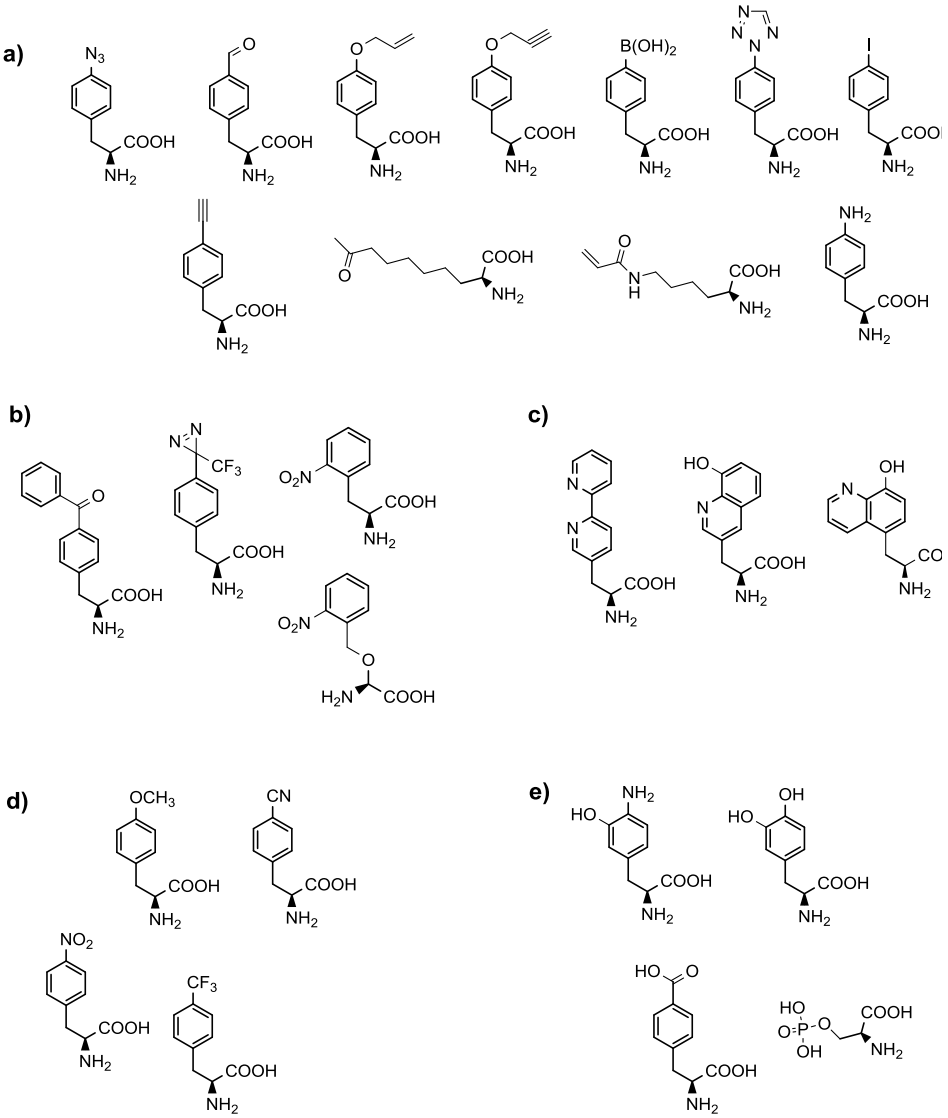

Figure 7. Overview of unnatural amino acids and their applications. **a)** Reactive chemical handles. **b)** Photo-reactive UAAs (photocrosslinkers and photo-caged). **c)** Metal chelators. **d)** Spectroscopic probes. **e)** Posttranslational modifications.

1.5 AIM AND OUTLINE OF THE THESIS

The aim of this thesis was to develop a novel methodology to create artificial metalloenzymes utilizing unnatural amino acids as a metal-binding moiety or catalytic residue. Use of this approach provides numerous advantages in comparison to the previously reported methods, e.g. an exquisite degree of control over the position without the need for specific ligand binding interactions, no need for chemical modification and/or subsequent purification steps while assembly of the AME is easily achieved by addition of the transition metal salt. This is particularly attractive since it should allow for rapid optimization of artificial metalloenzymes by genetic methods.

Therefore,

Chapter 2 discusses novel artificial metalloenzymes created by *in vivo* incorporation of the non-proteinogenic metal-binding amino acid (2,2´-bipyridin-5yl)alanine (BpyAla) into the LmrR scaffold. The initial studies of the resulting artificial metalloenzymes focus on its characterization and application in catalytic asymmetric Friedel-Crafts alkylation reactions.

Further research of this novel BpyAla-based artificial metalloenzyme is presented in **chapter 3** with focus on *in-silico* design of an enzyme to perform the challenging enantioselective water-addition reaction.

In **chapter 4,** (8-hydroxyquinolin-3-yl)alanine unnatural amino acid (HQAla) is explored as a novel metal-binding moiety for preparation of artificial metalloenzymes. Its incorporation into the LmrR, affinity for different metals and catalytic activity in several reactions were studied in this chapter.

In **chapter 5,** a different catalytic moiety, i.e. the aniline side-chain of *p*-aminophenylalanine, is exploited, and incorporated via expanded genetic code methodology. This gives a rise to a novel class of artificial enzymes with a nucleophilic moiety able to catalyze hydrazone-forming reactions. A detailed study of the novel artificial enzymes and their mechanism of reaction is presented.

In **chapter 6**, a novel scaffold for artificial metalloenzymes is explored, based on the *bc*PadR1 protein. *bc*PadR1 is a transcription regulator from the same family as LmrR, however exhibits some structural differences. The potential of this novel artificial metalloenzyme is tested in two different reactions, i.e Friedel-Crafts and tandem Friedel-Crafts alkylation/enantioselective protonation reaction and a mutagenesis design study of the opening of the pore of this protein is discussed.

Finally**, chapter 7** gives overall conclusions of the presented research with perspectives and outlook for the field of artificial enzymes and the use of unnatural amino acids in catalysis.

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