Artificial Metalloenzymes

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Enantioselective Artificial Metalloenzymes by Creation of a Novel Active Site at the Protein Dimer Interface**

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Natural metalloenzymes are a continuing source of inspiration for the design of bio-inspired catalyst. Key to their high catalytic efficiencies and excellent (enantio)selectivities are the second coordination sphere interactions provided by the protein scaffold. The emerging concept of hybrid catalysis is an effort to impart enzyme-like characteristics to homogeneous transition-metal catalysts by embedding catalytically active transition-metal complexes in a biomolecular scaffold, resulting in an artificial metalloenzyme.[1–4] Several elegant examples of artificial metalloenzymes, some of which are capable of performing highly enantioselective reactions, have been reported. However, the majority of these examples rely on a limited number of protein scaffolds that have a binding pocket that is large enough to bind the catalyst and still leave space for the substrates. Examples include scaffolds, such as avidin, streptavidin, bovine serum albumin (BSA), and apomyoglobin.[5] An alternative approach to the design of artificial metalloenzymes involves creation of a new active site at an appropriate position in a protein scaffold, which is not necessarily an existing active site or binding pocket.[6,7] Herein we present a novel concept for the creation of artificial metalloenzymes, which involves the creation of an active site on the dimer interface of the transcription factor LmrR. With this artificial metalloenzyme up to 97% *ee* was achieved in the benchmark copper(II)-catalyzed Diels–Alder reaction (Figure 1a).

Dimerization of proteins is an important phenomenon in nature and is used as a convenient way to increase the complexity of the protein structure and to control activity.[8] Indeed, quite regularly, active sites of dimeric proteins are composed of residues from both monomeric polypeptides. Protein dimerization is the result of a combination of intermolecular interactions, with a key contribution made by hydrophobic interactions. This situation makes the protein dimer interface an attractive microenvironment for the creation of a new active site: the chirality of the second coordination sphere, which is composed of the dimer interface residues, combined with the hydrophobic nature of the dimer interface, which will facilitate the binding of organic substrates, should allow high catalytic activity and enantioselectivity in the catalyzed reaction. In a first design we have attempted the creation of an artificial metalloenzyme using a dimeric hormone peptide as the scaffold.[9] Despite achieving good enantioselectivities using this metallopeptide as catalyst, the introduction of the transition-metal catalyst into the scaffold caused a significant disruption of the structure and loss of the dimerization affinity, that is, the catalysts predominantly existed in the monomeric form.

For the present study, the Lactococcal multidrug resistance Regulator (LmrR), a transcriptional repressor that regulates the production of a major multidrug ABC transporter in *Lactococcus lactis*, was selected as the protein scaffold.[10,11] LmrR is a dimeric protein, with a size of 13.5 kDa per subunit, and contains a typical β-winged helix-turn-helix domain with an additional C-terminal helix involved in dimerization. A particularly interesting aspect of LmrR is that at the dimer center a large flat hydrophobic pore is present (Figure 1c). It was envisioned that a new active site could be created in this hydrophobic pore, which is large enough to incorporate a metal complex while still leaving enough space for the substrates to bind.

For the construction of the artificial metalloenzyme a covalent anchoring strategy utilizing cysteine conjugation was chosen.[12] Since the protein is a homodimer, covalent attachment will lead to two metal complexes being incorporated in the protein: one on each monomer. Based on the crystal structure of LmrR, positions 19 and 89 were selected for the covalent attachment of a Cu^{II} complex because these positions are located at the far ends of the hydrophobic pore, thus minimizing the chance that the metal complexes will interfere with each other (Figure 1c). In computer modeling, manual docking of the Diels–Alder product **4a** (see Scheme 1) bound to the Cu^{II} phenanthroline into LmrR suggested that the pore is indeed large enough for the reaction to occur inside (Supporting Information, Figure S6).

The LmrR N19C and M89C mutants were prepared by standard site-directed mutagenesis techniques (QuickChange). These cysteine residues are unique because no naturally occurring cysteine residues are present in LmrR. In addition, a sequence encoding a Strep-tag was added to the

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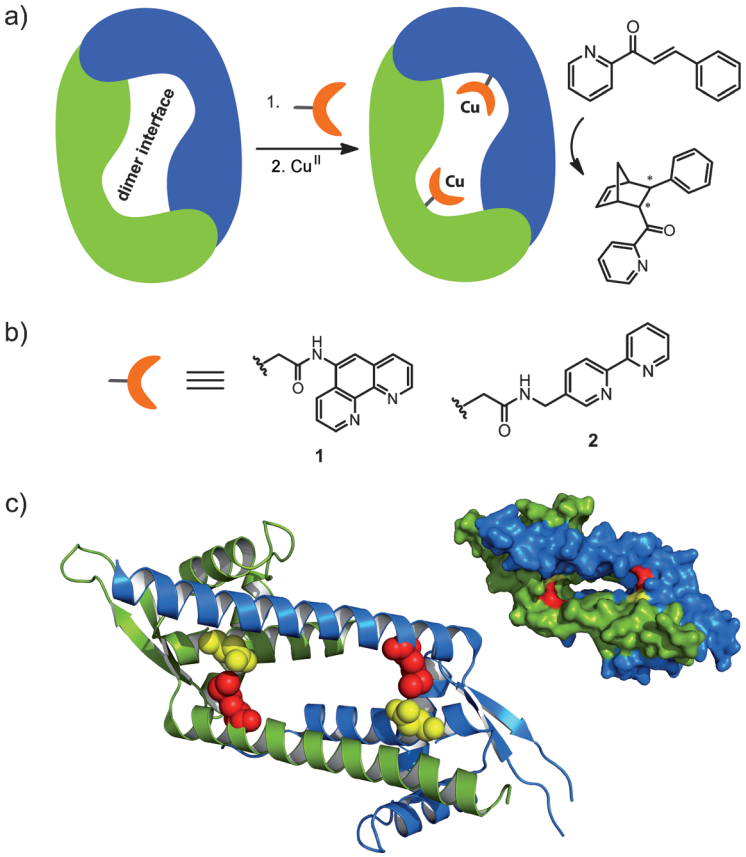


Figure 1. a) Schematic representation of the proposed artificial metalloenzyme in which a Cu^{II} complex is grafted on the dimer interface of a protein scaffold. b) Ligands used for grafting on the dimer interface. c) Pymol representations of dimeric LmrR in a ribbon and a space-filling model (protein data bank (pdb) code: 3F8B). Either position 89 (red) or 19 (yellow) were used for the covalent attachment of ligand **1** or **2**.

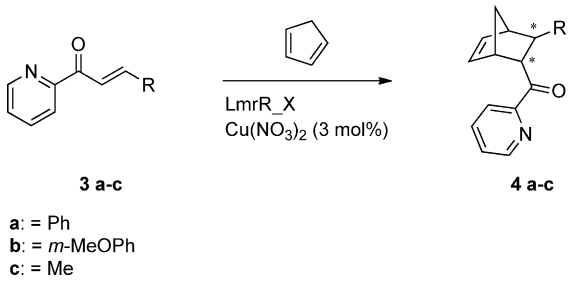
LmrR gene for purification purposes. The resulting constructs were expressed in *E. coli* BL21(DE3)C43 and subsequently purified by affinity chromatography (Strep-Tactin Sepharose column). Because LmrR is a transcription factor, an additional ion-exchange chromatography step using a Heparin column was necessary to remove residual bound DNA. The purity of the protein samples was determined by analytical SDS-PAGE (Figure S2). Typical yields of purified protein were 5–10 mg L^{-1} culture.

Phenanthroline- and bipyridine-based ligands (**1** and **2**, Figure 1 b), containing a bromo acetamide group for selective conjugation to cysteine, were synthesized. Ligand **1** was prepared by reaction of 1,10-phenanthroline-5-amine with bromoacetyl bromide (Scheme S1). For the synthesis of ligand **2** a classical Kröhnke synthesis was employed to make 5-methyl-2,2′-bipyridine, which was converted into 5-(aminomethyl)-2,2′-bipyridine by bromination, reaction with hexamethylenetetramine, and hydrolysis. Finally, reaction with bromoacetyl bromide resulted in ligand **2** (Scheme S1).

The conjugation of the phenanthroline- or bipyridine-based ligands to the LmrR mutants was achieved by treating LmrR with an eight-fold excess of ligand **1** or **2**. The reaction was carried out in the dark in degassed potassium phosphate buffer (50 mM, pH 7.75) containing 150 mM NaCl under nitrogen at 4 °C. After the reaction, excess of ligand was removed by preparative size exclusion chromatography. The coupling efficiencies were determined by the Ellman's test.[13] Typical coupling efficiencies were in the range of 80–90 %. The conjugation of compound **1** or **2** to LmrR M89C/N19C was further supported by electrospray ionization (ESI) mass spectrometry (Figure S4). In addition, the selective conjugation of ligand **1** to LmrR M89C was confirmed by a trypsin digest experiment, which shows that only the Cys89 residue was functionalized with ligand **1** (Figure S5).

The effect of the conjugation of ligands **1** or **2** to the LmrR scaffold on the quaternary structure was evaluated by analytical size exclusion chromatography. The LmrR ligand conjugates (LmrR_X) eluted as single band from a Superdex-75 10/300 GL size exclusion column with an apparent size of 29 kDa comparable to wild-type LmrR containing a Strep-tag. Also LmrR M89C_**1** containing Cu^{II} (LmrR M89C_**1**_Cu^{II}), in 3-(*N*-morpholine)propanesulfonic acid buffer (MOPS) containing 150 mM NaCl and in the presence of substrate **3a** (catalytic conditions) eluted with the same apparent size (Figure S3). These data show that the quaternary structure does not change significantly upon functionalization and thus LmrR_X is still dimeric.

The catalytic potential of LmrR_X_Cu^{II} was evaluated in the Diels–Alder reaction of azachalcone (**3a**) with cyclopentadiene as a benchmark reaction (Scheme 1).[14] The reactions were performed with 3 mol % of $Cu(H_2O)_6(NO_3)_2$ (30 μM) and with a slight excess (1.1 equiv) of the LmrR_X conjugate (taking into account the coupling efficiency) in MOPS buffer solution (20 mM, pH 7.0) containing 150 mM NaCl. These catalyst concentrations are significantly lower than what was used before in our DNA-[15] and peptide-based catalytic asymmetric Diels–Alder reactions.[9] The reaction was carried out for 3 days at 4 °C, and the Diels–Alder product **4a** was obtained as a mixture of *endo* and *exo* isomers as the only detectable products.



Scheme 1. Asymmetric Diels–Alder reaction catalyzed by LmrR_X/$Cu(NO_3)_2$ complexes in a MOPS buffered solution.

The conversion of **3a** using a Cu^{II} phenanthroline complex without the presence of LmrR was 20 %. When the reaction

Table 1: Results of Diels–Alder reactions catalyzed by copper LmrR_X complexes (Scheme 1).[a]

Entry	Catalyst	Product	Conversion [%]	*endo*:*exo*	*ee* (*endo*) [%]
1	LmrR_N19C_**1**_Cu^{II}	**4a**	24±3	92:8	53±5 (+)
2	LmrR_M89C_**1**_Cu^{II}	**4a**	93±4	95:5	97±1 (+)
3[b]	LmrR_M89C_**1**_Cu^{II}	**4a**	98±1	90:10	95±1 (+)
4	LmrR_M89C_**1**_Cu^{II}	**4b**	56±9	96:4	93±1 (+)
5	LmrR_M89C_**1**_Cu^{II}	**4c**	97±1	n.a.[c]	<5
6	LmrR_M89C_**2**_Cu^{II}	**4a**	55±8	63:37[d]	66±2 (−)
7	LmrR_M89C_V15A_**1**_Cu^{II}	**4a**	89±4	96:4	97±1 (+)
8	LmrR_M89C_D100E_**1**_Cu^{II}	**4a**	38±4	88:12	40±2 (+)
9	LmrR_M89C_D100E_**1**_Cu^{II}	**4b**	14±2	84:14	21±3 (+)
10	LmrR_M89C_Cu^{II}	**4a**	30±5	90:10	<5
11[e]	LmrR_9_Cu^{II}	**4a**	23±1	88:12	13±4 (+)
12[f]	LmrR_Phenanthroline_Cu^{II}	**4a**	29±8	90:10	28±7 (+)
13	Phenanthroline_Cu^{II}	**4a**	20±5	93:7	0
14	Phenanthroline_Cu^{II}	**4c**	full	95:5	0

[a] Typical conditions: 90% $Cu(H_2O)_6(NO_3)_2$ (3 mol%; 30 μM) loading with respect to LmrR_X in 20 mM MOPS buffer (pH 7.0), 150 mM NaCl, for 3 days at 4°C. Conversions and *ee* values are an average of two independent experiments, both carried out in duplicate. [b] Reaction carried out at room temperature. [c] *exo* peak could not be observed. [d] *ee exo* 90±2%. [e] Compound **9** was added 2:1 with respect to LmrR (wild-type). [f] Copper phenanthroline was added 2:1 with respect to LmrR (wild-type).

was carried out with LmrR_N19C_**1** an *ee* value of 50% was observed of the *endo* product (*endo*/*exo* 92:8), albeit also with a low conversion. Anchoring of the phenanthroline ligand to the 89 position, that is, using LmrR_M89C_**1**, resulted in a conversion of 93% and an excellent *ee* of 97% for the (+) enantiomer of the *endo* product (*endo*/*exo* 95:5) (entry 2, Table 1). Performing the reaction at room temperature only caused a small decrease in *ee* value (entry 3, Table 1).

The higher conversions obtained with the artificial metalloenzyme compared to Cu^{II}-phenanthroline alone indicate that the reaction is accelerated by the protein scaffold. Indeed, following the consumption of **3a** by UV/Vis spectroscopy showed that the reaction catalyzed by the artificial metalloenzyme is significantly faster than the reaction catalyzed by Cu^{II} phenanthroline alone (Figure S7). Addition of a fresh aliquot of reagents after the reaction was complete showed that LmrR_M89C_**2** was still active, albeit slightly less than before, indicating a small degree of inactivation over time (Figure S7b).

Interestingly, LmrR_M89C_**2**, which contains a conjugated 2,2′-bipyridine instead of a phenanthroline ligand, gave rise to 66% *ee* of the opposite, that is, the (−) enantiomer of the endo isomer of the Diels–Alder product **4a** (entry 6, Table 1). Hence, by judicious choice of the Cu^{II} binding ligand, both enantiomers of the Diels–Alder product can be accessed. Furthermore, with this artificial metalloenzyme significant amounts of the *exo* product were obtained also (*endo*/*exo* 63:37), with the *exo* product having 90% *ee*. No significant enantioselectivity was found in the control reaction performed with LmrR M89C having a free cysteine (without ligand **1** or **2**, entry 10, Table 1). Additionally, low enantioselectivities and conversions were found with wild-type LmrR supplemented with an analogue of **1**, that is, the propionamide derivative of 1,10-phenantroline-5-amine (**9**), and Cu^{II} phenanthroline (entry 12 and 13, Table 1). This result demonstrates that the covalent linkage of the ligand to the protein scaffold is required for selective catalysis. Using an excess of $Cu(H_2O)_6(NO_3)_2$ with respect to LmrR_M89C_**1** (ratio 2:1 Cu^{II}/monomer LmrR_M89C_**1**) resulted in precipitation of the protein.

Two residues in the pocket of LmrR_M89C_**1**, that is, Val15 and Asp100, were selected for a limited mutagenesis study because these residues are most likely situated in close proximity to the Cu^{II} complex. This selection was based on an X-ray structure of LmrR with a manually docked phenanthroline (Figure S6). The LmrR_M89C_V15A and LmrR_M89C_D100E mutants were prepared using standard QuickChange mutagenesis methods and the corresponding conjugates with ligand **1** were prepared and characterized as described above. The V15A mutation did not have an influence on the enantioselectivity of the catalyzed Diels–Alder reaction (entry 7, Table 1). In contrast, with the structurally conservative D100E mutation a significantly decreased conversion and *ee* value was obtained (entry 8 and 9, Table 1) suggesting that the catalyzed reactions are sensitive to the structure of the microenvironment of the pore of the LmrR scaffold.

The tolerance towards variation in the structure of the substrate was investigated using the α,β-unsaturated 2-acyl pyridine substrates **3b** and **3c**. When R = *m*-methoxyphenyl (**3b**) the same trend was observed as with azachalcone (**3a**): excellent *ee* values with LmrR_M89C_**1** and a significantly lower *ee* values with the D100E mutant (entries 4 and 9, Table 1). Using **3c**, which contains a methyl group at the β-position, full conversion was achieved but the Diels–Alder product was obtained without any significant *ee* value. The data obtained with this limited set of substrates suggests some substrate selectivity of artificial metalloenzyme, but more research is required to understand its structural origin.

The results show that the protein scaffold is not only a source of chirality for the reaction, but that it also causes an acceleration of the reaction rate, that is, the reaction is protein accelerated. Moreover, there appears to be a correlation between the rate and the enantioselectivity of the reaction, that is, the most enantioselective enzymes also gave rise to the highest conversions. Furthermore, both the conversion and *ee* values depend on the nature of the ligand, the position at which the ligand is anchored to the protein scaffold, and is also sensitive to mutation in the hydrophobic pocket. This demonstrates that the catalysis is dependent on the structure of the microenvironment around the catalytic Cu^{II} ion and these combined data strongly suggest that the reaction indeed takes place in the newly created active site in the hydrophobic pocket at the LmrR dimer interface. At present, the effect of the individual changes on the catalysis is difficult to rationalize, since LmrR shows some plasticity.[10] For this reason we are currently performing a detailed structural study of the LmrR-based artificial metalloenzyme.

In conclusion, we have presented a novel strategy towards the creation of an artificial metalloenzyme, which involves

grafting a new active site onto the dimer interface of the protein LmrR by conjugation of a bidentate ligand capable of binding Cu^{II} ions. The dimer interface provides the chiral microenvironment for the catalyzed reaction. The artificial metalloenzyme is capable of catalyzing Diels–Alder reaction with excellent enantioselectivities, that is up to 97% *ee*, and it was shown that the hydrophobic pocket of LmrR indeed functions as the active site where the reaction takes place. These results represent the first example of an artificial metalloenzyme in which the metal complex is linked covalently that gives rise to excellent enantioselectivities in catalysis. This novel approach towards the design of artificial metalloenzymes, significantly expands the possibilities and the number of potential scaffolds for artificial metalloenzyme design.

Experimental Section

Representative procedure for LmrR_X_Cu^{II} catalyzed reactions. To a solution of $Cu(H_2O)_6(NO_3)_2$ (30 μM) in 20 mM MOPS buffer (pH 7.0) at 0 °C was added LmrR_X (1.1 equiv based on $Cu(H_2O)_6(NO_3)_2$, variable concentrations depending on the coupling efficiency) to a final volume of 300 μL. A fresh stock solution (10 μL) of substrate in MOPS/CH_3CN was added (1 mM). After addition of freshly distilled cyclopentadiene (0.8 μL, 33 mM) at 0 °C, the reaction was mixed for 3 days by continuous inversion at 4 °C. The product was isolated by extraction with Et_2O (3 × 0.5 mL). The organic phases were dried (Na_2SO_4) and evaporated under reduced pressure to give the product. The conversion and *ee* value were determined by HPLC.

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