Supramolecular Assembly of Artificial Metalloenzymes based on the Dimeric Protein LmrR as Promiscuous Scaffold

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ABSTRACT: Supramolecular anchoring of transition metal complexes to a protein scaffold is an attractive approach to the construction of artificial metalloenzymes, since this is conveniently achieved by self-assembly. Here, we report a novel design for supramolecular artificial metalloenzymes that exploits the promiscuity of the central hydrophobic cavity of the transcription factor Lactococcal multidrug resistance Regulator (LmrR) as a generic binding site for planar coordination complexes that do not provide specific protein binding interactions. The success of this approach is manifested in the excellent enantioselectivities that are achieved in the Cu(II) catalyzed enantioselective Friedel-Crafts alkylation of indoles.

Artificial metalloenzymes open the way to engage the molecular recognition properties of biomolecules, such as proteins or DNA, in achieving enzyme-like activities and selectivities in transition metal catalysis.[1] While tremendous advances have been made already, the rational design of an artificial metalloenzyme for a given reaction requires knowledge of the relation between structure and activity that is still beyond the state of the art.[2] Indeed, the design of artificial metalloenzymes at present is focused first and foremost on the (re-)design of the metal binding site.[3] A popular approach involves the judicious positioning of a catalytically active transition metal complex within an existing protein scaffold.[1] This is often realized through supramolecular anchoring of the catalyst at a specific site in the protein, using metal complexes tethered via their ligand to high affinity binding motifs. This approach has been applied successfully in the design of a variety of artificial metalloenzymes, most notably the streptavidin/biotin system.[4,5] Yet, the nature of the tether and its position on the ligand place restrictions as to the structural flexibility and dynamics of the metal complex. As a consequence, the conformational space and second coordination sphere interactions that can be accessed during catalysis are also restricted.

Here, we report a novel design for supramolecular artificial metalloenzymes that exploits the extensive promiscuity of the central hydrophobic cavity of the protein Lactococcal multidrug resistance Regulator (LmrR) as a generic, moderate affinity binding site for planar coordination complexes that do not provide specific protein binding interactions. The success of this approach is manifested in the excellent enantioselectivities that are achieved in the Cu(II) catalyzed enantioselective Friedel-Crafts alkylation of indoles.

LmrR is a transcriptional repressor that is involved in the antibiotic resistance response of *Lactococcus lactis*.[6] It is a homodimeric protein with a size of 13.5 kDa per monomer and contains an unusual large hydrophobic pore at the dimer interface. Here, flat aromatic organic molecules can bind as is shown in X-ray and NMR structures of LmrR with various planar drugs bound.[7] Two tryptophan residues, one from each subunit, i.e. W96 and W96', play a key role in binding by sandwiching the organic molecules using π-stacking interactions (Figure 1). The LmrR structure has been reported to exhibit a broad and shallow conformation energy surface, in which the conformation can shift readily to accommodate structurally unrelated compounds without prerequisite specific binding interactions.[7b]

Recently, we have reported two new classes of artificial metalloenzymes containing a covalently linked Cu(II) complex at the dimer interface of LmrR.[8] These artificial metalloenzymes were shown to catalyze Diels-Alder, hydration and Friedel-Crafts alkylation reactions, with excellent ee's in the first and moderate ee's in the latter two reactions. These results demonstrated that the hydrophobic cavity of LmrR can accommodate many diverse substrates and reaction types. Encouraged by these results, it was decided to explore the supramolecular assembly of an LmrR-based artificial metalloenzyme, taking advantage of the promiscuity of the hydrophobic pocket at the dimer interface. We envisioned that Cu(II) complexes of planar aromatic ligands would be capable of binding partly or completely within the LmrR pocket, with the result that the catalyzed reaction takes place inside the chiral microenvironment provided by the LmrR hydrophobic pocket.

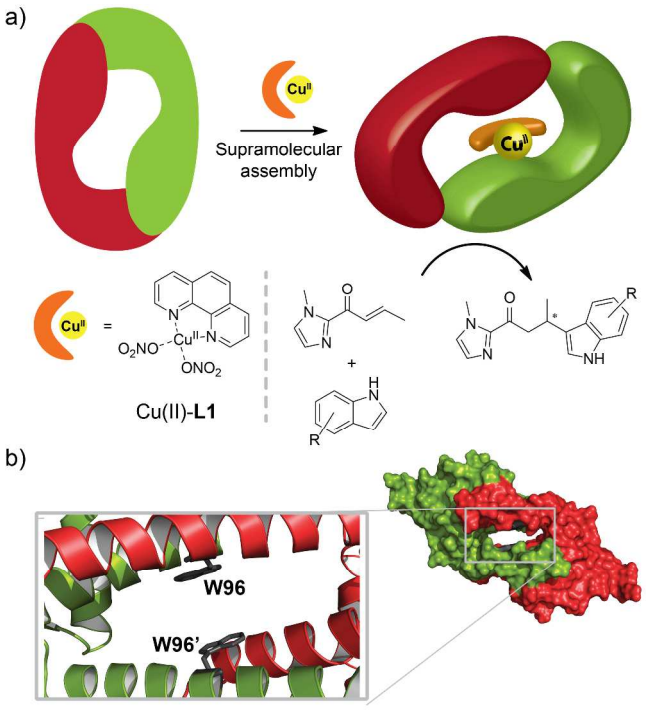
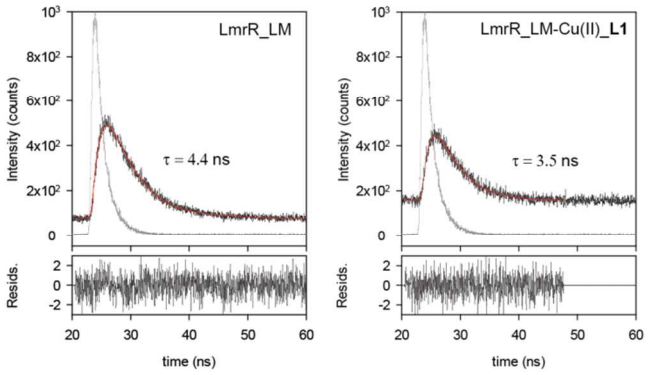


Figure 1. a) Schematic representation of the design of the supramolecular artificial metalloenzyme in which a Cu(II) complex is bound at the dimer interface of LmrR, followed by application in a catalyzed Friedel-Crafts alkylation. b) Space filled representation of dimeric LmrR (pdb 3F8B) and ribbon representation of the ligand binding pocket (inset). Tryptophan residues W96 and W96' are shown in gray. Pictures were generated with Pymol.

The LmrR variant used in this study, referred to as LmrR_LM, contains a C-terminal Strep tag to facilitate purification and two mutations in the DNA binding domain, i.e. K55D and K59Q, which facilitated expression and purification.[8b] A second mutant was prepared in which the tryptophan residues at the dimer interface were replaced by alanine, i.e. LmrR_LM_W96A. Both mutants were expressed in *E. coli* BL21(DE3)C43. Typical yields of purified protein were 20-30 mg L^{-1} culture.

The artificial metalloenzymes were prepared through self-assembly, by combining LmrR_LM with a Cu(II) complex of a planar aromatic ligand **L1-7** (Figure S1) in a buffered solution (20 mM MOPS, 150 mM NaCl, pH 7.0). The binding affinity of the Cu(II) complex to LmrR_LM was determined by titration through monitoring of the quenching of tryptophan fluorescence, taking into account the quenching of fluorescence of the tryptophan residues at the exterior of the protein, i.e. W67 and W119, (Figure S2), by using LmrR_LM_W96A as control (Figure S6). A dissociation constant (K_d) of 2.6±2 μM was determined for binding of Cu(II)-**L1** to LmrR_LM, which is significantly higher than the K_d reported for the tight-binding drugs H33342 and daunomycin (21±8 nM and 236±53 nM, respectively).[6] The binding of Cu(II)-**L1** to LmrR_LM_W96A mutant which does not contain tryptophan in the hydrophobic pocket, was significantly weaker: the K_d was determined to be 1 order of magnitude lower, i.e. 45 μM. Analytical size exclusion chromatography of the LmrR_LM⊂Cu(II)-**L1** and LmrR_LM_W96A⊂Cu(II)-**L1** hybrids was performed. Both eluted as a single band with an apparent molecular weight of 29 kDa, showing that the addition of Cu(II)-**L1** does not disrupt the homodimeric structure (Figure S7).[8]

The binding of Cu(II)-**L1** to LmrR_LM was studied further by fluorescence decay lifetime experiments. The tryptophan residues can be divided into two groups: those within the hydrophobic pocket (W96/W96') and those distant from the hydrophobic pocket (W67/W67'and W119/W119'). The observed fluorescence lifetime is a weighted sum of the individual lifetimes of both groups (note that these are too similar to be resolved reliably by a bi- or multi-exponential decay fit, given the much greater emission intensity of the tryptophans within the pocket, see SOI for further details), and was 4.4 ns in case of LmrR_LM (Figure 2, Figure S8). Upon saturation of LmrR_LM with Cu(II)-**L1**, i.e. with the latter at 44 μM, a decrease beyond experimental uncertainty in the fluorescence lifetime to 3.5 ns was observed. The final lifetime is close to the lifetime observed for LmrR_LM_W96A, i.e. the lifetimes for W67/W119, which is 3.7 ns (Figure 2). Combined, these data indicate that Cu(II)-**L1** binds predominantly in proximity to W96/W96', which strongly suggest it is bound at or near to the hydrophobic pore of LmrR (for a detailed discussion, see SOI)



Protein	[Cu(II)-**L1**) (μM)	Lifetime (ns)
LmrR_LM	0	4.4
LmrR_LM	44	3.5
LmrR_LM_W96A	0	3.7
LmrR_LM_W96A	44	3.7

Figure 2. Representative fluorescence decays (in black), IRF corrected exponential fits (in red) and residuals for LmrR_LM and LmrR_LM⊂Cu(II)-**L1**. In both cases the chi^2 values for the fits are ca. 1.01. Further addition of Cu(II) reduces emission intensity but has no additional effect on lifetime as expected for static quenching. The instrumental response function (IRF) was recorded using $BaSO_4$ (in gray).

The binding of a series of copper complexes of nitrogen based bidentate ligands (Cu(II)-(**L2**-**L7**)) (Table S1) to LmrR was studied also. The K_d for binding of these Cu(II)

complexes to LmrR_LM was found to be within the same order of magnitude as for Cu(II)-**L1**, suggesting that indeed, the hydrophobic pore of LmrR acts as a generic binding pocket and that significant specific interactions to the metal complex are not required,

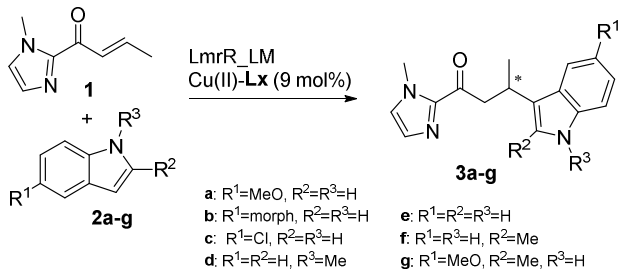
The potential of these new artificial metalloenzymes in catalysis was evaluated in the enantioselective vinylogous Friedel-crafts (FC) alkylation of 5-methoxy-1H-indole (**2a**) with 1-(1-methyl-1*H*-imidazol-2-yl)but-2-en-1-one (**1**) to generate **3a**.[9] This is a reaction that we previously showed to be compatible with dynamic catalytic assemblies.[9a] Artificial metalloenzymes were prepared *in situ* by self-assembly from 9 mol% of Cu(II)-**L1** (90 μM) with a slight excess (1.3 equiv.) of LmrR_LM in 3-(*N*-morpholine)propanesulfonic acid buffer (MOPS) (20 mM, pH 7.0), containing 150 mM NaCl, Based on the K_d more than 90% of Cu(II)-**L1** is bound to LmrR_LM under these conditions. The catalytic reaction was started by addition of 1 mM **1** and 5 mM indole **2a**. After 16 hours at 4 °C, a conversion of 57 % was observed and **3a** was obtained with an ee of 61 % in the (+) enantiomer (Table 1, entry 1). Interestingly, a strong dependence of the results of catalysis on the indole concentration was observed. When the concentration of **2a** was decreased 5 fold, *i.e.* to 1 mM, the conversion and ee increased to 84% and 75%, respectively (Table 1, entry 2). Further decreasing the concentration of indole to 0.5 mM, i.e. using a ratio of **1**:**2a** of 1:0.5, resulted in an ee of 84% (Table 1, entry 3) This suggests that the indole competes with Cu(II)-**L1** for binding to LmrR_LM,[10] i.e., at higher indole concentrations Cu(II)-**L1** is displaced from the hydrophobic pore of LmrR_LM. This results in an increased fraction of unbound Cu(II)-**L1** that catalyzes the reaction in a racemic fashion, causing a decrease in ee of **3a**.

Artificial metalloenzymes based on Cu(II)-(**L2**-**L7**) provided similar enantioselectivies in the catalyzed reaction. The presence of a ligand is required, since ee was not obtained when using LmrR_LM + $Cu(NO_3)_2$ (Table S2).

Using LmrR_LM_W96A ⊂ Cu(II)-**L1** as catalyst in the reaction of **1** and **2a** resulted in moderate conversion and <5% ee of **3a**. (entry 11). This is in agreement with the weaker binding affinity of Cu(II)-**L1** in the absence of W96/W96', *i.e.* under the conditions of catalysis only a small fraction of the Cu(II) complex is actually bound to the LmrR_LM_W96A.

The substrate scope of LmrR_LM ⊂ Cu(II)-**L1** was evaluated by variation of the 2-acyl imidazole and of the indole. The artificial metalloenzyme did not tolerate larger subsitutents on the β position of the enone (Table S3). In contrast, a broad scope of indoles **2** were accepted. Replacing the methoxy group at the 5 position of the indole with an *N*-morpholine moiety (**2b**) resulted in a similar ee, whereas a chloride at this position (**2c**) resulted in a lower ee (Table 1, entries 4-5). With *N*-methyl indole (**2d**) the ee decreased to 18% (table 1, entry 6). The best results were obtained with nonsubstituted indole (**2e**) and 2-methyl indole (**2f**), that is, full conversion of **1** and an excellent ee of 94% (R-enantiomer) and 93% for the corresponding products **3e** and **3f**, respectively (Table 1, entry 7-9). The double substituted indole **2g** gave results comparable to **2a**, albeit that additional unidentified products were detected (Table 1, entry 10, Figure S4). The ee's achieved are significantly higher than those obtained with our previous artificial metalloenzyme design containing a covalently linked bipyridine ligand.[8a]

Table 1. Results of Friedel-Crafts alkylation reactions catalyzed by LmrR-based artificial metalloenzymes



entry	indole	eq. indole	product	conversion (%)[b]	ee (%)[c]
*LmrR_LM ⊂ Cu(II)-**L1**:*					
1	**2a**	5	**3a**	57±7	61±8(+)
2	**2a**	1	**3a**	84±5	75±13(+)
3	**2a**	0.5	**3a**	90±2[d]	84±3(+)
4	**2b**	1	**3b**	94±4	82±1
5	**2c**	1	**3c**	19±8	64±7(+)
6	**2d**	1	**3d**	79±2	18±2(-)-R
7	**2e**	1	**3e**	full	94±0(+)-R
8	**2f**	1	**3f**	full	93±1
9	**2f**	0.5	**3f**	full[d]	93±1
10	**2g**	1	**3g**	56±25[e]	81±1
*LmrR_LM_W96A ⊂ Cu(II)-**L1**:*					
11	**2a**	1	**3a**	53±28	<5

[a] Typical conditions: 75% Cu(II)-**L1** (9 mol%; 90 μM) loading with respect to LmrR_LM or LmrR_LM_W96A, 1 mM **1**, 0.5-5 mM **2,** in 20 mM MOPS buffer (pH 7.0), 150 mM NaCl, at 4°C for 16 h (**2a**) or 64 h (other indoles). Conversions and *ee* values are an average of at least two independent experiments, both carried out in duplicate. [b] Conversions are determined by HPLC and are based on substrate **1**. [c] Sign of rotation and absolute configuration based on elution order in chiral HPLC.[9] [d] Conversion is based on substrate **2**.[11] [e] Additional unidentified products were detected (Figure S4).

The combined data of the binding studies, fluorescence lifetime experiments and catalysis demonstrate that the catalysis takes place in the hydrophobic pore of LmrR-LM and that tryptophan residues W96 and W96' in the newly created active site are of key importance to the catalysis. Possibly, they are involved in intercalation-like binding of the Cu(II) complex.[12] Alternatively, instead of binding the metal complex, it is also possible that either the enone or the indole substrates are bound between the tryptophan residues via π stacking, analogous to the observed binding mode of H33342 and daunomycin[6], and are thus posi-

tioned for the reaction, resulting in enantioselectivity. This model is in agreement with the observed preference in catalysis for planar conjugated molecules such as **1a** and unsubstituted indole **2e**. The observed competition between **2a** and Cu(II)-L1 in binding LmrR implies that both substrates and Cu(II) complex prefer to bind, at least partly, in the same location, which suggests significant dynamics during catalysis.

In conclusion, we have introduced a novel design of an artificial metalloenzyme, created by supramolecular binding of a catalytically active Cu(II) complex in the hydrophobic cavity the dimer interface of LmrR. A key element of the present design is the promiscuity of the hydrophobic cavity of LmrR that can accept many compounds, including substrates and catalytically active transition metal complexes, without requiring specific ligand - protein interactions, as is the case in most supramolecular artificial metalloenzyme designs. The success of this approach was manifested in the catalyzed asymmetric vinylogous Friedel-Crafts alkylation of indoles in water, giving rise to excellent ee's. The results of this study suggest that precise design of second coordination sphere interactions may not always be the most appropriate approach *a priori* to achieving high selectivity in artificial metalloenzyme catalysis, provided that substrates and metal complexes have enough freedom to find themselves the optimum orientation and interactions in a promiscuous chiral space. The present design is highly flexible and is envisioned to be adapted readily for binding other metal complexes and catalysis of other reactions. It is notable also that LmrR is the first scaffold that has been used successfully in multiple anchoring strategies, thus underlining the versatility of LmrR as a scaffold for artificial metalloenzymes.

ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures, characterization data and additional results of catalysis experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Due to the strong dependence on the indole (**2**) concentration, relatively large errors were sometimes observed in the conversions.

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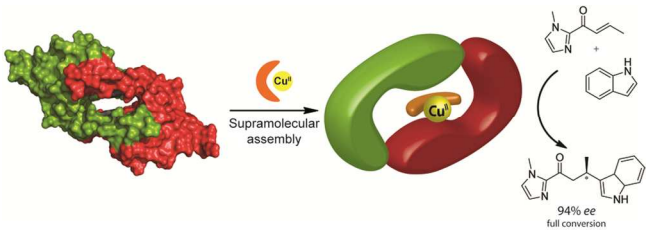

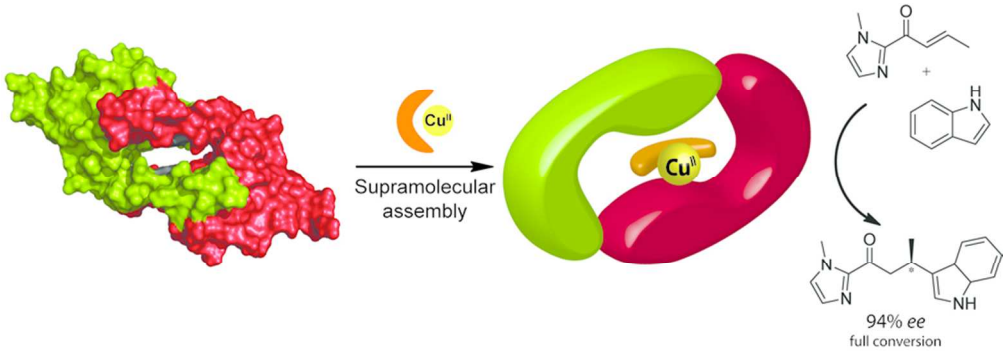

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