



Cite this: *Dalton Trans.*, 2017, **46**, 4325

Received 13th February 2017,  
Accepted 27th February 2017

DOI: 10.1039/c7dt00533d

rsc.li/dalton

## A metal ion regulated artificial metalloenzyme†

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Regulation of enzyme activity is essential in living cells. The rapidly increasing number of designer enzymes with new-to-nature activities makes it necessary to develop novel strategies for controlling their catalytic activity. Here we present the development of a metal ion regulated artificial metalloenzyme created by combining two anchoring strategies, covalent and supramolecular, for introducing a regulatory and a catalytic site, respectively. This artificial metalloenzyme is activated in the presence of  $\text{Fe}^{2+}$  ions, but only marginally in the presence of  $\text{Zn}^{2+}$ .

### Introduction

Control over enzymatic activity is vital for regulating the complex network of biosynthetic pathways in living cells. An important strategy employed by nature to regulate enzyme activity is allosteric regulation, that is binding of an effector molecule or metal ion at a site in the protein other than the active pocket.<sup>1–3</sup> As a result of this interaction, the catalytic activity of an enzyme and, hence, its activity in a biosynthetic pathway can be regulated by the presence or absence of chemical signals in the environment. Such allosteric regulation would also be desirable for controlling the activities of designer enzymes with new-to-nature activities. Additionally, such artificial allosteric enzymes could also be of interest for other applications, for example as sensors. In this work we present an artificial metalloenzyme that is selectively activated by a specific metal ion, that is  $\text{Fe}^{2+}$ , but not by addition of  $\text{Zn}^{2+}$ .

Artificial metalloenzymes are hybrid catalysts that incorporate non-natural metal cofactors into biological scaffolds, thereby giving access to enzyme-like catalysts with new-to-nature catalytic activities.<sup>4–7</sup> In recent years, this strategy has been utilized to create a wide variety of artificial metalloenzymes that display non-natural activities within a biological environment. However, examples of such hybrid enzymes that can be activated by mechanisms akin to allosteric control remain scarce. Indeed, the only example for this type of activation comes from Ward and colleagues, who has recently demonstrated that the activity of an artificial asymmetric trans-

fer hydrogenase could be upregulated by proteolysis when a natural protease is used as an external stimulus.<sup>8</sup>

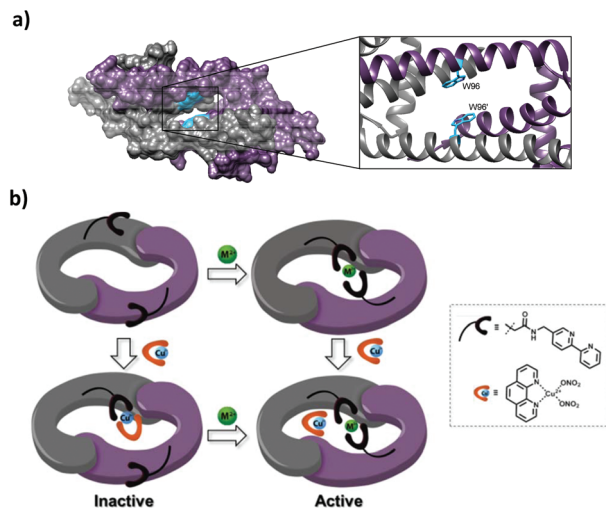
We have introduced a novel class of artificial metalloenzymes based on the transcriptional regulator LmrR (Lactococcal multidrug resistance Regulator). For this protein a new active site can be created within the hydrophobic cavity that is present at the dimer interface.<sup>9,10</sup> An attractive feature of LmrR as biomolecular scaffold for artificial metalloenzymes is that it is the only scaffold reported so far amenable to three different anchoring strategies for incorporation of non-natural cofactors: covalent and supramolecular anchoring and unnatural amino acid incorporation.<sup>11–14</sup> The resulting hybrid catalysts have been applied in a variety of catalytic reactions including enantioselective Diels Alder, water addition to  $\alpha,\beta$ -unsaturated ketones and Friedel–Crafts alkylation reactions.

For the creation of a metal regulated artificial metalloenzyme, we took advantage of the design versatility of LmrR and combined two anchoring strategies for binding transition metal complexes: incorporation of a catalytic site *via* supramolecular interactions and introduction of a regulatory site *via* covalent anchoring of a ligand. The incorporation of the catalytic site *via* the supramolecular approach is based on the binding of  $\text{Cu}^{2+}$  complexes of planar aromatic ligands (such as phenanthroline) between the two tryptophans located inside the hydrophobic pocket at the dimer interface of LmrR at positions 96 and 96' (Fig. 1a). This approach was applied to catalysis of the enantioselective Friedel–Craft alkylation of  $\alpha,\beta$ -unsaturated imidazoles to indoles using  $\text{Cu}^{2+}$  phenanthroline complexes, such as  $[\text{Cu}(\text{phen})(\text{NO}_3)_2]$ , obtaining good conversions and excellent enantioselectivities of >90% in several cases.<sup>14</sup>

The introduction of the regulatory site *via* covalent anchoring of a metal binding moiety to LmrR is based on the site-selective modification of the protein using unique cysteines as bioconjugation handles for alkylation reactions.<sup>11,12</sup> Since

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† Electronic supplementary information (ESI) available: Full experimental details and characterization, results of control and additional experiments. See DOI: 10.1039/c7dt00533d



**Fig. 1** (a) Surface representation of the front entrance of LmrR (PDB 3F8F).<sup>10</sup> Zoom in of part of the hydrophobic pocket at the dimer interface. Tryptophans in position 96 and 96' are shown in blue. (b) Schematic representation of the concept of the metal regulated artificial metalloenzyme.

LmrR is a homodimeric protein, the introduction of a ligand in a specific position *via* covalent anchoring will lead to functionalization of both monomers, resulting in a dimeric protein containing two metal binding moieties.

## Results and discussion

Our design of a metal ion regulated artificial metalloenzyme is depicted in Fig. 1b. In absence of an effector metal ion, the metal binding moieties covalently bound to LmrR will bind the catalytically active complex  $[\text{Cu}(\text{phen})(\text{NO}_3)_2]$ . As a result, the two free coordination sites on the  $\text{Cu}^{2+}$  ion required for substrate binding are blocked, rendering the artificial metalloenzyme inactive. Conversely, addition of transition metal ions that bind stronger than  $\text{Cu}^{2+}$ , would trap the metal binding moieties into stable chelate complexes, allowing the active  $\text{Cu}^{2+}$  complex to bind and activate the substrates (Fig. 1b).

Previous work with LmrR based metalloenzymes showed that enantioselective reactions take place inside the hydrophobic pocket in close proximity to the front entrance. Therefore it was envisioned that the formation of chelate metal complexes in this area might also have an effect on the catalysis.<sup>11,12</sup>

2-2'-Bipyridine was selected as metal binding moiety due to its ability to strongly bind late-first row transition metal ions with high affinity and in different stoichiometries.<sup>15–19</sup> The bipyridine ligands were introduced on solvent exposed positions on the  $\alpha 4$  helices, which delimit the front entrance of the hydrophobic pocket. Introduction of bipyridine ligands on these helices was envisioned to allow the formation of chelate complexes involving one bipyridine moiety of each monomer.

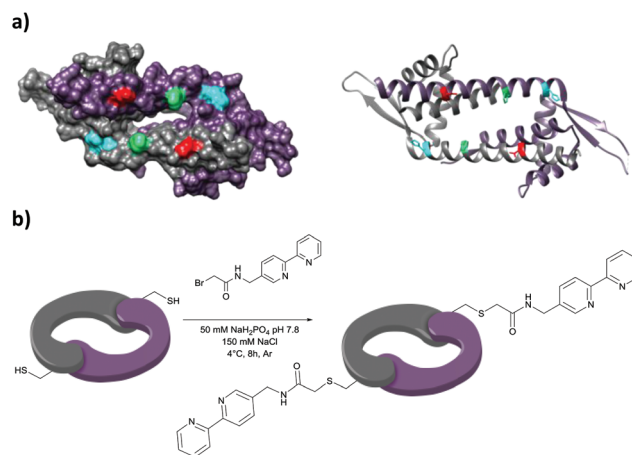
Positions 93 and 104, located in the central part of the front entrance, and position 86, which is located towards the edge of the pocket, were chosen for the introduction of the bipyridine ligands (Fig. 2a).

Plasmids encoding C-terminally Strep-tagged cysteines mutants of LmrR (F93C, E104C, H86C) were prepared by standard site-directed mutagenesis technique (Quick-Change) from a vector available from previous work.<sup>11</sup>

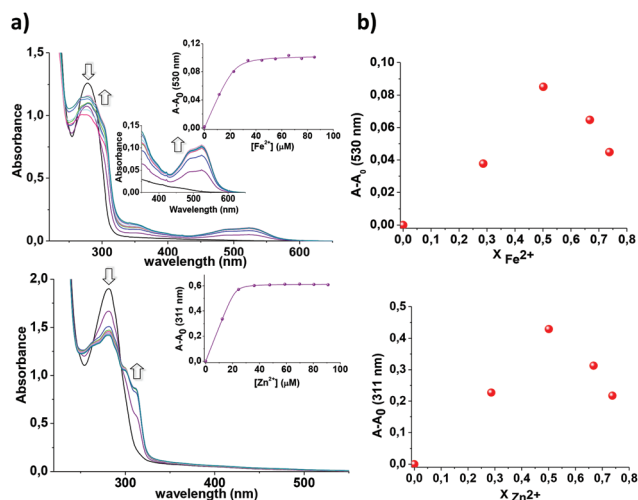
LmrR mutants were subsequently expressed in *E. coli* BL21 (DE3) C43 and purified by affinity chromatography (Strep-tactin Sepharose column) and cation exchange chromatography (Heparin column) to remove residual bound DNA. Target proteins were obtained in good yields ( $10\text{--}20\text{ mg L}^{-1}$ ) with excellent purity as judged by Tricine-SDS-PAGE, UPLC-MS and analytical size exclusion chromatography (Fig. S1†).

Bipyridine units were introduced by selective alkylation of cysteines with a bromo acetamide derivative of 2-2'-bipyridine (Fig. 2b).<sup>11,12</sup> UPLC-MS and Tricine-SDS-PAGE were used to confirm identity and purity of LmrR bipyridine conjugates and analytical size exclusion chromatography demonstrated that the dimeric structure of LmrR was not significantly altered by introduction of the metal binding moieties (Fig. S2–S4†).

With the LmrR bipyridine conjugates in hand, first the binding of these conjugates to  $\text{Zn}^{2+}$  and  $\text{Fe}^{2+}$  was studied by UV-visible titrations. These two ions are examples of divalent late-first row transition metal ions with different coordination properties. Upon addition of either metal ion, the appearance of a shoulder around 310 nm was observed, indicative of a change in the  $\pi\text{--}\pi^*$  transition of the bipyridine upon metal coordination (Fig. 3a and S5†). This red shift has previously been reported for bipyridine ligands in solution and for proteins containing a bipyridine moiety.<sup>18,22,23</sup> In the titration with  $\text{Fe}^{2+}$  also the appearance of two additional absorption bands between 490 and 530 nm was observed, which are characteristic of  $\text{Fe}^{2+}$  bipyridine complexes (Fig. 3a and S5†).<sup>17,19,24,25</sup> The resulting titration



**Fig. 2** (a) Surface and helix representation of LmrR front entrance. Residues H86, F93, E104 are shown in blue, green and red, respectively. (b) Reaction scheme of the synthesis of protein–metal binding moieties conjugates. Ligand used for alkylation reaction on LmrR cysteine mutants: *N*-([2,2'-bipyridin]-5-ylmethyl)-2-bromoacetamide (**1**).<sup>20,21</sup>



**Fig. 3** (a) UV-visible titrations of LmrR E104C\_bpy with  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (top) and  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (bottom). Titrations were performed in 50 mM  $\text{NaH}_2\text{PO}_4$  pH 7.0, 500 mM NaCl with 22.5  $\mu\text{M}$  LmrR\_bipyridine conjugate solutions (dimer) with subsequent additions from 1 mM solutions of metal salts in milliQ water. Inset containing the fitting of the titration data obtained with non-linear regression. (b) Job's plot graphs of LmrR E104C\_bpy with  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (top) and  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (bottom). Plots of  $\Delta A$  at 530 nm for  $\text{Fe}^{2+}$  and 311 nm for  $\text{Zn}^{2+}$  against the molar fraction ( $x$ ) of the  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$ , respectively. 100  $\mu\text{M}$  stock solutions of LmrR conjugates in 50 mM  $\text{NaH}_2\text{PO}_4$  pH 7.0, 500 mM NaCl and 200  $\mu\text{M}$  of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  or  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  in milliQ water. Total concentration in the cuvette was kept constant at 60  $\mu\text{M}$ .

curves could be fitted to a 1 : 1 binding model, corresponding to the formation of complexes containing one metal ion per LmrR dimer (Table S2†). The stoichiometry of the metal : protein complexes was further confirmed by Job's plot (Fig. 3b and S6†), in which the maximum complex formation was obtained with equimolar concentrations of metal ions and LmrR dimer. ICP-AES experiments performed after incubation of the LmrR\_E104C\_bipyridine conjugate with  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$ , followed by dialysis to remove unbound metal ions, also suggested the presence of one equivalent of metal ion per LmrR dimer (Table S4†).

Combined these observations suggest the formation of a chelate metal complex between one metal ion and two bipyridines in the LmrR scaffold. Exogenous water molecules or ligands supplied by amino acid side chains might complete the coordination sphere around the metal. The formation of a chelate complex between the two bipyridines and the metal ions was expected for the conjugates containing bipyridine units located in the central part of the front entrance (LmrR F93C\_bpy and LmrR E104C\_bpy), given the proximity of the two amino acids in the protein dimer (10–15 Å from the reported crystal structure).<sup>10</sup> However, for the conjugate in which the bipyridine units are located towards the edge of the hydrophobic pocket (LmrR H86C\_bpy) the formation of a chelate metal complex appeared unlikely due to the distance between the two residues in position 86 (28–30 Å).<sup>10</sup> Tentatively, chelation of the metal ions could be achieved due

to the known flexibility of the LmrR scaffold.<sup>10</sup> The cost for formation of these rather unfavoured complexes is reflected in a lower binding affinity for both regulatory metals complexes (Table S2†) and an apparent lower stability of the metal-bound protein, which was prone to precipitation. However, also the possibility of formation of chelate metal complexes with bipyridine units from separate LmrR dimers should be considered, as suggested by the presence of higher order aggregates in the size exclusion chromatography of some  $\text{Fe}^{2+}$  conjugates (LmrR E104C\_bpy and LmrR H86C\_bpy) (Fig. S4†).

The catalytic activity of the LmrR\_bipyridine conjugates and their metal complexes, was evaluated in the enantioselective, vinylogous Friedel–Crafts alkylation of 1-(1-methyl-1*H*-imidazol-2-yl)but-2-en-1-one (**1**) with 2-methyl-1*H*-indole (**2**).<sup>13,14,26–29</sup>

The metal complexes of LmrR bipyridine conjugates with  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  (LmrR\_bpy\_M<sup>2+</sup>) were prepared fresh prior to catalysis by incubation of 1 equivalent of the corresponding metal salt with 1 equivalent of the LmrR\_bpy conjugate (dimer) in 20 mM MOPS, pH 7.0, 500 mM NaCl (4 °C, 30 minutes). The mixtures were centrifuged to remove possible precipitate and the supernatant containing the LmrR\_bipyridine\_M<sup>2+</sup> complex was used for the supramolecular assembly of the artificial metalloenzyme. The artificial metalloenzymes were prepared *in situ* by self-assembly from  $[\text{Cu}(\text{phen})(\text{NO}_3)_2]$  (22  $\mu\text{M}$ ) with a slight excess (1.3 equivalents) of LmrR\_bipyridine\_M<sup>2+</sup> complex (30  $\mu\text{M}$ ) in 20 mM MOPS, pH 7.0, 500 mM NaCl. The mixture was incubated at 4 °C for 30 minutes and the catalytic reaction was initiated by addition of substrates **1** and **2** at a final concentration of 1 mM. Reactions were incubated at 4 °C for 72 h, after which the products were isolated and analyzed by chiral HPLC.

As previously reported, the reaction of the  $\alpha$ - $\beta$  unsaturated acyl imidazole **1** with 2-methyl indole **2** catalyzed by the assembly of  $[\text{Cu}(\text{phen})(\text{NO}_3)_2]$  with wt-LmrR is protein accelerated and results in excellent enantioselectivity up to 92% (Table 1, entries 1 and 2).<sup>14</sup> As expected, using the LmrR\_bipyridine conjugates in absence of any regulatory metal ion resulted in no significant catalytic activity of the artificial metalloenzymes with LmrR F93C\_bpy and LmrR E104C\_bpy, and low activity in case of LmrR H86C\_bpy (Table 1, entries 3–5). This is due to the fact that the free coordination sites of the  $\text{Cu}^{2+}$  ion in the  $[\text{Cu}(\text{phen})(\text{NO}_3)_2]$  complex are sequestered by the conjugated bipyridine ligands, thus preventing substrate binding (Fig. 1b). In case of LmrR H86C\_bpy, this sequestration is apparently not perfect, for reasons not understood at present.

Formation of metal complexes with either  $\text{Fe}^{2+}$  or  $\text{Zn}^{2+}$  prior to catalysis, resulted in almost no product formation for the conjugate LmrR F93C\_bpy (Table 1, entries 6 and 7). The observed lack of catalytic activity might be due to bulkiness of the chelate metal complexes located right in the middle of the front entrance of the hydrophobic pocket that does not allow the substrates to reach the active  $\text{Cu}^{2+}$  center located inside. This hypothesis is supported by the observation that no specific binding of the dye Hoechst 33342 (H33342), which is known to bind inside the hydrophobic pocket of LmrR *via* the

**Table 1** Results of vinylogous Friedel–Crafts alkylation reactions catalyzed by LmrR\_bpy conjugates and their respective Fe<sup>2+</sup> and Zn<sup>2+</sup> complexes

| Entry | Catalyst                                       | Yield <sup>a</sup> (%) | TON <sup>b</sup> | ee <sup>c</sup> (%) |
|-------|--|------------------------|------------------|---------------------|
| 1     | Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> (only) | 39 ± 4                 | 16 ± 2           | <5                  |
| 2     | wt-LmrR  | 58 ± 3                 | 24 ± 1           | 92 ± 1              |
| 3     | LmrR F93C_bpy                                  | ≤1                     | —                | n.d.                |
| 4     | LmrR E104C_bpy                                 | ≤1                     | —                | n.d.                |
| 5     | LmrR H86C_bpy                                  | 7 ± 1                  | 3 ± 1            | 81 ± 4              |
| 6     | LmrR F93C_bpy + Fe <sup>2+</sup>               | 2 ± 0                  | 1 ± 0            | n.d.                |
| 7     | LmrR F93C_bpy + Zn <sup>2+</sup>               | 2 ± 1                  | 1 ± 1            | n.d.                |
| 8     | LmrR E104C_bpy + Fe <sup>2+</sup>              | 34 ± 4                 | 14 ± 2           | 75 ± 6              |
| 9     | LmrR E104C_bpy + Zn <sup>2+</sup>              | 4 ± 0                  | 2 ± 0            | n.d.                |
| 10    | LmrR H86C_bpy + Fe <sup>2+</sup>               | 26 ± 6                 | 11 ± 2           | 88 ± 3              |
| 11    | LmrR H86C_bpy + Zn <sup>2+</sup>               | 14 ± 2                 | 6 ± 1            | 80 ± 10             |

Typical conditions: 2.2 mol% [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] (22 μM) loading with 1.3 eq. LmrR\_bpy conjugates (30 μM), 1 mM of substrate **1** and **2** in 20 mM MOPS buffer pH 7.0, 500 mM NaCl, for 72 h at 4 °C. All the results listed correspond to the average of two independent experiments, each carried out in duplicate. Errors are listed as standard deviations. <sup>a</sup> Yields were determined by HPLC and using 2-phenylquinoline as internal standard. <sup>b</sup> Turnover numbers (TON) were determined by dividing the concentration of product by the catalyst concentration. <sup>c</sup> For yields below 5% ee's were not determined.

front entrance, to LmrR F93C\_bpy was observed using fluorescence spectroscopy (Fig. S7†).<sup>10</sup>

We were pleased to find that upon incubation with the regulatory metal ions (Fe<sup>2+</sup> or Zn<sup>2+</sup>) prior to addition of the active [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] complex, two of conjugates, *i.e.* LmrR E104C\_bpy and LmrR H86C\_bpy, showed catalytic activity (Table 1, entries 8–10), albeit lower than with wt-LmrR. These results suggest that the activity of these artificial metalloenzymes can be regulated by the presence of metal ions.

It was observed consistently that activation with Fe<sup>2+</sup> yielded more active artificial metalloenzymes when compared to the addition of Zn<sup>2+</sup>: higher turnover numbers (TON) were achieved for the artificial metalloenzymes containing Fe<sup>2+</sup> complexes compared to the respective Zn<sup>2+</sup> complexes (Table 1, entries 8–11). It was hypothesized that the difference in activity might reflect the different interactions of the active transition metal complex ([Cu(phen)(NO<sub>3</sub>)<sub>2</sub>]) with the two types of metal complexes (Fe<sup>2+</sup> and Zn<sup>2+</sup> complex). Nevertheless, as described above, the Fe<sup>2+</sup> complexes of these two conjugates, in addition the dimeric form of LmrR, appeared to form higher order aggregates as well. At this moment it is not clear what effect these aggregates have on catalysis.

As this difference in activity is particularly pronounced for the LmrR E104C\_bpyridine conjugate, this variant was selected for further investigation. According to the Irving–Williams series, six-coordinate octahedral Cu<sup>2+</sup> complexes are thermodynamically more stable than other high spin divalent first-row transition metal complexes. Hence, in our design, the Cu<sup>2+</sup> from the active [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] complex could replace Zn<sup>2+</sup> from the high spin bipyridine complexes, forming a mixed complex with phenanthroline and the covalently bound bipyridine as ligands. As this additional interaction would prevent substrate binding, the resulting artificial metallo-

enzyme becomes inactive for the desired reaction. Conversely, Fe<sup>2+</sup> is known to predominantly form low spin complexes with bipyridine ligands, which are kinetically more stable and, thus, less prone to ligand substitution. Therefore, exchange of Fe<sup>2+</sup> by Cu<sup>2+</sup> will not occur and the artificial metalloenzyme will remain active.<sup>30,31</sup>

To test this hypothesis, reactions were performed using an excess of Fe<sup>2+</sup> or Zn<sup>2+</sup> ions. The LmrR\_E104C\_bpy conjugates were incubated with 1, 3 and 6 equivalents of metal ions and in case of Zn<sup>2+</sup> also 10 equivalents (with respect to LmrR dimer) before the artificial metalloenzyme was assembled by addition of [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>]. As expected, when additional Fe<sup>2+</sup> equivalents were added, no significant change in catalytic activity was observed. In contrast, when going from 1 to 10 equivalents of Zn<sup>2+</sup>, an increase in activity was observed from 7 to 12 TON. With increasing amounts of Zn<sup>2+</sup>, also an erosion of the ee was observed, which most likely is due to structural changes of the chiral active site as a result of non-specific Zn<sup>2+</sup> binding to the protein.

The possibility of metal exchange between the regulatory metal ion (Fe<sup>2+</sup> or Zn<sup>2+</sup>) and the catalytic metal ion (Cu<sup>2+</sup>) was further investigated by ICP-AES measurements. LmrR\_E104C\_bpy\_M<sup>2+</sup> complexes were incubated with Cu<sup>2+</sup> ions, followed by dialysis to remove non-bound metal ions (Fig. 4).

Quantification of the regulatory metal (Fe<sup>2+</sup> or Zn<sup>2+</sup>) bound to the protein after incubation with [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] did not show a change in the total amount of Fe<sup>2+</sup> or Zn<sup>2+</sup> (Table S3†) because metal exchange will result in the formation a mixed Cu<sup>2+</sup> phenanthroline bipyridine complex, while the regulatory metal might still be bound to the second bipyridine in the LmrR scaffold (Fig. 5a). In order to push the equilibrium towards a complete metal replacement, leading to a Cu<sup>2+</sup> bisbipyridine complex and the release of the regulatory metal ion,



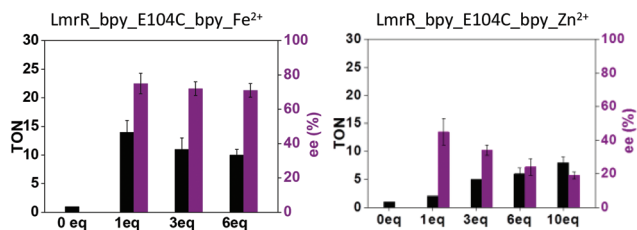


Fig. 4 Comparison of turnover numbers (black) and enantioselectivity (purple) in the vinylogous Friedel–Crafts alkylation reactions catalyzed by LmrR\_bpy\_E104C\_bpy conjugate and respective Fe<sup>2+</sup> and Zn<sup>2+</sup> complexes in presence of increasing concentration of Fe<sup>2+</sup> (left) and Zn<sup>2+</sup> (right). Same reaction conditions as in Table 1.

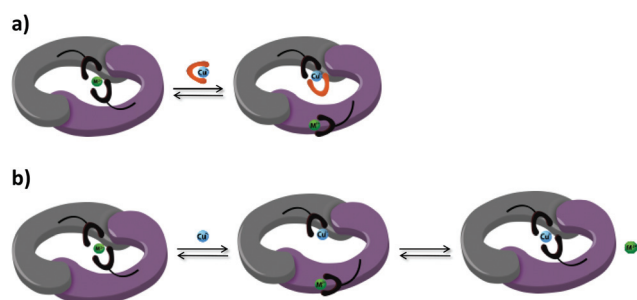


Fig. 5 Schematic representation of metal ion exchange between regulatory metal ion (M<sup>2+</sup>) and the catalytic metal ion (Cu<sup>2+</sup>) with [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] and Cu(NO<sub>3</sub>)<sub>2</sub>.

incubation of the LmrR\_bpy-M<sup>2+</sup> complexes with Cu(NO<sub>3</sub>)<sub>2</sub> was performed (Fig. 5b).

Quantification of the regulatory metal (Fe<sup>2+</sup> or Zn<sup>2+</sup>) bound to the protein after incubation with Cu(NO<sub>3</sub>)<sub>2</sub> showed a significant decrease in the amount of Zn<sup>2+</sup> bound to the protein (Table S3 and Fig. S8†), confirming the hypothesis of partial replacement of Zn<sup>2+</sup> with Cu<sup>2+</sup> with subsequent inactivation of the catalyst. The observation that the metal displacement reaction does not seem to happen for the Fe<sup>2+</sup> complex supports the formation of a stable low spin Fe<sup>2+</sup> bisbipyridine complex within the LmrR scaffold.<sup>31</sup>

Finally the reversibility of the designed metal regulated artificial metalloenzyme was tested by inverting the order of

addition of the regulatory and catalytic metal ions. LmrR\_bipyridine conjugates were first incubated with [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] at 4 °C for 30 minutes and then 1 equivalent of Fe<sup>2+</sup> or Zn<sup>2+</sup> salt was added. After incubation of the resulting mixture for 30 minutes substrates **1** and **2** were added at a final concentration of 1 mM. In parallel, exactly the same procedure was applied to the LmrR\_E104C\_bpy conjugates by incubating first the regulatory metal ions (Fe<sup>2+</sup> or Zn<sup>2+</sup>) and then the active catalyst [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>]. Reactions were incubated at 4 °C for 72 h, after which the products were isolated and analyzed by chiral HPLC. We were delighted to observe that catalytic activity (as well as enantioselectivity) could be restored to a large extent upon addition of Fe<sup>2+</sup> salt to the inactive metalloenzyme (Table 2). In contrast, addition of Zn<sup>2+</sup> salt did not restore the activity. This result suggests that selective regulation of activity of the designed artificial metalloenzyme by Fe<sup>2+</sup> ions is reversible.

## Conclusions

The results presented here show that we successfully designed, synthesized and characterized a metal ion regulated artificial metalloenzyme. By combining a regulatory site to bind an effector metal ion and an active site to recruit a catalytically active metal complex in the LmrR scaffold, the activity of the artificial metalloenzyme (LmrR\_E104C\_bpy) for a vinylogous Friedel–Crafts alkylation could be regulated by incubation with Fe<sup>2+</sup> ions. Reminiscent of allosteric regulation in natural enzymes, we achieved selective activation by Fe<sup>2+</sup> but not by Zn<sup>2+</sup>, taking advantage of the different coordination properties of these transition metals. This study represents the first example of a metal ion regulated artificial metalloenzyme and presents a significant advance toward controlling the activity of designer enzymes in hybrid bio-synthetic pathways.

## Acknowledgements

This work was supported by European Research Council (ERC Starting Grant 280010). Financial support from the Ministry of Education, Culture, and Science (Gravitation Program

Table 2 Effect of the order of incubation on the results of vinylogous Friedel–Crafts alkylation reactions catalyzed by the [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>]/LmrR\_bpy\_E104C\_bpy conjugate

| Entry | 1 <sup>st</sup> incubation                 | 2 <sup>nd</sup> incubation                 | Yield <sup>a</sup> (%) | TON <sup>b</sup> | ee <sup>c</sup> (%) |
|-------|--|--|------------------------|------------------|---------------------|
| 1     | [Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> ] | —  | ≤1                     | ≤1               | n.d.                |
| 2     | Fe <sup>2+</sup>                           | [Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> ] | 57 ± 14                | 23 ± 5           | 72 ± 14             |
| 3     | [Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> ] | Fe <sup>2+</sup>                           | 33 ± 5                 | 14 ± 2           | 78 ± 2              |
| 4     | Zn <sup>2+</sup>                           | [Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> ] | 13 ± 2                 | 6 ± 1            | 50 ± 1              |
| 5     | [Cu(phen)(NO <sub>3</sub> ) <sub>2</sub> ] | Zn <sup>2+</sup>                           | 11 ± 3                 | 5 ± 1            | 50 ± 13             |

Typical conditions: 2.2 mol% [Cu(phen)(NO<sub>3</sub>)<sub>2</sub>] (22 μM) loading with 1.3 eq. LmrR\_bpy conjugates (30 μM), 1 mM of substrates **1** and **2** in 20 mM MOPS buffer pH 7.0, 500 mM NaCl, for 72 h at 4 °C. All the results listed correspond to the average of two independent experiments, each carried out in duplicate. Errors are listed as standard deviations. <sup>a</sup> Yields were determined by HPLC and using 2-phenylquinoline as internal standard. <sup>b</sup> Turnover numbers (TON) were determined by dividing the concentration of product by the catalyst concentration. <sup>c</sup> For yields below 5% ee's were not determined.

no. 024.001.035) is gratefully acknowledged. The authors thank I. Drienovská and Dr L. Villarino for useful discussion and suggestions.

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