Dynamics of metal complex binding in relation to catalytic activity and selectivity of an artificial metalloenzyme

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Abstract

Structural dynamics is important in enzymes to achieve optimal complementarity to the activated complex of the catalyzed reaction and, hence, give rise to rate acceleration and (enantio)selectivity. Here, we present an artificial metalloenzyme based on the transcriptional regulator LmrR that exhibits a unique form of structural dynamics involving the positioning of its abiological metal cofactor. The position of the cofactor, in turn, was found to be related to the preferred catalytic reactivity, which is either the enantioselective Friedel-Crafts alkylation of indoles with β-substituted enones or the tandem Friedel-Crafts alkylation / enantioselective protonation of indoles with α -substituted enones. The artificial metalloenzyme could be specialized for one of these catalytic reactions by introducing a single mutation in the protein. The switching of catalytic activity by dynamic interconversion of the position of a metal cofactor has not been described for natural enzymes and, to date, appears to be unique to supramolecularly assembled artificial metalloenzymes.

Main text

Enzymes are remarkable catalysts, capable of catalyzing chemical transformations with high rates and selectivities. Key to this is their ability to provide structural complementarity of the active site to the activated complex of the catalyzed reaction.[1] The dynamics of the protein involve conformational changes, e.g. domain rearrangement, loop motions, partial folding/unfolding etc., crucial to reach the optimal structure of the active site. Those flexible regions are also frequently involved in the emergence of alternative active site structures and relate to promiscuous catalytic activities. Thus they are important targets for mutagenesis in the natural- or directed evolution of enzymes to improve the activity.[2–6]

A popular approach to achieve enzymatic catalysis of reactions that have no equivalent in nature involves the creation of artificial metalloenzymes, which are rationally designed hybrids of proteins with abiological catalytically active metal cofactors.[7–13] In this approach, the basal catalytic activity is supplied by the metal complex, whereas the second coordination sphere interactions provided by the protein scaffold are envisioned to contribute to rate acceleration and (enantio-)selectivity. Since the protein scaffolds used have not naturally evolved for the reaction of interest, usually the active site structure is far from optimal. Recent examples underscore that structural dynamics in artificial metalloenzymes can be important in this context, similar to natural enzymes.[14,15]

Here, we report that dynamics in the binding position of an abiological metal cofactor in an artificial metalloenzyme leads to alternative active site structures. We also show how different reactions catalyzed by the artificial metalloenzyme, and its substrate selectivity, are dependent on the position of the cofactor in the protein scaffold. Finally, by a single mutation the artificial metalloenzyme can be specialized towards either of these catalytic reactivities.

The design of the artificial metalloenzyme is based on the transcription factor Lactococcal multidrug resistance Regulator (LmrR), which is a homodimeric protein with a size of 13.5 kDa per monomer that contains an unusual large hydrophobic pore at the dimer interface.[16] This hydrophobic pore serves as a promiscuous binding pocket where planar aromatic molecules bind, as shown in X-ray and NMR structures of LmrR with various planar drugs bound.[16–18] Two tryptophan residues, one from each subunit, i.e., W96 and W96', play a key role in binding by sandwiching the guest molecule via π-stacking interactions. Previously, we have shown that this arrangement is attractive for the supramolecular self-assembly of a novel artificial metalloenzyme, by combining the protein LmrR with a Cu(II) complex with a planar aromatic ligand, like 1,10-phenanthroline (phen) (Fig. 1a).[19,20] LmrR showed a moderately strong affinity for Cu(II)-phen, with a dissociation constant (K_d) of 2.6 ± 2 μM. The importance of the central tryptophans for binding Cu(II)-phen is illustrated by the fact that in case of the mutant LmrR_W96A, the K_d was one order of magnitude lower, i.e., 45 μM.

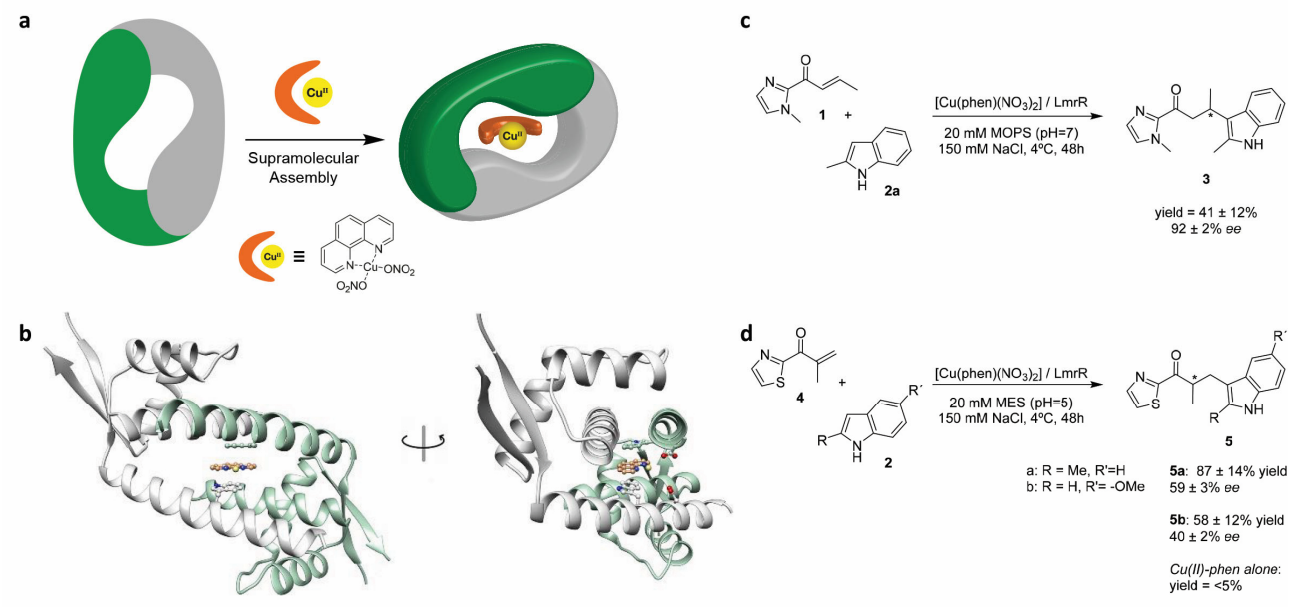


Figure 1. a) Schematic representation of the self-assembly of the artificial metalloenzyme. b) Crystal structure of the LmrR/Cu(II)-phen artificial metalloenzyme (PDB: 6R1L). Considerable disorder is observed in the binding mode of Cu-phen, as evidenced by its relatively weak associated electron density and high atomic B-factors. Disordered ligand binding is a general observation in crystal structures of LmrR and may be an inherent property of this protein. Unfortunately, the weak electron density around the copper, and its special position in the crystal on a crystallographic dyad, prohibited an unambiguous identification of its coordination geometry and ligands other than phenantroline.. c) catalyzed FC reaction (d) catalyzed FC/EP reaction. C and D: Typical reaction conditions: enone **1** or **4** (1mM), indole **2** (1mM) [Cu(II)-phen] (9 mol%; 90 µM) , LmrR (12 mol%; 120 µM) in 20 mM MOPS buffer pH 7.0 (FC reaction) or 20 mM MES buffer pH 5.0 (FC/EP reaction), 150 mM NaCl, at 4°C; Results are the average of at least two independent experiments, both carried out in duplicate. Error margins are listed as standard deviations.

The binding of Cu(II)-phen to LmrR was confirmed by X-ray crystallography, which showed the phenanthroline ligand of the complex sandwiched between W96 and W96', with the indole rings somewhat tilted with respect to each other and the Cu(II) complex (Fig. 1b). The Cu(II) ion is facing the front entrance of the pore. Two carboxylate side chains, from D100 and D100' are oriented towards the Cu(II) ion at a distance of ~5 Å and may interact with bound ligands, e.g. water, at the remaining coordination sites at the copper, albeit that these could not be identified with certainty. Protein residues other than W96 and D100 that surround the copper within a distance of 8 Å are predominantly hydrophobic, i.e., V15, A92, S97, V99 and I103 (and their equivalents from the dimer mate)

In this study, we focused on the application of the artificial enzymes in two catalytic reactions. First, the previously reported enantioselective vinylogous Friedel-Crafts alkylation of indoles with α, β unsaturated 2-acyl-imidazoles (FC reaction), which gives rise to excellent enantioselectivities, to up to 92% when catalyzed by LmrR/Cu(II)-phen (Fig. 1c).[19] The second reaction is the tandem Friedel-Crafts alkylation/enantioselective protonation reaction (FC/EP reaction, Fig. 1d). It involves the conjugate addition of indoles to α-substituted enones. In this case, the chirality is introduced not in the conjugate addition step, but in the protonation step and, hence, this reaction represents an enantioselective protonation in water, which is a highly challenging

reaction.[21] These two reactions share similiraties, but the chiral center is created in different elementary reaction steps.[22]

The conjugate addition of 5-methoxy-1H-indole (**2b**) to 2-methyl-1-(thiazol-2-yl)prop-2-en-1-one (**4**) was used as benchmark FC/EP reaction (Fig. 1d). The artificial metalloenzyme was prepared *in situ* by self-assembly from 9 mol% of [Cu(phen)$(NO_3)_2$] with a slight excess (1.3 equiv) of LmrR in MES buffer at pH 5.0. (Table S3). Under these conditions, the product was obtained in 58 % yield and 40 % ee. Interestingly, in absence of LmrR, so when using Cu(II)-phen alone, very low conversion was observed (Table S4, entry 2). This shows that the reaction is highly protein accelerated, that is, it requires the presence of LmrR to occur. Evaluation of the indole scope showed that the best results were obtained using 2-methyl-1H-indole (**2a**), with 87% yield and 59% *ee* (Table S3, entry 4).

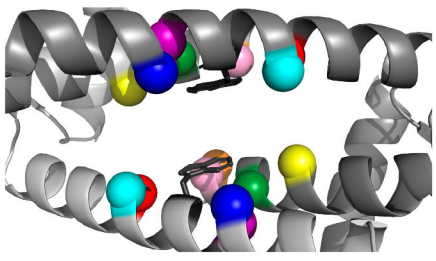
Mutagenesis of residues at various positions in the hydrophobic pocket in spatial proximity to W96 was performed to establish where catalysis of both these reactions occur and which residues are important for activity. This included positions at the front entrance, i.e. D100, F93 and A92, residues in the pocket interior, i.e. M8, Q12 and V99 and residues V15, E7, which are placed in the back entrance of the pocket. Most of these residues were probed by converting them to alanine (alanine scanning), except at position A92 were alanine was already present. In this case mutation to glutamate, A92E was performed.

All mutants were evaluated in both the FC reaction of 2-methyl-indole (**2a**) with *(E)*-1-(1-methyl-*1H*-imidazol-2-yl)but-2-en-1-one (**1**) and the tandem FC/EP reaction of **2a** with 2-methyl-1-(thiazol-2-yl)prop-2-en-1-one (4).

In the FC reaction significant effects on catalysis were observed in case of the front entrance mutants and the mutant M8A, which is located inside the pore (Fig. 2, Table S5). These results confirm that the reaction occurs in the pore, at the front entrance, close to the tryptophan residues where the Cu(II)-phen complex is bound. In most cases, the effect of the mutation was negative on both activity and enantioselectivity. Two mutations gave rise to significantly improved enantioselectivity: the mutation M8A resulted in a strong increase in the *ee* to 99% (Table S5, entry 11) and A92E gave rise to both complete enantioselectivity (>99 % *ee*) and a significantly increased product yield (Table S5, entry 6).

In contrast, almost all of these mutations had only a small effect on the results of the FC/EP reaction (Fig. 2b, Table S6). The only mutation that gave rise to a significant, negative, effect proved to be A92E; a decrease of the *ee* to 21 % was obtained (Table S6, entry 7). This was a surprising result since this same mutation proved to be the most beneficial for the FC reaction (vide supra).

a



b

Mutant:	ee (%) FC	ee (%) FC/EP
Wild type	92 ± 2	58 ± 2
D100A	46 ± 4	63 ± 1
F93A	51 ± 5	66 ± 2
A92E	> 99	21 ± 7
W96A	25 ± 9	64 ± 6
Q12A	98 ± 1	44 ± 6
V99A	95 ± 1	60 ± 4
M8A	99 ± 0	45 ± 1
V15A	94 ± 1	42 ± 1
E7A	96 ± 1	56 ± 1

c

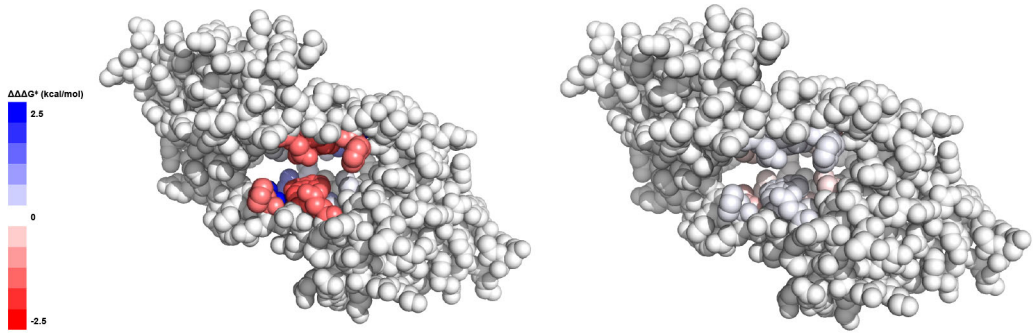


Figure 2. a) Close up of the hydrophobic pore of the LmrR. Residues used in the mutagenesis study are highlighted as spheres (color code as indicated). b) Effect of mutations in LmrR on the *ee* of the catalyzed FC reaction between **1** and **2a** and FC/EP reaction between **4** and **2a**. Colors represent the difference between the $\Delta\Delta G^{\ddagger}$ values calculated from the corresponding ee's as defined in (c); c) Visualization of the mutated residues, and the effects on enantioselectivity, onto the crystal structure of LmrR/Cu(II)-phen (metal complex omitted for clarity). The effect of the mutation on the *ee,* compared to the wild-type LmrR, is visualized as a heatmap where the colors represent the difference between the $\Delta\Delta G^{\ddagger}$ values calculated from the corresponding ee's ($\Delta\Delta\Delta G^{\ddagger} = \Delta\Delta G^{\ddagger}_{mutant} - \Delta\Delta G^{\ddagger}_{wild\ type}$), indicating an increase (blue) or decrease (red) of enantioselectivity of the reaction catalyzed by the mutant compared to the wild type LmrR.

The role of the hydrophobic pocket in catalysis was probed by inhibition studies using Hoechst H33342, which has been shown to bind with nanomolar affinity in the pocket, sandwiched between W96 and W96', analogous to binding of Cu(II)-phen as observed by X-ray crystallography.[16] Hence, H33342 can act as an inhibitor, blocking the binding of Cu(II)-phen, which in view of the fact that both reactions are protein accelerated, should have an effect on the results of catalysis. Indeed, addition of increasing amounts of H33342 up to 4 equivalents with respect to Cu(II)-phen caused a significant decrease in the enantioselectivity in the FC reaction, to 57% *ee*. In contrast, no significant effect was observed in the case of the FC/EP reaction (Table S7).

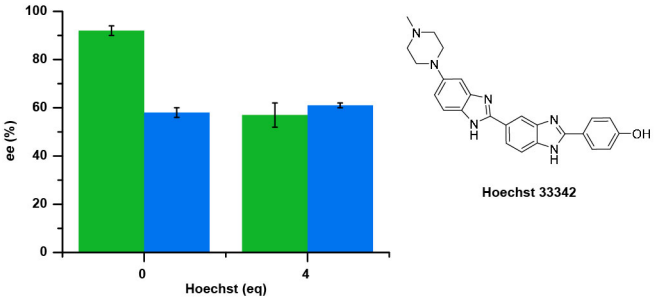


Figure 3. *ee* obtained in catalysis of the FC (green) and FC/EP (blue) reaction in presence of 0 and 4 equivalents of Hoechst 33342, with standard deviations shown.

Next, competition experiments were performed with the substrates **1** and **4** , using wild type LmrR and the mutants LmrR_W96A and LmrR_A92E (Figure 4, Figure S7). When 2-methylindole (**2a**) was combined with equimolar amounts of **1** and **4**, the corresponding products **3** and **5** were obtained in 66% and 17% yield, respectively, in the reaction catalyzed by LmrR/Cu(II)-phen, which corresponds to a selectivity of 80 % for the FC reaction. The enantioselectivity of the products was similar to that obtained in the independent experiments. Notably, when we carried out these competition experiments with the LmrR variant A92E, the selectivity for the FC reaction increased to 96 %; product **3** was obtained in 66% yield and 98% *ee*, while only trace amounts of nearly racemic product **5** were obtained. In contrast, when we used the mutant LmrR_W96A, the FC/EP reaction became the dominant activity, with a selectivity of 76 %; product **5** was obtained with 22 % yield and 69% *ee*. These results show how with one single mutation, either one of these catalyzed reactions can be made the dominant activity of the artificial metalloenzyme.

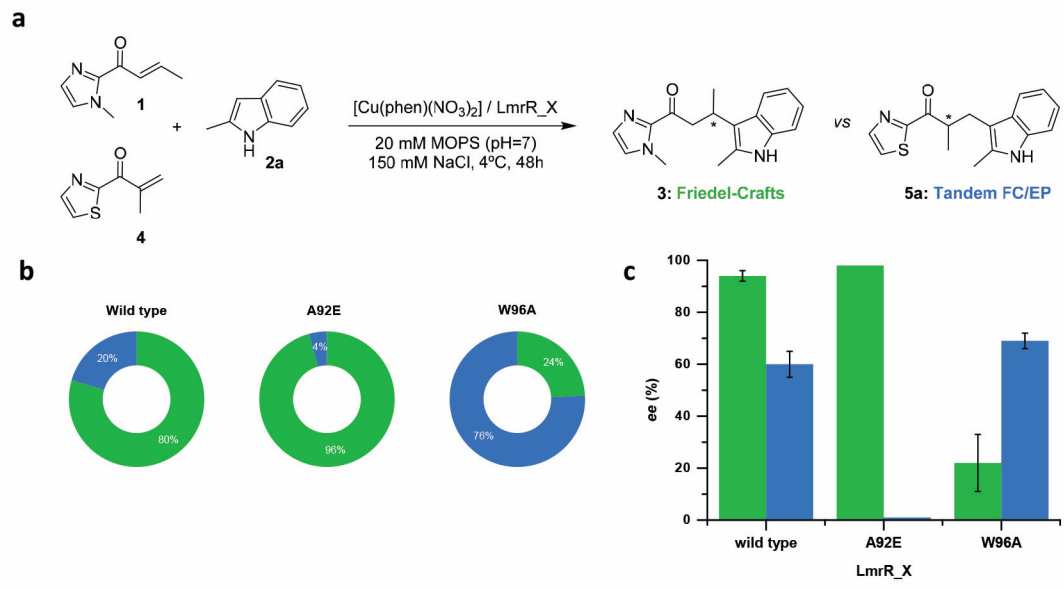


Figure 4. Competition experiment between FC reaction of **1** with **2a** (and FC/EP reaction of **4** with **2a**) catalyzed by LmrR/Cu(II)-phen, LmrR_A92E/Cu(II)-phen and LmrR_W96A/Cu(II)-phen. All substrates were present in equimolar amounts (1 mM). B) Relative product distribution (%) of the competing FC (green) and FC/EP reactions (blue). C) *ee* values for products of the FC and FC/EP reaction in the competition experiment catalyzed by LmrR mutants with standard deviations shown.

While the effect of the W96A mutation is clear, that is, it eliminates a crucial part of the Cu(II)-phen binding site, the role of the glutamate residue in the A92E mutant was less obvious. The binding affinity of the $[Cu(phen)(NO_3)_2]$ was determined (SI, Section VII) and a dissociation constant (K_d) of 65 ± 19 nM and 59 ± 16 nM was found at pH=7 and pH=5, respectively. This represents a two order of magnitude increase in binding affinity compared to WT LmrR (Table S8). Our initial hypothesis was that the carboxylate moieties would contribute to binding of the copper complex by interaction with the Cu(II) ion. For this reason, the corresponding glutamine mutant (i.e., A92Q) was prepared, since glutamine is sterically similar to glutamate, but is not a good ligand for Cu(II). However, the A92Q mutant also showed an increased affinity for Cu(II)-phen (K_d 103 ± 41 nM) (Figure S9). The increased affinity of the A92E mutant for Cu(II)-phen also allowed to determine the apparent catalytic efficiency of this improved mutant for the FC reaction: k_{cat}/K_M = 73.3 M^{-1} min^{-1}(Figure S10). This information could not be obtained for the wild type LmrR-based artificial metalloenzyme since, in case of the wild type protein, the binding affinity of the Cu(II)-phen complex is moderate. At higher concentrationsthis causes the substrates to start displacing the Cu(II)-phen complex, which leads to a significant decrease of the catalytic activity.[19] Unfortunately, due to substrate solubility issues, the individual Michaelis Menten parameters k_{cat} and K_M could not be determined.

Computation was then used to gain a better understanding about the effect of mutation A92E. For this purpose, the bis aqua form of the copper bound phenanthroline cofactor $[Cu(phen)(H_2O)_2]^{2+}$ was optimized via quantum calculations and embedded into the WT, A92E and A92Q variants of LmrR via protein-ligand docking (see details in Supporting Information). The best scored structures, which in all cases showed the copper-phenanthroline moiety at the centre of the cavity packed between tryptophans W96/W96', consistent with the X-ray structure, were submitted to 300 ns MD simulation.

The MD simulations for WT LmrR showed that hydrophobic interactions between A92 and V15 contribute to a somewhat closed arrangement of the active site, in which the indole rings of W96/W96' are slightly tilted with respect to each other, in agreement with the X-ray structure (Figure 5, left). Hence, the π-stacking interactions between the indole rings of residues W96/W96' and the phenanthroline ligand are not optimal. Hydrogen bonding interactions between the water ligands and residues D100/D100' contribute to maintain the positioning of the copper cofactor.

Instead, according to the MD simulations, the mutation of the alanine to glutamate in the A92E mutant disrupts the hydrophobic interaction and generates a hydrogen bonding network at the back of the active site, involving mainly residues N88 of helix α4 and N14, located at the opposite side of the cavity in helix α1 (Figure S12 and S13, left). This contributes to a different structural arrangement of the pore, with a parallel orientation of the W96/W96' indoles and an expanded hydrophobic free volume of the cavity (Figure S18, S19). The result is a better packing of the Cu(II)-phen complex by a dual π -stacking interaction from W96/W96'. Tentatively, this is related to the increased binding affinity, and causes the Cu(II)-phen being

positioned deeper inside the pore (Figure S20). While these structures are found frequently in the A92E mutant (40% of the MD simulation), they are virtually non-existent in case of the WT protein (1% of the MD simulation). Similar structures were observed for the A92Q mutant, albeit that these were less frequent than for A92E mutant (Supporting information). These results suggest that the A92E mutation does not have a direct effect on catalysis, but mainly has a structural effect, which translates a stronger binding of the Cu(II)-phen complex.

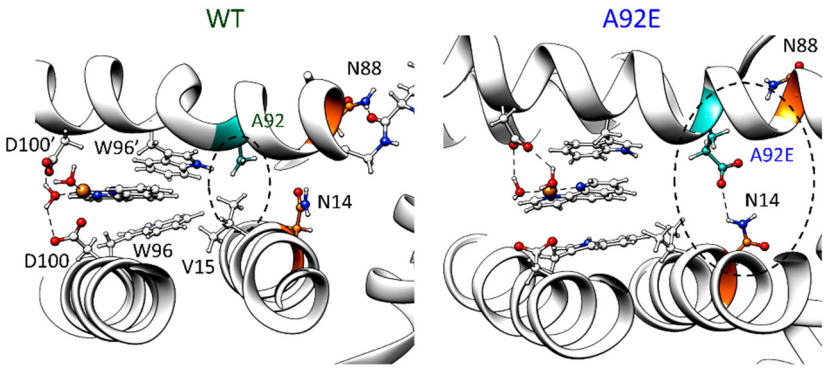


Figure 5. Comparison between representative structures of the pore along 300 ns of MD simulation for WT (left) and A92E (right) variants of LmrR with Cu(II)-phen bound. For WT, hydrophobic interactions between A92 and V15 promote closing of the active site, resulting in a not optimal π-stacking between both W96/W96' and the phenanthroline ligand of the Cu(II)-phen cofactor. In contrast, the A92E mutant enables polar interactions with N14, which contribute to the opening of the active site and a parallel orientation of the W96/W96' residues and, thus, cause a better binding of the phenanthroline ligand via π-stacking.

Combined, the data unambiguously shows that the two reactions, FC and FC/EP, do not occur at the same site in the LmrR protein. In other words, there are two – possibly more – different active sites, which each are more suited for either the FC or FC/EP reaction. The mutagenesis data and inhibition studies show that the FC reaction occurs in the hydrophobic pocket near the front entrance, close to the central tryptophans where the Cu(II)-phen complex preferentially binds. In contrast, the FC/EP reaction does not occur here. Yet, the fact that the reaction does not occur, or only very slowly, in the absence protein, combined with the enantioselectivity of product **5**, shows that the FC/EP reaction does occur at another position in or on the protein.

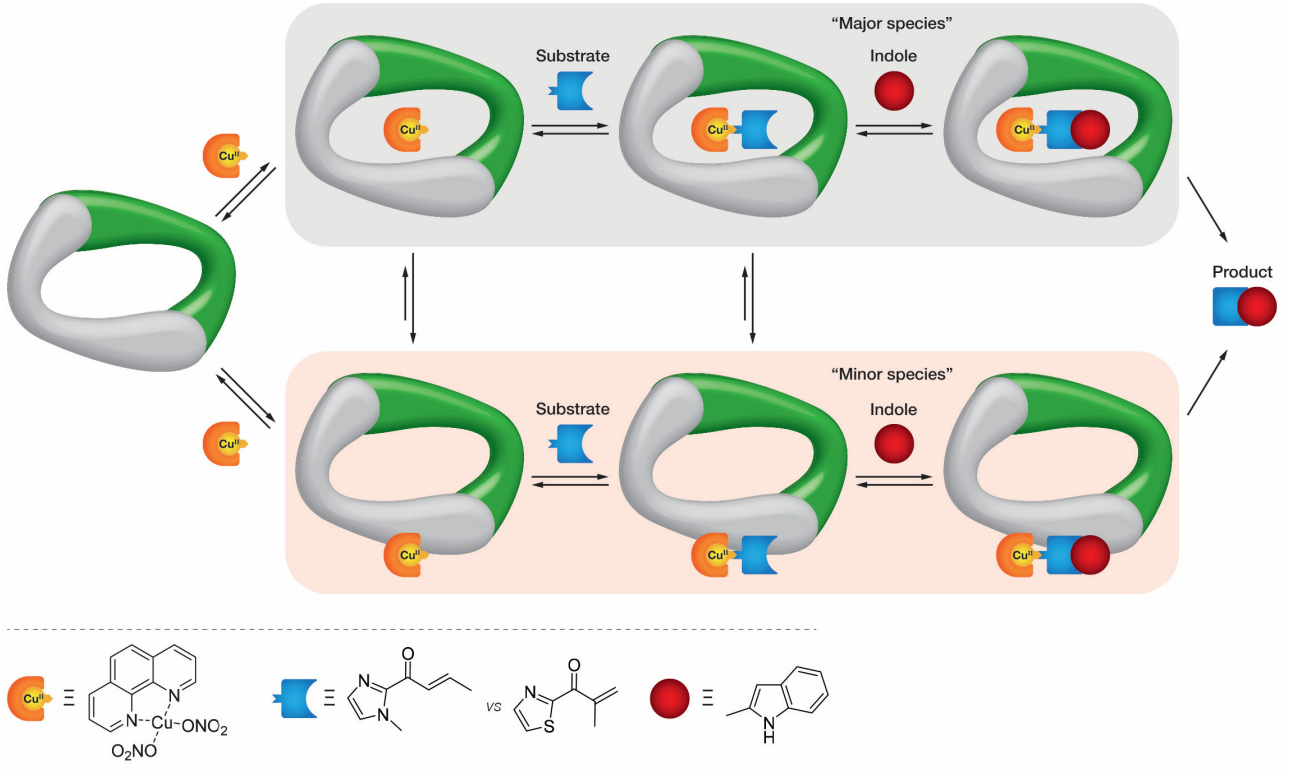


Figure 6. Schematic explanation of the catalytic promiscuity of LmrR-based artificial metalloenzymes. Most of the Cu(II)-phen complex will bind in the cavity between the two Trp residues W96/W96', where it can bind and activate the substrate to undergo conjugate addition by an indole nucleophile, resulting in formation of the product (top pathway, "major species") This pathway is preferred for the FC reaction. However, a small but non-neglible fraction of the Cu(II)-phen can bind at other positions in LmrR where it can also activate a substrate for reaction with the indole (lower pathway, "minor" species). This is the pathway that is favored for the FC/EP reaction.

This is a possibility in view of the moderate binding affinity of Cu(II)-phen for WT LmrR, which is in the micromolar range. This means that under the conditions of catalysis, most of the Cu(II)-phen is bound between the tryptophans (major species), but a non-neglible fraction of the Cu(II) complex does not bind there and, most likely, can interact at other positions of the protein, be it in the pore or on the surface of the WT LmrR (minor species) (Figure 6). This means that the catalyst is actually a mixture of copper complexes in a different environment and, depending on the reaction, it is one of these that is most reactive and may dominate the outcome of the catalyzed reaction. This is highly reminiscent of what was observed before in our work on salmon testes DNA-based catalysis.[23]

This behavior of the present artificial metalloenzymes can be rationalized by considering the Curtin-Hammett principle, which states that in case of competing pathways involving rapidly interconverting intermediates, the outcome of the reaction is solely determined by the relative kinetics of the pathways. In the present case this means that there are multiple intermediate LmrR/Cu(II)-phen_substrate complexes. In the case of the FC reaction, it is the major species, i.e. with the Cu(II)-phen_substrate complex bound between W96/W96', that reacts with high activity and enantioselectivity. However, in case of the FC/EP, this appears to be an

unproductive situation and does not result in reaction. In contrast, it is the minor substrate-bound complex that is bound at a different location, possibly on the protein exterior, that reacts much faster and, hence, is responsible for the observed catalysis.

The competition experiments support this hypothesis. Using LmrR, both the FC and FC/EP reaction are possible, but the former is preferred. Making a single mutation, A92E, results in a much higher binding affinity for the Cu complex, stabilizing the metal cofactor binding between the tryptophans, which is the most optimal conformation for the FC reaction. Hence, the reaction occurs almost exclusively via the major species (top pathway) and the lower pathway (via the minor species) is effectively shut down. This is reflected in the results: the A92E mutant catalyzes only the FC reaction and not the FC/EP reaction. Conversely, by removing the central tryptophans via the W96A mutation only the lower pathway can be followed, since it eliminates the preferred binding site for the Cu(II)-phen complex. The FC reaction is still possible, albeit with lower activity and selectivity. In this case, the FC/EP reaction is now the favored reaction, resulting in an increased yield and enantioselectivity of the FC/EP product.

In conclusion, the LmrR/Cu(II)-phen artificial metalloenzyme shows dynamic behavior in the positioning of its abiological metal cofactor, which , in turn, is related to the preferred catalytic reactivity: the FC or FC/EP reaction. In the WT LmrR/Cu(II)-phen, the FC reaction, which occurs in the hydrophopbic pore of the protein, is the main activity. However, it exhibits lower, but significant, levels of activity for another reaction, the FC/EP reaction, which does not occur at the same location in the LmrR pore, but at another site near or on the protein surface.. By only 1 mutation, A92E, this artificial metalloenzyme became almost fully selective for the FC reaction, while by another mutation, i.e. W96A, the FC/EP reaction becomes the dominant activity. The switching of catalytic activity by dynamic interconversion of the position of a metal cofactor has not been described for natural enzymes and, to date, appears to be unique to supramolecularly assembled artificial metalloenzymes. Thus, this study underlines the importance of structural dynamics as a key element of artificial enzyme design.

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Keywords

Artificial metalloenzymes, biocatalysis, structural dynamics, enzyme design, copper

Supporting Information

Detailed experimental procedures, characterization data for all new compounds and proteins, additional catalysis and biophysical data, x-ray crystallography data, details for molecular dynamics (MD) simulations, and DFT calculations.

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