In vivo Assembly of Artificial Metalloenzymes and Application in Whole-Cell Biocatalysis

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ABSTRACT:

Artificial metalloenzymes (ArMs), which are hybrids of catalytically active transition metal complexes and proteins, have emerged as promising approach to the creation of biocatalysts for reactions that have no equivalent in nature. Here we report the assembly and application in catalysis of ArMs in the cytoplasm of E. coli cells based on the Lactococcal multidrug resistance regulator (LmrR) and an exogeneously added copper(II)-phenanthroline (Cu(II)-phen) complex. The ArMs are spontaneously assembled by addition of Cu(II)-phen to E. coli cells that express LmrR and it is shown that the ArM containing whole cells are active in the catalysis of the enantioselective vinylogous Friedel-Crafts alkylation of indoles. The ArM assembly in E. coli is further supported by a combination of cell-fractionation and inhibitor experiments and confirmed by in-cell solid-state NMR. A mutagenesis study showed that the same trends in catalytic activity and enantioselectivity in response to mutations of LmrR were observed for the ArM containing whole cells and the isolated ArMs. This made it possible to perform a directed evolution study using ArMs in whole cells, which gave rise to a mutant, LmrR_A92E_M8D that showed increased activity and enantioselectivity in the catalyzed vinylogous Friedel-Crafts alkylation of a variety of indoles. The unique aspect of this whole-cell ArM system is that no engineering of the microbial host, the protein scaffold or the cofactor is required to achieve ArM assembly and catalysis. This makes this system attractive for applications in whole cell biocatalysis and directed evolution, as demonstrated here. Moreover, our findings represent important step forward towards achieving the challenging goal of a hybrid metabolism by integrating artificial metalloenzymes in biosynthetic pathways.
Introduction

Artificial metalloenzymes (ArM), which are hybrids of catalytically active transition metal complexes embedded in protein scaffolds, have emerged as a promising approach for biocatalysis of reactions that have no equivalent in nature.\textsuperscript{1, 2} This approach gives rise to rudimentary enzymes that can subsequently be tailored for the reaction of interest by employing the power of site-directed mutagenesis and/or directed evolution.\textsuperscript{3-5} For further developments, it is highly desirable to achieve the assembly and application of ArMs in bacterial cells. This will allow for whole cell biocatalysis, which is attractive from an economical perspective, is convenient for directed evolution and ultimately will be important towards achieving the goal of creating a hybrid metabolism, that is, a biosynthetic pathway augmented with new-to-nature chemistry.\textsuperscript{6} Yet, application in whole cells present some major challenges, which include assembly of the artificial enzyme from a heterologously expressed protein and an exogenously added metal complex and the mutual incompatibility and inactivation of transition metal complexes and biological components, in particular glutathione.\textsuperscript{7} Recently, the first reports of application of ArMs in cells appeared. In these studies, the above mentioned challenges were circumvented by creating the ArM in the periplasm or on the cell surface, where the GSH concentration is minimal and there are less barriers to achieving incorporation of the metal cofactor.\textsuperscript{8-13} Cytoplasmic assembly has been achieved for artificial metallo-heme enzymes, using bacteria containing co-expressed natural and engineered heme transporters.\textsuperscript{14-17} Here we report the spontaneous self-assembly of functional ArMs in the cytoplasm of \textit{E. coli} and subsequent application in whole cell biocatalysis and directed evolution.

Results and discussion

Design of artificial metalloenzymes

The design of the ArMs is based on the Lactococcal multi-drug resistance regulator (LmrR) protein from \textit{Lactococcus lactis}.\textsuperscript{18} LmrR is a transcription factor that forms a homodimer with a large hydrophobic pocket, known to promiscuously bind many small planar compounds.\textsuperscript{19} Previously we have shown that ArMs can be formed from LmrR by self-assembly upon addition of copper(II) phenanthroline (Cu(II)-Phen) complex.\textsuperscript{20, 21} The Cu(II)-Phen complex binds to LmrR with low micromolar affinity and is primarily located between the two central tryptophans, one from each monomer (W96/W96'). This ArM was found to be an excellent catalyst for the enantioselective vinylogous Friedel-Crafts alkylation of indoles. Moreover, mutagenesis of residues in the hydrophobic pocket showed significant effect on catalysis, with a few mutations, notably M8A and A92E giving rise to significant improvement of activity and selectivity of the catalyzed reaction.\textsuperscript{22}
Figure 1. (a) Schematic representation of self-assembly of Cu(II)-Phen/LmrR artificial metalloenzyme in E. coli cells and catalyzed enantioselective Friedel-Crafts alkylation reaction. (b) Results of catalysis of enantioselective Friedel-Crafts alkylation reaction of 1 with 2a by Cu(II)-Phen/LmrR and Cu(II)-Phen/SUMO in cell-free extract and whole cells. In vitro catalysis: 120 µM LmrR, 90 µM Cu(II)-Phen, 1 mM 1 and 2a in 20 mM MOPS, 150 mM NaCl, pH 7 at 4 °C for 30 min.; in vivo catalysis: E. coli C43(DE3) cells over-expressing LmrR (from a 0.5 mL overnight culture; OD600nm = 4), 90 µM Cu(II)-Phen, 1 mM 1 and 2a in catalysis buffer at 4 °C. Values are given as the average of independent duplicate experiments, each performed in duplo. Errors margins are standard deviations (c) Experimental design of the catalysis experiments with whole cells and supernatant. In solid boxes: values of copper content as determined by ICP-OES. In dashed boxes the samples that were used for catalytic reaction I, the Friedel-Crafts alkylation reaction of 1 with 2b and catalytic reaction II: the Friedel-Crafts alkylation reaction of 1 with 2a.

Catalysis in whole cells

To establish the tolerance of LmrR/Cu(II)-Phen against cellular components, we performed the catalysis in cell-free extracts. For this purpose, LmrR was expressed heterologously in E. coli C43(DE3) cells, the cells were lysed and the cell debris pelleted to yield cell free extracts, to which Cu(II)-Phen was added to self-assemble the ArMs. We observed significant catalysis and enantioselective product formation (Figure 1b). In contrast, expressing Small Ubiquitin-like MODifier
(SUMO) as a structurally unrelated control protein in combination with Cu(II)-Phen in cell free extract only gave rise to low yield of product and low enantioselectivity.

Next, we took the whole E. coli cells expressing LmrR and incubated them with Cu(II)-Phen. The cells were then washed to remove excess, unbound Cu(II)-Phen and incubated with substrates (Figure 1a). Remarkably, we still observed accelerated catalysis and enantioselective product formation. In absence of Cu(II)-Phen, or using SUMO instead of LmrR did not give rise to catalysis. Thus, it was confirmed that both LmrR protein and exogenously added Cu(II)-Phen are essential for catalysis.

To confirm that the catalysis occurs in the cells, we incubated E. coli cells expressing LmrR with Cu(II)-Phen for 1h and then pelleted the cells and washed with buffer to remove unbound Cu(II)-Phen. The copper content of the cells was quantified using inductively coupled plasma optical emission spectrometry (ICP-OES) (Figure 1c). The data shows that a significant portion of the copper added is retained by the cell fraction suggesting the cells did indeed take up the Cu(II)-Phen complex.

The cell and the supernatant fractions were both incubated with substrates 1 and 2b for the Friedel-Crafts Alkylation reaction. Low activity was observed in both cases, which is in part related to the fact that 2b is a less preferred substrate for LmrR/Cu(II)-Phen (vide infra), but only in the cell fraction significant enantioselectivity was observed in the product (Figure 1c). The cell fraction was then again subjected to the same procedure and then a fresh batch of substrates, in this case 1 and 2a, which is a preferred indole, was added. Again, enantioselective product formation was only observed from the cell sample. These results confirm that the ArM assembled in the cell is responsible for this catalysis. Moreover, it also shows that the ArM has not leaked out into the buffer/supernatant, indicating that the structural integrity of cells is preserved.

Next, a series of LmrR mutants, for which the activity and enantioselectivity is known were tested in the cell experiments. A good correlation was observed between activity and selectivity of the isolated ArM and that of the ArM in whole cells, even though the enantioselectivities in whole cells were generally lower than those found with isolated enzymes, most likely due to the background reaction (Figure 2a, Table S1). For example, the mutant A92E, which we reported as the most active and selective ArM to date for this reaction also gives significantly higher yield and ee than the wild type in the cell experiments. Similarly, mutations that had a detrimental effect on catalysis in isolated proteins also showed this effect in cells.

From previous work on the LmrR protein we know that it binds Hoechst 33342 and ethidium bromide with high affinity in its hydrophobic pocket and that they can inhibit catalysis by competing
for binding with Cu(II)-Phen and the substrates.\textsuperscript{22} Hoechst 33342 is cell permeable, whereas ethidium has difficulties crossing the double membrane barrier of \textit{E. coli} and is normally used to stain dead \textit{E. coli} cells, which have permeable cell membranes. Fluorescence microscopy confirmed that Hoechst 33342 was readily taken up by the LmrR expressing cells, whereas ethidium bromide was not (Figures S4-6). Indeed, using these dyes in combination with isolated LmrR/Cu(II)-Phen gave rise to a strongly reduced yield and enantioselectivity in catalysis in the reaction of 1 with 2b (Figure 2b). Incubating \textit{E. coli} cells expressing LmrR with Hoechst 33342 prior to addition of Cu(II)-Phen, we found that in catalysis the enantioselectivity of the product was significantly decreased. In contrast, no significant effect on the enantioselectivity was observed for cells incubated with ethidium bromide. This becomes especially apparent when expressing the enantioselectivity differences w/ and w/o inhibitor in $\Delta\Delta G^\ddagger$ (Figure 2b, inset).

We further investigated the cellular localization of Cu(II)-Phen/LmrR in \textit{E. coli} by performing cell fractionation experiments. These experiments showed that LmrR and Cu(II)-Phen/LmrR are localized exclusively in the cytoplasm of \textit{E. coli} (Figure 2c). This strongly suggests that the ArM assembles in the cytoplasm of \textit{E. coli}. For comparison, a variant of LmrR (called SRP-LmrR) containing a N-terminal periplasmic localization signal known to transport proteins to the periplasm via the SRP pathway, was constructed.\textsuperscript{23} As expected, SRP-LmrR and Cu(II)-Phen/SRP-LmrR were found to exclusively localize in the periplasm of \textit{E. coli}.

Combined these experiments support that the LmrR/Cu(II)-Phen ArM is assembled in the cytoplasm of \textit{E. coli} and that the structural integrity of the cellular membrane is maintained during the experiments.
Figure 2. (a) Enantioselectivity of the enantioselective Friedel-Crafts alkylation reaction of 1 with 2a, catalyzed by isolated Cu(II)-Phen/LmrR artificial enzyme mutants (dark blue) and Cu(II)-Phen/LmrR artificial enzyme mutants in whole E. coli cells (light blue). (b) Effect of addition Hoechst 33342 and ethidium bromide (EtBr) (4 equivalents compared to Cu(II)-phen) on the yield enantioselectivity of the reaction of 1 with 2b, catalyzed by isolated Cu(II)-Phen/LmrR artificial metalloenzymes and in whole cells. Values are given as the average of independent duplicate experiments, each performed in duplo. Errors are given as standard deviations. Inset: difference in enantioselectivity in the reaction 1 with 2b catalyzed by isolated Cu(II)-Phen/LmrR artificial metalloenzymes (dark blue bars) and in whole cells (light blue bars upon addition of inhibitors, compared to w/o inhibitor. Enantioselectivity differences are represented as $\Delta \Delta \Delta G^\dagger$, which is calculated using $\Delta \Delta \Delta G^\dagger = \Delta \Delta G^\dagger_{(w/o \ inhibitor)} - \Delta \Delta G^\dagger_{(w \ inhibitor)}$ and $\Delta \Delta G^\dagger = RT\ln(\text{er})$, in which er is the enantiomeric ratio: % major enantiomer/% minor enantiomer. (c) SDS PAGE of E. coli cell fractionation experiment to determine protein localization for LmrR and SRP-LmrR with and without Cu(II)-Phen. PF = periplasmic fraction; CF = cytoplasmic fraction.

In-cell NMR studies of ArM assembly

While the reactivity data is fully in agreement with in vivo assembly of the ArM, it does not provide direct evidence. For this reason, the assembly of the ArM in E. coli Lemo21 (DE3) cells was studied by in-cell NMR spectroscopy. We focused on the LmrR_A92E mutant, since this has a higher binding
affinity for Cu(II)-Phen.\textsuperscript{22} Due to high molecular crowding in \textit{E. coli} cells,\textsuperscript{24} in-cell solution-state NMR was not feasible.\textsuperscript{25} Instead, we employed dynamic nuclear polarization (DNP)-supported solid-state NMR (DNP-ssNMR) which can probe protein structure inside cells irrespective of protein size and molecular crowding at greatly enhanced sensitivity.\textsuperscript{26} For reference, we first obtained solution- and solid-state NMR evidence for \textit{in vitro} assembly of the ArM using near-complete resonance assignments (Figure 3a, see also Tables S5, S6) and via probing paramagnetic quenching effects exerted by Cu(II). 2D experiments in both soluble (Figures S9, S10) and microcrystalline (Figure S11) samples revealed site-selective paramagnetic quenching, along with faster longitudinal relaxation rates (R\textsubscript{1}) in the ssNMR spectra (Figure S12). Additionally, small chemical shift perturbations (CSPs) occurred in the residues away from the Cu(II)-Phen binding site (Figure S13), consistent with the structural plasticity exhibited by LmrR when bound to different compounds.\textsuperscript{19,27-29}

For the in-cell DNP-ssNMR experiments, LmrR-specific isotope labelling was achieved using the antibiotic rifampicin to suppress native \textit{E. coli} polymerases during T7 RNA polymerase mediated expression.\textsuperscript{30-33} The signal contributions from the cellular background were reduced further by eliminating the non-protein cellular background labelling using a specialized algal amino-acid mixture (devoid of Trp, Cys, Asn and Gln) for isotope labelling.\textsuperscript{34} Secondly, the cellular background was deuterated, which also leads to high DNP-enhancements on the molecule of interest.\textsuperscript{35} Lastly signal contributions from unincorporated, isotope labelled amino acids were removed by expressing in unlabelled medium during the last quarter of the expression time. The resulting in-cell DNP-ssNMR samples exhibited a ~100-fold increase in LmrR signals (Figure S14a) and allowed us to rapidly record different multidimensional ssNMR experiments. The 2D DNP-ssNMR spectra (Figure S15, S16) were in very good agreement with the \textit{in vitro} spectrum of LmrR. In line with our 2D data sets, analysis of the 3D data sets suggested qualitative agreement between our \textit{in vitro} assignments and the backbone correlations observed in cells. In spite of the increased NMR line width at low-temperature 400 MHz DNP conditions,\textsuperscript{36} we could obtain several spectral strips in the 3D experiment (see materials and methods) which unambiguously matched with the backbone assignments determined \textit{in vitro} (Figure 3b, Figures S17-19). We correlated the C\textsubscript{\alpha}, C\textsubscript{\beta} assignments to the side chains and the carbonyl chemical shifts to confirm the respective amino acid type (Figure 3b). The solution state NMR spectrum of the lysates further confirmed that well folded LmrR is the only labelled molecule in the sample, and additionally showed no visible signs of protein degradation (Figure S20).
Figure 3. *In vitro* and in-cell NMR studies of the LmrR_A92E/Cu(II)-Phen artificial metalloenzyme. a) Summary of NMR analysis: residues used for both solution- and solid-state NMR analysis are plotted on the crystal structure of LmrR (PDB ID: 3F8F) in yellow, green, pink and brown. Green residues were identified in the 3D $^{13}$C (DQ-SQ-SQ) DNP-ssNMR spectrum (b). Tryptophan (pink) and tyrosine (brown) residues are used for analysis in panel (c) where the selective reduction of NMR signal intensities for Trp aromatic signals confirms proper *in-cell* assembly of the ArM. In-cell experiments were performed in *E. coli* Lemo21 (DE3).
As a final test for assembly of the ArM in cells, we tracked the paramagnetic effect of adding Cu(II)-Phen upon Trp NMR resonances of LmrR. For this purpose, a modified expression protocol was used (SI Material and Methods NMR studies) that led to the labelling of all amino acids, including Trp. In the presence of Cu(II)-Phen, we observed a selective reduction of the Trp aromatic side-chain signal intensity, while Tyr residues, which are present far away from the Cu(II)-Phen binding region, do not display paramagnetic quenching effects (Figure 3a, 3c and Figure S21). Additionally, a clear increase in the longitudinal relaxation rate (R₁) was also observed (Figure S22) in full accordance with the spectral changes observed in vitro (Figure 3c, Figure S11, S12). To validate our DNP-ssNMR analysis, we finally analyzed the clear cell lysates of replicate cells used for in vivo DNP-ssNMR experiments using solution NMR (Figure S23). The CSPs due to Cu(II)-Phen binding largely followed a similar trend to in vitro CSPs (Figure S24) and we observed, a complete quenching of Trp96 side chain resonance (Figure S23, S25) suggesting tight binding to the protein, even after cell lysis.

Taken together, these results provide direct spectroscopic evidence for the in vivo assembly of the ArM. Moreover, the copper complex is predominantly present in the Cu(II) state. This is consistent with the observed activity in catalysis, further confirming that the reduction of Cu(II) to Cu(I),³⁷ due to cellular reductants, does not play a significant role.

**Directed evolution**

Having established that these LmrR based ArMs are assembled in vivo and can be employed in whole cell bio-catalysis, we aimed to exploit this for directed evolution of the ArMs. The reaction of enone 1 with 5-methoxyindole (2b), which is a less good substrate than 2-methylyndole (2a) for enantioselective vinylogous Friedel-Crafts alkylation, was selected for this purpose. An Alanine scan of residues in the hydrophobic pocket of LmrR was performed to identify positions of interest (Table S2). The trend of enantioselectivity from in vivo catalysis and activity from in vitro catalysis matched well for the Alanine mutants (Figure 2a).

LmrR_A92E was chosen as the starting point for directed evolution because it was shown to be significantly more active than LmrR.²² Site-saturation libraries with NDT codon degeneracy were designed for positions 8, 14, 88, 89, 93 and 100, which were identified in the alanine scan as positions of interest (Figure 4a). From one round of screening (in total ~ 250 clones) the mutant LmrR_A92E_M8D was found to show the highest activity and enantioselectivity (Table S10). Enzyme kinetics studies for the reaction of 1 and 2b confirmed that the catalytic efficiency (kₗcat/Kₗs) of LmrR_A92E_M8D was 185.9 M⁻¹min⁻¹, almost three times higher than that of the starting point, LmrR_A92E (73.3 M⁻¹min⁻¹) (Figure S28).
Figure 4. (a) Structure of LmrR/Cu(II)-Phen with residues that were randomized during the directed evolution study indicated as spheres. (b) Scope of the enantioselective Friedel-Crafts alkylation catalyzed by Cu(II)-Phen and Cu(II)-Phen/LmrR artificial metalloenzyme mutants. Conditions: 12 µM LmrR mutant, 9 µM Cu(II)-Phen, 1 mM 1 and 2a in 20 mM MOPS, 150 mM NaCl, pH 7 at 4 °C. Values are given as the average of independent duplicate experiments, each performed in duplo. Errors are given as standard deviations.

The scope of indoles was compared for ArMs based on three LmrR variants: LmrR, LmrR_A92E and LmrR_A92E_M8D (Figure 4b). A marked improvement in activity from LmrR to LmrR_A92E for the different indoles was found. This was further improved for the double mutant LmrR_A92E_M8D for all the indoles tested. It is remarkable to see that almost half of 2 is converted to product in thirty
minutes by LmrR_A92E_M8D. This demonstrates that directed evolution is a powerful tool for improving the catalytic properties of ArM.

**Discussion**

The remarkable and unexpected aspects of the current study are the straightforward *in vivo* assembly and apparent stability of the LmrR/Cu(II)-Phen artificial metalloenzymes in the cell’s cytoplasm, as evident from the catalysis data, inhibition studies, cell fractionation experiments and the in-cell NMR studies. *E. coli* cells over-expressing the protein LmrR are simply incubated with Cu(II)-Phen abiological cofactor, resulting in uptake of the abiological cofactor and *in vivo* ArM assembly. For almost all ArM catalysis *in vivo*, the ArM and/or the bacterial cell had to be engineered to transport and localize the protein in the periplasm or on the cell surface, to reduce exposure to glutathione that is detrimental to many transition metal complexes.7,8,13

Copper complexes are also expected to be readily reduced to Cu(I) and then be toxic to the cell due to the formation of reactive oxygen species (ROS). Yet, the combined results demonstrate unequivocally that the assembled LmrR/Cu(II) artificial metalloenzymes are stable and catalytically active in the cytoplasm of the cell. This was further verified by performing catalysis with isolated LmrR_A92E_M8D / Cu(II)-Phen ArM in the presence of increasing amounts of glutathione, which is usually the main culprit for deactivation and instability of metal complexes and ArMs in cellular environments. The results indeed show that the ArM is still active in the presence of moderate concentrations of GSH (1 mM) and only at higher concentrations (10 mM) a significant detrimental effect on catalytic activity is observed, albeit that activity and enantioselectivity is even then still observed (Table S3). We hypothesize that the Cu(II)-Phen complex bound to the front entrance of the binding pocket of LmrR is protected from GSH, and other cellular components. The front entrance where the Cu(II) ion is located has an overall negative charge due to the presence of multiple carboxylate rich residues. This may cause charge repulsion with glutathione and in this way protect the metal complex, as was recently reported also for a glycosylated albumin artificial metalloenzyme.38 Intriguingly, this is actually reminiscent of the biological role of the protein LmrR, which has evolved to rapidly bind a plethora of different toxic compounds entering the cell, as start of the cellular drug resistance response in Lactococcus lactis.18,28

In conclusion, we have demonstrated here that catalytically active artificial metalloenzymes can be self-assembled in the cytoplasm of *E. coli* from heterologously produced LmrR and an exogeneously added Cu(II)-Phen complex. The unique aspect of our system is that no extensive engineering of the microbial host, the protein scaffold or the cofactor is required, which makes this system attractive for applications in whole cell biocatalysis and directed evolution, as demonstrated here. Moreover,
this represents important step forward towards achieving a hybrid metabolism by integrating artificial metalloenzymes in biosynthetic pathways.6,14

**Competing Interests**

The authors declare no competing interests.

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