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PAPER

Multidrug resistance regulators (MDRs) as scaffold for the design of artificial metalloenzymes

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The choice of protein scaffold is an important element in the design of artificial metaloenzymes. Here, we introduce Multidrug Resistance Regulators (MDRs) from the TetR family as a viable class of proteins scaffolds for artificial metalloenzyme design. *In vivo* incorporation of the metal binding amino acid (2,2-bipyridin-5yl)alanine (BpyA) by stop codon suppression methods was used to create artificial metalloenzymes from three members of the TetR family of MDRs: QacR, CgmR and RamR. Excellent results were achieved with QacR Y123BpyA in the Cu^{2+} catalyzed enantioselective vinylogous Friedel–Crafts alkylation reaction with is ee's up to 94% of the opposite enantiomer that was achieved with other mutants and the previously reported LmrR-based artificial metalloenzymes.

Introduction

Artificial metalloenzymes have emerged as a powerful new approach to expand the repertoire of chemical reactions performed by enzymes.[1–4] The design comprises a transition metal catalyst embedded in a protein scaffold; this provides the chiral second coordination sphere interactions that might allow to achieve enzyme-like selectivities and activities. Hence, the choice of the protein scaffold is a key aspect of the design of artificial metalloenzymes.

We have introduced a class of artificial metalloenzymes based on the transcriptional regulator LmrR (Lactococcal multidrug resistance Regulator), a protein belonging to the PadR family of multidrug resistance regulators (MDRs).[5,6] MDRs are regulatory proteins involved in resistance mechanisms in bacteria where they regulate the expression multidrug (MD) efflux pumps. MDRs are generally homodimeric proteins, and they respond to the presence of structurally diverse small molecules that are substrates for the efflux pump of interest, such as antibiotics, polyaromatic compounds, lipophilic cations or disinfectants.[7] A common feature of MDRs is the presence of a promiscuous, mostly hydrophobic, binding pocket used for multidrug recognition. This makes them promising candidates for the design of artificial metalloenzymes. This feature has been successfully exploited in the case of LmrR, using a variety of anchoring approaches for ligands or metal complexes, to create artificial metalloenzymes that proved to be active in several catalytic enantioselective reactions.[8–11] One of the approaches that we have introduced involves the *in vivo* incorporation of non-natural metal binding amino acid for the creation of artificial metalloenzymes.[10] This strategy makes use of the amber stop codon suppression methodology, also known as the expanded genetic code method,[12] introduced by the Schultz group. It offers several advantages compared to classical anchoring strategies, including excellent control over the positioning of the ligand and the fact that no post-translational modification or additional purification of the artificial metalloenzyme is required.[13,14]

In this work we aimed to expand the scope of biomolecular scaffolds that can be used for the design of artificial metalloenzymes to different Multidrug Resistance Regulators, using *in vivo* incorporation of metal binding amino acids and their application in the Cu^{2+} catalyzed enantioselective vinylogous Friedel-Crafts alkylation of indoles. Expanding artificial metalloenzyme design to MDRs will give rise to a general platform of hybrid catalysts that are readily accessible and can be evaluated for a specific reaction using similar experimental protocols.

Results and discussion

The TetR family represents a large class of MDRs that can bind a wide variety of different compounds. Three proteins belonging to this family were selected for this study: QacR[15–18], CgmR[19] and RamR[20]. For these proteins crystal structures in complex with a variety of hydrophobic ligands, such as ethidium bromide, are available (Figure 1). QacR, CgmR and RamR are homodimers with a size around 20 KDa per subunit and they have similar quaternary structure consisting of 9 helices; the first three helices contain the DNA-binding domain and the last six the dimerization interface and the multi-drug binding pocket. The hydrophobic pockets of TetR proteins are rich in aromatic residues. Different from proteins belonging to the PadR family, TetR proteins comprise two drug binding pockets per protein dimer as the hydrophobic cavity is mostly created between helices of one monomer, with only few interactions from amino acids belonging to the second subunit.[17,19,20] The volume of the pockets is around 1000 $Å^3$ and the binding stoichiometry of the drugs with these proteins is variable: QacR only binds one drug molecule per protein dimer, while CgmR and RamR have been crystallized either with one or two drug molecules per dimer.[16,20,21] The promiscuity of QacR, which is one of the most studied members of the TetR family, is thought to be related to the fact that its binding pocket is comprised of multiple distinct overlapping "minipockets" that can host differently shaped ligands.[22] This is in contrast with LmrR, which can bind structurally unrelated compound by using the same binding pocket and adapting its overall conformation.

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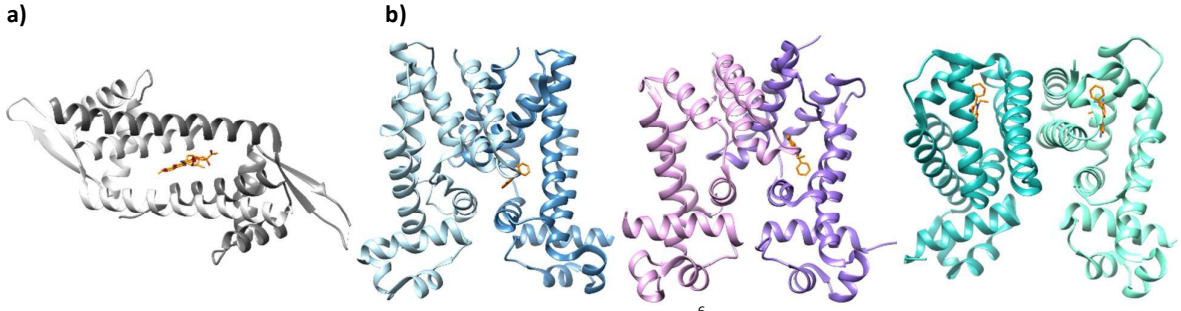

Figure 1. Helix representation of: a) LmrR in complex with daunomycin (PDB 3F8F[6]), b) from left to right QacR, CgmR and RamR in complex with ethidium (PDB 1JTY[16], 2ZOZ[21], 3VVY[20] respectively)

Based on the X-ray crystal structures of the three MDRs, four positions inside the hydrophobic pockets of each protein were selected for the introduction of (2,2-bipyridin-5yl)alanine (BpyA), the unnatural metal binding amino acid. [10,13,14,23–26] For QacR positions W61, Q96, Y103, and Y123 were chosen, for CgmR positions W63, L100, W113 and F147 and for RamR positions Y59, W89, Y92 and Y155 (Figure 2, S2-S4).

The metal-binding amino acid (2,2-bipyridin-5yl)alanine (BpyA) was synthesized according to previously reported procedures (see SI)[14,27] Plasmids encoding for QacR, CgmR and RamR containing a C-terminal strep-tag were ordered from a commercial source as codon optimized genes for expression in *E. coli*. A double mutant of QacR was prepared with the mutations C72A and C141S, which is here simply referred to as wt-QacR, to prevent disulfide bond formation and precipitation of the protein. This mutant is reported to have enhanced *in vitro* stability compared to the original protein, while retaining drug and DNA-binding ability.[28] Mutants containing the amber stop codon in the positions selected for the introduction of BpyA were prepared by standard site-directed mutagenesis techniques (Table S1) and these plasmids were co-transformed with pEVOL-BpyA, the plasmid containing the required orthogonal aminoacyl tRNA synthetase (aaRS) and tRNA genes, into *E. coli* BL21 DE3 (C43) cells. Large scale expression was performed in LB media in presence of 0.5 mM bipyridine alanine. With the exception of two mutants of CgmR (W63BPyA and W113BpyA) and one mutant from RamR (W89BpyA), which did not express, in general the target proteins were obtained in good yield, between 15 and 30 mg/L, and purity after affinity chromatography (Strep-tactin Sepharose column), as judged by Tricine-SDS-PAGE and UPLC-MS (Figure S4-S5). For all QacR variants, cation exchange chromatography (Heparin column) was performed to remove residual bound DNA (Figure S4). Wild type proteins and the BpyA containing mutants eluted as single peaks in analytical size-exclusion chromatography (Superdex-75 10/300 column) corresponding to the molecular weight of the dimers, indicating that the quaternary structure of the proteins was not significantly altered by introduction of the unnatural amino acid (Figure S6).

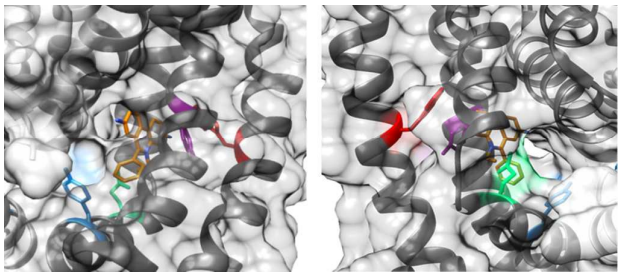

Figure 2: a) Hydrophobic pocket (front and back view) of QacR with ethidium bromide bound (PDB 3PM1[29]). Positions selected for the incorporation of the unnatural amino acid are highlighted: Y103 (light blue), Q96 (green), Y123 (purple), W61 (red).

QacR mutants Q96BpyA, Y103BpyA and Y123BpyA eluted from the Strep-Tag column having a pink color. This is usually indicative of the presence of Fe^{2+} bipyridyl complexes.[24,26,30] It is indeed possible that the proteins containing BpyA bind Fe^{2+} present in the cytoplasm or in the medium used for bacterial growth. UV-visible spectra indeed showed the typical absorption bands around 500 nm typical of Fe^{2+} bipyridyl complexes, as well as an unidentified band around 360 nm (Figure 3b and S7).[24,26,30–32] Alternatively, it is possible that $Fe(II)(BpyA)_3$ complex is formed in the medium and then binds to the protein in such a way that it is not removed during purification and dialysis, resulting in the spectroscopic signature observed. Treatment of these proteins with up to 50 mM EDTA followed by extensive dialysis, didn't lead to disappearance of the color. In any case, titration studies with Cu^{2+} showed that this iron contamination is very small and does not interfere with Cu^{2+} binding (vide infra).

The Cu^{2+} binding of the BpyA mutants of QacR, CgmR and RamR was investigated by UV-visible titrations. Upon addition of $Cu(NO_3)_2$ to the proteins, the appearance of a characteristic shoulder between 310 and 315 nm was observed, indicative of a change in the π–π* transition of the bipyridine moiety upon metal coordination (Figure 3).[10,13,30,33] Moreover, titration of the wild type proteins with $Cu(NO_3)_2$ didn't show similar changes (Figure S7).

The titration curves were fitted to a 1:1 binding model[‡] (Table S2) that corresponds to the binding of one Cu^{2+} ion per bipyridine moiety, indicating the binding of two Cu^{2+} ions per protein dimer (Figure 3 and S7). RamR mutant Y59BpyA precipitated upon addition of Cu^{2+}, therefore it was not possible to perform UV-visible titrations nor to use this mutant in catalysis.

The catalytic activity of the artificial metalloenzymes was evaluated in the Cu^{2+} catalyzed enantioselective vinylogous Friedel–Crafts alkylation of 2-methyl-1H-indole (**2**) with 1-(1-methyl-1H-imidazol-2-yl)but-2-en-1-one (**1**) .[10,11,34–36]

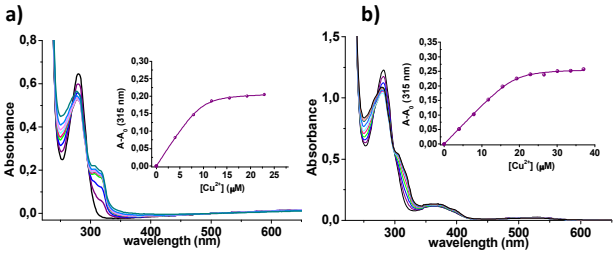

Figure 3: a) UV-visible titrations of a) QacR W61BpyA and b) QacR Y123BpyA with $Cu(NO_3)_2{\cdot}3H_2O$ Titrations were performed in 50 mM NaH_2PO_4 pH 7.0, 500 mM NaCl using 20 and 10 μM protein, respectively, and additions of aliquots of a 0.5 mM solutions of $Cu(NO_3)_2{\cdot}3H_2O$ in milliQ water. Insets containing the fitting of the titration data obtained by non-linear regression.

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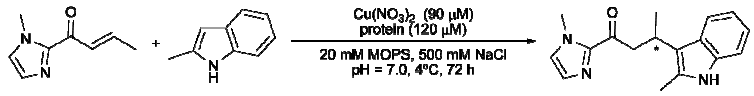
Table 1 Results of Cu^{2+} catalyzed vinylogous Friedel–Crafts alkylation reactions.



Entry	Catalyst	Yield (%)[a]	ee (%)[b]
1	$Cu(NO_3)_2$	67±10	<5
2[5]	LmrR M89BpyA_Cu^{2+}	92±4[c]	80± 2 (-)[d]
3	RamR_Cu^{2+}	68±3	14±0 (-)
4	RamR Y92BpyA_Cu^{2+}	42±6	38±4 (-)
5	RamR Y155BpyA_Cu^{2+}	28±5	<5
6	CgmR_Cu^{2+}	>95	17±2 (-)
7	CgmR L100BpyA_Cu^{2+}	10±2	10±7 (+)
8	CgmR F147BpyA_Cu^{2+}	9±1	13±4 (-)
9	QacR_Cu^{2+}	83±22	42±1 (-)
10	QacR_W61BpyA_Cu^{2+}	62±12	5±1 (-)
11	QacR_Q96BpyA_Cu^{2+}	58±11	56±2 (-)
12	QacR_Y103BpyA_Cu^{2+}	55±10	39±2 (-)
13	QacR_Y123BpyA_Cu^{2+}	82±5	94±1 (+)

Typical conditions: 9 mol % $Cu(NO_3)_2$ (90 μM) loading with 1.3 eq of protein (120 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 listed are standard deviations. [a] Yields were determined by HPLC and using 2-phenylquinoline as internal standard. [b] Sign of rotation was assigned based on the elution order in chiral HPLC by comparison to previous reports.[34,36] This value corresponds to conversion of substrate.[10] [d] Sign of the optical rotation of the major enantiomer obtained with this artificial metalloenzyme was assigned erroneously in previous report.[10]

The artificial metalloenzymes were prepared *in situ* by mixing of $Cu(NO_3)_2$ (90 μM) with a slight excess (1.3 equivalents) of BpyA containing proteins (120 μM in monomer) 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 each. The reactions were incubated under continuous inversion at 4°C for 72 h, after which the products were isolated and analyzed by chiral HPLC.

As previously reported, the reaction of the α-β unsaturated acyl imidazole **1** with 2-methyl indole **2** proceeds catalyzed by $Cu(NO_3)_2$ in absence of protein to give a racemic mixture of the products with a 67% yield (Table 1, entry 1).[5] In absence of $Cu(NO_3)_2$, using the protein alone, no significant background reaction was observed.

Unlike with LmrR-based metalloenzymes, the combination of $Cu(NO_3)_2$ with wild type QacR, RamR and CgmR, that are proteins not containing BpyA, resulted in formation of products with low to moderate enantiomeric excess (Table 1, entries 3, 6, 9). This suggests binding of Cu^{2+} ions to residues in the protein scaffold in some way that is not discernible by UV-visible spectroscopy, but does allow for enantioselective catalysis to occur.

The BpyA mutants of RamR and CgmR all showed inferior activity and enantioselectvitiy compared the corresponding wild type proteins (Table 1, entries 4, 5, 7, 8), albeit that in one case, i.e. with CgmR L100BpyA mutant, the opposite enantiomer of the product was obtained in excess.[10] Hence, in these cases the created novel active site is not optimal for the catalyzed reaction.

In case of QacR, three of the BpyA mutants showed similar or lower activity and selectivity compared to the wild type protein. However, one mutant, QacR Y123BpyA, showed superior catalytic behavior, giving rise to good yield and an excellent enantioselectivity of 94% of the (+) enantiomer (Table 1, entry 12). To date, this is the highest enantioselectivity value obtained for the Friedel Crafts alkylation with metalloenzymes created using *in vivo* unnatural amino acid incorporation.[‡‡] Interestingly, this QacR based artificial metalloenzymes also gives the opposite enantiomer than LmrR-based metalloenzymes, for which the (-) enantiomer was preferred.[10]

A limited substrate scope study of the QacR Y123BpyA_Cu^{2+} catalyzed reactions was performed using a selection of indole derivatives (Table 2). The catalyzed Friedel-Craft alkylation proved to be accelerated in presence of wt-QacR for all the substrates tested (Table 2, entries 2, 5, 8, 11). Moderate enantioselectivities were obtained in all cases, except for 5-methoxy indole (**2c**) (Table 2, entries 1-2, 4-5, 7-8, 10-11).

Indole (**2a**), 1-methyl indole (**2b**) and 5-chloro indole (**2d**) proved to be poor substrates for QacR Y123BpyA_Cu^{2+}, showing either no enantioselectivity (Table 2, entry 3) or hardly any conversion to the product (Table 2 entries 6, 12), respectively. In case of **2b** and **2d**, the opposite enantiomer was obtained. Interestingly, the fact that 2-methyl indole (**2**) gives the best results in the Friedel-Crafts alkylation reaction, both in term of activity and enantioselectivity, is similar to LmrR-based metalloenzymes.[10,11]

Conclusions

In conclusion, we have demonstrated that MDRs from the TetR family, due to their large hydrophobic and promiscuous binding pocket, are viable scaffolds for the creation of novel artificial metalloenzymes. By making use of *in vivo* incorporation of the metal binding amino acid BpyA, several Cu^{2+}-based metalloenzymes were created, which were catalytically active in the enantioselective vinylogous Friedel–Crafts alkylation reaction. Among the new artificial metalloenzymes developed one QacR mutant, that is, QacR Y123BpyA, showed outstanding performance resulting good conversion and excellent enantioselectivities up to 94%.

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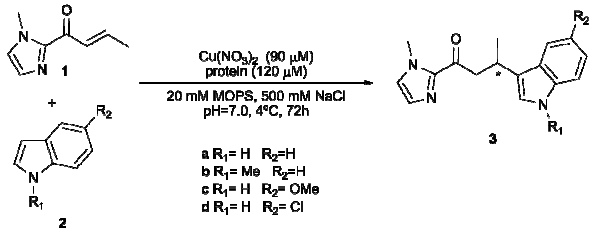
Table 2 Scope of vinylogous Friedel–Crafts alkylation reactions catalyzed by QacR Y123BpyA_Cu^{2+}



Entry	Indole	R	Catalyst	Product	Yield	ee (%)[c]
1			$Cu(NO_3)_2$		14±3	<5
2	**2a**	R_1=H R_2=H	QacR_Cu^{2+}	**3a**	33±13	27±3 (-)
3			QacR_Y123BpyA_Cu^{2+}		14±3	<5
4			$Cu(NO_3)_2$		6±2	<5
5	**2b**	R_1=Me R_2=H	QacR_Cu^{2+}	**3b**	20±9	58±2 (-)
6			QacR_Y123BpyA_Cu^{2+}		3±0	56±6 (+)
7			$Cu(NO_3)_2$		26±1	<5
8	**2c**	R_1=H R_2=OMe	QacR_Cu^{2+}	**3c**	58±17	<5
9			QacR_Y123BpyA_Cu^{2+}		26±3	12±5 (+)
10			$Cu(NO_3)_2$		3±1	<5
11	**2d**	R_1=H R_2=Cl	QacR_Cu^{2+}	**3d**	21±7	47±7 (-)
12			QacR_Y123BpyA_Cu^{2+}		5±2	33±3 (+)

Same reaction conditions as in Table 1 [a] Yields were determined by HPLC and using 2-phenylquinoline as internal standard. Errors listed are standard deviations. [b] Sign of rotation was assigned based on the elution order in chiral HPLC by comparison to previous reports [34,36]

Interestingly this mutant afforded the opposite enantiomer compared to other QacR mutants as well as to previous LmrR-based metalloenzymes, which is from the PadR family of MDRs.[10,11] This work illustrates that MDRs of the TetR family are an attractive class of scaffolds for artificial metalloenzyme design. Thus, when combined with our earlier work on LmrR, this shows that MDRs can be seen as a general platform for the design and construction of hybrid catalysts that readily available for evaluation in diverse catalytic reactions.

Acknowledgements

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Notes and references

[‡]Fitting of the titration curves for RamR Y92BpyA and CgmR L100BpyA were not performed due to precipitation of the protein after addition of more than 1 eq of Cu^{2+} or difficulties with reliably of fitting the data, respectively

[‡‡]Interestingly, this mutant also showed some background activity in absence of Cu^{2+}, albeit that the catalysis was significantly improved in the presence of $Cu(NO_3)_2$ (see Table S3) The origin of this background activity is not understood at present.

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