Artificial Metalloproteins for Binding and Stabilization of a Semiquinone Radical

Nathalie Ségaud, Ivana Drienovská, Juan Chen, Wesley R. Browne, and Gerard Roelfes*

Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

* Supporting Information

ABSTRACT: The interaction of a number of first-row transition-metal ions with a 2,2′-bipyridyl alanine (bpyA) unit incorporated into the lactococcal multidrug resistance regulator (LmrR) scaffold is reported. The composition of the active site is shown to influence binding affinities. In the case of Fe(II), we demonstrate the need of additional ligating residues, in particular those containing carboxylate groups, in the vicinity of the binding site. Moreover, stabilization of di-tert-butylsemiquinone radical (DTB-SQ) in water was achieved by binding to the designed metalloproteins, which resulted in the radical being shielded from the aqueous environment. This allowed the first characterization of the radical semiquinone in water by resonance Raman spectroscopy.

INTRODUCTION

Artificial metalloenzymes have emerged as a promising strategy to emulate the catalytic efficiency of natural metalloenzymes in new to nature reactions.1−12 In addition to their potential for application in synthesis, they are also valuable tools for the investigation of the role of the second coordination sphere in enzyme catalysis.3,13−18 Furthermore, designed metalloproteins may allow for the stabilization of highly reactive compounds, giving rise to increased lifetimes of these species. Some metal semiquinone intermediates have been identified in natural enzymes and synthetic mimic complexes.19,20 In one such example, DeGrado et al. recently reported the stabilization of the radical 3,5-di-tert-butylsemiquinone (DTB-SQ), which results from the one-electron oxidation of 3,5-di-tert-butylcatechol (DTB-C) (Scheme 1), in the interior of a de novo designed metalloprotein [DFsc-ZnII].21 o-Semiquinones are important radicals that natural metalloenzymes generate inside their pocket during the catalytic oxidation of catechols to quinones or in the oxidative cleavage of catechols.22−24 Notably, DTB-SQ rapidly decomposes in an aqueous environment; however, the coordination to zinc ions allows the radical to bind in the hydrophobic environment provided by DFsc and it is thus shielded from bulk water.

We have developed a class of artificial metalloenzymes based on multidrug resistance regulators such as the transcription factor lactococcal multidrug resistance regulator (LmrR).23−29 This homodimeric protein has a large hydrophobic pocket at the dimer interface that has proven suitable for the creation of novel active sites. Recently, we have reported the introduction of the metal-binding unnatural amino acid 2,2′-bipyridyl alanine (bpyA) in LmrR using stop codon suppression methodology (Figure 1).30 Upon binding of Cu(II) ions, the resulting artificial metalloenzymes were then employed successfully in Friedel−Crafts alkylation reactions.25

Here we report on the coordination chemistry of a variety of LmrR-based metalloproteins, involving different metal ions, and the binding and stabilization of o-semiquinone radicals.

RESULTS AND DISCUSSION

On the basis of X-ray crystal structures and our earlier studies,25,31,32 positions situated at the ends of the protein pocket were selected for the introduction of bpyA within the hydrophobic environment: positions V15, N19, and M89.

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LmrR is homodimeric, and it is important to note that every protein contains two bpyA residues in the hydrophobic pocket: one from each monomer. In the case of the mutant LmrR_M89X, positions V15 and F93, which are spatially close to the bpyA residue at position 89, were mutated to introduce additional dative ligands for the metal ion: i.e., carboxylate and imidazole moieties. These mutations were envisioned to result in binding sites that mimic the 2His-1 Carboxylate triad often found in nonheme iron proteins33 and the 3His motif observed in Zn(II) proteins.34 The diastereomers of these metal ions were evaluated. In the case of histidine mutation at position F93, the absorption spectrum of LmrR_M89X_F93H was unaffected in the presence of Fe(II); thus, its coordination was not observed. In contrast, mutation of this position to glutamate, i.e., LmrR_M89X_V15E_F93D. With these additional dative ligands present, Fe(II) binding was observed, as evidenced by the appearance of the 310 nm band in the absorption spectrum (Figure S5 in the Supporting Information). However, the titration curve with Fe(II) was not well-defined. Therefore, an additional carboxylate moiety was added to the coordination sphere by mutation of position V15 to glutamate: i.e., LmrR_M89X_V15E_F93D. With these additional dative ligands present, Fe(II) binding was observed, as evidenced by the appearance of the 310 nm absorption band and a well-defined titration curve, analysis of which showed a 1:1 metal ion to protein monomer binding ratio and a low K_d value of 1.67 μM (Figure S6 in the Supporting Information). Hence, it can be concluded that additional carboxylato ligands are important for achieving Fe(II) binding to the bpy moiety, likely by mimicking the 2 His-1 Carboxylate triad in the active site of natural Fe(II) enzymes. However, also with this mutant, Mn(II) coordination was still not observed.

The binding of the other metal ions to LmrR_M89X_V15E_F93D was investigated (Figure S7 and Table S4 in the Supporting Information) and this indicated that the mutations did not benefit Cu(II) and Ni(II) binding, with K_d values similar to those with LmrR_M89X: i.e., 0.3 and 0.65 μM, respectively (Table 1). The metal to monomer ratios were estimated to be 1:1 for Fe(II), Cu(II), and Zn(II), which correspond to the ratios established with LmrR_M89X. Interestingly, a 1:2 metal binding ratio was determined for Ni(II), which indicates that the coordination is different in LmrR_M89X in comparison to M89X_V15E_F93D. With Co(II), again precipitation was observed after addition of more than 1 equiv.

The influence of the position of bpyA on metal ion binding was studied with Zn(II) and the mutants LmrR_V15X and

Table 1. Dissociation Constants, Molar Absorptivities, and Metal Ion to Binding Site Ratios for the Different Metal Ions Determined with bpyA and LmrR Mutants

<table>
<thead>
<tr>
<th>ligand/protein</th>
<th>metal ion</th>
<th>K_d (μM)</th>
<th>ratio</th>
<th>ε (cm⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bpyA</td>
<td>Fe(II)</td>
<td>0.18 ± 0.18</td>
<td>1:2</td>
<td>27100</td>
</tr>
<tr>
<td></td>
<td>Cu(II)</td>
<td>0.00 ± 0.00</td>
<td>1:1</td>
<td>11690</td>
</tr>
<tr>
<td></td>
<td>Zn(II)</td>
<td>5.85 ± 0.95</td>
<td>1:2</td>
<td>23930</td>
</tr>
<tr>
<td>M89X</td>
<td>Co(II)</td>
<td>1.45 ± 0.82</td>
<td>1:3</td>
<td>32960</td>
</tr>
<tr>
<td></td>
<td>Ni(II)</td>
<td>0.49 ± 0.21</td>
<td>1:3</td>
<td>32840</td>
</tr>
<tr>
<td></td>
<td>Cu(II)</td>
<td>0.04 ± 0.05</td>
<td>1:1</td>
<td>15770</td>
</tr>
<tr>
<td></td>
<td>Ni(II)</td>
<td>0.05 ± 0.06</td>
<td>1:1</td>
<td>16410</td>
</tr>
<tr>
<td>M89X</td>
<td>Fe(II)</td>
<td>1.67 ± 0.93</td>
<td>1:1</td>
<td>13850</td>
</tr>
<tr>
<td>V15E</td>
<td>Cu(II)</td>
<td>0.31 ± 0.08</td>
<td>1:1</td>
<td>14050</td>
</tr>
<tr>
<td>F93D</td>
<td>Zn(II)</td>
<td>0.84 ± 0.39</td>
<td>1:1</td>
<td>12480</td>
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<tr>
<td></td>
<td>Ni(II)</td>
<td>0.65 ± 0.07</td>
<td>1:2</td>
<td>26390</td>
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<td>V15X</td>
<td>Zn(II)</td>
<td>1.35 ± 0.59</td>
<td>1:1</td>
<td>4120</td>
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<td>N19X</td>
<td>Zn(II)</td>
<td>1.52 ± 0.33</td>
<td>1:1</td>
<td>10660</td>
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<tr>
<td>M89X_F93D</td>
<td>Zn(II)</td>
<td>0.28 ± 0.07</td>
<td>1:1</td>
<td>13290</td>
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<tr>
<td>M89X_F93H</td>
<td>Zn(II)</td>
<td>1.41 ± 0.51</td>
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<td>14080</td>
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<tr>
<td>M89X_V15H_F93H</td>
<td>Zn(II)</td>
<td>1.75 ± 0.38</td>
<td>1:1</td>
<td>11670</td>
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</table>
N19X. In contrast to LmrR_M89X, well-defined behavior was observed with 1:1 metal to monomer ratios and low micromolar $K_d$ values (Figure 3 and Figure S8 in the Supporting Information). Interestingly, the molar absorptivities were almost twice as high for LmrR_N19X than for LmrR_V15X. The $K_d$ values were found to be comparable to that measured for LmrR_M89X_V15E_F93D (Table 1 and Table S5 in the Supporting Information). Thus, in contrast to LmrR_M89X, the proteins with bpyA at positions V15 and N19 do provide a good binding environment for Zn(II), which may be due to carboxylate ligands from D100 and E104 that are present in the vicinity (Figure S9 in the Supporting Information).

Finally, the influence of additional ligating residues in the vicinity of the M89X residue on Zn(II) binding was investigated for LmrR_M89X mutants. As previously mentioned, mutant M89X_V15E_F93D improved the binding of Zn(II). M89X_F93D, M89X_F93H, and M89X_V15H_F93H were prepared to provide carboxylate or imidazole groups, in order to complete the zinc coordination sphere. With all three mutants, formation of the band around 310 nm was observed and 1:1 metal to monomer ratios were determined (Figure 3 and Figure S8 in the Supporting Information). The lowest $K_d$ value was determined for M89X_F93D (0.28 μM, Table 1 and Table S5 in the Supporting Information), which was 1 order of magnitude lower than those obtained for the histidine mutants.

**UV−Vis Absorption Spectroscopy of Binding of DTB-SQ.** The semiquinone anion radical DTB-SQ was generated in aqueous medium by comproportionation between the catechol DTB-C and the quinone DTB-Q (Scheme 1). In aqueous media, only two-electron oxidation/reduction is observed and the semiquinone radical has not been detected\(^{39}\), a hydrophobic environment is thus essential to isolate it from water and stabilize it.

LmrR_M89X, without added metal ion, shows no ability to stabilize DTB-SQ: i.e., spectral features are not observed by UV−vis absorption and electron paramagnetic spectroscopy, indicating that no radical species was detected. In the presence of 0.9 equiv of metal ion (Cu(II), Zn(II), Co(II), and Ni(II)) with respect to the concentration in protein monomer, two absorption bands were observed in the near-IR region of the spectra (Figure 4, Figure S10 in the Supporting Information, and Table 2), although they are only weak for Co(II). These bands are similar to those observed with the designed metalloprotein [DFsc-ZnII\(_2\)] and, hence, are assigned to the...
radical semiquinone DTB-SQ (vide infra). The differences in absorbance are due to different yields in DTB-SQ, as observed by resonance Raman spectroscopy (vide infra). In comparison to Zn(II), about half the DTB-SQ is obtained with Cu(II) and Ni(II) and only 20% with Co(II). Attempts to determine the kinetics of semiquinone radical formation for the various metalloproteins were unsuccessful, which might be due to other processes such as binding being rate limiting.

In the presence of Fe(II), a different behavior was observed. While in the absence of DTB-C binding of Fe(II) was not observed (vide supra), two broad absorption bands centered at 790 and 617 nm were observed when DTB-C was present. However, this is characteristic for an Fe(III)-catecholate species, reminiscent of the intermediate identified in catechol oxidases. Such catecholate model complexes have been reported based on aminopyridine ligands, with two bands in the visible (500–580 nm) and NIR (800–950 nm) regions of their absorption spectra in organic solvent. Moreover, the absorption spectrum of [Fe(II)(cat)]19 in water shows only one band at 570 nm. The spectrum observed for LmrR_M89X in the presence of Fe(II) and DTB-C suggests that a bpy-Fe(III)-catecholate complex is generated under aerobic conditions, and catechol cleavage does not occur.

Similar behavior was observed with LmrR_M89X_V15E_F93D in the presence of 0.9 equiv of the different metal ions (Figure S11 in the Supporting Information). An Fe(III)-catecholate species was predominately formed with Fe(II), while DTB-SQ was detected with similar yields for the other metal ions. The absorbance maxima for the radical semiquinone absorption band were mainly red shifted in comparison to those measured with LmrR_M89X (Table 2), most probably due to the different binding environment offered by LmrR_M89X_V15E_F93D.

In an alternative approach, LmrR_M89X_V15D_F93D-Fe(II) was prepared by incubation with excess Fe(II) followed by dialysis to remove the excess of metal ion. Upon addition of DTB-C:DTB-Q, absorption bands centered at 745 and 803 nm were observed, indicative of DTB-SQ (Figure S12 in the Supporting Information and Table 2). In this case, a band at 614 nm was not observed, suggesting that the Fe(III)-catecholate species was not generated under these conditions. Thus, removal of free Fe(II) ions avoids the formation of Fe(III)-catecholate and only the radical semiquinone is observed.

The importance of the position of the binding site was determined for the Zn(II)-containing proteins (Figure S13 in the Supporting Information). All artificial zinc proteins showed absorption bands typical for DTB-SQ, although shifts in absorbance maxima of the DTB-SQ absorption band were observed (Table S6 in the Supporting Information), indicative of different binding environments in the different zinc proteins. Differences in absorbance were also observed, indicating that different yields in radical semiquinone can be achieved depending on the nature of the binding environment.

The stability of the radical semiquinone species was assessed for the LmrR_M89X metalloproteins after 24 h incubation with DTB-C:DTB-Q by performing dialysis against Tris buffer at pH 7. Interestingly, the intensities of the DTB-SQ absorption bands after dialysis were similar to those before dialysis (Figure S14 in the Supporting Information). In the case of Fe(II), the 617 nm band disappeared, indicating that the Fe(III)-catecholate species was removed and only the semiquinone species remained. Moreover, for each metal ion the band at ~310 nm was still observed in the UV spectra after dialysis of the artificial protein solution (Figure S14c), which shows that the metal ions are still bound to the bpy moiety. Surprisingly, the absorption spectra did not show changes even after storage of the solution at 5 °C for 4 days (data not shown). These results demonstrate the high stability of DTB-SQ in the hydrophobic pore of artificial metalloproteins.

**Spectroscopic Characterization of the Radical Semiquinone.** DTB-SQ stabilized by the different designed LmrR mutants and in the presence of the different transition-metal ions was further characterized by magnetic (EPR) and vibrational (resonance Raman) spectroscopy. EPR spectra at room temperature showed the presence of a broad signal at g = 2.003 for every semiquinone solution, which corresponds to a radical species similar to that reported by DeGrado et al. (Figure 5).

Resonance Raman spectra were recorded after addition of DTB-C:DTB-Q 1:1 solution in DMSO to LmrR_V15X_Zn(II), which showed higher yields in DTB-SQ in comparison to LmrR_M89X_Zn(II) (Figure 6). Over time, bands appeared at 1543, 1319, 1167, and 614 cm−1, which are assigned to C=C stretching of the aromatic ring, C=O stretching, C–H bending in plane, and ring breathing stretching, respectively. The observed Raman bands are different from those reported for an Fe(III)-catecholate complex, excluding that the species are metal catecholates.

Resonance Raman spectra on solutions containing LmrR_M89X or LmrR_M89X_V15E_F93D in the presence of different metal ions and after 24 h incubation with DTB-C:DTB-Q 1:1 were measured (Figure S16 in the Supporting Information). The same Raman shifts as with LmrR_V15X_Zn(II) were observed for all solutions, albeit with differences in intensity. This suggests that different conversions to the semiquinone were achieved depending on the metal ion used. Moreover, the bands were still observed after dialysis (Figure S17 in the Supporting Information), corroborating the results obtained by absorption spectroscopy.

**CONCLUSION**

Here we have shown that by in vivo incorporation of an unnatural amino acid (bpyA), a defined metal binding environment can be created within a protein scaffold. A variety of divalent metal ions can bind with good affinity. In those cases where initially no binding was observed, e.g. with Fe(II), this could be achieved by optimizing the binding site by introducing additional ligating residues in proximity of the bpyA residue, such as aspartate, glutamate, or histidine that are found in the binding sites of natural metalloproteins. Further spectroscopic

<table>
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<tr>
<th>Metal</th>
<th>λ_max(1) (nm)</th>
<th>λ_max(2) (nm)</th>
<th>λ_max(1) (nm)</th>
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<tr>
<td>Cu(II)</td>
<td>764</td>
<td>~850</td>
<td>765</td>
<td>~850</td>
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<td>Zn(II)</td>
<td>739</td>
<td>789</td>
<td>733</td>
<td>801</td>
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<tr>
<td>Co(II)</td>
<td>733</td>
<td>780</td>
<td>732</td>
<td>788</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>758</td>
<td>813</td>
<td>764</td>
<td>817</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>745</td>
<td>803</td>
</tr>
</tbody>
</table>

“Protein incubated with an excess of Fe(II) followed by dialysis to remove the excess of metal ions.”

Table 2. Absorbance Maxima of DTB-SQ Absorption Band Measured with LmrR_M89X and LmrR_M89X_V15E_F93D in the Presence of Transition-Metal Ions
analyses are required to determine the binding geometry of the different metals.

Reminiscent of natural enzymes, the artificial metalloproteins were shown to be capable of binding and stabilizing the radical semiquinone DTB-SQ. Binding this normally unstable species by LmrR_M89X with Fe(II) and Co(II), in comparison to the baseline. Absorption spectra of the corresponding solutions are presented in Figure S15 in the Supporting Information.

**Figure 5.** EPR spectra of DTB-SQ bound by LmrR_M89X with Cu(II), Ni(II), and Zn(II), and by LmrR_M89X_V15E_F93D with Fe(II) and Co(II), in comparison to the baseline. Absorption spectra of the corresponding solutions are presented in Figure S15 in the Supporting Information.

**Figure 6.** Resonance Raman spectra over time following addition of 30 μM monomer LmrR_V15X-Zn in solution (bottom spectrum) of 20 equiv of DTB-C:DTB-Q 1:1, over 200 min (top spectrum). Excitation was at 785 nm, and spectra are baseline corrected.

In its stabilization. The stabilization of radical species is the first step toward harnessing the chemistry of unstable radicals in water, potentially allowing future application in catalysis of radical reactions or one-electron-redox processes.

## EXPERIMENTAL SECTION

**Materials and Methods.** *E. coli* strains NEB5-alpha and BL21(DE3) (New England Biolabs) were used for cloning and expression. DNA sequencing was carried out by GATC-Biotech (Berlin, Germany). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Restriction endonucleases were purchased from New England Biolabs. Plasmid Purification Kit was purchased from QIAGEN. Pfu Turbo polymerase was purchased from Agilent. Strep-tactin columns were purchased from Iba-lifesciences. Chemicals were purchased from Sigma-Aldrich and used without further purification. The following metal salts were used: Cu(NO₃)₂·3H₂O, Zn(NO₃)₂·6H₂O, Ni(NO₃)₂·6H₂O, Mn(NO₃)₂·4H₂O, Co(NO₃)₂·6H₂O, Mn(NO₃)₂·4H₂O, and (NH₄)₂Fe(SO₄)₂·6H₂O.

All experiments are performed in freshly prepared Tris buffer pH 7 (50 mM Tris, 500 mM NaCl) at room temperature, unless otherwise specified. UPLC-MS spectra were measured on a Waters UPLC-MS instrument with C8 reversed phase column (Acquity BEH C8 1.7 μm 2.1 × 150 mm) in phosphate buffer pH 8 (50 mM NaH₂PO₄, 500 mM NaCl) with TQD detection. Exact masses of the proteins were extracted from the spectra by deconvolution using MagTran software. UV/vis absorption spectra were recorded at room temperature on a Jasco V-660 spectrophotometer. The concentration of the protein and bpyA was 30 μM in monomer in buffer pH 7 (50 mM phosphate buffer, 500 mM NaCl), unless otherwise specified. For kinetic experiments, absorbance was measured every 1 min over 900 min. Absorption maxima are ±2 nm. EPR spectra (X-band, 9.46 GHz) were recorded on a Bruker ECS106 spectrometer at room temperature. Experimental conditions: microwave frequency, 9.46 GHz; microwave power, 20 mW; 10 G field modulation amplitude; time constant 81.92 ms; scan time 83.89 s; three accumulations. Resonance Raman spectra were obtained with excitation at 785 nm using a PerkinElmer Raman station. Data were recorded and processed using Spectrum (PerkinElmer) and Spectrogryph. Samples were held in 10 mm path length quartz cuvettes.

**Molecular Biology.** Site-directed mutagenesis was used for preparation of all LmrR mutants. It was performed on the previously reported plasmids pET17b_LmrR_LM, pET17b_LmrR_LM_M89X, pET17b_LmrR_LM_M89X_V15E_F93D, and pET17b_LmrR_LM_M89X_F93H, according to the needed mutation. The primers required for the mutagenesis are summarized in Table S1 in the Supporting Information. The following PCR cycles were used: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 30 s, annealing at 58–63 °C for 30 s (depending on the Ta of the particular mutant), and extension at 72 °C for 4 min 30 s. The thermal cycle was repeated 16 times. The resulting PCR product was digested with restriction endonuclease DpnI for 1 h at 37 °C and transformed into the E. coli NEB5-alpha cells. Site-directed mutagenesis for the other mutants has been previously reported. In the text, the proteins have been named without the "LM" label.

**Protein Expression and Purification.** The plasmids pEVOL-bpyA and pET17b_LmrR_LM_X were cotransformed into E. coli BL21(DE3), and a single colony was used to inoculate an overnight culture of 10 mL of fresh LB medium containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol at 37 °C. A 2 mL portion (500X dilutions) of overnight culture was used to inoculate a fresh LB medium containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. When the culture reached an optical density at 600 nm of 0.8–0.9, the expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration 1 mM) and l-arabinose (final concentration 0.02%), and 100 mg/L of bpyA (racemic mixture, for synthesis see ref 2) was added. Expression was done overnight at 30 °C. Cells were harvested by centrifugation (6000 rpm, JA10, 20 min, 4 °C, Beckman), resuspended in washing buffer (50 mM NaH₂PO₄, 500 mM NaCl, pH 8.0), and sonicated...
Absorption spectra were measured at each step of the experiment. Solution in Milli-Q water. After 1 h of incubation, 5 mM DTB-C:DTB-Q 1:1 solution in DMSO was added, were analyzed by SDS-PAGE electrophoresis on 12% polyacrylamide resuspension bu-

Radical Stabilization Experiments. All experiments were run at room temperature and under aerobic conditions. To a solution of 30 μM of artificial protein in 250 μL of 50 mM Tris buffer pH 7 was added 0.9 equiv of the metal salt solution (2 μL from a 3.4 mM solution in Milli-Q water). After 1 h of incubation, 5 μL of a freshly prepared 15 mM DTB-C:DTB-Q 1:1 solution in DMSO was added, and the absorption at 735 nm was recorded every 15 h. The solution were then dialyzed overnight against 50 mM Tris buffer pH 7 at 4 °C. Absorption spectra were measured at each step of the experiment. Resonance Raman and EPR spectra were recorded at the end of the experiment.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorg-chem.7b02073.

List of primers, SDS-PAGE gels, UPLC mass spectra, additional UV/vis/NIR, EPR, and resonance Raman data, and additional LmrR structure representation (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail for G.R.: j.g.roelfes@rug.nl.

ORCID

Nathalie Séguad: 0000-0002-9221-1416
Wesley R. Browne: 0000-0001-5063-6961
Gerard Roelfes: 0000-0002-0364-9564

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS

bpyA, (2,2'-bipyridin-5-yl)alanine; bpy, bipyridyl; LmrR, lactococcal multidrug resistance regulator; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EPR, electron paramagnetic resonance; DMSO, dimethyl sulfoxide

REFERENCES


