Turning a Hyperthermostable Metallo-Oxidase into a Laccase by Directed Evolution

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Supporting Information

ABSTRACT: Multicopper oxidases are multifunctional enzymes that can be broadly divided into two functional classes: metallo-oxidases (with a robust activity toward metals, such as Cu(I) or Fe(II)) and laccases (with a superior catalytic efficiency for organic compounds). Laccases are green catalysts with an outstanding redox capability over a wide range of aromatic substrates using O2 as an electron acceptor and releasing water as reduced product. Hyperthermostable laccases are highly in demand for their robustness in biotechnological applications. In this study, a laboratory evolution approach was conducted to improve the specificity of the metallo-oxidase McmA from the hyperthermophilic bacterium Aquifex aeolicus for aromatic compounds. Four rounds of random mutagenesis of the mcoA-gene followed by high-throughput screening (∼94 000 clones) led to the identification of the 2B3 variant featuring a 2-order of magnitude higher catalytic efficiency (kcat/Km) than the wild-type enzyme for the typical laccase substrate ABTS (2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)) and additionally displaying a higher activity for phenolics and synthetic aromatic dyes. Notably, the recombinant 2B3 variant, unlike the wild-type, did not show temperature-dependent aggregation, exhibiting enhanced solubility and thus higher kinetic and thermodynamic thermostability. The structural basis of the altered substrate’s catalytic efficiency and increased solubility/thermostability of the 2B3 variant are discussed on the basis of the biochemical analysis of single and double mutations of wild-type and its variants. The hyper-robustness of the evolved enzyme reported here shows clear advantages for current applications and provides a powerful tool for generation of more efficient biocatalysts for specific applications because it is widely acknowledged that thermostable proteins have an enhanced mutational robustness and evolvability.

KEYWORDS: hyperthermophiles, enzyme specificity, protein aggregation, protein stability, ligninolytic enzymes, biorefineries

INTRODUCTION

Laccases are enzymes belonging to the large family of multicopper oxidases (MCOs) that are encoded in the genomes of organisms in all three Domains of Life — Archaea, Bacteria, and Eukarya. MCOs couple the oxidation of four molecules of one-electron donor substrate with the reduction of one molecule of dioxygen to H2O. These enzymes are distinguished by having three distinct copper sites, Cu types 1, 2, and 3; the oxidation of the reducing substrate occurs at the T1 Cu site, whereas the reduction of O2 occurs at the T2/T3 trinuclear cluster. The substrate specificity of MCOs is quite broad, and these enzymes are able to oxidize organic and inorganic substrates with varied specificity. One striking characteristic is the activity that only some MCOs, the so-called metallo-oxidases, exhibit toward metal ions, such as Cu(I) and Fe(II) as reducing substrate. Laccases, because of their wide range of aromatic substrates and lack of requirement for exogenous cofactors, have found application in a large number of biotechnological applications in several industrial fields. Additionally, laccases are the most promising ligninolytic enzymes, and therefore, it is expected that the number of laccase-based oxidation processes will increase significantly in the next few years in the lignocellulose biorefinery field, which represents a promising alternative source of renewable chemicals, materials, energy, and fuels.

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In recent years, we have focused our attention on the study and application of prokaryotic members of MCOs, the CuA-laccase from Bacillus subtilis and the metallo-oxidases, McoC from Campylobacter jejuni, McoA from the hyperthermophilic bacterium Aquifex aeolicus, and McoP from the archeon Pyrococcus aerophilum. McoA from A. aeolicus, which is encoded within a copper resistance operon in the genome of this bacterium, shows a notable thermostability \((T_{opt} = 75 ^\circ C)\) and thermostability (temperature values at the midpoint \((T_{mid})\) of 105, 110, and 114 \(^\circ C\)) and a remarkable specificity constant \((k_{cat}/k_{m})\) also called catalytic efficiency) for the oxidation of cuprous and ferrous ions. Therefore, the enzyme was designated a metallo-oxidase similar to CueO from E. coli or Fet3p from yeast. In the present study, we set up a directed evolution approach in order to increase the McoA’s catalytic efficiency for aromatic compounds because enzymes, from extremophiles and thermophiles, in particular, are promising for industrial applications due to their robustness required for specific industrial processes.

Directed laboratory evolution is a powerful protein engineering tool to tailor biocatalysts with improved features or new functions. By mimicking the principles of natural selection through iterative rounds of random mutagenesis and/or DNA recombination and screening, the time scale of evolution can be shortened to an experiment which can be conducted in the laboratory. One of the major technologies underlying synthetic biology is the use of directed evolution for creating novel biocatalysts aiming at the synthesis of chemicals or degradation of toxic pollutants able to replace current polluting agents. Directed evolution, guided the in vitro and in vivo approach in order to increase the McoA activity levels similar to well-studied laccases. Importantly, the evolved enzyme features higher thermostability than that of McoA from the hyperthermostable metallo-oxidase from the multicopper oxidase family into a laccase. An evolved variant was selected among different McoA variants were used as candidates to tailor biocatalysts with improved features or new applications due to their robustness required for specific industrial processes.

### MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Media.** E. coli strain DH5\(\alpha\) (Novagen) was used for routine propagation and amplification of plasmid constructs. E. coli Tuner (DE3, Novagen) and KRX (Promega) strains in which the cueO gene, which codes for the multicopper oxidase CueO, are inactivated (E. coli Tuner \(\Delta\)cueO::kan, KRX \(\Delta\)cueO::kan) were constructed by generalized phase P1vir transduction of a \(\Delta\)cueO::kan cassette from strain W3110 replacing the cueO gene as described in ref 34. E. coli Tuner \(\Delta\)cueO::kan or KRX \(\Delta\)cueO::kan were used to express the mcoA gene previously cloned in pET-21a (+) plasmid (Novagen) or its evolved mutant variants. In the Tuner strain, the mcoA gene is under the control of T7 promoter, and its expression is induced by isoprpyl \(\beta\)-D-1-thiogalactopyranoside (IPTG), whereas in the KRX strain, mcoA expression is under the control of the rhaP promoter, induced by rhamnose. Luria–Bertani medium (LB) or Terrific Broth medium (TB) were used for the maintenance and growth of E. coli strains, supplemented with appropriate antibiotics when required.

**Random Mutagenesis by Error-Prone PCR and Mutant Library Construction.** Random variations in the mcoA gene were introduced by ep-PCR. Primers \(\Delta\)Tuner-CCACCTAAAGGAGTAAACATATGGAGAGGC-3’ (mcoA-182D) and \(\Delta\)Tuner-GAAGTTGAATTTTCAACATATTGACACC-3’ (mcoA-1816R) were used for amplification (nucleotides for restriction sites of NdeI and EcoRI are underlined). ep-PCR was performed in a 50 \(\mu\)L final volume containing 3 ng of DNA template (plasmid pATF1, \(\Delta\) containing the mcoA gene or its evolved variant genes), 1 \(\mu\)M of primers, 200 \(\mu\)M of dNTPs, 1.5 mM MgCl2, Taq polymerase buffer, and 2.5 U of Taq polymerase (Fermentas). The effect of MnCl2 was tested at 0.1–0.25 mM concentrations. After an initial denaturation period of 5 min at 94 \(^\circ C\), the following steps were repeated for 25 cycles in a thermal cycler (MyCycler thermocycler, Biorad): 1 min at 94 \(^\circ C\), 2 min at 55 \(^\circ C\), and 2 min at 72 \(^\circ C\) followed by a final 10 min period at 72 \(^\circ C\). The amplified products were purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare). The final PCR products were digested with NdeI/EcoRI (Fermentas) and cloned into plasmid pET-21a (+) (Novagen). These pET-21a (+) plasmids expressing wild-type mcoA or its evolved variants were transformed into electrocompetent E. coli Tuner \(\Delta\)mcoA::kan cells.

**Construction of mcoA Variants Using Site-Directed Mutagenesis.** Single amino acid substitutions in the mcoA gene were created using the Quick change mutagenesis protocol developed by Stratagene. Plasmids containing the mcoA gene and genes coding for different McoA variants were used as templates with appropriated primers (Table S1). PCRs were carried out in 50 \(\mu\)L reaction volumes containing 3 ng of DNA template, 2 \(\mu\)M of primers, 200 \(\mu\)M of dNTPs, 1.5 mM MgCl2, Taq polymerase buffer, and 2.5 U of Taq polymerase (Fermentas). The effect of MnCl2 was tested at 0.1–0.25 mM concentrations. After an initial denaturation period of 5 min at 94 \(^\circ C\), the following steps were repeated for 20 cycles in a thermal cycler (MyCycler thermodicyer, Biorad): 1 min at 94 \(^\circ C\), 2 min at 55 \(^\circ C\), and 2 min at 72 \(^\circ C\) followed by a final 10 min period at 72 \(^\circ C\). The amplified products were purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare). The final PCR products were digested with NdeI/EcoRI (Fermentas) and cloned into plasmid pET-21a (+) (Novagen). These pET-21a (+) plasmids expressing wild-type mcoA or its evolved variants were transformed into electrocompetent E. coli Tuner \(\Delta\)mcoA::kan cells.

**High-Throughput Screening.** E. coli Tuner \(\Delta\)mcoA::kan cells harboring expression plasmids were grown overnight on solid TB medium supplemented with ampicillin (100 \(\mu\)g \(\mu\)L\(^{-1}\)), kanamycin (10 \(\mu\)g \(\mu\)L\(^{-1}\)), and 0.05 mM IPTG. Expression of the wild-type mcoA gene was initially tried using both the E. coli Tuner \(\Delta\)mcoA::kan and KRX \(\Delta\)mcoA::kan strains, but the use of the Tuner strain resulted in a 10 times higher number of transformants and was thus chosen for further characterization.

**Activity-on-Plate** High-Throughput Screening. E. coli Tuner \(\Delta\)mcoA::kan cells harboring expression plasmids were grown overnight on solid TB medium supplemented with ampicillin (100 \(\mu\)g \(\mu\)L\(^{-1}\)), kanamycin (10 \(\mu\)g \(\mu\)L\(^{-1}\)), and 0.05 mM IPTG. Expression of the wild-type mcoA gene was initially tried using both the E. coli Tuner \(\Delta\)mcoA::kan and KRX \(\Delta\)mcoA::kan strains, but the use of the Tuner strain resulted in a 10 times higher number of transformants and was thus chosen for further characterization.
buffer, pH 7.6, containing 5 mM MgCl₂ and 1 U mL⁻¹ of DNase I. Cells were disrupted by French Press (Thermo EFC), and lysates were centrifuged at 40 000 g for 2 h at 4 °C. Cell crude extracts were incubated at 80 °C for 20 min, and denatured protein was removed by centrifugation (12 000 g for 10 min). For 2B3, the resultant supernatant was loaded onto an ion exchange Q-Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.6. Elution was carried out with a two-step linear NaCl gradient (0.0–0.5 and 0.5–1 M) in the same buffer. Fractions were collected and assayed for MCO-activity. Active fractions were pooled, concentrated, and applied on a Superdex 200 HR 10/30 column equilibrated with 20 mM Tris-HCl buffer, pH 7.6, containing 0.2 M NaCl. Active fractions were pooled, and enzyme concentration was estimated using the Abs₂₈₀ nm value.

**UV–vis and CD Spectra.** UV–visible absorption spectra of purified enzymes were obtained at room temperature in 20 mM Tris-HCl buffer, pH 7.6, using a Nicolet Evolution 300 spectrophotometer from Thermo Industries. CD spectra in the far-UV region were measured on a Jasco-720 spectropolarimeter using a circular quartz cuvette with a 0.1 cm optical path length in the range of 190–260 nm. Protein content was 25 µM in 20 mM Tris-HCl buffer, pH 7.6.

**Apparent Steady-State Kinetic Analysis.** Maximal rates for oxidation of ABTS, syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP), guaiacol, ferrous ammonium sulfate ([NH₄]₂Fe(SO₄)₂·6H₂O), manganese chloride (MnCl₂), Mordant black 9 (MB9), Acid blue 62 (AB62), and Acid black 194 (AB194) (Table S2) were monitored at 40 °C using either a Nicolet Evolution 300 spectrophotometer or a Synergy2 microplate reader (BioTek). Oxidation of the various substrates was performed using the following conditions: in 100 mM sodium acetate buffer, pH 4, for ABTS (3 mM); in 100 mM sodium phosphate, pH 7, for SGZ (0.1 mM), 2,6-DMP (1 mM) and guaiacol (1 mM); in 100 mM sodium phosphate, pH 6, for MB 9 (2 mM), AB62 (2 mM), AB194 (2 mM) and in 100 mM MES buffer, pH 5, for ferrous ammonium sulfate (0.1 mM). Reactions were followed at 420 nm (ε_{ABTS} = 36,000 M⁻¹ cm⁻¹), 530 nm (ε_{SGZ} = 65,000 M⁻¹ cm⁻¹), 468 nm (ε_{2,6-DMP} = 49,600 M⁻¹ cm⁻¹), 470 nm (ε_{Guaiacol} = 49,600 M⁻¹ cm⁻¹), 550 nm (ε_{MB9} = 15,641 M⁻¹ cm⁻¹), 600 nm (ε_{AB62} = 10,920 M⁻¹ cm⁻¹), 570 nm (ε_{AB194} = 11,927 M⁻¹ cm⁻¹) and 315 nm (ε_{FeIII} = 2,200 M⁻¹ cm⁻¹). Cuprous oxidase activity was measured in terms of rate of oxygen consumption by using an oxygen electrode (Oxygraph; Hansatech) at 40 °C as previously described. Stock solutions of [Cu(I)({MeCN})₄]PF₆ were freshly prepared in argon-purged acetonitrile, and reactions were initiated by adding the substrate to an air-saturated mixture containing enzyme, 100 mM acetate buffer, pH 3.5. Apparent steady-state kinetic parameters (K_{M} and k_{cat}) were measured at 40 °C for ABTS (0.02–3 mM, pH 4), SGZ (2–75 µM, pH 7), Cu(I) (0.02–0.5 mM, pH 3.5), and Fe(II) (5–100 µM, pH 5), and kinetic data was fitted directly using the Michaelis–Menten equation (Origin software).

The enzymatic decolourisation of 3 anthraquinonic (Reactive blue 5, Disperse blue 1 and AB62) and 12 azo dyes (MB9, Mordant black 17, Mordant black 3, Acid black 194, Direct blue 1, Sudan orange G, Reactive red 4, Reactive black 5, Reactive yellow 145, Acid black 210, Acid orange 7 and Direct red 80) was monitored by measuring the differences between the initial and final absorbance at the λ_{max} for each dye. Reactions were performed at 37 °C in 96-well plates in 100 mM sodium phosphate, pH 6, with 2 mM of dye and 0.1 mg mL⁻¹ of enzyme. The dependence on pH was tested using Britton Robinson buffer.
(100 mM phosphoric acid, 100 mM boric acid and 100 mM acetic acid mixed with NaOH to desired pH range (4–8)).

**Enzyme Stability Assays.** Kinetic stability studies were performed as described previously. In brief, enzyme solutions were incubated at 80 °C in 20 mM Tris-HCl buffer, pH 7.6, and at fixed time intervals, sample aliquots were withdrawn and tested for activity at 40 °C. Protein aggregation was monitored by measuring static light scattering with a Carry Eclipse spectrophotometer at 500 nm as excitation and emission wavelengths. Differential scanning calorimetry (DSC) measurements were carried out by VP-DSC from MicroCal at a scan rate of 1 °C/min. The experimental calorimetric races were obtained with 0.2 mg mL⁻¹ of protein in 20 mM Tris-HCl buffer, pH 7.6 and 200 mM glycine buffer pH 3. The data were processed and fitted using Origin software supplied by the DSC manufacturer. The progress baseline-subtracted and concentration-normalized DSC curve was fitted to nontwo-state model with three transitions.

**Other Methods.** The copper content was determined through the trichloroacetic acid/bicinchoninic acid method of Brenner and Harris. Protein identification was performed by MALDI-TOF/TOF, and the data was provided by the UniMS – Mass Spectrometry Unit, ITQB/IBET, Oeiras, Portugal. The molecular mass of the purified recombinant 2B3 protein was determined on a gel filtration Superose 12HR10/30 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl. Ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), and aldolase (158 kDa) were used as standards.

## RESULTS AND DISCUSSION

**Validation of High-Throughput Screenings and Optimization of Error-Prone PCR.** An “activity-on-plate” high-throughput screening (HTS) was developed using *E. coli* overproducing wild-type McoA (Figure 1A). This approach, in spite of its qualitative nature, allows for the screening of a higher number of clones in a shorter period of time as compared to a standardized 96-well plate assay. Colonies showing a dark green or purple color (i.e., higher enzyme activity than wild-type) were rescreened using a quantitative activity-screening in 96-well plates. The differences in color are most likely due to the existence of two relatively stable and electrochemically reversible oxidation states of ABTS, namely, the cation radical, ABTS⁺ (green), and the dication, ABTS⁺⁺ (dark purple). Coefficients of variance (CV = standard deviation/mean × 100%) of ≈13% were achieved for the final OD₆₀₀ of cell cultures in 96-well plates as well as for total protein content of crude extracts. A CV for maximal activity of ≈20% was reached, in six different 96-well plates, ensuring the required reproducibility of the mcoA expression system to pursue the directed evolution studies in spite of the low activity detected (5 × 10⁻⁴ U mg⁻¹) in crude extracts. Four libraries of mcoA mutant genes were constructed using different MnCl₂ concentrations in the error-prone PCR (ep-PCR) protocol with Taq polymerase and screened using ABTS (Figure 1B–D). The concentration of 0.15 mM MnCl₂ was chosen for the creation of further libraries because it resulted in a significant variation in activity (Figure 1C) and the desired mutation rate of 1–4 amino acid substitutions per gene.

**Directed Evolution of McoA for Increased Catalytic Efficiency toward Aromatic Compounds.** Four rounds of directed evolution were performed, and a total of approximately 94,000 clones were screened for activity using ABTS as a substrate (Figure 2). Random mutagenesis through ep-PCR was used to generate library diversity, and libraries of ~20,000–30,000 variants were first screened using the “activity-on-plate” HTS. Variants identified showing enhanced activity were rescreened in 96-well plates (~100–700 variants) using end point (for those from the first to the third generation) or kinetic (using the fourth generation variants) enzymatic assays. The parental variants for the following generations were selected on the basis of increased activity in crude extracts (Figure 2). Variant 2B3 was selected in the fourth generation, and the combined mutations gave a 37-fold improvement in activity in crude extracts compared with native enzyme. A fifth round of directed evolution was conducted with libraries constructed by using either ep-PCR using the 2B3 variant gene or DNA shuffling (using three variants from the fourth generation, Figure 2). Unfortunately, even after several attempts, no variants with significantly improved enzymatic activity were identified. Therefore, the variant 2B3 obtained in the fourth generation was produced in *E. coli* (Δ cueO) in a batch scale, purified from the soluble fraction using a heat treatment step and kinetically and biochemically characterized.

Interestingly, the evolved 2B3 variant was produced in a soluble form, and purification from crude extracts resulted in 3-fold higher enzyme yield (2 mg L⁻¹; Figure S1) when compared to the recombinant wild-type McoA. This latter was mostly produced in *E. coli* in the form of inclusion bodies, which required purification using laborious in vitro refolding approaches and resulted in a final yield of 0.6 mg L⁻¹. Purified 2B3 protein migrated in SDS-PAGE as two major bands of ~35 and 59 kDa (Figure S1A); however, a single peak was eluted from exclusion size chromatography with the molecular mass of ~57 kDa, close to the theoretical value predicted from the mcoA gene sequence (59.5 kDa) (Figure S1B). Moreover, the two bands were identified as McoA using MALDI-TOF/TOF analysis with sequence coverage of 37% after tryptic digestion. Therefore, we infer that the ~59 kDa form in the SDS-PAGE represents the fully denatured form and the faster migrating ~35 kDa species most likely represents a partially unfolded form of the enzyme. In fact, only the 35 kDa form was seen in the SDS-PAGE denaturing buffer if the purified protein was left at room temperature, although the treatment of the protein preparation for 10 to 60 min at 100 °C caused the appearance of the ~59 kDa band (data not shown). Incomplete denaturation by SDS treatment has also been reported for some extremely thermostable enzymes.
was similar to the wild-type enzyme, rich in $\beta$-sheets with a negative peak at 216–224 (Figure S2B), reflecting the typical secondary structure found in MCOs. A secondary structure with 12% $\alpha$-helical, 28% $\beta$-strand, and more than 60% of turns and random coil structure was estimated via the CDSSTR method.\(^4\)

**ZBMcoA Variant Possesses Laccase-Activity.** The apparent steady-state kinetic parameters of the purified 2B3 variant were measured for two aromatic substrates, ABTS and SGZ, and for the metal ions, Cu(I) and Fe(II) (Table 1). The obtained values indicated that the laboratory evolution of McoA changed its enzymatic activity from a metallo-oxidase to a laccase; its specificity constant or catalytic efficiency for ABTS ($k_{cat}/K_m$) increased about 100-fold when compared to the wild-type enzyme and is 10-fold higher than that for Cu(I) oxidation. Moreover, the activity for Fe(II) oxidation was strongly diminished, and oxidation of the phenolic SGZ by this variant was significant, in contrast to the wild-type enzyme, which is inactive for this substrate in the absence of exogenous copper in the reaction mixture. Interestingly the catalytic efficiency for Cu(I) remains similar to the wild-type protein. The achieved catalytic efficiency for ABTS oxidation ($2.4 \times 10^5\text{ s}^{-1}\cdot\text{M}^{-1}$) was approximating that observed for the wild-type Cota-laccase ($2.2 \times 10^6\text{ s}^{-1}\cdot\text{M}^{-1}$) and of evolved laccases from *B. subtilis* ($7 \times 10^5\text{ s}^{-1}\cdot\text{M}^{-1}$), *Mycelophthora thermophile* ($1 \times 10^5\text{ s}^{-1}\cdot\text{M}^{-1}$), and *Pleutotus ostreatus* ($6 \times 10^6\text{ s}^{-1}\cdot\text{M}^{-1}$). In all cases, the reported improvement of the efficiencies ($k_{cat}/K_m$) were due to an increase in the $k_{cat}$ parameter. Maximal enzymatic activity in the 2B3 variant was independent of the presence of copper in the reaction assay, in contrast to what was previously reported for the wild-type McoA enzyme.\(^14\) The catalytic efficiency of the 2B3 variant was further investigated using a range of possible aromatic substrates including the phenolic compounds 2,6-DMP, guaiacol and a few anthraquinonic and azo dyes (Table 2 and Table S2). The 2B3 variant exhibited activity not only for both phenolics, but it also showed significant oxidation rates for synthetic dyes, similar to those achieved by Cota-laccase.\(^46\) Moreover, it oxidatively bleached 1S synthetic dyes at different extents in contrast to wild-type McoA enzyme, which exhibited decolorizing activity for only 7 synthetic dyes (Figure 3A). Noteworthy, the catalytic activity of the 2B3 variant using dyes as substrates is measurable over a wide range of pH values (from pH 4 to pH 8) (Figure 3B) in contrast to what has been reported for other

### Table 1. Apparent Steady-State Kinetic Parameters of Wild-Type McoA and Its Evolved 2B3 Variant for the Oxidation of Metal Ions and Organic Substances at 40°C in the Absence of Exogenous Cu(II) in the Reaction Mixture

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wild-type</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cu(I)</td>
<td>2.3 ± 0.3</td>
<td>10.10 ± 0.02</td>
<td>2.3 × 10$^4$</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>0.1 ± 0.008</td>
<td>10.008 ± 0.001</td>
<td>0.25 × 10$^4$</td>
</tr>
<tr>
<td>ABTS$^4$</td>
<td>2.2 ± 0.1</td>
<td>1 ± 0.1</td>
<td>3.3 × 10$^3$</td>
</tr>
<tr>
<td>SGZ$^4$</td>
<td>ND$^*$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>2B3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu(I)</td>
<td>5 ± 1</td>
<td>0.3 ± 0.1</td>
<td>2.0 × 10$^4$</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>ND$^*$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ABTS$^4$</td>
<td>211 ± 10</td>
<td>0.9 ± 0.1</td>
<td>2.4 × 10$^3$</td>
</tr>
<tr>
<td>SGZ$^4$</td>
<td>0.040 ± 0.001</td>
<td>0.008 ± 0.001</td>
<td>5.0 × 10$^3$</td>
</tr>
</tbody>
</table>

ND$^*$ — not detected. \(^{*}100$ mM sodium acetate, pH 3.5, \(^{*}1$00 mM MES buffer, pH 5. \(^{*}1$00 mM sodium acetate, pH 4. \(^{*}1$00 mM sodium acetate, pH 7. *No activity was detected for SGZ in the absence of exogenous Cu(II)); in the presence of 0.1 mM Cu(II), a residual activity of 0.0010 ± 0.0002 U mg$^{-1}$ was measured.

### Figure 2. Lineage of McoA variants generated in this study. In the 1st generation, a total of 29,831 clones were screened using 10 mM ABTS in the “activity-on-plate” HTS, and 657 variants were identified showing enhanced activity. These mutant strains were rescreened in 96-well plates, and the 1B7 variant was selected on the basis of its 2-fold increased activity in crude extracts when compared to the parental wild-type extract. Next, the 2nd generation was evolved from the 1B7 variant as “parent”. The ABTS concentration was adjusted to 4 mM, and 23,891 clones were screened from which 259 variants were rescreened in 96-well plates, and the 1B7 variant was selected on the basis of its 2-fold increased activity for ABTS as compared to its parent (2G6). In the 4th generation, the ABTS concentration was diminished to 2 mM, and the screening of 18,052 clones resulted in the selection of 108 variants. From these, variant 2B3 was selected on the basis of a 4-fold higher activity than the parental strain (1D11). New nonsynonymous mutations are indicated in bold and synonymous mutations are between brackets.
laccases; CotA-laccase shows an optimum at pH 8–9 and no detectable decolorization below pH 5,17 whereas the majority of fungal laccases show optimal for dye degradation in the acidic pH range (from pH 4 to 6).18–20

**2B3McoA Variant Is a Thermoactive and Hyper-thermostable Laccase.** The 2B3 variant reached an activity optimum at temperatures around 70–75 °C (Figure S3), similar to the one observed for the wild-type.14 It is remarkable that, even though the enzyme was repeatedly evolved for higher activity for ABTS, the specificity of the variants changed without loss of thermophily (i.e., showing only minor stability–activity trade-offs). This is most likely related to alterations in conformational flexibility to mediate new functions that occur without severely compromising the conformation governing the previously existing functions.51 Interestingly, the 2B3 variant was dramatically less aggregation-prone than wild-type McoA: the variant deactivates at 80 °C according to a first-order kinetics (Figure 4A and inset) unlike the wild-type enzyme where the activity decay at 80 °C occurs in two steps: (i) a first deactivation step that last for 1 h assigned to an aggregation process, resulting in an intermediate state with 50% of the initial activity, and (ii) the slowly deactivation of this intermediate (with a lifetime of 8 h) to the final denatured state.14 Static light scattering confirmed that the 2B3 variant did not aggregate up to 120 °C, the highest temperature tested (Figure 4B). Moreover, 50% of activity was retained after 5 h at 80 °C, 5- and 2-fold improvements when compared to the wild-type McoA and CotA-laccase, respectively.52 Therefore, the thermal deactivation of the 2B3 variant can be described by the classical Lumry–Eyring model (N ↔ U → D, where N, U, and D stand for the folded, the unfolded, and the denatured enzyme, respectively) in contrast to the kinetic partitioning between aggregation and unfolding observed for the wild-type.14 A similar large improve in the kinetic stability associated with increased resistance to aggregation of a FMN-dependent NADPH:dye/quinone oxidoreductase (azoredutase) variant created by directed evolution was recently reported.52

The thermodynamic stability probed by differential scanning calorimetry (DSC) showed that the 2B3 variant did not unfold up to 120 °C at pH 3. Wild-type McoA exhibits an endothermic peak at pH 3 that was fitted using a non-two-state model with three independent transitions, with melting temperatures (temperature at which 50% of the molecules are unfolded) at 105, 110, and 114 °C.12 Precipitation before unfolding was observed at pH values higher than 3 in McoA wild-type;12 however, we have not detected this behavior in the present study, and the thermal stability of the tertiary structure of both wild type and 2B3 were probed by DSC at pH 7.6. The DSC thermogram of wild type showed the typical protein aggregation fingerprint characterized by a drop in the baseline after the endothermic peak leading to 100% of irreversibility and T_m of ~77, 81, 84 °C could be roughly estimated (Figure 4C). The variant 2B3 did not aggregate after thermal unfolding and displayed three distinct endothermic transitions showing slightly higher T_m values of 82, 84, 86 °C (Figure 4C).

**Molecular Details of Mutations That Change McoA Substrate Specificity or Improve Enzyme Solubility.** The vast majority of nonsynonymous amino acid changes identified in the 2B3 variant are spread on or near the protein surface (nine out of ten), and eight of them occurred in loops (Figure S5), based on the McoA model structure.14 Four of the substitutions, located on surface loops, resulted in serine residues or in other residues bearing a hydroxyl group (F17S, F55S, P58S, and I199T), possibly increasing surface hydrophilicity. Two of these changes, F55S and P58S, are located in one loop, Ser41 to Gly60, are not modeled because no counterparts were found in the template structures.14 The analysis of the five synonymous mutations accumulated show that the codon exchanged in mutations G38G and G255G (GGT → GGC) resulted in similar frequencies of codon usage as compared with the wild-type. In contrast, the mutation E412E (GAA → GAG) leads to a codon that has a lower percentage of usage (31.7%) as compared with the wild-type codon (68.3%) and the two mutations Q380Q and
V444 V favored codon usage CAA (33.5%) → CAT (66.5%) and GTA (15.9%) → GTG (36.2%), respectively, which could potentially enhance the heterologous variants expression.

In order to investigate which nonsynonymous mutations would contribute to the improved properties of the 2B3 variant, nine single variants containing mutations accumulated in the evolution process were constructed from wild-type by site-directed mutagenesis (except for F17S due to technical constraints). These variants were overproduced in *E. coli* and partially purified after incubation of crude extracts at 80 °C for 20 min. The kinetic characterization of variants showed that all mutations, except P58S that resulted in a 3-fold lower activity, led to increased activity for ABTS as compared to wild-type (Table 3); notably, two mutations I441L and I119T, introduced in the first and third generation, respectively, yielded in 6 to 17 higher activity to the wild-type. Interestingly, all variants showed increased half-lives at 90 °C as compared to wild-type (Table 3). Moreover, except for M449T, where a two-step decay in activity was observed upon incubation at 90 °C, the remaining variants deactivates according to a first-order kinetics and therefore

![Figure 4](image-url)  
**Figure 4.** (A) Kinetic stability of wild-type (open circles) and the 2B3 variant (closed circles) at 80 °C. (B) Static light scattering of wild-type (open circles) and the 2B3 variant (closed circles). (C) Excess heat capacity of McoA and the 2B3 variant obtained from differential scanning calorimetry at pH 7.6. The thick line (experimental data of 2B3) was fitted with three independent transitions shown separately in thin lines.

![Figure 5](image-url)  
**Figure 5.** Mapping of the amino acids identified in the 2B3 variant using the model structure of the *A. aeolicus* McoA derived by comparative modeling techniques. Mutations acquired in the 1st generation are indicated in green, those from the 2nd generation in orange, from the 3rd in red, and from the 4th generation in purple.

<table>
<thead>
<tr>
<th>proteins</th>
<th>activity (U mg⁻¹)</th>
<th>lifetime of the 1st step/τ₁/₂ intermediate* at 90 °C (h)</th>
<th>τ₁/₂ at 90 °C (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.038 ± 0.004</td>
<td>0.65 ± 0.1/14 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>M449T</td>
<td>0.06 ± 0.01</td>
<td>0.25 ± 0.05/18 ± 2</td>
<td>-</td>
</tr>
<tr>
<td>I441L</td>
<td>0.2 ± 0.1</td>
<td>-</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>K245R</td>
<td>0.11 ± 0.01</td>
<td>-</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>R471G</td>
<td>0.07 ± 0.01</td>
<td>-</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>P58S</td>
<td>0.011 ± 0.001</td>
<td>-</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>I199T</td>
<td>0.6 ± 0.1</td>
<td>-</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Y172C</td>
<td>0.09 ± 0.01</td>
<td>-</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>V19A</td>
<td>0.05 ± 0.01</td>
<td>-</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>F55S</td>
<td>0.05 ± 0.01</td>
<td>-</td>
<td>9.9 ± 0.1</td>
</tr>
</tbody>
</table>

*The intermediate shows ~50% of the initial activity.

Table 3. Activity (Vmax) and Kinetic Stability (τ₁/₂ at 90°C) of Partially Purified Preparations of Wild-Type McoA and Single Variants
seemed to be able to individually contribute to the abolishment of protein aggregation in the wild-type McoA (Figure 4A).

In the next step, we have assessed the synergistic effect of single mutations, and for that we have constructed and characterized single and double variants of the hits of the second (2G6) and third generation (1D11) (Table 4); please note that the hit of the second generation (2G6) showed only one additional mutation in relation to the hit of the first generation (1B7). 1B7 from the first generation yielded a 10-fold improved activity for ABTS in comparison to the wild-type, most likely related with the observed positive effects of single M449T, K245R, and I441L substitutions in the activity for ABTS (Table 3). The replacement of R471 by a Gly in the second generation, K245R, and I441L substitutions in the activity for ABTS (Table 4). The single replacements of F17S, V19A, and F55S in the 1D11 variant resulted in 3-fold improvements in the activity for ABTS, and thus, we can propose the occurrence of a further synergistic interaction among these residues explaining the 20-fold increased activity measured in 2B3 in comparison to the 1D11 variant (Table 4). Conversely, 2B3 showed a 10-fold lower activity for Fe(II) as compared to those exhibited by 1D11 single F17S, V19A, and F55S variants (Table 4). Maintaining similar levels of activity for Cu(I) in the 2B3 variant and in the wild-type as well as the residual activity for ferrous iron oxidation (Table 4) suggest that in McoA these transition metals do not share the same substrate binding sites. The methionine-rich regions present in many bacterial MCOs, reminiscent of those found in copper homeostasis proteins, are considered “cuprous oxidase” motifs, although carboxylate residues close to the T1 Cu center as in the yeast Fet3p are viewed as “ferroxidase motifs”.

In the model structure of McoA, a methionine-rich segment (Phe 321-Val 363) was observed close to the T1 Cu site, and the maintenance of the Cu(I) activity in the outperforming 2B3 variant most likely related with the absence of substitutions in this Met-rich segment; however, the structural reasons for abrogating Fe(II) oxidation are not obvious at this point. In this context, the apparent critical role of V19A substitution as determinant of stability is worth mentioning for explaining the differences observed in the 2B3 variant unfolding mechanism. The differential thermogram of 1D11 with an additional V19A single mutation did not exhibit, similarly to 2B3 and unlike the other examined variants, the typical protein aggregation fingerprint (i.e., the drop in the baseline after the endothermic peak; Figure 6). This finding correlated well with the highest stability measured in the single V19A variant (t1/2 = 12 h at 90 °C, Table 3).

Overall, our results show that although the directed evolution strategy was based on occurrence of a considerable number of mutation events (i.e., after 4 cycles the final variant accumulated 15 mutations (5 silent)), all mutations introduced appear essential for the hit 2B3 variant properties, except eventually for the synonymous E412E and nonsynonymous P58S substitutions. Therefore, McoA seems to have essentially been evolved through sequential introduction of beneficial mutations. Mutations M449T, K245R, I441L, Y172C, V19A, F55S, and in

<table>
<thead>
<tr>
<th>proteins</th>
<th>Cu (I) (μm$^{-2}$)</th>
<th>Fe (II) (μM mg$^{-1}$)</th>
<th>ABTS (μm$^{-2}$)</th>
<th>lifetime of the 1st step/t1/2 intermediate* at 90 °C (h)</th>
<th>$t_{1/2}$ at 90 °C (h)</th>
<th>$T_m$ at pH 7.6 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.10 ± 0.01</td>
<td>3.2 ± 0.3</td>
<td>0.038 ± 0.004</td>
<td>0.7 ± 0.1/14 ± 3</td>
<td>~77, 81, 84</td>
<td></td>
</tr>
<tr>
<td>1B7</td>
<td>0.08 ± 0.01</td>
<td>3 ± 1</td>
<td>0.37 ± 0.04</td>
<td>0.5 ± 0.1/14 ± 1</td>
<td>~78, 82, 85</td>
<td></td>
</tr>
<tr>
<td>2G6</td>
<td>0.08 ± 0.01</td>
<td>6.6 ± 0.3</td>
<td>0.29 ± 0.03</td>
<td>0.5 ± 0.1/11.3 ± 0.4</td>
<td>~80, 83, 86</td>
<td></td>
</tr>
<tr>
<td>2G6-P58S</td>
<td>nd</td>
<td>3.9 ± 0.3</td>
<td>0.32 ± 0.03</td>
<td>0.5 ± 0.1/7.0 ± 0.1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>2G6-Y172C</td>
<td>nd</td>
<td>2.3 ± 0.4</td>
<td>0.17 ± 0.02</td>
<td>-</td>
<td>6 ± 1</td>
<td></td>
</tr>
<tr>
<td>2G6-I199T</td>
<td>nd</td>
<td>2 ± 1</td>
<td>1.7 ± 0.1</td>
<td>-</td>
<td>6 ± 1</td>
<td></td>
</tr>
<tr>
<td>2G6-I199T/1Y172C</td>
<td>nd</td>
<td>3.8 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>4.9 ± 0.1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>2G6-I199T/P58S</td>
<td>nd</td>
<td>5 ± 1</td>
<td>1.11 ± 0.04</td>
<td>-</td>
<td>5 ± 1</td>
<td>nd</td>
</tr>
<tr>
<td>1D11</td>
<td>0.10 ± 0.01</td>
<td>2.9 ± 0.3</td>
<td>1.35 ± 0.13</td>
<td>-</td>
<td>7 ± 1</td>
<td></td>
</tr>
<tr>
<td>1D11-F17S</td>
<td>nd</td>
<td>2 ± 1</td>
<td>4.7 ± 0.1</td>
<td>-</td>
<td>5.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>1D11-V19A</td>
<td>nd</td>
<td>1.5 ± 0.3</td>
<td>4.7 ± 0.2</td>
<td>-</td>
<td>5.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>1D11-F55S</td>
<td>nd</td>
<td>4 ± 1</td>
<td>4.7 ± 0.2</td>
<td>-</td>
<td>5.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2B3</td>
<td>0.09 ± 0.01</td>
<td>0.4 ± 0.1</td>
<td>21 ± 2</td>
<td>-</td>
<td>5.7 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

nd – not determined. The intermediate shows ~50% of the initial activity.
particular I199T have likely contributed individually or synergistically to the improvement of the oxidation rates for aromatic substrates. Similarly, the substitution of R471G and especially V19A, to smaller amino acid residues, with a potential benefit of releasing of conformational strain, appears particularly significant in the stabilization of the enzyme during the in vitro evolution.

CONCLUDING REMARKS

Laccases are the most promising enzymes for lignin degradation and successful valorization due to (i) the low cost of introducing O₂ as primary oxidant, and (ii) the possibility of controlling reactivity by increasing or quenching O₂ partial pressure in process conditions. Bacterial laccases, although less known and utilized biocatalysts compared to their fungal counterparts, are able to perform analogous reactions while benefiting from higher optimal temperature and pH-range, salt tolerance, and increased stability. Additionally, bacterial systems have well-established genetic and molecular biological tools allowing for higher enzyme production yields and application of protein engineering tools. The evolved 2B3 variant reported here shows considerable catalytic efficiency for ABTS comparable to other well-known laccases and coupled with its enhanced stability, solubility, and easiness of purification is potentially useful for biotechnological applications. It is notable that this variant is potentially an excellent candidate to be further evolved for new specificities and applications considering that the additional stability confers the ability to accommodate the often destabilizing mutations that are required for the acquisition of new and improved functions. Such studies will also allow for a better insight into the structure–function relationships within the multicopper oxidase family of enzymes including the features and mechanisms behind their multifunctionality. This has several general implications in the understanding of molecular recognition of ligands, for example, for drug discovery programs and for the engineering of proteins in the realm of biotechnology, including the field of synthetic biology.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00771.

Supplemental tables and additional figures (Tables S1 and S2; Figures S1–S3) (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES


Figure 6. Excess heat capacity of McoA (solid thin line) and its variants 1B7 (dashed thin line), 2G6 (dot line), 1D11 (dashed thin dot), 1D11-F17S (dashed dot thin line), 1D11-V19A (dashed thick line), 1D11-F55S (short dashed thin line), and 2B3 (solid thick line) obtained from differential scanning calorimetry at pH 7.6.