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Enhancing Mn(II)-binding and manganese peroxidase activity in a designed cytochrome *c* peroxidase through fine-tuning secondary sphere interactions

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Abstract

Non-covalent second shell interactions are important in controlling metal-binding affinity and activity in metalloenzymes, but fine-tuning these interactions in designed metalloenzymes has not been fully explored. As a result, most designed metalloenzymes have low metal-binding affinity and activity. Here we identified three mutations in the second coordination shell of an engineered Mn(II)-binding site in cytochrome *c* peroxidase (called MnCcP.1, containing Glu45, Glu37, and Glu181 ligands) that mimics the native manganese peroxidase (MnP) and explored their effects on both Mn(II)-binding affinity and MnP activity. First, removing a hydrogen bond to Glu45 through Tyr36Phe mutation, enhanced Mn(II)-binding affinity, as evidenced by a decrease of K_M of Mn(II) oxidation 2.8 fold. Second, introducing a salt bridge through Lys179Arg improved Glu35 and Glu181 coordination of Mn(II), decreasing K_M 2.6 fold. Third, eliminating a steric clash that prevented Glu37 from orienting towards Mn(II) resulted in an 8.6 fold increase in k_{cat}/K_M , arising primarily from a 3.6 fold decrease in K_M , with a K_M value comparable to that of the native enzyme (0.28 mM vs. 0.2 mM for *Pleurotus eryngii* MnP PS3). We further demonstrated that while the effects of Tyr36Phe and Lys179Arg mutations are additive, other combinations of mutations were antagonistic. Finally, we showed that these MnCcP variants are functional models of MnP that mimic its activity both in Mn(II) oxidation and degradation of a phenolic lignin model compound and kraft lignin. Our results offer molecular insight into the role of non-covalent

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ASSOCIATED CONTENT

Supporting Information

The supplemental material is available free of charge via the Internet at <http://pubs.acs.org>

Author Contributions

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interactions around metal binding sites for improving metal binding and overall activity; such insight can be applied to rationally enhance these properties in other metalloenzymes and their models.

Graphical Abstract

Keywords

Lignin degradation; protein engineering; protein design; secondary coordination; hydrogen bond

Introduction

Proteins have evolved to catalyze numerous reactions with high efficiency and specificity. Among them, metalloproteins are of particular interest as they are responsible for catalyzing some of the most difficult reactions on earth, such as water oxidation,¹ nitrogen fixation² and lignin degradation.³ Therefore, there has been long-standing interest in elucidating the structural features responsible for strong metal binding and efficient catalysis.^{4–15} Toward this goal, many biochemical and biophysical studies have identified conserved amino acid residues in metalloenzyme active sites. An effective way to verify the importance of these residues is to introduce them to a designed protein to determine whether they confer new activity.^{4–15} Despite progress made in this field, most designed metalloproteins display much lower binding affinity for metal ions and lower activity than native enzymes.¹⁶ One contributing factor is a lack of appreciation for the role of non-covalent secondary coordination shell interactions around the metal binding site in determining the metal-binding affinity and functionality, as most studies so far have focused mainly on reproducing the primary coordination sphere of native metalloproteins. Recently, we and others have explored the roles of electrostatics, hydrogen bonding, and hydrophobic interactions around the primary coordination sphere in fine-tuning the reduction potentials of metalloproteins.^{17–23} While reduction potentials are an important parameter in metalloprotein function, metal-binding affinity and enzymatic activity are just as—if not more—important; however, few studies have focused on exploring the roles of such secondary shell interactions in tuning these aspects.

Manganese peroxidase (MnP, EC 1.11.1.13)^{24, 25} is a heme peroxidase that also binds Mn(II) in the active site. MnP plays a key role in degradation of lignin, the second most abundant biopolymer on earth and an attractive potential source of energy and many commodity chemicals.²⁶ MnP oxidizes lignin indirectly via oxidation of Mn(II) to Mn(III) and secretion of Mn(III) to the extracellular environment.^{27–29} Secretion of Mn(III) is highly advantageous because it eliminates complicated heterogeneous catalytic kinetics and broadens the substrate scope of organisms possessing MnP. Similar to many other peroxidases, the reaction cycle of MnP begins with the oxidation of a heme cofactor by hydrogen peroxide (H₂O₂) (Scheme 1). It then returns to the resting state by two consecutive, one-electron oxidation steps, converting two equivalents of Mn(II) to Mn(III) during the process. The resulting Mn(III) exits the active site whereupon it is chelated by organic acids such as oxalate or lactate secreted by the organism. Mn(III) is a potent oxidizing agent that can activate lignin to initiate its degradation, mainly by generating radicals and oxidizing phenolic substrates.^{27–30}

Despite the demonstrated activity of MnP for lignin degradation, its industrial-scale application has been limited due to difficulties in obtaining sufficient quantities of functional protein and in genetic manipulation of the organisms that produce it.^{28, 31–33} There have been several reports on efficient conversion of lignin to fuel precursors using biosynthetic models or organic catalysts with considerable success.^{34–40} However, most of these studies require extreme conditions such as high temperature and pressure.^{40, 41} In a complementary approach, we and other groups have used easy-to-prepare proteins such as cytochrome *c* peroxidase (CcP)^{24, 25} or DypB⁴² as a scaffold to mimic MnP. CcP is a heme peroxidase found in baker's yeast that shares significant structural similarity with MnP, both in terms of the overall fold (Figure 1a) and the active site (Figure 1b), with the major difference being lack of a Mn(II)-binding site.^{43–45} The catalytic cycle of CcP is also similar to that of MnP, diverging mainly in the type of substrate oxidized (Scheme 1). In contrast to MnP, CcP can be overexpressed in large quantities in *E. coli* in its functional form.^{24, 46}

Previously, we have reported the successful construction of a Mn(II)-binding site in CcP by structural comparison of the Mn(II)-binding site in MnP (Figure 1c) with the corresponding region of CcP (Figure 1d) and making Gly41Glu, Val45Glu, and His181Asp mutations (called MnCcP).²⁴ The protein was then further optimized through replacing Asp181 by Glu, reverting Glu41 back to Gly, and mutating Asp37 to Glu (called MnCcP.1).⁴⁷ These designs allowed converting WTCcP that does not bind Mn(II) and that has no MnP activity into MnCcPs that are capable of binding Mn(II) and performing new MnP activity (Figure 1). In the process, we have identified factors contributing to MnP activity such as the nature of the reaction intermediates (e.g., Compound I' that contains a tryptophan radical in CcP vs. Compound I that contains a porphyrin π cation radical in MnP) and the roles of redox active amino acids (e.g., Trp51 in CcP), which can lead to a more stable Compound I' that has a slower rate of decay to Compound II and a less reactive Compound II. These factors directly influence the ability of CcP to oxidize organic substrates.^{48, 49} Making mutations of Trp and Tyr residues in CcP into corresponding non-redox active amino acids (e.g., Phe) has resulted in a MnCcP with higher MnP activity.^{48, 49}

Although these MnCcP model proteins were shown to bind Mn(II) and oxidize it to Mn(III), their catalytic efficiency ($0.6 \text{ mM}^{-1}\text{s}^{-1}$ for the most active variant) was still far lower than the native MnP ($390\text{--}4340 \text{ mM}^{-1}\text{s}^{-1}$) (Table S1 contains some relevant kinetic parameters for MnPs and their mimics). To improve the catalytic efficiency even further, rather than focusing mainly on improving k_{cat} as in previous studies,^{48, 49} we hypothesize that higher K_{M} of the variants is an important factor contributing to low activity; hence, in this study we focus on improving K_{M} of the reaction. We report herein a crystal structure of Mn(II)-bound MnCcP.1 (Figure 1). Based on structural comparisons between MnCcP.1 and MnP, we have identified key non-covalent interactions in the secondary coordination shell of Mn(II) for tuning Mn(II) binding and MnP activity, specifically mutations of Tyr36, Ile40, and Lys179 that allowed removal of hydrogen bonding (H-bonding) interactions, adding a salt bridge, and removing steric clashes and increasing flexibility, respectively. These mutations resulted in a protein with a ~9-fold increase of catalytic efficiency compared to our initial design and a K_{M} comparable to that of native MnPs. Finally, we show that MnCcP mutants studied here display degradation activity towards phenolic lignin model compounds and alkali-treated lignin.

EXPERIMENTAL PROCEDURE

Materials and reagents

All the materials and reagents used were purchased from either Sigma or Fisher chemicals unless stated otherwise.

Mutant protein generation, purification, and verification

The mutants were generated using PfuUltra II fusion DNA polymerase enzyme (Agilent) for site directed mutagenesis. PCR was performed on a plasmid containing MnCcP.1⁴⁷ and the following DNA sequences as forward primers and their complementary sequences as reverse primers (synthesized by IDT, Inc):

Tyr36Phe: 5'- GGG AAG ATG ACG AAT TTG ACA ACT ATA TAG GC

Ile40Gly: 5'-CGA ATA TGA CAA CTA TGG CGG CTA TGG GCC C

Tyr36Phe/Ile40Gly: 5'- GGG AAG ATG ACG AAT TTG ACA ACT ATG
GCG GCT ATG GGC CC

Tyr36Phe2: 5'- GGG AAG ATG ACG AAT TTG AAA ACT ATA TAG GC

Lys179Arg(His181Glu): 5'- CGC TCT GGG CAG AAC CGA GCT CAA G
-3'

The template plasmid containing the original MnCcP.1 gene was removed by treating the PCR solution with DpnI restriction enzyme (NEB), followed by transformation of the remaining plasmid containing the mutant into Turbo competent cells (NEB) using a protocol prescribed by the company. The plasmids were then purified using QIAprep Spin Miniprep Kit, and the mutations were confirmed by DNA sequencing (ACGT-Inc.).

Purified plasmids containing the mutant sequences were then transformed into BL21-DE3* competent *E. coli* cells (Invitrogen). Protein purification was performed based on a

previously developed protocol in our lab.^{24, 47} Briefly, the cells were grown in a 5 mL Luria Broth media starter culture (10 g bacto tryptone (BD), 5 g yeast extract (BD), and 10 g NaCl (Fisher Chemicals) in 1 L) at 37 °C for 8 hours. Two flasks of 2 L rich media (10 g bacto tryptone, 8 g of yeast extract, 5 g NaCl) were inoculated by 1 mL of starter culture and grown for 16 hours at 37 °C. The culture was then induced by 1 mM IPTG (GoldBio) for 4 hours at 37 °C. The protein was purified from cytosol by sonication using Misonix ultrasonic liquid processor. The processing time was 6 min with 6 s pulses on and 12 s pulses off and an amplitude of 70. The supernatant was then batch-bound to a DEAE column (GE Healthcare) and eluted with a gradient of KCl in a phosphate buffer. The protein was then passed down a Sephadex S-100 gel filtration column (GE healthcare) and an additional DEAE column following reconstitution with hemin. The masses of all proteins were verified by electrospray ionization mass spectroscopy (ESI-MS) with a Quattro II (Waters) ESI mass spectrometer maintained by the School of Chemical Sciences Mass Spectrometry Laboratory (Urbana, IL). Formic acid was added to ionize the protein upon injection.

Protein crystallization, data collection and structure determination

The protein samples were concentrated using a Centricon with a centrifugal filter that has 10,000 Da molecular weight cutoff (Millipore). Proteins were crystallized using a vapor diffusion hanging drop technique. A 1 μ L drop of 50 mg·mL⁻¹ protein solution with 1 mM MnSO₄ was placed on siliconized glass with 1 μ L of well solution. The drop was then equilibrated against 500 μ L of well solution of 0.2 M sodium iodide pH 7.0 (the number 10 from PEG/ion screen HR2-126, Hampton Research) and was incubated at 4 °C. Crystals for suitable X-ray analysis began to appear after 1 week and finished growth after 2–3 weeks. The crystals were soaked for several minutes in 30% PEG 400 in the well solution before mounting. The diffraction data were collected at beam-line X-29A of Brookhaven National Laboratory (BNL). The data were phased and refined using CCP4⁵⁰ and Shelx97 suites, respectively. Molecular replacement was used as phasing model using WT-CcP structure as a model (PDB ID: 2CYP). Crystallographic data are presented in Table S2.

Kinetic experiments

Kinetics experiments were performed on an Agilent 8453 diode array (Agilent) instrument by monitoring the increase in absorption at 270 nm due to increased production of Mn(III)-malonate (molar extinction coefficient = 8000 M⁻¹cm⁻¹ ⁵¹). The experiment begins by addition of 6–10 μ M protein, 400 μ L of different MnSO₄ concentrations in overall 3000 μ L of 500 mM malonate buffer, pH=4.5. Reactions started upon addition of 25 μ L of 10 mM H₂O₂. The data were then transferred to Origin 9.1 for analysis. The values of k_{cat} and K_M were analyzed based on Michaelis-Menten curve fitting.

The extinction coefficient of the Soret band for each protein was determined using the common pyridine hemochromogen assay as described previously.^{52, 53} Extinction coefficients are presented in Table S3.

Gas Chromatography-Mass spectrometry (GC-MS) studies

GC-MS studies were performed on a 6890N network GC system from Agilent Technology with a network GC system 5973 mass selective detector. Samples were extracted by ethyl

acetate and dried in a sodium sulfate column prior to injection. The measurement begins at an initial temperature of 50 °C for 3 min and ramped to 300 °C at 40 °C/min, pausing at the final temperature for 2.5 min.

Molecular dynamics and simulation

Molecular dynamics (MD) simulations were performed using NAMD2⁵⁴ and the results were analyzed and visualized using visual molecular dynamics (VMD)⁵⁵. The structures of the mutants were modeled based on the structures of MnCcP.1 and MnP (PDB IDs: 1YYD and 1MNP, respectively). PSF files were generated using the PSFGEN suite in VMD. Proteins were solvated in a water box with a side of 15 Å. TIP3 explicit water model⁵⁶ was used to describe water. The simulation systems were neutralized by addition of Na⁺ and Cl⁻ ions, with a final NaCl concentration of 0.1 M. Production MD simulations were performed after 1,000–2,000 steps of minimization and a 2-ns equilibration (2 fs/step) in an NPT ensemble.

HPLC analysis

Guaiacylglycerol-β-guaiacol ether, a phenolic lignin model compound dimer was synthesized and generously provided by Ms. Margaret Brown and Dr. Michele Chang in the University of California at Berkeley. The degradation reactions of the compounds were performed in 50 mM sodium acetate buffer pH=4.5 as previously described.⁵⁷ The reaction mixture contained 0.2 μM protein, 1 μM (final) model compound, 1 mM MnCl₂, and initiated with 1 mM H₂O₂. The reaction then stirred overnight at room temperature. Samples for HPLC analysis were prepared by centrifuging the reaction for 5 min at 20,850 x g in a bench top centrifuge. 10 μL of the samples were then injected into an Agilent Eclipse XDB-C18 column (4.6*150, 5u) and ran at 0.5 mL/min. The program was as follows:

A: water with 0.1% formic acid, B: Acetonitrile with 0.1% formic acid

0–2 min, 10% B; 8–11.5 min, 90% B; 12–16 min, 10% B

Reactions were also carried out in the absence of MnCl₂ as a control. The sample elution was monitored at 254 nm.

The reaction with kraft lignin (Lignin, alkali from Sigma-Aldrich) was carried out in 3 mL of 500 mM malonate buffer pH=4.5, by adding 100 μL of 0.5 mM protein to the reaction in the absence or presence of 200 μL of 500 mM MnSO₄. The reaction was initiated with 100 μL of 50 mM H₂O₂ and mixed by shaking the container gently for 84 hours at 30 °C or 24 hours at 37 °C. The samples were prepared for HPLC analysis by addition of 40 μL of 100% CCl₃COOH to 400 μL of the reaction. After mixing, the samples were centrifuged for 10 min at 4,000 rpm. Samples were then loaded onto the HPLC column. The sample elution was monitored at 275 nm.

The program was as follows:

A: water with 0.1% formic acid, B: methanol with 0.1% formic acid

0–10 min, gradient of 20–30% B; 10–24 min, gradient from 30% to 50% B;
24–50 min, gradient from 50% to 80% B (flow rate: 0.5 mL/min).

RESULTS AND DISCUSSION

Crystal structure of Mn(II)-bound MnCcP.1 and its comparison to MnP

In order to investigate structural features responsible for improving Mn(II) binding and MnP activity of the designed MnCcP, we obtained the X-ray crystal structure of MnCcP.1 containing Mn(II) at the active site (see crystallographic parameters in Table S2). An overlay of the crystal structures of MnCcP.1 and MnP indicates the close similarity of both the overall structural folds (Figure 1a) and the heme-binding sites (Figure 1b), including the proximal His and the distal His and Arg residues. This structural comparison strongly suggests that MnCcP.1 offers an excellent scaffold for engineering a Mn(II)-binding site to mimic MnP function.

The primary Mn(II) coordination shell in MnCcP.1, consisting of the heme propionate group and three carboxylate ligands (Glu37, Glu45, and Glu181), is also similar to that in MnP (Figure 2a). However, it is interesting that despite the similar position of the ligands with respect to Mn ion, their distances vary substantially (Figure 2b,c). Similarly long distances between Mn ligands and Mn ion have also been observed in the crystal structure of another Mn-bound CcP variant²⁵ (see figure S1). A careful inspection of the two Mn(II)-binding sites suggests several subtle differences between the carboxylate ligands to Mn(II) in the two proteins that can lead to the elongated Mn-ligand distances in MnCcP.1. The first difference that caught our attention was the positions of Glu37 in MnCcP.1 and Glu35 in MnP (Figure 2). While the relative positions of these carboxylate ligands are similar, Glu37 in MnCcP is separated from Mn(II) ion by 3.23 Å (Figure 2b) vs. only 2.40 Å in MnP (Figure 2c). Similarly, although Glu181 in MnCcP occupies the same position as Asp179 in MnP, their orientations differ, causing a significant increase in the Mn-O distance from 2.41 Å in MnP to 4.08 Å in MnCcP.1 (Figure 2). Another even more subtle difference is the position of the ligands in different structures of the same protein. A comparison of two crystal structures of Mn(II)-bound MnP (PDB ID: 1MNP⁵⁸, 1YYD⁴⁵) with that of Mn(II)-free MnP (PDB ID: 1YZP⁴⁵) suggests that Glu39 in MnP shows the greatest variability in position and therefore in its distance to Mn(II) ion among all ligands. In contrast, the corresponding Glu45 in MnCcP.1 retained its distance in all of our crystal structures (Mn(II)-free MnCcP.1, PDB ID: 2IA8⁴⁷; Co(II)-MnCcP.1 PDB ID: 2ICV⁴⁷; and Mn(II)-bound MnCcP.1, PDB ID: 5D6M). This consistency in distance among different structures of MnCcP.1 suggests that the Glu45 in MnCcP.1 cannot adopt the observed rotamer of Glu39 in MnP for coordinating to Mn(II). This difference between the rotamers at the same position of the two proteins resulted in a 1.84 Å difference in distance between the carboxylate ligands and Mn(II) (3.94 Å in MnCcP.1 vs. 2.10 Å in MnP, see Figure 2b,c). These observations suggest that subtle differences in the orientation of the ligand that coordinates to the metal ion and the resulting differences in geometry and metal-ligand distances may be responsible for the differences of Mn(II)-binding affinity and MnP activity between the designed MnCcP.1 and native MnP. To minimize or eliminate these differences, we sought to engineer the second-sphere interactions around these ligands in order to change the orientation of the ligands in MnCcP.1, as described below.

Improving Mn(II)-binding by Tyr36Phe mutation to remove a H-bond to Glu45 ligand of Mn(II)

Since crystallographic analysis indicated that Glu45 in MnCcP.1 is much more rigid and more distant from Mn(II) than the corresponding Glu39 residue in MnP, we sought a way to relieve the rigidity and move Glu45 closer to Mn(II) in MnCcP.1 for stronger coordination. Structural analysis of Mn(II)-MnCcP.1 suggested that Tyr36 is within a hydrogen bonding (H-bonding) distance to the carboxylate of Glu45 in MnCcP.1 at a distance of 2.83 Å (Figure 3a) while an analogous interaction is absent at the corresponding residue Glu39 in MnP. We hypothesized that this H-bond interaction may compete with Mn(II) coordination by stabilizing the rotamer of Glu45 in MnCcP.1 that is observed in the crystal structure, resulting in low flexibility and poor orientation of the ligand and thus weakening the ability of Glu45 to coordinate Mn(II). There have been multiple reports on conserved hydrogen bonds that help position active site residues or substrates in the proper orientation to fine-tune the catalytic efficiency of enzymes or to tune reduction potentials of metal centers.^{20, 59, 60} Inspired by such reports, we mutated Tyr36 to Phe in MnCcP.1. We chose Phe because it differs from Tyr only by the OH group that is responsible for the H-bonding interactions, thus minimizing other structural perturbations.

MnP activity assays showed that the Tyr36Phe mutation improved the K_M by 2.8 fold (Figure 4 and Table 1), suggesting that removing the H-bonding interactions to the primary coordinating ligand, Glu45, to secondary shell residues (Tyr36) did indeed improve binding affinity to Mn(II).

Improving Mn(II)-binding by Lys179Arg mutation to introduce a salt bridge to better orient Glu37 and Glu181 ligands to Mn(II)

To investigate a way to improve Mn(II) coordination by Glu37 and Glu181 and to increase MnP activity in MnCcP.1, we performed structure-based sequence alignment of MnPs from different organisms and found that they all share a conserved Arg positioned below Glu35 in MnP (Figure 3b). We hypothesized that this Arg may play a key role in proper positioning of the Glu35 and Asp179 ligands in MnP through the formation of a salt bridge. In fact, mutagenesis studies in MnP from *Phenorocheatea chrysosporium* showed that replacing this Arg residue by a negatively charged residue (Glu) or a neutral residue (Gln, Asn) decreased the catalytic efficiency (k_{cat}/K_M) by four orders of magnitude.⁶¹ In MnCcP.1, Lys179 is analogous to Arg181 in MnP. Our MD simulations on Lys179Arg-MnCcP.1 showed an enhanced salt bridge interaction between this residue and Glu37 (from 3.32 to 2.72 Å) (Figure 3c). Consistent with the design, Arg179 moved further away from Glu181 in MnCcP.1 by 0.5 Å, resulting in a reorientation of Glu181 toward Mn(II) so that their distance is closer by almost 2 Å. Both Lys and Arg have positive charges, and previous studies on native MnP have shown that mutation of Arg177 to Lys effects mostly K_M of the enzyme, presumably by destabilizing the optimal conformation for Mn(II) binding (Figure 4 and Table S1).⁶² Encouraged by the above findings, we mutated Lys179 to Arg, and we found that the resulting mutant had a 2.6 fold improvement in K_M (Table 1).

Improving Mn(II)-binding and oxidation activity by Ile40Gly mutation to relieve steric clashes in the ligand-containing loop

Further inspection of the overlaid structures of MnCcP.1 and MnP revealed that the loop containing Glu37 and Glu45 ligands in MnCcP.1 differs substantially from the corresponding loop containing Glu35 and Glu39 ligands in MnP, leading to different positions of these carboxylate ligands (Figure 2a). Close inspection of this loop suggested that the side chain of Ile40 in MnCcP.1 can cause a steric clash with Glu37 in MnCcP.1, preventing it from orienting towards Mn(II) (Figure 5a). In contrast, a Gly is present at the same position in many MnPs. Therefore, to relieve this steric clash, we made the Ile40Gly mutation so that the smaller size of Gly would provide the Glu37 and Glu45 ligands in the loop of MnCcP.1 the freedom to better coordinate to Mn(II) ion, decreasing K_M and consequentially increasing k_{cat}/K_M . MD simulations of Ile40Gly-MnCcP.1 showed that the distances between these ligands to Mn(II) decreased from 3.23 to 2.54 Å for Glu37 and from 3.94 to 2.34 Å for Glu45 (Figure 5b). As shown in Figure 4 and Table 1, this mutation resulted in the highest catalytic efficiency among all of the mutants described in this work, arising primarily from a 3.6 fold decrease in K_M . As a result of this engineering effort, the K_M of Ile40Gly-MnCcP (0.28 mM) is comparable to that of the native *Pleurotus eryngii* MnP PS3 (0.2 mM).⁶³ This mutant also showed a 2.4 fold increase in the catalytic activity (k_{cat}) which, together with the decrease in K_M , results in an 8.6-fold increase in the catalytic Mn(II) oxidation efficiency.

Additive effect of the mutations on the activity of MnCcP.1

To investigate whether the effects of these mutations are additive, we made all possible combinations of our mutants: Tyr36Phe/Lys179Arg, Tyr36Phe/Ile40Gly, Ile40Gly/Lys179Arg, and Tyr36Phe/Ile40Gly/Lys179Arg. As shown in Table 1 and Figure 6, the MnP activity of the Tyr36Phe/Lys179Arg double mutant is equal to the combined activities of Tyr36Phe and Lys179Arg single mutant within the experimental error. This result was somewhat expected, as the Tyr36Phe and Lys179Arg mutations are involved in secondary shell interactions to different ligands (Glu45 and Glu37/Glu181, respectively).

Based on the activity results shown in Figure 4, we found Ile40Gly to be the most effective single mutation. However, when combined with either of the other single mutations (Tyr36Phe or Lys179Arg), we found that the effects of the double mutations were not additive (Figure 6), suggesting that the effect of Ile40Gly mutation on catalytic efficiency was diminished by combination with either of the other two mutations. In the case of the Ile40Gly/Lys179Arg double mutant, the decrease in the catalytic efficiency compared with Ile40Gly was mainly due to a larger K_M (Table 1), suggesting that the Lys179Arg and Ile40Gly mutations work antagonistically toward enhancing Mn(II) binding. This explanation is reasonable because, while the Ile40Gly mutation increases the flexibility of the ligands in the loop to coordinate to Mn(II), the Lys179Arg mutation favors the formation of a salt bridge to the Glu37 ligand that rigidifies the loop. In contrast, while the Tyr36Phe/Ile40Gly double mutant displayed a K_M similar to Ile40Gly alone, the k_{cat} decreased by 2 fold. Since Tyr36 is positioned in the same loop as Ile40, and both mutations have the effect of increasing flexibility of the loop containing Glu37 and Glu45, it is possible that the combination of both mutations will lead to overall instability of the protein that lowers k_{cat} .

The triple mutant Tyr36Phe/Ile40Gly/Lys179Arg displays a k_{cat} similar to Ile40Gly alone, an effect consistent with the dominance of Ile40Gly for increasing k_{cat} in all variants. However, K_{M} of the triple mutant is higher than most other single and double variants except for Tyr36Phe/Lys179Arg. Such an increase in K_{M} compared to other variants can be explained by an ultimately antagonistic effect from the combination of the three mutations even though the binding is enhanced compared to the initial MnCcP.1 variant.

Activity assays of the combined triple mutant, Tyr36Phe/Ile40Gly/Lys179Arg-MnCcP.1

MnP exhibits its lignin depolymerization activity by oxidizing Mn(II) to Mn(III), which then diffuses out of the enzyme to degrade phenolic components of lignin, aided by organic acids secreted by the white rot fungus such as oxalate or malonate.^{27, 28, 64, 65} To demonstrate that our designed MnCcP.1 mutants display a similar activity toward lignin through Mn(III) released from the enzyme, we tested the activity of the triple mutant on a previously reported lignin model compound, guaiacylglycerol- β -guaiacol ether (figure 7a), and analyzed the resulting products by HPLC.⁵⁷ As with other hybrid MnPs,^{66–69} our mutant was able to degrade guaiacylglycerol- β -guaiacol ether in the presence of Mn(II) (Figure 7b). We also assessed the activity of the triple mutant on kraft lignin with HPLC using previously reported methods.⁷⁰ The protein showed degradation activity (Figure 7c). Even though the detected activity was low, these results indicate that our models are capable of lignin degradation. HPLC trace of alkali-treated lignin exposed to Tyr36Phe/Ile40Gly/Lys179Arg at different times monitored at 275 nm.

CONCLUSION

By making Tyr36Phe, Lys179Arg, and Ile40Gly mutations within a region beyond the primary coordination shell of the Mn(II)-binding site in MnCcP.1, we have enhanced Mn(II)-binding affinity and MnP activity of an engineered CcP (MnCcP.1) that mimics MnP by removing a hydrogen bonding interaction, adding a salt bridge, and relieving steric clashes in the second sphere. Our endeavor has resulted in a set of variants with enhanced catalytic efficiencies over the previous design, in particular Ile40Gly-MnCcP.1 with almost 9-folds increase in the catalytic efficiency. Even though such enhancement is relatively modest in comparison with native enzymes, it is a significant step in the realm of protein design, as most designed proteins reported so far display low catalytic efficiency. Excitingly, the Ile40Gly-MnCcP.1 mutant also displays a K_{M} value (0.28 mM) comparable to that of native *Pleurotus eryngii* MnP PS3 (0.2 mM),⁶³ which shares a Mn-binding site nearly identical to other MnPs, including *Phanerochaete chrysosporium* MnP (see figure S2). Achieving a K_{M} in a designed protein that is similar to native enzymes is a rare achievement and suggests that the geometry of the MnCcP Mn-binding site can be improved to better mimic that of native MnPs. More importantly, this work demonstrated that careful design of structural features in the second coordination sphere is critical to achieve the same level of K_{M} as in the native enzymes.

We have also shown that while the combination of some mutations (e.g., Tyr36Phe and Lys179Arg) is additive, others are not. The Tyr36Phe and Lys179Arg mutations with different target residues (Glu45 and Glu37, respectively) result in additive effects while

combinations of mutations that individually impact different aspects of ligand geometry at the same residues (e.g. Ile40Gly/Lys179Arg on Glu37) were found to be antagonistic. We further demonstrated that the designed CcP is a functional model of MnP that mimics its activity both in terms of Mn(II) oxidation and degradation of both a phenolic lignin model compound and alkali-treated lignin. These results indicate that the ability of primary ligands to coordinate Mn(II) is highly dependent on proper distance and orientation of the coordinating atoms, and even slight deviations from the optimal geometry can influence metal binding and activity. This study has deepened our understanding of the role of non-covalent interactions around the metal binding site and how to rationally modify them to improve metal binding and overall activity. The principles demonstrated by this study can be applied to enhance metal-binding affinity and activity of other enzymes and their biomimetic models.

Supplementary Material

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ABBREVIATIONS

CcP	cytochrome <i>c</i> peroxidase
MnP	manganese peroxidase
H-bond	hydrogen bond
H₂O₂	hydrogen peroxide
MD	molecular dynamics
VMD	Visual Molecular Dynamics
ESI-MS	electron spray ionization mass spectroscopy
HPLC	high performance liquid chromatography
GC-MS	gas chromatograph-mass spectroscopy

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Figure 1.

Structural comparison of MnP, CcP, and MnCcP.1. (a,b) Overlay of MnP (gray, PDB ID: 1YYD) and CcP (orange, PDB ID: 2CYP) using Stamp structural alignment shows high structural similarities (a), especially in the heme-binding site (b). (c,d) Comparison of Mn(II)-binding site in MnP (c) and corresponding site in MnCcP.1 (d). (e) An overlay of Mn(II) CcP.1 (cyan, PDB ID: 5D6M) and Mn(II)P (gray, PDB ID: 1YYD).

Figure 2.

(a) Structural comparison of the Mn(II) binding sites of MnP (gray, PDB ID: 1YYD) and MnCcP.1 (cyan, PDB ID: 5D6M). (b) Primary Mn(II) ligands in MnP. (c) Primary Mn(II) ligands in MnCcP.1. Heme is not shown in b and c. The red spheres in the figures are water molecules.

Figure 3.

(a) Structure of Mn(II)-MnCcP.1 showing Tyr36 and its interaction with Glu45. (b) Salt bridge between Arg177 and Glu35 in MnP (PDB Id: 1YYD). (c) Possible salt bridge between Lys179 and Glu37/Glu181 ligands in the Mn(II)-MnCcP.1 structure (PDB ID: 5D6M).

Figure 4.
Different secondary shell residue positions investigated in this study and their effect on activity and metal affinity of MnCcP.1

Figure 5. Mn(II)-binding site and corresponding distances between Mn(II) and Glu37 and Glu45 in (a) Mn(II)-MnCcP.1 crystal structure (PDB ID: 5D6M) and (b) MD simulated model of Ile40Gly-MnCcP.1 variant.

Figure 6. Catalytic efficiency of double and triple mutants of MnCcP.1. The observed experimental values are shown in solid red bars. On the left of each red column is shown the theoretical value if the effects of mutations were additive. The original MnCcP.1 value is shown in white.

Figure 7.
(a) Phenolic lignin model compound. (b) The HPLC trace of degradation of the phenolic lignin model compound by Tyr36Phe/Ile40Gly/Lys179Arg monitored at 254 nm. (c)

Scheme 1.
Reaction cycles of MnP and CcP.

Table 1

Kinetic parameters of different MnP mimics. All the reactions were performed at pH 4.5.

enzyme	K_M (mM) (fold decrease) *	k_{cat} (s^{-1}) (fold increase)	k_{cat}/K_M ($mM^{-1}s^{-1}$) (fold increase)
MnCcP.1	1.02	0.19	0.19
Tyr36Phe-MnCcP.1	0.37 (2.8)	0.10 (0.5)	0.28 (1.5)
Lys179Arg-MnCcP.1	0.40 (2.6)	0.19 (1.0)	0.47 (2.6)
Ile40Gly-MnCcP.1	0.28 (3.6)	0.46 (2.4)	1.63 (8.6)
Y36F/K179R-MnCcP.1	1.10 (0.9)	0.62 (3.3)	0.56 (2.9)
I40G/K179R-MnCcP.1	0.48 (2.1)	0.39 (2.0)	0.81 (4.3)
Y36F/I40G-MnCcP.1	0.28 (3.6)	0.28 (1.5)	1.02 (5.4)
Y36F/I40G/K179R-MnCcP.1	0.57 (1.8)	0.46 (2.4)	0.80 (4.2)

* The values in the parentheses are the factor of decrease or increase with respect to MnCcP.1.