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Modifications of laccase activities of copper efflux oxidase, CueO by synergistic mutations in the first and second coordination spheres of the type I copper center

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ABSTRACT

The redox potential of type I copper in the *Escherichia coli* multicopper oxidase CueO was shifted in the positive or negative direction as a result of the single, double, and triple mutations in the first and second coordination spheres: the formation of the NH···S^-(Cys500 ligand) hydrogen bond, the breakdown of the NH(His443 ligand)···O^-(Asp439) hydrogen bond, and the substitution of the Met510 ligand for the non-coordinating Leu or coordinating Gln. Laccase activities of CueO were maximally enhanced 140-fold by virtue of the synergistic effect of mild mutations at and at around the ligand groups to type I copper.

1. Introduction

Multicopper oxidases (MCOs) are a family of enzymes that couple the one-electron oxidation of four substrates with the four-electron reduction of dioxygen to water. The catalytic motif in MCOs includes a type I (T1) copper and the trinuclear center (TNC) comprised of a type II (T2) copper and a pair of type III (T3) coppers [1–3]. Overall enzymatic activities of MCOs are governed by many factors, diffusion of substrate on the protein surface to the binding site, the electron transfer from substrate to TNC via T1 copper [4–6], the binding and reduction of O_2 at TNC [7], and transfer of H^+ from bulk solvent to O_2 through the hydrogen bond network [8–14]. We have performed a variety of mutations on the cuprous oxidase, CueO from *Escherichia coli* and bilirubin oxidase from *Myrothecium verrucaria* aimed at exploring structure and function relationships of MCOs and applying them to biofuel cell and pigment formation [8,10–13].

In a previous study on CueO [6], we performed point mutations at Met510, the axial ligand to T1 copper to produce the mutants containing the T1 copper center found in fungal laccases with high redox potential or the T1 copper center found in phyotocyanins with low redox potential. The former Met510 to Leu mutation brought about the positive shift in the redox potential of T1 copper leading to an enhancement in enzymatic activities of CueO because the driving force of the electron transfer between substrate and T1 copper was increased, while that between T1 copper and TNC was decreased. In contrast, the latter Met510 to Gln mutation resulted in the shift in the redox potential of T1 copper toward negative direction leading to a drastic decrease in enzymatic activities because the electron transfer process from substrate to T1 copper became extremely unfavorable. We also performed analogous mutations on bilirubin oxidase, although an asparagine residue at the distal position played a role as the compensatory ligand to the coordination-unsaturated T1 copper center [15].

In addition to the mutations at Met510 in the first coordination sphere of T1 copper we performed mutations at the amino acids located in the second coordination spheres to tune the electron-donating abilities of Cys500 and His443 [16]. The sulfur atom in Cys500 is hydrogen-bonded with the main-chain NH group of Leu502 (Fig. 1) and plays a role to stabilize the structure of the T1 copper center, as evidenced by the result that the T1 copper site has become vacant by performing a mutation to delete this hydrogen bond [16]. The NH···S^- hydrogen bond also functions in the reduction of electron density on the sulfur atom and a positive shift in the redox potential of T1 copper. However, Pro444, which is highly conserved in a segment connecting T1 copper and TNC of most of MCOs, does not allow to form the second NH···S^- hydrogen bond. We have performed mutations at Pro444 with Gly, Ala,

Abbreviations: MCO, multicopper oxidase; T1, type I; TNC, trinuclear center; T2, type II; T3, type III; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BCA, bicinnchoninic acid; DAT, 2,5-diaminotoluene; DMP, 2,6-dimethoxyphenol; *p*-PD, *p*-phenylenediamine; *o*-PD, *o*-phenylenediamine; *p*-AP, *p*-aminophenol; ADPA, 4-aminodiphenylamine; CD, circular dichroism; EPR, electron paramagnetic resonance; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; HOPG, highly oriented pyrolytic graphite RCueO, recombinant CueO.

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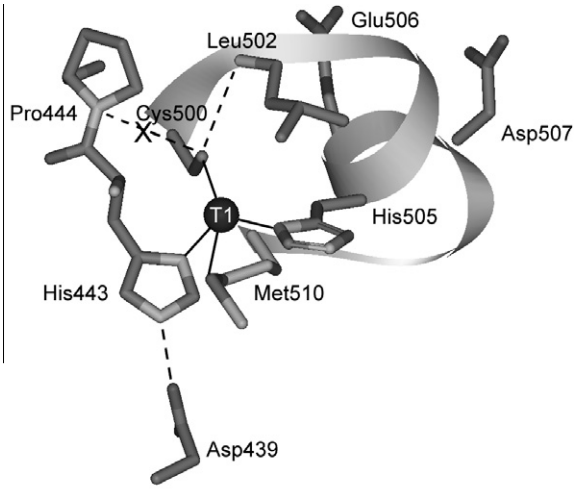

	444	510
CueO	HPFHIHGT	AHCHLLEHEDTGMML
Fungal Laccase	HPFHLHGH	FHCHIEFHLMNGLAI
Ascorbate Oxidase	HPWHLHGH	FHCHIEPHLHMGMGV
Ceruloplasmin	HTVHFHGH	LHCHVTDHIHAGMET
Bilirubin Oxidase	HPIHIHLV	FHCHNLIHEDHDMMA
Fet3p	HPFHLHGH	FHCHIEWHLLQGLGL
CotA	HPIHLHLV	WHCHILEHEDYDMMF

Fig. 1. Structure around the type I copper center in CueO (top) (This figure was generated from PDB entry 3OD3 using DS ViewerPro 5.0) and amino acid sequence for T1 copper ligands of CueO and some other MCOs for comparison (bottom).

Leu and Ile, and succeeded in enhancing enzymatic activity from that of the parent CueO by 10 times. An analogous mutation has been performed on blue copper proteins, amicyanin and pseudoazurin [17,18], leading to positive shifts in the redox potential, in turn a negative shift in azurin by the removal of one of two $NH\cdots S^{-}$ hydrogen bonds [19,20]. On the other hand, Fig. 1 indicates that the imidazole group of His443 coordinated to T1 copper is hydrogen-bonded with Asp439. By performing a mutation at Asp439 to remove this hydrogen bond the electron-donating ability of His443 towards T1 copper was decreased, leading to a positive shift in the redox potential of T1 copper and the 17-fold increase in the oxidizing activity of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

A preliminary double mutation at Asp439 and Pro444 aimed at a more advanced modification of CueO afforded the 40-fold increase in enzymatic activity [16]. In this communication we perform the double and triple modifications of Asp439, Pro444, and Met510 with an expectation to achieve further increases in enzymatic activities of CueO, although effect of each mutation at and at around the T1 copper center is not as significant as the mutation at the T1 copper center in blue copper proteins with much smaller molecular size [21].

2. Methods

2.1. Preparation of mutants

The plasmids for the double mutants, pUCCueO(D439A/M510L), pUCCueO(P444A/M510L), pUCCueO(D439A/M510Q), and pUCCueO(P444A/M510Q) were prepared with a QuikChange kit (Stratagene) using oligonucleotide primers for M510L and M510Q [6] and the template plasmids, pUCCueO(D439A) and pUCCueO(P444A) [16]. The plasmids for the triple mutants, pUCCueO(D439A/P444A/M510L) and pUCCueO(D439A/P444A/M510Q) were prepared using the plasmid for the double mutant, pUCCueO(D439A/P444A). *E. coli* BL21 (DE3) was transformed by electroporation with the mutant plasmids. Cultivations of the transformants and purifications of the mutant proteins were carried out as described previously [6,16]. Protein concentrations were determined using a BCA (bicinchoninic acid) protein assay reagent (Pierce) and from the absorption intensity at 280 nm, $\varepsilon = 73000\ M^{-1}cm^{-1}$.

2.2. Measurements

Activities of the mutants to oxidize ABTS and 2,5-diaminotoluene (DAT) were colorimetrically determined from absorption changes at 420 nm and 470 nm, respectively. One unit of activity is defined as the amount of enzyme to oxidize 1 μmol of substrates per min. In addition to activities for ABTS and DAT, those for 2,6-dimethoxyphenol (DMP, 477 nm), *p*-phenylenediamine (*p*-PD, 487 nm), *o*-phenylenediamine (*o*-PD, 430 nm), catecol (450 nm), guaiacol (436 nm), *p*-aminophenol (*p*-AP, 405 nm), *p*-methylaminophenol (*p*-MAP, 450 nm), and 4-aminodiphenylamine (ADPA, 405 nm) were also determined under substrate-saturated conditions in the presence or absence of 1 mM Cu(II) (Oxidizing activities of CueO are enhanced or newly emerged due to the labile regulatory Cu(II) ion bound to the substrate binding site [22]). At least three sets of data were averaged for kinetic parameters.

The averaged copper content in each mutant molecule was determined by atomic absorption spectroscopy on a Varian SpectrAA-50 spectrometer. Absorption spectra were measured on a JASCO V-560 spectrometer and circular dichroism (CD) spectra on a JASCO J-500C spectropolarimeter. X-band electron paramagnetic resonance (EPR) spectra were measured on a JEOL JES-RE1X spectrometer at 77 K. The total amount of the EPR detectable Cu^{2+} signals in a molecule of CueO and mutants has been determined using Cu^{2+}-*N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid (EDTA) as standard. Cyclic voltammetries of the mutants in 0.1 M acetate buffer solution (pH 5.5) and 0.1 M phosphate buffer (pH 7.0) were performed using a rotating disk electrode of highly oriented pyrolytic graphite (HOPG) as working electrode [16,23].

3. Results and discussion

3.1. Spectral properties of mutants

Double and triple mutants of CueO for combinations of the single mutations at Asp439 by Ala, Pro444 by Ala, and Met510 by Leu or Gln are prepared. Every mutant contained 3.8–4.2 copper atoms per a protein molecule except 3.4 copper atoms in a double mutant, P444A/M510Q (Table 1). These results and corresponding spectroscopic properties indicate that T1, T2 and T3 copper centers are fully occupied in every mutant except P444A/M510Q, which is a mixture of holo-proteins and partly copper-incorporated proteins as judged from spectra and low enzymatic activities (*vide infra*). Fully copper-incorporated P444A/M510Q could not be obtained by dialysis against Cu^{+} or Cu^{2+} ions or changes in cultivation condition.

Fig. 2 shows the absorption, CD, and EPR spectra of the mutants. While T1 copper gives the strong absorption band originated in the $Cys(S^{-}) \rightarrow Cu^{2+}$ charge transfer at ca. 610 nm, the absorption intensity has been decreased in many mutants especially in a series of mutants containing the Met510 to Leu mutation and D439A/P444A. However, the absorption intensities of these mutants were considerably increased after the reactions with the oxidizing agents such as hexachloroiridate(IV) (broken lines), indicating that not all but a considerably high population of T1 coppers had been expressed in the reduced form due to positive shifts in the redox

Table 1
Kinetic parameters for oxidations of ABTS and DAT, copper content, and redox potential of single, double and triple mutants.

	Kinetic parameters ABTS		DAT		Cu content	Redox potential
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	(Cu/protein)	E° (V vs. Ag|AgCl)[b]
rCueO	6.5[a]	0.98[a]	12	19	4.2	0.36
D439A	6.5[a]	16[a]	12	366	4.2	0.43
P444A	6.2[a]	8.7[a]	9.3	105	4.1	0.43
M510L	4.8[a]	6.7[a]	4.2	36	4.0	0.40
M510Q	7.1[a]	0.036[a]	–	–	4.1	0.23
D439A/P444A	0.73[a]	40[a]	6.7	233	3.9	0.46
D439A/M510L	1.3	137	5.5	832	4.1	0.39
D439A/M510Q	–	~0	N.D.	3.2	4.3	0.21
P444A/M510L	1.4	1.1	14	11	3.8	N.D.
P444A/M510Q	–	~0	N.D.	25	3.4	0.21
D439A/P444A/M510L	–	~0	–	~0	4.3	N.D.
D439A/P444A/M510Q	N.D.	0.1	N.D.	16	4.0	0.26

[a] These values have been reported in Ref. [16].
[b] Redox potential relative to Ag|AgCl(sat.), being 0.20 V negative value relative to normal hydrogen electrode.

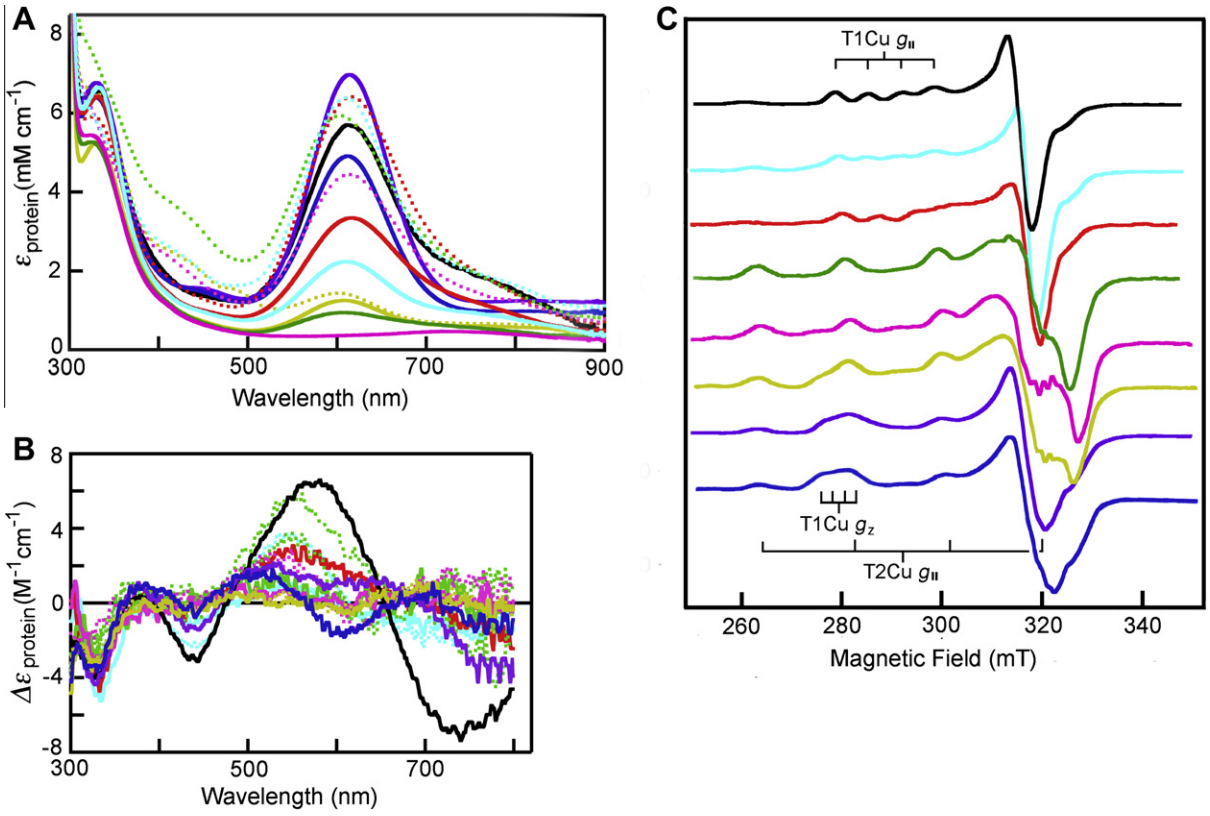

Fig. 2. Absorption (A), CD (B), and EPR (C) spectra of the rCueO (black), D439A/P444A (pale blue), D439A/M510L (red), P444A/M510L (green), D439A/P444A/M510L (pink), D439A/M510Q (purple), P444A/M510Q (yellowish green), and D439A/P444A/M510Q (blue) as isolated (full line) and treated with K_2IrCl_6 (broken line). Absorption and CD spectra were measured at room temperature and EPR spectra at 77 K with frequency 9.19 GHz, modulation 1.0 mT at 100 kHz, time 4 min, time constant 0.03 s for ca. 50 μM proteins in phosphate buffer, 0.1 M, pH 6.0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

potential. However, intensity of the 610 nm band of P444A/M510Q was not increased due to the low copper content. The *d–d* transition band of the mutants with the Met510 to Gln mutation performed a red-shift from ca. 800 nm to ca. 850 nm with a concomitant decrease in intensity, showing spectral features typical of T1 copper for phytocyanins [6,8,24].

CD spectra of CueO and mutants afford the charge transfer bands derived from TNC and T1 copper in the 330 nm and 400–650 nm, respectively and the *d–d* bands from all copper centers in the 650–800 nm regions (solid lines). The CD band at ca. 610 nm was inverted in sign for a series of M510Q mutants [6,8,24]. Intensities of the decreased CD bands due to positive shifts in the redox potential were recovered after the reactions with the oxidizing agents as in the corresponding absorption spectra (broken lines).

EPR spectra of CueO and mutants containing Met or Leu at the position 510 afford the signals from T1 copper (g_{II} = 2.22–2.23 and A_{II} = 6.9–8.4 × 10^{-3} cm^{-1}) and T2 copper (g_{II} = 2.24 and A_{II} = 18.4 × 10^{-3} cm^{-1}). The axial symmetry of the T1 copper center is reserved after the mutation of Met510 with Leu, although

the A_{II} value became slightly large ($A_Z = 8.4 \times 10^{-3}$ cm^{-1}) [6,15]. On the other hand, T1 copper in the M510Q mutants was significantly modified to give the rhombic signal ($g_z = 2.34$ and $A_z = 3.6 \times 10^{-3}$ cm^{-1}) due to the binding of the amide O atom in the side chain. These changes induced by the Leu and Gln mutations are in harmony with the EPR spectral features of the T1 copper in fungal laccases and phytocyanins, respectively [2]. In contrast to the mutations at Met510, the mutations at Asp439 and Pro444 in the second coordination spheres of T1 copper did not induce appreciable changes in the EPR spectra [16].

3.2. Enzymatic activities of the double mutants

Enzymatic activities of the mutants toward ABTS and DAT are tabulated in Table 1 together with averaged copper content in a protein molecule and the redox potential of T1 copper. Enzymatic activities of the mutants for other substrates are shown in Supplemental Table 1. In the previous mutations of CueO, we preliminarily prepared a double mutant D439A/P444A, which showed the 40-fold increase in the ABTS-oxidizing activity from that of CueO and 7 and 2.4-fold increases from those of the single mutants for Pro444 and Asp439, respectively [16]. This increase in activity shown by the double mutation was driven mainly from the increase in the k_{cat} value due to an integrated effect of the double mutation to positively shift the redox potential of T1 copper by 100 mV. Further, the K_m value of the double mutant became smaller compared to those of rCueO and single mutants, suggesting that the binding affinity of CueO to ABTS was also increased, although the effect was not as significant as the change in the redox potential. In this study we observed the increase in the oxidizing activity of the double mutant for DAT by 12 times from that of rCueO, the intermediate increase from those for the single mutants by 6 and 19 times, presumably because the electron transfer from DAT to T1 copper did not become as favorable as that for ABTS in D439A/P444A.

The double mutant D439A/M510L showed the 140-fold and 44-fold increases in the k_{cat} values for ABTS and DAT, respectively, from the activities of the unmodified CueO. Thus further increase in activities to exceed those by D439A/P444A is attained in this mutant. However, the shift in the redox potential toward positive direction was 30 mV from that of rCueO (Table 1) [16]. The K_m values for ABTS and DAT changed from 6.5 mM to 1.3 mM and from 12 mM to 5.5 mM, respectively, but apparently, these slight increases in the affinity of CueO to substrates are not the predominant factors for the drastic increases in the enzymatic activities because an analogous increase had been exhibited in the affinities of D439A/P444A ($K_m = 0.73$ mM for ABTS and 6.7 mM for DAT). Undoubtedly, polarity around the type I copper center should be decreased due to the Asp439 to Ala and Met510 to Leu mutations, if an unexpected structural change to enhance enzymatic activities has not been induced. Therefore, there may be other structural factors, which make the electron transfer steps involving T1 copper more preferable but are not reflected in absorption, CD and EPR spectra. Bulky organic substrates such as ABTS are not directly accessible to the binding site for cuprous ion due to the blocking effect by the helices 5–7 and a flexible segment [4]. Although we have not determined the crystal structure of D439A/M510L yet, the coordination bonds between T1 copper and the remaining three ligands will become stronger [2,25], and the electron transfer through the His-imidazole groups might become more favorable. Met510 is in the ligand loop harboring Asp507 (Fig. 1) [13], which is located in the end of the hydrogen bond network leading from the protein surface to TNC. Accordingly, the mutation at Met510 might affect accessibility of organic substrates to the region on the protein surface or the H^+ relay process to TNC. Enzymatic activities of the double mutant P444A/M510L were similar to those of the unmodified CueO presumably because the redox potential of T1 copper was remarkably shifted to positive direction as we could not determine the value. In contrast to the Met510 to Leu mutation, the Met510 to Gln mutation did not function in an increase in enzymatic activity due to the negative shift in the redox potential of T1 copper by 150 mV. No oxidizing activity of ABTS was observed for P444A/M510Q, while less copper incorporation into the mutant molecule might also be responsible for this result.

3.3. Enzymatic activities of the triple mutants

Since the double mutant, D439A/M510L exhibited the 140-fold increase in enzymatic activity, we prepared a triple mutant, D439A/P444A/M510L. However, T1 copper in the triple mutant could not be fully oxidized even with the strong oxidizing agents such as hexachloroiridate(IV), and a large fraction of T1 coppers was kept to be in the cuprous form due to the significant positive shift in the redox potential. Unfortunately, this situation for T1 copper resulted in the practical loss of enzymatic activities in spite of the successful modification of CueO because the electron transfer from T1 copper to TNS became thermodynamically very unfavorable. In order to avoid this too extensive shift in the redox potential of T1 copper, we prepared another triple mutant D439A/P444A/M510Q. However, the redox potential of this mutant was 0.26 V and high enzymatic activities were not attained, indicating that the negative shift in the redox potential due to the modification on the first coordination sphere was more significant in spite of the accumulated mutations in the second coordination sphere. Therefore, it appears that there is a limit to attain high enzymatic activities of CueO by combining the three different mutations in the first and second coordination spheres of T1 copper. To attain higher activities we will have to perform modifications on TNC to reach the redox potential of 0.82 V, at which potnetial O_2 is transformed into $2H_2O$.

3.4. Electrochemistry of mutants

Cyclic voltammetry of the mutants was performed under O_2 atmosphere using a rotating disk electrode. While the observed electric densities were as high as to show effective electrochemical communications between HOPG and protein molecules via T1 copper, the maximum cathodic electric density to reduce O_2 did not exceed that by CueO differing from the single mutant D439A

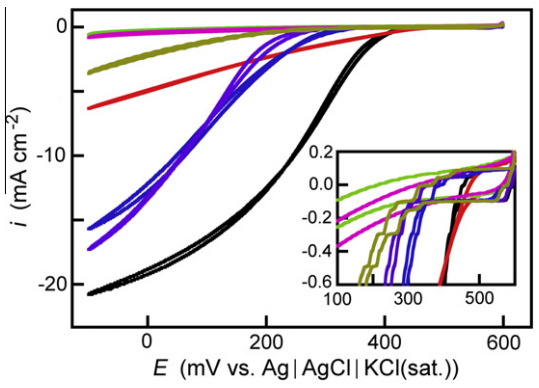

Fig. 3. Cyclic voltammograms of rCueO (black) and mutants, D439A/M510L (red), P444A/M510L (green), and D439A/P444A/M510L (pink) mutants with M510L mutation and D439A/M510Q (purple), P444A/M510Q (yellowish green), and D439A/P444A/M510Q (blue) mutants with M510Q mutation with a HOPG electrode under O_2. Measurement conditions: scan rate of 5 mV/s, rotation rate of 1000 rpm, 0.1 M acetate buffer pH 5.5, ionic strength of 0.5 (K_2SO_4), Pt counter electrode, Ag|AgCl|KCl(sat.) reference electrode, and temperature of 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3) [16,21]. For variants with the Met510 to Gln mutation the HOPG electrode afforded electric densities of the order of mA/cm^2, high enough for uses as sensor and biofuel cell. However, only D439A/M510L among mutants with high redox potentials afforded a high current density. Thus, electrochemical response and enzymatic activity of the mutants are not directly correlated with each other, and this fact implies that mutations to realize better electrochemical communication should also be performed on the protein surface to utilize as bioelectrocatalyst.

In summary, we performed synergistic modifications in the first and second coordination spheres of T1 copper in CueO to form and/or delete the hydrogen bond with the ligand groups and to replace the axial ligand. Due to accumulated mutations to modify the donating abilities of Cys and His residues coordinating T1 copper and the effect slightly enhances affinities of substrates to the protein surface, maximally 140-fold increase was attained in enzymatic activity. However, synergistic effect of mutations was not necessarily additive for many mutants, presumably because the reorganization energy of T1 copper was also influenced by the accumulation of artificial modifications [26]. Nevertheless, the present modifications will be applicable to other MCOs and nitrite reductase with evolutionally common ancestors and copper enzymes containing the redox active center.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.040.

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