Biochemical and Biophysical Research Communications 431 (2013) 393–397

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# 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

Kunishige Kataoka [a], Hiroki Kogi [a], Seiya Tsujimura [b], Takeshi Sakurai [a,*]

[a] Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan
[b] Faculty of Pure and Applied Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba 305-8573, Japan

ARTICLE INFO

*Article history:*
Received 28 December 2012
Available online 18 January 2013

*Keywords:*
CueO
Multicopper oxidase
Type I copper
Laccase activity
Protein engineering

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].

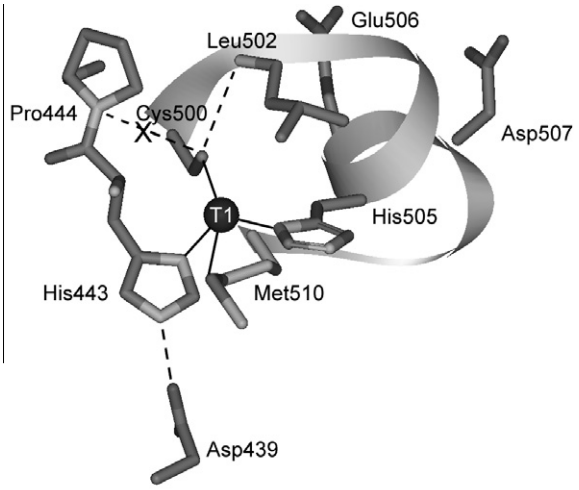
In a previous study on CueO [6], we performed point mutations at Met510, the axial ligand to T1 copper to produce the mutants containing the T1 copper center found in fungal laccases with high redox potential or the T1 copper center found in phyotocyanins with low redox potential. The former Met510 to 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.

* Corresponding author. Fax: +81 76 264 5742.
*E-mail addresses:* tsakurai@se.kanazawa-u.ac.jp, ts0513@kenroku.kanazawa-u.ac.jp (T. Sakurai).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.040




| | 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