Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 354 (2007) 21–26
BBRC
www.elsevier.com/locate/ybbrc

# Crystal structures of *E. coli* laccase CueO at different copper concentrations

Xu Li [a], Zhiyi Wei [a,b], Min Zhang [a,b], Xiaohui Peng [a], Guangzhe Yu [a], Maikun Teng [a], Weimin Gong [a,b,*]

[a] *Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China*

[b] *National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China*

Received 20 November 2006
Available online 22 December 2006

**Abstract**

CueO protein is a hypothetical bacterial laccase and a good laccase candidate for large scale industrial application. Four CueO crystal structures were determined at different copper concentrations. Low copper occupancy in apo-CueO and slow copper reconstitution process in CueO with exogenous copper were demonstrated. These observations well explain the copper dependence of CueO oxidase activity. Structural comparison between CueO and other three fungal laccase proteins indicates that Glu106 in CueO constitutes the primary counter-work for reconstitution of the trinuclear copper site. Mutation of Glu106 to a Phe enhanced CueO oxidation activity and supported this hypothesis. In addition, an extra α-helix from Leu351 to Gly378 covers substrate biding pocket of CueO and might compromises the electron transfer from substrate to type I copper.


*Keywords:* CueO; Laccase; Crystal structure; Oxidase activity; Trinuclear cluster; Copper concentration

Laccase (EC 1.10.3.2) is a polyphenol oxidase, which catalyzes oxidization of a wide range of substrates, such as methoxyphenol, aniline, and benzenethiol [1]. It belongs to the multicopper oxidase family that couples the four-electron reduction of dioxygen to water with the oxidation of substrates [2]. Both fungal and plant laccases contain four copper atoms and no other cofactors are implicated [3]. The copper containing sites have been divided into three classes based on their spectroscopic features, type I or blue copper, type II or normal copper, and type III or coupled binuclear copper center [4]. The type II and two type III coppers are usually described as the trinuclear copper cluster [5]. Recently, because of their potential applications in industry and environmental protection, laccases have gained increasing attention [6].

Based on current knowledge, fungal laccases possess oxidation activity 100-fold higher than bacterial ones. However, fungal laccases are much more difficult to be expressed in recombinant forms to meet industrial requirement. It is therefore applicable to define and optimize factors that affect bacterial laccase activity.

*Escherichia coli* CueO, a hypothetical bacterial laccase, is involved in copper tolerance *cue* (copper efflux) system in periplasm [7]. CueO expression is stimulated by exogenous copper ion and plays an important role in *E. coli* copper homeostasis [8]. Although the native function and *in vivo* substrate of CueO remain unclear, it is able to oxidize a range of substrates *in vitro*, including catechol, siderophore, and $Fe^{2+}$ ion. Several groups reported studies on structure and function of CueO, trying to reveal why the recombinant CueO is much less active than many fungal laccases. Unfortunately, no clear conclusion has been made to date.

In this study, a new method to produce more active CueO was proposed. In addition, the crystal structures of CueO at

* Corresponding author. Fax: +86 10 64888513.
*E-mail address:* wgong@ibp.ac.cn (W. Gong).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.116

different copper ion concentrations are reported. These structures clearly reveal that occupancies of Cu I and Cu II are dependent on exogenous copper concentrations, indicating that the lower activity of CueO may be caused by the lower affinity of Cu I and Cu II. Analysis of the copper cluster suggests that Glu106 decreases the micro-environmental hydrophobicity of the type II Cu. Consistent with this hypothesis, replacement of Glu106 by a Phe significantly increases the oxidase activity of CueO. On the other hand, compared to fungal laccases, an extra α-helix (Leu351-Gly378) was found near Cu I. This α-helix could be another negative effecter on the CueO laccase activity.

## Materials and methods

*Cloning, expression, and purification. cueO* gene was cloned into a modified pET-22b+ expression vector, and the CueO protein was induced by IPTG, and expressed in *E. coli* strain BL21 (DE3).

Harvested cells were lysed by osmotic shock method with buffer A (20 mM Tris–HCl, pH 7.5, 10 μM PMSF, and 30% m/v sucrose) and buffer B (20 mM Tris–HCl, pH 7.5, and 10 μM PMSF). Purification of CueO protein was carried out by ion exchange chromatography using DEAE–Sepharose column (Amersham Biosciences) followed by size-exclusion chromatography on a Superdex 75 column (Amersham Biosciences). The protein concentration was determined by Bradford method (Bio-Rad Protein Assay), using bovine serum albumin as standard [9]. The purified and concentrated CueO (20 mg/mL) was stored in 20 mM Tris–HCl (pH 7.5).

CueO* was prepared by adding $CuCl_2$ (final concentration 1 mM) into 1 mg/mL CueO protein solution after the DEAE–Sepharose chromatography. After addition of $CuCl_2$, some blue flocculent aggregation was formed in solution, while it was re-dissolved after 24-h incubation at 4 °C. The re-dissolved protein was purified through Superdex 75 column in the absence of copper ion, concentrated to 20 mg/mL, and stored in 20 mM Tris–HCl (pH 7.5).

CueO mutant E106F was generated from recombinant cueO pET-22b+ expression vector by site-directed mutagenesis with TaKaRa MutanBEST Kit (Takara Biotechnology) using the forward primer 5′-CACGGGCTGTTTGTACCGGGTGAAG-3′ and reverse primer 5′-CCAGTGCAACGTTGTCTCTTCCGTCAG-3′. E106F mutant CueO was expressed and purified using the same protocol as the native CueO.

*Enzymatic activity assays.* The oxidase activity of CueO was measured using 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) as the electron donor. The assay mixture (300 μL) contained 0.1 M sodium acetate buffer (pH 4.6), 0–100 mM $CuCl_2$, and 1 μL purified protein. Two millimolars of ABTS was added to start the reaction. Heat inactivated CueO (2 min, 97 °C) and the buffer without protein were used as controls. Absorbance change over time was monitered at 420 nm for ABTS ($\varepsilon_{ABTS} = 36000\ M^{-1}\ cm^{-1}$) and oxidation rates were calculated. Such enzymatic activity measurements were performed in triplicate at 25 °C on a U-2810 spectrophotometer (Hitachi High-Technologies Corporation). The specific activity was expressed in the format of μmol of ABTS oxidized/min/mg of protein.

*Crystallization and structure determination.* CueO crystals were grown in the presence of different concentrations of copper ion. Four types of crystals were used for X-ray diffraction. Details of each crystal growth condition are summarized in Table 1.

Prior to data collection, crystals were flash frozen in liquid nitrogen. Diffraction data of apo-CueO and 0.5-CueO were collected on Beamline 3WIA, Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, while that of 10-CueO and CueO* were collected at home facilities with a Rigaku RU2000 rotating $CuK_\alpha$ anode in Institute of Biophysics, Chinese Academy of Sciences.

The initial phases were obtained by molecular replacement using the program AMoRe with CueO structure (PDB code 1KV7) as the starting model [10,11]. Multiple cycles of refinement and model building were performed with the programs CNS and O (Table 1) [12,13]. In each case, 5% of the data were excluded for calculation of $R_{free}$. The positions of the copper ions and water were confirmed by $F_o - F_c$ and $2F_o - F_c$ maps. All figures were prepared using the program RasTop (Philippe Valadon, http://www.geneinfinity.org/rastop/).

## Results and discussion

### *CueO oxidase activity*

There was no detectable oxidase activity of apo-CueO without exogenous copper ion. The oxidase activity of

Table 1
Data processing and refinement statistics

| Crystal ID | Apo-CueO | 0.5-CueO | CueO* | 10-CueO |
|---|---|---|---|---|
| Crystallization condition (8 mg/mL purified protein, with 100 mM tri-sodium citrate dihydrate pH 5.6) | 16% PEG 4000 5% isopropanol | 10% PEG 4000 15% isopropanol | 20% PEG 4000 4% isopropanol | 17% PEG 4000 6% isopropanol |
| Space group | C222(1) | C222(1) | C222(1) | C222(1) |
| Cell parameters (Å) | $a = 69.3$, $b = 73.3$, $c = 183.7$ | $a = 69.6$, $b = 73.2$, $c = 184.3$ | $a = 69.4$, $b = 73.3$, $c = 189.9$ | $a = 69.1$, $b = 73.0$, $c = 189.4$ |
| Resolution range (Å) | 91.67–2.40 | 92.06–1.90 | 50–2.00 | 50–2.30 |
| Outer shell | 2.46–2.40 | 1.94–1.90 | 2.07–2.00 | 2.36–2.30 |
| Total reflections | 171,881 | 401,134 | 415,628 | 226,468 |
| Unique reflections | 18,395 | 35,855 | 29,496 | 20,806 |
| No. of solvent molecules | 226 | 416 | 323 | 236 |
| Completeness (%) (outer shell) | 98.1 (93.1) | 98.6 (82.9) | 88.6 (93.3) | 95.2 (91.2) |
| $R_{merge}$[a] (outer shell) | 7.9% (36.2%) | 10.9% (40.1%) | 9.3% (41.5%) | 9.3% (46.3%) |
| $\langle I/\sigma \rangle$ (outer shell) | 12.3 (4.1) | 6.7 (3.5) | 7.3 (3.7) | 7.5 (3.1) |
| $R_{factor}$[b]/$R_{free}$[c] | 16.5/22.3 | 16.5/20.5 | 17.7/23.3 | 15.7/23.3 |
| *RMS deviation from ideality* | | | | |
| Distance (Å) | 0.008 | 0.009 | 0.011 | 0.008 |
| Angle (deg) | 1.22 | 1.13 | 1.33 | 1.11 |

[a] $R_{merge} = \sum|(I_{hkl} - \langle I_{hkl}\rangle)|/\sum I_{hkl}$, where $\langle I_{hkl}\rangle$ is the average of $I_{hkl}$ over all symmetry equivalents.
[b] $R_{factor} = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$, where $F_{obs}$ and $F_{calc}$ are observed and calculated structure factors.
[c] $R_{free} = \sum_T||F_{obs}| - |F_{calc}||/\sum_T|F_{obs}|$, where T is a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.

apo-CueO was greatly dependent on the presence of excess copper ion. With 100 mM exogenous $CuCl_2$, apo-CueO achieved the maximal activity of 28.3 U/mg (Table 2). This number was similar to previously reported values [14]. Interestingly, CueO incubated with 1 mM copper ion over night during the purification process (named as CueO*) could oxidize ABTS with a specific activity of 12 U/mg, even in the absence of exogenous copper. When exogenous $CuCl_2$ was included, CueO* gained about 5-fold more activity than apo-CueO under the same exogenous copper concentration (Table 2). This was the highest activity of CueO reported so far. Fungal laccases does not need exogenous copper ion to perform their function. Therefore, our results suggested that the copper ions in the recombinant *E. coli* CueO could be missing or partially missing, and the re-constitution of copper binding could be slow.

### *Copper sites in four CueO structures demonstrate different occupancy*

To investigate copper binding in CueO, CueO was crystallized either in the presence of different concentrations of copper ion (0, 0.5, and 10 mM) or with pre-incubation of copper (CueO*). These four crystals were defined as apo-CueO, 0.5-CueO, 10-CueO, and CueO*, respectively. Their structures were determined by molecular replacement and finally refined to 2.4, 1.9, 2.3, and 2.0 Å resolutions, respectively. Although the space groups are different from the previously determined CueO structure [11], the new CueO crystal structures displayed no difference in the overall folding. In contrast, the binding modes of Cu I and Cu II showed significant variations. In apo-CueO, the occupancy of Type I copper was as low as 0.7 and type II copper was completely missing. Consequently, the electron transfer chain of laccase redox reaction was broken [15]. With higher exogenous copper concentrations, the occupancies of type I and type II copper ions increased to 1.0 and 0.5, respectively (Table 3). These data were consistent with the CueO copper reconstitution assays performed by optical and EPR spectroscopy, in which the copper occupancies increased when exogenous copper was added [16]. Based on the above observation, the inactivity of CueO in the absence of exogenous copper is mostly due to the missing of type II copper. In addition, even with high concentration of copper ions, the occupancy of type II copper in CueO structure can only reach 0.5. This indicates the binding affinity of Cu II was much lower than that in fungal laccases. Unlike bacterial ones, fungal laccases possess almost fully occupied type II copper, and their activities are independent of exogenous copper. Therefore, it may be possible to generate highly active bacterial laccases by increasing the type II copper affinity.

Roberts et al. observed the fifth labile copper bound to CueO in a surface region, which is rich in methionine residues and 7.5 Å from type I copper, in a crystal soaked in 10 mM $CuCl_2$ and 30% polyethylene glycol for 60 min [17]. Based on this observation, it was proposed that this labile copper could mediate electron transfer from substrate to type I copper, then accelerate the oxidization rate. In addition, the sixth copper is located on another side of molecule and far away from CueO functional domain. However, in our four structures, neither the fifth copper nor the sixth copper was observed. This is consistent with the previous EPR experiment, in which the spectrum did

Table 2
Copper dependent oxidation activity (U/mg)[a] of apo-CueO, CueO*, E106F, and E106F incubated with $Cu2^+$

| [$CuCl_2$] | 0 (μM) | 10 (μM) | 100 (μM) | 1 (mM) | 10 (mM) | 100 (mM) |
|---|---|---|---|---|---|---|
| Apo-CueO | 0.0 | 0.3 | 0.5 | 2.5 | 19.6 | 28.3 |
| CueO* | 11.8 | 17.0 | 34.0 | 67.9 | 109.0 | 143.0 |
| E106F | 0.8 | 0.9 | 1.9 | 9.8 | 28.6 | 68.3 |
| E106F* | 27.8 | 31.9 | 51.0 | 77.2 | 134.5 | 202.0 |

[a] Oxidation of ABTS in the absence and presence of $CuCl_2$ was examined, respectively. Each reaction was performed in 300 μL of 0.1 M sodium acetate (pH 4.6) containing about 1 nM each protein and 0–100 mM $CuCl_2$. Reactions were started by addition of ABTS (2 mM final concentration) and the absorbance was recorded at 420 nm at the indicated times. All data were performed in triplicate at 25 °C. Enzyme-specific activity was expressed in μmol of ABTS oxidized/min/mg of protein. CueO* and E106F* were pre-incubated with 1 mM $CuCl_2$ for 24 h before the activity assay.

Table 3
Copper occupancy factor and B factor for each copper site of four CueO crystals

| Crystal ID | Resolution (Å) | Ratio of CueO:$Cu^{2+}$ | Mean B factor (overall) | | Cu I | Cu II | Cu IIIA | Cu IIIB |
|---|---|---|---|---|---|---|---|---|
| Apo-CueO | 2.4 | 1:0 | 25.3 | Occupancy | 0.7 | None | 0.5 | 0.8 |
| | | | | B factor | 28.0 | None | 28.3 | 26.5 |
| 0.5-CueO | 1.9 | 1:4 | 19.7 | Occupancy | 0.8 | 0.2 | 0.3 | 0.9 |
| | | | | B factor | 23.6 | 18.7 | 23.0 | 16.2 |
| CueO* | 2.0 | 1:55 | 25.3 | Occupancy | 1 | 0.3 | 0.4 | 0.8 |
| | | | | B factor | 24.5 | 21.9 | 25.9 | 23.6 |
| 10-CueO | 2.3 | 1:75 | 25.9 | Occupancy | 1 | 0.5 | 0.6 | 0.8 |
| | | | | B factor | 23.5 | 29.3 | 23.5 | 25.0 |

not show more change after the fourth copper binding site was saturated [16].

### *Influence of Glu106 on CueO activity*

To investigate the structural differences around type II copper binding site in CueO and other fungal laccases, a structural comparison between CueO and three typical fungal laccases, which were isolated from *Trametes versicolor*, *Melanocarpus albomyces*, and *Rigidoporus lignosus* [18–20], has been performed.

The structural comparison indicates that the overall structures and copper coordination of the above four proteins are much conserved, with the amino acid residues and their steric conformations within 7 Å from the type II copper site identical. A tunnel is found connecting type II copper to the protein surface while Glu106 is located at the gate of the tunnel in CueO (Fig. 1). It is interesting that in the two highly active fungal laccases from *R. lignosus* (specific activity of 1200–5400 U/mg) and *T. versicolor* (specific activity of 214–343 U/mg) [21,22], the corresponding residue is a Phe. It is likely that this hydrophobic residue gating the tunnel prevents type II copper from exchange with solvent. While in CueO, the charged Glu106 would make type II copper solvent accessible and easy to be lost. Consistent with the above hypothesis, in *M. albomyces* laccase that displays an intermediate specific activity of 50–68 U/mg [23], the gating residue is a His.

To confirm this hypothesis, E106F mutant of CueO was constructed by site-directed mutagenesis method. Purified E106F mutant protein showed detectable oxidase activity to ABTS without exogenous copper ion. When $CuCl_2$ is added, the oxidase activity of E106F was 50–100% higher than that of the native CueO (Table 2). On the other hand, the activity of E106F, after incubation with 1mM $CuCl_2$ for 24 h, still showed exogenous copper dependence, indicating that Glu106 might not be the only factor affecting the copper binding in CueO.

### *Steric hindrance near type I copper site*

In proposed mechanism of laccase catalysis, electrons from substrate first transport to type I copper, then go though a 13 Å pathway formed by one cysteine and two histidines to type III coppers, and finally reduce molecular oxygen to water at type II copper [2]. According to this mechanism, the substrate should bind near the type I copper. It was recently confirmed by the structure of endospore coat laccase from *Bacillus subtilis*. The complex structure of this laccase and ABTS proved that the substrate pocket is very close to type I copper site, and the electron donor ABTS directly interacts with type I copper [24].

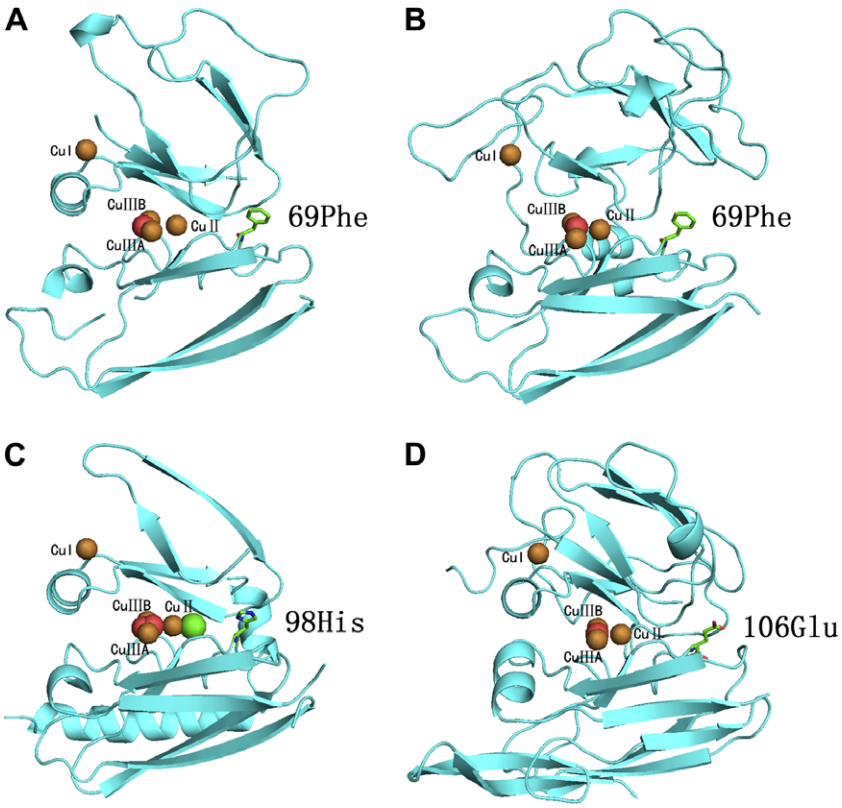


Fig. 1. Ribbon diagrams of solvent tunnel and trinuclear cluster in four different laccases. Brown, red, and green spheres represent copper ions, the bridging water molecule, and the chloric ion, respectively. (A) Phe69 residue and solvent tunnel of *Trametes versicolor* laccase. (B) Phe69 residue and solvent tunnel of *Rigidoporus lignosus* laccase. (C) His98 residue and solvent tunnel of *Melanocarpus albomyces* laccase. (D) Glu106 residue and solvent tunnel of CueO. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

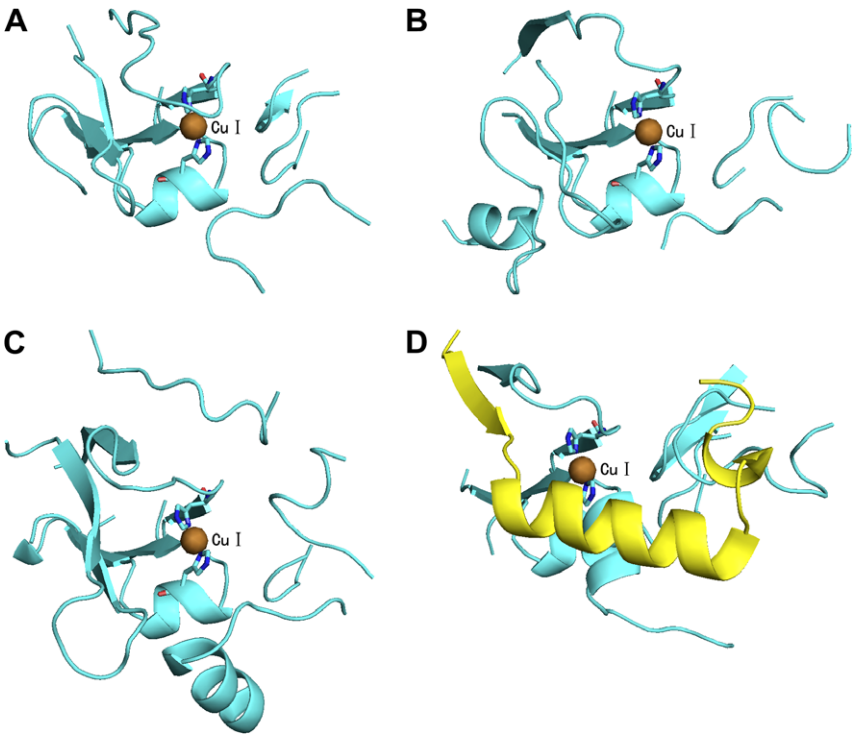


Fig. 2. Ribbon diagrams of four laccase substrate binding pockets. Brown spheres represent copper ions. (A) Substrate binding pocket motif of *Trametes versicolor* laccase. (B) Substrate binding pocket motif of *Rigidoporus lignosus* laccase. (C) Substrate binding pocket motif of *Melanocarpus albomyces* laccase. (D) Substrate binding pocket motif of CueO, α-helix from Leu351 to Gly378 is indicated in yellow. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

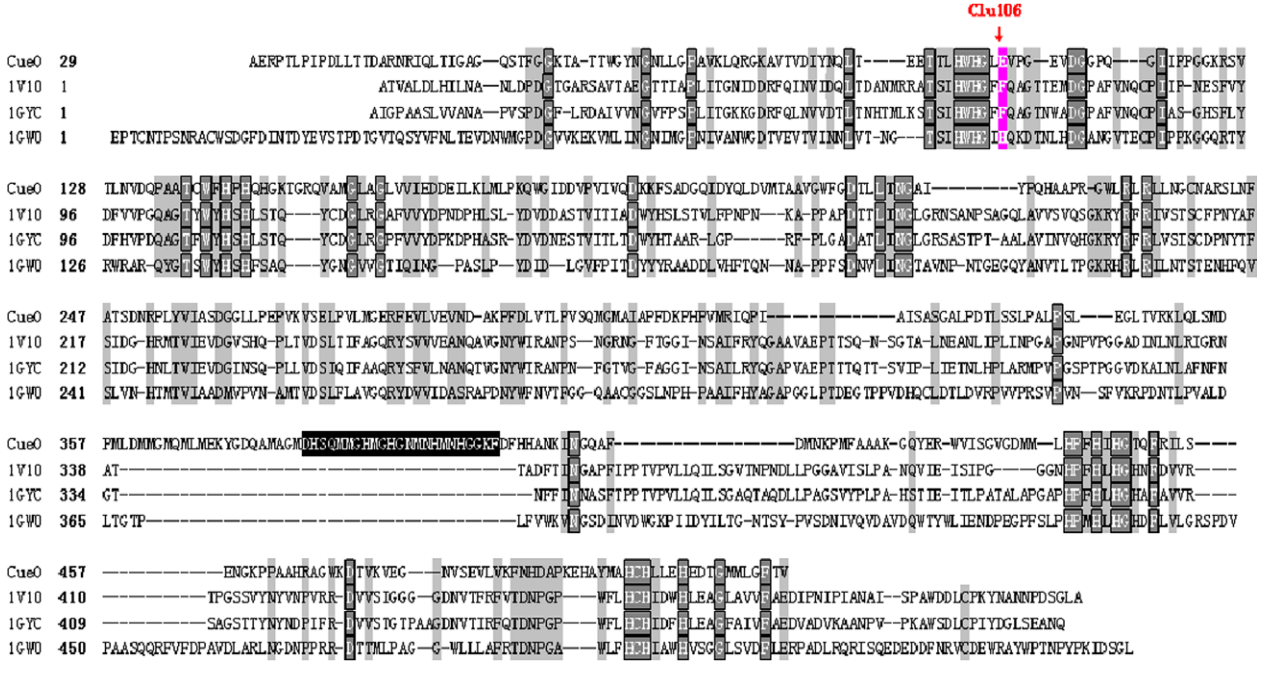


Fig. 3. Structure-based sequence alignment of CueO with four fungal laccases. 1V10 from *Rigidoporus lignosus*; 1GYC from *Trametes versicolor*; and 1GW0 from *Melanocarpus albomyces*. The residue numbering for each laccase is shown ahead of each sequence row. White letters on black rectangles represent the disordering residues in crystal structure. Fully conserved residues (white letters on gray background with black frame) and conservatively substituted residues (black letters on offwhite background) across this group of laccases are indicated. The pink line points out the position of Glu106 in CueO, Phe69 in *Trametes versicolo* and *R. lignosus* laccases, and His98 in *M. albomyces* laccase. The alignment is performed with the program O. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Structural comparison between CueO and three typical fungal laccases revealed an obvious difference in the substrate binding pocket. In fungal laccases, the substrate binding pockets are fully open and type I copper is exposed to solvent (Fig. 2A–C). However in CueO, an additional α-helix from Leu351 to Gly378 is located over type I copper and makes the substrate binding pocket smaller (Fig. 2D). Structure-based sequence alignment with the program O indicates this α-helix is absent in fungal laccases (Fig. 3) [13]. Although, the physiological substrate of CueO remains unknown, the active site structure of CueO suggests a smaller substrate compared to that of fungal laccases. The *in vitro* laccase substrates such as ABTS might not be preferred by CueO due to this extra α-helix. In order to obtain an engineered CueO with higher laccase activity, deletion of this extra α-helix might be a good approach.

This study clearly demonstrated the negative influence of Glu106 on CueO laccase oxidation activity. In addition, an extra α-helix (Leu351-Gly378) that might decrease the substrate binding affinity of CueO was also identified. These findings are of importance in production of highly active recombinant laccase for industrial and/or environmental applications. It is a bit disappointing that in our study, only a few fold increasing in oxidation activity was achieved. Further investigation is needed before real application of recombinant laccase can take place.

## Coordinates

The atomic coordinates for apo-CueO, 0.5-CueO, CueO*, and 10-CueO have been deposited in Protein Data Bank (PDB Accession code: 2FQD, 2FQE, 2FQF, and 2FQG).

## Acknowledgments

This work is supported by National Funding for Talent Youth (Grant No. 30225015), the 973 Program of the Ministry of Science and Technology (No. 2004CB720008 and 2006CB0D1705), the National Natural Science Foundation of China (Grant No. 10490193), and the Chinese Academy of Sciences (KSCX2-YW-R-61). We thank Dr. Yuhui Dong and Dr. Peng Liu in Institute of High Energy Physics and Mr. Yi Han in Institute of Biophysics for diffraction data collection.

## References

[1] F. Xu, Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition, Biochemistry 35 (1996) 7608–7614.
[2] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Multicopper oxidases and oxygenases, Chem. Rev. 96 (1996) 2563–2605.
[3] G. Felsenfeld, The determination of cuprous ion in copper proteins, Arch. Biochem. Biophys. 87 (1960) 247–251.
[4] E.I. Solomon, M.J. Baldwin, M.D. Lowery, Electronic structures of active sites in copper proteins: contributions to reactivity, Chem. Rev. 92 (1992) 521–542.
[5] D.J. Spira-Solomon, M.D. Allendorf, E.I. Solomon, Low-temperature magnetic circular dichroism studies of native laccase: confirmation of a trinuclear copper active site, J. Am. Chem. Soc. 108 (1986) 5318–5328.
[6] F. Xu, Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases, J. Biol. Chem. 272 (1997) 924–928.
[7] G. Alexandre, I.B. Zhulin, Laccases are widespread in bacteria, Trends Biotechnol. 18 (2000) 41–42.
[8] F.W. Outten, D.L. Huffman, J.A. Hale, T.V. O'Halloran, The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*, J. Biol. Chem. 276 (2001) 30670–30677.
[9] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[10] J. Navaza, AMoRe: an automated package for molecular replacement, Acta Crystallog. A 50 (1994) 157–163.
[11] S.A. Roberts, A. Weichsel, G. Grass, K. Thakali, J.T. Hazzard, G. Tollin, C. Rensing, W.R. Montfort, Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*, Proc. Natl. Acad. Sci. 99 (2002) 2766–2771.
[12] A.T. Brunger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, Crystallography and NMR system: a new software suite for molecular structure determination, Acta Crystallog. D 54 (1998) 905–921.
[13] T.A. Jones, J.Y. Zou, S.W. Cowan, M. Kjeldgaard, Improved methods for building protein models in electron density maps and the location of errors in the model, Acta Crystallog. A 47 (1991) 110–119.
[14] C. Kim, W.W. Lorenz, J.T. Hoopes, J.F.D. Dean, Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli* yacK gene, J. Bacteriol. 183 (2001) 4866–4875.
[15] W. Shin, U.M. Sundaram, J.L. Cole, H.H. Zhang, B. Hedman, K.O. Hodgson, E.I. Solomon, Chemical and spectroscopic definition of the peroxide-level intermediate in the multicopper oxidases: relevance to the catalytic mechanism of dioxygen reduction to water, J. Am. Chem. Soc. 118 (1996) 3202–3215.
[16] I. Galli, G. Musci, Sequential reconstitution of copper sites in the multicopper oxidase CueO, J. Biol. Inorg. Chem. 9 (2004) 90–95.
[17] S.A. Roberts, G.F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, W.R. Montfort, A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO, J. Biol. Chem. 278 (2003) 31958–31963.
[18] K. Piontek, M. Antorini, T. Choinowski, Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers, J. Biol. Chem. 277 (2002) 37663–37669.
[19] N. Hakulinen, L.L. Kiiskinen, K. Kruus, M. Saloheimo, A. Paananen, A. Koivula, J. Rouvinen, Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site, Nat. Struct. Biol. 9 (2002) 601–605.
[20] S. Garavaglia, M.T. Cambria, M. Miglio, S. Ragusa, V. Iacobazzi, F. Palmieri, C. D'Ambrosio, A. Scaloni, M. Rizzi, The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the t3 copper pair, J. Mol. Biol. 342 (2004) 1519–1531.
[21] R.P. Bonomo, G. Cennamo, R. Purrello, A.M. Santoro, R. Zappala, Comparison of three fungal laccases from *Rigidoporus lignosus* and *Pleurotus ostreatus*: correlation between conformation changes and catalytic activity, J. Inorg. Biochem. 83 (2001) 67–75.
[22] P.J. Collins, M.J. Kotterman, J.A. Field, A.D. Dobson, Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*, Appl. Environ. Microbiol. 62 (1996) 4563–4567.
[23] L.L. Kiiskinen, L. Viikari, K. Kruus, Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*, Appl. Microbiol. Biotechnol. 59 (2002) 198–204.
[24] F.J. Enguita, D. Marcal, L.O. Martins, R. Grenha, A.O. Henriques, P.F. Lindley, M.A. Carrondo, Substrate and dioxygen binding to the endospore coat laccase from *Bacillus subtilis*, J. Biol. Chem. 279 (2004) 23472–23476.