Promotion of Laccase Activities of *Escherichia coli* Cuprous Oxidase, CueO by Deleting the Segment Covering the Substrate Binding Site

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Total and partial deletions of the region covering the substrate-binding site of the *Escherichia coli* cuprous oxidase, CueO evidenced that the unique specificity for cuprous ion originates in the steric hindrance to interfere the access of laccase substrates towards the type I Cu site.

CueO involved in a Cu efflux system of *E. coli* oxidizes Cu^{I} to less toxic Cu^{II} in the periplasm.[1] CueO contains four catalytic Cu ions classified into type I Cu, type II Cu, and type III Cu, and accordingly, belongs to multicopper oxidase similarly to laccase, bilirubin oxidase, ascorbate oxidase, etc.[2] Type I Cu functions as the mediator of electron transfer from Cu^{I} as substrate to the trinuclear Cu center formed by a type II and a pair of type III Cu's, where O_2 accepts four electrons to be two water molecules.[3] In order to specifically bind substrate, multicopper oxidase has a crevice formed by loops attached to the ends of β-structures as the scaffold of the protein molecule.[4] An imidazole edge of the histidine residues coordinated to type I Cu is positioned at the side wall of the crevice to facilitate the electron transfer between substrate and type I Cu. In contrast, CueO has a large segment including helices 5–7, beneath which the substrate-biding site is formed by a set of amino acids, Met355, Asp360, Asp439, and Met441.[5] It is supposed that the oxidizing activities of CueO for the laccase-substrates such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,4-phenyldiamine (*p*-phenylenediamine, PPD), and 2-aminophenol (*o*-aminophenol, OAP) are very low due to this unique molecular architecture to interfere the access of potential organic substrates towards the type I Cu site.

In the present study, we totally or partly deleted the region between Pro357 and His406, which covers the substrate-binding site of CueO. The deleted region includes helices 5–7 and the very flexible region between helix 6 and helix 7 whose coordinates were not fixed in the crystal structure[5] (Figure 1). In order to obtain a definitive evidence about the role of the deleted region we prepared four mutants differing in the length of deletion. One is $\Delta\alpha$5–7CueO, in which the region between Pro357 and His406 is totally deleted. The second is $\Delta\alpha$6–7CueO, in which the helices 6 and 7 and the flexible region between them consisted of 25 amino acid residues are deleted (deleted Asp373–His406). The third is $\Delta\alpha$5CueO, in which helix 5 is deleted (deleted Pro357–Gly372). The forth is $\Delta\alpha$5–7CueO + 1/2α5, in which the N-terminal side of helix 5 is reserved (deleted Gln365–His406). In the case of $\Delta\alpha$5–7CueO, a GlyGly liker was used to connect the deleted ends separated ca. 0.5 nm not to deform the structure of the CueO scaffold. As for other three mutants, oligo-amino acids formed with Ala and Gly were designed as linkers to connect the deleted ends based on the crystal structure of CueO[5] (Supporting Information for mutant preparations).[9]

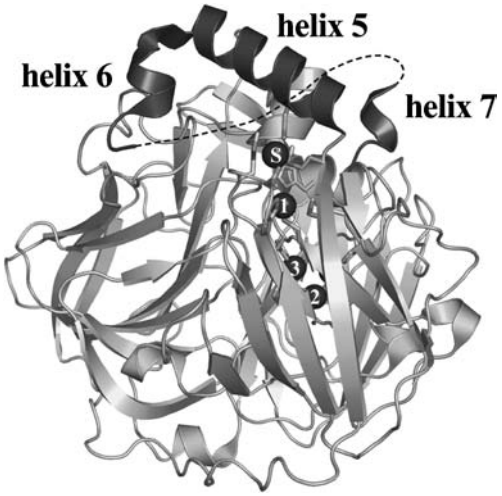

Figure 1. Structure of the wild-type CueO and the deleted region (helix 5, helix 6, the region without coordinates due to high mobility (broken line), and helix 7 figured using the protein data bank data, code 1N68 by PyMol. 1, 2, 3 and S represent type I Cu, type II Cu, type III Cu, and the Cu^{II} ion bound at the substrate-binding site by soaking a CueO crystal in $CuCl_2$. Asp360 as one of ligands for the substrate Cu is located in helix 5.

All mutants were successfully expressed and purified with yields of approximately 7 mg/dm^3 culture medium. On the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), these mutants gave a single band, and their estimated molecular masses, 48 kDa for $\Delta\alpha$5–7CueO, 49 kDa for $\Delta\alpha$6–7CueO, 51 kDa for $\Delta\alpha$5CueO, and 49 kDa for $\Delta\alpha$5–7CueO + 1/2α5, corresponded well with the calculated values, 48.7, 51.1, 53.1, and 49.8 kDa, respectively (Supporting Information, Figure 1).[9]

Each $\Delta\alpha$5–7CueO, $\Delta\alpha$6–7CueO, $\Delta\alpha$5–7CueO + 1/2α5, and $\Delta\alpha$5CueO afforded the strong charge-transfer band at 612 nm due to type I Cu, $Cys(S^-)_\pi \rightarrow Cu^{2+}$ with the molar extinction coefficients of 6000, 3600, 3600, and 4400, respectively (Figure 2A). The smaller values compared to that ($\varepsilon = 6300$ $M^{-1}\,cm^{-1}$) of the recombinant CueO (rCueO)[6] indicate that apo-proteins were also contained in agreement with the copper contents determined by atomic absorption spectroscopy, 3.7, 2.5, 2.5, and 3.1 per protein molecule, respectively (The Cu content in rCueO was 3.6 per protein molecule with the experimental error of ca. 10%.). The shoulder at around 330 nm indicated the presence of the paired type III Cu's bridged by a hydroxide,

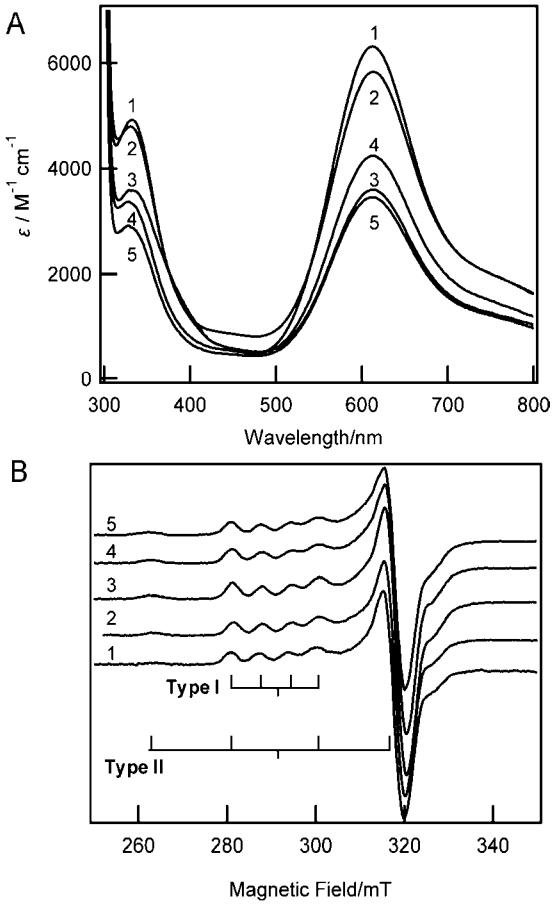

Figure 2. Absorption (A) and ESR (B) spectra of rCueO and mutants. 1, rCueO; 2, $\Delta\alpha$5–7CueO; 3, $\Delta\alpha$6–7CueO; 4, $\Delta\alpha$5CueO; 5, $\Delta\alpha$5–7CueO + 1/2α5. Measurement conditions: (A) 0.1 mol dm^{-3} potassium phosphate buffer (pH 6.0) at room temperature; (B) temperature, 77 K; power, 3.125 mW; frequency, 9.196 GHz; modulation, 100 kHz and 1 mT; sweep time, 4 min; amplitude, 125. Protein concentrations were determined using the BCA (bichinchoninate) protein assay reagent kit (Pierce, U.S.A.).

supporting that the trinuclear Cu center was accurately constructed in these mutants. In the electron spin resonance (ESR) spectra (Figure 2B), the signals derived from type I Cu and type II Cu were observed with the spin Hamiltonian parameters, $g_{II} = 2.24$ and $A_{II} = 6.7 \times 10^{-3}$ cm^{-1} for type I Cu and $g_{II} = 2.24$ and $A_{II} = 18.8 \times 10^{-3}$ cm^{-1} for type II Cu. These values were the same with those of rCueO.[6] Consequently, the present modifications of the CueO molecule were successful, and the copper active sites were not affected at all, although apo-proteins were present for $\Delta\alpha$6–7CueO, $\Delta\alpha$5–7CueO + 1/2α5, and $\Delta\alpha$5CueO. On the other hand, the total α-helix contents in rCueO and mutants were estimated based on the mean residue ellipticities, [θ] in the circular dichroism (CD) spectra at 222 nm.[7] However, it seemed to be difficult to obtain a series of reliable data reflecting slight changes in low α-helix content of $<10\%$.

Table 1. Enzyme activities of rCueO and mutants

Enzyme	ABTS[a,b] /unit mg^{-1}	PPD[a,c] /unit mg^{-1}	OAP /ΔA_{420} mg^{-1} min^{-1}
rCueO	0.45	0.53	0.15
$\Delta\alpha$5–7CueO	15	4.6	0.88
$\Delta\alpha$6–7CueO	1.9	1.2	0.76
$\Delta\alpha$5CueO	5.0	1.7	0.82
$\Delta\alpha$5–7CueO + 1/2α5	3.3	1.7	0.81

[a]One unit of activity was defined as the amount of enzyme that oxidized 1×10^{-6} mol of substrate per minute. [b]Determined colorimetrically by monitoring the oxidation of ABTS at 420 nm. [c]Determined colorimetrically by monitoring the oxidation of PPD at 487 nm.

The oxidizing activities of $\Delta\alpha$5–7CueO, $\Delta\alpha$6–7CueO, $\Delta\alpha$5–7CueO + 1/2α5, and $\Delta\alpha$5CueO for the laccase-substrates, ABTS, PPD, and OAP, were determined comparing with those of rCueO (Table 1). Total and partial deletions of the region covering the substrate-binding site from the rCueO molecule led to the increases in the laccase activities up to 30 times (If the content in apo-protein was taken into consideration, the increase was up to ca. 50 times.). These increases in the oxidizing activities for laccase-substrates were most prominent for the bulky ABTS. On the other hand, among the four mutants, $\Delta\alpha$6–7CueO showed the lowest activities, indicating that helix 5 is the most effective region to interfere the access of these organic substrates towards the type I Cu site. Nevertheless, the laccase activities promoted by the present modifications of CueO were lower than those of fungal laccases, while simple comparison was difficult because of the diverse reported values depending on the source of laccase.[8]

As conclusion, the segment deleted by the present study functions as the steric hindrance to interfere the access of laccase-substrates towards the type I Cu site of CueO and renders this enzyme the unique substrate specificity as cuprous oxidase.

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References and Notes

1 S. K. Singh, G. Grass, C. Rensing, W. R. Montfort, *J. Bacteriol.* **2004**, *186*, 7815.
2 G. Grass, C. Rensing, *Biochem. Biophys. Res. Commun.* **2001**, *286*, 902.
3 T. Sakurai, K. Kataoka, *Chem. Rec.*, in press.
4 M. E. Murphy, P. F. Lindley, E. T. Adman, *Protein Sci.* **1997**, *6*, 761.
5 S. A. Roberts, G. F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, W. R. Montfort, *J. Biol. Chem.* **2003**, *278*, 31958.
6 Y. Ueki, M. Inoue, S. Kurose, K. Kataoka, T. Sakurai, *FEBS Lett.* **2006**, *580*, 4069.
7 Y. H. Chen, J. T. Yang, H. M. Martinez, *Biochemistry* **1972**, *11*, 4120.
8 P. Baldrian, *FEMS Microbiol. Rev.* **2006**, *30*, 215.
9 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

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