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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/30348

An O-Centered Structure of the Trinuclear Copper Center in the Cys500Ser/Glu506Gln Mutant of CueO and Structural Changes in Low to High X-Ray Dose Conditions**

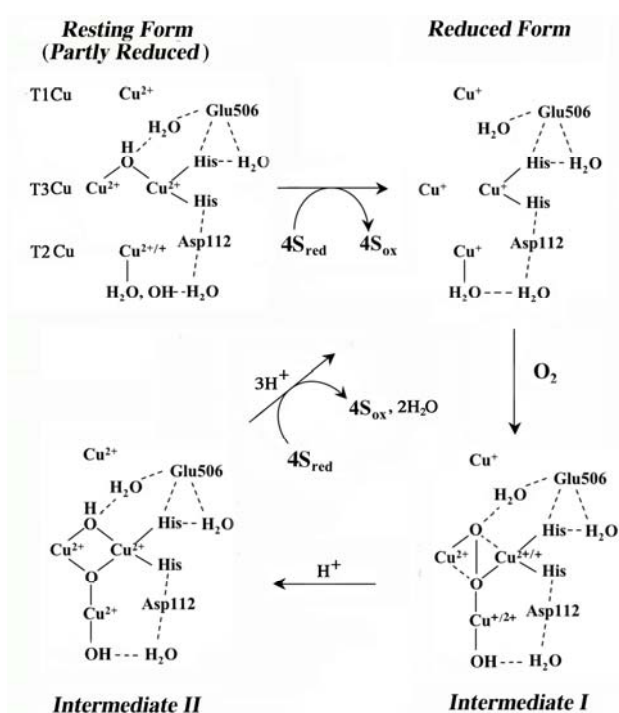
Hirofumi Komori, Ryosuke Sugiyama, Kunishige Kataoka, Yoshiki Higuchi, and Takeshi Sakurai*

CueO is a multicopper oxidase (MCO) involved in the copper efflux system of *Escherichia coli*.^[1,2] The type I copper (T1Cu) in CueO mediates the process of electron transfer from the substrate to the trinuclear copper center (TNC) which is composed of a type II copper site (T2Cu) and a pair of type III copper sites (T3Cus (T3aCu and T3bCu)). O₂, the final electron acceptor, binds to the TNC and is reduced to two water molecules.^[3-6] Special attention has been paid on this O₂ reduction mechanism of MCO to avoid to form or to release activated oxygen species. Only terminal oxidases such as cytochrome *c* oxidase catalyze this process except MCOs. Thus, MCOs have been expected to apply to biofuel cells as a cathodic enzyme.

Two reaction intermediates in the four-electron O₂ reduction process have been discovered and are known as, the intermediate I (peroxide intermediate) and intermediate II (native intermediate) (Figure 1). Intermediates I can be trapped during the course of reactions of the MCO derivatives such as: the Hg-substituted laccase at the T1Cu center,^[7] the mixed valent laccase with T1Cu in the cupric state and T2Cu and T3Cus in the cuprous state,^[8] CueO,^[9] bilirubin oxidase,^[10] and Fet3p,^[11] in which the T1Cu site is vacant as a result of the replacement of the Cys ligand with Ser. Since the intermediate I does not have an electron paramagnetic resonance (EPR) signal (Figure S1 in Supporting Information),^[7-11] it has been proposed that a peroxide species is bound to the TNC in a μ -1,2-peroxo-fashion, and accordingly, one of the three copper ions remains in the cuprous state. However, direct evidence to support the presence of peroxide or the oxidation state of the copper centers in the TNC has not been published. Thus, it

Figure 1. Proposed reaction mechanism of MCO. Intermediates I and II are also known as the peroxide intermediate and native intermediate, respectively.

is possible that the intermediate I is an artifact obtained in the reaction when performed under electron-deficient



conditions. On the other hand, the intermediate II is in the fully oxidized form which is reached at the end of a catalytic cycle of MCO, and it has been considered that an O₂²⁻ derived from O₂ is located at the center of the TNC.^[9,12-14] The three copper centers in the TNC are in the cupric state and interact magnetically. This gives rise to a broad $g < 2$ EPR signal which is detectable only at cryogenic temperatures.^[9,12-14] Analogous structures have also been found in the X-ray crystal structures of the resting laccases from *Lentinus tigrinus*^[15] and *Streptomyces coelicolor*,^[16] although the oxygen atom is not located within bonding distance of all three of the copper centers in the TNC. Thus, the proposed structures of the TNC remain controversial for all forms.



In this study, we determined the X-ray crystal structure of a double mutant of CueO. This Cys500Ser/Glu506Gln mutant is isolated predominately as intermediate I (~70%) with the remaining fraction in the conventional resting form without the central oxygen atom (~30%) as reported previously.^[9] The isolated form of Cys500Ser/Glu506Gln is predominately stabilized as intermediate I because the mutation of Cys500 leads to an one-electron deficiency with respect to full reduction of O₂, and because protons are not effectively transferred to intermediate I as a result of the mutation at Glu506 in the hydrogen bond network leading from the exterior of the CueO molecule to the TNC. In this study, we collected diffraction data of a single crystal of Cys500Ser/Glu506Gln at low to high X-ray dose conditions. In addition, we measured copper K-edge X-ray absorption spectra *in situ* to track facile changes in the redox state of copper centers caused by the presence of hydrated electrons produced by synchrotron radiation.

A set of seven X-ray diffraction datasets with 1.10 to 1.70 Å resolution and three copper K-edge X-ray absorption datasets were obtained at low to high X-ray dose conditions for a single crystal of Cys500Ser/Glu506Gln (Table S1 in Supporting Information). The crystal structure of the TNC represented by dataset 1 which was measured within the initial 9 s (Figures 2a (2FoFc map) and 2c (FoFo difference map)) shows that an oxygen atom is located inside copper triangle. In contrast, the conventional structure of the resting state of the TNC does not have an oxygen atom. A structure of the TNC with an inner oxygen atom has not been reported in the crystal structures of CueO and other MCOs,^[17,18] with the exception of laccases from *Lentinus tigrinus*^[15] and *Streptomyces coelicolor*.^[16] However, the inner oxygen atom is not bound to all three copper centers in these two examples. In the structure of dataset 1, the distances between the central oxygen atom and T2Cu, T3aCu, and T3bCu are 1.83 Å, 2.08 Å, and 2.26 Å, respectively, and the

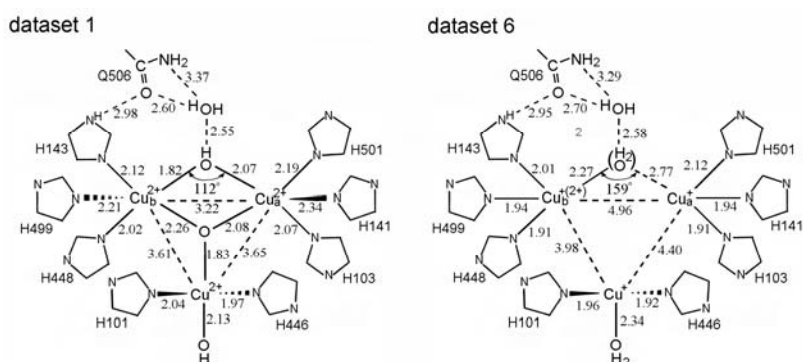


Figure 3. Bond distances (unit Å) and the bond angle between T3aCu-OH-T3bCu for dataset 1 and dataset 6. Three copper and oxygen atoms are essentially coplanar.

T3bCu angle identified thus far.

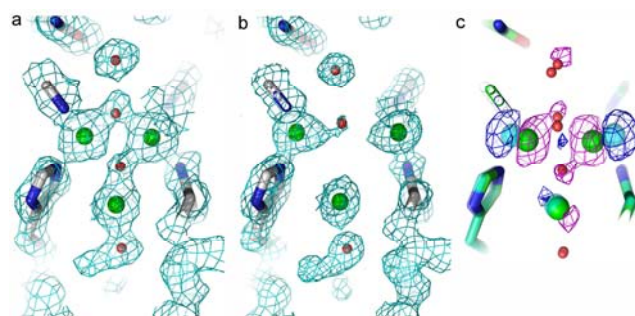


Figure 2. The crystal structure of the trinuclear copper center of the Cys500Ser/Glu506Gln CueO mutant. a: 2FoFc map (1.2σ) of dataset 1, b: 2FoFc map (1.2σ) of dataset 6, and c: FoFo difference map (5σ) for dataset 1 minus dataset 6 (red) and for dataset 6 minus dataset 1 (blue). Copper and oxygen atoms are shown with green or blue and red spheres, respectively.

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[**] We acknowledge financial support from a Grand-in-Aid for Scientific Research (23580131) from the Ministry of Education, Science, Sports and Culture of Japan (to K. K.). We also thank the beamline staff at BL26B2 for their kind help with X-ray data collection.

Supporting information for this article is available on the WWW under <http://www.angewandte.de>.

The other striking feature in the structure of the TNC in Cys500Ser/Glu506Gln is that the 2.34 Å bond length between T3aCu and His141 is significantly longer than the other two T3aCu-His bond lengths (2.07 Å and 2.19 Å). Among the three T3bCu-His bond lengths, the 2.21 Å bond length between T3bCu and His499, is longer than the other two (2.02 Å and 2.12 Å). Therefore, both T3aCu and T3bCu have distorted square pyramidal structures containing with a long vertex bond involving His141 and His499, respectively. A similar arrangement has been identified for the T3Cu centers in oxy-hemocyanin, in which a peroxide molecule occupies the equatorial plane in the $\mu\text{-}\eta^2\text{:}\eta^2$ -fashion.^[20] The square plane of T2Cu with a 2N2O coordination mode (including the central oxygen and an exogenous OH⁻) is essentially perpendicular to the TNC plane. An acetate ion derived from the precipitation buffer is partly bound to T2Cu for OH⁻ (seen in the deposited PDB data), supporting the unpublished fact that the wild type CueO shows higher enzymatic activities in acetate buffer by

presumably assisting the supply of protons in the course to form water molecules. A noteworthy feature of the substitution of Glu506 is that the amide group of the Gln side chain is hydrogen bonded to the OH⁻ bridged between T3Cu^s via a water molecule as observed in wild type CueO. The fact that this hydrogen bond exists in wild type CueO and that the reaction of the Glu506Gln mutant with O₂ stops at intermediate II^[9] provide strong support for a role of Glu506 in transferring protons to O₂ similarly to Glu487 in Fet3p.^[21] The T1Cu site in Cys500Ser/Glu506Gln showed a structure similar to that in the wild type CueO and the deletion mutant,^[17,18] while a water molecule was present in the place of T1Cu (Figure S2 in Supporting Information).

The copper K-edge X-ray absorption spectrum of Cys500Ser/Glu506Gln (blue line in Figure 4) measured *in situ* shortly after the collection of the X-ray diffraction dataset 1, did not have any 1s → 4p_z, ~8984eV features originating from cuprous ions.^[22] This indicates that all three copper centers were in the divalent state when dataset 1 was collected.

Reduction of the copper centers of the TNC occurs upon exposure of a single crystal to X-rays, as evidenced by the gradual propagation of the 1s → 4p_z feature at ~8984eV in the copper K-edge X-ray absorption spectra (green and red lines in Figure 4). Simultaneously, the electron density of

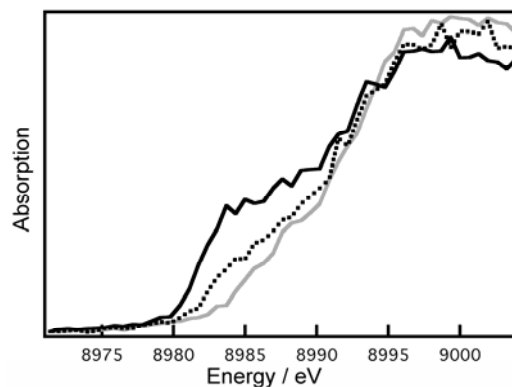


Figure 4. Copper K-edge X-ray absorption spectra obtained after collecting X-ray diffractions data for dataset 1, dataset 2, and dataset 6 shown in gray line, square dot and black line, respectively. The data acquisition time for each of the spectra was 8 sec, and the total X-ray exposure times before measurement of each were, 9 sec, 197 sec., and 2545 sec, respectively.

the central oxygen gradually decreases and finally disappears. The size of the copper-triangle was continuously enlarged during the progression from dataset 1 to dataset 6. The distance between T2Cu-T3aCu changed from 3.65 Å to 4.40 Å. Likewise, the distance between T2Cu-T3bCu changed from 3.61 Å to 3.98 Å, and the distance between T3aCu-T3bCu changed from 3.22 Å to 4.96 Å. The bond angle between T3aCu-OH-T3bCu widened significantly from 112° (dataset 1) to 159° (dataset 6).

Subtractions of dataset 1 and dataset 6 (Figures 2c, red for dataset 1 minus dataset 6 and blue for dataset 6 minus dataset 1) unequivocally shows that T3aCu shifts towards His141, and the bond length between T3aCu and His141 decreases from 2.34 Å to 1.94 Å (*vide supra*). T3aCu becomes three-coordinated with the dissociation of the bridging OH⁻ ligand. Therefore, it appears that T3aCu is fully reduced in dataset 6 which was collected with the highest X-ray dose. T3bCu also shifts to His499 and the bond length between them changes from 2.21 Å to 1.94 Å. However, T3bCu is four-coordinated with the binding of a H₂O molecule (the T3bCu²⁺-O(H₂O) distance of 2.27 Å). A portion of T3bCu is reduced and three-coordinated judged from the weakened electron density for the O atom, although it is difficult to estimate the content of cupric and cuprous ions in T3bCu (Only the four-coordinated structure is shown in Figure 3, dataset 6). T2Cu is reduced in dataset 6 and adopts the T-shape which is characteristic of a cuprous ion. The bond length between T2Cu and the oxygen atom outside the copper-triangle changes from 2.13 Å to 2.34 Å,

indicating the binding of a H₂O molecule. Another H₂O molecule formed would enter the outlet channel constructed between domain 1 and domain 3 with the change in the coordination number of T2Cu from 4 to 3. These changes in the structure of the TNC from dataset 1 to dataset 6 indicates that hydrogen bonding plays a significant role in the formation and release of water molecules, which are strongly coupled to the steps used to supply electrons and protons to intermediate II.

Since absorption, circular dichroism (CD), and EPR spectra of Cys500Ser/Glu506Gln (Figure S1 in Supporting Information) showed properties characteristic of intermediate I, T3Cu^s should be in the cupric state and T2Cu in the cuprous state before data collection is initiated.^[9] However, the crystal structure of Cys500Ser/Glu506Gln of dataset 1 does not indicate the peroxide-bound structure. Instead, the O-centered structure proposed for the intermediate II is visible (Figures 1, 2a and 2c). The *in situ* measured copper K-edge X-ray absorption spectra (Figure 4) show that all three copper ions are in the cupric state in the structure of dataset 1. One possible explanation for this conflict in the crystal structure of the TNC and the oxidation state of the copper centers and spectroscopic properties is that hydrated electrons are supplied to intermediate I soon after X-ray irradiation, and the TNC reaches the intermediate II form with the supply of an additional electron from T2Cu. This facile conversion of intermediate I (which has a deficiency of a single electron) to the fully oxidized intermediate II^[23] accompanied by full reduction of O₂ might have prevented us from detecting a peroxide species in resonance Raman spectra of *Rhus vermicifera* laccase,^[12] CueO,^[9] and bilirubin oxidase.^[10] Otherwise, Trp139, which is located ~4 Å from T3aCu or disulfide bonds might act as a source of the fourth electron which allows conversion of intermediate I to intermediate II during crystallization. Absorption features of intermediate I was gradually decreased in prolonged incubations of Cys500Ser/Glu506Gln in solution.

In conclusion, the present crystal structure analysis of the CueO mutant of Cys500Ser/Glu506Gln adopts the O-centered TNC structure proposed for the intermediate II (native intermediate) due to transformation of the peroxide-bound intermediate I structure by X-ray or incubation in solution. The O-centered smallest-sized TNC structure allows all three copper centers to magnetically couple^[9] as shown via spectroscopy and density functional theory calculation,^[6,13,24] in contrast to the larger TNC which contains an inner oxygen atom that fails to bind to all three copper centers in the resting state of laccases from *Lentinus tigrinus*,^[13] *Streptomyces coelicolor*,^[14] and *Trametes hirsuta*.^[25] Further, *in situ* measurements of low to high doses of illuminated X-rays and copper K-edge X-ray absorption spectra indicate non-equivalence in the reduction of the three copper centers in the TNC and their shifts in

location leading to the stepwise formation of and release of water molecules from their coordination spheres^[24].

Experimental Section

Cys500Ser/Glu506Gln was prepared as described previously (spectral data are shown in Figure S1 in Supporting Information),^[9] and crystallized by mixing an equal volume of the protein solution (7.8 mg/mL in 100 mM potassium phosphate buffer, pH 6.0) and 100 mM ammonium acetate, pH 4.6, 20% polyethylene glycol (PEG4000), 5% isopropanol at 20°C. X-ray diffraction and copper K-edge absorption datasets for a single crystal were collected at the beam line BL26B2 of SPring-8 using a wavelength of 0.8000 or 1.3700 Å and a detector MX225 (Marresearch) at 100 K. Determination of the unit-cell parameters and integration of reflections were performed using the program HKL2000^[26] and CCP4 programs.^[27] Diffraction from CueO mutant crystals extends to a resolution of 1.1 Å. The Cys500Ser/Glu506Gln mutant crystal belongs to space group $P2_1$, with unit-cell parameters $a = 50.44$, $b = 91.10$, $c = 53.23$ Å, $\beta = 102.87$. There is one monomer in the asymmetric unit of both crystals. Structure determination was performed by the molecular replacement method using a reported structure of CueO (PDB code:1KV7). Further model building and refinement of the structure were carried out using COOT,^[28] REFMAC,^[29] and SHELX programs.^[30] The progress and validity of the refinement process were checked by monitoring the R-free value for 5% of the total reflections.^[31] The data collection and refinement statistics are summarized in Table S1 in Supporting Information. Model geometry was analyzed using the MOLPROBITY program^[32] and no residue was found in the disallowed region of the Ramachandran plot. The figures were prepared by using PyMOL (<http://pymol.sourceforge.net>). The atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with accession codes, 3UAA, 3UAB, 3UAC, 3UAD, and 3UAE.

Received: ((will be filled in by the editorial staff))

Published online on ((will be filled in by the editorial staff))

Keywords: bioinorganic chemistry · enzyme catalysis · metalloprotein · X-ray absorption spectroscopy · X-ray diffraction

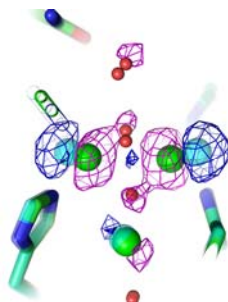
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Multicopper Oxidase

Hirofumi Komori, Ryosuke Sugiyama,
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Intermediate in dioxygen reduction:

The O-centered structure of the trinuclear copper center in a multicopper oxidase, CueO as an intermediate of the four-electron reduction of dioxygen was determined by *in situ* data collection of X-ray diffractions and copper K-edge spectra at low to high X-ray dose conditions.