Multicopper Oxidases

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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**

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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). O_2, the final electron acceptor, binds to the TNC and is reduced to two water molecules.[3–6] Special attention has been paid to this O_2 reduction mechanism of MCO to avoid the formation or release of activated oxygen species. Other than MCOs, only terminal oxidases such as cytochrome *c* oxidase catalyze this process. Thus, it is expected that MCOs can be applied as cathodic enzymes in biofuel cells.

Two reaction intermediates in the four-electron O_2 reduction process have been discovered and are known as intermediate I (peroxide intermediate) and intermediate II (native intermediate) (Figure 1). Intermediate I can be trapped during the course of reactions of 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. As intermediate I does not have an electron paramagnetic resonance (EPR) signal (see Figure S1 in the 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 is possible that the intermediate I is an artifact obtained in the reaction when performed under electron-deficient conditions. On the other hand, 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 O^{2-} derived from O_2 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 EPR signal ($g < 2$), 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.

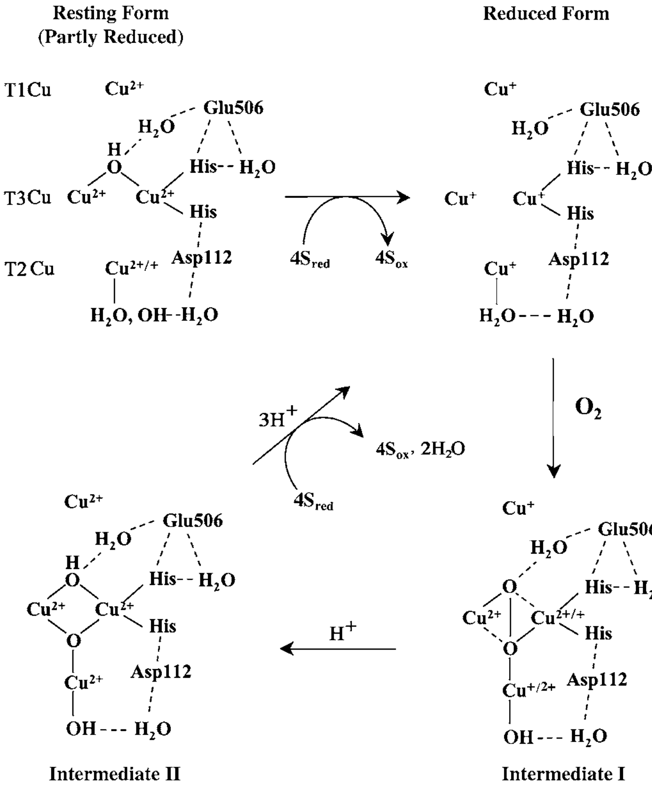

Figure 1. Proposed reaction mechanism of MCO. Intermediates I and II are also known as the peroxide intermediate and native intermediate, respectively.

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 ($\approx 70\,\%$) with the remaining fraction in the conventional resting form without

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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 a one-electron deficiency with respect to full reduction of O_2, 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 the Supporting Information). The crystal structure of the TNC represented by dataset 1, which was measured within the initial 9 seconds, (Figure 2a, $2F_OF_C$ map; and 2c, F_OF_O difference map) shows that an oxygen atom is located inside the 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 distances between the copper atoms of T2Cu–T3aCu, T2Cu–T3bCu, and T3aCu–T3bCu are 3.65 Å, 3.61 Å, and 3.22 Å, respectively (Figure 3). The central oxygen atom is strongly bound to all three Cu centers inside the smallest reported Cu triangle.[19] Two T3Cus are further bridged by an OH$^-$ group as reported in every structure of MCO (including CueO) and in a deletion mutant Δα5-7CueO.[17,18] In the Cys500Ser/Glu506Gln double mutant, the 112° angle between T3aCu-OH-T3bCu is the smallest T3aCu-OH-T3bCu angle identified thus far.

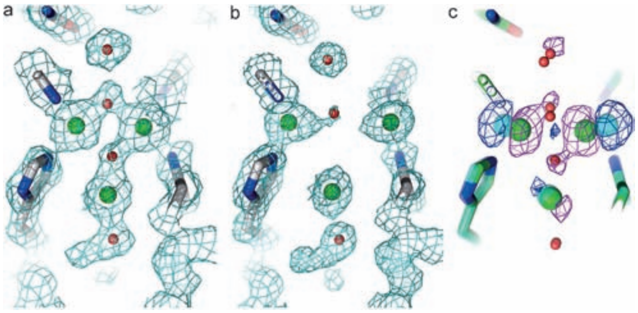

Figure 2. The crystal structure of the trinuclear copper center of the Cys500Ser/Glu506Gln CueO mutant. a) 2FoFc map (1.2σ) of dataset 1, b) 2FoFc map (1.2σ) of dataset 6, and c) FoFo difference map (5σ) for dataset 1 minus dataset 6 (red) and for dataset 6 minus dataset 1 (cyan). Copper and oxygen atoms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

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; Figure 3). 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 with a long vertex bond involving His141 and His499, respectively. A similar arrangement has been identified for the T3Cu centers in oxyhemocyanin, in which a peroxide molecule occupies the equatorial plane in the μ-η^2:η^2 fashion.[20] The square planar structure of T2Cu cooridinated to two nitrogen atoms and two oxygen atoms (including the central oxygen and an exogenous OH$^-$) is essentially perpendicular to the TNC plane. Instead of an OH$^-$ ion, an acetate ion, from the precipitation buffer, is partly bound to T2Cu (seen in the deposited PDB data), thus supporting the unpublished fact that the wild-type CueO shows higher enzymatic activities in acetate buffer by presumably assisting in the supply of protons in the course of the formation of water molecules. A noteworthy feature of the substitution of Glu506 is that the amide group of the Gln

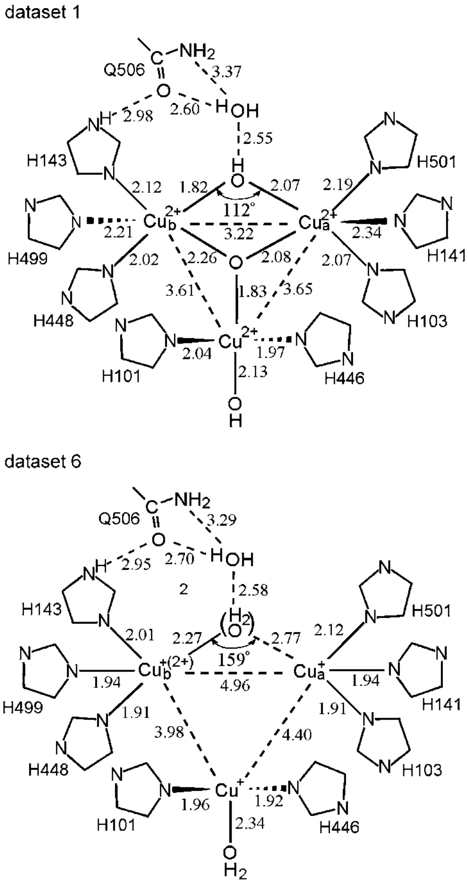

Figure 3. Bond distances (Å) and the bond angle between T3aCu-OH-T3bCu for dataset 1 and dataset 6. The three copper and oxygen atoms are essentially coplanar.

side chain is hydrogen bonded through a water molecule to the OH^- bridged between T3Cus, 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 O_2 stops at intermediate II[9] provide strong support to the hypothesis that Glu506 plays a role in transferring protons to O_2, similar to Glu487 in Fet3p[11] and Glu463 in bilirubin oxidase.[21] The T1Cu site in Cys500Ser/Glu506Gln showed a structure similar to that in the wild-type CueO and the deletion mutant,[17,18] but with a water molecule present in the place of T1Cu (Figure S2 in the Supporting Information).

The copper K-edge X-ray absorption spectrum of Cys500Ser/Glu506Gln (gray line in Figure 4) measured in situ shortly after the collection of the X-ray diffraction dataset 1, did not have any $1s\rightarrow4p_z$ features at approximately 8984 eV, originating from cuprous ions.[22] This finding indicates that all three copper centers were in the divalent state when dataset 1 was collected.

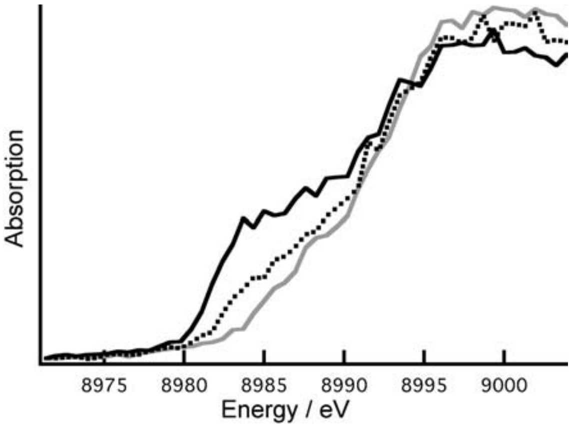

Figure 4. Copper K-edge X-ray absorption spectra obtained after collecting X-ray diffractions data for dataset 1, dataset 2, and dataset 6 shown by gray line, square dot, and black line, respectively. The data acquisition time for each of the spectra was 8 s, and the total X-ray exposure times before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

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 $1s\rightarrow4p_z$ feature at approximately 8984 eV in the copper K-edge X-ray absorption spectra (square dot and black lines in Figure 4). Simultaneously, the electron density of 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 (Figure 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 (see above). T3aCu becomes three coordinated upon 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 towards His499 and the bond length between them changes from 2.21 Å to 1.94 Å. However, T3bCu is four coordinated upon the binding of a H_2O molecule (the $T3bCu^{2+}–O(H_2O)$ distance of 2.27 Å). A portion of T3bCu is reduced and three coordianted, as 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 a 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, thus indicating the binding of a H_2O molecule. Another H_2O molecule formed would enter the outlet channel constructed between domain 1 and domain 3 with a 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 indicate that hydrogen bonding plays a significant role in the formation and release of water molecules, transformations which are strongly coupled to the steps used to supply electrons and protons to intermediate II.

As absorption, circular dichroism (CD), and EPR spectra of Cys500Ser/Glu506Gln (Figure S1 in the Supporting Information) showed properties characteristic of intermediate I, T3Cus 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 (Figure 1 and Figure 2a and c). 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 between 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) into the fully oxidized intermediate II[23] accompanied by full reduction of O_2 might have prevented us from detecting a peroxide species in resonance Raman spectra of *Rhus verniciferra* laccase,[12] CueO,[9] and bilirubin oxidase.[10] Otherwise, Trp139, which is located approximately 4 Å from T3aCu or disulfide bonds might act as a source of the fourth electron, thus allowing conversion of intermediate I into intermediate II during crystallization. The absorption features of intermediate I 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) owing 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 by spectroscopy and density functional theory caluculations,[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 nonequivalence in the reduction of the three copper centers in the TNC and their shifts in location, thus 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 the Supporting Information),[9] and crystallized by mixing an equal volume of the protein solution (7.8 mg mL^{-1} in 100 mM potassium phosphate buffer, pH 6.0) and 100 mM ammonium acetate (pH 4.6), 20 % polyethylene glycol (PEG4000) and, 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 Å 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 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 the 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.

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