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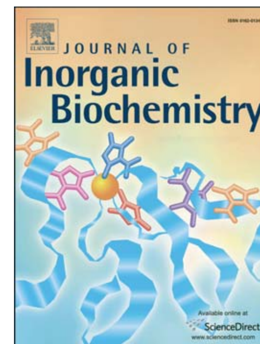

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A novel resting form of the trinuclear copper center in the double mutant of a multicopper oxidase, CueO, Cys500Ser/Glu506Ala

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ABSTRACT

A multicopper oxidase, CueO was doubly mutated at its type I copper ligand, Cys500 and an acidic amino acid residue located in the proton transfer pathway, Glu506, to Ser and Ala, respectively. Cys500Ser/Glu506Ala was mainly in a novel resting form to afford the absorption band at ca. 400 nm and an EPR signal with a highly anisotropic character derived from type III copper. However, Cys500Ser/Glu506Ala gave the same reaction intermediate (peroxide intermediate) as that from Cys500Ser and Cys500Ser/Glu506Glu.

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Multicopper oxidases (MCOs) harbor a type I copper (T1Cu) and a trinuclear copper center (TNC) comprised of a type II copper (T2Cu) and a pair of type III coppers (T3Cu) to oxidize substrate and reduce O_2, respectively [1,2]. T1Cu is responsible for the Cys to Cu(II) charge transfer band at ca. 600 nm and the d-d band at around 800 nm. T1Cu is also responsible for the EPR signal with an unusually small hyperfine splitting. TNC affords the band at ca. 330 nm derived from the antiferromagnetically coupled T3Cus and the EPR signal from T2Cu with a normal magnitude of hyperfine splitting. These properties of TNC arise from the OH^--bridged structure between T3Cus. However, other resting TNC structures related with the reaction mechanism have also been discovered in an increased number of MCOs and mutants [1 and references therein, 3,4].

We have performed studies on a MCO, *Escherichia coli* CueO [5-9] at the aims of revealing its structure-function relationships and of applying it to biofuel cells and pigment formations as catalyst [10,11]. The T1Cu site in CueO becomes unoccupied as a result of the mutation at the Cys ligand with Ser. The intermediate I (peroxide intermediate) can be trapped in the reaction of the Cys500Ser mutant with O_2 because one electron is deficient to form two water molecules [5]. On the other hand, the hydrogen bond network constructed with a conserved Glu residue (Glu506) and water molecules has been proved to function as a proton transport pathway from bulk water to TNC [6,8.9,12-14] (Fig. 1). Mutations of this Glu residue in CueO resulted in loss or reduction in enzymatic activities depending on amino acid introduced and also in the successful trapping of the intermediate II (native intermediate) due to the inability to transport protons to O_2. The double mutant, Cys500Ser/Glu506Gln as isolated was mainly in the intermediate I state and was EPR-silent (~0.1T2Cu was EPR-detectable per a protein molecule). On the other hand, considerably high enzymatic activities have been exhibited by the Glu to Ala mutant of CueO and bilirubin oxidase. The crystal structure of the CueO mutant exhibited that two water molecules occupied the space formed by

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the mutation, constructing a compensatory hydrogen bond network with only water molecules [9].

In the present study we report unique spectral and magnetic properties of a double mutant of CueO, Cys500Ser/Glu506Ala at a ligand to T1Cu, Cys500 and at Glu506. The reaction of the double mutant with O_2 has also been performed to study properties of the intermediate I in comparison with that formed from Cys500Ser and Cys500Ser/Glu506Gln.

Cys500Ser/Glu506Ala contained 3.2 Cu atoms in a protein molecule as determined from atomic absorption spectroscopy. Due to the absence of T1Cu the absorption bands at 600 nm disappeared, although the d-d band from TNC (ε_{745} ~400 $M^{-1}cm^{-1}$) was observable (Fig. 2A, full line). The band at 330 nm was considerably weakened (ε ~2000 $M^{-1}cm^{-1}$ relative to ~5200 $M^{-1}cm^{-1}$ for the wild type enzyme (dotted line)), but a novel shoulder band was unequivocally observed at ca. 400 nm. This absorption band disappeared upon reduction, indicating that it did not come from an impurity. The corresponding circular dichrosim (CD) spectrum (Fig. 2B, full line) afforded a negatively signed band at 400 nm in addition to the weakened negatively signed band at 330 nm and the d-d bands at ca. 700 nm and 860 nm which were inverted in sign from those of Cys500Ser (dashed line). The EPR spectrum of Cys500Ser/Glu506Ala (Fig. 2C, full line) afforded a rhombic Cu(II) signal (g_z = 2.40, g_y = 2.11, g_x = 2.04, A_z = 7.6 x 10^{-3} cm^{-1}, and A_x= 5.3 x 10^{-3} cm^{-1}) differing from the resting CueO and Cys500Ser (Fig. 2C dashed line). The EPR signal of T2Cu (g_{II} = 2.25, A_{II} = 19.6 x 10^{-3} cm^{-1}) was apparently minor. The total EPR-detectable Cu(II) in Cys500Ser/Glu500Ala was 2.2 per a protein molecule. Therefore, Cys500Ser/Glu506Ala is supposed to be in a unique resting state, both T3Cus to be magnetically isolated and T2Cu to be cuprous.

The reaction of the reduced Cys500Ser/Glu506Ala with O_2 gave the absorption and CD spectra typical of the intermediate I (full lines in Figs. 3A and 3B, in which the dotted and dashed lines are of Cys500Ser and Cys500Ser/Glu506Gln, respectively) [5]. Differing from Cys500Ser the bands at

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ca. 350 nm and 470 nm from Cys500Ser/Glu506Ala did not decay soon after the formation and kept the maximum intensities for ca. 50s. However, these bands derived from the intermediate I decayed with a considerably short life-time (inset in Fig. 3A) compared with those formed from Cys500Ser Cys500Ser/Glu500Gln. Freezing of Cys500Ser/Glu506Ala soon after the reaction with O_2 gave the weak T2Cu EPR signal (Supplementary Fig. 1, The total EPR-detectable Cu(II) was ~0.2 per a protein molecule), indicating that the main species is EPR-silent. The original spectral features were recovered with the decay of the intermediate.

Based on spectral property and enzymatic reactivity the resting form of TNC in Cys500Ser/Glu506Ala differs from that of the wild type enzyme. Detection of the highly rhombic Cu EPR signal suggests that it originates in T3Cus magnetically isolated [7,12]. The total EPR-detectable Cu(II) was 2.2 per a protein molecule. This fact excludes possibilities that the rhombic Cu-EPR signal came from Cu^{2+} ions incorporated into the vacant T1Cu site and from T2Cu with a strong distortion. Cu^{2+} ion has never been incorporated into the T1Cu site, of which Cys ligand is changed to Ser [2,5-9]. Since T2Cu and two N ligands are almost linearly arranged, the geometry of T2Cu does not significantly deviate from planer structures [3,7]. Two T3Cu signals with small A_{II} values (15.0 x 10^{-3} and 15.6 x 10^{-3} cm^{-1}) have been reported for a bilirubin oxidase mutant, His94Val, in which a His ligand to T2Cu was substituted by Val [15]. The Cu-OH-Cu structure will become more flexible because of the absence of the carboxyl group in the hydrogen bond network. In addition, two T3 Cus are not equivalent due to the unequal interactions with amino acids located in the outer-sphere. One of T3Cu (T3Cua) is in the d-π interaction distance with Trp139 (~4 Å). A His ligand to another T3Cu (T3Cub) is hydrogen bonded with Asp112, one of key amino acids to form water molecules [3,7]. This unequivalence between T3Cus might have become more peculiar due to the loss in magnetic interaction. Therefore, Glu506 may play multiple roles in the reduction of dioxygen by CueO.

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The origin of the 400 nm band is not known at the present stage, although a deformation on the Cu-OH-Cu structure might not be excluded. Small molecule and theoretical studies have indicated that the magnetic interaction between binuclear Cu centers is highly dependent on the angle, Cu-O-Cu [2]. An organic radical is excluded as the origin for the 400nm. A Tyr or Trp residue is not located within a distance to magnetically interact with T2Cu in CueO molecule.

TNC in Cys500Ser/Glu506Ala was in a unique resting form to give the new absorption band and rhombic EPR signal as a result of the absence of T1Cu and the modification of the hydrogen bond network. This resting form might have been realized from a modification of the hydrogen bond network leading from bulk waters to the OH^- ion bridged between T3Cus. In addition, the absence of T1Cu would have indirectly but profoundly affected the properties of TNC because the T1Cu site and TNC are closely connected each other though the His499-Cys500-His501 triad, His505-Glu506 diad (Fig. 1) and other peptide backbones. The present double mutation is the first case that T3Cus are detected in the EPR spectrum. In spite of this unique resting form, Cys500Ser/Glu506Ala exhibited the ordinary reactivity with O_2 to produce the intermediate I, excluding the possibility of unusual crosslinking of His residues to the three Cu centers in TNC.

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Appendix A. Supplemental data

Supplemental data to this article can be found on line at http://doi.org/10.1016/j.jinorgbio2014.0X.XXX.

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Figure Legends

Fig. 1. The active site of CueO figured using 1N68 in the protein data bank with a DS ViewerPro 5.0. The hydrogen bond network involving Glu506 and water molecules terminates at the OH^- ion bridged between T3Cua and T3Cub. T3Cua and T3Cub are not equivalent due to the unequal arrangement of the amino acid residues located in the outer-sphere of TNC.

Fig. 2. The absorption (A), CD (B), and EPR (C) spectra of Cys500Ser/Glu506Ala CueO (full line) together with those of the wild type CueO (dotted line) and Cys500Ser CueO (dashed line).

Fig. 3. Absorption (A) and CD (B) spectra of the intermediate I formed from the reaction of the reduced Cys500Ser/Glu506Ala CueO with dioxygen (full line) together with those from Cys500Ser/Glu506Gln CueO (dotted line) and Cys500Ser CueO (dashed line).

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Figure 1

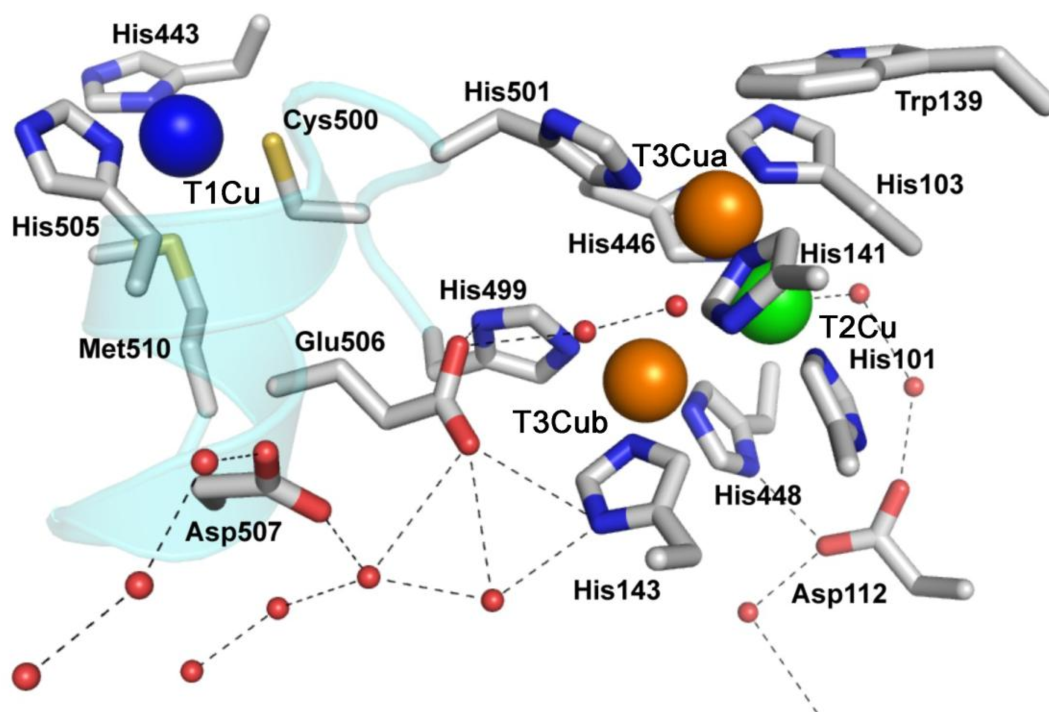

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Figure 2

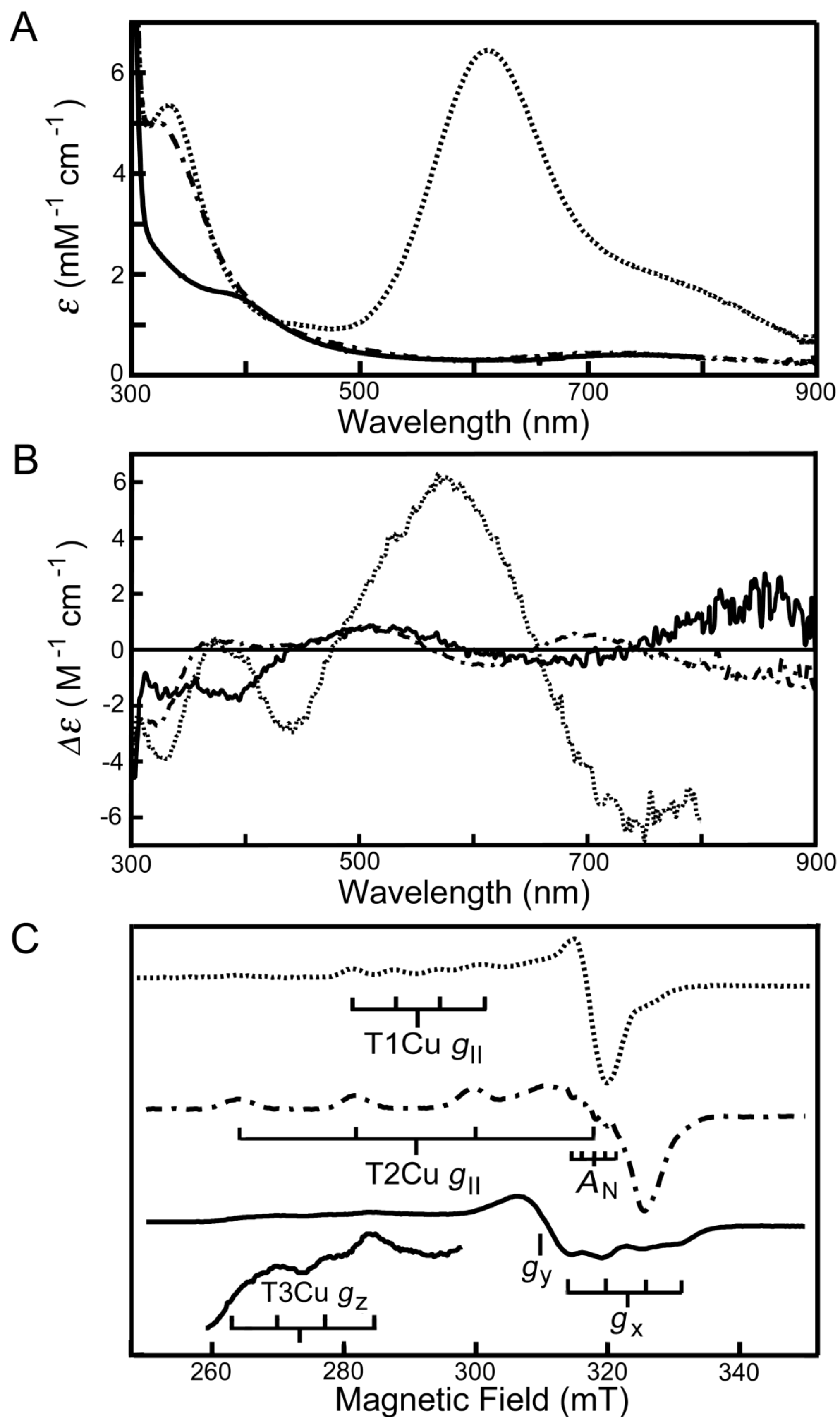
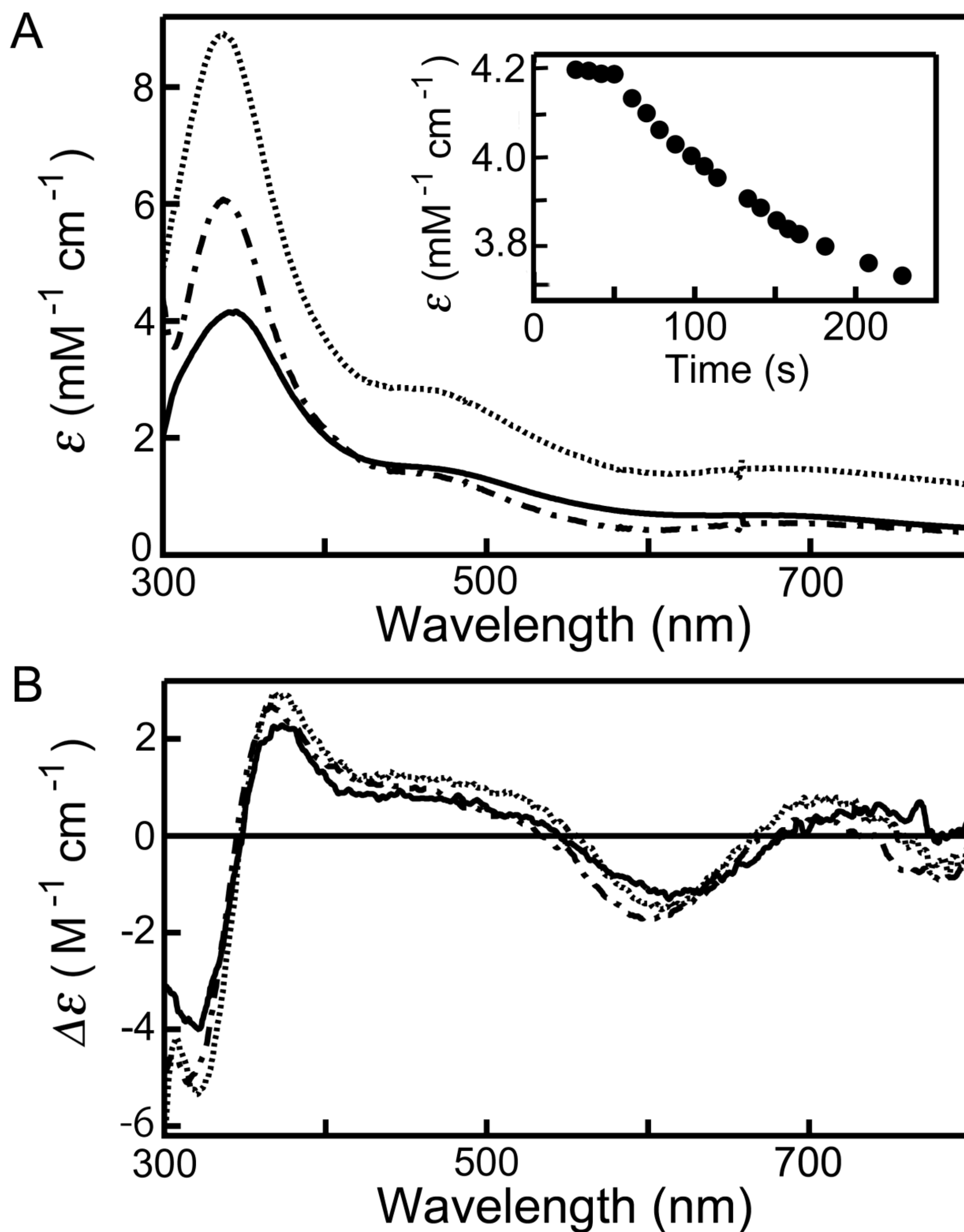


Figure 3



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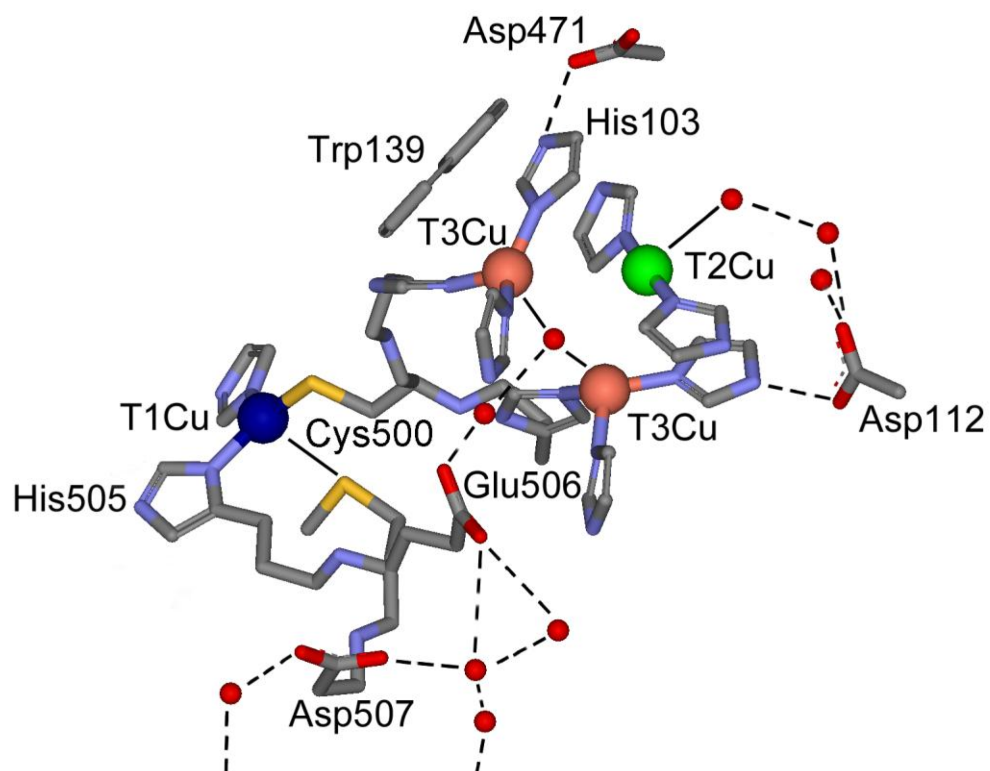

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Synopsis

The Cys500Ser/Glu506Ala mutant of CueO was mainly in a novel resting form to afford the new absorption band and EPR signal derived from type III coppers, although the reaction of it with O_2 gave the same reaction intermediate (peroxide intermediate) as that from Cys500Ser and Cys500Ser/Glu506Gln.

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Highlights

・CueO was mutated at its type I copper ligand and a Glu residue concerned in proton transport.

・Cys500Ser/Glu506Ala was in a novel resting form differing from other multicopper oxidases.

・Glu506 plays a dual role to assist the biding of O_2 and transfer of H^+ for H_2O formation.

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