

Modifications on the hydrogen bond network by mutations of Escherichia coli copper efflux oxidase affect the process of proton transfer to dioxygen leading to alterations of enzymatic activities

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Dear Editor

Biochemical Biophysical Research Communications

We have the pleasure for submitting our manuscript entitled “Modifications on the Hydrogen Bond Network by Mutations of *Escherichia coli* CueO Affect the Process of Proton Transfer to Dioxygen Leading to Peculiar Alterations of Enzymatic Activities” in Biochemical Biophysical Research Communications. CueO is the cuprous oxidase from *Escherichia coli* and concerns in a system to excrete excess copper to protect *E. coli* from oxidative damages. Together with laccase and related enzymes, CueO is a multicopper oxidase and has the type I copper to mediate the electron transfer from substrate and the trinuclear copper center comprised of type II copper and a pair of type III coppers, at which dioxygen is four-electron reduced without forming activated oxygen species. This conversion of dioxygen to water molecule is performed only by terminal oxidases and multicopper oxidases. Therefore, multicopper oxidases are expected to apply to cathodic enzyme for biofuel cells. We have trapped reaction intermediates by preparing mutants and derivatives of multicopper oxidases, which are electron deficient to fully reduce dioxygen and are difficult to relay protons to dioxygen. We revealed that Glu506 in CueO located adjacent to the trinuclear copper center is a key amino acid to relay protons to dioxygen, because this amino is located in the hydrogen bond network leading from the exterior of the CueO molecule to the trinuclear copper center. We performed systematic mutations at Glu506 and Asp507 to study proton relay mechanism in the course of the four-electron reduction of dioxygen by multicopper oxidases. We found that the conserved Glu506 is exchangeable with Asp. The Ala mutation was also functional presumably because one or two water molecules occupied the open space and the alternative proton relay network was constructed in contrast to the Ala mutations in cytochrome oxidases. The hydrogen bond network was also constructed by the Gln mutation, but Gln had not ability to relay protons because the amide group do not cycle between protonated and deprotonated forms.

The Ile mutation resulted in complete loss of enzymatic activity because an open space to accommodate a water molecule is not formed due to bulkiness in the side chain. Thus the present systematic mutations at Glu506 and also Asp507, which is located in a branch of the hydrogen bond network and was found to play a role to support the proton transfer, unequivocally showed clear results of the proton relay pathway in multicopper oxidase for the first time. Due to special attention to the four-electron reduction of dioxxygen by multicopper oxidases, we believe the present study attracts wide attention from researches studying on biochemistry, inorganic chemistry, electrochemistry and related fields, and this is the reason why we would like to publish the present study as rapidly as possible in BBRC.

Proton transfer pathway to dioxygen in CueO was identified.

Glu506 is the key amino acid to transport proton.

The Ala mutation at Glu506 formed a compensatory proton transfer pathway.

The Ile mutation at Glu506 shut down the hydrogen bond network.

**Modifications on the Hydrogen Bond Network by Mutations of *Escherichia coli* CueO Affect the Process of Proton Transfer to Dioxygen Leading to Peculiar Alterations of Enzymatic Activities**

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## ABSTRACT

CueO has a branched hydrogen bond network leading from the exterior of the protein molecule to the trinuclear copper center. This network transports protons in the four-electron reduction of dioxygen. We replaced the acidic Glu506 and Asp507 residues with the charged and uncharged amino acid residues. Peculiar changes in the enzyme activity of the mutants relative to the native enzyme indicate that an acidic amino acid residue at position 506 is essential for effective proton transport. The Ala mutation resulted in the formation of a compensatory hydrogen bond network with one or two extra water molecules. On the other hand, the Ile mutation resulted in the complete shutdown of the hydrogen bond network leading to loss of enzymatic activities of CueO. In contrast, the hydrogen bond network without the proton transport function was constructed by the Gln mutation. These results exerted on the hydrogen bond network in CueO are discussed in comparison with proton transfers in cytochrome oxidase.

*Keywords:* CueO, Multicopper oxidase, Proton transfer, O<sub>2</sub>-reduction, hydrogen bond network

## Introduction

CueO (copper efflux oxidase) from *Escherichia coli* (*E. coli*) [1,2] is a multicopper oxidase (MCO) containing a type I (T1) copper, a type II (T2) copper and a pair of type III (T3) coppers in the active site [3,4]. The T1 copper center is involved in oxidation of substrate and is responsible for the strong charge transfer band at 610 nm which arises from the Cys(S<sup>-</sup>) → Cu<sup>2+</sup> charge transfer. Electrons from substrate are transferred from the T1 copper to the trinuclear copper center (TNC) which is comprised of a T2 copper and T3 coppers through the His-Cys-His triad. TNC affords a band at about 330 nm which is derived from the charge transfer of the bridging of OH<sup>-</sup> to the T3 coppers. The enzyme converts O<sub>2</sub> into 2 molecules of H<sub>2</sub>O without forming or releasing activated oxygen species.

It has been proven that acidic amino acids located adjacent to TNC in MCOs such as CueO [5-7], laccase [8,9], bilirubin oxidase (BO) [10], Fet3p [11] and CotA [12] play an important role in the four-electron reduction of O<sub>2</sub>. Asp112, which is located within the hydrogen bond network extending between domain 1 and domain 3 in the CueO molecule is connected to the OH<sup>-</sup> coordinated to T2 copper via a water molecule (Fig. 1). This residue is involved in the binding of dioxygen at TNC [5]. On the other hand, Glu506 which is hydrogen-bonded to the OH<sup>-</sup> bridging the T3 copper sites via a water molecule (Fig. 1) plays a crucial role in supplying protons to O<sub>2</sub> through another hydrogen bond network leading from the exterior of the CueO molecule to TNC. A Glu residue corresponding to Glu506 in

CueO has also been identified in BO [7] Fet3p [13], and CotA [12]. Mutations of the Glu residue in CueO and BO by Gln produce significant reductions in enzymatic activities. From a Fourier transform infrared spectroscopy study [7], we showed that both Glu506 in CueO and Glu463 in BO cycle between the protonated and deprotonated forms with changes in the redox state. Furthermore, intermediate II [6] (the native intermediate [11]) could be trapped in the single turnover process of Glu506Gln presumably because this mutant is unable to transport protons to the O<sub>2</sub>-reduced species. The intermediate II has also been trapped in the reactions of *Rhus vernicifera* laccase and BO, although its lifetime could not be increased [8,11]. These results strongly suggest that the hydrogen bond network involving Glu506 plays a crucial role in relaying protons to TNC during the course of the conversion of O<sub>2</sub> to 2H<sub>2</sub>O by CueO. Fig. 1 indicates that this putative proton transport pathway is branched at a water molecule located between Glu506 and Asp507. One branch leads to the exterior of the CueO molecule via an arrangement of water molecules in a channel, but the other branch passes through Asp507 which is exposed to solvent. It is certain that protons are transported from solvent water molecules to TNC, although one of the branches or both are utilized in the four-electron reduction of O<sub>2</sub> at TNC.

In the present study, we describe mutations of Glu506 and Asp507 in CueO with the objective of revealing the roles of these residues and identifying the proton transfer pathway. The mutations of Glu506 with Asp, Ala, and Ile were performed to impose a variety perturbations on the hydrogen bond network, and to compare the results with data obtained from the previous Gln mutation which inactivates the hydrogen bond network with respect to proton transfer [6,7,14]. We expect that the hydrogen bond network is modified, but still highly functional in the Glu506Asp mutant. Furthermore, a space sufficient to accommodate one or two water molecules will become available within the Glu506Ala mutant according to the crystal structures of CueO [15] and the deletion mutant [16]. In contrast, the substitution of Glu506 by Ile with its bulky nonpolar side chain is expected to close the proton relay pathway and lead to loss of enzyme activity. By performing these mutations at Glu506 we not only intend to confirm whether a hydrogen bond network comprised only of water molecules functions properly but also to reveal why acidic amino acids are commonly utilized in the proton relay pathway in MCOs as well as in cytochrome oxidases [17]. With respect to Asp507, mutations with Ala and Asn are performed to determine whether this amino acid, which is not conserved in all MCOs, plays a significant role in the proton relay.

## **2. Materials and Methods**

### *2.1 Preparation of Mutants*



The genes for Glu506Ala/Asp/Ile and Asp507Ala/Asn were prepared with a QuikChange kit (Stratagene) using oligonucleotide primers below and the template plasmid pUCCueO as described [5-7]:

E506A(+), 5'-CTGGAGCATGCAGATACGGGG-3',  
E506A(-), 5'-CCCCGTATCTTCATGCTCCAG -3',  
E506D(+), 5'-CTGGAGCATGATGATACGGGG-3',  
E506D(-), 5'-CCCCGTATCATTCATGCTCCAG -3',  
E506I(+), 5'-CTGGAGCATATTGATACGGGG-3',  
E506I(-), 5'-CCCCGTATCAATATGCTCCAG -3',  
D507A(+), 5'-GAGCATGCCACGGGGATGATG-3',  
D507A(-), 5'-CATCATCCCCGTGGCTTCATGCTC-3',  
D507N(+), 5'-CTGGAGCATGAAAATACGGGGATG-3'  
D507N(-), 5'-CATCCCCGTATTTTCATGCTCCAG-3'.

*E. coli* BL21 (DE3) was transformed with the mutant plasmids with electroporation. Cultivations of the transformants and purifications of the mutant proteins were carried out as described previously [5-7]. Protein concentrations were determined using the BCA (bicinchoninic acid) protein assay reagent (Pierce) and also from the absorption intensity at 280 nm,  $\epsilon = 73000$  [5-7], resulting in less than 5% deviations in the two independent methods.

## 2.2 Measurements

Activities of the mutants to oxidize 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), which functioned as an electron donor, were colorimetrically determined from changes in the absorption of the oxidized product at 420 nm ( $\epsilon = 36000 \text{ M}^{-1} \text{ cm}^{-1}$ ) [18,19]. One unit of activity is defined as the amount of enzyme to oxidize 1 mmol of ABTS per min. The acetate buffer, 100 mM, pH 6.0, citrate buffer, 100 mM, pH 5.5, and phosphate buffer, 50 mM, pH 6.0 were used for enzyme assays of the CueO mutants.

The averaged total copper content in each mutant molecule was determined by atomic absorption spectroscopy on a Varian SpectrAA-50 spectrometer. Absorption spectra were measured on a JASCO V-560 spectrometer. Circular dichroism (CD) spectra were measured on a JASCO J-500C spectropolarimeter. X-band electron paramagnetic resonance (EPR) spectra were measured on a JEOL JES-RE1X spectrometer at 77 K. The total amount of the EPR detectable  $\text{Cu}^{2+}$  signals in a molecule of CueO and mutants has been determined using  $\text{Cu}^{2+}$ -*N,N,N',N'*-tetramethyleneacetic acid (EDTA) as standard with MnO as the external

standard to calibrate signal intensity.

### 3. Results and discussion

#### 3.1 Characterizations of the *Glu506Gln/Ala/Asp/Ile* and *Asp507Ala/Asn* Mutants of *CueO*

The *Glu506Asp*, *Glu506Ala*, *Glu506Ile*, *Asp507Ala*, and *Asp507Asn* were found to contain 4.1, 3.8, 4.3, 4.0, and 4.0 copper atoms per protein molecule, respectively (Table 1), indicating that within experimental error of 10%, each of the mutants contains four copper atoms as does recombinant *CueO* (4.1 Cu/protein molecule) and the *Glu506Gln* mutant (3.8 Cu/protein molecule). Therefore, it is apparent that the T3 copper centers and the T1 and T2 copper centers are not significantly affected by the mutations on the outersphere of the T3 copper centers.

Absorption, circular dichroism (CD) and electron paramagnetic resonance (EPR) spectra of the *Asp* (purple), *Ala* (red), and *Ile* (green) mutants at *Glu506* are shown in Figs. 2A-2C together with those of *CueO* (black) and the *Gln* mutant (flesh colored). Spectra of the *Asp507Ala* (pale blue) and *Asp507Asn* (blue) mutants are also included in Figs. 2A-2C. All mutants have an absorption band at ca. 610 nm derived from T1 copper which is similar to that of native *CueO* [6], although the CD spectral features derived from T1 copper of certain mutants were found to be slightly different in the range of 400-800 nm. On the other hand, the intensity of the band at ca. 330 nm was apparently decreased in both the absorption and CD spectra of the mutants. This indicates that the charge transfer band from OH<sup>-</sup> to the T3 coppers is indirectly affected as a result of modifications of the hydrogen bond with the bridging OH<sup>-</sup> group although the extent of the change is not dramatic. Furthermore, only *Glu506Ile* (green) was found to exhibit a slightly modified spectral feature in the near UV-region, presumably because a fraction of the intermediate II form was in the resting form [6-8].

All mutants exhibit EPR signals derived from the T1 and T2 copper centers without any change in the spin Hamiltonian parameters from those of *CueO* and *Glu506Gln* (T1 copper:  $g_{II} = 2.23$  and  $A_{II} = 6.9 \times 10^{-3} \text{ cm}^{-1}$ ; T2 copper:  $g_{II} = 2.24$  and  $A_{II} = 18.4 \times 10^{-3} \text{ cm}^{-1}$ ) [6] (Fig. 2C). The values for total EPR detectable Cu<sup>2+</sup> ions per protein molecule were found to be within the range of 1.8-2.0 in each of the mutants (Table 1). This indicates that both of the T1 and T2 copper centers are mostly in the cupric state. The T3 copper centers of the mutants remain undetectable by EPR with the exception of *Glu506Asp*. This indicates that the effects on the bridging OH<sup>-</sup> as a result of the mutations are not large enough to induce a significant change in the strong antiferromagnetic interaction between the T3 copper centers.

Specific activities of *CueO* and the *Glu506Asp*, *Glu506Ala*, *Glu506Gln*, *Glu506Ile*,

Asp507Ala and Glu507Asn mutants towards ABTS were found to be 0.42, 0.33, 0.35, 0.0026, ~0, 0.48, and 0.73 units/mg, respectively, in acetate buffer solutions (Table 1). The mutants exhibited weakened enzymatic activities in citrate and phosphate buffer solutions, but the shifts in activities were generally parallel as long as the measurements were obtained in the same buffer solution. Considerably high activities were identified for the Glu506Asp and Glu506Ala mutants, but negligibly low activity was identified for the Glu506Gln mutant and the Glu506Ile mutant was found to have no activity. The absorption at 420 nm continued to increase very slowly in the determination of the oxidizing activity of the Glu506Gln mutant. In contrast, the absorption at 420 nm for the Ile mutant leveled off after a slight initial increase to 1.0-1.5 electron oxidation equivalents of ABTS in the acetate buffer solution, although absorption changes were found to be below the detection limit in the citrate and phosphate buffer solutions (Fig. 3). The Asp507 mutants were found to have activities higher than that of the parent enzyme by 14-86%.

Effects of mutations at Glu506 and Asp507 are minimal for spectral properties of the mutants. Differences are seen only in the absorption intensity at ca. 330 nm because the OH<sup>-</sup> group bridging between the T3 copper centers is indirectly hydrogen-bonded with these acidic amino acid residues as indicated in Fig. 1. Since the properties of each copper center remain intact after the outersphere mutations, it is possible to assess the role of the hydrogen bond network which includes Glu506, Asp507, and water molecules constructed in the branched channel leading from the exterior of the CueO molecule to TNC

### *3.2 Roles of Glu506 and Asp507 Located in the Hydrogen Bond Network in the Four-Electron Reduction of O<sub>2</sub> to 2H<sub>2</sub>O*

Enzymatic activities of MCOs are determined by a variety of factors including the accessibility of substrates towards the binding site, differences in the redox potentials between substrate and T1 copper and between T1 copper and TNC, affinity of TNC to O<sub>2</sub>, and the mechanism of reduction of O<sub>2</sub> to two molecules of H<sub>2</sub>O which includes transport of four protons. There is no doubt that Glu506, which provides a connection between TNC and solvent water molecules is one of the dominant amino acid residues governing the O<sub>2</sub>-reduction process. We were able to trap the reaction intermediates from the reactions of the mutants at this amino acid residue [6], and enzyme activities were found to change according to the following order depending on the identity of the amino at the 506 position: Glu > Ala, Asp >> Gln > Ile~0.

The high activities shown by the Asp mutants, (79-113% of the activity of native CueO), were not unexpected. Although it is difficult to predict how the hydrogen bond network is

modified due to the shortening of one CH<sub>2</sub> group in the side chain, it is apparent that Glu506 is exchangeable with Asp. According to the alignment of the amino acid sequence and crystal structures of MCOs, Glu is highly conserved [3,4]. One exception is in human ceruloplasmin, which has Asp instead of Glu [20]. In the analogous Glu to Asp mutations for Fet3p and CotA, 116% and 6% activities relative to the respective parent enzyme were reported [12,13]. This diversity in activities shown by the Glu to Asp mutants in MCOs is a result of the hydrogen bond being highly directional. In line with the mutation at Glu506 in CueO, we made mutations at Asp112 located at the other side of TNC in a previous study, and observed 51% activity for the Asp112Glu mutant but only 10% activity for the Asp112Asn and Asp112Ala mutants [5]. This proves that Asp112 plays an important role in the binding of O<sub>2</sub> at TNC [5].

The Glu506Ala mutant was found to have high activity, (63-78% of native CueO) presumably because one or two water molecules can occupy the open space and a compensatory hydrogen bond network can be formed to effectively relay protons. An analogous Glu to Ala mutation has also been performed for Fet3p and this mutant was found to have 61% activity of the native enzyme, although the result was not discussed in detail [13].

In contrast to the Ala mutant, the Ile mutant exhibited no enzymatic activity. This is in line with our prediction (based on the crystal structures of CueO and the deletion mutant [15,16] that the bulky side chain of the Ile residue would shut down the hydrogen bond network. In addition, it is apparent that Glu506, which is located at the branch point of the hydrogen bond network is the key amino acid in the process of transporting protons to O<sub>2</sub>. In support of this proposal, it has been found that an analogous Ile mutation in BO (unpublished result) and an analogous Leu mutation in CotA [12] have essentially no activity. A water molecule is not visible between the Leu residue and the OH<sup>-</sup> bridging the T3 copper centers in the crystal structure of the Leu mutant of CotA [12]. It is unclear whether the absence of the water molecule represents an obligatory architectural modification induced by the mutation or an accidental result of the expression system.

We have observed only the initial oxidation of ABTS equivalent to ca. 1.5 electrons, but the absorption at 420 nm was found to level off. This limitation to incorporate more electrons into the Glu506Ile molecule suggests that electron transfer from substrate to TNC via the T1 copper center is closely coupled with the transport of protons from the exterior of the CueO molecule to TNC to retain a charge balance. The shutting down of the proton relay pathway at the mutated Ile residue may result in prevention of full transport of electrons to TNC [7]. Fig. 1 shows that His505 coordinated to T1 copper and Glu506 are arranged to

enable close communication during coupled transport of electrons and protons. The Asp and Ala mutants behave in a manner which is distinct from the Ile mutant and could be fully reduced with a slight excess of reducing agents as a result of compensatory hydrogen bond networks for relay of protons.

The previous Glu to Gln mutations of CueO and BO [6,7] resulted in drastic decreases in enzymatic activities and the detection of reaction intermediates. A recent crystallographic study on a double mutant Cys500Ser/Glu506Gln showed that the hydrogen bond network leading from the exterior of the protein molecule to TNC formed with the amide group in the side chain of the Gln residue [14]. However, the carboxyl group of the side chain of Glu is distinct from the amide group in the side chain of Gln which does not cycle between the protonated and deprotonated forms [7]. Therefore, the activity is very low in the Glu506Gln mutant. This very low activity might indicate that, to a minor extent, protons can diffuse from the exterior of the protein molecule to TNC. This presumably occurs through the channel formed at the interface between domain 1 and domain 3 [14]. Analogous mutations have been performed for bovine, *Pseudomonas denitrificans*, and *Rhodobacter sphaeroides* cytochrome oxidases in studies of scalar and vectorial proton transport mechanisms coupled with electron transfer [17]. Blocking of the transport of protons to the d-propionate group in the side chain of heme  $a_3$  and the binuclear heme  $a_3$ -Cu $_b$  via water molecule(s) due to the Glu to Gln mutations is a good analogy with respect to our current experiments on proton transport by CueO, although the results of the experiments on the Ala mutation of cytochrome oxidases are not unequivocal with respect to the possible contribution of a compensatory proton transfer pathway. The contrast between the results of the Ala mutations for CueO and cytochrome oxidase might be due to the difference in the length of the proton transport pathways. The proton transport pathway of MCOs which leads from the exterior of the protein molecule to TNC is not as long as that of the membrane bound-cytochrome oxidase which penetrates from the cytoplasmic side to the periplasmic side via the metal centers. MCOs might not incur a loss of function even if an acidic amino acid is absent from the hydrogen bond network.

It was found that the Asp507Asn mutation did not interfere with the transport of protons. Instead, the enzymatic activity was observed to increase by 63% to 86% from that of native CueO in a manner which depends upon the buffer solutions used. Since it is unclear how the Asn mutation favors the function of the adjacent branch of the hydrogen bond network, further studies are required to assess the role of Asp507, including whether the Asn mutation actually abolishes proton transfer through the branch or an unexpected local structural change occurs which favors proton transfer. Asp507 is located on the surface of the

protein. It is possible that the Asp507Ala mutation produced a wider space which would increase accessibility of solvent water molecules to Glu506.

In summary, the enzymatic activity of the Gln506 mutants of CueO decreases in the following order, Glu > Asp, Ala >> Gln > Ile~0. While it is possible to replace the Glu residue located in the proton relay pathway with Asp, its effectiveness differs among different MCOs. Ala allows water molecule(s) to occupy the open space and to form an alternative proton relay pathway. In contrast, Ile shuts down the hydrogen bond network with its bulky side chain, leading to complete loss of enzymatic activity. In the Gln mutant, an alternative hydrogen bond network was adopted but the ability to relay protons was essentially lost. The results of mutations at Glu506 and Asp507 indicated that Glu506, which is located at the root of the branched hydrogen bond network leading from the exterior of the CueO molecule to TNC plays a key role in relaying protons in the four-electron reduction O<sub>2</sub>. The main function of the network could be modified by mutations in the hydrogen bond network.

### **Abbreviations**

Copper efflux oxidase; CueO, Multicopper oxidase; MCO, *Escherichia coli*; *E. coli*, Type I; T1, Type II; T2, Type III; T3, Trinuclear copper center; TNC, bilirubin oxidase; BO, Circular dichroism; CD, Electron paramagnetic resonance; EPR, bicinchoninic acid; BCA, 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ABTS, *N,N,N',N'*-tetramethylethylenediamine; EDTA

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## Figure Legends

**Fig. 1.** The Active site of CueO showing the location of Glu506 and Asp507 in the hydrogen bond network with connections to the OH<sup>-</sup> bridging between the T3 copper centers and the exterior of the protein molecule. Drawn with DS ViewerPro for PDB code 1KV7.

**Fig. 2.** Absorption (A), CD (B), and EPR (C) spectra of CueO (black), Glu506Gln (flesh colored), Glu506Ala (red), Glu506Asp (purple), Glu506Ile (green), Asp507Ala (pale blue), and Asp507Asn (blue). Measurement conditions: approximately 0.1 mM protein in 0.1 M potassium phosphate buffer, pH 6 at room temperature for the absorption and CD spectra and at 77 K for the EPR spectra with 9.20 GHz microwave frequency, 5 mW of microwave power, 100 kHz and 1 mT of modulation, 4 min of sweep time, and 400 of amplitude. Asterisks indicate the signals presumably derived from uncoupled T3 coppers.

**Fig. 3.** Absorption change at 420 nm for the oxidation of ABTS by Glu506Gln. Conditions: ABTS 6 mM, Glu506Gln 0.318 nM, cell path 1 cm, temp. 25 °C. Citrate buffer 100 mM, pH 5.5.

**Table 1**

Characterizations of Glu506 and Asp507 Mutants of CueO.

Protein	Cu-content	EPR-detectable Cu <sup>2+</sup>	Specific activity in buffer solutions		
	(Cu/Protein)	(Cu <sup>2+</sup> /Protein)	Acetate <sup>a)</sup> (U/mg)	Citrate <sup>b)</sup> (U/mg)	Phosphate <sup>c)</sup> (U/mg)
rCueO	4.1	2.0	0.42	0.14	0.052
Glu506Asp	4.1	2.0	0.31	0.15	0.059
Glu506Ala	3.8	ND <sup>d)</sup>	0.33	0.088	0.040
Glu506Ile	4.3	1.8	0.013	~0	~0
Glu506Gln	3.8	ND	0.0056	0.0014	0.00082
Asp507Ala	4.0	1.9	0.48	0.18	0.065
Asp507Asn	4.0	1.9	0.73	0.26	0.084

a) 100 mM, pH 5.5, 25°C

b) 100 mM, pH 5.5, 25°C

c) 50 mM, pH 6.0, 25°C

d) not determined

Fig. 1

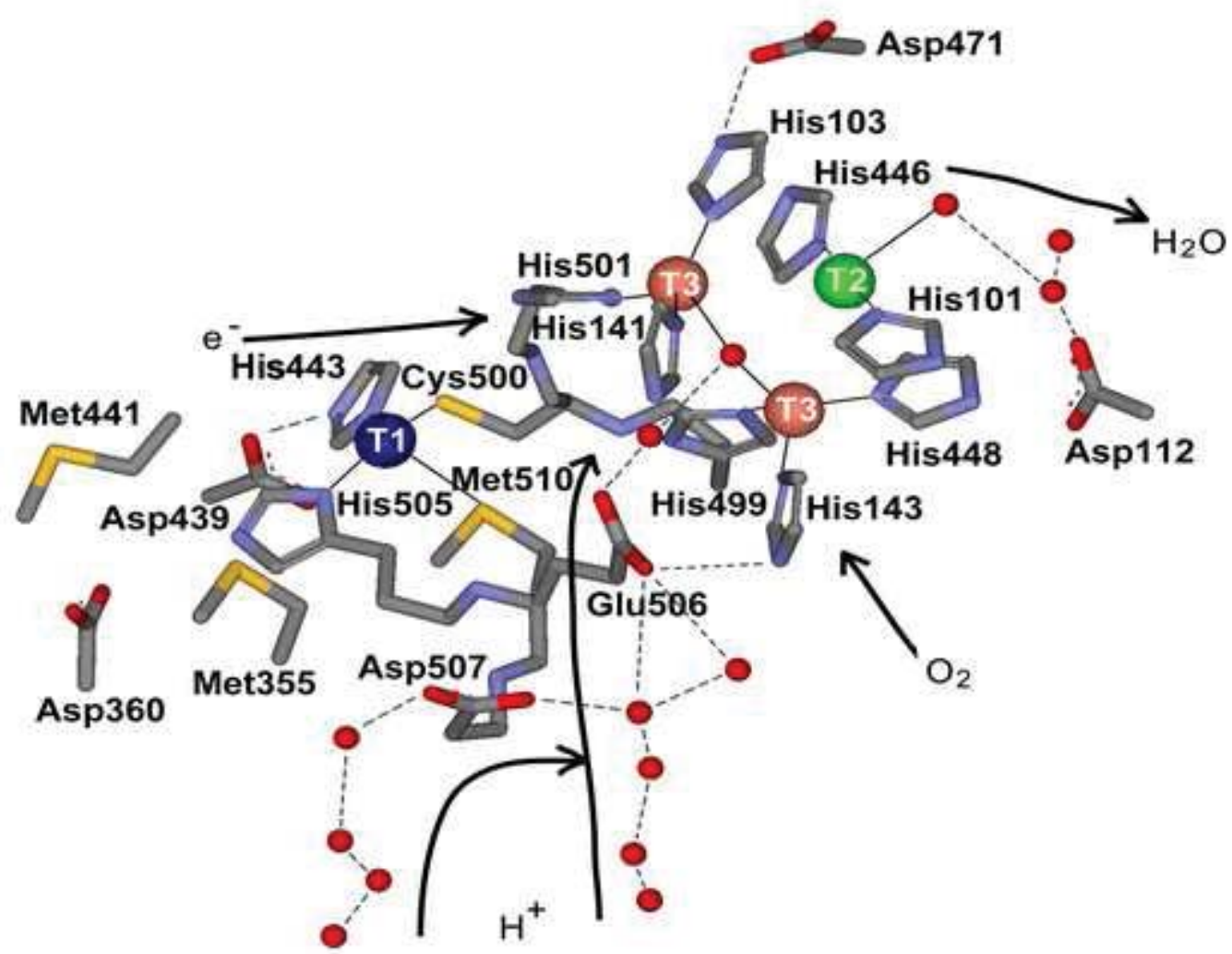


Fig. 2

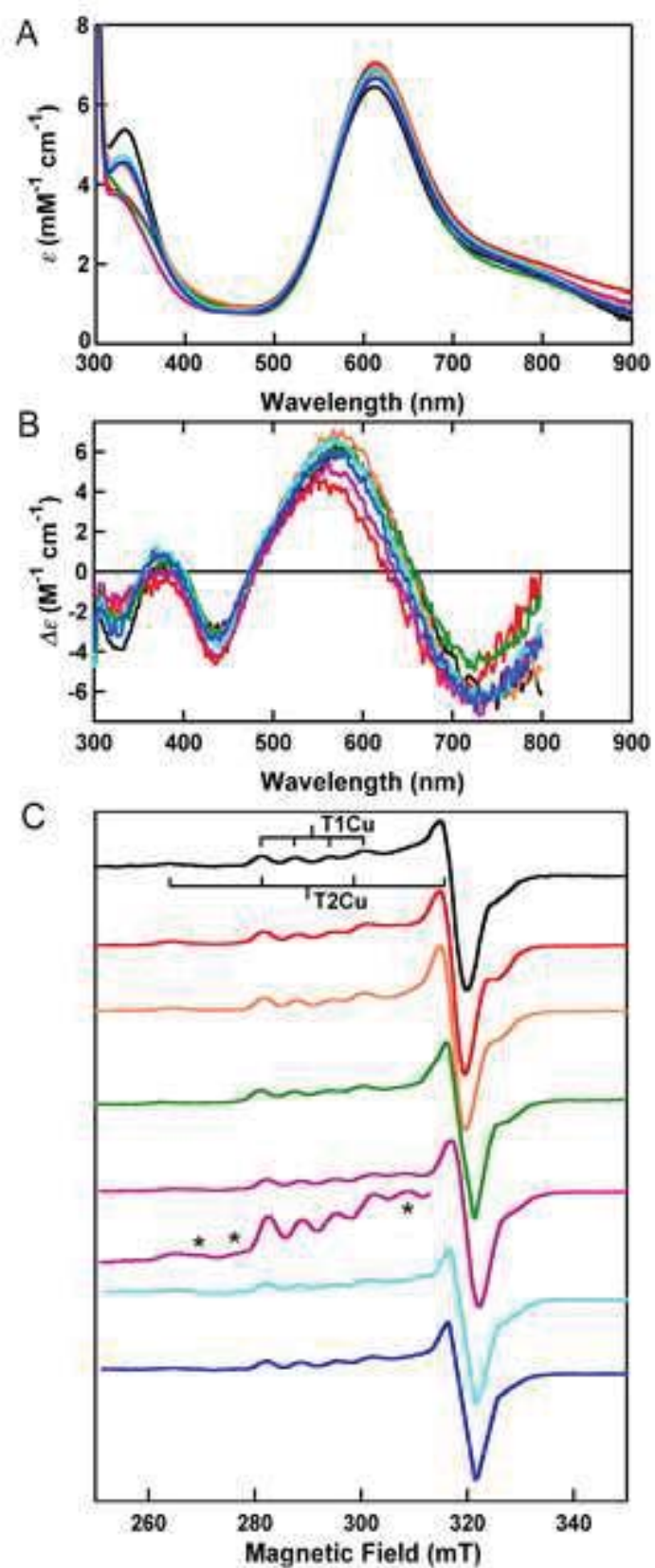


Fig. 3

