Study on dioxygen reduction by mutational modifications of the hydrogen bond network leading from bulk water to the trinuclear copper center in bilirubin oxidase

Elsevier Editorial System(tm) for Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: Study on dioxygen reduction by mutational modifications of the hydrogen bond network leading from bulk water to the trinuclear copper center in bilirubin oxidase

Article Type: Regular Article

Keywords: bilirubin oxidase; multicopper oxidase; hydrogen bond network; dioxygen reduction.

Corresponding Author: Dr. Takeshi Sakurai, Dr.

Corresponding Author's Institution: Kanazawa University

First Author: Hirotoshi Morishita

Order of Authors: Hirotoshi Morishita; Daisuke Kurita; Kunishige Kataoka; Takeshi Sakurai, Dr.

Dear Editor,

BBRC,

Bilirubin oxidase is a multicopper oxidase containing a type I Cu center to specifically oxidize bilirubin and related substrates and a trinuclear Cu center to perform four-electron reduction of dioxygen as the final electron acceptor. Special attentions have been paid on multicopper oxidases, since this class of enzymes has potential uses as electrocatalyst for biofuel cells and biosensor and catalyst to form a variety of dyes. In addition, bilirubin oxidase has been utilized for the clinical test of liver. In spite of wide attention and potential wide use of the enzyme, structural and functional studies on bilirubin oxidase have been limited.

The present study is on the proton transfer mechanism for dioxygen reduction by bilirubin oxidase. We performed mutation at Glu463 located in the hydrogen bond network leading from bulk water to the active site deeply buried inside protein molecule. By singly performing mutation at this amino acid and doubly performing mutation at a Cys ligand to type I Cu center in addition to the mutation at Glu463, we could trap two reaction intermediates, and characterized them in comparisons with those trapped in other multicopper oxidases.

We believe this study attracts wide attention from researches studying on biochemistry, bioinorganic chemistry, electrochemistry, biophysics and related fields including bioinspired chemistry, and contributes in the understanding of the four-electron reduction by multicopper oxidases and more widely, transport of protons in protein molecules. According to reasons as above we would like to publish our results in the world wide BBRC.

sincerely yours,

Takeshi Sakurai

***Highlights (for review)**

- Proton transport pathway in bilirubin oxidase was mutated
- Two intermediates in the dioxygen reduction steps were trapped and characterized.
- A specific glutamate for dioxygen reduction by multicopper oxidases was identified.

Study on dioxygen reduction by mutational modifications of the hydrogen bond network leading from bulk water to the trinuclear copper center in bilirubin oxidase

Hirotoshi Morishita, Daisuke Kurita, Kunishige Kataoka, Takeshi Sakurai*

Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

ABSTRACT

The hydrogen bond network leading from bulk water to the trinuclear copper center in bilirubin oxidase is constructed with Glu463 and water molecules to transport protons for the four-electron reduction of dioxygen. Substitutions of Glu463 with Gln or Ala were attributed to virtually complete loss or significant reduction in enzymatic activities due to an inhibition of the proton transfer steps to dioxygen. The single turnover reaction of the Glu463Gln mutant afforded the highly magnetically interacted intermediate II (native intermediate) with a broad g =1.96 electron paramagnetic resonance signal detectable at cryogenic temperatures. Reactions of the double mutants, Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala afforded the intermediate I (peroxide intermediate) because the type I copper center to donate the fourth electron to dioxygen was vacant in addition to the interference of proton transport due to the mutation at Glu463. The intermediate I gave no electron paramagnetic resonance signal, but the type II copper signal became detectable with the decay of the intermediate I. Structural and functional similarities between multicopper oxidases are discussed based on the present mutation at Glu463 in bilirubin oxidase.

Keywords: Bilirubin oxidase, Multicopper oxidase, Hydrogen bond network, Dioxygen reduction

Abbreviations: MCO, multicopper oxidase; T1Cu, type I copper; TNC, trinuclear copper center; T2Cu, type II copper; T3Cu, type III copper; BOD, bilirubin oxidase; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); *p*-phenylenediamine, *p*-PD; CD, circular dichroism; EPR, electron paramagnetic resonance; WT, wild type.

**Corresponding author*. Address. Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan (T. Sakurai), Fax: +81 76 264 5742.

E-mail address: tsakurai@se.kanazawa-u.ac.jp, ts0513@kenroku.kanazawa-u.ac.jp (T. Sakurai).

1. Introduction

Multicopper oxidases (MCOs) contain 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 (T3Cus) to oxidize substrate and reduce dioxygen as the final electron acceptor, respectively [1-3]. T1Cu is responsible for the absorption bands at ca. 600 nm and 700-900 nm originated in the charge transfer from the Cys ligand to Cu^{2+} and d-d transitions, respectively. T1Cu gives a peculiar electron paramagnetic resonance (EPR) signal with a small superhyperfine splitting. TNC affords a band at ca. 330 nm derived from the antiferromagnetically coupled T3Cus with an OH^- bridge and an EPR signal derived from T2Cu with a normal magnitude of superhyperfine splitting.

MCOs such as CueO (copper efflux oxidase) from *Escherichia coli* [4-6] and bilirubin oxidase (BOD) from *Myrothecium verrucaria* [7] have been extensively studied at the aims of revealing their structure-function relationships and applying them to cathodic enzymes for biofuel cell and clinical test [8-10]. The T1Cu site in CueO, BOD and *Saccharomyces cerevisiae* Fet3p became vacant by receiving mutations to its Cys ligand for Ser, and the intermediate I (peroxide intermediate) could be trapped because of the absence of the fourth-electron donor to dioxygen [11-13]. Other strategies to trap the intermediate I were to perform the reactions of the *Rhus vernicifera* laccase in a mixed valence state, $T1Cu_{ox}T2Cu_{red}T3Cus_{red}$ [14] and the Hg-substituted derivative at the T1Cu site with dioxygen [15].

On the other hand, studies on the intermediate II (native intermediate), a fully oxidized form with a multiple magnetic interaction, have initially been performed by trapping the reaction intermediate of the plant laccase [16,17] or by performing the mutation at the Met ligand for T1Cu in BOD to Gln [18]. Later, a hydrogen bond network leading from bulk water to TNC, which is constructed with water molecules and a conserved Glu residue, has been found in several MCOs (Fig. 1) [11,19-21]. Cycling of protonation and deprotonation in the side chain of the conserved Glu residue coupled with the change in redox state of BOD and CueO has been observed by FT-IR studies [22]. Mutations on the conserved Glu residue have been performed for CueO and Fe3p, resulting in the successful trapping and characterization of the intermediate II [11,19,21]. Mutations of this Glu residue with Gln led to practically complete loss in enzymatic activities, but its substitution with Ala exhibited considerably high enzymatic activities due to a formation of compensatory hydrogen bond network with only water molecules [19,23,24], while it was found that Asp located in one of other two channels leading to TNC also concerns profoundly in the formation of water molecules [25,26].

In the present study we performed the mutations at Glu463 in BOD with Gln and Ala and also at the Cys457 with Ser, and characterized the single and double mutants. Further, we studied the reactions of them in comparison with those of CueO and Fet3p to reveal the role of the Glu residue highly conserved in the hydrogen bond network leading from bulk water to TNC.

2. Materials and Methods

2.1. Preparation of mutants

The gene fragments to mutate Cys457 and Glu463 in *M. verrucaria* BOD were synthesized by PCR using the oligonucleotide primers shown below and pBSBO harboring the complete BOD gene cloned into pBluescript II (Stratagene) as a template (underlines indicate the sites of mutation).

Cys457Ser(+): 5'-ATCAACGCCGGTGCCGGTTGGACGCAC-3'
Cys457Ser(-): 5'-GTGCGTCCAACCGGCACCGGCGTTGAT-3'
Glu463Ala(+): 5'-CACAATTTGATTCACGCCGATCACGATATGATG-3'
Glu463Ala(-): 5'-CATCATATCGTGATCGGCGTGAATCAAATTGTG-3'
Glu463Gln(+):5'-CACAATTTGATTCACCAGGATCACGATATGATG-3'
Glu463Gln(-): 5'-CATCATATCGTGATCCTGGTGAATCAAATTGTG-3'

BOD mutants were purified as reported from transformants of *P. pastoris* GS115 with multicopy inserts which were screened for geneticin (G418) resistance [18].

Protein concentrations were determined using the Pierce BCA (bicinchoninic acid) Protein Assay Kit from absorption intensity at 280 nm, $\varepsilon = 115\ mM^{-1}cm^{-1}$. Deviations in the two independent methods were less than 5%.

2.2. Measurements

Enzymatic activities of the mutants to 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *p*-phenylenediamine (*p*-PD), bilirubin and ditaurobilirubin were determined from changes in absorptions of the oxidized products at 436 nm ($\varepsilon = 29.3\ mM^{-1}\ cm^{-1}$) and 477 nm ($\varepsilon = 14.7\ mM^{-1}\ cm^{-1}$) and of substrates at 440 nm ($\varepsilon = 68.5\ mM^{-1}\ cm^{-1}$) and 440 nm ($\varepsilon = 49.5\ mM^{-1}\ cm^{-1}$), respectively [27]. One unit of activity was defined as the amount of enzyme to oxidize one mmol of substrate per min. Buffer solutions used were 50 mM phosphate buffer (pH 6.5) for ABTS and *p*-PD, 50 mM Tris-H_2SO_4 (pH 8.0) for bilirubin, and 50 mM acetate buffer (pH 5.5) for ditautobilirubin.

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 using an Oxford cryostat at 6–77K. Averaged number of EPR detectable Cu^{2+} ions in a protein molecule was determined using Cu^{2+}-ethylenediaminetetraacetic acid (EDTA) as standard with MnO as external standard to calibrate signal intensities.

2.3. Intermediate formations and characterizations

Reactions to form intermediates I and II and spectral measurements have been performed as reported previously [11,12].

3. Results and Discussion

3.1. Characterizations of mutants

Single mutants, Glu463Gln and Glu463Ala, and double mutants, Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala of BOD gave a single band with 66kDa molecular mass on SDS-PAGE (not shown). Averaged Cu contents in the single and double mutants were 4 and 3, respectively, within the experimental error of 10% (Table 1). The single mutants, Glu463Gln and Glu463Ala exhibited very low enzymatic activities, although the corresponding Ala mutants of CueO and Fet3p showed considerably high enzymatic activities [19,23]. On the other hand, the double mutants did not show enzymatic activities due to the mutation at the T1Cu site [12].

In the absorption spectra of the single mutants the strong Cys to T1Cu charge transfer band was observed at 602 nm, although its intensity (ε = 4,700 $M^{-1}cm^{-1}$ for Glu463Gln and 4,000 $M^{-1}cm^{-1}$ for Glu463Ala) was decreased from that of the wild type (WT) enzyme (ε = 5,200 $M^{-1}cm^{-1}$) (Fig. 2A). Glu463Ala as isolated had lower absorption intensity (ε = 3,500 $M^{-1}cm^{-1}$), but the value was increased to 4,000 $M^{-1}cm^{-1}$ by the reaction with a strong oxidizing agent potassium hexachloroiridate(IV) (the spectrum after the reaction is shown in Fig. 2A), indicating that a fraction of T1Cus had been reduced. On the other hand, the double mutants, Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala showed pale blue color due to the absence of T1Cu. In the absorption spectra, only the d-d band derived from the copper centers in TNC was observable at ca. 700 nm in the visible region (Fig. 2A).

The absorption spectral features of Glu463Gln and Glu463Ala were also similar to that of the wild type (WT) BOD in the near-UV region (Fig. 2A). In contrast, Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala gave the weakened bands at 330 nm and 400 nm (Fig. 2A). The 330 nm band is characteristic of T3Cus bridged by an OH^-. However, the 400 nm band has not been observed in the resting MCOs and mutants. This 400 nm band disappeared with the reduction of the double mutants and did not appear after a single turnover, indicating that it was not derived from a certain impurity

with a heme group but from an indirect modification induced on TNC. The replacement of the carboxylate group in the side chain of the Glu residue to water molecules or the amide group in the side chain of Gln indirectly affects the hydrogen bond network, at which terminal the OH^- is located between T3Cus via a water molecule (Differing from the Ala mutant of CueO this water molecule is not seen in the crystal structure of BOD (Fig. 1) presumably because TNC is partly reduced [6]). Thus, the double mutations at Cys457 and Glu463 might have induced a change in the structure of TNC when isolated: distances between T3Cu-OH^-, the angle between T3Cu-OH^--T3Cu, or the donating ability of the bridged OH^- to T3Cus, leading to the shift in the band from 330 nm to 400 nm. This modification in the spectral properties of TNC has not been brought about from a change in the coordination mode of His ligands and OH^- (*vide infra* for the electron paramagnetic resonance spectra (Fig. 2C)).

CD spectra of the single mutants were very similar to that of the WT BOD over near-UV to visible regions (Fig. 2B). Therefore, reductions in absorption intensity of the 602 nm band for Glu463Gln and Glu463Ala are derived from an indirect effect on the S^- to Cu^{2+} charge transfer band induced by the mutations at Glu463. This is not unexpected since Glu463 is directly bound to His462 coordinated to T1Cu through the peptide bond (Fig. 1), and this may realize a close coupling of proton transfer and electron transfer, with which these amino acids are deeply concerned, respectively. The double mutants exhibited, on the other hand, much weakened absorption features in the near-UV to visible regions due to the absence of T1Cu. The features at > 450 nm might be coming from the d-d bands of the copper centers in TNC and the features at <450 nm (330 (-) and 400 (+) nm bands) from the charge transfer from His and/or OH^- to Cu^{2+}. Since both double mutants exhibit analogous CD spectral features, it appears that the structures of TNC are similar in them.

In the EPR spectra of the single mutants both of the T1Cu and T2Cu signals were observed, although the signal intensity of T1Cu was slightly decreased, suggesting a portion of T1Cu were reduced. On the other hand, the T1Cu signal disappeared in the double mutants, giving the T2Cu signal with a 5-line superhyperfine splitting for Cys457Ser/Glu463Ala. This indicats the binding of 2N(His) ligands to T2Cu (Fig. 2C, red line), while the superhyperfine splittings have not been observed in the WT BOD. On the other hand, the 7- and 5-lines of superhyperfine structures have been observed in the double mutants for a ligand to each T3Cu, His456Lys/His458Lys and His456Asp/His458Asp, which did not show the 330 nm band, respectively [28]. The total number of EPR detectable Cu^{2+} was 1.1 per a protein molecule, and accordingly, T2Cu ions were fully oxidized and magnetically isolated from T3Cus. On the other hand, an extra signal derived presumably from T3Cu was also observed for Cys457Ser/Glu463Gln (Fig. 2C, blue line) (averaged number of EPR detectable Cu^{2+} was 1.8 per a protein molecules, differing from 1.1 for Cys457Ser/Glu463Ala).

3.2. Intermediate I

Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala reduced with a slight excess of dithionite under Ar were reacted with dioxygen. The intermediate I formed from the double mutants (Figs. 3A and 3B) gave the absorption maxima at 340, 470 and 680 nm similarly to that formed from CueO, Fet3p, laccase [11,14-16] and the single mutant of BOD, Cys457Ser [12]. Absorption intensities of these bands, however, were lower than those derived from other MCOs except Cys457Ser BOD. The intermediate I was exponentially decayed as shown in the inlets for the absorption change at 340 nm ($t_{1/2}$ = 12 min for Cys500Ser/Glu463Gln and 18 min for Cys500Ser/Glu463Ala). The identical life-time was obtained from decays of the band at 470 nm.

CD spectra of the intermediate I were quickly measured over 300 to 500 nm (Fig. 3C). A negatively signed band and a broad positively signed band (presumably comprised of two positively signed bands) were obtained at 330 nm and 400-500 nm, respectively. The intermediate I derived from the mutants of CueO, Cys500Ser [11] and Cys500Ser/Glu506Gln and Cys500Ser/Glu506Ala (unpublished spectra) unequivocally exhibited the negatively signed band at 330 nm, positively singed band at 370 nm and a broad positively signed band at around 370 nm. Because of shorter life-times of the present intermediate I, CD spectra ware not well resolved, although it was certain that the intermediate I from all MCOs exhibits analogous CD spectral features.

EPR spectra of the intermediate I measured at 77K soon after the reactions (Fig. 3D) gave the weakened T2Cu signal (~ 0.3Cu^{2+} for Cys457Ser/Glu463Gln and ~ 0.1Cu^{2+} for Cys457Ser/Glu463Ala), but the T2Cu signal intensities increased with the decays of intermediate, indicating that the intermediate I is EPR-silent. It is not known, however, whether the decay process of the intermediate I is coupled with the electron transfer from T2Cu to peroxide or T2Cu is autooxidized after decomposition of the intermediate I. Two electrons are required to convert peroxide to two water molecules. Nevertheless, the double mutants reached the resting state after the present one-electron deficient process lacking in T1Cu, and the second cyclings of reaction were possible since the mutant molecules did not receive fatal damages.

3.3. Intermediate II

Glu463Gln was reduced with a slight excess of dithionite under Ar, and after the excess dithionite was decomposed Glu463Gln was reacted with dioxygen. Soon after the reaction with dioxygen the colorless Glu463Gln turned intense blue, indicating that T1Cu was oxidized. The EPR spectrum of the promptly frozen sample gave the full T1Cu signal, but the T2Cu signal was not detected (Fig. 4). Instead, the g = 1.96 signal, not easily saturated with high microwave powers (the inset in Fig. 4), was detected at

cryogenic temperatures (<20K) at around 340 mT, the magnetic field both T1Cu and T2 Cu never give signals, as has been reported in the reactions of the analogous mutants of CueO [11] and Fet3p [13] and in the rapidly frozen plant laccase [16,17]. This indicates that the novel EPR signal is derived from a highly magnetically interacted species. Intensity of the g = 1.96 signal was not very strong compared with those observed in other MCOs, CueO, Fet3p and plant laccase, but was detected in BOD for the first time. After dissolving the sample and standing it for 20 min on ice the g = 1.96 signal was not detected anymore, but the T2Cu signal was recovered. The absorption spectrum of the intermediate was not measured due to rapid disappearance of it at room temperature, while we could measure the absorption spectrum of the intermediate II derived from the T1Cu mutant, Met467Gln [12].

As for the origin of the g = 1.96 EPR signal, the O-centered TNC structure has been proposed [16,17], and the presence of the central O atom has been visualized for CueO as the X-ray crystal structure and also for some resting fungal laccases as a minor species [21,29-31]. On the other hand, the magnetically coupled TNC with an adjacent Tyr radical has been proposed for SLAC comprised of two domains [32]. However, a proximal Tyr residue is not located in the three domain MCDs, BOD, CueO, and Fet3p within a distance to magnetically interact with TNC. Therefore, more profound structural and kinetic studies are required to reveal the origin of the g = 1.96 signal transiently observed for the intermediate II.

3. 4 Summary

The EPR-silent intermediate I was trapped from the double mutants of BOD at Cys 457, a type I Cu ligand, and Glu463 located in the hydrogen bond network to transport protons to dioxygen. The life-time of the intermediate I was similar in both Gln and Ala mutants at Glu463, but was shorter compared to that formed from CueO and Fet3p. The broad g < 2 EPR signal derived from the intermediate II with a multiple magnetic interaction was detected at cryogenic temperatures by freezing Glu463Gln reacted with dioxygen. As a result of mutations at Cys457, a ligand to T1Cu and at Glu463, the highly conserved amino acid in the proton transfer pathway, BOD was found to follow the dioxygen reduction mechanism similar to those of CueO, Fet3p and plant laccase with three domain structures. Compared to other MCOs, however, the importance to locate the Glu residue at the 463 position is more profound for BOD in the reduction of dioxygen as reflected in significant reductions in the enzymatic activities of the mutants and considerably rapid decays of the reaction intermediates.

Acknowledgements

We would like to thank the Ministry of Education, Culture, Sports, Science and Technology of Japan

for financial support (fund no. 26288076).

References

[1] T. Sakurai, K. Kataoka, Multicopper Proteins in; K.D. Karlin, S. Itoh (Eds), Copper-Oxygen Chemistry, 2011, John Wiley & Sons, Hoboken, New Jersey, 2011.

[2] T. Sakurai. K. Kataoka, Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase, Chem. Rec. 7 (2007) 220-229.

[3] T. Sakurai. K. Kataoka, Structure and function of type I copper in multicopper oxidases Cell. Mol. Life Sci. 64 (2007) 2642-2656.

[4] F.W. Outten, D.L. Huffman, T.V. O'Halloran, The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*, J. Biol. Chem. 276 (2001) 30670-30677.

[5] C. Rensing, G. Grass, *Escherichia coli* mechanisms of copper homeostasis in a changing environment, FEMS Microbiol. Rev. 27 (2003) 197-213.

[6] H. Komori, T. Kajikawa, K. Kataoka, Y. Higuchi, T. Sakurai, Crystal structure of the CueO mutants at Glu506, the key amino acid located in the proton transfer pathway for dioxygen reduction, Acta Cryst. D70, (2014) 772-779.

[7] T. Sakurai and K. Kataoka, Bilirubin oxidase, bilirubin chemistry and disorders, eds., J. F. Jakub and F. Sedlacek, 2012, pp. 211-233, Nova Science, New York.

[8] Y. Miura, S. Tsujimura, S. Kurose, Y. Kamitaka, K. Kataoka, T. Sakurai and K. Kano, Direct electrochemistry of CueO and its mutants at residues to and near type I Cu for oxygen-reducing biochathode, Fuel Cells, 9 (2009) 70-78.

[9] Y. Kamitaka, S. Tsujimura, K, Kataoka, T. Sakurai, T. Ikeda, K. Kano, Effects of ligand mutation of the type I copper site in bilirubin oxidase on direct electron transfer-type bioelectrocatalytic reduction of dioxygen, J. Electroanal. Chem., 601 (2007) 119-124.

[10] N. Mano, Features and applications of bilirubin oxidase, Appl. Microbiol. Biotechnol. 96 (2012) 301-307.

[11] K. Kataoka, R. Sugiyama, S. Hirota, M. Inoue, K. Urata, Y. Minagawa, D. Seo, T. Sakurai, Four-electron reduction of dioxygen by a multicopper oxidase, CueO, and roles of Asp112 and Glu506 located adjacent to the trinuclear copper center, J. Biol. Chem. 284 (2009) 14405-14413.

[12] K. Kataoka, R. Kitagawa, M. Inoue, D. Naruse, T. Sakurai, H. Huang, Point mutations at the type I copper ligands, Cys457 and Met467, and the putative proton donor, Asp105, in *Myrothecium verrucaria* bilirubin oxidase and reactions with dioxygen, Biochemistry 44 (2005) 7004-7012.

[13] A.E. Palmer, L. Quintanar, S. Severance, T.-P. Wang, D.J. Kosman, E.I. Solomon, Spectroscopic characterization and O_2 reactivity of the trinuclear Cu cluster of mutants of the multicopper

oxidase Fet3p, Biochemistry 41 (2002) 6438-6448.

[14] G. Zoppellaro, T. Sakurai, H.-W. Huang, A novel mixed valance form of *Rhus vernicifera* laccase and its reaction with dioxygen to give a peroxide intermediate bound to the trinuclear center, J. Biochem. 129 (2001) 949-953.

[15] W. Shin, U.M. Sundaram, J.L. Cole, H.H. Zhang, B. Hedman, K.O. Hodgson, E. I. Solomon, Chemical and spectroscopic definition of the peroxide-level intermediate in the multicopper oxidases: Relevance to the catalytic mechanism of dioxygen to water, J. Am. Chem. Soc. 118 (1996) 3202-3215.

[16] H.-W. Huang, G. Zoppellaro, T. Sakurai, Spectroscopic and kinetic studies on the oxygen-centered radical formed during the four-electron reduction process of dioxygen by *Rhus vernicifera* laccase, J. Biol, Chem. 274 (1999) 32718-32724.

[17] S.-K. Lee, S.D. George, W.E. Antholine, B. Hedman, K.O. Hodgson, E.I. Solomon, Nature of the intermediate formed in the reduction of O_2 to H_2O at the trinuclear copper cluster active site in native laccase, J. Am. Chem. Soc. 124 (2002) 6180-6193.

[18] K. Kataoka, R. Kitagawa, M. Inoue, D. Naruse, T. Sakurai, H. Huang, Point mutations at the type I copper ligands, Cys457 and Met467, and the putative proton donor, Asp105, in *Myrothecium verrucaria* bilirubin oxidase and reactions with dioxygen, Biochemistry 44 (2005) 7004-7012.

[19] A.J. Augustine, L.Quintanar, C.S. Stoj, D.J. Kosman, E. I. Solomon, Spectroscopic and kinetic studies of perturbed trinuclear copper clusters: the role of protons in the reductive cleavage of the O-O bond in the multicopper oxidase Fet3p, J. Am. Chem. Soc. 129 (2007) 13118-13126.

[20] Z. Chen, P. Durao, C.S. Silva, M.M. Pereira, S. Todorovic, P. Hildebrandt, I. Bento, P. F. Lindley, L. O. Martins, The role of Glu498 in the dioxygen reactivity of CotA-laccase from *Bacillus subtilis,* Dalton Trans 39 (2010) 2875-2882.

[21] H. Komori, R. Sugiyama, K. Kataoka, Y. Higuchi, T. Sakurai, 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 does conditions, *Angew. Chem. Int. Ed*. 51 (2012) 1861-1864.

[22] M. Iwaki, K. Kataoka, T. Kajino, R. Sugiyama, H. Morishita, T. Sakurai, ATR-FTIR study of the protonation states of the Glu residue in the multicopper oxidases, CueO and bilirubin oxidase, FEBS Lett. 584 (2010) 4027-4031.

[23] T. Kajikawa, K. Kataoka, T. Sakurai, Modification 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, Biochem. Biophys. Res. Commun. 422 (2012) 152-156.

[24] H. Komori, T. Kajikawa, K. Kataoka, Y. Higuchi, T. Sakurai, Crystal Structure of the CueO

mutants at Glu506, the key amino acid located in the proton transfer pathway for dioxygen reduction, Biochem. Biophys. Res. Commun. 438 (2013) 686-690.

[25] Y. Ueki, M. Inoue, S. Kurose, K. Kataoka, T. Sakurai, Mutations at Asp112 adjacent to the trinuclear Cu center in CueO as the proton donor in the four-electron reduction of dioxygen, FEBS Lett. 580 (2006) 4069-4072.

[26] V. Brissos, Z. Chen, L. Matins, The kinetic role of carboxylate residues in the proximity of the trinuclear center in the O_2 reactivity of CotA-laccase, Dalton Trans. 41 (2012) 6247-6255.

[27] K. Kataoka, H. Komori, Y. Ueki, Y. Konno, Y. Kamitaka, S. Kurose, S. Tsujimura, Y. Higuchi, K. Kano, D. Seo, T. Sakurai, Structure and function of the engineered multicopper oxidase CueO from *Escherichia coli* – Deletion of the methionine-rich helical region covering the substrate-binding site, J. Mol. Biol. 373 (2007) 141-152.

[28] A. Shimizu, T. Samejima, S. Hirota, S. Yamaguchi, N. Sakurai and T. Sakurai, Type III mutants of *Myrothecium verrucaria* bilirubin oxidase, J. Biochem., 133 (2003) 767-772.

[29] K.M, Polyakov, T.V. Fedorova, E.V. Stepanova, E.A. Cherkashin, S.A. Kurzeev, B.V. Strokopytov, V.S. Lamzin, O.V. Koroleva, Acta Cryst. D65 (2009) 611-617.

[30] H. Komori, K. Miyazaki, Y. Higuchi, X-ray structure of a two-domain type laccase: a missing link in the evolution of multicoppper proteins, FEBS Lett. 583 (2009) 1189-1195.

[31] H. Komori, R. Sugiyama, K. Kataoka, K. Miyazaki, Y. Higuchi, T. Sakurai, New insights into the catalytic active-site structure of multicopper oxidases, Acta Cryst. D70 (2014) 772-779.

[32] A. Gupta, I. Nederlof, S. Sottini, A.W.J. Tepper, E.J.J. Groenen, E.A.J. Thomassen, G.W. Canters, Involvement of Ty2108 in the enzyme mechanism of the small laccase from *Streptomyces coelicolor*, J. Am. Chem. Soc. (2012) 18213-18216.

Figure Captions

Fig. 1. The active site structure of bilirubin oxidase. Small red spheres are H_2O or OH^- constructing the hydrogen bond network leading from bulk water to TNC. An OH^- between T3Cu's and a water molecule to connect the bridged OH^- and Glu463 are not seen presumably because TNC are partly reduced in the resting form as isolated or received the action of hydrated electrons due to strong X-ray radiation. DS viewerPro5.0 was used to model the PDB data 2Xll.

Fig. 2. Absorption (A), CD (B), and EPR (C) spectra of WT BOD (black), Glu463Gln (pale blue), Gln463Ala (orange), Cys457Ser/Glu463Gln (blue), and Cys457Ser/Glu463Ala (red). Asterisk indicates an impurity or a minor signal. Absorption and CD spectra were measured at room temperature, and EPR spectra at 77 K for ca. 10-20 μM protein in 0.1 M phosphate buffer, pH 6.0. Measurement conditions for EPR spectra: microwave, 9.2 GHz, 5mW; modulation, 100 kHz, 10 mT; amplitude, 200-400, time constant, 0.03 s.

Fig. 3. Reactions of the reduced Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala with O_2. (A): absorption spectrum of Cys457Ser/Glu463Gln soon after the reaction (blue) and decay at 340 nm (inset), (B): absorption spectrum of Cys457Ser/Glu463Ala soon after the reaction (red) and decay at 340 nm (inset), (C): CD spectra of Cys457Ser/Glu463Gln (blue), Cys457Ser/Glu463Ala (red) soon after reactions together with Cys457Ser/Glu463Gln and Cys457Ser/Glu463Ala before reduction the same spectra with those shown in Fig. 2B (dotted lines), and (D): EPR spectra of Cys500Ser/Glu463Gln (blue) and Cys500Ser/Glu463Ala (red) before (dotted lines) and after the reaction with O_2 (full lines) at 77K. Samples and measurement conditions are the same with the caption to Fig. 2 except the mutant concentrations, ca. 200 μM).

Fig. 4. EPR spectra of Glu463Gln before reduction, after reduction and reaction with O_2, and after incubation for 20 min at 0℃ and spectra at 200 mW microwave powers (from top to bottom). Inset shows the power saturation behavior of the signal at ca. 340 mT coming from the intermediate II. Sample and measurement conditions are the same with the caption to Fig. 3 except temperature at 6K and microwave power at 1 mW.

Table 1

Copper content and specific activities of WT BOD and its single and double mutants at Gln463

	Cu Content [Cu atom/protein]	Specific Activity [unit/mg][a]			
		ABTS	*p*-PD	bilirubin	taurobilirubin
WT BOD	4.0	114	14.1	35.9	7.5
Glu463Gln	3.8	0.012	0.013	0.011	0.013
Gln463Ala	3.7	0.74	0.38	0.21	1.19
Cys457Ser/Glu463Gln	2.8	~0	~0	~0	~0
Cys457Ser/Glu463Ala	2.8	~0	~0	~0	~0

[a]assay conditions in Methods.

Figure

Click here to download high resolution image

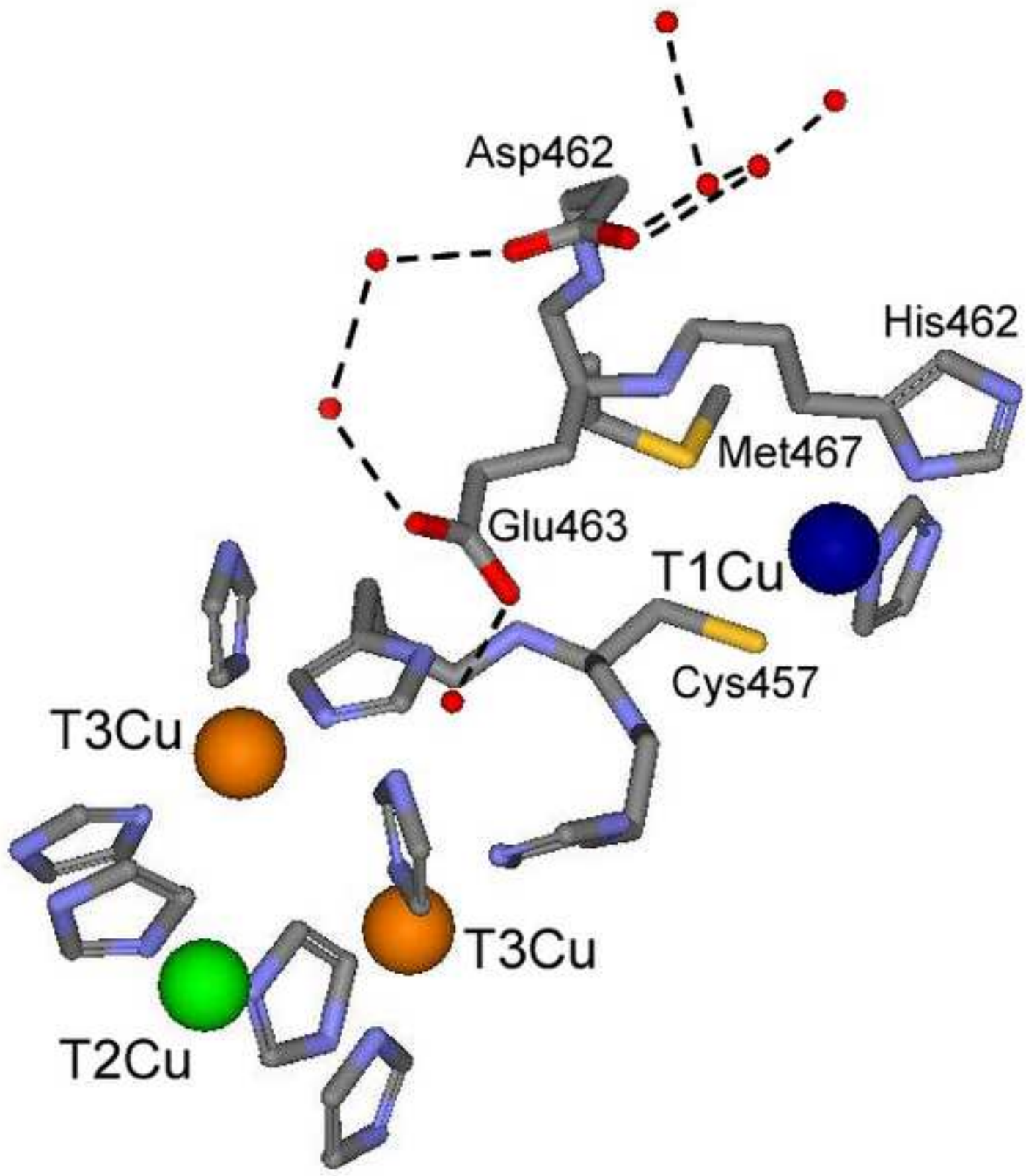

Figure 2

Click here to download high resolution image

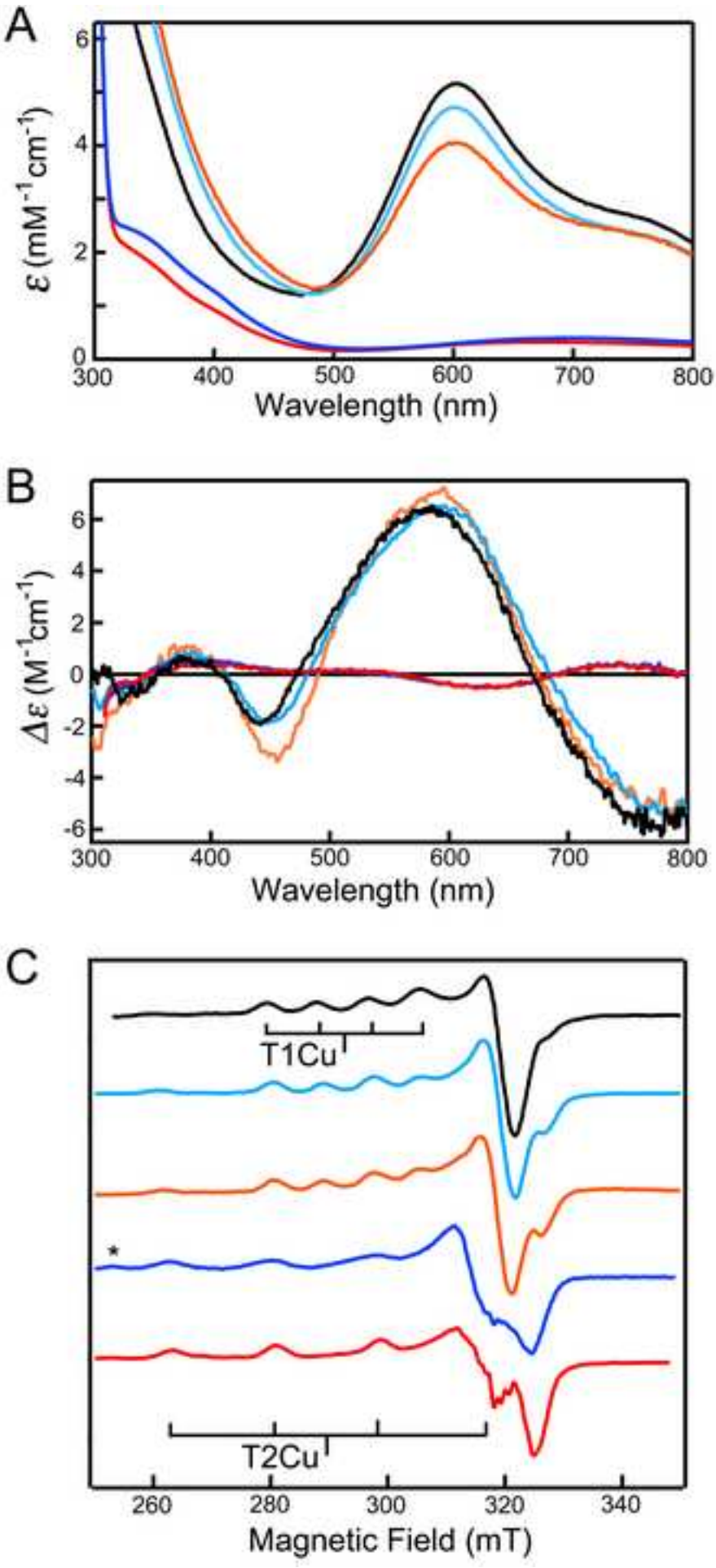

Figure 3

Click here to download high resolution image

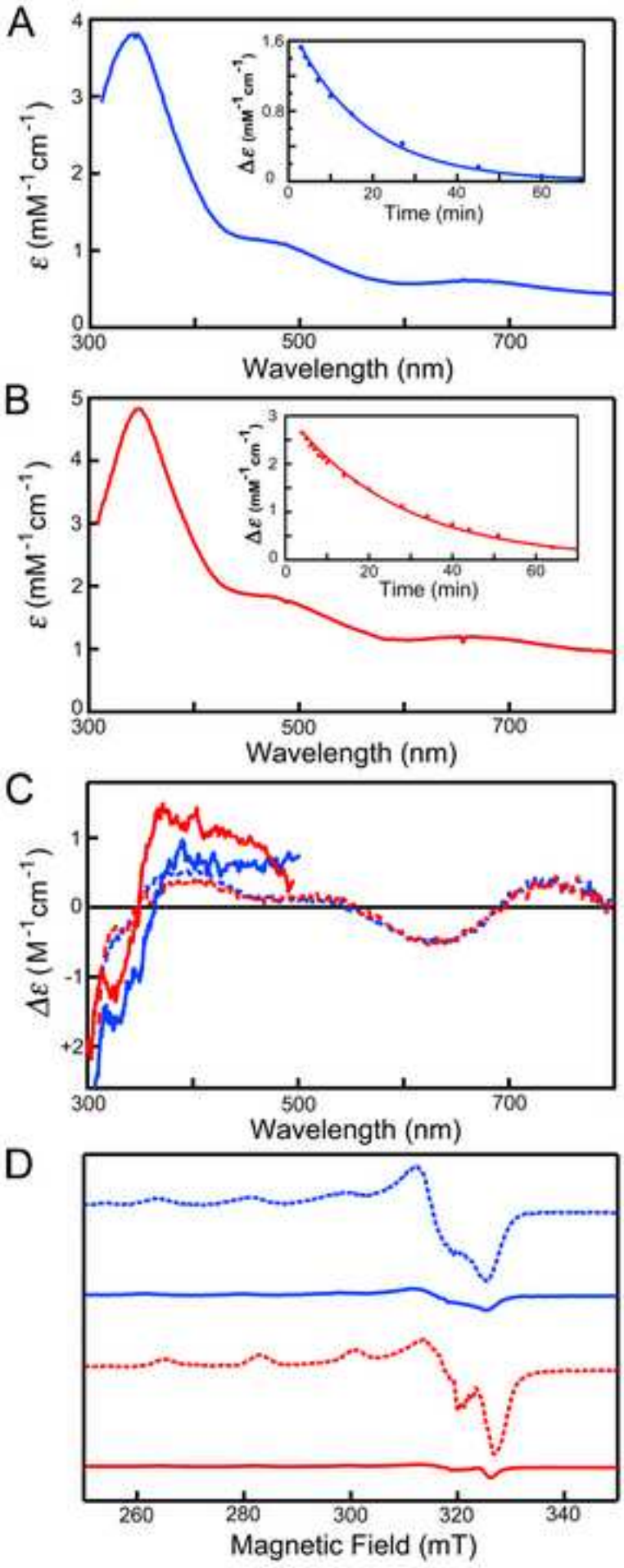

Figure 4

Click here to download high resolution image