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doi: 10.1002/tcr.20125

Basic and Applied Features of Multicopper Oxidases, CueO, Bilirubin Oxidase, and Laccase

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Abbreviated Title: Basic and Applied Features of Multicopper Oxidases

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ABSTRACT: Multicopper oxidases (MCOs) such as CueO, bilirubin oxidase, and laccase contain

four Cu centers, type 1 Cu, type II Cu, and a pair of type III Cu's in a protein molecule consisting of three domains with homologous structure to cupredoxin containing only type I Cu. Type I Cu mediates electron transfer between the substrate and the trinuclear Cu center formed by a type II Cu and a pair of type III Cu's, where the final electron acceptor O_2 is converted to H_2O without releasing activated oxygen species. During the process, O_2 is reduced by MCOs such as lacquer laccase and bilirubin oxidase, the reaction intermediate II with a possible doubly OH^- bridged structure in the trinuclear Cu center has been detected. The preceding reaction intermediate I has been detected by the reaction of the lacquer laccase in a mixed valence state, at which type I Cu was cuprous and the trinuclear Cu center was fully reduced, and by the reaction of the Cys → Ser mutant for the type I Cu site in bilirubin oxidase and CueO. An acidic amino acid residue located adjacent to the trinuclear Cu center was proved to function as a proton donor to these reaction intermediates. The substrate specificity of MCO for organic substrates is produced by the integrated effects of the shape of the substrate-binding site and the specific interaction of the substrate with the amino acid located adjacent to the His residue coordinating to the type I Cu. In contrast, the substrate specificity of the cuprous oxidase, CueO, is produced by the segment covering the Cu(I)-binding site so as to obstruct the access of organic substrates. Truncating the segment spanning helix 5 to helix 7 greatly reduced the specificity of CueO for Cu(I) and prominently enhanced the low oxidizing activity for the organic substrates, indicating the success of protein engineering to modify the substrate specificity of MCO.

Keywords: multicopper oxidase, metalloenzymes, Cu-protein, dioxygen reduction, protein engineering

Introduction

Cu sites in proteins have been classified into three classes according to their spectroscopic and magnetic features that reflect the geometric and electronic structure of the active site. The type I Cu site or blue Cu site contained in blue Cu proteins is related to electron transfer. The type II Cu site or non-blue Cu site contained in proteins such as superoxide dismutase and galactose oxidase is related to a variety of redox reactions. The type III Cu site or coupled binuclear Cu site contained in hemocyanin and tyrosinase is related to the binding and activation of dioxygen.

Multicopper oxidase (MCO) is a family of enzymes that normally contains four Cu atoms, a type I Cu, a type II Cu, and a pair of type III Cu centers in a protein molecule.[1] Type I Cu shows a strong absorption at ca. 600 nm (ε = 4,000 ~ 6,000 $M^{-1} cm^{-1}$) and a narrow hyperfine splitting ($|Az| < 9.5 \times 10^{-3}$ cm^{-1}) in the electron paramagnetic resonance (EPR) spectrum. Type I Cu in MCO is coordinated by a set of amino acids, 1Cys2His1Met or 1Cys2His1Phe/Leu, although Phe or Leu occupies the position of Met without coordinating to type I Cu. The ligand set, 1Cys2His1Gln, has not been found in MCO, as opposed to the blue Cu center in phytocyanins of higher plants, a class of blue Cu proteins. Type II Cu only gives the d-d bands usually masked by the strong absorptions caused by type I Cu in the visible region. However, its EPR signal shape is normal with $|A_Z| > 14 \times 10^{-3}$ cm^{-1}. Type II Cu is very unusual because of its three coordination with only 2His1H_2O/OH^- (No three coordinated-Cu(II) complex has not been known in the field of inorganic chemistry). Type III Cu's show a charge transfer band at ca. 330 nm due to the bridging OH^- between cupric ions. No EPR signal is given by coupled type III Cu's because of their strong antiferromagnetic interaction. Each type III Cu is coordinated by 3His in addition to the bridging OH^-. Type I Cu mediates the electron transfer between the substrate and the trinuclear Cu site formed by a type II Cu and a pair of type III Cu's, where a dioxygen molecule is converted into two water molecules.

Until recently, only laccase, ascorbate oxidase, and ceruloplasmin have been known as enzymes

belonging to the MCO family. However, many MCOs have been discovered and MCO is no longer a minor family of enzymes as was previously thought. Table 1 lists MCOs together with their sources, biological functions, expression systems, and PDB codes. Cu-containing nitrite reductase and coagulation factors such as factors V and VIII might also be included in MCO as subfamilies from a molecular evolution point of view (*vide supra*),[2] but they were excluded from Table 1.

Laccase was discovered as early as 1883 by Yoshida from latex of the lacquer tree (*Rhus vernicifera*) as a component to oxidize and polymerize urushiol and other phenol lipids.[3] However, it took about 50 years before laccase was established as a Cu-enzyme. Nevertheless, the main source of laccase is wood-destroying white-rot fungi. The biological roles of plant laccase and fungal laccase are contrastive: formation of lignin and degradation of lignin, respectively.[4] In addition, sequence homologies between plant and fungal laccases are not necessarily very high (*vide supra*). For example, the homology of amino acid sequences between lacquer laccase and cucumber ascorbate oxidase is higher than that between lacquer laccase and fungal laccases.[5] Laccase in insects functions in the formation of external cuticle.[6] CotA from *Bacillus subtilis* is a kind of laccase that functions in the formation of endospore coating to withstand exposure to a wide range of physical agents.[7] Bilirubin oxidase is also a class of laccase named for its high oxidizing activity of bilirubin.[8] Ascorbate oxidase is abundant in higher plants during the developmental period and is thought to function in cell division.[9] Phenoxazinone synthase, sulochrin oxidase, and dihydrogeodin oxidase are related to biosyntheses, but have not been studied in detail.[10] The biological role of ceruloplasmin, which is present in the serum of vertebrates, is primarily as a ferroxidase.[11] Recently, metal oxidases such as hephaestin[12] and Fet3p for Fe(II),[13] CueO (named after the *cueO* gene encoding a protein involved in the Cu efflux system of *Escherichia coli*) for Cu(I),[14] and MnxG, CumA, and MofA for Mn(II)[15] have been discovered. All of these MCOs are

related to the transport systems of metal ions. CueO, whose expression is controlled by CueR, a sensor of excess Cu, functions to convert Cu(I) to the less toxic Cu(II) in the periplasm together with CopA to transport Cu(I) across the inner membrane.

In this review, we mostly discuss our studies on multicopper oxidases such as lacquer laccase, bilirubin oxidase, CueO, fungal laccase, ceruloplasmin, and ascorbate oxidase, especially the first three MCOs for their structure, characterization, reaction mechanism, and application.

Structure of Multicopper Oxidases and Evolution

We determined the amino acid sequences of two isozymes of *R. vernicifera* laccase as the prototype enzyme of MCO from cDNA after its discovery more than a hundred years ago.[5] Considering that the biological roles of plant and fungal laccases are contrastive in addition to their considerably low sequence homologies, the fact that MCOs from plant and fungus were given the same name might be a cause of confusion. Nevertheless, the amino acids around the Cu binding sites of all MCOs are highly conserved (Fig. 1). Most MCOs consist of three domains, each of which has a structure homologous to that of cupredoxin (blue Cu proteins, named as compared with ferredoxin), the β–barrel scaffold.[2] In contrast, the so-called small laccase[16] is constructed by two domains and ceruloplasmin by six domains,[17] suggesting the diverse molecular evolution of MCO. In addition, it has been reported that a novel polyphenol oxidase, differing from tyrosinase and phenol oxidase, contains four Cu ions and might be classified as a laccase, although the sequence homology with laccases is low and the ligand set for the binding of Cu's is different from those of the laccases previously reported.[18] According to the crystal structures of MCO, the metal binding sites in MCO with three domains are positioned between domain 1 and domain 3. Ceruloplasmin has three repetitions of two domains only one of which (domains 1 and 6) has a complete set of Cu binding

sites to cause enzyme activity.[17] The other two two-domains sustain only the type I Cu site or a variant of the type I Cu site. Cu-containing nitrite reductase evolved from the common ancestor of MCO because it is a trimer of two domains analogous to the structure of cupredoxin.[19] The catalytic site analogous to one of the type III Cu sites in MCO is located between subunits, not being called a type III Cu but a type II Cu since it is isolated and EPR detectable.

Cu Binding Sites of Multicopper Oxidase

Fig. 2 shows the active site of CueO.[20] The His-Cys-His sequence peculiar to all MCOs directly connects the type I Cu and the type III Cu's. All Cu's in the trinuclear Cu centers are also closely connected to each other with the sequence His-X_n-His. Therefore, all Cu centers are closely connected to each other, and a perturbation on a Cu center is propagated to the other Cu centers. Type I Cu in MCO is usually coordinated by 1Cys2His1Met. However, in the case of fungal laccases, Phe or Leu occupies the position of Met and, accordingly, Cu is three coordinate. The low coordination number and the location of bulky hydrophobic amino acid in place of Met raise the redox potential of type I Cu, occasionally causing type I Cu to be in the cuprous state rather than in the cupric state. Different from blue Cu proteins, the coordination of Gln in place of Met has not been found for MCOs, and only site-directed mutageneses produced MCOs containing the type I Cu site whose redox potential shifted to a negative value.[21] This change is unfavorable for electron transfer between the substrate and type I Cu but is a valid change for trapping the reaction intermediates and increasing electric current when applied in electrochemistry (*vide infra*). The type I Cu site and the trinuclear Cu center are separated by ca. 13 Å with the sequence, His-Cys-His, which achieves the long-range electron transfer between the isolated Cu sites. According to the crystal structures of MCO, the distances between Cu centers in the trinuclear Cu center are

considerably diverse. However, there seems to be a tendency that the distances between the Cu centers are greater in the reduced form than in the resting form.[22] This might be derived from the necessity that the reduced trinuclear Cu center must accommodate dioxygen as the final electron acceptor. In accordance with this, anions such as azide, F^-, and Cl^- have been utilized as probes to explore the binding of O_2 towards the trinuclear Cu site, while the structures of these anion-bound forms were not necessarily the same for all MCOs.[22,23]

An acidic amino acid, Asp112 in the case of CueO, is positioned adjacent to the trinuclear Cu center (see Fig. 2). This amino acid interacts directly and indirectly with His residues coordinated to type II and III Cu's and a water molecule coordinated to type II Cu. This Asp or Glu conserved in all MCOs functions as the H^+ donor to the O_2-reduced species and profoundly affects the properties of the trinuclear Cu center as evidenced by mutation studies[21c,24] and kinetic studies[21c,25] to characterize the reaction intermediates.

Fig. 3 shows the absorption, circular dichroism (CD), and EPR spectra of CueO and bilirubin oxidase. Contributions from type I Cu are dominant in the absorption and CD spectra except for the contribution from type III Cu's at 330 nm. The d-d bands due to type II Cu are masked by the intense charge transfer bands due to type I and type III Cu ions. In the case of CueO, a clear band can be seen at 330 nm because the absorption at 280 nm is not very strong, and possibly, the Cu-O-Cu atoms are almost located in line.[20a,24, 26,] In the EPR spectra, both the type I and type II Cu's are detected and the type III Cu's are EPR-silent due to the strong antiferromagnetic interaction between them through the bridged OH^-. However, the expressed MCOs as isolated, which have not experienced reaction, sometimes show an extra Cu EPR signal with the $|Az|$ value, $\sim 10 \times 10^{-3}$ cm^{-1}, possibly originated in an uncoupled type III Cu in a mixed valence state, although turnover of the reaction gave the spectra due to the resting form of the MCO.[27] In accordance with this, possible delocalization of a radical on type I Cu and the S atom of the Cys residue depending

on pH was observed[28] as has also been observed for blue Cu proteins.[29] Changes in the EPR spectral features depending on pH and temperature are due to deprotonation from the coordinated H_2O to type II Cu and conformational change of the protein molecules, respectively. Bilirubin oxidase mutants, Cys457Ser and Met467Gln, afforded the spectra contributed from only the type II Cu and the spectra due to the modified type I Cu, respectively.[21c]

Superconducting quantum interference device (SQUID) measurement of ascorbate oxidase from cryogenic temperatures to room temperature suggested that the antiferromagnetic interaction between type III Cu's is not as strong as in the case of laccase because the effective magnetic moment continuously increased with increasing temperature.[30,31]

Extended X-ray absorption fine structure (EXAFS) of lacquer laccase showed that the oxidation state of all Cu centers are cupric in the resting form, but type III Cu's are in the cuprous state in the selectively type II Cu-depleted form, indicating that type III Cu's alone are not able to react with dioxygen even under air, in contrast to hemocyanin.[32]

Resonance Raman spectra of MCO are exclusively contributed by type I Cu.[33] A contribution from the trinuclear Cu center has not been observed because of the strong fluorescence.

Four-electron Reduction of Dioxygen at the Trinuclear Cu Center

During the process, O_2 is converted to H_2O, superoxide is formed first, followed by the formation of peroxide. When the third electron is supplied to the peroxide, the O-O bond is cleaved, and O^{2-} and $O^{\bullet}$ are formally formed. By accepting the forth electron, O_2 is converted to $2O^{2-}$. For the oxygenation reactions catalyzed by proteins containing only type II Cu or type III Cu's, these activated oxygen species are utilized in addition to the hydroxyl radical and hydroperoxide. However, terminal oxidase and multicopper oxidase directly convert O_2 to H_2O without releasing

activated oxygen species outside the protein molecules. Therefore, terminal oxidase and MCO do not couple with proteins such as superoxide dismutase (SOD) and catalase to scavenge the activated oxygen species. The active centers that are able to perform the four-electron reduction of dioxygen in the biological systems are only the heme-Cu center in the terminal oxidases and the trinuclear Cu center in the MCOs.

The reaction intermediates, which should be formed during the conversion of O_2 to $2H_2O$ by MCO, have not been detected for a long time in spite of numerous kinetic studies. We have succeeded in detecting an intermediate by the reaction of lacquer laccase.[25a] Soon after the reaction of the four-electron reduced laccase with O_2, the absorption at around 330 nm became stronger than that of the resting enzyme and began to decay. The difference spectrum between the spectrum obtained soon after the reaction and that of the resting laccase gave a transient spectrum with three bands at 370, 420, and 670 nm due to a reaction intermediate (intermediate II) (solid line in Fig. 4). This reaction intermediate II gave the peculiar EPR signals at g = 1.83 and 1.61 at cryogenic temperatures, indicating that a triplet state was involved. Decay of the intermediate species was dependent on pH, suggesting that a group with pKa = 5.4 was involved. This group, functioning as a proton donor to the intermediate II, was identified as an Asp residue adjacent to the trinuclear Cu center (corresponding to Asp112 of CueO in Fig. 2). Stopped-flow study of the formation of the intermediate II indicated that no preceding reaction intermediate could be detected because of the lifetime.[25b] Bilirubin oxidase also gave an analogous reaction intermediate.[21c]

The preceding intermediate (intermediate I) was detected by the reaction of the mixed valent laccase in which type I Cu was oxidized and the trinuclear Cu center was reduced.[25c] This mixed valent laccase was prepared by acting Cu(I) to the selectively type II Cu-depleted laccase, in which type I Cu was cupric and type III Cu's were cuprous, under strictly anaerobic conditions as long as half a day. The intermediate I gave the transient spectrum with the bands at 340, 475, and 680 nm

(dotted line in Fig. 4) showing a pH-dependent decay analogous to that by the intermediate II. This intermediate could be detected by shutting-dawn the electron transfer from the type I Cu. For bilirubin oxidase[21c] and CueO, whose heterologous expression systems have been constructed, a different approach has been performed to trap the reaction intermediate I. The vacant type I Cu site was constructed by mutating the Cys residue as a ligand to type I Cu with Ser. The reaction of the type I Cu-depleted enzymes in the reduced form with O_2 gave the EPR-silent reaction intermediate I.

The structures of these intermediates have not been determined in spite of their characterization studies since no data that directly indicate their structures has been obtained. The proposed structure and reaction scheme are shown in Fig. 5. There are two possibilities for the structure of the intermediate I: a peroxide form or a five-centered form involving a Cu(III). In contrast, $2OH^-$ is considered to doubly bridge the type III Cu's in the intermediate II, in which two radical centers are delocalized to give the triplet state as observed by the cryogenic EPR measurement. In accordance with these kinetic studies, crystallographic structures of CotA and mutants in the O_2^-- and O_2^{2-}-bound resting forms have recently been shown, suggesting that O_2 is able to be present at the trinuclear Cu center without being bound to Cu(II)'s when expressed.[34] The presence of O_2 at the trinuclear Cu center has also been reported for *Melanocarpus albomyces* laccase,[35] although further studies are required to assess whether these are artifacts and whether these structures properly reflect the reduction mechanism of O_2. We frequently found that MCO and the mutants were in states different from the resting states when expressed.[27] Mutations for the ligands in the trinuclear Cu center might ease detection of the reaction intermediates.[21a, 33]

The trinuclear Cu center, which is able to accommodate a three- to four-electron equivalent, is a device to convert dioxygen to water without releasing activated oxygen species such as superoxide, peroxide, and hydroxyl radical. Elucidation of the mechanism of MCO together with catalysis by

the heme-Cu center in terminal oxidases is expected to lead to the construction of fuel cells (*vide infra*).

Substrate Specificity of Multicopper Oxidase

The substrate specificity of MCO is considerably broad except for the metallo-oxidases for Fe(II), Cu(I), and Mn(II) (Table 1). Organic substrates are bound in the cleft at the "north side of the protein molecules. This substrate-binding site is constructed by assembling the loops attached to the β–barrel scaffolds. The shape of the site is the primary factor in causing the substrate specificity of MCO. Crystal structures of CotA docked with 2,2'-azino-bis(3-ethybenzothiazoline-6-sulfonic acid) (ABTS) and syringaldazine showed that these substrates were accommodated in the substrate-binding site in the folded forms.[36] A bilirubin-docked model of bilirubin oxidase also suggested the analogous folded binding of bilirubin at the substrate-binding site (Fig. 6).[21c] An acidic amino acid located adjacent to the type I Cu site is hydrogen bonded with an imidazole group coordinating to type I Cu to facilitate electron transfer from the substrate to the type I Cu.[37] The carboxy group in the side chain of the Asp residue was revealed to directly interact with 2,5-xylidine used as an inducer in the crystal structure of *Trametes versicolor* laccase.[38] Accordingly, this Asp must be one of the key amino acids to cause laccase activity. In the case of ascorbate oxidase, O-1 and O-2 of L-ascorbate were in hydrogen-bonding distance to the imidazole nitrogen of His512 coordinated to type I Cu and the nitrogen of Trp362.[39] A stacking interaction of the L-ascorbate ring with the aromatic ring of Trp163 also facilitates the binding of the substrate. Therefore, the substrate specificity of MCO for organic substrates is produced by the integrated effects of the shape of the substrate-binding site and the specific interaction of the substrate with the amino acid located adjacent to the His residue

coordinating to the type I Cu. The substrate-docked model of bilirubin oxidase is expected to be a clue to designing mutants for wider clinical use of the enzyme.

In contrast, cuprous oxidase, CueO, and the ferroxidases, Fet3p and ceruloplasmin, have specific substrate-binding sites consisting of 2Asp2Met, 1Glu2Asp1Gln, and 2Glu1Asp1His, respectively.[37a] As opposed to the catalytic metal centers, these ligand sets are not sufficient to bind the target metal ions, but are able to produce the proper labilities to bind the substrate and release a product. In addition, each substrate-binding site is buried inside the protein molecule. In particular, the substrate-binding site of CueO is insulated from bulk water with 50 amino acids including helices 5-7 (Fig. 7B; compare the difference with the "north side" of *Trametes versicolor* laccase, Fig. 7A).[20] To cause specificity for the metal ion as a substrate, an appropriate set of ligand groups and their steric arrangement are required to bind a cuprous ion and to release a cupric ion. Further, the pathway to transport substrate will also be important. Fourteen Met residues are present in the segment consisting of 50 amino acids. Met has frequently been found in the regions to function to transport Cu ions.

Full and partial deletions of this segment (the greenish-yellow region in Fig. 7A) confirmed that its presence is essential for CueO to function as cuprous oxidase, promoting the oxidizing activities for a variety of laccase substrates.[26] This has been a modification of CueO so as to return to an ancestral MCO.

Applications of multicopper oxidase

Lacquer laccase has been used as paint and adhesive for more than six thousand years in East Asia. These days, MCOs offer several advantages for biotechnological applications. Fungal laccases exhibit broad substrate specificity and thus are able to oxidize a broad range of organic compounds

including xenobiotics such as chlorinated phenols, synthetic dyes, pesticides, and polycyclic aromatic hydrocarbons. Indeed, they have been utilized for pulp delignification, textile dye bleaching, water or soil detoxification, etc. Bilirubin oxidase and ascorbate oxidase have been utilized for the clinical test of the function of liver and the pretreatment of sample to eliminate the inhibitory effect of ascorbate, respectively. By mutating the amino acids potentially located in the substrate-binding site, oxidations of the directly detected bilirubin and indirectly detected bilirubin could be kinetically discriminated, suggesting their application for the diagnosis of the origin of liver disorders.[40]

Formation of pigments by MCOs has been explored, and we have recently shown that bilirubin oxidase, CueO, and their mutants are promising catalysts for forming dyes that will not function as mutagens.[41] By deleting the region covering the substrate binding site of CueO, we demonstrated the prominent reduction in Cu(I) oxidizing activity and, in turn, the prominent increase in laccase activities for organic substrates.[26] Thus, it may not be too far into the future that we can prepare tailor-made MCOs with arbitrary specificity by modifying the substrate-binding site on an MCO scaffold.

Electron transfer between redox proteins and electrodes has been studied extensively due to their prospective applications in the field of biotechnology involving biosensors, bioreactors, and biofuel cells. However, the electrochemical applications of redox proteins have been limited to relatively small redox proteins such as cytochrom *c*, iron-sulfur proteins, and blue copper proteins, and there are only a limited number of examples for oxidoreductase.[42] MCOs and terminal oxidases are enzymes that are promising as catalysts for the bioelectrocatalytic, four-electron reduction of dioxygen to water. Activated oxygens leading to cell deterioration are not released when MCO is used as the cathodic enzyme. Bilirubin oxidase has been found to be the best enzyme for converting O_2 to H_2O as a cathodic enzyme in biofuel cell,[43] and its Met467Gln mutant with the lowered type I

Cu potential yields a higher electric current than does the wild-type enzyme.[44] Recently, Kano and Tsujimura in collaboration with the authors indicated that CueO gives the highest electric current density as a cathodic enzyme.[45] Protein engineering of MCO to tune the redox potential of Cu centers or to facilitate electric communication at the interface of the protein molecule and electrode is expected to increase the amount of electric current leading to practical use of MCO as a catalyst for biofuel cells, although searches for more stable MCOs with higher redox potential will be continued.

We thank Prof. Kenji Soda for giving us an opportunity to publish this review article, Prof. Kenji Kano and Dr. Seiya Tsujimura for their collaboration on the study of electrochemistry, and Dr. Hong-wei Huang for modeling the substrate-docked bilirubin oxidase. We also thank NEDO, Mandom Corporation, Toyota Motor Corporation, and Alfresa Farma for their financial support.

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Figure Captions

Fig. 1. Sequence alignment of amino acids to construct Cu binding sites and their neighbors of multicopper oxidases, CueO, *Myrothecium verrucaria* bilirubin oxidase (BO), *Rhus vernicifera* laccase (RvLc), zucchini ascorbate oxidase (AO), *Trametes versicolor* laccase (TvLc), *Coprinus cinereus* laccase (CcLc), Fet3p, CumA, CotA, *Streptomyces coelicolor* small laccase (SLAC), and human ceruloplasmin (hCp). Numbers and asterisks indicate the amino acids as ligands for each type of Cu and a potential proton donor for the reaction intermediates, respectively.

Fig. 2. Active site of CueO. Type I Cu, type II Cu, and type III Cu's are indicated by blue, green, and orange balls, respectively. The Cu(II) bound at the substrate-binding site is indicated by the red ball. The backbone peptides colored water blue, green, cyan, and yellow show that each Cu center is closely connected with each other. Asp112 is located behind the trinuclear Cu center, forming hydrogen bonds with the imidazoles coordinating to a type II and a type III Cu directly and indirectly through a water molecule. Calculated using PyMol for PDB data, 1N68.

Fig. 3. Absorption (A), CD (B), and EPR (C) spectra of CueO (solid line) and bilirubin oxidase (dotted line).

Fig. 4. Transient spectra of the reaction intermediate I (solid line) and the reaction intermediate II (dotted line) obtained from lacquer laccase.

Fig. 5. Proposed reaction scheme of the four-electron reduction of dioxygen by multicopper oxidase. As for reaction intermediate I, the peroxide form and a five-centered form with a Cu(III)

ion are possible. As for reaction intermediate II, the doubly OH^- bridged structure in the triplet state is most promising.

Fig. 6. Bilirubin-docked model of bilirubin oxidase formed using Discovery Studio Modeling (Accelrys). Two forms, colored yellow and blue, were suggested for the binding of bilirubin.

Fig. 7. Crystal structures of CueO (A) and *Trametes versicolor* laccase (B) calculated using PyMol with the PDB data, 1GYC and 1N68, respectively. The region covering the substrate-binding site of CueO, of which the region between helix 6 and helix 7 is not shown because of disorder, is colored greenish-yellow.

Takeshi Sakurai was born in 1950. He received his B.S. and M.S. degrees in polymer chemistry from Osaka University in 1973 and 1975, respectively, under the supervision of Professor Heimei Yuki. He then started his research in bioinorganic chemistry under the supervision of Professors Akitsugu Nakahara and Osamu Yamuchi at Osaka University where he received his Ph.D. in 1978. Soon after that, he became Assistant Professor at Osaka University and began to study metalloenzymes. During 1983 to 1984, he joined Professor Howard S. Mason's Group at Oregon Health Sciences University. In 1988, he moved to Kanazawa University as Associate Professor. In 1996, he moved to the Institute for Molecular Science (part of the Okazaki National Research Institutes), but returned to Kanazawa University in 1998 where he has been a Full Professor since 2000. Most of his research interests are on elucidating the reaction mechanism of proteins with multimetal centers such as multicopper oxidases and NO reductase and also on their applications.

Kunishige Kataoka was born in 1965. He received his M.Agr. degree from Kyoto University in 1991 under the supervision of Professor Kenji Soda. In 1993, he joined Professor Katsuyuki Tanizawa and Professor Toshio Fukui’s group as a research associate at the Institute for Scientific and Industrial Research, Osaka University. He received his Ph.D. degree from Osaka University in 1994. He then moved to Professor Shinnichiro Suzuki’s laboratory at the same university as an assistant professor and started his research on metalloproteins. In 2001, he joined Professor Takeshi Sakurai’s group at Kanazawa University as an associate professor. Most of his research interests are on the protein engineering of metalloproteins and application of the designed metalloproteins.

Graphical Abstract

Basic and Applied Features of Multicopper Oxidases, CueO, Bilirubin Oxidase, and Laccase

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The structure, spectroscopic property, and the reaction mechanism to perform the four-electron reduction of dioxygen have been studied for native multicopper oxidases such as laccase, CueO, and bilirubin oxidase and their mutants. Basic studies to explore their substrate specificities led to the promotion and creation of substrate specificities applicable for practical uses such as pigment formation and biofuel cells.

Fig. 1

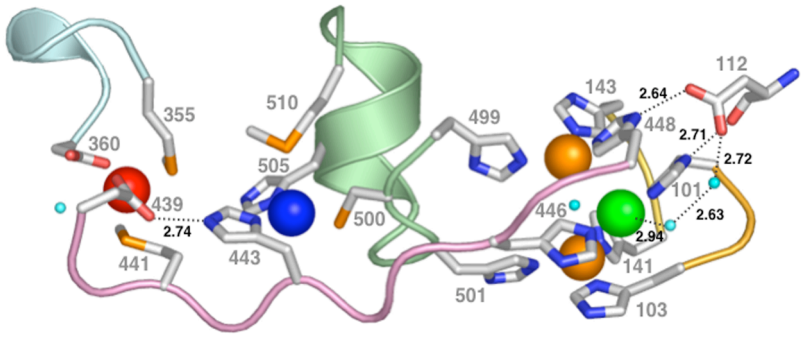


Fig. 2 Sakurai and Kataoka

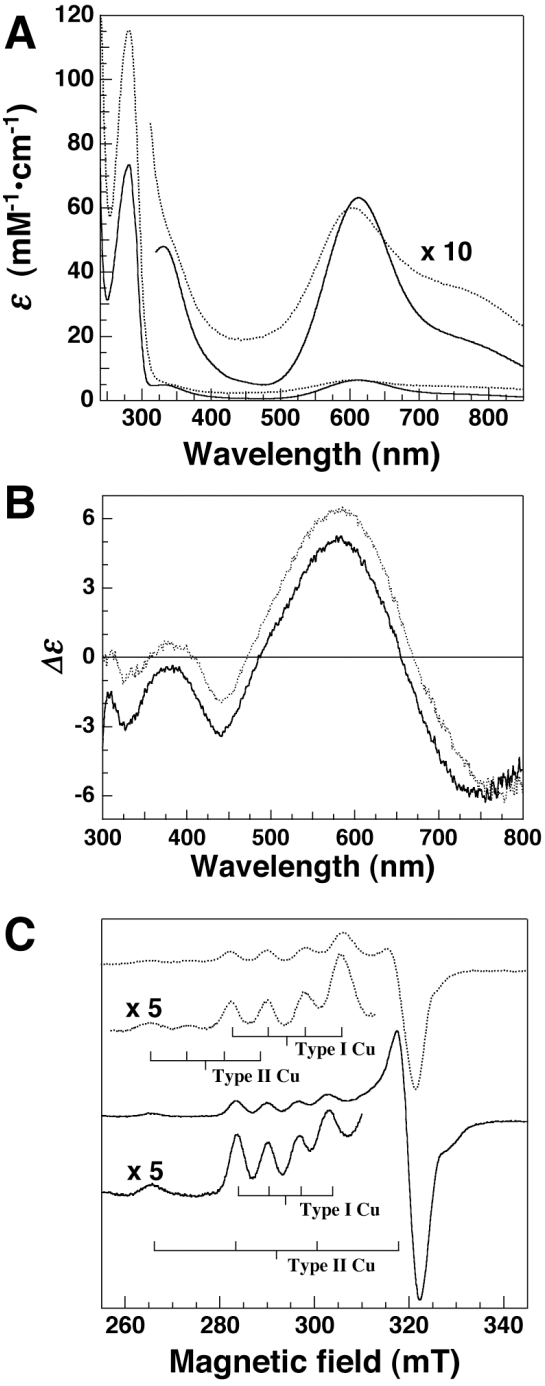
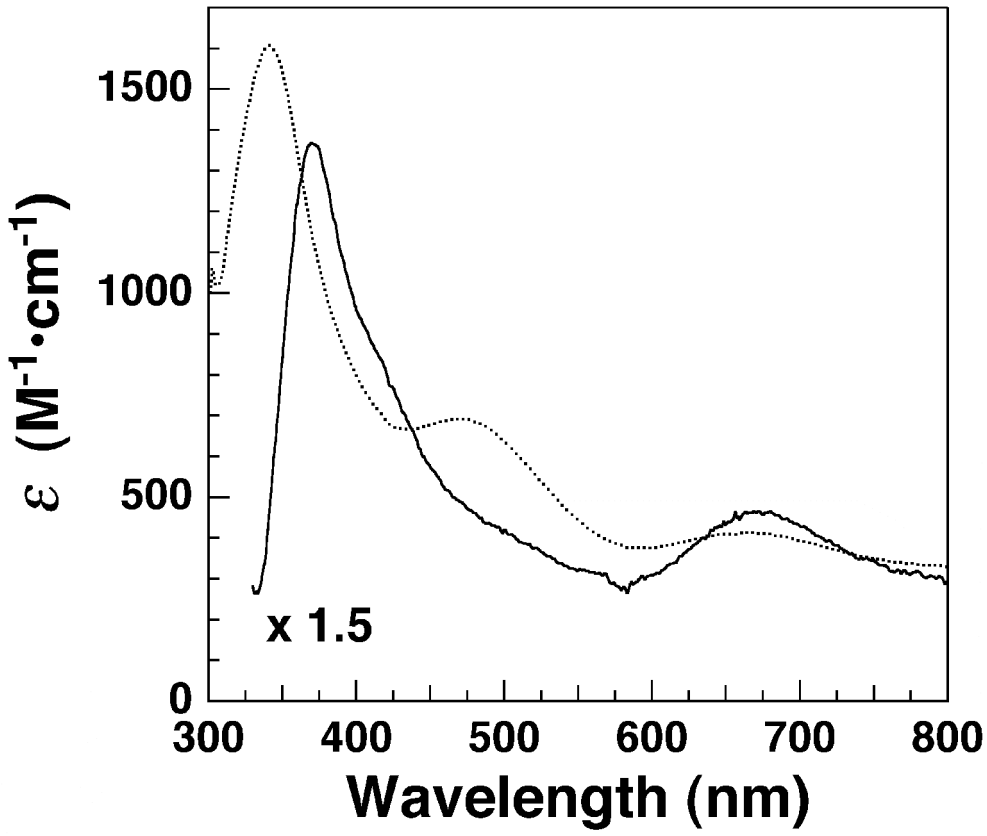

A
120
100
80
60
40
20
0
ε (mM-1•cm-1)
x 10
300
400
500
600
700
800
Wavelength (nm)
B
6
3
0
-3
-6
Δε
300
400
500
600
700
800
Wavelength (nm)
C
x 5
Type I Cu
Type II Cu
x 5
Type I Cu
Type II Cu
260
280
300
320
340
Magnetic field (mT)

Figure 4.



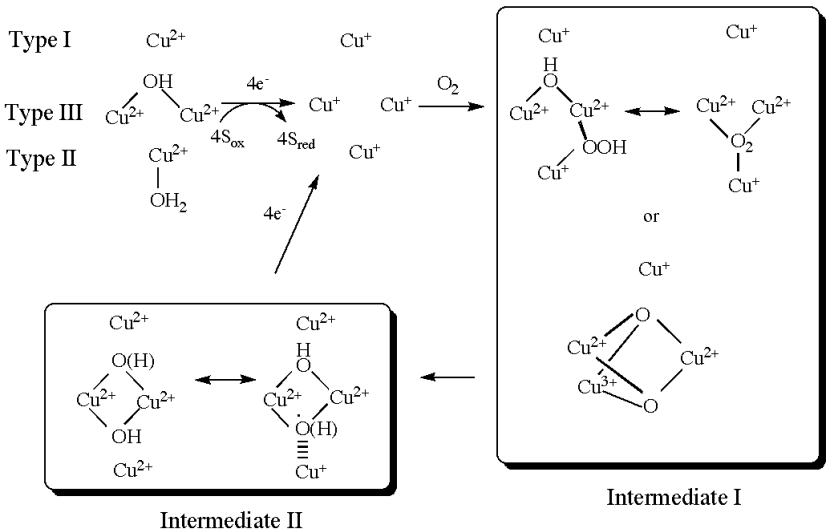
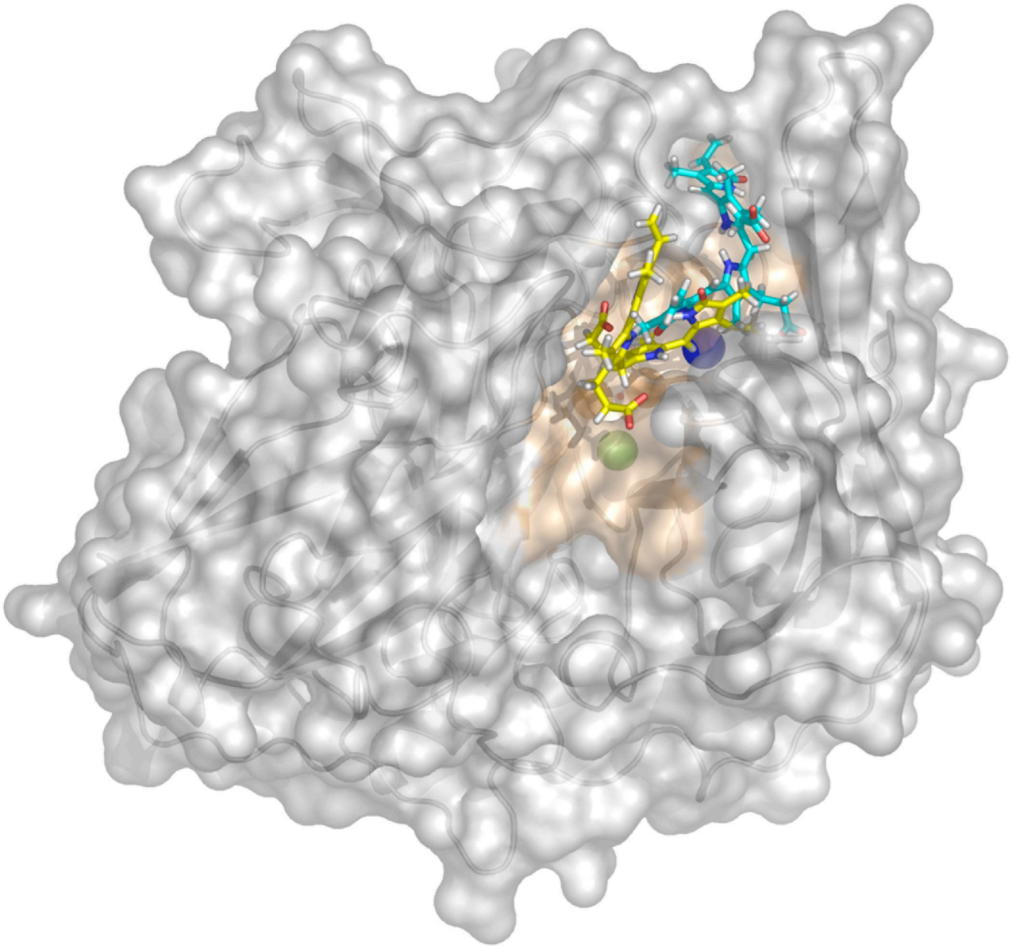
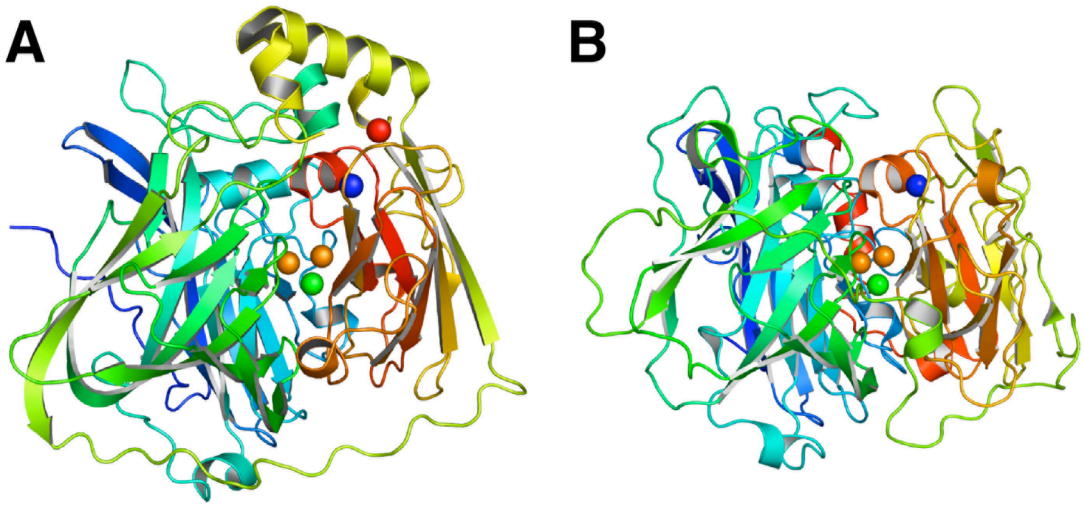


Fig. 5 Sakurai and Kataoka




A
B

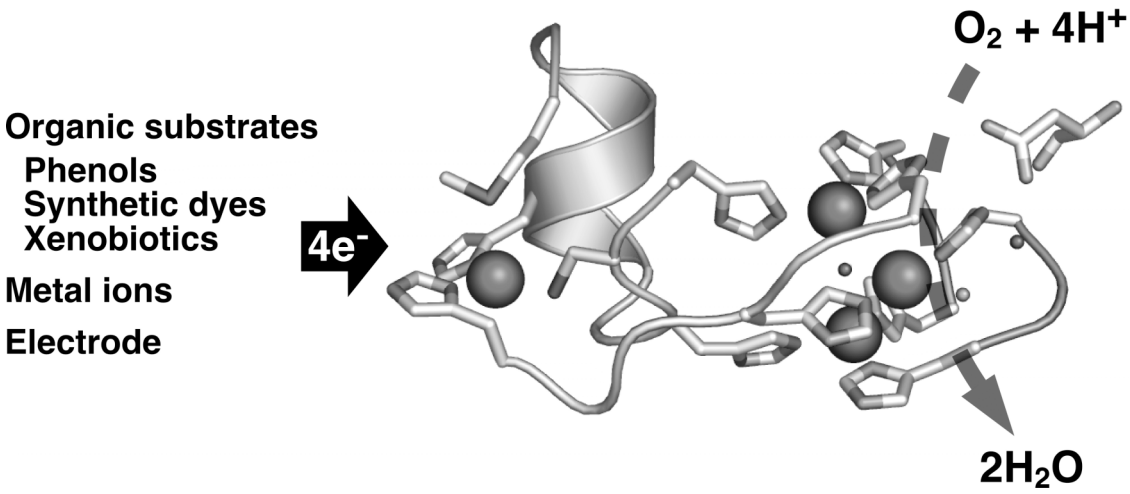

Organic substrates
Phenols
Synthetic dyes
Xenobiotics
Metal ions
Electrode
4e-
O2 + 4H+
2H2O

Table 1. Origin, Function, Expression System, and PDB Code of Multicopper Oxidase

Multicopper Oxidase	Origin	Function	Expression System	PDB Code
Laccase	*Rhus vernicifera*	Oxidation of Urushiol	-	-
	Coprinus cinereus	Degradation of Lignin	*Aspergillus oryzae*	1A65, 1HFU
	Coriolus hirsutus		-	preliminary
	Trametes versicolor		*Aspergillus oryzae*	1GYC.1KYA
	Melanocarpus albomyces		-	1GW0
	Rigidoporus lignosus		-	1V10
	Cerrena maxima			2H5U
	Manduca sexta	Formation of Cuticle	-	-
CotA	*Bacillus sabtilus*	Formation of Endospore Coat	*E. coli*	1GSK,1W8E, 1W6L, 1HKP, 1HKZ, 1HL0, 1HL1, 1OF0, 1OGR, 1UVW, 1W6W, 1W8E, 2BHF
Bilirubin Oxidase	*Myrothecium verrucaria*	Oxidation of Bilirubin	*Pichia pastoris* *Aspergillus oryzae*	-
CueO	*E. coli*	Oxidation of Cu(I)	*E. coli*	1N68, 1PF3, 1KV7, 2FQD, 2FQE, 2FQF, 2FQG
PcoA	*E. coli*	Efflux of Cu	*E. coli*	-
Fet3p	*Saccharomyces cerevisiae*	Oxidation of Fe(II)	*Saccharomyces cerevisiae*	1ZPU
CumA, MofA, MnxG	*Pseudomonas putida*	Oxidation of Mn(II)	-	-
Phenoxazinone Synthase	*Streptomyces lividans*	Biosynthesis of Antibiotic	-	2G23
Dihydrogeodin Oxidase and Sulochrin Oxidase	*Aspergillus terreus*	Biosynthesis of Grisans	*Aspergillus nidulans*	-
Ascorbate Oxidase	Higher Plant	Cell Division	-	1AOZ, 1ASO, 1ASP, 1ASQ, 1WA6
Ceruloplasmin	Vertebrate	Oxidation of Fe(II)	*Pichia pastoris*	1KCW, 2J5W
Hephaestin	Vertebrate	Transport of Fe(II)	BHKCell	-