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Short Conceptual Overview

Structure and molecular evolution of multicopper blue proteins

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Abstract

The multicopper blue protein family, which contains cupredoxin-like domains as a structural unit, is one of the most diverse groups of proteins. This protein family is divided into two functionally different types of enzymes: multicopper oxidase and nitrite reductase. Multicopper oxidase catalyzes the oxidation of the substrate and then reduces dioxygen. The structures of many multicopper oxidases are already known, and until recently they were classified into two main groups: the three- and six-domain types. Both function as monomers and have three spectroscopically different copper sites: Types I (blue), II, and III (tri-nuclear). Nitrite reductase is a closely related protein that contains Types I and II (mono-nuclear) coppers but reduces nitrite instead of dioxygen. Nitrite reductase, which consists of two domains, forms a homotrimer. Multicopper oxidase and nitrite reductase share similar structural architectures and also contain Type I copper. Therefore, it is proposed that they have a common ancestor protein. Recently, some two-domain type multicopper oxidases have been found and their crystal structures have been determined. They have a trimeric quaternary structure and contain an active site at the molecular interface such as nitrite reductase. These results support previous hypotheses and provide an insight into the molecular evolution of multicopper blue proteins.

Keywords: ascorbate reductase; blue copper; ceruloplasmin; laccase; multicopper oxidase; nitrite reductase; Type I copper.

Introduction

Copper is essential for life, through its role in various proteins (1). Among the bioelements, copper is thought to be a modern metal that is available mainly after the photosynthetic generation of an oxidizing environment. Many copper-containing enzymes participate in reactions involving molecular oxygen. During the evolution of copper-containing proteins, the number of domains in a subunit and the pattern of subunit assembly have varied from protein to protein, together with point mutations. The variation appears to promote the functional evolution of various copper-binding sites for an efficient catalysis of complex redox processes. In the course of evolution, unique copper sites were created for oxygen binding and electron storage.

Type I copper, called blue copper, has unique spectroscopic properties and was originally found in electron transfer proteins (2, 3). It has unusually high redox potential and assists electron transfer systems in photosynthesis and nitrate respiration (1, 4). The cupredoxin-like fold containing Type I copper is found as a structural unit in numerous proteins and is a typical motif for the multicopper blue proteins (MCBPs). The MCBP family is one of the most important copper-containing protein groups, which contains a diverse group of proteins containing 2–6 copper ions with anywhere from 300 to more than 1000 amino acid residues in a single peptide chain. They have a multidomain structure and are divided into two types of enzymes: multicopper oxidase (MCO) and nitrite reductase (NIR).

MCO uses the distinctive redox ability of copper to catalyze the oxidation of a wide range of substrates, followed by the reduction of dioxygen (O_2) to water (H_2O) (Figure 1) (5). MCO contains three different types of copper ions. All types of copper are involved in the transfer of electrons from the substrate to dioxygen, the final electron acceptor. MCO has cupredoxin-like domains as structural units and has been classified into two groups according to the number of domains: three-domain type (3dMCO) and six-domain type (6dMCO), while they possess very similar optical spectral properties related to the copper ions.

NIR is highly related to MCO but no longer reduces the dioxygen, instead functioning to reduce nitrite (NO_2^-) (Figure 1). Therefore, it is thought to be another distinct branch of MCO (6–8).

Since the first crystal structure of MCO was obtained in 1989, many crystal structures of 3dMCOs, 6dMCOs, and NIRs have been determined (8–10). They share similar structural architectures, but the oligomeric state of NIRs is completely different from MCOs. Therefore, it was widely believed that they have a common ancestor protein, which consists of consecutive cupredoxin-like domains (8, 11).

As the genome projects have given us an increasing amount of sequence data, new homologs of MCOs have been identified. In 2003, Nakamura et al. suggested a new class

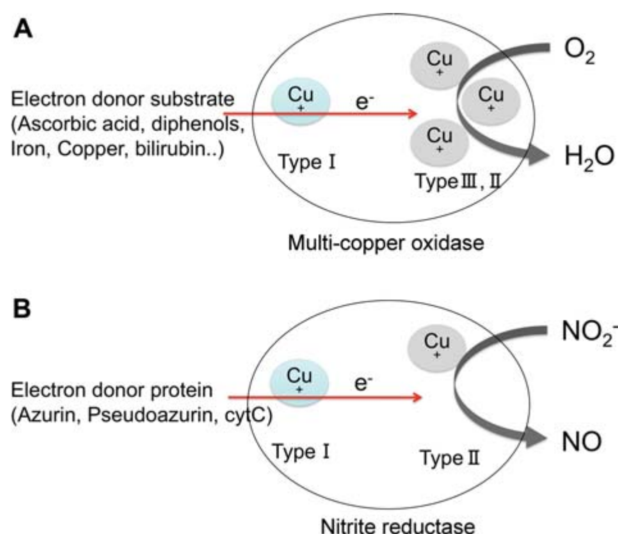

Figure 1 Schematic presentation of the catalytic mechanism of multicopper oxidase (A) and nitrite reductase (B).

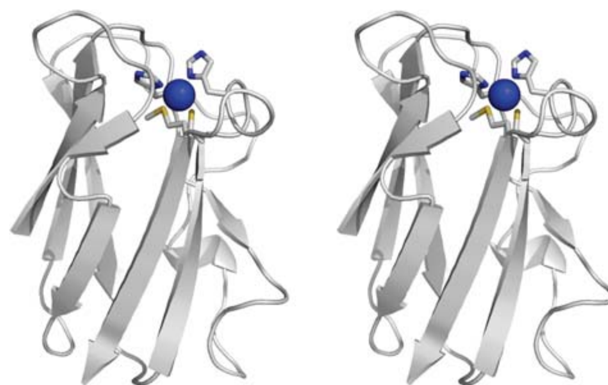

Figure 2 Structure of a cupredoxin fold, which has an eight-stranded Greek key β-barrel fold.
Type I coppers are shown in blue. It has a distorted tetrahedral coordination system coordinated by three strong ligands (one cysteine and two histidines) and one weaker ligand, a methionine. The figures were prepared by the PyMOL program (http://pymol.sourceforge.net), using the coordinate from PDB file 1PLC (Popar plastocyanin).

of two-domain type MCO (2dMCO) (12). On the basis of sequence alignments, several types of 2dMCOs have been predicted and are classified according to the location of the Type I copper-binding sites. At the same time, the novel types of MCOs, which have much smaller molecular weight than those of the typical 3dMCOs have been characterized biochemically (13–15). They are thought to lack the second domain and are considered 2dMCOs.

Recently, three crystal structures of 2dMCOs have been determined. All structures of 2dMCOs show the same trimeric architecture as that of NIR. Furthermore, 2dMCOs and NIRs have their active sites at a similar molecular interface. These results confirm the evolutionary relationship between MCOs and NIRs. In this study, we describe the molecular evolution of MCBPs on the basis of the three-dimensional structures of 2dMCOs.

Multicopper oxidase

MCO is a Type I (blue) copper-containing enzyme, which is involved in the oxidation of substrate, followed by the reduction of dioxygen (Figure 1) (5). MCOs are the only enzymes known to catalyze the four-electron reduction of dioxygen to water, except for cytochrome *c* (CYTC) oxidase, the terminal enzyme in the respiratory system. They all consist of cupredoxin-like domains, which have eight stranded Greek key β-barrel folds (Figure 2). The cupredoxin fold was originally found in small copper proteins, such as plastocyanin and azurin (2, 3). They contain Type I copper, exhibiting a bright blue color; they generally have high redox potential and function as an electron transfer protein. Until recently, the multicopper proteins were divided into two subfamilies, 3dMCO and 6dMCO, according to the number of cupredoxin-like domains.

Three-domain type MCO (3dMCO)

3dMCO, representing the main group of MCOs, consists of three cupredoxin-like domains. There are various types of 3dMCO, including laccases and several oxidases with specific substrates such as ascorbate, copper, iron or bilirubin. Laccases catalyzes the oxidation of a variety of aromatic compounds including diphenols. They have been found in a wide variety of plants, fungi, bacteria, and insects and have hence been studied extensively (16). Ascorbate reductase (AO), which exhibits a high specificity toward L-ascorbate, is one of the best characterized MCOs and the crystal structure was solved 20 years ago (9). Many crystallographic results are available for 3dMCOs (laccases from fungus (16–24), CueO (25), CotA (26), Fet3p (27), and phenoxazinone synthase (28)). They contain single peptide chains of around 500 amino acid residues with four copper ions in three distinct sites. They all have the same structural architecture with three sequentially arranged cupredoxin-like domains (Domains 1, 2, and 3) (Figure 3A). Each of them has eight conserved β-strands, constituting the core structure of each cupredoxin-like domain, which are connected by seven structurally unconserved regions (8). These unconserved regions are likely to be involved in the modulation of the substrate recognition and molecular stability in 3dMCOs.

Three types of coppers

Coppers in MCOs are classified into three types (I, II, and III) on the basis of optical and electromagnetic resonance (EPR) spectroscopic features. The spectroscopic features of a Type I copper are basically the same as those of electron transfer proteins. It has an absorption peak around 600 nm and a narrow hyperfine coupling in EPR spectroscopy. This copper has a distorted tetrahedral coordination system coordinated by three strong ligands (one cysteine and two histi-

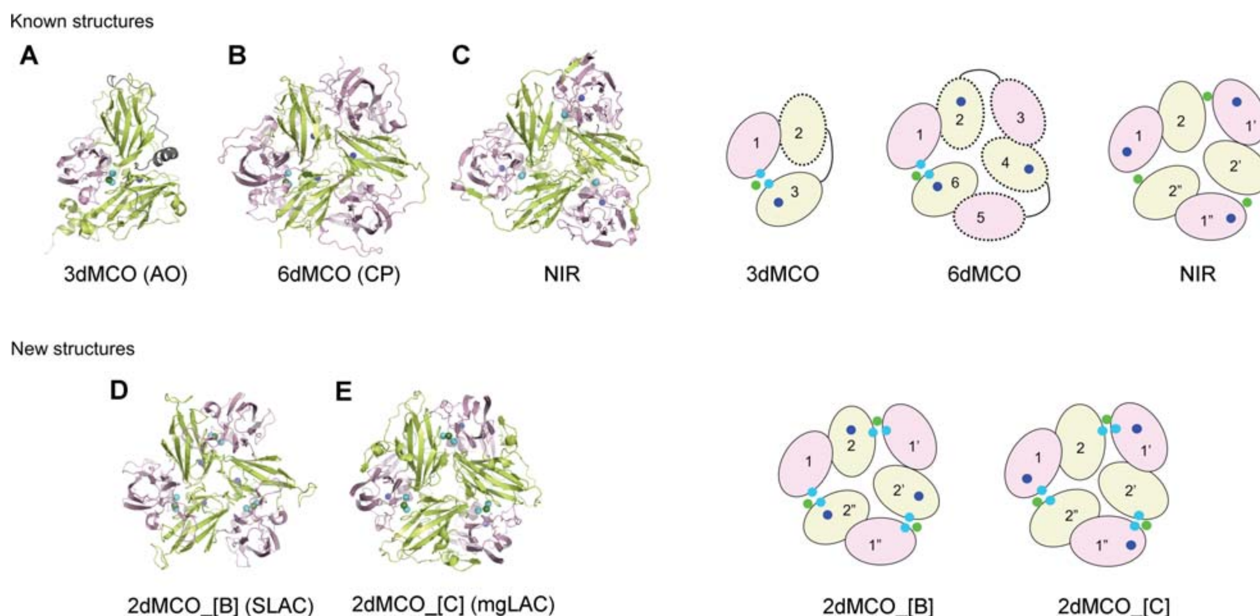

Figure 3 Overall structure of multicopper blue proteins, 3dMCO (AO) (A), 6dMCO (CP) (B), NIR (C), 2dMCO_[B] (SLAC) (D), and 2dMCO_[C] (mgLAC) (E).

The first (Class IV) and second (Class V) domains are shown in pink and light green, respectively. 3dMCO and 6dMCO are single chain proteins. NIR and 2dMCOs are trimers of two consecutive domains. Type I coppers are indicated by blue circles. The Type II and Type III coppers in MCOs are shown in green and cyan circles, respectively. The Type II coppers in NIR are shown in cyan circles. The figures were prepared by the PyMOL program, using coordinates from PDB files 2BW4 (NIR from *Achromobacter cycloclastes*), 1AOZ (AO from *Cucurbita pepo* var. *melopepo*), and 2J5W (CP from human).

dines) and one weaker ligand, typically a methionine (Figure 4A). The charge transfer transition between the copper and cysteine ligand is responsible for an extremely intense absorption, giving rise to the deep blue color of the enzyme. Type II copper has a much weaker absorption, broader hyperfine interactions, and is generally coordinated with two histidines and water–oxygen. Type III copper is an EPR non-detectable copper pair (antiferromagnetically coupled) and is usually coordinated by three histidines per copper and a bridging moiety, which is associated with an absorbance band at 330 nm (10). Types II and III form a tri-nuclear copper cluster, which is the active site for dioxygen reduction. The ligands for Types II and III are supplied symmetrically by the N-terminal (Domain 1) and the C-terminal domain (Domain 3) (Figure 4A). The distance between Type I copper and the tri-nuclear copper cluster connected through the sequence segment (His-Cys-His) is approximately 12 Å. The cysteine ligates the Type I copper and two histidines ligate the Type III coppers.

Catalytic mechanism of dioxygen reduction

3dMCOs receive electrons at the Type I copper site from an electron donor substrate and then transfer to the tri-nuclear copper cluster. The key element for the dioxygen reduction is the tri-nuclear copper cluster constructed by Types II and III, as shown in Figure 4A. The electrons are transferred through the highly conserved His-Cys-His tri-peptide to the tri-nuclear copper cluster. It has been proposed that the two-electron reduction mechanism functions through the two intermediates (5). However, the detailed catalytic mechanism of dioxygen reduction remains unclear. Copper sites have high-redox potential are easily reduced. In particular, the tri-nuclear copper cluster is very sensitive to X-ray radiation. The crystal structures probably represent the mixtures of the different stages of the catalytic reactions. Therefore, it is difficult to detect the intermediate states in the oxygen reduction pathway by X-ray crystallography (24). A detailed structural analysis still needs to be elucidated by other biophysical methods, such as neutron crystallography.

Substrate binding site

The substrate is oxidized by Type I copper. Although the electron transfer pathway and the tri-nuclear copper cluster are well conserved, the electron donor substrate binding sites of 3dMCOs are diverse. The Type I copper site is 7 Å beneath the surface of the molecule. The relatively large groove at the substrate binding site, which is constructed by the structurally unconserved regions, presumably contributes to its broad substrate specificity. CueO, a member of 3dMCO, is cuprous oxidase (25). The extra α-helical region of CueO covers the substrate-binding site and buries the Type I copper site deeply inside (Figure 5). It functions as an access barrier to the bulky organic substrates, which provides CueO with specificity as a cuprous oxidase (29). The structurally unconserved loop region of the second domain (Domain 2) of 3dMCOs also contributes to the substrate-binding surface. In addition to the overall molecular stability,

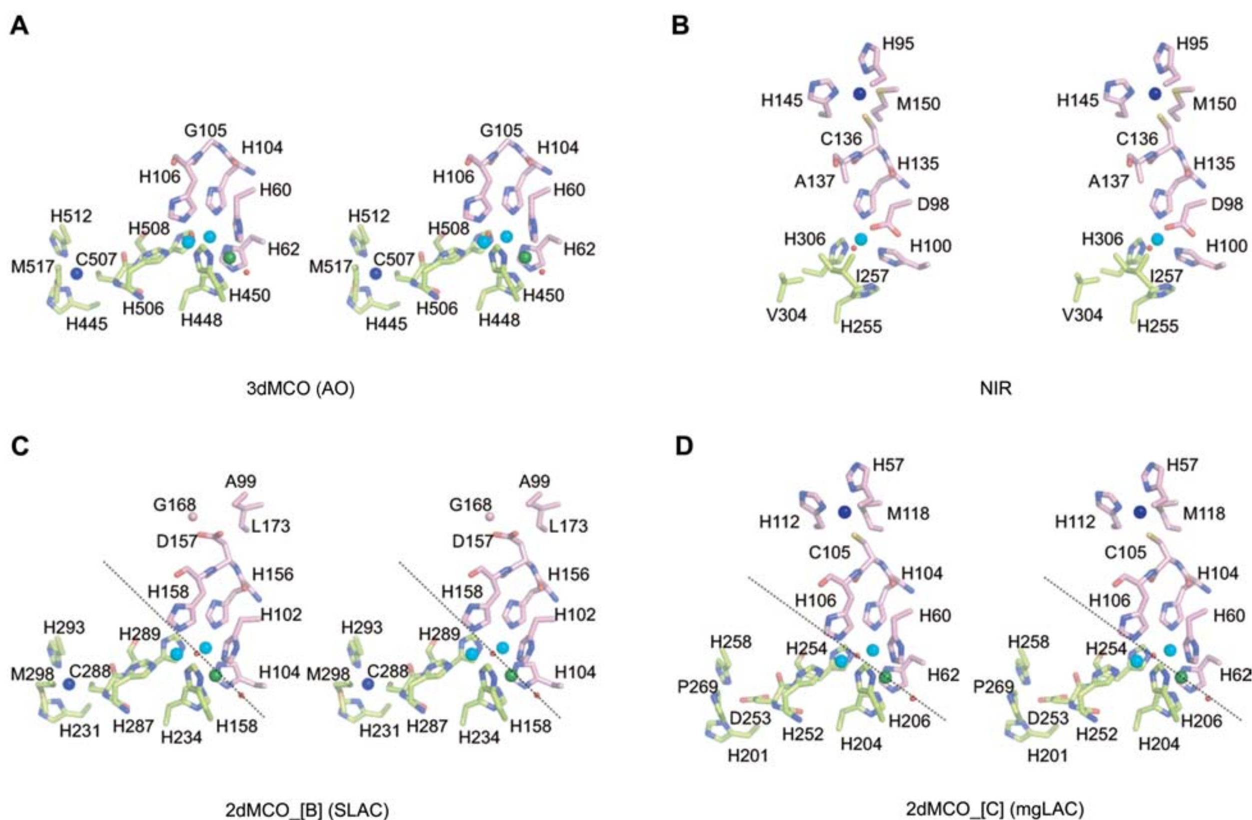

Figure 4 Stereo views of the copper-binding sites of multicopper blue proteins, 3dMCO (AO) (A), NIR (B), 2dMCO_[B] (SLAC) (C), and 2dMCO_[C] (mgLAC) (D).
Residues from the first domain (Class IV) are shown in pink and those from the adjacent second domain (Class V) are shown in light green. Type I copper atoms are depicted as blue spheres. The Type II and Type III coppers in MCOs are depicted in green and cyan spheres, respectively. The Type II coppers in NIR are depicted in cyan spheres. A local pseudo two-fold axis is drawn as a dotted line.

embellishment of the substrate-binding site would be the one of the important roles of Domain 2 in 3dMCOs.

Six-domain type MCO (6dMCO)

Ceruloplasmin (CP) is a human serum multicopper oxidase that is capable of oxidizing ferrous iron ($Fe2^{+}$) to ferric iron ($Fe3^{+}$) for transfer (30). The enzyme contains single peptide chain of 1046 amino acid residues (132 kDa) with six copper ions. CP is composed of six cupredoxin-like domains and belongs to 6dMCO. It is a monomeric protein and is folded in six sequentially arranged domains (Domains 1–6) with pseudo-threefold symmetry axis (Figure 3D). The six copper ions compose three Type I copper sites and one tri-nuclear copper cluster. Three Type I coppers are located in Domains 2, 4, and 6. The tri-nuclear copper cluster, formed by Types II and III, is located between the N-terminal domain

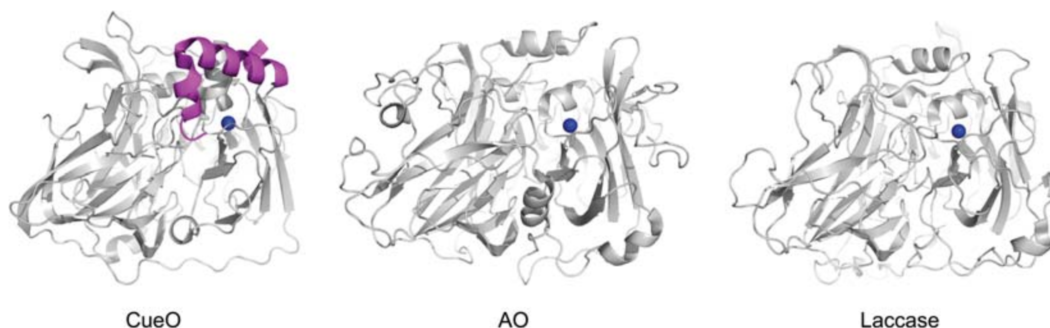

Figure 5 Structure of CueO, AO, and laccase.
Type I coppers are shown in blue. The extra α-helical region of CueO covering the substrate-binding site is shown in magenta. The figures were prepared by the PyMOL program, using coordinates from PDB files 1KV7 (CueO), 1AOZ (AO from *Cucurbita pepo* var. *melopepo*), and 1GW0 (laccase from *Melanocarpus albomyces*).

(Domain 1) and the C-terminal domain (Domain 6). A pair of Type III coppers between two domains plays the primary role in stabilizing CP. The Type I copper within Domain 6 is likely to be the electron acceptor from ferrous iron. The distance and the sequence segment (His-Cys-His) connecting tri-nuclear copper cluster and Type I copper within Domain 6 are similar to that of 3dMCO, suggesting a similar catalytic mechanism as 6dMCO. The three Type I sites are separated from each other by a distance of around 18 Å. This distance could allow internal electron transfer and could increase the probability for electron uptake; however, the roles of the other Type I copper domains (Domains 2 and 4) are unknown (31). The mosaic gene structure 6dMCOs were also found in blood coagulation factors V and VIII (32). They belong to the group of proteins related to MCOs.

Nitrite reductase (NIR)

There are two main categories of NIRs: heme-containing NIR and copper-containing NIR (CuNIR). They are not structurally related and contain different prosthetic groups. CuNIR contains copper ions as redox-active prosthetic groups and catalyzes one electron reduction of nitrite (NO_2^-) to nitric oxide (NO) (Figure 1). The enzyme contains a single peptide chain of approximately 300 amino acid residues. Crystal structures of several CuNIRs have been determined (CuNIR from *Achromobacter cycloclastes* (7), *Alcaligenes Xylosoxidans* (33), *Alcaligenes faecalis* (34), and *Rhodobacter sphaeroides* (35)). CuNIR consists of two cupredoxin-like domains (Domains 1 and 2), is similar to those of MCOs, and contains two copper sites (Figure 3C). The enzyme forms a stable homotrimer arranged in a head-to-tail manner around a three-fold symmetry axis as seen in CP (36, 37). The two copper ions in the monomer compose of one Type I copper site and one Type II copper site (Figure 4B). A Type I copper site is located in the N-terminal domain (Domain 1) and a Type II copper site is embedded in the intermolecular interface between two adjacent monomers. The similar structural features of CuNIR have also been observed in the soluble domain of the major anaerobically induced outer membrane protein (AniA) from pathogenic Neisseria gonorrhoeae (AniA) (38).

Mechanism of nitrite reduction

The Type I copper in the first domain (Domain 1) functions as an electron acceptor from electron donor proteins such as azurin, pseudoazurin, and CYTC and then transfers an electron to the Type II copper site, which catalyzes one electron reduction of nitrite to NO (33, 39–41). MCOs have eight histidines ligating to the tri-nuclear copper cluster at the active site; NIR contains only four histidines (Figures 4B and 6). The tetrahedral Type II copper site is formed by three histidines, one of which belongs to an adjacent monomer. The fourth histidine is located nearby, but is not involved in the ligation to the copper site. In addition to the fourth histidine, substitutions from potential copper ligand histidines to hydrophobic valine and isoleucine (Val304 and Ile257), and aspartate (Asp98) are likely to be responsible for the nitrite reduction. A hydrogen bond network including the aspartate and histidine residues around the Type II copper functions as the proton donor.

Although the oligomeric states are different between CuNIR and MCOs, the domain orientation of CuNIR is similar to those of MCOs. It is possible to superpose NIR on the N-terminal two domains (Domains 1 and 2) of AO. At this time, the C-terminal domain (Domain 3) and the tri-nuclear copper cluster of AO are superposed on the neighboring Domain 2 and the Type II mono-nuclear copper of CuNIR, respectively (Figure 4B). The relationship of two domains from neighboring monomers of CuNIR is the same as the three-dimensional relationship of Domains 1 and 3 of AO. Two copper sites are connected through the conserved sequence segment (His-Cys) with MCO (Figure 4B). The cysteine ligates the Type I copper and the histidine ligates Type II copper. These structural features indicate that they share common intramolecular electron transfer reactions.

N- and C-terminal extended NIR

Recently, the structure of a new type of CuNIR was reported (42). The NIR from a methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans* (HdNIR), has a larger molecular mass, with an additional cupredoxin-like domain at the N-terminus (N-terminal extended NIR as shown in Figures 6 and 7). HdNIR is composed of a 15-kDa N-terminal cupredoxin-like domain with a Type I copper binding site and a 35-kDa CuNIR motif. The core structure is the same with well-characterized CuNIR, a trimer of two consecutive cupredoxin-like domains. HdNIR is organized into a unique hexameric architecture, a dimer of the trimers, which is also maintained in solution. A head-to-head interaction between the extra N-terminal domain is observed in the hexameric formation of HdNIRs. Type I copper in the extra N-terminal domain is essential for dimerization of the trimers, which contributes molecular structural stabilities. Both Type I sites (in the extra N-terminal domain and in the 35-kDa CuNir motif) will accept an electron from electron donor proteins. However, the reduction of the Type I sites in the N-terminal domain seem to be unfavorable to catalytic reaction. Furthermore, another type of CuNIR with an additional domain like HdNIR was found in a genomic database (43). They are C-terminal extended NIRs with an attached CYTC-like domain. The CYTC-like domain could be acting as an electron donor to the Type I copper. The role of those additional domains is still unclear. Fusion of N-terminal cupredoxin-like and C-terminal CYTC-like domains would occur after the emergence of the well-characterized CuNIR (Figures 6 and 7).

Two-domain type MCO (2dMCO)

Owing to their broad substrate range, laccases are implicated in a variety of biotechnological applications related to the development of various industrial oxidative processes. It has stimulated efforts to discover new types of laccases. Among the newly identified laccases, some have small molecular

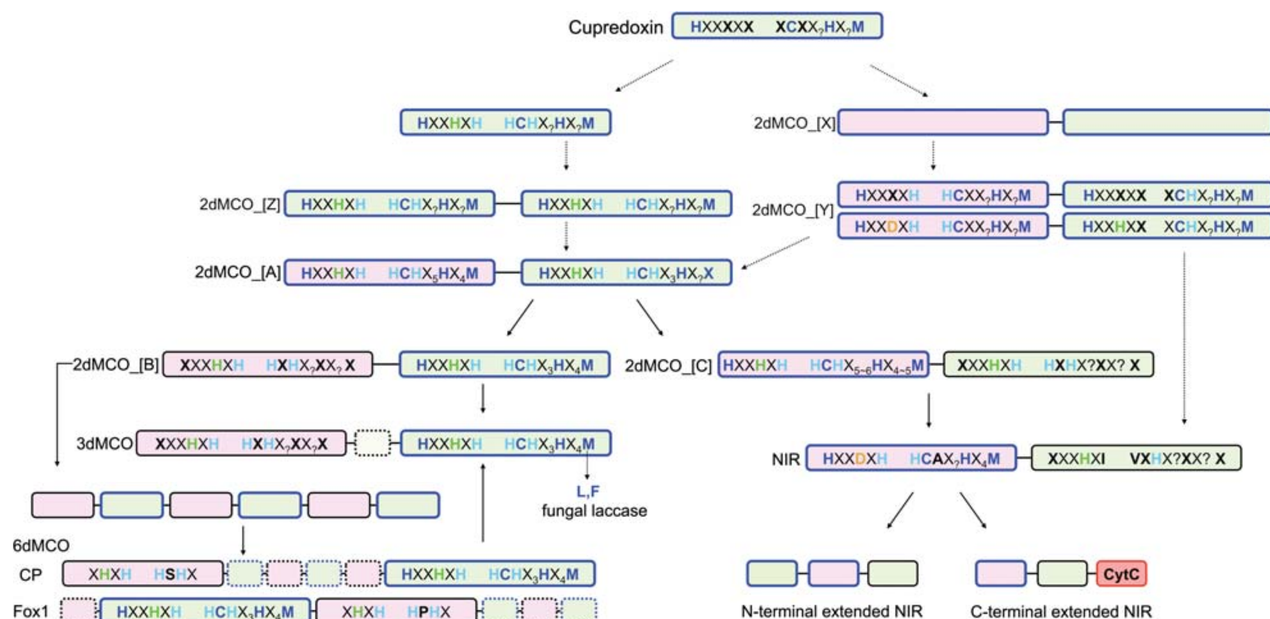

Figure 6 Proposed trajectory for the evolution of the domain structures of multicopper proteins.
The first (Class IV) and second (Class V) domains are shown in pink and light green. The residues binding to the Type I copper are shown in blue. The residues binding to Type II and Type III coppers in MCOs are shown in green and cyan, respectively. The Type II coppers in NIR are shown in cyan. The catalytic Asp residue for NIR is shown in orange. Blue outlines indicate the cupredoxin-like domains containing Type I copper. Dotted outlines indicate the domains lacking the Type II and Type III ligands. The CYTC-like domain is shown in red.

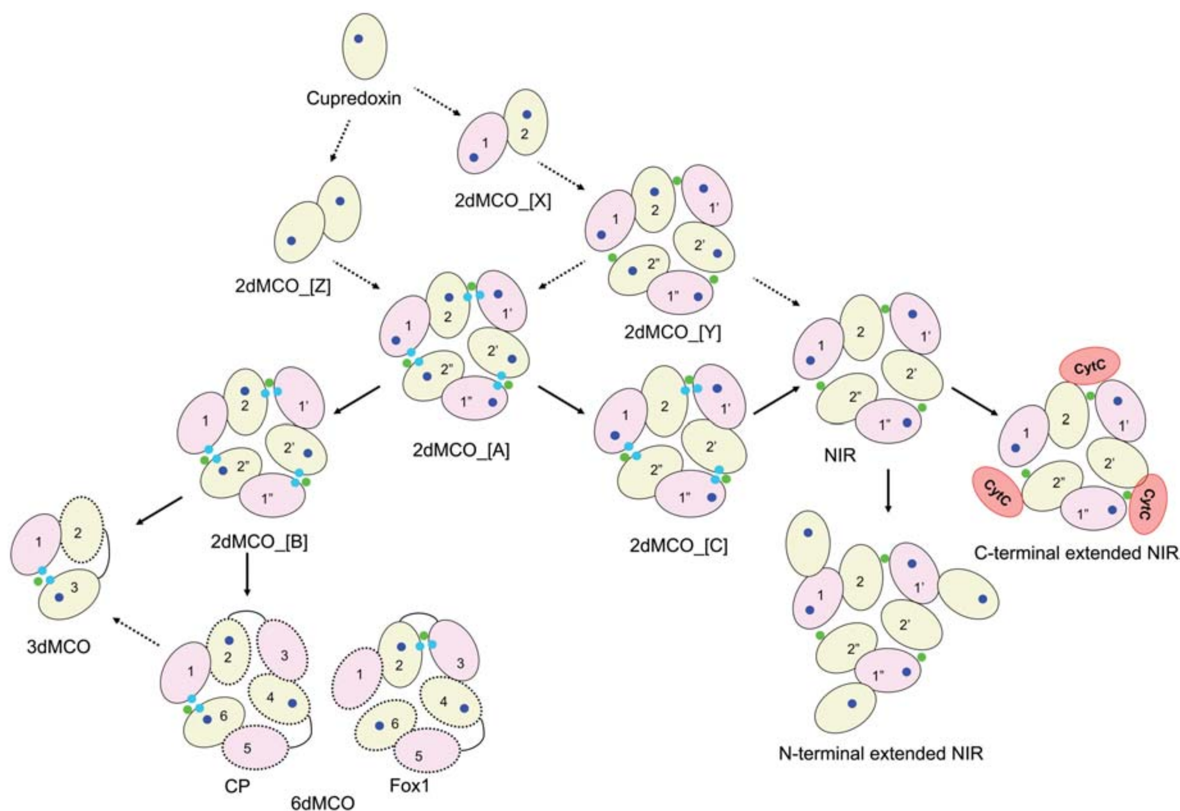

Figure 7 Schematic presentation of the molecular evolution of multicopper proteins. The color code is the same as those used in Figures 3 and 6.

weights between 30 and 40 kDa, whereas the majority of laccases found so far are 3dMCOs, which usually have a relative molecular mass of more than 50 kDa. The small molecular weight laccases identified to date are SLAC (small laccase from *Streptomyces coelicolor* (15)), EpoA from *Streptomyces griseus* (14), and mgLAC (laccase from metagenome of sludge) (44). A unique blue copper oxidase (BCO) was also purified from *Nitrosomonas europaea* more than 20 years ago (45). Biochemical studies revealed that BCO was trimeric and displayed laccase activities. However, a detailed characterization remained unclear for a long time. They share significant sequence similarities with each other and are grouped into the new type MCOs with two cupredoxin-like domains.

The crystal structures of the three types of 2dMCOs have been reported in 2009 (SLAC (46), BCO (47), and mgLAC (48)). They revealed a surprising homologous oligomeric state with NIR (Figure 3D and E). They all have a trimeric structure, uncommon in the most typical MCOs, in which a monomer contains four copper atoms (Type I copper and tri-nuclear copper cluster by Types II and III). Each monomer is composed of two consecutive cupredoxin-like domains (Domains 1 and 2) similar to that of NIR. The quaternary structure, consisting of six cupredoxin-like domains, is also similar to those of NIR and CP. An individual domain of 2dMCOs has the same topology as those of other MCOs and NIR. 2dMCOs contain a tri-nuclear copper cluster at the intermolecular interface, whereas a mono-nuclear Type II copper occupies this position in NIR (Figure 4C and D). Despite the overall structural similarity, the positions of Type I coppers are different in 2dMCOs. BCO and mgLAC have a Type I copper at the N-terminal domain (Domain 1), whereas SLAC has one at the C-terminal domain (Domain 2). The Type I copper site is important for substrate binding; therefore, the substrate-binding mode of BCO and SLAC are thought to be completely different from the other laccases. The physiological role of the 2dMCOs is still not clear; however, their substrate specificities are similar to three-domain type laccases.

Other small laccases, where molecular weight as a monomer is obviously smaller than typical 3dMCOs, have been purified from the fungi *Pleurotus pulmonarius* (49), *Tricholoma giganteum* (50), *Pleurotus eryngii* (51), and *Cantharellus cibarius* (52). These enzymes seem to consist of two domains. It was reported that some of them form homodimers to exploit the function; however, it is likely that they would need to assemble into the same homotrimer architecture as 2dMCOs. Interestingly, SLAC and mgLAC were also thought to be a dimer in solution by biochemical analysis as well as NIR, until their crystal structures had been solved (7, 15).

Molecular evolution of multicopper blue proteins (MCBPs)

Almost three decades ago, an evolutionary relationship between 3dMCOs and 6dMCOs was suggested based on typical Type I copper sequences (53). It was also predicted that the common ancestral oxidase would consist of only two cupredoxin-like domains by duplication of a single domain (11). Since then, the structure and sequence of NIR revealed that each of the two domains is similar to AO and small electron transfer proteins (6, 7). The sequence and structural similarities to AO and sequence similarity to CP lead to a plausible model for the domain structure of CP. The crystal structure of CP confirms that six domains of CP are arranged like those of the trimeric NIR, and the structure of AO can be thought of as three of the six domains of CP (30). Further sequence and structure based phylogenetic analysis suggested the possibility of an evolutionary and functional relationship between MCOs and NIR (8). Interestingly, the cupredoxin-like domains of NIR, AO, and CP were divided into two different classes, Class IV and Class V, based on structural similarity (8). The first domains (Domain 1) of NIR and AO and Domains 1, 3, and 5 of CP make up the one class (Class IV), and the remaining domains of CP, Domain 2 of NIR, Domains 2 and 3 of AO form another class (Class V) (Figure 3). The structural similarity of the cores of these domains does not correlate with copper content. This classification of the domains into two classes is consistent with the domain organization (Class IV and Class V domains are colored in pink and green, respectively, in Figures 3, 4, 6 and 7). Rusticyanin is a close relative of the Class V domains and the biochemical properties suggest that it is a possible candidate as the common ancestor (8, 54).

High conservation of copper-binding motifs in cupredoxin-like domains allows for easy identification of copper proteins based solely on the amino acid sequence. A recent increase in the amount of information available in genome databases has been used to explore the structural variation of MCOs (12, 55). In addition to well-characterized MCBPs, such as 3dMCO, 6dMCO, and NIR, three types of 2dMCOs were identified in genome databases, designated as Type [A], Type [B], and Type [C] in Figures 6 and 7. They are supposed to have a tri-nuclear copper cluster like 3dMCO and 6dMCO. Type [A] has Type I coppers in both cupredoxin-like domains, whereas Types [B] and [C] have Type I copper in C-terminal (Domain 2) and N-terminal (Domain 2) domains, respectively. The hypothetical 2dMCOs, Types [X] and [Y], were also proposed as ancestral proteins. Type [Y] is supposed to have a Type II mono-nuclear copper site like NIR, whereas Type [X] has no Type II/III coppers but does have Type I coppers. The proposed evolutionary pathway is from Type [X] to Type [A] through Type [Y]. In the course of evolution by duplication of the cupredoxin-like domain and by obtaining additional copper binding sites, the first ''prototype'' oxidase, Type [A], would be created (Figures 6 and 7). The loss of Type I copper appears to have occurred at multiple stages, and then Types [B] and [C] would be created as intermediate structures for 3dMCO, 6dMCO, and NIR. Structure analysis has confirmed that SLAC belongs to Type [B], and BCO and mgLAC belong to Type [C], respectively. It is likely that Type [C] is the closest to the ancestor of NIR; however, it still remains unclear whether NIR would

have evolved from the ''prototype'' oxidase Type [A] or directly from Type [Y] before the creation of the tri-nuclear copper cluster. It is reported that the structure of BCO is closer to that of 3dMCOs than NIRs (47). The Type II copper at the active site of NIR corresponds to that of the Type III copper of MCOs (Figure 4). To acquire the oxidase function of Type [A] from an NIR-like trimer, Type [Y], mutations to create an eight-histidine coordination system are required. An alternative evolutionary pathway is also proposed. The first ''prototype'' oxidase, Type [A], would be created by duplication of the cupredoxin-like domain, which already has the four histidines required for a tri-nuclear copper cluster, (Type [Z]), as shown in Figures 6 and 7 (56). It seems reasonable to consider that the evolution could be directed toward an asymmetric structure such as a mono-nuclear active site of NIR from a symmetric structure like the tri-nuclear cluster of Type [A]. In BCO and SLAC, the active site is close to symmetric and there are vestigial histidine residues at Domain 1, which no longer participate in copper binding (Figure 6). These vestiges indicate that Type [C] is most likely derived from the Type [A].

The position of Type I copper site does not seriously affect the intramolecular electron transfer to the tri-nuclear copper cluster, because the electron transfer pathway through the sequence segment (His-Cys) is conserved in all MCOs and NIRs. By contrast, the position of the Type I copper site should be crucial for substrate binding and specificity. In MCOs, only the Type [C] 2dMCO has Type I copper at the N-terminal domain (Domain 1), as NIR does. The electron donors for NIR are not small organic compounds but small electron transfer proteins. It is reported that BCO has nitrite reductase activity using cytochrome *c*-552 as an electron donor. There has been no experimental evidence showing that other Type [C] 2dMCOs, such as mgLAC, uses small electron transfer proteins as an electron donor and participates in nitrite reduction. The role of the position of Type I copper might be related to the selection of the electron donor; however, it remains to be elucidated.

Some fungus laccases have leucine or phenylalanine residues at the Type I copper site instead of the methionine typically found in Type I copper proteins, in which the Type I copper exhibits a planar triangular coordination with the above-mentioned single cysteine and two histidine residues (4, 17, 20). They have very high redox potential relative to other MCOs. By contrast, Stellacyanin, which is not an MCO, but a small electron transfer protein, has glutamine as an axial ligand (57), and has unusually low redox potential. Therefore, axial coordination of Type I copper has been considered to be one factor affecting the redox potential (58). Laccases have the capability to oxidize recalcitrant aromatic compounds and cover a wide range of redox potential. The variation of the axial coordination occurs in order to modulate the redox potential of Type I copper. The divergence of the Type I copper ligands could have emerged at a later stage in the evolution of MCO.

The tri-nuclear copper cluster of all structure-determined MCOs was located at the interface of the N-terminal domain and C-terminal domain. Recently, new type of 6dMCO, Fox1, has been found (56). Interestingly, Fox1 appears to have a tri-nuclear copper cluster at the interface of Domains 2 and 3. Fox1 also has three Type I coppers in Domains 2, 4, and 6, as seen in CP. These structural features could be the result of the fact that the evolutionary unit was the two domains (Figures 6 and 7).

Summary

Structural studies of 2dMCOs will help to extend our understanding of the molecular evolution of the MCBP family. 2dMCOs share important structural and functional aspects of MCOs, such as the positioning of the Type I site, and the electron transfer pathways between Type I copper and the active site. It seems that all MCBPs including 2dMCOs utilize similar intramolecular electron transfer steps, which force the molecular architecture to keep a common fold to ensure an optimal arrangement of the essential copper sites. The structures of 2dMCOs also reveal the intermediate structures between typical MCOs and NIRs. It suggests that MCBPs would have diverged from the two consecutive cupredoxin-like domains. The first step of evolution would be the duplication of the cupredoxin-like domain. The gene duplication of the cupredoxin-like domain would induce the dimer precursor, in which two domains are very similar, and the trimeric oligomerization would induce the symmetric interface suitable for the new copper binding site, like a tri-nuclear copper cluster. It increases the stability of the overall molecular structure and also results in the creation of the characteristic tri-nuclear copper cluster. The acquisition of a new type copper-binding site is a significant step for MCBP evolution. The homotrimeric architecture would be essential for the evolution of a new catalytic property.

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