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Review

Function and molecular evolution of multicopper blue proteins

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Abstract. Multicopper blue proteins (MCBPs) are multidomain proteins that utilize the distinctive redox ability of copper ions. There are a variety of MCBPs that have been roughly classified into three different groups, based on their domain organization and functions: (i) nitrite reductase-type with two domains, (ii) laccase-type with three domains, and (iii) ceruloplasmin-type with six domains. Together, the second and third group are often commonly called multicopper oxidases (MCOs). The rapid accumulation of genome sequence information in recent years has revealed several new types of proteins containing MCBP domains, mainly from bacteria. In this review, the recent research on the functions and structures of MCBPs is summarized, mainly focusing on the new types. The latter half of this review focusses on the two-domain MCBPs, which we propose as the evolutionary intermediate of the MCBP family.

Key words. Multicopper blue protein (MCBP); multicopper oxidase (MCO); blue-copper-binding site (BCB site); cupredoxin; nitrite reductase; laccase; ceruloplasmin; molecular evolution.

Introduction

Some metal ions are essential to life, and interest in the field of bioinorganic chemistry [1, 2] is growing, as increasing amounts of information about the sequences, structures, and functions of metalloproteins become available [3]. Copper is an essential element in all organisms, and there are many vital proteins that exert their functions by coordinating copper ions [4]. Despite the usefulness of copper as a redox mediator, stray copper ions can be harmful and the distribution of copper ions in living organisms is strictly regulated [5–7]. The copper homeostasis systems in bacteria (for reviews see refs 8, 9), *Escherichia coli* [10], *Enterococcus hirae* [11], cyanobacteria [12], and yeast [13] have been relatively well studied. Copper homeostasis in humans has also been extensively studied in relationship to human disorders, such as Wilson and Menkes disease [14]. The increasing number of structures of copper-transporting [15, 16] and storage proteins [17] determined in recent years has greatly enhanced our understanding of the detailed mechanisms of the metal regulation systems.

Multicopper oxidases (MCOs) are enzymes that utilize the unique redox property of the copper ion [18]. Among others, laccase [19], ascorbate oxidase [20], and ceruloplasmin [21, 22] are the most commonly recognized MCOs. Nitrite reductase is closely related to MCOs, in terms of both sequence and structural similarity, although

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it physiologically function as a 'reductase' [23]. In this review, we use the term 'multicopper blue proteins' (MCBPs) for the group of proteins including nitrite reductase and MCOs. MCBPs consist of tandem repeats of a homologous sequence domain, which shares distant homology to a single-domain cupredoxin-fold protein family, which includes plastocyanin, azurin, pseudoazurin, rusticyanin, stellacyanin, and amicyanin. A cupredoxin-fold domain usually carries a type 1 copper-binding site, which is responsible for the characteristic blue color of these proteins, and therefore the single-domain cupredoxin-fold proteins and MCBPs together are often called blue copper proteins, and the cupredoxin domain is called a blue copper-binding (BCB) domain. The BCB domain family includes a large number of quite diverse homologues [24], in terms of sequence similarity (as low as 10% sequence identity), while the overall structural fold of the domain, consisting of eight beta strands, is generally conserved.

With their distinctive blue color and other unique redox properties, blue copper proteins have attracted attention of many researchers, and have been studied quite extensively, even before the structural and/or sequence information became available. Reviews and books about this protein family have been published [18, 24, 25]. In the present review, we would like to focus on recent developments contributing to our understanding of the sequence-structure-function relationships of the MCBPs.

Rapid progress in recent genome analysis is providing a large number of protein sequences without any accompanying experimental characterization. The functions of these sequenced proteins can be annotated by homology with known proteins. Sometimes these sequences include novel ones, where the part of the sequence including functional residues or the domain organization is somehow different from the precedents. Such a 'variant' may possess a new kind of biological function, and in this review, some of these novel variants of MCBPs will be discussed. Among others, the emerging groups of two-domain MCBPs, which we propose as the evolutionary intermediates of MCBPs, are of particular interest, and we will focus on this type of protein in the latter part of the article.

Copper-binding sites of MCBPs

Historically, copper atoms coordinated in proteins have been classified into three types (type 1, type 2 and type 3) based on their spectroscopic properties [26]. In the UV–visible spectrum, type 1 (blue) copper shows maximum absorption around 610 nm, and type 3 copper shows maximum absorption around 330 nm. Type 1 and type 2 coppers are EPR (electron paramagnetic resonance) detectable, while the dinuclear type 3 coppers are EPR silent.

The type 1 copper-binding site consists of two histidines, one cysteine, and one methionine. The first three residues are essential for the blue copper-binding site and form a tight trigonal coordination with the copper ion, while the coordination of the fourth residue, an axial methionine, is rather distant and weaker, and this residue can be replaced with different amino acids, such as leucine or phenylalanine. Other than the type 1 copper-binding sites embedded within the domain, MCBPs possess copper binding sites between domains (interdomain copper- binding (IDCB) site), which consist of only histidine residues. In nitrite reductase, this interdomain site is a mononuclear type 2 copper coordinated by three histidines (two from the first domain and one from the second domain), and in the cases of MCOs such as laccase and ceruloplasmin, the interdomain site is trinuclear, composed of one type 2 and two type 3 coppers coordinated by eight histidines (four from each domain).

In MCBPs, the function of the blue copper is to accept an electron from (oxidize) a substrate, and the function of the interdomain copper is to donate the electron to (reduce) another substrate. Nitrite reductase accepts an electron from electron transporter proteins such as azurin and pseudoazurin to its blue copper, and reduces nitrite at the interdomain site. MCOs oxidize a wide variety of substrates, such as phenol, methoxyphenol, aromatic amines, polyaromatic compounds, and metal ions, in the vicinity of the blue copper, and donate electrons to molecular oxygen to convert it to water at the interdomain site.

The environment around the blue copper atom affects the electronic structure and the physical properties, such as the redox potential, of the blue copper. Blue copper proteins use several strategies to control the electronic environment of the copper ion. One of the methods is to modify the loop region around the metal-binding residues. There are several variations in the metal-binding loop of blue copper proteins, and the effects of switching this region from one protein to another have been reported [27, 28]. Another simple strategy taken by the blue copper proteins is to replace the axial ligand residue, which typically is a methionine. The classification of laccases according to the kind of axial ligand and its correlation with the physical properties has been discussed [29]. Detailed analyses of the electronic structure, from both theoretical and spectroscopic points of view, have been carried out with mutations of the axial residue in nitrite reductase [25, 30] and laccase [31].

Nitrite reductase (two domains)

Nitrite reductase consists of two consecutive MCBP domains. It forms a homotrimer with the IDCB sites shared between the chains as shown on the top right of figure 1 [32–34]. This scheme showing a hypothesis for the evolu-

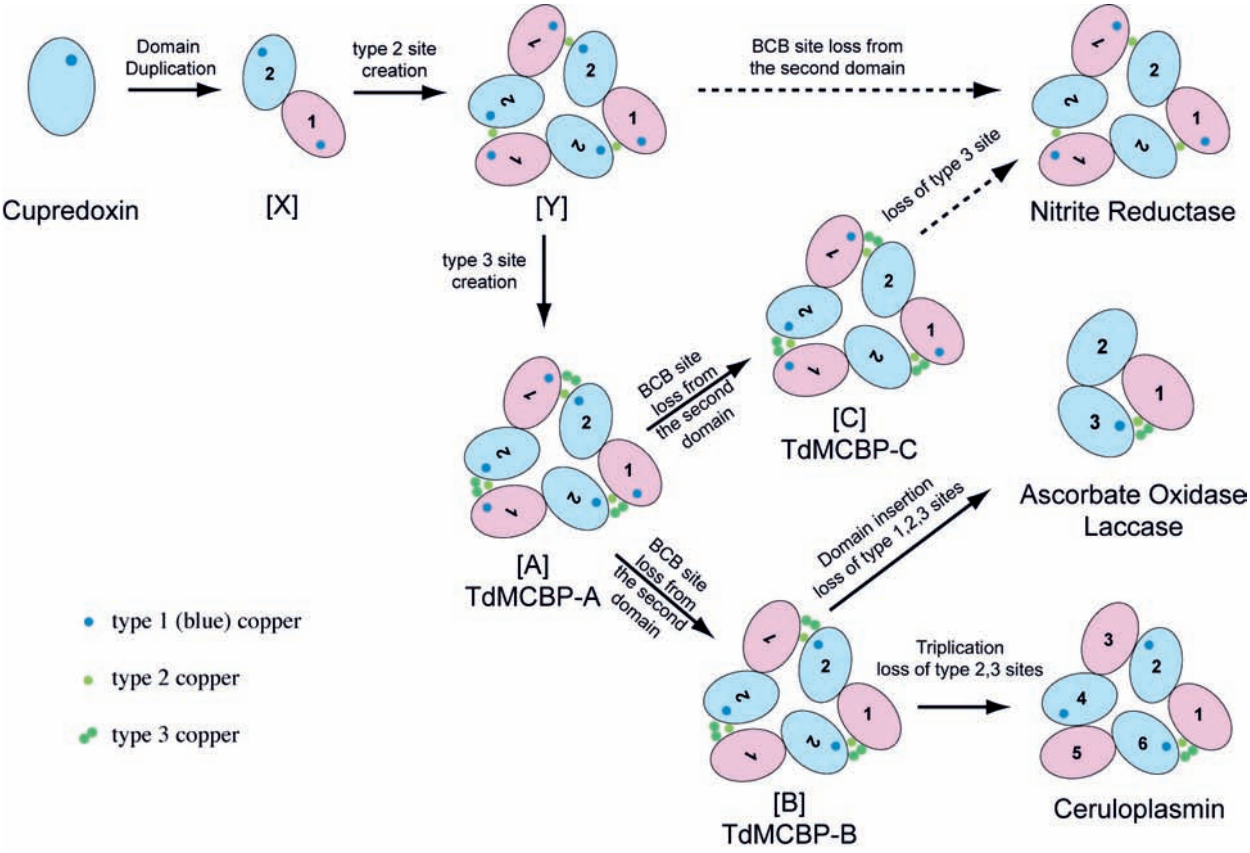

Figure 1. Schematic presentation of domain organization of the MCBPs. An oval shape indicates a BCB domain. Pink and blue colors show the domain classes IV and V [117]. Blue and green dots indicate type 1 and 2/3 copper atoms, respectively. Solid arrows indicate the postulated evolutionary pathways and dotted arrows indicate alternative evolutionary pathways. Conventional MCBPs are shown on the right. Five hypothetical proteins are shown with bracketed labels. Three of them ([**X**], [**Y**], and [**A**]) have BCB sites in both domains, whereas [**B**] and [**C**] have a BCB site only in the first and second domain, respectively. The hypothetical proteins [**A**], [**B**], and [**C**] have trinuclear IDCB sites, whereas [**Y**] has a mononuclear IDCB site. This figure was modified from the figure 2 in Nakamura et al. [118], and is used by courtesy of *FEBS Letters*.

tionary pathway of MCBPs will be described later. A phylogenetic tree including all of the known nitrite reductase sequences is shown in figure 2. Nitrite reductase is responsible for a crucial step (reduction of nitrite to nitric oxide) in the denitrification process, which is very important for the nitrogen distribution cycle in nature. To reduce the nitrite, the nitrite reductase accepts an electron from electron transporting proteins, azurin or pseudoazurin. The binding surface of pseudoazurin in the 152-kDa complex with nitrite reductase has been mapped based on the nuclear magnetic resonance (NMR) measurement [35]. A site-directed mutation of nitrite reductase to improve the interaction surface with pseudoazurin enhances the intermolecular electron transfer process [36].

Several nitrite reductases with an extra cupredoxin (plastocyanin-like) domain at the N-terminus have been found [37–39] (fig. 3). The function of this additional cupredoxin domain is not known. Crystallization of one of these proteins was reported recently and, the crystal structure should therefore soon be available [40]. There is also a protein (YP_054810) with another additional region (~500 amino acids) at the N-terminus, which is predicted to consist of ten transmembrane helices (fig. 3).

Two sequences (YP_105511 from *Burkholderia mallei* and YP_111458 from *B. pseudomallei*) of the nitrite reductases shown in figure 2 (underlined) have one of the histidines at the interdomain site mutated to arginine (fig. 4, NRMR). Since the histidine that binds the type 2 copper is considered to be essential for the function of nitrite reductase, these sequences may have altered functions, or may be using another histidine in the vicinity (most likely the conserved histidine at position 347 of NRMR in fig. 4) for the type 2 copper binding.

Recently, the high-resolution X-ray crystal structures of nitrite reductase with nitrite and with nitric oxide have been solved [41]. Nitric oxide coordinates the blue copper atom in the side-on manner. With this accurate structural information, the detailed molecular mechanism of nitrite reductase function has become clearer, as some of the essential residues for its function have now been identified.

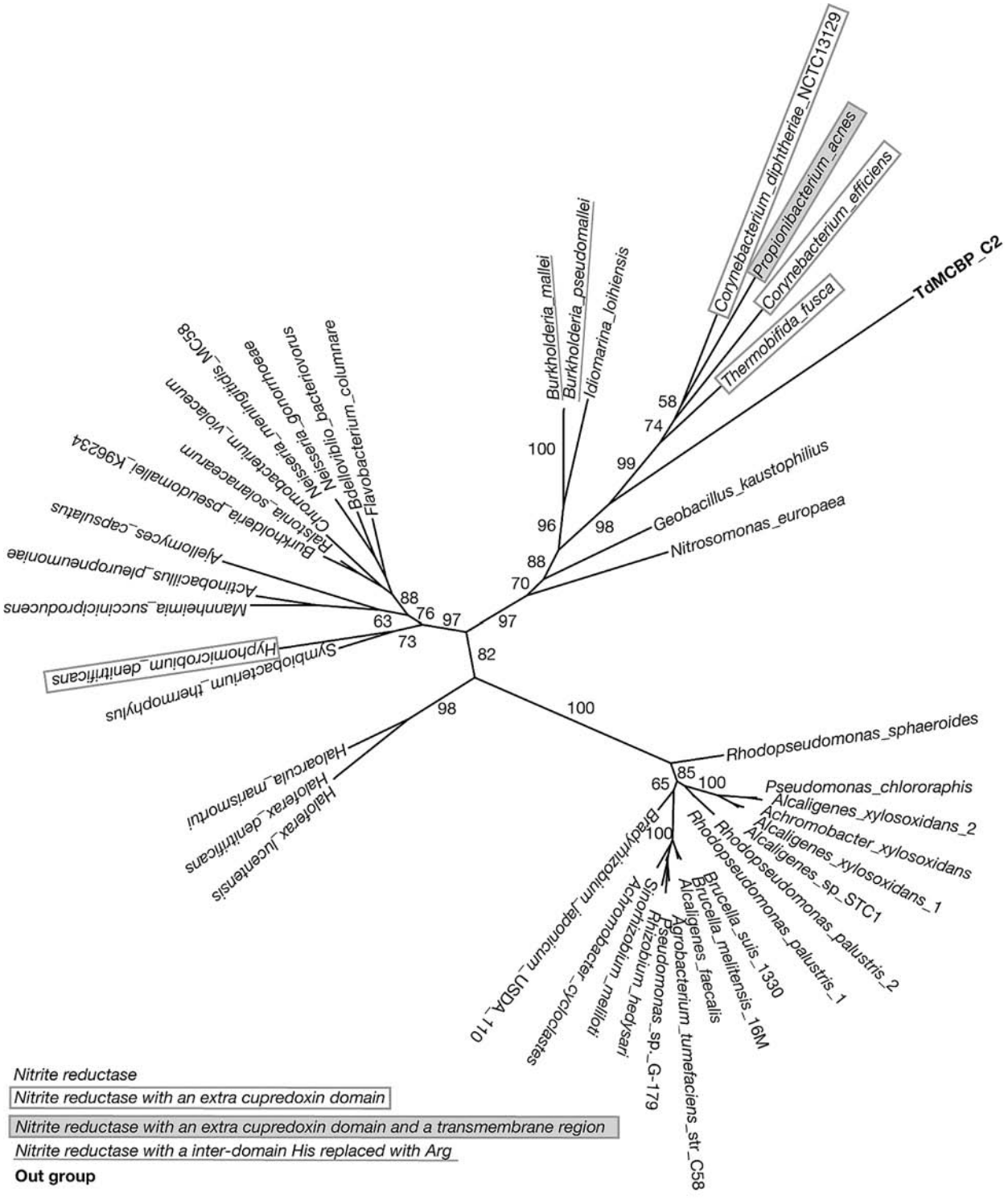

Figure 2. Phylogenetic tree of nitrite reductases. Each sequence is denoted by the name of the source organism. TdMCBP_C2 is included as an outgroup. Nitrite reductases with an extra cupredoxin domain at the N-terminus are boxed, and the one with an additional transmembrane helix is shaded. Nitrite reductases with a histidine residue at an interdomain site mutated into arginine are underlined. Numbers on branches are bootstrap values. The phylogenetic tree was drawn with the same method described in our previous publication [118]. The multiple sequence alignment used for the generation of the tree is available at http://yayoi.apr.jaeri.go.jp/qbg/ kenske/CMLS.

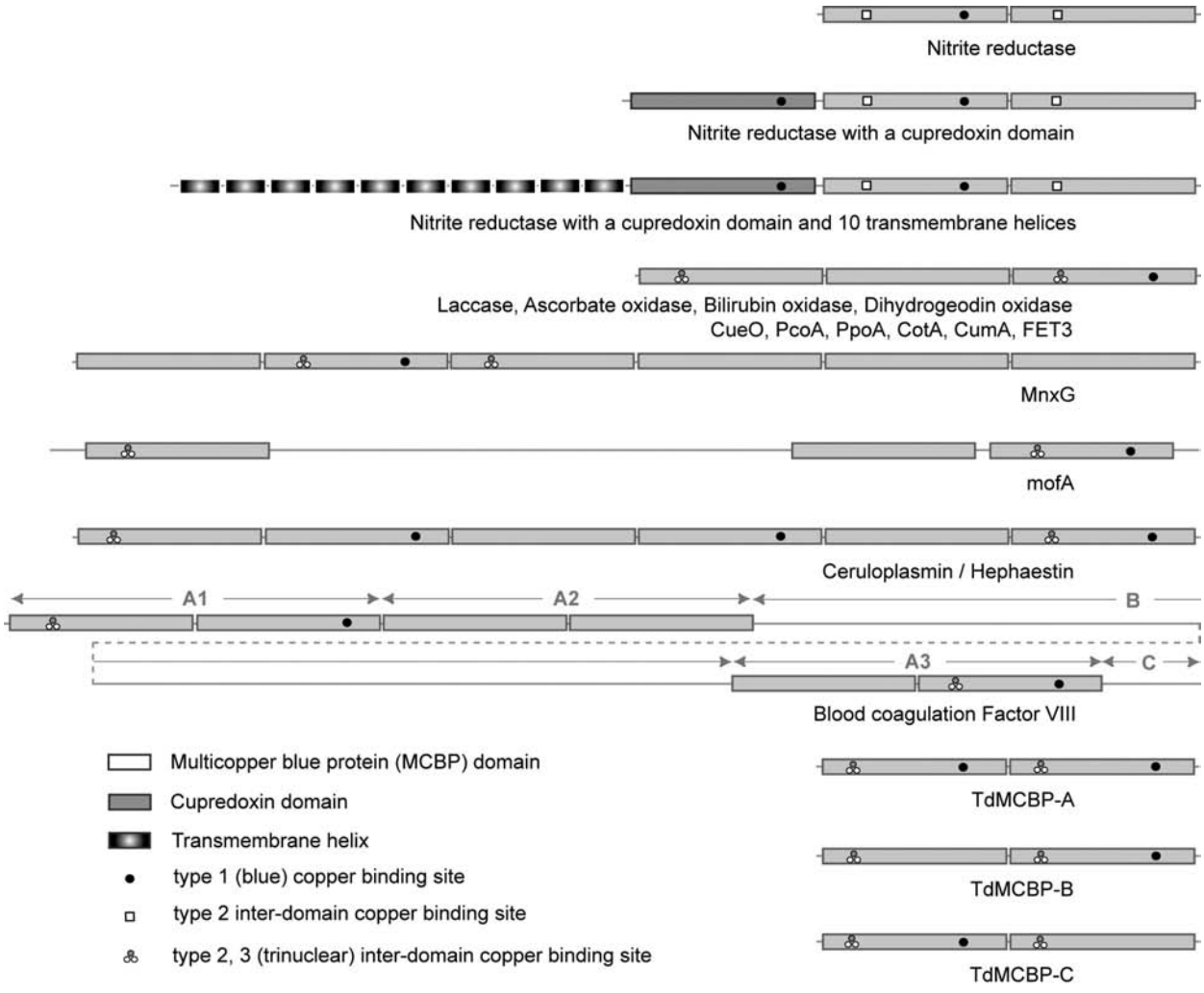

Figure 3. Schematic presentation of various domain organizations of MCBPs.

Three-domain MCBPs

Among the MCBPs, three-domain MCBPs form the most diverse group, in terms of their sequences, functions, and distribution in organisms. They are found in fungi, plants, and insects [42, 43] and are also widely distributed in bacteria [44–46]. The phylogenetic tree of the three-domain MCBPs can be roughly divided into three regions, with each region corresponding to the kind of source organism, i.e., bacteria, plants, and fungi (including insects) (fig. 5). The following sections describe, the recent findings for each of the three-domain MCBPs.

Laccase

Laccases are three-domain MCBPs found in fungi (*Polyporus*) and trees (Rhus). One of the functions of the enzyme is supposed to be the polymerization of lignans (phenol) to yield lignin (polyphenol), which form the cell wall of plants, and to decompose the lignin in the tree-rotting process by fungi. Although the name laccase originated from lacquer from plants, the three-domain MCBPs from other organisms, such as bacteria and insects, are generally called laccases, as long as they have an oxidase activity toward aromatic compounds.

Fungal laccase is a secreted enzyme that must function in various environments. Therefore, there are fungal strains that have evolved to be resistant against high temperature [47], high or low pH [48], and various harsh environmental conditions. Their ability to function in different conditions is also important in terms of industrial applications. Laccase has been used for many applications such as bleaching, organic synthesis, bioremediation, and in laundry detergent. For such purposes, the enzyme must have the ability to function under unusual conditions [49, 50]. For example, a detergent additive has to endure highly alkaline conditions. The optimal pH of

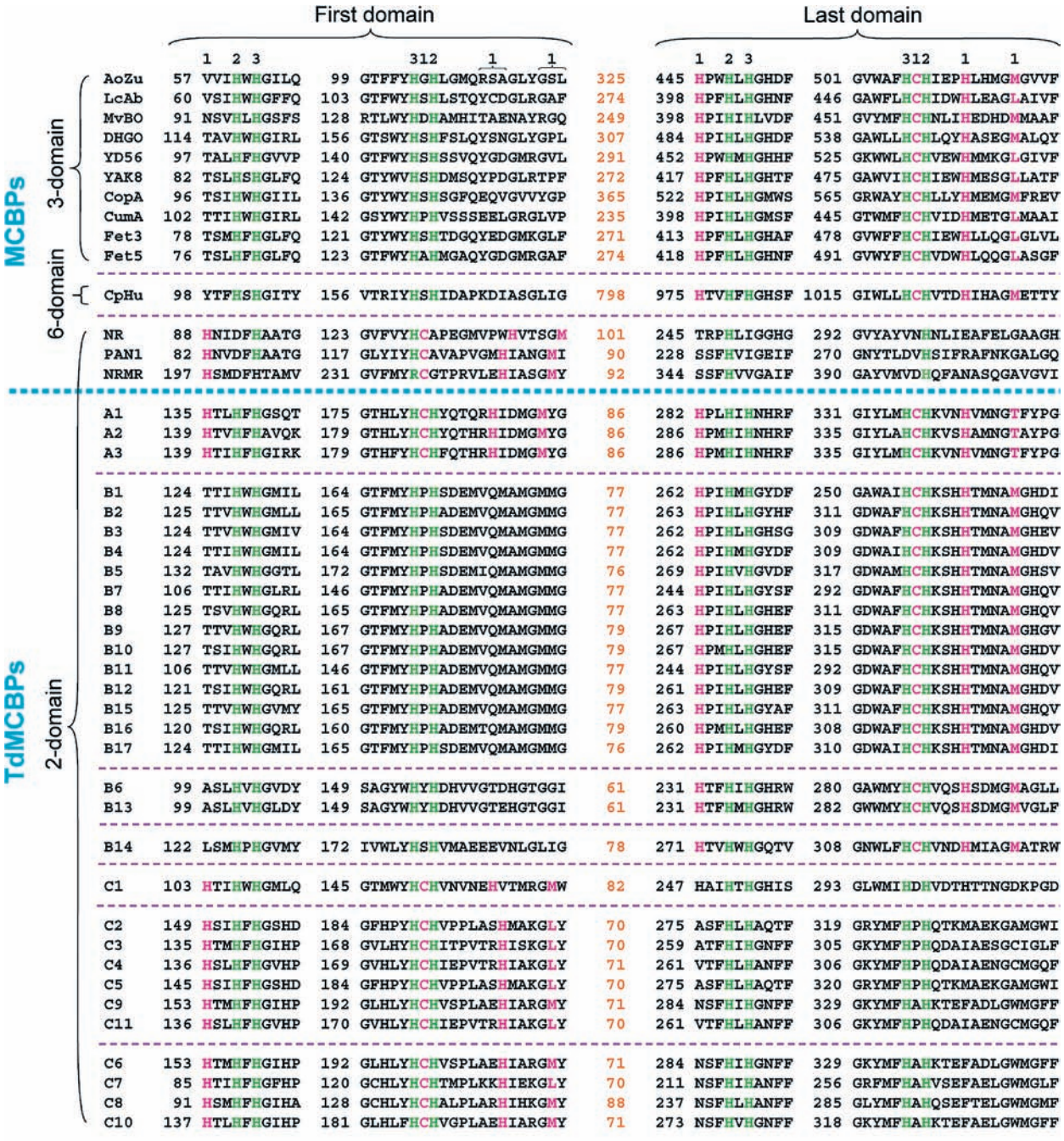

Figure 4. Sequence alignment around the copper-binding sites in MCBPs. The sequences in the upper part (above the blue dashed line) are of conventional MCBPs, and those in the lower part are of the two-domain MCBPs (TdMCBPs). The TdMCBPs are clustered into seven groups, according to sequence similarity, and are separated by purple dashed lines. The numbers 1, 2, and 3, above the alignments, indicate the consensus positions of the type 1, type 2 and type 3 copper-binding residues, respectively. The residues colored in red are of type 1 (blue) sites. The residues colored in green are of type 2/3 IDCB sites. Two sequence fragments, from both the first and last domains of the molecules, are indicated. In the cases of the three-domain and six-domain MCBPs, the last domain is the third and sixth domain, respectively. Numbers to the left of each sequence fragment show the first residue position of the fragment. The yellow number in the middle column is the number of residues between the second and third fragments. The sequence ID (SWISS-PROT ID or NCBI accession number), the common name (if any), and the origin of each sequence are listed below. AoZu: ASO_CUCPM, ascorbate oxidase from *Cucurbita pepo* var. *melopepo*; LcAb: LAC1_AGABI, laccase from *Agaricus bisporus*; MvBO: BLRO_MYRVE, bilirubin oxidase from *Myrothecium verrucaria*; DHGO: BAA08486, dihydrogeodin oxidase from *Aspergillus terreus*; YD56: YD56_YEAST, YD56 from *Saccharomyces cerevisiae*; YAK8: FIO1_SCHPO, YAK8 from *Schizosaccharomyces pombe*; CopA: CPA1_PSESM, CopA from *Pseudomonas syringae*; CumA: NP_743195, CumA from *Pseudomonas putida*; Fet3: FET3_YEAST, ferric reductase from *Saccharomyces cerevisiae*; Fet5: P43561, ferric reductase from *Saccharomyces cerevisiae*; CpHu: CERU_HUMAN, ceruloplasmin from *Homo sapiens*; NR: NIR_ACHCY, nitrite reductase from *Achromobacter cycloclastes*; PAN1: ANIA_NEIGO, PAN1 from *Neisseria gonorrhoeae*; NRMR: YP_105511,

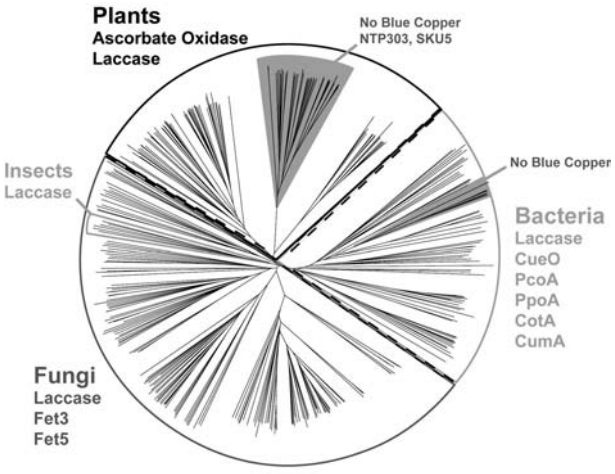

Figure 5. Phylogenetic tree of three-domain MCBPs. Branch regions were separated according to the kind of source organisms, i.e. plant, fungi, bacteria, and insect. Regions of sequence without type 1 copper-binding residues are shaded. The multiple sequence alignment and the construction of the phylogenetic tree were carried out using the ClustalW program (http://www.ebi. ac.uk/clustalw/).

laccase from *Rhus vernicifera* is 9.0, while most laccases work best in the pH range of 4.0 ~ 5.0 [51]. Understanding the mechanism of such an evolutionary adaptation may lead to the development of methods to introduce desired characteristics, such as thermal stability or durability under highly acidic/basic conditions, to other enzymes [52].

Extensive X-ray crystal structure analyses of laccases have been performed. The type 2 copper-depleted form was solved first [53, 54], and then structures with all four coppers per chain followed [55, 56]. One of the structures includes an aryl-amine bound in the substrate-binding pocket close to the type 1 site [57]. The latest structure provides a detailed picture of the type 3 copper-binding sites [58].

White laccase

An organism often contains several isoenzymes, which may have different biological roles, or different optimal functioning conditions. For example, *Coprinopsis cinerea* contains as many as eight paralogues of laccase [59]. One of the laccases extracted from cell culture of a white rot fungus, *Pleurotus ostreatus,* lacks the characteristic blue color of MCBPs, and has no type 1 (blue) copper [60]. Intriguingly, instead of the regular four copper atoms per chain, this white laccase contains two zinc ions, one iron ion and one copper ion, and yet it still exhibits the laccase activity. The amino acid sequence revealed that all of the copper-binding residues of laccase are conserved. Another white laccase, extracted from *Phellinus ribis*, binds two zinc ions, one manganese ion and one copper ion, and can oxidize various substrates [61]. Another strain of white laccase is found in *Volvariella volvacea* [62].

The presence of white laccase poses many questions. For example, if these transition metals are simply replacing the positions of copper in laccase, then which ion takes which position? Also, if some copper ions of laccase can be replaced with other metals, can the copper ions in other MCBPs, such as nitrite reductase, be replaced without losing the function?

For the first question, our current speculation is that the type 1 copper is replaced with Fe/Mn (so it loses the blue color), the type 3 coppers are replaced with zinc ions, and the type 2 copper remains.

Insect laccases

Intriguingly, laccase-type three-domain MCBPs also exist in insects. In the phylogenetic tree of the three-domain MCBPs, the laccases from insects form an independent group in the midst of the region of laccases from fungi (fig. 5). Two laccases from tobacco hornworm (*Manduca sexta*) and one from malaria mosquito (*Anopheles gambiae*) have been cloned and characterized [42]. The major difference between these laccases and those from fungi

Figure 4 (continued)

nitrite reductase from *Burkholderia mallei*; A1: NP_280081 from *Halobacterium* strain NRC-1; A2: YP_136013 from *Haloarcula marismortui* ATCC43049, A3: YP_134324 from *Haloarcula marismortui* ATCC43049, B1: Q92S43 from *Sinorhizobium meliloti*; B2: NP_534477 from *Agrobacterium tumefaciens* strain *C58*; B3: ZP_00283273 from *Burkhoderia fungorum*; B4: NP_768850 from *Bradyrhizobium japonicum* USDA 110; B5: ZP_00052601 from *Magnetospirillum magnetotacticum*; B6: BAB64332 from *Streptomyces griseus*; B7: ZP_00217852 from *Burkholderia cepacia* R18194; B8: ZP_00201899 from *Methylobacillus flagellatus* KT; B9: ZP_00244538 from *Rubrivivax gelatinosus* PM1; B10: AAP42069 from *Xanthomonas campestris* pv. vesicatoria; B11: ZP_00225043 from *Burkholderia cepacia* R1808; B12: ZP_00241821 from *Rubrivivax gelatinosus* PM1; B13: NP_630785 from *Streptomyces coelicolor* A3 (2); B14: NP_841583 from *Nitrosomonas europaea* ATCC 19718; B15: YP_110478 from *Burkholderia pseudomallei* K96243; B16: ZP_00361057 from *Polaromonas* sp. JS666; B17: NP_435809 from *Sinorhizobium meliloti* 1021; C1: NP_841001 from *Nitrosomonas europaea*; C2: NP_711736 from *Leptospira interrogans* serovar lai strain; C3: ZP_00328133 from *Trichodesmium erythraeum IMS 101*; C4: NP_487982 from *Nostoc* sp. PCC 7120.; C5: YP_002150 from *Leptospira interrogans* serovar *Copenhageni* str. Fiocruz L1-130; C6: AAP73980 from *Rhodococcus erythropolis*; C7: ZP_00187649 from *Rubrobacter xylanophilus* DSM 9941; C8: ZP_00196773 from *Mesorhizobium* sp. BNC1; C9: NP_926236 from *Gloeobacter violaceus* PCC 7421; C10: YP_121950 from *Nocardia farcinica* IFM 10152; C11: ZP_00159845 from *Anabaena variabilis* ATC. This figure was modified from figure 1 in Nakamura et al. [118], and is used by courtesy of *FEBS Letters*.

and plants is the longer amino-terminal sequence with several conserved cysteine, aromatic and charged residues. The main function of the laccase-type proteins in insects is supposed to be sclerotization of the cuticle in the epidermis [63]. The oxidative activity of this enzyme for various catecholic substrates has been observed [64, 65]. These laccase-type enzymes are mainly distributed in the epidermis of the insect. However, they are also found in the midgut, Malpighian tubules, and colleterial gland. The function of the enzyme in these internal organs is not yet understood. Recently, the expression of laccase in parasitoid wasp venom was identified [43].

Ascorbate oxidase

Ascorbate oxidase is a three-domain MCBP found in higher plants [20]. The biological function of ascorbate oxidase is not fully understood [66]. The expression of ascorbate oxidase is upregulated by light [67] and by mechanical wounds [68], and it is most likely to be responsible for defense against oxidants, related to the function of ascorbate, or vitamin C. The degradation process of ascorbate was investigated to shed light on the detailed biological role of ascorbate in plants [69].

The crystal structure of ascorbate oxidase was one of the first of the MCBPs to be solved [70, 71]. The substrate-binding site for ascorbate oxidase was predicted by a docking study, and the binding of an inhibitor to the same binding site was confirmed by ^{19}F NMR relaxation measurments [72].

While most laccases fold and function as a monomer, for ascorbate oxidase, the formation of a homodimer is necessary for both its stability and function. Equilibrium denaturation experiments in urea and guanidine hydrochloride revealed the presence of a partially unfolded dimeric state, and the dissociation into monomers was followed by the partial unfolding of the tertiary structure, indicating that the monomer itself is very unstable [73].

Ascorbate oxidase homologues with no blue copper-binding site

There is a group of plant proteins that share 25–30% sequence idendity with ascorbate oxidase, but lack the copper-binding residues (fig. 5). Since these proteins lack both BCB residues and IDCB residues, the function of these proteins should be different from that of regular multi-copper blue proteins.

Bp10 from *Brassica napus* (ASO_BRANA), expressed in developing pollen, shows 30% sequence identity to ascorbate oxidase, but the copper-binding residues are not conserved [74]. The *NTP303* gene is related to pollen tube growth and is expressed abundantly during pollen development [75]. The *SKU5* gene from *Arabidopsis thaliana* codes another protein homologous to ascorbate oxidase, but it has no copper-binding center [76]. This protein is involved in directional root growth.

These MCBP homologues without copper-binding sites are often annotated as pectin esterase in sequence databases. The X-ray crystal structure of pectin esterase consists of only β sheets, like the MCBP domain structure [77]. However, pectin esterase has a quite different fold from that shared by the MCBPs, and the annotation might be erroneous.

Single-domain cupredoxin homologues without a BCB site, called nodulins and dinodulins, are involved in root development [24, 78–80]. The similarity of their function with *SKU5* is interesting, although it may be just a coincidence, since the regression of the BCB site must have occurred independently in *SKU5* and nodulins.

There is a group of laccases without copper-binding sites in bacteria (fig. 5). Obviously, the regressions of copper-binding sites also have occurred independently for these proteins in bacteria and the ascorbate oxidases in plants.

Copper resistance by MCBPs (CueO, PcoA)

E. coli is a model organism, and the functions of its genes have been extensively studied. The regulation of copper homeostasis in *E. coli* has been analyzed, although the details are still unclear. Copper ions are needed in aerobic metabolism, but can be toxic even at low concentrations. Therefore, the distribution of copper ions has to be strictly regulated in the cell. There are five essential elements (CopA, CusCFBA, CueO, PcoABCD and PcoE) known to be responsible for copper homeostasis in *E. coli* [10]. One of these elements functioning under aerobic conditions involves an MCBP CueO, which is regulated by CueR. CueO can catalyze the oxidation of *p*-phenylenediamine, 2,6-dimethoxyphenol, and like some other three-domain MCBPs, it has ferroxidase activity [81]. The ferroxidase activity of MCBPs is essential to iron homeostasis in eukaryotes, from yeast (Fet3) to higher vertebrates (ceruloplasmin) as will be discussed later [22]. Also, in a fungus called *Cryptococcus neoformans*, the ferroxidase activity of laccase is suggested to be related with the resistance to the antifungal activity of alveolar macrophages [82]. The crystal structure of CueO has been solved [83]. The overall structure of CueO is similar to those of laccase and ascorbate oxidase, but it contains a methionine-rich region in the third domain. Also, the conformation of the trinuclear copper site is uniquely different, as compared to those of other MCBPs. The main mechanism of CueO in copper resistance is postulated to be the oxidation of the Cu^{+} ion to Cu^{2+} [84]. This process is effective for copper resistance because the Cu^{+} is more harmful than Cu^{2+} [85]. The cuprous oxidase activity of CueO is more than two- to fourfold higher than that of any other known three-domain MCBPs. CueO can also oxidize and disable the function of a natural ionophore

called enterobactin which enhances the toxicity of copper ions by functioning as a Cu^{2+} reducing agent [86]. CueO shows enhanced oxidase activity in the presence of extra copper ions. The involvement of the aforementioned methionine-rich region in forming the fifth copper-binding site is responsible for this enhanced activity in response to an increasing copper concentration [87]. In other words, this methionine-rich region works as a copper ion sensor, and boosts the oxidase activity when it detects the extra copper ion on its labile copper-binding site. This fifth copper-binding site in CueO consists of two methionines and two aspartic acids.

CueO forms a stable fold without copper ions, and it can incorporate copper ions after it folds. The order of copper incorporation into recombinant CueO was examined using optical and EPR spectroscopy [88]. The copper ion fills the type 1 (blue) copper site first, followed by the type 2 site and finally the type 3 site. The ability of the apo-protein to absorb copper ions from the environment is part of its copper resistance function.

Another copper resistance system, enhanced by the plasmid-based *pco*, includes the three-domain MCBP PcoA [89]. This system is expressed in response to an elevated copper concentration. PcoC is a periplasmic protein and is considered to be another essential component encoded by the *pco* genes [90]. PcoC is not an MCBP, and it has a type 2 copper-binding site. The crystal structures of two different complex forms of PcoC have been solved recently [91], and its protein-protein interactions using its methionine-rich region are enhanced in the presence of copper ions. A similar methionine-rich region is present in PcoA, and the mechanism of copper resistance in the *pco* system was proposed as follows. PcoC works as a copper chaperone and passes the environmental Cu^{+} ion over to PcoA. The Cu^{+} ion is oxidized to Cu^{2+} by PcoA and then excreted from the cell envelope.

Spore coat proteins (CotA)

Bacterial endospore coats provide enormous durability against chemical and physical insults from the surroundings and allow the bacteria to survive for thousands or even millions of years in harsh environments [92]. *Bacillus subtilis* forms an endospore coat that consists of over 30 kinds of polypeptide [93, 94]. One of the coat proteins is CotA, which is homologous to the laccase-type three-domain MCBPs. Due to its nature as an endospore component, it is highly stable and the half-life of its activity at 80 °C was measured to be 2–4 h [95]. A single amino acid substitution at the BCB site eliminates the oxidase activity, but does not prevent the assembly of CotA into the coat structure. *B. subtilis* is capable of oxidizing Mn^{2+} ions, but CotA and other coat proteins of *B. subtilis* are not involved in metal oxidation, unlike CumA, the spore coat protein of *Pseudomonas* described later. CotA might be responsible for the formation of a melanin-like pigment for protection against UV radiation [96]. The crystal structure of CotA has been solved [97, 98]. The overall fold of the protein is similar to those of other three-domain MCBPs. However, the interdomain loops of CotA have some extra regions, which provide tighter packing for structural stability.

PpoA

Marinomonas mediterranea is a melanogenic marine bacterium [99]. A polyphenol oxidase (PpoA) from this species is homologous to the three-domain MCBPs, and it carries copper-binding residues for the four copper atoms [100]. This enzyme also has a tyrosine hydroxylase activity. The expression of several products with truncated C-terminal regions indicated that the four copper atoms are necessary for the laccase activity, but they are not required for the tyrosinase activity. Since PpoA has a couple of histidine-rich regions near its N-terminus, it is possible that these histidines form a binuclear type 3 copper-binding site, which is responsible for the tyrosinase activity.

Manganese oxidase (CumA, MofA, MnxG)

Some of the endospore coat proteins of bacteria are capable of oxidizing Mn^{2+} ions [101]. Mn^{2+} is soluble in water, but its oxidized forms (Mn^{3+}, Mn^{4+}) are not, so the oxidation causes precipitation of the metal, a process which has been proposed to contribute to the accumulation of manganese nodules in the ocean. The purpose of metal ion oxidation by spore coat proteins is not well understood. One possible explanation is that it prolongs the viability of the cell. CumA is a three-domain MCBP similar to laccases, and its homologues are distributed in *Pseudomonas* strains [102]. Some *Pseudomonas* strains are capable of oxidizing Mn^{2+} and some are not. *Pseudomonas putida* has two three-domain MCBPs, CumA and CumB, encoded in the same operon. CumA contributes to the oxidation of Mn^{2+}, while CumB is necessary for the optimal growth of the cell.

Leptothrix discophora is also capable of oxidizing environmental Mn^{2+} and Fe^{2+}, and *Bacillus* SG-1 can oxidize Mn^{2+} [103]. *L. discophora* carries MofA (CAA81037) [101], and *Bacillus* SG-1 carries MnxG (T18209) [104, 105], which are both spore coat proteins consisting of domains homologous to MCBPs, but their domain organizations are quite unique.

Part of the MofA sequence shares about 20% sequence identity with some other bacterial three-domain MCBPs; however, MofA has a large insertion (~550 amino acids) between the second and third MCO domains (fig. 3). There are two sequences from *Geobacter* strains (ZP_00299982, NP_952447, ZP_00298767) with similar but shorter (~300 amino acid) insertions.

MnxG (T18209) consists of six consecutive MCBP domains, like ceruloplasmin. However, the positions of the copper-binding sites differ from those in ceruloplasmin. MnxG has the BCB site in the second domain (ceruloplasmin has it in the second, fourth, and sixth domains), and it has the trinuclear IDCB site between the second and the third domains (ceruloplasmin has it between the first and sixth domains) (fig. 3). Orthologues of MnxG exist in four other species (ZP_00265508: *P. fluorescens* PfO-1, NP_745627: *P. putida* KT2440, NP_952305: *Geobacter sulfurreducens* PCA, NP_840402: *Nitrosomonas europaea* ATCC 19718). The presence of trinuclear interdomain residues in two consecutive domains is unprecedented among the MCBPs, and thus MnxG may be relevant to one of the two-domain MCBPs (TdMCBP-C) described later in this review.

Six domain MCBPs

Ceruloplasmin and hephaestin

Ceruloplasmin and hephaestin play a vital role in iron metabolism in mammals, including humans, as do the Fet3 and Fet5 proteins in yeast [22, 106]. To be used in the formation of hemoglobin, iron has to be bound to transferrin, and to bind transferrin, the iron must be in the oxidized form. In blood serum, ceruloplasmin oxidizes Fe^{2+} to Fe^{3+}, so that the serum iron can be used for the formation of hemoglobin. Ceruloplasmin also works as a serum oxygen free-radical scavenger and an antioxidant [107].

Hephaestin, another six-domain MCBP in mammals, plays an important role in enteric iron absorption [108, 109].

The X-ray crystal structure of ceruloplasmin has been solved [110, 111]. Both ceruloplasmin and hephaestin consist of six consecutive MCBP domains. The second, fourth, and sixth domains have BCB sites, and the first and sixth domains share a trinuclear IDCB site and form a pseudo C3 symmetric structure with three MCBP domain pairs (fig. 1).

Ascorbate oxidase and laccase do not undergo significant structural changes upon the loss of copper ions, although prolonged copper absence may lead to their decomposition [112]. On the contrary, small-angle X-ray-scattering (SAXS) experiments suggested that ceruloplasmin undergoes a large conformational change between the apo- and holo-forms. Upon the loss of the copper ion, a large rearrangement of the central pair of domains occurs, indicating that the IDCB site of ceruloplasmin plays a crucial role in maintaining the attachment of the first and sixth domains [113].

Blood coagulation factors V and VIII

Like ceruloplasmin and hephaestin, blood coagulation factors V and VIII contain six MCBP domains. Unlike ceruloplasmin and hephaestin, however, these blood coagulation factors have a long (~900 amino acid) domain insertion (B domain) between the fourth and fifth MCBP domains, as well as two extra domains at the C-terminus (C1 and C2 domains). The three pairs of MCBP domains are called the A1, A2, and A3 domains. Factor VIII has BCB residues in the second and sixth MCBP domains, and interdomain residues in the first and sixth MCBP domains (fig. 3), while factor V does not have conserved copper-binding residues in its sequence. Blood coagulation factors undergo domain reorganization by posttranslational processing and their active forms consist of three separate subunits, the A1 and A2 subunits, and the subunit consisting of A3, C1, and C2 [24]. Recently, the crystal structure of factor V, consisting of two subunits (A1 and A3-C1-C2), was solved [114]. The structure has copper and calcium ion-binding sites, but neither is directly involved in the subunit interface. The crystal structure of blood coagulation factor V clearly shows that the subunits consisting of two MCBP domains can form a stable fold by themselves and can be associated to exhibit functional activity.

Two-domain MCBPs (TdMCBPs)

Evolutionary pathway of MCBPs

The evolutionary origin of MCBP domains is supposed to be a single-domain cupredoxin protein. Structure and sequence analyses revealed that among the single-domain cupredoxins, rusticyanin is the most closely related to MCBPs [115]. The multiplication of a cupredoxin domain followed by modifications, such as the creation of IDCB sites and substrate-binding sites, led to the formation of the contemporary MCBPs. With the accumulation of the sequences and X-ray crystal structures of these proteins, the evolutionary relationships among the MCBPs began to emerge, and several studies focusing on the analysis of the molecular evolution of these multidomain enzymes have been reported.

Based mainly on sequence analyses, Ryden and Hunt [116] proposed an evolutionary relationship of MCBPs. In their hypothesis, a pair of domains from the initial cupredoxin domain duplication was duplicated twice more to form a six-domain MCBPs such as ceruloplasmin, and a three-domain MCBP was formed by a single-domain addition to the double-domain protein.

As the significant similarities in the structures and functions of the trinuclear (type 2 and type 3) IDCB sites in laccase, ascorbate oxidase, and ceruloplasmin became clearer, it also became difficult to consider that these copper-binding sites developed independently. Therefore, Murphy et al. [117] proposed that they evolved one from another, i.e., ceruloplasmin lost three middle domains to form a laccase-type protein, or a domain duplication/

addition from a laccase-type protein led to the formation of ceruloplasmin.

To explain both the phylogenetic relationships deduced from sequence analyses and the similarity of the trinuclear copper-binding sites consistently, we hypothesized the presence of two-domain MCBPs with a conspicuous trinuclear IDCB site as evolutionary intermediates [118]. Three types of two-domain MCBPs were proposed as evolutionary intermediates leading to regular MCBPs ([A], [B] and [C] in fig. 1). The three kinds of intermediate all include trinuclear IDCB residues, and the difference among them is the location of the BCB residues. The type [A] intermediate has the BCB residues in both domains while the type [B] intermediate has the BCB residues only in the second domain, and the type [C] intermediate has the BCB residues only in the first domain. The difference represents the course of evolution during which the blue-copper binding sites have regressed, i.e., type [A] is most ancient with BCB residues in both domains, and type [B] and type [C] lost BCB residues from the first and the second domain, respectively. In our hypothesis shown in figure 1, the type [C] intermediate may be the direct ancestor of nitrite reductase so the close similarity between them is expected. Also, close relationships among the type [B] intermediate, laccase/ascorbate oxidase, and ceruloplasmin are expected.

Our original searches through the genome databases for these types of protein revealed 1 type [A], 6 type [B], and 4 type [C] sequences. Due to the growth of genome databases, the number of sequences has been increasing. At the present moment (December 2004), 3 type [A], 17 type [B] and 11 type [C] sequences can be found (fig. 4). Since the functions of these proteins are not well understood and are probably different from each other, we call these proteins 'two-domain MCBPs' (TdMCBP) in this article. Using this abbreviation, we use the notation for the type [A] proteins as TdMCBP-A1 to TdMCBP-A3. In the same manner, we denote the type [B] proteins as TdMCBP-B1 to TdMCBP-B17, and the type-[C] proteins as TdMCBP-C1 to TdMCBP-C11.

A phylogenetic analysis of these sequences, together with corresponding regions of nitrite reductase, ascorbate oxidase, and ceruloplasmin (fig. 6), shows that the type [B] sequences can be roughly separated into three groups (B6 and B13, B14 and others), and type [C] sequences fall into two groups (C1 and others). The phylogenetic tree also suggests that one of the type [B] groups (B14) is closely related to ceruloplasmin, and another group of type [B] (the cluster without B6, B13, and B14) is closely related to ascorbate oxidase. Also, one of the type [C] groups (C1) is closely related to nitrite reductase. These

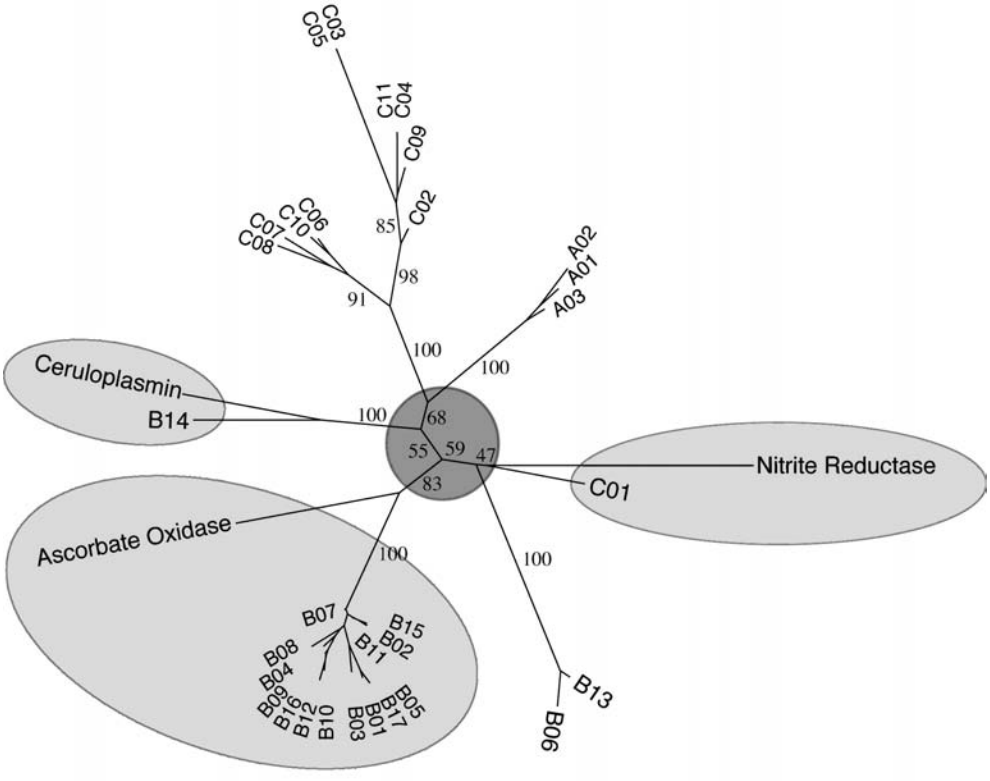

Figure 6. Phylogenetic tree of two-domain MCBPs, together with nitrite reductase, ascorbate oxidase, and ceruloplasmin. For ascorbate oxidase and ceruloplasmin, only the first and the last domains were used for the sequence alignment. Closely related groups are shaded together in ovals. Numbers on branches are bootstrap values. The region where the phylogenetic relationship has low reliability is shaded by a dark-gray circle. This figure was modified from figure 3 in Nakamura et al. [118], and is used by courtesy of *FEBS Letters*. The method used for the construction of the phylogenetic tree is described in the same article. The multiple-sequence alignment is available at http://yayoi.apr.jaeri.go.jp/qbg/kenske/ CMLS.

three pairs of kinship among the MCBPs are consistent with our proposed evolutionary relationship (fig. 1).

As we have seen in the case of blood coagulation factors, a protein consisting of two MCBP domains can fold independently and associate together to be functional. This implies that the common ancestor of ceruloplasmin and hephaestin, which share close sequence similarity with the blood coagulation factors, is likely to be one of the two-domain MCBPs.

When we reported the discovery of the two-domain MCBPs in the genome database, we did not realize that some of these proteins had been analyzed previously. We later found two experimental reports for the proteins that we recognized as TdMCBP-C1 and TdMCBP-B6. Another report characterizing TdMCBP-B13 appeared last year.

p-Phenylenediamine oxidase from *N. europaea*: TdMCBP-C1

The *p*-phenylenediamine oxidase (PPO) from *N. europaea*, which has the same sequence as TdMCBP-C1, was purified and identified as an 'unusual' blue copper oxidase almost 20 years ago [119]. The molecular weights of the native and monomer proteins were 127,500 and 40,100, respectively, which implies the formation of a homotrimer, as in the case of nitrite reductase. This protein exhibits very similar EPR spectra to those of laccase, which suggests the presence of type 1 and type 2 copper in a 1:1 ratio. The presence of an EPR-silent type 3 copper could not be confirmed. However, an optical absorbance at 300–350 nm, which may be due to a type 3 copper, was observed. The amino acid sequence shows the presence of a sufficient number of histidine residues to hold type 3 coppers at the interdomain binding sites.

Interestingly, this PPO (TdMCBP-C1) shares a similar chemical function with both laccase and nitrite reductase, which may imply the evolutionary relationship of these enzymes. TdMCBP-C1 and some of the laccases can oxidize *p*-phenylenediamine [120, 121]. Like nitrite reductase, TdMCBP-C1 can also catalyze the reduction of nitrite. However, TdMCBP-C1 can catalyze the reduction of nitrite only with cytochrome c-552 as the electron donor, while nitrite reductase can take an electron from other electron transfer proteins, such as pseudoazurin or azurin. Thus far, no laccase has been reported to reduce nitrite, and TdMCBP-C1 is the only MCBP with the trinuclear IDCB site known to be capable of reducing nitrite.

Usually, bacteria without nitrite reductase cannot survive in the presence of nitrate (NO_3^-) or nitrite (NO_2^-) [122]. Disruption of the nitrite reductase (*NirK*) gene in *N. europaea* lowers the tolerance to nitrite, but does not disable the production of nitrogen oxide (N_2O) [119, 123, 124]. This suggests that there is an alternative way to reduce nitrite, and TdMCBP-C1 possibly participates in this alternative route of denitrification. One of the *NirK* gene disruption experiments was carried out with *Bradyrhizobium japonicum*. Interestingly, this species has a gene with a sequence corresponding to that of TdMCBP-B3; however, it was unable to survive in the presence of nitrate or nitrite after the disruption of *NirK,* which suggests that TdMCBP-B3 is not capable of reducing nitrite.

EpoA: TdMCBP-B6

An extracytoplasmic phenol oxidase (EpoA) was identified in *Streptomyces griseus* [125]. The EpoA sequence corresponds to that of TdMCBP-B6 in our notation. Like some of the laccases and the PPO from *N. europaea* (TdMCBP-C1), it oxidizes *N,N′*-dimethyl-*p*-phenylenediamine. The oxidation activity of this enzyme increases in the presence of copper. EpoA was recombinantly expressed in *E. coli* and further characterized [126].

EpoA exhibits extraordinary stability against denaturing conditions. After a preincubation at 70° C for 60 min, the enzyme still retains 40% activity. The enzyme also shows high stability in the presence of SDS. Molecular-weight measurements by SDS-polyacrylamide gel electrophoresis revealed a 114-kDa band for the native protein. After boiling, a 38-kDa monomer band appeared. These results suggest that recombinant EpoA forms a homotrimer, like TdMCBP-C1 and the nitrite reductases.

Recombinant EpoA oxidizes a variety of aromatic substrates, such as *N,N′*-dimethyl-*p*-phenylenediamine, *p*-phenylenediamine, chlorogenic acid, dihydrophenylalanine (DOPA), pyrogallol, 4-aminoantipyrine, and *m*-methoxyphenol. However, compared to the wide range of laccase substrate selectivity, that of recombinant EpoA is narrower.

The UV absorption at around 600 nm indicates the presence of a type 1 copper. EPR spectroscopy revealed a laccase profile, which implies the presence of type 1 and type 2 copper atoms. Atomic absorption analyses indicated the presence of four copper ions per chain, which implies the presence of two type 3 copper atoms in each chain. The fact that the addition of extra copper enhances the stability of the trimer suggests that this protein forms a trimer complex by sharing interdomain copper ions between chains.

SLAC: TdMCBP-B13

The PPO from *N. europaea* and EpoA were first recognized as a kind of laccase with three MCBP domains. A close homologue of EpoA isolated from *S. coelicolor* was properly recognized as a two-domain MCBP [127]. The enzyme is named SLAC, for small laccase, because of its laccase activity and low molecular weight (32 kDa).

Like laccase, SLAC oxidizes a wide range of substrates, including *t*-butyl catechol, DOPA, syringaldazine (SGZ), 2,6-dimethoxy phenol (DMP), and $Fe(CN)_6^{4-}$.

UV-visible and EPR spectra revealed the presence of type 1 and type 2 copper atoms. In addition, UV-visible absorption at 330 nm suggested the presence of type 3 binuclear copper. Bicinchonicic acid (BCA) quantitation showed the presence of approximately four copper ions for each monomer. Like EpoA, the enzyme is remarkably stable against denaturing conditions. The optimum pH of SLAC is reported to be 9.4, which is the highest of all known laccases.

Gel filtration experiments revealed a 69-kDa band for the native protein and mass spectrometry indicated a mass around 32-kDa as a monomer, which suggests that this enzyme forms a homodimer, not a homotrimer like the other two TdMCBPs (C1 and B6) previously described. Also, the dimer band exhibits enzyme activity, while the monomer is inactive. The requirement of dimer formation for its stability and function resembles the characteristics of ascorbate oxidase, as discussed previously.

Small laccases in fungis

All of the TdMCBPs found so far in genome sequence analyses and experimental identifications are from bacteria. This may be due to the fact that the complete genomes have been solved for more bacteria than for other organisms. Considering that most laccases have been found in fungi and plants, some eukaryotes may also contain TdMCBPs. Recently, a homodimeric protein with laccase activity was isolated from yellow mushroom (*Cantharellus cibarius*) [128]. The molecular weight of this enzyme as a monomer was 46 kDa. A typical three-domain MCBP weighs 60–75 kDa and a two-domain MCBP weighs between 30–40 kDa. However, 46 kDa is between these molecular weight ranges. It is obviously too small for three domains, and considering that posttranslational modifications, such as glycosylation can increase the molecular weight, it could fall into the two-domain MCBP weight range. A low molecular-mass (43 kDa) laccase from a mushroom (*Tricholoma giganteum*) has also been isolated [129]. Another small dimeric laccase was isolated from a mushroom (*Pleurotus eryngii*) [130]. Its molecular weight (34 kDa) is too small for a three-domain MCBP, and is exactly in the range of a typical two-domain MCBP. Since all three of these enzymes possess laccase activity, they may represent the first TdMCBPs isolated from fungi. Further experimental characterization of these proteins is desired.

Perspectives and discussion

The experimental characterization of three two-domain MCBPs revealed the unpredicted features of these proteins. The characterization of other two-domain MCBPs, especially type A, may provide more interesting information, including new functions, structural properties, and clues to the molecular evolution process of MCBPs.

The presence of two-domain MCBPs strongly supports our hypothesis for the MCBP evolutionary pathway depicted in figure 1, but does not necessarily prove it. For example, we cannot completely exclude the possibility that the three-domain or six-domain blue protein first acquired the trinuclear copper-binding site and the other MCBPs evolved from this common ancestor. Phylogenetic analyses by sequence comparisons may provide a better clue to this problem; however, the sequence similarity among MCBP domains is generally low, and the difficulties in their alignment limit phylogenetic inferences from the sequence analyses. When the three-dimensional structures of two-domain MCBPs become available, more information can be gleaned by using the structural alignment method, and the phylogenetic inferences will be more reliable [131]. Further experimental analyses of the two-domain MCBPs, and especially their X-ray crystal structures, are desired.

One of the questions generated by the presence of SLAC and the other two-domain MCBPs is why the majority of laccases found in contemporary organisms have three domains, if two consecutive domains are sufficient to perform the redox function. This may be just a consequence of random selection, but other explanations are possible. For example, the additional domain may provide extra stability to the protein, thus allowing it to be functional under various environmental conditions, or it may provide the substrate-binding site so that the enzyme can modify its substrate specificity. It would be interesting to find out the role of these MCBP domains without copper-binding sites.

The MCBP domain family is one of the most diverse domain families, in terms of both sequence and function. The MCBP family provides a typical example of domain evolution, where a domain with a characteristic function (copper binding) is reused to modify, develop, and create a new function. Analyses of the evolutionary process of this enzyme family provide glimpses of the steps through which the proteins acquired new structures and functions. We are now trying to pursue the evolutionary analysis of the MCBPs from the viewpoint of bioinformatics in relation to the experimental data about their functional and structural properties. We hope the development of our approach will lead to a method that can be applied to the functional analysis or prediction of other protein families. The growing amount of genome sequence and protein structure information strongly promotes this kind of analysis. For this purpose, the collection of data from an unbiased selection of organisms, including fungi and insects as well as data from practically important target organisms, would be quite useful.

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