

Review

Structure and function of type I copper in multicopper oxidases

T. Sakurai* and K. Kataoka

Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920–1192 (Japan), Fax: +81 762645742, e-mail: ts0513@kenroku.kanazawa-u.ac.jp

Received 14 April 2007; received after revision 5 June 2007; accepted 26 June 2007
Online First 19 July 2007

Abstract. The type I copper center in multicopper oxidases is constructed from 1Cys2His and weakly coordinating 1Met or the non-coordinating 1Phe/1Leu, and it exhibits spectral properties and an alkaline transition similar to those of the blue copper center in blue copper proteins. Since the type I copper center in multicopper oxidases is deeply buried inside the protein molecule, electron transfers to and from type I copper are performed through specific pathways: the hydrogen bond between an amino acid

located at the substrate binding site and a His residue coordinating type I copper, and the His-Cys-His sequence connecting the type I copper center and the trinuclear copper center comprised of a type II copper and a pair of type III coppers. The intramolecular electron transfer rates can be tuned by mutating the fourth ligand of type I copper. Further, mutation at the Cys ligand gives a vacant type I copper center and traps the reaction intermediate during the four-electron reduction of dioxygen.

Keywords. Type I copper, trinuclear copper center, multicopper oxidase, blue copper protein, CueO, laccase, bilirubin oxidase.

Introduction

Nearly all organisms utilize copper as an essential trace element. Biological roles of copper include electron transfer, oxidation of organic substrates and metal ions, dismutation of superoxide, monooxygenation, transport of dioxygen and iron, and reduction of dioxygen, nitrite and nitrous oxide, *etc.* [1]. These functions of copper parallel those of iron due to their shared redox active properties. Regarding oxidation state, copper(I) and copper(II) states are utilized in biological systems, while the involvement of the copper(III) state has been erroneously suggested for galactose oxidase.

Copper in proteins has been classified into three groups based on their spectroscopic and magnetic

properties: type I copper, type II copper, and type III copper. Type I copper shows intense absorption at around 600 nm and narrow hyperfine splittings in the electron paramagnetic resonance (EPR) spectroscopy. Type II copper does not give strong absorption at 600–700 nm and shows hyperfine splittings of the normal magnitude in the EPR spectrum. Unlike type I copper and type II copper, type III copper is not detected in the EPR spectrum because of strong antiferromagnetic interaction. This classification of copper has been based on coppers contained in lacquer laccase, the prototype multicopper oxidase (MCO). On the other hand, copper in proteins containing only one type of copper has been classified into blue copper, non-blue copper or EPR non-detectable copper. These coppers have also been extensively called type I copper, type II copper, and type III copper, respectively.

* Corresponding author.

Table 1. Blue copper center, type I copper center and variant with their donor set and redox potential.

Type of Cu	Protein	Donor for Cu	Redox potential (mV vs normal hydrogen electrode)	
Blue Cu center	plastocyanin	1Cys2His1Met	350–370	
	azurin	1Cys2His1Met	266–305	
	pseudoazurin	1Cys2His1Met	260–269	
	amicyanin	1Cys2His1Met	294	
	rusticyanin	1Cys2His2Met	680	
	auracyanin	1Cys2His2Met		
	halocyanin	1Cys2His1Met	183 (highly pH dependent)	
	plantacyanin(CBP)	1Cys2His1Met	317	
	umecyanin	1Cys2His1Gln	283	
	mavicyanin	1Cys2His1Gln	285	
	stellacyanin	1Cys2His1Gln	180–260	
	Red Cu center	nitrosocyanin	1Cys2His1Glu1H ₂ O	85
	Cu _A	cytochrome <i>c</i> oxidase	2Cys2His1Met1Glu	260
nitrous oxide reductase		2Cys2His1Met1Trp	260	
Type I Cu center	plant laccase	1Cys2His1Met	394	
	ascorbate oxidase	1Cys2His1Met	344–370	
	CueO	1Cys2His1Met	364	
	PcoA	1Cys2His1Met		
	CumA, MofA, MnxG	1Cys2His1Met		
	EpoA	1Cys2His1Met		
	CotA	1Cys2His1Met	455	
	dihydrogeodin oxidase (sulochrin oxidase)	1Cys2His1Met		
	nitrite reductase	1Cys2His1Met	240–260	
	hephaestin	1Cys2His1Met		
	ceruloplasmin	1Cys2His1Met/1Leu	490, 580, >1000	
	phenoxazinone synthase	1Cys2His1Met		
	bilirubin oxidase	1Cys2His1Met	680	
	Fet3p	1Cys2His1Leu	433	
	fungal laccase	1Cys2His1Phe/Leu	550–785	

Blue copper protein is a class of copper proteins containing only a blue copper center in a relatively small-sized protein molecule of 9–23 kDa that functions in electron transfer [2–4], although the subgroup called phytoeyanin in higher plants is considered to function as a radical scavenger. Red copper protein (nitrosocyanin) is a variant of blue copper protein with a modified ligand set (see below) [5]. Table 1 lists the copper proteins containing the blue copper center and the type I copper center and their variants. Basically the same copper center, type I copper, is also present in MCOs containing four copper centers: a type I copper and a trinuclear copper center comprised of a type II copper and a pair of type III copper [6–8].

The function of both the blue copper center and the type I copper center is electron transfer. Since MCO is an oxidase, the electron transfer pathways to and from type I copper are different: electrons withdrawn from the substrate are transferred to the trinuclear copper center via type I copper and are used to convert dioxygen to water. On the other hand, blue copper proteins shuttle an electron between exogenous redox partners through the coordinating His imidazole group, although the directional electron transfer occurs in plant plastocyanin located on the thylakoid

membrane surface [9, 10] and only putatively occur in amicyanin.

Copper-containing nitrite reductase (NIR) involved in the anaerobic respiration system is a subgroup of MCOs, which contains a type I copper and a type II copper [11]. The type I copper in NIR is used for entry of electrons from a blue copper protein or cytochrome *c*. The type II copper in NIR is used as a binding and reducing site for substrates. Nitrous oxide reductase (N₂OR) and cytochrome *c* oxidase (COX) have a binuclear copper center, Cu_A, which is a variant of type I copper and iron-sulfur center and functions to facilitate entry of electrons towards the reduction centers of nitrous oxide (Cu_Z) and dioxygen (heme *a*₃-Cu_B), respectively [12].

In the present article, we review the structure and function of type I copper in MCOs and their variants in comparison with those of blue copper proteins and their variants. While a number of excellent reviews on blue copper proteins are available [2–4, 13–16], type I copper with extensive biological features has not yet been reviewed.

	2 3	*	3 3	1 2 3	3 1 3	1	1	
CueO	99 TLHWHGLEVPGE--VDGG		139 HPHQH GK	441 MLHPFH I HGT	497 MAFC H LLEHEDTG MML			
BO	92 SVVHLHGSFSRAA--FDGW		132 WYHDHAM	396 WTHPIH I HLV	454 MFHC H NLT HEDHD M MA			
RvLc	57 TIHWHGVKQPRNPWSDGP		102 WWHAHSD	431 TSHPMHLHGF	493 FLHC H FERHTTEGMAT			
CpAO	58 VIHWHGILQRGTPWADGT		102 FYHGHLG	443 ETHPWHLHGH	504 AFHC H IEPLHMG MGV			
TvLc	62 SIHWHGFFQKGTNWADGP		107 WYHSHLS	393 APHPFHLHGH	450 FLHC H IDFHLEAGFAV			
CcLc	62 SIHWHGLFQRGTNWADGA		107 WYHSHFG	394 GPHPFHLHGH	449 FFHC H IEFHLMNGLAI			
Fet3p	79 SMHFHGLFQNGTASMDGV		124 WYHSHTD	411 GTHPFHLHGH	481 FFHC H IEWHLLOGLGL			
CumA	94 TIHWHGIRLPLE--MDGV		142 WYHPHVS	389 YQHP I H LHM	588 MFHC H VIDHMETGLMA			
CotA	103 VVHLHGGVTPDD--SDGY		151 WYHDHAM	417 GTHPIH LHLV	489 VWHC H ILEHEDYDMMR			
SLAC	100 SLHVHGLDYEIS--SDGT		154 HYHDHVV	229 YYHTFHMHG H	285 MYHC H VQSHSMDGMVG			
hCp	99 TFHSHGITYYKE--HEGA		159 IYHSH I D	973 DLHTVHFHGH	1018 LLHC H VTDH I HAGMET			
			AxNIR	93 MPHNVDFHGA	133 VYHC +VPWHVVS GMSG	304' LNHN L (+, APEGM)		
			AcNIR	93 LLHNIDFHAA	133 VYHC +VPWHVTS G MNG	304' VNHN L (+, APEGM)		
			AfNIR	93 LMHNIDFHAA	133 VYHC +VPWHVVS G MNG	304' VNHN L (+, APPGM)		
			CrPc	35 FPHNIVFDED	81 GYYCE-P-HQGAGMVG			
			PnPc	35 FPHNIVFDED	81 SFYCS-P-HQGAGMVG			
			AcPAz	38 KGHNVETIKG	75 GVKCT-P-HYGMCMVG			
			PdAm	51 MPHNVHFVAG	89 DYHCT-P-H-P-FMRG			
			AxAzI	44 MGHNVVLTQK	109 AYFCSPFGHF-ALMKG			
			TfRc	83 FGH SFDITKK	135 YYVQIPGHAATGMFG			
			CsStc	44 NAHNVHEMET	86 YFVCTVGTGTHCSBGQKL			
			CpMv	42 KFHNVLQVDQ	82 YFLCGIPGHCQLGQKV			
			PdN ₂ OR	524 LTHGFTMGNY	560 YCQWFCHAL-HME--MRG			
			AcN ₂ OR	541 LTHGFTMGNH	577 YCQWFCHAL-HME--MRG			
			PdCOX	179 VIHAWTIPAF	214 QCSELCGIN-HAY--MPI			
			bCOX	159 VLHSAWVPSL	195 QCSEITCGSN-HSF--MPI			

Figure 1. Homology of amino acid sequences around the copper binding sites of multicopper oxidase (MCO), nitrite reductase (NIR), blue copper protein, nitrous oxide reductase and cytochrome *c* oxidase. 1, 2, 3, and asterisk represent type I copper ligand, type II copper ligand, type III copper ligand and proton donor, respectively. Plus represents the five amino acids shown in parenthesis. BO, bililubin oxidase; RvLc, *Rhus vernicifera* laccase; CpAO, *Cucurbita pepo* ascorbate oxidase; TvLc, *Trametes versicolor* laccase; CcLc, *Coprinus cinerius* laccase; SLAC, small laccase from *Streptomyces coelicolor*; hCp, human ceruloplasmin; AxNIR, *Alcaligenes xylosoxidans* NIR; AcNIR, *Achromobacter cycloclastes* NIR; AfNIR, *Alcaligenes faecalis* NIR; CrPc, *Chlamydomonas reinhardtii* plastocyanin; PnPc, *Populus nigra* plastocyanin, AcPAz, *Achromobacter cycloclastes* pseudoazurin; PdAm, *Paracoccus denitrificans* amicyanin; TfRc, *Thiobacillus ferrooxidans* rusticyanin; CsStc, *Cucumis sativus* stellacyanin; CpMv, *Cucurbita pepo* mavicyanin; PdN₂OR, *Paracoccus denitrificans* nitrous oxide reductase; AcN₂OR, *Achromobacter cycloclastes* nitrous oxide reductase; PdCOX, *Paracoccus denitrificans* cytochrome *c* oxidase; bCOX, bovine heart cytochrome *c* oxidase.

Structure of the type I copper center in MCOs

The ligand groups commonly coordinating the blue copper center and the type I copper center in MCO are 1Cys and 2His residues (Fig. 1, Table 1). Met is widely utilized as the fourth ligand, although the electron-donating ability of the thioether group is considerably lower compared to the cysteinate sulfur and the imidazole nitrogen [2, 16]. In phytocyanins from higher plants, however, Met is replaced by Gln containing an amide group in the side chain with a stronger electron-donating ability compared to the thioether group in the side chain of Met. The fourth ligand for the type I copper center is not present in fungal laccases, in which the uncoordinating Phe or Leu occupies the position [5–7]. An amide carbonyl group in the main chain approaches the blue copper center in azurin with a distance of ~3 Å [17–22]. Figure 2 compares the structure and properties of the type I copper center and the blue copper center together with their variants, the red copper center with

the coordination of 1Cys2His1Glu and a water molecule [5], and the coupled Cu_A center in N₂OR and COX [23–25].

Most of the blue copper proteins plastocyanin, azurin, pseudoazurin, amicyanin, rusticyanin, auracyanin, umecyanin, mavicyanin, plantacyanin (cucumber basic protein, CBP), and stellacyanin have been crystallized and their structural data are available [4, 16]. On the other hand, crystal structure analyses of MCOs comprised of more than 500 amino acids have been limited until recently. However, crystal data are now available for fungal laccases from *Coprinus cinereus* [26, 27], *Trametes versicolor* [28, 29], *Melanocarpus albomyces* [30, 31], *Rigidoporus lignosus* [32], and *Cerrena maxima* [33], CotA [34–36], CueO [37–39], Fet3p [40], phenoxazinone synthase [41], ascorbate oxidase [42, 43], and ceruloplasmin [44, 45] (Table 2), although plant laccase, bilirubin oxidase, PcoA, manganese oxidases such as CumA and MnxG, dihydrogeodin oxidase, and hephaestin have not yet been crystallized. The difficulty in crystallizing lac-

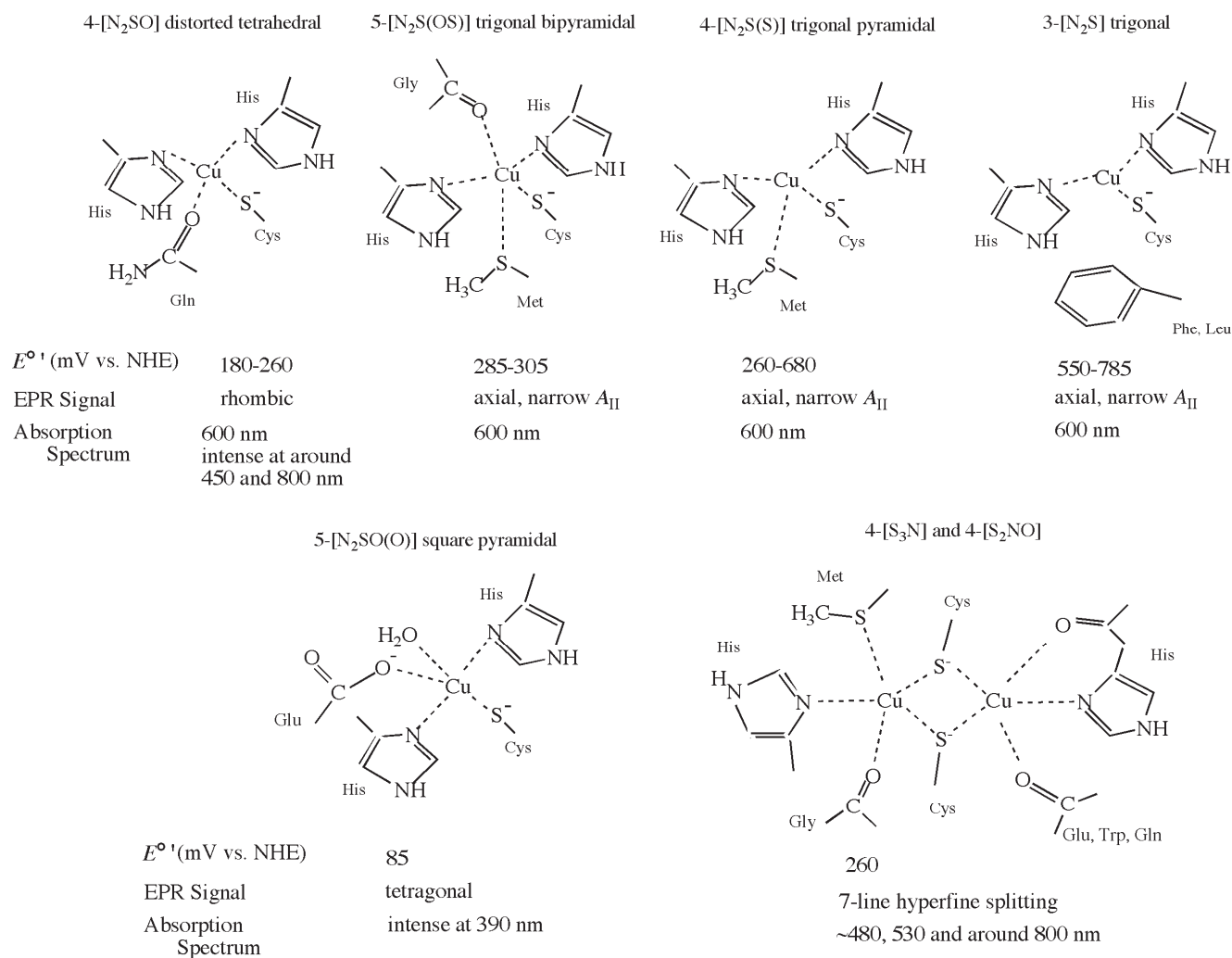


Figure 2. Structure and properties of the type I copper center and related copper centers: blue copper center, red copper center and Cu_A center.

quer laccase, the prototype MCO, comes from its high sugar content of ~50%. An expression system to obtain sugar-free lacquer laccase has not yet been constructed. The type I copper in MCO is coordinated by 1Cys2His1Met except for the fungal laccases and Fet3p, in which Phe or Leu occupies the position of Met. Crystal structure analysis of reduced MCO has only been performed for ascorbate oxidase, indicating that the space formed by the trinuclear copper center becomes wider to accommodate dioxygen [42]. Crystal structure analyses of ascorbate oxidase [42], CotA [35] and, *T. versicolor* laccase [29], to which substrates or inducers were bound (see below), demonstrated that the structure around the type I copper center was not noticeably perturbed upon binding of the substrate or inducer.

The MCO molecule consists of three domains, each of which has a folding pattern analogous to that of cupredoxin (blue copper protein) [28, 29] (Fig. 3). The

trinuclear copper center is located at the interface of domain 1 and domain 3, and the type I copper center at domain 3, being further from the protein surface when compared to the blue copper center in blue copper proteins. An exception to the general molecular structure of MCOs is ceruloplasmin. Ceruloplasmin consists of six domains: three repetitions of the two-domain unit [44–46]. This two-domain structure [47] is considered to be an ancient form of MCO from which the three-domain MCO evolved [11]. Two type I copper centers constructed from 1Cys2His1Met (domains 4 and 6) and one type I copper center constructed from 1Cys2His1Leu (domain 2) are present in ceruloplasmin, although only one trinuclear copper center is present at the interface between domains 1 and 6 [44, 45]. NIR, whose structure is a homo-trimer of two-domain monomers [48, 49], evolved from the common ancestor with ceruloplasmin.

Table 2. X-ray crystallographic data of multicopper proteins.

Multicopper protein	X-ray crystallographic characterization with Protein Data Bank (PDB) code or lit.
Ascorbate oxidase	ox. form (1AOZ), red. form (1ASO), peroxide-incubated (1ASP), azide-incubated (1ASQ), ascorbate-incubated [42]
CueO	ox. form (1KV7), Cu-soaked (1N68), M441L (1PF3), $\Delta\alpha 5-7$ (2YXV), $\Delta\alpha 5-7$ Cu-incubated (2YXW), low Cu amount form (2FQD, 2FQE, 2FOF, 2FOG)
CotA	ox. form (1GSK), red. form (2BHF), SGZ-incubated (1HKP, 1HL0), ABTS-incubated (1HKZ, 1HL1, 1OGR), EBS-soaked (1OF0), ABTS-adduct (1UVW), CuCl_2 -incubated (1W6L), azide-incubated (1W6W), peroxide-incubated (1W8E), M502L/F [63]
Phenoxazinone synthase	ox. form (2G23)
Fet3p	ox. form (1ZPU)
Fungal laccase	<i>Coprinus cinereus</i> : T2D (1A65, 1HFU) <i>Melanocarpus almomyces</i> : ox. form (1GW0, 2IH8, 2IH9) <i>Trametes versicolor</i> : ox. form (1GYC), 2,5-xylydine-complexed (1KYA) <i>Rigidoporus lignosus</i> : ox. form (1V10) <i>Cerrena maxima</i> : ox. form (2H5U)
Ceruloplasmin	ox. form (1KCW, 2J5W)
Nitrite reductase	<i>Alcaligenes faecalis</i> : ox. form (1AS6, 1AS7, 1QEO1, 2AVF), half-apo (1QE3), red. form (1AO8, 2FJS), nitrite-bound, (1AS8, 1SJM), NO-bound (1SNR), A98N (1ET5), H255D (1ET7, 1ET8), H255N (1J9S, 1JQT), D98N (1J9O, 1JQR), I257A (1L9O), I257G (1L9P), I257L (1L9O), I257M (1L9R), I257T (1L9S), I257V (1L9T), H145A (1NPJ, 1NPN), M150E (1NTD, 2AFN), D92E (1QE2), M150G (1ZDO, 1ZDS, 2B08), M168L (2BP0), M168Q (2BP8) <i>Alcaligenes xylosoxidans</i> : ox. form (1BO5, 1NDR, 1NDT), apo, high pH (1HAU, 1HAW), M144A (1GS6), H254F (1GS7), D92N (1GS8), W138H (1WA0), H313Q (1WA1, 1WA2), H129V (1WAE), nitrite-bound (1NDS) <i>Rhodobacter sphaeroides</i> : ox. form (1ZV2, 2A3T, 2DY2), M182T (1MZY, 1MZZ), H287A (1N70), nitrite-bound (2DWS, 2DWT) <i>Achromobacter cycloclastes</i> : ox. form (1RZP, 1RZP, 2BW4), nitrite-bound and T2D (1NIA~1NIE, 2BWI, 2NRD), NO-bound (2BW5, 2BWD), I257E (1KCB) <i>Hyphomicrobium denitrificans</i> : ox. form (2DV6)

$\Delta\alpha 5-7$, helices 5–7 were deleted; SGX, syrinagaldazine; EBS, 3-ethyl-2-[(2Z)-2-(3-ethyl-6-sulfo-1,3-benzothiazole-2(3H)-ylidene)hydrazino]-6-sulfo-3H-1,3-benzothiazole-1-ium; T2D, type 2 Cu depleted.

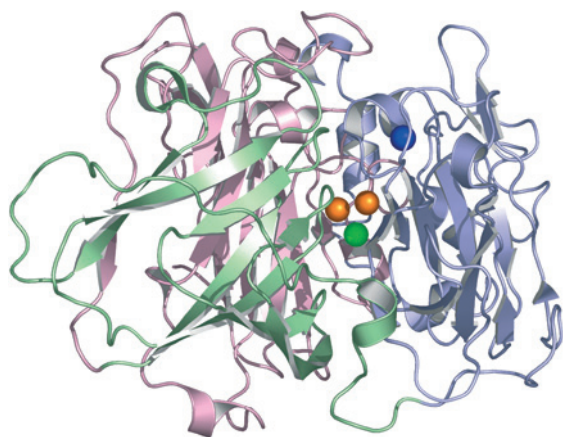


Figure 3. X-ray crystal structure of *Trametes versicolor* laccase whose three domains are colored light pink, light green, and light blue. Figure was constructed with PyMol for Protein Data Bank (PDB) data, 1GYC.

Figure 4 shows the structure of the copper site in an MCO, CueO (Fig. 4a) [37], together with those of NIR (Fig. 4c) [49] and mavicyanin (Fig. 4b) [50]. The backbone peptides show how the ligand amino acids are connected to each other. The architecture of the type I copper center and the blue copper center are analogous in that Cys, His and Met/Gln are located in

one loop and only another His is in the other loop. Type I copper is connected to type III coppers through the His-Cys-His sequence in MCO and with type II copper through the His-Cys sequence in NIR. Further, a peptide chain harboring the His ligands for type I copper and type II and III coppers (MCO) or type II copper (NIR) (magenta in Fig. 4a, c) indirectly connects the Cu sites separated by ~ 13 Å. The copper-ligand bond distances have been tabulated in the literature [16]. The structure of type I copper in most MCOs is trigonal pyramidal or distorted tetrahedral as in many blue copper proteins, but trigonal planar in fungal laccases and Fet3p (Fig. 2).

Spectroscopic properties of the type I copper center in MCOs

Absorption and circular dichroism spectra

The absorption spectral features of MCO are similar to those of blue copper proteins except for the presence of a band at ~ 330 nm derived from the trinuclear copper center, since the contribution from type I copper is significant over a wide range of wavelengths (Fig. 5a, absorption spectra of CueO and

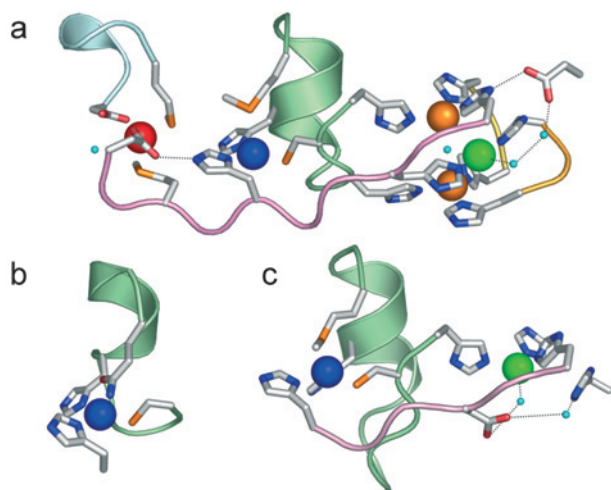


Figure 4. Structures of the active site of CeuO (*a*, PDB code, 1N68), mavicyanin (*b*, 1WS7), and AxNIR (*c*, 1BQ5) showing how ligand groups are correlated with each other.

green groups due to their relative absorption intensities at 450 and 600 nm [51, 52]. The Leu → His variant of a fungal laccase showed changes in type I copper from the blue site to the green site [53], while the Leu → Phe mutant did not show any notable change [54]. MCO mutants prepared until now are listed in Table 3.

In contrast to the absorption spectrum, the circular dichroism (CD) spectrum of MCO (Fig. 5b) is more complex. The sign of the charge transfer band at around 600 nm, $\text{Cys}(\text{S}_{\pi}^{-}) \rightarrow \text{Cu}^{2+}$, is positive in MCOs containing type I Cu with Met as the fourth ligand, but is negative in fungal laccases containing the three-coordinated type I Cu [55]. The charge transfer band, $\text{Cys}(\text{S}_{\sigma}^{-}) \rightarrow \text{Cu}^{2+}$ presents a shoulder at ~530 nm, although its presence is not recognized from the absorption spectrum. Oppositely signed bands coming from $\text{His}(\text{N}) \rightarrow \text{Cu}^{2+}$ and $\text{Met}(\text{S}) \rightarrow \text{Cu}^{2+}$ are observed

Table 3. Mutants of multicopper proteins.

Multicopper protein	Mutant	Reference
Ascorbate oxidase	T1: M544I, other site: A565I/S/V/L/G/T/M/N/D/E, F465W, M86I, I534F, A465L/F463W, S124G/V136L, A465L/F463W/M86I, A465W/F463I/M86I	[108]
Bilirubin oxidase	T1: M467F/L/N/G/H/R, C457A/V/S, T2: H94V/N, T3: H456,458V/K/D, H134,136V, Proton donor: D105E/N/A	[56, 61, 64], [70, 84]
Ceruloplasmin	T1: C1021S, L329M	[109]
CotA	T1; M502L/F	[63]
CueO	T1: C500S, M510L/Q/A/V, proton donor: D112E/A/N, other site: E106F, deletion mutant: $\Delta\alpha 5-7$, $\Delta\alpha 6-7$, $\Delta\alpha 5$, $\Delta\alpha 5-7+1/2\alpha$	[58, 107, 110]
Fet3p	T1: L494V/F/K/I/A/M, C484S, T1T3: C484S/H81N, T1T3: C484S/H126 N, T1T3: C484S/H128N T2: H81N, T3: H489F, proton donor: D94, Other site: E227A, D228A, E330A, M281A, M345A, M281A/M345A, E185A/D, D409A, D283A, D409A/E, E185A/D409A, D278Q, D279N, D312N, D315I, D319N, D320N	[57, 104] [111–116]
<i>T. villosa</i> laccase	T1: F463L/M, A461E/F463L	[65]
<i>M. thermophila</i> laccase	T1: L513F/H, other site: L509V/E467S/A468A	[53, 54]
<i>R. solani</i> laccase	T1: L470F, other site: L466V/E467S/A468G	[54]
Nitrite reductase	T1: M150Q/G/T/H/E/D, M182T, M144L, H145A/G, H139A, H135K, other site: W144L, Y203L, H133R, D98A/N/E, D92E/N, H255N/D/A/K/R, D129A/N, H287A, I289A, E46S, E58A, E89S, E133A, E118A, E160A, E197A, D201A, E204A, D205A, E112A/E197A/D201A, H287E	[62, 90], [117–132]

Data on nitrous oxide reductase and cytochrome *c* oxidase were not included.

azurin). The band at around 600 nm originating from the charge transfer $\text{Cys}(\text{S}_{\pi}^{-}) \rightarrow \text{Cu}^{2+}$ is most peculiar ($\epsilon=4500-6000$) [2–4, 6–8] and, accordingly, MCO is also called blue oxidase. The band at around 700–850 nm is now assigned to the d-d transitions as in the case of blue copper proteins. The band at ~450 nm assigned to the $\text{His}(\text{N}) \rightarrow \text{Cu}^{2+}$ charge transfer is rather weak for every MCO, although its intensity is considerably stronger for blue copper proteins showing the rhombic EPR signal. Copper-containing NIR molecules are classified into blue and

at 400–500 nm, while the latter band sometimes becomes noticeable when performing curve analysis. In contrast to the blue copper center and type I copper center with distorted tetrahedral or trigonal pyramidal structures and short Cu-S⁻(Cys) bonds (1.95–2.16 Å), the red copper center in nitrosocyanin from *Nitrosomonas europaea* with a square pyramidal structure and a longer Cu-S⁻(Cys) bond (2.26 Å) shows an intense absorption band at 390 nm ($\epsilon=4400$) [5]. The d-d bands of type I copper give a considerably stronger negatively signed CD band at around 800 nm,

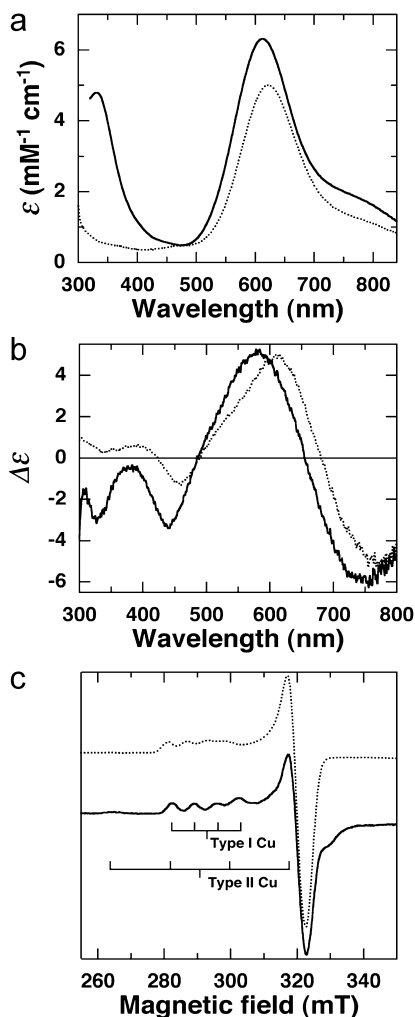


Figure 5. Absorption (a), CD (b), and EPR (c) spectra of a multicopper oxidase, CueO (solid line) and a blue copper protein, azurin 1 from *Alcaligenes xylosoxidans* (dotted line).

masking the contributions from d-d bands of type II and III coppers. These bands are only observable in the Cys to Ser mutant of the type I copper center in bilirubin oxidase [56], Fet3p [57] and CueO (unpublished) (see below). By mutating an Asp residue as a potential proton donor for dioxygen, which is positioned behind the trinuclear copper center, absorption at 330 nm was affected, although the band at 600 nm did not change [58]. Contributions from the trinuclear copper center to the CD spectrum at around 330 nm are not usually significant except for in CueO, since the OH⁻ group bridged between the type III coppers does not intrinsically have an asymmetric character. Cu_A in N₂OR is in the mixed-valence state, S = 1/2 [Cu(1.5),Cu(1.5)], and shows predominant S⁻ (Cys) → Cu²⁺ charge transfer bands at ~480 and 530 nm, as well as a class III mixed valence charge transfer band around 800 nm [59]. The magnetic CD spectrum is

similar to that of bovine heart COX [60], in which the contributions from the Cu_A center to the absorption spectrum are masked by the contributions from two heme centers.

EPR spectra

In the EPR spectra of MCOs both signals derived from type I copper and type II copper have been detected [6, 7] (Fig. 5c). Type I copper gives a signal with axial symmetry and the hyperfine splitting of $A_{II} \cong 10 \times 10^{-3} \text{ cm}^{-1}$. The rhombic type I copper signal as observed in blue copper proteins with the coordination of Gln has not been reported in authentic or recombinant MCOs, although it has been artificially obtained in the Met → Gln mutants of bilirubin oxidase [56, 61], CueO (unpublished) and NIR [62]. Mutations of Met → Phe/Leu for bilirubin oxidase (unpublished), CueO (unpublished) and CotA [63] resulted in the type I copper with the coordination found in fungal laccases, but the artificially prepared type I copper site became unstable due to the loss of a coordinating group. The modified type I copper site was not fully occupied with copper atoms, giving weakened EPR signal intensities. The mutation of Met → Gly for bilirubin oxidase [64] led to a decrease in the A_{II} value and increase in the g_{II} value, which is comparable to the change reported for mutations of the blue copper protein. The mutations of Phe/Leu → Met in fungal laccases also led to slight modifications in the EPR signal [65]. Since the type I copper and type II copper signals overlap in the EPR spectra of MCOs, simulations have been performed to determine the spin Hamiltonian parameters of type II copper. Another striking feature of the EPR spectra of MCO is that an uncoupled type III copper signal is occasionally observed for isolated recombinant MCOs [56, 61]. This takes place because the recombinant MCOs have not experienced a turnover and are in a state differing from that of the resting authentic MCO [66]. The red copper center gives an EPR signal more similar to normal copper ($A_{II}=13.8 \text{ mT}$) due to the coordination of Glu [67]. The Cu_A center of N₂OR and COX shows seven-line hyperfine splitting in the g_{II} regions of the EPR signal, indicating that two copper centers interact magnetically [68].

Resonance Raman spectra

The resonance Raman spectrum of MCO has not been studied in detail. Only the data for the resting MCOs and some mutants with excitations at their 600-nm band are available [69–71]. Their spectral features are very similar to those of blue copper proteins in that they have the clustered bands due to the mixing of the metal–ligand stretching. The Raman

shifts of the clustered bands decrease on the order Phe/Leu/Gly (vacant), Met, and Gln for the fourth ligand. Fluorescence does not allow excitation at the 330-nm band. The UV resonance Raman spectrum has also not yet been reported. The resonance Raman spectrum of N₂OR in three oxidation states (oxidized, semi-reduced, and reduced) discriminated the contribution from Cu_Z and Cu_A sites, showing vibrational modes between 220 and 440 cm⁻¹ [72].

X-ray absorption spectra

K-edge and extended X-ray absorption fine structure (EXAFS) spectra of MCOs have also not been widely measured. Since contributions from each copper site overlap, the average copper-copper distance in the trinuclear copper center can be obtained. The EXAFS spectra of the type II copper-depleted laccase [73] and ascorbate oxidase [74] show that their type III coppers are in the cuprous state even under air and are therefore different from the coupled copper centers in hemocyanin and tyrosinase. The EXAFS spectrum of Cu_A in N₂OR was very similar to that of COX and a copper-copper distance of ~2.4 Å was determined [75].

Properties of type I copper in MCOs

Redox potentials of the copper centers in MCOs have been determined by measuring the absorption spectrum and potential of the partly reduced states of MCO *in situ* [6, 16, 76–78]. The redox potential of type I copper ranges over ~360–780 (>1000) mV, which are higher redox potentials compared to those of blue copper proteins (184–680 mV) and red copper protein (85 mV) (Table 1). The redox potentials of type II copper and type III copper have been measured by changes in the EPR signal intensity of type II copper and the absorption at 330 nm, respectively.

The redox potential of type I copper in some MCOs has also been measured electrochemically [79–84], although a molecular mass higher than 50 kDa is exceptionally unfavorable for obtaining clear electrochemical responses. Direct electrochemistry using pyrolytic graphite as a working electrode or mediated electrochemistry using Au electrodes modified with an appropriate promoter by self assembly permit the measurement of the redox potential of type I copper, when the MCO molecules take the appropriate orientation on the electrode surface and electric communication between the electrode and type I copper becomes possible. Thus, the redox potential of the type I coppers of plant and fungal laccases [80], ascorbate oxidase [79], bilirubin oxidase [81, 84], CeuO [83] have been electrochemically determined,

although the association of these proteins and the electrode sometimes gave slightly different values from those determined by the titration method. The shorter the distance of type I copper from the protein surface, the better is the electrochemical response. However, since the type I copper site in MCO is located under the substrate-binding cleft, the electrochemistry of MCO is unfavorable compared to blue copper protein, in which the imidazole edge of the His ligand is exposed on the flat protein surface [85–88]. In the case of NIR, the electrochemical communication of type I copper with the electrode was effectively performed by using an Au electrode modified with a promoter [89] and still more effectively by using mediator proteins such as azurin and pseudoazurin, thus affording an analytical method to measure enzyme activity [90]. Figure 6 schematically illustrates these electrochemical processes of MCOs.

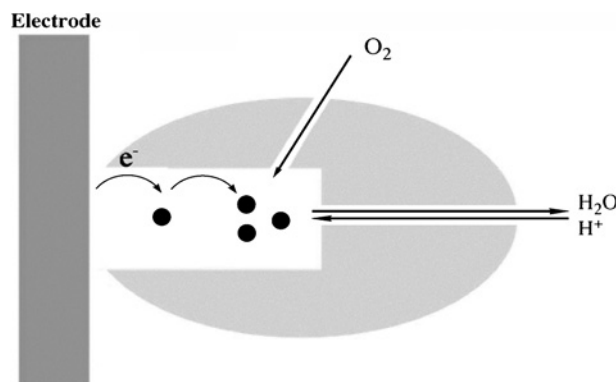


Figure 6. Direct electrochemical process of MCO, which involves an oriented association of the MCO molecule on the electrode surface, the electron transfer from electrode to type I copper center, the succeeding electron transfer to the trinuclear copper center, and the four-electron reduction of dioxygen with the concomitant supply of four protons.

Blue copper proteins show a so-called “alkaline transition” of their absorption wavelength, absorption intensity, and redox potential [91–94]. With increasing pH, especially at pH 8–11, the absorption maximum at ~600 nm shifts to shorter wavelengths and its absorption intensity decreases with the concomitant redox potential shifts to negative values. In some cases, the changes give sigmoidal curves, suggesting the involvement of deprotonation of amino acids [95]. Therefore, it has been suggested that deprotonation of a coordinated imidazole group or an amino acid hydrogen-bonded with a coordinated group [50] is the cause of the alkaline transition, although it has not been proved yet. Rather, negative data have been obtained from mutation studies (unpublished).

The alkaline transition of MCO has not been studied in detail but we have recently reported that bilirubin

oxidase shows a dramatic alkaline transition [96]. With increasing pH values higher than 7, the characteristic blue color faded reversibly and the intensity of the type I copper EPR signal also changed concomitantly with the absorption change. "Autoreduction" is supposed to be derived from a shift in the equilibrium between $\text{Cu}^{2+}\text{-S}^-$ and $\text{Cu}^+\text{-S}^\bullet$ depending on the pH [94]. When the radical center is delocalized on the sulfur atom, copper is formally in the cuprous state and S^\bullet may not give an EPR signal because of broadening. If protein deformation is the cause of the alkaline transition of blue copper protein and MCO, it would be difficult to explain why common changes take place. More information about the reduced state of MCO is required to understand the alkaline transition intrinsic to the blue copper center and the type I copper center.

Function of type I copper in MCOs

The function of type I copper in MCO is to withdraw an electron from the substrate and transfer it to the trinuclear copper center. This becomes possible because the redox potential of the substrate is more negative than that of type I copper (Fig. 7). When the Met ligand of the type I copper in bilirubin oxidase was replaced with Gln, the redox potential of type I copper shifted negatively by ~200 mV, giving rhombic type I copper EPR signal [56, 61, 70, 81, 84]. Enzyme activity for organic substrates dramatically decreased since the electron transfer from the substrate to type I copper became thermodynamically uphill, although the driving force for the succeeding electron transfer process from type I copper to the trinuclear copper center became steeply downhill. In connection with this, the ferroxidase activity of the Gln mutant increased more than 150% compared to the wild-type enzyme. An analogous negative shift in the redox potential, a spectral change related to type I copper, and a decrease in activity have all been observed in the Met150Gln mutant of NIR [62]. On the other hand, when Met was substituted with Phe and Leu, the redox potential of type I copper in CotA [63], bilirubin oxidase (unpublished), and CueO (unpublished) increased, as in the case of fungal laccases with a concomitant decrease in the enzyme activities. Met \rightarrow Phe and Leu mutations of the type I copper ligand in bilirubin oxidase led to the possible coordination of an Asn residue located adjacently to the type I copper site. This occurred to prevent the unstable three-coordinate state of copper. The crystal structure analysis of Met \rightarrow Phe/Leu mutants of CotA [63] demonstrated that the protein structure became considerably unstable and the type I copper center

was not fully occupied as in the cases of bilirubin oxidase and CueO. Therefore, the type I copper center in MCO might also contribute to increase stability of the protein structure. The Phe and Leu mutants showed prominent reductions in enzyme activities because the electron transfer between type I copper and the trinuclear copper center became unfavorable as illustrated in Figure 7. Effects of mutations at the trinuclear copper center and the putative proton donor concerned in the four-electron reduction of dioxygen have been reviewed elsewhere [8].

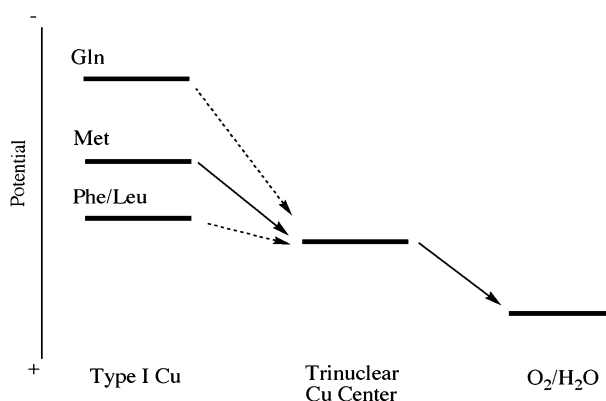


Figure 7. Redox potential diagram of MCO representing electron transfer from substrate to dioxygen and modification of the redox potential of the type I copper center by mutation.

The electron transfer processes of MCOs and NIR from the substrate to type I copper and from type I copper to the catalytic center have been studied with the stopped-flow technique, flash photolysis, and pulse radiolysis [37, 51, 52, 97–106]. The initial electron transfer process from the substrate to type I copper involves the association of substrate with the protein molecule, and accordingly, the proper selection of the substrate is extremely important to study the initial electron transfer process. Unlike the initial stage of the MCO reaction, the succeeding intramolecular electron transfer pathway from type I copper to the trinuclear copper center and the four-electron reduction of dioxygen [56, 102, 103] are not affected by how the reaction is started, although these processes will be accelerated under steady-state conditions in the presence of excess substrate [101]. The electrochemistry of MCO also involves the preceding association of the protein molecule with the electrode surface and electron transfer from the electrode to type I copper. The electrochemistry of the Met \rightarrow Gln mutant of bilirubin oxidase indicated that the intramolecular electron transfer between the type I copper center and the trinuclear copper center were accelerated as expected from the change in redox

potential [84]. The electric current density due to the electron transfer between the electrode surface and type I copper of fungal laccases [80, 82], bilirubin oxidase [81, 84] and CueO [83] dramatically increased in the presence of dioxygen, since dioxygen was effectively converted to water without forming activated oxygen species. CueO was found to be the most promising cathodic catalyst to construct a biofuel cell because of high stability and high electric current density. If the biofuel cell is used at pH 7, it is ideal that type I copper of MCO has a redox potential slightly lower than 0.815 mV, the potential at which dioxygen is converted to water. If MCO effectively functions at a more acidic pH, a higher voltage would be expected. MCOs that show more positive potentials have been explored to obtain larger voltage differences between the anodic catalyst and the cathodic catalyst.

The electron transfer from the type I copper center to the trinuclear copper center passes through the His-Cys-His triad. The electron transfer is performed using the through-bond mechanism, although a hydrogen bond between the O of Cys507 and the N^δ of His506 has been considered to be a short path in the case of ascorbate oxidase [100]. Therefore, the possible two electron transfer pathways leading to type III coppers might not be equivalent. In addition, it has been considered that the presence of the oxygen complexes significantly favors intramolecular electron transfer by affecting the driving force (*i.e.*, redox potential) [101].

Substrate specificity of MCOs

The specific binding of substrate and the electron transfer to the type I copper center is a process in which MCO functions as an oxidase. Unlike this process, which is specific to each MCO, the subsequent processes that transfer electrons from type I copper to the trinuclear copper center and convert dioxygen to water commonly take place in every MCO.

The substrate specificity of MCOs is quite broad except for the metallo-oxidases such as CueO, Fet3p, ceruloplasmin and manganese oxidases. Organic substrates are bound to the cleft at the "north" side of the protein molecules constructed by loops attached to the β -barrel scaffolds of the MCO domains (Fig: 3). The shape of the cleft is one of the critical factors that produce substrate specificity in MCOs. Bulky laccase substrates such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and syringaldazine were found to bind to the cleft of the substrate binding site in a folded form as indicated by X-ray crystallography of CotA [35] and the substrate-docked model of bilirubin oxidase [7]. An acidic amino acid is hydro-

gen-bonded with an imidazole group coordinating to type I copper, forming the electron transfer pathway. The side chain of Asp206 directly interacted with the 2,5-xylydine used as an inducer in the crystal structure of, *T. versicolor* laccase [29]. Accordingly, the Asp residue must be one of the key amino acids to produce laccase activity, giving a bell-shaped activity profile depending on pH. In the case of ascorbate oxidase, the two OH groups of L-ascorbate were within hydrogen-bonding distance of the imidazole nitrogen (NE2) of His512 coordinated to type I copper and the indole nitrogen (NE1) of Trp362 [42]. A stacking interaction of the L-ascorbate ring with the aromatic ring of another Trp also facilitated the binding of the substrate. Thus, the substrate specificity of MCO for organic substrates is produced by the integrated effect of the shape of the substrate-binding site and non-covalent interactions of the substrate with the amino acid(s) adjacent to the His residue coordinating to type I copper.

In contrast, cuprous oxidase, CueO, and ferroxidases, Fet3p and ceruloplasmin constitute their specific copper- or iron-binding site by assembling the ligand amino acids 2Asp2Met [37, 38] (Fig: 4), 1Glu2Asp1Gln [40], and 2Glu1Asp1His [44, 45], respectively, and affording a proper lability to the substrate metal ions. The substrate-binding site of CueO is insulated from bulk water by 50 amino acids including helices 5–7 [107]. Full and partial deletions of this segment produced oxidizing activities for a variety of laccase substrates depending on the extent of the deletion, affording evidence that helices 5–7 are essential for CueO to function as a cuprous oxidase. One of the Asp residues used as a ligand for the substrate is hydrogen-bonded with a His ligand coordinating type I copper, and thereby constructs the electron transfer pathway (Fig: 4) [37, 38]. An analogous hydrogen bond also has been found in Fet3p [40] and ceruloplasmin [44, 45]. This hydrogen bond is a universal contrivance to realize prompt electron transfer from the substrate to type I copper, which is buried inside the protein molecule.

In the case of NIR, the association with the redox partner is extremely important. The north side of NIR that accepts electrons from the cognate partner, blue copper protein (pseudoazurin, azurin) or cytochrome *c*, has a hydrophobic flat docking surface [48, 49]. In addition to the hydrophobic interactions, the fact that electrostatic interactions are also important for formation of a productive electron transfer complex between NIR and pseudoazurin, has been demonstrated by performing mutant preparations [125, 133], crystal structure analysis [134], and computational simulation [135].

Concluding remarks

The structure and properties of the type I copper center in MCO and NIR show a marked resemblance to the blue copper center in blue copper protein, while the type I copper center is closely connected with other copper site(s) through the amino acids intervening between them. The electron donor for type I copper is an organic substance or metal ion in the case of MCO and a protein molecule such as blue copper protein or cytochrome *c* in the case of NIR. The specific binding site of the substrate is adjacent to the type I copper center, and the electron transfer pathway is formed between the substrate binding site and the type I copper center by a hydrogen bond between an acidic amino acid and one of the His residues coordinating type I copper. The subsequent long-range electron transfer to the catalytic center is performed through the amino acids separating the type I copper center and the catalytic center. Mutations at or around the type I copper center change its redox potential and substrate specificity, leading to modifications of the oxidase function of MCO and to the development of MCO as an electro-catalyst for biofuel cells and a catalyst for dye formation and bleaching.

Acknowledgements. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (19350081 to ts), NEDO, Toyota Motor Corporation, and Mandom Corporation.

- Frausto da Silva, J. J. R. and Williams, R. J. P. (2001) The biological chemistry of the elements. The inorganic chemistry of life. 2nd edn. Oxford University Press, Oxford.
- Solomon, E. I., Szilagy, R. K., George, S. D. and Basumallick, L. (2004) Electronic structures of metal sites in proteins and models: contributions to function in blue copper proteins. *Chem. Rev.* 104, 419–458.
- Vila, A. J. and Fernandez, C. O. (2001) Copper in electron-transfer proteins. In: *Handbook on metalloproteins*, pp. 813–856, Bertini, I., Sigel, A. and Sigel, H. (eds.), Marcel Dekker, New York.
- Messerschmidt, A., Huber, R., Poulos, T. and Wieghardt, K. (eds.) (2001) Cupredoxins (type-1 copper proteins). In: *Handbook of metalloproteins*, vol. 2. pp. 1151–1241, John Wiley & Sons, Chichester.
- Lieberman, R. L., Arciero, D. M., Hooper, A. B. and Rosenzweig, A. C. (2001) Crystal structure of a novel red copper protein from *Nitrosomonas europaea*. *Biochemistry* 40, 5674–5681.
- Messerschmidt, A. (ed.) (1997) Multi-copper oxidases, World Scientific, Singapore.
- Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. (1996) Multicopper oxidases and oxygenases. *Chem. Rev.* 96, 2563–2605.
- Sakurai, T. and Kataoka, K. (2007) Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase and laccase. *Chem. Rec.* (in press).
- Guss, J. M. and Freeman, H. C. (1983) Structure of oxidized popular plastocyanin at 1.6 Å resolution. *J. Mol. Biol.* 169, 521–563.
- Mussiani, F., Dikiy, A., Semenov, A. Y. and Ciurli, S. (2005) Structure of the intermolecular complex between plastocyanin and cytochrome *f* from spinach. *J. Biol. Chem.* 280, 18833–18841.
- Nakamura, K. and Go, N. (2005) Function and molecular evolution of multicopper blue proteins. *Cell. Mol. Life Sci.* 62, 2050–2066.
- Kroneck, P. M. H. (2001) Binuclear copper, A. In: *Handbook of metalloproteins*. vol. 2. pp. 1331–1341, Messerschmidt, A., Huber, R., Poulos, T. and Wieghardt, K. (eds.), John Wiley & Sons, Chichester.
- De Rienzo, F., Gabdouliline, R. R., Wade, R. C., Sola, M. and Menziani, M. C. (2004) Computational approaches to structural and functional analysis of plastocyanin and other blue copper proteins. *Cell. Mol. Life Sci.* 61, 1123–1142.
- Nersissian, A. M. and Shipp, E. L. (2002) Blue copper-binding domains. *Adv. Protein Chem.* 60, 271–340.
- Machczynski, M. C., Gray, H. B. and Richards, J. H. (2002) An outer-sphere hydrogen-bond network constrains copper coordination in blue proteins. *J. Inorg. Biochem.* 88, 348–380.
- Gray, H. B., Malmstrom, B. G. and Williams, R. L. (2000) Copper coordination in blue proteins. *J. Biol. Inorg. Chem.* 5, 551–559.
- Li, C., Inoue, T., Gotowda, M., Suzuki, S., Yamaguchi, K., Kataoka, K. and Kai, Y. (1998) Structure of azurin-I from the denitrifying bacterium *Alcaligenes xylosoxidans* NCIMB 11015 at 2.45 Å resolution. *Acta Crystallogr. D Biol. Crystallogr.* 54, 347–354.
- Dodd, F. E., Hasnain, S. S., Abraham, Z. H. L., Eady, R. R. and Smith, B. E. (1995) Structure of a new azurin from the denitrifying bacterium *Alcaligenes xylosoxidans* at high resolution. *Acta Crystallogr. D Biol. Crystallogr.* 51, 1052–1064.
- Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M. and Canters, G. W. (1991) Crystal structure analysis of oxidized *Pseudomonas aeruginosa* azurin at pH 5.5 and pH 9.0. *J. Mol. Biol.* 221, 765–772.
- Lee, H., Dahms, T., Ton-That, H., Zhu, D.-W., Biesterfeldt, J., Lanthier, P. H., Yaguchi, M. and Szabo, A. G. (1997) Primary sequence and refined tertiary structure of *Pseudomonas fluorescens* holo azurin at 2.05 Å. *Acta Crystallogr. D Biol. Crystallogr.* 53, 493–506.
- Baker, E. N. (1988) Structure of azurin from *Alcaligenes denitrificans*. *J. Mol. Biol.* 203, 1071–1095.
- Chen, Z.-W., Barber, M. J., McIntire, W. S. and Mathews, F. S. (1998) Crystallographic study of azurin from *Pseudomonas putida*. *Acta Crystallogr. D Biol. Crystallogr.* 54, 253–268.
- Brown, K., Tegoni, M., Prudencio, M., Pereira, A. S., Besson, S., Moura, J. J., Moura, I. and Cambillau, C. (2000) A novel type of copper cluster in nitrous oxide reductase. *Nat. Struct. Biol.* 7, 191–195.
- Tsukihara, T., Aoyama, H., Nakashima, R., Yano, R. and Yoshikawa, S. (1995) Structure of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å. *Science* 269, 1069–1074.
- Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* 376, 660–669.
- Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Ostergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H. and Davies, G. J. (1998) Crystal structure of the type-2 copper depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nat. Struct. Biol.* 5, 310–316.
- Ducros, V., Brzozowski, A. M., Wilson, K. S., Ostergaard, P., Schneider, P., Svendsen, A. and Davies, G. J. (2001) Structure of the laccase from *Coprinus cinereus* at 1.68 Å resolution: evidence for different 'type 2 copper-depleted' isoforms. *Acta Crystallogr. D Biol. Crystallogr.* 57, 333–336.
- Piontek, K., Antorini, M. and Choinowski, T. (2002) Crystal Structure of a laccase from the fungus *Trametes versicolor* at

- 1.90-Å resolution containing a full complement of coppers. *J. Biol. Chem.* 277, 37663 – 37669.
- 29 Bertrand, T., Jolival, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. and Mougin, C. (2002) Crystal structure of a four-copper laccase with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry* 41, 7325 – 7333.
- 30 Hakulinen, N., Kruus, K., Koivula, A. and Rouvinen, J. (2006) A crystallographic and spectroscopic study on the effect of X-ray radiation on the crystal structure of *Melanocarpus albomyces* laccase. *Biochem. Biophys. Res. Commun.* 350, 929 – 934.
- 31 Hakulinen, N., Kiiskinen, L. L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A. and Rouvinen, J. (2002) Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nat. Struct. Biol.* 9, 601 – 605.
- 32 Garavaglia, S., Cambria, M. T., Miglio, M., Ragusa, S., Iacobazzi, V., Palmieri, F., D'Ambrosio, C., Scaloni, A. and Rizzi, M. (2004) The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. *J. Mol. Biol.* 342, 1519 – 1531.
- 33 Lyashenko, A. V., Bento, I., Zaitsev, V. N., Zhukhlistova, N. E., Zhukova, Y. N., Gabdoulkhakov, A. G., Morgunova, E. Y., Voelter, W., Kachalova, G. S., Stepanova, E. V., Koroleva, O. V., Lamzin, V. S., Tishkov, V. I., Betzel, C., Lindley, P. F. and Mikhailov, A. M. (2006) X-ray structural studies of the fungal laccase from *Cerrena maxima*. *J. Biol. Inorg. Chem.* 11, 963 – 973.
- 34 Enguita, F. J., Martins, L. O., Henriques, A. O. and Carrondo, M. A. (2003) Crystal structure of a bacterial endospore coat component: a laccase with enhanced thermostability properties. *J. Biol. Chem.* 278, 19416 – 19425.
- 35 Enguita, F. J., Marcal, D., Martins, L. O., Grenha, R., Henriques, A. O., Lindley, P. F. and Carrondo, M. A. (2004) Substrate and dioxygen binding to the endospore coat laccase from *Bacillus subtilis*. *J. Biol. Chem.* 279, 23472 – 23476.
- 36 Bento, I., Martins, L. O., Lopes, G. G., Carrondo, M. A. and Lindley, P. F. (2005) Dioxygen reduction by multi-copper oxidases; a structural perspective. *Dalton Trans.* 3507 – 3513.
- 37 Roberts, S. A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J. T., Tollin, G., Rensing, C. and Montfort, W. R. (2002) Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 99, 2766 – 2771.
- 38 Roberts, S. A., Wildner, G. F., Grass, G., Weichsel, A., Ambrus, A., Rensing, C. and Montfort, W. R. (2003) A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO. *J. Biol. Chem.* 278, 31958 – 31963.
- 39 Li, X., Wei, Z., Zhang, M., Peng, X., Yu, G., Teng, M. and Gong, W. (2007) Crystal structures of *E. coli* laccase CueO at different copper concentrations. *Biochem. Biophys. Res. Commun.* 354, 21 – 26.
- 40 Taylor, A. B., Stoj, C. S., Ziegler, L., Kosman, D. J. and Hart, P. J. (2005) The copper-iron connection in biology: structure of the metallo-oxidase Fet3p. *Proc. Natl. Acad. Sci. USA* 102, 15459 – 15464.
- 41 Smith, A. W., Camara-Artigas, A., Wang, M., Allen, J. P. and Francisco, W. A. (2006) Structure of phenoxazinone synthase from *Streptomyces antibioticus* reveals a new type 2 copper center. *Biochemistry* 45, 4378 – 4387.
- 42 Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. and Finazzi-Agro, A. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J. Mol. Biol.* 224, 179 – 205.
- 43 Messerschmidt, A., Luecke, H. and Huber, R. (1993) X-ray structures and mechanistic implications of three functional derivatives of ascorbate oxidase from zucchini. Reduced, peroxide and azide forms. *J. Mol. Biol.* 230, 997 – 1014.
- 44 Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A. and Lindley, P. (1996) The X-ray structure of human serum ceruloplasmin at 3.1 Å: Nature of the copper centres. *J. Biol. Inorg. Chem.* 1, 15 – 23.
- 45 Bento, I., Peixoto, C., Zaitsev, V. N. and Lindley, P. F. (2007) Ceruloplasmin revisited: structural and functional roles of various metal cation-binding sites. *Acta Crystallogr. D Biol. Crystallogr.* 63, 240 – 248.
- 46 Takahashi, N., Ortel, T. L. and Putnum, F. W. (1984) Single chain structure of ceruloplasmin: the complete amino acid sequence of the whole molecule. *Proc. Natl. Acad. Sci. USA* 81, 390 – 394.
- 47 Machczynski, M. C., Vijgenboom, E., Samyn, B. and Canters, G. W. (2004) Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Sci.* 13, 2388 – 2397.
- 48 Adman, E. T., Godden, J. W. and Turley, S. (1995) The structure of copper-nitrite reductase from *Achromobacter cycloclastes* at five pH values with NO₂⁻ bound and with type II copper depleted. *J. Biol. Chem.* 270, 27458 – 27474.
- 49 Inoue, T., Gotowda, M., Deligeer, Kataoka, K., Yamaguchi, K., Suzuki, S., Watanabe, H., Gohow, M. and Kai, Y. (1998) Type I copper structure of blue nitrite reductase from *Alcaligenes xylosoxidans* GIFU 1051 at 2.05 Å: resolution comparison of blue and green nitrite reductases. *J. Biochem.* 124, 876 – 879.
- 50 Xie, Y., Inoue, T., Miyamoto, Y., Matsuura, H., Kataoka, K., Yamaguchi, K., Suzuki, S. and Kai, Y. (2005) Structural reorganization of copper binding site involving Thr¹⁵ of mavecyanin from *Cucurbita pepo medullosa* (Zucchini) upon reduction. *J. Biochem.* 137, 455 – 461.
- 51 Suzuki, S., Kataoka, K., Yamaguchi, K., Inoue, T. and Kai, Y. (1999) Structure-function relationship of copper-containing nitrite reductase. *Coord. Chem. Rev.* 190 – 192, 245 – 265.
- 52 Suzuki, S., Kataoka, K. and Yamaguchi, K. (2000) Metal coordination and mechanism of multicopper nitrite reductase. *Acc. Chem. Res.* 33, 728 – 735.
- 53 Palmer, A. E., Szilagyi, R. K., Cherry, J. R., Jones, A., Xu, F. and Solomon, E. I. (2003) Spectroscopic characterization of the Leu513His variant of fungal laccase: effect of increased axial ligand interaction on the geometric and electronic structure of the type I copper site. *Inorg. Chem.* 42, 4006 – 4017.
- 54 Xu, F., Berka, R. M., Wahleithner, J. A., Nelson, B. A., Shuster, J. R., Brown, S. H., Palmer, A. E. and Solomon, E. I. (1998) Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. *Biochem. J.* 334, 63 – 70.
- 55 Sakurai, T. and Suzuki, S. (1997) Spectroscopy of cucumber ascorbate oxidase and fungal laccase in lit. In: Multi-Copper oxidases, pp. 225 – 250, Messerschmidt, A. (ed.), World Scientific, Singapore.
- 56 Kataoka, K., Kitagawa, R., Inoue, M., Naruse, D., Sakurai, T. and Huang, H. (2005) Point mutations at the type I copper ligands, Cys457 and Met467, and the putative proton donor, Asp105, in *Myrothecium verrucaria* bilirubin oxidase and reactions with dioxygen. *Biochemistry* 44, 7004 – 7012.
- 57 Palmer, A. E., Quintanar, L., Severance, S., Wang, T.-P., Kosman, D. J. and Solomon, E. I. (2002) Spectroscopic characterization and O₂ reactivity of the trinuclear copper cluster of mutants of the multicopper oxidase Fet3p. *Biochemistry* 41, 6438 – 6448.
- 58 Ueki, Y., Inoue, M., Kurose, S., Kataoka, K. and Sakurai, T. (2006) Mutations at Asp112 adjacent to the trinuclear copper center in CueO as the proton donor in the four-electron reduction of dioxygen. *FEBS Lett.* 580, 4069 – 4072.
- 59 Farrar, J. A., Neese, F., Lappalainen, P., Kroneck, P. M. H., Saraste, M., Zumft, W. G. and Thomson, J. A. (1996) The electronic structure of copper A: a novel mixed-valence dinuclear copper electron-transfer center. *J. Am. Chem. Soc.* 118, 11501 – 11514.
- 60 Farrar, J. A., Zumft, W. G. and Thomson, J. A. (1998) Cu_A and Cu_Z are variants of the electron transfer center in nitrous oxide reductase. *Proc. Natl. Acad. Sci. USA* 95, 9891 – 9896.

- 61 Shimizu, A., Sasaki, T., Kwon, J. H., Odaka, A., Satoh, T., Sakurai, N., Sakurai, T., Yamaguchi, S. and Samejima, T. (1999) Site-directed mutagenesis of a possible type I copper ligand of bilirubin oxidase; a Met467Gln mutant show stellacyanin-like properties. *J. Biochem.* 125, 662 – 668.
- 62 Kataoka, K., Yamaguchi, K., Sakai, S., Takagi, K. and Suzuki, S. (2003) Characterization and function of Met150Gln mutant of copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM1013. *Biochem. Biophys. Res. Commun.* 303, 519 – 524.
- 63 Durao, P., Bento, I., Fernandes, A. T., Melo, E. P., Lindley, P. F. and Martins, L. O. (2006) Perturbations of the T1 copper site in the CotA laccase from *Bacillus subtilis*: structural, biochemical, enzymatic and stability studies. *J. Biol. Inorg. Chem.* 11, 514 – 527.
- 64 Shimizu, A., Kwon, J.-H., Sasaki, T., Satoh, T., Sakurai, N., Sakurai, T., Yamaguchi, S. and Samejima, T. (1999) *Myrothecium verrucaria* bilirubin oxidase and its mutants for potential copper ligands. *Biochemistry* 38, 3034 – 3042.
- 65 Xu, F., Palmer, A. E., Yaver, D. S., Berka, R. M., Gambetta, G. A., Brown, S. H. and Solomon, E. I. (1999) Targeted mutations in a *Trametes villosa* laccase. *J. Biol. Chem.* 274, 12372 – 12375.
- 66 Sakurai, T., Zhang, L., Fujita, T., Kataoka, K., Shimizu, A., Samejima, T. and Yamaguchi, S. (2003) Authentic and recombinant bilirubin oxidase are in different resting forms. *Biosci. Biotechnol. Biochem.* 67, 1157 – 1159.
- 67 Arciero, D. M., Pierce, B. S., Hendrich, M. P. and Hooper, A. B. (2002) Nitrosocyanin, a red cupredoxin-like protein from *Nitrosomonas europaea*. *Biochemistry* 41, 1703 – 1709.
- 68 Neese, F., Zumft, W. G., Antholine, W. E. and Kroneck, P. M. H. (1996) The purple mixed-valence Cu_A center in nitrous-oxide reductase: EPR of the copper-63-, copper-65-, and both copper-65- and [¹⁵N]histidine-enriched enzyme and a molecular orbital interpretation. *J. Am. Chem. Soc.* 118, 8692 – 8699.
- 69 Woodruff, W. H., Dwyer, R. B. and Schoonover, J. R. (1988) Resonance Raman spectroscopy of blue copper proteins. In: *Biological applications of Raman spectroscopy*, Chapter 9, Spiro, T. G. (ed.), John Wiley & Sons, New York.
- 70 Shimizu, A., Samejima, T., Hirota, S., Yamaguchi, S., Sakurai, N. and Sakurai, T. (2003) Type III copper mutants of *Myrothecium verrucaria* bilirubin oxidase. *J. Biochem.* 133, 767 – 772.
- 71 Palmer, A. E., Randall, D. W., Xu, F. and Solomon, E. I. (1999) Spectroscopic studies and electronic structure description of the high potential type I copper site in fungal laccase: insight into the effect of the axial ligand. *J. Am. Chem. Soc.* 121, 7138 – 7149.
- 72 Alvarez, M. L., Ai, J., Zumft, W. G., Sanders-Loehr, J. and Dooley, D. M. (2001) Characterization of the copper-sulfur chromophores in nitrous oxide reductase by resonance Raman spectroscopy: evidence for sulfur coordination in the catalytic cluster. *J. Am. Chem. Soc.* 123, 576 – 587.
- 73 Cole, J. L., Tan, G. O., Yang, E. K., Hodgson, K. O. and Solomon, E. I. (1990) Reactivity of the trinuclear copper active site with dioxygen: an X-ray absorption edge study. *J. Am. Chem. Soc.* 112, 2243 – 2249.
- 74 Sakurai, T., Suzuki, A. and Sano, M. (1988) X-ray absorption study on the type II copper depleted cucumber ascorbate oxidase. *Inorg. Chim. Acta* 152, 3 – 4.
- 75 Charnock, J. M., Dreusch, A., Korner, H., Neese, F., Nelson, J., Kannt, A., Michel, H., Garner, C. D., Kroneck, P. H., M. and Zumft, W. G. (2000) Structural investigations of the copper A centre of nitrous oxide reductase from *Pseudomonas stutzeri* by site-directed mutagenesis and X-ray absorption spectroscopy. *Eur. J. Biochem.* 267, 1368 – 1381.
- 76 Nakamura, T. (1958) Purification and physico-chemical properties of laccase. *Biochim. Biophys. Acta* 30, 44 – 52.
- 77 Deinum, J. and Vanngard, T. (1973) The stoichiometry of the paramagnetic copper and the oxidation-reduction potentials of type I copper in human ceruloplasmin. *Biochim. Biophys. Acta* 310, 321 – 330.
- 78 Kawahara, K., Suzuki, S., Sakurai, T. and Nakahara, A. (1984) Characterization of cucumber ascorbate oxidase and its reaction with hexacyanoferrate(II). *Arch. Biochem. Biophys.* 241, 179 – 186.
- 79 Sakurai, T. (1996) Cyclic voltammetry of cucumber ascorbate oxidase. *Chem. Lett.* 481 – 482.
- 80 Yaropolov, A. I., Kharybin, A. N., Emneus, J., Marko-Varga, G. and Gorton, L. (1996) Electrochemical properties of some copper-containing oxidases. *Bioelectrochem. Bioener.* 40, 49 – 57.
- 81 Tsujimura, S., Kano, K. and Ikeda, T. (2005) Bilirubin oxidase in multiple layers catalyzes four-electron reduction of dioxygen to water without redox mediators. *J. Electroanal. Chem.* 576, 113 – 120.
- 82 Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A. I., Whittaker, J. W. and Gorton, L. (2005) Direct electron transfer between copper-containing proteins and electrodes. *Biosens. Bioelectron.* 20, 2517 – 2554.
- 83 Miura, Y., Tsujimura, S., Kamitaka, Y., Kurose, S., Kataoka, K., Sakurai, T. and Kano, K. (2007) Bioelectrocatalytic reduction of O₂ catalyzed by CueO from *Escherichia coli* adsorbed on a highly oriented pyrolytic graphite electrode. *Chem. Lett.* 36, 132 – 133.
- 84 Kamitaka, Y., Tsujimura, S., Kataoka, K., Sakurai, T., Ikeda, T. and Kano, K. (2007) Effects of axial ligand mutation of the type I copper site in bilirubin oxidase on direct electron transfer-type bioelectrocatalytic reduction of dioxygen. *J. Electroanal. Chem.* 601, 119 – 124.
- 85 Armstrong, F. A. (1990) Probing metalloproteins by voltammetry. *Structure Bonding* 72, 137 – 221.
- 86 Ikeda, O. and Sakurai, T. (1994) Electron transfer reaction of stellacyanin at a bare glassy carbon electrode. *Eur. J. Biochem.* 219, 813 – 819.
- 87 Sakurai, T. and Nose, F. (1995) Direct electrochemistry of blue copper proteins at Au electrodes modified with promoters. *Chem. Lett.* 1075 – 1076.
- 88 Sakurai, T., Nose, F., Fujiki, T. and Suzuki, S. (1996) Reduction and oxidation of blue copper proteins, azurin, pseudoazurin, umecyanin, stellacyanin, plantacyanin, and plastocyanin approached by cyclic and potential step voltammetries. *Bull. Chem. Soc. Jpn.* 69, 2855 – 2862.
- 89 Kohzuma, T., Shidara, S. and Suzuki, S. (1994) Direct electrochemistry of nitrite reductase from *Achromobacter cycloclastes* IAM 1013. *Bull. Chem. Soc. Jpn.* 67, 138 – 143.
- 90 Yamaguchi, K., Shuta, K. and Suzuki, S. (2005) Roles of Trp144 and Tyr203 in copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM 1013. *Biochem. Biophys. Res. Commun.* 336, 210 – 214.
- 91 Fernandez, C. O., Sannazzaro, A. I. and Vila, A. J. (1997) Alkaline transition of *Rhus vernicifera* stellacyanin, an unusual blue copper protein. *Biochemistry* 36, 10566 – 10570.
- 92 Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M. and Canters, G. W. (1991) Crystal structure analysis of oxidized *Pseudomonas aeruginosa* azurin at pH 5.5 and pH 9.0. A pH-induced conformational transition involves a peptide bond flip. *J. Mol. Biol.* 221, 765 – 772.
- 93 Yanagisawa, S., Banfield, M. J. and Dennison, C. (2006) The role of hydrogen bonding at the active site of a cupredoxin: the Phe114Pro azurin variant. *Biochemistry* 45, 8812 – 8822.
- 94 Sakurai, T. (2006) The alkaline transition of blue copper proteins, *Cucumis sativus* plastocyanin and *Pseudomonas aeruginosa* azurin. *FEBS Lett.* 580, 1729 – 1732.
- 95 Battistuzzi, G., Barsari, M., Loschi, L., Ranieri, A., Sol, M., Mondovi, B. and Marchesini, A. (2001) Redox properties and acid-base equilibria of zucchini mavecyanin. *J. Inorg. Biochem.* 83, 223 – 227.
- 96 Zoppellaro, G., Sakurai, N., Kataoka, K. and Sakurai, T. (2004) The reversible change in the redox state of type I copper in *Myrothecium verrucaria* bilirubin oxidase depending on pH. *Biosci. Biotechnol. Biochem.* 68, 1998 – 2000.

- 97 Andreasson, L.-E. and Reinhammar, B. (1979) The mechanism of electron transfer in laccase-catalyzed reactions. *Biochim. Biophys. Acta* 568, 145 – 156.
- 98 Hansen, F. B., Noble, R. W. and Ettinger, M. J. (1984) Absorbance and fluorescence stopped-flow kinetics of *Rhus vernicifera* laccase and the catalytic reaction sequence. *Biochemistry* 23, 2049 – 2056.
- 99 O'Neill, P., Fildes, E. M., Avigliano, L., Marcozzi, G., Ballini, A. and Finazzi-Agro, A. (1984) Pulse-radiolysis studies on the interaction of one-electron reduced species with blue oxidases. Reduction of type-2-copper depleted ascorbate oxidase. *Biochem. J.* 222, 65 – 70.
- 100 Kyritsis, P., Messerschmidt, A., Huber, R., Salmon, G. A. and Sykes, A. G. (1993) Pulse-radiolysis studies on the oxidized form of the multicopper enzyme ascorbate oxidase: evidence for two-intramolecular electron-transfer steps. *J. Chem. Soc. Dalton trans.* 731 – 735.
- 101 Farver, O., Wherland, S. and Pecht, I. (1994) Intramolecular electron transfer in ascorbate oxidase in enhanced in the presence of oxygen. *J. Biol. Chem.* 269, 22933 – 22936.
- 102 Huang, H., Zoppellaro, G. and Sakurai, T. (1999) Spectroscopic and kinetic studies on the oxygen-centered radical formed during four-electron reduction process of dioxygen by *Rhus vernicifera* laccase. *J. Biol. Chem.* 274, 32718 – 32724.
- 103 Zoppellaro, G., Huang, H. and Sakurai, T. (2000) Kinetic studies on the reaction of the fully reduced laccase with dioxygen. *Inorg. React. Mech.* 2, 79 – 84.
- 104 Stoj, C. S., Augustine, A. J., Zeigler, L., Solomon, E. I. and Kosman, D. J. (2006) Structural basis of the ferrous iron specificity of the yeast ferroxidase, Fet3p. *Biochemistry* 45, 12741 – 12749.
- 105 Suzuki, S., Furusawa, H., Kataoka, K., Yamaguchi, K., Kobayashi, K. and Tagawa, S. (2000) Intramolecular electron transfer process of native and mutant forms of blue copper-containing nitrite reductase from *Alcaligenes xylosoxidans*. *Inorg. React. Mech.* 2, 129 – 135.
- 106 Meyer, T. E., Marchesini, A., Cusanovich, M. A. and Tollin, G. (1991) Direct measurement of intermolecular electron transfer between type I and type III copper center in the multi-copper enzyme ascorbate oxidase and its type II copper-depleted and cyanide-inhibited forms. *Biochemistry* 30, 4619 – 4623.
- 107 Kurose, S., Kataoka, K., Otsuka, K., Tsujino, Y. and Sakurai, T. (2007) Promotion of laccase activities of *Escherichia coli* cuprous oxidase CueO by deleting the segment covering the substrate binding site. *Chem. Lett.* 36, 232 – 233.
- 108 Takeda, T., Itoh, H., Yoshioka, I., Yamamoto, M., Misaki, H., Kajita, S., Shirai, K., Kata, M., Shin, T., Murao, S. and Tsukagoshi, N. (1998) Cloning of a thermostable ascorbate oxidase from *Acremonium* sp. HI-25 and modification of the azide sensitivity of the enzyme by site-directed mutagenesis. *Biochim. Biophys. Acta* 1388, 444 – 456.
- 109 Bielli, P., Bellench, G. C. and Calabrese, L. (2001) Site-directed mutagenesis of human ceruloplasmin. Production of a proteolytically stable protein and structure-activity relationships of type 1 sites. *J. Biol. Chem.* 276, 2678 – 2685.
- 110 Li, X., Wei, Z., Zhang, M., Peng, X., Yu, G., Teng, M. and Gong, W. (2007) Crystal structures of *E. coli* laccase CueO at different copper concentrations. *Biochem. Biophys. Res. Commun.* 354, 21 – 24.
- 111 Askwith, C. C. and Kaplan, J. (1998) Site-directed mutagenesis of the yeast multicopper oxidase Fet3p. *J. Biol. Chem.* 273, 22415 – 22419.
- 112 Stoj, C., Augustine, A. J., Solomon, E. I. and Kosman, D. J. (2007) Structure-function analysis of the cuprous oxidase activity in Fet3p from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 282, 7861 – 7868.
- 113 Kwok, E. Y., Severance, S. and Kosman, D. J. (2006) Evidence for iron channeling in the Fet3p-Ftr1p high-affinity iron uptake complex in the yeast plasma membrane. *Biochemistry* 45, 6317 – 6327.
- 114 di Patti, M. C. B., Felice, M. R., De Domenico, I., Lania, A., Alaleona, F. and Musci, G. (2005) Specific aspartate residues in Fet3 control high-affinity iron transport in *Saccharomyces cerevisiae*. *Yeast* 22, 677 – 687.
- 115 Quintanar, L., Stoj, C., Wang, T.-P., Kosman, D. J. and Solomon, E. I. (2005) Role of aspartate in the decay of the peroxide intermediate in the multicopper oxidase Fet3p. *Biochemistry* 44, 6081 – 6091.
- 116 Blackburn, N. J., Ralle, M., Hassett, R. and Kosman, D. J. (2000) Spectroscopic analysis of the trinuclear cluster in the Fet3 protein from yeast, a multicopper oxidase. *Biochemistry* 39, 2316 – 2324.
- 117 Yamaguchi, K., Tsuda, A., Nojiri, M. and Suzuki, S. (2006) Effect of arginine at type 2 copper site on spectroscopic features and enzymatic activity of copper-containing nitrite reductase. *Chem. Lett.* 36, 140 – 141.
- 118 Fittipaldi, M., Wijma, H. J., Verbeet, M. P., Canters, G. W., Groenen, E. J. and Huber, M. (2005) The substrate-bound type 2 copper site of nitrite reductase: the nitrogen hyperfine coupling of nitrite revealed by pulsed EPR. *Biochemistry* 44, 15193 – 15202.
- 119 Hough, M. A., Ellis, M. J., Antonyuk, S., Strange, R. W., Sawers, G., Eady, R. R. and Hasnain, S. S. (2005) High resolution structure studies of mutants provide insights into catalysis and electron transfer processes in copper nitrite reductase. *J. Mol. Biol.* 350, 300 – 309.
- 120 Wijma, H. J., MacPherson, I., Farver, O., Tocheva, E. I., Pecht, I., Verbeet, M. P., Murphy, M. E. P. and Canters, G. W. (2007) Effect of methionine ligand on the reorganization energy of the type -1 copper site of nitrite reductase. *J. Am. Chem. Soc.* 129, 519 – 525.
- 121 Boulanger, M. J., Kukimoto, M., Nishoyama, M., Horinouchi, S. and Murphy, M. E. P. (2000) Catalytic roles for two water bridged residues (Asp-98 and His-255) in the active site of copper-containing nitrite reductase. *J. Biol. Chem.* 275, 23957 – 23964.
- 122 Wijma, H. J., Boulanger, M. J., Molon, A., Fittipaldi, M., Huber, M., Murphy, M. E. P., Verbeet, M. P. and Canters, G. W. (2003) Reconstitution of the type-1 active site of the H145G/A variants of nitrite reductase by ligand insertion. *Biochemistry* 42, 4075 – 4983.
- 123 Zhao, Y., Lukoyanov, D. A., Toropov, Y. V., Wu, K., Shapleigh, J. P. and Scholes, C. P. (2002) Catalytic function and local proton structure at the type 2 copper of nitrite reductase: the correlation of enzymatic pH dependence, conserved residues, and proton hyperfine structure. *Biochemistry* 41, 7464 – 7474.
- 124 Kataoka, K., Furusawa, H., Takagi, K., Yamaguchi, K. and Suzuki, S. (2000) Functional analysis of conserved aspartate and histidine residues located around type 2 copper site of copper-containing nitrite reductase. *J. Biochem.* 127, 345 – 350.
- 125 Kukimoto, M., Nishiyama, M., Tanokura, M., Adman, E. T. and Horinouchi, S. (1996) Studies on protein-protein interaction between copper-containing nitrite reductase and pseudoazurin from *Alcaligenes faecalis* S-6. *J. Biol. Chem.* 271, 13680 – 13683.
- 126 Prudencio, M., Eady, R. R. and Sawers, G. (2001) Catalytic and spectroscopic analysis of blue copper-containing nitrite reductase mutants altered in the environment of the type 2 copper centre: implications for substrate interaction. *Biochem. J.* 353, 259 – 266.
- 127 Boulanger, M. J. and Murphy, M. E., P. (2001) Alternate substrate binding models to two mutant (D98N and H255N) forms of nitrite reductase from *Alcaligenes faecalis* S-6: structure model of a transient catalytic intermediate. *Biochemistry* 40, 9132 – 9241.
- 128 Prudencio, M., Sawers, G., Fairhurst, S. A., Yousafzai, F. K. and Eady, R. R. (2002) *Alcaligenes xylosoxidans* dissimilatory nitrite reductase: alanine substitution of the surface-exposed histidine 139 ligand on the type I copper center prevents

- electron transfer to the catalytic center. *Biochemistry* 41, 3430 – 3438.
- 129 Olesen, K., Veselov, A., Zhao Y., Wang, Y., Danner, B., Scholes, C. P. and Shapleigh, J. P. (1998) Spectroscopic, kinetic, and electrochemical characterization of heterologously expressed wild-type and mutant forms of copper-containing nitrite reductase from *Rhodobacter sphaeroides* 2.4.3. *Biochemistry* 37, 6086 – 6094.
- 130 Veselov, A., Olesen, K., Sienkiewicz, A., Shapleigh, J. P. and Scholes, C. P. (1998) Electronic structural information from Q-band ENDOR on the type 1 and type 2 copper liganding environment in wild-type and mutant forms of copper-containing nitrite reductase. *Biochemistry* 37, 6095 – 6105.
- 131 Murphy, M. E. P., Turley, S., Kukimoto, M., Nishiyama, M., Hirinouchi, S., Sasaki, H., Tanokura, M. and Adman, E. T. (1995) Structure of *Alcaligenes faecalis* nitrite reductase and a copper site mutant M150E, that contains zinc. *Biochemistry* 34, 12107 – 12117.
- 132 Kukimoto, M., Nishiyama, M., Murphy, M. E. P., Turley, S., Adman, E. T., Horinouchi, S. and Beppu, T. (1994) X-ray structure and site-directed mutagenesis of a nitrite reductase from *Alcaligenes faecalis* S-6: roles of two copper atoms in nitrite reduction. *Biochemistry* 33, 5246 – 5252.
- 133 Kataoka, K., Yamaguchi, K., Kobayashi, M., Mori, T., Bokui, N. and Suzuki, S. (2004) Structure-based engineering of *Alcaligenes xylooxidans* copper-containing nitrite reductase enhances intermolecular electron transfer reaction with pseudoazurin. *J. Biol. Chem.* 279, 53374 – 53378.
- 134 Murphy, M. E. P., Turley, S. and Adman, E. T. (1998). On the mechanism of nitrite reductase: complex between pseudoazurin and nitrite reductase from, *A. cycloclastes*. In: *Biological electron transfer chains: genetics, composition, and mode of operation*, pp. 115 – 128, Canters, G. W. and Vijgenboom, E. (eds.), NATO Science Series, Kluwer Academic Publishers, Dordrecht.
- 135 Murphy, L. M., Dodd, F. E., Yousafzai, F. K., Eady, R. R. and Hasnain, S. S. (2002) Electron donation between copper containing nitrite reductases and cupredoxins: the nature of protein-protein interaction in complex formation. *J. Mol. Biol.* 315, 859 – 871.

To access this journal online:
<http://www.birkhauser.ch/CMLS>
