Type III Cu mutants of Myrothecium verrucaria bilirubin oxidase

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Type III Cu Mutants of *Myrothecium verrucaria* Bilirubin Oxidase

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Type III Cu ligand, His456 and His458, of *Myrothecium verrucaria* (MT-1) bilirubin oxidases (BO) [EC 1.3.3.5] were doubly mutated as to Lys, Asp, and Val. In spite of perturbation of the type III Cu centers, these mutants were pale blue or colourless when isolated. However, they became intense blue on reaction with reducing agents such as dithionite, ascorbate, hexacyanoferrate(II), and octacyanotangstate(IV) under air, or with an oxidizing agent such as hexacyanoferrate(III), indicating that they are in mixed forms when expressed in *Aspergillus oryzae*. His456.458Lys and His456.458Asp mutated as to potential coordinating groups showed weak BO and ferroxidase activities, while His 456.458Val mutated as to non-coordinating groups showed no enzyme activity at all.

Key words: bilirubin oxidase, multicopper oxidase, mutant, trinuclear Cu center.

Abbreviations: BO, bilirubin oxidase; SHE, standard hydrogen electrode.

Bilirubin oxidase (BO) [EC 1.3.3.5] catalyzes the oxidation of bilirubin to biliverdin, and further to a purple pigment (*1*). BO is a multicopper oxidase containing one type I Cu, one type II Cu and a pair of type III Cus per protein molecule (*2*, *3*). Type I Cu mediates electron transfer from a substrate to other Cu sites. Type II and type III Cus form the trinuclear center, to which dioxygen is bound and reduced to two water molecules. Type I and type II Cus are EPR-detectable but type III Cus are EPR-undetectable due to the strong antiferromagnetic interaction through a bridging hydroxo group. The nucleotide sequence of the gene encoding *Myrothecium verrucaria* BO (*4*) indicated that all ligands for the four Cu ions are conserved, similarly to those of other multicopper oxidases such as tree laccase (*5*), ascorbate oxidase (*6*), and ceruloplasmin (*7*) (Fig. 1). These multicopper oxidases have donor sets of 1Cys2His1Met for type I Cu, 2His1H_2O(or 1OH^-) for type II Cu, and 6His1OH^- (or O^{2-}) for type III Cus, whilst multicopper oxidases such as fungal laccase (*8*) and yeast Fet3 (*9*) lack the Met for type I Cu. While *M. verrucaria* BO has a Met for type I Cu, *Trachderma tsunodae* BO does not have it (*10*), and it is not known why there are two BOs with different redox potentials.

In previous papers we reported the successful preparation of a variety of *M. verrucaria* BO mutants as to every Cu site and their characterization (*2*, *3*). The direct mutations at the type I Cu site (Met467Gly, Met467Gln, Met467His, Met467Arg, Cys457Ala, Cys457Val, His462Gly, and His462Val) indicated that Met467 is not necessarily indispensable for formation of the type I Cu center, as shown by mutation studies on blue copper proteins such as plastocyanin and azurin (*11*, *12*). Mt467Gln gave a rhombic type I Cu similar to the blue copper centers in stellacyanin and mavicyanin (*3*). A conspicuous difference between the type I Cu mutants of BO and blue copper proteins was that some of the former exhibited BO and ferroxidase activities (Met467Gln showed higher ferroxidase activity than that of the wild type BO, although its BO activity was significantly decreased by the change in the redox potential of type I Cu). On the other hand, the effects of mutations on both spectroscopic properties and enzyme activities were more conspicuous, when the amino acids directly coordinating the trinuclear Cu center were mutated (*2*). His94Asp, His94Asn and His94Val have been prepared as type II Cu mutants, but a certain scrambling of the His residue(s) towards Cu ions took place in the trinuclear center, leading to the incorporation of less than three Cu ions in it. It is apparent that His is indispensable for the formation of this unusual three-coordinate Cu site. In line with this, the recombinant *Coprinus cinereus* laccase has been reported to lack the type II Cu ion, and one of the His residues to it switched to coordinate one of type III Cus (*13*). As a type III Cu mutant, we prepared a double mutant, His456.458Val (*2*). However, trinuclear center was empty and only the type I Cu site was occupied by a Cu ion. Another type III Cu mutant, His134.136Val, was expressed as an apo-protein.

In the present paper, we reported the preparation and characterization of mutants, His456.458Lys and His456.458Asp, in which 2 His for type III Cus were substituted by potential coordinating amino acids (Fig. 1).

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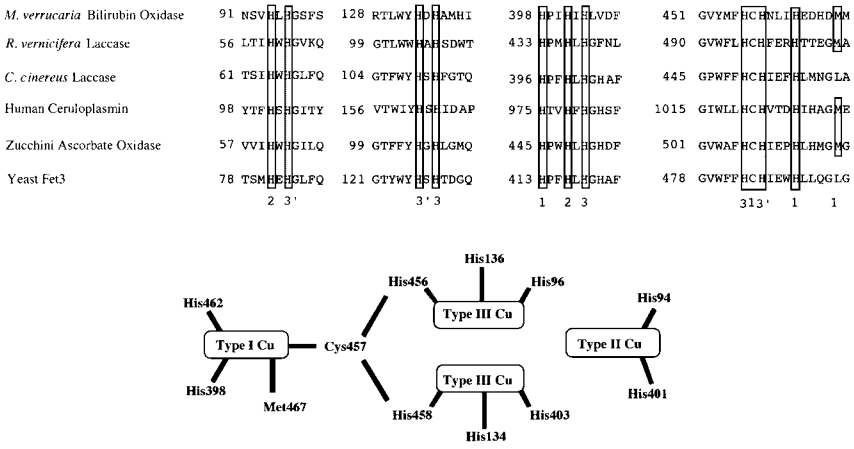

Fig. 1. **Sequence comparison of *M. verrucaria* bilirubin oxidase, *R. vernicifera* laccase, C. cinereus laccase, human ceruloplasmin, zucchini ascorbate oxidase and yeast Fet3 around the Cu-binding site, and topology of ligand groups in bilirubin oxidase.**

These His residues are at the two ends of the consensus sequence of multicopper oxidase, His-Cys-His. The enzyme activities of these mutants were also measured in comparison with those of the wild type BO.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis of Bilirubin Oxidase—The pNBC1 plasmid harbouring the *M. verrucaria* BO gene, diaminoacyl lipase (mdlA) promoter, and *Aspergillus oryzae* nitrate reductase gene (niaD) as a selection marker was constructed as reported earlier (*14*). In vitro mutagenesis was achieved by PCR using two primers, which have the recognition site of the restriction enzyme and sets of mutagenic oligonucleotides, as follows. The oligonucleotide primers used for mutageneses were from Sawaday Technology.

H456.458K(+): 5′-TACATGTTTAAATGCAAGAATTTG-ATTC-3′

H456.458D(–): 5′-AATCAAATTGTCGCAATCGAACAT-GTA-3′

The PCR products were cloned into pUC18 and sequenced to confirm the successful mutations. The mutated BO genes were cloned into pNBC1 for utilization as expression vectors, pNTIII-56V, pNTIII-56D, and pNTIII-56K for His456.458Val, His456.458Asp, and His456.458Lys, respectively. These plasmids were purified from *E. coli* cells by the alkali-SDS method, and 5 μg of DNA was introduced into the *A. oryzae* niaD strain by protoplast transformation (*15*). Transformants were cultured with a minimum volume of medium containing nitrate as the sole nitrogen source for 5 days and further cultured for 5 days in the medium containing soybean oil, as reported (*2*, *3*). The level of the expression products of the Val mutant was moderate but those of the Asp and Lys mutants were high, as ascertained by SDS-PAGE. Purification of the BO mutants was performed as reported elsewhere (the purities of the mutants were higher than 95% on SDS-PAGE with a colourless impurity free from metal ions) (*2*, *3*).

Assaying of Enzyme Activities—The BO and ferroxidase activities of the wild type enzyme and mutants were determined in Tris-H_2SO_4 buffer (pH 8.4) as the decrease in the absorbance at 440 nm and as the increase in the absorbance at 420 nm, respectively, as reported (*1*).

Redox Potential—The redox potential of type 1 Cu of the mutants was determined by potentiometry as the decrease in the absorption at 600 nm using a home-made optical cell attached to a reference Ag/AgCl electrode, a counter Pt electrode, and an inlet and outlet for Ar (*3*). Hexacyanoferrate(II), as a reductant, was added stepwisely using a gas-tight syringe under Ar.

Instruments and Spectroscopic Measurements—Absorption spectra were measured with a JASCO Ubest 50 spectrometer. The X- band EPR spectra were recorded on a JEOL JES-RE1X spectrometer at 77 K and on a Bruker ESP-300E spectrometer at 3–77 K. The EPR-detectable amount of Cu^{2+} was determined by the double integration method using Cu-EDTA as a standard. Signal intensities due to the differences in tuning conditions were calibrated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as an external standard (*16*). The total amounts of copper present in mutants were determined by atomic absorption spectroscopy with a Shimadzu AA-640-13 or a Varian SpectrAA-55B. Resonance Raman scattering was excited at 591 nm with an Ar^+ laser (Spectra Physics, 2017)–pumped dye laser (Spectra physics, 376) with a Rhodamine-6G, and detected with a triple polychrometor (JASCO, NR-1800) equipped with a CCD detector (Princeton Instruments). Resonance Raman measurements were carried out at ambient temperature with a spinning cell. Raman shifts were calibrated with CCl_4 and toluene, and the accuracy of the peak positions of the Raman bands was ±1 cm^{-1}. The mutated DNA segment was verified by sequencing with a DNA sequencer, Hitachi SQ-5500E.

RESULTS

His456.458Lys Mutant—Figure 2B shows the absorption spectrum of His456.458Lys as isolated (the absorption spectrum of the wild type BO is shown in Fig. 2A for comparison).

The absorption intensity of the band at 600 nm (broken line) was very low (ε = *ca.* 1,000) compared with that of the wild type BO (ε = *ca.* 5,000). However, when His456.458Lys, as isolated, was treated with hexacyanoferrate(II), octacyanotangstate(IV), or ascorbate under air, the absorption intensity of the band at 600 nm drastically increased to become ε = 5,800 per protein molecule after temporal bleaching of blue colour (solid line), indicating that *ca.* 80% of type I Cu had been reduced when isolated. Concomitantly, the absorption intensity of the shoulder at 330 nm also increased, although it was still

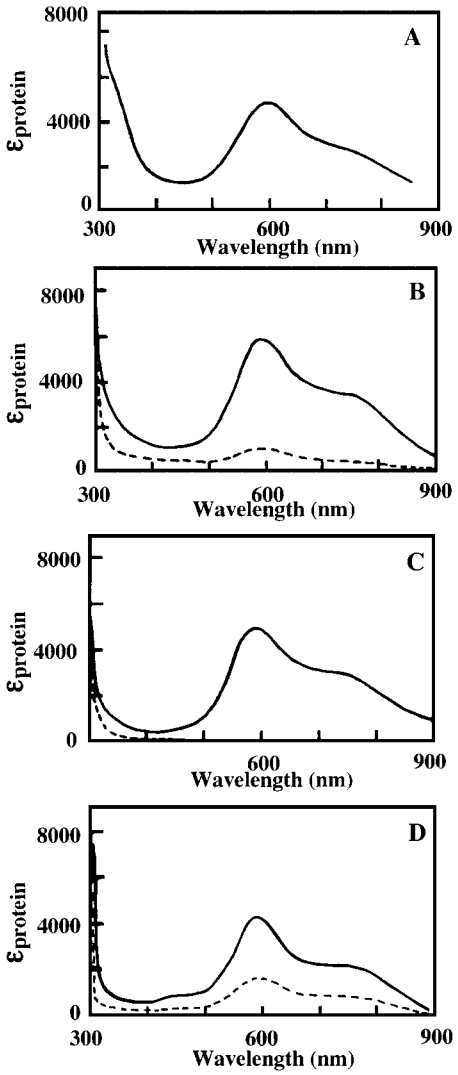

Fig. 2. **Absorption spectra of the wild type BO(A), His456.458Lys mutant as isolated (broken line) and as oxidized (solid line) (B), His456.458Asp mutant as isolated (broken line) and as oxidized (solid line) (C), and His456.458Val mutant as isolated (broken line) and as oxidized (solid line) (D).** All spectra were measured in phosphate buffer (pH 7.4). The oxidized spectra (solid lines) were obtained by treating mutants as isolated with hexacyanoferrate(III) and dialysis against buffer.

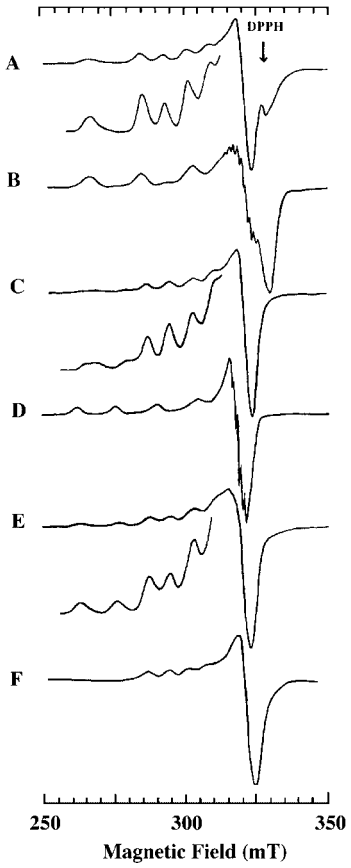

Fig. 3. **X-band EPR spectra of the (A) wild type BO, (B) His456.458Lys as isolated, (C) B as treated with hexacynaoferrate(III) and dialyzed against phosphate buffer (pH 7.4), (D) His456.458Asp as isolated, (E) D treated with hexacynaoferrate(III) and dialyzed against phosphate buffer (pH 7.4), and (E) His456.458 as isolated (F).** Measurement conditions: temperature, 77 K, microwave power, 5 mW, microwave frequency, 9.22 GHz, modulation, 5mT (100 kHz), amp, 500, time const., 0.1 s, sweep time, 8 min.

considerably weak compared with that of the wild type BO. The action of hexacyanoferrate(III) led to similar spectral changes.

The EPR spectrum of His456.458Lys, as isolated, is shown in Fig. 3B. The type II Cu signal (g_{II} = 2.24 and A_{II} = 17.7 × 10^{-3} cm^{-1}) was fully observed (Table 1) with five to seven superhyperfine splittings (A_N = 1.4 mT) due to the two to three N-coordinations (the EPR spectrum of the wild type BO, to which the type I and type II Cus fully contributed, is shown in Fig. 3A for comparison). The intensity of the I Cu EPR signal (g_{II} = 2.20 and A_{II} = 7.7 × 10^{-3} cm^{-1}) was very weak, contributing only 0.3 Cu^{2+} per protein molecule. However, when His456.458Lys, as isolated, was treated with hexacyanoferrate(II), hexacyanoferrate(III), octacyanotangstate(IV), or ascorbate under air, the signal intensity of type I Cu increased to become

Table 1. **Absorption and EPR spectral data for the wild type BO and type III Cu mutants.**

BO and mutants	ε_{600}	Type I Cu		Type II or Type III Cu		EPR-detectable Cu^{2+} per protein
		g_{II}	A_{II} (cm^{-1}) × 10^{-3}	g_{II}	A_{II} (cm^{-1}) × 10^{-3}	
Wild type	5,000	2.21	8.5	2.24	18.6	1.9
$His456.458Lys_{isolated}$	1,000			2.24	17.7	1.3
$His456.458Lys_{oxidized}$	5,800	2.20	7.7	2.26	16.7	2.9
$His456.458Asp_{isolated}$	0			2.32	14.2	1.0
$His456.458Asp_{oxidized}$	5,000	2.20	7.5	2.32	13.2	2.0
$His456.458Val_{isolated}$	1,900	2.20	7.2			0.4
$His456.458Val_{oxidized}$	4,200	2.20	7.2			1.0

Table 2. **BO and ferroxidase activities, total Cu contents and redox potentials of BO mutants.**

BO and mutants	BO activity (U/mg)	Ferroxidase activity	Total Cu (U/mg) per protein content	E^0 of type I Cu (mV) *vs.* SHE
Wild Type	30	440	4.0	470
His456.458Lys	0.01	1.08	3.7	470
His456.458Asp	0.01	0.10	2.2	470
His456.458Val	<0.01	<0.08	0.9	460

1.0 Cu^{2+} per protein molecule in parallel with the change in the absorption spectrum (Fig. 3C). Furthermore, a possible type III Cu signal with spin Hamiltonian parameters g_{II} = 2.31 and A_{II} = 11.4 × 10^{-3} cm^{-1} was observed at 269 (M_I = +3/2) and 275 (M_I = +1/2) mT in addition to the type I and type II Cu signals. The contribution of these signals other than that of type I Cu was 1.9 Cu^{2+} per protein molecule. Taking into account that the total amount of Cu in His456.458Lys was 3.7 per protein molecule (experimental error, 10%) (Table 1), about half of type III Cus became EPR-detectable through the action of oxidising or reducing agent.

The resonance Raman spectrum of His456.458Lys (Fig. 4) was obtained with excitation at 591 nm deviating 9 nm from the top of the blue band due to type I Cu. A cluster of bands similar to that for the wild type BO was observed over 350–500 cm^{-1}, indicating that the type I Cu was not perturbed profoundly by the mutation at remote site. The redox potential of type I Cu was the same for both His456.458Lys and the wild type BO (Table 2, E^0 = 470–480 mV). However, abilities to oxidize bilirubin (from 30 to 0.01 U/mg) and Fe^{2+} ion (from 440 to 1 U/mg) were drastically decreased (Table 2).

His456.458Asp Mutant—The His456.458Asp mutant, as isolated, was colourless, and no absorption band was observed except that at 280 nm (Fig. 2C, broken line). The EPR spectrum almost exclusively gave a Cu EPR signal with spin Hamiltonian parameters g_{II} = 2.32 and A_{II} = 14.2 × 10^{-3} cm^{-1}, and five superhyperfine splittings due to the 2 His residues (A_N = 1.4 mT) (Fig. 3D). Since the g_{II} value is considerably large and the A_{II} value is considerably small, the Cu^{2+} center is highly tetrahedrally hindered (*17*) and, accordingly, the observed signal might come from a magnetically uncoupled type III Cu with 2N2O coordinations. However, soon after treating this His456.458Asp mutant with hexacyanoferrate(II) under air or with hexacyanoferrate (III), a strong blue band appeared at 600 nm (ε = 5000) (Fig. 2C, solid line). Concomitantly, the full type I Cu signal with spin Hamiltonian parameters g_{II} = 2.20 and A_{II} = 7.5 × 10^{-3} cm^{-1} appeared (Fig. 3E). The EPR signal observed in Fig. 3D was still present, although it was broadened sparingly. (The A_{II} value became slightly small (13.2 × 10^{-3} cm^{-1}), while the g_{II} value did not change). It appears that the type I Cu in His456.458Asp had been in the cuprous state when isolated. The resonance Raman spectrum of His456.458Asp (Fig. 4) was similar to that of the wild type enzyme, as in the case of the His456.458Lys mutant (the strongest band shifted only 1 cm^{-1} from that of the wild type BO), supporting that the type I Cu center was practically unaffected by the mutation. The BO activity of His456.458Asp was very low (0.01 U/mg) but was not lost completely. The total Cu content of this mutant was 2.2 per protein molecule, indicating that only 1.2 Cu occupied the trinuclear center.

His456.458Val Mutant—Since the type I Cu was partly or fully reduced in the former mutants, as isolated, His456.458Val reported earlier (*2*) was also reacted with the oxidizing reagents. The colour of this mutant also changed to intense blue (ε = 1,900 to 4,200, Fig. 2D). The EPR signal intensity due to type I Cu (Fig. 3F, g_{II} = 2.20 and A_{II} = 7.2 × 10^{-3} cm^{-1}) increased from 0.4 to 1.0 per protein molecule (the EPR spectrum after oxidation is not shown because it was practically the same as in Fig. 3F except for the increase in intensity). Because the amount of Cu ion present in the protein molecule was 0.9 (error 10%), only the type I Cu site had been occupied by cupric and cuprous ions (Tables 1 and 2). The 330 nm band due to the bridged trinuclear center was completely absent. The redox potential of the type I Cu was very similar to that of the wild type BO (Table 2) (*2*, *3*). The resonance Raman spectrum (Fig. 4) also supported that the structure of the type I Cu was not strongly affected by the absence of Cus in the trinuclear center (the strongest band was shifted 4 cm^{-1} to the shorter wavenumber region by the mutation).

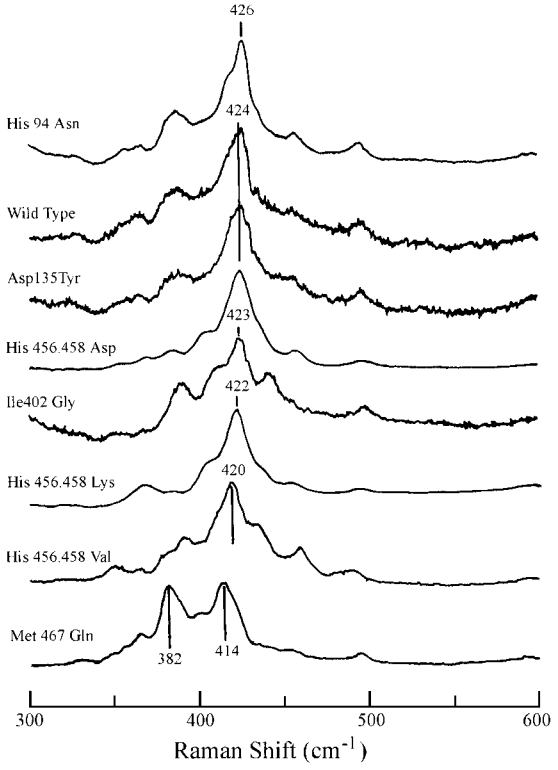

Fig. 4. **Resonance Raman spectra of the wild type BO, His456.458Lys, His456.458Asp, and His.456.458Val.** The spectra of other mutants, His94Asn, Asp135Tyr, Ile402Gly, and Met467Gln, are also shown in order from higher to lower energy for the strongest Raman line. Phosphate buffer (0.1 M, pH 6) was used for sample concentrations of *ca.* 0.08–0.8 mM.

DISCUSSION

Type I and Type III Cu centers are directly connected by a sequence, His-Cys-His, unique to multicopper oxidases. We substituted these residues, His456 and His458, in BO by potentially coordinating Lys and Asp and by non-coordinating Val (Fig. 1) to determine the roles of these amino acids in multicopper oxidase. Although expression of these mutants was successful, the amount of Cu ion incorporated in the Asp and Val mutants was less than four unexpectedly. The ability to hold Cu ions might have been significantly lowered or lost due to the lethal perturbations. Otherwise, the introduction of three Cu ions into the trinuclear center might have been inhibited in the process for production of the mature enzyme. In contrast, four Cu ions were incorporated in the Lys mutant, indicating that the substitution of the amino group for the imidazole group was not so fatal as to decrease the number of the incorporated Cu ions in the trinucelar center. Unexpected was that mutants were pale blue or colorless, differing from the wild type enzyme (broken lines in Fig. 2). The type II Cu EPR signal (g_{II} = 2.24, A_{II} =17.7 × 10^{-3} cm^{-1}) was almost exclusive in the His456.458Lys mutant when isolated (Fig. 3B). On the other hand, a highly tetrahedrally hindered Cu EPR signal with large g_{II} (2.32) and small A_{II} (14.2 × 10^{-3} cm^{-1}) values was observed for the His456.458Asp mutant. This signal might not originate from type II Cu but from a Cu(II) ion occupying a type III Cu site. The incorporation of the only one Cu ion in the trinucelar center and five superhyperfine splittings due to 2N2O coordination support this. Otherwise, the potential ligands in the trinuclear site scrambled for a Cu^{2+} ion as in the case of the crystal structure of *Coprinus cinereus* laccase, in which type II Cu had been depleted and His399, as one of the type II Cu ligands, had rotated the plane of the imidazole ring so as to coordinate one of the type III Cus (*13*).

When His456.458Lys and His456.458Asp reacted with $Fe^{III}(CN)_6$, a strong blue color similar to that of the wild type BO appeared spontaneously (Fig. 2, B and C). On the other hand, when these mutants reacted with $Fe^{II}(CN)_6$,$W^{IV}(CN)_8$ or ascorbate, the blue color disappeared. However, they soon became intense blue in the presence of dioxygen, giving the full EPR signal due to type I Cu (Fig. 3, C and E). The fact that the cuprous type I Cu in the expressed mutants is not oxidized under air indicates that the electron transfer from the reduced type I Cu is inhibited in the colorless form. However, the reduced type I Cu can be converted into the oxidized form on reduction of type II and III Cus in the presence of dioxygen. In the case of His456.458Val, only the oxidizing reagents were effective for full oxidization of the partly reduced type I Cu, giving absorption, EPR and resonance Raman spectra contributed only by type I Cu. The absence of a particular oxidizing reagent in the culture medium and *A. oryzae* as the host might have caused mutants to be expressed in mixed forms.

Analogous resonance Raman bands were observed over 350–500 cm^{-1} for all the wild type BO and mutants except Met467Gln, the type I Cu mutant (Fig. 4). These clustered bands were due to mixing of the metal-ligand stretching motions of type I Cu and were not due to the trinucelar center because the charger transfer band originating from type I Cu was excited (*22*). Therefore, the slight differences in the resonance Raman spectra of the wild type BO and mutants as to the trinuclear center indicate that the electronic state of type I Cu is not significantly perturbed by mutations in the remote center, although both Cu centers are directly connected by the sequence His-Cys-His.

The His456.458Lys mutant showed BO and ferroxidase activities, indicating that the electron from a substrate is finally transferred to dioxygen via type I Cu and the mutated trinuclear center (*18–21*). However, the drastic decreases in the enzyme activities of His456.458Lys would have been brought about by the change in the redox potential of the type III Cu centers (the substitution of Lys for His would lower the redox potential of type III Cu, significantly suppressing the rate of intramolecular electron transfer) and/or by lowering of the affinity of the trinuclear center towards dioxygen (Table 2). His456.458Asp contained only one Cu ion in its trinuclear center, but showed weak BO and ferroxidase activities. This indicates that electron transfer from type I Cu to the Cu ion accommodated in the trinucelar center and following oxidation with dioxygen are possible. This situation is similar to the reaction process of Cu-nitrite reductase containing two Cu ions [type I Cu and type II Cu (positioned at the site corresponding to type III Cu)], in that the intramolecular electron transfer from type I Cu is gated by the state of the type II Cu site (*23*). The His456.458Val mutant contained only one Cu ion at the type I Cu site, and did not show BO and ferroxidase activities, although the reduced type I Cu was easily oxidized through the action of hexacyanoferrate (III) to give a spectrum similar to those shown by plastocyanin and azurin without a shoulder at 330 nm.

Even if we fully oxidized the mutants previously, the enzyme activities did not increase. Since the recombinant BO showed strong activity (26 U/mg) similar to that of the authentic enzyme (30 U/mg), the drastic decreases in the activities of His456.458Lys were not due to a certain scrambling of the donor group in the trinuclear center but to interference with the reaction process. The binding ability of dioxygen as to the trinuclear center or the rate of dioxygen reduction would have been greatly decreased on the substitution of His by Lys. The substitution of His by Asp and Val was more fatal, leading to less or no Cu incorporation into the trinuclear center. Further structural and kinetic studies are in progress to reveal which process is significantly inhibited by doubly mutated type III Cu ligands.

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