Myrothecium verrucaria Bilirubin Oxidase and Its Mutants for Potential Copper Ligands†

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ABSTRACT: Bilirubin oxidase (EC:1.3.3.5) purified from a culture medium of *Myrothecium verrucaria* MT-1 (authentic enzyme) catalyzes the oxidation of bilirubin to biliverdin in vitro and recombinant enzyme (wild type) was obtained by using an overexpression system of the bilirubin oxidase gene with *Aspergillus oryzae* harboring an expression vector. The absorption and ESR spectra showed that both bilirubin oxidases are multicopper oxidases containing type 1, type 2, and type 3 coppers similar to laccase, ascorbate oxidase, and ceruloplasmin. Site-directed mutagenesis has been performed for the possible ligands of each type of copper. In some mutants, Cys457 → Val, Ala, His94 → Val, and His134.136 → Val, type 1 and type 2 copper centers were perturbed completely and the enzyme activity was completely lost. Differing from the holoenzyme, these mutants showed type 3 copper signals. However, the optical and magnetic properties characteristic of type 1 copper were retained even by mutating one of the type 1 copper ligands, i.e., a mutant, Met467 → Gly, showed a weak but apparent enzyme activity. A double mutant His456.458 → Val had only type 1 Cu, showing a blue band at 600 nm ($\epsilon = 1.6 \times 10^3$) and an ESR signal with very narrow hyperfine splitting ($A_{\|} = 7.2 \times 10^{-3}$ cm^{-1}). Since the type 2 and type 3 coppers are not present, the mutant did not show enzyme activity. These results strongly imply that the peculiar sequence in bilirubin oxidase, His456-Cys457-His458, forms an intramolecular electron-transfer pathway between the type 1 copper site and the trinuclear center composed of the type 2 and type 3 copper sites.

Copper in biological systems has been classified into three types according to their optical and magnetic properties (*1*). Type 1 copper (blue copper) shows several charge-transfer bands, in which the most peculiar one appears around 600 nm (Cys to Cu(II) charge transfer). The other bands appear at ca. 450 nm and ca. 750 nm. The hyperfine splitting ($A_{\|}$) in ESR spectrum is usually very narrow because of the highly covalent character of type 1 copper. On the other hand, type 2 copper (nonblue copper) does not show any strong charge-transfer bands in the visible region and its $A_{\|}$ value in ESR spectrum is normal. Type 3 coppers are not detectable by ESR because a pair of them are strongly antiferromagnetically coupled. A hydroxide ion is bridged between them, giving a strong absorption at 330 nm. Type 2 and 3 coppers form a trinuclear center to reduce dioxygen to two water molecules. Type 1 copper functions as the electron mediator from substrate to the trinuclear center.

The X-ray crystal structure analyses of ascorbate oxidase (*2, 3*) and ceruloplasmin (*4*) unequivocally indicate that the type 2 and a pair of type 3 coppers form the trinuclear center as shown in Figure 1. The type 1 copper site and the trinuclear center are separated by ca. 13 Å but are directly connected through the sequence His-Cys-His. The Cys sulfur coordinates to type 1 Cu, and the His imidazoles at the both ends coordinate to type 3 coppers. Type 1 copper is coordinated by 1Cys-2His-1Met, type 2 copper by 2His-1H_2O, and type 3 coppers by 3 His and a bridged hydroxide ion. Although type 3 Cu is usually ESR undetectable, it becomes detectable when perturbed. The hyperfine splitting thus measured is $(10-15) \times 10^{-3}$ cm^{-1}, the intermediate magnitude for type 1 Cu and type 2 Cu, indicating that the structure around type 3 Cu is tetrahedrally distorted from the normal tetragonal geometry.

Bilirubin oxidase (EC.1.3.3.5) catalyzes the four-electron reduction of molecular oxygen to water with concomitant oxidation of bilirubin to biliverdin in vitro. Biliverdin is finally changed to a purple pigment. *Myrothecium verrucaria* (MT-1) bilirubin oxidase (*5*) has been used to determine the total bilirubin in serum in the field of clinical pathology for the diagnosis and treatment of jaundice and hyperbilirubinemia. We have found that the bilirubin oxidase from *M. verrucaria* is a monomeric enzyme with molecular mass of

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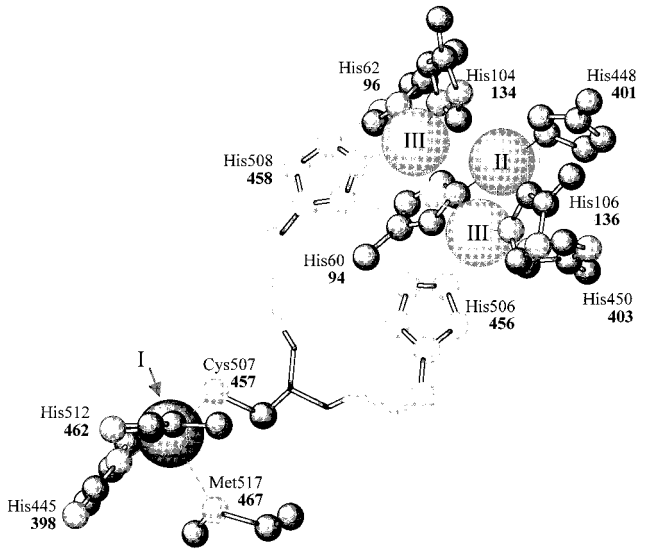

FIGURE 1: Copper binding site of ascorbate oxidase and presumed site for bilirubin oxidase. The 3-D structural data for ascorbate oxidase were obtained from the Protein Data Bank under PDB: 1aoz (*2*). Roman numerals indicate the copper types, and Arabic numerals indicate sequence numbers of amino acid residues. The upper number is for ascorbate oxidase, and the lower number in boldface is for bilirubin oxidase.

ca. 66 kDa. It contains *N*-linked and *O*-linked carbohydrate chains, and copper is essential for the enzyme activity.

cDNA of bilirubin oxidase was recently cloned, and the pre, pro sequences and the whole amino acid sequence of 534 residues were deduced (*6*). It appears that there is strong sequence homology between bilirubin oxidase and multicopper oxidases such as laccase, ascorbate oxidase, ceruloplasmin, etc., as shown in Figure 2 (*7*−*17*). The peculiar sequence characteristic of multicopper oxidase, His-Cys-His, is also present in bilirubin oxidase. In addition, all the intrinsic ligand groups for three types of copper are conserved in the sequence of bilirubin oxidase. Therefore, bilirubin oxidase should be a member of the multicopper oxidases as judged from the amino acid sequence, although no detailed spectroscopic and magnetic study has been performed. Furthermore, no genetic study such as mutation has been carried out for this enzyme.

In this paper, we report the expression of *M. verrucaria* bilirubin oxidase in *Aspergillus oryzae* using a secretory expression system. We also prepared several mutants, in which the potential ligands for three types of copper were changed, and we characterized them by absorption and ESR spectra. While a number of mutation studies have been reported for blue copper proteins containing only a blue copper (type 1 copper), azurin (*18*) and plastocyanin (*19*), and also for nitrite reductase containing a type 1 copper and a type 2 copper (*20*), mutations for multicopper oxidase have just started and no systematic data have been published on the effect of mutation on the ligand group of each type of copper site (*21*, *22*).

MATERIALS AND METHODS

Materials and Chemicals. The plasmid pBXba1 containing the cDNA of *M. verrucaria* bilirubin oxidase gene, plasmid pY1 containing the mono- and diaminoacyl lipase (mdlA) promoter (*23*), plasmid pSTA14 containing the *A. oryzae nia*D gene, and *A. oryzae nia*D mutant AO1.1 as a host cell strain were kind gifts of Amano Pharmaceutical Co., Ltd. Restriction enzymes and calf intestine alkaline phosphatase were purchased from Nippon Gene. The DNA ligation kit version 1, *Taq* DNA polymerase and the *Bca*BEST dideoxy sequencing kit were from Takara Shuzo. DEAE-Sephacel and Sephadex G-100 were from Pharmacia Biotech Inc.

Purification of Authentic Bilirubin Oxidase from Myrothecium verrucaria (MT-1). Bilirubin oxidase purified from the culture medium of *M. verrucaria* was kindly supplied by Amano Pharmaceutical Co. The lyophilized enzyme preparation was further purified by DEAE-Sephacel column chromatography in Tris−H_2SO_4 buffer (50 mM, pH 7.6) to homogeneity on polyacrylamide gel electrophoresis.

Purification of Recombinant Bilirubin Oxidase from Aspergillus oryzae. *A. oryzae* harboring an expression vector was cultured aerobically at 30 °C for 5−6 days in 1 L of soybean oil medium [3.0% (v/v), 0.5% (w/v) bacto-yeast extract (Difco), 0.5% (w/v) bonito extract (Kyokuto), 0.3% (w/v) sodium nitrate (Wako Pure Chemical Industries), 0.1% (w/v) potassium dihydrogenphosphate (Wako Pure Chemical Industries), 0.05% (w/v) potassium chloride, 0.05% (w/v) magnesium sulfate, 0.003% (w/v) cuprous sulfate, and 0.001% (w/v) ferrous sulfate]. After removal of the cells by filtration through a glass filter, ammonium sulfate was added to the filtrate up to 50% saturation under stirring, and the mixture was allowed to stand overnight. The precipitate was separated by a glass filter, the supernatant was made to 70% ammonium sulfate saturation under stirring, and the mixture was allowed to stand overnight again. The precipitate was collected by centrifugation and dialyzed against Tris−H_2SO_4 buffer (50 mM, pH 7.6). The supernatant was then applied to a DEAE-Sephacel column equilibrated with the same buffer. After the column had been washed, the enzyme was eluted with a linear gradient of 0−250 mM NaCl in the same buffer. The fractions containing bilirubin oxidase were pooled, and the protein solution was concentrated to about 5 mL by ultrafiltration using a PM-30 filter (Amicon). The sample was loaded on a Sephadex G-100 gel filtration column, and then the protein was eluted with the same buffer.

Assay of the Enzyme Activity. Bilirubin was purchased from Wako Pure Chemical Industries and used without further purification. The bilirubin oxidase activity was assayed as follows: 2.0 mL of 30 μM bilirubin dissolved in 0.2 M Tris−H_2SO_4 buffer (pH 8.4) was added to 0.2 mL of the enzyme solution, followed by incubation at 37 °C. Measurement of the absorbance decrease of bilirubin was carried out at 440 nm with a Jasco model Ubest-55 spectrophotometer. One unit was defined as the amount of enzyme which oxidized 1 μM bilirubin/min (*5*).

Plasmid Construction of the Expression Vector of Wild-Type Bilirubin Oxidase. Plasmid pNBC1 containing the bilirubin oxidase gene, mdlA promoter, and *A. oryzae nia*D selection marker gene was constructed in the following way: An *Xba* I fragment from pBXba1 was ligated into the same site of pY1. Into the *Hin* dIII sites of the resultant construct, the *Hin* dIII fragments containing the *nia*D gene from pSTA1 were ligated.

Site-Directed Mutagenesis of Bilirubin Oxidase. The in vitro mutagenesis was achieved by polymerase chain reaction (PCR) (*24*) using the two distant primers which have the restriction enzyme recognition site (bolded) and the sets of

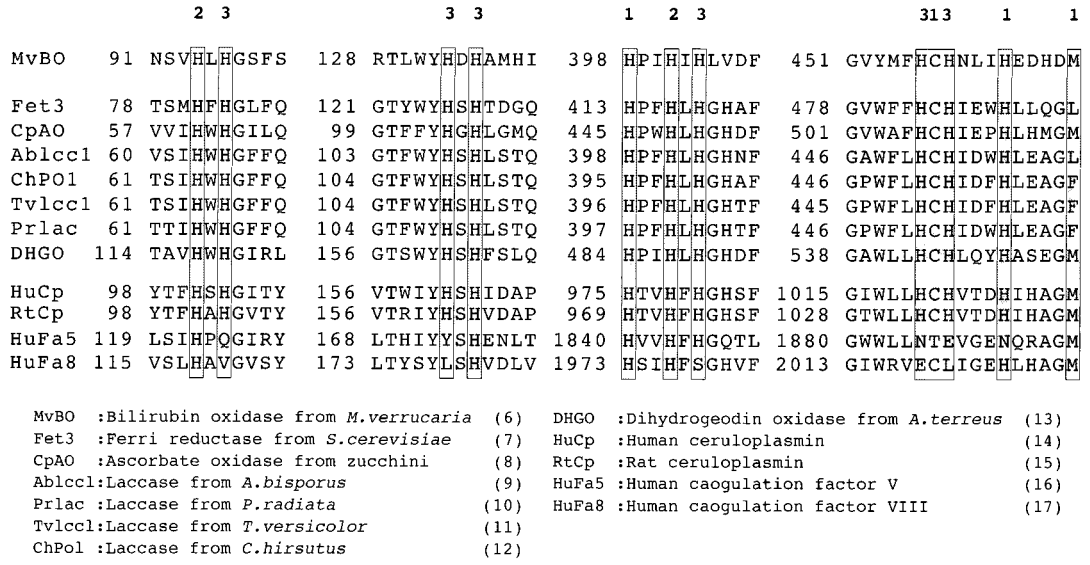

FIGURE 2: Presumed potential coordination sites with copper in bilirubin oxidase and homology of partial amino acid sequence between bilirubin oxidase and other copper proteins. The numbers 1, 2 and 3 indicate the potential coordination sites for types 1, 2, and 3 copper ions, respectively. The presumed amino acid residues for ligands are boxed.

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His94Val
 5'-GATCCATGCAGTACTACGGA-3'
 3'-CTAGGTACGTCATGATGCCT-5'

Distinct primers for His94Val
 5'-GCAAGCTTCCGGGAGTAAAT-3'
 3'-ATGTACAAGCACACGCAATTGAACTAAG-5'

His134.136Val
 5'-CCCTATGGTATGTCGACGTTGCTATG-3'
 3'-GGGATACCATACAGCTGCAACGATAC-5'

Cys457Val,Ala
 5'-CAAATTGTGGPCATGGAACA-3'
 3'-GTTTAACACCYGTACCTTGT-5'

His456.458Val
 5'-TACATGTTCGTGTGCGTTAACTTGATTC-3'
 3'-ATGTACAAGCACACGCAATTGAACTAAG-5'

Met467X
 5'-TTGATTCACGAGGACCACGATRKGATGGCTGCC-3'
 3'-AACTAACAGCTCCAGGTGCTATACYMCCGACGG-5'

Distinct primers for the above four mutageneses
 5'-TTCCATGGCCGAGCGTTACG-3'
 3'-ATACTGAACTTGGAGCTCAA-5'
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FIGURE 3: Nucleotide sequence of synthetic oligonucleotide primers used for mutagenesis. The recognition sites for restriction enzymes are described in boldface, and mutated codons are underlined.

mutagenic oligonucleotides (mutated codons are underlined) as in Figure 3. All oligonucleotide primers used for mutagenesis were chemically synthesized tailor-made products from Sawady Technology as shown in Figure 3. The PCR products were cloned into pUC18, and sequences were confirmed. The mutant partial bilirubin oxidase genes were cloned into pNBC1 plasmid and expressed as below.

Transformation and Expression of Mutants. Transformation of *Escherichia coli* and *A. oryzae* was done according to the methods of Hanahan (*25*) and Unkles (*26*), respectively. We constructed some expression vectors of the mutants. One of the constructions on His456.458Val (pNTIII-5.6V) is shown in Figure 4. These vectors contained the mono- and diaminoacyl lipase promoter and the *A. oryzae* nitrate reductase gene (*nia*D) as a selective marker. Plasmids of the expression vectors for the mutants were purified from *E. coli* cells using the alkali-SDS method, and 5 μg of DNA was introduced by protoplast transformation into the *A. oryzae nia*D strain. Transformants were cultured on the minimum medium containing nitrate as the sole nitrogen source. After cultivation for 5 days the transformants were picked up and isolated in test tubes. After an additional 5 days the transformants were cultured for 5 days in 3 mL of soybean oil medium. The level of the expression product was examined by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (*27*).

Spectroscopic Measurements. Absorption spectra of bilirubin oxidase were measured with a Jasco model Ubest-55 spectrophotometer. The X-band ESR spectra at 77 K were measured on a JEOL JES-RE1X spectrometer. The signal intensity of the ESR-detectable Cu^{2+} was determined by the double integration method using Cu−EDTA as a standard. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as an external marker to calibrate the estimation error of ESR signal intensities arising from the difference in tuning conditions (*28*). Some of the ESR spectra were simulated according to the previous work (*29*). CD spectra were measured on a JASCO J-600 automatic recording dichrograph at room temperature. The total amounts of coppers contained in the enzyme derivatives were determined by atomic absorption spectroscopy with a Shimadzu AA-640-13.

RESULTS

Mutagenesis and Protein Expression. We have previously reported the cloning of bilirubin oxidase cDNA from *M. verrucaria*, which included the pre,pro N-terminal signal sequences of 38 amino acids to express wild-type bilirubin oxidase under the control of mdlA promoter *A. oryzae*. The *M. verrucaria* signal sequences guided the expression of recombinant protein in *A. oryzae*. In this study, we obtained

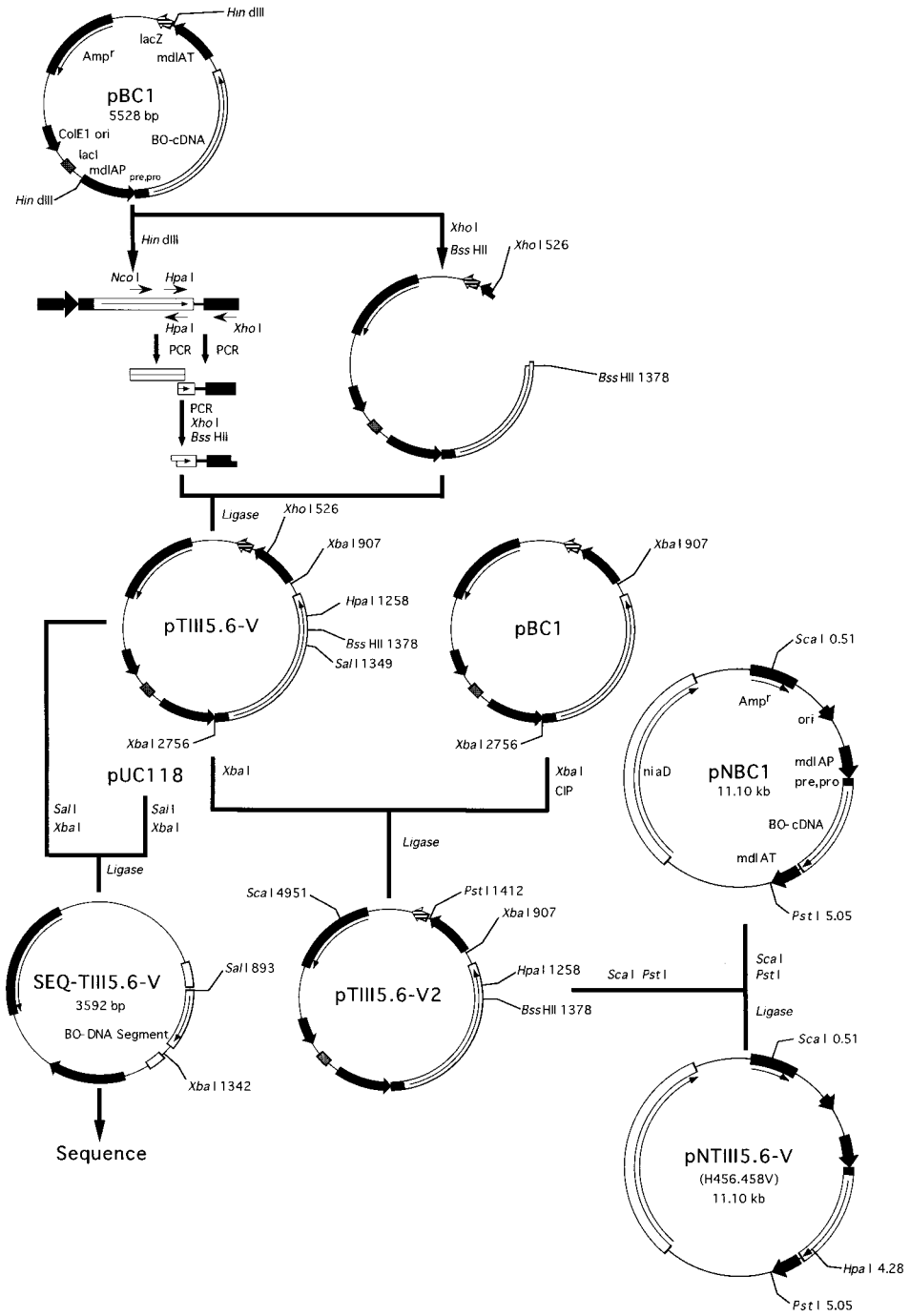

FIGURE 4: Construction procedure of the expression vector for mutant H456.458V. PCR was achieved by using mutation-introduced primer. The PCR product was digested with *Xba* I and *Bss* H II, substituted for pBC1 first, and then inserted in pUC-118 to check the nucleotide sequence. A selective marker was inserted in the expression vector (pTIII-5.6V) at the end of the construction procedure.

the mutated DNA segments using mutated PCR primers. As for the mutants on Met, we designed the PCR primers encoding four or five amino acids to express the plural mutated DNA segments except for Met468Val and Met468Arg. We constructed the mutant expression vectors, pNTII-H1V (His94Val), pNTIII-3.4V (His134. 136Val), pNTIII-5.6V (His456.458Val), pNTI-CV (Cys457Val), pNTI-CA (Cys457Ala), pNTI-H2V (His462Val), pNTI-MG (Met467Gly), pNTI-MR (Met467Arg), pNTI-MV (Met467Val), and pNM468G (Met468Gly). From these 10 vectors 6 transformants were expressed for the bilirubin oxidase mutants containing the same molecular mass as the wild-type bilirubin oxidase after cultivation for 5 days. However, the level of the mutant expression was remarkably different; namely, the yield from 1 L of culture medium was 80 mg for the wild-type enzyme, 15 mg for His94Val, 10 mg for His456.458Val, 5 mg for Met467Gly, 3 mg for His134.136Val and less than 2 mg for Cys457Ala and Cys457Val. For the other four mutants we tried to transform *A. oryzae* cells with the mutant vectors several times, but we did not find any expression product.

Authentic and Wild-Type Bilirubin Oxidases. The wild-type bilirubin oxidase purified from the culture medium of recombinant *A. oryzae* was subjected to automated amino acid sequencing. The *N*-terminal sequence was determined to be VAQIS, which was the same as that of the authentic

bilirubin oxidase from *M. verrucaria*. This wild-type bilirubin oxidase showed 24 units/(mg of protein) enzyme activity as high as 80% of the authentic enzyme. The authentic and wild-type bilirubin oxidases were expressed as glycoproteins. The molecular mass of the authentic bilirubin oxidase was determined as 66 kDa, and that of the wild-type bilirubin oxidase, as 63 kDa. The small discrepancy came from difference in the sugar content. Although the authentic bilirubin oxidase has two *N*-linked and some *O*-linked sugar chains (the number and positions of the *O*-linked sugar chains have not yet been fixed), the wild type bilirubin oxidase carries only two *N*-linked sugar chains (data not shown).

CD spectra of both authentic and wild-type bilirubin oxidases were almost identical, suggesting that the secondary structure of wild-type bilirubin oxidase was similar to that of the authentic enzyme (data not shown). In the absorption spectra both wild and authentic bilirubin oxidases showed an intense S^-(Cys) to Cu^{2+} charge-transfer band at 600 nm and a shoulder around 330 nm derived from OH^- to type 3 Cu's charge transfer (Figure 5). The former is typical of type 1 copper (blue copper), and the latter of type 3 coppers (ESR nondetectable coppers). The appearance of both bands unequivocally indicates that bilirubin oxidase is a multicopper oxidase. However, the absorption feature of the authentic bilirubin oxidase around 330 nm was more prominent. In contrast, the absorption intensity around 600 nm was weak as compared with that of the wild-type bilirubin oxidase (see Discussion). In addition, the charge-transfer band at ca. 450 nm (N(His) to Cu^{2+}) was not clear because of the intense 330 nm band.

The X-band ESR spectra of the authentic and wild type bilirubin oxidases are shown in Figure 6A,B, and their spin Hamiltonian parameters are listed in Table 1. Both type 1 and type 2 copper signals were apparently present, indicating that bilirubin oxidase is a member of the multicopper oxidases. A signal originating from a type 3 copper was slightly observable at 275 mT in the case of the wild type bilirubin oxidase, also producing a different feature at around 320 mT. Since type 3 Cu signal is usually rhombic, the *x* and *y* components affect the feature of this region when it is present. However, its signal was not intense as to be able to estimate the spin Hamiltonian parameters. Furthermore, the hyperfine splitting of type 2 copper was slightly different. These might be caused by a sugar moiety attached to an amino acid residue(s) near the active site, so as to give a perturbation on the protein structure, and/or by the possibility that the expressed wild-type bilirubin oxidase is in an equilibrium of the bridged and unbridged forms, as for type 3 copper sites (see Discussion). The total amount of the ESR detectable Cu^{2+} was 2.0 for both authentic and wild-type bilirubin oxidases according to the double integrations. Atomic absorption spectroscopy indicated that both authentic and wild type bilirubin oxidases contain 3.5 copper atoms/molecule. Considering the estimation errors of the protein concentration (gravimetry for the freeze-dried sample and Lowry method, 10%) and the atomic absorption spectroscopy (ca. 5%), we concluded that bilirubin oxidase contains 4 copper atoms per molecule similar to all other multicopper oxidases.

Effect of Metal Ion on Expression Level. Since we used the mdlA promoter, oil or fat was necessary to induce the expression of bilirubin oxidase. In addition, cupric ion (Cu^{2+}) was essential, although other divalent cations were not effective.

Mutagenesis for Potential Type 1 Cu Ligands. Bilirubin oxidase has only one Cys residue in a protein molecule. Because Cys is the most important amino acid residue to form type 1 copper, we mutated this residue first. Second, Met was noted because many mutation studies for blue copper proteins have targeted this amino acid to modify the spectroscopic and magnetic characters of type 1 copper as well as its electron-transferring ability. Thus, the site-directed mutagenesis was performed to exchange Cys457 for Val or Ala and Met467 for Gly, namely, the amino acids that cannot act as ligand towards the metal center.

The Cys457Val and Cys457Ala mutants showed no enzyme activity, while the Met467Gly mutant showed a weak but apparent enzyme activity (Table 2).

Absorption and ESR spectra of these mutants are shown in Figures 5 and 6, respectively. The Cys mutants exhibited no absorption band in the visible region, indicating that this Cys residue is undoubtedly a ligand for type 1 copper. On the other hand, the Met467Gly mutant showed absorption bands around 600, 430, and 330 nm. Although the small absorption band at 430 nm (N(imidazole) to Cu^{2+} charge transfer) was obscured for the authentic bilirubin oxidase, this band became prominent for the Met467Gly mutant probably because other two bands at ca. 600 nm and ca. 330 nm were weakened. In the ESR spectrum of this mutant (Figure 6C) both of the slightly modified type 1 and type 2 Cu signals were observable together with a weak type 3 copper signal. Although type 3 Cu's are usually undetected in the resting multicopper oxidases because of the strong antiferromagnetic interaction, they are sometimes detectable when the enzymes are modified, giving a 9−15 mT hyperfine splitting (*30*). The parameters are tabulated in Table 1. On the other hand, the mutants for Cys (C457A, C457V) showed only a weak type 2 Cu-like signal. CD spectra of these mutants were almost the same as that of wild-type enzyme (data not shown).

Mutagenesis for a Potential Type 2 Cu Ligand. His94Val was prepared as a mutant for type 2 Cu. The absorption spectrum of this mutant showed no band in the visible to near-UV regions (Figure 5F). The ESR spectrum of the mutants showed neither a type 1 nor a type 2 copper signal but showed two type 3 Cu-like signals (Figure 6F). Predictably, enzymatic activity was not observed for this mutant. However, CD spectrum of this mutant in the far-UV region was similar to that of wild-type bilirubin oxidase (data not shown).

Mutagenesis for Potential Type 3 Cu Ligands. We prepared two type 3 Cu mutants such as His134.136Val and His456.458Val. His456 and His458 are positioned at both ends of the His-Cys-His sequence, coordinating to different type 3 coppers (Figure 1). We expected that substitution of these residues with noncoordinating amino acid residues profoundly affects the character of the type 1 Cu site. The reason we doubly mutated the amino acid residues for type 3 coppers is to preserve the symmetry around type 3 copper centers. His134.136Val showed no enzymatic activity and also no optical absorption bands (Figure 5E), indicating that the mutation for these His residues fatally interfered with the formation of type 1 Cu site. The ESR spectrum of His134.136Val (Figure 6E) showed the existence of one type

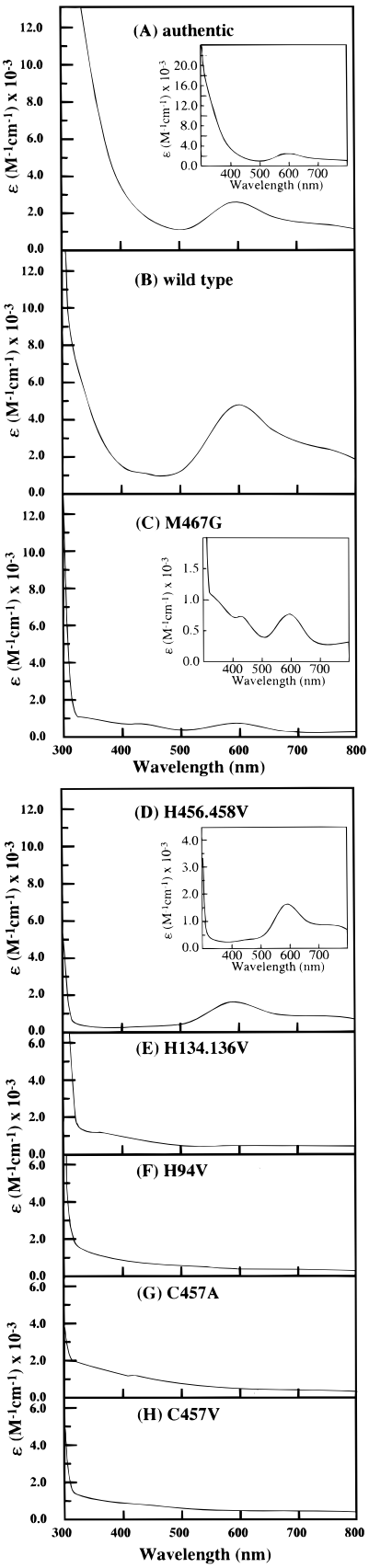

FIGURE 5: Absorption spectra of the authentic, wild type, and mutant bilirubin oxidases: (A) bilirubin oxidase from *M. verrucaria* (authentic); (B) recombinant bilirubin oxidase (wild type) expressed in *A. oryzae*. (C) Met467Gly mutant; (D) His456.458Val double mutant; (E) His134.136Val double mutant; (F) His94Val mutant; (G) C457A mutant; (H) C457V mutant. Inset: Same spectra are shown on curtailed (A) or amplified (C and D) scales.

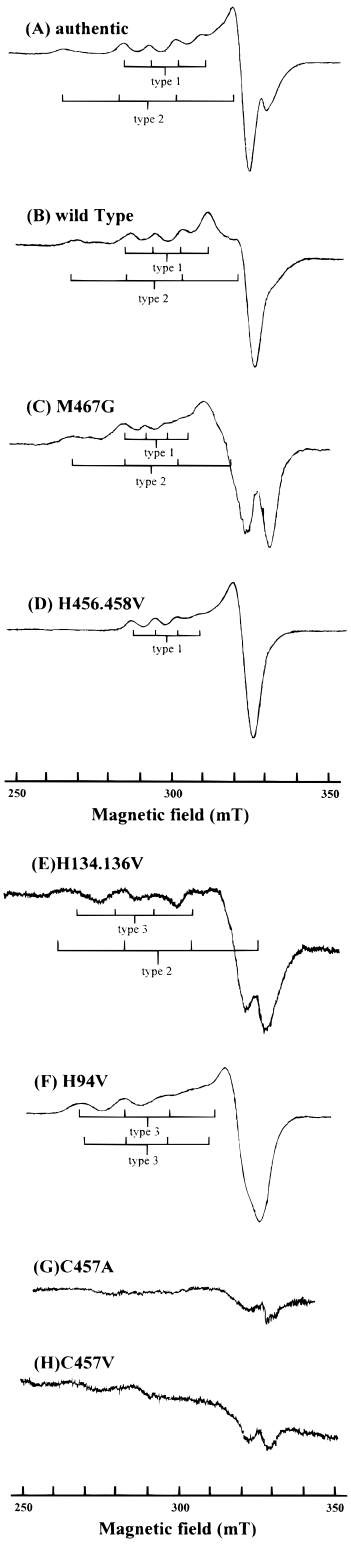

FIGURE 6: ESR spectra of the authentic, wild type, and mutant bilirubin oxidases: (A) bilirubin oxidase from *M. verrucaria*; (B) recombinant bilirubin oxidase expressed in *A. oryzae*; (C) Met467Gly mutant; (D) His456.458Val double mutant; (E) His134.136Val double mutant; (F) His94Val mutant; (G) Cys457Ala mutant; (H) Cys457Val mutant. The ESR spectra were obtained with a microwave power of 6.25 mW, modulation width of 9.210 G, time constant of 0.1 s, and sample temperature of 77 K.

2 and the modified type 3 coppers (see Discussion). The parameters for type 2 copper were similar to those of the wild-type bilirubin oxidase, but those for type 3 copper were different from those of His94Val (Table 1). On the other

Table 1: Parameters of ESR Spectra for Authentic, Recombinant Wild Type, and Mutant Bilirubin Oxidases

mutants	Cu type	$g_{\|\|}$	$10^3 A_{\|\|}$ (cm^{-1})
authentic	type 1	2.21	8.5
	type 2	2.24	18.6
wild type	type 1	2.21	8.3
	type 2	2.24	17.1
M467G	type 1	2.23	7.1
	type 2	2.24	17.3
H456.458V	type 1	2.20	7.2
H134.136V	type 2	2.22	19.9
	type 3	2.23	11.9
H94V	type 3	2.29	15.0
	type 3	2.28	15.6

Table 2: Enzymatic Activity, Copper Contents, and Absorbance Ratios of Authentic, Recombinant Wild Type, and Mutant Bilirubin Oxidases for Bilirubin Oxidation

mutants	specific activity (U/mg)	relative activity (%)	Cu content (atom/protein)	ϵ_{600} (M^{-1} cm^{-1})
authentic	30	100	3.5	2.6×10^3
wild type	24	80	3.5	4.8×10^3
M467G	0.074	0.3	2.1	0.8×10^3
H456.458V	*a*	*a*	0.9	1.6×10^3
H134.136V	*a*	*a*	0.6	ND[b]
H94V	*a*	*a*	1.3	ND
C457A	*a*	*a*	ND[b]	ND
C457V	*a*	*a*	ND	ND

[a] No enzymatic activity detected. [b] ND: not determined.

hand, the absorption spectrum of His456.458Val showed a prominent band derived from type 1 Cu near 600 nm but no shoulder at 330 nm (Figure 5D). This spectral feature is of simple blue copper proteins such as azurin, plastocyanin, etc. The ESR spectrum of His456.458Val showed only the type 1 copper signal but not the type 2 Cu signal. And this mutant showed no enzymatic activity. Atomic absorption spectroscopy showed that this mutant has 2 Cu's in a protein molecule, suggesting that one Cu is a type 1 Cu and the other possibly is a cuprous ion. The CD spectrum of type 3 Cu mutants in the far-UV region was similar to that of the wild-type enzyme (data not shown).

DISCUSSION

We succeeded in the overexpression of wild-type bilirubin oxidase in *A. oryzae* using the secretary expression system under the control of the mdlA promoter. The recombinant wild-type bilirubin oxidase showed copper content and absorption and CD spectra similar to those of the authentic bilirubin oxidase from *M. verrucaria*. The copper contents of both authentic and wild type bilirubin oxidases were determined to be 3.5 copper atoms per protein molecule (Table 2). Taking into account the experimental errors in determining the protein concentration and copper contents, the value of 3.5 for both authentic and wild type bilirubin oxidases indicates that 4 copper atoms are present in the active site, similar to other multicopper oxidases, such as laccase, ceruloplasmin, and ascorbate oxidase (ascorbate oxidase is a homodimer and has 4 coppers in a subunit). The molecular weight of wild type bilirubin oxidase was 63 kDa, being slightly smaller than 66 kDa for authentic bilirubin oxidase according to SDS−PAGE, although the molecular weight of bilirubin oxidase deduced from the base sequence of the cDNA was about 60 kDa. These discrepancies may be derived from the difference in the posttranslational glycosylation. The wild type bilirubin oxidase contains only two *N*-linked carbohydrate chains. On the other hand, the authentic bilirubin oxidase has *O*-linked carbohydrate chains in addition to the two *N*-linked carbohydrate chains (to be published elsewhere). The CD spectra in the far-UV region (200−250 nm) showed very similar spectral features abundant in β-sheet for both wild type and authentic bilirubin oxidases.

The absorption and ESR spectra of the authentic and wild-type bilirubin oxidases indicate the typical features of multicopper oxidase. In the absorption spectrum, two bands are observed at 600 and 330 nm. The former derives from type 1 Cu, and the latter, from the coupled type 3 Cu's (*31*). Although their absorption intensities were different between the authentic and wild-type bilirubin oxidases, it is considered that the discrepancy may be due to the indirect effect of carbohydrates on the spectra. While the absorption at ca. 450 nm (charge-transfer bands, N(His) and S(Met) → type 1 Cu^{2+}) was not clear for authentic bilirubin oxidase because of the stronger 330 nm band, it was clearly observed for the wild-type enzyme (Figure 5A,B). In addition, both of the ESR spectra of the authentic and wild-type bilirubin oxidases showed the existence of type 1 and type 2 coppers, indicating that bilirubin oxidase is a multicopper oxidase. Also a weak signal originating from type 3 was observed for wild-type bilirubin oxidase. A difference was observed in the $A_{\|\|}$ value of the type 2 Cu (18.6×10^{-3} cm^{-1} for authentic bilirubin oxidase and 17.1×10^{-3} cm^{-1} for wild type bilirubin oxidase). This slight difference in the parameters of type 2 copper signals might be caused by different attachments of sugars, which perturbed the protein conformation to modify the electronic and/or steric structure(s) of the type 2 copper site. The definition of type 3 Cu is based on that it is ESR undetectable because a pair of them are strongly antiferromagnetically coupled. However, a signal is sometimes observable when multicopper oxidases are stored in solution. But this is not the case for wild-type bilirubin oxidase because the ESR of a fresh sample was measured. The type 3 Cu signal has been also observed when multicopper oxidases are treated with exogenous small anions such as azide and fluoride ions (*30*). These anions have been used to reveal the structure and properties of the trinuclear center of multicopper oxidases. However, these anions are not present, and accordingly, it is supposed that the type 3 Cu's in wild-type bilirubin oxidase are in an equilibrium of bridged and unbridged forms. The weak absorption of the 330 nm band also supports this. The fact that wild-type bilirubin oxidase retained 80% authentic enzyme activity indicates that *M. verrucaria* enzyme was undoubtedly expressed in *A. oryzae* with less sugar moiety.

Mutations have been performed for blue copper proteins azurin (*32*−*34*) and plastocyanin (*35*) and nitrite reductase containing a type 1 Cu and a type 2 Cu (*36, 37*). However, mutation studies of for multicopper oxidases such as Fet3P (*21*) and laccase (*22*) have just started, and no systematic investigation has been performed on how the mutations will affect the structure of the active site and the enzymatic reaction.

When we replaced the one Cys in bilirubin oxidase, which is the origin of the intense blue color of bilirubin oxidase, by noncoordinating residues (Ala and Val), the resulting

mutants were colorless (Figure 5G,H) and showed no enzyme activity. Since the absorption band at 330 nm was not observed, it seemed unlikely that the intact trinuclear center was also formed. In line with this, very weak type 2 Cu-like signals were observed in the ESR spectra (Figure 6G,H). This indicates that a multicopper oxidase suffers a fatal blow when Cys for type 1 copper is mutated. In contrast, all absorption spectral features intrinsic to multicopper oxidase were, although modified, retained when Met was substituted with Gly. Charge-transfer bands due to type 1 Cu appeared at 600 and 430 nm. The former is due to the charge transfer from Cys to Cu^{2+}, and the latter, from His to Cu^{2+}. The charge-transfer band due to the coupled type 3 Cu's appears at 330 nm (*31*). The intensities of these bands decreased considerably compared with those of authentic and wild type bilirubin oxidases, but their appearance clearly indicates that the mutation for Met of the type 1 Cu site is not fatal, as it is in the mutation of blue copper proteins (*38*). Moreover, Met467Gly contains only about 2 copper atoms in a molecule (Table 2). We deduced that this decreased copper content may represent mixed populations of molecules with the full metal complement and partial or complete apo enzymes, because multicopper oxidase should contain 4 copper atoms to exhibit enzymatic activity. In fact, the apparent enzyme activity was observed in the Met467Gly mutant, although it was considerably lowered (0.3% of the authentic enzyme). The reduction of the enzyme activity may be derived from a change in the reduction potential of type 1 Cu. The replacement of Met by Gly may cause decreased hydrophobicity around the type 1 Cu site, lowering the redox potential of type 1 Cu (*32*). The intermolecular electron transfer from type 1 Cu to the trinuclear center becomes extremely perturbed by this change in the redox potential. It is well-known that the redox potential of all types of Cu's in multicopper oxidases are similar (*39*) (we are studying the redox property of bilirubin oxidase and mutants, and the results will be published elsewhere). In the ESR spectrum of the Met467Gly mutant the hyperfine splitting of type 1 Cu became slightly narrow and the $g_{\|}$ value slightly increased, indicating that the trigonal plane formed by 1 Cys and 2 His may be more tetrahedrally hindered. The analogous changes in both absorption and ESR spectra have been reported for the Met121Gly mutant of azurin (*38*). It is interesting that similar spectral changes take place for both blue copper protein and multicopper oxidase, when a ligand for type 1 copper (blue copper) is mutated. Met is not an indispensable amino acid to produce type 1 Cu characters as similarly reported from a series of mutant studies for Met121X (X = Gly, Ala, Val, Lue, Ile, His, Asn, Glu, and Asp) (*32*−*34*, *38*). The biological role of Met residue for the type 1 Cu site has been considered to tune the redox potential and also to uptake copper. As for the Met92Gln mutant of plastocyanin, the recombinant protein was purified mostly as an apo form (*40*). The type 1 copper site of Met150Glu mutant of nitrite reductase has been occupied by a zinc ion (*41*). The Met residue might also contribute to stabilizing the copper binding site and/or protein structure because the Met467Gly mutant was not very stable when stored in a refrigerator as a solution.

The mutation type 2 Cu (His94Val) was also found to be fatal for bilirubin oxidase. Atomic absorption spectroscopy indicates that only 1.3 Cu's are present in the mutant. We deduce that this decrease of the copper contents may be derived from the mixed populations of enzymes with the partial and complete apo forms. Furthermore, this mutant showed neither blue color due to type 1 Cu nor the 330 nm band due to the coupled type 3 Cu's (Figure 5F). The ESR spectrum indicated two type-3-like coppers with $A_{\|}$ values apparently narrower than that of type 2 copper (Figure 6F). Type 2 copper site is only three-coordinated by 2 His and a water or hydroxide ion. This unusual coordination chemistry as a cupric ion was found to be no longer possible to hold a cupric ion, when one of the two His residues was substituted with the noncoordinating residue. We mutated only a type 2 copper coordinated residue in His94Val, but this mutant showed no type 1 copper signals in the ESR and absorption spectra. This was caused by the change in oxidation state of type 1 copper, since we have already confirmed that cuprous ion (Cu^{+}) exists at the type 1 copper site in His94Val (to be published elsewhere). As for the coordination at the trinuclear center of the mutant, an intercross of ligand might also take place as has been reported for the X-ray crystal structure of the expressed laccase which contained only 2 copper ions in the trinuclear center (*42*).

The double mutations for the two His residues of type 3 coppers, i.e., His456.458Val and His134.136Val, showed contrasting results. The mutant His456.458Val gave the absorption and ESR spectra typical of blue copper proteins, features coming from only type 1 Cu. Both type 2 and type 3 coppers seemed to be absent in this mutant. To support this, the Cu content in this mutant was only 0.6 atom per protein molecule. This indicates that depletion of the coordinating group(s) in the trinuclear center is fatal for the formation of the trinuclear structure. (We have not specially treated the mutants by Cu. The similar decrease of the copper contents has been reported for mutants of nitrite reductase.) On the other hand, His134.136V showed no remarkable absorption band (Figure 5E). The ESR spectrum also supported the lack of type 1 copper. However, strong type 2 and type 3 copper-like signals with larger (19.9×10^{-3} cm^{-1}) and smaller (11.9×10^{-3} cm^{-1}) hyperfine splitting, respectively, were observed, indicating that the intact trinuclear center was not formed.

CONCLUSIONS

A new multicopper oxidase, bilirubin oxidase from *M. verrucaria*, was expressed in *A. oryzae* only with the loss of *O*-linked sugars and slight modifications of the spectral features. On the basis of the amino acid sequence comparison with those of the other multicopper oxidases, point mutations have been performed for all type 1, 2, and 3 coppers. Substitutions toward ligand groups with noncoordinating amino acids indicated that the target amino acids are really indispensable to form the intact active site and to show the enzyme activity. Since all coppers are closely linked by the specific architecture of the ligand groups directly or indirectly, mutations for a copper site were found to profoundly affect the formation of the other copper site(s). Nevertheless, the Met467Gly mutation indicated that a mutant with enzyme activity can be formed if we carefully select the target and replace the amino acid(s). In this study we changed the coordinating amino acid by the noncoordinating amino acid. Mutations by other coordinating amino acid(s) and also

mutations for the site adjacent to a coordinating amino acid residue are in progress.

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