Site-directed mutagenesis of a possible type I copper ligand of bilirubin oxidase; a Met467Gln mutant shows stellacyanin-like properties

J. Biochem. 125, 662-668 (1999)

Site-Directed Mutagenesis of a Possible Type 1 Copper Ligand of Bilirubin Oxidase; a Met467Gln Mutant Shows Stellacyanin-Like Properties[1]

Atsushi Shimizu,* Takashi Sasaki,* Jung Hee Kwon,* Akito Odaka,* Takanori Satoh,* Nobuhiko Sakurai,† Takeshi Sakurai,† Shotaro Yamaguchi,‡ and Tatsuya Samejima*,[2]

*Department of Chemistry, College of Science and Engineering, Aoyama Gakuin University, Chitosedai, Setagaya-ku, Tokyo 157-8572; †Division of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192 and Institute for Molecular Science, Okazaki National Research Institutes, Okazaki 444-8585; and ‡Research and Development Division, Amano Pharmaceutical Co., Ltd., Nishiharu, Nishi-kasugai, Aichi 481-0041

Received November 20, 1998; accepted December 11, 1998

In our previous paper, we reported a mutant of recombinant *Myrothecium verrucaria* bilirubin oxidase, in which the Met467 residue was replaced by Gly [Shimizu, A. *et al.* (1999) *Biochemistry* 38, 3034-3042]. This mutant displayed a remarkable reduction in enzymatic activity and an evident decrease in the intensity of the absorption band around 600 nm (type 1 charge transfer transition). In this study, we report the preparation of three Met467 mutants (Met467Gln, Met467His, and Met467Arg) and characterize their enzymatic activities, midpoint potentials, and absorption and ESR spectra. Met467His and Met467Arg show no enzymatic activity and a great reduction in the intensity of the absorption band around 600 nm. Furthermore, their ESR spectra show no type 1 copper signal, but only a type 2 copper signal; however, oxidation by ferricyanide caused the type 1 copper signal to appear. On the other hand, Met467Gln as expressed shows both type 1 and type 2 copper signals in its ESR spectrum, the type 1 copper atom parameters being very different from usual blue copper proteins but very similar to those of stellacyanin. The enzymatic activity of the Met467Gln mutant for bilirubin is quite low (0.3%), but the activity for potassium ferrocyanide is similar (130%) to that of the wild type enzyme. These results indicate that Met467 is important for characterizing the features of the type 1 copper of bilirubin oxidase.

Key words: blue copper protein, ESR, multicopper oxidase, site-directed mutagenesis, stellacyanin.

Bilirubin oxidase [EC 1.3.3.5] from *Myrothecium verrucaria* (MT-1) catalyzes the oxidation of bilirubin to biliverdin and then to a purple pigment *in vitro* (*1*). Bilirubin oxidase is a monomeric protein with a molecular mass of 60 kDa. It has recently been identified as a multicopper oxidase containing one type 1, one type 2, and one set of type 3 coppers (*2, 14*). Type 1 copper (blue copper) shows several charge transfer absorption bands in the visible region, of which the most peculiar band appears around 600 nm [Cys to Cu(II) charge transfer]. The hyperfine splitting in the ESR spectrum of type 1 Cu is usually very narrow because of its highly covalent character (*3*). Type 2 copper gives superficially normal optical and ESR spectra. Type 3 coppers are paired and strongly antiferromagnetically coupled to be diamagnetic, and accordingly are ESR-silent in the resting form. Multicopper oxidases such as laccase, ascorbate oxidase, and ceruloplasmin contain all three types of copper in a protein molecule, and their three dimensional structures have been determined by X-ray crystallography (*4-6*).

On the other hand, small blue copper proteins have only a type 1 copper atom. In many blue copper proteins, the coordination sphere of the type 1 copper is comprised of two nitrogen (two His) and two sulfur (Cys and Met) donors. However, the methionine is not invariant in all blue copper proteins but is replaced by Gln in stellacyanin, mavicyanin, and umecyanin (*7-9*) (see Fig. 1, A-C). It is an intriguing problem as to why only methionine residue show such ambiguity for the copper ligand among the four residues. Some investigators have tried to resolve this problem using site-directed mutagenesis, a new approach to the study of the metal binding sites of proteins, in particular blue copper proteins. The role of the side chain coordinating to the copper in blue copper proteins has been investigated by replacing a ligand by various other residues (*10*). Consequently, substitutions of Met with another residue cause a reduction in the uptake of copper ions, but the electron transfer activity and midpoint potential of the metal binding site show no remarkable changes (*11*). Furthermore, some Met mutants show two distinctive absorption spectral features depending on pH and temperature. For

[1] This work was supported in part by the Joint Studies Program (1997-1998) of the Institute for Molecular Science, Okazaki National Research Institute.

[2] To whom correspondence should be addressed. Tel: +81-3-5384-1111, Fax: +81-3-5384-6200

example, a Met121His mutant of azurin shows a strong 450 nm band and a weak 600 nm band, in which the copper coordination is classified as type 1.5, at pH values between 6 and 10, but at lower pH the mutant exhibits a strong 600 nm band and a rhombic type 1 ESR spectrum (*12*). This phenomenon is explained by the conversion from a non-coordinated to the coordinated form.

The cDNA of bilirubin oxidase was recently cloned and the pre.pro sequences and whole amino acid sequence of 534 residues were deduced from the base sequence of the cDNA (*13*). We then established a secretory expression system for bilirubin oxidase in *Aspergillus oryzae*, and prepared some mutants by site-directed mutagenesis. As a result, we found that the introduction of hydrophobic residues to the copper ligands causes considerable conformational change near the copper atoms and a lack of type 1 and/or type 2 coppers. On the other hand, when Met467 is replaced by Gly, three copper types are still retained, but there is a decrease in the enzyme activity, stability and absorbance at 600 nm (*14*). In this study we report the preparation of three Met467 mutants of bilirubin oxidase (Met467Gln, His, and Arg), and the results of the characterization of the ESR and absorption spectra and enzymatic activities of the mutants. These results provide further information concerning the role of Met residues in the multicopper oxidase.

(A)

MvBO	398	HPIHIHLVDF	451	GVYMFHCHNLIHEDHDM
Az	46	HNWVLSTAAD	106	EAYAYFCSFPGH-WAMM
Ste	46	HNVDKVTQKN	81	GQKYYICGVPKHCDLGQ
Ume	44	HDVAVVTKDA	79	GPQYYICTVGDHCRVGQ
Mav	44	HNVLQVDQEQ	79	GTFYFLCGIPGHCQLGQ

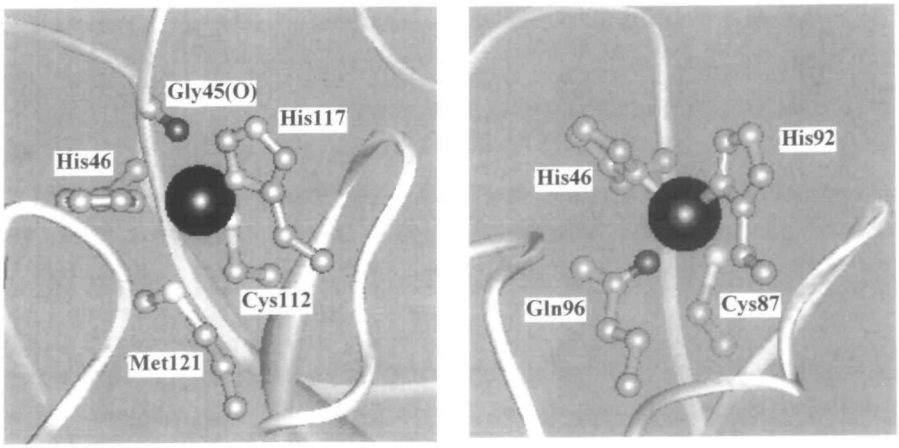
MvBO .Bilirubin oxidase from *M.verrucaria*
Az Azurin from *A denitrificans*
Ste Stellacyanin from *R. vernicifera*
Ume Umecyanin from *A. laphatifolia*
Mav Mavicyanin from *C pepo*

MATERIALS AND METHODS

Materials and Chemicals—Wild type bilirubin oxidase from *M. verrucaria* (MT-1) was obtained by expressing a cloned bilirubin oxidase gene in *A. oryzae* as described previously (*15*). Restriction enzymes were purchased from Nippon Gene. The DNA ligation kit ver.1™, Taq DNA polymerase, and the *Bca*BEST™ dideoxy sequencing kit were from Takara Shuzo. Oligodeoxynucleotides were products of Sawady Technology. DEAE-Sephacel and Sephadex G-100 were from Pharmacia Biotech.

Site-Directed Mutagenesis of Bilirubin Oxidase—The *in vitro* mutagenesis was achieved by the Polymerase Chain Reaction (PCR) using oligonucleotides encoding the desired mutation (*16*). Oligonucleotides encoding Met467 mutations were designed as hetero mix primers. The minus primer (5′-GGCAGCCATGYGATCGTGGACCTTC-3′), which encodes Met467 to His and Arg (in bold), has a *Sau*96I restriction site (underlined) and was used in conjunction with an opponent primer (5′-TTCCATGGCCG-AGCGTTACG-3′) containing an *Nco*I restriction site (underlined). The plus primer (5′-GAGGACCACGATCAAA-TGGCTGCC-3′), which encodes Met467 to Gln (in bold), has a *Sau*96I restriction site (underlined) and was used in conjunction with an opponent primer (5′-AACTCGAGG-TTCAAGTCATA-3′) containing an *Xho*I restriction site (underlined). The above two PCR products were purified and then mixed for a second PCR. Thermal cycling of the first PCR segments (92°C for 1 min, 59°C for 1.5 min, 45 s soak, a slow ramp to 72°C, and 72°C for 2 min) and the second PCR segment (92°C for 1 min, 59°C for 1.5 min, and 72°C for 2 min) was repeated 25 times. All second PCR products were digested with *Sal*I and *Xba*I and inserted into pUC18, then the mutated DNA segment was verified by sequencing with a DNA sequencer model SQ 3000 (Hitachi). The segment was then used to construct a mutant expression vector using the *Sal*I and *Xba*I restriction site.

Transformation and Expression of Mutants—The transformations of *Escherichia coli* and *A. oryzae* were carried out according to the methods of Hanahan (*17*) and Unkles *et al.* (*18*), respectively. We constructed some mutant expression vectors containing a mono- and di-aminoacyl



(B) Azurin **(C) Stellacyanin**

Fig. 1. **Sequence alignment of bilirubin oxidase and small blue copper proteins (A), and the three dimensional structure around the type 1 copper site of azurin (B) and stellacyanin (C).** The presumed amino acid residues in the type 1 copper ligands and the fourth ligands are boxed. The 3-D structural data for azurin and stellacyanin were obtained from the Protein data bank under PDB: 1AZU and 1JER, respectively.

lipase promoter and the *A. oryzae* nitrate reductase gene (*nia*D) as a selective marker. Plasmids of the mutant expression vectors were purified from *E. coli* cells by 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 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 more 5 days, the transformants were cultured for 5 days in 3 ml of soybean oil medium. The expression product levels were measured by SDS-polyacrylamide gel electrophoresis.

Purification of Recombinant Bilirubin Oxidase from A. oryzae—*A. oryzae* harboring an expression vector was cultured aerobically at 30°C for 5-6 days in 1 liter 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]. The cells were removed by filtration through a glass filter, and then ammonium sulfate was added to the filtrate to 50% saturation with stirring and the mixture was allowed to stand overnight. The precipitate was separated by a glass filter and the supernatant was brought to 70% ammonium sulfate saturation with stirring. The mixture was again allowed to stand overnight. 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. The column was washed, after which 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 placed on a Sephadex G-100 gel filtration column and the protein was eluted with the same buffer.

Oxidation Procedure—Purified mutants of bilirubin oxidase were oxidized with a solution of 50 mM $K_3[Fe(CN)_6]$ in 50 mM Tris-H_2SO_4 buffer (pH 7.8) under aerobic conditions. After about 24 h incubation, the reaction mixture was dialyzed against the same buffer without $K_3[Fe(CN)_6]$ for 3 or 4 days. All oxidation procedures were carried out at 4°C.

Assay of Enzyme Activity—Bilirubin was purchased from Wako Pure Chemical Industries and used without further purification. The bilirubin oxidase activity was assayed by dissolving 2.0 ml of 30 μM bilirubin in 0.2 M Tris-H_2SO_4 buffer (pH 8.4), to which was added 0.2 ml of enzyme solution; the mixtures were then incubated at 37°C. The decrease in the absorbance at 440 nm was measured with a Jasco model Ubest-55 spectrophotometer. One unit was defined as the amount of enzyme that oxidized 1 μM bilirubin per min (*1*). The oxidation of ferrocyanide was monitored by the increase in absorbance at 420 nm; other conditions were the same as for the bilirubin oxidase assay. The initial absorbance changes were used to calculate the initial reaction rate v. The apparent kinetic parameter K_m was determined by fitting the initial reaction rate (v) and substrate concentration to $v = V_{max} \times [\text{substrate}]/(K_m + [\text{substrate}])$, and the apparent k_{cat} was determined from $k_{cat} = V_{max}/[\text{bilirubin oxidase}]$.

Spectroscopic Measurements—Absorption spectra of bilirubin oxidase were measured with a Jasco model Ubest-55 spectrometer. X-band ESR spectra at 77 K were measured on a JEOL JES-RE1X spectrometer. ESR spectra were recorded at the following instrument settings: center field, 300 mT; field-sweep width, $\pm$50 mT around center field; field-modulation amplitude, 0.8 mT; modulation frequency, 100 kHz; time constant, 0.03 s; scan speed, 12.5 mT/min; and microwave power, 5 mW. The magnetic field was calibrated with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The signal intensity of ESR-detectable Cu^{2+} was determined by the double integration method using Cu-EDTA as a standard. The total amounts of coppers contained in the enzyme derivatives were determined by atomic absorption spectroscopy with a Shimadzu AA-640-13 instrument.

Electrochemical Measurements—The midpoint potential of type 1 copper in wild type and mutant bilirubin oxidases was calculated as follows. First, the protein concentration was adjusted to an optical density of 0.8 at 600 nm. Then, the absorption at 600 nm and the potential between a platinum electrode and a standard Ag/AgCl electrode was measured anaerobically using $[Fe^{II/III}(CN)_6]^{4-,3-}$ as a mediator in 50 mM Tris-H_2SO_4 buffer (pH 7.8) by titrating L-ascorbic acid. The ratios of the oxidized and reduced forms of bilirubin oxidase were plotted against potential and the potential at equal amounts of oxidized and reduced enzyme was determined as the midpoint potential.

RESULTS

Site-Directed Mutagenesis and the Purification of Met467 Mutants—In this study, we adopted the PCR method for the preparation of mutated DNA segments using mutated PCR primers. Three mutated expression vectors (pNTIM467Q, H, and R) were constructed for the transformation of *A. oryzae*, and the expression products were analyzed by SDS-PAGE. All three expressed proteins with the same molecular mass as the wild type enzyme after cultivation for 5 days. Purified Met467His and Met467Arg are very pale blue in color [$\varepsilon < 1{,}000\ cm^{-1} \cdot M^{-1}$ at 600 nm (Table I)] and show no enzyme activity (Table II). When we added $K_3[Fe(CN)_6]$ to the enzyme solution, the color of enzyme solution turned greenish, and the mixture was dialyzed to remove the oxidizing agent com-

TABLE I. **ESR and optical absorption parameters of bilirubin oxidase and Met467 mutants.**

Sample	Copper type	g_z	$A_z \times 10^3$ (cm^{-1})	ε_{600} ($M^{-1} \cdot cm^{-1}$)
Wild type	type 1	2.21	8.3	4,800
	type 2	2.24	17.1	
Met467Gly[a]	type 1	2.23	7.1	800
	type 2	2.24	17.3	
Met467Gln	type 1	2.27	5.0	6,000
	type 2	2.23	17.3	
Met467His(Red.)	type 2	2.32	14.2	700
Met467His(Oxid.)	type 1	2.21	7.6	4,800
	type 2	2.33	13.7	
Met467Arg(Red.)	type 2	2.32	14.2	400
Met467Arg(Oxid.)	type 1	2.21	7.5	4,000
	type 2	2.33	13.7	

[a]The data for M467G are taken from our previous work (Ref. *14*).

pletely. After dialysis, the enzyme solution was clear blue because the $K_3[Fe(CN)_6]$, which has an absorption band around 420 nm, was removed. On the other hand, Met467Gln was purified as a greenish colored protein in an enzymatically active form.

Optical Absorption Spectroscopy—Figure 2 shows the absorption spectra of the wild type and mutant bilirubin oxidases in the visible region at pH 7.8. The wild type bilirubin oxidase shows 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 charge transfer. Met467Gln has a slightly different spectrum between 700 and 900 nm from the wild type enzyme, suggesting that the structure near the ligands for the type 1 copper atom is similar to that of stellacyanin. The wild type enzyme shows a broad band around 750 nm, whereas Met467Gln shows a red-shift of the band toward 850 nm with decreased intensity. In addition, there is a slight increase in the intensity of the small band in the 400-500 nm region (Fig. 2B). Both the Met467His and Met467Arg mutants as isolated possessed no significant band in the visible region (Fig. 2, C and D), but treatment with $K_3[Fe(CN)_6]$ resulted in remarkable increases in visible absorption in the 500-850 nm region, indicating that the type 1 copper was reduced when expressed. However, these two oxidized mutants show no hump around 330 nm due to oxidized type 3 coppers. The prominent decreases in the enzyme activities of the Met467His and Met467Arg mutants are thought to be due to the incomplete introduction of copper ions in the type 3 copper sites as evidenced by the decrease in the copper content of the mutants (Table II).

ESR Spectroscopy—Figure 3 shows the ESR spectra of the wild type enzyme and its Met mutants. The ESR signals of the wild type enzyme are typical of multicopper oxidases showing signals from type 1 and type 2 coppers. A type 3 copper signal, which is usually undetectable, can also be slightly observed at 275 mT, suggesting that a portion of the type 3 coppers are in the unbridged form (Fig. 3A). In the EPR spectrum of the Met467Gln mutant, the $A_{/\!/}$ value of the type 1 copper signal appears greatly reduced (from 8.3×10^{-3} to 5.0×10^{-3} cm^{-1}). In addition, the g_x and g_y components are apparently different showing the rhombic character of a type 1 copper signal, although it is difficult to resolve because of the overlap of the type 2 copper signal. Nevertheless, these spectral features are similar to those of the Met121Gln mutant of azurin (*19*), Met92Gln of plastocyanin (*20*), mavicyanin (*21*), and stellacyanin (*22*), in which Met is substituted by Gln.

Figure 3, C and D, shows the ESR spectra of Met467His and Met467Arg, respectively, immediately after purification, suggesting the presence of only type 2 copper in the same field region as the wild type enzyme. Furthermore, we can observe the five spikes around the $g_{\perp}$ region originating from two His residues coordinated to a type 2 copper. The $A_{/\!/}$ values of these two mutants are quite typical of type 2 copper, but smaller than those of wild type bilirubin oxidase (Table I). After treatment with an oxidizing agent, the type 1 copper signal has been observed in the mutants

TABLE II. **Specific activities, midpoint potentials, and copper contents of wild type and mutant bilirubin oxidases.**

Sample	Bilirubin as substrate		Ferrocyanide as substrate		E° (V *vs.* NHE)	Copper contents
	units/mg	Relative activity (%)	units/mg	Relative activity (%)		
Wild type	24.0	100	430	100	570	3.5
Met467Gly[a]	0.074	0.31	0.56	0.13	n.d.	2.1
Met467Gln	0.062	0.26	580	135	430	4.0
Met467His	0.002	0.01	0.08	0.02	n.d.	2.0
Met467Arg	0.010	0.04	–	–	430	1.4

Dash indicates no enzymatic activity detected. n.d.: not determined. [a]The activity for bilirubin oxidase and the copper contents of M467G are taken from our previous work (Ref. *14*).

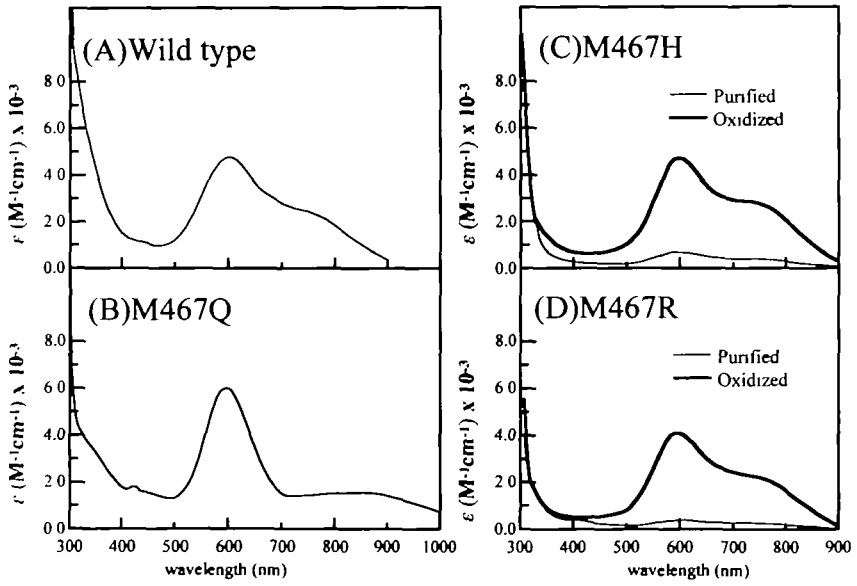


Fig. 2. **Absorption spectra of wild type and mutant bilirubin oxidases.** (A) Wild type bilirubin oxidase. (B) Met467Gln mutant. (C) Met467His mutant. (D) Met467Arg mutant. In (C) and (D), the thin lines indicate the absorption spectra of two mutants purified from the culture medium; the thick lines indicate enzyme solution oxidized with oxidizing agent.

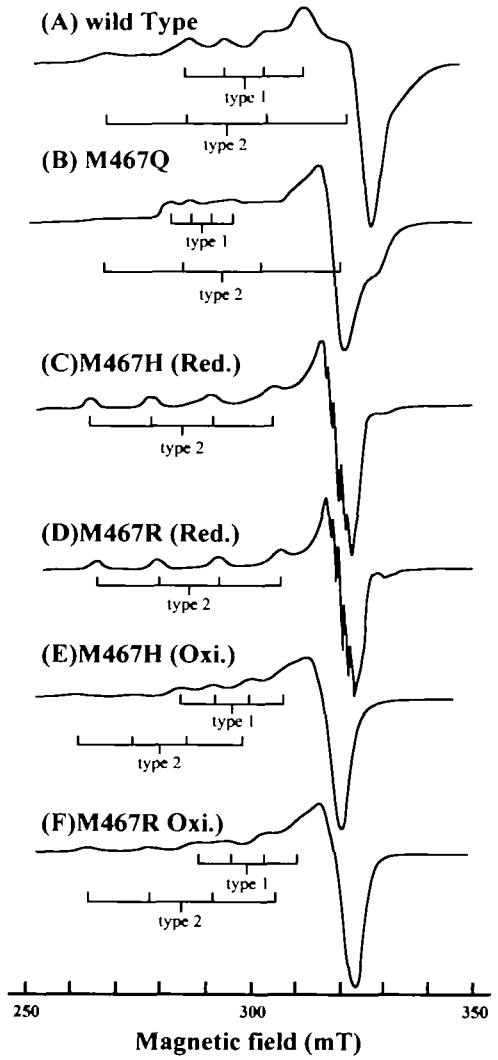


Fig. 3. **ESR spectra of wild type and mutant bilirubin oxidases.** (A) Wild type bilirubin oxidase. (B) Met467Gln mutant. Type 1 copper reduced form (C) and oxidized form (E) of Met467His mutant. Type 1 copper reduced form (D) and oxidized form (F) of Met467Arg mutant. The spectra were obtained with a microwave power of 6.25 mW, modulation width of 9.210 gauss, time constant 0.1 s, and sample temperature of 77 K.

together with the appearance of the blue band at 600 nm. The signals of the two mutants are similar and indicate slightly smaller $A_{\parallel}$ values than that of wild type bilirubin oxidase. Furthermore, this oxidative treatment causes a reduction of the $A_{\parallel}$ values of the two mutants.

Enzymatic Activities, Midpoint Potentials, and Copper Contents of Bilirubin Oxidase Mutants—The enzymatic activities of the bilirubin oxidase mutants were measured using bilirubin and potassium ferrocyanide as substrates. All three mutants show weaker enzymatic activities than that the wild type bilirubin oxidase when bilirubin is used as a substrate (Table II). Met467Gln has only about 0.3% of the bilirubin oxidizing activity of wild type. The activity is very low, but apparently present. In contrast, when ferrocyanide was used as the substrate, Met467Gln showed a remarkably high oxidation activity, almost identical to or exceeding that of the wild type. On the other hand, the activity of Met467His and Met467Arg is almost completely nonexistent. The kinetic parameters were determined for the wild type enzyme and Met467Gln using ferrocyanide as the substrate (Table III). The results reveal that Met467Gln retains ferrocyanide oxidation and O_2 reduction activities that are almost identical to those of the wild type.

TABLE III. **Kinetic parameters for ferrocyanide oxidation of wild type and Met467Gln mutant bilirubin oxidases.**

Enzyme	k_{cat} (s^{-1})	K_m (μM)
Wild type	393	113
Met467Gln	579	105

In particular, since the relationship between the enzymatic activity and midpoint potential of type 1 copper is important, we determined the midpoint potential as the electrode potential log([OX]/[Red]) is equal to 0. The ratio of oxidized type 1 copper to the reduced, [OX]/[Red], was obtained from the ratio of the absorbance, $(A_{600}-A^{r})/(A^{o}-A_{600})$, where A^{o} is the absorbance at 600 nm when type 1 copper is completely oxidized, and A^{r} is the absorbance at 600 nm when completely reduced. The midpoint potential for wild type bilirubin oxidase was 560 mV, whereas those for the type 1 copper in Met467Gln and Met467Arg were found to be 430 mV. It was difficult to determine the midpoint potential for Met467His and it is not reported here. In the presence of a small amount of reductant (ferrocyanide) under anaerobic conditions, the absorbance of the mutant solution at 600 nm decreased at first, but within a few minutes the absorbance returned to the original level seen before the addition of reductant. This phenomenon strongly indicates that electron transfer from type 1 copper to type 3 copper took place. Therefore, we also determined the oxidation potential of bilirubin, a substrate of bilirubin oxidase, to be about 500 mV. These results indicate that bilirubin is easily oxidized to biliverdin by wild type bilirubin oxidase, but only slightly oxidized by Met467Gln and Met467Arg, because the oxidation potential is too high compared to those of the mutants. We measured the copper contents of wild type and mutant bilirubin oxidases by atomic absorption spectroscopy (Table II). The copper content of Met467Gln was observed to be 4.0 atoms per molecule, which is almost identical to that of the wild type within experimental error, ensuring that all copper binding sites are fully occupied by copper ions. On the other hand, the copper contents of Met467His and Met467Arg were determined to be 2.0 and 1.4 atoms per molecule, respectively.

DISCUSSION

In this study, we succeeded in expressing in *A. oryzae* three mutants of *M. verrucaria* bilirubin oxidase, in which one of the type 1 copper ligands, Met467, was replaced by Gln, His, and Arg. All mutants were highly expressed in the culture medium (more than 50 mg/liter) and purified to an electrophoretically homogeneous state using two different columns. Met467Gln was purified as a holoenzyme and showed features contrasting with those of the Met121Gln mutant of azurin (*19*) and the Met92Gln mutant of plastcyanin (*20*), whose proteins accumulate as apoproteins. This difference in copper uptake may be due to the molecular size and shape of the proteins, *i.e.*, plastcyanin

and azurin are classified as blue copper proteins with molecular masses of 10–14 kDa. The type 1 copper in small copper proteins is not deeply burried inside the protein molecule as the copper ligand histidine imidazole edge is exposed to the solvent (*23*) and is easily removed from or reconstituted into the protein molecule under gentle conditions (*24*). On the other hand, the type 1 copper of multicopper oxidases is at the bottom of the substrate binding pocket (*4-6*), and, accordingly, it is difficult to remove the copper ion from the protein molecule (*25*). The absorption spectrum of Met467Gln is similar to that of stellacyanin (*22*). The analyses of the absorption spectra of blue copper proteins have revealed three visible absorption bands in the region between 550 and 700 nm, all indicative of Cys sulfur to the d_{x2-y2} orbital of copper charge transfer transitions and d-d transitions (*26*). The broad band around 750 nm in wild type bilirubin oxidase is decreased and red-shifted to around 850 nm in Met467Gln. This band has been assigned to the $d_{xz+yz}+(\pi(\mathrm{Cys}))$ to d_{x2-y2} transition. The ESR spectra of blue copper sites are usually classified as having axial symmetry, but in some cases as having rhombic symmetry. The relationship of the 3D-structural and ESR studies led to the conclusion that a copper site with rhombic ESR features would have a stronger ligand field in the axial direction than a copper site with axial symmetry. The ESR spectrum of type 1 copper in Met467Gln shows a rhombic spectrum with hyperfine splitting (5.0×10^{-3} cm^{-1}) smaller than in the wild type bilirubin oxidase (8.3×10^{-3} cm^{-1}), which indicates that the type 1 copper environment in Met467Gln is similar to that in stellacyanin and stabilizes the cupric form. This result supports the proposal that ligation of the O (amide) of Gln to type 1 copper occurs in both Met467Gln bilirubin oxidase and stellacyanin. The substitution of the S (thioether) of Met467 to the O (amide) of Gln may induce a more highly tetrahedral distortion of the copper site, leading to a decrease in the oxidation-reduction potential. We depict a scheme for the ligand configuration around the type 1 copper for Met467Gln together with schemes for the wild type and other mutants in Fig. 4. In fact, the oxidation-reduction potential of the type 1 copper in Met467Gln (430 mV) is lower than that of wild type bilirubin oxidase (560 mV). This effect seems to be strongly enhanced in stellacyanin, which has a potential of 184 mV, appreciably lower than the 240-400 mV reported for most blue copper proteins. The ESR spectra of Met467His and Met467Arg show typical type 2 copper signals before treatment with ferricyanide. Hyperfine splittings can also be observed. The five spikes of $A_\perp$ (*ca.* 1.2 mT) are assigned to the copper ligands of two ^{14}N atoms, and these signals are proof of the existence of two His residues. After oxidative treatment, new type 1 copper signals appear in the two mutants. This indicates that the type 1 copper of Met467His and Met467-Arg exists in the cuprous form before oxidation.

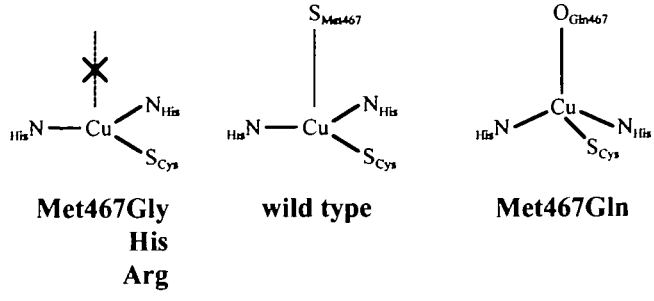


Fig. 4. **Schematic representation of the ligand configuration around type 1 copper of wild type, Met467 (Gly, His, and Arg), and Met467Gln.** An X in the left scheme indicates no ligation to the copper.

The enzymatic activity of Met467Gln is 300-fold smaller than that of wild type bilirubin oxidase when bilirubin is used as the substrate. This striking deterioration of activity may be caused by a decrease in the oxidation-reduction potential. The redox potential of bilirubin (500 mV) is higher than that of typical substrates for multicopper oxidases, *i.e.*, 430 mV and 0 mM for ferrocyanide and ascorbate, respectively. Therefore, Met467Gln is suitable for oxidizing substrates with midpoint potentials lower than 430 mV. To confirm this, we measured the enzymatic activity and rate of oxidation at various concentrations of ferrocyanide (Tables II and III). These analyses revealed that Met467Gln possesses a k_{cat} value of 579 s^{-1}, 1.35-fold greater than that of the wild type (393 s^{-1}). On the other hand, no significant change was observed in the K_m for ferrocyanide (113 and 105 μM for Met467Gln and wild type, respectively). These results indicate that substitution of Met467 by Gln affects only the redox potential of the type 1 copper site; the mutant still retains most of its ability to bind substrates and to reduce molecular oxygen. On the other hand, the enzymatic activity of Met467Gly is profoundly lower for ferrocyanide. This shows that the reduction of enzymatic activity of Met467Gly may be due not only to the change in the midpoint potential but also to a deficit of partial copper atoms. Furthermore, Met467His and Met467Arg show almost no enzymatic activity for either substrate in either the reduced or oxidized form. When azide ion, an inhibitor of multicopper oxidase, was added to a solution of multicopper oxidases or wild type bilirubin oxidase, the absorption increased around 400-500 nm, which may derive from the binding of azide ion(s) to type 3 copper (*27*). However, in the case of Met467His, no significant change was detected (data not shown). In addition, we also tried to reconstitute the copper ions for Met467His using cupric sulfate, but no change in enzymatic activity or the absorption spectrum around 300 nm was observed. Considering the results of enzymatic activities, absorption and, ESR spectra described above and the state of copper in the mutants (which is expressed as the type 1 reduced form), we conclude that these two mutants have no electron transfer activity from type 1 copper to trinuclear copper clusters caused by the deformation or loss of type 3 coppers.

For nitrite reductase containing type 1 and type 2 coppers, mutation of the type 1 copper ligand, Met150, to Glu induces the substitution of type 1 copper by Zn^{2+} ion (*28*). For *Pseudomonas aeruginosa* azurin, the wild type protein is expressed as a non-blue and zinc-occupied form in *E. coli* (*29*). In the case of bilirubin oxidase, the wild type and some mutants of its copper sites were also expressed in *A. oryzae* and studied in detail in our previous work (*14*); we showed these bilirubin oxidases to be expressed in blue or colorless forms depending on the mutated copper states. Wild type bilirubin oxidase and Met467Gly were expressed as blue and enzymatically active form. In this study, we

prepared three other mutants (Met467Gln, His, and Arg), and found that the copper content of Met467Gln is 4.0 atoms per molecule as in the native enzyme, but 2.0 and 1.4 atoms for Met467His and Arg, respectively. We assume that the reduction of the copper content may derive from the mixed population of enzymes with partial and complete apo forms, especially on a type 3 copper site as evidenced by the absorption spectra around 330 nm (Fig. 2, C and D).

In conclusion, the results obtained in this study are essentially consistent with the concept that the Met467 residue functions as a type 1 copper ligand in bilirubin oxidase.

REFERENCES

1. Tanaka, N. and Murao, S. (1985) Reaction of bilirubin oxidase produced by *Myrothecium verrucaria* MT-1. *Agric. Biol. Chem.* **49**, 843-844
2. Adman, E.T. (1991) Copper protein structure. *Adv. Protein Chem.* **42**, 145-197
3. Qiu, D., Dong, S., Ybe, J.A., Hecht, M.H., and Spiro, T.G. (1995) Variations in the type I copper protein coodination group: Resonance raman spectrum of ^{34}S-, ^{65}Cu-, and ^{15}N-labeled plastocyanin. *J. Am. Chem. Soc.* **117**, 6443-6446
4. Ducos, V., Brzowski, 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 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nat. Struct. Biol.* **5**, 310-316
5. Messerschmidt, A., Ladenstein, R., and Huber, R. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J. Mol. Biol.* **224**, 179-205
6. 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 center. *J. Biol. Inorg. Chem.* **1**, 15-23
7. Strange, R.W., Reinhammar, B., Murphy, L.M., and Hasnain, S.S. (1995) Structural and spectroscopic studies of the copper site of stellacyanin. *Biochemistry* **34**, 220-231
8. Schinina, M.E., Maritano, S., Barra, D., Mondovi, B., and Marchesini, A. (1996) Mavicyanin, a stellacyanin-like protein from zucchini peelings: primary structure and comparison with other cupredoxins. *Biochim. Biophys. Acta* **1297**, 28-32
9. van Driessche, G., Dennison, C., Sykes, A.G., and van Beeumen, J. (1995) Heterogeneity of the covalent structure of the blue copper protein umecyanin from horseradish roots. *Protein Sci.* **4**, 209-227
10. Chang, T.K., Iverson, S.A., Rodrigues, C.G., Kiser, C.N., Lew, A.Y.C., Germanas, J.P., and Richards, J.H. (1991) Gene synthesis expression, and mutagenesis of the blue copper proteins azurin and plastocyanin. *Proc. Natl. Acad. Sci. USA* **88**, 1325-1329
11. Hibino, T., Lee, B.H., and Takabe, T. (1995) Expression and characterization of Met92Gln mutant plastocyanin from *Silene pratensis*. *J. Biochem.* **117**, 101-106
12. Kroes, S.J., Hoitink, C.W.G., Andrew, C.R., Ai, J., Sanders-Loehr, J., Messerschmidt, A., Hagen, W.R., and Canters, G.W. (1996) The mutation Met121-His create a type-1.5 copper site in *Alcaligenes denitrificans* azurin. *Eur. J. Biochem.* **240**, 342-351
13. Koikeda, S., Ando, K., Kaji, H., Inoue, T., Murao, S., Takeuchi, K., and Samejima, T. (1993) Molecular cloning of the gene for bilirubin oxidase from *Myrothecium verrucaria* and its expression in yeast. *J. Biol. Chem.* **268**, 18801-18809
14. Shimizu, A., Kwon, J.-H., Sasaki, T., Satoh, S., 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
15. Yamaguchi, S., Takeuchi, K., Mase, T., and Matsuura, A. (1997) Efficient expression of mono- and diacylglycerol lipase gene from *Penicillium camembertii* U-150 in *Aspergillus oryzae* under the control of its own promoter. *Biosci. Biotech. Biochem.* **61**, 800-805
16. Higuchi, R., Krummel, B., and Saiki, R.K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351-7367
17. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557-580
18. Unkles, S.E., Campbell, E.I., Carrez, D., Grieve, C., Contreras, R., Fiers, W., Van den Hondel, C.A., and Kinghorn, J.R. (1989) Transformation of *Aspergillus niger* with the homologous nitrate reductase gene. *Gene* **78**, 157-166
19. Romero, A., Hoitink, C.W., Nar, H., Huber, R., Messerschmidt, A., and Canters, G.W. (1993) X-ray analysis and spectroscopic characterization of M121Q azurin (A Copper Site Model for Stellacyanin). *J. Mol. Biol.* **229**, 1007-1021
20. Hibino, T., Lee, B.H., and Takabe, T. (1995) Expression and characterization of Met92Gln mutant plastocyanin from *Silene pratensis*. *J. Biochem.* **117**, 101-106
21. Kataoka, K., Nakai, M., Yamaguchi, K., and Suzuki, S. (1998) Gene synthesis, expression, and mutagenesis of zucchini mavicyanin: the fourth ligand of blue copper center is Gln. *Biochem. Biophys. Res. Commun.* **250**, 409-413
22. Strange, R.W., Reinhammar, B., Murphy, L.M., and Hasnain, S.S. (1995) Structural and spectroscopic studies of the copper site of stellacyanin. *Biochemistry* **34**, 220-231
23. Hoitink, C.W., Driscoll, P.C., Hill, H.A., and Canters, G.W. (1994) ^{1}H and ^{15}N nuclear magnetic resonance assignments, secondary structure in solution, and solvent exchange properties of azurin from *Alcaligenes denitrificans*. *Biochemistry* **33**, 3560-3571
24. Marks, R.H. and Miller, R.D. (1979) A kinetic study of the reconstitution of azurin from Cu(II) and the apoprotein. *Arch. Biochem. Biophys.* **195**, 103-111
25. Morpurgo, L., Hartmann, H.J., Desideri, A., Weser, U., and Rotilio, G. (1983) Yeast copper-thionein can reconstitute the Japanese-lacquer-tree (*Rhus vernicifera*) laccase from the Type 2-copper-depleted enzyme via a direct copper(I)-transfer mechanism. *Biochem. J.* **211**, 515-517
26. Gewirth, A.A. and Solomon, E.I. (1988) Electronic structure of plastcyanin: excited state spectral features. *J. Am. Chem. Soc.* **110**, 3811-3819
27. Sakurai, T. and Takahashi, J. (1995) EPR spectra of type 3 coppers in *Rhus vernicifera* laccase and *Cucumis sativus* ascorbate oxidase. *Biochem. Biophys. Acta* **1248**, 143-148
28. Murphy, M.E., Turley, S., Kukimoto, M., Nishiyama, M., Horinouchi, 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
29. Nar, H., Huber, R., Messerschmidt, A., Filippou, A.C., Barth, M., Jaquinod, M., van de Kamp, M., and Canters, G.W. (1992) Characterization and crystal structure of zinc azurin, a by-product of heterologous expression in *Escherichia coli* of *Pseudomonas aeruginosa* copper azurin. *Eur. J. Biochem.* **205**, 1123-1129