Crystal Structures of Copper-depleted and Copper-bound Fungal Pro-tyrosinase: INSIGHTS INTO ENDOGENOUS CYSTEINE-DEPENDENT COPPER INCORPORATION*

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*Running title: Structural insights into tyrosinase maturation

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Background: Fungal tyrosinase maturation involves multiple processes of the dinuclear copper assembly and proteolytic activation.

Results: Structural examinations and mutational studies of the pro-tyrosinases revealed that three endogenous cysteines contribute to the copper incorporation.

Conclusion: The three highly flexible cysteines are essential for assembly of the active site across protein shell.

Significance: Elucidation of such copper incorporation process provides useful insights into metal homeostasis.

SUMMARY

Tyrosinase, a dinuclear copper monooxygenase/oxidase, plays a crucial role in the melanin pigment biosynthesis. The structure and functions of tyrosinase have so far been studied extensively, but its post-translational maturation process from the pro-form to the active-form has been less explored. In this study, we provide the crystal structures of Aspergillus oryzae full-length pro-tyrosinase in the holo- and the apo-forms at 1.39 Å and 2.05 Å resolution, respectively, revealing that Phe513 on the C-terminal domain is accommodated in the substrate-binding site as a substrate analog to protect the dicopper active site from the substrate access [Proteolytic cleavage of the C-terminal domain or deformation of the C-terminal domain by acid-treatment transforms the pro-tyrosinase to the active enzyme (Fujieda et al. in ChemBioChem (2012) 13,193-201, and Fujieda et al. in J. Biol. Inorg. Chem. (2013) 18, 19-26)]. Detailed crystallographic analysis and structure-based mutational studies have shown that the copper incorporation into the active site is governed by three cysteines; Cys92 which is covalently bound to His94 via an unusual thioether linkage in the holo-form and Cys522 and Cys525 of the CxxC motif located on the C-terminal domain. Molecular mechanisms of the maturation processes of fungal tyrosinase involving the accommodation of the dinuclear copper unit, the post-translational His-Cys thioether cross linkage formation, and the proteolytic
**C-terminal cleavage to produce the active-tyrosinase, have been discussed on the basis of the detailed structural information.**

Tyrosinase (Ty, EC 1.14.18.1), a dinuclear copper monooxygenase/oxidase, is a key enzyme in the melanin biosynthesis and is widely distributed in mammals, plants, fungi, and bacteria. The dinuclear copper center is involved in the catalytic reactions of tyrosinase, the hydroxylation of phenolic substrates (phenolase activity, Scheme 1) and subsequent oxidation of catechols to o-quinones (catecholase activity, Scheme 1) (1-4). Similar dinuclear copper center, so-called type-3 copper, also plays important roles in catechol oxidase catalyzing exclusively the latter reaction (catecholase activity), and in hemocyanin, which serve as the dioxygen carrier in the hemolymph of mollusks and arthropods (5). In the crystal structures of these proteins, six histidine residues, which are provided by a four-helix bundle in the copper-binding domain, coordinate to the two copper ions (three histidine imidazoles for each copper ion, CuA and CuB) in the active site (Fig. 1A and B) (6-12). In mushroom tyrosinase (12), sweet potato catechol oxidase (8), and octopus hemocyanin (7), one of the histidine imidazole groups coordinating to CuA is covalently bound to nearby cysteine via an unusual thioether linkage (Fig. 1), although its function still remains obscure.

In other copper-containing proteins, such as blue copper proteins, multi-copper oxidases, cytochrome c oxidase (CcO), dopamine β-hydroxylase (DβH), peptidylglycine α-hydroxylating monoxygenase (PAM), particulate methane monooxygenase (pMMO), quercetin 2,3-dioxygenase (2,3-QD), copper amine oxidase (AO), galactose oxidase (GO), and Cu/Zn superoxide dismutase (Cu/Zn-SOD), the copper cofactors also play crucial roles to dictate electron-transfer, two- and/or four-electron reduction of O₂, alkane-hydroxylation, dehydrogenation of alcohols and amines, and superoxide dismutation (13). However, copper is known to be highly toxic to cells due to its undesirable property in yielding the deleterious hydroxyl radical (14). Thus, copper content inside the cell is severely regulated by copper membrane transporter (Ctr) proteins, which transport copper from the extracellular medium to the cytoplasm (15). Therefore, amount of free copper ions available in the cytoplasm of the cell is very limited. In order to survive under such copper-depleted conditions, copper chaperones are often employed to deliver copper ions into the active site of copper proteins. In this case, copper ions are accommodated by using multiple cysteine and/or methionine units from the conserved motifs C-Xₐ-C (n = 1–3) and/or M-Xₐ⁻M (m = 1, 2), which are crucial for copper chaperone activity in the proteins (15,16).

For mammalian and yeast tyrosinases, it is thought that copper ions are transferred into the active site by Atox1 copper chaperone and copper-transporting ATPase (ATP7A), which catalyze copper-movement across the membranes in the Golgi apparatus on the secretion process (15,17). However, detailed mechanism of the copper-incorporation process has yet to be elucidated. Recently, a copper chaperone, so-called caddie protein (ORF378), was crystallized in the complex with the bacterial tyrosinase from **Streptomyces castaneoglobisporus** (9,18), where ORF378 binds to the active site of tyrosinase to help the copper trafficking. This result provides some insights into the molecular mechanism of the copper-incorporation process of type-3 copper proteins.

In the case of **octopus** hemocyanin, however, the dinuclear copper site is completely covered by additional shielding domain (C-terminal domain) (7), thus prohibiting the direct access of a copper chaperone to the active site. The situation may be similar with **fungal** tyrosinase and **plant** catechol oxidase, which also involve a C-terminal shielding domain as mentioned below. In these cases, a copper chaperone-like machinery may be equipped in the same protein to facilitate the copper-incorporation. This has been proved in
the present study using fungal tyrosinase from *Aspergillus oryzae*.

With regard to the C-terminal domain, it may play an important role to regulate the enzyme activity. Namely, C-terminal domain prohibits the substrate access to the enzyme active site and blocks the oxidase/oxygenase activity in order to avoid undesirable intracellular reactions of highly reactive quinonoid products. In support of this notion, several lines of evidences have indicated that a proteolytic enzyme *in vivo* cleaves a part of protein of the fungal pro-tyrosinase and plant pro-catechol oxidase to induce the enzymatic activity (19-21). Furthermore, the cloned cDNA clearly indicated the existence of such an additional extension in the C-terminal domain, although the isolated active forms of mushroom tyrosinase (PPO3) (12) and sweet potato catechol oxidase (8) lack the C-terminal domains in the crystal structures. On the basis of these results, it has been suggested that C-terminal domain of pro-tyrosinase is cleaved off by a proteolytic enzyme to produce the active form (Fig. 2B → C) after copper ions are transferred into the active site through protein shell (Fig. 2A → B). However, the structural information of pro-tyrosinase has yet to be available, so that little is known about the details of maturation processes including the copper incorporation and the proteolytic activation.

We have recently reported that the apo-form of recombinant pro-tyrosinase from *Aspergillus oryzae* (inactive precursor) can be over-produced from *E. coli* by using the full-length of cDNA (*melB*; 27 % identity to the full-length sequence of PPO3), and reconstitution of this apo-pro-tyrosinase with copper ion under aerobic conditions induces autocatalytic formation of His94-Cys92 cross-linkage in the enzyme active site (22). The holo-pro-tyrosinase thus formed has no catalytic activity, but the trypsin-treatment converts the holo-pro-form into the active-form of the enzyme, in which the C-terminal domain (Gly464–Ala616) is absent (cleaved) (23).

Here, we successfully determined the crystal structures of the holo-pro- and the apo-pro-forms of *melB* tyrosinase from *Aspergillus oryzae* at a 1.39 and a 2.05 Å resolution, respectively, to provide the first detailed structural information about the fungal tyrosinase containing the C-terminal domain. The structural data of holo-pro-form provides important insights into the role of the C-terminal domain not only as a reactivity regulation domain but also as a copper chaperone-like machinery. Comparison of the crystal structures between the apo-pro-form and the holo-pro-form together with the mutagenesis studies have elucidated the detailed molecular mechanism of the maturation process of tyrosinase; the incorporation of copper ions and autocatalytic formation of the His-Cys cross linkage.

**EXPERIMENTAL PROCEDURES**

*Expression of Tyrosinases and Seleno-methionine Substituted Tyrosinase–MelB*

Pro-form of tyrosinase was prepared by using pETH-melB plasmid as described previously (22,23). As for seleno-methionine substituted tyrosinase, expression plasmids were transformed into *Escherichia coli* B834 (DE3) pLysS competent cells. The transformants were grown in 2 L baffled Erlenmeyer flask of 0.4 L Se-Met core medium (Wako) broth containing 50 mg/L kanamycin, 4 % glucose, 5 mg/L vitamin B1, 50 mg/L L-SeMet, 1.2 g/L MgSO4, 24.4 mg/L FeCl3, 2 M HCl 50 µL/L, 4 % glucose, with shaking at 135 rpm at 25 °C until the cultures reached an *A*600 of 0.8. At this point, IPTG was added to the mixture (0.1 mM after addition) and the resulting mixture was allowed to continue being shaken for additional 24 h at 18 °C.

*Purification of Tyrosinase–* If not mentioned otherwise, the same purification protocol was used for all proteins. *MelB* pro-tyrosinase was purified as previously described with some modification (23). Harvested cells (ca. 10 g) from 1L culture were re-suspended in 40 mL of the binding buffer (0.3 M NaCl, 10 mM imidazole and 20 mM bis-Tris, pH 7.2) containing 0.2 mg/mL Lysozyme. After 15 min incubation on ice, the sample was sonicated for...
5 sec 120 times on ice (20 W output). After centrifugation at 30000g for 30 min, the clarified supernatant was loaded onto a 5 mL Ni-agarose (COSMOGEL His-Accept, Nacalai tesque) column previously equilibrated with the binding buffer, and the histidine affinity-tag fused protein was eluted with Ni-sepharose elution buffer (0.1 M imidazole, 0.3 M NaCl, 0.5 mM TCEP and 20 mM Bis-tris pH 7.2). The histidine affinity-tag free pro-tyrosinase was released from the fusion protein by incubation with 2 units HRV3C protease / mg protein on ice for over 16 h. The protein solution was then dialyzed against 10 mM Tris-HCl buffer, pH 8.2, containing 10 mM imidazole at 4 °C to remove the excess imidazole and NaCl. The dialyzed solution was loaded onto a 5 mL Ni-agarose column previously equilibrated with this dialysis buffer again to remove the released histidine affinity-tag protein. This eluent was subjected to anion-exchange chromatography by using a Q sepharose HP column (vol. 5 mL, GE Healthcare) with linear gradient elution of NaCl (10-200 mM) in 10 mM Tris-HCl buffer, pH 8.2. The fractions containing pro-tyrosinase were collected and concentrated by ultrafiltration using a VIVA SPIN 20 (Sartorius). Before crystallization, the purified protein solution was desalted using PD-10 column (GE Healthcare) and changed to 20 mM Bis-tris buffer, pH 7.2. The protein was stored at −80 °C until use. Purity was checked by SDS-PAGE.

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis experiments were performed on pETH-melB vector that contained melB cDNA. The pair of about 25 base mutagenic oligonucleotide shown in supplemental Table 1 was obtained from Integrated DNA Technology. Site-directed mutagenesis was carried out by the inverse PCR and following Dpn I digestion and self-ligation. For the mutant, the absence of undesired mutations on the melB gene was confirmed by DNA sequence analysis using a 3100 Genetic Analyzer (Applied Biosystems) after the construction of the vector.

Quantification of Protein and Copper Content–Protein concentration was determined by modified Lowry method using a DC protein assay kit (BIORAD) according to supplier’s manual. Copper concentration was determined by an ICP-AES on an ICPS-8100 (Shimadzu). The calibration curve of copper ion was made by using a copper ion standard solution (10.0 ppm).

Crystallization of Copper-bound Pro-tyrosinase (Holo-pro-tyrosinase)–The protein sample was concentrated to 6 mg/ml by ultrafiltration using a VIVA SPIN 20 and reserved at −80 °C. Crystals of holo-pro-tyrosinase were obtained by the hanging drop vapor diffusion method. Crystallization droplets were prepared by mixing the precipitant solution containing 18 % polyethylene glycol 3350, 50 mM NH₄F (1 µL) and 6 mg/ml tyrosinase solution (2 µL). Crystals were obtained at 4 °C in one day, and were soaked in the solution containing 25 % polyethylene glycol 3350, 50 mM NH₄F, and 20 mM Bis-tris at pH 7.2, for cryo-protection.

Crystallization of Copper-depleted Pro-tyrosinase (Apo-pro-tyrosinase)–The protein sample was concentrated to 17 mg/ml by ultrafiltration using a VIVA SPIN 20 and reserved at −80 °C. Apo-pro-tyrosinase was crystallized at 4 °C in similar condition as holo-pro-tyrosinase using the precipitant solution containing 20 % polyethylene glycol 3350, 50 mM KCl (1 µL) and 17 mg/ml tyrosinase solution (2 µL). For cryo-protection, solution containing 22.5 % polyethylene glycol 3350, 15 % ethylene glycol, 50 mM KCl, 20 mM Bis-tris at pH 7.2 was used.

Crystallization of Seleno-methionine Substituted Pro-tyrosinase–The protein sample was concentrated in similar condition as holo-pro-tyrosinase and crystallized at 4 °C using the precipitant solution containing the precipitant solution containing 20 % polyethylene glycol 3350, 50 mM NH₄F (1 µL) and 6 mg/ml tyrosinase solution (2 µL). The same solution as holo-pro-tyrosinase crystals was used for cryo-protection.

X-ray Data Collection–All diffraction data were collected at 100 K on the BL44XU beamline at the SPring-8 synchrotron facility.
Structural insights into tyrosinase maturation

Before flash cooling, crystals were directly soaked in a respective cryoprotectant solution for 20 minutes. X-ray diffraction images were collected using an MX225HE CCD detector (Rayonix, Illinois, USA) equipped with a Helix Technology cryo-system (Cryo Industries of America, New Hampshire, USA). All diffraction images were recorded on the CCD camera and the data were processed and scaled with the HKL-2000 program package. The data collection statistics are summarized in Table 2.

Phasing and Initial Model Building—We attempted to carry out molecular replacement using MOLREP (24). Mushroom tyrosinase (PDB entry: 2y9w) was selected for molecular replacement because of its 27 % amino acid similarity to melB tyrosinase. However, we were unable to obtain good solutions for the molecular replacement calculations. We also attempted to obtain the phase information using a copper single-wavelength anomalous dispersion (SAD) and multiple-wavelength anomalous dispersion (MAD) methods, but have failed. Finally, the structure of Se-Met pro-tyrosinase was solved by the SAD method using Se-Met-substituted pro-tyrosinase. Initial phases were calculated with the program AutoSolve (Figure-of-merit, 0.48), and the structure model was continuously built with the program Automodel in Phenix program package (25). The resulting model covered 935 residues of the two subunits in the asymmetric unit with 738 side chains assigned.

This procedure was iterated until the model did not further improve. During the refinement, the copper-ligand distances and the distance of His94-Cys92 cross linkage were loosely restrained with the ReadySet program. For apo-pro-tyrosinase, the structure was solved by molecular replacement using the final model of holo-pro-tyrosinase (chain A) as a search model, and the refinement procedure was same as the case of holo-pro-tyrosinase. In both structure, Ramachandran analysis with the Molprobity program (28) showed no outliers. Secondary structures were assigned by DSSP program (29).

RESULTS AND DISCUSSION

Copper-uptake Assays—Copper-binding Assays were performed in E.coli BL21(DE3) cytoplasm. Expression of pro-tyrosinase was performed as previously reported (22,23) except using the ZYM5052 medium (Its copper concentration was determined to be < 0.1 µM by ICP analysis) containing a various concentration of CuSO₄ (1 µM-3 mM). Harvested cells were washed with a 0.85 % NaCl aqueous solution to remove the remaining copper ions on the cell surface at least three times. After the purification as described above without the step of the anion-exchange chromatography, the copper content of pro-tyrosinase was determined by ICP-AES analysis. This experiment was performed at least two times for each mutants and copper concentration.

Overall Structure of Holo-form of Full-length MelB—The crystal structure of melB holo-pro-tyrosinase was determined by molecular replacement using the structure of the selenomethionine-substituted melB apo-pro-tyrosinase as a search model. Holo-pro-form was crystallized with one homodimer in the crystallographic asymmetric unit.
unit (space group P2_1). The final structures were refined to a resolution of 1.39 Å with excellent statistics (Table 2). Two protomers in the asymmetric unit are related by non-crystallographic two-fold axis (Fig. 3A), displaying an ellipsoid shape with dimensions of 95 Å × 65 Å × 45 Å (Fig. 3A). Elements of secondary structure (α for α-helix, α' for 3_10-helix, π for π-helix and β for β-strand) are identified and numbered sequentially in Fig. 4.

The full-length melB protomer consists of 621 amino acid residues deduced from cDNA sequence. In chain B of the crystal structure, most of the residues are well defined in the final electron density, except for some disordered loops in the flexible regions (Gln81-Pro88, Asn158-Gln160, Ser213-Ser222, Asp304-Gln316, Gly515-Gln527, and Gly515-Gln527, Table 3). On the other hand, the electron densities of three loops (Gln81-Pro88, Ser213-Ser222, and Gly515-Gln527) were observed in chain A. On the last loop, the backbone nitrogen atoms of Asp523(A), Lys524(A) and Cys525(A) of chain A are hydrogen bonded to Asp552(B') and Asn492(B') of the next protomer (Chain B'), the chain B molecule related by the crystallographic symmetry, respectively (Fig. 5). In addition, there is also a hydrogen bond between Asp523(A) and Asp552(B'). These results indicate that these hydrogen bonds fix one of the conformations of this loop (Gly515-Gln527) and further stabilized those of other two loops located in the neighbors (Gln81-Pro88 and Ser213-Ser222).

**Dimer Interface**

MelB pro-tyrosinase was found to exist mainly as a homodimer at physiological condition in our previous study (23). A very tight inter-protomer interaction was observed in the crystal structure of holo-pro-tyrosinase. The homodimer is glued together through extensive hydrophobic and charge-charge interactions, the accessible surface area buried in the interface is 1925 Å^2 (Fig. 3A). Thus, we propose that the structure obtained via tight association is a biologically functional dimer. The dimer of holo-pro-tyrosinase is formed between chains A and B in a side-to-side manner along a two-fold axis; thus, the substrate-binding pockets face alternate directions so as not to interfere with each other. The dimer interface can be classified into three regions (Fig. 3A, indicated by green and red rectangles). Region I (red region) predominantly involves hydrophobic interactions among leucine clusters (L259(A), L348(A), L350(A), L259(B), L348(B), and L350(B), Fig. 3B, red). The interface of region II (green region) is stabilized by hydrogen bonds between Tyr26 (A) and Asp245 (B), salt bridge between Glu163(A) and Lys349(B) and cation-π interaction between Arg165(A) and Tyr242(B). Furthermore, a tight hydrophobic interaction through van der Waals contacts situated at the heart of the region II (Thr171(A), Trp254(A), Leu255(A), Leu239, Tyr242(B), and Val246(B), Fig. 3B, green). Thus, both polar and hydrophobic interactions stabilize the association of the A and B chains.

**Domain Structure of Holo-form of Full-length MelB from Aspergillus oryzae**

Each protomer consists of two structural domains: the N-terminal copper-binding domain (Ser1–Phe463, indicated by blue) and C-terminal shielding domain (Gly464–Ala616, indicated by red) (Fig. 6A). Since two protomers are nearly identical to each other, we describe the monomer structure by referring mainly the chain-A molecule. The copper-binding domain comprises the central core structure (Arg19-Tyr435) and the peripheral loop region connecting the secondary structure elements (Fig. 4 and 6C). Although melB pro-tyrosinase having about 100 extra residues is much larger than mushroom tyrosinase (PPO3, PDB: 2Y9W), the core structure is almost identical to that of mushroom tyrosinase (Root mean square deviation (RMSD) value for Cα atoms of 351 matched residues is 2.5 Å, Fig. 4 and 6C). A significant structural difference is arisen between the loop regions surrounding the core structures (Fig. 6C). The melB core structure is also similar to those of the other type 3 copper proteins, sweet potato catechol oxidase (PDB: 1BT3, RMSD 3.3 Å, 261 matched residues), and functional unit of octopus
hemocyanin (PDB: 1JS8, RMSD 2.9 Å, 258 matched residues) (Fig. 6E and F). In these proteins including melB tyrosinase, a four-helix bundle of the core structure provides the highly conserved six histidine residues as the copper ligands (three histidine imidazoles for each copper ion, CuA and CuB) in the active site and one of these histidine (His94, melB numbering) is covalently bound to nearby cysteine (Cys92, melB numbering) via a thioether linkage (Fig. 6B, D and F).

The dinuclear copper center of melB tyrosinase is buried in the cleft of copper binding domain and is inaccessible to the exterior solvent due to the occlusion by the C-terminal domain as expected. The tip of the C-terminal domain is Phe513 residue, and its side chain is accommodated just above this dicopper active center as the “placeholder” for phenolic substrates (Fig. 6B). The phenyl ring of Phe513 stacks onto the imidazole ring of one of the CuB ligands (His332) at the distance of ~3.6 Å and the edge of the aromatic ring is close to CuB (~3.9 Å, Fig. 6B). Furthermore, one conformer of the side chain of Val359 residue (V359a) is also at a van der Waals distance (~3.5 Å) from the aromatic ring of Phe513 residues (Fig. 6B). Notably, Val359Ala mutant (V359A) could be expressed in the soluble fraction in E. coli, while His332Ala or Phe513Ala mutants (H332A and F513A) were expressed as inclusion body (Fig. 7), indicating that the π-stacking between Phe513 and His332 may strongly contribute to the stabilization of inter-domain interaction in melB pro-tyrosinase (Fig. 6A and B).

The C-terminal domain exhibits a seven-stranded antiparallel β-sandwich structure, whose topology is the truncated jellyroll motif (Fig. 8A and D). The C-terminal domain does not show any structural similarity to the caddie protein of S. castaneoglobisporus tyrosinase (Fig. 8C). By structural similarity search using the DALI server (30), the closest structural homologue was found in the shielding domain of octopus hemocyanin functional unit (Fig. 8B, PDB: 1JS8, RMSD 2.5 Å, 89 matched residues) in spite of little sequence identity (7 %), the copper-binding domain of which is also structurally similar to that of melB tyrosinase described above. Thus, the protomer can be superimposed to that of octopus hemocyanin not only with the copper-binding domain but also with the C-terminal domain (Fig. 6E). Consistent with the structural similarity, the holo-pro-tyrosinase has no catalytic activity, but the reduced-form of holo-pro-tyrosinase produced by the reduction of the as-isolated enzyme with hydroxylamine exhibits a reversible dioxygen-binding ability and its affinity toward dioxygen is almost equal to that of octopus hemocyanin (23). Leu2830 residue in the octopus hemocyanin structure occupied the similar position of Phe513 in melB tyrosinase (Fig. 6F), so that aromatic carbons of Phe513 might not have much influence on the dioxygen-binding ability.

The proteolytic digestion of the C-terminal domain having Phe513 “placeholder” leads to opening the entrance to the enzyme active site for substrate incorporation (Fig. 2B → C). In the copper-binding domain, the core region (Arg19–Tyr435) is followed by a linker consisting of 28-residues (Ala436–Lys463), which contains the five short helices (α29, α’30, α31, π32, and α33, Fig. 4 and 9). This linker region has an inflection point containing conserved Tyr–Gly sequence motif (Tyr452, Gly453), located in the π-helix (π32, Fig. 9). In the proteolytic activation in our previous study, C-terminal domain and the last part of this linker (α33) were digested (Glu458–Ala4616, see below) (23). The hydroxyl group of Tyr452 has hydrogen bonds to the side chains of the functionally conserved Asp147 and Arg377, enforcing this region of the linker to associate with the tyrosinase core domain (Fig. 9). Thus, this linker moiety is situated on the surface of monomer and α33-helix is bulged out from the surface of the copper-binding domain, so that the proteolytic enzyme can easily attack this region. This cleavage site (between Lys457 and Glu458) is also located next to the equivalent C-terminal position in the active mushroom tyrosinase (PP03).

Conserved Cysteines in MelB
Apo-pro-tyrosinase—As in the case of holo-pro-tyrosinase, melB apo-pro-tyrosinase exhibits a homodimeric structure. The crystal structure of apo-pro-form of melB was determined by molecular replacement using the structure of holo-pro-form as a search model. The final structure of apo-pro-form was refined to a resolution of 2.05 Å with good statistics (Table 2). Apo-pro-form melB tyrosinase was crystallized in the monoclinic space group of P2₁. Most of the residues are well defined in the final model in each protomer (Fig. 10A) and the crystallographic asymmetric unit has two sets of homodimers (Chains A, B and Chains C, D). Four protomer molecules are almost identical with each other and very similar to those of the holo-pro-form (Fig. 10B). In chain A, the six disordered loops and peptide side chains were observed (Lys82-Lys85, Gly155-Gln160, Ser213-Phe223, Asp304-Gln316, Ser516-Gly532, and Arg593-Asp597, Table S2), as is the case of chain B of holo-pro-form structure. The whole structures of apo-pro-form and holo-pro-form closely resemble each other, showing a 0.5 Å RMSD over all backbone atoms except disordered region (chain A and B of apo-pro-form crystal vs. chain A and B of holo-pro-form crystal).

In spite of the similarity of the whole structures, the positions of the three-conserved histidine residues (His94, His332, and His372) of apo-pro-form are different from those of the holo-pro-tyrosinase and a water molecule (Wat701) is present in place of the two copper ions (Fig. 10C, 11A and 11B). Wat701 is hydrogen-bonded to three Nε2 nitrogen atoms of the imidazole groups of His67, His94, and His103 (< 3.0 Å CuA-coordinating), but those of the other histidines (> 3.0 Å CuB-coordinating) are not within hydrogen-bonding distance. The side chains of His94 and His372 take different conformations from those of the apo-pro-form and exhibited higher average B-factors (33.3 and 19.5 Å², respectively) than those of the other four histidines (14.3 Å²). These results suggest that the side chain of these two histidine residues are flexible as compared to those of the others; His94 and His372 imidazoles exhibit no hydrogen-bonding interaction with other protein residues. The positions of side chains His332 and Phe513 deviate somewhat from those in holo-pro-tyrosinase due to the absence of the metal coordination (Fig. 11D). It should be noted that no electron density was found between Cys92 and His94 side chains (Fig. 11A), definitely demonstrating that the His94–Cys92 thioether linkage is absent in the apo-form and the covalent His–Cys bond is formed after the copper up-taken as reported in our previous paper (22). Furthermore, the side chain of Cys92 adopts dual conformations: one (Cys92a) is exactly the same as the conformation observed in the holo-form and the other one (Cys92b) faces to the direction to the CuA-binding site (Fig. 10C and 11A). In the latter conformation, S atom of Cys92 side chain is within bonding distance to CuA (< 2.5 Å), suggesting a direct interaction of the thiol (or thiolate) group of Cys92 and copper ion(s) during the maturation process.

It should be noted that the electron density of the surface region enveloping the CuA binding site is completely disordered in each protomer of apo-pro-tyrosinase. This area is composed of three loops (Loop 1-3; Lys82-Lys85, Ser213-Phe223, and Ser516-Gly532, Fig. 10B and 11C). From the amino acid sequence analysis of these flexible loops, a CxxC motif composed of the conserved two cysteines (Cys522 and Cys525, Figure 4 and 10B) are found on the loop 3 of the C-terminal domain. Such a motif is seen in the copper chaperone such as Atox1 and Ccc2 in yeast (15). Thus, we consider that the three cysteines (Cys92, Cys522 and Cys525) are involved in the copper up-taken process (Fig. 2A → B).

Assembling Process of Dinuclear Copper Center—To confirm this hypothesis, we have performed copper-uptake experiment from the E. coli cytoplasm by using Cys-to-Ala mutants. The extent of assembly of dinuclear copper center of as-isolated melB pro-tyrosinase practically depended on the CuSO₄ concentration in the medium (Fig. 12, black
circle). From this result, it was thought that the influx of the copper(II) ion into the *E. coli* cytoplasm might increase as the CuSO₄ concentration in the medium becomes higher. The redox state of the copper becomes monovalent cation (copper(I)) in the cytoplasm of *E. coli* like in the eukaryotic cells (31). Wild type of *melB* pro-tyrosinase was successfully metalated to full complement even at a low concentration of CuSO₄ (~ 1 mM). However, Cys522Ala and Cys525Ala mutants (C522A and C525A, Fig. 12, blue and green circle, respectively) needed more than one order of magnitude higher concentration of CuSO₄ to complete the assembly of dinuclear copper center, and full complement of double mutant (C522A/C525A, Fig. 12, orange circle) was not observed within the range of the concentration capable of *E. coli* to grow up (≤ 3 mM). Furthermore, Cys92-to-Ala mutants (C92A and C92A/C522A/C525A, Fig. 12, red and purple circle, respectively) have almost no ability to incorporate the copper ions from the cytoplasm of *E. coli*. Thus, it could be concluded that Cys92 residue as well as the C522xxC525 motif contributes to the copper-incorporation of *melB* apo-pro-tyrosinase (Fig. 2A → B).

From these results, we concluded that the C-terminal shielding domain functions not only to control the enzyme activity by prohibiting the substrate access but also to induce the copper-incorporation into the active site like copper chaperone. The completely disordered region of this shielding domain (loop 3) exists near CuA site and contains the two highly flexible cysteines (Cys522 and Cys525) within the distance range of about 5 – 15 Å from the sulfur atom of Cys92. This structural feature is reminiscent of the action of copper chaperones. The C522xxC525 motif may act as the linear bidentate ligand to copper (I) (Fig. 13B) and serve as a copper shuttle to Cys92, where the copper(I) ion may be transiently ligated by three cysteine residues (Fig. 13B → C). Another copper(I) ion may be incorporated in the similar manner to produce a dicopper(I) form of the reduced enzyme (Fig. 13D → E). During this process, Cys92 may switch the conformation between Cys92a and Cys92b, enhancing the copper(I) transport into CuA binding site (Fig. 13C → E). From the dicopper(I) species thus constructed (Fig. 13E), (μ-η¹:η¹-peroxido)dicopper(II) species is generated by the reaction with dioxygen (Fig. 13F). As demonstrated in our previous study, the His-Cys cross linkage formation proceeds autocatalytically involving the formation of this active species as a key intermediate to produce the met-pro-tyrosinase (dicopper(II), Fig. 13G) (22). As a result, two copper ions can be transported into the active site by the contribution of these cysteines (Cys92, Cys522, and Cys525).

**Maturation process of MelB tyrosinase**—In this study, we have obtained the detailed structural information of fungal pro-tyrosinase containing shielding C-terminal domain, which covers the entrance of the active site. We have confirmed that three cysteines (Cys92, Cys522, and Cys525) play significant roles in the copper incorporation process. Fungal tyrosinase exploits the thiol group of Cys92 for the metal incorporation as well as enhancement of the enzymatic activity as described in our previous paper (22). In this context, the His–Cys forming cysteines found in octopus hemocyanin and sweet potato catechol oxidase may also participate in the copper incorporation process (Fig. 1A and B). Although CxxC motifs like fungal tyrosinase are not conserved on the C-terminal domain of these proteins, we were able to find a disulfide bond in the proximity of their His–Cys cross-linkage (within 10 Å distance) on the copper-binding domain in each crystal structure (octopus hemocyanin, Cys2549-Cys2559 and sweet potato catechol oxidase, Cys27-Cys89), indicating that free thiol form of these disulfides may have similar functions to that of C522xxC525 motif of *melB* tyrosinase. Besides Cys92, two cysteines (Cys522 and Cys525) on the C-terminal domain are highly conserved among any fungal tyrosinases (Fig. 4), whereas the specific partner protein for tyrosinase as a copper chaperone has not been found in the fungi yet. Therefore, the...
fungal tyrosinase may assemble the dinuclear copper center by itself by using the internal three cysteines, directly transferring from copper permeases on the cytoplasmic membrane such as Ctr1/3 homologues (15) without the mediation by the external copper chaperones (Fig. 2A → B). However, the exact interaction between tyrosinase and such copper permeases are not known; therefore, the detailed molecular mechanism of the copper transfer between these proteins needs further investigation.

We have examined that the trypsin-treatment induces the activation of melB holo-pro-tyrosinase and explored its activation mechanism (Fig. 2B → C); the tryptic digestion induced cleavage of the C-terminal domain (Glu458-Ala616) with keeping the dimeric structure of the enzyme (23). As mentioned above, the interactions between subunits are located in the copper-binding domain predominantly, indicating that the dimeric structure retains after cleavage of C-terminal domain involving activation process. The core structure of copper-binding domain is almost identical to that of the active form of mushroom tyrosinase (PPO3) as well as catechol oxidase. Therefore, we could conclude that this cleavage process induces little conformational change of the active site but simply cause the removal of Phe513 from the active site (Fig. 9). To our knowledge, no protease specific to tyrosinase has been discovered yet, but the proteases with trypsin-like activity are common in fungi (32). Thus, such an enzyme may be involved in this proteolytic activation process of fungal tyrosinase (Fig. 2B → C).
REFERENCES


Structural insights into tyrosinase maturation


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FOOTNOTES
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1 To whom correspondence should be addressed: Shinobu Itoh, Department of Material and Life Science, Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan, Tel.:+81-6-6879-7932; Fax: +81-6-6879-7935; E-mail: shinobu@mls.eng.osaka-u.ac.jp and Genji Kurisu, Institute for Protein Research, Osaka University, 3-2 Yamada-oka, Suita, Osaka 565-0871, Japan, Tel.:+81-6-6879-8604; Fax: +81-6-6879-8606; E-mail: gkurisu@protein.osaka-u.ac.jp
1 The abbreviations used are: CcO, cytochrome c oxidase; DβH, dopamine β-hydroxylase; PAM, peptidylglycine α-hydroxylating monoxygenase; pMMO, particulate methane monoxygenase; 2,3-QD, quercetin 2,3-dioxygenase; AO, copper amine oxidases; GO, galactose oxidase; Cu/Zn-SOD, Cu/Zn superoxide dismutase; Ctr, copper membrane transporter; PPO3, polyphenol oxidase 3.

FIGURE LEGENDS

FIGURE 1. Three-dimensional structures of the dinuclear copper sites containing a His-Cys cross linkage. (A) Potato catechol oxidase (PDB: 1BT1) and (B) octopus hemocyanin (PDB: 1JS8). (C) A ChemDraw structure of 2-cysteinyl-histidine (His-Cys) cross linkage.

FIGURE 2. The schematic representation of fungal tyrosinase maturation process.

FIGURE 3. Whole structure and dimer interfaces of melB holo-pro-tyrosinase. (A) Homodimeric structure in the crystallographic asymmetric unit (Upper, top view; Middle, side view; Bottom, bottom view). Subunit interfaces of the dimeric structure have two regions at the dimer interface, as indicated by red (Region I) and green (Region II) rectangles. Two same interactions of Region II (green) exist in the interface (Upper) along a two-fold axis. The secondary structures are colored differently according to individual chains: chain A, blue; chain B, pink. (B) The magnified view of the two regions of dimer interface. Residues are shown as sticks. Dotted lines indicate closest contacts between atoms.

FIGURE 4. Multiple sequence alignment of amino acid sequences of fungal tyrosinase and secondary elements of melB (α for α-helix, α′ for 3₁₀-helix, π for π-helix and β for β-strand). The alignment was generated with ClustalW by using MEGA5 software. The conserved residues are marked with an asterisk and highlighted in rectangles (Green, Cu-coordinating histidines; red,
conserved cysteines; yellow, the placeholder; purple, YxY motif; gray, YG motif). The dashed line indicates residues not visible in the electron density. A.oryzae B: melB from Aspergillus oryzae (BD165761); A.bisporus: PPO3 from Agaricus bisporus (CAA59432); N.crassa: Neurospora crassa (CAE81941); P.nameko: Philiota nameko (AB275647); P.sanguineus: Pycnoporus sanguineus (AAX46018); L.edodes: Lentinula edodes (BAB71735).

FIGURE 5. Interaction between the flexible loop and the next protomer related by the crystallographic symmetry. (A) Two homodimeric structures of holo-pro-form related by the crystallographic symmetry. Colored loops in this circle are loop1 (Lys82-Lys85, red), loop2 (Ser213-Phe223, yellow), and loop3 (Ser516-Gly532, green) of holo-pro-form chain A. (B) Magnified view on the contacting region indicated by dotted rectangle. Residues are shown as. Dotted lines and small dashed line indicate the closest contacts between atoms, and the long dashed line indicates residues not visible in the electron density.

FIGURE 6. Crystal structure of protomer of melB holo-pro-tyrosinase. (A) Structure of the chain A molecule. The copper-binding domain (domain I, Ser1–Phe463) is colored in blue; the C-terminal domain (domain II, Gly464–Ala616) in red. Putative proteolytic site (Lys457) is labeled and shown in orange color. The N-terminus and C-terminus are indicated as N and C, respectively. The black arrow indicates Phe513 from the C-terminal domain covering the active site. (B) Active site structure viewing from the C-terminal domain. Phe513 is shown as a stick model colored in yellow. Dotted lines indicate closest contacts between atoms. (C) Superimposed structure of the copper-binding domain (blue) vs. mushroom tyrosinase (PDB: 2Y9W) (gray). (D) Superimposed structure of active site structure of melB (cyan) vs. mushroom tyrosinase (PDB: 2Y9W) (gray). (E) Superimposed structure of whole structure of melB (the copper-binding domain in cyan, and the C-terminal domain in red) vs. Octopus hemocyanin (PDB: 1JS8) (gray and yellow). (F) Superimposed structure of active site structure of melB (cyan and red) vs. Octopus hemocyanin (PDB: 1JS8) (gray and yellow). Green and red spheres indicate copper and water, respectively. Residues are shown as sticks.

FIGURE 7. SDS/12.5 % polyacrylamide gel electrophoresis. Precipitation (1, 3, 5, and 7) and supernatant (2, 4, 6, and 8) of cell extract of E.coli containing melB expression plasmid. Lane M, marker; lane 1 and 2, wild type; lane 3 and 4, V359A; lane 5 and 6, H332A; lane 7 and 8, F513A. Arrows indicates the bands of melB pro-tyrosinase.

FIGURE 8. The structure of C-terminal domains and caddie protein. (A) Structure of the C-terminal domain of melB holo-pro-tyrosinase. (B) Structure of the secondary domain of the functional unit of Octopus hemocyanin (PDB: 1JS8). (C) Structure of the caddie protein of S. castaneoglobisporus tyrosinase (PDB: 1WX2). (D) Superimposed structure of the C-terminal domain of melB (red) vs. shielding domain of octopus hemocyanin (PDB: 1JS8) (yellow). The dotted line indicates the residues not visible in the electron density.

FIGURE 9. Structure of the linker region between the core and C-terminal domains. Location of the proteolytic site in the molecule. Magnified view of the C-terminal region of the copper-binding domain shows the interactions between YG-motif and core region. Dashed lines show hydrogen bonds.

FIGURE 10. Crystal structure of melB apo-pro-tyrosinase. (A) Homodimeric structure of the apo-pro-form (gray and dark gray). (B) Subunit structures of apo-pro-form (chain A, gray) vs.
holo-pro-form (chain A, cyan, copper-binding domain; red, C-terminal domain). Dotted lines indicate the disordered loops colored in black and red (left) and dashed lines indicate disordered region (right). (C) Magnified view on the active site region of apo-pro-form (chain A, gray) and holo-pro-form (chain A, transparent cyan and sphere) indicated by the green rectangle. Green and red spheres indicate copper and water, respectively. Residues are shown as sticks and colored by atom type (carbon, as the respective structural element; nitrogen, blue; oxygen, red).

FIGURE 11. **Structural comparison of the holo-pro-form and apo-pro-form of melB.** (A) Free Cys92 and His94 side chains of apo-pro-form and (B) His94-Cys92 linkages of holo-pro-form covered with electron density map (σ level = 1.5). (C) Superimposition of apo-form chain A (gray) and holo-form chain A (blue). Dashed rectangle indicates the disordered region of apo-pro-form chain A. Colored loops in this rectangle are loop1 (Lys82-Lys85, red), loop2 (Ser213-Phe223, yellow), and loop3 (Ser516-Gly532, green) of holo-pro-form chain A. Dotted lines indicate the residues not visible in the electron density. (D) Superimposed structure of the active site region of apo-pro-form (chain A, gray) and holo-pro-form (chain A, blue and sphere). Residues are shown as stick models. Dotted lines indicate the closet contacts between atoms. Green spheres indicate copper ions.

FIGURE 12. **Copper content vs. CuSO₄ concentration.** Copper content of purified pro-tyrosinase from *E.coli* cultivated in the medium containing various concentration of CuSO₄. Wild type, black; C92A, red; C522A, blue; C525A, green; C522A/C525A, orange; C92A/C522A/C525A, purple.

FIGURE 13. **Schematic representation of putative copper incorporation process by pro-tyrosinase.**
### Table 1. Oligonucleotides used for site-directed mutagenesis

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\(^a\)The changed nucleotides are shown in capitals, and the codons corresponding to the amino acid residues to be changed are underlined.
### Table 2. Data Collection and Refinement Statistics

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*Values in parentheses are for the highest resolution shells*
### Table 3. Missing residues and atoms

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FIGURE 1
FIGURE 2

A C-terminal (Shield) Domain

Apo-pro-tyrosinase

Cu-binding Domain

Holo-pro-tyrosinase

Active tyrosinase

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FIGURE 3
Structural insights into tyrosinase maturation

FIGURE 5

A

Chain A

Loop1

Loop2

Loop3

Chain B

A'

B

K524

C525

C522

D492

D523

N552
Structural insights into tyrosinase maturation

FIGURE 7
FIGURE 11

Structural insights into tyrosinase maturation
FIGURE 12
SCHEME 1

\[
\begin{align*}
\text{Hydroxylation} & \quad \text{Phenolase Activity} \\
\text{Oxidation} & \quad \text{Catecholase Activity}
\end{align*}
\]
Crystal Structures of Copper-depleted and Copper-bound Fungal Pro-tyrosinase: Insights into endogenous cysteine-dependent copper incorporation
Nobutaka Fujieda, Shintaro Yabuta, Takuya Ikeda, Takuji Oyama, Norifumi Muraki, Genji Kurisu and Shinobu Itoh

J. Biol. Chem. published online June 7, 2013

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