Roles of Copper Ligands in the Activation and Secretion of Streptomyces Tyrosinase*

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The expression of the melanin operon (melC) of Streptomyces antibioticus requires the chaperone-like protein MelC1 for the incorporation of two copper ions (designated as CuA and CuB) and the secretion of the apotyrosinase (MelC2) via a transient binary complex formation between these two proteins. To investigate whether the copper ligand of tyrosinase is involved in this MelC1-MelC2 binary complex function, six single substitution mutations were introduced into the CuA and CuB sites. These mutations led to differential effects on the stability, copper content, and export function of binary complexes but a complete abolishment of tyrosinase activity. The defects in the tyrosinase activity in mutants were not because of the impairment of the formation of MelC1-MelC2 complex but rather the failure of MelC2 to be discharged from the copper-activated binary complex. Moreover, the impairments on the discharge of the mutant MelC2 from all the mutant binary complexes appeared to result from the structural changes in their apoforoms or copper-activated forms of the complexes, as evidenced by the fluorescence emission and circular dichroism spectral analysis. Therefore, each of six copper ligands in Streptomyces tyrosinase binuclear copper sites plays a pivotal role in the final maturation and the discharge of tyrosinase from the binary complex but has a less significant role in its secretion.

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase that catalyzes both the O-hydroxylation of monophenols and the oxidation of O-diphenols to O-quinones (1, 2). This enzyme is ubiquitous and is responsible for the biosynthesis of melanin from tyrosine (2). The primary structures of tyrosinase from Streptomyces (3–5), Neurospora crassa (6), Rana nigromaculata (7), Mus musculus (8, 9), and Homo sapiens (10, 11) have been determined and exhibit considerable heterogeneity. However, the catalytic domain of this enzyme from different sources all has a single binuclear copper center. In the last 20 years, substantial progress has been made to elucidate the role of the active site copper center involved in catalysis (for reviews see Refs. 2 and 12). Apart from the Streptomyces tyrosinase, the mechanisms by which copper ions incorporated into the various sources of apotyrosinase are largely unknown (13).

The structural gene (melC2) for the tyrosinase of Streptomyces antibioticus (14) or Streptomyces glaucescens (15) is part of a polycistronic operon (melC1), preceded by the melC1 gene, which encodes a conserved protein essential for the expression of melanin in Streptomyces (3, 14, 16). In a series of investigations, our results showed that the MelC1 protein plays the dual roles of regulating copper incorporation and promoting the secretion of apotyrosinase via a transient MelC1-MelC2 complex (16–19). Evidence was also provided that indicated a conformational transition of MelC2 during the copper activation (19). This function of MelC1 is reminiscent of that of the molecular chaperone involved in protein folding, assembly, secretion, and heat shock responses (20–22).

To gain insights into the molecular mechanism of the copper activation process in Streptomyces apotyrosinase, we recently set out to study the structure-function relationship of MelC1. Our results suggested that the signal peptide region as well as the histidine residues 102 and 117 of MelC1 played important roles in the activity of MelC1 (18, 19, 23). Their mutations affected either the copper incorporation process or the release of tyrosinase into the medium. To further delineate the copper metallocenter assembly process of the Streptomyces tyrosinase, we examined in this study the role of copper-binding ligands of tyrosinase in the activation of MelC1-MelC2 binary complex. Our results indicate that all copper ligands are crucial for the activation of tyrosinase. Their lesions result in the formation of defective binary complexes that severely affect the discharge of MelC2 from the complexes and also lead to a moderate effect on the secretion of MelC1 or MelC2 in some mutants.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Streptomyces lividans TK64 (SLP2, SLP3, pro-2, str-6) (24) was used as the host for recombinant plasmids. Plasmids pIJ702 (14) containing the thiostrepton resistance determinant (tsr) and the melanin operon (melC) was kindly provided by Prof. E. Katz (Georgetown University). Plasmid pIJ702-117 is a derivative of pIJ702 carrying a mutation in the upstream regulatory region of the melC operon that results in the overexpression of the melC operon (25). Standard media, culture conditions, and transformation procedures for Streptomyces were previously described (26).

Plasmid Construction and in Vitro Mutagenesis—The Altered Site system (Promega) was used for in vitro mutagenesis of the melC2 gene as specified by the manufacturer. Plasmid pSELC2 was constructed by subcloning the 1.4-kilobase SatI-EcoRV fragment of melC2 from pIJ702, into the SstI/SmaI digested pSELECT-1. Single-strand DNA from pSELC-2 was used as a template for site-directed mutagenesis. Oligonucleotides designed for site-directed mutagenesis are shown below. Each primer was designated by the position of the mutated amino acid in the MelC2 protein and by the one-letter symbols for the amino acids before and after the mutation. The mutated bases are shown in boldface and underlined type. All mutations were confirmed by DNA sequencing using the chain-termination method (27): H37Q, -TACACCAAGCA-GAACGCGTTCTC-3'; H53Q, -CACGCAGCCACTGATCCGCTG-3'; H62N, -CTGCTCTGAGACCGGATTTC-3'; H189Q, -GTCGAA-TCTGCAAGACGGGTTG-3'; H193Q, -CGGGGAGGTCTGCGG-3'; and H215Q, -GGCTGACACCGCTCAGC-3'. Construction of plIJ702-117 Mutant Derivatives—The 1.18-kilobase SatI-PvuII fragment containing each of the melC2 mutations on
pSEL2 derivatives was isolated and used to replace the corresponding segment on plj702-117. The derivatives produced were designated plj702S-H37Q, plj702S-H53Q, plj702S-H62N, plj702S-H189Q, plj702S-H193Q, and plj702S-H215Q, respectively.

**Analysis of Copper Ligands and Immunoblotting Analysis**—S. lividans TK64 harboring plasmid pIJ702-117 or its mutant derivatives were cultured in TSB medium (Difco) (50-ml culture) in the absence or presence of copper ion (100 μM) for 24 h at 30 °C. Preparation of mycelial extracts and culture supernatants, the assay of tyrosinase activity, and the detection of MelC1 and MelC2 proteins by immunoblot were described previously (18). The immunoblot was detected by the enhanced chemiluminescence method (ECL system, Amersham Pharmacia Biotech) using the horseradish peroxidase-conjugated antibody as the secondary antibody (23). The protein contents of the samples were determined by the Bradford method (28) using bovine serum albumin as the standard.

**Immunooaffinity Chromatography of the MelC1-MelC2 Complex**—The immunoochromatography of the MelC1-MelC2 complex has been described elsewhere (17, 18). Briefly, the culture supernatants (1 ml) were incubated with anti-MelC1 antibody resin (volume 150 μl), which had been pre-equilibrated with buffer A (0.1 M sodium phosphate buffer (pH 7.2), 0.25 M NaCl). After extensive washing with buffer A, protein was eluted with 0.1 M glycine-SCN buffer (pH 2.8). The collected fractions (volume, 200 μl) were immunoblotted with anti-MelC1 or anti-MelC2 antisera.

**The Effects of Copper Content**—The MelC1-MelC2 complex and tyrosinase were purified from the culture supernatants as described previously (17), and their copper contents were analyzed by using a polarized Zeeman effect atomic absorption spectrometer (Hitachi model Z-8200) (17). The detection sensitivity for the copper ion was in the range of 0.5–40 parts/billion.

**Fluorescence Spectroscopy**—The emission spectra of the purified MelC1-MelC2 complex (10 μg/ml) and its copper-activated form were measured at room temperature in a fluorometer (Hitachi model F-4010) with an excitation wavelength of 280 nm (excitation bandpass, 3 nm; emission bandpass, 10 nm).

**Circular Dichroism Spectroscopy**—All far-UV spectra were collected on a AVIV 60DS spectropolarimeter (AVIV Associates, Inc., Lakewood, NJ) in a 1-cm light path cell at 20 °C. Data were collected at a protein concentration of 1–2 μM. All protein samples were dialyzed against 5 mM sodium phosphate buffer (pH 7.2). Mean residue ellipticity, [θ]MRW (degree cm² dmol⁻¹), was determined from the formula [θ]MRW = ε/100n, where ε is the measured ellipticity in millidegrees, C is the protein concentration in mol/liter, l is the path length of the cuvette in cm, and n is the number of amino acid residues in the protein (29, 30).

**RESULTS**

**Alteration of the MelC2 Copper Ligands Blocked the Phoentypic Melanin Formation but Moderately Affected the Export of MelC1 or MelC2**—According to previous reports (4, 5, 31), the S. glaucescens tyrosinase contains 2 atoms of copper, CuA and CuB. His37, His52, and His62 are assigned to be the copper ligands for CuA, whereas His189 and His193 are for the CuB site (4, 5, 31). Because these copper ligands are also conserved in the S. antibioticus tyrosinase (Ref. 3 and Fig. 1), they may serve identical functions. In this study, we substituted each of these six histidine residues in MelC2 of the operon with a noncoordinating glutamine or asparagine residue using site-directed mutagenesis. Six such mutants were obtained: 1) His37 to Gln37 (mutant H37Q), 2) His53 to Gln53 (mutant H53Q), 3) His62 to Asn62 (mutant H62N), 4) His189 to Gln189 (mutant H189Q), 5) His193 to Gln193 (mutant H193Q), and 6) His215 to Gln215 (mutant H215Q).

When examined for melanin production on R2YE agar plates (containing 0.05% tyrosine), all mutants displayed Mel phenotype and showed no detectable tyrosinase activity (data not shown). The loss of tyrosinase activity in these mutant strains was not because of the reduction of intracellular MelC2 protein under different culturing conditions (with or without 100 μM copper ion) (Fig. 2, A and B). On the contrary, an increase of intracellular MelC2 protein (106–160% of wild type) was observed in all mutants except H53Q, where a slight reduction of MelC2 (78% of wild type) was observed when cultivating in the absence of copper supplement (Fig. 2A). Intriguingly, unlike MelC2, a completely different MelC1 expression pattern for the wild type and mutant strains was noted, depending on the culture condition. When cultured in supplemental copper ion (100 μM), both the intra- and extracellular levels of MelC1 in the wild type strain were markedly decreased as compared with those without copper ion supplement (compare lane W7 in Fig. 2B with the same lane in Fig. 2A). However, this is not the case for the mutant strains; their MelC1 levels for both cellular fractions under the copper ion supplement conditions accounted for a 5–17-fold increase over that of wild type, although in some cases such as those of the mutants H53Q, H62N, H189Q, and H193Q, their MelC1 levels in the absence of copper ion adversely decreased to 35–63% of the wild type (Fig. 2A).

The reduction of MelC1 in the wild type strain presumably resulted from the aggregation of MelC1 after released from the binary complex by the copper ion (17). The differential copper effect on the MelC1 levels in mutants as compared with the wild type may be indicative of a defect in their copper-activated complex. Moreover, the presence of multiple MelC1 species (14–15-kDa) in the intracellular fractions of the wild type and mutant strains (Fig. 2, A and B) might reflect the degradation or the different conformations of this intracellular protein as noted before (17–19). Additionally, quantitation of MelC1 and MelC2 exported by immunoblot suggested that in the wild type strain, approximately 56–73% of total cellular MelC1 and MelC2 were secreted to the medium, whereas in some mutants, especially mutants H53Q, H53Q, H62N, and H189Q, the secretion of MelC1 or MelC2 protein decreased to 60–77% of the wild type (Fig. 2, A and B). Thus, along with a block of tyrosinase activity, mutation of the copper ligand also elicited a moderate effect on the export of MelC1 or MelC2 protein.

**Effects of Copper Ligand Mutations on the Activation of MelC1-MelC2 Complex**—Our previous work (17) indicated that MelC1 forms a transient complex with apotyrosinase during the copper activation process and is discharged from the complex after the activation of tyrosinase. Because the copper ligand-defective mutants had lost their tyrosinase activity, it was likely that these mutations might have affected the binary...
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**Fig. 2.** Expression of MelC1 and MelC2 proteins in *Streptomyces* tyrosinase copper ligand-defective mutants. Streptomyces cultures were grown in TSB medium with (B) or without (A) supplemented copper ion (100 μM) for 24 h at 30 °C. The intracellular fractions were prepared by sonication. Extracellular proteins in the culture medium were precipitated by 5 volumes of cold acetone, and the recovered pellets were dissolved in the sampling buffer (39). Both fractions were analyzed in 13.5% PAGE containing 0.1% SDS and immunoblotted with both anti-MelC1 and/or anti-MelC2 antisera. Protein from 25 μl of cultures was applied to each lane. The positions for the MelC1 (C1) and MelC2 (C2) proteins are indicated with arrows. The band intensity (arbitrary units) of each MelC1 and MelC2 bands were determined densitometrically (Molecular Dynamic) and normalized to the wild type level. The percentages of secreted MelC1 and MelC2 were also indicated. W7, wild type, TK64 (pIJ702S-H215Q); H37N, TK64 (pIJ702S-H37Q); H53Q, TK64 (pIJ702S-H53Q); H62N, TK64 (pIJ702S-H62N); H189Q, TK64 (pIJ702S-H189Q); and H215Q, TK64 (pIJ702S-H215Q).

complex formation. Analysis of the MelC1-MelC2 complex formation in an anti-MelC1 antibody column showed that the MelC1 and MelC2 proteins from all the mutants cultured without copper ion supplement formed a complex like the wild type (Fig. 3, C and D, lanes marked with −). Nevertheless, when cultured in the presence of supplemented copper ion (100 μM), the MelC2 protein of all the mutant strains, unlike the wild type, was still retained by an anti-MelC1 antibody column (Fig. 3, C and D, lanes marked with +), suggesting that the mutant form of the binary complex was hardly dissociated by the added copper ion. The lack of retention of wild type MelC2 by the anti-MelC1 antibody column was not because of the lower quantity or absence of MelC2 after copper ion activation, because essentially the same amount of MelC2 was present in the loading or flow-through fraction from the wild type and mutant strains (Fig. 3, A and B). It was more likely because of the fact that MelC2 from the wild type was dissociated from the binary complex after copper activation (17).

This conclusion was further supported by fast protein liquid chromatography (FPLC)1 analysis of the *in vitro* copper-activated binary complex (Fig. 4). Although the purified wild type binary complex (retention time, 34.9 min) displayed a discharge of MelC2 (retention time, 36.6 min) from the complex after copper ion addition, no such phenomenon was observed in the complexes derived from the mutants. Apart from mutant H189Q, the retention time of all mutant binary complexes was identical to that of the wild type (34.9 min) and remained unchanged after copper addition. The retention time (36.3 min) for the H193Q binary complex was also independent of copper ion; however, its value was closer to that of MelC2 (Fig. 4, panel H193Q). This abnormal behavior of the H193Q binary complex was not because of the discharge of MelC2 from the complex (immunoblot analysis not shown) but rather implied a substantial change of this particular complex conformation.

Additionally, SDS-PAGE and immunoblot analysis showed that after *in vitro* copper activation, the wild type MelC1 markedly decreased as compared with that without copper activation, whereas the MelC1 of all the mutant forms remained the same (Fig. 5). Notably, this differential copper effect on the MelC1 of the wild type and mutants was in accord with the *in vivo* data (Fig. 2). Taken together, both *in vivo* and *in vitro* experiments strongly suggested that the copper activation process was defective in these copper ligand-defective mutants in such a way that the mutant MelC1 or MelC2 apparently could not be released from the complex after copper activation.

Analysis of the Copper Contents in the Binary Complexes of the Copper Ligand-defective Mutants—The failure to resolve the MelC2 from the copper-activated complexes of the mutants may be because of the mutational influence of the copper incorporation into the complexes. To assess this possibility, the copper contents of the purified binary complexes and their *in vitro* copper-activated species were examined. The purified complexes of the wild type and mutant strains contained essentially no copper ion (less than 0.09 atom/molecule) (Table I). *In vitro* activation led to the incorporation of approximately 2 atoms of copper/molecule of the complex in wild type, H189Q, and H215Q but only 1 atom of copper in the other four mutants. Therefore, whereas mutation at each of copper ligands in CuA site expectedly blocked copper incorporation into the CuA site, mutation at His189 and His215 in the CuB site did not affect the copper incorporation.

Analysis of the Copper-induced Conformational Change in the Binary Complexes of Mutant Strains—Because the binary complexes of all the mutants had copper ion(s) incorporation, their defects in the discharge of MelC2 after copper activation might instead result from the incompetent conformational change during copper activation. To examine this possibility, intrinsic fluorescence spectroscopy in combination with CD spectroscopy was used to probe conformational changes of the binary complexes from the wild type and mutant strains. The MelC1-MelC2 binary complex contains 37 aromatic amino acid residues, of which 30 are in the MelC2 protein (tryptophan 12, tyrosine 6, and phenylalanine 12). Approximately one-third of the aromatic amino acid residues (tryptophan 5, tyrosine 2, and phenylalanine 6) are located around the bimolecular copper sites in MelC2 (Fig. 1). We envisioned that the conformational change elicited either by copper ion incorporation or by the mutational effect might be revealed by the changes in the intrinsic fluorescence emission spectra. Fig. 6B showed that...
The positions for the MelC1 (for mutant strains are identical to those described in the legend of Fig. of protein were applied to each lane of the same panel. All designations MelC1 and/or anti-MelC2 antisera. For comparison, the same amounts cated. The presence of dimeric form of MelC1 in the wild type sample (17). The loaded samples (A), the flow-through (B), and the bound fractions (C and D) were analyzed by SDS-PAGE and immunoblotted with anti-MelC1 and/or anti-MelC2 antisera. For comparison, the same amounts of protein were applied to each lane of the same panel. All designations for mutant strains are identical to those described in the legend of Fig. 2. The positions for the MelC1 (C1) and MelC2 (C2) proteins are indicated. The presence of dimeric form of MelC1 in the wild type sample (marked with (C1)2 in lane WT of C) is consistent with previous results (17).

the intrinsic fluorescence emission intensity (excitation at 280 nm, maximum emission at 337–339 nm) of the wild type binary complex was quenched by 30% as a result of copper insertion. A similar finding was reported for the tyrosinase of Neurospora, for which an approximately 60% quenching was found upon copper insertion (32). Notably, the complexes from all the mutants except H53Q were similar quenching ranging from 35 to 48% after copper insertion (Fig. 6B). In contrast, a 1.5-fold enhancement of the fluorescence emission intensity was observed in the H53Q complex after copper activation. This indicated that the coordinated environment of the copper center in all the mutants, except H53Q, is similar to that in the wild type during copper insertion. However, none of intrinsic fluorescence emission intensity of the apoform of the mutant binary complex is identical to that of the wild type (Fig. 6A). All their fluorescence intensities were enhanced (1.1–1.6-fold of the wild type) and had a maximum enhancement in mutants H62N and H193Q, suggesting that the native conformation of the mutant binary complex is perturbed by mutation. Thus, the fluorescence spectroscopy study suggested that although copper insertion into the mutant binary complex yielded conformational changes, the conformations of the apoforms from these mutant complexes were distinct from that of the wild type, which might result in a defect in the copper activation process.

To ascertain whether the defect in copper activation of these mutants was because of gross structural perturbations, CD experiments were carried out. Fig. 7 showed the CD spectra for the mutants in the presence and absence of copper ion, which are superimposed with the spectra of the wild type binary complex in Fig. 8. The far-UV spectra (200–250 nm) of the wild type apoform was characterized by two minima with negative ellipticity near 208 and 230 nm, suggesting the presence of helical and other nonhelical structures including ß-turn and random coil (29, 30) (Fig. 7, panel WT). Apart from the intensity differences, these features were also present in the spectra of all the mutant apoforms. However, the CD spectra of H37Q and H193Q apoforms showed significant deviation from that of the wild type (Fig. 8, A and C), indicating minor secondary structural changes in these two mutants. Addition of copper ion to the apoform of the wild type binary complex led to a small, discernible decrease in the negative ellipticity near 208–225 nm (Fig. 7, panel WT), reflecting a decrease of helical content in the binary complex with a concomitant increase of other structures after copper activation (29, 30). When compared with the wild type, the mutants were less likely to show copper-induced spectral changes. This is especially the case for mutants H37Q, H62N, H189Q, and H215Q (Fig. 7). Only the mutant complex of H53Q or H193Q exhibited a significant copper-induced CD spectral change near the 220–235 nm (H53Q) or 200–220 nm (H193Q) region (Fig. 7). Furthermore, when compared with the apoform, the CD spectra of all the copper-activated mutant binary complexes differed considerably from that of the wild type (Fig. 8, compare panels B and D with panels A and C), suggesting that none of the copper-activated forms of mutant strains displayed the same conformational state as the wild type. Therefore, our results showed that although copper insertion into the mutant form of the binary complex might induce a structural change in certain mutants, these changes appeared dissimilar to those in the wild type. This incompetent binary complex might lead to the failure of the resolution of MelC2 from the copper-activated complex and of the production of active tyrosinase.

DISCUSSION

Tyrosinases are evolutionarily diverse enzymes, yet all of them possess a binuclear copper center consisting of six essential copper ligands. Of particular interest is the degree of equivalence of these two copper sites (CuA and CuB) and the mechanism of copper incorporation into this enzyme. Much of the available information on copper incorporation into tyrosinase has come from the studies of the Streptomyces system, the smallest representative of this class of enzyme known so far (13). In the present working model for the activation of Streptomyces tyrosinase, MelC1 functions as a chaperone that maintains the state of apotyrosinase that facilitates copper incorporation and secretion (17–19). Earlier works have shown that the alterations of either the signal peptide or several particular histidine residues (e.g. 102 and 117) of MelC1 affected the stability of the MelC1-MelC2 binary complex, resulting in a loss of tyrosinase activity or its secretion (18, 19, 23). Although these studies have delineated the role of the MelC1 in activation and secretion of tyrosinase, the relative importance of MelC2 in these two aspects remains uncharacterized.

In this study, we examined the role of copper ligands of MelC2 in the formation of the functional binary complex by introducing a single substitution mutation into each of six putative copper ligands in the CuA or CuB site of S. antibioticus tyrosinase (Fig. 1). All the mutants displayed Mol− phenotypes and had lost their tyrosinase activity. This result strongly
suggestions that these conserved histidine residues serve the same copper-binding function in *S. antibioticus* tyrosinase as in *S. glaucescens* tyrosinase. In fact, analysis of the copper contents of all copper ligand-defective mutants from these two tyrosinases supports this view. The tyrosinases from His\(^{189}\), and His\(^{215}\)-defective mutants of *S. glaucescens* and *S. antibioticus* all contain 2 copper atoms, whereas their Cu\(_A\) site and His\(^{193}\) mutations result in the loss of 1 copper atom (Ref. 31 and this work). This also implies that the lability of copper-histidine prosthetic group in the two copper sites of tyrosinase are different from one another. Conceivably, the two copper sites are structurally and functionally nonequivalent. Along this line, there are several other clues to support this view (also see Ref. 13). A comparison of amino acid sequences of tyrosinases from various sources, including *Streptomyces* (3, 4), *Neurospora* (6), *R. nigromaculata* (7), and mammals (8, 11) with a related, copper protein hemocyanin (33), shows strong sequence homology in regions containing the Cu A site (Fig. 1). In addition, the differential reactivity of two copper sites in mushroom tyrosinase with citrate, oxalate, or copper ion is in agreement with the different reactivities of the two copper sites of *S. antibioticus* tyrosinase. The data shown in this table represent the means ± S.D. of two to three determinations.

**TABLE I**

Copper content of the purified MelC1-MelC2 complex and its in vitro copper-activated products from wild type and copper ligand-defective mutant strains

| Sample | Protein concentration (nM) | Copper concentration (atom/molecule protein) |
|--------|---------------------------|---------------------------------------------|
| Tyrosinase | 25 | 51.3 ± 6.3 | 2.05 ± 0.25 |
| Wild type | 35 | 2.0 ± 1.4 | 0.06 ± 0.04 |
| H37Q | 120 | 11.3 ± 0.6 | 0.09 ± 0.01 |
| H53Q | 120 | 2.5 ± 0.3 | 0.04 ± 0.01 |
| H62N | 120 | 9.3 ± 2.8 | 0.08 ± 0.02 |
| H189Q | 60 | 0.63 ± 0.0 | 0.01 ± 0.00 |
| H193Q | 120 | 8.19 ± 0.5 | 0.07 ± 0.00 |
| H215Q | 120 | 16.1 ± 0.5 | 0.13 ± 0.00 |
| Wild type + Cu\(^+\) | 35 | 81.4 ± 8.7 | 2.33 ± 0.25 |
| H37Q + Cu\(^+\) | 120 | 110.2 ± 3.8 | 0.92 ± 0.03 |
| H53Q + Cu\(^+\) | 60 | 65.5 ± 5.2 | 1.09 ± 0.09 |
| H62N + Cu\(^+\) | 120 | 163.1 ± 5.4 | 1.36 ± 0.05 |
| H189Q + Cu\(^+\) | 60 | 136.2 ± 15.4 | 2.27 ± 0.26 |
| H193Q + Cu\(^+\) | 120 | 109.8 ± 1.1 | 0.92 ± 0.01 |
| H215Q + Cu\(^+\) | 120 | 283.1 ± 7.7 | 2.36 ± 0.06 |

The binary complex was incubated in 100 \(\mu\)M copper sulfate at 4 °C for 24 h, and the resulting mixture was dialyzed extensively against the distilled water.
with this notion (34–36). Finally, our observation of the differential impairment on the function or property (e.g. copper content, stability, secretion, or conformational status) of certain mutant binary complexes (e.g. H37Q, H53Q, and H193Q) can be ascribed to differential importance of copper ligands in maintaining the structural or functional integrity of the two copper sites.

If the two copper sites of Streptomyces tyrosinase are distinct from each other as inferred from our present study, such a feature of distinction may be also applicable to its copper activation process. If this is the case, it is pertinent to know whether the insertion of a copper ion into only one site (presumably the higher affinity one) is sufficient in eliciting a discharge of MelC2 from the complex. This working model for activation of apotyrosinase proposes the presence of MelC2 intermediate harboring only 1 atom of copper. However, our present study argues against this view. Although the introduced mutations in the CuA or CuB site resulted in differential levels of copper content (Table I), all mutants could not discharge MelC2 from their copper-activated complexes (Figs. 3–5), suggesting that neither one of the copper ion insertions alone can elicit a resolution of the MelC1-MelC2 binary complex and that insertion of two copper ions is necessary but not sufficient for the activation of tyrosinase. The defects of discharge of the mutant MelC2 from its binary complex appear to result from the alterations of the conformation of the binary complex, as evidenced by the changes in their fluorescence emission or CD spectra (Figs. 6–8). Therefore, an integrity of both copper centers is a determinant for formation and discharge of active tyrosinase. Although the two copper sites may have inherent structural and functional distinction, their roles in activation of tyrosinase are not distinguishable in this study. Interestingly, although these mutant proteins have deleterious effects on the copper activation process, most of their conformations are still competent for secretion (Fig. 2). This implicates that the binary complex conformation may exist in either activation or export subconformation, and presumably, the distinct features of these two subconformations are modu-
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Neurospora (37, 38) and mushroom (36). Involvement of copper metallothionein in the activation of the Neurospora tyrosinase has been suggested (37, 38). In mushroom, the differential kinetics for the incorporation of 2 copper atoms to the apotyrosinase has been found (36). The requirement of a chaperone protein MelC1 for the activation of Streptomyces tyrosinase raises the question as to whether this is also applicable to other tyrosinases. However, in view of the facts that 1) no MelC1 homologs have been found for other tyrosinase systems; 2) the apotyrosinase of Neurospora or mushroom can be readily reconstituted with copper ion in vitro without the presence of a chaperone protein (13, 38); and 3) the Streptomyces tyrosinase is the smallest representative of this class known so far, it is therefore likely that a MelC1-like chaperone function resides within the eukaryotic tyrosinase. In other words, a portion of the apoprotein sequence of these tyrosinases may perform similar functions as MelC1 for their copper center assembly. The fact that MelC1 can function as a cis-chaperone for activation of the apotyrosinase of Streptomyces supports this view. Therefore, the picture of the metallocenter assembly of tyrosinases from higher organisms may emerge through the identification of the protein moiety important for the copper incorporation into the active site of their respective apotyrosinases.

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Fig. 7. CD spectral change of MelC1-MelC2 complex during copper activation. Purified MelC1-MelC2 binary complex (1–2 μl) from each culture supernatant was incubated with or without 100 μM copper-sulfate for 24 h at 4 °C and then dialyzed against 5 mM phosphate buffer (pH 7.2) overnight. The resulting samples were analyzed identical to those described in the legend of Fig. 7.

Fig. 8. Comparison of the CD spectra of the apoform and copper-activated form of MelC1-MelC2 complexes from different strains. The CD spectra of the apoform (A and C) or copper-activated form (B and D) of CuA- or CuB-defective mutants were compared with those of wild type (WT). All data are from Fig. 7. All symbols are identical to those described in the legend of Fig. 7.
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