A Molecular Mechanism for Copper Transportation to Tyrosinase That Is Assisted by a Metallochaperone, Caddie Protein\(^*\)

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The Cu(II)-soaked crystal structure of tyrosinase that is present in a complex with a protein, designated “caddie,” which we previously determined, possesses two copper ions at its catalytic center. We had identified two copper-binding sites in the caddie protein and speculated that copper bound to caddie may be transported to the tyrosinase catalytic center. In our present study, at a 1.16–1.58 Å resolution, we determined the crystal structures of tyrosinase complexed with caddie prepared by altering the soaking time of the copper ion and the structures of tyrosinase complexed with different caddie mutants that display little or no capacity to activate tyrosinase. Based on these structures, we propose a molecular mechanism by which two copper ions are transported to the tyrosinase catalytic center with the assistance of caddie acting as a metallochaperone.

Among the many biological systems that employ transition metal ions for their function, it is a requirement that specific metal co-factors be transported into the correct metalloenzyme. Proteins known as “metallochaperones” play key roles in this process. Although a few structures of the metallochaperone have now been determined (1–6), the molecular mechanisms underlying specific metal transfers remain unclear with the exception of the metallochaperone involved in the copper transport to superoxide dismutase (1).

Tyrosinase (EC 1.14.18.1) belongs to a type-3 copper protein family harboring a catalytic center formed by dinuclear copper and catalyzes the ortho-hydroxylation of phenol and the subsequent oxidation of catechol to the corresponding quinone (7). The quinone product is a reactive precursor for the synthesis of melanin pigments. In mammals, this enzyme is responsible for skin pigmentation abnormalities, such as flecks and albinism (8). The development and screening of potent inhibitors of tyrosinase is of particular interest to the cosmetics industry. We have also recently reported from our laboratory that trilinolein contained in sake lees is a strong tyrosinase inhibitor (9).

Tyrosinase is classified into the same protein family as catechol oxidase and hemocyanin, although catechol oxidase lacks monoxygenase activity. Hemocyanin acts as an oxygen carrier in arthropods and mollusks. During catalysis, the type-3 copper center of tyrosinase adopts three redox forms (7). The deoxy form (Cu(I)-Cu(I)) is a reduced species, which binds dioxygen to yield the oxy form. In the oxy form, molecular oxygen binds in the form of peroxide in a \(\mu-\eta^{2}:\eta^{2}\) side-on bridging (Cu(II)-O\(_2\)\(^-\):Cu(II)) mode, which destabilizes the O–O bond and activates it. The met form (Cu(II)-Cu(II)) is recognized as the resting enzymatic form, where the Cu(II) ions are normally bridged with small ligands, such as water molecules or hydroxide ions. In these three redox forms of tyrosinase, the oxy form can catalyze both monoxygenase and oxidase reactions, whereas the met form lacks monoxygenase activity.

Many strains classified into the genus Streptomyces produce a melanin-like pigment (10). The melanin-synthesizing operon of Streptomyces antibioticus is composed of two genes that encode MelC1 and MelC2 proteins (11). It has been demonstrated that apotyrosinase (MelC2) forms a stable complex with MelC1 (12). Although apotyrosinase is not activated by copper added from the outside, the addition of copper ions to the purified complex gives rise to the incorporation of two copper ions. Furthermore, during the \textit{in vitro} activation of the MelC1-MelC2 complex, Cu(II)-bound MelC2 is discharged from the complex, but no trace of the released MelC1 protein is detectable. This suggests that the released MelC1 protein might form an aggregate to enable its separation from the protein complex. It is of interest to understand the molecular mechanism underlying the transactivation processes between MelC1 and MelC2.

We previously cloned a melanin-synthesizing gene from the chromosomal DNA of Streptomyces castaneoglobisporus HUT 6202 that produces a melanin pigment in high amounts. This gene forms an operon consisting of two cistrons (13), one being an open reading frame (ORF) consisting of 378 nucleotides designated \(orf378\) and the other a tyrosinase-encoding gene, designated \(tyrC\), which is located just downstream of \(orf378\). We refer to ORF378 as “caddie” because this protein may carry copper ions for tyrosinase.

We have succeeded in determining the three-dimensional structure of \(S.\) castaneoglobisporus tyrosinase in complex with \(\text{Cu(II)}\). The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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caddie is not only of interest from the viewpoint of structural biology but would also be useful in the design of novel tyrosinase inhibitors that block the maturation of these enzymes.

In our present study, structural changes in the active center of tyrosinase were crystallographically tracked by altering the Cu(II) soaking time. In addition, by preparing crystals of tyrosinase in complex with each of four caddie mutants whose capacity for the uptake of Cu(II) into the catalytic center is hindered, we were able to determine the copper-binding properties of these mutants. Based on these crystal structures determined at high resolutions, we propose a Cu(II)-transportation mechanism that is assisted by the tyrosinase-specific caddie protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Escherichia coli DH5α and BL21(DE3)-pLysS strains were used as hosts for cloning and protein expression, respectively.

Mutagenesis—The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate caddie mutants. The PCR primers used containing the desired single mutations (underlined) were as follows (sense only): 5'-CGGGCTGTCAGCTCGAGGTGATGCGAAGAC-3' (H82Q), 5'-GCGCTGTCAGCTGCTGGCAGAAGAC-3' (M84L), 5'-GCGCTGTCAGCCAGTACGACCCGGTGC-3' (H97Q), and 5'-GCGCTGTCAGCCAGTACGACCCGGTGC-3' (Y98F). The pET-orf378 plasmid (23) used for the expression of His6-tagged caddie, was amplified using sense and antisense primers. The original plasmid was removed by DpnI digestion, and the mutated plasmid was then amplified in E. coli. By using the mutated plasmid as a template, the region containing the T7 promoter and mutated orf378 gene was amplified with the forward primer, 5'-GCAC-GCATGGAAATTACGGACTACGACCTAC-3', and the reverse primer, 5'-CTATGACAAGCAAGACGTACGACCTAC-3' (the underlines in each case indicate the Spht site). The amplified fragment was digested with Spht and inserted into the same site in pET-tyrC (23), a plasmid used for the expression of His6-tagged tyrosinase, to generate a construct that coexpresses tyrosinase and a caddie mutant. A plasmid in which the direction of the tyrosinase and caddie genes is in the opposite orientation was chosen. The introduction of the mutation was confirmed by DNA sequencing analysis.

Preparation of the Complex—The overproduction and purification of tyrosinase complexed with wild type caddie or a caddie mutant was performed as described previously (23).

HPLC Analysis—Purified complexes (10 μM) were incubated in a 20 mM Tris-HCl buffer (pH 7.8) supplemented with 0.2 mM NaCl and 50 μM CuSO₄. After the aggregates generated in the samples were removed by centrifugation, the resulting supernatant fluid was applied to HPLC using Superdex 200 10/300 GL (GE Healthcare) equilibrated with a 20 mM Tris-HCl buffer (pH 7.8) containing 0.2 mM NaCl. The flow rate was set to 0.75 ml/min, and the elution profile was monitored at 280 nm.

Kinetic Analysis—The purified complexes (10 nM) were preincubated at 30 °C in a 10 mM sodium phosphate buffer (pH 4, 6, or 8) containing the given concentrations of CuSO₄. At specific times after a 1-ml portion of the solution was mixed with the same volume of a 100 mM sodium phosphate buffer (pH 6.28,
RESULTS

Effects of the Soaking Time on the Tyrosinase Activity Levels—The kinetics of tyrosinase complexed with the caddie protein were found to be multifaceted because the catalytic activity levels of this enzyme depended not only on the concentration of Cu(II) but also on the incubation time with the metal ions prior to the reaction. For the enzymatic kinetic study, the complex of tyrosinase with the caddie protein was preincubated for given times with CuSO4. HPLC analysis showed that, by incubation with Cu(II) at pH 7.8 for 2 h, a portion of tyrosinase was dissociated from the caddie protein as an active Cu(II)-bound form (Fig. 2A). On the other hand, it was difficult to observe the released caddie protein, probably due to the aggregation, as found in a previous study using the MelC1 protein and tyrosinase from S. antibioticus (12).

The oxidase activity of tyrosinase was measured using L-DOPA as a substrate (final concentration of 5 mM). The kinetics of tyrosinase complexed with the caddie protein after incubation with Cu(II) for 2 or 6 h, a 50-μL portion of the sample was injected into the column. B, time-dependent activation of tyrosinase by preincubation with 10 μM CuSO4. The effects of the pH of the incubation buffer were also investigated. Diamonds, squares, and triangles indicate the results obtained at pH 4, 6, and 8, respectively. C, Cu(II) requirement for the activation of tyrosinase. The purified complex was incubated at pH 6 or 8 with the given concentration of CuSO4, and the oxidase activity was then measured every 10 min. Maximal activities were plotted against the concentrations of CuSO4.

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the various concentrations of CuSO₄ every 10 min. It was found that when the concentration of Cu(II) was lower, a longer incubation time was necessary to attain the maximum activity. Fig. 2C shows the maximal activities plotted against the Cu(II) concentrations. The EC₅₀ values, which are defined as an effective concentration of Cu(II) producing 50% of the maximal oxidase activity, were determined to be 0.3 μM at pH 6 and 0.03 μM at pH 8. This also indicates that an alkaline pH is suitable for the transfer of Cu(II).

Effects of Soaking Time on the Manner of Cu(II) Binding—We have previously shown that one copper ion (designated CuA) in the catalytic center of tyrosinase is surrounded by His₃₈, His₅₄, and His₆₃ residues (14). Another copper (CuB) is surrounded by His₁₉₀, His₁₉₄, and His₂₁₆ residues. Fig. 3A shows the structure of the tyrosinase catalytic site obtained by soaking the crystal of the complex in a Cu(II)-containing solution for 6 months (Protein Data Bank code 2AHK). In the absence of the copper ion (Protein Data Bank code 1WXC), His⁵₄, which is a ligand of CuA, is clearly observed to adopt two conformations. One is similar to that found in the copper-binding state (B), and the side chain in the other conformation points toward the side chain of His⁹⁷ in the caddie protein (C).

**FIGURE 3. Structural models around the catalytic site of tyrosinase.** A, a structural model around the catalytic site of the met2 form of Cu(II)-bound tyrosinase is shown as a representative of Cu(II)-bound models. B, and C, structural models around the catalytic site of Cu(II)-free tyrosinase. As explained under “Results,” His⁵₄ of tyrosinase can adopt two conformations. One is similar to that found in the copper-binding state (B), and the side chain in the other conformation points toward the side chain of His⁹⁷ in the caddie protein (C).
TABLE 1

Data collection and refinement statistics

| Data set | ST1 | ST2 | ST3 | ST4 | ST5 | ST6 | ST7 | ST8 | ST9 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Crystal preparation | 20 h | 20 h | 40 h | 80 h | 80 h | 80 h | 80 h | 80 h | 80 h |
| Beam line | BL26B2 | BL41XU | BL26B2 | BL38B1 | BL41XU | BL26B2 | BL26B2 | BL26B2 | BL41XU |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ |
| Cell dimensions (Å) | 99.0 (92.3) | 99.8 (100) | 99.6 (99.1) | 100 (100) | 99.9 (99.9) | 99.8 (99.4) | 99.1 (97.4) | 98.2 (99.8) | 99.0 (92.3) |
| Resolution (Å) | 30.0–1.24 | 30.0–1.35 | 30.0–1.16 | 30.0–1.40 | 30.0–1.35 | 30.0–1.25 | 30.0–1.58 | 30.0–1.43 | 30.0–1.40 |
| Unique reflections | 98,110 | 78,103 | 121,708 | 70,002 | 78,628 | 96,860 | 74,585 | 62,640 | 69,218 |
| Redundancy | 7.1 (5.6) | 7.0 (6.9) | 7.1 (6.2) | 7.4 (7.1) | 5.4 (4.6) | 7.2 (6.4) | 7.0 (6.4) | 7.2 (6.9) | 4.4 (4.0) |
| Completeness (%) | 99.0 (92.3) | 99.8 (100) | 99.6 (99.1) | 100 (100) | 99.9 (99.9) | 99.8 (99.4) | 99.1 (97.4) | 98.2 (99.8) | 99.0 (92.3) |
| R₁(refined) (%) | 5.5 (36.8) | 4.8 (31.0) | 5.2 (36.7) | 5.1 (43.7) | 7.4 (42.5) | 5.1 (40.2) | 4.9 (24.8) | 4.5 (23.2) | 6.0 (35.0) |
| I/σ(I)refined | 38.7 (30.3) | 41.3 (43.3) | 40.6 (36.6) | 38.2 (30.0) | 14.5 (36.6) | 39.1 (34.9) | 41.8 (50.5) | 47.0 (50.5) | 23.1 (23.5) |

* Values in parentheses are for the highest resolution bin.

* Values are calculated by CNS (26).

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These (Wat₄–Wat₇) have been shown to be lined between the side chain of tyrosinase Asn⁹⁸ and the main-chain carbonyl of tyrosinase Asp⁴⁵. The side chain of the Tyr⁹⁷ residue in caddie is accommodated in the pocket of an active site of tyrosinase, like L-Tyr as a substrate. Both Wat₄ and Wat₇ form hydrogen bonds with the hydroxyl of caddie Tyr⁹⁸. In addition, Wat₅ is hydrogen-bonded to the side chains of His₃₈ and His₂₁₆ residues in tyrosinase, which are ligands of Cu⁴ and Cu² residues in tyrosinase Thr²⁰₃, the side-chain hydroxy of tyrosinase Ser²⁰⁶, and Wat¹. Wat¹ is present between the side chain of the tyrosinase His⁹₇, which is a ligand of Cu⁴, and the main-chain carbonyl of Gly²⁰⁴ in tyrosinase. On the other hand, when His⁵⁴ takes the latter conformation, six water molecules might be present at the catalytic site (Fig. 3C). In detail, two water molecules (Wat² and Wat⁶) disappear to avoid close contact with the side-chain of the tyrosinase His⁵⁴, instead, Wat⁸ is introduced between Wat³ and Wat⁴. In addition, the electron density of Wat⁴ is weaker when the side chain of the tyrosinase His⁵⁴ points toward the caddie His⁹₇ residue.

In a previous study (14), we reported that the caddie protein has two copper-binding sites (Fig. 1, A and B). A copper ion (designated Cu¹), which is most frequently identified in the Cu(II)-soaked crystals of tyrosinase in a complex with caddie, binds to the Nε atom from His⁹² of caddie. In addition, Cu² binds to the side-chain Nδ atom and the main-chain carbonyl from the His⁹⁸ and to the side-chain carboxyl from the Glu⁶⁷ residues of caddie, although these two residues are not always identified in an electron density map due to their high mobility. The Cu¹-binding site is located near to the C terminus of the α₂-helix of tyrosinase, which contains the Cu⁴-ligating His⁹⁸ residue. The negative charge on the caddie Glu⁶⁷ residue and the charge produced by a helix dipole effect may help the binding of the positively charged Cu(II) at this site. At this time, the Nδ atom of the His⁸² side chain forms a hydrogen bond with the Nδ atom of His⁹⁷ in caddie, mediated by the nitrate ion derived from a precipitant solution. However, when the crystal was soaked in a solution containing Cu(II) for 6 months (Protein Data Bank code 2AHK), neither Cu² nor nitrate ions were found, whereas another copper ion (designated Cu³), which adopts a trigonal planar coordination with the Nε atoms from the caddie His⁸² and His⁹⁷ residues and a sulfur atom from the caddie Met⁹⁴, alternatively emerged. To form the Cu³-binding site, the imidazole rings of His⁸² and His⁹⁷ should be rotated around the bond between their Cβ and Cγ atoms and then get closer to one another. Hence, it is impossible for both Cu¹ and Cu³ to coexist. The imidazole ring of caddie His⁹⁷ is located near the side chain of tyrosinase His⁵⁴. Notably, in Cu(II)-free tyrosinase complexed with caddie, the side chain of the tyrosinase His⁵⁴ residue partially (~50%) points toward the side chain of caddie His⁹⁷. These clustered His residues, which extend from the solvent-exposed His⁸² of caddie to the active center of tyrosinase, may bind to Cu(II) ions during the transfer of two metal ions to the catalytic center.

To evaluate the mechanism of copper transportation to tyrosinase assisted by caddie, we first analyzed the nature of Cu(II) binding in crystals soaked in a CuSO₄-containing solution for various lengths of time (Table 1). Information on the identified copper ions is summarized in Table 2. Two crystal structures (ST1 and ST2) were obtained by Cu(II) soaking for about 20 h. In ST1, electron densities derived from copper are found only at the Cu⁸ and Cu² sites (Fig. 4A and supplemental Fig. S1A). The occupancy of Cu² is about 0.4, whereas that of Cu⁸ is about 0.2. This result suggests that after Cu(II) is bound to the surface Cu¹ atom, the metal ion is transported to the tyrosinase active center. The side chain of the tyrosinase His⁵⁴ is disordered, as found in the Cu(II)-free structure. As a result, Wat¹ and Wat⁴ are absent, and the electron density of Wat⁷ is weak. On the other hand, in...
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TABLE 2
Information on the identified copper ions

| Data set | Occupation/equivalent B-factor | Distance (Å) |
|----------|--------------------------------|--------------|
| ST1      | CuA–1 0.21/25.2 CuA–2 0.42/21.4 | 3.4          |
| ST2      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST3      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST4      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST5      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST6      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST7      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST8      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |
| ST9      | CuA 0.25/11.2 CuA–1 0.62/17.9 CuA–2 0.82/13.5 | 3.2          |

ST2, electron densities from copper are mainly found at the CuA, CuB, and CuC sites (Fig. 4B and supplemental Fig. S1B). The occupancies of CuA, CuB, and CuC are about 0.3, 0.6, and 0.8, respectively. In addition, a weak electron density is evident at the CuD-binding site, and the occupancy was calculated to be about 0.2. To form the CuD site, the conformations of the side chains of the caddie His82, Met84, and His97 residues must be changed. Although the side chain of the caddie Met84 residue clearly adopts two conformations, the disordered structures of the caddie His82 and His97 residues were not confirmed, probably due to the low occupancy of the alternative conformations. The side chain of the tyrosinase His54 also adopts two conformations. Due to the disorder of His54, the electron densities at Wat3 and Wat4 are weak. The distance between CuA and CuB is 3.4 Å, and two molecules are present between these two copper atoms. The molecules that form the bridge are located near the sites of Wat1 and Wat4, although a hydrogen bond between the latter molecule and Wat4 is lost. The electron density of the molecule near the Wat4 site is much weaker than that near the Wat3 site. Although Wat3 cannot coexist with the CuA-oriented side chain of His54 due to the close contact, the bridging molecule near the Wat4 site seems to coexist with the side chain via an interaction with the CuA atom.

The electron densities from copper in ST3, which was obtained by Cu(II) soaking for about 40 h, are mainly observed at the CuA, CuB, and CuC sites (Fig. 4C and supplemental Fig. S1C). The electron density of CuB in ST3 is stronger than that in ST2. The occupancy was calculated to be about 0.8. Interestingly, the electron density of CuA is significantly elongated, suggesting that the Cu(II) ion is split between two binding sites (CuA–1 and CuA–2). CuA–1 is about 4.2 Å apart from CuB, whereas CuA–2 is about 3.4 Å apart from CuB. The occupancies of CuA–1 and CuA–2 were calculated to be 0.3 and 0.4, respectively. The electron density near the WatB site in ST3 is stronger than that in ST2. However, within ST3, the electron density near the WatB site is weaker than that near the WatA site. Furthermore, the side chain of the tyrosinase His54 is also disordered in ST3. CuA–1 is maximally coordinated to His38, His54, and His97, whereas CuA–2 is maximally coordinated to His38, His54, and two bridging molecules. Moreover, the electron density at the Wat1 site is weakened, and the tyrosinase Gly204 residue has two conformations. One is the same as that found in the Cu(II)-free structure, where its carbonyl oxygen is hydrogen-bonded to the N6 atom of the His97 residue via Wat1. As shown in Fig. 3A, a carbonyl oxygen of Gly204 in the other conformation is directly bound to His38.

In ST4 and ST5, which were obtained by Cu(II) soaking for about 80 h, strong electron densities from copper are found at the CuA–2, CuB, and CuC sites (Fig. 4D and E, and supplemental Fig. S1D, E). The occupancy of CuA–2 is in the range of 0.6–0.8, whereas that of CuB is in the range of 0.8 to 0.9. Although an additional copper was found at the CuD site after soaking for 6 months, the copper was mainly found at the CuC site in ST4 and ST5. Furthermore, the electron densities of the two bridging molecules are equally strong. On the other hand, the Wat2 density is lost. In accordance with the disappearance of Wat2, the hydroxyl oxygen of caddie Tyr98 is directly bound to the hydroxyl of tyrosinase Ser206, as found in the crystal structure obtained by Cu(II)-soaking for 6 months (Fig. 3A). Interestingly, the structures of the tyrosinase His54 and the caddie His97 residues are different in ST4 and ST5. In ST4, as found in ST1–ST3, the side chain of the tyrosinase His54 is disordered, and the side chain of the caddie His97 is hydrogen-bonded to that of the caddie His82 via a nitrate ion (Fig. 4D and supplemental Fig. S1D). As a result, the electron densities of Wat2 and Wat4 are not observed. On the other hand, in ST5, almost all of the side chain of the tyrosinase His54 points toward CuA–2 (Fig. 4E and supplemental Fig. S1E). Furthermore, the conformation of the side chain of the caddie His97 changes to point toward the CuB-binding site, although the electron density of CuD is not observed at the site. As a result, a large vacant space is generated between the tyrosinase His54 and the caddie His97 residues, and Wat3 and Wat6 are fully accommodated in the space.

Effects of Caddie Mutations on Tyrosinase Activity—The residues selected to mutate are His52, Met84, and His97, which are ligands of additional copper atoms (CuC and CuD) bound to caddie, and Tyr98, whose hydroxyl group participates in the hydrogen bond network around the active center. His52, Met84, His97, and Tyr98 were replaced by Gln, Leu, Gln, and Phe, respectively. The caddie mutants were thus named H82Q, M84L, H97Q, and Y98F, respectively. At first, the effects of these mutations on the Cu(II)-induced liberation of caddie from the complex were investigated by HPLC analysis (supplemental Fig. S2). We found that the H82Q, H97Q, and Y98F mutants were barely released from the complex, whereas the M84L mutant was more quickly released than the wild-type protein.

A kinetic experiment was also performed by using complexes of tyrosinase and the caddie mutants. When the oxidase activity was measured after incubation for a given time in the presence of 10 μM CuSO4 at pH 8, the activity of tyrosinase complexed with the H82Q or M84L mutants reached its maximum at 20 min, but that complexed with Y98F showed maximal activity at
30 min (Fig. 5A). Tyrosinase complexed with M84L or H82Q exhibited maximal activity comparable with wild type, whereas tyrosinase complexed with Y98F displayed a lower activity. On the other hand, the tyrosinase complexed with H97Q did not display oxidase activity. As discussed above, the dissociation of tyrosinase may be correlated with the aggregation of caddie
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FIGURE 5. Kinetic analysis of tyrosinase complexed with mutated caddie. A–C, time-dependent activation of tyrosinase by preincubation with 10, 1, and 0.1 μM CuSO₄, respectively, at pH 8. Diamonds, squares, triangles, crosses, and circles indicate the results of tyrosinase complexed with the wild-type, H82Q, M84L, H97Q, and Y98F caddie proteins, respectively. D, Cu(II) requirement for the activation of tyrosinase complexed with the wild-type, H82Q, M84L, and Y98F caddie proteins. The purified complex was incubated at pH 8 with the given concentration of CuSO₄ and the oxidase activity was then measured every 10 min. Maximal activities were plotted against the concentrations of CuSO₄ occurring after the copper transfer. Tyrosinase might not be dissociated from H97Q because the copper transfer was hindered in this mutated complex. However, the reasons why the dissociation rate of tyrosinase was altered by other mutations remain unclear. Furthermore, it was obvious that when the concentration of Cu(II) was low (1 or 0.1 μM), tyrosinase complexed with wild type or Y98F caddie has a slower maturation rate at the early phase (Figs. 5, B and C). On the other hand, the slow maturation phase was barely observed for tyrosinase when complexed with H82Q or M84L.

The maximal activities of tyrosinase complexed with the wild-type, H82Q, M84L, or Y98F caddie proteins were plotted against the concentration of CuSO₄ (Fig. 5D). It was revealed that tyrosinase complexed with the caddie mutants has very low activity in the presence of 0.03 μM CuSO₄, whereas tyrosinase complexed with wild-type caddie has about 60% of the maximum activity. In the mutated complexes, the concentration of copper ions required for the activation of tyrosinase was found to be 1 order higher than that in the wild-type complex. The ED₅₀ values of the H82Q and Y98F complexes were estimated to be 0.1 μM, whereas that of the M84L complex was 0.2 μM.

Effects of Caddie Mutations on the Crystal Structure of the Complex—We analyzed the crystal structure of tyrosinase in complex with each caddie mutant (Table 1). In the crystal structure of tyrosinase complexed with H82Q, which was soaked in a CuSO₄-containing solution for 80 h (ST6), strong electron densities were observed at the CuA and CuB sites. This indicates that two copper ions had been introduced into the active site in the slow maturation phase. As a result, Wat₈ is absent, whereas Wat₅ and Wat₆ are present at the dimer interface, although they are disordered. In ST6, the active-site geometry is also similar to that in ST7 (Fig. 4G). Additional copper with an almost full occupancy was found near the CuD-binding site (supplemental Fig. S1F), but the occupancy is very low (about 0.3). In the crystal structure of tyrosinase complexed with M84L, which was soaked in a CuSO₄-containing solution for 80 h (ST7), the active-site geometry is also similar to that in ST6 (Fig. 4G). Additional copper with an almost full occupancy was found at the CuC-binding site (supplemental Fig. S1G). From these structures, it is not clear why the concentration of Cu(II) required for the activation of tyrosinase was increased in the complexes with each of these caddie mutants, although the increased Cu(II) requirement is likely to be related to the destruction of additional copper-binding sites (the CuC and CuD sites in H82Q and the CuD site in M84L). Interestingly, in the crystal structure of tyrosinase complexed with H97Q, which was soaked in a CuSO₄-containing solution for 80 h (ST8), the electron densities from copper are found only at the CuB and CuC sites (Fig. 4H and supplemental Fig. S1H). The occupancy of CuB is about 0.8, whereas that of CuC is about 0.4. This is in agreement with the result that tyrosinase complexed with the H97Q caddie mutant showed very low activity. In ST8, the side chain of the tyrosinase His⁵⁴ adopts a single conformation that points toward CuA, and Wat¹ and Wat⁶ are present between the tyrosinase His⁵⁴ and caddie His⁹⁷ residues. Additional copper is found near the CuD-binding site (supplemental Fig. S1F), but the occupancy is very low (about 0.3). In the crystal structure of tyrosinase complexed with H97Q, which was soaked in a CuSO₄-containing solution for 80 h (ST7), the active-site geometry is also similar to that in ST6 (Fig. 4G). Additional copper with an almost full occupancy was found near the CuD-binding site (supplemental Fig. S1G). From these structures, it is not clear why the concentration of Cu(II) required for the activation of tyrosinase was increased in the complexes with each of these caddie mutants, although the increased Cu(II) requirement is likely to be related to the destruction of additional copper-binding sites (the CuC and CuD sites in H82Q and the CuD site in M84L). Interestingly, in the crystal structure of tyrosinase complexed with H97Q, which was soaked in a CuSO₄-containing solution for 80 h (ST8), the electron densities from copper are found only at the CuB and CuC sites (Fig. 4H and supplemental Fig. S1H). The occupancy of CuB is about 0.8, whereas that of CuC is about 0.4. This is in agreement with the result that tyrosinase complexed with the H97Q caddie mutant showed very low activity. In ST8, the side chain of the tyrosinase His⁵⁴ assumes one conformation that points toward the CuA-binding site. As a result, Wat⁸ is absent, whereas Wat⁵ and Wat⁶ are present at the dimer interface, although they are disordered. In addition, Wat¹ but not Wat² is present.
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ligand for the copper at either site, adopting a different conformation depending on which copper site is occupied. Similarly, CuD and CuK are mutually exclusive because the caddie HisE377 residue must adopt a different conformation. Furthermore, CuE and CuK are mutually exclusive because the tyrosinase His5H4 residue must adopt a different conformation. The partial occupancies of these coppers are also consistent with this concept. These results indicate that two copper ions are introduced from the CuC-binding site, which is located on the molecular surface, into the active center of tyrosinase via the CuD- and CuK-binding sites. In ST9, Wat1 and Wat2 remain at the active center. Furthermore, only one bridging molecule, which is positioned near the Wat3 site, was found between CuA1 and CuB. Although weak electron density was also found near the Wat8 site, the molecule assigned at the position is bound to neither CuA1 nor CuB.

**DISCUSSION**

Structural Changes at the Active Center of Tyrosinase—During the maturation of tyrosinase, the introduction of the two closely spaced copper ions into the catalytic center is of crucial importance because the electrostatic repulsion between these two ions seems to inhibit the maturation of this enzyme. We considered the possibility that the caddie protein may act as a metallochaperone to accommodate two copper ions in the tyrosinase catalytic center. In our present study, we carried out kinetic and crystallographic analyses to elucidate the mechanism underlying copper transport to tyrosinase assisted by caddie. When compared with the kinetic results obtained in solution, copper transfer was very slow in the crystals. However, the known structure of the tyrosinase binuclear copper center emerged later in the crystal structures, and the crystals, after a long soaking time, showed tyrosinase activity, suggesting that the crystallographic observations are functionally significant. In all probability, the mobility of the residues involved in the copper transfer, which is lowered in the crystals, may be necessary for fast copper transfer. Through crystallographic analysis, we evaluated the structural changes at the tyrosinase active center, as illustrated in Fig. 7.

In the ST1 structure obtained from a crystal soaked in a harvesting solution containing 1 mM CuSO4 for about 20 h, a copper ion was only introduced at the CuB-binding site, in which the copper took a tetrahedral coordination with the side chains of His190, His194, and His210 and a molecule near the Wat3 site (Fig. 7C). This indicates that the CuB-binding site is more stable than the CuA-binding site. In fact, the CuB-ligating residues have lower B-factors than the CuA-ligating residues. However, in ST2, which was also obtained from a crystal soaked for about 20 h, the electron densities of both CuA-2 and CuB could be clearly observed. Although the occupancy of CuB was calculated to be slightly higher than that of CuA-2, the sum of the occupancies of the two coppers was below 1.0. Assuming that the accommodation of the second copper in the active site is a rate-limiting step, it is reasonable to speculate that most complexes in the crystal contain one copper in the active center. Namely, at the early stages, CuA-2 and CuB appear to be mutually exclusive (Fig. 7, B, C, C’, and C”) because the electrostatic repulsion between the two coppers prevents the formation of a dicopper center. The partial copper occupancies of these two sites are also consistent with this. In ST2, as observed in the crystal structure of the Cu(II)-free complex, the electron density near the Wat8 site is lower than that near the Wat3 site, and the side chain of His54 is disordered. These results indicate that a copper ion is introduced at the CuA-2 site at first. The copper adopts a tetragonal coordination with the side chains of His388 and His54 and Wat3 and Wat8 (Fig. 7B). When the copper moves to the CuB site, the structures of the CuA-2-binding site are disrupted. The CuA-2-oriented side chain of His54 is too close to Wat8 if simultaneously occupied. Therefore, His54 takes the other conformation (Fig. 7C”), or Wat8 is released from active site (Fig. 7C”).

In ST3, which was obtained from a crystal soaked for about 40 h, the electron density from copper is observed at the CuA1, CuA-2, and CuB sites. The sum of the occupancies of CuA1, CuA-2, and CuB is higher than 1.0, indicating that tyrosinase molecules in the crystal may contain one or two copper ions in their active site. Moreover, as in ST2, the electron density near the Wat8 site is lower than that near the Wat3 site, and the side chain of His54 is disordered. When one copper is present at the active site, the metal ion is expected to be positioned at either the CuA-2 or the CuB site. On the other hand, when two coppers are present at the active site, the coppers are positioned at the CuA-1 and CuB sites (Fig. 7, E and E’). At this time, CuB adopts a tetrahedral coordination, whereas CuA-1 assumes a trigonal coordination with the Ne atoms of His388, His54, and His53. It is possible that one (E’) or two bridging molecules (E) are present between CuA-1 and CuB, although the former geometry seems to be more stable than the latter.

In the crystals soaked in a Cu(II)-containing solution for about 80 h (ST4 and ST5) or 6 months (Protein Data Bank code 2AHK), copper atoms with a higher occupancy are accommodated in the CuA-2 and CuB sites. Furthermore, two bridging molecules display equally strong electron densities. These results indicate that the second copper is accommodated in the CuA-1 site (Fig. 7E) and then moves toward the CuB-2 site (Fig. 7F). Hereafter, we designate the geometry containing two Cu(II) ions at the CuA-1 and CuB sites as the met1 form and the geometry containing two Cu(II) ions at the CuA-2 and CuB sites as the met2 form. The distance between CuA-1 and CuB is in the range of 4.0–4.5 Å, whereas the distance between CuA-2 and CuB is in the range of 3.2–3.5 Å. Two bridging molecules in the met2 form should be negatively charged hydroxide ions because the hydroxides seem to be important for weakening the electrostatic repulsion between the two coppers. On the other hand, one hydroxide may bridge the coppers in the met1 form. The second copper may be accommodated in the CuA-1 site after the conversion of one of two bridging water molecules to the hydroxide ion. Subsequently, together with the conversion of the other water molecule to the hydroxide, the copper may approach CuK, resulting in the generation of the met2 form.

An earlier EXAFS study on the met form of fungal tyrosinase suggests that the Cu–Cu distance is 3.4 Å (30), similar to the met2 form in this study. Furthermore, a previous x-ray absorption study on the met form of *S. antibioticus* tyrosinase (31), which is 82% identical to the *S. castaneoglobisporus* tyrosinase in terms of the amino acid sequence, suggests that
two cupric ions lie about 3.4 Å apart with two bridging oxygens. The dihydroxo-bridged dicopper(II) center found in our present study may be a characteristic geometry of the met form of tyrosinase. On the other hand, in the met form of catechol oxidase, two cupric ions lie about 2.9 Å apart with one bridging molecule, which is considered to be a hydroxide ion (19). Each of the two cupric ions adopts a trigonal pyramidal coordination with three His residues and one bridging oxygen. The preferred geometries of the met forms, which are different between the catechol oxidase and tyrosinase, must be determined by the scaffolding of the dicopper center comprising six His residues.

Interestingly, the electron densities of Wat$_1$ and Wat$_2$ were found to be weakened in a time-dependent manner, suggesting that the two water molecules were removed in accordance with the generation of the met2 form at the active site. Removal of these molecules from the active site seems to be entropically advantageous and accelerate the uptake of the second copper into the active site. After the generation of the met2 form, the side chain of the tyrosinase His$_{54}$ is still disordered (Fig. 7, F$^0$, and G) but was stabilized after the introduction of Wat$_5$ and Wat$_6$. Namely, if these water molecules are absent, the side chain is disordered to cover the vacant space. For the full introduction of Wat$_5$ and Wat$_6$, the side chain of the caddie His$_{97}$ must point toward the Cu$_D$-binding site. The introduction of the second copper to the active site is likely to be intrinsically coupled with the formation of the Cu$_D$-binding site and the introductions of Wat$_5$ and Wat$_6$. 

**FIGURE 7. Proposed structural changes at the active site of tyrosinase during Cu(II) incorporation.** In most cases, when the side chain of His$_{54}$ points toward the Cu$_A$-binding site, Wat$_8$ is absent. Two Cu(II) ions may be transported into the active site together with the structural change of the His$_{54}$ side chain (A’ to B and D’ to E). Wat$_8$ and Wat$_6$ are likely to be deprotonated together with the protonation of His$_{54}$ (D and F steps). Furthermore, according to the formation of the met2 form (G), Wat$_1$ and Wat$_2$ seem to be released from the catalytic center. ST1 and ST2 may represent the structures prior to the first deprotonation, and they contain A, A’, C, and C’ geometries and B, B’, and C’ geometries, respectively. In the ST3 crystal, some of the molecules seem to experience the first deprotonation, and the ratio of E’ geometry increases. In the ST4 crystal, some of the molecules seem to experience two deprotonation steps, and the ratio of F’ and G geometries increases. In the ST5 crystal, most of the molecules adopt a G geometry. Cu$_A$ is surrounded by His$_{38}$, His$_{54}$, and His$_{63}$, whereas Cu$_B$ is surrounded by His$_{190}$, His$_{194}$, and His$_{216}$. Cu$_A$ is closer to Cu$_B$ than Cu$_A$. It is noteworthy that when Cu$_A$ is bound to the active center, the coordination bond between His$_{54}$ and copper is lost.
To form the dihydroxo-bridged dicopper(II) center in tyrosinase, Wat³ and Wat⁴ must be converted to hydroxide ions by deprotonation. At the active center of tyrosinase, Wat³ forms a hydrogen bond with Wat⁶, and Wat⁶ is positioned near the Cu³-pointed side chain of the tyrosinase His⁵⁴. Hence, Wat³ and Wat⁶ may be converted to hydroxide ions as His⁵⁴ is protonated (Fig. 7, D and F). It is important to note also that the side chain of the tyrosinase His⁵⁴ in each of the two conformations interacts with the negatively charged side chain of the tyrosinase Asp⁴⁵. The positive charge generated on the His⁵⁴ residue may be relayed to the His⁹⁷ residue of caddie when the His⁵⁴ residue takes the other conformation. The charge may then move to the solvent molecule through a currently unknown route. This concept is also consistent with the experimental observation that an alkaline pH facilitates the activation of tyrosinase assisted by caddie. It is important to note also that the concentration of Cu(II) to activate tyrosinase complexed with the caddie Y98F mutant is higher than that in the case of tyrosinase complexed with wild-type caddie (Fig. 5D). Furthermore, the maximum activity of the Y98F complex, which is obtained under conditions of a high concentration of Cu(II), is lower than that of the wild-type complex (Fig. 5, A and D). At the active center of tyrosinase complexed with wild-type caddie, the hydroxyl oxygen of the caddie Tyr⁹⁸ forms hydrogen bonds with Wat¹ and Wat³, and Wat² and Wat⁴ further bond to Wat¹ and Wat⁶, respectively. The lack of hydroxyl of the caddie Tyr⁹⁸ residue results in an imperfect hydrogen bond network, which may be the reason for the low affinity toward Cu(II) of tyrosinase complexed with the Y98F caddie mutant. In fact, Wat¹ and Wat² remain at the active center, whereas a bridging molecule near the Wat⁶ site is absent in ST9.

Implications for the Mechanism of Cu(II) Transfer—Kinetic experiments demonstrated that mutations at the additional copper-binding site of caddie reduced the ability to activate tyrosinase. The effects of a mutation at His⁸², a ligand of Cu⁵ and Cu⁷, and Met⁸⁴, a ligand of Cu⁷, were moderate, whereas a mutation at His⁹⁷, a ligand of Cu⁵ and Cu⁶, completely abrogated tyrosinase activity. These results indicate that the binding of copper to the Cu⁵-binding site at the dimer interface is essential for Cu(II) transfer. On the other hand, binding to the Cu⁵- and Cu⁷-binding sites may be necessary for effective Cu(II) transfer.

As illustrated in Fig. 8, we propose a Cu(II) transfer mechanism assisted by caddie; first, a copper ion binds to the Cu⁵ site (Fig. 8B) and moves to either the Cu³- or the Cu⁶-binding site (Fig. 8C). The energy barrier to introduce the first copper into the active center may be low, and this step should progress without any assistance. Indeed, the transportation of a copper into the active site was observed even in the crystal structure of the inactive complex between tyrosinase and H97Q. To form a dicopper center in tyrosinase, a large energy barrier, which is formed by the electrostatic repulsion between the two copper ions, must be overcome. Therefore, a rate-limiting step for the maturation of tyrosinase is likely to be the uptake of the second copper into the active site.

The next step is the binding of the second copper to the Cu⁵-binding site (Fig. 8D), as indicated by ST2. Although it was difficult to trap the intermediate steps in the present crystallographic study, the second copper ion must be moved to the Cu⁶ site via the Cu⁶ site (Fig. 8E). At this time, because the coordination bond between Cu⁵-2 and His⁵⁴ is lost, the first copper completely moves toward the Cu⁶ site. The third copper ion then binds to the Cu⁵-binding site (Fig. 8F). This intermediate state may be contained in the crystal structure of tyrosinase in complex with Y98F. As reported above, the caddie mutant has a low ability to introduce the second copper into the active site of tyrosinase, probably due to the imperfect hydrogen bond network. It is noteworthy that the Cu⁶ site, formed by two ligands, has more difficulty fixing the copper than the Cu⁳ site, formed by three ligands. Hence, the second copper bound to the Cu⁶ site may easily move back toward the Cu⁵ site. The third copper ion, which is newly bound to the Cu⁶ site, may play a role in preventing Cu⁶ from moving back to the Cu⁵ site because the binding of the third copper blocks the formation of the Cu⁵ site.

Next, in accordance with the movement of the third copper to the Cu⁵ site, the second copper is introduced into the Cu⁵-1 site (Fig. 8G). This intermediate state is also likely to be contained in the crystal structure of tyrosinase complexed with Y98F. The second copper then moves to the Cu³-2 site, resulting in the formation of the met2 form. The formation of the met2 form is stimulated by the conversion of two water molecules (Wat¹ and Wat⁶) to the hydroxide ions and the release of two other water molecules (Wat¹ and Wat⁷), as reported above. Finally, two water molecules (Wat¹ and Wat⁶) are introduced into the space between the tyrosinase His⁵⁴ and caddie His⁹⁷ residues and complete the maturation of tyrosinase (Fig. 8H).

Relative to tyrosinase in a complex with wild-type caddie, tyrosinase complexed with the mutant caddie proteins H82Q or M84L requires a high concentration of copper ions for maturation (Fig. 5D), whereas the maturation rates within 10 min are high (Fig. 5, B and C). As reported above, the Cu⁵- and Cu⁶-binding sites seem to provide the route for the uptake of external copper to the Cu⁶ site, and the third copper bound to the Cu⁶ site may contribute to the transfer of the second copper to the Cu⁵ site. In these two mutated complexes, due to the destruction of Cu⁵- and Cu⁶-binding sites, the external copper may be more directly bound to the Cu⁵ site. As a result, copper should be transferred into the tyrosinase more quickly at an early stage under a high copper concentration. On the other hand, in a complex between tyrosinase and Y98F, the second copper may be transferred into the Cu⁶ site via the Cu⁵ and Cu⁷ sites, as in the wild-type complex. However, because the hydrogen bond network is imperfect, the affinity between the Cu(II) ions and tyrosinase is low (Fig. 5D).

Nitrate ions may block the binding of Cu⁷, but they will help the formation of the Cu⁵- and Cu⁷-binding sites. We investigated the effects of the nitrate ion on the kinetics of tyrosinase complexed with caddie. As a result, the nitrate ion reduced the Cu(II) concentration required for the activation of tyrosinase, indicating that this ion stimulates the Cu(II) transfer. As sug-
gested by our kinetic and crystallographic studies of tyrosinase
complexed with H82Q, the movement of the second copper
from the CuE site to the CuA site may occur without the assis-
tance of the third copper. However, for effective Cu(II) transfer,
the formation of an intermediate state, in which coppers bind to
the CuB, CuC, and CuE sites (Fig. 8 F), may be a crucial step,
and the nitrate may stabilize this state. Perhaps also, in the
presence of the nitrate ion, the movement of the second copper
from the CuE to the CuA site is not always coupled with the
movement of the third copper from the CuC to the CuD site
(F to G). The maturation of tyrosinase may be completed by the conversion of the met1 to met2 form and the introduction of two water molecules at the dimer interface (H). However, in
the crystalline state, a nitrate ion binds to the CuD site prior to the introduction of water molecules (I). Hence, His54 is usually disordered to fill the vacant space.
Additional ligands of Cu5, which were identified only when Cu5 strongly bound to caddie, are omitted from the figures.

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REFERENCES
1. Lamb, A. L., Wernimont, A. K., Pufahl, R. A., Culotta, V. C., O’Halloran,
T. V., and Rosenzweig, A. C. (1999) Nat. Struct. Biol. 6, 724–729

FIGURE 8. Proposed structural mechanism of Cu(II) transfer into tyrosinase assisted by caddie. Carbon atoms from the residues of tyrosinase and caddie
are represented by orange and cyan, respectively. Copper atoms and water molecules (or hydroxide ions) are represented by green and red spheres, respectively. In the copper-free form (A), the His54 of tyrosinase takes two conformations, and three water molecules (shown in pink) have an occupancy of less than 1.0. At the early stage, the first copper is introduced at the CuA-2 or CuB site. Pale green spheres in C and D indicate the copper atoms with low occupancy. The second copper is likely to move to the CuA-1 site in accordance with the movement of the third copper from the CuC to the CuD site (F to G). The maturation of tyrosinase may be completed by the conversion of the met1 to met2 form and the introduction of two water molecules at the dimer interface (H). However, in the crystalline state, a nitrate ion binds to the CuD site prior to the introduction of water molecules (I). Hence, His54 is usually disordered to fill the vacant space. Additional ligands of Cu5, which were identified only when Cu5 strongly bound to caddie, are omitted from the figures.
2. Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O’Halloran, T. V., and Culotta, V. C. (1999) *J. Biol. Chem.* **274**, 15041–15045
3. Wernimont, A. K., Huffman, D. L., Lamb, A. L., O’Halloran, T. V., and Rosenzweig, A. C. (2000) *Nat. Struct. Biol.* **7**, 766–771
4. Remaut, H., Safarov, N., Ciurli, S., and Van Beeumen, J. (2001) *J. Biol. Chem.* **276**, 49365–49370
5. Srinivasan, V., Netz, D. J., Webert, H., Mascarenhas, J., Pierik, A. J., Michel, H., and Lill, R. (2007) *Structure* **15**, 1246–1257
6. Srinivasan, V., Netz, D. J., Webert, H., Mascarenhas, J., Pierik, A. J., Michel, H., and Lill, R. (2007) *Structure* **15**, 1246–1257
7. Soloman, E. I., Sundaram, U. M., and Machonkin, T. E. (1996) *Chem. Rev.* **96**, 2563–2606
8. Oetting, W. S. (2000) *Pigment Cell Res.* **13**, 320–325
9. Jeon, H. J., Noda, M., Maruyama, M., Matoba, Y., Kumagai, T., and Sugiyama, M. (2004) *Protein Expr. Purif.* **34**, 202–207
10. Claus, H., and Decker, H. (2006) *Syst. Appl. Microbiol.* **29**, 3–14
11. Lee, Y. H., Chen, B. F., Wu, S. Y., Leu, W. M., Lin, J. J., Chen, C. W., and Lo, S. C. (1988) *Gene* **65**, 71–81
12. Chen, L. Y., Leu, W. M., Wang, K. T., and Lee, Y. H. (1992) *J. Biol. Chem.* **267**, 20100–20107
13. Ikeda, K., Masujima, T., Suzuki, K., and Sugiyama, M. (1996) *Appl. Microbiol. Biotechnol.* **45**, 80–85
14. Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., and Sugiyama, M. (2006) *J. Biol. Chem.* **281**, 8981–8990
15. Volbeda, A., and Hol, W. G. (1989) *J. Mol. Biol.* **209**, 249–279
16. Hazes, B., Magnus, K. A., Bonaventura, C., Bonaventura, J., Dauter, Z., Kalk, K. H., and Hol, W. G. (1993) *Protein Sci.* **2**, 597–619
17. Magnus, K. A., Hazes, B., Ton-That, H., Bonaventura, C., Bonaventura, J., and Hol, W. G. (1994) *Proteins* **19**, 302–309
18. Cuff, M. E., Miller, K. I., van Holde, K. E., and Hendrickson, W. A. (1998) *J. Mol. Biol.* **278**, 855–870
19. Klabunde, T., Eichen, C., Sacchettini, J. C., and Krebs, B. (1998) *Nat. Struct. Biol.* **5**, 1084–1090
20. Decker, H., Dillinger, R., and Tuczek, F. (2000) *Angew. Chem. Int. Ed. Engl.* **39**, 1591–1595
21. Decker, H., and Tuczek, F. (2000) *Trends Biochem. Sci.* **25**, 392–397
22. Decker, H., Schweikardt, T., and Tuczek, F. (2006) *Angew. Chem. Int. Ed. Engl.* **45**, 4546–4550
23. Kohashi, P. Y., Kumagai, T., Matoba, Y., Yamamoto, A., Maruyama, M., and Sugiyama, M. (2004) *Protein Expr. Purif.* **34**, 202–207
24. García-Molina, F., Muñoz, J. L., Varón, R., Rodríguez-López, J. N., García-Cánovas, F., and Tudela, J. (2007) *J. Agric. Food Chem.* **55**, 9739–9749
25. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307–326
26. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Grosse-Kunstleve, R. W., Jiang, S. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. D* **54**, 905–921
27. Brünger, A. T. (1992) *Nature* **355**, 472–475
28. Sheldrick, G. M., and Schneider, T. R. (1997) *Methods Enzymol.* **277**, 319–343
29. McRee, D. E. (1992) *J. Mol. Graph.* **10**, 44–46
30. Woolery, G. L., Powers, L., Winkler, M., Solomon, E. I., Lerch, K., and Spiro, T. G. (1984) *Biochim. Biophys. Acta* **788**, 155–161
31. Babcock, L., Spinazzé, R., della Longa, S., and Benfatto, M. (2007) *Arch. Biochem. Biophys.* **465**, 320–327

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