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Post-Translational His-Cys Cross-Linkage Formation in Tyrosinase Induced by Copper(II)−Peroxo Species

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Supporting Information

ABSTRACT: Autocatalytic formation of His-Cys cross-linkage in the enzyme active site of tyrosinase from *Aspergillus oryzae* has been demonstrated to proceed by the treatment of apoenzyme with Cu^{II} under aerobic conditions, where a $(\mu\text{-}\eta^2\text{:}\eta^2$-peroxo)dicopper(II) species has been suggested to be involved as a key reactive intermediate.

The variety of enzymatic functions are extended by post-translational modifications of amino acids, generating new cofactors in the enzyme active sites.[1] Notably, such modified amino acids have been frequently found in various copper oxidases and copper monooxygenases.[2] Tyrosyl-cysteine (Tyr-Cys in galactose oxidase)[3] and tyrosyl-histidine (Tyr-His in cytochrome *c* oxidase)[4] cross-linkages as well as 2,4,5-trihydroxyphenylalanine quinone (TPQ in copper amine oxidase)[5] and lysyl tyrosyl quinone (LTQ in lysyl oxidase)[6] cofactors have been discovered in the copper enzymes during the past decades. In most cases, the chemical modifications have been demonstrated to occur via an autocatalytic pathway without assistance by any other external proteins.[2a] Namely, the active-site tyrosines of copper amine oxidase and lysyl oxidase are converted to TPQ and LTQ cofactors, respectively, in dioxygen- and copper-dependent autocatalytic reactions.[7] The formation of Tyr-Cys in galactose oxidase also involves an autocatalytic oxidative coupling reaction just requiring a copper ion and dioxygen.[8] In these cases, however, copper-active oxygen species involved in the post-translational modification processes have yet to be fully elucidated.

By X-ray crystallographic analysis, His-Cys cross-linkage has been identified in the proximity of the Cu_A site of the dinuclear copper site of *Octopus* hemocyanin and sweet potato catechol oxidase (type III copper proteins) as shown in Figure 1.[9] Tyrosinase (Ty) is also a dinuclear copper protein widely distributed throughout mammals, fungi, and bacteria. Comparison of the amino acid sequence of molluscan hemocyanins to those of tyrosinases revealed that the three histidines and a cysteine in the proximity of the Cu_A site are highly conserved, suggesting the existence of a similar His-Cys cross-linkage in the active site of certain tyrosinases.[10] In this study, we have obtained firm experimental evidence for the autocatalytic formation of such a His-Cys cross-linkage in Ty from *Aspergillus oryzae*[11] and demonstrated that a $(\mu\text{-}\eta^2\text{:}\eta^2$-peroxo)dicopper(II) species is involved as a key intermediate for the post-translational His-Cys formation.

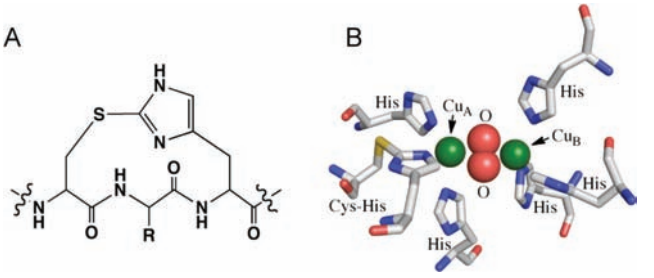

Figure 1. (A) Structure of 2-cysteinyl-histidine (His-Cys) cross-linkage. (B) Three dimensional structure of the copper center of oxy-form of *Octopus* hemocyanin (Protein Data Bank entry 1JS8).

First, copper quantification of a purified recombinant wild-type Ty (WT) and a cysteine 92 to alanine (C92A) mutant was performed by ICP-AES analysis. Their copper contents were determined to be 0.02 and 0.01 per protein, respectively, suggesting that these Tys were isolated as apo-forms. After incubation with excess $CuSO_4$ and following EDTA cleanup treatment, the copper contents of WT and C92A increased to 1.8 and 2.0, respectively (kinetic parameters of the enzymatic activity are presented in Figure S1 (Supporting Information [SI])). The thus obtained holo-WT exhibited a shoulder peak at 332 nm (ε = 3.9×10^3 M^{-1} cm^{-1}), which is a typical met-form feature

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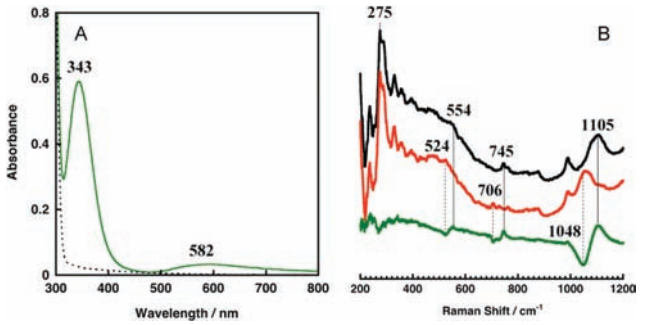
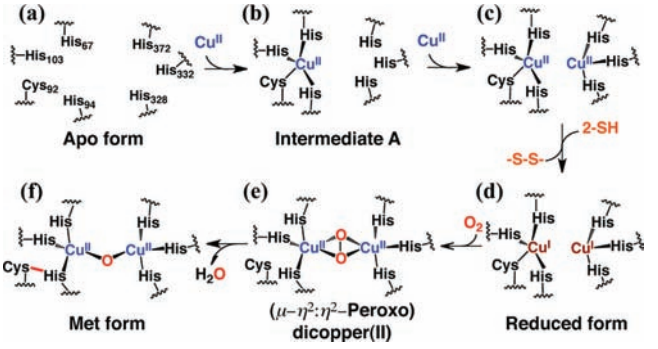


Figure 2. (A) UV−vis spectra of 35 μM holo-C92A (green) and apo-C92A (dotted black) in 50 mM phosphate buffer at pH 7.2 (B) Resonance Raman spectra of holo-C92A (250 μM, black, with $^{16}O_2$; red, with $^{18}O_2$; green, differential spectrum between the above two spectra ($^{16}O-^{18}O$).

(dicopper(II) resting state) (Figure S2A [SI], black line).[12] An *N*-terminal amino acid sequence of one of the trypsin-digested peptides of holo-WT (Figure S3 [SI]) was identified as GNPTGFX$_1$VX$_2$N, which corresponds to the residues 86−95 in the sequence of WT. The amino acids denoted as X_1 and X_2 were not identified in the sequence analysis and could be reasonably assigned to the sites of the modified cysteine 92 and histidine 94, respectively. This was confirmed by MALDI-TOF/MS analysis (Figure S4 [SI]), where a signal of the His-Cys linkage-containing peptide was observed in the peptide mass fingerprinting of holo-WT. These results indicate that the His(94)-Cys(92) cross-linkage formation occurs autocatalytically during the conversion of apo-WT to holo-WT (detailed characterization of the copper center of holo-WT is presented in ref 13). Formation of His-Cys cross-linkage was further examined by a free thiol (cysteine) quantification experiment (vide infra).

In contrast to holo-WT, which afforded a met-form by the aerobic Cu^{II} treatment, the same treatment of holo-C92A gave a characteristic UV−visible spectrum due to the (μ-η^2:η^2-peroxo)dicopper(II) species (oxy-form) with an intense absorption band at 343 nm ($\varepsilon = 1.6 \times 10^4$ M^{-1} cm^{-1}) together with an additional weak absorption band at 582 nm ($\varepsilon = 8.4 \times 10^2$ M^{-1} cm^{-1}) (Figure 2A, green line). These two bands have been assigned to the peroxo-to-copper(II) charge transfer (LMCT) transitions of the (μ-η^2:η^2-peroxo)dicopper(II) species,[12] the formation of which was confirmed by resonance Raman measurements as follows. There were three isotope-sensitive Raman bands at 745, 554, and 1105 cm^{-1} with $^{16}O_2$, which shifted to 706, 524, and 1048 cm^{-1}, respectively, upon $^{18}O_2$ substitution as shown in Figure 2B. These three bands have been assigned to symmetric O−O vibration and fundamental and overtone of asymmetric Cu−O−Cu vibrations of the (μ-η^2:η^2-peroxo)dicopper(II) core, respectively.[14] The additional intense Raman band at 275 cm^{-1}, which did not show isotope shift, has been assigned to the Cu−Cu vibration of the Cu_2O_2 core.[15] Reversible O_2-binding has also been demonstrated by repeated N_2 flushing (oxy-to-deoxy) and subsequent aeration (deoxy-to-oxy) (Figure S5B [SI]) as in the case of oxy-form of holo-WT (Figure S5A [SI]).[16] Thus, it became clear that the oxy-form of C92A having the (μ-η^2:η^2-peroxo)dicopper(II) species was generated even in the absence of a reductant such as NH_2OH. Such a different behavior in the aerobic Cu^{II}-treatment between WT (met-form formation) and C92A (oxy-form formation) may suggest that the peroxo species is involved as a key

Scheme 1



intermediate for the formation of His-Cys cross-linkage in WT as indicated in Scheme 1. Namely, the (μ-η^2:η^2-peroxo)-dicopper(II) species generated in WT is readily consumed in the following His-Cys cross-linkage formation, but in C92A the generated peroxo species remains unchanged because of the lack of Cys92.

In order to identify the electron donor for the reduction of dicopper(II) to dicopper(I), which is prerequisite for the formation of the (μ-η^2:η^2-peroxo)dicopper(II) species (Scheme 1 c → d), the numbers of free thiols (cysteine) were determined by colorimetric assay with Ellman's reagents. The number of free thiol in apo-C92A was approximately 5 per protein, which is consistent with the cysteine number deduced from the amino acid sequence of C92A (see Table S1 [SI]). On the other hand, holo-C92A (oxy-form) showed a free thiol number of 3, which recovered to 5 after TCEP [tris(2-carboxyethyl) phosphine] reduction of the protein (regeneration of free thiols from disulfide).[17] The results clearly demonstrated that two cysteines were used as the electron donor for the reduction of 2Cu^{II} to 2Cu^{I}, forming a disulfide bond in C92A. On the other hand, the free thiol number of apo-WT was 6, which is identical with the cysteine number deduced from the amino acid sequence of WT. The free thiol number of holo-WT (met-form) was reduced to 3, which increased to 5 by the TCEP reduction. Thus, in this case, one cysteine is irreversibly converted to the His-Cys thioether bond (Scheme 1 e → f), and two remaining cysteines are consumed as the electron donor for the reduction of 2Cu^{II} to 2Cu^{I}, forming a disulfide bond (Scheme 1 c → d).[18]

To further elucidate the formation process of His-Cys cross-linkage, kinetic studies were performed. When apo-C92A was treated with 2 equiv of Cu^{II} under aerobic conditions, the absorption bands at 343 and 582 nm due to the peroxo species increased in 2 h (Figure S6A [SI], Scheme 1a → e). The time course of the increase of A_{343} obeyed the first-order rate law (Figure S6B [SI]), suggesting a unimolecular reaction, and the first-order rate constant (k_{obs}^1) was determined to be 4.9×10^{-4} s^{-1} by the ordinary first-order plot (the inset of Figure S6B [SI]). On the other hand, the same treatment of apo-WT gave absorption bands at 388 and 502 nm (intermediate **A**) within a few seconds (Figure 3A, inset, Scheme 1a → b). This absorption band gradually disappeared, and a shoulder peak at 332 nm due to the met-form appeared in 2 h (Figure 3A) with an isosbestic point at 383 nm (Scheme 1b → f). The time course of the increase of A_{332} also obeyed the first-order rate law (Figure S7 [SI]), and the first-order rate constant (k_{obs}^2) was determined as 4.6×10^{-4} s^{-1} (the inset of Figure S7 [SI]). The good agreement

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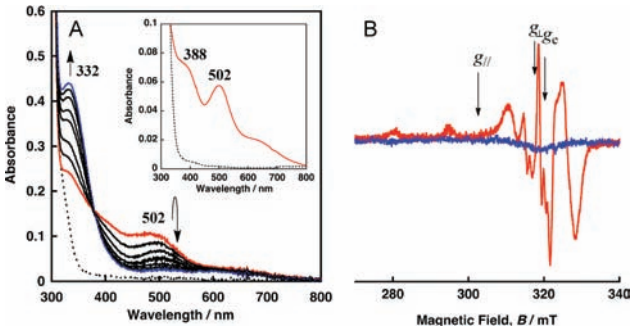

Figure 3. (A) UV−vis spectral change observed during the reconstitution of apo-WT (110 μM) with Cu^{II} in 50 mM phosphate buffer (pH 7.2) at 20 °C (dotted black: apo-WT). (Inset) UV−vis spectrum of intermediate **A** in 50 mM phosphate buffer (pH 7.2) at room temperature (dotted black: apo-WT). (B) ESR spectra of intermediate **A** (red, 175 μM) and holo-WT (blue, 175 μM) in 50 mM phosphate buffer (pH 7.2) at 77 K. $g_{||}$ and $g_{\perp}$ are axial g parameters for Cu^{II}, and g_e is the g value due to an organic radical species.

between k_{obs}^1 (formation rate of the oxy-form in C92A) and k_{obs}^2 (conversion rate of intermediate **A** to the met-form of WT) indicates that both processes involve the same rate-determining step that may be the reduction of dicopper(II) to dicopper(I) by the cysteines (Scheme 1c → d).[19]

Then, in order to get information about intermediate **A**, a titration of apo-WT by Cu^{II} ions was performed. The intensity of the absorption band at 502 nm due to an intermediate **A** increased with increasing the concentration of Cu^{II} up to about 1 equiv of Ty, while this band decreased by further addition of Cu^{II} ions (Figure S9 [SI]), suggesting that intermediate **A** might be a mononuclear copper(II) complex (Scheme 1b). The absorption spectrum of intermediate **A** was similar to those of the cysteinate type 2 copper centers of thiol-ligated copper-substituted carbonic anhydrase[20] and superoxide dismutase mutant[21] (tetragonal pyramidal geometry, N3S1 donor sets). Thus, the intermediate **A** may be trapped near the Cu_A site, where deprotonated Cys92 also coordinated to Cu^{II}. If so, the absorption band at 502 nm could be assigned to the cysteinate to Cu^{II} charge-transfer transition. The intermediate **A** exhibited a typical axial ESR spectrum of mononuclear Cu^{II} with a tetragonal geometry ($g_{||} = 2.14$ ($A_{||} = 16$ mT) and $g_{\perp} = 2.03$), although a minor signal at $g_e = 2.02$ derived from an amino acid free radical (≤3%) was observed at 77 K (Figure 3B, red line). Holo-WT (met-form) was almost ESR silent (Figure 3B, blue line), suggesting that the dicopper center is antiferromagnetically coupled as previously reported.[12]

In summary, we have demonstrated for the first time that the His-Cys cross-linkage formation proceeds autocatalytically in the type III copper protein tyrosinase without the aid of any other external catalysts. Comparison of the behavior in the aerobic Cu^{II} treatment between WT and C92A mutant has suggested that the (μ-η^2:η^2-peroxo)dicopper(II) species is involved as a key reactive intermediate for the His-Cys formation. Mechanistic details of the oxidative coupling reaction between His94 and Cys92 are now under investigation.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for additional spectroscopic and kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) When hydroxylamine (NH_2OH) was added to a holo-WT (met-form) solution under aerobic conditions, a characteristic spectrum of oxy-Ty exhibiting an intense absorption band at 347 nm ($\varepsilon = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$) and a weak band at 589 nm ($\varepsilon = 7.9 \times 10^2\ M^{-1}\ cm^{-1}$) due to the ($\mu$-$\eta^2$:$\eta^2$-peroxo)dicopper(II) species was obtained (Figure S2A [SI], green line). The formation of oxy-Ty was also confirmed by the resonance Raman spectra shown in Figure S2B (SI) (749, 540, 1080, and 273 cm^{-1} with $^{16}O_2$; 710, 514, 1025, and 273 cm^{-1} with $^{18}O_2$) and by reversible O_2-binding (Figure S5A [SI]).

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(18) In the crystal structures of *Octopus* hemocyanin (PDB code: 1JS8, Cys2549-Cys2559) and sweet potato catechol oxidase (PDB code: 1BT3, Cys27-Cys89), which have also His-Cys cross-linkage, there is a disulfide bond near the copper center (within 10 Å distance).

(19) The absorption bands at 388 and 502 nm due to intermediate **A** appeared within a few seconds when apo-WT was treated with two equiv of Cu^{II} under *anaerobic* conditions (Figure S8A [SI] red line). These absorption bands gradually disappeared in about 2 h (k_{obs}^{3}: 2.4×10^{-4} s^{-1}, Figure S8B (SI) to provide a featureless spectrum as shown in Figure S8A (SI) (green line), suggesting the reduction of dicopper(II) to dicopper(I) under anaerobic conditions (Scheme 1 c $\rightarrow$ d). The similarity of k_{obs}^{3} with k_{obs}^{1} and k_{obs}^{2} indicates that the reduction step from dicopper(II) to dicopper(I) is rate limiting in the overall reaction. In fact, aeration of WT reconstituted with 2 equiv of copper(I) significantly accelerated the formation of met-WT (more than 100-fold acceleration, see Figure S8 [SI]).

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