Copper, a mediator of redox chemistries in biology, is often found in enzymes that bind and reduce dioxygen. Among these, the copper amine oxidases catalyze the oxidative deamination of primary amines utilizing a type(II) copper center and 2,4,5-trihydroxyphenylalanine quinone (TPQ), a covalent cofactor derived from the post-translational modification of an active site tyrosine. Previous studies established the dependence of TPQ biogenesis on Cu(II); however, the dependence of cofactor formation on the biologically relevant Cu(I) ion has remained untested. In this study, we demonstrate that the apoform of the Hansenula polymorpha amine oxidase readily binds Cu(I) under anaerobic conditions and produces the quinone cofactor at a rate of 0.28 h\(^{-1}\) upon subsequent aeration to yield a mature enzyme with kinetic properties identical to the protein product of the Cu(II)-dependent reaction. Because of the change in magnetic properties associated with the oxidation of copper, electron paramagnetic resonance spectroscopy was employed to investigate the nature of the rate-limiting step of Cu(I)-dependent cofactor biogenesis. Upon aeration of the unprocessed enzyme prebound with Cu(I), an axial Cu(II) electron paramagnetic resonance signal was found to appear at a rate equivalent to that for the cofactor. These data provide strong evidence for a rate-limiting release of superoxide from a Cu(II)(O\(_2\)) complex as a prerequisite for the activation of the precursor tyrosine and its transformation for TPQ. As copper is trafficked to intracellular protein targets in the reduced, Cu(I) state, these studies offer possible clues as to the physiological significance of the acquisition of Cu(I) by nascent H. polymorpha amine oxidase.

Investigation of cu(I)-dependent 2,4,5-Trihydroxyphenylalanine Quinone Biogenesis in Hansenula polymorpha Amine Oxidase*

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Copper, an essential trace element, is frequently found as a cofactor in proteins that bind and/or catalyze the reduction of dioxygen (1). Copper homeostasis is highly regulated, and imbalances in cellular concentrations have been attributed to inherited disorders of copper metabolism, most notably Wilson disease and Menkes syndrome (2). The uptake, transport, and efflux of copper are orchestrated by a number of protein mediators (2). Among them, metal ion permeases on the plasma membrane bring copper from the environment into the cell, and metallothioneins buffer the copper ion concentration in the cytoplasm. In yeast, the cytoplasm has been estimated to be devoid of free copper, a scenario that necessitates the use of cytosolic metallochaperones (CCS, Atx1, and Cox17) that chelate and shuttle Cu(I) to distinct intracellular compartments and the protein target, superoxide dismutase (3, 4). Although there is now a wealth of knowledge regarding routes for the import of Cu(I) into the mitochondria and the trans-Golgi network for cupro-proteins that traverse the secretory pathway (2), an understanding of the pathways for the delivery of copper to proteins residing in other organelles is lacking.

Copper amine oxidase (CAO)\(^3\) is ubiquitous in Nature and found in various intracellular and extracellular locations within microbes, plants, and higher organisms. CAO catalyzes the oxidative deamination of primary amines to the corresponding aldehydes using a mononuclear copper center and the organic cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ) (5). One distinguishing feature of CAO is the ability to synthesize its quinone cofactor via the post-translational modification of a specific active site tyrosine (Tyr-405) in a self-processing reaction dependent on dioxygen and the neighboring copper ion (6). Previous investigations employing the amine oxidase from Hansenula polymorpha (HPAO) as a model system have examined in considerable detail the mechanism for TPQ biogenesis following the \textit{in vitro} reconstitution of the apoenzyme\(^4\) with Cu(II) (6–8); however, the relevance of Cu(I) in cofactor formation has remained unexplored. Given the prevailing paradigm for the trafficking of Cu(I) within the cell (2) and the apparent lack of a designated copper chaperone for HPAO, the pathway for the delivery of copper to this peroxisomal enzyme as well as the valence state of the metal inserted into the newly folded protein are brought into question. Herein we report the mechanism for Cu(I)-dependent TPQ biogenesis \textit{in vitro}, with the aim of gleaning insights into the acquisition of copper by nascent HPAO \textit{in vivo}.

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\(^3\) The abbreviations used are: CAO, copper amine oxidase; EPR, electron paramagnetic resonance; HPAO, \textit{H. polymorpha} amine oxidase; LMCT, ligand-to-metal charge transfer; TPQ, 2,4,5-trihydroxyphenylalanine quinone.

\(^4\) Apoenzyme refers to metal-free protein in which the precursor tyrosine (Tyr-405) is unprocessed. Mature enzyme refers to protein containing metal and in which the precursor tyrosine has been converted to TPQ.
EXPERIMENTAL PROCEDURES

General—Tetrakis(acetonitrile) copper(I) hexafluorophosphate was purchased from Aldrich. ApoHPAO was expressed in Escherichia coli and purified according to published methods (7, 9). All measurements were conducted in 50 mM HEPES, pH 7.0, unless otherwise noted.

UV-visible and Electron Paramagnetic Resonance (EPR) Spectral Analysis of Cu(I)-dependent TPQ Biogenesis—ApoHPAO and acetonitrile (HPLC grade) were separately purged with argon scrubbed by a basic solution of pyrogallol and subsequently transferred to an anaerobic glove box. A stock solution of tetrakis(acetonitrile) copper(I) hexafluorophosphate (10 mM) was freshly prepared in acetonitrile, and an equimolar amount (23 μl) was placed in an EPR tube (t, 0 h), capped with a rubber septum, removed from the glove box, and frozen immediately in a 2-methylbutane bath that had been cooled to liquid nitrogen temperature. The remaining portion of the washed protein was removed from the glove box and aerated with a steady stream of house air for 5 min. A small portion of the aerated sample was transferred to another cuvette, and TPQ formation was monitored on a Hewlett-Packard 8452A diode array spectrophotometer with a thermostated cell holder maintained at 25 °C. The remaining portion of the aerated protein sample was incubated in a 25 °C bath, and aliquots (250 μl) were transferred to EPR tubes and frozen at the designated times. EPR spectra were acquired on a Varian E-109 spectrometer at 6 K using the following instrument parameters: microwave frequency, 9.24 GHz; modulation amplitude, 16 G; microwave power, 0.5 milliwatt; time constant, 0.5 ms. Spin quantitation was performed following instrument parameters: microwave frequency, 9.24 GHz; modulation amplitude, 16 G; microwave power, 0.5 milliwatt; time constant, 0.5 ms. Spin quantitation was performed using a TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) standard. The total copper bound to HPAO was measured by inductively coupled plasma-atomic emission spectroscopy.

Metal Binding Stoichiometry—ApoHPAO in 50 mM HEPES, pH 7.0 (~26 μM), was anaerobically reconstituted with an equivalent of stock tetrakis(acetonitrile)copper(I) hexafluorophosphate (700 μM in acetonitrile) or CuCl₂ (700 μM in water) for 1 h and subsequently washed with anaerobic buffer to remove unbound metal. The remaining metal bound to HPAO was measured by inductively coupled plasma-atomic emission spectroscopy.

RESULTS AND DISCUSSION

Spectroscopic Analysis of Cu(I)-dependent TPQ Biogenesis—ApoHPAO was anaerobically reconstituted with an equivalent of Cu(II) and subsequently exposed to air to initiate TPQ biogenesis. When the reaction between the precursor tyrosine and dioxygen was monitored spectrophotometrically, the quinone cofactor was shown to appear with a rate of k_{TPQ} = 0.28 h⁻¹ and without the detection of a visible precursor species (Fig. 1). These properties contrast with Cu(II)-catalyzed cofactor biogenesis, which proceeded at a rate 17-fold faster and with the observation of the putative tyrosinate-Cu(II) ligand-to-metal charge transfer (LMCT) species (350 nm) (6, 7). However, analysis of the mature, TPQ-containing enzyme derived from the Cu(I)-dependent reaction revealed that the final protein product contained 0.26 eq TPQ and catalyzed the oxidation of methylvamine with a k_{cat} = 3.1 s⁻¹, values similar to those for the end product of the Cu(II)-dependent reaction (Table 1). Together, these data indicated that regardless of the valence state of copper supplied to apoHPAO, the mechanistically distinct pathways for TPQ biogenesis converge to yield the same final product (Fig. 2). Because the decay of the 350-nm intermediate is rate-limiting in Cu(II)-catalyzed TPQ biogenesis, the inability to detect this optical intermediate coupled with the diminished value for k_{TPQ} most plausibly indicated a new rate-limiting step further upstream in the Cu(I)-dependent reaction.

Isolation of Rate-limiting Step in Cu(I)-dependent TPQ Biogenesis—As a working hypothesis (Fig. 2), Cu(I) was proposed to undergo oxidation to Cu(II) and superoxide in the
Cu(I)-dependent TPQ Biogenesis in HPAO

initial stages of cofactor biogenesis (a transformation accompanied by a change in the magnetic properties of the metal). Thus, the nature of the rate-limiting step in Cu(I)-dependent TPQ formation was probed by EPR spectroscopy. As such, apo-HPAO was anaerobically reconstituted with Cu(I) and changes in copper valence monitored by time-resolved X-band EPR spectroscopy following aeration of the sample. As shown in Fig. 3, the addition of oxygen to Cu(I)-HPAO led to the growth of an axial Cu(II) EPR signal with spectroscopic properties similar to the mature CAO derived from the Cu(II)-dependent reaction (12). These data support the reaction scheme in which the Cu(I)-catalyzed reaction pathway for TPQ biogenesis converges with that for the Cu(II)-dependent reaction to yield a common end product protein (Fig. 2). Further analysis of the final product derived from the Cu(I)-dependent processing of the precursor tyrosine showed for every bound Cu(I) ion oxidized to Cu(II) (0.26 eq), a molecule of TPQ (0.26 eq) was produced. The 1:1 stoichiometry for the reaction products strongly indicated productive oxidative chemistry occurring at the active site as opposed to the oxidation of metal randomly bound on the surface of the protein. Most significantly, the rate constant for the conversion of Cu(I) to an EPR active Cu(II) signal ($k_{\text{Cu(II)}} = 0.29 \pm 0.06 \text{ h}^{-1}$) was found to be equivalent to that for the generation of TPQ ($k_{\text{TPQ}} = 0.28 \pm 0.01 \text{ h}^{-1}$) (Table 1 and Fig. 4) and was, therefore, concluded to be the rate-limiting step in Cu(I)-dependent TPQ formation. Because the Cu(II) EPR signal is expected to be detectable only after the release of the superoxide ligand, Cu(I)-catalyzed TPQ biogenesis was predicted to be limited by dioxygen binding, dioxygen reduction, or the dissociation of superoxide from the metal (Fig. 2). Chemically reduced CAOs have been shown to readily react with dioxygen (13) and bind CO (14). Moreover, rapid binding and reduction of dioxygen by Cu(I) model complexes (15) at cryogenic temperatures also indicate that neither process is likely to be the rate-limiting step in Cu(I)-initiated biogenesis, pointing instead to a slow dissociation of superoxide. Similarly, the dissociation of a small ligand (N₃) from the Cu(II) center in the precursor enzyme-azide complex was found to limit TPQ biogenesis in HPAO upon the introduction of air (16).

Cu(II)-Superoxo—Working under the assumption that Cu(I)-dependent TPQ biogenesis is limited by the dissociation of superoxide following the reduction of dioxygen, a Cu(II)-superoxo intermediate is predicted to accumulate during the course of the reaction (Fig. 2). It is conceivable that a Cu(II)-superoxo species could be relatively stable in a protected mononuclear site in the absence of a second electron donor (e.g. cysteine) or weak C-H bond (binding energy <80 kcal/mol) (18). The apparent lack of reactivity of the active site tyrosine with Cu(II)-superoxo is, however, a puzzle and may relate to its inaccessibility; during Cu(II)-initiated biogenesis, O₂ binding at a non-

### TABLE 1
Comparison of properties for HPAO following reconstitution with Cu(I) and Cu(II)

| Metal | Equivalent Cu | Equivalent TPQ | $k_{\text{TPQ}}$ | $k_{\text{Cu(II)}}$ | $k_{\text{cat}}$ |
|-------|---------------|----------------|------------------|---------------------|-------------|
| Cu(II) | 0.84 (0.06) | 0.33 (0.03) | 4.8 ± 0.2 | 3.2 ± 0.1 |
| Cu(I)  | 0.79 (0.05) | 0.26 (0.03) | 2.8 ± 0.01 | 2.9 ± 0.06 | 3.1 ± 0.1 |

- Bound copper normalized to protein concentration.
- Error reported as standard deviation of three independent measurements.
- TPQ content normalized to total copper added.
- From Ref. 7.
- TPQ content normalized to total bound copper.
- Rate of appearance of TPQ (480 nm). Standard error for fit to the equation for a single exponential is reported.
- Rate of appearance of Cu(II) EPR signal. Standard error for fit to the equation for a single exponential is reported.

![Fig. 2. Convergence of proposed mechanisms for Cu(I)- and Cu(II)-dependent TPQ biogenesis. Proposed intermediates outside the dashed lines are expected to be EPR silent. The steps contained within the dashed lines have been previously proposed for the Cu(III)-dependent process (6–8). The release of HO₂ is proposed to limit TPQ formation when initiated by Cu(I).](image-url)
metal site is the trigger for this tyrosine to migrate to the metal site (Fig. 2). Of the Cu(II)-superoxo model complexes described (19), end-on bound species exhibit strong LMCT bands at \( \approx 410 \) nm \( (3000–8000 \text{M}^{-1}\text{cm}^{-1}) \), whereas a single side-on species has been shown to exhibit only weak d-d transitions in the visible range \( (452 \text{and} 700 \text{nm}, \epsilon \approx 300 \text{M}^{-1}\text{cm}^{-1}) \) and an intense LMCT band at \( \lambda < 300 \text{nm} \). Although the use of high protein concentrations \( (7 \text{mg/ml}) \) precluded analysis of changes in the absorbance spectrum under 300 nm, no optical species in the visible range were shown to have a precursor-product relationship with respect to the band for the quinone cofactor \( (\lambda_{\text{max}} \approx 472 \text{nm}) \) (Fig. 1). Therefore, we speculate that Cu(I)-dependent TPQ formation in HPAO is limited by the dissociation of a side-on bound superoxide ion from copper, the absorbance signature of which is likely to be masked by the appearance of TPQ \( (\epsilon_{472} = 2400 \text{M}^{-1}\text{cm}^{-1}) \) (5). Because the metal coordination number in mature CAO decreases from 5 to 3 ligands following the chemical reduction of Cu(II) (13), it is easy to envision the binding of dioxygen to two vacant sites on Cu(I) in a side-on fashion in the unprocessed protein. The occupation of a metal binding site normally reserved for the O-4 hydroxyl of the precursor tyrosine would prevent the formation of the tyrosinate-Cu(II) LMCT complex prior to the dissociation of superoxide and, therefore, limit Cu(I)-catalyzed TPQ biogenesis in HPAO (Fig. 2).

**CONCLUSION**

We have shown that the in vitro reconstitution of apo-HPAO with Cu(I) supports TPQ biogenesis, albeit at a rate much slower than the Cu(II)-dependent reaction. As shown (Fig. 2), the bound Cu(I) ion is expected to readily reduce bound dioxygen to superoxide and a Cu(II) center, which is rendered chemically competent only upon the dissociation of the superoxide ligand. The newly unveiled cupric ion is subsequently coordinated by the precursor tyrosine \( (\text{Tyr-405}) \) to form the key tyrosinate-Cu(II) LMCT complex, an action proposed to activate the tyrosyl ring toward a direct reaction with triplet state dioxygen and thus facilitate the monooxygenation reaction \( (6–8) \). Although this and previous studies establish divalent copper as the actual catalyst in TPQ biogenesis, Cu(I) may still have biological relevance in this post-translational reaction in HPAO, a resident of the peroxisome. The accepted model for the delivery of matrix proteins to the peroxisome centers around mRNA translation on free polyribosomes in the cytosol and the subsequent translocation of folded proteins across a multiple protein complex on the peroxisomal membrane (20). Given the apparent absence of copper-transporting proteins on the peroxisomal membrane and low probability for the existence of a pool of free copper in the lumen of this oxidizing organelle, the acquisition of Cu(I) by newly folded HPAO in the cytosol prior to import into peroxisome is plausible. The maintenance of Cu(I)-HPAO in an inactive, cofactorless state for an extended period of time \( (t_{1/2}, 2.5 \text{h}) \) may serve a protective function in the cell. This strategy would prevent the generation of deleterious reduced oxygen species \( (i.e. \text{H}_2\text{O}_2) \), a product of amine oxidation and TPQ biogenesis, prior to the translocation of metallated HPAO into the lumen of the peroxisome.

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Cu(II)-dependent TPQ Biogenesis in HPAO

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