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Characterization of the Preprocessed Copper Site Equilibrium in Amine Oxidase and Assignment of the Reactive Copper Site in Topaquinone Biogenesis

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Abstract

Copper-dependent amine oxidases produce their redox active cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), via the Cu^{II}-catalyzed oxygenation of an active site tyrosine. This study addresses possible mechanisms for this biogenesis process by presenting the geometric and electronic structure characterization of the Cu^{II}-bound, prebiogenesis (preprocessed) active site of the enzyme *Arthrobacter globiformis* amine oxidase (AGAO). Cu^{II}-loading into the preprocessed AGAO active site is slow ($k_{\text{obs}} = 0.13 \text{ h}^{-1}$), and is preceded by Cu^{II} binding in a separate kinetically favored site that is distinct from the active site. Preprocessed active site Cu^{II} is in a thermal equilibrium between two species, an entropically favored form with tyrosine protonated and unbound from the Cu^{II} site, and an enthalpically favored form with tyrosine bound deprotonated to the Cu^{II} active site. It is shown that the Cu^{II}-tyrosinate bound form is directly active in biogenesis. The electronic structure determined for the reactive form of the preprocessed Cu^{II} active site is inconsistent with a biogenesis pathway that proceeds through a Cu^I-tyrosyl radical intermediate, but consistent with a pathway that overcomes the spin forbidden reaction of ³O₂ with the bound singlet substrate via a three-electron concerted charge-transfer mechanism.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/jacs.9b01922](https://doi.org/10.1021/jacs.9b01922).

Additional EPR fitting information, spectroscopic data not shown in the main text, and additional computational details (PDF)

Notes

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1. INTRODUCTION

Copper is utilized in a wide range of biological O_2 reactions.¹ Owing to the spin-forbiddenness of reactions between singlet organic substrates and $3O_2$ as well as the energetic favorability of O_2 reduction by Cu^I ,¹ the majority of biological copper–oxygen reactions involve the direct reduction of 3O_2 by Cu^I sites.² However, there are two known classes of enzymes that instead utilize mononuclear Cu^{II} centers to activate singlet organic substrates to react with 3O_2 . These are quercetin 2,4-dioxygenase, which catalyzes the $4e^-$ dioxygenation of flavone substrates (Scheme 1A),³ and the amine oxidase (AO) cofactor biogenesis reaction, in which a Cu^{II} center mediates the six electron oxidation of an active site tyrosine to form the cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ, Scheme 1B) or the closely related lysyl tyrosylquinone (LTQ).⁴

AOs are ubiquitous across the five kingdoms of life and utilize their post-translationally derived quinone cofactor to oxidize primary amines to aldehydes.⁵ The TPQ cofactor carries out this reaction via Schiff base formation and hydrolysis of amine substrates.⁶ This is followed by a regenerative step in which the cofactor reduces O_2 by two electrons and releases peroxide and ammonia to return to its catalytically active form. This regenerative step is mediated by the Cu^{II} , which reacts via a Cu^I -semiquinone radical charge transfer complex.^{7,8} Thus, the active site copper participates in two distinct reactions, the single turnover biogenesis formation of the TPQ from an active site tyrosine, and the catalytic oxidation of amines to aldehydes.

TPQ biogenesis is a Cu^{II} -dependent process that consumes two molecules of O_2 and involves the insertion of two oxygen atoms into the phenolic ring of an active site tyrosine (Scheme 1B).^{9–11} Although isotopic labeling studies (to determine whether the TPQ oxygens derive from O_2 or solvent) are not feasible due to rapid exchange,¹² crystallographic,¹³ mutant,¹⁴ and model¹⁵ studies have shown that the AO biogenesis reaction occurs through one monooxygenation and one hydration step (Scheme 1B). However, little is known about the mechanistic nature of these steps or the geometric and electronic structure adopted by the active site copper prior to or during the biogenesis reaction. The Cu^{II} -loaded, preprocessed active site structure is a topic of considerable ambiguity. While many biogenesis proposals invoke the reaction of a Cutyrosine complex, the crystal structure of preprocessed bacterial *Arthrobacter globiformis* AO (AGAO) shows copper coordinated by only three histidines, with the tyrosine to be processed observed at distances of 2.6 and 2.8 Å in the two separate asymmetric units, both of which are too far to

constitute a bond (Figure 1A).¹³ A similarly long metal–tyrosine bond length was measured in the crystal structure of cobalt substituted preprocessed AGAO.¹⁶ These structures provide no evidence for copper-coordinated solvent molecules, and the preprocessed CuAGAO structure shows two His592 positions with relative populations of 70% and 30%, as well as Cu–His distances that differ by as much as 0.4 Å between the two asymmetric units. Collectively, these features may reflect photoreduction of the copper, and do not provide a definitive preprocessed active site structure.

To date, the only spectroscopic intermediate observed in a wild-type amine oxidase is a 350 nm absorbance band in the yeast amine oxidase from *Hansenula polymorpha* (HPAO), and it is proposed to be a Cu^{II}-tyrosinate complex that forms after O₂ addition but prior to the first O₂ reaction and decays at the rate of TPQ formation.^{17,18} While a range of chemical and spectroscopic techniques have been employed to detect either Cu^I or organic radical in this intermediate and in the preprocessed site, neither has been observed for either species.¹⁹ In spite of the lack of evidence for Cu^I-radical formation, most biogenesis mechanistic proposals invoke Cu^I-tyrosyl radical character to explain O₂ reduction by the Cu^{II} active site.²⁰ The lack of evidence for Cu^I or organic radical buildup during biogenesis has been explained by invoking Cu^I-tyrosyl radical ground state character^{13,21} or a charge-transfer equilibrium with a Cu^I-tyrosyl radical state that is present in low concentration.^{22,23}

The basis for preprocessed AO reactivity toward O₂, and in particular whether the preprocessed Cu^{II} site has significant Cu^I-tyrosyl radical character or a low enough energy tyrosine to Cu^{II} CT for this to be accessible, are addressed in the present study of the holo, preprocessed enzyme. We define the geometric and electronic structure of the Cu^{II} center in AGAO that is active in biogenesis, and in doing so we address the Cu^I-tyrosyl radical and other possible mechanisms for activation of a singlet tyrosinate substrate for the reaction with ³O₂.

2. METHODS

2.1. Materials.

All reagents were purchased at the highest grade commercially available.

2.2. Preparation of Preprocessed AGAO.

Wild-type and mutant AGAO were purified in the metal-free (apo) form. Aliquots of apo preprocessed AGAO were taken from stock solutions and buffer exchanged into 25 mM MES (for pH 6–6.5), HEPES (for pH 7–8), or CHES (pH 8.5–9) using 30 kDa centrifugal filters. The enzyme was then degassed for a minimum of 30 min and transferred to a N₂ atmosphere glovebox before being loaded with 0.8 equiv of CuSO₄·5H₂O or 1.0 equiv of ZnCl₂ per enzyme active site unless otherwise noted. The enzyme was then incubated at room temperature in inert atmosphere for 6 h. Samples were cooled to 0 °C and then frozen in liquid nitrogen unless otherwise indicated. Magnetic circular dichroism (MCD) samples were prepared with 55% glycerol and 45% buffer by weight, loaded into 300 μL cells, and transparent glasses.

2.3. Preparation of Freeze-Quench Samples.

AGAO bio-genesis intermediates were prepared by mixing equal volumes of O₂ saturated buffer and 200 ± 20 μM holo, preprocessed enzyme directly into electron paramagnetic resonance (EPR) tubes and freezing in liquid nitrogen–ethanol slurries at the indicated time points. Variable-temperature freeze-quench intermediates were prepared by mixing holo, preprocessed AGAO samples with glycerol so that their freezing points were 1–2 K above their indicated freezing temperatures. The samples were then transferred into EPR tubes and anaerobically incubated at 1–2 K above the indicated temperature for 5 min before finally being rapidly frozen in liquid nitrogen, ethanol slurries. The absence of a glycerol induced effect on the preprocessed AGAO equilibrium was confirmed for the highest concentration of glycerol used.

2.4. EPR.

X-band EPR spectra were obtained with a Bruker EMX spectrometer, an ER 041 XG microwave bridge, and an ER4119HS resonator. A sample temperature of 77 K was maintained using a liquid nitrogen finger dewar. The X-band EPR parameters were as follows: freq ≈ 9.6 GHz, power ≈ 2 mW, rec. gain = 30, mod. freq = 100 kHz, mod. amp. = 4.00G, time constant = 327.68 ms, conversion time = 81.92 ms. Q-band EPR spectra were obtained at 77 K using an ER051QT microwave bridge, an ER 5106QT resonator, and an Oxford continuous-flow CF935 cryostat. The Q-band EPR parameters were as follows: freq 34.0 ≈ GHz, power = 0.1 mW, mod. amp. = 4.00G. EPR spin quantitation of the paramagnetic copper content was performed using a 0.250 mM standard solution of CuSO₄·5H₂O, 2 mM HCl, and 2 M NaClO₄. EPR simulations were performed using the EasySpin²⁴ Matlab software package.

2.5. UV–Vis.

UV–visible (UV–vis) absorption spectra were acquired on an Agilent 8453 diode array spectrophotometer.

2.6. CD and MCD.

CD and MCD data in the NIR (600–2000 nm) were collected on a Jasco spectropolarimeter (J730) operating with a liquid N₂-cooled InSb detector and in the UV–vis (300 nm–800 nm) on a Jasco J810 with an S20 PMT detector. The MCD spectra were recorded at 3 T, 5 T, and 7 T at temperatures between 3 K and 20 K. The sample temperature was measured with a calibrated Cernox resistor (Lakeshore Cryogenics) inserted into the MCD cell.

2.7. Stopped-Flow.

Stopped-flow was performed on an Applied Photophysics SX.18MV stopped-flow absorption spectrophotometer equipped with a photodiode array. The AGAO biogenesis reaction was performed by mixing room temperature O₂ saturated 25 mM buffer with 200 ± 30 μM holo, preprocessed enzyme.

2.8. DFT Calculations.

Spin-unrestricted density functional theory (DFT) calculations were performed using the Gaussian 09 software package²⁵ using the functionals B3LYP and BP86 with 0%, 10%, and 38% Hartree–Fock exchange,²⁶ the mixed triple- ζ /double- ζ basis set tzvp,²⁷ and a polarized continuum solvent correction (PCM, $\epsilon = 4.0$). Absorption spectra were calculated using time dependent DFT (TD-DFT). Calculations of the electronic g matrix were calculated with the ORCA code using the functional BP86 with 38% Hartree–Fock exchange and the basis set tzvp. Molecular orbital compositions from DFT single point calculations were calculated using the program QMForge²⁸ (c^2 and Mulliken population analyses (CSPA and MPA, respectively)).

3. RESULTS

3.1. Copper-Loading.

Anaerobic Cu^{II} loading into AGAO reveals two metal binding sites, a kinetically favored site that binds copper rapidly but reversibly, and a thermodynamically favored site that binds copper slowly but with a much higher affinity. Cu^{II} loading to apo-AGAO first leads to rapid ($>0.1 \text{ s}^{-1}$, estimated based on the full appearance of kinetic site features in the time between copper loading and spectroscopic measurement) formation of the kinetically favored binding site. This site has an axial EPR spectrum with four parallel hyperfine lines (Figure 2A), revealing that this Cu^{II} bound site is a single species (note that a small amount of thermodynamically favored site is present in the spectrum shown). Electronic spectroscopies (absorbance and CD) of the Cu^{II} loaded kinetic binding site show three distinguishing features: a moderately intense absorbance band at 28000 cm^{-1} , a 24500 cm^{-1} band that is negative in CD, and a set of bands in CD at 14000 cm^{-1} that are intense and derivative shaped (Figure 2B,C). The presence of CD intensity in the ligand-field (LF) ($\sim 14000 \text{ cm}^{-1}$) and charge transfer (24500 cm^{-1}) regions indicate that this Cu^{II} is bound to the protein.

Stoichiometric anaerobic Cu^{II} loading to apo AGAO leads to the rapid formation of the kinetic site followed by its slow decay. This decay occurs at the same rate that the thermodynamically favored site features grow in, and proceeds until complete conversion to the thermodynamically favored site has occurred (Figure 3A and B). In the conversion from the kinetic to thermodynamic site, the 28000 cm^{-1} kinetic site absorbance feature decreases while a weaker 22500 cm^{-1} band grows (Figure 3A). In the CD, a negative band at 10000 cm^{-1} grows in and the negative 24500 cm^{-1} band of the kinetic site is replaced by two positive bands at 22500 and 28000 cm^{-1} (Figure 3B). The rates of these processes were fit with a kinetic model that will be described later (Figure 3C). Over 6 h of anaerobic Cu^{II} loading, the single species axial EPR spectrum of the kinetic site is replaced by an EPR spectrum of the thermodynamically favored site whose features are distinct from those of Cu^{II} in the kinetically favored site, and which collectively reflect the presence of two thermodynamic site species (preprocessed Figure 4A) that are in equilibrium (vide infra).

Addition of excess Cu^{II} to preloaded AGAO shows that the thermodynamic site is distinct from the kinetic site. Following Zn^{II} loading into the thermodynamic site (based the lack of biogenesis upon subsequent aerobic Cu^{II}-loading and the crystal structure of Zn^{II} bound in

HPAO²⁹), aerobic additions of Cu^{II} result in the growth of the features of the kinetic site (along with a small amount of excess solution Cu^{II} in EPR, Figure 5A and B), demonstrating that the kinetic and thermodynamic species correspond to distinct metal binding sites. The intensities of the kinetic site features grow with increasing amounts of Cu^{II}, indicating that Cu^{II} in the kinetic site is in equilibrium with aqueous Cu^{II}. Additionally, with AGAO loaded anaerobically with one equivalent of Cu^{II} in the thermodynamic site, subsequent anaerobic additions of Cu^{II} result only in the growth of the 28 000 cm⁻¹ absorbance band associated with the kinetically favored site (Figure 5C). The EPR spectrum of stoichiometric Cu^{II} loaded anaerobic AGAO shows contributions from the thermodynamic and kinetic Cu^{II} sites (Figure 5D). Thus, the thermodynamic and kinetic forms correspond to separate, distinct sites. Cu^{II}-loading in HPAO shows similar Cu^{II}-loading kinetics with separate kinetic and thermodynamic binding sites.¹⁷ However, in the case of HPAO the kinetic binding site features decrease over time, even with the thermodynamically favored site occupied.

In order to distinguish whether the thermodynamic or kinetic site is active in biogenesis, the rate of topaquinone product formation was measured following two different Cu^{II} incubation times from experiments run in parallel. In both experiments, 0.8 equiv of Cu^{II} was added to anaerobic, apo-AGAO. Biogenesis was then initiated with O₂ after either 4 min or 12 h. Following the 12 h Cu^{II} incubation (after which only the thermodynamic site is populated), O₂ initiated biogenesis proceeds at a rate of $k_{\text{obs}} = 0.025 \pm 0.003 \text{ s}^{-1}$ (Figure 3D). For the 4 min Cu^{II} incubation time (populating less than 4% of the thermodynamic site, based on the Cu^{II} loading rate given in the model described below), the topaquinone biogenesis product appears approximately 700-times more slowly than for O₂ initiated biogenesis following a 12 h Cu^{II} incubation (Figure 3E). On this basis, the thermodynamic Cu^{II} binding site can be assigned as the site active in the O₂ biogenesis reaction, and the slow rate of biogenesis with Cu^{II} aerobically added to the apo enzyme is associated with the kinetics of the slow Cu^{II}-loading into the preprocessed active site. While a simple second order kinetic model gives moderate agreement with the Cu^{II}-loading data (SI Figure 7), this model is substantially improved by including the kinetic binding site equilibrium. The model given below provides the best fit to the Cu^{II}-loading and biogenesis data in Figure 3C and F:

$k_{\text{biogenesis}}$ was obtained from the O₂ reaction in the 12 h Cu^{II}-loading experiment, while K_{eq} for the kinetic site binding equilibrium with solution Cu^{II} and k_{loading} for the Cu^{II}-loading rate of the thermodynamic site were optimized using a differential evolution method.³⁰ This model is also consistent with the kinetics observed previously for a 30 min loading time but with 10-fold excess Cu^{II}.¹⁰

It is important to now emphasize that as noted above the anaerobic thermodynamic site exhibits a complex EPR spectrum having contributions from 2 Cu^{II} centers (Figure 4A–C), in a 4:1 ratio at 0 °C (vide infra) with a total Cu^{II} content from spin integration of 1.0 ± 0.2 per enzyme monomer. Reaction of this 4:1 mixture with O₂ (Figure 4A) converts it to a single Cu^{II} species with an EPR spectrum (Figure 4D) that is different from either form of

the preprocessed active site and is associated with the processed enzyme, showing that both of the Cu^{II} species are associated with the thermodynamic site.

3.2. Chemistry and Spectroscopy of the Preprocessed Sites.

Given the monophasic profile of topaquinone formation for preloaded, preprocessed AO (Figure 3F, red), either both species present in the thermodynamic site of preprocessed AGAO perform biogenesis at the same rate or the two forms are in an equilibrium rapid relative to the rate of biogenesis ($k_{\text{obs}} = 0.025 \text{ s}^{-1}$) and only one performs biogenesis. The preprocessed EPR spectrum does not change across the pH range in which the enzyme is viable 6–9 (Figure 6). Note that Figure 6A shows representative spectra with the error bars shown for the speciation in Figure 6B deriving from the variability over numerous experiments (see SI Figure 3). Thus, the two $\text{Cu}(\text{II})$ species in preprocessed are not in a pH equilibrium. However, the ratio between the two preprocessed forms is responsive to changes in temperature, with the less prevalent (minor) form increasing in concentration relative to the major form at lower temperatures (Figure 7). In samples rapidly frozen (relative to the rate of the major form to minor form interconversion, which we bracket to be between 0.025 and 0.2 s^{-1} , vide infra) from different incubation temperatures, the major form/minor form ratio changes from 4:1 at 0°C to almost 2:1 at -23°C (Figure 7). The thermodynamics of the major form to minor form conversion were determined from the temperature dependence of the equilibrium (obtained from van't Hoff fits to the variable temperature (rapidly frozen) EPR data (Figure 7B)) to be $H^\circ = -2.5 \pm 0.5 \text{ kcal/mol}$ and $S^\circ = -12 \pm 2 \text{ cal/Kmol}$, giving $T \Delta S^\circ = -3.5 \pm 0.7 \text{ kcal/mol}$ as the entropic contribution to the Gibbs free energy at room temperature (Figure 7). Thus, the preprocessed Cu^{II} is an equilibrium mixture with the major form entropically favored and the minor form enthalpically favored.

Fits of Q-band (SI Figure 2) and variable temperature rapidly frozen X-band EPR (Figure 7) spectra allowed EPR fits for the major and minor forms of the preprocessed equilibrium. The spectrum of the major form (Figure 4B and Table 1) is axial ($g_{\perp} = 0.033$) with a large $A_{\parallel}$ value ($|158| \times 10^{-4} \text{ cm}^{-1}$). The minor form has a rhombic EPR spectrum (Figure 4C and Table 1), with $g_{\perp} = 0.127$ and a small $A_{\parallel}$ of $|106| \times 10^{-4} \text{ cm}^{-1}$. Importantly, as shown in subsection 3.4, the EPR spectra of both forms reflect Cu^{II} centers with no significant ground state Cu^{I} -character or evidence of coupling to an organic radical.

3.3. Identification of Major and Minor Form Contributions to Preprocessed Spectroscopy.

The absorbance spectrum for the thermodynamic preprocessed site that is a mixture of major and minor species contains no bands more intense than $\epsilon \approx 100 \text{ M}^{-1} \text{ cm}^{-1}$ (approximating the site as a single species) below the $33\,000 \text{ cm}^{-1}$ protein cutoff (Figure 8A, absorbance). The MCD spectrum of this site (Figure 8A, MCD) has five distinct features between 7600 and $18\,400 \text{ cm}^{-1}$ (a positive feature at 7600 cm^{-1} , a negative feature at 9000 cm^{-1} , a positive feature at $12\,500 \text{ cm}^{-1}$, and two negative features at $15\,900$ and $18\,400 \text{ cm}^{-1}$, respectively) with large MCD to absorbance intensity ratios (C_0/D_0 , Table 2) that allow these to be assigned as LF transitions. Since a single Cu^{II} site can only have four ligand field bands, this reflects contributions from the two preprocessed active site Cu^{II} species. Based on variable temperature absorbance spectra of wild-type AGAO and MCD data on mutant AGAO

presented below, the LF region was fit with seven ligand field bands (#1–4 for the four LF transitions of the major form and #1'–3' for three LF bands of the minor form). The spectra in Figure 8A further identify the 22 500 cm⁻¹ band (4') as a charge transfer transition based on its low C_0/D_0 ratio (Figure 8A, Table 2 preprocessed). From Figure 8A MCD, two additional bands are present at 28 000 cm⁻¹ (minor form band #5') and 31 800 cm⁻¹ (major form band #5). These bands were assigned to the major and minor forms based on the Y382F mutant MCD data below, and are at reasonable energies for Cu^{II} LMCT transitions. Note that all preprocessed samples contained 1–2% TPQ, and thus, a band reflecting the presence 1% TPQ was included in the fits of the preprocessed spectra.

To further assist in the assignment of the major and minor forms, the Y382F variant, with the processing first-sphere tyrosine replaced by phenylalanine, was spectroscopically characterized in parallel to the wild-type preprocessed enzyme. This variant exhibits similar active site copper loading kinetics as the wild-type (SI Figure 7), i.e., a kinetic site that converts into a thermodynamically favored site, but yields a form that is unreactive toward dioxygen, as the tyrosine to be processed has been replaced by phenylalanine. The EPR spectrum of the Cu^{II} loaded, thermodynamically favored active site in Y382F shows a single, axial spectrum similar to that of the major component of the wild-type preprocessed spectrum (Figure 4E). Importantly, the MCD spectrum of Cu^{II} loaded Y382F AO contains 4 ligand field transitions between 8300 and 17900 cm⁻¹ (Figure 8B, Table 2 Y382F bands 1–4), whose energies and signs correlate well to the dominant LF bands in preprocessed (Figure 8A, Table 2 preprocessed bands 1–4). On this basis, the single form in Y382F is taken to correspond to the Cu^{II}-loaded wild-type preprocessed major form, requiring that the major component of the WT thermodynamic site cannot have a tyrosine coordinated to Cu^{II}, since this tyrosine is not present in Y382F. The small differences in g and A values observed between the major form and Y382F in these EPR spectra (Figure 4B and E) are likely due to changes in the hydrogen bonding architecture of the active site copper upon replacing a noncoordinated tyrosine with a non-H-bonding phenylalanine.

The strong correlation between the EPR and MCD spectra of the Y382F variant and the major component of the preprocessed equilibrium further allows assignment of the transitions associated with the minor and major forms of WT. The 9000 cm⁻¹ negative LF band (2', Figure 8A MCD) and the 22 500 cm⁻¹ positive charge transfer band (4', Figure 8A MCD) are present in the wild-type preprocessed MCD spectrum but absent in the Y382F spectrum (Figure 8A and B, Table 2), allowing these transitions to be assigned to the minor form. The 22 500 cm⁻¹ charge transfer band increases in absorbance intensity with decreasing temperature (SI Figure 8), supporting its assignment to the minor form of the preprocessed equilibrium. Given that the absorption intensity in this region is highly sensitive to the small amount (~1%) of used to quantify the ϵ of this band, yielding $390 \pm 200 \text{ M}^{-1} \text{ cm}^{-1}$ for a pure minor form Cu^{II} site. Additionally, the change in the intensity pattern of bands 1 and 2 between the wild-type preprocessed and Y382F MCD spectra (Figure 8A and B MCD), with band 1 half as intense as band 2 in Y382F and twice as intense as band 2 in wild-type preprocessed, reveals the presence of low energy minor form band at 7600 cm⁻¹ (1', Figure 8A). In the high energy region of the MCD spectra, the 30300 cm⁻¹ negative band in Y382F correlates to the 31800 cm⁻¹ band in preprocessed, allowing it to be assigned to the major form of the preprocessed equilibrium (Figure 8 and Table 2, band

#5), while the $28\,000\text{ cm}^{-1}$ negative band (minor form band #5') that is present in the preprocessed MCD is not present in Y382F, allowing it to be assigned to the minor form of the preprocessed equilibrium.

Given that the minor form is absent in the Y382F variant that does not have a nearby tyrosine, and that in wild-type AO the only residues within bonding distance of copper are tyrosine and histidine, the tyrosinate is the only possible donor for the $22\,500\text{ cm}^{-1}$ ligand-to-metal charge transfer transition (preprocessed band #4'). Thus, the minor form can be assigned as a tyrosinate-bound- Cu^{II} species that is present at 5% in the preprocessed enzyme at room temperature (and 20% at 0°C).

3.4. Assignment of the Spectroscopy of the Processed Form.

The processed enzyme active site has previously been shown to be a square pyramidal site with three histidine and two water ligands (Figure 1B),³¹ and provides useful reference below for analysis of spectroscopic features of the preprocessed forms. The EPR spectrum of the processed active site (Figure 4D, Table 1) is somewhat rhombic ($g_{\perp} = 0.053$) with a large $A_{\parallel}$ ($|160| \times 10^{-4}\text{ cm}^{-1}$). The MCD spectrum (Figure 8C) contains four bands between $10\,500$ and $15\,300\text{ cm}^{-1}$ assigned as ligand field transitions based on their large C0/D0 ratios and one higher energy negative MCD band at $33\,000\text{ cm}^{-1}$. The assignments of specific LF transitions and the negative $33\,000\text{ cm}^{-1}$ band is based on their MCD signs and TDDFT calculations (Table 2 processed).

3.5. Analysis of Preprocessed Minor Form Spectroscopy.

It will be shown in the subsection 3.7 section that the minor species is the relevant species in biogenesis, and thus its electronic structure is an important aspect of the activation of its bound tyrosinate ligand for biogenesis. Quantitative comparison of the spectroscopy of the minor form of the preprocessed enzyme with the Cu^{II} species in the processed form of AGAO reveals that the Cu^{II} site in the minor form has low covalency and little Cu^{I} radical character, similar to processed. In subsections 3.3 and 3.4 we identified three LF bands belonging to the minor form of the preprocessed active site and all four LF transitions belonging to the processed site. These bands are assigned based on their MCD signs and the energy order found in TD-DFT calculations (see subsection 3.8 section), as summarized in Tables 2 and 3.

The energies of the assigned LF transitions and the spin Hamiltonian g and A values from EPR were used to calculate ground (γ_{GS}^2) and LF excited state ($\gamma_{xz,yz}^2$ and γ^2) Cu^{II} d-minor preprocessed sites. Based on the rhombic splitting in the EPR spectra (i.e., g_x , g_y and A_x , A_y), the EPR parameters and hyperfine contributions $A^{\text{total}} = A^{\text{Fermi-contact}} + A^{\text{spin-dipolar}} + A^{\text{indirect-dipolar}}$ were fit with equations which include d_z^2 mixing into a Cu^{II} $d_x^2-y^2$ ground state,^{32,33} and are summarized in the SI. d-orbital character in molecular orbital $i(\gamma_i^2)$ and the ratio of $d_{x^2-y^2}:d_{z^2}$ (α/β) character in the ground state ($\alpha + \beta = 1$) were fit using the g -value equations:

$$\Delta g_z = \frac{-8\lambda\gamma_{\text{GS}}^2\gamma_{xy}^2\alpha^2}{E_{d_{xy}} - E_{\text{GS}}},$$

$$\Delta g_y = \frac{-2\lambda\gamma_{\text{GS}}^2\gamma_{\text{E}}^2(\alpha + \sqrt{3}\beta)^2}{E_{d_{xz}} - E_{\text{GS}}},$$

$$\Delta g_x = \frac{-2\lambda\gamma_{\text{GS}}^2\gamma_{\text{E}}^2(\alpha - \sqrt{3}\beta)^2}{E_{d_{yz}} - E_{\text{GS}}}$$

where the $E_d - E_{\text{GS}}$ are the d_i LF transition energies measured in MCD and the d_{xz}/d_{yz} LF state covalencies are treated as equal ($\gamma_{xz,yz}^2$). Given that only three of the four LF energies are resolved for the minor form of preprocessed in Figure 8A, the ratio of g_x and g_y was used to determine (α/β) . $A^{\text{indirect-dipolar}}$ values were then calculated using only the experimental g -values and the (α/β) coefficients obtained by fitting the above g -value equations. The $A^{\text{indirect-dipolar}}$ values obtained in this way include the covalent reductions in orbital angular momentum inherent to the experimental g -values, but do not account for ligand-orbital contributions to g -values, which are generally small and should be similar between the processed and preprocessed minor sites. The $A^{\text{Fermi-contact}}$ and $A^{\text{spin-dipolar}}$ terms were then obtained by subtracting $A^{\text{indirect-dipolar}}$ from the experimental hyperfine values and then separating their isotropic contribution (i.e., $A^{\text{Fermi-contact}}$) from the anisotropic ($A^{\text{spin-dipolar}}$) term. For both the processed and preprocessed minor forms, only one set of signs for the experimental hyperfine coupling constants lead to axial $A^{\text{spin-dipolar}}$ tensors, and were assigned accordingly. See SI 11 for further details.

The processed active site spectroscopy gives a ground state with 75% Cu-character, of which 98% is $d_x^2-y^2$ and 2% is d_z^2 . The minor form of the preprocessed active site has similar but slightly higher covalency reflected by its 70% ground state Cu-character, of which 93% is $d_x^2-y^2$ and 7% is d_z^2 . The 5% greater ground state d_z^2 character in the minor form accounts for its higher g and A -tensor rhombicity and smaller $g_{\parallel}$ -value relative to processed. From the SI, the smaller $A_{\parallel}$ -value of minor derives from a diminished magnitude of $A^{\text{Fermi-contact}}$, which reflects a combination of greater 4s ground state character (up to 2% relative to the processed site) and possibly also increased spin-polarization of the occupied valence orbitals,³⁴ which are expected given that 4s mixing and $3d_z^2$ character tend to correlate. Note that while $4p_z$ mixing is sometimes invoked to explain a small Cu $A_{\parallel}$, and indeed this would even lower the covalency we estimate, our calculations of preprocessed minor show no evidence for this mixing (vide infra). As the preprocessed minor form has similar ground state Cu^{II} character to the processed site which has histidine and water ligands that are not very covalent, the tyrosinate- Cu^{II} bond in minor is not significantly covalently delocalized onto the Cu^{II} center.

This is further evidenced by the low ϵ of the tyrosinate-Cu^{II} LMCT band, as charge transfer intensity reflects overlap between ligand donor and metal acceptor orbitals, in this case the tyrosinate out-of-plane π HOMO and Cu^{II} half occupied β -LUMO (vide infra). The low ϵ ($390 \pm 200 \text{ M}^{-1} \text{ cm}^{-1}$) and in italic for preprocessed AGAO. high energy ($22\,500 \text{ cm}^{-1}$) of this feature are atypical compared to those of previously characterized Cu^{II}-phenolate model complexes.³⁵ In the model complex with out-of-plane phenolate out-of-plane HOMO to Cu^{II} β -LUMO CT is at 14730 cm^{-1} with an ϵ of $2360 \text{ M}^{-1} \text{ cm}^{-1}$. As shown in SI Figure 9, the energy of this CT is determined by the energy of the β -LUMO and thus the ligand-field at the Cu^{II}. In going from the model to the preprocessed minor form of the active site, the coordination number increases from 4-coordinate tetrahedral to 5-coordinate square pyramidal and the His ligands are somewhat better donors than the pyrazolyls, thus accounting for the 7800 cm^{-1} shift of this CT transition to higher energy. The low ϵ of this feature reflects a low overlap between the tyrosinate HOMO donor and the Cu^{II} β -LUMO acceptor orbitals. Given the crystal structure and DFT calculations presented below, this decreased interaction relative to the phenolate-Cu^{II} model complexes arises from the tyrosinate being distorted out of the equatorial plane, and thus having poor overlap with the $d_{x^2-y^2}$ copper β -LUMO. Thus, the minor form of the preprocessed active site does not have particularly covalent bonds with its ligands including the tyrosinate. There is no experimentally measurable Cu^I-tyrosyl radical character in this ground state.

3.6. Analysis of Preprocessed Major form Spectroscopy.

Possible structures for the major component of the wild-type preprocessed Cu^{II} site are limited by its correlation to the Y382F variant, which necessitates that the major form does not include a tyrosinate-Cu^{II} bond. Over the accessible pH range, the uncoordinated tyrosine should be protonated. The preprocessed equilibrium is invariant to pH (Figure 6), and thus the major and minor forms of the preprocessed equilibrium must be related by an internal proton transfer. While it is plausible that the resultant deprotonated species in major is not bound to the Cu^{II}, this possibility can be eliminated by comparison to processed, oxidized AGAO. The LF manifold of processed (bands 1–4, Figure 8C, Table 2 processed) and the major preprocessed form (bands 1–4, Figures 8A, Table 2 preprocessed) are different. Given that the Cu^{II}-site in the minor form of the preprocessed equilibrium contains a deprotonated tyrosinate species, that in the major form this uncoordinated tyrosine is protonated, and that the major form does not correlate to the processed, 2-water, 3-histidine LF, the major form of the preprocessed equilibrium can reasonably be assigned as a Cu^{II}-OH species.

3.7. Determination of the Reactive Form of the Preprocessed Cu^{II} Site.

Upon reaction with O₂, both the major and minor forms of the Cu^{II} preloaded preprocessed AO disappear over the time course of the biogenesis reaction (0.025 s^{-1}). The monophasic profile of TPQ formation necessitates that either both forms are competent toward major and minor forms was addressed by obtaining EPR spectra of early time points in the biogenesis reaction. Within 3 s, when less than 3% of active sites have transformed to processed, the minor form of the equilibrium has diminished by greater than 75% relative to the major form that remains unchanged (Figure 9). This requires that the minor form of preprocessed AO reacts rapidly with O₂ in the biogenesis reaction, and that conversion of the major to minor form occurs slowly relative to the first step of biogenesis. Upper and lower bounds for the

rate of interconversion between the major and minor forms of the preprocessed equilibrium can be estimated from biogenesis kinetics. Given that both forms of the preprocessed equilibrium disappear over the course of biogenesis, the lower limit for the rate for the major to minor form conversion is the rate of biogenesis (0.025 s^{-1}). The upper limit for the major to minor form conversion can be estimated from the nearly complete disappearance of the minor form over the first 6 s of the biogenesis reaction (Figure 9), yielding an upper bound of 0.2 s^{-1} .

3.8. Correlation to Calculations.

Of the three forms of wild-type AGAO characterized in this study, the processed form provides the strongest handle for calibrating calculations, as its structure has been unambiguously assigned as square pyramidal with three histidine and two water ligands.²⁶ Combinations of second-sphere active site residues, numbers of second-sphere solvent molecules, and DFT functionals ranging from 0 to 38% Hartree–Fock (HF) mixing were tested using the tzvp basis set and a PCM with $\epsilon = 4.0$. The starting structure used was obtained from the crystal structure of processed AGAO (Figure 1B, PDB: 1IVX) with the residues truncated and constrained at their α -carbons. The best agreement between the calculated TPQ structure (Figure 10A) and the experimental parameters was achieved with BP86 with 38% HF, the inclusion of the three first-sphere histidines, two Cu^{II} -bound waters, TPQ, the second sphere Asn381 for the sterics to orient the TPQ, Tyr284 which provides a hydrogen bond to TPQ, and Asp598 which provides a strong hydrogen bond to the one first sphere histidine σ -bonded to Cu^{II} .²² Close agreement was achieved for all structural and spectroscopic parameters (Table 3) with the exception of $g_{\parallel}$, which was consistently calculated to be 0.05–0.1 too low across all tested functionals. This discrepancy is typical of DFT calculated copper g -tensors.³⁶ The total ground state Cu^{II} character of the processed active site was calculated to be 74.1%, which is close to the experimentally determined ground state Cu^{II} character of 75%.

This computational method was then applied to possible major and minor structures using atomic positions from the preprocessed AGAO crystal structure (Figures 1A and 10B and C, PDB: 1IVU). The two positions for His592 observed in this structure were both evaluated.²² Only the more heavily occupied position (shown in Figure 10B and C), the one that is coincident with the His592 position in processed, provided a reasonable correlation to experimental data for both forms of the preprocessed enzyme (in calculated vs experimental g -tensors and absorbance features). The PDB structure 1IVU only resolves one active site water, which is hydrogen bonding to Tyr284 and Tyr382. This water was found to be necessary for keeping Tyr382 within $\sim 3 \text{ \AA}$ of the active site Cu^{II} for structures lacking a Cu^{II} –Tyr382 bond, and thus was included in the major and minor form structures. A water molecule within bonding distance of the active site Cu^{II} was also included in the calculations of the preprocessed structures based on the presence of a second water in the processed active site, and was found to be necessary for generating structures in good agreement with the experimentally determined parameters for the preprocessed active site forms.

Minor form structures were calculated using the crystal structure coordinates for the residues and solvent molecules described above. This resulted in a five-coordinate complex with

tyrosinate and water bound equatorially at 2.0 and 2.1 Å distances, respectively, in an overall distorted square pyramidal structure (Figure 10B). The calculated *g*-tensor and absorbance profile for the minor form of the preprocessed equilibrium matched well with experiment (Table 3), while also properly reflecting the minimal Cu(I)-tyrosyl radical character of the minor form of the preprocessed active site. The copper β -LUMO was calculated to have 76.2% Cu d-orbital character, of which 8% was in d_z^2 . While this reflects a lower overall covalency than was experimentally determined for the minor form of the preprocessed active site, the covalency is mainly distributed between the three first sphere histidines, and not on the tyrosinate, as there is only 6.7% tyr-character in the ground state, with less than 1% spin density in the tyrosinate ligand. This is due to the position of the tyrosinate relative to the Cu^{II} β -LUMO, which is distorted out of the plane of the Cu^{II} $d_{x^2-y^2}$ orbital (Figure 11), and thus provides a weak σ -interaction to the Cu^{II} β -LUMO with its out-of-plane hydroxyl π -orbital. While the calculated covalency is lower than the experimentally determined value of 70%, it is nearly identical to the calculated ground state Cu-character of the processed form, thus supporting the experimental assignment that the minor preprocessed and processed forms are similarly covalent.

The tyr-to-Cu CT transition of the minor form (Figure 8A, band 4') experimentally observed at 22 500 cm⁻¹ was calculated to be at 25 380 cm⁻¹ (Table 3). These energies reflect vertical excitations, not the geometrically relaxed zero-point energy of the CT state. Geometry optimization of this excited state resulted in 21 kcal/mol relaxation, yielding a calculated energy of 43 kcal/mol for the adiabatic energy of the CT state above the ground state, and thus it is not thermally accessible.

The major preprocessed Cu^{II} structure was then calculated by moving the proton from the copper-bound water to the copper bound tyrosinate, resulting in the loss of the tyrosine ligand to yield a 3-His and 1-hydroxide, distorted, square-planar complex with His592–Cu–His433 and His431–Cu–OH bond angles of 148.1° and 165.1°, respectively, and a short Cu–OH distance of 1.86 Å (SI Figure 10). The energies of the LF excited states calculated for this structure were not in good agreement with the experimentally determined values (the calculated LF energy splitting was 2700 cm⁻¹ as opposed to the experimentally measured LF splitting of 10 800 cm⁻¹ as shown in Table 2).

Thus, additional major form structures were calculated using all possible permutations of two hydroxide, one water and one hydroxide, or two water molecules in the axial and equatorial positions. Structures with two hydroxide ligands produced very rhombic *g*-tensors and were thus disregarded. Furthermore, it was not possible to optimize 5-coordinate structures with one water and one hydroxide bound, as the water would dissociate from both the axial or equatorial position yielding 4-coordinate complexes. Alternatively, starting from the major form structure obtained by transferring the proton from the Cu^{II}-bound water to the tyrosine in the optimized minor structure and then moving the three histidine α -carbon positions 0.10 Å closer to the Cu^{II}, the geometry optimized to a structure with ligand-field energies and a *g*-tensor in better agreement with experimental values than for all other tested 4- and 5-coordinate major form structures (Table 3). The discrepancies between experimental and calculated spectroscopic values for the major preprocessed form are likely due to the exclusion of second sphere species that would affect the orientation of the hydroxide that is

coordinated to copper. Given that the histidine α -carbon coordinates vary by 0.2–0.4 Å between the preprocessed and processed crystal structures, the 0.10 Å contraction of these constraints in this major structure reflects a plausible conversion between the preprocessed equilibrium forms.

Compared to the structure optimized from crystallographic α -carbon coordinates of preprocessed, the contracted structure is more D_{2d} distorted (with bond angles of 151.4° and 152.2°) and has a slightly lengthened Cu–OH bond of 1.90 Å (vs 1.86 Å in the major form structure obtained with preprocessed α -carbon coordinates). This D_{2d} distortion of the 4-coordinate structure LF is responsible for the larger LF splitting as observed experimentally. In this enzyme structure the Tyr382 is positioned similarly to its position in the crystal structure, as it is stabilized by a hydrogen bond to a crystallographically resolved water that is also hydrogen bonding to the second sphere Tyr284.

The major and minor preprocessed Cu^{II} sites (SI Figure 9 and Figure 10B) geometry optimized with the same crystallographic α -carbon positions were used to calculate the thermodynamics of the interconversion between the major and the minor forms. The minor form is calculated to be enthalpically favored by 4.1 kcal/mol and entropically disfavored by –2.8 kcal/mol at 298 K relative to the major form. These numbers are in agreement with the experimental values of $H = -2.5 \pm 0.5$ kcal/mol and $-T S = 3.5 \pm 0.7$ kcal/mol for the major to minor form conversion.

4. DISCUSSION

Preprocessed AGAO contains three types of Cu^{II} species, one in the kinetic binding site distinct from the active site and two thermodynamically favored active site species. Given that the kinetic binding site is distinct from the active site, it is possible that it corresponds to the putative calcium binding site recently elucidated in the homologue ECAO.³⁷ The two thermodynamically favored Cu^{II} site species are in a temperature dependent equilibrium. The minor form of this equilibrium is enthalpically favored and entropically disfavored relative to the major form, so that at room temperature it is present at only 5% relative to the major form. The minor form has the tyrosinate residue to be processed bound to the Cu^{II} and is reactive toward O_2 . The major form lacks this tyrosinate- Cu^{II} bond and appears to shift to the minor form for the O_2 reaction.

The orientation of the tyrosinate- Cu^{II} bond in the minor form, which is distorted out of the equatorial plane, results in a low covalent interaction between tyrosinate and Cu^{II} with little Cu^I -tyrosyl radical character. This is observed experimentally from the weak ϵ of the tyrosinate- Cu^{II} LMCT band of the minor form, which reflects low overlap between the tyrosinate HOMO and Cu^{II} half occupied β -LUMO, and the quantitative correspondence between the LF energies and spin Hamiltonian parameters for the processed and minor preprocessed forms of the active site, which indicates that both have similar ground state copper character. Furthermore, the preprocessed minor form contains no thermally accessible tyrosinate to Cu^{II} charge transfer state, as the vertical excitation energy to this charge transfer state is measured at 22 500 cm^{-1} (64 kcal/mol), and DFT calculations give

an adiabatic energy of 43 kcal/mol, and thus too high to contribute to the biogenesis pathway.

In the initial phase of the AGAO biogenesis reaction, the minor form of the preprocessed site directly reacts with O₂, however, this interaction is not through Cu^I-tyrosyl radical character or a low energy CT state. This differs from proposed mechanisms from computational studies on AGAO bio-genesis.^{21,23} The major/minor equilibrium for tyrosine binding also differs from the accepted biogenesis mechanism for the yeast homologue HPAO.³⁸ This mechanism was based on the growth of a 350 nm band upon addition of O₂ assigned as a tyrosinate-Cu^{II} LMCT that appears to form more quickly than O₂ is consumed. Herein, we identified and characterized the tyrosinate-Cu^{II} form of the preprocessed active site, and found that its LMCT is at 440 nm (6000 cm⁻¹ lower in energy than the HPAO 350 nm band) and has a low molar ϵ of $390 \pm 200 \text{ M}^{-1} \text{ cm}^{-1}$ (as opposed to $3200 \text{ M}^{-1} \text{ cm}^{-1}$ for the HPAO 350 nm intermediate). It is thus unlikely that the HPAO 350 nm species is a tyrosinate-Cu^{II} form of the enzyme. A possible alternative explanation for the observed HPAO biogenesis kinetics is that an undetected amount of Cu^{II}-tyrosinate form is also present in HPAO prior to the addition of O₂, and that this form rapidly reacts with O₂ to form the 350 nm species, which would be a downstream intermediate in the reaction to form TPQ.

Given that a tyrosinate-Cu^{II} form of the preprocessed active site reacts rapidly with O₂, a mechanism not involving a Cu^I-tyrosyl radical must be considered. One such possibility is the 3-electron concerted mechanism first developed for ferric protocechuate 3,4-dioxygenase,³⁹ and subsequently determined to be possible for biomimetic Cu(II)-phenolate AO model complexes.³⁰ For the Cu^{II}-phenolate model complexes, the barrier for this concerted O₂ reaction mechanism was calculated to be as low as 11.2 kcal/mol. The concerted mechanism involves three simultaneous one electron transfers and results in the formal two electron reduction of ³O₂ by a singlet organic substrate and a concomitant change in the spin on Cu^{II} (Figure 12). As shown in Figure 12, this can be described as the transfer of two α -spin electrons to a bridging O₂, one from the phenolate HOMO and one from the Cu^{II}. However, the α -spin electron transferred out of the Cu^{II} will be concertedly compensated by the transfer of a β -spin electron to the Cu^{II} from the phenolate HOMO (ie the LMCT transition). The preprocessed minor structure is well oriented to carry out this concerted process, which requires that one of the two perpendicular O₂ π^* orbitals overlap with a d π orbital on the Cu^{II} and the second with the out-of-plane HOMO on the substrate. In preprocessed AO, both bonding interactions are achieved by O₂ replacing the equatorial water on the Cu^{II}. Using the preprocessed reactive structure (minor) determined in this study (the tyrosinate-Cu^{II} site in Figure 10B), the complete biogenesis reaction will be experimentally and computationally evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Chemical Schemes of the Two Known Cu^{II} -Substrate Activating Enzymes^a (A) Flavone dioxygenation by quercetinase and (B) tyrosine dioxygenation in the biogenesis of the amine oxidase active site.

Figure 1.

Crystal structures of the (A) AGAO holo preprocessed active site (PDB: 1IVU) and (B) AGAO holo processed (TPQ-containing) active site (PDB: 1IVX). The sets of two distances shown for the preprocessed active site are from the two asymmetric crystal units. The Cu–N_{His} distances are between 1.8 and 2.2 Å unless noted in the figure. Atoms: Cu, orange; C, green; N, blue; and O, red.

Figure 2.

(A) 77 K EPR (spectrum shown in dark blue, fit shown as red dashed line), (B) room-temperature absorbance, and (C) room-temperature CD of Cu^{II}-loaded kinetic binding site. The sample used for spectrum A was frozen 6 min following Cu^{II} addition. The ϵ and $\Delta\epsilon$ values in panels (B) and (C) were determined using the holo-kinetic site concentrations predicted by the K_{eq} in subsection 3.1. All samples were prepared in 25 mM pH 7.0 HEPES buffer. The concentrations of the samples are 46 μM for panel (A), 100 μM for panel (B), and 80 μM for panel (C), with 80% stoichiometric copper loading in each case. Gaussian fits to the spectra in panels (B) and (C) are shown in purple, with the summed fits shown with dashed red lines.

Figure 3.

Absorbance (A) and CD (B) profiles over 5 h following 0.8 equiv of Cu^{II} added to 100 μM (Abs) and 80 μM (CD) apo enzyme, respectively. (C) The percent of kinetic site present at each time point was determined by fitting each spectrum with pure kinetic and thermodynamic site spectra (shown in Supporting Information (SI) Figure 1). The fit shown was determined with the equilibrium binding kinetic equation given in subsection 3.1 section and shown with a dashed line. (D) Absorbance spectra over 15 min of wild-type AGAO biogenesis initiated with O₂ 12 h after the addition of 0.8 equiv of Cu^{II} to 60 μM apo, anaerobic enzyme. (E) Absorbance spectra over 2.6 h of wild-type AGAO biogenesis initiated with O₂ 4 min after the addition of 0.8 equiv of Cu^{II} to 60 μM apo, anaerobic enzyme. (F) The rate of TPQ (490 nm band) formation following the 12 h Cu^{II} incubation (shown in red) was determined by the linear first-order kinetic equation: $\log[\text{TPQ}]_{\text{time}} = \log((\epsilon_{\text{time}} - \epsilon_{\text{final}})/(\epsilon_{\text{initial}} - \epsilon_{\text{final}}))$. The rate of TPQ formation following the 4 min Cu^{II}

incubation (shown in blue) was determined using the equilibrium binding kinetic equation given in subsection 3.1. All samples were prepared in 25 mM pH 7.0 HEPES buffer.

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Figure 4.

77 K EPR Spectra of (A) the thermodynamic site of anaerobic, preprocessed wild-type AGAO (rapidly frozen from 1 °C); the simulated spectrum of the anaerobic preprocessed major form (80% of the simulated spectrum A); (C) the simulated spectrum of the anaerobic preprocessed minor form (20% of the simulated spectrum A); (D) aerobic processed AGAO; and (E) the thermodynamic site of the Y382F AGAO mutant. The blue and red vertical lines correlate hyperfine features between the preprocessed spectrum and the major and minor form fits, respectively. The red dashed spectra below (A), (D), and (E) are simulated fits. All samples were prepared in 25 mM pH 7.0 HEPES buffer.

Figure 5.

(A) Room temperature absorbance spectra following the additions of multiple equivalents of Cu^{II} to wild-type AGAO with Zn^{II} bound in the thermodynamic site. (B) 77 K EPR spectrum of wild-type AGAO with Zn^{II} bound in the thermodynamic site following the addition of 5 equiv of Cu^{II}. (C) Room temperature absorbance spectra following the anaerobic additions of multiple equivalents of Cu^{II} to wild-type apo-AGAO. (D) 77K EPR spectrum of wild-type AGAO following the anaerobic addition of 2 equiv of Cu^{II}. All samples were prepared in 25 mM pH 7.0 HEPES buffer.

Figure 6.

(A) 77 K EPR spectra of preprocessed AGAO at multiple pHs with Cu^{II} only in the thermodynamic (active) site. (B) Percent minor-form of the preprocessed equilibrium of thermodynamic (active) site Cu^{II} . Mean values shown with thick horizontal black lines. Individual values shown with green circles. Values were determined by fitting EPR spectra, and are shown with error bars. All pH 7.0 spectra are shown in SI Figure 3.

Figure 7.

(A) 77 K EPR spectra of preprocessed AGAO samples rapidly frozen from different incubation temperatures. Variable-temperature freeze-quench intermediates were prepared by mixing holo, preprocessed AGAO samples in 50 mM pH 8.5 CHES buffer with glycerol so that their freezing points were 1–2 K above their indicated freezing temperatures. The samples were then transferred into EPR tubes and anaerobically incubated at 1–2 K above the indicated temperature for 5 min before finally being rapidly frozen in liquid nitrogen, ethanol slurries. The absence of a glycerol induced effect on the preprocessed AGAO equilibrium was confirmed for the highest concentration of glycerol used (SI Figure 4). (B) van 't Hoff plot of variable temperature equilibrium data with best fit given as dashed red line. The data points represent the average of three replicates for all but the lowest temperature point (which is shown without error bars). Thermodynamic parameters are given for the conversion from the major preprocessed form to the minor preprocessed form.

These trends were confirmed in a different buffer and pH (HEPES, pH 7.8) and are shown in SI Figure 5. The fits to each spectrum shown above are shown in SI Figure 6.

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Figure 8.

Room temperature absorbance (top), room temperature CD (middle), and 5 K 7 T MCD (bottom) of (A) preprocessed AGAO, (B) holo Y382F, and (C) processed AGAO.

Experimental results are shown in blue, with bands assigned for Y382F, processed, and the major form of the preprocessed equilibrium shown in red and labeled with numbers, and bands assigned to the minor form of the preprocessed equilibrium shown in green and labeled with primed numbers, bands assigned to TPQ shown in purple, and bands assigned to a heme impurity indicated with an asterisk (*). Absorbance and CD spectra were collected on 100 μM samples at room temperature, with the exception of preprocessed AO CD, which was collected on an 80 μM sample. All samples were prepared in 25 mM pH 7.0 HEPES buffer. The summed fits to each spectrum are shown in SI Figure 6.

Figure 9. AGAO biogenesis monitored by freeze quench EPR. Biogenesis at pH 7.0 and 1 °C with 1.1 mM O₂ and 90 μ M Cu^{II} (in AGAO active site). All samples were prepared in 25 mM pH 7.0 HEPES buffer.

Figure 10.

Optimized geometries for the (A) processed, (B) preprocessed minor form, and (C) preprocessed major form of AGAO. Structures A and B utilized crystallographic α -carbon coordinates, while structure C utilized α -carbon coordinates in which the 3 His-residue α -carbons were moved 0.1 Å toward Cu^{II}.

Figure 11.
 β -LUMO for the minor form of the preprocessed equilibrium, showing the interaction between the Cu $d_{x^2-y^2}$ and tyrosinate out-of-plane hydroxyl p-orbital.

Figure 12.

Proposed concerted 3-electron mechanism for minor-preprocessed AGAO, with donor orbitals shown in red and acceptor orbitals shown in blue

Table 1.EPR Parameters for Wild-Type Preprocessed, Wild-Type Processed, and Y382F AG AO^a

Species	<i>g</i> -tensor	A-tensor
(B) preprocessed major	$g_z = 2.282$	$A_z = 158$
	$g_y = 2.057$	$A_y = 25$
	$g_x = 2.024$	$A_x = 18$
(C) preprocessed minor	$g_z = 2.264$	$A_x = 106$
	$g_y = 2.149$	$A_y = 61$
	$g_x = 2.022$	$A_x = 12$
(D) processed	$g_z = 2.298$	$A_z = 160$
	$g_y = 2.091$	$A_z = 12$
	$g_x = 2.038$	$A_x = 12$
(E) Y382F	$g_z = 2.293$	$A_z = 148$
	$g_y = 2.080$	$A_y = 16$
	$g_x = 2.041$	$A_x = 15$

^a A-values in 10^{-4} cm^{-1}

Table 2.Band Assignments for Preprocessed, Processed, and Y382F AGAO Absorbance, CD, and MCD Spectra^a

preprocessed			
band	energy (cm ⁻¹)	C_0/D_0	assignment
1 + 1'	7600		major and minor d_z^2 ligand-field transitions
<i>2'</i>	<i>10 500</i>	<i>-0.14</i>	<i>minor $d_{xz} + d_{yz}$ ligand-field transition</i>
<i>3'</i>	<i>12 200</i>	<i>0.19</i>	<i>minor $d_{xz} - d_{yz}$ ligand-field transition</i>
2	12 500	0.15	major d_{xz} ligand-field transition
3	15 900	-0.19	major d_{yz} ligand-field transition
4	18 400	0.12	major d_{xy} ligand-field transition
<i>4'</i>	<i>22 500</i>	<i>0.029</i>	<i>minor tyrosinate HOMO to Cu^{II} LMCT</i>
<i>5'</i>	<i>28 000</i>	<i>-0.011</i>	<i>minor tyrosinate HOMO-1 to Cu^{II} LMCT</i>
5	31 800	-0.02	major hydroxide HOMO to Cu^{II} LMCT
Processed			
band	energy (cm ⁻¹)	C_0/D_0	assignment
1	11 000	0.092	d_z^2 ligand-field transition
2	12 700	0.15	$d_{xz} + d_{yz}$ ligand-field transition
3	15 000		$d_{xz} - d_{yz}$ ligand-field transition
4	16000	-0.22	d_{xy} ligand-field transition
5	33 000		water HOMO to Cu ^{II} LMCT
Y382F			
band	energy (cm ⁻¹)	C_0/D_0	assignment
1	8300		d_z^2 ligand-field transition
2	10 800	0.12	d_{xz} ligand-field transition
3	15 300	-0.15	d_{yz} ligand-field transition
4	17 900	-0.12	d_{xy} ligand-field transition
5	30 300		hydroxide HOMO to Cu ^{II} LMCT

^aMajor form bands indicated in bold and minor form bands indicated in italic for preprocessed AGAO

Table 3.

Calculated Excited State Energies and g -Tensors for the Processed and Preprocessed Minor and Major forms of AGAO^a

	processed	Preprocessed minor	Preprocessed major
ligand-field energies (cm ⁻¹)	10 420, 13 610, 13 620, 14 470	7550, 9150, 12 450, 13 310	8750, 13 210, 14 260, 15 400
charge-transfer energies (cm ⁻¹)		25 380, 28 370	32 750
g -tensor	[2.248, 2.081, 2.068]	[2.259, 2.159, 2.026]	[2.226, 2.075, 2.052]

^aThe processed and preprocessed minor form calculations were performed on structures utilizing the crystallographic α -carbon positions, while the preprocessed major form calculations were performed on modified crystallographic α -carbon coordinates in which the 3 His-residue α -carbons were moved 0.1 Å toward Cu^{II}.