Reversible Dioxygen Binding and Phenol Oxygenation in a Tyrosinase Model System

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Abstract: The complex $[Cu_2(L\text{-}66)]^{2+}$ (L-66 = α,α'-bis{bis[2-(1′-methyl-2′-benzimidazolyl)ethyl]amino}-*m*-xylene) undergoes fully reversible oxygenation at low temperature in acetone. The optical [$\lambda_{max} = 362$ (ε 15000), 455 (ε 2000), and 550 nm (ε 900 $M^{-1}cm^{-1}$)] and resonance Raman features (760 cm^{-1}, shifted to 719 cm^{-1} with $^{18}O_2$) of the dioxygen adduct $[Cu_2(L\text{-}66)(O_2)]^{2+}$ indicate that it is a μ-η^2:η^2-peroxodicopper(II) complex. The kinetics of dioxygen binding, studied at $-78\,^{\circ}C$, gave the rate constant $k_1 = 1.1\,M^{-1}s^{-1}$, for adduct formation, and $k_{-1} = 7.8 \times 10^{-5}\,s^{-1}$, for dioxygen release from the Cu_2O_2 complex. From these values, the O_2 binding constant $K = 1.4 \times 10^4\,M^{-1}$ at $-78\,^{\circ}C$ could be determined. The $[Cu_2(L\text{-}66)(O_2)]^{2+}$ complex performs the regiospecific *ortho*-hydroxylation of 4-carbomethoxyphenolate to the corresponding catecholate and the oxidation of 3,5-di-*tert*-butylcatechol to the quinone at $-60\,^{\circ}C$. Therefore, $[Cu_2(L\text{-}66)]^{2+}$ is the first synthetic complex to form a stable dioxygen adduct *and* exhibit true tyrosinase-like activity on exogenous phenolic compounds.

Keywords: copper · oxidations · oxygenations · peroxo complexes · Raman spectroscopy

Introduction

Tyrosinase is a nearly ubiquitous copper-containing monooxygenase that has both catecholase and cresolase activity.[1] It is responsible for browning reactions that occur to perform diverse physiological functions in different organisms.[2] Tyrosinase reversibly binds dioxygen,[3] and the spectral characteristics of oxytyrosinase bear strong similarities to those of the oxy form of the dioxygen carrier hemocyanin:[4] absorption bands at 350 nm ($\varepsilon \sim 20000\,M^{-1}cm^{-1}$) and 590 nm ($\varepsilon \sim 1000\,M^{-1}cm^{-1}$), an O–O stretching frequency of $\sim 750\,cm^{-1}$, and a Cu–Cu distance of 3.6 Å, as shown by X-ray crystallography of hemocyanin.[5] These features are associated with a Cu_2O_2 core containing a side-on bridged (or μ-η^2:η^2) peroxide moiety.[5] Dioxygen binding to biomimetic Cu^I complexes, to form Cu_2O_2 adducts, has been reported for several systems.[6–11] These Cu_2O_2 cores contain either end-on μ-1,2 or side-on μ-η^2:η^2 peroxide coordination, or have a bis-μ-oxo arrangement. Some of them have been shown to be capable of performing hydroxylation reactions at C–H bonds of the ligand,[6, 8–10] or radical reactions at C–H bonds of exogenous substrates, including phenols.[11] These radical reactions are reminiscent of the nonphysiological, poorly efficient enzymatic activity exhibited by molluscan hemocyanin,[12a] while tyrosinase catalysis proceeds through nonradical pathways.[12b] We previously reported conversion of phenolic substrates to catechols by the complex $[Cu_2(L\text{-}66)]^{2+}$ (L-66 = α,α'-bis{bis[2-(1′-methyl-2′-benzimidazolyl)ethyl]amino}-*m*-xylene) and dioxygen at $-40\,^{\circ}C$ or room temperature.[13] Full incorporation of 18-oxygen from $^{18}O_2$ into the product was demonstrated.[13b] Here we show that $[Cu_2(L\text{-}66)]^{2+}$ undergoes fully reversible oxygenation at $-80\,^{\circ}C$ (Scheme 1), and that the resulting μ-peroxodicopper(II) adduct is involved in the regiospecific *ortho*-hydroxylation of an exogenous, electron-

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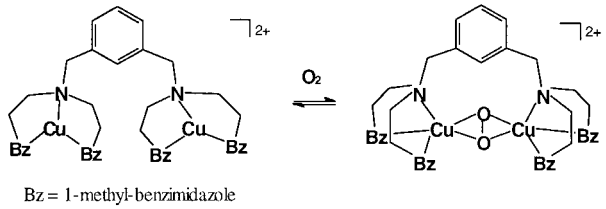

Bz = 1-methyl-benzimidazole

Scheme 1. The reversible oxygenation of $[Cu_2(L\text{-}66)]^{2+}$.

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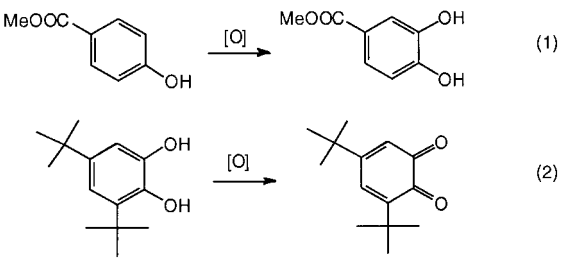
poor phenol to catechol [Eq. (1)] and oxidation of an electron-rich catechol to quinone [Eq. (2)].[13, 14] All these features are characteristic of tyrosinase.[1] Therefore, this system represents the first true tyrosinase model.



Results and Discussion

Oxygenation of $[Cu_2(L\text{-}66)]^{2+}$ carried out at about $-80\,^{\circ}C$ in acetone yields an intensely brown solution with $\lambda_{max} = 362$ (ε 15000), 455 (ε 2000), and 550 nm (ε 900 $M^{-1}cm^{-1}$) (Figure 1).[15] The stoichiometry of oxygen binding ($Cu:O_2 = 2:1$)

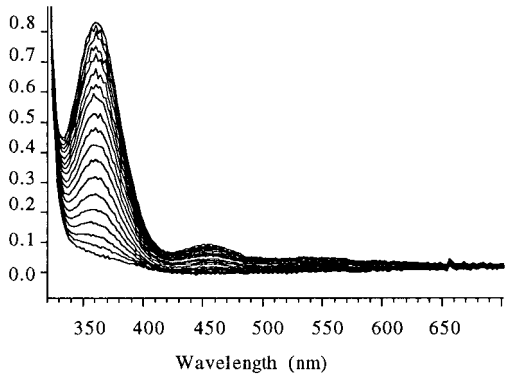


Figure 1. Optical spectra developed on oxygenating $[Cu_2(L\text{-}66)]^{2+}$ in acetone at $-78\,^{\circ}C$.

indicates that a peroxodicopper(II) complex, $[Cu_2(L\text{-}66)(O_2)]^{2+}$, is formed. The oxygenation reaction can be fully reversed at low temperature upon application of a vacuum, the process being accelerated by mild warming of the solution. After several oxygenation/deoxygenation cycles at $-80\,^{\circ}C$ no appreciable loss of absorption intensity from the oxygen adduct is observed. The pattern of three UV-visible bands developing upon oxygenation of $[Cu_2(L\text{-}66)]^{2+}$ bears resemblance with that of the dioxygen adducts formed at low temperature by the dinuclear complexes $[Cu_2(R\text{-}XYL)]^{2+}$ ($R \neq H$)[8b] and $[Cu_2(NnPY2)]^{2+}$[8c] and has been attributed to peroxide $\rightarrow Cu^{II}$ LMCT transitions of a side-on (μ-η^2:η^2)-bridging peroxide unit. The resonance Raman spectra of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ at $-80\,^{\circ}C$ in acetone exhibit the following features: with an excitation wavelength of $\lambda = 350.7$ nm four peaks are observed at 214, 256, 283 and 314 cm^{-1}, the strongest one at 283 cm^{-1} (Figure 2A). Upon warming, these peaks gradually decrease in intensity and have essentially disappeared at $-45\,^{\circ}C$. After subsequent cooling to $-80\,^{\circ}C$ the peaks reappear, demonstrating again the reversible formation of the peroxo complex (Figure 2B). No further

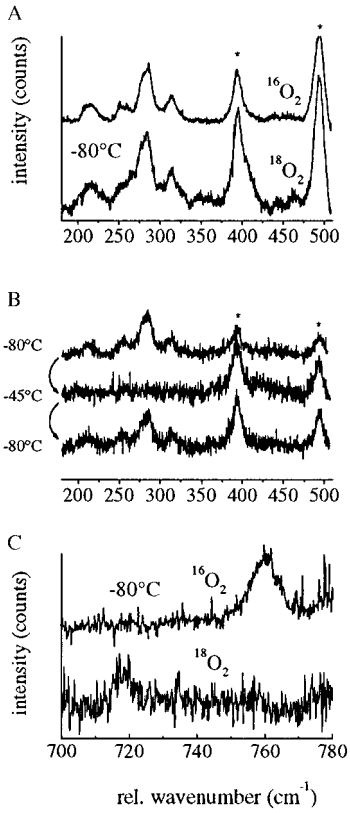


Figure 2. Resonance Raman spectra of a <1 mM solution of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ in acetone including $^{18}O_2$ data. A) $\lambda_{exc.} = 350.7$ nm, 1000 s, $-80\,^{\circ}C$. B) Temperature cycle $-80 \rightarrow -45 \rightarrow -80\,^{\circ}C$ ($\lambda_{exc.} = 350.7$ nm, 100 s spectra). C) Difference spectrum after O_2 addition showing the O–O stretching vibration at 760 cm^{-1} and 719 cm^{-1} for $^{16}O_2$ and $^{18}O_2$, respectively ($\lambda_{exc.} = 415.4$ nm, 1000 s, $-80\,^{\circ}C$). * = solvent peaks.

peaks could be detected with this excitation wavelength. However, when excited at $\lambda = 415.4$ nm an additional peak is observed at 760 cm^{-1}, which shifts to 719 cm^{-1} upon $^{18}O_2$ substitution (Figure 2C). Owing to its isotopic shift (41 cm^{-1}), this peak is assigned to the O–O stretch. The low frequency of this vibration, combined with the UV-visible spectral data, proves the presence of side-on (μ-η^2:η^2)-coordinated peroxide in $[Cu_2(L\text{-}66)(O_2)]^{2+}$.[16] Also typical for this binding geometry are the peaks at 256, 283, and 314 cm^{-1}, which do not shift upon $^{18}O_2$ substitution (Figure 2A); in resonance Raman spectra of hemocyanin these features have been assigned to Cu–(Ligand)N stretching vibrations.[17] An alternative assignment of the peak at 283 cm^{-1} to the symmetric Cu_2O_2 core stretch, which primarily involves copper motion, has been suggested.[16b]

The dioxygen binding process undergone by $[Cu_2(L\text{-}66)]^{2+}$ occurs in a single observable step; no evidence was found for an intermediate mononuclear copper(II)-superoxide species. The rate constant for adduct formation determined at $-78\,^{\circ}C$ was $k_1 = 1.1\,M^{-1}s^{-1}$, and that of release of O_2 from the Cu_2O_2 complex $k_{-1} = 7.8 \times 10^{-5}\,s^{-1}$. From these values the binding constant for dioxygen at $-78\,^{\circ}C$ is determined to be $K = (k_1/k_{-1}) = 1.4 \times 10^4\,M^{-1}$. This value is about three orders of magnitude smaller than the equilibrium constant reported for the oxygenation of $[Cu_2(H\text{-}XYL)]^{2+}$ in dichloromethane.[8a] The difference is almost entirely due to the lower value

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of k_1, possibly because the larger benzimidazole groups slow down the conformational rearrangement of the complex leading to the dioxygen adduct. The different bite angle of the five-membered benzimidazole ring, with respect to the six-membered pyridine ring, may also cause some difference in the stereochemistry of the two peroxo complexes. However, whereas the $[Cu_2(H\text{-}XYL)]^{2+}$ complex does not form a stable dioxygen adduct and undergoes a fast ligand hydroxylation reaction,[18] replacement of the pyridine donors of $[Cu_2(H\text{-}XYL)]^{2+}$ with benzimidazoles in $[Cu_2(L\text{-}66)]^{2+}$ disables the endogenous ligand hydroxylation. This is a necessary condition to perform the regiospecific *ortho*-hydroxylation of an exogenous phenolate.[13a]

When sodium 4-carbomethoxyphenolate is added to the solution of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ at $-80\,^{\circ}C$ (excess O_2 removed) no change of the UV-visible spectrum occurs. Upon raising the temperature to $-60\,^{\circ}C$ the intensity of the peroxide $\rightarrow Cu^{II}$ CT band at 362 nm diminishes to about 50%, due to thermal decay of the dioxygen adduct. In addition, a slow reaction takes place, as indicated by a further decrease in intensity of the 362 nm band and appearance of a new absorption feature near 340 nm (Figure 3). The spectrum that results after

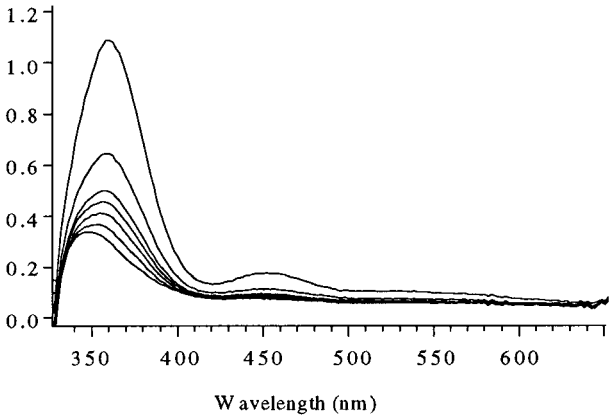

Figure 3. Changes in the absorption spectra for the reaction of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ with sodium 4-carbomethoxyphenolate at low temperature. In order of decreasing absorbance: a) $[Cu_2(L\text{-}66)(O_2)]^{2+}$ after the addition of phenolate at $-80\,^{\circ}C$; b) after equilibration at $-60\,^{\circ}C$; c) after 1 h, d) 2 h, e) 3 h, f) 4 h, and g) 5 h reaction at $-60\,^{\circ}C$.

several hours and which exclusively exhibits the 340 nm band is analogous to that observed for the catecholate adduct of $[Cu_2(L\text{-}66)]^{4+}$ in acetonitrile,[13a] and thus strongly suggests conversion of phenol into catechol mediated by the copper-dioxygen adduct. The further oxidation of catechol to quinone is demonstrated by the reaction of 3,5-di-*tert*-butylcatechol with $[Cu_2(L\text{-}66)(O_2)]^{2+}$ at $-60\,^{\circ}C$ (excess O_2 removed) (Figure 4). The decrease of the copper-peroxide band at 362 nm and the development of the quinone band at about 420 nm follow the same time profile ($k_{obs} = 2.8 \times 10^{-3}\,s^{-1}$ and $2.6 \times 10^{-3}\,s^{-1}$, respectively). The process shown corresponds to one catalytic cycle (2 catechol:1 O_2).[14]

The conversion of 4-carbomethoxyphenolate to catecholate by $[Cu_2(L\text{-}66)(O_2)]^{2+}$ at $-60\,^{\circ}C$ is also proven by workup of the reaction mixture followed by chemical analysis. Due to the thermal instability of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ at $-60\,^{\circ}C$ (see above)

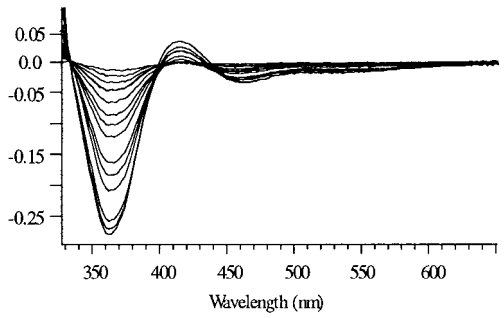

Figure 4. Difference spectra recorded during the reaction of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ and 3,5-di-*tert*-butylcatechol (50 mol equiv) at $-60\,^{\circ}C$ in acetone.

and slow rate of the hydroxylation at this temperature, side reactions between phenolate, catecholate ions, and acetone decrease the yield of catechol (about 20% with respect to the starting $[Cu_2(L\text{-}66)]^{2+}$ complex , i.e. about 40% with respect to $[Cu_2(L\text{-}66)(O_2)]^{2+}$, after 2 h). In contrast, clean oxygenation of the same substrate by $[Cu_2(L\text{-}66)]^{2+}$ and dioxygen was observed in acetonitrile at $-40\,^{\circ}C$.[13a] However, under those conditions the dioxygen adduct does not build up. Moreover, hydroxylation was achieved by exposing the Cu^{I}-phenolate adduct to dioxygen;[13] without substrate the reaction between $[Cu_2(L\text{-}66)]^{2+}$ and dioxygen at or near room temperature results in the di-μ-hydroxo-bridged dicopper(II) complex $[Cu_2(L\text{-}66)(OH)_2]^{2+}$.[19] In contrast, here the phenolate has been added *after* oxygenation of the Cu^{I} complex at low temperature, demonstrating conversion of the substrate into catechol in the presence of the peroxo complex. In this way, the $[Cu_2(L\text{-}66)]^{2+}$ complex is shown to be the first small-molecule system to form a stable dioxygen adduct *and* to mediate the hydroxylation of exogeneous phenols, in analogy to tyrosinase.

Experimental Section

The ligand L-66 and the corresponding dinuclear Cu^{I} and Cu^{II} complexes were prepared as described before.[13] Low-temperature UV/Vis spectra were recorded by using a HP 8452A diode array spectrophotometer in conjunction with a custom-designed immersible fiber-optic quartz probe (5 mm path length, Hellma) fitted to a Schlenk vessel with gas and vacuum inlets. The stoichiometry of oxygen binding to $[Cu_2(L\text{-}66)]^{2+}$ was determined by assaying the hydrogen peroxide produced by the addition of excess trifluoroacetic acid to the oxygenated solution at $-78\,^{\circ}C$. A small amount of this acidified solution was added to an aqueous phosphate buffer solution (100 mM) at pH 7 that contained an excess of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and horseradish peroxidase (40 nM). The peroxidase reaction produces the $ABTS^{\bullet+}$ radical cation, characterized by intense absorption bands at 414 nm (ε $3.6 \times 10^4\,M^{-1}cm^{-1}$)[20] and 660 nm ($\varepsilon$ $1.47 \times 10^4\,M^{-1}cm^{-1}$).[21] The yield of hydrogen peroxide found considering a $2Cu{:}1O_2$ stoichiometry was >90%. The dicopper(II) complex of L-66 gives no interference in this assay.

Raman spectra were recorded on a home-built spectrometer at Universität Mainz and consisted of an Acton SpectraPro-500i double monochromator equipped with 3600, 1800, and 600 groove per mm gratings and with a nitrogen-cooled, back-illuminated CCD camera (PI Instruments, 1100PB, 1100×330 PI Chip). The 3600 groove per mm grating was used for all measurements. Excitation source was a Spectra Physics 2080 Kr^{+} Laser (350.7 nm, 415.4 nm). Typically spectra were run with laser powers of 10 mW and acquisition times of 100 s and 1000 s. Spectral resolution was set to $<3.5\,cm^{-1}$. Low-temperature measurements were carried out in a liquid-

helium flow cryostat with a specially designed quartz sample rod (CryoVac). The sample rod contained vacuum and gas inlets as well as a silicon septum through which solutions could be injected under inert atmosphere. The Cu^I complex $[Cu_2(L\text{-}66)]^{2+}$ was prepared directly in the sample rod under Ar and after cooling to $-80\,^\circ C$, oxygen was bubbled through the solution for ≈ 15 min, generating the brown peroxo complex in solution. Concentrations of this species were estimated to be < 1 mM. ^{18}O-substitution experiments were performed by oxygenating the $[Cu_2(L\text{-}66)]^{2+}$ solution with $^{18}O_2$ gas (99%) obtained from Chemotrade.

The kinetics of dioxygen binding by $[Cu_2(L\text{-}66)]^{2+}$ were studied at 362 nm, upon injecting a small volume of a concentrated solution of the Cu^I complex in acetone into a large volume of the solvent saturated with dioxygen at low temperature. The kinetic constant k_{obs} was obtained from fitting of the data of absorbance versus time at $-78\,^\circ C$ to the equation for the reversible oxygenation equilibrium (even though at $-78\,^\circ C$ oxygenation of the complex is nearly complete), Equations (3) – (5), in which $[Cu^I{}_2]^0$ is the initial concentration of $[Cu_2(L\text{-}66)]^{2+}$.

$$Cu^I{}_2 + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Cu_2O_2 \tag{3}$$

$$[Cu_2O_2] = \frac{k_1[O_2][Cu^I{}_2]^0}{k_1[O_2] + k_{-1}}\{1 - \exp[-(k_1[O_2] + k_{-1})t]\} \tag{4}$$

$$k_{obs} = k_1[O_2] + k_{-1} \tag{5}$$

The solubility of dioxygen in acetone at low temperature was taken from the literature.[9b] The deoxygenation of $[Cu_2(L\text{-}66)(O_2)]^{2+}$ is slow at low temperature and was followed spectrally after applying a vacuum to the oxygenated solution and purging with an inert gas. Under these conditions, deoxygenation proceeded until the system equilibrated to the residual dioxygen pressure. To achieve full deoxygenation, application of a second vacuum/purge cycle would be necessary, but since this process causes base line problems in the optical readings we limited the spectral observation to the first cycle.

The phenol oxygenation experiments were carried out under Ar by adding a solution of sodium 4-carbomethoxyphenolate (0.024 mmol) in dry acetone (1 mL) to $[Cu_2(L\text{-}66)(O_2)]^{2+}$ (0.024 mmol, excess O_2 removed) in the same solvent (40 mL) at $-80\,^\circ C$. After 15 min, the reaction was allowed to proceed at $-60\,^\circ C$. Samples (10 mL) of the solution were withdrawn and immediately quenched in H_2SO_4 (0.2 M, 1 mL). The mixture was taken to dryness, and the residue was extracted with chloroform and analyzed by ^1H NMR and HPLC (after addition of a standard) as described previously.[13, 14] Total recovery of catechol plus unreacted phenol did not exceed 50%. By-products from side reactions are evident in the NMR spectra and HPLC traces.

Acknowledgments

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