Copper Monooxygenases

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# The First Catalytic Tyrosinase Model System Based on a Mononuclear Copper(I) Complex: Kinetics and Mechanism**

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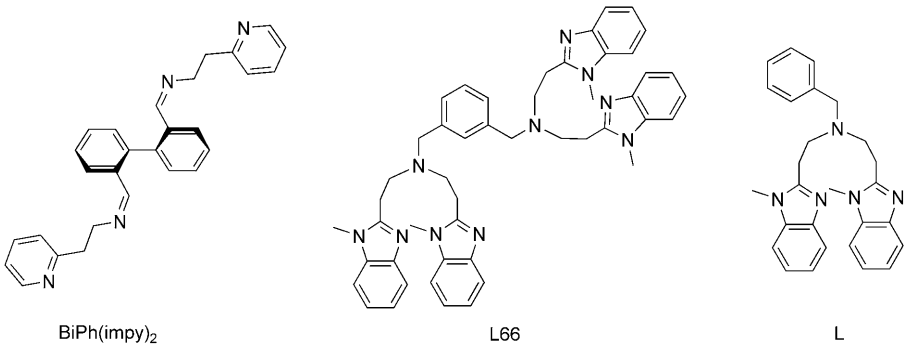
*Dedicated to Professor Martin Jansen on the occasion of his 65th birthday*

Tyrosinase is a ubiquitous enzyme mediating the *o*-hydroxylation of monophenols to catechols and the subsequent two-electron oxidation to quinones.[1] Its physiological function is the conversion of tyrosine into dopaquinone, which constitutes the first step of melanine synthesis.[2,3] As evident from spectroscopic data and a recent X-ray crystal-structure determination,[4] the active site of tyrosinase contains a binuclear copper center coordinated by six histidine residues. This type of active site (type 3) is analogous to the active sites of hemocyanin and catechol oxidase.[3] All of these copper proteins in their oxy states bind dioxygen in a characteristic side-on bridging structure whereby the $Cu^{I}$ centers in the deoxy states are converted into $Cu^{II}$ in the oxy state. Starting from the oxy state of tyrosinase, monophenols are converted into *o*-diphenols (monophenolase reaction), presumably in an electrophilic substitution reaction.[5] Subsequently, the *o*-diphenol (catechol) intermediates are converted into *o*-quinones. A second reactivity of the oxy site is the two-electron oxidation of external catechol substrates to *o*-quinones (diphenolase reactivity). Whereas tyrosinase exhibits both mono- and diphenolase reactions, the enzymatic activity of catechol oxidase is restricted to diphenolase reactivity.[6]

The reactivity of tyrosinase towards phenolic or aromatic substrates has successfully been reproduced with small-molecule copper complexes.[7–12] Whereas initially mainly aromatic hydroxylation reactions of the ligand framework by $\mu$-$\eta^2$:$\eta^2$-peroxo and bis-$\mu$-oxo dicopper cores were investigated,[13–17] the hydroxylation of external mono- and diphenolic substrates has more and more shifted into the focus of interest.[18–24] An additional challenge in this research area has been the synthesis of catalytic tyrosinase-model systems. Apart from a patent,[25] however, there are only two reports referring to this point. In 1990, Réglier et al. synthesized the ligand bis-2,2′-[2-(pyrid-2-yl)ethyl]iminobiphenyl (BiPh(impy)$_2$) which contains two pyridylethylimine sidearms bridged by a biphenyl spacer (Scheme 1).[21,26] It was shown that a mixture of this ligand with two equivalents of $Cu^{I}$ salt, 100 equivalents of 2,4-di-*tert*-butylphenol (DTBP-H), and 200 equivalents of triethylamine ($NEt_3$) leads to the catalytic generation of quinone with a turnover number (TON) of 16, monitored by the appearance of the optical absorption band of 3,5-di-*tert*-butyl-*o*-quinone (DTBQ) at approximately 400 nm. After one hour, the reaction stopped, presumably by formation of an oxo-bridged complex. A mechanism was proposed for this reaction, involving the formation of a binuclear peroxo complex and a catechol-bridged intermediate. Shortly afterwards Casella et al. described a catalytic model system of tyrosinase based on the ligand $\alpha,\alpha'$-bis{bis[2-(1-methylbenzimidazol-2-yl)ethyl]amino}-*m*-xylene (L66, Scheme 1).[18] In a stoichiometric mode (TON = 1) the system was found to lead to hydroxylation of carbomethoxyphenol to carbomethoxycatechol. With phenol groups containing electron-donating substituents such as two *tert*-butyl groups, TONs greater than 1 were observed.




***Scheme 1.*** Ligand BiPh(impy)$_2$ by Réglier et al., ligand L66 and ligand L by Casella et al.

Stoichiometric hydroxylation of phenols has also been shown for a mononuclear $Cu^{I}$ complex supported by L, the tridentate analogue of the ligand L66 (Scheme 1). In this reaction an involvement of the binuclear $\mu$-$\eta^2$:$\eta^2$ peroxo complex was demonstrated.[27] The question therefore arises

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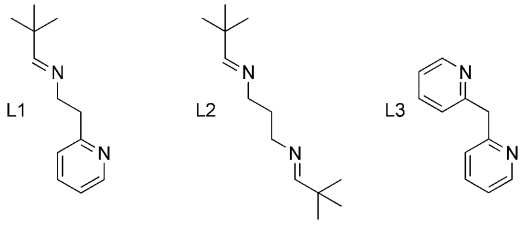
as to whether mononuclear copper(I) complexes are also capable of mediating the phenol hydroxylation reaction in a *catalytic* fashion.

To address this problem we synthesized the ligand L1 (Scheme 2) which represents one half of the binucleating, tetradentate ligand BiPh(impy)$_2$ with the imine function




***Scheme 2.*** Ligand set L1–L3 used in this study.[28]

terminated by a *tert*-butyl group. The Cu$^{I}$ bis(acetonitrile) complex derived from L1, [Cu$^{I}$(L1)(CH$_3$CN)$_2$](PF$_6$) (**1**) is also converted into the phenolato complex by the procedure developed by Casella;[18] that is, **1** was treated with one equivalent of DTBP-H and two equivalents of NEt$_3$, leading to a 1:1 mixture (**2**) of the neutral phenolato-triethylamine complex [Cu$^{I}$(L1)(DTBP)(NEt$_3$)] and HNEt$_3$PF$_6$.

Importantly, oxygenation of a mixture **1** or **2** with 50 equivalents of DTBP-H and 100 equivalents of NEt$_3$ in CH$_2$Cl$_2$ was found to catalytically lead to generation of DTBQ, as shown by the appearance of an absorption band of DTBQ at 407 nm (Figure 1). Based on an ε value of 1830 $M^{-1}cm^{-1}$, a TON of 18 per dicopper unit (see below) was calculated, almost identical to the result of Réglier et al. However, the reaction was found to be much slower, concomitant with a longer lifetime: whereas for the BiPh(impy)$_2$ system the reaction is completed in 1 h, the first 16 cycles for the Cu(L1) system take 10 h and 18 cycles are completed after 1 day.

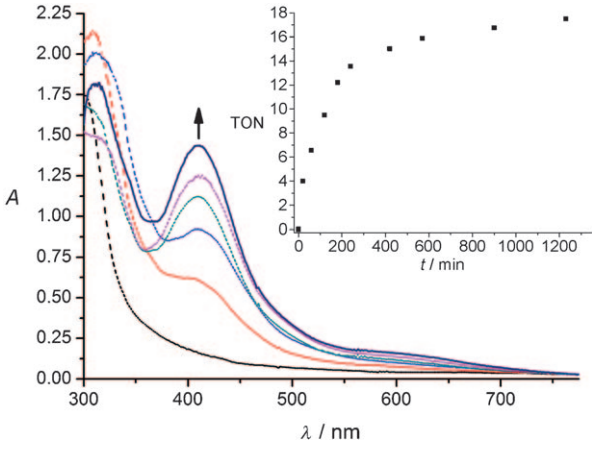



***Figure 1.*** UV/Vis spectroscopic monitoring of the catalytic formation of DTBQ by oxygenation of a 20 μM solution of **1** in CH$_2$Cl$_2$ in the presence of 50 equivalents DTBP-H and 100 equivalents NEt$_3$ during the first 6 h (dashed lines) and after 8 h and subsequent HCl quench/CH$_2$Cl$_2$ extraction (solid line); $l$ = 5 cm. Inset: Turnover number per dicopper unit as a function of time.

To eliminate absorptions from copper species, the reaction mixture was quenched after 8 h of oxygenation with an excess of HCl and extracted with CH$_2$Cl$_2$. The UV/Vis spectrum of the organic phase clearly exhibits the optical spectrum of DTBQ (Figure 1, solid line). The organic phase was investigated by NMR spectroscopy, which indicated a mixture of DTBQ (15 % of the starting amount of DTBP-H), DTBP-H (52 %), 3,5-di-*tert*-butyl-catechol (DTBC-H$_2$, 3 %) and the C–C coupling product 4,4′,6,6′-tetra-(*tert*-butyl)-2,2′-biphenol (30 %). This result is in good agreement with the TON for the formation of quinone determined from the 407 nm absorbance (Figure 1, inset).[29,30] In a control experiment a 1:1 mixture of DTBP-H/DTBC-H$_2$ was oxygenated in the presence of NEt$_3$ with subsequent HCl quench, leading to a 1:1 mixture of DTBP-H/DTBQ; the optical absorption and NMR spectra of DTBQ were found to be identical to those resulting from the copper-catalyzed oxygenation of DTBP-H.

To check the role of the mixed pyridine/imine donor set of L1, we additionally synthesized the ligands L2 and L3 containing two imine and two pyridine functions, respectively (Scheme 2).[28] Oxygenation of mixtures of [Cu(L2)]$^+$ or [Cu(L3)]$^+$ with 50 equivalents DTBP-H and 100 equivalents NEt$_3$, however, did not lead to the appearance of the 407 nm band, indicating that these complexes are catalytically inactive with respect to the oxygenation of monophenols. Clearly, only the combination of pyridine and imine is successful whereas either two pyridine or two imine donor functions per metal center do not provide the proper coordination for a catalytic action of the copper complex.

To study the mechanism of the Cu(L1)-mediated oxygenation of DTBP-H to DTBQ, the reaction was also run in a stoichiometric fashion. To avoid the presence of HNEt$_3^+$ in the solution, the parent copper phenolato triethylamine complex was prepared from **1** and NaDTBP/NEt$_3$, leading to a mixture (**2′**) of [Cu(L1)(NEt$_3$)(DTBP)] and NaPF$_6$.

A solution of **2′** in CH$_2$Cl$_2$ was oxygenated for 30 min without adding further NEt$_3$ or DTBP-H. The absorption spectrum of **2′** exhibits a shoulder at approximately 350 nm, corresponding to a phenolate→Cu transition (Figure 2, curve a). After oxygenation an absorption spectrum with a shoulder at 340 nm (Figure 2, curve b) is observed. The corresponding spectrum is similar to that of the catecholato complex of the L55 system by Casella et al.[31] The resulting intermediate (**4**) thus may contain catecholate coordinated to a binuclear copper complex. This assumption was supported by quenching the reaction mixture with HCl, and subsequent extraction with CH$_2$Cl$_2$. NMR spectroscopic analysis of the organic phase lead to a roughly 5:4:1 mixture of DTBP-H/DTBQ/DTBC-H$_2$, suggesting the presence of one catecholate and one phenolate in intermediate **4**.[32] Compound **4** was also isolated and characterized by elemental analysis, magnetic susceptibility ($\chi$), and mass spectrometry. Elemental analysis is compatible with a binuclear formulation of **4** as [Cu$_2$(L1)$_2$(OH)(DTBC)(DTBP)]·2 NaPF$_6$. The $\chi$ versus $T$ data, on the other hand, indicate only a weak antiferromagnetic interaction, suggesting that compound **4**, upon isolation, has split into two Cu$^{II}$ monomers, [Cu(L1)(DTBC)] + [Cu(L1)(DTBP)(OH)] (compound **4′**; see Supporting Information). Further evidence for this constitution is provided by

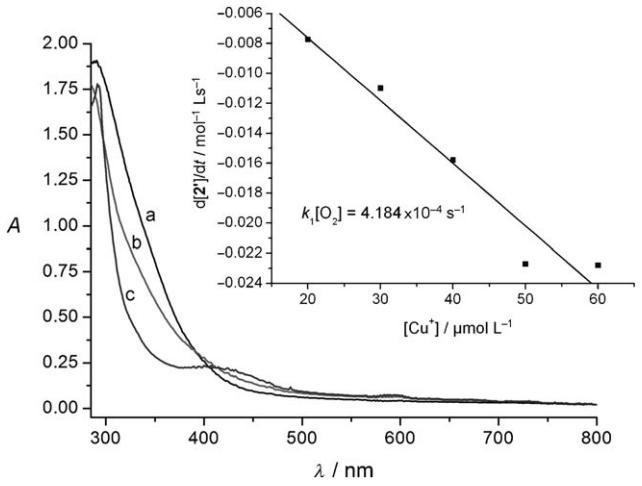



***Figure 2.*** 500 μM solution of **1** in $CH_2Cl_2$ containing 1 equivalent NaDTBP and 1 equivalent $NEt_3$ (a, **2′**), after oxygenation for 30 min (b, **4**) and subsequent addition of 200 equivalents $NEt_3$ and 100 equivalents DTBP-H under argon atmosphere (c); $l = 1$ cm. Inset: Kinetics of the oxygenation of **2′** to **4** at $\lambda = 310$ nm.

MALDI-TOF mass spectrometry (Figure 3, bottom); the high-resolution structure of the signal at approximately $m/z$ 477 is excellently reproduced by a 1:1 superposition of the signals of $[\{Cu(L1)(DTBC)\} + 3H]^+$ and $[\{Cu(L1)(OH)(DTBP)\} + 3H]^+$ (Figure 3, top) generated upon ionization of **4′**. The splitting of the μ-catecholato μ-hydroxo $Cu^{II}_2$ dimer into two monomers is related to the copper dibenzyl ethylene diamine (DBED) system where the corresponding intermediate was found to split into a $Cu^I$ aquo and a $Cu^{II}$ semiquinone complex upon addition of $H^+$.[23]

It thus has been shown by two methods (workup and NMR spectroscopic analysis of a solution containing compound **4** as well as direct isolation/analysis of compound **4**) that the product (catechol) yield in a stoichiometric run (phenol/Cu 1:1) is 50%. This result strongly supports a binuclear reaction course as shown in Scheme 3:

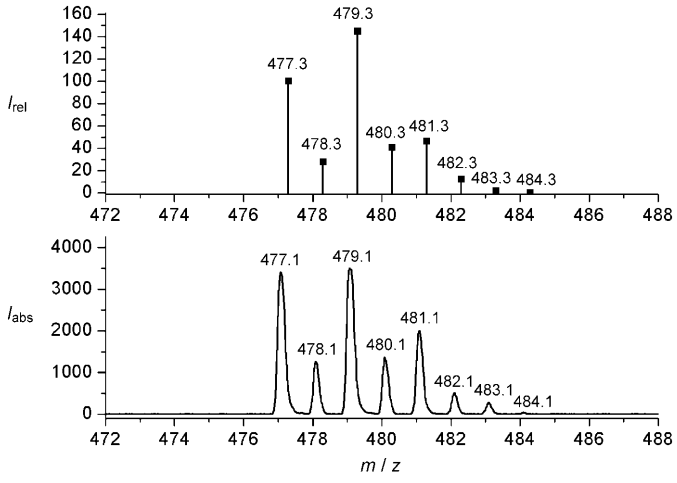



***Figure 3.*** High resolution MALDI-TOF spectrum of **4′** (bottom) and calculated isotope pattern for the two fragments of $[\mathbf{4'} + 6H]^{2+}$ (top; see text and Supporting Information for further details).

Oxygenation of the phenolato triethylamine $Cu^I$ complex **2** initially forms the binuclear μ-$\eta^2$:$\eta^2$ peroxo complex **3** containing two coordinated phenolates. In this complex only one of the bound phenolates can be hydroxylated, leading to an asymmetrically bridged μ-catecholato-μ-hydroxo complex,[33] intermediate **4**. Hydroxylation is thereby mediated by the σ*-orbital of the coordinated peroxide.[16] In a stoichiometric mode, the reaction stops at the level of **4**. Importantly, addition of an excess of DTBP-H/$NEt_3$ to **4** under anaerobic conditions leads to release of the substrate as quinone (Scheme 3 and Figure 2, curve c). Moreover, upon oxygenation of this mixture, the catalytic action of the system is resumed, as evident from the growing of the 407 nm band of DTBQ with time. This result indicates that both compound **2** and intermediate **4** are catalytically competent.

To gain further insight into the mechanism of the hydroxylation step, the conversion of **2'** into **4** was also studied kinetically. The reaction sequence can be described with the following kinetic scheme [Eq. (1)–(3)](see Supporting Information).

$$\mathbf{2'} + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Cu^{II}(L1)(OPh)(O_2^{-})] + NEt_3 \quad (1)$$

$$[Cu^{II}(L1)(OPh)(O_2^{-})] + \mathbf{2'} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathbf{3} + NEt_3 \quad (2)$$

$$\mathbf{3} \overset{k_3}{\rightarrow} \mathbf{4} \quad (3)$$

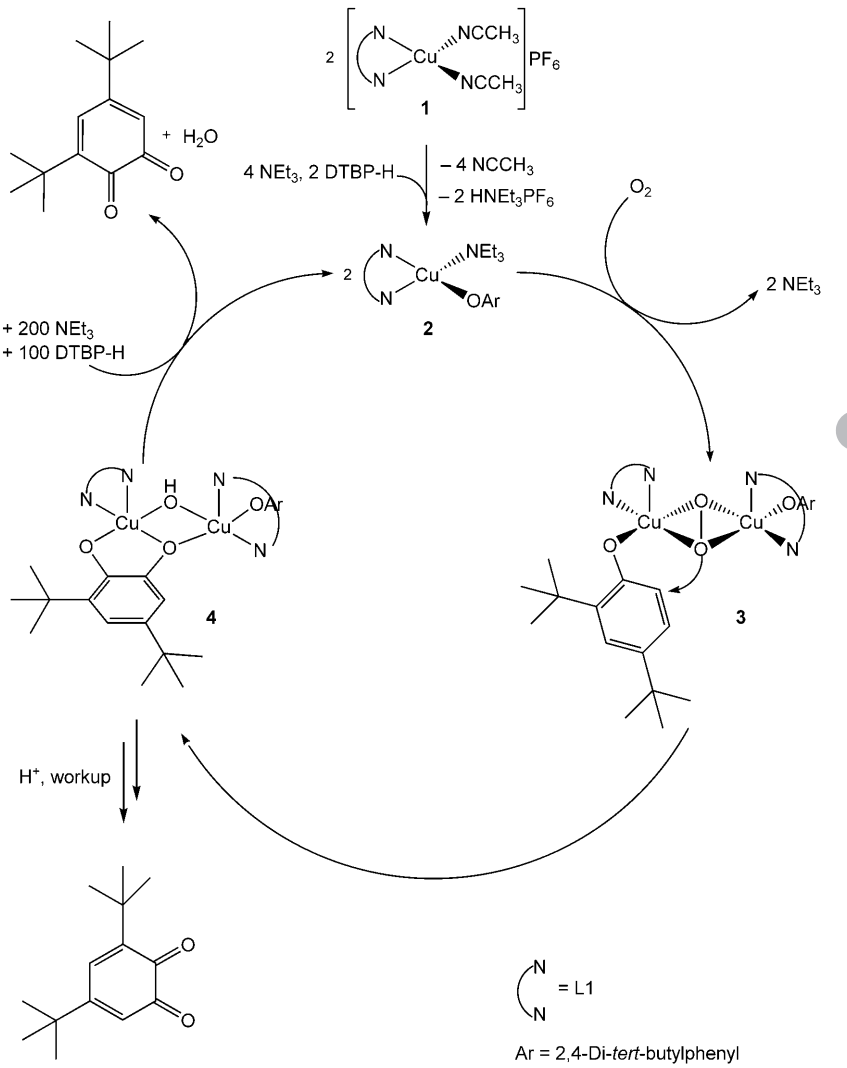
If both the superoxo complex $[Cu^{II}(L1)(OPh)(O_2^{-})]$ and the peroxo complex **3** are highly reactive, $k_{-1}$ and $k_{-2}$ can be assumed to be small compared to $k_1$, $k_2$, and $k_3$, and the product formation follows the rate law [Eq. (4)]:

$$\frac{d[\mathbf{4}]}{dt} = k_1[\mathbf{2'}]p(O_2) \quad (4)$$

This result is what is observed experimentally (Figure 2, inset). Although the hydroxylation reaction proceeds in a binuclear fashion, the reaction rate exhibits a first-order dependence on the copper concentration.[27] The described kinetics also accounts for the fact that no peroxo intermediate could be detected in our system.

The derived mechanistic cycle (Scheme 3) has also an interesting consequence with respect to the enzymatic reaction. In general, it is assumed that phenols bind to the oxy form of tyrosinase.[2] It has been difficult to understand, however, why addition of (protonated) phenols to synthetic binuclear peroxo complexes always leads to non-physiological radical-coupling products whereas the correct hydroxylation reaction is only observed after addition of (deprotonated) phenolates.[34] The question therefore arises as to whether phenols may become deprotonated in the enzyme prior to coordination to the oxy site. However, there is no residue available in the immediate vicinity of the binuclear copper center which could perform such a deprotonation, with the exception of a noncoordinating histidine that is sequentially positioned directly adjacent to a His residue coordinating to CuB (in *Streptomyces castaneoglobisporus* tyrosinase His215 and His216, respectively).[4,35] The way this




***Scheme 3.*** Proposed mechanistic cycle of the Cu(L1) system.

noncoordinated residue could be involved in a proton shuttle with the active site is, however, unclear. Alternatively, if phenol binding was coupled with release of bound catechol as quinone (cf. Scheme 3), the incoming phenol could be deprotonated by the bound hydroxide of the catecholate intermediate, forming water. A corresponding mechanism in the enzyme would 1) render a basic amino acid residue close to the binuclear copper center unnecessary under turnover conditions and 2) imply that under these conditions the phenol is already bound in the deoxy state. Experiments are underway to check whether this alternative scenario might be applicable to tyrosinase.[36]

In conclusion, the Cu(L1)/$NEt_3$ system is the first catalytic tyrosinase model that is based on a mononuclear $Cu^I$ complex. In a stoichiometric mode it allows the two consecutive stages of the tyrosinase reaction, phenol hydroxylation and product release as quinone, to be addressed individually. The combination of pyridine and imine donors is shown to be essential for the catalytic action while a bridge connecting the two bidentate ligands acts to increase the reaction rate. The Cu(L1)/$NEt_3$ system thus enables both a complete mechanistic understanding of the tyrosinase reaction and should lead to the development of further catalytic tyrosinase model systems. Detailed spectroscopic and quantum-chemical investigations will now be performed to obtain further insight into the elementary steps involved in the described catalytic cycle.

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