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How Does Tyrosinase Work? Recent Insights from Model Chemistry and Structural Biology**

Heinz Decker,* Renée Dillinger, and Felix Tuczek*

Introduction

The catalysis of oxygenation and oxidation reactions by tyrosinase and catecholoxidase is currently one of the most exciting topics of bioinorganic chemistry. These enzymes belong to the family of type 3 copper proteins which is involved in a variety of biological functions. Hemocyanins (Hcs) serve as O_2 carriers in the hemolymph of arthropods and molluscs,[1] catecholoxidases (COases) oxidize *ortho*-diphenols to *ortho*-quinones (diphenolase activity),[2] and tyrosinases mediate the hydroxylation of monophenols to *ortho*-diphenols as well as the subsequent oxidation to *ortho*-quinones (monophenolase activity).[3] Although they have been studied for over 100 years, these reactions and the observed functional differences are far from being understood on a molecular level.

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With the notable exception of tyrosinase, X-ray structures are available for all classes of type 3 copper proteins: the deoxy form of the Hc from spiny lobster (*Panulirus interruptus*),[4] the deoxy and oxy forms of the Hc from horseshoe crab (*Limulus polyphemus*),[5] the oxy form of the Hc from *Octopus dofleini*,[6] and the deoxy, met, and inhibitor-bound forms of the COase from sweet potato (*Ipomoea batatas*).[7] As expected, all of these proteins contain virtually the same binuclear copper active site which in its $Cu^I - Cu^I$ deoxy form reversibly binds O_2, leading to a binuclear Cu^{II} unit with O_2 as a side-on bridging (μ-η^2:η^2) peroxide group. Based on these structural data, a mechanistic understanding of the corresponding biochemical reactions now appears within reach. In this respect, the recent finding of mono- and diphenolase activity in activated Hcs[8] is of great interest, as it provides information regarding the orientation of monophenolic substrates within the protein pocket containing the binuclear copper active site.

Importantly, the growing structural insight into COase and tyrosinase activity is complemented by a significant amount of chemical and mechanistic information obtained from the study of synthetic low molecular weight analogues.[9] The current electronic-structural, spectroscopic, and chemical characterisation of the Cu_2O_2 cores present in these inorganic model systems constitutes a fascinating area of bioinorganic research in itself and will most probably be crucial for a further understanding of the oxygenation reactions mediated by tyrosinase. The present highlight first describes key results of these model studies and these are then contrasted with mechanistic proposals derived from structurally characterized proteins.

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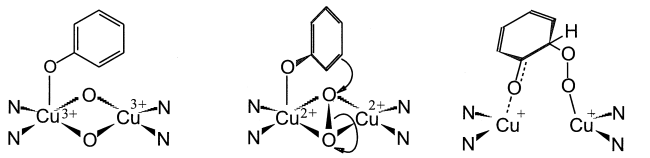
Cu-O_2 Model Chemistry: What is the Hydroxylating Species?

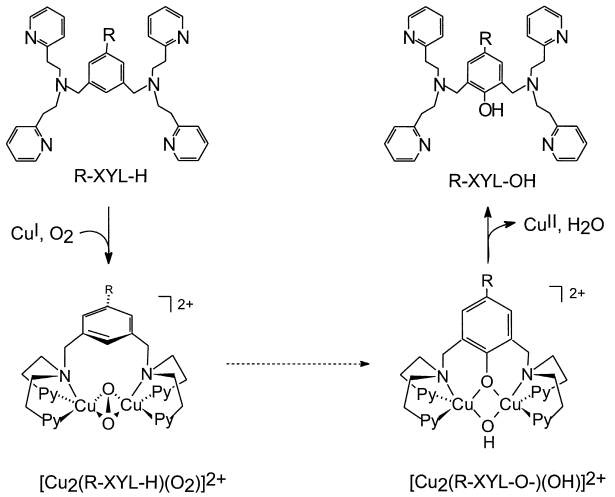
The mechanistically most demanding step of tyrosinase activity involves the introduction of a second OH group in the *ortho* position of the aromatic ring of a monophenol (such as tyrosine), coupled with the two-electron oxidation of the resulting catechol to the corresponding quinone (for example, dopaquinone). The primary question is whether splitting of the O-O bond occurs *prior to*, *along with*, or *after* attack by oxygen on the aromatic ring.[3a] In the first case, the hydroxylation would be mediated by a bis(μ-oxo) core (Scheme 1, left). The last scenario would lead to an intermediate aryl peroxide species (Scheme 1, right) which then



Scheme 1. Possible reaction mechanisms for the oxygenation of phenols by Cu_2O_2 centers.

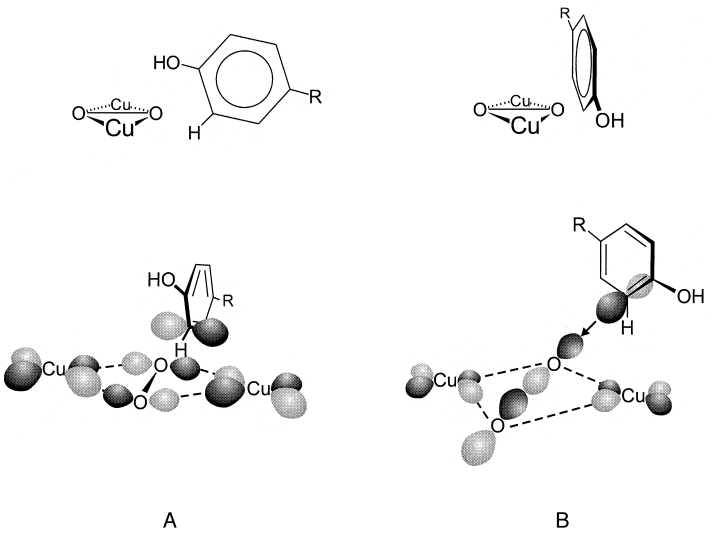
reacts with the diphenol (quinone). In the second case, the hydroxylation step would represent a case of electrophilic aromatic substitution (S_E) by peroxide with the π system being activated for this reaction by the first OH group (Scheme 1, center). The electrophilic character of the side-on bridging peroxide group has been explained by a large amount of charge donation into the Cu^{II} centers, making this the least negative form of peroxide.[3b, 10]

A prototypical example for this reactivity is provided by the binuclear copper complex $[Cu_2(R\text{-}XYL)]^{2+}$ (Scheme 2). In the binucleating ligand R-XYL-H, two bis[2-(2-pyridyl)ethyl]-amino (PY2) tridentate units are linked by a xylyl spacer which is hydroxylated upon reaction of the Cu^I complex with oxygen.[11] It has recently been proven through resonance Raman spectroscopy that the hydroxylation of the aromatic π system occurs via a side-on bound peroxide intermediate.[12] Importantly, both spectroscopy and molecular orbital theory



Scheme 2. Reaction pathway for ligand hydroxylation in the model system of Karlin. The hydroxylation proceeds via a μ-η^2:η^2 peroxide intermediate.

allowed participation of the bis(μ-oxo) structure Cu^{III}-$(O^{2-})_2$-Cu^{III} (Scheme 1, left) in the reaction to be excluded (see below). The results have been explained based on a S_E mechanism.[12] Within a frontier orbital approach, the authors consider two distinct orbital pathways for electrophilic attack of the peroxide onto the aromatic ring of the substrate; the HOMO of the arene ring can either interact with the lowest unoccupied molecular orbital (LUMO) of the side-on peroxide bridged structure, which has π^* character, or with the next unoccupied orbital at higher energy, which is σ^* type. Scheme 3 provides a look approximately along the Cu-Cu (top) or the O-O axis (bottom) for the corresponding "π^* pathway" (A) and the "σ^* pathway" (B), respectively. In the



Scheme 3. Substrate orientation and frontier orbital analysis for the hydroxylation of phenols by a μ-η^2:η^2 peroxide intermediate in the case of an electrophilic substitution. Two possible reaction pathways with corresponding orbital interactions are shown. A: The O-O axis is parallel to the aromatic ring plane of the substrate. B: The O-O axis is perpendicular to the aromatic ring plane of the substrate.

bottom views, the frontier orbital interactions are included schematically (note that only one p orbital of the HOMO of the aromatic ring has been included). The first possibility involves an approach of the substrate with the O-O axis parallel to the aromatic ring plane and a π interaction between the LUMO of the complex and the HOMO of the aromatic system (Scheme 3A). In the other case, the aromatic ring plane is perpendicular to O-O, and one C atom of the aromatic ring in *ortho* position to the OH group interacts in a σ fashion with peroxide (Scheme 3B). From geometric and electronic considerations Karlin et al. conclude that the hydroxylation of the xylyl ring in the present system is mediated by the side-on bridged peroxide adduct through the "π^* pathway".

In proteins so far only the presence of the μ-η^2:η^2 peroxide form has been established by crystallography and spectroscopy. However, the recent structural characterisation of the bis(μ-oxo) core in small-molecule model systems[13, 14] and the observation of its facile interconversion with the side-on bridging peroxide[15] raise the question: to what extent is this

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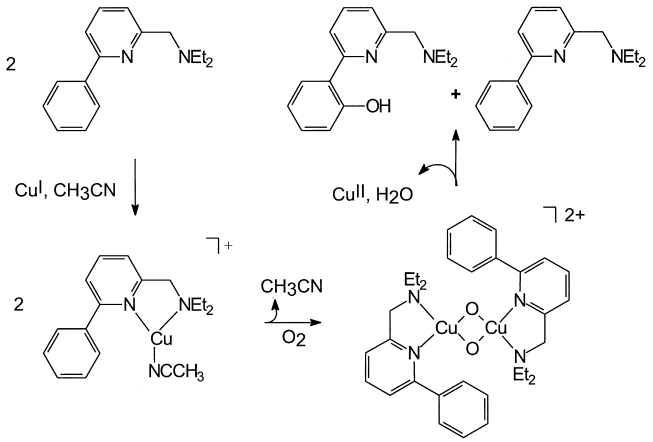
bis(μ-oxo) form capable of hydroxylating a phenolic substrate, namely does it participate in the enzymatic reaction? As a rule, the side-on peroxide group is more electrophilic than the bis(μ-oxo) form,[12] and the latter is more prone to H atom abstraction than the side-on coordinated peroxide.[16, 17] Aliphatic ligand hydroxylation reactions mediated by bis(μ-oxo)dicopper(III) and -dinickel(III) centers also proceed by the latter mechanism.[18] Experimental evidence for the fact that the bis(μ-oxo)dicopper(III) core is also able to oxygenate an aromatic substrate has recently been provided by Tolman and co-workers;[19] upon reaction of the ligand 2-diethylaminomethyl-6-phenylpyridine and Cu^{I} with O_2, an adduct is formed at low temperatures, with spectroscopic properties characteristic for a bis(μ-oxo) core. After warming the reaction solution, hydroxylation of an aromatic ring contained within the ligand system occurs (Scheme 4). This is in contrast



Scheme 4. Reaction pathway for ligand hydroxylation in the model system of Tolman. The hydroxylation proceeds via a bis(μ-oxo)dicopper(III) intermediate.

to earlier systems, where the bis(μ-oxo) core primarily decomposes under N-dealkylation of the respective ligand.[16] The rate of the hydroxylation reaction and the relative amounts of hydroxylated versus non-hydroxylated ligand strongly depend on the substituents in the phenyl ring in a way that suggests an electrophilic substitution process. The alternative mechanism, H atom abstraction, could be excluded in this case based on the absence of a deuterium effect on the reaction rate.

Enzymatic Systems: The Case of Exogenous Substrates

In both examples considered so far the substrate has been preorganized for hydroxylation as a part of the ligand system. What happens if those substrates forming "endogenous" ligands are replaced by external substrates being coordinated (and possibly converted) as "exogenous" ligands? Possible answers to this question are provided by the study of Cu_2O_2 model systems that react with external phenols. However, in almost all known cases unphysiological radical coupling products are formed in such reactions.[20] This is explained by the primary formation of phenoxyl radicals through H atom abstraction from the corresponding phenols by the Cu_2O_2 center. Complexes with side-on coordinated peroxide,[20a–e] as well as those having a bis(μ-oxo) core,[20e,f] show this reactivity. In the meantime however, one small-molecule system is also known that hydroxylates external phenols in a tyrosinase-like fashion.[21]

Alternatively, the coordination of substrates or corresponding analogues (inhibitors, etc.) to the active sites of structurally characterized type 3 copper proteins can be investigated. A prominent example for this second approach is provided by the recently resolved X-ray structure of a 39 kDa COase of sweet potato which features an open entrance to the active site (Figure 1 a).[7] In the met form, only CuB is free to coordinate a phenolic substrate. Importantly, the structural

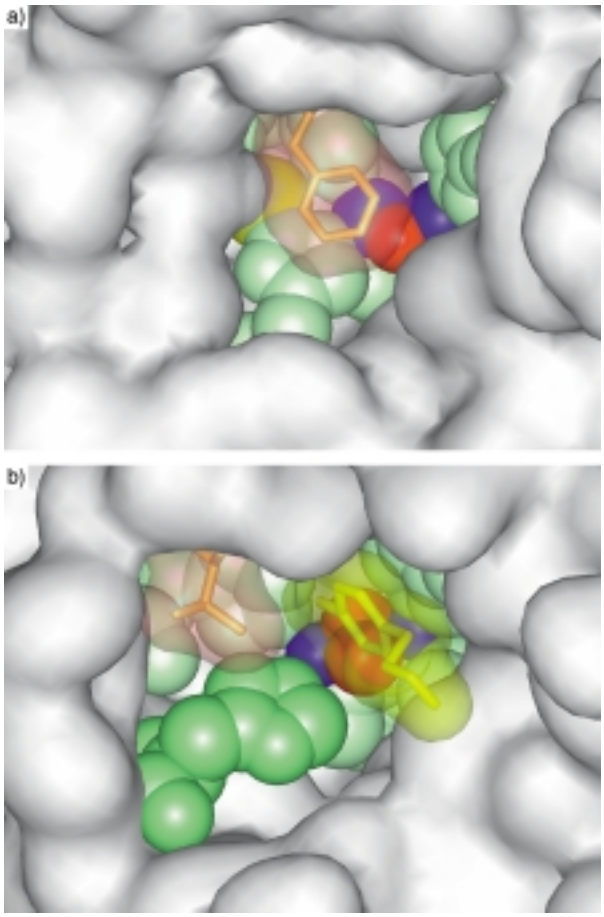


Figure 1. a) View of the active site of catecholoxidase in the met form. The two copper atoms (blue) are coordinated by six histidines (green). A stabilizing cysteine (dark yellow) is shown with van der Waals radius. CuA is shielded by a phenylalanine residue (orange). Bonding of diphenols is assumed to occur to CuB (see text). b) View of the substrate binding pocket and orientation of putative phenolic substrates (tyrosine) in hemocyanins. A putative tyrosine substrate (yellow) in the protein pocket of *Limulus* oxy-hemocyanin replaces Phe49 in the native structure (see text). Space-filling (transparent) atoms demonstrate that the substrate fits perfectly. Dioxygen (red) is bound in a μ-η^2:η^2 coordination.

characterisation of the inhibitor-bound form of this enzyme allowed a detailed proposal for the coordination of *ortho*-diphenols. Comparable structural insight has been obtained with respect to the bonding of monophenols from the study of "activated" hemocyanins. Most significant in this respect is the finding of mono- and diphenolase activity for spider Hc

after limited proteolysis with trypsin and chymotrypsin.[8c] This activation mechanism has been explained on the basis of the structurally closely related Hc from *Limulus polyphemus*;[5] the N-terminal peptide containing Phe 49, which is highly conserved among Hcs and phenoloxidases from arthropods, is pulled out of a cavity of the protein leaving a pocket and a free access for dioxygen and potential substrates to the copper active site (Figure 1 b). A spacefilling tyrosine (yellow) placed as a phenolic substrate into this pocket exactly like Phe49 demonstrates that not much freedom for other orientations is available. This is due to steric constraints through hydrophobic interactions between the phenyl ring of the substrate and the axial His 328 on CuB (green) and a Thr 351 residue next to CuA (orange, transparent). The resulting coordination geometry of the substrate shows that the phenyl ring is almost perpendicular to the Cu-O_2-Cu plane with close contacts (under 4 Å) of its hydroxyl group to CuA (blue) and of its ortho position to one of the two oxygen atoms of the peroxide ligand (red, Figure 2).[8c] This suggests bonding to CuA, in agreement with earlier concepts assuming direct coordination of the phenolic substrates to one Cu center, preferably CuA.[3a] Furthermore, the vicinity of the ortho position of the aromatic ring to O_2 may be essential for the hydroxylation reaction (see below). The comparison with the COase active site (Figure 1 a) shows that CuA is shielded by a phenylalanine residue (orange) in this case, which may be the reason for the lack of monophenolase activity for this enzyme.

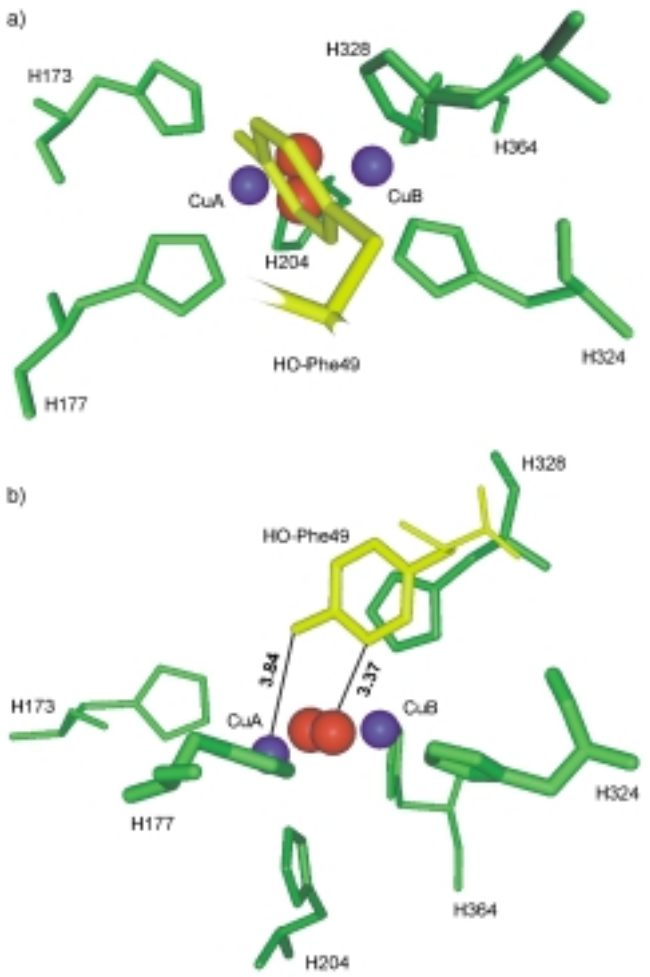


Figure 2. Top (a) and side view (b) of the coordination geometry of the hydroxylated Phe 49, a putative substrate according to Decker and Rimke,[8c] at the active site. Distances from the hydroxyl group and the ortho position of the benzyl ring to dioxygen and CuA are given in Å.

Looking back to the model system of Karlin, it is evident that from the two orbital pathways shown in Scheme 3 only the first reaction mode (A, π donation into the LUMO of the side-on peroxide structure) appears to be compatible with the substrate orientation presented in Figure 2. In contrast, the second possibility (B) is clearly distinct from this picture suggesting that the plane of the aromatic ring would not fit into the active-site pocket in this geometry. The same conclusion has also been reached by Karlin et al.[12] The proposed substrate bonding geometry raises a question concerning the time sequence of the hydroxylation step (attack of O_2) and possible bonding to CuA; upon primary coordination of the phenol to this center, ligand-to-metal charge transfer would ensue which would act to diminish the activation of the aromatic ring for electrophilic attack. Moreover, upon coordination of the phenolic substrate the charge donation of peroxide to copper would decrease leading to a less electrophilic O_2 ligand. Both factors would be counterproductive with respect to the electrophilic substitution reaction (hydroxylation). In addition to the above-discussed question as to which is the hydroxylating agent (side-on peroxide or μ-oxo), the problem needs to be addressed as to whether a phenolic substrate binds to one Cu center of the Cu_2O_2 unit *at all*, and if it does whether this is *before*, *after*, or *along with* hydroxylation.[3a, 22] Finally, it should be mentioned that radical reaction paths have also been discussed for tyrosinase, for example in an "early" mechanistic proposal by Kitajima and Moro-oka.[20c] A recent theoretical study of tyrosinase activity by Lind, Siegbahn, and Crabtree also draws the conclusion that a radical mechanism is a plausible alternative to the electrophilic substitution pathway.[23] The question of which mechanistic alternative actually applies to tyrosinase (or whether different mechanisms are found to explain the hydroxylation of monophenols) will remain a challenging problem of bioinorganic chemistry in the next few years.

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