Copper complexes as biomimetic models of catechol oxidase: mechanistic studies

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1 Introduction[†]

1.1 Copper-containing metalloproteins

Proteins containing copper ions at their active site are usually involved as redox catalysts in a range of biological processes, such as electron transfer or oxidation of various organic substrates. In general, four major functions of such proteins can be distinguished: (i) metal ion uptake, storage and transport; (ii) electron transfer; (iii) dioxygen uptake, storage and transport; (iv) catalysis. Initially, all copper proteins were classified based on their spectroscopic features, which led to the distinguishing of the type-1, type-2 and type-3 active sites. However, recent development of crystallographic and spectroscopic techniques enabled the discovery of other types of copper-containing active sites, and a current classification distinguishes seven different types of active site in the oxidized state of copper-containing proteins; they are briefly outlined below.

Type-1 active site

The copper proteins with the type-1 active site are commonly known as "blue copper proteins" due to their intense blue color. The latter is caused by a strong absorption at *ca.* 600 nm, corresponding to an LMCT transition from a cysteine sulfur to copper(II) ions.[1] These proteins are usually participating in electron transfer processes, and the most well-known representatives of this class include plastocyanin, azurin and amicyanin.[2] The type-1 active site is also found in some multicopper oxidases, which contain more than one copper sites, such as ascorbate oxidase, and in redox enzymes such as nitrite reductase. The coordination sphere around the copper center in the type-1 active site is constituted by two nitrogen donor atoms from two histidine residues, a sulfur atom from a cysteine residue and a weakly coordinated sulfur atom from, in most cases, a methionine residue (Figure 1.1, a). Instead of methionine, a glutamine or a leucine are known to be present in some cases.

[†] This chapter is based on: Koval, I.A., Gamez, P., Belle, C., Selmeczi, K., Reedijk, J., submitted to Chem. Soc. Rev.

Type-2 active site

The copper proteins containing the type-2 active site are also known as "normal" copper proteins, a name historically based on their EPR features which are similar to common Cu^{II} complexes containing an N,O chromophore with tetragonal geometry. The copper coordination sphere in these proteins is constituted by four N and/or O donor atoms in either square-planar or distorted tetrahedral geometry.[3,4] The examples of the proteins with this active site include copper-zinc superoxide dismutase, dopamine-β-hydroxylase, phenylalanine hydroxylase and galactose oxidase (Figure 1.1, b).[5] The proteins of this class are mostly involved in catalysis, such as disproportionation of $O_2^{\bullet -}$ superoxide anion, selective hydroxylation of aromatic substrates, C-H activation of benzylic substrates and primary alcohols oxidation.

Type-3 active site

This class is represented by three proteins, namely hemocyanin, tyrosinase and catechol oxidase. The active site contains a dicopper core, in which both copper ions are surrounded by three nitrogen donor atoms from histidine residues.[3,6] A characteristic feature of the proteins with this active site is their ability to reversibly bind dioxygen at ambient conditions. Hemocyanin (Figure 1.1, c) is responsible for dioxygen transport in certain mollusks and arthropods, whereas tyrosinase and catechol oxidase utilize dioxygen to perform an oxidation of phenolic substrates to catechols (tyrosinase) and subsequently to *o*-quinones (tyrosinase and catechol oxidase), which later on undergo polymerization with the production of the pigment melanin. The copper(II) ions in the *oxy* state of these proteins are strongly antiferromagnetically coupled, leading to EPR-silent behavior. The crystal structures of hemocyanin[7] and catechol oxidase[8] have been solved, whereas the exact structure of tyrosinase still remains unknown.

Type-4 active site

The copper site in these proteins is usually composed of a type-2 and a type-3 active sites, together forming a trinuclear cluster. In some cases, these proteins also contain at least one type-1 site and are in this case addressed as multicopper oxidases, or blue oxidases.[3] The trinuclear cluster and the type-1 site are connected through a Cys-His electron transfer pathway. The representatives of this class are laccase (polyphenol oxidase),[9-11] ascorbate oxidase (Figure 1.1, d)[12] and ceruloplasmin,[13] which catalyze a range of organic oxidation reactions.

Very recently, Lieberman and Rosenzweig[14] reported a 2.8 Å resolution crystal structure of methane monooxygenase, the enzyme encountered in metamophores, which are bacteria catalyzing the methane conversion to methanol. In the crystal structure, three copper centers have been found: a mononuclear center resembling a type-2 active site, and an unusual site, refined as dinuclear, in which two copper ions are located at a very short distance of 2.6 Å (Figure 1.1, e). In contrast to other multicopper oxidases, the dinuclear site is situated 21 Å apart from the mononuclear site. The oxidation states of the three copper ions are not clear, but the mononuclear copper center is believed to

give rise to an EPR signal, typical for the type-2 active sites. However, the presence of some Cu^{I} in the crystal structure was confirmed by X-ray absorption near edge spectra (XANES), which would suggest that at least one or both copper ions in the dinuclear site have +1 oxidation state.

The Cu_A active site

This type of active site is also known as a mixed-valence copper site. It contains a dinuclear copper core, in which both copper ions have a formal oxidation state +1.5 in the oxidized form. Both copper ions have a tetrahedral geometry and are bridged by two thiolate groups of two cysteinyl residues. Each copper ion is also coordinated by a nitrogen atom from a histidine residue. This site exhibits a characteristic seven-line pattern in the EPR spectra and is purple colored. Its function is a long-range electron transfer, and this site can be found, for example, in cytochrome *c* oxidase[15-17] and nitrous oxide reductase (Figure 1.1, f).

The Cu_B active site

This active site was detected close to an iron center in cytochrome *c* oxidase (Figure 1.1, g).[18] In this site, a mononuclear Cu ion is coordinated by three nitrogen atoms from three histidine residues in a trigonal pyramidal geometry. No fourth ligand coordinated to the metal ion was detected. The vacant position in the copper coordination sphere is directed towards the vacant position in the coordination sphere of the heme iron ion. Two metal ions are strongly antiferromagnetically coupled in the oxidized state. A copper-iron distance of 5.3 Å for *Paracoccus denitrificans* and 4.5 Å for bovine heart cytochrome *c* oxidase was found. The function of the Cu_B site is the four-electron reduction of dioxygen to water.

The Cu_Z active site

The Cu_Z active site consists of four copper ions, arranged in a distorted tetrahedron and coordinated by seven histidine residues and one hydroxide anion. This site was detected in nitrous oxide reductase (Figure 1.1, h) and is involved in the reduction of N_2O to N_2. The crystal structures of nitrous oxidase from *Pseudomonas nautica* and *Paracoccus denitrificans* were solved at resolutions of 2.4 Å and 1.6 Å, respectively.[19,20] The copper ions in the tetranuclear cluster are bridged by an inorganic sulfur ion,[21] which until recently was believed to be a hydroxide anion. The metal-metal distances between the Cu2 and Cu4 and Cu2 and Cu3 atoms are very short (*ca.* 2.5-2.6 Å) and can be thus regarded as metal-metal bonds, whereas the distances between the other copper centers are substantially longer (*viz.* 3.0-3.4 Å).[19] Three copper ions are coordinated by two histidine residues, whereas the fourth is coordinated by only one, forming thus a substrate binding site. The oxidation states of the copper ions in the resting state are still unclear, as the EPR spectra of this active site can be explained by two different oxidation schemes, *i.e.* $Cu^{I}_3Cu^{II}$ and $Cu^{I}Cu^{II}_3$, both resulting in four-line spectra.

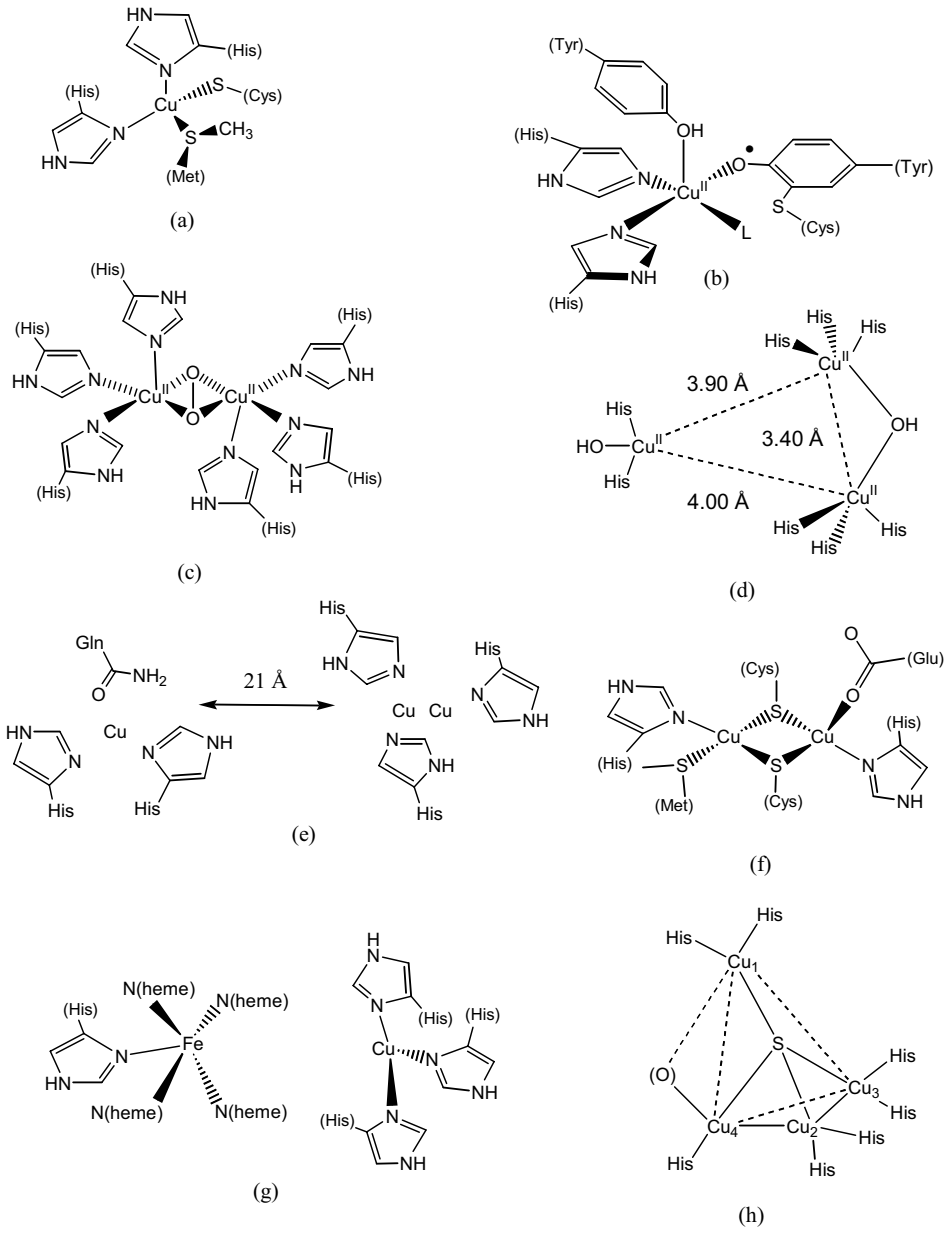


Figure 1.1. Schematic representations of the selected active sites of the copper proteins: plastocyanin[22] (type-1, a), galactose oxidase[23] (type-2, b), *oxy* hemocyanin[7] (type-3, c), ascorbate oxidase[12] (type-4, or multicopper site, d), methane monooxygenase[14] (multicopper site, e), nitrous oxide reductase[24] (Cu_A site, f), cytochrome c oxidase[18] (Cu_B site, g) and nitrous oxide reductase (Cu_Z site, h).[19]

1.2 Catechol oxidase: structure and function

1.2.1 General

Catechol oxidase (COx) is an enzyme containing the type-3 active site that catalyzes the oxidation of a wide range of *o*-diphenols (catechols), such as caffeic acid, to the corresponding quinones in a process known as a catecholase activity. The latter highly reactive compounds undergo an auto-polymerization leading to the formation of a brown polyphenolic pigment, *i.e.* melanin, a process thought to protect a damaged tissue against pathogens or insects.[25] COx's are found in plant tissues and in

crustaceans. The first COx was isolated in 1937.[26] Subsequently, they were purified from a wide range of vegetables and fruits (*e.g.* potato, spinach, apple, grape berry),[26] and more recently, from gypsy wort[27] and litchi fruit.[28] The purity of COx's was not always satisfactory due to a multiplicity of isozymes and forms, but improved purification protocols have been reported,[26] *e.g.* for COx from black poplar.[27]

The molecular weight of COx's varies, depending on the tissue and organism from which it has been extracted. Two ranges of molecular mass can sometimes be found, even in a single source: one in the range of 38-45 kDa, and another in the range of 55-60 kDa. This difference is possibly due to C-terminal processing.[29] Smaller enzymes with a molecular weight of about 30 kDa are also found, but they are generally described as proteolyzed derivatives of the purified mature protein.

In 1998, Krebs and co-authors have reported the crystal structures of the catechol oxidase isolated from *Ipomoea batatas* (sweet potato) in three catalytic states: the native *met* ($Cu^{II}Cu^{II}$) state, the reduced *deoxy* ($Cu^{I}Cu^{I}$) form, and in the complex with the inhibitor phenylthiourea.[8] An isolated monomeric enzyme with a molecular weight of 39 kDa was found to be ellipsoid in shape with dimensions of 55×45×45 $Å^3$. The secondary structure of the enzyme is primarily α-helical with the core of the enzyme formed by a four-helix bundle composed of α-helices α2, α3, α6 and α7. The helical bundle accommodates the catalytic dinuclear copper center and is surrounded by the helices α1 and α4 and several short β-strands. Each of the two copper ions is coordinated by three histidine residues contributed from the four helices of the α-bundle.

1.2.2 The structures of the active site

1.2.2.1 The *met* ($Cu^{II}Cu^{II}$) state

In the native *met* state, two copper ions are 2.9 Å apart. In addition to six histidine residues, a bridging solvent molecule, most likely hydroxide anion was refined in a close proximity to the two metal centers (CuA-O 1.9 Å, CuB-O 1.8 Å), completing the coordination sphere of the copper ions to a trigonal pyramid. These findings are in agreement with EXAFS data for the oxidized COx's from *Lycopus europaeus* and *Ipomoea batatas*, confirming the presence four N/O donor atoms and a $Cu^{II}…Cu^{II}$ distance of 2.9 Å in solution for both enzymes.[30,31] The apical positions are occupied by the His 109 and His 240 residues for CuA and CuB, respectively (Figure 1.2, left). EPR data reveal a strong antiferromagnetic coupling between the copper ions, therefore the presence of a bridging OH^- ligand between the copper(II) ions was proposed for the *met* form of the enzyme.

1.2.2.2 The reduced *deoxy* ($Cu^{I}Cu^{I}$) state

Upon reduction of the copper(II) ions to +1 oxidation state, the distance between them increases to 4.4 Å, while the histidine residues move only slightly, and no

significant change was observed for other residues of the protein.[8] Based on the residual electron density maps, a water molecule was positioned on a distance of 2.2 Å from the CuA atom. Thus, the coordination sphere around CuA ion is a distorted trigonal pyramid, with three nitrogen atoms from the histidine residues forming a basal plane, while the coordination sphere around CuB ion can be best described as square planar with one missing coordination site.

1.2.2.3 The adduct of catechol oxidase with the inhibitor phenylthiourea

Phenylthiourea binds to catechol oxidase by replacing the hydroxo bridge, present in the *met* form. The sulfur atom of phenylthiourea is coordinated to both copper(II) centers, increasing the distance between them to 4.2 Å (Figure 1.2, right). The amide nitrogen is weakly interacting with the CuB center (Cu-N distance of 2.6 Å), completing its square-pyramidal geometry. The dicopper core in catechol oxidase is found in the center of a hydrophobic pocket lined by the side chains of Ile 241, Phe 261, His 244 and Ala 264.[8] Upon phenylthiourea binding, the phenyl ring of Phe 261 and the imidazole ring of His 244 undergo a conformational change to form hydrophobic interactions with the aromatic ring of the inhibitor. These van der Waals interactions further contribute to the high affinity (IC_{50} = 43 μM, K_M = 2.5 mM for catechol substrate[30]) of this inhibitor to the enzyme.

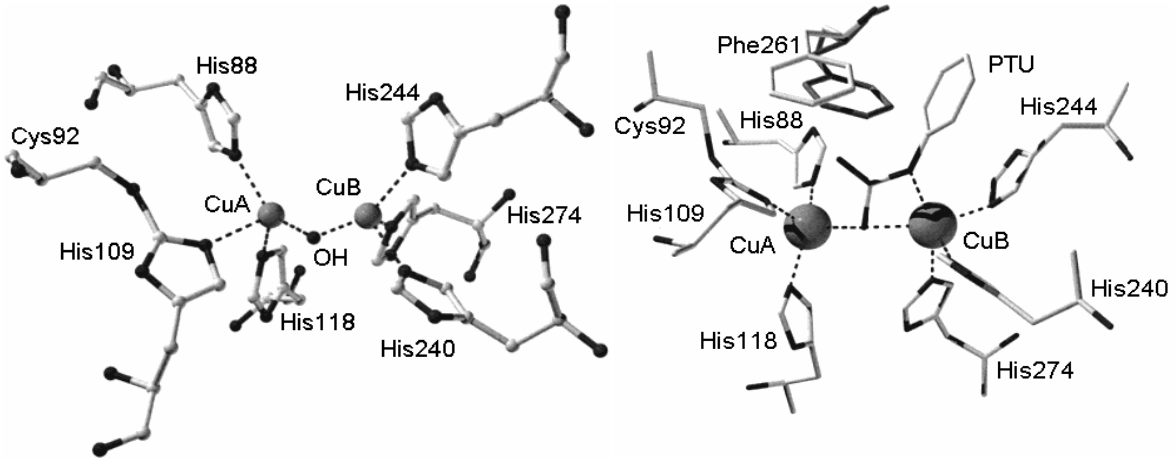


Figure 1.2. Left: coordination sphere of the dinuclear copper(II) center in the *met* state. Right: crystal structure of the inhibitor complex of catechol oxidase with phenylthiourea. Phe 261 is shown additionally in the orientation of native COx (in dark color) to show rotation of Phe 261 in the inhibitor complex (in light color). Redrawn after Krebs and co-workers.[29]

1.2.2.4 The dioxygen binding by the dicopper(I) center: *oxy* state

The *oxy* form of catechol oxidase can be obtained by treating the *met* form of the enzyme with dihydrogen peroxide. Eicken *et al.*[30] reported that the treatment of the 39 kDa catechol oxidase from *Ipomoea batatas* (ibCOx) with H_2O_2 leads to absorption bands at 343 nm (ε = 6500 $M^{-1}cm^{-1}$) and 580 nm (ε = 450 $M^{-1}cm^{-1}$), which reach

maximal development when 6 equivalents of dihydrogen peroxide are added (Figure 1.3). Similar results have been reported for COx's isolated from *Lycopus europaeus and Populus nigra.*[27] This type of UV-Vis spectra is characteristic for a μ-η^2:η^2-peroxo-dicopper(II) core, which was originally reported by Kitajima *et al.*[32] for a synthetic dinuclear copper model complex. The first strong absorption in the range of 335-350 nm is assigned to a peroxo O_2^{2-} (π_σ^*) → Cu^{II} ($d_{x^2-y^2}$) charge transfer, whereas the second weak band around 580 nm corresponds to a peroxo O_2^{2-} (π_v^*) → Cu^{II} ($d_{x^2-y^2}$) CT transition.[4,33]

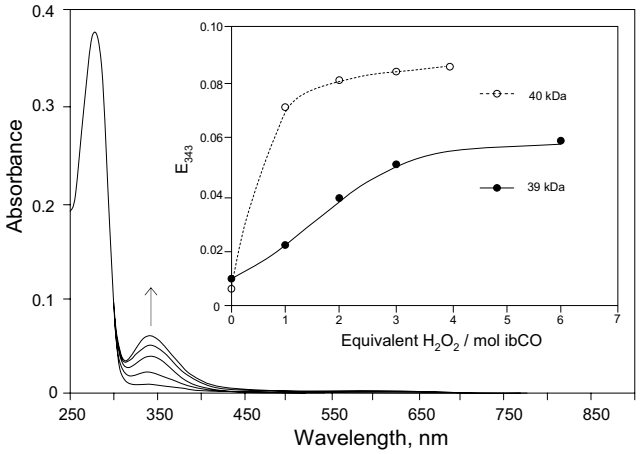


Figure 1.3. Titration of the 39 kDa ibCOx in 0.5 M NaCl, 50 mM sodium phosphate pH = 6.7 with H_2O_2. Insert: absorption at 343 nm without and after addition of one, two, three and six equivalents of H_2O_2. Redrawn after Krebs and co-workers.[30]

1.2.2.5 The covalent cysteine-histidine bond

An interesting feature of the dinuclear copper center in catechol oxidase is the unusual thioether linkage formed between the Cε atom of the histidine His 109, one of the ligands to CuA ion, and the cysteine sulfur atom of Cys 92. It should be noted that a thioether linkage has also been described for the type-2 copper enzyme galactose oxidase. In this structure, a covalent bond formed between the Cε carbon atom of a tyrosinate ligand and the sulfur atom of a cysteine residue was proposed to stabilize the tyrosine radical generated during catalysis.[23] There are also reports of this type of bond for a tyrosinase from *Neurospora crassa*,[34] as well as for several types of hemocyanins.[35-37] The absence of this unit in arthropod hemocyanins and in human tyrosinase does not, however, support its involvement in the electron transfer process. The crystal structure of CO reveals that this covalent bond puts additional structural restraints on the coordination sphere of the CuA ion. In particular, such restrains may help to impose the trigonal-pyramidal geometry (which can be also regarded as a distorted trigonal bipyramid with a vacant apical position) on the CuA ion in +2 oxidation state. This may in turn optimize the redox potential of the metal needed for the oxidation of the catechol substrate and may allow a rapid electron transfer in the

redox processes. Also, this thioether bond may prevent the displacement of His 109 and a didentate binding mode of the substrate to a single Cu^{II} ion.

1.2.3 Enzymatic reaction mechanism

Catechol oxidase catalyzes the oxidation of *o*-diphenols (catechols) to the respective quinones through four-electron reduction of dioxygen to water. Krebs and co-workers proposed a mechanism for the catalytic process, based on biochemical,[3,38] spectroscopic[30] and structural[8] data, as depicted in Figure 1.4. The catalytic cycle begins with the *met* form of catechol oxidase, which is the resting form of the enzyme. Because the *oxy* state of COx could be obtained only after the addition of H_2O_2, this form was excluded as the start situation. The dicopper(II) center of the *met* form reacts with one equivalent of catechol, leading to the formation of quinone and to the reduced *deoxy* dicopper(I) state. This step is supported by the observation that stoichiometric amounts of the quinone product form immediately after the addition of catechol, even in the absence of dioxygen.[8,39] Based on the structure of COx with the bound inhibitor phenylthiourea, the monodentate binding of the substrate to the CuB center has been proposed. Afterwards, dioxygen binds to the dicopper(I) active site replacing the solvent molecule bonded to CuA in the reduced enzyme form. Binding of the catechol substrate to the *deoxy* state prior to dioxygen binding seems less likely, as no substrate binding was observed upon treating the reduced by dithiothreitol enzyme with the high molar excess of catechol, indicating a low binding affinity of the substrate to the dicopper(I) center. UV-Vis spectroscopy and Raman data suggested that dioxygen binds in the bridging side-on μ-η^2:η^2 binding mode with a copper-copper separation of 3.8 Å, as determined by EXAFS spectroscopy.[30] The rotation of the side chain of Phe 261 in the enzyme opens the dicopper center to permit the binding of the catechol substrate. The observed binding mode of phenylthiourea and the modeled catechol-binding mode suggest that a simultaneous binding of catechol and dioxygen is possible. Superposition of the aromatic ring of the modeled catechol substrate and the phenyl ring of phenylthiourea places the coordinated catecholate hydroxylate group close to the coordinated amide nitrogen of the inhibitor and maintains the favorable van der Waals interactions observed in the inhibitor complex.[8] In this model, CuB is six-coordinated with a tetragonal planar coordination by His 240, His 244 and the dioxygen molecule. The CuA site retains the tetragonal pyramidal geometry with dioxygen, His 88 and His 118 in equatorial positions, His 109 in an axial position and a vacant sixth coordination site. In this proposed ternary COx-O_2^{2-}-catechol complex, two electrons can be transferred from the substrate to the peroxide, followed by the cleavage of the O-O bond, loss of water and the formation of the quinone product, together with the restoration of the *met* state, completing the catalytic cycle.

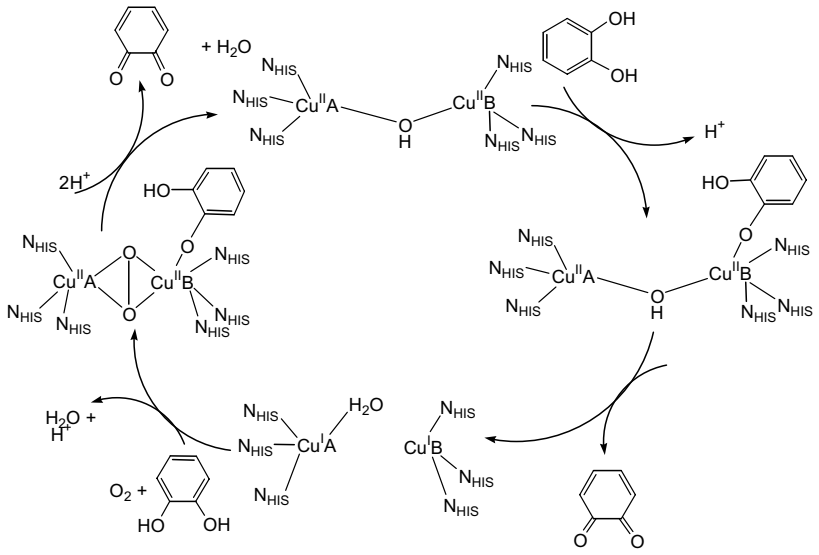


Figure 1.4. Catalytic cycle of catechol oxidase from *Ipomoea batatas*, as proposed on the basis of structural, spectroscopic and biochemical data. Two molecules of catechol (or derivatives thereof) are oxidized, coupled with the reduction of molecular oxygen to water. The ternary $COx\text{-}O_2^{2-}$-catechol complex was modeled, guided by the binding mode observed for the inhibitor phenylthiourea. Redrawn after Krebs and co-workers.[39]

A totally different mechanism of the catalytic cycle, however, was proposed by Siegbahn,[40] who applied a hybrid density functional theory for a quantum chemical study of the catalytic cycle. According to the author, the growing number of theoretical[41] and experimental[42,43] studies suggest that the active site of an enzyme, which is deeply buried in the low dielectric of a protein, as observed in catechol oxidase, should not change its charge during the catalytic cycle. However, in the mechanism, proposed by Krebs *et al.*,[8] the charge of the active site changes from +1 in the peroxo-dicopper(II)-catecholate adduct, to +3 in the met form. According to Siegbahn,[40] this in turn implies the availability of several external nearby bases, which could store protons, released during the cycle. At the same time, the X-ray crystal structure does not reveal the presence of such candidates in the region of the active site. Consequently, a different mechanism[40] was proposed by the author based on the DFT calculations, as depicted in Figure 1.5. The catalytic cycle starts from the deoxy dicopper(I) form. In order to maintain an overall charge + 1 of the active site, the author proposed a presence of a bridging hydroxide ligand between the two copper(I) ions,[44] in contrast to the X-ray crystallographic findings,[8] which suggest a presence of a water molecule, asymmetrically bonded to only one copper center. At the first stage, catechol binds to the deoxy form, transferring the proton to the bridging hydroxide with a consequent generation of the bridging water molecule between the metal centers. Afterwards, dioxygen displaces a water molecule, binding as a superoxide radical anion and resulting in the formation of the mixed-valence dicopper(II,I) species (step a). The superoxide then abstracts a hydrogen atom (a proton and an electron) from the bound

substrate. To release the quinone molecule, an electron is then transferred from the quinone radical to the Cu^{II} ion, leading to the restoration of the dicopper(I) state (steps b and c). The next step involves the cleavage of the O-O bond, which is accompanied by a transfer of two protons from the substrate and two electrons (from one of the Cu^{I} ions and the substrate) to the peroxide moiety (steps d, e). Altogether this leads to a product which can be best described as a $Cu^{II}Cu^{I}$ species with a quinone radical anion. The second electron transfer from the quinone radical to the Cu^{II} center leads to the restoration of the initial hydroxo-bridged dicopper(I) form.

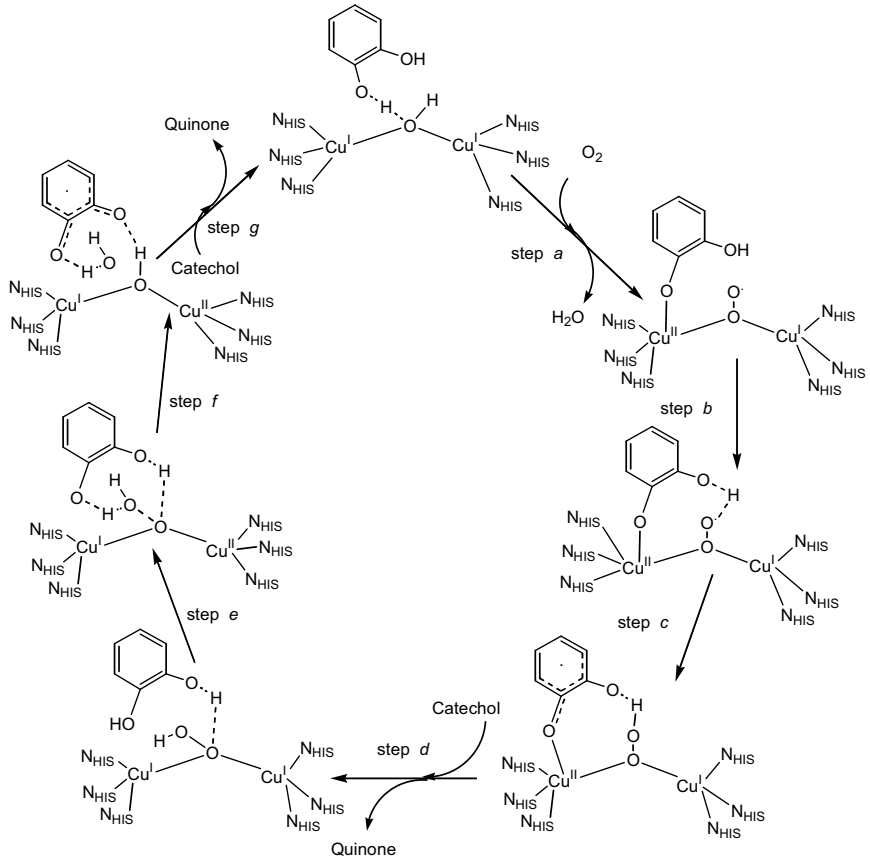


Figure 1.5. The mechanism of the catalytic cycle of catechol oxidase, as proposed by Siegbahn.[40]

However, it should be noted that at the present moment the latter mechanism is not supported by the experimental findings. In particular, an existence of a bridging μ-1,1-superoxide radical anion, the formation of which is proposed by the author, has never been reported in the literature.

1.3 Model systems of catechol oxidase

1.3.1 Historic overview

The ability of the copper complex to oxidize phenols and catechols has been well known for at least 40 years. For example, in 1964 Grinstead reported the oxidation of 3,5-di-*tert*-butylcatechol ($DTBCH_2$) to the respective 3,5-di-*tert*-butyl-*o*-

benzoquinone (DTBQ) with 55% yield in 75% aqueous methanol in the presence of 1% of copper(II) chloride.[45] In 1974, Thuji and Takayanagi reported the oxidative cleavage of catechol, leading to the formation of *cis,cis*-muconic acid, by dioxygen and copper(I) chloride in aqueous solution.[46] Rogić and Demmin have also studied the oxidation of catechol by copper(I) chloride and dioxygen in various solvent mixtures.[47] The reactions were usually carried out in pyridine in the presence of 5 molar equivalents of an alcohol (MeOH, EtOH, *i*-PrOH or *n*-BuOH). Depending on the reaction conditions, either muconic acid or its monoalkyl esters were obtained as products. However, in the presence of dichlorobis(pyridine)copper(II) in pyridine-methanol mixture under dioxygen, 4,5-dimethoxy-1,2-benzoquinone was isolated as the reaction product.

One of the pioneering mechanistic studies on catechol oxidation by copper(II) complexes was presented by Lintvedt and Thuruya.[48] In their study of the kinetics of the reaction of $DTBCH_2$ with dioxygen catalyzed by bis(1-phenyl-1,3,5-hexanetrionato)dicopper(II) complex, the authors showed that the overall reaction was first order in the substrate and second order in Cu^{II}, thus in fact confirming that the active reaction intermediate involved in the rate-determining step was a dicopper-catecholate species. Another interesting early mechanistic studies is the work of Demmin, Swerdloff and Rogić,[49] who emphasized the main steps in the catalytic process: (i) formation of dicopper(II)-catecholate intermediate; (ii) electron transfer from the aromatic ring to two copper(II) centers, resulting in the formation of *o*-benzoquinone and two copper(I) centers; (iii) irreversible reaction of the generated copper(I) species with dioxygen, resulting in copper(II)-dioxygen adduct, and (iv) the reaction of this adduct with catechol, leading to regeneration of the dicopper(II)-catecholate intermediate and formation of water as the byproduct.

Oishi *et al.* have reported the higher activity of dinuclear copper(II) complexes in the oxidation of $DTBCH_2$ in comparison to their mononuclear analogues,[50] thus confirming the earlier hypothesis of Lintvedt and Thuruya about the formation of the dicopper-catecholate intermediate in the catalytic process.[48] Furthermore, the authors reported a stoichiometric oxidation of $DTBCH_2$ in anaerobic conditions to the respective quinone by a number of mononuclear and dinuclear copper(II) complexes, which was consistent with the 1^{st} step of the mechanism proposed by Demmin, Swerdloff and Rogić.[49] They also made an interesting observation that mononuclear planar copper(II) complexes could not be reduced by $DTBCH_2$ and showed very little catecholase activity in comparison to the readily reducible complexes. Thus, the catalytic activity of the complexes appeared to correlate with their reduction potentials. Another interesting conclusion made by these authors was, that the catecholase activity of the dinuclear copper(II) complexes seemed to depend on the metal-metal distance; thus, the complexes for which the copper-copper separation was estimated to be more than 5 Å, showed very little catalytic activity. Therefore, the authors suggested the hypothesis of the catecholase activity being regulated by a steric match between the

dicopper(II) center and the substrate. The higher activity of dinuclear copper(II) complexes in catechol oxidation in comparison to the mononuclear copper(II) complexes has also been pointed out by some other authors, *e.g.* Malachowski[51] and Casellato *et al.*[52]

In 1985, the hypothesis about the formation of the dicopper-catecholate intermediate at the first stage of the catalytic reaction was further supported by Karlin and co-workers[53], who have succeeded in crystallizing the adduct of tetrachlorocatechol (TCC) with the dicopper(II) complex with a phenol-based dinucleating ligand (Figure 1.6, see Section 1.3.2.2.1 for details). However, almost at the same time Thompson and Calabrese[54] proposed that the catalytic reaction proceeds via the one-electron transfer from catechol to the copper(II) ion, resulting in the formation of a semiquinone intermediate species. The authors have prepared and characterized a bis(3,5-di-*tert*-butyl-*o*-semiquinonato)copper(II) complex by reaction of $[Cu_2(py)_4(OCH_3)_2](ClO_4)_2$ with $DTBCH_2$ in anaerobic conditions. Interestingly, they did not observe the simultaneous two-electron transfer yielding DTBQ and two copper(I) centers. The formation of the semiquinone species in the catalytic cycle was later reported by other authors.[55-57]

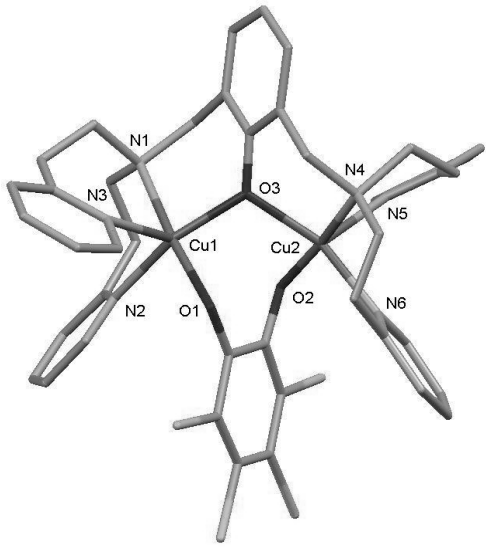


Figure 1.6. Crystal structure of the complex cation of $[Cu_2(L\text{-}O^-)(TCC)]^+$. LOH: 2,6-bis(*N,N*-bis(2-methylpyridyl)aminomethyl)phenol. The Cu…Cu distance is 3.248(2) Å. Redrawn after Karlin and co-workers.[53]

The determination of the structure of hemocyanin, another protein with the type-3 active site, in 1989,[7] and extensive studies on the enzyme tyrosinase, responsible for the conversion of L-tyrosine to L-DOPA, leading to melanin production, prompted the extensive studies on the synthetic models of the type-3 active site and their reactivity. In the early 1990s, a few research groups reported the formation of dihydrogen peroxide instead of water as a dioxygen reduction product in the catalytic oxidation of $DTBCH_2$ by the copper(II) complexes.[58,59] In order to explain their experimental results, Chyn and

Urbach proposed two different mechanisms for the catalytic cycle, as depicted in Scheme 1.1:[58]

(1)

$Cu^{II}...Cu^{II} + DTBCH_2 \longrightarrow Cu^{I}...Cu^{I} + DTBQ + 2H^{+}$ (fast)

$Cu^{I}...Cu^{I} + O_2 \longrightarrow Cu^{II}(O_2)^{2-}Cu^{II}$ (slow)

$Cu^{II}(O_2)^{2-}Cu^{II} + 2H^{+} \longrightarrow Cu^{II}...Cu^{II} + H_2O_2$ (fast)

(2)

Initial step:

$Cu^{II}...Cu^{II} + DTBCH_2 \longrightarrow Cu^{I}...Cu^{I} + DTBQ + 2H^{+}$

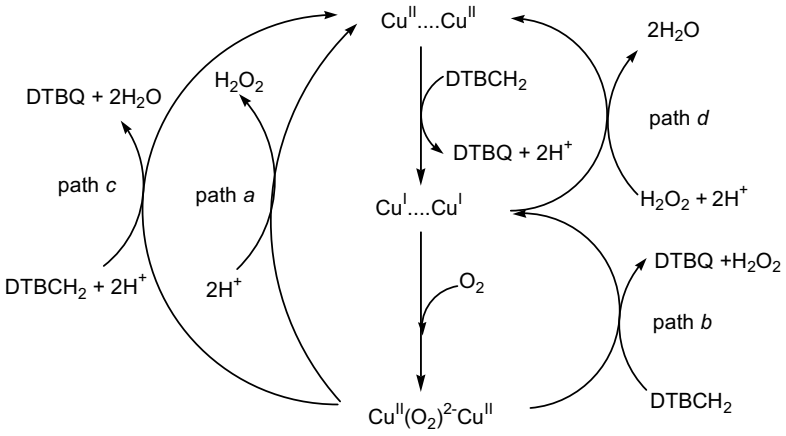
Redox cycle

$Cu^{I}...Cu^{I} + O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Cu^{II}(O_2)^{2-}Cu^{II}$

$Cu^{II}(O_2)^{2-}Cu^{II} + 2H^{+} \longrightarrow Cu^{II}...Cu^{II} + H_2O_2$

Scheme 1.1. Two possible mechanistic pathways resulting in the formation of H_2O_2 as a by-product, as proposed by Chyn and Urbach [58]

Rockcliffe and Martell have published numerous studies on catechol oxidation by dicopper(II) and peroxo-dicopper(II) complexes.[60-66] A rather significant attention has been devoted to the structure-activity relationship of the catalytically active compounds. Very detailed mechanistic studies on the catecholase activity of a series of structurally related dicopper(II) complexes have also been published by Casella and co-workers,[67-70] who reported that the catalytic reaction proceeds via a biphasic mechanism, in which a fast stoichiometric reaction between the dicopper(II) center and the catechol substrate is followed by a slower catalytic reaction. They have also grouped together different mechanisms earlier proposed for the catecholase activity of dicopper(II) complexes, as shown in Scheme 1.2.



Scheme 1.2. The possible reaction pathways in the catalytic cycle of catechol oxidation by dicopper(II) complexes, as proposed by Casella and co-workers. Redrawn after Casella and co-workers.[68]

However, despite the significant attention received by this topic and the large number of publications on the catalytically active copper(II) complexes, detailed mechanistic studies are unfortunately quite scarce.[58,59,67,68,71-73] As a consequence, the catalytic pathways proposed by different authors are often largely speculative in nature and sometimes controversial. Furthermore, it appears that very different methods to explore the catecholase activity and to study the reaction mechanism were applied by different research groups, which makes the corresponding results difficult to compare. An overview of the different approaches to study the reaction mechanism in respect to earlier reported works will be presented below.

1.3.2 Mechanistic studies: different approaches

1.3.2.1 General

The approaches used by different research groups to study the mechanism of catecholase activity of the copper(II) complexes can be roughly divided into four major groups. The first one is dealing with the substrate binding to the metal centers. This group includes a crystallographic and/or spectroscopic characterization of the adducts of the catechol(ate) or structurally related compounds with the copper complexes and studies on the interaction of the complexes with catechol in anaerobic conditions. The interest in this subject is enhanced by the currently disputed way of the substrate binding to the active site of catechol oxidase. The original assumption of the didentate bridging binding mode of the substrate[3] has been called into question by crystallographic findings for the native enzyme; these suggested an alternative mechanism with monodentate binding of the catechol to only one of the copper ions.[8,39]

The second group includes structure-activity relationship studies. These include the correlation of the catecholase activity of the complexes with the metal-metal distance in the dicopper(II) core, their redox potentials, ligand properties (electronic properties, basicity, sterical demands) and the nature of the bridging ligands between the two metal centers. For the sake of simplicity, pH-dependent studies were also included in this group, as the pH-influenced changes in the catalytic activity of the complexes are usually caused by the structural changes at the dicopper center. The third approach includes the kinetic studies on the catalytic reaction, *e.g.* the influence of the various factors (*e.g.* substrate, catalyst and dioxygen concentration, addition of dihydrogen peroxide *etc.*) on the reaction rates; and the proposals on the reaction mechanism based on these data.

Finally, the fourth group includes the examples of stoichiometric oxidation of catechol substrates by peroxo- or oxo-dicopper complexes, which are almost always proposed as intermediate species in the catalytic oxidation of catechol by copper(II) compounds.[8,64,65,67,68]

1.3.2.2 Substrate-binding studies

1.3.2.2.1 Structural characterization of dicopper-catecholate adducts

The various possible binding modes of catechol to the copper centers are summarized in Figure 1.7.

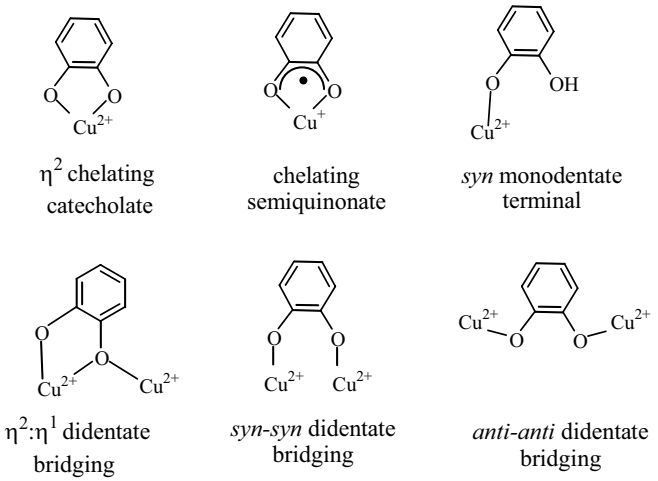


Figure 1.7. Different binding modes of the (deprotonated) catechol substrate to the copper centers.

The first crystallographically characterized adduct of a dicopper(II) complex with tetrachlorocatecholate was reported by Karlin and co-workers.[53] The compound was prepared by reacting tetrachloro-1,2-benzoquinone with the dicopper(I) precursor complex in dichloromethane. The catecholate anion binds as a bridging ligand in a *syn-syn* fashion to both copper(II) ions, resulting in a metal-metal separation of 3.248(2) Å. Both copper(II) ions adopt a square-pyramidal geometry, with the oxygen atoms of the catecholate anion occupying the basal plane, as depicted in Figure 1.6.

Other structurally characterized examples of catechol adducts with dinuclear copper(II) complexes were reported significantly later. Thus, Comba and co-authors[74] have reported the crystal structures of four different copper-tetrachlorocatecholate adducts, with three different modes of substrate coordination to the metal centers (Figure 1.8): as a monodentate, monoprotonated ligand (**1**), as a didentate fully deprotonated chelating ligand (**2** and **4**), and as a bridging deprotonated ligand between the two copper(II) centers (**3**, *anti-anti* binding mode). Interestingly, the authors reported that the highest catecholase activity was observed for the complexes which bound catecholate in a didentate bridging fashion, whereas mononuclear copper(II) complexes were found to be completely inactive.

Meyer and co-workers[75] have reported the structures of three dinuclear Cu^{II} complexes, in which the deprotonated tetrachlorocatecholate is bound to only one of the two copper(II) ions in a didentate chelating fashion (Figure 1.9). It is further linked via one or two hydrogen bridges to water molecules bound to the adjacent metal center. Interestingly, the copper-copper separation in the precursor dicopper(II) complexes

(Figure 1.10, complexes **3**$(ClO_4)_2$ and **4**$(ClO_4)_2$) exceeds 4 Å, which probably precludes the binding of the catecholate to both copper(II) ions.

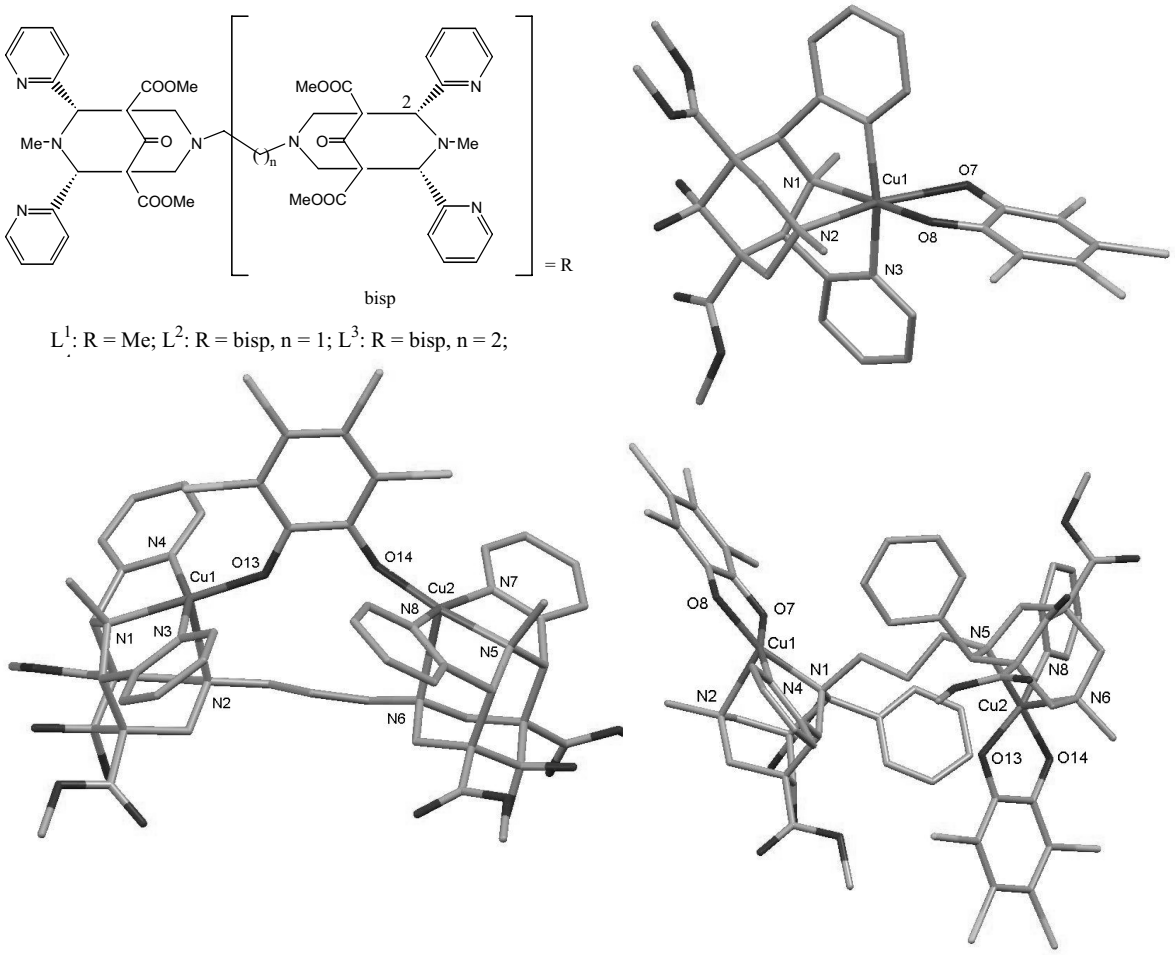


Figure 1.8. The structures of the bispidine ligands (left) and the X-ray crystal structure projections of $[Cu_2(L^1)(TCC)]$ (**2**, top, right), $[Cu_2(L^3)(TCC)]^{2+}$ (**3**, bottom, left) and $[Cu_2(L^4)(TCC)_2]$ (**4**, bottom, right). Redrawn after Comba and co-workers.[74]

An interesting example of the formation of mononuclear copper(II)-semiquinonate complexes was reported by Tolman and co-workers.[76] The authors reported the oxidation of $DTBCH_2$ and TCC by μ-η^2:η^2 peroxo-dicopper(II) and μ-oxo-dicopper(III) complexes, resulting in the dissociation of the dinuclear core and the formation of mononuclear copper(II)-semiquinonate adducts. Similarly to the earlier reported mononuclear copper-catecholate adducts, the semiquinonate ligand is occupying two places in the coordination sphere or the metal ion, with a ferromagnetic coupling realized between the unpaired electron of the Cu^{II} ion and the organic radical. Thompson and Calabrese[77] have reported the crystal structure of a Cu^{II}-semiquinonate complex, obtained by the interaction of a bis-methanolate-bridged copper(II) dimer with $DTBCH_2$ (see also Section 1.3.1). During this process, the dicopper(II) core undergoes a dissociation into two mononuclear units, with one electron being transferred from the

catecholate substrate to one of the two copper(II) ions, resulting in the formation of the Cu^{II}-semiquinonate and the reduced Cu^{I} mononuclear species.

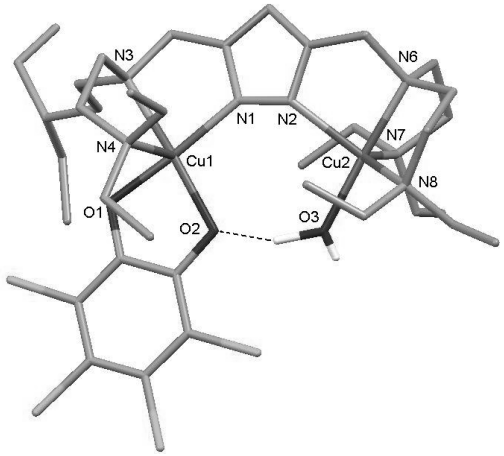


Figure 1.9. X-ray crystal structure of one of the dicopper(II)-catecholate adducts crystallized by Meyer and co-workers. The Cu…Cu distance is 4.4388(8) Å. Redrawn after Meyer and co-workers.[75]

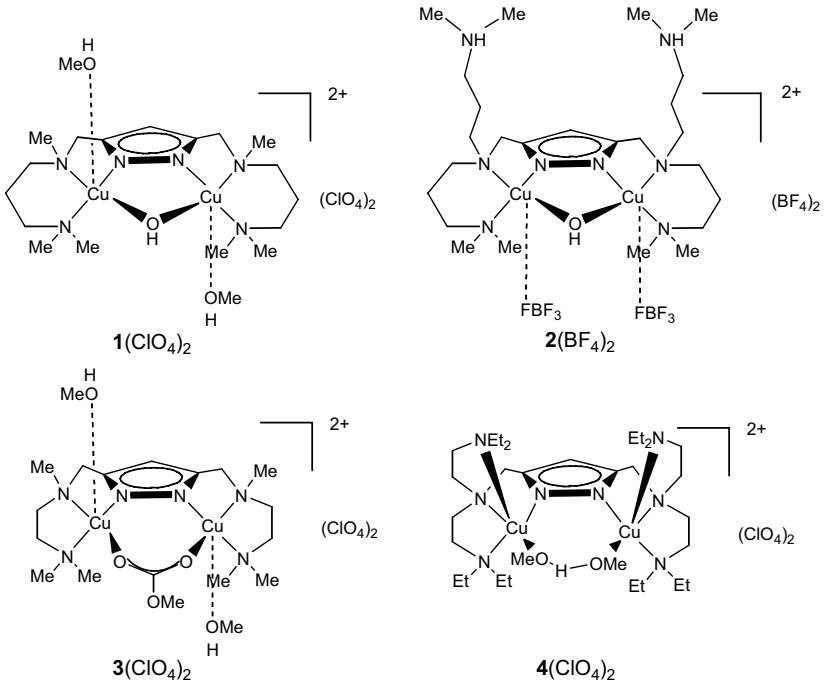


Figure 1.10. Schematic representations of the copper(II) complexes of the various pyrazolate ligands, prepared by Meyer and co-workers[75] (in the case of **1**, the analogous complex **1'**, which bears ethanol instead of methanol ligands, was analyzed crystallographically). The Cu…Cu distance is 3.540(1) Å for **1'**$(ClO_4)_2$, 3.447(2) Å for **2**$(BF_4)_2$, 4.088(1) Å for **3**$(ClO_4)_2$ and 4.553(1) for **4**$(ClO_4)_2$. Redrawn after Meyer and co-workers.[75]

1.3.2.2.2 Substrate binding to the metal centers followed by spectroscopic methods

Attempts to monitor the binding of the catechol substrate to the metal centers by spectroscopic methods, mostly UV-Vis spectroscopy, were undertaken by many authors. Thus, Reim and Krebs[78] titrated solutions of catalytically active and inactive

dicopper(II) complexes with the phenol-based ligands (Figure 1.11) with tetrachlorocatechol and followed the changes spectrophotometrically. Whereas the inactive complexes appeared to be completely indifferent to TCC, the reaction of the active complexes with the substrate was accompanied by the development of new bands in the 400-500 nm range, assigned to the catecholate → Cu^{II} charge transfer, and changes in the positions and extinction coefficients of the Cu^{II} d-d bands. These results indicated the binding of the substrate to the metal centers prior to the catalytic cycle for the active complexes and revealed that the inactive complexes did not interact with the substrate.

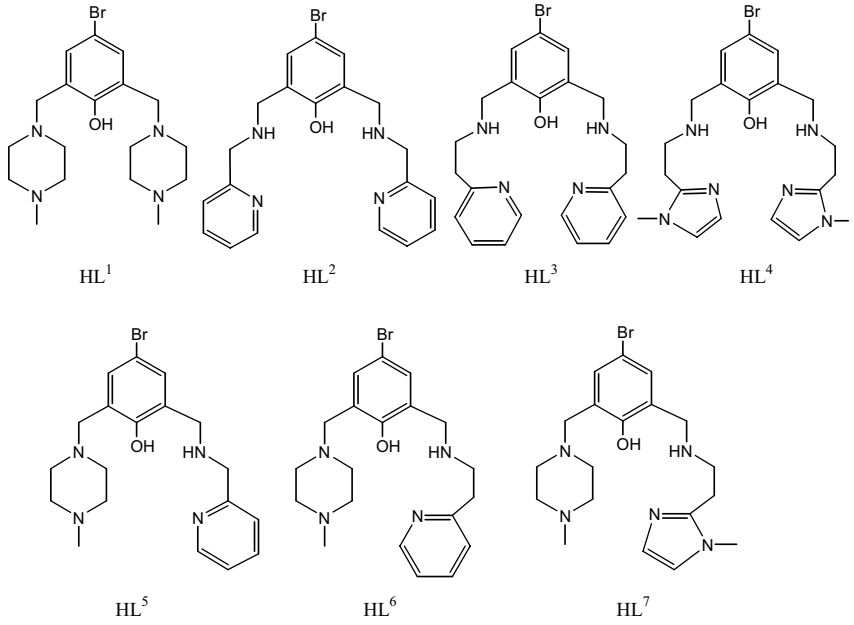


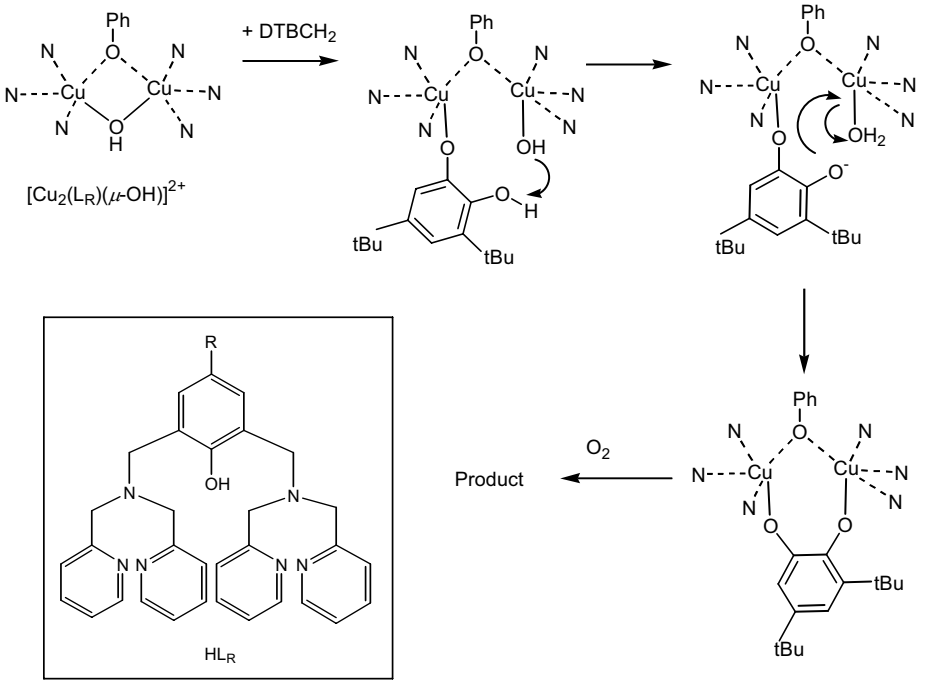
Figure 1.11. Pentadentate dinucleating phenol-based ligands prepared by Reim and Krebs. Only the complexes of the ligands HL^1, HL^5, HL^6 and HL^7 showed catecholase activity. Redrawn from Reim and Krebs.[78]

Jäger and co-authors have also studied the interaction of a series of the copper(II) complexes of aminocarbohydrate *β*-ketoenaminic ligands with TCC.[79] However, in this case both the active and inactive complexes were found to interact with TCC, although the spectra of the active compounds changed to a remarkably higher degree in comparison to the inactive molecules. The observed spectroscopic changes were rather consistent with those reported by Reim and Krebs:[78] the development of a new band at 480 nm along with the decrease of the d-d band of the Cu^{II} ion at 650 nm. Very similar results (a development of a new band in the 400-500 nm range and changes in the position and absorption of the d-d bands of the Cu^{II} ions) during the interaction of TCC with the dicopper(II) complexes of some dinucleating ligands (*e.g.* phenol-based) were also reported by Mukherjee *et al.*[80] Comba and co-workers[74] have reported the titration of the mononuclear and dinuclear complexes $[Cu_2(L^1)(solv)]^{2+}$, $[Cu_2(L^2)(solv)_2]^{4+}$ and $[Cu_2(L^3)(solv)_2]^{4+}$ (see Figure 1.8) with TCC

and showed that in the first case, a strong absorption band appeared at *ca.* 450 nm, whereas for dinuclear complexes, equilibriums between species with absorptions at *ca.* 450 nm and *ca.* 530 nm were established. The authors proposed that catecholate-bridged compounds are formed with $[Cu_2(L^2)(solv)_2]^{4+}$ and $[Cu_2(L^3)(solv)_2]^{4+}$, whereas a mononuclear catecholate complex is formed with $[Cu_2(L^1)(solv)]^{2+}$.

Very detailed studies on the substrate binding to the copper(II) complexes with the phenol-based dinucleating ligands were reported by Belle *et al.*[81] The authors studied the binding of TCC and $DTBCH_2$ (the binding studies of the latter compound were performed in anaerobic conditions) to a catalytically active μ-hydroxo-dicopper(II) complex with the phenol-based ligand HL_{OCH_3} (Scheme 1.3, insert) and its inactive bis-aqua-dicopper(II) analogue. In both cases, a new UV-Vis band at *ca.* 450 nm developed upon addition of TCC to the complexes, reaching its maximum when two molar equivalents of catechol were added to the solution. Thus, in both cases, a first substrate binding occurred, followed by a second one. EPR-spectroscopic measurements showed that in the case of the catalytically active hydroxo complex, the catechol binding results in the cleavage of the hydroxo bridge, leading to the evolution of the EPR-signal, in contrast to the EPR-silent initial complex. The stopped-flow studies allowed the determination of a kinetic constant of the fixation of the second equivalent of TCC by this complex, whereas the fixation of the first molar equivalent was found to be too fast to be determined. In the case of the inactive bis-aqua-dicopper(II) complex, the binding of TCC did not lead to any appreciable changes in the EPR-spectrum, and the fixation of two substrate molecules was too fast to be distinguished. The anaerobic studies on the $DTBCH_2$ binding to the complexes indicated that, in contrast to the natural enzyme, catechol is not oxidized stoichiometrically in the absence of dioxygen. However, electrochemical studies indicated that the binding of $DTBCH_2$ to the active hydroxo complex affects significantly its electrochemical behavior, leading to a complex being made more easily reducible and oxidizable. On the contrary, the electrochemical behavior of the inactive diaqua complex was only weakly affected by the binding of the substrate.

Based on these observations, the authors proposed a mechanism of the substrate binding to the dicopper(II) center, as depicted on Scheme 1.3, which reconciled two earlier proposed modes of the substrate fixation by the natural enzyme: a didentate bridging mode proposed by Solomon for the catecholase activity of tyrosinase,[3] and a monodentate asymmetric coordination, proposed by Krebs.[8] In this mechanism, the substrate first binds to only one copper center along with the concomitant cleavage of the hydroxo bridge. Then, the proton transfer from the second phenol group of catechol to the hydroxyl group bound to the second copper center occurs, resulting in the displacement of a water molecule and the bridging coordination of the catecholate.



Scheme 1.3. Proposed mechanism for the interaction between the dinuclear μ-hydroxo-copper(II) complexes and $DTBCH_2$. Insert: dinucleating ligands HL_R employed to prepare the copper(II) complexes: R = CH_3 (HL_{CH3}), F (HL_F), CF_3 (HL_{CF3}), and OCH_3 (HL_{OCH3}). Redrawn after Belle and co-workers.[81]

Casella and co-workers[82] have used inactive *p*-nitrocatechol (NCat) to isolate and spectroscopically characterize catecholate adducts of mononuclear and dinuclear copper(II) complexes. The authors prepared the complexes of the composition [Cu(L6)(NCat)] (L6 = *N,N*-bis[2-(1'-methyl-2'-benzimidazolyl)ethyl]amine), $[Cu_2(L66)(NCat)](ClO_4)_2$ (L66 = α,α'-bis{bis[2-(1'-methyl-2'-benzimidazolyl)ethyl]amino}-*m*-xylene, Figure 1.12) and $[Cu_2(L66)(NCat)_2]$ (the latter compound was studied only in solution), and reported their IR, Raman and UV-Vis spectra.

Based on the very similar spectroscopic features of [Cu(L6)(NCat)] and $[Cu_2(L66)(NCat)](ClO_4)_2$ (C-O stretch peak of the coordinated catecholate at 1265±2 cm^{-1} in the IR spectra and in the Raman spectra with the excitation length of 454.5 nm; bands at 293, 350 and 468 nm in the UV-Vis spectra), the authors proposed that in both compounds catecholate is bound in a similar chelating η^2 mode to one copper ion, eventually exhibiting an additional η^1 bridging coordination to a second copper atom in the dicopper(II) complex, as depicted in Figure 1.13. In addition, the second equivalent of catechol could bind to the dicopper complex, forming a bis-catecholate adduct, which also seems to indicate that the substrate is bound to only one metal center. In fact, these results seem to correlate with the observations of Belle *et al.*,[81] who also reported the successive binding of two catechol molecules to dicopper complexes and suggested the asymmetric coordination of the substrate.

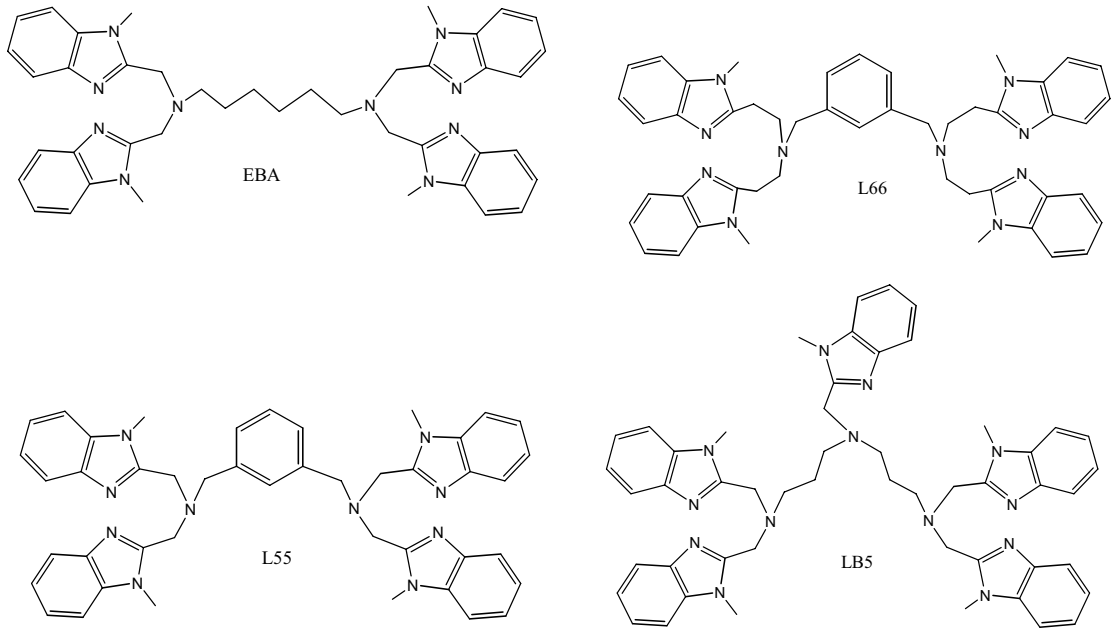


Figure 1.12. Structures of the ligands EBA, L55, L66 and LB5, prepared by Casella and co-workers.[67,68]

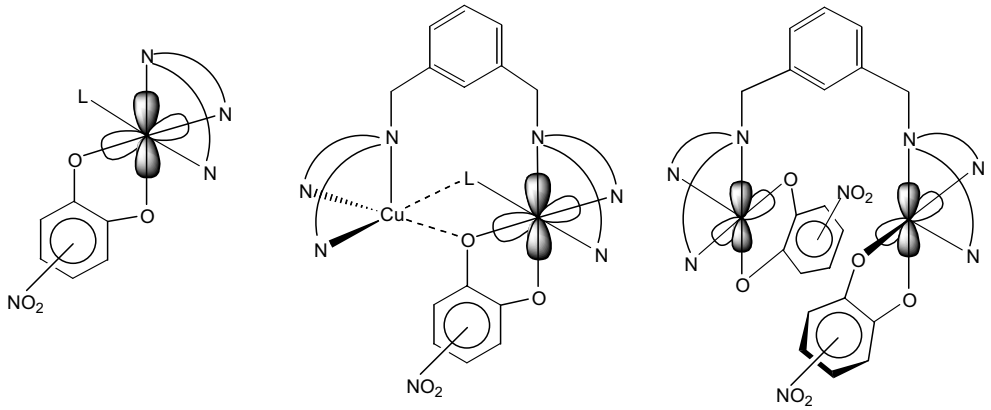


Figure 1.13. Structure proposals for [Cu(L6)(NCat)] (left), $[Cu_2(L66)(NCat)](ClO_4)_2$ (middle) and $[Cu_2(L66)(NCat)_2]$ (right). Redrawn after Casella and co-workers.[82]

1.3.2.2.3 Anaerobic interaction of catechol with copper(II) complexes

The stoichiometric oxidation of the catechol substrate by the dicopper(II) core, leading to the formation of quinone and dicopper(I) species, has often been proposed as the first step in the catalytic cycle.[8,68,79,83] Consequently, some examples of studies on anaerobic interaction of the copper(II) complexes with $DTBCH_2$ have been reported. In most cases, the reduction of the dicopper(II) core along with the release of the quinone molecule was indeed observed, in some cases only in the presence of catechol excess.[69-71,79,84] As an example, the spectroscopic changes observed upon treating the dicopper(II) complex $[Cu_2(L55)]^{2+}$ (L55 = α,α'-bis{bis[1-(1'-methyl-2'-

benzimidazolyl)methyl]amino}-*m*-xylene, Figure 1.12) with $DTBCH_2$, reported by Casella and co-workers,[70] are shown in Figure 1.14.

At -90 °C, the electron transfer from catechol to the dicopper(II) core is prevented, which enabled the authors to spectrophotometrically characterize the catecholate adduct with the complex (curve b, Figure 1.14). Similarly to earlier reported UV-Vis spectra of adducts with electron-poor catechols,[79-81] this species is characterized by weak absorptions at 345 and 440 nm, attributed to LMCT bands. Upon warming the reaction mixture to room temperature, the dicopper(II) core is reduced to the copper(I) state, and the molecule of quinone is released, easily monitored by the absorption at *ca.* 400 nm (curve c, Figure 1.14).

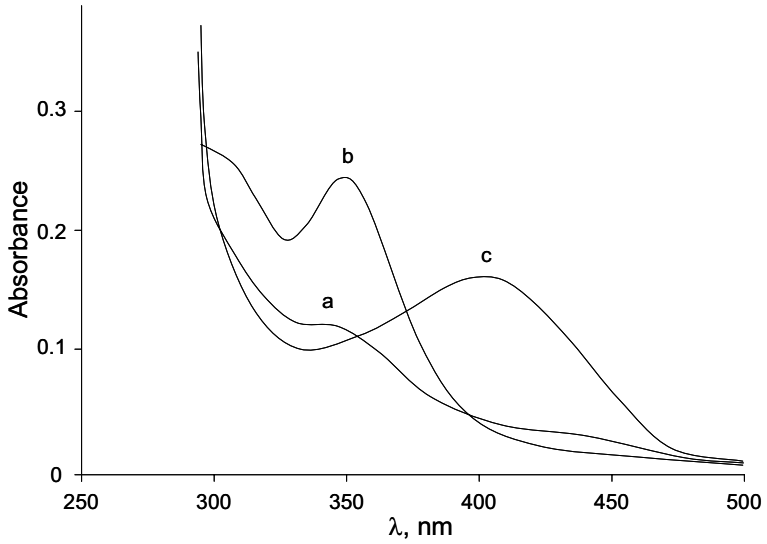


Figure 1.14. Electronic spectra recorded anaerobically in methanol solution at -90 °C of: (a) $[Cu_2(L55)]^{4+}$ (0.2 mM) and (b) its complex with $DTBCH_2$ (1.8 mM). Spectrum (c) shows the stoichiometric formation of DTBQ after warming the solution to room temperature (λ = 396 nm, ε = 1600 $M^{-1}cm^{-1}$). Redrawn after Casella and co-workers.[70]

Some exceptions from this type of behavior, however, have been reported. Thus, μ-hydroxo-dicopper(II) complexes with a series of phenol-based ligands reported by Belle *et al.* (Scheme 1.3, insert) do not oxidize $DTBCH_2$ in anaerobic conditions, but instead bind two equivalents of the substrate in two successive steps.[81] As discussed above, the parent complexes become more easily reducible and oxidizable upon binding of the first molecule of the substrate, whereas the binding of the second molecule hardly affects further the electrochemical behavior. A number of authors have reported that in case of the anaerobic catechol interaction with mononuclear copper(II) complexes, one-electron transfer takes place, leading to the formation of copper(I)-semiquinonate species.[57,85] The reaction of dinuclear copper(II) complexes, formed by the self-assembly of two mononuclear units, with catechol was found to result in the dissociation of the dicopper(II) core.[56,77] As a result, either a mononuclear copper(II)-catecholate adduct,[56,86]

or a copper(II)-semiquinonate product along with the reduced copper(I) co-product,[77] were formed.

It should be noted that some authors have reported the vanishing of the d-d and/or LMCT bands of the copper(II) complexes immediately after the substrate addition along with the appearance of the characteristic quinone absorption at 400 nm in the UV-Vis spectra also in the presence of dioxygen.[79,87,88] These changes were also attributed to the fast stoichiometric reaction between the complex and the substrate, leading to the reduction of the copper(II) centers and the release of one molar equivalent of the quinone, prior to the rest of the catalytic cycle.

1.3.2.3 Structure-activity relationship

1.3.2.3.1 Metal-metal distance vs. catecholase activity

The assumption that a steric match between the dicopper(II) center of a complex and catechol substrate is required for the catecholase activity has been published as early as 1980.[50] Consequently, the majority of the authors use a comparison of the metal-metal distances within a series of structurally related complexes to interpret the difference in their catecholase activities, if their crystal structures are available.[75,80,89] Taking into account that the copper-copper distance in the *met* form of the natural enzyme is very short (2.9 Å only), and comparing this value to that reported by Karlin and co-workers[53] for the *o*-catecholate-bridged dicopper(II) complex (*ca.* 3.25 Å, Figure 1.6), a conclusion can be drawn that the optimal copper-copper distance for the catecholase activity falls in a range of 2.9–3.2 Å. Kao *et al.*[89] have studied the catecholase activities within a series of oxy-bridged dicopper(II) complexes and showed that the complexes with the metal-metal distance, closest to that observed for the *met* form of catechol oxidase, display the best catalytic activity, as depicted in Figure 1.15.

Nevertheless, a large metal-metal separation in dicopper(II) complexes does not necessarily prohibit a catecholase activity. For example, Meyer and co-workers have reported the catalytic oxidation of $DTBCH_2$ by two dicopper(II) complexes with a metal-metal separation of 4.088 Å and 4.553 Å (Figure 1.10, complexes **3**$(ClO_4)_2$ and **4**$(ClO_4)_2$).[75] The catecholase activity of these complexes was found, however, to be significantly lower in comparison to their analogues with the shorter (*ca.* 3.5 Å) copper-copper distance (Figure 1.10, complexes **1**$(ClO_4)_2$ and **2**$(BF_4)_2$). Furthermore, Selmeczi *et al.* reported the catecholase activity of a dicopper(II) complex $[Cu_2(L^1)(CF_3SO_3)_2(H_2O)_4](CF_3SO_3)_2$ (L^1=1,3-bis{*N,N*-bis(2-[2-pyridyl]ethyl)}aminopropane, Figure 1.16), in which a copper-copper distance amounts to 7.840 Å.[71,72]

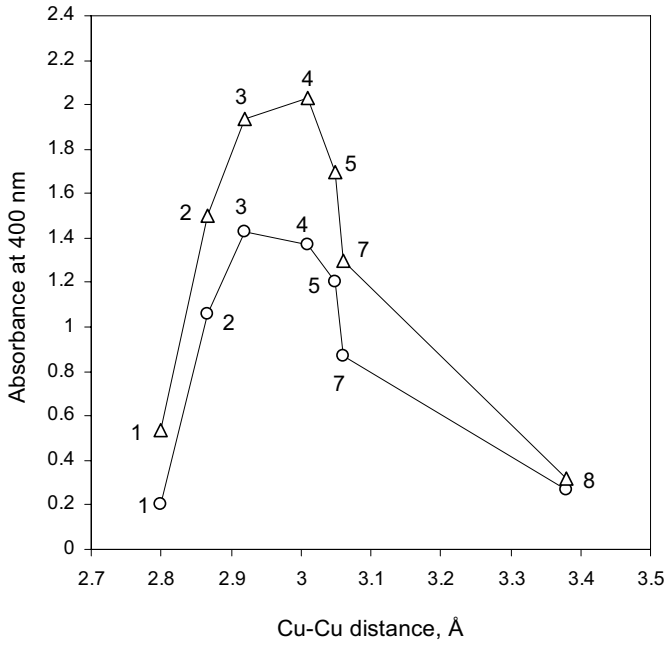


Figure 1.15. Plot of absorption of the quinone band at 400 nm (a) 30 min (– ○ –) and (b) 60 min (– Δ –) after addition of $DTBCH_2$ to the oxy-bridged complexes *vs.* copper-copper distance in these complexes. Redrawn after Kao *et al.*[89]

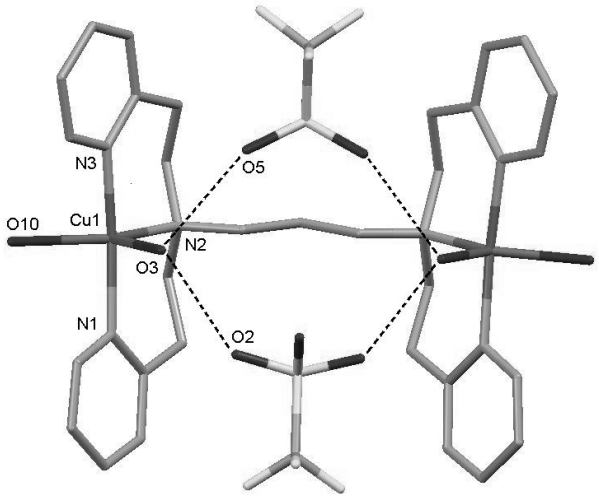


Figure 1.16. X-ray crystal structure of $[Cu_2(L^1)(CF_3SO_3)_2(H_2O)_4]^{2+}$ (L = 1,3-bis{*N*,*N*-bis(2-[2-pyridyl]ethyl)}aminopropane), prepared by Selmeczi *et al.*[71] The Cu…Cu distance is 7.8398(9) Å.

1.3.2.3.2 Electrochemical properties of the complexes vs. catecholase activity

Many research groups have attempted to correlate the redox properties of the copper(II) complexes with their catecholase activity.[67,75,78,80,88,90,91] However, a correlation between the two is not easily established. For example, Torelli *et al.*[91] reported that the inactive bis-aqua-dicopper(II) complex with the HL_{CH3} ligand (Scheme 1.3, insert) could be more easily reduced than its catalytically active μ-hydroxo-bridged analogue. On the other hand, the same authors reported the existence of a correlation between the

first reduction potentials of hydroxo-bridged dicopper(II) complexes with a series of dinucleating compartmental ligands HL_R ligands (Scheme 1.3, insert) and their catecholase activities.[90] The authors have changed the *para*-substituents on the phenol ring of dinucleating compartmental ligands HL_R (Scheme 1.3, insert) and showed that the presence of the strong electron-withdrawing CF_3 group in this position results in a completely inactive dicopper(II) complex. The complexes with *p*-CH_3, *p*-OCH_3 and *p*-F substituents were found to exhibit catecholase activity; furthermore, taking the methyl-substituted complex as a reference, a higher activity was observed in the presence of the electron-donating OCH_3 group, whereas the presence of an electron-withdrawing fluorine atom was found to inhibit the activity to a moderate extent.

Reim and Krebs[78,88] studied the electrochemical behavior of a series of dicopper(II) complexes with dinucleating phenol-based ligands (Figure 1.11) in acetonitrile solution, but observed only irreversible and ill-defined reduction steps. The reduction potentials were found to be very sensitive to the degree of protonation and/or the number of transferred electrons, thus no clear relationship between the redox properties of the complexes and their catecholase activity could be established.

Mukherjee *et al.* also reported the absence of an obvious correlation between the first reduction potentials of the doubly bridged dicopper(II) complexes with various endogenous and exogenous bridges and their catecholase activity.[80] However, Casella and co-workers succeeded in calculating the reaction rates for the two successive steps of the catalytic reaction (a fast stoichiometric reaction between a dicopper(II) complex and catechol and a slower catalytic reaction), and showed a clear dependence of the reaction rate in the first stoichiometric step on the Cu^{II}/Cu^{I} reduction potential.[67] As this step involves the electron transfer from the bound catecholate to the dicopper(II) center, this observation is fully understandable. On the other hand, as overall reaction rates obviously depend on many factors, *i.e.* the rate of the reoxidation of the dicopper(I) species by dioxygen, the rate of the catechol oxidation by the formed peroxo-dicopper intermediate *etc.*, it is hardly surprising that in the majority of cases, no straightforward correlation between the activity and the redox potential of a complex can be established.

1.3.2.3.3 The influence of the exogenous bridging ligands on the catecholase activity of dicopper(II) complexes

The nature of the bridging ligands between the copper centers in a complex plays an important role in its catecholase activity. The small bridging ligands can promote a short copper-copper distance within a dimetal core, required for the catecholate binding in a didentate bridging fashion, which is thought to be beneficial for catecholase activity. On the other hand, the substrate should effectively bind to the copper(II) ions and needs thus to be able to displace a present bridging ligand at the dimetal core. Furthermore, some bridging ligands, *e.g.* OH^- ion, can facilitate the deprotonation of catechol due to their ability to abstract the proton with the subsequent release of a water molecule. In general, it can be stated that such bridging ligands as

hydroxide,[75,80,91] alkoxide or phenoxide[79,80,87,89], imidazolate[92] and carboxylate[75,83,93,94] can be readily displaced by the incoming catecholate and thus promote the catecholase activity. On the other hand, strongly coordinated ligands, such as chloride and bromide, cannot be displaced by the substrate, resulting in catalytically inert compounds.[95,96]

Neves *et al.* studied the catecholase activity of dicopper(II) complexes with acetate bridging ligands in the presence of variable amounts of sodium acetate.[83] The authors reported the decrease of the reaction rates, in accordance with the hypothesis that the acetate competes with the incoming catecholate for a binding site in the copper coordination sphere, leading to inhibition effect. Krebs and co-workers[93] have recently published interesting studies on the catecholase activity of a series of dicopper(II) complexes with phenol-based compartmental ligands and double acetate bridges between the metal centers (Figure 1.17). The authors showed that the presence of the thiomorpholine substituent on the ligand facilitates the displacement of one acetate bridge, leading to higher catalytic activities (see Section 1.3.2.3.4 for details). These results indicate that the easiness of the bridging ligand displacement in general leads to higher catalytic activities, although it is obvious that this factor does not solely control the reactivity.

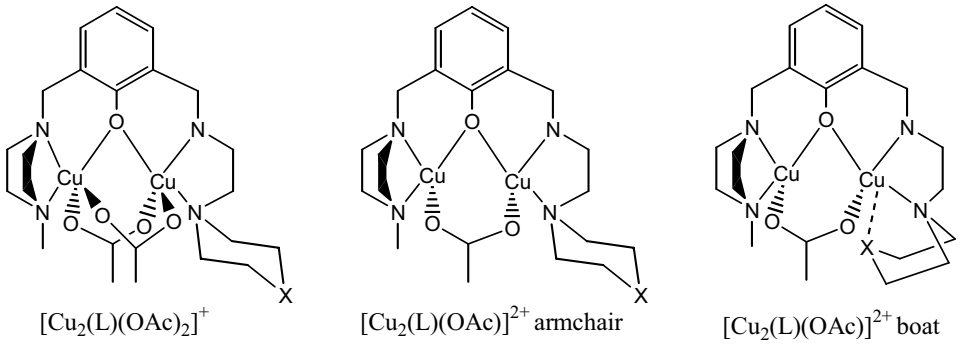


Figure 1.17. Structures of $[Cu_2(L)(OAc)_2]^+$ and the boat and chair conformations of $[Cu_2(L)(OAc)]^{2+}$ (with X = CH_2, O or S). Redrawn after Krebs and co-workers.[93]

On the other hand, Reedijk and co-authors[96] reported the interaction of chloro- and bromo-bridged dicopper(II) complexes of the phenol-based compartmental ligand Hpy2th2s (2,6-bis[*N*-(2-pyridylmethyl)-*N*-(2-thiophenylmethyl)aminomethyl]-4-methylphenol) with catechol substrates (see Chapter 4). In these complexes, both copper ions are pentacoordinated, with three positions in the coordination sphere occupied by the donor atoms of the ligand and the other two by the halogen ions, one bridging and one monocoordinated. Both complexes were found to be inactive in catechol oxidation; however, their titration with TCC indicated that in the chloride complex, one of the monocoordinated chloride anions could be substituted by the catechol substrate. The bridging chloro atom could not be exchanged with the catecholate anion. In the case of the bromide complex, neither monocoordinated nor bridging halogen anions could be substituted by TCC.

A few authors pointed out that the presence of two hydroxide, alkoxide or phenoxide bridges may lead to catalytically inactive complexes. Thus, Mukherjee *et al.* explains the inactivity of the complex $[Cu_2(L^5\text{-}O)_2(ClO_4)_2]$ (L^5-OH = 4-methyl-2,6-bis(pyrazolyl-1-ylmethyl)phenol) by the presence of two phenoxide bridges in its structure.[80] Similarly, Casella and co-workers showed that the active species in the catechol oxidation by the dicopper(II) complex with the ligand L55 (L55 = α,α'-bis{bis[1-1'-methyl-2'-benzimidazolyl)methyl]amino}-*m*-xylene, Figure 1.12) is a monohydroxo-bridged dicopper(II) species, whereas the bis(μ-hydroxo) species is essentially inactive.[70] However, these observations are not conclusive, as the examples of the catalytically active complexes with the double hydroxo,[56,80] alkoxo[79,87,89] and phenoxo[89] bridges have also been reported.

An interesting possible function of the bridging hydroxo group in the catecholase activity of a complex has been proposed by Reim and Krebs.[78] The authors investigated the catecholase activities of a series of dicopper(II) complexes with phenol-based compartmental ligands (Figure 1.11) and reported that the complex containing the exogenous μ-hydroxo bridge exhibits the highest catalytic activity. This appears to be caused by the fact that the bridging hydroxide group enforces the complex to adopt a very strained geometry, which makes it willing to exchange the μ-hydroxo bridged structural motif in favor of the bridging catechol coordination. In the presence of alternative bridging ligands with a larger bite distance, a more relaxed conformation is adopted, which in turn leads to a lower activity.[78]

1.3.2.3.4 The influence of the ligand structure on the catecholase activity of dicopper(II) complexes

Although many authors refer to the ligand properties to explain the results of the catecholase activity studies on the copper complexes, only a few detailed studies on changes in the ligand structure and their influence on the catecholase activity have been reported so far.

Krebs and co-workers[93] have prepared three asymmetric phenol-based compartmental ligands, one arm of which contained piperidine (L1), morpholine (L2) or thiomorpholine (L3) heterocycles (Figure 1.17), and studied the catecholase activity of their dicopper(II) complexes with two acetate bridges between the metal centers. The authors have found that the complex with the thiomorpholine substituent shows the highest catecholase activity, probably because the sulfur atom can displace one of the bridging acetate ligands and yield a free coordination site for the substrate binding. This hypothesis was confirmed by DFT calculations,[93] which were performed to determine the different reaction energies ($[LCu_2(OAc)_2]^+ \rightarrow [LCu_2(OAc)]^{2+} + OAc^-$) for all three monocation conversions into the corresponding monoacetate-bridged dications in their boat and armchair conformations (Figure 1.17). For the thiomorpholine system, the isomer with a boat conformation of the subunit was found to be 5.5 kcal mol^{-1} more stable than the corresponding armchair conformer, whereas for the morpholine system,

the energy difference was only 1.4 kcal mol^{-1}, and for the piperidine system, the armchair conformation was found to be significantly more stabilized. Furthermore, the thiomorpholine-containing structure was found to possess a Cu-S bond (R_{Cu-S} = 2.42 Å). These results indicate the ability of the sulfur atom in the ligand to displace a bridging ligand between the copper(II) centers, which in turn leads to higher catecholase activity of the system in question.

The ligand flexibility also plays a role in the activity of the resulting copper(II) complexes. Kandaswamy and co-workers have studied the catecholase activities of a series of copper(II) complexes with lateral macrodicyclic compartmental ligands (Figure 1.18) and reported the enhancement of the activity with the increase of the macrocyclic ring size.[97] The increase in ring size makes the system more flexible and favors the catalysis phenomenon.

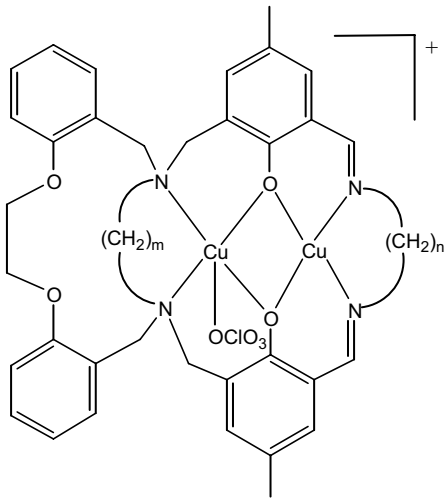


Figure 1.18. General structure of dicopper(II) complexes with macrodicyclic ligands. Redrawn after Kandaswamy and co-workers.[97]

On the other hand, the studies of Reim and Krebs on the catecholase activity of the dicopper(II) complexes with phenol-based compartmental ligands (see Figure 1.11) showed that only the complexes containing piperazine unit within their ligand framework exhibited catecholase activity.[78] This is perhaps related to the fact that the square-pyramidal coordination spheres of the copper(II) ions in these complexes are strongly distorted due to the coordination of the piperazine group. Thus, the presence of a certain substituent in a ligand framework can have a strong influence on the catalytic behavior of the corresponding copper complexes.

1.3.2.3.5 The influence of pH on the catecholase activity of dicopper(II) complexes

The natural enzyme exhibits catecholase activity only in a limited pH range (pH 5-8), with an optimum activity at pH 8, and an irreversible loss of activity below pH 4.0 and above 10.0.[27] Some authors have studied the influence of pH on the catecholase

activity of model copper complexes.[67,70,83,91,94] It should be noted that the changes in pH are often accompanied by the changes in the structure of a complex, leading to different catalytic behavior. Thus, Torelli *et al.*[90,91] have studied the pH-driven interconversions of dicopper(II) complexes with a series of phenol-based compartmental ligands (Scheme 1.3, insert) and found that the μ-phenoxo-μ-hydroxo-dicopper(II) complexes, which are stable at neutral pH values, can reversibly interconvert into the μ-phenoxo-bis-aqua-dicopper(II) and μ-phenoxo-bis(hydroxo)dicopper(II) species at lower and higher pH levels, respectively, as shown in Figure 1.19. Of these species, only the μ-hydroxo-dicopper(II) complexes exhibit catecholase activity. The possible reasons for that could be a short metal-metal distance (2.89 Å) in these complexes and the ability of the bridging hydroxo group to assist in the deprotonation of the incoming catechol substrate, facilitating its binding to the dicopper(II) center, as discussed above (Scheme 1.3).

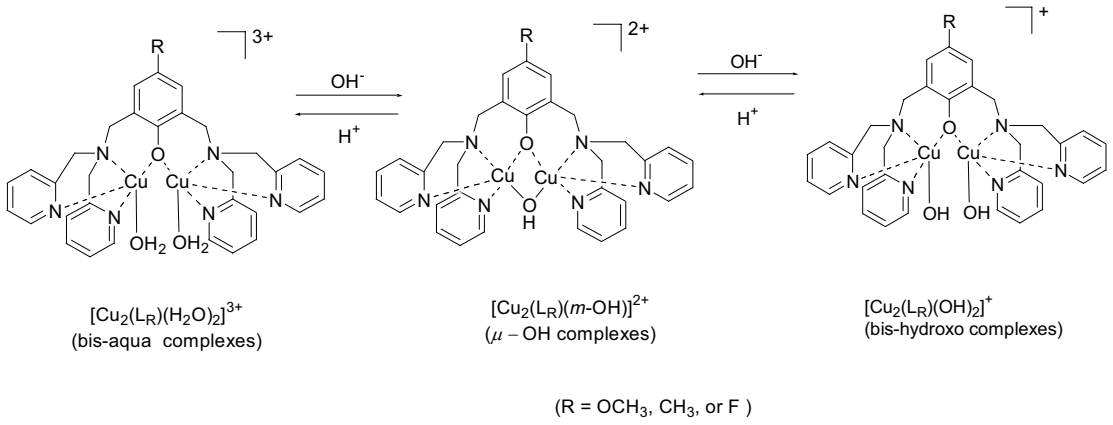


Figure 1.19. pH-driven interconversions of dicopper(II) complexes with phenol-based ligands HL_R. Redrawn after Belle and co-workers.[91]

Fernandez *et al.* have studied the catecholase activity of the dicopper(II) complex with the asymmetric ligand HTPPNOL (*N*,*N*,*N*'-tris-(2-pyridylmethyl)-1,3-diaminopropan-2-ol) at different pH values.[94] The pH-titrations indicated that above pH 8.0, the water molecule, coordinated to one of the two copper(II) ions in solution, undergoes a deprotonation with the formation of a hydroxide group (Figure 1.20). An increase of the activity was observed at pH 8.05, *e.g.* when the hydroxide-containing species is present in solution. The authors have also suggested that the hydroxide moiety assists in the deprotonation of the substrate, facilitating its binding to the dicopper(II) core. This assumption is consistent with the proposal of Belle *et al.*,[81] although the apical coordination of the hydroxide anion was proposed by Fernandez *et al.*, in contrast to the bridging coordination, as determined by the latter authors.

Casella and co-workers[68] have studied the catecholase activity of the dicopper(II) complexes $[Cu_2(LB5)]^{4+}$, $[Cu_2(L55)]^{4+}$ and $[Cu_2(L66)]^{4+}$ (Figure 1.12) in methanol solution and found that at neutral pH values, the complexes oxidized $DTBCH_2$ either

stoichiometrically, or with extremely low catalytic efficiency. Thus, the catalytic reactions were performed at pH 5.1, in the presence of a small amount of an aqueous buffer. At this pH, the contribution of the non-catalytic oxidation of $DTBCH_2$ was found to be negligible. A year later, the authors reported[67] the catecholase activity studies on the dicopper(II) complex $[Cu_2(EBA)]^{2+}$ (Figure 1.12) and the influence of pH on the catalytic behavior. The authors analyzed only the acidic pH range in order to prevent the possible substrate autoxidation and to increase the pH sensitivity. The studies were performed at two different substrate concentrations: the one that gave the highest reaction rate, and the one-fourth of this substrate concentration. While at lower catechol concentration the pH influence was negligible, at high substrate concentration the reaction rate in both phases (see above for the biphasic mechanism proposed by Casella and co-workers) increased with the pH with a saturation behavior (Figure 1.21).

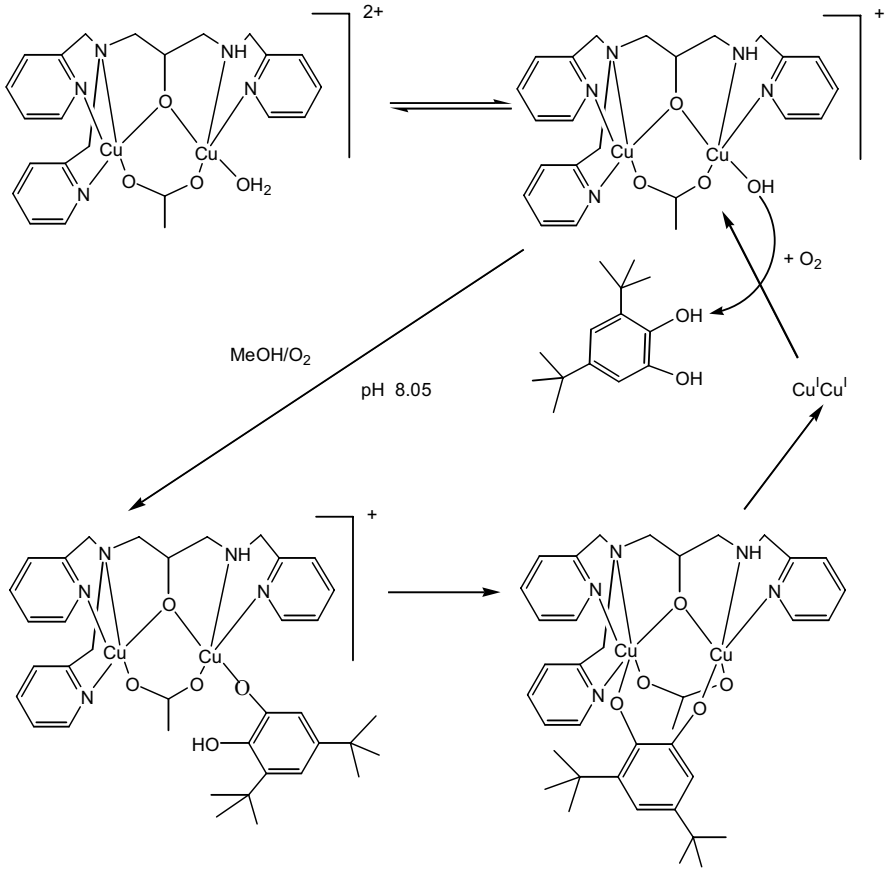


Figure 1.20. Mechanism of the interaction between the dinuclear copper(II) complex with the asymmetric ligand HTPPNOL and 3,5-di-*tert*-butylcatechol, as proposed by Fernandes *et al.*[94]

Later, Casella and co-workers[70] have reported the studies on the catecholase activity of the dicopper(II) complex with the ligand L55 in a mixed solvent of 75% methanol/glycerol (7/1 v.v.) and 25% (v/v) aqueous 50 mM Hepes buffer, which allowed to keep the pH of the solution close to neutral values. The studies on the pH influence on the catalytic reaction rates showed that the maximal rate was observed

around pH 7, whereas it dropped drastically above pH 7.5 (Figure 1.22, left). Earlier studies on the pH-driven interconversions[98] of this complex indicate that rate profile parallels the distribution curve of the monohydroxo species $[Cu_2(L55)(H_2O)(OH)]^{3+}$, while the bis($\mu$-hydroxo) species $[Cu_2(L55)(OH)_2]^{2+}$, which is dominant above pH 6.5, is catalytically inactive (Figure 1.22, right).

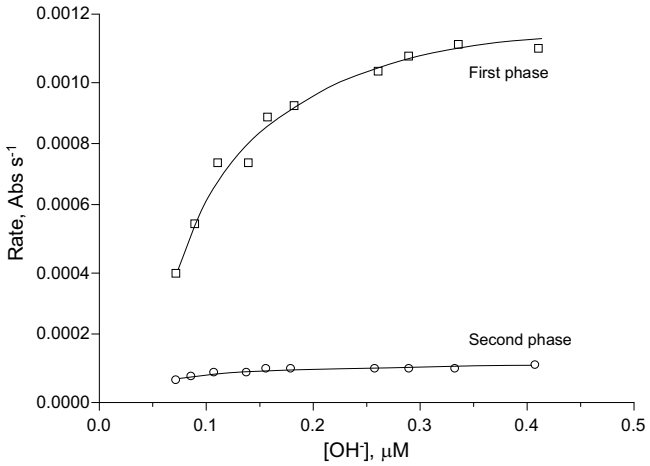


Figure 1.21. Dependence of the rate (as absorbance change at 396 nm *vs.* time) of the first and second phases of catalytic oxidation of $DTBCH_2$ by $[Cu_2(EBA)]^{4+}$ (14 μM) on the solution pH, as reported by Casella and co-workers.[67] The concentration of 3,5-di-*tert*-butylcatechol was 6 mM in all experiments. The reactions were performed in 30:1 mixture of methanol/aqueous phosphate buffer, the pH of which was varied from 3.4 to 5.3. Redrawn after Casella *et al.* [67]

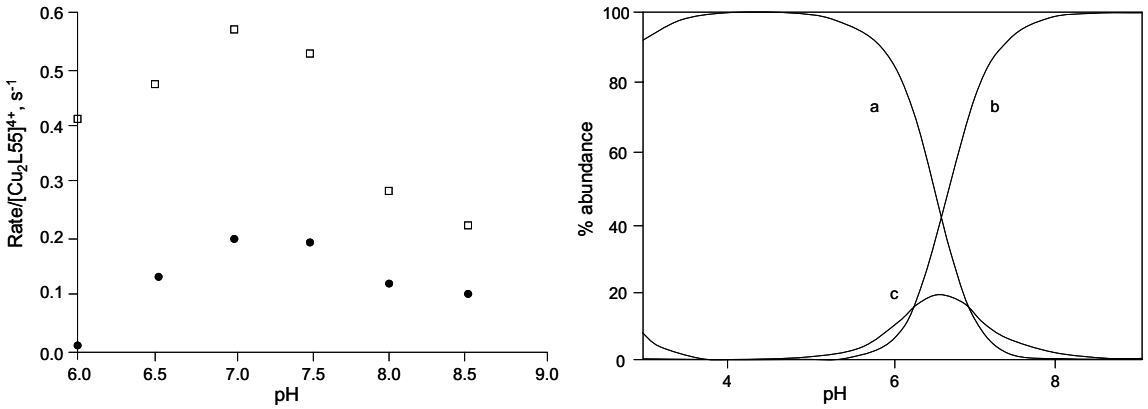


Figure 1.22. Left: rate dependence for the first (open squares) and second (solid circles) steps of the oxidation of $DTBCH_2$ (2 mM) catalyzed by $[Cu_2(L55)]^{4+}$ (6 μM) on the pH in the mixed solvent of 75% methanol/glycerol (7:1) and 25% (v/v) Hepes buffer (50 mM). Redrawn after Casella and co-workers.[70] Right: species distribution in the 2Cu/L55 system as a function of pH in acetonitrile/water solution: a) $[Cu_2(L55)(H_2O)_2]^{4+}$; b) $[Cu_2(L55)(H_2O)(OH)]^{3+}$; $[Cu_2(L55)(OH)_2]^{2+}$. Redrawn after Casella and co-workers.[98]

Thus, it appears that all authors have reached a similar conclusion: in case of pH-driven interconversions of (bis)aqua-, monohydroxo- and bis(hydroxo)-dicopper(II) species, the monohydroxo derivatives usually exhibit the highest catecholase activity, likely to be caused by the short metal-metal distance enforced by the bridging hydroxide anion, and its function in the substrate deprotonation, facilitating its binding to the catalytic core.

1.3.2.4 Kinetic studies

1.3.2.4.1 Dependence of the reaction rates on the complex and catechol concentration

Almost all reports on the catecholase activity of copper(II) complexes include the kinetic studies, *e.g.* the dependence of the reaction rates on the concentration of the substrate, catalyst, dioxygen and some additives, *e.g.* dihydrogen peroxide or kojic acid. It appears that in most cases, a simple Michaelis-Menten model is sufficient to describe the behavior or the catalytic system. This kinetic model, initially proposed for the enzymatic catalysis by Leonor Michaelis and Maud Menten in 1913, is based on the assumption that the catalyst and substrate reversibly react with each other to form an intermediate species prior to the substrate conversion, according to Scheme 1.4.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Scheme 1.4. The mechanism of the interaction of the enzyme (E) with the substrate (S), leading to the formation of the product P, according to the Michaelis-Menten model.

The reaction rate for this model is determined by the equation (1.1), also called Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_M + [S]} \quad (1.1)$$

In this equation, V_{max} corresponds to the limiting reaction rate, reached at a very high substrate concentration. Thus, a characteristic of a Michaelis-Menten system is the substrate saturation behavior, upon which the reaction rate asymptotically approaches a certain value with the substrate concentration increase, but never reaches it. K_M is a Michaelis constant, which corresponds to the substrate concentration at which the reaction rate is equal to one half of the maximal value, and is defined as $(k_2+k_{-1})/k_1$ (Figure 1.23).

A rearrangement of the equation (1.1) leads to the equation (1.2), from which a linear dependence of the reciprocal reaction rates on the reciprocal substrate concentration becomes obvious. By building a plot of 1/V *vs.* 1/[S], also known as a double reciprocal, or Lineweaver-Burk plot, and taking into account that $V_{max} = k_2$ [E],

the kinetic constants k_2 and K_M can be determined (Figure 1.23, right). The constant k_2 in this case corresponds to the turnover frequency, determined in s^{-1}.

$$\frac{1}{V} = \frac{[S] + K_M}{V_{max}[S]} = \frac{[S]}{V_{max}[S]} + \frac{K_M}{V_{max}[S]} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} \qquad (1.2)$$

Most of the studies on the catecholase activity of copper(II) model compounds, performed by the method of the initial reaction rates, showed that the catalytic reaction indeed shows a saturation behavior *vs.* the substrate (=catechol) concentration, making a Michaelis-Menten model applicable.[68,69,75,78-80,83,91,93,94] The observed Michaelis constants usually vary in a range of 10^{-4}-10^{-3} M, and k_2 values fall in a range of 10^{-2}-10^{-1} s^{-1}. Less studies report the dependence of the reaction rates on the catalyst (=complex) concentration.[68,78,80] Usually, a linear dependence is found, indicating that the reaction shows a first-order dependence on the catalyst.

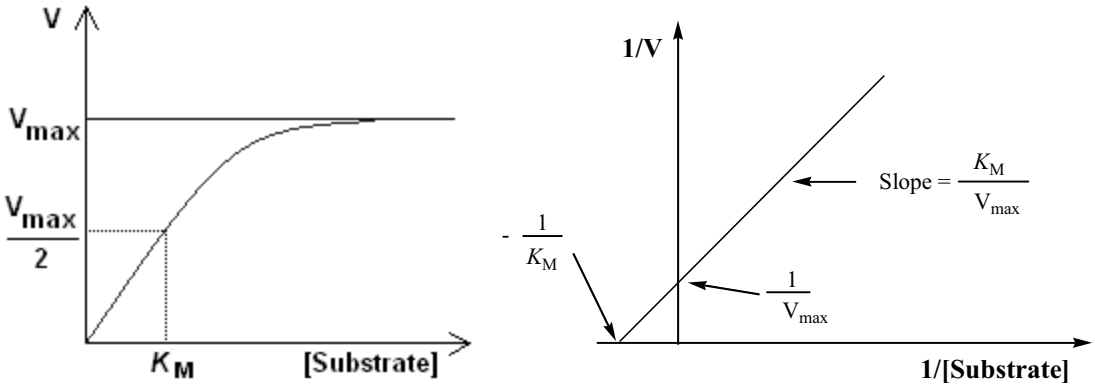


Figure 1.23. Left: the plot of the reaction rates *vs.* the substrate concentration according to the Michaelis-Menten model. Right: an example of a Lineweaver-Burk plot.

A few exceptions from a general trend have also been reported. Thus, in some cases the reaction rates were found to be independent on the substrate concentration.[68,71,75] This behavior can be explained by the presence of another rate-determining step in the overall catalytic cycle, for example, a reoxidation of the dicopper(I) species by molecular dioxygen, as proposed by Casella and co-workers.[68] In this case, the reaction rates are expected to depend on the concentration of molecular dioxygen. Unfortunately, the influence of dioxygen concentration on the reaction rates has been only studied in a few cases, but the works of Casella and co-workers[68] and Speier and co-workers[71] proved indeed that a strong dependence of the reaction rates on dioxygen concentration exists for the catalytic systems, showing a zero-order dependence on the catechol. For example, a three-fold increase in the reaction rate was reported by Casella for the complex $[Cu_2L66]^{4+}$, for which the reaction rates were found to be independent on the substrate concentration, when the solution was saturated with pure dioxygen instead of air.[68] The studies of Speier and co-workers[71] showed a clear

dioxygen saturation behavior for the complex $[Cu_2(L^1)(CF_3SO_3)_2(H_2O)_4](CF_3SO_3)_2$ (L^1=1,3-bis{*N,N*-bis(2-[2-pyridylethyl)}aminopropane, Figure 1.16), for which a zero-order dependence on the substrate has been reported (Figure 1.24).

Casella and co-workers,[67] who proposed a biphasic mechanism for the oxidation of catechol by dicopper(II) complexes (a fast stoichiometric oxidation of catechol by dicopper(II) core, followed by a slower catalytic reaction), have derived a kinetic equation for two consecutive steps in the catalytic cycle (equation 1.3), which allows to determine the reaction rate constants k_1 and k_2 of the first and the second phases. [DTBQ] and [Cat] correspond to the concentrations of DTBQ and the catalyst, respectively.

$$[3,5\text{-DTBQ}] = \frac{k_1[\text{Cat}]}{k_1 + k_2}\left\{2\,k_1 t - \frac{k_1 - k_2}{k_1 + k_2}[1 - \exp[-(k_1 + k_2) \times t]]\right\} \quad (1.3)$$

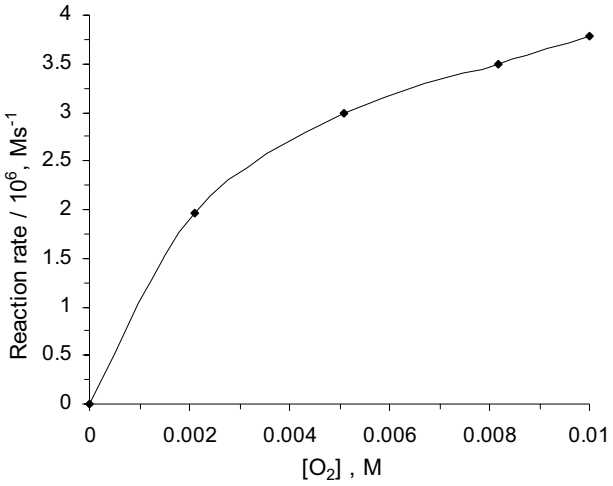
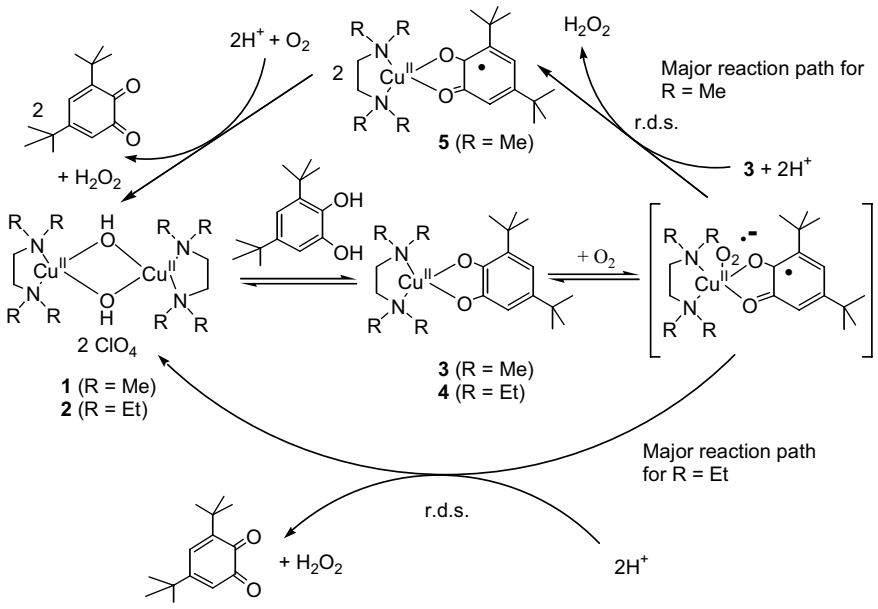


Figure 1.24. The dependence of the oxidation rate of $DTBCH_2$ catalyzed by $[Cu_2(L^1)(CF_3SO_3)_2(H_2O)_4](CF_3SO_3)_2$ on the dioxygen concentration. Conditions: $[[Cu_2(L^1)(CF_3SO_3)_2(H_2O)_4](CF_3SO_3)_2]$=0.125 mM, $[DTBCH_2]$=4.16 mM at 25 °C in MeOH. Redrawn after Speier and co-workers.[71]

Very recently, the same authors reported that in the case of catechol oxidation by the complex $[Cu_2(L55)]^{4+}$ (Figure 1.12), two steps of the catalytic cycle could be separated.[70] The use of stopped-flow technique allowed the determination of the reaction rate in the first stiochiometric phase, whereas the rate of the second step was studied in a time interval of 5-20 s after the beginning of the reaction. In order to prove the reliability of this method, the authors have also calculated the rates of the first and the second phases by fitting the development of the quinone absorbance with time to equation 1.3. In spite of the differences in the two methods of analysis, the results obtained were identical.

1.3.2.4.2 Dihydrogen peroxide formation during the catalytic reaction

The overall catalytic mechanism, reported by Casella and co-workers in 1998 (Scheme 1.2),[68] indicates that either water or dihydrogen peroxide can form as a side product in the catalytic oxidation of catechol by copper(II) complexes. The formation of dihydrogen peroxide in the reaction mixture has indeed been reported in a few cases;[58,59,71,75,83] however, it should be noted that the reports containing the studies aimed to definitely establish the mode of the dioxygen reduction to either water or dihydrogen peroxide are quite scarce. The exact way of dihydrogen peroxide formation is not fully understood. Curiously, in some cases the formation of dihydrogen peroxide is correlated with the detection of the semiquinone intermediate species in the catalytic reaction.[56,57,99] It is indeed plausible that the dihydrogen peroxide may form as a product of the oxidation of the copper(I)-semiquinone intermediate, as proposed by Kodera *et al.*[56] (Scheme 1.5). This mechanism can be rationalized as follows. In case of dicopper(II) complexes, the simultaneous reduction of two copper(II) centers to the copper(I) state results in the oxidation of one equivalent of catechol, leading to the release of one quinone molecule. In case of mononuclear copper(II) complexes (or dinuclear complexes, formed by self-assembly of two mononuclear units), only one electron transfer may occur, resulting in the formation of copper(I)-semiquinonate intermediate species. The reaction of such species with dioxygen may result in the two-electrons reduction of the latter, leading to the reoxidation of the copper(I) ion, a release of the quinone molecule and dihydrogen peroxide formation. Thus, only one molecule of catechol is being oxidized per such catalytic cycle, in contrast to the mechanism proposed for the natural enzyme[8] and for dicopper(II) complexes.[68]



Scheme 1.5. The proposed mechanism of the catechol oxidation, leading to dihydrogen peroxide formation as a byproduct, as proposed by Kodera *et al.* Redrawn after Kodera and co-workers.[56]

It is, however, not clear how dihydrogen peroxide can form upon catechol oxidation by dinuclear copper(II) complexes. According to the overall mechanism proposed by Casella, it may form either via the path a, or the path b (Scheme 1.2).[68] The studies of Ackermann *et al.*[75] showed that upon consumption of one mole of dioxygen, one molar equivalent of quinone was formed, which allowed the authors to propose that the catalytic cycle proceeds via the mechanism including the path a (the protonation of the dicopper(II)-peroxo species, leading to the dihydrogen peroxide release). On the contrast, the path b has been proposed by Selmeczi *et al.*[71] Although both paths are liable, another possibility can though exist. The studies of Ackermann[75] and Casella[82] on dicopper(II)-catecholate adducts indicate that the doubly deprotonated catecholate can bind to only one of the two copper(II) ions instead of the dinuclear bridging coordination, especially when the metal-metal distance is long. It is logical to assume that in this case, only one-electron transfer can occur, resulting in the formation of a mixed-valence $Cu^{I}Cu^{II}$-semiquinonate species. Its interaction with dioxygen may further proceed via the mechanism proposed by Kodera *et al.*[56] It is thus plausible that only one of the two copper ions plays a part in the electron transfer, whereas another has only a structural role. This is certainly an interesting possibility, as the examples of the enzymes containing two or more metal ions, only one of which plays a role in catalysis, are widely known in nature. Unfortunately, very limited information available on this subject does not allow accepting or discarding this option.

1.3.2.4.3 The influence of dihydrogen peroxide on the reaction rates

The studies on the influence of dihydrogen peroxide on the catalytic behavior have only been reported in a few cases.[67,68] In general, dihydrogen peroxide can participate in the catalytic cycle by reoxidizing the reduced dicopper(I) species to the copper(II) oxidation state, thus competing in this with dioxygen. Respectively, its influence is to a large extent defined by the sensitivity of the formed dicopper(I) intermediate to dioxygen. In case of slow reoxidation of this species by dioxygen, dihydrogen peroxide enhances the reaction rates, as the reduced species prefers to react with it instead of dioxygen. On the contrary, when the reoxidation proceeds very fast, the reaction rates are not significantly affected by dihydrogen peroxide addition. In fact, even a slight decrease of the reaction rates may be observed, perhaps caused by the conversion of the active dicopper(II) complex into a less reactive peroxide intermediate, according to the following reaction:

$$Cu^{II}\ldots Cu^{II} + H_2O_2 \rightarrow Cu^{II}(O_2)^{2-}Cu^{II} + 2H^{+}$$

Furthermore, the saturation behavior in dihydrogen peroxide can be observed.[68] This can be related to the fact that presence of dihydrogen peroxide changes the rate-determining step in the reaction. At low H_2O_2 concentration, a normal reoxidation of dicopper(I) species by dioxygen takes place. At higher concentrations, the copper(I)

species can be oxidized by both dioxygen and dihydrogen peroxide, whereas above a certain H_2O_2 concentration, only the latter reaction takes place.

1.3.2.4.4 The influence of the inhibitor kojic acid on the reaction rates

The only example of the studies on the influence of inhibitors on the catecholase activity of model copper complexes has been published by Casella and co-workers, who reported the inhibiting effect of kojic acid on the catecholase activity of dicopper(II) complexes with the ligands L55, L66 and EBA (Figure 1.12).[73] The inhibitor strongly binds to the dicopper(II) complex in the first stoichiometric step of the reaction and to the dicopper(II)-dioxygen adduct in the second step, preventing in both cases the binding of the catechol substrate. The inhibition was found to be of a competitive type. The latter means that in the presence of an inhibitor, a higher substrate concentration is required to achieve the same reaction rates that were reached in its absence resulting in a higher K_M. In case of a non-competitive inhibitor, the binding of the inhibitor to the catalyst molecule makes it inactive. The differences in Lineweaver-Burk plots for a competitive and a non-competitive inhibition mechanisms are shown in Figure 1.25.

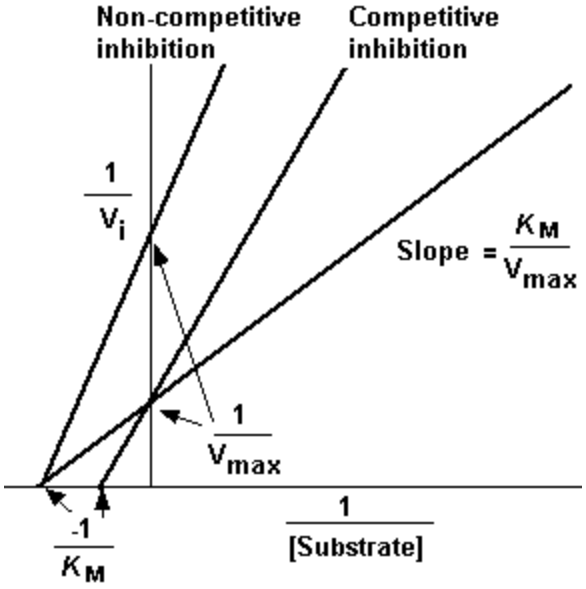


Figure 1.25. An example of Lineweaver-Burk plots for a competitive and a non-competitive inhibition.

Fitting the rate data considering a simple linear competitive inhibition mechanism according to the equation (1.4), the authors could determine the K_I parameter, characterizing the inhibition behavior:

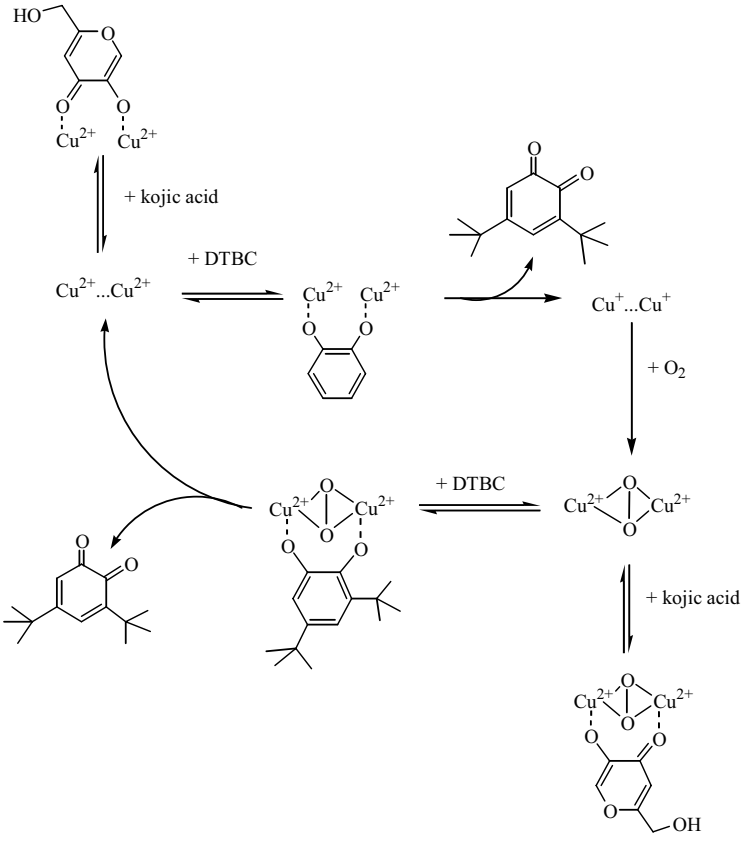
(1.4)

$$V = \frac{\frac{k_{cat}}{K_M}\,[\text{complex}]\;\varepsilon_{DTBQ}\,[\text{DTBC}]}{1 + \frac{1}{K_I}\,[\text{I}] + \frac{[\text{DTBC}]}{K_M}}$$

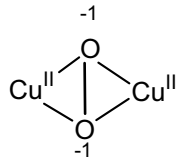
In this equation, [*I*] corresponds to the concentration of kojic acid, whereas K_M and k_{cat} are Michaelis constant and a turnover frequency determined in the absence of the inhibitor. The value $1/K_I$ corresponds in this case to the formation constants of the catalyst-inhibitor complexes. The inhibition mechanism, proposed by Casella and co-workers, is depicted in Scheme 1.6.

1.3.2.5 Stoichiometric oxidation of catechol by (per)oxo-dicopper complexes

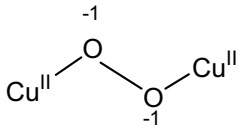
The formation of peroxo-dicopper species as a result of dioxygen binding to a reduced dicopper(I) intermediate and a subsequent oxidation of catechol by them has often been proposed[61,62,64,67,68,71,75] as a second (catalytic) stage of the catechol oxidation by model copper complexes. However, relatively few examples of the interaction of such species with catechol substrates are described in the literature. A schematic representation of the structures of previously reported dicopper-dioxygen cores is shown in Figure 1.26. Kitajima *et al.* reported the oxidative coupling of $DTBCH_2$ by a μ-η^2:η^2 peroxo complex $[Cu_2(HB(3,5\text{-}Me_2pz)_3](O_2)$ ($HB(3,5\text{-}Me_2pz)$ = tris(3,5-dimethylpyrazolyl)borate), leading to the formation of the C-C-coupled products.[100] Interestingly, no formation of *o*-benzoquinone was observed, unless exogenous dioxygen was introduced into the reaction mixture.



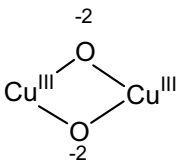
Scheme 1.6. The mechanism of model dicopper(II) complexes inhibition by kojic acid. Redrawn after Casella and co-workers.[73]



μ–η^2:η^2-peroxo -dicopper(II)



trans-μ–1,2-peroxo-dicopper(II)



bis-μ-oxo-dicopper(III)

Figure 1.26. Schematic representation of dicopper-dioxygen cores, reported in the literature.

Casella and co-workers reported a stoichiometric oxidation of $DTBCH_2$ to DTBQ by a μ-η^2:η^2 peroxo-dicopper complex with the ligand L66 (Figure 1.12).[101] The same type of reactivity was observed by Stack and co-workers for a bis μ-oxo-dicopper complex $[(L_{TMCHD})_2Cu^{III}{}_2(O)_2]^{2+}$ (TMCHD = *N,N,N′,N′*-tetramethyl-(*1R,2R*)-cyclohexanediamine).[102] The oxidation of $DTBCH_2$ by μ-η^2:η^2 and bis μ-oxo-dicopper complexes was also reported by Tolman and co-workers[76] with isolation of mononuclear copper(II)-semiquinonate complexes as a sole product of the reaction, the (per)oxo-dicopper species being generated by reaction of two essentially mononuclear Cu^I molecules with dioxygen. Rockcliffe and Martell also reported a number of examples on the stoichiometric oxidation of catechols to the respective quinones or dicarboxylic acids involving various dicopper-dioxygen complexes.[61,64-66] Unfortunately, these authors did not provide detailed information concerning the structure of the peroxo species. Although the end-on dioxygen-binding mode was proposed based on the results of molecular modeling,[63] the UV-Vis spectroscopic data,[63,66] reported by the authors, as well as the overall reactivity of the described peroxo species[62,65] suggest that dioxygen is bound in the μ-η^2:η^2 mode.

Very recently, Reedijk and co-workers[103] reported a stoichiometric oxidation of $DTBCH_2$ by the trans-μ-1,2-peroxo-dicopper(II) complex with the macrocyclic ligand [22]py4pz (= 9,22-bis(2-pyridylmethyl)-1,4,9,14,17,22,27,28,29,30-decaazapentacyclo-[22.2.1.$1^{4,7}$.$1^{11,14}$.$1^{17,20}$] triacontane-5,7(28),11(29),12,18,20(30),24(27),25-octaene) (Chapter 7). The stoichiometric oxidation was found to proceed in two steps through the formation of the intermediate species, characterized by the intensive absorption at 342 nm (ε =3960 $M^{-1}\cdot cm^{-1}$) in the UV-vis spectrum (Chapter 7). Based on the resonance Raman spectroscopic studies and the kinetic isotopic effect measurements, the authors proposed that the first step involves the proton transfer from the substrate to the nucleophilic peroxo core, resulting in the formation of μ-1,1-hydroperoxo-dicopper(II)-catecholate species, while the second step involves the oxidation of the bound substrate.

1.4 The scope of this thesis

This thesis is devoted to the studies on the model compounds of the type-3 active site copper proteins, in particular catechol oxidase. Chapter 1 presents a general overview of the copper proteins, the structure and properties of catechol oxidase, the copper enzyme with the type-3 active site, and discusses earlier reported model

compounds of this enzyme and the mechanistic studies, aimed to elucidate the mechanism of catechol oxidation by the natural enzyme and by model complexes. Chapter 2 deals with the development of the strategy of the synthesis of dinucleating asymmetric ligands to model the asymmetry of the dicopper core, found in the natural enzyme,[8] and reports the crystal structure and properties of the asymmetric complex $[Cu_2(py3asym)(H_2O)_{1.5}(NO_3)_{2.5}](NO_3)_{0.5}$ (Hpy3asym = 2-[*N,N*-bis(2-pyridylmethyl)aminomethyl]-4-methyl-6-[(2-pyridylmethyl)aminomethyl]phenol. In Chapter 3 the crystal structures and properties of a number of Cu^{II}, Mn^{II} and Co^{II} complexes with the ligand Hpy2ald (Hpy2ald = 3-[*N,N*-di(2-pyridylmethyl)aminomethyl]-5-methylsalicylaldehyde), which has been prepared as an intermediate in the synthesis of the asymmetric ligand Hpy3asym are reported. In Chapter 4 the synthesis of the symmetric phenol-based ligand Hpy2th2s (Hpy2th2s = 2,6-bis[*N*-(2-pyridylmethyl)-*N*-(2-thiophenylmethyl)aminomethyl]-4-methylphenol), bearing thiophene and pyridine substituents, as well as the properties of its two dicopper(II) complexes and their interaction with catechol model substrates are described. This ligand was designed to model an unusual thioether bond, discovered in a close proximity to one of the copper ions in the active site of catechol oxidase.[8] In Chapter 5, the synthesis of the asymmetric phenol-based ligand Hpy2th1as (Hpy2th1as = 2-[*N,N*-bis(2-pyridylmethyl)aminomethyl]-4-methyl-6-[2-thiophenylmethyl)-aminomethyl]phenol), containing a tertiary amine arm with two pyridine substituents in the 2 position of the phenol ring, and a secondary amine arm bearing a thiophene ring in the 6 position of the phenol ring, is reported. Two copper(II) complexes, obtained with this ligand, have been crystallographically and spectroscopically characterized, and their structural properties are discussed.

In Chapter 6 the paramagnetic ^1H NMR spectroscopy on the monohydroxo-bridged dicopper(II) complex $[Cu_2[22]py4pz)(\mu\text{-}OH)](ClO_4)_3\cdot H_2O$ (**1**) with the macrocyclic N-donor ligand [22]py4pz (9,22-bis(2-pyridylmethyl)-1,4,9,14,17,22,27,28,29,30-decaazapentacyclo-[22.2.1.$1^{4,7}$.$1^{11,4}$.$1^{17,20}$]triacontane-5,7(28),11(29),12,18,20(30),24(27),25-octaene) and its magnetic properties are reported. The catecholase activity of this dicopper(II) complex, as well as the synthesis and characterization of its reduced dicopper(I) analogue $[Cu_2([22]py4pz)](ClO_4)_2\cdot 2CH_3OH$ (**2**) and the trans-μ-1,2-peroxo-dicopper(II) adduct (**3**), including 3D structure of **2**, are discussed in Chapter 7. These three compounds represent models of the three states of the catechol oxidase active site: *met*, *deoxy* (reduced) and *oxy*. The dicopper(II) complex **1** catalyzes the oxidation of catechol model substrates in aerobic conditions, while in the absence of dioxygen a stoichiometric oxidation takes place, leading to the formation of quinone and the respective dicopper(I) complex. The dicopper(I) complex binds molecular dioxygen at low temperature, forming a trans-μ-1,2-peroxo-dicopper adduct, which was characterized by UV-Vis and resonance Raman spectroscopy, and electrochemically.

This peroxo complex stoichiometrically oxidizes a second molecule of catechol in the absence of dioxygen. A catalytic mechanism of catechol oxidation by **1** is proposed, and its relevance to the mechanisms earlier proposed for the natural enzyme and other copper complexes is discussed.

In Chapter 8 the mechanism of catechol oxidation by a copper(II) complex with the macrocyclic ligand [22]pr4pz (9,22-dipropyl-1,4,9,14,17,22,27,28,29,30-decaazapentacyclo [22.2.1.1^{4,7}.1^{11,14}.1^{17,20}]triacontane-5,7(28),11(29),12,18,20(30),24(27),25-octaene), containing a carbonate bridge between the copper(II) ions, is discussed. The complex has a tetranuclear structure in the solid state, with one macrocyclic unit accommodating two carbonate-bridged copper(II) ions, and two copper(II) ions of two different macrocyclic units being further doubly bridged by two oxygen atoms of the two carbonate anions. This tetranuclear cluster was found dissociate into two dinuclear units in solution at the concentration range, used for the catecholase activity studies. The dinuclear complex catalyzes the oxidation of $DTBCH_2$ to the respective quinone in methanol by two different mechanisms, one proceeding via the formation of a semiquinone intermediate species with the subsequent production of dihydrogen peroxide as a by-product, and another proceeding via the two electrons reduction of the dicopper(II) center by the substrate, with two molecules of quinone and one molecule of water generated per one catalytic cycle. Chapter 9 presents general conclusions and an overview of the perspectives for further research.

Chapters 1-8 of this thesis have either been published[96,103-107] or submitted for publication.[108-110]

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